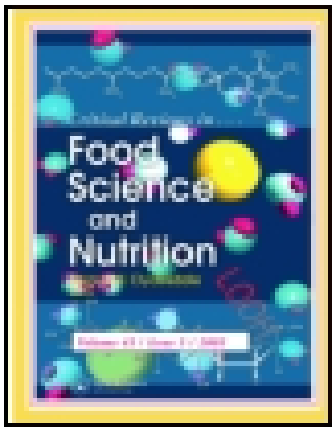

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A Review of Potential Marine-Derived Hypotensive and Anti-Obesity Peptides

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Title: A review of potential marine-derived hypotensive and anti-obesity peptides

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ABSTRACT

Bioactive peptides are food derived components, usually consisting of 3-20 amino acids, which are inactive when incorporated within their parent protein. Once liberated by enzymatic or chemical hydrolysis, during food processing and gastrointestinal transit, they can potentially provide an array of health benefits to the human body. Owing to an unprecedented increase in the worldwide incidence of obesity and hypertension, medical researchers are focusing on the hypotensive and anti-obesity properties of nutritionally-derived bioactive peptides. The role of the renin-angiotensin system has long been established in the aetiology of metabolic diseases and hypertension. Targeting the renin-angiotensin system by inhibiting the activity of angiotensin-converting enzyme and preventing the formation of angiotensin II can be a potential therapeutic approach to the treatment of hypertension and obesity. Fish-derived proteins and peptides can potentially be excellent sources of bioactive components, mainly as a source of ACE inhibitors. However, increased use of marine sources, poses an unsustainable burden on particular fish stocks, so, the underutilised fish species and by-products can be exploited for this purpose. This paper provides an overview of the techniques involved in the production, isolation, purification and characterization of bioactive peptides from marine sources, as well as the evaluation of the ACE inhibitory activity and bioavailability.

Keywords Angiotensin-converting enzyme, angiotensin II, bio-functional peptides, bioavailability, renin-angiotensin system, anti-obesity peptides

INTRODUCTION

Dietary proteins have been known for their wide range of nutritional, functional and biological properties. Nutritionally, proteins are recognized for their ample supply of amino acids that are primarily essential for physical development, body growth, maintenance and repair as well as for the proper functioning of body organs and cells. The nutritional quality of proteins depends on the structural conformation, amino acid content and the physiological utilization of the specific amino acids after digestion and absorption (Friedman, 1996). Whilst from a functional point of view, proteins contribute efficiently to the physicochemical and sensory properties of various protein-rich foods (Korhonen and Pihlanto, 2003), their biological properties relate to their satiating power (the feeling of fullness) (Astrup, 2005) and their ability to release bio-functional peptides during food processing or gastrointestinal transit. These peptides may serve as potential ingredients in functional or health-promoting foods (Korhonen and Pihlanto, 2003, Korhonen and Pihlanto, 2006, Shahidi and Zhong, 2008).

During the last 20 years, there has been an increasing scientific and commercial interest in the field of food-derived bioactive peptides owing to their ability to positively affect the major body systems, notably, the cardiovascular, digestive, endocrine, immune and nervous systems as well as minimizing the developmental risks of chronic diseases. Bioactive peptides, defined as food derived peptides (nutraceuticals), which will provide a health benefit to the host beyond nutritional value, have been known for their myriad of bio-functionalities, such as hypotensive, anti-oxidative, anti-microbial and opioid activities (Hartmann and Meisel, 2007, Möller et al., 2008), amongst other properties. Remarkably, within the parent protein matrix, bioactive peptides (usually of short-chain length of 3-20 amino acid residues) are inactive. Thus, in order

to exert a beneficial health effect, they must be released, resist the digestive conditions of the gastrointestinal tract (GIT) and subsequently be absorbed through the intestine where they enter the blood circulatory system and reach their site of action (Möller et al., 2008, Vermeirssen et al., 2004).

In view of the worldwide high occurrence of obesity and hypertension, changes in lifestyle, dietary approaches and pharmacological treatments, such as weight loss supplements and antihypertensive drugs, are broadly applied to treat and/or prevent the onset of these two phenomena. However, the available synthetic products, such as sibutramine (reductil), which is an appetite suppressant (Tziamalos et al., 2009) have often been associated with undesirable side effects, such as increased blood pressure, constipation, headaches, acute liver failure and insomnia (Slovacek et al., 2008, Thurairajah et al., 2005). Therefore, efforts are being emphasized on the production of functional foods with anti-obesity and anti-hypertensive properties. It has long been established that the hyperactivity of the renin-angiotensin system (RAS) plays an important role in the onset of obesity, metabolic diseases and hypertension (Weisinger et al., 2007). The most significant regulatory component of the RAS is angiotensin-converting enzyme (ACE), which converts angiotensin-I (ANG I) to angiotensin-II (ANG II). The latter is a physiologically important constituent that performs a number of functions, such as i) the secretion of aldosterone; ii) increase in renal sodium reabsorption, which ultimately results in an increase in the systolic blood pressure (Inagami, 1994) and iii) growth of adipose tissue (Goossens et al., 2003), which affects the endocrine and metabolic systems. An increase in adipocyte angiotensinogen (AGT) was observed in obese subjects in the study of Van Harmelen and colleagues (2000b). Moreover, it was shown that ANG II may promote adipocyte

hypertrophy by increasing the expression of fatty acid synthase (FAS) within fat cells (Jones et al., 1997).

Therefore, inhibiting the enzyme ACE may be therapeutically useful in lowering blood pressure, reducing body fat mass and improving overall body composition. Indeed, several studies have demonstrated the ability of bioactive peptides or ACE inhibitors to perform these functions, both *in vitro* and *in vivo* studies (Lee et al., 2010, Li et al., 2007, Mathai et al., 2008, Ohta et al., 1997). Moreover, different synthetic ACE inhibitors, such as captopril, enalapril and perindopril are extensively used as anti-hypertensive drugs in addition to reducing body fat mass (Mathai et al., 2008). However, due to their side effects, researchers are focusing more on the development of nutritionally-derived ACE inhibitory peptides, from various plant sources, such as apricot (Zhu et al., 2010), wheat (Matsui et al., 1999), rice (Li et al., 2007), garlic (Suetsuna, 1998), soy (Lo and Li-Chan, 2005) and mushroom (Lee et al., 2004). Animal sources of ACE inhibitory peptides include dried bonito (Fujita et al., 1995, Yokoyama et al., 1992), oyster proteins (Wang et al., 2008), egg white proteins (Liu et al., 2010, Yu et al., 2011), beef hydrolysates (Jang and Lee, 2005) and fish proteins (Fujita and Yoshikawa, 1999, Jung et al., 2006) as well as dairy milk proteins (Donkor et al., 2007).

This article aims to discuss the enzymatic cascade involved in the renin-angiotensin system and provides an overview of the techniques involved in the production, isolation and purification of bioactive peptides from marine sources as well as evaluation of the ACE inhibitory activity and bioavailability.

OVERVIEW OF RENIN-ANGIOTENSIN SYSTEM

In 1898, Robert Tigerstedt, a notable physiologist and his student, Per Bergman made an incredible discovery of the enzyme renin, which has led to the extensive research of the RAS and remains a landmark in its history (Marks and Maxwell, 1979). The RAS has long been identified as a regulatory system that is involved in a multitude of physiological and pathophysiological functions, including body fluid regulation, cardiovascular functions, sodium re-absorption from the kidney, increasing vascular tone and aldosterone secretion (Goossens et al., 2003, Inagami, 1994, Weisinger et al., 2007); all of which are contributing factors for hypertension and cardiovascular diseases (CVD). Recently, the RAS present in various tissues such as the adrenal cortex, kidney, heart, liver, retina, pancreas, vascular smooth muscle, brain and most importantly, the adipose tissue (de Kloet et al., 2010, Weisinger et al., 2007), has also been implicated in the aetiology of obesity (de Kloet et al., 2010, Goossens et al., 2003, Weisinger et al., 2007). The cascade of enzymatic reactions that occurs in the classical pathway of the RAS, as depicted in Figure 1, is further described.

The initial enzymatic reaction occurring in RAS involves the cleavage of the liver-generated glycoprotein prohormone, angiotensinogen into ANG I, by the enzyme renin (EC.3.4.23.25), secreted by the juxtaglomerular cells of the kidney (Li et al., 2004). The decapeptide ANG I (10 amino-acid sequence: DRVYHPFHL) reaches the lungs via circulation, where it is converted into the biologically active ANG II by the dipeptidyl carboxypeptidase, ACE (EC.3.4.15.1). The enzymatic cleavage occurs from the C-terminal Histidyl-Leucine (HL) peptide to produce the octapeptide ANG II, which is the central component of RAS. Besides the ACE-dependent pathways, alternate enzymes such as the serine proteases (Chymase) or Cathepsins D and G may also assist in the formation of ANG II and therefore may contribute to

heart failure or high blood pressure (Karlsson et al., 1998). Even though many studies have linked the formation of ANG II primarily by the catalytic action of ACE, it still remains unclear whether the synthesis of ANG II is due to the ACE-dependent pathways, the ACE-independent pathways or a combination of the two. As illustrated in Figure 1, ANG II is further cleaved by amino-peptidases into degradation products, such as angiotensin III [active (2-8) fragment of ANG II] and angiotensin IV [active (3-8) fragment of ANG II], which are predominantly involved in brain functions, particularly attributed to learning and memory, modulation of behavior and neuronal development (de Kloet et al., 2010, Goossens et al., 2003).

The complexity of RAS (Figure 2) was further realized in 2000, when a new human homologue of ACE, known as angiotensin-converting enzyme 2 (ACE2) was discovered from 5' sequencing of a human heart failure ventricle cDNA library (Donoghue et al., 2000, Tipnis et al., 2000). ACE2, a monopeptidase, hydrolyses the carboxyl terminal Leucine from ANG I to liberate angiotensin-(1-9) [ANG-(1-9)], which is then converted into angiotensin-(1-7) [ANG-(1-7)] by ACE2. ACE2 can also convert ANG II directly into angiotensin-(1-7) by cleaving the Pho^{7}-Phe^{8} amino acid bond of the octapeptide to produce heptapeptide ANG-(1-7), which is then, converted into angiotensin-(1-5) [ANG-(1-5)] by the zinc metallopeptidase ACE, due to the enzymatic degradation of the His^{6}-Pro^{7} dipeptide (Donoghue et al., 2000, Ferrario, 2006). The amino acid composition of the RAS components and the enzymatic cleavage occurring during the pathway are demonstrated in Figure 2. The synthesis of ANG-(1-7) is physiologically beneficial since it opposes many of the ANG II-mediated deleterious properties via the G-protein coupled receptor (GPCR) Mas.

Hypertension

Systolic and diastolic blood pressures (SBP and DBP) are the clinical parameters involved in the measurement of BP of an individual. An optimal BP of a healthy person is around 120 mmHg systolic pressure over 80 mmHg diastolic pressure. A chronic elevation in arterial pressure would result in high blood pressure (HBP), clinically termed as hypertension (≥ 140/90 mmHg) (Grundy et al., 2005). It is well established that overweight and/or obese individuals tend to develop an increase in BP. The mechanisms, however, remain elusive. Besides blocking the vasoconstrictor effects of ANG II from the RAS, current pharmacotherapy for hypertension includes i) beta-blockers, which reduce contractal force, ii) calcium-channel antagonists, which inhibit vascular smooth muscle contraction, and iii) diuretics, which reduce blood volume, thus reducing blood pressure (Staessen et al., 2001). It has also been postulated that an excessive body fat causes an imbalance between the proper functioning of adipose and lean tissues in the liver or skeletal muscle (Unger, 2003). This disordered functioning, eventually results in i) the production of cytokines (inflammatory markers), adipokines and angiotensin from adipocytes; ii) an increased activity of sympathetic nervous system (SNS) and iii) overexpression of the components of RAS, such as AGT, ACE and ANG II. These three parameters, consequently, cause sodium retention, extracellular fluid volume expansion and increased cardiac input; all of which, are fundamentally linked with the development of hypertension and are frequently observed in obese persons (Goossens et al., 2003).

Furthermore, the obesity-related activation of SNS may be partly mediated by the adipocyte-derived hormone, leptin (Hall et al., 2001), which increases proportionally with the degree of adiposity. An increase of leptin in hypertensive individuals is associated with elevated

plasma renin activity, aldosterone and angiotensin concentrations (Schorr et al., 1998). On the other hand, weight reduction by as little as 5% can potentially decrease RAS activation (Engeli et al., 2005), and reduce the level of hypertension. The link between adipose tissue RAS, blood pressure and obesity is well documented in animal models (Alonso-Galicia et al., 1996, Boustany et al., 2004).

Moreover, obese patients will often have a high plasma level of triglycerides (TG) or cholesterol, which may contribute to plaque formation or constrict arteries, thereby promoting the development of CVD (Eckel, 2007). Another potential cause of hypertension is the abnormality in insulin production. Besides its role in blood glucose management, insulin, a vasodilator, prevents sodium re-absorption in the kidney of healthy individuals (Reaven, 2003). However, in obese patients, insulin loses its vasodilatory effect and leads to excessive sodium and water re-absorption in the kidneys; a causative factor for hypertension. An increased plasma insulin level seems to increase atherogenic risk factors including plasma levels of low density lipoproteins (LDL), triglycerides and elevated SBP (Reaven, 2003). Obesity-induced hypertension is well associated with the onset of various types of CVD, such as stroke, myocardial infarction, heart failure and kidney diseases (DeFronzo and Ferrannini, 1991). An in-depth review on the mechanisms involved in the obesity-linked hypertension is provided by Rahmouni and colleagues (2005). The possible mechanisms of obesity-induced hypertension are schematically illustrated in Figure 3.

Correlation of RAS with Obesity and Body Fat

Many studies have correlated the different components (renin, AGT, ANG II, ACE and ANG II receptors) of RAS with body fat and adipose tissue, which is markedly expanded in obesity. For example, the study of Hainault and colleagues (2002) established the fact that adipocytes from obese Zucker rats displayed a significant increase in AGT content in adipose tissue, which was measured in culture medium mature fat cells. Their study reported that AGT over-secretion was correlated with the development of adipocyte hypertrophy (Hainault et al., 2002). Other studies have revealed the impact of high-fat diet on the level of RAS components in the adipose tissue. Male Sprague-Dawley rats gained weight upon consumption of high-fat diet, and were assessed for AGT expression in their adipose tissue and its plasma concentration (Boustany et al., 2004). Real time polymerase chain reaction demonstrated a 2-fold increase in AGT mRNA in retroperitoneal adipose tissue in obesity-prone rats compared to obesity-resistant rats. Moreover, an increase in plasma AGT concentration was observed in obesity-prone rats (Boustany et al., 2004).

Similarly, Van Harmelen et al. (2000a) studied the association of adipose AGT genes expression with human obesity. The reverse transcriptase polymerase chain reaction was used to measure the levels of subcutaneous adipose AGT mRNA and 18S ribosomal RNA in the obese participants. Their study concluded that AGT mRNA expression was about 2 times elevated in the obese men. Moreover, Northern Blot analyses demonstrated AGT expression in abdominal subcutaneous adipose tissue of nine obese subjects, aged 40-62 years old (Karlsson et al., 1998). Furthermore, Yasue and team (2010) discovered that adipose tissue-derived AGT was greatly increased in the obese human group. The above studies provide strong evidence for the notion

that adipocytes expressing AGT may affect adipogenesis, which is well correlated with the pathogenesis of obesity.

Decades ago, the presence of ACE in adipose tissue was debatable. Saye et al. (1993) showed that ACE inhibition failed to prevent ANG II production by isolated adipocytes, suggesting that an alternate enzyme produced the ANG peptides. However, a later experiment demonstrated the presence of ACE mRNA in both human adipose tissue and in cultured adipocytes (Engeli et al., 1999). Later on, more studies confirmed that the inhibition of ACE prevented the formation of ANG II. In a recent study of Jayasooriya et al. (2008), mice lacking ACE gene have improved energy expenditure, with reduced fat mass as well as improved glucose clearance. Likewise, Carter et al. (2004) hypothesized that ACE inhibition in aged rats improved their body composition and physical performance compared to control rats. Moreover, ACE inhibitors administered to hypertensive rats reduced their blood pressure considerably (Li et al., 2007). These studies confirmed the importance of ACE inhibition in relation to the prevention of obesity and obesity-related diseases.

Physiological Roles of ANG II in Obesity

Indeed, most of the biological effects of RAS are mediated by the peptide ANG II, by binding to either the angiotensin type I or type II receptors (AT_1R and AT_2R), in target tissues or organs (Weisinger et al., 1996). However, it was reported that most of the functional actions of ANG II are mediated by the AT_1R via intracellular activation of phospholipase, inhibition of adenylate cyclase and stimulation of tyrosine phosphorylation (Sharma et al., 2001). Conversely, the physiological properties of AT_2R remain less well-defined; nonetheless, it is generally

hypothesized that its activation opposed the effects transduced by AT_1R activation in different aspects (Yayama and Okamoto, 2008). Briefly, some of the reported functions of ANG II are further described. Whilst in the brain, ANG II acts as a neurotransmitter, in the liver, it stimulates the generation of new glucose molecules from glycogen (glycogenolysis) and non-carbohydrates substrates (gluconeogenesis), which may potentially lead to the development of insulin resistance or diabetes mellitus. Moreover, it acts as a potent vasoconstrictor by binding to specific cell surface receptors to produce contractile proteins and to promote sodium and fluid retention, which results in an increase in BP (Eriksson et al., 2002). The activity of ACE leads to production of ANG II and the degradation of bradykinin, a vasodilator, resulting in promotion of hypertension (Eriksson et al., 2002).

Furthermore, ANG II has the potential to stimulate the synthesis of inflammatory cytokines and growth factors (e.g. tumour-necrosis factor, IL-1, PAF, etc.), reactive oxygen species (ROS) and coagulation factor (PAI-1) via the AT_1R, which may potentially interfere with several steps of intracellular insulin signaling (Santos et al., 2008) and vascular damage (Virdis et al., 2011). Most importantly, in the adipose tissue, ANG II enhances the accumulation of fatty acids and triglycerides within the body and promotes fat cell proliferation, which is a common causative factor for obesity and metabolic syndrome (de Kloet et al., 2010, Mathai et al., 2008).

Adipocyte Differentiation

Adipose tissue, a passive reservoir for energy storage, in the form of triacylglycerol, has been an important source for ANG II secretion, which plays a major role in adipocyte growth and differentiation (Saint-Marc et al., 2001, Darimont et al., 1994). While adipogenic

differentiation is defined as the formation of new fat cells from precursor cells, adipocyte hypertrophy is the terminology used to describe an increase in the size of adipocyte cells due to excessive fat storage. ANG II has also been long recognized as a growth factor in a variety of cells and tissues (Sil and Sen, 1997). The mechanisms by which ANG II actually stimulates adipocyte differentiation remain complex and elusive, although, some suggestions have been made. Prostaglandin (PGI_2), a major metabolite of arachidonic acid in adipose tissue, has been associated with the onset of adipocyte differentiation. *In vitro* studies demonstrated that ANG II stimulated the release of PGI_2 from adipocytes, via the AT_2 receptor (Darimont et al., 1994, Saint-Marc et al., 2001). Moreover, evidence suggests that overexpression of ANG II increases the triglycerides content of adipocytes. This was demonstrated in 3T3-L1 and human adipose cells in the study of Jones and colleagues (1997). According to Weisinger et al. (2007), the mechanism by which ANG II increases triglycerides content of fat cells, is by increasing the activity of fatty acid synthase, an enzyme that is involved in lipogenesis and which catalyzes the synthesis of palmitate from acetyl coA and manolylcoA in the presence of nicotiamide adenine dinucleotide phosphate (NADPH) and glycerol-3-phosphate dehydrogenase (GPDH), the rate-limiting enzymes for triglyceride synthesis in adipose tissue. Furthermore, the stimulation of the so-called peroxisome proliferators-activated receptors (PPAR) by ANG II can also result in lipogenesis and differentiated cells (de Kloet et al., 2010).

Body Weight Regulation

The impact of ANG II on body weight regulation has been broadly investigated in animal models. Young rats receiving a 1-week intracerebro-ventricular (ICV) infusion of ANG II, for a

period of 10 days, were monitored for body weight change and food intake on a daily basis (Porter et al., 2003). It was observed that the ANG II-infused rats exhibited a decreased body weight gain and food intake compared to the control animals (Porter et al., 2003). These results were in line with the study of Brink et al. (1996), in which male Sprague-Dawley rats treated with ANG II infusion lost 18-26% of body weight by 1 week. Brink and colleagues (1996) postulated that ANG II produced weight loss through a pressor-independent mechanisms, which includes both marked anorexigenic and metabolic effects. Conversely, other studies have reported weight loss, overall change in body composition and improvement in glucose intolerance with the administration of ACE inhibitors, which blocks the synthesis of ANG II (Carter et al., 2004, Mathai et al., 2008, Weisinger et al., 2008). The roles of ANG II in body weight regulation are still controversial; however, there is enough scientific evidence to confirm the implication of ANG II in increasing body weight, food intake and energy expenditure as well as the potential of bioactive peptides to inhibit the formation of ANG II.

BIOACTIVE PEPTIDES

The extensive research and development in the field of bioactive peptides began since the 1950s when Mellander proposed that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants (Pihlanto-Leppälä, 2000) and the peptides were named caseinophosphopeptides (CPPs) (Korhonen and Pihlanto, 2003). Since that era, milk and milk-derived products have been the primary focus for the characterization of bioactive peptides (Rutherfurd-Markwick and Moughan, 2005). However, with increasing knowledge on the functionality of the biologically active peptides, intense efforts have been made in the last

two decades, to identify and quantify functional peptides from diverse plant and animal sources, with various physiological functions, as illustrated in Table 1 and Table 2, respectively. A number of commercial functional foods containing bioactive peptides are presently available on the markets. Some examples include i) Calpis®, which is a Japanese product fermented with bacterial culture of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* and contains bioactive peptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) derived from milk caseins, which have been claimed to exert antihypertensive properties; ii) Evolus®, developed in Finland, contained peptides VPP and IPP, which corresponds to β-casein and κ-casein fragments (Hartmann and Meisel, 2007); iii) Collactive^{TM}, a marine source of collagen and elastin, can be used as an anti-wrinkle ingredient; and iv) Nutripeptin^{TM}, another marine-derived bioactive compound, has been found to be effective in increasing satiety and weight loss response (Pszczola, 2009). Calpis®, Evolus® and Nutripeptin^{TM} are commercial products with Food for Specified Health Use (FOSHU) claims (Shimizu, 2012).

Bioactive peptides are food-derived functional protein sequences that, beyond their basic intrinsic nutritional value, confer a physiological effect on one or more body organs, when administered in adequate amounts (Möller et al., 2008). One of the main criteria for their application is that they must be safe for human consumption. These peptides, generally inactive when encrypted in the primary sequence of a large protein molecule, will exert their biological functions only after being liberated from their parent protein (Korhonen and Pihlanto, 2003). The release of the physiological peptides from the intact proteins may be achieved by three major ways, including i) *in vivo* during gastrointestinal transit by digestive (e.g. trypsin) or microbial enzymes, ii) *in vitro* during food processing such as ripening or fermentation by isolated

microbial enzymes (e.g. *Lactobacillus helveticus*) and iii) through the actions of enzymes derived from proteolytic microorganisms (Korhonen and Pihlanto, 2003, Shahidi and Zhong, 2008).

Upon release as their individual entities, these short-chain bioactive peptides may act as potent metabolism modulators (Korhonen and Pihlanto, 2003), regulatory compounds with hormone-like functionalities (Shahidi and Zhong, 2008) or play an important role in pathogenesis (Shi et al., 2004). Depending on their specific amino acid sequences, various food-based functional peptides may exert multiple physiological properties, indicating that specific peptide sequences may exhibit two or more different biological effects. The well-established physiological activities of the biofunctional peptides are illustrated in Figure 4. However, there are a few factors that may affect their proper functionality, namely, the innate amino acid composition, structural conformation, their amino acid sequence as well as their bioavailability or bio-accessibility (Vermeirssen et al., 2004). In order to stimulate a biological response, the peptides must be bioavailable, i.e., following digestion, they must be able to i) cross the intestinal epithelial cells and enter the blood circulatory system; ii) bind directly to specific epithelial cell-surface receptor sites and iii) produce local effects in the GIT (Korhonen and Pihlanto, 2003).

Techniques for the Release of Bioactive Peptides

Peptides with ACE inhibitory (ACE-I) activity have already been isolated from different food proteins (Yamamoto, 1997). A potentially successful food protein source to generate bio-functional peptides is expected to meet at least two major selection criteria, including i) the potential of use of underutilised proteins industrial by-products (e.g. underutilised fish species and their scales, skins, etc.), and ii) utilization of identified peptide sequence of specific

pharmaceutical interest. These two criteria can be implemented to generate high yields of characterized potent peptide with one or multiple bioactivities.

Bioactive peptides can be produced through enzymatic hydrolysis by digestive enzymes of the whole protein molecules. Simulated gastrointestinal digestion techniques, which can be a cost-effective strategy, have been, so far, the most common means of investigating the effects of digestive enzymes (pepsin, trypsin, pancreatin and other peptidases) on the delivery of potential biologically active peptides, such as the ACE-I peptides (Korhonen and Pihlanto, 2006, Lo and Li-Chan, 2005). Different combinations of proteinases including chymotrypsin, papain and thermolysin; commercial enzymes such as Alcalase, Flavourzyme and Neutrase; enzymes from bacterial and fungal sources; gene expression approaches; and chemical hydrolysis involving the applications of acids and/or alkali are potential technologies that have been used to produce bioactive peptides from various proteins as reviewed in recent articles (Korhonen and Pihlanto, 2006, Shahidi and Zhong, 2008). Furthermore, parameters such as degree of protein hydrolysis, the duration of hydrolysis, enzyme-substrate ratios and the pre-treatment of the protein prior to hydrolysis (Rutherfurd, 2010), are important to be considered in the production of bioactive peptides.

Isolation and Characterization Techniques of Bioactive Peptides

Prior to isolating and characterizing particular bioactive peptides, the selected protein food source must primarily be hydrolysed, for example, by enzymatic hydrolysis. This hydrolysis process allows the release of a crude peptide mixture, which should then be analysed for different bioactivities, in particular interest to the investigators. After determination of the

bio-functionalities, the released peptides or the hydrolysates are then fractionated mainly by ultrafiltration, which is based on peptide size (Korhonen and Pihlanto, 2006); or preparative-HPLC (Vermeirssen et al., 2005). The fraction demonstrating the highest bioactivity is further purified to isolate an individual peptide using different separations techniques, principally reverse-phase high-performance liquid chromatography (RP-HPLC) or gel permeation chromatography (Cao et al., 2010, Donkor et al., 2007, Jang and Lee, 2005, Jung et al., 2006, Wang et al., 2008). Purification may lead to more active fractions. Moreover, the characterization of individual peptide fraction is achieved using the combined techniques of mass spectrometry and protein sequencing. For instance, matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS) was used to analyse the sequence of the purified ACE-I peptide derived from rice hydrolyzate (Li et al., 2007).

Moreover, because bioavailability is a crucial determinant of the potency of identified ACE-I peptide, it is therefore crucial to determine whether the peptides are absorbed within the gut and transported into blood circulation. The most widely used assay to investigate the bioavailability and transport of purified ACE inhibitor involved the Caco-2 cell monolayer, which can potentially mimic the human small intestinal epithelial cells (Satake et al., 2002, Vermeirssen et al., 2004). Eventually, to identify the amino acid sequence of potent ACE-I peptide, automated Edman degradation on peptide/protein sequencer or MS/MS (Donkor et al., 2007) is usually applied. The flow diagram for the production, isolation, purification, identification and development of ACE-I peptides from several protein food sources is represented in Figure 5. Should the compound indicate effective *in vitro* bio-functionality, some researchers will then investigate the effects of the derived compound in animal models to study

the *in vivo* physiological aspects of the bioactive constituent. For example, the peptides are administered in spontaneously hypertensive rats (SHR) to investigate whether the potent ACE inhibitor will influence BP and cardiovascular health *in vivo* (Ramchandran and Shah, 2011).

Industrial Techniques Used for the Generation and Purification of Bioactive Peptides

Recently, food proteins-derived bioactive peptides have attracted mounting attention as potential candidates for various health-promoting functional foods. At present, milk- and marine-derived proteins are the best known source of such physiological ingredients; and they present promising area for developing new value-added food products. Owing to the increasing health-conscious consumers' interest in the consumption of these biological food components/products and commercialization's perspective, there has lately been, an emergence for the production of such peptides on an industrial or plant scale, rather than only laboratory scale. This can be exemplified by the study of Wang and others (2010). Oysters-derived oligopeptide-enriched hydrolysates, produced from the protease of *Bacillus* sp. SM98011 at laboratory level, and eventually scaled up to pilot (100 L) and plant (1000 L) levels with the same conditions, were found to exert antitumor activity and immunostimulating effects, when investigated in BALB/c mice (Wang et al., 2010). However, until recently, this general industrial scale production of bioactive peptides has been challenging owing to expensive production/processing cost, costly methods such as chromatography techniques, complex processing step, and limiting appropriate large-scale technologies (Korhonen and Pihlanto, 2003, Korhonen and Pihlanto, 2007, Pouliot et al., 2006).

It is important to note that from an industrial point view, the development of functional foods with added physiological value depends on i) cost-effective isolation and purification techniques, with the capacity to yield bioactive peptides within specific molecular weight range and with particular health benefits, ii) enrichment of bioactivity of the characterized peptide to such a level that when the product is consumed, a minimal active amount is enough to deliver its bioactivity to the target site; as well as iii) safety and organoleptic characteristics of the final product (Kim and Mendis, 2006, Korhonen and Pihlanto, 2006, Pouliot et al., 2006).

Pressure-driven membrane-based separation techniques have been, by far, the best potential available technology, that have been extensively used in the concentration of milk- and marine-derived bioactive peptides (Kim and Mendis, 2006, Murray and Fitzgerald, 2007, Pouliot et al., 2006). Examples of these separation methods comprise of microfiltration (MF), electro-membrane filtration (EMF), ultrafiltration (UF), reverse osmosis (RO) and diafiltration (DF) (Agyei and Danquah, 2011; Korhonen and Pihlanto, 2007; Pouliot et al., 2006, Saxena et al., 2009), of which, the latter three, are now industrially involved in the production of whey powder and whey protein concentrates (WPCs), yielding a protein content of 30-80% (Korhonen and Pihlanto, 2007).

In view to the isolation and enrichment of marine peptides exerting high ACE inhibitory and antioxidative activities, UF has been preferably used, using molecular weight cut-off membranes ranging from 1 to 10 kDa (Fujita et al., 2001, Hai-Lun et al., 2006, He et al., 2006, Je et al., 2005, Jeon et al., 1999, Rajapakse et al., 2005). Some of the proposed advantages of UF include i) cost-effectiveness, ii) ease to scale-up for commercial production, iii) high thermal stability and chemical sensitivity, iv) improvement of the efficiency of enzyme-catalysed

bioconversion, v) increase of production yield, and vi) yield of even product with required molecular weight properties (Korhonen and Pihlanto, 2006, Saxena et al., 2009). Additionally, the so-called membrane bioreactor technology, which involves the combination of enzymatic hydrolysis of marine proteins and peptide separation by UF, is currently considered as another potential processing scheme for the generation and isolation of marine-derived bioactive peptides, with beneficial health attributes (Byun and Kim, 2001, Guérard et al., 2010, Kim and Mendis, 2006).

According to Pouliot and colleagues (2006), in order to obtain specific fractions, which is rich in particular peptides of interest from the crude mixture, the coupling of membrane-processes with chromatographic techniques, may often deem necessary. Such chromatographic methods may include i) ion-exchange, ii) hydrophobic interaction, iii) size-exclusion, and iv) affinity chromatography. However, on an industrial scale, the processing costs and processing steps involved are two crucial factors that must be considered prior to industrializing these techniques. Consequently, semi- and preparative-scale chromatography may only be implied when high purity of the active peptides is critical for commercialization (Pouliot et al., 2006). Importantly, it is worthwhile taking into account that the potency of the targeted bioactive component will eventually designate the extent of purification that is required, prior to food formulation.

Subsequently, commercialization of these industrially-derived functional products can only be achieved if the latter has been claimed to exhibit a specific health effect by certain regulatory health claim policy. Japan, the birthplace of functional foods and the first country to adopt a legal system in relation to allowing claims on functional foods, introduced, in 1991, the

Food for Specified Health Use (FOSHU) licensing system, which was under the regulatory system called 'Foods with Health Claims' (Shimizu, 2002, Siró et al., 2008). Usually, after approval, a 'FOSHU' symbol will be displayed on the food label (Shimizu, 2002). As reported by Siró and others (2008), more than 500 products were labeled FOSHU in 2005. A list of commercially available FOSHU-approved milk- and marine-derived functional foods and food ingredients, carrying potent physiological bioactive peptides, with a specific health claim connected to the respective product, are already presented in recent articles (Guérard et al., 2010, Hartmann and Meisel, 2007, Korhonen and Pihlanto, 2006, Shimizu, 2012). These products are available on the Japanese, American and European markets.

ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDES

Mode of Actions of ACE Inhibition

ACE primarily deactivates the vasodilator bradykinin, while at the same time catalyzes the conversion of inactive ANG I into a potent vasoconstrictor, ANG II. Several studies have investigated the beneficial roles of bradykinin, which are predominantly mediated by the bradykinin type 2 receptor (BT_2R) (Erdös et al., 1999). In specific tissues and organs, bradykinin i) contracts the uterine and ileal smooth muscle, ii) enhances vascular permeability, iii) increases mucous secretion, iv) induces vasodilation by the nitric oxide-mediated pathways and by stimulating the arachidonic acid metabolites (Brown and Vaughan, 1998) and v) improves glucose utilization and insulin sensitivity (Duka et al., 2001). Therefore, in order to prevent the occurrence of deleterious health effects due to the degradation of bradykinin, ACE must be inhibited.

Certainly, ACE inhibition has played a fundamental role in the therapeutical treatment of hypertension and normal heart functioning. Recent studies have also demonstrated the role of ACE inhibition in adipogenesis inhibition, body weight control in addition to improvement of body composition, glucose intolerance and lipid metabolism (Jayasooriya et al., 2008, Mathai et al., 2008, Weisinger et al., 2008). In regards to hypertension, ACE inhibitors will chiefly maintain the balance between the vaso-constrictive and salt-retentive features of ANG II with the vaso-dilatory effects of bradykinin. This balance is particularly maintained by blocking or diminishing the production of ANG II whilst simultaneously reducing the degradation of bradykinin (Brown and Vaughan, 1998).

Classification of ACE Inhibitors

Depending on their inhibitory activity following pre-incubation with ACE, the ACE-I food peptides can be divided into three major groups. The first category is known as the 'true inhibitor type' peptides since their IC_{50} value is not affected when pre-incubated with ACE. The 'substrate type' inhibitors are the second group of peptides that are hydrolyzed by ACE resulting in weak inhibition. The third group includes the 'pro-drug type' peptides, which are converted to 'true inhibitor type' peptides by ACE or enzymes (proteases) of the digestive tract. In this regards, *in vivo* studies have demonstrated that only peptides belonging to the groups of true inhibitor or pro-drug type reduce SBP of SHR (Iroyukifujita et al., 2000).

Structure-Activity Correlation of ACE Inhibitory Peptides

The inhibition modes of ACE-I peptides have normally been determined by Lineweaver-Burk studies of enzymatic inhibition (Hai-Lun et al., 2006). These plots have shown that there are two types of inhibition; competitive and non-competitive inhibition. For instance, in the study of Hai-Lun and team (2006), the Lineweaver-Burk plot indicated that the three novel ACE-I peptides derived from *Acetes chinensis*, which is a Chinese underutilized marine shrimp species, were all competitive inhibitors. Competitive inhibition involves the binding of the inhibitor to the active site of the enzyme (ACE) to block its activity, while preventing the binding of the substrate (HHL) to the active site of the enzyme (Li et al., 2004). Interestingly, the presence of tryptophan (Trp), tyrosine (Tyr), proline (Pro) or phenylalanine (Phe) amino acids at the C-terminal of the peptide and branched-chain aliphatic amino acids at the N-terminal is usually suitable for the peptides to act as competitive inhibitors by binding with ACE (Cushman and Cheung, 1971).

Conversely, a non-competitive inhibitory mechanism, defined as the mechanism in which the inhibitor and the substrate may both be bound to the enzyme's active site, has also been observed in some peptides (Lee et al., 2010, Wang et al., 2008). A non-competitive inhibitor indicates that the inhibitor has an equal affinity for the enzyme and the enzyme-substrate complex. Moreover, ACE inhibition is also expressed as the IC_{50} value, which is the protein concentration in the sample (μg / mL) required to inhibit 50% of the ACE activity (Donkor et al., 2007). Several structural features, such as molecular mass, amino acid chain length, shape, hydrophobicity and charge can potentially influence the effectiveness of ACE-I peptides released from food protein sources (Meisel, 1997). It is also important to note that the active site of ACE, usually, cannot accommodate large peptide molecules. Furthermore, the overall hydrophobicity

of the peptide is also critical for its bioactivity. Due to their incompatibility to the active sites of ACE, hydrophilic peptides possess weak or no ACE-I activity. Inhibition of ACE is most preferably achieved by hydrophobic peptides which display high affinity to the active sub-sites of ACE (Li et al., 2004).

Bioavailability of ACE Inhibitory Peptides

Although the ACE-I potential of bioactive peptides can be measured *in vitro*, it is often a challenge to extrapolate a direct relationship between ACE-I activity *in vitro* and anti-hypertensive activity or anti-obesity (for e.g. body composition) in animal or human studies. In this regards, bioavailability is a crucial determining factor for the potency of the ACE-I peptide. This indicates that for the latter to exert its physiological effects *in vivo*, it must reach the target organ(s) in an intact form. Bioavailability of peptides is referred to the total amount of amino acids that can be absorbed and utilized by the cells. This implies that the functional components must remain active and resistant to the i) digestive conditions of the GIT, ii) brush border membrane peptidases, and iii) absorption through the intestinal epithelium where they enter the blood circulatory system (Vermeirssen et al., 2004). The resistance of peptides to these conditions is usually confirmed by i) hydrolysis with pepsin and pancreatin to mimic the GIT digestion process (chemically known as simulated artificial digestion) and ii) the epithelial intestinal cells (commonly termed as the Caco-2 cell monolayer). (Vermeirssen et al., 2004).

Caco-2 cell monolayers, derived from a human colon carcinoma, exhibit characteristics that resemble the human intestinal epithelial cells, such as a polarized monolayer, well-defined brush border on the apical surface and inter-cellular junctions. This cellular model has been used

to assess transport of the bioactive compound in both directions (apical to basolateral and vice-versa) across the cell monolayer (Hilgers et al., 1990, Le Ferrec et al., 2001). Peptides are rapidly metabolized by amino-peptidases into their constituent amino acids. However, the bioavailability studies have demonstrated that certain peptides are resistant to these physiological processes and can reach the circulation intact. This could possibly be due to the fact that several peptides are proline-rich, which exhibit the potential to resist digestion conditions. This can be exemplified by the peptide LHLPLP, which was hydrolysed to a shorter active form, HLPLP, by cellular peptidases prior to transport and absorption across the intestinal epithelial cells in a concentration-dependent manner (Quirós et al., 2008, Quirós et al., 2009). Moreover, the pentapeptide remained intact after one hour incubation with human plasma and remained unbound to plasma proteins. This suggests that the peptide HLPLP is readily absorbed and is resistant to proteases. It therefore may offer the possibility for an alternative peptide in the treatment or prevention of hypertension in humans.

In vitro Assay for Evaluation of ACE Inhibitory Activity

To study and develop effective ACE inhibitors from natural sources, a simple, rapid, sensitive and reliable analytical method as a means to monitor and pre-screen the enzymatic activity *in vitro* is desirable and crucial. *In vitro* ACE-I activity is generally measured by observing the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors. The broadly used method has always been based on the inventory principle of Cushman and Cheung (1971), which involved the ACE-mediated hydrolysis of the substrate Hippuryl-His-Leu (HHL) to hippuric acid (HA) and His-Leu (HL). Liberated HA is extracted

with ethyl acetate and measured spectrophotometrically at a wavelength of 228nm. High performance liquid chromatography (HPLC) (Wu et al., 2002) and fluorimetry using the substrate aminobenzoylglycyl-p-nitrophenylalanylproline (Sentandreu and Toldrá, 2006) are other commonly used techniques, which require longer analytical times to obtain reliable results. Moreover, the separation and quantification of HA from the unhydrolysed HHL and HL products by HPLC coupled with electrospray-mass spectrometry to avoid any confounding HHL that may be extracted in the ethyl acetate layer, was achieved in the study of Xiao and others (2006). Other techniques are available. For example, Curtis and colleagues (2002) employed liquid chromatography-dual mass spectrometry (LC-MS/MS) to analyze the ACE-I activity of the peptide LKPNM in Bonito muscle hydrolyzates. Despite all the various methods available, the spectrophotometric assay development by Cushman in the 1970s still remains the foundation for most of the ACE-I activity assays used today.

Sources of ACE Inhibitory Peptides

A myriad of potent ACE inhibitors are available and are usually categorized into two major types; the synthetic and the nutritionally-derived ACE inhibitors. Pharmaceutical inhibitors are biochemically based on the structure of the amino acid sequence, including three major categories: i) sulfhydryl-containing groups; ii) dicarboxyl-containing groups and iii) phosphorus-containing groups (Cushman and Ondetti, 1999). These anti-hypertensive drugs, such as captopril, enalapril and lisinopril, have effectively been used in the treatment of hypertension, congestive heart failure and diabetic neuropathy. Moreover, ramipril and quinapril exert effects such as plaque stabilization, enhancement of the effects of nitric oxide and

prostaglandins, regression of vascular smooth muscle proliferation, decreased coronary vasoconstriction and anti-macrophage function. These beneficial functions are due to ACE inhibition. (Wong et al., 2004). Some of the opposed effects of ACE inhibitors are dose-dependent and may also occur due to drug-drug and/or drug/nutrient interactions. Albeit their established therapeutical efficiency, their inherently associated side effects necessitate the urgent need for the research and development of innovative, safer, economical and natural alternatives.

MARINE-DERIVED ACE INHIBITORS

The discovery of ACE inhibitors from marine organisms started in the early 1990s, when dried bonito (*Katsuobushi*), a traditional Japanese seasoning made of bonito muscle, was examined for its potential to inhibit the activity of ACE. Yokoyama and colleagues (1992) hydrolyzed the dried bonito by various proteases and the *in vitro* ACE-I activity of the hydrolyzates was measured. Their results indicated that amongst all the other digests, the dried bonito hydrolyzed by thermolysin, of which 8 inhibitory peptides were identified by HPLC, demonstrated the highest *in vitro* ACE-I activity. However, in an *in vivo* study, a digest of dried bonito by gastrointestinal proteases failed to lower BP after a single oral administration in spite of a fairly high *in vitro* ACE inhibition (IC_{50} = 41µg/ml) (Fujita et al., 1995). As mentioned earlier, the parent peptide must be hydrolyzed to exert a health beneficial effect. This was confirmed in the *in vivo* study of Fujita and Yoshikawa (1999) where the efficiency of the isolated ACE-I peptide LKPNM (parent peptide) and LKP was compared after oral administration in SHR. It was found that LKPNM and captopril showed a maximum decline in BP after 4 h of administration, whereas LKP reduced BP after 2 h, indicating a more potent

inhibition of ACE than the parent peptide (Fujita and Yoshikawa, 1999). From their study, it can be deduced that ACE-I peptides isolated from the dried bonito exerted remarkably higher hypotensive activity *in vivo* but weaker activities *in vitro*, which was ascertained by using captopril as the reference drug. It is known that BP is regulated by the contraction of the vascular smooth muscle and endothelial function. The effect of dried bonito on rat isolated aorta was investigated (Kouno et al., 2005). The study demonstrated that ACE inhibition can have a direct action on vascular smooth muscle, involved in vasoconstriction BP regulation. Besides dried bonito, a variety of other fish species have been investigated for their potential anti-hypertensive bioactivity.

With marine species comprising approximately one half of the global biodiversity, the ocean offers a wonderful resource for novel compounds, which may serve in improving health of the worldwide population. Unfortunately, not all of the sea resources are adequately used. In recent years, over-exploitation of fishery resources has become a major concern worldwide. Many commercially important fish species are being overfished and existing valued species are becoming exhausted. According to the Food and Agriculture Organization (FAO), approximately 77% of the 143.6 million tons of fish and shellfish caught in 2006 was used for human consumption (Wilson et al., 2011). The remaining processing, such as skin, scales, fins, frames, viscera, shells, trimmings, are either thrown back in the sea or retained for production of fishmeal, fish oil, fertilizer, fish silage and animal feed (Korhonen and Pihlanto, 2006). The annual unintentional catch and discards have been reported to be 7.3 million tons (Food and Agriculture Organization, 2005), presenting a serious marine conservation problem (Blanco et al., 2007). Notably, much of these wasted and discarded stocks are highly nutritious, comprising

of high-quality proteins, vitamins, omega-3 fatty acids and important novel compounds, the bioactive peptides. These wasted species may, thus, constitute an incipient industry for valuable human food.

Consequently, there has been growing interest in exploring the possible uses of the fish by-products or the remaining raw materials, so that they can potentially be utilized rather than posing a waste and sustainability problem. For instance, sea bream scales were used to reproduce ACE-I peptides, which were analyzed for their ability to exert their hypotensive effects in SHR. The amino-acid sequences of the potential inhibitors were determined to be Gly-Tyr, Val-Tyr, Gly-Phe and Val-Ile-Tyr (Fahmi et al., 2004). Similarly, Je and co-workers (2004) hydrolyzed Alaska Pollack (salt water fish of the Cod family), which is usually discarded as an industrial by-product in the Korean fish processing plant, with pepsin to purify a novel ACE-I peptide, of amino acid sequence of Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala. Another study, involving the utilization of Alaskan Pollack skin, was carried out to investigate the relationship between ACE-I activity of the hydrolyzates and their structure (Byun and Kim, 2002). Theodore and Kristinsson (2007) investigated ACE inhibition of hydrolyzate made from extracted catfish muscle proteins. Their results indicated that hydrolyzates prepared from pure and uniform catfish isolate do show a potential for ACE inhibition, which may find a use as bioactive ingredients. Tilapia, another commercially important aquatic species, was used as a potential source of bioactive compounds because of the large quantities of waste generated, on a yearly basis. Raghavan and Kristinsson (2009) used tilapia protein hydrolyzate to evaluate its *in vitro* ACE inhibition capability and the outcomes of their study suggested that optimizing enzymes can be used to obtain peptides from tilapia with a high level of ACE inhibition.

Due to the fact that hypertension is affecting more than 15-20% of the adult population worldwide, researchers are attempting to use the most natural resources, such as yellowfin frame, oysters or shark meat, to develop anti-hypertensive bioactive compounds. Moreover, in order to exert an antihypertensive effect *in vivo*, the nutritionally-derived ACE inhibitor must be absorbed in their intact form from the intestine and further be resistant to plasma peptidases degradation to reach the target sites after oral or intravenous administration. Yellowfin sole frame protein, normally discarded as an industrial waste in the process of fish manufacture, has been hydrolyzed by digestive enzymes (such as α-chymotrypsin) to generate a potent ACE inhibitor (Jung et al., 2006). The purified non-competitive inhibitor, composing of 11 amino acids, mainly hydrophobic, was then orally administered in spontaneously hypertensive rats (SHR). Systolic blood pressure was measured at 1, 2, 3, 6 and 9 h and a significant reduction of 22 mmHg at 3 h after administration was observed. This is an indication that the isolated ACE-I peptide from yellowfin sole frame protein might be added as a food ingredient to develop functional food or may be used as a nutraceutical, with the potential of lowering BP (Jung et al., 2006).

From the coastal waters of China, *Acetes chinensis*, a marine shrimp, which is highly harvested annually, is however, not fully utilized for its high protein value, due to its small size; but rather used as flavoring agent in shrimp sauce. Cao and team (2010) endeavored to prepare ACE-I peptides from the shrimps' protein hydrolyzate using gastrointestinal proteases (pepsin) to examine whether the peptides will be resistant to the digestive enzymes. The fractionated peptide, Leu-His-Pro, exhibiting 92.7% ACE inhibition *in vitro*, was investigated for its *in vivo* anti-hypertensive activity in male SHR, as measured by tail-cuff method. Their findings demonstrated that after an oral administration of a high dose of 6 mg/kg of body weight of the

synthesized peptide, BP of SHR was significantly reduced by 31 ± 3.1 mmHg ($P < 0.01$) at 4 hour, 36 ± 2.8 mmHg ($P < 0.01$) at 6 hour and 29 ± 2.5 mmHg ($P < 0.01$) at 8 hour (Cao et al., 2010).

In the study of Wang and colleagues (2008), an ACE-I peptide was derived from oyster proteins by pepsin treatment. After separation and purification by gel filtration chromatography, RP-HPLC and LC-MS, the peptide sequence, which was determined to be a nonapeptide of Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe, was assayed for heat, pH and digestive enzymes stability. The outcomes indicated a peptide yield of 8.5%, with good heat and pH stability, as well as strong enzyme-resistance properties against digestive proteases. This further showed the excellent *in vivo* anti-hypertensive effect when orally administered in SHR at a dose of 20 mg/kg. This could, therefore, be served as a natural, cheap, safe and innovative source of hypotensive peptide (Wang et al., 2008). Table 3 lists the ACE-I peptides from different marine organisms and the treatment used to release these peptides.

ANTI-OBESITY PEPTIDES

Increasing epidemiological evidence is linking the prevalence of obesity to dietary factors. It is well established that high carbohydrate and/or high fat diets, coupled with a sedentary lifestyle, are the major lifestyle factors associated with a disordered lipid metabolism, which often results in high plasma levels of TG, low-density lipoproteins (LDL)-cholesterol and dysregulation of glucose. It has been recently recognized that protein is the most satiating macronutrient. Moderate intake of protein diet plays a crucial role in body weight loss and weight maintenance (Gerstein et al., 2004). The proposed mechanisms for weight loss include

increased satiety and thermogenesis, accretion of fat free mass and lowering food intake, resulting in decreased body weight (Veldhorst et al., 2008). However, little information is available on the mechanistic roles of nutritionally-derived ACE-I peptides in controlling food intake, lowering body weight and improving glucose intolerance. As discussed earlier, RAS blockade has been linked not only to hypotensive actions but also to improvements in abnormal glucose metabolism, which may result in diabetes.

In this regards, Otani and colleagues (2009) used the stroke-prone SHR, a well-characterized animal model of essential hypertension with high incidence of stroke, cardiac hypertrophy, kidney dysfunction and hyperglycemia, to investigate the effects of a Sardine-derived ACE-I peptide (SP) exhibiting an ACE inhibitory concentration of IC_{50} value of 62.4 µg/ml, on blood glucose level and blood pressure (Otani et al., 2009). SP-ACE inhibitor-treated animals showed i) a decreased ACE activity in the kidney, aorta and mesentery and lowered BP; ii) reduced glucose level after glucose loading, but no effect on insulin secretion, which suggested that SP administration improved glucose tolerance and insulin sensitivity in the stroke-prone animals. Fish protein hydrolyzates, not examined for their ACE inhibition potential, have been demonstrated to reduce plasma total proportion of LDL cholesterol and lower Acyl-CoA: cholesterol acyl-transferase activity in Zucker rats (Wergedahl et al., 2004). Whilst many pharmacological studies have been done, no naturally-derived ACE-I peptides have been tested as possible treatments for obesity and the biomarkers of metabolic diseases.

CONCLUSION

The unprecedented escalation in the food-related diseases, such as hypertension and obesity globally has increased consumers' perception about healthy eating habits and simultaneously has led to the development of functional food products that not only offer basic nutritional value but also physiological effects that can potentially prevent the onset of these detrimental health issues. The RAS has long been recognized for its role in the aetiology of these two major health phenomena, and this has prompted the discovery and production of various synthetic ACE inhibitors, which have been therapeutically beneficial in the treatment of hypertension. However, owing to their side effects, food-derived ACE inhibitors have been recognized as desirable alternatives. Various plant (garlic, mushroom, apricot) and animal (dried bonito, eggs, fish proteins) sources have been assessed for their ACE-I capability *in vitro*, in animal and human models. Moreover, some of the peptides, such as β-casein peptides from milk have also been assessed for their bioavailability. Owing to the fisheries over-exploitation issues, the use of marine wastes, such as skin, scales, frames, amongst others, have received much attention in recent years for their potential use as food ingredient and their potential use as a treatment for hypertension. However, limited information is available on the effects of fish-derived bioactive peptides, mainly with ACE-inhibitory potential on the markers of metabolic syndrome, including obesity, glucose intolerance and body composition improvement. Therefore, future studies must focus on these aspects.

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Figure Captions

Figure 1: The biochemical cascade of the classical renin-angiotensin system. The diagram clearly demonstrates that the synthesis of ANG II may be derived by two different pathways, namely the ACE-dependent pathway and other enzymes such as Chymase and the Cathepsins. The biological active component of RAS, ANG II, activates AT1 and AT2 receptors in various tissues, such as brain, heart, pancreas, kidney and adipose tissue to numerous important homeostatic systems of the body. The circulating ANG II is further cleaved into biologically active peptides ANG III and ANG IV by amino-peptidases (Ferrario et al., 2005, Mathai et al., 2011).

Figure 2: The complexity of the renin-angiotensin system, the amino acid composition and enzymatic cleavage of each angiotensin peptide. Angiotensin-Converting Enzyme 2 (ACE2), a novel homologue of the classical ACE, converts ANG II into angiotensin-(1-7), which acts as a

vasodilator through the Mas receptor, via the nitric oxide pathway, release of prostaglandins, inhibition of nor-epinephrine release and the activation of the vasodilator Bradykinin. ANG-(1-7) can also be indirectly formed from ANG I by the catalytic actions of the neutral Endopeptidases (NEPs) (Donoghue et al., 2000, Ferrario et al., 2005). This diagram illustrates the amino acid sequence of each of the angiotensin peptide involved in the functioning of the renin-angiotensin system. The Leu-Val dipeptide is cleaved by the enzyme renin to form ANG I. The His^{9}-Leu^{10} bond from ANG I is degraded by ACE to form the octapeptide ANG II. ACE2 cleaves ANG II at the Pho^{7}-Phe^{8} bond to synthesize ANG-(1-7). ACE then degrades the His^{6}-Pro^{7} dipeptide bond from ANG-(1-7) to produce ANG-(1-5) (Ferrario, 2006, Pihlanto-Leppälä, 2000).

Figure 3: Mechanisms implicated in the onset of hypertension. Obesity increases the activity of the sympathetic nervous system (SNS), which results in an increase in sodium (Na+) ions and water in the kidney. The components of RAS, such as AGT, ANG II and ACE are overexpressed and may act upon i) the arteries to cause vasoconstriction; ii) heart to constrict the vascular smooth muscle, which result in coronary heart disease; and iii) the adrenal cortex to secrete aldosterone, which increases the reabsorption of sodium and fluid in the kidney, resulting in high blood pressure. Moreover, the excessive body fat in obese individuals results in the overproduction of mineralcorticoid releasing factors (MCRFs) and increases the ability of oxidized derivatives of linoleic acid to synthesize more aldosterone, which in turn increases sodium and fluid retention. Furthermore, the active components of RAS activate the sympathetic nerve terminals to release norepinephrine, which causes vasoconstriction, to raise blood pressure.

Figure 4: Potential bio-functionalities of bioactive peptides. Bioactive peptides exert various established and novel biological functions, which may potentially protect the cardiovascular system, prevent the onset of metabolic syndrome, increase satiety, and maintain healthy nervous, immune and gastrointestinal systems as well as improving metabolism.

Figure 5: Diagrammatical representation of the processes involved in the production, isolation, purification, identification and characterization of bioactive peptides from natural protein food sources. Food proteins are hydrolyzed by selected proteases, such as pepsin to form the hydrolysate or to release the peptides. The latter is then assayed for potential different bioactivities, which are then purified and isolated. Isolated peptides are eventually re-assayed for the specific bioactivities and the most active peptide is sequenced by either MALDI-TOF or peptide sequencer to determine the amino acid sequence of the physiologically active peptide. Furthermore, the synthesized peptide undergoes a bioavailability assay, most commonly using the Caco-2 cell monolayer to investigate whether the peptide can be absorbed within the GIT. Some *in vivo* studies are also involved prior to development of the bioactive peptides as a food ingredient to be incorporated into functional foods or as a nutraceutical.

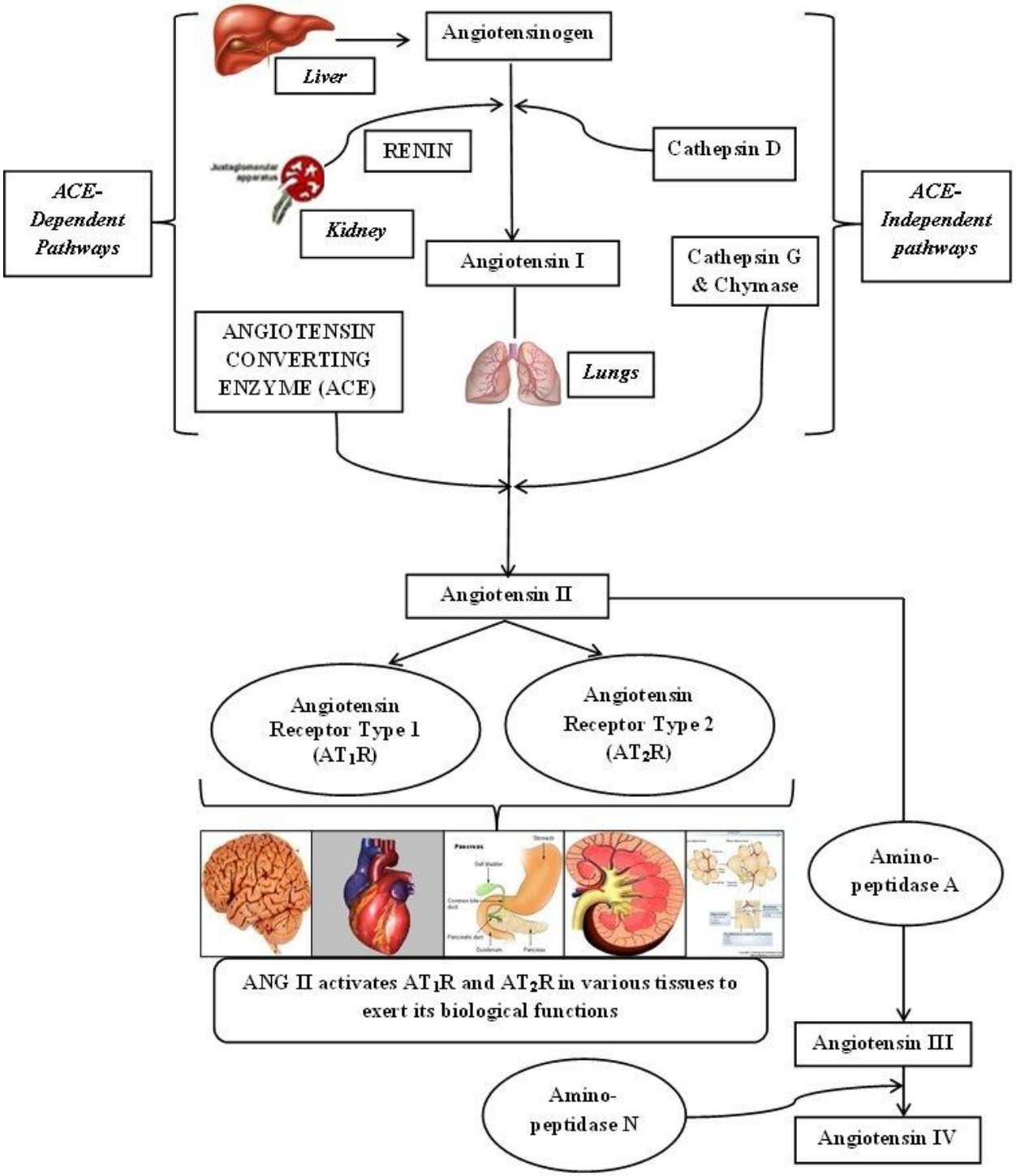


Figure 1

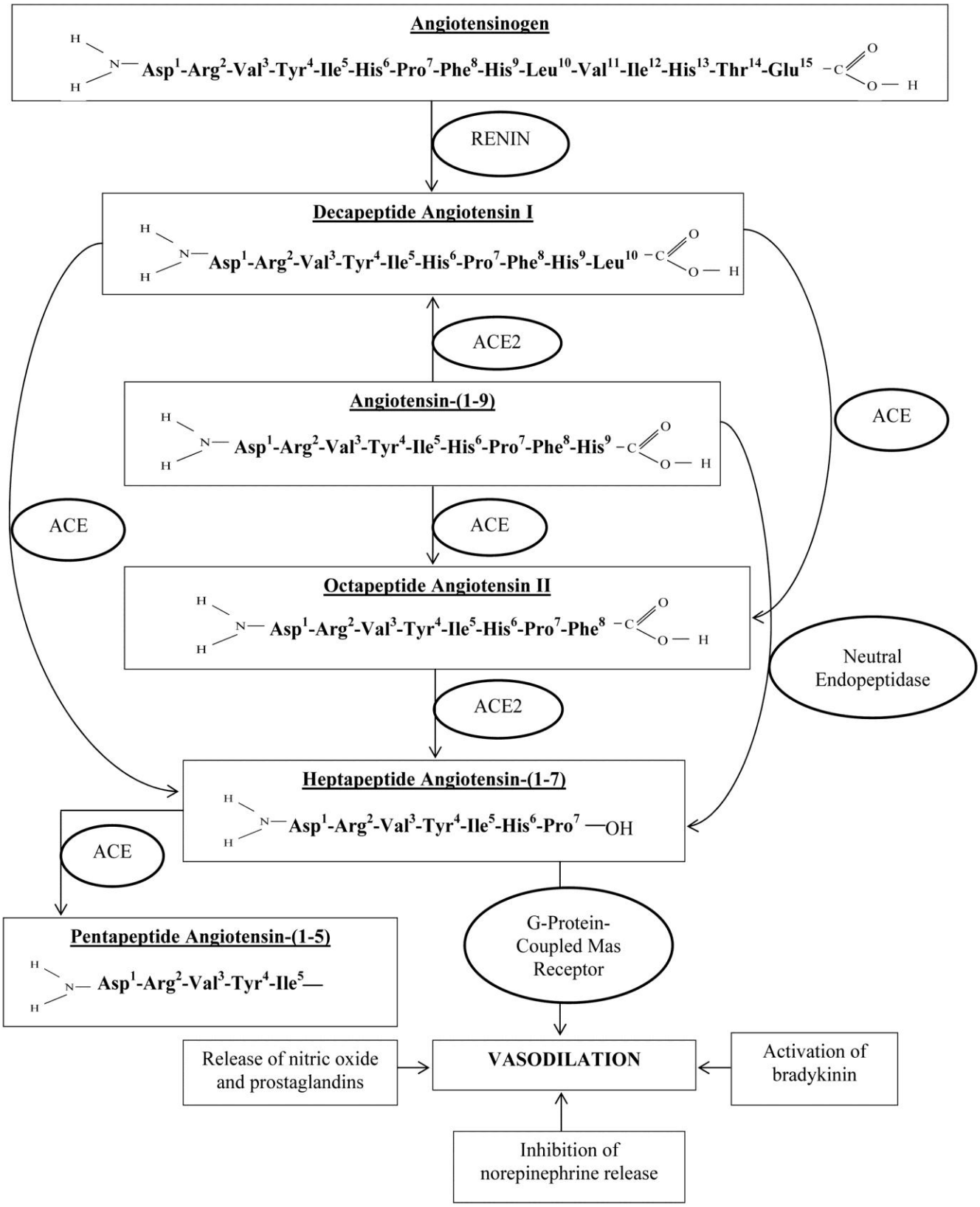
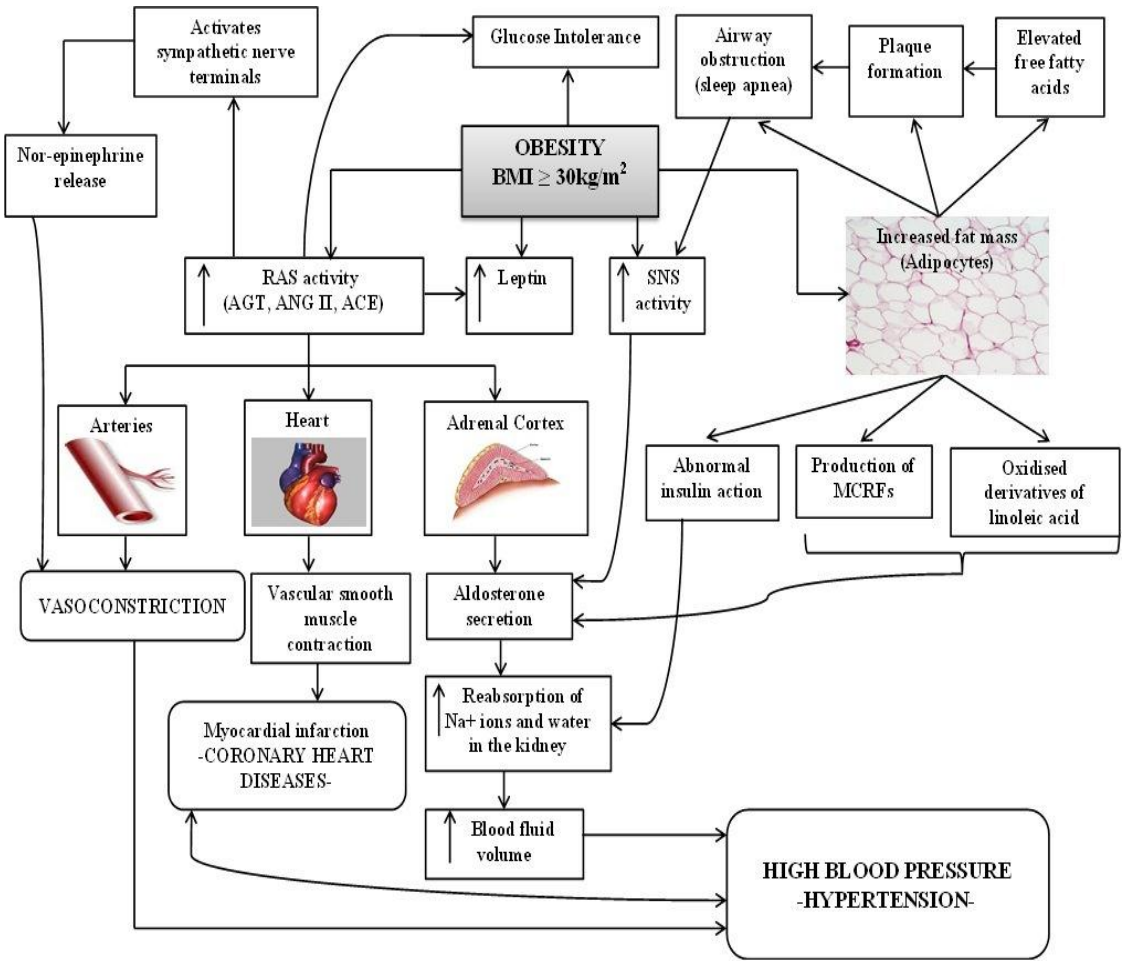


Figure 2

Figure 3



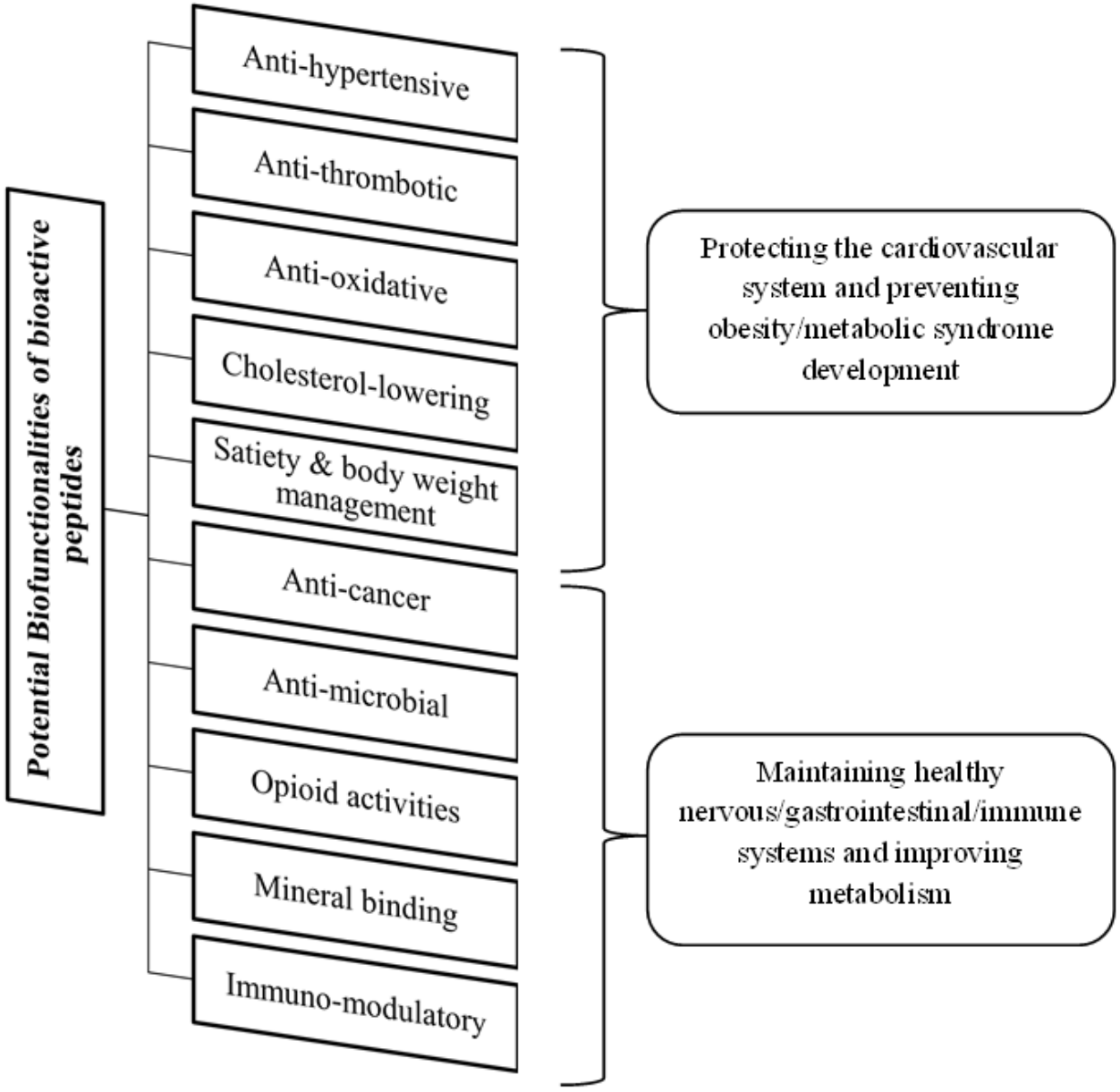


Figure 4

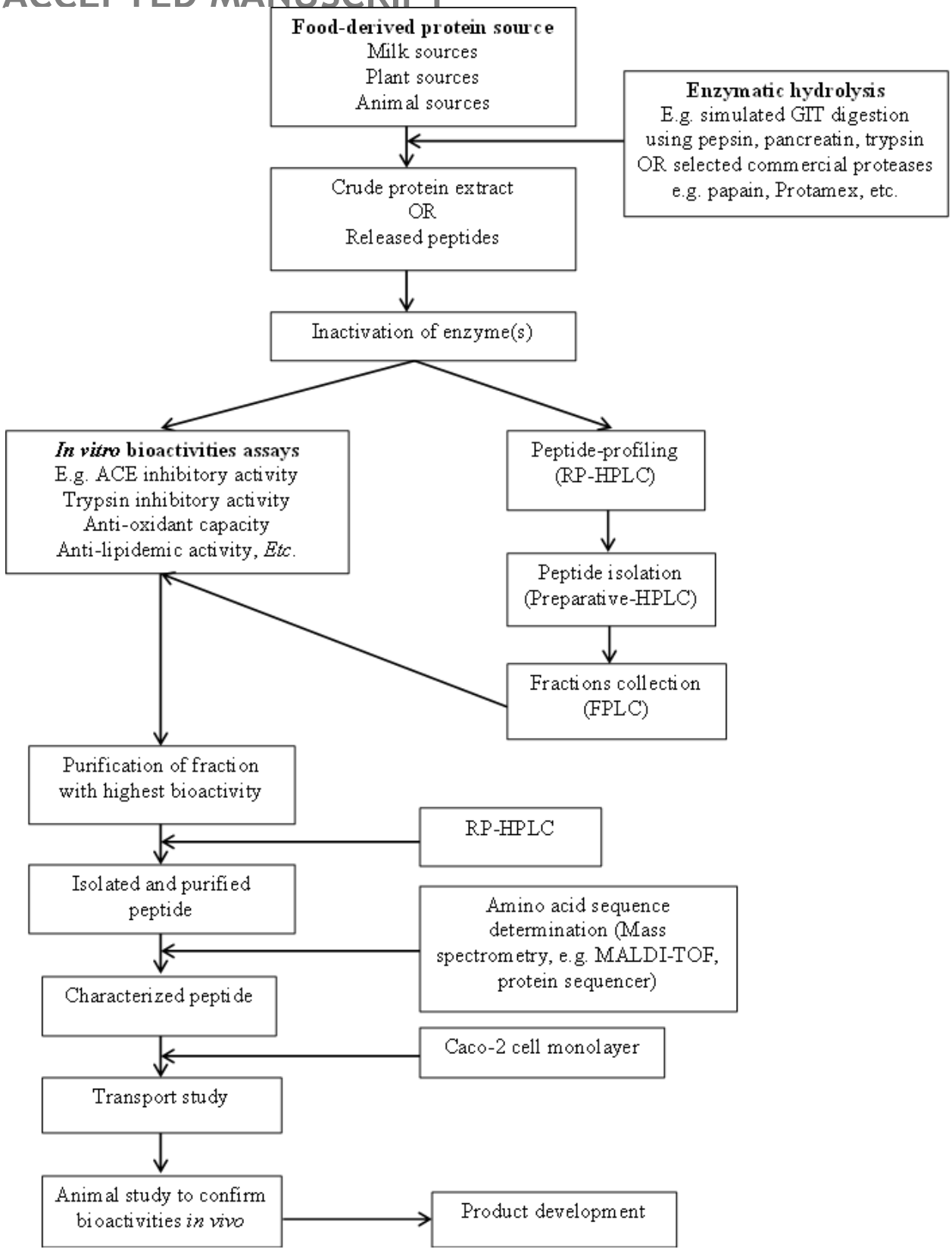


Figure 5

Table 1: Examples of plant food derived bioactive peptides and their potential physiological activities

Plant sources	Amino-acid sequences	Bio-functional properties	Study model	References
Defatted rice bran	-	Inhibition of pancreatic lipase activity (anti-lipidemic)	Male Sprague Dawley rats	(Tsutsumi et al., 2000)
Rice protein hydrolysate	Thr-Gln-Val-Tyr	Inhibition of angiotensin-I converting enzyme (anti-hypertensive)	Male spontaneously hypertensive rats	(Li et al., 2007)
Mushrooms (*Tricholoma giganteum*)	Gly-Glu-Pro	Inhibition of angiotensin-I converting enzyme (anti-hypertensive)	Male spontaneously hypertensive rats	(Lee et al., 2004)
Wheat germ	Ile-Val-Tyr	Inhibition of angiotensin-I converting enzyme (anti-hypertensive)	Male Sprague Dawley rats	(Matsui et al., 1999)
Defatted soybean meal	β-conglycinin	Appetite-suppressing effects	Human hepatoma cell line (Hep G2 cells)	(Nishi et al., 2003)
Soybean	-	Cholesterol-lowering effects	*In vitro* study	(Lovati et al., 2000)
Apricot-kernel protein hydrolysate	Gly-Phe		Spontaneously hypertensive rats	
Garlic		ACE inhibitory peptides		(Zhu et al., 2010)
		Inhibition of angiotensin-I converting enzyme (anti-hypertensive)		(Suetsuna, 1998)

- **: Not specified**

Table 2: Examples of animal food derived bioactive peptides and their potential physiological activities

Animal sources	Amino-acid sequences	Bio-functional properties	Study model	References
Dried bonito	Ile-Lys-Pro	Inhibition of angiotensin-I-converting enzyme (anti-hypertensive)	Male Wistar rats	(Yokoyama et al., 1992)
Chicken bone protein	-		*In vitro* study	(Cheng et al., 2008)
Beef hydrolysates	Val-Leu-Ala-Gln-Tyr-Lys	Inhibition of angiotensin-I-converting enzyme (anti-hypertensive)	*In vitro* study	(Jang and Lee, 2005)
	YAEERYPIL		*In vitro* study	(Davalos et al., 2004)
Egg white proteins	-	Inhibition of angiotensin-I-converting enzyme (anti-hypertensive)	*In vitro* study (human breast cancer lines)	(Picot et al., 2006)
Fish protein hydrolysate	-	Anti-oxidant capacity	STC-1 cells	(Cudennec et al., 2008)
Shrimp head	Gln-Tyr-Gly-Asn-Leu-Leu-Ser-Leu-Leu-Asn-Gly-Tyr-Arg	Anti-proliferative activity	*In vitro* study (Haemocytes)	(Battison et al., 2008)
American lobster	-	Cholesterol-lowering effects Appetite suppressant	*In vitro* study	(Lin and Li, 2006)
Squid skin gelatin		Anti-microbial activity Anti-oxidant capacity		

- **: Not specified**

Table 3: ACE-Inhibitory peptides derived from marine organisms: Sources, treatment used, amino acid sequences and IC_{50} value

Source	Treatment	Peptide sequence	IC_{50} value (μM)	Type of inhibition	Physiological activities	Study model	References
Alaska Pollack frame protein	Pepsin	Phe-Gly-Ala-Ser-Thr-Arg-Gly-Ala	14.7	Non-competitive	ACE inhibition	*In vitro*	(Je et al., 2004)
	α-chymotrypsin		28.7*			*In vivo*	
Yellow fin sole (*Limanda aspera*)		Met-Ile-Phe-Pho-Gly-Ala-Gly-Gly-Pro-Glu-Leu		Non-competitive	Hypotensive	10mg/kg /d 10wk, male	(Jung et al., 2006)
frame protein	Alcalase, Neutrase, pepsin, papain, α-chymotrypsin, trypsin		11.28			SHR rats	
Tuna frame protein hydrolysate		Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Trp-Ser-Pro-Pro-Lys-Try-Lys-Asp-Thr-Pro	66**	Non-competitive	Hypotensive	*In vivo* 10mg/kg /d 10wk, male rats	(Lee et al., 2010)
Oysters (*Crassostrea talienwhanensis Crosse*)	Pepsin	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	35.7	Non-competitive	Hypotensive	*In vivo* 20mg/kg /d 10wk, male rats	(Wang et al., 2008)
Pacific cod (*Gadus macrocephalus*) skin	Pepsin, trypsin and α-chymotrypsin		1.6	Non-competitive	ACE inhibition	*In vitro*	(Himaya et al., 2012)
	Pepsin	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro	63.9	Competitive	Hypotensive	*In vivo*	
Shrimps (*Acetes chinensis*)	Alcalase	Leu-His-Pro	9.64	Competitive	ACE inhibition	6mg/kg/d 10wk, male rats	(Cao et al., 2010)
Rainbow trout		Lys-Val-Asn-Gly-Pro-Ala-	0.00534***			*In vitro*	

(*Oncorhynchus mykiss*) muscle	Pepsin, chymotrypsin	Met-Ser-Pro-Asn-Ala-Asn	383.2	Competitive	ACE inhibition	*In vitro*	(Kim and Byun, 2012)
Rotifer (*Brachionus rotundiformis*)	Thermolysin	Asp-Asp-Thr-Gly-His-Asp-Phe-	51				
	Protamex	Glu-Asp-Thr-Gly-Glu-Ala-Met		Competitive	ACE inhibition	*In vitro*	(Lee et al., 2009)
Grass carp protein hydrolysates	Bromelain	Val-Ala-Pro	15.9	Competitive	ACE inhibition	*In vitro*	
	Alcalase	Leu-Phe	2.68			*In vitro*	(Chen et al., 2012)
Chum salmon muscle hydrolysate	Protease SM98011	Tyr-Asn	730	Mixed-type	ACE inhibition	*In vivo* 3μM/kg/d	(Ono et al., 2006)
Hard clam (*Meretrix lusoria*) muscle	Proteases	MEGAQEAQGD	7.5	Pro-drug	Hypotensive	Male SHR rats	
	Proteases		21.6	-	ACE inhibition	*In vitro*	(Tsai et al., 2008)
Sea cucumber (*Acaudina molpadioidea*)	Pepsin	Glu-Tyr		-	ACE inhibition	*In vitro* 2g/kg/d 9wk, male rats	(Zhao et al., 2009)
		Gly-Ile-Gly	0.62 ***		Hypotensive		
Shark meat hydrolysate	Alcalase	Val-Ile-Tyr	1.44***	-		*In vivo* 300mg/kg/d	
Chum salmon head	Protease S ‘Amano’ (from *Bacillus stearothermophilus*)	Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro	35.2 6.1 18.8 42.3 3.3 1.5 23.6	- - -	Hypotensive ACE inhibition	4wk, male rats *In vitro*	(Wu et al., 2008) (Ohta et al., 1997)
Sea bream scales		Thr-Phe-Pro-His-Gly-Pro	11.6	-	ACE		

		His-Trp-Thr-		-	inhibition		
Big eye		Thr-Gln-Arg	-	-		*In vitro*	(Fahmi
tuna dark	Bacterial			-		*In vivo*	et al.,
muscle	proteases	Val-Tyr		-		1mg/kg	2004)
(*Thunnus*	(*Bacillus*	Ile-Tyr		-		*18 wk*	
obesus)	*mojavensis*	Ala-Trp	0.060***	-	-ACE	*male rats*	
	A21)	Phe-Tyr			inhibition		
Seaweed		Val-Trp	0.332***	-	-		(Qian et
pipefish	Alcalase	Ile-Trp			Hypotensiv		al.,
muscle		Leu-Trp			e		2007)
protein						*In vitro*	
		Ala-His-Ser-	1.28***	-			
	Alcalase,	Tyr					
	papain						(Wijesek
Wakame						*In vitro*	ara et al.,
(*Undaria*				-			2011)
pinnatifida)		Gly-Pro-Leu-		-	ACE		
	Pepsin, papain	Gly-Leu-			inhibition		
		Leu-Gly-Phe-	27.9*			*In vitro*	
		Leu-Gly-Pro-		-			(Sato et
		Leu-Gly-					al.,
		Leu-Ser			ACE		2002)
					inhibition	*In vivo*	
Cuttlefish		Ala-Pro				*200*	
(*Sepia*	Thermolysin	Val-Arg				*mg/kg,*	
officinalis)			1.2			*400mg/k*	
protein				-	ACE	*g and*	
hydrolysate		-			inhibition	*800mg/k*	
						g	
Squid						*10-*	(Balti et
gelatin			-			*15wks,*	al.,
hydrolysate	Papain				-ACE	*male*	2010)
					inhibition	*SHR*	
			0.26***	-	-		
Atlantic		-			Hypotensiv	*In vitro*	
salmon					e	*In vivo*	(Aléman
(Salmo	Cryotin-F				-Anti-	*500mg/k*	et al.,
salar L.)	Flavouzyme		-		hyperlipide	*g and*	2011)
skin				-	mic	*2000mg/*	
hydrolysate	*Bacillus*					*kg*	
	licheniformis					*9wk,*	(Gu et
Jellyfish	alkaline	Ile-Trp				*male rats*	al.,
(*Rhopilema*	protease				-ACE	*In vitro*	2011)
esculentum)					inhibition		

protein hydrolysate	Protease	-	- Hypotensive	*In vitro* *In vivo* *30mg/kg male SHR*	(Liu et al., 2012)
Chum salmon muscle	-	-	-ACE inhibition	*In vitro* *In vivo* (Stroke-prone SHR) 1g/kg/d 14wk, male rats	(Ono et al., 2003)
Salmon muscle (*Oncorhynchus gorbuscha*)	-	-	ACE inhibition		
Tilapia	-	-	ACE inhibition		(Enari et al., 2008)
Sardine muscle	-	-	- Hypotensive -Lower blood glucose level -Decrease ACE activity in kidney, aorta, mesentery -No effects on insulin secretion		(Raghavan and Kristinsson, 2009) (Matsui et al., 1993) (Otani et al., 2009)
Sardine muscle	-	-			

* µg/ml

** µmol/L

*** mg/ml

-: Not specified