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QSAR- AIDED STUDY OF ANTIHYPERTENSIVE PEPTIDES FROM EGG PROTEINS

by

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DEDICATION

This thesis is dedicated to my supervisor Dr. Jianping Wu, whose amazing support I cannot do without.

ABSTRACT

Many bioactive peptides have been reported from various food proteins through the conventional activity-guided-purification approach; however, the rationale behind the selection of conditions for the production of the bioactive peptides has not been extensively explored. The purposes of the study were to provide the rationale behind the selection of conditions, and to develop an innovative strategy to explore the most potent peptides within egg proteins through an integrated QSAR and bioinformatics approach. Thermolysin-pepsin hydrolysate of ovotransferrin was predicted as the best condition for production of ACE-inhibitory peptides. Three predicted peptides, IRW, LKP and IQW, were successfully released from ovotransferrin. Simulated gastrointestinal incubation showed IQW was stable while IRW and LKP can be degraded into dipeptides (IR and KP respectively). Peptides produced from the study will have the potential to be developed as functional foods and nutraceuticals for the prevention of hypertension, a disease affecting ~ 31% of the adult population.

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LIST OF ABBREVIATIONS

2-ME - β-Mercaptoethanol

ACE - Angiotensin-Converting Enzyme

AHA - American Heart Association

Ang-I - Angiotensin I

Ang-II - Angiotensin II

ANOVA - Analysis of Variance

ARB - Angiotensin II Receptor Blocker

AT_1 - Angiotensin Receptor

b-ET - Big Endothelin

BSA - Bovine Serum Albumin

CCB - Calcium Channel Blocker

cGMP- cyclic guanosine monophosphate

DASH - Dietary Approach to Stop Hypertension

DBP - Diastolic Blood Pressure

DTT – Dithiothreitol

ECE - Endothelin-Converting Enzyme

ESI - Electrospray Ionization Technique

ET - Endothelin

FOSHU - Foods for a Specific Health Issue

GABA - Gamma-Aminobutyric Acid

GPCR - G-protein Coupled Receptors

HHL - Hippuryl-L-Histidyl-L-Leucine

LC-MS/MS - Liquid Chromatography Tandem Mass Spectrometry

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NCBI - National Center for Biotechnology Information

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

QSAR - Quantitative Structure and Activity Relationship

Q-TOF - Quadrupole Time-of-Flight

RAS - Renin-Angiotensin System

SBP - Systolic Blood Pressure

SHR – Spontaneously Hypertensive Rats

SOD - Superoxide Dismutase

VSM - Vascular Smooth Muscle

The one-letter amino acid codes used in text.

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

CHAPTER-1 LITERATURE REVIEW

1.1 FUNCTIONAL FOOD OVERVIEW:

It is well recognized that diet and health are intrinsically linked. The primary role of diet is to provide essential energy and nutrients to meet the functions of the body; recent biochemical advancements have provided evidence that, beyond meeting nutritional and energy needs, diet may modulate various functions in the body and have beneficial or detrimental roles against some chronic diseases or pathogenic disorders, such as cardiovascular diseases, obesity, diabetes, or even cancers (Nobes et al., 2009). Facing challenges such as an increase in life expectancy, an increasing incidence of chronic diseases and associated uncontrollable increases in the cost of health care, emerging functional foods are expected to play an important role in the prevention/management of chronic diseases, reducing health care costs and improving the quality of life (Roberfroid et al., 2000). The concept of functional food was first established in Japan in 1984, as foods for a specific health issue (FOSHU) (Siro et al., 2008). It has turned out rapidly to be a growing global trend in the last two decades. The global market for functional food is estimated to be at least $47.6 billion US, with the United States being the largest market segment (with an estimated market share of more than 50%), followed by Europe and Japan (Sloan et al., 2000). Functional foods have a market share of around 2–3% in the US food market, and this percentage is expected to be double by the end of the 2008 fiscal year (Siro et al., 2008). Functional foods may improve the general conditions of the body (e.g., pre- and

probiotics), decrease the risk of diseases (as, cholesterol-lowering products), and could even be used for curing some illnesses (Siro et al., 2008).

Bioactive peptides derived from various food proteins have attracted extensive attention due to their various physiological functions, such as inhibiting the activity of enzymes, modulating the functions of the immune system, enhancing mineral absorption, exhibiting antimicrobial, antibacterial, and antifungal activity, demonstrating antagonistic activity, and improving cardiovascular condition (Dziuba et al., 1999; Kitts and Weiler, 2003). They are latent in the parent protein sequence, but have the ability to exert physiological benefits in human beings, after being released by enzymatic hydrolysis, fermentation, or food processing (Dziuba et al., 1999). Examples of various types of bioactive peptides from food proteins and their respective activities are listed in Table 1.1.

1.1.1 Bioactive peptides in the body's defense system

1.1.1.1 Antimicrobial peptides: Antimicrobial peptides belong to an effective part of innate immunity that has evolved in most living organisms. Before the evolution of adaptive immunity, most living organisms survived against microbial challenges through this nonspecific immune system (Diplock et al., 1999). Antimicrobial peptides are small molecular weight fragments of proteins with broad spectrum activity against bacteria, viruses, and fungi. These evolutionarily-conserved peptides are usually positively charged and have both hydrophobic and hydrophilic side chains that enable the molecule to be soluble in aqueous environments, yet also enter lipid-rich membranes. Once in a target microbial membrane, the peptide kills target cells through diverse mechanisms

such as pore formation through electrostatic attraction (Izadpanah and Gallo, 2005).

Milk proteins are a good source of antimicrobial activities. Lactoferricins, derived from lactoferrin upon pepsin hydrolysis, are a well-known example (Tomita et al., 1994). Lactoferrin is now commercialized and well known for its antimicrobial activities. The disruption of normal membrane permeability is one of the mechanisms that can be partly responsible for the antibacterial activity of lactoferricins (Diplock et al., 1999). Lactoferricins act against a number of different Gram-positive and Gram-negative bacteria (such as *Escherichia*, *Helicobacter*, *Listeria*, *Salmonella*, and *Staphylococcus*), yeasts, and filamentous fungi (Diplock et al., 1999). Other antimicrobial peptides have also been isolated from two major milk proteins, α_s-1 and α_s-2 caseins (Meisel, 1998; Ganz, 2003). Egg white proteins, by nature, protect the embryo against various kinds of microbial invasions. Two major egg white proteins, lysozyme and ovotransferrin, are reported to have antimicrobial activities (Fitzgerald and Murray 2006). A 92-amino-acid-long peptide (OTAP-92) isolated from ovotransferrin was reported to exhibit antimicrobial activity through a membrane damage mechanism (Ibrahim et al., 2000). A peptide (IVSDGDGMNAW), isolated from egg lysozyme, showed antimicrobial activity against Gram negative bacteria *E. Coli* K-12 (Mine and Kovacs-Nolan, 2004).

1.1.1.2 Immunomodulatory peptides: Some specific peptides from egg proteins can enhance the function of immune cells. These groups of peptides are called immunomodulatory peptides. They can modulate the activity of natural

killer cells, lymphocyte proliferation, and cytokine regulation (Gautron et al., 2001). Moreover, immunomodulatory peptides may reduce allergic reactions in atopic humans and enhance mucosal immunity in the gastrointestinal tract (Gautron et al., 2001). Peptides derived from bovine milk protein can affect immune functions. Peptides derived from pancreatin and trypsin digests of α_{s1}-casein and β-casein were shown to inhibit the proliferation of murine spleen cells and rabbit peyer's patch cells (Otani and Hata, 1995). Peptides derived from αs1-casein have been shown to have a wide range of immunomodulatory activities, including suppression *in vitro* of mitogen-stimulated human peripheral blood mononuclear cell proliferation (Markwick, 2005), promotion of antibody formation, enhancement of phagocytosis *in vitro*, and *in vivo* protection against *Klebsiella pneumoniae* infection in mice (Markwick, 2005). An active peptide was obtained from an egg white protein after fermentation with *Saccharomyces cerevisiae* (yeast) (Kassaify et al., 2005). Oral administration of this product increased the non-specific activity of neutrophils. Peptides derived from a tryptic digest of egg proteins were shown to stimulate superoxide anions, which can trigger the nonspecific immune defense system (Mine and Kovacs-Nolan, 2004).

1.1.2 Bioactive peptides in the body's nervous system

Food-derived peptides with opioid activity were first discovered in the 1970s (Horiguchi et al., 2005). Opioid peptides can be obtained by *in vitro* enzymatic hydrolysis of various food proteins. Peptides with opioid activity mainly affect appetite, gastrointestinal motility, and behavior (Markwick, 2005). Most of the opoid peptides were derived from bovine milk protein (α-casein) which is known

as casomorphins, some peptides were also found in other milk proteins such as α-lactalbumin and β-lactoglobulin. Casomorphins were produced mainly during cheese ripening due to the proteolytic activity of some bacteria, while α-casomorphins are the most active among opioid peptides and are also categorized as "food hormones". This can also act as a histamine releaser (Korhonen and Pihlanto, 2003).

1.1.3 Bioactive peptides in the body's regulatory system

Some bioactive peptides play an important role in the body's regulatory system. Antihypertensive peptides derived from various food proteins are major types of this group. Detailed information about the antihypertensive peptides will be provided in the following part (section 1.4). Peptides that can regulate oxidative stress, antioxidant peptides are the other type. Some antioxidant peptides were identified in milk proteins such as α-lactalbumin and β-lactoglobulin (Kitts and Weiler, 2003). Wheat proteins and fish muscle proteins can also produce some antioxidant peptides after enzymatic hydrolysis (Teschemacher, 2003).

1.2 HYPERTENSION

Cardiovascular disease is the leading cause of death in many countries (Padwal et al., 2009). According to the World Health Organization (WHO), 17.5 million people died from cardiovascular diseases in 2005, representing 31% of all global deaths (Padwal et al., 2009). Hypertension, a medical term for high blood pressure, is one of the well-defined risk factors in cardiovascular disease and is defined as a condition in which systolic blood pressure is greater than 140 millimeters of

mercury (mmHg) and/or a diastolic pressure greater than 90 mmHg. In North America, 79.4 million people have some type of cardiovascular disease; of those 72 million have hypertension (Whelton, 2004). There are two main types of hypertension. Primary hypertension, or essential hypertension, represents 90–95% of cases, but the specific medical cause is unknown; it is reported that various factors such as obesity (Oshima et al., 1985), salt sensitivity (Padwal et al., 2009), renin homeostasis (Lopez and Murray, 1998), insulin resistance (Rosamond et al., 2007), sleep apnea (Calhoun et al., 2008), genetics (Haslam and James, 2005), age (Caprioli et al., 2008), and unbalanced diets, especially in terms of sodium chloride and protein intake (Dietze and Henriksen, 2008), might affect the occurrence of hypertension. Secondary hypertension is a result of the pathogenesis of different organs, such as in kidney disease, adrenal disorders, thyroid disorders, and tumors (Chapman and Sposito, 2008). Primary hypertension is regarded as one of the major 'lifestyle-related diseases,' and it has been demonstrated that lifestyle modifications such as reducing weight, controlled diet, such as the Dietary Approach to Stop Hypertension (DASH), physical activities, and moderate alcohol consumption, would reduce the risk of hypertension (Chapman and Sposito, 2008).

The renin-angiotensin system is one of the important pathways of the human body that plays a key role in controlling blood pressure. The main components of the renin-angiotensin system (RAS) are renin, angiotensin-converting enzyme (ACE), and angiotensinogen (Fig 1.1). Renin is synthesized in the human kidneys, stored in the afferent arterioles, and released in response to hemodynamic, neurogenic,

and ionic signals (Sealey and Laragh, 1990; Hackenthal et al., 1990). Renin is an aspartyl protease that has a very high specificity for angiotensinogen (Zhu et al., 2006). Angiotensinogen is a 58 kilodalton (KDa) protein synthesized and released from the liver (Nasjletti and Mason, 1972). Angiotensin-converting enzyme (ACE) [EC 3.4.15.1], which is also known as kinase II, is a metallo-protease with two zinc active catalytic sites. Renin cleaves angiotensinogen and releases amino terminal decapeptide angiotensin I [DRVYIHPFHL] (Ang I). ACE then cleaves Ang I and releases two carboxy-terminal amino acids (HL). The resulting octapeptide is called angiotensin II (Ang II) (Ng and Vane, 1967). Ang II is a potent vasoconstrictor, whereas Ang I is biologically inactive. Ang II directly stimulates vascular smooth muscle contraction, which increases systematic vascular resistance and elevates blood pressure. It also stimulates secretion of aldosterone from the adrenal cortex. Aldosterone produces kaiuresis and increases sodium and water retention in the body. Additionally, ACE also hydrolyzes and inactivates bradykinin [RPPGFSPFR], a potent vasodilator (Sealey and Laragh, 1990). Therefore, excessive ACE activity leads to high blood pressure or congestive heart failure through an increased rate of vasoconstriction. Thus, inhibition of ACE is the therapeutic target for antihypertensive drug development (Hackenthal et al., 1990).

The pharmacological ACE-inhibitors (i.e., captopril and enalpril) are widely used for the treatment of hypertension. However, these ACE inhibitors are associated with adverse effects including dry cough, hyperkalemia, hypotension, renal failure, decrease in white blood cells, and angioedema (Silverberg et al., 2002). Pregnant

women or patients suffering from kidney problems are urged to avoid synthetic ACE inhibitors (Pravenec and Petretto, 2008; Ben-Sira and Oliveira, 2007). Therefore, there is an interest in searching for non-pharmacological methods of prevention and management of hypertension. One of the major interests is the use of various food-derived peptides (e.g., vegetables, legumes, fish, milk, eggs) to prevent hypertension by inhibiting ACE activity. Bioactive peptides derived from food sources are generally regarded as a safer and more economical alternative for the prevention and treatment of hypertension (Champagne, 2006).

1.3 *In-vivo* MECHANISMS OF ANTIHYPERTENSIVE ACTIVITY

Blood pressure in the blood vessels is controlled by the contraction and relaxation of vascular smooth muscle; the two processes that are involved in this mechanism are known as vasoconstriction and vasodilatation, respectively.

Vasoconstriction is the narrowing of blood vessels resulting from the contraction of the muscular walls of the vessels. When blood vessels constrict, the flow of blood is restricted or decreased. The opposite of vasoconstriction is vasodilatation, in which the widening of blood vessels results from the relaxation of smooth muscle cells within the vessel walls. So, vasoconstriction and vasodilatation regulate and maintain the blood pressure in the body. Factors involved in the vasoconstriction and vasodilatation processes are produced in the endothelium (Sudano et al., 2006). The endothelium is the thin layer of epithelial cells (flat cells) that lines the heart, blood and lymph vessels, by forming an interface between the circulating blood in the lumen and the rest of the vessel wall. It is one of the largest and most extensive tissues in the human body (Sundano et al., 2006).

In the endothelium, ACE converts angiotensin I to angiotensin II. The angiotensin II binds with the angiotensin receptor (AT_1) in the endothelium and produces big endothelin (b-ET), which is converted to endothelin (ET) by the endothelin-converting enzyme (ECE). Earlier research showed that endothelins can work as a vasoconstrictory peptide (Hickey et al., 1985; Yanagisawa et al., 1988). Endothelin activates two ET receptors (A and B) located in vascular smooth muscle. This smooth muscle also contains AR that binds angiotensin II. Angiotensin II activates the activity of NADPH-oxidase, which is a major source of free radicals (O_2-) in vascular smooth muscles. These free radicals can damage the cell membrane of vascular smooth muscle (Sundano et al., 2006).

In contrast, vasodilatation or relaxation of smooth muscle occurs mainly via the production of nitric oxide (NO) in the endothelium. The yield of NO is regulated by the expression of nitric oxide synthase (NOS) as well as bradykinin receptors. NO causes vasodilatation by activating guanylate cyclase and increasing levels of cyclic guanosine monophosphate (cGMP) in vascular smooth muscle (VSM) (Sundano et al., 2006).

An imbalance of endothelium-derived relaxing and contracting substances disturbs the normal function of the vascular endothelium and results in an elevation of blood pressure. Therefore, exogenous therapies (ACE inhibition, angiotensin II receptor blockers, calcium channel blockers, etc) need to be administered to reduce high blood pressure or hypertension (Spieker and Luscher, 2005).

The clinical benefits of various antihypertensive therapies to reduce blood pressure are largely accepted. Nevertheless, developing a better understanding of the mechanisms underlying hypertensive vascular disease, as well as the actions of antihypertensive agents in the cellular matrix, offers the potential for a more targeted therapy that reduces the global risk of cardiovascular diseases.

Vasocontraction can happen through various regulatory pathways so antihypertensive substances should have a target specific morphologic, hemodynamic and physiological effect that can influence the vasodilatation process. Various antihypertensive drugs such as, ACE inhibitors, angiotensin II receptor blockers (ARBs), and, calcium channel blockers (CCBs) were able to interact in different pathways to achieve vasodilation (Yoshiko et al., 2008).

ACE inhibitors also inhibit the breakdown of bradykinin, a potent vasodilator. Another antihypertensive agent, angiotensin-II receptor blocker (ARB), resists the binding of angII with the receptor. As a result, the production of big endothelin and the concentration of ang II are prevented. Simultaneously, intact bradykinin via the receptor helps to increase NO production in the endothelium. These processes help to balance the contraction and relaxation of vascular smooth cells by limiting the oxidation of NADPH into free radicals (O_2-) and increasing the activation of cGMP by NO (Spieker and Luscher, 2005). These free radicals and others such as hydrogen peroxide cause membrane damage. The free radicals produced in the endothelium are converted into hydrogen peroxide by the action of superoxide dismutase (SOD). Antioxidants help to reduce the amount of free radicals in the cell (Spieker and Luscher, 2005). ACE- inhibitory peptides, such as

dipeptide (MY) which was isolated from sardine muscle, showed antioxidant activity in the human endothelium (Erdmann et al., 2006).

Two ACE-inhibitory peptides from ovalbumin (ovokinin I and II) also showed vasorelaxation activity and lowered blood pressure in SHRs after oral administration (Yoshikawa et al., 2000). See Fig 1.2 for a schematic diagram of various actions within the blood vessels which control blood pressure.

1.4 ACE-INHIBITORY PEPTIDES FROM DIFFERENT FOOD PROTEINS

Many ACE-inhibitory peptides have been reported in different food sources (e.g., food-grains, vegetables, fruits, seeds and legumes, milk and dairy products, seafood, meat, and egg) showing *in vitro* ACE-inhibitory activity as well as *in vivo* antihypertensive effects in animals (spontaneously hypertensive rats [SHRs]) (Champagne, 2006; Iwai and Matsue, 2007; Bakris et al., 2008; Opie, 1987) or even in human subjects (Williams et al., 2004). Peptides from various food sources will be discussed in the following sections.

1.4.1 ACE-inhibitory peptides from plant sources

ACE-inhibitory peptides from various plant sources are summarized in Table 1.2. ACE-inhibitory peptide (TQVY) was obtained from rice (Champagne, 2006). Another peptide from wheat (IAP) and another active peptide from barley (EVSLNSGYY) were isolated with antihypertensive activity (Chen et al., 2007; Kajihara et al., 2008). A hexapeptide (PSGQYY) was derived from corn protein hydrolysate prepared by pescalase, a serine protease from *Bacillus licheniformis* (Miguel et al., 2005). Grains, such as rice, wheat, rye, barley, cereal, and corn are

important constituents of daily human diet. A study also recently reported that whole grain diets can be successfully used to reduce blood pressure, as well as weight, in mildly hypercholesterolemic men and women (Williams et al., 2004). In addition to protein-derived peptides, other bioactive food components, such as dietary fiber, vitamins, minerals, phytochemicals, and sterol, may also contribute to the benefit (Seiber et al., 2008). For example, gamma-aminobutyric acid (GABA) in grains was shown to decrease blood pressure in SHRs and hypertensive humans (Shih and Chuang, 2007), and 2"-hydroxynicotianamine, isolated from buckwheat powder, showed a very high ACE-inhibitory activity (Potenza et al., 2007).

ACE-inhibitory peptides from soybean proteins have been extensively studied. ACE-inhibitory and free-radical scavenging properties were recognized in peptides derived from soybean protein hydrolysates (McCue et al., 2005; Farzamirad and Aluko, 2008). A pentapeptide, VLIVP, derived from 11S globulin from soybeans (*Glycine max*), was reported to have an IC_{50} value of 1.69 μM (Gouda et al., 2006). A fermented soybean product, known as *douchi*, contains a potent ACE inhibitor (Zhang et al., 2006). A fermented soybean paste from Korea, fermented by the fungi *Monascus koji*, *Aspergillus sojae*, or *Aspergillus oryze*, also exerted ACE inhibition *in vitro* and antihypertensive activity *in vivo*, and one bioactive tripeptide (HHL) was identified from the hydrolysate (Yang et al., 2003). A pea protein digest also exerted a transient but strong antihypertensive effect in SHRs after intravenous administration (Vermeirssen et al., 2005). A novel ACE inhibitory peptide, LRW, was also isolated from the garden pea (*Pisum sativum*)

(Wu et al., 2006). Peptides derived from alcalase hydrolysate of mung-bean protein also exhibited ACE- inhibitory activity (Li et al., 2006). Three peptides, KDYRL, VTPALR, and KLPAGTLF, were identified in this study. Eight ACE inhibitory peptides were isolated from 11s globulin protein of amaranth (Silva-Sanchez et al., 2008). Six peptides were identified from sesame hydrolysate but only LSA, LQP, and LKY showed high ACE inhibition *in vitro*; however, a reconstituted sesame peptide mixture of LSA, LQP, LKY, IVY, VIY, LVY and MLPAY showed a strong antihypertensive effect or SHRs (Nakano et al., 2006).

1.4.2 ACE-inhibitory peptides from animal sources

Many different peptides were also reported from the muscle proteins of pork, chicken, and various types of fish, as well as from milk and eggs, as summarized in Table 1.3 and Table 1.4.

1.4.2.1 ACE-inhibitory peptides from different meat sources: ACE-inhibitory peptides derived from muscle proteins of animals have been studied in pork, chicken, and beef (Table 1.3). ACE inhibitory peptides could be released by enzymatic hydrolysis of water-insoluble proteins (actin, myosin, and collagen), from porcine skeletal muscle, and from myosin (Arihara et al., 2001). Enzymatic hydrolysis was carried out by eight types of proteases (pepsin, R-chymotrypsin, trypsin, papain, ficin, pronase E, proteinase K, and thermolysin). Among them, thermolysin hydrolysate showed the highest ACE-inhibitory activity. Two potent peptides, MNPPK and ITTNP, were isolated from the crude hydrolysate. Thermolysin hydrolysates of the water-insoluble proteins and myosin from porcine skeletal muscle also exhibited antihypertensive effects after a single oral

administration to SHRs (Nakashima et al., 2002). Peptic hydrolysates of regulatory proteins of porcine, such as tropomyosin and troponin, also exhibited *in vitro* ACE-inhibitory activity. Among them, troponin hydrolysate showed a relatively strong activity (Katayama et al., 2003). Two peptides, RMLGQTPTK and a partial peptide of this ennea-peptide (1-7), RMLGQTP, were purified. Stability study of these peptides against gastrointestinal enzymes (pepsin, R-chymotrypsin, or trypsin) revealed that the ennea-peptide exerted a relatively high resistance to digestive proteases. Thus, it is conceivable that RMLGQTPTK may work *in vivo* as an ACE inhibitor. Seven potent ACE-inhibitory peptides were also derived from the thermolysin hydrolysate of chicken muscle. The ACE-inhibitory activity of these peptides was measured *in vitro*, with IC_{50}-values ranging from 0.21 to 14 μM (Fujita et al., 2000); four peptides—IKP, IKW, LAP, and LKP—showed *in vivo* antihypertensive activity in SHRs. However, FKGRYYP failed to exert antihypertensive activity, while IVGRPRHQG showed antihypertensive activity after oral administration, but not after intravenous administration. Therefore, IVGRPRHQG can be considered as a prodrug-type ACE inhibitor. It was digested by trypsin in the gastrointestinal (GI) tract and converted into the active peptide IVGRPR after oral administration. But an intravenous administration did not produce this conversion. Besides chicken breast muscle, ACE inhibitory peptides were also reported from chicken leg bone proteins (Cheng et al., 2008). A potent hexapeptide, VLAGYK, was identified from the hydrolysate of sarcoplasmic protein from beef rump (Jang and Lee, 2005).

1.4.2.2 ACE-inhibitory peptides from fish and different types of seafood: Fish are very popular in human diets. ACE- inhibitory peptides were derived from different types of fish, such as Alaska Pollack, salmon, sardines, and anchovies (Table 1.3). As an example, a novel ACE-inhibitory peptide, PTHILWGD, was isolated by acid extraction from tuna muscle (Kohama et al., 1988). Eight ACE-inhibitory peptides were isolated from dried bonito. Among them, a peptide, LKPNM, could be regarded as a prodrug-type ACE-inhibitory peptide, as its hydrolyzed product, LKP, exerted potent antihypertensive activities *in vivo* (Fujita et al., 1999). Peptides derived from salmon muscle also possessed antihypertensive effects on SHRs (Enari et al., 2008). Besides fish, some other types of invertebrate seafood, such as Antarctic krill, oysters, and pearl oysters, also exhibited ACE inhibitory activities (Katano et al., 2003; Kawamura et al., 1992; Wang et al., 2008). Fermented fish sauce, widely used in countries such as Korea, also exhibited a tendency to lower blood pressure in SHRs (Ichimura et al., 2003).

1.4.2.3 Antihypertensive activity of milk: Milk is considered one of the best sources of bioactive peptides (Maruyama et al., 1998). Different studies have been performed on the antihypertensive effect of milk protein-derived peptides. As the major milk protein, casein is composed mainly of three heterozygote, αs1-casein, αs2-casein, and β-casein, accounting for 12–15, 9–11, and 7–9 g/L in bovine milk respectively; these proteins are composed of 199, 209, and 222 amino acid residues in their sequences and have the potential to release about 20,000 kinds of peptides (Maruyama et al., 1998). Another major group of proteins after

casein separation in milk is whey proteins, which consist of β-lactoglobulin and α-lactalbumin (Mullally et al., 1996). Previous research demonstrated that *in vitro* incubation of milk proteins with gastrointestinal proteases (pepsin, trypsin, and chymotrypsin) resulted in the release of ACE inhibitory peptides (Yamamoto and Takano, 1999); ACE inhibitory peptides derived from milk proteins and their respective IC_{50} values are illustrated in Table 1.4.

Casein is very susceptible to proteolytic digestion (Maruyama et al., 1998). Tryptic digests of caseins revealed some ACE-inhibitory peptides such as FFVAPFPEVFGK and TTMPLW from αs1-casein, and AVPYPQR from β-casein (Maruyama et al., 1998). Proteolytic digestion of αs1-casein produces the peptide FFVAP, which is more active than the peptide KVLPVPQ, which was derived from β-casein (Maruyama et al., 1998). Different bacterial enzymes were also studied to digest milk proteins to yield ACE-inhibitory peptides. But most of them are long peptides and have very mild activity. A potent ACE-inhibitory peptide, KVLPVP, was found from β-casein, which is composed of six amino-acids (Maeno et al., 1996). Studies of ACE-inhibitory peptides from whey proteins are limited because β-lactogloblin, one of the major components of whey, has a very rigid structure and resists digestive proteases. However, some ACE-inhibitory peptides produced from whey proteins by enzymatic hydrolysis were reported (Chiba and Yoshikawa, 1991; Yamamoto and Takano, 1999). Three ACE-inhibitory peptides were identified after enzymatic hydrolysis of α-lactogloblin (Mullally et al., 1996). Several ACE-inhibitory peptides were also identified from β-lactogloblin (Mullally et al., 1996; Mullally et al., 1997). Apart

from this, a peptide, ALKAWSVAR, was identified from bovine serum albumin and exhibited *in vitro* ACE-inhibitory activity (Mullally et al., 1997).

ACE-inhibitory peptides can be produced from milk proteins through the proteolytic activity of different bacterial species used in the production of fermented milk products such as yogurt and cheese (Maruyama et al., 1987). Two well-known ACE-inhibitory peptides were purified from Calpis sour milk (the commercial fermented milk from Japan), which was fermented by *Lactobacillus helveticus* and *Sccharomyces cerevisiae*. These peptides were from β-casein, IPP, and VPP (Nakamura et al., 1995). Some lactic acid bacteria showed good growth in milk because they have a particular proteolytic system which decomposes the casein and lactose-digestive enzymes (Yamamoto et al., 1994). Extracellular proteases purified from the bacterial cell wall of *Lactobaillus helveticus* CP790 can also degrade casein (αs1-casein, αs2-casein, and β-casein) and produce ACE-inhibitory peptides (Yamamoto et al., 1994). Milk fermented by *Enterococcus faecalis* can also produce some ACE-inhibitory peptides (LHLPLP, LVYPFPGPIPNSLPQNIPP, VLGPVRGPFP, and VRGPFPIIV) (Miguel et al., 2006a). Two ACE-inhibitory peptides (LQP and MAP) were isolated from enzyme-modified cheese (Tonouchi et al., 2008); eight-month-aged Gouda cheese also exhibited a depressive effect on systolic blood pressure in SHRs and potent ACE-inhibitory activity. Two potent peptides (RPKHPIKHQ and YPFPGPIPN) showed the highest activity (Saito et al., 2000). Peptides from different milk sources that showed very high potency in ACE inhibition have already been commercialized in the market (Erdmann et al., 2008).

Milk proteins are high in calcium content; it is well documented in the literature that sufficient intake of calcium offers benefits by lowering high blood pressure. Increased dietary calcium intake has been reported to correct the low serum ionized calcium concentration ([Ca++]) and to result in a significant amelioration of the prevailing hypertension (Stern et al., 1984). Therefore, this is another possible mechanism of blood pressure lowering activity reported in fermented milk products (Seppo et al., 2003).

1.4.2.4 Antihypertensive activity of peptides from eggs: Eggs are an excellent source of well-balanced nutrients. Although egg contains more than 40 different kinds of proteins, the ACE-inhibitory peptides reported were mostly derived from ovalbumin (López-Fandiño et al., 2007); ACE-inhibitory peptides derived from egg proteins are listed in Table 1.4.

The peptic hydrolysate of ovalbumin could produce many ACE-inhibitory peptides. Three potent ACE-inhibitory peptides, FFGRCVSP, ERKIKVYL, and FRAHPFL, were isolated from ovalbumin with IC_{50} values of 0.4, 1.2, and 3.2 μM respectively (Lopez-Fandino et al., 2007). Ovokinin (FRAHPFL) is one of the most intensively studied vasorelaxing peptides from ovalbumin (Fujita et al., 1995a). Ovokinin lowered the blood pressure in SHRs significantly when administered orally (Fujita et al., 1995b). Another peptide (RAHPFL), lacking the N-terminus Phe (F) residue of ovokinin, was isolated from ovalbumin pepsin hydrolysate (Miguel et al., 2004). Under i*n vitro* conditions, this peptide showed weaker ACE-inhibitory activity (IC_{50} = 6.2 μM) than that of ovokinin (IC_{50} = 3.2 μM), but exhibited significant antihypertensive activity in SHRs (Miguel et al.,

2005). This hexapeptide (RAHPFL) was also isolated from chymotryptic digestion of ovalbumin; it has been shown to induce nitric oxide-dependent vasorelaxation in an isolated mesenteric artery as well as an antihypertensive effect in SHRs (Matoba et al., 1999). Another promising peptide, YAEERYPIL, was identified from pepsin ovalbumin hydrolysate. This peptide showed significant antihypertensive activity in SHRs at a dosage rate of 2 mg/kg (Miguel et al., 2006b; Potter et al., 2009), but was totally degraded after simulated gastrointestinal digestion, and its ACE-inhibitory activity decreased; surprisingly, however, one of its main fragments, YPI, significantly decreased blood pressure in SHRs at a dosage rate of 2 mg/kg (Nakaoka et al., 1987).

1.5 QSAR AND BIOINFORMATICS – A USEFUL TOOL IN BIOACTIVE PEPTIDE STUDIES

The quantitative structure and activity relationship (QSAR) is a mathematical relationship between the biological activity of a molecular system and its geometric and chemical characteristics. QSAR explores the relationship between any chemical structure and its corresponding biological activities through the construction of mathematical models; thus, QSAR can be used to evaluate the physicochemical or biological properties of a series of compounds based on the following relationship:

$$\text{Activity or Property} = f\,(\text{Chemical structure})$$

QSAR approaches, based on different molecular descriptors, are of major importance for bioorganic and medicinal chemistry (Estrada and Uriarte, 2001). Molecular descriptors are numerical values that characterize the properties of a particular molecule. They are used to build the complete dataset for QSAR

analysis, such as *z*-scale scores for amino acids (Hellberg et al., 1987). The value of different molecular descriptors (such as: hydrophobicity, charge etc) can be quantified by this *z*-scale scores. Drug discoveries and drug designs are mainly based on two fundamentals: "Lead Discovery" and "Lead Optimization." Lead discovery is a process of identifying active new chemical entities that can be subsequently modified or transferred into a clinically useful drug. By contrast, lead optimization is the synthetic modification of a biologically-active compound that fulfills all physiochemical or toxicological requirements of clinical usefulness (Estrada and Uriarte, 2001). Lead optimization in drug designing has been successfully achieved through various QSAR models. Many QSAR models have been developed to predict biological activities such as anti-HIV activity, enzyme inhibition, and toxicity (Tsai et al., 2004; Ruiz et al., 2003; Serra et al., 2001). Among these, toxicity prediction is very useful for drug development and has been successfully used by pharmaceutical industries (Tsai et al., 2004). QSAR models have been extensively used in studies of compounds with anticancer, anticoccidial, antifungal, or even anti-RNA-virus activities (Humberto et al., 2006; Humberto et al., 2007; Frasncisco et al., 2009; Humberto et al., 2005).

Apart from medicinal chemistry, QSAR has also been used in peptide research from various food proteins. The breakdown of proteins into smaller peptides during gastrointestinal digestion or food processing often influences the quality of ripened dairy products, fish, meat, and fermented foods. The texture, aroma, and physical properties of a food are affected by different protelolytic products or peptides (Pripp et al., 2005a). Peptides contribute to varying degrees to the taste

of foods. The most popular examples from the literature are soy sauce, ripened cheese, and various meat products, from which different peptide fractions have been isolated (Pripp et al., 2005a). Detailed studies on sensory peptide structure have mainly focused on bitter peptides. Earlier structure and bitterness studies of bitter peptides showed that hydrophobicity, rather than any specific amino acid sequence, is one of the crucial factors in the bitterness of peptides (Ney, 1979). But later QSAR studies showed that the bitterness of various peptides depends on the specific structure of the peptides along with the hydrophobicity (Gulyaeva et al., 2003). Some later work has suggested that the zigzag peptide backbone is also important for bitterness (Kim and Li-Chan, 2006).

Milk proteins are one of the major sources of various bioactive peptides; the activities of prolyl oligopeptidase (POP)-inhibitory peptides were predicted by using QSAR models (Pripp, 2006). Lactoferrin is a well-known protein from milk that exerts antimicrobial activity. QSAR studies have been conducted on antimicrobial peptides derived from lactoferrin (Strøm et al., 2002). A series of lactoferricin-like peptides was synthesized from human, bovine, murine, and carpine lactoferrin (Strøm, 2001). The results showed that net charge is one of the important parameters affecting antimicrobial activities. Later, a new QSAR study was performed on an extended set of lactoferricin derivatives using physicochemical bulk peptide descriptors (Strøm et al., 2001). Results from this study showed that net charge and micelle affinity were the most important structural parameters affecting antibacterial activity. Therefore a new QSAR model was developed using *z*-scales of amino acids. This model was successfully

used for the prediction of antibacterial activity (Lejon et al., 2001). Further investigation demonstrated the importance of the helical region of the peptide for antimicrobial activity (Nakai et al., 2003). QSAR models were also used to predict the ACE-inhibitory activity of peptides. A synthetic ACE-inhibitory dipeptide was first developed through QSAR research (Cushman et al., 1980). Amino acid descriptors were later developed for QSAR modeling and used for peptide studies. Interpretation with these descriptors of the resulting QSAR models has revealed that a highly active peptide should have a large, hydrophobic amino acid, an aromatic amino acid, and a polar functional group (Kim, Chi, Yoon, & Sung, 1998). A comparative QSAR analysis was done with the ACE inhibitory peptides from milk proteins (Pripp et al., 2004). Recently, prediction of ACE inhibition was successfully achieved, and QSAR models have been developed to predict ACE inhibitory activity (IC_{50} value) for a wide range of different polypeptides (2–10 amino acid) from various food proteins (Wu et al., 2006a; Wu et al., 2006b). These studies revealed that the ACE-inhibitory activity of a peptide is dependent on amino acids in the N- or C-terminal. Amino acids with bulky side chains as well as hydrophobic side chains were preferred for dipeptides. For tripeptides and aromatic amino acids at the C-terminal, charged amino acids in the middle and hydrophobic amino acids at the N-terminal are preferred. The resulting QSAR model explained that a highly active ACE-inhibitory peptide should have with a large, hydrophobic, aromatic amino acid with a polar functional group in the C-terminal position (Wu et al., 2006a).

Bioinformatics is a new tool of modern biological study. The protein sequences of many organisms have been described and their identities have already been submitted to public databases. Now that these protein sequences are freely available, it has become feasible to analyze—for various purposes and through different computational methods—the vast information on proteins (Dzuiba et al., 2004). This analysis gives us an idea of the nature or behavior of particular proteins. Thus, bioinformatics is often defined as the application of computational techniques to understand and organize the information associated with biological macromolecules. Bioinformatics is especially suitable in the determination of the structure-function relationship of proteins, in the identification of protein domains, and in the computer simulation of proteolytic processes (Dzuiba et al., 2004). Through the bioinformatics approach, various plant proteins have already been predicted as potent precursors of bioactive peptides (Dzuiba et al., 2004). G-protein coupled receptors (GPCR) are one of the important targets of drug development. Some potent biologically active peptides were studied for GPCR deorphanization through the bioinformatics approach (Jean et al., 2007). Proteolysis is an important step in the production of biologically active peptides. Hence, computer-simulated proteolysis, or *in silico* proteolysis, is one of the major requirements in this process**.** PeptideCutter, provided by the Expasy molecular biology server is such a computational tool. PeptideCutter takes a protein sequence from the SWISS-PROT and/or TrEMBL databases or any specific protein sequence defined by a user. It provides a selection of, or the whole list of, the different proteases and chemicals that can be used. The

algorithm was developed according to the specificity or cleavage site of the available enzymes (Keil, 1992).

1.6 EGG PROTEINS DERIVED PEPTIDES WITH ANTIHYPERTENSIVE ACTIVITY

In light of the adverse effects of pharmacological ACE inhibitors, the development of bioactive peptides from food proteins is a major concern. Extensive research has been done on milk proteins, but another "complete food of nature," the egg, has not been investigated completely. Eggs have more than 40 different kinds of proteins, with five major proteins (ovalbumin, ovotransferrin, ovomucin, ovomucoid, and lysozyme) in egg white and four major proteins (low density lipoprotein, high density lipoprotein, phosvitin, and livitin) in egg yolk. Most ACE-inhibitory peptide studies have been done with ovalbumin, so the lion's share of the egg proteins have not been studied yet.

Furthermore, the traditional approach to bioactive peptide research has been based on the concept of investigating one protein and one enzyme at a time. This conventional peptide discovery strategy generally requires multistep purifications to generate a single peptide. Therefore, to search for the most potent ACE-inhibitory peptides from egg proteins it would take years by the conventional hydrolysis-purification-characterization method, which is both labor-intensive and costly. Moreover, the traditional activity-guided purification method may not be able to produce all of the potent bioactive peptides from the hydrolysate. Many types of bioactive peptides have been reported in the literature; however, the rationale behind the choice of one particular condition (a particular enzyme or a particular protein) over the others has not been well justified in the previous

literatures. Therefore, there is a need to establish an innovative strategy to explore bioactive peptides to overcome the shortcomings of the conventional approach. The overall objective of the present study was to develop an innovative strategy to develop the most potent ACE-inhibitory peptides from egg proteins. The specific objectives of this research were:

- To predict the most potent ACE-inhibitory peptides from egg proteins through an integrated QSAR and bioinformatics approach;
- To release the predicted potent ACE-inhibitory peptides from egg protein hydrolysates under the enzymatic conditions predicted;
- To purify the ACE-inhibitory peptides identified in egg protein hydrolysate by multistep chromatographic separations;
- To characterize the stability of ACE-inhibitory peptides in simulated gastrointestinal digestion and to study the kinetics of ACE in the presence of inhibitory peptides.

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Table 1.1: Some examples of different bioactive peptides from various food proteins.

Activity	Origin	Encrypting protein	Peptide name/ sequence
Antimicrobial	Milk	Lactoferrin	Lactoferricin
		α, β -Casein	Casecidins
	Egg	Lysozyme	Not specified
		Ovotransferrin	OTAP-92
Antiangiogenic	Egg	Cystatin	Not specified
Immunomodulatory	Milk	α, β -Casein	Immuno-peptides
		α–LA	TTMPLW
	Rice	Rice albumin	GYPMYPLR
	Egg	Ovalbumin	Not specified
	Wheat	Wheat glutein	Immuno-peptides
Opioid	Milk	α, β -Casein	Casomorphins
		α–LA	α–Lactoferroxins
Antioxidant	Milk	α–LA, β–LG	MHIRL, YVEEL
	Wheat	Wheat germ protein	Not specified
	Fish	Sardine muscle	MY
ACE inhibitory			
	Egg	Ovalbumin	FRADHPPL
		Ovotransferrin	KVREGTTY
	Milk	α–LA, β–LG	Lactokinins
	Fish	Fish muscle	LKP,IKP
	Soybean	Soy protein	NWGPLV

* Modified from: Hartmann and Meisel, 2007

Table 1.2: Some examples of ACE-inhibitory peptides derived from plant sources

Origin	Sequence	IC_{50} (μM)
Rice	TQVY	18200
Barley	EVSLNSGYY	23
Wheat	IAP	2.7
Sesame	LVY	1.80
	LSA	7.81
	LQP	1.04
	LKY	0.78
	IVY	14.74
	VIY	4.50
	MLPAY	1.58
Maize	LRP	0.27
	LSP	1.7
	LQP	1.9
	VHLPP	18
	LPP	9.6
	SAYPGEITN	7.0
Spinach Rubisco	MRWRD	2.1
	MRW	0.6
	LRIPVA	0.38
	IAYKPAG	4.2
Wakame	VY	35.2
	IY	6.1
	AW	18.8
	FY	42.3
	VW	3.3
	IW	1.5
Sweet potato	TYCQ	2.3
Soybean	HHL	5.4
	PGTAVFK	26.5
	VLIVP	1.69
	NWGPLV	21
Pea	LRW	0.15
Mung bean	KDYRL	26.5
	VTPALR	82.4
	KLPAGTLF	13.4

Table 1.3: Some examples of ACE-inhibitory peptides derived from animal sources

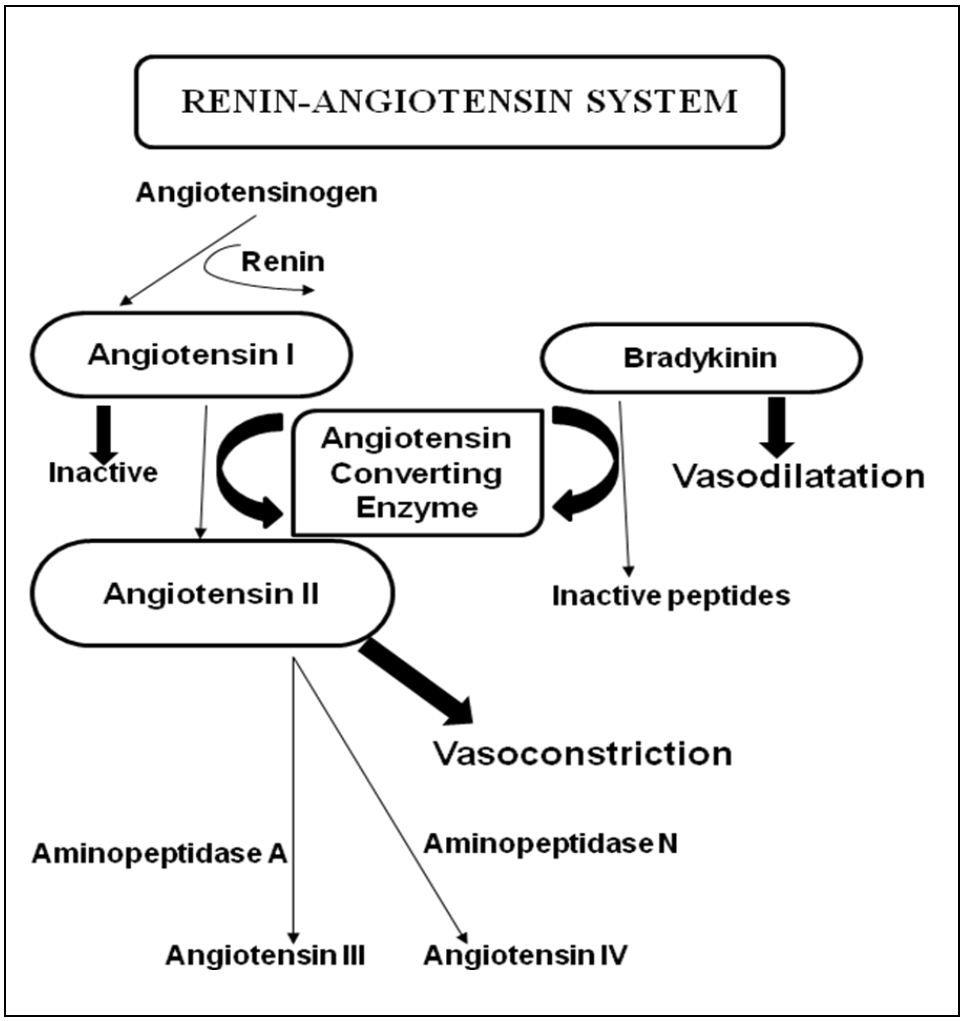
Origin	Sequence	IC_{50} (μM)
	Vertebrates	
Pork	ITTNP	549.0
	MNPPK	945.5
	MNP	66.6
	NPP	290.5
	ITT	678.2
	TTN	672.7
	TNP	207.4
Chicken	LKA	8.5
	LAP	3.2
	FQKPKR	14
	FKGRYYP	0.55
	IKW	0.21
Bonito	IKPLNY	43
	IVGRPRHQG	2.4
	IWHHT	5.8
	ALPHA	10
	FQP	12
	LKPNM	2.4
	IY	2.31
	DYGLYP	62
	IWH	3.5
	IKP	6.9
Salmon	WA	277.3
	VW	2.5
	WM	96.6
	MW	9.9
	IW	4.7
	LW	17.4
Sardine	MF	44.7
	RY	51
	MY	193
	LY	38.5
	YL	82
	VY	10
	GRP	20.1
	KY	1.63
	GWAP	3.86
Tuna	IY	3.7
	FQP	12
	ALPHA	10
Alaska pollack	GPL	2.65

	GPM	17.13
	Invertebrates	
Antartic krill	KLKFV	30
Oyster	LF	126
	VVYPWTQRF	66
Fresh-water calm	VKP	3.7
Hard calm	YN	51

Table 1.4: Some examples of ACE-inhibitory peptides derived from milk and egg

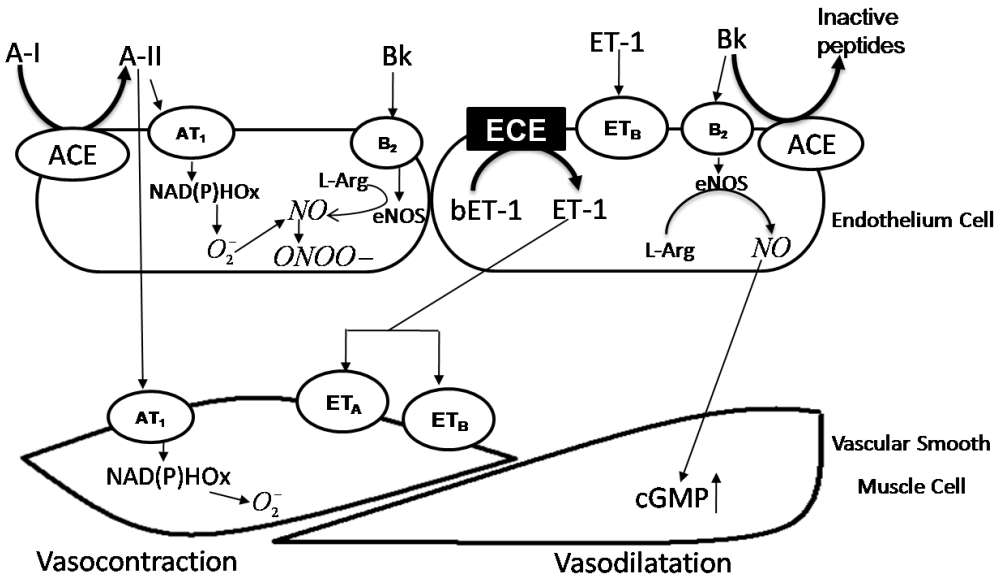
Origin	Protein	Sequence	IC_{50} (μM)
Milk	α_s1- casein	AYEYPE	106.0
		RPKHPIKHQ	13.1
		FFVAPFPEVFGK	77.2
		YKVPQL	22.2
		YP	720.1
		TTMPLW	16.3
		FFVAP	6.1
		TTMPLW	16.2
		PLW	36.01
		VAP	2.0
		FVAP	10.0
	α_s2-casein	AMPKPW	580.0
		MKPWIQPK	300.21
		TKVIP	399.95
	β-casein	KVLPVP	4.6
		VYP	288.12
		VYPFPG	221.0
		YPFPGPIPN	15.1
		IPP	5.0
		TPVVVPPFLQP	749.12
		VPP	9.0
		LQSW	500.1
		KVLPVP	5.0
		AVPYPQR	15.1
		IYPFVEPI	8.2
		LIYPFVEPIP	8.91
		TTMPLW	16
		PIPY	30.0
		YKVPQL	22
Egg	Ovalbumin	LW	6.8
		FRADHPFL	3.2
		RADHPFL	6.2
		FFGRCVSP	0.4
		FGRCVSP	6.2
		ERKIKVYL	1.2
		YAEERYPIL	4.7
	Ovotransferrin	KVREGT	9.1

Figure 1.1: Regulation of blood pressure via Renin-Angiotensin system



***** Modified from FitzGerald et al., 2004.

Figure 1.2: *In-vivo* mechanism of vasocontraction and vasodilatation



Mechanism of action of vasocontraction and vasodilatation : Angiotensin Converting Enzyme (ACE) converts angiotensin I (A-I) to angiotensin II (A -II) , A-II binds with angiotensin 1 receptor (AT_1) on enothelium cells as well as vascular smooth muscle cells, then A-II activates NAD(P)HOx and produces (O_2 -) which exerts vasocontraction. ACE also converts Bradykinin (Bk) to inactive peptides. Bk binds with bradykinin receptor (B_2) and activates nitric oxide synthase (eNOS), which converts L-Arg to nitric oxide (NO). NO exerts vasodilating effects on smooth muscle cell by activating cyclic guanosine monophosphate (cGMP). In endothelium cells A-II produces free radical (O_2 -) which scavenge NO and produces peroxynitrite (ONOO -), exerts vasocontracting effect by limiting the supply of NO to the vascular smooth muscle cells. Endothelin-Converting Enzyme (ECE) converts big-endothelium (bET-1) to endothelin-1 (ET-1). ET-1 exerts major vascular effects – vasocontriction and cell proliferation – through activation of specific receptor, enothelin receptor-A (ET_A) on vascular smooth muscle cells. In contrast, enothelin receptor-B (ET_B) mediate vasodilatory effects via release of NO, additionally it also removes ET-1 from plasma.

* Modified from Sundano et al., 2006 and Zaman et al., 2002.

CHAPTER-2 AN INTEGRATED QSAR AND BIOINFORMATICS APPROACH FOR NEW ANTI-HYPERTENSIVE PEPTIDES DISCOVERY

2.1 INTRODUCTION:

Cardiovascular disease is the leading cause of death in many developed countries. As one of the well-defined major risks for cardiovascular disease, hypertension afflicts 28.7% population in the USA, 22% in Canada and 44.2% in Europe (Kearney et al., 2004). Uncontrolled hypertension would likely lead to death. As a life-style related disease, many factors could affect the onset and development of hypertension. Currently, food protein-derived angiotensin converting enzyme (ACE) inhibitory peptides have been extensively studied due to their potential roles in blood pressure reduction and several of them have been commercialized for the prevention and management of hypertension (Hata et al., 1996).

ACE (dipeptidyl carboxypeptidase, EC 3.4.15.1) is a zinc metallopeptidase that plays a central role in blood pressure regulation through mainly the rennin-angiotensin system (Laragh et al., 1972). ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor; ACE also inactivates bradykinin, a potent vasodilator. Thus inhibition of ACE may lead to blood pressure reduction and an ACE inhibitory drug is the first line therapy for hypertension (Gravas and Gravas, 1999).

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Although synthesized antihypertensive drugs targeting inhibition of ACE have been proven successful in lowering high blood pressure, synthetic medications for hypertension are believed to be associated with certain adverse side effects such as cough and angioedema (Beltrami and Zimgale, 2006). The incidence of cough has been estimated at 5–20% of patients and may result in the discontinuation of treatment; angioedema affects 0.1–0.5% of patients, but can be life-threatening. Therefore, development of ACE inhibitory peptides from food proteins is under extensive research as an alternative for the prevention and treatment of hypertension (Fitzgerald et al., 2004, Miguel and López-Fandiño, 2005).

Many ACE inhibitory peptides have been found in various protein sources including egg proteins (Fitzgerald et al., 2004, Miguel and López-Fandiño, 2005, Miguel and Aleixandre, 2006). Ovokinin, an octapeptide FRAHPFL, was isolated from the peptic digestion of ovalbumin (Miguel and Aleixandre, 2006). Another hexapeptide corresponding to the 2-7 fragment of ovokinin was identified from the chymotryptic digest of ovalbumin (Fujita et al., 1995). Other egg protein-derived ACE inhibitory peptides are ESIINF, YRGGLEPINF, YAEERYPIL (Miguel et al., 2007, Miguel et al., 2007), and LVREGT (Lee et al., 2006). Several new peptides were recently identified in cooked eggs under simulated gastrointestinal digestion condition (Majumder et al., 2009). Despite the fact that an increasing number of new peptides were continuously reported in literature, the rationale behind the selection of the condition such as the choice of one enzyme over the others as well as one protein substrate over the others has not been well justified in literature. Furthermore, the current approach to bioactive peptide

research was built upon the concept of investigating one protein and one enzyme at a time and has long since reached its throughput limits. The conventional peptide discovery strategy generally requires multi-step purifications (which often take months) to generate a new sequence. For example, egg contains over 40 different kinds of proteins (Karlheinz, 2007; therefore, it would take years to extensively study bioactive peptides within egg proteins by the conventional hydrolysis-purification-characterization protocol, which is labour-intensive and costly. Moreover, the conventional activity-based purification steps might exclude potent but minor peptides in the hydrolysate. Therefore, there is a need to explore a new approach of peptide discovery.

Quantitative structure activity relationship (QSAR) studies the relationship between biological activity and chemical structure (Lin et al., 2008); the basic principle behind QSAR modeling is that the activity or a function of a specific chemical can be described by its molecular or physicochemical descriptors, e.g., electronic attributes, hydrophobicity, steric properties etc (Hansch et al., 1995). The discovery of many potent ACE inhibitory peptides from food proteins has greatly advanced our understanding of the structure and activity relationships of peptides. Our recently developed peptide structure and activity models have enriched the knowledge of the structure and activity relationship of bioactive peptides (Wu et al, 2006a, Wu et al., 2006b). It should be noted that the availability of massive protein sequences has prompted several studies to search for bioactive peptides in proteins using exact sequence match; however, methods using exact sequence match lack the ability to predict new peptides with improved

activities (Dziuba and Iwaniak, 2006). Our established QSAR models, on the other hand, have shown preliminary potential to predict new peptides with improved activity (Wu et al, 2006a, Wu et al., 2006b). Therefore, the objective of the study was to explore a new method of peptide discovery through an integrated QSAR and bioinformatics approach.

2.2 MATERIAL AND METHODS:

2.2.1 Materials

Egg ovotransferrin (Ovotransferrin 100) was gifted from Neova Technologies **(**Abbotsford, BC**,** Canada**).** Angiotensin converting enzyme (ACE, from rabbit lung), pepsin (porcrine gastric mucosa), thermolysin (*Bacillus thermoproteolyticus rokko*) and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma (Oakville, ON, Canada). All other chemicals were analytical grades.

2.2.2 In- silico proteolysis and ACE inhibitory activity prediction

Major egg white (ovalbumin, ovotransferrin, ovomucin, ovomucoid, lysozyme, avidin) and major egg yolk protein sequences (low density lipoprotein, high density lipoprotein, phosvitin) were obtained from the National Center for Biotechnology Information (NCBI) protein database (http://www.ncbi.nlm.nih.gov). *In silico* proteolysis was performed with the software Peptidecutter (http://www.expasy.ch/tools/peptidecutter/) available at ExPASy Molecular Biology Server using trypsin, thermolysin, pepsin and chymotrypsin individually or in combination. The IC_{50} values (defined as the concentration of the peptides that can inhibit 50% of the enzyme activity sample) of

the peptides were predicted by QSAR models using the SIMCA-P version +11 (Umetrics INC., Kinnelon, NJ) (Wu et al., 2006a, Wu et al., 2006b).

2.2.3 Ovotransferrin hydrolysis

All the digestions were carried out in a jacket beaker coupled with a water bath for temperature control and Ttitrando (Metrohm, Herisan, Switzerland) for pH control during the course of the hydrolysis. Aqueous solution (5%; w/v) of ovotransferrin was prepared; after heating the sample solution at 80^{o}C for 15 min with continuous shaking, the temperature and pH was adjusted to 55^{o}C and 8, respectively. The sample solution was first digested by thermolysin at constant temperature of 55^{o}C and pH of 8 for 3 h, and then the pH of the solution was adjusted to 2 to terminate the reaction by adding 1N HCl. After the temperature was adjusted to 37^{o}C, pepsin was added for another 3 h. The hydrolysis was terminated by raising the temperature to 95^{o}C and maintaining it for 15 min. The digest was subjected to centrifugation at 10,000 x g for 25 min (4°C) and the supernatant was obtained and freeze-dried for further analysis. Hydrolysates from thermolysin or pepsin were also prepared individually.

For sonication and reducing agent treatments, ovotransferrin sample solution (5%, w/v) was subjected to sonication in 4 pulses (60 Hz for 30 seconds for each pulse) using sonic 300 (ARTEK Systems Corporation, Farmingdale, NY, USA), or treated in the presence of β-mercaptoethanol (1:100, w/w, Ovotransferrin /β-mercaptoethanol) for 30 min at room temperature (22^{o}C) or 5 mM dithiothreitol (DTT) at 50^{o}C for 30 min with continuous stirring. β-mercaptoethanol was removed by raising the temperature 50^{o}C for 30 min with continuous stirring in the

hood while DTT was removed by extensively dialysis using 12 KDa membranes (Spectrum Laboratories, Rancho Dominguez, CA, USA). Hydrolysis was carried out as above after the treatments. All samples were prepared in duplicate.

2.2.4 ACE inhibitory activity

ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971) with slight modifications as reported (Majumder et al., 2009). The IC_{50} value was defined as the concentration of the peptides that can inhibit 50% of the enzyme activity. Each assay was performed in duplicate of five different concentrations of a given sample.

2.2.5 Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Identification of the predicted potent peptides in the hydrolysate was carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS). The analysis was carried out in Waters (Micromass) Q-TOF Premier (Milford, MA, USA) and sample was separated by Waters Atlantis dC18 (75 μm x 150 mm, 3μm) UPLC column (Milford, MA, USA). The dual solvent system is composed of solvent A as 0.1% formic acid in optima LC/MS grade water and solvent B as 0.1% formic acid in optima grade acetonitrile. Samples were prepared in solvent A; then 5 μL of sample was injected to the 5 μm trapping column. Sample was trapped for 2 min at a flow rate of 10 μL/min using 99% solvent A, followed by a gradient from 99% A to 90% A over 5 min, to 70% A over 30 min, to 60% A over 3 min and 5% A over 1 min at a constant flow rate of 0.350 μL/min. Then the flow rate was increased to 0.500 μL/min, hold at 5% A for 2 min, increased it to 98% A over 1

min, hold for another 27 min, and then decreased the flow rate to 0.350 μL/min over 1 min. Ionization was performed by electrospray ionization technique (ESI) by NanoLockspray ionization source in an appositive ion mode (capillary voltage at 3.80 kV and the source temperature at 100^{o}C). Peptide mass was detected through Q-TOF analyzer operated in a positive ion MS/MS mode. A MS/MS full-scan was performed for each sample with an acquisition m/z range of 0-1000 Da. The peptides were then characterised by using Peaks Viewer 4.5 software (Bioinformatics Solutions Inc., Waterloo, ON Canada) and the peptide sequences were identified from respective monoisotopic mass.

2.2.6 Determination of amino nitrogen

The amino nitrogen content of hydrolysed samples was measured using the ninhydrin method (ASBC, 1992). The samples were extracted by100 mM sodium phosphate at pH 8 at a ratio of 1:10 (w/v) for 1 h at room temperature and then centrifuged at 10000 × *g* for 10 min. 200 μL of supernatant was mixed with 100 μL of ninhydrin color reagent (Sigma, Oakville, Canada), and 0.3 g of fructose in 100 mL of distilled water, pH 6.7] and heated in a boiling water bath for 16 min. After cooling for 20 min at room temperature, 500 μL of the dilution solution (0.2% KIO3 in 40% ethanol) was added and the absorbance at 570 nm was measured. Glycine was used as a standard. Duplicate analyses were conducted for each sample.

2.2.7 Measurement of reactive and total sulfhydryl (SH) groups

Measurement of sulfhydryl groups was performed by the Ellman's reagent method (22). To 0.5 mL of treated protein samples, 4.5 mL of 0.1M Tris-glycine

buffer, pH 8.0 containing 0.01M EDTA (for reactive SH groups) was added and mixed. After incubation at 40^{o}C for 30 min, 125 μL of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) solution (4mg/mL) was added, mixed and then incubated for another 30 min at 20^{o}C. The absorbance at 412 nm was recorded and the content of sulfhydryl group was calculated as following:

$$\mu M\ SH/g = 73.53^{*}\ A_{412}{}^{*}(D/C)$$

where A_{412} is the absorbance at 412 nm, C is the sample concentration in mg/mL, and D is the dilution factor.

2.2.8 Statistical analysis

All statistical analysis was performed by one-way analysis of variance (ANOVA) using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC). The significant differences were determined using Duncan's multiple range test at $p < 0.05$ (Duncan et al., 1995).

2.3 RESULTS AND DISCUSSION:

2.3.1 ACE inhibitory peptides from in-silico digestion

Major egg proteins, such as ovalbumin, ovotransferrin, ovomucin, lysozyme, low-density lipoprotein, high-density lipoprotein and phosvitin, were subjected to various *in silico* digestions. More than 20,000 peptides were generated from a total of 75 *in silico* hydrolysates (Table 2.1). The IC_{50} value of the peptides was predicted by the QSAR models; of three most potent peptides, IRW (f7-9), LKP (f74-76) and IQW (f345-347) with predicted IC_{50} values of 0.59 μM, 1.4 μM and 2.8 μM, respectively, were identified in the thermolysin-pepsin hydrolysate of ovotransferrin. Therefore, ovotransferrin was decided as the most suitable protein

substrate and thermolysin-pepsin was the appropriate enzyme combination for the production of the potent peptides. The following procedure was to demonstrate if the predicted peptides could be released from the predicted condition established above.

2.3.2 Identification of predicted peptides in ovotransferrin hydrolysate

Ovotransferrin hydrolysate was prepared by thermolysin followed by pepsin; thermolysin and pepsin ovotransferrin hydrolysates were also prepared individually for comparison. As shown in Table 2.2, ACE inhibitory activity of the pepsin hydrolysate showed the weakest activity among the three hydrolysates but there was no significant difference between the thermolysin hydrolysate and the thermolysin-pepsin hydrolysate. The thermolysin-pepsin hydrolysate was then subjected to LC-MS/MS analysis to identify the potent peptide as found in the *in silico* digestion. As shown in Figure 2.1, two pentapeptides (IRWCT and IQWCA) and one quatrapeptide (LKPI), containing the sequences of the predicted peptides, were identified in the thermolysin-pepsin hydrolysate. It is known that thermolysin preferentially cleaves sites with bulky and aromatic residues (Ile, Leu, Val, Phe) at position P1'. P1' is a subsite nomenclature of enzymatic cleavage model created by Schechter and Berger (Schechter and Berger, 1967, Schechter and Berger, 1967) corresponding to the first C-terminal amino acid residue at the cleaved site. P1, correspondingly, is the first N-terminal amino acid residue at the cleaved site. Cleavage of thermolysin is favored with aromatic sites in position P1 but hindered in the presence of acidic residues in position P1 (Keil, 1992). IRW's neighboring amino acid sequence is shown as V_6IRWCTI_{12}; the bonds between

VI and TI are cleaved by thermolysin resulting in a peptide of IRWCT. The pepsin is expected to further cleave at the site of WC to release the predicted tripeptide of IRW but is hindered due to the presence of a disulfide bond formed between C_{10} and C_{45}. Therefore, our result showed that the predicted peptide did not present in the hydrolysate. Similar reason was applied to the unsuccessfully release of IQW. However, the reason behind the unsuccessfully liberation of LKP may be due to the fact that LKP is embedded in several disulphide bond-formed canopy which may block the accessible site of enzyme as shown in the 3-D structure of ovotransferrin (Figure 2.3); although it contains no cysteine (C) amino acid residue at its neighboring sequence as of $Y_{72}KLKPI_{77}$, LKP is embedded in the canopy formed by neighboring disulphide bonds of C_{10}-C_{45}, C_{20}-C_{36} and C_{115}-C_{197} (27). The bond between KL may be cleaved by either thermolysin or pepsin; however, the bond between PI is preferably cleaved by thermolysin. However, the presence of the canopy conformation formed by the disulfide bonds thus hinders the cleavage of thermolysin and therefore results in a quatrapeptide (LKPI).

It is known that ovotransferrin is a globular glycoprotein constituting of 686 amino acid residues with 15 disulphide bonds (Williams et al., 1982). It is very likely that the complexity of the rigid protein structure may hinder the enzymic access thus rendering unsuccessfully the predicted peptides. Our previous study showed that its proteolysis of ovotransferrin could be improved in *L. sanfranciscensis* sourdoughs due to a reduction of disulfide bonds (Loponen et al., 2008). It was also reported that ovotransferrin is highly sensitive to reducing

agents and undergoes autolytic cleavage under reductive conditions (Ibrahim et al., 2006). Therefore, methods that can disrupt the disulphide bonds in ovotransferrin may facilitate the release of the predicted peptides.

2.3.3 Effect of sonication and reducing agent treatments on the production of ACE inhibitory peptides

It was reported that sonication of food proteins could disrupt the protein structure (Jambark et al., 2009, Guzey et al., 2009). Ovotransferrin was pre-treated by sonication and two common reducing agents of β-mercaptoethanol and DTT, respectively, prior to hydrolysis. As showed in Table 2.3, the activities of sonication and DTT treated samples were enhanced over 20 times compared to the un-treated sample; the activity of β-mercaptoethanol treated sample showed less improvement compared to sonication and DTT treated samples due probably to an insufficient breakdown of disulfide bonds at a lower concentration and lower reaction temperature applied. Sonication-treated sample was subjected to LC-MS/MS analysis; as shown in Figure 2.2, all predicted peptides were identified and present in the sonication-treated hydrolysate. Although heating treatment is routinely employed prior to enzymatic hydrolysis as a means to improve the efficiency of hydrolysis, it seems that there are cleaved sites that are blocked even after the heat treatment mainly due to a high content of disulfide bonds in ovotransferrin. Our results showed that it is necessary to breakdown the disulphide bonds to allow release the predicted peptides from the parent protein.

To demonstrate whether the above treatments could affect the content of disulphide bonds in the samples, the content of the reactive SH groups was analyzed. A significant increase in the content of the reactive SH groups were

observed in the treated samples compared to the untreated sample (Figure 2.4a), which indicated that sonication or reducing agent treatments could breakdown the disulphide bonds of the proteins which was in a good agreement with our observation that the predicted peptides could only be released after breakdown of disulfide bonds in ovotransferrin. Free amino nitrogen content of a hydrolysate was used to estimate the amount of peptides produced from protein hydrolysis (Vaughan et al., 1962); our results showed that the amino nitrogen contents was increased significantly after the treatments (Figure 2.4b) which indicated an improvement of hydrolysis under the treatments.

The 3-D structure of ovotransferrin was analyzed by a molecular visualization program, Rasmol (Herbert J. Bernstein, NY, USA), to identify the specific position and the structure of the peptides. Rasmol competent Protein Data Bank (PDB; Accession code: 1OVT) file was chosen for the analysis (Kurokawa et al., 1995). Within the structure of ovotransferrin, we identified the position of predicted peptides and also visualized the locations of the disulphide bonds (Figure 2.3). Peptides IRW and LKP belong to the N-lobe while IQW belongs to the C-lobe. The C-terminals of both peptides (IRW and IQW) were adjacent to the cysteine residues to form the disulphide bonds whereas LKP was embedded within the complex canopy structure of N terminal of the whole protein. It was also observed that all these three peptides were a part of beta (β)-sheet structure of the parent protein; disulfide bond is known to play an important role in stabilizing β-sheet structure of proteins (Creighton, 1984).

Several egg protein ACE inhibitory peptides have been reported in literature; for example, FFGVRCVSP (0.4 μM), ERKIKVYL (1.2 μM), LW (6.8 μM), FRAHPFL (3.2 μM) were obtained from peptic digest of ovalbumin (Miguel and Aleixandre, 2006), and KVREET (9.1 μM) from chymotryptic digestion of ovotransferrin (Lee et al, 2006). In addition to LW, all reported egg protein ACE inhibitory peptides are relatively large peptides; it is known that short-chain peptides, especially di- and tri-peptides have a higher efficiency of absorption into the blood circulatory system that those of large ones (Mathews and Abidi, 1976). Therefore, small peptides as we reported may have higher bioavailability then larger peptides, although *in vivo* study is required to confirm this assumption. Both IRW and LKP contain aromatic and branched chain amino acid at their carboxyl terminals, positively charged amino acids at middle and hydrophobic amino acids at amino terminals, which are in a good alignment with our previously reported potent ACE inhibitory peptide structural requirements (Wu et al, 2006a); IQW, differing only the middle amino acid from IRW, on the other hand, showed 5 times weaker activity than that of IRW due to the lack of a positively charged amino acid residue in the middle position of the peptide which reinforcing the significance of the positively charged amino acid residue in determining the potency of peptides.

In conclusion, for the first time our study showed that potent bioactive peptides could be predicted through an integrated QSAR and bioinformatics approach. The development of this new approach of discovery of novel bioactive peptides could greatly facilitate the course of discovery new peptides from various food proteins.

Although we focus on ACE inhibitory peptides from egg protein in the study, the concept developed in the study can be applied to a wide range of bioactive peptide study at large.

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Table 2.1: Total number of peptides produced after in-silico digestion

Proteins	Di-peptides	Tri - peptides	Quatra-peptids	Penta-peptides	Hexa-peptides	Hepta-peptides	Octa-peptides	Ennea-peptides	Deca-peptides
Egg White	4334	3411	2052	1482	1106	868	679	507	388
Egg Yolk	1881	1505	900	616	488	391	288	219	190

Table 2.2: ACE inhibitory activity of the ovotransferrin hydrolysate without reducing agent treatment, different letters (a, b, c) defines the significant difference within different type of ovotransferrin hydrolysates ($p < 0.05$).

Enzymes Used	**IC_{50} (μg/ml)**
Thermolysin	201.6 ± 3.81^{b}
Pepsin	320.1 ± 5.06^{a}
Both enzymes (Thermolysin + Pepsin)	198.0 ± 1.21^{b}

* The statistical analysis was done by one-way analysis of variance (ANOVA) and the significant differences were determined using Duncan's multiple range test.

Table 2.3: ACE inhibitory activity of the ovotransferrin hydrolysate after treated with different reducing agents; different letters (a, b, c) defines the significant difference within different type of ovotransferrin hydrolysates ($p < 0.05$).

Ovotransferrin hydrolysate	**IC_{50} (μg/ml)**
Control	198.0 ± 1.21^{a}
Sonication	9.2 ± 3.11^{c}
2-ME	45.6 ± 8.41^{b}
DTT	10.7 ± 2.62^{c}

* The statistical analysis was done by one-way analysis of variance (ANOVA) and the significant differences were determined using Duncan's multiple range test.

*2-ME - β-Mercaptoethanol, DTT – Dithiothreitol

Figure 2.1: LC-MS spectra of hydrolyzed ovotransferrin without reducing agent treatments (I). Parts (II, III and IV) illustrated the LC-MS/MS spectra of the predicted peptide conjugate with other amino acid.

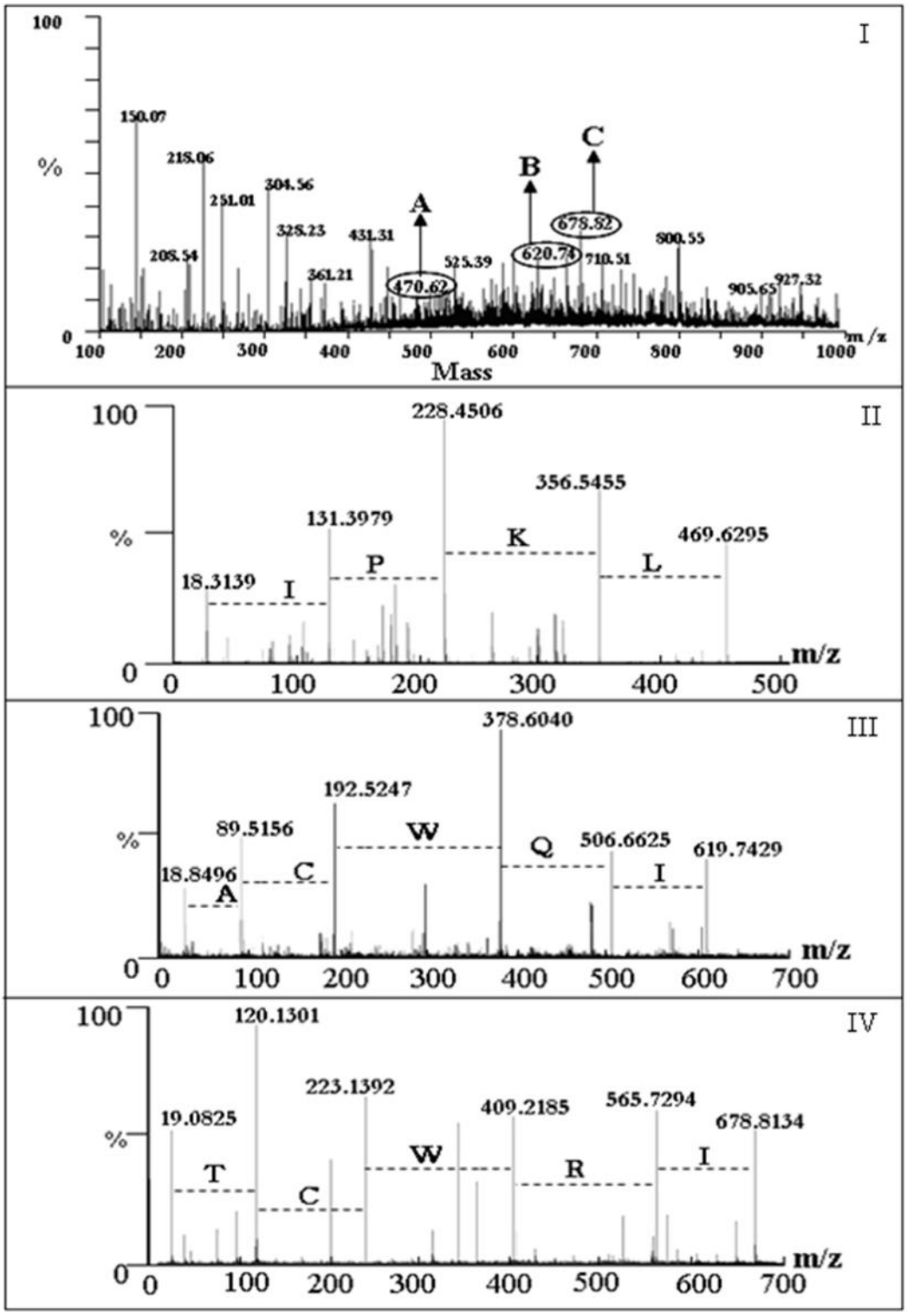


Figure 2.2: LC-MS spectra of hydrolyzed ovotransferrin after sonication treatment (I). Parts (II, III and IV) illustrated the sequence of three peptides LKP (A), IQW (B) and IRW (C) using their MS/MS spectrum by mono-isotopic mass of the amino acids.

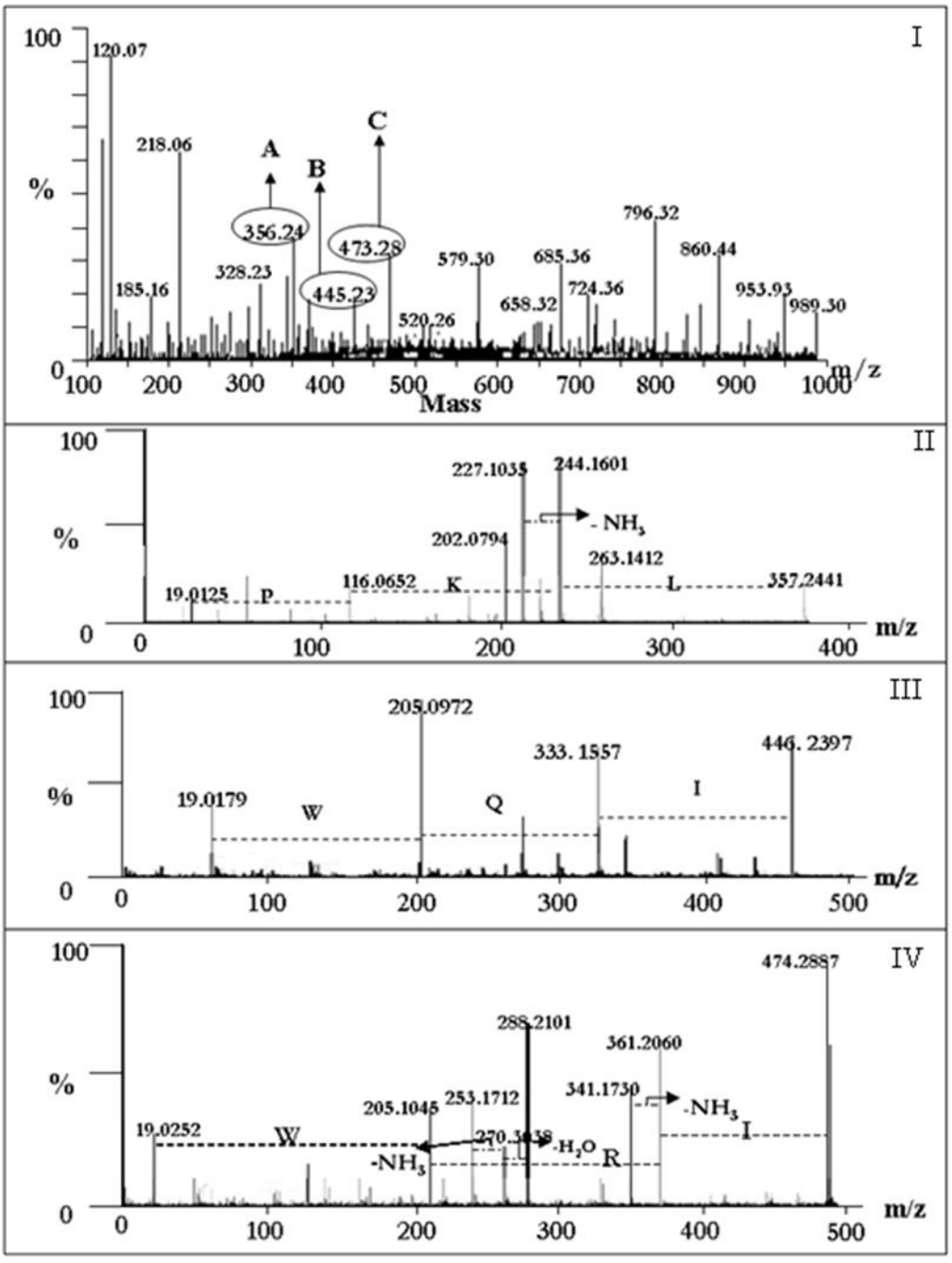


Figure 2.3: 3-D structure of ovotransferrin, golden part represents the N-lobe and green part represents C-lobe. Positions of potent peptides IRW, LKP and IQW were marked by blue, red and violet respectively. Corresponding cysteine residues and di-sulphide bonds were marked in black.

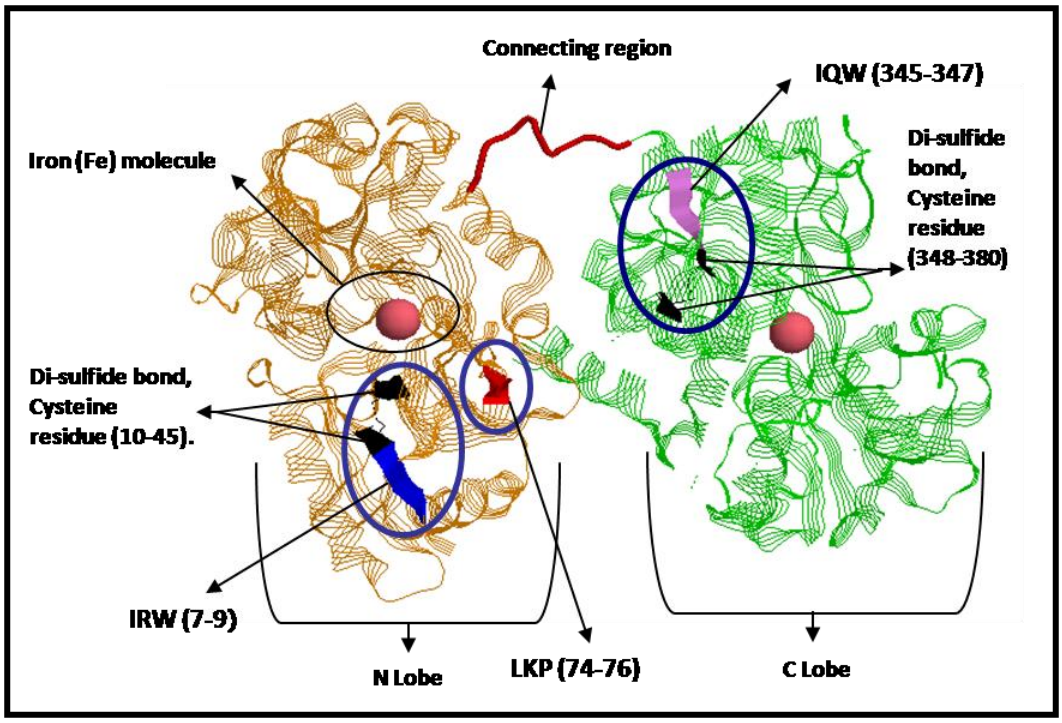
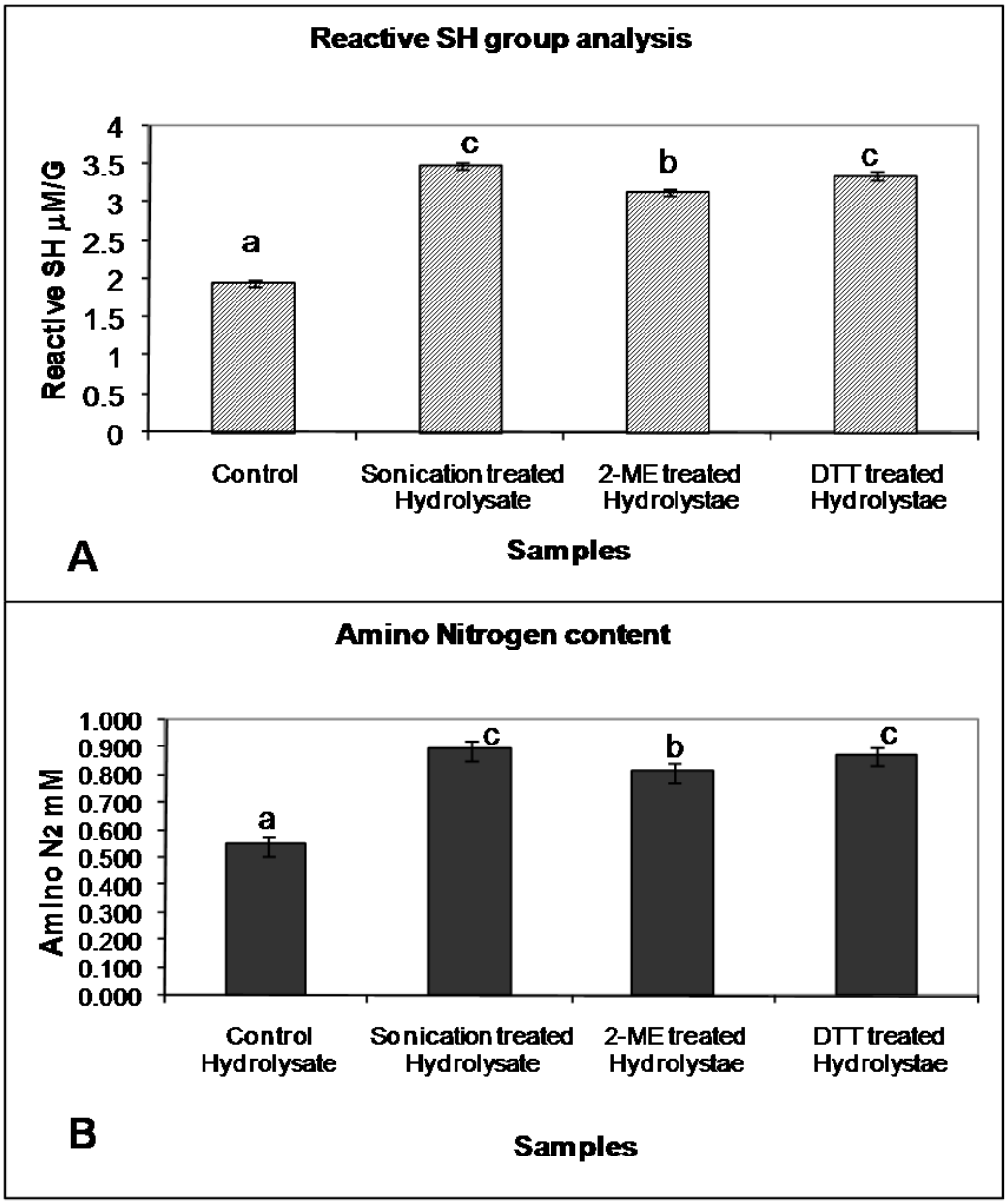


Figure 2.4: Effect of reducing agent treatment on amino N_2 content and reactive SH groups. Bars with different letters indicate significant differences after reducing agent treatment ($p < 0.05$).



* The statistical analysis was done by one-way analysis of variance (ANOVA) and the significant differences were determined using Duncan's multiple range test.

*2-ME - β-Mercaptoethanol, DTT – Dithiothreitol

CHAPTER-3 PURIFICATION AND CHARACTERIZATION OF ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY PEPTIDES DERIVED FROM ENZYMATIC HYDROLYSATE OF OVOTRANSFERRIN

3.1 INTRODUCTION:

High blood pressure or hypertension, defined as a systolic blood pressure (SBP) above 140 mmHg and/or a diastolic blood pressure (DBP) above 90 mmHg, is one of the major chronic diseases that affects up to 30% of the adult population in most of the countries; however, more than 50% of the hypertensive individuals are unaware of their conditions (Lewanczuk, 2008; Chockalinga, 2008). As a lifestyle related disease, lifestyle modifications and diet intervention have been proved useful in hypertension management (Hermansen, 2000). It is recommended by American Heart Association (AHA) that increased physical activity, a reduced salt (sodium chloride) intake, weight loss, moderate amount of alcohol consumption, no smoking, increased potassium intake and an overall healthy dietary pattern such as adoption of Dietary Approach to Stop Hypertension (DASH) diet are the effective ways to keep a healthy blood pressure. The DASH diet emphasizes fruits, vegetables and low fat diary products and other food products reduced in fat and cholesterol (Ignatius and Ernst, 2006). Substantial evidence from epidemiological as well as laboratory studies has revealed that hypertension is closely related to diet (Jung et al., 2006). Various bioactive components such as vitamin C, ω-3 fatty acids, as well as some traditional herbal extracts (ginkgo biloba, ginseng, kava, valerian and lemon

balm) have been demonstrated to have antihypertensive or blood pressure lowering effects (Hamer et al., 2005; Chen et al 2009).

Food protein-derived angiotensin converting enzyme inhibitory peptides or ACE-inhibitory peptides have been extensively studied recently as an alternative for prevention or management of high blood pressure (Fujita et al., 1999). These peptides are inactive in the original proteins, but can be released by proteolytic enzymes during gastrointestinal digestion or food processing (Meisel, 1997). It is thought that ACE inhibitory peptides function by inhibiting the activity of ACE, although other mechanisms have also been proposed (Wright and Musini, 2009). ACE is the key regulatory enzyme of blood pressure via the renin-angiotensin system; ACE converts angiotensin I (Ang-I), a decapeptide (DRVYIHPFHL), to a potent vasoconstrictory angiotensin II (Ang-II), an octapeptide (DRVYIHPF), and also inactivates vasodilatory bradykinin (Ondetti, Rubin and Cushman, 1977). Therefore, inhibiting ACE could reduce the circulating level of Ang-II and thus lower high blood pressure. Synthetic ACE-inhibitory drugs are widely used to control high blood pressure but are associated with adverse side effects such as dry cough, hyperkalemia, hypotension, renal failure decrease in white blood cells and angioedema (Bakris et al., 2008). Furthermore, it is recommended that patients suffering from kidney problems and pregnant women should avoid pharmacological ACE-inhibitory drugs lest they encounter various side-effects (Williams et al., 2004). Food protein-derived ACE-inhibitory peptides are reported to be safer than synthetic ACE-inhibitors (Chen et al., 2007). Many ACE inhibitory peptides have been found in various food protein sources such as milk,

soybean, egg etc (FitzGerald, Murray and Walsh, 2004, Miguel and López-Fandiño, 2005). Ovokinin (FRAHPFL), an octapeptide derived from ovalbumin, and its several analogues were the first peptides isolated from ovalbumin (Matoba et. al, 1999); oligopeptides from egg yolk protein and YAEERYPIL and RADHPFL from egg white protein were further reported to have ACE inhibitory activities (Yoshi et. al, 2001; Miguel and López-Fandiño, 2005; Miguel et. al, 2007).

Ovotransferrin, accounting for ~12% of egg white protein, is a globular glycoprotein with iron-binding property (Williams, 1982). It contains 686 amino acids, with a molecular weight of 77.90 kDa and an isoelectric point of 6.38. Ovotransferrin is composed of two lobes linked by an alpha helix structure; each lobe has the capability to reversibly bind one Fe^{3+} ion (Williams, 1982). Ovotransferrin is well known for its antimicrobial activity (Valenti and others 1983; Florence and Rehault, 2007). Recently, a prodrug-type ACE inhibitory peptide, KVREGT, was isolated from ovotransferrin (Lee et. al, 2006). Our previous study showed that ovotransferrin was the preferred substrate for the production of ACE inhibitory peptides through an integrated QSAR and bioinformatics approach; three potent peptides, IRW, IQW and LKP, were predicted and further identified in the hydrolysate (Majumder and Wu, 2009a). However, it is unknown whether the predicted peptides purified from the hydrolysate would have the same activity as the synthetic ones and whether the peptides would remain stable under simulated gastrointestinal condition. Therefore, the objectives of this study were to purify these three peptides (IRW,

IQW and LKP) from ovotransferrin hydrolysate, to characterize the stability of each peptide in simulated gastrointestinal condition, and to investigate the inhibitory kinetics to understand their interactions with ACE.

3.2 MATERIALS AND METHODS:

3.2.1 Materials

Egg ovotransferrin (Ovotransferrin 100) was obtained from Neova Technologies Inc. (Abbotsford, BC, Canada). Angiotensin converting enzyme (ACE, from rabbit lung), pepsin (porcrine gastric mucosa), thermolysin (*Bacillus thermoproteolyticus rokko*), pancreatin (porcine pancreas), mucosal peptidases (porcine intestine) and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma (Oakville, ON, Canada). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Acros Organics (Morris Plains, NJ, USA). Peptides (IRW, IQW and LKP) were customarily synthesized by Genescript Corp (Piscataway, NJ, USA); their purity (>95%) was confirmed by HPLC-MS.

3.2.2 Preparation of ovotransferrin hydrolysate

Ovotransferrin hydrolysate was prepared in a jacketed beaker coupled with a Lauda Water Bath (Brinkman, Missisauga, ON, Canada) for temperature control and a Ttitrando (Metrohm, Herisan, Switzerland) for pH control according to our previously described method (Majumder and Wu, 2009b). Ovotransferrin slurry (5%, w/v) was subjected to sonication in 4 pulses (60Hz for 30 seconds for each pulse) using Sonic 300 sonicator (ARTEK Systems Corporation, Farmingdale, NY, USA) prior to enzymatic digestion. Then the sample slurry was heated to 80^{o}C for

10 min with continuous shaking. The sample was first digested by thermolysin (0.5% w/w) at a constant temperature of 55^{o}C and pH of 8 for 3 h, and then the pH of the slurry was adjusted to 2 by adding 1N HCl to inactivate the enzyme. After the temperature was adjusted to 37^{o}C, pepsin (4% w/w) was added to the sample for another 3 h. The hydrolysis was terminated by raising the temperature to 95 ^{o}C and maintaining it at that level for 10 min. The digest was then subjected to centrifugation at 10,000 x g for 25 min and the supernatant was obtained and freeze dried for further analysis.

3.2.3 ACE inhibitory activity

ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971) with slight modifications (Majumder and Wu, 2009b). The IC_{50} value was defined as the concentration of the peptides that inhibited 50% of the enzyme activity. Each assay was performed in triplicate.

3.2.4 Measurement of peptide concentration

The peptide concentration of the fractions collected from chromatographic separations was determined by modified Lowry's protein assay (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as a standard.

3.2.5 Purification of ACE inhibitory peptides from hydrolysate

Freeze-dried hydrolysate sample was dissolved in 10 mM ammonium acetate (pH 4) buffer at a concentration of 5 mg/mL. After passing through the molecular weight cut-off 3000 Da ultra-filtration membrane, a 2 mL permeate was fractionated on a HiPreP 16/10 SP FF cation exchange column (16 × 100 mm, GE Healthcare Sweden) coupled with an ÄKTA explorer 10XT system. After

washing the column with 2 column volume (CV) of 10 mM ammonium acetate (pH 4), the sample was eluted with 5 CV of 0.5 M ammonium carbonate buffer at a flow rate of 5 mL/min. The elution was monitored at 280 nm. Fractions were collected, and freeze dried, and the ACE-inhibitory activity of the fractions was determined. Two potent fractions were further purified by reverse-phase-high-performance-liquid chromatography (RP-HPLC) on a Xbridge C_{18} column (10 mm x 150 mm, 0.5 μm, Waters Inc, Milford, MA, USA) coupled with a guard column (40 x10 mm, Waters Inc, Milford, MA. USA) attached to Waters 600 HPLC system. Instrumental control, data collection and data processing were carried out by Empower Version 2. Samples were automatically injected by a Waters 2707 autosampler at a volume of 900 μL. The elution was performed with a linear gradient from 98% solvent A (0.1%TFA in HPLC-grade water) and 2% solvent B (0.1% TFA in acetonitrile) to 45% solvent B over 55 min at a flow rate of 5 mL/min, followed by washing the column at 100% solvent B for 10 min before next run. The elution was monitored by a Waters 2998 photodiode array at 220 nm. Fractions were collected over 2 min-intervals from 5 min to 55 min (25 fractions). The fractions were concentrated using a vacuum-rotary evaporator at 35^{o}C, and peptide concentrations and ACE-inhibitory activities were determined. The active fractions were then subjected to the second HPLC chromatography using the same column with a different two-solvent system composing of 80% of solvent A (20mM sodium phosphate buffer with 0.05% TFA) and 20% of solvent B (20mM sodium phosphate buffer prepared in 80% acetonitrile with 0.05% TFA). The sample was injected at a volume of 400 μL (0.5mg/mL), eluted at a

flow rate of 3 mL/min, and monitored at 220nm. The active fractions were subjected to the third HPLC chromatography using the same column by a two-solvent system: solvent A (0.1%TFA in HPLC-grade water) and solvent B (0.1% TFA in acetonitrile) at a gradient from 15% B to 25%B over 6 CV at a flow rate of 1.5 mL/min. The most active fractions were further analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) to sequence the active peptide sequences.

3.2.6 Peptide sequence confirmation by LC-MS/MS

Peptide sequence was analyzed by a Waters (Micromass) Q-TOF Premier (Milford, MA, USA). The samples were separated on a Waters Atlantis dC_{18} (75 μm x 150 mm, 3 μm) UPLC column (Milford, MA, USA). The system solvents were solvent A (0.1% formic acid in optima LC/MS grade water) and solvent B (0.1% formic acid in optima grade acetonitrile). The samples were dissolved in solvent A; 5 μL of sample was injected into the 5 μm trapping column. The sample was trapped for 2 min at a flow rate of 10 μL/min using 99% solvent A, followed by a gradient from 99% A to 90% A over 5 min, to 70% A over 30 min, to 60% A over 3 min and finally 5% A over 1 min at a constant flow rate of 0.350 μL/min. Then the flow rate was increased to 0.500 μL/min, hold at 5% A for 2 min, increased it to 98% A over 1 min, hold for another 27 min, and then decreased the flow rate to 0.350 μL/min over 1min. Ionization was performed by electrospray ionization technique (ESI) by nanoLockspray ionization source in appositive ion mode (capillary voltage 3.80 kV and a source temp of 100^{o}C). The peptide mass was detected through Quadrupole Time-of-Flight (Q-TOF) analyzer

operated in a positive ion MS/MS mode. A MS/MS full-scan was performed for each sample with an acquisition range of m/z 0-1000 Da. The peptides were then characterised by using Peaks Viewer 4.5 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada) and the peptide sequences were identified from respective monoisotopic mass.

3.2.7 Inhibitory kinetics of ACE inhibitory peptides

Various concentrations of substrate (HHL) (7, 3.5, 1.7 and 0.8 mmol/L) were incubated with ACE in the absence and presence of 1, 0.5 and 0.125 mg/mL of peptides at 37^{o}C for 30 min. The type of enzyme kinetics was investigated by using the Lineweaver –Burk plots (Lineweaver and Burk, 1934). The Michaelis-Menten constant *Km* for the binding of the inhibitor to ACE was calculated. *Ki* was calculated according to the formula, corresponding to the X-axis intercept from the Lineweaver-Burk plots.

$$\frac{1}{v} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$

Where v = reaction rate; V_{max} = maximum reaction rate; [S] = substrate concentration and [I] = inhibitor concentration.

3.2.8 Stability of the peptides against ACE

To test the stability of the peptides against ACE, peptides (1, 0.5, 0.25, 0.125 and 0.0625mg/mL) prepared in 100 mM potassium phosphate buffer containing 300 mM NaCl were incubated with ACE for 1 h at 37°C before the substrate HHL was added for *in-vitro* ACE inhibitory activity assay. Comparison of the IC_{50}

values with and without pre-incubation with ACE indicates the types of inhibitors (Vercruysse et. al, 2009).

3.2.9 Effect of gastrointestinal protease on ACE inhibitory activity

To simulate the human gastrointestinal digestion process *in vitro*, a subsequent hydrolysis of the peptides was performed using pepsin and pancreatin followed by mucosal peptidase (Vercruysse et. al, 2009). The digestion in the stomach was simulated in an acidic condition by lowering the pH to 2 with 1 M HCl, incubating at 37°C for 2 h after the addition of pepsin (E/S: 2% w/w) . Then the pH was raised to 6.5 through the addition of 1 M NaOH; pancreatin (E/S: 2% w/w) was added for further digestion for 2.5 h at 37°C to simulate the small intestine phase. Porcine mucosal peptidases contain a general proteolytic and aminopeptidase activity similar to human enterocytes; digestion with the mucosal peptidases (E/S: 1/50, w/w) were performed at pH 7.4 and 37°C for 2 h. After hydrolysis, the pH was re-adjusted to 4 with 1 M HCl to inactivate the enzyme. Then the sample was centrifuged at 10,000 x g for 25 min at 4^{o}C. The clear supernatant was collected for further analysis. The *in vitro* ACE inhibitory activities of peptides after simulated gastrointestinal incubation were determined as described above. To evaluate the effect of hydrolysis on peptide sequences, samples were analyzed before and after incubation by Xbridge C_{18} column (3.0 mm x 250 mm, 0.5 μm, Waters Inc, Milford, MA. USA), eluted using a linear gradient elution consisting of two solvent systems: solvent A (0.1% TFA in HPLC grade water) and solvent B (0.09% TFA in HPLC grade acetonitrile). The concentration of solvent B was increased from 2% to 10% within 5 min, to 45%

over 45 min and to 100% over 10 min. The injection volume was 10 μL, the flow rate was 0.8 mL/min and sample elution was monitored at 220 nm using the photo diode array detector (Waters 2998, Waters Inc, Milford, MA. USA). Characterization of the peptides produced in the hydrolysate was performed by LC-MS/MS as described above.

3.2.10 Statistical analysis

All results were performed by one-way analysis of variance (ANOVA) using statistical analysis system software (SAS, version 9.0, SAS Institute, Cary, NC). The significant differences were determined using Duncan's multiple range test at $p < 0.05$ (Duncun, 1955).

3.3 RESULTS AND DISCUSSION:

3.3.1 Purification and identification of ACE-inhibitory peptides from ovotransferrin hydrolysate

The cation exchange chromatography of ovotransferrin hydrolysate resulted in three major peaks (A, B and C) (Fig. 3.1). The IC_{50} values of those three fractions were 254.0, 16.01 and 7.6 μg/mL, respectively; fraction A showed the weakest activity whereas fractions B and C showed higher potency. It should be noted that only fraction C showed an ACE-inhibitory activity greater than that of crude ovotransferrin hydrolysate, which was 9.2 μg/mL (Majumder and Wu, 2009a). Both fractions B and C were subjected to further fractionate using an Xbridge C_{18} RP-HPLC column. As shown in Figures 2 and 3, 25 fractions were collected for fractions B and C respectively; one fraction, B17 (Fig. 3.2), and two fractions, C16 and C25 (Fig. 3.3), were identified as the most active fractions. These three

fractions (B17, C16 and C25) were further subjected to the second RP-HPLC fractionation as shown in Figures 3.4, 3.5, and 3.6, respectively; fractions B17-7, C16-6, and C25-4 were identified as the most potent fractions and were subjected to the third RP-HPLC fractionation. The three most potent fractions, B17-7-2 (Fig. 3.7), C16-6-2 (Fig. 3.8) and C25-4-1 (Fig. 3.9) were obtained and used for peptide characterization.

Peptide sequences were analyzed by LC-MS/MS; the responsible peptides in the B17-7-2, C16-6-2 and C25-4-1 fractions were identified as IQW, IRW and LKP (Fig 3.10). These peptides, previously predicted through an integrated QSAR and bioinformatics, were successfully isolated from the hydrolysate by multiple chromatographic fractionations. The IC_{50} values of the purified peptides were comparable to those of the synthetic ones (Table 3.1). The LKP fraction had a higher impurity (purity: 76%) than the IQW (purity: 88%) and IRW fraction (purity: 89%), measured by UPLC chromatogram. However, it was observed that the impurity present in the LKP fraction did not affect the ACE-inhibitory activity which indicates that LKP is the major peptide for the activity observed. The activities of these newly identified peptides were comparable to the previously-identified peptides from ovalbumin, such as FFGRCVSP (0.4 μM), ERKIKVYL (1.2 μM) (Yoshikawa et. al, 2000; Fujita et al. 2000), YAEERYPIL (4.7 μM) and RADHPFL (6.2 μM) (Miguel et al., 2004). All three peptides, IQW, IRW and LKP, are true inhibitors because their IC_{50} values did not significantly change after incubation with ACE (Table 3.1). Interestingly, LKP was reported previously to exhibit an eight-fold stronger ACE-inhibitory activity than that of its

prodrug-type peptide, LKPNM (Fujita et. al, 1999). The present study was the first to report the ACE-inhibitory activity of the IRW and IQW peptide sequence.

3.3.2 Inhibitory kinetics study:

Lineweaver-Burk plots of ACE inhibitory peptides showed that the three peptides (IQW, IRW and LKP) were non-competitive inhibitors (Fig. 3.11). Competitive inhibitors bind with the enzyme on the active site and restrict its activity; therefore, a competitive-type inhibitor is usually reversible if sufficient substrate molecules are available to displace the inhibitor. In contrast, non-competitive inhibitors bind with the enzyme other than the active site which causes a conformational change of the enzyme; the enzyme is no longer able to bind with the substrate correctly due to this change (Perazella and Setaro, 2003). The Michaelis-Menten constant *Km* for the IQW, IRW and LKP peptides were 4.26, 2.65 and 3.09 mM respectively. The calculated *Ki* value for LKP was 1.89 μM which is quite similar to the value of 1.7 μM that was reported for a tuna derived octapeptide, PTHIKWGD (Kohama et al., 1989). The *Ki* values for IRW and IQW were 0.71 and 1.31μM respectively, which is less than that of LKP but is close to previously reported value of 0.74 μM of a tripeptide derived from pea (Wu et al., 2006) and 0.75 μM of decapeptide derived from snake venom (Cheung and Cushman, 1973). *Ki* is the dissociation constant which represents the affinity or binding of enzyme inhibitor complex; therefore, the lower the *Ki* value is the higher the affinity for the enzyme will be. In the case of non-competitive inhibitors, the IC_{50} value should be equal to or close to the *Ki* value (Cheng et al., 1973). In this study, we also observed that IC_{50} values are very close to the *Ki*

value of IRW and IQW; however, the IC_{50} value of LKP was higher than that of *Ki*.

3.3.3 Stability study for ACE inhibitory activity

To evaluate the stability of the peptides, the synthetic peptides were incubated under simulated gastrointestinal conditions using pepsin and pancreatin followed by mucosal peptidases (lower intestinal peptidase) and their digests were analyzed by RP-HPLC. The RP-HPLC chromatograms of IQW and LKP were not affected by simulated gastrointestinal incubation with pepsin and pancreatin as compared to the control but that of IRW was significantly changed (Fig. 3.12 A, B and C); *in vitro* ACE inhibitory activity analysis showed that IC_{50} values of IQW and LKP were not affected but that of IRW was increased from 0.31 μg/mL to 18.15 μg/mL (Table 3.2). The results implied that peptides of IQW and LKP, but not IRW, are resistant to gastrointestinal enzymatic degradation. The LC-MS/MS analysis of IRW gastrointestinal digest revealed a dipeptide of IR and a free amino acid of W (Fig. 3.12B). This result was expected due to presence of trypsin in pancreatin. It is known that trypsin preferentially cleaves Arg and Lys with a higher rate of cleavage for Arg at high pH at position P1 (Keil, 1992). P1 is a subside nomenclature of enzymatic cleavage model created by Schechter and Berger (Schechter and Berger, 1967, 1968) corresponding to the first N-terminal amino acid residue at the cleaved site. On the other hand, P1' is the first C-terminal amino acid residue at the cleaved site. Thus, IRW was hydrolyzed by pancreatin into a dipeptide of IR by cleaving the peptide bond between Arg (R) and Trp (W). Although LKP also has a susceptible cleavage site, Lys (K), the

presence of Pro (P) at position P1' blocks the action of the enzyme (Keil, 1992); therefore, LKP was resistant to trypsin. Further digestion of the respective peptide digests by mucosal peptidase showed that LKP was degraded into two peaks (Figure 3.12). The LC-MS/MS analysis of the LKP digest identified a dipeptide of KP and a free amino acid of L (Figure 3.12C); a significant decrease in activity was also observed as the IC_{50} value was increased from 1.39 to 27.69 μg/mL (Table 3.2). Although LKP showed degradation under simulated gastrointestinal conditions in the present study, intravenous administration of LKP to spontaneously hypertensive rats was reported to lower blood pressure by 30mm Hg at a dose of 30 μg/kg of body weight. But in oral administration, the minimum effective dose was 1.25 mg/kg (Fujita et al., 1999). Therefore, *in vivo* activity also depends on other factors such as the route of administration, and the food matrix and intestinal absorption of the peptides if taken orally. Our results suggested that IQW is more stable than both IRW and LKP in the gastrointestinal tract which implies that IQW might exert better antihypertensive effect *in vivo* than IRW and LKP.

3.4 CONCLUSIONS:

In the study, three highly potent peptides (IRW, IQW and LKP) were successfully purified from the thermolysin-pepsin ovotransferrin hydrolysate. All these peptides are noncompetitive inhibitors and all of them are true-type inhibitors. However, simulated gastrointestinal digestion could significantly reduce the ACE-inhibitory activity of IRW by pancreatin and of LKP by mucosal peptidase due to the generation of the less potent dipeptides and free amino acids as

confirmed by LC-MS/MS. IQW was found to be resistant to the simulated digestion. These results indicate that *in vivo* study of IRW and LKP may exhibit lower activity than that of IQW after oral administration; considering the fact that LKP has been proven to lower blood pressure *in vivo*, it is reasonable to deduce that the hydrolysate containing the peptides reported would be active *in vivo*. Validation of this hypothesis will be investigated by using an animal model.

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Table 3.1: Comparison of ACE inhibition (IC_{50} value) of three peptides

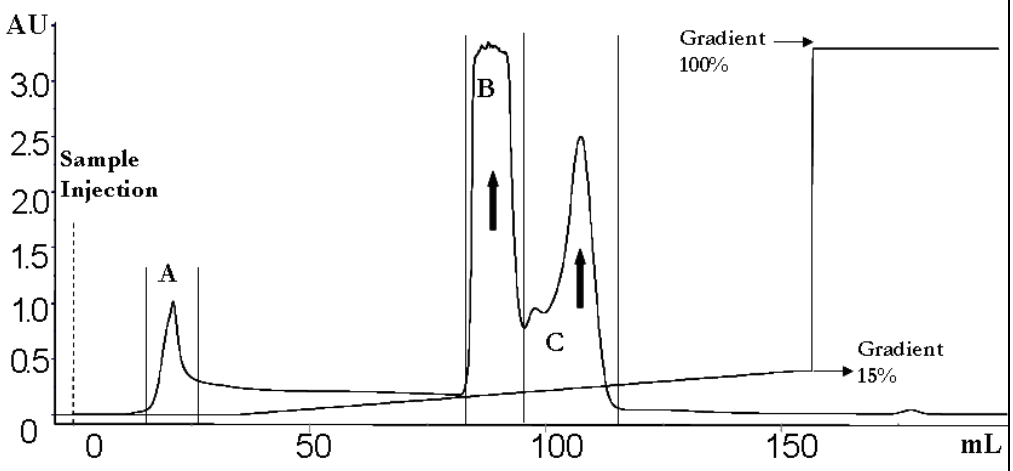
Peptides	**Predicted IC_{50} (μM)**	**Observed IC_{50} (μM)**		**Pre-incubation with ACE IC_{50} (μM)**	**Type of inhibitor**
		IC_{50} (μM) of synthetic peptides	**IC_{50} (μM) of purified peptides**		
IQW	1.41	1.56	1.54	1.57	TRUE
IRW	0.59	0.61	0.62	0.64	TRUE
LKP	2.81	2.93	2.94	2.97	TRUE

Table 3.2: Resistance of three peptides against digestive enzymes, different letters (a, b) within the same column show significant difference under simulated digestion ($p<0.05$).

Treatments	**Peptides IC_{50} (μg/mL)**		
	IQW	**IRW**	**LKP**
Non- hydrolyzed	0.71 [a]	0.31 [a]	1.34 [a]
Gastrointestinal digestion	0.71 [a]	18.15 [b]	1.39 [a]
Gastrointestinal + mucosal digestion	0.72 [a]	18.72 [b]	27.69 [b]

Figure 3.1: Cation exchange chromatogram of ovotransferrin hydrolysate using HiPreP 16/10 SP FF cation exchange column as described in materials and methods.

Total 3 fractions were collected and their ACE inhibitory activities were analyzed as in the appended table where fractions B and C were the active fractions.



ACE inhibitory activity of fractions

Fraction name	IC_{50} (μg/mL)
Fraction A	254.02
Fraction B	**16.50**
Fraction C	**7.61**

Figure 3.2: RP-HPLC chromatogram of fraction B in Figure 1 by Xbridge C18 column (10 mm x 150 mm, 0.5 μM) under linear gradient condition of 98% solvent A (0.1%TFA in water) and 2% solvent B (0.1% TFA in acetonitrile) to 45% solvent B over 55 min.

Total of 25 fractions were collected at 2 min interval (A) and ACE inhibitory activity was determined for each fraction (B). B17 (marked with arrow) showed the highest ACE inhibitory activity.

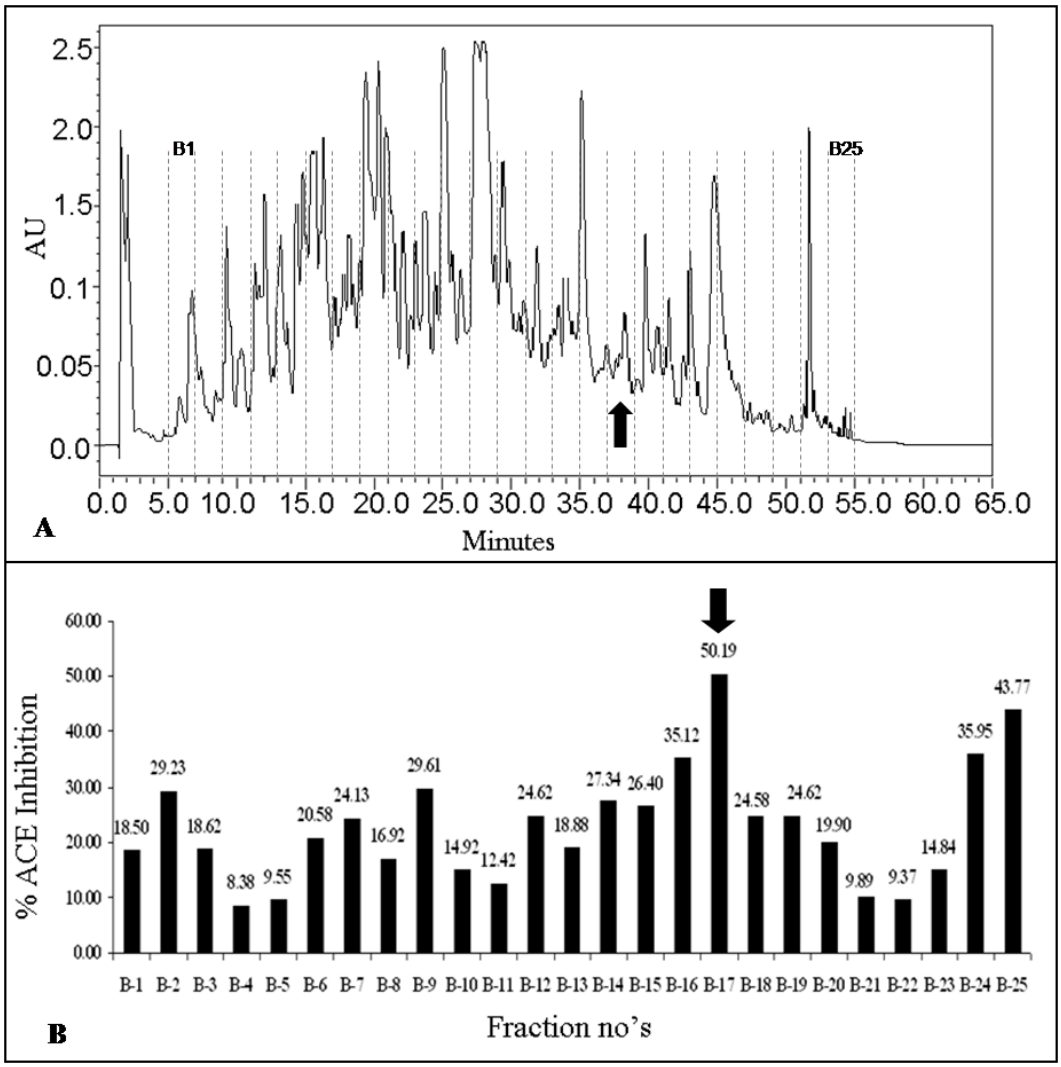


Figure 3.3: RP-HPLC chromatogram of fraction C in Figure 1 by Xbridge C18 column (10 mm x 150 mm, 0.5 μM) under linear gradient condition of 98% solvent A (0.1%TFA in water) and 2% solvent B (0.1% TFA in acetonitrile) to 45% solvent B over 55 min.

Total of 25 fractions were collected at 2 min interval (A) and ACE inhibitory activity was determined for each fraction (B). C17 and C25 (marked with arrow) showed the highest ACE inhibitory activity.

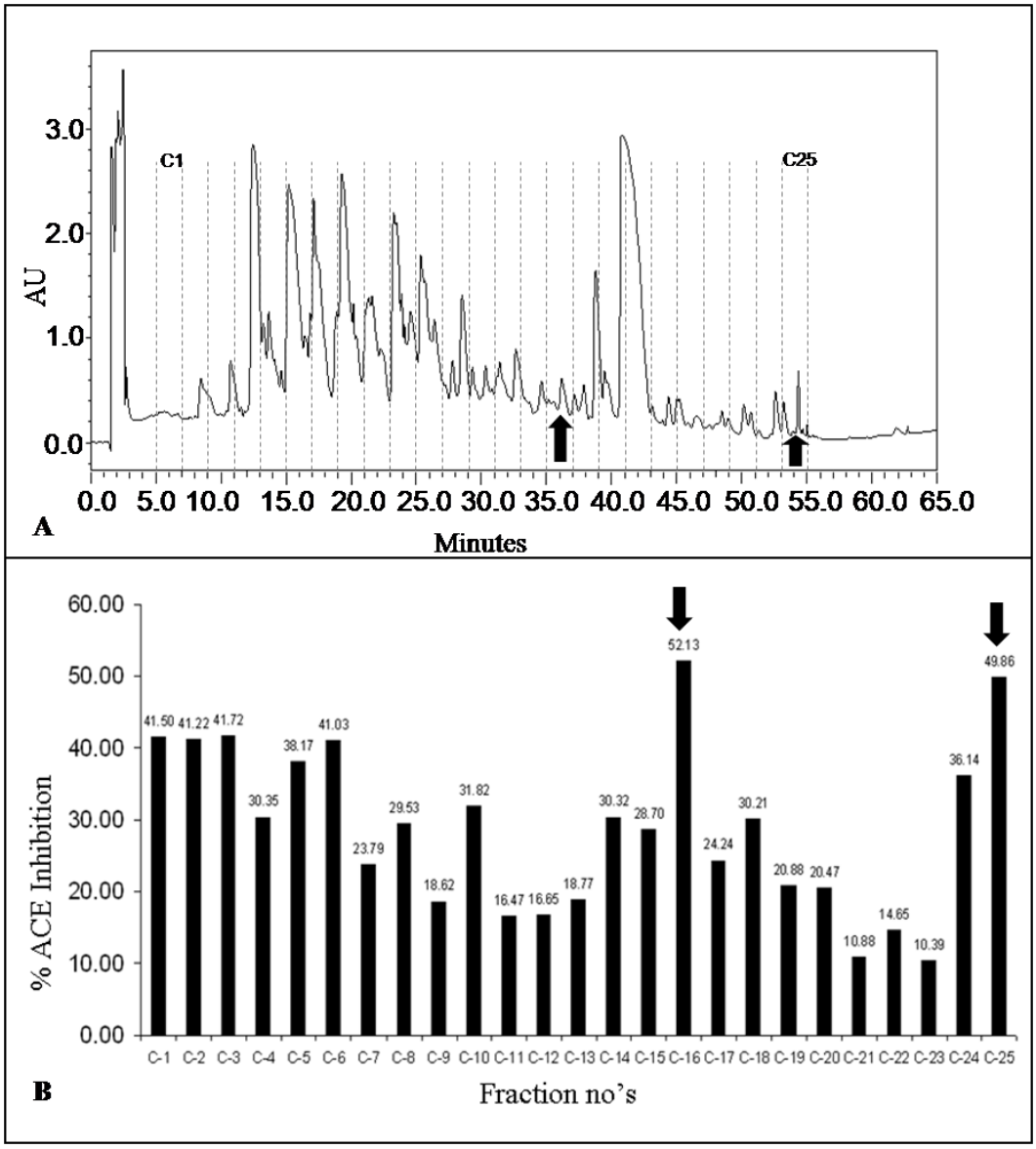
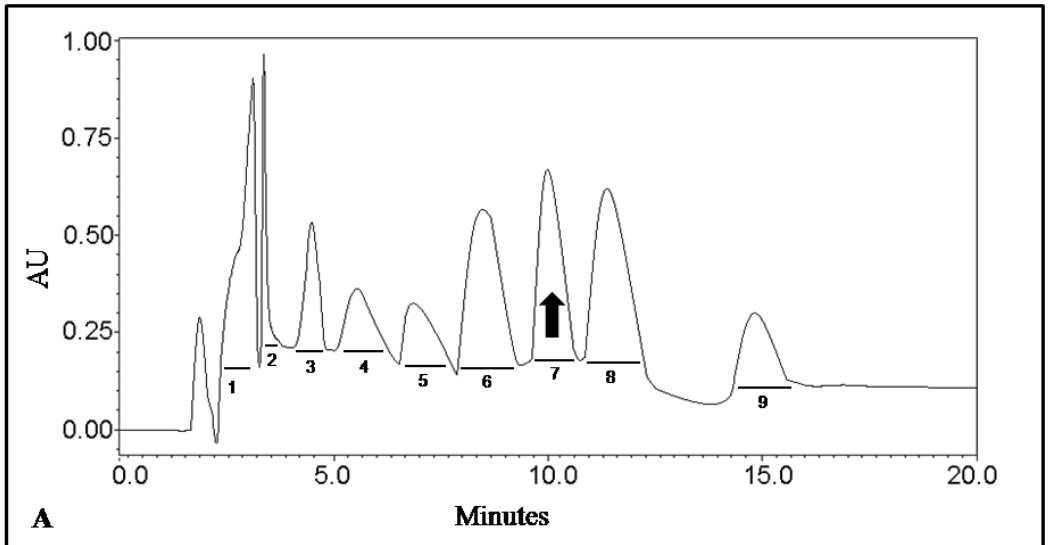


Figure 3.4: RP-HPLC chromatogram of fraction B17 by using Xbridge C_{18} column (10 mm x 150 mm, 0.5 μm) under isocratic condition of 80% solvent A (20 mM sodium phosphate buffer with 0.05% TFA) and 20% solvent B (20 mM sodium phosphate buffer prepared in 80% acetonitrile with 0.05% TFA).

Total 9 fractions were eluted (A) and B17-7 (marked with arrow) showed the highest ACE inhibitory activity (B).



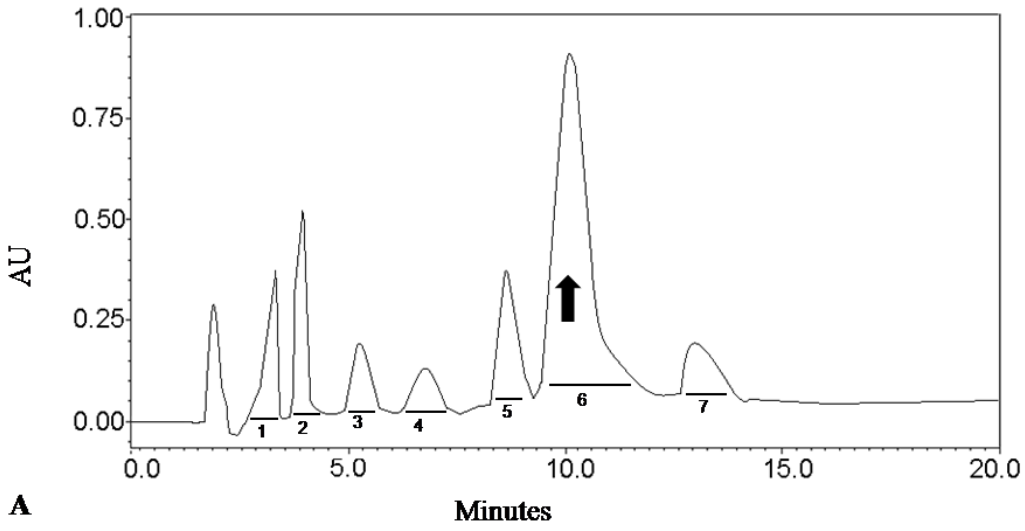
A

ACE inhibitory activity of fractions

Fraction name	% ACE inhibition
B17-1	2.84
B17-2	11.99
B17-3	17.45
B17-4	15.89
B17-5	19.01
B17-6	11.21
B17-7	**58.79**
B17-8	25.25
B17-9	22.41

B

Figure 3.5: RP-HPLC chromatogram of fraction C16 by using Xbridge C_{18} column (10 mm x 150 mm, 0.5 μM) under isocratic condition of 80% solvent A (20 mM sodium phosphate buffer with 0.05% TFA) and 20% solvent B (20 mM sodium phosphate buffer prepared in 80% acetonitrile with 0.05% TFA). Total 7 fractions were eluted (A) C16-6 (marked with arrow) showed the highest ACE inhibitory activity (B).



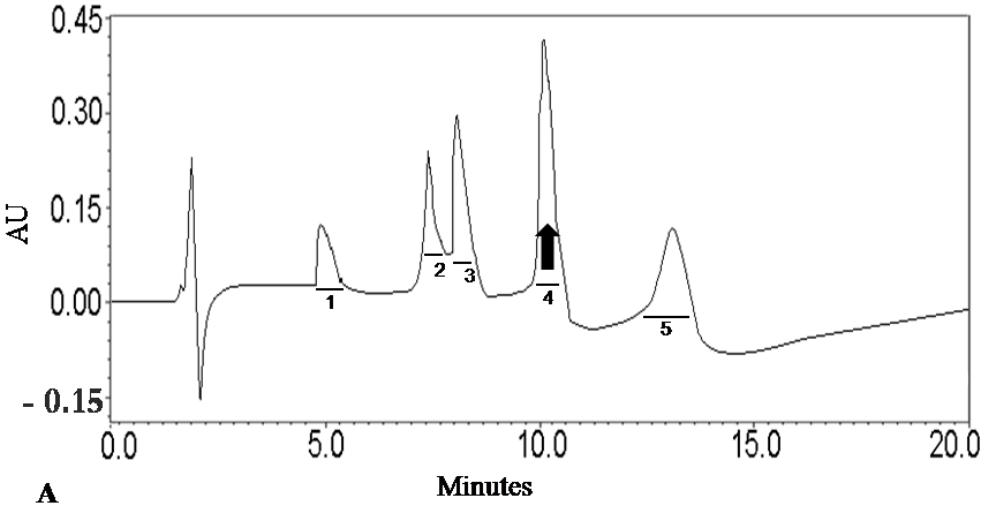
A

B

ACE inhibitory activity of fractions

Fraction name	% ACE inhibition
C16-1	11.99
C16-2	5.67
C16-3	13.55
C16-4	18.65
C16-5	15.67
C16-6	**65.60**
C16-7	18.51

Figure 3.6: RP-HPLC chromatogram of fraction C25 by using Xbridge C_{18} column (10 mm x 150 mm, 0.5 μM) under isocratic condition of 78% solvent A (20 mM sodium phosphate buffer with 0.05% TFA) and 22% solvent B (20 mM sodium phosphate buffer prepared in 80% acetonitrile with 0.05% TFA). Total 5 fractions were eluted (A) C25-4 (marked with arrow) showed the highest ACE inhibitory activity (B).



A

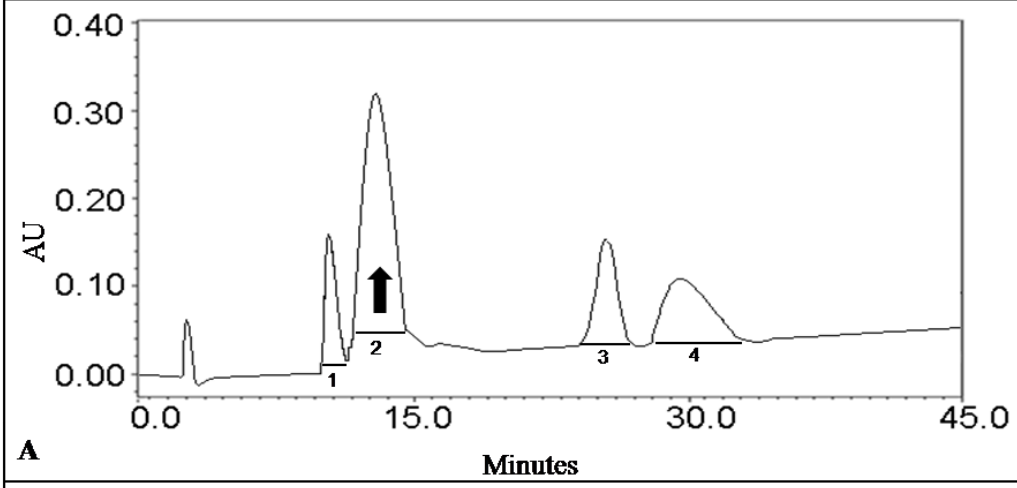
B

ACE inhibitory activity of fractions

Fraction name	% ACE inhibition
C25-1	6.17
C25-2	14.40
C25-3	16.81
C25-4	**54.18**
C25-5	14.89

Figure 3.7: RP-HPLC chromatogram of fraction B-17-1 by using Xbridge C_{18} column (10 mm x 150 mm, 0.5 μM) under linear gradient condition of 15% solvent B (0.1% TFA in acetonitrile) to 45% solvent B over 45 min.

Total 4 fractions were eluted (A) B17-7-2 (marked with arrow) showed the highest ACE inhibitory activity (B).



A

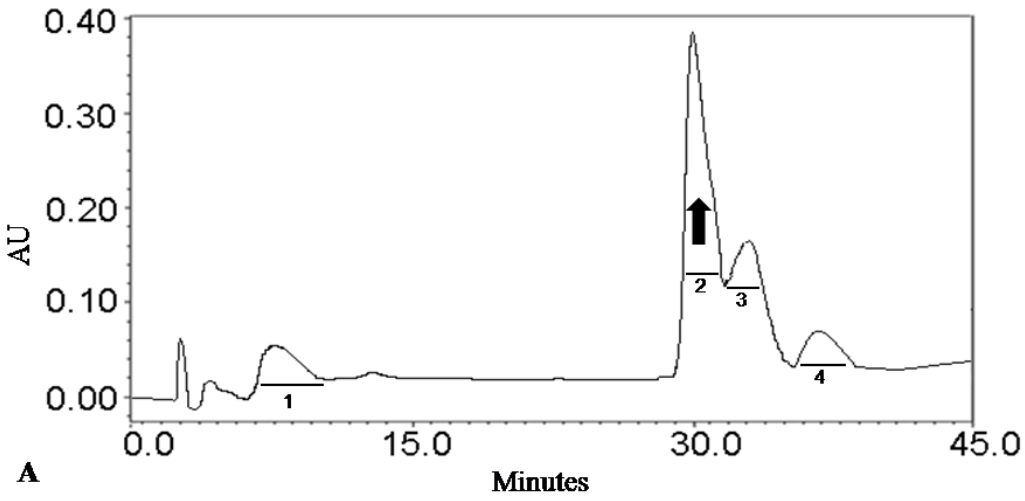
ACE inhibitory activity of fractions

Fraction name	% ACE inhibition
B17-7-1	2.02
B17-7-2	**75.31**
B17-7-3	9.11
B17-7-4	11.36

B

Figure 3.8: RP-HPLC chromatogram of fraction C16-6 by using Xbridge C_{18} column (10 mm x 150 mm, 0.5 μm) under linear gradient condition of 15% solvent B (0.1% TFA in acetonitrile) to 45% solvent B over 45 min.

Total 4 fractions were eluted (A) C16-6-2 (marked with arrow) showed the highest ACE inhibitory activity (B).



A

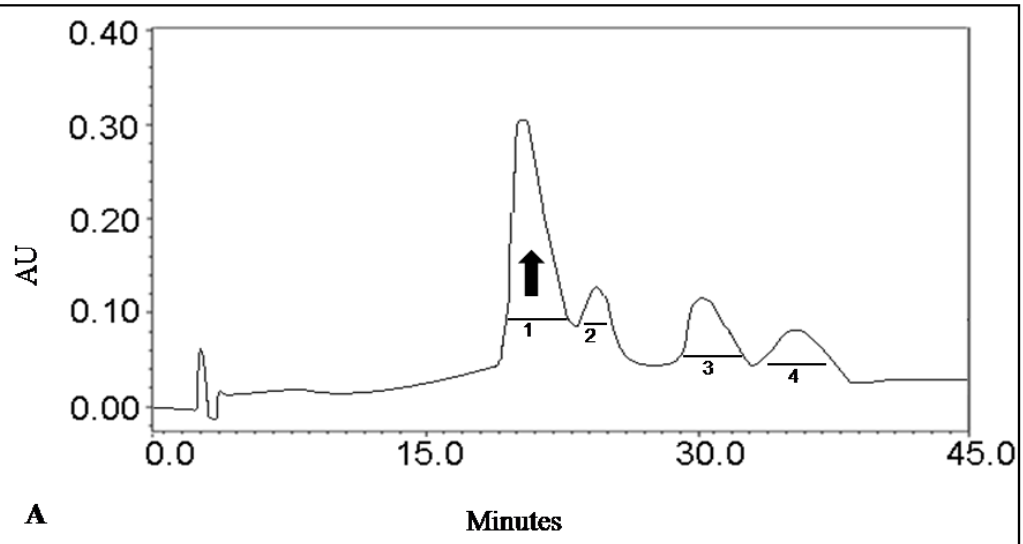
ACE inhibitory activity of fractions

Fraction name	% ACE inhibition
C16-6-1	11.32
C16-6-2	**86.20**
C16-6-3	16.90
C16-6-4	2.32

B

Figure 3.9: RP-HPLC chromatogram of fraction C25-4 by using Xbridge C_{18} column (10 mm x 150 mm, 0.5 μM) under linear gradient condition of 15% solvent B (0.1% TFA in acetonitrile) to 45% solvent B over 45 min.

Total 4 fractions were eluted (A) C25-4-1 (marked with arrow) showed the highest ACE inhibitory activity (B).



A

B

ACE inhibitory activity of fractions

Fraction name	% ACE inhibition
C25-4-1	**69.21**
C25-4-2	11.23
C25-4-3	2.36
C25-4-4	4.66

Figure 3.10: LC-MS spectrum of the purified fractions, (A) LC-MS spectrum of B17-7-2 (B) LC-MS spectrum and (C) LC-MS spectrum of C25-4-1

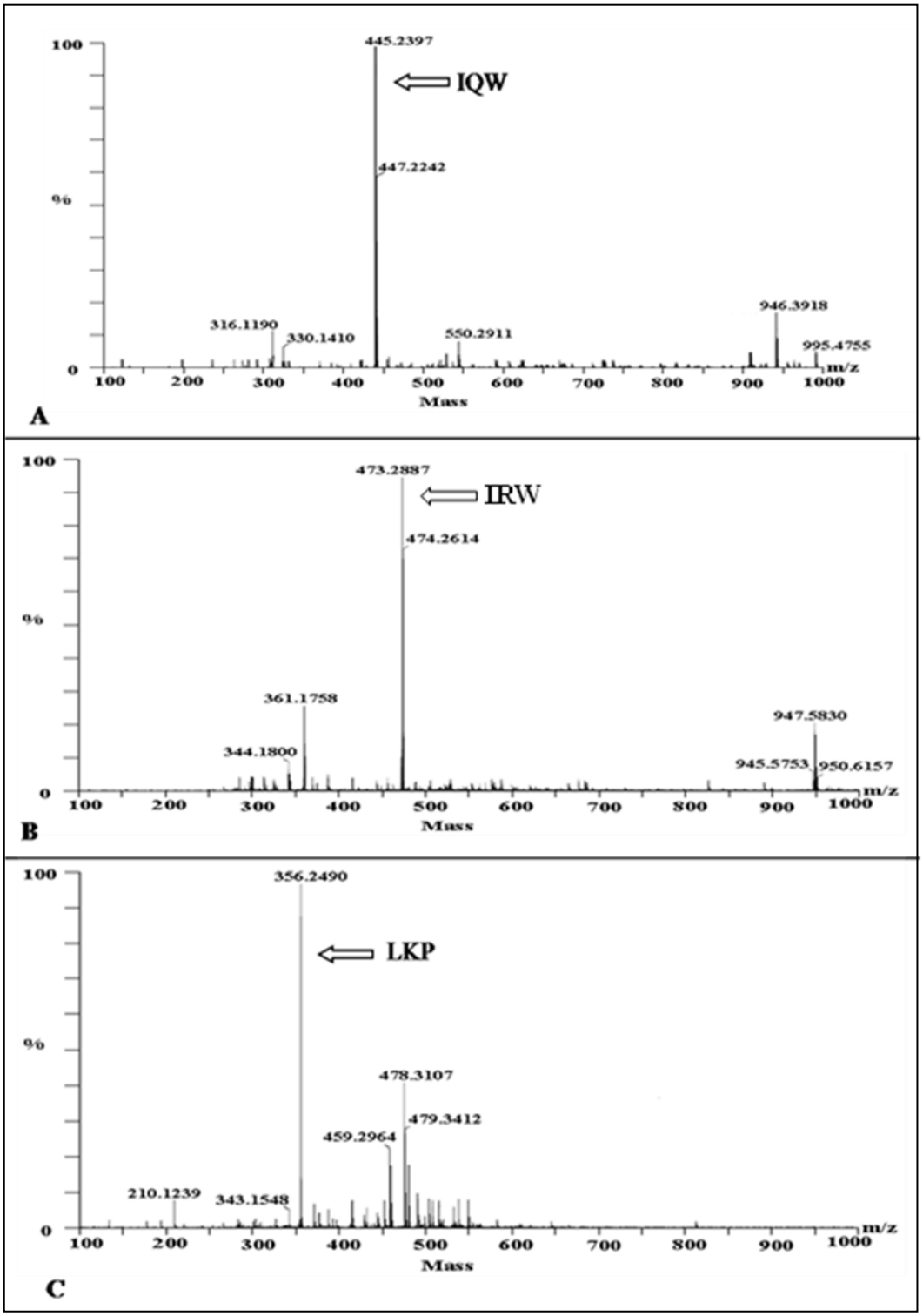
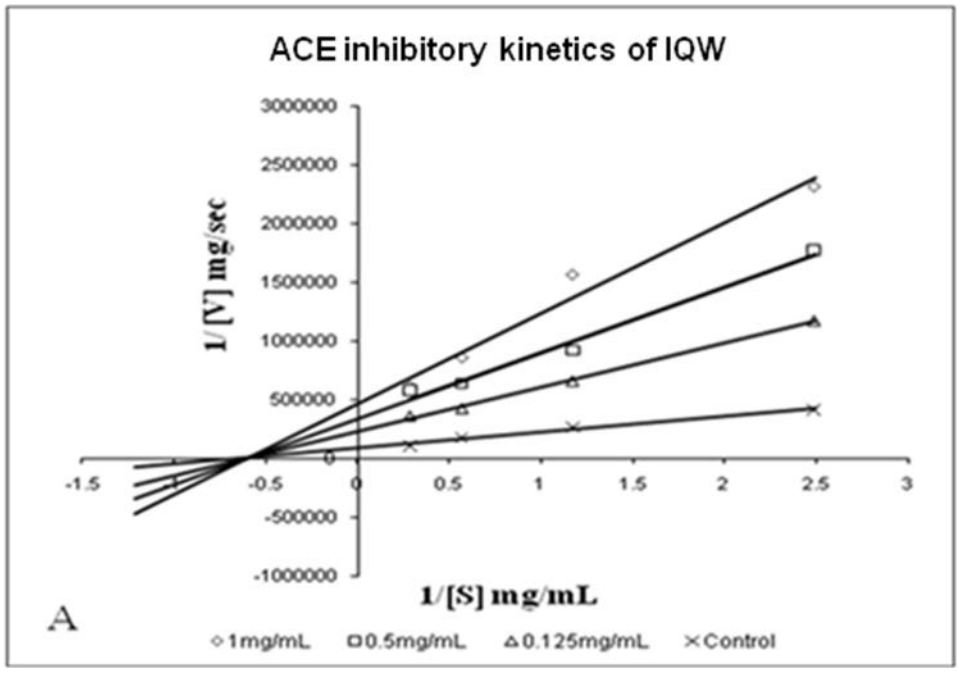
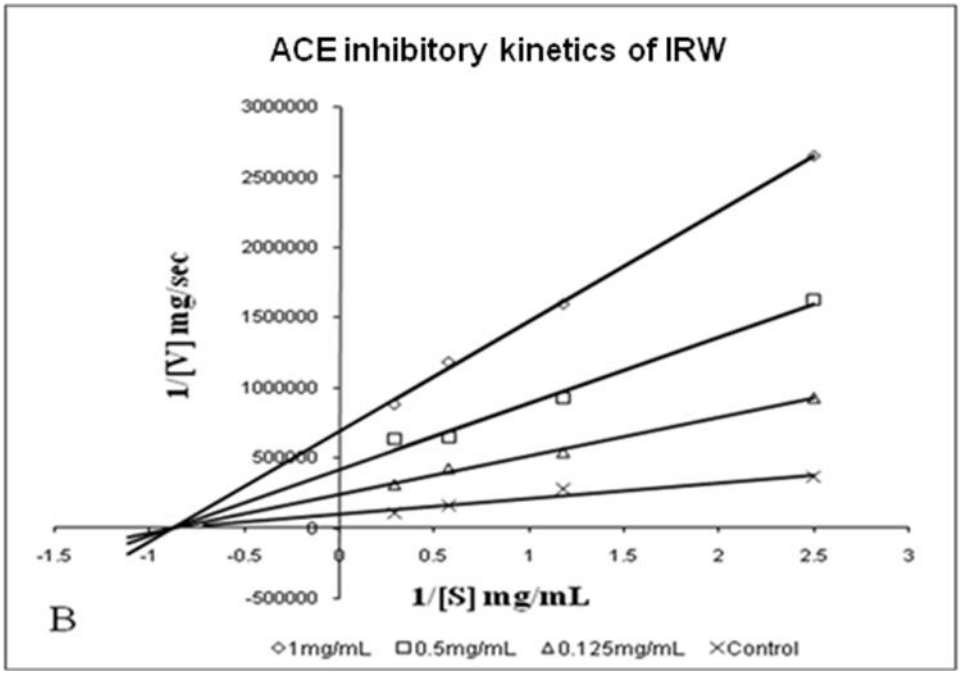
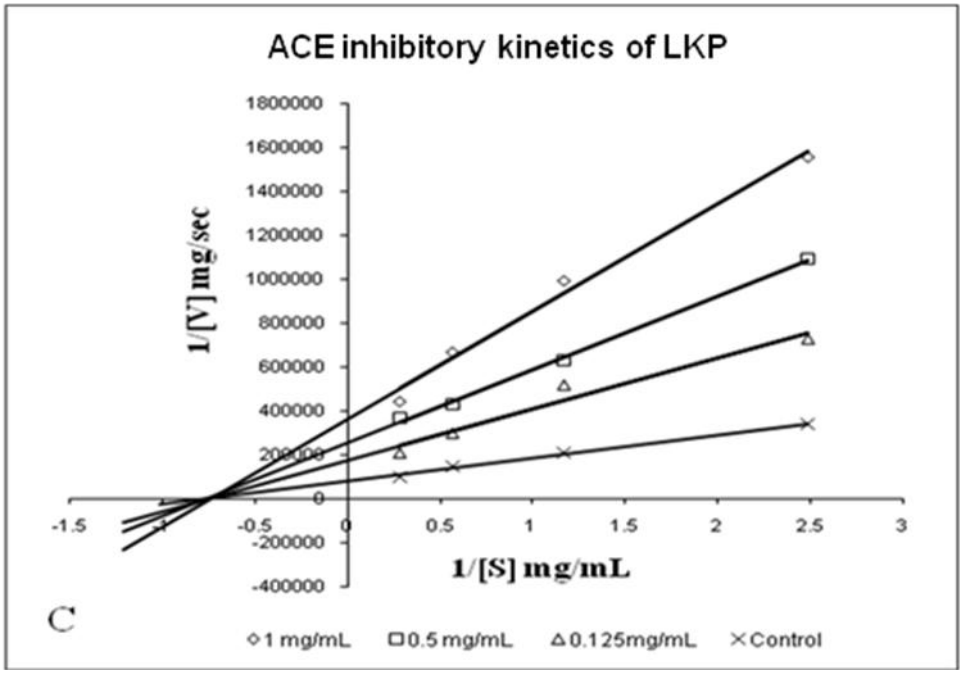


Figure 3.11: ACE inhibitory kinetics of IQW (A), IRW(B) and LKP (C).

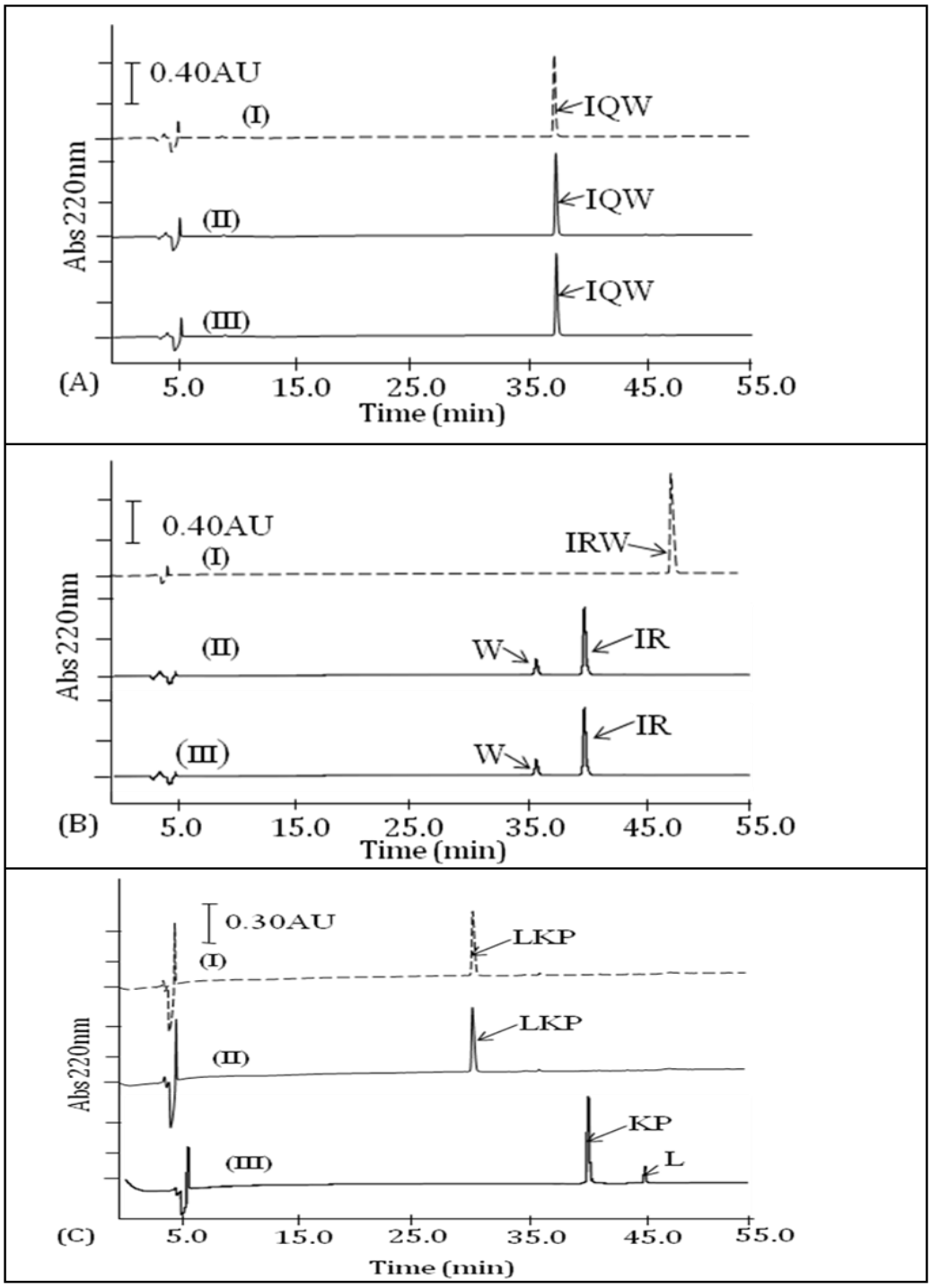






***** Each of the point is the means of nine individual determinations.

Figure 3.12 (A): RP-HPLC chromatogram of IQW (A), IRW (B), LKP (C) after gastrointestinal digestion, (I) is the chromatograms of the synthesized peptides, showed in dotted lines; (II) is the chromatograms of the digests after pepsin-pancreatin digestion; (III) is the chromatograms of the digests after further digestion with mucosal peptidase.



CHAPTER- 4 FINAL REMARKS

4.1 FUNCTIONAL FOODS: A NEW ALTERNATIVE IN THE TREATMENT AND PREVENTION OF CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) are the leading cause of mortality in the developed countries. Globally, CVDs represented 31% of the mortality burden and 10% of the total disease burden in 2000 (American Heart Association, 2000). CVDs are the diseases affecting the heart as well as blood vessels, thus leading to various complications such as arrhythmia, endocarditis, ischemic heart disease, etc. Hypertension is one of the underlying causes of CVDs (WHO, 2003). Medication through the inhibition of angiotensin converting enzyme (ACE) inhibition is one of the most recommended remedies for hypertension (Ng and Vane, 1967). Synthetic or pharmacological ACE inhibitors, such as captopril, enalapril and ramipril, commonly prescribed by physicians, exhibit effective reduction and help to control hypertension. However, patients may suffer from adverse effects such as dry cough, hyperkalemia, etc (Bakris et al., 2008). As hypertension is one of the lifestyle-related diseases, modifications in lifestyle such as a controlled diet, regular exercise, restriction of smoking and moderate consumption of alcohol can assist in the effective management of hypertension. Earlier research has already proven that hypertension is significantly related to diet, and a diet rich in fruits, vegetables and low fat diary products has a significant correlation with a decrease in high blood pressure (Nuñez-Cordoba et al., 2009). ACE-inhibitory peptides from various food sources have been studied intensively. In contrast to synthetic ACE-inhibitory drugs, food protein-derived

peptides are reported to be safer in both animal and human studies (Chen et al., 2007, Kajihara et al., 2008, Potenza et al., 2007, Miguel et al., 2005; Hata et al., 1996). Therefore, ACE-inhibitory peptides can be used as an alternative for the prevention and treatment of hypertension. Functional foods containing ACE inhibitors have been already commercialized on the market as shown in Table 4.1.

4.2 A SUMMARY OF PRESENT RESEARCH

Eggs are a good source of bioactive peptides. Recent studies have shown that peptides derived from egg white proteins could significantly reduce the blood pressure through ACE inhibition. Some potent ACE-inhibitory peptides have already been identified from different egg proteins; peptic digest of ovalbumin generated some potent peptides, FFGRCVSP (IC_{50}: 0.4μM), ERKIKVYL (IC_{50}:1.2μM), LW (IC_{50}: 6.8) and FRAHPFL (IC_{50}: 3.2μM) (Miguel et al., 2006). Another peptide KVREGT (IC_{50}: 9.1μM) is the only ACE-inhibitory peptide derived previously from ovotransferrin (Lee et al., 2006). In this study we identified three potent ACE-inhibitory peptides from ovotransferrin. The ACE-inhibitory activities of the newly identified peptides are IRW (IC_{50}: 0.6μM), IQW (IC_{50}: 1.41), and LKP (IC_{50}: 2.8μM). According to a previous study, peptide which consists of 2-10 amino acid residues can exhibit more potency than longer peptides (Byun and Kim, 2002). Moreover, short-chain peptide fragments can easily pass through the gastrointestinal tract, and can also easily be absorbed into the body. It is also well accepted now that small peptides, such as di- or tri-peptides, have much lower osmolarities than free amino acids. So in terms of bioavailability, these new peptides can exhibit much potency. According to the

structural requirements for ACE-inhibitory peptides, a potent peptide prefers a hydrophobic amino acid at the N-terminal, and an aromatic amino acid at the C-terminal (Hata et al., 1996). For tri-peptides, the presence of a positively-charged amino acid in the middle position is one of the major requirements. Interestingly, all three of these newly-identified peptides are tripeptides and have hydrophobic amino acids at the N-terminal. But only IRW has an aromatic and branched chain amino acid at the C-terminal and a positively-charged amino acid in the middle. In the case of the two other peptides, IQW lacks a positively-charged amino acid in the middle and LKP lacks an aromatic amino acid at the C-terminal. Thus, IRW exhibits more ACE-inhibitory activity than IQW and LKP. All of these newly identified peptides are non-competitive inhibitors and true-type inhibitors. In simulated gastrointestinal (GI) conditions IRW degraded and produced IR; as well, LKP degraded and produced KP. However, IQW was more resistant to degradation and remain stable against GI enzymes.

In conclusion, an integrated QSAR and bioinformatics approach has been successfully developed in this study. Three peptides were identified and characterized. According to the database of antihypertensive peptides (BIOPEP) [www. uwm.edu.pl/biochemia/index_en.php] (Dziuba et al., 1999) and literature, we found that IRW and IQW have not been reported whereas LKP was reported from a prodrug-type penta-peptide, LKPNM (Fujita and Yoshikawa et al., 1999). Comparing to the traditional method which is labor intensive, time consuming and costly, this new approach shows advantages in efficiency and productivity in terms of time and cost savings.

4.3. IMMPLICATIONS OF THE PRESENT STUDY

Driven by the increasing consumers' awareness of a link between food and health, the marketing share of functional foods and nutraceuticals has received tremendous growth in recent years. In addition to their basic nutritional values, functional foods and nutraceuticals are believed to have various health benefits. This study showed that one of egg white proteins, ovotransferrin, could generate a number of potent ACE inhibitory peptides which have implications for the prevention of hypertension. It is well known that synthetic ACE inhibitors are associated with adverse effects including dry cough, hyperkalemia, hypotension, renal failure, decrease in white blood cells, angioedema etc (Silverberg et al., 2002); ACE-inhibitory peptides reported from the present study will provide an alternative for the prevention of hypertension. Developing functional foods from eggs as in the present study will also help to diversify the use of eggs. Finding from the present study can also be used by the egg industry to educate consumers about the benefits of egg which would improve the knowledge between egg and human health and thus contribute to a sustainably growing egg industry in Canada.

4.4. RECOMMENDATIONS FOR FUTURE RESEARCH

- *In vivo* studies of these ACE-inhibitory peptides are essential for more scientific evidence. Therefore, verification of the antihypertensive properties of these peptides in animal models, followed by clinical trials, is needed to estimate the ultimate efficacy of the active peptides.

- Study on biochemical pathways of the peptides in cells will provide a better understanding of the mechanism underlying the antihypertensive activity of these peptides.

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Table 4.1: Commercially available nutraceuticals/ functional foods enriched with ACE-inhibitory peptides

Product name	**Source of the product**	**Peptide sequence**	**Company, country**
Calpis™ sour milk	Milk	VPP and IPP	Calpis Food Industry, Japan
Katsubushi Oligopeptide	Dried Bonito	LKPNM	Nippon Supplement Inc., Japan
BioZate	Whey protein	Not known	Davisco Food International Inc., USA
Evolus	Milk	VPP and IPP	Valito Ltd., Finland
C12 Peption	Milk	FFVAPFPEVFGK	DMV, Netharlands