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Review

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Cardioprotective Cryptides Derived from Fish and Other Food Sources: Generation, Application, and Future Markets

Leticia Mora[†] and Maria Hayes*[,§]

[†]Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avenida Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[§]Food BioSciences Department, Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland

ABSTRACT: The primary function of dietary protein is to provide amino acids for protein synthesis. However, protein is also a source of latent bioactive peptides or cryptides with potential health benefits including the control and regulation of blood pressure. Hypertension or high blood pressure is one of the major, controllable risk factors in the development of cardiovascular disease (CVD), and it is also implicated in the development of myocardial infarction, heart failure, and end-stage diabetes. Cryptides can act on various systems of the body including the circulatory, gastrointestinal (GI), nervous, skeletal, and respiratory systems. A number of studies carried out to date have examined the health benefits of food protein isolates and hydrolysates. This review provides an overview of existing blood pressure regulating peptides and products derived from fish and other protein sources and hydrolysates. It discusses the methods used currently to generate and identify cryptides from these sources and their application in food and pharmaceutical products. It also looks at the current market for protein-derived peptides and peptide-containing products, legislation governing their use, and the future development of research in this area.

KEYWORDS: *renin angiotensin aldosterone system, cryptides, antithrombotic, ACE and ACE2 inhibitory, renin inhibitory, platelet activating factor acetylhydrolase inhibitory, blood−brain barrier, bioavailability, European Food Safety Authority, hydrophilic interaction liquid chromatography*

INTRODUCTION

Proteins are found in animal muscle and mammary fluids, in cereals and plants, and in fish and shellfish heads, frames, fins, tails, skin, and guts.[1] Three types of muscle protein are found in fish muscle; the sarcoplasmic, myofibrillar, and stromal proteins.[2] These proteins may serve as source material for the generation of bioactive peptides with potential health effects. In addition, fish skin is a rich source of collagen protein, which is also a known source of bioactive peptides. Bioactive peptides or cryptides are food-derived peptides that exert a hormone-like effect that has a positive physiological action, beyond that of basic human nutrition, following consumption.[3,4] Cryptides contain between 3 and 20 amino acid residues, and their bioactivities are based on the inherent amino acid composition and location within the peptide sequence.[5] They are inactive in the sequences of their parent proteins but may be released by a number of methods (Figure 1) including enzymatic hydrolysis using commercial, proteolytic enzymes,[6] gastrointestinal (GI) digestion,[7] by Generally Recognized As Safe (GRAS) bacteria such as lactobacilli during fermentation of protein,[8] or during food processing.[9] To exert a positive health effect, cryptides must cross the intestinal barrier and survive enzyme degradation in the GI tract following consumption. Cryptides are often multifunctional and can exert several beneficial physiological effects at different target sites when liberated in the human body. Depending on their amino acid sequence, they may be involved in biological functions including prevention of hypertension, opioid agonists or antagonists, and immunomodulatory, antithrombotic, antioxidant, anti-cancer, or antimicrobial activities.

Hypertension is defined as a sustained increase in blood pressure and is a controllable risk factor in the development of cardiovascular disease (CVD). Classically, the control of hypertension is associated with the renin−angiotensin−aldosterone system (RAAS) as well as the nitric oxide (NO) system and the sympathetic nervous system (SNS) system. Key enzymes within the RAAS include renin (EC 3.4.23.15), which acts on angiotensinogen produced by the liver to yield angiotensin-I, and angiotensin-I-converting enzyme (ACE) (EC 3.4.15.1), which catalyzes the conversion of inactive angiotensin I into the vasoconstrictor angiotensin II. The number of hypertensive adults worldwide is predicted to increase by about 60% to a total of 1.56 billion in 2025. Hypertension prevention, treatment, and control are therefore global, high-priority public health challenges. The relationship between blood pressure (BP) and cardiovascular risk appears to be positive, graded, and continuous.[10,11] Several potent synthetic peptides are in use for the clinical treatment of high blood pressure. These include the sulfhydryl-containing agents including Captopril (trade name Capoten), the first ACE inhibitor, Zofenopril, and dicarboxylate-containing agents including Enalapril, Ramipril, Quinapril, Perindopril, Lisinopril, and Benazepril. Phosphonate-containing agents including Fosinopril are also used.[12]

However, synthetic ACE inhibitors have certain side effects including cough, taste disturbances, skin rashes, high cost, and drug−drug interactions[12] as well as fetopathy if used during the second and third trimesters of pregnancy.[13] Therefore, much research has focused on the search for safer, nontoxic, and

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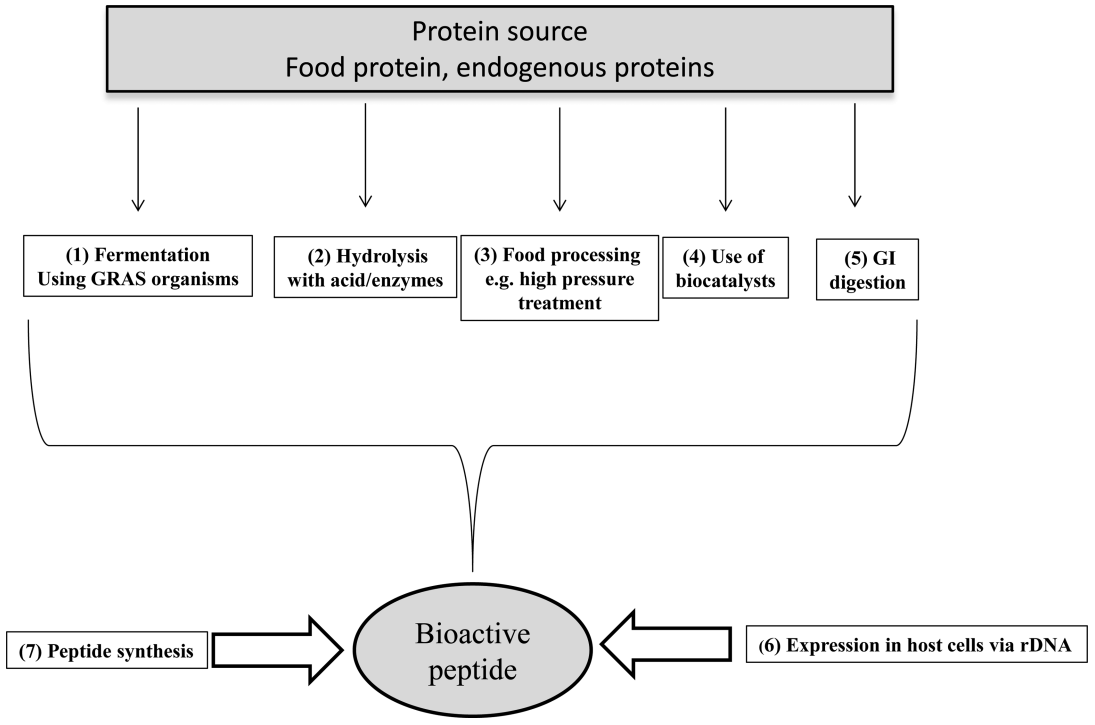

Figure 1. Routes to production of bioactive peptides from food protein sources.

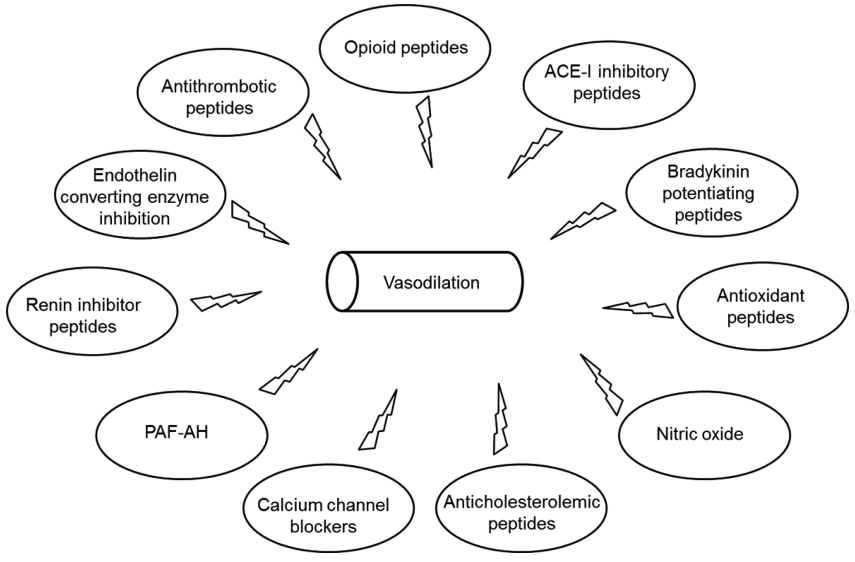

Figure 2. Peptides that can influence blood pressure regulation and vasodilation derived from food protein sources.

economical ACE inhibitors as alternatives to these synthetic drugs. During recent decades, a number of studies have looked at food therapy and dietary approaches for the prevention of chronic, lifestyle-related diseases, including hypertension.[14] Antihypertensive peptides with ACE or renin inhibitory activities are cheaper to produce with less stated side effects.[15] Fish meat consumption is often discussed as a factor in the prevention of heart disease as it is correlated with an increased dietary intake of omega-3 fatty acids.[16] However, fish protein is also a potential source of cardioprotective cryptides with ACE, renin, and phospholipase A_2 (PAF-AH) inhibitory and other activities. This review discusses the different pathways for blood pressure regulation in the circulation system and describes cardioprotective peptide generation from fish proteins. It also discusses the markets and future potential of muscle-derived heart health peptides, and the latest regulations regarding their use and generation.

BLOOD PRESSURE REGULATING ENZYMES AND THE RAAS

The RAAS and the SNS play major roles in the control of blood pressure.[17] In addition, a number of other factors may affect blood pressure due to the action of regulatory peptides including opioid, bradykinin potentiating, calcium channel blockers, and endothelial converting peptides (see Figure 2). Not all opioid or antioxidant peptides affect blood pressure. However, the opioid peptides endomorphin 1 and 2 were found to decrease systemic arterial pressure previously,

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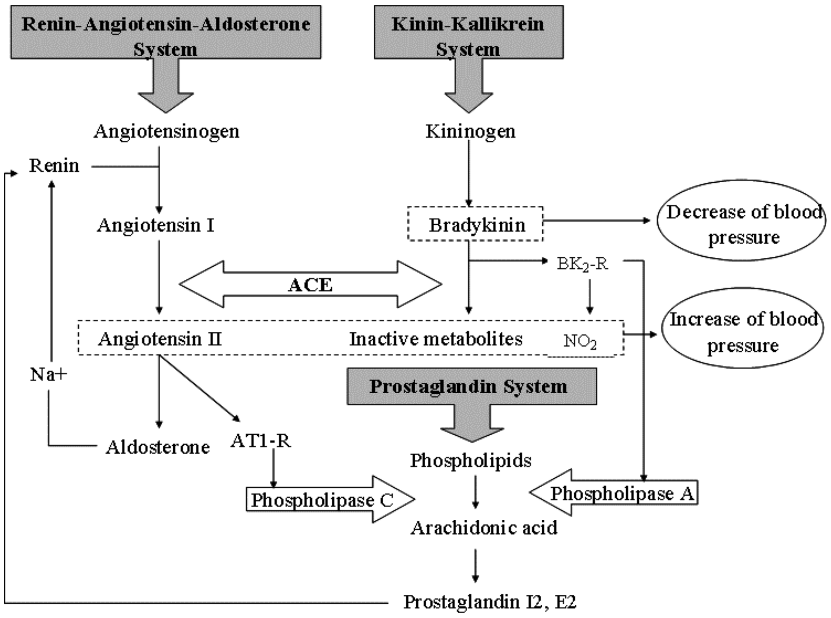

Figure 3. Classical renin–angiotensin aldosterone system (RAAS) and its relationship with the kinin–kallikrein and prostaglandin system. RAAS represents a cascade of enzymatic reactions. The huge precursor molecule of angiotensin II (Ang-II), angiotensinogen, is cleaved by renin, resulting in the still inactive decapeptide angiotensin I (Ang-I), which is then further cleaved by the membrane-bound metalloproteinase angiotensin-converting enzyme (ACE) to give the main effector hormone of the RAAS, the octapeptide Ang-II. In 2000, the first known homologue of ACE, ACE2, was identified. ACE2 catalyzes the generation of Ang-1–9 from An- I and of Ang-7 from An- II. It is not inhibited by ACE inhibitors. ACE2 metabolizes Ang-II to give the vasodilator Ang-1–7, which has been interpreted to mean that ACE2 provides a counterbalance, preventing overactivity of the classic RAAS.

although the mechanism of action is unknown.[18] Furthermore, it is hypothesized that low antioxidant levels may increase coronary heart disease. More research is needed to elucidate the role of antioxidative peptides and their protective functions in humans.[19] The classical RAAS (Figure 3) is a hormonal cascade that functions in the homeostatic control of arterial blood pressure. It was thought to be a straightforward cascade containing one substrate (angiotensinogen), two enzymes (ACE and renin), two peptides, angiotensin I (Ang-I) and angiotensin II (Ang-II), and one receptor (AT1). In reality, RAAS is a multiple effector system that consists of more than two dozen peptidases, a dozen Ang fragments, and at least six receptors.[20] The substrate angiotensinogen is released into the circulation from adipose tissue, and the liver and is degraded by the enzyme renin, which originates in the kidney. This action generates the inactive decapeptide angiotensin I, which is converted at the endothelial surface of blood vessels by the enzyme ACE into angiotensin II, the primary effector molecule of the RAAS.[20] Furthermore, evidence is now available that several RAAS components are expressed in cells from different organs of the body including the kidney.[21] In 2000, a homologue of ACE was discovered and called ACE2. Evidence indicates that ACE2 negatively regulates the activated RAAS by degrading angiotensin II to the heptapeptide Ang-(1–7). The enzymes ACE, renin, and ACE2 are key enzymes in the RAAS and therefore in the control of arterial blood pressure. Furthermore, the peptide Ang-A with the amino acid sequence Ala-Arg-Val-Tyr-Ile-His-Pro-Phe metabolizes Ang-II to Ang-III and causes a vasoconstrictive effect dependent on AT_1 receptors. Furthermore, Ang-M metabolizes Ang-III to Ang-IV and aspartyl aminopeptidase, which metabolizes Ang-I to Ang-2–10. They are suitable targets for functional food therapeutics for potential cardiovascular disease prevention.

ACE (EC 3.4.15.1). ACE, which is also known as kinase II, was first isolated in 1956 and termed the hypertension-converting enzyme.[22] It is a zinc metallopeptidase that requires chlorine for activation.[23] In the RAAS, ACE cleaves the decapeptide angiotensin I (Ang-I) into the octapeptide angiotensin II (Ang-II) by removal of the C-terminal dipeptide His-Leu. Ang-II is a potent vasoconstrictor and stimulates the release of aldosterone and antidiuretic hormone or vasopressin and increases retention of salt and water.[20] These activities raise blood pressure. ACE also inactivates the vasodilatory protein bradykinin and kallidin in the kallikrein–kinin system. ACE cleaves bradykinin (1–7) into the shorter fragment bradykinin (1–5).[24] ACE is also involved in the impairment of nitric oxide bioavailability.[20] Furthermore, ACE is thought to be involved in the functioning of the brain and nervous system as it can hydrolyze neuropeptides including encephalin, substrate P, luteinizing release hormone, and neurotensin.[25] ACE may also play a role in the gastrointestinal system as it hydrolyzes the hormones cholecystokinin and gastrin.[26] Furthermore, the ACE inhibitor perindopril ameliorates cognitive impairment and may have beneficial effects on Alzheimer's disease,[27] although ACE itself is thought to prevent the formation of Alzheimer amyloid β-peptide.[20]

There are over 10 synthetic ACE inhibitors available commercially including Captopril.[20] Drug development of ACE inhibitors is based on the knowledge that somatic ACE has two active sites and that these are not identical. However, ACE inhibitors developed to date show little discrimination between the active sites.[23] A number of studies have looked at isolation of ACE inhibitory peptides from food sources. ACE inhibitory peptides usually have large, bulky side chains, aromatic amino acids at the C-terminal end of the peptide, and nonpolar or positively charged amino acids at the N-terminus.[28] In tripeptides, aromatic amino acids are preferred at

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Table 1. Marine- and Food-Derived Cryptide Sequences with Confirmed in Vitro and in Vivo Cardioprotective Effects

source material	bioactive peptide sequence	bioactivity	enzymes or chemicals used	IC_{50} value	ref
marine animal and fish					
dried bonito (katsuobusi)	Ile-Lys-Pro	ACE inhibition	Thermolysin	0.32 μM	39
Branchionus rotundiformis (rotifers)	Asp-Asp-Thr-Gly-His-Asp-Phe-Glu-Asp-Thr-Gly-Glu-Ala-Medt	ACE inhibition	Alcalase	9.64 μM	40
Corbicula fluminea (freshwater clam)	Val-Lys-Pro and Val-Lys-Lys	ACE inhibition	Protamex	0.043 mg/mL	41
Crassostrea gigas (fermented oyster sauce)	not given	ACE inhibition	fermented	2.45 mg/mL	42
Crassostrea talienwhanensis (oyster protein)	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	ACE inhibition	pepsin	66 μmol/L	43
Ctenopharyngodon idella (grass carp fish)	not given	ACE inhibition	desalted fish scale	0.13 mg/mL	44
Limanda aspera (yellowfin sole frame protein)	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	ACE inhibition	none	28.7 μg/mL	45
Mytilus edulis (blue mussel)	Glu-Val-Met-Ala-Gly-Asn-Leu-Tyr-Pro-Gly	ACE inhibition	fermented	19.34 μg/mL	46
sardine muscle	not given	ACE inhibition	*Bacillus licheniformis* alkaline protease	0.015 mg protein/mL	47
sea bream scales	Gly-Tyr, Val-Tyr, Gly-Phe, Val-Ile-Tyr	ACE inhibition	alkaline protease	not given	48
shark meat	Cys-Phe, Glu-Tyr, Met-Phe, Phe-Glu, Cys-Phe, Glu-Tyr, Phe-Glu	ACE inhibition	Protease SM98011	0.4 mg/mL	49
Theragra chalcogramma (Alaska pollock skin)	Gly-Pro-Leu, Gly-Pro-Met	ACE inhibition	Alcalase, Pronase, Collagenase	2.6 and 17.13 μM	50
tuna frame protein	Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro	ACE inhibition	pepsin	11.28 μM	51
Barbus callensis (freshwater fish skin)	not given	DPP-IV and PEP inhibition	Esperase	not given	52
macro- and microalgae					
Undaria pinnatifda (wakame)	Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly, Tyr-Asn-Lys-Leu	ACE inhibition	pepsin	213, 64.2, 90.5, 21 μM	53
Palmaria palmata	Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala	renin inhibition and antihypertensive in SHR	papain	3.34 mM	54
Palmaria palmata	Ile-Leu-Ala-Pro, Leu-Leu-Ala-Pro, Met-Ala-Gly-Val-Asp-His-Ile	DPP-IV inhibition	Corolase PP	43−139 μM	55
Palmaria palmata	Asn-Ile-Gly-Lys	PAF-AH inhibition	papain	2.32 mM	56
Chlorella vulgaris	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe	ACE inhibition	pepsin	29.6 μM	57
animal					
Biceps fermoris (beef rump) sarcoplasmic proteins	Val-Leu-Ala-Gln-Tyr-Lys	ACE inhibition	Thermolysin and proteinase A	23.11−24.15 μg/mL	58
bovine brisket sarcoplasmic proteins	multiple peptides	ACE inhibition and antioxidant activity	papain	not given	59
bovine liver sarcoplasmic proteins, 10 kDa fraction	multiple peptides	antioxidant activity	Thermolysin	not given	60
porcine skeletal muscle myosin muscle	Met-Asn-Pro-Pro-Lys, Ile-Thr-Thr-Asn-Pro, Met-Asn-Pro, Asn-Pro-Pro, Pro-Pro-Lys, Ile-Thr-Thr	ACE inhibition	Thermolysin	66.6, 290.5, 1000, 678.2, 672.7 μM	61
dairy and egg					
Bos grunniens (yak) milk casein	Pro-Pro-Glu-Ilu-Asn, Pro-Leu-Pro-Leu-Leu	ACE inhibition	Alcalase	0.25−0.29 mg/mL	62
chicken egg yolk	Arg-Ala-Asp-His-Pro-Phe-Leu, Ile-Val-Phe	antihypertensive in rats		not given	63
fermented dairy drink Calpis	Ile-Pro-Pro, Val-Pro-Pro	ACE inhibition and antihypertensive in humans	*Lactobacillus helveticus* and *Saccharomyces cervisiae*	reported	5
fermented dairy drink Evolus	Ile-Pro-Pro, Val-Pro-Pro	ACE inhibition and reduction of arterial stiffness*	*Lactobacillus helveticus*	reported	5
bovine whey protein isolate BioZate	cysteine peptides	ACE inhibition and antihypertensive	whey hydrolyzed protein	not given	64
bovine casein protein isolate C12 peptide	Phe-Pro-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	antihypertensive in prehypertensive humans	bovine casein hydrolysate	not given	65
plants, legumes, and cereals					
Cicer arietinum (chickpea)	six inhibitory peptides	ACE inhibition	Alcalase	0.011−0.021 mg/mL	66
Amaranth hypochondriacus	Ala-Leu-Gln-Pro, Val-Ile-Lys-Pro	ACE inhibition		6.32, 175 μM	67
Brassica oleracea (broccoli)	Tyr-Pro-Lys	ACE inhibition	organic solvents	10.5 μg protein/mL	68
Allium sativum (garlic)	Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe, Asn-Phe	ACE inhibition	water extraction	66.3, 72.1, 3.74, 32.6, 130.2, 277.9, 46.3 μM	69
Fagopyrum esculentum (buckwheat)	Gly-Pro-Pro	ACE inhibition		6.25 μg protein/mL	70

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the C-terminus of the peptide, positively charged amino acids for the central position, and hydrophobic amino acids at the N-terminus for bioactivity. The IC_{50} values obtained for food-derived ACE inhibitors are usually a thousand-fold higher than synthetic Captopril.[29]

ACE2 (EC 3.4.17.23). ACE2 belongs to the metalloproteinase family of enzymes but has a single active site and was discovered in 2000 by two research groups.[30] It has a substrate preference for hydrolysis between proline and a basic or hydrophobic C-terminal residue.[30] It functions to counterbalance ACE and increases Ang-(1−7) formation. ACE2 may regulate blood pressure through bradykinin and apelin, and it is known that ACE2 deficiency accentuates vascular atherosclerosis and inflammation.[31] Compared with ACE inhibitor use, ACE2 is an endogenous regulator of RAAS, and activation of ACE2 would produce Ang-(1−7). ACE2 activity is commonly measured by hydrolysis of quenched fluorescent substrates in the absence or presence of an ACE2-specific inhibitor, such as the commercially available inhibitor DX600.[32] Screening marine and food protein sources for ACE2 activators is a potential option for identification of blood pressure and hypertension regulators.

Renin (EC 3.4.23.15). Renin secretion control is a key and rate-limiting step within the RAAS. Renin cleaves angiotensinogen to form the inert decapeptide Ang-I. Angiotensinogen is produced mainly from the liver but is also found in the brain, heart, adrenal, ovary, and adipose tissue.[33] In 2010 meta-analysis of randomized controlled trials with AT1 receptor blockers found that their use led to an increase in new cancer diagnosis. Direct renin inhibition may be an alternative pharmacological approach to RAAS inhibition. The renin inhibitor Aliskiren has been approved for use in Europe and the United States from 2007.[34] A number of dipeptides with renin inhibitory activities have been isolated previously from pea[35] and seaweed.[54] Their bioactivities were observed in vitro and in animal models. Saponins from soybean have also been documented as having renin inhibitory activities in vitro.[36]

COMMERCIALLY AVAILABLE MARINE PROTEIN HYDROLYSATES WITH HEALTH BENEFITS

Commercialization of cardioprotective food products is dependent on the availability of scientific data from in vivo animals and human models that positively demonstrate their contribution to blood pressure control. In Europe, the European Food Safety Authority (EFSA) governs the use of novel food and health claims made on food products and ingredients. Regulation 1924/2006/EC of December 20, 2006, on nutrition and health claims made on foods is a claim to protect consumers by prohibiting the use of misleading information on packaging. Therefore, food producers have to justify the use of a health claim and need to fully substantiate the claim scientifically. To address this, the bioactivity of functional ingredients needs to be characterized as fully as possible.

Guerard and colleagues[37] provide an overview of commercially available fish protein hydrolysates. Commercially available functional food products for human use developed from fish protein include Stabilium 200, an Atlantic fish autolysate (www.yalacta.com), and PROTIZEN, a white fish hydrolysate (www.copalis.fr), both carrying a relaxing effect. Other products include Nutripeptin, a cod hydrolysate, for lowering glycemic index (www.copalis.fr), Seacure, a fish fillet hydrolysate obtained by fermentation using a marine microorganism for improving gastrointestinal health (www.propernutrition.com (USA)), and Fortidium LIQUAMEN, a fish autolysate of white fish (*Molva molva*) (www.biothalassol.com), which claims to reduce oxidative stress and lower glycemic index. Other fish protein hydrolysates with health benefits include a hydrolyzed sardine protein, VALTYRON, manufactured using enzymatic hydrolysis (a protease) of sardine muscle and subsequent water and ethanol extraction of peptides. The Japanese company Senmi Ekisu Co., Ltd., obtained GRAS status for its hydrolyzed sardine protein from the FDA recently. This hydrolysate contains a dipeptide (Val-Tyr) with known ACE inhibitory and antihypertensive benefits. Furthermore, EFSA issued a positive opinion regarding the safety of the hydrolyzed Californian sardine protein hydrolysate product VALTYRON. EFSA concluded that VALTYRON produced by the Japanese company Senmi Ekisu Co., Ltd., was safe for use as a food ingredient at use levels of up to 0.6 g/serving. Significant reductions in blood pressure were observed following administration of single doses of up to 6 g of a sardine peptide hydrolysate product in subjects that exhibited higher than normal blood pressure at the start of the study and not in normotensive individuals. Indeed, EFSA issued a positive opinion regarding the safety of hydrolyzed sardine protein for use in cereal and other food products in 2010.[71]

Peptide sequences obtained from fish sources, muscle tissue, dairy and eggs, and plants are shown in Table 1.[38−70]

MARINE PROTEIN DERIVED CARDIOPROTECTIVE PEPTIDES

ACE Inhibitory Peptides. ACE inhibitory peptides are extensively used in therapy against hypertension.[72] Many ACE inhibitors have been isolated as enzymatic digestion products from food sources such as milk,[72] wheat,[73] soybean,[74] and maize.[75] Several studies have identified ACE inhibitory peptides from fish muscle proteins including salmon and chum salmon,[76] yellowfin sole,[44] sardine,[46] bonito,[78] tuna,[80] and shrimp.[80] Indeed, the most documented fish product with ACE inhibitory effects is dried bonito bowel, a seasoning used in Japan made from thin slices of boiled, dried bonito.[81,82] The pentapeptide corresponding to the amino acid sequence Leu-Lys-Pro-Asn-Met is responsible for the ACE inhibitory activity, and this peptide demonstrates a prolonged blood pressure lowering effect following oral administration.[82] This peptide is also found in traditional Japanese food known as katsuobushi. Katsuobushi is fermented, smoked skipjack tuna (*Katsuwonus pelamis*).[82] Sardine protein hydrolysate is widely documented as a source of ACE inhibitory peptides. The most active ACE inhibitory sardine peptide is the dipeptide VY. This peptide demonstrated a significant antihypertensive effect on mildly hypertensive patients as well as in spontaneously hypertensive (SPH) rats, previously.[84] Indeed, a thermolysin digest of katsuobushi was approved as a food for special health use (FOSHU) in Japan.[85]

The ACE inhibitory effects of fish sauces developed from salmon, sardine, and/or anchovy have also been reported. For example, Bordenave et al.[76] reported that 50 μg of powder consisting of autohydrolyzed protein of sardine and cod head wastes inhibited ACE by 30%. Shrimp autohydrolyzed waste in a similar quantity inhibited ACE by 57%. Furthermore, a study carried out by Fahmi and colleagues[47] showed that a sea bream hydrolysate showed excellent ACE inhibitory activity, and the peptides responsible for this action were determined as Gly-Tyr, Val-Tyr, Gly-Phe, and Val-Ile-Tyr. Freshwater fish muscle

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proteins and their hydrolysates offer huge potential as novel sources of natural bioactive peptides with ACE inhibitory activity.[83] Recently, two ACE inhibitory peptides were isolated from snakehead fish sarcoplasmic protein hydrolysates.[86] Enzymatic hydrolysis of sarcoplasmic protein was performed using commercial enzymes including Alcalase. The peptides had the amino acid sequences Leu-Tyr-Pro-Pro-Pro and Tyr-Ser-Met-Tyr-Pro-Pro with ACE inhibitory IC_{50} values of 1.3 and 2.8 μM, respectively. These peptides showed no cytotoxicity when tested using human embryonic fibroblast cell line (MRC-5) and human hepatocarcinoma cell line (HepG2).[86] ACE inhibitory peptides have also been isolated from fish skin proteins previously. For example, the ACE inhibitory peptide GPL was isolated from Alaska pollock skin gelatin hydrolysate.[49]

Fish-Derived Antioxidant Peptides. The increased production of reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals in combination with the outstripping of endogenous antioxidant defense mechanisms is thought to be an important cause of disease development including cancer, gastric ulcers, Alzheimer's, and arthritis. Oxidation of foods is one of the major causes of food deterioration, especially of lipids and proteins. In the food and pharmaceutical industries, many synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, *tert*-butylhydroquinone, and propyl gallate are used to retard peroxidation processes. However, the use of these synthetic antioxidants is strictly controlled due to potential health hazards caused by such compounds. This fact has caused an increase in the interest afforded to the isolation and characterization of natural antioxidants.

In relation to heart health, antioxidant peptides may play a positive role as oxidation by ROS and RNS is also thought to play an important role in the initiation or progression of several vascular diseases such as ischemic reperfusion, which is a common occurrence in cardiovascular surgery patients. It results in inflammation and oxidative damage of tissue due to the return of blood supply after a period of ischemia.[58] Several bioactive peptides including carnosine, anserine, L-carnitine, and the lipid conjugated linoleic acid (CLA) were identified as possessing antioxidant regulating properties.[59] Furthermore, commercial enzyme cocktails can be used to generate hydrolysates containing antioxidant peptides from different fish muscle protein sources.[60] These antioxidant peptides can act at different levels in the oxidative sequence, preventing the formation of free radicals or introducing substances that compete for the existing radicals. The antioxidant properties of peptides derived from different proteins are mainly influenced by the antioxidant properties of the amino acids contained in the peptidic sequence, including histidine, tyrosine, methionine, lysine, and tryptophan.

Protein hydrolysates from muscles of several fish species including tuna dark muscle (*Thunnus tonggol*),[61] round scad (*Decapterus maruadsi*),[62] yellow stripe trevally (*Selaroides leptolepis*),[63] Pacific hake (*Merluccius productus*),[64] tilapia (*Oreochromis niloticus*),[65] silver carp (*Hypophthalmichthys molitrix*),[66] grass carp (*Ctenopharyngodon idellus*),[66] houndshark (*Mustelus mustelus*),[67] mackerel (*Scomber austriasicus*),[68] Alaska pollock (*Theregara chalcogramma*),[41] and brown-tripe red snapper (*Lutjanus vitta*),[69] all have reported antioxidant activities. A report comparing the antioxidant activity of Atlantic salmon, Coho salmon, Alaska pollock, and southern blue whiting hydrolysates, generated using pepsin, pancreatin, and thermolysin enzymes, demonstrated antioxidant activities when hydrolysates were assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay. Alaska pollock pancreatin hydrolysate showed the lowest antioxidant values, whereas all other hydrolysates demonstrated similar antioxidant activities when assayed.[70]

Recently, a peptide identified from a croaker (*Otolithes ruber*) muscle protein hydrolysate with pepsin, trypsin, and α-chymotrypsin, found to contain the bioactive peptide sequence KTFCGRH, exhibited antioxidant activity in vitro against the radical scavenging efficiency of DPPH and hydroxyl radicals.[87] The identified peptide also proved effective when tested in vivo and neutralized ethanol-induced oxidative stress in rats.[87] Peptides VCSV and CAAP were purified and identified from α-chymotrypsin hydrolysate of flounder fish muscle, which showed radical scavenging activity against the DPPH free radical (IC_{50} values of 111.32 and 26.89 μM, respectively) and high cytoprotective activities against 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) in Vero cells.[88] Recently, Udenigwe and Aluko used partial least-squares (PLS) models to elucidate specific positive and negative contributions of individual amino acid residues and groups to antioxidant activities of proteins. They found that the effects depend on the oxidative assay system used, but on the basis of the results they found that previously reported antioxidant amino acid residues, especially histidine, had a negative effect on the antioxidant properties in the DPPH and H_2O_2 assays.[89]

Opioid Peptides. Opioid peptides bind to opioid receptors to produce morphine-like effects. Opioid receptors exist in the central and peripheral nervous systems, in the endocrine system, and in the immune system of mammals, as well as the intestinal tract. Natural opioid peptides include endorphins, enkephalins, and dynorphins.[90,91]

They are known to regulate various endocrine systems, including the hypothalamic−pituitary−adrenocortical (HPA) axis, and opioid peptides are thought to be responsible for the phenomenon of stress-induced analgesia.[92] Activation of opioid receptors results in adenylate cyclase inhibition, potassium channel activation, and calcium channel inactivation.[93] These receptors are also involved in the regulation of circulation, which can affect blood pressure.[94,95]

The first opioid peptides were identified in the late 1970s and were called "exorphins" due to their structural similarity to the endogenous ligands (endorphins and enkephalins) that interact with the opioid receptors found in the human body (μ-, κ-, and δ-).[96] Individual opioid receptors are responsible for specific bioactivities. For example, the μ-receptor is responsible for emotional behavior and suppression of intestinal motility,[97] the κ-receptor for satiety and food intake, and the δ-receptor for emotional behavior.[97] Opioid peptides derived from foods are of considerable interest as they are considered stable compared to endogenous opioid peptides, which are easily degraded by enzymes. Furthermore, food-derived opioid peptides have weaker bioactivities and therefore fewer side effects than those associated with endogenous opioid peptides, which include addiction and high dependency.[98−101]

The first opioid peptides derived from food proteins were of milk protein origin. The tetrapeptide β-lactorphin (YGLF) was originally released from the milk protein α-lactalbumin by enzymatic hydrolysis and showed an antihypertensive effect after oral administration to spontaneously hypertensive rats (SHR), with maximum reductions of blood pressure (BP) of 23 ± 4 mmHg in systolic BP. The vascular effects of another

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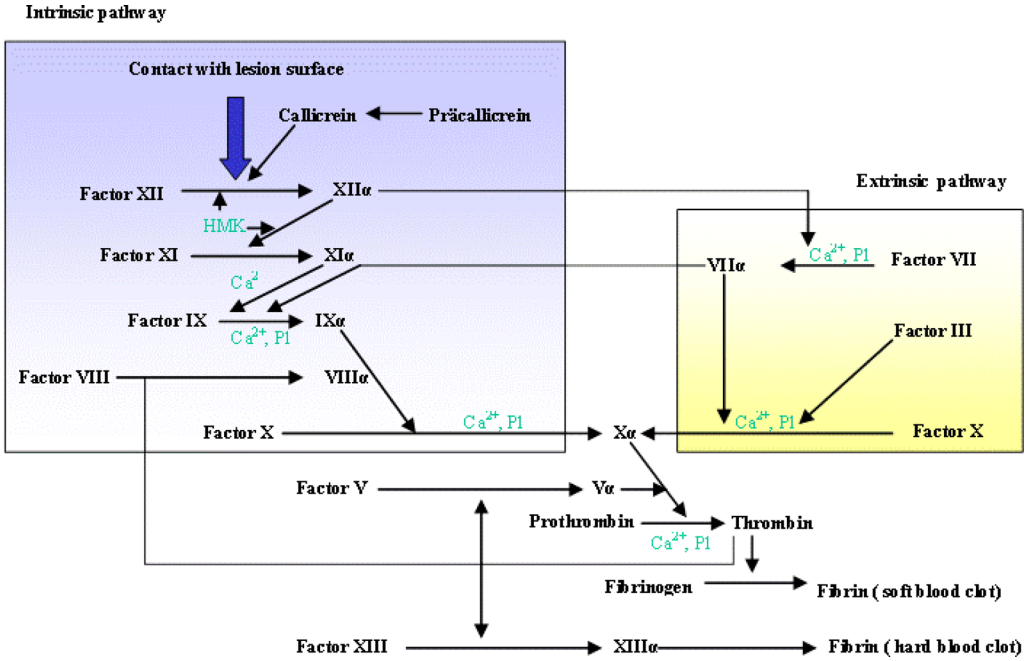

Figure 4. Coagulation cascade. In the intrinsic and extrinsic pathway model, initiation of coagulation is divided into two parts, the extrinsic pathway, which is thought to be responsible for the initial generation of activated factor X (factor Xa), and the intrinsic pathway, which leads to amplification of factor Xa generation. Factor Xa occupies a point where the intrinsic and extrinsic pathways converge and is central to the coagulation cascade.

structurally related tetrapeptide, α-lactorphin (Tyr-Leu-Leu-Phe), which originates from milk protein α-lactoglobulin, were also studied together with α-lactorphin (Tyr-Gly-Leu-Phe) effects. α-Lactorphin produced an endothelium-dependent vasorelaxation effect with nitric oxide synthase (NOS) inhibition. The α-lactorphin peptide also enhanced endothelium-independent vasorelaxation. This study concluded that α-lactorphin may stimulate the opioid receptors, which in turn release NO, causing the vasorelaxative effect.[100,101]

β-Casomorphins are well characterized in the literature.[101] Casomorphins are involved in regulation functions of the gut.[79] They enhance water and electrolyte absorption and act as antidiarrheal agents.[101] The casein-derived peptide casoxin D (Tyr-Val-Pro-Phe-Pro-Pro-Phe) has also been reported to have a hypotensive effect via opioid receptors, causing a vasodilatory relaxing effect in canine mesenteric artery strips.[101] Fish protein hydrolysates are known to accelerate calcium absorption[77] and provide satiety. They include the fish-derived peptide calcitonin/calcitonin gene related peptide (CGRP) and cholecystokinin-like peptides.[102] CGRP is a neuropeptide that acts as a potent arterial and venous vasodilator in humans.[102] Its capacity to increase heart rate as well as its ability to decrease food intake is well documented.[101,102] Guerard and colleagues generated CGRP molecules from a *Pollachius virens* byproduct hydrolyzed with Alcalase and treated with the microbe *Saccharomyces cerevisiae*.[103,104] Indeed, two commercially available products exist that carry relaxing effect claims.[37]

Antithrombotic Effect. An important complication that frequently occurs in patients with heart disease is the predisposition to develop thrombosis. Thrombosis is linked to platelet aggregation and small levels of fibrinolysis. Peptides that inhibit blood platelet aggregation and enhance fibrinolysis are strongly recommended to prevent thrombosis.

Blood coagulation is important in the cessation of blood loss from a damaged blood vessel (also known as hemostasis). A number of factors are involved in blood coagulation. The coagulation cascade consists of two pathways that are interconnected (Figure 4). These include the intrinsic pathway, which is also known as the contact activation pathway, and the extrinsic pathway or tissue factor pathway that leads to fibrin formation. When damaged, a platelet and fibrin-containing clot to prevent further bleeding covers the blood vessel. Both the intrinsic and extrinsic pathways activate the "final common pathway" of factor X, thrombin, and fibrin. Coagulation factors are generally serine proteases, transglutaminases (factor XIII), or glycoproteins (FVIII and FV), helping factors include calcium ions (Ca^{2+}), and phospholipid membranes. Heparin is an important anticlotting agent, which is often isolated from the small intestine of pigs or bovine lung tissue.

Food-derived peptides that inhibit blood platelet aggregation can be useful natural ingredients for the prevention of thrombosis. Thus, the search for new antithrombotic agents has mainly been focused on fruits and vegetables and marine carbohydrates including fucoidans and carrageenan.[105−110] Recently, collagen and its component proteins gelatin, collagen, and gelatin hydrolysates have been examined as potential natural antithrombotic ingredients. Indeed, a tetrapeptide, Asp-Gly-Glu-Ala (D-G-E-A), derived from type I collagen was found to inhibit collagen-induced platelet aggregation.[111] Khiari et al. studied the structure of antithrombotic peptides isolated from mackerel skin gelatin hydrolysates generated with pepsin, and observed antithrombotic activity due to the presence of the tripeptide FGN with a molecular weight of 337 Da.[112]

With regard to muscle sources, a recent study compared the effect of long-term intake of purified vegetable and animal proteins (casein, pork, egg white, chicken, white and red fish, soybean, and potato) and powders from whole vegetable and animal meats (soybean, pork, chicken, and horse mackerel) on thrombotic tendency and found that there was no difference in thrombotic tendency between the vegetable and animal protein diets with the exception of pork protein, which showed a prothrombotic effect.[113] In contrast, soybean powder showed an

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antithrombotic effect, which was mainly due to inhibition of atherogenesis rather than to platelet inhibition.[113] More recently, it was published that papain-hydrolyzed pork peptides showed antithrombotic activity using the shear-induced thrombosis test in vivo and a laser-induced thrombosis test in the muscle carotid artery in vivo.[114] However, the amino acid sequence of papain-hydrolyzed peptide responsible for the observed bioactivity was not determined.

Anticholesterolemic Effect. Elevated blood pressure, hypertriglyceridemia, hyperglycemia, and obesity are main factors of metabolic syndrome. These risk factors have a correlation with cardiovascular diseases and are also associated with an increased tendency to develop type 2 diabetes mellitus.[115] Hypercholesterolemia and low levels of high-density lipoprotein cholesterol are strongly associated with increases in blood pressure, which is known to promote atherosclerosis. This process leads to cardiovascular diseases and can cause myocardial infarction, stroke, and peripheral vascular disease. Thus, hypercholesterolemia is usually associated with high levels of low-density lipoprotein (LDL) and lower concentrations of functional, high-density lipoprotein (HDL). These balances are mostly genetically determined but can also be influenced by body build, medications, and food.

The hydrolysis of meat and fish proteins generates peptides that could alter the free and total amount of cholesterol in plasma and, thus, influence blood pressure values and development of cardiovascular diseases. Fish protein hydrolysates have been reported to reduce plasma total cholesterol, increasing the proportion of HDL cholesterol and decreasing the activity of the coenzyme group acetyl coenzyme A (acyl-CoA) in the liver of Zucker rats.[116] Acyl CoA is a group of essential coenzymes in the balance between carbohydrate and fat metabolism. Two acyl CoA molecules can be condensed to create acetoacetyl CoA, which participates in the pathway of cholesterol synthesis. More recently, a combination of fish oil and fish protein hydrolysates in the diet was reported to reduce the plasma cholesterol level and increase the hepatic total cholesterol concentration. This is probably due to the action of the acyl-CoA coenzyme, which increases cholesterol esterification.[117] The hydrolysis of marine waste/byproducts such as freshwater clam residual meat together with insoluble dietary fiber separated from whole *Gracilaria* showed a bile acid-binding capacity and inhibited the micellar solubility of cholesterol in vitro.[118] This study was subsequently conducted in vivo, and results suggested that a combination of the freshwater clam hydrolysate and the insoluble dietary fiber from *Gracilaria* could be used to develop new functional foods for the prevention of hypercholesterolemia.[119]

Calcium Channel Blocking Effects. Calcium channel blockers interact with voltage-gated calcium channels in cardiac muscle and blood vessel walls, reducing intracellular calcium and consequently lowering vasoconstriction. In fact, it has been reported that peptides that bind and solubilize calcium can be considered to be beneficial in the prevention of dental caries, osteoporosis, hypertension, and anemia. During the past decade, some peptides derived from hoki and Alaska pollock frame were reported as calcium channel blockers,[120] and their bioactivities were demonstrated in rat models.[120−122]

Endothelin-1 (ET-1) and Endothelin Converting Enzyme (ECE) Inhibitory Effect. The endothelin system also has an increasingly recognized role in blood pressure regulation. The vasoconstriction peptide ET-1 is released from endothelin-1 by the activation of endothelin converting enzyme. The hyperactivation of the endothelin system has been implicated in the pathogenesis of several cardiovascular disorders such as pulmonary and arterial hypertension, heart failure, myocardial infarction, restenosis, and Chagas cardiopathy.[123]

Indeed, the more potent vasoconstrictive effect of ET-1 over angiotensin II was previously reported. However, only a few studies have focused on the potential effect of food-derived peptides on the endothelin system. Okitsu and colleagues[124] found ECE inhibitory peptides in pepsin digests of bonito pyrolic appendix and beef. Later, the influence of ACE inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, generated through tryptic digestion of bovine β-lactoglobulin, on the release of ET-1 by endothelial cells was investigated,[125] and basal ET-1 release from these cells was reduced by 29% in the presence of 1 mM of the peptide, compared to 42% for 0.1 mM Captopril. More recently, the potential of bovine lactoferrin hydrolysates generated by trypsin and proteinase K digestions as a source of ECE inhibitors was studied.[125,126] The peptides Gly-Ile-Leu-Arg-Pro-Tyr and Arg-Glu-Pro-Tyr-Phe-Gly-Tyr exerted in vitro inhibitory effects on ECE activity.

Useful Computational Methods in the Study of Cryptides. *In Silico Analysis.* Bioinformatics is a useful tool in the study of proteins. Bioinformatics is the computational analysis, modeling, and prediction of biological data. In silico analysis is the use of computational tools and databases to predict the release of bioactive peptides from parent proteins and the prediction of suitable enzymes for the release of bioactive peptides. It is also useful for predicting bioactivities associated with a given peptide sequence. Previously, Vermeirssen and colleagues[127] quantitatively evaluated pea and whey proteins as precursors for ACE inhibitory peptides. This was achieved by means of establishing an ACE inhibitory peptide database, containing about 500 reported sequences and their IC_{50} values. More recently, Iwaniak and Dziuba developed BIOPEP and Pôle Bioinformatique Lyonnais analysis tools.[128,129] These provide valuable data on food protein precursors as sources of bioactive peptides combined with the structural aspects of bioactivities encrypted in proteins. In silico analysis has been used in a number of studies to date to predict the release of bioactive peptides from protein sources including milk[129] cereals,[130] meat,[131] endogenous human proteins,[132] and other food proteins.[133] Bioinformatics and in silico analysis of fish proteins coupled with mass spectrometry technologies could prove useful in the future identification of useful fish-protein derived bioactive peptides.

Computational prediction of peptide bioactivity is also a useful tool for streamlining the search for active peptide sequences. PLS modeling is a widely used descriptive and predictive chemometrics approach for quantitative structure−activity relationship (QSAR) studies. It assists the user in elucidating how variation of molecular structures affects bioactivity of therapeutic agents, especially when working with high numbers of descriptor variables compared to the number of observations. This technique was used in QSAR studies of food-derived peptides and helped to identify bitter peptides[133] and ACE inhibiting peptides[134] previously. The bioactive peptide predictor PeptideRanker can prove useful in identifying among a set of peptides those that are more likely to be bioactive. This allows the focus of experimental screening on this subset.[135]

Technologies for the Identification of Bioactive Peptides. Technologies including mass spectrometry (MS) coupled to

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liquid chromatography (LC) are some of the commonly applied technologies for bioactive peptide analysis. Many bioactive peptides contain two or three amino acid residues. These di- and tripeptides are usually identified using de novo sequencing or by matching the accurate mass and retention time to standards. Commonly used database search strategies using MS/MS spectra of peptide ions include SEQUEST and MASCOT, which are designed for proteomics applications. Other commercial databases used include the NIST 12 MS/MS library, the Wiley MS/MS library, and online spectral databases including METLIN (http://metlin.scripps.edu) and MassBank (http://www.massbank.jp/?lang=en). These libraries are suitable for the identification of oligopeptides greater than four amino acids in length. Recently, Tang and colleagues developed a new library suitable for the identification of di- and tripeptides from complex mixtures and untargeted analysis.[135] This method involved the separation of peptides using hydrophilic interaction (HILIC) liquid chromatography. To facilitate di- and tripeptide identification, a database consisting of all the predicted MS/MS spectra from 400 dipeptides and 8000 tripeptides was created, and a search tool, PEP search, was developed and housed at the MyCompoundID Web site (www.mycompoundid.or/PEP).[136] PEP Search in MyCompoundID searches an MS/MS spectrum against a database of di/tripeptides. This database consists of 400 dipeptides and 8000 tripeptides and their theoretical MS/MS fragments. PEP Search also provides an option to use dimethyl labeling confirmation. This option enables the search against the 8400 di/tripeptide database plus a library that consists of 8400 dimethyl-labeled di/tripeptides and their theoretical MS/MS fragments.[136] Peptide databases including BIOPEP are useful for peptide search operations also.

Bioavailability and Safety of Bioactive Peptides. For a bioactive peptide to be delivered to its active site, it is necessary to fully understand the barriers of the GI tract and the blood−brain barrier (BBB). The GI tract has site-specific absorption based on the peptide consumed and regional differences in pH, residence time, surface area, and enzyme activities.[137,138] For cardioprotective peptides to be active and to give a positive effect in the human body, they must be able to cross the intestinal mucosa and reach the systemic circulation. The most well-known antihypertensive and ACE inhibitory peptides, Ile-Pro-Pro and Val-Pro-Pro, contain a double proline at their C-terminal end and can therefore survive GI transit intact and brush border peptidase bioactivities. Transport across the intestinal epithelium may be either active or passive, and the mechanism of transport depends on the physicochemical properties of the peptide and the length of the peptide.[138] Furthermore, the RAAS has been described in the brain, and an interconnection between neurotransmitters and the brain RAS affects behavior and neurological diseases, for example, Parkinson's and Alzheimer's diseases. For a bioactive peptide with cardioprotective capabilities to exert beneficial effects within the brain RAAS, it would need to cross the BBB. The BBB prevents entry into the brain of many substances.[139] This is due to the epithelial-like tight junctions within the brain capillary endothelium.[139] The BBB is anatomically and functionally distinct from the blood−cerebrospinal fluid barrier.[140] However, it is documented that lipid mediated the free diffusion of substances with a molecular mass under 400 Da, which form less than eight hydrogen bonds and may cross the BBB.[139,140]

Protein is an essential part of the human diet. Bioactive peptides encrypted within native fish muscle proteins and released using food-processing methods including fermentation are an attractive option for use in food therapy for the treatment of high blood pressure. Despite their decreased potency, cardioprotective marine-derived peptides offer a suitable alternative to synthetic drugs for the prevention of high blood pressure and the maintenance of normotensive BP. However, further detailed mechanistic studies on how these antihypertensive peptides interact in humans must be carried out to elucidate the blood pressure lowering mechanisms of action involved. Safety issues including potential toxicity also warrant further research. It is important also that complete physicochemical, technofunctional, and bioactive properties, including potential allergic reactions, are considered prior to production of new fish muscle derived antihypertensive food products.

AUTHOR INFORMATION

Corresponding Author

*(M.H.) Phone: +353 (0) 1 8059957. Fax: +353 (0) 1 8059500. E-mail: maria.hayes@teagasc.ie.

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Notes

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ABBREVIATIONS USED

AAPH, 2,2-azobis(2-amidinopropane) dihydrochloride; Ang-I, angiotensin I; Ang-II, angiotensin II; Ang-A, angiotensin A; Ang-M, angiotensin M; ACE, angiotensin converting enzyme-I; ACE2, angiotensin converting enzyme-2; BBB, blood−brain barrier; BP, blood pressure; CGRP, calcitonin gene-related peptide; CVD, cardiovascular disease; CLA, conjugated linoleic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ET-1, endothelin-1; ECE, endothelin-1 converting enzyme; GI, gastrointestinal; EFSA, European Food Safety Authority; FDA, U.S. Food and Drug Administration; FOSHU, foods of specified health use; GRAS, Generally Recognized As Safe; HDL, high-density lipoprotein; HILC, hydrophilic interaction liquid chromatography; MRC-5, human embryonic fibroblast cell line; HepG2, human hepatocarcinoma cell line; NOS, nitric oxide synthase; PLS, partial least-squares; QSAR, quantitative structure−activity relationship; ROS, reactive oxygen species; RNS, reactive nitrogen species; RAAS, renin angiotensin aldosterone system; SHRs, spontaneously hypertensive rats; SFDA, state food and drug administrations

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