CHAPTER 4

Characterization of Bioactive Peptides Obtained from Marine Invertebrates

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Contents

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Abstract

Bioactive peptides as products of hydrolysis of diverse marine invertebrate (shellfish, crustacean, rotifer, etc.) proteins are the focus of current research. After much research on these muscles and by-products, some biologically active peptides were identified and applied to useful compounds for human utilization. This chapter reviews bioactive peptides from marine invertebrates in regarding to their bioactivities. Additionally, specific characteristics of antihypertensive, anti-Alzheimer, antioxidant, antimicrobial peptide enzymatic production, methods to evaluate bioactivity capacity, bioavailability, and safety concerns of peptides are reviewed.

I. INTRODUCTION

Bioactive peptides with beneficial effects for human health have been found that are marine in origin. Several bioactive peptides were isolated from various invertebrate marine organisms. Marine invertebrates are animals that inhabit a marine environment and are invertebrates (they lack a vertebral column). In order to protect themselves, they may have evolved a shell or a hard exoskeleton, but this is not always the case. Invertebrate muscle cells, however, comprise two major cell classes: striated and smooth. Striated muscle can be subdivided according to the type of striation into transversely striated (like that of vertebrate striated muscle) and obliquely striated. As bioactive peptides have already been discovered in various vertebrate muscles, and as the structures of vertebrate and invertebrate muscles are very similar, invertebrates/insects may be new sources of bioactive peptides. The basic proteins of vertebrate and invertebrate muscles are actin, myosin, and collagen (Oota and Saitou, 1999). Components of proteins in marine foods contain sequences of bioactive peptides which could exert a physiological effect in the body. Moreover, some of these bioactive peptides have been identified to possess nutraceutical potentials that are beneficial for the promotion of human health. Recently, the possible roles for food-derived bioactive peptides in reducing the risk of cardiovascular diseases have been explored (Erdmann *et al.*, 2008). Bioactive peptides usually contain 3–20 amino acid residues, and their activities are based on their amino acid composition and sequence (Pihlanto-Leppala, 2000). 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. Marine-derived bioactive peptides have been obtained widely by enzymatic hydrolysis of marine proteins and have been shown to possess many physiological functions including antimicrobial (Liu *et al.*, 2008), antioxidant (Mendis *et al.*, 2005), and antihypertensive activities (Lee *et al.*, 2011). The goal of this chapter is not to present a complete panorama of

marine bioactivity peptides but to show that new materials in marine substances provide new solutions for tackling some of the major public health problems of the twentieth century. These include hypertension, oxidation damage, Alzheimer's disease (AD), and antibacterial issues. These are correlated with aging populations, especially in advanced countries.

II. PREPARATION OF BIOACTIVE PEPTIDES FROM INVERTEBRATES

There is great potential in the marine bioprocessing industry for converting and utilizing products from most marine organisms and marine food products as valuable functional ingredients. There has been increasing interest in the utilization of marine invertebrates, and novel bioprocessing technologies are being developed to isolate bioactive substances from marine food products. The substances contain antihypertensive, antioxidatives, and anti-Alzheimer's properties, and can be used as functional foods and nutraceuticals. Development of these functional ingredients involves certain biotransformation processes through enzyme-mediated hydrolysis in batch reactors. Membrane bioreactor technologies equipped with ultrafiltration membranes are recently emerging for the bioprocessing and development of functional ingredients. It is considered a potential method for utilizing marine food products efficiently (Kim and Mendis, 2006; Kim and Rajapakse, 2005; Kim *et al*., 2006). Biologically active peptides can be generated from precursor proteins in multiple ways, including enzymatic hydrolysis (either by digestive enzymes or enzymes derived from microorganisms and plants) and microbial fermentation (Kim *et al*., 2001). Enzymatic hydrolysis is a particularly famous method for obtaining bioactive peptides from organism tissue by *in vivo* hydrolysis of protein sources using appropriate proteolytic enzymes. The physicochemical conditions of the reaction media, such as temperature and pH of the protein solution, must then be adjusted in order to optimize for the activity of the enzyme used. Proteolytic enzymes from microbes, plants, and animals can be used for the hydrolysis process of marine proteins to develop bioactive peptides (Simpson *et al*., 1998). Further, alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin have been used for the hydrolysis of marine invertebrate muscle under optimal conditions of pH and temperature (Lee *et al*., 2009). Moreover, one of the most important factors for producing bioactive peptides with the desired functional properties is the molecular weight (MW) of the bioactive peptide (Deeslie and Cheryan, 1981). Therefore, for efficient recovery and in order to obtain bioactive peptides with both the desired molecular size and functional properties, the use of an ultrafiltration

membrane system is suitable. An ultrafiltration membrane system equipped with the appropriate MW cutoff is effective in separating peptides with desired MWs from fish protein hydrolysates (Jeon *et al*., 1999). In order to obtain functionally active peptides, a common method used sorts enzymes, which allows for sequential enzymatic digestion. Moreover, the ultrafiltration membrane system has been able to obtain serial enzymatic digestions using a multistep recycling membrane reactor combined with the ultrafiltration membrane system to separate fish protein hydrolysates based on their MWs (Fig. 4.1) (Byun and Kim, 2001). Additionally, 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 (Byun and Kim, 2001; Kim and Mendis, 2006). This membrane bioreactor technology equipped with ultrafiltration membranes is a newly emerging technology for the development of bioactive compounds and is considered a potential method for utilizing marine proteins as value added nutraceuticals with beneficial health effects.

Bioactive peptides can be extracted and purified with these technologies, which vary from simple to complex. Following this, the isolation of bioactive peptides, oligosaccharides, fatty acids, enzymes, water-soluble minerals, and biopolymers for biotechnological and pharmaceutical applications is possible. Further, some of these bioactive peptides have been identified to possess nutraceutical potentials that are beneficial for human health.

III. ANTIHYPERTENSIVE ACTIVITY

A. The mechanism of hypertensive

The renin–angiotensin system (RAS) constitutes one of the most important hormonal systems in the physiological regulation of blood pressure. Indeed, deregulation of the RAS is considered a major factor in the development of cardiovascular disease, and blockade of this system offers an effective therapeutic regimen. Originally defined as a circulation or endocrine system, multiple tissues expressed as a complete local RAS are compelling evidence for angiotensin I and angiotensin II, the primary peptides that can affect the system. ACE is a dipeptidyl carboxypeptidase that catalyzes the conversion of angiotensin I (decapeptide) to angiotensin II (octapeptide), inactivates the antihypertensive vasodilator bradykinin, and increases blood pressure (Fig. 4.2). Inhibition of ACE activity leads to a decrease in the concentration of angiotensin II and consequently reduces blood pressure (Skeggs *et al*., 1957).

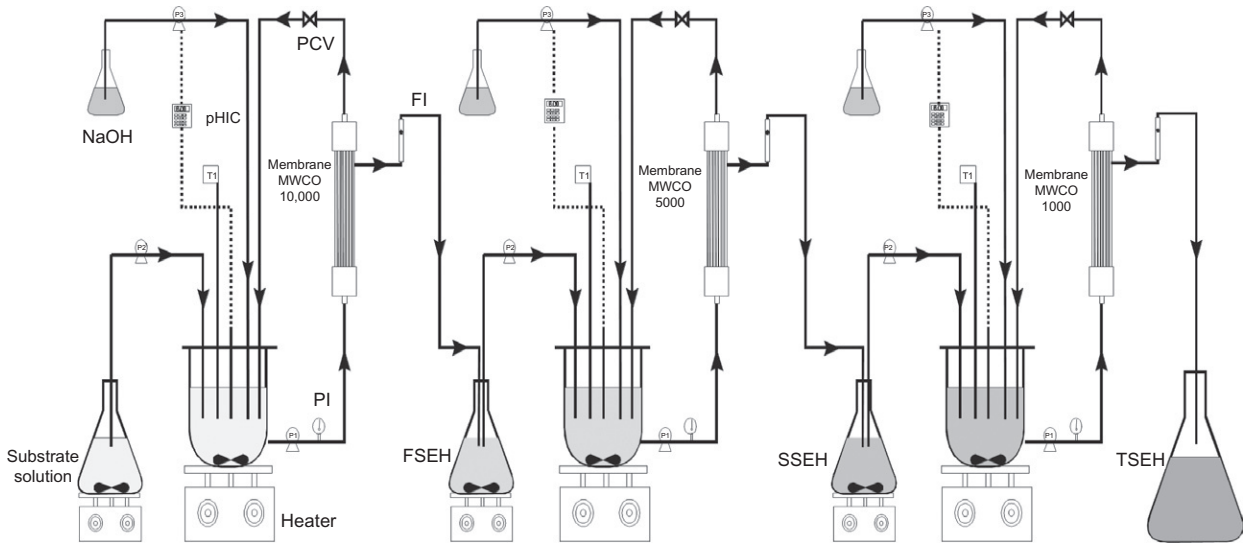

FIGURE 4.1 Schematic diagram of the three-step recycling membrane reactor for production and separation of enzymatic hydrolysates. PI, pressure indicator; FI, flow indicator; pHIC, pH indicator controller; FSEH, first hydrolysates; SSEH, second hydrolysates; TSEH, third hydrolysate.

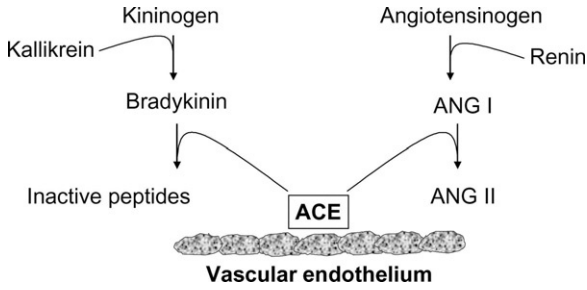

FIGURE 4.2 Schematic diagram of the renin–angiotensin system and kallikrein–kinin system. The angiotensin-converting enzyme is strategically poised to regulate the balance between ang II and bradykinin. ACE, angiotensin-converting enzyme; ang, angiotensin (Borer, 2007).

B. Shellfish

As ACE-inhibiting peptides have already been mentioned with regard to various vertebrate muscles, and as the structure of vertebrate and invertebrate muscle is very similar, invertebrates may be new sources of ACE-inhibiting peptides. However, while many ACE-inhibitor peptides have been derived from vertebrate muscle, very few studies on the subject have been conducted.

Two studies reporting on ACE-inhibitory peptides detected in them in hydrolysates of oyster. Among hydrolysis of oyster proteins with 11 proteases, hydrolysis with denazyme AP (from *Aspergillus oryzae*) (Matsumoto *et al.*, 1994) resulted in Table 4.1. The hydrolysate showed an inhibitory activity against ACE with an IC_{50} of 550 μg/ml. After separation by column chromatography, the most active fraction had an IC_{50} of 85 μg/ml. The peptide isolated from this fraction was identified as Leu-Phe (IC_{50}=126 μM). Katano *et al.* (2003) hydrolyzed pearl oyster meat with an alkaline protease. The hydrolysate was orally administrated to spontaneously hypertensive rats (SHRs) and resulted in a significant decrease of systolic blood pressure (SBP). Four active peptides were isolated and identified as Phe-Tyr, Ala-Trp, Val-Trp, and Gly-Trp. Further, the oyster, *Crassostrea talienwhanensis Crosse* (Wang *et al.*, 2008a), was known as a source of the ACE-inhibitory peptide. In these peptides, the amino acid sequence of *C. talienwhanensis* contains ACE-inhibitory peptides that are not present in the other amino acid sequences: Val-Val-Tyr-Pro-Trp-Try-Glu-Arg-Phe (Table 4.1). Particularly, for the invertebrate *C. talienwhanensis*, unique ACE-inhibitory peptides were found that were different from sardine muscle (Table 4.1) (Matsufuji *et al.*, 1994). Another study of oysters by Huang *et al.* (2011) separated the highest ACE-inhibitory peptide and the calculated the relative MW distributed between 1842 and 1396 Da. Moreover,

TABLE 4.1 ACE-inhibitory peptides derived from shellfish: origin, amino acid sequence, enzyme used for hydrolysis, and IC_{50} value

Origin	Amino acid sequence	Enzyme	IC_{50} (μM)	References
Oyster, *Pinctada fucata martencii*	Leu-Phe	Denazyme AP	126	Matsumoto *et al.* (1994)
Oyster, *Crassostrea talienwhanensis Crosse*	Val-Val-Tyr-Pro-Trp-Tyr-Glu-Arg-Phe	Pepsin	66	Wang *et al.* (2008a,b)
Freshwater clam, *Corbicula fluminea*	Val-Lys-Lys Val-Lys-Pro	Protamex	1,045 3.7	Tsai *et al.* (2006)
Fermented oyster sauce, *Crassostrea gigas*	Val-Lys-Lys	–	1,045	(Je and Kim, 2005)
Fermented blue mussel, *Mytilus edulis*	Glu-Val-Met-Ala-Gly-Asn-Leu-Tyr-Pro-Gly	–	81.91	Je *et al.* (2005a)

Shiozaki *et al.* (2010) hydrolyzed oyster (*Crassostrea gigas*) muscle, and the purified ACE-inhibitory activity peptide that resulted due to synergism was the hypertension-affecting peptide. One potent ACE-inhibitory peptide, Asp-Leu-Thr-Asp-Tyr. This raises the research objective to evaluate invertebrate species, such as mollusks, as potential sources of ACE-inhibitory peptides. In hydrolysates of the protein of the freshwater clam, *Corbicula fluminea*, hydrolyzed with Protamex, ACE-inhibitory activity was observed. A gastrointestinal digestion resulted in the highest ACE-inhibitory activity (Tsai *et al.*, 2006). This freshwater clam hydrolysate (peptide concentration 5 mg/ml) was used as a drink administered to SHRs for 8 weeks. The SBP and diastolic blood pressure of the SHRs were significantly reduced by 22.0 and 13.2 mmHg, respectively. Further, the antihypertensive activity of the purified peptide present in fermented oyster sauce, *C. gigas* (Je and Kim, 2005), was evaluated by measuring the change of SBP at 1, 2, 3, 6, and 9 h after oral administration of 10 mg/kg of body weight. There was no change in SBP in the controlled group investigated. *C. gigas*, with a recorded SBP reduction of 19 mmHg at 3 h after administration, was observed, and the activity was maintained for 6 h. Captopril lowered SBP significantly from 1 to 6 h after administration of the drug. The other fermented blue mussel, *Mytilus edulis*, was highest in ACE-inhibitoryactivity (Je *et al.*, 2005b). The IC_{50} value of the purified ACE-inhibitory peptide was 19.34 μg/ml, and the 10 amino acid residues of the N-terminal sequence were Glu-Val-Met-Ala-

Gly-Asn-Leu-Tyr-Pro-Gly (Table 4.1). The purified peptide was evaluated for antihypertensive effects in SHRs following oral administration.

C. Marine zooplankton

Marine zooplankton (interstitial animals <1 mm) is recognized as one of Earth's most diverse communities, and yet is one of the least known. Generally, larval fish have a high demand because of their high dietary protein. Hence, their food requires high protein content and a rapid growth rate. Rotifers have a lot of amino acid pools for producing high content proteins (Rønnestad *et al.*, 2003).

The ACE-inhibitory peptide was isolated from the marine zooplankton rotifer (*Brachionus rotundiformis*). The ACE-inhibitory peptide from the rotifer was hydrolyzed using various commercial enzymes. Among the various hydrolysates, the Alcalase hydrolysate had the highest ACE-inhibitory activity; the IC_{50} value of Alcalase hydrolysate for ACE-inhibitory activity was 0.63 mg/ml. The ACE-inhibitory peptide from *B. rotundiformis* was fractionated using Sephadex G-25 and octadecyl silica column (Lee *et al.*, 2009). After a three-step purification process, the purified ACE-inhibitory peptide was identified as being 14 amino acid residues of Asp-Asp-Thr-Gly-His-Asp-Phe-Glu-Asp-Thr-Gly-Glu-Ala-Met, with a MW of 1538 Da (IC_{50}=9.64 μM). This peptide effectively lowered blood pressure after oral administration in SHRs at a dose of 50 mg/body weight (kg) (Fig. 4.3). This peptide exhibited maximal blood pressure reduction 3 h after oral administration and was more effective than oral administration of captopril. This peptide showed long-lasting antihypertensive activity since it contains ACE-inhibitory peptides with a variety of maximally effective times. In another study, the ACE-inhibitory peptide was purified and characterized from the enzymatic hydrolysate of a freshwater rotifer (*B. calyciflorus*) fractionation through gel chromatography and HPLC system (Lee *et al.*, 2010a). The ACE-inhibitory peptide was identified as being seven amino acid residues of Ala-Gln-Gly-Glu-Arg-His-Arg by N-terminal amino acid sequence analysis. The IC_{50} value of the purified ACE-inhibitory peptide was 47.1 μM.

D. Crustaceans

ACE-inhibitory peptides from invertebrates such as crustaceans have been reported. The sequential hydrolysis of defatted Antarctic krill muscle via pepsin and trypsin resulted in an ACE-inhibitory extract. The active peptide isolated from the extract was found to be Lys-Leu-Lys-Phe-Val, showing an IC_{50} value of 30 μM (Kawamura *et al.*, 1992). The other crustacean, *Acetes chinensis*, used the protease from *Bacillus* sp.

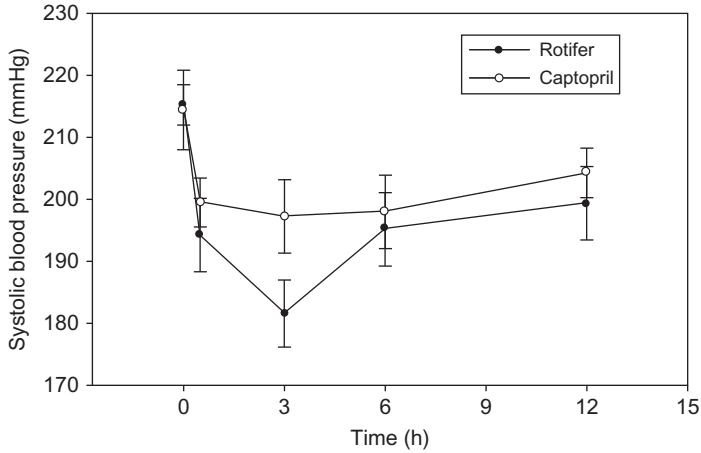

FIGURE 4.3 Antihypertensive activities of ACE-inhibitory peptides after single oral administration in SHRs. SHRs were administered captopril (○), the ACE-inhibitory peptide from rotifer (●) at a dose of 50 mg/kg. Changes in systolic blood pressure were expressed as mean±SE (n=5). Statistical analyses were done by Student t-test ($P<0.05$) (Skeggs *et al.*, 1957).

SM98011 to digest this kind of shrimp and found that the oligopeptide-enriched hydrolysate possessed antioxidant activity and high ACE-inhibitory activity with an IC_{50} value of 0.97 mg/ml (He *et al.*, 2006). The five peptides with high ACE-inhibitory activity were purified from the shrimp hydrolysates, and their sequences were identified by amino acid composition analysis and MW analysis. Three of them, Phe-Cys-Val-Leu-Arg-Pro, Ile-Phe-Val-Pro-Ala-Phe, and Lys-Pro-Pro-Glu-Try-Val, were novel ACE-inhibitory peptides (Table 4.2). Their IC_{50} values were 12.3, 3.4, and 24.1 μM, respectively. To test the ACE-inhibitory activity of the peptides after they were digested by digestive enzymes *in vivo*, derived peptides from Phe-Cys-Val-Leu-Arg-Pro and Ile-Phe-Val-Pro-Ala-Phe were synthesized based on their amino acid sequences and the cleavage sites of digestive enzymes. The IC_{50} values of the derived peptides were determined, and the result showed that except for Val-Pro-Ala-Phe, Phe-Cys and Phe-Cys-Val-Leu, the ACE-inhibitory activity of the other nine derived peptides did not significantly change when compared with their original peptides. Surprisingly, five peptides had lower IC_{50} values than their original peptides, particularly for Arg-Pro (IC_{50} value=0.39 μM), which was about 30 times lower than its original peptide and nearly the lowest IC_{50} value overall for ACE-inhibitory peptides. It was found that the inhibitory activity of the peptide was intensified by 3.5 times from IC_{50} 15.9 to IC_{50} 4.5 μM after incubation with gastrointestinal proteases. The ACE-inhibitory peptide from *Acaudina molpadioidea* showed a clear

TABLE 4.2 ACE-inhibitory peptides derived from invertebrates: origin, amino acid sequence, enzyme used for hydrolysis, and IC_{50} value

Origin	Amino acid sequence	Enzyme	IC_{50} (μM)	References
Antarctic krill	Lys-Leu-Lys-Phe-Val	Pepsin+trypsin	30	Kawamura *et al.* (1992)
Shrimp, *Acetes chinensis*	Phe-Cys-Val-Leu-Pro	Protease from *Bacillus* sp. SM98011	12.3	He *et al.* (2006)
	Ile-Phe-Val-Pro-Ala-Phe		3.4	
	Lys-Pro-Pro-Gln-Try-Val		24.1	
	Tyr-Leu-Leu-Phe		172	
	Ala-Phe-Leu		65.2	
Shrimp, *Pandalopsis dispar*	Rich Tyr, Phe, Leu, Ile, Val, Lys	Alcalase	100–200 μg/ml	Cheung and Li-Chan (2010)
		Protamex	70μg/ml	
Fresh sea bream	Val-Ile-Tyr	Alkaline protease	7.5	Fahmi *et al.* (2004)
	Val-Tyr		1.6	
Sea cucumber, *Acaudina molpadioidea*	Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp	Bromelain+Alcalase	15.9	Zhao *et al.* (2009)

antihypertensive effect in SHRs at a dosage of 3μM/kg. Cheung and Li-Chan (2010) produced hydrolysates of shrimp, *Pandalopsis dispar*, by Alcalase and Protamex, which possessed strong ACE-inhibitory activity (IC_{50}=100–200μg/ml and 70μg/ml, respectively). Further, ACE inhibition was positively correlated (r^2=0.87) with bitterness of the hydrolysates. Fractionation by size-exclusion chromatography revealed that the bitter substances, which also showed strong ACE inhibition, were <3kDa in size and contained many hydrophobic residues, including Tyr, Phe, Leu, Ile, Val, and Lys. Despite the bitterness, these hydrolysates may have potential health benefits arising from their potent ACE-inhibitory activity.

E. Other invertebrates

Fahmi *et al*. (2004) produced the hydrolysate of collagen obtained from a sea bream scale and evaluated it with respect to ACE-inhibitory activity. The scales were hydrolyzed using an alkaline protease treatment by which 92% of the peptides were degraded to form hydrolysates. In addition, using SHRs, the oral administration of 300mg of the peptides (kg of body weight)$^{-1}$ d^{-1} was shown to decrease blood pressure significantly (P<0.05). Four peptides that demonstrated high ACE-inhibitory activities were isolated from the hydrolysate of the scales using chromatographic methods. The amino acid sequences of inhibitory peptides were determined to be Gly-Tyr, Val-Tyr, Gly-Phe, and Val-Ile-Tyr. Among the purified peptides, Val-Ile-Tyr had the highest ACE-inhibitory activity (IC_{50}=7.5μM) followed by Val-Tyr (IC_{50}=16μM). The sea cucumber, *A. molpadioidea*, was a novel ACE-inhibitory peptide, showing very low similarities to other ACE-inhibitory peptide sequences and was sequenced as Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp (Zhao *et al*., 2009).

Byun and Kim (2001) reported that the aromatic amino acids at the C-terminal and aliphatic amino acids at the N-terminal were designated peptide binding sites for ACE as a competitive inhibitor. However, Maria *et al*. (2009) reported that the casein hydrolysate had a hydrophobic character and low MW. More specifically, they were rich in the amino acids Pro, Val, and Phe, which contributes to the correct location of the peptide in the active site of ACE. This is most likely due to the rigid structure of this residue. In addition, their study suggested that the Pro, Val, and Phe at the C-terminus may contribute to ACE-inhibiting activity. In this chapter, the purified ACE-inhibitory peptides revealed were Pro, Val, and Phe at the C-terminal, where they exhibited strong ACE-inhibitory activity. The tripeptides with Pro, Val, and Phe hydrophobic amino acids at the C-terminal have high ACE-inhibitory activity because of the interaction between three subsites at the active site of ACE (Pihlanto-Leppala, 2000). ACE-inhibitory activity correlation studies have indicated

that ACE binding is strongly affected by the C-terminal tripeptide sequence of the substrate, and that the tripeptide could interact with ACE subsites S1, S′1, and S′2 (Pihlanto-Leppala, 2000). Therefore, marine invertebrate-derived ACE-inhibitory peptides have a potential use as functional ingredients in nutraceuticals and pharmaceuticals due to their effectiveness in both prevention and treatment of hypertension in addition to nutritive value. Some antihypertensive synthetic commercial drugs are known to produce side effects such as an abnormal elevation of the blood pressure after administration; however, marine invertebrate-derived bioactive peptides are well tolerated by the body and are not expected to cause any harmful side effects. In addition, cost-effective safe drugs can be produced from marine bioactive peptides, but further studies are needed with clinical trials for these marine-derived antihypertensive peptides.

IV. ANTI-ALZHEIMER'S ACTIVITY

A. Pathogenic mechanism of AD: Amyloid cascade hypothesis

According to recent epidemiologic studies, AD, a protein misfolding disease, is recognized as a major problem in public health in industrialized countries. This progressive neurodegenerative disease generally occurs after the age of 65 with prevalence around 2–4% at age 70 and somewhere between 30% and 50% by age 90 (Holtzman, 2002). It begins with short-term memory loss and continues with more widespread cognitive dysfunctions. The ultimate cause of the disease is unknown. Macroscopic changes found in brains with AD include shrinkage of the gyri, widening of the sulci, and enlargement of the ventricles along with two major microscopic lesions: extracellular amyloid plagues and intracellular neurofibrillary tangles (Giorgio *et al*., 2006). The main constituent of the accumulated amyloidic plaques in the brain is β-amyloid peptide (Aβ). After the cloning of the amyloid precursor protein (APP), it was revealed that Aβ must be excised from the middle of its larger precursor protein (Kang *et al*., 1987). The two necessary proteolytic cleavage events, one at the N-terminus by an enzyme termed ''β-secretase'' and one at the C-terminus by an enzyme termed ''γ-secretase'' have attracted a lot of attention. This is understandable because Aβ formation is the initial step in the hypothetical amyloid cascade (Hardy and Allsop, 1991) and is thus supposed to be ultimately responsible for the pathology of AD. Moreover, the necessity of proteolytic cleavage for Aβ generation immediately suggests the existence of two potential therapeutic intervention targets which could be addressed using standard protease inhibition approaches (John *et al*., 2003). Consequently, APP processing and Aβ generation have been

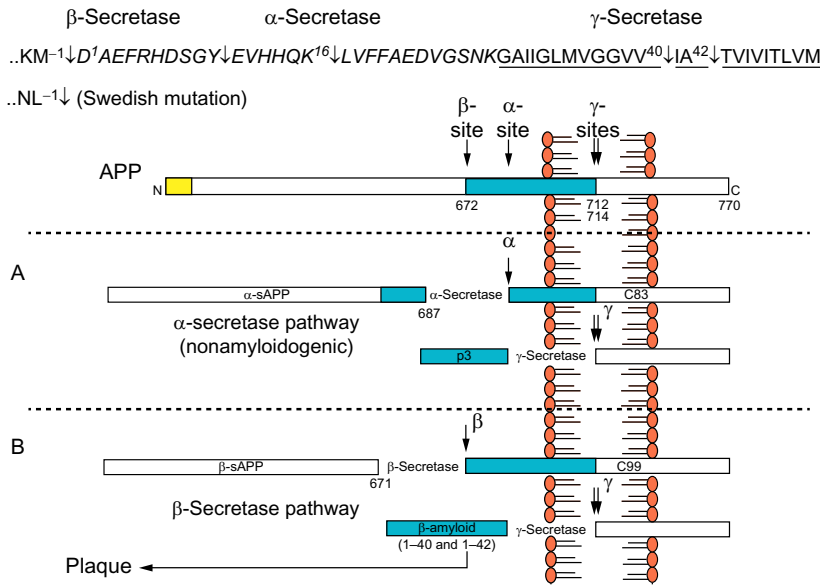

FIGURE 4.4 Schematic overview of APP processing by α-, β-, and γ-secretases (John *et al.*, 2003). The top panel shows the amino acid sequence of APP upstream of the transmembrane segment (underlined, bold) and encompasses the sequences of Aβ1-40 and Aβ1-42. The β-secretase cleaves at Asp^{1} and Tyr^{10}. The α-secretase cleaves at Lys^{16}, and the γ-secretase cleaves at Val^{40} and/or Ala^{42}. Below the sequence is a representation of APP emphasizing its membrane localization and the residue numbers of interest in β- and γ-secretase processing. (A) The nonamyloidogenic α-secretase pathway in which sAPPα and C83 are generated. Subsequent hydrolysis by the γ-secretase produces a p3 peptide that does not form amyloid deposits. (B) The amyloidogenic pathway in which cleavage of APP by the β-secretase to liberate sAPPβ and C99 is followed by γ-secretase processing to release β-amyloid peptides (Aβ1-40 and Aβ1-42) found in plaque deposits.

studied in a variety of systems by many investigators and their results are summarized in Fig. 4.4. At least three distinct protease activities are involved in processing the membrane protein APP along two major pathways: the α-secretase and the amyloid-forming β-secretase pathways.

A relatively small minority of APP molecules enter the β-secretase pathway in which β-secretase cleaves APP and releases a soluble fragment, sAPPβ. The C-terminal membrane-bound C99 peptide is then cleaved by γ-secretase within the transmembrane domain, and two major isoforms of 40 and 42 amino acid lengths with different C-termini, Aβ40 and Aβ42, are generated. Based on the amino acid sequence, β-secretase is predicted to be a type I transmembrane protein with the active site on the lumenal side of

the membrane where β-secretase cleaves APP. β-Secretase is an attractive treatment for the AD target (John *et al.*, 2003).

B. Marine zooplankton

For the β-secretase inhibitor, many groups have focused on the identification of inhibitors using high-throughput screening of compound collections and natural product extracts. The peptidic β-secretase (aspartic protease, memapsin-2) inhibitor OM 99-1 and other aspartic protease inhibitors have been developed (Ghosh *et al.*, 2000). OM 99-2, an eight-residue transition state inhibitor (Hu *et al.*, 2006), and OM 00-3, a more potent eight-residue transition state inhibitor (Turner *et al.*, 2001), have also been developed. Chitosan derivatives from crab shells exhibited weak β-secretase inhibition (Byun *et al.*, 2005; Je and Kim, 2005). Catechins from green tea (Jeon, *et al.*, 2003), ellagic acid from pomegranate (Kwak *et al.*, 2005), hispidin from mycelial cultures of *Phellinus linteus* (Park *et al.*, 2004), and several compounds isolated from *Sanguisorbae radix* (Lee *et al.*, 2005) have all been studied as β-secretase inhibitors. In contrast, efforts to discover naturally occurring β-secretase inhibitors have been relatively limited. Several hydroxyl containing inhibitors have been reported (Cumming *et al.*, 2004). Byun *et al.* (2009a) studied β-secretase activity of six hydrolysates from marine zooplankton and rotifers with different enzymes (Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin). In their study of the β-secretase-inhibitory activity, the highest IC_{50} value was exhibited by tryptic hydrolysate at 0.21 mg/ml (Fig. 4.5). The IC_{50} value of the purified β-secretase-inhibitory activity peptide was 183 μM. The β-secretase-inhibitory peptide was identified as a sequence of

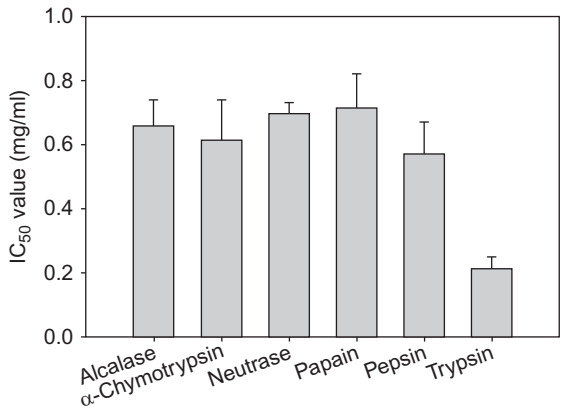

FIGURE 4.5 β-Secretase-inhibitory activity of various enzymatic hydrolysates from marine zooplankton, rotifer (Byun *et al.*, 2009a).

four amino acids, Gly-Arg-Gln-Lys by Q-TOF MS/MS. In addition, β-secretase-inhibitory peptides Gly-Arg-Gln-Lys, Gly-Arg-Gln, Gly-Arg, Arg-Gln-Lys, and Gln-Lys were synthesized by the solid-phase method. Among the five synthesized peptides, Arg-Gln-Lys had the highest β-secretase-inhibitory activity for 150.7 μM.

V. ANTIOXIDANT ACTIVITY

A. Oxidative stress

Oxidation is a vital process in all living organisms even though its side effect is the production of free radicals. Free radicals arise naturally during metabolism and during respiration in aerobic organisms, being by-products of normal reactions such as the production of calories, the degradation of lipids, the catecholamine response due to stress, and inflammatory processes (Wang *et al*., 2008b). During normal body reactions, such as respiration, reactive oxygen species such as superoxide anion radicals (O^2), hydroxyl radicals (OH•), and radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are formed (Gülçin, 2009, 2010). When free radicals are produced in excess or are produced and not eradicated, they can attack the closest molecules, subtracting electrons and starting a chain reaction in which the new molecule with a missing electron attacks other molecules (Kaur and Kapoor, 2001). It is well documented that free radical formation is implicated in many human diseases, such as health disease, stroke, arteriosclerosis, diabetes, and cancer (Dávalos *et al*., 2004). In the human body, a variety of enzymatic antioxidants, such as catalase, superoxide dismutase, and glutathione peroxidase, helps maintain some control over oxidation processes (Dimitrios, 2006). However, when an excess of free radicals is formed, the body's inherent protective antioxidant enzymatic system may become overpowered and it can cause destructive and lethal cellular effects by oxidizing lipids, proteins, DNA, and enzymes (Philanto, 2006).

B. Shellfish

As antioxidant peptides are rarely present in marine invertebrates, they must be released from the parent protein by hydrolysis with enzymes. Various enzymes have been used to release peptides from muscle proteins. To date, different muscle proteins have been extracted, hydrolysed, and their antioxidant activities studied, which is among all invertebrate muscles the most similar to vertebrate skeletal muscle. Various studies have been conducted to investigate the antioxidant properties of hydrolysates or bioactive peptides from marine invertebrate sources like oysters

(*C. gigas*; Qian *et al.*, 2008b), fermented mussel sauce (*M. edulis*; Jung *et al.*, 2005; Rajapakse *et al.*, 2005), and blue mussel (*Mytilus coruscus*; Jung *et al.*, 2007). Table 4.3 presents a list of studies on the antioxidant activities as well as structural properties of peptides and hydrolysates.

Qian *et al.* (2008b) hydrolyzed gastrointestinal digestions of oysters by gastrointestinal proteases and the purified peptide with the amino acid sequence Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu (MW 1.60 kDa) and exhibited higher activity against polyunsaturated fatty acid (PUFA) peroxidation than that of the native antioxidant, α-tocopherol. The free radical scavenging assay conducted using electron spin resonance (ESR) spectroscopy clearly exhibited that it scavenged hydroxyl radicals and superoxide radicals at IC_{50} values of 28.76 and 78.97 μM, respectively. Jung *et al.* (2005) investigated the use of fermented blue mussel sauce (FBMS). The antioxidative activities of FBMSs were investigated and compared with that of a natural antioxidant with α-tocopherol standing as a reference. Using consecutive chromatographic methods, the antioxidative peptide with a molecular mass of 620 Da was purified from a 6-month-fermented sauce, and its sequence of the peptide was Phe-Gly-His-Pro-Tyr. In addition, 64.8 mM of the purified peptide could scavenge 89.5% of hydroxyl radicals in radical scavenging assay using ESR spectroscopy. Rajapakse *et al.* (2005) used fermented mussel, which was identical with Jung *et al.*'s (2005) FMBs. It and the hepta-peptide sequence, His-Phe-Gly-Asp-Pro-Phe-His (MW 962 Da), were found to be highly effective for radical scavenging. FMBs could scavenge superoxide, hydroxyl, carbon-centered, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals for 21, 34, 52, and 96 μM by IC_{50} values, respectively. Moreover, FMBs exhibited a strong lipid peroxidation inhibition at 54 μM concentration, and it was higher than α-tocopherol. Jung *et al.* (2007) also gastrointestinally digested blue mussel (*M. coruscus*) muscle by pepsin, trypsin, and α-chymotrypsin. The potent antioxidant peptide, which was identified as Leu-Val-Gly-Asp-Glu-Gln-Ala-Val-Pro-Ala-Val-Cys-Val-Pro (MW 1.59 kDa), exhibited higher protective activity against PUFA peroxidation than the native antioxidants, ascorbic acid, and α-tocopherol.

C. Marine zooplankton

In another study conducted on marine zooplankton (Byun *et al.*, 2009b), antioxidant activity was measured for the DPPH radical of hydrolysates produced by Alcalase, α-chymotrypsin, Neutrase, papain, pepsin, and trypsin. To identify antioxidant peptides, peptic hydrolysate was purified using consecutive chromatographic methods, and antioxidant peptides were identified to be Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala (MW 1076 Da) and Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His (MW 1033 Da) by Q-TOF ESI mass spectroscopy. IC_{50} values of purified peptides were

TABLE 4.3 Antioxidant activity peptides derived from invertebrates: origin, amino acid sequence, enzyme used for hydrolysis, and IC_{50} value

Origin	Amino acid sequence	Radical	Scavenging activity, IC_{50} (μM)	References
Oyster, *Crassostrea gigas*	Leu-Lys-Gln-Glu-Leu-Glu-Asp-Leu-Leu-Glu-Lys-Gln-Glu	Hydroxyl Superoxide	28.76 78.97	Qian *et al*. (2008b)
Fermented blue mussel, *Mytilus edulis*	Phe-Gly-His-Pro-Tyr	Hydrl	64.8 (RAS at 89.5%)	Jung *et al*. (2005)
	His-Phe-Gly-Asp-Pro-Phe-His		34 21 96	Rajapakse *et al*. (2005)
Gastrointestinal digests of *Mytilus coruscus*	Leu-Val-Gly-Asp-Glu-Gln-Ala-Val-Pro-Ala-Val-Cys-Val-Pro	Superoxide	31.44 (RAS at 75.04%)	Jung *et al*. (2007)
Rotifer, *Brachionus rotundiformis*	Leu-Leu-Gly-Pro-Gly-Leu-Thr-Asn-His-Ala	DPPH	189.8	Byun *et al*. (2009b)
	Asp-Leu-Gly-Leu-Gly-Leu-Pro-Gly-Ala-His	DPPH	167.7	
Rotifer, *Brachionus calyciflorus*	Gly-His-Asp-Gly-Tyr-Glu-Pro-Leu-Ser-Ser	DPPH	100.8	Lee *et al*. (2010b)

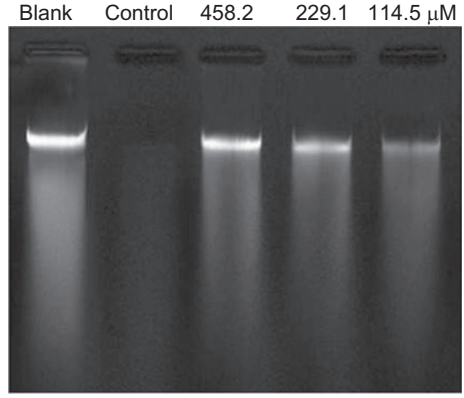

FIGURE 4.6 Protective effect on oxidative DNA damage of purified peptide from freshwater rotifer protein at various concentrations. Blank: untreated sample and H_2O_2, Control: distilled water instead of sample (Lee *et al.*, 2010b).

189.8 and 167.7 mM, respectively. In freshwater rotifers (Lee *et al.*, 2010b), the IC_{50} value of the purified antioxidant peptide was 100.8 μM. The antioxidant peptide was identified as a sequence of 10 amino acids, Gly-His-Asp-Gly-Tyr-Glu-Pro-Leu-Ser-Ser (MW 1091 Da), by N-terminal amino acid sequence analysis. The purified peptide exhibited an inhibitory effect against induced DNA oxidation (Fig. 4.6). Hydroxyl radicals have been known to cause oxidative damage in DNA structures by relaxing the DNA or denaturing it.

The results of this study strongly suggest that purified peptides can prevent oxidative damage to DNA when the DNA is exposed to OH• generated by the Fenton reaction. With the purified peptide of protective activity against DNA oxidation, a clear dose-dependent effect was observed. In this result, the effect of purified peptide was to protect hydroxyl radical-induced DNA damage.

D. Other invertebrates

Purple sea urchin (*Strongylocentrotus nudus*) gonads were treated separately with neutral protease, papain, pepsin, and trypsin (Qin *et al.*, 2011). The resultant hydrolysates were fractionated using a series of ultrafiltration membranes (MWCO 10, 5, 3, and 1 kDa). Five fractions were prepared from each hydrolysate, and the corresponding MW ranges were below 10 kDa, 5–10 kDa, 3–5 kDa,1–3 kDa, and below 1 kDa. The peptide fractions were evaluated for antioxidant activity by using the DPPH assay and reducing power assay. Results indicated that all peptide fractions possessed DPPH radical scavenging capacities and reduced power in a dose-

dependent manner. For all four hydrolysates, fractions below 1 kDa exhibited the highest DPPH radical scavenging capacity. The fractions below 1 kDa were prepared with neutral protease, papain, and pepsin, and the 1–3-kDa fraction prepared with trypsin showed the highest reducing capacity among corresponding hydrolysates.

Antioxidative properties of the peptides are more related to their composition, structure, and hydrophobicity (Chen *et al*., 1998). Tyr, Trp, Met, Lys, Cys, and His are examples of amino acids that cause antioxidant activity (Wang and De Mejia, 2005). Amino acids with aromatic residues can donate protons to electron-deficient radicals. This property improves the radical-scavenging properties of the amino acid residues (Rajapakse *et al*., 2005). It is proposed that the antioxidative activity of His-containing peptides is in relation with the hydrogen-donating, lipid peroxidation, radical trapping, and/or the metal ion-chelating ability of the imidazole group (Rajapakse *et al*., 2005). On the other hand, the SH group in cysteine has an independently crucial antioxidant action due to its direct interaction with radicals (Qian *et al*., 2008a). In addition to the presence of proper amino acids, their correct positioning in the peptide sequence plays an important role in antioxidant activity of peptides (Rajapakse *et al*., 2005).

Rajapakse *et al*. (2005) purified antioxidant peptides following the structure of His-Phe-Gly-Asp-Pro-Phe-His from the digestion of fermented blue mussel *M. edulis*. It was revealed that the antioxidant activity of a peptide was more dependent on the His segment in the N-Pro-Phe-His domain. According to the results from the same study, the His sequence displayed the greatest antioxidative activity with rotifer *B. rotundiformis* (Byun *et al*., 2009b). Table 4.3 provides information regarding the effect of amino acid compositions and their correct positioning in peptide sequences.

VI. ANTIMICROBIAL ACTIVITY

Antimicrobial peptides (AMPs) are a group of molecules exhibiting antimicrobial activity *in vitro*. In nature, they constitute an important part of the innate immune system in animals where they participate in the neutralization and elimination of intruding microorganisms (Zasloff, 2002). This chapter aims to discuss AMPs from marine invertebrates, mainly emphasizing the challenges and perspectives of purifying such peptides. In addition, different aspects concerning their antimicrobial potential are discussed. For a more general description of these molecules, recent review articles are available (Otero-Gonzalez *et al*., 2010; Smith *et al*., 2010). In this chapter, AMPs are considered as peptides/proteins between 1 and 12 kDa,

exhibiting a profound activity against microorganisms *in vitro*, and which are coded by single genes and ribosomally synthesized. Many low MW, extensively modified, and cyclic peptides have been characterized from sponges and tunicates. These peptides are presumably produced nonribosomally or by associated microorganisms and will therefore not be implemented in this chapter. Peptides with antimicrobial activity but obtained from larger proteins or classified with other functions are also omitted. However, the distinction between AMPs and some other peptides showing antimicrobial activity is rather unclear.

Marine-derived AMPs are well described in the hemolymph of the many marine invertebrates (Tincu and Taylor, 2004), including spider crabs (Stensvag *et al.*, 2008), oysters (Liu *et al.*, 2008), American lobsters (Battison *et al.*, 2008), shrimp (Bartlett et al., 2002), and green sea urchins (Li *et al.*, 2008). Liu *et al.* (2008) isolated a novel peptide *CgPep33* with high inhibitory activity against bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and fungi such as *Botrytis cinerea* and *Penicillium expansum* from the Pacific oyster (*C. gigas*) via enzymatic hydrolysis, and purified with DEAE Sephadex A-25 ion exchange, Sephadex G-25 gel filtration, and HPLC. *CgPep33* inhibited the *in vitro* growth of *B. cinerea* by 50% at 20–40 μg/ml and by 100% at 120 μg/ml. More interestingly, the IC_{50} values of this peptide against the above bacteria and fungi were 18.6–48.2 μg/ml (Liu *et al.*, 2007, 2008). Bartlett *et al.* (2002) reported that arthropod AMPs, while characterized primarily from insects, also have been isolated from crustaceans. Expressed sequence tag analysis of hemocyte complementary DNA libraries from two species of shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*, revealed transcripts with strong sequence similarity to an 11.5-kDa antibacterial peptide which identified Pro-Arg-Pro amino acid sequence. Further, an active peptide, Arg-Arg-Trp-Trp-Cys-Arg-X (X is an amino acid or an amino acid analog), against the herpes virus has been isolated from the enzymatic hydrolysate of oyster *C. gigas.* This peptide showed high inhibitory activity on the herpes virus (Zeng *et al.*, 2008). Zeng *et al.* (2008) obtained four fractions of hydrolysates according to MW <1, 1–5, 5–10, and higher than 10 kDa. Among them, the peptide purified from 5- to 10-kDa fractions had a higher biological activity than other fractions. The need to discover new antimicrobial substances is important due to the progressive development of resistance by pathogenic microorganisms against conventional antibiotics (Kim and Wijesekara, 2010). Marine invertebrates, representing an enormous genetic and biological diversity, have proven to be a rich source for discovering potent AMPs with novel and unique structural motifs. Therefore, it might be suggested that these AMPs have potent capacities for new antibiotic development in the pharmaceutical and food industries as novel antimicrobial agents.

VII. OTHER BIOLOGICAL ACTIVITY

The countless skin pathologies are correlated with exposure to UV light. Skin exposure to UV radiation induces critical effects for photodamage, which is characterized by distinct alterations in the composition of the dermal extracellular matrix resulting in wrinkles, laxity, coarseness, a mottled pigmentation, and histological changes that include increased epidermal thickness and connective tissue alteration (Kondo, 2000; Rittie and Fisher, 2002). The connective tissue of skin is made up of dozens of biomolecules including collagens, proteoglycans, and glycoproteins. Moreover, the breakdown of balance of the combination between these components leads to the detrimental effect in dermal fibroblasts like photoaging. Several investigations have described the deficiency of collagen in photoaged skin due to the inhibition of synthesis and the degradation mediated by matrix metalloproteinases (MMPs).

Kwon *et al*. (2007) revealed the antiwrinkle effects of peptides derived from collagens isolated from the starfish *Asterias amurensis*. The purified collagen peptides were fractions (F1: 116 kDa; F2: 100 kDa; F3: 58 kDa; F4: 43 kDa; F5: 24 kDa), and these peptides were reduced to 34.8% for MMP-1 expression of UVA-induced human normal fibroblasts at 1.0 mg/ml. The starfish *A. amurensis*' antiwrinkle collagen peptides had superior antiwrinkle effects of approximately 20 kDa. Barnacle (Mannello *et al*., 2003) (*Balanus amphitrite*) larvae MMPs showed biochemical characteristics different from those of vertebrate MMPs but common to other gelatinases from marine invertebrates; they were unaffected by several protease inhibitors and insensitive to specific activators' inhibitors of vertebrate MMPs.

VIII. CONCLUSION

In recent years, the marine environment has been shown to provide extremely rich biological active compounds. One of the hydrolysates derived from the marine invertebrate seems to possess many biological activities. Nowadays, major public health problems (including hypertensive, antioxidant, AD, and antibacterial) are in most cases treated by medicines containing synthetic drugs, which can cause serious side effects. Naturally occurring bioactivity peptides derived from marine organism proteins that are consumed daily can be used as components for functional foods or nutraceuticals. In this chapter, more particularly, marine invertebrates as a source of bioactive peptides were considered. Marine invertebrate muscle itself can act as a biologically active food through digestion with appropriate enzymes.

This suggests that products with bioactive peptides derived from marine invertebrates can meet the needs of marine organism-derived products due to health and/or religious reasons. From such a viewpoint, hydrolysates or bioactive peptides from marine invertebrates can be interesting sources of bioactivity peptides in the treatment of chronic diseases.

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