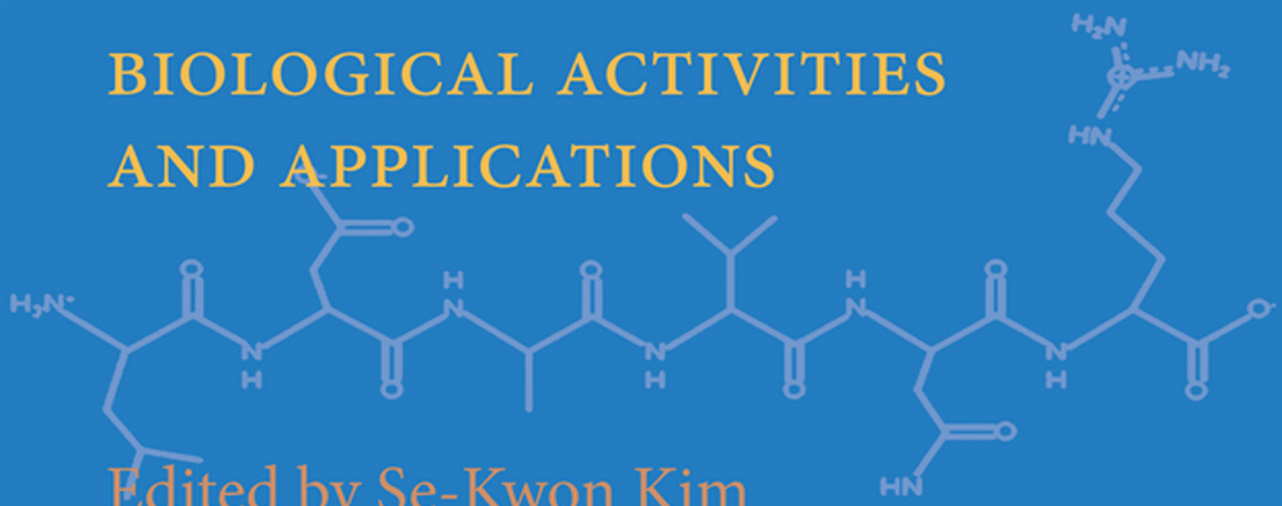
Marine Proteins and Peptides

BIOLOGICAL ACTIVITIES AND APPLICATIONS



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Biological Activities and Applications

Se-Kwon Kim
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1 Marine-derived Peptides: Development and Health Prospects

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1.1 INTRODUCTION

The role of protein in the human diet has been acknowledged recently worldwide. Dietary proteins have become a source of physiologically active components, which have a positive impact on the body's function after gastrointestinal digestion. Bioactive peptides may be produced by one of three methods: solvent extraction, enzymatic hydrolysis and microbial fermentation of food proteins. Marine-derived bioactive food proteins and biopeptides are often effective in promoting health and lead to a reduction in the risk of disease. Recently, much attention has been paid by consumers to natural bioactive compounds as functional ingredients. Hence, it can be suggested that marine-derived bioactive food proteins and biopeptides are alternative sources for synthetic ingredients that can contribute to consumers' well-being, as a part of functional foods, pharmaceuticals and/or cosmetics. Furthermore, they can be utilized in other industries such as medicine, animal feed, printing, textile and so on. This chapter presents an overview of the development, health effects, industrial perspectives and commercial trends of marine-derived bioactive food proteins and biopeptides used in the food, pharmaceutical and cosmetic industries.

1.2 DEVELOPMENT OF MARINE PEPTIDES

Enzymatic hydrolysis of marine-derived proteins allows preparation of bioactive peptides, which can be obtained by *in vitro* hydrolysis of protein substrates using appropriate proteolytic enzymes. The physicochemical conditions of the reaction media, such as the temperature and pH of the protein solution, must then be adjusted in order to optimize the activity of the enzyme used. Proteolytic enzymes from microbes, plants and animals can be used for the hydrolysis process of marine proteins in order to develop bioactive peptides. Enzymatic hydrolysis is carried out under optimal conditions to obtain a maximum yield of peptides. For example, α-chymotrypsin, papain, Neutrase and trypsin have been applied to the hydrolysis of tuna dark muscle under optimal pH and temperature conditions for each by Qian *et al*. (2007).

One of the most important factors in producing bioactive peptides with desired functional properties for use as functional materials is their molecular weight (Deeslie & Cheryan, 1981). Therefore, for efficient recovery and in order to obtain bioactive

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peptides with a desired molecular size and functional property, an ultrafiltration membrane system can be used. This system's main advantage is that the molecular-weight distribution of the desired peptide can be controlled by adoption of an appropriate ultrafiltration membrane (Cheryan & Mehaia, 1990). In order to obtain functionally active peptides, it is normal to use three enzymes in order to allow sequential enzymatic digestion. Moreover, it is possible to obtain serial enzymatic digestions in a system using a multistep recycling membrane reactor combined with an ultrafiltration membrane system to separate marine-derived bioactive peptides (Jeon *et al*., 1999). This membrane bioreactor technology has recently emerged for the development of bioactive compounds and has potential for the utilization of marine proteins as value-added neutraceuticals with beneficial health effects.

1.3 HEALTH BENEFITS OF MARINE PEPTIDES

Marine-derived antihypertensive peptides have shown potent antihypertensive effect with angiotensin-I-converting enzyme (ACE)-inhibition activity. The potency of these marine-derived peptides has been expressed as an IC_{50} value, which is the the ACE-inhibitor concentration that inhibits 50% of ACE activity. The inhibition modes of ACE-catalyzed hydrolysis of these antihypertensive peptides have been determined by Lineweaver–Burk plots. Competitive ACE-inhibitory peptides have been reported most frequently (Lee *et al*., 2010; Zhao *et al*., 2009). These inhibitors can bind to the active site in order to block it or to the inhibitor-binding site remote from the active site in order to alter the enzyme conformation such that the substrate no longer binds to the active site. In addition, a noncompetitive mechanism has been observed in some peptides (Qian *et al*., 2007; Suetsuna & Nakano, 2000). Numerous *in vivo* studies of marine-derived antihypertensive peptides in spontaneously hypertensive rats have shown potent ACE-inhibition activity (Fahmi *et al*., 2004; Zhao *et al*., 2009).

Recently, a number of studies have observed that peptides derived from different marine-protein hydrolysates act as potential antioxidants; these have been isolated from marine organisms such as jumbo squid, oyster, blue mussel, hoki, tuna, cod, Pacific hake, capelin, scad, mackerel, Alaska pollock, conger eel, yellow fin sole, yellow stripe trevally and microalgae (Kim & Wijesekara, 2010). The beneficial effects of antioxidant marine bioactive peptides in scavenging free radicals and reactive oxygen species (ROS) and in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation are well known. The inhibition of lipid peroxidation by marine bioactive peptide, isolated from jumbo squid, has been determined by a linoleic acid model system; its activity was much higher than α-tocopherol and was close to the highly active synthetic antioxidant BHT (Mendis *et al*., 2005b).

Marine-derived antimicrobial peptides have described in the hemolymph of many marine invertebrates (Tincu & Taylor, 2004), including the spider crab (Stensvag *et al*., 2008), oyster (Liu *et al*., 2008), American lobster (Battison *et al*., 2008), shrimp (Bartlett *et al*., 2002) and green sea urchin (Li *et al*., 2008). Antibacterial activity has been reported in the hemolymph of the blue crab, *Callinectus sapidus*; it was highly inhibitory to Gram-negative bacteria (Edward *et al*., 1996). Although there are several reports of antibacterial activity in seminal plasma, few antibacterial peptides have been reported in the mud crab, *Scylla serrata* (Jayasankar & Subramonium, 1999).

The anticoagulant marine bioactive peptides have rarely been reported, but have been isolated from marine organisms such as marine echiuroid worm, starfish and blue mussel. Moreover, marine anticoagulant proteins have been purified from blood ark shell and yellow fin sole. The anticoagulant activity of these peptides has been determined by prolongation of activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TP) assays and compared with heparin, the commercial anticoagulant.

Biologically active marine peptides are food-derived peptides that exert a physiological, hormone-like effect beyond their nutritional value, and have a possible role in reducing the risk of cardiovascular diseases by lowering plasma cholesterol level and show anti-cancer activity through a reduction in cell proliferation on human breast-cancer cell lines. Moreover, calcium-binding bioactive peptides derived from pepsin hydrolysates of the marine fish species Alaska pollock (*Theragra chalcogramma*) and hoki frame (*Johnius belengerii*) can be introduced to Asians with lactose indigestion and intolerance as an alternative to dairy products (Kim & Wijesekara, 2010).

1.4 CONCLUSION

Marine-derived proteins and bioactive peptides have potential for use as functional ingredients in neutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of diseases. Moreover, cost-effective and safe drugs can be produced from marine bioactive proteins and peptides. Further studies and clinical trials are needed for these bioactive proteins and peptides.

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2 Bioactive Proteins and Peptides from Macroalgae, Fish, Shellfish and Marine Processing Waste

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2.1 INTRODUCTION

The marine environment, which makes up more than 70% of the earth's surface, represents a vast, relatively untapped resource for biofunctional compound mining. To date, numerous nitrogenous components (protein, peptides and amino acids) with diverse biological activities have been identified in macroalgae, fish and shellfish. Furthermore, macroalgae, fish, shellfish and marine processing waste contain significant quantities of high-quality protein (10–47% (w/w)), which represents a good candidate raw material for further biofunctional peptide mining.

Significant quantities of waste are generated annually from onshore processing of fish and shellfish and during the processing of aquacultured fish and shellfish. For example, in Norway 800 000 metric tonnes of byproducts were generated by fish processing industries in 2009 (Rustad *et al*., 2011). It has been estimated that up to 25% of fish and shellfish can end up as waste. In general, this waste material consists of trimmings, viscera, fins, bones, head, skin, undersized fish and shellfish, damaged shellfish and shells. These waste components contain significant levels of protein with potential biofunctional and technofunctional properties. The mining and subsequent exploitation of marine byproducts/waste streams for components with bioactive properties represents a specific strategy for added-value generation. Furthermore, it provides a solution to the legal restrictions, high costs and environmental problems associated with disposal of such waste material. However, regulations concerning the treatment, storage and transport of fish and shellfish byproducts must be carefully adhered to if these raw materials are to be used as sources of functional food ingredients.

2.2 MACROALGAL, FISH AND SHELLFISH PROTEINS: POTENTIAL SOURCES OF BIOACTIVE HYDROLYSATES AND PEPTIDES

Macroalgal, fish and shellfish proteins represent a vast resource for the mining of novel biofunctional peptides with specific or multifunctional activity. To date, numerous bioactive peptides have been characterised from these protein-rich marine sources. Furthermore,

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given their high structural diversity, proteins produced by macroalgae, fish and shellfish potentially contain a range of as yet undiscovered novel bioactive peptides encrypted within their primary structure(s).

2.2.1 Macroalgal Proteins

Marine macroalgae, or seaweeds as they are commonly known, are a diverse group of marine organisms that have developed complex biochemical pathways to survive in highly competitive marine environments. Macroalgae are classified into three groups according to their pigmentation: Phaeophyceae (brown), Rhodophyta (red) and Chlorophyta (green). To date, seaweed has mainly been exploited as a source of food in Asian countries and as a source of technofunctional polysaccharides (agar, carrageenan and alginates) in the Western world. However, macroalgae have begun to emerge as an alternative dietary source of protein and as a reservoir of potential bioactive proteinaceous components.

The protein content of marine algae varies to a large extent with species and season. In general, the highest levels of protein are found in the red species (maximum 47% (w/w) dry weight), with moderate to low levels found in green (9–26% (w/w) dry weight) and brown (3–15% (w/w) dry weight) phyla (Fleurence, 2004). In some red seaweed, protein levels can be as high as 35% (w/w) (*Palmaria palmata* (dulse)) and 47% (w/w) (*Porphyra tenera* (nori)), while the green alga *Ulva pertusa* (anori) can contain up to 26% (w/w) protein (Fleurence, 2004; Wong & Cheung, 2000). Most brown seaweed, with the exception of *Undaria pinnatifida* (wakame) and *Alaria esculenta*, which contain protein levels in the range 11–24 and 9–20% (w/w) respectively, has a maximum protein content of 15% (w/w) (Burtin, 2003; Fleurence, 2004; Morrissey *et al*., 2001). In general, macroalgal proteins contain all essential amino acids, high levels of glutamic and aspartic acid (7.9–44.0% (w/w)) and low levels of threonine, lysine, tryptophan, histidine and the sulfur amino acids cysteine and methionine (Fleurence, 2004). However, the concentrations of those amino acids found at low levels are in general higher than the levels found in terrestrial plants (Galland-Irmouli *et al*., 1999). Furthermore, some macroalgal species contain high levels of specific amino acids, such as *Palmaria palmata*, glycine; *Porphyra* sp. and *Chondrus crispus*, arginine; *Ulva armoricana*, proline and *Ulva pertusa*, arginine; *Laminaria digitata*, alanine; *Undaria pinnatifida*, alanine, glycine, arginine, leucine, valine, lysine and significant levels of methionine (Augier & Santimone, 1978; Dawczynski *et al*., 2007; Fleurence, 2004; Fleurence *et al*., 1995; Fujiwara-Arasaki *et al*., 1984; Galland-Irmouli *et al*., 1999; Morgan *et al*., 1980; Young & Smith, 1958).

The levels of protein and the corresponding amino acid profiles in macroalgae vary significantly with season. These fluctuations are believed to be linked with a number of variables, such as: geographical location, nutrient supply, environmental conditions (such as light irradiation, water temperature and salinity) and fluctuations in carbohydrate levels (Galland-Irmouli *et al*., 1999; Marinho-Soriano *et al*., 2006; Martínez & Rico, 2002; Morgan & Simpson, 1981a,1981b). Furthermore, variations in seaweed protein amino acid profiles indicate variations in the types of proteins or enzymes present in algal tissues at different times of the year. For example, the storage phycobiliprotein, phycoerythrin, was shown to increase in *Palmaria palmata* during the winter months and early spring—times when seawater nitrogen resources were plentiful—and to decrease during the summer and early autumn months—when seawater nitrogen levels were at their lowest and nitrogen was required for growth (Galland-Irmouli *et al*., 1999).

2.2.2 Fish and Shellfish Proteins

The protein content of raw fish flesh and shellfish can range from 17 to 22% (w/w) and from 7 to 23% (w/w), respectively (Murray & Burt, 2001). Myofibrillar, sarcoplasmic and stroma proteins are the three main groups of fish and shellfish muscle proteins. In general, myofibrillar proteins (structural proteins) account for 65–75% (w/w) of the total protein in fish and shellfish muscles (Venugopal, 2009). These high-salt soluble proteins consist primarily of myosin, actin, tropomyosin, m-protein, alpha-actinin, beta-actinin, c-protein and troponin T, I and C, of which myosin and actin account for 65–78% (w/w) (Vareltzis, 2000). Additionally, invertebrate muscle contains paramyosin, a protein not found in vertebrate myofibrils (Vercruysse *et al*., 2005). The level of paramyosin can vary significantly from one shellfish myofibril to another. Levels in the range 3–19% (w/w) have been reported for scallops, squid and oysters, and 38–48% (w/w) for oyster and clam smooth-muscle adductor muscles (Venugopal, 2009). Furthermore, paramyosin contains significant quantities of glutamic acid, arganine and lysine, and low levels of proline (Belitz *et al*., 2004; Venugopal, 2009).

Sarcoplasmic (water or low-salt buffer soluble) proteins constitute approximately 15–35% (w/w) of the total muscle protein. In general, this protein fraction consists primarily of myoglobin, haemoglobin, cytochrome proteins and a wide variety of endogenous enzymes. However, the level of each protein can vary significantly between species. Some molluscs, for instance, contain no haemoglobin (Belitz *et al*., 2004). Furthermore, compositional differences have been reported between fish- and mammalian-derived myoglobin (Belitz *et al*., 2004). Fish-derived myoglobin was shown to contain cysteine, whereas the mammalian equivalent lacked this residue.

Stroma or connective-tissue proteins consist primarily of collagen and elastin (Belitz *et al*., 2004; Venugopal, 2009). In general, muscle proteins contain approximately 3% (w/w) stroma proteins. However, in some fish, such as shark, ray and skate, stroma proteins can account for 10% (w/w) of total muscle protein. Collagen is the single most abundant protein found in fish species (Kim & Mendis, 2006). It is present in bone, skin, tendons, cartilage and muscle. This triple-helix protein and its partially hydrolysed coiled form, gelatin, contain repeated glycine-proline-hydroxyproline-glycine-X-X amino acid sequences and in addition to glycine, proline and hydroxyproline are rich sources of valine and alanine (Kim & Mendis, 2006; Vercruysse *et al*., 2005). Currently, the collagen and gelatin used in functional foods, cosmetics and pharmaceutical applications come from bovine and porcine sources (Benjakul *et al*., 2009b; Venugopal, 2009). However, marine-derived gelatin is an important alternative source for religious reasons and it combats consumer concerns associated with the emergence of bovine spongiform encephalopathy (BSE) and foot and mouth diseases (Kim & Mendis, 2006).

Waste derived from the fish and shellfish industries contains significant quantities of protein. In general, this waste consists of trimmings, fins, frames, heads, skin and viscera, undersized fish and shellfish, and shellfish that have been rejected during grading, due for example to broken shells or excessive fouling (Kim & Mendis, 2006). It has been estimated that 10–20% (w/w) of total fish protein can be found in waste components (Kristinsson, 2008). In addition to trimmings, frames and heads contain residual meat and thus are a good source of muscle proteins. Furthermore, frames, fins and skin are an excellent source of collagen and gelatin (Venugopal, 2009). Rejected shellfish, shells with attached meat and head components are the main waste products arising from the

shellfish industry. On average, this waste can account for up to 30–45% (w/w) of the unprocessed weight and represents an excellent source of protein.

The conversion of waste into high-value functional ingredients not only can add value to existing marine resources but also provides industry with a method for dealing with the high cost and legal restrictions associated with the disposal of such waste.

2.3 ENZYMATIC HYDROLYSIS OF MACROALGAL, FISH AND SHELLFISH PROCESSING WASTE PROTEINS: BIOACTIVE PROTEIN HYDROLYSATES AND PEPTIDES

Dietary proteins have been shown to contain peptide sequences that can influence physiological parameters in the body. Some of the parameters modified include blood pressure, insulin and glucose homeostasis, blood cholesterol level and immune function. In general, bioactive peptides are 2–20 amino acids in length and are released from the parent protein during gastrointestinal digestion and/or food processing.

While a number of proteolytic enzymes have been characterised in macroalgae, the endogenous proteolytic system appears to be less developed than that in other marine organisms such as fish and shellfish. Peptides are produced from fish and shellfish muscle proteins during normal post mortem storage by the action of inherent proteolytic enzymes such as calcium-activated calpains and lysosomal cathepsins (Bauchart *et al*., 2007). Fermented fish products are a popular food source in many Asian countries. During this fermentation process (up to 18 months) fish proteins are hydrolysed by intrinsic muscle proteinases, digestive-tract proteinases and proteinases produced by halophilic bacteria. This fermentation process can result in products with varying consistencies and qualities. Furthermore, due to legal criteria set for potential hazards such as halophilic pathogen bacteria, high salt content and formation of biogenic amines (histamine), domestic and international marketing of these products is becoming difficult. Furthermore, fish proteins can also be hydrolysed using acid or alkali. However, products produced by chemical hydrolysis have limitations on their use as food ingredients. Enzymatic hydrolysis of macroalgal, fish and shellfish processing waste proteins with proteolytic preparations from plant, animal or microbial sources also has the capability to release an array of peptides with potential biofunctional properties in a highly controlled environment.

To date, fish protein hydrolysates have primarily been used for the production of low-value animal feeds, aquaculture (fish and shellfish) feeds, flavours and ingredients for food supplementation (Thorkelsson & Kristinsson, 2009; Venugopal, 2009). Furthermore, fermented fish sauces and pastes are used as staples or condiments in South East Asian, Scandinavian and Eskimo cultures (Fitzgerald *et al*., 2005). However, a growing body of scientific evidence demonstrates that many marine-derived protein hydrolysates and peptides, including macroalgal, fish and shellfish processing waste byproducts, may play a role in the prevention and management of certain chronic diseases, such as cardiovascular disease (CVD), diabetes, cancer and obesity-related chronic conditions, and thus can be used as functional food ingredients (Harnedy & FitzGerald, 2011; Kim *et al*., 2008; Kim & Wijesekara, 2010). A summary of macroalgae-derived protein hydrolysates and peptides exhibiting biofunctional activity is given in Table 2.1. These include angiotensin-I-converting enzyme (ACE)-inhibitory, antihypertensive, antioxidant, antitumour, antityrosinase, anticoagulant, calcium precipitation-inhibitory, antimutagenic and plasma and hepatic cholesterol-reducing, blood sugar-lowering and superoxide dismutase (SOD)-like activities. Tables 2.2 and 2.3

Table 2.1 Biological activity associated with macroalgal protein hydrolysates and peptides. Adapted from Harnedy & FitzGerald (2011).

Macroalga	Biological activity	Peptide(s) sequence	Reference(s)
Codium fragile	Anticoagulant	-	Athukorala *et al.* (2007)
Costaria costata	ACE inhibitory	-	Lee *et al.* (2005)
Costaria costata	Antioxidant	-	Lee *et al.* (2005)
Costaria costata	Antitumor	-	Lee *et al.* (2005)
Costaria costata	Antityrosinase	-	Lee *et al.* (2005)
Ecklonia cava	ACE inhibitory	-	Cha *et al.* (2006)
Ecklonia cava	Antioxidant	-	Heo *et al.* (2003a,2003b, 2005), Kim *et al.* (2006)
Enteromorpha prolifera	ACE inhibitory	-	Lee *et al.* (2005)
Enteromorpha prolifera	Antioxidant	-	Lee *et al.* (2005)
Enteromorpha prolifera	Antitumor	-	Lee *et al.* (2005)
Enteromorpha prolifera	Antityrosinase	-	Lee *et al.* (2005)
Grateloupia filicina	ACE inhibitory	-	Lee *et al.* (2005)
Grateloupia filicina	Antioxidant	-	Lee *et al.* (2005)
Grateloupia filicina	Antitumor	-	Lee *et al.* (2005)
Grateloupia filicina	Antityrosinase	-	Lee *et al.* (2005)
Hizikia fusiformis	ACE inhibitory	GKY, SVY, SKTY	Suetsuna, 1998b)
Ishige okamurae	Antioxidant	-	Heo & Jeon (2008), Heo *et al.* (2003b, 2005)
Laminaria japonica	Antioxidant	-	Park *et al.* (2009)
Palmaria palmata	Antioxidant	-	Wang *et al.* (2010)
Polysiphonia urceolata	ACE inhibitory	-	He *et al.* (2007)
Porphyra tenera	ACE inhibitory	-	Lee *et al.* (2005)
Porphyra tenera	Antioxidant	-	Lee *et al.* (2005)
Porphyra tenera	Antitumor	-	Lee *et al.* (2005)
Porphyra tenera	Antityrosinase	-	Lee *et al.* (2005)
Porphyra yezoensis	ACE inhibitory	-	Saito & Hagino (2005), Suetsuna (1998a), Suetsuna & Saito (2001)
Porphyra yezoensis	ACE inhibitory	IY, MKY, AKYSY, LRY	Suetsuna (1998a)
Porphyra yezoensis	Antihypertensive	-	Suetsuna & Saito (2001)
Porphyra yezoensis	Antihypertensive	AKYSY	Saito & Hagino (2005)
Porphyra yezoensis	Antimutagenic	-	Suetsuna & Saito (2001)
Porphyra yezoensis	Ca^{2+} precipitation inhibition	-	Suetsuna & Saito (2001)
Porphyra yezoensis	Plasma and hepatic cholesterol lowering	-	Suetsuna & Saito (2001)
Porphyra yezoensis	Improved hepatic function	-	Suetsuna & Saito (2001)
Porphyra yezoensis	Blood sugar reducing	-	Suetsuna & Saito (2001)
Porphyra yezoensis	Antioxidant	-	Suetsuna & Saito (2001)
Porphyra yezoensis	SOD- like activity	-	Suetsuna & Saito (2001)
Sargassum coreanum	Antioxidant	-	Heo *et al.* (2003b, 2005)
Sargassum fullvelum	Antioxidant	-	Heo *et al.* (2003b, 2005)
Sargassum horneri	Antioxidant	-	Heo *et al.* (2003b, 2005), Park *et al.* (2004)
Sargassum horneri	Anticoagulant	-	Athukorala *et al.* (2007)
Sargassum thunbergii	Antioxidant	-	Heo *et al.* (2003b, 2005), Park *et al.* (2005)

(*continued overleaf*)

Table 2.1 (*continued*)

Macroalga	Biological activity	Peptide(s) sequence	Reference(s)
Scytosiphon lomentaria	Antioxidant	-	Ahn *et al.* (2004), Heo *et al.* (2003b, 2005)
Undaria pinnatifida	ACE inhibitory	-	Sato *et al.* (2002a,b), Suetsuna & Nakano (2000)
Undaria pinnatifida	ACE inhibitory	IKY,[a] KY,[a] IK,[a] YKY,[a] KYY,[a] KY,[a] KFY,[a] KF,[a] FY,[a] YNK,[a] NKL,[a] NK,[a] KL,[a] KW, KF, VW, VF, IW, VY	Suetsuna *et al.* (2004), Suetsuna & Nakano (2000)
Undaria pinnatifida	Antihypertensive	-	Kajimoto *et al.* (2002), Sato *et al.* (2002b)
Undaria pinnatifida	Antihypertensive	VY, IY, AW, FY, VW, IW, LW, AIYK, YKYY, KFYG, YNKL, YH, KY, FY, IY	Sato *et al.* (2002a), Suetsuna & Nakano, 2000), Suetsuna *et al.* (2004)
Undaria pinnatifida	Antioxidant	-	Je *et al.* (2009)

[a]Synthetic peptide. All other peptides were purified from macroalgal protein hydrolysates.

summarise bioactive hydrolysates and peptides as reported in the literature from fish waste and shellfish, respectively. Fish waste-derived hydrolysates and peptides display antioxidant, ACE-inhibitory, antihypertensive, anticoagulant and calcium-binding activity. Shellfish-derived hydrolysates and peptides display antioxidant, antihypertensive, antimicrobial, ACE-inhibitory, appetite-suppressant and human immunodeficiency virus (HIV)-1 protease-inhibitory activity.

2.3.1 *In Vitro* and *In Vivo* Cardioprotective Activity

Hypertension, or high blood pressure, is one of the major independent controllable risk factors for CVD. It contributes to an estimated 7.1 million deaths annually, and with increasing longevity worldwide the prevalence of hypertension in people aged 20 years and older is estimated to reach 1.56 billion by 2025 (Kearney *et al*., 2005). Furthermore, individuals with hypertension are at a greater risk of stroke, coronary artery disease, heart failure, vascular disease and chronic renal failure.

Peripheral blood pressure is regulated by a number of biochemical processes in the body. These include the renin–angiotensin system (RAS), the kinin–nitric oxide system, the neutral endopeptidase system and the endothelin-converting enzyme system (FitzGerald *et al*., 2004). Treatment of hypertension and heart failure has focused on therapeutic manipulation of the RAS pathway, and in particular inhibition of ACE (EC 3.4.15.1). This carboxydipeptidase converts the inactive decapeptide angiotensin I to angiotensin II, a potent vasoconstrictor (Meisel *et al*., 2006). Furthermore, ACE inactivates the vasodilator bradykinin. Synthetic ACE inhibitor drugs, such as captopril, enalapril, alcacepril and lisinopril, are widely used for the treatment and prevention of hypertension. Although these synthetic inhibitors are remarkably effective as antihypertensive drugs, they can cause adverse side effects, such as cough, allergic reactions, taste

Table 2.2 Biological activity associated with fish waste-derived protein hydrolysates and peptides. Adapted from Harnedy & FitzGerald (2012). Copyright 2012, with permission from Elsevier.

Common name	Scientific name	Origin	Biological activity	Peptide(s) sequence	Reference(s)
Cod	-	Frame	Antioxidant ACE inhibitory	-	Jeon *et al.* (1999)
Cod	*Gadus macrocephalus*	Skin	Antioxidant ACE inhibitory	TGGGNV TCSP	Ngo *et al.* (2011)
Cod	*Gadus macrocephalus*	Skin	Antioxidant ACE inhibitory	-	Himaya *et al.* (2012)
Herring	*Clupea harengus*	Whole, Body, Head, Gonads	Antioxidant	-	Sathivel *et al.* (2003)
Hoki	*Johnius belengerii*	Skin	Antioxidant	HGPLGPL	Mendis *et al.* (2005b)
Hoki	*Johnius belengerii*	Bone	Calcium binding		Jung *et al.* (2005b)
Hoki	*Johnius belengerii*	Frame	Antioxidant	GSTVPERTHPACPDFN	Kim *et al.* (2007)
Hoki	*Johnius belengerii*	Frame	Ca-binding	VLSGGTTMYASLYAE	Jung & Kim (2007)
Pollack	-	Skin	Antioxidant	GE-(Hyp)-GP-(Hyp)-GP-(Hyp)-GP-(Hyp)-G, GE-(Hyp)-GP-(Hyp)-GP-(Hyp)-GP-(Hyp)-G	Kim *et al.* (2001)
Pollack	*Theragra chalcogramma*	Skin	ACE inhibitory	GPL, GPM	Byun & Kim (2001)
Pollack	*Theragra chalcogramma*	Frame	ACE inhibitory	FGASTRGA	Je *et al.* (2004)
Pollack	*Theragra chalcogramma*	Frame	Antioxidant	LPHSGY	Je *et al.* (2005d)
Pollack	*Theragra chalcogramma*	Frame	Ca-binding	VLSGGTTMAMYTLV	Jung *et al.* (2006a)
Salmon	*Salmo salar* L.	Skin	ACE inhibitory	AP, VR	Gu *et al.* (2011)
Sea Bream	-	Scale	ACE inhibitory	GY, VY, GF, VIY	Fahmi *et al.* (2004)
Snapper	*Priacanthus macracanthus*	Skin	Antioxidant	-	Phanturat *et al.* (2010)
Snapper	*Lutjanus vitta*	Skin	Antioxidant	-	Khantaphant & Benjakul (2008)
Sole	-	Skin	Antioxidant	-	Giménez *et al.* (2009)

(*continued overleaf*)

Table 2.2 (*continued*)

Common name	Scientific name	Origin	Biological activity	Peptide(s) sequence	Reference(s)
Sole	*Limanda aspera*	Frame	Antioxidant	N-terminal RPDFDLEPPY	Jun *et al.* (2004)
Sole	*Limanda aspera*	Frame	Antioxidant	-	Jun *et al.* (2004)
Sole	*Limanda aspera*	Frame	Antihypertensive	MIFPGAGGPEL	Jung *et al.* (2005a)
Sole	*Limanda aspera*	Frame	Anticoagulant	-	Rajapakse *et al.* (2005a)
Tuna	-	Frame	Antioxidant	VKAGFAWTANQQLS	Je *et al.* (2007)
Tuna	-	Frame	Antihypertensive	GDLGKTTTVSNWSPP KYKDTP	Lee *et al.* (2010)
Yellowtail	-	Bone	Antioxidant ACE inhibitory	-	Morimura *et al.* (2002)
Yellowtail	-	Bone	Antioxidant ACE inhibitory	-	Ohba *et al.* (2003)
Yellowtail	-	Scale	Antioxidant ACE inhibitory	-	Ohba *et al.* (2003)

[a]-: not specified

Table 2.3 Biological activity associated with shellfish-derived protein hydrolysates and peptides. Adapted from Harnedy & FitzGerald (2012). Copyright 2012, with permission from Elsevier.

Common name	Scientific name	Origin	Biological activity	Peptide(s) sequence	Reference(s)
Clam	*Meretrix lusoria*	Muscle	ACE inhibitory	VRK YN	Tsai *et al.* (2008)
Krill	*Mesopodopsis orientalis*	Fermented product	Antioxidant	-	Faithong *et al.* (2010)
Krill	-	Muscle	ACE inhibitory	KLKFV	Kawamura *et al.* (1992)
Lobster	*Pleuroncodes planipes*	Shell waste	Anticancer	-	Kannan *et al.* (2011)
Mussel	*Mytilus edulis*	Fermented sauce	Antihypertensive	EVMAGNLYPG*	Je *et al.* (2005c)
Mussel	*Mytilus edulis*	Fermented sauce	Antioxidant	HFGBPFH	Rajapakse *et al.* (2005c)
Prawn	*Penaeus japonicus*	Muscle	Antioxidant	IKK, FKK FIKK	Suetsuna (2000)
Oyster	*Crassostrea gigas*	Fermented sauce	Antihypertensive	Not given (MW 592.9Da)	Je *et al.* (2005b)
Oyster	*Crassostrea gigas*	Muscle	Antimicrobial	-	Liu *et al.* (2008)
Oyster	*Pinctada fucata martencii*	Muscle	ACE inhibitory	FY AW VW GW	Katano *et al.* (2003)
Oyster	*Crassostrea talienwhanensis Crosse*	Muscle	ACE inhibitory	VVYPWTQRF	Wang *et al.* (2008)
Oyster	*Crassostrea gigas*	Muscle	HIV-1 protease inhibitors	LLEYSI LLEYSL	Lee & Maruyama (1998)
Sardine	*Harengula zunasi*	Head, bone, gut	Anti-anaemia	-	Shang-gui *et al.* (2004)
Shrimp	*Penaeus setiferus*	Shell waste	Anticancer	-	Kannan *et al.* (2011)
Shrimp	*Penaeus aztecus*	Head	Appetite suppressant	-	Cudennec *et al.* (2008)
Shrimp	*Acetes chinensis*	Whole shrimp	ACE inhibitory	FCVLRP IFVPAF KPPETV YLLF AFL	Hai-Lun *et al.* (2006)

(*continued overleaf*)

Table 2.3 (*continued*)

Common name	Scientific name	Origin	Biological activity	Peptide(s) sequence	Reference(s)
Shrimp	*Plesionika izumiae Omori*	Whole shrimp	Antihypertensive	VWYHT VW	Nii *et al.* (2008)
Shrimp	*Acetes vulgaris/ Acetes sp.*	Fermented product	Antioxidant	-	Faithong *et al.* (2010)
Shrimp	*Metapenaeus monoceros*	Shell waste	Antioxidant	-	Manni *et al.* (2010)
Shrimp	*Litopenaeus vannamei*	Cephalothorax	ACE inhibitory	-	Benjakul *et al.* (2009a)
Shrimp	*Litopenaeus vannamei*	Cephalothorax	Antioxidant	-	Benjakul *et al.* (2009a)
Squid	*Dosidicus gigas*	Skin	Antioxidant	FDSGPAGVL NGPLQAGQPGER	Mendis *et al.* (2005a)
Squid	*Dosidicus eschrichitii Steenstrup*	Skin	Antioxidant	-	Lin & Li (2006)
Squid	-	Skin	Antioxidant	-	Giménez *et al.* (2009)
Squid	*Dosidicus gigas*	Muscle	Antioxidant	NADFGLNGLEGLA NGLEGLK	Rajapakse *et al.* (2005b)

[a]*: partial sequence of a 6.5 kDa peptide (N-terminal region). b-: not specified

disturbances and skin rashes, among others. Therefore, the use of naturally occurring components as therapeutic agents for the treatment and management of hypertension has gained great interest in recent years. A wide variety of ACE-inhibitory peptides with potent activity *in vitro* have been identified and characterised from macroalgae, shellfish and marine processing waste proteins. The majority of the ACE-inhibitory peptides shown in Tables 2.1, 2.2 and 2.3 have relatively short sequences and low molecular masses. Although the structure–activity relationship of ACE-inhibitory peptides has not yet been fully established, several structural features have been identified which appear to influence the ACE-inhibitory action of peptides. It seems that competitive inhibitory peptides with hydrophobic (aromatic or branched side chain) residues in the last three C-terminal positions preferentially bind to ACE (Murray & FitzGerald, 2007). The majority of ACE-inhibitory di- and tripeptides in Tables 2.1, 2.2 and 2.3 contain Tyr, Phe, Trp, Lys, Val and Ile, and when present Val and Ile are at the N-terminal position. In general, these peptides display ACE IC_{50} values in the range 1.5–45.0 μM. The *in vitro* potency of these peptides is comparable to the potency of the ACE inhibitory peptides derived from other food protein sources (Meisel *et al*., 2006). The potency of an ACE-inhibitory peptide is usually expressed as an IC_{50} value, which is equivalent to the concentration of peptide that inhibits ACE activity by 50%. Careful control and reporting of enzyme units, in addition to reporting of IC_{50} values for positive controls such as captopril, is essential for comparison of the different ACE-inhibitory values that have been reported from different laboratories.

Information obtained from *in vitro* inhibition and simulated gastrointestinal studies is only an indicator of a peptide's potential to act as an hypotensive agent *in vivo*. Usually the first step in investigating the antihypertensive potential of an ACE-inhibitory protein hydrolysate or peptide *in vivo* is an animal study using spontaneously hypertensive rats (SHR). A number of macroalgal, shellfish and marine-derived waste protein hydrolysates and peptides have shown hypotensive effects in SHR. In general, the reduction in systolic blood pressure (SBP) following oral administration of 10 mg/kg shellfish and marine-derived peptides was on average of the order of 25 mmHg compared to controls (Je *et al*., 2005c; Jung *et al*., 2005a; Lee *et al*., 2010; Nii *et al*., 2008). This reduction in SBP was similar to that of captopril. ACE-inhibitory tetrapeptides (YKYY, KFYG and YNKL) derived from the macroalgal species *Undaria pinnatifida* were shown to significantly reduce SBP at an ingestion level of 50 mg/kg (Suetsuna & Nakano, 2000). Furthermore, dipeptides from the same species were shown to reduce SBP by 14 mmHg (VY, FY and IW) and 21 mmHg (IY) following oral administration at a dose of 1 mg/kg (Suetsuna *et al*., 2004; Suetsuna & Nakano, 2000). Protein hydrolysates and peptide fractions derived from oyster proteins, sea bream scale collagen and *Porphyra yezoensis* proteins have also shown antihypertensive activity in SHR (Fahmi *et al*., 2004; Katano *et al*., 2003; Saito & Hagino, 2005; Suetsuna, 1998a; Suetsuna & Saito, 2001; Wang *et al*., 2008). However, variations in sample type, dosage and duration of administration make it difficult to compare these hydrolysates in terms of SBP reduction. While a number of shellfish and marine-derived ACE-inhibitory peptides have shown hypotensive effects in SHR studies, there is a lack of information in relation to human intervention studies. However, macroalgal protein-derived hydrolysates and peptides exhibited promising cardioprotective effects in two human studies. *Undaria pinnatifida* protein hydrolysates in a jelly format were fed at two doses to two groups of mildly hypertensive subjects over an 8-week period. A significant reduction in SBP compared to controls was observed after 8 weeks for the group consuming 300 mg/day and after 6 weeks for the group consuming

500 mg/day (11 mmHg) (Kajimoto *et al*., 2002). In a second study, a significant antihypertensive effect was observed after 8–12 weeks when 1.6 g Nori (*Porphyra yezoensis*) oligopeptides was consumed per day for 12 weeks by subjects with high-normal blood pressure (Kajimoto *et al*., 2004).

Inhibition of blood clot formation and the lowering of plasma and hepatic cholesterol levels can significantly reduce the risk associated with CVD. Blood clotting or coagulation is a complex biochemical pathway triggered to prevent excessive bleeding when a blood vessel is injured (Schaffer *et al*., 1991). Once the injury has healed, the body naturally dissolves the clot. Unwanted clot formation is however detrimental and may result in stroke, heart attack, pulmonary embolism or deep vein thrombosis. Heparin, a highly sulfated polysaccharide, is one of the most common anticoagulant agents used for the treatment of such blood clots (Wijesinghe & Jeon, 2012). However, there are some well-documented side effects, such as excessive bleeding, associated with the clinical use of heparin as an anticoagulant agent. A number of protein hydrolysates and peptides derived from macroalgae and marine-derived waste components exhibit anticoagulant activity (Tables 2.1 and 2.2), (Athukorala *et al*., 2007; Rajapakse *et al*., 2005a). However, to date no research appears to have been carried out to assess the anticoagulant efficacy of these proteinaceous components *in vivo*. In contrast, a number of animal studies have shown that feed containing *Porphyra yezoensis* protein hydrolysates can significantly lower total cholesterol, free cholesterol, triglyceride and phospholipid levels in rats and reduce plasma total cholesterol, triglycerides and low-density lipoprotein (LDL) levels in mice (Suetsuna & Saito, 2001). As with soy protein, where according to an approved health claim an intake of 25 g of soybean per day can lower serum cholesterol levels by 5–10%, these sea vegetable-derived protein hydrolysates may promote cardiovascular health by aiding in the treatment or prevention of hyperlipidaemia or hypercholesterolaemia (Nagaoka, 2006).

2.3.2 Oxidative Stress

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) such as superoxide anion (O^{-2}) and hydroxyl (OH^{-1}) radicals and the inability of a biological system's endogenous antioxidant defence mechanisms (enzymatic and non-enzymatic) to readily detoxify and deal with reactive intermediates. Oxidative stress has implications in many chronic diseases, including heart disease, stroke, arteriosclerosis, diabetes and cancer (Dávalos *et al*., 2004). Furthermore, deterioration in food quality, arising from oxidation of unsaturated fatty acids, is a major concern for the food industry. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and propyl gallate are added to food products to retard lipid oxidation. However, the utilisation of these synthetic antioxidants must be rigorously controlled due to potential adverse side effects (Shahidi & Zhong, 2005). Currently, there is a growing interest in the use of natural antioxidant agents with little or no known harmful health effects to combat oxidative stress and prolong the quality of food.

Macroalgae, as previously mentioned, are a diverse group of marine organisms that have developed complex biochemical pathways to survive in highly competitive marine environments. Harsh environmental factors, such as high irradiation, desiccation, freezing, low temperature, heavy metals and salinity fluctuations, promote the production of excess ROS within macroalgal cells (Cornish & Garbary, 2010). In response, seaweeds synthesise

intrinsic antioxidant components and stimulate the production of endogenous antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx). The range of natural antioxidant components with potential functional food applications produced by seaweed is reviewed elsewhere (Cornish & Garbary, 2010; Tierney *et al*., 2010). Numerous protein hydrolysates generated by direct hydrolysis of macroalgal cells in aqueous solutions via a range of food-grade proteolytic enzyme preparations exhibit antioxidant activity *in vitro* (Table 2.1). The mechanisms by which macroalgal hydrolysates exert antioxidant activity include radical scavenging, transition metal chelating, lipid peroxidation inhibition and H_2O_2-induced DNA damage protective effects.

While the precise relationship between peptide structure and antioxidant activity has not yet been elucidated, the type, position and hydrophobicity of amino acids present in the peptide are thought to play essential roles. Several amino acids, such as histidine, leucine, tyrosine, methionine and cysteine, are generally accepted as antioxidants. These improve radical scavenging activity by donating protons to electron-deficient radicals (Mendis *et al*., 2005a; Sarmadi & Ismail, 2010). As shown in Tables 2.2 and 2.3, several shellfish and fish waste-derived peptides containing such amino acids exhibit radical scavenging activity (Je *et al*., 2005d; Kim *et al*., 2007; Mendis *et al*., 2005a,b; Rajapakse *et al*., 2005b,c). Furthermore, the gelatin-derived peptides HGPLGPL and GE(Hyp)GP(Hyp)GP(Hyp)GP(Hyp)GP(Hyp)G exhibit potent lipid peroxidation-inhibiting activity (Kim *et al*., 2001; Mendis *et al*., 2005b). Both of these peptides contain the characteristic repeating glycine–proline sequence associated with gelatin. Hydrophobic amino acids such as glycine, valine, alanine, proline and hydroxyproline are found in abundance in gelatin, and it is the presence of these amino acids that is believed to exert such a potent lipid peroxidation inhibition effect (Kim & Wijesekara, 2010). Hydrophobic amino acids have high affinity for lipid systems. Therefore, oil-soluble radicals (e.g. hydrophobic peroxyl radicals) generated during oxidative attack of unsaturated fatty acids are believed to be neutralised by hydrophobic amino acid-containing antioxidant peptides. Moreover, two squid muscle-derived peptides containing the same dipeptide (GP) repeating sequence have also been shown to inhibit free radical-mediated oxidation of linoleic acid (Mendis *et al*., 2005a).

Protein hydrolysates and peptides derived from macroalgal and marine waste have also shown antioxidant activity in a number of cell model systems. Hydrolysates generated from *Laminaria japonica* stimulated the production of the endogenous antioxidant enzymes CAT, GPx and glutathione S-transferase (GST)) in H_2O_2-treated cells (Park *et al*., 2009). Furthermore, cellular antioxidative enzyme (SOD, CAT and GPx) levels were significantly elevated in a human hepatoma cell model following induction with a purified antioxidant peptide, HGPLGPL, from a Hoki skin hydrolysate (Mendis *et al*., 2005b).

Oxidative stress may also play a role in neurodegenerative diseases such as Alzheimer's and Parkinson's disease. In particular, reactive quinones in the brain produced by tyrosinase are believed to be linked to Parkinson's disease (Schurink *et al*., 2007). Tyrosinase (EC:1.14.18.1) is a multifunctional copper-containing enzyme present in plant and animal tissues that catalyses the hydroxylation of a monophenol (tyrosine) and the oxidation of o-diphenols to the corresponding o-quinone (Chang, 2009). This is the key enzyme involved in the biosynthesis of the pigment melanin and in the browning that occurs in vegetables and fruit upon bruising or long-term storage. Various dermatological disorders result in the accumulation of excessive levels of epidermal pigmentation and a number of cosmetic preparations contain chemical antityrosinase inhibitors for the treatment

of hyperpigmentation. Partially purified hydrolysates derived from the macroalgae *Enteromorpha prolifera* and *Porphyra tenera* have been reported to inhibit tyrosinase *in vitro* (Lee *et al*., 2005).

A need currently exists to demonstrate the efficacy of antioxidant and antityrosinase protein hydrolysates and peptides derived from macroalgae, shellfish and marine waste in carefully controlled human-intervention studies. Furthermore, studies are needed to assess the potential of marine-derived peptides as natural lipid peroxidation-inhibiting agents or natural biopreservatives for the prevention of browning in food systems.

2.3.3 Other Biofunctionalities

As shown in Tables 2.1, 2.2 and 2.3, protein hydrolysates and peptides with appetite-suppressing, blood sugar-reducing, calcium-binding, calcium precipitation-inhibiting, antimicrobial and HIV protease-inhibiting activity have also been identified in macroalgae, shellfish and marine processing waste.

Cholecystokinin (CCK) is an important hormone which regulates appetite and gastric emptying. Quantification of CCK can be used as a biomarker for assessing satiety within the body. Low-molecular-weight peptides (1.0–1.5 kDa) from shrimp-head protein hydrolysates have been identified as having a significant stimulatory effect on the release of CCK in intestinal endocrine cells (Cudennec *et al*., 2008). Furthermore, *Porphyra yezoensis* protein hydrolysates incorporated in feed aided in the reduction of plasma glucose levels in rats (Suetsuna & Saito, 2001). It is commonly accepted that intervention is needed to combat the rise in obesity and associated chronic diseases. The prevalence of obesity has reached epidemic proportions worldwide. In the USA and UK, levels are predicted to increase by 65 million and 11 million, respectively, by 2030. Chronic obesity-related diseases such as type II diabetes, CVD and cancer are also predicted to escalate within this timeframe. Products containing proteinaceous biofunctional ingredients targeted at prevention may have application in the management of obesity.

Components which bind and solubilise minerals such as calcium can be considered physiologically beneficial in the prevention of osteoporosis, dental caries, hypertension and anaemia (Korhonen & Pihlanto, 2006). Hoki and pollock frame-derived peptides and a semipurified hydrolysate derived from *Porphyra yezoensis* aid in calcium binding and inhibition of calcium precipitation *in vitro* (Jung & Kim, 2007; Jung *et al*., 2005b, 2006a; Suetsuna & Saito, 2001). Furthermore, fish meal rich in hoki frame phosphopeptides has been shown to increase calcium bioavailability in osteoporosis model rats to the same level as a commercially prepared casein oligophosphopeptide preparation (Jung *et al*., 2006b). Potential applications of such marine-derived phosphopeptides include dairy-free functional ingredients for people who are lactose intolerant or for the prevention and management of caries and osteoporosis.

The unstoppable, ongoing emergence of resistant microbes worldwide has rapidly accelerated the search for novel antimicrobial compounds to replace and/or supplement conventional antibiotics. Furthermore, the food industry is looking for additional safe biopreservatives to replace existing synthetic antimicrobial components. Nisin, a polycyclic antibacterial peptide, is an example of such an agent used in the food industry. Marine organisms by their very nature have innate defence mechanisms to deal with water-based pathogens. In general, nitrogenous components produced by macroalgae, fish and shellfish to combat such an attack come in the form of endogenous non-protein-derived peptides (Table 2.4). In most cases, these peptides have similar structural characteristics, being

Table 2.4 Endogenous bioactive peptides derived from marine sources. Adapted from Harnedy & FitzGerald (2012). Copyright 2012, with permission from Elsevier.

Common name	Scientific name	Peptide name	Bioactivity	Reference(s)
Bass	*Morone chrysops/ Morone saxatilis hybrid*	Moronecidin	Antimicrobial Antifungal	Lauth *et al.* (2002)
Bass	*Morone chrysops/ Morone saxatilis hybrid*	Hepcidin	Antimicrobial	Shike *et al.* (2002)
Bluefish	*Oncorhynchus mykiss*	Glutathione	Antioxidant	Jia *et al.* (1996)
Catfish	*Parasilurus asotus*	Parasin	Antimicrobial	Park *et al.* (1998)
Crab	*Carcinus maenas*	Crustin/crustin-like	Antimicrobial	Brockton & Smith (2008), Relf *et al.* (1999), Schnapp *et al.* (1996)
Crab	*Callinectes sapidus*	Callinectin	Antimicrobial	Khoo *et al.* (1999)
Crab	*Tachypleus tridentatus, Limulus polyphemus*	Tachyplesin	Antimicrobial Antifungal Antiviral	Miyata *et al.* (1989), Murakami *et al.* (1991), Ohta *et al.* (1992)
Crab	*Tachypleus tridentatus*	Polyphemusin	Antimicrobial Antifungal Antiviral	Masuda *et al.* (1992), Miyata *et al.* (1989), Nakamura *et al.* (1988), Ohta *et al.* (1992)
Crab	*Tachypleus tridentatus*	Big defensin	Antimicrobial	Saito *et al.* (1995)
Crab	*Tachypleus tridentatus*	Tachycitin	Antimicrobial Antifungal Agglutinating	Kawabata *et al.* (1996)
Crab	*Tachypleus tridentatus*	Tachystatin	Antimicrobial Antifungal Haemolytic	Osaki *et al.* (1999)
Crab	*Hyas araneus*	Arasin	Antimicrobial	Stensvag *et al.* (2008)
Crayfish	*Pacifastacus leniusculus*	Astacidin	Antimicrobial	Lee *et al.* (2003)
Dogfish	*Scyliorhinus canicula*	Insulin	Antidiabetic	Anderson *et al.* (2002)
Flounder	*Pleuronectes americanus*	Pleurocidin	Antimicrobial	Cole *et al.* (1997)
Loach	*Misgurnus anguillicaudatus*	Misgurin	Antimicrobial	Park *et al.* (1997)

(*continued overleaf*)

Table 2.4 (*continued*)

Common name	Scientific name	Peptide name	Bioactivity	Reference(s)
Lobster	*Homarus gammarus*, *Panulirus japonicas*	Crustin/crustin-like	Antimicrobial	Hauton *et al.* (2006, Pisuttharachai *et al.* (2009)
Lobster	*Homarus americanus*	Crustin/crustin-like	Antimicrobial	Battison *et al.* (2008)
Mackerel	*Scomber scombrus*	Glutathione	Antioxidant	Jia *et al.* (1996)
Mackerel	*Scomber austriasicus*	Carnosine	Antioxidant	Fujita & Yoshikawa (2008), Wu *et al.* (2003)
Mackerel	*Scomber austriasicus*	Anserine	Antioxidant	Fujita & Yoshikawa (2008), Wu *et al.* (2003)
Mussel	*Mytilus edulis*	Defensin	Antimicrobial Antifungal	Charlet *et al.* (1996)
Mussel	*Mytilus galloprovincialis*, *Mytilus edulis*	Defensin-like	Antimicrobial Antifungal Antiprotozoan Antiviral	Hubert *et al.* (1996), Mitta *et al.* (1999b, 2000a), Roch *et al.* (2004), Romestand *et al.* (2004)
Mussel	*Mytilus galloprovincialis*, *Mytilus edulis*	Mytilin	Antimicrobial	Charlet *et al.* (1996), Mitta *et al.* (2000b)
Mussel	*Mytilus galloprovincialis.*	Myticins	Antimicrobial	Mitta *et al.* (1999a)
Mussel	*Mytilus edulis*	Mytimycin	Antifungal	Charlet *et al.* (1996)
Mussel	*Mytilus edulis*	MEAP	Anticoagulant	Jung & Kim (2009)
Mussel	*Mytilus edulis*	-	Opioid	Leung & Stefano (1983)
Salmon	*Salmo salar*	Protamine	Antimicrobial Antifungal	Islam *et al.* (1984), Kamal & Motohiro (1986), Uyttendaele & Debevere (1994)
Salmon	-	Calcitonin	Calcium-Binding	Kanis (2002)
Sea hare	*Dolabella auricularia*	Dolastatin	Antiproliferative Opioid Antifungal	Madden *et al.* (2000), Pettit *et al.* (1998), Turner *et al.* (1998), Vaishampayan *et al.* (2000), Woyke *et al.* (2001)
Sea slug	*Pleurobranchus forskalii*	Keenamide A	Anticancer	Wesson & Hamann (1996)
Shark	*Sphyrna lewini*	Insulin	Antidiabetic	Anderson *et al.* (2002)
Shrimp	*Penaeus vanname*, *Penaeus setiferus*	Penaeidin	Antimicrobial Antifungal	Cuthbertson *et al.* (2002) Destoumieux *et al.* (1997, 1999)
Shrimp	*Penaeus vannamei*, *Penaeus stylirostris*	-	Antifungal	Destoumieux-Garzón *et al.* (2001)

Shrimp	*Penaeus monodon*, *Fenneropenaeus indicus*, *Litopenaeus vannamei*, *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*	Crustin/ crustin-like	Antimicrobial	Amparyup *et al.* (2008), Antony *et al.* (2010), Bartlett *et al.* (2002), Rattanachai *et al.* (2004), Sun *et al.* (2010, Supungul *et al.* (2008), Vargas-Albores *et al.* (2004), Vatanavicharn *et al.* (2009), Zhang *et al.* (2007)
Soapfish	*Grammistes sexlineatus*, *Pogonoperca punctata*	Grammistin	Antimicrobial Haemolytic Ichthyotoxic	Shiomi *et al.* (2000, 2001), Sugiyama *et al.* (2005), Yokota *et al.* (2001)
Sole	*Pardachirus marmoratus*	Pardaxin	Antimicrobial Haemolytic	Oren & Shai (1996)
Trout	*Pomatomus saltatrix*	Glutathione	Antioxidant	Bauchart *et al.* (2007)
-	*Bryopsin* sp.	Kahalalide F	Antitumor Antiviral Antibacterial Antifungal	Ciruelos *et al.* (2002), El Sayed *et al.* (2000), Faircloth *et al.* (2000), Galan *et al.* (2006), Hamann & Scheuer (1993), Hamann *et al.* (1996), Martín-Algarra *et al.* (2009), Pardo *et al.* (2008), Paz-Ares *et al.* (2006), Provencio *et al.* (2006), Suárez *et al.* (2003)
-	*Bryopsin* sp.	Kahalalide A	Antibacterial	El Sayed *et al.* (2000)
-	*Galaxaura filamentous*	Galaxamide (1)	Antiproliferative	Xu *et al.* (2008)
-	Red macroalga	Almazole D	Antibacterial	N'Diaye *et al.* (1996)
-	Red macroalgae	GABA analog	Neurotransmitter	Morse (1991)
-	*Ulva fasciata*	Carnosine	Antioxidant	Shiu & Lee (2005)
-	*Ulva fasciata*	Glutathione	Antioxidant	Shiu & Lee (2005)
-	*Ulva* sp.	SECMA 1	Mitogenic	Ennamany *et al.* (1998)

- : not specified

cationic and amphiphilic. However, a novel cysteine-rich antimicrobial peptide, *CgPep33*, exhibiting activity against Gram-positive and Gram-negative bacteria and fungi, has been isolated from enzymatic hydrolysates of *Crassostrea gigas* oyster protein (Liu *et al*., 2008). To date, no *in vivo* or food-stability studies have been performed to determine the therapeutic and food-preservation efficacy of marine-derived antimicrobial peptides.

Finally, HIV-1 protease-inhibitory peptides, LLEYSI and LLEYSL, have been identified from thermolysin digests of oyster proteins (Lee & Maruyama, 1998). These peptides have potential application as antiviral agents for the control of HIV-1 infection by inhibiting HIV-1 protease, an enzyme central to retrovirus HIV-1 replication.

2.4 ENDOGENOUS BIOACTIVE PEPTIDES FROM MACROALGAE, FISH AND SHELLFISH

Several linear, cyclic peptides, depsipeptides and peptide derivatives have been characterised from marine resources including macroalgae, fish and shellfish. In addition to the antimicrobial peptides mentioned in Section 2.3.3, peptides with antioxidant, mitogenic, anticancer, antiproliferative, antiviral, antiprotozoan, calcium-binding, cytotoxic, opioid, antidiabetic, anticoagulant, haemolytic and agglutinating activity have also been reported. The specific details concerning these peptides are summarised in Table 2.4. Carnosine, anserine and glutathione are antioxidant peptides found in high quantities in fish muscle (Bragadóttir, 2001). Furthermore, the histidyl dipeptide carnosine and the tripeptide glutathione have been identified in macroalgae. The latter peptide is involved in the inherent enzymatic antioxidant defence system (ascorbate–glutathione cycle) (Fleurence, 2004; Shiu & Lee, 2005).

Galaxamide 1 and Kahalalide F are two endogenous peptides produced by macroalgae that exhibit potential anticancer activity. Galaxamide 1, a peptide isolated from *Galaxaura filamentous*, has shown antiproliferative activity against human renal-cell carcinoma GRC-1 and human hepatocellular carcinoma HepG2 cell lines (Xu *et al*., 2008). Furthermore, Kahalalide F, a cyclic depsipeptide isolated from the mollusc *Elysia rufescens* but originated from its foodstuff, the green alga *Bryopsin* sp., has been shown to display potent cytotoxic activity against cell lines from solid tumours, including prostate, breast and colon carcinomas, neuroblastoma, chondrosarcoma and osteosarcoma (Hamann & Scheuer, 1993; Hamann *et al*., 1996; Suárez *et al*., 2003). The anticancer activity of this cyclic depsipeptide has been assessed in three phase II clinical trials. While the compound was show to stabilise the tumours, the studies were discontinued after the first stage due to lack of significant antitumour activity (Galan *et al*., 2006; Martín-Algarra *et al*., 2009; Paz-Ares *et al*., 2006; Provencio *et al*., 2006). However, in one of the trials an advanced lung-cancer tumour in one of the patients showed a partial response to Kahalalide F (Provencio *et al*., 2006).

2.5 BIOACTIVE PROTEINS FROM MACROALGAE, FISH AND SHELLFISH

Poor permeability through biological membranes due to molecular size, physical and chemical instability, degradation by intrinsic proteolytic enzymes, aggregation, adsorption

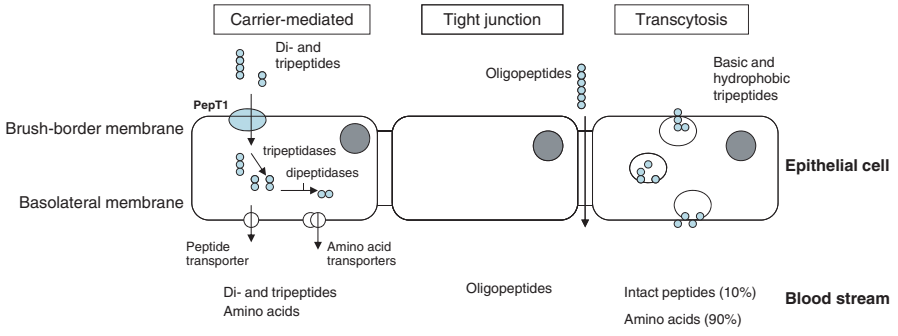

Fig. 2.1 Peptide transport routes across intestinal cells.

and immunogenicity are some of the limitations that affect the bioavailability of bioactive proteins and peptides. In order for a bioactive component to exert a modulating effect *in vivo*, it needs to reach its target site in an intact form. In general, peptides with two to six amino acids are more readily absorbed across the gastrointestinal tract than are whole proteins (Grimble *et al*., 1986). On ingestion, proteins and peptides may be hydrolysed by gastrointestinal enzymes such as pepsin, trypsin and chymotrypsin. Peptides can then be further degraded by brush-border peptidases and intracellular peptidases during their passage across intestinal epithelial cells into the bloodstream. There are three major routes by which this takes place (Fig. 2.1): (1) PepT1 carrier-mediated transport; (2) tight-junction paracellular diffusion; and (3) endocytosis–exocytosis (Young & Mine, 2009). PepT1 specifically transports di- and tripeptides from the apical side of the epithelial membrane into the cell by H^+ coupling. Once inside the cell, peptides may be degraded to amino acids by cytoplasmic peptidases and amino acids are transported across the basolateral membrane into the bloodstream by amino acid transporters. In some cases, peptides may remain intact if their structural characteristics render them resistant to attack by cytoplasmic peptidases. In paracellular diffusion, oligopeptides are transported passively via pores at the tight junctions in an intact form. Peptides can also be transported in and out of the cell by endocytosis and exocytosis. This route involves engulfing the peptide at the apical side of the epithelial membrane inside a vesicle and transporting it to the basolateral membrane, where the peptide is expelled. However, peptides can be hydrolysed to amino acids within this vesicle. In general, basic and hydrophobic peptides are transported by this method (Young & Mine, 2009). PepT1 carrier-mediated transport and tight-junction paracellular diffusion offer the best mode of transport of intact peptides across intestinal epithelial cells into the bloodstream. Furthermore, peptides can be degraded in the blood by serum peptidases. As a result, peptides, and more specifically proteins, may not reach their target site in an intact form.

However, some marine-derived proteins have shown biological activity in animal models following oral administration. It may be that these active agents are broken down into fragments. Such protein molecules include lectins from the macroalgal species *Eucheuma* sp., *Bryothamnion triquetrum*, *Bryothamnion seaforthii* and *Amansia multifida*. Lectins are a structurally diverse group of carbohydrate-binding proteins found in a wide range of organisms (Hori *et al*., 2000). They are involved in a number of biological processes, such as host–pathogen interactions, cell–cell communication, induction of apoptosis, cancer metastasis and differentiation (Calvete *et al*., 2000; Ziółkowska & Wlodawer, 2006). ESA-2, a lectin isolated and characterised from *Eucheuma serra*,

has been shown to suppress colonic carcinogenesis in mice following oral administration (Hori *et al*., 2007). Furthermore, lectins from this species of macroalga have been shown to exhibit strong mitogenic activity against mouse and human lymphocytes and are reported to be cytotoxic against several cancer cell lines, such as colon cancer (Colo201) and cervical cancer (HeLa) cells—they inhibited the growth of 35 human cancer cell lines (Hori *et al*., 2007; Kawakubo *et al*., 1997, 1999; Sugahara *et al*., 2001). Lectins with mitogenic activity have also been isolated from *Soleria robusta* (Hori *et al*., 1988). These monomeric glycoproteins have been shown to be active against mouse spleen lymphocytes and inhibited the growth of mouse leukaemia cells L1210 and mouse FM3A tumour cells (Hori *et al*., 1988). Lectins from several species of marine alga—*Bryothamnion triquetrum*, *Bryothamnion seaforthii* and *Amansia multifida*—have shown acetic acid-induced abdominal contraction inhibitory activity in Swiss mice (Neves *et al*., 2007; Viana *et al*., 2002). Other bioactive properties exhibited by macroalgal lectins include antibiotic, anti-inflammatory, antiadhesion, anti-HIV activity and human platelet aggregation inhibition activities (Harnedy & FitzGerald, 2011).

The phycobiliproteins (phycoerythrin, phycocyanin, allophycocyanin and phycoerythrocyanin) are another group of interesting bioactive proteins found in red algae. These molecules are highly fluorescent compounds. As a result, phycobiliproteins, in particular phycoerythrin, have wide-ranging applications in biotechnology, being utilised in fluorescent immunoassays, fluorescent immunohistochemistry assays, biomolecule (protein, antibody, nucleic acid) labelling and fluorescent microscopy (Aneiros & Garateix, 2004; Sekar & Chandramohan, 2008). In addition, phycobiliproteins are used as natural colourants for foods such as chewing gum and dairy products and for cosmetics such as lipsticks and eyeliners (Sekar & Chandramohan, 2008). While most research has been performed with phycobiliproteins from the blue-green microalga *Spirulina* and the unicellular red alga *Porphyridium*, it is possible that phycobiliproteins from red macroalgae may have similar activities. Phycobiliproteins from *Spirulina* and *Porphyridium* exhibit antioxidant, anti-inflammatory, neuroprotective, hypocholesterolaemic, hepatoprotective, antiviral, antitumour, liver-protecting, atherosclerosis-treatment, serum lipid-reducing and lipase-inhibition activities (Harnedy & FitzGerald, 2011; Sekar & Chandramohan, 2008). Bioactive proteins with anticoagulant, antimicrobial, immunostimulatory, anticancer and hypocholesterolaemic properties have also been identified in fish and shellfish (Harnedy & FitzGerald, 2011; Jung *et al*., 2002; McFadden *et al*., 2003; Nagashima *et al*., 2003; Patat *et al*., 2004; Riggs *et al*., 2002; Smith *et al*., 2000). The majority of these proteins exhibit antimicrobial activity and are believed to be produced as part of an organism's defence mechanism against microbial attack. However, if marine-derived bioactive proteins are to be incorporated into foods as biofunctional ingredients, a critical requirement exists that the biological activity of these proteinaceous components be assessed in human studies.

2.6 COMMERCIAL PRODUCTS CONTAINING MARINE-DERIVED BIOACTIVE PROTEIN HYDROLYSATES AND PEPTIDES

Substantial scientific validation of the health-promoting effects of biofunctional ingredients is required before a health claim can be made that has European Food Safety Authority (EFSA), Food and Drug Administration (FDA) and Foods for Specified Health Use (FOSHU) approval. Numerous products containing fish-protein hydrolysates/peptides

as functional ingredients have been given FOSHU status in Japan (Table 2.5), including Valtyron® and Lapis Support™, both of which are marketed as having hypotensive effects. The 'Sardine Peptide product' Valtyron®, generated using a commercially available food-grade alkaline protease from *Bacillus licheniformis*, has been incorporated into 33 different products, including soft drinks, jelly, powdered soup and dietary supplements (EFSA Panel on Dietetic Products, 2010). These products, in particular the dipeptide VY, have been shown to significantly reduce SBP in mildly hypertensive patients (Kawasaki *et al*., 2000). Furthermore, Valtyron® has recently been passed as safe by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) for use as a novel food ingredient at a level of 0.6 g/serving (EFSA Panel on Dietetic Products, 2010).

A hypotensive 'Peptide Soup' product generated from katsuobushi (bonito) muscle has also been awarded FOSHU status. The bioactive peptide therein, LKPNM, has been shown to exhibit antihypertensive effects in mildly hypertensive subjects (Fujita *et al*., 2001; Fujita & Yoshikawa, 2008). This product is commercially available in Japan in beverage format (soup and tea), as a powdered ingredient and in tablet format, in which it is marketed as Peptide ACE 3000 (http://www.nippon-sapuri.com/english/index.html, last accessed 08/10/12). Furthermore, the tablet format is sold in the USA under the trade names Vasotensin™ and PeptACE™ and in Canada as Levenorm™ (Thorkelsson & Kristinsson, 2009). Two other marine-derived peptides with FOSHU-approved anti-hypertensive claims are the Wakame peptide (YNKL), which is sold as a peptide jelly, and the Nori peptide (AKYSY), which is sold under the trade name Peptide Nori S (http://en.item.rakuten.com/kenkoex/noripepu30/, last accessed 08/10/12).

Several marine-derived proteinaceous components are sold in Europe and North America as food supplements without approved health claims. These include Stabilium® 200, Protizen®, Collagen HM, Glycollagen®, Marine Cartilage Powder, Protein M^+, PeptiBal™, AntiStress 24, Nutripeptin™, Seacure® and Fortidium Liquamen®. A blue ling autolysate Stabilium® 200 and the fish hydrolysates Protizen® and AntiStress 24 claim to have relaxing effects (Crocq *et al*., 1980; Guerard *et al*., 2010; Le Poncin & Lamproglou, 1996; Thorkelsson & Kristinsson, 2009). In addition to Protizen®, Copalis Sea Solutions markets products containing marine-derived collagen hydrolysates that aid joint health. Collagen HM, which contains fish skin collagen-derived oligopeptides (<3.6 kDa), is recommended as a food supplement for the promotion of cartilage, bone and skin regeneration, while Glycollagen®, Marine Cartilage Powder and Protein M^+ are sold as supplements to soothe joint pain. Glycollagen®, Marine Cartilage Powder and Protein M^+ also contain chondroitin sulfate and glucosamine. These two mucopolysaccharides have been shown to aid joint regeneration *in vivo* (Reginster *et al*., 2001; Wildi *et al*., 2011). PeptiBal™, an ultrafiltrated shark-protein hydrolysate product, has been reported to have immunomodulatory effects in human clinical trials. A statistically significant increase in IgA production was observed in a randomised double-blind placebo-controlled study (Boutin *et al*., 2012). Nutripeptin™, Seacure® and Fortidium Liquamen® are also sold as food supplements. Nutripeptin™ is marketed as having postprandial blood glucose-lowering activity, Seacure® is sold as a supplement for the improvement of gastrointestinal health and Fortidium Liquamen® is commercialised as having multifunctional effects including antioxidant, antistress and glycaemic index-lowering activities (Guerard *et al*., 2010).

A number of factors need to be taken into account when producing marine-derived protein hydrolysates/peptides at an industrial scale. Two strategies can be adopted for the hydrolysis of marine proteins: hydrolysis of fish and shellfish muscle and marine

Table 2.5 Commercially available marine protein, protein hydrolysate and peptide products. Adapted from Harnedy & FitzGerald (2012). Copyright 2012, with permission from Elsevier.

Product	Activity	Source	Manufacturer
PeptACE™	Antihypertensive	Bonito peptides	Natural Factors Nutritional Products Ltd, Canada
Vasotensin®	Antihypertensive	Bonito peptides	Metagenics, USA
Levenorm®	Antihypertensive	Bonito peptides	Ocean Nutrition Canada Ltd, Canada
Peptide ACE 3000	Antihypertensive	Bonito peptides	Nippon Supplement Inc., Japan
Lapis Support	Antihypertensive	Sardine peptides	Tokiwa Yakuhin Co. Ltd, Japan
Valtyron®	Antihypertensive	Sardine peptides	Senmi Ekisu Co. Ltd, Japan
Wakame jelly peptide	Antihypertensive	*Undaria pinnatifida*	Riken Vitamin, Japan
Peptide Nori S	Antihypertensive	*Porphyra yezoensis*	-, Japan
Stabilium® 200	Relaxing	Fish autolysate	Yalacta, France
AntiStress 24	Relaxing	Fish hydrolysate	Forté Pharma Laboratories, France
Protizen®	Relaxing	Fish hydrolysate	Copalis Sea Solutions, France
Collagen HM	Improves joint health	Fish skin collagen hydrolysate	Copalis Sea Solutions, France
HyadroColla™ Fish hydrolysed collagen	Antiageing	Tilapia collagen hydrolysate	Fenchem, China
Glycollagen®[a]	Soothes joint pain	Skate cartilage collagen hydrolysate	Copalis Sea Solutions, France
Marine cartilage powder[b]	Soothes joint pain	Skate cartilage collagen hydrolysate	Copalis Sea Solutions, France
Protein M+[b]	Soothes joint pain	Fish collagen hydrolysate	Copalis Sea Solutions, France
PeptiBal™	Immunomodulatory	Shark protein hydrolysate	InnoVactiv Inc., Canada
Nutripeptin™	Lowers glycaemic index	Cod hydrolysates	Nutrimarine Life Science AS, Norway
Seacure®	Improves gastrointestinal health	Pacific whiting hydrolysate	Proper Nutrition, USA
Fortidium Liquamen®[b]	Antioxidant, lowers glycaemic index, antistress	Fish autolysate	Biothalassol, France

[a]Also contains chondroitin sulfate and glucosamine.
[b]Also contains fish oil and vegetable oil.

waste directly with food-grade proteolytic enzymes, such as Alcalase®, Neutrase®, Flavourzyme® and Protamex®, and pre-extraction of protein prior to hydrolysis (Giménez *et al*., 2009; Guerard *et al*., 2010; Hai-Lun *et al*., 2006). Membrane-separation techniques such as ultrafiltration and nanofiltration seem to provide the most suitable industrially relevant technology for the enrichment of peptides within specific molecular-weight ranges and are currently used for the industrial production of ingredients containing bioactive peptides (Korhonen, 2009; Korhonen & Pihlanto, 2007). Membranes with molecular mass cut-off values in the range 1–10 kDa are primarily used for the fractionation of peptides (Hai-Lun *et al*., 2006; Je *et al*., 2005a; Jeon *et al*., 1999; Rajapakse *et al*., 2005b).

Furthermore, electromembrane filtration, which involves the use of charged membranes and membrane bioreactor technology combining enzymatic hydrolysis of marine proteins and peptide separation by ultrafiltration, is being considered as a potential method for the generation and fractionation of marine-derived bioactive peptides (Kim & Wijesekara, 2010). In general, *in vitro* bioactivity assay-directed purification is the approach taken to fractionate and purify peptides with specific biofunctional activities. However, if specific peptides are to be purified, specific chromatographic techniques may need to be coupled with membrane processing (Pouliot *et al*., 2006). The extent to which a peptide needs to be purified is dependent on the potency of the bioactive component, and on an industrial level the cost of purifying specific bioactive peptides must be weighed against the value of the purified or semipurified product. As a result, high-cost semi- and preparative-scale chromatography may only be utilised if highly purified peptides are required for commercialisation (Pouliot *et al*., 2006).

2.7 CONCLUSION

Marine-derived waste, fish and shellfish are a relatively untapped source of new bioactive proteinaceous compounds, and more efforts must be made to fully exploit their potential for use and for delivery to consumers in food products. However, in order for marine bioactive peptides or protein hydrolysates to be utilised as health-promoting functional food ingredients, aspects such as large-scale production, compatibility with different food matrices, gastrointestinal stability, bioavailability and long-term stability must be addressed. Carefully controlled human-intervention studies are needed to demonstrate the efficacy of bioactive components *in vivo*. Furthermore, there is a need for more detailed understanding of the mechanisms by which different peptides/hydrolysates may mediate their physiological effects. In order to fully understand the relationship between exposure in the body and physiological effect, metabolomic and nutrikinetic studies may have to be performed. Finally, marketing of bioactive health-promoting functional ingredients requires scientific validation before a health claim can be made with European Food Safety Authority (EFSA), Food and Drug Administration (FDA) and Foods for Specified Health Use (FOSHU) approval.

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3 Lectins with Varying Specificity and Biological Activity from Marine Bivalves

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3.1 INTRODUCTION

3.1.1 Bivalves

Bivalves are one of the most common classes in the modern fauna under Phylum: Mollusca. The current estimated total number of bivalve species is approximately 9200. These species are placed within 1260 genera and 106 families. A large number of bivalves, such as oysters, scallops, mussels and clams, are found to be present in marine and fresh water. They are taxonomically categorized in five major subdivisions: (1) *Protobranchia* (a small group of deep-sea clams); (2) *Pteriomorphia* (mussels, scallops and oysters); (3) *Anomalodesmata* (a small group of marine clams); (4) *Palaeco heterodonta* (freshwater mussels); and (5) *Heterodonta* (most marine clams).

3.1.1.1 Mussels

'Mussel' is the common name used for members of several families of clams or bivalvia mollasca from seawater and freshwater habitats. The word 'mussel' is also used for many freshwater bivalves, including freshwater pearl mussels. Freshwater species inhabit lakes, ponds, rivers, creeks and canals all over the world, except in the polar region. Marine mussels have their habitat in different regions of sea. They are largely present in the low and mid intertidal zone in temperate seas globally. Other species of marine mussel live in tropical intertidal areas, but not in huge numbers as in the temperate zones. Certain species of marine mussel prefer to inhabit quiet bays, while some prefer sea surf. Some species colonize hydrothermal depths of sea. The South African white mussel burrows into sandy beaches. Systematists type blue mussels (Fig. 3.1) based on three closely related taxa: *Mytilus edulis*, *Mytilus galloprovincialis* and *Mytilus tossulus*. *M. edulis* are abundant in both coasts of the North Atlantic, *M. galloprovincialis* cover the Mediterranean (they are also known as the Mediterranean mussel) and *M. tossulus* is the main native intertidal mussel in the North Pacific; in North America it is found from California to Alaska, and in Asia from Hokkaido northwards. It is also abundant in the Baltic Sea in Europe. Green mussels (*Perna viridis*) are coastal bivalves, typically, occurring at depths of less than 10 m. The native range of green mussel broadly encompasses the Asia-Pacific

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Fig. 3.1 Blue mussel.

and Indo-Pacific regions, including parts of coastal Australia, Japan, the Caribbean and North and South America. Atlantic and Caribbean occurrences of *P. viridis* have been reported from Trinidad and Tobago, Jamaica and Venezuela. Horse mussels, *Modiolus modiolus*—chiefly subtidal in deeper waters—are widely distributed throughout British coasts. Their beds are extensively found on northern and western coasts but are absent south of the Irish Sea and the Humber Estuary. They inhabit soft sediments or coarse grounds or are found attached to hard-forming clumps or extensive beds or reefs.

3.1.1.2 Oysters

The word 'oyster' is used as a common name for a number of distinct groups of bivalve mollusks that live in marine or brackish habitats. They are available across a large area in the USA, including the bay and estuaries along the coast of the Gulf of Mexico from Apalachicola, Florida to the coast of Galveston, Texas in the west. Large beds of edible oysters are also found in Japan and Australia. The Pacific oyster, *Crassostrea gigas*, an estuarian species, is also found in intertidal and subtidal zones. These prefer to stay on hard or rocky surfaces in shallow waters up to 40 m deep, but are also found in muddy and sandy beaches. They are located from Japan to the west coast of the USA. The eastern oyster, *Crassostrea virginica*, is abundant in shallow bays, lagoons and estuaries. It is distributed from Canada to Mexico. The European flat oyster, *Ostrea edulis*, a native of Europe, occurs naturally from Norway to Morocco in the northeastern Atlantic and in the whole of the Mediterranean basin. The flat oyster, *Ostrea chilensis*, is found in two regional populations separated by 7400 km of uninterrupted South Pacific Ocean. It is located throughout the nearshore water of New Zealand to depths of 550 m and in the shallow subtidal zone of southern Chile (Foighil *et al*., 1999). The pearl oyster of the genus *Pinctada* is widely distributed across the world. It is found in seas of the tropical and subtropical regions. *Pinctada fucata* inhabit temperate, subtropical and tropical coral

reefs and are widely distributed in the Indo-Pacific area, down to northern Australia, throughout the equator and extending up to the southernmost region of Japan. Although a number of species of pearl oyster have been identified, only a few have been known to produce pearls of good quality and commercial value. There are three species of *Pinctada*: *P. maxima*, *P. magaritifera* and *P. fucata*. The latter is located in the Red Sea, Persian Gulf, India, China, Korea, Japan, Venezuela and the Western Pacific Ocean. Although six species of pearl oyster occur in Indian waters, only *P. fucata* grows in pearl fisheries in the Gulf of Mannar and Gulf of Kutch.

3.1.1.3 Clams

'Clam' is one of the common names of various species of marine bivalve mollusk, but it is not used as a general term to cover edible clams that live and burrow in sand or mud. *Tridacna maxima* (Fig. 3.2) refers to the small range of all giant clam species. These are found in the oceans surrounding East Africa, South East Asia and the Pacific islands. The giant clam *Tridacna gigas* is the largest living bivalve mollusk and is an endangered species. It is native to the shallow coral reefs of the South Pacific and Indian Oceans. It is also found off the shores of the Philippines and in the South China Sea, in the coral reefs of Malaysian Borneo. *T. gigas* lives in coral sand and can be seen at depths of 20 m. Its range covers the Indo-Pacific, but populations are gradually becoming extinct in many areas where it was once dominant, due to extensive fishing—its meat is a delicacy in Japan, France, South East Asia and many Pacific islands, and its shell is used decoratively. *Tridacna corcea* is a species of bivalve in the Tridacnidae family. It is located in Australia, Indonesia, Japan, Malaysia, the Northern Mariana Islands, Papua New Guinea, the Philippines, Singapore, Thailand and Vietnum. The southern giant clam, *Tridacna derasa*, is a an extraordinarily large saltwater clam. It is distributed around Australia, Fiji, Indonesia, Palau, Papua New Guinea, the Phillipines, Tonga and Vietnam, found on the outer edges of reefs at depths between 4 and 10 m.

3.1.1.4 Scallops

A scallop is another bivalve mollusk of the family Pectinidae. These are regarded as cosmopolitan bivalves, found in all the world's oceans. Atlantic bay scallops,

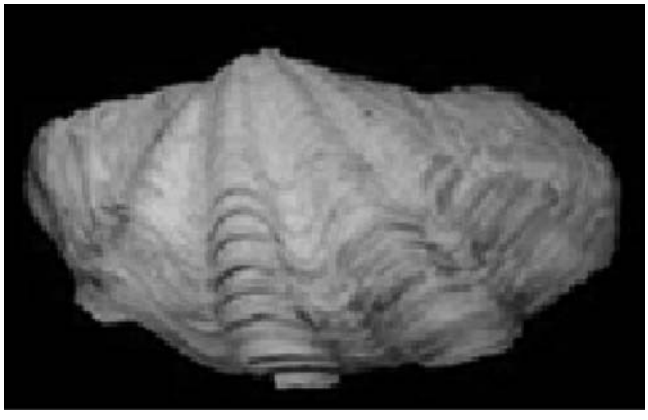

Fig. 3.2 *Tridacna maxima.*

Argopecten irradians, dwell in bays of high salinity where sea grass meadow is common. They are distributed from Galveston down to the Laguna Madre along the Texas coast.

3.1.1.5 Cockles

'Cockle' is the common name for a group of small edible saltwater clams—marine bivalve mollusks in the family Cardiidae—found throughout the world; there are more than 200 living species. Various species of cockle live in sandy beaches. The common cockle, *Cerastoderma edule*, is widely distributed along the coastline of Northern Europe, ranging west to Ireland. The dog cockle, *Glycymeris glycymeris*, has a similar range and habitat to the common cockle. The blood cockle, *Anadara granosa*, in the family Arcidae, is extensively cultured from Southern Korea to Malaysia.

3.1.2 Innate Immunity of Invertebrates

A fundamental aspect of immunity lies in the ability to distinguish self from non-self. Unlike vertebrates, invertebrates lack antibody-mediated humoral immunity in their system. However, they possess innate immunity: by virtue of the innate immune response, they are believed to possess host-defense mechanisms which protect them from various pathogenic infections in the aquatic environment (Beutler *et al*., 2004; Hoebe *et al*., 2004; Locker *et al*., 2004). The unique feature of the innate immune response is the ability to recognize highly conserved structures called pathogen-associated molecular patterns (PAMP) found in a large group of organisms. In the innate immune system there are receptor molecules that recognize PAMPs, known as pathogen-recognizing receptors (PRRs). These are a group of proteins, also called pattern-recognition proteins (PRPs) or pattern-recognition molecules (PRMs), that trigger protective immune responses (Iwanaga & Lee, 2005). There are six PRPs in invertebrates, including bivalves: peptidoglycan-binding protein, β-1,3-glucan-binding and lipopolysaccharide-binding protein, toll-like receptor, thioester-containing protein, C-type lectin and galectin. The C-type lectins are dominant in invertebrates, including bivalves. They possess at least one carbohydrate-recognition domain (CRD), by which they recognize oligosaccharide structures on the cell surface of bacteria and manifest several biological events: cell adhesion, signal transduction, clearance of pathogens by phagocytosis (Kondo *et al*., 1992), cytotoxic effects (Ma *et al*., 1999), activation of the prophenol oxidase system (proPO) (Yu *et al*., 2000) and nodule formation (Koizumi *et al*., 1999).

3.1.3 Importance of Bivalve Mollusks

Study of bivalve lectins is essential to gaining a better understanding of the immune mechanism of bivalve mollusks, which can provide insight into disease control in mollusk aquaculture, the maintainence of a healthy environment and economic growth.

Blue mussels are filter feeders and play a vital role in estuaries by removing bacteria and toxins. *Mytilus edulis* is commonly harvested for food throughout the world, from both wild and farmed sources. The pearl oyster has long been a source of pearls worlwide. However, natural pearls are extremely rare, found in only 1 in 10 000 mussels. The shells of native Tennessee mussels are ideal for the lucrative cultured pearls industry.

In comparison to research on C-type lectins in insects and crustaceans involved in the immune defense against microbial infection, study of C-type lectins in bivalves is not so common. The Zhikong scallop, *C. farreri*, is an important bivalve cultivated on the north

coast of China due to its market economy. It has a proven innate immune response against various Gram-negative and Gram-positive bacteria. Song and his groups have detected C-type lectin genes (Cflec-1, Cflec-2, Cflec- 3 and Cflec-4) from *C. farreri* (Wang *et al*., 2007; Yang *et al*., 2011; Zheng *et al*., 2008, 2009 a,b).

Marine resources, particularly cyanobacteria, algae, invertebrate animals and fish, are considered a great treasure for the production of a variety of potential bioactive compounds. They are mostly natural organic compounds, such as fatty acids, polysaccharides, proteins, peptides and enzymes. Marine natural products, due to their pharmacological functions, have found application in the pharmaceutical industry in the development of new antiviral, antimicrobial, anticancer, anti-human immunodeficiency virus (HIV) and anti-inflammatory drugs (Smith *et al*., 2010; Vo & Kim, 2010). In the modern era of drug discovery, lectins from marine sources, due to their recognition of glycoprotein, glycolipids, proteoglycans and bacterial lipopolysaccharide in certain cell types, participate in several physiological and pathological functions, including host–pathogen interactions, cell–cell communications, protein trafficking and defence against foreign invaders (Gabius, 1997). This behavior makes them potential candidates for diagnostics and therapeutics and thus opens up a new avenue for drug discovery.

3.2 LECTINS

Lectins are a group of carbohydrate-binding proteins or glycoproteins of nonimmune origin omnipresent in nature, found everywhere from microbes to humans, capable of agglutinating cells or precipitating glycoconjugates in solution.

3.2.1 Bivalve Lectins

A great variety of bivalve mollusks, such as mussels, clams, oysters, scallops and cockles, live alongside various bacteria in aquatic environments, marine and fresh water. They protect themselves against pathogenic infection through their innate immune system, which utilizes several biomolecules for their defense, including phenoloxidase (PO), lectins, protease inhibitors, antimicrobial peptides, toll receptors and other humoral factors. Invertebrates, including bivalve mollusks, commonly utilize lectins, which are PRRs or PRPs expressed as an array of glycans conserved on the surfaces of pathogens. Like many other invertebrate animals, bivalves contain mostly C-type lectins, which because of their sugar-recognizing properly bind to a variety of sugars exposed on the microbial surface, leading to a cascade of reaction-causing phagocytosis. Therefore, lectins appear to play a crucial role in the host-defense mechanism of bivalve mollusks, both by recognizing and binding to pathogenic microorganisms and by opsonizing for phagocytic hemocytes.

3.2.1.1 C-type Lectins

Most invertebrates contain C-type lectin, which as the PRP, recognizing and binding to terminal sugars on glycoproteins and glycolipids, is one of the major candidates in the host-defense system (Yu & Kanost, 2000). Bivalve mollusks exhibit physiological functions by involving their hemolymph lectins, which act as opsonins for phagocytosis by the hemocytes and non-self-recognition receptors (Olafsen, 1995; Vasta *et al*., 1994; Wang *et al*., 2007). C-type lectins are a family of Ca^{+2}-dependent carbohydrate-binding proteins that have at least one CRD of 130 amino acid residues, present in invertebrates

(Drickmer, 1988; Drickmer & Taylor, 1993). They occasionally play a significant role in binding PAMPs in bacterial surfaces, which are decorated by carbohydrate structures, resulting in their clearance from the host system (Yu & Kanost, 2003). Each CRD contains a double loop, which is stabilized by two highly conserved disulfide bridges. There are four Ca^{+2} binding sites, among which Ca^{+2} binding site 2 participates in carbohydrate binding (Zelenky & Gready, 2005). Based on CRD domain structures, the C-type lectin superfamily is classified into 17 subtypes: (1) lecticans, (2) the ASGR group, (3) collectins, (4) selectins, (5) NK receptors, (6) the macrophage mannose receptor group, (7) Reg proteins, (8) the condrolectin group, (9) attractin, (10) SEEC, (11) the tetranectin group, (12) polycystin I, (13) CBCP, (14) the thrombomodulin group, (15) DGCR2, (16) Bimlec and (17) EMBP. Reg-protein C-type lectins have only one C-type lectin domain (CTLD) and a short N-terminal peptide, while the C-type lectins in other groups have more than one CTLD.

3.2.1.2 Galectins

Galectins are a family of carbohydrate-binding proteins (lectins) which bind β-galactoside sugar without the presence of Ca^{+2} and have a conserved sequence motif in their CRD. In mammals, 15 galectins have been characterized and sequenced (Almkvist & Karlsson, 2004; Liu & Rabinovich, 2005). They possess a conserved sequence motif within their CRD, consisting of 130 amino acid residues. Galectins can be subdivided into three types based on their structural motif features. The prototypes contain a single CRD domain of molecular weight 14–18 kD. The members of this subtype are galectin-1, -2, -7, -10, -13, -14 and -15. The chimera type has only one member: galectin-3, of molecular weight 26–30 kD. It consists of a single CRD domain with a collagenous domain. Both types of galectin form homodimer. The third type, the tandem-repeat type, is composed of two CRDs linked by a hinge peptide. Galectin-4, -8, -9 and -12 belong to this type (Barondes *et al*., 1994; Saussez & Kiss, 2006). Galectins are believed to participate in diverse physiological functions, such as development, differentiation, immunity, apoptosis and metastasis of cancer cells.

3.3 ISOLATION, MOLECULAR CHARACTERIZATION AND CARBOHYDRATE SPECIFICITY OF BIVALVE LECTINS

Lectins are carbohydrate-binding proteins or glycoproteins of 'nonimmune origin' that agglutinate cells and precipitate glycoconjugates in solution (Goldstein *et al*., 1980). They are present intra- and intercellularly in all forms of living thing, from single-cell protozoa to higher plants and animals. Because of their binding affinity for carbohydrates, they are widely used as preparative and analytical tools in biochemistry, cell biology, immunology, histology and glycobiology. In bivalve mollusks, lectins are present in different parts of the body, including the tissues, gill, gonad, mantle, foot muscle and hemolymph and hemocytes, and they are believed to be involved in their internal host-defense mechanisms. The most stricking aspect of marine bivalve lectins is their antimicrobial property. With a view to understanding their opsonic property against *Vibrio sp*. and *Perkinsus sp*., extensive research has been carried out on bivalve lectins since the 1990s. (Kang *et al*., 2006; Kim *et al*., 2006, 2008a,b; Olafsen *et al*., 1992; Ordas *et al*., 2000; Song *et al*., 2011; Takahashi *et al*., 2008; Tamplin & Fisher, 1989; Tasumi & Vasta, 2007;

Tunkijjanukijj *et al*., 1997). Lectins have been isolated and characterized from many marine mollusk species: PPL from *Pteria penguin* (Naganuma *et al*., 2006), chiletin from the flat oyster, *Ostrea chilensis* (Minamikawa *et al*., 2004), perlucin and perlustrin from abalone, *Haliotis laevigata* (Weiss *et al*., 2000), MBA from *Macoma birmanica* (Adhya *et al*., 2009a), gigalin E and H from the Pacific oyster, *Crassostrea gigas* (Olafsen *et al*., 1992), modiolin E and H from the horse mussel, *Modiolus modiolus* (Tunkijjanukij *et al*., 1997), tridacnin from the giant clam, *Hippopus hippopus* (Puanglarp *et al*., 1995), MCl and MCL-4 from the Manila clam, *Ruditapes philippinarum* (Bulgakova *et al*., 2004; Takahashi *et al*., 2008), CGL from the sea mussel, *Crenomytilus grayanus* (Belogortseva *et al*., 1998), one lectin from the Japanese pearl oyster, *Pinctada fucata martensii* (Suzuki & Mori, 1989), and three lectins from the sperm and one from the hemolymph of *Mytilus edulis* (Takagi *et al*., 1994; Renwrantz & Stahmer, 1983). Some of these acted as opsonins (Olafsen *et al*., 1992; Renwrantz & Stahmer, 1983) and agglutinated various bacteria (Adhya *et al*., 2009a; Naganuma *et al*., 2006; Olafsen *et al*., 1992; Tunkijjanukij *et al*., 1997, 1998). In recent years, analyses of the biological significance of the lectin genes of marine mollusks have been carried out (Adhya *et al*., 2010; Espinosa *et al*., 2010; Gourdine & Ravin, 2007; Kim *et al*., 2008 a,b; Wang *et al*., 2007; Yamaura *et al*., 2008; Zhang *et al*., 2009a,b, 2010; Zheng *et al*., 2008; Zhu *et al*., 2009).

Three isoforms of galactose/N-acetylgalactosamine-specific lectins were isolated from the hemolymph withdrawn by dissecting the adductor muscles of *Anadara granosa* clam (Adhya, 2007). *A. granosa* hemolymph separated into four fractions: AGL-IA (*Anadara granosa* lectin–IA), AGL-IB, AGL-IV and a hemolysin, AGL-III, by gel filtration on Sephadex G-100. Of these, AGL-IA and -IV were purified by affinity chromatography on an arabinogalactan matrix. AGL-IB was purified by successive affinity chromatography on arabinogalactan and fetuin-sepharose columns (Fig. 3.3). The molecular weights of AGL-IA, AGL-IB and AGL-IV were 375, 260 and 33 kD, respectively. AGL-IA and AGL-IV agglutinated rabbit and pronase-treated human erythrocytes, whereas AGL-IB agglutinated only rabbit erythrocytes. Interestingly, AGL-III caused hemolysis of pronase-treated human erythrocytes but agglutinated rabbit erythrocytes. The hemolytic activity of

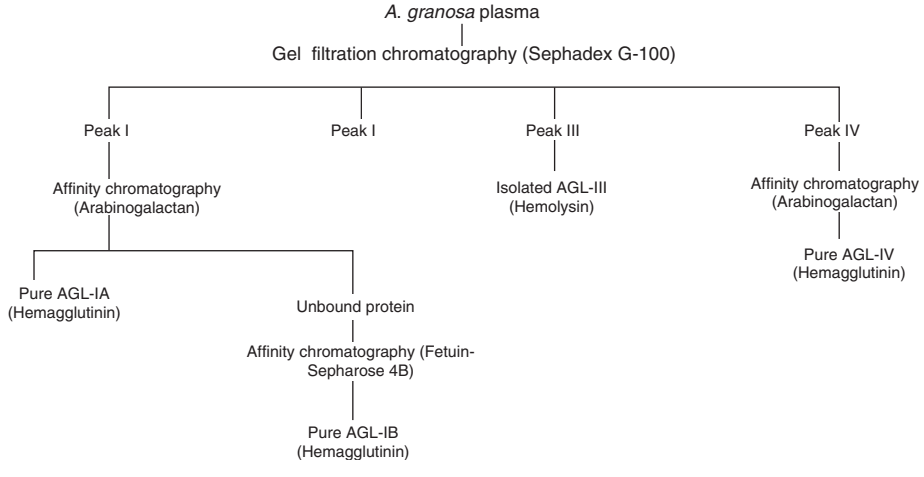

Fig. 3.3 Purification scheme of *Anadara granosa* lectin.

AGL-III was dependent on Ca^{2+}, Mg^{2+} and Mn^{2+}, whereas the hemagglutinating activity of three other lectins was Ca^{2+}-dependent. There were subtle differences in the carbohydrate specificities of these four lectins. AGL-IA and AGL-IV reacted more with Me-αGal than Me-βGal; in contrast, AGL-III bound more with Me-βGal than with Me-αGal and AGL-IB was specific for Me-αGal only. Table 3.1 depicts the molecular properties and carbohydrate specificities of three lectin isoforms and a hemolysin from *A. granosa* hemolymph. Dam *et al*. (1992) purified a galactose-binding lectin, Anadarin P, from the plasma of the blood clam, *Anadara granosa*, by affinity chromatography on asialofetuin Sepharose 4B matrix. This has a molecular mass of 130 kD, consisting of two noncovalently bound subunits of Mr 17 and Mr 16 kD. Anadarin MS, another lectin from the hemolymph of *A. granosa* was purified by affinity chromatography on heparin Sepharose 4B followed by gel filtration on Sepharose 6B. The purified lectin is pentameric, of molecular mass 300 kD, and is composed of noncovalently attached identical subunits of 60 kD. Anadarin MS agglutinated rabbit erythrocytes—but not those of humans—in the presence of Ca^{2+} and infective promastigotes of *Leishmania donovani* exclusively, suggesting it can be used as a biomarker for this parasite (Dam *et al*., 1994). A unique lectin that binds to N-glycolylneuraminic acid from the foot muscle of *A. granosa* was also isolated (Dam *et al*., 1993); this was proven to be tetramer of molecular mass 254 kD, consisting of four subunits: two each of Mr 65 kD and two of 62 kD. The activity of the lectin was inhibited by porcine mucin containing N-glycolylneuraminic acid. It is pertinent to believe that multiple lectins present in the circulation, each with different carbohydrate-binding specificities, are involved in the immune system, allowing recognition of a wide variety of invading organisms. Several mollusks with multiple lectins have been reported, as summarized in Table 3.2. In conclusion, these three isoform lectins with subtle galactose-binding specificity warrant further study to elucidate their significance in the *A. granosa* immune system.

Adhya *et al*. (2009a,b) isolated a novel lectin from *Macoma birmanica* and characterized its detailed carbohydrate specificity and antibacterial activity. *M. birmanica* is abundant along the whole northeast coast of the Bay of Bengal, including deltaic Sunderban, West Bengal and India, and inhabits the intertidal mud flat. The agglutinin was purified from the foot muscle of *M. birmanica* by successive ammonium sulfate precipitation and affinity chromatography on GlcNAc-Sepharose 4B column, and designatd as MBA. Interestingly, unlike marine bivalve lectins, the hemagglutinating activity of MBA was found to be Ca^{2+}-independent and of Mr 47 kD by SDS-PAGE and 50 kD by gel filtration on Sepharose 12HR10/30. MBA also showed Mr of 46.6 kD with electron spray ionization mass analysis (Fig. 3.4). By hemagglutination-inhibition assay and ELISA-inhibition assay of binding between biotinylated MBA and immobilized asialoagalacto porcine thyroglobulin using various saccharides, the pattern of monosaccharide specificity was found to be in the order Me-βGlcNAc > Me-αGlcNAc > Me-αMan > Me-α/βGlc > GlcNAc > Man > Glc > Me-αGlc, Me-βMan $\gg$ Gal (Table 3.3) (Adhya *et al*., 2009b). The binding affinity of MBA for β-GlcNAc and α-Man cluster-containing glycoproteins is summarized in Table 3.4. Among the glycoproteins, MBA showed strong binding with β-GlcNAc exposed-complex-type N-linked glycans; the maximum was for the polyvalent GlcNAc(β1-2)-Man-α1-containing asialoagalacto glycoform of porcine and bovine thyroglobulins (PTG and BTG) (Adhya *et al*., 2009a) and human apotransferrin. Compared to N-linked glycans, BSM and its asialo derivative, containing mostly core-1-type O-linked mucin-type chains, showed very negligible binding (Adhya *et al*., 2009a). Strong binding with fetuin was observed due to three complex-type N-linked glycans present in the

Table 3.1 Molecular properties and carbohydrate specificities of *A. granosa* lectins.

Properties	AGLIA	AGLIB	AGLII	AGLIV
Molecular mass (kD)				
SDS-PAGE (reduced)	60, 47, 30	65, 32		30
SDS-PAGE (non-reduced)	260, 74, 30	105,52		30
Native PAGE	375	260		33/24[a]
Erythrocytes specificity				
Hemagglutination	Rabbit > human	Rabbit	Rabbit	Rabbit > human
Hemolysis	No	No	Human	No
Bivalent cation requirment	Ca^{+2}	Ca^{+2}	$Ca^{+2}/Mg^{+2}/Mn^{+2}$	Ca^{+2}
Hemagglutination-inhibition	1. Gal > GalNAc	1. Gal = GalNAc	1. Gal = GalNAc	1. Gal = GalNAc
Carbohydrate specificity	2. Me-α-Gal > Me-β-Gal	2. Me-α-Gal	2. Me-β-Gal > Me-α-Gal	2. Me-α-Gal > Me-β-Gal
Monosaccharides	3. —OH at C-4 important	3. —OH at C-4, C-6 important	3. —OH at C-1, C-4, C-6 important	3. —OH at C-4 important
	4. Hydrophobic group increased inhibiting activity	4. Hydrophobic group decreased inhibiting activity	4. Hydrophobic group increased inhibiting activity	4. Hydrophobic group decreased inhibiting activity
Polysaccharide	Arabinogalactan	No	Arabinogalactan	Arabinogalactan
Glycoproteins				
N-glycan	Yes	Yes	No	Yes
O-glycan	Yes	Yes	No	Yes
Cluster glycosidic effect	Yes	Yes	No	Yes

[a]ESI-MS analysis.

Table 3.2 Biological role of multiple lectins in bivalve molluscs.

Species	Lectin	Carbohydrate specificity	Biological functions	Reference
R. philippinarum	MCL	GalNAc, PSM	Bind *Perkinsus* species	Bulgakov *et al., (*2004)
	MCL-4	GalNAc, PSM	Have opsonizing and bacteriostatic properties	Takahashi *et al.*, (2008)
	MCL-Gal	Gal, GalNAc	Induced upon *Perkinsus* infection and agglutinated *Vibrio tapetis*	Kim *et al.*, (2008a)
	MCL-3	GalNAc, BSM	Induced upon *Perkinsus* and *V.tapetis* infection	Kim *et al.*, (2008b)
	Mc-sialec	Sialic acid	Expression increased upon *Perkinsus* and *V. tapetis* infection	Adhya *et al.*, (2010)
C. gigas	Gigalin H	Sialic acid	Agglutinated *Vibrio anguillarum*	Hardy *et al.*, (1977)
	Gigalin E	Sialic acid	Agglutinated *Vibrio anguillarum*	Olafsen *et al.*, (1992)
	CgClec-1			
	CgGal	β Gal	Not induced by bacterial infection	Yamaura *et al.*, (2008)
A. irradians	AiLec		Increased expression upon bacterial challenge	Zhu *et al.*, (2009) Zhao *et al.*, (2007)
	AiCTL-6		Agglutinated bacteria	Zhang *et al.*, (2011)
C. ferrari	Cflec-1		Upregulated upon bacterial challenge	Wang *et al.*, (2007)
	Cflec-2	Man	Agglutinated *Staphylococcus haemolyticus* and suppressed the growth of *E. coli* TOP10F′	Zheng *et al.*, (2008)
	Cflec-3	Man	Agglutinated bacteria	Zhang *et al.*, (2009a)
	Cflec-4		Induced in heamocytes after bacterial challenge	Zhang *et al.*, (2009b)
M. edulis	Agglutinin	Mucin	Acts as opsonins	Renwrantz and Stahmer, (1983)
	MeML		Play role for capture of food particle and in particle sorting mechanism	Espinosa *et al.*, (2010)
H. laevigata	Perlucin Perlustrin		May promote the nucleation and the growth of $CaCO_3$ crystals	Weiss *et al.*, (2000)

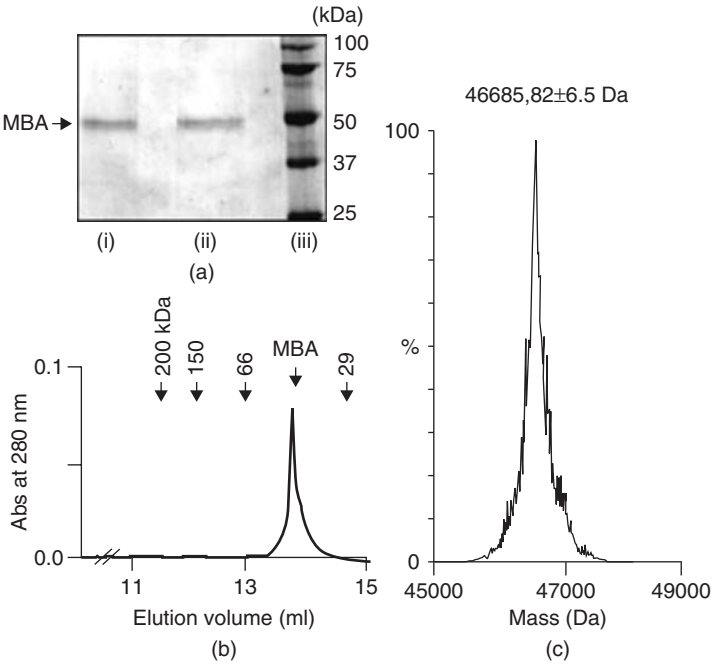

Fig. 3.4 (a) SDS-PAGE of MBA. Purified MBA was subjected to SDS-PAGE (10%), lane (i) nonreduced MBA, (ii) reduced MBA, (iii) protein standards (Bio-Rad). (b) Determination of the molecular weight of MBA by gel filtration on Sepharose 12 HR 10/30 column. (c) Electron-spray mass spectrum of MBA.

molecule. The carbohydrate specificity of MBA was further confirmed by inhibiting the binding of MBA with immobilized asialoagalacto PTG using mono- and oligisaccharides, polysaccharides and glycoproteins.

Among the oligosaccharides of Glc, Man and GlcNAc tested, the tetramannose core of N-linked glycans [curve 1 (Fig. 3.5); Man(α1–3)[Man(α1–3)Man(α1–6)]Man was most potent, being 15.3 and 19.8 times more reactive than GlcNAc and Man, respectively. The lower the length of mannosyl core of N-glycan, the lower the reactivity, in the order: tetramannose [curve 1; Man(α1–3)[Man(α1–3)Man(α1–6)]Man] > trimannose [curve 3; Man(α1–3)[Man(α1–6)]Man)] > mannobiose [curve 8; Man(α1–3)Man] > Man [curve 10], indicating the importance of (α1–3) and (α1–6) linkages between mannose residues. It could be concluded that the CRD of MBA can accommodate oligosaccharides; this is in contrast to banana lectin, which has a small combining site, accommodating only terminal single glucosyl or mannosyl residue (Mo *et al*., 2001). *Psathyrella velutina* lectin has a high affinity toward Me-βGlcNAc, recognizing only terminal β-GlcNAc residue (Endo *et al*., 1992). The inner chitobiose core [GlcNAc(β1–4)GlcNAc] of N-linked glycan was not recognized by MBA; this is in contrast to similarly Man/GlcNAc-specific lectin, codakine from marine bivalve *Codakia orbicularis* (Gourdine *et al*., 2008). These results summarize the major carbohydrate recognition factors of MBA as: (1) GlcNAc, Man and Glc are the most important sugars for MBA recognition, and the reactivity order is: Me-βGlc-NAc > Me-αGlcNAc > Me-αMan > Me-βGlc > GlcNAc > Man > Glc > Me-αGlc, Me-βMan >> Gal/GalNAc (nonreactive). The carbohydrate recognition profile of MBA is analogous to other MBL or MBL-like defense molecules (PRPs)

Table 3.3 Amounts of various saccharides giving 50% inhibition of binding between biotinylated MBA (10 ng) and immobilized asialoagalacto PTG (25 ng).

Sugar inhibitors	Quantity giving 50% inhibition[a] (nmol)	Relative potency[b]
Man(α1–3)[Man(α1–3)Man(α1–6)]Man	4.7×10^2	15.3
Me-βGlcNAc	6.9×10^2	10.4
Man(α1–3)[Man(α1–6)]Man	1.3×10^3 (extrapolated)	5.5
Me-αGlcNAc	3.4×10^3	2.1
Me-αMan	3.9×10^3	1.8
Me-βGlc	6.4×10^3	1.1
Glc(β–3)Glc	6.5×10^3	1.1
Man(α1–3)Man	6.8×10^3	1.1
GlcNAc	7.2×10^3	**1.0***
Man	9.3×10^3	0.8
Glc	10.0×10^3	0.7
Glc(α1–6)Glc(α1–4)Glc	$>1.2 \times 10^3$ (48.1%)[c]	—
Glc(α1–6)Glc	$>2.5 \times 10^3$ (47.5%)	—
3-0Me-Glc	$>10.0 \times 10^3$ (46.8%)	—
Glc(α1–1α)Glc	$>10.0 \times 10^3$ (46.4%)	—
Glc(β1–6)Glc	$>2.5 \times 10^3$ (45.8%)	—
Gal(α1–6)Glc	$>10.0 \times 10^3$ (41.7%)	—
Glc(α1–2β)Fru	$>5.0 \times 10^3$ (40.8%)	—
Glc(α1–4)Glc	$>5.0 \times 10^3$ (40.5%)	—
Me-αGlc	$>10.0 \times 10^3$ (40.2%)	—
Me-βMan	$>10.0 \times 10^3$ (36.8%)	—
Gal	$>10.0 \times 10^3$ (13.6%)	—
LRha	$>10.0 \times 10^3$ (11.7%)	—
Gal(β1–4)GlcNAc	$>3.1 \times 10^2$ (11.5%)	—
$[GlcNAc(\beta1–4)]_4$	$>1.2 \times 10^3$ (11.0%)	—
GalNAc	$>10.0 \times 10^3$ (10.8%)	—
Gal(β1–4)GlcNAc(β1-0-octy]	$>3.1 \times 10^2$ (11.5%)	—
$[GlcNAc(\beta1–4)]_3$	$>1.0 \times 10^3$ (8.5%)	—
Gal(β1–4)Glc	$>5.0 \times 10^3$ (7.8%)	—
2-Keto-deoxyoctanoate	$>1.0 \times 10^3$ (5.4%)	—
GlcNAc(β1–4)GlcNAc	$>5.9 \times 10^2$ (4.8%)	—
1.6-Anhydro-Glc	$>10.0 \times 10^3$ (0%)	—
ManNAc	$>10.0 \times 10^3$ (0%)	—
DFru	$>10.0 \times 10^3$ (0%)	—
DFuc	$>10.0 \times 10^3$ (0%)	—
LFuc	$>10.0 \times 10^3$ (0%)	—
LAra	$>5.0 \times 10^3$ (0%)	—
Glc(β1–4)Glc	$>5.0 \times 10^3$ (0%)	—
Neu5Ac	$>3.7 \times 10^2$ (0%)	—
p-NO_2Ph-αGlc	$>1.2 \times 10^2$ (0%)	—
p-NO_2Ph-βGlc	$>1.2 \times 10^2$ (0%)	—
p-NO_2Ph-βGlcNAc	$>5.0 \times 10^2$ (0%)	—
p-NO_2Ph-αGlcNAc	$>2.5 \times 10^2$ (0%)	—

*Bold '**1.0**' signifies the potency of GlcNAc that was taken as unity. Therefore, relative potency of other glycans was calculated with respect to it.

[a]The inhibitory activity was estimated from the curves (Fig. 3.5) and is expressed as the quantity of sugars (nmol) giving 50% inhibition.

[b]Relative potency of sugars was compared with respect to GlcNAc (taken as 1.0).

[c]Amounts in brackets specify the percentage of inhibition that did not reach 50%.

Table 3.4 Binding of biotinylated MBA (20 ng) with immobilized glycoproteins.[a]

Glycoproteins (polyvalent or cluster glycans)	Amount[b] (ng)	Maximum binding[c]
Asialoagalacto fetuin [GlcNAc(β1–2)Man(α1–]	22.00	3.0
Asialoagalacto PTG [GlcNAc(β1–2)Man(α1–]	22.67	3.0
Asialoagalacto apotransferrin [GlcNAc(β1–2)Man(α1–]	26.19	3.0
Asialo fetuin [Gal(β1–3/4)GlcNAc(β1–2)Man(α1–]	26.75	3.0
Asialo PTG [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	27.86	3.0
PTG [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	33.53	3.0
Fetuin [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	34.20	3.0
Asialo apotransferrin [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	37.35	2.9
Apotransferrin [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	41.71	2.9
SBA [Man(α1–2/3/6)Man(α1–]	80.15	2.8
Asialoagalacto human AGP [GlcNAc(β1–2)Man(α1–]	194.54	2.3
Asialo human AGP [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	254.92	2.2
Human AGP [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	602.42	1.7
Asialo BSM [GalNAc(α1–0)Ser/Thr]	>1000.0	–
BSM [sialyl GalNAc(α1–0)Ser/Thr]	>1000.0	–

[a]Glycoproteins are used in serial dilution mode, ranging from 1.0 ug to 0.95 ng.
[b]Amount of glycoproteins required for binding that corresponds to 1.5 unit absorbance at 492 nm (A492).
[c]Maximum binding of MBA with immobilized glycoproteins in terms of A492.

involved in the innate immune response (Gadeva *et al*., 2001; Weis *et al*., 1992) and establishes its biological importance as a PRP molecule. (2) The presence of a −NAc group at the equatorial C-2 of hexose, −OH group at C-4 and C-6 and pyranose ring conformations are the important recognition factors for MBA. (3) MBA recognizes both internal and external Glc, Man and GlcNAc residues. (4) MBA recognizes cluster or polyvalent GlcNAc(β1–2) Man(α1– > cluster or polyvalent Man(α1–2/3/6)Man(α1– or Man(α1–3/6)Man(β1–. These polyvalent glycotopes present on the macromolecules generated a great enhancement in affinity with MBA up to 10^5 times that of monomeric GlcNAc (Table 3.5). (5) MBA can also recognize the internal β-GlcNAc and α-Man residue in glycoproteins, though to a lesser extent than the exposed form.

Many lectins of varying carbohydrate specificities have been purified and characterized with emphasis on their biological properties from the hemolymph of bivalve mollusks. A C-type lectin from the Manila clam, *Ruditapes philippinarum*, was purified from the body extracts by mucin-sepharose followed by ion-exchange chromatography on DEAE-Toyperl and gel filtration with sepharose column (Bulgakov *et al*., 2004). Manila clam lectin (MCL) had a molecular mass of 138 kD and showed three covalently attached sugar units of 74, 34 and 30 kD. MCL forms dimer in solution and a Ca^{+2}-dependent lectin. In hemagglutination-inhibition assay, MCL did not bind to any monosaccharide except N-acetyl-D-galactosamine (GalNAc), maximum inhibition being with GalNAc-containing porcine mucin, which gives a hint that it reacts with O-linked glycans.

Kim *et al*. (2006) observed that the Manila clam (*R. philippinarum*) synthesized lectin, which they termed MCL, in the hemocytes upon infection with the protozoan parasites, *Perkins olseni*. The newly synthesized lectin was secreted into hemolymph as a precursor protein of molecular mass 74 kD. It was noted that the greater the degree of infection, the greater the hemagglutinating activity. The hemagglutination titer was more that 1000-fold greater than the noninfected clam. This was confirmed by Western blotting of the body fluid of infected and noninfected clams. MCL was expressed highly in infected-clam body fluid, with the appearance of three protein

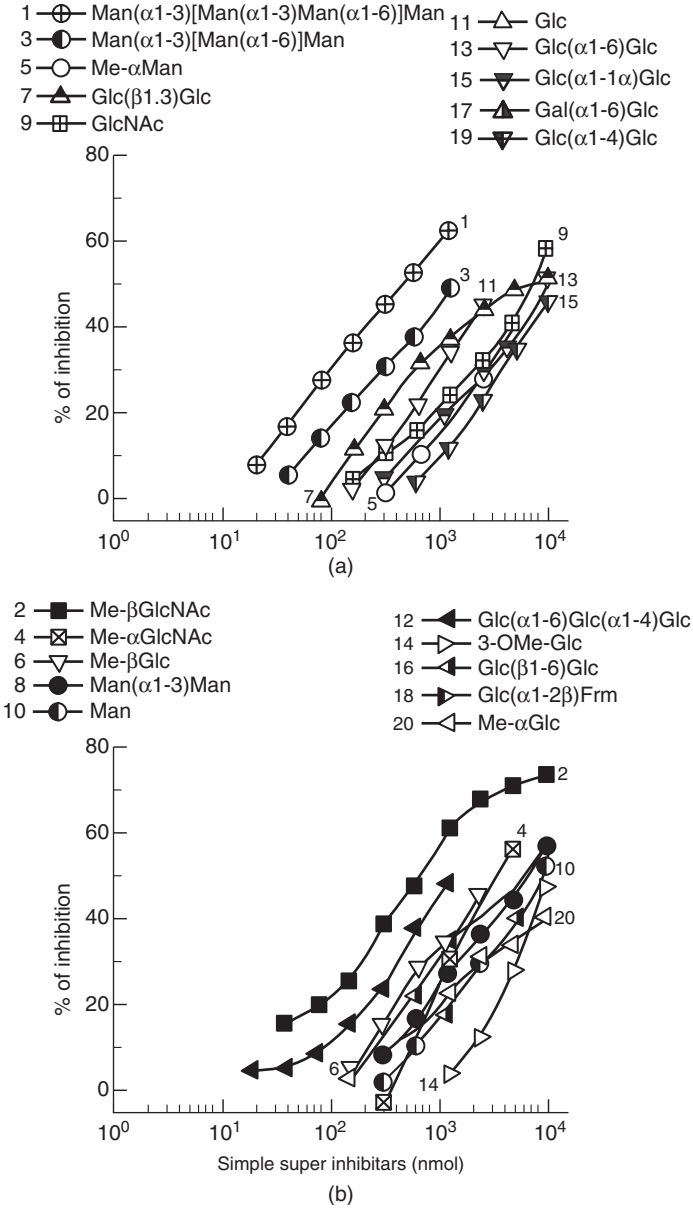

Fig. 3.5 Inhibition of binding between biotinylated MBA (10 ng) and immobilized asialoagalacto PTG (25 ng) by different sugars. After 45 minutes' incubation of serially diluted sugars and biotinylated MBA at room temperature, a binding assay was performed with immobilized asilaloagalacto PTG. 50% inhibition of binding was calculated from the absorbance at 492 nm (A492) after reaction termination at 1 hour.

Table 3.5 Amounts of various glycoproteins and polysaccharides giving 50% inhibition of binding between biotinylated MBA (10.0 ng) and immobilized asialoagalacto PTG (25.0 ng).

Polyvalent of cluster inhibitors	**Quantity giving 50% inhibition[a] (ng)**	**Relative potency[b]**
Asialoagalacto apotransferrin [GlcNAc(β1–2)Man(α1–]	11.2	1.5×10^5
Asialoagalcto fetuin [GlcNAc(β1–2) Man(α1–]	19.5	8.7×10^4
Asialoagalacto PTG [GlcNAc(β1–2)Man(α1–]	24.5	6.9×10^4
Asialo fetuin [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	26.8	6.3×10^4
Asialoagalacto BTG [GlcNAc(β1–2)Man(α1–]	30.6	5.5×10^4
Asialo PTG [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	31.2	5.4×10^4
Asialo apotransferrin [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	36.4	4.7×10^4
Asialo BTG [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	72.8	2.3×10^4
PTG [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	95.5	1.8×10^4
SBA [Man(α1–2/3/6)Man(α1–]	1.0×10^2	1.7×10^4
Apotransferrin [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	1.4×10^2	1.2×10^4
Fetuin [sialyl Gal(β1–3/4)GlcNAc(β1–2)Man(α1–]	1.6×10^2	1.1×10^4
BTG [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	2.2×10^2	7.7×10^3
Asialoagalacto AGP [GlcNAc(β1–2)Man(α1–]	2.7×10^2	6.3×10^3
HRP [Man(α1–3/6)Man(β1–4)Man(α1–]	4.1×10^2	4.1×10^3
LBL [Man(α1–2)Man(α1–]	6.2×10^2	2.7×10^3
GlcNAc	1.7×10^6	**1.0**
Asialo AGP [Gal(β1–4)GlcNAc(β1–2)Man(α1–]	>1000(49.0%)[c]	–
AGP [sialyl Gal(β1–4)GlcNAc(β1–2)Man(α1–]	>1000(47.9%)	–
Ovalbumin [Man(α1–2)Man(α1–]	>1000(13.9%)	–

The bold '**1.0**' signifies the potency of GlcNAc taken as unity. Therefore, the relative potency of other glycans was calculated with respect to this.

[a]The inhibitory activity was estimated from the respective curves and is expressed as the quantity of inhibitor (ng) giving 50% inhibition.

[b]The relative potencies of glycoproteins and polysaccharides are compared with GlcNAc (taken as 1.0).

[c]The amounts in brackets specify the percentage of inhibition that did not reach 50%.

bands of 74, 34 and 30 kD, whereas noninfected clams showed very little expression of lectin. This phenomenon was suggested to be due to the expression of more lectin by the clam for host defense. Western-blotting analysis revealed the validity of this result. It is established that invertebrates, upon microbial infection, produce some important compounds that are essential to combating invaders, resulting in phagocytosis or clearance: antimicrobial peptides, lysosomal enzymes and lectins. Takahashi *et al*., (2008) isolated an isoform of MCL (MCL-4) from the plasma of the Manila clam (*Ruditapes philippinarum*). MCL-4 showed molecular mass of 70 kD with native PAGE, while with SDS-PAGE under reducing and nonreducing conditions it was found to be composed of two nonidentical subunits of 58 and 43 kD. Like many other bivalve lectins, MCL-4 exhibited self-agregation of molecular mass 147 kD, as demonstrated by gel filtration and the highest agglutinating and specific activity. The N-terminal sequence (LSCESSADCG) of MCL-4 has sequence similarity with those of other MCLs (MCL-1 of 74 kD (LSCGSSADGGEGNI) and that of 50 kD (LSCGSSADGGEG), isolated by Bulgakov (2004)). MCL-4 showed opsonizing and bacteriostatic properties but did not show agglutinating activity with *Alteromonus halopaktis* (IAM 12919), *Vibrio tubiashii* (ATCC19106) and *Marinococcus halophilus* (IAM12844).

A galectin was cloned and identified from the Manila clam, *R. philippinarum* (Kim *et al*., 2008a), designated as McGal. The galectin is a tandem-repeat type, since it is

composed of two CRDs. It shows sequence similarities with the CRDs of other galectin family members, found to be highest in galectin-4 and -9. McGal has 56% amino acid sequence homology to the bi-CRD galectin of amphioxus and similarity to the CRD of the eastern oyster, *C. virginica* (Tasumi & Vasta, 2007). It has the receptor for the protozoan parasite *Perkinsus marinus*. By RT-PCR, the mRNA of McGal was found to be distributed among different tissues: highly present in mantle and gill and with lowest expression in hemocyte (Fig. 3.6). This indicates that McGal is produced in several tissues. Immunohistochemical analysis by confocal microscopy of the tissues of different organs with anti-McGal antibody, FITC-labeled secondary antibody and Giemsa staining showed fluorescence in multiple organs, specifically the gill, foot and mantle (Fig. 3.7). McGal was found to be distributed mainly on the outer surfaces of the adductor and foot, whereas in the mantle it appeared as a punctured pattern. Some Giemsa-stained cells also showed the presence of McGal. Like other galectins, McGal binds significantly to Gal, GalNAc and galectins containing oligosaccharides and mucin.

Sialic acid-binding lectins are widely present in several invertebrate hemolymphs of phyla mollusca and arthropoda, such as snails (Basu *et al*., 1986), crabs (Chattopadhyay & Chatterjee, 1993; Pramanik *et al*., 2010), insects (Yu & Kanost, 2003), abalone (Wang *et al*., 2008) and Pacific oysters (Hardy *et al*., 1977). Adhya *et al*. (2010) investigated the expression and localization of sialic acid-binding lectin, designated MCsialec, in various tissues of *Perkinsus*-infected and noninfected Manila clams by RT-PCR and immunohistochemical techniques. They detected the presence of MCsilec mRNA in various tissues, namely the adductor, foot, gill, mantle, pulp and siphon, plus hemocytes from both infected and noninfected clams. Interestingly, the expressed level of lectin was always found to be higher in tissues of the infected compared to the noninfected clams. Lei *et al*. (2011) cloned the cDNA library and sequence analysis of sialic acid-specific lectin from *V. philippinarum*. They quantified the expression of SABL mRNA in *V. philippinarum* by RT-PCR in different tissues, with Manila clam being highest in

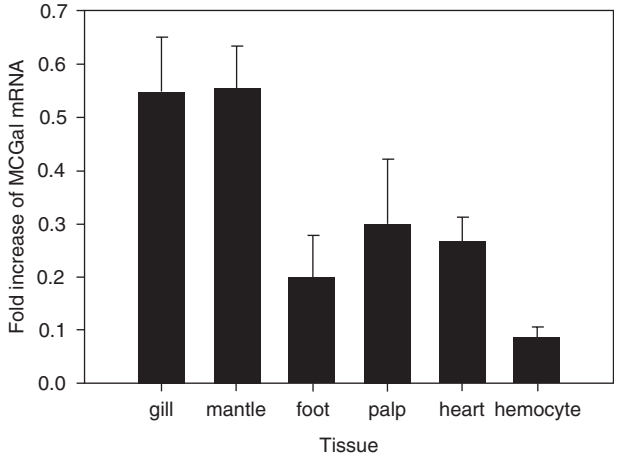

Fig. 3.6 Tissue distribution of McGal. Total RNA was extracted from Manila clam tissues, and double-stranded cDNA was synthesized by reverse transcription. The expression of McGal mRNAs was examined by RT-PCR. The β-actin gene was used as a control. Each triplicate experiment was repeated three times and the mean and standard deviations of nine reactions were plotted.

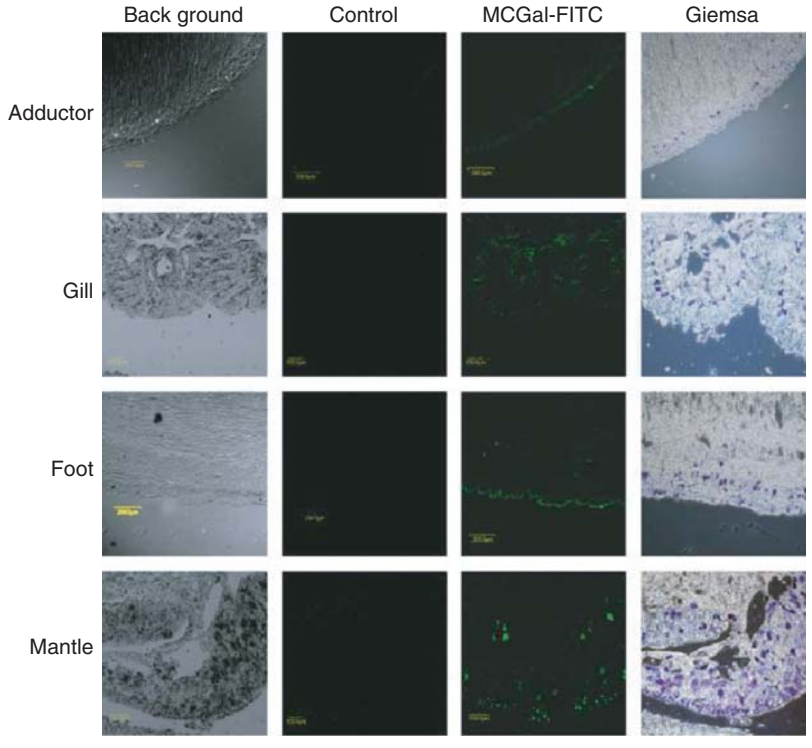

Fig. 3.7 Localization of McGal. Tissues were fixed with formaldehyde and embedded in paraffin wax. McGal was detected with the anti-McGal rabbit polyclonal antibody, followed by the addition of FITC-labeled anti-rabbit secondary antibodies. The immunoreactivities were examined by confocal laser microscopy. McGal was detected as a green coloration. Gimsa staining was carried out for the detection of hemocytes in series section. Hemocytes were detected as a purple coloration.

mantle: 150-fold more than hepatopancreas. Codakine, a C-type Ca^{+2}-dependent lectin, is purified from the gill of the tropical clam, *Codakia orbicularis*, by affinity chromatography on a mannose-Sepharose column (Gourdine *et al*., 2002). The specificity of codakine was determined by a binding study of fluorescence-tagged lectin with a glycan array using microcalorimetry, which showed high affinity towards a biantennary complex-type N-glycan (Gourdine *et al*., 2008).

Tridacnin from *Tridacna maxima* clam was purified from hemolymph and its molecular properties were studied. Tridacnin forms aggregate of Mr 470 kD and has been reported to constitute subunits of Mr 10, 20 and 40 kD. It shows significant specificity for Galβ 1–6 Gal, galactobiose, besides a number of mono- and oligosaccharides. Tridacnin agglutinates a variety of vertebrate erythrocytes in the presence of Ca^{2+} (Baldo & Ulhenbuck, 1975; Baldo *et al*., 1978; Ulhenbuck *et al*., 1979).

A C-type lectin chiletin was isolated from the hemolymph of the flat oyster, *Ostrea chilensis*, using Sepharose 6L-6B followed by anion-exchange chromatography (Mono Q HR 5/5). Chiletin required Ca^{2+} to agglutinate sheep erythrocytes analogous to *C. virginica* hemolymph lectin in Ca^{2+}-dependent agglutination. By SDS-PAGE, chiletin produced two bands of Mr 12 and 24 kD under reducing and nonreducing conditions (Minamikawa *et al*., 2004). Chiletin forms aggregates and remains in five different

conformations in native condition, one being enormously large—640 kD—and another very small—1 kD only. Some aggregates were resolved with 8M urea into covalently and noncovalently attached subunits. Two-dimensional electrophoresis of the purified chiletin and the whole hemolymph followed by Western blot proved chiletin consisted of a 12 kD monomeric protein. The molecular complexity of these protein molecules was caused by either post-translation modifications or multiple genes. Self-aggregating complexes (500–1600 kD) of gigalin H and gigalin E from Pacific oyster, *C. gigas*, were similarly dissociated by 8M urea into 21.0, 22.5 and 33.0 kD subunits, and a similar phenomenon was observed when the eastern oyster, *C. virginica*, was treated with 5M guanidine—HCl. A galactose-specific lectin from the hemolymph of the pearl oyster (*Pinctada fucata martensii*) was purified successively by affinity chromatography on bovine submaxillary mucin-conjugated Sepharose 4B, anion-exchange chromatographyon Mono Q and Superose 6B gel filtration (Suzuki & Mori, 1989). The lectin existed as an aggregate of 440 kD, which dissociated into a single subunit of Mr 20 kD with SDS-PAGE under nonreducing condition (Suzuki & Mori, 1989). Two sialic acid-specific lectins from the hemolymphs of the Pacific oyster, *C. gigas*, were isolated by affinity chromatography on bovine submaxillary mucin conjugated Sepharose 4B following linear-gradient elution by NaCl; these were named gigalin E and gigalin H (Hardy *et al*., 1977). Gigalin E agglutinates horse erythrocytes, while gigalin H agglutinates human erythrocytes. A novel lectin was isolated from the mantle of the penguin wing oyster, *Pteria penguin*, by affinity chromatography on BSM-Sepharose 4B gel matrix followed by cation-exchange chromatography on a HiTrap SP column with a linear gradient of NaCl (Naganuma *et al*., 2006). The lectin is a monomer of Mr 21 kD, determined by SDS-PAGE, gel filtration and matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry. *Pteria penguin* lectin (PPL) forms dimer in the presence of a high salt concentration (500 mM NaCl), determined by dynamic light scattering (DLS), assuming that PPL attains globular conformation of mass 42 kD, whereas by DLS without NaCl, PPL corresponds to its monomer of 21 kD. This suggests that PPL exists in a higher-order conformation in the presence of a high salt concentration. PPL agglutinates several vertebrate erythrocytes, including human ABO types; the strongest hemagglutination was observed with rabbit erythrocytes. Among the mono- and oligosaccharides, PPL showed high binding affinity towards galactose, methylgalactoside and lactosamine. The detailed carbohydrate binding specificity of PPL, examined by frontal-affinity chromatography (FAC) using 37 different pyridylaminated oligosaccharides, showed strong binding with lactose, lactosamine, T-disaccharide and Gal(α1-4)Gal, which are present in N-linked and O-linked glycans and glycolipid glycans (Naganuma *et al*., 2006). These strong affinities might be due to polyvalent interaction of PPL with the oligosaccharides present on glycoproteins. From the amino acid sequence derived by protein sequence analysis and cDNA cloning, PPL showed two tandemly repeated homologus domains containing 44% internal homology. PPL showed sequence homology with rhamnose-binding lectins from fish eggs and a galactose-binding lectin from sea urchin eggs, covering nearly 50% homology (Naganuma *et al*., 2006). A novel gene of sialic acid-binding lectin from the Hong Kong oyster, *Crassostrea hongkongenins*, was cloned. The recombinant lectin showed specificity to sialoglycoprotein fetuin. The lectin inhibited the growth of both Gram-positive and Gram-negative bacteria (He *et al*., 2011).

A C-type lectin specifically synthesized in epithelial-layer monocytes of the digestive gland and the palial organ of *C. virginica* was isolated by Jing and his group (2011) and named CvML. Sequence analysis of CvML showed similarities with C-type lectin

present in the digestive gland of *C. gigas* (Jing *et al*., 2011). CvML consists of a single peptide and one CRD that contains a YPD motif with conserved sites, WID and DCM for Ca^{2+} binding. mRNA transcripts of CvML were detected in the gills, labial palps, mantle and digestive gland, but not in hemocytes. Its expression was significantly high on starvation, bacterial bath exposure and injection of bacteria assessed by quantitative RT-PCR. This group also demonstrated that mucosal lectin from blue mussel, *M. edulis* (MeML), after 5 days starvation resulted in lectin expression in gills and labial palps (Pales Espinosa *et al*., 2010). The implication of lectin–carbohydrate interaction in establishing a relationship between microalgae and *C. virginica* in particle selection in feeding bivalves was demonstrated (Pales Espinosa *et al*., 2008). These investigations gave an insight into the role of these two bivalve lectins in particle capture and selection.

A heterogenous sialic acid-binding lectin, modiolin, has been purified from the hemolymph of the horse mussel, *Modiolus modiolus*. It forms aggregates of 100–1300 kD. By SDS-PAGE under reducing conditions, modiolin dissociates into three nonidentical subunits of 14.0, 17.5 and 20.0 kD, respectively (Tunkijjanukij & Olafsen, 1998). By using 2 DE with immobilized pH gradients, modulin showed three subunits of Mr 14.0, 17.5 and 20.0 kD with different isoelectric points (pI ~5.1 and ~5.5, pH 5.5 and pH 4.9, respectively). By stepwise centrifugation of affinity-purified lectin, modiolin fractionated in high- and low-molecular-weight fractions. The fractions of Mr ≥100 kD showed binding of both horse and human erythrocytes (modiolin H and E) activity, whereas the fractions collected between Mr ≤30 and 100 kD showed modiolin E activity, and the fraction with the lowest molecular weight, between ≤5 and 10 kD, showed activity towards horse erythrocytes. Hemocytes of horse mussel are the source of hemolymph lectins, like other invertebrates generally. The cells of the hemocytes were shown to be immunoreactive with antihemolymph antibody and protein A-gold labeling, and the lectin was localized in large granular cells in hemocytes. A lectin named 'CGL' was isolated from marine mussel, *Crenomytius grayamus*, tissues by affinity chromatography on acid-treated Sepharose 6B followed by gel filtration on Sephacryl S-200. CGL consists of a single subunit of Mr 18 000 kD in SDS-PAGE under reducing and nonreducing conditions. Its activity is independent of the divalent cations and agglutinates human erythrocytes irrespective of ABO blood-group system. CGL has binding specificity for GalNAc/Gal or those containing glycoproteins, the best being asialo-BSM (Belogortseva *et al*., 1998). Blue mussel, *Mytilus edulis*, was isolated by affinity chromatography on asialomucin-Sepharose.

Many investigators (Wang *et al*., 2007; Zhang *et al*., 2009a,2009b; Zheng *et al*., 2008) have cloned and characterized C-type lectins: from Zhikong scallop, *C. farreri* (Cflec-1,2,3, 4 and 5), and from bay scallop, *Argopeceten irradians* (AiCTL-1, 2, 3, 4, 5 and 6) (Zhang *et al*., 2011; Zhu *et al*., 2008). Zhikong scallop, *Chlamys farreri*, is one of the bivalves cultivated on the northern coast of China for commercial exploration. Scallops, like other bivalve mollusks, prevent themselves from bacterial attack through a set of PRRs. Some of these have been identified in *C. farreri*: β-1,3-glycan and lipopolysaccharide-binding protein (Su *et al*., 2004), thioester-containing protein (Zhang *et al*., 2007) and C-type lectins (Wang *et al*., 2007; Zhang *et al*., 2008). C-type lectin from Zhikong scallop, *Chlamys farreri* (Cflec-1), cloned cDNA using expressed sequence tag (EST) and RACE techniques (Wang *et al*., 2007). The expression pattern of Cflec-1 transcripts in infected and noninfected scallop was measured by RT-PCR. Cflec-1 mRNA transcripts were detected in gill and gonad tissues, and little expressed in mantle and hemolymph. No expression was detected in hepatopancreas, kidney and adductor muscle

of healthy scallops. However, expression of mRNA transcript in *V. anguillarum*-injected scallop was observed to be very high in all tissues compared to healthy scallops. The recombinant Cflec-1 expressed in *Escherichia coli* BL21 (DE3)−pLysS as a fusion protein (Wang *et al*., 2007). Another lectin from *C. farreri*, *Cflec*-2, was cloned by EST and RACE analysis. From deduced amino acid sequence and conserved CRD, Cflec-2 was considered a member of the C-type lectin superfamily. Zhang *et al*. (2009) indentified a multidomain C-type lectin gene from *C. farreri*, Cflec-4, by cDNA cloning and EST analysis. C-type lectins with the Ca^{+2}-dependent carbohydrate binding property involved in the host defense mechanism occur generally in invertebrates (Dodd & Drickamer, 2001; Schulenburg *et al*., 2008; Shagin *et al*., 2002). From the deduced amino acid sequence, Zhang and his groups showed that Cflec-4 is very common to the C-type lectins. They confirmed Cflec-4 lectin as a member of the C-type family by performing cDNA cloning, sequence study, phylogenetic analysis and potential tertiary structures. A novel C-type lectin from the bay scallop, *Argopecten irradians* (AiCTL-7), was cloned by EST analysis and rapid-amplification-of-cDNA-ends (RACE) study (Kong *et al*., 2011). From the deduced amino acid sequence of AiCTL-7, its CRD showed homology not only with other CRDs of bivalve C-type lectins but with fish lectin CRDs too. The mRNA transcripts of AiCTL-7 were primarily detected in hepatopancreas, but also a little in the kidney, gonad, hemocytes, heart and adductor of normal healthy scallops. The relative expression level of AiCTL was found to be highly increased in hepatopancreas and hemocytes on challenging with *Pichia pastoris* and the Gram-negative bacteria *Listonella anguillarum*. The recombinant protein, rAiCTL-7, agglutinated *P. pastoris* in the presence of Ca^{2+} and the agglutination was found to be inhibited by mannose. Galactose and β-1,3-glucan were found to be noninhibitory. Like AiCTL-7, another novel C-type lectin gene from *A. irradians* was cloned (AiCTL-6) by fluorescent quantitative RT-PCR. The mRNA of AiCTL-6 was found chiefly in hepatopancreas and gill and was very little expressed in other tissues. When the scallops were challenged by *L. anguillarum*, mRNA expression of AiCTL-6 was found to be upregulated.

3.4 BIOLOGICAL FUNCTIONS OF BIVALVE LECTINS

Lectins are well-recognized molecules which display defensive roles in both invertebrates and vertebrates. Invertebrate lectins' ability to react with bacteria, causing phagocytosis, shows an opsonic effect (Olafsen, 1988; Vasta, 1991). Several invertebrate lectins act as mediators of non-self-recognition in the innate immune response against microbial invasions and foreign substances (Epstein *et al*., 1996). The endogenous lectins in the cockroach hemolymph are capable of acting as non-self-recognition molecules for a wide range of microorganisms (Wilson *et al*., 1999). Immunolectin-2 from *Manduca sexta* acts as a pattern-recognition protein specific for Gram-negative bacteria through its interaction with LPS (Yu & Kanost, 2000). Lectins derived from hemocytes and hemolymph of the Japanese horseshoe crab, *Tachypleus tridentatus*, offer defense against invading microbes and foreign substances (Kawabata & Iwanaga, 1999). In bivalves, lectins have been identified mainly in hemolymph and hemocytes; besides this, they are also present in various organs of bivalves and are largely involved in host defense mechanisms against pathogenic invasion. Bivalve lectins interact with different varieties of bacteria due to their specific recognition of carbohydrates present on the bacterial cell surface. After recognition and binding, lectins function to remove bacteria and phagocytosis, thus acting as opsonins.

It is evidenced that a large variety of invertebrates, such as horse mussel, *Modiolus modiolus* (Tunkijjanukijj *et al*., 1997), Pacific oyster, *C. gigas* (Olafsen *et al*., 1992), black tiger prawn, *Penaeus monodon* (Ratanapo *et al*., 1992) brown shrimp, *Penaeus californiensis*, (Vargas-Albores *et al*., 1993), freshwater prawn, *Macrobrachium rosenbergii* (Vazquez *et al*., 1996), edible crab, *Scylla serrata* (Chattopadhyay *et al*., 1996), crustacean, *Liocarcinus depurator* (Fragkiadakis & Stratakis, 1997), solitary ascidian, *Halocynthia roretzi* (Azumi *et al*., 1991), and *Aplysi*a sp., sea hare (Zipris *et al*., 1986), cause bacterial agglutination. The involvement of tridacnin, a C-type lectin in the hemolymph of the giant clam, *Hippopus hippopus*, in digesting the clam's symbiotic algae has been proposed (Uhlenbruck, 1991). Heparin-binding lectin, Anadarin MS from the plasma of *Anadara granosa*, demonstrated agglutinating activity towards the infective promastigotes of *Leishmania donovani* (Dam *et al*., 1994).

It has been reported that the Manila clam (*Ruditapes philippinarum*), upon infection with the protozoan parasite *Perkinsus olseni*, binds to the surface of hypnospores and expresses in hemocytes seven different lectins involved in the innate immune response (Kang *et al*., 2006). Although bivalves hold bacteria within their bodies, they do not suffer with disease: they remain active in maintaining defense to keep themselves healthy. Antibacterial effects in hemolymph are believed to be due to acid phosphatase and lysozyme, which may be released by degranulation of hemolymph cells into serum. Manila clam is highly reactive with *Vibrio fluvialis* (strain AQ-00010 and strain AQ-002) mannan containing β-(1-2) and β-(1-6)-linked D-mannose (MIC 4.5×10^{-3} and 9×10^{-3} mg/ml), *Atteromonus atlanticus* mannan (MIC 9.8×10^{-3} mg/ml) and *Marinomonus communis* mannan (MIC 5×10^{-3} mg/ml), whereas inhibition by yeast mannan (MIC 1.5×10^{-3} mg/ml), and *Candida albicans* (MIC 1.25 mg/ml) is poor. This is due to the presence of a highly branched mannose chain in *Vibrio* compared to a linear mannose chain in yeast mannan. Glucan from *Pseudoalteromonas haloplanktis* is very poor (MIC 2.5 mg/ml). Mannan with β-(1-2) and β-(1-6)-linked mannose residues is common in the cell walls of bacteria and yeast and can act as a surface receptor for MCL.

M. birmanica agglutinin (MBA) interacts with both Gram-positive (*Bacillus subtilis*, *Staphylococcus. aureus*) and Gram-negative (*Escherchia coli* and *Vibrio parahaemolyticus*) bacteria in a dose-dependent manner (Adhya *et al*., 2009a). The binding potency of MBA with marine bacteria, *V. parahaemolyticus*, is stronger than that of the other three bacteria tested. Again, *E. coli* interacts with MBA more strongly than the two Gram-positive bacteria. The binding specificity was demonstrated by inhibition assay using GlcNAc and asialoagalacto porcine thyroglobulin (AA PTG). The observed interaction of MBA with both Gram-negative and Gram-positive bacteria was inhibited significantly by the sugar ligand (GlcNAc) of the agglutinin, which suggested that the interaction occurred through the CRD of the agglutinin. Further support for the N-linked glycan affinity of MBA was confirmed by inhibiting the lectin–bacteria interaction with AA PTG. The major cell-wall component of the Gram-negative bacteria *E. coli* O142 and *V. parahaemolyticus* is lipopolysaccharide, which is basically composed of membrane-bound lipid-A moiety attached to a polysaccharide chain of variable length and variable composition with bacterial strain.

MBA interacts with the terminal β GlcNAc residue of the polysaccharide of LPS of *E. coli* (Landersjo *et al*., 1997). Since KDO is a noninhibitory sugar of MBA, the binding of MBA with *E. coli* O142 and *V. parahaemolyticus* is exclusively for the GlcNAc moiety. All Gram-positive bacteria possess a peptidoglycan layer composed of repeating units of GlcNAc and MurNAc linked by short polypeptide chains. This points to the fact that

MBA, which shows specificity towards GlcNAc, interacts with *B. subtilis* and *S. aureus*. It has also been reported that mannose-binding lectin can bind *S. aureus* (Shi *et al*., 2004). MBA shows growth suppression of *V. parahaemolyticus*. The gigalins (H and E) from oyster (*C. gigas*) act as opsonins to stimulate *in vitro* phagocytosis of the marine bacterium *V. anguillarum* (Olafsen *et al*., 1992). The lectin from *C. virginica* agglutinates a large variety of bacterial species (Fisher & DiNuzzo, 1991). Likewise, TPL-1 and TPL-2 isolated from horseshoe crab, *T. tridentatus*, hemolymph recognize both Gram-positive and Gram-negative bacteria—*S. pneumoniae* R36A, *V. parahaemolyticus* and *E. coli* Bost-12—in a dose-dependent manner (Chen *et al*., 2001) The specificity of binding with these bacteria was demonstrated by inhibition with GlcNAc or at -NAc moiety of the GlcNAc-MurNAc cell-wall peptidoglycans (Chen *et al*., 2001). Though most lectins exhibit antibacterial activity by carbohydrate recognition, there are some exceptions. Lectin from Chinese shrimp, *Fenneropenaeus chinensis*, Fc-hsL, displayed antibacterial activity to *B. subtilis*, *B. cereus*, *B. thuringiensis*, *E. coli* and *Klebsiella pneumoniae*, but no agglutination activity could be detected when Fc-hsL was incubated with these bacteria (Sun *et al*., 2008). Sialic acid-binding lectins from invertebrates have also been known to mediate the innate immune response by causing bacterial agglutination or phagocytosis through binding to sialic acid exposed on the bacterial cell surface (Iwanaga & Lee, 2005). Considering these findings, it can be postulated that MBA might be involved in a similar fashion in the recognition and targeting of invading pathogens as part of an immune-like response through the prevention and control of microbial infection.

Modiolin, a lectin from *M. modiolus*, shows a strong antibacterial effect against several serotypes of *Vibrio* pathogens: *V. anguillarum*, *V.ordalii*, *V. salmonicida*, *V. viscosus* and *V. wodanis*. Such a property is also exhibited by *M. modiolus* hemolymph, but with less potency; it is even inactive towards some serotypes. However, modiolin has no such effect on *Alteromona* sp. (Tunkijjanukijj & Olafsen, 1998).

C-type lectins, which are most common in invertebrates, including bivalves, are the most important candidates. Many are involved in the immune response through their interaction with certain Gram-negative and Gram-positive bacteria. There are some reports of lectins involved in the recognition and clearance of different pathogens in mollusks, such as Zhikong scallop, *Chlamys farreri* (Wang *et al*., 2007; Zheng *et al*., 2008). Cflec-1 agglutinated Gram-negative bacteria *E.coli* JM 109 *in vitro* in the presence of Ca^{2+}, which was completely inhibited by TBS-EDTA. No agglutination of Gram-positive bacteria *M. luteus* and *C. lipolytica* was observed with Cflec-1. This indicates that Cflec-1 recognized the Gram-negative bacteria cell-surface carbohydrates. Another lectin, Cflec-2, from the same bivalve, was also reported to agglutinate *Staphylococcus haemolyticus* and inhibit the growth of *E. coli* TOP10F (Zheng *et al*., 2008). The recombinant Cflec-2 (rCflec-2) lectin exhibited agglutination of *Staphylococans hemolyticus* even in the presence of TBS-EDTA, suggesting that Cflec-2-induced agglutination, unlike that of Cflec-1, was Ca^{2+}-independent, and that the agglutination was inhibited by mannose. There have been reports of many C-type Ca^{2+}-independent lectins, such as OLABL from *Shishamo smelt* (Hosono *et al*., 2005), *Anguilla japonica* lectin from Japanese eel (Tasumi *et al*., 2002), immulectin-2 from tobacco hornworm, *Manduca* sexta (Yu *et al*., 2006), and Pjlec from the hemolymph of shrimp, *Penaeus japonica* (Yang *et al*., 2007). C-type lectin from bay scallop, *Argopectan irradians*, hemocytes has been proposed to be involved in the immune responses (Zhu *et al*., 2008). Interestingly, no agglutination of *L. anguillarum* has been observed by *A. irradians* C-type lectin-7 (AiCTL-7), though with its challenge the AiCTL-7 expression level was found to be remarkably upregulated in hepatopancreas

and hemocytes. Similarly, rAiCTL-6 agglutinated the Gram-negative bacteria *E. coli* TOP 10F and the Gram-positive bacteria *M. luteus* and *S. aureus*.

PPL agglutinated different strains of *E. coli* (Gram-negative) and *S. aureus* and *B. subtilis* (Gram-positive); the strongest binding was observed with *E. coli* K12. Agglutination was inhibited by LPSs of smooth strains of *E. coli*, such as *E. coli* 055, K12 and K235, and also *Shigella flexineri* A1. Of these, S-LPS of *E. coli* K12 and K235 and *S. flexineri* strongly inhibited the agglutination. In contrast, almost no inhibition was shown with the Re mutant strain of *E. coli* K12. These results indicate that PPL reacted with LPSs of O-antigens and core polysaccharides. Eastern oyster, *C. virginica*, hemolymph also agglutinated *V. cholera* serovars and biovars. The galectin identified from *C. virginica* hemocytes, CvGaL, recognized *Perkinsus marinus* and made its way into the hemocytes (Tasumi & Vasta, 2007). In accordance with the established fact that bacterial challenge augments the production of lectins in different organs of bivalves (Kang *et al*., 2006; Kim *et al*., 2008a; Perrigault *et al*., 2009; Zhu *et al*., 2008), CvML expressed in *C. virginica* gills with the injection of *V. alginolyticus*. However, a significant increase was observed following bath exposure to the same bacteria. The bath exposure provided signaling which initiated CvML regulation, whereas bacterial injection into the circulation did not upregulate CvML-producing mucocytes. It can be concluded that mucus from palial organs seems to play a key role in the interactions of waterborne particles. These findings support the participation of CvML in the capture or selection of particles and in oyster mucosal immunity.

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4 Digestive Enzymes from Marine Sources

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4.1 INTRODUCTION

The marine environment is by far the largest habitat on Earth, with oceans covering ~70% of its surface. Within this environment, habitats range from tropical sunlit surface waters to ocean trenches 11 000 m deep where pressures exceed 100 MPa. While salinity is fairly uniform at 3.5%, temperatures range from sea ice in the polar regions to temperatures in excess of 100 °C at deep-sea hydrothermal vents. Therefore, the marine habitat has been and continues to be a source of unique natural products possessing useful characteristics for biotechnological applications (Bhatnagar & Kim, 2010).

Among these products are enzymes, and the marine environment can be considered almost unexplored in terms of the enzymatic activities to be found. These enzymes can offer properties related to their habitat that are of great utility from a general biotechnological perspective. For example, most marine enzymes have cold-adapted properties that are useful in food and feed processing, such as their high catalytic activity at low temperatures and their inactivation at moderate temperatures (Diaz-Lopez & Garcia-Carreno, 2000). Additionally, marine-based enzymes are valuable as food ingredients and in food processing due to their specificity, diverse properties, salt tolerance and high activity at mild pH values (Okada & Morrissey, 2007; Shahidi & Janak Kamil, 2001).

A marine enzyme may be a unique protein molecule not found in any terrestrial organism, or it may be a known enzyme from a terrestrial source but with novel properties. In addition to microorganisms such as bacteria, fungi and actinomycetes, many other marine organisms such as fish, prawns, crabs, snakes, plants and algae have been studied. Properties such as high salt tolerance, hyperthermostability, barophilicity, cold adaptivity and ease of large-scale cultivation are of key interest to scientists (Debashish *et al*., 2005).

The main challenges to the use of marine-derived enzymes are their limited availability depending on harvest, the instability of the raw material and their potentially poor economic advantages, depending on the technologies used for extraction, potential markets and the quality of any byproducts (Haard *et al*., 1994). A potential biotechnological approach to these challenges appears in the transfection and overexpression of fish enzyme

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genes in select marine microorganisms (Simpson, 2000). Major sources of marine enzymes are byproducts produced as a result of fish and shellfish processing, such as the viscera, heads, skin, bones, exoskeletons and shells (Rosalee *et al*., 2007).

4.2 BIODIVERSITY AND AVAILABILITY

The global total production of fish, crustaceans and mollusks has continuously increased, reaching 144.6 million tonnes in 2009. Table 4.1 lists 70 of the main species captured in recent years. By far the most-caught species globally is the anchovy, at about 7.9 million tonnes. This is followed by the skipjack tuna, Atlantic herring, Alaska pollock and chub mackerel. Capture production provides around 90 million tonnes, while aquaculture production has continued growing, at an annual average rate of 6.1%, to 55.7 million tonnes (FAO Yearbook, 2011). Carps are the most cultured species in the world, with 40% of production. Other major cultured groups include shellfish (oysters, clams, mussels and scallops), other freshwater fish (especially tilapias), shrimp, prawn and salmon (Table 4.2). About 84% of total fishery production is for direct human consumption. The remaining 16%, or 22.8 million tonnes, is destined for non-food products, mainly the manufacture of fishmeal and fish oil. However, about one-third of the catch of fish in the world is thrown back into the sea (Raa, 1997). Furthermore, processing discards from fisheries account for as much as 70–85% of the total weight of the catch, and these have generally been dumped in-land or hauled into the ocean (Shahidi, 1994). Approximately 30% of total landings may be considered underutilized, bycatch, unconventional or unexploited (Venugopal & Shahidi, 1995). Hence, the management of fishery resources and new product development using underutilized species, bycatch and processing discards is very important in the world's fishing industry (Shahidi, 1994; Simpson & Haard, 1987a). Probably more than 50% of the remaining material from the total fish capture is not used as food, involving almost 32 million tonnes of waste (Kristinsson & Rasco, 2000). Although there are many approaches to further utilization of these resources, interest has been expressed in isolating value-added components from such raw material, including enzymes from the digestive tract (Venugopal *et al*., 2000).

4.3 MARINE BIOCATALYSTS

Among the most prominent current uses for treated fish waste are collagen and antioxidant isolation for cosmetics, biogas/biodiesel, fertilizers, dietetic applications (chitosan), food packaging (gelatin, chitosan) and enzyme isolation (Arvanitoyannis & Kassaveti, 2007). Waste-management research concerning fishery- and seafood-related industries is a rich source of information about clever and commercially useful exploitation of marine enzymes from various sources, especially marine animals. Interesting examples are: (1) the use of scallop mantle in the production of collagen and collagen peptides useful in the cosmetics industry, providing an improved hair damage-restoring product over conventional bovine peptides; (2) biomedical application of squid β-chitin obtained from squid pen; and (3) application of the digestive juice of abalone and scallop, which is rich in digestive enzymes, to the degradation of crystalline cellulose from plant-source byproducts into glucose (Takahashi & Fukunaga, 2008).

Table 4.1 Major species captured by world fishery.

Rank	Common name	Scientific name	AP	Rank	Common name	Scientific name	AP
1	Peruvian anchovy	*Engraulis ringens*	7.838	36	Haddock	*Melanogrammus aeglefinus*	0.334
2	Alaska pollock	*Theragra chalcogramma*	2.742	37	Chum salmon	*Oncorhynchus keta*	0.333
3	Skipjack tuna	*Katsuwonus pelamis*	2.502	38	Southern rough shrimp	*Trachypenaeus curvirostris*	0.330
4	Atlantic herring	*Clupea harengus*	2.379	39	Daggertooth pike conger	*Muraenesox cinereus*	0.323
5	Chub mackerel	*Scomber japonicus*	1.841	40	Japanese jack mackerel	*Trachurus japonicus*	0.318
6	Chilean jack mackerel	*Trachurus murphyi*	1.675	41	Pacific herring	*Clupea pallasii*	0.305
7	Blue whiting	*Micromesistius poutassou*	1.544	42	Kawakawa	*Euthynnus affinis*	0.305
8	Japanese anchovy	*Engraulis japonicus*	1.344	43	Short mackerel	*Rastrelliger brachysoma*	0.297
9	Largehead hairtail	*Trichiurus lepturus*	1.329	44	North Pacific hake	*Merluccius productus*	0.296
10	Yellowfin tuna	*Thunnus albacares*	1.143	45	Yesso scallop	*Patinopecten yessoensis*	0.292
11	Sardine pilchard	*Sardina pilchardus*	1.069	46	Hilsa shad	*Tenualosa ilisha*	0.290
12	Atlantic cod	*Gadus morhua*	0.820	47	American sea scallop	*Placopecten magellanicus*	0.278
13	Jumbo flying squid	*Dosidicus gigas*	0.755	48	Cape horse mackerel	*Trachurus capensis*	0.277
14	European sprat	*Sprattus sprattus*	0.638	49	Indian mackerel	*Rastrelliger kanagurta*	0.273
15	Argentine shortfin squid	*Illex argentinus*	0.609	50	Longtail tuna	*Thunnus tonggol*	0.245
16	Atlantic mackerel	*Scomber scombrus*	0.598	51	Albacore	*Thunnus alalunga*	0.242
17	Akiami paste shrimp	*Acetes japonicus*	0.593	52	Giant tiger prawn	*Penaeus monodon*	0.237
18	Araucanian herring	*Strangomera bentincki*	0.532	53	Bombay-duck	*Harpadon nehereus*	0.229
19	European anchovy	*Engraulis encrasicolus*	0.505	54	Nile tilapia	*Oreochromis niloticus*	0.227
20	Pacific saury	*Cololabis saira*	0.499	55	Southern African anchovy	*Engraulis capensis*	0.223
21	Saithe pollock	*Pollachius virens*	0.453	56	Narrow-barred Spanish mackerel	*Scomberomorus commerson*	0.221
22	California pilchard	*Sardinops caeruleus*	0.450	57	Pacific sandlance	*Ammodytes personatus*	0.220
23	Pink salmon	*Oncorhynchus gorbuscha*	0.433	58	Atlantic horse mackerel	*Trachurus trachurus*	0.212
24	Bigeye tuna	*Thunnus obesus*	0.426	59	Japanese pilchard	*Sardinops melanostictus*	0.211
25	Gulf menhaden	*Brevoortia patronus*	0.422	60	Pacific thread herring	*Opisthonema libertate*	0.209
26	Capelin	*Mallotus villosus*	0.412	61	Japanese jack mackerel	*Trachurus japonicus*	0.206
27	Japanese flying squid	*Todarodes pacificus*	0.408	62	Bonga shad	*Ethmalosa fimbriata*	0.205
28	Indian oil sardine	*Sardinella longiceps*	0.395	63	Atlantic menhaden	*Brevoortia tyrannus*	0.193
29	Northern prawn	*Pandalus borealis*	0.392	64	Okhotsk atka mackerel	*Pleurogrammus azonus*	0.192
30	Round sardinella	*Sardinella aurita*	0.368	65	Goldstripe sardinella	*Sardinella gibbosa*	0.174
31	Argentine hake	*Merluccius hubbsi*	0.365	66	Yellowstripe scad	*Selaroides leptolepis*	0.168
32	Nile perch	*Lates niloticus*	0.360	67	Blue swimming crab	*Portunus pelagicus*	0.165
33	Gazami crab	*Portunus trituberculatus*	0.357	68	Bigeye scad	*Selar crumenophthalmus*	0.158
34	Yellow croaker	*Larimichthys polyactis*	0.356	69	Sockeye-red salmon	*Oncorhynchus nerka*	0.151
35	Pacific cod	*Gadus macrocephalus*	0.341	70	Mote sculpin	*Normanichthys crockeri*	0.138

AP, annual production (million tonnes), average of last 5 years. Prepared from FAO Yearbook (2011).

Table 4.2 Major species produced by world aquaculture.

Species	AP	Growth ratio
Hypophthalmichthys molitrix	3.787	0.070
Ctenopharyngodon idellus	3.682	0.123
Ruditapes philippinarum	2.942	0.173
Cyprinus carpio	2.902	0.041
Hypophthalmichthys nobilis	2.187	0.130
Penaeus vannamei	2.130	0.284
Oreochromis niloticus	2.115	0.198
Catla catla	1.914	0.273
Carassius carassius	1.913	0.089
Salmo salar	1.371	0.054
Labeo rohita	1.270	0.222
Crassostrea gigas	0.682	0.006
Penaeus monodon	0.678	0.005
Sinonovacula constricta	0.662	0.022
Chanos chanos	0.648	0.026
Oncorhynchus mykiss	0.638	0.021
Megalobrama amblycephala	0.558	0.021
Eriocheir sinensis	0.474	0.039
Ictalurus punctatus	0.433	0.026
Anadara granosa	0.405	0.005
Cirrhinus mrigala	0.363	0.088
Mylopharyngodon piceus	0.333	0.027
Procambarus clarkii	0.305	0.079
Channa argus	0.299	0.032
Silurus asotus	0.298	0.025
Perna viridis	0.275	−0.004
Anguilla japonica	0.244	0.009
Patinopecten yessoensis	0.230	0.000

AP, annual production (million tonnes), average of last 10 years. Prepared from FAO Yearbook (2011). Growth ratio is the slope of the lineal regression to data.

4.3.1 Salt and pH Tolerance

The salinity of seawater is about 3.5% ($\geq$0.6 M NaCl), while halophilic organisms are considered moderate if they can grow at salt concentrations between 0.85 and 1.7 M NaCl and extreme if they require concentrations above 1.7 M (Marhuenda-Egea & Bonete, 2002). The synthesis and production of polyhydroxyalkanoates by halophiles is one of the current biotechnological topics related to these organisms (Quillaguamán *et al*., 2010). Interestingly, in the range of sodium chloride concentration below the value characterizing moderate halophiles and more common in marine organisms, enhancement of thermostability can be observed (Demirjian *et al*., 2001). Thermal-stability studies have been performed on numerous proteins from several hyperthermophiles, and glutamate dehydrogenase (GDH) is one of the most thoroughly studied enzymes in this domain, used as a model for understanding the molecular mechanisms involved in thermostability. An example is the enzyme trypsin from the intestine of the carnivorous fish smooth-hound (*Mustelus mustelus*). This enzyme shows high proteolytic activity at very high (30%) NaCl concentration and demonstrates its potential for protein hydrolysis at high salt content (Bougatef *et al*., 2010), confirming its role as a possible biotechnological tool in the fish-processing and food industries.

4.3.2 Barophilicity

The depth of the oceans is estimated to be ~4000 m on average; ocean life must therefore be able to withstand considerable pressure. Vertebrate fish have been observed at ~10 000 m in the deepest ocean sites, while metabolism in piezophiles (barophiles) has been shown to be viable at artificial pressures of an order of magnitude higher. In addition to living at high pressures, piezophilic organisms are typically psychrophilic or thermophilic in nature, due to the cold temperatures of the deep ocean and the proximity to hydrothermal vents (Trincone, 2011).

4.3.3 Cold Adaptivity

The considerable potential of enzymes that act at low temperatures for biotechnological exploitation is well known: these biocatalysts might be utilized as additives in the detergent and food industries, or in bioremediation processes, by minimizing energy consumption, reducing the risk of microbial contamination and avoiding the high-temperature instability of reactants or products. The use of psychrophilic enzymes could be advantageous due to their high specific activity (allowing reduction of the amount of enzyme needed) and their easy inactivation, which prevents prolonged action when it is not desired (Trincone, 2011).

4.4 DIGESTIVE ENZYMES

The marine environment is extremely diverse and a large number of enzymes still require identification, isolation and application. Some useful enzymes have already been isolated and applied in the food industry, including digestive proteolytic enzymes such as gastric, serine and cysteine and extremophilic enzymes such as thiol proteases, lipases, polyphenol oxidases (PPOs), chitinolytic enzymes, muscle proteases, transglutaminase.

The activity of digestive enzymes within the gut is affected by feeding habits. Herbivorous fish usually possess a longer gut than carnivores and a digestive enzymatic profile that is adapted to their diet, with a predominance of carbohydrases (amylases, disaccharidases), allowing the hydrolysis of polysaccharides. Conversely, carnivorous fish have a short intestine, with higher levels of proteases and a minor percentage of amylase and lipase (Caruso *et al*., 2009).

4.4.1 Digestive Proteases

Proteases extracted from marine organisms currently have a limited use, in part due to a lack of basic knowledge regarding these specific enzymes but also due to consumer attitudes toward their source: fish and seafood discards (Simpson, 2000). However, digestive proteases from marine sources have received growing interest due to their high enzymatic activities at low temperatures and the increasing availability of raw materials such as viscera (Okada & Morrissey, 2007). Fish viscera are a rich source of digestive enzymes, such as pepsin, trypsin, chymotrypsin and gastricsin, and several methods for their large-scale recovery have been developed (Klomklao, 2008; Haard & Simpson, 1994; Reece, 1988).

Due to their ability to hydrolyze proteins, proteases can significantly alter the texture of food products. One of their most economically important applications is the tenderizing

of meat after rigor mortis (Whitaker, 1996). They can also be used to enhance the texture of cereals and baked products, remove membranes from organs and egg sacks (thereby improving the drying and quality of egg products), ripen cheeses, remove skin from fish and squid and recover bone proteins (Haard *et al*., 1994; Simpson, 2000). In shrimp, proteases can be used to loosen shells from the meat, recover flavor compounds for use in surimi- and cereal-based extrusion products and recover carotenoprotein (up to 80% of the protein and 90% of the astaxanthin in shell waste) (Haard *et al*., 1994). Stomachless marine organisms, such as crayfish, cunner and puffer, also contain digestive proteases that can be used to inactivate PPO and/or pectin-esterases in fruit juices (Shahidi & Janak Kamil, 2001). Based on the nature of catalytic site, digestive proteases from the marine world are further classified into four categories: acid/aspartyl (gastric) proteases, serine proteases, cysteine or thiol proteases and metalloproteinases (Simpson, 2000).

4.4.1.1 Acid/Aspartyl Proteases

The acid or aspartyl proteases are a group of endopeptidases characterized by high activity and stability at acidic pH. They are referred to as 'aspartyl' proteases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid residues (Whitaker, 1994). In the EC system, all the acid/aspartyl proteinases from marine animals have the first three digits in common, as EC 3.4.23. Three common aspartyl proteinases that have been isolated and characterized from the stomach of marine animals are pepsin, chymosin and gastricsin (Simpson, 2000).

Pepsin is an aspartic endopeptidase that has been found in the gastric fluid of numerous marine and freshwater species (Shahidi & Janak Kamil, 2001) and in fish viscera. This enzyme has an extracellular function as the major gastric proteinase. Pepsin, secreted as a zymogen (pepsinogen), is activated by the acid in the stomach (Clarks *et al*., 1985). It is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35 kDa (Simpson, 2000). However, the pepsins from marine animals were reported to have molecular weights ranging from 27 to 42 kDa. The molecular weights of two pepsins (I and II) from orange roughy stomach were estimated to be approximately 33.5 and 34.5 kDa, respectively (Xu *et al*., 1996). Sanchez-Chiang *et al*. (1987) reported that the molecular weights of two pepsins from salmon stomach were estimated to be approximately 32 and 27 kDa by gel filtration. The molecular weights of two pepsins from polar cod stomach were estimated by SDS-PAGE to be approximately 42 and 40 kDa (Arunchalam & Haard, 1985). Klomklao *et al*., (2007) reported that pepsins A and B from the stomach of pectoral rattail (*Coryphaenoides pectoralis*) had apparent molecular weights of 35 and 31 kDa, respectively, as estimated by SDS-PAGE and gel filtration on Sephacryl S-200.

Pepsins and pepsin-like enzymes can be extracted from the digestive glands of marine animals such as Atlantic cod (*Gadus morhua*) (Brewer *et al*., 1984), capelin (*Mallotus villosus*) (Gildberg & Raa, 1983), polar cod (*Boreogadus saida*) (Arunchalam & Haard, 1985), sardine (*Sardinos melanostica*) (Noda & Murakami, 1981) and Monterey sardine (*Sardinops sagax caerulea*) (Castillo-Yanez *et al*., 2004). Several methods are suggested for the purification of pepsins from marine animals by Klomklao (2008). Cold-adapted pepsins from Atlantic cod (*Gadus morhua*) are being recovered in Norway; they have commercial applications in the cheese industry for cold renneting of milk and in the fish-feed industry to assist in digestion (Simpson, 2000).

Pepsin activity is highly dependent on pH values, temperature and the type of substrate. Hemoglobin is the substrate most frequently used for the determination of pepsin activity

(De Vecchi & Coppes, 1996; Klomklao *et al*., 2004). Klomklao (2008) mentions several determinations reported previously in the literature.

A second type of acid protease of interest is chymosin, which is typically present in the digestive compartment of young ruminant stomachs. Chymosin has been identified in the stomachs of marine organisms such as carp and harp seals (Shahidi & Janak Kamil, 2001). Cheddar cheese prepared with seal chymosins have been reported to have higher sensory scores than cheese made with calf rennet (Simpson, 2000). Chymosin has been described as an acid proteinase with some distinctive characteristics compared to other acid proteinases. For example, it is most active and stable around pH 7 and has a relatively narrower substrate specificity (Simpson, 2000). Digestive proteinases with chymosin activity were isolated as zymogens from the gastric mucosa of young and adult seals (*Pagophilus groenlandicus*) (Shamsuzzaman & Haard, 1984) by a series of chromatographies, including DEAE-Sephadex A-50, Sephadex G-100 and Z-D-Phe-T-Sepharose gel. The enzyme had an optimal pH range of 2.2–3.5 for hemoglobin hydrolysis. The chymosins from marine animals did not hydrolyze the specific synthetic substrate for pepsin (i.e. *N*-acetyl-L-phenylalanine diiodotyrosine (APD)) and were also more susceptible to inactivation by urea (Simpson, 2000).

Gastricsin is a gastric protease that has been identified in marine organisms and has similar properties to pepsin. However, it differs from pepsin in structure and certain catalytic properties (Simpson, 2000). Two gastricsin isozymes were purified and characterized from the gastric juices of hake (Sanchez-Chiang & Ponce, 1981). The optimum pH for the hydrolysis of hemoglobin by hake gastricsins was 3.0, which was similar to that of mammalian gastricsins. Hake gastricsins were stable up to pH 10 but rapidly inactivated at higher pH values (Sanchez-Chiang & Ponce, 1981). The latter property would appear to distinguish the gastricsins from pepsins (Simpson, 2000).

4.4.1.2 Serine Proteases

Digestive serine proteases are present in the pyloric ceca, the pancreatic tissues and the intestines of animals and have been reported in numerous species of Archaea (Eichler, 2001). Serine proteases are inactive at acidic pH and have high activity under neutral to slightly alkaline conditions (Simpson, 2000). Although fish serine proteases are quite similar to their mammalian counterparts, they have been reported to be more active under alkaline rather than neutral conditions (Shahidi & Janak Kamil, 2001). These enzymes have been described as a group of endoproteinases with a serine residue in their catalytic site. This family of proteases is characterized by the presence of a serine residue together with an imidazole group and aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The proteases in the serine subclass all have the same first three digits: EC 3.1.21. Some of the most well-known serine proteases from marine sources include trypsins, chymotrypsins, collagenases and elastases.

Trypsins (EC 3.4.21.4), mainly members of a large family of serine proteases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues (Klomklao *et al*., 2006). Trypsins play major roles in biological processes that include digestion and activation of zymogens of chymotrypsin and other enzymes (Cao *et al*., 2000). Trypsins from marine animals resemble mammalian trypsins with respect to their molecular size (22–30 kDa), amino acid composition and sensitivity to inhibitors. Their pH optima for the hydrolysis of various substrates are from 7.5 to 10, while their temperature optima for hydrolysis of those substrates range from 35 to 65 °C (De Vecchi & Coppes, 1996).

Trypsin-like enzymes can be found in both cold- and warm-water marine organisms, such as stomachless bone fish (*Carassius auratus gibelio*) and sardines (Shahidi & Janak Kamil, 2001). They can inactivate enzymes such as PPO, giving them potential application in the food industry for the prevention of undesired color changes in PPO-containing products such as shrimp and fruit (Haard *et al*., 1994). Trypsins and other digestive proteases have also been isolated from crustaceans and mollusks in an organ called the hepatopancreas, which is a combination of the mammalian liver and pancreas (Shahidi & Janak Kamil, 2001). A popular marine source for trypsins is the Atlantic cod, which contains cold-adapted enzymes that have catalytic activity between 4 and 55 °C and are sensitive to inactivation by autolysis, low pH and/or moderate heat (65 °C or above). Research conducted over the past 2 decades has helped to develop industrial methods for the extraction of cold-adapted trypsins from fish-processing byproducts such as the pyloric cecum of cod. These trypsins have promising applications in areas of food processing that require protein digestion at low temperatures in order to avoid undesirable chemical reactions and bacterial contamination (Gudmundsdottir & Palsdottir, 2005). For example, cod trypsins have been used for low-temperature curing of herring (Matjes) and in squid fermentation, thereby accelerating the ripening process, which traditionally takes about 1 year (Haard *et al*., 1994; Simpson, 2000). A number of studies carried out on trypsins from fish viscera are described in a review article by Klomklao (2008).

Trypsins from marine animals tend to be more stable at alkaline pH but are unstable at acidic pH. On the other hand, mammalian trypsins are most stable at acidic pH (Simpson, 2000; Klomklao *et al*., 2006a). Trypsin from tongol tuna spleen showed high stability in the pH range 6–11, but inactivation was more pronounced at pH values below 6 (Klomklao *et al*., 2006). Klomklao *et al*., (2007) also reported that skipjack tuna-spleen trypsins were stable in the pH range 6–11 but were unstable at pH below 5. The stability of trypsins at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez *et al*., 2005). Trypsin might undergo denaturation under acidic conditions, where conformational changes take place and the enzyme cannot bind to the substrate properly (Klomklao *et al*., 2006).

Chymotrypsin is another member of a large family of serine proteases that functions as a digestive enzyme. Chymotrypsins have been isolated and characterized from marine species such as anchovy (Heu *et al*., 1995), Atlantic cod (Asgiersson & Bjarnason, 1991) and Monterey sardine (Castillo-Yanez *et al*., 2006). In general, these enzymes are single-polypeptide molecules with molecular weights between 25 and 28 kDa. They are most active within the pH range 7.5–8.5 and are most stable at around pH 9 (Simpson, 2000). Chymotrypsin has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophan and leucine (Simpson, 2000).

Another type of serine protease, elastase, is produced by the pancreas and is meant for the digestion of proteins such as elastin, a fibrous protein found in connective tissues. Elastase is an intestinal protease that operates at alkaline pH and has been isolated from marine animals such as carp, catfish and Atlantic cod (Shahidi & Janak Kamil, 2001; Simpson, 2000). Shotton (1970) reported that elastase can digest a wide variety of other proteins in addition to native elastin. Gastric elastase enzymes have been recovered from marine and freshwater fish species such as carp (Cohen & Gertler, 1981), catfish (Clark *et al*., 1985) and Atlantic cod (Asgeirsson & Bjarnason, 1993; Asgeirsson *et al*., 1998; Gildberg & Overbo, 1990; Raa & Walther, 1989). Furthermore, Yoshinaka *et al*., (1985) reported pancreatic elastase activity in several freshwater and marine fish species. Asgeirsson &

Bjarnason (1993) observed lower average hydrophobic character amino acid residues in cod elastase compared with porcine elastase and concluded there were weaker interactions in the hydrophobic core of the molecule, leading to its better adaptation for low-temperature environments.

A fourth category of marine serine protease is the digestive collagenases, which have been isolated from the digestive organs of many fish and from the hepatopancreas of marine invertebrates such as crab, prawn and lobster (Haard *et al*., 1994; Shahidi & Janak Kamil, 2001). Collagenases are thought to be one of the principal compounds responsible for the flesh mushiness observed in the seafood industry following handling and storage; these enzymes therefore have potential applications as meat tenderizers in the manufacturing of high-quality meat and meat products (Haard *et al*., 1994).

Collagenolytic serine proteases differ from muscle collagenases, which belong to zinc metalloenzyme proteases, and their physiological function in several organisms is attributed to their digestive power (Kristjansson *et al*., 1995). These enzymes differ from true muscle collagenases and are known to hydrolyze the triple type-I, -II and -III tropocollagen molecules due to existing differences in their active sites (Zefirova *et al*., 1996). They display both trypsin-like and chymotrypsin-like activities (Haard, 1994; Haard & Simpson, 1994; Zefirova *et al*., 1996). Collagenolytic serine proteases have been isolated and characterized from the digestive tracts of various fish and aquatic invertebrates, as mentioned by Shahidi & Janak-Kamil (2001). These enzymes are most active in the pH range 6.5–8.0 and are inactivated at pH values lower than 6 (Haard & Simpson, 1994). Furthermore, Zefirova *et al*., (1996) reported that practical use of these enzymes is restricted due to their low heat stability; it has also been shown that such collagenases lose their activity even at 40 °C. Several groups have identified collagenolytic serine proteases as being responsible for the autolysis of crustacean muscle tissues during post-harvest storage (Baranowski *et al*., 1984; Kawamura *et al*., 1984; Salem *et al*., 1970).

4.4.1.3 Cysteine or Thiol Proteases

Thiol or cysteine proteases are a group of endopeptidases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (−SH) group furnished by the active-site cysteine residue to be intact, hence the name 'thiol' or 'cysteine' proteases (Mihalyi, 1978). The thiol proteases are inhibited by heavy-metal ions and their derivatives, as well as by alkylating and oxidizing agents (Mihalyi, 1978). The first three digits common to thiol proteases are EC 3.4.22. Like the gastric proteases, digestive cysteine or thiol proteases are active at acidic pH and inactive at basic pH. They are important components of the hepatopancreas of many marine crustaceans and are responsible for over 90% of the protease activity in the hepatopancreas in short-finned squid (*Illex illecebrosus*) (Raksakulthai & Haard, 2001).

Cathepsin B is one example of a marine-derived digestive thiol protease. Only a few marine sources have been identified for cathepsin B, including surf clam (*Spisula solidissima*), horse clam (*Tresus capax*) and mussel (*Perna perna* L.) (Simpson, 2000). Generally, cathepsin B from marine animals is a single polypeptide chain, with molecular sizes ranging from 13.6 to 25.0 kDa. Cathepsins from different species display maximum activity over a broad pH range of 3.5–8.0. Cathepsin B is activated by Cl^- ions and requires sulfhydryl-reducing agents or metal-chelating agents for activity (Zeef & Dennison, 1988).

Cathepsin L from carp hepatopancreas was purified using ammonium sulfate precipitation and a series of chromatographies, in which the enzyme had an affinity toward

Concanavalin A and Cibacron Blue F3GA (Aranishi *et al*., 1997). Its homogeneity was established by a native PAGE. Two protein bands corresponding to molecular weights of 30 and 24 kDa were found on SDS-PAGE. The enzyme exhibited a maximal activity against Z-Phe-Arg-MCA at pH 5.5–6.0 and 50 °C. Both tested cysteine protease inhibitors, TLCK and chymostatin, markedly inhibited its activity, whereas the other serine protease inhibitors and the metalloproteinase inhibitor showed no inhibitory activity on the enzyme (Aranishi *et al*., 1997). Cathepsin S from the hepatopancreas of carp (*Cyprinus carpio*) was purified by ammonium sulfate fractionation followed by SP-Sepharose, Sephacryl S-200 and Q-Sepharose (Pangkey *et al*., 2000). The molecular weight of purified protease was 37 kDa, estimated by SDS-PAGE; this protease hydrolyzed Z-Phe-Arg-MCA but not Z-Arg-MCA. The optimal temperature and pH for the hydrolysis of Z-Phe-Arg-MCA were 37 °C and 7. This protease activity was inhibited by E-64, leupeptin, 5-5′-dithiobis (2- nitro-benzoic acid) and *p*-tosyl-lys-chloromethylketone.

4.4.1.4 Metalloproteinases

The metalloproteinases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations (Simpson, 2000). Chemical-modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteinases (Whitaker, 1994). The metalloproteinases are inhibited by chelating agents such as 1,10-phenanthroline, EDTA and sometimes the simple process of dialysis. The metalloproteinases have been characterized from marine animals (e.g. rockfish, carp and squid mantle) but have not been found in the digestive glands, except in muscle tissue (Simpson, 2000). Metalloproteinases do not seem to be common in marine animals (Simpson, 2000). However, Sivakumar *et al*., (1999) purified collagenolytic metalloproteinase with gelatinase activity from carp hepatopancreas by ammonium sulfate fractionation and gel-filtration chromatography. The enzyme had a molecular weight of 55 kDa and was active against native type-I collagen. Optimum temperature and pH were 25 °C and 7.0–7.5. The activity of the active form of the enzyme was strongly inactivated by 10 mM EDTA.

4.5 LIPASES

Lipases or acylglycerol acylhydrolases (EC 3.1.1.3) are mainly produced in the pancreas and catalyze the hydrolysis of triacylglycerols into free fatty acids, mono- and/or diacilglycerols and glycerol at the lipid–water interface. Some findings suggest that fish may possess a mammalian-type pancreatic lipase and colipase system and homologues of bile salt-activated lipase. Mammalian-type pancreatic lipase has been isolated from rainbow trout (*Oncorhynchus mykiss*) pancreas (Leger *et al*., 1977) and from sardine (*Sardinella longiceps*) hepatopancreas (Mukundan *et al*., 1986).

Both sn-1,3-specific and nonspecific lipases have been found in fish (Gjellesvik *et al*., 1992, 1994; Leger, 1985). Lipase preparations (acetone-ether powders) of scallop (*Patinopecten yessoensis*) hepatopancreas have high specificity for the sn-1(3)-position of long-chain triacylglycerol molecules; therefore, scallop hepatopancreas represents a potential source of 1,3-specific lipases (Itabashi & Ota, 1994). A nonspecific lipase predominates in the intestine of Arctic charr (*Salvelinus alpinus* L.). Interestingly, lipolytic

activity has been detected along the whole intestinal tract of Arctic charr, cod (Lie *et al*., 1987), *Chanos chanos* (Borlongan, 1990) and turbot (Koven *et al*., 1994).

Marine fish require polyunsaturated n-3 fatty acids, namely eicosapentanoic acid (20:5n-3) (EPA) and docosahexanoic acid (22:6n-3) (DHA), as essential fatty acids for normal growth (Ackman & Kean-Howie, 1995; Bell *et al*., 1987). Fatty acid digestibility in fish decreases with increasing chain length and increases with greater degree of unsaturation (Gjellesvi *et al*., 1992; Kove *et al*., 1994; Lie *et al*., 1985). Polyunsaturated fatty acids (PUFAs), followed by monounsaturated fatty acids (MUFAs) and then saturated fatty acids, are good substrates for fish digestive lipases (Gjellesvik, 1991; Lie *et al*., 1985). Marine sources of lipases include Atlantic cod, seal, salmon, sardines, Indian mackerel and red sea bream. Lipases have valuable applications in the food industry because of their distinct specificities and their ability to catalyze processes such as esterification, hydrolysis and exchange of fatty acids in esters (Shahidi & Wanasundara, 1998). These characteristics provide numerous opportunities for the use of marine lipases in the fats and oils industry, such as in the production of triglycerides enriched with n-3 LC-PUFAs. Production of these triacylglycerols using commercially available lipases from nonmarine sources is generally difficult because nonmarine lipases either are less specific than marine lipases or have different specificities than is desired (Shahidi & Janak Kamil, 2001). A marine lipase isolated from Atlantic cod was shown to preferentially hydrolyze LC-PUFAs over shorter-chain fatty acids (Lie & Lambertsen, 1985), and lipase-assisted hydrolysis of seal blubber oil and menhaden oil has been used for enrichment of acylglycerols with n-3 LC-PUFAs (Shahidi & Wanasundara, 1998). The pH optimum of most lipases lies between 7 and 9, although there are a few exceptions. Lipases are active over a very wide temperature range of −20 to 65 °C, but the more usual range is 30–45 °C (Jensen, 1982).

The presence of homologues of bile salt-activated lipase involved in triglyceride hydrolysis has been reported in fish species such as the anchovy (*Engraulis mordax*), striped bass (*Morone saxatilis*), pink salmon (*Oncorhynchus gorbuscha*) (Leger, 1985), leopard shark (*Triacus semifasciata*) (Patton *et al*., 1977), rainbow trout (*Salmo gaidnerii*) (Tocher & Sargent, 1984) and dogfish (Raso & Hultin, 1988). The properties of cod (*Gadus morhua*) lipase have been compared with those of mammalian bile salt-activated lipases (Gjellesvik, 1991). In addition, mammalian-type bile salt-activated lipase has been isolated from cod pyloric caeca (Gjellesvik *et al*., 1992), red sea bream (*Pagrus major*) hepatopancreas (Iijima *et al*., 1998) and Atlantic salmon (*Salmo salar*) pancreas (Gjellesvik *et al*., 1994).

4.5.1 Phospholipases

It is known that juvenile and adult fish are able to synthesize phospholipids (Geurden *et al*., 1995). Dietary sources of phospholipids such as phosphatidylcholine and phosphatidylinositol, rich in ω3 PUFAs, are commonly required by fish (Cowey & Sargent, 1979). Despite their importance, documentation of fish muscle phospholipases is poor due to difficulties in their isolation and characterization (Aaen *et al*., 1995). Purification of PLA_2s from pollock (*Pollachius virens*) (Audley *et al*., 1978), pyloric caeca (Iijima *et al*., 1997), red sea bream (*Pagrus major*) hepatopancreas (Ono & Iijima, 1998), trout (*Salmo gairdneri*) liver (Neas & Hazel, 1985) and cod (*Gadus morhua*) muscle (Aaen *et al*., 1995) has been reported.

4.5.2 Chitinolytic Enzymes

Enzymes with chitinolytic activity include 'true' chitinases and lysozymes (Clark *et al*., 1988). True chitinases are further divided into chitinase (EC 3.2.1.14) and chitobiase (EC 3.2.1.30) (Clark *et al*., 1988; Danulat & Kausch, 1984). Chitinase is an endo-β-*N*-acetylglucosaminidase that produces random hydrolysis of β-1,4-linkages in poly- and oligosaccharides of *N*-acetylglucosamine (Clark *et al*., 1988; Fang *et al*., 1979; Flach *et al*., 1992). The dimer of β-1,4-linked *N*-acetylglucosamine (chitobiose) is the main digestion product of chitinase activity. Chitobiase (exo-*N*-acetyl-β-D-glucosaminidase or NAGase) specifically hydrolyzes the β-1,4-linkages of such dimers to give single units of *N*-acetylglucosamine (Clark *et al*., 1988; Flach *et al*., 1992; Lindsay, 1984).

Lysozymes have a similar distribution in nature to chitinases and are generally defined as *N*-acetylmuramylhydrolases (EC 3.2.17); they are able to promote lysis of the cell walls of Gram-positive bacteria (Clark *et al*., 1988; Fang *et al*., 1979) due to the hydrolysis of the β-1,4 linkage between C-1 of *N*-acetylmuramic acid and C-4 of *N*-acetylglucosamine in the cell wall (Koga *et al*., 1999).

In nature, chitinolytic enzymes are utilized for the degradation of chitin during molting of insects and crustaceans and as digestive aids, as they are able to disrupt the exoskeletons of prey, allowing access to the soft inner tissues. The genes for these chitinolytic enzymes have been cloned from several different organisms (Shahidi & Abuzaytoun, 2005). Chitinases have been identified in the digestive tracts of numerous fish, in shellfish and shellfish waste, in squid liver and in octopus saliva. Chitinases have a wide range of potential applications in the food industry; for example, they can replace hydrochloric acid in the conversion of chitin into commercially available oligomeric units, resulting in products with more consistent characteristics (Shahidi & Janak Kamil, 2001).

Chitinolytic enzymes are a prime tool for the conversion of chitin into oligomeric units without the use of chemical depolymerization, in which concentrated hydrochloric acid is employed, which results in commercial products with inconsistent physicochemical characteristics. Research on the preparation of chitin and chitosan oligomers and the study of their physiological activities has continuously attracted much attention in the food and pharmaceutical fields, due to their versatile antimicrobial and antitumor activity and immune-enhancing and protective effects against some infectious pathogens (Shahidi *et al*., 1999). Therefore, chitinolytic enzymes from fish and aquatic invertebrates have a broad range of potential applications in different areas. Furthermore, they may be used as bacteriolytic enzymes, mainly due to their ability to degrade peptidoglycan components of the cell walls of bacteria. Enzymes and lysozymes with antibacterial properties have been recovered from Arctic scallop (*Chlamys islandica*) (Raa, 1997) and several fish species in recent years. Generally, lysozymes isolated from marine invertebrates and fish differ from lysozymes of warm-blooded animals and plants, and at low temperatures may be up to several hundred times more active. Marine lysozymes have a potential antibacterial action due to the more acidic nature of the molecule (Raa, 1990).

4.5.3 Transglutaminase

Transglutaminase (EC 2.3.2.13) is an endogenous fish enzyme utilized in the food industry for the breakdown of specific compounds. Transglutaminase is unique in that it is able to modify protein functions by promoting crosslinks (Ashie & Lanier, 2000), which is known to reinforce the thermal myosin gelation that occurs during surimi production.

They form intra- and intermolecular crosslinks between myosin heavy chains in a process known as 'setting' (Lee *et al*., 1997; Nakahara *et al*., 1999; Seki *et al*., 1990). This setting phenomenon, also called 'suwari', is one of the most important processes in the manufacture of surimi-based products. Transglutaminase catalyzes an acyl-transfer reaction between γ-carboxamide groups of glutamine residues in proteins, polypeptides and a variety of primary amines. When the ε-amino group of lysine and lysyl residue acts as an acyl acceptor, ε-(γ-glutamyl) lysine crosslinks are formed (Kumazawa *et al*., 1996).

Transglutaminase activity has been found in the muscle of red sea bream (*Pagrus major*), rainbow trout (*Oncorhynchus mykiss*), atka mackarel (*Pleurogrammus azonus*) (Muruyama *et al*., 1995; Yasueda *et al*., 1994), scallop (*Patinopecten yessoensis*), botan shrimp (*Pandalus nipponensis*) and squid (*Todarodes pacificus*) (Nozawa *et al*., 1997), and in the liver of walleye pollock (*Theragra cHogramma*) (Kumazawa *et al*., 1996). Since the activity of endogenous fish transglutaminase decreases rapidly after capture and is almost completely destroyed by freezing, in order to obtain high-quality surimi production should be carried out at the sea. However, the production cost at sea is much higher than that of onshore production (An *et al*., 1996). In order to overcome this problem, commercially available transglutaminases may be used in the surimi industry. Activa®, for example, may be added to surimi of low grade in order to improve the elastic properties of kamaboko by forming ε-(γ-glutamyl) lysine crosslinks in the gels (Oshima, 1996). The effects of transglutaminase on crosslinking during surimi production have been investigated using transglutaminase inhibitors (Kumazawa *et al*., 1995); since transglutaminase generally requires the presence of calcium for full activity, EDTA and NH_4Cl have been used as transglutaminase inhibitors—EDTA is a calcium-chelating agent that inhibits the setting of protein molecules (Kumazawa *et al*., 1995).

On the other hand, Chen *et al*., (2003) reported a microbial transglutaminase that catalyzes gel formation from gelatin. Unlike typical gelatin-based gels, transglutaminase-catalyzed gels are thermally irreversible, presumably due to the strong crosslinking ability of this enzyme. The addition of chitosan was reported to promote gel strength and result in more rapid gel formation.

4.6 INDUSTRIAL APPLICATIONS

The use of marine-based enzymes has not been completely explored, despite their offering several advantages over other enzyme sources. To date, a limited number of applications have been developed for marine enzymes, some of which are listed in Table 4.3 and discussed in this section.

In their review, Zambonino-Infante & Cahu (2007) report recent data on dietary modulation of digestive enzymes, with a particular emphasis on the molecular and hormonal mechanisms controlling enzyme expression in larvae. These data show that nutrition can affect many mechanisms controlling fish larvae development. The larval stage represents a transitional period in which both ontogeny and growth cause substantial changes in structure, physiology, size and body shape. The authors refer to how the dietary modulation of pancreatic and intestinal enzymes involved in protein digestion can provide useful information concerning the nature and molecular form of a dietary protein supply adequate for larval stages. These proteolytic enzymes are mainly located at three different levels of the digestive tract: the stomach (pepsin), pancreas (trypsin, chymotrypsin, elastase) and intestine (membranous and cytosolic enzymes).

Table 4.3 Some food-industry applications of marine enzymes.

Application	Enzyme	Source
Cold renneting milk clotting	Pepsin	Cold-adapted marine organisms
Extraction of carotenoprotein from shrimp processing wastes	Trypsin	Atlantic cod, bluefish pyloric ceca
Removal of skin, scales and membranes from fish	Proteolytic and glycolytic enzymes	Cod viscera
Caviar production	Pepsin	Atlantic cod, orange roughy
Milk stability improvement	Trypsin	Greenland cod
Cheese manufacture	Chymosyn	Harp seal
Preparation of fish-protein hydrolysates and concentrates	Proteolytic enzymes	Shark viscera, tuna pyloric ceca
Ripening and fermentation of fish product	Cathepsins	Cod viscera, squid liver
Production of structured lipids with polyunsaturated fatty acids	Lipase	Sardine viscera, tuna

Anxo Murado *et al*. (2009) report the recovery of proteolytic and collagenolytic activities from rayfish (*Raja clavata*) viscera wastes. Initially, different gastrointestinal-tract byproducts (stomach, duodenum section including pancreas, final intestine) were evaluated. The extracts from the proximal intestine yielded the highest values of both enzymatic activities. Optimal conditions for protease activity quantification were established at pH 6, 40 °C and incubation time less than or equal to 20 minutes. Overcooled acetone was found to be best option for recovery of enzymatic activities. The authors demonstrated the usefulness of viscera waste from rayfish in obtaining proteolytic and collagenolytic activities in order to upgrade fishing-industry byproducts.

Lipases occupy a place of prominence among biocatalysts due to their novel and multifold applications in the food industry related to oleochemistry, biotechnology and nutrition (Hou, 2002). Modification of fats and oils for the preparation of specific structured triacylglycerols and phospholipids containing the n-3 PUFAs has been of great interest (Bornscheuer *et al*., 2003; Lyberg *et al*., 2005). Apparently, long-chain PUFAs are not preferred substrates for most of the commercially available lipases (Senanayake & Shahidi, 2002), while lipases from cod (*Gadus morhua*), rohu (*Labeo rohita Hamilton*), sea bream (*Pragus major*), atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynus mykiss*) have shown high specificity for these fatty acids (Gjellesvik *et al*., 1992; Halldorsson *et al*., 2004; Iijima *et al*., 1998; Lie & Lambertsen, 1985). Noriega-Rodríguez *et al*. (2009) screened the lipolytic activity of a semipurified lipolytic enzyme (SLE) from the viscera of sardine (*Sardinops sagax caerulea*) on the lipolysis of olive, Menhaden and sardine oil. A lipolytic enzyme was partially purified from the crude extract of sardine viscera by fractional precipitation followed by ultrafiltration (30 kDa). The main tissues found in sardine viscera were pyloric caeca (19.0% (w/w)), digestive tract (13.0% (w/w)), liver (4.8% (w/w)) and pancreas (1.5% (w/w)). The results showed that pancreas had the highest lipolytic activity. There were no significant differences in lipolytic activity between pyloric caeca, intestine and liver ($P < 0.05$). Specific activity of the SLE increased 47-fold after extraction and fractionation, with a yield of 0.34% calculated for the whole viscera weight. The lipolytic activity of SLE from sardine viscera increased threefold when sardine oil was used as substrate. The results of this study confirm the potential importance of lipases from marine sources.

Nasri *et al*. (2011) studied some biochemical characteristics of crude alkaline protease extracts from the viscera of goby (*Zosterisessor ophiocephalus*), thornback ray (*Raja clavata*) and scorpionfish (*Scorpaena scrofa*), in order to investigate their applications in the deproteinization of shrimp wastes. At least four caseinolytic protease bands were observed in zymograms of each enzyme preparation. The optimum pHs for enzymatic extraction of *Z. ophiocephalus*, *R. clavata* and *S. scrofa* were 8.0–9.0, 8.0 and 10.0, respectively. Interestingly, all of the enzyme preparations were highly stable over a wide range of pH, from 6.0 to 11.0. The optimum temperatures for enzyme activity were 50 °C for *Z. ophiocephalus* and *R. clavata* and 55 °C for *S. scrofa* crude alkaline proteases. Proteolytic enzymes showed high stability towards non-ionic surfactants (5% Tween 20, Tween 80 and Triton X-100). In addition, crude proteases of *S. scrofa*, *R. clavata* and *Z. ophiocephalus* were found to be highly stable towards oxidizing agents, retaining 100, 70 and 66%, respectively, of their initial activity after incubation for 1 hour in the presence of 1% sodium perborate. These enzymes were, however, highly affected by the anionic surfactant SDS. The crude alkaline proteases were tested for the deproteinization of shrimp waste in the preparation of chitin. All proteases were found to be effective in the deproteinization of shrimp waste. The protein removals after 3 hours of hydrolysis at 45 °C with an enzyme/substrate ratio (E/S) of 10 were about 76, 76 and 80% for *Z. ophiocephalus*, *R. clavata* and *S. scrofa* crude proteases, respectively. These results suggest that enzymatic deproteinization of shrimp wastes by fish endogenous alkaline proteases could be applicable to the chitin production process.

Rossano *et al*. (2011) mention that the enzymes extracted from whole crustaceans *Munida* (fam. *Galatheideae*, ord. *Decapodi*), mainly serine proteases, showed high caseinolytic and moderate clotting activities. These species were fished in the southern Adriatic Sea and their proteolytic activities were characterized and tested for potential application in cheese manufacture. Analysis by 2D zymography of the digestive enzymes extracted from *Munida* hepatopancreas showed the presence of several isotrypsin- and isochymotrypsin-like enzymes in the range of 20–34 kDa and pH of 4.1–5.8. Moreover, specific enzymatic assays showed the presence of aminopeptidases and carboxypeptidases A and B. Overall, optimum activity was achieved at pH 7.5 and 40–45 °C. Caseinolytic activity, determined both spectrophotometrically and by SDS gel electrophoresis, indicated higher activity on β-casein than on α-casein. Miniature Cheddar- and Pecorino-type cheeses were manufactured by adding starter, rennet and *Munida* extracts to milk. Reverse-phase HPLC and MALDI-ToF mass spectrometry showed a more complex pattern of proteolytic products in cheeses made using *Munida* instead of chymosin. *Munida* extracts were found to degrade the chymosin-derived β-casein fragment f193-209, one of the peptides associated with bitterness in cheese.

It is important to mention that most of this field has been explored in Japan, the UK and Denmark, with the Icelandic Fisheries Laboratory developing the most important and significant research.

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5 Kamaboko Proteins as a Potential Source of Bioactive Substances

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5.1 INTRODUCTION

The term 'functional foods' or 'food for specified health uses' was first introduced in Japan in the mid 1980s and refers to processed foods that contain ingredients which aid specific bodily functions in addition to being nutritious. These ingredients are officially approved by the Consumer Affairs Agency of the Government of Japan as having physiological effects on the body (Table 5.1). Food for specified health uses is intended to be consumed for the maintenance and promotion of health or for special health uses. As of November 21, 2011, 971 products are licensed as food for specified health uses in Japan (Fig. 5.1) (http://www.caa.go.jp/foods/index.html). The following types of food for specified health uses are available (http://www.caa.go.jp/foods/index.html): standardized food for specified health uses (standards and specifications are established for foods which have sufficient evidence of their health benefits; a food is approved when it meets the standards and specifications) (Fig. 5.2a); reduction of disease risk food for specified health uses (the reduction of disease risk claim is permitted when reduction of disease risk is clinically and nutritionally established in an ingredient) (Fig. 5.2a); and qualified food for specified health uses (food with a health function that is not substantiated by scientific evidence but which shows a certain effectiveness will be approved as qualified food for specified health uses) (Fig. 5.2b). The market is growing at a rate of about 10% per year, and the industry was claimed to be worth about ¥600 billion in 2009 (Fig. 5.3) (http://www.caa.go.jp/foods/index.html). With respect to public health, the ratio of intestinal regulation is decreasing and that of natural fat and body fat is increasing year on year (Fig. 5.4).

The functional foods category is not recognized legally in the USA. Many organizations have proposed definitions for this area of food and nutrition science. The Institute of Medicine of the National Academies, Food and Nutrition Board defines functional foods as any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains.

The Richardson Centre for Functional Foods and Nutraceuticals is dedicated to the discussion, discovery and development of functional foods and nutraceuticals, with a focus

Marine Proteins and Peptides: Biological Activities and Applications, First Edition. Edited by Se-Kwon Kim.

Table 5.1 Approved foods for specified health uses in Japan and their principal ingredients.

Specified health uses	Principal ingredients exhibiting health functions
Modification of gastrointestinal conditions	Oligosaccharides, lactose, bifidobacteria, lactic acid bacteria, dietary fiber, ingestible dextrin, polydextrol, guar gum, psyllium seed coat
Blood cholesterol levels	Chitosan, soybean protein, degrared sodium alginate
Blood sugar levels	Indigestible dextrin, wheat albumin, guava tea polyphenol, L-arabiose
Blood pressure	Lactotripeptide, casein dodecaneptide, tochu leaf glycoside (geniposidic acid), sardine peptide
Dental hygiene	Paratinose, maltitiose, erythrytol
Cholesterol plus gastrointestinal conditions, triacylglycerol plus	Degraded sodium alginate, dietary fiber from psyllium seed husk
Mineral absorption	Calcium citrated malate, casein phosphopeptide, hem iron, fracuto-oligosaccharide
Ostepgenesis	Soybean isoflavone, milk basic protein
Triacylglycerol	Middle-chain fatty acid

Fig. 5.1 Example of a food for specified health uses.

(a) (b)

Fig. 5.2 Seals for food for specified health uses. (a) Seal for standardized food for specified health uses and reduction of disease risk food for specified health uses. (b) Seal for qualified food for specified health uses.

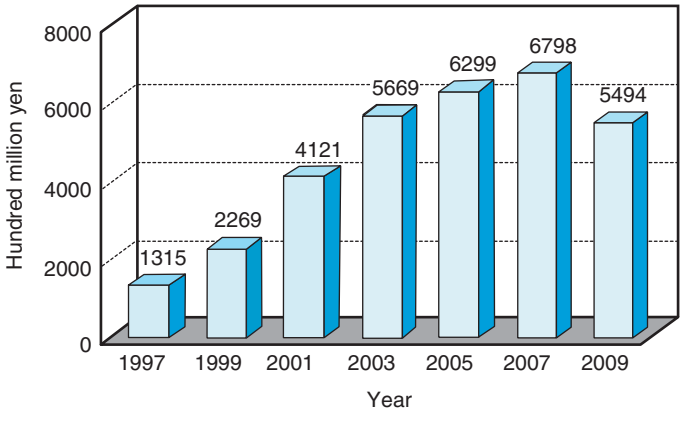

Fig. 5.3 Changes in the market size of food for specified health uses.

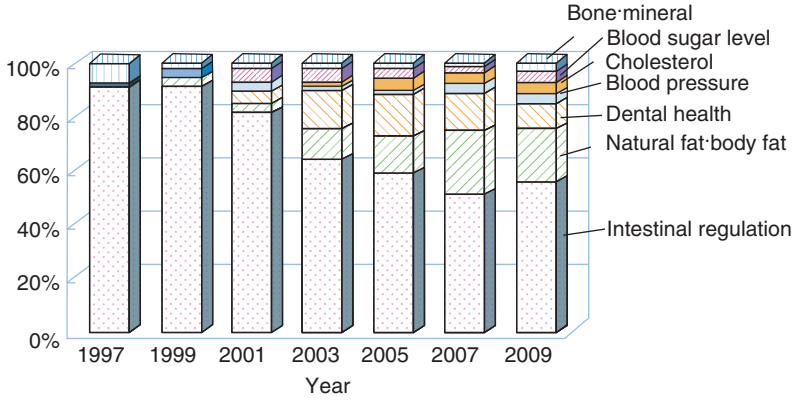

Fig. 5.4 Changes in percentage composition by public health use.

on the crops of the Canadian Prairies. Its center investigates functional food ingredients by examining the efficacy of novel bioactive materials such as plant sterols. The New Zealand Institute for Plant & Food Research has a dedicated research team for the study of functional foods. Its focus is in on both whole food and food extracts, as well as the gut-health and immune-function role of natural fruits and vegetables. It also works with mood foods and examines the delivery mechanisms behind components in foods and beverages designed to enhance mental performance, brain function and cognitive ability. The Functional Food Centre at Oxford Brookes University is the UK's first research center dedicated to functional food. It is known internationally for its work on the glycemic index and is the largest testing center in Europe. It provides customer-focused research and consultancy services to the food and nutrition industry, the UN and government agencies in the UK and overseas. Its research and consultancy portfolio not only concentrates on the scientific characteristics of food and nutrition but also integrates the scientific and social aspects of food. The center also focuses on areas such as satiety, dietary interventions, female nutrition and aging.

Health-conscious baby boomers have made functional foods the leading trend in the US food industry. But it is difficult to estimate the size of the functional food market globally because of the difficulty in understanding the adoption of functional food in diverse regions of the world.

Functional foods is one of the several areas of the food industry that has experienced rapid growth in recent years (Roberts, 2009). It is estimated that the global functional food market will reach US$176.7 billion in 2013, with a compound annual growth rate of 7.4%. The functional food sector will experience 6.9% compound annual growth rate, the supplement sector will rise by 3.8% and the functional beverage sector will be the fastest-growing segment, with 10.8% compound annual growth rate (Roberts, 2009). This kind of growth is fueled not only by industrial innovation and development of new products that satisfy the demand of health-conscious consumers but also by health claims covering a wide range of health issues. More significant is the potential of functional foods to mitigate disease, promote health and reduce health care costs. Malnutrition – both over- and undernutrition – continues to affect populations in developed and developing countries, resulting in increased disease and often catastrophic loss of life.

Foods can be modified by the addition of bioactive peptides, phytochemicals, omega-3 highly unsaturated fatty acids and probiotics and/or prebiotics (Berner & O'Donnell, 1998).

5.2 CREATION OF HEALTHIER AND SAFER FOODS

Enzymes are a type of protein and the perfect tools for food formulators. They are specific in their action on substrates relative to the chemical and physical processing of foods. They safely and effectively enable food technologists to selectively modify lipids, carbohydrates and proteins in complex food systems in order to create foods that taste good, improve health and are safe to consume. By selectively modifying a specific protein within a complex food system that contains lipids, carbohydrates and other proteins, an enzyme that breaks a specific peptide bond may prove to be more effective than a process such as the addition of a chemical oxidant and heating, which might affect the entire food substrate in various and often uncontrolled ways. Moreover, enzymes are usually regarded as label-friendly processing aids in foods, unlike with many chemical additives.

As food processors design new products to satisfy the growing consumer need for healthy and safe foods, enzymes often prove the preferred means of achieving specific objectives.

5.3 ENZYMATIC MODIFICATION OF FOOD PROTEINS

Proteolytic enzymes have been used to modify food proteins in order to improve texture, flavor, nutritional quality and functionality (Aubes-Dufau *et al*., 1995; Cabrera-Chávez *et al*., 2010; Jeon *et al*., 1999; Pedersen *et al*., 2005). The use of exopeptidases reduces bitterness in enzymatically modified cheese, while bacterial proteases restore emulsification properties to heat-denatured soy proteins. Proteolytic enzymes are being used to improve the health-and-safety aspects of food proteins (Dong *et al*., 2008; Jao & Ko, 2002, Je *et al*., 2005). Proteases are providing food technologists the tools to improve the palatability of reformulated low-carb/high-protein foods. Enzymatically modified proteins are replacing lipids and carbohydrates by providing mouth feel, texture and viscosity, and brothy notes. Proteins with altered fat- and water-binding properties have enhanced the ability to replace lipids and carbohydrates in food products. Enzymes can contribute to the healthiness of foods.

Proteases are also used to produce bioactive peptides with various functionalities, reduce the allergenicity of food proteins and protect food quality. The human body produces a large number of bioactive peptides, which function as biological messengers, stimulating or suppressing a wide range of physiological responses. It is suggested that specific peptides derived from food proteins also act as messengers and play a significant role in maintaining and promoting health and preventing disease (Je *et al*., 2007; Kang *et al*., 2011; Kim *et al*., 2007; Klompong *et al*., 2007; Mendis *et al*., 2005; Nagai & Nagashima, 2006; Nagai *et al*., 2005a,b, 2006, 2008; Qian *et al*., 2008; Ranathunga *et al*., 2006). These bioactive peptides can be produced *in vitro* by enzymatic hydrolysis of food proteins, such as eggs (Manso *et al*., 2008; Sakanaka & Tachibana, 2006; Sakanaka *et al*., 2004), wheat gluten (Kong *et al*., 2007; Sivri *et al*., 1999; Wang *et al*., 2008), beans (Li *et al*., 2008; Moure *et al*., 2006), bovine protein (Kim *et al*., 2001), porcine protein (Chang *et al*., 2007; Saiga *et al*., 2003; Yu *et al*., 2006), silk protein (Wu *et al*., 2008) and fish and seafood proteins (Morris *et al*., 2007; Sheih *et al*., 2009; Thiansilakul *et al*., 2007).

5.4 KAMABOKO

Kamaboko is one of the most popular and traditional fish products of Japan. Great progress has been made since the 1960s in developing and utilizing a large amount of Alaska pollock in the production of kamaboko, as well as in frozen surimi. Frozen surimi is an intermediate foodstuff with a high potential for long-term shelf life, for distribution over a wide area and for the production of various textured products. A variety of devices and new techniques have been introduced to the fish jelly industry in Japan. There are now many kinds of kamaboko, such as chikuwa, datemaki, flavior kamaboko (kani-kamaboko), naruto-maki, hanpen and tempra (satsuma-age) available in food markets around the world (Fig. 5.5). However, in Japan the annual production of kamaboko is drastically decreasing year by year, down to about 440 000 tons in 2010 (Table 5.2) (http://www.zenkama.com/). Recently, studies into its health effects have been vigorously performed in an attempt to increase the home consumption of kamaboko.

Fig. 5.5 Representative selection of commercially produced kamaboko.

Table 5.2 Changes of the annual production of commercially produced kamaboko in Japan.

Year	Total production (tons)	Yaki-chikuwa	Kamaboko	Age-kamaboko	Yude-kamaboko	Flavour-kamaboko	Other
1976	1 034 262	258 882	362 469	327 068	84 519	-	1 324
1981	823 729	174 377	288 920	269 211	73 184	-	18 037
1986	891 486	199 861	241 669	290 979	85 621	-	73 356
1987	854 568	195 351	228 350	276 209	81 025	-	73 633
1988	836 787	189 297	228 942	271 488	59 797	68 952	18 311
1989	836 229	190 451	231 411	277 618	56 307	60 688	19 754
1990	830 486	184 713	233 010	273 563	55 152	58 011	26 037
1991	829 121	181 693	223 021	279 607	54 148	65 270	25 382
1992	795 376	174 735	213 266	270 459	49 991	59 321	27 604
1993	774 472	169 607	209 152	265 960	47 541	55 493	26 719
1994	762 942	172 579	194 306	264 952	47 487	57 242	26 194
1995	756 713	173 445	186 486	265 346	45 918	59 365	26 153
1996	734 720	169 559	178 326	258 698	44 837	59 036	24 264
1997	725 146	166 940	171 186	258 927	43 818	58 136	26 139
1998	707 619	159 807	165 157	258 110	44 333	56 544	23 668
1999	691 628	164 065	161 061	252 891	42 445	52 292	18 874
2000	654 327	159 830	153 391	235 807	38 213	50 971	16 115
2001	648 093	153 285	155 255	233 304	40 394	50 451	15 404
2002	640 263	145 962	152 212	230 658	41 779	50 591	19 061
2003	614 497	141 530	142 326	223 357	36 956	49 618	20 710
2004	590 611	137 238	136 826	212 172	33 285	53 607	17 483
2005	589 099	139 343	131 943	211 477	32 676	55 894	17 766
2006	586 965	131 732	127 586	217 862	34 153	54 517	21 115
2007	554 026	121 584	125 462	204 757	32 211	-	70 012
2008	536 679	119 751	119 351	200 180	33 154	-	64 243
2009	491 328	104 405	104 925	189 861	31 700	-	60 438
2010	439 827	90 911	92 087	171 288	28 440	-	57 100

5.5 CHEMICAL PROPERTIES OF KAMABOKO

With about 90% of consumers believing that some foods have health benefits that go way beyond nutrition – and contain natural components that can help prevent and even cure disease – it is not surprising that individual foods and commodities that market their intrinsic healthiness or leverage individual nutritional assets have been among the greatest success stories in the functional food area (FMI, 2002; IFIC, 2002).

Mounting evidence supports the observation that functional foods containing physiologically active components, from either plant or animal sources, may enhance health. However, it should be stressed that functional foods are not magic bullets or universal panaceas for poor health habits.

Kamaboko contains about 60–80% water by weight (Table 5.3). The relatively large amounts of water are considered to be captured in the fine network structure of the protein, contributing to the characteristic texture, *ashi*. Chemically, kamaboko comprises water (54–76%), proteins (8–16%), lipids (0.5–12.0%), carbohydrates (7–18%) and salts (1–3%) (Table 5.3) (New Standard Tables of Food Composition in Japan, 2011). The lipid contents of kamaboko are lower than those of ham and sausage prepared from animal meat. It is well known that kamaboko is a high-protein and low-calorie food. In recent years, health-conscious consumers have increasingly sought functional foods in an effort to control their own health and well-being. Therefore, highly unsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and calcium-enriched kamaboko have been produced.

5.6 EXPRESSION OF HEALTH THE FUNCTION OF KAMABOKO PROTEINS

As has been mentioned, kamaboko contains about 8–16% protein (New Standard Tables of Food Composition in Japan, 2011). It can effectively produce bioactive peptides with various functionalities via proteolytic enzymes. Commercially produced kamaboko (donated by the All-Japan Kamaboko Makers Association; shiranuki-kamaboko, datemaki, yaki-chikuwa, hanpen, satsuma-age, kani-kamaboko) was digested by six kinds of proteolytic enzyme: pepsin, trypsin, papain, thermolysin, pronase E and chymotrypsin. High yields of hydrolysates were obtained – about 10.5–30.5% on a wet-weight basis – and the protein content was about 118.4–532.9 μg/mg hydrolysate powder. Generally, the yield of pepsin hydrolysate was high among these hydrolysates. As an example, the amino acid composition of the hydrolysates from the All-Japan Kamaboko Makers Association kamaboko is expressed as residues per 1000 total residues in Table 5.4. Glutamic acid is the most abundant amino acid, making up about 16–19% of all amino acids; it is formed by sugars contained in frozen surimi materials. Lysine (86–140 residues), aspartic acid (102–114 residues), alanine (68–96 residues), glycine (61–78 residues), and leucine (47–86 residues) are also highly abundant. In total, these five amino acids account for about 50% of all amino acids in the hydrolysates from commercially produced kamaboko (Table 5.4).

Table 5.3 Chemical composition of commercially produced kamaboko.

Species	Energy (kcal/100 g)	Water (g/100 g)	Proteins (g/100 g)	Lipids (g/100 g)	Carbohydrates (g/100 g)	Ash (g/100 g)	Salt (g/100 g)
Mushi-kamaboko	95	74.4	12.0	0.9	9.7	3.0	2.5
Sumaki-kamaboko	90	75.8	12.0	0.8	8.7	2.7	2.2
Yakinuki-kamaboko	103	72.8	16.2	1.0	7.4	2.6	2.4
Datemaki	196	58.8	14.6	7.5	17.6	1.5	0.9
Tsumire	113	75.4	12.0	4.3	6.5	1.8	1.4
Naruto	80	77.8	7.6	0.4	11.6	2.6	2.0
Hanpen	94	75.7	9.9	1.0	11.4	2.0	1.5
Satuma-age	139	67.5	12.5	3.7	13.9	2.4	1.9
Kani-kamaboko	90	75.6	12.1	0.5	9.2	2.6	2.2
Fish ham	158	66.0	13.4	6.7	11.1	2.8	2.3
Fish sausage	161	66.1	11.5	7.2	12.6	2.6	2.1

Table 5.4 Amino acid composition of enzymatic hydrolysates from kamaboko derived from walleye pollack surimi (residues/1000).

Amino acid	Pepsin	Trypsin	Papain	Thermolysin	Pronase E	Chymotrypsin
Asp	102	105	112	113	104	114
Thr[a]	51	46	47	39	42	46
Ser	55	52	49	41	44	46
Glu	172	188	159	170	159	164
Gly	65	61	66	70	78	76
Ala	86	92	77	87	68	96
1/2Cystine	7	5	5	11	10	8
Val[a]	56	52	49	33	48	41
Met[a]	30	25	35	45	43	38
Ile[a]	46	39	41	26	41	36
Leu[a]	86	85	72	47	62	48
Tyr	27	22	29	41	13	33
Phe[a]	28	21	30	42	40	38
Lys[a]	86	105	118	140	135	125
His[a]	18	16	18	13	22	13
Arg	50	55	48	38	39	33
Pro	35	32	43	44	53	44

[a]Essential amino acid.

5.7 ANTIOXIDATIVE ACTIVITIES OF KAMABOKO PROTEINS

The linoleic acid model system is used to evaluate the effect at the initiation stage of lipid peroxidation (Nagai & Nagashima, 2006). The antioxidative activities of enzymatic hydrolysates prepared by six kinds of proteolytic enzyme from commercially produced kamaboko were estimated. As shown in Table 5.5, the activity of each hydrolysate tended to increase with increasing concentration of these hydrolysates. The activities of the hydrolysates from the All-Japan Kamaboko Makers Association kamaboko and of hanpen were high among commercially produced kamaboko tested. The hydrolysates prepared by papain, pronase E and chymotrypsin had significantly higher antioxidative activities (Table 5.5). Papain hydrolyzes the peptide bonds with basic amino acids such as arginine, glutamic acid, glycine, histidine, lysine and tyrosine residues. Pronase E has a wide range of substrate specificities as it is a mixture of many kinds of protease. Moreover, chymotrypsin cleaves the peptide bonds of the C-terminus of hydrophobic amino acids such as alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan and valine. It is suggested that the large quantities of peptide produced by hydrolysis contribute to the antioxidative activities. On the other hand, it is also suggested that the higher activities of the hydrolysates from satsuma-age are due to the synergistic effects of the peptides produced by enzymatic hydrolysis and the Maillard-reaction products as melanoidin.

The hydroxyl radical is the most reactive free radical and is formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper and iron (Fig. 5.6). It has the highest 1-electron reduction potential and can react with everything in a living organism at second-order rate constants of 10^{9-10} mol/s (Fig. 5.6). Hydroxyl radicals react with lipids, polypeptides, proteins and DNA – especially thiamine

Table 5.5 Antioxidative activities of enzymatic hydrolysates from commercially produced kamaboko.

Species(mg/ml)	Activity (%)						
	Kamaboko*	***Shiro-yakinuki***	***Datemaki***	***Yaki-chikuwa***	***Hanpen***	***Satsuma-age***	***Kani-kamaboko***
Pepsin							
1	18.9	59.5	31.9	59.5	60.8	23.1	44.9
10	65.8	72.5	72.5	69.0	73.3	62.8	61.2
100	82.3	84.1	83.7	76.5	79.4	82.3	75.9
Trypsin							
1	53.6	65.2	0.0	43.5	61.9	30.2	63.4
10	70.6	73.8	64.4	70.6	76.4	71.4	63.3
100	82.6	81.4	70.6	88.4	93.4	93.0	88.4
Papain							
1	42.5	38.5	10.2	47.4	44.5	31.7	44.0
10	68.9	48.7	55.8	69.1	92.7	63.0	61.7
100	91.7	85.3	75.6	82.6	96.2	92.2	92.3
Thermolysin							
1	35.6	57.5	38.1	65.7	67.6	41.3	54.1
10	73.5	74.6	63.3	68.3	72.9	74.5	60.4
100	94.0	86.7	81.6	55.8	89.7	93.3	86.1
Pronase E							
1	47.2	55.0	13.9	89.6	54.6	27.7	32.0
10	67.7	68.9	69.1	69.1	71.1	62.9	63.0
100	92.2	82.8	83.8	87.2	92.6	87.1	89.8
Chymotrypsin							
1	41.5	54.1	16.8	56.5	65.4	47.9	35.1
10	72.0	71.8	60.5	69.8	70.7	63.4	65.8
100	95.7	84.9	80.6	87.0	93.0	86.3	87.1
Ascorbic acid (1 mM)	66.6						
Ascorbic acid (5 mM)	93.6						
α-tocopherol (1 mM)	98.0						

*Gifted from the All-Japan Kamaboko Makers Association.

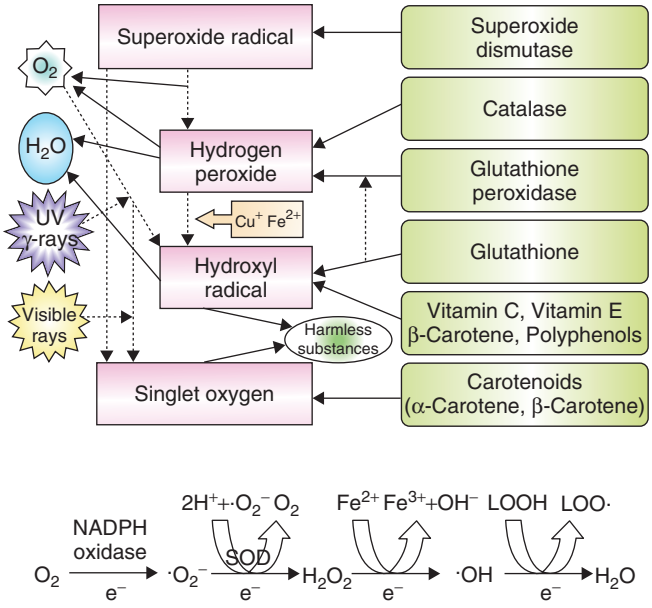

Fig. 5.6 Mechanism of the production and scavenging of reactive oxygen species.

compounds – and can add across a double bond, resulting in the hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as with oxygen, to give peroxyl radical, or else can decompose to phenoxyl-type radicals via water elimination (Lee *et al*., 2004).

The hydroxyl radical scavenging activities of the enzymatic hydrolysates from commercially produced kamaboko (gifted from the All-Japan Kamaboko Makers Association) were estimated using the Fenton reaction system. Shiro-yakinuki kamaboko perfectly scavenged this radical for 100 mg/ml (Table 5.6) and possessed a remarkably high scavenging activity (followed by that of satsuma-age). Yaki-chikuwa, hanpen, and kani-kamaboko have moderate activities, as does α-tocopherol (final concentration of 1 mM). The activity was low in all of the hydrolysates prepared from datemaki. In general, trypsin hydrolysates of commercially produced kamaboko showed high scavenging activity against hydroxyl radical. This suggests that the hydroxyl radical scavenging activity is due to the peptides having basic amino acid residues such as arginine, histidine and lysine.

The superoxide anion is an initial free radical formed by mitochondrial electron transport systems. Mitochondria generate energy using a four-electron chain reaction, reducing oxygen to water (Fig. 5.6). Some of the electrons escaping from the mitochondrial chain reaction directly react with oxygen and form superoxide anion, which plays an important role in the formation of other reactive oxygen species in living systems, such as hydrogen peroxide, hydroxyl radical and singlet oxygen (Fig. 5.6) (Lee *et al*., 2004). The superoxide radical has been implicated as playing a crucial role in ischemia-reperfusion injury (Radi *et al*., 1991).

The superoxide anion radical scavenging activities of the enzymatic hydrolysates from commercially produced kamaboko were investigated using the xanthine-xanthine oxidase system. The kamaboko was gifted by the All-Japan Makers Association;

Table 5.6 Hydroxyl radical scavenging activities of enzymatic hydrolysates from commercially produced kamaboko.

Species (mg/ml)	Activity (%)						
	Kamaboko*	***Shiro-yakinuki***	***Datemaki***	***Yaki-chikuwa***	***Hanpen***	***Satsuma-age***	***Kani-kamaboko***
Pepsin							
1	3.5	2.7	0.0	0.0	5.6	0.0	0.0
10	18.2	29.4	1.0	7.9	5.4	12.6	12.8
100	75.2	100.0	11.7	40.9	34.6	91.9	70.9
Trypsin							
1	0.2	9.5	0.0	0.0	4.0	0.0	16.5
10	20.7	50.0	0.0	20.8	12.6	13.4	16.6
100	68.5	100.0	21.9	88.9	54.3	100.0	71.9
Papain							
1	3.0	12.8	0.0	0.0	4.0	0.0	4.8
10	29.3	51.9	0.0	19.5	14.4	24.2	16.3
100	67.8	100.0	20.4	57.3	50.1	100.0	71.0
Thermolysin							
1	14.4	14.2	0.0	2.8	3.4	0.0	12.0
10	28.1	48.5	0.0	21.2	12.4	5.7	24.8
100	59.7	100.0	28.2	67.7	53.3	70.8	66.7
Pronase E							
1	12.8	13.2	2.1	5.3	1.6	0.0	12.2
10	26.2	34.4	0.0	23.4	15.0	24.7	26.0
100	62.1	100.0	27.4	72.0	46.3	94.4	68.5
Chymotrypsin							
1	1.4	4.7	0.0	3.3	5.4	0.0	1.0
10	20.1	40.3	15.2	11.8	9.9	7.6	21.9
100	60.1	100.0	26.4	62.4	40.8	96.7	73.2
Ascorbic acid (1 mM)	13.2						
Ascorbic acid (5 mM)	17.6						
α-tocopherol (1 mM)	67.6						

*Gifted by the All-Japan Kamaboko Makers Association.

yaki-chikuwa, hanpen and satsuma-age showed extremely high scavenging activity (Table 5.7). The activities of the hydrolysates from shiro-yakinuki kamaboko, datemaki and kani-kamaboko were moderate, as was that of α-tocopherol (final concentration 1 mM). This suggests that pepsin and trypsin hydrolysates from commercially produced kamaboko inhibit xanthine oxidase activity and suppress the production of superoxide anion radical.

DPPH is a stable nitrogen-centered free radical. Its color changes from violet to yellow when it is reduced by either hydrogen or electrondonation. Substances which perform this reaction can be considered antioxidants and therefore radical scavengers (Brand-Williams *et al*., 1995). DPPH radical scavenging activity is known to correlate well with the inhibitory capacity of lipid peroxidation of test compounds (Rekka & Kourounakis, 1991). DPPH has been widely used to investigate the free radical scavenging ability of various food samples.

DPPH radical scavenging activities of the enzymatic hydrolysates from commercially produced kamaboko were measured. Most of the kamaboko was gifted by the All-Japan Kamaboko Makers Association. Datemaki, yaki-chikuwa, hanpen, and satsuma-age possessed strong activity (Table 5.8). The activity of the hydrolysate from kani-kamaboko was moderate, while that from shiro-yakinuki kamaboko was low. On the other hand, the thermolysin hydrolysates strongly scavenged this radical; this suggests that many species of peptide were produced by thermolysin with the broad substrate-specificity scavenged DPPH radical. Moreover, papain and pronase E hydrolysates showed high scavenging activities against this radical in every kamaboko.

5.8 ANGIOTENSIN I-CONVERTING ENZYME-INHIBITORY ACTIVITIES OF KAMABOKO PROTEINS

Hypertension is a significant public problem worldwide (Fujita *et al*., 2012). One of the factors affecting blood pressure in mammals is angiotensin I-converting enzyme (ACE). ACE catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II and deactivates the vasodilator nonapeptide bradykinin (Fig. 5.7) (Haartmann & Meisel, 2007). ACE inhibitors reduce blood pressure by decreasing peripheral vascular resistance and stabilizing renal function, making them useful in reducing the progress of diabetic nephropathy (Sarafidis *et al*., 2007). Therefore, finding new sources of ACE inhibitors, especially in food resources, is of great interest.

The ACE-inhibitory activities of enzymatic hydrolysates from commercially produced kamaboko were investigated in each enzyme. The hydrolysates of shiro-yakinuki kamaboko showed extremely high inhibitory activities (Table 5.9), similar to those of fermented foods such as cheese and natto (Okamoto *et al*., 1995). Those peptides with high ACE-inhibitory activities from yaki-chikuwa and satsuma-age could be obtained using any of the hydrolytic enzymes. Pepsin and thermolysin were good enzymes for the preparation of peptides with high ACE-inhibitory activities from commercially produced kamaboko. On the other hand, the hydrolysates from kani-kamaboko had low activity regardless of enzymes.

Antioxidant compounds play an important role as health-protective factors. Most of the antioxidant compounds in a typical diet derive from plant sources and food proteins and belong to various classes of compound, with a wide variety of physical and chemical properties. They can delay or inhibit lipid oxidation by inhibiting the initiation or

Table 5.7 Superoxide anion radical scavenging activities of enzymatic hydrolysates from commercially produced kamaboko.

Species (mg/ml)	Activity (%)						
	Kamaboko*	***Shiro-yakinuki***	***Datemaki***	***Yaki-chikuwa***	***Hanpen***	***Satsuma-age***	***Kani-kamaboko***
Pepsin							
1	2.1	0.0	9.6	21.2	7.0	0.0	0.0
10	20.3	0.0	13.2	19.3	21.4	0.0	0.0
100	37.4	11.3	65.5	75.5	82.7	59.2	4.8
Trypsin							
1	9.6	19.8	0.0	2.0	2.7	0.0	0.0
10	35.9	26.6	3.4	39.6	61.7	0.0	0.0
100	51.9	30.6	9.8	25.9	87.8	37.6	60.1
Papain							
1	10.3	0.0	0.0	18.0	21.0	0.0	0.0
10	42.3	0.0	0.0	27.0	64.6	0.0	0.0
100	60.9	0.0	40.6	59.5	90.1	52.8	46.7
Thermolysin							
1	25.6	5.8	0.0	32.0	5.9	0.0	0.0
10	68.0	11.7	0.0	13.1	23.9	0.0	0.0
100	94.9	22.6	32.8	60.1	62.6	22.4	12.9
Pronase E							
1	30.3	3.0	0.0	17.3	18.5	0.0	0.0
10	29.7	6.8	0.0	31.5	41.9	0.0	0.0
100	87.0	14.7	31.8	65.8	54.5	29.6	42.2
Chymotrypsin							
1	21.8	8.1	0.0	20.5	8.1	0.0	0.0
10	23.9	10.5	0.0	22.1	77.3	0.0	0.0
100	88.0	12.7	39.9	63.3	77.3	92.0	3.6
Ascorbic acid (1 mM)	13.3						
Ascorbic acid (5 mM)	90.0						
α-tocopherol (1 mM)	52.5						

*Gifted by the All-Japan Kamaboko Makers Association.

Table 5.8 DPPH radical scavenging activities of enzymatic hydrolysates from commercially produced kamaboko.

Species (mg/ml)	Activity (%)						
	Kamaboko*	***Shiro-yakinuki***	***Datemaki***	***Yaki-chikuwa***	***Hanpen***	***Satsuma-age***	***Kani-kamaboko***
Pepsin							
1	0.0	3.8	0.0	0.0	0.0	0.0	0.0
10	11.8	4.9	0.0	0.0	6.5	3.2	0.5
100	66.2	10.5	20.9	20.9	63.9	65.3	33.7
Trypsin							
1	0.0	3.8	0.0	0.0	0.0	0.0	0.0
10	5.1	5.8	0.0	0.0	23.8	25.3	11.6
100	53.1	11.9	55.9	55.9	80.8	62.7	63.0
Papain							
1	0.0	4.7	0.0	0.0	0.0	0.0	0.0
10	29.2	6.4	0.0	0.0	39.7	31.3	20.2
100	75.1	29.9	63.8	63.8	76.5	74.2	69.7
Thermolysin							
1	0.0	2.9	0.0	0.0	0.0	0.0	0.0
10	39.7	23.6	2.8	2.8	13.9	28.7	9.6
100	70.6	49.8	81.3	81.3	85.2	68.6	61.7
Pronase E							
1	0.0	2.8	0.0	0.0	0.0	0.0	0.0
10	24.5	4.1	2.9	2.9	13.3	17.7	12.1
100	71.3	14.9	80.9	80.9	73.6	73.6	56.2
Chymotrypsin							
1	0.0	4.4	0.0	0.0	0.0	0.0	0.0
10	34.9	2.3	0.9	0.9	21.0	20.8	11.4
100	75.5	24.6	70.1	70.1	81.0	57.2	62.5
Ascorbic acid (0.1 mM)	5.6						
Ascorbic acid (1 mM)	94.7						
α-tocopherol (1 mM)	94.7						

*Gifted by the All-Japan Kamaboko Makers Association.

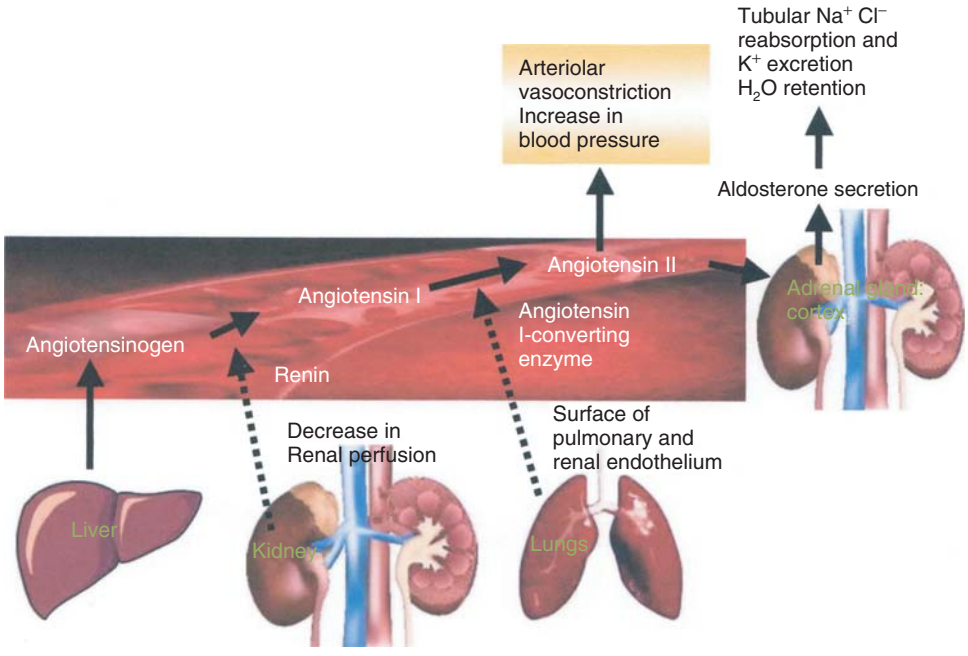

Fig. 5.7 Renin-angiotensin-aldosterone system.

Table 5.9 ACE inhibitory activities of enzymatic hydrolysates from commercially produced kamaboko.

Species	IC_{50} (mg protein/ml)					
	Pepsin	**Trypsin**	**Papain**	**Thermolysin**	**Pronase E**	**Chymotrypsin**
Kamaboko*	1.47	1.47	1.56	1.89	1.72	2.49
Shiro-yakinuki	0.075	0.064	0.100	0.074	0.040	0.060
Datemaki	0.718	3.71	2.51	0.794	1.12	1.55
Yaki-chikuwa	0.833	0.716	0.815	0.884	0.920	1.46
Hanpen	1.45	1.66	17.4	0.98	9.34	1.50
Satsuma-age	0.94	0.97	1.22	1.02	0.86	1.13
Kani-kamaboko	10.1	9.06	8.93	9.28	8.83	11.7

*Gifted by the All-Japan Kamaboko Makers Association.

propagation of oxidizing chain reactions and are involved in scavenging free radicals. Kamaboko is a protein-rich foods. The enzymatic hydrolysates exhibit highly antioxidative activity and high scavenging activities against superoxide anion radicals, hydroxyl radicals and DPPH radicals. In addition, these hydrolysates possess high ACE-inhibitory activity. In general, food proteins taken in the body are first digested in the stomach by pepsin, then the hydrolysates are further digested in the intestines by trypsin, chymotrypsin and carboxyl protease. Moreover, these hydrolysates are absorbed in the body system, where their functions are performed. In recent years, some researchers have studied the functional properties of kamaboko products, obtaining useful information on the following subjects: reduction of the effects of oxidative stress by commercially produced kamaboko in the brain of spontaneously hypertensive rats and stroke-prone spontaneously

hypertensive rats; prevention of the effects of dietary kamaboko products, peptides or hydrolysates on the development of tumors via angiogenesis inhibition; improvement of the nutritional status or immunity activation; inhibition of dietary kamaboko products on the progression of colon cancer and improvement of serum cholesterol metabolism of rats; suppression of the hypertrophy on adipocytes and promotion of the energy production of white adipocytes by the investigation of 3T3-L1 fibroblast cells and wister rats; prevention of the effects of kamaboko on the increase of liver weight induced by a high-fat diet and the development of a fatty liver induced by taking too much fat in foods in mice; learning of improvement effects and resistance to forgetfulness via drugs such as scopolamine and cycloheximide on kamaboko-fed mice; prevention of the kamaboko peptides produced by trypsin digestion against dementia diseases such as the Alzheimer's disease via the investigation of cultured astroglial cells derived from rat fetal brains using enzyme immunoassay methods (All-Japan Kamaboko Association, 2010).

5.9 CONCLUSION

From these findings, it can be seen that the intake of kamaboko proteins and its peptides derived by enzymatic digestion contribute to enhance and promote human health. Kamaboko may become useful in preventing certain lifestyle-related diseases. With further investigation, consumption of commercially produced kamaboko is likely to increase.

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6 Biological Activities of Fish-protein Hydrolysates

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6.1 INTRODUCTION

The production of fish-protein hydrolysates (FPHs) has been envisaged as an alternative to the upgrading of underutilised fish species and fish byproducts. The first studies on the preparation of fish peptones for microbiological growth media date back to the 1940s (Tarr, 1942; Tarr & Deas, 1949). FPHs were mainly intended for animal or human nutrition and also as a nitrogen source for microbiological growth media. Their production represented an improvement in fish ensiling, where the hydrolysis process did not depend on the endogenous fish-protease activity. However, the physiological regulation functions evidenced by peptides obtained from different protein sources (Lee *et al*., 1983; Maruyama *et al*., 1985; Oshima *et al*., 1979; Yashiro *et al*., 1985) led to a renewed interest in protein hydrolysates. These bioactive peptides are inactive when included in the amino acid sequence of the parent protein but can exhibit physiological activities after they have been released by the action of proteases during gastrointestinal digestion or food processing.

This chapter reviews protein-derived bioactive peptides obtained from the proteins of fish, shellfish and other aquatic organisms. Particular attention is given to the amino acid sequences of the bioactive peptides isolated and purified from the protein hydrolysates obtained under different hydrolysis conditions.

6.2 ANGIOTENSIN I-CONVERTING ENZYME INHIBITORS

Cardiovascular disease (CVD) is the leading cause of death in people over 60 years of age (Mackay & Mensah, 2006). High blood pressure is one of the leading risk factors for the onset of CVD in developed countries. One way to reduce blood pressure is to use angiotensin I-converting enzyme (ACE) inhibitors. ACE is a dipeptide-liberating carboxypeptidase (M2 family metallopeptidase) secreted by pulmonary and renal endothelial cells and is present in the cell surface of vascular endothelial cells and in organ-specific sites (heart, brain, kidney, placenta, bone marrow, pancreas and testis) (Ehlers & Riordan, 1989). This enzyme is the main component of the body's renin-angiotensin system (RAS) and mediates the extracellular volume and arterial vasoconstriction. The secretion of renin, an aspartic protease, by the granular cells of the kidney is stimulated by a decrease

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in arterial blood pressure or sodium chloride levels in the ultrafiltrate of the nephron or by sympathetic nervous system activity (Pratt *et al*., 1988). Circulating renin cleaves angiotensinogen to produce angiotensin I (the decapeptide DRVYIHPFHL), which is cleaved by ACE to yield angiotensin II (DRVYIHPF), a potent vasoconstrictor. ACE also inactivates bradykinin, a vasodilator, which has a depressor action in the RAS. Moreover, ACE also hydrolyses neuropeptides as enkephalins, neurotensin and substance P, which may interact with the cardiovascular system. Another protease, prolyl endopeptidase, may also cleave angiotensin I or angiotensin II into the heptapeptide Ang (1–7), which exhibits biological activity as vasodilatation (Ferrario *et al*., 2005). The inhibition of ACE activity with pharmaceuticals was hence the main target in the treatment of hypertension, congestive heart failure and myocardial infarction. Different types of synthetic inhibitors have been designed, but they all have adverse side effects. However, ACE-inhibitory peptides derived from proteins have not shown these side effects. As mentioned by Gildberg *et al*. (2011), the content of inhibitory peptides in protein hydrolysates depends on the substrate protein, the proteolytic enzyme used and the hydrolytic conditions. According to Maruyama *et al*. (1989), the structure and activity of ACE-inhibitory peptides are related to the isolation techniques used and their source, but the relationship between the structure and the ACE-inhibitory activity of peptides has not yet been clearly established. However, it has been proposed that di- and tripeptides with high inhibitory activity have aromatic (Phe, Trp, Tyr) or proline residues or hydrophobic amino acids at their C-terminus (Gobbetti *et al*., 2004) and a branched aliphatic residue at their N-terminus (Ile or Val). Active oligopetides often have a sequence of three hydrophobic residues at their C-terminus (Li *et al*., 2004; Meisel *et al*., 2006). Wu *et al*. (2006) developed two models to predict the IC_{50} (concentration of peptide mediating a 50% inhibition of activity) of the ACE-inhibitory activity of di- and tripeptides. They concluded that amino acid residues with both bulky side chains and hydrophobic side chains are preferred for dipeptides. For tripeptides, aromatic residues are the most favourable for the C-terminus, hydrophobic residues for the N-terminus and charged amino acids for the middle position. It was also observed that some inhibitory peptides possessed a C-terminal glutamate residue, which may chelate the zinc ion present in the active site of ACE (Wei *et al*., 1992).

The first report evidencing the presence of peptides in sardine and hairtail protein hydrolysates exhibiting inhibitory activity against ACE was published in 1986 (Suetsuna & Osajima, 1986). Later, Suetsuna *et al*. (1988) reported the ACE-inhibitory activity in hydrolysates prepared from several fish and shellfish species. Ukeda *et al*. (1991, 1992) identified three ACE-inhibitory peptides (LKL, VLAGF and KVLAGM) in sardine-muscle hydrolysate and tested their effect in normotensive rats. Suetsuna *et al*. (1991), Sugiyama *et al*. (1991) and Matsui *et al*. (1993) also detected ACE-inhibitory peptides in sardine hydrolysates. Ariyoshi (1993) identified five ACE-inhibitor peptides in sardine hydrolysates; the most potent was LKVGVKQY (IC_{50} 11.6 μM). Matsufuji *et al*. (1994) identified 11 ACE-inhibitor peptides in sardine hydrolysates; the dipeptide KW had the maximal inhibitory activity (IC_{50} 1.63 μM) and all those peptides identified inhibited ACE competitively, except for MY, which showed noncompetitive inhibition. Seki *et al*. (1995) identified the inhibitory peptide VY in sardine hydrolysates which was resistant to gastrointestinal proteases. Tuna muscle and tuna byproducts have been used as a raw material for the preparation of FPH. Kohama *et al*. (1988) isolated an ACE-inhibitory peptide from tuna-muscle hydrolysate and characterised its amino acid sequence as PTHIKWGD. The thermolysin hydrolysate of dried bonito (*katsuobushi*) showed the most potent inhibitory activity against ACE and the following inhibitory peptides were

identified: IKPLNY, IVGRPRHQG, IWHHT, ALPHA, FDF, LKPNM, IY and DYGLYP (Yokoyama *et al*., 1992). Matsumura *et al*. (1993a) also reported the isolation of six ACE-inhibitory peptides from bonito-bowel autolysate; their amino acid sequences were YRPY (IC_{50} 320 μM), GHF (IC_{50} 1100 μM), VRP (IC_{50} 22 μM), IKP (IC_{50} 25 μM), LRP (IC_{50} 1.0 μM) and IRP (IC_{50} 1.8 μM). In a second study, Matsumura *et al*. (1993b) discovered two ACE-inhibitory peptides in an autolysate of bonito bowels with the sequences GVYPHK and IRPVQ. Oral administration of these two peptides decreased blood pressure in spontaneously hypertensive rats (SHR), as reported by Karaki *et al*. (1993). Fujii *et al*. (1993) obtained a partially purified autolysate of bonito bowels which decreased the systolic blood pressure (SBP) of SHR. The pepsin digest of dried, salted skipjack tuna showed the highest ACE-inhibitory activity among the various protein hydrolysates prepared (Astawan *et al*., 1995). Four inhibitory peptides were isolated and characterised as VAWKL (IC_{50} 31.97 μM), WSKVVL (IC_{50} 156.28 μM), SKVPP (IC_{50} 74.22 μM) and CWLPVY (IC_{50} 22.20 μM). Fujita & Yoshikawa (1999) isolated eight kinds of ACE-inhibitory peptide from *katsuobushi* and found that one (LKPNM, IC_{50} 2.4 μM) was hydrolysed by ACE to produce LKP (IC_{50} 2.4 μM), suggesting that LKPNM can be regarded as a prodrug-type ACE-inhibitory peptide. It was also demonstrated that these peptides exert high antihypertensive activities *in vivo*. The pepsin-derived hydrolysate prepared from tuna dark muscle exhibited the highest ACE-inhibitory activity (Qian *et al*., 2007). An inhibitory peptide structure with 13 amino acids (MW = 1581 Da, IC_{50} 21.6 μM) was identified. It acts as a noncompetitive inhibitor and had an antihypertensive effect after oral administration to SHR. Je *et al*. (2009) used skipjack tuna liver to prepare protein hydrolysates as both first- and second-step hydrolysates. All hydrolysates had similar ACE-inhibitory properties of around 36%. A potent ACE-inhibitory peptide, composed of 21 amino acids (2482 Da, IC_{50} 11.28 μM), was isolated from a tuna-frame hydrolysate (Lee *et al*., 2010). This peptide acts as a noncompetitive inhibitor and its oral administration to SHR indicated that it can decrease SBP significantly.

Byun & Kim (2001) identified two tripeptides (GPL, IC_{50} 2.6 μM; GPM, IC_{50} 17.13 μM) from Alaska pollock-skin hydrolysates with high ACE-inhibitory activity. Morimura *et al*. (2002) obtained yellow tail-bone hydrolysates with high potential for decreasing blood pressure (IC_{50} 0.16, 0.41 mg/ml). Obha *et al*. (2003) also obtained hydrolysates from fish byproducts (bones, scales) exhibiting ACE-inhibitory activity (IC_{50} 0.6–2.8 mg/ml). Je *et al*. (2004) identified an ACE-inhibitory peptide from a peptic Alaska pollock-frame hydrolysate with IC_{50} 14.7 μM and the amino acid sequence FGASTRGA. An ACE-inhibitory peptide with the sequence GLLP was isolated from dried Alaska pollock (*Hwangtae*) hydrolysates (Cho *et al*., 2008). Gelatin hydrolysates prepared from the refiner discharge of an Alaska pollock surimi processing plant were fractionated by ultrafiltration (UF) and the highest ACE-inhibitory activity (IC_{50} 0.21 mg/ml) was observed with the 3 kDa filtrate (Park *et al*., 2009).

A hydrolysate prepared from upstream salmon proteins showed high inhibitory activity against ACE (Ono *et al*., 2003). A significant SBP reduction was recorded in SHR after oral administration of this hydrolysate. A total of six inhibitory peptides were identified, as WA, VW, WM, MW, IW and LW, with IC_{50} varying from 2.5 to 227.3 μM. The same authors (Ono *et al*., 2006) demonstrated the antihypertensive effect of salmon hydrolysates on SHR. Enari *et al*. (2006) studied the effect of a drink containing salmon peptide on high-normal blood pressure and mild hypertensive subjects. The results revealed that SBP was reduced in the test food group. In another study (Enari *et al*., 2008), the antihypertensive effect of salmon hydrolysate was examined in SHR; 20 active di- and tripeptides were

identified and the dipeptide IW had the strongest ACE-inhibitory activity (IC_{50} 1.2 μM) and showed a digestive resistance by *in vitro* assay. Gu *et al*. (2011) also reported the presence of ACE-inhibitory peptides in salmon-skin hydrolysates. A total of 11 peptides were identified and the dipeptides AP (IC_{50} 0.060 ± 0.001 mg/ml) and VR (IC_{50} 0.332 ± 0.005 mg/ml) exhibited the highest ACE-inhibitory activity.

Cinq-Mars & Li-Chan (2007) optimised the ACE-inhibitory activity of Pacific hake hydrolysates using response-surface methodology (RSM). The hydrolysate fraction with molecular weight lower than 10 kDa had IC_{50} 44 ± 7 μg/ml. In another study with the same fish species, Cinq-Mars *et al*. (2008) showed that simulated gastrointestinal digestion of FPH and ultrafiltrate (10 kDa molecular-mass cutoff) had similar ACE-inhibitory activity (IC_{50} 90 μg/ml). These results indicated that FPH peptides act as 'prodrug-type' inhibitors. It was also shown that they exhibit a competitive inhibitory mode. It was demonstrated (Samaranayaka *et al*., 2010) that the hydrolysates from this fish species showed substrate-type inhibition of ACE with IC_{50} 161 μg peptide/ml. This activity remained unchanged after *in vitro*-simulated gastrointestinal digestion, although individual UF fractions showed decreased or no activity.

Lee *et al*. (2011) isolated two ACE-inhibitory peptides from skate skin α-chymotrypsin hydrolysate. Their sequences were PGPLGLTGP (975.38 Da, IC_{50} 95 μM) and QLGFLGPR (874.45 Da, IC_{50} 148 μM) and both acted as noncompetitive inhibitors. Yellowfin sole-frame proteins were hydrolysed with α-chymotrypsin, and an ACE-inhibitory peptide with 11 amino acids was characterised (IC_{50} 28.7 μg/ml). This peptide acted as a noncompetitive inhibitor and its antihypertensive activity was demonstrated in SHR (Jung *et al*., 2006c). Protein hydrolysates prepared from cod-frame proteins and further fractionated by UF membranes showed excellent ACE-inhibitory activity (Jeon *et al*., 1999). Cod-liver proteins were hydrolysed by α-chymotrypsin (Choi *et al*., 2000). Two peptides exhibiting ACE-inhibitory activity were identified as MIPPYY (IC_{50} 10.9 μM) and GLRNGI (IC_{50} 35.0 μM). Fahmi *et al*. (2004) isolated and characterised four ACE-inhibitory peptides from sea bream-scale collagen. Their sequences were identified as GY, VY, GF and VIY. It was also shown that the oral administration of the unpurified hydrolysate to SHR significantly decreased blood pressure. Shark-meat hydrolysate showed ACE-inhibitory activity (Wu *et al*., 2008). The three novel inhibitory peptides (and their respective IC_{50}) were CF (1.96 μM), EY (2.68 μM) and FE (1.45 μM). Hydrolysates prepared from sardine, cod and shrimp byproducts (Bordenave *et al*., 2002), blue whiting (Geirsdottir *et al*., 2011), channel catfish (Theodore & Kristinsson, 2007), brownstripe red snapper (Khantaphant *et al*., 2011a), tilapia skin (Zeng *et al*., 2005), Atlantic and Coho salmon, Alaska pollock and southern blue whiting (Nakajima *et al*., 2009) and saithe (Halldorsdóttir *et al*., 2011) also exhibited ACE-inhibitory activity.

Balti *et al*. (2010a,) working with cuttlefish hydrolysates, reported that the highest ACE inhibition (IC_{50} 1 mg/ml) was displayed by the hydrolysate with the highest degree of hydrolysis (DH) (13.5%). In a second paper they reported the isolation of 11 ACE-inhibitory peptides from cuttlefish hydrolysates (Balti *et al*., 2010b). The structures of the most potent peptides were identified as AHSY (IC_{50} 11.6 μM), GDAP (IC_{50} 22.5 μM), AGSP (IC_{50} 37.2 μM) and DFG (IC_{50} 44.7 μM). It was shown that the former peptide was stable against gastrointestinal proteases *in vitro*. It was also demonstrated that this peptide acts as a noncompetitive inhibitor. Hydrolysates prepared from gelatin of the inner and outer tunics of giant squid possessed ACE-inhibitory activity. The most potent inhibitor peptide had a molecular weight of approximately 1400 Da and

IC_{50} 90 μM. Leucine and glycine were present in this peptide at the second and third positions from the carboxyl terminus (Alemán *et al*., 2011).

An underutilised shrimp was used by He *et al*. (2006a) to prepare protein hydrolysates. Five ACE-inhibitory peptides were purified and three of them–FCVLRP (IC_{50} 12.3 μM), IFVPAF (IC_{50} 343 μM) and KPPETV (IC_{50} 24.1 μM)–were novel peptides. These were competitive inhibitors, and they probably maintain a high ACE-inhibitory activity even if they are digested *in vivo*. Nii *et al*. (2008) identified two ACE-inhibitory peptides in the hydrolysates prepared from Izumi shrimp. Wang *et al*. (2008a), working with the same shrimp species, produced protein hydrolysates by fermentation with *Lactobacillus fermentus*. The fermented sauce presented high ACE-inhibitory activity and three peptides were purified; their sequences were DP, GTG and ST, with IC_{50} values of 2.15 ± 0.02, 5.54 ± 0.09 and 4.03 ± 0.10 μM. Benjakul *et al*. (2009) reported the preparation of Mungoong, an extract paste from the cephalotorax of white shrimp, using Flavourzyme. The product obtained with Flavourzyme added exhibited higher ACE-inhibitory activity than the traditional Mungoong. Cao *et al*. (2012), also working with the same shrimp species, optimised the peptic hydrolysis parameters using the RSM. The hydrolysate had an ACE-inhibition value of IC_{50} 1.17 mg/ml. Cheung & Li-Chan (2010) optimised the preparation of shrimp-byproduct hydrolysates possessing ACE-inhibitory activity. The IC_{50} of Alcalase and Protamex hydrolysates were 100–200 and 70 μg/ml, respectively. Gildberg *et al*. (2011) identified two novel antihypertensive tripeptides, FSY and FTY, in desalted shrimp-protein hydrolysate. Positive results were obtained in a feeding trial with SHR when 60 mg hydrolysate/kg body weight/day was given. A peptide inhibiting ACE activity from Antarctic krill-protein hydrolysate was characterised as KLKPV with IC_{50} 30 μM (Kawamura *et al*., 1992).

Lee *et al*. (2002) isolated peptides inhibiting ACE from the hydrolysate of Manila clam proteins. The maximum inhibitory activity was observed in one fraction that had an IC_{50} of 0.748 μg. Later, Lee *et al*. (2005), again working with Manila clam, characterised the amino acid sequence of an ACE-inhibitory peptide as LLP. Suetsuna (2002) also identified antihypertensive peptides in peptic hydrolysates from the short-necked clam and pearl oyster. Katano *et al*. (2003) obtained oyster hydrolysates exhibiting ACE-inhibitory activity which reduced the SBP of SHR. Four inhibitory peptides were isolated and their amino acid sequences were identified as FY, AW, VW and GW. Chung *et al*. (2006) obtained Protamex oyster hydrolysates with high ACE-inhibitory activity (IC_{50} 1.16 mg/ml). Tsai *et al*. (2006) identified two peptides with high antihypertensive activity (VKP, IC_{50} 3.7 μM and VKK, IC_{50} 1045 μM) from Protamex hydrolysates of freshwater clam muscle. The positive antihypertensive effect of this hydrolysate was demonstrated in SHR. An ACE-inhibitory dipeptide of a hard clam hydrolysate was characterised (YN, IC_{50} 51 μM) and showed mixed-type inhibitor kinetics (Tsai *et al*., 2008). Protein hydrolysates prepared from solid wastes of freshwater clam showed strong ACE-inhibitory activity (IC_{50} 0.23 mg/ml). The peptides with molecular weight less than 1 kDa possessed the strongest inhibition and their inhibitory pattern was found to be competitive (Sun *et al*., 2011). In the pepsin hydrolysate prepared from oyster, a peptide exhibiting ACE-inhibitory activity was purified and identified (Wang *et al*., 2008b). This peptide, with the sequence VVYPWTQRF, had an IC_{50} value of 66 μM and strong enzyme-resistant properties against gastrointestinal proteases. Kinetics experiments also demonstrated that this peptide was a noncompetitive inhibitor.

Protein hydrolysates prepared from gelatin sea cucumber exhibited ACE-inhibitory activity (IC_{50} 0.35 mg/ml) and a peptide with 840 Da (IC_{50} 0.0142 mg/ml) was isolated.

A fraction with MW < 1 kDa significantly reduced the blood pressure of renal hypertensive rats (Zhao *et al*., 2007). In a second study, Zhao *et al*. (2009) identified an inhibitory peptide as MEGAQEAQGD from a gelatin sea cucumber hydrolysate. Its inhibitory activity was intensified from IC_{50} 15.9 to IC_{50} 4.5 μM after incubation with gastrointestinal digestion. It also showed an antihypertensive effect in SHR.

The optimal conditions for the production of ACE-inhibitory peptides from the collagen of the giant red sea cucumber were studied by Liu *et al*. (2011). The collagen hydrolysates had molecular weights less than 6.5 kDa, an IC_{50} 0.43 ± 0.04 mg/ml and were resistant to gastrointestinal enzymes. Collagen hydrolysates inhibit the activity of ACE via a competitive mechanism.

Lee *et al*. (2009) purified and characterised an ACE-inhibitory peptide from a rotifer. The Alcalase hydrolysate had the highest ACE-inhibitory activity compared to the other hydrolysates. An inhibitor peptide with 14 amino acids (1538 Da, IC_{50} 9.64 μM) was identified which acts as a competitive inhibitor against ACE.

Ascidian tunicate flesh muscle was used to obtain hydrolysates (Ko *et al*., 2012) exhibiting ACE-inhibitory activity. A noncompetitive inhibitory peptide was isolated and identified as AHIII (565.3 Da, IC_{50} 37.1 μM). Tamari (2005) prepared protein hydrolysates of the hot-water extract from a sea anemone. Two phosphopeptides exhibiting ACE-inhibitory activity were isolated; they had IC_{50} values of 0.55 and 0.33 mg protein/ml.

The presence of ACE inhibitory peptides in several fish sauces was also reported (Je *et al*., 2005a,b; Okamoto *et al*., 1995).

Sørensen *et al*. (2004) detected the presence of inhibitory activity against prolyl endopeptidase in hydrolysates from Atlantic cod, salmon and trout. Prolyl endopeptidase can cleave angiotensin I and II into Ang (1–7), which is a vasodilatator.

6.3 ANTIOXIDATIVE PROPERTIES

The deterioration of foods due to lipid oxidation is of great concern in the food industry. The resultant oxidised compounds are responsible for changes in food quality, particularly in sensory attributes as colour, flavour and texture, and losses of nutritional value. Oxidation processes have also been related to several diseases, such as cancer, coronary heart and Alzheimer's disease (Diaz *et al*., 1997). Reactive oxygen species (ROS) (hydroxyl radicals, peroxyl radicals, superoxide anion and peroxynitrite) are generated during normal physiological conditions. Endogenous antioxidants (enzymes, Se, tocopherol, vitamin C and peptides) are also present in tissues and organs; these prevent the deleterious effects of ROS. However, the balance between ROS and antioxidants in human body can change with advancing age and as a result of other factors (environmental pollutants, fatigue, excessive caloric intake, high-fat diets). Thus, the need to use dietary antioxidants to increase the amount of antioxidants in the body has been recognised, leading to the development and marketing of many dietary antioxidant supplements. Nevertheless, there is concern about the safety of synthetic antioxidants with regards to human health (Madhavi *et al*., 1996). As a consequence, the search for natural antioxidants has gained increasing interest in recent decades. Among the wide variety of natural antioxidants available are some amino acids and protein hydrolysates. All amino acids can generally interact with free radicals, but the most effective are those containing nucleophilic sulfur-containing side chains (Cys and Met) or aromatic side chains (Trp, Tyr and Phe), which can easily donate hydrogen atoms. However, peptides exhibit

higher antioxidative activity (AOA) than free amino acids, as a result of their unique chemical composition and physical properties (Elias *et al*., 2008). Thus, protein hydrolysates possessing antioxidant properties have been prepared from many plants (soybean, rice bran, quinoa seed, canola) and animal sources (milk casein, egg yolk, porcine miofibrillar proteins, aquatic products). One of the first works demonstrating the AOA in sardine myofibrillar protein hydrolysates was published in 1990 (Hatate *et al*., 1990). As mentioned by Raghavan *et al*. (2011), the antioxidant nature of FPH is mainly dependent on peptide size and amino acid composition, including such factors as the nature of functional groups, the hydrophilic–hydrophobic amino acid balance and the partitioning capacity of the peptides. The molecular weight of the majority of antioxidant peptides is in the range between 500 and 1800 Da, as mentioned by several authors (Kim *et al*., 2007; Klompong *et al*., 2009; Je *et al*., 2005c; Jun *et al*., 2004; Ranathunga *et al*., 2006; Ren *et al*., 2008; Wu *et al*., 2003). On the other hand, hydrophobic amino acid residues (Val or Leu) were identified in these peptides at the N-termini and Pro, His, Tyr, Trp, Met and Cys in their sequences (Elias *et al*., 2008).

AOA has been detected in protein hydrolysates produced from a variety of fish products, including fish muscle, fish, crustacean and cephalopod byproducts and underutilised species. The works by Suetsuna (1999) and Suetsuna & Ukeda (1999) were the first to identify several antioxidative peptides in the muscle of dried bonito (VKL, VVKL, VKV, PKAV, IKL, VPSGK, EAK, FVAGK, KAI and KD) and sardine (LQPGQGQQ), respectively. Wu *et al*. (2003) also prepared protein hydrolysates from mackerel muscle exhibiting antioxidant properties. Ranathunga *et al*. (2006) purified and characterised the peptide LGLNGDDVN (928 Da) from conger eel-muscle hydrolysate. A peptide fraction with a molecular weight of approximately 1400 Da possessed the strongest AOA. Protein hydrolysates prepared from round scad fillets possessed an AOA that slightly decreased during storage at 4 and 25 °C (Thiansilakul *et al*., 2007a). Thiansilakul *et al*. (2007b), working with the same fish species, prepared hydrolysates from raw or defatted muscle using Alcalase or Flavourzyme. They concluded that the type of protease, DH and defatting process prior to hydrolysis influenced the AOA of hydrolysates.

Ren *et al*. (2008) reported the preparation of grass carp-protein hydrolysates with various proteases. The highest AOA was found in Alcalase hydrolysates and a peptide was purified and identified as PSKYEPFV (966.3 Da). Silver carp-protein hydrolysates prepared with Alcalase and Flavourzyme possessed AOA related to its DH, hydrolysis time and molecular weight (Dong *et al*., 2008). Samaranayaka & Li-Chan (2008) demonstrated that FPH with AOA may be prepared using parasitised Pacific hake. The hydrolysate obtained by autolysis possessed an AOA comparable to BHA and α-tocopherol. Klompong *et al*. (2009), working with yellow stripe trevally, obtained antioxidant peptides with 656 and 617 Da isolated from Alcalase and Flavourzyme hydrolysates, respectively. Bougatef *et al*. (2009) described the preparation of smooth hound-muscle protein hydrolysates with various gastrointestinal proteases. The hydrolysate produced with low-molecular-weight protease showed notable AOA and its fraction with MW < 3500 Da displayed the highest AOA. Loach meat was used to prepare hydrolysates with papain and Protamex. The products obtained presented AOA influenced by the DH and the protease used (You *et al*., 2009). The *in vitro* digestion of the hydrolysate obtained with papain from the same fish species was studied (You *et al*., 2010a) and it was found that the final gastrointestinal digests had a higher AOA when compared to the blank. A purified antioxidant peptide was identified as PSYV (464.2 Da) and showed a 9.14-fold higher scavenging activity for hydroxyl radical compared with the crude protein hydrolysate

(You *et al*., 2010b). Tilapia muscle was used to prepare protein hydrolysates (Foh *et al*., 2010a,b). The highest AOA was measured in the Alcalase hydrolysate fraction, which also had the lowest-molecular-weight peptide size (180–2000 Da). The muscle proteins of two marine species were used to prepare hydrolysates with three proteases (Naqash & Nazeer, 2010). The purified peptides from trypsin hydrolysates did not show any cytotoxic effect for Vero lines and exerted a significant antiproliferative effect on $HepG_2$ cell lines. Pepsin hydrolysates prepared from two other Indian marine fish (ribbon fish and seela fish) showed the capacity to effectively scavenge free radicals (Rajaram & Nazeer, 2010). Nalinanon *et al*. (2011) obtained ornate threadfin bream hydrolysates using pepsin skipjack tuna. Antioxidant peptides with molecular weights of approximately 1.3 kDa exhibited the highest ABTS radical-scavenging activity. It was not affected by *in vitro* pepsin hydrolysis, and further digestion with pancreatin enhanced this activity. Khantaphant *et al*. (2011b) showed that hydrolysates prepared from brownstripe red snapper mince with previous membrane separation followed by washing presented higher antioxidant activities compared to those from untreated mince. In a second study (Khantaphant *et al*., 2011a) it was concluded that protein hydrolysates prepared by a two-step hydrolysis process showed the highest antioxidative activities. They remained constant or increased after digestion in a gastrointestinal-tract model system. Papain hydrolysates (HP) from prepared grass carp exhibited higher radical-scavenging activity and reducing power than those prepared with Alcalase. The metal-chelating activity of HP significantly increased after *in vitro* gastrointestinal digestion (Li *et al*., 2012).

A large number of studies on the preparation of protein hydrolysates from underutilised species and fish byproducts exhibiting antioxidative properties have been published. The report by Amarowicz & Shahidi (1997) was one of the first papers on the preparation of capelin hydrolysates exhibiting AOA. Fish byproducts from a variety of species have also been used to prepare FPH possessing AOA (Batista *et al*., 2010; Centenaro *et al*., 2011; Kim *et al*., 2007; Klompong *et al*., 2007; Je *et al*., 2005c,d; Jun *et al*., 2004; Raghavan *et al*., 2008; Sathivel *et al*., 2003; Šližytė *et al*., 2009; Theodore *et al*., 2008). Sardinelle-byproduct hydrolysates prepared with crude enzyme extracts from sardine exhibited the highest antioxidant activity. Seven antioxidant peptides were identified: LHY, LARL, GGE, GAH, GAWA, PHYL and GALAAH. LHY displayed the highest DPPH radical-scavenging activity (63% at 150 μg/ml) (Bougatef *et al*., 2010). Whole sand eel was used to obtain protein hydrolysates that exhibited AOA. An antioxidant peptide was isolated and identified as IVGGFPHYL (1189 Da) with EC_{50} 22.75 μM (Lee *et al*., 2011).

Tuna processing generates large amounts of byproducts and their utilisation in producing protein hydrolysates has been regarded as an alternative to upgrading. Je *et al*. (2007) identified an antioxidant peptide with 14 amino acids (MW 1519 Da) in the peptic hydrolysate of tuna backbone. A peptide from the dark muscle of bigeye tuna was also purified (Je *et al*., 2008). Tuna liver was used to prepare protein hydrolysates through a two-step hydrolysis process with commercial proteases. The results showed that the second hydrolysis increased AOA (Je *et al*., 2009). Ahn *et al*. (2010), also working with tuna liver, obtained hydrolysates with excellent AOA, which was dependent on the enzymes used. All fractionated hydrolysates inhibited acetylcholinesterase activity, which is involved in Alzheimer's disease. Hsu (2010) used tuna dark muscle to obtain protein hydrolysates using orientase (OR) and protease XXIII (PR). Both hydrolysates possessing AOA were fractionated and two antioxidative peptides from OR and PR were characterised as LPTSEAAKY (978 Da) and PMDYMVT (756 Da). The tuna cooking juice was also used to produce protein hydrolysates. The first study was reported by

Jao & Ko (2002), who were able to isolate seven antioxidative peptides with sequences comprising four to eight amino acid residues. Hsu *et al*. (2009) also prepared protein hydrolysates from tuna cooking juice with OR and identified three oxidative peptides (Table 6.1).

Ngo *et al*. (2010) described the preparation of Nile tilapia-scale gelatin hydrolysates using various commercial proteases. Alcalase-derived hydrolysate possessed the highest AOA compared to the other hydrolysates and an antioxidant peptide showing high radical scavenging activity was identified as DPALATEPDPMPF (1382.57 Da). Fish skin is an abundant byproduct of the fish industry and represents a good source of fish gelatin. It has thus been used for the extraction of collagen and gelatin and also in the preparation of protein hydrolysates. The work by Kim *et al*. (1996) is one of the first references to the isolation and characterisation of antioxidative peptides from yellowfin sole-skin hydrolysates. It was demonstrated that fish gelatin can be processed into biologically active peptides (Kim *et al*., 2001). The hydrolysate prepared from hoki-skin gelatin with trypsin exhibited the highest radical-scavenging activity. A peptide with the sequence HGPLGPL (797 Da) was identified. It was also observed that the antioxidative enzyme levels in culture human hepatoma cells were increased in the presence of this peptide (Mendis *et al*., 2005a). Kim *et al*. (2007) purified and characterised an antioxidant peptide from hoki frame with a molecular mass of 1801 Da and 16 amino acids. The AOA was measured in the skin gelatin hydrolysates from chum salmon (Nagai *et al*., 2006), brownstripe red snapper (Khantaphant & Benjakul, 2008), tuna (Gómez-Estaca *et al*., 2009), sole (Giménez *et al*., 2009a), Alaska pollock (Jia *et al*., 2010), bigeye snapper (Phanturat *et al*., 2010) and Korean rockfish (Kim *et al*., 2011). Sampath Kumar *et al*. (2011) characterised two antioxidant peptides from horse mackerel skin (NHRYDR, 856 Da) and croaker skin (GNRGFACRHA, 1101.5 Da).

The presence of AOA in protease digest of prawn muscle was demonstrated (Suetsuna, 2000) and pepsin digest showed the most potent AOA. Three antioxidant peptides were isolated from the active peptidic fraction and their structures were identified as ILL, FLL and FILL. Discards from shrimp processing were utilised to prepare protein hydrolysates with Alcalase (Guérard *et al*., 2007). RSM was used and the optimum conditions for obtaining the strongest AOA were: pH 9.7, 66.2 °C and 68.1 Anson units (AU)/kg crude protein. Fermentation is traditionally applied in East Asian countries in food preservation. Proteins are hydrolysed by microbial or indigenous proteases, generating bioactive peptides and thus increasing the biological properties of these food products. The antioxidative activities in fermented products have been studied by several authors. Rajapakse *et al*. (2005a) identified a hepta-peptide sequence, HFGBPFH (962 kDa), in fermented mussel sauce. Jung *et al*. (2005a) also identified an antioxidant peptide (FGHPY, 620 Da) in a similar product. He *et al*. (2006b) reported the enhancement of AOA of shrimp product through the addition of crude proteases from *Bacillus* sp. Similarly, Binsan *et al*. (2008) showed that a paste prepared from the cephalothorax of white shrimp contained stable antioxidant peptides over a wide pH and temperature range. Manni *et al*. (2010) reported that protein hydrolysates obtained during enzymatic isolation of chitin from shrimp wastes exerted remarkable antioxidant activities. The antioxidative activities of some Thai traditional fermented shrimp and krill products (kapi, jaloo and koong-som) were studied by Faithong *et al*. (2010). It was shown that the water-soluble fraction from kapi exhibited the highest AOA, and this fraction of all products was highly stable over a wide pH range and after heating at 40–100 °C for 15–60 minutes.

Table 6.1 Biological activity associated with protein hydrolysates from marine sources.

Source	Scientific name	Origin	Amino acid sequence	IC_{50} (μM)	Reference
ACE-inhibitory activity					
Alaska pollack	*Theragra chalcogramma*	Yellowish dried Alaska pollack	GLLP	0.23 μg/ml	Cho *et al.* (2008)
Salmon	*Oncorhynchus gorbuscha*	Muscle	VL	50	Enari *et al.* (2008)
		Muscle	IL	50	Enari *et al.* (2008)
		Muscle	LI	240	Enari *et al.* (2008)
		Muscle	VF	50	Enari *et al.* (2008)
		Muscle	IF	50	Enari *et al.* (2008)
		Muscle	LF	50	Enari *et al.* (2008)
		Muscle	IW	1.2	Enari *et al.* (2008)
		Muscle	LW	50	Enari *et al.* (2008)
		Muscle	FY	50	Enari *et al.* (2008)
		Muscle	YF	50	Enari *et al.* (2008)
		Muscle	IVL	180	Enari *et al.* (2008)
		Muscle	VIL	180	Enari *et al.* (2008)
		Muscle	LVL	180 mM	Enari *et al.* (2008)
		Muscle	FIA	180 mM	Enari *et al.* (2008)
		Muscle	AFL	50 mM	Enari *et al.* (2008)
		Muscle	IVF	50 mM	Enari *et al.* (2008)
		Muscle	FVL	50 mM	Enari *et al.* (2008)
		Muscle	VIF	50 mM	Enari *et al.* (2008)
		Muscle	YLV	160 mM	Enari *et al.* (2008)
		Muscle	IVW	1.0 mM	Enari *et al.* (2008)
Salmon	*Salmo salar*	Skin	AP	0.060 mg/ml	Gu *et al.* (2011)
			VR	0.332 mg/ml	Gu *et al.* (2011)
Shark	-	Muscle	CF	1.96	Wu *et al.* (2008)
		Muscle	EY	2.68	Wu *et al.* (2008)
		Muscle	MF	0.92	Wu *et al.* (2008)
		Muscle	FE	1.45	Wu *et al.* (2008)
Skate	*Raja kenojei*	Skin	PGPLGLTGP	95	Lee *et al.* (2011)
			QLGFLGPR	148	Lee *et al.* (2011)

Tuna	*Thunnus obesus*	Dark muscle	WPEAAELMMEVDP	21.6	Qian *et al.* (2007)
Tuna	-	Frame	GDLGKTTTVSNWSPPKYKDTP	11.28	Lee *et al.* (2010)
Shrimp	*Acetes chinensis*	Whole shrimp	DP	2.15	Wang *et al.* (2008a)
		Whole shrimp	ST	4.03	Wang *et al.* (2008a)
		Whole shrimp	GTG	5.54	Wang *et al.* (2008a)
Shrimp	*Plesionika izumiae*	Whole shrimp	VWYT	-	Nii *et al.* (2008)
			VW	-	Nii *et al.* (2008)
Shrimp	*Pandalus borealis*	Dried protein hydrolysate	FSY	7.7[a] 2.2[b]	Gildberg *et al.* (2011)
			FTY	274.6[a] 59.3[b]	Gildberg *et al.* (2011)
			PSY	1790[a] 1065[b]	Gildberg *et al.* (2011)
Cuttlefish	*Sepia officinalis*	Muscle	AHSY	11.6	Balti *et al.* (2010b)
			GDAP	22.5	Balti *et al.* (2010b)
			AGSP	37.2	Balti *et al.* (2010b)
			DFG	44.7	Balti *et al.* (2010b)
			AVV	66.6	Balti *et al.* (2010b)
			GVHHA	71.8	Balti *et al.* (2010b)
			FGG	82.5	Balti *et al.* (2010b)
			GHG	122.0	Balti *et al.* (2010b)
			IAV	153.4	Balti *et al.* (2010b)
			AGS	527.9	Balti *et al.* (2010b)
			AGSS	672.1	Balti *et al.* (2010b)
Hard clam	*Meretrix lusoria*	Meat	YN	51	Tsai *et al.* (2008)
Oyster	*Crassostrea gigas*	Meat	VVYPWTQRF	66	Wang *et al.* (2008b)
Sea cucumber	*Acaudina molpadioidea*	Body wall	MEGAQEAQGD	15.9	Zhao *et al.* (2009)
Rotifer	*Brachionus rotundiformis*	Whole body	DDTGHDFEDTGEAM	9.64	Lee *et al.* (2009)
Ascidian tunicate	*Styela clava*	Flesh tissue	AHIII	37.1	Ko *et al.* (2012)

(*continued overleaf*)

Table 6.1 (*continued*)

Source	Scientific name	Origin	Amino acid sequence	IC_{50} (μM)	Reference
Antioxidative properties					
Croaker	*Otolithes ruber*	Skin	GNRGFACRHA	-	Sampath Kumar *et al.* (2011)
Grass carp	*Ctenopharyngodon idellus*	Muscle	PSKYEPFV	-	Ren *et al.* (2008)
Hoki	*Johnius belengerii*	Frame	ESTVPERTHPACPDFN	41.37[c] 17.77[d]	Kim *et al.* (2007)
Horse mackerel	*Magalaspis cordyla*	Skin	NHRYDR	-	Sampath Kumar *et al.* (2011)
Loach	*Misgurnus anguillicaudatus*	Muscle	PSYV	17.0 mg/ml[c] 2.64 mg/ml[d]	You *et al.* (2010b)
Sardinelle	*Sardinella aurita*	Byproducts	LHY	-	Bougatef *et al.* (2010)
			GALAAH	-	Bougatef *et al.* (2010)
			GAWA	-	Bougatef *et al.* (2010)
			LARL	-	Bougatef *et al.* (2010)
			GGE	-	Bougatef *et al.* (2010)
			GAH	-	Bougatef *et al.* (2010)
			PHYL	-	Bougatef *et al.* (2010)
Sand eel	*Hypoptychus dybowskii*	Whole body	IVGGFPHYL	22.75	Lee *et al.* (2011)
Tilapia	*Oreochromis niloticus*	Scale	DPALATEPDPMPF	8.82[c] 7.56[d]	Ngo *et al.* (2010)
Tuna	-	Backbone	VKAGFAWTANQQLS	-	Je *et al.* (2007)
Tuna	-	Dark muscle	LPTSEAAKY	-	Hsu (2010)
			PMDYMVT	-	Hsu (2010)
Tuna	-	Cooking juice	PVSHDHAPEY	-	Hsu *et al.* (2009)
			PSDHDHE	-	Hsu *et al.* (2009)
			VHDY		Hsu *et al.* (2009)
Mussel	*Mytilus coruscus*	Meat	LVGDEQAVPAVCVP	-	Jung *et al.* (2007)
Oyster	*Crassostrea gigas*	Meat	LKQELEDLLEKQE	28.76[d]	Qian *et al.* (2008)
Rotifer	*Brachionus rotundiformis*	Whole body	LLGPGLTNHA	189.8	Byun *et al.* (2009)
			DLGLGLPGAH	167.7	Byun *et al.* (2009)

Antiproliferative activity					
Tuna	*Thunnus tonggol*	Dark muscle	LPHVLTPEAGAT	-	Hsu *et al.* (2011)
			PTAEGGVYMVT	-	
Antimicrobial and antiviral activity					
Half-fin anchovy	*Setipinna taty*	Muscle	MLTTPPHAKYVLQW	-	Song *et al.* (2012)
		Muscle	SHAATKAPPKNGNY	-	Song *et al.* (2012)
		Muscle	PTAGVANALQHA	-	Song *et al.* (2012)
		Muscle	QLGTHSAQPVPF	-	Song *et al.* (2012)
		Muscle	VNVDERWRKL	-	Song *et al.* (2012)
		Muscle	LATVSVGAVELCY	-	Song *et al.* (2012)
		Muscle	NPEFLASGDHLDNLQ	-	Song *et al.* (2012)
		Muscle	PEVVYECLHW	-	Song *et al.* (2012)

[a]N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly substrate
[b]Hippuryl-His-Leu substrate
[c]DPPH radical-scavenging activity;Hydroxyl radical-scavenging activity.

The AOA of giant squid-muscle hydrolysates was studied in different *in vivo* oxidative systems (Rajapakse *et al*., 2005b). Two antioxidant peptides were purified from the tryptic hydrolysate fraction with molecular weight below 3 kDa. They were characterised as NADFGLNGLEGLA (1307 Da) and NGLEGLK (747 Da). In a second work, it was also demonstrated that tryptic hydrolysates from jumbo squid exhibited strong lipid peroxidation inhibition (Mendis *et al*., 2005b). Two antioxidant peptides were purified and characterised as FDSGPAGVL (880.18 Da) and NGPLQAGQPGER (1241.59 Da). The cell viability in oxidation-induced human lung fibroblasts was enhanced following the treatment with the two peptides. Lin & Li (2006) prepared hydrolysates from jumbo flying squid gelatin with several proteases. The hydrolysate prepared in two steps with Properase E followed by pepsin exhibited the best radical-scavenging activity. The UF fraction (MW < 2000 Da) had high yield and radical-scavenging activity. Giménez *et al*. (2009a) observed that gelatin hydrolysates obtained by Alcalase from giant squid had higher antioxidant properties than the gelatin extracted from this raw material. It was demonstrated that the addition of gelatin hydrolysates prepared from the same squid species to gelatin films improved their antioxidant properties (Giménez *et al*., 2009b). In another work by Alemán *et al*. (2011) on the preparation of gelatin hydrolysates from giant squid, it was shown that all fractions obtained by UF and size-exclusion chromatography exhibited higher reducing power than the crude hydrolysate. The presence of Leu residues in the peptide sequence (GPLGLLGFLGPLGLS) seemed to play an important role in their AOA. Balti *et al*. (2011) studied the preparation of protein hydrolysates from cuttlefish mantle. They concluded that the AOA of hydrolysates obtained increased with increasing DH. The major amino acids of these hydrolysates were histidine and arginine.

Jung *et al*. (2007b) isolated and identified an antioxidative low-molecular-weight (1.59 kDa) peptide with 14 amino acids from a mussel protein hydrolysate. Qian *et al*. (2008) purified a potent antioxidative peptide (Table 6.1) from the *in vitro* oyster-muscle gastrointestinal digest with a molecular mass of 1.60 kDa.

Two antioxidant peptides were isolated from a marine rotifer peptic hydrolysate (Byun *et al*., 2009). Their amino acid sequences were identified as LLGPGLTNHA (1076 Da) and DLGLGLPGAH (1033 Da).

In order to improve the AOA of FPH, Guérard & Sumaya-Martinez (2003) demonstrated that Maillard reaction products (MRPs) prepared with protein hydrolysates and glucose possessed an antiradical-scavenging activity 75% higher than that of the starting hydrolysate. It was also shown that MRPs from ribose and tuna-protein hydrolysates had an antiradical activity 11-fold higher than MRPs prepared with glucose (Sumaya-Martinez *et al*., 2005).

6.4 ANTICANCER ACTIVITY

A large number of peptides with anticancer activity have been indicated from various sources (Shahidi & Zhong, 2008). However, the studies on antiproliferative activity (APA) derived from FPH are very limited. A peptide fraction inducing apoptosis in a human lymphoma cell line (U937) was separated from anchovy sauce (Lee *et al*., 2003). In a second study, Lee *et al*. (2004) found a peptide fraction in anchovy sauce exhibiting APA, which was composed of Ala and Phe and had an estimated molecular weight of 4409 Da. Picot *et al*. (2006) measured the APA of 18 commercial fish hydrolysates on two human breast cancer cell lines. Three blue whiting, three cod, three plaice and one

salmon hydrolysate possessed significant growth inhibitors against the two cancer lines. Protein hydrolysates obtained from tuna dark muscle with papain and PR were tested for their APA (Hsu *et al*., 2011). Peptide fractions with molecular weights ranging from 390 to 1400 Da possessed the greatest APA against the human breast cancer cell line MCF-7. The amino acid sequences of the two antiproliferative peptides were identified (Table 6.1). The APA of pepsin hydrolysates from half-fin anchovy was demonstrated in DU-145 human prostate cancer, 1299 human lung cancer and 109 human oesophagus cancer cell lines. In addition, it was recognised that peptide sterilisation increased its APA (Song *et al*., 2011a).

6.5 ANTIMICROBIAL AND ANTIVIRAL ACTIVITY

Antimicrobial peptides (AMPs) are usually low-molecular-weight peptides isolated and purified from a variety of living organisms, in which they are involved in natural defence mechanisms against pathogens. Since their first discovery at the beginning of the 1980s (Steiner *et al*., 1981), more than 700 molecules have been identified However, AMPs may also be obtained from food proteins by enzymatic hydrolysis (Bolscher *et al*., 2006; Daoud *et al*., 2005; Dionysius & Milne, 1997; Pellegrini *et al*., 2004; Pihlanto-Leppälä *et al*., 1999). The work by Liu *et al*. (2008) is one of the first reports describing the isolation of a cysteine-rich AMP by digestion of oyster. A pepsin hydrolysate prepared from half-fin anchovy displayed broad antibacterial spectra *in vitro* and against *Escherichia coli* (Song *et al*., 2010). Song *et al*. (2011b) used RSM to optimise the antibacterial activity of pepsin hydrolysates from half-fin anchovy. The optimised hydrolysate inhibited the growth of *E. coli* CGMCC 1.1100, *Pseudomonas fluorescens* CICC 20225, *Proteus vulgaris* CICC 20049 and *Bacillus megaterium* CICC 10324. In another study, Song *et al*. (2012) isolated and characterised the peptide fraction from the peptic hydrolysate of half-fin anchovy with antibacterial activity against *E. coli*. Five cationic peptides and three anionic peptides were identified and their secondary structures were also predicted (Table 6.1).

The presence of two peptides inhibiting HIV-1 protease was detected in an oyster hydrolysate prepared with thermolysin (Lee & Maruyama, 1998). The amino acid sequences of the peptides were determined as LLEYSI and LLEYSL. An active peptide against herpes virus was isolated from oyster first digested by alcalase followed by bromelin (Zeng *et al*., 2008).

6.6 CALCIUM-BINDING PEPTIDES

The presence of compounds which bind and solubilise calcium prevents its precipitation as calcium phosphate salts and consequently increases its availability for absorption. Several studies have demonstrated the presence of binding peptides in protein hydrolysates from hoki frame (Jung *et al*., 2005b, 2006b; Jung & Kim, 2007; Kim *et al*., 1999) and Alaska pollock backbone (Jung *et al*., 2006a).

6.7 APPETITE SUPPRESSION

Gastrin and cholecystokinin (CCK) are secretagogue molecules which exhibit a large spectrum of activities. Gastrin is a peptide hormone that stimulates the secretion of gastric

acid and aids gastric mobility. CCK is a peptide hormone that controls the emptying of the gall bladder and pancreatic enzyme secretion. It also regulates intestinal mobility, satiety signalling and the inhibition of gastric acid secretion. The presence of gastrin/CCK-like molecules has been detected in protein hydrolysates obtained from sardine, cod, blue whiting and shrimp byproducts (Cancre *et al*., 1999; Cudennec *et al*., 2008; Ravallec-Plé *et al*., 2000, 2001; Ravallec-Plé & Van Wormhoudt, 2003).

6.8 ANTICOAGULANT ACTIVITY

Several studies aimed at identifying novel anticoagulants in FPH have been reported. Of note are the reports on the isolation of active peptides or proteins from starfish (Koyama *et al*., 1998), echiuroid worm (Jo *et al*., 2008) and blue mussel (Jung & Kim, 2009). The purification of marine anticoagulant proteins from blood ark shell (Jung *et al*., 2001), yellowfin sole (Rajapakse *et al*., 2005c) and granulated ark (Jung *et al*., 2007a) has also been reported.

6.9 IMMUNOSTIMULANT ACTIVITY

The use of dietary immunostimulants has attracted particular interest as an alternative to vaccines in improving fish health. Kitao & Yoshida (1986) showed that intraperitoneal injection of low-molecular-weight peptides in rainbow trout may induce protection against infectious fish diseases. In a challenge experiment with Atlantic salmon fry fed with FPH, no protection was obtained against infection by *Aeromonas salmonicida* (Gildberg *et al*., 1995). Further experiments with Atlantic salmon fry concluded that low-molecular-weight peptide fractions may stimulate the activity of fish macrophages (Bøgwald *et al*., 1996; Gildberg *et al*., 1996). In another study with Atlantic cod fry fed with commercial feed supplemented with *Carnobacterium divergens* alone or in combination with immunostimulating peptides, no protective effect was observed. Murray *et al*. (2003) tested the effect of various supplements, including FPH, on the innate immune functions of juvenile coho salmon. Fish from each diet group were challenged with *Vibrio anguillarum* but no differences were found in survival among the various fed groups. In another experiment, a commercial fermented fish protein (FFP) was evaluated on its mucosal immune response in a murine model (Duarte *et al*., 2006). It was generally concluded that FFP was an immunomodulating food with the capacity to enhance nonspecific host-protection mechanisms.

6.10 HYPOCHOLESTEROLEMIC ACTIVITY

The cholesterol-lowering effect of soy protein has been demonstrated in numerous studies (Anderson *et al*., 1995; Carroll & Kurowska, 1995) but those carried out on the hypolipidemic effect of fish proteins are very limited (Bergeron & Jacques, 1989; Zhang & Beynen, 1993). The cardioprotective effect of protein hydrolysates prepared from salmon frames was demonstrated by Wergedahl *et al*. (2004). The cholesterol-lowering effect of these hydrolysates involved a decrease of plasma cholesterol in Zucker rats by reduction of the activity of acyl-CoA:cholesterol acyltransferase. Lin *et al*. (2010) showed that

freshwater clam hydrolysates had higher bile acid-binding capacity than the water extract of freshwater clam meat. It was also demonstrated that a combination of the freshwater clam hydrolysate and marine red alga (*Gracilaria* tenuistipitata) powder had a synergistic effect on bile acid-binding capacity.

6.11 HORMONE-REGULATING PROPERTIES

Calcitonin gene-related peptide (CGRP) is a 37-residue neuropeptide involved in a variety of physiological functions, including vasodilatation. It has positive inotropic and chronotropic effects on the heart, is implicated in gastric acid secretion, and can also inhibit the proliferative response of T lymphocytes to mitogens and macrophage activation. The presence of CGRP-like molecules was reported in protein hydrolysates from cod, sardine, shrimp and shark byproducts (Fouchereau-Peron *et al*., 1999; Martínez-Alvarez *et al*., 2007; Ravallec-Plé *et al*., 2001; Rousseau *et al*., 2001; Šližytė *et al*., 2009).

6.12 OTHER BIOLOGICAL ACTIVITIES

Bernet *et al*. (2000) concluded that the commercial Gabolysat PC60 showed diazepam-like effects on the stress responsiveness of the rat pituitary adrenal axis and on sympathoadrenal activity.

Dong *et al*. (2005) reported that hydrolysates from *Saurida elongata* contained significant *in vivo* anti-anaemia activity on experimental anaemia models induced by blood loss or cyclophosphamide damage to the haematogenic mechanism.

Fitzgerald *et al*. (2005) examined the potential bioactivity of a commercially available fermented protein prepared from Pacific hake. They concluded that this product possessed biological activity when assessed in a variety of models of gut integrity and repair. The product was studied in *in vivo* and *in vitro* models by Marchbank *et al*. (2009) and it was concluded that it reduced apoptosis and the gut-damaging effects of indomethacin.

Table 6.1 provides a summary of the most recent works on peptides derived from protein hydrolysates from marine sources that display biological activity.

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7 Biological Activities of Proteins and Marine-derived Peptides from Byproducts and Seaweeds

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7.1 INTRODUCTION

Diet therapy and lifestyle modifications are the two most important tools commonly employed to effectively improve human health. Any food component that has the ability after digestion to enhance health is a potential candidate for disease prevention and health maintenance. Bioactive peptides are amino acid sequences which display a variety of beneficial physiological activities once consumed that go beyond those of basic human nutrition (Vercruysse *et al*., 2005). The first food-derived bioactive peptide was identified in 1950, when Mellander reported that casein-phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants (Mellander, 1950). Bioactive peptides range from 2 to 30 amino acids in length, are usually less than 3 kDa in size and often have a myriad of functionalities, including antimicrobial (Andavan *et al*., 2010; Kim *et al*., 2009; Plaza *et al*., 2007), immunomodulatory (Yang *et al*., 2009), opioid (Pihlanto-Leppälä, 2000), angiotensin converting enzyme (ACE)-inhibitory (Wijesekara & Kim, 2010), renin-inhibitory (Fitzgerald *et al*., 2011), platelet activating factor acetyl-hydrolase (PAF-AH)-inhibitory (Wilson *et al*., 2011), prolyl endopeptidase (PEP)-inhibitory (Wilson *et al*., 2011), α-amylase-inhibitory (Li *et al*., 2004), anticoagulant (Kim & Wijesekara, 2010) and satiety-inducing (Anderson *et al*., 2011) activities. These peptide sequences are inactive within the parent protein sequence and must be cleaved before bioactivities are observed. They are cleaved from the parent protein by a number of methods, including enzyme hydrolysis, fermentation with lactic acid bacteria (LAB) or proteolytic bacteria, acid/base hydrolysis, excessive heating and autolysis methods, and gastrointestinal digestion (Hayes *et al*., 2006). Bioactive peptides have been isolated and characterised from a number of food sources, including milk (Hayes *et al*., 2006), eggs (Miguel *et al*., 2006), fish (Gimenez *et al*., 2009; You *et al*., 2009), meat (Di Bernardinin *et al*., 2011; Ryan *et al*., 2011), soy (Rho *et al*., 2009) and other vegetable sources, including marine macroalgae (seaweeds or sea vegetables) (Fitzgerald *et al*., 2011) and microalgae (Kang *et al*., 2012).

Regarding new bioactive peptide products developed for human benefit, the focus has been on prevention of hypertension and infectious diseases. Indeed, many of the bioactive peptides discovered to date are derived from dairy proteins such as casein and whey (Pihlanto-Leppälä, 2000; Rival *et al*., 2001). The most notable of these peptides include the ACE-inhibitory tripeptides, isoleucine-prolyl-proline (IPP) and valine-prolyl-proline

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(VPP), which can be found in the Japanese product Calpis® (which has obtained Foods of Specified Health Use (FOSHU) status) (Yamamoto *et al*., 2003) and the *Lactobacillus helveticus* fermented milk product produced by Valio of Finland (which also contains these tri-peptides but does not have a European Food Safety Authority (EFSA), article 13.5 health claim) (Ricci-Cabello *et al*., 2012). Other antihypertensive peptides discovered to date include the milk ingredient C_{12} peptide manufactured by the Dutch ingredients company DMV International, which is derived from casein protein and directly inhibits diastolic and systolic blood pressure (Townsend *et al*., 2004). Antimicrobial peptides have also been observed, including isracidin (Lahov *et al*., 1996), caseicins A and B (Hayes *et al*., 2006) and the marine-derived antimicrobial peptide Kahalalide F, known to be an anti-HIV agent, which is a cyclic depsipeptide isolated from the sacoglossan mollusc, *Elysia rufescens*, but most likely derived from *Bryopsis* sp., its green algal diet (Smit, 2004).

The marine environment is home to macroalgae, microalgae, shell, pelagic and white fish, cyanobacteria, sea urchins, tunicates and other species. These sources are potential reservoirs for unique proteins and peptides. Indeed, peptides with different bioactivities, such as antiproliferative, antioxidant and antimicrotubule activities, have been isolated from marine sources, specifically algae and cyanobacteria (Lordan *et al*., 2011; Smit, 2004). Moreover, byproducts of the marine processing industries, including skin, bone, trimmings, viscera and blood, may also be considered reservoirs for potential bioactive protein and peptide generation (Rustad & Hayes, 2012). Byproducts may be defined as products generated during marine processing that are not regarded as saleable but which can be recycled after treatment (Cudennec *et al*., 2008). In marine seafood processing, discards and byproducts constitute approximately 75% of the total weight of the catch, and solid wastes generated from seafood factories may range from 30 to 85% of the weight of landed fish (Shahidi, 1994). Valuable proteins, including gelatine and collagen, may be retrieved from byproducts such as fish skins and frames. Purified peptides isolated from marine byproduct sources have been shown to display antioxidant and cytotoxic activities in several human cancer cell lines, including HeLa, AGS and DLD-1 (Suarez-Jimenez *et al*., 2012), as well as other bioactivities including ACE inhibition (Wijesekara & Kim, 2010).

Much work has been carried out on the types of protein fraction that may be produced from fish byproducts (Kristinsson & Rasco, 2000). Early work on the chemical recovery of proteins from byproducts used chemical hydrolysis to produce fish-protein concentrates in order to increase the biological availability of protein (Rustad & Hayes, 2012). While marine hydrolysates have demonstrated good nutritional properties, the aim of this chapter is to discuss the bioactive properties of marine hydrolysates and also peptides isolated from macroalgal sources, including the interesting carbohydrate-binding proteins known as lectins and how these bioactive ingredients may be exploited for health maintenance. The chapter also discusses current and future methods for the generation and screening of bioactive peptides.

7.2 BIOACTIVE PEPTIDES

Peptides mediate a number of physiological functions in the human body. Bioactive peptides are defined as amino acid sequences between 2 and 30 amino acids in length that have a positive impact on health by acting with hormone-like effects on several systems within the human body (Mills *et al*., 2011). The bioactivities are encrypted within the

parent protein and peptides are released through several different mechanisms, including hydrolysis with enzymes, food processing and manufacturing using acids, alkali and heat, microorganisms and others (Mills *et al*., 2011). Bioactive peptides are also a source of nitrogen and amino acids (Harnedy & FitzGerald, 2011) (see Table 7.4b). Regardless of origin, once released, bioactive peptides must reach the target organ or receptors in the intestinal lumen intact and must survive enzymatic degradation. Bioactivity is based on the inherent amino acid sequence and composition (Adessi & Soto, 2002). Many peptides contain multifunctional bioactivities and certain sequences that exert different activities may be considered 'strategic zones' that are partially protected from further breakdown by proteolysis (Meisel, 2004). For example, a 'strategic zone' is located in bovine and human β-casein f (60–70), and this sequence is protected from proteolysis by its high hydrophobic amino acid content and the presence of proline (Pro) residues (Meisel, 2004).

7.3 MARINE-DERIVED BIOACTIVE PEPTIDES

Sources of marine-derived bioactive peptides include marine animals such as tunicates, sponges and molluscs, marine byprocessing waste streams, including pelagic, white and shellfish, and macroalgae and microalgae. Biologically active peptides with antiproliferative, antioxidant, antimicrotubule and antimicrobial activities have been isolated from a number of algae and cyanobacteria. Bioactive peptides from marine animal sources have been reviewed recently by Suarez-Jimenez *et al*. (2012). This group has reported the various peptides from ascidians, tunicates and molluscs. The structural characterisation of peptides from these sources shows that the peptides contain unusual amino acid residues which may be responsible for their enhanced bioactivities. For example, squirts produce the compound *Didemnin*, which was first isolated from the Caribbean tunicate *Tridemnum solidum*. *Didemnin* B has potent antitumour activity and has demonstrated antiproliferative activity against human prostatic cancer cell lines (Suarez-Jimenez *et al*., 2012). *Aplidine*, a cyclodepsipeptide isolated from the tunicate *Aplidium albicans*, was shown to have anticancer activity against human cancer cell lines including melanoma and lung cancers (Broggini *et al*., 2003). It is thought to work through inhibition of protein synthesis.

7.4 ISOLATION AND CHARACTERISATION OF MARINE-DERIVED BIOACTIVE PEPTIDES

7.4.1 Byproducts and Fish-protein Hydrolysates

The isolation of bioactive peptides from marine proteins of macroalga and marine byproduct origin is the focus of this chapter. Marine-protein hydrolysates were studied in detail in recent years, and the liberation of bioactive peptides encrypted within marine 'food' proteins through enzymatic hydrolysis, fermentation and other methods is the accepted standard in the generation of functional-food bioactive peptide ingredients (Di Bernardini *et al*., 2011; Meisel, 2004). Essuman (1992) defined fermented fish as any fishery product that has undergone degradative changes through microbiological or enzymatic activity in the presence or absence of salt. Although produced on a worldwide scale, fermented fish products are most popular in Asia and Africa (Table 7.1), and their production in aiding to develop a sustainable fish processing industry could be developed

Table 7.1 Fish protein hydrolysates from European and African countries. Modified from Hall (2010), Essuman (1992).

Country of origin and use	Local name	Fish species used	Duration of fermentation
Cambodia	Prahoc	Freshwater fish species (*Cyprinidae*)	Variable
Indonesia	Ketjap-ikan	*Ctenops* spp.	Variable
Indonesia	Trassi	Shrimp	Variable
Japan	Qunaga	*Katsuwonus pelamis*	Variable
Philippines	Patis	*Sardinella perforate*, *Leiognathus* spp., any fish or shrimp species	6–12 months
Philippines	Balao-balao	Shrimp paste—varies in amount of salt added	Typically 4 days
Burundi	Ndagala	All fish species	2–5 days
Senegal	Guedi, tambadiang, yet	All fish species	Overnight—2 days
Ghana	Momone, koobi, kako, ewule	All fish species	Overnight—3 days
Scandinavian countries	Gaffelbitar	Atlantic herring (*Clupea harengus*)	12–18 months
Scandinavian countries	Tidbits	Atlantic herring (*Clupea harengus*)	12–18 months
Swedish origin	Surstromming (sour herring)	Atlantic herring (*Clupea harengus*)	12–18 months
Norwegian and Swedish origin	Rakfisk	Trout (*Salmo trutta*)	3–12 months
Iceland	Hakarl	Shark	Variable
Germany (of Scandinavian origin)	Gravad lacks	All fish species	Variable

further, particularly in Europe. Several potential human physiological bioactivities are often associated with fermented protein products, and bioactive peptides have been isolated from fish muscle (Kim *et al*., 2012). Antioxidant peptides have been identified from vertebrate muscle of fish, fish byproducts and squid (see Table 7.4a). In addition, studies have been performed concerning the antioxidant activities of silver and grass carp muscle hydrolysates (Dong *et al*., 2008). Hydrolysis was performed using the enzyme Alcalase® for 1.5–2.0 hours.

Enzymatic hydrolysis is ordinarily employed to liberate the bioactive peptide sequence from the parent protein. Acid and alkaline hydrolysis methods may also be used, but certain amino acids can be destroyed at high pH, particularly tryptophan, serine and threonine (Thorkelsson *et al*., 2009). Commercially available enzymes including alcalase, trypsin, pepsin, papain, pancreatin, pepsin, thermolysin and others have been used to liberate bioactive peptides from a variety of marine-protein sources (Di Bernardini *et al*., 2011). The pH and temperature of the hydrolysate are closely observed during the hydrolysis process to ensure that undesirable products are not obtained (Thorkelsson & Kristinsson, 2009). Marine byproduct-derived hydrolysates studied to date have exhibited good nutritional properties. The chain length of the peptides is of importance as it impacts on the organoleptic and functional characteristics of the hydrolysate and also affects bitterness, solubility and emulsifying capacity (Rustad, 2007). For example, powders of fish-protein hydrolysate (FPH) produced from fish viscera where the gall bladder was in place were

Table 7.2 Factors that affect the quality, sensory and functional properties of fish protein hydrolysates (FPHs).

Factor	Processing parameter
Chilled storage	Gutting of fish
Type	Chilled storage
Enzyme state of raw material	Enzyme hydrolysis length control
Stability of raw material	pH control
Removal of internal organs	Temperature control

more bitter in taste than powders derived without gall bladders, indicating that sorting of byproducts is necessary to obtain FPHs with acceptable sensory profiles (Dauksas *et al*., 2004). Bile acids impact negatively on FPH sensory profiles (Dauksas *et al*., 2004). In order to remove these, cationic compounds such as soy protein, black beans or wheat gluten are used. These complex the bile acids and are subsequently easily removed. Cholestyramine resin is often used to bind bile acids (Rustad *et al*., 2011).

Factors such as the source, quality, safety and stability of the byproduct materials are important considerations when generating protein hydrolysates containing bioactive peptides. Control of spoilage due to growth of pathogenic and spoilage bacteria can be obtained by chilling, storing the products at low temperatures and implementing good manufacturing practice (GMP) (Ioannis *et al*., 2009). Gutting of live demersal fish improves the quality and storage life. Histamine formation in fish such as mackerel and herring is also a problem but may be overcome by chilling (Thorkelsson & Kristinsson, 2009). The type of raw material, the enzyme activity of the raw material and the state of degradation, along with the choice of enzyme and the process conditions employed, determine the yield and properties of the resulting fish hydrolysate (Thorkelsson & Kristinsson, 2009) (Table 7.2).

7.4.2 Macroalgal Protein and Peptide Hydrolysates

Macroalgae are a diverse group of marine organisms which survive in harsh, competitive environments where variability in salinity, tidal and solar radiation, competition for space and nutrients, and grazing stresses are commonplace (Olson & Lubchenco, 1990). They can survive due to their unique and complex metabolic pathways. Macroalgae are classified into three higher taxa—Class Phaeophyceae (brown algae), Phylum Rhodophyta (red algae) and Phylum Chlorophyta (green algae)—based on their pigmentation (Chan *et al*., 2006). They are a rich source of unique bioactive compounds, including peptides. The protein content of macroalgae varies with species and with the site and time of harvesting (FitzGerald *et al*., 2011; Fleurence, 1999; Harnedy & FitzGerald, 2011). A number of studies report that the protein content of macroalgae varies between 3 and 47% (w/w) dry weight (FitzGerald *et al*., 2011; Fleurence, 1999; Harnedy & FitzGerald, 2011). In general, the red species contain the highest amounts of protein and are therefore the most suitable seaweeds for protein isolation and peptide generation (Fleurence, 1999). Two red macroalgal species, *Palmaria palmata* (commonly known as dulse, dillisk or creathnach) and *Porphyra tenera* (commonly known as nori, sleabhac, laver or sloke), contain up to 47% protein (w/w) dry weight (Mabeau & Fleurence, 2003). Furthermore, red macroalgae contain high amounts of aspartic and glutamic acid, and *Palmaria palmata* and *Porphyra* species have been reported to contain high amounts of the amino

acids glycine and arginine in particular (Dawczynski *et al*., 2007). The green algae *Ulva lactuca* and *Enteromorpha intetinalis* are reported to contain up to 25 and 18% protein (w/w) dry weight, respectively (Harnedy & FitzGerald, 2011). *Ulva armoricana* contains high amounts of proline and *U. pertusa* contains high amounts of arginine (Hwang *et al*., 2008). The highest protein content in *Palmaria palmata* was recorded in winter and spring, when 24.5% was recovered (Fleurence *et al*., 1999; Harnedy & FitzGerald, 2011). Macroalgae contain two families of protein known for their bioactivities: the lectins and the phycobiliproteins.

7.5 LECTINS

Lectins were first described in 1888 by Stillmark, working with castor bean extracts (Vasconcelos *et al*., 2004). Marine-derived lectins were first reported by Boyd (1970). Lectins are agglutinins; they are proteins that bind carbohydrates without initiating their further modification through associated enzymatic activities. They are primarily found in protein bodies in cells, in proteins made in the endoplasmic reticulum and transported via the Golgi apparatus. They have dissimilar structures and are therefore classified according to their functionality rather than their structure. They have a wide variety of bioactivities, including induction of apoptosis (Büssing *et al*., 1996), cancer metastasis, host–pathogen interactions, cell–cell communication, activity against the human immunodeficiency virus (HIV) and other antiviral activities (Ziolkowska & Wlodawer, 2006) (Table 7.3). Lectins are useful for the detection of disease-related alterations of glycan synthesis and in blood-group typing (Naeem, 2007). Lectins also play an important role in the recognition and adherence of gametes during sexual reproduction (Kremp & Anderson (2007). They are resistant to high temperatures and pHs and have no odour, making them suitable candidates for microbiocide drug development (Ziolkowska & Wlodawer, 2006).

Table 7.3 Macroalga-derived lectins and associated bioactivities.

Lectin activity	Macroalgal source	Reference
Cytotoxic	*Soleria robusta*	Hori *et al.* (1988)
Mitogenic	*Eucheuma serra*	Kawakubo *et al.* (1997)
Mitogenic	*Eucheuma amakusaensis*	Kawakubo *et al.* (1999)
Mitogenic	*Eucheuma cottonii*	Kawakubo *et al.* (1999)
Cytotoxic	*Eucheuma serra*	Sugahara *et al.* (2001)
Antibacterial	*Eucheuma serra*	Liao *et al.* (2003)
Antibacterial	*Galaxaura marginata*	Liao *et al.* (2003)
Antinociceptive	*Bryothamnion triquetrum*	Viana *et al.* (2002)
Antinociceptive	*Bryothamnion seaforthii*	Viana *et al.* (2002)
Antinociceptive	*Amansia multifida*	Neves *et al.* (2007)
Antinociceptive	*Hypnea cervicornis*	Bitencourt *et al.* (2008)
Anti-inflammatory	*Hypnea cervicornis*	Bitencourt *et al.* (2008)
Anti-HIV	*Griffithsia* sp.	Mori *et al.* (2005)
Human platelet aggregation inhibition	*Hypnea japonica*	Matsubara *et al.* (1996)
Antiadhesion	*Bryothamnion triquetrum*	Teixeira *et al.* (2007)
Antiadhesion	*Bryothamnion seaforthii*	Teixeira *et al.* (2007)
Mitogenic	*Soleria robusta*	Hori *et al.* (1988)

7.5.1 Isolation of Lectins

The algal material should be freeze-dried or frozen in liquid nitrogen prior to homogenisation for optimum lectin isolation (Yang *et al*., 1996). The addition of detergents, such as Tween 80, phosphate buffered saline (PBS) and others, to the extraction medium and diluents enhances extraction and enables detection of the lectins (Brechtel *et al*., 2001). Affinity chromatography may be used to isolate lectins. A general affinity complex such as yeast mannan-cellulofine, which facilitates the isolation of purified lectin, may be used for biochemical characterisation (Rogers & Hori, 1993). Lectins' carbohydrate-binding specificity ensures that they are useful in immunological and histochemical studies, such as characterisation of glycoconjugates or probing of cell-surface sugars. As all biological membranes contain glycoconjugates, lectins can be used to study a wide variety of living organisms (Roger & Hori, 1993).

Red algal lectins exist as three types: low-molecular-weight molecules which bind glycoproteins but not monosaccharides and have no requirement for divalent cations; lectins which bind monosaccharides and related small molecules but have no divalent cation requirements; and larger lectins with molecular weights greater than 64 kDa which bind monosaccharides in the presence of divalent cations (Rogers & Hori, 1993). Only green algal lectins capable of forming oligomers have the capacity to bind monosaccharides (Ambrosio *et al*., 2003).

A lectin from the red marine alga *Hypnea musciformis* was purified by extraction with 20 mM PBS, precipitation with 70% saturated ammonium sulfate, ion-exchange DEAE-cellulose chromatography and RP-HPLC (Nagano *et al*., 2005). The 9.3 kDa polypeptide agglutinated erythrocytes. Mannose-binding lectins, such as cyanovirin-N derived from the blue-green algae *Nostoc ellipsosporum*, have shown high-affinity interactions with the envelope glycoprotein gp120 of HIV, enhancing its potential as an anti-HIV microbicide (Nagano *et al*., 2005; Ziolkowska & Wlodawer, 2006).

7.6 PHYCOBILIPROTEINS

Phycobiliproteins are economically important, water-soluble, fluorescent, coloured proteins found in blue-green and red algae and characterised by a tetrapirrolic ring covalently attached to their structure (Harnedy & FitzGerald, 2011; Sekar & Chandramohan, 2008). They play an important role in photosynthesis in algae. They are either blue-coloured phycocyanobilin (PCB), red-coloured phycoerythrobilin (PEB), yellow-coloured phycourobilin (PUB) or purple-coloured phycobiliviolin (PXB), also known as cryptoviolin (Sekar & Chandramohan, 2008). The tetrapirrolic ring is partly responsible for the functional properties associated with these proteins; bioactivities associated with phycobiliproteins include hepatoprotective, anti-inflammatory and antioxidant activities (Harnedy & FitzGerald, 2011; Sekar & Chandramohan, 2008). Phycobiliproteins include phycoerythrin, phycocyanin, allophycocyanin and phycoerythrocyanin. Phycoerythrin is found at levels of up to 12% (dry weight) in the red algal *P. palmata* and *Gracilaria tikvahiae* (Martínez & Rico, 2003). Phycoerythrin, along with other phycobiliproteins, is of economic importance as it spontaneously fluoresces both *in vitro* and *in vivo* and therefore has a number of applications in biotechnology (Sekar & Chandramohan, 2008). For example, phycoerythrin is used in flourescent immunoassays and biomolecule labelling (Glazer, 1994). Other phycobiliproteins, such as phycocyanin, are used as

natural food colourants (Harnedy & FitzGerald, 2011). Indeed, two companies in Tokyo market phycocyanin as a food colourant. There have been 17 patents for the therapeutic applications of phycobiliproteins submitted worldwide (Harnedy & FitzGerald, 2011).

The primary use of phycobiliproteins is as natural dyes. They are used as colourants in chewing gums, soft drinks, dairy products and cosmetics (Deshmurkh & Puranik, 2012). In addition, phycobiliproteins have been reported to have a number of bioactivities and are documented as exhibiting antioxidant, antitumour, anti-inflammatory, serum lipid-reducing and lipase-inhibiting activities (Harnedy & FitzGerald, 2011; Sekar & Chandramohan, 2008). The primary organisms exploited for the production of phycobiliproteins are *Spirulina* for phycocyanin and the red alga *Phorphyridium* for phycoerythrin (Deshmurkh & Puranik, 2012; Harnedy & Fitzgerald, 2011; Sekar & Chandramohan, 2008).

7.6.1 Isolation of Phycobiliproteins

The extraction of phycobiliproteins from different algal sources has been extensively studied due to their economic importance. Extraction protocols usually include the selection of a suitable source of phycobiliprotein, such as the protein-pigment molecules from blue-green and red micro- and macroalgae, followed by disruption of the algal cells and release of the phycobiliprotein of interest. Moraes and colleagues (2011) extracted C-phycocyanin, a natural blue dye used in the food and pharmaceutical industries, from *Spirulina platensis* wet biomass. A number of extractions were carried out using six different methods, including chemical (organic and inorganic acid treatment), physical (freezing and thawing, sonication, homogenisation) and enzymatic (lysozyme treatment) methods (Moraes *et al*., 2011). Extraction using an ultrasonic bath in the presence of glass pearls in the biomass proved to be the most efficient method, giving yields of C-phycocyanin 56% greater than that obtained using the common freeze–thaw method (Harnedy & FitzGerald, 2011; Moraes *et al*., 2011).

7.7 OTHER AMINO ACIDS AND PEPTIDES PRESENT IN AND DERIVED FROM MACROALGAE

Marine algal proteins are a potential source of novel bioactive peptides with unique bioactivities (FitzGerald *et al*., 2011; Fleurence *et al*., 1999; Harnedy & FitzGerald, 2011; Tierney *et al*., 2010). Bioactive peptides, particularly ACE-inhibitory peptides, have been isolated from a variety of macroalgal species. For example, water extracts of 10 green and 19 brown algae extracted at 70 °C displayed ACE-inhibitory activities (Cha *et al*., 2006; Harnedy & FitzGerald, 2011; Jung *et al*., 2006a; Sato *et al*., 2002; Suetsuna and Nakano, 2000; Tierney *et al*., 2010). Hydrolysis of these water extracts with commercially available enzymes increased ACE-inhibitory activities and reduced the IC_{50} values (Cha *et al*., 2006). In addition, a pepsin hydrolysate of *Porphyra yezoensis* also displayed ACE- and calcium precipitation-inhibitory, antimutagenic and antioxidant activities (Suetsuna & Saito, 2001). The accessibility of the chosen enzyme to the parent protein is an important factor to take into consideration when generating bioactive protein hydrolysates. One solution is to extract the parent protein from the macroalgal species chosen. Alternatively, polysaccharide-degrading enzymes may be used to solubilise fibre and allow access to the parent protein (Harnedy & FitzGerald, 2011). Algal compounds,

including fibre, phenolic compounds and trypsin inhibitors, may affect enzymatic digestion (Harnedy & FitzGerald, 2011; Samarakoon & Jeon, 2012). However, it is difficult to characterise to completion peptides contained within macroalgal hydrolysates as at present not all proteins from macroalgal sources are sequenced.

7.8 MEMBRANE PROCESSING

Protein hydrolysates generated from macroalgae or from marine processing waste streams are complex matrices that contain a large number of hydrolysed protein fractions. In order to isolate the bioactive peptides responsible for the observed functionality of the hydrolysates, it is necessary to fractionate the hydrolysates using membrane technologies and other devices for the separation of bioactive peptides. These include conventional pressure-driven processes and non-pressure-driven processes that combine an electrical field as a driving force with porous membranes for selective separation (Bazinet & Firdaous, 2009). Membrane separation is based on the selective permeability of one or more of the liquid constituents through the membrane. Pressure is usually the main driving force applied in ultrafiltration, microfiltration and nanofiltration (Bazinet & Firdaous, 2009). Membranes are selective barriers that allow for transport of certain components and retention of others. Ultrafiltration is the method of choice for the enrichment of bioactive peptides from protein hydrolysates. Several studies have described the use of ultrafiltration for the isolation of bioactive peptides. One example is the generation of antithrombotic peptides from caseinomacropeptide using membrane reactors. Membrane filtration has been used in the generation of phosophopeptide-enriched mixtures.

Nanofiltration is also useful for the separation of marine-derived peptides, due to the suitable cut-off of nanofiltration membranes and their electrochemical effects. Nanofiltration was used in the generation of peptide fractions from sweet whey to separate and isolate peptidic fractions with a molar mass range of 500–1800 Da. Nanofiltration could also be applied in the separation of bioactive peptides from marine fish and macroalgal hydrolysates. Indeed, several hydrolysates from different substrates have been fractionated using nano- and ultrafiltration in this way. One example is the fractionation of hot washing waters of cod-frame proteins (Jeon *et al*., 1999; Picot *et al*., 2010), Alaskan pollock-frame proteins (Je *et al*., 2005) and jumbo squid-skin gelatine (Mendis *et al*., 2005a,b; Picot *et al*., 2010).

7.9 BIOACTIVITIES OF MARINE-DERIVED PEPTIDES – INHIBITING PROTEASES FOR HEALTH

Proteases are proteolytic enzymes that are responsible for the breakdown of proteins through hydrolysis of peptide bonds (Turk, 2006). They are classified according to their mechanism of action as serine, cysteine or threonine proteases or as metallo, aspartic or glutamic proteases. They specifically cleave protein substrates from the N- or C-termini, or in the middle of the molecule in the case of endopeptidases (Turk, 2006). Protease signalling pathways are strictly regulated and any upset of protease activity can cause pathologies such as cardiovascular, cancer and inflammatory diseases as well as neurological disorders and osteoporosis (Turk & Stoka, 2007). They are therefore important targets for drugs and functional-food molecules such as peptides. Examples include renin and ACE.

Protease biology is important in the identification and validation of biomarkers for drug discovery and development, especially in cancer diagnosis. For example, the serine protease plasminogen activator and its inhibitor PAI1 are markers for breast cancer, and the serine protease kallikrein 3, also known as prostate-specific antigen (PSA), is the major diagnostic marker for prostate cancer (Turk, 2006). Inhibition of protease enzymes by food-derived peptides also plays an important role in the control of blood pressure within the renin–angiotensin–aldosterone system (RAAS).

7.10 HEART-HEALTH BIOACTIVE PEPTIDES

Bioactive peptides are known to play an important role in the prevention of high blood pressure, a well-known risk marker for cardiovascular disease (CVD) (Kitts & Weiler, 2003; Vermeirssen *et al*., 2004; Wijesekara & Kim, 2010). CVD is the leading cause of death in Western society and ranks in the top five causes of death in less-developed countries. A number of enzymes have a key role in the control of blood pressure within the RAAS in the human body (Dayane *et al*., 2012). The RAAS (Fig. 7.1) is involved in the regulation of blood pressure and salt–water balance. Activation of the RAAS is a key step in the progression of hypertension in the human body and may result in chronic heart failure (Remuzzi *et al*., 2005). Abnormal activation of the RAAS has been associated with the pathogenesis of cardiovascular, lung and renal diseases, including hypertension,

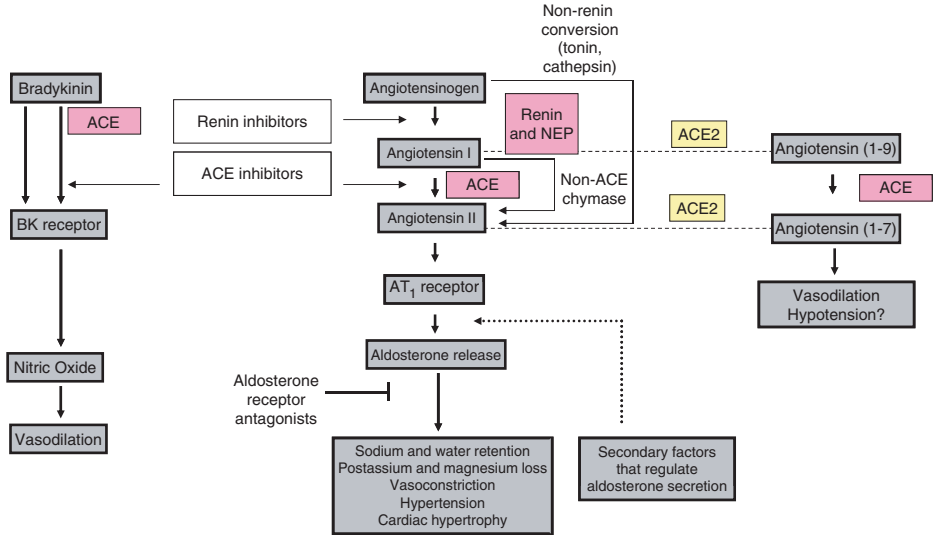

Fig. 7.1 The renin-angiotensin-aldosterone system (RAAS). The RAAS plays a key role in the regulation of blood-pressure control and salt–water and electrolyte balance, with effects on cardiovascular and renal functions. The enzyme renin is the rate-limiting step. It cleaves angiotensinogen to generate the decapeptide angiotensin I (Ang I). Angiotensin-converting enzyme (ACE) cleaves Ang I to form the octapeptide angiotensin II (Ang II), a key regulator of the RAAS. Ang II acts through the AT receptors (Ang II receptor type I and Ang II receptor type II). Angiotensin I also serves as a substrate for ACE and ACE2. ACE2 acts on different peptide substrates and removes a single residue from Ang I to give Ang (1–9). ACE2 cleaves a single residue from Ang II to yield Ang (1–7). ACE2 protects against acute respiratory distress syndrome (ARDS).

myocardial infarction and heart failure, as well as acute respiratory distress syndrome (ARDS), a severe form of acute lung injury (ALI) (Imai *et al*., 2008).

7.10.1 ACE Inhibition

ACE inhibitors are a major protease inhibitor success story and sales of ACE-inhibitory drugs exceed US$6 billion annually (Turk, 2006). ACE is an important target in blood-pressure regulation since it performs the last step in the biosynthesis of the vasoconstricting octapeptide angiotensin II within the RAAS (Carluccio *et al*., 2001). ACE also destroys the vasodilating peptide bradykinin. ACE inhibitors share a number of features: they have low molecular mass, they usually contain a metal-chelating group that binds to the zinc moiety in the enzyme's active site and they target S1, S1′ and S2′ subsites of ACE with high affinity. The C-terminal domain of ACE is primarily responsible for the conversion of angiotensin I into angiotensin II, whereas the N-terminal domain is involved in haemoregulation. Several food protein-derived ACE-inhibitory peptides have been isolated from sources including milk (Hayes *et al*., 2007), soy (Nakamori, 2010; Norris *et al*., 2012; Wu *et al*., 2006) and terrestrial plants (Udenigwe & Aluko, 2012). Indeed, several ACE-inhibitory peptides have also been isolated from marine byproducts including cod frame, pollock skin, sea bream scales, yellow sole frame, tuna frame, clam, krill, mussels and prawns (Howell & Kasase, 2010). ACE-inhibitory peptides are usually short-chain peptides (di- or tripeptides) that carry polar amino acid residues, such as proline (Norris *et al*., 2012). Structure–activity relationship studies carried out to date suggest that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, indicating that peptides which contain hydrophobic amino acids at these positions are potent ACE inhibitors (Hayes *et al*., 2007). ACE-inhibitory peptides are often produced as prodrugs, ensuring oral bioavailability (Jimsheena & Gowda, 2010). Some of the rules governing ACE activity are shown in Table 7.4a. Examples of ACE-inhibitory prodrug-type peptides include C_{12} peptide, with the sequence Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys, produced by DMV (Ricci-Cabello, 2012) (Table 7.4).

Several seaweed species are rich in proteins. The protein content in seaweeds varies from species to species, with *Palmaria palmata* reported to contain up to 47% protein, depending on the site and season of collection (Fleurence *et al*., 1999; Harnedy & FitzGerald, 2011). A number of bioactive peptides with antioxidant and ACE-inhibitory activities have been reported recently (Tierney *et al*., 2010). *Undaria pinnatifida* (commonly called 'wakame') was reported to have *in vitro* ACE-inhibitory and *in vivo* antihypertensive activity due to bioactive peptides. Furthermore, wakame was found to be the source of four dipeptides with demonstrated antihypertensive activities in spontaneously hypertensive rats (SHR) (Suetsuna *et al*., 2004). The dipeptide IY was reported to decrease systolic blood pressure (SBP) by 21 and 33 mmHg at doses of 1 and 50 mg/kg, respectively (Suetsuna *et al*., 2004). Furthermore, antihypertensive peptides were isolated from *Porphyra yeoensis* (Nori) and the microalga *Spirulina platensis* (Tierney *et al*., 2010). The peptide IQP, derived from *Spirulina platensis* protein, demonstrated antihypertensive effects in SHR (Lu *et al*., 2010). Sato and colleagues (2002) identified seven different ACE-inhibitory peptides in a butanol fraction of an *Undaria pinnatifida* hydrolysate, which was generated using the enzyme protease S 'Amano'®. Peptides reported included the dipeptides Val-Tyr, Ile-Tyr, Phe-Tyr and Ile-Trp, which were found to decrease blood pressure significantly when administered at a dose of 1 mg/kg of body weight. Previous to the study by Sato and colleagues (2002), the peptides Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly and Tyr-Asn-Lys-Leu were generated from *Undaria pinnatifida*

Table 7.4 (a) ACE-inhibitory peptides from marine muscle sources.

(a)

Peptide sequence	Bioactivity	Fish source	Reference
LKVGGKGY	ACE-inhibitory	Sardine	Suetsuna & Osajima (1986)
MF	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
RY	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
MY	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
LY	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
YL	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
IY	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
KW	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
VF	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
GRP	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
RFH	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
AKK	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
RVY	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
GWAP	ACE-inhibitory	Sardine	Matsufuji *et al.* (1994)
PTHIKWGD	ACE-inhibitory	Tuna	Kohama *et al.* (1988)
VAWKL	ACE-inhibitory	Tuna	Astawan *et al.* (1995)
WSKVVL	ACE-inhibitory	Tuna	Astawan *et al.* (1995)
SKVPP	ACE-inhibitory	Tuna	Astawan *et al.* (1995)
CWLPVY	ACE-inhibitory	Tuna	Astawan *et al.* (1995)
VW	ACE-inhibitory	Pelagic thresher	Nomura *et al.* (2002)
IKW	ACE-inhibitory	Pelagic thresher	Nomura *et al.* (2002)
VTR	ACE-inhibitory	Pelagic thresher	Nomura *et al.* (2002)
FRVFTPN	ACE-inhibitory	Pelagic thresher	Nomura *et al.* (2002)
MW	ACE-inhibitory	Pelagic thresher	Nomura *et al.* (2002)
WA	ACE-inhibitory	Salmon	Ono *et al.* (2003)
WM	ACE-inhibitory	Salmon	Ono *et al.* (2003)
VW	ACE-inhibitory	Salmon	Ono *et al.* (2003)
MW	ACE-inhibitory	Salmon	Ono *et al.* (2003)
IW	ACE-inhibitory	Salmon	Ono *et al.* (2003)
LW	ACE-inhibitory	Salmon	Ono *et al.* (2003)
FL	ACE-inhibitory	Salmon	Ono *et al.* (2006)
DW	ACE-inhibitory	Salmon	Ohta *et al.* (1997)
GIG	ACE-inhibitory	Salmon	Ohta *et al.* (1997)
YRPY	ACE-inhibitory	Bonito	Matsumura *et al.* (1993)
GHF	ACE-inhibitory	Bonito	Matsumura *et al.* (1993)
VRP	ACE-inhibitory	Bonito	Matsumura *et al.* (1993)
LRP	ACE-inhibitory	Bonito	Matsumura *et al.* (1993)
IRP	ACE-inhibitory	Bonito	Matsumura *et al.* (1993)

(*continued overleaf*)

Table 7.4 (b) Structural elements and amino acid content observations associated with peptides with different bioactivities.

(b)

Bioactive function	Structural elements	Observations	Reference
Angiotensin I converting enzyme (ACE) inhibition	Pro or hydroxy-Pro as C-terminus Pro, Lys or Arg as C-terminus Tyr or Phe as C-terminus Aromatic or branched side-chain residues preferred Aliphatic, basic and aromatic residues preferred in the penultimate position Aromatic, proline and aliphatic residues preferred in the ultimate positions Positive charge of arginine at the C terminus also preferred for inhibition	Usually resistant to degradation by digestive enzymes Preferred C-terminal residues with contribution to ACE-inhibitory potency Contribution to the antioxidant potency	Vermeirssen *et al.* (2004) Matsufuji *et al.* (1994) Suetsuna *et al.* (1998) Hayes *et al.* (2007)
Antioxidant activities	High amounts of His and hydrophobic amino acids Peptides with Pro-His-His sequence	Pro-His-His peptides show the greatest antioxidant activities Important for antithrombotic activity	Chen *et al.* (1996) Fiat *et al.* (1989)
Hypocholesterolemic	Low ratios of methionine-glycine and lysine-arginine in the dietary protein High in hydrophobic amino acid residues	Correlates positively with an antithrombotic effect Favours a hypocholesterolemic effect	Chabance *et al.* (1995) Making *et al.* (1986) Erdmann *et al.* (2008)
Anti-obesity	Peptide length Multiple Arg residues	Influences CCK-releasing activity, which is different with each protein Necessary condition for CCK release through direct binding to intestinal cells	Erdmann *et al.* (2008) Erdmann *et al.* (2008) Nishi *et al.* (2001)
Antithrombotic activity	Ile, Lys and Asp residues of casoplatelin	Important for antithrombotic activity	Chabance *et al.* (1995) Erdmann *et al.* (2008)

hydrolysed with pepsin. The IC_{50} values for these peptides were between IC_{50} 21 and IC_{50} 213 μm (Suetsuna & Nakano, 2000).

In order to detect ACE inhibitors present in or derived from macroalgal proteins, a reliable and reproducible assay is required. ACE inhibition is commonly measured using synthetic tripeptides and quantification of the released N-terminal amino acid (Cushman & Cheung, 1971). Most methods are based on the assay developed by Cushman and Cheung (1971), which is based on the cleavage of hippuryl-L-histidyl-L-leucine (HHL). N-terminal hippuric acid (HA) is released after cleavage of the dipeptide from the C-terminus, which is catalysed by ACE. HA is then quantified spectrophotometrically at a wavelength of 228 nm or else can be measured using HPLC or capillary electrophoresis. For example, He and colleagues (2007) used CE to screen a marine-protein hydrolysate enriched in peptides with ACE-inhibitory activity.

Other substrates, such as furanacryloyl-prolyl-glycyl-glycine (FA-PGG), may be used when screening for ACE inhibitors. FA-PGG is hydrolysed and separated into the related amino acid, FA-Phe, and the dipeptide, Gly-Gly (Lahogue *et al*., 2010). The absorbance is measured at 34 nm (Lahogue *et al*., 2010). A modern application of the FA-PGG method involves separation of the FA-PGG hydrolysis product 2-furylacryloyl-Phe by reverse phase chromatography and FAP detection using UV-titration. This method is reported as being more sensitive and economical (Lahogue *et al*., 2010). Sentandreu & Toldrá (2006) also developed a fluorescence-based protocol for quantifying ACE activity.

7.10.2 Renin Inhibition

The enzyme renin is an aspartic protease that catalyses the initial rate-limiting step in the RAAS and converts angiotensinogen to angiotensin I (Verdecchia *et al*., 2008). It is thought that direct inhibition of renin activity provides better control of elevated blood pressure than ACE inhibition, as it prevents the production of angiotensin I, which can be converted to angiotensin II via ACE-independent pathways catalysed by chymase (FitzGerald *et al*., 2011; Kumar *et al*., 2009). Early renin inhibitors were substrate analogues which were degraded quickly and had low bioavailability. Although a peptidic transition-state analogue, the drug Zankiren (A-72517; Abbott), when tested, showed improved bioavailability and proteolytic stability in animal models (Turk, 2006).

The health-promoting potential of food-derived compounds is a subject of interest, as new and underutilised food sources, such as seaweeds, are continually investigated for their bioactive properties. To date, no renin-inhibitory peptides have been isolated from macroalgae (FitzGerald *et al*., 2011), but they have been isolated from terrestrial plant sources, including soy and beans (Udenigwe *et al*., 2012). Within the Irish research programme known as NutraMara (www.nutramara.ie), one aim is to identify renin-inhibitory peptides from seaweeds such as *Palmaria palmata* (FitzGerald *et al*., 2011).

7.10.3 PAF-AH Inhibition

Human plasma PAF-AH is a Ca^{2+}-independent phospholipase A_2 of haematopoietic origin associated with low- and high-density lipoproteins (LDLs and HDLs); it degrades PAF and oxidises phospholipids (Stafforini, 2009). Plasma PAF-AH activity is essential for the metabolism of PAF and oxidised phospholipids: both bioactive lipids involved in the pathophysiology of atherosclerosis (Eisaf, 2003). PAF-AH is produced by cells of

monocyte/macrophage origin, as well as by T cells and mast cells, all of which are present in atherosclerotic lesions (Hurt-Camejo *et al*., 2001). PAF-AH degrades PAF and PAF-like lipids by hydrolysing its acetate moiety in the *sn*-2 position of the phospholipids, and therefore it has been suggested in the literature that PAF-AH is anti-atherogenic (Nasopoulou *et al*., 2011). However, PAF-AH cannot bind to LDL cholesterol in mice because of differences in amino acid composition in the 114–117 domain. Indeed, an increasing body of evidence indicates that PAF-AH is a risk factor or a marker for CVD, including stroke and coronary artery disease (Madjid *et al*., 2010).

7.10.4 PEP Inhibition

PEP, once referred to as post-proline cleaving enzyme, plays a role in the breakdown and metabolism of biologically active peptides containing proline, such as oxytocin, vasopressin, substance P, bradykinin, neurotensis and angiotensins (Wilson *et al*., 2011). These peptides play important roles in the functioning of organs, including the brain, and have been implicated as potential contributors to the development of neurodegenerative conditions such as Alzheimer's disease (AD) (Lambeir, 2011). Brain expression of PEP is altered by the ageing process and hippocampal PEP activity is significantly increased in transgenic AD mice compared to wild-type littermates (Hiltunen *et al*., 2009; Wilson *et al*., 2011). Furthermore, specific inhibitors of PEP have antiamnesic effects, and some have been synthesised as antiamnesic drugs.

PEP (EC 3.4.21.26) is a highly conserved serine protease enzyme that cleaves peptide bonds at the carboxyl side of proline residues in proteins with a relatively small molecular weight containing the recognition sequence X-Pro-Y, where X is a peptide or protected amino acid and Y is either an amide, a peptide, an amino acid, an aromatic amine or an alcohol (Williams, 2004).

Studies have suggested that PEP could be related to neurodegeneration and disturbances in memory and cognition. Amyloid β plaques are commonly found in the brains of AD patients (Hiltunen *et al*., 2011). A study by Amor and colleagues (2003) isolated PEP-inhibitory compounds from *Syzygium samarangense* (Blume) Merr. & L. M. Perry (common name 'makopa'), a plant native to the Philippines. Furthermore, *Ginkgo biloba* leaves were examined for antiamnestic constituents/PEP inhibitors and the results showed significant PEP inhibition (Lee *et al*., 2004; Wilson *et al*., 2011). PEP activity-guided fractionation and column chromatography of the MeOH extracts of *G. biloba* leaves resulted in the isolation of 6-(8′Z-pentadecenyl)salicylic acid and 6-(10′Z-heptadecenyl)salicylic acid (Lee *et al*., 2004). The PEP-inhibitory activity of unsaturated fatty acids was also examined by Park *et al*. (2006), who looked at the effects on PEP activity of mono- and polyunsaturated fatty acids found in vegetable seeds and fatty fish. Food-derived PEP inhibitors exist and have been isolated from a variety of sources. For example, peptides from hydrolysates of fish proteins and from cheeses were analysed for inhibition of PEP from porcine muscle. Muscles of cod, salmon and trout were homogenised and incubated at pH 4.0 with pepsin and then at pH 7.5 with trypsin to obtain FPHs. The peptide fractions from fish hydrolysates, fish autolysates and water-soluble extracts of cheeses inhibited PEP in hydrolysing Z-Gly-Pro-amido-methylcoumarin (Sørensen *et al*., 2004). Inhibition by peptides from rakfisk was negligible. Pepsin + trypsin hydrolysates from the three fish species contained PEP-inhibitory peptides with a broad range of apparent hydrophobicities and apparent molecular masses (Sørensen *et al*., 2004).

7.10.5 Factor Xa

Arterial and venous thromboses are major causes of death and the incidence of these events increases with age. Several anticoagulant treatments are currently available, including low-molecular-weight heparin (LMWH) and fondaparinux, which are parenteral anticoagulants effective in the initial treatment stages of venous and arterial thrombosis (Gross & Weitz, 2008). These require injection, however, and therefore there is a need for new, oral anticoagulants (Gross & Weitz, 2008). Oral anticoagulants are usually direct inhibitors of thrombin or factor Xa (FXa). FXa is a serine endopeptidase composed of two disulfide-linked subunits. It is an attractive target for anticoagulant treatment, as it is the primary and rate-limiting source of amplification in the coagulation cascade (Weitz, 2011).

FXa leads to blood-clot formation by converting prothrombin to thrombin through the prothrombinase complex. It is generated from zymogen factor X via the intrinsic and extrinsic pathways (Fig. 7.2) and is the rate-limiting step in the propagation of thrombin generation. In the presence of Ca^{2+} ions, FXa forms prothrombinase with factor Va on the phospholipid membrane of the activated platelets. FXa has emerged as an attractive target for drug discovery in thromboembolic diseases (Weitz, 2011).

7.10.6 Antioxidant Peptides

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced as a result of the metabolism of oxygen and nitrogen. ROS and RNS can cause damage

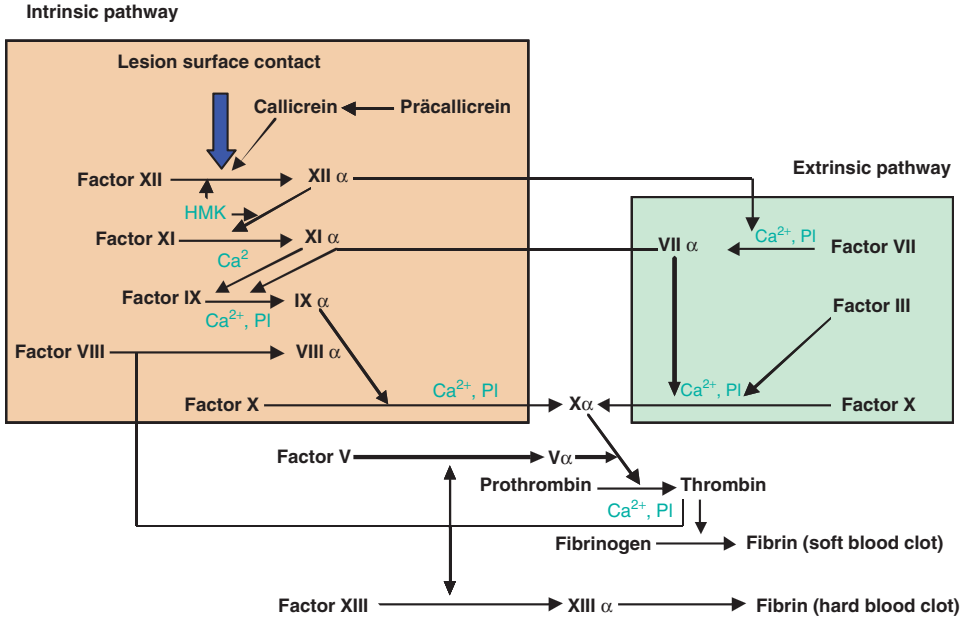

Fig. 7.2 The coagulation cascade and Factor Xa (FXa). FXa is a good target for new oral anticoagulants. Factor X is positioned at the convergence of the extrinsic and intrinsic pathways of coagulation and, when activated, one molecule of FXa can generate more than 1000 thrombin molecules.

to cellular components in the human body and are thought to be a major reason for the development of diseases such as diabetes, cancer, myocardial infarction, renal failure and several inflammatory conditions, including osteoporosis. Macroalgae produce bioactive compounds as a result of the stressful environments in which they exist, and these compounds tend to have a broad polarity range. Bioactive compounds such as polyphenols, phycobiliproteins and vitamins are antioxidants and are often isolated using hydrophilic solvents. Fat-soluble fractions from macroalgae often contain antioxidants such as carotenoids and α-tocopherol. Several studies have reported the isolation of antioxidants from macroalgae and marine FPHs. The antioxidant capacity of a number of Phaeophycean species, including *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosus* and *Laminaria hyberborea* (Gunnerus), was assessed using the total phenol content (TPC) assay (Tierney *et al*., 2010). Antioxidant crude extracts were generated using 70% acetone, and antioxidant capacity mean values of between 12 and 24.2 g PGE/100 g were reported (Tierney *et al*., 2010).

Endogenous enzymatic and non-enzymatic antioxidants are present in fish muscle and can be either hydrophilic (contained in the cytosol) or lipid-soluble (Kopec *et al*., 2012). The dipeptides carnosine and anserine are known hydrophilic antioxidants. Wu *et al. (*2003) reported the generation of carnosine and anserine from a number of fish species, including tuna, skipjack, salmon and eel. Another study looked at the antioxidant activity of collagen and keratin samples from different animal sources (Ohba *et al*., 2003). Keratin proteins are the major structural proteins in epithelial cells, and their role is to act as a framework for epithelial cells which sustain mechanical and nonmechanical stresses (Coulombe & Omary, 2002; Di Bernardini *et al*., 2011). In this study, collagen was extracted from scale and bone from yellowfish, as well as from other non-fish sources including cow collagen. The peptide FGHPY, which has antioxidant activities, was isolated from a blue mussel (*Mytilus edulis*) fermentate. Moreover, the antioxidant peptide, LVGDEQAVPAVCVP, was isolated by Jung and colleagues (2005) from the mussel species M*ytilus coruscus*. Peptides containing the amino acids histidine, tyrosine and methionine are thought to have enhanced radical scavenging and therefore antioxidant activities, as these amino acids have been reported as radical scavengers (Saiga *et al*., 2003). A schematic showing an approach to the generation of bioactive peptides from muscle sources is given in Fig. 7.3.

The antioxidant activity of marine-derived hydrolysates or peptides can be assessed using *in vitro* and *in vivo* methods. *In vitro* antioxidant capacity assays can be divided into two categories: assays based on hydrogen atom-transfer (HAT) reactions (Huang *et al*., 2005) and assays based on electron-transfer (ET) reactions (Huang *et al*., 2005). The HAT-based assays usually involve the use of a synthetic free-radical generator, an oxidisable molecular probe and an antioxidant (Huang *et al*., 2005). Quantification is obtained from kinetic curves derived from competitive reaction kinetics (Huang *et al*., 2005). HAT assays include oxygen radical absorbance capacity assay (ORAC) and the total trapping antioxidant parameter assay (TRAP). ET-based assays assess the capacity of a potential antioxidant (in this case, an FPH, marine macroalgal-derived hydrolysate or purified peptide) to reduce an oxidant, which changes colour when reduced (Huang *et al*., 2005). The degree of colour change is correlated with the sample's antioxidant activity (Huang *et al*., 2005). An overview of HAT- and ET-based assays and how they work can be found in two papers: Di Bernardini *et al*. (2011) and Tierney *et al*. (2010).

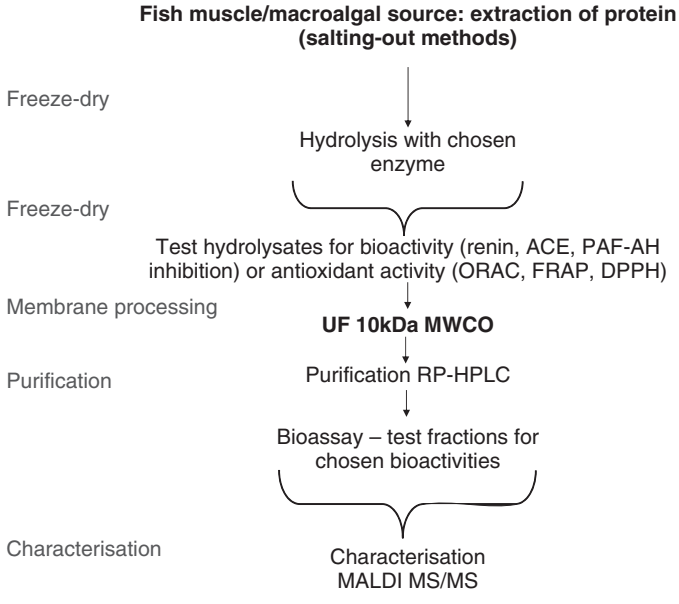

Fig. 7.3 A schematic approach to the generation of bioactive peptides from fish muscle sources.

7.11 COMMERCIALLY AVAILABLE BIOACTIVE PEPTIDES

The dairy and soy industries dominate the protein and peptide ingredients market, and the market for marine proteins and peptides is small. Seafood flavours are the traditional products. Other products include collagen/gelatine, which accounts for approximately 1–3% of global production. A number of commercial FPH products are currently available, including PEPTIDE ACE 3000 in Japan and Vasotensin® and PeptACE™ in Canada (Arasoni *et al*., 2009). All of these products contain the Katsuobushi oligopeptide, which is manufactured by hydrolysis of dried bonito. Other products include SECURE®, a white FPH concentrate which claims to support the gastrointestinal tract and assist in the regulation of bowel function. Furthermore, the peptide product Nutripeptin®, produced in Norway from codfish, is claimed to reduce blood sugar levels. Other commercially available products are listed in Table 7.5.

7.12 CONCLUSION

In the EU, regulation (EC) N_ 1924/2006 of the European Parliament and of the Council of Nutrition and Health Claims Made on Foods was adopted in December 2006. Article 13 covers the health claims of functional foods, and approval from EFSA (European Food Safety Authority) is required in order for a functional food product to utilise a claim in its marketing. In this article, 'health claims' means 'any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health' (Hayes, 2011). The Japanese Ministry of Health, Labour and Welfare set up Foods for Specified Health Use (FOSHU) in 1991 as a regulatory system by which to

Table 7.5 Commercially available fish-protein hydrolysate (FPH) products.

Product name and source	Claimed functional bioactive peptide(s)	Health claim	Source	Manufacturer(s)
Milk peptide products				
Calpis sour milk	Val-Pro-Pro, Ile-Pro-Pro	Reduces blood pressure	β-casein and κ-casein proteins	Calpis Co., Japan
Evolus calcium-enriched fermented milk drink	Val-Pro-Pro, Ile-Pro-Pro	Reduces blood pressure	β-casein and κ-casein proteins	Valio, Finland
BioZate whey-protein-isolate hydrolysate	β-lactoglobulin fragments	Reduces blood pressure	β-lactoglobulin fragments	Davisco Foods, USA
BioPure GMP whey-protein isolate	κ-casein f(106–169) glycomacropeptide	Prevents blood clotting, dental caries and antimicrobial activities	κ-casein f (106–169)	Davisco Foods, USA
BioPure Alphalactalbumin whey-protein isolate	α-lactalbumin	Helps sleep and memory	α-lactalbumin	Davisco Foods, USA
Prodilet F/200 lactium-flavoured milk drink	αs1-casein f(91–100) Tyr-Leu-Gly Tyr-Leu-Glu-Gln-Leu-Leu-Arg	Reduces stress effects	αs1-casein fragments	Ingredia, France
Festivo fermented low-fat hard cheese	αs1-casein f(1–9), αs1-casein f(1–7), αs1-casein f(1–6)	No health claims made to date	αs1-casein fragments	MTT Agrifood Research, Finland
C12 peptide hydrolysate	Milk peptide sequence	Reduces blood pressure	Phe-Phe- Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	DMV International, The Netherlands
Cystein Peptide ingredient	Milk proptein-derived peptide	Aids sleep and energy levels	Not given	DMV International, The Netherlands
Capolac ingredient	Caseinophosphopeptide	Aids mineral absorption	Not given	Arla Food Ingredients, Sweden
PeptoPro ingredient	Casein-derived peptides	Improves athletic performance and muscle recovery	Casein-derived peptides	DSM Food specialties, The Netherlands
Vivinal alpha ingredient	Whey-derived peptide	Aids sleep and relaxation	Whey-derived peptides	Borculo Domo Ingredients, The Netherlands
Lowpept	Casein-derived peptides	Reduces blood pressure	Casein-derived peptides in cheese powders	Innaves, Spain

(*continued overleaf*)

Table 7.5 (*continued*)

Product name and source	Claimed functional bioactive peptide(s)	Health claim	Source	Manufacturer(s)
Marine peptide products				
Vasotensin®	Bonito-derived pro-peptides (LKP, IKP, IRP)	Reduces blood pressure	Bonito-derived peptides	Metagenics, US
Levenorm®	Bonito-derived pro-peptides (LKP, IKP, IRP)	Reduces blood pressure	Bonito-derived peptides	Ocean Nutrition Canada, Ltd., Canada
PeptACETM	Bonito-derived pro- peptides (LKP, IKP, IRP)	Reduces blood pressure	Bonito-derived peptides	Natural Factors Nutritional products Ltd., Canada
Peptide ACE 3000	Bonito-derived pro-peptides (LKP, IKP, IRP)	Reduces blood pressure	Bonito-derived peptides	Nippon Supplement Inc., Japan
Lapis Support	Sardine-derived pro-peptides (LKP, IKP, IRP, MY)	Reduces blood pressure	Sardine-derived peptides	Tokiwa Yaakuhin Co. Ltd., Japan
Valtyron®	Sardine-derived pro-peptides (LKP, IKP, IRP, MY)	Reduces blood pressure	Sardine-derived peptides	Senmi Ekisu Co., Ltd., Japan
Stabilium® 200	High concentration of peptides similar to pituitary-gland peptides derived from blue whiting	Relaxing effect	Blue whiting-derived peptides	Yalacta, France
Protizen®	Fish autolysate	Relaxing effect	Fish autolysate	Copalis Sea Solutions, France
AntiStress24	Fish autolysate	Relaxing effect	Fish autolysate	Forté Pharma Laboratories, France
Nutripeptin TM	Cod hydrolysate-derived peptides	Lowers glycaemic index	Cod hydrolysate derived peptides	Nutrimarine Life Sciences, Norway
Seacure®	Pacific whiting hydrolysate-derived peptides	Improves GI health	Pacific whiting hydrolysate	Proper Nutrition, USA
Fortidium Liquamen®	Fish autolysate-containing vegetable oils	Antioxidant effects, antistress effects	Fish autolysate and vegetable oil	Biothalassol, France
Soy peptide products				
Peptein soy peptide drink	Soy protein-derived peptides	Energy release	Soy protein-derived peptides	
CholesteBlock	Soft drink containing soy-derived peptides bound to phospholipids	Hypocholesterolemic	Soy-derived peptides bound to phospholipids	Kyowa Hakko, Japan

approve statements made on functional food labels dealing with health claims. In addition, in China, an application for a health claim for a food product undergoes substantive examination before receiving approval from the State Food and Drugs Administration (SFDA) (Hayes, 2011).

Including FPHs and peptides and/or macroalgal-derived bioactive peptides as functional ingredients in foods requires several steps regarding their isolation and characterisation, safety and quality assurance and, of course, economically viable scale-up procedures. Although the generation of bioactive proteins and peptides from marine byproducts can be a costly venture, its potential is increasing due to expanding regulations regarding the disposal of marine byproducts at sea and in landfill. Not only might utilisation of byproducts be economically beneficial to marine processors, it is also an environmentally beneficial and sustainable approach towards fishing.

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8 Ability of Diverse Marine Invertebrate Lectins to Regulate Cell Functions

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8.1 INTRODUCTION

The biological macromolecule protein serves important functions in living systems through precise interaction with various ligand molecules such as proteins, nucleotides, lipids and glycans. Glycans, which consist of monosaccharides, are notable for being 'the third chain of living systems', following polypeptides and polynucleotides. Shorter glycans with less than 20 monosaccharides, such as D-galactose (Gal), *N*-acetyl D-glucosamine (GlcNAc), D-glucose (Glc), *N*-acetyl D-galactosamine (GalNAc), D-mannose (Man), L-fucose (Fuc) and *N*-acetyl D-neuramic acid (NeuAc), connected by glycosidic bonds with α- or β-anomer linkages through hydroxyl groups, are called 'oligosaccharides' (Fig. 8.1).

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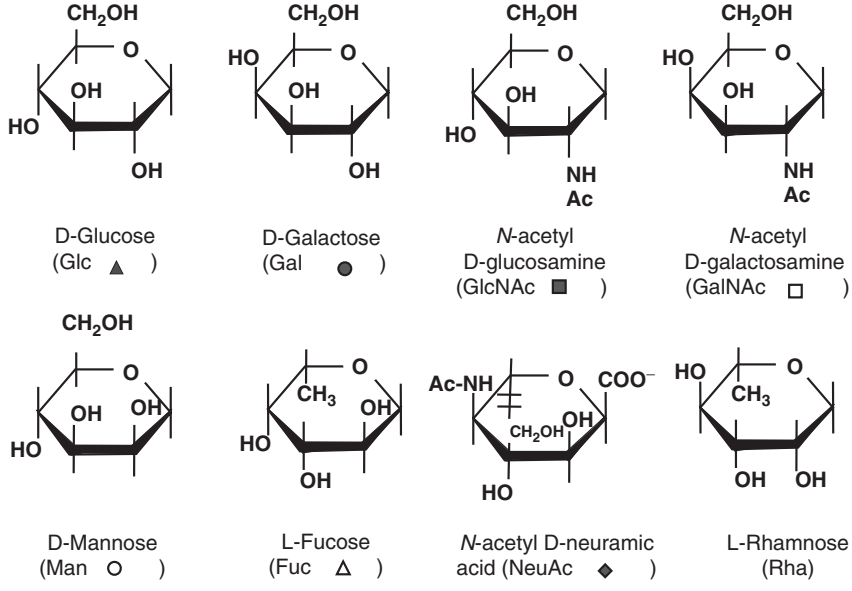

Fig. 8.1 Oligosaccharide structures. Nomenclature follows the Human Disease Glycomics/Proteome Initiative (Wada *et al.*, 2007) and the Consortium for Functional Glycomics (www.functionalglycomics.org).

Oligosaccharide structures are produced by glycosyltransferases and glycosidases in the endoplasmic reticulum and Golgi body. They are composed of monosaccharides, anomer linkages and a number of branches and chains. As a post-translational modification, oligosaccharide chains may be covalently linked to polypeptides to form glycoproteins or proteoglycans, or to ceramides (consisting of sphingosine and a fatty acid) to form glycosphingolipids. The diverse structures of oligosaccharides affect the quality of glycoproteins and provide a molecular basis of glyco-code through noncovalent binding with oligosaccharides via carbohydrate–carbohydrate interaction (Eggens *et al.*, 1989; Hakomori, 2004) or with lectins (carbohydrate-binding proteins) via carbohydrate–protein interaction (Ghazarian *et al.*, 2011; Lee, 1992).

Lectins are found in a wide variety of organisms. They were first discovered through the extraction of castor beans by H. Stillmark in Estonia in 1888 as a hemagglutinin. The hemagglutination activity resulted from binding of a protein in the beans with oligosaccharides on the surface of erythrocytes. Following this initial discovery, many carbohydrate-binding proteins were isolated from a variety of sources, including plants, lower animals, and microorganisms. Such proteins were called 'lectins' because of their ability to select (= *legere* in Latin) different types of cells according to different specificities of interaction with oligosaccharide structures. Some plant-seed lectins have been found to stimulate the cell proliferation of lymphocytes in bone-marrow transplantation and to specifically agglutinate malignant cells transformed by oncoviruses. Certain lectins on the fimbriae or envelope of infectious bacteria and viruses play essential roles in attachment to specific oligosaccharides on host cells.

Since the late 1960s, lectins have also been found in higher animals. Animal lectins play significant roles in cell adhesion, immunity and metabolism. Improvements in affinity

chromatography technology for the purification step have allowed the development of advanced molecular biological studies of lectin structure and function. Various lectin families based on a characteristic structure, such as C (divalent cation required)-type, F (fucose-binding)-type, galectin (β-galactose-specific lectin), intelectin, siglec (sialic acid-binding lectin with IgG motif) and SUEL (sea urchin egg lectin)-type, have been identified. The existence of these families emphasizes the essential role of lectins as receptors that can decipher the complex glyco-codes of oligosaccharides in living systems. Precise glycan-binding properties of lectins are beginning to be elucidated, and novel lectins of great medical value are being discovered. Lectins are thus promising targets for investigative and therapeutic research.

Characteristic lectin structure families have been identified in marine invertebrates, in addition to other representative lectin families (Gokudan *et al*., 1999; Ozeki *et al*., 1991). Some structure homologues of marine invertebrate lectins with important functions have been isolated from higher vertebrates, including humans (Lelianova *et al*., 1997; Sugita *et al*., 1998). The ancestors of the present-day marine invertebrates first emerged in the Cambrian era, when the diversity of organisms on the earth exploded. Various essential or valuable genes have apparently been maintained during evolution and are still present in the modern species. Glycomic investigation of lectins and oligosaccharides in marine invertebrates is therefore expected to produce important findings regarding the mechanisms and potential applications of lectins.

In this chapter, we describe three aspects of marine invertebrate lectins based on our glycomic studies of glycan-binding properties. Section 8.2 describes a lectin from the feather star, a close relative of the sea lily, as a molecular device to separate somatic and iPS cells. Sections 8.3 and 8.4 describe two lectins isolated from Mediterranean mussel and catfish eggs that have identical glycan-binding properties but completely different primary structures and different regulatory effects on Burkitt's lymphoma cells.

8.2 DOES A FEATHER STAR LECTIN HAVE A ROLE IN REGENERATIVE BIOLOGY?

8.2.1 The First Evidence of Lectin in the Feather Star (Phylum Echinodermata, Class Crinoidea)

Echinoderms are classified as deuterostomes and have a common evolutionary origin with the phylum Chordata; they therefore provide a useful model for studies of vertebrate lectins. There are two subphyla of echinoderms: (i) Eleutherozoa, containing the classes Asteroidea (starfish), Echinoidea (sea urchin), Holothuroidea (sea cucumber) and Ophiuroidea (brittle star), and (ii) Pelmatozoa, containing the class Crinoidea (sea lily and feather star). Until recently, all lectins isolated from echinoderms had come from sea urchins (Alliegro & Alliegro, 1991, 2007; Giga *et al*., 1987; Ozeki *et al*., 1991), starfish (Kakiuchi *et al*., 2002) and sea cucumbers (Gowda *et al*., 2008), and none from the subphylum Pelmatozoa. Crinoidea is considered the most primitive echinoderm class and is useful for investigation of the evolution of the basic body plan of deuterostomes. Fossil study has shown that the Crinoidea appeared and diversified during the Ordovician period in the Paleozoic age. They evolved biological properties differing from those of other echinoderms: while other echinoderms lost their ganglion-like nerve centers during their evolution, the Crinoidea retained theirs (Nakano *et al*., 2009), providing the ability to regenerate their arms (Shibata *et al*., 2010). For these reasons, the Crinoidea are

useful models for studies of regeneration and the evolution of deuterostomes (Kondo and Akasaka 2010; Nakano *et al*., 2009).

Recently, the Japanese feather star (*Oxycomanthus japonicus*) has been successfully cultivated artificially on a large scale (Shibata *et al*., 2008) and has been promoted as an experimental animal model through the National Bio-Resources Project of Japan (www.nbrp.jp). Homogenate supernatants of *O. japonicus* showed strong hemagglutination against human and rabbit erythrocytes, and this activity was inhibited by the co-presence of a serum glycoprotein from fetal calf (asialofetuin) or porcine submaxillary mucin. Feather star lectin was purified using an asialofetuin-agarose affinity column with 4 M urea after collection of fractions with hemagglutination activity using anion ion-exchange chromatography (IEC). It was shown to be a divalent cation-independent tetrameric lectin consisting of four 14 kDa polypeptides and was named OXYL (*O. japonicus* lectin) (Fig. 8.2a) (Matsumoto *et al*., 2011).

8.2.2 Type-2 N-Acetyllactosamine Recognition of Oxyl Identified by Frontal-affinity Chromatography Technology

Frontal-affinity chromatography technology (FACT) was originally developed as an analytical procedure by which to evaluate the weak and reversible molecular interaction around $K_D = 10^{-4}$ to 10^{-7} M between proteases and their inhibitors (Kasai & Ishii, 1978a, 1978b). It has been refined as an excellent glycomic methodology tool for evaluating the glycan-binding properties of lectins (Hirabayashi *et al*., 2002, 2003). The device consists of a mini-column with affinity gel (200 µl) immobilized by highly concentrated lectin between a high-performance liquid chromatography (HPLC) pump and a fluorescence detector. Several pmol of each pyridylamine group-labeled (PA) oligosaccharide are injected into the column (Fig. 8.3). If the PA-oligosaccharide is recognized by the lectin immobilized in the affinity column, delays in the elution front peak detected by the fluorescence (emission and excitation wavelengths are 380 and 310 nm, respectively) comparable to the negative-controlled PA-sugar, glycan-binding profiles of each lectin are integrated as a graph. This system has been used to document characteristic and distinct glycan-binding profiles of D-galactose-binding lectins purified from marine invertebrates of the phyla Porifera, Annelida and Mollusca (Fujii *et al*., 2011; Kawsar *et al*., 2008, 2009, 2011; Naganuma *et al*., 2006), although the lectins were purified by the same procedure.

Each of 25 kinds of PA-oligosaccharide (Fig. 8.4) involving serum glycoprotein-type oligosaccharides (001–025 and M59) and glycosphingolipid-type oligosaccharides (026–045) was administered to the OXYL-immobilized column in the FACT system. According to the characteristic glycan-binding profile, OXYL had affinity for a branched type-2 *N*-acetyllactosamine structure (Fig. 8.5a). OXYL specifically bound to type-2 *N*-acetyllactosamine structure (**041**: Galβ1-4GlcNAcβ1-3Galβ1-4Glc) but did not bind to type-1 *N*-acetyllactosamine (**042**: Galβ1-3GlcNAcβ1-3Galβ1-4Glc). The affinity for the type-2 *N*-acetyllactosamine chain increased according to the number of branches as follows: mono- (**041**) < bi- (**001**) < tri- (**002**) and tetra- (**004**) antennary complex-type oligosaccharide. The *N*-acetyl group at the C-2 position in GlcNAc of Galβ1-4GlcNAc was essential, as OXYL lost its affinity to lactose (**026**: Galβ1-4Glc) substituted for the hydroxyl group from the *N*-acyl group at the C-2 position of GlcNAc. The hydroxyl group at the C-3 position of the GlcNAc residue was also essential, because Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc (**045**: lacto-*N*-fucopentaose III), which L-Fuc bound

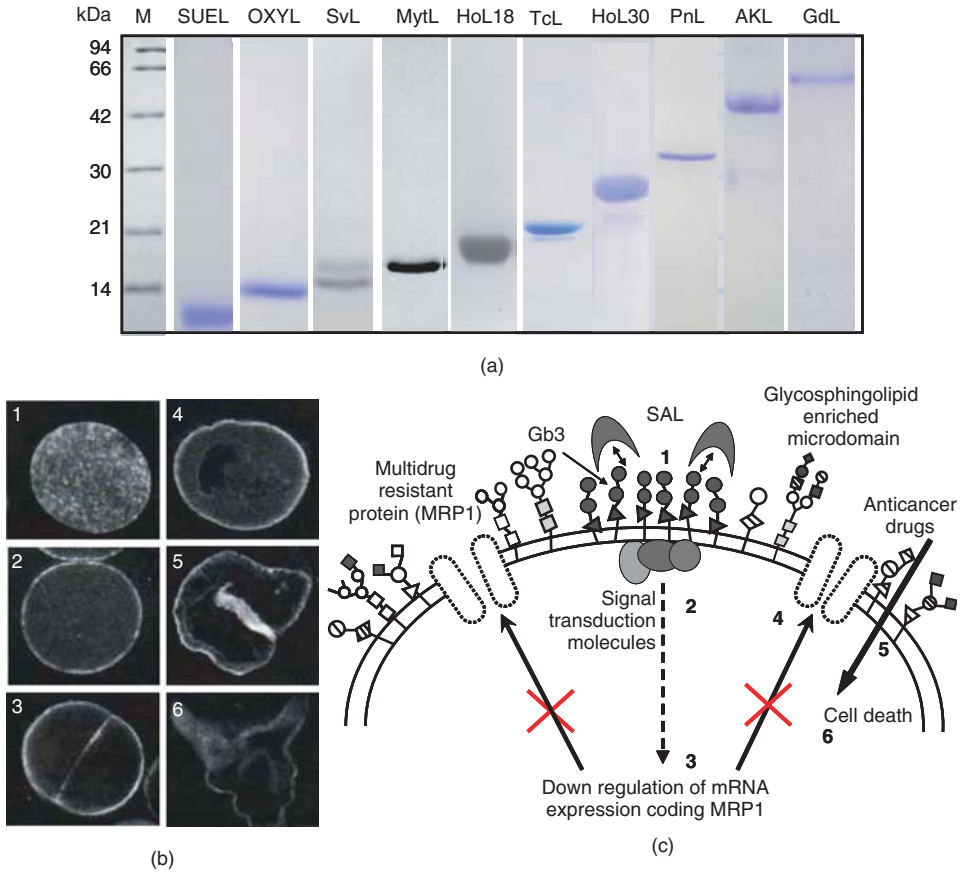

Fig. 8.2 Diversification of marine invertebrate lectins. (a) D-galactose and its derivative glycan-binding lectins purified from marine invertebrates. SUEL: 11.5 kDa sea urchin egg lectin (Ozeki *et al.*, 1991); OXYL: 14 kDa feather star lectin (Matsumoto *et al.*, 2011); SvL: 15 and 17 kDa black mussel (*Septifer virgatus*) lectin; MytiL: 17 kDa Mediterranean mussel (*Mitylus galloprovincialis*) lectin; HOL18: 18 kDa black sponge (*Halichondria okadai*) lectin (Matsumoto *et al.*, 2012); TcL: 22 kDa coronate moon turban (*Turbo (Lunella) coreensis*) lectin (Fujii *et al.*, 2011); HOL30: 30 kDa black sponge (*H. okadai*) lectin (Kawsar *et al.*, 2008); PnL: 32 kDa Pacific annelid (*Perinereis nuntia*) lectin (Kawsar *et al.*, 2009a); AKL: 56 kDa sea hair (*Aplysia kurodai*) egg lectin (Kawsar *et al.*, 2009b); GdL: 60 kDa bladder moon shell (*Glossaulax didyma*) lectin (Fujii *et al.*, 2009). (b) Localization of SUEL according to early development of purple sea urchin. 1: before fertilization; 2: after fertilization; 3: two-cell stage; 4: blastula stage; 5: gastrula stage; 6: pluteus larva (Ozeki *et al.*, 1995). (c) Schema of the SAL-Gb3 pathway for the depletion of MRP1 mRNA expression: 1: SAL molecules; 2: signal transduction; 3: downregulation of gene expression of mRNA coding MRP1; 4: depletion of MRP1 transporter on cell membrane; 5: increase of membrane permeability and incorporation of anticancer drugs; 6: induction of cell death.

to the C-3 position of GlcNAc in type-2 *N*-acetyllactosamine, did not bind to the lectin. The hydroxyl group at the C-6 position in Gal of Galβ1-4GlcNAc was also essential, because the Neu5Acα2-6Galβ1-4GlcNAc- structure (**022–025**) did not bind. Nevertheless, NeuAcα2-3Galβ1-4Glc- (**M59**) bound to the lectin. These results suggest that OXYL essentially recognized the enclosed regions in type-2 *N*-acetyllactosamine (Fig. 8.5b).

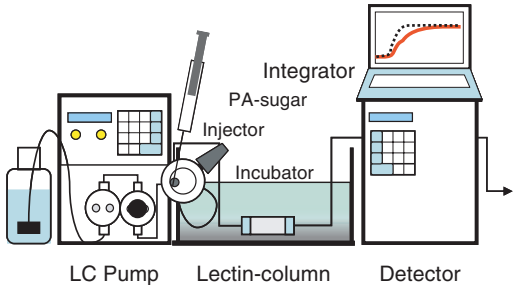

Fig. 8.3 Instruments of frontal-affinity chromatography technology (FACT). The system consists of a liquid chromatography pump, lectin-immobilized affinity column, fluorescence detector and integrator. Twenty-five different PA-oligosaccharides (5 pmol in 2 ml; listed in Fig. 8.4) and inert-controlled PA-rhamnose (Fig. 8.1) are manually injected in the affinity column at a flow rate of 250 µl/min. The lectin column and sample loop are kept at 20 °C in the incubator. The elution volume of PA-oligosaccharides is detected by fluorescence (excitation 310 nm/emission 380 nm). The retardation volume ($V - V_0$) of each PA-oligosaccharide (V) in relation to the control PA-rhamnose (V_0) is estimated for the glycan-binding profile.

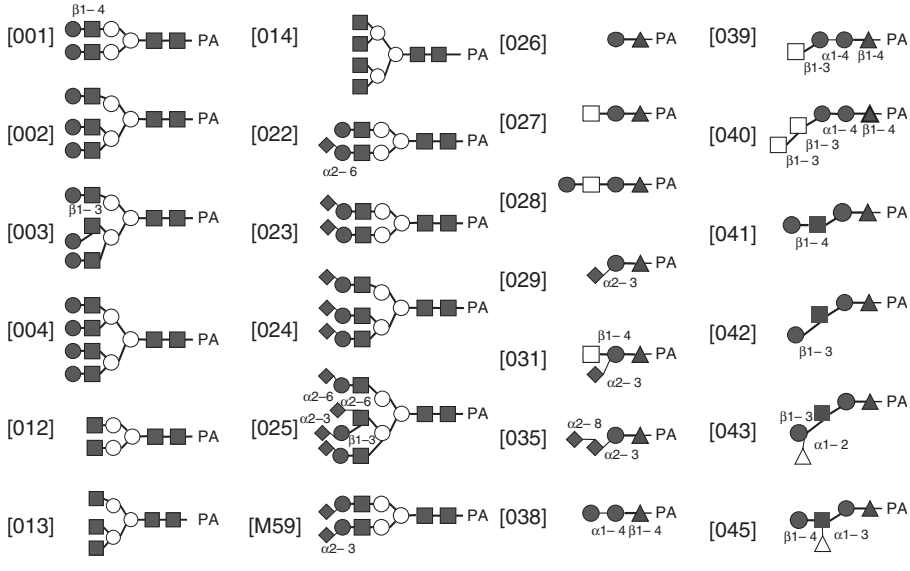

Fig. 8.4 Twenty-five PA-labeled oligosaccharides. Symbol nomenclature is according to Fig. 8.1. The inserted α- and β- numbers indicate the type of glycosidic bond connecting the monosaccharides. 001–025 and M059: oligosaccharides derived from glycoproteins. 026–045: oligosaccharides derived from glycosphingolipids.

8.2.3 Biomedical Aspects of OXYL for Regenerative Biology

Recent studies have shown that structural changes in oligosaccharides occur on the surface of induced pluripotent stem cells (iPS cells) derived from somatic cells (Tateno

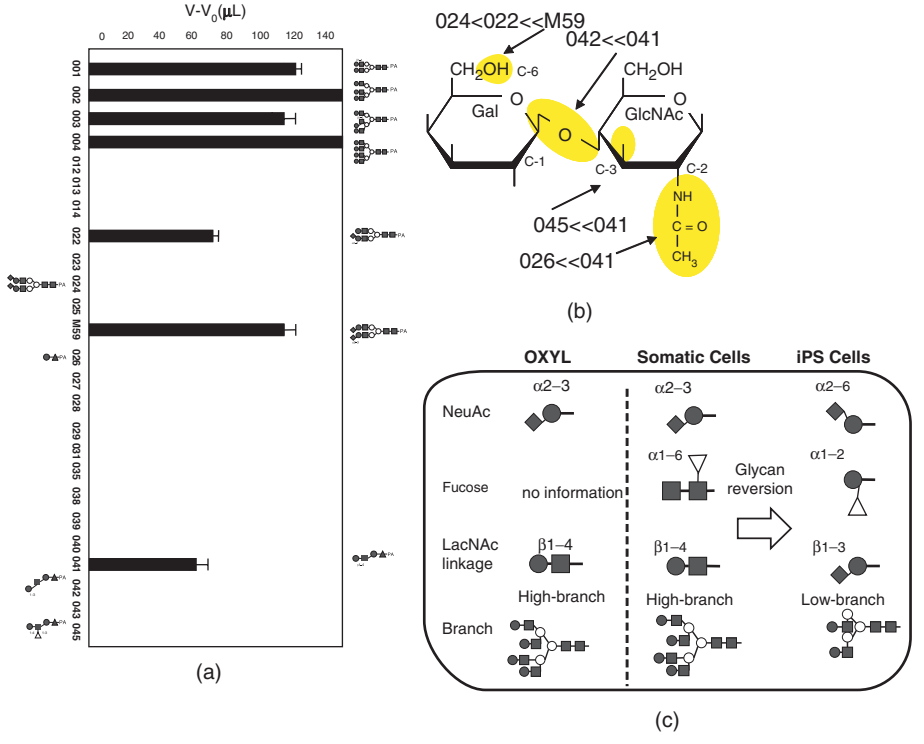

Fig. 8.5 Glycan-binding profile of a novel 14 kDa feather star lectin. (a) Glycan-binding profile of OXYL isolated by FACT analysis. The numbers on the vertical axis represent oligosaccharides (see the list of PA-glycans in Fig. 8.4). The horizontal axis indicates the relative intensity of the difference between the elution front volumes of each PA-oligosaccharide (V) and PA-rhamnose (V_0). The height of the bars was calculated from $V - V_0$. (b) Presumptive structure of type-2 *N*-acetyllactosamine recognized by OXYL. Essential hydroxyl groups that bind to the lectin are indicated by shaded circles based on FACT results. (c) Comparison of the glycan-binding profile of OXYL to the alteration of glycosylation properties during the transformation of somatic to iPS cells (Tateno *et al.*, 2010a).

et al., 2010a). Glycan-binding profiles indicate that NeuAcα2-6Gal, Galβ1-4GlcNAc (type-2 *N*-acetyllactosamine), Fucα1-6GlcNAc and branched complex-type oligosaccharides of the somatic cells were altered to NeuAcα2-3Gal, Galβ1-3GalNAc (type-1 *N*-acetyllactosamine), Fucα1-2Gal and low-branched complex-type oligosaccharides by reprogramming of iPS cells (Fig. 8.5c). The glycan-binding properties of feather star lectin promoted selective binding affinity for oligosaccharides on somatic cells (Fig. 8.5c). This lectin displayed a strong binding affinity for branched complex-type oligosaccharides, 10–100 times greater than that of other lectins (Matsumoto *et al.*, 2011). These properties of feather star lectin are presumably related to somatic cell sorting and the regeneration of cells from iPS cells. The physiological role of OXYL is a fascinating demonstration of the lectin function connecting this "living fossil" with vertebrates (including humans). Advances in immunohistochemistry and molecular biology will further clarify this topic.

8.3 A NOVEL LECTIN FROM THE MEDITERRANEAN MUSSEL INDUCES APOPTOSIS AND GLYCOSPHINGOLIPID INTERACTION

8.3.1 Progress in Mussel Genome Databases

Mollusks are regarded as important aquatic and biological resources. The marine bivalve mussel *Mytilus galloprovincialis* (family Mytilidae), which originated in the Mediterranean, is now found worldwide. It filters debris containing heavy metals and pathogenic microorganisms in sea water and has a defense system that can adapt to different environments. Recent work on genome databases has led to the establishment of expressed sequence tag (EST) libraries for bivalves (Kinoshita *et al*., 2011; Venier *et al*., 2011a, 2011b). One such EST library, MytiBase, established from *M. galloprovincialis*, provides valuable information for investigating the mechanisms of developmental and defensive systems. Studies using EST libraries have elucidated the dynamics of bivalve defenses against pathogen infection and suggested that lectin-encoding genes respond strongly to environmental stimuli (Venier *et al*., 2011b), resulting in up- or downregulation of the expression level of lectin-encoding mRNA (Kim *et al*., 2006; Li *et al*., 2011; Tasumi & Vasta, 2007). Characteristic structures of C-type (Espinosa *et al*., 2010), F-type (Gorbushin & Iakovleva, 2011) and siglec-type (Li *et al*., 2011) lectins have been found in mussels, suggesting that currently unknown lectins have important functions in Mytilidae. On this basis, we attempted to isolate a novel lectin from *M. galloprovincialis* and elucidate its biological properties.

8.3.2 Shorter Carbohydrate-binding Motif Candidate Found in α-Galactose-binding Lectin in Mussel

We purified a novel 17 kDa α-galactose-binding lectin from *M. galloprovincialis*. In contrast to other known mollusk lectins, it bound to tissues of the mantle and was solubilized by homogenization in a buffer containing the α-galactoside melibiose. The lectin appeared to be associated with endogenous lectins in mussel tissue. The supernatant after removal of sugar displayed strong hemagglutination activity. The lectin was purified using a melibiose-immobilized affinity column and appeared as a 17 kDa polypeptide under both reducing and nonreducing conditions (Fig. 8.2a). It appeared to recognize α-galactoside preferentially over β-galactoside. The complete primary structure of this mussel lectin, determined by Edman degradation and mass spectrometry, provides considerable novel information in regard to animal lectins (Fujii *et al*., 2013). The lectin has 149 amino acids and a triple-tandem structure consisting of 50 amino acids. Its N-terminus is acetyl threonine and its primary structure has no similarity to that of any known primary structure in the database. The structure consists of three tandem repeating domains, with 50% identity within the polypeptide (Fig. 8.6). In each domain, the basic amino acid residues Lys, His and Arg (Δ) are highly conserved around the polypeptide whereas, acidic amino acid residues such as Asp and Glu ($\bullet$) are conserved on the C-terminal side. The remarkable number of conserved amino acid residues shown in the polypeptide—such as Glu, His, Asp and Arg—are coincidentally also seen in many lectins as essential amino acids for binding with carbohydrates (Kasai & Hirabayashi, 1996; Naismith & Field, 1994; Ogawa *et al*., 2011; Vakonakis *et al*., 2008; Weis & Drickamer, 1995). Gel-permeation chromatography of the lectin indicated the presence of a monomer with hemagglutination activity under physiological conditions. These are valuable findings in regard to animal

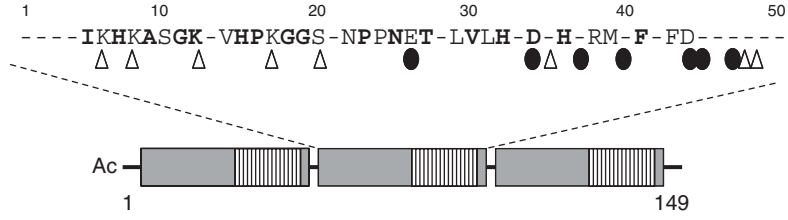

Fig. 8.6 Characteristic primary structure of 17 kDa α-galactoside binding lectin isolated from M. galloprovincialis. A polypeptide consisting of 149 a.a.'s with an acetylated N-terminus was recovered. There were three repeating subdomains each consisting of about 50 a.a.'s within the polypeptide. Common a.a. residues that appeared in 3 or 2 subdomains in the polypeptide are indicated by bold or regular letters, respectively. The locations of basic a.a.'s (△) and acidic a.a.'s (•) within the polypeptide are indicated. Regions enriched in acidic a.a.'s are indicated by stripes.

lectins because they suggest the existence of a carbohydrate-binding motif consisting of only 50 amino acids. Known animal lectins require from 100 (in the case of SUEL) to 130 (in the case of galectins and C-type lectins) amino acids for carbohydrate binding (Drickamer, 1988; Kasai & Hirabayashi, 1996; Ozeki *et al*., 1991). The development of a synthetic peptide with carbohydrate-binding activity based on the lectin primary structure could lead to a new drug for glycan-dependent therapy.

8.3.3 Globotriaosyl Ceramide (Gb3)-dependent Cytotoxicity of the Mussel Lectin

The P^k blood-group antigen of the glycosphingolipid globotriaosylceramide (Gb3; Galα1-4Galβ1-4Glc-ceramide), also known as CD77, acts as a target of both verotoxins and the HIV adhesion molecule gp120 (Lingwood *et al*., 2011). Gb3 is also highly expressed on the glycosphingolipid-enriched microdomain (GEM) of Burkitt's lymphoma cells (Hakomori & Handa, 2002; Wiels *et al*., 1981, 1984) (Burkitt's is a malignant lymphoma caused by herpesvirus infection). FACT analysis indicated that the 17 kDa α-galactose-binding mussel lectin preferentially recognizes Gb3 oligosaccharide (Fig. 8.7a, **038**:Gb3).

The administration of the mussel lectin to Burkitt's lymphoma Raji cells (0–50 μg/ml) caused reduction of cell viability in association with reduced concentration of the lectin, according to trypan blue exclusion assay (to detect apoptosis) and WST assay (to detect the growth of cells) (Fig. 8.7b, upper graph, black bars). However, viability was not reduced in erythroleukemia K562 cells, which do not express Gb3 (Fig. 8.7b, upper graph, white bars). The lectin-dependent cytotoxicity of Burkitt's lymphoma cells was more significantly inhibited by the co-presence of an α-galactoside (melibiose), which mimics Gb3 on the cell surface, than by the co-presence of a β-galactoside (lactose) (Fig. 8.7b, lower graph).

Administration of the lectin to Raji cells induced both inversion of phosphatidylserine and a loss of the integrity of the cell membrane, as assessed by anti-annexin V antibody binding and propidium iodate incorporation by fluorescence-activated cell sorting (FACS). These results demonstrate that the mussel lectin has a unique primary structure with short lectin motifs and promotes glycan-dependent apoptosis.

The primary structure identified in this mussel lectin is currently unique among known animal lectins. However, we expect that genetic and molecular homologues will be found in related organisms; *e.g.*, in the SUEL lectin family (Section 8.4). Many SUEL lectins—even a member isolated from pearl shell *Pteria penguin*

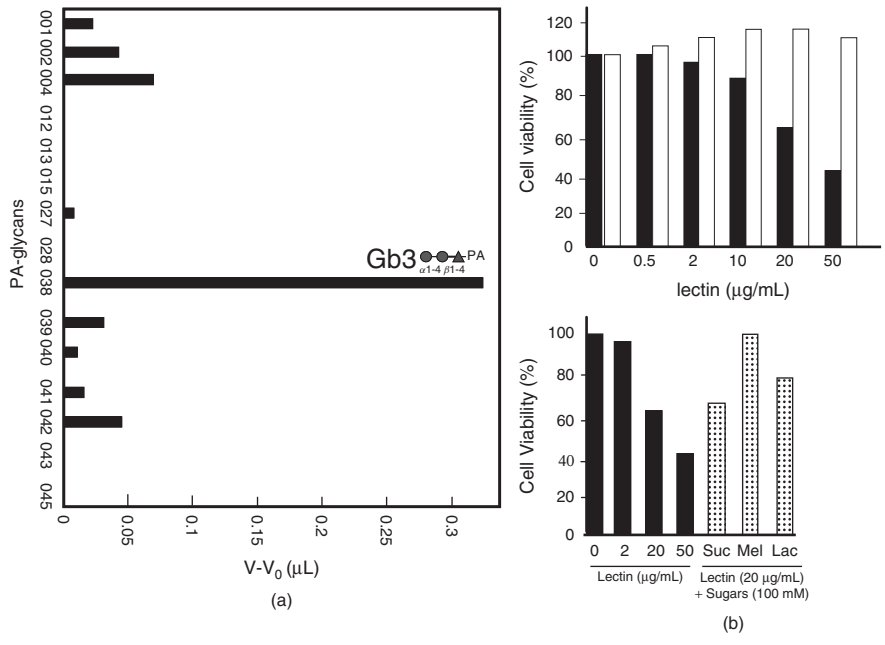

Fig. 8.7 Glycan-binding property and lectin-dependent cytotoxicity against Burkitt's lymphoma cells. (a) Glycan-binding profile of the lectin isolated from *M. galloprovincialis* by FACT analysis. (b) Glycan-dependent cell viability reduction activity of the mussel lectin against Burkitt's lymphoma Raji cells. Cell viability and number of living cells were determined using tetrazolium salt WST-8. Raji cells were treated with the lectin (0–50 μg/ml). A black bar (or circle) and gray bar (or circle) indicate the cell viability (or absorbance) of the Raji cells and K562 cells, respectively. Specific inhibition of the cell viability reduction activity of the mussel lectin was achieved by the addition of saccharides (Suc, sucrose; Mel, melibiose; Lac, lactose). A 100 mM concentration of saccharide was added for each experiment. A black bar (or circle) and slashed bar (or circle) indicate the cell viability (or absorbance) of Raji cells.

(Naganuma *et al*., 2006)—recognize Gb3 as does the mussel lectin. Comparison of the cell regulatory mechanisms of different Gb3-binding lectins will provide invaluable knowledge for glycobiology. An 18 kDa Gal/GalNAc-binding lectin with biochemical properties similar to those of the mussel lectin, isolated from the bivalve *Crenomytilus grayanus* (Belogortseva *et al*., 1998), may belong to the same lectin family. Studies of mollusks are expected to be increasingly important in the advancement of glycomics and glycosciences.

8.4 DOWNREGULATION OF THE GENE EXPRESSION OF AN ABC TRANSPORTER BY A NOVEL LECTIN-GLYCOSPHINGOLIPID PATHWAY INVOLVING A SUEL-TYPE LECTIN DOMAIN

8.4.1 The D-galactoside/L-rhamnose-binding SUEL-type Lectin Family

In this final section, we introduce the D-galactoside/L-rhamnose-binding SUEL-type lectin family. A D-galactoside-binding disulfide-bonded dimer lectin with a 11.5 kDa

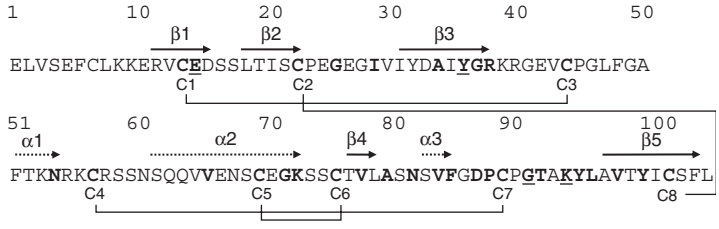

Fig. 8.8 Primary structure of SUEL from *Anthocidaris crassispina*. Bold capitals represent consensus sequences among all the SUEL-type lectin families and C2–C9 are disulfide bonds within the polypeptide. Uniquely, C1 is linked to another subunit, to make a homodimer. Whole (β1–5) and dotted (α1–3) arrows indicate areas of the β-sheet and α-helix, respectively. Underlined amino acids are essential for carbohydrate binding.

polypeptide was originally reported from unfertilized eggs of the Japanese purple sea urchin (*Anthosidaris crassispina*) by Sasaki & Aketa (1981). Immunohistochemical studies indicated that the lectin underwent rapid changes in localization before vs. after fertilization (Sasaki & Aketa, 1981). Ten years after its discovery, we elucidated the unique primary structure of the lectin (Ozeki *et al*., 1991). It consists of 105 amino acids, containing nine Cys and no Met, His or Trp (Fig. 8.8). It had no similarity with any other known primary structures for several years, until vertebrate SUEL homologues were found during cloning of a receptor (latrophilin) in mouse brain that binds to a neurotoxin from spiders (latrotoxin) (Lelianova *et al*., 1997), and to a rhamnose-binding lectin isolated from eggs of various fish species (Tateno *et al*., 1998). These lectins were organized into a characteristic structure family containing proto (SUEL), chimera (lattrotoxin receptor) and tandem (fish egg lectin) types.

According to EMBL nomenclature (www.embl.org), this new structure is called D-galactoside/L-rhamnose-binding SUEL-type lectin and SUEL-type domain (http://www.uniprot.org/uniprot/P22031). The positions of the disulfide bonds (Cys^{14}–Cys^{44}, Cys^{23}–Cys^{102}, Cys^{57}–Cys^{89} and Cys^{70}–Cys^{76}) and some amino acids (Glu^{15}, Gly^{26}, Ile^{29}, Ala^{34}, Tyr^{36}, Gly^{37}, Arg^{38}, Asn^{54}, Val^{66}, Gly^{72}, Lys^{73}, Ala^{80}, Asn^{82}, Val^{84}, Phe^{85}, Asp^{87}, Pro^{88}, Gly^{91}, Thr^{92}, Lys^{94}, Tyr^{95}, Leu^{96}, Val^{98} and Tyr^{100}) in SUEL polypeptides are predicted to be highly conserved within the SUEL-lectin family (Ogawa *et al*., 2011; Tateno, 2010). In the SUEL sequence, four amino acids (Glu^{15}, Tyr^{36}, Gly^{91} and Lys^{94}) that are essential to carbohydrate binding are also conserved (Vakonakis *et al*., 2008). Over 1000 sequences from protein and genomic informatics data that have similarity to the SUEL-lectin domain, including 60 defined molecules containing lectin, latrophilin and the galactosidase in animals and plants, are registered in the EMBL database (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR000922), suggesting that proteins with a SUEL-type lectin domain play a wide variety of roles in essential biological phenomena.

8.4.2 Novel Downregulation of Gene Expression of an ABC Transporter by the Lectin–Glycosphingolipid Pathway

SAL is a SUEL-type lectin isolated from catfish (*Silurus asotus*) egg (Hosono *et al*., 1999). It is a 32 kDa molecule containing three SUEL lectin domains in the polypeptide. Globotriaosylceramide (Gb3) is identical to the α-galactoside-binding mussel

(*M. galloprovincialis*) lectin, described in Section 8.3. When SAL was administered to Burkitt's lymphoma Raji cells, the cells displayed cell cycle arrest at the G1 phase, decreased cell size, and increased membrane pore size (*i.e.*, permeability) (Nitta *et al.*, 2007). SAL did not have a direct cytotoxic effect on the cells. However, the cytotoxic effect on the cells by doxorubicin (Adriamycin), an anticancer drug known to inhibit the activity of DNA polymerase and topoisomerase, was 1.5 times higher for co-treatment with SAL than for the drug alone (Sugawara *et al.*, 2004). These results suggest that SAL may have increased membrane permeability and accelerated the influx of the drug into the cell through binding with Gb3 on the cell.

Specific cell membrane components are involved in the excretion of chemicals and water for the promotion of malignant cell survival, and transporter-channel proteins in cancer cells actively excrete anticancer drugs (Loo *et al.*, 2006). Multidrug-resistance proteins (MRP) and multidrug-resistance associated proteins (MRAP) are representative ATP-binding cassette (ABC) transporter-channel proteins (Loo & Clarke, 2008). The expression level of these proteins appears to affect cell membrane permeability and may support the resistance of malignant cells against anticancer drugs. On this basis, we hypothesize that the expression of the transporter proteins on Burkitt's lymphoma cells increases membrane permeability through binding between SAL and Gb3. The elucidation of these molecular mechanisms will promote our understanding of the functions of animal lectins in the control of gene expression. For this purpose, we investigated the alteration of ABC transporter expression on the cell surface through SAL–Gb3 interaction.

Three anticancer drugs—vincristine, etoposide and cisplatin—were found to kill Raji cells at concentrations of 1 μg/ml, 1 μg/ml and 10 μg/ml, respectively, when applied once after 48 hours of incubation. Burkitt's lymphoma cells were sensitive to vincristine at lower concentrations (0.1–1.0 μg/ml). SAL alone did not kill the cells, even at a concentration of 25 μg/ml (Fig. 8.9a, shaded column). However, Raji cells were killed by the administration of vincristin at the less-than-lethal concentration of 10 ng/ml with co-treatment of SAL at 6–25 μg/ml (Fig. 8.9a, shaded columns). SAL-dependent cytotoxicity with low-concentration anticancer drugs was specifically eliminated by co-treatment with the α-galactoside melibiose (Galα1-6Glc) but not with the β-glucoside chitobiose (GlcNAcβ1-4GlcNAc). These results indicate that SAL enhanced the effect of the anticancer drugs at low concentrations through binding with Gb3. The lectin specifically agglutinated Raji cells in a glycan-dependent manner. Erythroleukemia K562 cells, which do not express Gb3, were not agglutinated by SAL (Fig. 8.9b).

Real-time polymerase chain reaction (RT-PCR) results indicated that Raji cells specifically expressed mRNA encoding MRAP called MRP1, a type of ABC-transporter channel protein (Fig. 8.9c, inset). Quantitative RT-PCR showed that the mRNA of MRP1 on the cells was decreased in a time-dependent (0–48 hours) manner after treatment with SAL (25 μg/ml), and the decrease was specifically inhibited by the co-presence of melibiose (Fig. 8.9c). However, the time-dependent disruption of the gene expression of a common cell-surface antigen on Raji cells, CD45, did not decrease 48 hours after SAL treatment. The decrease of MRP1 on the cell surface after SAL treatment was confirmed by an immunological procedure using anti-MRP1 monoclonal antibody against MRP1 and FACS. This procedure indicated that the expression of mRNA encoding MRP1 transporter on the cell surface was depleted by SAL treatment in a dose-, time- and glycan-dependent manner. The role of MRP1 in controlling the effect of vincristin was confirmed by small-interfering (si) RNA technology: the sensitivity to vincristin was significantly higher in MRP1 knock-down cells.

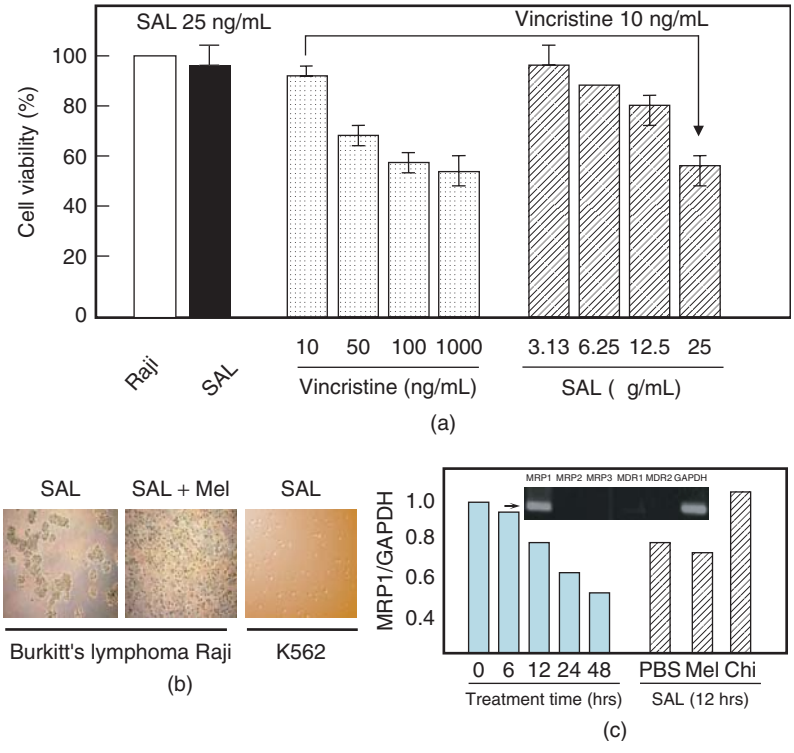

Fig. 8.9 Enhancement of anticancer drug effects against Raji cells by SAL. (a) Cell viability (%) (vertical axis) was quantified by trypan blue exclusion assay. Raji (open column): no lectin or anticancer drug. SAL (black column): lectin alone (25 μg/ml). Vincristine (0.01–1.00 μg/ml) was administered to cultured Raji cells for 48 hours at various concentrations (dotted column). After incubation of cells with various concentrations of SAL (3.13–25.00 μg/ml) for 24 hours, 10 ng/ml of vincristine was administered for 48 hours (shaded column). Disaccharides — 20 mM of melibiose (Mel) or chitobiose (Chito) — were co-administered with SAL against Raji cells for 24 hours, then each drug was administered for 48 hours (striped column). The solid lines indicate the enhancement of cytotoxicity in the SAL-treated cells in comparison to the untreated Raji cells. (b) SAL-Gb3-dependent downregulation of MRP1 mRNA. Quantitative RT-PCR shows the expression of MRP1 at the mRNA level by normalizing the GAPDH among Raji cells. Cells were treated with PBS, melibiose or chitobiose (white columns: PBS, Mel, Chito), and with SAL (25 μg/ml) in the presence or absence of saccharides (gray columns: PBS + SAL, Mel + SAL, Chito + SAL). (c) Expression of mRNA-encoding ABC-transporter superfamilies in Raji cells. RT-PCR was conducted for 30 cycles and amplified cDNA was detected by ethidium bromide staining. GAPDH was employed as a negative control. The arrow indicates the amplified MRP1-encoding cDNA.

Taken together, these results indicate that SAL, a SUEL-type lectin purified from catfish eggs, did not show cytotoxicity by itself, but uniquely downregulated both gene and protein expression levels of MRP1—an ABC-transporter protein—on cells, through binding with Gb3 a component of glycosphingolipid-enriched microdomains (GEMs) (Steelant *et al*., 2002). The enhanced effect of low-concentration anticancer drugs against Burkitt's lymphoma cells may have resulted from downregulated MRP1 gene expression through a lectin-glycosphingolipid pathway, leading to increased membrane permeability and the incorporation of anticancer drugs (Fujii *et al*., 2012). This novel pathway may be exploitable for the development of new oncotherapeutic strategies or drugs.

Recent studies indicate the importance of globo-series GSLs containing Gb3 in the expression of ABC-transporter genes. The upregulation of both P-glycoprotein and globo-series glycosphingolipids was shown to be promoted by the addition of doxorubicin and the transfection of siRNA of glucosylceramide synthase (Liu *et al*., 2010). The silencing of Gb3 in GEMs by siRNA of Gb3-synthase induced downregulation of both β-catenin and P-glycoprotein, indicating that globo-series glycosphingolipids such as Gb3 in GEMs enhances cSrc and β-catenin signaling to trans-activate *MDR1* gene expression. The complex of transcription factors consisting of β-catenin and Tcf-4 presumably binds to the promoter region of the MDR1 gene and enhances upregulation of the mRNA, resulting in the accumulation of large amounts of globo-series glycosphingolipids by glucosylceramide synthase.

Taken together, these findings indicate that Gb3 plays an important role in the expression of ABC-transporter genes through cSrc family kinases, which are known to be located in the same vicinity as GEMs, and thereby facilitates cell signal transduction. SAL exists as a noncovalently bound trimer with a triple-tandem repeat structure in each polypeptide, consisting of three carbohydrate-binding domains (CRDs) (Fig. 8.2c). If trimeric-SAL is able to mask the enriched quantity of Gb3 in a GEM through all nine CRDs, it may be able to cause the depletion of *MRP1* gene expression (cf. the effect of the mussel lectin described in Section 8.3, which caused direct apoptosis with a polypeptide containing three CRDs). The evidence suggests that the various cell regulatory functions are caused by each of the lectins, with the multiple parameters (as diversified glycan-binding properties and binding constants) occurring through multivalency.

8.5 PERSPECTIVES ON STUDIES OF INVERTEBRATE LECTINS AND THEIR DIVERSE PROPERTIES

It is unclear so many galactose-binding lectins have been found in marine invertebrates compared to vertebrates, although some oligosaccharides express D-galactose residues at nonreducing terminals not found in vertebrates. The authors have purified ten D-galactose-binding lectins and related saccharide-binding lectins with totally different molecular masses using the same purification procedure (Fig. 8.2a). The glycan-binding profiles of these lectins have been categorized into different types, including complex-type oligosaccharide-binding (type 1): feather star lectin, OXYL (Matsumoto *et al*., 2011), sponge lectin, HOL30 (Kawsar *et al*., 2008), annelid lectin, PnL (Kawsar *et al*., 2009a) and bladder moon shell lectin, GdL (Fujii *et al*., 2009); glycosphingolipid-binding (type 2): sea urchin egg lectin, SUEL (Ozeki *et al*., 1991), black mussel lectin, SvL and Mediterranean mussel lectin, MytiL; and complex-type oligosaccharide- and glycosphingolipid-binding (type 3): coronate moon turban lectin, TcL (Fujii *et al*., 2011) and sea hair lectin, AKL (Kawsar *et al*., 2011). These different types can be utilized to develop glycan-dependent diagnostics by applying them in advanced analytical systems (Tateno *et al*., 2010b) and in research focused on the regulation of cell functions and the creation of rich sources of marine lectins. Elucidation of the physiological functions of these different types of lectins is also essential to the progress of glycobiology.

Many reports on lectins isolated from invertebrates have been concerned with innate immunity. The role of SUEL appears to be different, as it is located in cortical granules in the cytoplasm of unfertilized eggs. After fertilization, SUEL quickly rearranges itself in the hyaline layer in the periphery of the fertilized egg. Immunohistochemical studies

indicate that SUEL is located in the extracellular matrix of the blastula and primitive gut in the gastrula and finally disappears in the pluteus larva (Fig. 8.2b). The primitive gut has important functions in both the incorporation of nutrients from food and protection against harmful outside organisms (pathogens). Lectins thus appear to be crucial molecules in the early development of deuterostomes, which is similar to early human development. The C21orf63 mRNA that encodes a sequence homologous to SUEL-type lectin was recently found to be ubiquitously expressed in human tissues (Mitsunaga *et al*., 2009). Such enigmas regarding the functions of endogenous lectins will be elucidated by further studies using cutting-edge approaches such as bioinformatics, and by discovery of structural relatives of the novel mussel lectin in the genomes of higher animals.

Marine invertebrate lectins and their structural families display fascinating potential and provide attractive and useful models of glycan–lectin interaction in the regulation of cell functions. These lectins can be used as a basis for minimally invasive therapies involving the control of gene expression (Fig. 8.2c). Many yet-hidden keys to glycobiology and life sciences in relation to medicine will be revealed by further studies of marine invertebrate lectins.

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9 Routes in Innate Immunity Evolution: Galectins and Rhamnose-binding Lectins in Ascidians

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9.1 ANIMAL LECTINS

The term 'lectin' is commonly used to encompass a wide variety of carbohydrate-binding proteins, widely distributed in viruses, prokaryotes and eucaryotes (Vasta & Ahmed, 2008). The first animal lectins were isolated by Noguchi in early 1900 from *Limulus polyphemus* and *Homarus americanus*; many years later, Watkins & Morgan (1952) proposed a sugar-specific binding (L-fucose) property for the eel lectin. Animal lectins are grouped in various molecular families, differing in carbohydrate-recognition domain (CRD) structure and organization (Gabius, 1997; Kilpatrick, 2002; Loris, 2002; Vasta *et al*., 2004). They are involved in a variety of key biological processes, ranging from development (Kaltner & Stierstorfer, 1998; Kilpatrick, 2002) to immune responses (Arason, 1996; Vasta *et al*., 1994). Protein–carbohydrate interactions are the basis of a mechanism for signaling functions, cell communication and self–non-self recognitions and are critical in the establishment and maintenance of highly specific mutualistic associations in organism–microbe complexes (Sharon & Lis, 1993). In this respect, mutual benefit (symbiosis or commensalism) depends on the maintenance of a tightly regulated balance, whereas colonization of tissues beneficial to the microbe can lead to the loss of host fitness (pathogenesis), unless host-defense responses are able to eliminate the foreignness (Casadevall & Pirofski, 2000). Microheterogeneity, originating from multiple lectin gene copies, allelic variation or post-translational modifications of the gene products, expands the molecular diversity and recognition capabilities. The molecular repertoire may provide a broad non-self-recognition capacity for an efficient innate immune recognition system based on recognition of carbohydrate moieties.

Galectins, rhamnose-binding lectins, C-type lectins, fucolectins, P-type lectins and L-type lectins are some examples of animal lectins (Kilpatrick, 2002; López *et al*., 2011; Shirai *et al*., 2009).

9.2 ASCIDIANS

Ascidians are invertebrate chordates constituting the most-studied and richest in species class of the subphylum Tunicata or Urochordata, which, together with Cephalochordata

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and Vertebrata, forms the phylum Chordata. The body of the sessile adult is lined by an epidermis and covered with the tunic. The chordate features—notochord, neural tube, muscular tail and pharynx provided with gill slits—are present in the swimming larva and disappear at metamorphosis, with the exception of the pharynx, which extends into the body (Berril, 1955; Burighel & Cloney, 1997). Recent phylogenetic analysis suggests that ascidian could be the sister group of vertebrates (Delsuc, 2006; Tsagkogeorga *et al*., 2009).

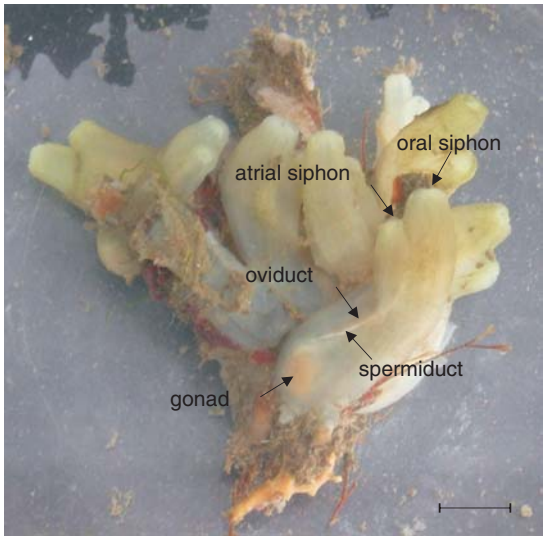
The solitary species *Ciona intestinalis* and the colonial species *Botryllus schlosseri* are important model organisms for developmental and evolutionary biology studies, including immunobiology. *C. intestinalis* is widely distributed in the coastal areas of all temperate seas and grows in dense aggregations on any floating or submerged substrate or other fouling organism (Fig. 9.1a). It is an insufficient hermaphroditic broadcast spawner, with a cylindrical soft body attached on a substrate by the posterior end, while at the anterior side the oral and atrial siphons provide for the water flux through the pharynx. The pharyngeal sac occupies a wide body region and is formed by bars and vessels containing hemolymph and hemopoietic nodules. Undifferentiated cells can proliferate and differentiate the hemocyte lines (Peddie *et al*., 1995). The genome has been fully sequenced and many transcript sequences of various developmental stages, from embryo to adult, are available in databases (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html).

The compound ascidian *Botryllus schlosseri* (Fig. 9.1b) is a reliable model organism for a variety of studies, ranging from sexual and asexual reproduction to immunobiology (Manni *et al*., 2007). The complete genome is not yet available and its transcriptome is less known than that of *Ciona*; however, the number of expressed sequence tags (ESTs) available in databases is progressively increasing due to the efforts of various research groups. Zooid individuals are grouped in star-shaped systems, are enveloped by a common tunic and share the colonial vascular system, with peripheral and radial vessels connecting zooids, buds and budlets. Colonies reproduce asexually and three blastogenetic generations are usually present in a colony: adult, filtering zooids; buds on zooids; and budlets on buds (Manni *et al*., 2007). A weekly (at 20 °C) generation change or take-over (TO) allows the cyclical renewal of the colony. Colonial developmental phases lying more than 1 day from the preceding and following TO are collectively referred to as 'midcycle' (MC) (Lauzon *et al*., 1992; Manni *et al*., 2007).

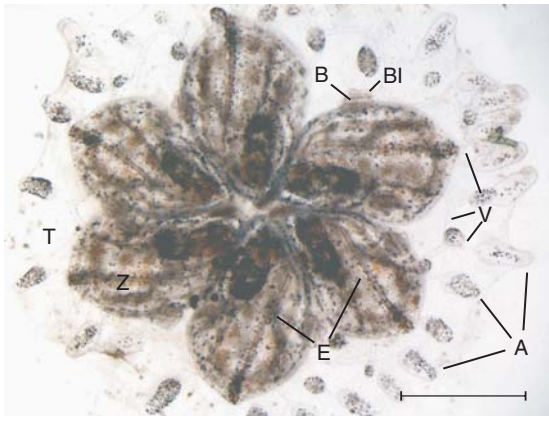
9.2.1 Inflammatory Responses of the Solitary Ascidian *C. intestinalis*

Various hemocyte types circulating in the pharynx vessels and scattered in a not-vascularized tunic matrix are involved in *C. intestinalis* innate immunity. Particulate or soluble materials inoculated into the tunic challenge a local inflammatory-like response due to a massive infiltration of hemocytes, which release several products and encapsulate the affected tissue (Arizza & Parrinello, 2009; Cammarata & Parrinello, 2009; Cammarata *et al*., 2008; Parrinello, 1981; Parrinello *et al*., 1984a,1984b). In addition, hemocytes are protagonists of the pharynx inflammatory reaction due to lipopolysaccharide (LPS) inoculation (Parrinello, 1996; Parrinello *et al*., 2007; Pinto *et al*., 2003; Shida *et al*., 2003; Vizzini *et al*., 2007, Vizzini et al., 2008). Humoral factors named '*tunicate*-cytokines' can stimulate cell proliferation and modulate hemocyte activities (Beschin *et al*., 2001; Parrinello *et al*., 2007).

Recent progress in the genome sequencing and cDNA/EST production (Azumi *et al*., 2003; Terajima *et al*., 2003a,b) derived from hemocytes (Shida *et al*., 2003; Terajima *et al*., 2003) has contributed to the study of immunity-gene expression and function. Genes for



(a)



(b)

Fig. 9.1 (a) Aggregation of *C. intestinalis* specimens. Scale bar: 2 cm. (b) Ventral view of a *B. schlosseri* colony. Zooids (**Z**), buds on zooids (**B**) and budlets on buds (**b**) are embedded in the common tunic (**T**). The endostyle (**e**) is clearly visible, and many vessels (**V**) of the common circulation connecting all the zooids can be seen; along the periphery they end with blind ampullae (**A**). Scale bar: 1 mm.

tumor necrosis factor-like proteins (*Ci*TNFα) (Parrinello *et al*., 2008), C3-like complement factor and CiC3-1a-like fragment (*Ci*C3 and *Ci*C3a) (Pinto *et al*., 2003), mannose-binding lectin-like (*Ci*MBL, Bonura *et al*., 2009) and a component of the CAP protein family (cysteine-rich secretory proteins, antigen 5 and pathogenesis-related 1 proteins) can be promptly expressed following LPS inoculation (*Ci*CAP) (Bonura *et al*., 2010).

9.2.2 *B. schlosseri* Immune Responses

B. schlosseri immune responses include phagocytosis and cytotoxicity, mediated by circulating immunocytes, and finely regulated by cytokines and lectins released by

immunocytes themselves (Ballarin, 2008; Menin & Ballarin, 2008). In addition, an inflammatory reaction, including the selective recruitment, extravasation and degranulation of cytotoxic cells, with the consequent release of phenoloxidase, a cytotoxic enzyme, and of its polyphenol substrata, is usually observed during the nonfusion reaction between contacting, genetically incompatible colonies (Ballarin *et al*., 2008).

9.2.3 Ascidian Lectins

A wide literature reports on lectin families in tunicates. Multiple lectins of diverse specificities indicate that a very complex lectin repertoire is involved in innate immunity (Queseberry *et al*., 2003; Sharon & Lis, 2007). Several C-type (Ca-dependent) lectins, both soluble and integral membrane proteins, have been found in ascidians, including mannose-binding lectins, provided with a collectin-like structure, and selectins (Bonura *et al*., 2009; Green *et al*., 2006; Raftos *et al*., 2001; Vasta *et al*., 1999). A ficolin homolog functions like mammalian collectins (Sekine *et al*., 2001); pentraxins are acute-phase proteins that, like MBL and other collectins, have opsonic, mitogenic and complement-activation properties (Vasta *et al*., 1986a,b). In ascidians, a conserved lectin-dependent pathway of complement may be activated and a C3-like component is cleaved by lectin-associated serine proteases (*Ci*MASPs) (Ji *et al*., 1997; Nonaka & Azumi, 1999; Marino *et al*., 2002; Miyazawa *et al*., 2001; Sekine *et al*., 2001; Vasta *et al*., 1999). The *Ci*C3a fragment exerts *in vitro* chemotactic activity toward hemocytes (Pinto *et al*., 2003). Interestingly, a *B. schlosseri* lectin type contains both a C-type lectin domain and an immunoglobulin-like domain (Pancer *et al*., 1997), similar to the fibrinogen-related proteins from molluscs (FREPS) involved in defense (Adema *et al*., 1997). Finally, a lytic mechanism based on soluble phospholypases A2 (sPLA2) and β-galactoside-specific lectins is exerted by *C. intestinalis* hemocytes against the K562 cell line and mammalian erythrocytes (Arizza *et al*., 2011).

9.3 GALECTINS

Galectins are widely distributed throughout the animal kingdom (from protists to mammals) and are expressed in a variety of organs and tissues (Cooper & Barondes, 1999). They form a molecular family composed of Ca^{+2}-independent soluble lectins (also known as galaptins or S-lectins)—defined by a conserved CRD that binds β-galactoside-containing glycans—and present a conserved β-sandwich structure formed by six S1–S6 and five F1–F5 strand sheets. The S4–S6 strands contain the conserved carbohydrate-binding amino acids (Hirabayashi & Kasai, 1993; Loris, 2002; Rabinovich *et al*., 2007; Rapoport *et al*., 2008; Rini & Lobsanov, 1999).

Galectins are probably the most conserved and ubiquitous lectin family. The first galectin was identified and characterized from the electric organs of the eel *Electrophorus electricus* (Teichberg *et al*., 1975), and more than 15 galectins have been identified in mammals involved in the regulation of innate and adaptive immune responses. Members of the galectin family have been found in birds, amphibians, fish, nematodes, sponges and some fungi (Houzelstein *et al*., 2004; Vasta, 2012).

Galectins are nonglycosilated proteins that lack a signal peptide and share a conserved sequence motif in their CRDs consisting of about 130 amino acid residues (Ogawa *et al*., 2011). They bind to β-galactosides (such as lactose and N-acetyllactosamine) either in free form or as components of glycoproteins or glycolipids. Based on their structural

features, galectins can be classified into three subfamilies: **prototype** (mono-CRD, single carbohydrate-binding domain), which exists as monomer; **tandem-repeat type** (bi-CRD; two distinct carbohydrate-binding domains on a single chain, joined by a random-coil linker); and **chimera type** (carbohydrate-binding domain and a regulatory N-terminal domain with collagen-like sequences). Functional multivalency, allowing the crosslinking of glycan ligands, improves galectin activity and cellular effects. A noncovalently linked dimeric form of the prototype galectin is required for effective binding and signaling through the cell surface (Levroney *et al*., 2005). The activity of tandem-repeat galectins results from the constitutive bivalency, but the ability of the flexible linker domain to form dimers may increase their potency (Earl *et al*., 2011). It is maintained that association in the regulatory domain of chimera type in the presence of multivalent carbohydrate ligands could result in oligomerization (Ahmad *et al*., 2004). Garner & Baum (2008) proposed a model in which cell function may be 'fine-tuned' by modulating galectins, affecting membrane-domain organization and signaling-threshold setting, and increasing receptor residency time at the cell surface. In addition, cell function can be modulated by protein glycosylation, in which galectins regulate apical sorting of glycoproteins (Rodriguez *et al*., 1999).

These galectin structures are conserved among invertebrates and vertebrates, although the number of genes can vary markedly even between closely related species (Cooper, 2002; Kaltner & Gabius, 2012; Vasta, 2012). They play important roles in morphogenesis, cell proliferation control, cell death, tumor progression (modulating tumor growth and metastasis) and diverse other pathological processes (Brewer *et al*., 2002; Gabius & Wu, 2008; Paulson *et al*., 2006; Sato & Rabinovich, 2008). Galectins have been shown to participate in a plethora of immune responses by acting intracellularly and extracellularly as cytokines, growth-inhibitory factors, death triggers and survival inducers (Liu *et al*., 2008; Sato & Rabinovich, 2008). In this regard, they have been related to inflammatory responses, including a direct interaction with pathogens and modulation of phagocyte function (Vasta, 2012). Extracellular galectins exhibit low-affinity bivalent or multivalent interactions with glycans and form multivalent complexes with cell-surface glycoprotein receptors (Garner & Baum 2008) that can induce various cellular responses and regulate a variety of cell functions, including proliferation, cell adhesion, migration, cell motility and apoptosis (Hernandez & Baum, 2002; Rabinovich & Toscano, 2009). Intracellular galectins can participate in signaling pathways and alter biological responses, including cell differentiation, production of cytokines and other inflammatory mediators (Liu *et al*., 2012). Galectin involvement in some *in vivo* processes has been discovered, or supported through studies of genetically engineered mouse strains, each deficient in a given galectin. Current evidence also suggests that galectins may be therapeutic targets or employed as therapeutic agents (Parsonage *et al*., 2006; Yang *et al*., 2008).

9.3.1 Molecular Features, Structure and Evolution of *C. intestinalis* Galectins

In order to elucidate the chordate galectin evolutionary history, Houzelstein *et al*. (2004) exploited the location of two galectin-encoding genes (*Ci*Lgals-a and *Ci*Lgals-b) on the EST and JGI *C. intestinalis* database, the exon–intron organization and the sequence comparison of CRDs. The N-terminal and C-terminal CRDs may have diversified into two different subtypes (F4-CRD and F3-CRD), defined by exon–intron structures that are always encoded by three exons and identified on the basis of the second CRD exon

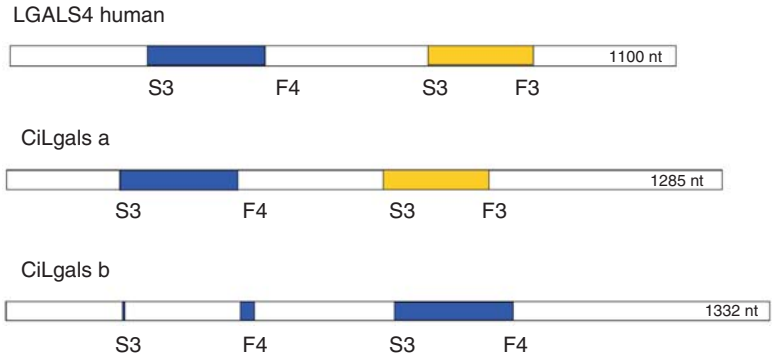


Fig. 9.2 Comparison of the typical human bi-CRD with *C. intestinalis* gene organization. Exons are represented as boxes. The W exons, which contain the highly conserved tryptophan residue of galectins, are either dark gray where they encode S3 to F4 sequences (F4 subtype), or are light gray where they encode S3 to F3 sequences (F3 subtype).

(Fig. 9.2), the F4-CRDs ending within the sequence encoding the F4 β strand (S3-F4) and the F3-CRDs within the sequence encoding the F3 β strand (S3-F3). The phylogenetic tree built with the deduced amino acid sequences suggests that the ascidian biCRD galectin gene originated from a first duplication of a mono-CRD gene, probably before chordate evolution. Various domain structures, found in mollusks and hydrozoans, suggest that an ancestor CRD gene contributed in proteins with diversified functions (Fig. 9.3). An oyster (*Crassostrea virginica*) galectin, involved in defense, contains four galectin CRDs in the

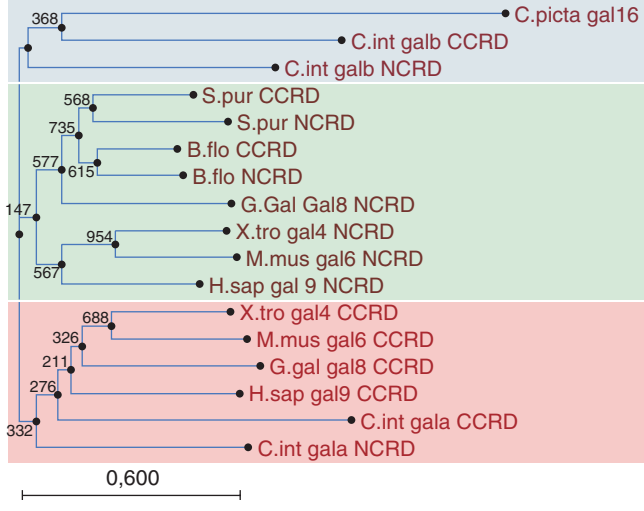


Fig. 9.3 Phylogenetic tree of CRDs from *Ci*Lgals-a, *Ci*Lgals-b, vertebrate bi-CRD galectins amino acid sequences (C. picta: *Clavelina picta*; C. int: *Ciona intestinalis*; S. pur; *Strongylocentrotus purpuratus*; B. flo: *Branchiostoma floridae*; G. gal: *Gallus gallus*; X. tro: *Xenopus tropicalis*; M. mus: *Mus musculus*; H. sap: *Homo sapiens*). The tree was constructed by the neighbor-joining method and bootstrap analysis. The bootstrap value indicates the percentage of time that a particular node occurred in 1000 trees generated by bootstrapping the galectin CRD sequences. Bar 0.600 (number of amino acid-residue substitutions for site).

same polypeptide chain (Tasumi & Vasta, 2007). A nematocyst tubule nematogalectin, with an N-terminal GlyXY domain, can form a collagen triple helix followed by galectin CRD (Hwang *et al*., 2010). Galectins isolated from the sponge *Geodia cydonium* form tetramers (Hirabayashi *et al*., 2002).

The sequence of duplication and divergence events could explain the different chordate galectins (Cooper *et al*., 2002; Houzelstein *et al*., 2004), while multiple lectin gene copies, allelic variation or post-translational modifications of the gene products expand the molecular diversity and recognition capabilities. Vizzini *et al*. (2011) analyzed the sequenced cDNA and CRD sequences and supported the predicted bi-CRD *Ci*Lgals-a and *Ci*Lgals-b gene organization and the chromosome localization (chromosome 4q and chromosome 6q, respectively). The *Ci*Lgals-a transcript (1285 nt) encodes a 289-amino acid sequence with a deduced molecular size of 32 kDa, and the *Ci*Lgals-b transcript (1332 nt) encodes a 318-amino acid sequence (37 kDa). Like vertebrate cytosolic galectins, they do not contain a signal peptide and presumably are released through a nonclassical secretory pathway (Vizzini *et al*., 2011). The major sequence-similarity and identity percentages were found with chicken galectin 8 (*Ci*Lgals-a) and mouse galectin 6 (*Ci*Lgals-b).

The *Ci*Lgals-a exhibits the F4-CRD-linkers-F3-CRD arrangement typical of the vertebrate Bi-CRD Lgals genes. Otherwise, the *Ci*Lgal-b presents a specific F4-CRD-linker-F4-CRD organization. Moreover, the *Ci*Lgals-a and *Ci*Lgals-b CRD sequences were aligned with F4-CRD-linkers-F3-CRD vertebrate bi-CRD galectins, the cephalochordate *Branchiostoma floridae*, the echinoderm *Strongylocentrotus purpuratus* gal RL30 galectin and the ascidian *Clavelina picta* mono-CRD gal16 (Vizzini *et al*., 2011). The phylogenetic tree shows that vertebrate N-CRDs and C-CRDs are grouped into two distinct clusters, indicating an early domain divergence after a duplication event. Interestingly, the divergence between vertebrate N-CRD and C-CRD was greater than that between N-CRD and C-CRD of the examined deuterostome invertebrates. Moreover, the N-CRD cluster includes both *S. purpuratus* and *B. floridae* N-CRD and C-CRD, while the C-CRD lineage includes *Ci*Lgals-a C-CRD and N-CRD. Both *Ci*Lgals-b C-CRD and N-CRDs are derived from an S3-F4 gene organization (F4-CRD-linker-F4-CRD). The close relationship between *Ci*Lgals-b and the sequenced mono-CRD of *Clavelina picta* (F4 subtype) supports a duplication event for the ascidian bi-CRD galectin origin. On the basis of the known galectin molecular structures, molecular models (homology-modeling process) have been performed and the following overlapping was found (Fig. 9.4): the *Ci*Lgals-a N-CRD can be superimposed on the human Gal 9 C-CRD (about 40% identity); the *Ci*Lgals-a C-CRD on the human Gal 3 C-CRD (30% identity); the *Ci*Lgals-b N-CRD on the human Gal 9N-CRD (30% identity); the *Ci*Lgals-b C-CRD on the human Gal 4 C-CRD (about 28% identity).This procedure disclosed that the *Ci*Lgals-a and b domains and human CRDs share a structural model that includes two antiparallel β-sheets composed of five and six β-strands. The *Ci*Lgals-a N-CRD and C-CRD, as well as the *Ci*Lgalsb N-CRD structures, appear to be suitable for binding to β-galactosides. On the other hand, the *Ci*Lgals-b C-CRD is so divergent that a distinct functional role cannot be excluded. In addition, while the CiLgals-a can be superimposed on the gal 4 (2wsuC) domain, the CiLgals-b cannot. Finally, *Ci*Lgals-a and *Ci*Lgals-b can form distinct oligomers. Specific antibodies identified two different subunits of 42 kDa (*Ci*Lgals-a) and 38 kDa (*Ci*Lgals-b), which could form 73 and 70 kDa dimers, respectively. Protein–protein interaction in *Ci*Lgals oligomerization is an intriguing issue that merits further investigation. In this respect, multiple galectins have been found in *Clavelina picta*, with various subunit sizes (4, 15, 33 and 37 kDa) (Ahmed & Vasta, 1994b).

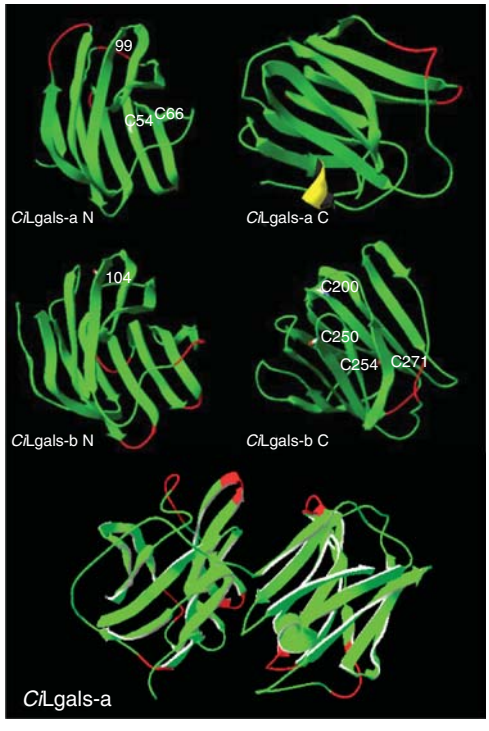


Fig. 9.4 *Ci*Lgals-a, *Ci*Lgals-b CRDs of *C. intestinalis*, modeled with the more structural significant template of galectin domains. *Ci*Lgals-a N terminal: 3nv1A (Human Gal-9 C-Terminal CRD 39.85% identity); *Ci*Lgals-a C terminal: 2xg3A (Human Gal-3 29.71% identity); *Ci*Lgals-b N terminal: 2zhnA (Human Gal-9 N-Terminal CRD (30.35% identity); *Ci*Lgals-b C terminal 1x50A (Human Gal-4 C-Terminal CRD 27.40% identity). All sequence (Human Gal-4 C 2wsuC). Cysteins are indicated by position numbers.

9.3.2 Involvement of Galectins in *C. intestinalis* Inflammatory Response

It is known that, in vertebrate organisms, pathogens upregulate the expression of galectin genes (Klyosov, 2008; Sato & Rabinovich, 2008). LPS locally inoculated into the *C. intestinalis* body wall permeates the pharynx tissue surrounding the inoculation site and stimulates a local inflammatory reaction, which rapidly upregulates the expression of the *Ci*Lgals-a and b genes. Real-time polymerase chain reaction (PCR), *in situ* hybridization and immunohistochemistry disclosed that, in the pharynx, both galectins are constitutively expressed at a low level. However, they are inducible; the expression is little increased as an effect of the medium inoculation, but significantly enhanced by LPS inoculation. The *Ci*Lgals-a gene is highly expressed, reaching its maximum level within 24 hours post inoculation. Conversely, the *Ci*Lgals-b reaches the highest expression within 1 hour, then decreases, with a secondary increase at 24 hours post inoculation. Afterwards (72 hours), both *Ci*Lgals-a and *Ci*Lgals-b gene expressions lowered at control levels. *In situ* hybridization assays showed that both genes were expressed by two granulocyte types (unilocuolar and multilocuolar hemocytes) (Fig. 9.5). The specific riboprobes are localized in the nucleus and in the surrounding cytoplasm, while the specific antibodies show that both the galectins are mainly associated with

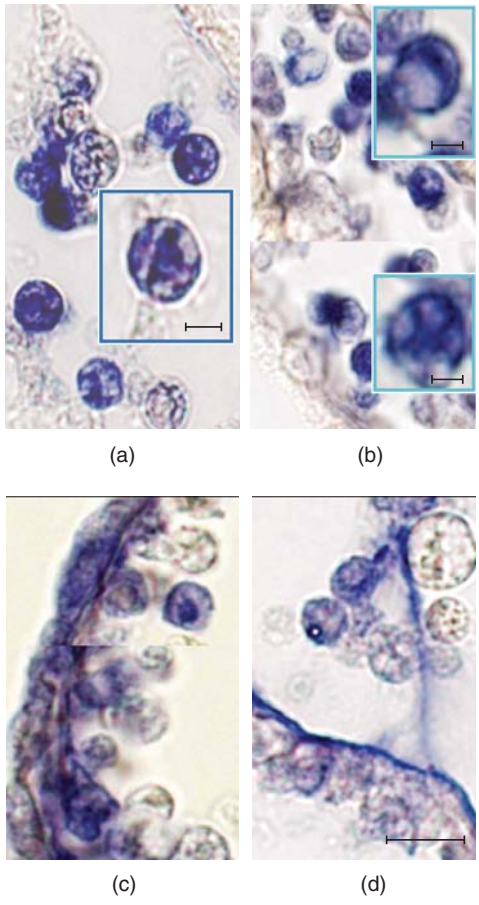


Fig. 9.5 Histological sections from *C. intestinalis* pharynx. *In situ* hybridization with *Ci*Lgals-a (a) and *Ci*Lgals-b (b) ripoboprobe. (a,b) Immunohistochemistry with anti-*Ci*Lgals-a (c) and anti-*Ci*Lgals-b (d) antibody. (c) Sham ascidian at 24 hours after inoculation with marine solution; hemocytes at 24 hours. (d) Sham ascidian at 24 hours after inoculation with marine solution; hemocytes at 24 hours. Scale bar: 10 μm; insets: 5 μm.

granule and nucleus envelopes, and less frequently are components of granule content (Fig. 9.5). Immunoblotting analysis disclosed *Ci*Lgal-a (74 and 43 kDa) and *Ci*Lgal-b (71 and 39 kDa) in the pharynx-extract supernatants sampled at 24 hours post LPS inoculation. In addition, both galectins appeared to be components of the endothelium basal membrane. The amino acid sequence alignments also disclosed the vertebrate galectin sequence signature (HNPRN and WG-EE). In the *Ci*Lgals-a, the seven key residues known to be directly involved in galactoside binding (H-N-R N W- -EE) are conserved in the N-CRD, whereas in the C-CRD only one key residue is substituted with a conservative amino acid. The *Ci*Lgals-b contains only five conserved key residues in the N-CRD, whereas the *Ci*Lgals-b C-CRD displays only three out of seven key amino acids. Due to the differences between signature sequences involved in sugar binding, the possibility exists that *Ci*Lgals-b has a minor role in pharynx inflammatory response, as also indicated by the minor immunohistochemical staining with anti-*Ci*Lgals-b antibody.

Although it has not been clarified whether in the hemolymph serum various types of D-galactose-specific lectin might be opsonins, galectin-like molecules (Ca^{+2}-independent binding and D-galactoside specificity) with opsonic properties can be enhanced in response to LPS (Parrinello *et al*., 2007). Finally, it is of interest that inducible galectins may evoke danger signals (alarmins) in inflammatory responses (Sato & Rabinovich, 2008; Sato *et al*., 2008).

9.4 RHAMNOSE-BINDING LECTINS

More than 20 years ago, Ozeki *et al*. (1991) described a new lectin, named SUEL, with specificity for D-galactosides, which is abundant in the eggs of the sea urchin *Anthocidaris crassispina* and is now recognized as the first described member of a new family of animal lectins, the rhamnose-binding lectins (RBLs). RBLs are Ca^{2+}-independent lectins with specificity for rhamnose and galactosides (galactose can easily antagonize rhamnose and bind the RBL CRD), which are particularly abundant in teleosts and other aquatic invertebrate species, such as annelids and bivalves among protostomes and ascidians among chordates (Ogawa *et al*., 2011). All RBLs do not show amino acid sequence similarity to known lectin families, do not require Ca^{2+} for carbohydrate recognition and share the presence of one or multiple CRDs with a unique α/β fold, about 100 amino acids long, with eight highly conserved cysteine residues engaged in four disulfide bridges with characteristic topology (Jimbo *et al*., 2007; Terada *et al*., 2007). In addition, conserved motifs, such as YGR, DPC and KYL, are also found in their CRDs (Terada *et al*., 2007).

As regards the role of RBLs, it has been proposed that they are involved in the regulation of carbohydrate metabolism, control of fertilization and cytotoxicity. In addition, an enhancing effect on cell proliferation has been reported for RBLs of human dermal fibroblasts (Faury *et al*., 2008), whereas fish lectins increase their expression in response to inflammatory stimuli, enhance phagocytosis, acting as opsonins, and induce the synthesis and release of pro-inflammatory cytokines (Ji *et al*., 2009; Lam & Ng 2002; Ogawa *et al*., 2011; Tateno *et al*., 2002a; Terada *et al*., 2007; Watanabe *et al*., 2009).

The ability to recognize and bind lipopolysaccharisides and lipoteichoic acid and agglutinate both Gram-positive and Gram-negative bacteria has been described in trout RBLs, suggesting an antibacterial activity (Matsui *et al*., 1994; Tateno *et al*., 2002a). In addition, RBLs have also been found in the cortex of teleost eggs, as well as in the skin mucus, further confirming their protective role. A putative natural ligand of fish RBL is the glycosphingolipid globotriasylceramide (Gb3), abundant in membrane lipid rafts (Ogawa *et al*., 2011; Watanabe *et al*., 2009).

The RBL CRD appeared early in metazoan evolution and is found in a variety of proteins with different domain architectures, from basal metazoans (e.g. cnidarian rhamnospondins) to mammals (e.g. polycystic kidney disease 1-like, axon-guidance receptor EVA-1 and latrophilin); all these proteins and RBLs constitute the RBL superfamily of proteins, which contain RBL CRDs in their sequences as a domain structure (López *et al*., 2011; Ogawa *et al*., 2011; Schwarz *et al*., 2007; Vakonakis *et al*., 2008).

9.4.1 RBLs in *B. schlosseri*: Biochemical and Molecular Features

The hemolymph of the colonial ascidian *B. schlosseri* contains soluble lectin(s) able to agglutinate yeast cells and rabbit erythrocytes (Ballarin *et al*., 1999). In an attempt to

characterize this material and obtain the amino acid and nucleotide sequences of the protein(s) and the transcript(s), a full-length cDNA library from *Botryllus* colonies was prepared, from which five sequences were identified, each with a single, complete open reading frame, representing five isoforms of a novel ascidian RBL, referred to as *Bs*RBL. All of them encoded putative proteins of 118 amino acids, belonging to type-V RBLs (Watanabe *et al*., 2009), containing a single CRD of 81 amino acids. The predicted protein sequences showed full conservation of the eight cysteines involved in the four disulfide bridges, as well as of the YGR, DPC and KYL motifs which characterize RBL CRDs (Fig. 9.6a). Four of the five sequences were identified and confirmed by biochemical analysis, after purification of colony homogenates. The positions of the disulfide bonds were verified after sequential hydrolysis with V8-endoprotease and formic acid and MS analysis (Fig. 9.6b) (Gasparini *et al*., 2008).

The five isoforms differ in a few of their amino acids and have slightly different sizes, as indicated by MS and HLPC analysis. Electrophoretic analysis under reducing conditions showed a single band of 11 kDa, in good agreement with the expected molecular masses of *Bs*RBLs, whereas bands of 6, 12.4 and 17.5 kDa were observable under nonreducing conditions (Fig. 9.6c), which suggests the presence of oligomers, with the three bands representing the monomeric, the dimeric and the trimeric form, respectively.

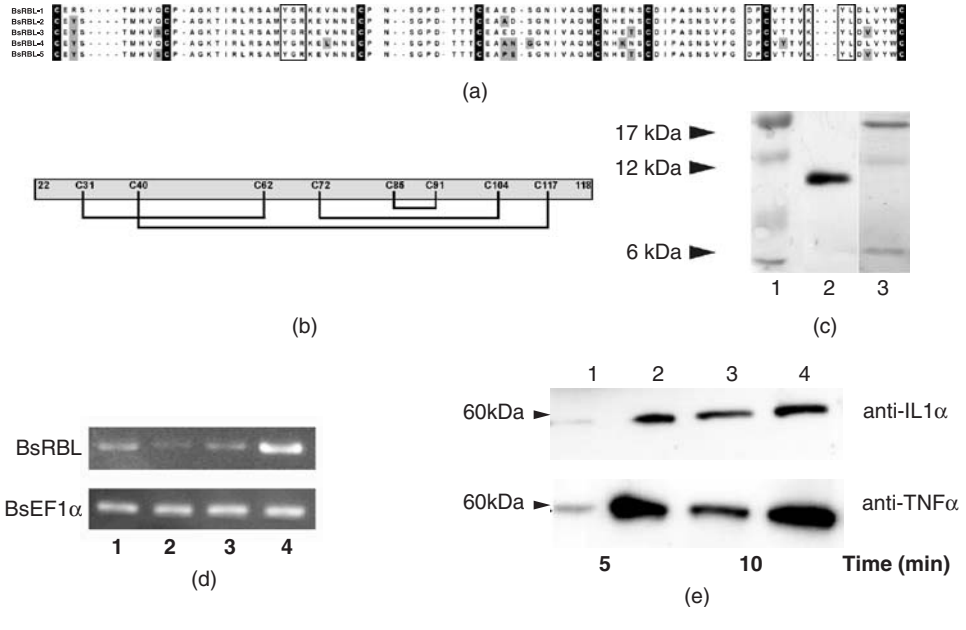


Fig. 9.6 (a) Multiple alignment of the five known isoforms of *Bs*RBL. Conserved cysteine residues are shaded black, amino acid variations are shaded grey, boxes indicate conserved motifs. (b) Aligmnent of the five isoforms of *Bs*RBL, showing the locations of the disulfide bonds. Grey area above the alignment: CRD; grey area below the alignment: N-terminal signal peptide. Highly conserved motifs of RBLs are boxed. (c) SDS-PAGE analysis of purified *Bs*RBL. Lane 1: reference molecular weights; lane 2: strong denaturing conditions; lane 3: mild denaturing conditions. (d) Semiquantitative-PCR analysis of *Bs*RBL expression during colonial blastogenetic cycle, compared with *Bs*EF1α expression. Lane 1: beginning of a new cycle; lanes 2 and 3: midcycle (MC); lane 4: take-over. (e) Immunoblot analysis, with anti-IL1α and anti-TNFα antibodies, of culture media from haemocytes incubated for 5 and 10 minutes in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of *Bs*RBL.

The formation of oligomers was reported in other RBLs (Ng *et al*., 2003; Ozaki *et al*., 1983; Ozeki *et al*., 1991; Tateno *et al*., 1998) and is required in order to explain the agglutinating activity of *Bs*RBLs, which, like SUEL, are endowed of a single CRD. However, unlike SUEL, all the cysteines of *Bs*RBLs are involved in the formation of the disulfide bridges, so that the lectins cannot be linked by covalent bonds in their multimeric form. The lower size (6 kDa) of the monomer under mild denaturing conditions with respect to that observed under reducing conditions (11 kDa) may be the result of the compact configuration of the protein, imposed by the presence of the disulfide bridges. Results from gel chromatography support this hypothesis, as four peaks are observable, with apparent sizes of 3.5, 7.5, 16.8 and 33.5 kDa, probably representing the monomeric, dimeric, tetrameric and octameric forms, respectively (Franchi *et al*., 2011; Gasparini *et al*., 2008). The three-dimensional structure of *Bs*RBLs (Franchi *et al*., 2011) supports the formation of oligomers through noncovalent interactions between monomers.

Residues 1–21 represent an N-terminal signal peptide, which is removed prior to secretion as it is never found in tryptic peptide analysis. No consensus sequences for N-linked glycosylation are found, suggesting the absence of sugars in our lectin, in accordance with the majority of RBLs (Terada *et al*., 2007).

A phylogenetic tree built with the RBL sequences in databases clearly showed that *Bs*RBLs were located within the protochordate cluster representing the sister group of vertebrate RBLs (Gasparini *et al*., 2008).

9.4.2 RBLs in *B. schlosseri*: Synthesis and Immune Roles

BsRBLs are constitutively secreted, as they are always found in colony homogenates. Immunocytochemistry with an antibody against the native form of *Bs*RBL indicates that professional phagocytes are the lectin-secreting cells, and they probably release *Bs*RBLs

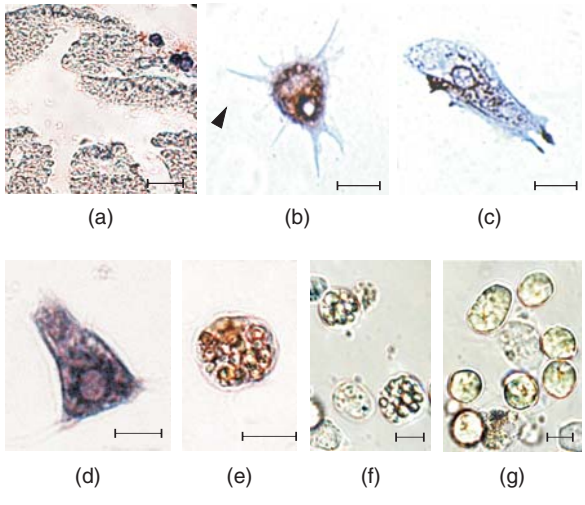


Fig. 9.7 (a–c) Immunohistochemical analysis on colony sections (a), at the level of the stomach of an adult zooid and on hemocyte monolayers (b,c), showing that phagocytes are the sole cells labeled by *Bs*RBL. (d,e) *In situ* hybridization on hemocyte monolayers with antisense probe for *Bs*RBL; only phagocytes are labeled (d), whereas morula cells are unlabelled (e). (f,g) Living morula cells in the presence (f) and absence (g) of *Bs*RBL. Scale bar: 50 μm in (a), 10 μm in (b–g).

with an apocrine-like modality (Fig. 9.7a,b,c) (Ballarin *et al*., 2000). This result was confirmed by *in situ* hybridization with an antisense riboprobe of 460 nucleotides, as staining was restricted to hemolymph and circulating phagocytes were the only labeled cells (Fig. 9.7d) (Franchi *et al*., 2011).

*Bs*RBLs change their expression during the colonial blastogenetic cycle: during the take-over a significant increase in the percentage of cells immunopositive to the anti-RBL antibody was observed with respect to MC. The increase was mostly due to labeled phagocytes, but additional immunopositive cells were also labeled on their cell surface. Semiquantitative PCR confirmed the higher expression of BsRBL at take-over (Fig. 9.6d) (Franchi *et al*., 2011). The rise in the frequency of immunopositive cells at take-over is probably related to the massive apoptosis occurring in the tissues of old zooids during this phase of the colonial blastogenetic cycle. Previous studies (Cima *et al*., 2010) indicate that phagocytes change their morphology and behavior during the take-over, and 20–30% of circulating hemocytes undergo cell death. Therefore, the increase in immunolabeled cells probably has a twofold origin, deriving both from the increase in the number of phagocytes synthesizing *Bs*RBL, which justifies the higher quantity of lectin mRNA at take-over, and in part from the interaction of *Bs*RBL with the surfaces of senescent cells, as coating by *Bs*RBL may either induce apoptosis in effete cells expressing complementary glycolipids, analogously to what is reported for catfish-egg RBL (Kawano *et al*., 2009; Shirai *et al*., 2009), or represent an 'eat me' signal for the clearance of dying cells by phagocytes.

*Bs*RBLs can agglutinate rabbit erythrocytes (this feature was exploited for their identification during purification), yeast cells and some bacterial strains, both Gram-positive and Gram-negative, in a Ca^{2+}-independent manner (Franchi *et al*., 2011). The recognition of red blood cells and yeast cells is probably mediated by the recognition of galactosides exposed on their surfaces, as endogenous rhamnose is rare in eukaryotes (Tymiak *et al*., 1993); the agglutination of bacteria fits the reported ability of RBLs to recognize components of the bacterial cell walls, such as lipopolysaccharides and lipoteichoic acid (Shiina *et al*., 2002; Tateno *et al*., 2002a). *Bs*RBLs do not exert any inhibitory effects on bacterial growth (Franchi *et al*., 2011) and their activity towards microbial cells likely consists in increasing their visibility to phagocytes and their clearance, through phagocytosis or encapsulation. Indeed, *Bs*RBLs can act as opsonins by increasing *in vitro* phagocytosis of target yeast cells (Ballarin *et al*., 1999, 2000; Gasparini *et al*., 2008). When exposed to the same *Bs*RBL concentration, the morphology of phagocytes changed to a more amoeboid shape and respiratory burst was induced, with the consequent production of reactive oxygen species with microbicidal activity (Franchi *et al*., 2011). At lower concentrations, *Bs*RBL exerts a chemotactic effect on phagocytes; this disappears at higher concentrations.

When incubated *in vitro*, *Botryllus* hemocytes release immunomodulatory molecules in the culture medium (i.e. cytokines, in the broad sense of the term), which are recognized by antibodies raised against mammalian cytokines IL1α and TNFα, observable as a single band of 60 kDa in immunoblot analysis (Fig. 9.6e). Upon exposure to *Bs*RBL, the band markedly increased its intensity with respect to controls (incubation in filtered seawater) after 5 minutes of incubation (Franchi *et al*., 2011). Previous studies indicated that these molecules are synthesized and released by cytotoxic morula cells and act as cytokines on both morula cells, stimulating their migration, and phagocytes, enhancing the phagocytosis of foreign cells and the synthesis and release of *Bs*RBLs (Menin & Ballarin, 2008; Menin *et al*., 2005).

The incubation of hemocytes with 60 µg/ml or higher *Bs*RBL led to a significant ($p < 0.001$) increase in the degranulation of cytotoxic morula cells (Fig. 9.7f), with a

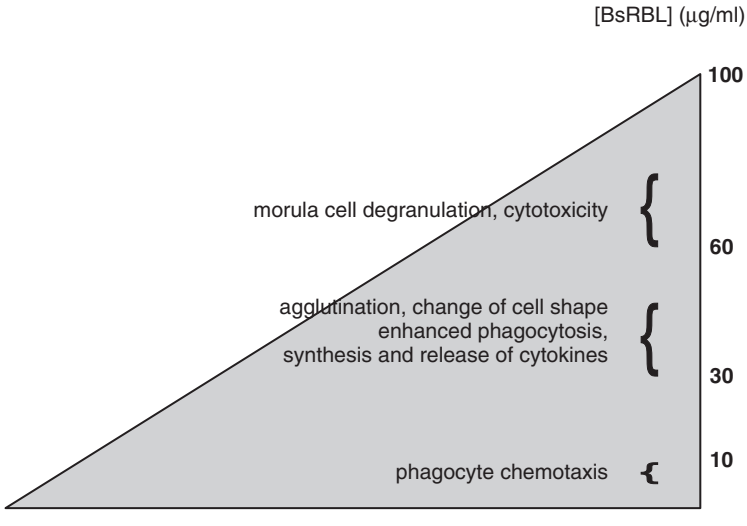
consequent significant ($p < 0.05$) increase of phenoloxidase activity in the culture medium (Franchi *et al*., 2011).

9.4.3 *Bs*RBL as a Multifuntional Molecule

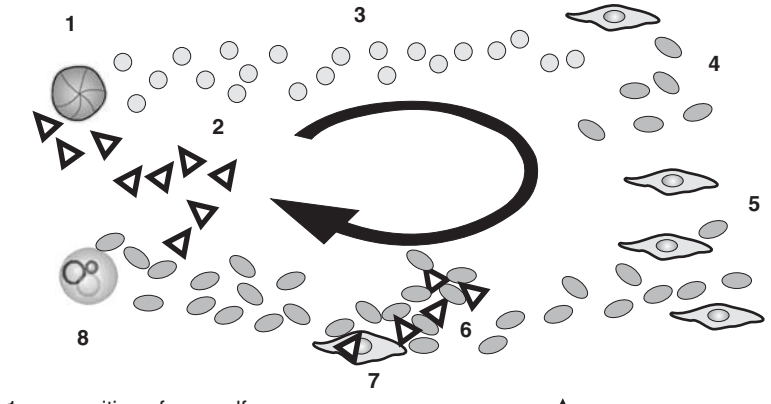
Collectively, all the results indicate that *Bs*RBLs exert multiple roles in immunosurveillance and immunomodulation. During an immune response, morula cells are the first hemocytes to sense foreign molecules and release the cytokines recognized by the anti-IL1α and anti-TNFα antibodies (Menin & Ballarin, 2008). The latter, acting in a paracrine way, induce the synthesis of *Bs*RBL by a limited number of phagocytes: this allows the migration and activation of additional phagocytes towards the infection area, as *Bs*RBL exerts chemotactic activity at low concentrations. The increased number of activated phagocytes leads to a rise of the lectin concentration, which allows the agglutination of foreign cells, favoring their clearance through phagocytosis or encapsulation, and activates morphology changes in phagocytes, rendering them more prone to phagocytosis and triggering their respiratory burst, which helps in killing microbes through the production of reactive oxygen species. In addition, analogously to the reported induction of pro-inflammatory cytokine synthesis by RBL in trout cultured cells (Ogawa *et al*., 2011; Watanabe *et al*., 2009), *Bs*RBL potentiates the synthesis and release of cytokines by morula cells and, consequently, with a positive feedback, the progressive increase of its local concentration. When microbes are fully eliminated, the synthesis and release of cytokines by morula cells and, consequently, the secretion of lectin, probably ends. If the infection persists, as phagocytes cannot eliminate the foreign cells, the concentration of *Bs*RBL reaches high levels in the circulation: this probably acts as an endogenous danger signal (Matzinger, 2002) and induces both cell death by apoptosis and morula cell degranulation. This event releases the enzyme phenoloxidase, which, acting on polyphenol substrata (also released by morula cells), induces necrotic death of all the cells within a certain area around the source of phenoloxidase (PO), thus decreasing the probability of survival of foreign cells. Fig. 9.8 summarizes these concepts.

9.5 CONCLUSION

The outer surface of the cell is covered by sugar molecules, which can be attached to proteins or fats. Each cell type has a unique collection of sugars, which change as the cell develops. Glycans modify the behavior of the cell by responding to external stimuli, are critical to cell–cell communications, affect the adhesion within organs, are involved in self/not-self recognition and affect susceptibility to disease, promptly interacting with various bacterial and viral intruders (see Sharon & Lis, 2007). Glycans display a complex and branched structure, further complicated by the multiplicity of their possibilities of combination and interaction, generated by carbohydrate permutation, anomeric status, glycosidic linkage position, size of the ring and branching (Kaltner & Gabius, 2012). The immense theoretical diversity leads to the glycome definition (collective identity of all the carbohydrates in a cell), which exceeds the proteome complexity (Gabius, 2008; Hirabayashi *et al*., 2001). A pattern of enzymes (glycosyltransferases, members of multigene superfamilies) modifies sugars or attaches them to proteins and lipids destined to the cell surface or extracellular compartments. Although a lectin enzymatic activity cannot be denied (Kylosov, 2008), enzymes bind to glycans to form with them multivalent



(a)



1 - recognition of non-self
2 - synthesis and secretion of cytokines
3 - activation of phagocytes
4 - synthesis and secretion of BsRBL
5 - phagocyte chemotaxis
6 - non-self opsonisation and agglutination
7 - phagocytosis of non self
8 - morula cell degranulation and cytotoxicity

non-self material
morula cell cytokines
*Bs*RBL

(b)

Fig. 9.8 (a) *In vitro* effects of various concentrations of purified *Bs*RBL on hemocytes. (b) Sketch of proposed interactions between phagocytes and morula cells, as indicated by our results. Upon recognition of non-self molecules, morula cells release cytokines, which stimulate morula cell chemotaxis, *Bs*RBL synthesis and secretion by phagocytes. *Bs*RBL in turn contributes to recruitment of phagocytes to the infected site, enhances phagocytosis and the release of cytokines by morula cells and, at high concentrations, induces morula cell degranulation and consequent release of PO, which is responsible for cytotoxicity.

(multipoint, multicontact) noncovalent complexes without chemical modification of the ligands. In this respect, the sugar–protein recognition pattern represents an evolutionary conserved mechanism involved in a wide variety of biological processes. Accordingly, the interest given to invertebrate key models is increasing as various aspects of the lectin structure, lectin–glyconjugate interactions, signaling pathways and functions begin to be explored. cDNA and genome sequencing has facilitated the discovery of new lectins and made functional studies possible.

Ascidian lectins include galectins and rhamnose-binding proteins. Since the spectrum of galectin activities also encompasses their involvement in establishing certain features of the malignant phenotype, it is of interest to examine the evolutionary routes that have led to diversified expressions. On the other hand, RBLs constitute a new lectin family, differing from galectins in their sequence and CRD structure. Although they have not yet been found in mammals, multidomain molecules containing RBL-CRD, involved in fundamental adhesion mechanisms, have been described in various metazoans, from cnidarians to vertebrates, humans included. However, issues concerning the signaling pathways activated by the lectins upon binding to their receptor(s) require further investigation. A putative RBL receptor in fish has been identified in the Gb3 molecule, but the possibility exists that other receptors can interact with RBL-CRDs. Thus, on the grounds of immunosurveillance and growth/invasion-regulatory activity, ascidian lectins represent a promising field of study.

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10 Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part I — Upstream Processes

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10.1 INTRODUCTION: BIOACTIVE PEPTIDES — PRODUCTION AND FUNCTIONALITIES

The use of food-grade microorganisms to enrich food with bioactive peptides via proteolysis has recently been highlighted and appraised by researchers, clinicians and food manufacturers (Agyei & Danquah, 2011, 2012c). Upon oral administration and/or absorption, bioactive peptides may induce several physiological effects, such as antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory effects, under *in vivo* and *in vitro* conditions (Gibbs, 2004; Hartmann & Meisel, 2007; Korhonen, 2009; Korhonen & Pihlanto, 2006; Yang *et al*., 2009). Thus, the biological potency of bioactive peptides in the major body systems continues to be a major research endeavour (Möller *et al*., 2008) and forms the basis of application in the consumer industries (see Table 10.1).

Despite the clinical and nutritional importance of bioactive peptides, bioprocesses and production methodologies are not fully optimised for industrial-scale titres (Agyei & Danquah, 2011). An economically feasible bioprocess for the manufacture of bioactive peptide requires that the necessary ingredients and processes should be economical and cost-effective. For example, enzymes for use in bioactive peptide manufacture should preferably satisfy such criteria as ease of availability, extraction and purification; variety in proteolytic specificity; and hardiness with use under unfavourable conditions. The lactobacilli are good sources of enzymes which meet these criteria, since they are equipped with a complex proteolytic system consisting of proteinases and peptidases with varied activities and specificities (Agyei & Danquah, 2011). Also, proteinases of lactobacilli are able to hydrolyse over 40% of the peptide bonds of α_{s1}- and β-casein, giving rise to oligopeptides with 4–40 amino acid residues (Kunji *et al*., 1996). Several of the known bioactive peptides have amino acid residues within this range (Korhonen & Pihlanto, 2006). Consequently, the lactobacilli are good candidates for the generation of bioactive peptides. Factors such as strain selection and the influence of nutritional and environmental parameters on lactobacilli proteolysis thus markedly influence the release of encrypted bioactive peptides from proteins (Minervini *et al*., 2003).

Bioactive peptides are produced via the proteolytic action of microorganisms on proteins or by *in vitro* enzymatic hydrolysis of proteins with enzymes of gastrointestinal or microbial origin (Fig. 10.1). With adequate control of hydrolyses, this method results in

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Table 10.1 Application of bioactive peptides in the major consumer industries.

Bioactivity	Area of application	Industry
Antimicrobial peptides	Natural preservatives	Food Nutraceuticals and functional foods Pharmaceuticals Cosmetics
Antimicrobial Angiotensin-converting enzyme (ACE)-inhibitory Immunomodulatory	Therapeutic products	Pharmaceuticals
Immunomodulatory	Immunonutrition Food-fortification programs	Nutraceuticals and functional foods

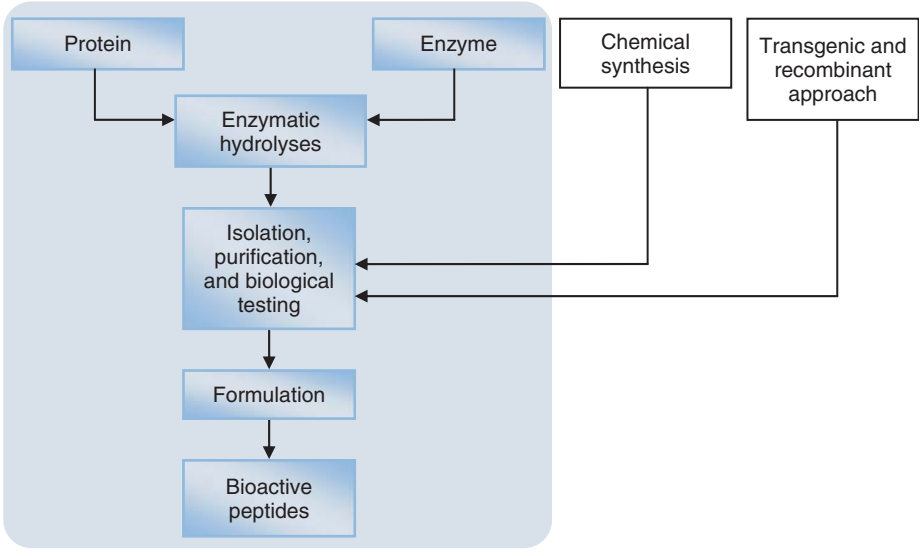

Fig. 10.1 Schematic for the production of bioactive peptides by enzyme hydrolyses *in vitro* (selected region).

breakage of the peptide bonds in proteins and subsequent generation of smaller peptides with little or no free amino acids. Although proteins can be hydrolysed by alkaline and acid treatments equally, these methods are not recommended, because, whereas alkaline hydrolysis causes the racemisation or destruction of certain amino acids at high pH (Neklyudov *et al*., 2000), acid treatment destroys tryptophan and hydrolyses asparagine and glutamine to their respective conjugate acids (Walker & Sweeney, 2002). The digestive and microbial proteases, including alcalase, trypsin, pepsin, chymotrypsin, pancreatin, pepsin, thermolysin and cell-envelope proteinases (CEPs), are among the most widely used enzymes (Korhonen & Pihlanto, 2006). It is important that enzymatic hydrolyses be carried under optimum pH and temperature conditions, in order to ensure maximum activity and reduce the formation of undesirable products (Kim & Wijesekara, 2010).

Other approaches to the production of bioactive peptides include transgenic, recombinant and synthetic methods (Marx, 2005). However, these production technologies are prohibitive for large-scale applications, due to cost (Hancock & Sahl, 2006). Bioactive peptides could also be produced naturally from dietary proteins during gastrointestinal transit, but production through such routes is uncontrolled and it might generate insufficient quantities to stimulate physiological responses in adult humans (Gauthier *et al*., 2006). The development of commercially viable processes capable of up-scaling bioactive peptide production is therefore crucial.

10.2 LACTOBACILLI METABOLISM

The lactic acid bacteria (LAB), or the 'lactics', have been used for centuries in fermented food products, such as dairy, vegetables and meat. Due to their long history of use in cultured foods with no adverse effects, they have been assigned a 'GRAS' (Generally Regarded as Safe) status (Kaushik *et al*., 2009). They are nontoxic and nonpathogenic (Gupta *et al*., 2002a). Their ability to improve the quality, safety and nutritional content of food is based on their reduction of carbohydrates and on their production of many antimicrobial agents, such as organic acids, hydrogen peroxide and proteinaceous low-molecular-weight-like bacteriocins (Vesterlund *et al*., 2004). The lactics are also important commercially in the processing of meats, alcoholic beverages and vegetables, including sausage, cured hams, wine, beer, fortified spirits, pickles and sauerkraut. However, although the LAB serve beneficial roles in the food industry, they can occasionally become a nuisance by producing off-flavours via contamination of products (Carr *et al*., 2002).

The LAB consist of a number of genera, with *Lactobacillus* being the largest and having the widest industrial and technological application (Axelsson, 2004). There is a high level of biochemical, physiological and phenotypic heterogeneity among the lactobacilli. The broadly interested reader is referred to Carr (2002), Axelsson (2004) and Vogel (2008) for a more comprehensive review of LAB and their metabolic processes. The focus of this chapter is on the metabolism of genus *Lactobacillus*, with emphasis on the proteolytic potential of the well-known species in order to highlight their potential use in the production of bioactive peptides.

10.3 THE PROTEOLYTIC SYSTEM OF THE LACTOBACILLI

It is generally believed that LAB have a very limited capacity to synthesise amino acids using inorganic nitrogen sources. They are therefore dependent on preformed small peptides and amino acids being present in the growth medium as a nitrogen source (Atlan *et al*., 1990; Axelsson, 2004; Kunji *et al*., 1996; Picon *et al*., 2010; Tsakalidou *et al*., 1999). Most LAB generally require complex or enriched media for growth. It is well established that most LAB are auxotrophic for between 4 and 14 amino acids and that the amino acid requirement is species- and strain-dependent (Kunji *et al*., 1996). The requirement for a particular amino acid may be the result of mutations in the genes for amino acid biosynthesis and/or the downregulation of these genes or the enzymes involved (Chopin, 1993). Moreover, the amino acid auxotrophy of some LAB species has been attributed to a lack of key fermentative enzymes necessary for amino acid metabolism. For

example, the obligately homofermentative lactobacilli lack the phosphoketolase enzyme, which explains their inability to synthesise the aromatic amino acid family and histidine from their precursors D-erythrose-4-phosphate and ribose-5-phosphate, respectively. This fermentative pattern could explain the degree of amino acid auxotrophy in the species *L. delbrueckii* subsp. *lactis*, which is prototrophic for glutamine, glycine, threonine, aspartic acid, asparagine, proline and alanine (Hebert *et al*., 2004).

Protease production is an inherent property of all organisms, but only those microbes that produce a substantial amount of extracellular protease have been exploited commercially. Of these, the lactobacilli have been the most widely exploited, owing to their biotransformation and caseinolytic properties (Gupta *et al*., 2002a). The basis of their use in the dairy industry stems from the fact that for optimal growth, these bacteria must be able to degrade milk proteins, since the concentration of free amino acids and peptides present in milk is not sufficient for the growth of LAB (Tsakalidou *et al*., 1999). Protein degradation and subsequent utilisation of the degradation products therefore requires a complex proteolytic system consisting of proteinases, peptidases and amino acid and peptide carriers. The structural components of the proteolytic systems are threefold, on the basis of their function: (1) proteinases that breakdown caseins to peptides, (2) peptidases that degrade peptides and (3) transport systems that translocate the breakdown products across the cytoplasmic membrane (Kunji *et al*., 1996; Liu *et al*., 2010) (Fig. 10.2). In addition to their role in bacterial growth in proteins such as milk, the proteinases also contribute to the development of texture and organoleptic characteristics of fermented milk products and may cause the release of bioactive peptides, which can contribute to health improvements beyond basic nutrition (Espeche Turbay *et al*., 2009). Research efforts aimed at understanding the proteolytic system of the lactics are therefore imperative, considering the numerous fields of technological application of lactobacilli proteases.

Our knowledge of the proteolytic system of the LAB is largely derived from studies with *Lactococcus* species. Among the LAB, the CEPs of *Lactococcus lactis*, subspecies

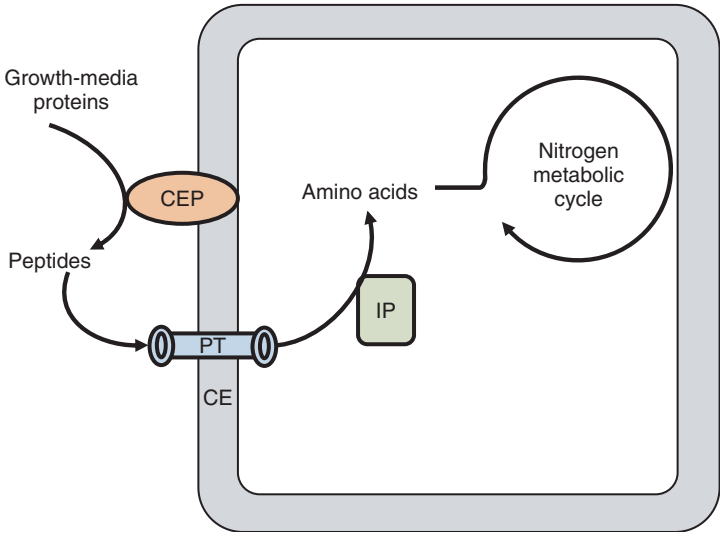

Fig. 10.2 Simplified structural schematic of the proteolytic system of lactobacilli. CEP, cell membrane-associated proteinase; PT, peptide transporters; IP, intracellular peptidases; CE, cell envelope.

SK11 and Wg2, have been the most extensively characterised, both genetically and biochemically, and are also the best documented (Kunji *et al*., 1996). These enzymes represent two of the several types of lactococcal CEP that have been distinguished on the basis of their specificity toward α-, β- and to a lesser extent κ-caseins. The primary substrates for CEP_I are β- and to a lesser extent κ-casein, while CEP_{III} uses αs_1-, β- and κ-caseins (Kunji *et al*., 1996; Oberg *et al*., 2002; Oommen *et al*., 2002). In addition to casein specificity, lactobacilli exhibit an extremely wide range of proteolytic activity, which is usually species- and/or strain-specific. Studies have also demonstrated that the proteinase enzymes from *Lactobacillus* spp. can be loosely grouped into a CEP_I/CEP_{III}-type classification (Kunji *et al*., 1996). A further, more detailed classification of CEPs into seven groups on the basis of their ability to cleave the chymosin-derived peptide α_{s1}-casein (f1–23) has also been proposed (Oberg *et al*., 2002).

10.4 SOURCES OF PROTEASES AND ADVANTAGES OF MICROBIAL PROTEASES

Enzymatic hydrolysis of proteins is the most common way of producing bioactive peptides (Korhonen & Pihlanto, 2006). This approach requires the two major raw materials: proteins and proteolytic enzymes. Byproducts of food protein processing, such as minced meat, fish meal, skins, bones, pulse cakes and protein wastewaters, are relatively cheap, thus their usage in the production of bioactive peptides will largely be conducive to a reduction in production cost (Agyei & Danquah, 2011). Alternatively, bioactive compounds may be formed from components present in the waste by microbial fermentation and/or enzyme hydrolysis, thus maximising profitability and providing an avenue for efficient waste disposal (Wilson *et al*., 2011; Yang *et al*., 2009). Proteolytic enzymes, on the other hand, are usually obtained from plants, gastrointestinal animals and microbial organisms. Microbial proteases obtainable from the *Bacillus* spp., *Bifidobacterium* and LAB are the most widely used for industrial processes (Ferrero, 2001) and present several advantages over proteases from other sources. The advantages of the lactobacilli proteinases include the following (Agyei & Danquah, 2011):

1. They are safe and nontoxic, which gives less concern for safety, especially when their products are intended for human food or drug-based application.
2. They have minimal nutritional requirements and a short maturation time for cells, which means a reduction in production costs.
3. Their production is influenced by culture growth conditions, making it possible to manipulate their yield and properties.
4. Their location in the lactobacilli cell, which is produced and borne on the cell-envelope surface, makes harvesting and enzyme purification relatively cheaper and less laborious.
5. They have a broad biochemical diversity of the microorganisms. Many of these can be subjected to genetic manipulation in order to improve enzyme yield. Recent developments in culturing procedures and taxonomic profiling of microorganisms have provided avenues to exploit different proteolytic enzymes produced by proteolytic starters and nonstarters.
6. Not only are the microorganisms diverse, so are their enzyme products. The plethora of proteases produced by lactobacilli offers varied enzymatic activities and specificities. There are the CEPs and a host of intracellular peptidases (endopeptidases,

aminopeptidases, tripeptidases and dipeptidases) (Khalid & Marth, 1990). Added to this, some *Lactobacillus helveticus* strains produce and express more than one CEP, with differences in protein cleavage patterns (Oberg *et al*., 2002). For the purposes of bioactive peptide production, the diversity in enzyme types and specificities ensures the generation of numerous peptides, each with unique potential bioactivities.

7. Microbial proteins have a longer shelf life and can keep longer under less than ideal conditions without significant loss of activity (Gupta *et al*., 2002a).

10.5 MARINE LACTOBACILLI

The marine environment is rich in nutrient and organic matter and thus supports microbial growth. Some lactobacilli are adapted to living in seawater, from which they can be isolated and subcultured (Rajaram *et al*., 2010). Others are usually found living in symbiotic relationships with other marine animals and are thus usually isolated from gut, muscles or shells. A large percentage of marine *Lactobacillus* species are detected in Pacific oysters (*Crassostrea gigas*) (Shiflett *et al*., 1966); the prevalent species include *L. paracasei*, *L. johnsonii*, *L. plantarum*, *L. pentosus*, *L. paraplantarum*, *L. parabuchneri* and *L. rhamnosus* (Lee *et al*., 2010). Marine-derived lactobacilli are a rich source of useful enzymes for food and pharmaceutical applications. Most are useful in the production of bacteriocins with antagonistic effect on fish pathogens (Lee *et al*., 2010). Captured in Table 10.2 are some bioactive peptides produced from purified proteinase or via lactobacilli fermentation. Among the lactobacilli, the proteinase system of *Lactobacillus casei* is the best studied (Khalid & Marth, 1990; Kojic *et al*., 1991; Tsakalidou *et al*., 1999). The proteolytic systems of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Laloi *et al*., 1991), *Lactobacillus sanfrancisco* CB1 (Gobbetti *et al*., 1996), *Lactobacillus helveticus* (Martín-Hernández *et al*., 1994) and *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC 178 and CRL 581 (Espeche Turbay *et al*., 2009; Tsakalidou *et al*., 1999) have also been studied. Other strains, such as *Lactobacillus delbrueckii* subsp. *lactis* 313 ATCC 7830, have been studied to a lesser degree (Agyei & Danquah, 2012b). Research aimed at fully characterising the proteolytic systems of all known lactobacilli is a major endeavour, as the achievement of high yields of bioactive peptides, under process and economic optimum conditions, hinges upon it.

10.6 PROTEINASE PRODUCTION REQUIREMENTS

10.6.1 Cell-Line Acquisition

The screening of proteinase-producing microorganism is the first step in obtaining microbial proteinases for industrial purposes. Although there are many microorganisms that produce proteases in nature, for industrial purposes it is convenient to find highly proteolytic strains. Some *Lactobacillus* proteinases have relatively broad specificity, resulting in a large number of possible cleavage sites on proteins (Oberg *et al*., 2002). For example, *L. helveticus* CNRZ32 (Blanc *et al*., 1993; Gilbert *et al*., 1997; Pederson *et al*., 1999; Sadat-Mekmene *et al*., 2011; Yamamoto *et al*., 1998) and *L. delbrueckii* subsp. *bulgaricus* ACA DC235 (Stefanitsi *et al*., 1995) have each been found to express two different cell-surface proteinases. Highly proteolytic strains or strains with peculiar specificities can

Table 10.2 Bioactive peptides produced from purified proteinase or via lactobacilli fermentation.

Peptide sequence/name	Bioactivity	Fermentation by/ proteinase sourced from	Reference
Tyr-Lys-Val- Pro-Glu-Leu	ACE inhibitory	*Lb. helveticus* CP790	(Murray and FitzGerald, 2007)
Ile-Pro-Pro	Antihypertensive	*Lb. delbrueckii* ssp. *lactis* CRL 581	(Hebert *et al.*, 2008)
Uncharacterized peptides	Immunomodulatory	*Lb. casei* strain GG + pepsin/trypsin	(Sütas *et al.*, 1996)
Tyr-Pro	Antihypertensive	*Lb. helveticus* CPN4	(Yamamoto *et al.*, 1999)
Tyr-Pro-Phe-Pro, A la-Val-Pro-Tyr-Pro-Gln-Arg, Thr-Th r-Met-Pro-Leu-T rp	Opioid, Antihypertensive, Immunostimulatory	*Lactobacillus GG* enzymes + pepsin & trypsin	(Korhonen and Pihlanto, 2006)
Val-Pro-Pro and Ile-Pro-Pro	Antihypertensive	*Lb. helveticus JCM1004*	(Pan *et al.*, 2005)
Ser-Lys-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile	Antihypertensive	*Lb. delbrueckii* ssp. *bulgaricus*	(Korhonen, 2009)
β-casein f184-210	Antibacterial activity	*Lb. helveticus* PR4	(Minervini *et al.*, 2003)
Ala-Arg-H is-Pro-His-Pro-H is-Leu-Ser-Phe-Met	Antioxidative	*Lb. delbrueckii* ssp. *bulgaricus* IFO13953	(Korhonen and Pihlanto, 2006)

f, fraction.

be identified by consulting the published literature. Strains for lactobacilli can be obtained from commercial organisations, academic groups and research institutes, or from a variety of culture collections such as the German Collection of Microorganisms and Cell Cultures (DSMZ), the American Type Culture Collection (ATCC), the National Collection of Industrial and Marine Bacteria (NCIMB) and the National Center for Agricultural Utilization Research (NCAUR), among others.

In microbial biotransformation, the production of bioproducts from wild strains of source organisms often does not support industrial-scale titres (Shirley, 1999). Moreover, the enzymes must have high catalytic activity and stability in an industrial environment. Therefore, molecular techniques may be required to improve proteinase production in the selected strains and thus engineer enzymes with the desired properties for all kinds of processes.

These are achieved by either conventional mutagenesis (ultraviolet (UV) or chemical exposure) or recombinant DNA technology (rDNA) to selectively generate mutants that exhibit higher proteinase yields (Gupta *et al*., 2002b). Over the past few decades, rDNA techniques have changed bulk enzyme production dramatically (Hodgson, 1994). The cloning of genes that encode key regulatory metabolic enzymes and proteinases should therefore be of special interest in attempts at genetic manipulation of lactobacilli. The main

objective of cloning bacterial enzyme genes has been the overproduction of enzymes for various commercial applications (Rao *et al*., 1998). The prospects for genetic manipulation of lactobacilli are bright, since some remarkable milestones have been achieved in developments of gene-exchange system via vector development, gene cloning, transfection, conjugation and transformation (Chassy, 1987). Other methods such as directed evolution are gaining popularity as biotechnological tools for the 'creation' of enzymes with useful desired properties (Hodgson, 1994; Otten & Quax, 2005).

10.6.2 Production (Growth) Media Selection

The expression of many extracellular products such as exopolysaccharides and proteinases, by lactobacilli is largely dependent on cell growth, which is directly linked with the composition of the growth medium. Most lactobacilli exhibit a complex nutritional requirement for growth and proteinase production, and usually consist of carbohydrate and nitrogen sources in the right ratio, vitamins, nucleotides, salts and other supplements. This nutrient requirement is usually satisfied during growth in complex growth media (containing peptone, meat and yeast extract, as well as other undefined compounds) or by the use of a chemically defined medium (CDM). A CDM is essential to the design of reproducible biochemical, physiological and genetic studies of cell growth kinetics, as well as the regulation of proteinases (Hebert *et al*., 2004). Despite these advantages, a typical CDM contains several constituents—usually over 20 nutrients—and has the added drawback of increased cost, since all the vitamins, minerals, bases and amino acids are used in extracted and purified forms. Thus, research aimed at studying the minimal growth requirements of lactobacilli is a worthy venture, as it will allow the development of lactobacilli growth media with small numbers of constituents and low costs.

10.6.2.1 Carbon Sources

The type and concentration of carbon in a growth medium are important variables that influences the proteinase activity of lactobacilli. Several authors have studied the utilisation of sugars by lactobacilli for numerous applications, and simultaneous carbohydrate utilisation has been demonstrated in a few different species (Chervaux *et al*., 2000; Kim *et al*., 2009, 2010; Schiraldi *et al*., 2003). However, little is known concerning the effect of the carbon source on the proteinase activity of lactobacilli, and the few studies that have been reported largely focus on genetic control mechanisms. Biochemical studies relevant to validating and confirming the link between carbohydrate metabolism and proteinase synthesis are scarce (Agyei & Danquah, 2012a).

When bacteria are exposed to a mixture of carbon sources they choose the substrate that yields the maximum profit for maximum survival (Titgemeyer & Hillen, 2002). The repression of secondary carbohydrate utilisation is achieved through several mechanisms, which are collectively termed carbon catabolite repression (CCR). This is controlled by inducer exclusion and genetic repression by the catabolite control protein (CcpA) and by repressor proteins (Kim *et al*., 2009). Genes encoding CcpA and CcpA-like proteins have been described from a number of lactobacilli and the genetic organisation of ccpA genes is always in the order of *pepQ-ccpA*, meaning that *pepQ*—which encodes a prolidase—and *ccpA* are always divergently transcribed (Mahr *et al*., 2000). The fact that *ccpA* in LAB is always linked to a divergently transcribed *pepQ* gene encoding a proline-specific peptidase suggests that expression of this and perhaps other *pep* or *prt* genes may be regulated by

CcpA and may therefore be coordinated with carbon regulation, thereby linking carbon utilisation to proteolysis (Titgemeyer & Hillen, 2002).

The amino acid auxotrophy of some *Lactobacillus* species is the basis of proteolysis in peptide-rich media and has been attributed to the lack of key fermentative enzymes in the sugar metabolic cycle. For example, the obligately homofermentative lactobacilli lack the phosphoketolase enzyme, which explains their inability to synthesise the aromatic amino acid family and histidine from their precursors D-erythrose-4-phosphate and ribose-5-phosphate, respectively (Hebert *et al*., 2004).

Catabolite control of PepQ has been demonstrated in *L. delbrueckii* subsp. *lactis* DSM 7290, where the enzyme activity was twofold higher with cells grown in lactose, as compared to in the presence of glucose (Schick *et al*., 1999). Agyei & Danquah (2012a) have also demonstrated in *L. delbrueckii* subsp. *lactis* 313 that different sugars stimulate the production of different cell-surface proteins, with a significant effect on cell proteinase activity. This shows that greater amounts of proteinase can be obtained from lactobacilli with minimal effort, simply by optimising medium carbon compositions.

10.6.2.2 Nitrogen Sources

The amino acid auxotrophy exhibited by most lactobacilli indicates that cell growth depends on an ability to produce enough proteinases by which to hydrolyse proteins in the growth medium to smaller units. Thus, by inference, profuse growth should be expected in a peptide-rich medium, with a subsequent increase in proteinase yields. However, studies have shown that this is not the case. For the lactobacilli, CEP activity levels are controlled by the peptide content of the growth medium. In their study with *Lactobacillus delbrueckii* subsp. *lactis* CRL 581, Hebert *et al*. (2008) observed that the maximum cell proteinase activity was observed in a minimal defined medium, whereas in the presence of casitone, casamino acids or yeast extract the synthesis of proteinase was inhibited 99-, 70- and 68-fold, respectively. Also, low-molecular-weight (<3 kDa) peptides extracted from casitone have been shown to significantly affect proteinase yields from *Lactobacillus helveticus* CRL 1062 (Hebert *et al*., 2000).

In another study, the proteinase activities of several strains of the thermophilic lactobacilli *L. delbrueckii* subsp. *lactis* and *L. helveticus* were remarkably reduced when cells were grown in peptide-rich medium MRS broth or in a CDM supplemented with casitone. However, when cells were grown in a synthetic medium containing free amino acids the proteinase activity was remarkably enhanced. Further, although peptides substantially affected the cell-envelope proteinase activities of thermophilic lactobacilli, they had no effect on the peptidase activity, and their effect on the proteinase was strain-dependent (Hébert *et al*., 2002). This observation was accounted for by the presence of uncharacterised repressing factors in MRS medium and casitone.

Several genetic control mechanisms have been studied using *Lactococcus lactis* as the model organism, in order to establish a link between nitrogen metabolisms and cell proteolyses in the LAB. The expression of the divergently transcribed genes (*prtP* and *prtM*) involved in proteinase production of *Lactococcus lactis* SK11 is controlled at the transcriptional level by the peptide content of the growth medium (Marugg *et al*., 1995). The genes *prtP* and *prtM* are required for the production of active serine proteases (Kok, 1990), and their level of expression was high in whey permeate growth medium containing relatively low concentrations of peptides (Marugg *et al*., 1995).

CodY is a pleiotropic transcriptional regulator conserved in lactobacilli and other low-G+C Gram-positive bacteria. Two distinct signals have been shown independently to

influence the activity of this regulator: the level of intracellular guanidine triphosphate, GTP (as in *Bacillus subtilis*), and the level of intracellular branched-chain amino acids (BCAA), isoleucine, leucine and valine (as in *Lactococcus lactis*). The difference in the function of CodY between *B. subtilis* and *L. lactis* seems to reflect the difference in the physiology between these two bacteria and thus their proteolytic properties (Petranovic *et al*., 2004). It has also been demonstrated that CodY is responsible for the repression of several transcriptional units of the lactococcal proteolytic system (including *prtP*), when cells are grown in the presence of rich nitrogen sources, such as casein hydrolysates, casitone or casamino acids (Hebert *et al*., 2008). The variation in activity of CodY as influenced by growth conditions such as nutrient availability demonstrates the effect of nitrogen source on cell proteolyses.

Further, in *Lactobacillus helveticus* the casein hydrolytic pattern (form polyacrilamide gel electrophoresis) differs between cells grown in peptide-rich media and those grown in milk, and this suggests that the biosynthesis of cell-surface proteinases with different cleavage specificities is medium-dependent and medium-induced (Gilbert *et al*., 1997). Thus, the type and concentration of nitrogen available in the growth medium has a significant effect on the genetic and biochemical pathways that lead to the production of proteinases in lactobacilli.

10.6.2.3 Carbon/Nitrogen Ratio

Because the type and concentration of nitrogen and carbon substrate in a growth medium influence the production of proteinases in lactobacilli, high productivities of proteinases require carbon and nitrogen to be present in the right ratio. The production of many other bioproducts in complex media has been shown to be dependent on the carbon/nitrogen (C/N) ratio, including exopolysaccharides (Degeest & De Vuyst, 1999), plasmids (Danquah & Forde, 2007), bacteriocins (Mataragas *et al*., 2004) and α-amylase (Singh *et al*., 2011). Usually, lower C/N ratios are beneficial for cell growth. An increase in C/N ratio increases proteinase production due to better cell growth, but this happens only until a certain plateau value is reached, after which proteinase yields begin to fall again. The decrease in proteinase yield at high C/N ratios is probably due to high osmotic stress, caused by a high carbon concentration. The C/N ratio and its effect on proteinase yield is an important parameter to consider in the design of growth media for proteinase production.

10.6.2.4 Metal-ion Requirement

Most proteolytic bacteria show variation in their requirement for salts for growth as well as in enzyme formation. Calcium (II) ions are important for proteinase synthesis in lactobacilli. Whereas Ca^{2+} content does not improve proteinase yield via metabolic mechanisms, it is important to the stability of proteinases during cell growth (Rahman *et al*., 2003). Proteinases in lactobacilli are cell-envelope bound and are therefore exposed to the susceptibility of degradation or shedding into the growth medium. Research has shown that calcium ions are directly involved in the active moiety of the enzyme to the anchor sequence (Coolbear *et al*., 1992). The functional involvement of calcium ions in the active conformation of the proteinase has been demonstrated and calcium ions have been shown to protect the enzyme against autoproteolytic release (Exterkate, 1995). The Ca^{2+} ions are weakly bound in proteinases and their removal initiates a structural rearrangement in the proteinase domain, causing an intramolecular autoproteolytic event which truncates the proteinase at the C-terminal end, leading to the release of the enzyme (Exterkate, 2000; Martín-Hernández *et al*., 1994; Kunji *et al*., 1996). Thus, the addition of

lower concentrations of Ca^{2+} in the growth medium of lactobacilli will inhibit proteinase degradation or autoproteolysis, since calcium plays a role in inducing and stabilising active conformations of the bound proteinases on cell surfaces (Coolbear *et al*., 1992; Exterkate, 1995; Siezen, 1999). Hebert *et al*., (2008) have used a minimal defined medium supplemented with 5 mM of $CaCl_2$ in characterisation studies of *Lactobacillus delbrueckii* subsp. *lactis* proteinases.

10.6.3 Process Optimisation for Growth and Proteinase Production

In addition to growth-media requirements, fermentation conditions such as incubation temperature, broth pH, agitation and the presence of oxygen also show a significant impact on proteinase synthesis.

10.6.3.1 Culture pH

As in most microorganisms, growth and enzyme production by lactobacilli are strongly dependent on extracellular pH (Espeche Turbay *et al*., 2009; Tsakalidou *et al*., 1999). Proteinase production by microbial strains is affected by extracellular pH via either pH-dependent control of protease gene expression (Young *et al*., 1996) or culture pH strongly controlling cell growth by influencing many enzymatic processes and transport of various components across the cell membranes (Ellaiah *et al*., 2002). The optimum pH range that promotes increased yields of proteinases is usually between 7.0 and 5.5, depending on whether fermentation occurred under controlled or uncontrolled pH conditions (Agyei & Danquah, 2012b; Espeche Turbay *et al*., 2009). The metabolic processes of most protease-producing microorganisms show a close relationship between proteinase syntheses, the utilisation of nitrogenous compounds and changes in pH. Thus, during fermentation, pH variation may indicate kinetic information about the enzyme production, such as the onset and consummation of the enzyme production (Kumar & Takagi, 1999). Moreover, there often exists a difference between the pH optimum for growth and that for proteinase production. This demonstrates that although bacterial growth and its enzyme production are greatly controlled by environmental conditions such as temperature and pH, the optimum conditions for enzyme synthesis and for optimum growth may differ (Das Mohapatra *et al*., 2009).

10.6.3.2 Incubation Temperature

Temperature affects all the physiological activities in a living cell and is an important environmental factor in the control of growth, microbial activities and the normal functioning of a cell's enzymes. The primary role temperature plays is to change the rates of the biochemical reactions necessary for cell growth; there is thus a link between enzyme synthesis and energy metabolism, which is controlled by temperature (Kumar & Takagi, 1999). The optimal temperature for growth of LAB varies between genera, from 20 to 45 °C (Panesar *et al*., 2010). For most bacteria there exists a linear relationship between the square root of the growth rate (μ_{max}) and the temperature (Fig. 10.3). This simple equation has been used successfully to predict the effect of temperature on the growth of a wide range of bacteria (Mackey & Kerridge, 1988; Membré *et al*., 2005). The temperature, obtained by extrapolation, at which the growth rate is zero is defined as T_o and may be a cardinal characteristic property of an organism growing under defined nutrient conditions (Mackey & Kerridge, 1988).

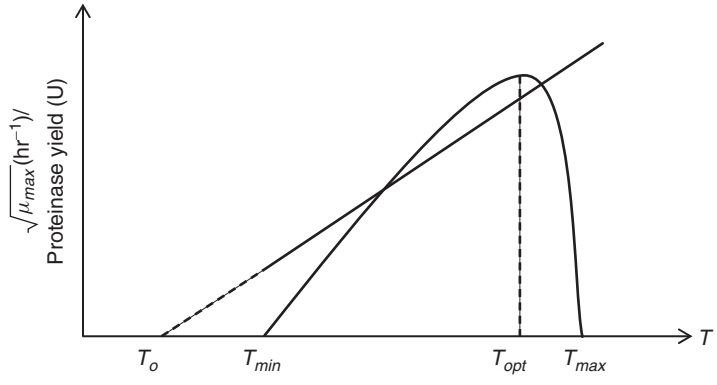

Fig. 10.3 Variation in the proteinase yield (parabola) and the square root of the growth rate, μ_{max} (straight line), as influenced by temperature of growth, T, for lactobacilli.

Further, proteinase activity increases linearly with temperature up to an allowed maximum and then decreases sharply (Fig. 10.3, parabola). Agyei *et al*. (2012) made this observation in their work with *L. delbrueckii* subsp. *lactis* 313. No proteinase activity was observed at temperatures 30 °C and below, whereas optimum proteinase yield was recorded at 45 °C. The decrease in proteinase activity beyond the optimum is likely due to thermal inactivation of biosystems at temperatures higher than the optimum; that is, the progressive enzyme distortion with respect to its biologically active conformation induced by thermal agitation (Perego *et al*., 2003; Sampaio *et al*., 2006). Therefore, although a high incubation temperature results in high cell growth rates, beyond the optimum temperature, T_{opt}, it does not necessarily give a higher protease yield. Because its effect on proteinases varies from organism to organism, critical points such as T_o, T_{opt} and T_{max} have to be elucidated for each organism in order to aid the control of proteinase via temperature manipulation.

The results of recent studies seem to suggest that inducing stress via fluctuating conditions such as osmosis, pH and temperature may improve bioproduct synthesis from bacteria. For example, temperature fluctuation has been used to improve the volumetric and specific yields of plasmid DNA by *E. coli* (Ongkudon *et al*., 2011). Proteinase synthesis, like plasmid synthesis, is induced and controlled by temperature, indicating that proteinase yields might also be improved via temperature-induction techniques. For example, heat-shock responses have been linked to the regulation of some peptidases in *E. coli* (Gottesman, 1996). Further, temperature fluctuation and its effect on the synthesis of inducible proteins have been demonstrated in lactobacilli such as *L. plantarum* (De Angelis *et al*., 2004) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Gouesbet *et al*., 2002). Consequently, temperature fluctuation and its effect on proteinase yield must be established for the different *Lactobacillus* species.

10.6.3.3 Gaseous Regime (Aeration)

LAB are generally phylogenetically intermixed with the aerobic and facultatively anaerobic genera of the of Gram-positive bacteria (Axelsson, 2004). The effect of a gaseous regime on proteinase production by lactobacilli is directly related to the bacterial growth at the indicated dissolved-oxygen level of the specified gaseous conditions. Usually, the dissolved-oxygen level of a fermentation culture is controlled by three mechanisms:

(1) varying the aeration rate; (2) varying the agitation speed of the bioreactor; or (3) using an oxygen-rich or oxygen-deficient gas phase as the oxygen source (Kumar & Takagi, 1999).

Differences in cell growth rate and cell density under different gaseous compositions have been demonstrated in several lactobacilli. For example, *L. plantarum* observes optimum growth under aerobic conditions (Murphy & Condon, 1984), whereas *L. delbrueckii* subsp. *lactis* 313 observes optimum growth under anaerobic conditions (Agyei & Danquah, 2012b).

During growth, lactobacilli adapt to various conditions and change their metabolism accordingly in order to favour pathways of efficient carbohydrate utilisation by altering pyruvate metabolism. During growth under microaerophilic and/or anaerobic conditions, the enzyme pyruvate-formate lyase catalyses the reaction of pyruvate and CoA to formate and acetyl CoA. This results in an increased overall ATP/glucose yield, as well as a change from the normal homolactic to a heterolactic-mixed acid fermentation, with consequent high culture acidification. This pathway is operational in several lactobacilli, such as *L. delbrueckii*, *L. plantarum* and *L. casei*, and results in high cell densities and high levels of organic acids, due to efficient glucose assimilation (Agyei & Danquah, 2012b; Kandler, 1983). However, in the presence of oxygen, reduced growth levels are observed for some lactobacilli. This has been attributed to the action of pyruvate oxidase, which is involved in the oxidation of pyruvate to acetyl phosphate, acetate and an equimolar amount of hydrogen peroxide (Hickey *et al*., 1983; Kandler, 1983). The low yields of ATP/glucose and the production and accumulation of toxic hydrogen peroxide in the culture medium lead to a slow growth rate and low cell density (Batdorj *et al*., 2007; Sakamoto & Komagata, 1996). Agyei & Danquah (2012b) have demonstrated that cell growth profile rate and proteinase-yield kinetics for cells grown under anaerobic conditions differ from those grown under microaerophilic conditions. The gaseous regime for the growth is therefore an important parameter to consider during proteinase production from lactobacilli.

10.6.3.4 Agitation Speed

For many lactobacilli, higher agitation speeds during growth generally result in higher biomass, due to generation of additional ATP and enhancement of fluid–particle mass transfer (Gupta *et al*., 2010). In addition to ensuring homogeneity of the fermentation broth, agitation also creates an aerobic milieu in the culture broth. Consequently, several studies have reported higher cell-biomass yields for lactobacilli grown at high agitation speeds or under micro-aeration conditions (Gupta *et al*., 2010; Murphy & Condon, 1984; Tango & Ghaly, 1999). However, Agyei & Danquah (2012b) have demonstrated that for *L. delbrueckii* subsp. *lactis* 313, vigorous agitation and other factors that promote aerobic conditions inhibit cell biomass yields. On the other hand, specific proteinase formation due to biomass (Yp/x) is significantly enhanced at higher agitation speeds of about 150 rpm. At high agitation speed, aeration of the culture medium is enhanced, which can lead to sufficient supply of dissolved oxygen in the media and/or enhanced nutrient uptake by bacteria, thus resulting in increased proteinase yields (Sepahy & Jabalameli, 2011). Thus, micro-aeration conditions favour proteinase synthesis, whereas inadequate aeration and nutrient uptake can be a cause of reduced proteinase yield. The effect of high culture agitation speeds on protease yields is well documented for several proteases that produce bacteria species (Potumarthi *et al*., 2007; Sepahy & Jabalameli, 2011), as well as some bacillus species (Saurabh *et al*., 2007). However, extremely high agitation speeds can be detrimental to proteinase yields, as seen in some studies (Agyei *et al*., 2012; Sepahy &

Jabalameli, 2011). Very high agitation rates can damage bacterial cells, thereby reducing proteinase yields. Also, at very high agitation speeds, CEPs are highly susceptible to autoproteolytic release into culture medium.

10.6.3.5 Inoculum Conditions

In food microbiology, the inoculum level significantly affects the changes in the physicochemical, biochemical and microbiological properties of food during fermentation (Visessanguan *et al*., 2006). In the same way, the proteinase yields of lactobacilli are greatly impacted by inoculum conditions such as density and age. Usually, proteinase yields increase with an increase in inoculum levels until a maximum point, beyond which they begin to decrease (Agyei *et al*., 2012). Seeding of cultures with low levels of inoculum often prolongs fermentation duration and achievement of high proteinase yields. On the other hand, very high inoculum levels also result in a decrease in the cell growth rate, perhaps due to the increasing limitation of key nutrients, and accumulation of greater amounts of growth-inhibitory metabolites, especially for cells at higher densities. A further explanation arises from reports that discuss cell–cell chemical signalling (quorum-sensing effects), which affects growth initiation under stressful conditions for cells at higher cell densities (Koutsoumanis & Sofos, 2005).

Moreover, inoculum can be conditioned to favour high proteinase yields either by using exponential phase cell as the inoculum or by preparing the inoculum in specialised media. Sakellaris & Gikas (1991) have reported that casein has the ability to stimulate proteinase production in pH-controlled fermentations of some *Lactobacillus* species. Thus, by preparing inoculum in casein, higher levels of proteinase yields can be induced from certain *Lactobacillus* species.

10.6.3.6 Time Course for Proteinase Production

For most microorganisms, the production of proteases exhibits a characteristic relationship with regards to the growth phase of the organism (Kumar & Takagi, 1999). Since lactobacilli are proteolytic for several amino acids, it follows that the synthesis of proteinase is largely constitutive (Gupta *et al*., 2002a). There are no or low proteinase yields during the lag phase. Proteinase production increases with cell growth until the middle-to-late exponential phase and then decreases gradually until the early stationary growth phase (Agyei & Danquah, 2012b; Tsakalidou *et al*., 1999). Some amount of enzyme activity is detected in the stationary phase, but it is usually low—the result of denaturation caused by prolonged enzyme exposure to very low pH (Espeche Turbay *et al*., 2009). Also, at the stationary phase, the protein machinery of a cell probably shifts from proteinase production to the production of stress-induced proteins such as surface-layer proteins (Agyei & Danquah, 2012b). Elucidating the proteinase production profile with cell growth is very important in identifying the ideal harvesting time for maximum proteinase activity.

10.7 EFFECT OF FERMENTATION MODES ON CELL GROWTH AND PROTEINASE PRODUCTION

The usefulness of lactobacilli in food processing and in the manufacture of bioproducts is largely dependent on the physiological state of the cells during fermentation, which is in turn influenced by the fermentation mode, growth conditions and growth parameters. It is therefore important to maintain proper control of starter production in order to prevent

poor fermentation yields and improve the product quality. This control may be achieved by studying the process parameters of the different fermentation modes (whether batch, fed-batch or continuous) and their effects on the growth kinetics of the bacteria and the cell physiological state during growth and product synthesis (Rault *et al*., 2009).

In batch fermentation, the materials required for fermentation are loaded on to the fermentor and sterilised before beginning the process in a partially closed system. The materials that enter and exit the system are the gas exchanges and pH control solutions. Products are removed at the end of the process. Economically, batch processes are easy to set up and maintain. If contamination occurs, only one batch is affected. Batch fermentation remains the most commonly used approach in most industrial-scale processes (Bouguettoucha *et al*., 2009). It is, however, characterised by substrate and product inhibition kinetics, which limits the growth rate and productivity of cultures (Boonmee *et al*., 2003; Rault *et al*., 2009). Also, product quality may vary with the different batches as conditions change with time and the fermentor is in an unsteady-state system.

With fed-batch fermentation, cells are first grown under a batch regime until a certain point in time (usually the attainment of the exponential growth phase), when the setup is fed with a solution of fresh substrates without removing the spent culture fluid. Fed-batch processes are useful in achieving a high product yield based on biomass over a relative large span of time.

For continuous fermentation, nutrients and substances (e.g. pH control solutions) are added and products and spent media or cells are removed continuously at rates at which organisms are held in the exponential growth phase. A steady state can be attained with a continuous fermentation mechanism which allows the determination of the relationship between microbial behaviour and the culture environmental conditions. The characteristic features of continuous systems—the continuous operation of the bioreactors at a dilution rate exceeding the maximum specific growth rate and the reduced inhibitory effects of the substrate, product or both—lead to improvements in the system's efficiency and increased productivity (Tyagi *et al*., 1992). The continuous fermentation mechanism is useful for reducing production costs and improving the process efficiency and product yield (Vasconcelos *et al*., 2004). Continuous production is more advantageous than the batch processes because the unproductive time employed in charging, discharging, cleaning, sterilisation and so on is avoided or minimised.

A number of studies have been published on improving lactobacilli biomass by exploiting novel bioreactor systems which permit media supplementation and/or the exchange of medium to prevent product or waste accumulation and therefore alleviate growth inhibition and achieve high cell density. However, these are largely optimised for products like lactic acid, and not for proteinases. Consequently, the literature is very scanty on the effects of different fermentation modes on proteinase production in the *Lactobacillus* species.

Fermentation activities for most of the literature references used in this chapter were conducted in batch mode (e.g. Agyei & Danquah, 2012b; Espeche Turbay *et al*., 2009; Hebert *et al*., 1997, 2004, 2008; Tsakalidou *et al*., 1999) but the focus of these studies was not on comparing fermentation modes and their effects on proteinase yields. On the other hand, some literature exists for *Lactococus lactis*, which can be approximated for the lactobacilli. Marugg *et al*. (1995) have reported that the expression of *prtP* gene promoter is only affected marginally by the growth rate for batch cultures of *Lactococus lactis* SK11. However, in continuous cultures at increasing dilution rates ($0.05 < D < 0.5\,h^{-1}$), a maximally threefold decrease in *prtP* expression levels was observed.

Laan *et al*. (1993), on the other hand, observed a different growth-rate dependency for *Lactococus lactis* Wg2 proteinase gene expression, where the expression level in continuous cultures was found to be maximal at a dilution rate of $0.23\,h^{-1}$ but decreased at higher dilution rates. This implies that the different fermentation modes trigger different responses in proteinase synthesis levels among the LAB. In order to scale up proteinase production from lactobacilli to the industrial level, biochemical engineering strategies need to be applied to obtain high yields of proteinases in a bioreactor. The literature is replete with bioreactor systems and fermentation modes that have been used to improve the yields of products other than proteinases. These include various stirred-tank reactors (STRs), cyclone reactors, gas-lift reactors, shaken ceramic flasks, compact, submerged membrane bioreactors (Ramchandran *et al*., 2012) and fed-batch, semi-batch and chemostat fermentations. Research studies are needed to establish the kinetics of proteinase synthesis by lactobacilli for these systems.

10.8 CELL SYSTEMS FOR PROTEINASE PRODUCTION

Microbial products are usually produced by either free or immobilised cells and the use of immobilised cells as industrial catalysts offers more advantages than batch fermentation processes (Adinarayana *et al*., 2005). The use of immobilised biocatalysts (whole cells or enzymes) is advantageous because such biocatalysts display better operational stability, higher catalysis efficiency and higher cell density, as well as allowing reusability, during continuous fermentation (Adinarayana *et al*., 2005; Norton *et al*., 1994). Research information is abundant on the use of immobilised systems for the production of *Bacillus* proteases (Gupta *et al*., 2002b), as well as for the production of lactic acid from lactobacilli (Norton *et al*., 1994). However, in spite of the considerable commercial interest, relatively little work has been carried out on this subject (Alekseiva *et al*., 1998). *Lactobacillus* cell morphology reveals that the proteinases are cell envelope-bound and not strictly 'extracellular' products, and thus do not lend themselves to production by immobilised cells. The merits of the use of immobilised cells ought therefore to be weighed against the demerits. The cell system which incorporates lower costs and higher proteinase yields would be preferred.

10.9 STATISTICAL METHODS AND MATHEMATICAL MODELS

The conventional sequential method used for optimisation studies is often cumbersome, tedious and time-consuming, and requires a large number of experiments when a large number of parameters are being studied. In addition, it does not consider the effect of different interactions of various parameters (Vellanki *et al*., 2009). The use of statistical designs, however, applies a mathematical framework that covers all experimental factors, with a minimum number of experiments required to achieve useful results (Beg *et al*., 2002). Using an appropriate statistical approach, optimisation can be carried out by the simultaneous control of many factors (fermentation conditions and process parameters). For example, the Plackett–Burman factorial design is often used in preliminary studies to select variables that can be fixed or eliminated in further optimisation processes. It allows for the screening of main factors from a large number of process variables

(Reddy *et al*., 2008). Response-surface methodology, on the other hand, is used in many biotechnology processes to evaluate and study the interactions between different process parameters (Vellanki *et al*., 2009). Mathematical models also exist to study microbial growth or the kinetic constants of microbial enzymes (Beg *et al*., 2002). These offer better understanding of the fermentation process and its optimisation. The Gompertz model is an efficient mathematical method of quickly estimating and describing the microbial growth parameters in easy-to-understand and technologically relevant terms (Tomás *et al*., 2010; Zwietering *et al*., 1990). Other models, such as Luedeking–Piret, provide both a 'growth-associated' and a 'non-growth-associated' term for product yield (Luedeking & Piret, 2000). They were originally designed for products such as lactic acid but can be modified and extended for proteinase production mechanisms in lactobacilli. The use of properly designed models with multifactor analyses will help the design and scale-up of proteinase yields.

10.10 CONCLUSION

Proteinases are an important biocatalyst for the production of biotechnological products such as bioactive peptides, and microbes represents the preferred source of these enzymes due to their rapid growth, the limited space required for their cultivation and their genetic pliability. The vast diversity of lactobacilli proteinases represents an industrially feasible avenue for exploitation in biotechnological applications. However, continued improvement of proteinase yields will depend on a number of factors: identification of highly proteolytic strains, improvement of proteinase yields via optimisation of media-component and process parameters and efficient product recovery. Successfully addressing these issues will require a multidisciplinary approach, encompassing fields such as microbiology, statistics and mathematical modelling and bioprocess engineering.

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11 Production of Lactobacilli Proteinases for the Manufacture of Bioactive Peptides: Part II — Downstream Processes

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11.1 INTRODUCTION: CELL RECOVERY

The various process engineering techniques necessary to increase proteinase synthesis by lactobacilli have been discussed in Chapter 10. This chapter deals with the remaining downstream processes: recovery, isolation and purification strategies.

After fermentation is complete, the cells can be separated from the culture medium via centrifugation using continuous disc centrifuges or filtration using vacuum rotary drum filters. In order to enhance the separation, the cells can be flocculated by flocculating agents, which neutralise the charges on the microbial cell surfaces and alter the ionic environment. This aids the formation of larger flocs or agglomerates, which in turn accelerate solid–liquid separation. The cell flocculants commonly used are organic polyelectrolytes, inorganic salts and mineral hydrocolloids (Kumar & Takagi, 1999). Following this, harvested cells are washed with appropriate buffer, such as sterile phosphate-buffered saline or 0.15 M saline supplemented with 10–20 mM $CaCl_2$. The resulting cell pellet is then ready for the subsequent stages of the process, in which proteinases are extracted.

It is important that the cell-recovery stages be undertaken under low-temperature conditions. The lowering of the temperature to below 5 °C helps prevents microbial contamination, as well as maintaining enzyme activity and stability (Kumar & Takagi, 1999).

11.2 ISOLATION: PROTEINASE-EXTRACTION METHODOLOGIES

Usually, no cell disruption is necessary to recover proteinases from lactobacilli. That is because during lactobacilli fermentation, the proteinases are expressed and anchored on to the cell membrane. This is why the proteinases are referred to as cell envelope-associated proteinases, or cell envelope-bound proteinases, cell wall-bound proteinases, cell-surface proteinases (generally abbreviated as CEPs) or lactocepins (Siezen, 1999). Their position on the cell surface makes harvesting and purification of the proteinases relatively less laborious and cheaper (Agyei & Danquah, 2011). The CEPs in bacteria have

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been shown to display five different functional multidomain structures on the cell surface; beginning at the N-terminus, these are PrtP (present in *Lactococcus lactis*), PrtB (present in *Lb. delbrueckii* subsp. *bulgaricus*), PrtH (present in *Lb. helveticus*), ScpA (present in *Streptococcus pyogenes*) and Csp (present in *Streptococcus agalactiae*) (Gilbert *et al*., 1997; Pastar *et al*., 2003; Siezen, 1999).

The prepro domain (PP domain) has been shown to be important for the secretion and activation of proteinase, whereas the PR domain is the catalytic serine protease domain, which comprises several sequences homologous to subtilases and an internal domain (I domain). The large A domain common to all CEPs immediately follows the PR domain. The B domain is found only in the lactococci and lactobacilli, and the helix spacer domain (H domain) is present in PrtP and PrtH following the A domain. The W domain (cell-wall spacer) is present in all five types of proteinase. The W domain of PrtP and streptococcal CEPs precedes a typical cell-wall anchor (AN domain), while PrtH and PrtB lack the C-terminally positioned AN domain and bind the cell wall by means of the W domain itself (Pastar *et al*., 2003; Siezen, 1999).

Generally the total cell envelope-associated proteins in the lactobacilli consist of ribosomal proteins; permeases of the glycolytic pathway (Sánchez *et al*., 2009); the crystalline surface-layer (S-layer) proteins, which are responsible for cell protection, adhesion and cell-surface recognition (Deepika *et al*., 2009; Lortal *et al*., 1992; Schar-Zammaretti *et al*., 2005); and the CEPs responsible for hydrolysis of proteins to peptides (Espeche Turbay *et al*., 2009; Tsakalidou *et al*., 1999).

Elucidating the most suitable method for the extraction of cell-envelope proteinases from lactobacilli is an important undertaking as the use of a particular extraction agent produces yields that are species- and/or strain-dependent, and no single method works for all lactobacilli. A suitable method should release large quantities of CEPs of high specific activity and a low degree of cell lysis, in order to avoid contamination of CEPs with intracellular enzymes. The mechanism of action of the different CEP-extracting agents is based on their ability to disrupt the molecular interactions anchoring cell-surface proteins (including proteinases) to the cell envelope, such as the S-layer, peptidoglycan layer and phosphlipid bilayer. Some of the methods used for the extraction of CEPs from lactic acid bacteria (LABs) are shown in Table 11.1. Broadly, the widely used extraction methods for LABs consist of: (1) Ca^{2+}-free buffers such as Tris–HCl or phosphate-buffered saline; (2) low-concentration detergents such as sodium dodecyl sulfate (SDS); (3) chaotropic agents such as LiCl and guanidine hydrochloride; and (4) enzymes (muramidases).

11.2.1 Ca^{2+}-Free Buffers

The most extensively used method for releasing CEPs from LAB cells is to wash or incubate the cells in a calcium-free buffer (Exterkate, 1990; Fira *et al*., 2001; Tsakalidou *et al*., 1999). It has been observed that the release of lactococcal CEPs from the cell surface occurs spontaneously in a Ca^{2+}-free buffer. The removal of relatively weakly bound calcium in CEP initiates a structural rearrangement in the proteinase domain. This causes an intramolecular autoproteolytic event, which truncates the proteinase at the C-terminal end, causing the release of the enzyme (Exterkate, 2000; Kunji *et al*., 1996; Martín-Hernández *et al*., 1994). However, although this method is simple and gives low levels of cell lysis (Tsakalidou *et al*., 1999), it leads to a reduction in the enzyme activity and thermal stability of the CEP released (Exterkate, 2000). Moreover, Martín-Hernández *et al*.

Table 11.1 Agents for the extraction of surface proteins from some lactobacilli species.

Agent	Conc.	Incubation		LAB species studied	Reference
		Time (min)	Temperature (°C)		
Guanidine-HCl	4 M	30	37	*L. acidophilus* W	Bhowmik *et al.* (1985)
Sodium dodecyl sulfate	0.2% (w/v)	30	37	*L. acidophilus* W	Bhowmik *et al.* (1985)
Urea	8 M	60	37	*L. acidophilus* W, *L. rhamnosus* (strains E/N, Oxy, and Pen)	Bhowmik *et al.* (1985), Jarocki *et al.* (2010)
Lithium Chloride	1 M, 5 M	60	30	*L. rhamnosus*	Jarocki *et al.* (2010)
Glycine (pH 2.2)	0.2 M	15	4	*L. fermentum* BR11	Turner *et al.* (1997)
Na-phosphate buffer (pH 7)	50 mM	120	30	*L. delbrueckii* subsp. *lactis* ACA-DC 178	Tsakalidou *et al.* (1999)
Lysozyme in buffer	800 U/ml			*L. rhamnosus* GG	Sánchez *et al.* (2009), Macedo *et al.* (2003)
NaOH	10 mM	30[a]	37	*L. rhamnosus* GG	Sánchez *et al.* (2009)
Mutanolysin (with or without lysozyme)	42 U/ml			*L. plantarum*	Macedo *et al.* (2003)
Lysozyme + sucrose in NH_4CO_3		30[a]	37	*L. rhamnosus* GG	Sánchez *et al.* (2009)

[a]Incubation done with gentle agitation.

(1994) observed that no CEPs are released via washing or incubation of *L. delbrueckii* and *L. casei* cells in calcium-free buffer, unless the cells were treated previously with lysozyme. In their work, Atlan *et al*. (1989) observed that repeated washing of *L. bulgaricus* CNRZ 397 cells with Ca^{2+} buffer could not release a metallo-enzyme from the cell wall, and they explain that this is due to the rather close and tight association of the proteinase with the peptidoglycan wall (Laloi *et al*., 1991). In another study, the activity, release and stability of CEP from *L. delbrueckii* subsp. *lactis* CRL 581 were not affected by the presence of calcium ions (Espeche Turbay *et al*., 2009). This indicates that the effect of Ca^{2+} on CEPs is strain-dependent. This property is probably dependent on the structure of the CEP domains on the cell surface (Siezen, 1999).

11.2.2 Chaotropic Agents

Chaotropic ions favour the transfer of apolar groups to water and thus provide a means for the resolution of proteins and for increasing the water solubility of particular proteins (Hatefi & Hanstein, 1969). The action of chaotropic agents is related to their effect on the structure and lipophilicity of water, since they dissociate aggregated proteins by increasing the solubility of hydrophobic regions of proteins in aqueous environments (Clinkenbeard *et al*., 1995). Thus chaotropic agents interfere with noncovalent interactions between surface proteins and bacterial surfaces.

11.2.2.1 Urea

Microbial cell-surface proteins can also be extracted by employing the chaotropic properties of detergents and salts. For example, Jarocki *et al*. (2010) observed that the use of urea releases large quantities of surface-associated proteins from *L rhamnosus*. Urea is a strong protein denaturant and disrupts the noncovalent bonds in the proteins, thus working through a solvation mechanism. Urea exerts its effect directly by binding to the protein and/or indirectly by altering the solvent environment (Bennion & Daggett, 2003). Direct urea interactions involve hydrogen bonding to the polar moieties of the protein; particularly peptide groups of the S-layer proteins and peptidoglycan layer. Subsequently, this leads to disruptions of intramolecular hydrogen bonds of the protein. Since urea acts on both the S-layer and the peptidoglycan layer, it has been demonstrated to exhibit the best results with the highest mean activity and specific activity (Hua *et al*., 2008). In comparison with other extraction agents, urea is known to release larger quantities of cell surface-associated proteins without significantly affecting the morphological structure or viability of cells (Jarocki *et al*., 2010).

11.2.2.2 Lithium Chloride

Lithium chloride is a mild extraction agent and predominantly attacks only the surface layer. The lithium ions attack the S-layer protein subunits, which are noncovalently linked to each other, and disintegrates them into monomers by cation substitution (Schär-Zammaretti & Ubbink, 2003). Sánchez *et al*. (2009) have shown treatment of *Lb. rhamnosus* GG (LGG) cells with 5 M LiCl to be the most suitable method for the extraction of surface proteins in LGG, since this allows the extraction of some additional cell surface-associated proteins compared to other extraction methods. The use of LiCl is an established method for selective surface-molecule solubilisation with maintained cell integrity (Hussain *et al*., 1999).

11.2.2.3 Guanidine Hydrochloride

Guanidine–HCl is a chaotropic agent that denatures proteins by disrupting their secondary structure, leading to the formation of random coils, which become further displaced by guanidine ions. The guanidinium ion is a strong base with a pKa of 13.6 (Perrin, 1972). Consequently, it is able to disrupt the noncovalent interactions between S-layer monomer units. Because it is such a strong base, the solution may also hydrolyse the glycoproteins of the S-layer monomer, peptidoglycan layer and phospholipid bilayer through hydroxide nucleophilic attack of the carbonyl group in the peptide linkages. Bowmik *et al*., (1985) have remarked that guanidine hydrochloride solution is the best extraction agent by which to obtain large quantities of cell-surface proteins from *L. acidophilus* strains. Guanidine–HCl is also used in medical microbiology extraction of indicator proteins from the cell walls of pathogenic microorganisms, due to its protein-dissolution properties (Russell & Facklam, 1975).

11.2.2.4 Glycine

Glycine is the smallest amino acid, with an isoelectric point of 6.06. At a pH of 2.2, the solution mostly contains the cationic acid ion of glycine (Brown & Poon, 2010). Since it is a polar cationic molecule, it is thought to disrupt the S-layer protein-monomer noncovalent interactions. Glycine at 0.2 M has been used in the preparation of a virtually pure 32 kDa protein from *Lb. fermentum* BR11 (Turner *et al*., 1997). However, glycine at the same concentration is unable to release cell-surface proteins from *Lb. fermentum* 104R (Rojas *et al*., 2002). This shows that some of the extraction agents are very strain-specific.

11.2.3 Low-Concentration Detergent

11.2.3.1 Sodium Dodecyl Sulfate

SDS is an anionic denaturing agent and is thus capable of disintegrating the S-layer protein subunits of lactobacilli cell-surface proteins into monomers (Schär-Zammaretti & Ubbink, 2003). It denatures secondary and non-disulfide-linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. This causes a breakdown of the cell membrane due to emulsification of surface lipids and proteins and the disruption of the polar interactions that hold it together (Brown & Poon, 2010). Based on its mechanism of action, SDS can be quite a harsh extraction agent and may irreversibly denature proteinases when used at higher concentrations. It is generally effective at concentrations between 0.1 (Bhowmik *et al*., 1985) and 2% (v/v) (Chagnaud *et al*., 1992). The use of SDS in proteinase extraction from lactobacilli cells may require an initial physical or chemical treatment to enhance extraction efficiency. For example, Chagnaud *et al*. (1992) have reported that extraction of cell-surface proteins from lactobacilli with SDS alone shows improved effectiveness if the cells are sonicated prior to SDS treatment.

11.2.3.2 Triton X-100, CHAPS and DTT

Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) are milder detergents that are not effective in releasing large quantities of cell-surface protein but are however useful for retaining the protein's native conformation during subsequent purification steps (Bhowmik *et al*., 1985; Wu *et al*., 2009). This property makes them useful additives in mixtures of extraction agents.

11.2.4 Use of Enzymes (Muramidases)

Proteinase extraction has also been achieved via the hydrolytic properties of lysozyme and/or mutanolysin for the hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan cell wall. This causes the release of all cell wall-bound proteins. Atlan *et al*. (1990) obtained an excellent yield of proteinases from *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 when they coupled lysozyme/mutanolysin treatment with cold-temperature shock, and the dual treatment did not cause significant cell lysis. However, based on the mechanism of their action, the use of muramidases without proper control of process conditions (such as incubation time and temperature) could result in significant levels of cell lysis. Another downside to the use of lysozyme is that lysozyme exhibits an antibacterial activity against some Gram-positive bacteria. This antimicrobial activity is independent of its enzymatic activity (McKenzie & White, 1991).

11.2.5 Use of a Cocktail of Extractant

Proteinases can also be solubilised with cocktails or combination of two or more extraction agents. The use of a combination of methods provides the advantage that the demerits of one component can be counterbalanced by the merits of another. There is also the possibility of synergistic action between the components, which can enhance activities and thus reduce overall cost. Rojas *et al*. (2002) have used 1 M lithium chloride–lysozyme solution (comprising 0.1 M Tris, 0.015 M NaCl, 0.05 M $MgCl_2$ and 40 μg/ml of lysozyme) to extract cell-surface proteins from *L. fermentum* 104R. Sánchez *et al*. (2009) have also used lysozyme buffer (containing 800 U/ml lysozyme and 0.5 M sucrose in 30 mM ammonium bicarbonate) to release cell surface-associated proteins from *L. rhamnosus* GG.

11.2.6 Factors Affecting Enzyme Extraction

11.2.6.1 Cell Lyses

During the extraction of proteinases, cell lyses leading to the release of intracellular substances is a nuisance and must be kept to the allowed minimum. This is because intracellular enzymes contaminate, and may also compromise, the activity of crude proteinases. They also increase the cost of proteinase purification. Assessment of the performance of an extraction agent should include its effect on cell lysis. The release of intracellular enzyme is characterised by perforation or lysis of the cell during enzyme extraction. Cell lysis or cell death is quantified by measuring the activity of one or more intracellular enzyme(s), such as lactate dehydrogenase, β-galactosidase or intracellular peptidases (such as lysyl-aminopeptidase). Cell lysis can also be verified by running and comparing the SDS-PAGE profile of cell-surface extracts and total cell protein extracts. Moreover, viable bacterial counts made before and after extraction treatments will give an indication of cell death.

11.2.6.2 Incubation Time, Temperature and pH

During extraction, the pH of extractant, the incubation temperature and the time are important factors to control, for economic reasons. For example, too long an incubation time will contribute to a high processing cost. Further, the efficiency of an extractant depends partly on these factors. The native chemical and functional properties of proteins

imply that interaction with solubilising agents is largely influenced by temperature and pH, which, if uncontrolled, could result in a compromise of the activity and stability of proteinases.

All extraction agents are used at an optimal temperature, which is usually equal to or less than the fermentation temperature. Other authors have reported the use of cold shock to aid the release of proteinase in *L. delbrueckii* subsp. *bulgaricus* (Atlan *et al*., 1990).

It has also been reported that the attachment of some cell-surface proteins to viable cells is pH-dependent, and release of surface proteins into the bacterial surroundings is pronounced at neutral or slightly alkaline pH (Antikainen *et al*., 2007; Sánchez *et al*., 2009). This pH dependency for the attachment/release of cell surface-bound proteins implies that extraction agents must be used at the optimal pH; that is, the pH at which protein solubilisation is enhanced and enzyme activity is maintained.

11.3 PURIFICATION OF ENZYMES

Captured in Table 11.2 are a number of proteinases from different lactobacilli and a summary of their purification techniques. There are challenges for the bioprocess industry in downstream processes for the separation and fractionation of proteins, which are due to the complexity of biosuspensions, the selectivity and low filtration velocity of targeted molecules and the low concentration of product (Saxena *et al*., 2010). Also, in protein purification, not only should a downstream purification scheme achieve the required purity and recovery levels of the desired product in a safe, reliable and reproducible manner, but this must be done in an economically viable and cost-effective manner (Desai *et al*., 2000). This requires the exploitation of several alternative purification routes (Fig. 11.1) and selection of the most optimal for the proteinase of interest.

Often, the enzyme activity for crude cell-free extract is low. It is therefore important to concentrate the extract down via the removal of water. This is achieved by membrane separation technologies such as ultrafiltration (UF), which is used largely for the recovery of enzymes (Kumar & Takagi, 1999). Other techniques for protein concentration include salting out with sodium sulfate or ammonium sulfate (Gupta *et al*., 2002a; Kumar & Takagi, 1999; Macedo *et al*., 2003) and the use of organic solvents such as acetone (Kumar & Takagi, 1999) and ethanol (Gupta *et al*., 2002a). Enzyme precipitation can also be achieved by the use of water-soluble, neutral polymers such as polyethylene glycol (Kumar & Takagi, 1999). Through these concentration steps, some level of purification is also achieved.

Primary and intermediate purification stages are performed on clarified and/or concentrated product for the purpose of removing impurities such as media components, DNA, viruses and endotoxins. The actual purification of the enzyme requires a combination of one or more techniques, usually high-performance tangential flow filtration (HPTFF), high-performance liquid chromatography (HPLC) and UF techniques. Other methods, including foam fractionation, aqueous two-phase systems and dye-ligand chromatography, have been employed on a small scale and are currently awaiting scale-up (Gupta *et al*., 2002a).

The purification process is finally completed by the polishing stage, in which trace impurities and contaminants are removed, leaving behind an active and safe product suitable for formulation or utilisation.

Table 11.2 Characterization of proteinases of lactobacilli.

Strain	Molecular weight	Structure/ proteinase type	CEP type	Purification technique(s)	Optimum pH	Optimum temperature (°C)	Metal-ion inducers	Reference
Lb. delbrueckii subsp. *lactis* ACA-DC 178	–	Serine- proteinase	Type I	No purification — crude proteinase used	6	40		Tsakalidou *et al.* (1999)
Lb. casei NCDO 151	–	Serine proteinase		DEAE-sephacel ion-exchange chromatography	7	40		Ezzat *et al.* (1988)
Lb. casei NCDO 151	–	Cysteine proteinase		DEAE-sephacel ion-exchange chromatography	7.5	45	–	Ezzat *et al.* (1988)
Lb. helveticus	–	Serine proteinase	–	Sephacryl gel-filtration chromatography	7	40		Ezzat *et al.* (1993)
Lb. casei HN14		Serine proteinase	Type I	No purification — crude proteinase used	–	–	–	Kojic *et al.* (1991)
Lb. paracasei subsp. *paracasei* NCDO 151	135 kDa and 110 kDa[a]	–	–	Anion-exchange; hydrophobic interaction; chromatofocusing; gel-filtration chromatography	–	–	–	Nas & Nissen-Meyer (1992)
Lb. casei NCDO 151	150 kDa	Serine proteinase	–	(Mono Q HR 5/5) Anion-exchange FPLC; chromatofocusing (Mono P, HR 5/20);	5.6 (with casein as substrate)	35–37	Ca^{2+}, Co^{2+}	Næs *et al.* (1991)
Lb. delbrueckii ssp. *bulgaricus* CNRZ 397	170 kDa	Cysteine proteinase		Ultrafiltration; ion-exchange chromatography	5.5	42		Laloi *et al.* (1991)
Lb. helveticus L89	180 kDa	Serine proteinase	Type I/III mixed-type variant	Hydrophobic interaction chromatograph; diafiltration	7	50	–	Martín-Hernández *et al.* (1994)
Lb. helveticus CP790	45 kDa	Serine proteinase	Type III	Ion-exchange chromatography	6.5	42	–	Yamamoto *et al.* (1993)
Lb. rhamnosus BGT10	154 kDa	Serine proteinase	–	No purification — crude proteinase used	6.5	–	–	Pastar *et al.* (2003)
		Serine proteinase		Ion-exchange gel filtration	7.5–8	42		Zevaco & Gripon (1988)

[a]As determined by SDS-PAGE.

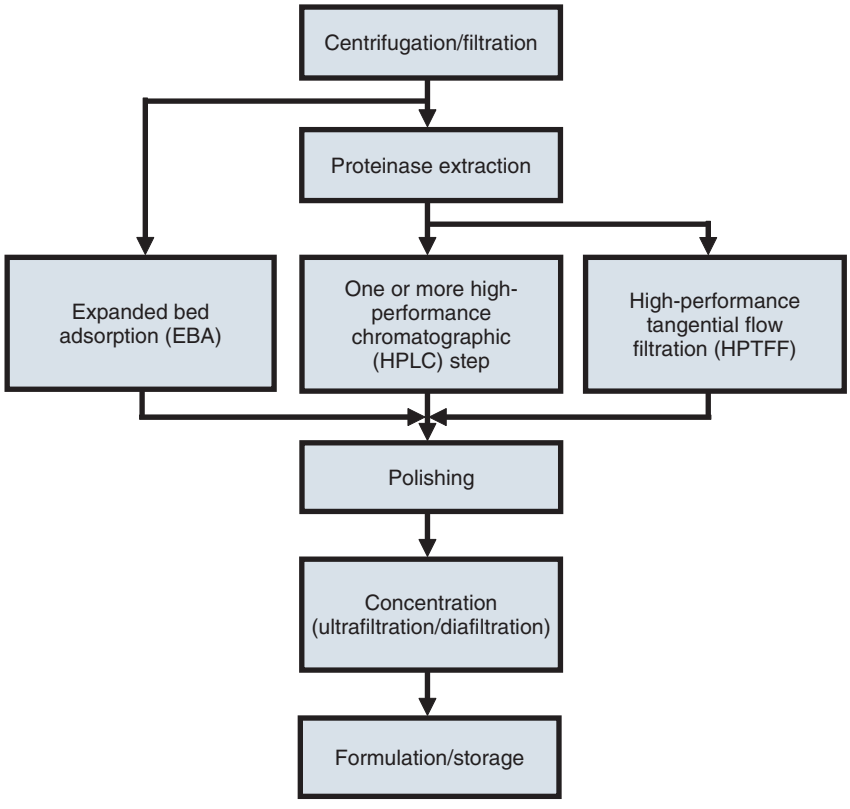

Fig. 11.1 Schematic of downstream purification operations.

11.3.1 High-Performance Tangential Flow Filtration

HPTFF is a highly selective technology that can be used to separate charged species of similar or different size using semipermeable membranes (van Reis *et al*., 1997, 1999). It is a two-dimensional purification method that exploits differences in both size and charge characteristics of biomolecules. HPTFF technology can provide protein concentration, buffer exchange and purification in a single-unit operation; processes that are performed using a combination of quite a number of separate chromatography and UF steps. Also, by the control of filtrate flux and device fluid mechanics, this technique eludes to a great degree the problem of fouling. The performance of HPTFF has been shown to be reproducible and robust. Usually, the charged membrane used in HPTFF can be regenerated and reused many times without loss of selectivity or throughput. There are also the added advantages of the elimination of one or more chromatographic steps, yield improvement and a significant reduction in the cost of product purification (Lebreton *et al*., 2008).

It has been demonstrated that significant improvements in performance and maximal protein selectivity in HPTFF can be achieved simply by controlling buffer pH and ionic strength. Saksena & Zydney (1994) have shown that selectivity for the filtration of bovine serum albumin (BSA) and immunoglobulin G (IgG) is increased from a value of 2 to more than 30 simply by adjusting the pH from 7 to 4.7 and lowering the solution ionic

strength. Van Reis *et al*. (1999) demonstrated the possibility of exploiting the membrane charge to further enhance performance. They reported, in a study in which the effects of membrane charge and buffer pH were explored, that purification factors up to 990-fold were obtained with yields of 94%. Further, Rao *et al*. (2007) have demonstrated the ability to use small charged affinity ligands with biospecific binding characteristics to enhance protein separation by HPTFF. HPTFF therefore lends itself as a promising technique to proteinase purification, based on its success rates in the purification of other protein systems. Further, based on the aforementioned advantages and high throughput values, HPTFF can be used in initial, intermediate and final purification stages and can easily be utilised in even industrial-scale systems with reduced production costs (van Reis *et al*., 1999).

11.3.2 High-Performance Liquid Chromatography

Although protein separations can be achieved by a variety of techniques, chromatographic separations are by far the most widely used (Jones & Baines, 2000; Kennedy, 2001). Liquid-column chromatographic techniques have been used for many decades in analytical biochemistry. The introduction of HPLC in the mid-1970s saw the use of extremely high pressures and smaller-particle packing materials. This development has completely revolutionalised liquid chromatography, improving efficiency, purification, separation, identification and quantification, as well as reducing analysis time and allowing for the separation of samples at the microscale (MacNair *et al*., 1997). In HPLC, the different interactions and/or binding mechanisms between a solid-phase resin and the target molecule in the feedstream affect the separation or purification of biomolecules (Desai *et al*., 2000). The well-known HPLC types and their potential in the separation of proteinases are described in this section.

11.3.3 Ion-exchange Chromatography

Ion-exchange chromatography (IEC) is one of the most commonly used HPLC modes. Due to its rapid separation, easy sanitisation, lack of organic-solvent requirements and wide selection of available stationary phases, IEC remains one of the most prominent methods in the separation and purification of most charged biomolecules that are soluble within an aqueous system (Ongkudon & Danquah, 2010). IEC separates on the basis of ionic interaction between molecules of different charge and charges of stationary matrix. Elution of bound molecules from the column is done by an increasing salt or pH gradient. IEC is ideal for proteinases because it offers binding and elution at relatively mild conditions, allowing the retention of biological activity (Desai *et al*., 2000).

The ion-exchange matrix for IEC can contain either anion exchanger (positively charged) or cation exchanger (negatively charged), determined by the presence and type of charged ligand groups. Some common functional groups used in ion-exchange matrices are diethylaminoethyl (DEAE), quarternary aminoethyl (QAE) and quarternary ammonium (Q) functional groups for anion-exchange chromatography; and carboxymethyl (CM), sulfopropyl (SP) and methyl sulfonate (S) functional groups for cation-exchange chromatography. The use of anion exchangers for proteinase purification is very common (Table 11.2).

11.3.4 Size-exclusion Chromatography

Size-exclusion chromatography (SEC) separates proteins according to their effective molecular size. It is also referred to as 'gel permeation' or 'gel filtration'. The resins used involve porous silica and polyacrylamide crosslinked with agarose gel matrices. These can be modified to give separation resins that possess specific surface and structural properties, and still maintain high mechanical strength and chromatographic properties.

The common applications of SEC include protein concentration, fractionation, desalting and buffer exchange. With SEC, the analyte does not interact with the chromatographic system. This aids the retention of biological activity, while separating the impurities (Desai *et al*., 2000). SEC is one of the most widely used chromatographic steps for proteinase purification (see Table 11.2).

11.3.5 Hydrophobic-interaction Chromatography

Hydrophobic-interaction chromatography (HIC) is a salt-mediated separation of proteins adsorbed on to a weakly hydrophobic support matrix. That is, HIC exploits the interaction between the hydrophobic moiety of proteins and the bulk aqueous environment, in the presence of a neutral salt, to facilitate the adsorption and elution of proteins of interest. Elution is usually achieved by reducing the ionic strength of the mobile phase, in a decreasing salt gradient (Queiroz *et al*., 2001). HIC is largely used in industrial operations and also as an analytical technique for the separation of several biotechnological products, such as homologous proteins, antibodies, recombinant proteins and nucleic acids (Desai *et al*., 2000; Mahn *et al*., 2009). It shows a high level of resolution and can be used as an orthogonal method for the purification of complex protein mixtures (Mahn *et al*., 2009). It is also considered the ideal next step after ammonium sulfate precipitation via elution at high ionic strength (Cramer & Jayaraman, 1993; Nfor *et al*., 2011). Further, since relatively weak van der Waals forces are the major contributing factor to the hydrophobic interactions in HIC, it follows that there is low structural damage to the protein, and that its biological activity is maintained, with HIC versus affinity chromatograypy (AC), IEC or reversed-phase HPLC (rHPLC) (Queiroz *et al*., 2001).

Because HIC exploits the surface hydrophobicity of proteins, it is affected by certain characteristics of the mobile phase: temperature, pH, salt concentration and salt type (Nfor *et al*., 2011). These are important parameters in improving the performance of HIC. Further, certain authors have increased protein hydrophobicity through the addition of short tags of hydrophobic peptides to the target protein, in order to enhance HIC selectivity (Lienqueo *et al*., 2007). Other parameters for consideration in optimising HIC are the temperature of the chromatographic system and the properties of the stationary phase (such as the chemical nature of the matrix, the hydrophobic ligand type and the degree of substitution of the resin) (Mahn *et al*., 2009). Preparative HIC has been used in the purification of several lactobacilli proteinases (Table 11.2)

11.3.6 Reversed-phase HPLC (rHPLC)

As the name suggests, in rHPLC the mobile phase is significantly more polar than the stationary phase. It is the most popular chromatographic technique, due to its speed, high resolution, robustness and reproducibility, and the stability of its microparticulate bonded

phase (Ballschmiter & Wößner, 1998; Desai *et al*., 2000; Dorsey & Dill, 1989). The actual mechanism of action and the binding interaction observed in rHPLC are not well understood (Dorsey & Cooper, 1994). However, it is usually thought that proteins bind to the matrix at a low organic concentration and elute at increasing organic concentrations, depending on the protein hydrophobicity (Desai *et al*., 2000). Gradient elution is often used because gradient elution achieves both concentration and purification in a single step, which is beneficial, especially for dilute proteinase samples (Gu & Zheng, 1999). It is also very powerful in the separation of proteins that differ at only one amino acid and in conformational isomers of peptides (McNay & Fernandez, 2001).

The key parameters that affect an RPC separation are the stationary phase, organic solvent, elution gradient, ion-pairing agents, pH and temperature (Desai *et al*., 2000; Melander *et al*., 1979).

rHPLC is a good chromatographic technique for final polishing of proteins. However, because it is particularly useful for small polypeptides and proteolytic fragments, it is not very efficient for proteinases with high molecular weights. Also, the use of rHPLC leads to protein denaturation, due to the effect of strong adsorption and the organic modifiers needed for desorption. rHPLC is also accompanied by solvent disposal issues (McNay & Fernandez, 2001), which make it unrecommendable for use in process-scale proteinase purification.

11.3.7 Affinity Chromatography (AC)

AC is a highly specific and selective technique which separates biomolecules based on their peculiar biological or chemical interaction with ligands (Desai *et al*., 2000). It is usually the method of choice for the purification of high-value proteins and biologics. It offers a means of separating and purifying any given protein in one step, directly from crude solution. Thus, this approach eludes the product loss (and its attendant cost) associated with multistep bioprocessing. Additionally, AC has simple scale-up procedures and the unique ability to stabilise analyte, and it performs a concentration role when the starting material is dilute (Jones & Baines, 2000).

The common ligands used in AC are usually natural high-molecular-weight biomolecules (such as peptides, saccharides) coupled on to adsorbents. Many of these natural ligands are plagued with limitations, such as high cost, poor chemical and biological stability and ligand leakage (which lead to product contamination). In addition to the fickle nature of some affinity ligands, AC is plagued with the high costs of enzyme supports (Kumar & Takagi, 1999). However, research continues to churn out designed synthetic ligands that are resistant to chemical and biological degradation (Jones & Baines, 2000). This will increase the popularity of AC invaluably in the downstream processing of important proteins such as proteinases.

For the isolation of proteinases, the substrate may serve as a ligand: a competitive reversible inhibitor or an allosteric proteinase activator. This approach is gaining momentum and has been applied in the isolation of certain enzymes other than proteinases (Eijsink *et al*., 1991; Govrin & Levine, 1999; Peters & Fittkau, 1990). For example, the synthetic inhibitor para-aminobenzamidine has been used as the affinity ligand for the removal of trypsin-like serine proteases (Nakamura *et al*., 2003). Other authors have also designed efficient and specific-affinity ligands for the isolation of serine proteases by preparing a peptide derivative which imitates the structure of natural substrates but contains a bond resistant to proteolysis (Kuznetsova *et al*., 1997). The isolation of cysteine

proteases by the use of a fungal cysteine protease inhibitor has also been reported (Sabotič *et al*., 2012).

11.3.8 Methacrylate Monoliths as Stationary Phase in Chromatography

In chromatography, a stationary phase or support is employed for ligand immobilisation (Kline, 1993). Support materials must have such properties as a large specific area, high rigidity, suitable particle form, hydrophilic character and high permeability, and they must also be insoluble in the system where the target molecule is found (Hermanson *et al*., 1992).

Although a significant number of bead-based particulate-separation sorbents have been in existence since the development of chromatographic techniques, there are many limitations to their use. For example, particle-based sorbents function by diffusion, which gives very low binding capacity for large biomolecules (Ongkudon & Danquah, 2010). There is also the problem of interparticular volume, slow mass transfer and decreasing in separation efficiency due to broadening of the chromatographic zones from the large void volume between the packed particles (Vlakh & Tennikova, 2007). The introduction of monolithic solid supports is a giant leap forward, since these are able to circumvent the setbacks of particle-based sorbents, becoming the most popular and most successfully explored sorbents.

A monolith is a continuous phase consisting of a piece of highly porous organic or inorganic solid material. The methacrylate-based monoliths are polymeric macroporous materials, made by radical co-polymerisation of glycidyl methacrylate and ethylene glycol dimethacrylate (GMA/EDMA). Additionally, the pore size of a monolith support can be optimised to allow all of the mobile phase to flow through. Consequently, mass transport is by convection (rather than diffusion). The pore size of the monolith also helps in providing anchorage for both ligand attachment and biomolecule mobility (Ongkudon & Danquah, 2010), which allow the separation of biomolecules at extremely high flow rates without the loss of efficiency and capacity and lead to very short operation times (Vlakh & Tennikova, 2007). Further, scaling up and scaling down of monolithic support is simple. However, the use of methacrylate monolithic support for enzyme purification is in its infancy and therefore requires urgent research attention.

11.3.9 Expanded-bed Adsorption

In most protein-purification systems, the liquids contain suspended particulates, as observed with whole cells in fermentation broth. Thus, downstream processing should include at least one unit operation for the removal of suspended particulates. In some cases, the removal of particulates is attended with high cost, reduction in product yield and technical difficulties (as in the case of particulate removal from viscous liquids and particles of submicron size). It is also near to impossible to apply particulate-containing materials to a fixed, packed bed of adsorbent, as in the case of HPLC adsorbents, since this results in an increase in the pressure drop across the bed, as well as the formation of a plug of trapped solids near the bed inlet. A promising solution to the problem of applying particulate-containing material to a packed bed is the use of a fluidised or expanded bed (Chase, 1994). Expanded-bed adsorption (EBA) combines the effects of centrifugation, filtration, concentration and purification into one step,

thereby increasing yield and cutting down processing time (Desai *et al*., 2000; Hubbuch *et al*., 2005; Kennedy, 2001; Thömmes, 1997). The use of fluidised beds for the direct extraction of proteins from whole fermentation broths has received much research attention in the past few decades (Chase, 1994; Gailliot *et al*., 1990; Hjorth, 1997).

EBA works in a five-step cycle: creation of bed sediment; expansion and equilibration of adsorbents; sample application and washing; elution; and cleaning-in-place. Factors that are critical for the successful operation of EBA are the choice of adsorbent and the design of the apparatus in which the separation is performed (Chang & Chase, 1996; Chase, 1994). Although EBA has made significant strides towards product recovery in large volumes of feedstock, there is still a need to develop new adsorbents with highly stable and specific ligands that give enhanced protein-binding capacities (Hjorth, 1997).

11.4 ENZYME CONCENTRATION AND STORAGE

The concentration and storage of proteinases are the likely steps following purification. Concentration of purified proteinases can be achieved by appropriate HPLC techniques, dialysis, UF and precipitation and/or freeze-drying, followed by reconstitution in a smaller volume of buffer. Notably, pressure-driven separation techniques such as UF are inexpensive, result in little loss of enzyme activity and offer both purification and concentration in the same step (Kumar & Takagi, 1999). The setback with the use of membrane-based separation kits is the fouling or membrane clogging due to the formation of precipitates by the final product. This can usually be alleviated or overcome by treatment with detergents, proteases or acids and alkalies (Kumar & Takagi, 1999).

The storage of proteinases must be accomplished in a suitable environment that will not compromise activity. Storage temperature and pH and the buffer are some parameters to consider. There might be a need for the addition of the necessary cofactors to maintain enzyme activity.

11.5 CHARACTERISATION OF PROTEINASE

11.5.1 ENZYME-ACTIVITY DETERMINATION

Quantitative determination of proteinase activity is achieved primarily by methods based on the enzyme's ability to hydrolyse casein or synthetic chromogenic substrates. Several synthetic chromogenic substrates (such as amino acyl 4-nitroanilide and β-naphthylamides derivatives) have been used for the estimation of proteinase activity (Exterkate, 1990). They are made of functional methoxy and/or succinyl groups bound to a chain of amino acid residues, with the whole bulk bound to an aniline group at the *para* position. The methoxy and/or succinyl functional groups help increase the solubility of the substrate in aqueous medium. Enzymatic cleavage of 4-nitroanilide substrates yields 4-nitroaniline, which gives a yellow colour and absorbs light at 410 nm.

11.5.1.1 Use of Casein in the Estimation of Proteinase Activity

The basis of using casein as a suitable substrate in the estimation of proteolytic activity can be classed into three groups. First, β-casein can be labelled with radioactive ^{14}C to produce ^{14}C-methylated β-casein, which is used as the substrate. The reaction mixture, comprising the substrate, enzyme and a suitable buffer such as TRIS-maleate, is allowed

to proceed for a fixed period of time, after which the reaction is stopped with an organic acid. The reaction mixture is allowed to incubate for some time, then organic acid-soluble aliquots are taken and their radioactivity is measured by liquid-scintillation counting. The number of counts per minute (cpm) or disintegrations per minute (dpm) obtained is proportional to the extent of hydrolysis. One unit of proteinase is usually defined as the amount of enzyme that releases 1 dpm/minute per millilitre (Laloi *et al*., 1991).

Second, casein and the enzyme extract are incubated in a suitable buffer. After incubation, the reaction mixture is centrifuged and the reaction is stopped by the addition of a chaotropic agent, such as organic acid (trichloroacetic acid) or ninhydrin. After centrifugation, the concentration of soluble products is quantified by an appropriate method, such as that of Lowry *et al*. (1951), from which an arbitrary unit (AU) of the proteolytic activity can be defined (Fira *et al*., 2001).

Third, the number of primary amino groups released by the enzyme is estimated with o-phtaldialdehyde (OPA). OPA reacts with primary amino groups and a thiol compound (e.g. dithiothreitol) to form a compound that will absorb light at 340 nm (Nielsen *et al*., 2001). The proteinases of some lactobacillus species do not hydrolyse 4-nitroanilide substrates (Laloi *et al*., 1991), thus the use of casein in the estimation of proteinase activity offers an advantage over the use of 4-nitroanilide substrates. Polyacrylamide-gel electrophoresis (PAGE) is also commonly used, qualitatively, for the estimation of proteinase activity. The disappearance of intact casein is used as an indicator of the extent of proteolysis. Newer methods used to monitor and quantify, respectively, the degradation of casein and the appearance of breakdown products include HPLC, free-solution capillary electrophoresis and micellar electrokinetic capillary chromatography (Oommen *et al*., 2002).

11.5.2 Proteinase Kinetic Parameters

In enzyme catalyses, key parameters such as V_{max} (maximum rate), K_m (the substrate concentration at half v_{max}), K_{cat} (turnover number) and E_a (activation energy) are enzyme-specific as well as substrate- and environment-specific. Studying the kinetics of proteinases can unravel their catalytic mechanism, how their activity can be controlled and how the enzyme can be inhibited. Information about kinetic parameters of the proteinase of interest is therefore of utmost importance in an enzyme-based process, since the knowledge thereof is essential to designing enzyme reactors or quantifying the applications of the enzyme under different conditions (Gupta *et al*., 2002a).

11.5.3 Optimum Conditions for Proteinase Activity

In order to fully characterise the proteinase obtainable from lactobacilli, it is important to elucidate the optimum conditions (such as pH, temperature, and ionic strength) of enzyme activity. Such information is needed to decide the best storage conditions by which to delay or avoid the loss of enzyme activity.

The optimum pH range of alkaline proteases is generally between 5.5 and 7.5. Some few lactobacillus proteinases have an optimum pH of 8 (Zevaco & Gripon, 1988). The optimum temperature of lactobacilli proteinases ranges from 35 to 50 °C. The release of some lactobacilli proteinases is more pronounced at higher temperatures (around 40 °C) but the enzymes are more stable at cold temperatures (4 °C) (Espeche Turbay *et al*., 2009).

The thermal stability of proteinase is another important property. It helps determine whether they will remain active at the temperature conditions found during the manufacture of a product of interest. For example, in the dairy industry an important characteristic of dairy starter is its ability to grow and produce acid(s) at high temperatures. Dairy products such as Cheddar cheese require higher-than-normal processing temperatures of 42 °C (Hickey *et al*., 1986). Lactobacilli and proteinases intended for use in such products must demonstrate their ability to survive the high temperature for a sustained period of time. Thermal stability is usually determined by diluting an enzyme into an assay buffer and pre-warming it at a fixed inactivation temperature. Fractions are then taken at different times of incubation and immediately chilled for proteinase-activity assay (Atlan *et al*., 1989). The gradient of a graph of residual activity with time will give an indication of how thermally stable the proteinase is.

11.5.4 Molecular-mass Estimation, Metal-ion Inducers and Inhibitors

The requirement for metal ions, inhibitors and the molecular weight of proteinase are also worth determining, as these help classification of the proteinases. The molecular mass of most proteinases ranges between 45 and 180 kDa. This range of values is higher than those observed for the *Bacillus* proteases, which are usually between 15 and 30 kDa. Further, most alkaline proteases require a divalent cation such as Ca^{2+}, Mg^{2+} or Mn^{2+}, or a combination, for maximum activity (Kumar & Takagi, 1999). The importance of Ca^{2+} in the stabilisation of proteinases during *Lactococcus* cell growth has been demonstrated (Rahman *et al*., 2003). However, for most lactobacilli, proteinase activity and stability have been shown to be independent of the presence of Ca^{2+} ions in the medium (Espeche Turbay *et al*., 2009; Laloi *et al*., 1991). In addition, most lactobacilli proteinases are inhibited by phenylmethylsulfonyl fluoride (PMSF) or diisopropylfluorophosphate, both of which are specific inhibitors of serine-type proteinases.

11.5.5 Substrate Specificity

Lactobacilli proteinases vary in their activity towards synthetic substrates and native proteins such as casein. Substrate specificity is elucidated via proteinase hydrolysis of specific synthetic dipetides or peptides with labelled amino acid residues. It can also be found via the hydrolysis of native proteins (such as casein) followed by liquid chromatography–mass spectrometry analysis of the peptides fractions.

In the *Lactococcus*, proteinase types have been distinguished on the basis of their specificity toward α-, β-caseins and to a lesser extent κ-casein. CEP_I proteinases predominantly hydrolyse β-casein, and to a lesser extent κ-casein, while CEP_{III} uses αs_1-, β-caseins and κ-casein (Kunji *et al*., 1996, Oberg *et al*., 2002; Oommen *et al*., 2002). A mixed-type $CEP_{I/III}$ classification also exists for some lactobacilli (Kunji *et al*., 1996). The proteolysis of α_{s1}-casein (f1–23) by the lactobacilli forms a basis for the classification of proteinases into seven groups (Oberg *et al*., 2002). Most lactobacilli proteinases are able to hydrolyse the Gln_9-Gly_{10}, Gln_{13}-Glu_{14} and Leu_{21}-Arg_{22} bonds in α_{s1}-casein (f1–23) and the Ser_{15}-Leu_{16}, Glu_{42}-Asp_{43}, Leu_{192}-Tyr_{193}, Pro_{206}-Ile_{207} Met_{156}-Phe_{157}, Ser_{161}-Val_{162} and Leu_{192}-Tyr_{193} bonds in β-casein (Hebert *et al*., 2008).

Knowledge of the substrate specificity of a proteinase helps in classification as well as its directed use.

11.6 SOLVENT AND ENZYME ENGINEERING FOR ENHANCED STABILITY AND SPECIFICITY

It is well known that the tertiary structure of an enzyme determines its catalytic role. Thus, manipulating the tertiary structure may be a useful tool for the improvement of enzyme stability and catalytic behaviour. The stability of enzymes under other-than-usual conditions is also a major factor in their industrial application, necessitating a consistent search for methods for the preparation of stable enzymes.

The study of protein structures and functions has shown that when enzymes are subjected to hostile environments (such as in organic solvents), they exhibit new behaviours, increase in stability and can catalyse reactions that were impossible in water (Gupta *et al*., 2002b). Some other methods used for stabilising enzymes are chemical modification, chemical crosslinking, immobilisation in hydrophobic solvents and polyelectrolyte complexes, use of lyoprotectants and protein engineering (Gupta *et al*., 2002b; López-Gallego *et al*., 2005; Wong & Wong, 1992; You & Arnold, 1996).

11.7 CONCLUSION

The production and downstream processing of lactobacilli proteinases is an ongoing research endeavour, and various types of proteinase have been characterised and have had their potential industrial applications explored. One of the most pressing bioprocess challenges in the area of production of pharmaceutical/biological materials is that of isolation and purification of the finished products. Although several isolation and purification methods exist for proteins, they must all be weighed out based on their merits and demerits. From the already established molecular and particle separations using membranes, centrifugation and phase-partitioning techniques, combinatoric techniques can be developed to ensure proteinase purification at the minimum allowed cost and high product purity. The industrial-scale use and commercialisation of lactobacilli proteinases will require the inputs of scientists, engineers, controls specialists, quality experts and economists. Further, with the important role and prospect of lactobacilli proteinases in industry, more targeted research is needed to aid in the development, purification and characterisation of robust enzymes with desired properties for wider industrial purposes.

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12 Recovery of Proteins and their Biofunctionalities from Marine Algae

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12.1 INTRODUCTION

Marine organisms possess a useful prolific source of secondary metabolites. The biodiversity of marine organisms and associated enormous functional ingredients serve as a practically unlimited resource in the development of pharmaceutical products (Leary *et al*., 2009). Marine algae have been the subject of increasing demand compared to other organisms, due to their chemical diversity (Kim & Wijesekara, 2010). They are an evolutionarily adaptive organisms in the marine environment. Abiotic factors such as light intensity, temperature, nutrients and salinity levels have gained a much influence over their biological functions. Extreme fluctuations in the climates, according to the seasons lead to expose competitive environment conditions. Marine algae are primary producers and sit at the bottom of the food chain, serving other organisms (Plaza *et al*., 2008). Therefore, it is logical to consider that algae can bear rich sources of primary constituents.

Marine algae can be classified into two broad groups: 'macroalgae', or seaweeds, and 'microalgae'. Macroalgae are multicellular organisms with a diverse array of shapes and sizes, and can be further divided according to their pigmentation as red (Rhodophyceae), green (Chlorophyceae) and brown (Phaeophyceae) algae (Garson, 1989; Smit, 2004). Microalgae are microscopic organisms largely consisting of phytoplankton. They can be subdivided again into blue-green algae (Cyanobateria), diatoms (Bacillariophyta) and dinoflagellates (dinophyceae). Biochemically and ecologically significant differences have been found between marine algae, providing a wide diversity and a broad spectrum of secondary metabolites (Plaza *et al*., 2009).

There is a long tradition behind the consumption of marine algae in the human diet (Miroslav & Zorica, 2008). Ancient people believed that marine algae could provide a steady enhancement of life expectancy and improvement in long-term health. Marine algae do have an unusual bredth of nutritional qualities, including proteins and peptides, polysaccharides, polyunsaturated fatty acids (PUFAs), minerals, vitamins and pigments (Becker, 2004, 2007; Burtin, 2003). They are useful research tools and their natural products are practicable in the treatment of human disease (Imhoff *et al*., 2011). Thus, researchers are constantly being undertaken into the bioactivities of marine algae and their applicability to human health. This chapter discusses marine algal proteins and the

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harvesting of their desirable bioactive peptides, examining both their current use and future prospects.

12.2 IMPORTANCE OF PROTEOLYTIC ENZYME-ASSISTED EXTRACTIONS

In recent years, rich protein sources have been examined in an attempt to elucidate the compositions and bioactive constituents that provide their health-promoting effects (Korhonen & Pilanto, 2006). Marine algae have examined their physiologically active metabolites, focusing on proteins, and proteolytic extractions in particular (Heo *et al*., 2005c). In order to explore the potentiality of bioactive resources and the nature of their chemical constituents, separation, isolation and characterization techniques have been employed. The isolation of biologically active components from the enzymatic extracts produced a high yield of high purity compared to organic solvent extractions. Several examples of the isolation of peptides from toxic solvent residues in the target compounds have been reported by researchers. Currently, toxic solvent residues can cause problematic toxicity in *in vitro* and *in vivo* assays in the food and pharmaceutical industries (Kim & Wijesekara, 2011). Attempts to access the inner cellular matrix of marine algae is facilitated by breaking down the cell wall since, a highly sophisticated technique. Mechanical techniques such as ultrasound sonication and the pulverization of lyophilized materials by grinding may also be helpful.

Proteases are used to digest proteins into smaller polypeptides. Most research studies have shown bioactive peptides encrypted in algal proteins (Harnedy & FitzGerald, 2011). Therefore, the physiologically active peptides derived from marine algae are inactive in the parent proteins and only become active when released by proteolytic hydrolyzation (Kim & Wijesekara, 2011). There are three kinds of protease used in enzymatic hydrolyzation: (1) proteolytic digestive enzymes, including pepsin, α-chymotrypsin and trypsin, used by animals; (2) proteolytic enzymes derived from plants or microorganisms; and (3) bacterial or microbial proteases and peptidases found in fermentation (Korhonen & Pihlanto, 2006) (Fig. 12.1). The encrypted bioactive peptides range in size from about 2 to 20 amino acid constituents and can be harvested either by each of these proteolytic hydrolyzations or a serial combination of all three together. Furthermore, adjustment of the physicochemical environment can be managed to release numerous bioactive peptides *in vitro* from the desired protein sources. Conversely, bioactivity-guided protein hydrolysates may be further employed to fractionate from ultramembrane filtration (cut-off molecular mass can be selected as 30, 10 or 5 kDa, as appropriate). In addition, sequential chromatographic techniques have been utilized (reverse-phase high-performance liquid chromatography (RP-HPLC), ion-exchange or gel filtration chromatography and gas chromatography) for further fractionations or purifications up to the optimum level (Chabeaud *et al*., 2009). Spectrophotometric methodologies are used to characterize the molecular structures and molecular masses of bioactive peptides along the chromatographic steps. Taken together, these systematic procedures are a noteworthy and potentially limitless approach to employing serial combinations of enzyme-assisted extractions to the production of bioactive peptides (FitzGerald & Murray, 2007; Harnedy & FitzGerald, 2011). As a consequence, bioactive marine algal protein hydrolysates or peptides can be thought of as good candidates for future use.

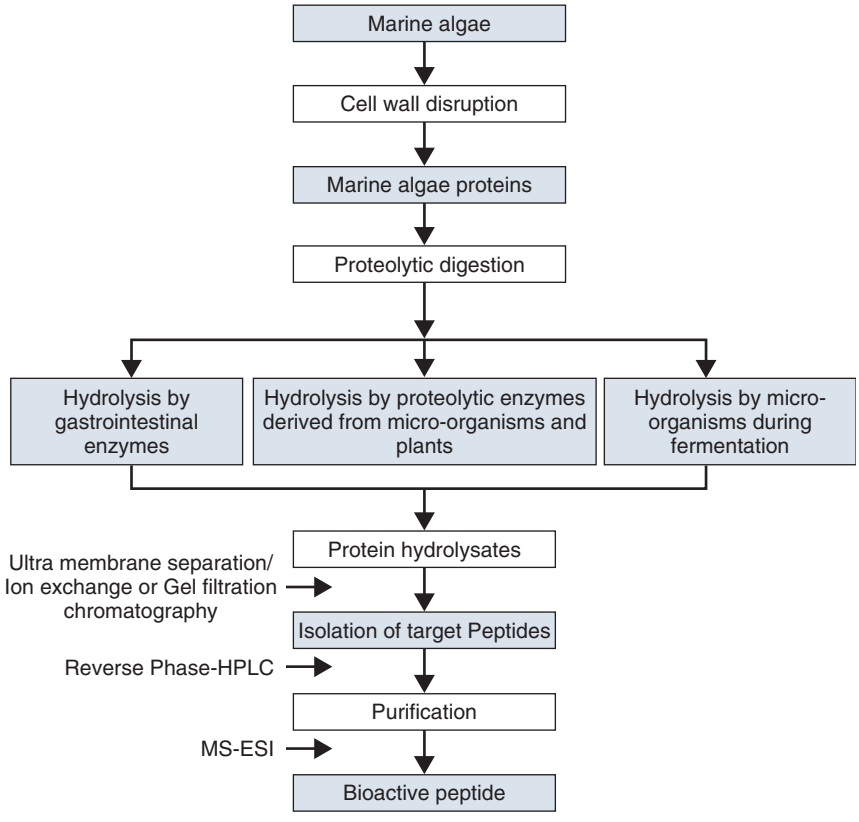

Fig. 12.1 Schematic diagram of the isolation of bioactive peptides from marine algae by proteolytic enzyme-assisted extraction.

12.3 MARINE-ALGAL FUNCTIONAL PROTEINS AND PEPTIDES WITH BIOACTIVITY

12.3.1 Antioxidant Proteins and Peptides

Antioxidants are considered as an important molecular components in protecting the human body and food-processing systems from various toxic, reactive oxygen and nitrogen species (Ahn *et al*., 2004). These molecules can able to scavenge free radicals generated in chain reactions and play a significant role in biological processes by being oxidizing themselves. Endogenous, antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase and non-enzymatic antioxidants including vitamin C, α-tocopherol and selenium, are considered pharmacologically effective sources against oxidative stress and damage. Imbalances between the endogenous antioxidants and reactive oxygen species (ROS) lead to serious health issues and disorders, such as cancer, cardiovascular disease (CVD), hypertension, diabetes mellitus, inflammatory diseases and neurodegenerative diseases as well as aging (Valko *et al*., 2007). Therefore, the need for a novel antioxidant for human health is an important topic for many researchers (Ngo e*t al*., 2011). The same interest has been shown for bioactive marine peptides. Marine

algae in particular are considered a potential candidate for the provision of antioxidant metabolites to the pharmaceutical industry (Heo *et al*., 2005a, 2005b, 2006; Karavita *et al*., 2007; Kim *et al*., 2006).

The determination of antioxidant activities has been carried out and published in many research studies, using various *in vitro* methods, such as DPPH (1,1-diphenyl-2-pricrylhydrazyl) radical, alkyl radical, hydrogen peroxide and superoxide anion radical. These radical scavenging activities have been monitored predominantly by electron-spin resonance (ESR) spectrophotometer (Heo *et al*., 2005b, 2006). In addition, protease-based enzyme-assisted extractions have been used to investigate the antioxidant activities of marine algae (Heo *et al*., 2003). According to this study, five commercially available proteases including Protamex, Kojizyme, Nutrase, Flavourzyme and Alcalase have been used to provide proteolytic digestion against seven species of brown seaweed such as *Ecklonia cava*, *Scytosiphon lomentaria*, *Ishige okamurae*, *Sargassum fullvelum*, *Sargasum coreanum, Sargasum horneri* and *Sargassum thunbergii* around the Jeju-Do coasts in Korea. *E. cava* and *S. lomentaria* showed pronounced antioxidant activity as inhibitors of lipid peroxidation in linoleic acid against Alcalase and Neutrase extracts. Heo & Jeon (2008) have reported that the strong hydrogen peroxide scavenging effects of *I. okamurae* against protease. Furthermore, the prepared concentrations (140 μg/ml) of *I. okamurae* by proteases including Alcalase, Flavourzyme, Kojizyme and Protamex were shown to have remarkably high scavenging activities of 91.62, 93.41, 96.27 and 93.71%, respectively. Four separate studies (Ahn *et al*., 2004; Je *et al*., 2009; Shiu & Lee, 2005; Wang *et al*., 2010) have reported that proteolytic enzyme extracts from *S. lomentaria*, *P. palmate*, *U. pinnatifida* and *U. fasciata* show ROS-scavenging activity. Antioxidant effects of seaweed have been revealed as a result of both bioactive peptides and protein hydrolysates. Seaweeds have not so far been found to isolate bioactive peptides with the antioxidant effects. A high content of polysaccharides and polyphenols make an impermeable barrier to access into the seaweed proteins. This might be the reason why reports are scarce concerning macroalgae peptides as opposed to microalgae.

A few years ago, a potent antioxidative peptide was isolated from the waste of *Chlorella vulgaris* proteins by pepsin hydrolyzation (Sheih *et al*., 2009b). The isolated and purified peptide (VECYGPNRPQF), which had a low molecular mass (1309 Da), exhibited significant tolerance against gastrointestinal enzymes. Furthermore, the purified peptide from algal protein shows higher ABTS-radical scavenging (IC_{50} 9.8 ± 0.5 μM) and superoxide-radical scavenging (IC_{50} 7.5 ± 0.12 μM) activities than standard antioxidants such as trolox, ascorbic acid and BHT. Kang *et al*. (2011) have reported a pronounced detectable antioxidant activity by the benthic diatom *Navicula incerta*. Moreover, enzymatic hydrolysates have been tested by electron spin-trapping techniques for free-radical scavenging effects on DPPH, hydroxyl and superoxide radicals, and the highest IC_{50} values were recorded as 196 μg/ml (pepsin), 102.0 μg/ml (α-chymotrypsin) and 196.0 μg/ml (neutrase), respectively. On the other hand, the water-soluble protein pigment, C-phycocyanin, from *Spirulina plantensis*, was reported to have an inhibitory effect against CCl_4-induced lipid peroxidation in the liver of rats *in vivo* (Bhat & Madyastha, 2000). As reported, the radical-scavenging activity (IC_{50} value 5.0 μm) of C-phycocyanin was detected against peroxyl radicals *in vitro* as well.

Most studies have focused on the hydrolysis of food proteins by proteolytic enzymes. Antioxidative peptides are associated with 5∼11 amino acid residues, including hydrophobic amino acids, proline, histidine, tyrosine and tryptophan (Samaranayaka & Li-Chan, 2011). This has been proven in many substances, including milk protein (Pihlanto, 2006),

giant-squid muscle (Rajapakse *et al*., 2005), conger-eel muscle (Ranathunga *et al*., 2006), bullfrog skin (Qian *et al*., 2008a), oyster (Qian *et al*., 2008b) and tuna dark-muscle byproducts (Hsu, 2010).

Table 12.1 presents a list of different enzymes that have been used in making these protein hydrolysates and peptides from marine microalgae. The selection of suitable enzymes and digestion conditions, such as ideal pH, temperature and hydrolysis time, for the optimal activity of enzymes is essential to obtaining the desired peptides from protein hydrolysates of marine algae. Consequently, many publications have reported antioxdative properties. However, the isolation and characterization of antioxidative peptides from marine algae is rarely being published compared to those in other marine organisms.

12.3.2 Antiproliferative Proteins and Peptides

Cancer is an increasing threat to the global population and the leading cause of death in economically developed countries, and the second-leading cause in developing countries (Ezzati *et al*., 2002). There are about 12.7 million current cancer cases and 7.6 million deaths are estimated to have occurred in 2008 (Jemal *et al*., 2011). Breast cancer in females and lung/bronchus cancer in male are the most frequently diagnosed types of cancer in both developing and developed countries. In fact, these two types of cancer are the leading causes of death in both sexes worldwide. However, a significant portion of the global burden of cancers could be prevented through awareness of cancer-causing behaviors, early diagnosis and implementation of treatments.

Finding cancer-cure therapeutics is given a priority by many scientists. Cancer can be characterized as abnormal, uncontrolled growth of the cells in any part of the body. A tumor or mass of cells can be initiated by changes in or damages to the genetic materials in the cells by environmental or internal factors. These continuously multiplying cells then spread from one part of the body to another. Identification of this mechanism is an important method of controlling the spread (metastasis). Chemotherapy is a promising approach to the prevention and cure of cancers. However, cancers are continuously found to have developed resistance to the effects of chemotherapy. A few years ago, some data on peptides were found to show pronounce effect for the development of anticancer therapeutics (Sehgal, 2002). Recently, several peptides have been derived from thrombospondin, collagens, chemokines and coagulation cascade proteins and tested for inhibitory activity against microvessel formation in preclinical and clinical studies of cancer (Rosca *et al*., 2011). Another study on the use of peptides as ligands for the prevention of tumors has been announced. In this study, a novel peptide (PC5-2: TDSILRSYDWTY) was identified from the phage-displayed peptide library as having the ability to target non-small-cell lung cancer (NSCLC), and it demonstrates improved therapeutic efficacy for animal models (Chang *et al*., 2009).

Marine algae might be good candidates for harvesting bioactive peptides against cancers. Microalgae have shown a strong effect and the potential for antiproliferative activity. Recently, Chen *et al*. (2011) reported that the protein waste of *C. vulgaris*-derived peptide has been shown to inhibit the solar ultraviolet B (UVB)-induced matrix metalloproteinase-1(MMP-1) level in skin fibroblast cells. According to this study, 10 or 5 mg/ml of *C. vulgaris* peptides diminished the UVB-induced level of MMP-1 and cysteine-rich 61 (CYR61) mRNA expression in monocyte chemoattractant protein-1 (MCP-1) production. In another study, a purified glycoprotein (ARS-2; amino acid sequence DVGEAFPTVVDALVA) from hot water-extracted *C. vulgaris* showed

Table 12.1 Peptide sequences with possible bioactivity derived from marine microalgae.

Marine microalga	Possible bioactivity	Proteolytic enzyme and fermenting micro-organism	Identified peptide sequence	IC_{50} value[a]	Reference	Country
Chlorella vulgaris	Antioxidative: superoxide radical	Pepsin	Val-Glu-Cys-Iyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	7.5 μM	Sheih *et al.* (2009a)	Taiwan, ROC
Chlorella vulgaris	ACE-inhibitory	Pepsin	Val-Glu-Cys-Iyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	29.6 μM	Sheih *et al.* (2009b)	
Chlorella vulgaris	Antiproliferation	Pepsin	Val-Glu-Cys-Iyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	70.7 μM	Sheih *et al.* (2010)	
Chlorella vulgaris	ACE-inhibitory	Pepsin	Ile-Val-Val-Glu Ala-Phe-Leu Phe-Ala-Leu Ala-Glu-Leu Val-Val-Pro-Pro-Ala	315.3 μM 63.8 μM 26.3 μM 57.1 μM 79.5 μM	Suetsuna & Chen (2001)	Japan
Spirulina platensis	ACE-inhibitory	Pepsin	Ile-Ala-Glu Phe-Ala-Leu Ala-Glu-Leu	34.7 μM 26.2 μM 57.1 μM	Suetsuna & Chen (2001)	Japan
Navicula incerta	Antioxidative:					
	DPPH	Pepsin	Acidic amino acids: Glu-, Asp-, Lys-, Arg-	196 μg/ml		
	Hydroxyl Superoxide	α-chymotrypsin Neutrase		102 μg/ml 196 μg/ml	Kang *et al.* (2011a)	Korea
	Hepatic fibrosis-inhibitory effect	Papain	Pro-Gly-Trp-Asn-Gln-Trp-Phe-Leu Val-Glu-Val-Leu-Pro-Pro-Ala-Glu-Leu		Kang (2011)	Korea
Pavlova lutheri	Myofibroblast differentiation	*Candida rugopelliculosa*	Met-Pro-Gly-Pro-Leu-Ser-Pro-Leu		Ryu (2011)	Korea

[a]IC_{50} value: the concentration of peptide required to inhibit 50% of activity.

antitumor activities. Hot-water extracts of *C. vulgaris* also reduced the enzyme level of superoxide dismutase (SOD) against liver cancer-induced rats in a dose-dependent manner (Sulaiman *et al*., 2006). Furthermore, Sheih *et al*. (2010) reported a strong antiproliferative activity by the protein waste of *C. vulgaris* against the post-G1 cell-cycle arrest in AGS cells. The isolated peptide VECYGPNRPQF and protein hydrolysate exhibited growth-inhibition activities with IC_{50} values of $70.7 \pm 1.2\,\mu g/ml$ and 1.74 ± 0.3 mg/ml against the AGS cell line, respectively.

On the other hand, phycobiliproteins including C-phycocyanin and B-phycoerythrin, pigment proteins in marine algae (red and blue-green algae), can be described as significant components in exerting antiproliferative activities *in vitro*. A significant diminution (49%) of the proliferation has been reported against the human chronic myeloid leukemia cell line (K562) by the isolated C-phycocyanin (50 µM for 48 hours) by *S. platensis* (Subhashini *et al*., 2004). Cells treated with 25 and 50 µM of C-phycocyanin for 48 hours showed 14.11 and 20.93% reduction by flow cytometric analysis in sub-G0/G1 phase, respectively. In addition, Minkova *et al*. (2011) showed that B-phycoerythrin isolated from *Porphyridium cruentum* was effective in the inhibition of tumor-cell proliferation on Graffi myeloid tumor cells in a dose-dependent manner *in vitro*. Taken together, these findings indicate that protein hydrolysates and peptides from *C. vulgaris* and phycobiliproteins from some marine algae have application for the development of pharmaceuticals against cancers.

12.3.3 Antihypertensive Proteins and Peptides

CVDs are increasing and represent a major health problem worldwide. Hypertension is the main risk factor for CVDs. The estimated total number of adults suffering hypertension greater than 25% in 2000 was 972 million in both economically developed and developing countries. This figure is predicted to increase by 60% to a total 1.56 billion adults in 2025 (Kearney *et al*., 2005). Several other disorders, including valvular heart disease, coronary heart disease, cardiothoracic heart failure and atrial fibrillation are also associated with CVDs (Veenhuyzen *et al*., 2004). Therefore, identification of the mechanism of the renin–angiotensin system (RAS) and exploration of bioactive peptides from marine algae are important avenues of research in CVD prevention.

The RAS has two key roles in clinical hypertension (Weber, 1999): inhibition of angiotensin-I generation from angiotensinogen by rennin, and blocking of the conversion of angiotensin-II from angiotensin-I by angiotensin-I-converting enzyme (ACE-I) (Verdecchia *et al*., 2008). ACE inhibitors are a commonly available and widely prescribed medicine for hypertension (Riordan, 2003). Recently, there has been a growing demand for isolation of antihypertensive peptides from marine algae. In order to investigate these, gastrointestinal proteases are used for proteolytic digestion (Table 12.2).

Athukorala & Jeon (2005) investigated the ACE-inhibitory activities of seven selected species of macroalga: *E. cava*, *I. okamurae*, *S. fulvellum*, *S. horneri*, *S. coreanum*, *S. thunbergii* and *S. lomentaria*, after being digested by commercial proteases. In this study, Flavourzyme-digested *E. cava* showed potent ACE-inhibitory activity, with an IC_{50} of 0.3 µg/ml, compared to 0.05 µg/ml for the commercial ACE-inhibitor captopril. In another study, pepsin extract of *U. pinnatifida* exhibited a high ACE-inhibitory activity along the isolated peptides Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly and Tyr-Asn-Lys-Leu, with IC_{50} values of 213.0, 64.2, 90.5 and 21.0 µM, respectively (Suetsuna & Nakano, 2000). The same seaweed (*U. pinnatifida)* has been subjected to ACE-inhibitory

Table 12.2 ACE-inhibitory peptides with IC_{50} values derived from marine-macroalgal proteins.

Marine macroalga	Proteolytic enzyme used	Peptide sequence	IC_{50} value[a]	Reference
Undaria pinnatifida	Pepsin	Ala-Ile-Tyr-Lys	213 μM	Suetsuna & Nakano (2000)
		Tyr-Lys-Tyr-Tyr	64.2 μM	
		Lys-Phe-Tyr-Gly	90.5 μM	
		Tyr-Asn-Lys-Leu	21 μM	
Undaria pinnatifida	Protease S	Val-Tyr	35.2 μM	Sato *et al.* (2002)
		Ile-Tyr	6.1 μM	
		Ala-Trp	18.8 μM	
		Phe-Tyr	42.3 μM	
		Val-Trp	3.3 μM	
		Ile-Trp	1.5 μM	
		Leu-Trp	23.6 μM	
Undaria pinnatifida	Hot-water extract	Tyr-His	5.1 μM	Suetsuna *et al.* (2004)
		Lys-Trp	10.8 μM	
		Lys-Tyr	7.7 μM	
		Lys-Phe	28.3 μM	
		Phe-Tyr	3.7 μM	
		Val-Trp	10.8 μM	
		Val-Phe	43.7 μM	
		Ile-Tyr	2.7 μM	
		Ile-Trp	12.4 μM	
		Val-Tyr	11.3 μM	
Porphyra yezoensis	Pepsin	Ile-Tyr	2.69 μM	Suetsuna (1998)
		Met-Lys-Tyr	7.26 μM	
		Ala-Lys-Tyr-Ser-Tyr	1.52 μM	
		Leu-Arg-Tyr	5.06 μM	
Porphyra yezoensis	Pepsin	Ala-Lys-Tyr-Ser-Tyr		Saito & Hagino (2005)

[a]IC_{50} value: the concentration of peptide required to inhibit 50% of activity.

peptides by proteolytic digestion from the protease S 'Amano' enzyme (Sato *et al*., 2002). Isolated four dipeptides, including Val-Tyr, Ile- Tyr, Phe-Tyr and Ile-Trp (IC_{50} values of 35.2, 6.1, 42.3 and 42.3 μM) were significantly reduced blood pressure after a single oral-administration dose (1 mg/kg) in spontaneously hypertensive rats (SHR). Suetsuna *et al*. (2004) further studied the ACE-inhibitory effect of the hot water-extracted *U. pinnatifida* and isolated 10 kinds of dipeptides: Tyr-His, Lys-Trp, Lys-Tyr, Lys- Phe, Phe-Tyr, Val-Trp, Val-Phe, Ile-Tyr, Ile-Trp and Val-Tyr. A well-known edible red alga, *Porphyr yezoensis* was hydrolyzed by the pepsin proteolytic enzyme, and isolated the antihypertensive peptides Ile-Tyr, Met-Lys-Tyr, Ala-Lys-Ser-Tyr and Leu-Arg-Tyr, with IC_{50} values 2.69, 7.26, 1.52 and 5.06 μM, respectively (Suetsuna, 1998). In another study, the same macroalga had subjected to isolate the oligopeptides (Ala-Lys-Tyr-Ser-Tyr) by pepsin hydrolysate (Saito & Hiroshi, 2005). He *et al*. (2007) have shown that protein hydrolysates of the red seaweed *Polysiphonia urceolata* and microalga *S. platensis* possessed ACE-inhibitory activities with IC_{50} values of less than 1.0 mg/ml. Interestingly, *S. platensis* hydrolysates obtained ACE-inhibitory activity with IC_{50} values of 0.17 and 0.22 mg/ml against Protamex and SM98011, respectively.

Industrial protein waste from *C. vulgaris* has been used to hydrolyze by pepsin, and the ACE-inhibitory activity was determined. In fact, the isolated peptide (Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe) showed an ACE-inhibitory effect (IC_{50} value of 29.6 μM) greater than that of other protein hydrolysates (Sheih *et al*., 2009a). Another group of researchers isolated antihypertensive peptides from the pepsin hydrolysates of two microalgae, *C. vulgaris* and *S. platensis*. As reported, *C. vulgaris* has produced a few ACE-inhibitory peptides, including Ile-Val-Val-Glu, Ala-Phe-Leu, Phe-Ala-Leu, Ala-Glu-Leu, and Val-Val-Pro-Pro-Ala, with IC_{50} values of 315.3, 63.8, 26.3, 57.1 and 79.5 μM, respectively. *S. platensis* likewise produced the peptides Ile-Ala-Glu, Ile-Ala-Pro-Gly and Val-Ala-Phe, with ACE-inhibitory IC_{50} values of 34.7, 11.4 and 35.8 μM, respectively (Suetsuna & Chen, 2001).

Considerable attention has been devoted to the use of marine algal peptides in combating hypertension. Most ACE studies have focused extensively on *in vitro* and *in vivo* rat models (SHR). However, marine peptides may still show lower efficacy in preventing and the treating hypertension than the commercial drugs. This may soon be overcome by implementing the biospecificity and stability of the ACE-inhibitory peptide-based structure–activity relationship or by molecular modeling techniques against hypertension, obesity and diabetic mellitus.

Evidence suggests that marine algae provide a complete source of proteins, with peptides encrypted in the native protein and released by proteolytic digestion or microbial fermentation. Comprehensive analyses of the algal proteins and bioactive peptides have occurred and there may be advantage to their use in functional foods and nutraceuticals (Chacón- Lee *et al*., 2010; Guil-Guerrero *et al*., 2004), pharmaceuticals (Dominic & Danquah, 2011) and cosmetics (Sekar & Chandramohan, 2008; Stolz & Obermayer, 2005).

12.4 MARINE-ALGAL PROTEINS: POTENTIAL SOURCES FOR FUTURE APPLICATIONS

12.4.1 Nutraceutical Value

Protein is one of the key nutrients used to regulate basic physiological and biological functions in the human body (Shao & Hathcock, 2008). After the consumption, food proteins are broken down into smaller peptides in the stomach during digestion by protease enzymes. Then they provide essential amino acids, which humans cannot synthesize inside the body. These essential amino acids function in bile–acid conjugation, osmoregulation, retinal and neurological development, immune functions and maintenance of calcium levels (Shao & Hathcock, 2008). Protein composition and content vary between macroalgae and microalgae. In general, microalgae contain a higher protein content than seaweed. The protein content also varied according to the species, season and environmental conditions (Burtin, 2003). The nutritional qualities of algal proteins depend significantly on the composition, contents, proportions and bioavailability of the amino acids (Becker, 2007; Cerna, 2011).

High protein contents are found in red and green seaweeds (10–47% of dry weight); for example, in some red seaweed, *Palmaria palmate* (dulse) and *Porphyra tenera* (nori) proteins make up 21–47 g/100 g dry weight (Rupérez & Saura-Calixto, 2001), while in the green seaweed *Ulva pertusa*, proteins form 20–26 g/100 g dry weight (Fujiwara *et al*., 1984). Brown seaweeds only contain low protein contents (3–15%

of the dry weight) (Fleurence, 1999): for example, in some brown seaweed, such as *Laminaria japonica*, *U. pinnatifida* and *Hizikia fusiforme*, proteins make up about 7–16 g/100 g dry weight (Jurkovic *et al*., 1995; Kolb *et al*., 1999). The bioavailability of the seaweed proteins can sometimes be inhibited by the entrapped nature of the cellular matrix. However, physical processes, enzymatic extractions and microbial fermentation techniques have been used to liberate these proteins (MacArtain *et al*., 2007). The high content of essential amino acids and their bioavailability have been reported in red algae verities (Dawczynski *et al*., 2007), while the nutritional values of these essential amino acids, including histidine, leucine, valine, lysin and methionnine, were reported in many seaweeds and were similar to those in ovalbumin proteins. The levels of isoleucine and threonine amino acids found in *P. palmate* were similar to those in leguminous protein (Fleurence, 1999). However, both glutamic acid and aspartic acid constituents make up a large fraction of amino acids in the proteins of most species of seaweed (MacArtain *et al*., 2007).

On the other hand, marine microalgae have been recognized as a rich source of amino acids, comprising highly valuable proteins (6–71% of dry weight). In certain microalgae species, including *Chlorella vulgaris*, *Dunaliella salina*, *Chlorella pyrenoidosa*, *Arthrospira maxima*, *Anabaena cylindrica* and *Spirulina platensis*, proteins range from 40 to 60% of dry weight and demonstrated a superior quality to conventional vegetable proteins (Beker, 2007). Spolaore *et al*. (2006) have compared the protein contents of general food sources with different microalgae. According to their estimations, all tested marine microalgae, including *C. vulgaris*, *D. salina*, *Spirulina maxima*, *A. cylindrical*, *Clamydomonas rheinhardii* and *Porphyridium cruentum*, showed protein contents in the range 43–71% of dry matter, equal to or higher than the available protein percentages in meat (43%), milk (26%), rice (8%), soybean (37%) and baker's yeast (39%). Another study evaluated the nutritional compositions of three microalgae: *S. platensis*, *C. vulgaris* and *Isochrisis galbana*. The protein contents, energy values, mineral elements and fatty acid compositions met the guidelines for Recommended Dietary Allowances (RDA) of the Food and Nutrition Board of the United States National Academy of Sciences (Tokuşoglu & Ünal, 2003). Therefore, the associated functional nutrients of the microalgae would be the innovative food sources that provide beneficial health effects (Plaza *et al*., 2003). In contrast to macroalgae, microalgae have gained much attention with the cultivation and development of markets worldwide. The utilization of microalgae and biotechnology has been increased along with the development of nutraceuticals (Pulz & Gross, 2004).

The manufacture of microalgal biomasses for the production of food supplements and food additives has been marketed in recent years. For example, noodles, bread, biscuits, candy, ice cream, bean curds and liquid foods such as health drinks, soft drinks, tea, beer and spirits have been supplemented with bioactive functional ingredients from *Spirulina* and *Chlorella* (Liang *et al*., 2004). There is a concern about the consumption of microalgae, and it would be beneficial to boost their health effects. Moreover, the nutritional value of algal proteins may open up a new field of research in the future, which will become even more diverse and economically competitive.

12.4.2 Pharmaceutical Value

Nowadays, there is a huge demand for the isolation of bioactive peptides from marine organisms. In fact, endogenous marine peptides have opened up a new scenario for the development and commercialization of therapeutic agents (Dominic & Danquah, 2011).

It would be a great boon to discover the source of marine algal proteins in the search for new therapeutic agents. Consumer interest regarding the role of dietary proteins in controlling and influencing health may help to commercialize the bioactive peptides from marine algae. On the other hand, the pharmaceuticals industry is based on the exploration of new lead compounds and innovative drugs. However, the cost of this is very high and estimated expenditures to bring a new drug to market are more than 802 million US dollars (Frank, 2003). However, it takes 10–12 years to gain approval for a new drug. In recent years, the pharmaceutical companies have therefore looked at the utilization of marine algae with respect to their bioactive peptides. With these, investigations into new isolation methodologies under optimized conditions would become a cost- and time-efficient process. In addition, peptides have shown a number of other advantages, such as being toxicologically safe and having high bioactivity and biospecificity to research targets and the development of clinical trials. Furthermore, research has shown that the therapeutic activity of peptides with low-level toxicity and little or no accumulation in body tissues is compromised by structural diversity (Marx, 2005). Fig. 12.2 shows the physiological importance of the peptides derived from marine algae for therapeutic uses. These act as mimic hormones, with fewer side effects than small molecular drugs (Dominic & Danquah, 2011). Bioactive peptides alter physiological functions and have a positive impact through binding to specific receptors and interacting with target cells or inhibiting enzyme actions (FitzGerald & Murray, 2007; Kitts & Weiler, 2003). There is thus a continually increasing demand for the isolation of new functional proteins or bioactive peptides from marine algae (Harnedy & FitzGerald, 2011).

Recently, the isolation and characterization of marine algal-derived peptides with hepeto-protective (Kang et al., 2012), anticoagulative (Athukorala *et al*., 2007), immune-modulatory (Morris *et al*., 2007) and anticholesterol (Shibata *et al*., 2007) bioactivities has

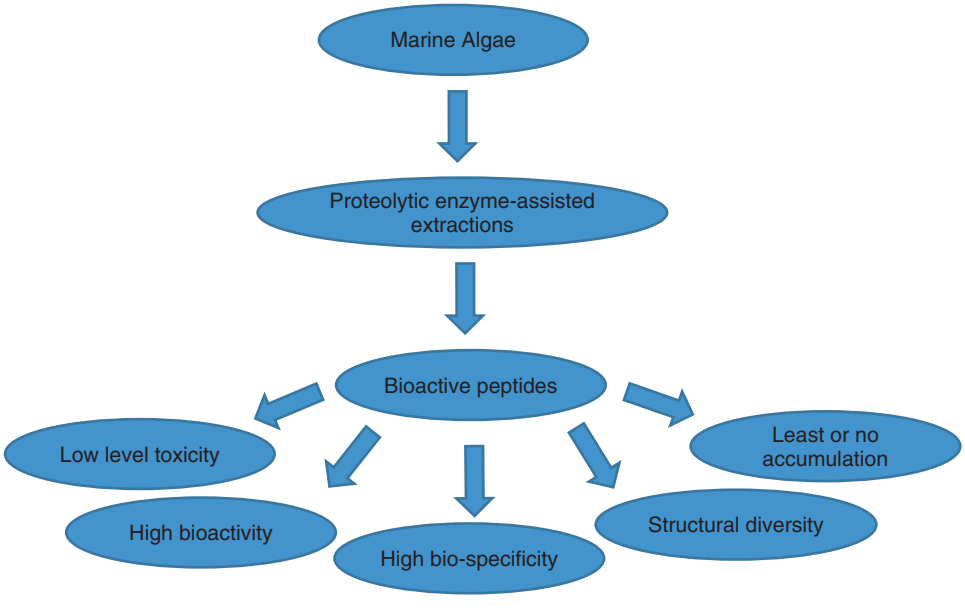

Fig. 12.2 Physiological importance of bioactive peptides derived from marine algae.

been reported. Moreover, these bioactivities have been described as the multifunctional properties of the proteins from marine algae. However, a variety of benefits attributed to bioactive peptides have not been established so far. Some peptides are relatively unstable, with a low half-life and low ability to cross membrane barriers. The difficulties in understanding their mechanism of action must be considered (Hancock & Sahl, 2006). Structure–activity relationships and molecular modeling can overcome those problems and help expand our knowledge of the bioactive peptides. Improvement of the structures, stabilities and capacities in human model systems must be further discussed.

12.4.3 Cosmetic Value

There has been much interest in the use of marine algal products in cosmetics (Raja *et al*., 2008). The potential for preventing aging and enhancing the appearance and protection of human skin is of particular note (Kim *et al*., 2008). In addition, other functional nutrients, such as vitamins, phytochemicals, enzymes, antioxidants and essential oils, are also desirable (Kim *et al*., 2008). The use of algal proteins can improve nourishment of the hair and skin while helping maintain moisture. Hagino & Masanobu (2003) have stated that porpyra species, wakame seaweed protein hydrolysates and peptides contribute to major physiological activities in cosmetics applications.

Some microalgae species are becoming established in the face and skincare market. Extracts from *Arthrospira* and *Chlorella* species are well known in anti-aging creams, anti-irritant products and refreshing or regenerative care products (Stolz & Obermayer, 2005). A protein-rich extract from *Arthrospira* species has been used to repair the signs of early skin aging, for example (Protulines, Exsymbol S.A.M., Monaco). Another (Dermochlorella, Codif, St Malo, France) uses extracts from *C. vulgaris* to stimulate collagen synthesis in the skin. It is claimed that tissue regeneration and wrinkle reduction are provided by *C. vulgaris* proteins. In addition, B-phycoerythrin has shown heat-stability and pH-tolerant characteristics, and it is applied in natural pink and purple colorants for lipsticks, eyeliners and formulations for cosmetic products (Viskari & Colyer, 2003). B-phycoerythrin has numerous other pharmacological activities, including antioxidant, anti-inflammatory, neuroprotective, antiviral, antitumor, hepetoprotective, serum lipid-reducing and liver-protecting (Sekar & Chandramohan, 2008). A noteworthy is that the formulations of cosmerceutical products and applications were manipulated up to certain extend from the proteins and its derivatives of marine algae.

12.5 CONCLUSION

Recent studies have supported the idea that marine algae-derived proteins play a vital role in promoting health and nutrition in humans. It is suggested that marine-algal proteins are a promising approach to exploration of the bioactive peptides as new chemical entities in the pharmaceutical industry. A comprehensive analysis has provided wide acceptance of their use as functional foods with medicinal value. Taken together, marine algae-derived proteins and peptides have the potential to act as active ingredients in future nutraceutical, pharmaceutical and cosmetic applications. However, in order to create a platform for the commercialization of marine-algal proteins and peptides, cost- and time-efficient screening, isolation, purification, production and marketing strategies must be developed.

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13 Fish Gelatin: A Versatile Ingredient for the Food and Pharmaceutical Industries

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13.1 INTRODUCTION

Gelatin (sometimes spelled 'gelatine') is one of the most versatile ingredients used to improve the elasticity, consistency and stability of foods and for several pharmaceutical applications. It is a polypeptide derived by hydrolytic degradation of collagen, the principal component of animal connective tissue. Gelatin has traditionally been extracted from the skin and bone collagens of certain mammalian species, primarily cows and pigs. Although it has a wide range of applications, strong concerns still persist among consumers over its use, mainly due to religious sentiments (both Judaism and Islam forbid the consumption of any pork-related products, while Hindus do not consume cow-related products) and safety considerations, especially since the outbreak of bovine spongiform encephalopathy (BSE; mad cow disease) in the 1980s. These sociocultural and safety concerns have led to intensive research to identify and develop alternatives to mammal-derived gelatin.

Gelatin from marine sources has been looked upon as a possible alternative to bovine and porcine gelatin. Seafood processing leads to the generation of a large biomass of fish waste (e.g. skin, bones, scales and fins), which is generally discarded (~7.3 million tons/year) (Kelleher, 2005). Morrissey *et al*. (2005) reported that the solid waste from surimi processing constituted up to 70% of the original raw material, depending on the method used to extract meat from the carcass. Consequently, efforts have been initiated to investigate an increased utilization of collagenous fish waste for the production of gelatin (Gilsenan & Ross-Murphy, 2000; Holzer, 1996; Wasswa *et al*., 2007). Fish gelatin is acceptable for Islam, and can be used with minimal restrictions in Judaism and Hinduism. Furthermore, the commercial use of fish skin and bones, which are otherwise discarded, is a good waste-management practice, leading to additional economic benefit.

Though fish gelatin has been highlighted as a better alternative to mammalian gelatins from ethical and religious points of view, qualities such as a low melting point and low gel strength have affected its commercial applications. Therefore, the production of fish gelatin is still in its infancy, contributing only about 1% of the annual world gelatin production (Arnesen & Gildberg, 2006).

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Gelatin possesses the following basic properties:

- It is capable of forming and stabilizing hydrogen bonds with water molecules to form a stable three-dimensional gel.
- It is nontoxic, widely used in foodstuffs and acceptable for use worldwide (GRAS status).
- It is readily soluble in biological fluids at body temperature and biocompatible with different physiological environments.
- It is a good film-forming material, having potential to produce strong, flexible, edible and biodegradable film.

Several comprehensive and successive reviews are available on this subject, illustrating the biomedical and nutraceutical applications of marine gelatin (Kim & Mendis, 2006), the potential of fish-processing byproducts in the gelatin industry (Wasswa *et al*., 2007), possible gelatin alternatives for the food industry (Karim & Bhat, 2008), fish gelatin as an alternative to mammalian gelatin (Karim & Bhat, 2009), valorization of fish gelatin from marine byproducts (Ferraro *et al*., 2010) and the functional and bioactive properties of gelatin from alternative sources (Gomez-Guillen *et al*., 2011). Being a unique hydrocolloid with exceptional characteristics, marine gelatin finds a broad range of applications in both the food and pharmaceutical industries. It has been extensively employed as a gelling, thickening, foaming and emulsifying agent. Gelatin is also considered a highly digestible dietary food, ideal for certain types of diet, particularly as a nutritional supplement. Consequently, gelatin is classified as a food in its own right and is not subjected to food-additives legislation in Europe.

13.2 STRUCTURAL FEATURES OF FISH GELATIN

Gelatin, produced by partial hydrolysis of collagen, is a heterogeneous mixture of water-soluble proteins of high molecular weight, in the range between 80 and 250 kDa. The broad molecular-weight distribution of gelatin is probably due to its production process, which usually produces single α-chains, two α-chains covalently crosslinked to give β-chains and three covalently crosslinked α-chains, called γ-chains (Haug *et al*., 2004). The average molecular weight of one α-chain is reported to be between 80 and 125 kDa, the β form is 160–250 kDa and the γ form 240–375 kDa. These three types of chain have been identified by electrophoresis and chromatography in both mammalian and fish gelatins (Gomez-Guillen *et al*., 2002). According to Johnston-Banks (1990), the sum of intact α and β gelatin fractions, together with their peptides, is proportional to the gel strength, while the viscosity, setting rate and melting point increase with increasing amount of high-molecular-weight (greater than γ) fraction.

The wide range of potential functionality of fish collagen and gelatin is due to wide variation in their amino acid composition. Glycine is the most dominant amino acid, constituting about 27% of the total amino acid pool (Arnesan & Gildberg, 2002; Tabata & Ikada, 1998). Proline and hydroxyproline come second in abundance, and play a key role in maintaining the thermal stability of collagen. The total amount of these two amino acids is higher in mammalian gelatin (20–24%) than in fish gelatin (16–20%). Fish gelatin is different from mammalian gelatin in properties such as melting and gelling temperatures and gel strength, due to differences in its amino acid composition, especially with respect to proline and hydroxyproline content. Furthermore, the levels of proline and

hydroxyproline vary significantly among fish species (Balian & Bowes, 1977; Gudmundsson & Hafsteinsson, 1997). The amount of an amino acid, especially hydroxyproline, depends on the environmental temperature in which the fish lives, and this affects the gel strength and melting point of the gelatin. Gelatin derived from fish species living in cold environments has a lower content of hydroxyproline and exhibits lower rheological properties than that from fish living in warm environments. This is because hydroxyproline is involved in interchain hydrogen bonding, which stabilizes the triple helical structure of collagen. Cold-water fish species are also reported to contain higher levels of hydroxyamino acids, serine and threonine (Balian & Bowes, 1977). Gelatin from tilapia, a warm-water fish species, contains higher levels of amino acids than cold-water fish gelatin (Grossman & Bergman, 1992).

The amino acid composition is mainly dependent on the source species (Eastoe & Leach, 1977), while the molecular-weight distribution and isoionic point greatly depend on the extraction process used (Muller & Heidemann, 1993). During conversion of collagen to gelatin, the inter- and intramolecular bonds linking collagen chains, as well as some peptide bonds, are broken. The more severe the extraction process, the greater the extent of hydrolysis of peptide bonds, and therefore the higher the proportion of peptides with molecular weight less than α. The isoionic point of gelatin is determined by the type of pretreatment and gelatin is classified into two types based on its isoionic point. Type A, with isoionic point of 7–9, is obtained by acid pretreatment. Type B, with isoionic point of 4 or 5, is produced by deamination during alkaline pretreatment (Veis, 1964; Ward & Courts, 1977).

13.3 IMPROVEMENT OF FUNCTIONAL PROPERTIES

Though fish waste has been considered as a potential source for the production of gelatin, mammalian gelatin is usually preferred because of its superior functional properties (Cho *et al*., 2005; Choi & Regenstein 2000), particularly high gelling and melting temperatures. Researchers have investigated various approaches to modifying fish gelatin in an effort to improve its functionality: blending of fish gelatin with other biopolymers, such as κ-carrageenan, chitosan and pectin (Chen *et al*., 2003; Haug *et al*., 2004; Uresti *et al*., 2003); addition of plasticizers, such as glycerol, sorbitol, sucrose, polyethylene glycol (Tanaka, *et al*., 2001) and solutes (Fernandez-Diaz *et al*., 2001; Haug *et al*., 2004); and addition of chemical crosslinking agents, such as glutaraldehyde, formaldehyde and glyoxal (Bigi *et al*., 2001; De Carvalho & Grosso, 2004), or enzymes, such as microbial transglutaminase (Yi *et al*., 2006). As the chemical crosslinkers are toxic, the use of enzymes as crosslinking agents has gained in significance.

Electrolytes generally have a decisive influence on the biophysical properties (swelling, solubility, gelation, viscosity and water binding capacity) of a protein, and the behavior will depend on the ionic strength and pH. The effects of different salts on the rigidity or melting temperature of warm-blooded animal gelatins have been known for a long time (Harrington & Von Hippel 1961). However, studies on the effect of salts on the viscoelastic properties of fish-skin gelatins were only initiated in 2000 by Sarabia *et al*. Although salts generally extended the setting time of gelatins, this group found that the melting temperatures were increased considerably, mainly by the addition of $MgSO_4$.

Non-electrolytes, such as sugars and glycerol, usually increase the strength of gelatin gels. Studies carried out by Naftalian & Symons (1974) indicated that the stabilization

of sugars is a structural effect that operates in the order of sucrose, D-galactose and D-glucose. These workers suggested that the stabilization is due to hydrogen bonding. The properties of gelatins can also be enhanced enzymatically. Transglutaminase is an enzyme that catalyses an acyl-transfer reaction between γ-carboxyamide groups of glutamine residues as 'acyl donors' and 3-amine groups of lysine residues as 'acyl acceptors'. The reaction results in the formation of 3-(γ-glutaminyl) lysine intra- and intermolecular crosslinking bonds in the proteins (De Jong & Koppelman, 2002). Crosslinked bonding between γ-glutamine and lysine residues due to the presence of transglutaminase has been reported to increase melting and gelling temperatures and gel strength, which could lead to the formation of products with enhanced rheological and functional properties (Fernandez-Diaz *et al*., 2001; Kolodziejska *et al*., 2004).

The gel strength and melting point of fish gelatin can also be increased by incorporation of co-enhancers such as magnesium sulfate, sucrose and transglutaminase (Koli *et al*., 2011a). The addition of co-enhancers at different combinations as per Box–Behnken design results in gel strength and melting points in the range 150.5–240.5 g and 19.5–22.5 °C, respectively. Based on these results, mathematical models were developed by following response-surface methodology (RSM) to determine optimal concentrations and the predicted maximum gel strength and melting point. Through the addition of co-enhancers at the optimal concentrations in verification experiments, the gel strength and melting point could be improved from 170.0 to 240.89 g and from 20.3 to 22.7 °C, respectively. The models developed help to produce desirable properties of fish gelatin suitable for wide range of applications in the food industry. Further, there is a tremendous scope for enhancing the functional properties of fish gelatin extracted from cold-water fish in particular, which are known to possess a low gel strength and melting point (Table 13.1).

In a preliminary investigation carried out by Bhat & Karim (2009), the impact of ultraviolet (UV) irradiation on the gel strength, viscosity and thermal properties of fish gelatin was determined by exposing the gelatin samples (dry granules) to UV for 30 and 60 minutes. Irradiated samples exhibited significant improvement in the gel strength, a marked reduction in viscosity and significant change in the melting enthalpy. The results indicated good prospects for the employment of UV radiation as an alternative method of improving some of the quality attributes of fish gelatin.

13.4 APPLICATIONS IN THE FOOD INDUSTRY

Gelatin has been extensively used in the food industry, especially in confectionery, low-fat spreads, dairy products, bakery products, meat products, beverages, desserts and ice cream (Table 13.2). It is utilized in confections mainly to provide chewiness, texture and foam stabilization; in low-fat spreads to provide creaminess, fat reduction and mouth feel; in dairy to provide stabilization and texturization; in bakery items to provide emulsification, gelling and stabilization; in meat products to provide water binding; in beverages to provide clarification by removing haze-causing materials; and in desserts and ice cream to provide gelation and emulsification (Johnston-Banks, 1990; Schrieber & Gareis, 2007).

The suitability of a gelatin for a particular application depends largely on functional properties such as gel strength, viscosity and melting point (Stainsby, 1987). In turn, the functional properties of gelatin depend on their chemical and structural features, particularly the molecular-weight distribution and amino acid composition (Muyonga

Table 13.1 Variation in yield, gel strength and melting point of fish gelatin extracted from different sources.

Fish	Source of gelatin	Yield (%)	Bloom strength (g)	Melting point (°C)	Reference
Red tilapia (*Oreochromis niloticus*)	Skin	11.75	487.61	-	See *et al.* (2010)
Yellow fin tuna (*Thunnus albacares*)	Skin	-	426	24.3	Cho *et al.* (2005)
Sole (*Solea vulagaris*)	Skin	8.3	350	19.4	Gomez-Guillen *et al.* (2002)
Megrim (*Lepidorhombus boscii*)	Skin	7.4	340	18.8	Gomez-Guillen *et al.* (2002)
Nile tilapia (*Oreochromis niloticus*)	Skin	-	328	-	Songchotikunpan *et al.* (2008)
Pangasius catfish (*Pangasius sutchi*)	Skin	10.78	324.53	-	See *et al.* (2010)
Cobia (*Rachycentron canadum*)	Dried skin	18.47	319	-	Amiza & Siti Aishah (2011)
Amur sturgeon (*Acipenser schrenckii*)	Skin	19.6	316	19.6	Mehdi *et al.* (2011)
Snakehead (*Channa striatus*)	Skin	16.57	311.18	-	See *et al.* (2010)
Catfish (*Clarias batrachus*)	Skin	27.79	278.72	-	See *et al.* (2010)
Grass carp (*Ctenopharyngodon idella*)	Scales	-	276	26.9	Zhang *et al.* (2011)
Flying gurnard (*Dactylopterus volitans*)	Skin	-	275	-	Abbey *et al.* (2008)
Tilapia	Skin	-	273	25.4	Zhou *et al.* (2006)
Grass carp (*Ctenopharyngodon idella*)	Skin	19.83	267	26.8	Kasankala *et al.* (2007)
Tilapia spp.	Skin	-	263	-	Grossman & Bergman (1992)
Bigeye snapper (*Priacanthus macracanthus*)	Skin	6.41	254.10	-	Benjakul *et al.* (2009)
Channel catfish (*Ictalurus punctaus*)	Skin	-	243–256	23–27	Liu *et al.* (2008)
Striped catfish (*Pangasius sutchi fowler*)	Skin	11.17	238.9	26.2	Jamilah *et al.* (2011)
Cobia (*Rachycentron canadum*)	Frozen skin	14.24	237	-	Amiza & Siti Aishah (2011)
Adult Nile perch (*Lates niloticus*)	Skin	16.0	229	26.3	Muyonga *et al.* (2004)
Bigeye Snapper (*Priacanthus tayenus*)	Skin	7.93	227.73	-	Benjakul *et al.* (2009)
Young Nile perch (*Lates niloticus*)	Skin	12.3	222	21.4	Muyonga *et al.* (2004)
Brownbanded bamboo shark (*Chiloscyllium punctatum*)	Skin	19.06–22.81	206–214	-	Kittiphattanabawon *et al.* (2010)
Blacktip shark (*Carcharhinus limbatus*)	Skin	21.17–24.76	206–214	-	Kittiphattanabawon *et al.* (2010)
Hoki (*Macruronus novaezelandiae*)	Skin	17.4	197	16.6	Mohtar *et al.* (2010)
Cuttlefish (*Sepia officinalis*)	Skin	2.21	181	-	Balti *et al.* (2011)
Silver carp *(Hypophthalmichthys molitrix)*	Skin Fins	6.5–7.5	176–184	26–20	Tavakolipour (2011)

Table 13.1 (*continued*)

Fish	Source of gelatin	Yield (%)	Bloom strength (g)	Melting point (°C)	Reference
Tiger-toothed croaker (*Otolithes ruber*)	Skin	7.56	170	20.36	Koli *et al.* (2011b)
	Bone	4.57	150	19.5	
Greater lizardfish (*Saurida tumbil*)	Skin	10.74	159.1	-	Taheri *et al.* (2009)
	Bone	5.08	135		
Hake *(Merluccius merluccius)*	Skin	6.5	150	12–13	Gomez-Guillen *et al.* (2002)
Pink perch (Nemipterus japonicus)	Skin	5.57	140	19.23	Koli *et al.* (2011b)
	Bone	3.55	130	19.0	
Sin croaker (*Johnius dussumieri)*	Skin		124.9	18.5	Cheow *et al.* (2007)
Channel catfish (*Ictalurus Punctatus*)	Head Bone	3.95–8.43	117–282	13.3–18.4	Liu *et al.* (2008)
Big eye Snapper (*Priacanthus hamrur)*	Skin	4.0	108	16.8	Binsi *et al.* (2009)
Salmon (*Salmo salar*)	Skin	-	108	-	Arnesen & Gildberg (2007)
Alaska pollock (*Theragra chalcogramma*)	Skin	-	98	21.2	Zhou *et al.* (2006)
Cod (*Gadus morhua*)	Skin	7.4	90	13.8	Gomez-Guillen *et al.* (2002)

Table 13.2 Functional properties of gelatin and their utility in different products.

Functional property	Products
Gelation	Water-gel desserts Jelly confectionery Dairy products Desserts
Emulsifying property	Toffees Low-fat margarines Salad dressings Whipped cream Baked products Cheese spreads Fruit chews
Texturizing property	Flavoring syrups Canned syrups Dairy products
Film-forming property	Hard and soft capsules Edible packaging materials Bioactive coatings
Water-holding capacity	Meat products Canned hams
Stabilizing property	Frozen desserts Icings and glazes Ice creams
Clarifying property	Beverages
Whipping property	Marshmallow Aerated confectionery
Foaming property	Gelatin patch for wound bandage

et al., 2004). It has been well established that the melting and gelling point of gelatin correlate with the proportion of the amino acids proline and hydroxyproline in the original collagen (Gilsenan & Ross-Murphy, 2000).

The cationic nature of gelatin makes it usable as a clarifying aid to remove haze-causing materials from beverages such as wine, fruit juices and beer. In an acidic solution, gelatin molecules have a positive charge. Wine, fruit juices and vinegar are natural media for the settling effect of type-B gelatin, because the haze-causing materials, such as yeast particles, tannins and resins, have a negative charge. These haze-causing materials have the tendency to stay in suspension. The addition of gelatin helps to speed up precipitation and settling. The positively charged gelatin and the negatively charged haze are attracted and combined to form an agglomerate that settles to the bottom of the tank (Jacobson, 2006).

13.4.1 Gelatin Gels

The ability to form a gel is one of the most important properties of gelatin. It is a unique protein, having solubility in water and the ability to form a thermoreversible gel with a

melting temperature close to body temperature. As a result, the single largest use of gelatin in food products is in water-gel desserts. The need to analyze the characteristics of a gel has resulted in the concept of gel strength, also known as bloom value. Bloom value is a measurement of gelling power and the firmness or strength of the resulting gel. The bloom value of a gelatin is defined as the weight in grams required to push a 12.5 mm-diameter plunger to a depth of 4 mm into a gel surface of 6.67% (w/w) concentration matured at 10 °C for 16–18 hours. The higher the bloom value, the stronger the gel. Based on its gel strength or bloom value, gelatin is categorized in terms of low (<150), medium (150–220) and high (220–300) bloom (Johnston-Banks, 1983).

Gelatin-based gels and other desserts have a melt-in-the-mouth characteristic lacking in other biopolymers. A literature survey shows that gelatin has been extracted from several warm-water and cold-water fish and from other marine sources (Table 13.1). The data generated on gel strength and melting point clearly demonstrate the variation in fish gelatin with respect to these important functional properties. The skin of warm-water fish such as tilapia, yellowfin tuna, sole and megrim is a potential material for gelatin production, and gelatin obtained from these sources can be used in products requiring very high gel strength. On the other hand, cold-water fish, such as cod and Alaska pollock, have very low gelling and melting points. Though gel strength is considered a useful indication of the quality of gel dessert, it is preferred that gelatin of lower gel strength has a melt-in-the-mouth characteristic. Fish gelatin, especially cold-water fish gelatin with low gel strength and low melting point, has been reported to accelerate flavor release over that in high-gel-strength gelatin, because the melting point of the latter is above normal body temperature (Choi & Regenstein, 2000; Zhou & Regenstein, 2007).

Gelling is also regarded as the 'water-absorbing property' of a given product. Gelatin swells when placed in water, absorbing 5–10 times its own volume. It is highly prized for its ability to gel with water during the production of table jellies. An interesting application of this gelation property is the addition of gelatin to canned ham before cooking. On cooking, the exudates from the meat are absorbed by the gelatin and appear as a gel when the can is opened.

While studying instrumental textural characteristics of gels prepared from gelatin extracted from grouper skin, as compared to gelatin from mammalian sources (bovine and porcine), Rahman & Al-Mahrouqi (2009) observed a notable textural behavior of fish gelatin. At 10% concentration, the textural characteristics of fish-skin gelatin gels were significantly lower than those of the gels prepared from bovine and porcine gelatins. However, in the case of fish-skin gelatin gels, all texture-profile-analysis (TPA) attributes (hardness, cohesiveness, adhesiveness, springiness and gumminess) of 10% gels showed significant differences from those of 20 and 30% gels. But in the case of bovine and porcine samples, such generic trends were not observed. In view of its concentration-dependent mechanical gelling characteristics, fish gelatin can be utilized in preparing different types of gels as per the requirement for diverse products.

Recently, a new application for fish gelation has been found: as an additive in surimi processing. Surimi is washed minced fish meat used as the raw material for seafood analogs such as crabmeat substitutes. The most important attributes of surimi are gelling and whiteness. Fish gelatin has been evaluated as an additive in surimi, in order to improve the mechanical and functional properties of gels. The results indicate that fish gelatin may be used as a functional additive to produce Alaska pollock surimi gels, but it is not recommended for FA-grade surimi (Hernández-Briones *et al*., 2009).

13.4.2 Food Emulsions

Its amphoteric nature and the presence of hydrophobic zones on its peptide chain lead gelatin to show strong emulsifying and emulsion-stabilizing properties (Cole, 2000). Consequently, it has been proven that gelatin has the ability to form a strong adsorbed layer at the interface to prevent droplet coalescence. Because of this property, it is widely used in the manufacture of toffees and water-in-oil emulsions such as low-fat margarine, salad dressings and whipped creams. A low-fat spread has been prepared using fish gelatin and pectin (Cheng *et al*., 2008).

The influence of molecular weight on the ability of fish gelatin to form and stabilize oil-in-water emulsions was examined by Surh *et al*. (2006). Low-molecular-weight fish gelatin (LMW-FG, w55 kDa) and high-molecular-weight fish gelatin (HMW-FG, 120 kDa) were used to prepare 20% (w/w) corn oil-in-water emulsions, which were found to be moderately stable to creaming, with the majority of particles distributed throughout the volume of the sample and only a small fraction (of presumably large droplets) creaming. Further, LMW-FG emulsions showed a better creaming stability than HMW-FG emulsions, even though the continuous-phase viscosity was less, which was attributed to a depletion flocculation effect (Surh *et al*., 2006). The emulsions remained fairly stable when they were subjected to thermal treatments (30 and 90 °C for 30 minutes), high salt concentrations (250 mM NaCl) and various pH values (3–8), demonstrating the potential of fish gelatins as emulsifiers in food products.

13.4.2.1 Oxidatively Stable Emulsions

A major potential advantage of proteins as emulsifiers in foods is their ability to protect polyunsaturated lipids from iron-catalyzed oxidation (Hu *et al*., 2003). At pH values below their isoelectric point (pI), proteins form positively charged interfacial membranes around oil droplets that electrostatically repel any Fe^{2+} and Fe^{3+} ions present in the aqueous phase. Thus, iron is prevented from catalyzing oxidation of the polyunsaturated lipids contained within the droplets. One of the major limitations of using food proteins for this purpose is that most of them have pI in the pH range 4.5–5.5, so cationic emulsion droplets can only be produced at relatively low pH values (Damodaran, 1996). On the other hand, the aqueous phase of many food products has a pH > 5, and therefore emulsions created using most protein emulsifiers would contain anionic droplets that are prone to lipid oxidation, because cationic iron ions would be attracted to the droplet surfaces (McClements & Decker, 2000).

The relatively high isoelectric point (pI > 7.0) of type-A gelatin makes it possible to create oil-in-water emulsions that have a positive charge over a wider range of pH values than are produced by conventional protein emulsifiers, such as soy, casein or whey proteins (Dickinson & Lopez, 2001). Consequently, type-A gelatin may be suitable for creating oil-in-water food emulsions with high oxidative stability, since it could repel iron ions from oil-droplet surfaces over most of the pH range typically found in foods.

Attachment of 5% oxidized phenolic compounds to cuttlefish-skin gelatin enhanced antioxidative activity with no detrimental effect on the emulsifying properties of the resulting gelatin. Use of gelatin modified with oxidized tannic acid could effectively inhibit lipid oxidation of menhaden oil-in-water emulsion (Aewsiri *et al*., 2009). The efficacy is dose-dependent. Therefore, cuttlefish-skin gelatin modified with oxidized tannic acid could be used as an emulsifier with antioxidative activity in emulsion systems.

13.4.3 Nutritional Supplements

The use of gelatin in the nutraceutical industry is widespread. Gelatin not only serves as an excipient but also acts as an excellent and economical source of multiple amino acids. Even though gelatin lacks the amino acid tryptophan, it can still serve as a valuable protein supplement. Studies have shown that up to 50% of muscle protein in a meat product can be replaced with gelatin while keeping the same nutritive value. Gelatin is the foundation of many nutritional and arthritis/joint-care formulas. Further, it is also considered to be a highly digestible dietary component, ideal for inclusion in certain dietary supplements. Hence it is widely used for nutritional bars and protein drinks. Gelatin is also recommended for enhancement of protein levels in foodstuffs, and especially in body-building foods. In addition, gelatin is used to reduce carbohydrate levels in foods formulated for diabetic patients (Gans, 2007).

Hydrolyzed gelatin, also known as 'nongelling' gelatin, is cold water-soluble and still retains the other functional properties of conventional gelatin. It is mainly used for protein drinks, protein energy bars and nutritional formulas. Fish gelatin, particularly cold-water fish gelatin, with low gelling properties assumes importance as an ideal ingredient for incorporation into nutritional supplements.

13.4.4 Biodegradable Edible Films for Food Packaging

The protection of foods by packaging has traditionally been based on provision of an inert barrier to the outside environment (Rooney & Yam, 2004). Packaging materials provide physical protection and create proper physicochemical conditions for products, which are essential to obtaining a satisfactory shelf life. The packaging system, based on a proper choice of packaging materials with appropriate barrier and mechanical properties, prevents product deterioration due to physicochemical or biological factors and maintains overall quality during storage and handling. After their useful life, it is desirable for packaging materials to biodegrade in a reasonable time period without causing environmental problems. Biopolymer-based packaging materials have some beneficial properties in improving food quality and extending the shelf life through minimization of microbial growth in the product.

Natural biopolymers have an advantage over synthetic polymers in that they are biodegradable and renewable as well as edible. However, their relatively poor mechanical and water-vapor barrier properties are a major limitation to their industrial use. Protein and polysaccharide films are generally good barriers against oxygen at low to intermediate relative humidity and have good mechanical properties; however, their barrier against water vapor is poor due to their hydrophilic nature. Research efforts have been focused on the modification of natural biopolymer-based films to improve their mechanical and water-vapor barrier properties (Rhim & Perry, 2007).

Gelatin is one such versatile biomaterial obtained from skin and bones generated as waste during animal slaughter and fish processing (Ahmad *et al*., 2011). It is known for its excellent film-forming ability (Hoque *et al*., 2010). Gelatin-based film coating or packaging can maintain the quality of foods during storage, due to its barrier to oxygen and light and its prevention of dehydration and lipid oxidation (Jongjareonrak *et al*., 2011). It serves not only as a barrier to moisture, water vapor, gases and solutes but also as a carrier of some active substances. Biopolymer films may also serve as gas and solute barriers and complement other types of packaging by improving the quality and

Table 13.3 Fish gelatin-based edible films for food packaging.

Skin source	Plasticizer	Reference
Tilapia	Glycerol	Nur Hanani *et al.* (2012)
Cod, haddock and pollock	Glycerol	Krishna *et al.* (2012)
Cuttlefish (*Sepia pharaonis*)	Glycerol	Hoque *et al.* (2011a, 2011b)
Bigeye snapper (*Priacanthus tayenus*)	Extract from seaweed	Rattaya *et al.* (2009)
Giant squid (*Dosidicus gigas*)	Glycerol, sorbitol	Giménez *et al.* (2009a)
Alaska pollock (*Theragra chalcogramma*) and Alaska pink salmon (*Oncorhynchus gorbuscha*)	-	Chiou *et al.* (2009)
Baltic cod (*Gadus morhua*)	Trans glutaminase, EDC	Piotrowska *et al.* (2008)
Baltic cod (*Gadus morhua*)	Trans glutaminase, EDC	Staroszczyk *et al.* (2012)
Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*)	Glycerol, sorbitol, ethylene, glycol, PEG 200, PEG 400	Jongjareonrak et al. (2006b)

extending the shelf life of foods. Furthermore, biopolymer films are excellent vehicles for incorporating a wide variety of additives, such as antioxidants, antifungal agents, antimicrobials, colors and other nutrients (Wong *et al*., 1994).

Among the different plasticizers as mentioned in Section 13.4, glycerol and sorbitol are widely utilized to improve the mechanical properties of fish-gelatin films (Tables 13.3, 13.4 and 13.5). Vanin *et al*. (2005) concluded that glycerol was compatible with gelatin and showed the highest plasticizing effect on the mechanical properties of the film, producing a flexible and easy-handling film with no phase separation. Sobral *et al*. (2001) reported that the effects of sorbitol on the water-vapor permeability, mechanical and thermal properties of edible films based on gelatin produced a reasonable plasticizing effect on the puncture force. They also reported that the water-vapor permeability increased with the increase in sorbitol content, and the increase in sorbitol content did not reduce the formation of junction zones in the films.

13.4.4.1 Biocomposite and Nanocomposite Films

Edible films can be prepared from protein, polysaccharides, lipids or the combination of these components (Cao *et al*., 2007; Cuq *et al*., 1997). Starch-based films provide a minimal barrier to moisture because of the hydrophilic properties of the starch polymers. Films of protein or polysaccharides have overall suitable mechanical and optical properties but are highly sensitive to moisture and exhibit a poor water-vapor barrier (Guilbert *et al*., 1996). Therefore, composite films and coatings can be formulated which consist of several biopolymers. For example, the lipid component in the film formulation can serve as a good barrier to water-vapor permeability (Garcia *et al*., 2000) and polysaccharides and proteins can be reasonably effective as gas barriers (O_2 and CO_2) (Arvanitoyannis *et al*., 1996; Baldwin *et al*., 1995). Lipid films are more resistant to moisture, but vulnerable to oxidation. Therefore, the new trend is to combine different biopolymers for food packaging and coatings (Gomez-Guillen *et al*., 2008).

Table 13.4 Fish gelatin-based biocomposite films.

Skin source	Plasticizer	Copolymer	Reference
Skin of walleye pollock (*Theragra chalcogramma*)	-	Chitosan	Liu *et al.* (2012)
Tuna skin	Glycerol and sorbitol	Chitosan	Gómez-Estaca *et al.* (2011)
Skin of cod, haddock and pollock	Glycerol and sorbitol	Lignosulfonate from eucalyptus wood	Núñez-Flores *et al.* (2012)
Commercial fish gelatin	Glycerol and sorbitol	Lignin	Ojagh *et al.* (2011)
Cuttlefish (*Sepia pharaonis*)	Glycerol	Mungbean (*Phaseolus aureus*) protein isolate	Hoque *et al.* (2011c)
Commercial cod gelatin	Glycerol	Soy protein isolate	Guerrero *et al.* (2011)
Tilapia fish skin	-	Gellan and κ-carrageenan	Pranoto *et al.* (2007)
Fish gelatin	Glycerol and sorbitol	Sago starch	Al-Hassan & Norziah (2012)
Blue shark (*Prionace glauca*) skin	Glycerol	Stearic and oleic acids	Limpisophon *et al.* (2010)
Cod (Gadus morhua) skin	Glycerol and sorbitol	Sunflower oil	Pérez-Mateos *et al.* (2009)

Table 13.5 Fish gelatin-based active films for food preservation.

Skin source	Plasticizer	Bioactive ingredient	Reference
Unicorn leatherjacket (*Aluterus monoceros*)	Glycerol	Bergamot (*Citrus bergamia*) oil and lemongrass (*Cymbopogon citratus*) oil	Ahmad *et al.* (2012)
Tuna	Sorbitol and glycerol	Extracts from oregano (*Origanum vulgare*) and rosemary (*Rosmarinus officinalis*)	Gómez-Estaca *et al.* (2009a)
Sole (*Solea* spp.) and catfish	Sorbitol and glycerol	Extracts from seeds of borage (*Borago officinalis*), BHT and α-tocopherol	Gómez-Estaca *et al.* (2009b)
Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*)	Glycerol	BHT and α-tocopherol	Jongjareonrak *et al.* (2011)
Tuna (*Thunnus tynnus*)	Glycerol	Extracts from Murta (*Ugni molinae* Turcz) leaves	Gómez-Guillén *et al.* (2007)
Cuttlefish (*Sepia pharaonis*)	Glycerol	Extracts from cinnamon, clove and star anise	Hoque *et al.* (2011d)
Giant squid (*Dosidicus gigas*)		Hydrolysates from squid gelatin	Giménez *et al.* (2009b)

Several biopolymers, such as chitosan, lignin, protein isolates and carrageenan, have been tested by various research groups for their potential to produce better barrier and mechanical properties in fish gelatin-based films (Table 13.4). Al-Hassan & Norziah (2012) determined the physical and mechanical properties of edible films based on blends of sago starch and fish gelatin plasticized with glycerol or sorbitol (25% (w/w)). The morphology study of the sago starch/fish gelatin films showed smoother surfaces, with decreasing protein in the samples, for both plasticizers. Differential scanning calorimetry scans showed that plasticizers and protein incorporated with sago-starch films reduced the glass transition temperature (Tg) and melting temperature (Tm) and the melting enthalpy (DHm). A single Tg was observed in the study, indicating compatibility between the sago starch and fish gelatin polymers.

Owing to the ability of nanosized material fillers to significantly improve polymer properties when compared with polymer alone or microscale composites, emerging nanotechnology tools have been applied to the development of nanocomposite films. Potential improvements include enhanced mechanical strength, weight reduction, increased heat resistance and improved barrier properties (Ray & Okamoto, 2003). Accordingly, in order to obtain packaging films with high mechanical strength and high barrier properties, gelatin films have been filled with layered silicates. The effect of clay content, homogenization RPM and pH on the mechanical and barrier properties of fish gelatin/nanoclay composite films has been investigated. The addition of 5% (w/w) nanoclay increased the tensile strength from 30.31 ± 2.37 MPa to 40.71 ± 3.30 MPa. The 9 g clay/100 g gelatin film exhibited the largest improvements in oxygen and water barrier properties (Bae *et al*., 2009).

13.4.4.2 Active Films for Food Preservation

Nowadays, consumers are more health-conscious about pursuing a natural lifestyle and expect products that reflect their interests and values. Marine supplements that are made from renewable resources are increasingly becoming a priority for these consumers. Over the years, increasing attention has been given to active packaging, which is a group of packaging technologies that perform some role in the preservation of food other than to provide an inert barrier (Brody *et al*., 2001). Active packaging is a kind of food-preservation system in which bioactive compounds are incorporated into the package. Various bioactive ingredients, such as antioxidants and antimicrobials in the form of plant extracts or essential oils, have been evaluated for their protective and preservative roles (Table 13.5). Release of bioactive ingredients in a controlled manner to the food contributes to shelf-life extension and quality retention.

Since oxidation is commonly initiated at the food surface, antioxidant-releasing packaging is a promising means by which to protect food surfaces from rancidity. A slow-release mechanism provides a continuous replenishment of antioxidant to the food. Oxidation of lard has been effectively retarded by covering it with fish-skin gelatin films of bigeye snapper and brownstripe red snapper species incorporated with BHT and α-tocopherol (Jongjareonrak *et al*., 2011). Essential oils derived from natural sources have been added to films as antimicrobial agents (Pereda *et al*., 2011). Essential oils categorized as GRAS (generally recognized as safe) by the US Food and Drug Administration (FDA) (Persico *et al*., 2009) can be considered potential alternatives to synthetic additives (Valentao *et al*., 2002). Essential oils, especially from bergamot and lemongrass, appear to be interesting natural compounds with great potential for use in foodstuff preservation. *In vitro* studies have revealed significant antimicrobial effects of these essential oils (Lv *et al*., 2011).

Thus the incorporation of bergamot and lemongrass essential oils into gelatin films offers the possibility not only of imparting antimicrobial activity but also of improving physicochemical properties. In general, essential oils—the odorous volatile products of an aromatic plant's secondary metabolism—are well known as antimicrobial agents that can be used to control food spoilage and foodborne pathogenic bacteria (Kalemba & Kunicka, 2003). Essential oil-based antimicrobial packaging has high potential for food applications (Emiroglu *et al*., 2010).

Gelatin films incorporated with bergamot and lemongrass oil at various concentrations as a glycerol substitute were prepared and characterized by Ahmad *et al*. (2012). Significant changes of molecular organization and higher intermolecular interactions among gelatin molecules were found in the film structure, as determined by FTIR. Thermogravimetric analysis (TGA) demonstrated that films with added bergamot and lemongrass oil exhibited enhanced heat stability, with a higher degradation temperature, compared with control film. Scanning electron microscopic (SEM) images revealed the presence of micropores in the essential oil-incorporated films, which contributed to physical properties of the resulting films. Thus, gelatin films incorporated with bergamot and lemongrass oil can be used as active packaging, and their properties can be modified by the choice of essential oil added.

13.5 APPLICATIONS IN THE PHARMACEUTICAL INDUSTRY

Gelatin is routinely used in medical applications, because of its biodegradability and biocompatibility in the physiological environments (Young *et al*., 2005). It is widely used for the preparation of hard and soft capsules, for encapsulation of drugs and bioactive molecules, as an antioxidative hydrolysate, as a carrier in controlled drug delivery, as a component in dietary health supplements and in wound care. Other pharmaceutical applications include its use in tablets, emulsions, ointments, medical glue, salves, granulation and syrups. Gelatin is also utilized as a matrix for implants, in injectable drug-delivery microspheres and in intravenous infusions (Pollack, 1990; Rao, 1995; Saddler & Horsey, 1987). It has also been reported that live attenuated viral vaccines used for immunization against measles, mumps, rubella, Japanese encephalitis, rabies, diphtheria and tetanus contain gelatin as a stabilizer (Burke *et al*., 1999).

Several patents exist on the utility of fish gelatin in various pharmaceutical applications, such as hard and soft capsules, colloidal compositions, medical glue and sealants (Table 13.6). Gelatin surgical sponges are used to stanch surgical bleeding. These sponges are completely resorbed in the body. Because of its low melting point close to human body temperature, gelatin is used in the preparation of suppositories, which are small plugs of medication designed to melt at body temperature within a body cavity other than the mouth, especially the rectum, urethra or vagina, where they melt and release their active substance.

Gelatin is also used as a colloid in plasma-substitute solutions, which are used to control the circulating blood volume in the management of shock resulting from hemorrhages or burns. In such applications, the size of the gelatin molecules is controlled to prevent gel formation and to maintain sufficient osmotic pressure. Fish gelatin, especially nongelling gelatin, may be an ideal ingredient for these purposes.

Table 13.6 Patents on the utility of fish gelatin in food and pharmaceutical industries.

Product/use	Reference
Hard capsule	Yang (2007)
Film-forming composition for hard capsule	Park *et al.* (2007)
Soft gel capsules	Haug & Draget (2011)
Chewable soft capsule	Rowe & Garnett (2005)
Flavored gelatine capsule	Opheim (2002)
As a carrier in pharmaceutical composition	Murray *et al.* (2004)
Fish gelatin-based nanoparticles	Bahl *et al.* (2011)
Encapsulation using fish gelatin	Soper (1997)
Particulate drug microencapsulated with gelatin	Apfel *et al.* (1991)
Fat-soluble composition of colloidal fish gelatin	Berneis & Schuler (1995)
Hydrocolloid system containing an active pharmaceutical agent	Scott *et al.* (2006)
Fish-maw glue product	Zhang *et al.* (2004)
Crosslinking medical glue	Gaissmaier & Ahlers (2009)
Hemostatic dressings and sealants	Preiss-Bloom *et al.* (2010)
Fish-scale collagen and its production process	Wu & Chai (2006)
Partly hydrolyzed fish gelatin for treatment of arthrosis, osteoporosis etc.	Bonanomi & De Gregorio (2004)
Food products with a reduced fat content	Grossman *et al.* (1993)
Fish gelatin as an ingredient in drug tablets	Hansen *et al.* (2002)

13.5.1 Fish Gelatin-based Hard and Soft Capsules

The film-forming property of gelatin has been extensively utilized in the manufacture of pharmaceutical capsules. Because gelatin shrinks with great force on drying, polyhydric alcohols (such as glycerol, sorbitol, polyethylene glycerol (PEG), mannitol, mantitol and their combinations) are added to modify the adhesion and flexibility of the dry film. Park *et al*. (2007) patented a process describing the preparation of a film-forming composition for hard capsules composed of fish gelatin. The problem caused by the low gelling nature of fish gelatin was overcome by crosslinking with the transglutaminase.

There are currently two types of capsule: two-piece hard gelatin capsules, used for powders, and one-piece soft gelatin capsules, used for liquids. These capsules need to be robust enough for high-speed filling machinery while also having elasticity for filling and softening characteristics that allow the capsule to seal rapidly after filling. Investigation of the thermomechanical properties of fish gelatin when mixed with glycerol and water, the standard mixture used for soft capsules, shows it to have similar behavior to bovine and porcine gelatin (Gomez-Guillen *et al*., 2008).

The soft-capsule shell is usually made with a gelatin with poor gel strength, necessitating the use of either cold-water fish gelatin or a mixture of cold and warm fish gelatin/κ-carrageenan. The capsule shell comprises at least 40% (w/w) cold-water fish gelatin relative to the total weight of gelatin. Further, the films of fish gelatin, particularly of cold-water fish gelatin possessing low gel strength, have lower water-vapor permeability, which can be useful in reducing water loss in gel-encapsulated drugs and in refrigerated and frozen food systems (Avena-bustillos *et al*., 2006).

13.5.2 Antioxidative Fish-gelatin Hydrolysates

In view of the reported antioxidant activity of gelatin hydrolysates, several researchers have investigated hydrolysis of fish gelatin by both enzymatic and thermal hydrolysis methods. In a pioneering work, Kim *et al*. (2001) produced fish-gelatin hydrolysates from Alaskan pollock by enzymatic hydrolysis. Subsequently, gelatin hydrolysates have been prepared by several researchers from different marine sources, such as hoki fish (Mendis *et al*., 2005a), cobia skins (Yang *et al*., 2008), giant squid (Mendis *et al*., 2005b) and bullfrog (Qian *et al*., 2008). Most of the gelatin hydrolysates investigated in these studies showed antioxidant activity. Recently, gelatins obtained from marine sources such as the skins of tuna and halibut and the tunics of jumbo flying squid were hydrolyzed by Alcalase to produce antioxidant peptides (Aleman *et al*., 2011). This enzymatic hydrolysis yielded an increase in the antioxidant capacity of the gelatins when measured by the ferric-reducing antioxidant power (FRAP) assay and the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical-scavenging method.

By following thermal hydrolysis, Yang *et al*. (2008) have reported antioxidative gelatin hydrolysates from cobia (*Rachycentron canadum*) in which alkali-pretreated skin was extracted in a retort (121 °C) for 30 minutes in order to obtain a retorted skin gelatin hydrolysate (RSGH). The molecular-mass distributions and antioxidant activities of cobia RSGH and enzyme-treated RSGHs (ET-RSGHs) derived from bromelain, papain, pancreatin and trypsin digestion were characterized. The molecular-mass distribution of the RSGH ranged mainly between 20.0 and 0.7 kDa, and those of ET-RSGH between 6.5 and 0.7 kDa. The same research group (Yang *et al*., 2009) produced RSGH from tilapia-skin gelatin by following a face-centered, central-composite design, and found that retorting time had significant effects on α,α-diphenyl-bpicrylhydrazyl (DPPH) scavenging by RSGHs. Based on the response surface models of DPPH scavenging ($R^2 = 0.977$) and inhibition of lipid peroxidation ($R^2 = 0.967$), the predicted maxima were 79.4% for DPPH scavenging activity and 77.3% for lipid peroxidation inhibition, demonstrating tilapia RSGH to be a potential source of natural antioxidant.

13.5.3 Collagen Peptides

Collagen peptide is a hydrolyzed form of collagen or gelatin with a molecular weight in the range 1–5 kDa and the same amino acid distribution as that of collagen/gelatin. It contains di-, tripeptides and free amino acids that are easily absorbed in the body, and the presence of Pro-Hyp and Pro-Hyp-Gly peptides stimulates collagen formation during the wound-healing process.

Oral consumption of collagen peptides has been reported to provide beneficial effects to the body, particularly in improving joint health and hastening the rate of ulcer healing. Collagen peptides as a food supplement may improve low bone mineral density in people suffering from malnutrition and degenerative joint diseases (Wu *et al*., 2004). Reports also indicate that consumption of collagen peptides can thicken hairs (Scala *et al*., 1976), improve nail disorders such as brittle nails, increase the size of collagen fibrils in the Achilles tendon (Minaguchi, *et al*., 2005), induce fibroblast density and enhance the formation of collagen fibrils in the dermis and so on (Matsuda *et al*., 2006).

13.5.3.1 Fish-scale Collagen Peptides

Wu & Chai (2006) patented an enzymatic method for producing collagen peptides from fish scales. The effect of daily ingestion of fish-scale collagen peptides (FSCPs) derived

from the scales of *Tilapia zillii* on skin damage induced by repeated UV-B irradiation was examined by Tanaka *et al*. (2009). Ingestion of FSCP at 0.2 g/kg/day was reported to suppress UV-B-induced decreases in skin hydration, hyperplasia of the epidermis and soluble type-I collagen, demonstrating the beneficial role of FSCPs as a dietary supplement in suppressing UV-B-induced skin damage and photoaging.

The FSCPs have also been prepared by Chai *et al*. (2010), using a patented protocol (Wu & Chai, 2006) to hydrolyze tilapia (*Oreochromis* sp.) scales. FSCPs were found to stimulate fibroblast cell proliferation and procollagen synthesis in a time- and dose-dependent manner. The transdermal penetration capabilities of the fractionationed FSCPs were evaluated using the Franz-type diffusion cell model. The heavier FSCPs (3.5 and 4.5 kDa) showed higher cumulative penetration capability as compared to the lighter FSCPs (2.0 and 1.3 kDa). The FSCPs, particularly the heavier ones, have been concluded to efficiently penetrate the stratum corneum to the epidermis and dermis, activate fibroblasts and accelerate collagen synthesis.

13.5.4 Carriers in Controlled Drug Delivery

Although various biodegradable nanoparticles of natural polymers such as starch (Hamdi *et al*., 1998) and chitosan (Andrianov & Payne, 1998) are widely used as drug carriers in controlled drug-delivery systems, gelatin nanoparticles represent a promising carrier system for controlled drug delivery. Gelatin is a natural macromolecule and has several advantages over synthetic polymers that make it a suitable material for use as a nanoparticulate carrier. Considerable work has been performed using gelatin as a carrier system for the delivery of drugs and bioactive molecules (Mladenovska *et al*., 2002).

The interest in gelatin as a potential source of nanomaterials is based on the fact that it is biodegradable, nontoxic and easy to crosslink and to modify chemically (Jahanshahi *et al*., 2008). It is also inexpensive and easy to sterilize (Schwick & Heide, 1969).

The addition of a chemical crosslinker such as glutaraldehyde gives gelatin stability, shape and a raised circulation time *in vivo* as compared to unmodified particles (Jahanshahi *et al*., 2008; Jameela & Jayakrishnan, 1995). This structural change improves its performance, properties and characteristics, for example giving it insolubility at elevated temperatures, reduced swelling in water and lower permeability to cell membranes (Levy *et al*., 1982), making it an ideal candidate for carrying drugs.

Fish gelatin, particularly that obtained from cold-water fish sources in the nongelling and nonhydrolyzed form, was used as a carrier in a pharmaceutical composition designed to release the active ingredient rapidly on contact with a fluid (Murray *et al*., 2004). The composition can be used for oral administration, where it releases the active ingredient in the oral cavity on contact with saliva.

13.6 CONCLUSION

Although fish gelatin may not be able to completely replace mammalian gelatin, it can still become a versatile ingredient, offering novel and viable properties and meeting the demands of the global halal/kosher market. Unlike the invariable composition of land-based mammalian gelatin, fish gelatin can be extracted from the skins, bones and scales of cold- or warm-water fish, providing a lot of variation in composition, particularly with respect to amino acid content, and allowing diverse applications

in the food and pharmaceutical industries. Further, fish gelatins are available in both gelling and nongelling forms. Nongelling fish gelatins find applications in the microencapsulation of bioactive/flavor compounds and as stabilizers and film-forming, binding and clarifying agents.

Though kosher gelatin fetches a premium price over the non-kosher version, its availability is still rather low, revealing vast scope for its production and use. The most important challenges of fish gelatin are its fishy odor and poor rheological properties, limiting its utility to a few products. Development of improved processes for eliminating the odor and enhancing the desirable functional and rheological properties by incorporating co-enhancers would certainly make fish gelatin a versatile ingredient for both the food and the pharmaceutical industry.

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14 Health Effects of Antioxidative and Antihypertensive Peptides from Marine Resources

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14.1 INTRODUCTION

The health benefits of a diet rich in seafood are well recognised. Seafood (fish and shellfish) consumption is known to protect against development of cardiovascular diseases (CVD), particularly coronary heart disease (CHD) and CHD mortality (de Leiris *et al*., 2009; He *et al*., 2004; Marik & Varon, 2009; Saremi & Arora, 2009), as well as against several other diseases and medical conditions, including inflammatory diseases such as arthritis and inflammatory bowel diseases (Calder, 2008; James & Cleland, 1997; Rosell *et al*., 2009; Ruggiero *et al*., 2009), beneficial outcomes in foetal and infant development (Carlson, 2009; Cohen *et al*., 2005; Eilander *et al*., 2007; Helland *et al*., 2003; Hibbeln *et al*., 2007; McCann & Ames, 2005; Ryan *et al*., 2010) and psychological disorders (Appleton *et al*., 2010; Freeman *et al*., 2006; Lin & Su, 2007; Liperoti *et al*., 2009). The fatty acid profile of seafood is considered favourable and its beneficial effects have mainly been attributed to the n-3 polyunsaturated fatty acids (n-3 PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, other components in seafood may also contribute to the observed health effects, either synergistically with n-3 PUFA or through separate mechanisms. Seafood is considered an important source of minerals, such as selenium, iodine and zinc. Fatty fish is rich in vitamin D, and vitamin B_{12} is more abundant in seafood than in terrestrial food sources (von Castel-Roberts *et al*., 2010).

The quality of fish proteins is also considered excellent as they contain all essential amino acids and are easily digestible (Friedman, 1996). In recent years, increased attention has been directed towards the potential health benefits of peptides of marine origin. These peptides, either released during gastrointestinal digestion or manufactured *ex vivo*, may provide beneficial health effects in addition to those of n-3 PUFA. Some peptides have been found to exhibit antioxidative and antihypertensive properties. Herein these properties of marine-derived peptides are reviewed, and their health benefits are evaluated.

14.1.1 Origin of Peptides

Bioactive peptides usually consist of 2–30 amino acids (Pihlanto-Läppäla, 2001) and their activity is based on the composition and sequence of these. While some peptides are present naturally as such in foods (Samaranayaka & Li-Chan, 2011), the majority

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are incorporated in proteins. Peptides are inactive within the parent protein and need to be released during fermentation, food processing, *in vitro* enzyme-catalysed proteolysis or digestion. Most of the reported bioactive peptides are produced by *in vitro* enzymatic hydrolysis and this process enables the manufacture of bioactive peptides with desired properties. The properties of the peptides depend on an array of variables, such as hydrolysis time, temperature, enzyme, enzyme/substrate ratio, pH and pretreatment (Kim & Wijesekara, 2010; Udenigwe & Aluko, 2012). During fermentation, the action of microbes and their endogenous proteolytic enzymes releases peptides that may be purified. However, *in vitro* enzymatic hydrolysis gives more consistent molecular-weight profiles and peptide composition, and is often preferred due to better control of the peptides produced and the shorter time required to obtain a similar degree of hydrolysis to that found in natural fermentation (Samaranayaka & Li-Chan, 2011).

14.2 ANTIOXIDATIVE PEPTIDES

14.2.1 Antioxidants and Health Effects

Oxidation is a vital process for all living organisms. During metabolism and respiration, reactive oxygen species (ROS) are constantly and unavoidably produced. These include superoxide anion radicals (O_2^-), hydroxyl radicals (OH) and non-free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In excess, ROS can exert oxidative damage to proteins, lipids and DNA by subtracting electrons, thus starting chain reactions, in which the new radicals attack other molecules (Kaur & Kapoor, 2001). All living organisms have antioxidants to counter oxidation and the potential harm of oxidative components. Antioxidants are, in biological terms, 'natural substances that delay, prevent or repair oxidative damages to a target molecule' (Halliwell & Gutteridge, 2007). Imbalance between ROS and antioxidants may cause oxidative stress, which is associated with several diseases, including CVD (Lakshmi *et al*., 2009), diabetes (Yang *et al*., 2011), cancer (Cerutti & Trump, 1991) and neurodegenerative disorders (Katzman & Saitoh, 1991), and ageing (Sohal & Orr, 2012). Consumption of dietary antioxidants is now recognised to be effective in increasing the body's antioxidant load, which is considered particularly important when the diet contains a high amount of PUFA. However, epidemiological data on natural antioxidant intake and disease prevention are controversial and inconclusive. One recent meta-analysis investigating the effects of intake of antioxidant supplements did not find any preventative effect in relation to all-cause mortality (Bjelakovic *et al*., 2012), while another study did clearly indicate beneficial health effects (Wild *et al*., 2004). Although the impact of antioxidants on various health outcomes is inconclusive, current investigations in this area may provide further evidence of such effects.

14.2.1.1 Cardiovascular Diseases

The correlation between oxidative stress and CVD is now generally accepted. Lakshmi *et al*. (2009) have reviewed the biochemical evidence concerning the link between ROS and CVD, including atherosclerosis, hypertension, heart failure and ischemia/reperfusion injuries. Endothelial dysfunction is an early event in the development of atherosclerosis. The endothelium is the inner layer of the blood vessels. It regulates vascular-tone permeability and the flow of nutrient substances, biologically active molecules and blood

cells (Cines *et al*., 1998). In addition, it produces a variety of regulator mediators, including nitric oxide (NO), which is a potent vasodilator with antiplatelet, antiproliferative, permeability-decreasing and anti-inflammatory properties (Cines *et al*., 1998). Atherogenesis is initiated when the endothelial monolayer is activated by stimuli such as dyslipidemia, hypertension or pro-inflammatory mediators (Nahrendorf *et al*., 2012). The activated endothelium release less NO, and leukocytes are captured on the endothelial lining by increased exposure of leukocyte adhesion molecules. Endothelial dysfunction is manifested in a reduction in the endothelial barrier properties and an increase in endothelial permeability. The arrested leukocytes transmigrate into the subendothelial layers. Development of atherosclerotic plaque also involves both elevated plasma cholesterol levels and proliferation of smooth-muscle cells. Low-density lipoproteins (LDLs) are readily oxidised by hydroxyl radicals and become oxidised-LDLs (ox-LDLs). Ox-LDLs are absorbed by sub-endothelial leukocyte-derived macrophages, which form foam cells in the arterial intima. Accumulation of such foam cells results in the formation of 'fatty streak'. Vascular smooth-muscle cells migrate from the media into the intima and their proliferation contributes to the formation of plaque. Both ROS and ox-LDLs are also believed to play a critical role in hypertension, although the underlying mechanisms remain unexplored (Lakshmi *et al*., 2009). ROS may inactivate NO and thereby cause vasoconstriction (Cines *et al*., 1998), and also trigger mechanisms for oxidative damage to macromolecules, such as LDLs, which has been observed to be increased in hypertensive subjects. Oxidative stress is increased in heart failure and may contribute to structural and functional changes that characterise disease progression. The evidence for association between oxidative stress and CVD is clear, but the use of antioxidant therapy to prevent such diseases has given mixed results (Jialal & Devaraj, 2003; Patterson *et al*., 2000). Several recent studies have, however, indicated that dietary antioxidants in combination with n-3 PUFA reduce atherogenesis in animal atherosclerosis models (Eilertsen *et al*., 2011, 2012; Verschuren *et al*., 2011).

14.2.1.2 Diabetes Mellitus

Diabetes mellitus is a group of diseases characterised by hyperglycaemia that is caused by absolute or relative insulin deficiency or insulin resistance (Yang *et al*., 2011). Around 170 million individuals are affected by this disease worldwide (Quinn, 2001) and it is expected to alter the lives of 366 million individuals over the next 25 years if the obesity level remains constant (Wild *et al*., 2004). There are two forms of diabetes mellitus: type 1 and type 2. Type-1 or insulin-dependent diabetes is manifested early in life. It is caused by a failure in the pancreatic production and release of insulin, and may lead to long-term complications. Type-2 or non-insulin-dependent diabetes results from insulin resistance. This form is by far the most dominant, representing more than 80% of all cases, and globally the incidence is increasing (Yang *et al*., 2011). Oxidative stress may directly promote the onset of type-2 diabetes by decreasing insulin sensitivity and injuring the insulin-producing β-cells in the pancreas (Yang *et al*., 2011). In addition, ROS are also believed to modify a number of signalling pathways within the cell, which can ultimately lead to insulin resistance (Maiese *et al*., 2007). Elevated glucose levels can result in an increased production of ROS (You *et al*., 2002). Although antioxidants act in many different ways to inhibit ROS, studies using antioxidants in diabetes-mellitus patients have given inconclusive results. Novel and more-powerful antioxidants need to be studied before it can be concluded whether antioxidants may be used in the treatment of diabetes and diabetic complications.

14.2.1.3 Neurodegenerative Disorders

Alzheimer's disease is the major dementing disease of the elderly, and age is the main risk factor (Katzman & Saitoh, 1991). The key symptoms are deficits in cognitive areas and language and progressive weakening of the memory. The brain is particularly vulnerable to oxidative damage due to its large utilisation of oxygen, high amount of easily oxidisable PUFA, high amount of pro-oxidative transition metals and low antioxidant content (Butterfield *et al*., 2002). As we age, the plasma and cellular antioxidant potential gradually diminishes and the absorption of nutrients, including antioxidants, becomes less effective (Elmadfa & Meyer, 2008). Evidence supporting the hypothesis that oxidative stress is linked to Alzheimer's disease has been reviewed (Markesbery, 1997) and our current understanding is that a brain affected by Alzheimer's disease is under intense oxidative stress, manifested by lipid peroxidation, free-radical formation, protein oxidation and DNA/RNA oxidation (Butterfield *et al*., 2001). Such oxidative stress will lead to neurodegeneration and neuronal cell death. The consensus has been that nutritionally derived antioxidants may provide one approach to delaying the onset and progression of this disease (Butterfield *et al*., 2002). The antioxidative capacity (AOC) of marine peptides has, however, not been studied in direct relation to Alzheimer's disease.

14.2.1.4 Cancer

Cancer is one of the most abundant diseases worldwide. In 2008, there were 12.6 million new cases of cancer, approximately 28.8 million people living with cancer and 7.6 million deaths from cancer worldwide (IARC, 2010). The development of the disease is characterised by three phases: initiation, promotion and progression. In the initiation phase, the normal cell regulatory process is challenged by exogenous and endogenous factors (carcinogens). Most such challenges are neutralised by the organism's detoxification and DNA-repair systems, but occasionally these fail and mutations of the DNA occur. In the promotion phase, the mutated cells grow under the influence of several promotion factors and may develop into precancerous lesions, which in turn can develop into invasive lesions of cancer. Progression is an irreversible process characterized by rapid cell division, increased invasiveness and metastasis.

There is an established opinion that nutrition and dietary factors can contribute to protect against the development of cancer (World Cancer Research Fund/American Institute for Cancer Research, 2007). Due to their ability to serve as free-radical scavengers, antioxidants from fruits and vegetables have been suggested to be especially health-beneficial. This assumption has been supported by some epidemiological studies, but others have found no significant correlation. There are also large differences between types of cancer, and often between sexes (Annema *et al*., 2011; Aune *et al*., 2011; Boffetta *et al*., 2010; Buchner *et al*., 2011; Epplein *et al*., 2010; Heinen *et al*., 2012; Key, 2011; Paluszkiewicz *et al*., 2012). Several randomised controlled trials (RCTs) have been performed into the use of antioxidants in pharmacological doses as cancer-prevention agents. A recent review of such clinical trials concluded that there is no evidence in favour of using antioxidants in this role (Goodman *et al*., 2011).

14.2.2 Antioxidant Function

Several antioxidants and antioxidant systems are present *in vivo* to counter the production of ROS. The inherent defence against oxidative stress involves mechanisms that prevent free radicals from causing damage and mechanisms that repair or mitigate this

damage (Lakshmi *et al*., 2009). Three main reaction mechanisms apply to antioxidants: free-radical scavenging, quenching of singlet oxygen and metal chelation. Free-radical scavengers can reduce lipid, protein and DNA oxidation by inhibiting the initiation phase through scavenging of free radicals (preventive antioxidants) or by inhibiting the propagation phase of protein or lipid peroxidation through scavenging of lipid alkoxyl, lipid peroxyl and protein carbonyl radicals (chain-breaking antioxidants). Quenching of singlet oxygen is an interaction between $^1O_2$ and the quenching agent (antioxidant). This process can either involve transference of access energy from $^1O_2$ to the antioxidant or a chemical reaction in which $^1O_2$ adds to the antioxidant-forming endoperoxides. Metal-ion chelators inhibit the activity of transition metals with two or more valence states, such as iron and copper. Antioxidants of physiological importance can be of both endogenous and exogenous origin. They are often classified as high-molecular (mostly proteins and enzymes) or low-molecular (smaller endogenous compounds and most exogenous compounds, such as vitamins and plant phenols). The different antioxidants can act through one or multiple reaction mechanisms; for instance, antioxidant enzymatic activity by superoxide dismutase (SOD) metabolises superoxide (O_2^-) to hydrogen peroxide (H_2O_2), whereas glutathione peroxidase and catalase in turn degrade H_2O_2 to water and oxygen. Exogenous antioxidants, such as tocopherols and plant phenols, can act both as free-radical scavengers and as quenchers, while ascorbic acid can act both as a quencher and as a metal chelator.

14.2.2.1 Antioxidative Effects of Proteins, Peptides and Amino Acids

The antioxidant activity of proteins and peptides has been reviewed (Elias *et al*., 2008), including both mechanisms of action and their role in foods. The AOC of proteins is unique in the way that they can act as multifunctional antioxidants and can inhibit several different oxidation pathways, including inactivation of ROS (Fang *et al*., 2002), scavenging of free radicals (Guiotto *et al*., 2005) and chelation of pro-oxidative transition metals (Seth & Mahoney, 2001), in which the amino acid residues histidine, glutamic acid, aspartic acid and phosphorylated serine and threonine are particularly active metal chelators. Another mechanism by which proteins and peptides can act as antioxidants is the nonradical reduction of hydroperoxides to relatively nonreactive hydroxides (Garner *et al*., 1998; Pryor *et al*., 1994). Methionine is thought to be central in this process, through the action of a two-electron transfer from the sulfide of the methionine's thioether group (Garner *et al*., 1998). Peptides are usually considered to be more potent antioxidants than free amino acids, due to the stability of the resultant peptide radical, which does not initiate or propagate further oxidative reactions (Elias *et al*., 2008). The free-radical scavenging potential of an amino acid is dependent on its functional side chain. Theoretically, all 20 amino acids in proteins are potentially oxidisable and can act as antioxidants. The most reactive amino acids are usually those containing either nucleophilic sulfur-containing side chains (taurine, cysteine or methionine) or aromatic side chains (tryptophan, tyrosine, and phenylalanine) from which hydrogen is easily abstracted (Elias *et al*., 2008). The AOCs of amino acid residues are, however, limited by the tertiary structures of the peptides and proteins, since many amino acids with AOCs are buried in the core of the peptide or protein, where they are inaccessible to pro-oxidants. An approach to increasing the AOC is tertiary disruption of proteins or peptides, which may expose amino acids within the parent protein. This can be achieved by food processing, fermentation or gastrointestinal digestion.

14.2.3 Evaluation of Antioxidative Capacity

Evaluation of AOCs of different food sources has received much attention due to the increased acceptance that antioxidants are inversely correlated with several diseases (Diplock, 1994). Dietary antioxidants include radical chain-reaction inhibitors, $^1O_2$ quenchers, metal-ion chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors. The antioxidant assays can be divided into *in vitro* chemical and biological assays, *in vivo* animal models and human clinical experiments. On the basis of the chemical reactions involved, *in vitro* chemical assays can be classified into assays that measure the ability of an antioxidant to quench free radicals by hydrogen atom-transfer (HAT) or electron-transfer (ET) reactions (Huang *et al*., 2005). Both HAT and ET assays are intended to measure the radical-scavenging activity. Generally, HAT assays involve a synthetic free-radical generator, an oxidisable probe and an antioxidant to be measured. Quantification is determined from kinetic curves after monitoring the competitive reaction kinetics (Huang *et al*., 2005). It is assumed that HAT assays more closely reflect *in vivo* trials in that the hydrogen transfer is a key mechanism in the radical chain reactions. Oxygen-radical absorbance capacity (ORAC), total trapping antioxidant parameter (TRAP) and crocin bleaching are examples of HAT assays. ET assays measure the ability of an antioxidant to reduce an oxidant probe, which normally changes colour when reduced (Huang *et al*., 2005). These methods involve only two components in the reaction mixture: an oxidant probe and an antioxidant. An ET occurs from the antioxidant to the oxidant, resulting in a reduced oxidant and an oxidised antioxidant. The reducing capacity is correlated to the degree of colour change of the reduced probe and represents the antioxidant capacity. Ferric-reducing antioxidant power (FRAP), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 2′ azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and total phenols by Folin–Ciocalteu reagent (FC) are examples of ET assays. The assays differ from each other in terms of substrates, probes, pH and quantification methods, and it is therefore challenging to compare the results from the different assays (Frankel & Meyer, 2000). Also, the same assays may differ in results due to variable endpoints used, variable concentrations of reagents and reactants and variable choices of the standard or reference compound used to calibrate the assay. The complexity of food composition makes it cost-demanding and inefficient to separate each antioxidant compound and study it separately. To date there is no standardised method to evaluate the AOC of a substance, and it is often considered wise to execute several antioxidant methods when documenting AOC.

14.2.3.1 In Vitro Chemical Studies

Marine organisms are rich sources of structurally diverse bioactive compounds with various biological activities (Ngo *et al*., 2011), and interest in the development of antioxidants from these sources has been increasing. A number of studies, using different assays for the evaluation of AOC, have documented the AOCs of peptides derived from marine organisms through processing, fermentation or gastrointestinal digestion (Table 14.1). Natural fermentation has been used for the hydrolysis of pacific hake and fermented blue mussel and mussel sauce (Jung *et al*., 2005; Rajapakse *et al*., 2005b; Samaranayaka & Li-Chan, 2008). The amino acid sequence of the antioxidative peptide in fermented blue-mussel sauce was determined to be Phe-Gly-His-Pro-Tyr (Jung *et al*., 2005). Enzymatic hydrolysis of proteins using proteolytic enzymes is more widely used,

Table 14.1 Marine-derived peptide sources evaluated for antioxidative capacity (AOC).

Source of peptides or hydrolysates	*In vitro* method used in measuring	Enzyme(s)	Reference
Pacific hake (*Merluccius productus*)	DPPH, ABTS, ORAC, iron chelation, LPAS, Caco-2	Autolysis	Samaranayaka & Li-Chan (2008), Samaranayaka *et al.* (2010)
Tuna cooking juice (*Katsuwonus pelamis*), liver (*Thunnus obesus*), dark muscle byproduct	DPPH, H_2O_2 and OH· scavenging capacity, reducing power, DNA damage	Protease XXIII, alcalase, flavourzyme, neutrase, protamex, cooking	Ahn *et al.* (2010), Hsu (2010), Jao & Ko (2002)
Capelin whole fish	B-carotene-linoleate model system	Alcalase	Amarowicz & Shahidi (1997)
Dried bonito	Methyl linolate model system		Suetsuna (1999)
Sardine muscle	ESR, O_2· and OH· scavenging	Pepsin	Suetsuna & Ukeda (1999)
Alaska-pollack (*Theragra chalcogramma*) skin, frame protein	TBA, LAPS, DPPH, reducing power, DPPH	Alcalase, pepsin, collagenase, mackerel intestine crude enzyme, flavourzyme, protamex, neutrase, papain, pepsin + pancreatin, thermolysin	Je *et al.* (2005d), Jia *et al.* (2010), Kim *et al.* (2001), Nakajima *et al.* (2008)
Hoki (*Hignuys belengerii*) frame, skin	LAPS, DPPH (ESR), OH· and O_2· scavenging activity, CCRS, Hep3B cells, DNA damage	Pepsin, trypsin,papain, a-trypsin, alcalase, neutrase	Je *et al.* (2005a), Kim *et al.* (2007), Mendis *et al.* (2005b)
Mackerel (*Scomber austriasicus*) fillet	DPPH, reducing power, LAPS	Autolysis, protease N	Wu *et al.* (2003)
Yellowfin-sole (*Limanda aspera*) frame	LAPS	Pepsin and mackerel-intestine crude enzyme	Jun *et al.* (2004)
Jumbo-squid (*Dosidicus gigas*) skin, giant squid	OH· and CCRS, metal chelation, LAPS	Trypsin, a-chymotrypsin, pepsin	Mendis *et al.* (2005a), Rajapakse *et al.* (2005a)
Blue mussel (*Mytilus edilus*)	Hydroxyl radical and CCRS, superoxide anion radical, DPPH, metal chelation, LAPS	Autolysis	Jung *et al.* (2005), Rajapakse *et al.* (2005b)
Chum-salmon (*Oncorhynchus keta*) cartilage and skin	Hydroxyl radical and CCRS, superoxide anion radical, DPPH	Boiling	Nagai *et al.* (2006)

(*continued overleaf*)

Table 14.1 (*continued*)

Source of peptides or hydrolysates	*In vitro* method used in measuring	Enzyme(s)	Reference
Atlantic salmon (*Salmo salar*)	DPPH	Pepsin, pepsin + pancreatin, thermolysin	Nakajima *et al.* (2008)
Coho salmon (*Oncorhynchus kisutch*)	DPPH	Pepsin, pepsin + pancreatin, thermolysin	Nakajima *et al.* (2008)
Whiting (*Micromesistius australis*)	DPPH	Pepsin, pepsin + pancreatin, thermolysin	Nakajima *et al.* (2008)
Yellow stripe trevally (*Selaroides leptolepis*)	DPPH, metal chelation, reducing power	Alcalase, flavourzyme	Klompong *et al.* (2007)
Herring (*Clupea harengus*) press juice	ORAC, LDL, cellular model	Gastrointestinal digestion	Gunnarsson *et al.* (2006), Sannaveerappa *et al.* (2007)
Conger eel (*Conger myriaster*)	LAPS, OH· and carbon-centred radical-scavenging capacity (ESR)	Alcalase, α-chymotrypsin, trypsin, pepsin	Ranathunga *et al.* (2006)
Tilapia protein	FRAP, TEAC, isoluminol-enhanced chemiluminescence	Amano A2, amano N, flavourzyme, neutrase	Raghavan *et al.* (2008)
Channel catfish	FRAP, ORAC, DPPH, TBARS, metal-chelating activity	Protamex	Theodore *et al.* (2008)
Oyster (*Crassostrea gigas*, *Crassostrea talienwhannensis*)	LAPS, O_2· and OH· scavenging capacity (ESR), protection against DNA damage, DPPH, reducing power	Gastrointestinal digestion, papain, neutrase, alcalase	Dong *et al.* (2010), Qian *et al.* (2008)
Algae (*Chlorella vulgaris*)	TEAC, ORAC, DPPH, O_2·, OH· scavenging capacity, DNA and cell damage	Pepsin	Sheih *et al.* (2009b)
Cod (*Gadus morhua*)	DPPH, liposomes	Protamex	Slizyte *et al.* (2009)
Shrimp (*Pandalus borealis*, *Litopenaeus vannamei*)	ORAC, ABTS, FRAP, DPPH	Gastrointestinal digestion	Binsan *et al.* (2008), Jensen *et al.* (2009)
Saithe (*Pollachius virens*)	ORAC	Gastrointestinal digestion	Jensen *et al.* (2009)
Loach (*Misgurnus anguillicaudatus*)	DPPH and OH· scavenging capacity, LAPS, Cu-ion chelating activity	Papain	You *et al.* (2010)

LAPS, linoleic acid peroxidation system; CCRS, carbon-centred radical scavenging; DPPH, 2,2′-diphenyl-1-picrylhydrazyl-radical scavenging-capacity assay; ORAC, oxygen-radical absorbance-capacity assay; FRAP, ferric-reducing antioxidant-power assay; TEAC, trolox-equivalent antioxidative-capacity assay.

and provides an improved control of hydrolysis time, enzymes and molecular weight. In a study performed by Slizyte *et al*. (2009), different hydrolysis times of cod were tested. DPPH radical-scavenging activity increased with increased degree of hydrolysis, whereas iron-induced lipid-oxidation inhibition was not affected. In another study, Theodore *et al*. (2008) discovered that DPPH radical-scavenging activity and the reducing power of hydrolysed Channel catfish decreased, while the ORAC, metal-chelating ability and TBARS-inhibitory activity increased, with increasing degree of hydrolysis. They also showed that low-molecular-weight peptides had higher ORAC values and metal-chelating activities, whereas high-molecular-weight peptides had a higher FRAP and DPPH radical-scavenging activity. Dong *et al*. (2010) learned that oyster peptide with a molecular weight below 1 kDa possessed the highest overall AOC, whereas results on hoki (Je *et al*., 2005a) and mackerel (Wu *et al*., 2003) showed that peptides with molecular weights over 1 kDa possessed the highest AOC. The low AOC of some fractions has been explained by the presence of some prooxidative amino acids (Amarowicz & Shahidi, 1997). The antioxidative peptides released during cooking of seafood protein, such as tuna and salmon, have also been evaluated (Jao & Ko, 2002; Nagai *et al*., 2006). Tuna cooking juice showed a high DPPH radical-scavenging activity, comparable to those of the synthetic antioxidants butylated hydroxytoluene (BHT) and L-ascorbic acid at the same concentrations. After simulated gastrointestinal digestion of hoki, loach and oyster (Kim & Mendis, 2006; Qian *et al*., 2008; You *et al*., 2010), peptides with AOC were obtained, and it was demonstrated that the simulated gastrointestinal digestion resulted in peptides with higher AOCs than those produced by other proteases (Kim & Mendis, 2006). Gastrointestinal digestion of herring-press juice (Sannaveerappa *et al*., 2007), saithe and shrimp (Jensen *et al*., 2009) showed that AOC, measured by ORAC, increased during digestion.

Utilisation of marine wastes and byproducts has received increased attention lately. Peptides from hydrolysed tuna liver have been documented to exhibit AOC and protective effects against hydroxyl radical-induced DNA damage (Ahn *et al*., 2010). Skin from Alaska pollock (Jia *et al*., 2010; Kim *et al*., 2001), hoki (Mendis *et al*., 2005b) and squid (Mendis *et al*., 2005a) has been used as a source for antioxidative peptides. Peptides exhibiting AOC were characterised as being composed of less than 16 amino acid residues. Algae waste hydrolysed using pepsin showed an AOC in both HAT and ET assays demonstrating it to be a good source of antioxidative peptides (Sheih *et al*., 2009b). It was also demonstrated to resist gastrointestinal digestion, which is essential for a physiological impact.

14.2.3.2 In Vitro Biological Studies

As a supplement to *in vitro* chemical assays, cell-culture model systems should be applied prior to animal studies and human clinical trials. Like *in vitro* chemical studies, use of cultured-cell model systems allows for rapid and inexpensive screening of antioxidative compounds. However, these models also take into account the cellular physiological conditions, bioavailability, metabolism and bioactivity of the compound and are adequate for use prior to animal and human studies. Such biological models are of particular importance as the studies to date have demonstrated that the antioxidant mechanism in human health promotion goes beyond the antioxidant activity of scavenging free radicals (Liu, 2004). Few studies have been published on the AOC of marine peptides using cellular antioxidant models. Gunnarsson *et al*. (2006) extracted ROS from human monocytes, which

were detected by isoluminol-enhanced chemiluminescence. They demonstrated that the addition of herring press juice inhibited the ROS-derived chemiluminescence signal. It has also been demonstrated that a dipeptide from sardine muscle, Met-Tyr, has a protective effect against oxidative stress on endothelial cells (Erdmann *et al*., 2006). This protective effect is due to stimulation of the antioxidant defence protein HO-1 and ferritin expression in the cells.

14.2.3.3 Animal Studies

Documentation from animal studies is usually demanded before clinical human trials can take place. Animal studies have documented antioxidative effects following intake of marine peptides. Boukortt *et al*. (2004) conducted an animal study on streptozotocin-induced diabetes in spontaneously hypertensive rats (SHR), in which they evaluated the antioxidative status after intake of a fish-protein hydrolysate. Antioxidative enzyme activity and antioxidant substances were determined in organs (liver, kidney and heart) and vitamin C was analysed in plasma. After 2 months' intake of the fish-protein hydrolysate, the antioxidant status had increased significantly compared to the control group. Recently, extract from the marine crustacean *Erugosquilla massavensis* was documented to enhance antioxidative status in rat (Fahmy & Hamdi, 2011). Carbon tetrachloride-induced oxidative stress in rat liver was studied in rats fed the crustacean extract. After 9 days the malondialdehyde levels were decreased, whereas the activities of glutathione-S-transferase and -catalase and the levels of reduced glutathione had increased.

14.2.3.4 Human Clinical Trials

Human clinical trials are conducted to confirm both the bioavailability and the function of antioxidants in the body. Results from human *in vivo* studies are essential before any dietary recommendations can be given or approval can be granted to use health claims in functional foods or nutraceutical formulations. Up to now, most of the bioactivities in marine peptides have been demonstrated *in vitro* or in animal-model systems. Different biomarkers can be used in measuring the ability of dietary antioxidants to protect lipids, proteins and DNA from oxidative damage (Collins, 2005; Griffiths *et al*., 2002). Parra *et al*. (2007) studied the oxidative stress level in individuals subjected to energy-restricted diets that were isocaloric and had the same dietary macronutrient distribution, but with different fat and protein sources. The four diets were cod-based, salmon-based, fish oil-supplemented and fish-restricted (control). In the control and fish-oil diets, lean meat was used as a protein source. In contrast to the other energy-restricted diets, the cod-based diet was found to significantly increase plasma total AOC and decrease oxidative stress, measured as plasma malondialdehyde. In a cross-over study conducted by Lindqvist *et al*. (2009), herring was given to overweight men 5 days a week during a 6-week period, followed by 6 weeks with chicken or lean pork and a 12-week wash-out period. The herring diet did not result in increased ox-LDL or decreased AOC, which was suggested by the authors to be due to potent antioxidants from the fish muscle. In a pilot human clinical trial, Marchbank *et al*. (2008) studied the protective effect of a protein hydrolysate from Pacific hake (*Merluccius productus*) on small-intestinal injury as a side effect of the nonsteroidal anti-inflammatory drug indomethacin. The hydrolysate reduced the degree of small-intestinal injury, and the authors suggested that glutamine present in the fish-protein hydrolysate may have contributed to antioxidative activity via stimulation of glutathione production.

14.3 ANTIHYPERTENSIVE PEPTIDES

14.3.1 Antihypertensive Peptides and Health

Hypertension, or elevated blood pressure (BP), is a chronic condition that requires the heart to work harder in order to ensure adequate blood circulation. It is a major, although controllable, independent risk factor for CVD (Harris *et al*., 1985) and is associated with cardiovascular events such as myocardial infarction, stroke and heart failure. Hypertension is an important health issue in both industrialised and developing countries (WHO, 2011), estimated to affect 1.56 billion individuals by 2025 (Kearney *et al*., 2004). The renin–angiotensin system is one of the hormone systems regulating the BP. In the event of decreased blood volume or flow through the kidneys, renin is secreted by kidney cells and acts on angiotensinogen to form angiotensin 1. Angiotensin-converting enzyme (ACE), first discovered in 1953, exhibits a variety of functions in the body, and plays an important role in the regulation of blood pressure by converting the inactive angiotensin 1 into the potent vasoconstrictor angiotensin 2 (Goodfriend *et al*., 1996). ACE also inactivates the potent vasodilator bradykinin (Witherow *et al*., 2001). Compounds with ACE-inhibitory activity were first obtained from snake venom (Ondetti *et al*., 1971). Their utility in the treatment of hypertension was evident. Synthetic ACE inhibitors such as captopril, enalapril, alcacepril and lisinopril are traditionally used to treat hypertension and heart failure (Ondetti & Cushman, 1977), but are often associated with side effects such as cough, taste alterations, skin rashes and renal dysfunction (Atkinson & Robertson, 1979). This has resulted in an increased interest in finding natural inhibitors, and numerous studies have documented antihypertensive and ACE-inhibitory effects in different food sources (Fujita *et al*., 2000; Wijesekara & Kim, 2010). Antihypertensive treatment of patients with both diabetic and nondiabetic nephropathy has been proven to maintain the glomerular filtration rate (Parving *et al*., 1985). It has been suggested that ACE inhibitors may reduce renal dysfunction by mechanisms independent of their direct BP-reducing effects (Anderson *et al*., 1989; Lewis *et al*., 1993). However, clinical trials with sufficient power and duration are needed to confirm this hypothesis (Daien *et al*., 2012).

14.3.2 Function of ACE Inhibitors

ACE is a dipeptidyl carboxypeptidase (EC 3.4.15.1) within the class of zinc proteases. It is widely but unevenly distributed in mammalian tissue, particularly located in the vascular endothelial lining of the lungs (Lee *et al*., 2010). It acts as an exopeptidase, cleaving dipeptides from the C-terminus of various oligopeptides (Curtiss *et al*., 1978; Ondetti & Cushman, 1982). The inactive decapeptide angiotensin 1, with the amino acid sequence Asp-Arg-Val-Try-Ile-His-Pro-Phe-His-Leu, is converted to the active octapeptide angiotensin 2, Asp-Arg-Val-Try-Ile-His-Pro-Phe, by cleavage of the dipeptide His-Leu by ACE (Cushman & Cheung, 1971). This results in an increase in BP through vasoconstriction, increased systemic resistance and stimulated secretion of aldosterone, resulting in increased sodium and water reabsorption in the kidneys (Skeggs *et al*., 1956). ACE also inactivates the vasodilative nanopeptide bradykinin in the kallikrein–kinin system (Cobb *et al*., 2004).

A compound's ACE-inhibitory potency is often expressed as its IC_{50} value: the inhibitor concentration required to inhibit 50% of ACE activity. Both competitive (Je *et al*., 2005b; Zhao *et al*., 2009) and noncompetitive (Lee *et al*., 2010; Qian *et al*., 2007,

2008; Sheih *et al*., 2009a; Suetsuna & Nakano, 2000) ACE inhibitors have been reported. Competitive ACE inhibitors bind to and block the active site of the enzyme or a distinct inhibitor-binding site, separated from the active site, to alter the enzyme conformation so that the substrates no longer binds to the active site (Wijesekara & Kim, 2010). Peptides with tryptophan, proline or phenylalanine at the C-terminus or branched-chain aliphatic amino acids at the N-terminus are suitable to act as competitive inhibitors of ACE (Cushman & Cheung, 1971). Hydrophobic amino acids in the N-terminus may also contribute to the inhibitory activity (Rho *et al*., 2009). Noncompetitive ACE-inhibitory peptides may combine with an ACE molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not (Wijesekara & Kim, 2010). Wu *et al*. (2006) proposed that the most favourable tripeptide sequence consists of hydrophobic amino acids at the N-terminus, positively charged amino acids in the middle position and aromatic amino acids at the C-terminus. Dietary ACE-inhibitory peptides may be classified into three groups (Fujita *et al*., 2000): the first comprises true inhibitors, which are not changed by preincubation with ACE; the second comprises substrates for ACE which are converted to weaker or inactive peptides by ACE; and the third comprises the so-called prodrug peptides, which are converted to true inhibitors by ACE or by gastrointestinal proteases. Only the ACE inhibitors in the first and third group exert antihypertensive activity after oral administration (Fujita & Yoshikawa, 1999; Fujita *et al*., 2000).

14.3.3 Evaluation of ACE-inhibitory Effect

Evaluation of the antihypertensive effects of ACE inhibitors from marine sources (see Table 14.2) covers *in vitro* studies, animal studies and human clinical trials. The *in vitro* studies are fairly numerous, while substantially less animal studies have been carried out. Only a few human clinical trials have been conducted, limiting the amount of solid evidence that oral intake of peptides has BP-reducing (antihypertensive) effects.

The method most commonly used to evaluate the ACE-inhibitory capacity of a marine peptide *in vitro* is that of Cushman & Cheung (1971), with some modifications. This method is based on hydrolysis of hippuryl-histidyl-leucine (HHL) by ACE, resulting in hippuric acid (HA) and histidyl-leucine as products. The reaction is carried out at pH 8.3 and terminated by HCl. The end product, HA, has been extracted into ethyl acetate and quantified from its absorbance at 228 nm. The extraction of HA is time-consuming, complicated and requires many steps, in which the HA can be contaminated by ethyl acetate. An alternative high-performance liquid-chromatography (HPLC) method for direct analysis of an ACE mixture with complete separation of HHL and HA has therefore been developed (Wu *et al*., 2002). Other high-throughput and rapid-screening capillary-electrophoresis methods for the evaluation of ACE-inhibitory capacity have also been developed (He *et al*., 2007; Hillaert & Van den Bossche, 2001). Capillary-electrophoresis methods has been considered more suitable for the measurement of ACE-inhibitory capacity because they are faster, more automated and need less sample, substrates and other reagents compared to the HPLC method (He *et al*., 2007).

Orally administered peptides are, like dietary proteins and peptides, hydrolysed by protelytic and peptidolytic enzymes present in the gastrointestinal tract. Only peptides resistant to *in vivo* hydrolysis or with remaining ACE-inhibitory activity after gastrointestinal digestion will have any potential as antihypertensive agents. SHR is the most frequently used animal model for the evaluation of the *in vivo* BP-reducing effect of

Table 14.2 Overview of ACE-inhibitory peptides from marine sources.

Source	Enzymes	Amino acid sequence	IC50 (μM)	Reference
Alaska Pollack skin (*Theragra chalcogramma*)	Alcalase, proteinase, collagenase	Gly-Pro-Leu	2.6	Byun & Kim (2001)
Alaska pollack frame (*Theragra chalcogramma*)	Pepsin	Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala	14.7	Je *et al.* (2004)
Sardine muscle	Alkaline protease	Lys-Trp	1.63	Matsufuji *et al.* (1994)
Pacific hake (*Merluccius Productus*)	Protamex		44 μg/ml	Cinq-Mars & Li-Chan (2007)
Pacific hake (*Merluccius Productus*)	Pepsin, pancreatin		161 μg/ml	Samaranayaka *et al.* (2010)
Tuna muscle (*Neothunnus macropterus*)	Acid extract	Pro-Thr-His-Ile-Lys-Trp-Gly-Asp	2.0	Kohama *et al.* (1988)
Big eye tuna muscle (*Thunnus obesus*)	Pepsin	Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro	21.6	Qian *et al.* (2007)
Atlantic salmon (*Salmo salar*)	Pepsin, pepsin + pancreatin, thermolysin		5 mg/ml	Nakajima *et al.* (2008)
Coho salmon (*Oncorhynchus kisutch*)	Pepsin, pepsin + pancreatin, thermolysin		3.7 mg/ml	Nakajima *et al.* (2008)
Whitting (*Micromesistius australis*)	Pepsin, pepsin + pancreatin, thermolysin		3.6 mg/ml	Nakajima *et al.* (2008)
Alaska Pollack (*Theragra chalcogramma*)	Pepsin, pepsin + pancreatin, thermolysin		2.9 mg/ml	Nakajima *et al.* (2008)
Big eye tuna frame	Pepsin	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro	11.28	Lee *et al.* (2010)
Dried bonito	Termolysin	LKPNM	2.4	Fujita & Yoshikawa (1999)
Chum salmon (Oncorhynchus Keta)	Termolysin	Phe-Leu	13.6	Ono *et al.* (2006)
Shark meat	Protease	Phe-Glu Cys-Phe Glu-Try	1.45 1.96 2.68	Wu *et al.* (2008)

(*continued overleaf*)

Table 14.2 (*continued*)

Source	Enzymes	Amino acid sequence	IC50 (μM)	Reference
Shrimp (*Acetes chinensis*)	Protease	Arg-Pro	0.39	Hai-Lun *et al.* (2006)
Shrimp (*Pandalus borealis*)	Unknown	Phe-Ser-Tyr	2.2, 7.7	Gildberg *et al.* (2011)
Shrimp (*Acetes chinesis*)	Pepsin	Leu-His-Pro	1.6	Cao *et al.* (2010)
Shrimp (*Acetes chinesis*)	Lactobacillus fermentum S605	Asp-Pro Gly-Thr-Gly Ser-Thr	2.15 5.54 4.03	Wang *et al.* (2008b)
Hard clam (*Meretrix lusoria*)	Protamex	Tyr-Asn	51	Tsai *et al.* (2008)
Sea cucumber (*Acaudina molpadioidea*)	Bromelain, alcalase	Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp	15.9	Zhao *et al.* (2009)
Wakame (*Undaria pinnatifina*)	Pepsin Protease	Tyr-Asn-Lys-Leu Ile-Trp	21 1.5	Suetsuna & Nakano (2000) Sato *et al.* (2002)
Oyster (*Crassostrea talienwhanensis Crosse)*	Pepsin	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	66	Wang *et al.* (2008a)
Oyster sauce (*Crassostrea gigas*)	Fermentation		145	Je *et al.* (2005b)
Anchovy fish sauce	Fermentation	Lys-Pro	22	Ichimura *et al.* (2003)
Microalgae (*Chlorella vulgaris*)	Pepsin	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe	29.6	Sheih *et al.* (2009a)
Fish scales	Protease	Val-Ile-Tyr	7.5	Fahmi *et al.* (2004)
Yellowfin sole (*Limanda aspera*)	α-chymotrypsin	Met-Ile-Phe.Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	22.3	Jung *et al.* (2006)
Rotifer (*Brachionus rotundiformis*)	Alcalase	Asp-Asp-Thr-Gly-His-Asp-Phe-Glu-Asp-Thr-Gly-Glu-Ala-Met	9.64	Lee *et al.* (2009)
Blue-mussel sauce (*Mytilus edulis*)	Fermentation	Glu-Val-Met-Ala-Gly-Asp-Leu-Tyr-Pro-Gly	2.98	Je *et al.* (2005c)
Salmon (*Salmo salar*)	Pepsin, trypsin, α-chymotrypsin		0.7 μg/mU	Dragnes *et al.* (2009)
Cod (*Gadus morhua*)	Pepsin, trypsin, α-chymotrypsin		1.6 μg/mU	Dragnes *et al.* (2009)

dietary components and food-derived hydrolysates. The BP-modifying effect of oral administration of peptides and hydrolysates can be evaluated after a single dose or following long-term administration.

RCTs in humans are regarded as the ultimate choice for the evaluation of the efficacy of a pharmacological treatment. In human trials, BP-lowering effects may be evaluated in clinically healthy, mildly hypertensive or normotensive subjects. Subjects are randomised into treatment groups or control groups, receiving the treatment to be evaluated or a placebo. Blood pressure is measured prior to, during and after the intervention. A post-treatment analysis can also be included.

14.3.3.1 In Vitro Studies

Since the discovery of the first naturally occurring peptide with ACE-inhibitory capacity (Oshima *et al*., 1979), a number of studies have been conducted on peptides. The diversity and availability of marine resources has led to an intense search for bioactive compounds from the marine environment, and the characterisation is growing rapidly. Differences in the methods used to evaluate ACE-inhibitory capacity and more specific conditions, such as the concentration of enzyme, substrate, incubation time, extraction of HA and presentation of results, make comparison of the ACE-inhibitory capacities of different protein sources and between research groups difficult (Gildberg *et al*., 2011). Despite this issue, comparisons are made, and may give an indication of the relative effectiveness of different peptides. The first reported marine ACE-inhibitory peptide was an octapeptide from tuna muscle with the amino acid sequence Pro-Thr-His-Ile-Lys-Trp-Gly-Asp (Kohama *et al*., 1988). Its IC_{50} value was evaluated to be 2 μM. Peptides from tuna frame and muscle have also been shown to have moderate noncompetitive ACE-inhibitory effects, of 11.28 and 21.6 μM, respectively (Lee *et al*., 2010; Qian *et al*., 2007). Clam has been demonstrated to show a mixed type of inhibition pattern (Tsai *et al*., 2008), with an IC_{50} value of 51 μM. As described, ACE inhibitors can be classified into three groups. True inhibitor peptides have been isolated from sardine, which did not lose the effect after incubation with gastrointestinal enzymes and preserved its IC_{50} value of 1.63 μM (Matsufuji *et al*., 1994). The inhibitory capacity of sea cucumber was enhanced three times after incubation with gastrointestinal enzymes, exhibiting an IC_{50} value of 4.5 μM after digestion, and was characterised as a prodrug type inhibitor (Zhao *et al*., 2009). Fujita & Yoshikawa (1999) also demonstrated a prodrug-type inhibitor from dried bonito, Leu-Lys-Pro-Asn-Met (LKPNM). Originally it exhibited an IC_{50} value of 2.4 μM, but after hydrolysation by ACE to LKP, it increased its capacity eightfold. A substrate-type ACE inhibitor was discovered in Pacific hake, exhibiting an IC_{50} value of 161 μg/ml (Samaranayaka *et al*., 2010). Other marine sources of peptides, such as microalgae, wakame and rotifers, have also been studied in order to document novel ACE-inhibitory peptides (Lee *et al*., 2009; Sheih *et al*., 2009a; Suetsuna & Nakano, 2000). Shark meat exhibited IC_{50} values ranging from 1.45 to 2.68 μM (Wu *et al*., 2008). Fermented fish sauce, oyster sauce and blue-mussel sauce are used as seasonings, particularly in South East and East Asian countries. Of these, blue-mussel sauce seems to have the highest capacity (IC_{50} 2.98 μM) (Je *et al*., 2005c) and oyster sauce the lowest (145 μM) (Je *et al*., 2005b). Evaluation of oyster protein showed that its IC_{50} was 66 μM (Wang *et al*., 2008a). Annually, 30 million tons of seafood waste is dumped worldwide. Utilisation of these wastes (or byproducts) can potentially increase the economic value of the catch and simultaneously

reduce pollution. Marine byproducts are potentially rich in bioactive peptides and much effort has been made in the search for these. Dragnes *et al*. (2009) investigated the ACE-inhibitory capacity of different parts of cod and salmon, demonstrating that salmon stomach, frame and kidney exhibited as good ACE-inhibitory capacity as salmon muscle. Cod milt, roe and blood exhibited equal ACE-inhibitory capacity to cod muscle. Fahmi *et al*. (2004) documented the IC_{50} of a tripeptide from fish scales to be 7.5 μM. Another tripeptide, from Alaska pollock, was documented to exhibit an IC_{50} value of 2.6 μM. Frame-protein hydrolysate from the same species exhibited less activity (14.7 μM). The ACE-inhibitory peptides must be absorbed from the intestine in active forms in order to have a hypotensive effect. It is known that small di- and tripeptides are easily absorbed in the intestine (Hara *et al*., 1984). In a study conducted by Samaranayaka *et al*. (2010), it was demonstrated that the peptides did not display any ACE-inhibitory capacity after permeation through Caco-2 cells, due to a physical limitation or the possibility that the active peptides did not pass through the Caco-2-cell membrane.

14.3.3.2 Animal Studies

Several studies have been conducted on the effect of a diet rich in ACE-inhibitory peptides on SHR. Studies involving a single oral administration of peptides (10 mg/kg body weight) from tuna frame, tuna muscle, yellowfin sole and oyster have shown a significant suppressive effect on systolic BP, comparable to that of captopril, with a maximum effect 3 hours after administration (Je *et al*., 2005b, 2006; Lee *et al*., 2010; Qian *et al*., 2007). Wakame also lowered the BP to a comparable degree to captopril (Suetsuna & Nakano, 2000). It has been demonstrated that although different peptides exhibit different *in vitro* capacities, their BP-lowering effects in SHR are all almost the same (Suetsuna & Nakano, 2000). An ACE-inhibitory peptide from sea cucumber with an amino acid sequence of Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp and captopril as a positive control was administered to SHR in an oral dose of 3 μM/kg body weight (Zhao *et al*., 2009). The peptide was comparable to captopril, and both significantly reduced the BP compared to the negative control (Zhao *et al*., 2009). The same effect was shown for a nonapeptide isolated from oyster protein. After 9 days of daily administration of 20 mg/kg body weight, the effect was similar to that of captopril at a dosage of 2 mg/kg body weight (Wang *et al*., 2008a).

14.3.3.3 Human Clinical Trials

The marine peptide katsuobushi oligopeptide (KO), from dried and processed bonito, has been used in a human clinical trial, in which mildly hypertensive and borderline patients were provided with 1.5 g/day KO (Fujita *et al*., 2001). The results revealed that after 5 weeks of administration, 62% of test subjects had a significant decrease in BP. Systolic BP decreased by 10.5 mmHg and diastolic BP decreased by 5.75 mmHg compared to the placebo group. After cessation, the BP returned slowly to the baseline level. No side effects, such as dry cough, were registered. In another 4-week trial on 29 hypertensive individuals, Kawasaki *et al*. (2000) found that BP decreased significantly after oral administration of the Val-Tyr peptide, derived from enzymatic hydrolysis of sardine. Despite a number of *in vitro* studies confirming the ACE-inhibitory capacity of different marine peptides, there is a lack of human studies supporting most of these findings. Before nutritional guidelines can be generally applied, and before new nutraceuticals are developed, such documentation of the hypotensive effect in humans is required.

14.3.4 Comparison of the ACE-inhibitory Capacities of Nonmarine Peptides and Commercial Products

Research on ACE-inhibitory peptides has progressed further than that on antioxidative peptides, and many ACE-inhibitory peptides have been discovered and commercialised. Milk proteins of terrestrial animal origin are an extensively studied source of bioactive peptides and their ACE-inhibitory effects have been documented in several publications (Urista *et al*., 2011). They have been documented in porcine muscle by Katayama *et al*. (2007, 2008), with IC_{50} values between 26.2 and 552.5 μM. In beef muscle, the documented IC_{50} value ranged between 50.5 and 117.0 μM (Jang *et al*., 2008), and chicken has been demonstrated to exhibit IC_{50} values between 2.4 and 14.0 μM (Fujita *et al*., 2000). Captopril is one of many synthetic ACE-inhibitor drugs. In various studies it has been shown to exhibit an IC_{50} value several times greater than that of marine peptides (Fujita & Yoshikawa, 1999; Tsai *et al*., 2008), but other studies have shown that the ACE-inhibitory capacity of marine peptides is comparable to that of captopril (Wang *et al*., 2008a; Zhao *et al*., 2009). Natural peptides with antihypertensive capacity may thus be promising as alternatives to synthetic ACE inhibitors, and they most likely do not exhibit any side effects.

14.4 CONCLUSION

Interest in the development of bioactive peptides from the marine environment has been increasing. Marine organisms are rich sources of structurally diverse bioactive peptides with various biological activities, and several studies have documented both antihypertensive and antioxidative effects. Bioactive peptides have from 3 to 20 amino acids and may provide beneficial health effects in addition to their classical nutritional value. Within the parent proteins, peptides are ineffective, and they must be released during *in vitro* enzymatic hydrolysis, fermentation, processing or digestion. Whereas antioxidative peptides may provide a beneficial effect in the gut, preventing unwanted food oxidation, ACE-inhibitory peptides need to be absorbed in the intestine in order to enter the bloodstream and exert a physiological effect at the target site. According to theoretical knowledge, the antioxidants can prevent oxidative stress linked to several diseases, such as CVD, diabetes, neurodegenerative disorders and cancer, as well as ageing. Results from human clinical trials, however, have been scarce and inconclusive. Further research and more clinical trials are therefore essential before a final conclusion can be made. In contrast, ACE-inhibitory peptides have demonstrated a BP-reducing effect in multiple human trials, and several commercial products from marine sources are now on the market.

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15 Potential Novel Therapeutics: Some Biological Aspects of Marine-derived Bioactive Peptides

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15.1 INTRODUCTION

The sea has been identified as a treasure chest that hides a great potential for new compounds that can be used as therapeutics (de Vries & Beart, 1995). Mankind has been using marine resources with potent biological activities for many thousands of years (Kaul & Daftari, 1986). Marine resources have been identified as the largest incompletely explored resource remaining on earth (Aneiros & Garateix, 2004). More than 70% of the earth's surface is covered by the sea, and owing to the inherent difficulties of exploring deep marine habitats, many bioactive marine compounds are yet to be identified and characterized. Thermal vents in the deep oceans have been identified as a particular biodiversity hotspot. The need for new drugs/therapeutics is ever increasing as disease diagnosis improves, aided by the various high-throughput molecular genetics-based tools. Furthermore, the advancement of novel techniques in biological science, with high throughput and highly sensitive equipment, has made new discoveries more likely, with greater repeatability. Therefore, the recent trend has been to focus on marine bioresources as a source of novel therapeutics (Aneiros and Garateix, 2004).

In the last 4 decades, a great deal of literature has focused on identifying possible bioactive compounds, including bioactive peptides, from various marine resources, including sea anemones, corals, seaweeds, bacteria, sponges, mollusks, ascidians and so on (Mayer & Gustafson, 2003, 2004, 2006, 2008; Mayer *et al*., 2007, 2009; Ortega-Morales *et al*., 2008; Sato *et al*., 2001; Sugumaran & Robinson, 2010; Tanabe *et al*., 2007; Zheng *et al*., 2011). In very early literature, some of the activities described come from the crude or semipurified lysates of the different marine organisms (Kaul & Daftari, 1986). Owing to this, the precise compound which gives the effect cannot be identified accurately. But most recent studies are more focused on purifying the individual compound, characterizing it and looking at its effect, so that effect can be clearly defined (Aneiros & Garateix, 2004). On the other hand, the world is moving towards more 'green' concepts in day to day life, from energy generation to therapeutics; therefore, there is an increased interest in discovering novel bioactive compounds via 'green chemistry' (Bohlin *et al*., 2010). Many such identified and characterized bioactive peptides show direct effects as

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anticancer/tumor substances (Mayer & Gustafson, 2004, 2006, 2008), while some have demonstrated anti-inflammatory characteristics (Goetz *et al*., 2004; Hong & Secombes, 2009; Krishnaveni & Jayachandran, 2009; Rao *et al*., 2002; Wang *et al*., 2010; Wijesekara & Kim, 2010). Furthermore, certain other bioactive peptides have shown positive effects in controlling chronic and nontransferable diseases (Grabley & Thiericke, 1999; Nakao & Fusetani, 2007; Wijesekara & Kim, 2010), while some have demonstrated certain anti-HIV properties as well (Gunasekera *et al*., 1994; Tziveleka *et al*., 2003; Vo & Kim, 2010). The reasons such totally different substances can be obtained from the marine biota may be due to the various habitats in which different species (algae, bacteria, fungi, actinomycetes, sponges etc.) are discovered, where they need specialized mechanisms to defend themselves from predators, to find food and to tolerate extreme environmental conditions. It is pretty evident that most of the organisms that have been used to extract bioactive substances come from extreme marine environments where they have very competitive and aggressive relationships. Such different situations from the terrestrial norm demand certain unique and specific survival mechanisms, aided by very many potent active substances. Many of the already identified substances produced by the marine biota are nondietary and nonnutritive, and mainly play roles in self-defense mechanisms and in counteracting natural predators in their habitats (Banerjee *et al*., 2008). In particular, the cyclical and linear peptides that have been described from many marine bioresources have broadened our knowledge of potent substances with the possibility for use as novel therapeutics, as antibacterial, antifungal, antimalarial, cytotoxic/anticancer drugs, as enzyme inhibitors and as ion-channel blockers with novel pharmacokinetic properties (Aneiros and Garateix, 2004). But the process by which a newly found substance is brought up to a therapeutic level is very rigorous. Therefore, many of the bioactive substances reported can be identified as the results of descriptive studies, and very few have been published with comprehensive functional bioassays. Owing to this fact, a huge knowledge gap exists in the route to making these findings available as therapeutics.

A large number of bioactive compounds have been characterized from the phylum *Porifera* (sponges) and many of these belong to the metabolites such as alkaloids, terpenoids, macrolides, nucleoside derivatives and polyethers, while few are peptides or proteins. Anticancer compounds such as Monanchocidins (Makarieva *et al*., 2011), Metachromines (Ovenden *et al*., 2011), Spongouridine and Spongothymidine, Geodiamolids, Arenastatin, Phakellistatins and Theonellamides (Gulavita *et al*., 1993, 1995; Pettit, 1997; Pettit *et al*., 2008; Sato *et al*., 2001) have also been derived from various commensalm bacteria existing in sponges or directly from the different sponge species. Furthermore, the sponges have yielded potent antimicrobials, antiinflammatories, neurotoxins, hepatoxins, cardiac stimulants (Aneiros & Garateix, 2004) and even certain compounds have shown potent HIV inhibition (Aneiros & Garateix, 2004; Bewley *et al*., 1998; Coleman *et al*., 1999; Rashid *et al*., 2000) Therefore, sponges have become one of the centers of attention in the search for bioactive peptides from marine resources. More details on sponge-derived bioactive substance can be found later in this chapter.

Numerous bioactive peptides have been derived from the *Ascedians*. Didemin is a very prominent cytotoxic *Ascidians*-derived compound (Geldof *et al*., 1999). Some fungi and bacteria from marine deposits have also yielded specific bioactive substances. Many of the *Aspergillus* species can produce Asperolides compounds that are known for their cytotoxic effects (Sun *et al*., 2012).

Phylum *Mollusks* is one of the most highly researched phyla in relation to the search for novel bioactive substance. The bioactive substances from *Mollusks* are well known for

their potent cytotoxic/anticancer effects. Conus toxins come from the genus *Conus*, and the animals of this genus produce well-known conotoxins, which are a valuable source of neurophamacologically active peptides (Jacobsen *et al*., 1999; McIntosh *et al*., 1999; Olivera *et al*., 1985, 1999a, 1999b). Sea anemones are also known for sodium polypeptide toxins or neurotoxins (Bruhn *et al*., 2001).

Seaweeds have also been used as a valuable resource for the isolation of numerous bioactive peptides. Many of have shown mitogenic, antineoplastic and antioxidant effects. Interestingly, even though the marine biota includes large teleost fish and other mollusks, and various classes of large animals, very little attention has been paid to any potent bioactive substances that can be identified from them. Recently, many more potent substances have been described with anticoagulant, antihelminthic, antiviral, antituberculosis and antiprotozoal effects (Bruhn *et al*., 2001; Mayer *et al*., 2009). This chapter will evaluate some of the marine proteins and peptides that have been isolated and their biological activities, giving special emphasis to their bioactivity and potential use as novel therapeutics for very common human complications, such as hypertension, cancers and viral infections.

15.2 MARINE-DERIVED PROTEINS AND BIOPEPTIDES WITH ANTIHYPERTENSIVE ACTIVITY

Hypertension, or high blood pressure, is considered a significant health problem worldwide, a major risk factor in cardiovascular diseases (CVDs). In addition to numerous preventive and therapeutic drug treatments, important advances have been achieved in the identification of dietary compounds that may contribute to cardiovascular health. Among these compounds, peptides with antihypertensive properties have received special attention in the recent past. Angiotensin-I-converting enzyme (ACE) plays an important physiological role in the regulation of blood pressure by converting angiotensin I to angiotensin II, a potent vasoconstrictor. Therefore, the inhibition of ACE activity is a major target in the prevention of hypertension. ACE-inhibitory peptides were first discovered in snake venom (Ferreira *et al*., 1970) and since then numerous synthetic ACE inhibitors have been produced, with Captopril being the most common. Inhibitory peptides isolated from enzyme hydrolysates of many food proteins show *in vitro* ACE-inhibitory activity and *in vivo* activity in spontaneously hypertensive rats (SHR) (Vercruysse *et al*., 2005). Marine-derived peptides have shown significant ACE-inhibition activity *in vivo*(Qian *et al*., 2007; Rho *et al*., 2009) and a single oral administration (10 mg/kg body weight) of peptide has shown a strong suppressive effect on systolic blood pressure (SBP) of SHR. This antihypertensive activity was similar to that of Captopril. More importantly, no side effect has been observed in rats after administration of antihypertensive peptide. In addition, these marine bioactive peptides exhibit antihypertensive activity *in vivo*, rather than *in vitro*. The exact mechanisms underlying this phenomenon have not yet been identified. However, it has been suggested that bioactive peptides have higher tissue affinities and are subject to a slower elimination than Captopril (Fujita & Yoshikawa, 1999).

In *in vitro* studies, the potency of these marine-derived peptides to inhibit ACE activity has been expressed as an IC_{50} value, which is the ACE-inhibitor concentration. Moreover, the inhibition modes of ACE-catalyzed hydrolysis of these antihypertensive peptides have been determined by Lineweaver–Burk plots. According to Lineweaver–Burk-plot studies, competitive ACE-inhibitory peptides have most frequently reported (Je *et al*., 2005b; Zhao *et al*., 2009). These inhibitors can bind to the

active site to block it, or to the inhibitor-binding site, which is remote from the active site, to alter the enzyme conformation such that the substrate no longer binds to the active site. Moreover, tryptophan, tyrosine, proline or phenylalanine at the C-terminal and branched-chain aliphatic amino acids at the N-terminal are suitable to allow peptides to act as competitive inhibitors by binding with ACE (Cushman & Cheung, 1971). In addition, a noncompetitive mechanism has also been observed in some peptides (Suetsuna, 2000), which means that the peptide can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not. The hydrophobicity of the N-terminus, which is one of the common features of ACE-inhibitory peptides, may contribute to the inhibitory activity (Rho *et al*., 2009). ACE-inhibitory peptides are generally short-chain peptides which often carry polar amino acid residues like proline. Furthermore, structure–activity relationships among various peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides, which contain hydrophobic amino acids at these positions, are potent inhibitors. Table 15.1 shows the amino acid sequence of ACE-inhibitory peptides derived from marine proteins, their origins, the enzyme used for hydrolysis and their IC_{50} values.

15.2.1 'Katsuobushi' Peptides

The most documented product with ACE-inhibitory effects is dried bonito bowel ('katsuobushi'), a traditional Japanese food (Fujii *et al*., 1993; Fujita *et al*., 1995; Yokoyama *et al*., 1992). Thermolysin hydrolysate of bonito bowels has shown *in vitro* ACE-inhibitory activities (Yokoyama *et al*., 1992) that increased 16-fold on treatment with ultrafiltration and chromatography (Fujii *et al*., 1993). The hydrolysate reduced SBP in SHR rats and humans (Fujita *et al*., 1995). LKPNM peptide, isolated from dried bonito, is a prodrug ACE inhibitor that is activated eightfold by ACE itself and shows a prolonged effect after oral administration (Fujita & Yoshikawa, 1999; Yoshikawa *et al*., 2000). According to a clinical trial done using hypertensive and borderline-hypertensive human subjects, LKPNM containing hydrolysate reduces SBP by 12.55 ± 1.5 mmHg (Yokoyama *et al*., 1992). This clearly shows that the thermolysin hydrolsate of dried bonito is sufficiently well absorbed in humans to effectively reduce the SBP of hypertensive and borderline-hypertensive subjects *in vivo*. The hydrolysate has been given Foods for Specified Health Use (FOSHU) approval by the Ministry of Health and Welfare in Japan (Fujita & Yoshikawa, 1999). Recent findings suggest that the antihypertensive mechanism of dried bonito peptides involves direct action on vascular smooth muscle in addition to ACE-inhibitory activity (Kouno *et al*., 2005).

15.2.2 Sardine Peptides

Sardine-protein hydrolysate is the second most documented ACE inhibitor from fish proteins. ACE-inhibitory peptides from fish sources were first identified in sardine meat over 20 years ago (Suetsuna, 1986). Sardine-protein hydrolysate has shown inhibitory effects in both *in vitro* and *in vivo* tests on SHR before and after *in vitro* digestion (Marchbank *et al*., 2008, 2009; Matsui *et al*., 1993; Sugiyama, 1991). The most active sardine peptide is a dipeptide VY that has a significant antihypertensive effect on mildly hypertensive people as well as on SHR (Kawasaki *et al*., 2000). In another study (Bougatef *et al*.,

Table 15.1 Marine proteins and peptides with anti-ACE activities.

Raw material	Enzyme	Peptide sequence	IC_{50} value	References
Katsuobushi (dried bonito bowels)	Thermolysin	IKPLNY, IVGRPRHQG, IWHHT ALPHA, FQP, LKPNM; IY DYGLYP, LKP, IWH, IKP IVGRPR, GYPHK; IRPVQ	43, 2.4, 5.8, 10, 12, 2.4, 2.31, 62, 0.32, 3.5, 6.9, 300 (μM)	Fujii *et al.* (1993), Fujita *et al.* (1995, 1999), Karaki *et al.* (1993), Kouna *et al.* (2005), Matshumura *et al.* (1993), Yokojama *et al.* (1992), Yoshikawa *et al.* (2000)
Sardine	Alcalase, chymotrypsin	MF, RY, MY, LY, YL, IY, VF, RFH, AKK, RVY, GWAP, KY, VY	44.7, 51, 193, 38.5, 82, 10.5, 43.7, 330, 3.13, 205.6, 3.86, 1.63, 10 (μM)	Bordenave *et al.* (2002), Bougatef *et al.* (2008), Kawasaki *et al.* (2000), Matsufuji *et al.* (1994), Matsui *et al.* (1993, 2002), Matsumoto *et al.* (2004), Sugiyama *et al.* (1991)
Viscera of sardinelle	*Bacillus licheniformis* NH1 *Aspergillus clavatus* ES1, crude enzyme extract from sardine		63.2 ± 1.5% at 2 mg/ml	
Skipjack tuna	Pepsin	VAWKL;WSKVVL SSKVPP;CWLPVY		Astwan *et al.* (1995)
Cod frames from tuna	Crude proteinase from *Pyloric caeca*			Jeon *et al.* (1999)
Alaska pollock skin	Alcalase, pronase E, collagenase	GPL, GPM	2.66, 17.13 μM	Byun & Kim (2001)
Alaska pollock frame	Pepsin	FGASTRGA	14.7 μM	Je *et al.* (2004)
Alaska pollack surimi	Pronase, flavourzyme		0.49 mg/ml	Park *et al.* (2009)
Salmon	Thermolysin	IVF and FIA, IW	1.2 μM	Hiroyuki *et al.* (2008)
Chum salmon		WA, VW, WM, MW, IW and LW	277.3, 2.5, 96.6, 9.8, 4.7 and 17.4 μM	Ono *et al.* (2003)

(*continued overleaf*)

Table 15.1 (*continued*)

Raw material	Enzyme	Peptide sequence	IC_{50} value	References
Oyster and pearl oyster	Denazyme AP, alkaline protease, trypsin	LF, FY;AW;VW;GW DLTDY	126 μM 66 μM 0.0874 mg/ml	Matsumoto *et al.* (1994), Katano *et al.* (2003), Je *et al.* (2005), Wang *et al.* (2008) Kazuhiro *et al.* (2010)
Fermented mackeral				Itou & Akahane (2004), Kouji Itou *et al.* (2007)
Alaska pollock frame, Alaska pollock surimi	Pepsin	FGASTRGA		Je *et al.* (2004)
Shrimp peptides	*Lactobacillus fermentum* enzymes	VPAF; FC; FCVL	0.39 μM 3.37 mg/ml	Hai-Lu *et al.* (2006), Wang *et al.* (2010)
Acetes chinensis	Protease from *Bacillus* sp. SM98011	FCVLRP;IFVPAF; KPPETV	0.97mg/ml 12.3, 3.4 and 24.1 μM	
Kamaboko	Gastrointestinal proteases, protein proteases			Nagai *et al.* (2006)
Yellow fin sole	α-chymotrypsin	MIFPGAGGPEL, IAW, YNR	28.7 μg/ml	Jung *et al.* (2006)
Big eye tuna dark muscle	Pepsin	WPEAAELMMEVDP	21.6 μM	Qian *et al.* (2007)
Tuna frame peptides	Pepsin	GDLGKTTTVSNWSPPKYKDTP	11.28 μM	Lee *et al.* (2010)
Tuna broth	Orientase			Hwang & Ko (2004)
Fermented blue mussel	Natural fermentation		19.34 μg/ml	Je *et al.* (2005)
Shark peptides	Protease SM98011 from *Bacillus* sp. SM98011	EY, FE and CF; CF, QY, MF and FQ	1.45 μM	Lee *et al.* (2008)
Sea brim	Alkaline protease		0.57 mg/ml	Fahmi *et al.* (2004)
Macroalagae peptides				Suetsuna & Nakano (2000), Sheih *et al.* (2009), Ciarán *et al.* (2011)

A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine (Phe); G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagines; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

2008), a crude enzyme extract from the viscera of sardinelle (*Sardinella aurita*) exhibited inhibitory activity towards ACE. Protein hydrolysates were obtained by treatment with Alcalase®, chymotrypsin, crude enzyme preparations from *Bacillus licheniformis* NH1 and *Aspergillus clavatus* ES1, and crude enzyme extract from sardine (*Sardina pilchardus*) viscera. The resulting hydrolysate contained a high concentration of peptides displaying low hydrophobicity, with molecular masses between 200 and 600 Da. The alkaline protease extract from the viscera of sardine produced hydrolysate with the highest ACE-inhibitory activity ($63.2 \pm 1.5\%$ at 2 mg/ml). Further, the degree of hydrolysis and the inhibitory activity against ACE increased with increasing proteolysis time. The protein hydrolysate generated with alkaline proteases from the viscera of sardine was fractionated, and eight major fractions ($P_1 - P_8$) were obtained. P_4 fraction was found to display a high ACE-inhibitory activity. The IC_{50} values for sardinelle-byproduct protein hydrolysates and fraction P_4 were 1.2 ± 0.09 and 0.81 ± 0.013 mg/ml, respectively. In another study, sardine autolysate and cod-head hydrolysate powder (50 µg) showed the ability to inhibit nearly 30% of ACE activity (Bordenave *et al*., 2002).

15.2.3 Salmon Peptides

Salmon peptide digested from salmon muscle shows a strong inhibitory activity against ACE. *In vivo* studies using SHR showed that a single intravenous administration of salmon peptide at a dose of 30 mg/kg body weight significantly reduced SBP against control. Further, a double-blind, placebo-controlled, parallel-group study determined the efficacy of the salmon peptide in mild hypertensive subjects. SBP was significantly reduced 4 weeks after 1.0 g salmon peptide intake, and 2 weeks after the intake finished, compared to the value before ingestion. From separation techniques, active di- and tripeptides were identified, including Ile-Val-Phe and Phe-Ile-Ala as two new ACE-inhibitory tripeptides. Ile-Trp had the strongest ACE-inhibitory activity (IC_{50} = 1.2 µM) *in vitro*, and contributed 5.2% to the total ACE-inhibitory activity (Enari *et al*., 2008). Following oral administration of a thermolysin hydrolysate from chum salmon (*Oncorhynchus keta*, salmon family) muscle, the SBP of SHR was significantly reduced when compared to that of controls (Ono *et al*., 2003). The amino acid sequences of the six dipeptides identified from the thermolysin digest of chum salmon were WA, VW, WM, MW, IW and LW. The IC_{50} values of these six peptides were determined as 277.3, 2.5, 98.6, 9.8, 4.7 and 17.4 µM, respectively. In another study (Ono *et al*., 2006), the peptide Phe-Leu, isolated from chum salmon, showed ACE-inhibitory activity with an IC_{50} value of 13.6 µM, with noncompetitive inhibition. The reverse-sequence dipeptide Leu-Phe also showed ACE-inhibitory activity. However, Leu-Phe was much less inhibitory than Phe-Leu, with an IC_{50} value of 383.2 µM. In addition, the inhibition mode was competitive. The relationship between dipeptide sequence and ACE-inhibition properties in Trp-containing dipeptides found in a previous study was also investigated. It was found that peptides with Trp as the C-terminal residue (Ala-Trp, Val-Trp, Met-Trp, Ile-Trp and Leu-Trp) showed noncompetitive inhibition. On the other hand, reversed-sequence peptides with Trp at the N-terminal were competitive inhibitors, except Trp-Leu. These results indicate that the sequence of ACE-inhibitory dipeptides can affect both inhibitory potency and inhibition mechanisms.

15.2.4 Mackeral Peptides

In fermented mackerel products (*narezushi*), the concentration of peptides required to inhibit 50% of the ACE activity in the assay media (IC_{50}) was remarkably decreased

with a rapid increase in peptide contents. SBP in SHR decreased between 2 and 4 hours after a single oral administration of >10 mg peptide/kg *narezushi* extract, and recovered to the initial level by 8 hours thereafter. The SBP decreased at seven successive daily doses of 10 mg/kg *narezushi* extract and then recovered to the initial level 5 days after stopping a total of 10 daily administrations. The extract was administered to 5-week-old SHR for 70 days and SBP decreased 21 days after starting and continued decreased for 28 days after the end of administration. The peptide-rich fraction from *narezushi* extract had a powerful antihypertensive effect, whereas the other fraction had a similar but weak effect (Itou & Akahane, 2004; Itou *et al*., 2007).

15.2.5 Shrimp Peptides

Acetes chinensis is an underutilized shrimp species that thrives in the Bo Hai Gulf of China. Oligopeptide-enriched shrimp hydrolysate digested from this shrimp species has shown high ACE-inhibitory, activity with an IC_{50} value of 0.97 mg/ml. Five peptides with high ACE-inhibitory activity were purified from the shrimp hydrolysates and three of them—FCVLRP, IFVPAF and KPPETV—were novel. Their IC_{50} values were 12.3, 3.4 and 24.1 μm, respectively. Lineweaver–Burk plots for the three novel peptides showed that they are all competitive inhibitors (Wang *et al*., 2008). In another study, desalted protein hydrolysate from northern shrimp (*Pandalus borealis*) showed ACE-inhibitory activity both *in vitro* and *in vivo*. Measurements by two independent methods showed higher *in vitro* ACE-inhibitory activity—IC_{50} = 0.075 and 0.035 mg/ml, respectively—than was earlier reported in comparable hydrolysates. Two novel ACE-inhibitory tripeptides—Phe-Thr-Tyr (IC_{50} = 275 and 59 μM) and Phe-Ser-Tyr (IC_{50} = 7.7 and 2.2 μM)—were detected in the hydrolysate. An introductory *in vivo* feeding trial with SHR indicated positive *in vivo* results when the rats were given 60 mg hydrolysate/kg body weight per day. Although further *in vivo* studies are necessary to verify the antihypertensive potential, the very high *in vitro* ACE-inhibitory activity reveals that shrimp-protein hydrolysate is a promising candidate for nutraceutical application (Asbjørn *et al*., 2009).

15.2.6 Alaska Pollock Peptides

The peptides extracted from the frame protein of Alaska pollock, which is normally discarded as an industrial byproduct, show ACE-inhibitory activity (Je *et al*., 2004). Pepsin-hydrolyzed frame protein was separated into five fractions according to molecular weight. The most active ACE-inhibitory peptides were found in the fraction of less than 1 kDa. From this fraction, a novel peptide was isolated, with an amino acid sequence of FGASTRGA and an IC_{50} value of 14.7 μM. Proteolytic digestion of gelatin extracts from Alaska pollock (*Theragra chalcogramma*) skin shows a high ACE-inhibitory activity. Gelatin extracts were hydrolyzed by serial protease treatments in the order alcalase, pronase E, collagenase. Two catalytically active peptides—Gly-Pro-Leu and Gly-Pro-Met—were separated and showed IC_{50} values of 2.6 and 17.13 μM, respectively (Byun & Kim, 2002). In another study, gelatin hydrolysates with a high ACE-inhibitory activity were fractionated from Alaska pollock surimi refiner discharge, and ACE-inhibitory activity was 0.49 mg/ml. Fractionation produced the highest inhibitory activity of 0.21 mg/ml (Park *et al*., 2009).

15.2.7 Yellow Fin Sole Peptides

The normally discarded yellow fin sole (*Limanda aspera*)-frame protein was identified as another source of ACE-inhibitory peptides (Jung, 2006). Using α-chymotrypsin, a peptide with a molecular mass of 1.3 kDa and 11 amino acids was isolated, with an amino acid sequence of MIFPGAGGPEL. Lineweaver–Burk plots suggested that YFP acts as a noncompetitive inhibitor of ACE, with an IC_{50} value of 28.7 µg/ml. It was demonstrated that this peptide reduced the SBP of SHR over 9 hours when administrated at 10 mg/kg animal body weight. The SBP of SHR was reduced by 22 mmHg at 3 hours, a reduction comparable with that of the group treated with Captopril. Reduction in SBP remained after 9 hours, suggesting that MIFPGAGGPEL is an effective antihypertensive agent *in vivo*.

15.2.8 Oyster Peptides

Hydrolysis of oyster proteins with proteases and hydrolysis with denazyme AP (from *Aspergillus oryzae*) (Matsumoto *et al*., 1994) produced a hydrolysate that could inhibit ACE with an IC_{50} value of 550 µg/ml. After separation the most active fraction (Leu-Ple) showed IC_{50} value of 85 µg/ml. Trypsin hydrolysate of oyster (*Crassostrea gigas*) showed ACE-inhibitory activity. The hydrolysate significantly suppressed SBP and ACE activity in SHR following a one-shot oral administration and a long-term feeding experiment lasting 9 weeks. Each hydrolysate from oyster tissue showed ACE-inhibitory activity, indicating the hypotensive effect was due to synergism. One potent ACE-inhibitory peptide, Asp-Leu-Thr-Asp-Tyr, was identified from the hydrolysate of the striate muscle, and the peptide exhibited hypotensive activity *in vivo*. Protease digestion analysis suggested that Asp-Tyr could be the real effector of this pentapeptide *in vivo* (Shiozaki *et al*., 2010). Further, the fermented oyster sauce showed ACE-inhibitory activity, and its IC_{50} value was 2.45 mg/ml. The purified inhibitor from fermented oyster sauce had an IC_{50} value of 0.0874 mg/ml and exhibited competitive inhibition against ACE. The purified peptide was evaluated for its antihypertensive effect in SHR following oral administration. Rat blood pressure significantly decreased after inhibitor injection (Je *et al*., 2005a, 2005b). Fermented pearl oyster sauce also showed a significant decrease in SBP in SHR. Four active peptides were identified: Phe-Tyr, Ala-Trp, Val-Trp and Gly-Trp.

15.2.9 Tuna Peptides

ACE-inhibitory peptide was isolated from tuna dark-muscle hydrolysates prepared by alcalase, neutrase, pepsin, papain, α-chymotrypsin and trypsin, respectively. Among these, the pepsin-derived hydrolysate exhibited the highest ACE-inhibitory activity. Pepsin hydrolysate was reported to contain the ACE-inhibitory peptide WPEAAELMMEVDP, with a molecular mass of 1581 Da and an IC_{50} value of 21.6 µM (Qian *et al*., 2007). Employing Lineweaver–Burk plots, the authors determined that the peptide formed enzyme–substrate–inhibitor and enzyme–inhibitor complexes to lower the efficiency of ACE *in vitro*. Administration of this peptide to SHR resulted in a maximum decrease in SBP between 3 and 6 hours. The SBP of test animals remained lower than that of control animals ($\sim$15 mmHg) after 10 hours, proving that WPEAAELMMEVDP

is an effective hypotensor *in vivo*. In another study, the peptic hydrolysate of tuna-frame protein exhibited potent ACE-inhibitory activity (Lee *et al*., 2010). After separation, a potent ACE-inhibitory peptide was identified from tuna-frame protein (PTFP), that was composed of 21 amino acids: Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr Pro (MW 2482 Da, IC_{50} 11.28 μm). Lineweaver–Burk plots suggest that PTFP acts as a noncompetitive inhibitor against ACE. Furthermore, the antihypertensive effect in SHR also revealed that oral administration of PTFP can decrease SBP significantly ($P < 0.01$). A protein hydrolysate prepared from tuna broth showed potent inhibitory activity against ACE, with an IC_{50} value of 12.52 mg/ml. Further fractionation produced an active fraction with an IC_{50} value of 0.21 mg/ml. This fraction was rich in basic amino acids and aromatic amino acid peptides with a molecular mass less than 565 Da (Hwang & Ko, 2004).

15.2.10 Shark Peptides

Shark-meat hydrolysate obtained with protease SM98011 digestion showed high ACE-inhibitory activity, with an IC_{50} value of 0.4 mg/ml. Further separation of hydrolysate resulted in four peptides with high ACE-inhibitory activity. Their sequences were identified as Cys-Phe, Glu-Tyr, Met-Phe and Phe-Glu. Cys-Phe, Glu-Tyr and Phe-Glu were confirmed to be novel ACE-inhibitory peptides, with IC_{50} values of 1.96, 2.68 and 1.45 μM, respectively (Wu *et al*., 2008).

15.2.11 Algae Peptides

A peptide fraction with activity against ACE was separated from the peptic digest of protein prepared from wakame (*Undaria pinnatifida*). Further separation yielded four tetrapeptides with ACE-inhibitory properties. These tetrapeptides were identified as Ala-Ile-Tyr-Lys (IC_{50} 213 μM), Tyr-Lys-Tyr-Tyr (64.2 μM), Lys-Phe-Tyr-Gly (90.5 μM),and Tyr-Asn-Lys-Leu (21 μM). *In vivo* studies showed that each tetrapeptide decreased blood pressure significantly (Sagar *et al*., 2010). Macroalgae are more commonly known as seaweeds and have for centuries been consumed whole among the East Asian populations of China, Korea and Japan. Peptides derived from marcoalgae are proven to have hypotensive effects in the human circulatory system (Fitzgerald, 2011). In another study, pepsin hydrolysate of microalgae (*Chlorella vulgaris*) protein waste produced a hendecapeptide with ACE-inhibitory activity. Edman degradation revealed its amino acid sequence to be Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe. Inhibitory kinetics revealed a noncompetitive binding mode with an IC_{50} of 29.6 μM, suggesting a potent amount of ACE-inhibitory activity compared with other peptides from the microalgae protein hydrolysates, which have a reported range between 11.4 and 315.3 μM (Sheih *et al*., 2009).

15.2.12 Other Marine Peptides with Potent Anti-ace Properties

Other species/raw materials with protein hydrolysates that have documented ACE inhibition are cod frames (Jeon *et al*., 1999) and heads (Bordenave *et al*., 2002), hard clam (Tsai, 2008), rotifer (Lee, 2009), fermented blue mussel (Je *et al*., 2005b), skipjack tuna (Astwan *et al*., 1995), sea bream scale (Fahmi, 2004), sea cucumber gelatin hydrolysate (Zhao *et al*., 2007), fermented surimi (Shan *et al*., 2007) and hydrolysed kamabo (Nagai *et al*., 2006).

15.3 ANTICANCER EFFECTS OF MARINE-DERIVED BIOACTIVE PEPTIDES

Nature has provided numerous bioactive compounds as therapeutics against various human cancers. There is a vast array of potent substances that have not yet been described but which possess a rich potential as therapeutics. Many of the compounds described in Section 15.2 have been isolated from various terrestrial and marine organisms, including a range of species from microorganisms, plants and higher-order animals. The marine biota accounts for a wide range of bioactive substances/peptides (Banerjee *et al*., 2008).

As the popular trend has turned towards the use of natural products, despite advances in the formulation of synthetic compounds using high-throughput equipment, so the demand for naturally derived therapeutics has increased. The ocean covers almost two-thirds of the earth's crust, and there is thus an enormous resource base available in the marine biota still to be explored in search of potent bioactive compounds. Due to the ever-increasing incidence of life-threatening human cancers, the search for antitumor compounds has become a very heated field in natural-products chemistry, and advancements in technology have paved the way for novel discoveries. It is very encouraging to observe that many of the marine organism-derived antitumor compounds have entered phase-I and -II clinical trials with promising outputs. Didemnin was the first marine-derived antitumor biopeptide entered in human clinical trials in the USA, and other anticancer peptides such as dolastatins, hemiasterlin, cemadotin, kahalalide F, soblidotin, didemnins, aplidine and so on have since been enrolled (Rawat *et al*., 2006). Table 15.2 summarizes some of the anticancer bioactive compounds derived from marine organisms that have been entered into clinical trials or are at the stage of preclinical evaluation. Owing to the cytotoxic nature of the isolated compounds, most have been further studied in search of anticancer drugs or therapeutics (Simmons *et al*., 2005). This section will briefly evaluate the potent anticancer effects of some very prominent and promising marine-derived bioactive peptides and proteins.

15.3.1 Didemin B and Aplidine

A bioactive depsipeptide, Didemnin B, that was isolated from a symbiotic cyanobacteria living in Carribian tunicate, *Tridemnum solidium*, was the very first marine-derived bioactive compound to enter into clinical trials as an anticancer agent, paving the way for hundreds of other compounds (Rinehart *et al*., 1981, 1988a,b). Didemnin B has expressed a broad range of antitumor effects against cervical, breast, myeloma, ovarian and lung cancers (Rawat *et al*., 2006; Sakai *et al*., 1992; Simmons *et al*., 2005). The mode of action of didemin B is interruption of protein synthesis through binding to the palmitoyl protein thioesterase. Aplidine (Dehydrodidemnin B) is very similar to Didemnin B but has a side chain which differs from Didemnin B's. It was developed against leukaemia and was synthesized by replacing the –OH group in the cyclic structure of Didemnin B. Didemnin imposes its cyctotoxic effects by inhibiting the synthesis of DNA, RNA and protein, which is a unique feature among anticancer drugs (Vera & Joullie, 2002). However, this effect leads to higher cellular toxicity (neuromuscular toxicity), no objective response and severe fatigue in patients after treatment, so clinical trials were terminated by the US National Cancer Research Institute.

Table 15.2 Anticancer bioactive peptides/proteins from marine organisms.

Organism	Bioactive compound	Chemical classification	Biological target
Cymbastella sp.	HTI-286	Linear peptide	Tubulin
Dolabella auricularia	Soblidotin (Dolastatin 10 derivative)	Linear peptide	Tubulin
Dolabella auricularia	Dolastatin 10	Linear peptide	Tubulin
Dolabella auricularia	Dolastatin 15	Linear peptide	Tubulin
Dolabella auricularia	Synthadotin/ILX651 (Dolastatin 15 derivative)	Linear peptide	Tubulin
Dolabella auricularia	Cemadotin/LU103793 (Dolastatin 15 derivative)	Linear peptide	Tubulin
Elysia rufescens	Kahalalide F	Cyclic depsipeptide	Lysosomes and erbB pathway
Tridemnum solidum	Dehydrodidemnin B	Cyclic depsipeptide	Ornithine decarboxylase
Tridemnum solidum	Didemnin B	Cyclic depsipeptide	FK-506 bp
Aplidium albicans	Dehydrodidemnin B/aplidin	Depsipeptide	Fas, MKP-1, cyclin D1, cdk4 , p21, Rac1, JNK
Jaspis digonoxea	LAF-389 (Bengamide B derivative)	ε-lactam peptide derivative	Methionine
Lyngbya majuscula	DMMC	Cyclic depsipeptide	Tubulin
Diazona angulata	Diazonamide	Cyclic peptide	Tubulin
Didemnin cucliferum	Vitilevuamide	Cyclic peptide	Tubulin
Micromonospora marina	Thiocoraline	Depsipeptide	DNA-polymerase
Nostoc sp.	Cryptophycins	Depsipeptide	Tubulin
Jaspis sp.	Jasplakinolide	Cyclic depsipeptide	Actin, caspase-3, CD11,CD14
Geodia corticostylifera	geodiamolides H	Cyclic peptides	F-actin filaments
Lyngbya majuscula	Laxaphycins A and B	Cyclic depsipeptides	Topoisomerase II
Symplocasp	Symplocamide A	Depsipeptide	Proteasome
Aspergillus insulicola	Azonazine	Dipeptide	NFκB
Dysidea arenaria	Arenastatin A	Cyclic depsipeptide	Tubulin
Spongia sp.	Spongidepsin	Cyclic depsipeptide	Actin

The Didemnin B derivative Aplidine possesses both inhibition of protein synthesis and antiangiogenic characteristics, making it an ideal drug for many solid cancers (Crews *et al*., 1994; Vera & Joullie, 2002). The cytotoxicity of Aplidine is lower than that of its precursor, and its mode of action is a little different to that of its original source. Aplidine exerts its effects on tumor cells via induction of oxidative stress, inhibiting ornithine decarboxylase, inhibiting synthesis of DNA and arresting the cell cycle (Molinski *et al*., 2009). Furthermore, Aplidine can activate p38 mitogen-activated protein kinases (MAPKs) and the JNK pathway via activation of Rac1, a small GTPase (Cuadrado *et al*.,

2003; Gonzalez-Santiago *et al*., 2006), and it can inhibit the secretion of VEGF as well (Biscardi *et al*., 2005). Few phase-II clinical trials have been conducted with Aplidine to test against metastatic melanoma, non-Hodgkin's lymphoma, prostrate cancers or bladder cancers (Molinski *et al*., 2009). Its side effects are very mild and it shows effectiveness even against cases that were unresponsive for other treatments (Simmons *et al*., 2005). Aplidine was marketed as a drug but later had to withdraw due to low demand.

15.3.2 ω-Conotoxin MVIIA

Conotoxins were the very first cytotoxic compounds to be identified as marine-derived bioactive compounds with very potent cytotoxicity (Olivera *et al*., 1985). The compound ω-conotoxin MVIIA, was first isolated from the marine snail *Conus magus*. It has linear peptides with 25 amino acids and can now be completely synthesized by the chemical means (Molinski *et al*., 2009). The mode of action of ω-conotoxin MVIIA in pain relief is to block the N-type voltage-sensitive calcium channels (NVSCCs) during the conduction of nerve signals (Olivera *et al*., 1987; Rivier *et al*., 1987). Recently, a few synthetic ω-conotoxin MVIIA types were tested as drugs, and Ziconotide, which has high potency as an antinociceptive substance, was the first marine-derived drug to get US Food and Drug Administration (FDA) approval. Following successful clinical trials, ω-conotoxin MVIIA ziconotide was marketed under the trade name 'Prialt' as an analgesic for the treatment of severe chronic pains (Garber, 2005). Many more ω-conotoxin MVIIAs are now being tested due to its success, and hopefully at least one more conotoxin-derived product will hit the market as an effective analgesic in the next few years.

15.3.3 Hemiasterlin/HTI-286

Hemiasterlin was also isolated from a marine sponge, *Hemiasterella minor*, as an oligopeptide (Gamble *et al*., 1999). Later, another isomer of the same compound was described (Molinski *et al*., 2009). All the hemiasterlins (A, B and C) exert their action on tumor cells by binding to tubulins and effectively arresting mitotic division (Anderson *et al*., 1997) and by inducing apoptosis via cytotoxicity. The synthetic analog compound of hemiasterlin, HTI-286, has greater potency than its precursor. Due to its side effects, such as alopecia, pain and nausea, clinical trials with HTI-286 were halted, but this compound still possesses high potency against many human solid tumors. Therefore, the search for/synthesis of a similar analog with less side effects is a very promising approach to the use of hemiasterlin as anticancer drug.

15.3.4 Dolastatins

Several preclinical and clinical evaluations have been completed on dolastatins. These small linear peptides were originally isolated from a marine mollusk, *Dolabella auricularia*. The dolastatins have shown primarily anticancer effects against live cancers, breast cancers, certain forms of leukemia and solid tumors. The general mode of action of all the dolastatins is via the inhibition of mitosis by interference with tubulin formation. After initial isolation, many synthetic compounds similar to dolastatins ILX651 and LU103793 were derived (Mayer *et al*., 2009). Soblitodin (TZT-1027) was one such promising compound. It exhibits high potency as an anticancer compound (Kobayashi *et al*., 1997; Watanabe *et al*., 2006) and has been tested against various types of human solid tumor,

with promising outcomes. Natural dolastatins and their derivatives can exert potent antimitotic effects through inhibition of the microtubule assembly and polymerization of the tubulin (Bai *et al*., 1990). Dolastatin 10 has demonstrated potent *in vitro* mitotic inhibitory activity against human melanoma, sarcoma and ovarian cancer cells (Aherne *et al*., 1996; Pettit, 1997) and it has been entered into many phase-II clinical trials against various human cancer types (Molinski *et al*., 2009). Among the dolastatins and their derivatives that have been investigated, the dolastatin 10 derivative TZT-1027 and the dolastatin 15 derivative ILX651 have shown the fewest side effects in clinical trials, while stabilizing tumor growth to result in a partial response (Tamura *et al*., 2007; Yamamoto *et al*., 2009). Even though several phase-II clinical trials have been completed with different dolastatin derivatives, none has proven sufficiently effective for use as an anticancer drug. Phase-II clinical trials using dolastatin 10 have been completed, and they show some promising results against Waldenstorm's macroglobulinemia, chronic lymphocytic leukemia and indolent lymphoma. Further studies with high-throughput screening will accelerate the discovery of dolastatin derivatives with utility as anticancer drugs.

15.3.5 Kahalalide F

Kahalalide F is a dehydroaminobutyric acid-containing peptide that was initially isolated from the mollusk *Elysia rufescens* (Simmons *et al*., 2005). The compound can induce apoptosis by disrupting the lysosome membrane in target cell types. A further effect of DNA-synthesis inhibition has also been described, making this peptide a promising anticancer agent. It has been entered into phase-I and -II clinical trials against non-small-cell lung cancer, prostrate cancers and melanomas, but further trials (phase III) are needed in order to release it as a drug to the market.

15.3.6 Cryptophycins

Cryptophycin A was originally isolated from the syanobacteria *Nostoc* sp. (Shih & Teicher, 2001), and later many of its isomers were characterized and its name was changed to cryptophycin-1 (Crp-1) (Molinski *et al*., 2009). Cyrptophycins are macrocyclic depsipeptides in nature. They have also been found in the marine sponge *Dysidea arenaria*. Natural and synthetic cryptophycins have shown potent anticancer effects against both carcinoma and adenocarcinoma types via destabilization of microtubules and induction of hyperphosphorylation of anti-apoptotic-protein B-cell leukemia/lymphoma-2 (BCL-2), inducing apoptosis (Lu *et al*., 2001). Cryptophycin-52, a synthetic derivative of Crp-1, was entered to phase-I clinical trials in the USA, using patients with solid tumors, but due to decreased responsiveness it was later dropped. Novel cryptophycin derivatives like cryptophycin-249 and cryptophycin-309 are now being tested for their potential effects against solid tumors (Liang *et al*., 2005).

15.3.7 Neovastat/AE-941

Neovastat was first derived from shark-cartilage extract (Falardeau *et al*., 2001). The mechanism of action of the compound was proven to be via antiangiogenic effects by inhibition of vascular endothelial growth-factor activity during angiogenesis. The compound can also inhibit the metastasis of the cancers by disrupting the extracellular matrix and inducing endothelial cell-specific apoptosis (Falardeau *et al*., 2001). The drug has entered

into phase-III clinical trials against non-small-cell lung cancer and renal-cell carcinoma, and has shown some promising results, stabilizing progression and relieving pain. Moreover, the compound's anti-angiogenic activity has been in focus for the treatment of multiple myeloma. Due to the multiple effects of AE-941, it has huge potential for use as an effective anticancer drug. However, since it has to be extracted from shark fins, some conservation aspects must be considered, as long-term exploitation might lead to eradication.

15.3.8 Vitilevuamide

This cyclic peptide was first isolated from one ascidian, *Didemnum cuculiferum*, and later from another ascidian named, *Polysyncraton lithostrotum*. Vitilevuamide can inhibit the polymerization of tubulin and then cause arrest of the target cells in the G2/M phase of mitotic cell division (Edler *et al*., 2002). The compound shows potent effects against certain luekemia types but still has not been entered into any clinical trials.

15.3.9 Thiocoraline

Thiocoraline is a thiodepsipeptide that was isolated from the actinomycete *Micromonospora marina*. Its active ingredient exerts its effect on cancer cells by arresting the cells in the G1 phase via inhibition of the DNA-polymerase enzyme (Negri *et al*., 2007). The compound has shown potent antitumor effects on colon-cancer cells in preclinical trials, and more in-depth studies are needed so it can proceed to clinical trial.

15.3.10 Jasplakinolide

Jasplakinolide is also known as 'jaspamide' and was first isolated from *Jaspis* sp. It is found in marine sponges (Andavan & Lemmens-Gruber, 2010) and shows very potent antitumor activity. Its mode of action is mainly via interference with actin polymerization in the tumor cells, hampering cell division. In preclinical screenings, jaspamide could inhibit the colony formation of myeloid leukemia cells. Furthermore, it can increase the expression of CD14 and CD11, while downregulating CD34 in the cancer cells (Fabian *et al*., 1995). Interestingly, jasplakinolide was found to be a very effective apoptosis inducer *in vitro* in various transformed cell lines, including human leukemia Jukurt T-cells and HL-60, which is a promyelotic cell line, by induction of the caspase-3-like protease-dependent pathway (Odaka *et al*., 2000). Therefore, this compound shows great promise as an effective antileukemic drug.

15.3.11 Conclusion

Though the discovery of novel compounds that possess anticancer activities happens every day, it is noticeable that only a very few proceed to the level of clinical trails. Many of the studies published are mainly restricted to simple descriptions, rather than looking for real bioactivities. Continuation of such work is lacking in many instances. A summary of the anticancer biopepeitdes discussed in this section can be found in Table 15.2. Although we must appreciate that in order to take an identified substance to market as an anticancer drug it must undergo a very painstaking procedure, with the development of high-throughput systems in both genomics and proteomics, we need to accelerate this process by incorporating multidisciplinary systems. Encompassing all possible interactomes

in human cellular physiology, biology, chemistry, physics and nanotechnology will yield more fruitful results than going through a linear-model approach, looking at only one aspect of the cellular processes in the treatment of novel compounds, which has been proven to be very inefficient. Furthermore, bringing scientists, institutions and companies into an open-access platform through the use of advanced communication technologies will greatly reduce the waste of time, money and the abilities of the people involved in the noble task of discovering novel anticancer therapeutics.

15.4 ANTIVIRAL BIOACTIVITIES OF MARINE-DERIVED BIOACTIVE PEPTIDES

Viral infections are becoming more and more common all around world, and the during the last 2 decade we have experienced several pandemic flu/influenza infections, severe acute respiratory syndrome (SARS), swine flu and bird flu cases. There has been a worldwide increase in human immunodeficiency virus (HIV) infections with the opening of most Asian and Sub-Saharan economies. Therefore the demand for antiviral compounds is also on the rise, and even during the last influenza outbreak, the world was in short supply of essential antiviral drugs, which became a controversial health issue (Smith *et al*., 2011). Owing to a virus's ability for rapid evolution, development of resistance to existing drugs has also been a profound concern (Danve-Szatanek *et al*., 2004; Morfin & Thouvenot, 2003; Sauerbrei *et al*., 2010; Vijaykrishna *et al*., 2011). Therefore, there is an urgent need to continue with the research and development on antiviral pharmaceuticals.

Examining the recent literature, several attempts have been made to isolate compounds with antiviral properties from marine sources, as marine sources are rich with a wide range of biologically active compounds and their metabolites (Gul & Hamann, 2005; Mayer *et al*., 2009; Molinski *et al*., 2009). In fact, the very first successfully used antiviral drug, acyclovir, was synthesized based on the arabinosyl nucleoside from a marine sponge, *Tethya cripts* (Elion *et al*., 1977, 1999). Even though many antiviral compounds have been derived from marine sources, only a few marine biopeptides have been identified as having antiviral activity. This section evaluates certain selected marine-derived peptides from different marine organisms for their antiviral activities and the feasibility of using them as therapeutics for viral infections.

15.4.1 Papuamides

The cyclic depsipeptides papuamide A, B, C and D were first isolated from the marine sponges *Theonella mirabilis* and *Theonella swinhoei*, which can be found in Papua New Guinea waters, by two different groups of scientists (Sagar *et al*., 2010). Very recently, papuamides E and F were also described from another sponge species, *Meloplus sp*. (Prasad *et al*., 2011). Papuamides A and B show potent anti-HIV activity by inhibiting viral entry to the cells (Andjelic *et al*., 2008; Este & Telenti, 2007), and a viral membrane-targeted direct viricidal activity by papuamide A has also been proposed (Andjelic *et al*., 2008). Recently, the total chemical synthesis of papuamide B was reported to be possible. This allows extensive evaluation of the bioactive compound without the need to extract it from marine sources, which would require a large number of sponges.

15.4.2 Callipeltin A

This active biopeptide, with four amino acids in its structure, was first isolated from a sponge belonging to genus *Callipelta* (Vo & Kim, 2010). Callipeltin A shows inhibition of cytopathic effects due to HIV-1 infections. More recent findings show its cytotoxic effects as well (Kikuchi *et al*., 2011). The compound is still under preclinical evaluation.

15.4.3 Neamphamide A

This novel anti-HIV depsipeptide, containing 11 amino acid residues, was isolated from the marine sponge *Neamphius huxlei*. The active compound shows cytoprotective effects against HIV-1 infection (Oku *et al*., 2004).

15.4.4 Mirabamides

Mirabamides A, B, C and D were isolated from the marine sponge *Siliquariaspongia mirabilis*. Recently, some other mirabamides (E–H) were isolated from another sponge, *Stelletta clavosa* (Lu *et al*., 2011). Mirabamides A–D have shown inhibition of HIV-1 viral fusion, specifically via interaction with viral-envelope glycoproteins (Plaza *et al*., 2007)

15.4.5 Cyanovirin-N

Cyanovirin-N (CV-N) is a small protein belonging to the lectin family. It was first isolated from a cyanobacterium, *Nostoc ellipsosporum*. Unlike many other antiviral compounds, CV-N shows antiviral affects against a plethora of viruses, including influenza, ebola, hepatitis and herpesvirus (Keeffe *et al*., 2011). But it is most well known for its inhibitory action against HIV-1, via binding to glycans on the envelope glycoprotein gp120 and then very effectively preventing viral entry (Bewley *et al*., 1998; Tsai *et al*., 2004). Furthermore, CV-N can prevent the replication and cytopathic effects of retroviruses and HIV-2 as well. Many successful preclinical trials have been conducted using CV-N, with promising outcomes, but clinical trials are needed in order to derive an effective version.

15.4.6 Microspinosamide

The cyclic depsipeptide microspinosamide was first identified from extracts of *Sidonops microspinosa*. The depsipeptide has a potent anti-HIV effect (Rashid *et al*., 2001), showing inhibitory activity against the cytopathic effect during the HIV-1 infection process.

15.4.7 Griffithsin

Griffithsin (GRFT) is a lectin-family protein that was isolated from the red-algae species *Griffithsia* sp. (Mori *et al*., 2005). The recombinant protein of GRFT shows potent antiviral activities *in vitro* and has raised interest in exploration of GRFT'ss further use as an antiviral therapeutic. The mode of action of GRFT as an anti-HIV-1 compound derives from its ability to inhibit viral entry to the host cells by binding to the viral glycoproteins

gp41 and gp120. The peptide derivative of griffithsin, grifonin (GRFN-1) (Micewicz *et al*., 2010), also displays anti-HIV-1 activity by inhibition of viral entry to the cells (Alexandre *et al*., 2011, 2012). GRFT is not only important as an HIV entry inhibitor but can also prevent cell-to-cell fusion in order to stop the transmission of the virus (Emau *et al*., 2007). Furthermore, GRFT has been found to act against hepatitis C virus (HCV) infection by interfering with cell-to-cell transmission through interaction with viral-envelope proteins and viral-receptor CD81 (Meuleman *et al*., 2011). Preclinical studies with mice models have shown very promising outcomes, indicating GRFT to be a good candidate for a potent antiviral agent, but further preclinical and subsequent clinical trials are needed.

15.4.8 Conclusion

There are hundreds of bioactive components described as possessing antiviral activities in the literature, but the major focus has been on anti-herpes simplex virus (HSV-1) and anti-HIV-1 compounds. HIV-1 infections are increasing at an alarmingly high rate all over the world and the absence of a permanent cure has attracted many scientists to the search for potent antiviral chemicals against it (Vo & Kim, 2010). With the evolution of drug-resistant HIV-1 with high cytotoxicity, many of the compounds discovered cannot be proceeded to clinical trial. Other than the proteins and peptide compounds mentioned above, manzamine A, dragmacidin F, 4-methylaaptamine, microspinosamide, phlorotannins, chitin, chitosan, chitooligosaccharides, sulfates and polysaccharides have been extensively studied using various cell models *in vitro* (Vo & Kim, 2010; Yasuhara-Bell & Lu, 2010). On the other hand, the relative abundance of the marine organisms that produce antiviral compounds is very low, and some of the chemical derivatives have not been as effective as the original isolated compound. To date, only four anti-HSV-1 compounds or derivatives have been able to come to market—acyclovir, ara-A and ara-C, Avarol and Azidothyminide—and only two potent anti-HIV-1 agents have reached preclinical development—avarol and cyanovirn. Nonetheless, the development of novel high-throughput systems and screening procedures will invariably help to screen and chemically synthesize the active compounds on a large scale, as is demanded by clinical trials. Such platforms will enable fast screening and cut short the time from discovery of a molecule to clinical trial. But for the compounds which we may not be able to synthesize chemically, we will have to adopt novel biotechnologies and nanotechnologies in order to scale up production/extraction from the marine organisms. Overall, marine organisms and marine-derived compounds will play an important and definitive role in the war against the viruses in years to come, and it may be vital to conserve such habitats globally in order to reap the optimal benefits.

15.5 THE FUTURE OF MARINE PEPTIDES AS THERAPEUTICS

As we have discussed, taking examples from the most-sought-after fields in drug discovery—drugs against hypertension, cancers and viral infections—hundreds of novel marine proteins and peptide compounds have been discovered and continue to be discovered every day all around the world. With the ever-increasing world population, globalization, increase in access to novel diagnostics and increasing income of many Asian, South American and African nations, the demand for drugs is on the rise. There

will inevitably be a chance of over-exploitation of the available marine resources in our search for novel therapeutics. This issue will be further aggravated by environmental risk factors such as global warming and marine pollution. Therefore, we need to put more effort into focusing on those compounds that have already been discovered, by doing more research in order to bring them up to the level of therapeutics, rather than searching for novel substances. As we have described in this chapter, in order to purify a protein or peptide from the original marine source, a huge quantity of that original source is needed. This particular constraint had driven researchers away from working on the same compound in large numbers in depth. Therefore, we need to generate novel aquaculture techniques and biotechnologies encompassing the green technologies in order to produce these sources effectively and with more manageable properties, while at the same time having a minimal impact on the environment. It is essential to find the means of fully or partly synthesizing the deired product as well.

Scientists must also begin a process of changing from a linear approach to a nonlinear, more dynamic approach, looking towards translation of discoveries into medicine-type applications, rather than purely academic work. Collaboration between academic institutes and pharmaceutical companies, together with basic scientists and clinicians, will definitely speed up the process with many elaborate success stories. Since novel high-throughput, highly sensitive, low-cost-per-sample and user-friendly machines and techniques will push the limits of the scientist to very high standards, we can foresee a bright future for novel drug discoveries from marine proteins and peptides, where many of these compounds are available as safe, effective and low-cost therapeutics for the general population all around the world.

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16 Hormone-like Peptides Obtained by Marine-protein Hydrolysis and Their Bioactivities

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16.1 INTRODUCTION

Heads, skin, scales, viscera, backbones, trimmings and sawdust represent the main residues derived from the fish-processing industry. Although some of these wastes have traditionally been used as fishmeal or fertiliser (Benjakul & Morrisey, 1997), they are rarely upgraded if not rejected into the sea, causing a heavy environmental impact. However, fish waste constitutes a valuable source of protein, which could be hydrolysed by mild processing techniques to obtain biologically active peptides, some of them having the potential to offer specific health benefits to consumers. Considerable attention is paid to angiotensin-I-converting enzyme (ACE) peptides, as they constitute a safe and economical alternative to synthetic ACE inhibitors used to treat hypertension (Wijesekara *et al*., 2010). Antimicrobial, antioxidative, antithrombotic and antiproliferative peptides can also be found in protein hydrolysates derived from marine sources, as has been widely reported (Alemán *et al*., 2011; Himaya *et al*., 2012; Najafian & Babji, 2012; Ngo *et al*., 2011; Picot *et al*., 2006; Rajapakse *et al*., 2005; Stensvag *et al*., 2008; Thorkelsson *et al*., 2008). These bioactive peptides thus have the potential to be used as nutraceuticals in the formulation of functional foods, and could hence reduce the population need for drugs and other medical therapies.

Regulatory peptides with hormone-like activity (hormone-like peptides) or with the ability to modulate the concentration of certain hormones or cytokines in blood could also be obtained by enzymatic hydrolysis. The biological activity of the hormone-like peptides is usually mediated by their interaction with G-protein-coupled receptors on the cell surface and further activation of the ligand receptor-signal transduction pathway, thus regulating different physiological functions of the body (Teschemacher, 2003). Although milk and soybean proteins are the most important sources of hormone-like peptides (Teschemacher, 2003; Yoshikawa *et al*., 2003), fish proteins could also serve as a hormone-like-peptides reservoir. Interestingly, some authors have found neuropeptide-immunorelating molecules and molecules capable of binding to specific hormone receptors on cell membranes in fish-protein hydrolysates (Fouchereau-Peron *et al*., 1999; Martínez-Alvarez *et al*., 2007, 2008; Slyzite *et al*., 2009). In addition, the hormone-like effects

[1]This Centre has implemented and maintains a Quality Management System which fulfils the requirements of ISO standard 9001:2008.

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of some orally administered fish-protein hydrolysates have been observed in vertebrates. However, scientific evidence of these marine-peptide effects is severely limited, and very few hormone-like peptides have been identified to date.

This chapter provides an overview of the hormone-like effects of different protein hydrolysates derived from marine sources, as well as of the occurrence of potential hormone-like and hormone-releasing fragments encrypted within the sequences of different proteins from commercial species of fish, crustaceans and molluscs. The potential use of these peptides as ingredients in functional foods or in fish feeds is also contemplated, as well as their physiological properties, both beneficial and detrimental.

16.2 GROWTH HORMONE-RELEASE PEPTIDES

Growth hormone (GH) and insulin-like growth factors (IGF-I and IGF-II) play an important role in growth regulation in vertebrates. GH is released from the pituitary gland and shows the capacity to stimulate growth, cell reproduction and regeneration in vertebrates. IGFs are mainly produced in the liver upon stimulation by GH, and both IGF-I and IGF-II are responsible for tissue growth in vertebrates (Thissen *et al*., 1999). These hormones can be used to grow fish in aquaculture, and in fact chronic administration or injection of GH and IGF-I may stimulate growth and food intake in fish (Chen *et al*., 2000; Lin *et al*., 2000). Nonetheless, production of large quantities of purified GH is expensive and difficult, and supplementation of GH-releasing molecules in fish feeds is more cost-effective, since fish can produce higher levels of GH on their own. In this sense, incorporation of fish protein in fish feed has been related to increments in growth performance, whereas partial or total replacement of fish meal by plant-protein sources has been associated with low growth performance, low feed intake and low plasma IGF-I levels (Aksnes *et al*., 2006; Gómez-Requeni *et al*., 2004; Mundheim *et al*., 2004). These differences ascribed to the protein source result in changes in the plasma levels of the essential amino acids Lys and Met, nucleotides, anserine and taurine, and lead to significant differences in IGF-I and GH regulation (Aksnes *et al*., 2006; Espe *et al*., 2008; Hevrøy *et al*., 2007).

The hydrolysis degree of proteins used in fish feeds may affect growth performance. Fish-protein hydrolysates generally show a higher beneficial effect on growth performance than intact fish proteins, but only at low inclusion levels (Hevroy *et al*., 2005). Aksnes *et al*. (2006) have highlighted the importance of small-molecular-weight compounds from fish hydrolysates to the growth and feed efficiency of rainbow trout, and recently Zheng *et al*. (2011) have also suggested the importance of the molecular-weight profile of the hydrolysate to growth. Specifically, Zheng *et al*. (2011) observed that feeding juvenile flounders a diet including an ultra-filtered pollock hydrolysate can significantly increase plasma IGF-I levels and liver IGF-I mRNA expression.

Some fish-protein hydrolysates has been reported to include molecules with the ability to exert an effect in animals similar to that of the epidermal growth factor (EGF), which stimulates the proliferation of epidermal and epithelial tissues (Carpenter, 1979). Specifically, Fitzgerald *et al*. (2005) and Marchbank *et al*. (2008) have described, in animal gastric-damaging models, the capacity of an orally administered commercial protein hydrolysate of Pacific whiting (Seacure®) to induce the same biological repair-promoting responses as EGF. Hydrolysates including these EGF-like molecules could provide a novel and inexpensive approach to the prevention and treatment of the harmful effects of nonsteroidal anti-inflammatory drugs on the bowel, where therapy is currently suboptimal.

16.3 OPIOID-LIKE PEPTIDES

Recent studies have provided evidence that exogenous peptides obtained by enzymatic hydrolysis of milk, plant or blood proteins may play an active role in the nervous system after ingestion (Iwaniak & Minkiewicz, 2008; Meisel & Bockelmann, 1999; Teschemacher, 2003; Yoshikawa *et al*., 2003). These natural opioid-like peptides have attracted attention in the food industry as they can elicit effects on motivation, emotion, behaviour, stress, appetite and pain in vertebrates. Opioid-like peptides seem capable of mimicking the effects of body opioid hormones by interacting with specific opioid receptors in the nervous, endocrine and immune systems, as well as in the intestinal tract of the mammalian organism. Although opioid-like peptides possess a weaker activity than endogenous opioid peptides, this is compensated for by the high peptide concentration in the gastrointestinal tract following ingestion. It makes them less likely to cause the deleterious effects often associated with opioids, such as tolerance, dependence and addiction, allowing hydrolysates, including opioid-like peptides, to be used a natural and safe alternative to opioid drugs. Although fish protein is not the main source of exogenous opioid-like peptides, some marine hydrolysates are currently commercially available in Europe and the USA as food supplements with anxiolytic properties. Nonetheless, there are only a few clinical studies showing scientific evidence of the opioid-like effect of these marine-protein hydrolysates. The first evidence of the opioid-like effect of fish-protein hydrolysates was found by Crocq *et al*. (1978, 1980), who reported the anti-anxiety effect of Stabilium® (a dietary supplement obtained from enzymatic autolysis of blue ling viscera) in asthenic patients dealing with severe anxiety. The anti-anxiety effect of Stabilium® has been further described in college students exposed to stressful conditions (Dorman *et al*., 1995), and its positive effect on behavioural equilibrium and concentration in stressed rats has also been proved (Le Poncin, 1996a). Another commercial hydrolysate derived from marine proteins, named Seaforce®, has been reported to improve short- and long-term memory, as well as the capacity to sustain attention in humans with anxious–depressive symptoms (Le Poncin, 1996b). The commercial fish-protein hydrolysate Gabolysat PC60® has shown the ability to suppress rises in plasma adrenalin levels and to reduce adrenocorticotropic hormone (ACTH) release in animals under stress conditions (Bernet *et al*., 2000). The gabolysat derivative Stabilium 200® and the pollock hydrolysate Protizen® may elicit a potent anxiolytic effect in stressed rats (Messaoudi *et al*., 2008; www.copalis.fr). The commercial hydrolysate Antistress 24® has been described as a nutritional complement with the capacity to regulate adrenaline and cortisol secretion (www.fortepharma.com), and the fish hydrolysate Procalm® may manage canine anxiety disorders and improve dog well-being (www.copalis.fr).

Although the mechanisms underlying the anxiolytic-like activity of fish-protein hydrolysates remain unknown, various hypothesis have been proposed. It is possible that the anxiolytic effect is promoted by the presence of high quantities of specific free amino acids, such as glutamic acid, glutamine, tyrosine, lysine, arginine or taurine, in the hydrolysate. These amino acids are known for having anxiolytic-like and/or antidepressant-like activity (Kong *et al*., 2006; Smriga & Torii 2003; Srinongkote 2003; Zhang & Kim 2007). Glutamic acid is a gamma amino butyric acid (GABA) precursor and may hence increase GABA levels in the hippocampus and hypothalamus, thereby reducing ACTH release and anxiety under stress conditions (Jones & Gillham, 1988). Glutamine may also increase GABA levels (Wang *et al*., 2007), improving mood in patients with depression (Young *et al*., 1993). Tyrosine is the physiological precursor

of catecholamine synthesis, and its administration has been reported as an effective treatment for depression in humans, although its effect is controversial (Gelenberg *et al*., 1980). Lysine, combined with arginine, may decrease corticosterone and cortisol levels in the plasma of stressed animals, thus reducing anxiety (Smriga & Torii 2003; Srinongkote 2003). Taurine may also produce an anxiolytic-like effect in mice, as observed by Chen *et al*. (2004) and Kong *et al*. (2006).

The anxiolytic effect of several fish-protein hydrolysates might also be promoted by peptides released during the enzymatic hydrolysis. These peptides could be structurally similar to pituitary- and hypothalamic-stimulating peptides and could act as precursors of hormones and neurotransmitters such as GABA, encephalin and endorphin. Although none of these peptides has been identified as yet, they could have an identical sequence to that of other opioid-like peptides previously identified in scientific literature, and could be detected within a given protein sequence by bioactive peptide databases as BIOPEP (Minkiewicz *et al*., 2008). Interestingly, the BIOPEP database has detected several fragments with demonstrated opioid-like effects within the sequences of several proteins abundantly found in the most common commercial marine species (Table 16.1). These peptides (YL, GP, PG, PGP, GPGG, PLG and NAGA) could be released during enzymatic hydrolysis and might be responsible for the demonstrated opioid-like activity of some fish-protein hydrolysates. Regarding peptide YL, it is found in the primary structures of osteonectin (a glycoprotein located in fish bones), β-actin and different types of collagen fibre found in several marine species. YL may exhibit a dose-dependent anxiolytic effect in mice following oral administration (Kanegawa *et al*., 2010). This effect is comparable to that of diazepam and is mediated by the activation of serotonin 5-HT_{1A}, dopamine D_1 and $GABA_A$ receptors, although YL does not act as an agonist of these receptors. Tripeptides with a YL sequence in the N-termini have also been found to exhibit anxiolytic activity (Kanegawa *et al*., 2010). Another opioid-like peptide, NAGA, is present in the primary structure of type-I collagen in picked dogfish (Table 16.1) and has shown narcotic-like pharmacological activity in mice after intracerebroventricular administration, possibly through interaction with the brain opioid receptor (Kosaka *et al*., 1985). He *et al*. (1994) have also studied the antinociceptive effect of NAGA, and associated this effect with the release of met-enkephalin and leu-enkephalin in rat brains. The glyprolines PGP, GP, GPGG and PG also show interesting opioid-like activity and may be found within the sequence of collagen chains of several marine species, as well as within myosin and actin chain sequences in different marine species. PGP and GP may exert a corrective effect on stress-induced impairments in rat behaviour (Badmaeva *et al*., 2006; Kopylova *et al*., 2007), and GP may also suppress the analgesic action of morphine in rats (Ray & Dey, 1982). GP, PG, PGP and GPGG may potentiate memory-consolidation processes in the central nervous system (Ashmarin, 1998), and PGP, PG and GP have also shown the ability to diminish visceral pain sensitivity in rats in a dose-related manner (Samonina *et al*., 2000). Another interesting fragment, PLG, is encrypted within the sequence of alpha-II collagen (type XI) of Atlantic salmon (Table 16.1), and shows a similar sequence to that of melanocyte-stimulating hormone release-inhibiting factor-1 (MIF-1), although MIF-1 is amidated at its C-terminus. In experiments performed with rats, PLG has exerted a similar antagonistic activity to that of MIF-1 on the development of tolerance to the cataleptic effects of haloperidol (Bhargava, 1981; Khan *et al*., 2010; Mycroft *et al*., 1987).

Table 16.1 Hormone-like peptides detected within the sequences of different proteins in common fish and mollusc species by the BIOPEP database. The sequence of the proteins screened is available in the protein-sequence databases of the National Centre for Biotechnology Information (NCBI) and the Swiss Institute of Bioinformatics (Swiss-Prot). GP, PG, and PGP also show immunomodulating activity.

Protein	Protein chain	Common name	Opioid peptides	Glucose uptake-stimulating peptides	Immunomodulating peptides
Osteonectin		*Atlantic cod*	YL	VL, LV, IV, LI, II, LL	
		Halibut	YL	VL, LV, IV, LI, II, LL	
		Gilt-head bream	YL	VL, LV, IV, LI, II, LL	
Actin	*alpha*	*Atlantic salmon*	GP, PG	VL, LV, IV, IL, II, LL	GVM
	alpha	*Halibut*	GP, PG	VL, IV, IL, II	
	alpha	*Gilt-head bream*	GP, PG	LV, IV, IL, LL	
	alpha	*Rainbow trout*		VL, IV, IL, II, LL	
	alpha	*Atlantic cod*	GP, PG	VL, LV, IV, IL, II, LL	GVM
	alpha	*Alaska pollock*	GP, PG	LL, LV, IV, IL, LL	GVM
	alpha	*Atlantic herring*		VL, LV, IV, II, LL	GVM
	alpha	*European seabass*	GP, PG	VL, LV, IV, IL, II, LL	GVM
	beta	*Atlantic salmon*	YL, GP, PG	VL, LV, IV, IL, II, LL	GVM
	beta	*Halibut*	PG	VL, IV, IL, II, LL	GVM
	beta	*Gilt-head bream*	YL, PG	VL, IV, IL, II, LL	
	beta	*Rainbow trout*	YL, PG	VL, LV, IV, IL, II, LL	GVM
	beta	*Atlantic cod*	YL	VL, IV, IL, LL	
	beta	*Picked dogfish*	PG	VL, II	
	beta	*Chub mackerel*	YL	VL, IV, IL, LL	GVM
	beta	*European pilchard*	YL	VL, IV, IL, LI, II, LL	
	beta	*Japanese jack mackerel*	YL, GP, PG	VL, LV, IV, IL, II, LL	GVM
	beta	*European seabass*	YL, GP, PG	VL, LV, IV, IL, II, LL	GVM
	--	*Blue mussel*	YL	VL, IV, IL, LL, YL	
	--	*Kuruma prawn*	YL, GP, PG	VL, LV, IV, IL, LL	GVM
	beta	*Pacific white shrimp*	YL, GP, PG	VL, LV, IV, IL, LL	GVM
	--	*Cuttlefish*	YL, PG	VL, IV, IL, LL	

(*continued overleaf*)

Table 16.1 (*continued*)

Protein	Protein chain	Common name	Opioid peptides	Glucose uptake-stimulating peptides	Immunomodulating peptides
Myosin	*heavy*	*Pacific bluefin tuna*	YL, GP, PG	VL, LV, IV, IL, LI, LL	YG, GFL, EAE, RKP
	heavy	*Atlantic bluefin tuna*		VL, LV, IL, LI, LL	EAE
	heavy	*Kuruma prawn*	YL, PG	VL, LV, IV, IL, LI, II, LL	EAE, YG, GLF
	heavy	*European seabass*	YL, GP, PG	VL, LV, IL, LI, II, LL	YG, EAE, RKP, GLF
	heavy	*Rainbow trout*		VL, LV, IV, IL, LL	EAE
	heavy	*Cuttlefish*	YL, PG	VL, LV, IV, IL, LI, II, LL	YG, EAE, GFL, GLF
	heavy	*Alaska pollock*	YL, PG	VL, LV, IL, LI, II, LL	YG, EAE, RKP, GLF
	heavy	*Blue mussel*			EAE
	light	*Japanese anchovy*	GP, PG, PGP	VL, IV, II, IL, LL	
	light	*Rainbow trout*		VL, IV	EAE, RKP
	light	*Atlantic horse mackerel*		VL, IL, II	EAE, GLF
	light	*Gilt-head bream*	GP	VL, IV, II, LL	
	light	*Chub mackerel*		VL, IV, LL	GLF
Collagen	*I (alpha-3)*	*European seabass*	YL, GP, PG, GPGG, PGP	VL, II, LL	GVM
	I (alpha-1)	*Rainbow trout*	YL, PG, GP, GPGG, PGP	VL, IV, II, LL	YG, GVM
	I (alpha-1)	*Gilt-head bream*	GP, PG, PGP	VL, II	YG
	I (alpha-2)	*Picked dogfish*	NAGA, GP, PG, GPGG, PGP	VL, LV, IL, LI, II, LL	GFL
	XI (alpha-l)	*European seabass*	YL, GP, PG	VL, LV, IV, IL, LI, II, LL	
	XI (alpha-2)	*Atlantic salmon*	PLG, GP, PG, GPGG, PGP	VL, LV, IV, IL, LI, LL	YG, EAE

16.4 IMMUNOMODULATING PEPTIDES

Research concerning the role of functional peptides in the immune system is quite new but seems promising. Different fish-derived peptides have elicited interesting immunostimulating effects in animals, although relevant clinical studies in this field are scarce. The potential use of these hydrolysates is today mainly focused on animal farms, specifically on aquaculture, as they can enhance stress and disease resistance in cultured fish. Immunomodulating hydrolysates can be incorporated into feed supplements, representing an alternative method to antibiotic use in controlling infectious diseases in fish farms (Bagny *et al*., 2000).

The immunostimulating effects of several fish protein hydrolysates can be produced by enhanced lymphocyte proliferation, natural killer (NK)-cell activity and cytokine regulation. Nonetheless, the specific mechanisms underlying this immunostimulating effect remains uncertain. Fermented fish-protein concentrate from orally administered Pacific whiting and Chum-salmon hydrolysate may enhance T-helper cells (T_h1 and T_h2) to produce increased amounts of various cytokines (IL-2, IFN-γ, IL-5, IL-6) in rats (Duarte *et al*., 2006; Yang *et al*., 2009). Release of the cytokines interleukin-2 (IL-2) and interferon-gamma (IFN-γ) would then induce a strong enhancement of the cytotoxic activity of the NK cells, as has been observed in rats administered Chum-salmon hydrolysate (Yang *et al*., 2009). However, high doses of the hydrolysate show just the opposite effect. This Chum-salmon hydrolysate may also increase the number of $CD4^+$ cells (T_h cells) and enhance the antigen-presentation effect of macrophages (Yang *et al*., 2009). Nonetheless, it does not seem to directly affect macrophage activity. Another fish-protein hydrolysate derived from cod muscle has been reported to strongly enhance the production of reactive oxygen metabolites in Atlantic salmon macrophages (Bøgwald *et al*., 1996; Gildberg *et al*., 1996).

The main peptides responsible for the immunomodulating activity of some marine-protein hydrolysates have not been identified. However, it is possible that this activity results from the presence of certain peptides with proven immunomodulating activity, such as GVM, GLF, GFL, YG, EAE, GP or PG, in the hydrolysates (Table 16.1). The immunostimulating peptide GVM (Xu, 1998) may remain inactive within the sequence of actin and collagen chains of different species of fish and mollusc (Table 16.1). GLF is encrypted within the sequence of myosin heavy chains of European seabass, cuttlefish and Alaska pollock, and in myosin light chains of two different species of mackerel (Atlantic horse mackerel and chub mackerel). This peptide may stimulate phagocytic activities *in vitro* and protect mice against *Klebsiella pneumoniae* infection (Migliore-Samour *et al*., 1992). Its immunomodulating activity can be mediated by interaction with specific receptors on plasmatic membranes of polymorphonuclear leukocytes and monocytes. This interaction initiates phosphoinositide breakdown and finally triggers a rapid and massive production of superoxide anion O_2^- (respiratory burst). GFL is another immunomodulating peptide which may remain inactive within the sequence of myosin heavy chains of Pacific bluefin tuna and cuttlefish, as well as in the collagen type I of picked dogfish. Although GFL lacks immunomodulating activity, it can potentiate the stimulating effect of GLF on polymorphonuclear leukocytes (Migliore-Saumur *et al*., 1992). YG is a partial sequence in the primary structures of myosin and collagen chains of several fish species, and may stimulate proliferation of human peripheral blood lymphocytes *in vitro* (Kayser & Meisel, 1996). Moreover, YG has been reported to inhibit the development of infections in patients with pre-AIDS (Hadden, 1991). Peptide EAE (thymosin-like peptide) is encrypted within

the sequence of type-XI collagen of Atlantic salmon, and within the sequence of myosin chains of different commercial fish and mollusc species (Table 16.1), showing the ability to stimulate lymphocytes in a mixed lymphocyte culture assay (Ciardelli *et al*., 1981). Glyprolines GP and PG are abundant in collagen and exhibit important immunostimulating activity. Both dipeptides may stimulate superoxide anion generation in neutrophils pretreated with chemotaxis stimulator, as observed by Watanabe *et al*. (1994). In contrast, RKP has been described as an immunosuppressive peptide. It is present within the sequence of myosin chains of Pacific bluefin tuna, European seabass, Alaska pollock and rainbow trout (Table 16.1), and may decrease the humoral immune response in mice in a dose-dependent manner (Wieczorek *et al*., 1994).

Other peptides might also indirectly stimulate the immune system when exerting other bioactivities. Specifically, ACE-inhibitory peptides could increase the bradykinin content in blood after ACE inhibition, and this increment could lead to increased immunostimulation and neurotransmission, stimulation of macrophages, enhancement of lymphocyte migration and cytokine secretion (Paegelow & Werner, 1986). In addition, opioid-like peptides could bind to opioid μ-receptors on T-lymphocytes and human phagocytic leukocytes, exerting an immunomodulating effect (Faith *et al*., 1984; Lopker *et al*., 1980).

16.5 GLUCOSE UPTAKE-STIMULATING PEPTIDES

The development of new therapeutic agents to improve glucose metabolism and prevent and inhibit type-2 diabetes mellitus-related complications is greatly significant. A few fish-protein hydrolysates have shown *in vivo* glucose uptake-stimulating activity and could be used in hyperglycaemia management in addition to regular therapy. These glucose uptake-stimulating hydrolysates can ameliorate glucose tolerance either by stimulating glucose uptake via a different mechanism to that of insulin or by increasing insulin sensitivity in target cells.

The cod hydrolysates Nutripeptin® and Peptide N® are described as being able to lower postprandial blood glucose and reduce insulin resistance in individuals suffering from persistent high blood-glucose levels (www.nutripeptin.no; www.celergen.ch). These products are food use-approved. Another hydrolysate, derived from wild marine fish collagen, has recently been found to improve insulin sensitivity and hence glucose metabolism in patients with type-2 diabetes mellitus (Zhu *et al*., 2010). Oral administration of this collagen hydrolysate has been associated with changes in serum levels of three adipocyte-secreted hormones closely implicated in the pathogenesis of diabetes: leptin, resistin and adiponectin. Serum levels of leptin and resistin were shown to decrease after oral administration of the protein hydrolysate in diabetic patients, whereas adiponectin levels rose markedly in diabetics after prolonged administration. Oral administration of this fish-protein hydrolysate was also associated with a sharp decrease in levels of serum high-sensitivity C-reactive protein (hs-CRP), free fatty acids and cytochrome P450, which are associated with the development of insulin resistance and type-2 diabetes mellitus (Asfandiyarova *et al*., 2006; Doi *et al*., 2005). Furthermore, serum levels of prostacyclin, also involved in the development of diabetes (Ylikorkala & Viinikka, 1981), decreased after the prolonged administration of this fish hydrolysate.

The precise mechanism underlying the action of protein hydrolysates on insulin sensitivity is still to be determined. Some dipeptides characterized by the presence of branched-chain amino acids may stimulate glucose uptake and glycogen synthesis rate

after oral administration (Morifuji *et al*., 2009) and could be released during enzymatic hydrolysis of marine proteins (Table 16.1). The glucose uptake-stimulating effect of these branched chain-containing peptides may be different to that reported by Zhu *et al*. (2010), as peptides may stimulate glucose uptake in skeletal-muscle cells and isolated muscles via the phosphoinositide 3-kinase (PI3-kinase) and atypical protein kinase C (aPKC) pathways; that is, by pathways different to that of insulin (Morifuji *et al*., 2009).

Finally, it is worth mentioning that ACE inhibitors in protein hydrolysates can also increase glucose metabolism by improving insulin sensitivity and insulin secretion (Henrikssen *et al*., 1998; Uehara *et al*., 1994). The ACE-inhibitory peptides found in some fish hydrolysates may promote higher serum levels of bradykinin and increase glucose transport and insulin sensitivity (Henriksen & Jacob, 2003; Henriksen *et al*., 1998; Ishimura *et al*., 2009; Uehara *et al*., 1994), as well as the expression of adiponectin, hence providing a higher insulin sensitivity (Rasouli & Kern, 2008). A similar relationship between the occurrence of ACE-inhibitory peptides in a sardine hydrolysate and the suppression of rising blood-glucose levels after glucose loading was observed by Otani *et al*. (2009) in animal studies.

16.6 SECRETAGOGUE AND CALCIOTROPIC ACTIVITIES

Calcitonin gene-related peptide (CGRP) is a neurohormone involved in an increasing number of biological activities. CGRP is one of the most potent vasodilators among endogenous substances known to date, active throughout the cardiovascular system, although the most noticeable vasodilation effects occur in the microcirculation. In addition to its great potency as a vasodilator, CGRP can increase the rate and force of heart contraction (Bell & McDermott, 1996) and suppress gastric acid secretion, decreasing food intake (Hughes *et al*., 1984; Sun *et al*., 2010). CGRP is also involved in the regulation of bone remodelling (Huebner *et al*., 2008).

Specific radioimmunoassay has shown the presence of CGRP-immunorelated molecules in protein hydrolysates from shrimp, sardine, cod, dogfish and saithe (Fouchereau-Peron *et al*., 1999; Martínez-Alvarez *et al*., 2007, 2008). Specific radioreceptorassays have also demonstrated the presence of CGRP biologically related molecules (CGRP-like molecules) in protein hydrolysates from sardine, shrimp, saithe, dogfish, cod and North Atlantic lean fish (Fouchereau-Peron *et al*., 1999; Picot *et al*., 2010; Ravallec-Plé *et al*., 2001; Rousseau *et al*., 2001; Slizyte *et al*., 2009). These CGRP-like molecules displayed affinity for specific G-protein-coupled receptors on rat-liver plasma membranes, and some were also able to exhibit either agonist (Martínez-Alvarez *et al*., 2007, 2008) or antagonist activity (Rousseau *et al*., 2001). CGRP-like molecules from saithe and dogfish hydrolysates have been isolated and sequenced; they are eight residues long and show homology with fragments of actin and collagen, respectively (Martínez-Alvarez *et al*., 2008, 2012).

The CGRP-like peptides may exert certain health-enhancing effects of interest to the pharmaceutical and food industries in the near future. Although the effects of the CGRP-like molecules have not been tested in animal or clinical studies, it is possible that these peptides could bind to cell-surface receptors on osteoclasts and thus have a role in calcium metabolism.

Gastrin and cholecystokinin (CCK) are small-intestinal hormones belonging to the secretagogue family. Although gastrin and CCK share a common amino-terminal sequence

(-Trp-Met-Asp-Phe-NH_2), they exert different functions in the body. Gastrin stimulates gastric acid secretion and epithelial cell proliferation (Dockray, 2004), while CCK is a family of peptide hormones which controls both the emptying of the gallbladder and pancreatic enzyme secretion. Rehfeld (2004) has also reported the capability of CCK to work as a growth factor and neurotransmitter. Peptides of the gastrin/CCK family mediate physiological activities by acting on CCKA and CCKB receptors. CCKA receptors are found in pancreatic acini and have a greater affinity for sulfated CCK than for gastrin, while gastrin CCKB receptors are found in gastric mucosa and the brain and have similar affinities for gastrin and CCK (Shulkes & Baldwin, 1997).

Both gastrin and CCK represent hormones of economic interest in the preparation of animal feeds due to their capacity to stimulate digestion (Beinfeld, 1995). In recent years, molecules immunologically related to gastrin and CCK have been obtained by controlled hydrolysis of fish and shellfish wastes. These molecules may exert biological activities similar to those of gastrin/CCK and could potentially be incorporated as a sustainable complement in animal feed. Gastrin/CCK-immunoreactive molecules have been identified in hydrolysates of cooked sardine wastes (Cancre *et al*., 1999; Ravallec-Plé *et al*., 2001), yellowfin tuna stomach (Guerard *et al*., 2001) and skins from North Atlantic lean fish (Picot *et al*., 2010). Moreover, gastrin/CCK-like molecules of approximately 1000 Da with the capacity to stimulate amylase release in AR4-2J cells have been found in protein hydrolysates of cod muscle and shrimp heads (Ravallec-Plé *et al*., 2003). Slizyte *et al*. (2009) have also reported the presence of gastrin/CCK-immunoreactive molecules in different protein hydrolysates derived from cod backbones, observing significant differences in the concentrations of these molecules, depending on both the quality of the raw material used and the hydrolysis time. Although the effect of gastrin/CCK-like molecules on animal feed has not yet been tested, the demonstrated beneficial effect of protein hydrolysates on the growth of juvenile carp and seabass (Cahu *et al*., 1999; Carvalho *et al*., 1997) could be related to the occurrence of such molecules. Nonetheless, the role of gastrin/CCK-immunoreactive molecules in digestion is still controversial and may depend on factors such as protein sources, hydrolysis conditions, doses, animal species and so on.

Some peptides derived from fish proteins might also regulate food intake by stimulation of CCK secretion in the intestine. Only a few *in vitro* studies have reported the ability of marine-protein hydrolysates to stimulate CCK secretion in culture cells. Cudennec *et al*. (2008) have described the effect of three protein hydrolysates from blue whiting and brown shrimp on CCK secretion in enteroendocrine STC-1 cells. These CCK-stimulating peptides may show apparent molecular weights ranging from 1000 to 1500 Da and may stimulate CCK secretion in a dose-dependent manner. Cudennec *et al*. (2012) have further observed that orally administered blue whiting hydrolysate may generate significant effects on short-term food intake in rats, reducing the increase in body weight. Lower food intake may be correlated with an increase of CCK and glucagon-like peptide-1 (GLP-1) circulating rate.

16.7 LIMITATIONS ON THE USE OF HORMONE-LIKE PEPTIDES AS NUTRACEUTICALS

The discovery of hormone-like molecules which might regulate vital biological functions in our organism has aroused interest in their therapeutic use. Unfortunately, the use of linear peptides as nutraceuticals is greatly limited by their poor metabolic stability, as they

are rapidly degraded after oral ingestion in the gut by digestive enzymes. Moreover, linear peptides show low bioavailability, which is due in part to their inability to readily penetrate across biological barriers. Nonetheless, experimental investigations have demonstrated that dipeptides and tripeptides, and also collagen-derived peptides, especially those with C-terminal Pro or Hyp residues, may cross the intestinal barrier and enter the circulation in quantitatively significant amounts (Moskowitz, 2000; Roberts *et al*., 1999). Some large peptides (10–51 amino acids) might also be absorbed intact through the intestine, although increased chain length appears to diminish the gut absorption of polypeptides (Roberts *et al*., 1999). Some proline- or hydroxiproline-containing peptides are also particularly resistant to intestine proteolysis and enterocytes, and may cross the intestinal barrier, as observed in studies performed *in vivo* after ingestion of collagen hydrolysate (Aito-Inoue *et al*., 2007; Iwai *et al*., 2005; Meilman *et al*., 1963; Ohara *et al*., 2007; Watanabe-Kamiyama *et al*., 2010). Another important limitation on the use of certain peptides as nutraceuticals is their low penetration across the blood–brain barrier. Nonetheless, some Pro-containing peptides may cross the blood–brain barrier and directly affect central-nervous structures involved in the organism's response to stress factors (Badmaeva *et al*., 2006; Kopylova *et al*., 2007). Finally, the biological potencies of absorbed peptides, as well as the quantities of peptide that must be administered into the gut in order to produce a biological effect, is also an important limitation on the use of protein hydrolysates as nutraceuticals.

16.8 FURTHER DEVELOPMENT AND RESEARCH NEEDS

The main challenge to the use of marine-protein hydrolysates as a source of hormone-like peptides is how to ensure good-quality raw materials and consistent composition in all batches following enzymatic hydrolysis. Fish wastes usually show wide variability in their composition and deficient microbiological quality, and these facts could enormously limit the successful application of fish wastes in health-related practices on a large scale.

Much work must also be done to clearly demonstrate the hormone-like effect of fish-protein hydrolysates in rats and humans. First, research into hormone-like peptides from marine sources will have to be developed according to a set of fixed experiments. High-throughput screenings, using ligand competition assays or alternative methods, will be very useful in evaluating both the occurrence of hormone-like peptides in enzymatic extracts and their affinity for specific receptors. The peptide pool in a protein hydrolysate might include diverse molecules with agonist or antagonist activity, and extensive research on the activation or blockade of effector systems (adenylate cyclase, K^+ channels, etc.) after receptor binding will be similarly necessary. In addition, the hormone-like peptides should be isolated and identified. Measurement of the activity of the synthetic peptide with an amino acid sequence identical to that of the peptide of interest should be performed to confirm the sequence of the potential bioactive peptide. Moreover, the biological activity of the hormone-like peptides, or the hydrolysates thereof, might be tested in animals prior to human assay. The instability of most of the peptides in the gastrointestinal tract, together with their difficulties crossing biological barriers, is the main obstacle to making the oral administration of peptides feasible, and different strategies for improving the stability and bioavailability of peptides should be considered in order to overcome these problems (Adessi & Soto, 2002). Exceptionally, some short Pro-containing oligopeptides (e.g. PG, GP and PGP) might be used unprotected, as they are resistant to proteases and

are usually absorbed easily compared to longer ones. Finally, clinical studies showing scientific evidence of relevant effects in humans will be necessary before the use of hormone-like peptides as nutraceuticals can be considered. Prevention of peptide effects by specific antagonists and specific antibodies should also be demonstrated, along with the coincidence of time/effect and time/concentration courses.

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17 Antimicrobial Activities of Marine Protein and Peptides

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17.1 INTRODUCTION

Antimicrobial peptides (AMPs) are known as host defense peptides. They play major roles in the innate immune system in both vertebrates and invertebrates, and protect against a wide variety of bacterial, fungal, viral and other pathogenic infections. Since the first discovery of AMPs in insects (Hultmark *et al*., 1980), more than 1500 sequences which encode for AMPs or putative AMPs have been published in databases (http://aps.unmc.edu/AP/main.php). In recent decades, these natural antibiotics have attracted an increasing amount of attention due to their promising role as therapeutics or drug leads (Giuliani *et al*., 2007; Hadley & Hancock, 2010; Hughes & Fenical, 2010).

AMPs are characterized as short amino acid sequences (10–100 AA), having a net positive charge and being amphiphilic in their active forms. The AMP structures obtained have allowed identification of five major classes of peptides: (1) α-helical, (2) cysteine-rich (defensin-like), (3) β-sheet containing, (4) peptides with an unusual composition of regular amino acids and (5) bacterial or fungal peptides containing uncommon modified amino acids.

The marine environment differs substantially from terrestrial and freshwater habitats. Recently, a great deal of interest has been expressed regarding marine-derived antimicrobial peptides, because of their exigent, competitive and aggressive environment. Marine organisms exist in close proximity with microbes. The estimated density of bacteria in seawater and in sediment is in the order of 10^5-10^7/ml and 10^8-10^{10}/g, respectively (Austin, 1988). This constant pressure from potentially harmful microbes, combined with the evolutionary success among marine organisms, suggests that the immune effectors found in these organisms are highly effective in bacterial killing/inhibition. Interestingly, the expression of AMPs in insects and marine organisms differs markedly. Insects activate the production of AMPs after bacterial exposure (Lemaitre *et al*., 1997), while marine organisms seem to express their natural antibiotics constitutively (Muñoz *et al*., 2002; Smith *et al*., 2008; Sperstad *et al*., 2010).

To date, around 70 and 40 different AMPs or AMP-families have been characterized from marine fish and invertebrates, respectively (Rajanbabu *et al*., 2011; Sperstad *et al*., 2011). Of these, only a very small number of AMPs belongs to an AMP family already characterized in terrestrial species, namely defensin (Charlet *et al*., 1996). The vast majority have novel primary structures and are either species-specific or confined to certain

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taxa, including Chordata (Rajanbabu *et al*., 2011), Cnidaria (Ovchinnikova *et al*., 2006), Annelida (Ovchinnikova *et al*., 2004), Chelicerata (Iwanaga, 2002), Tunicata (Lee *et al*., 1997), Mollusca (Mitta *et al*., 2000), Crustacea (Smith *et al*., 2008) and Echinodermata (Li *et al*., 2008).

17.2 PREPARATION, PURIFICATION AND CHARACTERIZATION

17.2.1 Preparation and Purification

Marine AMPs may be produced by one of the following methods: solvent extraction, enzymatic hydrolysis or a genetic/*in silico* approach (Fig. 17.1). Most purification schemes for marine AMPs are based on multistep strategies, with a methodology dictated by the features of most AMPs: small size, cationic charge and amphipathic nature. In general, most purification schemes contain: (1) an extraction/precipitation step combined with centrifugation and/or ultrafiltration, removing particulate matter and larger proteins; (2) a preparative purification step, using methods such as size exclusion chromatography (SEC, also known as gel permeation chromatography or gel filtration), ion-exchange chromatography (IEC) and solid-phase extraction (SPE), to remove inorganics (salts), anionic proteins, fatty material and other bioassay-interfering compounds; and (3) one or several analytical separation steps using reversed-phase high-performance liquid chromatography (RP-HPLC), which separate the bioactive peptides from inactive ones based on differences in hydrophobicity (Fig. 17.2). The presence of bioactive peptides in the fractions obtained is usually tested after each step in these protocols, a procedure which has led to the term 'bioassay-guided purification' (Sperstad *et al*., 2011).

The solvent-extraction system is mainly used at laboratory scale. This technique has several drawbacks, including low selectivity, low extraction efficiency, solvent residue and environmental pollution. Large quantities of highly purified peptides are required to meet the needs of basic research and clinical trials. Compared with isolation from natural sources, enzymatic hydrolysis and the recombinant approach offer the most cost-effective means for large-scale peptide manufacture. The enzymatic hydrolysis method is preferred in the food and pharmaceutical industries because the other methods can leave residual organic solvents or toxic chemicals in the products. Antimicrobial peptides usually exist below a molecular weight of 10 kDa and are encoded within the sequences of native protein precursors, but may also be generated *in vitro* by enzymatic hydrolysis (Bulet *et al*., 2004; Liu *et al*., 2008; Reddy *et al*., 2004).

The genetic or *in silico* approach is an alternative strategy for AMP discovery (Fig. 17.1). This is by far the most frequently used method today, and it has expanded several AMP families based on sequences from purified peptides (e.g. penaeidin, crustin, defensin, cathelicidin). The advantages of this strategy are its relative ease of performance and its high rate of success for finding new AMPs (Patrzykat & Douglas, 2003). Using this strategy, the diversity of AMP sequences can be explored and conserved motifs can be revealed. It is important, however, to be aware of the challenges related to such an approach. First, it is not possible to predict whether the translated peptide *in vivo* contains post-ribosomal modifications, or in the case of cysteine residues, to assign the correct disulfide connections. Candidates must be synthesized or expressed recombinantly, and subsequently screened against selected microorganisms, to truly assign them as antimicrobials (Sperstad *et al*., 2011). Furthermore, this strategy relies on

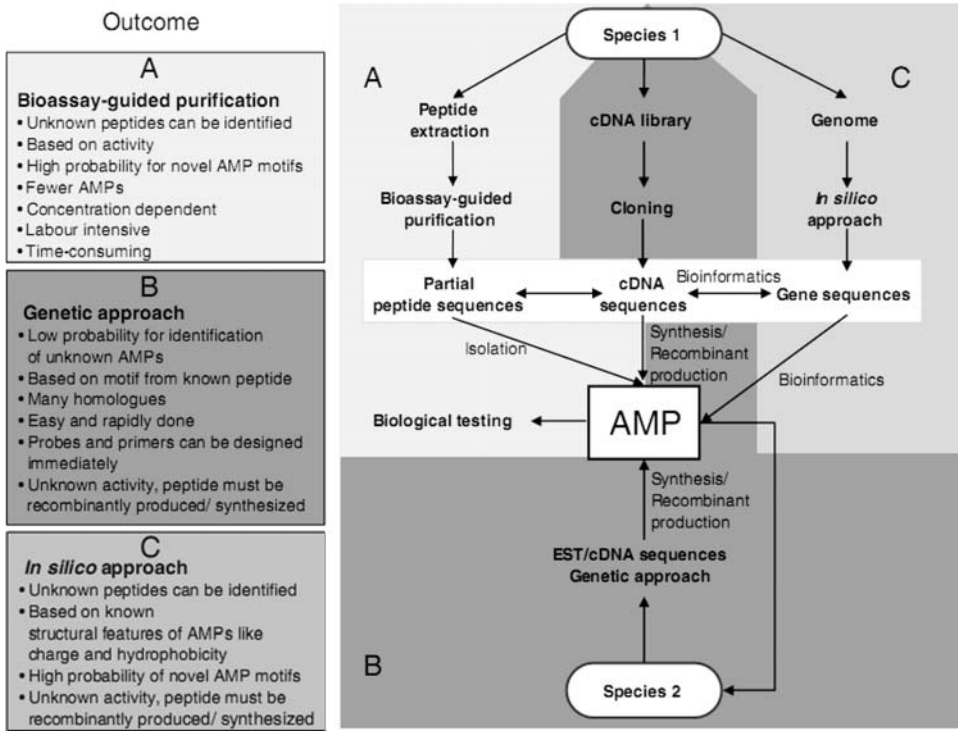

Fig. 17.1 Overview of the differences and challenges of isolating marine antimicrobial peptides using (a) the traditional bioassay-guided purification approach, (b) the genetic approach and (c) the *in silico* approach. Reprinted from Sperstad *et al*. (2011). Copyright 2011, with permission from Elsevier.

already known characteristics of AMPs (e.g. size, cationicity, amphipathicity), which methodically will exclude peptides that have other features, such as a negative net charge (Harris *et al*., 2009). Therefore, bioassay-guided purification will continue to be of importance in the discovery of novel marine AMPs.

17.2.2 Characterization

Liquid chromatography followed by tandem mass spectrometry detection (LC–MS/MS) is commonly used to identify peptide sequences. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis is also useful for generating peptide profiles of protein hydrolysates or semipurified fractions (Najafian & Babji, 2012). Solving the secondary structure of an AMP has been considered an important step in understanding its function and will be useful in developing potent peptides for pharmaceutical applications. While global structure analysis of AMPs using low-resolution techniques like CD and FTIR usually provides quick information on the experimental conditions under which a conformational change occurs, atomistic level-resolution three-dimensional structures can provide high-resolution information on peptide–peptide and peptide–membrane interactions (see Fig. 17.3). Such high-resolution structural information is powerful in elucidating the role of individual amino acids in the formation of oligomers in solution or in a membrane environment, and in providing insights into the mechanism of cell lysis (Gottler & Ramamoorthy, 2009).

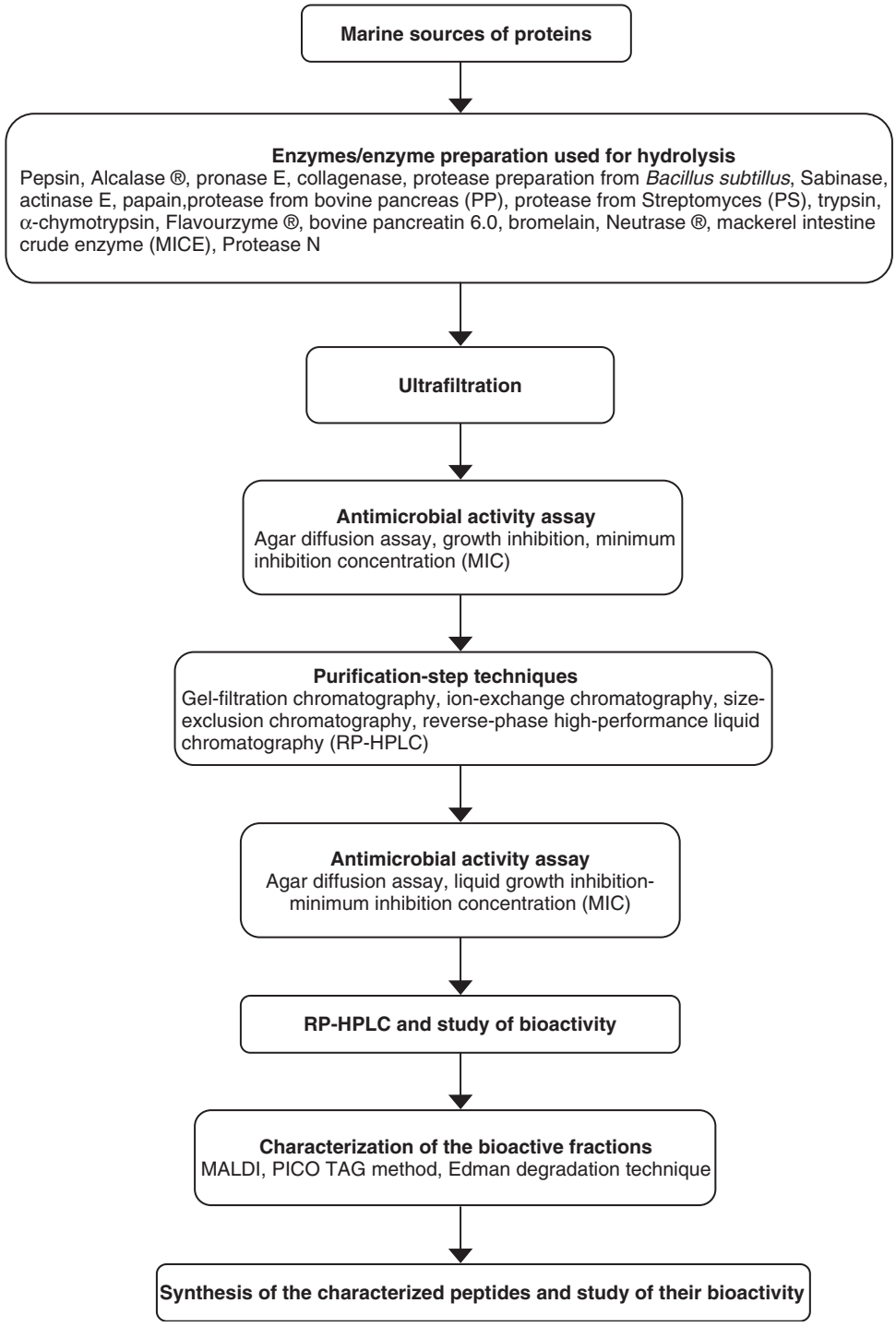

Fig. 17.2 Schematic diagram representing methods used for the isolation and characterization of antimicrobial peptides from marine protein sources. Reprinted from Bernardini *et al*. (2011). Copyright 2011, with permission from Elsevier.

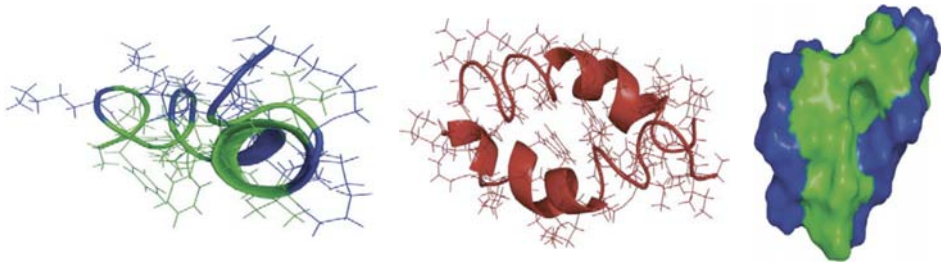

Fig. 17.3 Monomeric (a) and antiparallel dimeric (b) helical structures of AMP MSI-78 determined by NMR experiments in a membrane environment. Reprinted with permission from Porcelli, F., Buck-Koehntop, B., Thennarasu, S. *et al*. (2006) Structures of the dimeric and monomeric variants of magainin antimicrobial peptides (MSI-78 and MSI-594) in micelles and bilayers by NMR spectroscopy. Biochem, 45, 5793–5799. Copyright 2006, American Chemical Society. (c) A surface representation showing the hydrophobic interface (light gray) and hydrophilic exterior (dark gray) of the dimeric helical structure. The formation of a dimer is a key step in its activity. Since the dimer has more hydrophilic surface exposed for the membrane interaction and hydrophobic residues are not exposed outside, the selectivity of the peptide toward negatively charged (both Gram-positive and Gram-negative) bacterial membranes is increased. Therefore, the toxicity of the peptide is further reduced. Reprinted from Gottler, L. M. & Ramamoorthy, A. (2009). Copyright 2009, with permission from Elsevier.

17.3 IN VITRO ANTIMICROBIAL STUDIES

17.3.1 Antimicrobial Activity

The antimicrobial potential of any compound is reflected by the test strains selected for activity measurements. When choosing the strategy of bioassay-guided purification, the amount of purified material usually does not permit a broad primary screening, and thus the test strains have to be selected carefully (Sperstad *et al*., 2011). The standard bacteria and fungi used for the antimicrobial assays include the Gram-negative bacteria *Pseudomonas solanacearum* and *Xanthomonas Campestris* pv. Vesicatoria, the Gram-positive bacterium *Bacillus subtilis* and the fungi *P. capsici*, *B. cinerea*, *Verticillium dahliae* and *F. omysporum* (Zhao *et al*., 2012). Clearly, the strains selected for the primary screening determine its outcome.

Several methods for testing the antimicrobial activity of hydrolysates or peptides have been used. The agar-diffusion assay (or inhibition-zone assay) is a common method used to test the antimicrobial activity of peptidic hydrolysates and peptides (Hickey *et al*., 2003). The antimicrobial effect of a peptidic hydrolysate or a peptide increases in accordance with the diameter of the zone of inhibition formed (Hayes *et al*., 2006). Peptidic solutions can be placed in wells made in the agar (Hickey *et al*., 2003). An accurate way to study the antimicrobial activity of hydrolysates and/or peptides is to determine their minimum inhibitory concentration (MIC). The MIC value defines the lowest concentration of an antimicrobial that inhibits 100% of the growth of a microorganism, and is usually determined by liquid-growth inhibition in a 96-well-plate spectrometry method (Fogaca *et al*., 1999). A novel polypeptide antimicrobial activity was isolated and characterized from loach (*Misgurnus anguillicaudatus*) using Sephadex G-50 gel filtration, DEAE-52 cellulose IEC and an improved polyacrylamide-gel electrophoresis, together with electroelution (Dong *et al*., 2002).

17.3.2 The Effects of AMPs on Bacterial Cells

Many experimental techniques have been utilized to study antimicrobial peptides and their effects on bacterial cells. One of the most well established and most direct methods to monitor the effect of a given antimicrobial peptide on a lipid bilayer is differential scanning calorimetry (DSC). The most common approach for the study of lipid–peptide interactions involves codissolving both components in an organic solvent. The organic solvent is then evaporated, leaving a lipid–peptide film. This film is rehydrated in buffer to form aqueous suspensions of peptide and lipids. The type of peptide–lipid suspension formed in such an experiment depends on the lipid species, but usually multilamellar vesicles are obtained using this technique. DSC can be used to determine whether a peptide interacts preferentially with one lipid species over another and can also determine the effect of a peptide on the lamellar to nonlamellar phase-transition temperature (TH) (Haney *et al*., 2010).

Nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction transmission electron microscopy (TEM) are also used to examine the polymorphism induced by an antimicrobial peptide. Because of their small size, the solution structure of an antimicrobial peptide bound to detergent micelles is commonly determined using NMR spectroscopy and the resulting structure is linked to other experimentally determined characteristics of the peptide (Haney *et al*., 2007). Other NMR techniques have been developed to examine the behavior of an antimicrobial peptide in phospholipid bilayers (Haney & Vogel, 2009).

X-ray diffraction is another experimental technique that is commonly used to examine polymorphic changes in the lipid bilayer. This method is particularly useful for examining cubic lipid phases where a peptide induces the formation of an ordered three-dimensional lattice that will diffract X-rays in a particular pattern depending on the space group occupied by the cubic lipid phase. X-ray diffraction is a powerful technique because it allows the cubic structures induced by antimicrobial peptides to be characterized in detail (Staudegger *et al*., 2000).

TEM was carried out to inspect the effects of AMP protegrin-1 on bacteria. The effects of a large volume increase on bacterial cells are seen most dramatically in microscopy images of protegrin-1-treated bacteria. Fig. 17.4 shows TEM images of *E. coli* strain ML-35p after several minutes of exposure to protegrin-1 at a concentration of 25 mg/ml protegrin-1. A rupture in the outer envelope of the organism in Fig. 17.4 leads to extrusion of the cellular membrane through the cell wall, followed by massive leakage of cell contents (Bolintineanu *et al*., 2010).

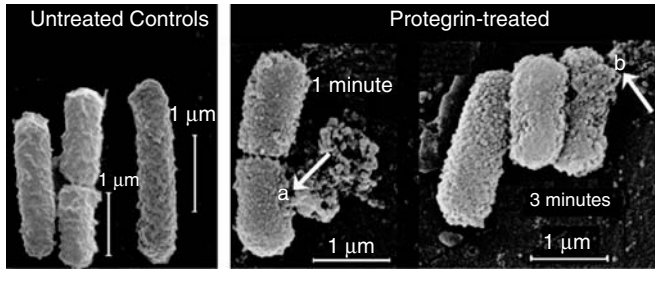

Fig. 17.4 Transmission electron microscopy (TEM) images of *E*. coli treated with protegrin-1. The arrows point to rupture sites in the bacterial membrane, where the cellular membrane and the intracellular contents are being extruded. Reprinted from Bolintineanu *et al*. (2010). Copyright 2010, with permission from Elsevier.

17.4 ANTIMICROBIAL MECHANISMS

The mechanism of antimicrobial peptides is being actively studied and the available information continues to grow. The majority of experiments to date have focused primarily on the interaction of cationic peptides with model membrane systems. Additional studies have also been conducted on whole microbial cells, predominantly utilizing membrane potential-sensitive dyes and fluorescently labeled peptides. These have indicated that all antimicrobial peptides interact with membranes and tend to divide peptides into two mechanistic classes: membrane-disruptive and non-membrane-disruptive. An alternative perspective is that as a group, cationic antimicrobial peptides have multiple actions on cells, ranging from membrane permeabilization to cell-wall and division effects to macromolecular synthesis inhibition, and that the action responsible for killing bacteria at the minimal effective concentration varies from peptide to peptide and from bacterium to bacterium for a given peptide (Friedrich *et al*., 2000).

17.4.1 Membrane-disruptive Mechanism

Membrane-disruptive peptides are generally reported to be of the α-helical structural class. Three mechanistic models, the 'barrel-stave', 'micellar-aggregate' and 'carpet' models, have been developed to explain membrane disruption.

17.4.1.1 'Barrel-stave' Model

In the barrel-stave model, the amphipathic peptides reorient perpendicular to the membrane and align (like the staves in a barrel) in a manner in which the hydrophobic side chains face outwards into the lipid environment while the polar side chains align inward to form transmembrane pores (Powers & Hancock, 2003) (see Fig. 17.5). The pore acts as a conductance channel that disrupts transmembrane potential and ion gradients, leading to a leakage of cell components and cell death. Dissipating the transmembrane electrochemical gradient causes a loss of the bacterial cell's ability to synthesize ATP, and the increase in water and ion flow that accompanies loss of the permeability barrier leads to cell swelling and osmolysis. This model requires peptides to be sufficiently long to traverse the hydrophobic core of the bilayer and implies direct contact between peptides upon channel formation (Sato & Feix, 2006).

17.4.1.2 'Micellar-aggregate' Model

The micellar-aggregate model suggests that the peptides reorient and associate in an informal membrane-spanning micellar or aggregate-like arrangement, and further indicates that collapse of these micellar aggregates can explain translocation into the cytoplasm (Matsuzaki *et al*., 1997).

The initial step involved in membrane permeation and micellization requires a change in the curvature of the membrane. Membrane-lytic peptides can be classified into two groups in terms of their effect on membrane curvature (Epand *et al*., 1995):

1. **Negative-curvature inducers (class-L peptides)**. A reciprocal wedge model was suggested by Tyler *et al*. (1993) in order to explain the ability of class-L peptides to lyse cells. In this model, a class-L peptide folds into an amphipathic α-helical structure upon association with phospholipids, such that the polar face of the peptide is associated with the polar head groups of the phospholipids. The lipid-associated peptide, when viewed in cross-section, is an inverted wedge shape, in which the hydrophobic face of the helix forms the apex. This structure is expected to force the alkyl chains of the phospholipid apart, inducing a negative curvature on the membrane.

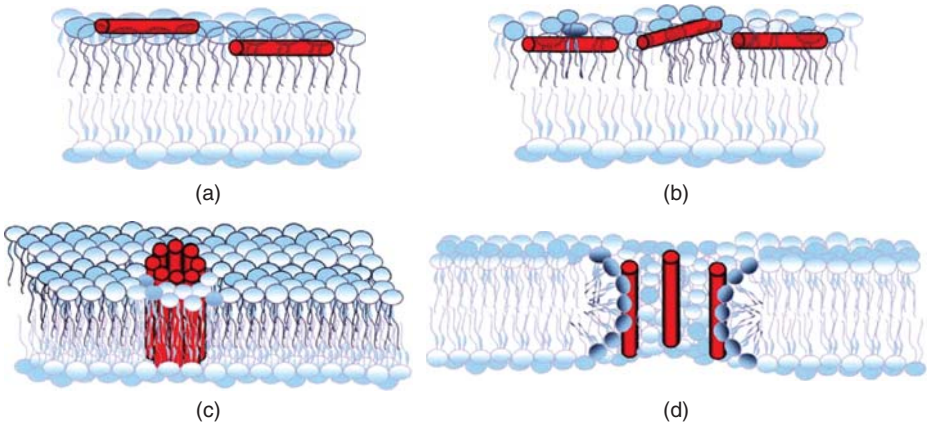

Fig. 17.5 Models of transmembrane-channel formation. (a) Peptide α-helices (cylinders) initially associate parallel to the membrane surface, either superficially (left) or embedded just below the aqueous interface. (b) Peptides continue to accumulate at or near the bilayer surface, disrupting lipid packing and causing membrane thinning. This step may or may not involve peptide–peptide aggregation. Once a critical peptide/lipid ratio is reached, peptides either insert into the membrane as a barrel-stave type pore (c) or induce the localized formation of toroidal pores (d). Reprinted from Sato, H. & Feix, J. B. (2006). Copyright 2006, with permission from Elsevier.

2. **Positive-curvature inducers (class-A peptides)**. Class A peptides have been shown to cause micellization of bilayers (Matsuzaki *et al*., 1993). A high positive curvature in a dimension perpendicular to the bilayer plane is required to stabilize a pore structure toward the formation of a micelle (Fig. 17.6).

17.4.1.3 'Carpet' Model

A conceptually different model for the way in which AMPs disrupt membranes is the carpet mechanism (see Fig. 17.7). In this model, peptides accumulate at the bilayer surface like a carpet. Above a threshold concentration of monomers, the membrane is permeated and disintegrated in a detergent-like manner, without the formation of discrete channels (Gazit *et al*., 1995, 1996; Steiner & Andreu, 1988). This mechanism was first proposed by Steiner and Andreu (1988), based on the observation that, at the concentration needed to obtain 50% cell killing, cecropin A was present in sufficient amounts to completely cover the bacterial cell surface. Subsequent studies on the porcine cecropin P1 were also interpreted in terms of a carpet mechanism (Gazit *et al*., 1995). Cecropin P1 studies using ATR-FTIR spectroscopy indicated that it incorporated parallel to the surface of the PE/PG membrane and did not change the order parameters of the acyl chains, suggesting the peptide did not translocate into the hydrocarbon core (Gazit *et al*., 1996).

Regardless of which model is correct, the net result of membrane disruption will be the rapid depolarization of the bacterial cell, leading to rapid cell death, with total killing occurring within 5 minutes for the most active peptides (Friedrich *et al*., 1999). It should be noted that each of these models might be correct depending on the peptide examined, such that certain peptides may function through a barrel-stave mechanism, while others may function through a micellar-aggregate or a carpet mechanism.

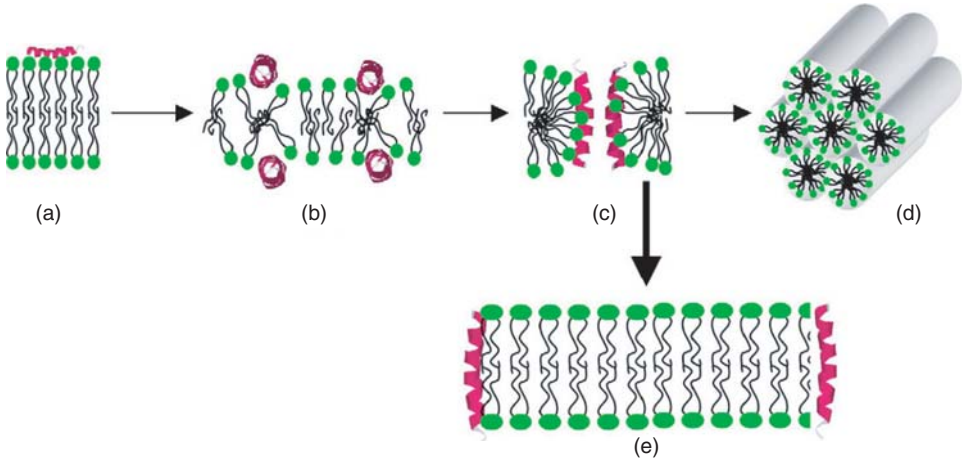

Fig. 17.6 Mechanism of membrane disruption by antimicrobial peptides. (a) NMR studies have shown that antimicrobial peptides are unstructured in solution and form a helix in a membrane environment. The amphipathic peptide is aligned near the surface of the membrane. (b) A positive-curvature strain induced by the peptide was determined from ^{31}P solid-state NMR on POPE bilayers and from differential scanning calorimetry experiments on DiPoPE bilayers. (c) Formation of toroidal pores was determined from solid-state NMR studies. (d) Solid-state NMR studies revealed the formation of a normal hexagonal phase structure of lipids at higher concentrations of antimicrobial peptides. (e) Solid-state NMR experiments revealed that few- week-old samples exhibited the formation of bicelles and then micellization due to the detergent-like behavior of the peptide (Hallock & Lee, 2003; Ramamoorthy *et al*., 2006). Reprinted from Gottler & Ramamoorthy (2009). Copyright 2009, with permission from Elsevier.

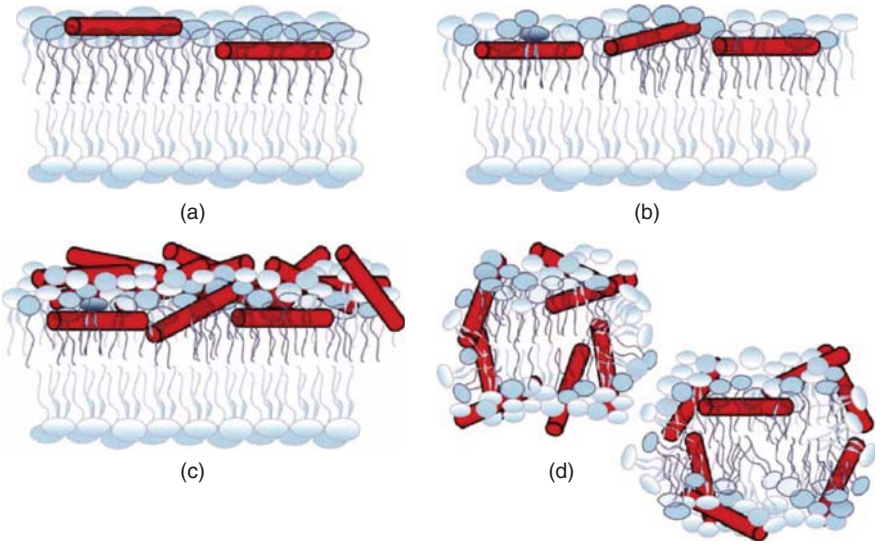

Fig. 17.7 Model of membrane disruption by the carpet mechanism. (a,b) As in channel formation, peptide α-helices (cylinders) initially (a) bind and (b) accumulate in an orientation parallel to the membrane surface. (c) Continued accumulation of membrane-bound peptide associated with the phospholipid head groups, eventually covering (i.e., carpeting) the bilayer. (d) Detergent-like membrane disintegration. Reprinted from Sato, H. & Feix, J. B. (2006). Copyright 2006, with permission from Elsevier.

17.4.2 Non-membrane-disruptive Mechanism

Some peptides can induce transcriptional changes within bacteria and translocate into the bacterial cytoplasm, and there may be a role for these peptides in a non-membrane-disruptive application.

Regardless of the mechanism used by an antimicrobial peptide to disrupt bacterial growth, antibiotic molecules must interact with biological membranes and induce changes in the structure of the bilayer. It was observed early on that membrane active peptides have the ability to alter the lipid polymorphism of a bilayer, and Epand *et al*. (1997) outlined five factors that contribute to this interaction: hydrophobicity, charge, conformation and self-association, steric effects and the mode of bilayer insertion. The hydrophobicity of the peptide will determine how far the peptide will partition into the bilayer. The charge on the peptide determines which lipid head groups the peptide will interact with. The structure of the peptide usually contributes to an amphipathic distribution of amino acids by clustering hydrophobic residues away from the charged residues. The steric effects induced by binding of the peptide to the lipid might change the packing interactions of the lipids. Finally, the mode of insertion into the plasma membrane will also determine the effect of the peptide on the polymorphic changes seen in the lipid bilayer (Haney *et al*., 2010).

To date, studies focused on the mechanism of action have concentrated primarily upon the chemical and structural properties of peptides and relatively little interest has been shown in other factors. Specifically, membrane components may play a significant role in the activity of peptides. Indeed, studies focused upon the translocation of other cationic compounds have revealed major contributions from non-bilayer-forming lipids, suggesting the importance of these compounds in the mechanism of action of antimicrobial peptides. The diversity of lipids among microorganisms may very well explain the differences in activity of a single peptide between these species, and thus further study of the interactions between antimicrobial peptides and lipids is required so we can propose an accurate mechanism of activity for each peptide and organism.

17.5 APPLICATIONS AND PROSPECTS IN FOOD PRESERVATION

The low toxicity characteristics and the antimicrobial activity of AMPs against a broad spectrum of bacterial species makes them promising candidates for the treatment of bacterial infections, the immune system and disease control. All AMPs show direct antibacterial or bacteriostatic functions against several Gram-negative and -positive strains. The histone-derived H2B peptide from Atlantic cod was reported to have antimicrobial functions against the fish pathogens *Aeromonas hydrophilia* and *Saprolegnia* spp. (Bergsson *et al*., 2005). The antiparasitic functions of the β-hemoglobin peptide family AMP against *Ichthyophthirius multifiliis*, which causes ichthyophthiriosis in channel catfish, has been demonstrated (Ullal & Noga, 2010). The grouper peptide, epinecidin-1, shows antimicrobial function against *Trichomonas vaginalis*, an anaerobic, flagellated protozoan that causes trichomoniasis (Pan *et al*., 2009).

AMPs with activity against plant pathogens such as *Fusarium moniliforme*, *Penicillium* sp., *Botrytis cinerea*, *Phytophthora infestans* and *Trichoderma* sp. have been reported by Powell *et al*. (1995) and Cavallarin *et al*. (1998). Besides their use in plant protection, antimicrobial peptides have been proposed as protective agents in postharvest

preservation. The synthesized peptides PAF19 and PAF26 have been shown to inhibit *in vitro* growth of strains of *Penicillium italicum*, *P. digitatum* and *Botrytis cinerea* (Lopez-Garcia *et al*., 2000). The antifungal peptides retarded blue- and green-mold diseases of citrus fruit and gray mold of tomato fruit under controlled inoculation conditions (Lopez-Garcia *et al*., 2003). In addition, a cecropin A-based peptide has been shown to inhibit germination of *Colletotrichum coccodes* at 50 μM. Expression of the antifungal peptide in yeast inhibited the growth of germinated *C. coccodes* spores and decay development in tomato fruit (Jones *et al*., 2002). The oyster peptide, *CgPep33*, shows antimicrobial function against *Botrytis cinerea* and inhibition of the gray-mold disease of harvested strawberries (Liu *et al*., 2008).

Many peptides with clinically proven efficacy have orthologs in fish species, and these can provide the structural diversity for similar peptides. In addition, mediation of the antimicrobial function of the peptide epinecidin-1was demonstrated against the diploid fungus, *Candida albicans*, a causal agent of opportunistic oral and genital infections in humans (Pan *et al*., 2009). AMPs can serve as a source for noncontaminated coatings of food packages. The use of AMPs killed both Gram-negative and Gram-positive bacteria, revealing that they have diverse applications. The multiple functions of AMPs may help with investigations of the underlying molecular mechanisms between different physiological processes. As an example, the iron-regulatory and antimicrobial functions of hepcidins helped elucidate the relationship between iron homeostasis and pathogenic infections. The cost of mimicking peptide synthesis using present methods is high for nonclinical purposes, and investigating cost-effective strategies for the large-scale synthesis of peptides will provide a platform for the wider application of fish AMPs. In conclusion, exploitation of fish AMPs will provide the source of uniquely structured antimicrobial drugs in the future, and these peptides can be used as models to investigate molecular pathogenesis.

Although promising as broad-spectrum antibiotics, AMPs are susceptible to proteolysis *in vivo* by endogenous or bacterial proteases, which may considerably diminish their effectiveness for intravenous applications. Studies on Leishmania pinpoint leishmanolysin as the preventative factor in AMP-induced apoptosis of the bacteria (Kulkarni *et al*., 2006). Attempts to overcome this problem by increasing the dose of the AMP often lead to toxic side effects, most notably lysis of red blood cells, which has been attributed to nonspecific hydrophobic interactions between the peptide and the eukaryotic cell membrane (Zelezetsky & Tossi, 2006). Improvements to the stability and/or activity of AMPs would be significant to their potential applications, such as acylated analogs (Radzishevsky *et al*., 2005) and non-natural amino acid analogs (Meng & Kumar, 2007). Incorporation of the 12-carbon lauryl group or amino lauryl group to the N-terminus of AMPs resulted in increased hemolytic activity and no substantial gains in antibacterial activity (Radzishevsky *et al*., 2005). Non-natural amino acid analogs, specifically fluorinated amino acids, including hexafluoroleucine and pentafluor-ophenylalanine (Gottler, 2008), have been substituted into AMPs for the hydrophobic residues leucine, isoleucine and phenylalanine (Gottler & Ramamoorthy, 2009; Meng & Kumar, 2007).

High manufacturing cost is another major obstacle to the wide application of antimicrobial peptides. In the last decade, various systems and approaches that aim at increasing efficiency and lowering cost have been developed. However, the current cost remains far from commercially acceptable. The use of smaller fusion partners that are specifically designed for peptides, combined with expression optimization and high-cell-density fermentation, should be able to further improve the efficiency. Even if such an approach does not provide an ultimate solution to the high cost, the systems/protocols developed

should nevertheless benefit the recombinant production of antimicrobial peptides, as well as other therapeutically valuable bioactive peptides (Li, 2011).

17.6 CONCLUSION

Nowadays many antimicrobial peptides have been obtained from marine organisms, but the need to discover new antimicrobial substances is still urgent, due to the progressive development of resistance by pathogenic microorganisms against conventional antibiotics. Marine-derived antimicrobial peptides are considered promising candidates, having a broad-spectrum antimicrobial activity. It might be suggested that these antimicrobial peptides have potent capacities for new antibiotic development in the pharmaceutical as well as the food industries, as novel antimicrobial agents (Kim & Wijesekara, 2010). To enable this widespread use, it will be important to have convenient methods for the extraction, purification and identification of these bioactive peptides, and to discover new methods of increasing efficiency and lowering cost.

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18 Production and Antioxidant Properties of Marine-derived Bioactive Peptides

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18.1 INTRODUCTION

Lipid peroxidation is of great concern to the food industry and consumers because it is a principal cause of quality deterioration in lipid-containing foods during processing and storage, resulting in the production of rancid odours and unpleasant flavours, changes of colour and texture, and generation of potentially toxic compounds such as malondialdehyde (MDA) and cholesterol oxidation products (COPs). Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate have been widely used to inhibit oxidative reactions and extend the shelf-life of food products. However, the use of synthetic antioxidants as food additives is under strict regulation and is even prohibited in some countries due to the potential health hazards of these compounds (Hettiarachchy *et al*., 1996). Therefore, the search for natural antioxidants as effective alternatives to synthetic ones is gaining great interest worldwide.

Moreover, accumulated scientific evidence indicates that reactive oxygen species (ROS) and free radical-meditated reactions can induce oxidative damage to vital biomolecules in tissue cells, eventually leading to many chronic diseases, such as cardiovascular diseases, diabetes mellitus, Alzheimer's, certain types of cancer and other neurodegenerative and cognitive disorders. Antioxidants could attenuate this oxidative damage through a direct scavenging effect on several free radicals and/or by boosting the body's natural antioxidant defence system.

In addition to plant-derived natural antioxidants, bioactive peptides generated from the protein hydrolysis of various animal and plant sources, such as soy proteins (Chen *et al*., 1995a), casein (Gómez-Ruiz *et al*., 2008; Kim *et al*., 2007a; Suetsuna *et al*., 2000), whey proteins (Hernández-Ledesma *et al*., 2005), porcine myofibrillar proteins (Saiga *et al*., 2003), wheat gluten (Wang *et al*., 2007) and egg-white and egg-yolk proteins (Dávalos *et al*., 2004), have been demonstrated to possess noteworthy antioxidant activities. The use of protein hydrolysates or peptides in developing functional foods offers additional advantages over other natural antioxidants, since they also enhance the nutritional value and technofunctional properties of the food products.

Many studies have been performed to identify antioxidant peptides from fish protein. Owing to the increasing concern about the over exploitation of limited biological

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resources, environmental pollution and the need for effective and value-added utilisation of the unused or underutilised fish species and byproducts from the fish processing industries, many researchers emphasise exploration of the potential application of protein hydrolysate from fish processing byproducts as alternative sources of antioxidants. An increasing number of potent antioxidant peptides have been identified, both from hydrolysates of fish-muscle proteins such, as giant squid (Rajapakse *et al*., 2005a), grass carp (Ren *et al*., 2008), yellow stripe trevally (Klompong *et al*., 2009) and ornate threadfin bream (Nalinanon *et al*., 2011), and from fish processing byproducts, including Alaska pollock frame (Je *et al*., 2005b) and skin gelatin (Kim *et al*., 2001), yellowfin sole frame (Jun *et al*., 2004) and skin gelatin (Kim *et al*., 1996), hoki frame protein (Kim *et al*., 2007b), tuna cooking juice (Hsu *et al*., 2009), tuna dark muscle byproduct (Hsu *et al*., 2010) and bigeye tuna head (Yang *et al*., 2011) (Table 18.1). These peptides show great potential as natural antioxidants, with broad applications in the functional foods, nutraceuticals and pharmaceuticals industries and in improving public health and preventing ROS-related chronic diseases.

This chapter presents and discusses recent advances in the search for potential antioxidant peptides from marine sources, with an emphasis on technological approaches to the production of bioactive peptides and current progress in understanding the antioxidant mechanism and structure–activity relationship.

18.2 PRODUCTION OF ANTIOXIDANT PEPTIDES

Marine organisms are rich in structurally diverse bioactive compounds with various biological activities and have become important sources of antioxidant peptides. A growing body of scientific evidence shows that many protein hydrolysates and peptides derived from marine sources, including fish, molluscs, crustaceans and marine processing waste or byproducts, can promote human health and aid in the prevention of chronic disease (Kim & Wijesekara *et al*., 2010; Kim *et al*., 2008). However, antioxidant peptides are inactive within the sequences of parent proteins (Najafian *et al*., 2012) and must be released and activated by processing through different techniques. Basically, antioxidant peptides can be generated from precursor proteins by microbial fermentation and enzymatic hydrolysis (Korhonen *et al*., 2006).

18.2.1 Microbial Fermentation

Fermentation is one of the oldest methods of food preservation and is popular in East Asian countries such as China, Japan and Korea, where seafood products such as fermented fish, shellfish sauces and pastes are used as staples or condiments (Fitzgerald *et al*., 2005). It is believed that fermentation can increase the nutraceutical value and shelf life of foods, possibly due to fragmentation of proteins to bioactive peptides by the action of microbes and endogenous proteolytic enzymes. Fermented food products are a good source of peptides and amino acids, which provide their typical flavour and taste (Faithong *et al*., 2010; Rajapakse *et al*., 2005; Sathivel *et al*., 2003).

Antioxidant activities associated with the fermentation of marine animals have been reported. Blue mussel was fermented with 25% (w/w) NaCl at $20 \pm 0.5\,^{\circ}C$, producing the antioxidant peptide FGHPY (molecular weight 620 Da) from 6-month-fermented sauce, which could scavenge 89.5% of the hydroxyl radical in a radical-scavenging

Table 18.1 Antioxidant peptides from marine sources.

Protein source	Preparation	Peptide	Antioxidative activity	Reference
Conger eel (*Conger myriaster*)	Trypsin	Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn (928 Da)	Free-radical scavenging	Ranathunga *et al.* (2006)
Alaska pollock frame	Mackerel-intestine crude enzyme	Leu-Pro-His-Ser-Gly-Tyr (672 Da)	Free-radical scavenging	Je *et al.* (2005b)
Grass carp muscle	Alcalase 2.4L	Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (966.3 Da)	Free-radical scavenging	Ren *et al.* (2008)
Tuna backbone	Pepsin	VKAGFAWTANQQLS (1519 Da)	Free-radical scavenging Inhibition of lipid peroxidation	Je *et al.* (2007)
Tuna dark muscle	Orientase or protease XXIII	Leu–Pro–Thr–Ser–Glu–Ala–Ala–Lys–Tyr (978 Da) Pro–Met–Asp–Tyr–Met–Val–Thr (756 Da)	Free-radical scavenging	Hsu (2010)
Tuna cooking juice	Orientase	Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr (1305 Da) Pro-Ser-Asp-His-Asp -His-Glu (938 Da) Val-His-Asp-Tyr (584 Da)	Free-radical scavenging Inhibition of lipid peroxidation	Hsu *et al.* (2009)
Yellowfin sole (*Limanda aspera*)-frame protein	Pepsin and mackerel-intestine crude enzyme	Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr (1300 Da)	Inhibition of TBAS formation	Jun *et al.* (2004)
Sardinelle (*Sardinella aurita*)	Crude enzyme extract from viscera of sardine	Leu-His-Tyr (431.2 Da)	Free-radical scavenging Inhibition of lipid peroxidation	Bougatef *et al.* (2010)
Horse mackerel viscera	Pepsin, trypsin and α-chymotrypsin	Ala–Cys–Phe–Leu (518.5 Da)	Free-radical scavenging Inhibition of lipid peroxidation	Sampath Kumar *et al.* (2011)

(*continued overleaf*)

Table 18.1 (*continued*)

Protein source	Preparation	Peptide	Antioxidative activity	Reference
Hoki (*Johnius belengerii*) frame	Pepsin	Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn (1801Da)	Free-radical scavenging, Inhibition of lipid peroxidation	Kim *et al.* (2007)
Hoki-skin gelatin	Trypsin	His-Gly-Pro-Leu-Gly-Pro-Leu (797 Da)	Free-radical scavenging Inhibition of lipid peroxidation	Mendis *et al.* (2005b)
Giant squid muscle	Trypsin	Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da)	Free-radical scavenging Inhibition of lipid peroxidation	Rajapakse *et al.* (2005)
Squid skin (*Dosidicus gigas*)	Trypsin	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu (880.18 Da) Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg (1241.6 Da)	Hydroxyl and carbon-centred-radical scavenging	Mendis *et al.* (2005a)
Squid tunic (*Dosidicus gigas*)	Alcalase	Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser	Free-radical scavenging Ferric-reducing power	Alemán *et al.* (2011)
Blue mussel (*Mytilus edulis*)	Fermented	His-Phe-Gly-Asp-Pro-Phe-His (962 Da)	Free-radical scavenging Lipid-peroxidation inhibition	Rajapakse *et al.* (2005)
Oyster (*Crassostrea gigas*)	*In vitro* gastrointestinal digestion	Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu (1600 Da)	Free-radical scavenging Lipid-peroxidation inhibition	Qian *et al.* (2008)
Sea cucumber (*Stichopus japonicas*)	Trypsin	Gly-Pro-Glu-Pro-Thr-Gly-Pro-Thr-Gly-Ala-Pro-Gln-Trp-Leu-Arg (1563 Da)	Free-radical scavenging	Zhou *et al.* (2012)

assay (Jung *et al*., 2005). The antioxidant peptide HFGBPFH (molecular weight 962 Da) was also produced, and exhibited strong scavenging effects against superoxide, hydroxyl and DPPH radicals (Rajapakse *et al*., 2005). Some traditional Thai fermented shrimp and krill products, including jaloo and koong-som, also possessed DPPH and ABTS radical-scavenging activity and concentration-dependent ferric-reducing antioxidant power (Faithong *et al*., 2010).

18.2.2 Enzymatic Hydrolysis

Enzymatic hydrolysis is the most commonly applied method of producing antioxidant peptides. Hydrolysis leads to the production of tailor-made bioactive peptides with desired functional and biological properties (Kristinsson *et al*., 2000a; Moure *et al*., 2005). In addition, it can improve the technofunctional properties of proteins, such as solubility, emulsification, gelation and water-holding capacity. It has also been proposed as a way to reduce allergenicity of food proteins, in particular betalactoglobulin and ovalbumin (Moure *et al*., 2005).

18.2.2.1 Enzymatic Hydrolysis by Commercial Enzymes

At present, many commercial proteases from animal, plant and microbial sources have been experimentally used for the production of antioxidant peptides, including trypsin, chymotrypsin, pepsin, Alcalase, Properase E, Pronase, collagenase, Bromelain and Papain. The types of enzyme used in protein hydrolysis are very important because they dictates the cleavage patterns of the peptide bonds (Shahidi *et al*., 2008). Protease specificity affects the size, amount and amino acid composition of the peptides, which in turn influences the biological activity of the hydrolysates (Chen *et al*., 1995; Jeon *et al*., 1999; Wu *et al*., 2003a). Virgibacillus sp. SK33 proteinase, Alcalase, pepsin and trypsin were used to prepare the hydrolysates of threadfin bream surimi wastes, including frame, bone and skin, and the results showed that pepsin-hydrolysed FBS at a 5% degree of hydrolysis (DH) had the highest antioxidant activity based on chemical and biological assays (Chompoonuch *et al*., 2012). Similarly, among protein hydrolysates of silver carp byproducts prepared by Alcalase, Flavourzyme, Neutrase, Papain, pepsin, Protamex and trypsin, pepsin hydrolysate again had the highest free radical-scavenging activity (Zhong *et al*., 2011). However, giant squid muscle was hydrolysed with three digestive enzymes (pepsin, trypsin and α-chymotrypsin) and tryptic hydrolysate exhibited the highest lipid-peroxidation inhibition ability (Rajapakse *et al*., 2005a). In our previous study, pepsin-hydrolysed collagen from scallop skirt exhibited higher superoxide anion radical activity than the other hydrolysates (trypsin and Papain) (Liu *et al*., 2009). Recently, we reported the antioxidant activity of protein hydrolysates from *Mercenaria mercenaria* and *Ruditapes philippinarum* prepared by three enzymatic methods (Flavourzyme, a combination of Alcalase and Flavourzyme, and Protamex and Flavourzyme). Hydrolysate prepared by the combination of Protamex and Flavourzyme had the highest radical-scavenging activity on DPPH and hydroxyl radicals. Increasing the DH resulted in enhanced antioxidant activity (Mu *et al*., 2012).

The optimisation of enzymatic hydrolysis conditions, including time, temperature, pH and enzyme/substrate ratio, is critical for the activity of the enzyme (Najafian *et al*., 2012). It also affect the production yield and antioxidant properties of the peptides. For example, Papain hydrolysate of the sarcoplasmic protein from the muscle of grass carp was obtained at an enzyme/substrate ratio of 0.79%, an incubation time of 5.69 hours and

an incubation temperature of 52.15 °C, which showed the maximum antioxidant activity (Ren *et al*., 2008).

18.2.2.2 Enzymatic Hydrolysis by Autolysis or Self-prepared Enzymes

Apart from commercial proteases, autolytic digestion and enzymatic extracts from fish viscera have been used to produce antioxidant peptides. Depending on the raw materials used, endogenous proteases such as trypsin, chymotrypsin, pepsin and other types of enzyme from fish viscera and digestive tracts, as well as lysosomal proteases or catheptic enzymes in fish or other invertebrate muscle cells, may contribute to the breakdown of proteins during autolysis and have some unique properties for industrial application (Klomklao *et al*., 2005; Kristinsson *et al*., 2000b).

The application of enzymes from the viscera and digestive tracts of different marine animals has been reported. Alaska pollock-frame protein, which is normally discarded as an industrial byproduct, was hydrolysed with mackerel-intestine crude enzyme (Je *et al*., 2005b). Protein hydrolysates from ornate threadfin bream (*Nemipterus hexodon*) muscle were prepared using skipjack tuna pepsin (Nalinanon *et al*., 2011). Furthermore, the combined heads and viscera of sardinelle hydrolysates with crude extract from sardine viscera resulted in production of the hydrolysate with a high antioxidant activity (Bougatef *et al*., 2010).

18.2.3 Purification and Identification of Antioxidant Peptides

The identification and quantification of individual antioxidant peptides present in a desired food complex is important, but separating the antioxidant peptides from the mixtures is expensive and time-consuming. It is therefore necessary to apply a convenient method to quantify the antioxidant effectiveness of a whole food or of partially purified antioxidant peptides.

The marine-derived peptides exhibit different antioxidant activities depending on their molecular weight and amino acid sequence. Ultrafiltration (UF) membranes, ion-exchange membranes and column chromatography can be used to separate peptides with the desired molecular weights and functional properties from protein hydrolysates (Je *et al*., 2005a; Jeon *et al*., 2000).

UF is a membrane separation technology used to separate proteins and peptides in solution on the basis of size. Suspended solids and solutes of high molecular weight are retained, while water and low-molecular-weight solutes pass through the membrane. In recent years, UF has been used for the separation and purification of marine antioxidant peptides. UF membrane reactor systems with different molecular-weight cut-off (MWCO) membranes (such as 10, 5 and 3 kDa) were often used to fractionate crude hydrolysates according to their molecular size (Rajapakse *et al*., 2005a; Ren *et al*., 2008).

Ion-exchange chromatography (IEC) is a frequently used method for the separation and purification of peptides, which separates proteins and peptides based on differences in their charge. Many researchers use the SP-BY Sephadex C-25 cation-exchange chromatography for the separation of antioxidant active components, such as Alaska pollock frame (Je *et al*., 2005b) and giant squid muscle (Rajapakse *et al*., 2005a).

Gel chromatography (GC) is another method for separating proteins and peptides based on differences in size as they pass through a gel chromatographic medium.

Sephadex G-25 gel-column chromatography is often used for the separation of antioxidant peptides.

One of the most useful methods for further separation and purification of peptides is high-performance liquid chromatography (HPLC), which is usually used in conjunction with other analysing equipment, including an ultraviolet (UV) detector or mass spectrometer. Commercially available reversed-phase columns allow rapid separation and detection of their hydrophilic and hydrophobic characteristics (Shahidi *et al*., 2008).

The types of antioxidant peptide in the crude hydrolysates are diverse, and antioxidant components are more sensitive to environmental conditions than are intact proteins. Therefore, separation and purification of antioxidant peptides by the use of a single method is usually difficult to achieve. At present, different separation techniques are commonly combined to obtain the most active peptides. These potent antioxidant peptides usually contain 3∼20 amino acid residues, with molecular weights of about 1000 Da (Table 18.1). In addition, liquid chromatography followed by tandem mass-spectrometry detection (LC-MS/MS) or matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometric analysis is commonly used to identify these peptide sequences.

Recently, a number of studies have demonstrated that peptides derived from different fish-protein hydrolysates act as potential antioxidants. Alaska pollock-frame protein hydrolysate by mackerel intestine crude enzyme was fractionated on a molecular basis using a UF membrane bioreactor system. The fraction exhibiting the highest antioxidant activity was further purified by consecutive chromatographic methods on an SP-Sephadex C-25 column, Sephadex G-25 column and HPLC on an octadecylsilane column. The sequence of the purified peptide was Leu-Pro-His-Ser-Gly-Tyr and its molecular weight was 672 Da (Je *et al*., 2005b). In another study, grass carp-muscle hydrolysate prepared with Alcalase 2.4L was purified using UF and consecutive chromatographic methods, including IEC, multilayer-coil high-speed counter-current chromatography and gel-filtration chromatography, and the purified peptide was identified as Pro-Ser-Lys-Tyr-Glu-Pro-Phe-Val (966.3 Da) using reversed-phase high-performance liquid chromatography (RP-HPLC) connected on-line to an electrospray ionisation mass spectrometer (Ren *et al*., 2008). The antioxidant peptides are also produced from collagen and gelatin of different fish species. Two peptides with strong antioxidant activity were isolated from the gelatin hydrolysate from Alaska pollock-skin hydrolysates and further purified using consecutive chromatographic methods including gel filtration on a Sephadex G-25 column, IEC on an SP-Sephadex C-25 column and HPLC on an ODS column. The isolated peptides, Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly and Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly, were composed of 13 and 16 amino acid residues, respectively (Kim *et al*., 2001).

The proteins of molluscs, custaceans, echinodermata and cnidarians are also important sources of antioxidant peptides. The peptide sequence (Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser) from squid inner and outer tunics was identified by LC–MS/MS (Alemán *et al*., 2011). The antioxidant peptide Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu, isolated from oyster (*Crassostrea gigas*), exhibited higher activity against polyunsaturated fatty acid peroxidation than did α-tocopherol (Qian *et al*., 2008). Sea cucumber (*Stichopus japonicas*) protein was hydrolysed by trypsin, and the antioxidant peptide purified by one-step IEC and RP-HPLC was sequenced as GPEPTG-PTGAPQWLR (Zhou *et al*., 2012). All these purified peptides exhibited strong antioxidant activity *in vitro*.

18.3 ANTIOXIDANT MECHANISM AND STRUCTURE–ACTIVITY RELATIONSHIP

18.3.1 Antioxidant Mechanism of Bioactive Peptides

Although there are many reports on the potential antioxidant activities of marine-derived bioactive peptides, the precise mechanisms underlying their antioxidant effects have not been fully elucidated and appear to be far more complex and subtle than previously realised. In general, the overall antioxidant activities of peptides have been ascribed to the cooperative or synergistic effects of a variety of properties. For instance, it was postulated that the mode of antioxidant action of soybean peptides might involve multiple mechanisms (Chen *et al*., 1998), including hydrogen donation, scavenging of hydroxyl radicals, transition-metal ion chelation and active-oxygen quenching. Other investigators pointed out that the marked oxidation-inhibitory effect of soy-protein hydrolysates could also be linked to their ability to form a protective membrane around the lipid droplets, thus preventing the penetration and diffusion of lipid-oxidation initiators (Hirose & Miyashita, 1999; Peña-Ramos & Xiong, 2002). Because of their amphoteric nature and structural flexibility, smaller peptides diffuse more rapidly across the water–oil interface than intact proteins by adsorbing or loosely and nonspecifically binding to the phospholipid membrane in the liposome where the initial oxidation takes place. It is well known that the partitioning behaviours of different classes of antioxidant have a great impact on their ability to protect membrane lipids from deleterious oxidation in a variety of biological and food model systems. The specific positioning of antioxidant peptides may allow them to effectively scavenge free radicals generated in both aqueous and lipid phase of different model systems, suggesting their potential application in emulsion-type food products as well as in the prevention of lipid peroxidation of cell membranes.

In addition, bioactive peptides have been shown to confer significant protective effects against oxidative damage of important cellular components through direct scavenging of free radicals or by increasing the expression of antioxidant defence proteins or enzymes. Antioxidant peptides derived from fish gelatin or collagen have been reported in studies to enhance cell viability and protect living cells against ROS-induced oxidative damage (Gómez-Guillén *et al*., 2011; Mendis *et al*., 2005a). It is thought that direct scavenging of different free-radical species and their subsequent transformation into less toxic products is a major mechanism for their antioxidant effects. The antioxidant peptides isolated from the gelatin hydrolysate of Alaska pollock skin were shown to exert an inhibitory effect on tert-butyl hydroperoxide (t-BHP)-induced oxidative injury in rat liver (Kim *et al*., 2001). Similar antioxidant effectiveness was reported for two purified peptides from jumbo squid-skin gelatin against t-BHP-induced cell death in human-lung fibroblasts (Mendis *et al*., 2005a). Both purified peptides increased cell viability in a concentration-dependent manner. It was hypothesised that the hydrophobic nature of peptides facilitates effective quenching and elimination of free radicals in cellular systems by keeping them in close proximity with cell-membrane lipids that are highly susceptible to oxidative damage. Erdmann *et al*. (2006) demonstrated that a dipeptide Met-Tyr derived from sardine-muscle protein could diminish free-radical formation in human endothelial cells. The protection was associated with the stimulated expression of the antioxidant defence proteins ferritin and haeme oxygenase-1 (HO-1), a cytoprotective enzyme that catalyses the degradation of haeme. In D-galactose-induced ageing of ICR mice, Protamex hydrolysates of jellyfish collagen displayed promising intracellular free-radical scavenging

effects by upregulating the protein-expression levels of antioxidant enzymes (Ding *et al*., 2011). Oral administration of the hydrolysates caused a significant increase in the activities of antioxidant enzymes including glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), accompanied by a decrease in serum and hepatic MDA levels. The antioxidant potential of casein hydrolysates was evaluated in human Jurkat T-cells (Lahart *et al*., 2011; Phelan *et al*., 2009). Different casein hydrolysates generated with proteases of different specificities displayed varying degrees of antioxidant activity. No apparent correlation was found between the DH and antioxidant capacity. It was postulated that the amino acid sequence, peptide mass and conformational structure of antioxidant peptides may be more important than DH in determining their cellular uptake. The possible mechanisms of antioxidant action include their ability to increase cellular catalase activity and reduce glutathione (GSH) level. But casein hydrolysate supplementation did not affect the SOD activity. It was also observed that some of the casein hydrolysates reduced cell viability and prevented the proliferation of human Jurkat T-cells in a dose-dependent manner. However, pre-incubation of Jurkat cells with casein hydrolysates at lower concentrations ($\leq$1% (v/v)) had no appreciable effect on cell-membrane integrity (Phelan *et al*., 2009). In a recent study, Vo *et al*. (2011) observed that the peptide isolated from Nile tilapia gelatin hydrolysates was able to inhibit H_2O_2-induced ROS generation in the murine microglial cell line BV-2 in a dose- and time-dependent manner. It also exerted a protective effect on DNA damage induced by hydroxyl radicals generated through Fenton reaction (Fe^{2+}/H_2O_2). The ability of the purified peptides to interfere with oxidative processes in cellular systems suggests their potential for use as natural additives and/or dietary supplements for the prevention of oxidative stress-related diseases, such as diabetes, cardiovascular diseases and cancer.

18.3.2 Structure–activity Relationship of Antioxidant Peptides

Neither the antioxidant mechanism nor the structure–activity relationship of bioactive peptides (including antioxidant peptides) has been fully elucidated to date. However, some general features have been revealed. Many antioxidant peptides seem to share some common structural characteristics, which include a relatively short peptide residue length (e.g. 3–16 amino acids), often presenting hydrophobic amino acid residues in the sequence. Some purified antioxidant peptides included hydrophobic amino acids (Val or Leu) at the N-terminal positions and Pro, His, Tyr, Trp, Met and Cys in the sequences (Chen *et al*., 1996; Elias *et al*., 2008; Uchida & Kawakishi, 1992), and a few contain mainly acidic acid residues (Glu, Asp) (Saiga *et al*., 2003).

18.3.2.1 Molecular Weights of Peptides

The molecular weight of a peptide has been demonstrated to be one of the most important factors in producing protein hydrolysates with desired functional and antioxidant properties (Ranathunga *et al*., 2006). Many researchers report that oligopeptides with 2–10 amino acids are more potent antioxidants than their parent native proteins or polypeptides (10–50 amino acids, or more) (Chen *et al*., 1995b; Ranathunga *et al*., 2006; Wu *et al*., 2003a). The higher antioxidant potential of low-molecular-weight peptides has been supposed to be due to their easier access to lipid radicals and ability to quench them more effectively than large peptides and proteins, which inhibits the free radical-mediated propagation of lipid peroxidation (Ranathunga *et al*., 2006).

The peptic hydrolysate of hoki-frame proteins was fractionated into four molecular-weight fractions (5–10, 3–5, 1–3 and <1 kDa) using UF membranes with different MWCOs (Kim *et al*., 2007b). Among the four groups, the lower molecular-weight fraction (1–3 kDa) was found to be the most effective inhibitor of lipid peroxidation, comparable to that of a standard antioxidant, α-tocopherol. The free radical-scavenging activities of the 1–3 kDa fraction were also higher than those of other fractions. Similar results were obtained by Ranathunga *et al*. (2006), who stated that the lowest molecular-weight fraction (<1 kDa) from the tryptic hydrolysate of conger eel-muscle protein possessed the strongest antioxidant potency, exhibiting about 79% inhibition of linoleic acid peroxidation in the linoleic acid model system. In another study, peptides with molecular weights between 390 and 1400 Da showed the strongest antioxidant capacity among all the hydrolysate fractions from tuna dark-muscle byproduct (Hsu, 2010).

However, other authors have observed that the antioxidant activities of the hydrolysate decrease gradually after reaching a maximum level at a certain DH. Wu *et al*. (2003a) reported that the total amounts of peptides released by the hydrolysis of mackerel with Protease N increased dramatically during the first 10 hours of hydrolysis and gradually decreased thereafter. Accordingly, the ability of the hydrolysate to inhibit linoleic acid peroxidation reached a maximum after 10 hours of hydrolysis and then declined up to 25 hours. The isolated oligopeptide with a molecular weight of 1.4 kDa exhibited stronger antioxidant activity than two small-sized peptides (0.9 and 0.2 kDa). Similarly, Li *et al*. (2007) found that the radical-scavenging activity of porcine skin-collagen hydrolysate by a cocktail mixture of proteases increased initially with increasing DH but decreased when the DH reached 85%. The peptide fraction from squid-gelatin and hoki-frame hydrolysates with the lowest molecular weight has been reported to exhibit the weakest radical-scavenging activity (Alemán *et al*., 2011; Je *et al*., 2005).

In order to produce a stronger antioxidant hydrolysate or to preserve the inherent antioxidant properties of the parent proteins, the extent of hydrolysis must be carefully controlled within certain optimum ranges. An intensive degradation of these proteins can lower the antioxidant capability (Peña-Ramos & Xiong, 2002; Zhou *et al*., 2012). The significant decreases in antioxidant activities of the hydrolysate when the DH reaches higher levels can be attributed to the fact that most of the proteins or peptides are further degraded into free amino acids, which have little or no antioxidant activity. In addition to acting as an efficient free-radical scavenger, a protein hydrolysate can form a protective membrane around the lipid droplets, deterring the penetration and diffusion of lipid-oxidation initiators into the interior of the lipids (Hirose & Miyashita, 1999; Wiriyaphan *et al*., 2012). To function as a physical barrier around oil droplets, proteins or peptides must possess a certain structural integrity. Extensive hydrolysis could impair a peptide's ability to act as a protective barrier and prevent oxidants from reaching the lipid phase of the liposomes (Kong & Xiong, 2006; Peña-Ramos & Xiong, 2002).

It is worth noting that various peptides with different sizes and amino acid sequences may display varying antioxidant efficiencies in different antioxidant assay systems. Nalinanon *et al*. (2011) compared the antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream produced by skipjack tuna pepsin. The strongest ABTS and DPPH radical-scavenging activities were observed in hydrolysates with 20% DH, whereas the hydrolysate with 30% DH exhibited the highest Fe^{2+}-chelating ability. In order to maximise their functions as free-radical scavengers and metal-ion chelators, the hydrolysis conditions must be optimized to prepare hydrolysates with optimal DH.

In another study, the antioxidant activity of hydrolysates formed from brownstripe red snapper muscle by two-step hydrolysis were evaluated by assaying for DPPH and ABTS radical-scavenging activity, ferric-reducing antioxidant power (FRAP) and ferrous ion-chelating ability (Khantaphant *et al*., 2011). During the first stage of hydrolysis, all hydrolysates with 40% DH showed higher radical-quenching activities than those with lower DH and higher peptide chain length, while the secondary antioxidant potential, as measured by the chelating ability, was lowered. Therefore, the peptides present in this hydrolysate might function more effectively as primary, chain-breaking antioxidants. Furthermore, there was no apparent correlation between DPPH and ABTS radical-scavenging activity for the peptides prepared by two-step hydrolysis. The result indicate that the same peptide may have different scavenging capacities toward different types of free radical.

18.3.2.2 Hydrophobicity

Peptides derived from different protein sources with increased hydrophobicity have been reported to possess strong antioxidant activities. Several studies have shown that the high content of hydrophobic amino acids is mainly responsible for the potent radical-scavenging and lipid-peroxidation inhibitory activities of peptide fractions from jumbo squid-skin gelatin, hoki-skin gelatin and giant squid muscle (Mendis *et al*., 2005a, 2005b; Rajapakse *et al*., 2005a). For protein hydrolysates and peptides, an increase in hydrophobicity will potentially increase their concentration at water–lipid interfaces and allow close contact with lipid molecules, thus facilitating the scavenging of lipid-derived radicals through direct proton donation (Chen *et al*., 1995b; Rajapakse *et al*., 2005a; Saiga *et al*., 2003; Sun *et al*., 2012). Furthermore, hydrophobic amino acids may increase the affinity of the peptide for hydrophobic cellular targets such as the long-chain polyunsaturated fatty acids (LC-PUFAs) of biological membranes and subsequently enhance its reactivity in living cells (Chen *et al*., 1998; Mendis *et al*., 2005a).

Murase *et al*. (1993) reported that N-(long-chain-acyl) histidine and N-(long-chain-acyl) carnosine were more effective than intact histidine and carnosine in preventing oxidation of phosphatidylcholine liposomes induced by ferrous ion and ascorbic acid. The enhancement in antioxidant activity of the modified compounds was tentatively attributed to the increased hydrophobicity caused by long-chain acyl groups, thereby facilitating better interaction of carnosine and His with peroxyl radical. In another study, histidine-containing peptides did not show any appreciable antioxidant effect against the oxidation of methyl linoleate in an AMVN-induced aqueous system, while N-(long-chain-acyl) histidine exhibited good inhibitory activity (Chen *et al*., 1998). In general, the hydrophobicity of peptides increases with an increase in the alkyl chain length. The histidine-containing peptides, due to the lack of hydrophobic peptide moiety, are unable to interact properly with the hydrophobic peroxy radical.

Hydrophobicity has also been implicated as one of the most important features for marine-derived antioxidant peptides. Rajapakse *et al*. (2005a) isolated two potent antioxidant peptides (Asn-Ala-Asp-Phe-Gly-Leu-Asn-Gly-Leu-Glu-Gly-Leu-Ala (1307 Da) and Asn-Gly-Leu-Glu-Gly-Leu-Lys (747 Da)) from giant squid muscle (Table 18.1). Both peptides significantly inhibited lipid peroxidation in the linoleic acid emulsion system, with higher activity than that of the natural antioxidant α-tocopherol and close to that of the strong synthetic antioxidant BHT. Sequence analysis revealed a high prevalence of hydrophobic amino acids such as Gly, Leu and Ala, representing more than 75% of the sequence. Furthermore, the hydrophobic diamino acid sequence (–Gly-Leu-) was frequently observed in both peptides, which was expected to further

contribute to the inhibition of lipid peroxidation. In another study, a nonapeptide (Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn) isolated from conger eel was found to be highly effective in scavenging several species of free radical (Ranathunga *et al*., 2006) (Table 18.1). It also exerted a strong inhibitory effect on lipid peroxidation, which was superior to that of α-tocopherol. The observed antioxidant activities of this nonapeptide could be related to the presence of hydrophobic amino acid residues, representing about 55% of the sequence. Kim *et al*. (2007b) purified a potent antioxidant peptide, APHPH (Glu-Ser-Thr-Val-Pro-Glu-Arg-Thr-His-Pro-Ala-Cys-Pro-Asp-Phe-Asn, 1801 Da) from the hydrolysate of hoki-frame protein. APHPH was found to be more effective than α-tocopherol in preventing lipid peroxidation and efficiently quenched different sources of free radicals. In the sequence of APHPH, hydrophobic amino acid residue composed approximately 44% of the peptide sequence, which increased its accessibility to hydrophobic targets and exerted a better scavenging effect on lipid-derived radicals.

18.3.2.3 Amino Acid Composition and Sequence

In addition to the molecular weight and hydrophobicity of the peptides, the presence of some important amino acid residues and their specific positioning within the peptide sequence play an important role in antioxidant activity (Chen *et al*., 1996; Kim *et al*., 2001; Mendis *et al*., 2005b; Suetsuna *et al*., 2000).

A number of studies have observed a high correlation between certain amino acid residues and the antioxidant activity of peptides. The importance of these amino acid residues is believed to be related to their unique structural features. Aromatic amino acids such as Tyr, His, Trp and Phe and hydrophobic amino acids including Val, Leu and Ala, as well as Met and Gly, have been reported to be critical for the antioxidant activities of peptides, although some of these amino acids, such as Gly, Met and Trp, have also been reported to show pro-oxidative effects under certain experimental conditions (Chen *et al*., 1998; Mendis *et al*., 2005b; Rajapakse *et al*., 2005b).

Aromatic amino acids such as Tyr and Phe are generally considered effective free-radical scavengers. The antioxidant activity of Tyr is believed to be caused by the presence of a phenolic hydroxyl group in its aromatic structure. It can act as a chain-breaking antioxidant, following a hydrogen-atom transfer (HAT) mechanism, as observed in most phenolic antioxidants (Ou *et al*., 2002). At the same time, its antioxidative stability can remain intact, via resonance structures. Dávalos *et al*. (2004) reported that the synthetic peptide Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu exhibit superior radical-scavenging activity. The ORAC-FL value of this peptide was almost sixfold higher than that of α-tocopherol. It was postulated that the presence of Tyr at the N-terminal position contributed greatly to the observed scavenging activity. To comfirm the role of Tyr in the antioxidant activity of the peptide, two shorter derived fragments, Tyr-Pro-Ile-Leu and Tyr-Pro-Ile, were synthesised, and their antioxidant activity was evaluated. As expected, both exhibited lower radical-scavenging capacities. Similarly, the strong antioxidant activity of purified peptide from fermented mussel sauce was suggested to be due to the presence of two aromatic amino acids and two His residues in the peptide sequence (Rajapakse *et al*., 2005b).

It is well known that acidic and/or basic amino acid residues play an important role in the chelation of Fe^{2+} and Cu^{2+} by antioxidant peptides. More specifically, carboxyl and amino functional groups in the side chains of the acidic and basic amino acids are mainly responsible for the metal-chelating capacity of these peptides (Saiga *et al*., 2003). Chan & Decker (1994) postulated that peptides containing basic amino acid residues (His and Lys)

function as electron acceptors, taking on electrons from free radicals formed during the oxidation of unsaturated fatty acids and terminating the reaction. Studies carried out on porcine myofibrillar proteins showed that the isolated peptides from papain hydrolysate contained mainly acidic amino acid residues. These acidic amino acids may interact with metal ions through their charged residues and inactivate the pro-oxidant activity of metal ions (Saiga *et al*., 2003). Ranathunga *et al*. (2006) reported that the presence of two acidic Asp residues and two Gly residues in conger eel antioxidative peptide (CEAP) might partly contribute to its potent radical-scavenging activity.

In addition, Gly and Pro have been shown to enhance the radical-scavenging capacity of some peptides (Alemán *et al*., 2011; Chen *et al*., 1996, 2011). The smallest amino acid, glycine, possesses only a single hydrogen atom as its side chain, and therefore it confers a higher degree of flexibility to the peptide backbone than the other amino acids. The unusual cyclic structure of Pro and its conformational flexibility are responsible for its biological function in peptides and proteins. Pro residues tend to interrupt the secondary structure of the peptide and impose conformational constraints on its backbone, through both the cyclic nature of the rigid pyrrolidine ring and the steric effect (Alemán *et al*., 2011; Rajapakse *et al*., 2005a).

However, several investigators have reported that some antioxidant peptides do not contain any of these favourable amino acid residues. The constituent amino acids of the identified peptides mixed with the same concentration have generally shown little or no antioxidant activity (Ren *et al*., 2008; Suetsuna *et al*., 2000; Zhang *et al*., 2009). The amino acid sequence of a peptide may be more important in determining its antioxidant effectiveness than a special amino acid in the sequence. The stability of any resultant peptide radicals that do not initiate or propagate further oxidative reactions also plays an important role (Elias *et al*., 2008).

The positioning of amino acids with hydrophobicity, such as Leu or Val, at the N-terminus has been shown to be important to the antioxidant activity of a peptide (Chen *et al*., 1995a; Elias *et al*., 2008; Park *et al*., 2001; Ranathunga *et al*., 2006). It is assumed that the long aliphatic side-chain group of Leu will favour interactions between peptides and the acyl chains of susceptible fatty acids. But it has also been reported that the elimination of Leu from the N-terminus has no effect on activity (Klompong *et al*., 2009). Suetsuna *et al*. (2000) observed that a casein-derived radical-scavenging peptide and its synthetic mimics, with Leu and Pro at the C-terminal, exhibited higher radical-scavenging potentials. The strong superoxide anion radical-scavenging activity of the peptide Tyr-Phe-Tyr-Pro-Glu-Leu was attributed mainly to the dipeptide of the C-terminal amino acid Glu-Leu, although deletion of N-terminal Tyr, Tyr-Phe and Tyr-Phe-Tyr led to a loss of activity. Mendis *et al*. (2005b) described the significance of Leu for a high radical-scavenging activity when present at the C-terminus. Furthermore, the potent antioxidant peptide (His-Gly-Pro-Leu-Gly-Pro-Leu) from fish-skin gelatin was characterised by two distinctive repeating amino acid residues (Gly-Pro), placing Leu or His at the other position (Table 18.1).

18.3.2.4 Histidine-containing Peptides

The antioxidant activity of histidine-containing peptides has been well documented and extensively reviewed (Bougatef *et al*., 2010; Park *et al*., 2001; Uchida & Kawakishi, 1992). These peptides exert their antioxidant effect through various mechanisms, including transition-metal ion chelation, hydroxyl-radical scavenging and the quenching of singlet oxygen and other active oxygen species (Chen *et al*., 1998; Saito *et al*., 2003).

In the literature, there are many studies that report the strong antioxidant properties of several biologically important, histidine-containing dipeptides, including carnosine (β-alanyl-L-histidine), anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (λ-aminobutyryl-L-histidine). Carnosine and anserine have been shown to be highly efficient in preventing lipid peroxidation in linoleic acid systems (Wu *et al*., 2003b). Moreover, they also exhibit high reducing power and free radical-scavenging and copper ion-chelating activities *in vitro*. These dipeptides can be used as natural antioxidants in various muscle food systems in order to extend the shelf life. The addition of 1.5% carnosine effectively prevents rancid odour development and maintains the redness in frozen salted ground pork (Decker & Crum, 1991). The presence of carnosine (50 mM) inhibits lipid oxidation in beef patties during storage in modified atmosphere ($O_2/CO_2/N_2$, 70/20/10 (%)) (Sánchez-Escalante *et al*., 2003). However, several other studies have revealed no or even pro-oxidant effects in carnosine. Carlsen *et al*. (2002) reported that carnosine did not show the ability to deactivate ferrylmyoglobin, an important pro-oxidant in muscle tissue and meat. It was therefore ineffective in retarding lipid peroxidation in cooked pork meat. In another study, carnosine was found to have significant antioxidant activity, especially in oxidising systems based on Fe^{2+}, Fe^{3+} or Cu^{2+} ions (Mozdzan *et al*., 2005). But the addition of histidine and alanine to carnosine, which mimics partial carnosine hydrolysis, led to a substantial decrease in DNA protection. The pro-oxidant effect was likely caused by the release of free histidine after carnosine hydrolysis.

Although the structure–activity relationship of His-containing peptides has not yet been clearly stated, the activity is believed to be mainly caused by the proton-donation ability, lipid peroxyl radical-scavenging ability and/or metal ion-chelating ability of the imidazole ring of histidine (Klompong *et al*., 2009; Mendis *et al*., 2005b). The capability of histidine-related compounds to inhibit iron-mediated lipid peroxidation may be associated with their ability to coordinate with iron and form a complex, thus preventing the reduction of Fe^{3+} to Fe^{2+} (Erickson & Hultin, 1992). The interactions between the metal ions and the histidine imidazole ring were confirmed by nuclear magnetic resonance (NMR) analysis, which showed the loss of the C-2 and C-4 peaks of the imidazole ring. In another study carried out on jumbo squid-skin gelatin peptides (Mendis *et al*., 2005a), two purified peptides (Phe–Asp–Ser–Gly–Pro–Ala–Gly–Val–Leu (P1) and Asn–Gly–Pro–Leu–Gln–Ala–Gly–Gln–Pro–Gly–Glu–Arg (P2)) did not show any metal ion-chelating effect. The relatively lower percentage of histidine residues in their sequences seemed to explain their poor chelating ability.

As indicated in several studies, the differences in the antioxidant potencies of individual His-containing peptides may be related to their lipophilic properties. His-containing peptides are generally more hydrophobic than His itself, and an increase in antioxidant activities was observed for N-(long-chain-acyl) His-containing compounds (Murase *et al*., 1993). The hydrophobicity of the compounds was important for accessibility to the hydrophobic targets. The high antioxidant activities of His-containing dipeptides may be related to their increased accessibility towards lipophilic fatty acids or peroxyl radical through the increased hydrophobicity caused by long-chain acyl groups. In addition, the presence of the Ala residue and the peptide bond between β-alanine, His and 1-methylhistidine has also been suggested to partially contribute to the antioxidant effect of these dipeptides (Mozdzan *et al*., 2005; Wu *et al*., 2003b). It is worth noting that specific positioning of His in the peptide sequence has a great impact on the antioxidant activity. Chen *et al*. (1998) investigated the metal ion-chelating activities of 22 synthetic His-containing peptides. In general, increasing the number of His residues in the sequence

enhanced their affinity to bind metal ions. The peptides containing His residues at the N-terminus exhibited higher chelating ability than did those with His residues at the C-terminus. The significance of N-terminal His was also supported by a previous study on the antioxidant activity of synthetic peptide mimics in a linoleic acid peroxidation system (Chen *et al*., 1996). A complete loss of activity was observed after deletion of the terminal His residue. The addition of the hydrophobic amino acids Pro and Leu to the N-terminus of a dipeptide His-His significantly improved the antioxidant activity of the peptides. These new peptides also showed synergistic antioxidant effects with nonpeptide antioxidants. Similarly, the N-terminal His was suggested to contribute significantly to the higher radical-scavenging activity of peptide (His-Gly-Pro-Leu-Gly-Pro-Leu) isolated from hoki-skin gelatin, as compared to other peptides (Mendis *et al*., 2005b) (Table 18.1).

18.3.2.5 Peptide Conformation and Amino Acid Configuration

Peptide conformation has been suggested to be one determinant factor in the antioxidant activity of peptides, showing either synergetic or antagonistic effect in comparison to the effects exerted by free amino acid mixture. Hernández-Ledesma *et al*. (2005) isolated several potent antioxidant peptides from β-lactoglobulin A (β-Lg A) hydrolysate using corolase PP. One of the peptides, Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile, exhibited better radical-scavenging activity than the synthetic antioxidant BHA. However, the ORAC−FL value of this peptide was lower than that of the amino acid Trp or of the equimolar mixture of the corresponding amino acids, indicating that the peptidic bond or structural peptide conformation might impair the free radical-scavenging activity of the amino acid residues. Moreover, the presence of other amino acids in a peptide sequence could lead to antagonism among them, which might explain the reduced radical-scavenging activity of the peptide. On the other hand, the ORAC−FL value of another identified peptide Tyr-Val-Glu-Glu-Leu was found to be approximately twofold higher than that of those measured for their equimolar amino acid mixtures. In a later study, the same authors again noted that all the synthesized β-Lg-derived peptides possessed higher peroxyl radical-scavenging capacities than did equimolar mixtures of free amino acids (Hernández-Ledesma *et al*., 2007). In this case, the peptidic bond or structural peptide conformation appears to have positive effects on the hydrogen-donor capacity of the constitutive amino acids. More recently, various protein hydrolysates made from the larvae of the cotton leafworm, *Spodoptera littoralis*, were shown to possess similar degrees of antioxidant capacity independent of the enzyme employed (Vercruysse *et al*., 2009). The similarity in antioxidant activity suggestsd that peptide conformational changes might have no net effect, due to both synergistic and antagonistic effects of different peptides present in crude hydrolysates. Another explanation could be that the presence of certain amino acids is more important in determining the antioxidant property than is the structural conformation of the peptides.

In addition, the configuration of amino acid residues in peptides can also have a profound impact on antioxidant activity. Substitution of the second L-His with D-His in a tripeptide sequence (Pro-His-His) resulted in decreased antioxidant activity (Chen *et al*., 1996). It was speculated that the proper positioning and orientation of the imidazole ring is key to determining the proton-donation capacity of the constitutent peptides.

Based on these discussions, it is reasonable to conclude that our knowledge of the antioxidant mechanisms of bioactive peptides is incomplete. Antioxidant activity seems to be attributed to the integrative effects of these antioxidant mechanisms, rather than

to the individual actions of a peptide. Further studies are needed to provide a better understanding of the precise mechanisms underlying the observed antioxidant effects.

18.4 INDUSTRIAL APPLICATIONS AND PERSPECTIVES

Marine organisms are rich sources of protein for human consumption and the byproducts from seafood processing are good materials for the production of antioxidant peptides. Globally, more than 100 million tonnes of fish and shellfish are caught annually, with only 75% being utilised for human consumption (Shahidi, 1994). A large quantity of byproducts and discards, including skins, heads, viscera and backbone, are generated from the seafood processing industry. For example, the yield of catfish when processed as whole fillets is around 45%, generating about 55% byproducts (Yin *et al*., 2009). In 2000, a total of 251 000 metric tonnes of byproducts were created by Norwegian cod fisheries alone, of which 114 000 tonnes were dumped, while 137 000 tonnes were utilised. Only 33 000 tonnes of the byproducts were used for human consumption, which accounts for 13% of the total (Shahidi, 2009). In the southern part of the USA, processing of catfish in 2007 was about 225 000 metric tons (NASS, 2008), and the byproducts were about 123 750 metric tons. But a large part of the byproducts are dumped and not used. The protein content of byproducts is very high, for example cod frames have 16.9% protein (Liaset *et al*., 2000). These seafood byproducts can be used as food for human consumption, and they are biologically active, with nutritional and medical benefits, which can increase their market value (Alemán *et al*., 2011).

Marine-derived hydrolysates and peptides with antioxidant properties can be used as food additives to inhibit lipid oxidation in various food products, such as fish products, frozen foods and drinks. They also possess unique technofunctional properties, such as high solubility, low viscosity and resistance to gel formation. Antioxidants can prevent or delay food oxidation, fat rancidity, change of food colour, loss of vitamins and unsaturated material damage, and they can be directly added to food to improve its quality or extend its shelf life during storage and transportation. Many synthetic antioxidants are still widely used in the food industries. However, their use is becoming more strictly regulated in many countries, due to their potential health hazards (Bernardini *et al*., 2011). Much research has shown that marine-protein hydrolysates and peptides possess potent antioxidant activity in various lipid or food systems and can be used as natural antioxidants with little or no side effects. One promising application of antioxidant peptides is in seafood processing. Fish products contain a variety of unsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are extremely unstable when exposed to light and high temperatures, and gradually break down into aldehydes and ketones, resulting in quality deterioration of fish products. Hydrolysates and peptides can not only effectively control lipid oxidation during storage but also improve flavour and increase nutritional value. Hard clams are steamed and boiled as raw material, and the liquids obtained are made into hard clam essence. The free amino acid, nucleotide-related compound and dipeptide content and the antioxidant activity of hard clam essence are significantly higher than those of commercial freshwater calm (Zheng *et al*., 2011). A patent describes a process of incorporation of salmon-protein hydrolysates into smoked salmon fillets and successful inhibition of lipid oxidation during storage (Hagen *et al*., 2004). Another good application example is silver carp antioxidant peptide, which has been demonstrated to be effective in retarding lipid peroxidation of Sierra fish (*Scomberomorus niphonius*) during refrigerated storage.

Marine-derived antioxidant peptides may have great potential to be used as active ingredients in functional foods, food supplements and the cosmetic and pharmaceutical industries. Antioxidant peptides can eliminate excess free radicals in the body and prevent free radical-induced diseases (Mendis *et al*., 2005b). A new health food has been prepared by the fermentation of Tilapia meat by lactic acid bacteria, and showed strong antioxidant and ACE-inhibitory activity (Chen, 2004). A tetrapeptide (Val-Trp-Trp-Trp) purified from mackerel hydrolysates not only shows *in vitro* antioxidant activity but is also effective in the prevention of low-density lipoprotein (LDL) oxidation, which induces atherosclerosis disease (Zhou, 2008). Furthermore, collagen polypeptides from jellyfish and cod skin might prevent mice skin from UV irradiation and moderate skin photo-ageing through antioxidation. They also inhibit mclanogenesis of melanoma B16 through clearance of free radicals and some molecular regulation. Therefore, collagen polypeptides may have good anti-photo-ageing action and be suitable for application in cosmetics and medicines (Dong, 2007; Zhang, 2009).

An increasing number of bioactive peptides with antioxidant capacity have been separated, purified and identified from a variety of protein hydrolysates. But the exact mechanism underlying the antioxidant effects is not fully understood. Different antioxidant assays have been used in different studies, which makes it difficult to directly compare the results across different laboratories. Standardised methods are urgently required. In addition, the use of a combination of methodologies based on different mechanisms is recommended, in order to build a more complete picture of the antioxidant capacity of peptides. The antioxidant effect of peptides is system-dependent, and it is important to conduct studies in appropriate models which closely mimic the targeted food or biological system. Antioxidant peptides show a wide range of disease prevention and therapeutic effects, but most studies focus on *in vitro* chemical assays. Well-designed *in vivo*, animal and human clinical studies should be carried out to systematically evaluate their health benefits and potential risks. The impact of processing conditions on stability should be addressed. More work is needed to understand these petides' bioavailability *in vivo*. Stability and absorption in the gastrointestinal tract and the effects of metabolic transformation and degradation on the antioxidant activity of peptides all warrant further investigation.

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19 Marine Peptides and Proteins with Cytotoxic and Antitumoral Properties

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19.1 INTRODUCTION

Cancer is a collection of diseases with multiple causes (e.g. genetic mutations and/or viral infection) in which cells with diverse genetic backgrounds and extracellular environments, as well as altered key regulatory networks, display aberrant proliferation, migration, invasion and/or differentiation. Two consequences of the highly polymorphic nature of oncological diseases are the need for: (1) a battery of tests to diagnose cancer accurately and (2) a specific therapeutic strategy to counteract the particular form of cancer afflicting each patient. As the genetic backgrounds of cancer cells can be quite different—e.g. rearrangement, gain or loss of chromosomes (reviewed by Albertson *et al*., 2003)—it is common for a given therapy to fail even if it is often used to treat a given form of cancer successfully (ACS, 2012). This state of affairs underlines the constant need for improvement of existing therapies or the discovery of new drugs able to provide higher survival rates and lower the impact of side effects on the quality of life of cancer patients.

19.2 CURRENT PIPELINE OF ONCOLOGICAL DRUGS BASED ON NATURAL PRODUCTS

Between 1981 and 2006, natural products (NPs) and slight variations thereof accounted for 28% of the drugs approved by the US Food and Drug Administration (FDA). The impact of NPs on drug discovery can be further emphasized if the proportion of approved drugs based on chemical skeletons found in nature (24%) is also taken into account (Butler & Newman, 2008). Despite these numbers, between 1997 and 2006 the FDA did not approve a single NP to treat oncological patients (Bailly, 2009). However, in 2007 this dismal picture began to change when several NP-based antitumoral drugs came to the fore. One was trabectedin (Fig. 19.1) (ET-743/Yondelis®), a marine-derived alkaloid with DNA alkylating properties that was approved by the European Agency for the Evaluation

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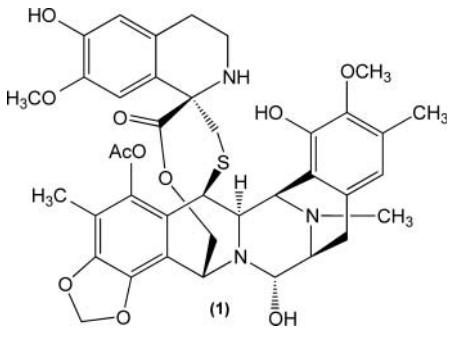

Fig. 19.1 Trabectedin.

of Medicinal Products (EMEA) for treatment of soft-tissue sarcoma (Mayer *et al*., 2010; Soares *et al*., 2007). Two years later, trabectedin was also approved for therapy of patients with ovarian carcinoma (Mayer *et al*., 2010). Supplementary clinical trials have indicated that trabectedin could be used to treat other forms of cancer, in particular breast cancer and renal carcinoma (Molinski *et al*., 2009).

19.3 CURRENT PIPELINE OF MARINE PEPTIDES WITH ANTITUMORAL ACTIVITY

To date, the FDA and EMEA have approved six additional drugs based on marine NPs, three of them intended to treat cancer, namely the nucleoside analog cytarabine (Ara-C), the macrolide erubilin mesylate (E7389) and brentuximab vedotin (SGN-35) (Mayer, 2012). The latter is an antibody conjugated with monomethyl auristatin E (Doronina *et al*., 2008), a synthetic variant of the marine acyclic peptide dolastatin 10 (Fig. 19.2), which was originally isolated from the marine shell-less mollusk *Dolabella auricularia* (Pettit *et al*., 1987). Interestingly, other synthetic dolastatin analogs are or have been in clinical trials, namely soblidotin (TZT-1027; Schöffski *et al*., 2004), tasidotin (ILX651; Bai *et al*., 2009), glembatumumab vedotin (CDX-011; Naumovski & Junutula, 2010), h1F6-mcMMAF (SGN-75; Oflazoglu *et al*., 2008) and ASG-5ME (Arnett *et al*., 2011), the latter three of which are formulated as antibody–auristatin conjugates. In addition, two marine peptides with ester bonds (depsipeptides), elisidepsin (Fig. 19.3) (PM02734 / Irvalec®) and plitidepsin (Fig. 19.4) (dehydrodidemnin B / Aplidin®), are undergoing phase-II and phase-III clinical trials (Barboza *et al*., 2012; Ling *et al*., 2009). Phase-I

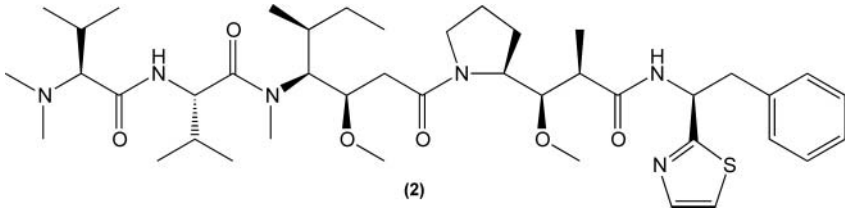

Fig. 19.2 Dolastatin 10.

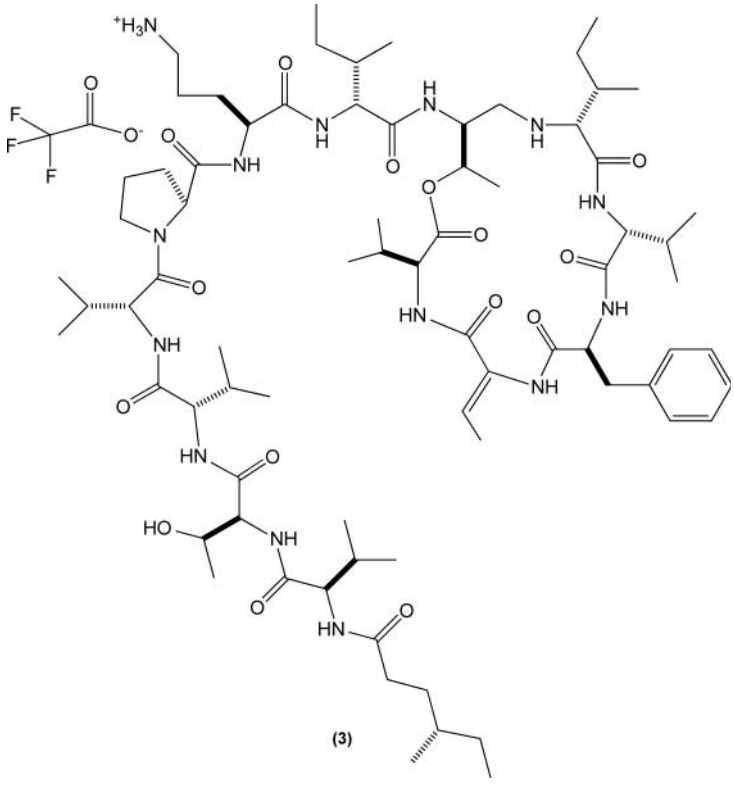

Fig. 19.3 Elisidepsin.

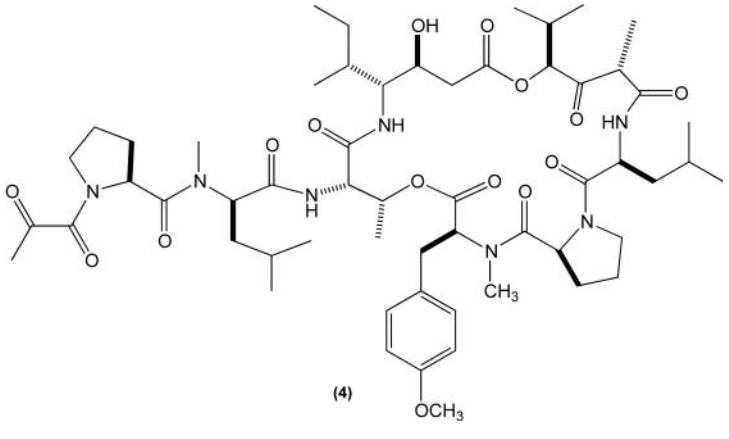

Fig. 19.4 Plitidepsin.

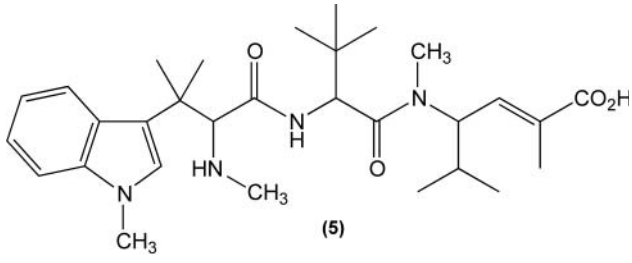

Fig. 19.5 Hemiasterlin.

clinical trials with E7974, an analog of hemiasterlin (Fig. 19.5), have recently been concluded with interesting results concerning antitumoral activity against refractory solid tumors (Rocha-Lima *et al*., 2012).

19.4 MAJOR BIOLOGICAL SOURCES OF MARINE CYTOTOXIC PEPTIDES AND PROTEINS

Sponges (Porifera) and cyanobacteria have been, by far, the major contributing taxa for the array of peptides and proteins displaying potential antitumoral activity that have been isolated from marine sources in the last 3 decades (Tables 19.1, 19.2 and 19.3). Other important taxonomical groups are mollusks and chordates (e.g. ascidians, tunicates and fish). However, mounting evidence that commensal, symbiotic or ingested cyanobacteria and green algae are the actual biological sources of a few bioactive peptides that were originally isolated from sponges and mollusks has been gathered. Examples are the cytotoxic peptides didemnin B (Fig. 19.6), dolastatin 10 (Fig. 19.2), kahalalide F (Fig. 19.7) and wainunuamide (Dunlap *et al*., 2007; Hamann *et al*., 1996; Luesch *et al*., 2000, 2001; Tabudravu *et al*., 2001). For this reason, molecular methods such as metagenomics have been proposed and used to pre-screen marine invertebrates (e.g. sponges, gorgonians, tunicates and bryozoans) for photosynthetic symbionts and genes coding for enzymes involved in peptide biosynthesis (Dunlap *et al*., 2007). Another molecular method, single-cell genome amplification, is preferred when entire operons coding for enzymes of peptide biosynthetic pathways need to be cloned from complex microbial assemblages (Grindberg *et al*., 2011).

19.5 STRUCTURAL MOTIFS IN CYTOTOXIC PEPTIDES

As a result of the promising antitumoral bioactivities of marine peptides, a number of peer-reviewed papers describing the isolation and characterization of novel cytotoxic peptides (Tables 19.1 and 19.2) and polypeptides (Table 19.3) from marine biological sources have been published in the last 2 decades.

Although the structure of cytotoxic marine peptides can vary considerably in terms of size and complexity, a few basic structural types can be recognized, namely: (1) acyclic (depsi)peptides with up to 20 amino acid residues and modifications thereof (Table 19.1); (2) mono-, bi- and tricyclic (depsi)peptides (Table 19.2); and (3) polypeptides with more

Table 19.1 Cytotoxic marine acyclic (depsi)peptides.

Name	Source			Reference
	Species	Phylum	Family	
Belamide A	*Symploca* sp.	Cyanobacteria	Phormidiaceae	Simmons *et al.* (2006)
Bisebromoamide	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Teruya *et al.* (2009)
Carbamin A	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	McPhail *et al.* (2007)
Dolastatin 10[a]	*Dolabella auricularia*	Mollusca	Aplysiidae	Bai *et al.* (1990)
Dragonamide A, C–D	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Gunasekera *et al.* (2008); Jiménez & Scheuer (2001)
Efrapeptin J	*Tolypocladium* sp.	Fungi	Clavicipitaceae	Hayakawa *et al.* (2008)
Efrapeptin G	*Acremonium* sp.	Fungi	Hypocreaceae	Boot *et al.* (2006)
Epinecidin-1	*Epinephelus coioides*	Chordata	Serranidae	Lin *et al.* (2009)
Hemiasterlin	*Hemiasterella minor*	Porifera	Hemiasterellidae	Talpir *et al.* (1994)
Hemiasterlin	*Auletta* sp.	Porifera	Axinellidae	Gamble *et al.* (1999)
Hemiasterlin	*Siphonochalina* sp.	Porifera	Callyspongiidae	Gamble *et al.* (1999)
Koshikamide A	*Theonella* sp.	Porifera	Theonellidae	Araki *et al.* (2005)
Lucentamycin A	*Nocardiopsis lucentensis*	Actinobacteria	Nocardiopsaceae	Cho *et al.* (2007)
Milnamide A	*Auletta cf. constricta*	Porifera	Axinellidae	Crews *et al.* (1994)
Milnamide D	*Cymbastela* sp.	Porifera	Axinellidae	Chevallier *et al.* (2003)
Mitsoamide	*Geitlerinema* sp.	Cyanobacteria	Pseudanabaenaceae	Andrianasolo *et al.* (2007)
Polytheonamides A–B	*Theonella swinhoei*	Porifera	Theonellidae	Hamada *et al.* (2005)
Proximicin A	*Verrucosispora maris*	Actinobacteria	Micromonosporaceae	Fiedler *et al.* (2008), Schneider *et al.* (2008)
Psammaplin A	*Psammaplysilla* sp.	Porifera	Pseudoceratinidae	Jiang *et al.* (2004)
Sintokamide A	*Disidea* sp.	Porifera	Dysideidae	Sadar *et al.* (2008)
Somocystinamide A	*Lyngbya majuscula/ Schizothrix* sp.	Cyanobacteria	Oscillatoriaceae	Nogle & Gerwick (2002)
Styelin D	*Styela clava*	Chordata	Styelinae	Taylor *et al.* (2000)
Tasiamide	*Symploca* sp.	Cyanobacteria	Phormidiaceae	Williams *et al.* (2002a)
Virenamides A–C	*Diplosoma virens*	Chordata	Didemnidae	Carroll *et al.* (1996)
Yaku'amide A–B	*Ceratopsion* sp.	Porifera	Raspailiidae	Ueoka *et al.* (2010)

[a]The actual source is ingested cyanobacteria from genera such as *Lyngbya* or *Symploca* (Luesch *et al.*, 2000, 2001).

Table 19.2 Cytotoxic marine cyclic (depsi)peptides

Name	Source			Reference
	Species	Phylum	Family	
Apratoxin A–G	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Gutiérrez *et al.* (2008), Tidgewell *et al.* (2010)
Arenastatin A (cryptophycin-24)	*Dysidea arenaria*	Porifera	Dysideidae	Morita *et al.* (1997)
Aurilide B-C	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	Han *et al.* (2006)
Axinastatin 1–2	*Axinella* sp.	Porifera	Axinellidae	Pettit *et al.* (1994)
Azumamide A	*Mycale izuensis*	Porifera	Mycalidae	Nakao *et al.* (2006)
Bistratamide A,D[a]	*Prochloron* sp.	Cyanobacteria	Prochloraceae	Degnan *et al.* (1989), Foster *et al.* (1992)
Callipeltin A–B	*Callipelta* sp.	Porifera	Neopeltidae	Zampella *et al.* (1996)
Callyaerin A–E, G–H	*Callyspongia aerizusa*	Porifera	Callyspongiidae	Ibrahim *et al.* (2010)
Calyxamide A–B	*Discodermia calyx*	Porifera	Theonellidae	Kimura *et al.* (2012)
Celebeside A	*Siliquariaspongia mirabilis*	Porifera	Theonellidae	Plaza *et al.* (2009)
Coibamide A	*Leptolyngbya* sp.	Cyanobacteria	Pseudanabaenaceae	Medina *et al.* (2008)
Cycloxazoline	*Lissoclinum bistratum*	Chordata	Didemnidae	Hambley *et al.* (1992)
Desmethoxymajusculamide C	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	Simmons *et al.* (2009)
Diazonamide	*Diazona angulata*	Chordata	Diazonidae	Cruz-Monserrate *et al.* (2003), Knowles *et al.* (2011)
Didemnin B[b]	*Tridemnum solidum*	Chordata	Didemnidae	Rinehart *et al.* (1981)
Discodermin E	*Discodermia kiiensis*	Porifera	Theonellidae	Ryu *et al.* (1994a, 1994b)
Doliculide[c]	*Dolabella auricularia*	Mollusca	Aplysiidae	Bai *et al.* (2002)
Geodiamolides A–G	*Geodia* sp.	Porifera	Geodiidae	Sonnenschein *et al.* (2004)
Grassypeptolide	*Lyngbya confervoides*	Cyanobacteria	Oscillatoriaceae	Kwan *et al.* (2008)
Guineamides B–C	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	Tan *et al.* (2003)
Halicylindramides	*Halicondria cilindrata*	Porifera	Halichondriidae	Li *et al.* (1995)
Haligramide A–B	*Haliclona nigra*	Porifera	Chalinidae	Rashid *et al.* (2000)
Hantupeptin A	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	Tripathi *et al.* (2009)
Hectochlorin	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	Marquez *et al.* (2002)
Homophymines	*Homophymia* sp.	Porifera	Neopeltidae	Zampella *et al.* (2009)

Jasplakinolide (Jaspamide)[d]	*Jaspis johnstoni*	Porifera	Coppatiidae	Braekman *et al.* (1987)
Kahalalide F[e]	*Elysia rufescens*	Mollusca	Plakobranchidae	Hamann & Scheuer (1993)
Keenamide A	*Pleurobranchus forskalii*	Mollusca	Pleurobranchidae	Wesson & Hamann (1996)
Koshikamide B	*Theonella* sp.	Porifera	Theonellidae	Araki *et al.* (2008)
Kukekahilide	*Philinopsis speciosa*	Mollusca	Aglajidae	Takada *et al.* (2012)
Lagunamide C	*Lyngbya majuscula*	Cyanobacteria	Oscillatoriaceae	Tripathi *et al.* (2011)
Largazole	*Symploca* sp.	Cyanobacteria	Phormidiaceae	Taori *et al.* (2008)
Laxaphycin B	*Anabaena torulosa*	Cyanobacteria	Nostocaceae	Bonnard *et al.* (2007)
Lissoclinamide 1	*Prochloron didemni*	Cyanobacteria	Prochloraceae	Degnan *et al.* (1989)
Lyngbyabellin A–C, J	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Williams *et al.* (2003a)
Mechercharmycin A	*Thermoactinomyces* sp.	Firmicutes	Thermoactinomycetaceae	Kanoh *et al.* (2005)
Microcionamide A–B	*Clathria (Thalysias) abietina*	Porifera	Microcionidae	Davis *et al.* (2004)
Microsclerodermin F–I	*Microscleroderma* sp.	Porifera	Scleritodermidae	Qureshi *et al.* (2000)
Neopetrosiamide A	*Neopetrosia* sp.	Porifera	Petrosiidae	Austin *et al.* (2010), Williams *et al.* (2005)
Obyanamide	*Lyngbya confervoides*	Cyanobacteria	Oscillatoriaceae	Williams *et al.* (2002b)
Orbiculamide A	*Theonella* sp.	Porifera	Theonellidae	Fusetani *et al.* (1991)
Pahayokolide A	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Berry *et al.* (2004)
Palau'amide	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Williams *et al.* (2003d)
Pattelamide D	*Prochloron didemni*	Cyanobacteria	Prochloraceae	Schmidt *et al.* (2005), Williams & Jacobs (1993)
Phakellistatin 1	*Phakellia* sp.	Porifera	Axinellidae	Pettit *et al.* (1993)
Phakellistatin 1	*Stylotella aurantium*	Porifera	Axinellidae	Pettit *et al.* (1993)
Piperazimycin	*Streptomyces* sp.	Actinobacteria	Streptomycetaceae	Miller *et al.* (2007)
Plitidepsin	*Aplidium albicans*	Chordata	Polyclinidae	Rinehart *et al.* (1987)
Polydiscamide A	*Discodermia* sp.	Porifera	Theonellidae	Gulavita *et al.* (1992)
Rolloamide A	*Eurypon laughlini*	Porifera	Raspailiidae	Williams *et al.* (2009)
Sansalvamide A	*Fusarium* sp.	Ascomycota	Nectriaceae	Belofski *et al.* (1999), Dias *et al.* (2005)
Scleritodermin A	*Scleritoderma nodosum*	Porifera	Scleritodermidae	Schmidt *et al.* (2004)
Scopularides A and B	*Scopulariopsis brevicaulis*	Ascomycota	Microascaceae	Yu *et al.* (2008)
Spiruchostatin A–B	*Pseudomonas* sp.	Proteobacteria	Pseudomonadaceae	Yurek-George *et al.* (2007)
Symplocamide	*Symploca* sp.	Cyanobacteria	Phormidiaceae	Linington *et al.* (2008)

(*continued overleaf*)

Table 19.2 (*continued*)

Name	Source			Reference
	Species	Phylum	Family	
Tasipeptin A–B	*Symploca* sp.	Cyanobacteria	Phormidiaceae	Williams *et al.* (2003c)
Theonellamides A–E	*Theonella* sp.	Porifera	Theonellidae	Matsunaga & Fusetani (1995)
Theonellapeptolides	*Theonella swinhoei*	Porifera	Theonellidae	Kobayahsi *et al.* (1991)
Theopapuamide A	*Theonella swinhoei*	Porifera	Theonellidae	Plaza *et al.* (2009), Ratnayake *et al.* (2006)
Thiocondrilines	*Verrucosispora* sp.	Actinobacteria	Micromonosporaceae	Wyche *el al.* (2011)
Thiocoraline A	*Micromonospora marina*	Actinobacteria	Micromonosporaceae	Erba *et al.* (1999)
Ulithyacyclamide	*Lissoclinum patella*	Chordata	Didemnidae	Fu *et al.* (1998)
Ulongamides	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Luesch *et al.* (2002)
Ulongapeptin	*Lyngbya* sp.	Cyanobacteria	Oscillatoriaceae	Williams *et al.* (2003b)
Urukthapelstatin A	*Mechercharimyces asporophorigenens*	Firmicutes	Thermoactinomycetaceae	Matsuo *et al.* (2007)
Veraguamides	*Oscillatoria margaritifera*	Cyanobacteria	Oscillatoriaceae	Mevers *et al.* (2010)
Vitilevuamide	*Didemnum cuculiferum*	Chordata	Didemnidae	Edler *et al.* (2002)
Vitilevuamide	*Polysyncranton lithostrotum*	Chordata	Didemnidae	Edler *et al.* (2002)
Wainunuamide[e]	*Stylotella aurantium*	Porifera	Halichondriidae	Tabudravu *et al.* (2001)
Wewakpeptins A-B[f]	*Lyngbya semiplena*	Cyanobacteria	Oscillatoriaceae	Han *et al.* (2005)
Zygosparamide	*Zygosporium masonii*	Ascomycota	Not assigned	Wang *et al.* (2008)

[a]The biological source was originally assigned to ascidians *Lissoclinum bistratum* and *L. patella* (see cited references).
[b]The symbiotic cyanobacterium *Synechocystis trididemni* is most likely the true biological source (Dunlap *et al.*, 2007).
[c]The actual source is ingested cyanobacteria from genera such as *Lyngbya* or *Symploca* (Luesch *et al.*, 2000, 2001).
[d]The actual source might be the myxobacterium *Chondromyces crocatus* (Radjasa *et al.*, 2011).
[e]The actual source is most likely ingested green algae of the *Bryopsis* genus (Hamann *et al.*, 1996).
[f]The actual source is most likely a cyanobacterium of the *Oscillatoria* genus (see cited reference).

Table 19.3 Marine polypeptides with cytotoxic activities.

Name	Source			Reference
	Species	**Phylum**	**Family**	
C-phycocyanin	*Spirulina platensis*	Cyanobacteria	Pseudanabaenaceae	Pardhasaradhi *et al.* (2003)
MML	*Meretrix meretrix*	Mollusca	Veneridae	Ning *et al.* (2009)
Pardaxin	*Pardachirus marmoratus*	Chordata	Soleidae	Huang *et al.* (2011)
PG155	*Prionace glauca*	Chordata	Carcharhinidae	Zheng *et al.* (2007)
Shrimp Anti-lipopolysaccharide factor	*Penaeus monodon*	Arthropoda	Penaeidae	Lin *et al.* (2010)

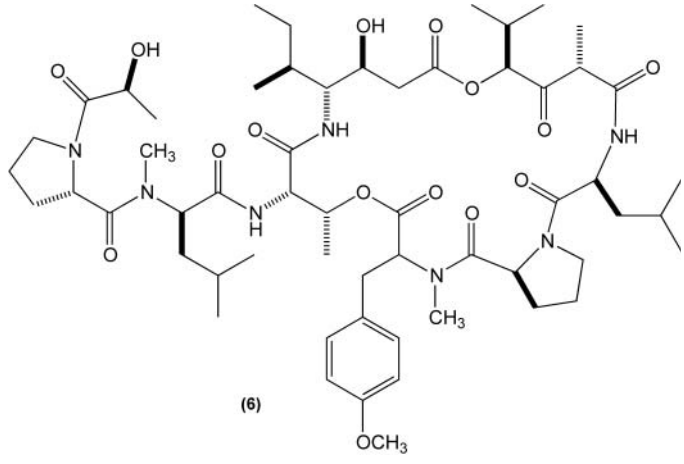

Fig. 19.6 Didemnin B.

than 20 amino acid residues, which can attain secondary and tertiary structures typical of proteins (Table 19.3).

Many of the (depsi)peptides contain amino acids with post-translational modifications such as carbamoylation or *N*- and *O*-methylation or are amino acids not found in proteins, in which the amino and/or carboxylic acid groups are not bonded to the α-carbon. Examples are the 2-(3-amino-2-hydroxy-5-oxypyrrolidin-2-yl)propionic acid found in the lactone-containing cyclic peptide koshikamide B (Fig. 19.8) (Winder *et al*., 2011) and the 4-amino-3-hydroxy-5-methylhexanoic acid, an unusual γ-amino-β-hydroxy acid present in the acyclic depsipeptide lyngbyabellin D (Fig. 19.9) (Williams *et al*., 2003a). These modifications may increase the chemical and biological half-life of the peptide by preventing its degradation and improving its bioavailability in the target tissues (Vagner *et al*., 2008). Another interesting feature found in bioactive peptides is the presence of heterocyclic structures (e.g. thiazoles and triazoles) on their backbones, which may improve their structural stability and rigidity without loss of cytotoxicity (Davies *et al*., 2012).

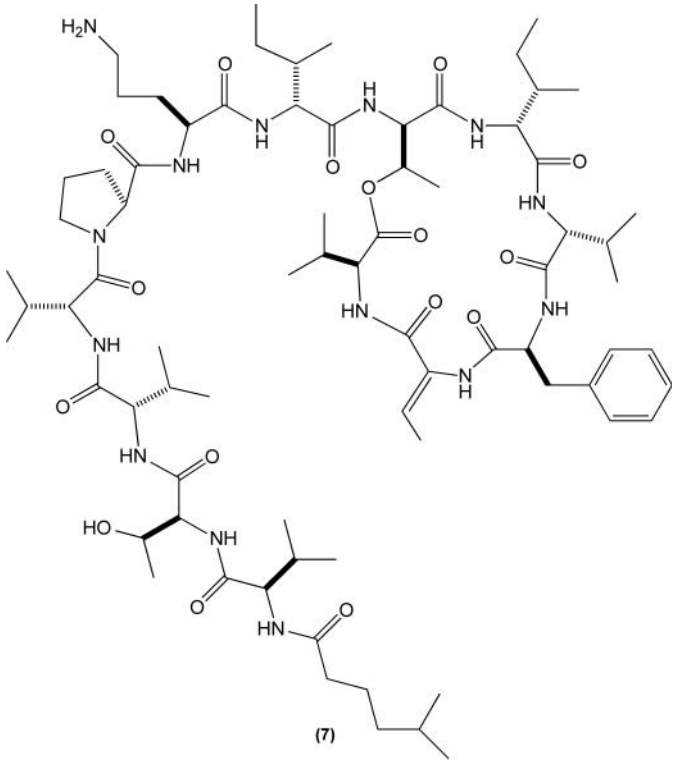

Fig. 19.7 Kahalalide F.

Interestingly, on some occasions, linearization of naturally occurring cytotoxic cyclic peptides does not affect their bioactivity, either (Simmons *et al*., 2009).

19.6 CYTOTOXIC ACYCLIC PEPTIDES

Dolastatin 10 (Fig. 19.2) and dolastatin 15 (Fig. 19.10) are acyclic pentapeptides that display the ability to disrupt the polymerization of microtubules, preventing cells from entering mitosis and inducing apoptosis (Ali *et al*., 1998; Bai *et al*., 1990). However, early clinical trials revealed that dolastatin 10 caused bone-marrow toxicity and mild peripheral sensory neuropathy (Pitot *et al*., 1999). Moreover, dolastatins are prone to the development of drug resistance mediated by the P-glycoprotein (P-gp) efflux system (Haustedt *et al*., 2006), a possible reason for the lack of a significant effect in cancer patients in phase-II clinical trials (reviewed by Molinski *et al*., 2009). This led to the development of analogs of dolastatin 10 (e.g. soblidotin/TZT-1027/auristatin PE) and dolastatin 15 (e.g. tasidotin/synthadotin/ILX651). Like dolastatin 10, soblidotin binds to the vinca domain of the β-tubulin, interfering with the GTP-exchange mechanism necessary for microtubule assembly (Cormier *et al*., 2008). The mechanisms of microtubule-dynamics inhibition by tasidotin are not as clear, but a recent study suggests that the active form is a metabolic product of the peptide and that the intracellular levels of tasidotin decrease rapidly in

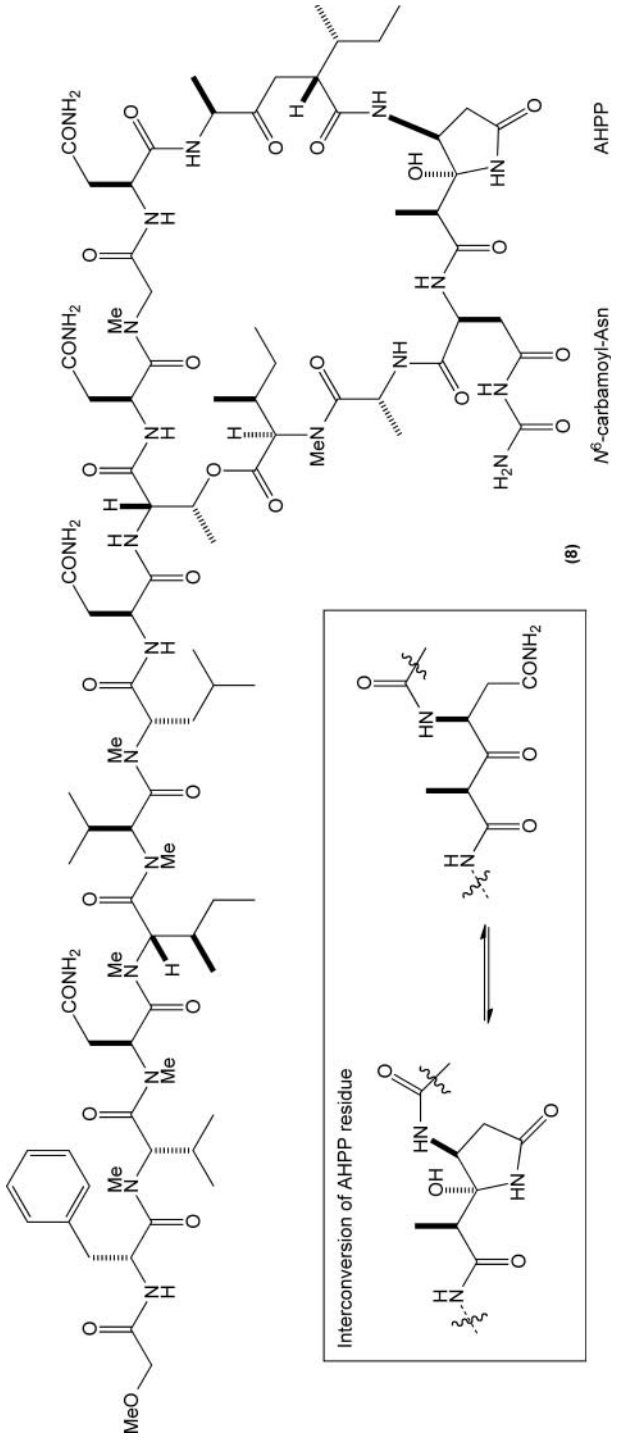

Fig. 19.8 Koshikamide B.

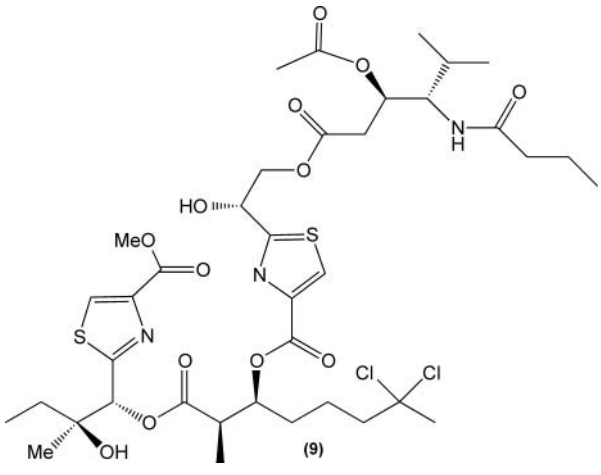

Fig. 19.9 Lyngbyabellin D.

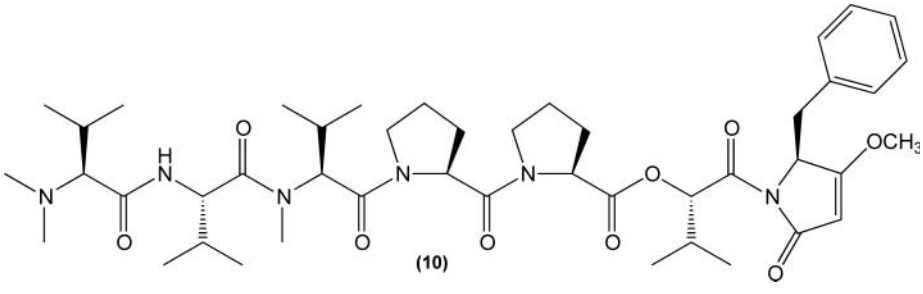

Fig. 19.10 Dolastatin 10.

less than 24 hours (Bai *et al*., 2009). On the other hand, auristatins have been conjugated with monoclonal antibodies (mAb) to improve delivery and tolerability. The mechanism of action seems to be related to the ability of the conjugated mAb to be internalized by facilitated uptake and the ability of endogenous peptidases to cleave the linker and deliver the free drug to the cellular target (Doronina *et al*., 2008).

Hemiasterlins (Fig. 19.5) are a class of acyclic tripeptide that were originally isolated from the sponges *Hemiasterella minor* (Talpir *et al*., 1994) and *Cymbastela* sp. (Coleman *et al*., 1995), although they were also later found in other species, namely *Auletta* sp. and *Siphonochalina* sp. (Gamble *et al*., 1999). The backbone of hemiasterlins is composed of highly modified amino acids, namely methylated tryptophan, *tert*-leucine and *N*-methyl homo vinylogous valine, a structure analogous to other cytotoxic tripeptides—milnamides—found in the same sponges (Chevallier *et al*., 2003; Crews *et al*., 1994). Hemiasterlins exert their antimitotic action by preventing tubulin polymerization and arresting cells at the G_2–M transition of the cell cycle (Anderson *et al*., 1997). As hemiasterlins are associated with *in vivo* toxicity (Coleman *et al*., 1995), researchers looked for more promising analogs. This search culminated in the discovery of the *N*-isopropyl-D-pipecolic acid derivative E7974, a poor substrate

for the P-gp efflux pump, which decreased the chance of drug-resistance development. Interestingly, unlike dolastatin 10, E7974 binds to α-tubulin instead of β-tubulin (Kuznetsov *et al*., 2009), overcoming a second mechanism of drug resistance present in cancer cells carrying mutant forms of the latter protein. Moreover, the same authors showed that E7974 is able to induce apoptosis. To this day, E7974 remains a promising candidate for the treatment of several forms of solid tumor, as shown by recent clinical trials (Rocha-Lima *et al*., 2012).

In spite of the fact that the biosynthetic pathway of the hemiasterlins has not been found as yet, their structural similarity with dolastatins (Haustedt *et al*. 2006) suggests that their actual biogenic source might be bacterial symbionts of sponges. In fact, a panoply of cytotoxic acyclic peptides has been isolated not only from marine cyanobacteria (reviewed by Liu & Rein, 2010) but also from marine actinobacteria; for example, lucentamycin A (Cho *et al*., 2007) and the proximicins (Fiedler *et al*., 2008). The latter seem to act by increasing levels of p53 and cyclin kinase inhibitor p21, two key regulatory proteins of the cell cycle that possess tumor-suppressor activity (Schneider *et al*., 2008).

19.7 CYTOTOXIC CYCLIC PEPTIDES

The diversity of cytotoxic cyclic peptides (Table 19.2) is far greater that of their acyclic counterparts (Table 19.1). This enormous structural variation is due to the different ways peptides can be synthesized, by either ribosomal (RBP) or nonribosomal (NRBP) biosynthetic pathways, and then modified. Recently it has been suggested that the cyclic peptides be named based on the proteolytic cleavage of precursor peptides synthesized via RBP as cyanobactins (Donia *et al*., 2008). Cyanobactins include marine cytotoxic peptides such as axinastatins, bistramides, dolastatin I, lissoclinamide 1, mollamides, pattelamides, phakellistatins and ulithiacyclamide (reviewed by Sivonen *et al*., 2010). However, the cytotoxic cyclic peptides currently going through clinical trials (plitidepsin and elisidepsin) are both depsipeptides that are most probably synthesized via NRBP.

The cyclic depsipeptide didemnin B (Fig. 19.6) was the first marine-derived NP to undergo clinical trials targeted at oncological patients. However, the toxicity of didemnin B rendered it unusable for further drug development. As a result, researchers focused on plitidepsin (Fig. 19.4), as it was already known that small changes in the structure of peptides of the didemnin class could lead to different cytotoxic and clinical outcomes (reviewed by Simmons *et al*., 2005).

Plitidepsin is a cyclic depsipeptide composed of a six-unit macrocycle (leucine, proline, threonine, *N*-methyl-*O*-methyl-tyrosine, 3-hydroxy-4-amino-5-methyl-heptanoic acid and 3-oxo-4-hydroxy-2,5-dimethylhexanoic acid) and a three-unit tail (*N*-methyl-leucine, threonine and piruvil-proline). Plitidepsin was originally isolated from the tunicate *Aplidium albicans* (Rinehart *et al*., 1987). It has been suggested, however, that the true biological source of plitidepsin is a cyanobacterium, as the closely related cytotoxic peptide didemnin B is synthesized by the cyanobacterial symbiont *Synechocystis trididemni* (Dunlap *et al*., 2007). Lower doses of plitidepsin displayed strong *in vivo* antitumoral activities in mice bearing B16 melanoma, P388 leukemia, Ehrlich carcinoma and Lewis lung carcinoma when compared to other didemnin-related compounds (Sakai *et al*., 1996; Urdiales *et al*., 1996). *In vitro* plitidepsin induces cell-cycle arrest at G_1 and G_2/M, and at higher concentrations stimulates apoptosis in melanoma cells, presumably via activation of the Rac1 GTPase, c-JNK and p38 mitogen-activated protein kinase (Muñoz-Alonso *et al*., 2008).

Other mechanisms of action in several cancer cell lines have been reviewed by Mayer & Gustafson (2008). In phase-II clinical trials, plitidepsin showed relatively low toxicity to bone marrow and displayed promising results in patients with relapsed and refractory multiple myeloma when used together with dexamethasone (Mateos *et al*., 2010).

As with the didemnin class of peptides, the kahalalide peptides possess one macrocycle and tails of varying lengths. Kahalalide F (KF) (Fig. 19.7) is a tridecadepsipeptide consisting of a six-unit macrocycle (phenylalanine, threonine, *allo*-isoleucine, two valines and one unusual amino acid, dehydroaminobutyric acid) and a seven-unit tail (L-ornithine, proline, threonine and several valines, one of which bonded to a short fatty acid, 5-methylhexanoic acid) (Hamann and Scheuer, 1993). It was initially found that KF was able to decrease *erbB2* steady-state transcript levels in a screening involving 49 000 compounds and the National Cancer Institute (NCI) 60-cell line panel (Wosikowski *et al*., 1997). However, a recent study suggests that the action mechanism of the peptide is actually related to the depletion of ErbB3 in KF-sensitive cell lines. Furthermore, resistant cells can be rendered more sensitive to this peptide when ErbB3 expression is enhanced ectopically (Janmaat *et al*., 2005). ErbB2 (HER-2/neu) and ErbB3 (HER-3) belong to the ErbB tyrosine kinase receptor family, being able to form homo- and heterodimers upon ligand binding. ErbB overexpression has been linked to drug resistance and tumor metastasis and has been associated with several forms of cancer (e.g. breast, ovarian and colon cancer). This might be a consequence of the key role that this family of receptors plays in the regulation of proliferation, differentiation, adhesion, invasiveness, inhibition of apoptosis and malignant transformation (Chow *et al*., 2011). Notwithstanding the fact the action mechanism of KF is not fully understood, it is currently known that this peptide is able to inhibit a regulatory pathway downstream of ErbB2/ErbB3, namely the phosphatidylinositol 3-kinase/Akt-signaling pathway (Janmaat *et al*., 2005). Moreover, this depsipeptide is able to induce necrosis-like cell death (oncosis) rather than apoptosis. The oncolytic process might be related to the pleiotropic changes observed in the shape and permeability of plasma and lysosomal membranes in KF-treated cells, which can display ruptured plasma membranes and enlarged lysosomes and vacuoles (reviewed by Gao & Hamann, 2011).

KF linear solid-phase synthesis (López-Macià *et al*., 2001) led to the development of an array of synthetic derivatives by Hamann's and Albericio's groups, which showed the importance of a structured macrocycle and the L/D configuration of the α-carbon of different amino acids to cytotoxic activity (Gao & Hamann, 2011). More recently, elisidepsin (Fig. 19.3), a synthetic peptide structurally very similar to KF, has gained particular interest for its favorable therapeutic index (Ling *et al*., 2009). As suggested of its natural counterpart, elisidepsin seems to act by altering cellular membranes. Herrero *et al*. (2008) have reported that overexpression of a sphingolipid fatty acyl 2-hydroxylase in *Saccharomyces cerevisiae* and human cell lines rendered the cells more sensitive to the peptide, suggesting that 2-hydroxy fatty acid-containing ceramides are important to the action of elisidepsin.

19.8 CYTOTOXIC (POLY)PEPTIDES OBTAINED BY ENZYMATIC HYDROLYSIS OF SEAFOOD

There is a recent tendency to look for (poly)peptides with cytotoxicity against cancer cell lines by isolating peptides generated by enzymatic hydrolysis of food products (reviewed by Udenigwe & Aluko, 2012). For example, Alemán *et al*. (2011) and Hsu *et al*. (2011)

have shown that (poly)peptides derived from the hydrolysis of squid gelatin and tuna dark muscle can inhibit the proliferation of cancer cell lines *in vitro*. An *in vivo* study using hydrolysates of the oyster *Crassostrea gigas* indicated that such an approach can yield peptides with antitumoral properties in BALB/c mice (Wang *et al*., 2010). However, further studies are clearly needed to understand how feasible this methodology is in generating drugs for oncological therapy in humans.

19.9 CYTOTOXIC POLYPEPTIDES

As shown by results obtained with food hydrolysates, polypeptides seem to possess anticancer properties (Table 19.3). This possibility had already been proposed in 1983, in a patent application by Dainippon Ink & Chemical Co., which suggested that phycobilins had antitumoral activity *in vivo*. Two decades later, it was shown that C-phycocyanin, an abundant water-soluble bluish heterodimeric phycobilin used by cyanobacteria to capture light for photosynthesis, inhibits cancer-cell proliferation *in vitro* via apoptosis, down-regulation of Bcl-2 and generation of radical oxygen species (Pardhasaradhi *et al*., 2003; Subhashini *et al*., 2004).

Using a genomic approach, Somboonwiwat *et al*. (2005) have cloned the gene coding for the shrimp (*Penaeus monodon*) anti-lipopolysaccharide factor (SALF), a 98-amino-acid polypeptide with known antibacterial properties. This enabled another team to test whether a smaller synthetic cyclic peptide based on SALF could enhance the antitumoral activity of cisplatin in HeLa cells. Indeed, this shorter version of SALF not only increased the cisplatin effect on cell-proliferation inhibition but also induced apoptosis on its own and inhibited tumor growth *in vivo* (Lin *et al*., 2010). Similarly, apoptosis induction in human fibrosarcoma cells has been ascribed to another antimicrobial polypeptide, pardaxin, which was originally isolated from the secretions of the Red Sea Moses sole (Huang *et al*., 2011).

19.10 CONCLUSION

The wealth of knowledge concerning (poly)peptides with cytotoxic and antitumoral activities that has been gathered in the last 3 decades is astounding. Approval of novel marine peptides for the treatment of oncological patients is therefore very likely to come in the next few years. So far the isolation of marine peptides has had a very limited range of biological sources, but there is a current trend toward widening this search to other taxa, such as fish and nonphotosynthetic bacteria. Moreover, molecular-biology tools such as metagenomics and single-cell genome amplification may soon provide a better understanding of how bioactive peptides are synthesized in nature. The cloning of complete biosynthetic pathways has revealed that bacteria have developed a way to generate an entire collection of peptides by means of cassettes that encode hypervariable amino acid sequences flanked by more conserved regions, which, in turn, recruit modifying enzymes. This knowledge has recently been used to produce a member of the pattelamide family of marine cytotoxic peptides (trunkamide) in *Escherichia coli* via genetic engineering (Donia *et al*., 2008). The combination of DNA recombinant technology and combinatorial chemistry based on marine peptides should result in novel and very promising anticancer drugs in the near future.

19.11 ACKNOWLEDGMENTS

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20 ACE-inhibitory Activities of Marine Proteins and Peptides

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20.1 INTRODUCTION

ACE, or kininase II, is a dipeptidyl carboxy peptidase (EC 3.4.15.1) found in various tissues in the body and is integral to the moderation of blood pressure and normal heart function (Shalaby *et al*., 2006). In the rennin-angiotensin system, ACE catalyzes the conversion of the inactive form of angiotensin I (Ang I) to the potent vasoconstrictor angiotensin II (Ang II). Additionally, ACE is involved in the deactivation of the hypotensive peptide, bradykinin (Ondetti *et al*., 1977). Ang II is a documented potent vasoconstrictor which acts directly on vascular smooth-muscle cells. It is also responsible for the expansion of vascular volume via sodium retention and fluid retention (Biron *et al*., 1961; Brown *et al*., 1998; Folkow *et al*., 1961; Padfield *et al*., 1977). Bradykinin is responsible for uterine and ileal smooth-muscle contraction, enhanced vascular permeability, activation of peripheral and C-fibers, and increases in mucous secretion (Brown *et al*., 1998; Proud *et al*., 1988). Furthermore, and more notably, bradykinin contributes to vasodilation by advancing the assembly of arachidonic acid metabolites, nitric oxide, and endothelium-derived hyperpolarizing factor in the vascular endothelium (Brown *et al*., 1998; Vanhoutte *et al*., 1989). Therefore, ACE inhibitors function by maintaining the balance between the associated vasoconstrictive and salt-retentive attributes of Ang II and the vasodilatory effect of bradykinin. This balance is maintained by decreasing the production of Ang II and reducing the degradation of bradykinin (Brown *et al*., 1998). While synthetic ACE inhibitors, such as Captopril, function by directly blocking the action of ACE, ACE-inhibitory peptides function by reacting with ACE, thus leaving ACE unavailable to cleave Ang I and preventing the production of the vasoconstrictor Ang II (Ahhmed *et al*., 2010). In the past decade, it has been reported that food proteins are a viable source of ACE-inhibitory peptides *in vitro* and that they may potentially be incorporated into nutraceutical products in order to exert antihypertensive effects *in vivo* (Jang *et al*., 2005, 2008; Qian *et al*., 2007).

Specific inhibitors of ACE have been shown to be useful as antihypertensive drugs. Many synthetic ACE inhibitors, including Captopril, Enalapril, Lisinopril, and others, are available for clinical use (Brown *et al*., 1998; Raia *et al*., 1990). ACE inhibitors are well tolerated by most patients, but some undesirable side effects may occur, such as cough, lost of taste, renal impairment, and angioneurotic edema (Antonios *et al*., 1995). The peptides derived from food proteins are considered to be milder and safer compared with synthetic

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drugs; furthermore, these peptides usually have multifunctional properties and are easily absorbed. Therefore, there is interest in research into inhibitors derived from food protein.

Since the discovery of ACE inhibitors in snake venom (Ferreira *et al*., 1970), many studies have been directed toward enzymatic hydrolysates of different food proteins. These food protein sources include casein (Gobbetti *et al*., 2000; Silva *et al*., 2005), rapeseed (Marczak *et al*., 2003), mushroom (Lee *et al*., 2004), whey protein (Pihlanto *et al*., 2000; Vermeirssen *et al*., 2004), porcine and chicken muscle (Arihara *et al*., 2001; Fujita *et al*., 2000), soybean (Kuba *et al*., 2005; Wu *et al*., 2002), and sake and sake lees (Saito *et al*., 1994). In addition, some ACE inhibitors have also been reported in marines animals such as cod (Kim *et al*., 2000), bonito (Matsumura *et al*., 1993), tuna (Kohama *et al*., 1988), sardine (Matsui *et al*., 1993), and sea cucumber (Zhao *et al*., 2009).

20.2 DETERMINATION OF ACE-INHIBITORY PEPTIDE ACTIVITY

Methods of determination of ACE-inhibitory peptide activity can be divided into *in vitro* and *in vivo* approaches. The ACE-inhibitory activity of peptides is usually tested by *in vitro* assays. However, it is necessary to perform *in vivo* assays to ensure that peptides identified in *in vitro* systems are bioavailable following ingestion and that they can reach a target site and administer a response in a living system.

20.2.1 *In Vitro* ACE-Inhibition Assay

In order to facilitate the identification and isolation of ACE-inhibitory peptides, establishment of a simple, sensitive, and reliable *in vitro* inhibition assay is desirable. Numerous methods for the measurement of ACE activity have been reported, including spectrophotometric, fluorometric, radiochemical, high-performance liquid chromatography (HPLC), and capillary electrophoresis approaches. Various substrates are suitable for measuring ACE activity. Among them, synthetic peptides hippuryl-histidyl-leucine (HHL) and furanacryloyl-phenylalanylglycyl-glycine (FAPGG) are the most commonly used, but other peptides can be employed, such as fluorescent molecules for specific detection and quantification (Carmel *et al*., 1978; Sentandreu *et al*., 2006).

In most of the previously cited works, the assay used for ACE activity was based on the method developed by Cushman & Cheung (1971). The amount of hippuric acid (HA) formed from HHL by the action of ACE is extracted with ethyl acetate and the concentration is determined by a spectrophotometric assay. Modifications of this method have been reported, in which the ethyl acetate extraction was replaced by a specific binding of His-Leu with 2,4,6-trinitrobenzene sulfonate (TNBS) (Matsui *et al*., 1992) or a specific reaction of HA with benzene sulfonyl chloride (Li *et al*., 2005). Although this assay has been very useful for decades, it has some limitations, such as the required extraction of the product from the reaction mixture with an organic solvent, which is an additional source of error. Therefore, Zhao *et al*. (2009) established a reverse-phase HPLC (RP-HPLC) analysis method using acetiontrile–water isocratic elution. This method has proved to be convenient, accurate, and suitable for the analysis of food-derived ACE-inhibitory peptide activity *in vitro*.

Another method was described by Holmquist *et al*. (1979), using FAPGG as substrate. This method, involving measurement of the absorbance decrease due to substrate hydrolysis by the action of ACE, was adopted for testing of the ACE-inhibitory activity of

peptides (Vermeirssen *et al*., 2002). Different modifications were also reported with fixed time conditions (Murray *et al*., 2004), using either 96-wells microtiter plate (Otte *et al*., 2007) or HPLC determination (Anzenbacherova *et al*., 2001). Veronique *et al*. (2010) established a sensitive, extraction-free HPLC method using N-(3-[2-furylacryloyl)-Phe-Gly-Gly (FAPGG) as substrate. This method relies on the ultraviolet (UV)-titration of the peptide 2-furylacryloyl-Phe (FAP), resulting from the hydrolysis of the FAPGG after a chromatographic separation on a reverse-phase column. The experimental conditions (enzyme/substrate ratio, incubation time, NaCl concentration) are optimized for linearity, sensitivity, and precision. The assay is adequate for the study of ACE inhibition by Captopril and peptides.

20.2.2 Antihypertensive-Activity Assay *In Vivo*

For the practical purpose of using food materials as physiological modulators, it is necessary to confirm the antihypertensive effect of orally administrated ACE-inhibitory peptides on renal hypertensive rats (RHR) or spontaneously hypertensive rats (SHR).

RHR are created by two kidney and one clip renal hypertension operation. Tribromoethanol-anesthetized rats (SPF) have a 0.4 mm silver wire clip inserted in their left renal artery. Tribromoethanol has minimal effects on cardiovascular function in rats (Huang *et al*., 1998). The clip is placed on the artery after blunt dissection is used to remove the overlying tissue. The abdominal musculature and skin incision are closed in layers by standard techniquesm with absorbable suture and autoclips. To avoid infection, penicillin (20–30 kunit) is injected into the belly of the rats. The rats are kept warm and monitored for any sign of morbidity post-surgery before transport to the animal room.

Zhao *et al*. (2007) reported that sea cucumber-gelatin hydrolysate was used as a drink administered to RHR for 1 month. Their systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly reduced. The ACE-inhibitory peptides from the sea cucumber-gelatin hydrolysate may have resistance to gastrointestinal enzymes and can be absorbed in their intact active form to lower blood pressure.

SHR are the most commonly used experimental animals in antihypertensive-activity assay. Zhao *et al*. (2009) investigated the antihypertensive effect of a purified sea cucumber peptide in SHR by measuring changes in SBP at 1–6 hours after oral administration (a dosage of 3 μM/kg per rat). The results showed that the purified ACE-inhibitory peptide produces a clear antihypertensive effect in SHR. When this is compared, the ACE-inhibitory peptide isolated from sea cucumber hydrolysate had only slightly lower antihypertensive effect *in vivo* than Captopril, and its activity lasted longer. This indicates that the ACE inhibitor possesses higher *in vivo* activity than the efficacy levels extrapolated from *in vitro* activities. Similar effects can be found in the case of ACE-inhibitory peptides derived from other protein digests (Fujita *et al*., 1999). This phenomenon may be attributed to a higher affinity of these peptides for tissue and a slower elimination than is found in a synthetic compound such as Captopril.

20.3 ACE-INHIBITORY PEPTIDES FROM MARINE SOURCES

ACE-inhibitory peptides have been found in various marine species, including fish (Joseph *et al*., 2011), sea cucumber (Zhao *et al*., 2009), seaweeds (Suetsuna *et al*., 2000, 2001), and others. There have been many reports of crude marine-protein hydrolysates containing

ACE-inhibitory peptides. Following the hydrolysis of a marine protein with a commercial enzyme, ACE-inhibitory peptides are released (Theodore *et al*., 2007). Most inhibitory peptides are reported to be short-chained and polar, and contain few hydrophobic amino acids in their sequence (Samaranayaka *et al*., 2010).

20.3.1 ACE-Inhibitory Peptides from Fish Sources

ACE-inhibitory peptides from fish sources were first identified in sardine meat over 20 years ago (Suetsuna *et al*., 1989). Since then, ACE-inhibitory peptides have been found in various fish species, including shellfish, tuna, bonito, salmon, and sardine (Fujita *et al*., 1999; Hai *et al*., 2006; Matsufuji *et al*., 1994; Ono *et al*., 2003; Qian *et al*., 2007; Yokoyama *et al*., 1992).

A crude enzyme extract from the viscera of sardine was used to hydrolyze the protein contained within the head and viscera of the fish species known as sardinelle. The resulting hydrolysate contained a high concentration of peptides that displayed low hydrophobicity, with molecular masses between 200 and 600 Da (Bougatef *et al*., 2008). Pacific hake fish protein subjected to simulated gastrointestinal digestion was also reported to contain ACE-inhibitory activity.

The frame protein of Alaska Pollock, which is normally discarded as an industrial byproduct, was mined for ACE-inhibitory peptides (Je *et al*., 2004). The frame protein was first hydrolyzed with pepsin and then separated into five fractions according to molecular weight. The most active ACE-inhibitory peptide was found in the fraction <1 kDa. From this fraction, a novel peptide was isolated, with the amino acid sequence FGASTRGA and an IC_{50} value of 14.7 μM. The normally discarded yellowfin sole-frame protein was identified as another source of ACE-inhibitory peptides (Jung *et al*., 2006). Using α-chymotrypsin, a peptide with a molecular mass of 1.3 kDa was isolated, with the corresponding amino acid sequence MIFPGAGGPEL.

The pepsin hydrolysate of bigeye tuna dark muscle was reported to contain the ACE-inhibitory peptide WPEAAELMMEVDP, with a molecular mass of 1581 Da and an IC_{50} value of 21.6 μM (Qian *et al*., 2007).

Following the hydrolysis of shark meat with the protease SM98011, four ACE-inhibitory peptides were identified (Wu *et al*., 2008). Three were reported as being novel; their amino acid sequences were EY, FE and CF, and they had IC_{50} values of 1.98, 2.68 and 1.45 μM, respectively.

Fujita *et al*. (1999) developed a thermolysin hydrolysate from 'Katsuo-bushi', a traditional Japanese food processed from dried bonito. This hydrolysate was administered to 30 hypertensive and borderline-hypertensive human subjects in a small-scale clinical trial. It contained the previously reported ACE-inhibitory peptide LKPNM (Yokoyama *et al*., 1992). Following 8 weeks of administration, the SBP of the subjects was reduced by 12.55 ± 1.5 mmHg. This clearly demonstrates that the thermolysin hydrolsate of dried bonito is sufficiently well absorbed in humans to effectively reduce the SBP of hypertensive and borderline-hypertensive subjects *in vivo*. The hydrolysate has been approved as Foods for Specified Health Use (FOSHU) by the Ministry of Health and Welfare in Japan (Fujita *et al*., 1999).

20.3.2 ACE-Inhibitory Peptides from Sea Cucumber

There are more than 900 kinds of sea cucumber in the world, of which only about 40 are edible. Some kinds are low-value, such as the *Molpadida*, which includes *Acaudina*

molpadioidea. *A. molpadioidea* is rich in proteins and is familiar in the coastal region of China, but isn't fully exploited and utilized. Therefore, conversion of *A. molpadioidea* to highly valued economic products is greatly desired.

Zhao *et al*. (2009) describe the extraction and characterization of a novel ACE-inhibitory peptide that can be used as an antihypertensive drug from *A. molpadioidea* protein. Body-wall protein from the sea cucumber (*A. molpadioidea*) was hydrolyzed sequentially with bromelain and alcalase. The hydrolysate was fractionated into two ranges of molecular weight (PH-I > 2 kDa; PH-II < 2 kDa) using an ultrafiltration-membrane bioreactor system. The PH-II brought about a high ACE-inhibitory activity. An ACE-inhibitory peptide was isolated from the PH-II using the chromatographic methods, including gel filtration, ion-exchange chromatography (IEC), and RP-HPLC. The purified ACE-inhibitory peptide was a novel peptide, sequenced as MEGAQEAQGD, and showed very low similarity with other ACE-inhibitory peptide sequences. It was found that the inhibitory activity of the peptide was intensified 3.5 times, from IC_{50} 15.9 μM to IC_{50} 4.5 μM, after incubation with gastrointestinal proteases. The ACE-inhibitory peptide from *A. molpadioidea* showed a clear antihypertensive effect in SHR at a dosage of 3 μM/kg. A summary of ACE-inhibitory peptides derived from marine sources is given in Table 20.1.

20.4 TYPES OF ACE-INHIBITOR PEPTIDE

An important factor when discussing ACE-inhibitory peptides isolated from food proteins is the discrepancy between the ACE-inhibitory activity of peptides *in vitro* and their antihypertensive effect *in vivo* (Arihara *et al*., 2006; Vercruysse *et al*., 2005). ACE-inhibitory peptides can be classified into three groups (Fujita *et al*., 2000): the first comprises true inhibitors, whose activity is not changed by pre-incubation with ACE; the second comprises substrates for ACE, which convert them to inactive peptides; the third comprises the prodrug peptides, which are converted into true inhibitors by ACE or gastrointestinal proteases, resulting in increased activity. *In vivo* studies have demonstrated that only true inhibitors and prodrugs have the ability to lower blood pressure (Fujita *et al*., 1999, 2000).

The stability of MEGAQEAQGD, from *Acaudina molpadioidea*, against gastrointestinal proteases *in vitro* was examined in order to predict its antihypertensive effect *in vivo* (Zhao *et al*., 2009). The ACE-inhibitory activity of this peptide showed a change after *in vitro* incubation with gastrointestinal proteases. It was found that the inhibitory activity of the peptide was intensified by 3 times, from IC_{50} 15.9 μM to IC_{50} 5.3 μM, after pepsin digestion. The inhibitory activity was further increased from IC_{50} 15.9 μM to IC_{50} 4.5 μM following chymotrypsin digestion. Trypsin digestion did not change the activity. So, the peptide from *Acaudina molpadioidea* can be considered prodrug-type ACE inhibitor because its inhibitory activity was intensified by a factor of 3.5 after pepsin and chymotrypsin digestion. Intensification of the ACE-inhibitory activity was found after gastrointestinal proteases treatments, which indicates that the peptide may have a potential antihypertensive effect.

20.5 STRUCTURE–ACTIVITY RELATIONSHIPS OF ACE-INHIBITORY PEPTIDES

The mode of action of the majority of ACE-inhibitory peptides is thought to be as a competitive substrate for ACE. The structure–activity relationships of ACE-inhibitory

Table 20.1 ACE-inhibitory peptides derived from marine sources (He *et al*. (2004; Joseph *et al*. (2011).

Source	Amino acid sequence	Enzyme	Reference
Bonito	IKPLNY, IVGRPRHQG, IWHHT, ALPHA, DYGLYP, FQP	Thermolysin	Yokoyama *et al.* (1992)
	LKP, IWHHT, IKP		Fujita *et al.* (1999)
	IVGRPR		Iroyukifujita *et al.* (2000)
	LKPNM, IY		Yokoyama *et al.* (1992), Fujita *et al.* (1999)
Salmon	WA, VW, WM, MW, IW, LW	Thermolysin	Ono *et al.* (2003)
Sardine	MF, RY, MY, LY, YL, IY, VF, GRP, RFP, AKK, RVY	Alcalase	Matsufuji *et al.* (1994)
Tuna	PKYKDTP	Pepsin	Lee *et al.* (2010)
Alaska pollock	FGASTRGA	Pepsin	Je *et al.* (2004)
Yellowfin sole	MIFPGAGGPEL	α-chymotrypsin	Jung *et al.* (2006)
Shark	EY	Protease SM98011	Wu *et al.* (2008)
	FE	Protease SM98012	
	CF	Protease SM98013	
Sea cucumber	MEGAQEAQGD	Bromelain + alcalase	Zhao *et al.* (2009)
Wakame	AIYK, YKYY, KFYG,YNKL	Pepsin	Suetsuna *et al.* (2000)
Chlorella vulgaris, *Spirulina platensis*	IVVE, AFL, FAL, AEL, IAPG, VAF	Trypsin	Suetsuna *et al.* (2001)

peptides have not yet been confirmed, due to the variety of amino acid sequences identified to date (Arihara *et al*., 2006; Li *et al*., 2004).

Zeng *et al*. (2005) have reported that the average molecular weight of ACE-inhibitory peptides from aquatic products is below 1500 Da. Jung *et al*. (2006) revealed that yellowfin-frame protein hydrolysate is fractionated into two fractions, of high and low molecular weight, by ultrafiltration. The low-molecular-weight fraction has a more potent ACE-inhibitory activity than its high-molecular-weight counterpart. Matsui *et al*. (1993) reported that, with respect to alkaline protease hydrolysate derived from sardine muscle, ACE-inhibitory activity markedly increased with increasing proteolysis.

As reported by Cushman *et al*. (1981), the active sites of two domains of ACE are structurally and functionally homologous with a dipeptidyl carboxypeptidase, and the zinc coordination geometry is critical for their hydrolytic action. However, the two catalytic sites are differentially activated by chloride ions and the physiological substrate Ang-I is

preferentially bound to the C-domain catalytic site. The substrate also makes a contribution to the chloride-mediated activation of the active site. Therefore, these differences indicate that despite the higher level of primary sequence homology, structural and functional differences exist between two active sites of C and N domains. Three subsites, S_1 (antepenultimate), S'_1 (penultimate), and S'_2 (ultimate), with unshared distinct characteristics for the binding of carboxyl-terminal amino acids of the substrate or inhibitor, are located on two homologous active sites. For the inhibitor–enzyme binding and interaction, three main subsites on the active site of the enzyme, with different amino acid sequences, should be bound with the substrate. Binding of the inhibitor or the natural substrate to the enzyme takes place predominantly via the carboxyl-terminal tripeptide residues. C-terminal amino acid residues of the inhibitor, such as Pro and Phe, are reported to be favorable to ACE inhibition. Ala and Val are more favorable to the antipenultimate position (S_1), while Pro and Leu are most favorable to the the ultimate position (S'_2) with regard to the affinity exerted on the ACE (Cushman *et al*., 1981; Jung *et al*., 2006). Hydrophilic peptides possess weak or no ACE-inhibitory activity, since hydrophilic peptides are incompatible with the active sites of ACE. Indeed, inhibition of ACE is achieved by hydrophobic peptides that display high affinity to the active subsites of ACE (Li *et al*., 2004; Maruyama *et al*., 1987; Matsui *et al*., 2006).

20.6 CONCLUSION

Although ACE-inhibitory peptides from marine sources are weaker in ACE inhibition than synthetic drugs, the peptides derived from food sources are eaten daily. Considering the low molecular mass of the peptides, coupled with their low allergenicity, the potential of ACE-inhibitory peptides for use in functional foods or antihypertensive drugs aimed at mitigating hypertension would seem to merit further clinical studies in humans. However, the antihypertensive activity will depend largely on the processing and preparation conditions, and finally also on the peptide structure itself. More research is needed to clarify these issues.

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21 Isolation and Biological Activities of Peptides from Marine Microalgae by Fermentation

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21.1 INTRODUCTION

Bioactive material metabolites are synthesized by organisms for self-defense and other purposes; it can be used as a source of both energy and biofunctional nutrition to promote health and reduce the risk of chronic disease (Biesalski *et al*., 2009; Denny & Buttriss, 2007). Much evidence has been provided concerning the bioactive substances produced by natural marine materials, with a variety of species and characteristics (Barrow & Shahidi, 2008; Kim & Wijesekara, 2010). The wide diversity of marine organisms is thus recognized as a rich source of bioactive materials, including polysaccharides, phenolic compounds and bioactive peptides. Marine-derived protein in particular has a positive impact on body function and condition, and may ultimately influence human health, if provided by a safe, reliable and consistent oral delivery system. However, natural marine protein faces application difficulties due to absorption and the supply of raw material. Therefore, development of a biotechnology procedure which can enhance hydrolysis and bioactivity is essential.

Bioprocesses such as roasting, water/solvent extraction, enzyme and microwave-assisted hydrolysis have been routinely used for extraction and hydrolysis from natural materials. However, the use of these procedures has several defects, including high energy input, high economic cost and toxicological effects. A method must be found to enhance the hydrolysis, bioactivity, safety and economical efficiency of these processes. Fermentation is known to have a good safety and economic profile and to enhance the physiochemical properties of protein, essential amino acids, essential fatty acids, vitamins, polyamines, carbohydrates and numerous antioxidants and phytosterols. Kim *et al*. (2004) have reported that the amount of liquiritigenin in fermented licorice increased up to 400 times over that of control. The hydrolytic action of fermentation may improve biofunctional or bioactive protein and peptide with hormone- or drug-like activity, which eventually modulates physiological function through binding interactions to specific receptors on target cells, leading to induction of physiological responses (Fitzgerald *et al*., 2006). Therefore, much attention has been paid to fermented proteins and peptides (Chin, 2007; Ghorai *et al*., 2009; Wilkins *et al*., 2007). This chapter

Marine Proteins and Peptides: Biological Activities and Applications, First Edition. Edited by Se-Kwon Kim.

provides an overview of marine bioactive protein and peptide fermentation by microbial hydrolysis as a functional ingredient source.

21.2 UTILIZATION OF FERMENTATION TO HYDROLYZE PROTEIN

Marine-derived micro-organisms can access molecules via fermentation, breaking them down to usable small units and binding them with specific receptors, leading to induction of physiological responses. Generally, fermentation can improve the stability, digestibility and bioavailability of nutrients through microbial hydrolysis. Fermented materials are known to have acidic pH, which is microbiologically safe and allows them to be stored for longer periods (Boralkar & Reddy, 1985; Knuckles *et al*., 1985). The significant increment in the soluble fraction and the reduction in the level of antinutrients to a safe level may also change the levels of nutrients.

Many kinds of substance, such as carbohydrates, proteins and lipids, can undergo fermentation, for a variety of applications. Bioactive proteins and peptides in particular have attracted attention for their structural, compositional and sequential properties. Peptides are inactive within the sequence of the parent protein and can be released by microbial hydrolysis (Boralkar & Reddy, 1985; Knuckles *et al*., 1985). Therefore, they can be used as potential modulators of various regulatory processes in the body. The actual effect of these peptides is determined by their amino acid composition and sequence level. The peptides are released by various micro-organisms, such as *Candida lipolytica*, *Candida rugopelliculosa* and *Rhizopus oligosporus*. They have high value as pharmaceutical and neutraceutical agents (Hesseltine & Wang, 1980; Paredes-López & Harry, 1988).

Numerous peptides and peptide fractions with bioactive properties beneficial to human health have been isolated from fermented dairy products. These properties include immunomodulatory, antihypertensive, anticancer, hypocholesteremic, antimicrobial (including bacteriocins), mineral-binding, opioid, peptidase-inhibitory and bone-formation activities (Agerbaek *et al*., 1995; Ennahar *et al*., 1999; Gobbetti *et al*., 2004; Hernández *et al*., 2005; Kawase *et al*., 2000; LeBlanc *et al*., 2002, 2004, 2005; Lorenzen & Meisel, 2005; Matar & Goulet, 1996; Meisel *et al*., 2006; Narva *et al*., 2004; Richelsen *et al*., 1996; Rokka *et al*., 1997; Smacchi & Gobbetti, 1998; Sütas *et al*., 1996; Takano, 1997, 1998).

21.3 MICROALGAE AS A SOURCE OF PROTEIN

Recently, reports have focused on the numerous beneficial applications of proteolytic fermentation. For example, optimal cultivation of microalgae such as *Botryococcus braunii*, *Dunaliella primolectal*, *Chlorella* sp. and *Nannochloris* sp. has been getting attention as one of the largest producers of biomass in the marine environment, and has been reported as an unconventional source of protein (Becker, 2004; Bhadury & Wright, 2004). It is thought that microalgae can be developed as useful functional materials. The amino acid patterns of the microalgal proteins show high nutritive value and good availability of essential amino acids.

A well-established fermentation and processing technique for proteolytic yeast make this organism suitable for several biotechnological purposes. It is important to select a

substance with beneficial biological activity for proteolytic fermentation. Microalgae are one of the largest producers of biomass, forming the base of the food chain in the marine environment (Bhadury & Wright, 2004). They are therefore indispensable in the commercial rearing of various species of marine animal as a food source for all growth stages of bivalve molluscs, for the larval stages of some crustacean species and for the very early growth stages of some fish species. The biodiversity of microalgae is enormous and they represent an almost untapped resource. The increase in the world's population and predictions of an insufficient protein supply have led to a search for new and effective alternative protein sources and the microalgal biomass appears to be a good candidate for this purpose (Becker, 2004; Cornet, 1998). Microalgae are able to enhance the nutritional content of conventional food preparations and hence to positively affect the health of humans. The high protein content of various microalgal species is one of the main reasons to consider them for this role (Cornet, 1998; Soletto *et al*., 2005). In addition, the amino acid pattern of almost all algae compares favorably with that of other food proteins. As the cells are capable of synthesizing all amino acids, they can provide thosee amino acids essential for humans (Guil-Guerrero *et al*., 2004). However, in order to provide a complete characterization and determine the amino acid content of microalgae, information on the nutritive value of the protein and the degree of availability of amino acids must be given (Becker, 1988). Many metabolic studies have confirmed the capacity of microalgae to act as a novel source of protein: the average quality of most algae examined is equal or even superior to that of conventional high-quality plant proteins (Becker, 2004).

21.4 METABOLITES OF PROTEOLYTIC HYDROLYSIS BY FERMENTATION

Fermentation, usually by the action of microorganisms, is a rapid and reproducible method for the production of bioactive peptide and amino acids though the breakdown and reassembly of protein (Aaslyng *et al*., 1998; Baek *et al*., 2010; Hartmann & Meisel, 2007). Amino acids and peptides released from these proteins are ultimately transferred into molecules obtained from the breakdown of those same proteins and changes in amino acid and peptide composition are prominent.

The fermentation process affects various metabolites, including amino acids, small peptides, nucleosides, urea-cycle intermediates and organic acids. Kang *et al*. (2011) have reported that metabolites (phenylalanine, glutamic acid, leucine, adenine, citrulline, arginine, glutamine, γ-aminobutyric acid, proline, acetylornithine, valine, pipecolic acid, methionine, citric acid, xanthine, tyrosine, isoleucine, Glu-Tyr, Ser-Pro, tryptophan, Glu-Phe and Leu-Val-Pro-Pro) were altered following periods of fermentation on a PLS-DA score plot. Amino acid composition changed following fermentation, suggesting that microorganisms might use it as a preferred nitrogen source, providing a unique taste and nutritional and functional quality (Kada *et al*., 2008; Kang *et al*., 2011). Metabolomic profiling of *doenjang* (Korean traditional fermented soybean) by NMR-PCA and GC-MS demonstrated that the amino acids predominantly produced, included alanine, valine, leucine, isoleucine, proline, glutamine, phenylalanine and lysine, showed remarkable increases in number during the latter stages of fermentation (Namgung *et al*., 2010; Yang *et al*., 2009). These metabolite changes produced by fermentation can be expected to alter the functional bioavailability.

Table 21.1 Amino acid composition of fermented *C. rugopelliculosa* on microalgal *P. lutheri*.

Amino acid	Pre-fermentation content (%)	Post-fermentation content (%)
Asp	9.86	11.61
Thr	4.26	2.91
Ser	4.06	5
Glu	12.96	11.22
Pro	4.84	6.8
Gly	5.8	8.28
Ala	8.51	5.82
Val	6.58	5.56
Ile	4.51	3.57
Leu	9.28	7.26
Tyr	1.68	2.58
Phe	5.54	6.64
His	3.55	3.96
Lys	5.93	2.91
Arg	3.29	6.15
Met	4.38	4.94
Cys	3.35	3.66
Trp	1.61	1.11

Ryu *et al*. (2012) evaluated the total amino acids and free amino acids produced by fermentation of the microalga *P. lutheri* using an amino acid analyzer (Table 21.1 and 21.2). Their results indicate that monitoring of the changes in metabolites during microalgal fermentation might be an important biomarker for the production of beneficial microalgae and the construction of the microalgal fermentation metabolic pathway.

21.5 HYDROLYZED MICROALGAL PEPTIDE APPLICATION

There are many reports that peptides or associated peptide sequences capable of mediating physiological effects may be generated through proteolysis. It worth noting that bioactive peptides may be released from proteins through a combination of hydrolysis and fermentation. Bioactive peptides produced by these process techniques have the potential to be used as nutraceuticals in the formulation of functional foods, and can hence reduce the population need for drugs and other medical therapies.

Application of hydrolysis microalgal peptide capable of regulating vital biological functions has led to interest in their therapeutic use, despite the microalgae having various beneficial effects on nutra- and pharmaceutical agents. Recently, some reports have demonstrated that hydrolysis microalgal peptides have such biological effects as antioxidant activity, liver protection and wound healing (Kang *et al*., 2011; Ryu *et al*., 2012).

There has been much research into hydrolysis microalgal peptides of benefit to human health, examining their biological activities and development techniques. Hydrolysis-derived peptides from microalgae have to be developed using advanced techniques. Statistical analysis capable of applying central composite design (CCD)-based response

Table 21.2 Free amino acid composition of fermented *C. rugopelliculosa* on microalgal *P. lutheri*.

Free amino acid	Pre-fermentation content (%)	Post-fermentation content (%)
Phosphoserine	0.023	0.061
Taurine	0.13	0.291
Phosphoethanolamine	-	-
Urea	-	-
L-aspartic acid	0.112	0.293
L-threonine	0.173	0.567
L-serine	0.084	0.266
Asparagine	-	-
L-glutamic acid	0.232	0.847
L-α-aminoadipic acid	-	-
L-proline	0.25	0.17
L-glycine	0.12	0.273
L-alanine	0.614	1.292
L-citrulline	0.204	0.017
L-α-aminobutyric acid	-	-
L-valine	0.365	1.07
L-cystine	-	-
L-methionine	0.04	0.114
L-isoleucine	0.261	0.742
L-leucine	0.493	1.072
L-tyrosine	0.129	0.455
L-phenylalanine	0.346	0.735
β-alanine	-	-
DL-β-aminoisobutyric acid	-	-
γ-aminobutyric acid	0.097	0.304
L-orinithine	0.069	0.214
L-lysine	0.245	0.456
1-methyl-L-histidine	-	-
L-histidine	0.011	0.03
3-methyl-L-histidine	0.002	0.024
L-carnosine	-	-
L-arginine	0.007	0.05

surface methodology (RSM) to an analysis of the effects of the process parameters must be optimized for the search for usable microalgal peptides. Detailed understanding of the beneficial effects of microalgal proteins and peptides based on molecular mechanisms must be developed.

21.6 CONCLUSION

There is a great deal of interest in the use of microalgal protein as a source of nutraceuticals and pharmaceuticals. Hydrolysis biotechnology, and especially fermentation, can increase their stability, digestibility and bioavailability. The possibility of designing new bioactive peptides by fermentation and microbial hydrolysis is promising. Further research is needed into the application of marine microalgae and their activities.

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22 Antioxidant Activities of Marine Peptides from Fish and Shrimp

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22.1 INTRODUCTION

Oxidation is a vital process in all living organisms. During normal body reactions, such as respiration, reactive oxygen species (ROS), such as superoxide anion radicals, hydroxyl radicals, and non-free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are formed (Bernardini *et al*., 2011). When free radicals are produced in excess or are not eradicated, they can attack the closest molecules by subtracting electrons, starting a chain reaction in which the new molecule with a missing electron attacks still other molecules. It is well documented that free-radical formation is implicated in many human diseases, such as hearth disease, stroke, arteriosclerosis, diabetes, and cancer. Lipid oxidation in foods is one of the major causes of food deterioration (Bernardini *et al*., 2011), leading to rancidity and shortening of shelf life. Oxidation of proteins in foods is influenced by lipid oxidation, where lipid-oxidation products react with proteins, causing their oxidation (Viljanen *et al*., 2004).

Antioxidants play a vital role in both food systems and the human body, reducing oxidative processes. In food systems, antioxidants are useful in retarding lipid peroxidation and secondary-lipid-peroxidation product formation. They further reduce protein oxidation and the interaction of lipid-derived carbonyls with proteins, which leads to an alteration of protein functionality (Elias *et al*., 2008). In the human body, endogenous antioxidants, including enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and various nonenzymatic compounds such as selenium, atocopherol, and vitamin C help to protect tissues and organs from oxidative damage caused by ROS and reactive nitrogen species (RNS) such as hydroxyl radicals, peroxyl radicals (•OOR), superoxide anion, and peroxynitrite (Wojcik *et al*., 2010). Apart from these, amino acids, peptides, and proteins also contribute to the overall antioxidative capacity of cells and towards maintaining the health of biological tissues. Peptides such as carnosine, anserine, and glutathione are well known for their endogenous antioxidative activity (Babizhayev *et al*., 1994). Use of dietary antioxidants has been recognized as potentially effective in promoting human health by increasing the body's antioxidant load (Samaranayaka & Li-Chan, 2011).

In recent years, a considerable amount of research has also focused on the liberation of antioxidant peptides encrypted within food proteins, with a view to utilizing such peptides as functional food ingredients aimed at health maintenance. Interestingly, within the parent protein sequence, the peptides are inactive and thus must be released to exert an effect.

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These bioactive peptides are usually 2–20 amino acid residues in length, although some have been reported to have more than 20 amino acid residues (Ryan *et al*., 2011).

Even though there have been few *in vivo* or *in situ* studies conducted to date, *in vitro* studies using various chemical assays have indicated the potential of these food-derived peptides to act as antioxidative agents and control various oxidative processes in the human body, as well as in food. The multifunctional nature of peptidic antioxidants, having the ability to impart other bioactivities such as antihypertensive, opioid, and cholesterol-lowering capacity (Samaranayaka & Li-Chan, 2011), makes them more attractive candidates than nonpeptidic antioxidants as dietary ingredients in promoting human health.

Shrimp and fish provide valuable sources of protein for many populations around the world; furthermore, shrimp and fish proteins offer huge potential as novel sources of bioactive peptides. To date, bioactive peptides displaying antihypertensive, antioxidant, antimicrobial, and antiproliferative effects have been found in the hydrolysates of shrimp and fish proteins (Bernardini *et al*., 2011; Huang *et al*., 2011; Ryan *et al*., 2011; Sarmadia & Ismaila, 2010).

The objectives of this paper are to review the reported antioxidative peptide production from marine fish and shrimp, to examine the possible mechanisms of antioxidative peptides in exerting their activity in food, to conduct a critical evaluation of the methods used in assessing their antioxidative potential, and finally, to present a perspective on food applications of marine shrimp- and fish-derived peptidic antioxidants.

22.2 PRODUCTION, ISOLATION, AND PURIFICATION OF ANTIOXIDANT PEPTIDES

Fish and shrimp have gained particular interest as potential antioxidative protein hydrolysates or peptide sources, mainly due to the abundance of raw materials in the form of processing discards and underutilized species, in conjunction with research findings indicating an array of other biological activities for fish- and shrimp-protein hydrolysates and specific peptide sequences derived from these sources, including antihypertensive, immunomodulatory, neuroactive, antimicrobial, and mineral- and hormone-regulating properties (Samaranayaka & Li-Chan, 2011).

While numerous methods have been utilized to release bioactive peptides from food proteins, enzymatic hydrolysis of whole protein is the most widely used technique. The physicochemical conditions of the reaction media, including time, temperature, pH, and enzyme/substrate ratio, must be optimized for the activity of the enzyme. The type of enzyme used in enzymatic protein hydrolysis affects the cleavage patterns of the peptide bonds (Shahidi & Zhong, 2008). Use of exogenous enzymes is preferred in most cases to the autolytic process (i.e. use of endogenous enzymes present in the food source itself), due to the shorter time required to obtain a similar degree of hydrolysis (DH), as well as better control of the hydrolysis, resulting in more consistent molecular-weight profiles and peptide compositions (Ryan *et al*., 2011). Industrial food-grade proteinases derived from microorganisms, such as Alcalase, Flavourzyme, and Protamex, and enzymes from plant (e.g. papain and bromelain) and animal (e.g. pepsin and trypsin) sources have been widely used in producing antioxidative peptides (Table 22.1). Nevertheless, the use of endogenous enzymes has also been reported in the literature, especially for the production of various antioxidative fish-protein hydrolysates (Table 22.1). Depending on the raw

Table 22.1 Antioxidative protein hydrolysates and amino acid sequences derived from fish and shrimp.

Source organism	Enzyme used	Amino acid sequence	Reference
Sardine muscle	Pepsin	LQPGQGQQ	Suetsuna & Ukeda (1999)
Mackerel fillet	Protease N	-[a]	Wu *et al.* (2003)
Capelin whole fish	Alcalase	-	Amarowicz & Shahidi (1997)
Conger eel muscle	Trypsin	LGLNGDDVN	Ranathunga *et al.* (2006)
Round scad muscle	Alcalase 2.4l, Flavourzyme 500l	-	Thiansilakul *et al.* (2007)
Yellow stripe trevally hydrolysate	Alcalase 2.4l, Flavourzyme 500l	-	Klompong *et al.* (2007)
Royal jelly protein	Protease N	AL, FK, FR, IR, KF, KL, KY, RY, YD, YY, LDR, KNYP	Guo *et al.* (2009)
Yellowfin sole-frame protein	Pepsin and mackerel-intestine crude enzyme	RPDFDLEPPY	Jun *et al.* (2004)
Herring (*Clupea harengus*) press juice	Simulated gastrointestinal digestion	-	Sannaveerappa *et al.* (2007)
Hoki (*Johnius belengeri*)-skin gelatin	Trypsin	HGPLGPL	Mendis *et al.* (2005)
Hoki (*Johnius belengeri*)-frame protein	Pepsin	ESTVPERTHPACPDFN	Kim *et al.* (2007)
Ornate threadfin bream muscle	Pepsin	-	Nalinanon *et al.* (2011)
Alaska pollock-skin gelatin	Alcalase, Pronase E	GEOGPOGPOGPOGPOG2, GPOGPOGPOGPOG2	Kim *et al.* (2001)

(*continued overleaf*)

Table 22.2 (*continued*)

Source organism	Enzyme used	Amino acid sequence	Reference
Alaska pollock frame	Mackerel-intestine crude enzyme	LPHSGY	Je *et al.* (2005)
Alaska pollock skin	Neutrase Flavourzyme Alcalase, protamex	-	Jia *et al.* (2010)
Tuna (*Katsuwonus pelamis*) liver	Two-step hydrolysis: Alcalase/Flavourzyme, Neutrase/Flavourzyme, Protamex/Flavourzyme	-	Ahn *et al.* (2010)
Tuna (*Thunnus obesus*) dark-muscle byproduct	Orientase, Protease XXIII	LPTSEAAK, YPMDYMVT	Hsu (2010)
Tuna-backbone protein	Pepsin	VKAGFAWTANQQLS	Je *et al.* (2007)
Pacific hake muscle	Validase BNP, Flavourzyme 500	L PLFQDKLAHAK, AEAQKQLR	Samaranayaka (2010)
Shrimp (*Acetes chinensis*)		-	Cao *et al.* (2009)
Prawn (*Penaeus japonicus*) muscle	Pepsin	IKK, FKK, FIKK	Suetsuna (2000)
Shrimp (*Penaeus monodon* and *Penaeus indicus*) waste	Alcalase 2.4l	-	Dey & Dora (2011)
Shrimp processing byproducts	Alcalase 2.4l		Huang *et al.* (2011)
Shrimp processing discards	Alcalase 2.4l	-	Guerard *et al.* (2007)

[a]Amino acid sequence not identified.

material, endogenous enzymes such as trypsin, chymotrypsin, pepsin, other enzymes of the viscera and digestive tract, and lysosomal proteases or catheptic enzymes in fish or other invertebrate muscle cells may contribute to the breakdown of proteins during autolysis (Kristinsson & Rasco, 2000). Peptides with antioxidative properties can be prepared from Alaska pollock (*Theragra chalcogramma*) and yellowfin sole (*Limanda aspera*) frame proteins by using a crude enzyme mixture taken from mackerel intestine (Je *et al*., 2005; Jun *et al*., 2004), while the increased level of cathepsin L-like proteases present in parasitized Pacific hake (Merluccius productus) muscle has been successfully used to produce fish-protein hydrolysates with antioxidative properties (Ryan *et al*., 2011; Samaranayaka *et al*., 2010).

The crude protein hydrolysate may be further isolated by passing it through ultrafiltration (UF) membranes, in order to obtain a more uniform product with the desired range of molecular masses (Pihlanto-Leppälä & Korhonen, 2003). In large-scale production of hydrolysates, membrane technology may also be coupled with enzymatic hydrolysis in a continuous process, reducing the cost by eliminating the need to use heat or pH adjustment to inactivate the enzymes at the end of hydrolysis (Guérard, 2007). Low-molecular-mass membrane cutoffs are useful for concentrating antioxidative peptides from the remaining higher-molecular-mass components, including undigested polypeptide chains and enzymes. Other techniques such as nanofiltration, electrodialysis, ion-exchange membranes, or column-chromatographic methods can be used to further concentrate and purify antioxidative peptides (Pihlanto-Leppälä & Korhonen, 2003; Poulin *et al*., 2007).

Overall, the most useful method for peptide separation or purification is high-performance liquid chromatography (HPLC), preceded by one of the other separation techniques. Commercially available reversed-phase columns are widely used for the rapid separation and detection of their hydrophilic and hydrophobic characteristics (Shahidi & Zhong, 2008). Ferreira *et al*. (2007) reported that peptides with different surface hydrophobicities can be separated by reversed-phase columns with a polystyrene–divinylbenzene copolymer-based packing. Therefore, choosing the right pore size for optimal separation of peptides is very important, with the wrong pore size resulting in poor resolution. The ligand on the gel also plays an important role in obtaining effective separation (Najafian & Babji, 2012). Another essential factor in effective separation is obtaining an appropriate hydrophobicity of the gel. The appropriate pore size, hydrophobicity, particle size, and column size should be combined to achieve high recovery and resolution in the isolation of peptides and proteins (Kuriyama *et al*., 2005). Once hydrolysates are separated into peptidic fractions, these fractions are further tested for their antioxidant activities, and the most active fractions are sequenced using mass-spectrometry techniques including MALDI-TOF (matrix-assisted laser-deionization time-of-flight), ESI (electrospray ionization), and Edman degradation (Bernardini *et al*., 2011). Fig. 22.1 shows procedure for the production, isolation and purification of antioxidant peptides fish and shrimp.

22.3 METHODS USED TO MEASURE ANTIOXIDANT ACTIVITY

The identification and quantification of individual antioxidants present in a desired food complex is important, but separating the antioxidative compounds from these mixtures and studying each one individually is expensive and inefficient. Therefore, it is necessary

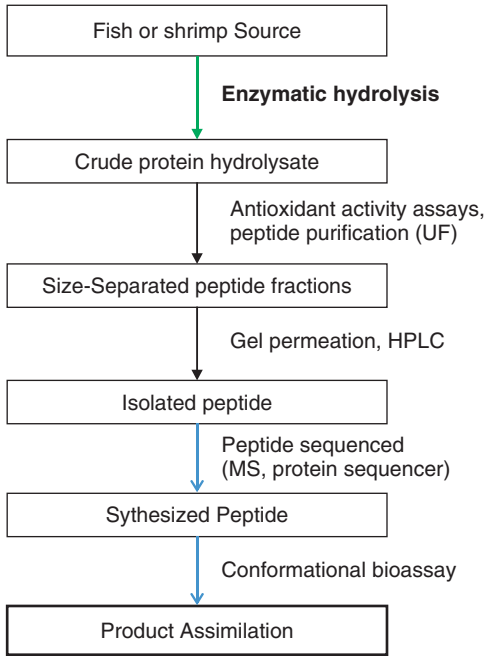

Fig. 22.1 Procedure for the production, isolation, and purification of fish and shrimp antioxidant peptides. Adapted from Arihara & Ohata (2006) with slight modifications. Reproduced by permission of Taylor & Francis Group.

to have a convenient method by which to quantify the antioxidative effectiveness of a whole food or of partially purified antioxidant peptides (Najafian & Babji, 2012).

Antioxidant assays can be divided into *in vitro* and *in vivo* types (Bernardini *et al*., 2011). The antioxidant activity of hydrolysates or peptides is usually tested by *in vitro* assays; it is necessary to perform *in vivo* assays to ensure that peptides identified by *in vitro* systems are bioavailable following ingestion and can reach a target site and administer a response in a living system (Vermeirssen *et al*., 2004).

22.3.1 *In Vitro* Chemical Assays

On the basis of the chemical reactions involved, *in vitro* antioxidant-capacity assays can be primarily classified into two groups: assays based on hydrogen atom transfer (HAT) (Huang *et al*., 2005) and assays based on electron transfer (ET) (Huang *et al*., 2005).

The HAT-based assays usually involve the use of a synthetic free-radical generator, an oxidizable molecular probe, and an antioxidant (Huang *et al*., 2005). Quantification is obtained from kinetic curves derived from competitive reaction kinetics (Huang *et al*., 2005). These assays include the oxygen-radical absorbance-capacity assay (ORAC) and the inhibition of linoleic acid autoxidation. The ORAC assay is an example of a HAT-based assay in which an antioxidant and a substrate (fluorescein probe) compete kinetically for hydroxyl radicals (Jimenez-Alvarez *et al*., 2008). In this assay, as the reaction progresses, the antioxidant compound present limits the decrease in fluorescence, which is a measure of the extent of damage to the fluorescein probe. The inhibition of linoleic acid

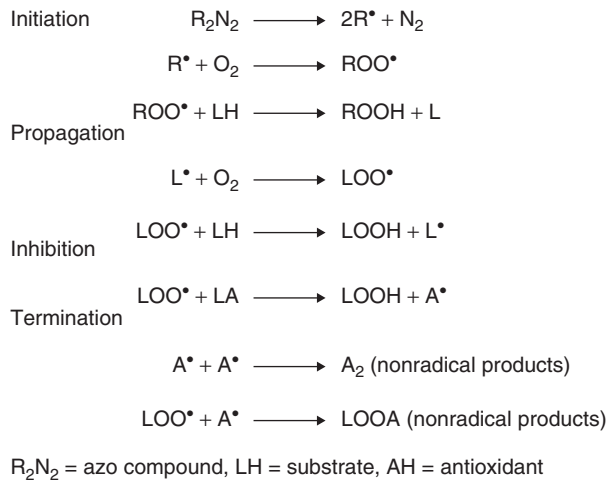

Fig. 22.2 Steps in the autoxidation process and the action of antioxidants. From Najafian & Babji (2012) Peptides, 33, 178–185. Copyright 2012, with permission from Elsevier.

autoxidation by antioxidants is another example of a HAT reaction. Fig. 22.2 shows the major steps involved in lipid autoxidation, which are initiated by an azo compound, the action of inhibition, and termination of antioxidants in the radical-generating process.

ET-based methods measure the capacity of an antioxidant to reduce an oxidant, which changes color when reduced (Huang *et al*., 2005). The degree of color change is correlated with the sample's antioxidant activity (Huang *et al*., 2005). These assays measure the radical-scavenging activity of an antioxidant. They include the trolox-equivalence antioxidant-capacity assay (TEAC), the ferric ion-reducing antioxidant-power assay (FRAP), the 'total antioxidant potential' assay, which uses the copper (II) complex (Cu (II)) as an antioxidant, and the 2,2-diphenyl-1-picrylhydrazyl assay (DPPH). Other radical-scavenging assays, such as hydroxyl radical-scavenging activity, superoxide radical-scavenging activity, and peroxyl radical-scavenging activity, have also been used (Kim *et al*., 2009).

Antioxidant activity can also be calculated by studying the capacity of a substrate to inhibit or halt lipid oxidation in complex food systems, and the most common such assays include the thiobarbituric acid reactive substance (TBARS) and the peroxide-value (PV) assays (Bernardini *et al*., 2011). TBARS measures the concentration of malondialdehyde (MDA), the most abundant product of lipid oxidation, along with other secondary oxidation products in a sample (Antolovich *et al*., 2002). Some volatile secondary oxidation compounds of lipid oxidation, such as propanal and hexanal, are analyzed by SPME-GC, which is more specific than TBARS. PV assays represent the total peroxide and hydroperoxides oxygen content of lipids and can be studied in a linoleic acid system, with the hydroperoxides determined by the ferric thiocyanate method (Antolovich *et al*., 2002). Additionally, the study of the carbonyl values (COV) can be used to study the degree of lipid oxidation. This measures the secondary decomposition products of oxidation, such as aldehydes and ketones (Farhoosh & Reza Moosavi, 2008).

In order to have a comprehensive view of the antioxidant activity of a hydrolysate or peptide, it is necessary that a number of antioxidant assays are used, as in most of the

studies reported in this review. To get a broad estimation of their antioxidant activity, hydrolysates and/or peptides should be assayed for antioxidant activities using one HAT-based assay, one ET-based assay, and one assay that takes account of the protective effect against lipid oxidation (i.e. TBARS or PV) (Zulueta *et al*., 2009).

22.3.2 *In Vitro* Biological Assays and *In Vivo* Assays

In vitro cultured-cell-model systems allow for rapid, inexpensive screening of antioxidative compounds for their bioavailability, metabolism, and bioactivity, as compared to expensive and time-consuming animal studies and human clinical trials. Use of cell-culture models for antioxidant research is particularly important because the studies to date have demonstrated that the mechanism of action of antioxidants in human health promotion goes beyond the antioxidant activity of scavenging free radicals (Liu & Finley, 2005).

Once the antioxidative potential of a food constituent of interest is established using *in vitro* assay methods, animal studies and human clinical trials can be conducted to confirm the bioavailability and the desired biological function. Various biomarkers are used in measuring the ability of dietary antioxidants to protect lipids, proteins, and DNA from oxidative damage (Samaranayaka & Li-Chan, 2011). The results from these *in vivo* assays are an essential part of gaining approval from federal agencies for a dietary component to be used in functional-food and nutraceutical formulations (Samaranayaka & Li-Chan, 2011).

22.4 ANTIOXIDANT ACTIVITY OF PEPTIDES

Previous studies on peptides with antioxidant activity isolated from marine fish and shrimp byproducts are listed in Table 22.1. To date, several different proteins from marine fish and shrimp have been extracted and hydrolyzed and had their antioxidant activities studied.

22.4.1 Antioxidant Peptides from Fish Sources

Wu *et al*. (2003) investigated the antioxidant activity of free amino acids and peptides from mackerel (*Scomber austriasicus*) muscle. Mackerel meat was hydrolyzed with protease N for up to 25 hours at a temperature of 50 °C. The most abundant free amino acids in nonhydrolyzed mackerel were H, Tau, and K, and the concentration of these free amino acids increased during the hydrolysis, with H concentration reaching a maximum at 10 hours, after which time it decreased (Wu *et al*., 2003). The total free amino acids increased during the first 15 hours and subsequently remained unaltered. Carnosine and anserine (extracted from the hydrolyzed mackerel) were not found in the nonhydrolyzed mackerel, but they were present in the protease N hydrolysate at all the hydrolysis time points (Wu *et al*., 2003). The antioxidant activity (determined in a linoleic acid peroxidation system, by the DPPH and reducing-power assay method) of the hydrolysates increased from 0 to 10 hours (where it reached a peak) and then decreased gradually (Wu *et al*., 2003). The 10-hour hydrolysate was studied for its molecular-weight distribution using gel-filtration chromatography on a Sephadex G-25 column. Three main fractions of approximately 1400, 900, and 200 Da were detected and tested for their antioxidant activity. Peptides

with a molecular weight of about 1400 Da possessed stronger *in vitro* antioxidant activity than those of 900 and 200 Da (Wu *et al*., 2003).

Klompong *et al*. (2007) studied the antioxidant activity of yellow stripe trevally (*Selaroides leptolepis*)-muscle hydrolysates. Yellow stripe trevally meat and defatted meat were hydrolyzed with Flavourzyme 500 L at pH 7 and a temperature of 50 °C and Alcalase 2.4 L at pH 8.5 and a temperature of 60 °C for 20 minutes at different substrate–enzyme concentration ratios (Klompong *et al*., 2007). The antioxidant activity of the hydrolysates was tested by the DPPH assay, the metal-chelating activity assays, and the reducing-power assay (determined by incubating samples with potassium ferricyanate). The antioxidant activity of the two hydrolysates was found to depend on the enzymes used and the hydrolysis conditions. The alcalase hydrolysate showed high DPPH radical-scavenging activity, which decreased with increasing DH. The metal-chelating activity of both hydrolysates increased with increasing DH (Klompong *et al*., 2007).

Thiansilakul *et al*. (2007) found that the antioxidative activity of hydrolysates from round scad (*Decapterus maruadsi*) muscle with DH of 20, 40 and 60%, prepared using Alcalase or Flavourzyme, was related to the type of proteinase, DH, and defatting process prior to hydrolysis. At the same DH, Flavourzyme exhibited a higher DPPH radical-scavenging activity and reducing power, but a lower Fe^{2+}-chelating ability than Alcalase. Flavourzyme from isopropanol-defatted muscle with 60% DH was extracted using different solvents, including hexane (E_1), dichloromethane (E_2), ethyl acetate (E_3), and residual (R) fractions were obtained. Among all fractions, E_2 and E_3 exhibited the highest DPPH radical-scavenging activity and reducing power. Flavourzyme with 60% DH and E_2 at 1000 ppm exhibited antioxidant activity in linoleic oxidation and lecithin liposome systems, and the results were comparable to butylated hydroxytoluene (BHT) at 100 ppm.

Herring-body hydrolysate (HBH), herring-gonad hydrolysate (HGH), herring-head hydrolysate (HHH), and whole-herring hydrolysates (WHH) were produced from *Clupea harengus* (Sathivel *et al*., 2003) and studied for their antioxidant activities. All three protein sources were hydrolyzed with Alcalase for 60 minutes at pH 8 and a temperature of 50 °C, and the antioxidant activity of the hydrolysates was identified using the inhibition of linoleic acid peroxidation by the thiocyanate method (Sathivel *et al*., 2003). The WHH and HBH hydrolysates exhibited higher antioxidant activity than the HHH hydrolysate (Sathivel *et al*., 2003).

Amarowicz & Shahidi (1997) reported that the antioxidant activities of four peptide fractions were separated from protein hydrolysates of capelin (*Mallotus villosus*) using Sephadex G-10 gel-filtration column chromatography. The antioxidant activity of each fraction was determined in a β-carotene-linoleate model system. One isolated fraction possessed a notable antioxidant activity, two others had a weak efficacy, and the fourth exerted a pro-oxidant effect. Two-dimensional thin-layer chromatography (TLC) of isolated fractions gave spots with both antioxidant and pro-oxidant activities. Two pro-oxidant compounds were separated from the hydrolysate by preparative TLC using silica-gel plates. One compound exhibited a maximum absorption at 254 nm, while the other absorbed at 260 nm.

The tryptic hydrolysate of conger eel (*Conger myriaster*)-muscle protein was fractionated according to molecular weight using an UF membrane system (Ranathunga *et al*., 2006). The lowest molecular-weight fraction (<1 kDa) with higher antioxidative properties was purified using consecutive chromatographic techniques and designated ‘conger eel antioxidative peptide’ (CEAP). Its sequence was LGLNGDDVN (molecular mass

928 Da). CEAP performed better than the natural antioxidant α-tocopherol for the prevention of lipid peroxidation *in vitro*. Additionally, it scavenged hydroxyl radicals and carbon-centered radicals at IC_{50} values of 74.1 and 78.5 μM, respectively. Therefore, the peptides derived from conger eel-protein hydrolysates are responsible for higher antioxidative properties and molecular weights, as well as for the presence of hydrophobic amino acids. In addition, Mendis *et al*. (2005) obtained the peptide (sequence: H-G-P-L-G-P-L) from hoki (*Johnius belengerii*)-skin gelatin hydrolyzed by trypsin, suggesting the peptide's inhibition of lipid peroxidation could be related to its large hydrophobicity, which would favor interaction between the peptide and fatty acids, and also to the presence of the N-terminal H, which has been shown previously to exert antioxidant protection.

Gelatin from Alaska pollock (*Theragra chalcogramma*) skin was extracted and hydrolyzed in three steps. The extract was hydrolyzed with Alcalase at pH 8 and a temperature of 50 °C, ultrafilterated with a 10 kDa MWCO membrane, and then hydrolyzed with pronase E at pH 8 and a temperature of 50 °C (Kim *et al*., 2001). This second hydrolysate was ultrafiltered through a 5 kDa MWCO membrane and hydrolyzed with collagenase at pH 7.5 and a temperature of 37 °C. The collagenase hydrolysate was ultrafiltrated with a 1 kDa MWCO membrane (Kim *et al*., 2001). The three molecular-weight fractions, 10, 5, and 1 kDa, were tested for their antioxidant activities in a linoleic acid peroxidation system using the TBARS method. The pronase E hydrolysate showed the highest antioxidant activity, and was further separated and purified by consecutive chromatographic techniques, with sequences G-E-Hyp-G-P-Hyp-G-P-Hyp-G-P-Hyp-G-P-Hyp-G and G-P-Hyp-G-P-Hyp-G-P-Hyp-G-P-Hyp-G (Kim *et al*., 2001). These isolated peptides were tested with the TBARS method and the peptide G-P-Hyp-G-P-Hyp-G-P-Hyp-G-P-Hyp-G showed the highest antioxidant activity (Kim *et al*., 2001).

Je *et al*. (2005) studied the antioxidant activity of Alaska pollock (*T. chalcogramma*)-frame proteins hydrolyzed with mackerel-intestine crude enzyme (MICE) for 12 hours at pH 10 and a temperature of 50 °C. Hydrolysate was ultrafiltered with five different MWCO membranes (30, 10, 5, 3, and 1 kDa) and the antioxidant activity of the filtrates was studied with the protection of linoleic acid peroxidation method. The 1 kDa filtrate showed the highest antioxidant activity and was purified using consecutive chromatographic techniques (Je *et al*., 2005). The sequence of the antioxidant peptide, determined by Edman degradation with a protein sequencer, was L-P-H-S-G-Y (molecular mass 672 Da) (Je *et al*., 2005). The antioxidant activity of the peptide could be attributed to the H and Y residues, because of the reported chelating and lipid radical-trapping ability of the imidazole ring of H and the hydrogen-donor capacity of Y (Je *et al*., 2005).

Je *et al*. (2007) studied the hydrolysis of tuna-backbone protein using different proteases (alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin) for the production of antioxidant peptide. The antioxidant activities of hydrolysates were evaluated using lipid-peroxidation inhibition assay and direct free radical-scavenging activity by an electron spin resonance (ESR) spectrometer. Peptic hydrolysate exhibited the highest antioxidant activity compared to other hydrolysates. The peptic hydrolysate was purified using consecutive chromatographic methods and the antioxidant peptide was identified to be VKAGFAWTANQQLS (1519 Da) by Q-TOF ESI mass spectroscopy. The antioxidant peptide from tuna-backbone protein (APTBP) significantly inhibited lipid peroxidation in a linoleic acid emulsion system and quenched free radicals (DPPH, hydroxyl, and superoxide) in a dose-dependent manner. Moreover, APTBP did not show any cytotoxic effect against MRC-5 and ECV304 cell lines.

Sannaveerappa *et al*. (2007) reported the antioxidative effect of herring (*Clupea harengus*) light-muscle press juice (PJ) against hemoglobin (Hb)-mediated oxidation of washed cod mince during ice storage. The PJ was fractionated into low-molecular-weight (LMW-PJ, >1 kDa) and high-molecular-weight (HMW-PJ, >1, >3.5, and >50 kDa) fractions. It was preheated (10 minutes at 100 °C) and tested with or without removing heat-coagulated proteins. Whole-herring PJ and the LMW-PJ fraction significantly extended the oxidation lag phase of controls, from 2 up to 8 and 7 days, respectively. The HWM-PJ fractions were significantly less efficient than the whole and LMW-PJ samples, giving only 3.5–4.5 days of lag phase. Heat-treated PJ, with and without the heat-coagulated proteins, gave 7 and 5 days of oxidation lag phase, respectively.

Nalinanon *et al*. (2011) studied the functional properties and antioxidant activities of protein hydrolysates prepared from ornate threadfin bream (*Nemipterus hexodon*) muscle, using skipjack tuna pepsin, with different DH (10%, 20%, and 30%). The emulsifying and foaming properties of hydrolysates were governed by their DH and concentrations. Hydrolysates with 20% DH had the highest scavenging activities for ABTS and DPPH radicals. However, the chelating activity of hydrolysates for ferrous ion increased as DH increased. Size-exclusion chromatography of the hydrolysate with 20% DH using Sephadex G-25 revealed that antioxidative peptides with a molecular weight of approximately 1.3 kDa exhibited the highest ABTS radical-scavenging activity. *In vitro* simulated gastrointestinal digestion indicated that ABTS radical-scavenging activity of the antioxidative peptides was not affected by pepsin hydrolysis, while further digestion by pancreatin enhanced the activity. Therefore, protein hydrolysate from the muscle of ornate threadfin bream produced by skipjack tuna pepsin can be used as a promising source of functional peptides with antioxidant properties.

Hsu (2010) used two commercial enzymes, orientase (OR) and protease XXIII (PR), to hydrolyze tuna dark muscle byproduct for up to 6 hours, and evaluated the antioxidative properties of the hydrolysates. The 60-minute OR and 120-minute PR hydrolysates possessed the highest antioxidative activities. The protein hydrolysates were then subjected to a Sephadex G-25 gel-filtration chromatography, and the molecular weights of the peptide fractions which showed the highest antioxidative activity ranged from 390 to 1400 Da. The peptide fractions were further isolated using two-step HPLC (HPLC-1 and HPLC-2). The amino acid sequences of the two antioxidative peptides from OR and PR hydrolysates were Leu-Pro-Thr-Ser-Glu-Ala-Ala-Lys-Tyr (978 Da) and Pro-Met-Asp-Tyr-Met-Val-Thr (756 Da), respectively.

Ahn *et al*. (2010) found that the functional peptides obtained from tuna-liver protein by enzymatic hydrolysis using Alcalase, Neutrase, and Protamex following Flavourzyme hydrolysis showed excellent antioxidant activities against DPPH, hydrogen peroxide, and hydroxyl radical scavenging and reducing power. The hydrolysates were further fractionated into four categories based on their molecular weights by UF membranes. The antioxidant activities of the fractions depended on the bioassay employed. Moreover, the authors confirmed the antioxidant ability against hydroxyl radical-induced DNA damage by measuring the conversion of supercoiled pBR322 plasmid DNA to the open, circular form.

Jia *et al*. (2010) found that Protamex was the most efficient enzyme for preparing antioxidant peptides from Alaska pollock skin. The optimal hydrolysis conditions were: hydrolysis time, 8 hours; enzyme–substrate ratio, 2 : 1000; skin/water ratio, 1 : 6; temperature, 55 °C; pH 6.0. Under these conditions, the highest yield of peptides was 83.44%, with 85.95% of the hydrolysate being mainly composed of oligopeptides with molecular weights ranging from 180 to 1000 Da. The hydrolysate showed DPPH radical-scavenging

activity, with an IC_{50} value of 2.5 mg/ml and a reducing power of 0.14 at 1 mg/ml: 53.8% of that of reduced glutathione at the same concentration.

Jun *et al*. (2004) hydrolyzed yellowfin sole (*Limanda aspera*)-frame proteins using eight enzymes – Alcalase, α-chymotrypsin, MICE, Neutrase, papain, pepsin, pronase E, and trypsin – for 6 hours. Hydrolysates were tested for their antioxidant activities by studying the inhibition of linoleic acid peroxidation using the TBARS and ferric thiocyanate methods. The pepsin hydrolysate showed the highest antioxidant activity but the lowest DH, while MICE had the highest DH. Yellow sole-frame proteins were then hydrolyzed with MICE for 3 hours at pH 10 and a temperature of 50 °C and with pepsin for 3 hours at pH 2 and a temperature of 37 °C. The hydrolysates were subsequently filtered through 30, 10, 5, 3, and 1 kDa MWCO membranes. The 1 kDa filtrate showed the highest activity and was purified by consecutive chromatographic techniques. The amino acid sequence of an antioxidant peptide was identified as R-P-D-F-D-L-E-P-P-Y (Jun *et al*., 2004). Its antioxidant activity could be explained by the presence of Y, which is reported to have protective affects against the peroxidation of lipids (Jun *et al*., 2004).

Kim *et al*. (2007) isolated an antioxidant peptide from hoki (*J. belengerii*)-frame protein hydrolysate. They hydrolysed frame proteins separately with six different enzymes – pepsin, trypsin, papain, α-chymotrypsin, Alcalase, and Neutrase – and tested the hydrolysates for their antioxidant activity using lipid peroxidation-inhibition assay, DPPH assay, and hydroxyl and superoxide radical-scavenging activity. The peptic hydrolysate presented the highest antioxidant activity and was ultrafiltrated with various MWCO membranes (10, 5, 3, and 1 kDa). The 1–3 kDa fraction showed the highest antioxidant activity and was purified further using fast liquid-protein chromatography (FPLC) and reverse-phase HPLC (RP-HPLC). An antioxidant peptide, E-S-T-V-P-E-R-T-H-P-A-C-P-D-F-N (molecular weight 1801 Da), was identified and sequenced using ESI mass spectrometry (Kim *et al*., 2007).

22.4.2 Antioxidant Peptide from Shrimp Sources

Generally, the byproducts from shrimp processing account for approximately 30–40% of the total yield, and this waste has real potential for pollution and disposal problems. Several researchers (Guerard *et al*., 2007; He *et al*., 2006) have suggested these byproducts have the potential to produce functional protein hydrolysates. Research into antioxidant protein hydrolysates and peptides obtained from shrimp sources is still limited, focusing mainly on the preparation and antioxidant activity of the crude shrimp hydrolysates.

Protein hydrolysates are prepared by hydrolysis of shrimp (*Penaeus monodon* and *Penaeus indicus*) waste for 90 minutes using Alcalase and following the pH-stat method. The antioxidative activities of shrimp-waste protein hydrolysates (SWPH) were assessed to determine their FRAP, ABTS, and DPPH radical-scavenging activities, which increased linearly with increasing concentration of protein hydrolysate up to 5 mg/ml, maintaining good correlation. SWPH showed high stability over wide ranges of pH (2–11) and temperature (up to 100 °C for 150 minutes), with an activity >80% retained. Protein-hydrolysate solution at a concentration of 5 mg/ml significantly lowered the TBA values of croaker fish fillet and maintained a yellowish skin color compared to an untreated control sample over 10 days of refrigerated storage at 4 °C. SWPH also restricted the increase of PV and FFA values in croaker fish fillet within acceptable limits (Dey *et al*., 2011).

Shrimp processing byproducts (SPB) were digested by six proteases (trypsin, pepsin, neutrase, Protamex, Flavourzyme, and Alcalase) to produce antioxidative peptides. The

Alcalase hydrolysate had the highest DH and DPPH radical-scavenging activity. The effect of defatting on the DH and DPPH radical-scavenging activity of the Alcalase hydrolysate was significant, with the DH decreasing and the DPPH radical-scavenging activity increasing. The antioxidative activity of Alcalase hydrolysate was also investigated using several *in vitro* assays, including DPPH and ABTS radical-scavenging assays, reducing-power assay, and chelating activity. The antioxidative activity of the hydrolysate was obviously concentration-dependent. The SPB Alcalase hydrolysate exhibited notable DPPH and ABTS radical-scavenging activity, with IC_{50} values of 500 and 7.4 μg/ml, respectively. The hydrolysate showed 38.9% chelating activity at 120 μg/ml. The SPB Alcalase hydrolysate was a potential source of natural antioxidants (Huang *et al*., 2011).

The muscle of the prawn *Penaeus japonicus* was hydrolyzed by various proteases and the antioxidant activity of its hydrolysates was examined. Pepsin digest showed the most potent antioxidant activity. Three antioxidant peptides have been isolated from the active peptidic fraction by ion-exchange chromatography, gel filtration, and ODS HPLC. Their structures were identified as Ile-Lys-Lys, Phe-Lys-Lys, and Phe-Ile-Lys-Lys (Suetsuna, 2000).

The hydrolysis parameters required to obtain a hydrolysate of *Acetes chinensis* with potent radical scavenging activity using Alcalase 2.4 l were optimized by response surface methodology (RSM). The results showed that the optimum conditions were: temperature, 57 °C; pH 8.0; enzyme–substrate ratio, 2.6 AU 100 g/shrimp; hydrolysis time, 3 hours. The DH was 26.32%, the hydroxyl radical-scavenging activity was up to 88.12%, and the DPPH radical-scavenging activity was 35.61%. Gel-column-filtration chromatography by a Sephadex G-25 column yielded five fractions, with the most potent free-radical-scavenging-activity fraction having a molecular weight of 207–915 Da, an IC_{50} for hydroxyl radical of 0.03 mg/ml, and an IC_{50} for DPPH radical of 8.86 mg/ml (Cao *et al*., 2009). Guerard *et al*. (2007) also optimized the hydrolysis parameters of wild prawn (a mix of *Penaeus braziliensis* and *Penaeus subtilis*) processing discards by RSM.

22.5 ANTIOXIDANT MECHANISMS OF PEPTIDES

In general, all 20 amino acids found in proteins can interact with free radicals if the energy of the free radicals is high (e.g. hydroxyl radicals) (Elias *et al*., 2008). The most reactive include the nucleophilic sulfur-containing amino acids Cys and Met, the aromatic amino acids Trp, Tyr, and Phe, and the imidazole-containing amino acid His. However, free amino acids are not generally found to be effective as antioxidants in food and biological systems, and extensive proteolysis of food proteins has in fact been reported to result in decreased antioxidative activity (Samaranayaka & Li-Chan, 2011). The higher antioxidative activity of peptides compared to free amino acids is attributed to the unique chemical and physical properties conferred by their amino acid sequences, especially the stability of their resultant peptide radicals, which do not initiate or propagate further oxidative reactions (Elias *et al*., 2008).

Several mechanisms have been postulated for the antioxidative properties of peptides, including metal-ion chelation, free-radical scavenging, and aldehyde adduction (Samaranayaka & Li-Chan, 2011). The majority of the antioxidative peptides derived from food sources have molecular weights ranging from 200 to 1800 Da (Samaranayaka & Li-Chan, 2011); moreover, they often include hydrophobic amino acid residues such as

Val or Leu at the N-termini of the peptides, and Pro, His, Tyr, Trp, Met, and Cys in their sequences (Elias *et al*., 2008; Guo *et al*., 2009; Hsu, 2010).

In addition, the amino acid composition, sequence, and size play an important role in the antioxidative potential of a peptide (Samaranayaka & Li-Chan, 2011). It is evident that different amino acid residues and peptide sequences are responsible for the inhibition of oxidative reactions that are initiated by different types of free radical or pro-oxidant, such as metal ions, as well as in different molecular environments (for example, aqueous, lipid, or emulsion systems, different pH conditions, or the presence of other compounds in food matrices or biological systems). Although a general idea of the importance of specific amino acid residues and peptide sequences for antioxidative action can be obtained based on literature findings, it will really be necessary to first identify the target use of a particular peptide of interest, and then select appropriate assays and model systems with which to establish the antioxidative potential, considering possible mechanisms of action in the envisioned application. The use of different antioxidative assays as well as variations of the same assay by different research groups makes it difficult to compare antioxidative potentials and the mechanisms of reported peptide sequences. In addition to the peptides derived from food proteins, the proteins themselves have the ability to act as antioxidative agents by inactivating ROS, scavenging free radicals, chelating pro-oxidative transition metals, reducing hydroperoxides, and enzymatically eliminating specific oxidants (Elias *et al*., 2008).

22.6 APPLICATIONS AND PROSPECTS

Protein hydrolysates and peptide fractions can be added as functional ingredients in food systems to reduce oxidative changes during storage. Several studies report that the antioxidative activities of protein hydrolysates and isolated peptides prepared from sources such as hoki-skin gelatin, tuna backbone, and Pacific hake fillet are superior to that of α-tocopherol and in some cases similar to or higher than those of commonly used synthetic antioxidants such as BHA and BHT (Je *et al*., 2007; Mendis *et al*., 2005; Samaranayaka & Li-Chan, 2011). Synergistic effects of some antioxidative peptides with tocopherols in food and model systems have also been reported (Jun *et al*., 2004; Kim *et al*., 2001).

Incorporation of a fish-protein hydrolysate preparation made by autolysis of arrowtooth flounder protein into a coating of salmon fillets slowed down the lipid oxidation process (Sathivel, 2005). Further, a brine solution containing salmon fish-protein hydrolysate injected into smoked salmon fish fillets was shown to reduce lipid oxidation, measured as 2-thiobarbituric acid-reactive substances (TBARS) during 6 weeks of cold storage (4) and 8 months of frozen storage (−18) (Hagen & Sandnes, 2004).

It is important to study the technofunctional properties of active peptide fractions and how these can retain their antioxidant activities in different targeted food matrices. Antioxidant peptides have the ability to interact with other components of the food matrix, such as carbohydrates and lipids, by Maillard reaction, and may also lose or improve their activity during food-processing operations such as thermal processing (Samaranayaka & Li-Chan, 2011). Some research has shown that the Maillard reaction is a chemical method that can improve the antioxidant activity of some protein hydrolysates, such as hydrolysates of mechanically deboned chicken residue, casein peptides, soy hydrolysate, and peanut hydrolysate (Dong *et al*., 2011; Liu *et al*., 2010; Su et al., 2011; Sun *et al*., 2010).

Further, particular antioxidant peptide sequences of interest may also have synergistic or antagonistic effects with other antioxidants and/or trace metals present in food and

biological systems, and even act as pro-oxidants under certain conditions. These factors should be carefully taken into consideration when looking for possible applications of antioxidant peptides.

It will be important to identify the forms in which antioxidative peptides can be incorporated into food matrices. Compared to pure isolated peptides, crude or semipurified peptide extracts will be more economically feasible for use in food products. Furthermore, crude extracts may contain several different peptides that can act synergistically to exert antioxidative action. On the other hand, other components in crude extracts, such as pigments and trace lipids, may cause color and flavor problems (Samaranayaka & Li-Chan, 2011).

The most challenging task in antioxidant-peptide research at present is to investigate antioxidant activity *in vivo* and the bioavailability of these peptides. To our knowledge, information about antioxidant activity *in vivo* is still scarce. In addition, an important task for the production of functional foods containing antioxidant peptides is to enhance their bioavailability from fish and shrimp sources through the addition/fortification of isolated or enriched fractions of bioactive peptides. The production of bioactive peptides during food processing, for example by the use of specific bacterial enzymes or genetically transformed microorganisms, is of interest for future research. Moreover, genetically modified proteins will be designed to carry multiple copies of bioactive sequences (Park *et al*., 2006).

While peptides have excellent antioxidant activity and great potential as food additives, they may not be suitable in all food applications (Elias *et al*., 2008). A major concern associated with the use of peptides as antioxidants is the potential issue of allergenicity, especially with proteins derived from shrimp. Also of concern are bitterness and other potential organoleptic problems, as well as the stability of antioxidative peptides during food processing and storage. Peptides could also cause problems if they alter the texture (e.g. increased viscosity or gelation) or color (light scattering or Maillard reactions) of the food product. Finally, certain Maillard-reaction products have been implicated as carcinogenic, making their addition to foods problematic.

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23 Fish-elastin Hydrolysate: Development and Impact on the Skin and Blood Vessels

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23.1 INTRODUCTION

Elastin is one of the major fibrous constituents of the extracellular matrix of fish, birds, animals, and human. Elastin gives elasticity to tissues, while collagen, another major fibrous constituent of the extracellular matrix, gives tissue mechanical strength. Elastin is rich in elastic tissues, such as aorta, skin, lung, ligament, and so on (Mithieux & Weiss, 2005; Vrhovski *et al*., 1998). Elastin is synthesized as a soluble protein of approximately 70 kDa, referred to as 'tropoelastin', by fibroblasts, smooth-muscle cells, vein endothelial cells, condrocytes, and so on (Sandberg *et al*., 1969; Smith *et al*., 1972). It is deposited on microfibrils and forms an elastic fiber. The microfibrils are formed by co-aggregation of fibrillin (Trask *et al*., 2000) and microfibrils-associated glycoprotein (MAGP) (Brown-Augsburger *et al*., 1996), which are glycoproteins secreted into the extracellular matrix by fibroblasts. The microfibrils appear to provide a scaffold for the deposition of elastin. To organize deposition of tropoelastin on microfibrls, fibulin-5 (also known as 'developmental arteries and neural crest EGF-like': 'DANCE') is necessary, transcription of which is enhanced by tropoelestin mRNA (Suruga *et al*., 2004). Fibulin-5 interacts with tropoelastin and has an RDG motif, a ligand of integlin on the cell, which determines microlocalization of the elastin in the extracellular matrix (Hirai *et al*., 2007; Midwood & Schwarzbauer, 2002). The tropoelastin molecules in elastic fibers are crosslinked and converted to an insoluble form. Lysyloxidase plays a significant role in crosslinking of the elastin molecules, catalyzing the formation of aldehyde from lysine residues in collagen and elastin precursors (Mithieux & Weiss, 2005; Narayanan *et al*., 1978; Vrhovski *et al*., 1998). The lysine residue-derived aldehyde (allysyl residue) reacts with the amino group of unmodified lysine residue, which induces crosslinks in collagen and elastin. Desmosine (Des) and isodesmosine (Ide) are known elastin-specific lysine-derived crosslinks (Mithieux & Weiss, 2005; Vrhovski *et al*., 1998).

In addition to providing elasticity to tissues, elastin also plays a significant role in tissue development. Li *et al*. (1998) demonstrated that tropoelastin gene-knockout mice undergo lethal intima-media thickness in the aorta without damage to the endothelial cell.

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It has also been demonstrated that elastin interacts with fibroblast, smooth-muscle cell, endothelial cell, and so on via elastin-binding protein (Hinek *et al*., 1988, 1992; Mecham *et al*., 1991).

Elastin in tissues remains insoluble to most protein solvents. Thus, elastin has been isolated in an insoluble form after hot-alkaline extraction using 0.1 M NaOH at 98 °C (Lansing *et al*., 1952; Lowry *et al*., 1941; Richard *et al*., 1902). It is difficult to characterize elastin in tissue on the basis of molecular weight, isoelctric point, and so on due to insolubility. Thus, the purity of an elastin preparation has been evaluated by compositional analysis. Elastin is rich in Gly (30–45% (mol/mol)), Ala, Val, and Pro and contains the elastin-specific crosslinking amino acids desmosine (Des) and isodesmosine (Ide). On the other hand, it consists of smaller amounts of hydroxyproline (Hyp) (1%) than of collagen (10%) (Starcher & Galione, 1976).

There are episodes during which ingestion of skin, cartilage, and placenta extracts, which are rich in extracellular matrix, improve skin and joint conditions. Recently, it has been demonstrated that ingestion of collagen hydrolysate increases the moisture content of the skin of healthy women and moderates knee-joint pain in osteoarthritis patients (Matsumoto *et al*., 2006; Schauss *et al*., 2012; Takase *et al*., 2011). The occurrence of food-derived collagen peptides such as Pro-Hyp in human peripheral blood has been demonstrated (Ichikawa *et al*., 2010; Iwai *et al*., 2005). The Pro-Hyp enhances the growth of fibroblast (Shigemura *et al*., 2009) and synthesis of hyaluronic acid (Ohara *et al*., 2010), which have been suggested to be mechanisms for the beneficial effects of collagen hydrolysate.

Elastin-rich organs such as artery have a long history of use as a food. However, purified elastin and its derivatives have not been commonly used as food ingredients. Recently, an enzymatic hydrolyaste of elastin has been prepared from mammalian tissues on an industrial scale and used as a food ingredient. It has been suggested that ingestion of such elastin hydrolysate might improve the condition of the skin (Hayakawa *et al*., 2009). Recent consumer preference for products of marine origin over those from terrestrial animals has led to the production of elastin hydrolysate from fish. This chapter introduces the development of enzymatic hydrolysate of fish elastin and its potential beneficial activities in the skin and blood vessels, and also discusses its underlying mechanism.

23.2 STARTER MATERIALS FOR FISH-ELASTIN HYDROLYSATE

In order to screen suitable starter materials for the production of fish elastin, the Des and Ide contents of HCl hydrolysates of various fish organs have been examined. Skipjack (*Katsuwonus pelamis*), yellowtail (*Seriola quinqueradiata*), tuna (*Thunnus thynnus*), and Atlantic salmon (*Salmo salar*) were used for the screening (Nakaba *et al*., 2006). The amino acid compositions of various organs of skipjack are shown in Table 23.1. Only the bulbus arteriosus contains significant amounts of Des and Ide. The bulbus arteriosus is a fish-specific elastic tissue and plays a significant role in stabilizing blood flow from the heart (see Fig. 23.1). In Table 23.2, the Des, Ide, and Hyp contents of the bulbus arteriosus in various fish species are shown. In all cases, significant amounts of Des and Ide are present. On the other hand, significant amounts of Hyp, a post-translationally modified amino acid rich in collagen, are observed in all organs (Table 23.1). As shown in Fig. 23.2, skipjack bulbus arteriosus is rich in elastic fibers, while skin is rich in collagen

Table 23.1 Amino acid composition of HCl hydrolysates of skipjack organs (residues/1000 residues).

Amino acid(s)	Skin	Ordinal muscle	Dark muscle	Bulbus arteriosus	Heart	Liver	Pancreas	Kidney	Colon	Pyloric caeca	Ovary	Branchi	Stomach
Gly, Ala, Val, Pro	502.9	264.4	283.7	595.1	295.3	302.4	308.9	308.5	335.8	312.8	311.5	491.0	329.8
Asp, Asn	56.4	90.5	92.1	39.0	39.7	91.4	100.5	86.5	87.2	90.8	88.4	56.5	89.4
Glu,Gln	70.4	104.2	113.3	55.9	111.1	95.3	62.7	62.4	82.3	89.6	98.5	78.8	115.3
Des, Ide	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Hyl	4.8	0.0	0.0	0.3	0.2	0.2	0.0	0.3	2.6	0.0	0.9	11.9	2.8
Hyp	48.4	0.4	0.9	0.7	1.1	0.9	0.0	1.8	14.4	0.0	5.7	68.9	13.1

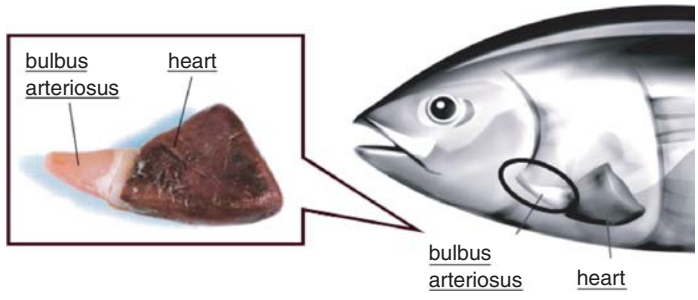

Fig. 23.1 Bulbus arteriosus of skipjack.

Table 23.2 Contents of Des, Ide and Hyp in the bulbus arteriosus of some fish species (mg/g).

Amino acid	Skipjack	Yellowtail	Tuna	Atlantic salmon
Des	0.6	0.4	0.4	0.5
Ide	0.5	0.6	0.4	0.5
Hyp	4.1	3.5	6.5	5.1

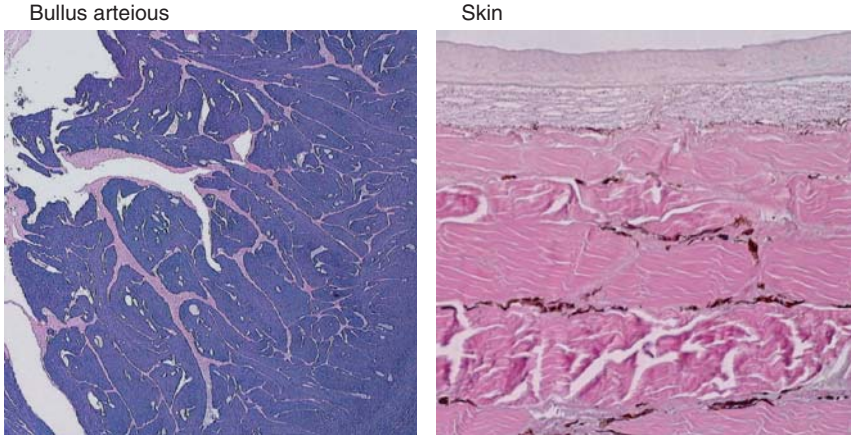

Fig. 23.2 EVG staining of sections from skipjack bulbus arteriosus and skin.

fibers. These facts indicate that fish bulbus arteriosus can serve as a starter material for elastin. In the following experiments, skipjack bulbus arteriosus has been used for the production of elastin hydrolysate, due to the availability and abundance of resource.

23.3 PREPARATION OF SKIPJACK-ELASTIN HYDROLYSATE

Enzymatic hydrolysate of elastin has been prepared from skipjack bulbus arteriosus on an industrial scale (Nakaba *et al*., 2006). Briefly, the bulbus arteriosus is collected from

Table 23.3 Amino acid composition of skipjack elastin hydrolysate (residues/1000 residues).

Amino acid	Skipjack elastin hydrolysate	Literature value (elastin from yellowtail)
Asp	21	18
Thr	68	63
Ser	25	32
Glu	37	33
Gly	440	400
Ala	85	130
Val	72	57
Cys	1	<0.6
Met	4	6.4
Ile	12	12
Leu	37	37
Tyr	30	38
Phe	21	31
Hyl	0	0
His	3	3.5
Lys	8	11
Ide	0.3	0.4
Des	0.2	0.4
Arg	25	21
Hyp	7	8.5
Pro	102	99

skipjack and washed with 0.01 M NaOH at 4 °C to remove the soluble proteins and then heated at 80–90 °C in water to remove the collagen. The residue is suspended in water to approximately two volumes of wet weight of the starting material and digested with Protin SD-AC10F (Taiwa Kasei, Shiga, Japan) and protease N-G (Amano Enzyme, Nagoya, Japan) at enzyme/substrate (wet weight) ratios of 1 : 100 and 2 : 1000, respectively. Digestion is performed at 50 °C. The digest is filtered with a filter press and ceramic filter (0.2 μm) and spray-dried. The amino acid composition of the final product is shown in Table 23.3. It contains a high content of Gly (440 residues/1000 residues) and small contents of Hyp, Des, and Ide. The overall amino acid profile is that of typical fish elastin (Sage & Gray, 1979). These facts indicate that the product consists of peptides from highly purified elastin. Size-exclusion chromatographic analysis reveals that more than 70% of the peptides in the product are less than 1000 Da. The elastin hydrolysate has a slightly yellow appearance and is soluble in water up to 50% (w/v). Solution at 1% is odorless and tasteless. This product (katsuwo elastin) can be obtained commercially from Hayashikane Sangyo Co. Ltd. (Shimonoseki, Japan).

23.4 IMPACT OF INGESTION OF SKIPJACK-ELASTIN HYDROLYSATE ON SKIN CONDITIONS

Skin consists of collagen (70% in protein) and a small amount of elastin (2–4%). However, elastin plays a significant role in providing elasticity and lifting the skin. The elastin content in skin decreases with aging and through exposure to ultraviolet rays, which may

induce wrinkles and sagging. In some cases, ingestion of elastin hydrolysate might suppress such unwelcome changes. Human trials have been performed in order to demonstrate this effect of skipjack-elastin hydrolysate (Nakaba *et al*., 2007; Shiratsuchi, 2012).

Japanese women in their 30s claiming obvious wrinkles in the eye area and dryness and sag of the skin (35.8 ± 2.6 years old) were enrolled in the trial. Subjects were divided into an elastin hydrolysate-supplemented group (n = 10) or a nonsupplemented group (n = 10). The nonsupplemented group received collagen hydrolysate, chondroichin sulfate, and hyaluronic acid, which had been demonstrated to improve skin conditions in previous studies (Matsumoto *et al*., 2006; Takase *et al*., 2011). The composition of ingredients in both samples per day is shown in Table 23.4. Subjects were asked to take the sample at bedtime and not to change their eating and exercising habits, and were advised to avoid excess sunlight. They were asked to visit a laboratory on the first day and 4 and 8 weeks after the start of the trial. After washing their faces in the normal way, the subjects stayed in an air-conditioned room (temperature $22 \pm 1\,^{\circ}C$ and humidity $50 \pm 10\%$) for 30 minutes. Skin firmness and elasticity were evaluated by a Cutometer 300™. These parameters imply skin softness/firmness and the ability of the skin to return to its original state, respectively. Maximum amplitude, minimum amplitude, and gross elasticity of the cheek were also determined. As shown in Fig. 23.3, both groups significantly decreased their maximum and minimum amplitudes in a time-dependent manner. These data indicate that firmness of the skin increased through the trial, which could be attributed to a seasonal effect; the trial began on October 20 and finished December 14. After 8 weeks, the elastin hydrolysate-supplemented group showed a higher maximum amplitude than the control group ($p = 0.076$), which suggests that supplementation with elastin hydrolysate moderated skin firmness in the winter (Fig. 23.3). On the other hand, there is no significant difference in gross elasticity between the two groups.

Fine lines and wrinkles on the face were observed using a skin replica. A fine line is a crevice in the surface of the skin that only delves into the epidermal layer. A wrinkle is a crevice that delves into the dermal and subcutaneous layers. Fine lines on the cheek before and 8 weeks after the trial are shown in Fig. 23.4 (left). Before the trial, fine lines are not obvious and fine-line shape and the area surrounded by fine lines are irregular. After ingestion of the elastin hydrolyaste-supplemented sample for 8 weeks, these items became obvious and regular. As shown in Fig. 23.5, the number of fine lines and volume of fine-line crevices on the cheek increased in both groups, which indicates that fine lines on the epidermal layer became regularly oriented. After 8 weeks, the skin texture of the elastin hydrolysate-supplemented group significantly improved on those criteria compared to controls.

As shown in Fig. 23.4 (right), wrinkles in the eye area became less obvious with ingestion of the elastin hydrolysate-supplemented sample for 8 weeks. Fig. 23.6 shows

Table 23.4 Composition of ingredients in skipjack elastin hydrolysate-supplemented and nonsupplemented individuals per day (mg).

Skin ingredient	Elastin hydrolyaste-supplemented group	Elastin hydrolysate nonsupplemented group
Elastin hydrolysate	100	0
Collagen	1000	1000
Chondroitin sulfate	100	100
Hyaluronic acid	30	30

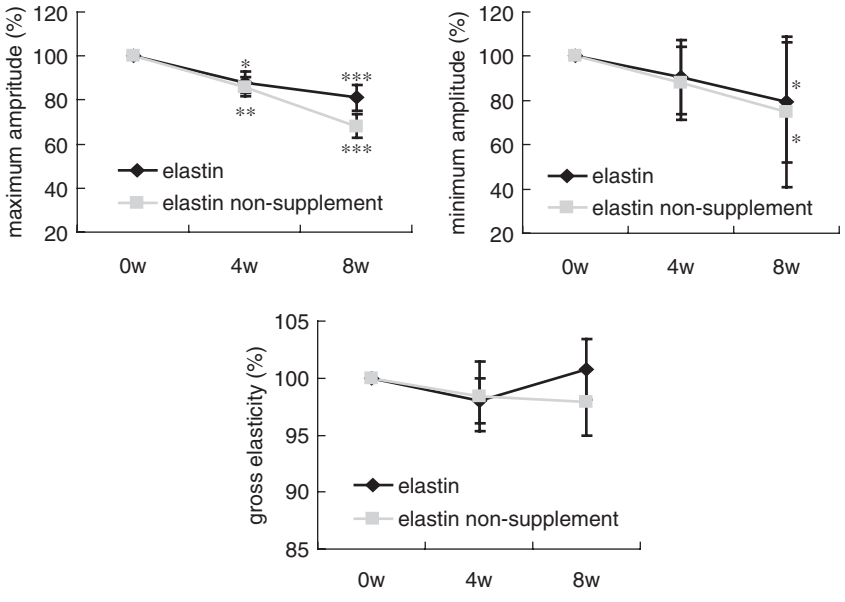

Fig. 23.3 Effect of supplementation of skipjack-elastin hydrolysate on skin dynamics. Skipjack-elastin hydrolysate was supplemented into a mixture of collagen hydrolysate, chondroichin sulfate, and hyaluronic acid. Dunnet's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus 0 weeks. Data are presented as mean ± SE.

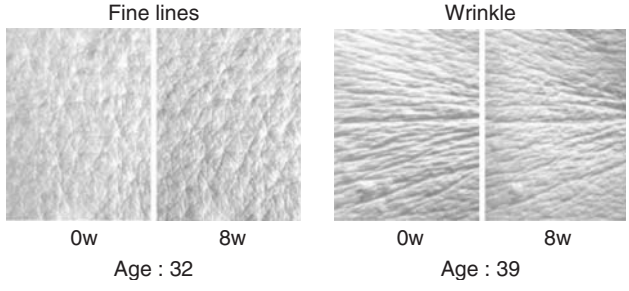

Fig. 23.4 Replica of the skin of subjects before and after ingestion of skipjack-elastin hydrolysate-supplemented sample for 8 weeks.

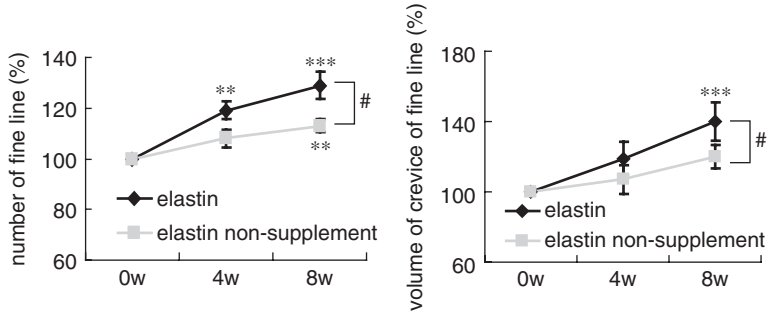

Fig. 23.5 Improvement of fine line on the skin by skipjack-elastin hydrolyaste-supplemented and nonsupplemented samples. Dunnet's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus 0 weeks. *t*-test: #, $p < 0.05$ versus elastin nonsupplement. Data are presented as mean ± SE.

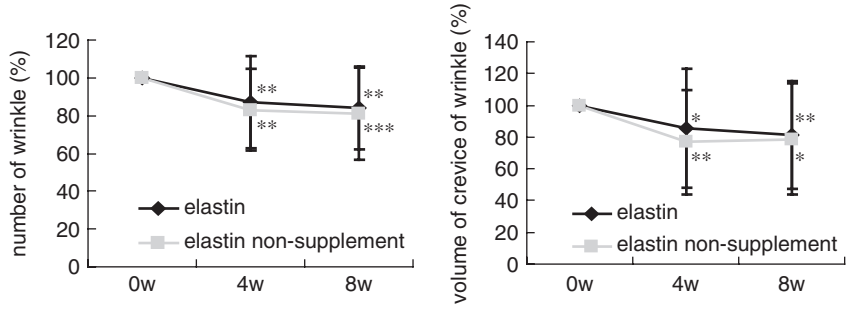

Fig. 23.6 Improvement of wrinkle by skipjack-elastin hydrolyaste-supplemented and nonsupplemented samples. Dunnet's test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus 0 weeks. Data are presented as mean ± SE.

that the number of wrinkles and the volume of wrinkle crevices significantly decreased 4 weeks after the start of the trial in both groups. There were no significant differences in improvement of winkles between the two groups.

Improvements in subjective skin conditions on ingestion of elastin hydrolysate-supplemented and nonsupplemented samples are summarized in Fig. 23.7. The elastin hydrolysate-supplemented group improved in the following items 4 weeks after the start of the trial: drying of the whole face, drying of the eye area and mouth area, dull skin, badness of smoothing make-up. In addition, fine wrinkles, tight sensations of face washing, sagging, and dark circles under the eyes were significantly improved 8 weeks after the start of the trial. The elastin hydrolysate nonsupplemented group also improved in dull skin (4 weeks), sagging, and badness of smoothing make-up (8 weeks). Compared to the elastin hydrolysate nonsupplemented group, the elastin hydrolysate-supplemented group significantly improved in fine wrinkles 8 weeks after the start of the trial. No obvious harm was attributable to the ingestion of either sample throughout the trial.

This trial started in fall and finished in winter. Generally, skin conditions become worse in winter than in fall. Nevertheless, the trial demonstrates that ingestion of a sample consisting of collagen hydrolysate, chondroichin sulfate, and hyaluronic acid improves

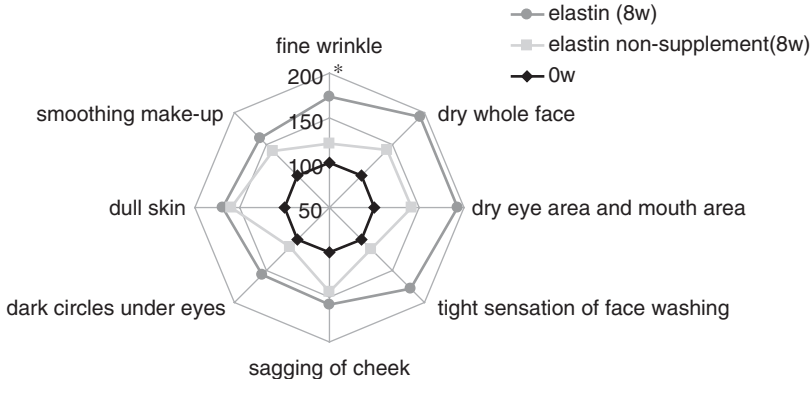

Fig. 23.7 Improvement of subjective skin conditions by skipjack-elastin hydrolyaste-supplemented and nonsupplemented samples. *t*-test (elastin versus elastin nonsupplement): *, $p < 0.05$.

some subjective and objective skin conditions in women, which supports the previous studies demonstrating the beneficial effects of collagen hydrolysate on skin conditions (Matsumoto *et al*., 2006; Takase *et al*., 2011). This trial also reveals that the addition of 100 mg of elastin hydrolysate to the collagen hydrolysate-based sample enhances the beneficial effects on women's skin.

To examine the effect of ingestion of skipjack-elastin hydrolysate alone, another trial was performed. Japanese women in their 30s claiming obvious wrinkles in the eye area, sagging of the skin, and poor blood circulation were enrolled. Subjects were divided into elastin-hydrolysate (35.7 ± 3.2 years old) and placebo (35.6 ± 3.0) groups. The placebo did not consist of collagen hydrolysate, chondroichin sulfate, and hyalronic acid in this case. The composition of ingredients in both daily samples is shown in Table 23.5. This trial started in January and finished in February.

As shown in Fig. 23.8, the skin gross elasticity significantly improved in the elastin-hydrolysate group 4 weeks after the start of the trial. Such change was not observed in the placebo group. There was no significant difference in the gross elasticity between the two groups. As shown in Fig. 23.9, wrinkles in the eye area tended to be less obvious in the elastin-hydrolysate group than in the placebo group.

Cheek skin blood flow was measured by a Laser Doppler Perfusion Imager (PeriScan PIM II). As shown in Fig. 23.10, the face skin blood flow significantly decreased in the placebo group 4 weeks after the start of trial, which could be attributed to seasonal effect. However, no significant decrease of blood flow was observed in the elastin-hydrolysate group.

Improvements in subjective skin conditions by ingestion of the skipjack-elastin hydrolysate alone are summarized in Fig. 23.11. The elastin-hydrolysate group showed

Table 23.5 Composition of ingredients in elastin-hydrolysate and placebo samples (mg per day).

Ingredient	Elastin-hydrolysate group	Placebo group
Elastin hydrolysate	75	–
Microcrystalline cellulose	87	21
Lactose	132	270
Sucrose esters	6	9

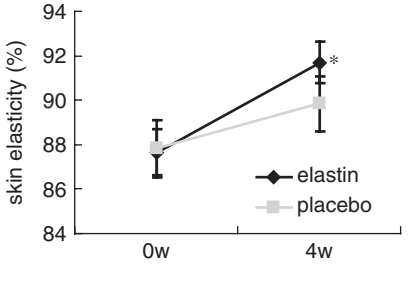

Fig. 23.8 Improvement of skin elasticity by skipjack-elastin hydrolysate alone versus placebo. Paired *t*-test (0 versus 4 weeks): *, $p < 0.05$. Data are presented as mean ± SE.

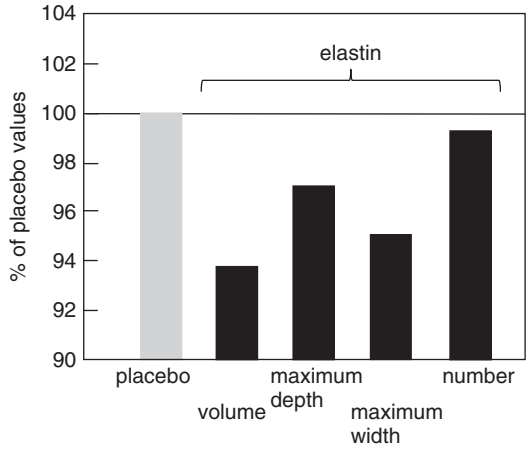

Fig. 23.9 Improvement of wrinkles by skipjack-elastin hydrolysate alone versus placebo.

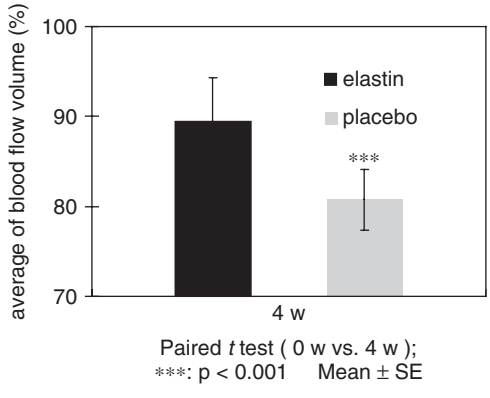

Fig. 23.10 Comparison of cheek blood-flow volumes 4 weeks after trial.

improvements in the following items compared to the placebo group: drying of the eye area and mouth area, badness of smoothing make-up, and long-lasting make-up. In comparison to the start of the trial, in most items except for flexibility of skin, subjective skin conditions improved through ingestion of the elastin hydrolysate, especially badness of smoothing make-up and long-lasting make-up.

These two human trials demonstrate the potential of elastin hydrolysate for skin-care food ingredients. Relatively small doses (75 mg/day) of elastin hydrolysate alone show beneficial effects. Furthermore, addition of elastin hydrolysate to the conventional supplement consisting of collagen hydrolysate, chondroichin sulfate, and hyaluronic acid enhances its beneficial activities. These facts suggest that skipjack-elastin hydrolysate is a promising food ingredient for improving skin condition. However, the beneficial effects of elastin hydrolysate may depend on the skin condition and eating habits of the subject,

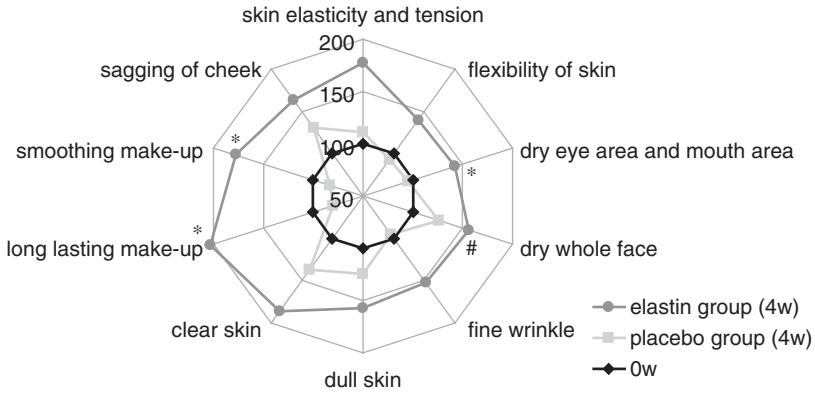

Fig. 23.11 Improvements of subjective skin conditions by skipjack-elastin hydrolysate alone versus placebo. *t*-test (elastin group versus placebo group): *, $p < 0.05$; #, $p < 0.1$.

seasonal conditions, and on on. In addition, optimization for the formulation of elastin hydrolysate and other ingredients is necessary.

23.5 IMPACT OF SKIPJACK-ELASTIN HYDROLYSATE ON BLOOD VESSELS

In addition to skin, blood vessels, especially the aorta, consist of elastin, which gives them elasticity and serves as a medium for pressure-wave propagation. Elastic fibers form lamella structures with smooth muscle in the tunica media of the aorta. The elastic fiber generally becomes thin and fragments with aging, which decreases the elasticity of the aorta. On the other hand, the tunica intima of the aorta generally thickens and accumulates collagen fibers with aging, which makes the aorta stiff. These unwelcome aging phenomena may hinder blood flow and increase blood pressure, and consequently increase the risk of atherosclerosis and myocardial infarction. As mentioned in Section 23.4, ingestion of skipjack-elastin hydrolysate improves skin elasticity, which has led to an examination of its effect on blood-vessel properties.

Two open-label studies using high and low doses (400 and 75 mg/day) of skipjack-elastin hydrolysate were performed. A total of 46 subjects (31 males and 15 females aged 43.1 ± 11.8 years) were enrolled in the high-dose trial. For the low-dose trial, 50 subjects were recruited. Of these, 25 subjects showing higher vascular aging scores were selected and enrolled (18 males and 7 females aged 45.8 ± 10.8 years). The compositions of the test samples are shown in Table 23.6. Subjects were asked to take the sample with water at bedtime and not to change their eating and exercise habits.

The hemodynamics was evaluated by acceleration plethysmography, in which blood content is detected by reflection of an infrared ray from a red cell using a finger clip and the blood's pulse waves from the heart are recorded. Acceleration plethysmography is obtained by the second derivative of the waveform of the plethysmography; it consists of a, b, c, d, and e waves. The ratios of b/a and d/a waves represent elasticity (a high

Table 23.6 Composition of ingredients in high- and low-dose trials (mg per day).

Ingredient	High dose	Low dose
Elastin hydrolysate	400	75
Microcrystalline cellulose	90	87
Lactose	–	132
Sucrose esters	10	6

value means elastic) and peripheral resistance of the artery (minus value; a small minus value means high resistance), respectively. By using these parameters, waveform index 1 (W1) is obtained: W1 = d/a − b/a. A vascular aging score is calculated formulas follows: (W1*ave*—W1*indiv*)/ W1*std* × 10 + 50, where W1*ave* is the average of W1, W1*indiv* is the individual value of W1, and W1*std* is the standard deviation of W1 (Shinohara *et al*., 2005; Takada & Okino, 2004). Additionally, blood biochemical parameters were examined.

The results of the hemodynamics in the high-dose study are shown in Fig. 23.12. The b/a value significantly decreased at 4 months, which indicates an increase in blood-vessel elasticity. On the basis of d/a value, peripheral resistance decreased at 5 months. Consistent with this, the vascular aging score decreased in a time-dependent manner and significantly decreased at 3 months compared to the baseline at the start of the study. The improvement was marked in subjects in their 40s and 50s. No significant changes in blood biochemical parameters and body weight were observed through the trial (data not shown), which indicates the safety of skipjack-elastin hydrolysate even with long-term ingestion.

The results of the low-dose trial are shown in Fig. 23.13. The blood-vessel elasticity (b/a) and peripheral resistance (d/a) were significantly improved at 16 weeks and

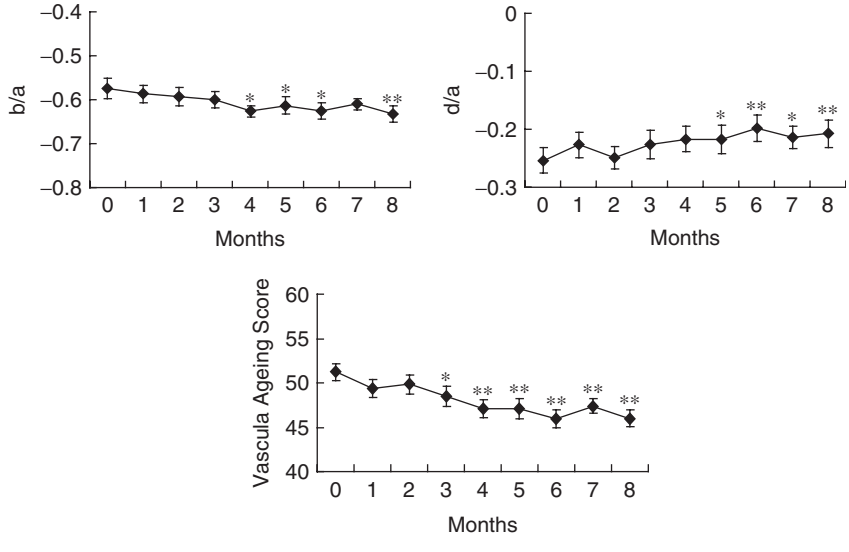

Fig. 23.12 Improvements of hemodynamics by a high dose of skipjack-elastin hydrolysate. Data are presented as mean ± SE (n = 46). Asterisks on values indicate significant difference (*, $p < 0.05$; **, $p < 0.01$). Statistical evaluation was analyzed by one-way ANOVA with Dunnett *post hoc* test.

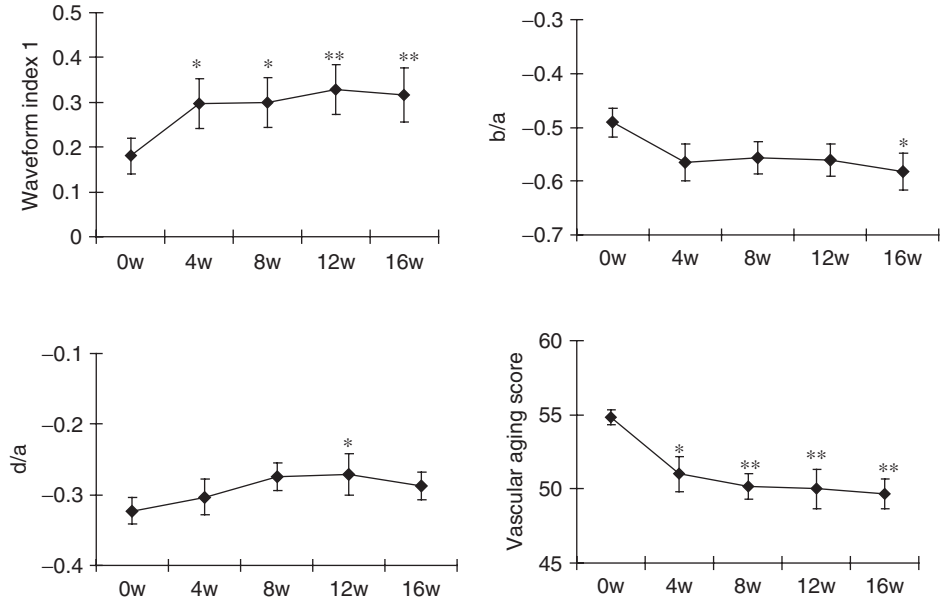

Fig. 23.13 Improvements of hemodynamics by a small dose of skipjack-elastin hydrolysate. Data are presented as mean ± SE (n = 25). Asterisks on values indicate significant difference (*, $p < 0.05$; **, $p < 0.01$).

12 weeks, respectively. W1 significantly improved at 4 weeks ($p < 5\%$) and 12 weeks ($p < 1\%$). The vascular aging score also significantly improved at 4 weeks ($p < 5\%$) and 8 weeks ($p < 1\%$).

There is a limitation in that these two trials did not use a placebo group. However, they suggest that oral ingestion of skipjack-elastin hydrolysate, even at relatively low doses (75 mg/day), could increase the elasticity of the blood vessel without adverse effects. Therefore, skipjack-elastin hydrolysate has the potential to suppress blood-vessel aging, which might reduce the risk of atherosclerosis. There is a tendency for a high vascular aging-score response in older subjects. Well-controlled trials using a placebo group should therefore be carried out to prove the blood-vessel anti-aging efficacy of skipjack-elastin hydrolysate.

23.6 SAFETY OF SKIPJACK-ELASTIN HYDROLYSATE

The skipjack bulbus arteriosus has a long history of use as a food in areas where skipjack is landed and processed. Therefore, the elastin hydrolysate from skipjack bulbus arteriosus can be considered safe. To confirm its safety, the acute toxicity and mutagenicity of skipjack-elastin hydrolysate were examined (Nakaba, 2009).

Single-dose oral toxicity to female BrlHan:WIST rat was evaluated by OECD Guideline for Testing of Chemicals 420 (2001). The rats received either skipjack-elastin hydrolysate (2000 mg/kg body weight/day) or vehicle (water) for 14 days. No deaths or obvious adverse effects were observed throughout the experiment period. There was no significant difference in body weight between test and control groups. Necropsy showed no obvious adverse effect from ingestion of skipjack-elastin hydrolysate.

Mutagenicity of the skipjack elastin hydrolysate was evaluated by umu test. DNA-damaging agents activate the umu operon. In this method, expression of the umu operon in *Salmonella typhimurium* is detected by β-galactosidase activity under the operon. Skipjack-elastin hydrolysate was used as intact and after treatment by s9mix: liver homgenate from rat with cofactors. 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) and d2-aminoanthracene (2-AA) were used as a positive control and DMSO as a negative control. AF-2 and S9mix-treated 2-AA increased β-galactosidase activity in a dose-dependent manner. No significant increase of β-galactosidase activity was observed with the addition of skipjack-elastin hydrolysate between 9.77 and 5000 μg/well (data not shown). In the human trials mentioned in Sections 23.4 and 23.5, no significant adverse effects were observed. Together with the animal experiment and umu test, it can be concluded that skipjack-elastin hydrolysate is a safe food ingredient.

23.7 IDENTIFICATION OF FOOD-DERIVED ELASTIN PEPTIDE IN HUMAN BLOOD

The human trials mentioned in Sections 23.4 and 23.5 demonstrate that ingestion of skipjack-elastin hydrolysate enhances the elasticity of the skin and blood vessels and could suppress aging of these organs. These facts suggest that ingestion of skipjack-elastin hydrolysate may increase elastin content in these organs. However, for a long time no one could explain the underlying mechanism for these effects, as it was believed that the peptides were degraded into amino acids and lost their apparent biological activity. In 2005, food-derived collagen peptides were identified in human peripheral blood (Iwai *et al*., 2005). The content of these peptides is unexpectedly high (10–100 μM) for a few hours after ingestion of collagen hydrolysate (5–20 g) (Ichikawa *et al*., 2010; Iwai *et al*., 2005; Ohara *et al*., 2007). In addition, some food-derived collagen peptides in human blood, such as Pro-Hyp and Hyp-Gly, have been demonstrated to enhance the growth of fibroblast (Shigemura *et al*. 2009, 2011) and increase synthesis of hyaluronic acid (Ohara *et al*., 2010). Fibroblast plays a significant role in the synthesis of extracellular matrix compounds. Therefore, the biological activities of food-derived collagen peptides might at least partially explain the beneficial effects from ingestion of collagen hydrolysate: increase of face skin moisture, improvement of subjective condition of the skin, moderation of osteoarthritis, and so on (Matumoto *et al*., 2006; Schauss *et al*., 2012; Takase *et al*., 2011). These facts led to an assumption that food-derived elastin peptide(s) with biological activity occur in the human blood, and research has been undertaken in search of these peptides (Shigemura *et al*., 2012).

Four healthy volunteers (average age 50.5 ± 7.9 years old) fasted for 12 hours before ingestion of skipjack-elastin hydrolysate (10 g/60 kg body weight). Plasma obtained from their venous blood was then deproteinized by the addition of three volumes of ethanol. The peptides in the ethanol-soluble fraction were fractionated by size-exclusion chromatography (SEC). The peptides in the SEC fractions were derivatized with phenyl isothiocyanate and the resultant phenyl thiocarbamyl (PTC) peptides were resolved by reversed-phase high-performance liquid chromatography (HPLC). As shown in Fig. 23.14, one specific peak appeared in SEC fractions 37–39 only after ingestion of the skipjack-elastin hydrolysate. This peak was identified as PTC-Pro-Gly by Edman degradation analysis.

To determine the Pro-Gly content of plasma after ingestion of skipjack-elastin hydrolysate, six healthy volunteers (average age 37.4 ± 14.8 years) fasted 12 hours and

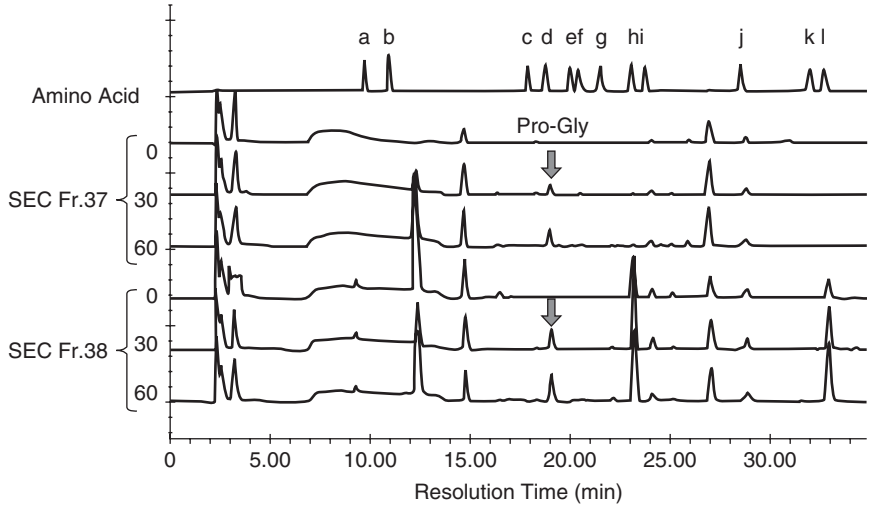

Fig. 23.14 Detection of Pro-Gly in the plasma from a subject 30 and 60 minutes after ingestion of skipjack-elastin hydrolysate. Plasma ethanol-soluble fraction was fractionated by size-exclusion chromatograpgy (SEC). SEC fraction 37 and 38 were derivatized with PITC and resolved by reversed-phase high-performance liquid chromatography (HPLC).

ingested skipjack-elastin hydrolysate (10 g/60 kg body weight). The ethanol-soluble fractions of the plasma were prepared following the same procedure as in the previous study above. Pro-Gly in the ethanol-soluble fraction was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The resultant derivative was resolved and determined by LC-MS/MS (Shigemura *et al*., 2012). As shown in Fig. 23.15, Pro-Gly reached maximum values (18 μM) 30 minutes after ingestion and was still detected 240 minutes after ingestion. Before ingestion, only negligible amounts of Pro-Gly were detected. Pro-Gly has not been detected in human blood after ingestion of enzymatic hydrolysates of collagen (Ichikawa *et al*., 2010; Iwai *et al*., 2005), soy protein or milk whey proteins

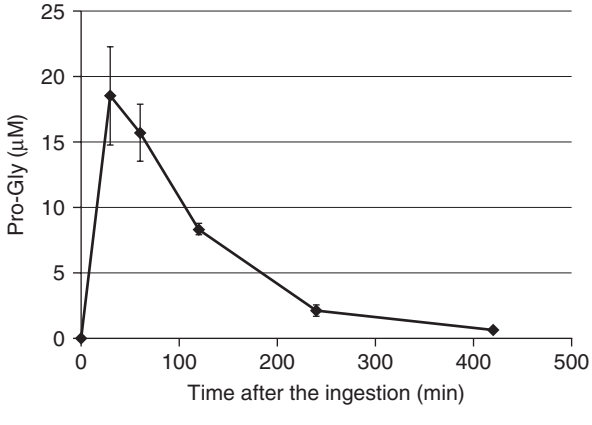

Fig. 23.15 Plasma Pro-Gly levels after ingestion of skipjack-elastin hydrolysate (10 g/60 kg body weight).

(unpublished data). Pro-Gly can thus be concluded to be a food-derived elastin peptide. The maximum value of Pro-Gly 30 minutes after ingestion of skipjack-elastin hydrolysate (10 g/60 kg of body weight) is comparable to that of food-derived collagen peptides such as Pro-Hyp, but is extensively higher than that (nM level) of angiotensin-converting enzyme (ACE)-inhibitory peptides such as Ieu-Pro-Pro (Foltz *et al*. 2007), Val-Tyr (Matsui *et al*., 2002), and so on. Pro-Gly can be derived from repeated motifs in fish elastin: Val-Pro-Gly-Val-Gly, Val-Pro-Gly (He *et al*., 2007; Miao *et al*., 2007). From the motifs Val-Gly, Gly-Val, Val-Pro could potentially be released and absorbed into the blood circulation system. However, LC-MS/MS analysis reveals that only negligible amounts of these peptides are present in human blood after ingestion of skipjack-elastin hydrolysate. These peptides might be degraded during preparation of the hydrolysate and/or in the human digestive tract and blood circulation system. Taking these facts together, Pro-Gly is a major food-derived elastin peptide that can be absorbed into the blood circulation system.

23.8 EFFECT OF FOOD-DERIVED ELASTIN-PEPTIDE PRO-GLY ON CELLS

In order to link the biological activity of ingestion of skipjack-elastin hydrolysate and the occurrence of Pro-Gly in human blood, the effects of Pro-Gly on fibroblast and vein endothelial cells have been examined (Shigemura *et al*., 2012).

A normal human dermal fibroblast (NHDF) suspension in DMEM containing 1% FBS was poured into 96 well plastic plates ($2 \times 10^4 \times$ cell/well) and the plate was placed in a humidified incubator at 37 °C under 5% CO_2. After 24 hours, 0.0, 0.1, 1.0 and 10.0 μg/ml of Pro-Gly were added to the medium. Another 3 days later, the elastin synthesis was determined by enzyme-linked immunosorbent assay (ELISA) as the concentration of tropoelastin. As shown in Fig. 23.16, addition of Pro-Gly enhanced the synthesis of elastin in a dose-dependent manner in the plasma levels of Pro-Gly. On the other hand, Pro-Gly did not significantly affect the growth of NHDF on the plastic plate. In addition, Pro-Gly did not affect the growth of mouse-skin primary cultured fibroblast on collagen gel (data not shown). It has been demonstrated that Pro-Hyp and Hyp-Gly, which are food-derived collagen peptides in human blood, can enhance the growth of mouse-skin primary cultured fibroblast on collagen gel. These facts indicate that Pro-Gly, a food-derived

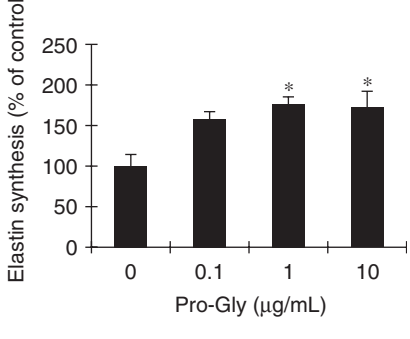

Fig. 23.16 Enhancement of elastin synthesis by human skin fibroblast with addition of Pro-Gly.

elastin peptide, acts on fibroblast in a different manner and gives a different outcome to the food-derived collagen peptides, which may explain why supplementation of skipjack-elastin hydrolysate in a collagen-hydrolysate preparation shows better effects on skin condition than the collagen hydrolysate alone.

Endothelial cells in the blood vessel tunica intima play a significant role in controlling blood flow, coagulation, and blood-vessel permeability. The functions and growth of the blood-vessel endothelial cells generally deteriorate with aging, smoking, hyperglycemia, hyperlipidemia, and so on. The human trials mentioned in Sections 23.4 and 23.5 suggest that ingestion of skipjack-elastin hydrolysate may improve blood flow and blood-vessel conditions. Thus, the effect of Pro-Gly on the growth of vein endothelial cells was examined. Addition of Pro-Gly into media slightly but significantly enhanced the growth of human umbilical-vein endothelial cells (data not shown). Although this observation remains in a preliminary state, it suggests the possibility that the food-derived elastin peptide Pro-Gly might directly act on blood-vessel endothelial cells.

These *in vitro* experiments clearly demonstrate that the food-derived elastin peptide Pro-Gly acts on fibroblasts, increasing elastin synthesis, which may at least partially explain the increase in the elasticity of the skin on ingestion of skipjack-elastin hydrolysate. The detailed mechanism underlying the improvement in subjective skin condition and wrinkles remains to be elucidated. The action of Pro-Gly on other cell types—keratinocyte, endothelial cells, and so on—and its effect on the production of other extracellular-matrix compounds should also be examined.

23.9 CONCLUSION

Enzymatic digests of elastin hydrolysates from animal tissues have been suggested to exert beneficial effects on skin conditions upon ingestion. However, little was known of the occurrence and function of fish-elastin hydrolysates. In order to obtain a suitable starter material for the production of fish-elastin hydrolysate, the contents of the elastin-specific amino acids desmosine and isodesmosine and the collagen-specific amino acid hydroxyprline were evaluated. The bulbus arteriosus was found to be rich in desmosine and isodesmosine and was thus selected to produce fish-elastin hydrolysates. The skin and other organs predominantly contain collagen as an extracellular-matrix compound. Elastin hydrolysate has been prepared from skipjack bulbus arteriosus on an industrial scale and is commercially available.

A couple of human trials have demonstrated that skipjack-elastin hydrolysate exerts beneficial effects on the skin and blood vessels in combination with a collagen-hydrolysate preparation and alone. Both subjective and physicochemical properties of skin and blood flow are improved upon ingestion of skipjack-elastin hydrolysate at a relatively low dose (75 mg/day). No adverse effect attributable to the ingestion of skipjack-elastin hydrolysate has been observed in these human trials. Acute toxicity and mutagenicity tests have also demonstrated its safety.

To explore the mechanism underlying the beneficial effects of skipjack-elastin hydrolysate, a food-derived elastin peptide in the human blood was screened. Consequently, Pro-Gly was identified. The content of Pro-Gy in human plasma is extensively higher than the reported values for the ACE-inhibitory peptide but is comparable to the values for food-derived collagen peptides such as Pro-Hyp and Hyp-Gly. Pro-Gly enhances synthesis of elastin by human dermal fibroblast and the growth of human

umbilical-vein endothelial cells. On the basis of these facts, we have proposed the hypothesis that the food-derived elastin peptide Pro-Gly is responsible for the beneficial activities of skipjack-elastin hyrdolysate.

The results of the human trials and the occurrence of food-derived elastin peptide with biological activities in human blood indicate that fish-elastin hydrolysate is a promising food ingredient for improving skin and blood-vessel conditions. This makes it to improving quality of life and preventing life-threatening diseases in aged people. Well-designed human trials should be carried out to confirm the beneficial activities of skipjack- and other fish-elastin hydrolysates and to discover their optimal doses and ideal combinations with other food ingredients. In addition, *in vitro* studies to elucidate the mechanism of the effects of Pro-Gly on cells are necessary.

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24 Free Radical-scavenging Activity of Marine Proteins and Peptides

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24.1 INTRODUCTION

The oceans, covering more than 70% of the earth's surface, are an enormous resource for the discovery of potential therapeutic agents. In recent decades, numerous novel compounds with interesting biological activities have been found from marine organisms, which have been applied in pharmaceuticals, the food industry and other fields. Therefore, marine organisms, especially novel biologically active substances such as proteins and peptides, have attracted a great deal of attention due to their potential effects in improving health and reducing disease risk (Blunt *et al*., 2006; Vo *et al*., 2010). These proteins and peptides have been obtained from algae, fish, mollusks, crustacean, shellfish and marine byproducts, including substandard muscles, viscera, skins and trimmings from manufactories. Marine bioactive proteins and peptides, based on their structural properties and amino acid compositions and sequences, have been shown to display a wide range of biological functions, such as antioxidant, antimicrobial, anticancer, anticoagulant, antihypertensive, immunological and mineral-binding effects (Betoret *et al*., 2011; Elias Kellerby & Decker, 2008; Rajanbabu & Chen, 2011).

The pressures of work and environmental pollution become higher with every passing day, leading to stress for many people. Oxidative stress forms reactive oxygen species (ROS), and if the concentration of ROS increases in a cell, it will cause cell or tissue injury, leading to cell death (Kang *et al*., 2005). This has a direct or indirect relationship with the oxidative processes of cellular components and plays an important role in various diseases, such as cancer, arthritis, inflammation, Alzheimer's, hypertension, diabetes and aging (Calabrese *et al*., 2005; Je *et al*., 2004; Ngo *et al*., 2008). This chapter focuses on the free-radical activities of proteins and peptides extracted from marine sources, as well as on some methods for assaying antioxidant activities.

24.2 FORMATION OF FREE RADICALS AND METHODS OF ASSAYING ANTIOXIDANT ACTIVITY

24.2.1 Formation of Free Radicals

ROS and reactive nitrogen species (RNS) play a significant role in the pathogenesis of free-radical formation in human cells. They are clusters of atoms, one of which contains

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an unpaired electron in its outermost shell, which is an extremely unstable configuration. 'Excited states' and radicals quickly react with other molecules or radicals to achieve a stable configuration. Therefore, free radicals are very short-lived, with half-lives in milli-, micro- or nanoseconds. ROS and RNS are both produced to help maintain the homeostasis of live cells in normal healthy tissues and both play an important role as cell-signaling molecules. Most cells can produce hydrogen peroxide (H_2O_2), nitric oxide (NO), peroxyl radical (ROO•), superoxide anion radical ($O_2^{\cdot -}$), hydrogen radical (•H) and hydroxyl radical (•OH) on demand. Free radicals have an important beneficial role and attend biochemical pathways and some mechanisms of apoptosis or defective cells. In addition, they also have a role in the killing of microorganisms and cancer cells by macrophages and cytotoxic lymphocytes. Oxygenases (e.g. cyclooxygenases, COX; lipoxygenase, LOX) and nitric oxide synthases (e.g. neuronal NOS, nNOS; inducible NOS, iNOS; endothelial NOS, eNOS) play a role in many regulatory functions through cell signals (Ngo *et al*., 2010; Valko *et al*., 2007). A great deal of research shows that ROS and RNS may act as secondary messengers to modulate signaling pathways (Dröge, 2002; Yoshikawa *et al*., 2000). If too many ROS/RNS are formed, they cause cell death molecular damage and tissue injury, leading to various diseases. However, antioxidant agents formed by enzyme such as superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase and so on (Ngo *et al*., 2012), or by non-enzymes such as natural products, peptides, proteins, carbohydrates and vitamins, help in redox cycling to balance the ROS/RNS in the system (Ngo *et al*., 2011; Valko *et al*., 2007). Therefore, marine organisms help isolate peptides and proteins to supplement foods and prevent diseases caused by free radicals.

24.2.2 Methods of Assaying Antioxidant Activity

24.2.2.1 Antioxidant Activities Using Chemical Tests

Reducing power Reducing power may be due to a hydrogen-donating ability (Shimada *et al*., 1992) and is generally associated with the presence of reductions which react with certain precursors of peroxide to prevent peroxide formation (Xing *et al*., 2005).

The reducing power of a sample is determined according to the method described by Yen & Chen (1995), with a minor modification. Briefly, 1 ml of reaction mixture, containing different concentrations of sample in phosphate buffer (0.2 M, pH 6.6), is incubated with potassium ferricyanide (1% (w/v)) at 50 °C for 20 minutes. The reaction is terminated by trichloroacetic acid (TCA) solution (10% (w/v)) and the mixture is centrifuged at 3000 rpm for 10 minutes. The supernatant is then mixed with distilled water and ferric chloride (0.1% (w/v)) solution and the absorbance is measured at 700 nm. An increased absorbance of the reaction mixture indicates increased reducing power (Ngo *et al*., 2010a).

Ferrous ion-chelating assay The degradation of lipid hydroperoxides forms peroxyl and alkoxyl radicals by stimulation of lipid peroxidation and accelerates peroxidation of ferrous ions (Fe^{2+}). The metal-chelating capacity is significant because it reduces the concentration of the catalyzing transition metal in lipid peroxidation. Ferrous ions can catalyze and produce superoxide anion to form more harmful hydroxyl radicals. Ferrozine can form complexes with ferrous ions quantitatively, and the presence of chelating agents in the red color of the complex is decreased by disruption of the complex formation. Therefore, the chelating activity can be estimated from the color reduction (Yuan *et al*., 2006).

The ferrous ion-chelating activity is measured according to the method Singh & Rajini (2004). Extracts are mixed with 0.1 mM $FeCl_2$ and then allowed to stay at room temperature for 30 seconds. The reaction is initiated by the addition of 0.25 mM ferrozine over 10 minutes, and the absorbance is measured at 562 nm wave length.

$$\text{Chelating ability (\%)} = (1 - \text{A sample/A control}) \times 100\%$$

Where A sample and A control stand for the absorbance of the sample and no sample, respectively.

DPPH Free Radical-scavenging activity Assay The principle of scavenging the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical model, which is widely used to test antioxidant activities, is based on reduction of the stable DPPH solution (purple) in the presence of a hydrogen-donating antioxidant, leading to the formation of the nonradical form, DPPH-H (yellow). The scavenging effect of the sample on DPPH free radical is measured according to the method of Shimada (1992), with a minor modification. The sample (1 ml) is added to 2 ml of 0.1 mM DPPH in 50% ethanol. The mixture is incubated for 30 minutes at the room temperature in the dark, then the absorbance of the solution is measured at 517 nm with a spectrophotometer. Scavenging DPPH-radical activity is calculated according to the following equation:

$$\text{Scavenging effect (\%)} = (1 - \text{A sample/A control}) \times 100\%$$

Where A sample and A control stand for the absorbance of the sample and no sample, respectively.

Hydroxyl Radical-scavenging Activity Assay Hydroxyl radicals are considered the most reactive oxygen radicals. The Fenton reaction, which forms hydroxyl radicals and ferric ion (Fe^{3+}), is a standard method used to determine the hydroxyl radical-scavenging effect of antioxidants. The hydroxyl radical assay is measured by Fenton reaction (Xie *et al*., 2008), with a minor modification. Briefly, the reactive mixture contains 1 ml of samples of various concentrations, 0.5 ml of Safranine-O (0.36 mM), 0.5 ml of EDTA-Fe^{2+} (2 mM) and 1.0 ml of H_2O_2 (3.0%). The mixture is incubated for 30 minutes at room temperature and its absorbance is measured at 520 nm. Hydroxyl radicals form a crimson color, so the absorbance change of the reaction mixture indicates the hydroxyl radical-scavenging effect. The hydroxyl radical-scavenging activity is calculated according to the following equation:

$$\text{Scavenging effect (\%)} = [(\text{A sample} - \text{A blank})/(\text{A control} - \text{A blank})] \times 100$$

where A sample is the absorbance of the sample, A blank is the absorbance of the blank (distilled water, instead of the samples) and A control is the absorbance of the control group (phosphate buffer, instead of H_2O_2).

Superoxide Radical-scavenging Assay Superoxide anions are a precursor to active free radicals, which are normally formed first in cellular oxidation reactions. Although they are not highly reactive, they can produce other ROS such as hydrogen peroxide, hydroxyl radical and singlet oxygen. Furthermore, superoxide anion radicals and their derivatives can cause damage in lipids, proteins and DNA. Therefore, it is of great important to scavenge

superoxide anion radicals (Xie *et al*., 2008). The scavenging effects of polysaccharide fractions on superoxide radicals are assayed by photoreduction of nitroblue tetrazolium (NBT) (Shu & Lung, 2008), with some modifications. Reaction mixtures of 3 ml contain the following reagents at final concentration: 60 μM phenazine methosulfate (PMS), 468 μM nicotinamide adenine dinucleotide (NADH), 150 mM NBT, and various concentrations of samples. The mixture is reacted at 20 °C for 10 minutes and then the absorbance is measured at 560 nm. Each value is expressed by the mean of triplicate measurements with standard deviation. The capacity to scavenge the superoxide radical is calculated using the following equation:

$$\text{Scavenging effect } (\%) = (1 - \text{A sample}/\text{A control}) \times 100\%$$

where A sample and A control stand for the absorbance of the sample and no sample, respectively.

24.2.2.2 Antioxidant Activities Using ESR Assay

Scavenging Ability on DPPH Radicals The DPPH radical-scavenging activity is measured using the method described by Nanjo *et al*. (1996). A solution of 30 μl of each sample (or distilled water as control) is added to 30 μl DPPH (60 μM) in methanol solution. After mixing vigorously for 10 seconds, the solution is transferred into a 100 μl quartz capillary tube and the scavenging activity of the sample on DPPH radical is measured using a JES-FA ESR spectrometer. The spin adduct is measured on an ESR spectrometer exactly 2 minutes later. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 30 seconds (Ngo *et al*., 2010a).

Scavenging ability on hydroxyl radicals Hydroxyl radicals are generated by iron-catalyzed Haber–Weiss reaction (Fenton-driven Haber–Weiss reaction) and rapidly reacted with nitrone spin-trap DMPO, 5,5-dimethyl-1-pyrroline-N-oxide (Je *et al*., 2004). The resultant DMPO-OH adducts are detected using an ESR spectrometer. Various concentrations of sample (20 μl) are mixed with DMPO (0.3 M, 20 μl), Fe_2SO_4 (10 mM, 20 μl) and H_2O_2 (10 mM, 20 μl) in a phosphate-buffer solution (pH 7.2) and then transferred into a 100 ml quartz capillary tube. After 2.5 minutes, the ESR spectrum is recorded using an ESR spectrometer. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 1 mW; amplitude, 1 × 200; sweep time, 30 seconds. The radical-scavenging activities of the samples are calculated as scavenging percentage by $H\ (\%) = (h_o - h_1) \times 100/h_o$, where h_o and h_1 are ESR signal intensities in the absence and the presence of sample, respectively (Ngo *et al*., 2010a).

Scavenging ability on superoxide radicals Superoxide anion radicals are generated by an ultraviolet (UV)-irradiated riboflavin/EDTA system (Guo *et al*., 1999). The reaction mixture, containing 0.3 mM riboflavin (20 μl), 1.6 mM EDTA (20 μl), 800 mM DMPO (20 μl) and indicated concentrations of FMP solution (20 μl), is irradiated for 1 minute under UV lamp at 365 nm and then transferred to a 100 μl quartz capillary tube of the ESR spectrometer for measurement. The experimental conditions employed are as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1 × 1000; sweep time, 1 minutes. Superoxide radical-scavenging ability is calculated as $H(\%) = (h_o - h_1) \times 100/h_o$; where h_o and h_1 are ESR signal intensities in the absence and the presence of sample, respectively.

Scavenging ability on alkyl radicals Alkyl radicals are generated by 2,2′-azobis-(2-amidinopropane)-hydrochloride (AAPH). A phosphate-buffered saline (PBS, 20 µl) reaction mixture containing 10 mM AAPH (20 µl), 10 mM α-(4-pyridyl-1-oxide)-N-t-butylnitrone (4-POBN, 20 µl) and sample (20 µl) at various concentrations is incubated for 30 minutes at 37 °C in a water bath (Hiramoto *et al*., 1993) and then transferred to a 100 ml quartz capillary tube. The spin adduct is recorded using an ESR spectrometer. Experimental conditions are as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; sweep time, 30 seconds (Ngo *et al*., 2010a).

24.3 FREE RADICAL-SCAVENGING ACTIVITY OF MARINE PROTEINS AND PEPTIDES

In order to protect the oxidation of cellular biomolecules (lipid, protein and DNA) in biological systems, equilibrium exists between oxidant formation and endogenous antioxidant defense mechanisms. If this balance is disturbed, it can produce oxidative stress, potentially leading to damage in plants and humans (Grabmann, 2005; Kang *et al*., 2005). This state of oxidative stress can result in injury to all important cellular components; thus, excessive generation of ROS and RNS in tissues can cause cell death. Furthermore, ROS and RNS have direct or indirect relationships with the oxidation of cellular biomolecules and play an important role in the regulation of the normal physiological processes of the human body and of many difficult therapeutic diseases (Calabrese *et al*., 2005; Park *et al*., 2004; Perry *et al*., 2000; Valko *et al*., 2007). Therefore, there has been must interest in finding antioxidant compounds from natural resources in order to overcome the radical-mediated deleterious effects in biological systems. Many biological compounds, including peptides, carbohydrates and natural products, have been identified as potent radical scavengers. In particular, peptides and proteins from marine sources present readily in seafood and help promote human health. Although there are many synthetic antioxidants, such as hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG), currently used in food, they are potential health hazards and must be strictly controlled (Branen, 1975). Hence the importance of research into natural antioxidants as safe alternatives (Park *et al*., 2004).

Much research has shown that marine organisms such as algae (Sheih *et al*., 2009), fish (Je *et al*., 2005; Slizyte *et al*., 2009), crustaceans and mollusks contain a great deal of peptides and proteins (Jung *et al*., 2005; Qian *et al*., 2008; Rajapakse *et al*., 2005; Ranathunga *et al*., 2006). Peptides and proteins with higher antioxidant activities are isolated for use as functional foods or nutrient foods. Recently, a number of algae, including macro- and microalgae, have been determined to have capacity against free radicals. The protein content is highest in red macroalgae, estimated at about 10–47% of dry weight (Fleurence, 1999). In contrast, brown macroalgae contains a low amount of protein (about 3–15% of dry weight). Further, there are qualitative differences between proteins from red and green macroalgaes. Macroalgae protein content significantly depends upon the season, with the lowest protein value being found during summer and early autumn and the highest during winter and early spring. Protein levels also depend upon location (Fleurence, 1999; Galland-Irmouli *et al*., 1999). Microalgae also have high protein content, and both macroalgae and microalgae contain essential amino acids; however, variations in their concentrations are known to occur (Galland-Irmouli *et al*., 1999). Leucine, phenylalanine

and valine are the major essential amino acids necessary for human health. Histidine is also found (Taboada *et al*., 2010). Peptides purified from *Chlorella vulgaris* demonstrate significant protective effects against cellular damage by ROS (Sheih *et al*., 2009). Phycobiliprotein, one of the major proteins in *Spirulina platensis* and *Porphyridium*, has been shown to possessantioxidant effects (Plaza *et al*., 2009).

The properties, structures and production methods of bioactive peptides and proteins are of great interest to scientists. Marine bioactive peptides are usually produced by one of the following methods: solvent extraction, enzymatic hydrolysis and microbial fermentation of proteins. The enzymatic-hydrolysis method is preferred because of the account of lack of residual organic solvents or toxic chemicals in the product. For example, antioxidant peptides isolated from oyster (*Crassostrea gigas*) or by enzymatic hydrolysis of Nile tilapia (*Oreochromis niloticus*) gelatin are not cyctotoxic to either mouse macrophages (RAW 264.7) or normal human-lung fibroblasts (MRC-5) and exhibit a protective effect against DNA damage by free radicals (Ngo *et al*., 2010b; Qian *et al*., 2008). Fish proteins derived from processing byproducts can be widely enzymatically hydrolyzed to improve their nutritional and functional characteristics, retard deterioration and remove toxic or inhibitory ingredients from waste residues of the seafood industries. The physicochemical conditions, including temperature, pH and reaction time, must be adjusted in order to optimize the activity of the enzyme and obtain a maximum yield (Shahidi & Janak Kamil, 2001).

Protein hydrolysate is produced by breaking down enzyme proteins into peptides, peptones and amino acids. Bioactive peptides isolated from various fish-protein hydrolysates have shown numerous bioactivities (Jun *et al*., 2004; Rajapakse *et al*., 2005a) and the antioxidant peptides derived from marine sources have been determined to have antioxidant activity by various *in vitro* methods, including DPPH, hydroxyl, alkyl and superoxide anion-radical scavenging effects, which have been detected by chemical or ESR spectroscopy and intracellular free radical-scavenging assays. The beneficial effects of antioxidant marine bioactive peptides are well known in scavenging of ROS and RNS and in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Sampath Kumar *et al*., 2012). In a linoleic acid-model system, lipid peroxidation is inhibited by peptide from jumbo squid. Its antioxidant effect is much higher than that of α-tocopherol and similar to that of the synthetic antioxidant BHT (Mendis *et al*., 2005). Qian *et al*. (2008) studied peptide from oyster, which exhibited higher protective activity against polyunsaturated fatty acid peroxidation than the natural antioxidant α-tocopherol. In addition, the antioxidant activity of a compound has the specific oxygen-scavenging, metal-chelating and free radical-scavenging ability to remove all ROS/RNS formed during peroxidation. Peptides isolated from marine fish proteins have greater antioxidant properties than α-tocopherol in various oxidative systems (Jun *et al*., 2004). The antioxidant activities of bioactive peptides are mainly caused by the presence of hydrophobic amino acids, and one or more residues of histidine, proline, methionine, cysteine, tyrosine, tryptophan and phenylalanine are believed to enhance the activities of the antioxidant peptides; in particular, the N-terminal histidine may contribute higher antioxidant activity to the peptides (Chen *et al*., 1996; Dávalos *et al*., 2004; Hernandez-Ledesma *et al*., 2005).

The most bioactive marine proteins and peptides used in foods are collagen and gelatin, both of which can be extracted from fish and seafood byproducts (Rasmussen & Morrissey, 2007). Collagen and gelatin are rich in nonpolar amino acids (>80%) such as glycine, alanine, valine and proline (Kim & Mendis, 2006). Collagen can be extracted from the skin, bones, cartilage and ligaments of fish-processing byproducts. It is lightly

hydrolyzed to form gelatin, which has a unique gel-forming ability (Rasmussen & Morrissey, 2007) and is used as a food supplement to increase the texture, water-holding capacity and stability of several food products. At present, gelatin can be extracted from the skins of flatfish, cold-water fish and alternative sources such as squid and octopus (Choi & Regenstein, 2000; Rasmussen & Morrissey, 2007). Fish gelatin has a better release of aroma and shows a higher digestibility than other animal proteins, because of its high percentage of glycine and proline, and has a characteristic melt-in-the-mouth property; therefore, it has great potential for application in the food and pharmaceutical industries (Gómez-Guillén *et al*., 2002).

Shahidi *et al*. (1995) demonstrated that capelin-fish protein hydrolysate added to minced pork muscle at 0.5–3.0% reduced by 17.7–60.4% the formation of secondary oxidation products including thiobarbituric acid reactive substances (TBARS). However, the use of peptides as food supplements is limited by the bitter taste of protein hydrolysates and by undesirable side effects such as reducing biological activity through molecular alteration during food processing and interaction with other food ingredients. Therefore, Shahidi *et al*. (1995) treated fish-protein hydrolysate with activated carbon to reduce its bitterness. A future challenge for food technologists will be to develop nutraceuticals and functional foods from this product. Furthermore, researchers of peptides produced from capelin protein (Amarowicz & Shahidi, 1997), prawn muscle (Suetsuna, 2000), mackerel protein (Wu *et al*., 2003), yellowfin sole-frame protein (Jun *et al*., 2004), jumbo squid skin (Mendis *et al*., 2005), hoki-frame protein (Kim *et al*., 2007), tuna-backbone protein (Je *et al*., 2007), hydrolysate of bullfrog skin (Qian *et al*., 2008), marine rotifer (Byun *et al*., 2009) and Nile tilapia (Ngo *et al*., 2010b) have reported the presence of significant free radical-scavenging activities (Table 24.1). The antioxidant activity of a peptide is closely related to its amino acid constituents and sequence (Chen *et al*., 1998; Erdmann *et al*., 2008; Pihlanto-Leppala, 2000). These short chains of amino acids are released

Table 24.1 Antioxidant peptides derived from marine organisms.

Source	Amino acid sequence	Ref.
Prawn	IKK, FKK, and FIKK	(Suetsuna, 2000)
Yellowfin sole	RPDFDLEPPY	(Jun *et al.*, 2004)
Squid	NADFGLNGLEGLA	(Rajapakse *et al.*, 2005)
	NGLEGLK	(Rajapakse *et al.*, 2005)
	FDSGPAGVL	(Mendis *et al.*, 2005)
	NGPLQAGQPGER	(Mendis *et al.*, 2005)
	GPLGLLGFLGPLGLS	(Alemán *et al.*, 2011)
Alaska Pollack	LPHSGY	(Je *et al.* 2005)
Blue mussel	HFGDPFH	(Rajapakse *et al.*, 2005b)
Conger eel	LGLNGDDVN	(Ranathunga *et al.*, 2006)
Hoki	ESTVPERTHPACPDFN	(Kim *et al.*, 2007)
Tuna	VKAGFAWTANQQLS	(Je *et al.*, 2007)
Oyster	LKQELEDLLEKQE	(Qian *et al.*, 2008)
Bullfrog skin	LEELEEELEGCE	(Qian, Jung & Kim, 2008)
Chlorella vulgaris	VECYGPNRPQF	(Sheih *et al.*, 2009)
Rotifer	LLGPGLTNHA	(Byun *et al.*, 2009)
	DLGLGLPGAH	(Byun *et al.*, 2009)
Sardinelle	LHY	(Bougatef *et al.*, 2010)
Nile tilapia	DPALATQPDPMPF	(Ngo *et al.*, 2010)
Seaweed pipefish	QLGNLGV and SVMPVVA	(Ryu *et al.* 2011)

during gastrointestinal digestion, food processing and fermentation and become active, while they remain inactive within the sequence of their parent protein. It is believed that the antioxidant peptides possess some metal-chelation or hydrogen/electron-donating activity, which could make them interact with free radicals and terminate the radical chain reaction or prevent their formation. Recent fidings from *in vitro* studies demonstrate the relationship between the structure of peptides, proteins and antioxidant activity; the Glu-Leu residue in peptides was reported to play an important role in radical scavenging (Jun *et al*., 2004) However, the sequence of the known antioxidant peptide Gln-Gly-Ala-Arg does not contain any of the previously mentioned proton-donating amino acid residues (Li *et al*., 2007). More research must be conducted into the structure–function relationship of peptides.

The molecular weight of the bioactive peptides is one of their most important functional properties. Wu *et al*. (2003) separated three peptide fractions from mackerel hydrolysate by size-exclusion chromatography. The fraction with molecular weight ~1400 Da possessed a stronger antioxidant activity than those of 900 and 200 Da according to various different antioxidant measurements, including inhibition of linoleic acid, scavenging effect on DPPH free radical and reducing power. Rajapakse *et al*. (2005a) isolated two peptides by ultrafiltration of giant squid (*Dosidicus gigas*). The fractions had molecular weights of 1307 and 747 Da and exhibited different antioxidant effects in different *in vitro* oxidative systems. However, the exact mechanism of action of peptides as free-radical scavengers is not clearly known, and must be elucidated by modern methods and further research.

24.4 CONCLUSION

Bioactive proteins are a part of our daily food intake. Their free radical-scavenging effects on the human body mainly take place in the lumen and mucosa of the digestive tract, after they are degraded to form peptides. In recent years, peptides and proteins isolated from marine organisms such as algae, invertebrates and vertebrates have been found to have various antioxidant activities and health-beneficial effects. They are assumed to be safe and efficient agents in the prevention or treatment of chronic diseases. Study of the peptides derived from marine organisms will contribute to the generation of novel functional foods and pharmaceutical products. Although a large number of antioxidant effects have been described in peptides and their structures have been clearly determined, no clinical research involving humans has yet been performed. The mechanism of the free radical-scavenging activity of marine peptides and proteins is not well known, and it will be necessary to undertake further research to demonstrate and elucidate their antioxidant activities.

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25 Marine-derived Bioactive Peptides: Their Cardioprotective Activities and Potential Applications

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25.1 INTRODUCTION

Marine biotechnology is the application of biotechnology tools to marine resources. It encompasses those efforts that involve the marine resources of the world, either as the source or the target of biotechnology applications. Biotechnology is the application of science and technology to living organisms, as well as parts, products and models thereof, in order to alter living or nonliving materials for the production of knowledge, goods and services. In the case of marine biotechnology, the living organisms derive from marine resources.

The ocean encompasses about 71% of the surface of our planet, but over 90% of the biosphere (since organisms are found throughout the water column), and represents the greatest extremes of temperature, light and pressure encountered by life. Adaptation to these harsh environments has led to a rich marine bio- and genetic diversity, with potential biotechnological applications related to drug discovery, environmental remediation, increase of seafood supply and safety and development of new resources and industrial processes (Mayekar *et al*., 2012).

Research into the pharmacological properties of marine natural products has led to the discovery of many compounds considered worthy of clinical application. There is great potential in bioprospecting from the sea, and marine natural-products research has just started to bloom. Today, marine sources have the highest probability of yielding natural products with unprecedented carbon skeletons and interesting biological activities. New habitats, such as deep ocean sample sand symbiotic systems, still have great possibilities for future research.

This chapter examines bioactive peptides which have properties relevant to cardiovascular health, including effects on blood pressure, atherosclerosis, oxidative stress, hemostasis and lipid metabolism.

Marine Proteins and Peptides: Biological Activities and Applications, First Edition. Edited by Se-Kwon Kim.

25.2 CARDIOVASCULAR DISEASES AND NUTRACEUTICALS

Acute myocardial infarction remains the leading cause of death in most Western and Asian countries. In Japan, several reports have demonstrated that the incidence of acute myocardial infarction has increased with the rapid progression of longevity and westernized lifestyle, despite recent substantial developments of appropriate diagnoses and therapeutics (Rumana *et al*., 2008). The underlying mechanisms of acute myocardial infarction include disruption of vulnerable plaques in the coronary arteries, subsequent formation of coronary thrombus and occlusion of the coronary arteries. Since atherosclerotic damage is present throughout the entire coronary artery system in patients with acute myocardial infarction (Asakura *et al*., 2001), the concept that there are vulnerable patients, not vulnerable plaque, is widely accepted (Naghavi *et al*., 2003). For these vulnerable patients, treatment with aspirin and statins achieves a reduction in mortality as well as in the occurrence of acute myocardial infarction.

If acute myocardial infarction does develop, acute-phase therapy of thrombolysis or precutaneous coronary intervention results in a marked improvement in prognosis, as has been well shown in the 20 years in which these interventions have been applied. As a consequence, the in-hospital death rate of patients with an acute myocardial infarction has decreased from approximately 20% to 5% during these 20 years. This better treatment of the acute phase of acute myocardial infarction has prevented patients with large infarct size in particular from cardiovascular death. However, as these patients easily develop post-infarction heart failure in the chronic phase, the prevention of post-infarction cardiovascular remodeling is mandatory. Several medications, such as angiotensin-converting enzyme (ACE) inhibitors (Pfeffer *et al*., 1992), aldosterone-receptor antagonists (Hayashi *et al*., 2001) and beta blockers (Dargie, 2001), have been used for the prevention of post-infarction heart failure in patients who have suffered from an acute myocardial infarction. However, these medications alone are unable to sufficiently prevent the progression and occurrence of post-infarction heart failure and left-ventricular remodeling, suggesting that the development of new therapies is required.

Recently, various cell therapies have been aggressively developed (Dimmeler *et al*., 2005), but we will have to wait several years to know their efficacy. One possibility concerns cell therapy or regenerative therapy using stem cells. Another is further adjunctive drug therapy in the acute phase in patients with myocardial infarction.

On the other hand, adjunctive drug therapies other than accepted medication have also been explored (Armstrong *et al*., 2007). Any treatment method that reduces infarct size following acute myocardial infarction is likely to be effective in suppressing the progression of post-infarction heart failure. However, no further beneficial adjunctive therapy in the acute phase of acute myocardial infarction besides ACE inhibitors, aldosterone-receptor antagonists and beta blockers has been established so far, though its developed is readily expected.

25.3 SOURCES OF MARINE PEPTIDES

Marine organisms are rich sources of structurally diverse bioactive compounds with various biological activities (Kim & Wijesekara, 2010). The importance of marine organisms as a source of novel bioactive substances is growing rapidly. With marine species

comprising approximately one-half of the total global biodiversity, the sea offers an enormous resource for novel compounds (Aneiros & Garateix, 2004; Barrow & Shahidi, 2008). Moreover, very different kinds of substance have been procured from marine organisms, because they live in very exigent, competitive and aggressive surroundings, different in many aspects from the terrestrial environment—a situation that demands the production of quite specific and potent active molecules. The marine environment serves as a source of functional materials, including polyunsaturated fatty acids (PUFAs), polysaccharides, minerals and vitamins, antioxidants, enzymes and bioactive peptides (Kim & Wijesekara, 2010; Kim *et al*., 2008; Pomponi, 1999).

There has been considerable activity in the area of marine natural products over the last 2 decades. To date, approximately 16 000 marine natural products have been isolated from marine organisms and reported in about 6800 publications. In addition, there are approximately another 9000 publications which covers syntheses, reviews, biological-activity studies, ecological studies and so forth on the subject of marine natural products. In the year 2003, over 656 marine natural products were isolated from marine organisms and reported in 243 research papers. Chemical investigation of different phyla of marine organisms has yielded different classes of compound (Ireland *et al*., 1987).

Recently, much attention has been paid to unraveling the structural, compositional and sequential properties of bioactive peptides (Kim & Wijesekara, 2010). Marine bioactive peptides may be produced by one of three methods: solvent extraction, enzymatic hydrolysis and microbial fermentation of food proteins. The enzymatic hydrolysis method is preferred, especially in the food and pharmaceutical industries, because of the lack of residual organic solvents and toxic chemicals in its products (Kim & Wijesekara, 2010). Bioactive peptides are inactive in the sequences of their parent proteins, but can be released by enzymatic hydrolysis (Kim *et al*., 1999; Lahl & Braun, 1994). They usually contain 3–20 amino acid residues and their activities are based on their amino acid composition and sequence (Pihlanto-Leppala, 2001). They have been detected in many different food sources (Dziuba *et al*., 1999; Pihlanto-Leppala *et al*., 1998). Depending on their amino acid sequence, they may possess various biological functions, including antihypertensive, immunomodulatory, antithrombotic, antioxidant, anticancer and antimicrobial activities, opioid agonism or antagonism and nutrient utilization (Clare & Swaisgood, 2000; Elias *et al*., 2008; Kim & Wijesekara, 2010). Some bioactive peptides have demonstrated multifunctional activities based on their structures and other factors, including hydrophobicity, charge and microelement-binding properties (Cho *et al*., 2008; Korhonen & Pihlanto-Leppala, 2003).

Marine bioactive peptides have been widely produced by enzymatic hydrolysis of marine organisms (Je *et al*., 2008; Slizyte *et al*., 2009). However, enzymatic hydrolysis is already carried out in the production of fermented marine food sauces, so bioactive peptides can be purified from them without further hydrolysis (Je *et al*., 2005a,b; Kim & Wijesekara, 2010). In addition, several bioactive peptides have been isolated from marine processing byproducts (Kim & Wijesekara, 2010; Kim *et al*., 2000). Marine-derived bioactive peptides have been shown to possess many physiological functions, including antihypertensive or ACE-inhibitory (Byun & Kim, 2001; Je *et al*., 2005b), antioxidant (Kim *et al*., 2007; Mendis *et al*., 2005), anticoagulant (Jo *et al*., 2008; Rajapakse *et al*., 2005a) and antimicrobial (Liu *et al*., 2008; Stensvag *et al*., 2008) activities. Moreover, some of these bioactive peptides may have potential for human health promotion and disease risk reduction (Kim & Wijesekara, 2010; Shahidi & Zhong, 2008). Thus possible

role of food-derived bioactive peptides in reducing the risk of cardiovascular disease has been well demonstrated (Erdmann *et al*., 2008).

Increasing consumer knowledge about the link between diet and health has raised awareness and demand for functional-food ingredients and nutraceuticals (Kim & Wijesekara, 2010). Bioactive peptides derived from marine organisms and marine fish-processing byproducts have potential in the development of functional foods (Shahidi, 2007). Hence, marine-derived bioactive peptides can be used as versatile raw materials for the production of nutraceuticals and pharmaceuticals for human use.

25.4 DEVELOPMENT OF MARINE BIOACTIVE PEPTIDES

A bioactive peptide generated from a particular protein is dependent on two factors: (1) the primary sequence of the protein substrate and (2) the specificity of the enzyme(s) used to generate it. Furthermore, different peptides can be generated by both acid and alkaline hydrolysis, although this approach is generally not compatible with food-ingredient manufacturing strategies. Although the structure–activity relationship of many bioactive peptides has not yet been fully established, several structural features have been identified which appear to influence their biological action. With ACE-inhibitory peptides, for example, binding of the peptide to ACE is strongly influenced by the presence of tyrosine, phenylalanine, tryptophan, proline, lysine, isoleucine, valine, leucine and arginine (Lopez-Fandino *et al*., 2006; Murray and FitzGerald, 2007). Positively charged residues are associated with the activity of antimicrobial peptides (Lopez Exposito & Recio, 2006). Amino acid residues such as histidine, leucine, tyrosine, methionine and cysteine are associated with radical-scavenging activity, while hydrophobic amino acids such as proline and hydroxyproline appear to play a role in the inhibition of lipid peroxidation (Mendis *et al*., 2005; Sarmadi & Ismail, 2010).

25.5 OXIDATIVE STRESS

The increased generation of reactive oxygen species (ROS) such as superoxide anion (O^{2-}) and hydroxyl (OH^{-}) radicals, in conjunction with the overpowering of endogenous antioxidant defense mechanisms (enzymatic and non-enzymatic), is another causative factor for the initiation of chronic diseases. These diseases include heart disease, stroke, arteriosclerosis, diabetes and cancer (Davalos *et al*., 2004). Furthermore, lipid peroxidation is a major cause of deterioration in the quality of foods (rancidity and 'off-flavors') (Di Bernardini *et al*., 2011). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate are added to food products to retard lipid oxidation. However, there are safety concerns over their use (Shahidi and Zhong, 2005). As a result, there has been increasing interest in the use of natural antioxidants, which have little or no side effects.

The exact mechanism by which peptides display antioxidant activity is not fully understood. However, research has shown that protein hydrolysates and peptides can act as radical scavengers, as well as transition-metal chelators, and exert antioxidant activities against enzymatic (lipoxygenase-mediated) and non-enzymatic peroxidation of lipids and fats (Erdmann *et al*., 2008; Sarmadi & Ismail, 2010). Furthermore, peptides have been shown to induce specific genes encoding for endogenous non-enzymatic

antioxidant components (heme oxygenase-1 and ferritin) and enzymatic systems in cell-culture models (Erdmann *et al*., 2006). Marine-derived protein hydrolysates and peptides produced from processing waste, mollusks and crustaceans have been shown to exert antioxidant activity *in vitro*.

While the exact structure–antioxidant activity relationship of peptides has not yet been established, the type, position and hydrophobicity of amino acids present in a peptide are thought to play an essential role. Amino acids such as histidine, leucine, tyrosine, methionine and cysteine are believed to enhance radical-scavenging activity by donating protons to electron-deficient radicals (Mendis *et al*., 2005; Sarmadi & Ismail, 2010). Several peptides with radical-scavenging activity derived from marine waste frame and skin, mollusks and crustaceans contain amino acids associated with proton donation (Je *et al*., 2005; Kim *et al*., 2007). Furthermore, lipid peroxidation-inhibition activities similar to the natural potent antioxidant α-tocopherol are seen with peptides derived from gelatin (Kim *et al*., 2001; Mendis *et al*., 2005). This potent lipid peroxidation-inhibition activity is thought to be associated with the presence of hydrophobic amino acids, which have high affinity for lipid systems. Oil-soluble radicals (i.e. hydrophobic peroxyl radicals) generated during oxidative attack of unsaturated fatty acids such as linoleic acid are believed to be neutralized by hydrophobic amino acid containing antioxidant peptides. Gelatin contains an abundance of hydrophobic amino acids such as glycine, valine, alanine, proline and hydroxyproline, and potentially could contain a range of peptides with potent lipid peroxidation-inhibiting activity (Kim & Mendis, 2006). The lipid peroxidation-inhibiting peptides isolated from gelatin include HGPLGPL and GE(− Hyp)GP(Hyp)GP(Hyp)GP(Hyp)GP(Hyp)G, both of which contain the characteristic repeating glycine–proline sequence associated with gelatin. Two peptides, NADFGLNGLEGLA and NGLEGLK, isolated from squid-muscle hydrolysates also contain this unusual di-amino acid (GP) repeating sequence. These peptides also inhibit the free radical-mediated oxidation of linoleic acid (Rajapakse *et al*., 2005).

25.6 ANTIHYPERTENSIVE ACTIVITY

Blood pressure is controlled by a number of different interacting biochemical pathways and can be increased or decreased depending on which pathway predominates at any given time. Classically, blood-pressure control has been associated with the renin–angiotensin system, which plays an important role in regulating arterial pressure (Dostal & Baker, 1999). Renin converts angiotensinogen in the liver to the decapeptide angiotensin I, which in turn undergoes proteolytic cleavage to the biologically active octapeptide angiotensin II. The latter step is carried out by ACE, which is highly expressed on vascular endothelium, particularly in the lungs. ACE belongs to the class of zinc proteases that require zinc and chloride for their activation. It converts the biologically inactive angiotensin I to the potent vasoconstrictor and cardiovascular trophic factor angiotensin II (Dubay *et al*., 1993). Angiotensin II has many important actions, including increasing arterial pressure, increasing sodium and fluid retention, enhancing sympathetic adrenergic function and causing cardiac and vascular remodeling. For the most part, these actions are mediated by the plasma membrane AT1 and are generally opposed by the type-2 receptor (AT2) (Messerli *et al*., 1996). The ability of ACE inhibitors and AT1-receptor antagonists to influence cardiovascular status in hypertensive conditions is consistent with an important role for the renin–angiotensin system in physiological and pathophysiological states (Mazen

et al., 2005). Thus, inhibition of this enzyme or AT1 is believed to lower blood pressure. At present, the renin–angiotensin system is a key target for drugs combating hypertension.

Hypertension is a significant health problem worldwide. It is one of the major controllable risk factors associated with cardiovascular-disease events such as myocardial infarction, heart failure and end-stage diabetes (Dezsi, 2000). Various synthetic ACE inhibitors are widely used to treat cardiovascular disorders (Jaspard *et al*., 1992). Conventional antihypertensive drugs cause various adverse effects, so cheaper, safer alternatives are desirable. Some natural or synthesized peptides which act on the renin–angiotensin system have the ability to reduce blood pressure. At present, a higher dietary protein intake seems to have a favorable influence on blood pressure in hypertensive individuals. The Dietary Approaches to Stop Hypertension trial demonstrated that a diet rich in fruits, vegetables and low-fat dairy products will reduce blood pressure effectively (Allen *et al*., 2002). Increasing consumer knowledge of the link between diet and health has raised awareness and demand for functional-food ingredients and nutraceuticals. This is leading to a mindset of self-medication, often driven by the desire to avoid undesirable side effects associated with the consumption of organically synthesized chemical drugs and to avoid the increasing cost of drug therapy. It is well recognized that apart from their basic nutritional role, many food proteins contain encrypted within their primary structures peptide sequences capable of modulating specific physiological functions. The application of specific foods or food components to the prevention and/or treatment of disease is of particular relevance to the management of hypertension (Yoshiji *et al*., 2001). Although other mechanisms play a role, ACE inhibition by bioactive peptides released from food proteins may cause these antihypertensive effects. Indeed, peptides which are derived from food have certain advantages. Several studies in spontaneously hypertensive rats (SHR) suggest a significant suppression of the development of hypertension with a diet rich in ACE-inhibitory peptides (Moskowitz, 2002). A number of research reports have also demonstrated the antihypertensive effect of ACE-inhibitory peptides or foods containing these bioactive compounds in hypertensive patients (FitzGerald & Meisel, 2000). Overall, this points to the fact that ACE-inhibitory peptides, as part of a food product or as nutraceuticals, may be of functional interest in both the treatment and the prevention of hypertension. ACE-inhibitory peptides have lower ACE-inhibitory activity *in vitro* than the ACE-inhibitory drugs, but do not have their harmful side effects. They also lower the cost of health care (Michel, 2004). These peptides need to be considered hypotensive agents. Increasingly, research is exploring the relation between these peptides and their antihypertensive effects.

25.7 ANTICOAGULANT ACTIVITY

Blood coagulation is processed by coagulation factors in order to stop the flow of blood though the injured vessel wall whenever an abnormal vascular condition and exposure to non-endothelial surface at the site of vascular injury occurs. As endogenous or exogenous anticoagulants interfere with the coagulation factors, blood coagulation can be either prolonged or stopped (Jung *et al*., 2001). These anticoagulants are used for therapeutic purposes, for example as a cure for hemophilia.

Heparin has been identified and used for more than 50 years as a commercial anticoagulant and is widely used for the prevention of venous thromboembolic disorders. However, several side effects have been identified, such as the development of thrombocytopenia,

hemorrhagic effect and ineffectiveness in congenital or acquired antithrombin bound to fibrin (Pereira *et al*., 2002). Moreover, heparin is found only in very low concentrations in pig intestine and bovine lungs, from where it is primarily extracted. Therefore, the necessity of discovering alternative sources of anticoagulant has arisen.

The anticoagulant marine bioactive peptides have rarely been reported, but they have been isolated from marine organisms such as marine echiuroid worm (Jo *et al*., 2008), starfish (Koyama *et al*., 1998) and blue mussel (Jung & Kim, 2009). Moreover, marine anticoagulant proteins have been purified from blood ark shell (Jung *et al*., 2001) and yellow fin sole (Rajapakse *et al*., 2005a). The anticoagulant activity of these peptides has been determined by prolongation of activated partial-thromboplastin-time (APTT), prothrombin-time (PT) and thrombin-time (TP) assays and compared with that of heparin, the commercial anticoagulant. The anticoagulant peptide Gly-Glu-Leu-Thr-Pro-Glu-Ser-Gly-Pro-Asp-Leu-Phe-Val-His-Phe-Leu-Asp-Gly-Asn-Pro-Tyr-Ser-Leu-Tyr-Ala-Asp-Ala-Val-Pro-Arg, isolated from marine echiuroid worm, effectively prolonged the normal clotting time on APTT from 32.3 ± 0.9 to 192.2 ± 2.1 seconds in a dose-dependent manner, with IC_{50} 42.6 μg/ml (Jo *et al*., 2008). This peptide binds specifically with clotting factor FIXa, a major component of the intrinsic tense complex, and inhibits molecular interaction between FIXa and FX in a dose-dependent manner. Moreover, the anticoagulant peptide Glu-Ala-Asp-Ile-Asp-Gly-Asp-Gly-Gln-Val-Asn-Tyr-Glu-Phe-Val-Ala-Met-Met-Thr-Ser-Lys, derived from blue mussel, showed prolongation of clotting time to 321 ± 2.1 seconds on APTT (from 35.3 ± 0.5 seconds in control) and to 81.3 ± 0.8 seconds on TT (from 11.6 ± 0.4) (Jung & Kim, 2009). In addition, a protein derived from blood arch shell prolonged the APTT clotting time from 32 to 325 seconds, and 2.8 μg/ml of heparin prolonged clotting time on APTT, PT and TT to more than 300 seconds (Jung *et al*., 2001). These marine-derived anticoagulant peptides are also noncytotoxic.

25.8 CONCLUSION

Marine-derived bioactive peptides have potential for use as functional ingredients in nutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of cardiovascular diseases. Moreover, cost-effective and safe natural health products can be produced from marine bioactive peptides, though further studies and clinical trials are needed.

The marine world has become an important source of therapeutic agents with novel mechanisms of actions. Even though thousands of new molecules are discovered every year, only a small number of candidates are incorporated in clinical trials. The problem is creating a sustainable supply of these compounds from natural resources. Various strategies have been developed to tackle this issue, such as mariculture or aquaculture of source organisms, synthetic analogues of active compounds and fermentation of the microorganisms producing the compounds. Another possible solution is the use of genetic engineering to transfer the genes encoding the synthetic enzymes that produce the desired compound to microorganisms that can be grown in huge quantities. Development of these products and services, as well as the fundamental research from which they must be derived, will be enhanced by greater reliance on interdisciplinary sciences such as pharmacology, chemical ecology, molecular biology, genomics, metagenomics, computational and combinatorial chemistry and biology. In the future, marine plants, animals and microorganisms will be the basis of new technological products and services.

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26 Biological Activities of Marine Bioactive Peptides

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26.1 INTRODUCTION

Marine organisms can serve as a source of functional materials, such as polyunsaturated fatty acids, polysaccharides, minerals and vitamins, antioxidants, enzymes and bioactive peptides. In particular, marine bioactive peptides have attracted a great deal of attention due to their potential effects in promoting health and reducing disease risk. These peptides have been produced by fish, algae, mollusc, crustacean and marine byproducts. Marine bioactive peptides can exhibit diverse activities, including antioxidant, anticancer, antihypertensive, antimicrobial, anticoagulant, opioid agonistic, immunomodulatory, prebiotic, mineral-binding and hypocholesterolemic effects (Betoret *et al*., 2011; Blunt *et al*., 2006; Rajanbabu and Chen, 2011; Vo *et al*., 2010).

Components of proteins in marine foods contain sequences of bioactive peptides that can exert a physiological effect in the body. Some of these peptides have been found to possess nutraceutical potentials of benefit in human health promotion. Moreover, the possible roles of marine bioactive peptides in reducing the risks of diseases have been reported. Bioactive peptides generally contain 3−20 amino acid units, and the amino acid composition and sequence can affect the activity of biopeptides. These short chains of amino acids are inactive within the sequence of the parent protein, but can be released during gastrointestinal digestion, food processing or fermentation (Erdmann *et al*., 2008; Pihlanto-Leppala, 2001).

Bioactive peptides can be obtained from *in vitro* enzymatic hydrolysis of different marine resources using appropriate proteolytic enzymes. Proteolytic enzymes from fish and aquatic invertebrates can be used for the hydrolysis of marine products to develop bioactive peptides and applied in the food industry. The physicochemical conditions (temperature and pH) of the reaction media must be adjusted to optimize the activity of the enzyme used (Shahidi & Janak Kamil, 2001). The crude proteinase is extracted from the pyloric caeca of tuna for enzymatic hydrolysis of cod frame protein under optimal pH and temperature conditions of the relevant enzymes to obtain a maximum yield. The molecular weight of the bioactive peptides is one of the most important factors in releasing peptides with desired functional properties (Deeslie & Cheryan, 1981). An ultrafiltration membrane reactor system is thus a suitable method for the production of bioactive peptides with specific functional properties and desired molecular size characteristics. This system's main advantage is that the molecular weight distribution of the desired functional

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peptide can be controlled by the adoption of an appropriate ultrafiltration membrane. In order to obtain functionally active peptides, a three-enzyme system for sequential enzymatic digestion can be used. Moreover, it is possible to obtain serial enzymatic digestions in a system by use of a multistep recycling membrane reactor combined with an ultrafiltration membrane system to separate marine-derived bioactive peptides (Doyen *et al*., 2011). This membrane bioreactor technology has recently emerged for the development of bioactive compounds and has potential in the utilization of marine proteins as value-added neutraceuticals with beneficial health effects. This chapter thus focuses on the biological activities of peptides derived from marine resources and their potential health-beneficial applications in the functional foods, neutraceutical and pharmaceutical industries.

26.2 PHYSIOLOGICAL PROPERTIES OF MARINE BIOACTIVE PEPTIDES

26.2.1 Antioxidant Activity

Oxidation is a vital process in aerobic organisms, particularly in vertebrates and humans, although it leads to the formation of free radicals. The formation of reactive oxygen species (ROS), including free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals (•OH) and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), is an unavoidable consequence of the body's normal use of oxygen during respiration. Free radical-mediated modification of DNA, proteins, lipids and small cellular molecules is associated with a number of pathological processes, including diabetes, atherosclerosis, arthritis, cataractogenesis, pulmonary dysfunction, muscular dystrophy, inflammatory disorders, ischemia-reperfusion tissue damage and neurological disorders such as Alzheimer's disease. Antioxidants may have a positive effect on human health as they can protect the human body against deterioration by free radicals and ROS (Butterfield *et al*., 2006).

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been used to retard peroxidation processes. However, the use of these synthetic antioxidants must be strictly controlled due to their potential health hazards. The search for a safe natural antioxidant alternative to synthetic products is therefore important in the food industry. Recently, the use of natural antioxidants found in food and other biological substances has attracted significant interest, due to their presumed safety and nutritional and therapeutic values (Ajila *et al*., 2007; Park *et al*., 2001). A number of studies have shown that peptides derived from various marine-protein hydrolysates, such as blue mussel (Jung *et al*., 2005), squid (Rajapakse *et al*., 2005), fish (Slizyte *et al*., 2009), conger eel (Ranathunga *et al*., 2006) and microalgae (Sheih *et al*., 2009), act as antioxidants. The antioxidant activity of bioactive peptides derived from marine sources has been determined by different *in vitro* methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), carbon-centered, hydroxyl and superoxide anion radical scavenging activities, which have been detected by electron spin resonance (ESR) spectroscopy and by intracellular free radical scavenging assays. The beneficial effects of antioxidant marine bioactive peptides are well known in scavenging ROS and free radicals and in preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation (Sampath Kumar *et al*., 2011). A bioactive peptide from jumbo squid inhibited lipid peroxidation in the linoleic acid model system and its activity was much higher than that of α-tocopherol, and close to

that of the highly active synthetic antioxidant BHT (Mendis *et al*., 2005). Moreover, the bioactive antioxidant peptide from oyster (*Crassostrea gigas*) exhibited higher protective activity against polyunsaturated fatty acid peroxidation than did the natural antioxidant α-tocopherol (Qian *et al*., 2008).

The antioxidant activity has been suggested to be due to either specific scavenging of oxygen-containing compounds or metal-chelating ability (scavenging of radicals formed during peroxidation). In addition, peptides isolated from marine fish proteins have greater antioxidant properties than does α-tocopherol, in various oxidative systems (Jun *et al*., 2004). The antioxidant activities of bioactive peptides are mainly due to the presence of hydrophobic amino acids, some aromatic amino acids and histidine. Gelatin peptides are rich in hydrophobic amino acids, and the abundance of these amino acids favors a higher emulsifying ability. Hence, marine gelatin peptides possess greater antioxidant effects than do peptides derived from other proteins, because of their high percentage of glycine and proline (Mendis *et al*., 2005). Antioxidant bioactive peptides derived from marine sources may thus have great potential for use as pharmaceuticals, neutraceuticals and substitutes for synthetic antioxidants. For example, Shahidi and colleagues (1995) clearly demonstrated that capelin fish protein hydrolysate, when added to minced pork muscle at a level of 0.5–3.0%, reduced the formation of secondary oxidation products, including thiobarbituric acid reactive substances (TBARS), by 17.7–60.4%. However, the bitter taste of protein hydrolysates prevents their use as food additives and their bioactivity may be reduced by molecular alteration during food processing or by interaction with other food ingredients. As a remedy to this bitterness, Shahidi and colleagues (1995) treated fish protein hydrolysate with activated carbon, which removed the bitter peptides. The challenge for food technologists will be to develop functional foods and nutraceuticals without the undesired side effects of the added peptides.

26.2.2 Antihypertensive Activity

Hypertension is a major health issue, estimated to affect about 20% of the world's adult population. The peptides regulating blood pressure are potent inhibitors of angiotensin-I-converting enzyme (ACE). Among the processes related to hypertension, ACE plays a critical role in the regulation of blood pressure, as it promotes conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II. ACE belongs to a class of zinc proteases that require both zinc and chloride for their enzymatic activity. Therefore, in the development of drugs for the control of high blood pressure, inhibition of ACE is considered a useful therapeutic approach (Shahidi & Zhong, 2008).

Currently, many natural ACE-inhibitory peptides have been isolated from different food proteins, such as cod frame, pollock skin, sea bream scales, yellowtail bone and scales, yellow sole frame, tuna frame and clam, krill, mussel, oyster and shrimp (Table 26.1). A great interest has thus arisen in bioactive peptides, which could be applied to the prevention of hypertension and the initial treatment of mildly hypertensive individuals (Guang & Phillips, 2009).

The competitiveness against ACE activity of different antihypertensive peptides has been determined kinetically using Lineweaver–Burk plots. Generally, the mechanism of action of antihypertensive peptides is different from that of synthetic drugs. Synthetic drugs basically indiscriminately block ACE by interfering with its action, while ACE-inhibitory peptides compete with ACE. ACE converts angiotensin-I to angiotensin-II by cleaving off a small peptide. Synthetic drugs work by directly blocking the action of

Table 26.1 ACE-inhibitory peptides derived from marine organisms.

Source	Enzyme	Amino acid sequence	IC_{50} (μM)	Reference
Alaska pollack	alcalase + pronase + collagenase	LGP	0.72	Byun & Kim (2001)
Wakame	protease	IW	1.50	Sato *et al.* (2002)
Sea bream	protease	VIY	7.50	Fahmi *et al.* (2004)
Alaska pollack	pepsin	FGASTRGA	14.7	Je *et al.* (2004)
Shrimp	protease	IFVPAF	3.4	Lun *et al.* (2006)
Bonito	protein	IKW	0.4	Hasan *et al.* (2007)
Hard clam	protamex	YN	51.00	Tsai *et al.* (2008)
Salmon muscle	alcalase + papain	IW	1.20	Enari *et al.* (2008)
Rotifer	alcalase	DDTGHDFEDTGEAM	9.64	Lee *et al.* (2009)
Sea cucumber	bromelain + alcalase + protease	MEGAQEAQGD	15.90	Zhao *et al.* (2009)
Microalga	pepsin	VECYGPNRPQF	29.60	Sheih *et al.* (2009)
Tuna frame	pepsin	GDLGKTTTVSNWS PPKYKDTP	11.28	Lee *et al.* (2010)

IC_{50}:50% inhibitory concentration.

ACE. ACE reacts with the antihypertensive peptides, instead of attacking angiotensin-I. Antihypertensive peptides relax the arterial walls and reduce fluid volume by inhibiting the formation of angiotensin-II. Therefore, antihypertensive peptides actually improve heart function and increase blood and oxygen flow to the heart, liver and kidneys. Many studies have shown that tryptophan, tyrosine, phenylalanine or proline at the C-terminal and branched-chain aliphatic amino acids at the N-terminal are suitable for peptide binding to ACE as a competitive inhibitor (Li *et al*., 2004).

In addition, a noncompetitive mechanism has also been observed in some peptides, which it has been suggested combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not. For example, YLYEIAR (Nakagomi *et al*., 1998) and LIY (Nakagomi *et al*., 2000) have been found to act as noncompetitive inhibitors. The hydrophobicity of the N-terminus, which is one of the common features of ACE-inhibitory peptides, may contribute to the inhibitory activity. ACE-inhibitory peptides are generally short-chain peptides, often carrying polar amino acid residues such as proline. Furthermore, structure–activity relationships among various peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides which contain hydrophobic amino acids at these positions are potent inhibitors (Qian *et al*., 2007).

Numerous *in vivo* studies of antihypertensive peptides derived from marine sources in spontaneously hypertensive rats (SHR) have shown potent ACE-inhibitory activity (Zhao *et al*., 2009). In general, the reduction in systolic blood pressure (SBP) following oral administration (10 mg/kg of body weight) of peptides was on average 25 mmHg compared to controls (Je *et al*., 2005; Lee *et al*., 2010). This antihypertensive activity was similar with captopril, a commercial antihypertensive drug. Protein hydrolysates derived from oyster proteins and sea bream scale collagen have also exhibited antihypertensive activity in SHR (Fahmi *et al*., 2004; Wang *et al*., 2008). However, variations in sample type, dosage and duration of administration make it difficult to compare these hydrolysates in terms of SBP reduction.

26.2.3 Anti-human Immunodeficiency Virus Activity

Human immunodeficiency virus type-1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS), a major human viral disease, with about 33.2 million people infected worldwide to date. Numerous studies have reported that marine bioactive peptides can be used as anti-HIV components in functional foods or nutraceuticals and pharmaceuticals due to their therapeutic potential in the treatment or prevention of infectious diseases (Table 26.2).

Lee & Maruyama (1998) searched for HIV-1 protease-inhibiting substances from oyster *Crassostrea gigas*. Two peptides inhibiting HIV-1 protease, LLEYSI and LLEYSL, were isolated from the hydrolysate of oyster proteins prepared with thermolysin. LLEYSI and LLEYSL exhibited strong inhibition of HIV-1 protease at IC_{50} (50% inhibitory concentration) values of 20 and 15 nM, respectively, and behaved as competitive inhibitors for HIV-1 protease with *Ki* values of 13 and 10 nM, respectively. Lee & Maruyama (1998) have found that the length of amino acid sequence and the presence of C-, N-terminal hydrophobic amino acids in these peptides are important to their inhibitory activity.

Sponges have traditionally been known as a source of novel bioactive peptides. The novel structural features and diverse biological activities of these peptidic metabolites have generated considerable interest. Mirabamides from the marine sponge *Siliquariaspongia mirabilis* have been shown to potently inhibit HIV-1 fusion. Among mirabamides, mirabamide A was found to powerfully inhibit HIV-1 in neutralization and fusion assays with IC_{50} values of 40 and 140 nM, respectively, while mirabamides C and D were shown to have lesser effects (IC_{50} values between 140 nM and 1.3 μM for mirabamide C and 190 nM and 3.9 μM for mirabamide D). Furthermore, mirabamides inhibited HIV-1 at the level of membrane fusion, presumably through interactions with HIV-1 envelope glycoproteins (Plaza *et al*., 2007). Celebesides A and theopapuamide B have been isolated from sponges of the same *Siliquariaspongia mirabilis*. Celebesides A is a cyclic depsipeptide incorporating a polyketide moiety and five amino acid residues, among which are the unusual amino acids phosphoserine and 3-carbamoyl threonine. Theopapuamide B is a undecapeptide comprising two previously unreported amino acids, 3-acetamido-2-aminopropanoic acid and 4-amino-2,3-dihydroxy-5-methylhexanoic acid. Theopapuamide B was active in the neutralization assay, with an IC_{50} value of 0.8 μg/ml, while celebesides A displayed an inhibition of HIV-1 entry with an IC_{50} value of 1.9 μg/ml. In addition, the anti-HIV activity of celebesides A correlates with the presence of phosphoserine residue but is absent in the inactive theopapuamide (Plaza *et al*., 2009). However, this hypothesis has been ruled out by evidence given in the study of Zampella *et al*. (2008). A novel anti-HIV cyclodepsipeptide from the marine sponge *Homophymia* sp. is homophymine A, containing an amide-linked 3-hydroxy-2,4,6-trimethyloctanoic acid moiety and 11 amino acid residues, including four unusual residues: (2*S*,3*S*,4*R*)-3,4-diMe-Gln, (2*R*,3*R*,4*S*)-4-amino-2,3-dihydroxy-1,7-heptandioic acid, L-ThrOMe and (2*R*,3*R*,4*R*)-2-amino-3-hydroxy-4,5-dimethylhexanoic acid. Obviously, homophymine A lacks β-methoxytyrosine residue, which is replaced by an *O*-methyl threonine residue, but homophymine A is reported to potentially exhibit cytoprotective activity against HIV-1 infection, with an IC_{50} value of 75 nM. The antiviral activity found in homophymine A rules out the hypothesis that β-methoxytyrosine is essential for antiviral activity.

In a similar trend, depsipeptides isolated from a number of marine sponges have been identified to be active as HIV inhibitors. Neamphamide A, a novel HIV-inhibitory depsipeptide obtained from the marine sponge *Neamphius huxleyi*, exhibited a potent

Table 26.2 HIV-1 inhibitory effects of marine peptides.

Source	Peptide name	Activity	Potency	Reference
Marine sponge Callipelta	Callipeltin A	Inhibit cytopathic effects induced by HIV-1	EC_{50}: 0.01 μg/ml	Zampella *et al.* (1996)
Oyster Crassostrea gigas	LLEYSI LLEYSL	Inhibit HIV-1 protease	IC_{50}: 20 nM 15 nM	Lee & Maruyama (1998)
Marine sponge *Theonella mirabilis* *Theonella swinhoei*	Papuamide A Papuamide B	Inhibit HIV-1 infection	EC_{50}: 4 ng/ml	Ford *et al.* (1999)
Marine sponge *Sidonops microspinosa*	Microspinosamide	Inhibit cytopathic effect of HIV-1 infection	EC_{50}: 0.2 μg/ml	Rashid *et al.* (2001)
Marine sponge *Neamphius huxleyi*	Neamphamide A	Against HIV-1 infection	EC_{50}: 28 nM	Oku *et al.* (2004)
Marine sponge Siliquariaspongia mirabilis	Mirabamide A Mirabamide C Mirabamide D	Inhibit HIV-1 neutralization and fusion	IC_{50}: 0.04 and 0.14 μM 0.14 and 1.3 μM 0.19 and 3.9 μM	Plaza *et al.* (2007)
Marine sponge *Homophymia* sp	Homophymine A	Against HIV-1 infection	IC_{50}: 75 nM	Zampella *et al.* (2008)
Marine sponge *Siliquariaspongia mirabilis*	Celebeside A Theopapuamide B	Block HIV-1 entry Neutralize HIV-1	IC_{50}: 1.9 μg/ml 0.8 μg/ml	Plaza *et al.* (2009)

IC_{50}:50% inhibitory concentration; EC_{50}:50% effective concentration.

cytoprotective activity against HIV-1 infection, with an EC_{50} (50% effective concentration) of 28 nM (Oku *et al*., 2004). Similar to neamphamide A, Callipeltin A, a novel anti-viral and anti-fungal cyclodepsipeptide from sponge of the genus *Callipelta*, exhibited the inhibition of cytopathic effects on CEM4 lymphocytic cell lines infected with HIV-1 at EC_{50} value of 0.01 μg/ml (Zampella *et al*., 1996). The general structure of callipeltin A, with the N-terminus blocked and the C-terminus lactonized with a threonine residue, is similar to that of a family of potent antitumor and antiviral didemnins which possess anti-HIV activity.

On the other hand, the novel cyclic depsipeptides papuamides A and B have been isolated from the sponges *Theonella mirabilis* and *Theonella swinhoei* (Ford *et al*., 1999). They contain not only unusual amino acids, including β-methoxytyrosine, 3-methoxyalanine, 3,4-dimethylglutamine, 2-amino-2-butenoic acid and 2,3-diaminobutanoic acid residues, but also the first peptides derived from marine sources reported to contain homoproline and 3-hydroxyleucine residues. They also contain a previously undescribed 2,3-dihydroxy-2,6,8-trimethyldeca-(4*Z*,6*E*)-dienoic acid moiety N-linked to a terminal glycine residue. They have been reported to block the infection of human T-lymphoblastoid cells by HIV-1 sub(RF) *in vitro*, with an EC_{50} of approximately 4 ng/ml. The papuamides A can block at the early stage of the viral life cycle, but not in HIV-1 envelope glycoprotein (Andjelic *et al*., 2008). Papuamide B also inhibits viral entry by interaction of this peptide with phospholipid present on the viral membrane at a concentration of 710 nM (Sagar *et al*., 2010). Another anti-HIV candidate is the microspinosamide, a new cyclic depsipeptide incorporating 13 amino acid residues isolated from the sponge *Sidonops microspinosa*. This is the first naturally occurring peptide to contain a β-hydroxy-*p*-bromophenylalanine residue. Microspinosamide inhibited the cytopathic effect of HIV-1 infection in an XTT-based *in vitro* assay, with an EC_{50} value of approximately 0.2 μg/ml (Rashid *et al*., 2001). Accordingly, sponge-derived peptides are indicated to be promising candidates for the design of novel strong inhibitors of viral infection.

26.2.4 Anticoagulant Activity

Blood coagulation is processed by coagulation factors in order to stop the flow of blood though the injured vessel wall whenever an abnormal vascular condition and exposure to non-endothelial surfaces at sites of vascular injury occur. As endogenous or exogenous anticoagulants interfere with the coagulation factors, the blood coagulation can be prolonged or stopped. These anticoagulants have been used as convenient tools for the exploration of the complex mechanisms of coagulation cascade. Coincidentally, the importance of research into anticoagulants also arose from therapeutic purposes, such as a cure for hemophilia. Heparin has been identified and used for more than 50 years as a commercial anticoagulant, and it is widely used for the prevention of venous thromboembolic disorders. However, several side effects have been identified, such as the development of thrombocytopenia, hemorrhagic effects, ineffectiveness in congenital or acquired antithrombin deficiencies and an inability to inhibit thrombin bound to fibrin. Moreover, heparin is available in very low concentrations in pig intestine or bovine lungs, from where it is primarily extracted. Therefore, the necessity of discovering alternative sources of anticoagulants has arisen with the demand for safer anticoagulant therapy (Kim & Wijesekara, 2010).

Although the anticoagulant marine bioactive peptides have rarely been reported, they have been found in marine organisms such as marine echiuroid worm (Jo *et al*., 2008), starfish (Koyama *et al*., 1998), blue mussel (Jung *et al*., 2009), yellowfin sole (Rajapakse *et al*., 2005) and blood ark shell (Jung *et al*., 2007). The anticoagulant activity of the bioactive peptides has been determined by prolongation of activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TP) assays and compared with that of heparin, the commercial anticoagulant. Rajapakse *et al*. (2005) attempted to identify a novel marine fish protein with anticoagulant and antiplatelet properties from protein sequences of yellowfin sole (*Limanda aspera*) muscle following enzymatic proteolysis. The new anticoagulant protein (12.01 kDa) inhibits coagulation *in vitro* by forming an inactive complex with FXIIa in the contact system, and the significance of the anticoagulant protein acts as a platelet aggregation inhibitor. In addition, the anticoagulant peptide EADIDGDGQVNYEEFVAMMTSK isolated from blue mussel showed prolongation of 321 ± 2.1 s clotting time on APTT (from 35.3 ± 0.5 s of control) and of 81.3 ± 0.8 s clotting time on TT (from 11.6 ± 0.4 s of control) (Jung *et al*., 2009). The anticoagulant peptide GELTPESGPDLFVHFLDGNPSYSLYADAVPR derived from marine echiuroid worm effectively prolonged the normal clotting time on APTT from 32.3 ± 0.9 to 192.2 ± 2.1 s in a dose-dependent manner with IC_{50} of 42.6 μg/ml (Jo *et al*., 2008). This peptide binds specifically with clotting factor FIXa, a major component of the intrinsic tenase complex. In addition, it inhibited molecular interaction between FIXa and FX in a dose-dependent manner. A protein derived from blood arch shell prolonged the APTT from a 32 s control clotting time to 325 s and 2.8 μg/ml of heparin, while a commercial anticoagulant prolonged the APTT by more than 300 s on APTT, PT and TT (Jung *et al*., 2001). However, these marine-derived anticoagulant peptides are noncytotoxic and have potential for use as functional ingredients in nutraceuticals or pharmaceuticals (Table 26.3).

26.2.5 Other Biological Activities

Marine peptides have also been found to exhibit antiobesity, anticancer, antidiabetic and calcium-binding activities. Obesity—an excessive body weight in the form of fat—has

Table 26.3 Other biological activities of marine peptides.

Source	Peptide name	Potency	Reference
Yellowfin sole	TDGSEDYGILEIDSR	anticoagulant	Rajapakse *et al*. (2005)
Echiuroid worm	GELTPESGPDLFVHFLDGNPSYSL YADAVPR	anticoagulant	Jo *et al*. (2008)
Blue mussel	EADIDGDGQVNYEEFVAMMTSK	anticoagulant	Jung *et al*. (2009)
Starfish	Plancinin	anticoagulant	Koyama *et al*. (1998)
Granulated ark	HTHLQRAPHPNALGYHGK	anticoagulant	Jung *et al*. (2007)
Shrimp head		appetite suppressant	Cudennec *et al*. (2008)
Shark	Insulin	antidiabetic	Anderson *et al*. (2002)
Sea hare	Dolastatin	antiproliferative	Madden *et al*. (2000)
Tuna muscle	LPHVLTPEAGAT; PTAEGGVYMVT	antiproliferative	Hsu *et al*. (2011)
Sea slug	Keenamide A	anticancer	Wesson & Hamann (1996)
Salmon	Calcitonin	Ca-binding	Kanis (2002)
Hoki frame	VLSGGTTMYASLYAE	Ca-binding	Jung & Kim (2007)
Shrimp	TCH	Ca-binding	Huang *et al*. (2011)
Pollack frame	VLSGGTTMAMYTLV	Ca-binding	Jung *et al*. (2006)

become one of the most serious public health problems. Several lines of study are searching for efficient agents and potential targets for antiobesity therapeutics. Cholecystokinin, a biomarker associated with satiety, is identified as one such promising target (Szewczyk *et al*., 2003). Meanwhile, low-molecular-weight peptides (1–1.5 kDa) from shrimp head protein hydrolysates have been found to be effective for stimulating cholecystokinin release in STC-1 cells (Cudennec *et al*., 2008). These peptides are thus suggested as a promising functional food against obesity via regulation of cholecystokinin release.

Cancer is a major cause of mortality worldwide and its incidents are rapidly increasing from year to year. According to recent studies, the anticancer activity of marine peptides has been evidenced by induction of apoptosis and inhibition of cell proliferation *in vitro* and *in vivo*. These peptides have been obtained from sea slug (Wesson & Hamann, 1996), sea hare (Madden *et al*., 2000), squid (Alemán *et al*., 2011), anchovy sauce (Lee *et al*., 2003), cod, plaice, salmon (Picot *et al*., 2006), tuna dark muscle (Hsu *et al*., 2011), fish backbone (Naqash & Nazeer, 2011) and shrimp shell (Kannan *et al*., 2011). Moreover, Wergedahl and colleagues (2004) have revealed that protein hydrolysate of salmon is able to reduce the risk of cardiovascular diseases by lowering the plasma cholesterol level and inhibiting the activity of Acyl-CoA:cholesterol acyltransferase in Zucker rats.

Components which bind and solubilize minerals such as calcium can be considered beneficial in the prevention of dental caries, osteoporosis, hypertension and anemia. Notably, some peptides derived from hoki and Alaska pollock frame proteins are known for their calcium-binding capability (Jung & Kim, 2007). Moreover, the improved calcium retention with hoki phospho-peptide intake was observed in osteoporosis-model rats to the same level as a commercially prepared casein oligophospho-peptide preparation (Jung *et al*., 2006). Calcium-binding peptides derived from marine sources may have applications as dairy-free functional food or beverage ingredients for people with lactose intolerance, as anticarcinogenic ingredients or as agents for reducing the risk of osteoporosis.

26.3 CONCLUSION

Much attention has been paid recently to marine compounds as potential safe and efficient agents in the prevention or treatment of chronic diseases. Consequently, a large number of bioactive agents from marine organisms have been identified through a variety of specific assay systems and screening approaches. Interestingly, marine peptides have been identified by their various biological activities and health-beneficial effects. Extensive studies of marine organism-derived peptides will contribute to the generation of novel functional foods and pharmaceutical products. Thus, marine peptides are believed to be a valuable source of bioactive compounds that can be introduced into the food and pharmaceutical industries.

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27 Shark Fin Cartilage: Uses, Extraction and Composition Analysis

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27.1 INTRODUCTION

The proteins belonging to the collagen family are widely distributed in animal tissues. A translucent elastic fibre embedded in shark fin's fleshy tissues is called elastoidin, or ceratotrichia, which is an unusual type of intracellular structural protein. It is classified as one of the collagen complexes, consisting of collagen and noncollagenous protein (Galloway, 1985). The name 'elastoidin' was given to this fibrous protein by Krukenberg in 1885 (Damodaran *et al*., 1956). Protein types and amino acid profiles of elastoidin extracted from shark fin are different from those of the collagen and elastin obtained from other animals (Kimura, 1992).

Elastoidin fibres are packed tightly on either side of the cartilaginous radials of the shark fin and extend to its edge, giving it some stiffness (Alexander, 1974). Shark-fin elastoidin has different characteristics from typical collagen fibre and can be distinguished from it in several respects. In particular, it does not change into gelatine when boiling (Gross, 1963). It also has high tensile strength in load-bearing connective tissues (Wainwright *et al*., 1976) and stiffness in wet or dry form (Rajaram *et al*., 1981).

Many countries target shallow-water sharks for their meat and fins. At present, annual shark production is around 629 182 tonnes. Indonesia (17%), India (11%), Spain (9%), Taiwan (7%) and Mexico (6%) are leading contributors, but many other countries contribute less than 5% of worldwide production (Lack & Sant, 2009). There are some preferred species (blue shark, hammerhead shark, dusky shark) for fins, but the fins of all shark species can be used for cartilage extraction. Due to the high value and limited amount of elastoidine extracted from shark fins, artificial shark fins have been made from other types of collagen and starch or plant gums (Chou *et al*., 1998; Vannuccini, 1999).

There has been very little scientific researches into the properties and chemical composition of shark-fin elastoidin in different shark species. This chapter gathers what information is available and reviews it scientifically, facilitating industrial understanding.

27.2 HISTORY

Characteristics such as elasticity and palatability have made shark-fin soup a delicacy in southern China, and it is commonly available in Chinese restaurants all over the world.

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It is reported in writings of the Ming Dynasty (1368–1644), when the quest to locate exotic and health-promoting foods by emperors and noblemen was met by the use of shark fins. As only a small quantity could be obtained from a large fish, fins were considered noble and precious, fit for the tables of emperors. By the Qing Dynasty (1644–1911), shark fins had become a traditional part of formal banquets. Today, fins are still served at dinner parties to express the host's respect for their guests, usually at weddings and other important functions (Vannuccini, 1999).

27.3 USES

27.3.1 Shark-fin Rays or Elastoidin

Shark fins are prized because of their fibrous cartilage, which has claimed health benefits and the ability to make a dish tender and delicate. Shark fins do not have any special taste; therefore, the fin must be cooked with various other ingredients in order to acquire any flavour. Usually crab, chicken or abalone mushroom is mixed into the soup.

The benefits of shark-fin fibrous cartilage, as documented by old Chinese medical books, include rejuvenation, appetite enhancement, nourishment of the blood, provision of vital energy and enhancement of the kidneys, lungs, bones and many other parts of the body (Vanucini, 1999). It is believed that shark fin is lipid-lowering, anti-arteriosclerosis and anticoagulant and that it prevents cardiovascular-system diseases. Some shark-fin traders claimed that consuming shark-fin fibrous cartilage daily maintained their youthful appearance, since the fins had spent so many years exercising in the sea. However, these health properties of shark fin, whether based on tradition or on scientific theory, have not been proven by accurate research studies carried out by qualified health care professionals.

27.3.2 Chondroitin Sulfate

Fin cartilage that remains after making shark-fin soup or extracting elastoidin fibre is used in the preparation of chondroitin sulfate (Fig. 27.1) for use in foods, cosmetics and medicines (Tamai *et al*., 2011). Chondroitin sulfate is a linear heteropolymer comprising D-glucuronic acid bound to *N*-acytyl-D-galactosamine by a β1-3 bond, which forms a repeated disaccharide structure. This consistent disaccharide regularly contains one molecule of *O*-sulfate per disaccharide unit. Chondroitin sulphate has many different forms and properties according to its type (A, C, D types) and the cartilages from which it is extracted.

Many researchers have examined the effects of chondroitin sulfate. Soldani *et al*., (1991)) reported that it protects existing cartilage from degradation by inhibiting the action of enzymes that attack it and stimulating the production of proteoglycans, glycosaminoglycan and collagen, all of which make new cartilage. Conrozier (1998) claimed that it helps to prevent the loss of chondrocytes due to ageing. Chondroitin sulfates increase the production of hyaluronic acid, which thickens joint fluid, providing it with better cushioning capability (Nishikawa *et al*., 1985), and has a mild anti-inflammatory effect (Ronca *et al*., 1998). Shark-fin cartilage powders or capsules are marketed as dietary supplements and claimed to combat and/or prevent a variety of illnesses. However, the benefits of this supplement have not been significantly proven or reviewed by the US Foods and Drugs Administration (FDA).

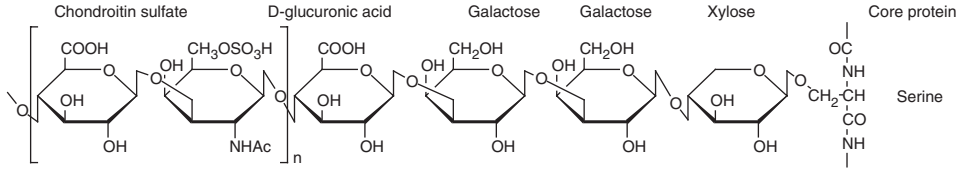

Fig. 27.1 Structure of chondroitin sulfate peptide. After Tamai *et al*. (2011). Reproduced by permission of Taylor & Francis Group.

27.4 SHARK-FIN PROCESSING

A complete set of shark fins consists of a caudal fin (upper and lower lobes), two pectoral fins, a first dorsal fin, a second dorsal fin, a pelvic fin and an anal fin (Fig. 27.2). There is high commercial demand for the lower lobe of the caudal fin, the two pectoral fins and the first dorsal fin, as these have higher amounts of thick elastoidin fibres. A shark fin has very little muscle tissue. There is a membrane, and sometimes a fatty layer under the skin, covering a bundle of collagen fibres spread out like a fan. In most fins these fibres are supported by a cartilaginous platelet in the centre. This platelet is absent in the caudal fin. The collagen fibres of the fin are rounded at the base, tapering to fine points at their extremities, giving the appearance of needles (Vannuccini, 1999). These elastoidin are commonly known as shark-fin cartilage, fin needles or fin rays.

Shark fins (wet or dry) are soaked for 12–24 hours in 3% food-grade acetic acid or coconut vinegar. The soaking time depends on the size and prior preservation technologies, such as drying or freezing. Soaking makes the skin soft. The softened skin is then removed carefully using a wire brush or a knife, without damaging or detaching the cartilaginous fibre or the fin needles, and washed with chilled water twice. The meat attached to the fin and cartilaginous base plate is removed carefully by trimming, without disturbing the fin's shape, using a sharp knife. Proper training is needed to achieve a good result in this step. After that, the fin is washed with chilled water twice, dipped in 5% hydrogen peroxide solution for 30 minutes and washed with water again. It is then dried at a controlled temperature (40–50 °C) in a mechanical dryer for a few days. The fin is turned occasionally, to facilitate uniform drying and prevent browning and curling.

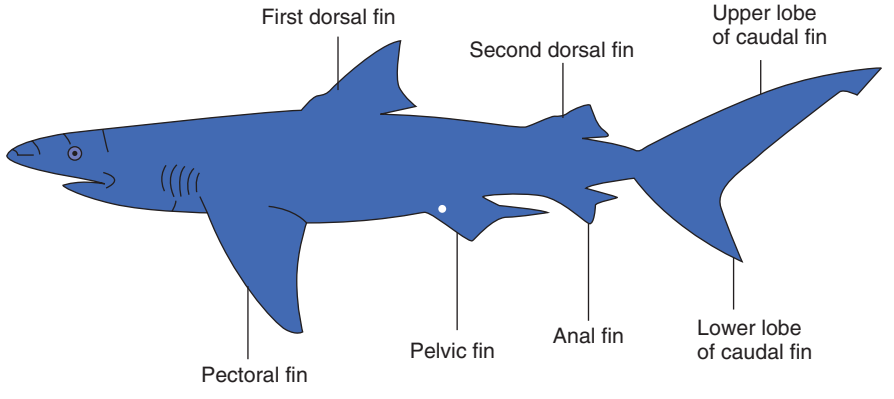

Fig. 27.2 Lateral view of a shark and its fins.

Sun drying can be also used. Excessive heat or even sun drying can result in browning and scorching of the product. This drying step also helps remove excessive H_2O_2 from the final product. The product is off-white to yellowish-white.

The data presented in Table 27.1 show that the quantity of processed fin yielded depends on the shark species and the type of fin. The lower lobe of the caudal fin has a high market value as it contains a high amount of elastoidin and no cartilaginous base plate. Hammerhead shark fin is particularly sought after as it has the highest total fin yield. Variations in the size of the fin needles are vast. Generally, the larger the fin, the longer and thicker the fin needles. The caudal fin is the largest shark fin and therefore yields the thickest and longest fin needles, followed by the first dorsal fin and then the pair of pectoral fins. The fin needles from the second dorsal fin, the pair of pelvic fins and the anal fin are considered to be of much lower quality (Vanucini, 1999).

27.5 EXTRACTION OF ELASTOIDIN AND CHONDROITIN SULFATE

Shark-fin needles or rays can be obtained directly from the wet fins or processed fins. If processed dry fins are used, they must be soaked in water for up to 12 hours, depending on their size. The fins are first boiled in water for 5–10 minutes to facilitate loosening of the fin needles from the attached membrane tissues. At this stage, the fin needles stand prominently and small ones begin to detach from the bundle. The fins are then transferred to chilled water and kept there for 15 minutes. Separation of the fin needles from the membrane can be done by pressing or kneading the base of the fin strand. The needles are further cleaned by rubbing and drying at $< 40\,^{\circ}C$ to remove attached tissues. Mesh filtering is used to collect the cleaned fin needles. After cleaning, fin needles have a very smooth, glittering surface and are white or whitish-gold in appearance. The separated fin needles are freeze-dried, cryogenically ground into a fine powder or made into a capsule form, according to their final use.

The cartilaginous base plate removed at the trimming stage contains chondroitin sulfate bound to a peptide from its core protein. This base plate is dried, powdered, decolourized, deodorized and prepared, along with the skull, spine and the bone under the cheeks, for sale in the health-food market.

27.6 COMPOSITION ANALYSIS

Nutritionally, shark fin has a high protein content (about 90%), irrespective of species. Dried processed shark fin contains 10–13% moisture and 0.16–1.00% ash (Table 27.2) (Jayasinghe *et al*., 1998). Irrespective of species, the fat content of processed shark fin is negligible (0.3–0.5%). Glycogen content varies between 0.33 and 0.87% depending on the shark species. The energy value of processed shark fins varies from 4850 to 5520 cal/g (Jayasinghe *et al*., 1998), although some sources make this somewhat lower, at 3500 cal/g (Vanucini, 1999).

Shark fins contain reasonable amounts of vitamins and minerals. Per 100 g, shark fin contains 250 mg calcium, 225 mg potassium, 117 mg phosphorus, 79.6 mg sodium, 11 mg iodine, 17 mg magnesium, 5 mg iron, 2.25 mg zinc, 72.5 µg selenium, 0.05 mg copper and 0.1 mg manganese.

Table 27.1 Processed fin (wet raw fin to processed dry fin) yield % of differernt shark species.

Type of fin	Shark species				
	Silky (*Carcharhinus falciformis*)	**Hammerhead (*Sphyrna lewini*)**	**Blue (*Prionace glauca*)**	**Oceanic white tip (*Carcharhinus longimanus*)**	**Thresher (*Alopias superciliosus*)**
Pectoral fin	12.55±0.78	11.46±0.31	7.55±0.64	12.35±1.21	5.22±0.35
First dorsal fin	17.56±0.67	29.65±0.85	15.12±0.42	14.52±0.57	2.24±0.21
Second dorsal fin	14.95±0.53	6.35±0.44	15.22±0.62	17.24±0.71	1.57±0.23
Lower lobe of caudal fin	27.75±1.13	25.18±1.36	27.54±0.98	25.26±1.03	4.97±0.53
Pelvic fin	12.53±0.71	9.85±0.51	15.13±0.86	5.25±0.46	2.47±0.22
Anal fin	9.56±0.34	15.16±0.65	9.35±0.75	27.22±1.24	2.64±0.08
Total fin yield (%)	14.0^{b} ±0.87	17.6^{a} ±0.79	12.7^{b} ±0.84	13.5^{b} ±0.92	2.0^{c} ±0.32

Each value in the table represents the mean ± standard deviation of triplicate analysis of the sample. Means within each row with different superscripts are significantly ($p < 0.05$) different.

Table 27.2 Biochemical composition and energy values of processed fins from different shark species.

Parameter	Shark species				
	Silky (*Carcharhinus falciformis*)	**Hammerhead (*Sphyrna lewini*)**	**Blue (*Prionace glauca*)**	**Oceanic white tip (*Carcharhinus longimanus*)**	**Thresher (*Alopias superciliosus*)**
Moisture (%)	$11.31^{b}\pm0.01$	$13.05^{a}\pm0.02$	$11.12^{b}\pm0.02$	$10.33^{b}\pm0.02$	$11.72^{b}\pm0.01$
Ash (%)	$0.53^{b}\pm0.00$	$1.01^{a}\pm0.02$	$0.16^{b,c}\pm0.01$	$0.24^{b,c}\pm0.02$	$0.46^{b}\pm0.03$
Total nitrogen (%)	$14.27^{a}\pm0.03$	$14.47^{a}\pm0.02$	$13.42^{b}\pm0.02$	$14.85^{a}\pm0.01$	$14.45^{a}\pm0.03$
Total protein (%)	$89.19^{a}\pm1.23$	$90.44^{a}\pm1.13$	$83.87^{b}\pm1.13$	$92.81^{a}\pm1.11$	$90.31^{a}\pm1.18$
Nonprotein nitrogen (%)	$0.46^{a,b}\pm0.01$	$0.34^{b}\pm0.00$	$0.33^{b}\pm0.00$	$0.52^{a}\pm0.05$	$0.13^{c}\pm0.02$
Fat (%)	Nil	Nil	Nil	Nil	Nil
Acid-insoluble ash (%)	Nil	Nil	Nil	Nil	Nil
Glycogen (%)	$0.73^{a}\pm0.02$	$0.87^{a}\pm0.07$	$0.78^{a}\pm0.01$	$0.65^{a}\pm0.03$	$0.33^{b}\pm0.02$
Energy value (cal/g)	4884	4863	5142	5264	5525

Each value in the table represents the mean ± standard deviation of triplicate analysis of the sample. Means within each row with different superscripts are significantly ($p < 0.05$) different.

Table 27.3 Amino acid content of processed fins of diferent shark species.

Amino acid	**Amino acid concentration, μmol/g**				
	Silky shark (*Carcharhinus falciformis*)	**Hammerhead shark (*Sphyrna lewini*)**	**Blue shark (*Prionace glauca*)**	**Oceanic White tip (*Carcharhinus longimanus*)**	**Thresher (*Alopias superciliosus*)**
Aspartic acid	145	340	320	585	125
Glutamic acid	195	550	430	705	185
Serine	190	195	330	35	230
Glycine	2115	1845	2885	2095	2150
Histidine	240	325	295	260	255
Arginine and threonine	435	535	595	630	455
Alanine	640	800	900	815	610
Proline	1220	1570	1560	550	1320
Tyrosine	260	320	385	390	245
Valine	160	250	240	265	165
Methionine	80	85	105	160	75
Cysteine	50	25	35	115	50
Isoleucine	110	160	150	190	115
Leucine	100	170	140	185	95
Phenylalanine	110	170	145	165	115
Lysine	120	185	280	270	115
Total amino acids	*6170*	*7525*	*8795*	*7415*	*6305*
Total essential amino acids	*1355*	*1880*	*1950*	*2125*	*1390*
Essential amino acid % (of total amino acids)	*28.1*	*33.3*	*28.5*	*40.2*	*28.3*

The protein component of shark fin has been reported to include both collagen and noncollageneous protein (Gross, 1963; Galloway, 1985). The collagen component of elastoidin is assumed to consist of three identical α-chains (Woodhead-Galloway *et al*., 1978). However, Kimura *et al*. (1986) analysed collagen from elastoidin by SDS-gel electrophoresis and confirmed that these appeared as a single $\alpha 1$-chain band together with crosslinked bands due to β and γ. In further purification steps it was noticed that degradation of β and γ formed crosslinked bands, providing the evidence for the occurrence of an $(\alpha 1)_3$ homotrimer alone in elastoidin. The same authors also studied the amino acid contents of both collagen and purified $\alpha 1$-chain. In another study, Chou *et al*. (1998) reported that electropherograms of proteins from natural dried shark fin were rich in three types of protein, but one type is easily degraded due to heat.

The amino acid profile of processed shark fin contains high glycine levels (approximately 2000 µmol/g), followed by alanine (approximately 600–900 µmol/g) and proline (approximately 550–1570 µmol/g), irrespective of species (Table 27.3). Many researchers have made similar observations concerning these amino acids, and all have noted the absence of the essential amino acid tryptophan (Chou *et al*., 1998: Damodaran *et al*., 1956; Jayasinghe *et al*., 1998; Kimura *et al*., 1986). Incidentally, elastoidine $\alpha 1$-chain is unique in having a higher level of isoleucine than of leucine, because the molar ratio of isoleucine to leucine is always below 1.0 for collagen $\alpha 1$-chain types in other animals (Kimura *et al*., 1986). The same tendency was observed for shark-fin elastoidine from five different shark species inhabiting the Indian Ocean (Damodaran *et al*., 1956; Jayasinghe *et al*., 1998). Essential amino acids make up 25–40% of total amino acids in shark fin. These data show that there is not much difference in the amino acid content and other biochemical compositions of processed shark fins between species.

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28 Marine Bioactive Peptide Sources: Critical Points and the Potential for New Therapeutics

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28.1 INTRODUCTION

The oceans cover more than 70% of the earth's surface, with marine species comprising approximately half of the total global biodiversity (Pangestuti & Kim, 2011). Hence, the wide diversity of marine organisms is increasingly being recognized as a rich source of functional materials, such as polysaccharides, phenolic compounds, natural pigments, fatty acids, and bioactive peptides (Shahidi, 2008; Shahidi & Alasalvar, 2011; Shahidi & Janak Kamil, 2001). Recently, marine bioactive peptides have opened a new perspective on pharmaceutical developments (Aneiros & Garateix, 2004). Bioactive peptides are specific protein fragments that have a positive impact on a body's function or condition and may ultimately influence human health. However, they must be provided by a safe, reliable, and consistent oral delivery system (Ryu *et al*., 2010a). The discovery of the bioregulatory role of different endogenous peptides in an organism and the understanding of the molecular mechanisms of action of some bioactive peptides obtained from natural sources against specific cellular targets have contributed to a belief that peptides have promise as drug candidates. The health benefits of marine bioactive peptides have been reviewed by Kim & Wijesekara (2010). Marine-derived bioactive peptides have been shown to possess many physiological functions, including antihypertensive or angiotensin-I-converting enzyme (ACE) inhibition, antioxidant, anticoagulant, and antimicrobial activities. Moreover, some of these bioactive peptides may have potential for human health promotion and disease risk reduction (Kim & Wijesekara, 2010; Shahidi & Zhong, 2008). Thus the possible role of marine food-derived bioactive peptides in reducing the risk of cardiovascular disease has been well demonstrated.

Marine bioactive peptides may be produced by several methods, including solvent extraction, enzymatic hydrolysis, and microbial fermentation. In the food and pharmaceutical industries, the enzymatic hydrolysis method is preferred because of the lack of residual organic solvents, toxic chemicals, and microbial contaminations in its products (Wijesinghe & Jeon, 2011). Enzymatic hydrolysis of proteins allows the preparation of bioactive peptides, which can be obtained by *in vitro* hydrolysis of protein sources using appropriate proteolytic enzymes. Proteolytic enzymes from microbes, plants, and animals can be used in the hydrolysis of marine proteins to develop bioactive peptides

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(Aneiros & Garateix, 2004). The physicochemical conditions of the reaction media, such as the temperature, hydrolysis time, substrate/enzyme ratio, and pH of the protein solution, must then be adjusted in order to optimize the activity of the enzyme used. Therefore, for efficient recovery and to obtain peptides with the desired biological activity and functional properties, a suitable method of enzyme optimization is needed. Fermentation, as one of the oldest food-preservation techniques specifically practiced in Asian countries, is believed to enhance the nutraceutical value of fermented foods and improve their shelf life. Breakdown of food proteins by microbial proteases to produce bioactive peptides may be one reason for the development of such properties during fermentation. Therefore, interest has grown in the identification of biological activities in enzyme hydrolysis and fermented foods, including marine algae.

The biological activities, nutritional value, and potential health benefits of marine bioactive peptides have been intensively investigated and reviewed. In spite of extensive studies and reviews on the nutritional value and potential health benefits of marine bioactive peptides, there is little available literature on the various sources of marine bioactive peptides, their development, and their prospects as new therapeutic agents. Therefore, this chapter focuses on the biological roles of marine algae and presents an overview of marine bioactive peptide sources, development, and prospects.

28.2 MARINE BIOACTIVE PEPTIDE SOURCES

28.2.1 Seaweeds

In recent years, seaweeds have served as an important source of bioactive natural substances. Many metabolites isolated from seaweeds have been shown to possess biological activity and potential health benefits. Therefore, a new trend has been to isolate and identify bioactive substances derived from seaweeds. One particularly interesting feature of seaweeds is their richness in bioactive peptides. However, it is important to take into account differences in protein content between species and also within species collected at different locations and during different seasons. Generally, the protein fraction of brown macroalgae is low (3–15% of dry weight) compared with that of green (10–26% of dry weight) and red macroalgae (35–47% of dry weight) (Fitzgerald *et al*., 2011).

These first-generation proteins extracted from seaweeds include the lectins and phycobiliproteins. Lectins are proteinaceous substances that are widely distributed in animals, plants, and microorganisms (Rogers & Hori, 1993). Seaweed lectins differ from higher-plant lectins in a variety of properties. In general, seaweed lectins have lower molecular masses than higher-plant lectins and have no affinity for simple sugars but are more specific for complex oligosaccharides. Most seaweed lectins do not require divalent cations for their biological activity and they occur mainly in monomeric forms, with a high proportion of acidic amino acids and isoelectric points from four to six. In seaweeds, lectins play an important role in recognition and adherence of gametes during sexual activity. Since they were first reported more than 5 decades ago, numerous studies have indicated that marine algae contain lectins capable of binding specific carbohydrates to produce unique biological activities, such as the aggregation of erythrocytes, yeasts, bacteria, and various unicellular algae (Boyd & Shapleigh, 1954). In addition, lectins from the red alga *Perocladiella capillacea* show potent analgesic and anti-inflammatory properties in rodent models (Silva *et al*., 2010).

Phycobiliproteins are water-soluble fluorescent proteins. They are used as accessory or antenna pigments for photosynthetic light collection, absorbing energy in portions of the visible spectrum (450–650 nm) (Sousa *et al*., 2006). Phycobilliproteins are the principal photoreceptors for photosynthesis in cyanobacteria, red algae, and cryptomonads (Glazer, 1994). In many algae, phycobiliproteins are arranged in subcellular structures called phycobilisomes, which allow the pigments to be arranged geometrically in a manner that helps optimize the capture of light and transfer of energy. The colors of the phycobiliproteins arise from the presence of covalently attached prosthetic groups—bilins—which are linear tetrapyrroles derived biosynthetically from heme via biliverdin. There are three major categories of phycobiliprotein: phycocyanins, allophycocyanins, and phycoerythtins. Phycoerythrins are the most abundant, being found in many red algae species (Bogorad, 1975; Glazer, 1994). Phycobiliproteins are used as a natural dye in the food (C-phycocyanin) and cosmetics (C-phycocyanin and R-phycoerythrin) industries. They are used as colorants in many other food products, such as fermented milk, ice creams, desserts, milk shakes, and cosmetics (Sekar & Chandramohan, 2008). Despite their lower stability in heat and light, phycobiliproteins are considered more versatile than gardenia and indigo, showing a bright blue color in jelly gum and coated soft candies. Recently, Yabuta *et al*. (2010) demonstrated antioxidant activity in phycoerythrobilin derived from *Porphyra* sp.

Digestion of seaweed proteins by proteolytic enzymes has led to the discovery of many antihypertensive peptides. Hypertension was identified as a cardiovascular risk factor in the late 1950s and still remains a public health issue today. It is often called a ‘silent killer’ because people with hypertension can be asymptomatic for years and then have a fatal heart attack or stroke (Suetsuna *et al*., 2004b). Among processes related to hypertension, ACE plays an important role in the regulation of blood pressure., as it promotes the conversion of angiotensin-I to the potent vasoconstrictor angiotensin-II and inactivates the vasodilator bradykinin, which has a depressor action in the renin–angiotensin system. Therefore, inhibition of ACE is considered a useful therapeutic approach in the treatment of hypertension (Wijesekara & Kim, 2010). Several studies have demonstrated the release of ACE-inhibitory peptides from seaweed proteins by proteolytic enzymes and hot-water extraction (Sato *et al*., 2002; Suetsuna, 1998; Suetsuna & Nakano, 2000; Suetsuna *et al*., 2004a). Antihypertensive peptides derived from seaweeds have shown potent ACE-inhibitory activities (Table 28.1). The abilityy of these seaweed-derived peptides to inhibit ACE activity is expressed as their IC_{50} value: the ACE-inhibitor concentration required for 50% inhibition of ACE activity. Structure–activity relationships among various peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate, and it is suggested that peptides which contain hydrophobic amino acids at these positions are potent ACE inhibitors (Wijesekara & Kim, 2010).

28.2.2 Seahorses

Seahorses are teleosts (bony fish), complete with gills, fins, and a swim bladder. They belong to the Syngnathidae family (from the Greek words ‘syn’, meaning ‘together’ or ‘fused’, and ‘gnathus’, meaning ‘jaws’), which also includes pipefish and seadragons (Lourie *et al*., 1999). Seahorses belong to the genus *Hippocampus* (from the Greek for ‘horse’ and ‘monster’). They are highly adapted to live among seagrass beds, mangrove roots, and coral reefs in shallow coastal tropical and temperate waters. Seahorses have long been used as one of the most famous materials in traditional medicine (Zhang *et al*.,

Table 28.1 Antihypertensive peptides derived from seaweeds.

Sequence	Source	Extraction	IC_{50}	Reference
VY	*Undaria pinnatifida*	Pronase S hydrolysis	35.2 μM	Sato *et al.* (2002)
IY	*Undaria pinnatifida*	Pronase S hydrolysis	6.1 μM	Sato *et al.* (2002)
AW	*Undaria pinnatifida*	Pronase S hydrolysis	18.8 μM	Sato *et al.* (2002)
FY	*Undaria pinnatifida*	Pronase S hydrolysis	42.3 μM	Sato *et al.* (2002)
VW	*Undaria pinnatifida*	Pronase S hydrolysis	3.3 μM	Sato *et al.* (2002)
IW	*Undaria pinnatifida*	Pronase S hydrolysis	1.5 μM	Sato *et al.* (2002)
LW	*Undaria pinnatifida*	Pronase S hydrolysis	23.6 μM	Sato *et al.* (2002)
YH	*Undaria pinnatifida*	Hot water	5.1 μM	Suetsuna *et al.* (2004)
KY	*Undaria pinnatifida*	Hot water	7.7 μM	Suetsuna *et al.* (2004)
FY	*Undaria pinnatifida*	Hot water	3.7 μM	Suetsuna *et al.* (2004)
IY	*Undaria pinnatifida*	Hot water	2.7 μM	Suetsuna *et al.* (2004)
AIYK	*Undaria pinnatifida*	Pepsin hydrolysis	213 μM	Suetsuna *et al.* (2000)
YKYY	*Undaria pinnatifida*	Pepsin hydrolysis	64.2 μM	Suetsuna *et al.* (2000)
KFYG	*Undaria pinnatifida*	Pepsin hydrolysis	90.5 μM	Suetsuna *et al.* (2000)
YNKL	*Undaria pinnatifida*	Pepsin hydrolysis	21 μM	Suetsuna *et al.* (2000)
IY	*Porphyra yezoensis*	Pepsin hydrolysis	2.65 μM	Suetsuna *et al.* (1998)
MKY	*Porphyra yezoensis*	Pepsin hydrolysis	7.26 μM	Suetsuna *et al.* (1998)
AKYSY	*Porphyra yezoensis*	Pepsin hydrolysis	1.52 μM	Suetsuna *et al.* (1998)
LRY	*Porphyra yezoensis*	Pepsin hydrolysis	5.06 μM	Suetsuna *et al.* (1998)

2003). *Hippocampus trimaculatus* is the most highly valued and heavily traded species for traditional-medicine purposes in many Asian countries (Fig. 28.1).

Traditional medicine may by defined as health practices, approaches, knowledge, and beliefs incorporating plant-, animal-, and mineral-based medicines, spiritual therapies, and manual techniques and exercises, applied singly or in combination to treat, diagnose, and prevent illnesses or maintain well-being (Shaikh & Hatcher, 2005). Traditional medicine is important for billions of people worldwide. For many in developing countries, traditional medicine is the only available form of medical care. Seahorses are one of the most important components of traditional Chinese medicine, and are used in other traditional medicines, such as Jamu (Indonesia), Hanyak (South Korea), Kanpo (Japan), and folk medicines in Malaysia, Vietnam, and Brazil. Several studies have demonstrated the health benefits of seahorses, including antifatigue, anti-aging, antioxidant, Ca^{2+}-channel-blocking, antineuroinflammatory, and anticancer effects (Himaya *et al*., 2011; Qian *et al*., 2008b). Recently, Ryu *et al*. (2010a,2010b) reported that bioactive peptides derived from seahorses inhibit collagen release, matrix metalloproteinase (MMP), and inflammatory responses in MG-63 and SW-1353 cells.

The proximate contents of several seahorse species have been investigated. A summary of proximate analyses of several seahorses species is presented in Table 28.2. Seahorses generally contain high amounts of crude protein content (>70%) (Lin *et al*., 2008, 2009). Proximate analysis also reveals that protein is the highest proximate content in seahorses, suggesting that they can be hydrolyzed to obtain bioactive peptides.

28.2.3 Seaweed Pipefish

Seaweed pipefish (*Syngnathus schlegeli*), a marine teleost fish, is well known for its special medicinal composition. Like seahorses, seaweed pipefish are one of the most famous and expensive materials in traditional Chinese medicine (Wijesekara *et al*., 2010). In the last

Fig. 28.1 *Hippocampus trimaculatus*. One special features of this species is the 'three spots' on its dorsolateral trunk surface.

3 years, our research groups have successfully identified four bioactive peptides derived from seaweed pipefish (Ryu *et al*., 2011; Wijesekara *et al*., 2010). The sequence, molecular weight, and biological activity of seaweed pipefish bioactive peptide are presented in Table 28.3. Furthermore, the antioxidative activity of two new peptides derived from seaweed pipefish—SPP-1 (QLGNLGV) and SPP-2 (SVMPVVA)—in human dermal fibroblasts (HDFs) has been investigated. Both peptides showed a significant hydroxyl-radical scavenging activity when tested by electron spin resonance (ESR) technique. The

Table 28.2 Proximate compositions of seahorse species.

	***Hippocampus kuda*[a]**	***Hippocampus trimaculatus*[b]**	***Hippocampus kellogi*[a]**
Moisture	74.58 ± 0.31	73.9 ± 0.8	73.81 ± 0.46
Crude protein	70.70 ± 2.12	78.5 ± 2.8	78.31 ± 1.74
Crude lipid	1.71 ± 0.12	1.2 ± 0.1	1.22 ± 0.25
Ash	20.92 ± 1.78	18.3 ± 2.2	16.54 ± 1.06

Results presented as percentage dry weight.
[a]Lin *et al.* (2008).
[b]Lin *et al.* (2009).

Table 28.3 Bioactive peptides derived from seaweed pipefish.

Peptide	Sequence	Molecular weight	Biological activity
SPP-1	QLGNLGV	809.97 Da	Inhibits oxidative stress
SPP-2	SVMPPA	807.9 Da	Inhibits oxidative stress
-	TFPHGP	744 Da	Antihypertensive
-	HWTTQR	917 Da	Antihypertensive

peptides effectively suppressed H_2O_2-induced radical oxygen species (ROS) production and DNA damage in HDF cells. Furthermore, the two peptides increased the expression levels of the intracellular antioxidant enzymes SOD1, GSH, and catalase, and at the cellular signaling level, SPPs block NF-κB activation, which may lead to a reduction of oxidative stress-mediated damage to HDF cells. These finding indicate the potential antioxidant effects of SPPs in response to H_2O_2 stimulation.

Collagen is a group of naturally occurring proteins. It is abundant in most invertebrates and vertebrates. It is the main protein of connective tissue and represents about one-quarter of the total protein content in many multicellular animals (Berillis *et al*., 2011). For industrial purposes, collagen is extracted mainly from the skin and bone of cattle and pigs. Highly infectious and contagious diseases such as mad cow disease (bovine spongiform encephalopathy, BSE), transmissible spongiform encephalopathy (TSE), and foot and mouth disease (FMD) have limited the industrial application of collagen derived these sources, as there is a possibility of transmission to humans (Senaratne *et al*., 2006). In addition, the collagen extracted from pigs cannot be used due to religious barriers. Therefore, many scientists have focused their efforts on the search for alternative sources of collagen. They have found that the skin, bone, fin, and scales of freshwater and marine fish, chicken skin, squid skin, octopus arms, marine sponge and bull frog skin can be used as alternatives. Seaweed pipefish contains a large quantity of collagen (Khan *et al*., 2009). Khan *et al*. (2009) isolated collagens of pipefish and characterized their various physicochemical properties, such as subunit composition, denaturation temperature, UV–vis spectra, and amino acid content in order to establish basic data for applications in industrial fields. Therefore, seaweed pipefish have potential as an important source of collagen without the threat of BSE and TSE.

28.2.4 Sea Cucumber

Sea cucumbers are soft-bodied wormlike echinoderms that belong to the class *Holothuroidae*. They are considered an important food in Asian countries, including the Philippines, Indonesia, Malaysia, Japan, Korea, and China (Himaya *et al*., 2010). In addition to their food use, sea cucumbers are also used in traditional-medicine applications in East Asia, for the treatment of asthma, hypertension, rheumatism, anemia, and sinus congestion. Moreover, the high eicosapentaenoic acid (EPA) content of sea cucumbers has been characterized as inducing tissue repair and wound healing. Solvent extracts and compounds isolated from sea cucumbers show a variety of biological activities, including antifungal, anticancer, hemolytic, cytostatic, antioxidant, and immunomodulatory effects.

Zhao *et al*. (2007) successfully hydrolyzed gelatin from a sea cucumber (*Acaudina molpadioidea*) using bromelain and alcalase and identified an ACE-inhibitory peptide. The

purified ACE-inhibitory peptide, with a molecular weight of 840 Da, consisted of five main amino acids (EDPGA), with an IC_{50} value of 0.0142 mg/ml (Zhao *et al*., 2007). Furthermore, two peptides purified from intestinal extracts of another sea cucumber (*Holothuria glaberrima*), were able to act as neurotransmitters in echinoderms. Their sequences are GFSKLYFamide and SGYSVLYFamide (Díaz-Miranda *et al*., 1992). FSKLYFamide was shown to be present in nerve fibers that apparently innervate various muscle systems. Diaz-Miranda *et al*. (1992) studied the potential neurotransmitter role of this peptide by assaying its effects on the contractility of visceral and somatic muscles. In nanomolar concentrations, GFSKLYFa induces a relaxation of the muscle tension in the intestine. A similar effect is observed on the longitudinal muscle bands of the body wall of the sea cucumber. The relaxing action of GFSKLYFa is dose–dependent, suggesting that it is mediated by receptors present in the muscle cells. In addition, GFSKLYFa induces the relaxation of the acetylcholine-contracted intestine. Díaz-Miranda & García-Arrarás's (1995) study provides additional evidence indicating that GFSKLYFa might be a neurotransmitter acting at the neuromuscular junctions of the sea cucumber *Holothuria glaberrima*.

28.2.5 Sponges

Sponges are sessile filter feeders. They were the first metazoans to evolve on earth (Müller *et al*., 2011). Sponges have traditionally been known as a source of novel bioactive metabolites such as terpenoids, alkaloids, macrolides, polyethers, nucleoside derivatives, and so on. Synthetic analogues of the C-nucleosides Spongouridine and Spongothymidine isolated from a Caribbean sponge led to the development of cytosine arabinoside, an anticancer compound. A decade ago, attention was directed at the search for bioactive peptides in sponges—a well-established sector in research into marine natural products (Aneiros & Garateix, 2004).

Bioactive peptides from sponges tend to have unprecedented structures compared to such compounds from other sources. They are often cyclic or linear peptides containing unusual amino acids which are either rare in terrestrial and microbial systems or even totally novel. They also frequently contain uncommon condensations between amino acids. Matsunaga *et al*. (1985) isolated the first bioactive peptide, discodermin A, from the marine sponge *Discodermia kiiensis*. This peptide contains the rare *tert*-leucine and cysteic acid, in addition to several D amino acids. Until this discovery, *tert*-leucine was only known as a constituent amino acid of peptidic antibiotics isolated from actinomycetes (Matsunaga *et al*., 1985).

28.2.6 Shellfish

Fish and shellfish sauces are greatly appreciated in food products in South East Asian countries such as Indonesia, Thailand, China, Japan, and Korea, where the annual production is about 250 000 metric tons. Fish and shellfish sauces have been produced by fermentation of anchovy, large-eyed herring, oyster, blue mussel, shrimp, and so on.

In Korea, the production of shellfish was estimated to be about 15 000 metric tons in 2002. In this country, fermented shellfish sauce is traditionally prepared from a mixture of shellfish by fermentation over 20% (w/w) NaCl for 6–12 months. The fermented shellfish condiments are used commercially as flavoring agents in various soups and sauces. They include a large amount of nucleic acids, amino acids, and peptides, which provide their nutrition and taste. Their biophysiological functions have thus been investigated and

identified in Asia. Two ACE-inhibitor peptides derived from fermented blue mussels and oysters have been identified. They have been evaluated for their antihypertensive effect in spontaneously hypertensive rats (SHR) following oral administration. Blood pressure significantly decreased after peptide ingestion (Je *et al*., 2005a,b). In addition, *in vitro* gastrointestinal digestion was employed to obtain a potent antioxidative peptide from the protein of oyster (*Crassostrea gias*). The amino acid sequence of this peptide was determined to be LKQELEDLLEKQE (1.60 kDa). The peptide exhibited higher activity against polyunsaturated fatty acid peroxidation than that of the native antioxidant α-tocopherol (Qian *et al*., 2008a,b). Furthermore, the hepta-peptide sequence, HFGBPFH, with molecular weight 962 kDa, derived from mussel sauce was found to be highly effective for radical scavenging. It was named 'mussel-derived radical scavenging peptide' (MRSP). Under assay conditions, MRSP could scavenge superoxide (98%), hydroxyl (96%), carbon-centered (91%), and DPPH radicals (72%) at 200 lg/ml concentration, with respective IC_{50} values of 21, 34, 52, and 96 μM. In addition, MRSP exhibited a strong lipid-peroxidation inhibition at 54 μM concentration: higher than that of α-tocopherol (Rajapakse *et al*., 2005).

28.2.7 Marine Byproducts

According to the Food and Agriculture Organization of the United Nations (FAO), capture fisheries and aquaculture supplied the world with about 110 million tonnes of fish for human consumption in 2006, providing an apparent per capita supply of 16.7 kg, which is among the highest on record. Marine capture fisheries contribute over 50% of the total world fish production. Apparently, a considerable amount of total catch is discarded as processing leftovers, including trimmings, fins, frames, heads, skin, and viscera. Therefore, there is great potential to convert and utilize more of these byproducts as valuable materials. Up till now, the majority of fisheries byproducts have been employed to produce fish oil, fishmeal, fertilizer, pet food, and fish silage. Most of these recycled products possess low economic value. Recent studies have identified a number of bioactive compounds from remaining fish-muscle proteins, collagen and gelatin, fish oil, fish bone, internal organs, and shellfish and crustacean shells. Generally, a far greater profitability and health benefit can be obtained by producing human consumables. These byproducts can be extracted and purified using both simple and complex technologies to produce bioactive peptides.

Fish internal organs represent a rich source of enzymes, and many exhibit high catalytic activity at relatively low concentrations. Considering the specific characteristics of these enzymes, fish-processing byproducts are currently used for enzyme extraction. A range of proteolytic enzymes, including pepsin, trypsin, chymotrypsin, and collagenases, are commercially extracted from marine fish viscera on a large scale (Kim & Wijesekara, 2010). Marine organisms have adapted excellently to diverse extreme environmental conditions, such as high salt concentration, low or high temperature, high pressure, and low nutrient availability. Therefore, fish proteinases are reported to possess useful properties, such as high catalytic efficiency at low temperatures, low sensitivity to substrate concentrations, and great stability across a broad pH range. These characteristics have made them suitable for various applications in many food-processing operations. Even though marine fish-derived enzymes do not have direct applications in the field of functional foods or nutraceuticals, they can be utilized to produce bioactive components on a large scale. Several studies have reported attempts to obtain crude enzyme mixtures

from the internal organs of fish species and to utilize them for the isolation of bioactive components from fish-protein hydrolysates (Kim & Mendis, 2006). In addition, chitinases and chitosanases can also be isolated from the digestive tract and other organs of some marine fish species. These enzymes promote the recovery of chitin and chitosan from marine byproducts, which are necessary for a wide array of biomedical applications. Use of these enzymes will be more economical for the isolation of bioactive compounds from marine shell waste.

28.3 CRITICAL POINTS AND THE POTENTIAL FOR NEW THERAPEUTICS

The use of marine species in traditional medicine has demonstrated great potential. However, conservation concerns must be borne in mind. Syngnathids (seahorses, pipefish, and pipehorses) are an important component of health treatments in numerous countries and their trade provides income for many fishers, pharmacists, and merchants. The World Health Organization considers traditional medicine to be a valid form of health care and thus concentrates its efforts on ensuring the sustainability of marine resources for this sector just as it would for any other form of consumption. At present, the designation of many seahorse species and some pipefish species as Vulnerable on the International Union for Conservation of Nature Red List argues that consumption should be adjusted to reduce pressure on wild populations. This means that while these species aren't endangered, their populations are facing trouble. In the last 5 years, the Syngnathid population has fallen by 50%. Trade is now restricted by CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora), but many of the biggest consumers have opted out of the convention and are still collecting seahorses in record numbers.

Two avenues of research aimed at maximizing peptide drug delivery in our bodies have been addressed: first, enhancement of peptide transport following systemic administration, and second, alternative routes of administration. Here, one must be cautious and consider the pitfalls inherent in trying to develop peptides as drugs, including issues of stability, permeability, and specificity. Stability is peptide structure-dependent and can be enhanced by amino acid substitutions, D-amino acid replacements, lipophilic extensions, and peptide cyclization (Gozes, 2001). Peptide receptor characterization leads to rational drug design and small-molecule library screening (Gozes *et al*., 2005). Taken together with specificity evaluation (i.e. blockade with a specific antagonist and side-effect monitoring), research efforts should lead to novel drug discovery. Furthermore, the development of peptides as drugs is increasingly attracting the attentions of pharmaceutical companies. It is estimated that there are more than 40 marketed peptides worldwide and hundreds more in some stage of preclinical or phased clinical development. The advantages of the use of peptides as drugs include their high specificity, potency, and activity. For late-stage research or clinical trials, it is not uncommon for many grams, or even kilograms, of high-quality peptides to be needed. Therefore, after identification of bioactive peptides derived from marine organisms, such peptides can be synthesized in order to prevent marine-animal extinction.

28.4 CONCLUSION

Marine organisms are a valuable source of bioactive peptides and may be introduced for the preparation of novel functional ingredients in foods. They also represent a good

approach to the treatment or prevention of chronic diseases. Recently, much attention has been paid by consumers to natural bioactive compounds as functional ingredients in foods. It can thus be suggested that bioactive peptides offer a good alternative source of synthetic ingredients for use in new functional foods. Furthermore, the wide range of biological activities associated with bioactive peptides derived from marine organisms has the potential to expand their health value in the food and pharmaceutical industries.

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29 Applications of Marine-derived Peptides and Proteins in the Food Industry

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29.1 INTRODUCTION

Consumption of seafood in human nutrition is well documented from ancient times. Seafood represents a diverse group of marine fish, crustaceans, mollusks, algae, seaweed and marine microorganisms. The chemical composition of seafood varies greatly among the species and from one individual to another, depending on age, sex, environmental factors and season. Among the different nutrients available in seafood, proteins plays a critical role in the growth and development of the human body, maintenance and repair of damaged tissues and production of enzymes and hormones, which aids in metabolism and maintains homeostasis (Nunes *et al*., 2011). The protein content range between 10 and 25% for most seafood species, accounting for 80–90% of the energy provided per 100 g of lean fish species, and is of good quality due to its high digestibility and specific amounts, availability and relative proportions of essential amino acids (Nunes *et al*., 2011). Recently, marine protein and bioactive peptides (BAPs) have gained much attention for their therapeutic effects, such as antihypertensive, antiproliferative, anticoagulant, calcium-binding, anti-obesity and antidiabetic activities (Ngo *et al*., 2012).

Apart from its nutritive and health-promoting value, seafood contains a vast variety of specific and unique substances not found in the terrestrial animals, or which are known from terrestrial sources but have novel properties. As the marine species make up approximately half of the total global biodiversity, the ocean offers an enormous resource for novel compounds, including proteins and peptides with specific bioactivities (Aneiros & Garateix, 2004; Kim & Wijesekara, 2010). Living in diverse, competitive and aggressive surroundings, very different in many aspects from the terrestrial environment, with a wide range of temperatures, salinities, pHs and pressures, allow these organisms to secrete unique substances either as part of a defensive mechanism or as an adaptation to the particular environment. Extraction of these substances for use in pharmaceutical developments and the food industry has gained much attention in the past decade (Aneiros & Garateix, 2004). Among the different bioactive proteins and peptides found from these marine sources, enzymes, collagen and gelatin, antifreeze proteins (AFPs), antimicrobials,

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antioxidants and other BAPs with different bioactivities are found to have a variety of food applications which can be exploited either now or in the future in order to obtain specific characteristics that cannot be accomplished by the already available commercial ingredients. For instance, cold-adaptive enzymes extracted from marine sources have been successfully employed as biocatalysts where mild reactions are necessary with a wide range of pH, for the manufacture a range of fish products such as surimi, fish sauce, ripened fish and fish-protein hydrolysate, as well as for the extraction of certain bioactive substances such as chitin and chitosan, collagen and pigments from fish-processing discards. Recently, seafood proteins have also gained attention as an alternative source of BAPs, due to their positive impact on nutrition and health (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006; Samaranayake & Li-Chan, 2011). AFPs extracted from polar fish species have been successfully utilized to minimize the recrystalization of ice in the frozen dairy and meat industry. Marine-derived gelatin has been identified as an alternative to mammalian gelatin for use in food products, as it possesses similar characteristics to porcine gelatin (Karim & Baht, 2009). It is also possible to isolate safe, natural, novel antioxidants from marine sources such as fish and algal biomass (Mendis *et al*., 2005b). Several antimicrobial peptides which play an important role in the natural defense mechanisms of animals against pathogenic microorganisms have been isolated from a variety of marine animals, such as crab, oyster and shrimp.

The production and utilization of marine-derived proteins and peptides not only satisfies the needs of food manufacturers and consumers but also serves as an effective means of utilizing some of the byproducts of the fishing industry. This chapter focuses on the unique features, extraction methods, possible applications and advantages and disadvantages of marine proteins and peptides in order to provide a comprehensive look at these invaluable food ingredients, their future industrial applications and possible directions for further studies.

29.2 MARINE-DERIVED PROTEINS AND PEPTIDES USED IN THE FOOD INDUSTRY

29.2.1 Antioxidants

In terms of its effects in the human body, an antioxidant can be defined as ‘a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen, on normal physiological functions’ (Huang *et al*., 2005). In terms of foods, antioxidants are compounds which are able to delay, retard or prevent auto-oxidation processes (Di Bernardini *et al*., 2011; Mielnik *et al*., 2003). Antioxidant compounds in food play an important role in human health. Scientific evidence suggests that they may help reduce the risk/symptoms of various diseases, including cardiovascular diseases, cancer, neurological decline and diabetes (Wootton-Beard & Ryan, 2011). Many studies have investigated the antioxidant properties of a number of plant and animal sources, such as rice bran (Revilla *et al*., 2009; Santa-Maria *et al*., 2010), alfalfa leaves (Xie *et al*., 2008) and egg yolk (Sakanaka & Tachibana, 2006). Antioxidants such as vitamins A, C and E, carotenes, phenolic acids, phytate, phytoestrogens, minerals and trace elements (e.g. iron and zinc), coenzyme Q, lipoic acid and glycolic acid in these foods scavenge free radicals such as peroxide, hydroperoxide and lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Fardet *et al*., 2008; Prakash *et al*., 2012; Santa-Maria *et al*., 2010).

The use of synthetic antioxidants in food systems, such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA), tert-butylhydroquinone (TBHQ) and propyl gallate (PG), needs to be replaced with natural antioxidants, since synthetic antioxidants have been found to be toxic and carcinogenic in animal models (Kim & Wijesekara, 2010; Li *et al*., 2007). In the last decade, waste from the fish and seafood industries has been used to develop high-quality functional byproducts that can be used as food for human consumption, or even to produce nutritional and medical benefits (Aleman *et al*., 2011). Furthermore, marine algal biomass and alga-derived compounds are considered a valuable source of new secondary metabolites that have novel activity in biological systems and are rich sources of novel antioxidants (Li *et al*., 2007; Ragubeer *et al*., 2010). Numerous studies have shown that peptides derived from different marine-protein hydrolysates act as potential antioxidants (Kim & Wijesekara, 2010), including those isolated from giant squid (*Dosidicus gigas*) (Mendis *et al*., 2005a; Rajapakse *et al*., 2005a), blue mussel (*Mytilus edulis*) (Rajapakse *et al*., 2005b), oyster (*Crassostrea gias*) (Qian *et al*., 2008), hoki (*Johnius belengerii*) (Kim *et al*., 2007; Mendis *et al*., 2005b), tuna (Je *et al*., 2007), cod (*Gadus morhua*) (Slizyte *et al*., 2009), Pacific hake (*Merluccius productus*) (Samaranayaka & Li-Chan, 2008), Alaska pollock (Cho *et al*., 2008), mackerel (*Scomber austriasicus*) (Wu *et al*., 2003), conger eel (*Conger myriaster*) (Ranathunga *et al*., 2006), yellowfin sole (*Limanda aspera*) (Jun *et al*., 2004), yellow stripe trevally (*Selaroides leptolepis*) (Klompong *et al*., 2009) and microalgae (Sheih *et al*., 2009).

The exact mechanism underlying the antioxidant activity of a peptide is not fully understood, but various studies have shown that peptides are lipid-peroxidation inhibitors, free-radical scavengers and transition-metal-ion chelators (Gomez-Guillen *et al*., 2011). Peptide antioxidative properties are related to their amino acid composition, structure and hydrophobicity. Marine-derived gelatin peptides show higher antioxidant activity than peptides from meat protein, probably because of their higher percentage of Gly and Pro (Rajapakse *et al*., 2005a). Apart from the presence of proper essential amino acids, correct positioning of amino acids in the peptide sequence and peptide confirmation have also been identified as influencing antioxidant capacity, showing both synergistic and antagonistic effects as far as the antioxidant activity of free amino acids is concerned. Furthermore, antioxidant activity is strongly related to peptide molecular weight (Gomez-Guillen *et al*., 2011). Some of the antioxidant peptide sequences isolated from marine sources and their potential activities are listed in Table 29.1.

29.2.2 Antifreeze Proteins

AFPs can be defined as a diverse group of polypeptides found in animals, plants and microbes, especially in fish inhabitants of ice-laden sea water, which provides protection from freezing. AFP lowers the freezing temperature of a solution noncolligatively without affecting the melting temperature, which can prevent the freezing of body fluids (Liu *et al*., 2007). It is generally accepted that AFPs inhibit further ice growth (recrystalization) through adsorption to their flat ice-binding surfaces on to particular planes of ice crystals, which prevents cell damage during freezing–thaw cycles (Fletcher *et al*., 2001; Liu *et al*., 2007). AFPs are known to be produced by the liver and from the epidermis of teleost fish as the first line of defense against ice propagation into the fish (Fletcher *et al*., 2001).

AFPs found from the marine sources have been categorized into two distinct groups: antifreeze glycoproteins (AFGPs) and nonglycoproteins. Based on their structural diversity, nonglycoproteins have been further classified as type-I, type-II, type-III and type-IV

Table 29.1 Antioxidant peptides derived from various marine sources. Adapted and modified from Gomez-Guillen *et al*. (2011).

Source	Amino acid sequence[a]	Preparation	Activity	Reference
Alaska pollock-skin gelatin	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly	Serial digestion (Alcalase, Pronase E, collagenase)	Inhibition of lipid peroxidation	Kim *et al*. (2001)
	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly		Increase of cell viability exposed to tert-Butyl hydroperoxide (*t*-BHP)	
Squid (*Dosidicus gigas*)-skin gelatin	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu	Trypsin	Radical scavenging	Mendis *et al*. (2005a)
	Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg		Increase of cell viability exposed to *t*-BHP	
Squid (*Dosidicus gigas*)-tunic gelatin	Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-Pro-Leu-Gly-Leu-Ser	Alcalase	Radical scavenging Ferric-reducing power	Aleman *et al*. (2011)
Fish (*Jonius belengerii*)-skin gelatin	His-Gly-Pro-Leu-Gly-Pro-Leu	Trypsin	Radical scavenging Inhibition of lipid peroxidation Increase of antioxidative enzyme levels in hepatoma cells	Mendis *et al*. (2005b)
Tuna backbone	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-Gln-Leu-Ser	Pepsin	Radical scavenging Inhibition of lipid peroxidation	Je *et al*. (2007)

[a]Three-letter amino acid code.

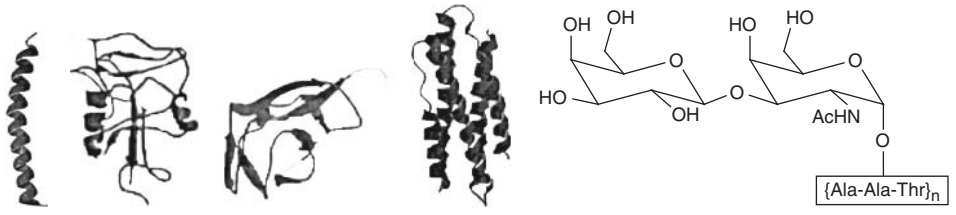

Fig. 29.1 Molecular structures of different types of AFP: (a) type I; (b) type II; (c) type III; (d) type IV; (e) AFGP.

Table 29.2 Comparison of fish AFPs. Adapted from Fletcher *et al*. (2001), Harding *et al*. (2003) and Jorgensen *et al*. (2008).

Type	Fish species	Molecular mass (kDa)	Molecular characteristics
Type I	Right eye flounder Longhorn sculpin Shorthorn sculpin Winter flounder	3.3–4.5	Single, long, alanine-rich, amphipathic α-helices
Type II	Sea raven Rainbow smelt Atlantic herring	11–24	Cysteine-rich globular proteins with secondary structures consisting of five disulfide bonds
Type III	Ocean pout Atlantic eel pout	6.0–6.5	Globular proteins consisting of β-strands with unique flattened surfaces
Type IV	Longhorn sculpin	12	Aggregations of four α-helix protein bundles rich in glutamate and glutamine
AFGP	Northern cod Northern notothenioid	2.6–3.3	Ala-Ala-Thr repeated; disaccharide-containing glycoprotein

kDa, kilodalton.

AFPs (Fig. 29.1). In contrast, AFGPs are a group of at least eight structurally related proteins first isolated from the blood serum of northern Notothenioids. Each AFGP consists of a number of repeating units of $(\text{Ala-Ala-Thr})_n$, with minor sequence variations, which leads to eight different structurally related subtypes (Harding *et al*., 2003). Comparison of fish AFPs is given in Table 29.2.

29.2.3 Antimicrobials

Antimicrobial peptides are usually small (<10 kDa), cationic and amphipathic peptides of variable length, sequence and structure. These components play an important role in the natural defenses of most living organisms against invading pathogens such as Gram-positive and Gram-negative bacteria, protozoa, yeast, fungi and viruses (Reddy *et al*., 2004). The major classes of antimicrobial peptide include: (1) α-helices, (2) β-sheets and

Table 29.3 Marine-derived antimicrobial peptides: their sources and amino acid sequences or major amino acid(s). Adapted and modified from Kim & Wijesekara (2010).

Source	Amino acid sequence or major amino acid(s)	Reference
Oyster	Cys, Leu, Glu, Asp, Phe, Tyr, Ile and Gly	Lee & Maruyama (1998), Liu *et al.* (2007, 2008)
	Leu-Leu-Glu-Tyr-Ser-Ile and Leu-Leu-Glu-Tyr-Ser-Leu	
	Arg-Arg-Trp-Trp-Cys-Arg-X (where X is an amino acid or an amino acid analog)	Zeng *et al.* (2008)
American lobster	Gln-Tyr-Gly-Asn-Leu-Leu-Ser-Leu-Leu-Asn-Gly-Tyr-Arg	Battison *et al.* (2008)
Shrimp	Pro-Arg-Pro	Bartlett *et al.* (2002)
Green sea urchin	Cys	Li *et al.* (2008)

small proteins, (3) peptides with thio-ether rings, (4) peptides with an overrepresentation of one or two amino acids, (5) lipopeptides and (6) macrocycliccystine knot peptides (Tincu & Taylor, 2004). Several antimicrobial peptides have been isolated from a variety of animals (both vertebrates and invertebrates) and plants, as well as from bacteria and fungi (Reddy *et al*., 2004).

Marine-derived antimicrobial peptides are well described in the hemolymph of the many marine invertebrates (Kim & Wijesekara, 2010; Tincu & Taylor, 2004) and have been isolated from several marine invertebrates, including chelicerates, mollusks and crustaceans (Stensvag *et al*., 2008), such as horseshoe crab (Iwanaga, 2002), green sea urchin (Li *et al*., 2008), spider crab (Stensvag *et al*., 2008), oyster (Liu *et al*., 2007, 2008), shrimp (Bartlett *et al*., 2002), American lobster (Battison *et al*., 2008), common periwinkle (Defera *et al*., 2009), marine polychaeta lugworm (Ovchinnikova *et al*., 2004) and marine snail (*Rapana venosa*) (Dolashka, 2011). Table 29.3 gives some examples of marine-derived antimicrobial peptides.

29.2.4 Bioactive Peptides

BAPs can be defined as specific protein fragments derived from plant and animal sources which have a positive impact on body functions or conditions, possess nutritional benefits and may ultimately influence health (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006). Research in the field of BAPs has intensified in the past 2 decades, as extensively reviewed by Korhonen & Pihlanto (2003). BAPs are reported to be produced upon *in vitro* or *in vivo* gastrointestinal digestion, food fermentation and enzymatic hydrolysis during food processing (Korhonen & Pihlanto, 2006). Recently, seafood proteins, especially those from fish proteins, have gained much attention as an attractive source of BAPs, due to the abundance of raw materials in the form of processing discards or byproducts and under-utilized fish species (Samaranayake & Li-Chan, 2011). Fish-protein hydrosylates (FPHs) and peptides produced by different means or inherently found in various seafood sources have been associated with a diverse range of bioactive properties, including antihypertensive, antioxidative, immunomodulatory, neuroactive, hormone-regulating, antimicrobial and mineral-binding effects (Guerard *et al*., 2005; Samaranayake & Li-Chan, 2011). BAPs isolated from different marine fish, invertebrates and other sources are listed in Table 29.4.

Table 29.4 Bioactive peptides isolated from marine organisms upon enzymatic hydrolyzation.

Bioactivity	Species	Isolated organ	Amino acid sequence[a]	Enzyme involved	Reference
ACE-inhibitory	Sardine	Fish muscle, hair tail	N/A	Denazyme AP	Suetsuna & Osajima (1986)
		Muscle	MF; RY; MY; LY; YL; IY; VF; GRP; RFH; AKK; RVY; GWAP; KY	Alcalase	Matsufuji *et al.* (1993)
			VY	Alcalase	Kohama *et al.* (2000)
	Bonito	Muscle	IKPLNY; LKPNM; DYGLYP	Thermolysin	Yokoyama *et al.* (1992)
		Muscle	IVGRPRHQG; FQR; IY; IWHHT; ALPHA	Thermolysin	Yokoyama *et al.* (1992)
		Muscle	LKP; IKP; IWH	Thermolysin	Fujita & Yoshikawa (1999)
		Muscle	IVGRPR	Thermolysin	Fujita *et al.* (2000)
	Salmon	Muscle	WA; VW; WM; MW; IW; LW	Thermolysin	Ono *et al.* (2003)
	Tuna	Muscle	PTHIKWGD	Acid protease	Yokohama *et al.* (1992)
	Alaska pollock	Skin	GPL; GPM	Alcalase, protease. collagenase	Byun & Kim (2001)
	Chlorella vulgaris	Alga-protein waste	VECYGPNRPQF	Pepsin	Sheih *et al.* (2009)
	Oyster	Muscle	LF	Denazyme AP	Matsumoto *et al.* (1994)
	Oyster	Muscle	KLKFV	Pepsin + trypsin	Katano *et al.* (2003)
	Aceteschinensis	Whole cells	FCVLRP; IFVPAF; KPPETV		Lun *et al.* (2006)
	Sea cucumber	Body-wall proteins	MEGAQEAQGD	bromalein + alcalase	Zhao *et al.* (2009)
	Rotifer	N/A	DDTGHDFEDTGEAM	Alcalase	Lee *et al.* (2009)
Immunomodulatory	Atlantic cod	Stomach	N/A	Acidic proteases	Gilberg *et al.* (1996)
		muscle	N/A	Acidic pretease	Bøgwald *et al.* (1996)
Ca-binding	Alaska pollock	Backbone	N/A	Pepsin	Jung *et al.* (2006)
	Hoki	Skeleton	N/A	Pepsin	Jung *et al.* (2007)
Anticoagulant and antiplatelet effects	Yellowfin sole	muscle	Yellowfin sole anticoagulant protein (YAP)	N/A	Rajapakshe *et al.* (2005)

[a]One-letter amino acid code.

29.2.5 Enzymes

Enzymes are proteins or polypeptides and may be defined as substrate-specific biocatalysts that accelerate the rate of a particular reaction. Enzyme technology has become an integral part of the food industry, enabling the production of a wide range of products for human consumption, due to its beneficial and industry-friendly characteristics, including substrate specificity, high catalytic activity at very low concentrations and high activity at mild pH and temperature (Shahidi & Kamil, 2001). Marine enzymes have food applications since they may be unique protein molecules not found in any terrestrial organism, or else known from terrestrial sources but with novel properties (Hathwar *et al*., 2011). Fish internal organs, especially fish viscera, are rich sources of enzymes and many exhibit high catalytic activity at a relatively low concentration. Due to their specific characteristics, fish-processing byproducts are currently used for enzyme extraction (Kim & Mendis, 2006). The optimal pH and temperature of proteases obtained from different body portions vary within and between fish species (Hathwar *et al*., 2011). Several proteases, lipases, chitinases and transglutaminases (TGs) have been isolated from different fish, invertebrates and marine microorganisms.

29.2.6 Proteases/Proteinases/Protein-degrading Enzymes

Proteases hydrolyze the peptide bonds that link amino acids in the polypeptide chain, which form the backbone of protein molecules, and can be either exopeptidases or endopeptidases (Hathwar *et al*., 2011). Protease sales account for about 60% of all industrial sales in the world (Samuel *et al*., 2012). A range of proteolytic enzymes, including trypsin, trypsin-like enzymes, chymotrypsin, collagenases, elactases and carboxylpeptidases, are commercially extracted on a large scale from marine fish-processing discards, especially from fish viscera (Hatwar *et al*., 2011; Kim & Mendis, 2006; Shahidi & Kamil, 2001). Fish proteases are reported to possess the best properties, such as higher catalytic efficiency at low temperatures, lower sensitivity to substrate concentrations and greater stability across a broader temperature range (Kim & Mendis, 2006). These promising characteristics are used in the food industry to produce a wide variety of products with mild reactions. Proteases from marine sources can be classified on the basis of their optimal pH (acid, neutral or basic), their similarities to well-characterized proteases (trypsin-like, chymotrypsin-like, chymosin-like and cathepsin-like), their substrate specificity or their mode of catalysis (serine, cysteine, aspartyl and metalloproteases) (Hathwar *et al*., 2011). Some proteases isolated from marine fish and invertebrates are illustrated in Table 29.5.

29.2.7 Lipid-degrading Enzymes

Seafood is considered an important source of lipases (Nakkarike *et al*., 2011), which break down fats and oils, including triacylglycerols (TAG), into a variety of free fatty acids (FFAs), diacyleglycerols and monoglycerols by engaging with reactions such as esterification, hydrolysis and exchange of fatty acids in esters. The diversity and effectiveness of seafood lipases can be attributed to factors such as food adaptations, migrations and seasonal variations (Hathwar *et al*., 2011). Isolation and characterization of lipases from different marine organisms, including microorganisms, has been well documented. Lipases have been isolated from cod, mackerel and salmon, and lipase activity in marine fish is mainly detected in muscle, gut and liver (Hathwar *et al*., 2011; Shahidi & Kamil, 2001).

Table 29.5 Marine-derived proteases: their sources and characteristics. Adapted and modified from Hathwar *et al*. (2011), Shahidi & Kamil (2001) and Zhao *et al*. (2011).

Protease type	Subclass	Molecular weight (kDa)	Optimum pH	Sources
Serine proteases	Trypsin and trypsin-like enzymes	22.5–24.0	7.5–10.0	Anchovy, Greenland cod, Atlantic cod, capelin, mullet, sardine, catfish, starfish, crayfish, cunner, Atlantic salmon, krill
	Chymotrypsin	25–28	7.8–8.0	Anchovy, Atlantic cod, capelin, herring, rainbow trout, spiny god fish
	Elactase	24.8–28.0	5–9	Atlantic cod, bluefin tuna catfish, dover sole, eel, sea bass, monk fish, yellow tail, teleost tuna
	Collagenase	25–125	6.5–8.0	Fiddler crab, cray fish, king crab, Atlantic cod
Aspartate proteases	Pepsin	27–42	2–4	Atlantic cod, capelin, Greenland cod, Polar cod, sardine, American smelt, dog fish, Monterey sardine, Arctic cod, European eel, sea bream, Mandarin fish, Albacore tuna, palometa, blue-fin tuna, orange roughy
	Pepsinogen	N/A	N/A	Bluefin tuna, shark
	Chymosin	33.8	2.2–3.5	Harp seals
	Gastricin	32.3–33.9	3	Atlantic cod, salmon, hake
Cysteine proteases	Calpain	108	7.5	Sea bass
	Cathepsin	13.6–39.5	3.5–8.0	Horse clam, mussel, surf clam
Metalloproteases	Collagenase	25–125	6.5–8.0	Crab, lobster, prawn, rockfish, Atlantic cod

kDa, kilodalton.

Two subgroups of lipase—bile salt-activated lipase (BAL) and phospholipase (PL)—have been identified from the hepatopancreas of red sea bream (*Pagus major*) (Igima *et al*., 1998) and from pollock, sea bream, trout liver and cod muscle, respectively. More importantly, BAL isolated from red sea bream has been reported to efficiently hydrolyze ethyl esters of polyunsaturated fatty acids (PUFAs), including arachidonic acid and eicosapentaenoic acid (EPA) (Igima *et al*., 1998).

29.2.8 Carbohydrate-transforming Enzymes

Marine organisms feed on seaweed and produce a mixture of carbohydrate-degrading enzymes (Harthwar *et al*., 2011). Two main enzymes have been documented: alginate lyases and chitinases. Alginate lyase is a complex polymer of α-L-guluronate and its C5 epimer β-D-mannuronate which falls into two categories: mannuronate and guluronatelyases. This enzyme has been isolated from several marine algae, invertebrates and microbes (Samuel *et al*., 2012); for instance, alginate lyase has been identified from the gut, gland, style and hepatopancreas of the marine mollusk *Dolabella auricular* (Solander, 1968).

Marine organisms produce at least 2.3 million metric tons of chitin per year (Samuel *et al*., 2012). Chitinases are endo-β-*N*-acetylglucoseaminidases which randomly hydrolyze β-1 $\rightarrow$ 4- linkage in poly- and oligosaccharides of *N*-acetylglucoseamine, while producing chitobiose as the main product. Chitobiose is further hydrolyzed by chitobiase into single units of *N*-acetylglucoseamine (Harthwar *et al*., 2011). Chitinases have been identified from the digestive tract of Dover sole (Clark *et al*., 1988), liver of Japanese common squid (*Todarodes pacificus*) (Matsumiya *et al*., 2002), prawn (*Penaeus vannamei*) (Xie *et al*., 2004) and hepatopancreas of northern shrimp (*Pandalus borealis*) (Esaiassen *et al*., 1995).

29.2.9 Miscellaneous-type enzymes

While a variety of enzymes have been isolated from marine organisms, the food applications of just two will be discussed here.

TG is an endogenous fish enzyme that catalyzes acyl-transfer reactions between γ-carboxamide groups of glutamine residues in proteins, polypeptides and primary amines. Seafood TG is generally a monomeric protein (Hathwar *et al*., 2011). TGs have been identified from red sea bream (Yasueda *et al*., 1994), scallop (*Patinopectin yessoensis*) muscle, botan shrimp (*Pandalus nipponensis*), squid (*Todarodes pacificus*), carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), atka mackerel (*Pleurogrammus azonus*) (Nozawa *et al*., 1997), tread-fin bream (*Nemipterus* sp.) liver (Hemung & Yongsawatdigul, 2008) and Japanese oyster (*Crassostera gigas*) (Kumazawa *et al*., 1997).

Lipoxygenase (LOX) is a dioxygenase that catalyzes the oxygenation of PUFA containing a *cis*,*cis*-1,4-pentadiene system to hydroperoxides. LOX activity is reported in a few marine organisms, including coral, sea urchins, grey mullet and starfish eggs. LOX from shrimp has shown increased reactivity with PUFA substrate, with increased unsaturation (Hathwar *et al*., 2011).

29.3 COLLAGEN AND GELATIN

Collagen, the main component of connective tissue, is a fibrous protein found abundantly in all multicellular animals (Swatschek *et al*., 2002; Zhu *et al*., 2010). Collagen fibers

Table 29.6 Amino acid content of some fish gelatins versus pork gelatin (residues/1000 total amino acid residues). Adapted from Karim & Bhat (2009).

Amino acid	Cod skin	Alaska pollock skin	Hake	Megrim	Tilapia skin	Pork skin
Ala	96	108	119	123	133	112
Arg	56	51	54	54	47	49
As	52	51	49	48	48	46
Cys	0	0	-	-	0	0
Glx	78	74	74	72	69	72
Gly	344	358	331	350	347	330
His	8	8	10	8	6	4
Hyl	6	6	5	5	8	60
Hyp	50	55	59	60	79	91
Ile	11	11	9	8	8	10
Leu	22	20	23	21	23	24
Lys	29	26	28	27	25	27
Met	17	16	15	13	9	4
Phe	16	12	15	14	13	14
Pro	106	95	114	115	119	132
Ser	64	63	49	41	35	35
Thr	25	25	22	20	24	18
Trp	0	0	-	-	0	0
Tyr	3	3	4	3	2	3
Val	18	18	19	18	15	26
Imino acid	156	150	173	175	198	223

are essentially inextensible and therefore provide mechanical strength, and through that strength confer and maintain form while allowing flexibility between various organs of the body (Bailey *et al*., 1998). So far, up to 27 variants of collagen have been reported (Gomez-Guillen *et al*., 2011). According to the nature of their aggregated forms (morphological differences), the majority of collagens can be classified into four major groups: thick, striated fibers; nonfibrous networks; nonstriated filamentous collagen; and fibril-associated collagen. These collagen molecules consist of closely related but genetically distinct proteins which possess a basic structure of three polypeptide chains, each with a glycine-X-Y repeat (where X is mostly proline and Y is mostly hydroxiproline), forming tightly bound triple helices (mainly stabilized by intra- and interchain hydrogen bonding) which subsequently aggregate to form various types of supporting structure (Bailey *et al*., 1998; Gomez-Guillen *et al*., 2011). The triple helix is approximately 300 nm in length, with a molecular weight of approximately 10^5 kDa (Karim & Bhat, 2009). The composition of collagen encompasses all 20 amino acids. However, there are some differences in the amino acid compositions of collagens derived from different sources (Table 29.6). Amino acid composition has been found not to vary greatly between marine-fish gelatin preparations, although some differences in imino acid and Ala content and in hydroxylation degree can be detected (Gomez-Guillen *et al*., 2002). The properties of a collagen vary according to the raw material used to create it. For example, the physical and chemical properties of collagen isolated from cattle skins are substantially different from those of fish skins (Sadowska *et al*., 2003).

Gelatin, which is widely used in the biomedical, pharmaceutical, cosmetic and food industries, is a soluble protein compound obtained by partial hydrolysis of collagen.

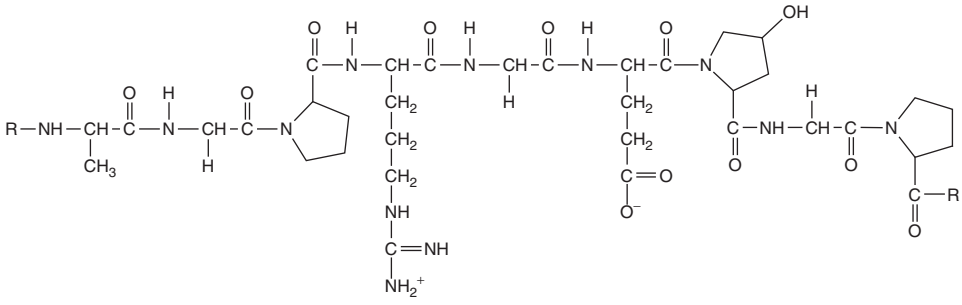

Fig. 29.2 Molecular structure of gelatin.

Heat denaturation easily converts collagen into gelatin (Gomez-Guillen *et al*., 2011; Kim & Mendis, 2006; Liu *et al*., 2009). The amino acid composition of gelatin is very close to that of its precursor collagen (Karim & Bhat, 2009). However, gelatin contains relatively high amounts of some nonpolar amino acids (>80%), such as glycine, proline and alanine (Kim & Mendis, 2006; Mendis *et al*., 2005). The molecular structure of gelatin is illustrated in Fig. 29.2.

29.4 EXTRACTION AND ISOLATION OF MARINE-DERIVED PROTEINS AND PEPTIDES

29.4.1 Extraction of Antioxidants

Antioxidant assays can be divided into *in vitro* and *in vivo* methods. The antioxidant activity of hydrolysates or peptides is usually tested by *in vitro* assays. However, it is vital to perform *in vivo* assays to ensure that peptides identified by *in vitro* systems are bioavailable following ingestion and can reach a target site and administer a response in a living system (Di Bernardini *et al*., 2011).

Many different commercial enzymes (such as pepsin, collagenase, protease preparation from *Bacillus subtillus*, trypsin and papain) and combinations of enzymes (such as a cocktail of protease from bovine pancreas, *Streptomyces* and *B. polimixa*) have been used to generate hydrolysates from different animal muscle sources, isolated muscle proteins and marine byproducts. After ultrafiltration using 10, 5, 3 and 1 kDa molecular-weight cutoff (MWCO) membranes, hydrolysates are tested for antioxidant activity by one or several assay methods, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, thiobarbituric acid reactive substance assay (TBARS), metal-chelating activity, hydroxyl radical-scavenging activity, superoxide radical-scavenging activity, peroxyl radical-scavenging activity, peroxide value or carbonyl values. When antioxidant activity is detected, further purification steps are required before the BAP sequence can be identified. Purification techniques used to date include ion-exchange chromatography, gel-filtration chromatography and high-performance liquid chromatography (HPLC). HPLC is the most commonly used method, often preceded by one of the other separation techniques. Once hydrolysates are separated into peptidic fractions, these fractions are further tested for their antioxidant activities and the most active are sequenced by mass-spectrometry

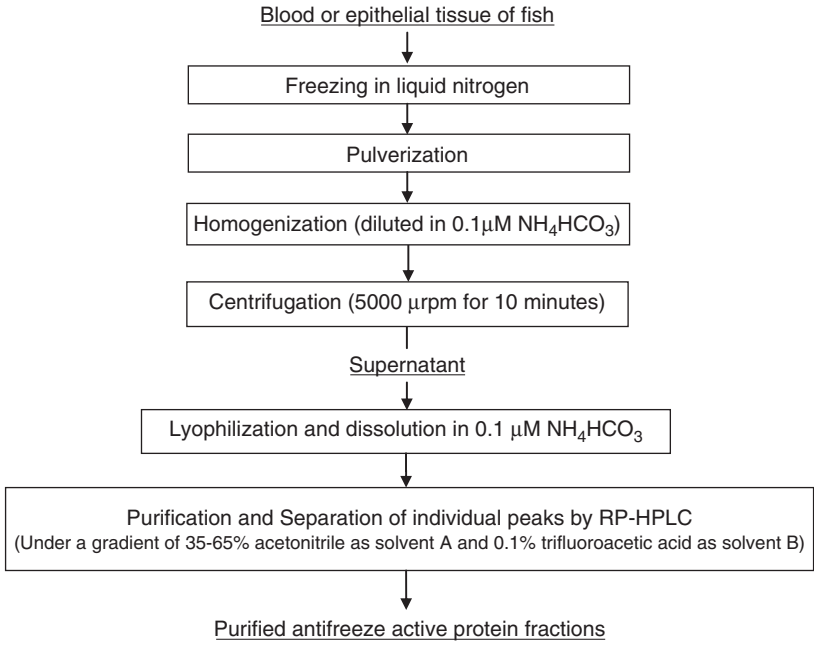

Fig. 29.3 Schematic diagram of the recovery of AFP from polar fish skin and blood.

techniques, including MALDI-TOF (matrix-assisted laser-deionization time-of-flight), ESI (electrospray ionization) and Edman degradation (Di Bernardini *et al*., 2011).

29.4.2 Extraction of Antifreeze Proteins

Fish inhabitants of ice-laden cold climates (see Table 29.2) are the most promising source of AFPs, from either their blood or their epithelial tissue/skin. In order to recover AFPs, the blood or skin is frozen in liquid nitrogen, pulverized and homogenized in an alkaline medium, and the supernatant is separated after centrifugation followed by lyophilization (Fig. 29.3). The lyophilized supernatant is then dissolved in the same alkaline medium and subjected to preparative chromatography (Evans & Fletcher, 2004).

Adsorption on ice has been suggested as an alternative procedure by Kuiper *et al*. (2003). In this method, ice is allowed to grow on a cold finger (Fig. 29.4a), which is a hollow structure constructed from brass tubing and silver solder connected to a temperature-programmable water bath containing a mixture of water and ethylene glycol (3 : 1 by volume) through insulated plastic tubes. A sample containing AFPs (diluted in <100 mM salt and of <1 mg/ml of a total volume of 80–120 ml) is kept in a beaker (150 ml) in solution. The temperature is brought down close to $0\,^{\circ}$C by placing the beaker in ice prior to assembly of the cold apparatus in a well-insulated polystyrene foam (Fig. 29.4b). Before immersion of the cold finger in the AFP solution, the finger's temperature is brought down to $-0.5\,^{\circ}$C by coating it with a thin film of ice through immersion in chilled, distilled water containing a few ice crystals to nucleate freezing. After 10 minutes, when a thin shell of clear ice has formed around the bottom of the cold finger, it is lowered into the pre-chilled solution, with about 15 mm clearance from

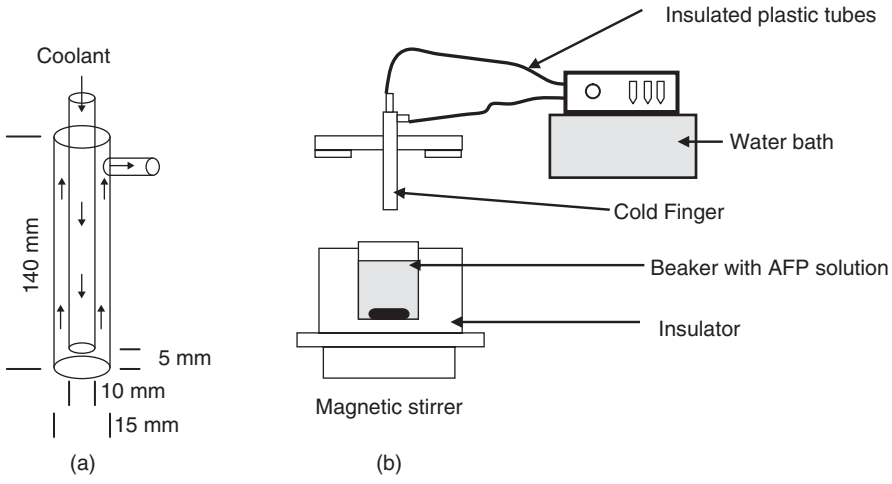

Fig. 29.4 Cold finger (a) and cold apparatus (b) used to recover antifreeze proteins from marine organisms. Adapted from Kuiper *et al*. (2003).

the bottom to facilitate rotation of the magnetic stir bar. The temperature is lowered gradually from -0.5 to $-2\,^{\circ}$C over 16 hours in order to freeze approximately 50% of the original solution through continuous supply of coolant, allowing the shell of ice to slowly grow into a hemisphere while the sample continuously mixes. Upon achievement of a satisfactory ice growth, the cold finger is separated from the liquid portion with adhered ice crystals, washed and detached by setting the cooling flow to $+1\,^{\circ}$C. The adsorbed proteins are then lyophilized/ultrafiltered, and if necessary an additional chromatographic separation is undertaken (Kuiper *et al*., 2003).

29.4.3 Extraction of Antimicrobials

The isolation and characterization of antimicrobial peptides from marine organisms and byproducts has not been well developed compared to that of the antioxidants. However, in general, the production and characterization of antimicrobial substances from various sources of protein is similar to that of antioxidant compounds, except in the use of antimicrobial-activity assays instead of antioxidant-activity assays, such as agar-diffusion assay, liquid-growth inhibition and minimum inhibition concentration (MIC). Among these tests, the agar-diffusion assay or inhibition-zone assay is the most widely used method by which to test the antimicrobial activity of peptidichydrolysates and peptides (Di Bernardini *et al*., 2011).

29.4.4 Extraction and Isolation of Bioactive Peptides

Enzymatic hydrolysis is the most common method of producing BAPs from marine sources, where the precursor proteins are hydrolyzed by using endogenous proteolytic enzymes already present in the muscle or viscera of fish or shellfish and/or by adding exogenous enzymes from other sources (Guerard, 2007). Peptides with the desired characteristics are separated by an ultrafiltration membrane system equipped with the appropriate MWCO levels (Kim & Mendis, 2006).

29.4.5 Extraction of Enzymes

Fish visceral discards are a rich source of digestive enzymes (Nakkarike *et al*., 2011; Shahidi & Kamil, 2001). Any enzyme-recovery process, regardless of the type of enzyme, should follow three steps: (1) dissection and homogenization of samples to disrupt the cells and release the enzyme; (2) solubilization of the enzyme in an appropriate buffer to control the pH and maintain enzyme stablility; and (3) centrifugation of the mixture to remove the cell residues from the enzyme. Temperature plays a significant role in crude enzyme recovery, and the extraction procedure should be conducted at a low temperature (0–4 °C) in order to minimize protein denaturation and autolysis of the enzymes (Zhao *et al*., 2011). Apart from this conventional method, enzyme recovery from a crude fraction can be carried out using other methods, namely gel-filtration chromatography, ammonium sulfate precipitation and dialysis (Nakkarike *et al*., 2011) and a two-phase aqueous system (ATPS) (Zhao *et al*., 2011). An ATPS allows extraction of an enzyme using two different aqueous phases, including a polymer and a salt solution in which all the impurities (soluble cells, blood, polysaccharides and pigments) are dissolved in the salt phase, resulting in a high purity of extracted enzyme. Moreover, an ATPS is advantageous because it can obtain enzymes through low-speed centrifugation, it provides an excellent environment for maintenance of the native structure and stability of the enzyme and polymers, it is rapid and the salts can be recycled (Zhao *et al*., 2011).

29.4.6 Extraction of Collagen and Gelatin

The skins or hides of farm animals such as cattle and swine have been used to isolate collagen for industrial applications for many years. Other common raw materials for collagen and gelatin extraction are the bones, tendons and cartilages of mammalian farm animals. Pig skin was the first raw material used for the manufacture of gelatin, in the 1930s, and it continues to be the most important material for large-scale industrial production today (Gomez-Guillen *et al*., 2011; Swatschek *et al*., 2002). However, the safety of collagen products isolated from livestock has become questionable due to the recent concerns regarding zoonotic diseases such as mad cow disease/bovine spongiform encephalopathy (BSE). Cultural and religious taboos (such as halal and kosher laws in Muslim and Jewish communities) also have an impact. Therefore, there is an increasing demand for alternative collagen sources, such as the skin, bones and scales of sea animals such as fish, shrimp, crabs and marine sponges (Cheow *et al*., 2007; Kim & Mendis, 2006; Sivakumar *et al*., 2000; Swatschek *et al*., 2002; Zhu *et al*., 2010).

In general, collagen is extracted by acid treatment and solubilized without altering its triple-helix structure. It is possible to convert collagen into soluble gelatin by heating it in alkali, which cleaves the hydrogen and the covalent bonds that stabilize the triple-helix configuration and convert it into a coiled conformation, producing a gelatin state. Therefore, hot-water treatment is used as a general procedure for the solubilization of collagen in skin and extraction of gelatin (Kim & Mendis, 2006). Partially purified collagen (e.g. without acid solubilization) from marine sources may possess improved functional properties, enabling possible industrial applications (Kim & Park, 2005). The optimum conditions for extraction are dependent on the source/raw material and the type of collagen. Extraction conditions, including temperature, time and pH, can slightly affect the properties of the extracted gelatin (Kim & Mendis, 2006; Kim *et al*., 1994), as the extraction process influences the length of the polypeptide chains. The pretreatment method and the properties and preservation method of the starting raw material can

also affect the properties of the gelatin (Karim & Bhat, 2009). Collagen/gelatin has been extracted from the fresh skin, bones and connective tissues of various marine organisms, including both cold-water (such as cod, hake, Alaska pollock and salmon) and warm-water (such as tuna, shark) fish species, echinoderms (sea cucumber), squid, sea sponges and crustaceans (Table 29.7). It seems likely that even precooked (Aewsiri *et al*., 2008) and preserved marine fish skins (smoked, salted and marinated) could be used to extract gelatin (Kolodziejska *et al*., 2008), as could solid waste (whole fish, fish muscles, skin, bone, trace amounts of scale fragments) and refiner discharge from marine industries such as surimi processing (Gomez-Guillen *et al*., 2011; Kim & Park, 2004, 2005).

Regardless of the raw material used, all gelatin-manufacturing processes consist of three main stages: (1) pretreatment of the raw material; (2) extraction of the gelatin; and (3) purification and drying. Different procedures, such as mincing, stirring, filtration, washing, centrifuging, freezing and lyophilization, are involved in the extraction process (Kolodziejska *et al*., 2008). Two different types of gelatin, with differing characteristics, can be produced according to the method by which the collagens are pretreated. Type-A gelatin (isoelectric point at pH 6–9) is produced from acid-treated collagen and type-B gelatin (isoelectric point at approximately pH 5) is produced from alkali-treated collagen. Mild acid pretreatment of the raw material and mild temperature conditions during the extraction process are generally used in preparing certain marine-derived gelatins, such as fish gelatin (Karim & Bhat, 2009).

29.5 FOOD-RELATED APPLICATIONS OF MARINE-DERIVED PROTEINS AND PEPTIDES

29.5.1 Food Applications of Antioxidants

Antioxidants play a vital role in both food systems and the human body by reducing oxidative processes. In food systems, antioxidants are useful in retarding lipid-peroxidation and secondary lipid-peroxidation product formation, and thus help to maintain the flavor, texture and, in some cases, color of the food product during storage (Samaranayaka & Li-Chan, 2011).

Marine algae represent a vast resource, the potential of which is not yet fully realized. Some algal species possess induced biological activities, associated with proteins, protein hydrolysates and peptides, which affect more than just their nutritional values as antioxidants (Kim *et al*., 2006; Samarakoon & Jeon, 2012). Marine algal proteins, with or without antioxidant properties, have been used extensively in the food industry in a wide range of products, from chewing gums to dairy items (Bermejo *et al*., 2002). Antioxidative peptides isolated from *Chlorella vulgaris*, a popular edible microalga in Japan, have quenched a variety of free radicals, including hydroxyl radical, superoxide radical and peroxyl radical, *in vitro*. They have also demonstrated significant protective effects on DNA and prevent cellular damage caused by hydroxyl radicals (Sheih *et al*., 2009).

The potential applications of marine-derived antioxidants in the food industry face many challenges. For example, the possible bitter taste of protein hydrolysates may prevent their use in many products as food additives (Kristinsson & Rasco, 2000). Capelin (*Mallotus villosus*) protein hydrolysate, when added to minced pork muscle at a level of 0.5–3.0%, can reduce the formation of secondary oxidation products, including TBARS, while increasing cooking yield by 4%. Treatments with activated carbon were able to

Table 29.7 Marine organisms used in the isolation of collagen and gelatin.

Marine organism	Body part	Collagen/gelatine type	Reference
Giant red sea cucumber (*Parastichopus californicus*)	Skin and connective tissues	Pepsin-solubilized collagen	Liu *et al.* (2009)
Brownstrip red snapper (*Lutjanus vitta*)	Skin	Acid-solubilized collagen and pepsin-solubilized collagen	Jongjareonrak *et al.* (2005)
Bigeye snapper (*Priacanthus tayenus*)	Skin	Acid-solubilized collagen	Kittiphattanabawon *et al.* (2005)
Jumbo flying squid (*Dosidicus eschrichitii* Steenstrup)	Skin	Gelatin	Lin & Li (2006)
Giant squid (*Dosidicus gigas*)	Skin	Gelatin	Gimenez *et al.* (2009) and Uriarte-Montoya *et al.* (2011)
Hoki (*Johnius belengerii*)	Skin	Gelatin	Mendis *et al.* (2005)
Baltic cod (*Gadus morhua*)	Skin, head and backbone	Gelatin	Kolodziejska *et al.* (2008) and Sadowska *et al.* (2003)
Alaska pollock (*Theragra chalcogramma*)	Skin	Gelatin	Chiou *et al.* (2006) and Zhou & Regenstein (2005)
Sin croaker (*Johnius dussumieri*)	Skin	Gelatin	Cheow *et al.* (2007)
Shortfin scad (*Decapterus macrosoma*)	Skin	Gelatin	Cheow *et al.* (2007)
Alaska pink salmon (*Oncorhynchus gorbuscha*)	Skin	Gelatin	Chiou *et al.* (2006)
Atlantic salmon (*Salmo salar*)	Skin	Gelatin	Arnesen & Gildberg (2007)
Harp seal (*Phoca groendlandica*)	Skin	Gelatin	Arnesen & Gildberg (2002)
Atlantic cod (*Gadus morhua*)	Soft connective tissues and bones in the head	Gelatin	Arnesen & Gildberg (2006)
Pagrus major	Scales	Type-I collagen	Ikoma *et al.* (2003)
Deep-sea redfish (*Sebastes mentella*)	Skin, scales and bone	Type-I collagen	Wang *et al.* (2008)
Lizardfish (*Saurida* spp.)	Scales	Gelatin	Wangtueai & Noomhorm (2009)
Cuttlefish (*Sepia pharaonis*)	Dorsal and ventral skin	Gelatin	Aewsiri *et al.* (2009)
Shark (*Isurus oxyrinchus*)	Cartilage	Gelatin	Cho *et al.* (2004)
Blue shark (*Prionace glauca*)	Skin	Gelatin	Limpisophon *et al.* (2009)

reduce the bitterness of fish-protein hydrolysate (Shahidi *et al*., 1995). Furthermore, the bioactivity of protein hydrolysates may be reduced by molecular alteration during food processing or interaction with other food ingredients (Moller *et al*., 2008). The challenge for food technologists will be to develop functional foods and nutraceuticals without the undesired side effects of the added peptides (Kim & Wijesekara, 2010).

Peptides isolated from Alaska pollock skin gelatin (Kim *et al*., 2001) and squid skin gelatin (Mendis *et al*., 2005b) demonstrate beneficial effects against oxidant injuries in rat liver cells and human lung fibroblasts, respectively. Furthermore, peptides isolated from hoki-skin gelatin enhances the expression of antioxidative enzymes such as glutathione peroxidase, catalase and superoxide dismutase in human hepatoma cells *in vitro* (Mendis *et al*., 2005b). Gelatin peptides contain mainly hydrophobic amino acids and an abundance of these amino acids favors a higher emulsifying ability. Thus, marine gelatin-derived peptides are expected to exert higher antioxidant effects among other antioxidant peptide sequences (Kim & Wijesekara, 2010; Mendis *et al*., 2005b). Marine-derived BAPs with antioxidative properties may therefore have great potential as a substitute for synthetic antioxidants in the food industry (Kim & Wijesekara, 2010).

29.5.2 Food Applications of AFPS

The incorporation of AFPs in food products is a new area and is currently limited to a few publications and patents. The unique functional properties of AFPs—lowering of the freezing point without affecting the noncolligative properties, inhibition of recrystalization at very low concentrations (<0.1 μg/ml) and their very high noncolligative activity (500×) compared to colligatively active substances—have piqued the interest of food scientists (Feeney & Yeh, 1998). Moreover, intake of AFPs in the diet is likely already to be substantial in most northerly and temperate regions, which seems to have great applicability to the food industry. However, application of AFPs in the food industry will likely depend on the cost. AFPs from natural sources may have low feasibility for food applications, but synthesized AFP analogs and mass-produced transgenic AFPs may have appeal as a cheaper option.

29.5.2.1 Use of AFPs in the Cryopreservation of Fruits and Vegetables

Freezing can be defined as the process of bringing the temperature of food below its freezing point in order to prolong its shelf life (Zhu & Ramaswamy, 2010). Freezing or low-temperature storage of fruits and vegetables is important. However, freezing of vegetables and fruits may drastically affect their textural and other sensory properties. The cellular structure of food from living tissues such as meat, fish, fruits and vegetables comprises delicate cell walls and membranes, with water within and between them (Potter & Hotchkiss, 1995). During freezing, liquid water is transformed into ice crystals. The formation of ice crystals believed to consist of two processes: nucleation and recrystalization. Intracellularly and extracellularly formed ice crystals cause undesirable changes in texture and exterior quality, especially when freezing is carried out at a slower rate that enables the extracellular formation of large ice crystals, which tend to squeeze the cell structure as they grow and result in a product with severe textural breakdown upon thawing. In contrast, rapid freezing processes minimize both recrystalization and cellular and tissue damage, which is desirable for a high-quality product (Jørgensen *et al*., 2008; Zhu & Ramaswamy, 2010). However, rapid freezing methods are very costly and are

unsuitable for some perishables; for example, rapid freezing causes the 'wooly texture' found in peaches (Fennema, 1973).

Introduction of AFP, with its ability to inhibit recrystalization, may be a better and more cost-effective solution to freeze-induced defects. It has been reported that AFPs can be added physically into food by mixing, injection, soaking, vacuum filtration or gene transfer. The latter method is most suited to fruits and vegetables, due to the benefits of transgenic plants. Incorporation of AFP genes into plants is inherited by future generations, providing a very cost-effective solution (Hightower *et al*., 1991; Jørgensen *et al*., 2008).

Hightower *et al*. (1991) have successfully produced transgenic tomato plants in which the tissues contain a chimetic gene called *spa-afa5*, which encodes a fusion protein between truncated *Staphylococcal protein A* and *afa3* AFP from winter flounder. This transgenic tissue is reported to inhibit recrystalization of ice, enabling the frozen storage of tomatoes.

29.5.2.2 Use of AFPs in the Meat Industry

Intracellularly produced large ice crystals are a prominent defect in the frozen meat industry which result in drip and loss of nutrients during thawing. AFPs may be useful in avoiding or minimizing such defects. There is some clear evidence of reduced ice-crystal sizes in bovine and ovine meat soaked in solutions containing AFPs derived from Antarctic cod and winter flounder at 0.1–1.0 mg/ml concentration and frozen at $-20\,^{\circ}$C. However, this has the disadvantage of a long soaking time of up to 2 weeks, which enhances the deterioration of meat (Payne *et al*., 1994). The preslaughter administration of AFPs has been suggested in an attempt to avoid this problem, with a reduced drip loss and ice-crystal size observed in lamb meat administered AFP from Antarctic cod 1–24 hours preslaughter and stored frozen at $-20\,^{\circ}$C. Moreover, the smallest ice crystals were obtained in lambs injected to a final concentration of 0.01 μg/kg AFGP 24 hours prior to slaughter (Payne & Young, 1995). These results suggest that the damage due to frozen storage of meat can be reduced by the application of AFPs and AFGPs.

29.5.2.3 Use of AFPs in Surimi Production

The raw materials of surimi production are often kept under refrigerated conditions in order to minimize autolysis and microbial activity (Boonsupthip & Lee, 2003). Muscle proteins can be subjected to protein degradation during cold storage. Moreover, finished products are usually kept under frozen conditions in order to maximize their shelf life. However, this causes freeze-induced denaturation of muscle proteins, which ultimately affects the gel-forming ability of constituent proteins; for instance, the gel strength of red hake (*Urophycis chuss*) was reduced to 46% after being stored in ice for 3 days, and to 63% when stored in chilled seawater (Lee, 1986). The quality of surimi strongly depends on the gel-forming ability of constituent muscle proteins. Cryopreservatives such as sucrose and sorbitol are being used in the food industry to minimize freeze-induced protein denaturation. However, commercially available cryopreservatives can cause undesirable sweetness in some products (Boonsupthip & Lee, 2003). Such adverse defects can be avoid or minimized by the utilization of AFPs to preserve the gel-forming ability of fish muscle, which is one of the most important quality determinants of surimi products.

Boonsupthip & Lee (2003) have reported that a type-III AFP preserved the Ca^{2+} ATPase activity of actomyosin obtained from tilapiya fish muscle during frozen and chilled storage. AFP also helped to retain the Ca^{2+} ATPase activity of actomyosin at a much higher level than did conventional cryopreservatives during frozen storage, with

a concentration of 50 g/l AFP giving the maximum activity. Ca^{2+} ATPase activity was reduced by 80% and 50% in controls and samples with conventional cryopreservatives, respectively, after chilled storage for 3 days, but was found to remain unchanged in AFP-treated samples. The Ca^{2+} ATPase activity of actomyosin affects its gel-forming capacity (Carvajal *et al*., 1999), AFPs can also be utilized for preservation of the gel-forming properties of fish muscle and even more thermally stable muscle proteins, such as beef, rabbit and poultry.

29.5.2.4 Use of AFPs in Ice-cream Manufacture

The formation of large ice crystals due to recrystalization is one of the major quality defects of ice-cream manufacture caused by temperature fluctuations during cold storage. These large ice crystals destroy the texture and taste experience of the ice cream. Incorporation of AFPs has been suggested as a natural ice growth inhibitor in ice-cream manufacture, where they are known as 'ice recrystallizing proteins' (Regand & Goff, 2006; Jorgensen *et al*., 2008). Natural AFPs purified from cold-water ocean pout have been used as preservatives in ice cream, coating the fine ice crystals that give the ice cream its smooth texture and preventing it from recrystalizating during storage and delivery (Goodshell, 2009).

Unilever, a British-Dutch global company, is the largest producer of ice cream and frozen novelties in the USA. It has manufactured ice-cream products, such as Breyer's Light Double-Churned, Creamy Chocolate Ice Cream and Good Humour Ice Cream, which incorporate a genetically modified (GM) ice-structuring protein (ISP) originating from ocean pout (*Macrozoarces americanus*) AFPs. The final products contain this ISP at a level of 0.01% of final volume, which enables the development of low-calorie, low-fat ice creams that taste like full-fat products. The ISP is added to the ice-cream mix and tends to lower the temperature at which ice crystals form and eliminate the granular texture resulting from refreezing (Reidhead, 2006).

29.5.2.5 Use of AFPs in Aquaculture

Aquaculture can be defined as the economic culturing of aquatic biota by humans. The adverse environmental conditions of the polar regions, where the temperature drops to $-1.9\,^{\circ}C$, is an obstacle to fish farming, especially during winter (Jørgensen *et al*., 2008). For instance, Atlantic salmon (*Salmo salar*) freeze to death if they come in contact with ice at water temperatures below $-0.7\,^{\circ}C$ (Garth *et al*., 1988). Moreover, freeze-intolerant fish migrate during such adverse conditions, which is detrimental to the livelihood of people who depend on the fisheries. Incorporation of AFP genes into freeze-intolerant fish enables commercial fish farming during winter and extends the range of aquaculture in the polar regions.

Garth *et al*. (1988) transferred AFP genes from freeze-tolerant winter flounder (*Pseudo pleuronectes americanus*) into the genome of Antarctic salmon by microinjection of linearized DNA into fertilized eggs, where it hybridized in the salmon genome. In another study, AFP genes from winter flounder were injected into rainbow trout, and the generation of cold resistance was observed. Such treated rainbow trout, which in nature is freeze-intolerant, could survive in temperatures as low as $-1.4\,^{\circ}C$ (Hew & Ewart, 2002). However, low levels of gene integration and inheritance are common drawbacks involved in the creation of transgenic organisms, which present limitations in the commercial aquaculture industry (Jorgensen *et al*., 2008).

29.5.3 Food Applications of Antimicrobials

Marine-derived antimicrobial peptides may be efficient in controlling postharvest diseases of fruit and vegetables as an alternative to fungicides. A novel peptide, *CgPep*33, isolated from enzymatic hydrolysates from the Pacific oyster (*Crassostrea gigas*) has been reported to significantly reduce gray mold disease incidence in strawberry fruit after harvest (Liu *et al*., 2007). This disease is common in many other fruits and vegetables with economic value, such as apples, grapes, blackberries, beans, cucumbers, and lettuce, and marine-derived antimicrobial peptides may have potential for utilization in controlling postharvest losses in these crops. The antimicrobial peptide derived from American lobster (*Homarus americanus*) exhibits bacteriostatic activity against some Gram-negative bacteria and both protozoastatic and protozoacidal activity against two scuticociliate parasites, *Mesanophrys chesapeakensis* and *Anophryoides haemophilia* (Battison *et al*., 2008), which can cause significant mortality in lobsters maintained in holding facilities at cold temperatures (Cribb *et al*., 1999). The synthetic PC-hepc (a gene) derived from the marine fish *Pseudosciaena crocea* demonstrates a wide spectrum of antimicrobial activity against the principal fish pathogens which affect the important economic species of marine-cultured fish, such as *Aeromonas hydrophila*, *Vibrio parahaemloyticus*, *V. alginolyticus* and *V. harvryi* (Wang *et al*., 2009). Therefore, marine-derived antimicrobial peptides for potential for use in the fish industry. Furthermore, growth inhibition of *Escherichia coli* (some serotypes of which can cause serious food poisoning in humans) by the antimicrobial peptide *CgPep*33, derived from oyster (*C. gigas*), has also been reported (Liu *et al*., 2008).

29.5.4 Food Applications of BAPS

Several food applications of BAPs derived from marine organisms have been reported in the literature, which have the added advantage of their neuraceutical properties, in addition to their nutritional value. More importantly, they do not have any of the side effects caused by artificially synthesized drugs.

29.5.4.1 BAPs with Antihypertensive Effects

Antihypertensive peptides are the most studied group of BAPs and are widely available as food supplements in Japan. ACE-inhibitory peptides inhibit the activity of angiotensin converting enzyme (ACE), which is associated with the renin–angiotensin system and elevates blood pressure by inactivating the vasodilator bradykinin and converting angiotensin-I into the vasoconstrictor angiotensin-II. Inhibition of ACE elevates the level of bradykinin and decreases the level of angiotensin-II, thereby lowering the blood pressure (FitzGerald *et al*., 2004). Dried bonito (*Katsuobushi*) is a Japanese traditional food product processed from bonito muscle upon enzymatic hydrolysis. In a study by Yokoyama *et al*. (1992), bonito muscle was hydrolyzed by various proteases and its maximum ACE-inhibitory activity (IC_{50} = 29 μg/ml) was found in the thermolysin digest. The high ACE-inhibitory activity of this digest was mainly attributed to two peptides: LKPNM and LKP, which had IC_{50} values of 2.4 and 0.32 μM, respectively. An IC_{50} value can be defined as the concentration of inhibitor needed to inhibit the original ACE activity by 50%. Moreover, two peptides have exhibited antihypertensive activity in hypertensive and borderline-hypertensive subjects. Of these, LKPNM has been officially approved as a Food for Specified Health Use (FOSHU) in Japan and as a new dietary supplement ingredient by the US Food and Drug Administration (FDA)

(Samaranayake & Li-Chan, 2011; Vercruysse *et al*., 2005). 'Peptide Soup', another product manufactured by Nippon in Japan, also contains bonito-derived peptides and is claimed to have hypertensive effects (Samaranayake & Li-Chan, 2011).

In another study, a sardine-protein hydrolysate incorporated into commercially available vegetable drink was evaluated for its antihypertensive activity (Kawasaki *et al*., 2002). The hydrolysate, which contained the dipeptide VY (Val-Tyr), was tested using subjects with mild hypertension, high–normal blood pressure and normal blood pressure. Each subject was served 195 g of vegetable drink containing 0.5 g sardine peptides once a day for over 13 weeks as an oral administration. At the end of the test period, a significant decrease in systolic blood pressure was reported in subjects with mild hypertension or high–normal blood pressure. Furthermore, diastolic blood pressure also significantly decreased with ingestion of sardine peptide. More importantly, not a single adverse effect was observed in either hypertensive or normotensive subjects during the test period (Kawasaki *et al*., 2002).

The ACE-inhibitory peptides Ala-Pro, Lys-Pro and Arg-Pro, along with another five proline-containing dipeptides with weak ACE-inhibitory activity, have been identified from naturally fermented anchovy fish sauce. Oral administration of Lys-Pro exhibits a tendency to lower the blood pressure of spontaneously hypertensive rats (SHR) while stimulating insulin secretion by cultured RNm5F insulinoma cells (Ichimura *et al*., 2003). It may have the same effects in hypertensive patients and if so can be promoted as a functional food. Je *et al*. (2005) isolated an ACE-inhibitory peptide from oyster sauce fermented for 6 months at 20 °C with an IC_{50} value of 0.0874 mg/ml. In the same study, antihypertensive effects were observed in SHR upon oral administration of the purified peptide, with the blood pressure being significantly decreased. These naturally available ACE-inhibitory peptides are a good source of natural drug for hypertension treatment, as an alternative to artificially synthesized antihypertensive drugs, and do not cause any of the latter's side effects (Saito, 2008).

29.5.4.2 Bioactive Peptides with Beneficial Effects on the Gastrointestinal Tract

Seacure is a commercially available fermented fish product made from Pacific whitening or hake (*Merluccius productus*) by controlled fermentation of highly proteolytic *Hansenula* yeast culture. The product contains 75–80% protein constituents, with considerable amounts of free amino acids (14–16% glutamine, 10% asparagines and 10% lysine), and has been claimed to be an inexpensive approach for the prevention and treatment of the injurious effects of nonsteroidal, anti-inflammatory drugs and other ulcerative conditions of the bowel (Fitzgerald *et al*., 2005).

29.5.4.3 Bioactive Peptides with Anti-anxiety Effects

Stabilium is a commercially available food product containing garum armoricum. It is made from fish viscera of the large blue fish found at depths of 1000–1500 feet off the coast of the Armonican peninsula of France (Anon, 2008; Ash, 2009). Garum armoricum was used by the ancient Celts (3rd century BC) of Armorica as a food supplement in order to improve their resilience to physical and emotional stress. There is some evidence to show that the Romans also used this supplement to avoid anxiety, especially before wars (Ash, 2009).

Another commercial product manufactured with garum armoricum, Stabilium 200 is rich in small peptides (5–8 amino acids), similar to the hypophysiothropic hypothalamic

peptides, which certain neurotransmitters and act as a response to physiopathological problems (Ash, 2009). These small peptides either act as hormone precursors or are similar to anti-anxiety drugs such as encephalins, endorphins and gamma-aminobutyric acid (Anon, 2008). Droman *et al*. (1995) reported a significant decrease in average anxiety scores upon oral administration of garum armoricum in a test conducted using 54 college students of 18–25 years of age. The college students were considered an excellent test group because of the anxiety associated with academic stress. In another double-blind, placebo-controlled study conducted by Le-Poncin (1996), oral administration of Stabilium was reported to enhance memory and mental clarity, as demonstrated by Wechesler's Test of Memorization and Rey's Test, as well as to possess anti-anxiety properties in asthenic/fatigue patients dealing with severe anxiety.

Benzodiazepine is a popular anti-anxiety drug prescribed by physicians to deal with stressful conditions. Although modern benzodiazepine derivatives are still widely prescribed, use of this drug has been significantly reduced due to its long-term side effects (Ash, 2009). Natural sources of anti-anxiety effects such as garum armoricum may be potent substitutes for expensive drugs in supporting the body during stressful conditions, both mental and physical, as they have no known side effects.

29.5.5 Food Applications of Marine-derived Enzymes

29.5.5.1 Use of Marine-derived Enzymes for the Extraction of Collagen from Fish-processing Byproducts

Collagen is extracted conventionally by an acid-solubilization process (ASP), in which it is solubilized in an acid while other non-acid-soluble materials are removed (Zhao *et al*., 2011). Pepsin can break down crosslinkages in the telopeptide regions of collagen without harming its secondary structure and therefore the use of pepsin in this process can effectively enhance the yield of collagen (Benjakul *et al*., 2010; Zhao *et al*., 2011).

Several authors have shown that collagen can be effectively extracted from fish skin by using digestive fish enzymes instead of acid. For instance, the recovery of collagen from unicorn leather jacket (*Aluteruus monocerous*) skin was 8.48 and 8.40% using tuna and yellow fin tuna peptides, respectively (Ahmad & Benjakul, 2010). According to Nalinanon *et al*. (2007), the extraction of collagen from defatted skin of big-eye tuna (*Priacanthus tayenus*) was increased by 18.74% when the pepsin extracted from the same fish was used at a level of 20 kUnits/g. Moreover, a considerable amount of collagen was recovered from the skin of thread fin bream (*Nemipterus* spp.) using pepsin extracted from albacore tuna, skipjack tuna and tongol tuna compared to the amount recovered by ASP (Nalinanon *et al*., 2008).

29.5.5.2 Use of Marine-derived Enzymes as Rennet Substitutes in Cheese Manufacture

Rennet extracted from calf stomach has been utilized for the curdling of cheese since ancient times. Substitution of rennet is a hot topic among food scientists since the number of calves slaughtered per year has decreased while the demand for cheese has increased, which has led calf rennet to become relatively expensive (Fox, 1982; Han, 1993). Several milk-clotting enzymes from various plant, animal and microbial sources have been reported. Despite microbial rennet being approved for food applications, it has certain limitations due to its broad specificity and temperature stability which result in a low yield of curd and excessive proteolysis, causing off-flavors during aging (Shahidi & Kamil, 2001).

The purified form of calf rennet, chymosin, is likely to be a good option as it eliminates the limitations associated with microbial rennet because it is relatively unstable and loses most of its activity during completion of milk-clotting. Therefore, the action of other proteases does not necessarily lead to curd formation or give rise to low yields and undesirable textures or flavors (Haard *et al*., 1982; Han, 1993). Chymosin is a highly specific endoproteinase that only splits K-casein into a glycomacropeptide and para-K-casein by selectively cleaving the 105–106 bond between phenylalanine and methionine; other proteolytic enzymes, including pepsin, are less specific and give rise to a number of degradation products with bitter tastes (Fox, 1981; Shahidi & Kamil, 2001).

Some gastric proteases with the same characteristics as chymosin have been reported. Successful efforts have been made in the last few decades to use gastric mucosal extracts of harp seal, cod acid proteases and tuna proteases as alternatives to calf rennet. Shamsuzzaman & Haard (1984) reported a crude mixture of mucosa extracts containing proteases A, B, C and D from harp seal (*Phoca groenlandica*) clotted milk over a wide pH range. Cheese prepared with seal gastric protease showed significantly higher sensory scores than cheese coagulated with calf rennet (Shamsuzzaman & Haard, 1985). Further, tuna proteases were able to clot milk effectively at pH 5.5–6.4, which is similar to the pH of commercial rennet (Tavares *et al*., 1997). Seal and cod gastric proteases have also demonstrated a milk-clotting ability (Han, 1993; Han and Shahidi, 1995). Cheddar-type cheeses have been manufactured using enzymes extracted from the marine crustacean *Munida*, which show a higher extent of degradation of β-casein than cheeses made using chymosin as a coagulant (Rossano *et al*., 2005).

Substitution of rennet from alternative sources means cheesemakers are no longer dependent upon enzymes recovered from slaughtered calves, kids and lambs and enables the production of cheese for a reasonable price. Despite these promising results, cheese production based on fish pepsin has not yet been commercialized (Zhao *et al*., 2011).

29.5.5.3 Use of Marine-derived Enzymes for the Removal of the Oxidized Flavor of Milk

Cardboard flavor is an undesirable phenomenon in the dairy industry caused by the milk oxidation process (Simpson, 1984). Treatement with trypsin is reported to improve stability against oxidative rancidity (Fox, 1982), with both bovine and Greenland cod trypsins having similar effects at concentrations greater than 0.0013%. However, trypsin from Greenland cod has the advantage over bovine trypsin that it can be inactivated by mild heat treatments, including pasteurization, which prevent subsequent hydrolysis of milk proteins by residual enzyme (Simpson & Harrd, 1987). Therefore, the nature of the thermal instability of trypsin to cold-adapted fish upon mild heat treatment has great potential in avoiding oxidative rancidity in dairy products.

29.5.5.4 Use of Marine-derived Enzymes for the Production of Ripened Fish and Fish Sauce

Fish ripening is a complex enzymatic process caused by endogenous intestinal proteases that transform muscle proteins and their products into a range of taste-active peptides and free amino acids, while creating a soft-textured product with unique organoleptic properties. The resultant free amino acids are reported to undergo complex reactions with lipid- and carbohydrate-transformed products, while enzyme-induced changes in the fish muscle-tissue structure cause redistribution of lipids (Shenderyuk & Bykowski, 1990). Dependence on naturally available proteases may itself affect the ripening process and

the quality of final product, due to seasonal variations in enzymes, such as sexual maturity, fat content and enzyme activity (Shahidi & Kamil, 2001). Therefore, supplementation of the enzymes may aid in the production of a quality product.

Lee *et al*. (1982) have reported that addition of trypsin from Greenland rock cod (*Gadus ojac*) or cathepsin C from squid liver accelerates the autolytic fermentation of brined Atlantic short-finned squid (*Illex ilecebrosus*), with a marked increase in sweetness and taste. In another study, trypsin recovered from fish sauce made from cod viscera was used to mature herring (Gildberg & Xian-Quan, 1994). Kolodziejska *et al*. (1992) have reported that squid liver extract treated at 4 or 20 °C causes significant hydrolysis of proteins in the squid mantle, with 65 and 40% decreases in toughness upon cooking, respectively.

Fish sauce can be defined as an amber-colored, liquefied, fermented product high in free amino acids which has a distinctive flavor produced by hydrolysis of fish proteins using halotolerent endogenous enzymes in the presence of a high salt concentration (20–30%). Production of fish sauce is a popular preservation method in countries such as Thailand, Cambodia, Malaysia, Philippines and Indonesia, which have high ambient temperatures and extensive coast lines. Gastric proteases, including trypsin-like enzymes and cathepsin B, are more likely to be important in fish-sauce production, resulting in high amounts of soluble proteins during fermentation and adding distinctive flavors (Shahidi & Kamil, 2001). Anchovy sauce is a liquefied fermented product produced by the hydrolysis of fish proteins in the presence of a high salt concentration. The enzyme present in anchovy intestine and muscle tissue, Cathepsin L, could play a significant role in the production of anchovy sauce by shortening the fermentation time (Heu *et al*., 1997). Furthermore, hepatopancreas proteinases from jumbo squid (*Dosidicus gigas*) have been discussed as a potential source for the preparation of fish sauce and the fermentation of brined squid with an enhanced flavor (Cardenas-Lopez & Haard, 2009).

Several fish proteases, including trypsin, chymotrypsin and squid hepatopancreas extract, have been successfully utilized in fish-sauce production. The duration of fermentation in fish-sauce production can be decreased to 2 months from the usual 6–12 months upon supplementation with proteolytic enzymes including trypsin and chymotrypsin extracted from herrings, without any adverse effect on the sensory qualities of the final product (Chaveesak *et al*., 1993). This method is advantageous over the accelerated method described by Gildberg *et al*. (1984), which uses a low salt concentration (5%) at pH 4 for the initial fermentation, leading to an inferior quality and bitter taste in the final product. In another study, minced squid hepatopancreas extract (2.5%) was successfully incorporated into the production of fish sauce from male capelin (*Mallotus villosus*). Consumer preference was found to be greater for fish sauce with hepatopancreas extract than for the same preparation treated with fungus-derived proteinases (Raksakulthai *et al*., 1986).

However, supplementation of exogenic enzymes in order to accelerate fermentation may have adverse effects on the desired end quality because of the residual enzymatic activity, if the remaining enzymes are not inactivated after the desired quality is obtained. This is not a problem in the conventional method of fish-sauce processing, where enzymes become inactive after a long period of fermentation (Shahidi & Kamil, 2001).

29.5.5.5 Use of Marine-derived Enzymes for the Production of Fish-protein Hydrolysate

Fish-protein hydrolysate can be prepared from cheap pelagic fish, byproducts of the fish processing industry, bycatch from trawlers and underutilized fish species. Hydrolyzation

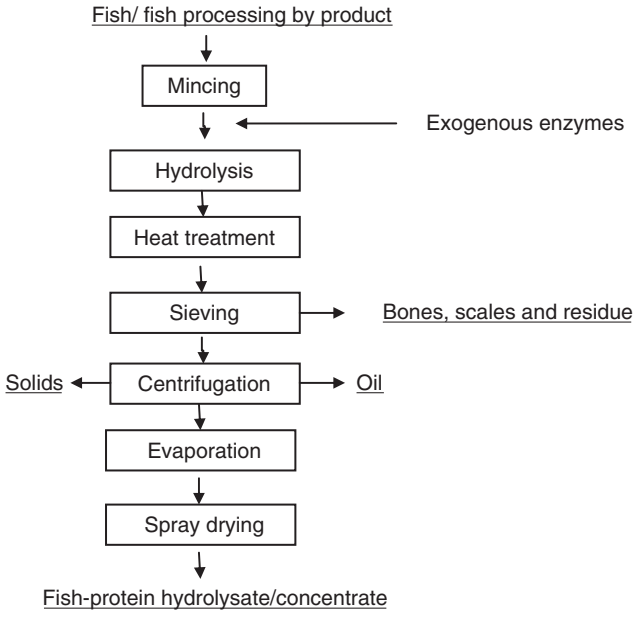

Fig. 29.5 General procedure for the manufacture of fish-protein hydrolysate/concentrate. Adapted from Gildberg (1993).

of the precursor proteins can be achieved either by digestive enzymes present in the fish itself or by adding exogenous proteases from other sources (Nakkarike *et al*., 2011). The common procedure for manufacturing fish-protein hydrolysate is illustrated in Fig. 29.5. During processing, minced fish is hydrolyzed by endogenous or exogenous enzymes and incubated for a sufficient length of time at an optimal temperature, according to the enzyme used. The enzyme activity is inactivated by a heat treatment after the desired characteristics are obtained. The hydrolyzed liquor is then centrifuged and evaporated, and the fish-protein concentrate is obtained (Gildberg, 1993). The properties of the resultant fish-protein hydrolysate depend on the choice of enzymes and the process conditions (Nakkarike *et al*., 2011). The solubility of the fish proteins depends on the incubation time and the amount of enzyme added (Quaglia & Orban, 1987). Prolonged hydrolysis enhances the production of short-chain amino acids, which lack the functional properties of native proteins (Venugopal & Shahidi, 1995). Certain digestive enzymes, namely pepsin, trypsin and chymotrypsin, have been employed for fish-protein hydrolysate preparations, along with some microbial enzymes with the added advantages of a wide variety of catalytic activities and greater pH and temperature stabilities (Nakkarike *et al*., 2011).

Fish-protein hydrolysates have a wide range of potential food applications: they can be used as flavor enhancers, functional ingredients, nutritive additives to foods of low protein quality, milk replacers for young calves and media for the isolation of BAPs (Gildberg, 1993; Kim & Mendis, 2006; Nakkarike *et al*., 2011).

29.5.5.6 Use of Marine-derived Enzymes to Improve the Thickness and Gelling Ability of Surimi

TG has been used to produce various food items, such as restructured meat and seafoods, new-textured sausages, fish cakes, retort-resistant soy bean curd, long-life noodles and

volume-improved bread (de Souza, 2011; Motoki & Kumazawa, 2000). TG derived from marine sources has industrial applications due to its effect on the functionality of proteins, inducing crosslinking of the protein molecules which ultimately improves their physiochemical and functional properties (Motoki & Kumazawa, 2000). The gelation of surimi largely depends on appropriate interactions between adjacent myosin molecules (Haejung *et al*., 1996). Moreover, endogenous TG plays an important role in gel-strength enhancement of surimi (Motoki & Kumazawa, 2000). Benjakul & Visessanguan (2003) investigated the effect of setting induced by endogenous TG in two species of bigeye snapper—*Priacanthus tayenus* and *Priacanthus macracanthus*—on gel properties and protein crosslinking in surimi manufacture. Setting at either 25 or 40 °C, prior to heating at 90 °C, resulted in an increase in both breaking force and deformation of surimi from both species, particularly when setting time increased. In addition, the breaking force and deformation of surimi from both species increased markedly with the addition of calcium chloride, and decreased considerably in the presence of EDTA, *N*-methylmaleimide and ammonium chloride. The results confirmed that endogenous TG played an important role in the gel enhancement of surimi from both species of big-eye snapper.

In another study, the effects of setting at 25 °C on textural properties and crosslinking of myofibrillar proteins in surimi produced from threadfin bream (*Nemipterus bleekeri*), big-eye snapper (*Priacanthus tayenus*), barracuda (*Sphyraena jello*) and bigeye croaker (*Pennahai macrophthalmus*) were investigated. The results suggested that setting at 25 °C for an appropriate length of time may be a promising means by which to improve the gelling properties of surimi produced from tropical fish (Benjakul *et al*., 2003). Moreover, Haejung *et al*. (1996) have suggested that the gelation process and gel strength of surimi can be improved by endogenous fish TG.

29.5.5.7 Use of Marine-derived Enzymes for the Enrichment of PUFAs in Fish Oil

PUFAs are fatty acids that contain more than one double bond in their backbone. They can be subdivided into n-6 (or omega-6, ω-6) series, which are synthetically derived from linoleic acid (18 : 2 n-6), and n-3 series, which are derived from α-linolenic acid (18 : 3 n-3) (Yamashima, 2012). The three major n-3 PUFAs are α-linolenic acid (18 : 3 n-3, ALA), eicosapentaenoic acid (20 : 5 n-3, EPA) and docosahexaenoic acid (22 : 6 n-3, DHA). Numerous health benefits have been attributed to EPA and DHA in relation to cardiovascular diseases, central-nervous-system and mental-health diseases, inflammation and immune functions (Fraeye *et al*., 2012).

Fish is the richest dietary source of EPA and DHA (Welch *et al*., 2010). Long-chain n-3 PUFA-enriched glycerides are considered the most desirable way of administering dietary PUFAs to humans (Lin *et al*., 2006). PUFA ethyl esters can be transformed into PUFA-enriched glycerides (Lyberg & Adlercreutz, 2008). PUFA enrichment can be carried out using a number of methods, namely molecular distillation, low-temperature crystallization, supercritical-fluid extraction, urea complexation and enzymatic methods such as lipase-catalyzed hydrolysis and lipase-catalyzed esterification (Shahidi & Wanasundara, 1998). Lipase-catalyzed synthesis of n-3 PUFA-enriched glycerides is considered a promising method due to its being environmentally friendly and having mild reaction conditions, and the high efficiency and high regiospecificity of some lipases (Lyberg & Adlercreutz, 2008).

Long-chain n-3 PUFAs are highly vulnerable to destruction by oxidation or *cis-trans*-isomeration during processing, due to the extreme pH and temperature conditions. Traditional chemical-modification processes for fats and oils generally involve rather

extreme conditions and the need for mild, enzymatic conditions is recognized in the marine oleochemical industry (Vilhelmsson, 1997). Most commercially available lipases specifically target the short-chained PUFAs and not the long-chained PUFA. However, lipolytic enzymes isolated from Atlantic cod (*Gadus morhua*) are reported to be specific to long-chained PUFAs (Lie & Lambertsen, 1985). Moreover, lipase activity related to esterification, hydrolysis or exchange of fatty acids in esters provides an opportunity to produce TGA enriched with omega-3 PUFA from fish oil (Nakkarike *et al*., 2011).

29.5.5.8 Use of Marine-derived Enzymes for the Deskinning and Descaling of Fish and Squid

Deskinning is an important step in fish fillet production, in which the fish's skin is gently removed without causing any damage to the flesh, since the value and quality of the fish depends on its exterior quality (Benjakul *et al*., 2009). Mechanical deskinning is rough and has a high risk of damaging the flesh and thus increasing wastage.

Enzymatic deskinning can improve the edible yield over that of mechanical deskinning (Benjakuel *et al*., 2009; Gildberg *et al*., 2000). Several enzymes derived from fish have been successfully employed in deskinning. Acid proteases obtained from cod viscera have been found to be effective in the deskinning of herring, for example (Joakimsson, 1984; Trincone, 2010), and Tschersich & Choudhury (1998) have used crude protease extract from minced arrow-tooth flounder to remove pollock skin. Kim *et al*. (1994) have described a method of deskinning filefish using collagenases from the digestive organs of fish.

There are some fish species in which the skin is very hard to remove, such as sharks and skates. Enzymatic deskinning may be a solution in this situation, too. It has been reported that only the outer skin of squid can be removed mechanically, leaving the rubbery, tough inner cover (Simpson, 2012). Strom & Raa (1991) have developed a gentle procedure for the deskinning of squid using its own digestive enzymes; earlier, Leuba *et al*. (1987) developed a method for deskinning squid skin using squid liver extract.

Descaling is also an important processing step in fresh fish markets, such as the Japanese sashimi market. Moreover, descaling is essential to the leather industry. In both cases, gentle descaling is responsible for maintaining quality. Enzymatic descaling is a more gentle process than mechanical descaling, in which the soft-flesh species are difficult to descale (Simpson, 2012).

29.5.5.9 Potential Food Applications of Marine-derived Lipases

Marine-derived lipases have great potential to be used in the food industry as substitutes for commercial lipases. Their possible uses are listed in Table 29.8. Fungi, especially the *Candida* species, are the most promising candidate for the commercial preparation of lipases, due to their ability for batch fermentation and low-cost purification (Aravindan *et al*., 2007). However, cold-adapted lipases derived from marine organisms, especially those from cold climates, may have added advantages over lipases of terrestrial organisms as they can be active and perform the same reactions at mild temperature conditions and across a wide range of pHs.

29.5.6 Food Applications of Marine-derived Collagen and Gelatin

At the industrial level, gelatin quality is determined by gel strength, viscosity, melting or gelling temperature, water content and microbiological safety. Marine sources such

Table 29.8 Potential applications of marine-derived lipases. Adapted from Aravindan *et al*. (2007).

Industry	Action	Product of application
Dairy processing	Hydolysis of milk fat, cheese ripening and modification of butter fat	Development of flavoring agents in milk, cheese and butter
Bakery industry	Flavor improvement	Shelf-life propagation
Beverage industry	Improved aroma	Production of alcoholic beverages (sake and wine)
Food-dressing manufacture	Quality improvement	Mayonnaise, dressings and whippings
Functional foods	Transesterification	Functional-food processing
Meat and fish processing	Flavor development	Fat removal, sausage production
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono- and diglyceride production

as fish skins have significant potential for the production of high-quality gelatin with melting and gelling temperatures over a much wider range than mammalian gelatins and with sufficiently high gel strength and viscosity (Boran *et al*., 2010). However, the fishy odor and inferior rheological properties of marine gelatin may have a negative impact on its industrial applications (Cheow *et al*., 2007; Gomez-Guillen *et al*., 2007). In the food industry, gelatin is utilized in confections, low-fat spreads and dairy, baked and meat products to provide specific product properties/functional properties, such as chewiness, texture, creaminess, fat reduction, stabilization, emulsification, gelling, water binding and mouth feel (Karim & Bhat, 2009; Kim & Park, 2005; Mendis *et al*., 2005). Food-grade fish gelatin is reported to have no or reduced risk to fish-allergic patients (Hansen *et al*., 2004), demonstrating its potential wide application in the food industry. Furthermore, gelatin has been reported to be one of the first materials used as a carrier of bioactive components (Gomez-Guillen *et al*., 2011).

29.5.6.1 Edible Films and Coatings from Marine-derived Gelatin

In general, an edible film is a thin, continuous layer of edible material. There is an increasing demand for edible and biodegradable films or materials with the potential to extend the shelf life and improve the quality of almost any food system by serving as mass-transfer barriers to moisture, oxygen, carbon dioxide, lipid, flavor and aroma between food components and the surrounding atmosphere. Increasing concern over health, nutrition, food-safety and environmental problems has also increased interest in edible and biodegradable films and materials (Al-Hassan and Norziah, 2012; Jongjareonrak *et al*., 2006). Edible films can be prepared from renewable sources such as proteins, polysaccharides, lipids or the combination of these components. However, proteins have been most extensively selected for the development of edible films, due to their availability, relatively low cost, nutritional value and unique film-forming ability, and the superior

mechanical and barrier properties of the films they produce (Cao *et al*., 2007; NurHanani *et al*., 2012; Ou *et al*., 2004).

Among the proteins, gelatin has been identified as the most suitable material for developing edible films, due to its abundance and biodegradability (Jongjareonrak *et al*., 2006). Furthermore, gelatin has excellent film-forming and good mechanical properties and is unique among hydrocolloids in forming thermoreversible films, with a melting point close to body temperature, which is particularly significant in food and pharmaceutical applications (Cao *et al*., 2007). In general, fish gelatin possesses excellent film-forming properties (Karim & Bhat, 2009). However, while the quality of a food-grade gelatin depends to a large extent on its rheological properties (mainly gel strength and viscosity), it is also determined by other characteristics, particularly transparency, absence of color and flavor and ease of dissolution (Gomez-Guillen *et al*., 2007). These properties can vary significantly with the raw materials used in isolating marine-derived gelatin. For example, sole- and megrim-skin gelatins have been reported to demonstrate a gel-strength value approximately five times higher than that of cod-skin gelatin (Gomez-Guillen *et al*., 2002). Marine gelatin with low gel strength produces an extremely soft gel with a low cohesive force (Gomez-Guillen *et al*., 2002), which can negatively affect its functional properties, such as its film-forming ability. However, such disadvantages can be reduced by various approaches, including the use of enzymes, addition of salts and application of ultraviolet (UV) radiation (Baht & Karim, 2008, 2009).

Collagen and gelatin films have been used in both the food and the pharmaceutical industries (Jongjareonrak *et al*., 2006; Sobral *et al*., 2001). Edible films have been successfully produced from marine sources such as brownstripe red snapper (*Lutjanu svitta*) and bigeye snapper (*Priacanthus macracanthus*) (Jongjareonrak *et al*., 2006), surimi-processing wastes (Al-Hassan &Norziah, 2012), cod (Krishna *et al*., 2011; Perez-Mateos *et al*., 2009), haddock and pollock (Krishna *et al*., 2011), tuna (Gomez-Guillen *et al*., 2007, 2009), cuttlefish (Hoque *et al*., 2010), Atlantic halibut (Carvalho *et al*., 2008), giant squid (Gimenez *et al*., 2009) and blue shark (Limpisophon *et al*., 2009). These films have good physical and mechanical properties, as acting as transparent, effective barriers to water vapor, gas and UV light. Although the inherent fishy odor of marine-derived gelatin may limit its application as an edible film and coating in certain foods, it is ideal for use with marine foods.

Microencapsulation is defined as the technology of packaging solids, liquids or gaseous materials in miniature, sealed capsules that release their contents at controlled rates under the influence of specific conditions (Anal & Singh, 2007). Microencapsulation can play a role in releasing functional components from a food product or bioactive packaging when it is ingested and masking the taste and odor of strongly flavored food components (Bao *et al*., 2009; Gomez-Guillen *et al*., 2011). Gelatin is one of the most commonly used proteins for the encapsulation of food ingredients by spray-drying (Gharsallaoui *et al*., 2007). Like conventional gelatin, it is possible that marine-derived gelatin may also possess beneficial characteristics such as entrapment of functional components in a carrier food matrix during microencapsulation and protection against oxidation or degradation during storage. Further, the low gelling temperature of fish gelatin can cause advantages in the microencapsulation process (Karim & Bhat, 2009). Fish gelatin has been used in the microencapsulation of vitamins, colorants and other pharmaceutical additives such as azoxanthine. It is also possible to microencapsulate food flavors such as vegetable oil, lemon oil, garlic, apple or black pepper using warm-water fish gelatin, and soft gel capsules produced from fish gelatin have been widely used as nutrition supplements

(Karim & Bhat, 2009). However, the use of marine-derived gelatin in microencapsulated food ingredients has not yet been well documented in the scientific literature.

29.5.6.2 Marine-derived Gelatin as a Gelling Agent in Food Products

The most widespread single use of gelatin in food products is in water-gel desserts, due to its unique melt-in-the-mouth property. Desserts made from different gelatins may show variety in texture and gel-melting behavior, offering new product-development opportunities in the food industry (Gomez-Guillen *et al*., 2011; Zhou & Regenstein, 2007). Cold-water fish gelatin can be used in frozen or refrigerated products that are consumed soon after removal from the fridge or defrosting (Karim & Bhat, 2009). Desserts made from Alaskan pollock gelatin (by increasing gelatin concentrations or using gelatin mixtures) have been found to be similar to desserts made from high-bloom pork-skin gelatin. The lower melting temperature in gel desserts made from fish gelatins accelerates flavor release (Zhou & Regenstein, 2007). Flavored fish-gelatin dessert gel products have been reported to possess less undesirable off flavors and off odors and a more desirable release of flavor and aroma than the same desserts made with an equal-bloom-value, but higher-melting-point, pork gelatin (Choi & Regenstein, 2000).

The gelatin produced from the skins of cold-water fish has a gelling temperature below 8 °C, which provides fresh application opportunities. For example, such a gelatin is the basis for light-sensitive coatings in the electronics trade and is a good medium for precipitating silver halide emulsions as it allows the process to be carried out at lower temperatures. On the other hand, the same feature limits the use of such a gelatin as a gelling component in food production (Kolodziejska *et al*., 2004). A possible means of manipulating the characteristics of such low-gelling gelatins to achieve greater similitude with the properties of mammalian gelatins is to induce enzymatic crosslinking through the action of TG, which is usually of microbial origin. Increasing concentrations of TG in megrim-skin gelatin gels have been reported to raise the melting temperature and increase the elasticity and cohesiveness, but due to excessively rapid gel-network formation there is a concomitant lowering of gel strength and hardness. However, for gelatin extracted from Baltic cod skins, the addition of TG has proven to be effective in producing stable gels at room temperature, as well as after heating in boiling water for a period less than 30 minutes (Gomez-Guillen *et al*., 2011; Kolodziejska *et al*., 2004).

Apart from treatment with TG, it is also possible to improve these functional properties by the addition of $MgSO_4$ and glycerol, without modification of quality, color or transparency. Depending on the intrinsic properties of the protein molecules, the behaviors of the gelatin–ingredient complexes will vary, making it possible to obtain a great variety of characteristics, which is important in industrial applications (Fernandez-Diaz *et al*., 2001).

Polysaccharides such as κ-carrageenan and gellan (Pranoto *et al*., 2007) have also been reported to increase the gel strength of fish gelatin without losing their functional properties. Reducing drip loss and impairing juiciness while improving sensory and physicochemical properties in frozen fish or meat products when thawed or cooked seems to be possible with the use of marine-derived solubilized collagen and gelatin (Gomez-Guillen *et al*., 2011). The application of appropriate amounts of fish gelatin as an additive in surimi processing has been reported to improve water retention in Alaska pollock surimi gels (Hernandez-Briones *et al*., 2009). The gel-forming capability of threadfin bream (*Nemipterus japonicus*) mince was substantially increased by adding gelatin (0.5%) from the skin of bigeye snapper (Binsi *et al*., 2009). Fish gelatins with lower melting points can

be considered good alternatives to conventional gelatin as they can prevent unattractive clumping in yogurt (Karim & Bhat, 2009). The combination of fish gelatin with hydrocolloides such as pectin has been used to produce low-fat spreads (Cheng *et al*., 2008).

Although such benefits of marine-derived fish gelatin have been recorded in the food industry, commercial interest in marine-derived fish gelatin is still relatively low compared to that for conventional gelatin isolated from land animals.

29.6 CONCLUSION

In the past few decades, significant advances have been made in the extraction and characterization of proteins and peptides from marine sources, including enzymes, collagen and gelatin, AFPs, antimicrobials, antioxidants and various other BAPs. Numerous BAPs with potential applications in the food industry have been reported, mainly from fish sources; however, other marine organisms such as crustaceans have not been extensively studied. Although certain marine proteins and peptides, such as gelatin from fish and waste from seafood processing, have been well characterized, their application in the food industry seems limited at present. Future research on marine proteins and peptides should be focused on producing high-quality end products with reduced contamination, enabling their direct use in food-manufacturing operations, developing Hazard Analysis & Critical Control Point (HACCP) systems related to isolation procedures, developing high-yield–low-cost extraction methods, improving functional properties to fit into food grades, undertaking clinical studies to investigate potential health benefits from incorporation into food matrices and further isolating and characterizing nonhazardous and novel bioactive compounds that can be utilized in developing functional foods with good microbial, physiochemical and sensory properties.

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30 Processing and Industrial Aspects of Fish-scale Collagen: A Biomaterials Perspective

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30.1 INTRODUCTION

Collagen is the most abundant protein in mammals, making up about 25–35% of total body protein (Lullo *et al*., 2002). It is the core component of connective tissues and is found in bones, tendons, ligaments, eye lenses, skin, and corneas, playing diverse structural and functional roles (Lullo *et al*., 2002). The extracellular matrices (ECMs) of most tissues are constituted predominantly of collagen, where it provides strength and stability (Aumailley & Gayraud, 1998). In humans, there are around 28 different types of collagen molecule, as well as about the same number of other members of the collagen super family (Kadler *et al*., 2007; Myllyharju *et al*., 2004; Ricard-Blum & Ruggiero, 2005). Collagen can be grouped based on its structure and supramolecular organization into fibril-forming collagens, fibril-associated collagens (FACIT), network-forming collagens, anchoring fibrils, transmembrane collagens, basement-membrane collagens and other types with unique functions in each specific tissue. Amongst these, fibril-forming collagen is the most predominant type, making up almost 90% of the total collagen population (Gelsea *et al*., 2003). Since many disorders are caused by damage to the ECM collagen structure, use of collagen as a biomaterial for tissue engineering, implant-device materials and drug delivery is a rapidly emerging field. However, a widely acceptable source of collagen that fulfills the demand for molecular analysis is still evasive. This chapter presents a brief overview of the collagen molecule and the various sources available, before going on to concentrate on the potential of fish to serve as an environmentally conducive, economically viable and immunologically acceptable source of collagen.

30.2 STRUCTURE AND COMPOSITION OF COLLAGEN

The composition of collagen makes it a unique protein, characterized by the presence of high amounts of hydroxyproline. Collagen has repeating Gly-X-Y motif, where Gly is glycine, and the X and Y positions are occupied by any of the other amino acids. However, these positions are mostly occupied by the imino acid proline, and the Y position is normally altered by post-translational modification to 4-hydroxyproline (Brodsky

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& Ramshaw, 1997). The other amino acids also show preferences for their occurrence in the X and Y positions. For example, large aliphatic and aromatic residues rarely occur in the Y position, due to steric constrains on the packing of the molecule (Ramshaw *et al*., 1998). Arg is the most frequently present amino acid in this position, probably due to its capacity to stabilize the triple helix (Yang *et al*., 1997). Glycine, which is present at every third position, makes the collagen structure compact. The stability of the collagen triple helix is related to the total content of proline and hydroxyproline, which together make up about 20% of the total amino acids in human fibrillar collagens (Hulmes, 2008). 4-hydroxyproline helps in triple-helix stabilization through intramolecular hydrogen bonding and the hydroxyl groups show an electron-withdrawing (inductive) effects which stabilizes the exo-pucker of the (4R) stereoisomer in the Y position (Brodsky *et al*., 2005). Additionally, hydroxylysine is a substrate for O-linked glycosylation and performs an important role in the crosslinking of collagen, thereby affecting fibril formation and other protein–protein interactions. The degree of lysyl hydroxylation varies between types of collagen, being relatively high in collagens IV and VI and in embryonic tissues. Interestingly, lysines in the Y position of the Gly-X-Y triplet can only be hydroxylated (Hulmes, 2008). Furthermore, the degree of crosslinking in collagen fibers differs in different tissue types according to the degree of hydroxylation of telopeptide lysine in the crosslinks and the rate of collagen metabolism (Hulmes, 2008). The triple-helical structure of the collagen molecule was first proposed by G. N. Ramachandran (1956). The triple-helical motif can form both the major and the minor part of the molecule, depending on the genetic type of collagen. Other regions of collagen also contain different noncollagenous domains (Hulmes, 2008). Structurally, the collagen molecule is made of three α-chains, with a pitch of 18 amino acids per turn. The orientation of the chains determines the type of collagen molecule: either a homotrimer (three identical chains, as in collagens II, III, VII, VIII and X) or a heterotrimer (two or more different chains, as observed in collagens I, IV, V, VI, IX and XI) (Hofmann *et al*., 1978). In the collagen structure, three left-handed polyproline II-like helices are wound to form a supercoiled triple-helical, right-handed rope-like structure (Brodsky & Ramshaw, 1997). The three chains are also staggered by one residue relative to one another (Fraser *et al*., 1979). The fibril-forming collagen molecules have an uninterrupted triple helix of approximately 300 nm length and 1.5 nm in diameter, followed by short extra helical telopeptides. The telopeptides which do not adopt triple-helical conformations due to the absence of repeating Gly-X-Y units account for 2% of the molecule and play a vital role in fibril formation (Kadler *et al*., 1996).

30.3 SYNTHESIS OF COLLAGEN

Different types of collagen molecule have similar structures, but their biosynthesis pathways are slightly different. Collagen biosynthesis has been studied in great detail with regard to the fibrillar collagens. It is a complex process involving numerous intracellular and extracellular steps, which contribute to the structural and biomechanical properties of the fibrils. Fibrillar collagen is synthesized as procollagen in the lumen of the rough endoplasmic reticulum, transported to the Golgi apparatus and secreted into the extracellular space through secretory vesicles. After synthesis in the nucleus, mRNA is translated by ribosomes to form pre-pro-peptide, which enters the rough endoplasmic reticulum guided by signal peptides. Subsequently, the first phase of post-translational modifications is initiated in the endoplasmic reticulum by cleavage of the signal peptide from

pre-pro-peptide, leading to pro-peptide. Hydroxylations of proline and lysine residues then take place, followed by glycosylation on to the hydroxyl groups of lysine residues. Disulfide bonding, prolyl *cis–trans*-isomerization also takes place at this stage. Propeptide then twists tightly towards the left, giving rise to a triple helix. The procollagen thus formed is then transported to the Golgi complex for further modifications, wherein oligosaccharides are added and packaged into secretory vesicles destined for the extracellular space. Outside the cell, enzymes remove the loose ends of procollagen, forming the tropocollagen. Finally, aldehyde groups are added to lysines and hydroxylysines, which will eventually undergo covalent bonding between tropocollagen molecules, resulting in the formation of collagen fibrils (Hulmes, 2008).

30.4 TYPE-I COLLAGEN

Type I is the major fibril-forming collagen, found in tendons, skin, artery walls, the endomysium of myofibrils, fibrocartilage and the organic part of bones and teeth of all animals. It is present throughout the body, except in cartilaginous tissues (Kadler *et al*., 1996). Type-I collagen shows versatility as a structural material, providing elasticity, energy storage, stiffness and toughness. It is a triple helix with two identical $\alpha 1$ chains and one $\alpha 2$ chain. It provides substantial biomechanical properties to tissues in the form of load-bearing capacity, tensile strength and torsional stiffness, either by itself or after calcification (Gelsea *et al*., 2003). The biomechanical scaffold for cell attachment and anchorage of macromolecules is mainly provided by type-I collagen, allowing for the maintenance of well-defined tissue structures (Kadler *et al*., 1996). The mechanical properties of the tissues are further dependent upon alignment of these crosslinked collagen fibers. Collagen forms a rod-like molecule from which reactive groups such as lysine, glutamic acid and hydroxyl project radially aligned parallel to the fiber, causing strong intermolecular or interfibrillar crosslinking. Crosslinked groups are additionally responsible for the enhancement in the mechanical strength of fibers and the increase in the denaturation temperature, by preventing molecules and fibers from sliding past each other under load. Further, due to the decrease in accessibility for enzymes and the reduced hydration of the fibers, they become less susceptible to enzymatic degradation (Avery & Bailey, 2008).

30.4.1 Sources

At present, type-I collagen for biomedical application is principally obtained from rat tail (O'Leary & Wood, 2003) or from bovine (Hsu *et al*., 1999), porcine (Huang *et al*., 2005) or equine (Angele *et al*., 2004) species. Collagen from different species differs notably in its chemical, physical and biological properties (Angele *et al*., 2004). Rat-tail tendon was one of the earliest sources of type-I collagen to be used for biomedical applications. Type-I collagen extracted from bovine achilles tendon is widely used in dental applications, especially in implant dentistry (Techatanawata *et al*., 2011). Chicken skin is another source of collagen that is used in biomedical research (Cliché *et al*., 2003). In some cases, human collagen is also used. Bovine collagen, though potentially useful, always carries the risk of spreading infectious diseases, such as bovine spongiform encephalopathy (BSE) or transmissible spongiform encephalopathy (TSE). Collagen obtained from porcine skin or bone is sometimes not usable as a food component due to aesthetic or religious beliefs.

Hence there is a growing need for alternative sources of collagen, for which processing wastes, including the skin, bone, scale and fin of both freshwater and marine fish, octopus arm, bullfrog skin and squid skin, have emerged as substitutes (Senaratne *et al*., 2006). Researchers have also tried isolating collagen from cockroach, pearl oyster, starfish and alligator (Francois *et al*., 1980; Lee *et al*., 2009; Mizuta *et al*., 2002; Wood *et al*., 2008). Work from Hokkaido University has found that collagen obtained from soft-shelled turtles is similar to porcine collagen, but it is less suitable for tissue-engineering applications as tissue growth in the matrix is slower. An investigation at Ocean University of China reported the antioxidative properties of jellyfish-derived collagen. Both unaltered and an altered version, called collagen hydrolysate, have been analyzed, and both have been found to combat ultraviolet (UV) damage (Zhuang *et al*., 2009).

30.4.2 Advantages and Disadvantages

30.4.2.1 Advantages

- abundant availability;
- convenient purification process;
- noncytotoxicity;
- biodegradability;
- biocompatibility;
- synergistic with bioactive components;
- high tensile strength and minimal expressibility;
- hemostatic property, which promotes blood coagulation;
- presence in a number of different forms;
- high mechanical strength.

30.4.2.2 Disadvantages

- isolation of the pure form is costly;
- exhibits variability in crosslink density and fiber size when isolated;
- hydrophilic, leading to swelling;
- variability in enzymatic degradation rate as compared with hydrolytic degradation;
- complex handling properties (Friess, 1998; Fujioka *et al*., 1998; Jerome & Ramshaw, 1995; Rao, 1995).

30.4.3 Biomedical Applications

By virtue of their superior biocompatibility, collagen-based materials are best suited for soft-tissue engineering applications (Soiderer *et al*., 2004). They have been useful in preparing collagen paste for soft-tissue augmentation (Wallace *et al*., 1988), low-porosity sheets to act as adhesion barriers (Edwards *et al*., 1997), gel for ophthalmic application (Vore *et al*., 1994) and molded scaffold for meniscal repair.

Collagen scaffolds have also found applications in *in vitro* cell-behavior studies, including studies of cell migration and proliferation, differentiation and phenotype expression. Collagen hydrogels are convenient scaffolds for membrane access, which is required in electrophysiological protocols (Ma *et al*., 2004; O'Shaughnessy *et al*., 2003; Xu *et al*., 2009). Collagen-based scaffolds are equally useful in nervous-system models,

for visualization of motor-neuron myelinization by schwann cells (Gingras *et al*., 2008), invasiveness of cancer cells (Che *et al*., 2006; Sabeh *et al*., 2009) and interaction between cancerous and other cell types in three-dimensional space. Collagen-based biomaterial implantation becomes almost indispensable in cases where osteochondral defects reach higher volumes or autografts cannot be used due to practical or pathological constrains. Collagen-based wound dressings have found application for decades as burn coverings and ulcer treatments (Doillon & Silver, 1986; Peters, 1980; Yannas *et al*., 1982). More recently, sophisticated and innovative tissue-engineered skin models using melanocytes (Regnier *et al*., 1997) have been developed for capillary-like networks (Tremblay *et al*., 2005), dendritic cells (Bechetoille *et al*., 2007), sensory innervations (Blais *et al*., 2009; Caissie *et al*., 2006), adipose tissue (Trottier *et al*., 2008) and tissue reproducing psoriatic or sclerotic phenotypes (Corriveau *et al*., 2009; Jean *et al*., 2009). Studies are being conducted to solve urethral strictures and other genitourinary disorders using acellular collagen scaffolds as bladder-augmentation systems (Akbal *et al*., 2006; el-Kassaby *et al*., 2008; Farahat *et al*., 2009; Fiala *et al*., 2007; Liu *et al*., 2009). A number of collagen-based medical devices are used in ophthalmology as grafts for corneal replacement, suture materials, bandage lenses, punctual plugs and viscous solutions for use as vitreous replacements or protectants during surgery (DeVore, 1995). Gene delivery using collagen-based pallets is an important proposition. Biodegradable collagen-based nanoparticles and nanospheres are thermally stable, and hence easy to sterilize (Rossler *et al*., 1995). In addition, nanoparticles are easily taken up by the reticuloendothelial system (Marty *et al*., 1978), enabling enhanced uptake of exogenous compounds, such as anti-HIV drugs, into a number of cells, particularly macrophages (Bender *et al*., 1996). Collagen is also used as an implantable carrier for bone-inducing proteins, such as bone morphogenetic protein 2 (rhBMP-2) (Reddi, 2000), and collagen itself has been used as a bone substitute, due to its osteoinductive activity (Murata *et al*., 1999). Collagen gel as a human skin substitute has proven a great success in tissue engineering, leading to the development of bioengineered tissues, such as blood vessels, heart valves and ligaments (Auger *et al*., 1998).

30.5 RECOMBINANT COLLAGEN

Due to its easy and cheap availability, animal-extracted collagen has been widely used as a biomaterial (Lee *et al*., 2001), but it can pose the threat of infection and in many cases, due to prevailing impurities, induce immune reactions (Lynn *et al*., 2004). Recombinant collagen has been developed to solve the problems faced during the extraction of collagen from animal sources (Myllyharju, 2000). It presents a safe, predictable and chemically defined source for biomedical applications. Due to their similar primary and tertiary structures, recombinant human collagens have similar properties and degrees of stability to natural human collagens. They can be produced from transfected mammalian cells, insect cells, yeast, *Escherichia coli*, transgenic tobacco, mice (Olsena *et al*., 2003) and transgenic silkworm (Tomita *et al*., 2003).

Recombinant collagen, especially type I, has been extensively investigated for drug-delivery (Olsena *et al*., 2003) and bone, skin, cartilage and periodontal ligament tissue-engineering applications, showing good success rates in laboratory experiments (Yang *et al*., 2004). Collagen-based hydrogels fabricated from recombinant human collagen have been found to be suitabile for human cornea regeneration due to their optical clarity and

nutrient permeability, and the fact they possess adequate strength for implantation. When implanted, these hydrogels induce stable regeneration of corneal tissue, including corneal cells and nerves (Wenguang *et al*., 2008). Recombinant human collagen, in combination with recombinant bone morphogenetic protein, forms an efficient, porous, sponge-like scaffold for bone repair, and can even serve as a basis for engineering skin, cartilage and periodontal ligament, depending upon requirements (Yang *et al*., 2004). The recombinant process is also used for types of collagen that have rare occurrence in natural tissues and are hence difficult to extract using conventional techniques, but which possess properties advantageous for certain clinical applications (Spiro *et al*., 2002).

30.6 FISH'S POTENTIAL AS AN ALTERNATIVE SOURCE OF COLLAGEN

Though it appears to be a promising approach, recombinant-collagen production is a tedious process, requiring highly skilled personnel, and is often very expensive. Considering the limitation of other sources, fish emerge as a potentially important source of collagen. Fish has been incorporated in the human diet since antiquity and today fish and its derivatives are among the most widely marketed products worldwide. Globally, several developing countries are net exporters of fishery products, which are a major source of foreign exchange. The total economic impact of capture fisheries is US$225–240 billion—three times the economic activity of the fisheries industry alone. There has been significant growth in fish production in India in recent years, making it the third largest producer of fish and the second largest producer of freshwater fish in the world. Fish production during 2008–09 was 76.2 lakh tonnes, comprising 29.8 lakh tonnes of marine fish and 46.4 lakh tonnes of inland fish (http://dahd.nic.in/dahd/division/fisheries.aspx).

There is no significant report on the possibility of disease transmission from fish sources. Fish skin and scales are major byproducts of the fish-processing industry, causing wastage and pollution; fortunately, they also provide a valuable source of collagen. Extraction of collagen from fish is economical and efficient. Pati *et al*. (2012) have found that collagen obtained from freshwater fish is highly biocompatible and exhibits considerable cell viability, comparable to that of bovine sources.

This section presents a comparative analysis of the extraction and characterization of collagen from fish sources, which will be useful not only to biomaterial scientists but also to the fishery industries, entrepreneurs and industrialists looking to add value to the flourishing industry of fish processing.

30.6.1 Isolation of Collagen From Various Parts of the Fish

The isolation procedure for collagen extraction from different parts of the fish is solely dependent on the composition and architecture of their native ECMs. These have been summarized schematically in Fig. 30.1.

30.6.1.1 Scale

Cycloid-type fish scales are completely devoid of bone. They show high availability and thus emphasis is given to them. Fish scales are primarily composed of calcium-based minerals and a fibrous inner layer mainly made of collagen. Thus, decalcification of

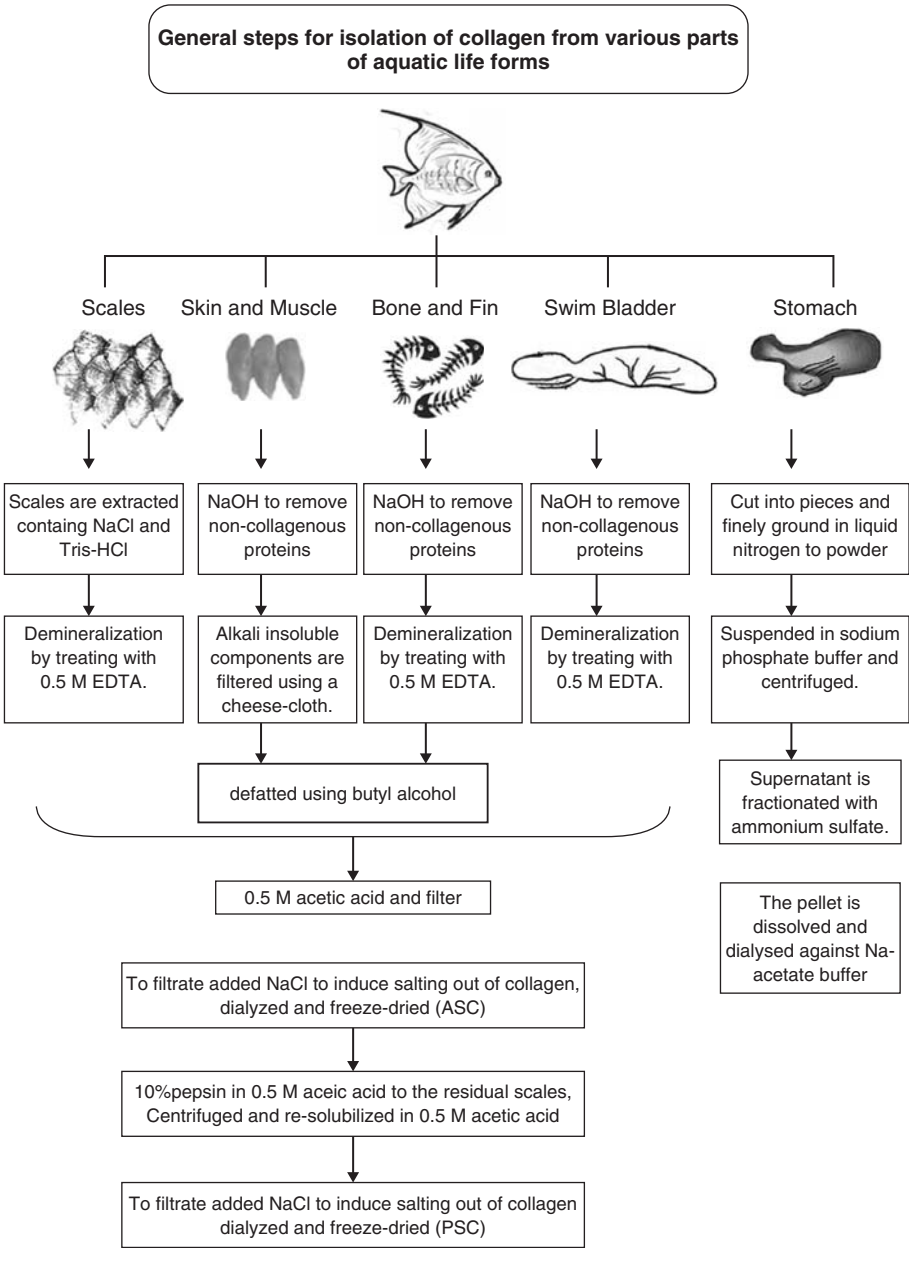


Fig. 30.1 Schematic of the collagen isolation procedure from different fish components.

the scale is essential for dissolution of collagen from the mineral particles (Pati *et al.*, 2010). There are several reports on the isolation of collagen from fish scales of various sources—such as freshwater and marine—where isolation is performed mainly through demineralization of the scales. All preparative procedures are carried out at 4 °C in order to reduce problem of collagen degradation. The scales are thoroughly washed first with

distilled water and then in a solvent system containing NaCl, Tris–HCl and EDTA at pH 7.5 in order to remove unwanted proteins from the surfaces. Demineralization of the scales is accomplished by treatment with 0.5 M EDTA solution at pH 7.4. The demineralized scales are then washed again with distilled water and treated with 0.5 M acetic acid solution at pH 2.5. The insoluble part of is then filtered out. NaCl is added to the filtrate to a final concentration of 0.9 M in order to induce salting-out of the collagen. The suspension is centrifuged and the precipitate resolubilized in 0.5 M acetic acid. The final solution is dialyzed using a dialysis membrane against 0.1 M acetic acid and distilled water and then freeze-dried. Collagen obtained by this method is termed 'acid-solubilized collagen' (ASC).

A second type of collagen, known as 'pepsin-solubilized collagen' (PSC), is obtained by adding 10% pepsin in 0.5 M acetic acid to the residual scales and then filtering the solution. The process is repeated twice for better extraction. NaCl is added to the filtrate to a final concentration of 0.9 M, and the solution is then centrifuged and resuspended in 0.5 M acetic acid. The final solution is dialyzed using a dialysis membrane against 0.1 M acetic acid and distilled water, each for 24 hours, and freeze-dried.

Pati *et al*. (2010) followed this procedure to extract collagen from rohu and catla. The same procedure was also used to isolate collagen from the fish scales of black drum, sheepshead seabream (Ogawa *et al*., 2004), *Cyprinus carpio* (Duan *et al*., 2009), deep-sea red fish (Wang *et al*., 2008), *Pagrus major* and *Oreochromis niloticas* (Ikoma *et al*., 2003). Collagen from the cartilage of brownbanded bamboo shark and blacktip shark has also been isolated following similar procedures (Kittiphattanabawon *et al*., 2010).

In the case of carp, the scales are washed with successive changes of distilled water and 0.5 M sodium acetate for 5 days. The skull is then cut into small pieces and decalcified for 6 days with EDTA at pH 7.5 containing phenylmethylsulfonylfluoride and N-ethylmaleimide. The EDTA solution is renewed once a day (Kimura *et al*., 1991).

30.6.1.2 Skin and Muscle

To obtain collagen from fish skin, it is cut into small pieces, treated with NaOH to remove noncollagenous proteins and washed with distilled water until the water becomes neutral or slightly alkanine. The alkali-insoluble components are subsequently filtered using a cheesecloth. Skin is defatted using butyl alcohol and treated with 0.5 M acetic acid. Further, collagen is extracted from the skin following the same procedure used for scales, as reported for brownstripe red snapper (Jongjareonrak *et al*., 2005), bigeye snapper (Kittiphattanabawon *et al*., 2005), largefin longbarbel catfish (Zhang *et al*., 2009), balloon fish (Huang *et al*., 2011), brown-backed toadfish (Senaratne *et al*., 2006), ornate threadfin bream (Nalinanon *et al*., 2011), striped catfish (Singh *et al*., 2011), ocellate puffer fish (Nagaia *et al*., 2002), squid (*Ommastrephes bartrami*) (Mingyan *et al*., 2009), unicorn leatherjacket (Ahmad & Benjakul, 2010), bamboo shark (Kittiphattanabawon *et al*., 2010), black drum and sheepshead seabream (Ogawa *et al*., 2003). Nagai *et al*. (2010) used 10% ethanol to remove the fat content from the skin of surf smelt, while ether and hexane have been used for similar purposes in channel catfish (Liu *et al*., 2007). Noncollagenous proteins are removed from the skin of deep-sea red fish by soaking it in NaCl with Tris–HCl at pH 7.5 (Wang *et al*., 2008), while NaCl with EDTA is used for the removal of noncollagenus proteins from *Amiurus nebulosus* (Chen *et al*., 2011) and Nile perch (Muyonga *et al*., 2004).

To extract fat from *Cyprinus carpio* (Duan *et al*., 2009), deproteinized skins are soaked in detergent overnight and then washed repeatedly with cold distilled water. Salting-out

is done by adding NaCl in the presence of 0.05 M tris (hydroxymethyl) aminomethane at pH 7.0. This salting process is also used in Argentine shortfin squid (Zhang *et al*., 2011), bamboo shark (Kittiphattanabawon *et al*., 2010) and unicorn leatherjacket (Ahmad *et al*., 2010). Sometimes NaCl is used with Tris–HCl for salting-out, as in bigeye snapper (Kittiphattanabawon *et al*., 2005; Zhang *et al*., 2011), ocellate puffer fish (Nagaia *et al*., 2002), paper nautilus (Nagai & Suzuki, 2002a), common minke whale (Nagai *et al*., 2008) and surf smelt (Nagai *et al*., 2010).

In the case of largefin longbarbel catfish, pigments are effectively removed by bleaching the defatted skin with 3% H_2O_2 solution (Zhang *et al*., 2009), while noncollagenous proteins are removed from octopus skin by sodium acetate and acetic acid (Kimura *et al*., 1981). Singh *et al*. (2011) precipitated collagen by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M tris(hydroxymethyl) aminomethane, at pH 7.0, in striped catfish (Singh *et al*., 2011). In hake and trout, the skin is homogenized with 0.5 M acetic acid and centrifuged. In order to precipitate 0.5 M formic acid is added for 1 minute, followed by a PSC extraction with pepsin in 0.5 M formic acid (Montero *et al*., 1990).

30.6.1.3 Bone

Bone is composed of calcium, phosphorus and collagen proteins, some special carbohydrates and lipids. It is first deproteinized by adding NaOH and then repeatedly washed with cold distilled water. Decalcification is carried out by adding 0.5 M EDTA at pH 7.4. After washing the residues with distilled water, fat is removed with 10% butyl alcohol. Collagen is further extracted from the bone in the same manner as in fish scale. This procedure is useful for black drum, sheepshead seabream (Ogawa *et al*., 2004), bigeye snapper (Kittiphattanabawon *et al*., 2005), tiger puffer (Mizuta *et al*., 2005), *Cyprinus carpio* (Duan *et al*., 2009) and bighead carp (*Hypophthalmichthys nobilis*) (Liu, 2012). In deep-sea redfish, fat is removed from the bone by soaking in 10 volumes of hexane (Wang *et al*., 2008).

30.6.1.4 Fin

Fins are first treated with NaOH to remove noncollagenous proteins. The residue is then washed with distilled water and decalcified using EDTA at pH 7.4. The insoluble matter is extracted with 0.5 M acetic acid. Collagen is further extracted from the fin in the same way as from scale (Liu, 2012; Nagai, 2004).

30.6.1.5 Swim Bladder

The swim bladder is first cut into small pieces and treated with NaOH to remove noncollagenous protein. The residue is then repeatedly washed with distilled water to remove alkali. Collagen is further extracted from the swim bladder in the same manner as for scale (Bama *et al*., 2010; Liu, 2012).

30.6.1.6 Stomach

Collagen isolation from the stomach is reported for bigeye snapper, in which the stomach is first cut into small pieces and finely ground to powder in liquid nitrogen, according to the method of Klomklao *et al*. (2004). The stomach powder is then suspended in sodium phosphate buffer at pH 7.2 and centrifuged. The pH of the supernatant is adjusted to 3 with 1 M HCl. The suspension is centrifuged and the supernatant is fractionated with ammonium sulfate. The pellet is further dissolved and dialyzed against Na-acetate buffer (Nalinanon *et al*., 2007).

30.6.1.7 Variations from Usual Processes in the Isolation of Fish Collagen

For some specific types of fish, the general steps in collagen isolation are modified as described in this subsection.

In grass carp, in order to remove noncollagenous proteins and pigments the scales are first soaked in distilled water and then microwave-treated. They are then decalcified with aqueous citric acid solution and the insoluble components are extracted with citric acid containing pepsin. Although in the method adopted by Li *et al*. (2008), the yield of PSC from grass carp scales was lower than in earlier reports, the researchers claimed that their preparative method is superior in two aspects. Soaking in distilled water for 5 hours and subsequent microwave treatment for a short time, followed by decalcification with 8% citric acid, reduces the pretreatment time from 7 days to 24 hours, without breaking the collagen structure. The color of the extracted collagen is purely white. Additionally, the collagen extracted by this method is safer, since all the solvents are edible and the calcium can be reclaimed.

In some extraction procedures, in order to terminate the pepsin reaction, dialysis is performed against sodium phosphate buffer, as documented for brownstripe red snapper, leather jacket, cuttlefish, sea cucumber, ocellate puffer fish, paper nautilus, common minke whale, cat fish, squid (*Illex argentines*), dogfish shark, jellyfish mesogloea, Atlantic salmon and starfish.

Variations in the procedure are also observed in the extraction of tiger puffer. PSC in this process is salted out by adding NaCl to a final concentration of 2.0 M, and then extracted with 0.5 M acetic acid containing ammonium sulfate overnight. After centrifugation, the supernatant is collected as a type-V collagen fraction and 0.5 M acetic acid containing ammonium sulfate is added to the precipitate. After centrifugation, the supernatant is combined with the type-V collagen fraction, while the resultant precipitate is collected as a type-I collagen fraction (Mizuta *et al*., 2005).

To extract collagen from flatfish skin, the centrifuged residue obtained after pepsin treatment is heated with distilled water in an autoclave and centrifuged. The pellet is rinsed by hot distilled water. The supernatant from centrifugation and the filtrate obtained from rinsing are then combined and lyophilized (Heu *et al*., 2010).

In order to remove lipids and pigments from the skin of Baltic cod, researchers used the method described by Gudmundsson & Hafsteinsson (1997). At first the skin is treated with a solution containing NaOH, H_2SO_4 and citric acid. After each extraction, the skin is washed with water until it reaches pH 7. By following the method of Montero *et al*. (1995) for the removal of pigments, the skin is repeatedly homogenized with NaCl solution. It is then soaked in NaCl solution at room temperature for 24 hours and bleached with H_2O_2 solution in 0.01 M NaOH. However, some research groups have isolated collagen by washing the raw material with water and disinfecting with H_2O_2. It is then extracted with 0.5 M citric acid solution. Collagen dissolved in citric acid is purified by dialysis in distilled water, and precipitated collagen fibers are dehydrated with acetone (Sadowska *et al*., 2003).

The skin of dogfish shark is treated differently in order to isolate collagen. Collagen solution extracted from the skin of an adult female exhibits significant cloudiness, which cannot be readily clarified by centrifugation. To overcome this, the lyophilized preparation is extracted with sodium chloride. A portion of the collagen is dissolved and separated by centrifugation, dialyzed exhaustively against 0.5 M acetic acid and lyophilized. Samples are stored at 5 °C over calcium chloride (Lewis & Piez, 1964).

PSC from horse mackerel is extracted with 0.5 M acetic acid containing ammonium sulfate. However, in yellow sea bream, a lesser percentage of ammonium sulfate is adopted for the fractionation of collagen, since minor collagens cannot be precipitated with a higher percentage of ammonium sulfate. After centrifugation, the supernatant is collected as S-fraction, while 0.5 M acetic acid containing ammonium sulfate is added to the precipitate, which is re-centrifuged. The resultant precipitate (P-fraction) is dissolved in and dialyzed against sodium acetate, containing NaCl and urea, and applied to a column of phosphocellulose P-11 that has been equilibrated with the same buffer. Ammonium sulfate is added to the S-fraction. The suspension is stirred, left standing overnight and centrifuged. The resultant precipitate is dissolved in and dialyzed against sodium phosphate, pH 6.8, containing 2 M urea, and applied to a column of phosphocellulose P-11 that has been equilibrated with the same buffer (Yata *et al*., 2001).

In order to obtain collagen from the body wall of sea cucumber, it is dissected and the samples are stirred in distilled water. Water is then replaced with NaCl, ethylenediaminetetraacetic acid, mercaptoethanol and Tris–HCl, and the mixture is stirred for 3–4 days. This causes collagen fiber bundles in the body walls to completely disaggregate into collagen fibrils. The suspension is then filtered through cheesecloth and the filtrate is centrifuged. NaOH solution is added to remove noncollagenous substances. After gentle stirring for a couple of days, the suspension is centrifuged. The precipitate is washed with distilled water and lyophilized. The supernatant is suspended in 0.5 M acetic acid and solubilized by limited pepsin digestion. Salting-out is done with NaCl to precipitate collagen, which is centrifuged, dialyzed and lyophilized (Park *et al*., 2012).

For the isolation of collagen from marine sponges, the cut pieces are first extracted in a solvent containing Tris–HCl buffer, EDTA, urea and 2-mercaptoethanol at pH 9.5. After 24 hours of continuously stirring at room temperature, the viscous extract is centrifuged. Collagen is precipitated from the supernatant by adjusting the pH to 4 with acetic acid and then collected by centrifugation. The pellet is resuspended in distilled water, centrifuged and lyophilized (Swatscheka *et al*., 2002).

Starfish tissues are first treated with $Ca(OH)_2$ to remove noncollagenous proteins and pigments. The small pieces of tissue are suspended in acetic acid and digested with pepsin (Lee *et al*., 2009).

The mesogloea of jellyfish is used by some researchers to isolate collagen. The mesogloea is cut into small pieces and extracted with 0.5 M acetic acid, then filtered and squeezed through a cheesecloth. It is dialyzed, centrifuged and dissolved in 0.5 M acetic acid. Salting-out is done in order to obtain ASC, while PSC is obtained by subjecting the solution to limited pepsin digestion (Park *et al*., 2012).

Muscle tissues (mantle and adductor) from pearl oyster are dissected out, homogenized in NaOH and extracted with Tris–HCl, pH 7.5, containing guanidine hydrochloride. After centrifugation, the supernatant is dialyzed and the resultant precipitate is collected by centrifugation and lyophilized. The insoluble matter is washed thoroughly with distilled water, and subsequently digested with porcine pepsin (Mizuta *et al*., 2002).

Although collagen has been isolated from different parts of fish, extraction from scales is relatively convenient. Scale is an exogenous part which can be easily separated and washed. All other parts of the fish are edible, while the scale is a waste material and discarded, creating environmental pollution. A calcified tissue, it is composed of collagen and mineral (calcium phosphate apatite), while other parts of the fish have fat, glycosaminoglycans (GAGs), proteoglycans and other biomolecules, along with collagen and/or minerals. Isolation of collagen from fish scale therefore involves fewer processing

and purification steps. This makes collagen extraction from scale economically lucrative. However, a variety of other factors must be considered before fish-scale collagen can be employed commercially for biomedical applications.

30.6.2 Comparative Characteristics of Fish Scale-derived Collagen

30.6.2.1 Yield

The total yield of collagen varies with the type of extraction method and the conditions used, such as homogenization, shaking, mixing, initial acid solubilization and duration (Muralidharan *et al*., 2011). The yield is also affected by variations in collagen composition among different fish species (Rodziewicz-Motowidło *et al*., 2008).

In general, the yield of ASC is found to be lower than that of PSC (as shown in Table 30.1). The scales and skins of different fish are not completely solubilized with 0.5 M acetic acid, but when the residues obtained from ASC are subjected to limited pepsin digestion in 0.5 M acetic acid, the collagen becomes readily soluble. The reason for the increase in collagen yield with the addition of pepsin in acid is attributed to the increasing solubilization of collagen, which is only partially soluble in 0.5 M acetic acid. Crosslinking of the collagen molecule is covalent, due to aldehyde-group condensation at the telopetide region and to intramolecular crosslinking bringing about a decrease in the solubility of collagen (Burghagen, 1999; Foegeding *et al*., 1996). The higher yield of PSC is probably due to the fact that with limited pepsin digestion, cleavage of crosslinked molecules takes place in the telopeptide region, maintaining the integrity of the triple helix (Bama *et al*., 2010), so that collagen with predominant monomeric molecules can be solubilized. Decalcification with EDTA solution also increases the solubility of collagen, and a subsequent yield in 0.5 M acetic acid and a highly viscous solution is obtained (Nagai, 2004). Another method is to re-extract with with 0.5 M acetic acid for a further few days. All residues are then solubilized and highly viscous solutions are obtained. It has been observed that the collagen and the noncollagenous protein content of cod skin varies according to the fishing seasons. Albumins and globulins are degraded, while the collagen content in skin increases during starvation (Aberoumand, 2012).

30.6.2.2 Amino Acid Analysis

Amino acid composition is an important characteristic of collagen and serves to identify different types. Amino acid analysis of fish skin, scale and bones reveals that amino acid composition varies among species due to differences in their living environments, particularly habitat temperature. Variations between compositions of ASC and PSC are also evident in some of species.

As expected, glycine is the most abundant amino acid found in the collagen from fish scales, muscle and skin. It occurs uniformly at every third residue, except in the first 14 amino acid residues from the N-terminus and the first 10 from the C-terminus (Burghagen, 1999; Foegeding *et al*., 1996; Wong, 1989). The presence of high amounts of glycine is a characteristic feature of collagen molecules. Both ASC and PSC exhibit high levels of proline, hydroxyproline, alanine and glutamic acid, while levels of methionine, isoleucine, tyrosine and hydroxylysine are very low. Cysteine is mostly not detected in either freshwater or marine sources, yet some researchers claim the cysteine level is high in freshwater fish scales (Zhang *et al*., 2011). In many of the fish species, tryptophan is undetectable, while the amount of histidine is almost negligible.

Table 30.1 Yield of collagen from different fish sources.

Species	%Yield		Reference
	ASC	PSC	
Freshwater origin			
Silver carp	-	75 (outer skin)	Rodziewicz-Motowidło *et al.* (2008)
Carp(*Cyprinus carpio*)	41.3 (skin) 1.35 (scale) 1.06 (bone)		Rui Duan *et al.* (2009)
Grass carp		46.6 (scales)	Nagai *et al.* (2008)
Amiurus nebulosus	62.05 (skin)	97.44 (skin)	Chen *et al.* (2011)
Labeo rohita	5 (scales)		Pati *et al.* (2010)
Catla catla	5 (scales)		Pati *et al.* (2010)
Cat fish		35 (swim bladder)	Bama *et al.* (2010)
Channel catfish	25.8 (skin)	38.4 (skin)	Liu *et al.* (2007)
Stripped catfish	5.1 (skin)	7.7	Singh *et al.* (2011)
Nile perch		63.1 (young) 58.7 (adult)	Muyonga *et al.* (2004)
Big head carp		5.1 (fin) 2.7 (scale) 60.3 (skin) 2.9 (bone) 59 (swim bladder)	Liu (2012)
Oreochromics niloticus	2 (scale)		Ikoma *et al.* (2003)
Marine origin			
Japanese sea bass	51.4 (skin) 5.2 (caudal fin)	36.4 (ASC from caudal fin)	Nagai (2004)
Seaweed pipefish	5.5	33.2	Khan *et al.* (2009)
Flatfish	57.3 (skin)	85.5 (skin)	Heu *et al.* (2010)
Deep-sea red fish	47.5 (skin)	92.2 (skin)	Nagai *et al.* (2008)
Ocellate puffer	10.7 (skin)	44.7 (skin)	Nagaia *et al.* (2002)
Brown-backed toadfish		54.3 (skin)	Ikoma *et al.* (2003), Huang *et al.* (2011)
Pagrus major	2 (scale)		Ikoma *et al.* (2003)
C. arakawai		62.9(arm)	Nagai *et al.* (2008)
Leather jacket (*Odonus niger)*	46.48 (muscle) 50 (skin, bone)	70.94 (skin) 66 (bone) 64 (muscle)	Muralidharan *et al.* (2011)
Balloon fish	4 (skin)	19.5 (skin)	Huang *et al.* (2011)
Brownbanded bamboo shark	1.04 (skin)	9.59 (skin)	Kittiphattanabawon *et al.* (2010)
Blacktip shark	1.27 (skin)	10.30 (skin)	Kittiphattanabawon *et al.* (2010)
Largefin longbarbel catfish	16.8 (skin)	28 (skin)	Zhang *et al.* (2009)
Black drum	2.3 (skin)	15.8 (skin)	Ogawa *et al.* (2003)
Sheepshead seabream	2.6 (skin)	15.8 (skin)	Ogawa *et al.* (2003)
Brown-stripe red snapper	9 (skin)	4.7 (skin)	Jongjareonrak *et al.* (2005)
Ornate threadfin bream	24.9 (skin)		Nalinanon *et al.* (2011)
Minke whale (unesue)	0.9	28.4	Nagai *et al.* (2008)
Cuttle fish	33 (outer skin)	35 (outer skin)	Nagai *et al.* (2001)
Scallop mantel		0.4	Shen *et al.* (2007)
Skipjack tuna	42.3 (bone)		Nagai & Suzuki (2000a)

(*continued overleaf*)

Table 30.1 (*continued*)

Species	%Yield		Reference
	ASC	PSC	
Yellow sea bream	40.1 (bone)		Nagai & Suzuki (2000a)
Chub mackerel	49.8 (skin)		Nagai *et al.* (2008)
Bull head shark	50.1 (skin)		Nagai *et al.* (2008)
Horse mackerel	43.5 (bone)		Nagai & Suzuki (2000a)
Paper nautilus	5.2 (outer skin)	50 (outer skin)	Nagai & Suzuki (2002a)
Atlantic salmon	23.7 (muscle) 5.8 (insoluble collagen)	70.5 (muscle)	Aidos *et al.* (1999)
Surf smelt	24 (skin)	-	Nagai *et al.* (2010)
Unicorn leatherjacket	4.19 (skin)	7.56 (skin)	Ahmad & Benjakul (2010)
Baltic cod	42.5 (skin)	71 (skin)	Sadowska *et al.* (2003)
S. lycidas	2	35	Aberoumand (2012)
N. crepidularia	0.48(GSC)	1.28	Aberoumand (2012)
Skate	35.6(skin)		Huang *et al.* (2011)
Archaeogastropod	0.48(GSC)	1.28	Palpandi *et al.* (2010)
Other marine species			
Purple sea urchin	35	-	Nagai *et al.* (2008)
Jellyfish		46.4 (exumbrella) 35.2 (rhizostomous) 35.2 (mesoglea)	Nagai *et al.* (2008)
Squid (*Illex argentinus*)		18.5 (skin)	Zhang *et al.* (2011)
Squid (*Ommastrephes bartrami*)	36.2	16.4 (skin)	Mingyan *et al.* (2008)
Starfish		5.8 (body wall)	Lee *et al.* (2009)

The imino acid content of collagen directly influences its thermal stability (Muyonga *et al*., 2004), while the animal's habitat influences its imino acid content (Rigby, 1968). Stabilization of the triple helix depends largely on the degree of hydroxylation of proline residues (Ramachandran, 1988). The hydroxyl groups of hydroxyproline and hydroxylysine increases the stability of collagen by interchain hydrogen bonding via a bridging water molecule and by direct hydrogen bonding to a carbonyl group (Wong, 1989). Therefore, the extent of hydroxylation of proline and lysine directly influences the thermal stability of the collagen molecule. The higher the imino acid content, the higher the thermal stability of the collagen (Table 30.2) (Muyonga *et al*., 2004). In general, proline is present in higher amounts than hydroxyproline in fish collagens (Kimura *et al*., 1988; Yata *et al*., 2001). Fish collagen has a lower imino acid content than does mammalian collagen. The proline and hydroxyproline content is generally lower in fish collagen than in animal collagen (Muralidharan *et al*., 2011). The content of hydroxyproline, proline and arginine is greater in skin than in bone, but conversely glycine and hydroxylysine contents are greater in collagen obtained from bone than from skin. The collagen obtained from hot- and warm-water fish has a higher total imino acid and hydroxyproline content than that from cold- and ice-water organisms (Liu, 2012). It has been reported that the relative proportions of hydroxyproline and proline in muscle type-I collagen are somewhat higher than those of skin collagen from several fish species (Kimura *et al*., 1988). Serine and

Table 30.2 Hydroxyproline, imino acid and denaturation temperature of collagens from different fish sources.

Fish	Hydroxyproline content	Total imino acid content	Td (°C)	Reference
Freshwater origin				
Grass carp	94	104	28.4 (skin) 32 (scale)	Li *et al.* (2008)
Amiurus nebulosus	87.2	210.5	22 (skin)	Chen *et al.* (2011)
Labeo rohita	83	201	35(scale)	Pati *et al.* (2010)
Catla catla	84	130	35(scale)	Pati *et al.* (2010)
Largefin longbarbel catfish	74 (ASC)	213 (ASC)	32.1 (ASC) (Tm) 31.6 (PSC)	Zhang *et al.* (2009)
Silver carp	84	119	34.5 (skin)	Rodziewicz-Motowidło *et al.* (2008)
Carp (*Cyprinus carpio*)	76 (skin)	190 (skin)	31.7 (skin)	Duan *et al.* (2009), Nagaia *et al.* (2002), Zhang *et al.* (2011)
	80 (bone)	192 (bone)	32.5 (muscle)	
	109 (PSC of scale)	231 (PSC of scale)	29 (PSC of scale)	
	89 (ASC of scale)	199 (ASC of scale)	32.9 (ASC of scale)	
Stripped catfish	86 (ASC) 91 (PSC)	206 (ASC) 211 (PSC)	35.35 (ASC of skin) 35.38 (PSC of skin)	Singh *et al.* (2011)
Channel catfish	73 (ASC) 75.9 (PSC)	170.9 (ASC) 177.2 (PSC)	32.5	Liu *et al.* (2007)
Big head carp	58.8 (fin) 56.2 (scale) 73.2 (skin) 73.8 (bone) 80.5 (swim bladder)	166 (fin) 156 (scale) 165 (skin) 174 (bone) 175 (swim bladder)	35.5 (fin) (Tm) 35.2 (scale) 35.7 (skin) 36.4 (bone) 37.3 (swim bladder)	Liu (2012)
Marine origin				
Leather jacket (*Odonus niger*)	71.1 (skin) 87.2 (bone) 88.2 (muscle)	161.1 (skin) 190.3 (bone) 190.4 (muscle)	27–28 (skin) 31–32 (bone) 30–32 (muscle)	Muralidharan *et al.* (2011)

(*continued overleaf*)

Table 30.2 (*continued*)

Fish	Hydroxyproline content	Total imino acid content	Td (°C)	Reference
Ocellate puffer	67	170	28	Nagaia *et al.* (2002)
Sea bass	82 (fin)	193 (fin)	29.1 (fin) 30 (bone) 26.5 (skin)	Nagaia *et al.* (2002)
Seaweed pipefish	82 (ASC) 87 (PSC)	185 (ASC) 193 (PSC)	34.8 (ASC) 35.7 (PSC)	Khan *et al.* (2009)
Flatfish	79.5 (ASC) 77.3 (PSC)	189.7 (ASC) 186.4 (PSC)	26.6 (ASC of skin) 26.7 (PSC of skin)	Heu *et al.* (2010)
Deep-sea red fish	64 (skin) 65 (scale) 61 (bone)	165 (skin) 160 (scale) 163 (bone)	16.1 (skin) 17.7 (scale) 17.5 (bone)	Wang *et al.* (2008)
Brown-backed toadfish	77	170	28 (skin)	Senaratne *et al.* (2006)
Pagrus major	73	180	29.7 (scale)	Ikoma *et al.* (2003)
Chum salmon			19.4 (skin) 20.6 (muscle)	Nagaia *et al.* (2002)
Oreochromics niloticus	83	193	35.5 (scale)	Ikoma *et al.* (2003)
Balloon fish	71 (ASC of skin) 78 (PSC of skin)	179 (ASC of skin) 174 (PSC of skin)	29.01 (ASC of skin) 30.01 (PSC of skin)	Huang *et al.* (2011)
Brownbanded bamboo shark	94 (ASC)	203 (ASC)	36.7 (ASC)	Kittiphattanabawon *et al.* (2010)
	94 (PSC)	204 (PSC)	35.9 (PSC)	
Blacktip shark	91 (ASC)	196 (ASC)	36.2 (ASC of skin) (Tm)	Kittiphattanabawon *et al.* (2010)
	91 (PSC)	197 (PSC)	34.5 (PSC)	
Bigeye snapper	77 (skin)	193 (skin)	28.6 (skin)	Nalinanon *et al.* (2007), Zhang *et al.* (2009)
	68 (bone)	163 (bone)	32.5 (ASC) (Tm) 31.5 (PSC)	

Black drum	84.6 (bone)	191.6 (bone)	34.8 (ASC of skin) (Tm)	Masahiro Ogawa *et al.* (2004), Ogawa *et al.* (2003)
	80.5 (skin)	198.4 (skin)	35.1 (PSC of skin) (Tm) 34.2 (ASC of skin) 35.8(PSC of skin) 33.5 (bone)	
Sheepshead seabream	88.5 (bone)	194.5 (bone)	33.5 (ASC of skin) (Tm)	Masahiro Ogawa *et al.* (2004), Ogawa *et al.* (2003)
	88.2 (skin)	201.6 (skin)	33.6 (PSC of skin) (Tm) 34 (ASC of skin) 34.3 (PSC of skin) 34.8 (bone)	
Brown stripe red snapper	81 (ASC) 86 (PSC)	212 (ASC) 221 (PSC)	30.5 (ASC of skin) (Tm) 30.4 (PSC)	Jongjareonrak *et al.* (2005)
Ornate threadfin bream	84	188	33.35 (skin) (Tm)	Nalinanon *et al.* (2011)
Minke whale	78	199	31.5	Nagai *et al.* (2010)
Hake	69	191	10	Wang *et al.* (2008)
Cuttle fish	90 (PSC of outer skin)	188 (PSC of outer skin)	27 (PSC of outer skin)	Nagai *et al.* (2001)
Tiger puffer	73.1	185.6	28.4	Bae *et al.* (2008)
Unicorn leatherjacket	81	190	35.8 (Tm)	Ahmad *et al.* (2010)
Dogfish shark	59	158	14.3 (skin) (Tm)	Lewis & Piez (1964)
Yellow sea bream	75.5	186.2	29.5 (bone)	Nagaia *et al.* (2002)
Common horse mackerel	70.2	185.2	29.5 (bone)	Nagaia *et al.* (2002)
Surfsmelt	64	172	32.5 (skin)	Nagai *et al.* (2010)
Silver-line grunt	71 (ASC) 73.6 (PSC)	187.2 (ASC) 191.3 (PSC)	28.1 (ASC) 27.7 (PSC)	Noitup *et al.* (2005)
Albacore tuna	60.3 (ASC) 63.9 (PSC)	174.3 (ASC) 177.2 (PSC)	28.8 (ASC) 28.6 (PSC)	Noitup *et al.* (2005)

(*continued overleaf*)

Table 30.2 (*continued*)

Fish	Hydroxyproline content	Total imino acid content	Td (°C)	Reference
Other marine species				
Squid (*Illex argentinus*)		179	23.2 (Tm)	Zhang *et al.* (2011)
Squid (*Ommastrephes bartrami*)	84 (ASC)	180 (ASC)	29.4 (skin)	Mingyan *et al.* (2009)
	89 (PSC)	187 (PSC)	52.8 (Tm)	
Starfish	61	158	24.7 (body wall)	Nagai *et al.* (2001)
Jellyfish	54 (PSC of mesoglea)	133 (PSC of mesoglea)	28 (mesoglea) 26 (exumbrella)	Nagai *et al.* (2010)

threonine are present in higher amounts in all fish collagens than in mammalian collagen (Neuman, 1949). The increase in the hydroxyl groups in fish collagen is attributed to the presence of larger numbers of aliphatic hydroxy amino acid residues, balanced by the loss of hydroxyl groups of hydroxyproline residues. This balancing effect results in similar proportions of hydroxyl groups in fish and mammals (Eastoe, 1956). The methionine content is also variable in fish collagens (Beveridge & Lucas, 1944), ranging from nearly 300% the mammalian content in cods to 60% in lung fish (Eastoe, 1956).

Tilapia has the highest imino acid content among fish collagens, at 25.4% (Chen *et al*., 2011), followed by Nile perch skin (Muyonga *et al*., 2004). Collagen from jellyfish has higher amounts of glutamine, glutamic acid and alanine, but contains less proline. Jellyfish collagen also contains cysteine and cystine. Pepsin digestion of jellyfish collagen yields small amounts of tyrosine, indicating that it is a tellocollagen of high purity (Songa *et al*., 2006). Grass carp collagen contains more glycine than *Pagrus major*, Japanese sea bass, *Oreochromis niloticas*, blackdrum, red sea bream, sheepshead seabream and sardine. Brown-banded bamboo shark cartilage has a high serine content but contains less alanine and arginine than blacktip shark cartilage (Kittiphattanabawon *et al*., 2010).

30.6.2.3 SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis is a rapid and convenient method by which to identify the type and structure of a collagen. Type-I collagen is made of three polypeptide chains, known as α-chains, which form a triple-helical structure stabilized by hydrogen bonds. Each α-chain is composed of more than 1000 amino acid residues and has a molecular weight of around 100 kDa, yielding a total molecular weight of 300 kDa for the tropocollagen molecule (Damodaran & Paraf, 1997). The triple helix of type-I collagen is normally a heterotrimer with two different subunits, α_1 and α_2. Generally, collagen has a chain composition of $(\alpha_1)_2\alpha_2$, but $\alpha_1\alpha_2\alpha_3$ is sometimes present. However, the α_3 subunit is mostly not detected by normal electrophoretic procedures as it migrates in a similar way to α_1 (Kimura, 1992). β dimers and γ trimers representing inter- and intramolecular crosslinked components of collagen are also found. The β component confirms the presence of intermolecular crosslinks, while the γ component indicates intramolecular crosslinking (Lewis & Piez, 1964). Differences in molecular weight among the fish collagens are probably due to their different sources. In some species, variation exists in the electrophoretic mobility of ASC and PSC, while in others mobility is found to be similar. Variation in mobility is due to cleavage of some telopeptide regions by partial pepsin hydrolysis. The ratio of α_1 to α_2 chains of collagens from different parts of the same species also shows variation, possibly because the α_3 chain, if present, has an indistinguishable molecular mass from the α_1 chain and thus cannot be separated from it (Wang *et al*., 2008). Similar electrophoretic patterns of ASC and PSC treated with β-mercaptoethanol confirm the absence of a disulfide bond. The ratio of band intensities of crosslinked components (β and γ components) to total noncrosslinked chains (α_1 and α_2) is higher in ASC than in PSC, suggesting that ASC has more intra- and intermolecular collagen crosslinks. The reason could be that pepsin cleaves the crosslink containing teleopeptide, converting the β components to two α_1 chains (Kittiphattanabawon *et al*., 2010). With increasing animal age, the number of high-molecular-weight crosslinked molecules in collagen increases (Foegeding *et al*., 1996). Additionally, starving fish have more crosslinked collagen than do well-fed ones (Love *et al*., 1976; Sikorski *et al*., 1990). However, fish-skin collagen has an extremely low crosslinking rate and a highly crosslinked molecule is rarely found (Cohen-Solal *et al*., 1981). Several reports reveal that well-nourished myocommata are thickened during

starvation, with mechanically stronger collagen containing more intermolecular crosslinks than are found in undernourished fish (Love *et al*., 1976; Sikorski *et al*., 1990).

It is to be noted that $(\alpha 1)_2\alpha_2$ heterotrimer is seen in the collagen of the common minke whale (Nagai *et al*., 2008), *Ommastrephes bartrami* (Mingyan *et al*., 2009), Argentine shortfin squid (Zhang *et al*., 2011), paper nautilus (Nagai & Suzuki, 2002b), carp scale (Zhang *et al*., 2011), Nile perch (Muyonga *et al*., 2004), leather jacket (Muralidharan *et al*., 2011), blackdrum seabream, sheepshead seabream (Ogawa *et al*., 2004), Baltic cod, grass carp, sardine (Li *et al*., 2008), brown-backed toad fish (Senaratne *et al*., 2006), silver carp (Rodziewicz-Motowidło *et al*., 2008), Japanese sea bass, bullhead shark, skipjack tuna, ayu (Nagai & Suzuki, 2000a), *S. japonicas* (Cui *et al*., 2007), balloon fish (Huang *et al*., 2011), hake, trout, bigeye snapper (Kittiphattanabawon *et al*., 2005), starfish (Lee *et al*., 2009), brownbanded bamboo shark, blacktip shark (Kittiphattanabawon *et al*., 2010), pearl oyster (Mizuta *et al*., 2002), octopus (Kimura *et al*., 1981), ocellate puffer fish (Nagaia *et al*., 2002), channel catfish (Liu *et al*., 2007), flatfish (Heu *et al*., 2010), tiger puffer (Mizuta *et al*., 2005), seaweed pipefish (Khan *et al*., 2009), *Amiurus nebulosus* (Chen *et al*., 2011), cat fish (Bama *et al*., 2010), *Labeo rohita*, *Catla catla* (Pati et al., 2010), ornate threadfin (Nalinanon *et al*., 2011), surf smelt (Nagai *et al*., 2010), deep-sea red fish (Wang *et al*., 2008), walleye pollock, largefin longbarbel catfish (Kittiphattanabawon *et al*., 2010), unicorn leather jacket (Ahmad & Benjakul, 2010), stripped catfish (Singh *et al*., 2011) and brownstripe red snapper (Jongjareonrak *et al*., 2005). All these fish show the presence of inter- and intracrosslinked components, β dimmers and γ trimers.

Nagai *et al*. (2001) found that cuttle fish ASC shows only a single α band, α_1, while PSC comprises two α chains, α_1 and α_2. The skin of chub mackerel, bone of horse mackerel and yellow sea bream show only a single α_1 band, although α_2 may be present but in an insignificant amount (Nagai & Suzuki, 2000a).

30.6.2.4 Subunit Composition

To separate the subunits of collagen and to identify presence of chains other than α_1, such as α_3, which are inseparable by SDS-PAGE, column chromatography is used. Different α-chains constitute different collagen types. Specific cell-surface receptors recognize distinct collagen subtypes. Thus subunit-composition analysis is important in knowing the distinct type of collagen that will interact with a specific type of integrin. $\alpha_1\beta_1$ and $\alpha_2\beta_1$, members of the integrin family, are the best-known collagen receptors. It has been reported that mutations which affect cell spreading on type-I collagen have no effect on type-IV collagen, which indicates that the two collagen subtypes are recognized by different mechanisms (Heino, 2000).

By this method, the presence of α_3 and α_4 chains in fish collagen can be detected. Ocellate puffer fish skin (Nagaia *et al*., 2002), octopus skin (Kimura *et al*., 1981), cuttle fish (Nagai *et al*., 2001), squid (Mizuta *et al*., 1994a,1994b; Shadwick, 1985), sardine scales, red sea bream, Japanese sea bass (Nagai *et al*., 2004), purple sea urchin test (Nagai & Suzuki, 2000b), common minke whale unesu (Nagai *et al*., 2008) and starfish (*Asterias amurensis*) (Lee *et al*., 2009) have collagen-chain compositions of $(\alpha_1)_2\alpha_2$ heterotrimer, indicating that isolated collagen may be similar to that of mammalian collagen, such as porcine skin.

Kimura *et al*. (1987) have reported that α_3 chain is widely distributed in teleosts, such as eel, sardine, chum salmon, rainbow trout, carp, anger, Alaska pollock, cod, halfbeak, common mackerel, tilapia, red barracuda, northern dab and file fish. Scientists have also reported the existence of α_3 chain in edible jellyfish exumbrella (Nagai *et al*., 1999),

rhizostomous jellyfish mesogloea (Nagai *et al*., 2000), ayu bone (Nagai & Suzuki, 2000c), paper nautilus outer skin (Nagai & Suzuki, 2002a), *Callistoctopusar akawai* arm (Nagai *et al*., 2001), surf smelt skin (Nagai *et al*., 2010), diamondback squid (Nagai, 2004) and brown-backed toadfish (Senaratne *et al*., 2006). Hence, it is evident that the α_3 chain is widely distributed in both teleost skin and marine invertebrate skins. Thus, this collagen may have useful biomedical application.

The subunit structure of $(\alpha_1)_3$ homotrimer exists extensively in invertebrates (Kimura & Tanaka, 1983; Mizuta *et al*., 1992). It is also found in the collagen of *Stichopus japonicus*, caudul fin of Japanese sea bass (Nagai, 2004), top shell muscle collagen (Kimura & Tanaka, 1983), sea urchin (Cluzel *et al*., 2000) and horse mackerel bone collagen (Nagai & Suzuki, 2000c, 2002a). Rhizostomous jellyfish mesogloea has a subunit composition of $\alpha_1\alpha_2\alpha_3\alpha_4$ (Nagai *et al*., 2000). The collagen from pipefish (Khan *et al*., 2009) and ornate thread fin (Nalinanon *et al*., 2011) has two molecular forms: $(\alpha_1)_2\alpha_2$ and $\alpha_1\alpha_2\alpha_3$ heterotrimer.

Brown-banded bamboo shark and blacktip shark may have collagen of two types, type I and type II (Kittiphattanabawon *et al*., 2010). Type-I collagen exihibits two α_1 chains and one α_2 chain, while type-II collagen comprises three identical α_1 chains (Foegeding *et al*., 1996; Wong, 1989). Shark cartilage contains type-I and type-II collagen in a 1 : 2 ratio (Rama & Chandrakasan, 1984). Since in brownbanded bamboo shark the band intensity of α_1 chain is not twofold higher than that of α_2 chain, it is hypothesized that the α_2 component might dimerize into the β component and form β_{12} dimer (Hwang *et al*., 2007). Hence, a much lower-band intensity of α_2 chain is detected by SDS-PAGE. Similar results have also been reported for type-I collagen from other elasmobranches (Bae *et al*., 2008; Hwang *et al*., 2007).

Some collagen subunit variations are seen specifically in the case of carp, with the coexistence of $(\alpha_1)_2\alpha_2$ and $\alpha_1\alpha_2\alpha_3$ heterotrimers as the major and minor molecular forms of type-I collagen (Kimura *et al*., 1991). Scale and bone collagens of carp are richer in α_3 chains than are skin and muscle collagen, while swim-bladder collagen contains no α_3 chain and exists solely as $(\alpha_1)_2\alpha_2$ heterotrimers (Miyauchi & Kimura, 1990; Piez *et al*., 1963). These properties play a crucial role in determining the application for which collagen extracted from a particular source can be used.

30.6.2.5 Circular Dichroism

The circular dichroism (CD) spectrum is a characteristic method for identifying the secondary structures of proteins and measuring the denaturation temperature of collagen. The CD spectrum of fish scales and bones gives a positive peak at around 220 nm and a negative peak at around 197–199 nm, which are both typical of the collagen triple-helix structure (Engel, 1987; Heidemann & Roth, 1982). Minor deviations in elasticity are observed among different fish species, suggesting discrepancies among collagen structures. All collagens extracted from fish skins show apparently biphasic thermal transitions (Ogawa *et al*., 2003) identical to those in animal skins (Brown *et al*., 2000; Sato *et al*., 2000). The existence of biphasic thermal transitions suggests that these collagen possess either at least two different inner domains or two different collagen molecules with different stabilities, such as the coexistence of $(\alpha_1)_2\alpha_2$ (type-I collagen) and $(\alpha_1)_3$ (type-II collagen) or of tropocollagen and atelocollagen (Ogawa *et al*., 2004).

The bone and scale collagen isolated from the subtropical fish black drum and sheepshead seabream show CD spectra at the range with apparently biphasic thermal transitions. When plotted on a transition curve, the first transition contributes less than

the second, but for bone collagen the first begins at 28 °C and for scale collagen it begins at 26 °C. In both bone and scales, the second transition appears at 33 °C for blackdrum and 32 °C for sheepshead. The transition is completed at around 38 °C for all collagen species (Ogawa *et al*., 2004). The CD spectrum for PSC of squid skin shows a positive peak at around 220 nm and a large negative peak near 208 nm (Assad *et al*., 2011). Pati *et al*. (2010) have reported that rohu and catla show rotatory maxima at 221 nm and minima at 205 nm over the temperature range 25–40 °C and a consistent crossover point (zero rotation) at about 212 nm, which are characteristic of the triple-helical conformation of collagen. Due to denaturation of the collagen triple-helical structure, the $[h]_{221}$ value decreases with temperature, and 36.5 °C is indicated as the denaturation temperature for both species. The CD spectrum of silver carp shows a rotatory maximum at 221 nm, a minimum at 199 nm and a consistent crossover point (zero rotation) at 213 nm (Rodziewicz-Motowidło *et al*., 2008), while *O. niloticas* and *P. major* shows rotatory maxima at 221 nm and minima at 191 nm, and consistent crossover points (zero rotation) at 212 nm (Ikoma *et al*., 2003).

The CD spectrum of PSC from grass carp at different temperatures falls in the range 190–250 nm, demonstrating a rotatory maximum at 221.2 nm and a negative peak at 197.5 nm (Li *et al*., 2008). The ratio of positive peak intensity to negative (Rpn) is characteristic of the triple-helical conformation of collagen in solution. The CD lines of grass carp scales at 20 and 30 °C overlap completely, and the Rpn value of PSC is 0.114 and changes little before and after holding for 10 minutes at 20 and 30 °C, which indicates the steadiness of the collagen solution at these temperatures. On complete denaturation, the positive peak at 220 nm completely disappears and the negative band shifts towards higher wavelengths (Usha & Ramasami, 2004). In the case of partially denatured collagen, absorption bands of lower intensities show red shift in their crossover points and a decrease in the ratio of the intensity of the long wavelength band to the intensity of the short wavelength band (Usha & Ramasami, 2005). Heating collagen to 35 °C for 10 minutes significantly decreases the intensities of positive and negative peaks and of the the Rpn values, due to conformational changes in the collagen molecule, but alteration of the native triple-helical collagen structure does not take place as the ratio is close to the native structure. When the temperature increases to 40 °C, the positive peak decreases sharply, with a parallel drop in the Rpn values of PSC drops. After holding for 10 minutes, the positive peak completely disappears and the negative band shows red shift due to the breakdown of the triple-helical structure. The changes in CD spectra before and after holding at 35 and 40 °C indicate that the change in collagen structure near T_d shows a time-dependent transition. The temperature at which the triple-helix structure of collagen is disintegrated into random coils is called the denaturation temperature, T_d (Hao & Li, 1999). When the temperature is raised above 50 °C, all of the CD lines appear very similar, the positive peak is not observed and the negative peak shifts to 200.0–201.5 nm, indicating the destruction of the collagen's secondary structure.

30.6.2.6 Thermal Stability

The denaturation temperature of collagen has significant implications for its biomedical applications, since it affects its processing properties. Denaturation of collagen is always accompanied by changes in several of its physical attributes, such as sedimentation, viscosity, light scattering, diffusion and optical activity (Gurdak *et al*., 2006; Sai & Babu, 2001; Usha & Ramasami, 2004). The denaturation temperature of fish collagen correlates with both the fish's body temperature and its environmental temperature (Rigby, 1968).

The tendency of marine organisms to have lower T_d than those of land animals is correlated with their environmental and body temperatures (Rigby, 1968). Moreover, the T_d of collagen from warm-water fish is higher than that of cold-water fish (Takahashi & Yokoyama, 1954).

The denaturation temperatures of the collagens can also be measured by fractional viscosity and is the temperature at which the fractional viscosity is 0.5 (Zhang *et al*., 2011). A high imino acid content and a high degree of hydroxylation of proline increase T_d (as shown in Table 30.3) (Kittiphattanabawon *et al*., 2005). Burjanadze (2000) has shown that the thermostability of collagen varies nonlinearly with its hydroxyproline content, and the dependence is hyperbolic. However, it has been concluded that it is the total Gly-Pro-Hyp sequence content which is probably the dominating factor influencing collagen thermostability. Hydroxyproline is important in maintaining the stabilization of the trimmers in collagen. It has been demonstrated that the body and environmental temperature govern the stability of collagen and thus also its T_d value (Rigby, 1968). In general, the T_d value of ASC is 1–2 °C lower than that of PSC. The T_d values of collagens extracted from different parts of a fish also show variation. The T_d values of bone and muscle collagen are higher than those of skin collagen by 3 or 4 °C. Bone and muscle, being internal parts of the fish, have higher body temperatures than the skin and contribute higher T_d values (Muralidharan *et al*., 2011).

It can be seen that the T_d of collagen obtained from the skin of largefin longbarbel catfish is higher than that of grass carp skin. However, since both are temperate freshwater fish and their living environments are similar, it seems that the denaturation temperature of collagen is also influenced by the presence of scales. The scales of teleosts are formed by the deposition of calcium in dermis. Thus, it is assumed that the calcium content in the dermis of grass carp scales will be higher than that of largefin longbarbel catfish without scales, and the association of collagen with calcium in dermis appears to influence the properties of collagens from the two fish (Zhang *et al*., 2009).

In addition, T_d is found to be higher when measured in deionized water than in acetic acid. This suggests that due to the repulsion of collagen molecules in acidic solutions, the intramolecular hydrogen bonds stabilizing the collagen triple-helix structure are disrupted

Table 30.3 FTIR spectra peak location and assignment for standard collagen.

Region	Standard	Assignment	Reference
Amide A	3289	NH stretch coupled with hydrogen bond	Sai & Babu (2001)
Amide B	2920	CH_2 asymmetrical stretch	Abe & Krimm (1972)
	2853	CH_2 symmetrical stretch	Abe & Krimm (1972)
Amide I	1644	C═O stretch/hydrogen bond coupled with COO^- stretch	Payne & Veis (1988)
Amide II	1537	NH bend coupled with CN stretch	Krimm & Bandekar (1986)
	1450	CH_2 bend	Jackson *et al.* (1995)
		COO— symmetrical stretch	Jackson *et al.* (1995)
		CH_2 wagging of Proline	Jackson *et al.* (1995)
Amide III	1260	NH bend coupled with CN stretch	Payne & Veis (1988)
	1078	C—O stretch	Jackson *et al.* (1995)
	1021	C—O stretch	Palpandi *et al.* (2010)

in the presence of acetic acid (Singh *et al*., 2011). The T_d obtained by differential scanning calorimetry (DSC) gives T_{max} (thermal transition temperature). Marine lifeforms have a lower T_{max} than do porcine and bovine collagen. However, striped catfish has a higher T_{max} than does pig-skin collagen (37 °C) (Ikoma *et al*., 2003), closer to that of calf-skin collagen (40.8 °C) (Komsa-Penkova *et al*., 1999).

30.6.2.7 FTIR Spectroscopic Study

Different band absorptions are attributed to the differences in molecular structure of collagens from different sources. Being a protein molecule, the amide-I, amide-II and amide-III regions of collagen are directly related to its polypeptide configuration. Intermolecular crosslinks cause absorption around 1660 cm^{-1} (Prystupa & Donald, 1996), while the random coil state is characterized at lower wavenumbers (Payne & Veis, 1988).

The peaks of amide I and amide II of rohu are found at higher frequencies than those of catla, indicating a higher degree of molecular order for rohu than for catla. The existence of hydrogen bonding in each collagen can be seen by shifting the amide-II band position to a lower frequency (Pati *et al*., 2010).

The Fourier-transform infrared (FTIR) spectra of ASC and PSC from flatfish skin reveal identical amide-I and amide-II peaks, indicating no differences in the degree of molecular order. ASC from flat fish has higher amide-A, amide-I and amide-III transmissions peaks, while its amide III is lower than in PSC from flatfish skin (Heu *et al*., 2010).

The IR spectra for collagens from the skin, scales and bones of deep-sea redfish exhibit slight variations, indicating differences in their secondary structures. Analysis of amide-I and-II peaks also indicates that skin has a higher degree of molecular order than do scales and bones, as these peaks show shift to higher frequencies. Deconvolution of the amide-I band shows that it consists of three components. The band intensity at 1633 cm^{-1} of skin (43.5%) is attributed to the unwinding of peptide chain and is much higher than those of scales (31.5%) or bone (33.4%). This indicates that due to the higher hydrogen bond in scales and bones, made evident by the amide-A band, the extent of peptide chain unwinding of scales and bones is low compared to that of skin. The lower intensity of the band at 1660 cm^{-1} of skin (22.6%) is attributed to a much lower extent of hydrogen bond than in scales (55.9%) and bones (39.9%). This band also suggests that there are more and/or stronger hydrogen bonds in scales and bones. It is in accordance with the amide-A band. Another considerable difference is the higher intensity of the band at 1696 cm^{-1} in skins, which is a consequence of the extent of intermolecular crosslinking and is analyzed from the intensity ratio of 1696/1660 cm^{-1}. The ratio observed in skins is much higher compared to that in scales and bone, proving that the extent of intermolecular crosslinking is much greater in skin. Generally, the triple-helical structures of skin, scales and bones isolated by acetic acid after pretreatment are well maintained, but the molecular structures of collagens from different tissues show slight differences. Skin possesses a higher degree of intermolecular crosslink and molecular order, and the extent of peptide-chain unwinding is also higher, which is attributed to the existence of less hydrogen-bond association than in scale and bone (Wang *et al*., 2008).

The FTIR spectrum also indicates the differences in functional groups and inter/intramolecular interaction obtained from the PSC of unicorn leather jacket skin, which may be due to pepsin affecting PSC to some extent. ASC contains a greater nonhelical portion of the telopeptides, which is reflected in the lower intramolecular H-bond between C=O of the peptide backbone and the adjacent hydrogen donor, in comparison with PSC. However, slight differences are also observed between the two types of collagen at the

telopeptide region (Ahmad & Benjakul, 2010). Further, some differences between the secondary structural components, such as α-helix, β-sheet, β-turn and other random coils between the two types from the skin of the common mink whale are also reported (Nagai *et al*., 2008).

A stronger hydrogen bond is seen in PSC compared to ASC in stripped catfish, from the amide-I and -II bands. This result is in accordance with the amide-A band. The higher hydrogen bonding in PSC triple helix bestows a higher structural order of collagen, attributed to pepsin disruption of the nonhelical portions of telopeptide regions. However, a slight change in the structure of PSC is also noticed, due to the removal of the telopeptide region by pepsin, but the triple-helical structure is still predominant with the stronger bonds. Peaks at around $1000\,cm^{-1}$, which represent the phosphate groups at 872, 1401 and $1450\,cm^{-1}$ and correspond to carbonate anions, are also preserved, indicating an organic (type-I collagen) and inorganic (calcium-deficient hydroxyapatite) composite composition of scales (Lina *et al*., 2011).

As a consequence of the removal of telopeptides by pepsin during the extraction of PSC in brownbanded bamboo shark cartilage, a loss of reactive amino acids, including lysine, hydroxylysine and histidine, for inter- and intramolecular crosslinking localized at the telopeptide occurs, shifting the amide-I peak to lower a wavenumber (Foegeding *et al*., 1996; Wong, 1989). As a consequence, its amide-I peak is shifted to the lower wavenumber (Kittiphattanabawon *et al*., 2010).

30.7 EMERGING APPLICATIONS OF TYPE-I COLLAGEN

Collagen, a major component of ECM, has been extensively used for diverse biomedical applications, essentially due to its structural integrity and peptide cues, which support biological compatibility, and its biodegradation into physiologically functional moieties. Low antigenicity and favorable mechanical, haemostatic and cytocompatible properties make it an attractive candidate for drug delivery and regenerative medicine (Parenteau-Bareil *et al*., 2010; Pati, 2011). Further, collagen provides appropriate signals to regulate various cellular processes essential for neo-tissue formation. With increasing life expectancy, management of geriatric diseases, especially chronic/diabaetic wounds, pressure/venous ulcer and osteoporosis, is becoming critical. Depletion of collagen in native ECM is the major cause of delayed healing, which may be therapeutically intervened with using collagen-based bioengineered products. New forms of collagen-based product are rapidly emerging for the development of bioengineered skin, bone, cartilage, tendon, ligament and so on. The application of collagen in the biomedical field can be best appreciated by considering its role in wound healing.

30.7.1 Type-I Collagen for Wound Healing and Skin Tissue Engineering

Collagen, being the major structural/functional protein of dermal ECM, has been frequently chosen as a material for skin-graft development (Ruszczak, 2003). Mainly synthesized by fibroblasts, at least six different types of collagen are present in human skin, including type I ($\sim$70%), type III ($\sim$10%) and trace amounts of types IV, V, VI and VII (Uitto *et al*., 1989). Collagen plays a major role in wound repair, as most of the higher vertebrates heal by collagenous scar-tissue formation. In chronic wounds, elevated levels

of inflammatory cells and proteases break down the ECM components and growth factors. Among various proteases, overexpression of matrix metaloprotease (MMP) degrades not only the nonviable collagen in the wound but also the freshly laid functional collagen.

Recent evidence about the ECM environment and its role during wound healing have given impetus to its wide-scale application in regenerative medicine. In a wound, collagen mainly acts as a support matrix for the restoration of continuity, structure and function to injured connective tissue. In the early stages of wound healing, primarily collagen III is laid down, which is predominantly replaced by collagen I with healing progression. Collagen deposition and remodeling lead to scar-tissue formation, which improves the tensile strength of the wound to ~20% that of the uninjured tissue in 3 weeks' time. However, collagen being a brittle substitute for intact connective tissue, the healed wound attains only 70% of original strength at the end of healing (Rangaraj *et al*., 2011). The linear orientation and fibrillar shape of collagen contribute to its tensile strength. Moreover, interfiber crosslinks between collagen molecules in adjacent bundles give rise to additional tensile strength, which stabilizes the skin against shear forces (Steinberg & John, 2009).

One of the key strategies in achieving wound closure in chronic conditions includes inhibition or deactivation of elevated levels of MMPs, thereby facilitating granulation tissue formation. Collagen-based wound-dressing systems are ideal for tackling issues of MMP overexpression as they act as 'sacrificial substrates' in the wound. Moreover, collagen cleavage products are chemotactic to a number of cells essential for wound healing. Recent evidence shows that topically placed collagen matrices activate inflammatory cells and facilitate vascularization in healing tissues. Due to their hydrophillic nature, collagen molecules provide surface geometries suitable for cell adherence (Purna & Babu, 2000). Rat Genome Database (RGD) sequences of collagen molecules also present specific high-affinity focal adhesion points to fibronectins present on fibrogenic cells' surfaces. Three-dimensional collagen scaffolds induce fibroblastic proliferation and subsequent granulation tissue formation. Collagen-based dressings absorb wound exudates and maintain a conducive healing environment.

30.7.2 Commercially Available Collagen-based Wound Dressings/Skin Substitutes

There is a wide variety of collagen-based products, in which protective polymer-based coatings, active agents and growth factors are integrated with collagen. These products have different modes of action and fulfill the following criteria to make them suitable for wound-healing applications:

- Maintain a moist environment in the wound and manage exudates.
- Act against exogeneous organisms and pollutants.
- Promote mass transfer and thermoregulation.
- Are elastic, flexible and nonantigenic.
- Are easy to apply and remove.
- Are patient-friendly and provide comfort.

These dressings are available in a variety of sizes, shapes and compositions and are suited to specific clinical conditions. Table 30.4 summarizes the currently commercially available wound-healing products. However, most of these products contain collagen from bovine or porcine sources, which restricts their widescale acceptability and use for the reasons mentioned in this chapter. Collagen derived from fish can be used as a way of addressing these health-care issues.

Table 30.4 Commercially available collagen based wound dressing/skin tissue engineering products.

Product /company	Key components	Collagen source/type	Clinical conditions	Advantages	Limitations
Apligraf (Organogenesis)	Neonantal fibroblasts, keratinocyte, collagen matrix	Bovine, type I	Diabetic foot ulcers, venous leg ulcers	Morphologically similar to human skin Delivers cell, matrix, protein to wound Safe, not clinically rejected Low risk of disease transmission in spite of being allogenic	Limited shelf life (10 days) Cannot be used on patients with hypersensitivity or allergy to bovine products
Alloderm (Life Cell Corp)	Acellular dermal matrix derived from cadaveric skin	Human	Hernia, abdominal wall repair, breast reconstruction, postmastectomy, orthopedic/urinogenital/dental surgeries	Eliminates need for palatal surgery Reduces patient reluctance to follow through with surgical treatment Available in two thicknesses, can be applied to multiple convenient sites	Removal may be required in 2–3 weeks Allergic reaction reported in some patients Infection due to contamination is very common Aseptic techniques are crucial during handling, rehydration and implantation Graft failure due to poor recipient interfacing For epithelial covering, autograft is essential Cannot be used on infected wounds
Biogbrane (Smith and Nephew)	Silicone film with nylon fabric embedded within it Collagen chemically bound to nylon fabric	Porcine, type I	Burns	Wound closed and protected Silicon barrier decreases risk of infection Rapid wound closure along inner surface Easy wound visualization, convenient application Flexible, durable, nonallergic, stored at room temperature, long shelf life (3 years) Reduces pain compared to conventional therapies	Cannot support homeostasis, so can be applied only after early stages of wound healing Not very effective against full-thickness skin burn

(*continued overleaf*)

Table 30.4 (*continued*)

Product /company	Key components	Collagen source/type	Clinical conditions	Advantages	Limitations
Biostep, Biostep Ag (Smith and Nephew)	Porcine collagen/gelatin/ alginate/EDTA/ carboxymethyl cellulose/with or without silver	Porcine	Chronic wounds	Presence of EDTA allows/prevents destruction of viable collagen in wound bed Only product that contains both collagen and denatured collagen, which augments faster healing Longer wear time (6 days) than other collagen-based products Due to CMC and alginate, more absorbent Presence of Ag in Biostep Ag has bactericidal action	N/A
Biopad (Argentum Medica)	100% collagen in lyophilized form Converts to gel in wound bed	Equine (horse)	Ulcer and skin lesions	Allow contact with entire wound bed in gel form Topical use to control minor bleeding Exudates management Promotes cell migration and wound closure Inhibition of MMPs	Cannot be used on grossly or clinically diagnosed infected wounds Cannot be used on patients with a family history of autoimmune diseases, anaphylactoid reactions or known hypersensitivity to meat products
Catrix (Lescarden Inc.)	Collagen in form of fine white powder	Bovine, type I	Nonhealing ulcers (e.g. bedsore, venous ulcer, diabetic ulcer), stasis ulcer, pressure ulcer (stage I–IV), burns, dermatitis, post-surgical treatments, abrasions, skin conditions with periostomal care	Accelerated wound healing Easy, painless application and lower treatment cost Protects against infection, maintains wound bed moisture and controls exudates Has hemostatic effect Less chance of accidental infection and no adverse effects	N/A

Cellerate RX (Wound Care Innovations)	Activated hydrolyzed collagen fragments with sizes that are fractions of those of native collagen molecules Available in gel/powder	Bovine, type I	Diabetic/pressure ulcers, ulcers due to reduced blood supply, traumatic/superficial/surgical wounds, first- and second-degree burns	Delivers collagen to body in readily usable form Faster action and healing Minimizes chances of scarring Reduces pain by occluding nerve endings Easy application, patient-friendly, no special storage requirements	N/A
Colactive/ Colactive Ag (Smith and Nephew)	Collagen/sodium alginate Colactive Ag additionally has low dose of silver (8 ppm)	Porcine, USP-grade	Advanced wounds	Pliable, absorbent, maintains moist environment necessary for granulation tissue formation and wound closure Can be cut and applied in wound as per size requirements. Can also be placed in layers in case of deep wounds Easy removal from wound Colactive Ag has antibacterial action due to Ag content	N/A
Dermagraft	Cryopreserved neonatal human dermal fibroblast/polyglactin mesh/collagen/matrix proteins/growth factors/cytokines	Human	Full-thickness diabetic foot ulcers	Longer shelf life Rapid wound closure	Cannot be used in ulcers with sinus tracts Cannot be used on infected wounds
EZDerm (Brennan Medical Inc.)	Xenograft with collagen crosslinked with an aldehyde	Porcine	Partial-thickness wounds, full-thickness wounds before grafting, skin ulcers, abrasions, toxic epidermal necrolysis, autograft protection	Protective covering Easily conforms to shape and contour of wound site, flexible, occlusive Minimizes heat/moisture loss Decreases pain Long shelf life (18 months)	Cannot be used on full-thickness burns Cannot be used on clinically infected wounds Potential immune response

(*continued overleaf*)

Table 30.4 (*continued*)

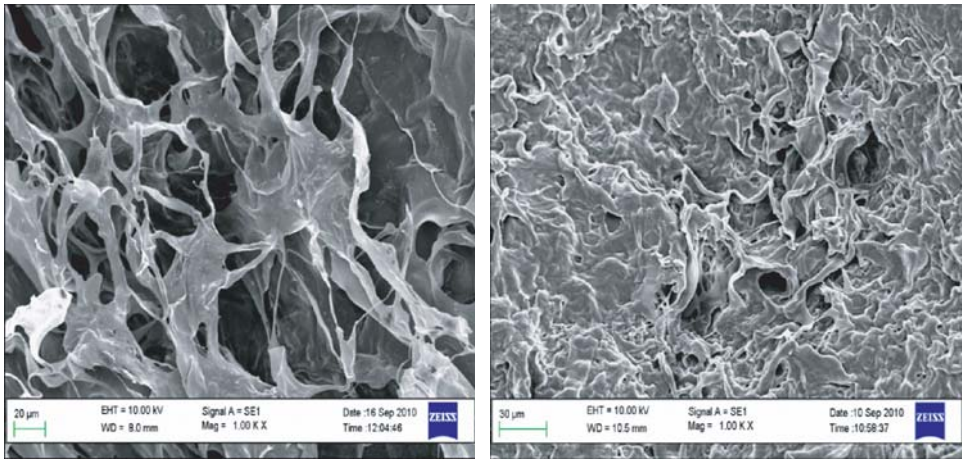
Product /company	Key components	Collagen source/type	Clinical conditions	Advantages	Limitations
Fibracol Plus (Johnson & Johnson)	Collagen/alginate	N/A	Full/partial-thickness traumatic or surgical wounds, first- or second-degree burns, pressure/diabetic/venous ulcers or ulcers due to mixed vascular etiologies	Maintains structural integrity in wound Nonadherent to wound so can be removed easily without pain. The wound does not retain the fiber after removal of dressing Flexible, can be cut into specific size according to the need of the patient Absorbs exudates from wound bed Can be applied for compression therapy	Cannot be used on patients with c third-degree burns or active vasculitis
Integra (Integra Life/Sciences)	Polysiloxane membrane/collagen/glycosaminoglycan	Bovine tendon, type I	Deep partial- and full-thickness burns	Immediate wound coverage Ultra-thin split-thickness skin grafting Good barrier protection, retains moisture Available in injectable form	Two-step procedure Operative removal of upper polysilicone layer is essential
GraftJacket (Wright Medical)	Collagen, elastin, hyaluronan, fibronectin, blood-vessel channels	Human cadaveric	Soft-tissue coverage over bones, tendons and other deeper structures	Rapid revascularization and healing Minimizes contamination and infection risk	N/A
Oasis (Healthpoint)	Acellular collagen matrix	Porcine small intestine submucosa	Partial/full-thickness diabetic pressure and venous wounds/burns	Long shelf life (1.5 years)	Potential immune response
Promogran/ Promogran Prisma (Johnson & Johnson)	Oxidized regenerated cellulose/collagen Prisma has silver content		Venous leg ulcers, diabetic foot ulcers, pressure ulcers Promogran Prisma can be used if there are signs of infection	Can 'kick start' healing in case of stalled wound Reduces pain	Cannot be used on patients with full-thickness burn injuries or active vasculitis

Puracol (Medline Industries Inc.)	Collagen matrix	Bovine	Chronic wounds	Destroys elastases, helps restore chemical balance in wound Readily absorbs exudates Easy application, can be cut into any size	N/A
Transcyte (Advanced Biohealing, Inc.)	Silicone outer membrane/ cryopreserved fibroblasts/nylon mesh/collagen/ growth factors/matrix proteins	Porcine/ human	Temporary cover for surgical full-thickness wounds or deep partial-thickness burns prior to autografting, mid-dermal to indeterminate-depth burn wounds	Protection from infection Transparent; wound visualization possible	Temporary covering; may need to be replaced in 2–3 weeksAutograft needed for epithelial covering Cannot be applied on infected wound

Information obtained from company websites and product catalogues; N/A = not available.

To date, only a few companies have concentrated on commercialization of marine collagen-based wound-healing products and cosmetic consumables. Of them, Klinipharm (Frankfurt, Germany) is significant due to its ongoing research into bioactive properties of marine sponges and product development for wound healing, treatment of psoriasis, ulcerative colitis, neurodermatitis, Crohn's disease and cosmetic applications (www.klinipharm.com). Eleana, KliniPharm's skin-care and wellness range, includes marine collagen alongside other bioactive compounds such as hyaluronic acid, antioxidants, phytonutrients and so on, and focuses on aesthetic and anti-aging dermatology. Another company in New Zealand, Health & Herbs International Ltd, is involved in developing hydrolyzed marine collagen-based dietary capsules, Radiance Ageless Beauty for beneficial effects on skin, including support of dermal integrity, maintenance of hydration, antioxidant protection and cleansing (www.healthandherbs.co.nz). Similar marine collagen-based dietary pills are commercialized by Nutriteck, Quebec, Canada (Nutriteck Marine Collagen) for cosmetic benefits (www.nutriteck.com). Other significant commercially available marine collagen-based skin-care products include Rose Plus Marine Collagen Complex (The Organic Pharmacy, UK; www.theorganicpharmacy.com), Marine Collagen Toner (Nam Sing Biotechnology Corp., Taiwan), Marine Collagen Mask (DermaPro, Valencia, Canada; www.dermapro.com) and Marine Collagen (Finn, Canada; www.finncanada.com). The novelty of the marine collagen-based cosmetics developed by Finn, Canada lies in their collagen source: the company mainly uses collagen extracted from the skin of wild ocean salmon. An alkaline-based extraction method is adapted to obtain superior-quality collagen with a higher mineral and amino acid content. No hatchery fishes are used, in order to avoid the harmful effects of hormones. Salmon-skin collagen, having a low melting temperature (18 °C), is beneficial for cosmetics as it easily melts on the skin (37 °C body temperature) and is readily absorbed. On the other hand, bovine or porcine collagen-based cosmetic products have a higher melting temperature (38 and 40 °C, respectively) and often fail to be absorbed into human skin.

In our laboratory, isolation of type-I collagen from the scales of freshwater fish such as rohu, catla and mrigel and of tropical-water fish such as hilsa is carried out. A recent study by Pati *et al*. (2012) shows promising characteristics of type-I collagen isolated from rohu and catla scales for tissue-engineering applications. The collagen extracted has been found to be nonimmunogenic and to have a denaturation temperature close to that of mammalian collagen (Pati *et al*., 2010). Three-dimensional self-assembled fibrous collagen scaffolds are obtained by freeze-drying (Fig. 30.2a). The scaffolds support the adhesion and growth of 3T3 fibroblast cells, thus proving their cytocompatible nature (Fig. 30.2b). Two-dimensional collagen films are also prepared for applications in tissue engineering and healing of chronic wounds, including venous and diabetic ulcers (Dhara *et al*., 2011) (Fig. 30.3 and Fig. 30.4). Freeze-dried nano/microfibrous collagen scaffolds with directional alignment are prepared using special kinds of mold for bone, tendon and ligament tissue regeneration (Fig. 30.4). Coating of tissue-culture flasks with fish-scale collagen and subsequent 3T3 fibroblast shows superior cellular response in comparison to uncoated flasks. Fish-scale collagen is also used for the development of cell-incorporated collagen gels with potential applications in skin tissue engineering. This collagen has been further blended with polymers such as PVA in aqueous solvents and electrospun to produce nanofibers of 250∼300 nm. The nanofibrous membranes support significant cell attachment and proliferation and are highly cytocompatible in nature. Their matrices are reported to be very effective as accelerators in early-stage wound healing. Crosslinked



(a) (b)

Fig. 30.2 Collagen scaffold (a) without and (b) with attached 3T3 cells at 1000× magnification.

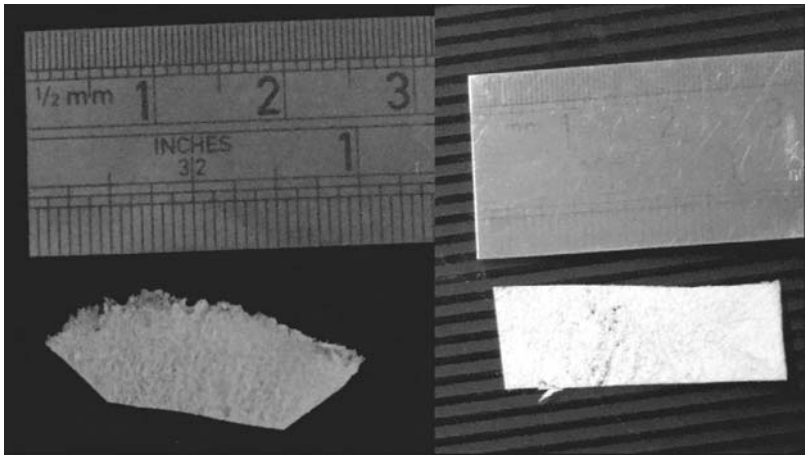
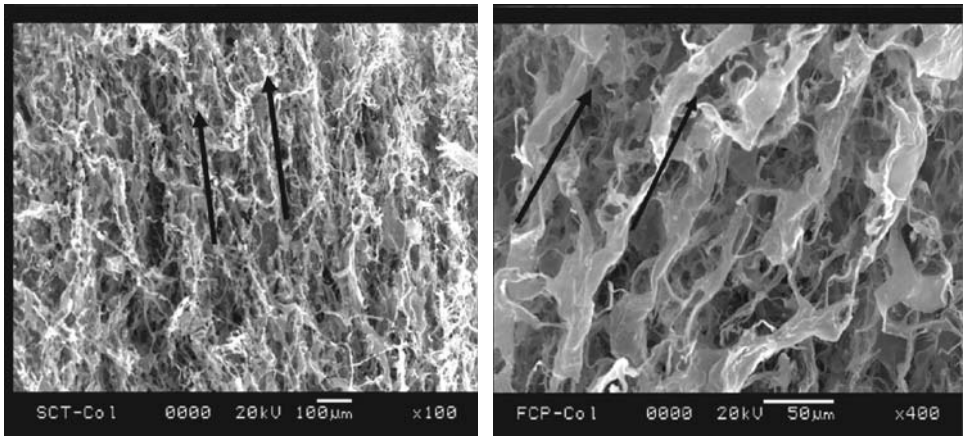


Fig. 30.3 Representative digital images of collagen film.

collagen nanofibers that mimic ECM proteins, particularly type-I collagen, are good candidates for biomedical applications, including wound dressing and use as scaffolds for tissue engineering. Type-I collagen is currently being used in conjunction with chitosan nanofibers to prepare nano/microfibrous layered scaffolds for wound-healing applications and the development of skin-equivalent models.

30.8 CONCLUSION

This chapter has presented and compared results from the literature concerning the isolation and characterization of collagens from fish species and their potential applications. Major emphasis has been given to those processing steps and characterizations required



(a) (b)

Fig. 30.4 Collagen scaffold with directional alignment at (a) 100× (b) 400× magnification. Arrows show direction of alignment.

for consideration of the commercial exploration of fish collagen as a source of biomaterials for various tissue-engineering and drug-delivery applications. It can be concluded that fish collagen is capable of providing a valuable product of far-reaching medical significance.

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31 Properties, Biological Advantages and Industrial Significance of Marine Peptides

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31.1 INTRODUCTION

Though industrialization is inevitable, as it enhances comfort and satisfaction, and development of marine products is unavoidable, as it meets the growing needs of the world population, it is equally important that the environment be preserved. The severity of the situation has led researchers worldwide to explore devices and technologies aimed at stopping contamination of environmental resources. Hence, environmental protection is becoming a challenging global problem.

A vital element in this work was the invention of a new technology for fish-viscera silage processing. Today there is an increasing demand for fish raw material, but no significant growth in the annual world fish harvest. The basic concepts in peptide manufacture are to develop the molecular structure of the protein, to develop the structure and function of protein fragments and to produce tens of millions of proteins with the desired physiological function. A peptide has a strong biological activity. Its basic constituents are amino acids, with twenty amino acids in the human body. Each peptide has a unique amino acid composition, which determines its function.

Large numbers of marine fish species have been identified with potential nutraceutical and medicinal values. Consequently, a number of bioactive compounds have been identified, including fish-muscle proteins, peptides, collagen and gelatin, fish oil and fish bone. According to their source, peptides can be classed as either endogenous or exogenous.

About 30% of the worldwide sale of drugs is based on marine natural products. Though recombinant proteins and peptides account for increasing sales rates, the superiority of low-molecular-mass compounds in human disease therapy remains undisputed, mainly due to their superior compliance and bioavailability properties.

31.1.1 Marine Resources used for Peptide Synthesis

Marine animals are emerging as a leading resource for the identification and extraction bioactive peptides (Kim *et al*., 2008). These peptides have been described as a source of pharmaceutical products with beneficial effects of humans. Beyond their basic nutritional roles, peptides are involved in many processes in living organisms, based on their

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function as hormones, neuropeptides, alkaloids, antibiotics, toxins and regulation peptides. Studies are underway to explore further sources of these marine peptides, especially from underutilized marine-processing byproducts. Their bioavailabilities, possible physiological functions and mechanisms of action are also being investigated.

Atlantic herring fisheries are of great importance on the south-west coast of Norway. This species has not perviously been studied for its peptide, so we have chosen to concentrate on it in this chapter. In recent years, overexploitation of fishery resources has become a major concern worldwide. According to data published by the United Nations Food and Agriculture Organization (FAO), approximately 77% of the 143.6 million tons of fish and shellfish caught in 2006 was used for human consumption. Historically, pelagic fish, and in particular herring, has been of great importance to the population along the west coast of Norway, with herring fisheries being an important contributor to this part of the country's economy. The majority of the pelagic fish caught is used for human consumption. Large quantities of the catch go to export, and in 2009 herring contributed to 30% of the total export of Norwegian seafood. Residual material in this context includes trimming, fins, frames, heads, skin and viscera. Fish-processing waste and byproducts of all types from fisheries in the European Union total about 5.2 million tons per year (Ferraro *et al*., 2010). With such a quantity of residual material available, there is increasing interest in finding new uses for it within the fishery sector (Mahro & Timm, 2007). Recent research has shown that fish residual material contains a range of components with interesting biological activity (Kim & Mendis, 2006). There is great potential to utilize it as a starting material for the generation of more valuable products.

31.1.2 Industrial Significance of Marine Peptides

Much attention has been paid to unraveling the structural, compositional and sequential properties of bioactive peptides. Marine bioactive peptides may be produced by one of three methods: solvent extraction, enzymatic hydrolysis, and microbial fermentation of food proteins. The enzymatic-hydrolysis method is preferred in the food and pharmaceutical industries because of the lack of residual organic solvents and toxic chemicals in its products. Bioactive peptides are inactive in the sequence of their parent proteins, but can be released by enzymatic hydrolysis (Kim *et al*., 1999). They usually contain 3–20 amino acid residues, and their activities are based on their amino acid composition and sequence. They have been detected in many different food sources (Dziuba *et al*., 1999).

Innovations in the Nordic seafood industry will increasingly involve transforming it from a traditional supplier of raw materials for food processing into a high-tech industry producing high-value products such as functional foods and other health products. There is increasing demand for complete utilization of the catch, in order to minimize waste of this valuable but limited resource. Marine bioprospecting is a fast-growing field, where scientists search for valuable compounds in the marine environment. These compounds can serve as active ingredients in functional foods and other health-related products, as lead drugs for the pharmaceutical industry and as a natural replacement for antibiotics in aquaculture and farming, to name but a few possible uses. A large group of such compounds, the bioactive peptides, are receiving increasing attention for their antibacterial, antifungal, antiviral, antioxidant, anticancer and angiotensin-converting enzyme (ACE)-inhibitory activities. These peptides have been found in both underutilized marine species (including sponges, echinoderms and mollusks) and commonly utilized species (such as cod, pollock and flounder).

31.2 MARINE-PEPTIDE PROPERTIES

Research into development of bioactive peptides from common food products has increased steadily in recent decades. This research has mainly been focused on the dairy industry, and many products based on bioactive peptides from milk proteins have been marketed (Silva & Malcata, 2005). Such products contains two peptides that have what is called ACE-inhibitory activity, which has been shown to lower blood pressure. The market for dairy functional foods has grown exponentially in the last few years and the potential for profits from these products is considerable.

The bioactive peptides from marine protein sources are much less advanced. Still, new methods for isolating high-quality protein from fish are very promising, especially with regards to adding value to cuts, frames and other byproducts from the fishing industry (Kristinsson & Rasco, 2000). Although the focus has mainly been on upgrading this protein from animal feed to human consumption, studies on bioactive peptides from fish are increasing.

One of the most important factors in producing bioactive peptides with desired functional properties is their molecular weight (Deeslie & Cheryan, 1981). Therefore, the efficient recovery and to obtain bioactive peptides with a desired molecular size and functional properties, the use an ultrafiltration membrane system is advised. This system has the main advantage that the molecular-weight distribution of the desired peptide can be controlled by adoption of an appropriate ultrafiltration membrane (Jeon *et al*., 1999).

In recent years, great attention has been paid to the bioactivity of marine natural products, due to their potential pharmacological utilization. Marine plants and animals have adapted to all sorts of environments and are constantantly under tremendous selection pressure, including space competition, predation, surface fouling and reproduction, a situation that demands the production of quite specific and potent active molecules. Many of these organisms have important medicinal properties, with most antibacterial agents being isolated from the marine environment. Marine bioactive peptides are widely produced by enzymatic hydrolysis of marine organisms (Slizyte *et al*., 2009). Fermented marine food sauces have already undergone enzymatic hydrolysis in their production, so bioactive peptides can be purified from these products without further hydrolysis (Je *et al*., 2005). In addition, several bioactive peptdies have been isolated from marine processing byproducts (Jun *et al*., 2004).

Compounds of marine origin are diverse in structure, from simple linear peptides to complex macrocyclic polyethers. Studies have shown that the determined ascidians are a rich source of bioactive peptides, many of which contain cysteine. These cysteine residues, in the form of disulfide bridges or five-membered heterocyclic rings, seem to constrain the peptides to specific conformations (Ireland *et al*., 1988). The marine environment is a rich source of functional materials including polyunsaturated fatty acids (PUFAs), polysaccharides, minerals and vitamins, antioxidants, enzymes and bioactive peptides (Kim *et al*., 2008). The bioactive peptides (in particular small, natural bioactive peptides with molecular weights lower than 10 kDa) in Atlantic herring (*Clupea harengus* L.) byproducts such as skin and more general residual materials, for example, represent a source of health-enhancing components. Some bioactive peptides have demonstarted multifuntional activities based on their structures and other factors, including hydrophobicity, change and microelement-binding properties (Cho *et al*., 2008).

31.3 INDUSTRIAL DEVELOPMENT OF MARINE BIOACTIVE PEPTIDES

New strategies in marine natural product chemistry are necessary if it is to compete successfully with combinatorial chemistry and meet the demand for several hundred thousands of test samples to be submitted to high-throughput screening (HTS). Today, pharmaceutical companies spend approximately US$350 million to develop a new drug (Grabley & Thiericke, 1999). Many of the reported bioactive peptides are multifunctional and can exert more than one effect. These peptides usually contain 2–20 amino acid residues per molecules. Many research groups and companies have therefore focused their attention on converting the remaining proteins into bioactive peptides by enzymatic treatment, thus generating various hydrolysates containing short peptides (Kim & Mendis, 2006; Kristinsson & Rasco, 2000; Ryan *et al*., 2011). As dairy products are widely accepted by consumers, the delivery of nutraceuticals through dairy products has received a great deal of attention. Since the incorporation of marine-derived active ingredients into dairy products causes minimal change in their physicochemical properties, marine-derived substances have great potential for use as nutraceuticals in the dairy industry (Vidanarachchi *et al*., 2012). This has great economical and ecological benefit for the fishery industries in the worldwide context of marine-resources depletion (Cudennec *et al*., 2012).

31.3.1 Enzymtic Methods in Peptide Synthesis

During the production of functional foods containing bioactive peptides, methods must be developed to enhance their bioavailability. Functional or novel foods are created by fortifying or adding enriched fractions of the bioactive peptide to the product. Bioactive peptides may be produced from precursor protein substrates by a number of methods, such as using specific Generally Recognized as Safe (GRAS) proteolytic bacterial enzymes (Hayes *et al*., 2007) or enzymatic hydrolysis with digestive enzymes such as trypsin and pepsin (Miguel *et al*., 2009). For many years, numerous processes based on enzymatic hydrolysis at various pHs, addition of plant or bacterial enzymes after inactivation by heating of the endogenous enzyme present in the raw material, or the action of endogenous enzymes have contributed to the degradation of marine-byproduct proteins in order to produce fractions with biological activities.

Marine bioactive peptides have been widely produced by enzymatic hydrolysis of proteins derived from marine animals (Sheih *et al*., 2009). Hydrolysis can generally be carried out in a thermostatic stirred-batch reactor in which the hydrolysis conditions (pH, temperature, enzyme concentration and stirring speed) are adjusted to optimize the activity of the enzyme. An intial mixing is usually carried out to adjust the pH and temperature to the desired values. In order to obtain a hydrolysate with high stability with reduced pro-oxidants, the raw materials can be washed to remove heme protein and lipids. Antioxidants can be added before hydrolysis. After hydrolysis, the reaction is teminated by inactivation of the protease by heat treatment or pH adjustment. Combining pH and temperature adjustments avoids the creation of harsh conditions, which might affect the resulting hydrolysate or peptides. The reaction mixture, which contains both the peptides and unhydrolyzed debris, is then centrifuged or filtered. The supernatant or filtrate is concentrated or dried. The hydrolysate can be an excellent source of peptides with functionalities and bioactivities determined by the types of protease, pretreatment of the raw materials, the condition of hydrolysis and so on. Several approaches have

been implemented to enhance the bioactivity of the peptides produced, such as the use of multistep hydrolysis with different proteases (Phanturat *et al*., 2010). Moreover, it is possible to obtain serial enzymatic digestions in a system by using a multistep recycling membrane reactor combined with an ultrafiltration membrane system to separate marine-derived bioactive peptides (Kim & Mendis, 2006). This membrane-bioreactor technology has recently emerged for the development of bioactive compounds and has potential for the utilization of marine proteins as value-added nutraceuticals with benefial health effects.

In order to obtain functionally active peptides, a three-enzyme system can be used for sequential enzymatic digestion. It is possible to obtain serial enzymatic digestions in a system by using a multistep recyling membrane reactor combined with an ultrafiltration membrane system to separate marine-derived bioactive peptides (Kim & Mendis, 2006). Enzymatic hydrolysis of proteins allows the preparation of bioactive peptides obtained by *in vitro* hydrolysis of protein sources using appropriate proteolytic enzymes. The physicochemical conditions of the reaction media, such as the temperature and pH of the protein solution, must then be adjusted in order to optimize the activity of the enzyme used. Proteolytic enzymes from microbes, plants and animals can be used to hydrolyze marine protein and develop bioactive peptides (Simpson *et al*., 1998). Furthermore, α-chymotrypsin, papain, neutrase and trypsin have been used for the hydrolysis of tuna dark muscle under optimal pH and temperature conditions of the respective enzymes (Qian *et al*., 2007).

31.3.2 Chemical Methods in Peptide Synthesis

Peptide synthesis is the chemical process of coupling the carboxyl group of one amino acid to the amino group of another. Usually, chemical techniques are used to synthesize peptides of up to 40 amino acids in length. Peptide synthesis can be classified on the basis of the techniques used and the type of final product. Liquid-phase peptide synthesis can further be divided into two types: step-by-step peptide synthesis with subsequent addition of one amino acid at one position from the C-terminal to the N-terminal and block synthesis with coupling of polypeptide fragements. Liquid-phase peptide synthesis is used in large-scale industrial peptide production. In contrast, in solid-phase peptide synthesis the polypeptide chain is covalently bound via a linker to porous insoluble bed particles. The first stage of the technique consists of peptide-chain assembly via protected amino acid derivatives on a polymeric support. The second stage is the cleavage of the peptide from the resin support and the concurrent cleavage of all side chain-protecting groups to produce crude free peptides. Currently, two protecting groups are commonly used in solid-phase peptide synthesis: Fmoc or 9-fluorenylmethyl carbamate and t-Boc or di-tert-butyl di carbonate. Fmoc chemistry is known for generating peptides of a higher quality and in greater yield than t-Boc chemistry. Impurities in t-Boc-synthesized peptides are mostly attributed to cleavage problems, dehydration and t-butylation. The advantage of Fmoc is that it is cleaved under very mild basic conditions (e.g. piperidine) but is stagble under acidic condtions. After base treatment, the nascent peptide is typically washed and then mixed with an activated amino acid and coupled with the next amino acid. After coupling, noncoupled reagents can be washed away. Then the protecting group on the N-terminus of the nascent peptide can be removed, allowing additional amino acid or peptide materials to be added to the nascent peptides in a similar fashion. After cleavage from the resin, the peptides are usually purified by reverse-phase high-performance liquid chromatography (RP-HPLC) using columns such as C-18, C-8 and C-4. The primary advantage of solid-phase peptide synthesis is its high yield.

31.3.3 Chromatography Methods in Peptide Synthesis

Chromatography offers a variety of methods for the separation of peptides, based on charge, hydrophobicity, size and molecular recognition. Chromatography is a powerful technique for achieving high degrees of purity. It has been well established in biotechnological industries as a production-scale unit operation and an analytical tool for monitoring the quality of raw materials and end products and the purification efficiency of sequential downstream operations. Much effort has been made to develop selective column chromatography methods that can isolate and purify bioactive peptides. However, less repoted are the advances in preparative chromatography of bioactive peptides. This will play an increasingly improtant role in the cost-efficient and enviromentally acceptable manufacture of bioactive peptides. Purification of peptides may be performed via various separation techiques used separately or in combination. Some, like crystallization or precipitation, are fairly well established, although they are constantly being improved. Others, like liquid–liquid extraction, chromatography and electrophoresis, are being developed with a view to being scaled up in the near future.

31.4 BIOLOGICAL APPLICATIONS OF MARINE PEPTIDES

Bioactive peptides are of commercial interest as components of functional foods and nutraceuticals with certain health claims. The biological activity of an extract of marine organism or isolated compound can be assessed in several ways. Due to the limited amount of material generally available and the high cost of biolgical testing, it is impossible in any laboratory to examine all permutations of drug–animal interaction in order to unmask the drug potential of a material. Besides, the candidate drug has to pass through rigorous toxicological evaluation and clinical trials before it reaches the clinician's armamentarrium. A fair understanding of biological, toxicological and clinical evaluation is essential to those interested in searching for potential drugs in marine organisms. Investigations to date have mainly focused on species caught in great quantities, such as pollock and cod, where ACE-inhibitory activity has been found (Je *et al*., 2004). Protein from food is a source of numerous bioactive peptides with antihypertensive, opioid, antithrombotic and immune-modulating activities. The use of fish-processing byproducts as a substrate provides a novel approach to the potential discovery of high-value bioactive products.

In the past 20 years, an emerging area of research has been the identification of bioactive compounds present in marine byproducts. Bioactive compounds are described as 'food-derived components that in addition to their nutritional value exert a physiological effect in the body' (Vermeirssen *et al*., 2004). In order to enhance the hydrolysis process, the raw materials may be subjected to size reduction by homogenization. The bones, scales or skin are added into the mixture containing the protein substrate, which is first adjusted to a pH of the desired value. The newly formed peptides can retain the biological properties of the native protein or can show new properties. Using appropriate proteolytic enzymes through the control of process parameters such as pH, time and enzyme/substrate ratio, it is possible to produce hydrolysates whose components present some intesting biological properties.

The discovery of the bioregulatory role of different endogenous peptides in the marine organism, as well as the understanding of the molecular mechanisms of action of some new bioactive peptides obtained from natural sources on specific cellular targets,

has contributed to the development of peptides as promising lead drug candidates. Peptides discovered from marine organisms induce cell death by different mechanisms, including apoptosis, alteration of the tubulin–microtubule equilibrium and inhibition of angiogenesis. This finding has increased our knowledge of new potent cytotoxic and other properties with novel chemical structures associated with the original mechanisms of pharmacological activity. Marine peptides are thus a good new choice from which to obtain lead compounds in biomedical research (Zheng *et al*., 2011). Furthermore, no side effect is reported in rats after adminstration of antihypertensive peptide derived from big eye tuna. In addition, marine antihypertensive peptides exhibit antihypertensive activity *in vivo* rather than *in vitro*. The exact mechanisms underlying this phenomenon have not yet been identified, but it has been suggested that bioactive peptides have higher tissue affinities and are subject to a slower elimination than captoprill (Fujita & Yoshikawa, 1999). Marine-derived bioactive peptides have potential for use as functional ingredients in nutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of hypertension. Moreover, cost-effective and safe natural health products can be produced from marine bioactive peptides. Further studies and clinical trials are needed.

Marine natural products have been a source of new leads for the treatment of many deadly diseases, such as cancer and acquired immunodeficiency syndrome (AIDS). A number of marine peptides have been isolated in recent years which exhibit potent biological activities, and many of the compounds show promising anticancer activity (Rawat *et al*., 2006). Marine-derived bioactive peptides have been shown to possess many physiological functions, including antihypertensive and ACE-inhibitory (Je *et al*., 2005), antioxidant (Kim *et al*., 2007), anticoagulant (Jo *et al*., 2008) and antimicrobial (Stensvag *et al*., 2008) activities. Furthermore, depending on the amino acid sequence, they may be involved in variouous biological functions, such as antihypertension, antithrombotic, antioxidant, antioxidant, anticancer and antimicrobial activities, in addition to nutrient utilization (Elias *et al*., 2008). The potency of a marine-derived antihypertensive peptide's ACE-inhibitory activity is expressed as an IC_{50} value, determined by Lineweaver–Burk plot. Competitive ACE-inhibitory peptides are most frequently reported (Lee *et al*., 2010).

Bioactive peptides derived from various fish-muscle proteins have shown biological activities including antihypertensive, antibacterial, anticoagulant, anti-inflammatory and antioxidant effects, and hence may have potential for use in the biomedical and food industries. Since the natural sources are recognized as safe for human consumption, the active substances produced in the diverse group of marine organisms have a wide role to play in the nutraceutical industry. These marine-derived active ingredients include certain polysaccharides, polyphenols, bioactive peptides, PUFAs and carotenoids known to have anticancer, anti-inflammatory, antioxidant, antiobese, hypocholesteroleic, antimicrobial, prebiotic and probiotic activity, enabling them to be applied as nutraceuticals. Numerous *in vivo* studeis of marine-derived antihypertensive peptides in spontaneously hypertensive rats (SHR) have shown potent ACE-inhibitory activity (Lee *et al*., 2010). These peptides can potentially by released during gastrointestinal digestion or food processing. They are believed to promote immunomodulatory, antimicrobial, antioxidant, antithrombotic, hypocholesterolemic and antihypertensive activities (Erdmann *et al*., 2008; Ryan *et al*., 2011).

Fish-derived bioactive peptides represent a source of health-enhancing components. They can potentially be released during gastrointestinal digestion or food processing. The peptides are considered to promote various activities, including immunomodulatory, antimicrobial, opiate-like, antioxidant, antithrombotic, hypocholesterolemic and

antihypertensive effects (Kim & Mendis, 2006; Rajanbabu & Chen, 2011; Ryan *et al*., 2011). Many of the reported bioactive peptides are multifunctional and can exert more than one of these activities. These peptides usually contain 2–20 amino acid residues per molecules. Antimicrobial peptides and proteins form an important means of host defense in eukaryotes. In addition to their role as endogenous antibiotics, antimicrobial peptides have functions in inflammation, wound repair and regulation of the adaptive immune system (Ravichandran & Fredrick, 2012).

31.5 CONCLUSION

Many marine peptides display a great deal of pharmacological activity via multiple targets. It is conceivable that the marine environment increases the diversity of natural products. It is also possible that a great many organisms with useful peptides have not yet been found, since the study of marine peptides is still in its infancy. Compared with the peptides found from other sources, there is more diversity in the style/class of marine peptides, which seem to have utility for biomedical research. Therefore, there is great potential to utilize more of these byproducts in the marine bioprocessing industry as starting material for the generation of valuable products.

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32 Muscle Proteins of Fish and Their Functions

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32.1 INTRODUCTION

The ocean is a rich repository of natural resources. Various species of fish are widely used for nutritional supplements, animal feed and fertilizer (Gehring *et al*., 2011). Lipids, carbohydrates and protein are essential elements of energy production for most of physiological processes and activities. There is considerable variation in fish's ability to use the nutrient groups as energy sources (Gatlin *et al*., 2002). Among other nutrients, it is well known that the protein content in many different types of fish is high (Aberoumand *et al*., 2011).

All proteins in fish species are chains of chemical units, called amino acids, linked together to make one long molecule. Fish protein can provides a good combination of amino acids highly suited to the nutritional requirements of humans and compares favorably with that provided by meat, milk and eggs. Among 20 types of essential amino acid, lysine and methionine are present in especially high concentrations in fish protein.

There are three main groups of fish-muscle tissue protein (Sotelo *et al*., 2000): structural proteins, sarcoplasmic proteins and connective-tissue proteins. Structural proteins constitute approximately 70–80% of the total amount of protein and comprise actin, myosin, tropormyosin and actomyosin. Myoalbumin, globulin and enzymes belong to the sarcoplsmic proteins and constitute around 25–30% of the total protein. These proteins are enzymes that participate in cell metabolism. Finally, connective-tissue proteins constitute only 3% of the protein in teleostei and about 10% in elasmobranchii. (http://www.fao.org/docrep/v7180e/v7180e05.htm#4.3).

32.2 FISH MUSCLES

The main component of most seafood products is muscle (Sotelo *et al*., 2000). As with all vertebrates, fish muscle can be divided into three basic types: skeletal, smooth and cardiac.

Skeletal muscles, which are a major component of most fish's muscle mass, are voluntary, and fish use them to move their bones and fins. Smooth muscles are involuntary and are responsible for operation of the internal organs, such as stomach and intestines,

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and of the circulatory system. Cardiac muscles, often called heart muscles, also work automatically and operate the heart.

32.2.1 Skeletal Muscle

The body of most species of fish consists almost entirely of skeletal muscles. There are different types of fish muscle: red, pink and white. Most fish have a mixture of two or all three types. Although each muscle maintains its individual characteristics, the red and white muscle types of salmonid fishes are mixed to form a mosaic type. The color of these muscles relates to the amount of hemoglobin present, with red muscle having the largest number of capillaries and a high hemoglobin content, enabling a rich oxygen supply. Red muscle is used for steady, constant-effort swimming and is present in active fish, particularly those which live in the oceans, such as tuna.

White muscle, or fast muscle, has thicker fibers than red muscle and possesses litter vascularization, and so it has a much reduced blood flow, and therefore a reduced oxygen-transport ability. Most white-muscle activity is anaerobic (glycogen is converted to lactate). White-muscle fibers can produce high-intensity actions that are up to 2.7 times faster than those of red muscle, but they are more energetically inefficient. White muscle can only work for a brief time and then becomes exhausted. Until that point, it requires a supply of glycogen. All this means that white muscles are used during burst swimming.

Pink muscle is sort of between red and white and is good for continued swimming efforts lasting a few tens of minutes at a relatively high speed, but not to the extent of red muscle.

32.3 MYOGLOBIN AND MYOFIBRILLAR PROTEINS OF FISH MUSCLE

The skeletal muscles of fish are arranged one behind the other in broad vertical bands called myomeres. Myomere is one of the key ingredients of the fish axial muscle. Various types of body and swimming styles within fish species are dependent upon how myomeres are metamerically arranged (James *et al*., 1998). Fish muscle fiber constantly contracts and expands to produce work during swimming. White and red fibers are the main types of muscle fiber making up a fish musculature. Most of the musculature normally consists of anaerobic white fibers, while red fibers are found in a thin strip lying right below the lateral line (Bone *et al*., 1966).

Myoglobin, a monomeric globular heme protein found mainly in muscle tissue, is responsible for binding with iron and oxygen (Faustman and Phillips., 2001). Its concentration in muscle tissue is related to a variety of factors, such as fiber type, muscular activity, and the age of the animal (Haard *et al*., 1992).

For surimi processing, residual myoglobin and hemoglobin play an essential role in the whiteness of post mortem fish muscle, which is one of the fundamental elements for determining the quality of surimi gels (Chen *et al*., 2002). Hemoglobin is usually easily dissipated by harvest, handling and freezer storage, while myoglobin is maintained by the muscle's intracellular structure (Haard *et al*., 1994). Although these two types of heme protein become less soluble as the fish muscle ages or deteriorates, both can generally be removed by washing the fish, leading to increased whiteness of the resulting surimi

(Chen *et al*., 2003). Myofibrillar protein is a basic ingredient of most mammal muscle, accounting for nearly 60% of the total protein content (Tornbrg *et al*., 2005). These proteins are an important part of meat processing, as they are responsible for quality of the meat product.

32.4 SARCOPLASMIC PROTEIN

The endoplasmic reticulum (ER) is a cell organelle in eukaryotic organisms which forms an interconnected network of tubules, vesicles, and cisternae (Porter *et al*., 1945). Rough ER synthesizes proteins, while smooth ER synthesizes lipids and steroids and regulates calcium concentration (Levine *et al*., 2006). The energy turnover of skeletal muscle is associated with the regulation of calcium concentration by the sarcoplasmic reticulum (SR).

Fish sarcoplasmic proteins account for 20–50% of muscle protein and comprise several enzymes associated with energy-producing metabolism in the muscle. The SR is a special type of smooth ER. It is a complex membranous network and plays an important role in calcium-ion supplies in smooth and striated muscle (Martonosi *et al*., 1983). Chemically, calcium ions have an effect on the muscle contraction and relaxation cycle. The increased concentration of calcium ion in sarcoplasm stimulates calcium-dependent ATPase and enhances mitochondrial oxidative metabolism. Finally, ATP synthesis is promoted (Franzini-Armstong *et al*., 1994).

32.5 ANTIFREEZE PROTEINS

In order to survive the frigid waters of the Arctic Ocean, which exist at temperatures below the freezing point of their body fluids, Antarctic fish have developed an antifreeze protein (AFP) that helps them avoid freezing (Kandaswamy *et al*., 2011; Moriyama *et al*., 1995). AFPs are known to be a class of polypeptides produced by certain fish, plants, fungi and bacteria to permit their survival in extremely cold environments through their ability to adsorb on to the surfaces of ice crystals (Davies *et al*., 2002). AFPs inhibit ice-crystal growth by binding to small ice crystals and decreasing the freezing temperature of the water (Fletcher *et al*., 2001). Fish differ from other organisms in that only they have the consensus sequence or structure for an ice-binding domain. In addition, the structures of some AFPs change with the temperature (Davies *et al*., 2002).

There may be several commercial applications of these AFPs. Compounds isolated from fish AFPs are more effective in preventing freezing than current widely used chemical antifreezes at the same concentrations. The effect of fish AFPs in inhibiting freezing suggests that they might be used to prevent freezing of food in several applications.

32.5.1 Types of AFP

AFPs in fish come in two different kinds, namely antifreeze glycoproteins (AFGPs) and AFP. AFP is classified as AFP I, II, III and IV, according to its structure (Duman *et al*., 1974).

AFGPs consists of a varying number of tripeptide repeats $(Ala\text{-}Ala\text{-}Thr)_n$, with a disaccharide attached as a glycoside to each hydroxyl oxygen of the Thr residues. AFGP

is the main component of protein in the blood serum of Antractic fish such as notothenioids and cod, and a constituent part of this protein allow the fish to survive in sub-polar ocean.

Type-I AFPs are made up of alanine-rich, amphipathic helices. Type-II cysteine-rich AFPs are globular proteins with mixed secondary structures. Type-III AFPs are composed of short β-strands and one helix turn, which gives them a unique flat-faced globular fold. Type-IV AFPs are alanine-rich helix-bundle proteins (Davies *et al*., 1997).

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33 Marine-derived Collagen: Biological Activity and Application

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33.1 INTRODUCTION

33.1.1 Molecular Structure of Collagen

Collagen is a simple protein built only from amino acids. It belongs to the fibrous proteins, or more precisely to fibrin proteins. It is built from small protein parts in the shapes of spiral chains, which consist of 19 amino acids. The collagen molecule consists of three polypeptide chains twined around one another as in a three-stranded rope. Each chain has an individual twist, which are layered in opposite directions, forming a helix (Piez, 1984). Five of these twisted chains crosslink and form a collagen microfibril. A graphical illustration of the development of a collagen fiber is given in Fig. 33.1. The basic collagen molecule is rod-shaped, with a length and width of about 3000 and 15 Å, respectively, and an approximate molecular weight of 300 kDa (Nimni & Harkness, 1988; Traub & Piez, 1971).

33.1.2 Different Types of Collagen and Their Distribution

Collagen is the most common structural protein, accounting for approximately 30% of body protein in all vertebrates. Furthermore, 90% of the extracellular protein in the tendon and bone and more than 50% in the skin consists of collagen (Piez, 1985). Although most of the scaffolding in mammals is composed of collagen, the collagenous spectrum ranges from the Achilles tendons to the cornea, including ligaments, blood vessels, and teeth. Hence, different collagen types are necessary to confer distinct biological features to different tissues. Collagen comprises a family of genetically distinct molecules with unique triple-helix configurations of three polypeptide subunits known as α-chains (Kucharz, 1992; Miller, 1998). Currently, at least 29 types of collagen have been isolated (Albu, 2011), which vary in the lengths of their helices and the nature and size of their nonhelical portions. The 13 most prominent types are categorized in Table 33.1.

Type-I collagen is predominant in higher-order animals, especially in the skin, tendon, and bone. It is a compound of three chains, two of which are identical (termed α 1(I)) and one of which has with a different amino acid composition (α-2(I) chain). Rarely, it can present as a trimer built of three α-1(I) chains. Type-II collagen is essentially unique

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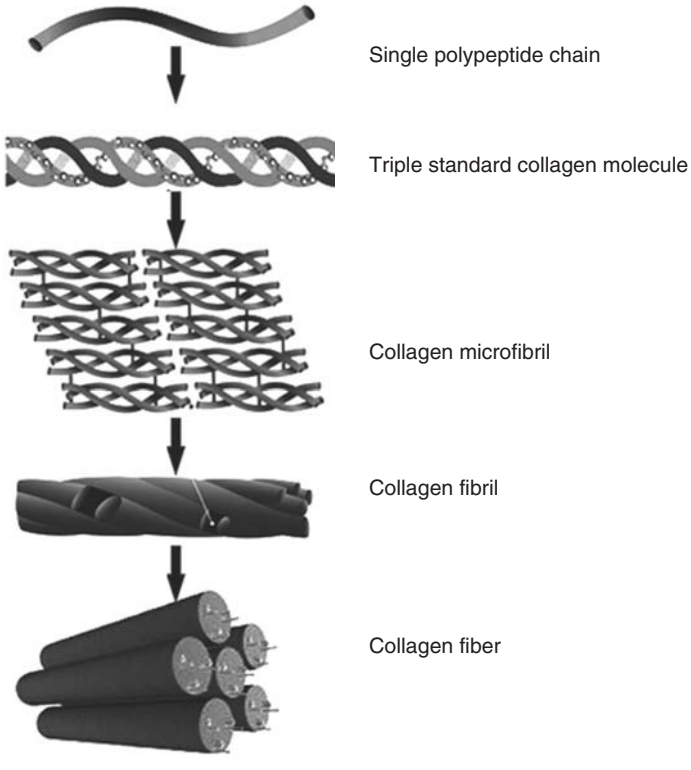

Fig. 33.1 Molecular structure of collagen.

Table 33.1 Body distribution of collagen types. Based on Friess, W. (1998) Collagen—biomaterial for drug delivery. European Journal of Pharmaceutics and Biopharmaceutics 45:113–136 (modified from Kucharz (1992)).

Collagen type	Tissue distribution
I	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dentin, dermis, tendon
II	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	Large vessels, uterine wall, dermis, intestine, heart valve, gingival (usually coexists with type I, except in bone, tendon, cornea)
IV	Basement membranes
V	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingival
VI	Descemet's membrane, skin, nucleus pulpous, heart muscle
VII	Produced by endothelial cells, Descemet's membrane
VIII	Skin, placenta, lung, cartilage, cornea
IX	Cartilage
X	Hypertrophic and mineralizing cartilage
XI	Cartilage, intervertebral disc, vitreous humour
XII	Chicken embryo tendon, bovine periodontal ligament
XIII	Cetal skin, bone, intestinal mucosa

to hyaline cartilage. The α-1(II) subunit is believed to be similar to α 1(I). Type III is found in limited quantities (approximately 10%) in association with type I. Thus, type-III collagen can be a minor contaminant of type-I collagen prepared from skin (Piez, 1985). In addition, blood vessels predominantly contain type III. Collagen types I, II, and III have large sections of homologous sequences, independent of the species. Type IV is a highly specialized form found only as a loose fibrillar network in the basement membrane. The other interstitial collagen types occur in small quantities and are associated with specific biological structures (Kucharz, 1992; Miller, 1984, 1998).

33.1.3 Degradation of Collagen

Collagen is particularly resistant to attacks by neutral proteases, probably due to its function as the primary structural protein in the body (Stricklinand Hibbs, 1988). Fibrils, as aggregates of collagen molecules, are degraded from the outside. Collagenase binds tightly to triple helices at or near the surface, while molecules in the interior become accessible to enzymes in the course of the progressive exterior degradation (Welgus *et al*., 1980). The various types of collagen show different susceptibilities to collagenolytic degradation. After the triple helix is cracked, further degradation of the collagen molecule is facilitated by enzymes such as gelatinases and nonspecific proteinases, which cleave the primary fragments into small peptides and amino acids (Woolley, 1984) (Fig. 33.2).

33.1.4 Functions of Collagen

The structures, properties, and functions of the collagens have been reviewed in detail by Van der Rest & Garrone (1991) and Bateman *et al*. (1996). Collagen fibrils have important structural functions in the mechanical properties of tissues such as tendon, skin and bone. They are also involved in numerous biological functions, from the early stages of development to tissue repair. In addition, collagen exhibits biodegradability, weak antigenicity (Maeda *et al*., 1999), and superior biocompatibility compared to other

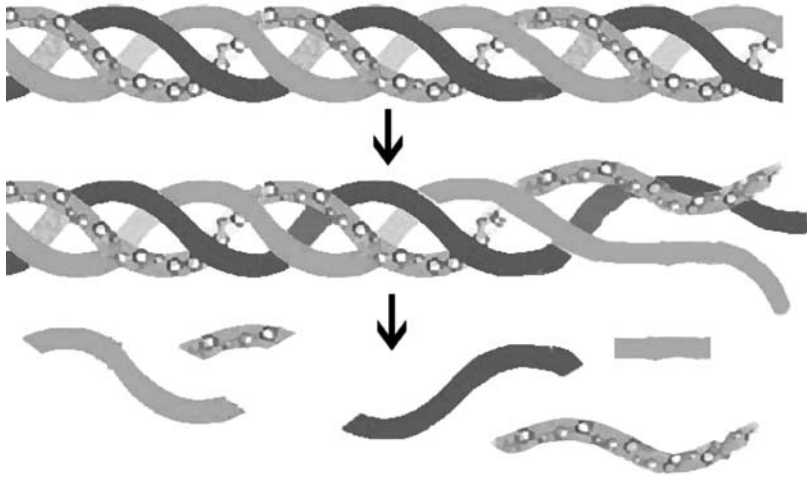

Fig. 33.2 Degradation of collagen.

natural polymers, such as albumin and gelatin. Since collagen is an important biomaterial, it is used in diverse fields, including the food, cosmetics, and biomedical industries.

33.2 SOURCES OF MARINE COLLAGEN

For industrial purposes, collagen is extracted mainly from the skin and bone of cattle and pigs. Highly infectious and contagious diseases such as mad cow disease (bovine spongiform encephalopathy, BSE), transmissible spongiform encephalopathy (TSE), and foot and mouth disease (FMD) have limited the use of collagen derived from these sources as there is a possibility of transmission to humans (Guille *et al*., 2000). The collagen extracted from pigs also cannot be used in the food industry due to religious barriers. Therefore, many scientists have focused their efforts of finding alternative novel sources of collagen, including poultry byproducts, marine organisms, and marine byproducts. They have found that the skin, bone, fin and scales of both freshwater and marine fish, chicken skin, squid skin, jellyfish, octopus arms, marine sponge, and bull frog skin can all be used as alternative sources (Bilgen *et al*., 1999; Kimura *et al*., 1983; Li *et al*., 2004; Morales *et al*., 2000; Muyonga *et al*., 2004; Nagai, 2004; Nagai and Suzuki, 2000, 2002; Nagai *et al*., 1999, 2000, 2001, 2002, 2004a; Sadowska, 2003; Swatschek *et al*., 2002).

33.2.1 Fish

Among collagen alternatives, fish provides the best source of raw material because of its high availability, zero risk of disease transmission, lack of religious barriers, and possibility of a higher collagen yield. Various fish species and their collagen sources are listed in Table 33.2. Byproducts from fish processing and by-catch of unutilized and underutilized fish species are also a promising source of collagen. Processing discards from fisheries, including skin, bones, scales, and fins, account for as much as 85% of the total weight of catch (Shahidi, 1994). Collagen is the main component in the skin (Sikorski & Borderias, 1994), which can be collected separately from other byproducts.

About 75% of the total weight of brown-backed toadfish (*Lagocephalus gloveri*) is discarded in the form of skin, bones, fins, heads, guts, and scales during processing (Shahidi, 1994). Shahidi (1994) discussed the possibility of reutilizing fish-processing waste in the production of collagen-like biomaterials, which has the potential to increase the economic value of the fish. Like recombinant and vertebrate collagens, fish collagens form the typical fibrillar structures, but they differ at the primary amino acid sequence level. The total amino acid content of fish collagen and gelatin is significantly lower than in other species; the amount of hydroxyproline is only 62% of that found in calf-skin collagen (Piez & Gross, 1960). As a result, fish collagen has a significantly lower melting point, which could affect performance at body temperature. Another potential drawback to the use of fish collagen and gelatin in pharmaceutical preparations is the occurrence of allergic reactions (Piez & Gross, 1960).

33.2.2 Crustaceans

The musculature of several crustaceans has been shown to contain collagenous proteins (Yoshinaka *et al*., 1989). Furthermore, Yoshinaka *et al*. (1990) have reported the

Table 33.2 Sources of marine fish-derived collagen.

Fish	Source	Reference
Ayu (*Plecoglossus altivelis*)	Bones	Nagai & Suzuki (2000)
Bigeye snapper (*Priacanthus tayenus*)	Skin	Kittiphattanabawon *et al.* (2005)
Black drum (*Pogonia cromis*)	Skin	Ogawa *et al.* (2004)
Brown-backed toadfish (*Lagocephalus gloveri)*	Skin	
Bullhead shark (*Heterodontus japonicas*)	Bones and fins	Nagai & Suzuki (2000)
Catla (*Catla catla*)	Scales	Pati *et al.* (2010b)
Chub mackerel (*Scomber japonicas)*	Skin, bones, and fins	Nagai & Suzuki (2000)
Chum salmon (*Oncorhynchus keta*)	Skin	Pei *et al.* (2010)
Cuttle fish (*Sepia lycidas*)	Outer skin	Nagai *et al.* (2004)
Horse mackerel (*Trachurus japonicas)*	Skin, bones, and fins	Nagai & Suzuki (2000)
Japanese sea bass (*Lateolabrax japonicas*)	Fins	Nagai & Suzuki (2000)
Nile perch (*Lates niloticus*)	Skin	Muyonga *et al.* (2004)
Pagrus major	Scales	Ikoma *et al.* (2003)
Oreochromis niloticas	Scales	Ikoma *et al.* (2003)
Puffer fish (*Takifugu rubripes*)	Skin	Nagia *et al.* (2002)
Rohu (*Labeorohita*)	Scales	Pati *et al.* (2010b)
Sheepshead sea bream (*Archosargus probatocephalus*)		Ogawa *et al.* (2004)
Skipjack tuna (*Katsuwonus pelamis*)	Skin, bones, and fins	Nagai & Suzuki (2000)
Yellow sea bream (*Dentextumifrons*)	Skin, bones, and fins	Nagai & Suzuki (2000)

purification and characterization of genetically distinct types of prawn-muscle collagens. Sivakumar *et al*. (1997) have also reported the simple purification and physicochemical characterization of a homotrimeric type-V collagen from marine prawn (*Penaeus indicus*). Similarly, Sivakumar *et al*. (2000) reported a comparative analysis of abdominal- and leg-muscle collagens of the marine crab (*Scylla serrata*). Studies on crustacean collagens by Kimura *et al*. (1969) were confined primarily to their content and biochemical characteristics.

Other collagen sources categorized under crustaceans include the muscle of kuruma prawn (*Penaeus japonicas*) (Mizuta *et al*., 1991) and the skin of diamondback squid (*Thysanoteuthis rhombus*) (Nagai, 2004a).

33.2.3 Mollusks

Nagai *et al*. (1999) studied the preparation and characterization of edible jellyfish-exumbrella collagen. Jellyfish are often considered gelatinous animals (mostly consisting of water and a developed collagen-rich mesogloea). However, little information is available on collagen in calcified tissues such as fin, scale and bone in mollusks. On the other hand, their increasingly frequent outbreaks generate ecological and economic consequences, from the formation of ocean jellyfish to beach closures; given these conditions, it is suitable to utilize them as a source of collagen. Various jellyfish species (*Aurelia aurita*, *Cotylorhiza tuberculata*, *Pelagia noctiluca*, and *Rhizostoma pulmo*) have been analyzed as a source of collagen. Dong *et al*. (2011) have also identified sea cucumber (*Stichopus japonicas*) flesh a as potential source.

33.2.4 Sponges

Sponges represent another alternative source of collagen and gelatin. Sponges contain both fibrillar and nonfibrillar collagens, as is the case in vertebrates (Exposito *et al*., 1991). Collagen from sponges is biochemically distinct from vertebrate collagens in that it is highly insoluble and therefore difficult to manipulate. Additionally, sponge collagen is highly glycosylated; most of the lysine residues are found in the consensus sequence for hydroxylation and are subsequently glycosylated. An efficient process for the extraction of collagen from the marine sponge *Chondrosia reniformis Nardo* has been reported.

Collagen yields of 30% (dry-weight collagen to dry-weight sponge) were obtained using a dilute basic extraction medium. The collagen was shown to have potential uses in cosmetic formulations, causing a slight increase in skin hydration. Tough bundles of collagen, called natural collagen fibers, were isolated from different marine sponges, including various *Ircinia* species, *Chondrosia reniformis*, and *Suberites domuncula*, which showed great applications in pharmaceutical technology, cosmetics, and nutrition, and had a high potency in tissue regeneration, especially after injuries (Pallela *et al*., 2011).

33.3 APPLICATIONS OF MARINE COLLAGEN

33.3.1 Cosmeceutical Applications

Proteins and peptides are an important category of cosmeceutical ingredient. Collagen is the major structural protein found in human skin. Its structural and physiological properties have been successfully used in moisturizing, nourishing, antiwrinkle and anti-aging agents. Cosmecuetical products with collagen can be applied to many skin surfaces, including the face and neck. Marine fish skin, bone, and fin byproducts have been used widely for the extraction of cosmeceutical collagen (Kim & Mendis, 2006). Marine collagen has been used as an oral supplement to support not only the skin but also nail and hair health. It contains the amino acid hydroxyproline, a critical building block for collagen synthesis.

33.3.1.1 Use of Marine Collagen in Anti-aging/Antiwrinkle Agents

By the age of 40, the natural synthesis of collagen in humans decreases. This decreased amount of collagen accounts for the emergence of fine lines on the skin, which gradually develop into wrinkles. Collagen supplements can help regain the skin's original glow and to revitalize it, especially collagen obtained from marine sources. Products with marine collagen are helpful in treating fine lines and wrinkles and rejuvenating the skin. Marine collagen helps in maintaining skin strength and elasticity by initiating synthesis of new collagen in the underlying layers (Bauza *et al*., 2004). As the fiber's density increases in the skin, the emergence of fine lines and wrinkles decreases (Glaser, 2004). Collagen leads to renewal of the cells and minimizes contraction of the facial muscles, which is one of the main causes of wrinkles. Pei *et al*. (2008) investigated the protective effect of marine-collagen peptides on the skin of aged mice induced by D-galactose. The results showed that marine-collagen peptides may play a protective role in skin aging by improving the activity of antioxidants.

It is certain that oxidative stress can lead to cell and tissue damage, resulting in age-related cognitive decline. Bioactive peptides derived from fish skin have antioxidant function in D-galactose-induced Sprague–Dawley rats. Hence, it is reasonable to hypothesize that fish protein may be a valuable source of neuroprotective peptides. Pei *et al*. (2010)

investigated the effects of marine-collagen peptide, compounds of low-molecular-weight peptides derived from chum salmon (*Oncorhynchus keta* sp.) skin by enzymatic hydrolysis, on the cognition of aged C57BL/6J mice. Certain cellular and molecular mechanisms of marine-collagen peptides regulated the plasticity of the brain and its ability repair itself in response to the effects of aging.

The collagen hydrolysates derived from squid skin greatly inhibited polyphenol oxidase activity *in vitro* and decreased the production of lipofuscin (a brown pigment characteristic of aging) in fruit fly (Liu *et al*., 2010). The anti-aging effect of collagen hydrolysates might be related to their antioxidant activities. These results suggest that the collagen hydrolysate from squid skin exhibits anti-aging activity in fruit fly and may be used as a neutraceutical ingredient in functional foods, cosmetics, and other industries for health-care purposes.

33.3.1.2 Use of Marine Collagen to Prevent Environmental Damage to Skin

Unlike other organs, the skin is in direct contact with the environment and therefore undergoes aging as a consequence of environmental damage (Fisher *et al*., 2002). Sun-exposed or photo-aged skin is typically coarse and rough, with deep lines and wrinkles and irregular pigmentation (Jenkins, 2002). In addition, free-radical damage causes wrinkles by activating the metalloproteinase that breaks down collagen. Several factors start this cascading process, including exposure to UV radiation in sunlight, smoking, and exposure to air pollution. A number of factors, including adverse environmental conditions, can cause skin dryness. Therefore, accepted practice is that dry skin can be improved by hydrating the outermost layer with a humectant or occlusive agent, smoothing the rough surface with an emollient, and normalizing the stratum corneum by miniaturization (Swatschek *et al*., 2002). Numerous cosmeceuticals have been tested in the treatment of sensitive skin, skin affected by rosacea, and the redness associated with inflammation. Consumer-driven demand has led to the development of products that counteract the signs of aging skin, decrease erythema, and even out tone and pigmentation. These cosmeceuticals can help protect the skin from photo damage and in some ways repair it, through stimulation of new collagen production. Using them in conjunction with sunscreens and prescription retinoids may enhance their results, especially when used as an adjunct to rejuvenating procedures. They can also help to increase the tolerability of retinoids by improving the epidermal barrier.

33.3.1.3 Use of Marine Collagen in Moisturizing Products

Collagen is the main marine-derived protein used in the cosmeceutical industry to increase the moisturizing property of a product. Moisturizers are employed to treat dry skin. Lipids work on the principle of occlusion, whereas humectants such as collagen attract water in the stratum corneum. In contrast to low-molecular-weight humectants, such as urea and glycerine, higher-molecular-weight humectants such as collagen are not absorbed by the stratum corneum but rather stay on the skin surface. Moreover, they bind water by hydration and increase the degree of skin humidity (Elsner *et al*., 1994). Swatschek *et al*. (2002) found that collagen possesses moisturizing effects which hydrate the skin by binding to water, and it is commonly used in the formulation of cosmetic products for skin care.

Collagen thus represents an excellent film-building polymer in cosmetics. According to the study carried by Garrone *et al*. (1975), marine sponge (*Chondrosia reniformis*) can be used as an alternative collagen source. Following physicochemical characterization of the isolated material, a possible application as a moisturizer in cosmetic preparations was

investigated using noninvasive *in vivo* measurement techniques. Studies on the cortex of the sponge *Chondrosia reniformis* showed that the hydroxyproline content approximately corresponded to a 40% collagen content. Due to its physicochemical properties, especially its striking insolubility, any attempt to incorporate isolated sponge collagen homogeneously into cosmetics failed. On the other hand, the centrifuged sponge extract was easy to incorporate in any tested formulation.

Recently, peptide collagen isolated from fish has shown inhibitory activity against DNA polymerase. It therefore has potential for use in anti-inflammatory skin-care cosmeceuticals. Gelatin is a heterogeneous mixture of high-molecular-weight water-soluble proteins and is a partially hydrolyzed form of collagen. Enzymatically hydrolyzed fish-skin gelatin has shown increased antioxidant activity (Kim *et al*., 2001; Mendis *et al*., 2005).

33.3.2 Biomedical Applications

Collagen-based biomaterials have been widely used in medical applications. The primary reasons for the usefulness of collagens are excellent biocompatibility, weak antigenicity, high biodegradability, and good hemostatic and cell-binding properties compared with synthetic polymers (Lee *et al*., 2001; Nimni, 1988). Collagen can form fibers with extra strength and stability through its self-aggregation and crosslinking (Lee *et al*., 2001). It is commonly used in the medical and pharmaceutical industries as a carrier molecule for drugs (drug-delivery system), proteins, and genes (Lee *et al*., 2001). In particular, microfibrous collagen sheets are used as promising drug carriers for the treatment of cancer (Satoet *et al*., 1996). They also find use as surgical sutures (Miller *et al*., 1964) and hemostatic agents (Browder & Litwin, 1986), and in tissue-engineering as basic matrices for cell-culture systems (Kemp, 2000) and replacement/substitutes for artificial blood vessels and valves (Chvapil *et al*., 1993).

Collagen was extracted by pepsin digestion from the swim bladder of catfish (*Tachysurus maculatus*) processing wastes. According to the electrophoretic pattern, the swim bladder of the fish consisted of comparable amounts of two $\acute{\alpha}$-chain-sized components, designated $\acute{\alpha}$ 1, $\acute{\alpha}$ 2, and a β component, and its collagen was crosslinked with chitosan. The collagen–chitosan sheet was characterized and showed potential for use in the medical field (Bama *et al*., 2010).

33.3.2.1 Use of Marine Collagen as a Drug-delivery System

Collagen's use as a drug-delivery system is very comprehensive and diverse. The attractiveness of collagen as a biomaterial rests largely on the view that it is a natural material of low immunogenicity and is therefore seen by the body as a normal constituent rather than as foreign matter (Peiz, 1984). Collagen can be processed into a number of forms, such as sheets, tubes, sponges, powders, fleeces, injectable solutions, and dispersions, all of which have found use in medical practice (Chvapi *et al*., 1973; Gorham, 1991). It can be extracted into an aqueous solution and molded into various forms of delivery system, such as collagen shields in ophthalmology (Kaufman *et al*., 1994), sponges for burns/wounds (Rao, 1995), microspheres, mini-pellets, and tablets for protein delivery (Lucas *et al*., 1989), gel formulations in combination with liposomes for sustained drug delivery (Fonseca *et al*., 1996), controlling materials for transdermal delivery (Thacharodi & Rao, 1996), nanoparticles for gene delivery (Rossler *et al*., 1995), and basic matrices for cell-culture systems. Furthermore, it has been used in films for the delivery of human

growth hormone, immunostimulants, tetracycline, and growth factors (Minabe *et al*., 1989) ,and as aqueous injection for local cancer treatment (Davidson *et al*., 1995).

Medical devices based on marine-derived collagen shields have numerous applications in ophthalmology as grafts for corneal replacement, suture materials, bandage lenses, punctual plugs, and viscous solutions for use as vitreous replacements or protectants during surgery (Devore, 1995). One of the most widely studied drug-carrier applications of marine collagen is as inserts and shields for intraocular drug delivery to the corneal surface or the cornea itself.

Collagen sponges have been very useful in the treatment of severe burns and as dressings for many types of wound, including pressure sores, donor sites, leg ulcers, and decubitus ulcers, as well as in *in vitro* test systems (Geesin *et al*., 1996).

33.3.2.2 Use of Marine Collagen in Bioengineering

Due to its excellent biocompatibility and safety, the use of marine-derived collagen in biomedical applications has been rapidly growing and has expanded to bioengineering areas, including polymer scaffolds (Angele *et al*., 2004; Ma *et al*., 2004; Riesle *et al*., 1998). Polymer scaffolds are central to tissue-engineering technology because they direct a variety of cellular processes based on their structural and biochemical properties (Chen *et al*., 2002; Yang *et al*., 2001). The strategy for scaffold use in bioengineering is illustrated in Fig. 33.3. The materials used for scaffold fabrication not only determine such physical properties as biocompatibility, biodegradability, and mechanical stability but also provide the appropriate signals for directing the cellular processes that lead to tissue formation (Yang *et al*., 2001). For this, collagen has become one of the most favored materials in artificial extracellular-matrix tissue-engineering applications, especially marine-derived collagen.

Marine-collagen skin substitutes or replacements are currently optimized for clinical applications. Collagen-populated hydrated gels can be used as a therapeutic option for the treatment of burn patients or chronic wounds (Auger *et al*., 1998). These skin substitutes are produced by culturing keratinocytes on a matured dermal equivalent composed of fibroblasts included in a collagen gel. New biotechnological approaches have been developed to prevent contraction and promote epithelial-cell differentiation (Auger *et al*., 1998).

Jellyfish collagen was found to have an amino acid composition typical of collagen from other sources, with glycine the most abundant amino acid. Song *et al*. (2006) revealed that jellyfish collagen did not induce a significant cytotoxic effect and had higher cell viability than other biomaterials, including bovine collagen, gelatin, hyaluronic acid, and glucan. Jellyfish-collagen scaffolds also had a highly porous and interconnected pore structure, which is useful for high-density cell seeding, an efficient nutrient, and an oxygen supply to cells cultured in the three-dimensional matrices. The findings of *in vivo* studies indicate that jellyfish-collagen scaffolds have potential as safe collagen materials for tissue-engineering applications.

Pati *et al*. (2010b) isolated collagen from the scales of rohu (*Labeo rohita*) and catla (*Catla catla*) and evaluated it using thermogravimetric analysis. Its most promising feature was is its close denaturation temperature to that of mammalian collagen, which significantly boosts its applicability. This isolated collagen may well find application in the biomedical and pharmaceutical fields for the construction of tissue-engineering scaffolds, wound-dressing systems, and drug-delivery devices.

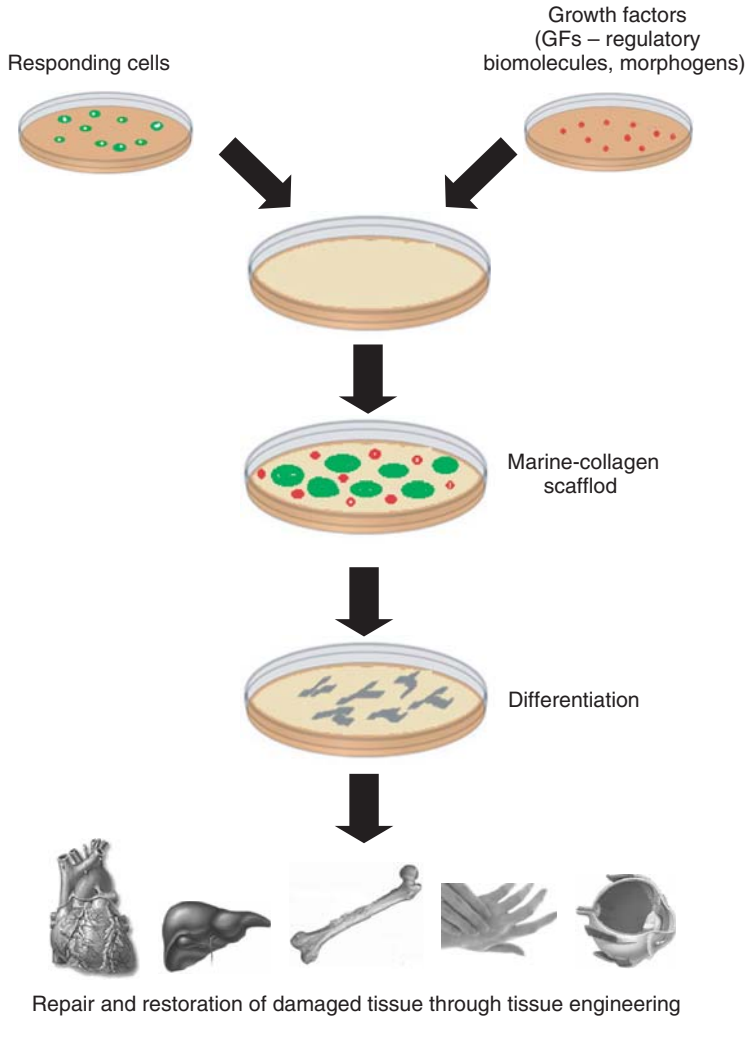

Fig. 33.3 Strategies for marine collagen use in tissue engineering.

Bone substitutes are one of the more common bioengineering uses of marine-derived collagen. Bone tissue engineering aims to mimic the natural process of bone formation by delivering a source of cells and/or growth factors in a scaffold matrix to induce cellular attachment, migration, proliferation, and osteoblastic differentiation. In bone tissue engineering, collagen scaffolds play an essential role in supporting bone regeneration. Green *et al*. (2003) reported the fiber skeleton of natural marine sponge and suggested its application for tissue-engineered bone. The skeletons of *Poriferans* appear to have unique properties that make them appealing as potential bioscaffolds for cell-based bone tissue engineering. These properties include the fibrous skeleton, the collagenous composition, the ability to hydrate to a high degree, and the possession of open, interconnected channels created by a porous structured network. Therefore, Green *et al*. (2003) demonstrated that *Poriferans* are able to induce osteoblast attachment, proliferation, migration, and differentiation *in vitro*.

Another bioengineering application of collagen is in artificial blood vessels and valves. Induced immune response using jellyfish-derived collagen was comparable to that caused by bovine collagen. The feasibility of using jellyfish collagen for tissue-engineered vascular grafts in a pulsatile perfusion bioreactor was studied by Jong *et al*. (2007). Collagen generally has a relatively low mechanical strength for application in blood vessels. In order to improve the mechanical properties of porous collagen scaffolds, Jong *et al*. (2007) fabricated tissue-engineered scaffolds for blood vessels composed of porous jellyfish-collagen tube and reinforced by biodegradable polymers such as polylactide-co-glycolide(PLGA) fibers. It was shown that the jellyfish-collagen/PLGA scaffolds had good mechanical properties, were durable under the mechano-active system, and possessed tissue compatibility with smooth-muscle and endothelial cells for vascular tissue-engineering applications.

Similarly, collagen derived from chum salmon (*Oncorhynchus keta*) was crosslinked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) during collagen fibrillogenesis and applied to an *in vitro* cell culture in order to evaluate its potential use as a scaffold for vascular tissue engineering. Human umbilical vein endothelial cells (HUVECs) were cultured on the crosslinked salmon-collagen fibrillar gel (EDC-SC gel), and their growth rates and cytokine production levels were studied by Nagani *et al*. (2008).

Collagen is also in demand as a skin replacement. Collagen-based implants have been widely used as vehicles for the transportation of cultured skin cells or as drug carriers for skin replacement and burn wounds (Harriger *et al*., 1997; Boyce, 1998). Reconstituted type-I collagen is suitable for use as a skin replacement and in treatment of burn wounds because of its mechanical strength and biocompatibility (Rao, 1995).

Trash-fish collagen can be exploited effectively as it has good thermal properties for pharmaceutical and biomedical applications. These applications include treatment of pain associated with osteoarthritis, hypertension, use in tissue engineering, implants in humans, inhibition of angiogenic diseases, and so on (Rehn *et al*., 2001). Trash fish is also used as a dermal filler and hemostat, and for drug delivery, skin substitutes, expandable intra-arterial stents, and cell-attachment substrates (Senaratne *et al*., 2006). Moskowitz (2000) suggested that ingestion of collagen/gelatin hydrolysates reduces pain in patients suffering from osteoarthritis, and hydrolyzed collagen has been involved in cartilage-matrix synthesis.

Hypertension is one of the most common lifestyle-related diseases and has become one of the most significant world health problems in recent years. Angiotensin-I-converting enzyme (ACE) plays an important physiological role in regulating blood pressure. It raises blood pressure by converting the inactive decapeptide angiotensin-I to the potent vasoconstrictor octapeptide angiotensin-II, as well as inactivating the vasodilating nonapeptide (Richard *et al*., 2004). Therefore, it is feasible to suppress blood-pressure elevation by inhibiting the catalytic action of ACE. In addition to plant and animal sources, some ACE-inhibitory peptides have been obtained from collagen from marine animals, such as cod (Kim *et al*., 2000), sea cucumber (Zhao *et al*., 2007), shrimp (Zhang *et al*., 2009), and salmon (Ono *et al*., 2006).

33.3.3 Food and Nutraceutical Applications

Naturally occurring bioactive extracts or single compounds of collagen and gelatin, which are believed to be of benefit to human health, have spawned an important and dynamic new area of research, resulting in substantial advances in nutritional knowledge. There is also growing awareness that diet affects overall health. The marine environment represents a

relatively untapped source of functional ingredients with application to various aspects of food processing, storage, and fortification. Marine-derived compounds, including collagen and gelatin, have potential use as functional food ingredients for health maintenance and the prevention of chronic diseases.

Matsumoto *et al*. (2010) investigated the use of collagen peptide in the food and beverage industry. Collagen peptide is composed of oligopeptides, which have the ability to enter the blood, making it suitable for daily intake. It can be consumed directly as a food or beverage product or indirectly as a raw material or intermediate product in food or beverage production. Specific examples include beverages such as soft drinks, carbonated drinks, nutritional beverages, fruit beverages, and milk beverages; frozen desserts such as ice cream, ice sherbet, and shaved ice; noodles; confectioneries such as chewing gum, caramel, chocolate, tablet sweets, snacks, baked goods (e.g. biscuit), jelly, jam, and cream; fish/livestock processed foods such as minced and steamed fish, hamburger, ham, and sausage; dairy products such as processed milk, fermented milk, yoghurt, butter, and cheese; fats and oils and fat- and oil-processed foods such as salad oil, margarine, mayonnaise, shortening, whipped cream, and dressing; seasonings such as sauce and baste; and soup, stew, curry, bread, jam, salad, and Japanese pickles (Matsumoto *et al*., 2010).

33.3.3.1 Use of Marine Collagen to Improve the Textural Properties of Meat Products

Collagen is a quantitatively major constituent of the muscle connective tissue of multicellular animals. It has been reported to have an important food function in developing meat texture. A close relationship has been reported between the texture and the collagen content of muscles of many aquatic species (Mizuta *et al*., 1994). Moreover, enzymatic degradation of collagen has been revealed to be responsible for the post-mortem tenderization of fish meat during chilled storage (Sato *et al*., 1997; Shigemura *et al*., 2003). Sato *et al*. (1986) found collagens in the muscle of marine animals play a key role in maintaining meat texture. Sivakumar *et al*. (2000) also reported the same application after a comparative analysis of abdominal- and leg-muscle collagens of the marine crab (*Scylla serrata*).

The most prevalent marine proteins used in foods are collagen, gelatin, and albumin, all of which can be extracted from fish and seafood byproducts (Rasmussen & Morrissey, 2007). Collagen and gelatin are unique proteins in that they are rich in nonpolar amino acids (>80%) such as glycine, alanine, valine, and proline. Collagen derived from species living in warmer environments (e.g. tuna) has higher contents of proline and hydroxyproline, giving it a higher melting point and superior thermal stability compared to that from fish in cooler environments (e.g. cod) (Ferraro *et al*., 2010).

33.3.3.2 Use of Marine Collagen and Gelatin to Improve the Sensory Properties of Meat Products

Collagen and gelatin derived from marine source are used to improve sensory properties such as the texture, juiciness, mouth feel, elasticity, consistency, and stability of different types of food. Collagen and gelatin are different forms of the same macromolecule: gelatin is the partially hydrolyzed form of collagen. Fish-derived collagen and gelatin hydrolysates are used widely in foods, in the manufacture of glues, and in several other industrial applications (Soper, 1997).

Heat denaturation easily converts collagen into gelatin. It has a unique gel-forming ability (Rasmussen & Morrissey, 2007) and is used as a food additive to improve the texture, water-holding capacity, and stability of several food products (Rustad, 2003).

Traditionally, gelatin has been derived from beef or pork; however, marine gelatin can also be extracted from the skins of flatfish, cold-water fish species, and alternative sources such as squid and octopus (Choi & Regenstein, 2000). Gelatin possesses a characteristic melt-in-the-mouth property, which makes it suitable for a wide range of applications in the food and pharmaceutical industries; in particular, fish gelatin has a better release of aroma and shows a higher digestibility than animal gelatin (Guillen *et al*., 2002).

The volume of gelatin used annually by the food industry worldwide is both considerable and growing. Gelatin is used not only for its functional properties but also to increase protein content. It can enhance the elasticity, consistency, and stability of food products, and it forms an outer film to protect against drying, light, and oxygen. There is considerable interest in the use of fish skins for two reasons: to make use of the byproducts from fish processing and to provide an alternative to mammalian gelatin, whose consumption is rejected in some cultures. Several marine species have been examined as a source of raw material for edible gelatin, including tilapia (Grossman & Bergman, 1992), megrim (Montero & Guillen 2000), cod (Gudmundsson & Hafsteinsson 1997), lumpfish (Osborne *et al*., 1990), and conger eel and arrow squid (Kim & Cho, 1996).

33.3.3.3 Use of Marine Collagen in Packaging Materials and Muscle Products

Collagen has been utilized to produce edible casings for the meat-processing industries (sausages/salami/ snack sticks), films, and coatings. Edible films serve as gas and solute barriers, improving the quality and shelf life of muscle foods. They have been used as a delivery system for antioxidants in poultry and applied directly to poultry meat surfaces and processed meats to prevent microbial growth, salt rust, grease bleeding, handling abuse, water transfer, moisture loss, and oil adsorption during frying. Despite these successes, gelatin lacks strength and requires a drying step in order to form more durable films (Ruban, 2009). Modern consumers are demanding that food packaging materials be more natural, disposable, potentially biodegradable, and recyclable (Lopez-Rubio *et al*., 2004). Suitable examples include bio-based polymers, bioplastic, and biopolymer packaging products made from raw materials originating in agricultural or marine sources (Cha & Chinnan, 2004).

Currently, the meat industry uses collagen films in the processing of meat products such as sausages. When heated, intact collagen films can form an edible film that becomes an integral part of the meat product (Cutte & Miller, 2004). These commercially available collagen caseins have been purported to reduce shrink loss, increase the permeability of smoke to the meat product, increase juiciness, allow for easy removal of nets after cooking or smoking, and absorb fluid exudates (Ruban, 2009).

33.3.3.4 Use of Marine Collagen in Functional Foods

Fibrillar collagens form a metazoan-specific family, and are highly conserved from sponge to human. On the other hand, the increase in the jellyfish population has led us to consider this marine animal a natural product for food and medicine (Addad, 2011).

Various physiological and pharmacological effects are exerted through the oral intake of foods and beverages, such as the curing of joint diseases (e.g. osteoarthritis, chronic rheumatism), the alleviation of osteoporosis, the prevention of arteriosclerosis and hypertension, the accelerated curing of wound sites, the curing of dermatological diseases (e.g. eczema, skin roughness, atopic dermatitis, pigment deposition), the improvement of moisture-retaining properties of the skin, the improvement of skin aging (e.g. wrinkles,

pigmented spots, dullness, sag, keratinization), the prevention of hair aging (e.g. gray hair, hair loss, thinning hair), and the prevention of ulcers (Matsumoto *et al*., 2010).

Marine-collagen protein compounds of proline and hydroxyl proline-rich oligopeptides have been derived from chum salmon (*Oncorhynchus keta*) skin, which is usually a waste product (Kim *et al*., 2001). Pei *et al*. (2010) demonstrated that dietary administration of marine-collage protein could facilitate acquisition of spatial learning and increase the passive avoidance ability. Furthermore, they found that these results were probably due to its antioxidative activity and neuroprotective effect. In other words, the protection of marine-collage protein against oxidative stress to the brain may have application in ameliorating impairments to learning and memory.

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34 Marine Antifreeze Proteins: Types, Functions and Applications

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34.1 INTRODUCTION

The sea-water temperature throughout the year in polar regions and during the winter in the North Atlantic Ocean usually remains at or below $-1.9\,^{\circ}C$, which is lower than the freezing point ($-0.7\,^{\circ}C$) of the body fluid of a typical marine fish (DeVries, 1969; DeVries & Wohlschlag, 1969). Therefore, most temperate and tropical fish would freeze to death in Antarctic winter sea water, whereas most polar species would survive. This survival is attributed to the presence of antifreeze proteins (AFPs) in the serum of these polar species (DeVries *et al*., 1970, 1971).

AFPs were first discovered in Antarctic fish more than 4 decades ago (DeVries, 1969; DeVries & Wohlschlag, 1969). Since then, a number of fish species in ice-laden sea water, and even northern temperate oceans, have been reported to express and secrete AFPs in their blood plasma. The presence of these AFPs lowers the freezing point of the blood plasma, enabling the fish to survive at subzero temperatures. The solutes in the serum can colligatively depress the freezing point down to $-0.7\,^{\circ}C$, and the additional presence of AFPs can depress it noncolligatively another $-1\,^{\circ}C$, eventually negating freezing of the body fluids (DeVries & Wohlschlag, 1969). This phenomenon is possible because AFPs are able to bind to small ice crystals in the blood and inhibit their further growth. This inhibition lowers the freezing points of solutions and creates a gap between the melting and freezing points. This phenomenon is referred to as 'thermal hysteresis' (TH) and is a way of expressing the activity of AFPs quantitatively (Fig. 34.1).

The TH phenomenon can be explained by an adsorption-inhibition mechanism (Raymond & DeVries, 1972, 1977), in which AFP adsorbs (or binds) on to the surface of ice irreversibly, allowing the ice to grow outward in a curved (i.e. convex) manner between the bound AFPs (Fig. 34.2). The convex shape increases the vapour pressure of the ice to that of the surrounding solution, which halts further growth (Kristiansen & Zachariassen, 2005; Wilson, 1993; Wilson & Leader, 1995).

The binding of AFPs to the ice surface also accompanies a modification of the ice-crystal shapes (Fig. 34.3a), which display unique ice morphologies depending on what planes the AFPs bind to (Davies & Hew, 1990). TH activity and ice-crystal morphology can be readily examined using a nanolitre osmometer. For most fish AFPs, the observed TH activity is typically approximately $1\,^{\circ}C$.

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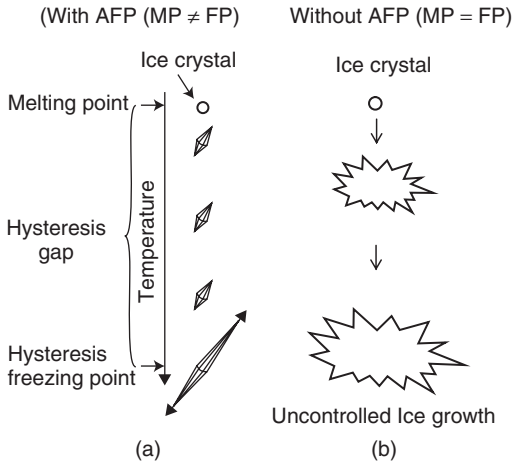

Fig. 34.1 (a) Illustration of the phenomenon of thermal hysteresis, as expressed in diluted samples of body fluid from polar fish. Reprinted from Kristiansen, E and Zachariassen, KE (2005) The mechanism by which fish antifreeze proteins cause thermal hysteresis. Cryobiology 51(3):262–280, Copyright 2005, with permission from Elsevier. (b) AFPs in the blood plasma inhibit the growth of ice to a certain degree. However, in most solutions without AFP the melting and freezing point are equal, such that ice starts growing as soon as the temperature drops below melting point.

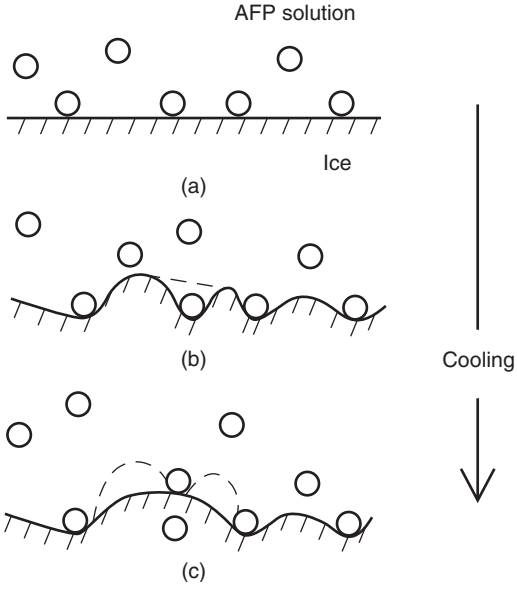

Fig. 34.2 Inhibition of ice-crystal growth following AFP adsorption. (A) AFP (open circles) in solution and in contact with the ice front (hatched line) at 0 °C. (b) The ice–water interface at undercooling approaching the non-equilibrium freezing point, where the curvature between bound AFPs leads to ice-growth inhibition through the Kelvin effect. The dotted line represents the overgrowth of a bound AFP. (c) Binding of an AFP from solution to the point of overgrowth in (b). Here the dotted line represents subsequent stabilization of the ice–water interface around the newly bound AFP. This figure is based on the two-dimensional representations of ice-growth inhibitions displayed in Knight & DeVries (1994) and Knight (2000). Republished with permission of Annual Reviews, Inc., from Fletcher, GL *et al*. Antifreeze proteins of teleost fishes. Annual Review of Physiology 2001;63:359–390; permission conveyed through Copyright Clearance Center, Inc.

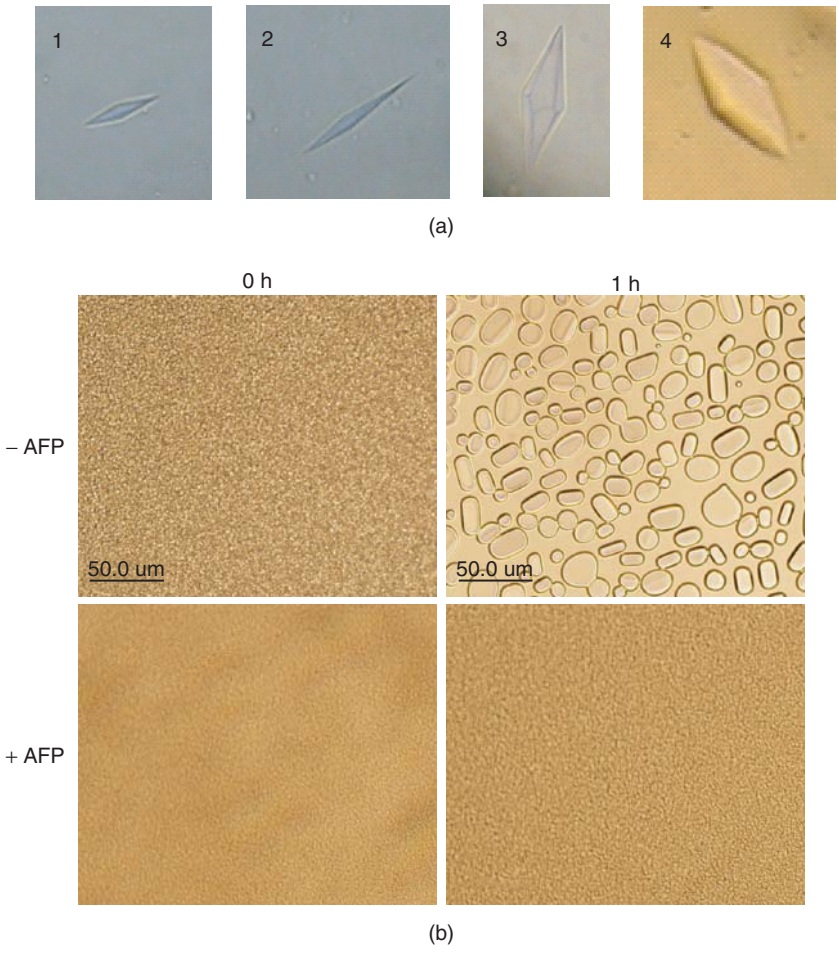

Fig. 34.3 (a) Shapes of ice crystal observed in the presence of AFGP (1), type-I AFP (3), and type-III AFP (4). A bipyramidal shape of crystal growth is observed in the presence of AFP due to the binding of AFPs to the specific planes of ice. A high concentration of AFGP leads to the speculate growth of ice (2). (b) Ice recrystallization inhibition (RI) assay of 30% sucrose solution in the absence (upper panel) and presence (lower panel) of type-I AFP (10 mg/ml). 2 µl of sample in 30% sucrose between two cover slides was placed in the cold stage. The sample was flash-frozen by lowering the temperature to −80 °C at a rate of 90 °C/minute, then warmed to −6 °C and monitored for 1 hour. Note that larger crystals were grown in the absence of AFP as a result of the recrystallization process in the upper panel, while no discernible changes took place through the action of AFP in the lower panel.

Another property of AFPs is ice recrystallisation inhibition (RI). Ice recrystallisation is a process in which the growth of larger ice grains occurs at the expense of the growth of smaller ones. Normally, very rapid freezing produces polycrystalline grains of a small size with a high internal energy due to imperfections and strains, which can promote recrystallisation (Knight *et al*., 1984, 1995). This high internal energy acts as a driving force for migration of the grain boundary, resulting in larger and fewer ice grains and reducing the free energy of the system (Fig. 34.3b). The larger ice crystals grown by this energetically favourable phenomenon can be detrimental or lethal to overwintering

organisms experiencing temperature fluctuations diurnally and in the spring thaw. The RI mechanism of AFPs is also ascribed to their affinity to ice. If sufficient AFPs are present at the supercooled interface of the ice-grain matrix, they bind to the ice grains and halt their growth, as previously described, eventually preventing recrystallisation.

Extensive studies have shown that AFPs from fish, insects and plants are very effective in ice RI. This seems to protect membranes from freezing injury and thus helps organisms survive in extremely cold environments (Knight & Duman, J.G. 1986; Knight *et al*., 1984; Sidebottom *et al*., 2000; Smallwood *et al*., 1999; Worrall *et al*., 1998). The intriguing activity of AFPs has drawn interest from academia and industry for its potential in cryopreservation, food preservation, transgenic technology, cryosurgery and other applications (Barrett, 2001; Ben, 2001; Bouvet & Ben, 2003; Davies *et al*., 1989; Fuller, 2004; Harding *et al*., 2003; Hew *et al*., 1992; Wohrmann, 1996).

In this chapter, we will delineate the types of AFP found in marine species and discuss their applications.

34.2 TYPES OF MARINE AFP

Marine AFPs include AFPs from fish and those from other marine species. Fish AFPs can be further classified according to their structural features. To date, five structurally distinct fish AFPs are known: a group of proteins termed 'antifreeze glycoproteins' (AFGPs) and AFP types I, II, III and IV (Davies & Hew, 1990). Based on current sequence information, nonfish AFPs seem to have sequential similarity to one another (Fig. 34.5). The characterisation of AFPs is summarised in Table 34.1, and the structures of representative AFPs are presented in Fig. 34.4.

34.2.1 AFGPS

AFGPs were isolated from the blood plasma of Antarctic notothenioids and later from Arctic fish (DeVries *et al*., 1970; Osuga & Feeney, 1978). Strikingly, these AFGPs were discovered from different areas and different fish species but show similar structural and functional characteristics.

AFGPs consist of a tripeptide repeat (Ala-Ala-Thr) with a disaccharide β-D-galactosyl-(1$\rightarrow$3)-α-N-acetyl-D-glactosamine moiety attached to the hydroxyl groups of the threonyl residues (Fig. 34.4a). Based on the number of repeating units, AFGPs can be classified as AFGPs 1–8 in order of decreasing molecular mass. For example, AFGP1 has 50 repeating units and a molecular weight of 33 700 Da, while AFGP8 has 4 repeating units, corresponding to 2600 Da. Occasionally, variations occur in the amino acid composition of smaller AFGPs (AFGP7 and 8), with the first Ala replaced by a Pro and/or Thr by Arg.

The TH activity of the AFGPs is proportional to the number of repeating units: the larger AFGPs 1–5 are much more active than the smaller AFGPs 7 and 8 (Ahlgren *et al*., 1988; Burcham *et al*., 1984; Feeney & Yeh, 1978; Kao *et al*., 1986; Knight *et al*., 1984). This discrepancy is due to the difference in the molecular masses, and not merely to the substitution of the proline in the repeating unit of the smaller AFGPs (Schrag *et al*., 1982). Even a very low concentration ($\sim 10^{-12}$ M) of AFGP has been shown to strongly prevent the recrystallisation of ice, implying that these proteins could be used in cryopreservation (Knight *et al*., 1984).

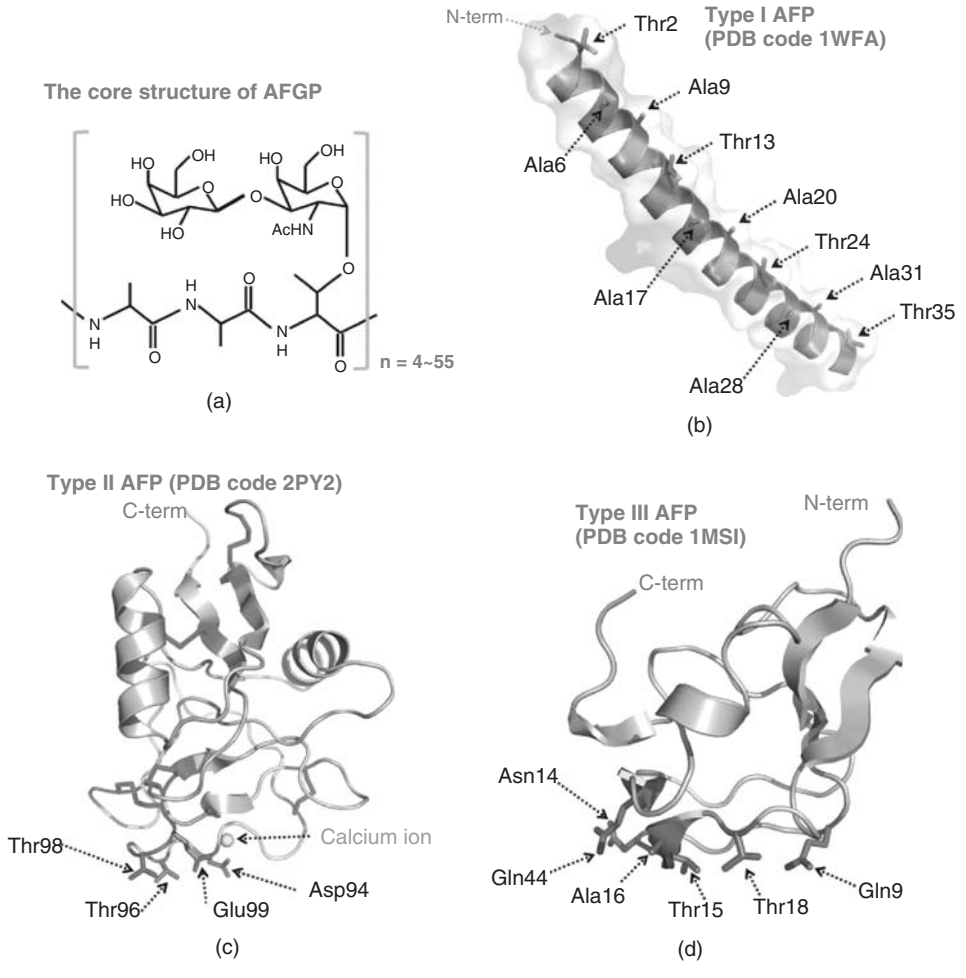

Fig. 34.4 Representative structures for the five types of fish AFP. AFPs from fish have been classified as AFGP, Type I, Type II, Type III, and Type IV according to their structural differences. (a) The core structure of AFGP is composed of three amino acids (alanine-alanine-threonine) glycosylated with the β-D-galactosyl-(1,3)-α-N-acetyl-D-galactosamine. Some small AFGPs have proline or arginine instead of alanine in the repeated tripeptide unit. (b) Overall structure of Type-I HPLC6 AFP. The proposed Thr-Ala ice-binding face is labeled. (c) Cartoon representation of the calcium-dependent Type-II AFP (PDB code 2PY2), showing the calcium-binding site and ice-binding site. The bound Ca^{2+} is shown as a sphere with four disulfide bonds. (d) Cartoon representation of Type-III AFP, showing the side chains of the ice-binding face. (e) Homology-model structure of Type-IV LS-12 AFP generated using the apolipoprotein A-I structure template (PDB code 2A01) and Phyre modeling server (Kelley & Sternberg, 2009). The amino acid sequence of Type-IV LS-12 AFP shows a 29% identity and 46% similarity to apolipoprotein A-I protein. (f) Structural model of an ice-binding protein produced by the sea-ice diatom, *Navicula glaciei* (NglIBP) (Janech *et al*., 2006), generated using the SWISS-MODEL homology-modeling Web server (http://swissmodel.expasy.org/). The crystal structure of an ice-binding protein from Arctic yeast (LelBP: Protein Data Bank entry code 3UYU) was used as a template structure because its amino acid sequence displays a 57% sequence similarity and 39% sequence identity with the NglIBP (Lee *et al*., 2012). These molecular-modeling data suggest that the β-helical fold provides a conserved ice-binding platform, forming a flat binding surface and common Thr-containing motif.

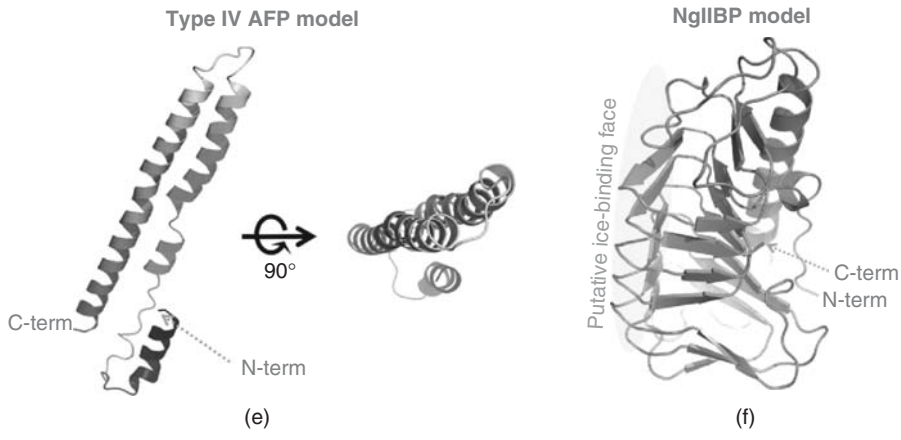

Fig. 34.4 *(continued)*

Studies on a series of synthetic AFGPs by Tachibana *et al*. (2004) revealed that the carbohydrate moiety is essential for AFGP activity, as previously proposed (Komatsu *et al*., 1970), and that the acetamide groups at the C2 position of the reducing hexosamines, the O-glycosidic linkages between the sugars and the peptide chain, and the g-methyl group of the threonyl residue are essential for function. In addition, a structural analysis of synthetic AFGPs with three repeating units using nuclear magnetic resonance (NMR) spectroscopy showed that these proteins folded into a left-handed helix similar to the previously predicted polyproline type II (PPII) helix (Bush & Feeney, 1986; Bush *et al*., 1981). Interestingly, in this synthetic AFGP structure the disaccharide moieties are on the same side of the molecule, thus forming a hydrophilic face, whereas the hydrophobic face is clustered into the one other face of the molecule by the methyl groups of the Ala residues and the acetylmethyl groups of the GalNAc residues. This structure is similar to that of the amphiphilic α-helix of the type-I AFP (Sicheri & Yang, 1995), emphasising the importance of amphiphilicity to antifreeze activity (Bush *et al*., 1981; Franks & Morris, 1978). However, unlike other AFPs, little is known about the mechanism of action of AFGPs in terms of how they inhibit ice growth.

34.2.2 Type-I AFP

The winter flounder type-I AFP is the most well-characterised AFP (Devries & Lin, 1977; Hew *et al*., 1978). Type-I AFP HPLC6 consists of 37 amino acids, in which an 11-amino-acid unit is tandemly repeated three times. The repeat sequence is Thr-X_2-Asx-X_7, where X is dominated by alanine (Davies *et al*., 1982; Devries & Lin, 1977; Pickett *et al*., 1984). The three-dimensional structure of the winter flounder HPLC6, the first structurally determined AFP (Yang *et al*., 1988), displays an amphipathic α-helix, confirming an earlier study (Ananthanarayanan & Hew, 1977). The long rod-shaped α-helix is stabilised by N- and C-terminal capping structures and by a salt bridge formed by Lys18 and Asp22 (Sicheri & Yang, 1995). In early studies, type-I AFP was proposed to bind to ice primarily through hydrogen bonds formed by regularly arrayed Thr and Asx residues. This model was supported by the distinct binding patterns of type-I AFP in ice-etching experiments. In this type of experiment, the etching pattern on a single ice-crystal

Table 34.1 Structural features of AFGP, Type I, Type II, Type III, Type IV AFPs, and NgIIBP.

	AFGP	Type I	Type II		Type III	Type IV	NgIIBP
			Calcium dependent	Calcium independent			
PDB code	NA[a]	1WFA	2PY2	2ZIB	1MSI	NA[a]	NA[a]
UniProtK B reference	P02732	P04002	Q91992	A0ZT93	P19614	P80961	Q108N3
Source	*Pagothenia borchgrevinki*	*Pseudopleuronectes americanus*	*Clupea harengus*	*Brachyopsis rostratus*	*Macrozoarces americanus*	*Myoxocephalus octodecim-spinosis*	*Navicula glaciei*
Number of amino acid residues	31	82	147	168	66	128	242
Disulfide bond	No disulfide bridge	No disulfide bridge	5 disulfide bridges	5 disulfide bridges	No disulfide bridge	No disulfide bridge	No cysteine residue
Signal sequence[b]	No	Yes (residues 1–23)	Yes (residues 1–18)	Yes (residues 1–18)	No	Yes (residues 1–20)	Yes (residues 1–20)
Secondary structure	Extended helix	1 α-helix	3 α-helices and 9 β-strands	2 α-helices and 5 β-strands	4 α-helices and 9 β-strands	4 α-helix	2 α-helices and 21 β-strands
Characteristics of three-dimensional structure	(Ala-Ala-Thr) repeat with O-linked Gal-GalNAc disaccharides	Alanine rich	Homology to the C-type lectin domain	Homology to the C-type lectin domain	Globular protein and rich in β-structure	Four helix bundle structure and homology to the N-terminal domain of a human apolipoprotein	Right handed β-helical structure
TH activity	1.2 °C at 40 mg/ml	0.91 °C at 7 mM	0.2 °C at 0.2 mM	0.6 °C at 0.2 mM	0.6 °C at 0.5 mM	0.5 °C at 2 mM	1.6 °C at 0.01 mM

[a]NA, not available.
[b]Signal peptide cleavage site was predicted using SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/).

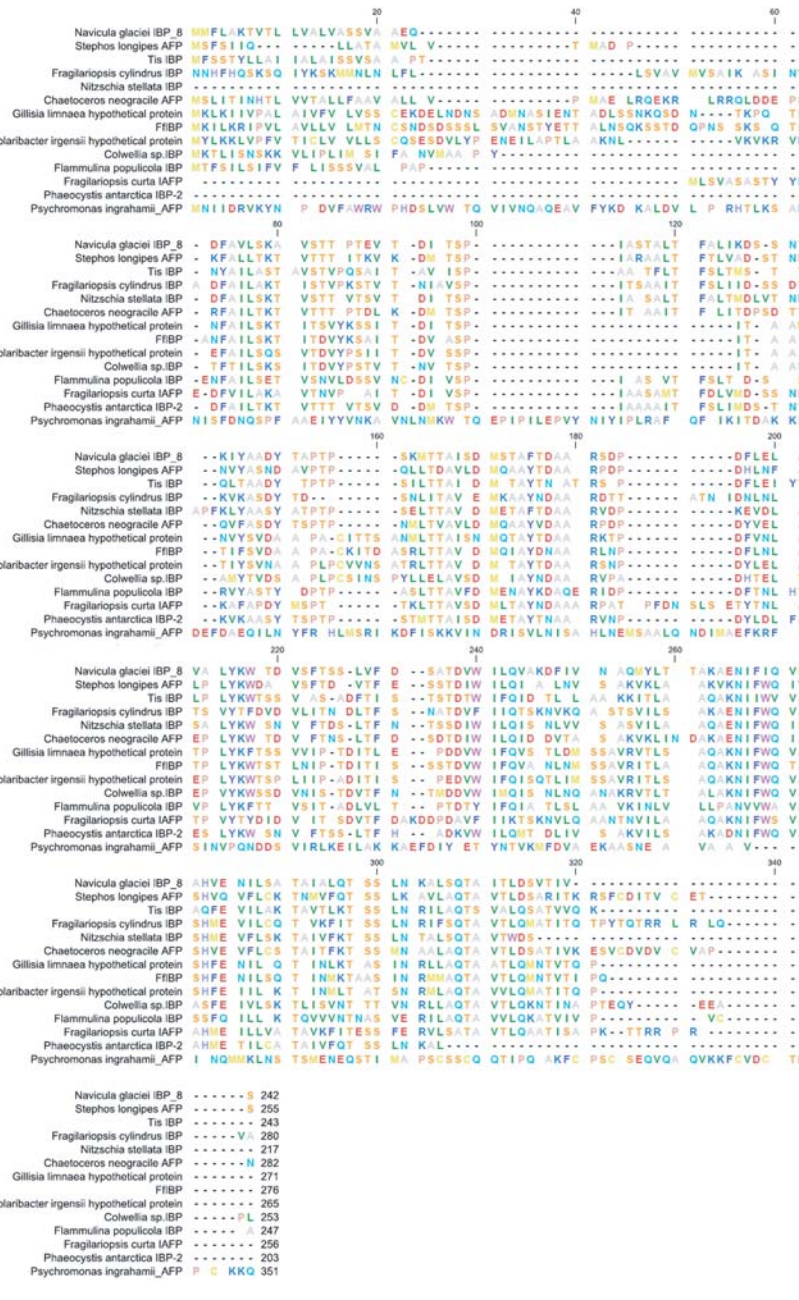

Fig. 34.5 Alignment of other marine AFPs. Accession numbers for the sequences are *Navicula glaciei*, AAZ76251 (Navicula_glaciei IBP_8); *Stephos longipes*, ACL00837 (Stephos longipes AFP); *Typhula ishikariensi*, BAD02892 (TisIBP); *Fragilariopsis cylindrus*, ACX36851 (Fragilariopsis cylindrus IBP); *Nitzschia stellate*, AEY75833 (Nitzschia stellata IBP); *Chaetoceros neogracile* ACU09498 (Chaetoceros neogracile AFP); *Gillisia limnaea*, ZP_09667540 (Gillisia limnaea hypothetical protein); *Flavobacterium frigoris*, ZP_09896943 (FfIBP); *Polaribacter irgensii*, ZP_01118128 (Polaribacter irgensii hypothetical protein); *Colwellia* sp., ABH08428 (Colwellia sp. IBP); *Flammulina populicola*, ACL27143 (Flammulina populicola IBP); *Fragilariopsis curta*, ACT99635 (Fragilariopsis curta AFP); *Phaeocystis Antarctica*, AEY75839 (Phaeocystis antarctica IBP-2); *Psychromonas ingrahamii 37*, YP_944155 (Psychromonas ingrahamii_AFP). Parenthesized are the names used in the alignment.

hemisphere grown in the presence of AFP indicates the ice planes to which the AFP is bound (Knight *et al*., 1991, 1993; Yeh & Feeney, 1996). However, experiments using variants of type-I AFP reveal that Asx and the hydroxyl group of Thr are not essential for binding to ice (Chao *et al*., 1997; Haymet *et al*., 1998; Loewen *et al*., 1999; Zhang & Laursen, 1998). The conserved Ala-rich, hydrophobic face of type-I AFP seems to be the ice-binding site (Baardsnes *et al*., 1999).

Recently, hyperactive type-I AFP was identified from the same species of fish. It previously eluded detection because of its extremely low abundance (Marshall *et al*., 2004, 2005). This AFP comprises 195 amino acids, 60% of which are alanine. Its structure has not yet been determined, but other spectroscopic data indicate that it is also α-helical in nature. Ultracentrifugation indicates that it is a dimer, and modelling predictions have led to the proposal that it is an amphipathic homodimer in which the monomer is a 195 amino acid α-helix. Each monomer contains a number of alanine patches, which appear similar to the ice-binding face of type-I AFP. The large surface area created by the alanine patches allows this protein to bind to a number of different ice planes, making it 10∼100-fold more active than type-I AFP. This idea is supported by the fact that type-I AFP binds to pyramidal ice planes and modifies the ice into a bipyramidal shape, whereas hyperactive type-I AFP binds to multiple ice planes and induces them to adopt a lemon-like shape (Patel & Graether, 2010).

34.2.3 Type-II AFP

Type-II AFP has been found from sea raven (*Hemitripterus americanus*), American rainbow smelt (*Osmerus mordax*), Atlantic herring (*Clupea harengus*) and longsnout poacher (*Brachyopsis rostratus*) (Bush *et al*., 1981). Compared to other types of fish AFP, type-II AFPs have higher molecular masses. They possess several cysteine residues that form disulfide bonds (Gronwald *et al*., 1998; Nishimiya *et al*., 2008a).

The Ca^{2+}-dependent type-II AFP from herring has a globular structure with two α-helices and nine β-strands (Liu *et al*., 2007). Its structure reveals a low sequence identity (20%) to C-type lectins, which are Ca^{2+}-dependent sugar-binding proteins, but a similar tertiary structure (Sonnichsen *et al*., 1995), supporting the idea of the evolution of type-II AFPs from sugar-binding lectins. Site-directed mutagenesis elucidated that Thr96, Thr98, Asp94 and Glu99 are crucial to ice-binding; these residues are either involved in the binding to calcium or are in the vicinity of the Ca^{2+}-binding site. Studies of this Ca^{2+}-dependent AFP show that the binding of Ca^{2+} is important to the formation of ice-binding sites (Ewart *et al*., 1996, 1998). In Ca^{2+}-independent type-II AFPs from sea raven and longsnout poacher, the residues involved in Ca^{2+} binding in the herring AFP are replaced with other residues (Gronwald *et al*., 1998; Nishimiya *et al*., 2008a).

These two different type-II AFPs seem to bind to different ice planes; however, their ice-binding sites share planar features. Recently, Japanese smelt (*Hypomesus nipponensis*), a mid-latitude freshwater species, was shown to have type-II AFP. Interestingly, this protein binds to Ca^{2+} but shows activity without it (Yamashita *et al*., 2003).

34.2.4 Type-III AFP

Type-III AFP was first found in Atlantic eelpout (*Macrozoarces americanus*) (Hew *et al*., 1984) and subsequently in Antarctic and Arctic eelpouts (Cheng & DeVries, 1989; Schrag *et al*., 1987), wolffish (*Anarhichas lupus*) (Cheng & DeVries, 1989; Hew *et al*., 1988;

Scott *et al*., 1988) and notched-fin eelpout (*Zoarces elongatus*) (Nishimiya *et al*., 2005). Type-III AFPs are 63–66 residues in length, and their molecular masses range from 6.5 to 7.0 kDa. They are compactly folded globular proteins with two or three antiparallel β-strands arranged as an orthogonal β-sandwich and one double-stranded antiparallel sheet (Chao *et al*., 1993; Sonnichsen *et al*., 1993). In addition to the findings from biochemical studies, the high-resolution X-ray and NMR structures of type-III AFPs indicate that a conserved alanine is present in the flat ice-binding surface (Chao *et al*., 1994; Davies & Hew, 1990; Jia *et al*., 1996; Sonnichsen *et al*., 1993, 1996). Site-directed mutagenesis shows that substitution of the alanine with bulkier residues in the ice-binding surface causes disruption of the planar ice-binding surface and its ability to dock to the ice, due to sterical hindrance, thereby decreasing the activity of the protein (DeLuca *et al*., 1996, 1998a, 1998b). Replacement of other residues (Gln9, Asn14, Ala16, Thr18 and Gln44) in the ice- binding site also tends to result in a loss of activity and a change in the ice morphology (Graether *et al*., 1999). Sonnichsen *et al*. (1996) suggested that type-III AFPs bind to the prism plane of ice and thus inhibit its growth. Moreover, they are active in a broad spectrum of pHs, which is advantageous for downstream applications.

34.2.5 Type-IV AFP

Type-IV AFP was identified from the blood plasma of longhorn and shorthorn sculpin (*Myoxocephalus octodecimspinosis* and *Myoxocephalus scorpius*) and from pyrosequencing the genome of Antarctic fish (Deng *et al*., 1997; Gauthier *et al*., 2008; Lee *et al*., 2011). Sequence analysis indicates that this class of AFP shares 29% identity with apolipoprotein and contains a high helix content (Deng & Laursen, 1998; Deng *et al*., 1997; Gauthier *et al*., 2008). The model for type-IV AFPs is an α-helical bundle structure, as shown in Fig. 34.4e. Since type-IV AFP exists at low concentrations (less than 100 µg/ml) in the blood plasma and is prone to aggregation when concentrated, it has been the least characterised among fish AFPs. In addition, type-IV AFP exhibits TH and modifies ice-crystal morphology. However, these activities occur at very low levels and are insufficient to protect fish from freezing.

34.2.6 Other Marine AFPS

In addition to fish, many other marine organisms also produce AFPs. Those thriving under the sea ice in a number of areas have been examined for their ice-binding activity. Dozens of sea-ice algae (Gwak *et al*., 2010; Janech *et al*., 2006; Krell *et al*., 2008; Raymond *et al*., 2009) and bacteria (Raymond *et al*., 2007, 2008), and even a copepod (Kiko, 2010), are known to produce AFPs. In order to adapt to cold temperatures, these organisms appear to secrete ~25 kDa AFPs into the surrounding environment. Sequence alignments show that these AFPs display high identity to one another (Fig. 34.5), supporting the hypothesis of horizontal gene transfer between bacteria and higher organisms such as microalgae and crustaceans. The list has been steadily increasing: currently, a handful of AFPs are extensively characterised or are being characterised from *Colwellia* (Hanada *et al*., 2011), *Flavobacterium frigoris* (Hak Jun Kim, personal communication), *Navicula glaciei* (Janech *et al*., 2006), *Fragilariopsis cylindrus* (Bayer-Giraldi *et al*., 2011; Uhlig *et al*., 2011) and *Chaetocero neoglacile* (Gwak *et al*., 2010). These AFPs seem to be moderately active or hyperactive, showing a distinct burst growth of ice crystals when the temperature reaches the nonequilibrium freezing point. This burst pattern is indicative

of hyperactive AFPs (Doucet *et al*., 2000; Tyshenko *et al*., 1997). In the near future, the properties of these AFPs will be elucidated in more depth.

34.3 PREPARATION OF FISH AFPS

34.3.1 Preparation of AFPS from Natural Sources

For early studies on function and application, most AFPs were readily prepared from fish blood serum. As an example, a total of 7.3 mg of type-II AFP was purified from 127.4 g of Japanese smelt muscle by ammonium sulfate precipitation, gel filtration and anion-exchange chromatography (Yamashita *et al*., 2003). Type-III AFP was found from Arctic and Antarctic eelpouts (zoarcidae) (Hew *et al*., 1984, 1988; Li *et al*., 1985; Nishimiya *et al*., 2005; Schrag *et al*., 1987) and wolfish (zoarcoidei) (Scott *et al*., 1988). This protein is rich, particularly in the winter season, in the serum of ocean pout (*Macrozoarces americanus*), at the level of ~20 mg/ml (Fletcher *et al*., 1985). Type-IV AFP was isolated from longhorn sculpin by gel filtration and C18 reverse-phase high-performance liquid chromatography (RP-HPLC) columns to yield about 80 μg/ml serum (Deng *et al*., 1997), which is the lowest level among all types of AFP. AFGPs were discovered in the plasma of deep-sea Antarctic notothenioids and northern cods (DeVries, 1971, 1986; Feeney & Yeh, 1978). In the blood of the Antarctic *Trematomas borgrevinki* and *Dissostichus mawsoni*, 25 mg/ml AFGP (75% from AFGPs 6–8 and 25% from AFGPs 1–5) was identified. Recently, a novel form of AFP was discovered from the Arctic yeast *Leucosporidium* sp. AY30 and purified by ice affinity using the cold-finger method (Lee *et al*., 2010).

AFPs are present in organs, including liver, gill, skin epithelia, muscle and heart (Brown & Sonnichsen, 2002; Fletcher *et al*., 2001; Gong *et al*., 1996; Nishimiya *et al*., 2008b). More interestingly, AFP activity has been found in fish fillets, minced fish and even dried fish commercially available from food markets (Nishimiya *et al*., 2008b). In most cases, the AFP resources are from Arctic fishing, but a Japanese research group has examined over 160 species of cold-adapted Japanese fish and detected AFP activity in at least 50, indicating that AFP can also be found in nonpolar coastal waters (Nishimiya *et al*., 2008b). In addition, this group was successful in purifying 75 mg of AFP from 100 g of fish muscle; 73 mg from 100 g of muscle and head; 90 mg from 100 g of muscle, head and heart; and 107 mg from 100 g of muscle, head, heart and entrails.

Even though the industrial sector has proposed a number of potential applications for AFPs, a substantial portion of production still depends on natural resources. Hence, AFP and AFGP resources are limited to live fish serum and possibly fish muscle. According to a commercial company, 1–4 g of AFP was purified from 1 l of fish blood (A/F Protein Canada, St Jones, Canada).

34.3.2 AFP-expression Systems

As with other proteins, the mass expression of AFP has been attempted using recombinant protein-expression systems in order to solve the bottleneck to industrial application. Heterologous production of AFP may be a breakthrough in obtaining a large amount of AFP with a high purity and at a lower cost. In previous studies, AFPs have been expressed successfully in bacterial-, yeast-, insect- and plant-expression systems.

Among the AFPs, type I is the smallest and simplest molecule; consequently, relatively more expression studies have been conducted upon it. Winter flounder type-I AFP

HPLC6 was expressed by *Escherichia coli* JM105 host bacterial cells, and the recombinant protein was secreted into the culture medium to levels of 16 mg/l (Tong *et al*., 2000). The protein was purified by a single-step RP-HPLC and used to characterise the protein. The researchers found that the rAFP was present in both the periplasmic space and the cytosol in very low amounts, suggesting that secretion of AFP out of the cell is not the bottleneck to AFP production. Another group successfully expressed winter flounder skin-type type-I AFP in JM105 cells to produce almost 20 mg/l culture (Lin *et al*., 1999). They defined the role of the α-helical structure in the ice-binding motif residues in type-I AFPs by site-directed mutagenesis studies. Another group was successful in producing winter flounder type-I AFP in inclusion bodies in AD494(DE3) cells. No protein degradation was evident, and the recombinant protein was refolded to yield 100 mg/l culture (Solomon & Appels, 1999). Shorthorn sculpin skin-type type-I AFP was expressed in BL21(DE3) cells (Low *et al*., 1998). Recombinant grubby sculpin type-I AFP GS-5 was expressed in *Saccharomyces cerevisiae* W303-1A, or baker's yeast, and the resulting yeast cells showed increased freeze tolerance (Panadero *et al*., 2005). *Lactococcus lactis* was used to express a recombinant type-I AFP analogue and secreted extracellular rAFP up to 98 mg/l (Yeh *et al*., 2008, 2009). Synthetic winter flounder type-I AFP was expressed in potato, and the transgenic plant leaves acquired a freeze-tolerance phenotype, as revealed by electrolyte-release analysis (Wallis *et al*., 1997). Other research groups have produced type-I AFPs as AFP multimers, AFPs with multiple amino acid repeats and chimeric proteins in order to overcome the small molecular size (Driedonks *et al*., 1995; Peters *et al*., 1989; Warren *et al*., 1993).

Heterologous expression of type-II AFP has barely been achieved, due to its large molecular mass, the presence of several cysteine residues and the dependence of its protein stability on disulfide bonds (Gronwald *et al*., 1998; Nishimiya *et al*., 2008a). However, some type-II AFPs have been expressed in recombinant cells (Gronwald *et al*., 1998; Liu *et al*., 2007; Loewen *et al*., 1997). Ca^{2+}-dependent herring type-II AFP was expressed in *S. cerevisiae* for use in clarifying the evolutionary relationship between type-II AFPs and sugar-binding lectins (Liu *et al*., 2007). Sea raven type-II AFP (SRAFP) was recombinantly expressed using a *Pichia pastoris*- and *E. coli*-expression system and purified by nickel-agarose and FPLC anion-exchange chromatography in order to solve its three-dimensional structure (Gronwald *et al*., 1998; Loewen *et al*., 1997); almost 30 mg/l recombinant type-II AFP was produced from the *P. pastoris*-expression system (Loewen *et al*., 1997).

Type-III AFP has a broad range of pH stability and is readily expressed in recombinant host cells. Antarctic eelpout type-III AFP RD3 was produced in BL21(DE3) cells and used to define the three-dimensional structure of the active domain (Holland *et al*., 2007). Ocean pout type-III AFP variant rQAE m1.1 was generated in K38 cells in order to carry out structural studies upon it using NMR spectroscopy (Chao *et al*., 1993).

Type-IV AFP has rarely been expressed in heterologous cells. The reasons have not been clarified, but type-IV AFPs may be toxic to host cells or difficult to express. Recently, however, type-IV AFPs from two Antarctic fish, *Pleuragramma antarcticum* and *Notothenia coriiceps*, were expressed as His-Tag fusion proteins in BL21(DE3) cells using a pCold expression vector (Lee *et al*., 2011). The recombinant proteins were purified from the soluble fraction using Ni-affinity chromatography, but insoluble precipitates occurred at concentrations greater than 0.5 mg/ml. The same aggregation has been observed in other research on type-IV AFP expression (Gauthier *et al*., 2008).

A novel form of AFP discovered from the Arctic yeast *Leucosporidium* sp. AY30 (LeIBP) was successfully expressed using the *E. coli*- and *P. pastoris*-expression systems in order to solve its three-dimensional structure and to characterise it (Lee *et al*., 2012; Park *et al*., 2011, 2012). The yields from a 1 l culture of purified protein were 24.5 and 61.2 mg from the bacterial and *Pichia* systems, respectively (Park *et al*., 2012).

The expression of AFPs could be further enhanced through the development of bioprocessing, as shown in other protein-expression systems (Chen *et al*., 2010; Guerfali *et al*., 2010; Han *et al*., 2011; Li *et al*., 2007; Tang *et al*., 2004). Codon optimisation might be another option for enhancing recombinant AFP expression efficiency or minimising the problems with the poor expression of heterologous proteins (Kim & Lee, 2006; Lee *et al*., 2010; Wang *et al*., 1994; Zhou *et al*., 2004). The codon usage of certain AFPs might be considerably biased in heterologous expression host cells; therefore, the relatively low frequency of native codons may need to rectified based on the results of the optimisation process.

34.3.3 Chemical Synthesis of AFGP

Natural sources are limited and are not sufficient to prepare AFGPs for basic research or application studies. Moreover, high-purity AFGPs are required in large quantities for commercial use. Thus, in the last decade, chemical synthesis has been considered for their production. Given that AFGP is a glycoprotein, chemical-synthesis strategies have not been easy to establish. Synthesis has been attempted in two different ways: solution-phase (Anisuzzaman *et al*., 1988) and continuous flow solid-phase techniques (Tseng *et al*., 2001). The solution-phase method, which glycosylates a tripeptide and then polymerises the peptide, produces oligomer mixtures that need to be further separated. The solid-phase route, which uses a glycosylated Thr precursor in the elongation of the peptide backbone, provides defined-length oligomers with various sequences (Harding *et al*., 2003). In 1996, the first synthetic AFGP (with a molecular mass 6000–7300 Da) was reported. This was a glycotripeptide polymerised (n = 10–12) using diphenylphosphoryl azide (DPPA) (Tsuda & Nishimura, 1996). The tripeptide-based synthesis adapted Thr glycosidation as its last step (Maeji *et al*., 1986; Anisuzzaman *et al*., 1988). To generate high-molecular-mass AFGPs, a solid-phase synthesis method using Fmoc-chemistry was suggested (Tseng *et al*., 2001). In other research, structurally modified AFGP analogues were synthesised to facilitate the synthesis procedure (Eniade & Ben, 2001; Eniade *et al*., 2001). The Thr residue was replaced with Lys to build more stable C-glycosides in place of the O-glycosides, but the thermal-hysteresis activity of these proteins was not examined. Recently, a homodimeric AFGP analogue was synthesised and was revealed to possess antifreeze activity (Ahn *et al*., 2012). This study also verified the relationship between antifreeze activity and the number of hydroxyl groups in the carbohydrate, which is linked to the Thr residue.

34.4 AFP APPLICATIONS

34.4.1 Cryopreservation

Two unique AFP properties, TH and RI, can be of benefit to various aspects of medicine, biotechnology, cell biology and the feed industry. The noncolligative freezing-point

depression can be used to improve hypothermic storage, while RI plays an important role in cryopreservation by protecting membranes from freezing injury. There is an ongoing demand for novel cryoprotectants (CPAs). The high number of CPAs used during the cryopreservation of cells and tissues ensures protection from freezing damage but unfortunately also displays toxicity (Fuller, 2004). In this regard, AFPs are emerging as a new candidate for less toxic or nontoxic CPAs, since they function effectively at much lower concentrations. In this section, we will outline the current status of the development of AFP-based preservation or cryopreservation techniques.

The routine cryopreservation of cell lines is well established. However, a number of factors may affect the survival of cells and tissues after cryopreservation, including freezing and warming rates, the state and type of cells, storage temperature, storage duration, and the type and concentration of CPAs. These factors make the cryopreservation of some cells and tissues difficult to reproduce (Fuller, 2004; Karow, 1981; Mazur, 1984). The major bottleneck to cryopreservation, aside from CPA toxicity, is ice recrystallisation during the thawing process (Pegg, 2001). The recrystallisation process accompanies physical damage to cell membranes, leading to cell death. Hence another reason AFPs have been investigated as possible additives or alternatives to cryopreservation solutions, since AFPs effectively behave as recrystallisation inhibitors (Doucet, *et al*., 2000; Knight & Duman, 1986; Knight *et al*., 1995; Raymond & Fritsen, 2001; Raymond & Knight, 2003). A large number of investigations have been performed on the cryopreservation of various cell types, oocytes, tissues, embryos and organs using AFPs, but they have provided conflicting results (Barrett, 2001; Wang, 2000).

Some cases that have shown promising hypothermic storage and cryopreservation effects are bovine and porcine oocytes (Arav *et al*., 1993; Rubinsky *et al*., 1991), mouse oocytes (O'Neil *et al*., 1998), porcine embryos (Chen *et al*., 1995), intact livers (Lee *et al*., 1992; Rubinsky *et al*., 1994), red blood cells (RBCs) (Carpenter & Hansen, 1992; Chao *et al*., 1996; Kang & Raymond, 2004), vertebrate and invertebrate cell lines (Koushafar *et al*., 1997; Tursman & Duman, 1995), oyster (*Crassostrea gigas*) oocytes (Naidenko 1997), carp sperm (Karanova *et al*., 1997), ovine sperm (Upreti *et al*., 1996), sheep embryos (Baguisi *et al*., 1997), the heart (Amir *et al*., 2003, 2004a, 2004b, 2005), bull sperm (Prathalingam *et al*., 2006), sea bream embryos (Robles *et al*., 2006), chimpanzee (*Pan troglodytes*) sperm (Younis *et al*., 1998), islet cells (Matsumoto *et al*., 2006), platelets (Tablin *et al*., 1996) and *in vivo*-matured metaphase II oocytes (Jo *et al*., 2011). These successful applications may be attributable to another unforeseen property of AFPs: their interaction with the cell membrane (Arav *et al*., 1993, 1994; Hays *et al*., 1996; Negulescu *et al*., 1992; Rubinsky *et al*., 1990, 1991), although this property is not unique to them (Wu & Fletcher, 2001). In early studies, Rubinsky, Arav and colleagues proposed that AFPs bind to the cell membranes of oocytes and embryos and block potassium and calcium ion channels, thereby inhibiting ion leakage (Arav *et al*., 1993, 1994; Negulescu *et al*., 1992; Rubinsky *et al*., 1990, 1991).

Further investigations with model membranes showed that AFGPs prevented leakage of dielaidoylphosphatidylcholine (DEPC), dielaidoylphosphatidylethanolamine (DEPE) and dielaidoylphosphatidylglycerol (DEPG) (Hays *et al*., 1996; Wu & Fletcher 2001; Wu *et al*., 2001), and that AFGP7–8 offered limited protection to spinach thylakoid membranes and dimyristoylphosphatidylcholine (DMPC) membranes with the addition of varying amounts of galactolipids as they cooled through the phase-transition temperature during chilling (Tomczak *et al*., 2001). Type-I AFP was also shown to interact with the acyl chains of lipids in a mixture of DMPC and the plant thylakoid lipid digalactosyldiacylglycerol

(DGDG) (Tomczak *et al*., 2002), and prevented zwitterionic DEPC liposomes (Wu & Fletcher, 2001).

In some instances, AFGP1–5 and type-I AFP were found to induce leakage from and to be fusogenic to the liposomesin in a concentration-dependent manner (Tomczak *et al*., 2001). AFP did not prevent negatively charged DEPG before cooling, or stop DEPE liposomes from leaking as they were cooled through their phase-transition temperature. In addition, Hincha *et al*. (1993) showed that AFPs actually increase leakage from cryopreserved thylakoid membranes. Collectively, these results demonstrate that protection against freezing damage depends not only on the type of AFP but also on membrane lipid compositions (Larese *et al*., 1996; Wu & Fletcher, 2001).

The negligible or negative effects of AFPs have also been reported. They include those observed for equine embryos (Lagneaux *et al*., 1997), human RBCs in glycerol (Ishiguro & Rubinsky, 1998), livers (Soltys *et al*., 2001), mouse embryos (Mezhevikina & Karanova, 1995; Shaw *et al*., 1995), isolated rat hearts (Wang *et al*., 1994), plant cells (Wang *et al*., 1999, 2002) and ram spermatozoa (Payne *et al*., 1994a; Upreti *et al*., 1996).

34.4.1.1 Red Blood Cells

In terms of transfusion, RBCs are important to both military and civilian communities. They were first stored using citrate and glucose in 1916 (Rous & Turner, 1916), and efforts were soon made to enhance the storage efficiency (Heaton *et al*., 1994; Hess, 2006; Hess & Greenwalt, 2002; Hill *et al*., 2001). However, the maximal storage period in a nonfrozen condition is only 6 weeks using the current preservation solutions, which are composed of sugars, citrate, phosphate, adenine and saline (Dumont & AuBuchon, 2008). Biochemical reactions are suspended under a frozen state at $-196\,^{\circ}$C, so cryopreservation may be another option for the preservation of RBCs. Indeed, frozen RBCs can be transfused for up to 10 years, according to the US Food and Drug Administration (FDA), so cryopreservation is a feasible way to prolong the storage period. Nonetheless, RBC cryopreservation involves several issues relating to cryodamage: solution effects, extra/intracellular ice formation and dehydration. There have been many reports on cryoprotectants as the solution to the problems raised by RBC cryopreservation (Meryman & Hornblower, 1972; Rowe *et al*., 1968; Smith, 1950). Glycerol, hydroxyethyl starch (HES), polyvinyl pyrrolidone (PVP), dextran and serum albumin are regarded as extracellular cryoprotectants. These additives have solved the cryodamage problems to a significant degree, but the issue of ice recrystallisation remains. Currently, AFP is the only biomaterial known to minimise this problem. Research has found that AFPs from microalgae, fish and yeast have RBC-cryopreservation effects through inhibition of ice recrystallisation in the presence of glycerol or HES (Carpenter & Hansen, 1992; Chao *et al*., 1996; Kang & Raymond, 2004; Lee *et al*., 2012). Clinical studies of cryopreserved RBCs using AFPs have not yet been reported, but it is anticipated that the results of transfusion *in vivo* studies will be promising.

34.4.1.2 Tissues and Organs

Extending the duration of the viable hypothermic (subzero nonfreezing) storage of human organs for transplantation will have a significant impact upon transplantation technology (Maathuis *et al*., 2007; McAnulty, 2010). The time for hypothermic storage varies depending on the type of organ. For example, the heart can be stored for 4–6 hours,the kidney for 48–72 hours and the liver for 12–24 hours. However, during cold storage the organs gradually deteriorate (Maathuis *et al*., 2007; McAnulty, 2010). A few reports show that

the TH activity of AFPs plays an important role in minimising cold-induced injury (Amir *et al*., 2003; Brockbank *et al*., 2009; Lee *et al*., 1992).

Several studies show that AFGP may be deleterious to cardiomyocytes or the heart. Wang *et al*. (1994) examined hypothermic (0 °C) and freezing (−1.4 °C) storage of rat hearts in the presence of 10 mg/ml AFGP, and under these conditions the hearts failed to beat. This was supported by a study (Mugnano *et al*., 1995) which indicated that needle-like ice formed in the presence of 10 mg/ml AFGP and that this ice penetrated the cardiomyocytes on freezing (−4 °C), leading to cell death.

In contrast, some investigations using animal organs have met with limited success (Amir *et al*., 2003, 2004b; Brockbank *et al*., 2009; Lee *et al*., 1992; Rubinsky *et al*., 1994). Lee *et al*. (1992) preserved isolated rat livers perfused with AFGP prior to hypothermic storage at a subzero temperature and showed that these livers produced bile at a higher rate and leaked a lower amount of enzymes compared to the untreated ones. Amir *et al*. (2003) showed the feasibility of subzero cryopreservation of mammalian hearts using type-I and type-III AFPs. Rat hearts were treated with AFPs, preserved at −1.1 to −1.3 °C for 2–6 hours and heterotopically transplanted. Electron microscopy of the transplanted hearts showed that they maintained myocyte structure and mitochondrial integrity. In a subsequent experiment performed by the same group, the rat hearts were preserved for prolonged period (24 hours) at a subzero temperature (−1.3 °C; this nonfreezing temperature was chosen because AFPs have a TH activity of ∼1 °C). The preserved hearts showed that improved survival, haemodynamics and reduced cell death were strongly related to treatment with the AFPs and that this method is likely superior to the standard preservation at +4° C (Amir *et al*., 2004a, 2004b, 2005; Brockbank *et al*., 2009).

Intriguingly, these results indicate that AFPs with even weak TH activity (∼1 °C) can be useful for hypothermic preservation at below-zero temperatures. Therefore, it is worthwhile using hyperactive AFPs possessing 10–30 times higher TH activity for hypothermic preservation. This particular approach is currently under study.

34.4.2 Cryosurgery

At higher concentrations, AFPs damage RBCs by inducing the ice to form a needle-like shape that is able to destroy the cells (Carpenter & Hansen, 1992). This result may be of no use to the other applications mentioned here, but it is apparently beneficial to cryosurgery, which is a minimally invasive surgical technique that adopts freezing to destroy undesirable tissues, such as cancers (Koushafar & Rubinsky, 1997; Koushafar *et al*., 1997; Rubinsky, 2000). Preliminary tests conducted by Rubinsky and his colleagues indicated that the use of 10 mg/ml type-I AFP causes complete destruction of human primary prostatic adenocarcinoma cells through the formation of intracellular ice (Koushafar & Rubinsky, 1997; Koushafar *et al*., 1997). An *in vivo* study using mice with subcutaneous tumours suggested that injection of type-I AFP into the tumours prior to freezing may give better destruction (Muldrew *et al*., 2001; Pham *et al*., 1999).

34.4.3 Food Preservation

Biological materials can pose any number of potential risks when applied to a new field of application. In one study, a novel protein included in foods produced an allergic reaction (Bindslev-Jensen *et al*., 2003). Even if a biomaterial is itself evaluated as innovative, its biosafety should be assessed prior to application. AFPs have many potential applications,

as already mentioned, but their biosafety should be approved before these are discussed. Since AFPs have been found in diverse organisms across the planet, including bacteria, yeast, plants, fish and animals (Brown & Sonnichsen, 2002; Davies & Hew, 1990; DeVries, 1969; Ewart *et al*., 1999; Fletcher *et al*., 2001; Hew *et al*., 1980, 1984, 1986, 1988; Li *et al*., 1985; Nishimiya *et al*., 2005; Schrag *et al*., 1987; Yamashita *et al*., 2003), it should be feasible to consider that the ingestion of AFP may be normal in human life through food intake activity. Recently, AFPs were subjected to a biosafety examination, and their safety was confirmed. Ice-structuring protein type III HPLC12 (ISP), which was renamed from 'type-III AFP', was accepted by Food Standards Australia New Zealand (FSANZ) and the FDA as a food ingredient (FSANZ, 2005). This protein was regarded as Generally Recognized As Safe (GRAS). The Philippines, Hong Kong, Mexico and Indonesia have also approved ISP for food use. A recent novel LeIBP recombinantly expressed from *Pichia* has also been subjected to preclinical studies (personal communication). The toxicological assays were conducted by a CRO (contract research organization) in South Korea, and its biosafety was confirmed. Considering all of this, AFPs should be safe to use in a broad range of applications.

Several studies have shown that food texture preservation is related to nutrient preservation through reduction of cellular damage, which occurs during the freezing, storage, transport and thawing of food (Feeney & Yeh, 1998; Griffith & Ewart, 1995; Wang, 2000). These studies also reported that AFPs preserved the texture of a high-quality product by inhibiting ice recrystallisation. The cellular-structure damage driven by freezing can be minimised by using AFPs rather than sugars to lower extracellular solution concentrations, which reduces dehydration during freezing (Griffith & Ewart, 1995). The potential resulting from AFP's ice RI properties has been applied to ice-cream preservation. Ice cream undergoes fluctuating temperatures in storage and transit, and thus the retail product can have a coarse texture. AFP's ice RI activity keeps ice crystals rather tiny (less than 15 μm), which affectss the quality of an ice cream. Private food companies have launched AFP-containing dairy products, including ice cream and yogurt (Eskimo from Twin POP, Unilever from Breyers and Haagen-Dazs from Edy's). It has been surmised that AFPs can be introduced to food by mixing, injection, soaking, vacuum infiltration or gene transference (Griffith & Ewart, 1995).

Bovine and ovine muscle was soaked in a solution containing up to 1 mg/ml type-I AFP or AFGP, which resulted in reduced cryodamage (Payne *et al*., 1994b). AFGP was injected into lamb meat prior to slaughter, and its effect was identified, resulting in reduced drip loss and ice-crystal size within the meat (Payne & Young, 1995). Canola leaves were vacuum-infiltrated with 1 mg/ml type-I AFP, and the nucleation temperature was lowered by 1.8 °C (Cutler *et al*., 1989). The AFP gene was introduced into a lactic acid bacterial genome for yogurt fermentation (Fletcher *et al*., 1997). The use of transgenic microorganisms allows a subsequent AFP purification procedure to be avoided. In addition, food preservation by AFPs at low temperatures has the beneficial effect of slowing down the growth of any contaminating microflora (Rothwell, 1985).

34.4.4 Transgenic Studies

Introduction of new traits into an organism through a transgene has become routine in bacteria, plants and animals. AFPs' unique trait has made them one of the most interesting targets for transgenic studies (Hew *et al*., 1995). It is fairly reasonable to think that introduced AFPs might endow cold or freeze resistance to transgenic organisms.

Stable lines of freeze-resistant salmon were established by microinjecting type-I AFP into eggs (Hew *et al*., 1992), although the transgenic offspring expressed only low levels of AFP. Later, Hew *et al*. (1999) showed tissue-specific and stable seasonal expression of AFP in transgenic salmon. Type-III AFP was microinjected into goldfish (*Carassius auratus*) and successfully transferred and expressed, providing significant cold tolerance to the transgenic fish (Wang *et al*., 1995). In addition, the AFP gene promoter turned out to be useful for exogenous gene expression (Du *et al*., 1992a, 1992b). Transgenic Atlantic salmon with growth hormone (GH) under the control of an AFP gene promoter from ocean pout were generated and showed a dramatic increase in growth rate (Butler & Fletcher, 2009; Du *et al*., 1992a, 1992b; Hobbs & Fletcher, 2008). However, despite the successful generation of transgenic fish, the expression levels of AFP and GH were so low that the effect of the freeze resistance and growth enhancement was less than expected (Zbikowska, H.M. 2003).

Since Cutler *et al*. (1989) showed that leaves vacuum-infiltrated with type-I AFP had a depressed freezing point, many attempts to establish transgenic plants have been made: type-I AFP to tomato (Hightower *et al*., 1991), tobacco (Hightower *et al*., 1991; Kenward, K.D. *et al*., 1993) and potato (Wallis *et al*., 1997); codon-optimised type-I AFP to wheat (Khanna & Daggard, 2006); type-II AFP to tobacco (Kenward, *et al*., 1999); carrot AFP to Arabidopsis (Meyer *et al*., 1999) and tobacco (Worrall *et al*., 1998); and insect AFPs to tobacco (Holmberg *et al*., 2001; Wang *et al*., 2008), Arabidopsis (Huang *et al*., 2002) and mushroom (Guo *et al*., 2005). In these studies, fish AFPs improved plant cold tolerance unsatisfactorily, but insect AFPs, which are the hyperactive counterpart to fish AFPs, seemed to have a better effect.

Transgenic *Drosophila melanogaster* of type-I (Peters *et al*., 1993; Rancourt *et al*., 1987), type-II (Duncker *et al*., 1996) and type-III (Rancourt *et al*., 1990) AFPs secreted functionally active protein to its haemolymph, but these transgenic genes did not appear to enhance the survival of adult fruit flies under hypothermic conditions. However, Nicodemus *et al*. (2006) showed that unlike fish AFPs, beetle *Dendroides canadensis* AFP could confer increased cold tolerance to transgenic *Drosophila* at above-freezing temperatures.

34.5 CONCLUSION

A few companies are currently running businesses based on AFP technology: A/F Protein (www.afprotein.com), ProtoKinetix (www.protokinetix.com), Ice Biotech (www.icebiotech.com), AquaBounty (www.aquabounty.com) and Nichirei (www.nichirei.co.jp). However, applied research into AFPs has lagged far behind the basic research. The bottleneck to application is the supply of a large amount of high-quality AFP. It is hoped that the recent effort to produce AFPs from natural sources and to chemically synthesise AFGP and its derivatives will pave the way to application in cryopreservation in the near future.

AFPs from marine organisms are increasing in number. Therefore, research into marine AFPs will grow out of that into fish AFPs. Many of the newly identified marine AFPs are more hyperactive than those from fish. This hyperactivity is thought to be useful, especially to hypothermic storage and transgenic technologies. Protein engineering, synthetic biology and structural biology will all help tailor existing AFPs to make them appropriate for medical and commercial applications.

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35 Antimicrobial Peptides in Marine Mollusks and their Potential Applications

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35.1 INTRODUCTION

Gene-encoded antimicrobial peptides (AMPs) are an ancient group of defense molecules in plants, invertebrates and vertebrates, although they have recently been found to be multifunctional anti-infective agents. They are ubiquitously found in all life forms, including unicellular (Pag & Sahl, 2002) and multicellular organisms (Bulet *et al*., 2004). AMPs are inducible upon microbial infection or under stress (sometimes), and can be expressed constitutively in certain cells or tissues (Brogen *et al*., 2005). In general, they act rapidly against target microorganisms as a first line of defense and are involved in antimicrobial function through neutralization and elimination of invading pathogens (Bulet, 2004; Hancock, 2006).

AMPs are one of the potential candidates for controlling antibiotic-resistant strains (superbugs). They can be used to overcome the major problems associated with antibiotic use, such as development of antibiotic-resistant strains and environmental and public-health issues. Development of antibiotics has been reduced drastically in recent years and alternatives are being actively sought (Hancock & Patrzykat, 2002). Therefore, AMPs have seen growing interest in their use as antibiotic alternatives and in other various applications in health-management strategies (Giuliani *et al*., 2007). They are considered effective immune modulators with a broad range of therapeutic capacities (Noga, 2010). Recent studies have demonstrated the importance of several AMPs as high biochemical diversity, broad specificity against microorganisms (Cho, 2009), anticancer activities (Lee, 2008), wound-healing effects (Murphy, 1993), regulation of cell proliferation, extracellular matrix production, anti-endotoxin activities and cellular immune responses (Mookherjee, 2007; Bals, 2003).

Mollusk is the second largest phylum (next to Arthropod), comprising 23% of all named marine organisms. Around 52 000 marine mollusk species have been reported and the estimated species diversity might be 100 000–200 000 (Bouchet, 2006). In 1996, the first report of AMPs in mollusks was described in blue mussel (*Mytilus edulis*) (Charlet *et al*., 1996). Hubert *et al*. (1996) have isolated a new AMP (MGD-1) with antibacterial properties from Mediterranean mussel (*M. galloprovincialis*). Several AMPs have been reported in other mollusks, and the most of them are important aquaculture species. Although the species diversity of marine mollusks is very large, only a small proportion has been investigated for the presence of AMPs. Identification of the functional

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characterization of different groups of AMPs from marine mollusks can be applied in aquaculture to prevent and control pathogenic microorganisms that cause diseases to these mollusks, as well as for the development of therapeutic agents for other animals. Two recent reviews have focused on AMPs in invertebrates (Sperstad *et al*., 2011) and defensin- and cysteine-rich peptides in mollusks (Diaz *et al*., 2010). In this review, the main AMPs isolated from different marine mollusks and their structures and antimicrobial and bioactive properties have been summarized.

35.2 CHARACTERISTICS OF AMPS

The gene-encoded AMPs are polypeptides (small proteins) of less than 30 kDa molecular mass (Bulet *et al*., 2004). Characteristics that affect the biological activity and specificity of AMPs are associated with the size, sequence, charge, hydrophobicity, amphipathicity, conformation and structure of the peptide (Brogden, 2002). The gene-encoded feature is also essential for applying tools in biotechnology to develop or modify these AMPs. Most AMPs are membrane-active, positively charged (cationic) and amphipathic peptides with high cysteine residues. However, anionic AMPs have been reported, such as Maximin H5 in amphibians (Lai *et al*., 2002) and Dermcidin in humans (Schittek *et al*., 2001). The cationic and hydrophobic features of AMPs are important in promoting their binding to negatively charged bacterial and fungal membranes via electrostatic forces. This could facilitate the forming of amphiphatic structures which penetrate into the microbial cells and thereby enable the antimicrobial function (Hultmark, 2003; Reddy, 2004).

AMPs can be classified into three major classes based on their amino acid sequences and secondary structures: (1) linear peptides with α-helix conformations; (2) cyclic and open-ended cyclic peptides (one to four disulfide bonds) with hairpin-like, β-like sheet or α-helix/β-sheet mixed structures; and (3) peptides with higher representations of specific amino acids (Boman, 1988). Based on the strategy used for their antimicrobial activities, AMPs can be classified into two main groups: (1) peptides that disrupt membranes and (2) peptides that bind intracellular components of bacteria. Both groups can kill an organism within a very short period of time (Brogden, 2002). In this regard, AMPs show alternative pathogen-elimination mechanisms to antibiotics, which is a key reason for the extreme difficulty bacteria have in developing resistance to them. With regard to mollusks, most identified AMPs are cationic peptides and contain high cysteine residues (Table 35.1).

35.3 DIVERSITY OF AMPS IN MARINE MOLLUSKS

The marine environment is very rich in microbial pathogens. It has been estimated that there might be up to 10^6/ml bacteria and 10^9/ml virus in sea water (Ammerman *et al*., 1984). Due to their filter-feeding habit, mollusks can easily accumulate different types of pathogenic microorganism from their environment (Pruzzo *et al*., 2005). Therefore, AMPs play a very important role in the internal defense mechanisms of mollusks, controlling a broad spectrum of marine pathogens (Zasloff, 2002). Various techniques, such as chromatography, gene cloning and sequencing, have been applied to isolating the gene-encoded AMPs. Most of the mollusk AMP sequences are available in public databases

Table 35.1 Comparison of abalone abhisin with selected histone-derived AMPs.

AMP property	Abhisin	Scallop AMP	Buforin I	Hipposin
Number of amino acids	40	39	39	51
Amino acid identity with abhisin	–	92%	80%	67%
Total hydrophobic ratio	27%	25%	28%	31%
Total net charge	+13	+13	+13	+15
Protein-binding potential (Boman index) kcal/mol	2.82	2.79	3.08	2.43
Activity	Gram (G)+, G– bacteria, fungi	G+, G– bacteria, fungi	G+, G– bacteria, fungi	G+, G– bacteria
Source	Disk abalone N-terminal domain of histon H2A	Scallop N-terminal domain of histon H2A	Asian toad N-terminal domain of histon H2A	Atlantic halibut N-terminal domain of histon

Antimicrobial peptide sequences were analyzed using an antimicrobial peptide database.

such as NCBI (http://www.ncbi.nlm.nih.gov) and the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php).

Production of AMPs is important if their functional activities are to be evaluated, as by chromatography (native peptide), recombinant expression and purification and synthetic peptide production. Isolation of AMPs from various tissues or from hemolymph (serum) of mollusks has shown tissue-specific variability. Mostly AMPs act as strong barriers to invading pathogens and commonly localized in epithelia in animals. However, several studies have confirmed that AMPs are expressed and stored in hemocytes of mollusks and released into hemolymph (plasma) upon pathogenic infection or under certain stress factors. Among marine mollusks, AMPs have been mainly characterized in class bivalve (mussels, oysters and clams) (Zhao *et al*., 2007). Different groups of molluscan AMPs have been reported, with the most common being the defensin isoforms (Diaz *et al*., 2010). Defensins are one of the major groups of AMPs in mollusks. Most share common features of the arthropod defensin family (Bulet *et al*., 2004). All of the AMPs isolated in mollusks have presented at least one defensin isoform, suggesting that defenisn might be common in mollusks. AMPs isolated and characterized from mussels, oysters, clams, abalones and some other minor species are summarized in this section (Table 35.2).

35.3.1 AMPS in Mussels

The existence of different types of AMPs has been reported in two mussel species, *M. edulis* and *M. galloprovincialis*. Mussel AMPs can be classified into four major groups: (1) mussel defensins, (2) mytilins, (3) myticins and (4) mytimycin (Mitta *et al*., 2000).

The first mussel AMPs were isolated from the blood (hemolyph) of blue mussel (*M. edulis*), which belongs to the cysteine-rich defensin family called ‘mussel defensin A and B’. In antibacterial assays, both mussel defensin A and mussel defensin B have shown greater inhibition of the Gram-positive (G^+) bacteria *Micrococcus luteus* than of

Table 35.2 Antimicrobial peptides in marine mollusks.

Mollusk species	Main source	Type of AMP	Amino acids (mature peptide)	Peptide size (kDa)	Cysteine residues	Antimicrobial activities	Reference
Mussels							
Mytilus edulis	Hemolymph	Mussel defensin A	37	4.3143	6	Antibacterial	Charlet *et al.* (1996)
M. edulis	Hemolymph	Mussel defensin B	35	4.3924	6	Antibacterial	Charlet *et al.* (1996)
M. edulis	Hemolymph	Mytilin A	34	3.7737	8	Antibacterial	Charlet *et al.* (1996)
M. edulis	Hemolymph	Mytilin B	34	3.9743	8	Antibacterial	Charlet *et al.* (1996)
M. galloprovincialis	Hemolymph	MGD-1	38	4.4180	8	Antibacterial/ antifungal	Hubert *et al.* (1996)
M. galloprovincialis	Hemocytes	MGD-2	60	6.9390	8	Antibacterial/ antifungal	Mitta *et al.* (1999)
M. galloprovincialis	Hemocytes	Mytilin B	34	3.8770	8	Antibacterial/ Antifungal/ antiviral	Mitta *et al.* (2000) Roch *et al.* (2007)
M. galloprovincialis	Hemocytes	Mytilin C	31	4.2870	7	Antibacterial/ antifungal	Mitta *et al.* (2000)
M. galloprovincialis	Hemocytes	Mytilin D	33	3.9730	8	Antibacterial/ antifungal	Mitta *et al.* (2000)
M. galloprovincialis	Hemocytes	Mytilin G1	36	4.1180	8	Antibacterial/ antifungal	Mitta *et al.* (2000)
M. galloprovincialis	Hemocytes/plasma	Myticin A	40	4.4380	8	Antibacterial	Mitta *et al.* (2000)
M. galloprovincialis	Hemocytes	Myticin B	40	4.5620	8	Antibacterial/ antifungal	Mitta *et al.* (2000)
M. edulis	Hemolymph	Mytimysin	32	6.5000	12	Antifungal	Charlet *et al.* (1996)
Oysters							
Crassostrea virginica	Gill	AOD	38	4.2650	6	Antibacterial	Seo *et al.* (2005)
Crassostrea gigas	Mantle	Cg-Defm	43	4.6340	8	Antibacterial/ antifungal	Gueguen *et al.* (2006)
C. gigas	Hemocytes	Cg-Defh1	43	4.7600	8	Antibacterial	Gonzalez *et al.* (2007), Schmitt *et al.* (2010)

C. gigas	Hemocytes	Cg-Defh2	43	4.6700	8	Antibacterial	Gonzalez *et al.* (2007), Schmitt *et al.* (2010)
C. gigas	Hemocytes	C*g*-BigDef1	94	11.000	6	N.A.	Rosa *et al.* (2011)
C. gigas	Hemocytes	C*g*-BigDef2	87	9.8500[a]	6	N.A.	Rosa *et al.* (2011)
C. gigas	Hemocytes	C*g*-BigDef3	87	9.7800[a]	6	N.A.	Rosa *et al.* (2011)
Abalones							
Haliotis discus discus	Hemocytes, gills	Defensin	48	4.902[a]	6	N.A.	De Zoysa *et al.* (2010)
Haliotis discus hannai	Liver and kidney	Defensin (hd-def)	42	4.323[a]	6	N.A.	Xuguang *et al.* (2008)
Haliotis discus discus	Synthetic peptide	Abhisin	40	4.3200[a]	0	Antibacterial/ antifungal	De Zoysa *et al.* (2009)
Clams							
Ruditapes philippinarum	Hemocytes	Defensin-like	N.A.	N.A.	N.A.	N.A.	Kang *et al.* (2006)
Ruditapes decussatus	Hemocytes	Clam myticin 1	80	8.9000[a]	8	N.A.	Gestal *et al.* (2007)
R. decussatus	Hemocytes	Clam myticin 2	78	8.7630[a]	8	N.A.	Gestal *et al.* (2007)
R. decussatus	Hemocytes	Clam myticin 3	78	8.7320[a]	8	N.A.	Gestal *et al.* (2007)
R. decussatus	Hemocytes	Clam Mytilin	78	9.1220[a]	8	N.A.	Gestal *et al.* (2007)
Venerupis philippinarum	Hemocytes	VpBD	74	8.9220[a]	6	Antibacterial	Zhao *et al.* (2010)
R. philippinarum	Hemocytes	MCdef	44	4.9711	8	Antibacterial	Adya *et al.* (2012)
Scallops							
Chlamys farreri	Hemocytes	Histone-derived AMP	39	4.5000	0	Antibacterial/ antifungal	Li *et al.* (2007)
Argopecten irradiants	Hemocytes, gills	Big defensin (AiBD)	84	9.2200	6	Antibacterial/ antifungal	Zhao *et al.* (2007)
Sea hare							
Dolabella auricularia	Skin, mucus	Dolabellanin B2	33	3.8725	4	Antibacterial, antifungal	Liijima *et al.* (2003)

[a]Molecular masses of AMPs are predicted values base on the amino acid sequence.
N.A., not available.

the Gram-negative (G^-) bacteria *Escherichia coli* (Charlet *et al*., 1996). Another two defensin molecules (MGD-1 and MGD-2) have been characterized from plasma and homocytes of *M. galloprovincialis*. Both molecules consist of eight cysteines (Mitta *et al*., 1999a). MGD-1 and MGD-2 are considered new members of the molluscan defensin family, due to the presence of two extra cysteines over the six in mussel defensin A and B. Peptide sequence analysis shows that MGD-1 and MGD-2 are more similar to arthropod defensins. The three-dimensional structure revealed by nuclear magnetic resonance (NMR) analysis shows that MGD-1 has a conserved CS$\alpha\beta$ structural motif (Yang *et al*., 2000). MGD-1 and MGD-2 have antibacterial effects against *M. luteus* and antifungal activity against *Fusarium oxyporum* (Mitta *et al*., 1999b).

Mytilins consists of five isoforms: A, B, C, D and G1. Mytilin A and B have been isolated from *M. luteus* (Charlet *et al*., 1996), while B, C, D and G1 have been extracted from acid extracts of *M. galloprovincialis* hemocytes (Mitta *et al*., 2000b). Mytilin B identified from *M. galloprovincialis* is similar to that from *M. luteus*. Both are considerably different mytilins A, C and D in that they have 34 amino acids, a mature peptide and an additional stretch of 48 C-terminal residues rich in acidic amino acids such as aspartic and glutamic. The additional C-terminal sequence is not a common characteristic of molluscan AMPs reported in previous studies. Mytilin C, D and G1 display antimicrobial activities against selected Gram-positive, -negative bacteria and the fungus *F. oxysporum*. Only Mytilin C has shown antiprtozan activities against *Perkinsus marinus*, which is the causative agent of Dermo disease in oysters.

The Myticins (A, B and C) are isolated from two mussels: *M. galloprovincialis* (A and B) and *M. trossulus* (C) (Mitta *et al*., 2000c).

Mytimysin contains 12 cystine residues and shows inhibition of the filamentous fungi *Neurospora crassa* and *Fusarium culmorum* (Charlet *et al*., 1996).

These muscle defensins, MGDs, mytilins and myticins have shown greater antibacterial activity against Gram-positive bacteria than against Gram-negative. Production of active, mature AMPs involves several sequential steps using proteasses. Initially, AMPs are synthesized as pre-propeptides. They are then cleaved from the signal sequence and modify the proregion through various proteases. Mollusk AMPs, such as mussel mytilin (Mitta *et al*., 1999a), myticin (Mitta *et al*., 1999b) and MGDs (Mitta *et al*., 2000a), have shown this phenomenon.

35.3.2 AMPS in Oysters

The first oyster defensin (AMP) was purified from the gill extract of American oyster (*Crassostrea virginica*) (AOD) using chromatography techniques. AOD has shown great antibacterial activity against both Gram-positive (*Lactococcus lactis subsp lactis*, *Staphylococcus aureus*) and G-negative bacteria of *E. coli* D31 and *Vibrio parahemolyticus* (Seo *et al*., 2005). Pacific oyster (*Crassostrea gigas*) consists of another three defensins (Cg-Defm, Cg-Defh1 and Cg-Defh2) clustered in three separate groups (Schmitt *et al*., 2010). Cg-Defm has been isolated from the mantle tissue of *C. gigas* (Gueguen *et al*., 2006), while Cg-Defh1 and Cg-Defh2 are isolated from hemocytes. Synthesis of *Cg-defm* is different from that of mussel defensin, since there is no N-terminal pro-region or C-terminal precursor extension in Cg-defm. All of these *C. gigas* recombinant defensins have greater activity at low concentrations against Gram-positive strains of *Micrococcus lysodeikticus*, *Bacillus megaterium*, *Staphylococcus aureus*, *S. simulans*, *S. hemolyticus*, *Mycobacterium maritypicum* and *Brevibacterium stationis*. On the other hand, all three defensins are

weakly active against Gram-negative bacteria such as *Vibrio splendidus*, *V. aestuarianus*, *V. angularum*, *V. nigripulchtritudo* and *E. coli*. Comparative antibacterial-spectrum analysis has been conducted with these three defensins, using their recombinant peptides, and it has been concluded that all of them are mainly active against Gram-positive bacteria, with Cg-Defm and Cg-Defh2 being more potent than Cg-Defh. Cg-Def2 has shown more potent activities than Cg-Defh1. Moreover, it has been shown that all oyster defensins can inhibit peptidoglycan biosynthesis by binding to lipid II (Schmit *et al*., 2010). These results suggest the evolutional conservation of bacterial cell-wall precursor lipid II-binding residues in oyster defensins. Recently, studies have been carried out to determine the bacterial (*S. aureus*) growth-inhibition mechanisms of oyster defensin Cg-Defm, Cg-Defh1 and Cg-Defh2, taking into consideration membrane integrity, inhibition of peptidoglycan biosysntheis and activity of lipid II binding (Schmitt *et al*., 2010). From the results, it has been suggested that the first action of oyster defensin mechanisms is binding to lipid II, followed by inhibition of peptidoglycan biosynthesis.

Very recently, three *C. gigas* big defensin isoforms (C*g*-BigDef1, C*g*-BigDef2 and C*g*-BigDef3) have been identified using a high-throughput gene-expression approach (Rosa *et al*., 2011). Moreover, several isoforms related to C*g*-BigDef1, C*g*-BigDef2 and C*g*-BigDef3 have been discovered by analyzing GigasBase (http://www.ifremer.fr/GigasBase), suggesting that big defensins might be multi-isoform AMPs in oyster and that they may exist in other mollusks as well. The antimicrobial activities of oyster big defensins have not been investigated as yet, but tranacriptional upregulation of C*g*-BigDef1 and C*g*-BigDef2 was noted upon bacterial challenge. These findings indicate that defensins are the major AMPs in oyster. Nonetheless, other AMPs must also be screened in this commercially important mollusk.

35.3.3 AMPS in Abalones

Abalone is a commercially important marine mollusk with a characteristic single shell. It belongs to the family *Haliotidae* (Lee & Vaequier, 1995). At present, only three AMPs have been reported from two abalone species, namely the disk abalones *Haliotis discus discus* and *Haliotis discus hannai*. *H. discus discus* defensin has a mature peptide (49 amino acids) with the characteristic features of AMPs, including net positive charge (+5), higher hydrophobic residues (46%) and six cysteine residues (De Zoysa *et al*., 2009). The predicted three-dimensional structure of *H. discus discus* defensin shows similarity to that of mosquito (*Anopheles gambiae*) defensin, with 42.8% sequence identity. The *H. discus discus* defensin molecule consists of an α-helical part (AHCLVK) and three crosslinked disulfide bonds at $^{27}C_1-C_4{}^{58}$, $^{44}C_2-C_5{}^{63}$ and $^{48}C_3-C_6{}^{65}$. *H. discus discus* defensins shares a classical disulfide linkage structure (C_1-C_4, C_2-C_5, C_3-C_6) similar to that found in invertebrate defensins. However, the number of cysteine residues presented in abalone defensin stands in contrast to that in mussel defensins (MGD1 and MGD2) and oyster defensin (CgDef), with two additional cysteine residues in the latter peptide sequences (Gueguen *et al*., 2006). It is expressed constitutively in hemocytes and other major tissues. Defensin gene transcripts have been induced in hemocytes, gills and the digestive tract in a tissue-specific manner by bacterial infection, which is an indirect indicator of the induction of mRNA expression against pathogens in those peptides. The antimicrobial activities of two defensins of *H. discus discus* and *H. discus hannai* have not been investigated.

Another AMP developed from abalone is histone-derived AMP, known as Abhisin (De Zoysa *et al*., 2009). This is an endogenous AMP derived from the N-terminal region

of histone H2A in disk abalone (*Haliotis discus discus*). Abhisin shows the characteristic AMP features, including 4.32 kDa molecular mass, +13 net positive charge, 27% hydrophobic ratio, 2.82 kcal/mol protein-binding potential and a linear α-helical structure without cysteine. Synthetic abhisin exhibits antimicrobial activity, especially at higher concentrations, against pathogenic *Listeria monocytogenes*, *V. ichthyoenteri* and *P. ovale*, which represent Gram-positive, Gram-negative bacteria and fungi, respectively. However, stronger antimicrobial activity is displayed against fungi than against bacteria. Histone H2A transcription has been significantly induced in abalone gills and digestive tract tissues at an early stage (3 hours) of bacterial challenge. These results suggest that abhisin is a potential antimicrobial agent and that its precursor histone H2A may be involved in the innate immune-defense system in abalone. In frog, histone H2A derived from buforin IIb is cytotoxic to cancer cells but not to normal eukaryotic cells (Lee *et al*., 2006). In preliminary studies, synthetic abhisin has shown inhibition of cancer cells. Abhisin treatment (50 µg/ml) decreases the viability of THP-1 leukemia cells by approximately by 25%, but not in normal fibroblast vero cells, suggesting that abhisin may have selective cytotoxicity to cancer cells over normal cells. The outer surface of the plasma membranes of cancer cells contain negatively charged gangliosides, while the surfaces of the normal plasma membranes of mammalian cells are generally composed of neutral zwitter-ionic phospholipids and sterols (Lehrer *et al*., 1993). This selective cytotoxicity against cancer cells has been explained as an interaction between positively charged buforin IIb and negatively charged gangliosides. A similar mechanism may be involved in reducing the cell viability of THP-1 cells, since both abhisin and buforin IIb have similar peptide residues.

Representatives of histone-derived AMPs from mollusks and vertebrates are compared in Table 35.2. This comparison suggests that mollusks may have similar antimicrobial and other therapeutic properties, since they all share some common characteristics.

35.3.4 AMPS in Clams

Clams are another economically important marine bivalve in aquaculture. The first evidence of clam AMPs was reported in Manila clam (*Ruditapes philippinarum*) (Kang *et al*., 2006). There were five express sequence tags (ESTs) in the *Perkinsus olseni*-challenged clam cDNA library, which showed similarity to defensin-like APMs of mussel MGD-1. The clam myticins (isoform 1, 2 and 3) and mytilin cDNA sequences have been isolated from hemocytes of carpet-shell clam (*Ruditapes decussates*) by suppression subtractive-hybridization technique (Gestal *et al*., 2007). These peptides show similarity with mussel myticin and mytilin molecules. No antimicrobial activity has been reported from clam myticins and mytilins, but transcription of these AMPs was induced by *Vibrio anguillarum* challenge, indicating that they may cause bacterial inhibition at the protein level. A recent study describes the Manila clam defensin (MCdef), which encodes a 44-amino-acid mature peptide with eight cysteines and a 4.9711 kDa molecular mass (Adhya *et al*., 2012). The MCdef cDNA sequence was originally cloned using the RNA from hemocytes, but it has shown transcriptional expression in other tissues, such as abductor muscle, gill, mantle, foot, palp and siphon. These results indicate that AMPs are expressed in nonimmune tissues or cells in mollusks in a similar way to other vertebrate animals.

The predicted three-dimensional structure of MCdef is matched with *C. gigas* defensin, showing overlap of the helix and β-sheet regions and other residues, but not of the amino acid insertions in the loop areas. The antibacterial activity of MCdef has been investigated with a broad range of bacteria, and strong inhibition of *Streptococcus iniae* and

S. aureus was shown at 1.25–2.50 μM. However, MCdef activity was low against Gram-negative *V. logei* and *V. salmonicida*. Moreover, a big defensin-family AMP has been cloned using cDNA constructed from hemocytes of *V. anguillarum*-challenged *Venerupis philippinarum* (Zhao *et al*., 2011). AMP prediction analysis has shown that *V. philippinarum* big defensin (VpBD) has the characteristic features of helix structure, net positive charge (+5) and hydrophobic residues (36%). Six cysteines of VpBD have been arranged in a consensus pattern of C-X6-C-X3-C-X13(14)-CX4-C-C, as reported in horseshoe crab and bay scallop big defensin. The bacterial growth-inhibition activity of recombinant VpBD is confirmed against various bacteria, both Gram-positive (*S. aureus*, *M. luteus*, *Bacillus* sp.) and Gram-negative (*V. anguillarum*, *V. ichthyoenteri*, *Pseudomonas putida*, *Proteus mirabilis*, *Enterobacter* sp.). Moreover, mRNA expression of VpBD is upregulated in hemocytes after vibrio challenge, showing that the activation of transcription towards the production of protein has antibacterial effects. As with other mollusks, most clam AMPs have not been investigated for a wide range of pathogenic agents or other therapeutic applications.

35.3.5 AMPS in Other Mollusks

The first mollusk big defensin (AiBD) has been cloned from bay scallop (*Argopecten irradians*) by a combination of expressed sequence tag (EST) and rapid amplification of cDNA ends (RACE) techniques (Zhao *et al*., 2007). The mature peptide of AiBD consists of 84 amino acids, with a predicted molecular mass of 9.22 kDa. Its N-terminal hydrophobic domain has a positive net charge (+4) and its C-terminal defensin contains a net charge of +6, which are important for maintaining amphipathic characteristics. It has been suggested that the defensin domain may attach to the negatively charged bacterial membrane of AiBD, while the hydrophobic sequence could promote the permeability of the bacterial membranes. AiBD mRNA expression has been detected in hemocytes and gills and is induced by bacterial challenge (*V. anguilarum*). Recombinant big defensin has shown strong antimicrobial activities against Gram-positive bacteria (*Micrococcus lysodikicus*, *S. aureu*) and yeast but was moderate or weak against Gram-negative bacteria (*V. anguillarum*, *V. splendidus*). The first histone H2A-derived AMP has been developed in mollusks using the cDNA of histone H2A of scallop (*Chlamys farreri*). The N-terminal fragment containing 39 amino acids of scallop H2A is recombinantly purified. It shows that scallop histone-derived AMP has 2.5 times more antibacterial activity against Gram-positive (*M. luteus*) than against Gram-negative (*V. splendidus*, *V. anguillarum* and *V. vulnifi*cus) bacteria. Furthermore, it shows fungicidal activity when inducing recombinant protein expression in yeast (*Pichia pastoris*) (Li *et al*., 2007). However, identification of AMPs in other mollusk species is very limited and large-scale screening programs are needed.

35.4 APPLICATIONS OF MOLLUSK-DERIVED AMPS

AMPs from marine mollusks have been investigated only in limited species, such as mussels, oysters, clams and abalones. Most of them are commercially important in aquaculture. Different AMP members (isoforms) of a single family can be expressed or present in different tissues (e.g. defensins in plasma, hemocytes, mantle and digestive tract). It is necessary to screen a wide range of mollusk species in different habitats and tissues in order to isolate different families of AMPs. Also, AMPs may not be constitutively

expressed in mollusks and determination of the inducible factors required for higher production of AMPs would be greatly benefited by the isolation and purification of AMPs on a large scale. Alternatively, cloned gene sequences of AMPs could be used as a template for constructing synthetic AMPs or producing recombinant peptides, which would allow understanding of their structure and bioactive function. Post-translational modification is important for AMP activity, especially for evaluation of antimicrobial properties. Eukaryotic expression systems are more efficient than bacterial expression systems at producing more active recombinant AMPs, since they can achieve the correct disulfide bond linkages during translational modifications.

It is reported that synthetic fragments of mussel Mytilin B (C10C) show antiviral activities against white-spot-syndrome virus, which causes mortality of *Palaemon serratus* shrimp (Roch *et al*., 2008). This suggests their potential use as antiviral agents in aquaculture. Such small peptides will be easy to synthesize on a large scale for application to biotechnological developments. Some defensins from plants and insects have shown antifungal activity via membrane-disruptive agents or fungal glucosylceramides (Thevissen *et al*., 2004). However, very few mollusk defensins or AMPs have been tested for anitifungal activities, which will be required to expand development of antifungal agents. Some vertebrate AMPs such as buforin II have anticancer properties. Some mollusk AMPs, such as abhisin, have high identity with buforin II and show cytotoxicity against THP-1 leukemia cells. Therefore, further investigation of mollusk AMPs for anticancer properties will be required.

Development of anti-infective drugs using AMPs has several advantages and some disadvantages or limitations. A broad spectrum of activity, rapid action against deadly pathogens and potentially low levels of induced resistance are some of the advantages. Disadvantages include susceptibility to proteolysis, reduced activity based on salt, pH and so on, systemic and local toxicity, allergy after repeated application, confounding biological functions (angiogenesis) and higher manufacturing costs. These have limited the clinical application of some AMPs.

Future research should focus on isolation of AMPs from different mollusks, which will help to diversify the different AMP families and their potential therapeutic applications in the pharmaceutical industry. This information will allow comparative studies of various AMPs from mollusks of different phyla and thus increase our understanding of their evolutionary origin. More importantly, it will facilitate further understanding, development, design and synthesis of more efficient, broad-spectrum AMPs from marine mollusks.

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36 Protein Hydrolysates and Bioactive Peptides from Seafood and Crustacean Waste: Their Extraction, Bioactive Properties and Industrial Perspectives

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36.1 INTRODUCTION

The concept of marine organisms as a source of new substances is of growing importance. The biological and chemical diversity of the marine environment provides an unlimited source of new active materials for the development of bioactive compounds. Marine organisms contain high-value-added compounds such as minerals, lipids, amino acids, polysaccharides and proteins. These compounds have unique biological and chemical features and, most importantly, their highest concentration is often found in parts of the marine organisms that are commonly discarded and considered as waste. Marine-organism waste materials such as heads, viscera, skin, tails, offal and blood, as well as seafood shells, exhibit a variety of high-value-added substances that can be exploited in improving and supporting human health and which can make excellent sources of pharmaceutical and neutraceutical ingredients, with specific effects against various diseases. More than 91 million tons of fish and shellfish are caught every year. The annual discard from fishing and aquaculture is estimated to be approximately 25% of the total catch (Fishery Statistic Data, Eurostat, 2006; Rustad, 2003). Fish filleting, salting and smoking generate the major part of solid waste and byproducts (50–75% of the fish). Fish canning is regarded as the second major source of solid waste and byproducts (30–65% of the fish) (AWARENET, 2004). Crustacean and mollusk processing also generates significant amounts of solid residues. It is estimated that only 43% of catches are utilized as products for human consumption (Archer *et al*., 2005).

The management of marine waste has been a major problem, with a great influence on the environment. Discharge of waste and byproducts by fisheries and seafood industries is on the rise, driven by both a net increase in fishery and seafood product consumption and the changing consumer trend towards ready-to-eat (RTE) products. Proteins, polysaccharides and lipids, including the minerals from seafood, possess unique features, some

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of which are a consequence of specific environmental factors prevailing in the marine environment (Shahidi, 1997). Byproducts generated from the seafood industries typically range between 20 and 60% of the starting raw material. Offal, heads, shells and tails are the major residues, while skin, bones, blood and frames are the second major residues. Treated marine waste has various applications, among which the most important are animal feed, biodiesel/biogas, dietary products (chitosan), natural pigments (after extraction), food-packaging applications (chitosan), cosmetics (collagen), enzymes, soil fertilizer and moisture maintenance in foods. Marine waste is now utilized for the isolation of bioactive compounds, especially proteins and peptides, whose potential applications greatly increase their value. Recent research has discovered various bioactive compounds in fish-muscle proteins, collagen and gelatin, fish oil, fish bone, internal organs and shellfish and crustacean shells. Seafood has been found to be a well-balanced source of proteins and peptides. It contains high level of minerals and trace elements, such as selenium and iodine, and high levels of vitamins, such as A, D and B_{12} (Zhang *et al*., 1993). Proteins and peptides obtained from seafood and its waste are nutritionally valuable and easily digestible, with a well-balanced amino acid composition (Venugopal *et al*., 1996).

Bioactive compounds from marine waste can be isolated and purified using various technologies, both simple to complex. Such compounds include bioactive peptides, oligosaccharides, fatty acids, enzymes, water-soluble minerals and biopolymers for biotechnological and pharmaceutical applications. In marine waste, biologically active peptides are of prime importance, due to their unique impact on human health. Various hydrolytic enzymes have been isolated from microbes, plants and animals for the hydrolysis of marine-waste products. These protein hydrolysates differ in their physicochemical properties and biological activities depending on their amino acid sequences and molecular weights. Membrane filtration by an ultra/nanofiltration membrane system with a suitable molecular weight cut-off is employed in separating peptides with desired molecular weights from marine-waste protein hydrolysates. Protein hydrolysates from the byproducts of several marine species have been analyzed for their nutritional and functional properties, with a focus on the possibility of obtaining biologically active peptides.

36.2 OVERALL CHEMICAL COMPOSITION OF SEAFOOD AND CRUSTACEANS

Seafood and crustaceans are primarily composed of muscles, shells and bones. These marine animals do not require extensive connective tissue or bones like other animals, because their body weight is supported by water. Marine animals like fish are cold-blooded and their muscle composition is different from that of warm-blooded animals as their body temperature reflects the water temperature.

Seafood and crustaceans are a rich source of nutrients. The macronutrients found in seafood and crustaceans include protein and lipids. All other nutrients found in seafood are considered micronutrients and are of minor significance. Fish contains 63–84% water, 14–24% protein and 0.5–17.0% lipid by weight, depending upon the species. The primary source of fish protein is muscle, and the protein quality is comparable to that of other animal proteins, for example from milk, eggs and beef. The muscle of fish and shellfish has very little connective tissue and is readily hydrolyzed upon heating, resulting in a product that is tender. Seafood contains lipid in the form of triglycerides or triacylglycerols. Triglycerides contain the long-chain polyunsaturated fatty acids 20:5ω3 eicosapenoic acid

(EPA) and 22:6ω3 docosahexanoic acid (DHA). The macronutrients in crustaceans and mollusks differ from those in bony fish. Mollusks contain more carbohydrates than fish. Seafood is a good source of vitamins such as A, D, B_6 and B_{12}. It also contains minerals in abundance, especially iodine and selenium. This chapter will mainly focus on proteins, peptides, amino acids and protein hydrolysates from seafood waste.

Fish- and shellfish-muscle proteins are classified into three main groups: sarcoplasmic, myofibrillar and stroma proteins. Fish muscles are arranged in the form of blocks, called myotomes. Each myotome is linked to the next by a thin layer of collagenous connective tissue called myocommata. This connective tissue is very thin and easily breakable. Fish is composed of three major components: white or ordinary tissue, red or dark muscle and connective tissue. Each type of muscle exhibits a different function and structure, but all are mainly composed of proteins, lipids and water, as shown in Table 36.1. White muscles contain more protein than dark muscles.

Fish skin and bones contain high bulk amounts of collagen and gelatin (Kim & Mendis, 2006), except in the muscles. Fish collagen and gelatin are receiving a great deal of attention due to their diverse applications, including in the food, pharmaceuticals, cosmetics and biomedical industries. Marine-derived processing waste has also been found to contain large amounts of these structurally diverse proteins. In general, this waste consists of undersized fish and shellfish, trimmings, fins, frames, heads, skin and viscera. Cod head, which accounts for approximately 20% (w/w) of the fish weight, consists of 55% (w/w) muscle, 20% (w/w) bone, 10% (w/w) gill, 5% (w/w) skin and 4% (w/w) eyes. It is a good source of collagen and other proteins (Venugopal, 2009). The discarded head and shell after shellfish processing account for approximately 30–45% (w/w) of the unprocessed weight and contain a good amount of protein and peptides, especially in shrimp and prawn.

Collagen is triple-helix protein that is rich in nonpolar amino acids such as glycine, valine, proline, alanine and hydroxyproline (Gomez-Gillen *et al*., 2002; Kim & Mendis, 2006). Its primary sequence contains repeated glycine-proline-hydroxyproline-glycine-A-A amino acid sequences (Vercruysse *et al*. 2005). Gelatin is the hydrolyzed form of collagen and can easily be converted by heat denaturation. It is a soluble protein compound

Table 36.1 Comparison of protein contents in raw, edible seafood, including bones, cartilage and shells. Adapted from Hui *et al*. (2006).

Species	Protein (%)
Cod	17–19
Herring	18–20
Mackerel	17–19
Sardine	15–17
Salmon	19–21
Skate	14–16
Tuna	22–24
Crab	19–20
Prawn	17–18
Octopus	29–30
Crayfish	16–17
Mussel	23–24
Oyster	18–19

obtained by partial hydrolysis of collagen, the main fibrous protein constituent of bones, cartilage and skin; therefore, the source and type of collagen and the age of the animal are intrinsic factors influencing the properties of a gelatin (Hinterwaldner, 1977; Regenstein & Zhou, 2007). Fish gelatin is different from mammalian gelatins in properties such as melting and gelling temperatures and gel strength (Karim & Bhat, 2009). The difference is due to the different amino acid compositions, especially proline and hydroxyproline. These two amino acids are responsible for the stability of the collagen structure. Proline and hydroxyproline form hydrogen bonds which stabilize the triple-helix structure by sharply twisting the collagen.

The myofibrilar proteins from marine vertebrates (e.g. fish) and invertebrates (e.g. mollusks and crustaceans) are very similar, with myosin, actin and collagen present in both (Toppe *et al*., 2007). Differences in quantities and amino acids are likely to occur, depending on the species. Clustal W sequence alignments of actin sequences from marine vertebrate species such as salmon and invertebrate species such as oyster show high sequence homology (Vercruysse *et al*., 2005). Some proteins, such as paramyosin, are only present in mollusks and crustaceans and not in marine fish. Paramyosin ranges from 3 to 19% (w/w) for scallops, squid and oysters (Venugopal, 2009). It contains high levels of glutamic acid (up to 23% w/w), followed by arginine (12% w/w) and lysine (9% w/w), with trace amounts of proline (Venugopal, 2009).

Crawfish waste is an excellent source of protein (23.5% on a dry-weight basis). Crawfish chitin contains residual amino acids, which are evidence of the presence of proteins covalently bound to it (Meyers *et al*., 2008). The chemical composition of crawfish whole meal and shell is illustrated in Table 36.2.

Among crustaceans, shrimp has been explored the most extensively by various researchers. Shrimp is divided into two portions: edible (muscle or meat, 65% (w/w)) and inedible (heads, shells and tails, 35% (w/w)) (Meyer, 1986). Of the edible portion, the approximate composition of raw shrimp meat is 75–80% water, 18–20% protein and about 1% fat. Cooked meat contains 65–70% water, 25–30% protein and about 1% fat. As for the inedible portion, research shows that shrimp waste contains substantial amounts of nutrients. For example, Cao *et al*. (2008, 2009) report that the crude protein, ash and fat values of *Penaeus vannamei* head are 60.3%, 19.9% and 7.9%, respectively,

Table 36.2 Chemical composition of crawfish whole meal and shell. Adapted from Meyers *et al*. (1986).

Composition	Whole meal	Shell
Crude protein (%)	35.8	16.9
Fats (%)	9.9	0.6
Fiber (chitin) (%)	16.5	23.6
Ash (%)	38.1	63.6
Minerals		
Ca (%)	12.3	24.8
P (%)	0.8	1.0
K (%)	1.0	0.1
Mg (%)	0.2	0.3
Mn (ppm)	545	200
Fe (ppm)	1610	180
Astaxanthin (ppm)	78	108

Table 36.3 Amino acid composition of proteins from shrimp (*Penaeus vannamai*) head.

	Free amino acid	Amino acid
Asparagine	15.40	50.89
Threonine	16.30	19.20
Serine	9.64	15.67
Glutamic acid	20.45	67.41
Proline	12.99	29.02
Glycine	18.62	43.30
Alanine	20.71	32.5
Cystine	3.57	2.14
Valine	17.37	28.75
Methionine	7.14	13.57
Isoleucine	14.87	23.57
Leucine	25.76	37.50
Tyrosine	15.36	18.88
Phenylalanine	19.69	26.16
Lysine	24.55	34.20
Histidine	7.28	11.74
Arginine	30.00	31.43
Tryptophan	4.33	5.63

on a dry-weight basis. Much research has been conducted on the protein content of shrimp waste. *Metapenacus affinis* contains 10.81 g protein/100 g (Zhu *et al*., 2003) and *Penaeus vannamai* 13.8 g protein/100 g (Cao *et al*., 2009). The amino acid composition of *Penaeus vannamai* head is shown in the Table 36.3.

Protein hydrolysates from shrimp head contain abundant amounts of amino acid. Arginine, leucine, proline, isoleucine and glutamic acids are mostly found in *Penaeus monodon* and *Penaeus vannamei*. The main free amino acids of shrimp meat (muscle) are arginine, glycine, porline and glutamine, while those of shrimp byproducts are arginine, phenylalanine, tyrosine, proline, threonine and leucine (Heu *et al*., 2002).

The main endogenous enzymes found in crustaceans and marine fish are protease, α-amylase, lipase, catalase, peroxidase, alkaline phosphatase, polyphenol oxidase, chitinase and hyuluronudase (Haard, 1995; Sutthitham, 2001). The enzyme in shrimp meat (*Litopenaeus vannamei*) is aspartic proteinase (Eakpetch *et al*., 2008).

36.3 EXTRACTION OF PROTEIN HYDROLYSATES AND BIOACTIVE PEPTIDES FROM SEAFOOD AND CRUSTACEAN WASTE

Numerous bioactive proteins and peptides have been reported from fish sources but those from other marine organisms such as mollusks and crustaceans still need to be explored. Marine byproducts from onboard and onshore processing plants might also be utilized as valuable raw materials for marine proteins and peptides.

Fish skin and bones can be used as sources for the extraction of collagen and gelatin. There are several methods for the isolation of proteins and peptides from marine waste, based on the demand, nature and amino acid composition and sequence of the protein.

In addition to nutrition, these bioactive peptides also have pharmaceutical and health applications. Bioactive peptides and protein hydrolysates can be produced from marine waste by solvent extraction, enzymatic hydrolysis or microbial fermentation. Enzymatic hydrolysis is preferred in the food and pharmaceutical industries as the other methods produce residual organic solvents or toxic chemicals in the final product. Bioactive peptides are inactive within the parent protein, but as they are released by enzymatic hydrolysis they may present various physiological functions based on their structure and amino acid composition.

Extraction is an important process in the food, flavor, fragrance and pharmaceutical industries, allowing beneficial natural compounds to be collected from many plant and animal sources. It involves a separation process, consisting in the separation of a substance from a matrix. The traditional is Soxhlet extraction, but this requires large quantities of solvents and is very time-consuming. Novel extraction methods used in some industries include microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and supercritical-fluid extraction (SFE) (Wang & Weller, 2006).

Sarcoplasmic proteins can be extracted from minced meat by filtering the supernatant and using the resulting pellet for myofibrillar extraction (Sanz *et al*., 1999). The pellet is then suspended in phosphate buffer containing Triton X-100 and homogenized. After centrifugation, the pellet is washed three times by suspending it in the same buffer system in order to remove the muscle protease. It is then suspended in phosphate buffer containing potassium iodide and undergoes centrifugation again. A washed pellet is obtained.

Recently, an alkali solubilization method has been developed for the extraction of myofibrillar proteins. In this method, the minced meat is homogenized with nine parts of cold de-ionized water and the pH is adjusted to 11.0 using sodium hydroxide 2 M solution in order to solubilize the proteins (Raghavan *et al*., 2009). The mixture is then subjected to centrifugation and incubated at 4 °C for 30 minutes. The supernatant is filtered through double-layered cheesecloths. The pH of the supernatant is then adjusted to 5.5 by 2 M HCl in order to precipitate the myofibrillar proteins.

An alkaline extraction method completed by acid precipitation is the most common conventional method used for the recovery of protein from meat, meat-processing and fish- and seafood-processing byproducts. Both vertebra and rib-bone protein have the best recovery at solution of 1 : 10 (v/v). Dialysis produces better recovery from acid precipitation if the protein concentration in a mixed solution is low. If the concentration is high, acid precipitation gives better results (Boles *et al*., 1992, 2000). According to Omana *et al*. (2010), increasing the pH increases the yield of protein extract. Protein yield was found to be highest at pH 12 for all types of sample. Higher pH gives more yield because it allows the solvent to enter and contact the target compounds. However, higher pH also causes protein denaturation. pHs of 10.5 and 11 have been found not to affect the properties of various proteins from animal sources, which remain in the same native conformation as in the raw material. The emulsification capacity of a protein is higher at pH 11, with little difference between samples. The foam-expansion property increases with increasing alkalinity.

The extraction solvent used should be suitable for the target compound. Different solvent systems give different yields and extract compositions. For example, the normal solvent for plant sources is hexane, but alternative solvents can be used if there are health, environmental or safety concerns. However, these alternative solvents sometimes result in a lower recovery due to a decline in molecular affinity between solute and solvent. Also, alternative solvents can sometimes be more expensive (Li *et al*., 2004).

Gelatin is produced from marine waste by acid or alkaline treatment of bones and skin, which give gelatin of type A and type B, respectively. Gelatin is used extensively today in functional foods and pharmaceuticals. Various methods are employed for the extraction of gelatin from marine waste. For example, skin is cleaned with sodium hydroxide (0.8 M) and water, then treated with sodium hydroxide (0.2 M) and acetic acid (0.05 M). It is kept in distilled water at 45 °C for 18 hours. Muyonga *et al*. (2004) introduced a method in which bones are pretreated by tumbling in water at 35 °C and then demineralized by 3% (v/v) HCl at a moderate temperature (20–25 °C) until they lose their hard cores. The gelatin is extracted at various temperatures (50, 60 and 70 °C) and then boiled for 5 hours.

Collagen and gelatin extraction has been reported from megrim skin (Montero & Gomez-Guillen, 2000), Alaska pollock skin (Zhou & Regenstein, 2004), sin croaker and shortfin scad skin (Cheow *et al*., 2007), grass carp skin (Kasankala *et al*., 2007), shark cartilage (Cho *et al*., 2004), yellowfin tuna skin (Cho *et al*., 2005), bigeye snapper and brown-stripe snapper skin (Jongjareonrak *et al*., 2006), channel catfish skin (Liu *et al*., 2008; Yang *et al*., 2007) and Nile perch skin and bone (Muyonga *et al*., 2004). However, utilization of fish scales for gelatin extraction has only been reported from sardine fish (Harada *et al*., 2007) and Asian carp (Wang & Regenstein, 2009), while collagen has been extracted from the scales of sea bream and red tilapia (Ikoma *et al*., 2003), sardine, red sea beam and Japanese sea bass (Nagai *et al*., 2000), deep-sea redfish (Wang *et al*., 2008) and carp (Duan *et al*., 2009).

The skin of Nile perch (*Lates niloticus*) is treated with sulfuric acid (pH 2.5–3.0) at a ratio of 1 : 2 (w/v) in order to extract gelatin (Muyonga *et al*. 2004). The skin from adult fish produces a gelatin with a lower gel-setting time and higher viscosity than that from young fish. Jongjareonrak *et al*. (2006) used an alkaline process to pretreat bigeye snapper (*Priacanthus macracanthus*) and brown-stripe red snapper (*Lutjanus vitta*) skins in order to remove debris, then used an acid process to extract the protein. Benjakul *et al*. (2009) also studied the characteristics of the gelatin from bigeye snapper skin in two species: *Priacanthus tayenus* and *P. macracanthus*. Pretreatment involved an alkali process followed by an acid process, with continuous stirring for 2 hours at 45 °C. The amino acid composition of both gelatins had a range of 186–187 mg/g sample. The optimal conditions for the extraction of gelatin from the skin of yellowfin tuna (*Thunnus albacares*) were a concentration of 1.89% (w/v) NaOH for about 3 days followed by extraction with hot water at 58 °C for 5 hours.

Hydrolysis (hydro = water, lysis = splitting) is a physical, chemical or biological reaction in which a larger compound is broken down into smaller constituents by the addition of molecules of water. One fragment of the original compound gains an H^+ and the other the remaining OH^-. Hydrolysis can be catalyzed by enzymes, metal salts, strong acids such as concentrated hydrochloric acid (6 M HCl) or concentrated sulfuric acid (H_2SO_4) and strong bases such as 6 M sodium hydroxide (NaOH). Although enzymes are used as catalysts to speed up the reaction, they can be eliminated at the end.

Proteins are the main organic compounds found in animal cells. Their building blocks are amino acids. Amino acids link with the peptide bonds and fold together to form the protein. A peptide bond is formed by the removal of a molecule of water (H_2O) or by condensation reaction. Thus, the H^+ and OH^- ions must be replaced by additional water when the bond is broken. Generally, this reaction occurs in the digestive process and through hydrolysis. Proteins are broken down into polypeptides, smaller peptides and free amino acids and are utilized by the cells. The product of protein hydrolysis is a

protein hydrolysate. The solution containing the pieces of the protein (smaller peptides and free amino acids) is called a protein hydrolysate solution.

Protein hydrolysates have been produced from many sources, such as soy bean, mung bean, wheat, sesame meal and seafood waste (fish processed waste, shrimp head, etc.). Protein hydrolysates have many applications in a variety of industries, including human nutrition, cosmetics and pharmaceuticals, and have been used to enhance the functional, nutritional and organoleptic values of food.

There are many methods by which to hydrolyze proteins into their components, including chemical (acid and alkaline hydrolysis), biological (lactic acid fermentation, enzymatic hydrolysis and autolysis) and physical (microwave and ultrasound extraction). Seafood protein hydrolysates are used in various food ingredients and for various food applications, including in fish and other food formulations, seafood flavors, ingredients for functional foods, food supplements and cosmetics (Kristinsson, 2006). They can be produced by heating with acid/base or by the addition of proteolytic enzymes. The latter method is preferred because enzymes remain active under mild pH and temperature conditions, whereas acid hydrolysis by hydrochloric or sulfuric acid oxidizes cysteine and methionine, destroys some serine and threonine, and converts glutamine and asparagine to glutamate and aspartate, respectively (Bucci & Unlu, 2000, as cited by Manninen, 2004).

36.3.1 Acid–alkaline Hydrolysis of Protein Hydrolysates and Peptide Extraction

Chemical methods of protein hydrolysis are classified into two groups: acid hydrolysis and alkaline hydrolysis. Generally, acid or base is considered a catalyst and can be eliminated at the end of the reaction.

Acid hydrolysis is normally carried out under high-temperature conditions (at 110–120 °C) and over a long period (18–96 hours). Hydrochloric acid is frequently used as it increases the rate of peptide-bond cleavage over that of sulfuric acid at the same concentration (6 M). Hydrochloric acid works quickly and its hydrolysate product has a highly acceptable savory profile. Excess hydrochloric acid in hydrolysates is removed by evaporation. The protein is hydrolyzed by 6 M sulfuric acid under reflux condition for 12–24 hours. Optimal conditions are 6 M HCl over 24 hours. The yield and the highest degree of hydrolysis (DH) of sesame-meal protein hydrolysates were 53.21 and 50.45%, respectively. Some hydrolysis chemicals can be applied to shrimp byproducts, including sodium sulfite (Mizani *et al*., 2005), formic acid (Nwanna *et al*., 2004) and 1 M HCl (Kjartansson *et al*., 2006).

Alkaline hydrolysis (or saponification) is a chemical process that uses a strong alkaline in water at temperatures up to 180 °C to hydrolyze or decompose raw materials of ash. Sodium hydroxide or potassium hydroxide are generally used to break down biological materials such as carbohydrates, lipids and proteins into sugars, soaps and amino acids, respectively. Alkaline hydrolysis is commonly used for ester-linked lipids. The ester-hydrolysis products are alkanol plus either free alkanoic acid at low pH or alkanoates at moderate or high pH. Normally, this method is applied to total lipid extracts. However, it can also be applied to solid materials such as biomass, extracted residues and sediments.

Hydrolysis by base is conducted by adding calcium, sodium and potassium to solubilized heated protein at 80–130 °C. The drawback of this technique is the formation of undesirable substances such as lysioalaine, ornithinoalanine, lanthionine and ß-amino

alanine. In addition, some amino acids, such as serine and threonine, are destroyed during the process (Pasupuleti & Braun, 2010).

Acid–alkaline hydrolysis methods are simple in operation, have a low cost and short hydrolysis time and are applicable to industrial processes (Gao *et al*., 2006). However, the use of strong acids or strong base makes the hydrolysis process ecologically unacceptable (Bueno-Solano *et al*., 2009) and reduce the nutritional qualities of the products. Furthermore, sodium hydroxide (NaOH) cannot properly be used to hydrolyze animal proteins because a strong solution of NaOH can destroy some of the amino acids, such as serine, cystein, histridine and threonine. D-form amino acids are generally resistant to strong acid and strong alkali and can be simply produced by chemical methods, but L-form amino acids are easily destroyed by these types of acid and alkali. They also form toxic substances such as lysine-alanine (Clemente, 2000).

36.3.2 Biological Methods of Extraction

Biological methods of protein hydrolysis are classified into three groups: fermentation, enzymatic hydrolysis using exogenous enzymes and autolysis by endogenous enzymes.

36.3.2.1 Fermentation

Lactic acid fermentation is an ecofriendly process used to reduce strong acids and alkalis for protein hydrolysis. It has recently been used to hydrolyze many sources of protein, such as crayfish shell (Batista 1999), red crab shell (Jung *et al*., 2005), scampi byproducts and shrimp byproducts. The protein hydrolysates from shrimp byproducts following lactic fermentation have a high nutritional content (Bueno-Solano *et al*., 2009).

36.3.2.2 Enzymatic Hydrolysis

Most of the initial work on seafood-protein and other protein hydrolysis was performed in the 1960s, largely on fish-protein concentrate (FPC) (Kristinsson and Rasco, 2000). Enzymatic hydrolysis of seafood and other fish biomass has been employed as an alternative approach to the conversion of underutilized biomass into edible protein products, instead of animal feed or fertilizer. Enzymatic hydrolysis is used to produce protein hydrolysate in order to avoid severity treatments such as chemical and physical treatment (Clemente, 2000). The product of enzymatic hydrolysis has high functionality and nutritive values (Gao *et al*., 2006). It affects factors such as percentage of enzyme, protease type and water-to-substrate ratio (Cheung & Li-Chan, 2010). Moreover, many researchers have shown that pH and temperature are also affected.

Proteases (proteinases, peptidases or proteolytic enzymes) are enzymes that are involved in proteolysis. They digest protein into small polypeptides or free amino acids by destroying the peptide bonds. Proteolytic enzymes can be split into endopeptidases and exoproteases. Exoproteases hydrolyze the peptide bonds at the terminal peptide bond and release the amino acids from either the N or the C side. Endopeptidases break down the peptide bond with protein molecules at a random side. Protein hydrolysates contain free amino acids and small polypeptides. Large polypeptides (more than 12 amino acids) are not found. In hydrolysis, the endopeptidases first digest the proteins, and then the exoproteases achieve a more complete degradation. Normally, enzymes are specific to substrates. For example, neutrases have many catalyzing sites at which to break soybean proteins into peptides, while trypsin has catalyze sites only at the bonds formed by the

Table 36.4 Enzymes used to extract protein hydrolysates and peptides from seafood waste. Reprinted from Najafian & Babji (2012). Copyright (2012), with permission from Elsevier.

Enzyme	Buffer	pH	Temperature (°C)
Alcalase	0.1 M PB[a]	7.0	50
α-chymotrypsin	0.1 M PB	8.0	37
Papain	0.1 M PB	6.0	37
Pepsin	0.1 M GHB[b]	2.0	37
Neutrase	0.1 M PB	8.0	50
Trypsin	0.1 M PB	8.0	37

[a]Phosphate buffer.
[b]Glycine–HCl buffer.

carboxyl group of lysine or argenine and has a higher specificity than neutrase (Zhao & Hou, 2009).

The proteases have been used for the generation of various bioactive peptides from proteins. Proteinases from animal, plant and microbial sources have been used for enzymatic protein hydrolysis. Many bioactive peptides have been experimentally produced under specific conditions using various commercial proteases, as shown in Table 36.4.

Fish skin, bones and fins can be used as sources of collagen. Tuna fish collagen and gelatin were extracted from tuna fish waste using tuna bone powder by treating it with food-grade enzymes (Herpandi *et al*., 2011).

Kristinsson *et al*. (2006) conducted a study with the aim of evaluating the biochemical and functional properties of salmon-muscle protein by hydrolyzing it with various alkaline poteases. The exogenous proteases, such as alcalase, flavourzyme, corolase and an endogenous enzyme extract, were added at the same activity level. The findings may be beneficial in two ways:

1. Discovery of new ways of utilizing byproducts from the growing Atlantic salmon and trout aquaculture industry.
2. Provision of alternative methods of utilizing byproducts from wild Pacific salmon.

The findings of this study can be applied to any fish waste. The food-grade enzyme preparations were chosen for their highly functional hydrolysates. They used various enzymes, such as alcalase (endoproteinase from *Bacillus licheniformis*), flavourzyme (endoproteinase and exopeptidase from *Aspergillus oryzae*), corolase (endoproteinase from *Bacillus subtilis*) and an endogenous enzyme mixture extracted from the salmon pyloric ceca. The substrate was prepared by homogenizing salmon-muscle mince with distilled water to a protein substrate of 7.5% (w/v), producing 300 g.

The commonly used food-grade proteolytic enzymes are extracted from either plant, animal or microbial origins (Herpandi *et al*., 2011). Commercially available food-grade enzymes such as neutase, protease, papian, trypsin, alcalase, bromelain, protemex and flavourzyme have been used in the hydrolysis of seafood waste to extract protein hydrolysates and peptides. The optimal range of proteases is gained at 50 °C (He *et al*., 2007).

The first step of fish-protein hydrolysate production involves mincing and homogenizing raw material in order to obtain a uniform consistency and ensure good enzyme

access (Slizyte *et al*., 2005). The temperature and pH of minced marine proteins are adjusted to optimal conditions so as to allow adequate digestion by the enzyme. Either endogenous or exogenous enzyme can be used. Endogenous proteolytic enzymes present in the muscle or viscera of fish, including pepsin, trypsin and chymotrypsin, are active at very low temperatures, which minimizes microbiological and quality problems. Nevertheless, variations in enzyme level and activity may result in a lower yield. Exogenous protease can be obtained from microorganisms, both animal (pepsin and trypsin) and plant (papain, bromelain and ficin) (Guerard, 2006). Exogenous enzymes derived from nonpathogenic microorganisms have been widely used commercially. A combination of endo- and exopeptidases is usually used, due to the reduction in the time required to achieve a similar DH and the better control of hydrolysis, which allows a more consistent molecular-weight profile and peptide composition to be obtained (Cinq-Mars & Li-Chan, 2007). The digestion is allowed to proceed for a duration ranging from less than 1 hour up to several hours, depending on the activity of the enzyme, the temperature and other factors such as the target molecular weight range of the resultant peptides. Care must be taken during hydrolysis or the formation of small and highly soluble peptides which lack the functional properties of native protein will take place (Venugopal, 2009). As reported by Thorkelsson *et al*. (2009), the type of enzyme, DH and amino acid sequence affect the properties of bioactive peptides. These researchers concluded that small peptides (2–8 amino acid residues) possess high ACE-inhibitory activity, whereas larger peptides (5–14 amino acids residues) show high antioxidative properties *in vitro*. In order to terminate the hydrolysis reaction, heat treatment or pH adjustment is applied, depending on the enzyme type. The solid is separated from the mixture. Centrifugal separation, including separators, decanters and clarifiers, is applied. The supernatant or hydrolysate is adjusted to neutral pH and dehydrated into powder form.

The advantages of enzymatic hydrolysis are accelerated hydrolysis reaction, mild reaction conditions, minimized undesirable reaction and a reduction in waste-product formation (Simpson *et al*., 1997). However, disadvantages include enzyme production, enzyme recovery and a slow reaction rate (Gao *et al*., 2006). Furthermore, because of the presence of bile whole fish and fish viscera, a bitter taste can be found in the protein hydrolysate (Rustad, 2003).

36.3.2.3 Autolysis

An economical and efficient alternative method for the recovery of protein is autolysis. This involves endogenous enzymes (lipases, cathepsin, phosphorylases and gut enzymes) available in seafood and its waste. These endogenous enzymes degrade the tissues when fish and crustaceans spontaneously die. Thus, autolysis depends on the action of these enzymes on their shelves (Rustad, 2003). Autolysis is related to proteolytic reactions involving soluble endogenous enzymes and the insoluble substrate from fish and crustacean waste. The effect of autolysis conditions depends on the temperature of incubation, pH and substrate concentration. The optimal conditions for autolysis of shrimp waste are substrate concentration 23% (w/v), pH 7.85 and temperature 50 °C. At these conditions, a higher DH, close to 45% (w/w), is obtained (Cao *et al*., 2008). Protein recovery from autolysis of shrimp head by gradual temperature rise (5 °C every half an hour, from 40 to 70 °C) is higher than for autolysis at a constant temperature. The maximum protein recovery to be obtained was 87.4% and its maximum DH was 48.6%.

The rapid rate of autolysis of white shrimp (*Penaeus vannamai*) with a gradual temperature rise increased from 45 to 60 °C and then slowed down. This indicates that the

Table 36.5 Protein hydrolysates obtained from shrimp head under different temperature conditions. Reprinted from Cao *et al*. (2009). Copyright 2009, with permission from Elsevier.

Temperature	Protein content	Protein recovery
40 °C	84.4 ± 0.56	43.6 ± 0.28
50 °C	82.1 ± 0.71	73.6 ± 0.42
60 °C	85.7 ± 0.43	50.3 ± 0.37
Gradually increasing	84.8 ± 0.53	87.4 ± 0.31

proper temperature for most endogeneous enzymes in shrimp head waste is between 45 and 60 °C, and their DH varies up to 48.6% after 180 minutes of incubation with gradually increasing temperate (Cao *et al*., 2009). The protein recovery from shrimp waste using autolysis under gradually increasing temperate condition (87.4 ± 0.31%) is higher than that using a commercial enzyme operation (alcalase treatment) (68.5%) (Gildberg & Stenberg, 2001). The crude protein content of shrimp head before autolysis of white shrimp was 13.8 g/100 g (Cao *et al*., 2009). Protein recovery and protein content were higher under gradually increasing temperature conditions than at constant temperatures. The approximate compositions of shrimp-head autolysis hydrolysates obtained under different temperature conditions are shown in Table 36.5.

36.3.3 Physical Methods of Extraction

In recent years, ultrasound has been used in food process engineering and green technology. The most common ultrasound apparatus uses the electrostrictive transformer principle. This is based on elastic deformation of ferroelectric materials in a high-frequency electrical field, caused by polarization of the mutual attraction of the molecules in the surrounding area. It uses sound waves instead of electromagnetic waves, and has frequencies higher than 20 kHz, which are mechanical vibrations in a gas, liquid and solid (Wang & Weller, 2006). Sound waves pass through the medium and cause continuous wave-type motions, generating longitudinal waves (Povey & Mason, 1998). When an ultrasound wave passes through a liquid medium, small vacuum bubbles are formed in the liquid phase. These bubbles are brought to aggressive destruction when they reach a critical size. The destruction of the extracted material cells creates high shearing phenomena in the liquid phase, increasing pressure and intense local heating on the liquid–gas interface and causing turbulence. The shear forces can destroy the cell-wall structure and break fibrous, cellulosic materials into small particles. Ultimately, the process releases intracellular materials into the solvent.

The amount of energy released by cavitation depends on the kinetics of the bubble generated and disruption of the bubbles. This energy increases the surface tension at the interface and lessens with the vapor pressure of the liquid. Nevertheless, the efficiency of the UAE process depends on factors such as superficial tissue disruption, pretreatment of the tissue prior to extraction, the location of the components to be extracted inside the materials, the nature of the tissue being extracted and the nature of the component being extracted (Chittapalo, 2009).

Ultrasound increases the extraction efficiency and rate and stabilizes the target compounds. It requires a low temperature and reducing solvent use. The marked increase

in the very local temperature enhances the solubility of the analytes in the solvent and drives their diffusion from the sample matrix to the solvent. On the other hand, increasing the local pressure will facilitate both penetration of the solvent into the sample matrix and the transfer of compounds. Moreover, the implosion of cavitation bubbles can hit the surface of the solid matrix and disintegrate the cells. As a result of these effects, less extraction time is required compared to other conventional extraction processes (Esclapez *et al*., 2011).

Frequency is the most important ultrasound aspect for the yield and the kinetics. On the other hand, the effects depend on the characteristic of the plant material to be extracted. The yield is different for each bioactive compound (Romdhane & Gourdon, 2002). When designing an ultrasonic extractor, the important thing to consider is the distribution of the ultrasound wave inside the extractor. Ultrasonic intensity decreases as the distance from the radiating surface increases.

Besides all these issues, the influence of solvents, the ratio between sample and the solvent, the particle size of the extracted materials, the temperature of extraction and the reactor design must all be considered. Moreover, the target of UAE is not always a larger yield, but may be cost savings and a lower energy consumption. The UAE process can be used as a critical pretreatment prior to extraction to get maximum oil yields from apricot, almond and rice bran. Liu *et al*. (2004) found that it can also increase the yield extracted from soybean. Ultrasound can also raise kinetics and improve the quality of the extraction product. It takes less time than the conventional method. Using ultrasound extraction can promote extraction processes by increasing mass transfer between plant materials and the solvent. Many thermally sensitive food products and ingredients, such as polyphenolics, polysaccharide and anthocyanins, can be extracted by ultrasonic extraction, include oil, herbal, protein and bioactive compounds from animals and plants (Vikhu *et al*., 2008). The aroma yields from aged brandies using ultrasonic extraction are higher than those from the conventional method. Moulton & Wang (1982) reported that protein recovery from soy protein using ultrasound continuous batch (54%) was higher than that using alkaline extraction (23%). Ultrasonic extraction of egg yolk was more effective in extracting lutein than tradition saponification solvent extraction. Zhou *et al*. (2011) reported that the optimum conditions under which to extract peanut protein using ultrasonic microwave-assisted extraction were an extraction time of 500 seconds, pH 9.73, a ratio of meal to solvent of 1 : 28 and 72 W microwave power. The extraction rate of peanut protein under these conditions was 88.37% and the protein content was 88.95%. Moreover, Liu *et al*. (2003) showed that under optimal conditions (time 30 minutes, ultrasonic frequency 60 kHz, temperature 30 °C), the yield of peanut-protein flour was 37.27% and the protein recovery of the finished product was 95.45%. The optimal conditions for protein extraction from brewer's spent grain (BSG) were an ultrasonic power of 88.2 W for 100 ml of extracts and an extraction time of 81.4 minutes. The protein recovery was 104.2 mg/g BSG (Tang *et al*., 2010).

Studies and publications on UAE methods for animal materials are less common than those for plant materials. Those that do exist include chitin extraction from freshwater prawn shells (Kjartansson *et al*., 2006) and lutein extraction from egg yolk using ultrasonication. The yield of chitin extract decreased during UAE because of depolymerization of the extract in the waste water. However, the process was not affected by the degree of acetylation. Though there is huge potential for the extraction of protein and peptides using UAE methods, there has not been much research on its use with seafood and fish materials. Hu *et al*. (2011) reported that ultrasound-assisted solvent extraction of protein

hydrolysate from defatted mackerel could reduce low bitter taste and obtain a nitrogen recovery of 88.6%.

In our laboratory, we have recently conducted research into the extraction of proteins from shrimp head by the UAE method (Nava Rose, 2012). This study was aimed at producing value-added protein hydrolysate from shrimp head (*Penaeus vannamei*) by two different methods: autolysis and UAE. Both are considered ecofriendly technologies. The chemical compositions of autolysis hydrolysate and UAE hydrolysate were investigated. The ground shrimp heads were incubated at temperatures of 40, 55, 70, 85 and 95 °C and time intervals of 20, 40, 60 and 90 minutes using the autolysis method. Similarly, they were treated by ultrasonication at powers of 80, 120, 160 and 200 W and for time intervals of 5, 10, 20 and 40 minutes. The highest-protein-content hydrolysate produced by autolysis was 8.03% (w/w), at 95 °C for 90 minutes, while the highest produced by ultrasonication was 7.26% (w/w) at 200 W for 40 minutes. The maximum DH from autolysis was 52.81% while that from UAE was 45.05%. The protein hydrolysates extracted from shrimp head had their physical and functional properties compared. It was found that they were similar in emulsifying activity index (EAI) and emulsifying stability index (ESI) profile. However, they were different in color, protein content and molecular weight.

The advantages of using ultrasound in food technology are a simplified manipulation, reduction of processing cost, increased production, high reproducibility, elimination of process steps, more effective mixing, faster energy and mass transfer, selective extraction, environmental friendliness, faster start-up and reduced time consumption. However, there are many disadvantages too. For example, the energy use was normally higher than that for conventional processes, a large amount of solvent was used and a filtration step was required (Chemat *et al*., 2011).

36.4 CHARACTERIZATION OF FISH-PROTEIN HYDROLYSATES AND BIOACTIVE PEPTIDES

In quantitative work on protein hydrolysis, its progression can be evaluated by its DH. DH is defined as the percentage of hydrolyzed peptide bonds during a hydrolytic process. This process is related to the proteolytic enzymes and the extent of hydrolysis. When peptide bonds are destroyed, free carboxyl groups and free amino acid groups are released, as shown in Fig. 36.1. There are many methods by which to determine the progress of hydrolysis, such as formol titration, ninhydrin reaction, fluorescamine reaction and TNBS reaction.

The molecular weight of a peptide is essential to its biological activity. Therefore, products of fermentation or hydrolysis are further concentrated and peptides are separated according to their different molecular weights by membrane filtration, including utrafiltration, microfiltation, nanofiltration and reverse osmosis. The principles of the different separations are not fundamentally different except in term of the size of the molecules retained. An ultrafiltration membrane of 20 kDa or above is used to separate peptides from nonhydrolyzed proteins (Thorkelsson & Kristinsson, 2009). As cited by Vandanjon *et al*. (2007), fractionation of peptide hydrolysates according to their molecular weight is successful when an ultrafiltration membrane of 4–8 kDa is applied. Membranes with a low molecular cutoff, approximately 0.2–0.3 kDa, are suitable for the concentration of a peptide solution, while a nanofiltration membrane in a diafiltration mode can be used to purify the solution, including desalinating and partially deodorizing it. Chabeaud *et al*. (2009)

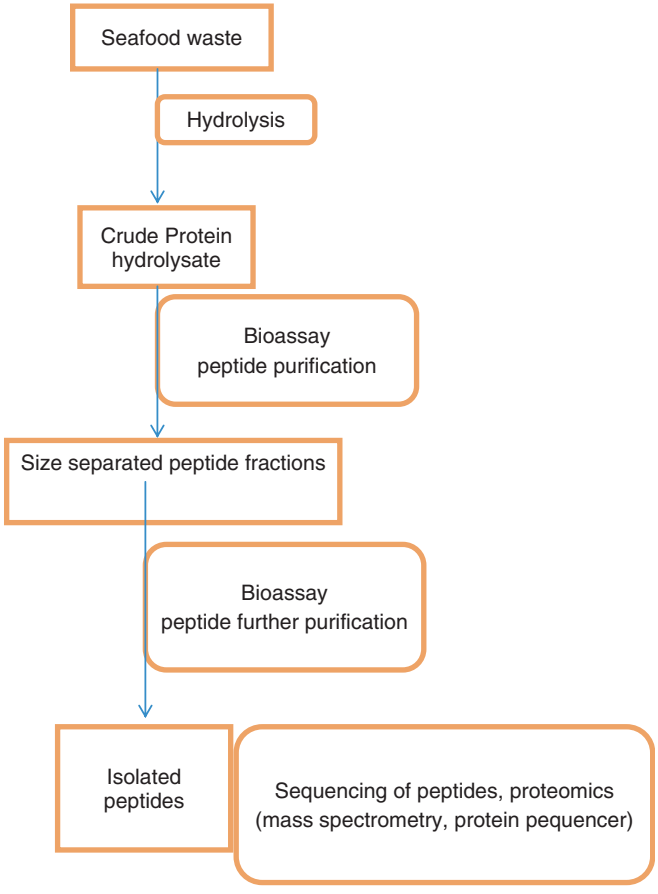

Fig. 36.1 General scheme of peptide production.

reported that low-molecular-weight antioxidant peptides with molecular-weight cutoffs of less than 2 kDa can be separated from saithe (*Pollachius virens*) hydrolysates by passing them through a 4 kDa modified polyethersulfone (m-PES) membrane, while a 8 kDa polysulfone (PS) membrane in sequence with a 4 kDa m-PES exhibits high performance in the separation of small- and medium-size peptides. Vandanjon *et al*. (2009) successfully refined peptides from white-fish hydrolysates by using a fractionation–concentration sequence coupling ultrafiltration and nanofiltration membranes. He *et al*. (2007) conducted research into 12 kinds of marine protein material, including fish, shrimp, seashell, algae and seafood wastes, hydrolysed by different proteases. The protein hydrolysates obtained from marine waste were subjected to rapid screening of peptides with ACE-inhibitory activity using capillary electrophoresis. The capillary-electrophoresis method achieved the same sensitivity as the high-performance liquid chromatography (HPLC) method. However, the capillary-electrophoresis method was faster and more economical.

Bourseau *et al*. (2009) described two-stage processes for the fractionation of fish-protein hydrolysate using ultrafiltration and nanofiltration, respectively. Two commercially available fish-protein hydrolysates were used: Prolastin (FPH1) and MariPep C

(FPH2). The study describes the impact of ultrafiltration/nanofiltration fractionation on the hydrolysates with different degrees of hydrolysis, with peptides and free amino acids in molecular-weight ranges between 1 and 7 kDa. Fractionation was performed under 'realistic' conditions for an industrial process by processing the protein hydrolysate solutions with a high peptide content. Peptidic profiles obtained by size-exclusion chromatography followed by fast-permeation liquid chromatography (SEC-FPLC) offered a valuable tool for analyzing the impact of the fractionation on the peptide composition of the fish protein hydrolysate.

Sophisticated processing techniques such as fast protein liquid chromatography (FPLC) can be used to analyze and purify mixtures of fish sauce and hydrolysates. Separation is achieved according to the different affinities of the mixture components for the mobile phase and the stationary phase. The column used in FPLC separates macromolecules based on size, charge distribution, hydrophobicity, reverse-phase or biorecognition. Ion exchange (charge exclusion) and gel filtration (size exclusion) are the most commonly used methods. After passage through the column, the protein or peptide concentration in the effluents is measured by detectors at a wavelength of 280 nm. Commercially available reversed-phase columns allow rapid separation and detection of the hydrophilic and hydrophobic amino acids (Najafian & Babji, 2012). Protein hydrolysates and peptides with different hydrophobicities are separated by reverse-phase columns using a polystyrene-divinylbenzene copolymer-based packing (Ferreira *et al*., 2007). Other chromatographic techniques such as liquid chromatography with tandem mass-spectrometry detection (LC-MS/MS) are usually used to identify the peptide sequences. Matrix-assisted laser-desorption ionization mass-spectrometry with time of flight (MALDI-TOF) analyzers and electrospray mass-spectrometry (ESI-MS) with quadrupole mass spectrometers are useful not only in obtaining peptide profiles of protein hydrolysates or semipurified fractions but also in accurately measuring masses of proteins of 100 kDa or more (Careri & Mangia, 2003). As reported by Rajapakse *et al*. (2005), MALDI-TOF mass-spectroscopic and SDS-PAGE analysis were successful in identifying the purified protein of yellowfin sole that possessed anticoagulant activity. Nazeer *et al*. (2012) investigated the antioxidant activity of hydrolysate from croaker. They fractionated the hydrolysate by passing it through FPLC on a DEAE anion-exchange column, then purified it by loading it on to a Sephadex G-25 gel-filtration column. Identifications of the peptides was conducted by electrospray-ionization tandem mass-spectrometry (ESI-MS/MS).

36.5 FUNCTIONAL AND BIOACTIVE PROPERTIES OF PROTEINS AND PEPTIDES FROM SEAFOOD AND CRUSTACEAN WASTE

Protein components in food and waste products contain sequences of bioactive peptides that can exert physiological effects in the body (He *et al*., 2004). These short chains of amino acids are inactive within the sequence of the parent protein, but can be released during gastrointestinal digestion, food processing or fermentation. Protein hydrolysates and bioactive peptides isolated from various marine wastes have shown numerous bioactivities, such as antihypertensive, antithrombotic, immunomodulatory and antioxidative activities. The bioactive peptides produced from autolysis can be utilized to develop pharmaceuticals and nutraceuticals (Cao *et al*., 2009).

Protein hydrolysates are widely used as protein complements in energy drinks, food flavors and the preparation of diets for sick adults and children. Protein hydrolysates have been used in energy drinks, sports nutrition, weight-control diets and geriatrics products. Clinically, protein hydrolysates are used to cure pancreatitis, ulcerative colitis, Crohn's disease and phenylketonuria (PKU) (Clemente, 2000). The protein hydrolysates and peptides obtained from enzymatic hydrolysis show better biological activity as antioxidants and antihypertensive agents than those derived from fish-muscle protein (Byun & Kim, 2001; Kim *et al*., 2001, 2007; Mendis *et al*., 2005). Glycine-proline-alanine is the repeat sequence found in gelatin hydrolysates and the peptides in their structures. It is presumed that the observed antioxidative and antihypertensive properties of gelatin peptides are associated with their unique amino acid compositions. These peptides have also shown enhanced absorption of dietary calcium (Kim *et al*., 1999). Protamine contains arginine (more than 80%) and is found in the testicles of fish. It can inhibit the growth of *Bacillus* spores and is applied as an antimicrobial in food preservation and processing. Hydrolysis improves its intestinal digestibility and hence enhances its bioavailability. The functional properties of a protein or peptide depend on its source, production and environmental parameters. The major functional properties of peptides isolated from marine waste are water-binding capacity, oil-holding capacity, viscosity, foaming properties, emulsifying properties, gelation and solubility.

36.5.1 ACE-inhibitory Activity

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor and causes an increase in blood pressure. With inhibition of ACE activity, there is decrease in conversion of angiotensin I to angiotensin II and consequently blood pressure decreases to normal. Synthetically available ACE inhibitors, including Captopril, Enalapril and Listinopril, are effective as antihypertensvie agents but have remarkable side effects. The potency of an ACE-inhibitory component is generally expressed as the concentration of the component which inhibits 50% of ACE activity, denoted by IC_{50}. The natural ACE inhibitors are found to be safer and more effective, particularly with new developments in the use of marine waste and the isolation of ACE-inhibitory peptides. More than 200 kinds of ACE-inhibitory peptide have been reported in hydrolysates from diverse food proteins digested using different proteases (He *et al*., 2004). These peptides are generally extracted from milk protein (Nakamura *et al*., 1995), muscle protein (Arihara *et al*., 2001) and plant proteins (Wu *et al*., 2002). Branched and aromatic amino acids in the peptides are important to the inhibition of ACE activity. ACE prefers substrates or competitive inhibitors that contain branched amino acid residues at the N-terminal position and aromatic amino acid residues at the C-terminal position (Cheung *et al*., 2010).

Kim *et al*. (2001) have reported that some peptides derived from fish show antihypertensive activity that inhibits the action of ACE even more than many other natural peptides. He *et al*. (2007) conducted screening of marine-protein hydrolysates enriched in peptides with ACE-inhibitory activity. The marine species studied included fish, shrimp, seashell, algae and seafood wastes. The IC_{50} values ranged from 0.17 to 501.7 mg/ml, and were mainly affected by both the marine-protein resources and the enzymes used. Hydrolysates with the lowest IC_{50} values were from shrimp (*Acetes chinensis*), shark meat and mackerel bone, indicating the beneficial peptides for the production of ACE-inhibitory peptides by proteolysis. The protein hydrolysates and peptides from these marine sources

were found to contain high amounts of branched and aromatic amino acids, such as isoleucine, valine, phenylalanine and tyrosine, which suggests their higher antihypertensive potentials.

The frame protein of Alaska Pollock, which is normally discarded as an industrial byproduct, was explored for ACE-inhibitory peptides (Je *et al*., 2001). It was first hydrolyzed with pepsin and then separated into five fractions according to molecular weight. The most active ACE-inhibitory peptides were found in the fraction of about 1000 Da. Chinese soft-shelled turtle hydrolysate showed a limited inhibition effect against ACE, with an IC_{50} value of 280 μg/ml, while its graded fraction of molecular weight <5000 Da, obtained through an ultrafiltration membrane, exhibited better activity (IC_{50} = 190 μg/ml) (Liu *et al*., 2012).

In addition to antihypertensive potential, protein hydrolysates generated from fish and shellfish exhibit anticoagulant and antiplatelet activity. A 12 kDa single-chain monomeric peptide with anticoagulant potential was purified from yellow sole frame-protein hydrolysates (Rajapakse *et al*., 2005). Sardine protein hydrolysate has been found to have ACE-inhibitory effects. The most common sardine peptide is dipeptide VY, which has a significant antihypertensive effect in mildly hypertensive people (Kawasaki *et al*., 2000). Other protein hydrolysates extracted from seafood waste include cod frames and heads (Jeon *et al*., 1999; Bordenave *et al*., 2002); Shrimp head (Bordenave *et al*., 2002), salmon (Ono *et al*., 2003), fermented pearl oyster sauce (Katano *et al*., 2003), fermented mackerel (Itou & Akahane, 2004), sea bream scales (Fahimi *et al*., 2004) and fermented surimi (Shan *et al*., 2007).

The ACE-inhibitory peptides isolated from fermented fish sauce show a tendency to lower the blood pressure of spontaneously hypertensive rats (SHR) (Ichimura *et al*., 2003). ACE-inhibitory peptides such as alanine-proline, lysine-proline, arginine-proline, glycine-proline, glutamine-proline, threonine-proline, valine-proline, glycine-isoleucine and asparagine-phenylalanine have been isolated from fermented anchovy sauce, while the peptide sequences alanine-proline, glycine-proline, threonine-proline, valine-proline, asparagine-proline, asparatic acid-methionine, asparatic acid-leucine, alanine-valine and glycine-valine were obtained from fermented sardine sauce, and the ACE-inhibitory peptides with sequences alanine-proline, arginine-proline, glycine-proline and alanine-glycine-proline have been isolated from fermented bonito sauce.

36.5.2 Antioxidative Functions

Free radicals are formed during the oxidation process in aerobic organisms, particularly in vertebrates and humans. The formation of reactive oxygen species (ROS), including free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-) and non-free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen (^1O), is an unavoidable consequence of the body's normal use of oxygen during respiration. These free radicals, including ROS and reactive nitrogen species, cause diseases; therefore, the removal or inactivation of such radicals in order to reduce the risk of disease is recommended. Unsaturated fatty acids generally react with these free radicals to become oxidized. The radicals interact with molecular oxygen to form fatty acids-peroxy radicals, and after reaction with the hydrogen atoms from adjacent unsaturated fatty acids, produce a hydroperoxide and new lipid radicals.

Many animal- and plant-based bioactive compounds have been found to possess antioxidant activity, including those from fish and crustacean byproducts. A number of antioxidant peptides composed of 3–20 amino acid residues have been identified from these proteins. The peptides containing the most of these amino acids, such as tyrosine, lysine, methionine, histidine and tryptophan, are generally thought to be antioxidants (Je *et al*., 2007). The antioxidant activities of peptides obtained from bigeye tuna backbone and dark muscle were evaluated and shown to inhibit lipid peroxidation in a linoleic acid emulsion system, and to quench free radicals (DPPH, hydroxyl and superoxide) in a dose-dependent manner. The hydrolysates obtained after hydrolysis with peptic enzymes showed the highest effect. Jao & Ko (2002) reported the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity of peptides and free amino acids from hydrolyzed tuna-cooking-juice and mackerel-muscle hydrolysates. Those hydrolysates containing the peptides that exhibited antioxidant activity were obtained from the mackerel (Wu *et al*., 2003). The hydrolysates released after hydrolysis by protease N inhibited the autoxidation of linoleic acid, quenched the free radical α,α-diphenyl-β-picrylhydrazyl (DPPH) and reduced Fe^{3+} to Fe^{2+}. The smaller peptides, with a molecular weight of 1400 Da, showed the strongest antioxidant activity.

Je *et al*. (2005) studied the antioxidant activities of protein hydrolysates extracted from Alaska pollock frame protein (APF). The peptides with a low molecular weight (about 1000 Da) had the ability to quench 35% of available hydroxyl radicals at a peptide concentration of 53.6 μM. In another study, the same group (Je *et al*., 2009) extracted peptides from tuna liver using falvourzyme, alcalase, protamax and nutrase. All hydrolysates exhibited significant antioxidant activity against DPPH and hydroxyl, showed hydrogen peroxide-scavenging and ferrous-chelating capacity and reducing power, and protected against hydroxyl radical-induced oxidative DNA damage.

Different antioxidant peptides were identified in the hydrolysates of protein from the sardinelle-processing waste stream (Bougatef *et al*., 2010). All the peptides had less than 600 Da molecular weight and the ability to scavenge 63% of available DPPH radical at a peptide concentration of about 150 µg/ml.

Hoki-skin gelatin was hydrolyzed by the digestive enzymes pepsin, trypsin and chymotrypsin, with the tryptic hydrolysates displaying the highest level of antioxidant activity (Mendis *et al*., 2005). After addition of peptide to a linoleic acid peroxidation system, inhibition of lipid peroxidation was found to be greater than that of the control α-tocopherol, and to be as effective as that of the synthetic antioxidant butylated hydroxytoluene (BHT).

The protein hydrolysates obtained from cod backbone have the potential to enhance product stability by preventing oxidative deterioration (Šližytė *et al*., 2005). Their DPPH-scavenging activity showed that the antioxidative activity of hydrolysates could be due to their ability to scavenge lipid radicals. The ability of hydrolysates to inhibit iron-induced lipid oxidation was not influenced by the time of hydrolysis.

36.5.3 Antimicrobial Activities

Antimicrobial peptides, generated after enzymatic hydrolysis of seafood protein, usually have less than 50 amino acids, of which nearly 50% are hydrophobic and have a molecular weight below 10 kDa (Bulet *et al*., 2004; Reddy *et al*., 2004). Antimicrobial peptides obtained from fish waste have been found to have both bacteriocidal and

bacteriostatic functions against Gram-negative and Gram-positive organisms. A novel 20-residue antimicrobial peptide from the skin of yellow catfish with the amino acid sequence GKLNLFLSRLEILKLFGVAL has been found to have broad-spectrum antimicrobial (without hemolytic) activity (Su, 2011). Protamine is a basic peptide containing arginine (>80%) found in the testicles of fish. Protamine has the ability to prevent the growth of *Bacillus* spores and is used as an antimicrobial agent in food preservation and processing. Another antimicrobial peptide obtained in the skin homogenate of *Epinephelus fario* using a trypsin digest (Zhang *et al*., 2008) was purified by ion-exchange and gel-filtration chromatography. The homogenate showed activity against various bacteria (*Vibrio parahemolyticus*, *vibrio fluvialis*, *Pasteurella multocida*, *Escherechia coli*, *Aeromonas hydrophila* and *Pseudomonas aeuroginosa*).

36.5.4 Emulsification Properties

There is increasing demand for new food products of high nutritional quality. Protein isolate represents an interesting ingredient for food formulations that will contribute not only good nutrition but also useful functional properties. In recent years, plant proteins have been increasingly added to foods because they contain an abundance of functional properties. Most studies have been conducted either with plant proteins or with dairy proteins.

Emulsions form a major part of many processed food formulations. It is well known that proteins are surface-active molecules that are commonly used to stabilize emulsions in food products such as mayonnaise, sauce, yoghurt and some beverages. During emulsification, protein molecules are rapidly adsorbed on to the newly formed droplet surfaces and reduce the interfacial tension and provide a protective coating. Consequently, protein facilitates the formation of oil droplets, improves emulsion stability and produces desirable physicochemical properties in oil-in-water emulsions. Many researchers have investigated the properties of oil-in-water emulsions formed by different proteins and determined parameters such as droplet size, interfacial properties, rheological properties and microstructures. These parameters play important roles in emulsion stability and rheological behavior, thus influencing the quality of the emulsion. Moreover, the emulsifying capacity of a protein emulsifier is strongly dependent on physicochemical conditions. Oil volume fraction, protein concentration, pH and the presence of ions are all important factors influencing protein emulsifying properties.

Protein hydrolysates can be used as emulsifying agents in foods such as spreads and salads dressing and in emulsified products such as luncheon meats and sausages (Badal & Kiyoshi, 2001). The emulsifying activity index (EAI) expresses the emulsifying property of a protein in the oil–water interface area (m^2). Pearce & Kinsella (1978) reported that the EAI values of soy-protein concentrate or soy-protein isolate reduced when the degree of hydrolysis of the protein increased. These results confirmed that the emulsifying properties of a protein will be destroyed by extensive hydrolysis of that protein (Zhao & Hou, 2009). Moreover, the EAI of a soy-protein isolate hydrolysate or soy-protein concentrate hydrolysate treated with neutrase is lower than that of one treated with trypsin at the same degree of hydrolysis. This justifies the claim that enzymes play a significant role in the EAI of protein hydrolysates.

Chalamaiah *et al*. (2010) extracted the protein hydrolysates from underutilized meriga (*Cirrhinus mrigala*) fish egg using the commercial Alcalase and papain enzymes. The degree of hydrolysis was 62% for Alcalase and 17.1% for papain, after 90 minutes' digestion at 50–55 and 60–65 °C, respectively. The protein content of Alcalase-produced

hydrolysate was higher (85%) than that of papain hydrolysate (70%). The authors revealed that the hydrolysates had good fat-absorption capacity (0.9 and 1.0 g/g sample for Alcalase and papain, respectively), foam capacity (70 and 25%) and emulsifying capacity (4.25 and 5.98 ml/g hydrolysate). The peptides in the protein hydrolysates had an average molecular weight of >10 kDa, indicating the usefulness of these bioactive peptides.

A series of hydrolysis trials have been carried out using backbones from fresh and frozen cod hydrolysed for different times (10, 25, 45 and 60 minutes) (Šližytė *et al*., 2009). A higher yield with lighter and less yellowish dry-protein hydrolysate powders and better emulsification properties was obtained with the fresh, raw cod backbones. A longer hydrolysis time gave a higher yield, increased degree of hydrolysis and decreased water-holding capacity. Among the times tested, 25- and 45-minute hydrolysis demonstrated the best emulsification properties. Protein hydrolysates made from fresh backbones (after 10 minutes of hydrolysis) created significantly more emulsions than hydrolysates obtained from frozen backbones. This can be explained by the peptide size, which is the key factor in emulsification capacity. The smaller the size of the peptide, the less able it is to form stable emulsions. The peptide size distribution indicates that hydrolysates that have undergone 10 minutes of protein hydrolysis have a significant portion of peptides with more than 20 residues, ranging in molecular weight from approximately 1000 to 80 000 Da, while those that have undergone 60 minutes of hydrolysis have more peptides with a molecular weight less than approximately 1000 Da. Cutting the backbones did not influence the emulsification capacity of protein hydrolysates from frozen raw material. Fresh backbones from pre-rigor filleted fish produced protein hydrolysates with better emulsification capacity than backbones from post-rigor filleted fish. The same tendency was observed after both 10 and 60 minutes of hydrolysis. Even though small peptides diffuse rapidly and absorb at the interface, they are less efficient at stabilizing emulsions, because they cannot unfold and reorient at the surface. Generally, peptides should have a minimum chain length of more than 20 residues in order to function as good emulsifiers. There is an optimum size for good emulsifiers.

36.6 CONCLUSION

Seafood-processing discards and other raw materials account for approximately 75% of the total weight of a catch. Valuable components such as lipids, proteins, collagen and gelatin, enzymes, peptides and minerals can be obtained from this raw material. Recent studies have identified a number of bioactive compounds in seafood and marine-fish raw materials and shellfish and crustacean shells. These compounds can be extracted and purified using technologies of varying complexity. Development of new technologies for the extraction of new bioactive compounds from marine-processing raw materials may increase the value of what is currently considered waste.

Proteins and other macromolecules are often added to a food in order to improve its quality or functional properties. The functional properties of these proteins and peptides depend on their source, production and environmental parameters. The production of protein hydrolysates has been also used as a tool for lipid recovery from byproducts. Because some proteins possess a range of dynamic functional properties, seafood and crustacean proteins exhibit a wide spectrum of physicochemical behaviors. The major functional properties of protein hydrolysates and peptides isolated from marine waste are water-binding capacity, oil-holding capacity, viscosity, foaming properties, emulsifying

properties, gelation and solubility. With a great amount of research on the extraction, isolation and purification of proteins and peptides from these seafood and crustacean byproducts, some biologically active compounds have been identified and shown to be applicable to various biological functions. More studies must be conducted on hydrolysis by biological methods and the purification of peptides extracted from seafood and crustacean waste in order to avoid any toxic effects. Biological and enzymatic methods will also avoid the environmental problem and lead to almost zero chemical residual in final products. These high-value-added compounds will become profitable, due to their beneficial roles in human health and livestock production. Their extraction and the development of new technologies for their recovery and purification will thus go a long way towards ensuring the long-term sustainability of marine activities.

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37 Production and Health Effects of Peptides from Fish Proteins

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37.1 INTRODUCTION

The marine environment has a tremendous biodiversity and is the source of a plethora of bioactive compounds with great potential for use as food ingredients and pharmaceuticals. Recent studies have confirmed that most fish species possess active compounds and serve as a source of functional materials. Fish catch is mainly used for human consumption and other minor uses, such as meal production and bait. Many studies of the waste from the fisheries industry have been conducted, with a great amount of research looking at ways of increasing the utilization of fish-muscle protein, which has been found to possess potential bioactive materials such as gelatin, collagen, peptides and various fatty acids, including EPA and DHA (Wassawa *et al*., 2007).

Recent studies have reported a number of bioactive peptides from various fish sources. They possess short sequences approximately 2–20 amino acids in length that exert physiological benefits when consumed *in vivo*. They can be produced by proteolytic hydrolysis using commercially available enzymes, including trypsin, chymotrypsin and alcalase, or proteolytic microorganisms and fermentation methods (Vercruysse *et al*., 2005). After digestion, bioactive peptides can be absorbed in the intestine and enter the blood stream directly, which ensures their bioavailability *in vivo* and a physiological effect at the target site (Erdmann *et al*., 2008). A growing literature demonstrates the wide spectrum of effects of peptides from marine organisms, including antihypertensive, immunomodulating, antithrombotic, antioxidative, anticancer, and antimicrobial (Je *et al*., 2007; Vercruysse *et al*., 2005; Yang *et al*., 2010). A number of bioactive compounds have been isolated from fish-muscle proteins, collagen and gelatin, fish oil, fish bone and fish internal organs (Je *et al*., 2005; Jeon & Kim, 2002; Kim *et al*., 2001). Generally, far more profit is obtained by producing human consumables, and the highest profitability is currently expected from bioactive compounds. These can be extracted and purified using both simple and complex technologies, including preparation and isolation for biotechnological and pharmaceutical applications. Furthermore, some of these bioactive compounds have been identified as possessing nutraceutical potential of benefit to human health. Therefore, new technologies must be developed to aid in their discovery. This chapter discusses

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trends in the development of marine fish-protein bioactive peptides and their biological activities relating to human health.

37.2 SOURCES OF FISH PEPTIDES

In recent years it has been increasingly acknowledged that dietary proteins provide a rich source of bioactive peptides that can promote human health by reducing the risk of chronic diseases. Fish skin, bones, muscle, scales and residual minced meat, the byproducts of the fish-processing industry, can effectively be converted into high-protein or bioactive peptides.

37.2.1 Muscle-protein Peptides

Discarded fish bones and cutoffs may contain considerable amounts of muscle proteins. These are nutritionally valuable and are easily digestible with a well-balanced amino acid composition (Venugopal *et al*., 1996). Therefore, fish proteins derived from seafood-processing byproducts can be hydrolyzed enzymatically to recover protein. Protein hydrolysates from several marine species have been analyzed for their nutritional and functional properties and the possibility of obtaining biologically active peptides has been explored (Benkajul & Morrissey, 1997). Moreover, skipjack tuna muscle (Kohama *et al*., 1988), sardine muscle (Bougatef *et al*., 2008), and shark meat (Wu *et al*., 2008) have been used to separate potential peptides. Effective oligopeptides were obtained from chum salmon prepared by enzymatic hydrolysis (Yang *et al*., 2011).

37.2.2 Peptides from Fish-skin Collagen and Gelatin

Gelatin derived from fibrous protein collagen is the principal constituent of animal skin, bone, and connective tissue. Fish-skin waste could be used as a potential source of collagen and gelatin. Zhu *et al*. (2010) evaluated the effect of collagen peptides on markers of metabolic nuclear receptors. Hoki-skin gelatin was hydrolyzed by the digestive enzymes pepsin, trypsin, and α-chymotrypsin with tryptic hydrolysates to obtain bioactive peptides (Mendis *et al*., 2004). Moreover, Klompong *et al*. (2009) purified peptides from yellow stripe trevally using enzyme-assisted methods with Alcalase and Flavourzyme.

37.2.3 Fish Bone as a Potential Peptide Source

The organic component of fish bone represents collagen (30%), while the inorganic component mainly consists of calcium phosphate and hydroxylapatite (around 60–70%) (Aronove *et al*., 2007; Barakat *et al*., 2008). Gelatin is traditionally produced from bones by acid or alkaline treatment. Muyonga *et al*. (2004) described a method in which bones are pretreated by tumbling them in warm (35 °C) water and then demineralized using 3% HCl to get gelatin.

37.2.4 Peptides from Other Body Parts

Antimicrobial peptides mainly present in the mucous layer and eliminate pathogenic bacteria before they enter the skin barrier. Several antimicrobial peptides have been isolated from marine fish, such as Pleurocidins from winter flounder (*Pleuronectes americanus*)

(Walbaum), American plaice (*Hippoglossoides platessoides*) (Fabricius), and Atlantic halibut (*Hippoglossus hippoglossus*) (Cole *et al*., 1997; Douglas *et al*., 2003). Intestinal antimicrobial peptides were derived from hagfish (*Eptatretus burger*) (Girard) (Hwang *et al*., 1999), chrysophsins from the gills of red sea bream (Iijima *et al*., 2003), and piscidin or moronecidin from the gills and skin of striped bass (Lauth *et al*., 2002; Silphaduang & Noga, 2001).

37.3 PRODUCTION OF FISH PEPTIDES

Peptides are encrypted in the primary structures of plant or animal proteins as inactive amino acid sequences. However, bioactive peptides can be produced by fermentation, food processing, and enzyme-assisted proteolysis *in vitro* or in the digestive tract after human consumption of protein-containing diet (Aluko, 2007; Hartmann & Meisel, 2007). Since most of these protein hydrolysates and peptides have demonstrated potent bioactivity compared to their parent proteins, hydrolysis of peptide bonds is important in liberating them. Several factors affect their bioactive properties, including the enzymes used for hydrolysis, processing conditions, and their resulting size. In many studies, peptides are obtained by *in vitro* enzymatic hydrolysis or fermentation (Fig. 37.1).

The most commonly used method for producing bioactive peptides from whole-muscle protein is enzymatic hydrolysis. Optimized conditions for time, temperature, and pH enzyme/substrate ratio should be used to obtain a maximized yield. The type of enzyme used in enzymatic hydrolysis is very important as it overrules the cleavage patterns of the peptide bonds (Shahidi & Zhong, 2008). Various proteases have been used for the production of peptides from fish proteins; Qian *et al*. (2007) used α-chymotrypsin, papain, neutrase, and trypsin for the hydrolysis of tuna dark muscle.

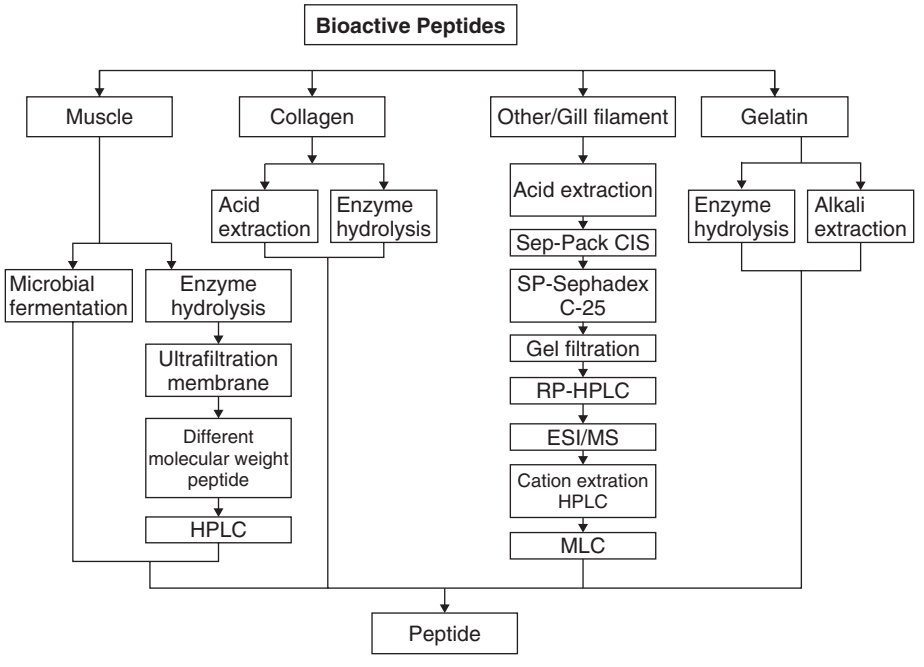

Fig. 37.1 Methods of production of bioactive peptides from various fish sources.

Antimicrobial peptides have been purified from the gill filaments of sea beam by acid extraction (Iijima *et al*., 2003). Gill filaments were crushed in liquid nitrogen and the resulting powder was boiled in water. The extraction was then performed with hydrocholoric acid, trifluoroacitic acid, formic acid and NaCl. Finally, the extract was purified using a Sep-Pack C18 cartridge, SP-Sephadex C-25 resin, gel filtration, reversed-phase high-performance liquid chromatography (RP-HPLC), ESI-MS/protein sequencer, cation-exchange high-performance liquid chromatography (CE-HPLC), and micellar liquid chromatography (MLC). Several studies have used acid extraction to isolate antimicrobial peptides from various fish body parts (Lauth *et al*., 2002; Zhang *et al*., 2009). Extraction media containing sodium acetate, Triton X-100, and phenylmethylsulfonyl fluoride also have been used for the extraction of antimicrobial peptides from fish (Park *et al*., 1998).

Collagen is generally extracted with acid treatment and solubilized without altering its triple helix. However, thermal treatment cleaves hydrogen and covalent bonds, which stabilizes the triple-helix configuration and converts it into a coiled conformation, resulting in a gelatin state (Djabourov *et al*., 1993). Fish-skin gelatin is processed into peptides by enzymatic hydrolysis. Various commercial proteases, such as Alcalase (Dong *et al*., 2008) and Flavourzyme (Thiansilakul *et al*., 2007a,b), have been used to obtain antioxidative peptides by hydrolyzing various fish-protein sources. Montero *et al*. (1995) also described a method by which to extract gelatin from fish skin. The skin is cleaned with 0.8 M sodium hydroxide and water, then treated with 0.2 M sodium hydroxide and 0.05 M acetic acid. Finally, the mixture is kept in distilled water at 45 °C for 18 hours. Muyonga *et al*. (2004) outlined a method by which to get gelatin from bones, in which the bones are pretreated by tumbling them in warm (35 °C) water and then demineralized using 3% HCl, at ambient temperature (20–25 °C), until they lose their hard cores (9–12 days). The solution is then covered and boiled at various temperatures (50, 60, and 70 °C) for 5 hours.

Methods have been developed for the separation and purification of peptides with different molecular weights. An ultrafiltration membrane system equipped with appropriate molecular-weight cutoffs has been found effective (Jeon *et al*., 2000).

37.4 HEALTH-PROMOTING ABILITY OF FISH PEPTIDES

Peptides isolated from various fish-protein hydrolysates show different biological activities, such as antihypertensive, antioxidative, antithrombotic, immunomodulatory, anticoagulant, antimicrobial, and calcium-absorbing effects, as shown in Fig. 37.2. The specific bioactivity of a fish peptide against various diseases depends basically on its structural properties such as chain length and the physicochemical characteristics of the amino acid residues, including hydrophobicity, molecular charge, and side-chain bulkiness (Pripp *et al*., 2004). Even if the activity of these naturally obtained peptides is lower than that of the synthetic compounds, they show several advantages over the synthetic compounds, including the safety of the natural product, low health cost, and the additional nutritional benefits of the peptides as a source of beneficial and essential amino acids.

37.4.1 Antioxidant Activity

Synthetic food antioxidants have been widely applied in the food industry to both preserve food and fortify its nutritional value, and interest in their use in human health is increasing.

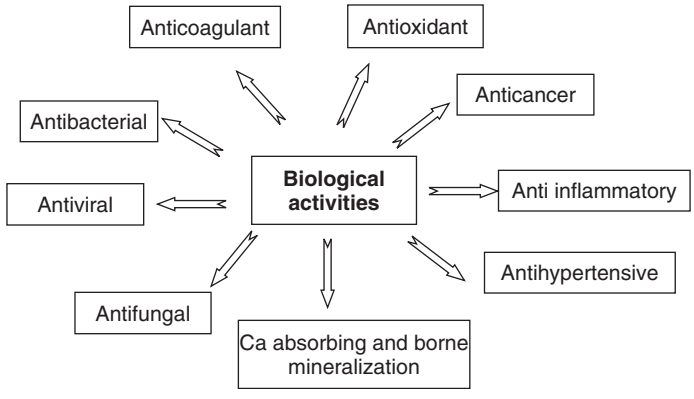

Fig. 37.2 Biological activities of fish peptides.

There is abundant information on the antioxidant properties of protein hydrolysates and peptides from fish sources in various oxidative reaction systems (Table 37.1). Currently there is interest in finding natural antioxidative substances that have no side effects, and those identified have the potential for safe and nonhazardous use against the complications arising from the oxidation of biomolecules.

The hydrolysates of mackerel were found to contain peptides that exhibited antioxidant activity *in vitro* (Wu *et al*., 2003). Peptides released by the hydrolysis of mackerel with Protease N inhibited the autoxidation of linoleic acid, quenched the free radical α,α-diphenyl-β-picrylhydrazyl (DPPH), and reduced Fe^{3+} to Fe^{2+}. The strongest antioxidant fraction of mackerel-protein hydrolysate contained small peptides and free amino acids and had a molecular weight of 1400 Da.

Je *et al*. (2007) purified an antioxidative peptide from tuna-backbone protein and identified it as VKAGFAWTANQQLS (1519 Da). This was very important with regard to the functional foods. It was reported that the derived peptides were good radical scavengers and had antioxidant activity.

An antioxidant peptide has been isolated from hoki-frame protein by gastrointestinal digestion (Kim *et al*., 2007). A number of enzymes have been used for the hydrolysis

Table 37.1 Bioeffects of fish-protein peptides.

Species/substrate	Bioeffect	Reference
Hoki-frame protein	Antioxidant	Kim *et al.* (2007)
Conger eel-muscle protein	Antioxidant	Ranatunga *et al.* (2006)
Alaska pollock-frame protein	Antioxidant	Je *et al.* (2005)
Alaska pollock backbone	Calcium bioavailability	Jung *et al.* (2006)
Jumbo squid-skin gelatin	Antioxidant	Mendis *et al.* (2005)
Large yellow croaker	Antimicrobial	Zhang *et al.* (2009)
Red sea bream	Antimicrobial	Iigima *et al.* (2003)
Bony fish Atlantic salmon	Antimicrobial	Dougles *et al.* (2003)
Fish-protein hydrolysate	Anticoagulant	Rajapaksha *et al.* (2005)
	Anti-breast cancer	Picot *et al.* (2006)
Anchovy sauce, whole fish	Human lymphoma cancer	Lee *et al.* (2003)
Tuna dark muscle	Breast cancer	Hsu *et al.* (2011)

process and the isolated peptides showed strong antioxidant activities in various model systems. Moreover, the peptides from Alcalase- and Flavourzyme-digested yellow stripe trevally were shown to prevent oxidative damage to DNA using the Fenton reaction. A possible reason for this is that the hydrolysates chelated Fe^{2+}, thus preventing it from reacting with H_2O_2 and forming hydroxyl radicals (Klompong *et al*., 2009).

Gelatin peptides have repeated unique Gly-Pro-Ala sequences in their structures and it is presumed that their observed antioxidative properties can be associated with this. Fish-skin gelatins have been reported to give rise to biologically active peptides with high antioxidant activity due to their radical-scavenging, metal-chelating, and reducing-power effects, or due to lipid-peroxidation inhibition (Mendis *et al*., 2005b).

The biological properties of peptides are to a large extent influenced by their molecular weights and conformational structures, and also by their processing methods and conditions. In contrast, the amino acid compositions of the hydrolysates resulting from hydrolysis with different types of enzyme are very similar to those of the parent gelatins, as reported by Kim *et al*. (2001) for Alaska pollock-skin gelatin and Gómez-Guillén *et al*. (2010) for tuna and giant squid-skin gelatins.

A dipeptide Met-Tyr, derived from sardine muscle (Matsufuji *et al*., 1994), stimulates expression of the antioxidant defense protein heme oxygenase-1 (HO-1) in a concentration-dependent manner. Previous findings revealed that HO-1 protein expression is accompanied by the induction of a secondary antioxidant protein, ferritin. In a current study, the effect of Met-Tyr on the expression of the antioxidant stress proteins HO-1 and ferritin in endothelial cells derived from the human umbilical vein and their contribution to the decrease in radical formation that occurs under the influence of this dipeptide were studied and their potential activity was reported (Erdmann *et al*., 2004). Yang *et al*. (2011) isolated an oligopeptide from chum salmon with antioxidant and antiradical activities in radiation-injured mice. Oligopeptides from chum salmon significantly increased white blood-cell-counts after irradiation and reduced the radiation-induced oxidative damage. These effects may be caused by augmentation of the activities of antioxidant enzymes, such as SOD and GSH-Px, reduction of lipid peroxidation (MDA level) in liver, and protection against radiation-induced apoptosis.

Various peptides have been isolated from seafood byproducts of the fisheries industry, such as tuna backbone (Lee *et al*., 2010). Tuna backbone was hydrolyzed using various proteases, such as alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin, in order to obtain an antioxidative peptide (Je *et al*., 2007). Seven novel antioxidative peptides were identified from protein hydrolysates recovered from the waste stream of sardinelle processing, with amino acid sequences LARL, GGE, LHY, GAH, GAWA, PHYL, and GALAAH and a molecular weight <600 Da (Bougatef *et al.*, 2010). It was reported that the peptide LHY displayed the highest DPPH radical-scavenging activity, with an ability to scavenge 63% of available DPPH radical.

Several mechanisms have been presented to explain the antioxidative activity of peptides, including metal ion-chelating ability, radical-scavenging activity, and aldehyde adduction (Zhou & Decker, 1999). Most of the food-derived peptides have a low molecular weight and possess hydrophobic residues with Lue and Val in their N-termini and Pro, His, Tyr, Trp, Met, and Sys in their sequences (Chen *et al*., 1995). These amino acid residues can increase the presence of peptides at the water–lipid interface and promote radical-scavenging activity at the lipid phase (Ranatunga *et al*., 2006). The

mechanism of His-containing peptides might be attributable to the hydrogen-donating ability, lipid peroxyl radical-trapping ability, and metal-chelating ability of the imidazole group (Chan & Decker, 1994).

37.4.2 Antihypertensive Activity

Hypertension is a major risk factor for cardiovascular diseases and is estimated to affect one-third of the Western population (Lopez-Fandino *et al*., 2006). Lifestyle modifications are much more important than synthetic drugs in effectively lowering blood pressure. Even small decreases in blood pressure result in significantly lower risks for cardiovascular diseases. Foods containing angiotensin I-converting enzyme (ACE)-inhibitory peptides may be a part of this lifestyle approach, as they have proven to be effective in both the prevention and the treatment of hypertension (Je *et al*., 2009).

Peptide quantitative structure–activity relationship modeling has been used to predict peptide structures with high ACE-inhibitory activity. Using physicochemical descriptors, Pripp *et al*. (2004) emphasized that increased side-chain hydrophobicity at the carboxy terminus and decreased side-chain length of the penultimate amino acid exemplified the ACE-inhibitory potential of peptides up to six amino acids in length. Moreover, ACE appears to be strongly influenced by the C-terminal sequence of the peptides. It has hence been suggested that proline, lysine, and arginine are the preferred amino acid at the C-terminal residue and thus contribute to ACE-inhibitory potency (Meisel, 1997). Furthermore, studies with spontaneously hypertensive rats (SHR) revealed that dipeptides with a C-terminal tyrosine residue produced a slow but prolonged decrease in systolic blood pressure compared to dipeptides with phenylalanine at the C-terminal. In contrast, dipeptides with a C-terminal phenylalanine caused a more rapid reduction in systolic blood pressure and a shorter duration of action (Suetsuna, 1998).

ACE-inhibitory peptides have been separated from the skin of skate by Lee *et al*. (2011). The purified peptides showed IC_{50} values of 95 and 148 μM respectively for the two peptides isolated. Further, Lineweaver–Burk plots indicated that the peptides act as noncompetitive inhibitors. Gu *et al*. (2011) separated a low-molecular-weight ACE peptide from Atlantic salmon skin with enzymatic hydrolysis using Alcalase and Papain. The salmon-skin collagen peptides obtained had high protein contents ($91.20 \pm 1.03\%$) and low molecular weights, 90.79% of which were less than 1000 Da. Recently, many inhibitory peptides against ACE have been reported as natural alternative biofunctional peptides that are safer than the existing artificial ACE-inhibitory compounds on the market, which show some side effects.

Fish-protein hydrolysates produced using commercially available proteases have been shown to possess combined ACE-inhibitory and antioxidant capacities (Je *et al*., 2009; Samaranayake *et al*., 2010), and it has been postulated that these two activities could contribute to their antihypertensive effect *in vivo* (Hou *et al*., 2003). Yellowfin sole frame has been used as the source of some ACE inhibitors (Jung *et al*., 2006), and studies have been conducted on sardinelle-protein byproducts in order to isolate ACE-inhibitory peptides (Bougatef *et al*., 2008).

Many of the salmon protein-derived peptides relate to salmon muscle (Enari *et al*., 2008; Ono *et al*., 2006). One *et al*. (2006) isolated Phe-Leu as a strong ACE-inhibitory

peptide from chum salmon muscle with thermolysin ($IC_{50} = 0.004$ mg/ml) and Leu-Phe from pink salmon muscle with papain (IC_{50} = 0.086 mg/ml) (Enari *et al*., 2008).

As previously mentioned, gelatin peptides have repeated unique Gly-Pro-Ala sequences in their structure which are presumed to be associated with their antihypertensive properties.

37.4.3 Antimicrobial Activity

Antimicrobial peptides are widely distributed throughout the animal kingdom. Three isoforms of a novel C-terminally amidated peptide—chrysophsin-1, chrysophsin-2, and chrysophsin-3—consist of 25, 25, and 20 amino acids, respectively, and are highly cationic, containing an unusual C-terminal RRRH sequence. They were isolated from the gills of red sea bream (Chrysophrys (Pagrus) major) (Iijima *et al*., 2003). These peptides show potent antimicrobial activity against Gram-negative and Gram-positive bacteria and act as nonspecific defense substances in fish skin. They fold into amphipathic secondary structures, typically α-helices and β-sheets. Such peptides can kill bacteria via either physical, chemical, or biological processes.

A novel 25-residue linear antimicrobial peptide named pleurocidin was isolated from the skin mucous secretions of the winter flounder (Cole *et al*., 1997). Pleurocidin is predicted to assume an amphipathic α-helical conformation similar to that of many other linear antimicrobial peptides and exhibits antimicrobial activity against *Escherichia coli* in a bacterial cell-lysis plate assay. Moreover, a study with skate skin included the production of collagen and the purification of an antimicrobial peptide (Cho *et al*., 2004).

Salampessy *et al*. (2010) isolated two antibacterial peptide fractions (9 and 12) from bromelainhydrolysate derived from leather jacket (*Meuchenia* sp.) insoluble muscle proteins. Fraction 12 showed higher minimum inhibitory concentration against *Bacillus cereus* and *S. aureus*, while fraction 9 only showed slight activity against *B. cereus*. Additional fractionation, on an analytical C-18 column, showed that many other peptides, such as the cationic antibiotic polymyxin, could account for the high minimum-inhibitory-concentration value.

Antimicrobial peptides are biomolecules employed in defense against bacteria by animals and plants (Brogden, 2005). They are short-chain, positively charged peptides secreted by organisms and are composed of 12–45 amino acid residues. When the antimicrobial peptides bind to the outermost leaflet of a negatively charged bacterial membrane by the aid of hydrophobic and electrostatic interactions, they fold into amphipathic secondary structures, typically α-helices and β-sheets. These peptides can kill bacteria via either physical, chemical, or biological processes. Many models are suggested to explain the antibacterial mechanisms of peptides, including the Shai–Matsuzaki–Huang (SMH) model, barrel-stave model, carpet model, and toroidal-pore model. Moreover, many theoretical and experimental models have been used to explain the antimicrobial mechanisms of cell lysis by antimicrobial peptides, including the molecular-mean-field theory (LaRocca *et al*., 1999), dynamics simulations (Leondiadou *et al*., 2006), and coarse-grained simulations (Lopez *et al*., 2005). Most of these approaches support the SMH model, which suggests that a physical hole in the membrane is stable and is an effective mechanism of antimicrobial activity.

37.4.4 Anticoagulant Effect

Enzymatically prepared fish-muscle peptides have also shown anticoagulant and antiplatelet properties tested *in vitro*, and the results suggest the capability of fish peptides to inhibit coagulation factors in the intrinsic pathway of coagulation (Rajapakse *et al*., 2005). A peptide purified from yellowfin sole, with 12.2 kDa molecular weight, activated coagulation factor XII by forming an inactive complex regardless of Zn^{2+} mediation (Rajapakse *et al*., 2005). This anticoagulant peptide acts to antagonize platelet-membrane glycoprotein integrin in order to arrest platelet aggregation.

37.4.5 Anticancer Effect

Proteins, peptides, and amino acids have been reported to show antitumor or antiproliferative activities. However, the antiproliferative activity of peptides derived from fish protein has rarely been studied. Picot *et al*. (2006) reported that hydrolysates obtained from three blue whiting, three cod, three plaice, and one salmon showed significant inhibition against two human breast-cancer cell lines, MCF-7/6 and MDA-MB-231. The hydrolysates contained a complex mixture of free amino acids, peptides of various sizes (ranging up to 7 kDa), and, in a lower proportion, lipids and sodium chloride. Moreover, two antiproliferative peptides were purified from tuna dark muscle by enzymatic hydrolysis with papain and protease against human breast-cancer cell line MCF-7 (Hsu *et al*., 2011). The amino acid sequences of the two antiproliferative peptides were Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr (1206 Da) and Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr (1124 Da), and both showed significant activity in a dose-dependent manner. Further, antiproliferative hydrophobic peptide (440.9 Da) derived from anchovy was able to induce apoptosis in human U937 lymphoma cells through an increase in caspase-3 and caspase-8 activity (Lee *et al*., 2003, 2004). Tilapia (*Oreochromis mossambicus*) hepcidin TH2-3 was evaluated in several tumor cell lines and found to inhibit human fibrosarcoma cell (HT1080 cell line) proliferation and migration. Chang *et al*. (2011) also investigated an antimicrobial peptide, TH1-5, with the view of finding antitumor activity in cancer cells, including the human cervix adenocarcinoma cell (HeLa), human hepatocellular carcinoma cell (HepG2), human fibrosarcoma cell (HT1080), *Cercopithecus aethiops* kidney cell (COS-7), and human kidney cell (WS-1).

37.4.6 Ca-absorbing and Bone-mineralization Ability

Kim *et al*. (1999) and Jung *et al*. (2005) reported that fish peptides are capable of accelerating calcium absorption. Furthermore, researchers have identified that fish-protein hydrolysates possess hormone-like peptides and growth factors which accelerate calcium absorption (Fouchereau-Peron *et al*., 1999). These peptides are capable of binding to cell-surface receptors on osteoclasts and affect calcium metabolism by decreasing the number of osteoclasts. Therefore, they could be used in the treatment of osteoporosis and Paget's disease.

In addition, gelatin peptides have been shown to accelerate the absorption of dietary calcium in animal models, increasing calcium bioavailability (Kim *et al*., 1998). Jung

et al. (2006) reported that fish-bone peptide II (FBP II) could inhibit the formation of insoluble Ca salts at neutral pH. During the experimental period, Ca retention was increased and the loss of bone mineral was decreased by FBP II supplementation in ovariectomized rats. The levels of femoral total Ca, bone-mineral density, and strength were also significantly increased by the FBP II diet, to levels similar to those in the casein-phosphopeptide diet group.

37.4.7 Others Activities

Acidic peptide fractions from Atlantic cod hydrolysate have shown strong immunostimulatory effects, and treatment of these peptides has stimulated the oxidative burst of Atlantic salmon leucocytes (Gildberg *et al*., 1996). Basically, immunomodulators enhance the production of oxygen metabolites in macrophages responsible for these oxygen metabolites, which determines the oxidative burst. Oxidative-burst reactions are of major importance to the bactericidal power of phagocytes. Boutin *et al*. (2012) conducted a study on the immunological effects of fish-protein supplementation in healthy adults.

Some peptides isolated from various fish and fermented fish have been reported to show prolyl endopeptidase-inhibitory activity (Sorensen *et al*., 2004). Peptides obtained from salmon, trout, cod, and Norwegian fermented trout muscle have been investigated for their potential inhibitory activity against prolyl endopeptidase and found to be good sources.

The intake of certain protein hydrolysates is more effective in lowering serum cholesterol than the intake of intact protein. Hosomi *et al*. (2010) showed that the hypocholesterolemic effect of fish protein and peptides was mediated by increased fecal acidic and neutral sterol excretions, which was due to the digested products of fish protein and peptides having reduced micellar solubility of cholesterol and increased bile acid-binding capacity. Further, they confirmed that intake of fish peptide is more effective in the suppression of lipid absorption than intake of intact fish protein.

37.5 FUTURE TRENDS OF PEPTIDES FROM FISH PROTEINS

Technological advances have created a cost- and time-efficient platform on which to screen a large number of compounds for their pharmacological and medicinal potencies. These technologies give insightful indications of the feasibility of transforming a new chemical entity into a drug and reduce the requirement for more extensive clinical trials. Due to their inherent bioactive properties, peptides might be useful as therapeutic or prophylactic agents. The production of peptide therapeutics from marine food proteins is therefore a promising approach for exploitation by the pharmaceutical industry.

37.6 CONCLUSION

Marine fish proteins and peptides have recently attracted great attention due to their potential biological activities and natural abundance. They are underutilized natural resources that have the potential to be used in many industries. Moreover, discarded seafood-processing wastes have been recognized as potential materials for the derivation of

bioactive peptides for use in various fields. These bioactive peptides have been reported to have a range of biological activities, including antioxidant, antihypertensive, anticoagulant, antibacterial, and calcium-absorbing abilities.

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