Structure and Function Study of Food Protein-Derived ACE Inhibitory Peptides

By

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ABSTRACT

Hypertension affects one third of the population worldwide. It is a multifactorial, polygenic disorder which involves the interplay among various risk factors. A number of scientific studies have demonstrated that renin-angiotensin system (RAS), oxidative stress and inflammation interact with each other in regulating the hypertensive response and targeted organ injury. Inhibiting angiotensin I-converting enzyme (ACE) is the key target for controlling high blood pressure. Due to the inevitable adverse side effects of antihypertensive drugs, food-derived compounds, especially food protein-derived peptides have emerged as an alternative strategy in the prevention and management of hypertension. The objectives of this thesis were to 1) identify potent ACE inhibitory peptides from various food proteins by *in silico* digestion and quantitative structure-activity relationship (QSAR) modeling and to 2) explore their additional antioxidative and anti-inflammatory activities in endothelial cells.

A systematic evaluation was performed on 15 food commodities as potential sources of ACE inhibitory peptides. *In silico* digestion of proteins by thermolysin generated 5709 peptides containing 2 to 6 amino acid residues, and their ACE inhibitory activity was predicted using QSAR modeling. Meat proteins from pork, beef and chicken contain the largest number of potent peptides (IC_{50} < 10 μM), followed by proteins from milk, egg, soybean and canola, whereas proteins from fish (with the exception of salmon) and cereals (oat and barley) contain the least number of potent peptides. This study has

demonstrated that proteins from livestock meat, milk, egg, soybean and canola are good sources of ACE inhibitory peptides.

Soybean, bovine latoferrin and spent hen were then selected to validate the prediction. Three tripeptides, LSW (IC_{50}=2.7 μM), IVF (IC_{50}=63.3 μM) and LLF (IC_{50}=63.8 μM) with potent ACE inhibitory activity were characterized from soybean hydrolysates, while LRP (IC_{50}=1.2 ± 0.05 μM) and IWHHT (IC_{50}=9.93 ± 0.65 μM) were characterized from bovine lactoferrin and spent hen, respectively. IWHHT was not resistant to simulated gastrointestinal digestion and produced a mixture of IW, IWH, HHT and HT. IW showed a more potent ACE inhibitory activity (IC_{50}=2.0 ± 0.06 μM). LSW, LRP and IWHHT were all predicted to be released from corresponding food proteins in the computational study.

LRP significantly inhibited tumor necrosis factor-alpha (TNF-α) induced vascular inflammation by reducing the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) at 50 μM in endothelial cells. LRP, IWHHT, IWH and IW all significantly reduced the level of superoxide when the cells were subject to serum withdrawal to induce oxidative stress. While IWHHT and IWH significantly reduced the expression of VCAM-1 only in TNF-α induced inflammation.

This thesis demonstrated the potential of various food proteins as sources of ACE inhibitory peptides. The ACE inhibitory peptides, with additional antioxidative and anti-inflammatory activities identified in the thesis may enhance their antihypertensive effects by interacting with different elements essential in the pathogenesis of hypertension.

PREFACE

This thesis is an original work done by Yuchen Gu and has been writing according to the guidelines provided by the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Alberta Livestock Meat Agency Inc. (ALMA) and Natural Sciences and Engineering Research Council (NSERC) of Canada to Dr. Wu.

This thesis consists of seven chapters. Chapter 1 provided a general introduction, hypothesis and objectives of the thesis. Chapter 2 was literature review. Chapter 3 has been published as "QSAR-aided *in silico* approach in evaluation of food proteins as precursors of ACE inhibitory peptides" in *Food Research International*. I was responsible for performing the *in silico* study and writing the draft of the manuscript. Chapter 4 has been published as "LC-MS/MS coupled with QSAR modeling in characterising of angiotensin I-converting enzyme inhibitory peptides from soybean proteins" in *Food Chemistry*. Chapter 5 is in preparation for submission as "Bovine lactoferrin derived ACE inhibitory tripeptide LRP also shows antioxidative and anti-inflammatory activities in endothelial cells". Chapter 6 is in preparation for submission as "Spent hen-derived ACE inhibitory peptide IWHHT also shows antioxidative and anti-inflammatory activities in endothelial cells". Finally, chapter 7 provided concluding remarks and future research directions.

Dr. Jianping Wu contributed to experimental design, data interpretation and manuscript edits. I was responsible for literature search required for the study, experimental designs, performing experiments, data collection and analysis, and writing the first draft of the

manuscript. Dr. Kaustav Majumder contributed to experimental design and data interpretation in chapter 3.

DEDICATION

This thesis is dedicated to my parents Rongxing Gu and Jianfang Huang, for their understanding, encouragement and support.

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CHAPTER 1- General Introduction and Thesis Objectives

1.1 General introduction

Cardiovascular diseases (CVD) are the major health concern in the world (WHO, 2011). Hypertension, defined as persistent elevation of systolic blood pressure (SBP) greater than 140 mmHg and/or diastolic blood pressure (DBP) greater than 90 mmHg, is one major type of CVD. It has been estimated that around one in three people worldwide has elevated blood pressure (WHO, 2012).

Hypertension results from a complex interaction among genetic, physiological and environmental elements. The available evidence from a wealth of studies has indicated a cooperative role of oxidative stress and inflammation in regulating the hypertensive response. Aberrant production of reactive oxygen species (ROS) stimulated by increased activity of the renin-angiotensin system (RAS) potentiates inflammatory responses, augmenting blood pressure elevation and cardiovascular control organ damage. Conversely, inappropriate activation of inflammatory responses in vascular, renal and/or neuronal tissues results in significant elevation of local oxidative stress, leading to pathologic increase in blood pressure (Crowley, 2014).

Antihypertensive drugs such as diuretics, beta-adrenergic receptor blockers, calcium channel blockers and angiotensin I-converting enzyme (ACE) inhibitors are widely used (Widmaier, Raff & Strang, 2006). However, the adverse effects such as dry cough and skin rashes commonly presenting in these drugs have substantially affected the adherence to and persistence with antihypertensive regimens (Israili & Hall, 1992; Sesoko & Kaneko, 1985). Meanwhile, studies have established that a healthy life style, such as

increased physical activity, reduced salt intake, weight loss, moderate amount of alcohol consumption, quitting smoking, together with adoption of the Dietary Approach to Stop Hypertension (DASH) diet is an effective way to maintain healthy blood pressure (Zarraga & Schwarz, 2006). Some foods have been specifically found to be beneficial (Huang, Davidge & Wu, 2013). Food-derived compounds, especially biologically active proteins/peptides have thus emerged as an alternative to synthetic drugs in the management of hypertension.

So far, antihypertensive peptides have been found in a number of food proteins (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012; Patrycja, Alegre & López, 2015). Food proteins are inexpensive and readily available. Bioactive peptides from food resources are generally considered to be safe because they are mainly produced from natural resources using food-grade agents and processes. Therefore, the research on food-derived bioactive peptides has been seen as an effective way to enhance the economic value of a food product beyond its nutritional value and to expand its market share (Bigliardi & Galati, 2013; Udenigwe, 2014).

1.2 Hypothesis and objectives

Studies have revealed that specific molecular properties play an important role in ACE inhibitory activity, based on which the characteristics of ACE inhibitory peptides have been established via quantitative structural-activity relationship (QSAR) modeling. The computer-based digestion and well-established protein data bases, make it possible to perform a systematic evaluation of food proteins as potential sources of ACE inhibitory peptides through QSAR modeling. Development of hypertension is a complex process involves interplay of oxidative stress and inflammation. Taken together, **it was**

hypothesized that 1) the presence of ACE inhibitory peptides in various food proteins could be predicted and characterized from QSAR modeling coupled with LC-MS/MS, and 2) ACE inhibitory peptides may show antioxidant and anti-inflammatory activities. The specific objectives of this study were to:

1. Perform a systematic evaluation of various food commodities as potential resources of ACE inhibitory peptides by QSAR modeling.

2. Validate the prediction of QSAR modeling by characterizing the potent ACE inhibitory peptides from three selected food proteins (soybean, bovine lactoferrin and spent hen).

3. Evaluate the antioxidant and anti-inflammatory effects of these peptides in endothelial cells.

To achieve these goals, the endothelial cell line EA.hy926 was used for *in vitro* cell culture studies. The EA.hy926 cell line is established by fusing human umbilical vein endothelial cells with the permanent human cell line A549. The cell line has been screened for human factor VIII-related antigen to confirm its preserved features as endothelial cells (Edgell, McDonald & Graham, 1983) and has been widely used for studying vascular endothelium (Bouïs, Hospers, Meijer, Molema & Mulder, 2001; Drabarek, Dymkowska, Szczepanowska & Zablocki, 2012; Armoza, Haim, Basiri, Wolak & Paran, 2013). Brief descriptions of each chapter are given below.

Chapter 2 provides a literature review on several topics related to this thesis including: a) the role of RAS, oxidative stress and inflammation in the pathogenesis of hypertension

and their interaction; b) the production and activity of food-derived antihypertensive peptides and their underlying mechanism.

Chapter 3 performed a systematic evaluation of 15 food commodities as potential resources of ACE inhibitory peptides using a QSAR-aided *in silico* approach. The aim of this study is to establish the rationale for choosing the appropriate protein to produce ACE inhibitory peptides. *In silico* digestion of proteins by thermolysin was predicted to generate 5709 peptides containing 2 to 6 amino acid residues, which could be further digested by pepsin and trypsin to release more peptides. Peptides were divided into three categories based on their predicted activity. The results showed that meat proteins from pork, beef and chicken contained the largest number of potent peptides (IC_{50} < 10 μM), followed by proteins from milk, egg, soybean and canola, whereas proteins from fish (with the exception of salmon) and cereals (oat and barley) contained the least number of potent peptides. This study demonstrated that proteins from livestock meat, milk, egg, soybean and canola are good source of ACE inhibitory peptides.

Chapter 4 characterized the potent ACE inhibitory peptides from soybean using LC-MS/MS coupled with the QSAR model. A soybean protein hydrolysate digested by thermolysin showed an IC_{50} value of 53.6 μg/mL, which decreased slightly to 51.8 μg/mL after adding pepsin, and increased to 115.6 μg/mL after adding trypsin. A total of 34 peptides were characterized. Five novel tripeptides, IVF, LLF, LNF, LSW, LEF and one dipeptide, LW, were predicted to have IC_{50} values lower than 10 μM. The five tripeptides were synthesized and their ACE inhibitory activity was determined. Among them, LSW (IC_{50}=2.7 μM), IVF (IC_{50}=63.3 μM) and LLF (IC_{50}=63.8 μM) showed potent ACE inhibitory activity. The results showed that: (a) LC-MS/MS coupled with QSAR

modelling was an efficient way of screening potent ACE inhibitory peptides (b) soybean is an excellent source for ACE inhibitory peptides and more potent ACE inhibitory peptides are yet to be purified from soybean protein.

Chapter 5 identified the potent ACE inhibitory peptides from bovine lactoferrin and explored their potential antioxidative and anti-inflammatory activities. A bovine lactoferrin hydrolysate digested by thermolysin showed an IC_{50} value of 31.0 ± 1.1 μg/mL, which was not affected by further pepsin and trypsin digestion. LRP with an IC_{50} value of 1.2 ± 0.05 μM in ACE inhibitory test was characterized from lactoferrin digested by thermolysin-pepsin-trypsin and was resistant to simulated GI digestion. The previous computational study had predicted the presence of LRP released from lactoferrin by thermolysin and its resistance to pepsin and trypsin digestion (chapter 3). Further tests showed that LRP was effective in the down-regulation of cytokine-induced inflammatory protein expression, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1). LRP also significantly reduced the level of superoxide in endothelial cells, indicating its ability to suppress the generation of superoxide and/or scavenge superoxide in vascular endothelium. These results indicate the potential application of bovine lactoferrin against hypertension.

Chapter 6 identified potent ACE inhibitory peptides from spent hen and explored their potential antioxidative and anti-inflammatory activities. IWHHT (IC_{50}=9.93± 0.65 μM) was obtained from spent hen hydrolysate digested by thermolysin. This peptide had been predicted from our previous computational study (chapter 3). Simulated GI digestion showed that IWHHT was hydrolyzed by pancreatin to produce a mixture of peptides. Further tests demonstrated that IWHHT and its GI digestion products were effective in

down-regulation of cytokine-induced inflammatory protein expression, as well as suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium. These results indicate the potential application of spent hen-derived peptides in lowering blood pressure.

Chapter 7 provided a general discussion about the foundation and the key findings of this thesis, as well as recommended future studies.

The findings from this thesis demonstrated that: 1) ACE inhibitory peptides could be predicted and characterized through QSAR modelling and LC-MS/MS and 2) ACE inhibitory peptides may show antioxidative and anti-inflammatory activities.

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CHAPTER 2 - Literature Review

Cardiovascular disease is the leading cause of death in the world. According to the report released by World Health Organization in 2011, an estimated 17.3 million people died from cardiovascular disease in 2008, accounting for 30 % of all global deaths, and this number will increase to 23.3 million by 2030 (World Health Organization, 2011).

Hypertension, with systolic blood pressure over 140mmHg and/or diastolic blood pressure over 90mmHg, is affecting a majority of people in the world. According to the World Health Statistics 2012 report, around one in three people worldwide has raised blood pressure (World Health Statistic, 2012). Hypertension causes serious damage to a number of organs. The most affected organ is the heart. In the early phases of the disease, the left ventricle of the heart could develop an adaptive increase in muscle mass in response to an elevated arterial pressure. This is an effective way to maintain the function of the heart. With time, however, changes in the organization and properties of myocardial cells will result in decreased contractility and even heart failure. The presence of hypertension also enhances the risk of atherosclerosis, heart attack, kidney damage, and rupture of a cerebral blood vessel, leading to localized brain damage (Widmaier, Raff & Strang, 2006a).

2.1 The pathogenesis of hypertension

Theoretically speaking, hypertension could result from an increase in cardiac output or in total peripheral resistance, or both. In reality, an increase in total peripheral resistance caused by abnormally reduced arteriolar radius is the major reason in most well-established hypertension (Widmaier, Raff & Strang, 2006a). Since hypertension is a multifactorial, complex polygenic disorder, which involves many organ systems

including heart, kidney and brain, a number of risk factors have been proposed to be responsible for the pathogenesis of hypertension including: (a) activation of the sympathetic nervous system, (b) up-regulation of the renin-angiotensin-aldosterone system (RAS), (c) long term increased levels of sodium intake and insufficient intake of potassium and calcium, (d) endothelial dysfunction and deficiency of vasodilators, (e) vascular remodeling due to inflammation, oxidative stress and other factors (Hall, et al., 2012). A detailed discussion of all possible risk factors is beyond the scope and this review will only focus on three well recognized risk factors: renin-angiotensin system (RAS), oxidative stress and inflammation.

2.1.1 The role of RAS in the pathogenesis of hypertension

The RAS has been recognized to play a key role in regulating blood pressure. There are systemic RAS and tissue RAS. The Systemic RAS involves circulating renin and ACE to produce angiotensin II following a sequential enzymatic cleavage. Renin, an aspartyl protease produced by the juxtaglomerular apparatus in the kidney and secreted into the circulation, hydrolyzes angiotensinogen to generate a decapeptide, angiotensin I. Angiotensinogen is a serum α2-globulin that is released constitutively into the blood from the liver. The next step involves the cleavage of the dipeptide, His-Leu, from the C-terminus of angiotensin I by endothelial ACE to generate angiotensin II, which is a potent vasoconstrictor. Besides that, ACE also inactivates bradykinin, which is a potent vasodilator.

In contrast to systemic RAS, tissue RAS is characterized by the presence of all RAS components in a single tissue. It has been found in many tissues including heart, kidney, brain, pancreas and adipose tissues (Paul, Poyan Mehr & Kreutz, 2006). It is controlled

independently from the systemic RAS. Studies have indicated that tissue RAS is involved in regulating renal and cardiovascular physiology and appears to augment circulating angiotensin II signalling (Bader, 2010; Navar, Kobori, Prieto & Gonzalez-Villalobos, 2011). Elevated tissue levels of RAS components have also been observed in cardiovascular disease without blood pressure elevation, such as atherosclerosis, myocardial infarction, cardiac failure, diabetes and kidney disease (Ribeiro-Oliveira, Nogueira, Pereira, Boas, Dos Santos & Simoes e Silva, 2008).

Angiotensin II mediates its biological function through binding with two G-protein-coupled receptors: angiotensin II type I receptor (AT_1R) and angiotensin II type II receptor (AT_2R). AT_1R shares partial homology with AT_2R in amino acid constitution (Grady, Sechi, Griffin, Schambelan & Kalinyak, 1991) and it is the dominant subtype after birth (Horiuchi, Akishita & Dzau, 1999). The two receptors also differ in their action. AT_1R is associated with vasoconstriction, inflammation, growth and fibrosis, while AT_2R is associated with apoptosis and vasodilation (Touyz & Schiffrin, 2000; Henrion, Kubis & Lévy, 2001). Studies have indicated that the stimulation of AT_1R interferes with AT_2R expression (De Paolis et al., 1999; Miura & Karnik, 2000).

The binding of angiotensin II to AT_1R stimulates the formation of reactive oxygen species (ROS). This is mainly achieved by inducing activation of NADPH oxidase to produce superoxide and hydrogen peroxide (H_2O_2) within vascular cells (Griendling, Minieri, Ollerenshaw & Alexander, 1994). Superoxide and H_2O_2 regulates redox-sensitive signalling molecules including mitogen-activated protein kinases (MAPKs), tyrosine kinases and transcription factors (NF-κB, AP-1 and HIF-1), important in inflammation, cell growth and fibrosis (Nathan, 2003; Torres & Forman, 2003).

Angiotensin II also signals through AT_1R stimulation of cell growth via phosphorylation of MAPKs and tyrosine kinases, leading to vascular remodeling by enhanced growth, inflammation, fibrosis and constriction (Frank, Eguchi, Yamakawa, Tanaka, Inagami & Motley, 2000; Touyz, He, Deng & Schiffrin, 1999; Touyz, Yao, Viel, Amiri & Schiffrin, 2004). Another important mechanism involved in the vasoconstriction of angiotensin II is the activation of RhoA and its downstream target Rhokinase, which has been implicated in the pathophysiology of hypertension and other vascular diseases (Loirand, Guerin & Pacaud, 2006; Loirand & Pacaud, 2010). The vascular remodeling and inflammation due to these angiotensin II-mediated signalling events leads to vascular injury in hypertension.

Besides the constriction of arterioles in vascular system, another physiological role of angiotensin II involved in regulating and changing blood pressure is the stimulation of aldosterone. Aldosterone is a steroid hormone and is a major factor which determines the rate of tubular sodium reabsorption. This greatly influences the total-body sodium, which is positively related to the plasma volume and blood pressure (Widmaier, Raff & Strang, 2006b).

Angiotensin II can be further cleaved to form a group of smaller peptides by angiotensin-converting enzyme 2 (ACE2). These angiotensin II-derived peptides in general mediate opposite effects to those of angiotensin II. It has been shown that decreased production of angiotensin II-derived peptides is associated with vasoconstriction, vascular remodelling and oxidative injury in hypertension and other diseases (Dilauro, Zimpelmann, Robertson, Genest & Burns, 2010; Imai, Kuba, Ohto-Nakanishi & Penninger, 2010).

2.1.2 The role of oxidative stress in the pathogenesis of hypertension

Links between oxidative stress and hypertension was proposed over twenty-five years ago, when researchers found that generation of superoxide was stimulated in the brain of cats with acute hypertension induced by intravenous infusion of vasoconstrictor agents (Wei, Kontos & Christman, 1985). Oxidative stress occurs when the rate of oxidants formation overcomes the capacity of antioxidant defenses in human body. The major products generated from the accumulation of oxidants are ROS, a family of molecules with high reactivity and ability to act as oxidants in redox reactions, including superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2).

So far, numerous experiment and clinical trials have been carried in an attempt to reveal the possible correlations between oxidative stress and hypertension. Previous experimental studies have indicated ROS involvement in the pathogenesis of hypertension. NADPH oxidase, which is the primary source of superoxide in vascular system (Cave et al., 2006), has been intensively studied. NADPH oxidase contains two membrane-bound subunits, p22phox and gp91phox. A couple of gp91phox analogues have also been discovered, including Nox1 (Suh et al., 1999). Nox1-deficient mice have reduced vascular superoxide production, and blood pressure elevation in response to angiotensin II was hindered (Gavazzi, Banfi, Deffert, Fiette & Schappi, 2006). Yet overexpression of Nox1 led to increased blood pressure elevation in response to angiotensin II (Dikalova et al., 2005). In clinical trials, ROS production is increased in vascular smooth muscle cells from hypertensive patients and this is associated with up-regulation of vascular NADPH oxidase (Touyz & Schiffrin, 2001, Touyz, 2005). Antihypertensive treatment in patients with hypertension reduced levels of oxidative

stress, improved the activity of antioxidant enzymes in both whole blood and peripheral mononuclear cells (Saez et al., 2004). Antioxidants such as vitamin C reduced blood pressure and arterial stiffness in patients with diabetes (Mullan, Young, Fee & McCance, 2002).

A couple of mechanisms with respect to how ROS induce hypertension have been proposed. One major mechanism involved is vascular remodeling, which is characterized by hypertrophy of the arterial wall and an increased wall-to-lumen ratio and associated decreased arterial distensibility. In general, oxidative stress promotes vascular smooth muscle cell proliferation and hypertrophy, collagen deposition and alterations in activity of matrix metalloproteinase (MMPs), which cause thickening of the vascular media and arterial remodeling (Touyz, 2000). Another major mechanism proposed is endothelial dysfunction. Chronic dysfunction of the endothelium caused by inactivation of NO by superoxide anion to form peroxynitrite has been regarded as a fundamentally important underlying mechanism in hypertension (Panza, Casino, Kilcoyne & Quyyumi, 1993; Cai & Harrison, 2000). NO is one of the most important vasodilator in the vascular system. Low NO bioavailability can upregulate vascular cell adhesion molecule (VCAM-1) (Khan, Harrison, Olbrych, Alexander & Medford, 1996) and leukocyte adhesion (Radomski, Palmer & Moncada, 1987a), which play an important role in the initiation of the inflammation process and invasion of the vessel wall. Reduction in NO can also cause an increase in platelet aggregation (Radomski, Palmer & Moncada, 1987b), which contributes to thrombogenicity. Peroxynitrite contributes to the degradation of endothelial NO synthase (eNOS) cofactor tetrahydrobiopterin BH_4 (Milstien & Katusic, 1999), leading to the uncoupling of eNOS, which is the primary source of vascular NO

(Förstermann, Closs, Pollock, Jakane, Schwarz, Gath & Kleinert, 1994). Peroxynitrite is also an important mediator of oxidation of LDL, which promotes atherosclerosis (Griendling & FitzGerald, 2003).

Elevated level of superoxide production has important implications for vascular injury and vascular disease that extend beyond its reaction with NO. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide and produce H_2O_2, which is the main source of H_2O_2 in vascular tissue (Touyz, 2000). H_2O_2 is typically associated with lipid and protein oxidation in the presence of transition metals. Studies have shown that H_2O_2 elicits specific responses in different vascular cells including the proliferation of vascular smooth muscle cells (VSMC) (Rao & Berk, 1992) and increased endothelin-1 expression and activation of proapoptotic signals in endothelial cells (EC) (Kähler et al., 2000; Chen, Vita, Berk & Keaney, 2001).

It should be noted that although experimental studies have strongly supported a correlation between oxidative stress and hypertension, human studies have been less consistent (Briones & Touyz, 2010). Clinical studies have demonstrated increased ROS levels in plasma and urine of patients with different types of hypertension (Ward, Hodgson, Puddey, Mori, Beilin & Croft, 2004). In patients with essential hypertension, there are higher levels of superoxide and H_2O_2 in VSMC from resistance arteries and enhanced angiotensin II-stimulated redox signalling compared with cells from normotensive, healthy subjects (Touyz & Schiffrin, 2001). However, clinical studies have been inconsistent in regard to whether reducing oxidative damage by antioxidant results in decreased blood pressure. Small clinical studies have reported the blood pressure-lowering effects of antioxidants (Holowatz & Kenney, 2007), however, large clinical

studies have failed to demonstrate a beneficial effect of antioxidants on blood pressure or cardiovascular injuries. Although the actual reasons are complex, it indicates that there is no conclusive evidence to show that antioxidants alone are potent blood-pressure lowering agents (Briones & Touyz, 2010).

2.1.3 The correlation between RAS and oxidative stress

When the local and/or systemic RAS is activated, the production of ROS, especially superoxide is increased. In an experimental study, the production of superoxide was increased in the aortic segment of rats with angiotensin II-induced hypertension (Rajagopalan, Kurz, Münzel, Tarpey, Freeman, Griendling & Harrison, 1996). In patients with chronic artery disease, the accumulation and the activity of ACE was increased (Diet, Pratt, Berry, Momose, Gibbons & Dzau, 1996) and increased production of superoxide mainly due to NADPH oxidase was associated with increased clinical risk for coronary artery disease (Guzik et al., 2000).

NADPH oxidase was first found in phagocytes, and consists of cytosolic (p47phox, p67phox or homologues) and membrane-bound (p22phox and gp91phox) that forms a functional enzyme complex on activation. Later on, it became evident that NADPH oxidase also exists in vascular cells including VSMC (Ushio-Fukai, Zafari, Fukui, Ishizaka & Griendling, 1996), endothelial cells (EC) (Bayraktutan, Draper, Lang & Shah, 1998; Bayraktutan, Blayney & Shah, 2000) and cardiac fibroblasts (Chamseddine & Miller, 2003). A new family of homologous gp91phox isoforms was found to be expressed in vascular NADPH oxidase. These are now termed Noxs, with gp91phox known as Nox2. Nonphagocytic NADPH oxidase generates superoxide in a slow and

sustained manner while the production can be augmented by specific stimuli such as Angiotensin II (Cave et al., 2006).

Angiotensin II was first reported to stimulate NADPH oxidase activity in cultured VSMC (Griendling, Minieri, Ollerenshaw & Alexander, 1994) and subsequently in EC (Zhang, Schmeisser, Garlichs, Plotze, Damme, Mugge & Daniel, 1999; Li & Shah, 2003) and fibroblasts (Pagano, Chanock, Siwik, Colucci & Clark, 1998). The mechanism involved may depend on the type of cells, as angiotensin II-induced oxidase activation appears to involve Nox1 in VSMC (Lassegue et al., 2001), while in EC it critically relies on Nox2 (Wang et al., 2001). Available evidence has also indicated that Angiotensin II is involved in transcriptional regulation of oxidase subunits. For instance, angiotensin II upregulates Nox1 in VSMC (Lassegue et al., 2001), and Nox2 (Rueckschloss, Quinn, Holtz & Morawietz, 2002) and Nox 4 (Yamagishi, Nakamura, Ueda, Kato & Imaizumi, 2005) in EC. In VSMC (Touyz et al., 2002) and EC (Rueckschloss, Quinn, Holtz & Morawietz, 2002), chronic exposure to angiotensin II also upregulates other subunits of NADPH oxidase.

Upon activation, NADPH oxidase plays an important role in hypertension and other cardiovascular diseases induced by angiotensin II. A study on rats with angiotensin II-induced hypertension has shown that treatment with liposome-encapsulated SOD reduced blood pressure by 50 mm Hg in angiotensin II–infused rats while having no effect on blood pressure in control rats. Liposome-encapsulated SOD also enhanced *in vivo* hypotensive responses to acetylcholine and *in vitro* responses to endothelium-dependent vasodilators in rats treated by angiotensin II (Laursen et al., 1997). In mice lacking $p47^{phox}$ (a key subunit of NADPH oxidase), the hypertensive responses to angiotensin II

infusion was blunted and the formation of superoxide in EC and VSMC was not increased compared to wild- type angiotensin II-infused mice (Landmesser et al., 2002). These studies have provided evidence that hypertension caused by chronically elevated angiotensin II is mediated in part by superoxide. The correlation between angiotensin II and NADPH oxidase was described in Figure 2.1.

2.1.4 Immune responses in the pathogenesis of hypertension

A wealth of experiments has indicated the critical role of immune responses in the pathogenesis of hypertension (Muller et al., 2000; Muller et al., 2002). Epidemiologic studies have demonstrated that in patients with hypertension, the levels of the inflammatory cells, and the mediators organizing the cell entry into cardiovascular control organs are increased. Patients with modest elevations in blood pressure or prehypertension have elevated circulating levels of inflammatory cytokine tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), and leukocytes in comparison to normotensive subjects (Chrysohoou, Pitsavos, Panagiotakos, Skoumas & Stefanadis, 2004). Another inflammatory cytokine interleukin-6 (IL-6) has also been positively correlated with blood pressure in cross-sectional studies (Chae, Lee, Rifai & Ridker, 2001). In addition, the rising level of adhesion molecules which propagate the recruitment of leukocytes to the inflamed tissues have been found in patients with hypertension (Chae, Lee, Rifai & Ridker, 2001; Stumpf et al., 2005).

It has been shown that end-organ damage in hypertension can be limited by broad pharmacologic blocking of pro-inflammatory signaling pathway. A well described example is nuclear factor kappa B (NF-κB), which triggers a couple of key inflammatory mediators at gene transcription level upon activation. Immunosuppressive treatments

against NF-κB activation and translocation to the nucleus has shown the capacity to limit renal damage in hypertension and mitigate blood pressure elevation in some models (Henke et al., 2007; Muller et al., 2000; Muller et al., 2002).

Cells of the immune system also appear to have a profound influence on blood pressure in experimental hypertension. Mice lacking functional lymphocytes have a blunted response to angiotensin II simulated hypertension, which can be restored by adoptive transfer of T lymphocytes. These T cells may promote hypertension by potentiating vascular dysfunction (Guzik et al., 2007). The inflammatory response of T cells can be suppressed by regulatory T cells, which is an important "self-check" function of the immune system to avoid excessive reactions. In contrast to T cells, regulatory T cells have been found to protect blood pressure elevation and target organ damage induced by angiotensin II (Kvakan et al., 2009; Barhoumi et al., 2011).

Circulating inflammatory cells are attracted by chemokines to penetrate across the endothelium lining of the blood vessels and enter the cardiovascular control organ where they mediate the hypertensive response. Adhesion molecules expressed by both inflammatory cells and endothelial cells facilitate cell adhesion and subsequent transendothelial migration. Hypertension up-regulates chemokines and adhesion molecules such as VCAM-1 and ICAM-1 in the renal and systemic vasculature. Although the role of chemokines and adhesion molecules in regulating blood pressure is not clear, blocking their corresponding receptors mitigates renal and vascular damage in hypertension, indicating their potential regulatory role in tissue injury responses during hypertension (Mervaala et al., 1999; Henke et al., 2007).

Recruitment of inflammatory cells into targeted organs triggers local inflammatory responses including cytotoxic activity and the generation of cytokines. This is because leukocyte-endothelial cells adhesion triggers the production of ROS, mainly by a membrane-associated enzyme NADPH oxidase to generate superoxide. The normal function of NADPH in neutrophils or macrophages is to generate ROS to attack foreign invaders. However, inappropriate activation of this innate defense in response to hypertensive stimulus could cause misdirected oxidative stress and potentiate blood pressure elevation. Superoxide generates hydrogen peroxide with the assistance of superoxide dismutase (SOD) (Weiss, 1989; Robinson, 2009). Hydrogen peroxide contributes to the generation of hypochlorous acid (HOCL), which is also a potent oxidizing agent (Lau & Baldus, 2006). HOCL activates a variety of leukocyte-derived proteases including elastase, collagenase and gelatinase, which elicit proteolysis of the vascular wall and the interstitial matrix (Weiss, Peppin, Ortiz, Ragsdale &Test, 1985; Granger, Grisham & Kvietys, 1994). Although the presence of antioxidants and protease inhibitors in extracellular fluid may potentially limit the cytotoxicity, the sequestered microenvironment created on the leukocyte-endothelial cell interface allows neutrophil-derived oxidants and proteases to overwhelm plasma antioxidants and protease inhibitors (Granger, & Senchenkova, 2010). In addition to cytotoxic activities, various cytokines are produced to shape the local inflammatory response including TNF-α (Muller, Shagdarsuren, Park, Dechend, et al., 2002; Elmarakby, Quigley, Pollock & Imig, 2006), interleukin-1 (IL-1) (Boesen, Sasser, Saleh, Potter, et al., 2008) and interleukin-6(IL-6) (Lee, Sturgis, Labazi, et al., 2006), which contribute to target organ damage and blood

pressure elevation. The interplay among RAS, oxidative stress and inflammation was illustrated in Figure 2.2.

2.2 The pharmacological treatment of hypertension

The majority of drugs used to treat hypertension all act in ways that reduce cardiac output and/or total peripheral resistance. These drugs fall into five categories as follows: (a) diuretics, which increase urinary excretion of sodium and water to decrease cardiac output, (b) beta-adrenergic receptor blockers, which mainly reduce cardiac output, (c) calcium channel blockers, which reduce the entry of calcium into vascular smooth muscle cells and cause them to contract less strongly to lower total peripheral resistance, (d) ACE inhibitors, which inhibit the formation of angiotensin II or block its receptors leading to arteriolar vasodilation and a reduction of total peripheral resistance, (e) drugs that antagonize one or more components of the sympathetic nervous system to reduce total peripheral resistance (Widmaier, Raff & Strang, 2006a).

Although effective control of blood pressure is essential to hypertensive patients and significantly reduces the risks of other cardiovascular events such as stroke and coronary heart disease (MacMahon & Rodgers, 1994), the control of hypertension among treated patients in actual clinical practice is less than optimal (Bangalore & Ley, 2012; Elliott, 2009). Besides the cost of medicines and medical care, one major factor affecting the control of blood pressure is the adherence and persistence with antihypertensive regimens (Mazzaglia et al., 2009).

Adherence is defined as the appropriate use of therapy, including taking medications at the appropriate frequency and dose. One important aspect of adherence is the persistence with drug therapy, which is defined as the continuing use of a medication(s) for the

specified treatment period (Burnier, 2006; Halpern et al., 2006). Barriers to treatment adherence are multidimensional. One of them is drug tolerability. The five major classes of antihypertensive drugs have been associated with adverse metabolic effects such as electrolyte, glucose/insulin, lipid and uric acid disturbances, especially diuretics and beta-blockers (Preuss & Burris, 1996). Patients are also subject to skin rashes, dry cough and taste disturbances (Atkinson & Robertson, 1979). These adverse effects associated with antihypertensive drugs hinder their beneficial actions in patients. Another major barrier is the complexity of the drug regimen. The five major classes of antihypertensive drugs have specific properties, advantages and limitations that should be considered (Mancia et al., 2007), while they have complementary mechanisms of action (Ruschitzka, 2011). Consequently, different classes of antihypertensive drugs have been combined to manifest their antihypertensive effects and minimize their adverse effects. According to Clinical Outcomes Utilizing Revascularization and Aggressive Drug Evaluation study of patients with cardiovascular diseases, around 80% of patients were on at least three medications and over 50% patients were on at least four medications (Maron et al., 2010). In a study on 85,000 patients it was found that treatment adherence levels dropped 22% from single-drug therapy to four-drug therapy (Fung, Huang, Brand, Newhouse & Hsu, 2007), indicating that the complexity of drug therapy significantly affects treatment adherence.

2.3 Food-derived peptides with biological activities

The value of food protein was once evaluated only for its nutritional quality for providing essential amino acids and energy. The recognition of the presence of biological active peptides encrypted in the protein sequences with a wide range of biological functions has

given rise to intensive research into their potential applications in the prevention and treatment of various diseases.

One of the major drivers of bioactive peptide research is the popular perceived notion that protein raw materials are readily available and inexpensive, ranging from primary food proteins to by-products of the food industry. The current wide-spread health burden has increased consumers' health-awareness and stimulated the pursuit of functional food for health promotion (Sirò, et al., 2008). This is widely thought to be due to the perception that food-derived peptides are generally safe as they are mainly produced from natural resources using food-grade agents and processes. In addition, bioactive peptide research may be seen as an effective way to enhance the economic value of food product beyond its nutritional value and expand the market share (Bigliardi & Galati, 2013; Udenigwe, 2014).

Bioactive peptides are usually not active within the primary protein structure and must be released intact to exert their physiological function. The generation of bioactive peptides is mostly performed by enzymatic hydrolysis using endogenous or exogenous enzymes during food consumption by gastric digestion or food processing. They can also be generated by the hydrolysis of microbial enzymes during fermentation. So far, a couple of physiological functions of bioactive peptides has been reported, including: a) anticancer activity (Haubner & Decristoforo, 2009; Kobayashi, Suzuki, Kanayama & Terao, 2004), b) antihypertensive activity (Matoba, Usui, Fujita & Yoshikawa, 1999), c) cholesterol-lowering activity and antiobesity activity (Sagara et al., 2004; Wang, Jones, Ausman & Lichtenstein, 2004; Wang & de Mejia, 2005), d) antioxidative activity (Szeto,

2006; Takenaka, Annaka, Kimura, Aoki & Igarashi, 2003), e) antimicrobial activity (Gálvez, Abriouel, López & ben-Omar, 2007).

2.4 Antihypertensive peptides from food resources

Peptides derived from various food resources have been reported to possess antihypertensive activity. Milk and dairy products are the most intensively studied food resources and a number of peptides derived from milk and dairy product have demonstrated blood pressure-lowering effect in experimental studies and even in clinical trials. For example, RYLGY and AYFYPEL from hydrolysis of casein by pepsin, reduced the systolic blood pressure of spontaneously hypertensive rats (SHR) by 25 mmHg and 20 mmHg respectively at a dosage of 5mg/kg (Contreras, Carrón, Montero, Ramos & Recio, 2009). Other food resources could also be potent sources of antihypertensive peptides, such as egg (Miguel, Manso, Aleixandre, Alonso, Salaices & López-Fandiño, 2007b; Miguel, Aleixandre, Ramos, Katayama, Xu, Fan & Mine, 2006), meat (Fujita, Yokoyama & Yoshikawa, 2000; Nakashima, Arihara, Sasaki, Mio, Ishikawa & Itoh, 2002), sardine (Matsui, Tamaya, Seki, Osajima, Matsumoto & Kawasaki, 2002; Wang, Watanabe, Kobayashi, Tanaka & Matsui, 2010) and soybean (Shin et al., 2001; Wu & Ding, 2001; Wu & Ding, 2002).

2.4.1 Production of food-derived antihypertensive peptides

The market for functional food products have expanded in recent years. The need to increase the values of protein-rich food commodities and exploit the beneficial effect of food-derived peptides has promoted research into improving the approach for discovering and producing antihypertensive peptides. The classical approach typically involves selecting food material based on research interest, followed by protein isolation and

enzymatic hydrolysis with proteases that show preference in generating peptides with antihypertensive activity. Subsequent fractionation and purification of protein hydrolysates can result in enriched fractions with potent antihypertensive activity. Eventually, antihypertensive peptides can be identified from the resulting fractions by mass spectrometry. This method is time-consuming, and single potent peptide may not be discovered due to potential additive or synergistic effects of different components of protein hydrolysates.

In recent years, the bioinformatics approach has been developed in an attempt to circumvent some challenges of the classical approach. This approach involves applying computer-based simulation to predict the potential products after the proteolysis of the selected proteins. The protein sequences can be obtained from databases, such as the UniProt Knowledgebase (UniProtKB) and the National Center for Biotechnology Information (NCBI). The simulation of proteolytic specificities of enzymes can be achieved by using bioinformatics software such as BIOPEP and ExPASy PeptideCutter to generate peptide profiles *in silico*. This method has been applied to evaluate various food proteins as potential resources of ACE inhibitory peptides (Gu, Majumder & Wu, 2011) and dipeptidyl peptidase (DPP)-IV inhibitors (Lacroix & Li-Chan, 2012) and for sourcing sustainable protein precursors of bioactive peptides (Udenigwe, Gong & Wu, 2013). The activity of peptides generated by *in silico* digestion can be predicted using quantitative structural activity relationship (QSAR) modeling. Although laboratory experiments are needed to validate the prediction, the bioinformatics approach is helpful in optimizing the protein hydrolysis to produce peptides with the highest activity and

reducing the cost in their manufacturing (Carrasco-Castilla, Hernάndez-Ἀlvarez, Jiménez-Martínez, Gutierrez-López & Dάvila-Ortiz, 2012).

A number of enzymes are commercially available and can be used to produce bioactive peptides, including trypsin, chymotrypsin, thermolysin, pepsin, proteinase K. and papain. A wealth of studies have demonstrated the release of ACE inhibitory and/or antihypertensive peptides from food proteins by enzymes (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012; Patrycja, Alegre & López, 2015). Enzymatic hydrolysis can be affected by different factors defining the nature of enzymes and substrates and the potential impact of inhibitors. Therefore, high pressure (Chicón, Belloque, Recio & Lopez-Fandiño, 2006; Chicón, Lopez-Fandiño, Quirós & Belloque, 2006), heat-denaturing (Hernández-Ledesma, Ramos, Recio & Amigo, 2006) and ultrasound (Jia, Ma, Zhao, Wang, Tian, Luo & He, 2010) have been applied to improve enzymatic hydrolysis by modifying protein structure.

Microbial fermentation has become an alternative way to produce ACE inhibitory peptides and/or antihypertensive peptides in recent years. During this process, peptides are released by microbial activity of fermented food or via the hydrolysis of enzymes derived from the micro-organism. Among the enzymes originated from micro-organism, lactic acid bacteria (LAB) proteases are well-characterized (Korhonen & Pihlanto, 2006) to include a cell-wall proteinase and several specific intracellular enzymes such as endopeptidases, aminopeptidases, dipeptidases and tripeptidases. They have been used to release some of the well-known antihypertensive peptides such as VPP and IPP (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995).

Protein hydrolysates are often fractionated by using membrane and chromatography techniques. Peptides with a specific range of molecular weight can be obtained by using ultrafiltration. Various chromatography approaches can be selected to fractionate protein hydrolysates based on the physicochemical properties of peptides. For instance, ion exchange column is used to fractionate peptides with a characteristic net charge, and reverse phase HPLC is for peptides with various degrees of hydrophobicity. Besides these, affinity chromatography was applied to purify ACE inhibitors via immobilizing ACE on a glyoxyl-agarose and immobilized copper (Megías, Pedroche, del Mar Yust, Alaíz, Girón-Calle, Millάn & Vioque, 2006).

2.4.2 Structure-activity relationship

The search for food-derived antihypertensive peptides usually starts with identifying peptides with ACE inhibitory activity. The identification of the role of specific molecular properties in the ACE inhibitory activity of peptides can make an important contribution in the production of nutraceuticals and functional foods. Although the full mechanism of the interaction between the peptides and ACE is not known, some structural characteristics of ACE inhibitory peptides have been established. Peptides which inhibit ACE usually contain hydrophobic amino acid residues, especially those with aliphatic chains such as Gly, Ile, Leu, and Val in the N-terminal, and amino acid residues with cyclic or aromatic rings (Pro, Tyr, Trp) in the C-terminal. Studies also found that binding to ACE is strongly dependent on the C-terminal tripeptide sequences of the substrate as ACE appears to prefer hydrophobic amino acid residues at the three C-terminal positions. However, positively charged guanidine and ε-amine group derived from Arg and Lys significantly contribute to the increased ACE inhibitory activity, indicating a possible

interaction between the substrate and anionic binding site instead of the catalytic site of ACE (Iwaniak, Minkiewicz & Darewicz, 2014).

A principal component analysis of 98 dipeptides with ACE inhibitory activity collected in the BIOPEP database found that the highest positive correlations were related to the bulkiness of the C-terminal amino acid residue (Iwaniak et al., 2014). A QSAR study on dipeptides and tripeptides with potent ACE inhibitory activity collected from previous publications indicated that dipeptides were composed of amino acid residues with hydrophobic and bulky side chains, while tripeptides usually contained an aromatic amino acid residue in the first position from the C-terminal, a positively charged amino acid residue in the second position, and an hydrophobic amino acid residue in the third position (Wu, Aluko & Nakai, 2006).

2.4.3 Absorption of antihypertensive peptides

Digestion of protein starts in the stomach by pepsin in acidic pH. The polypeptides produced from the digestion are further hydrolyzed by pancreatic proteases in the luminal phase of the small intestine, resulting in a mixture of oligopeptides and free amino acids. The free amino acids account for a minor portion of the product and can be absorbed across the intestinal brush border membrane by amino acid transport. The oligopeptides, which is the major product at this point, are further cleaved by the peptidases present in the intestinal brush border membrane. There are different types of peptidases such as aminopeptidases, endopeptidase and dipeptidase. All these proteases and peptidases contribute to the hydrolysis of oligopeptides to generate a mixture predominantly consisting of free amino acids and di- and tripeptides.

It has been found that proline and hydroxyproline-containing peptides are generally resistant to gastrointestinal digestion. In addition, peptides containing two proline residues at the C-terminal are even resistant to proline-specific peptidase (Vanhoof et al. 1995; FitzGerald & Meisel, 2000).

The di- and tripeptides can be absorbed intact across the intestinal brush border membrane by a specific peptide transport system which uses a transmembrane electrochemical proton gradient as the driving force and has broad substrate specificity (Yang et al. 1999). Small peptides are absorbed more rapidly than free amino acids (Webb, 1990) and they are usually hydrolysed to free amino acids in the cytoplasm by various intracellular peptidases inside the enterocytes. Eventually, the amino acids are transported though the basolateral membrane and enter the portal circulation (Ganapathy & Leibach, 1999).

Intact peptides may also be transported from the intestinal lumen into the blood circulation. It has been proposed that peptides can be transported intact across the intestinal mucosa via paracellular transport or transcellular transport. The mechanisms for intestinal transport of peptides are as follows: (a) paracellular transport in which peptides pass the tight junctions between cells, (b) passive diffusion in which peptides diffuse via the transcellular route, (c) endocytosis in which cells absorb peptides by engulfing them, (d) carrier-mediated transport in which peptides are transported by peptide transporter (Vermeirssen, Van Camp & Verstraete, 2004). The gastrointestinal digestion of bioactive peptides was illustrated in Figure 2.3.

The absorption of peptides is vitally important and directly related to their bioavailability. The Caco-2 cell monolayer is the most widely used model in studying the intestinal

transport of peptides. One study on the antihypertensive peptide VPP found a significant amount of intact VPP was transported though the Caco-2 cell monolayer (Satake, Enjoh, Nakamura, Takano, Kawamura, Arai & Shimizu, 2002). The tripeptide was also found to be present in the abdominal aorta and it exerted an antihypertensive effect after oral intake in SHR (Masuda, Nakamura & Takano, 1996). Subsequent study indicated that the transport of VPP is not mediated by peptide transporter PepT1 and paracellular transport appeared to be the main mechanism of intestinal absorption (Satake et al., 2002). However, a more recent study on the intestinal absorption of VPP showed that VPP were poorly absorbed and quickly eliminated in humans (Wuerzner, Peyrard, Blanchard, Lalanne & Azizi, 2009). The contradicting results arising from the reported observations has indicated that although *in vitro* study is a good starting point and is based on biological mechamism, *in vivo* study and clinical trials are essential. Besides VPP, another peptide VY, originally derived from sardine muscle, reached a maximum level in human plasma in 2 h after a single oral administration in normotensive human subjects. A 10-fold greater increase in plasma concentration was achieved compared with baseline concentration at the highest dose. The increased peptide level in plasma was suggested to be due to the saturation of peptide transporter (Matsui et al., 2002). In addition, the presence of other food components might have a significant influence on the susceptibility to peptidase degradation (Charman, Porter, Mithani & Dressman, 1997).

2.4.4 Mechanisms of action

RAS is the major target for developing antihypertensive agents, in particular through the inhibition of ACE. Various food proteins have been evaluated for their potential application as precursors of ACE inhibitory peptides (Gu, Majumder & Wu, 2011). The

most well-known two peptides, VPP and IPP were identified from sour milk fermented by starter containing *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. The two tripeptides were regarded as the major contributors to the ACE inhibitory activity of sour milk (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995). The blood pressure lowering activity of these two peptides have been studied in animals and in human (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995; Geleijnse & Engberink, 2010). ACE inhibitory peptides have been extensively reviewed (Iwaniak, Minkiewicz & Darewicz, 2014; Aluko, 2015). Besides, peptides targeted on other components of RAS have also been reported. Milk-derived RPYL and LIWKL reduced the blood pressure of spontaneously hypertensive rats (SHRs) through inhibiting angiotensin II binding to AT_1R (Fernandez-Musoles, Castello-Ruiz, Arce, Manzanares, Ivorra & Salom, 2014). Recently, our lab reported the ability of egg-derived IRW to up-regulate the expression of ACE2 in the vasculature of SHRs (Majumder, Liang, Chen, Guan, Davidge & Wu, 2015).

Antioxidants, mostly phenolic compounds, have long been incorporated into the antihypertensive therapy (Huang, Davidge & Wu, 2013). The role of antioxidants in treating hypertension in general involves reducing oxidative stress by scavenging free radicals, chelating metal ions, inhibiting redox active transcription factors and pro-oxidant enzymes, and inducing antioxidant enzymes (Huang, Davidge & Wu, 2013). As discussed before, elevated oxidative stress leads to overproduction of superoxide and other ROS, resulting in reduced bioavailability of NO, which is implicated in endothelial dysfunction. Thus antioxidants could be beneficial for mitigating endothelial dysfunction. Peptides with antioxidative activity have been identified from various protein sources,

however most reports were based on chemical assay, which lacks of physiological relevance, making it impossible to extrapolate the results to *in vivo* condition (Chakrabarti, Jahandideh & Wu, 2014). Hence it is of significant important to evaluate the bioactivity of antioxidative peptides under physiological conditions and to establish their protective role in diseases. However, there are limited reports with regard to the effect of antioxidative peptides *in vivo*. Long term administration (17 weeks) of egg white hydrolysate to SHRs has shown to improve the capacity of plasma antioxidants and to reduce the level of oxidative stress in aortic tissues (Manso, Miguel, Even, Hernández, Aleixandre & López-Fandiño, 2008). Studies on endogenous peptides have also confirmed the beneficial actions of antioxidative peptides on cardiovascular system. Glutathione (GSSH) was found to reverse the impaired relaxation of SHR aortas to acetylcholine to a comparable magnitude to other antioxidants, aminotriazole and ascorbic acid, at the same concentration of 0.04 mM/kg BW (body weight) (Akpaffiong & Taylor, 1998). ACE inhibitory peptides with additional antioxidative activity were also reported. A dipeptide MY from sardine muscle reduced the formation of free radicals in human endothelial cells by stimulating the expression of home oxygenase-1 (HO-1), an antioxidant defense protein independent of its ACE inhibitory activity (Erdmann, Grosser, Schipporeit & Schröder, 2006). Two bovine casein-derived ACE inhibitory peptides, RYLGY and AYFYPEL, showed potent radical scavenging activity. However, how their antioxidative activity contributed to antihypertensive activity against SHR was not elucidated (Contreras, Carrón, Montero, Ramos & Recio, 2009).

Several ACE inhibitory peptides also showed anti-inflammatory activities. VPP and IPP were reported to decrease the expression of NF-κB subunit gene in rat aorta, but to

increase the expression of the endothelial nitric oxide synthase (eNOS) gene (Ehlers, Kivimaki, Turpeinen, Korpela & Vapaatalo, 2011), implying their protective role in endothelial function. eNOS generates NO in endothelial cells and NO is the key vasodilator and vital mediator in maintaining endothelial function and blood pressure. Egg ovotransferrin-derived antihypertensive peptides IRW also significantly suppressed TNF-α induced inflammation and superoxide generation (Majumder, Chakrabarti, Davidge & Wu, 2013). This peptide also increased NO-mediated vasodilation in mesenteric arteries of SHR animals apparently through increasing eNOS expression (Majumder, Chakrabarti, Morton, Panahi, Kaufman, Davidge & Wu, 2013). The anti-inflammatory and anti-oxidative activities of IRW, together with its role in improving endothelial function, contributed to its antihypertensive activity. Other peptides reported with NO-generating activity includes ovokinin (2-7) from egg white ovalbumin (Matoba, Usui, Fujita &Yoshikawa, 1999), carnosine as an endogeneous dipeptide (Gariballa &Sinclair, 2000), and flaxseed protein hydrolysate containing arginine-rich peptides (Udenigwe & Aluko, 2012). All these peptides showed endothelium-dependent vasorelaxation and stimulation of NO formation, whereas endothelium-independent vasorelaxation may also present.

It has also been reported that some peptides bind to opioid receptors, which present in the central nervous system and in peripheral tissues and are involved in the regulation of circulation and blood pressure. One example is YGLF, formed by proteolysis of α-lactalbumin with pepsin and trypsin, bound to opioid receptor and produced an endothelium-dependent relaxation of mesenteric arteries in SHR that was inhibited by an eNOS inhibitor. These observation indicates the capacity of YGLF to stimulate peripheral

opioid receptors and subsequent NO release, which lead to vasodilation (Nurminen et al., 2000; Sipola, Finckenberg, Korpela, Vapaatalo & Nurminen, 2002).

Those examples are not surprising, given the fact that the components in RAS are also engaged in other system implicated in the pathogenesis of hypertension. For instance, angiotensin II stimulates NADPH oxidase activity and modulates the expression of endothelin 1, leading to decreased bioactivity of NO and endothelial dysfunction. Consequently, the *in vivo* mechanism of blood pressure regulation could be different from ACE inhibition and the antihypertensive effect of peptides could be manifested by the interaction of peptides with other elements essential in the pathogenesis of hypertension.

2.5 General conclusion

Food-derived peptides have emerged as a novel alternative strategy to current pharmaceutical therapeutics. Regarding hypertension, ACE is a key enzyme in regulating blood pressure and ACE inhibitory peptides from a wide range of food sources have been reported. Molecular properties are essential in ACE inhibitory activity, based on which the potency of ACE inhibitory peptides can be predicted via QSAR modeling. A systematic review of plant and animal food proteins as potential sources of ACE inhibitory activity will contribute to establishing the rationale in the selection of protein sources.

Hypertension develops from a complex interaction of genetic and environmental factors. The pathophysiology of hypertension involves the interplay among RAS, oxidative stress and inflammation. This multidirectional relationship of risk factors implies that antihypertensive agents with a single activity can barely achieve the goal of controlling

blood pressure. A number of ACE inhibitory peptides identified from food sources also showed additional biological activities, which may enhance their actions in the treatment of hypertension.

The *in vivo* activity of peptides can be influenced by bioavailability. In-depth studies are necessary to investigate the underlying mechanisms of these peptides in animal models and clinical trials. Eventually, the long-term safety of these peptides needs to be evaluated before commercialization.

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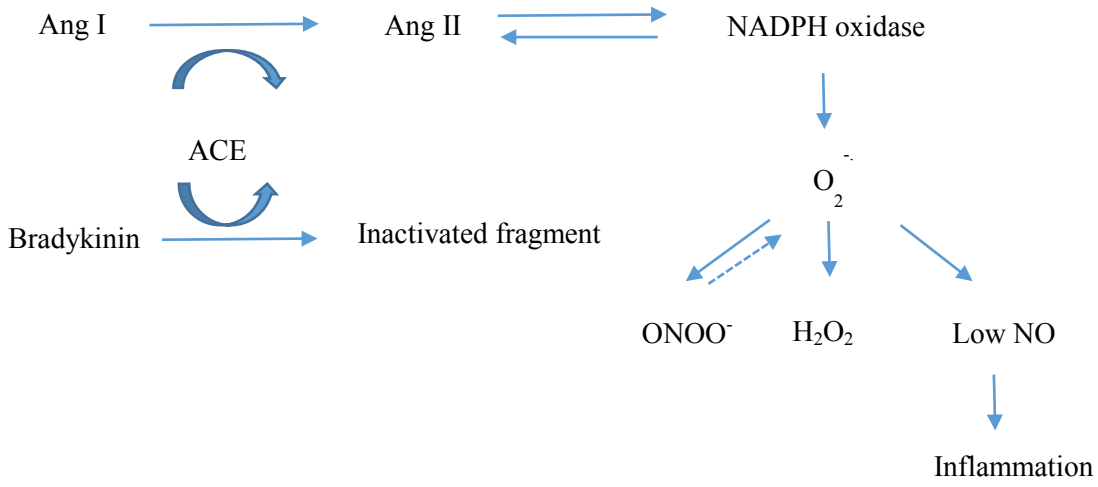

Figure 2.1. **Scheme for relation between ACE action and NADPH oxidase.** ACE hydrolyzes angiotensin I to produce angiotensin II and inactivates bradykinin. Angiotensin II stimulates the activity of NADPH oxidase to generate superoxide. Superoxide reacts with NO to form peroxynitrite, which reduces the bioactivity of NO, contributing to inflammation. Superoxide is subject to dismutation to form hydrogen peroxide in the presence of SOD. Conversely, NADPH oxidase is engaged in hypertensive responses induced by angiotensin II.

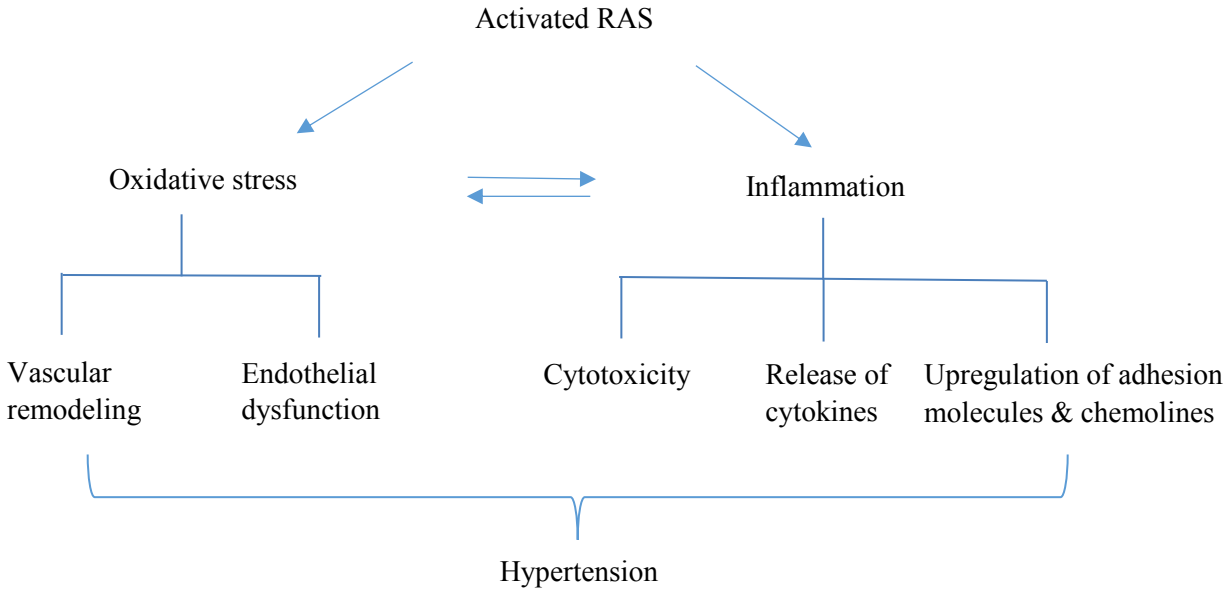

Figure 2.2. The role of oxidative stress and inflammation in the pathophysiology of hypertension. Oxidative stress and inflammation can be initiated by activated RAS. ROS cooperates with immunity in mediating hypertensive responses. Immune cells triggers aberrant elaboration of ROS in pathophysiological conditions, while misdirected oxidative stress in cardiovascular control organs elicits inflammatory responses, augmenting targeted organ damage. Oxidative stress causes vascular remodeling and endothelial dysfunction, whereas inflammation leads to cytotoxicity, release of various types of cytokines and upregulation of the expression of chemokines and adhesion molecules.

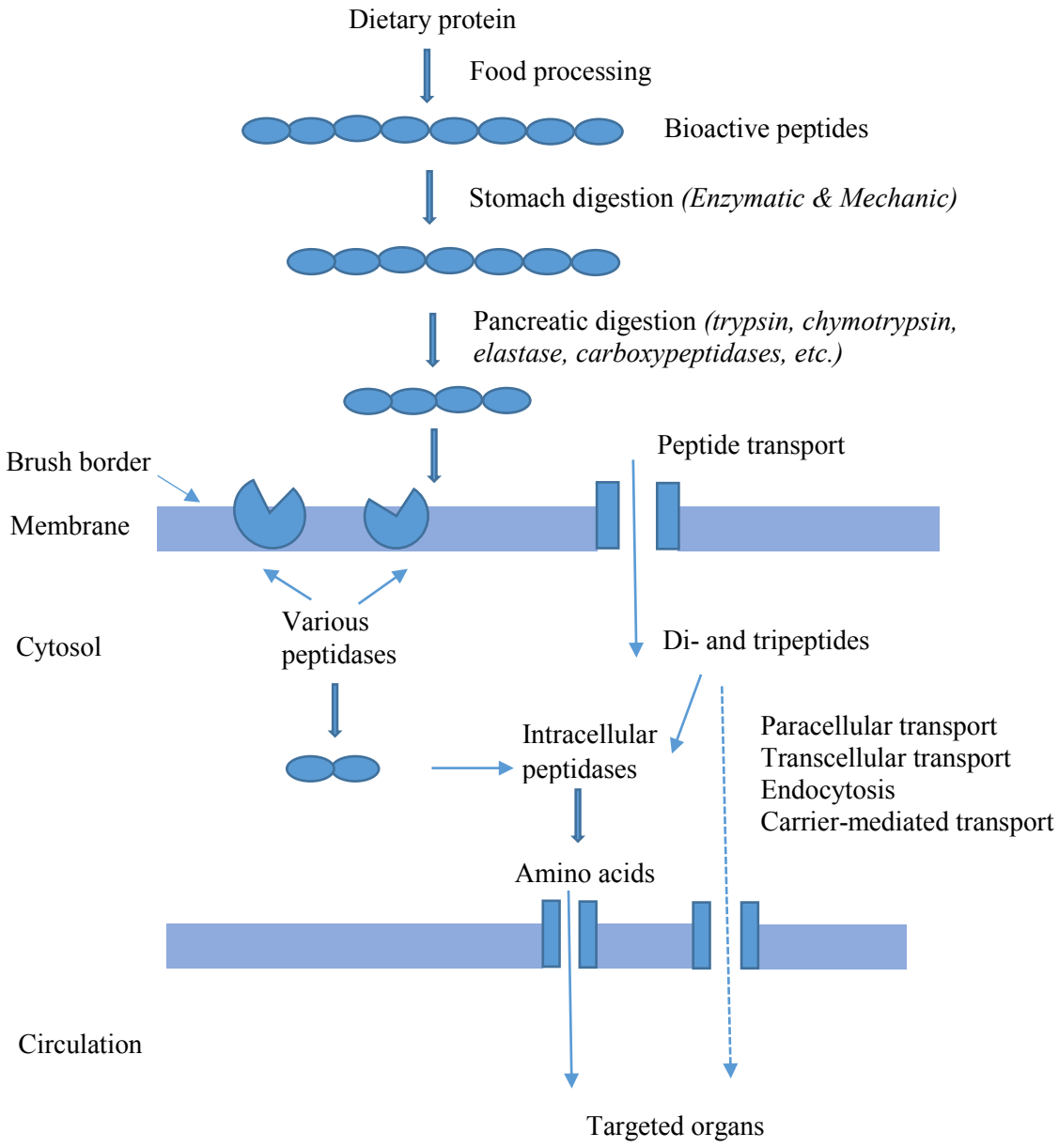

Figure 2.3. The gastrointestinal digestion of bioactive peptides. Bioactive peptides released from a dietary protein undergo luminal and membranous phases of digestion before entering the circulation. Di- and tripeptides can be absorbed intact across the intesetinal brush border membrane and may potentially be transported intact into the blood circulation through various mechanisms.

CHAPTER 3 - QSAR-aided in silico approach in evaluation of food proteins as precursors of ACE inhibitory peptides

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3.1 Introduction

Bioactive peptides play a central role in many physiological processes. A large number of bioactive peptides have been isolated from bacteria, fungi, plant, animal and human body that involve in signal transduction, enzyme inhibitory, antibiotic, and antiviral activities (Karelin, Blishchenko, & Ivanov, 1998). These peptides are becoming increasingly important as starting points for the development of drugs and drug-related compounds (Komajda & Wimart, 2000). It is also a fact that bioactive peptides derived from food proteins contribute greatly to the content of functional foods. Generally speaking, ingestion of proteins from a variety of food sources is considered to be essential for growth, maintenance and energy supply. Current literature supports that bioactive peptides sequestered within the primary structure of proteins can be released by action of proteases, or food processing, thus exerting potential health benefits *in vivo* (Miyoshi, Ishikawa, Kaneko, Fukui, Tanaka, & Maruyama, 1991; Ariyoshi, 1993; Korhonen & Pihlanto, 2003). It is now well accepted that food protein-derived bioactive peptides have vast potential for improving human health and preventing chronic diseases through their impacts on the body's gastrointestinal, defense, regulatory, and nervous systems (Yamamoto, Ejiri, & Mizuno, 2003; FitzGerald, Murray, & Walsh, 2004; Mine & Shahidi, 2006; Shimizu & Son, 2007).

Among various bioactive peptides, angiotensin converting enzyme (ACE)-inhibitory peptides are among the most extensively studied ones over the last few decades (Ariyoshi, 1993; Korhonen & Pihlanto, 2003; FitzGerald et al., 2004; Vercruysse, Van Camp, & Smagghe, 2005; Hong, Ming, Yi, Zhanxia, Yongquan, & Chi, 2008). As a key enzyme in the regulation of blood pressure and body fluid, ACE converts angiotensin I to the

potent vasoconstrictor, an octapeptide called angiotensin II, and also hydrolyzes bradykinin, a vasodilatory agent (Yang, Erdos, & Levin, 1970). Therefore, excessive levels of ACE activity could lead to increased vasoconstriction and consequently, the development of high blood pressure and associated pathological symptoms. Inhibition of ACE activity is key to the management of hypertension (Atkinson & Robertson, 1979). However, synthetic ACE inhibitory drugs are reported to have side effects such as dry cough, taste disturbances and skin rashes, as well as alterations in serum lipid metabolism (Atkinson & Robertson, 1979; Sesoko & Kaneko, 1985; Israili & Hall, 1992). Therefore, food protein-derived ACE-inhibitory peptides are considered as a safer alternative to commercial ACE-inhibitory drugs (Hartmann & Meisel, 2007; Hong, Ming, Yi, Zhanxia, Yongquan, & Chi, 2008; Geleijnse & Engberink, 2010).

Although at its infancy, accumulated information on various ACE inhibitory peptides has greatly advanced our understanding of their structure and activity relationships (Pripp, Isaksson, Stepaniak, & Sorhaug, 2004; Wu, Aluko, & Nakai, 2006; Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009). Our previous study showed that both the type of amino acid residues and the sequences determine the potency of ACE inhibitory peptides; for example, the presence of hydrophobic amino acids and positively charged amino acids contribute to enhanced inhibitory potency (Wu, Aluko, & Nakai, 2006). Knowledge on the structure and activity relationship is important in facilitating our process in exploring the potent ACE inhibitory peptides from food proteins. Our recent study using QSAR prediction has led to characterization of three novel potent peptides, IRW, IQW and LKP, from eggs (Majumder & Wu, 2010). However, production of potent food-derived ACE inhibitory peptides remains to be a continuous challenge due to lack of

rationales in the selection of both enzyme and substrate (Li, Le, Shi, & Shrestha, 2004). *In silico* techniques have been successfully applied in searching ACE inhibitory peptides based on known sequence matching from available databases (Cheung et al., 2009; Iwaniak & Dziuba, 2009; Vercruysse, Smagghe, van der Bent, van Amerongen, Ongenaert, & Van Camp, 2009), whereas lacking the power for predicting the activity of new sequences. It also should be noted that many previous studies on ACE inhibitory peptides focused on one protein at one time whereas a systematic study of various proteins has not been attempted. Therefore the rationale for selection of protein sources has not been defined. The objectives of the study were to evaluate the potential of food proteins from major food commodities as precursors in preparing ACE inhibitory peptides, and thus to establish the rationale for choosing the appropriate substrate proteins.

3.2 Materials and methods

3.2.1 Proteins

Proteins from 15 major food commodities, egg, soybean, milk, pork, beef, chicken, pea, oat, canola, barley, bonito, tuna, Atlantic salmon, Chum salmon, Pollock, were selected for the study. Major egg white protein sequences were obtained from the National Center for Biotechnology Information (NCBI) protein database (http://www.ncbi.nlm.nih.gov). The other protein sequences were obtained from ExPASy Proteomics Server (http://ca.expasy.org/sprot/) and the information on proteins was summarized in supplemental Table 1.

3.2.2 *In Silico* proteolysis

In silico proteolysis was performed by using the Peptidecutter software (http://www.expasy.ch/tools/peptidecutter/) available at ExPASy Molecular Biology Server with thermolysin, and/or in combinations with pepsin and trypsin to study their stability against simulated gastrointestinal tract enzymes.

3.2.3 ACE inhibitory activity prediction

Peptide sequences were converted into X-matrix by using z-scales (Hellberg, Sjostrom, Skagerberg, & Wold, 1987; Hellberg et al., 1991). In brief, the amino acid at the amino terminus was designed as *n*1, and its properties were described as *n*1*z*1, *n*1*z*2, and *n*1*z*3; the amino acid adjacent to the amino terminus was designed as *n*2, and its properties were described as *n*2*z*1, *n*2*z*2, and *n*2*z*3, etc. Activity of each peptide was then predicted by our previously reported QSAR models (Hellberg et al., 1991; Wu et al., 2006) using the SIMCA-P software version +11 (Umetrics INC., Kinnelon, NJ). Activity was expressed as IC_{50} values, defined as the concentration of the peptides that can inhibit 50% of the enzyme activity.

3.3 Results and discussion

3.3.1 Peptide profiles

Different proteases have been used to prepare bioactive peptides. Previous studies have repeatedly shown that thermolysin digestion of food proteins could release potent ACE inhibitory peptides (Miyoshi, Ishikawa, Kaneko, Fukui, Tanaka, & Maruyama, 1991; Yokoyama, Chiba, & Yoshikawa, 1992; Yano, Suzuki, & Funatsu, 1996; Fujita, Yokoyama, & Yoshikawa, 2000; Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001;

Cheung et al., 2009; Udenigwe, et al., 2009; Majumder & Wu, 2010). Thermolysin cleaves bonds with bulky and aromatic residues (Ile, Leu, Val, Ala, Met, Phe) in position P1' (Keil, 1992); cleavage by thermolysin is favored with aromatic sites in position P1 but hindered with acidic residues in position P1. Therefore, peptides generated by thermolysin digestion usually contain bulky and aromatic residues, which were known to be important for the potency of peptides (Ariyoshi, 1993; Li et al., 2004; Wu et al., 2006a; Wu, Aluko, & Nakai, 2006b; Hong, Ming, Yi, Zhanxia, Yongquan, & Chi, 2008).

In silico digestion of food proteins from 15 food commodities by thermolysin generated 5709 peptides ranging from 2 to 6 amino acid residues. Peptides were categorized into 3 groups based on predicted activity: potent peptides with IC_{50} values less than 10 μM, moderate active peptides with IC_{50} values greater than 10 but less than 50 μM, and weak peptides with IC_{50} values greater than 50 μM. Our analysis showed that meat proteins from pork, beef and chicken had the highest number of potent peptides, followed by egg, soybean and canola, while fish (with the exception of salmon) and cereals (oat and barley) had the lowest number of potent ACE-inhibiting peptides (Fig 1A). Meat proteins from pork, beef and chicken also had the highest total number of peptides, followed by egg, soybean and canola, while fish (with the exception of salmon) and cereals (oat and barley) had the lowest total number of peptides.

In order to test their resistance to gastrointestinal enzymes, peptides produced *in silico* by thermolysin were further processed by pepsin and trypsin subsequently. The results indicated that the total number of peptides increased from 5709 to 6619 and 8439 for thermolysin-pepsin and thermolysin-pepsin-trypsin, respectively. However, the number of potent peptides decreased as further digestion was applied, showing that some peptides

derived from thermolysin hydrolysis are not resistant to gastrointestinal digestion (with the exception of egg) (Fig 1B and 1C). The distribution of potent peptides obtained from the different food protein sources followed the same pattern as in the thermolysin hydrolysis when pepsin and trypsin were applied. Based on our *in silico* digestion and QSAR prediction, our results indicated that meat, egg, soybean, canola and pea are potential good sources, whereas cereals and fish (with the exception of salmon) are poor sources of ACE inhibitory peptides. Generally speaking, the raw material of plant protein source is cheaper than that of animal protein; therefore there is advantage in preparing plant-derived peptides in this regard. However, cost of protein extraction from raw material is another economic factor. It should be noted that ACE inhibitory peptides from milk proteins, such as VPP and IPP, have already been commercialized.

3.3.2 ACE inhibitory peptides from plant proteins

Plant derived ACE inhibitory peptides were reviewed recently (Guang & Phillips, 2009). Thermolysin was used to prepare pea protein (Wu et al., 2006a; Wu et al., 2006b), and recently in oat (Cheung et al., 2009) and flaxseed (Udenigwe, et al., 2009). Peptides from thermolysin digestion of soy protein have not been reported. Soy protein was reported to have benefits for cardiovascular diseases and various ACE inhibitory peptides were characterized from soy products and hydrolysates while thermolysin preparation of soy ACE inhibitory peptides have not been reported (Gouda, Gowda, Rao, & Prakash, 2006). *In silico* digestion of soybean proteins by thermolysin resulted in 29 new potent peptides ($IC_{50} < 10$ μM), while more than half of them are not resistant to the gastrointestinal (GI) enzymes (Table 1). IC_{50} values from soybean-derived ACE inhibitory peptides ranged from 1.69 to 153 μM; the most potent three peptides are DLP (IC_{50}=4.8 μM) (Wu & Ding,

2002), HHL (IC_{50}=2.2 μM) (Shin et al., 2001) and VLIVP (IC_{50}=1.69 μM) (Gouda et al., 2006). Our *in silico* study indicated that thermolysin might release more potent ACE inhibitory peptides from soybean proteins than other enzymes reported in literature. Some peptides were detected in various subunits of the proteins in our study. For instance, VIP (IC_{50}=9.2 μM) was found to be present in α', α and β chains of β-conglycinin as well as one subunit of glycinin, A_3B_4; AWW (IC_{50}=6.5 μM) presented in glycinin G1 and glycinin G2 and AFPGS (IC_{50}=3.7 μM) presented in α and β chain of β-conglycinin. It should be noted that the combination of thermolysin and pepsin generated new potent peptides such as VRF (IC_{50}=1.3 μM) from β chain of β-conglycinin and ARF (IC_{50}=3.0 μM) from α, α' chain of β-conglycinin. VRF was previously predicted through quantitative structure-activity relationship modeling. An IC_{50} value of 24.0 μM was reported for the synthesized peptide (Wu, Aluko & Nakai, 2006).

Canola is a farm-gate crop in Canada. Two potent peptides, VSV (IC50=0.15μM) and FL (IC_{50}=1.33 μM), derived from both napin and cruciferin, were reported (Wu et al., 2008). Our analysis showed that there are more potent peptides from thermolysin digestion of canola proteins than the reported ones. 34 potent peptides were produced by *in silico* digestion of canola and nearly half of them are resistant to GI enzymes (Table 1). Although potent ACE inhibitory peptides from napin were reported from previous study, all potent peptides were obtained from thermolysin digestion of cruciferin in this study. Two peptides, VVPGC and PQW, were found in various subunits of cruciferin, with predicted IC_{50} value of 8.6 μM and 7.7 μM, respectively.

Our previous study has predicted two potent peptides, LRW and FW, from pea proteins through quantitative structure-activity relationship modeling and their synthesized forms

exhibited IC_{50} values of 0.23 μM and 5.9 μM, respectively (Wu et al., 2006a; Wu et al., 2006b). We further confirmed that both FW and LRW could be released by thermolysin digestion of legumin A and legumin A2 theoretically, with the other 18 potent peptides generated from major pea proteins by *in silico* digestion with thermolysin (Table 1). There are only 4 peptides remained after GI hydrolysis in this study. They are FRC (IC50= 7.0 μM), LVP (IC_{50} value= 9.9 μM), IDPNG (IC_{50}= 8.4 μM) and VIP (IC_{50}= 9.2 μM), whereas the rest of them were degraded after GI digestion. For example, VRKGQ (IC_{50}= 1.5 μM), obtained from thermolysin digestion of legumin J, could be degraded into two smaller peptides by GI hydrolysis, VR and GQ, whose IC_{50} values were increased to 98 μM and 1910 μM, respectively. Besides, new peptides were also produced along with the GI digestion. YW (IC_{50}= 8.2 μM) was obtained from a peptide consisting 17 amino acids after further digestion with pepsin. YW was also previously predicted from our QSAR study and its synthetic peptide was reported to have an IC_{50} value of 43.6 μM (Wu et al., 2006a).

Oat and barley are two important cereals in Western Canada. Study on preparing ACE inhibitory peptides from these two commodities is rare with the exception of a recent study by Cheung et al who reported some matched sequences from thermolysin digestion (Cheung, Nakayama, Hsu, Samaranayaka & Li-Chan, 2009). In our study, 6 potent peptides from oat proteins were generated from 12S globulin by *in silico* digestion of thermolysin and two of them, IEPQG and LKP, with the predicted IC_{50} values of 5.3μM and 2.8μM, respectively, were resistant to GI enzymes (Table 1). LKP was previously characterized from chicken with an IC_{50} value of 0.32 μM and it could reduce the systolic blood pressure of spontaneously hypertensive rats (SHR) by 18 ΔmmHg after oral

administration in 4 hours (60 mg/kg) (Fujita et al., 2000). It seems that thermolysin digestion of oat protein might have *in vivo* activity. There is no report on ACE inhibitory peptides from barley protein. In our study, 3 potent peptides were predicted from *in silico* study of B hordein, and two of them, VRMPQ and LQPHQ, showed resistance to the GI enzymes, with IC_{50} values of 2.7 μM and 6.8 μM, respectively. Three new potent peptides, one peptide FW (IC_{50}= 3.9 μM) from B hordein, and two peptides LQQGG (IC_{50}=8.6μM) and ATF (IC_{50}=9.6μM) from D hordein), were further released by GI enzymes.

3.3.3 ACE inhibitory peptides from milk proteins

ACE inhibitory peptides were most extensively studied in milk proteins (FitzGerald et al., 2004; Hong, Ming, Yi, Zhanxia, Yongquan, & Chi, 2008; Geleijnse & Engberink, 2010). *In silico* digestion by thermolysin released 26 potent peptides from milk and a major proportion of them could resist GI hydrolysis, whereas LW and LRP from α_{S1} casein and lactoferrin with the predicted IC_{50} values of 4.4 μM and 2.3μM, respectively were not resistant to GI digestion (Table 2). ACE inhibitory peptides were reported largely from caseins, including the well-know tripeptides, IPP and VPP, which are commercialized in several fermented milk products (FitzGerald et al., 2004; Geleijnse & Engberink, 2010). A casein hydrolysate containing a C12 peptide, FFVAPFEVFGK, has also been marketed (FitzGerald et al., 2004). Two peptides, LLF and LQKW, obtained from thermolysin digestion of β-lactoglobulin, showed IC_{50} values of 3.5μM and 82.4μM, respectively (Hernandez-Ledesma, Amigo, Recio, & Bartolome, 2007; Hernandez-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007), and YGLF, derived from α-lactalbumin, have been confirmed to be able to reduce both systolic and diastolic blood pressure of SHR and

Wistar Kyoto rats (Nurminen et al., 2000). Our study showed that lactoferrin, a well - known protein for its diverse biological activities including antimicrobial, antiviral and antioxidant activities (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002), possesses the most potent peptides in milk including VRW, with a predicted IC_{50} value of 0.6 μM (Supplemental table 3). Lactoferricin-related peptides were reported to have ACE inhibitory activity (Centeno et al., 2006). It should be noted that lactoferricin-related peptides are rich in hydrophobic and positively charged amino acid residues, which were postulated to play an important role in the potency of ACE inhibitory peptides.

3.3.4 ACE inhibitory peptides from livestock meats

ACE inhibitory peptides from animal muscle proteins were reviewed (Vercruysse et al., 2005). High similarity in sequence was found among major proteins from pork, beef and chicken, which may accounts for the presence of many potent peptides with the same primary sequences (Table 3). For instance, pork, beef and chicken share the same sequences of actin alpha cardiac muscle and actin alpha skeletal muscle, which are the two major types of actin in skeletal and cardiac muscle. A peptide of IWHHT, with predicted IC_{50} value of 0.8 μM, was found in both of the two proteins. This sequence had previously been obtained from dried bonito with an IC_{50} value of 5.8 μM and was reported to reduce systolic blood pressure in SHR by 60 mmHg after intravenous administration (10mg/kg) (Fujita et al., 2000). However, our test showed that this peptide was susceptible to GI hydrolysis and the product, WHHT, possess a much lower ACE inhibitory activity with a predicted IC_{50} value of 310.8 μM. Most peptides produced by thermolysin hydrolysis were not resistant to GI enzymes; however, new peptides could be obtained from subsequential digestion of pepsin and trypsin. IHETT, for instance, with an

IC_{50} value of 2.7 μM, was released from a combination of thermolysin, pepsin and trypsin.

ACE inhibitory peptides, GPL (IC_{50}=2.55 μM) and GPV (IC_{50}=4.67 μM), were also obtained from bovine skin gelatin hydrolysate (Kim, Byun, Park, & Shahidi, 2001). Thermolysin was used to digest pork skeletal muscle proteins but resulted in peptides with weak ACE inhibitory activity, such as ITTNP (IC_{50}=549μM) and MNPPK (IC_{50}=945.5μM) (Arihara et al., 2001), whereas potent ACE inhibitory peptides were characterized from chicken muscle proteins, such as LKA (IC_{50}=8.5 μM), LKP (IC_{50}=0.32 μM) and IKW (IC_{50}=0.21 μM). LKP and IKW have shown to be able to decrease systolic blood pressure of SHR by 18 mmHg and 17 mmHg, respectively after oral administration in 4 hours (60 mg/kg) (Fujita et al., 2000). Our study showed the possibility of releasing more potent antihypertensive peptides from pork, beef and chicken. *In silico* digestion of the three commodities by thermolysin released 85, 66, 46 potent peptides from pork, beef and chicken, respectively (Supplemental table 4). IKP (IC_{50}= 2.3μM) presented universally in all three proteins. This peptide has been obtained from bonito and its IC_{50} value was tested to be 1.6 μM. *In vivo* study indicated that IKP could reduce the systolic blood pressure of SHR by 20 mmHg after 6 hours (Fujita et al., 2000). This peptide was found to be resistant to further GI digestion. Less than one-fourth of the potent peptides obtained from thermolysin digestion resistant to GI hydrolysis while incorporating GI enzymes might lead to producing new potent peptides. IEF, for example, was found after digestion of myosin light chain of chicken by a combination of thermolysin, pepsin and trypsin with a predicted IC_{50} value of 3.8 μM.

3.3.5 ACE inhibitory peptides from fish

ACE inhibitory peptides from salmon, sardine, bonito and tuna were reviewed (Vercruysse et al., 2005). A number of potent peptides were characterized from thermolysin digestion of bonito (Yokoyama et al., 1992; Wu et al., 2006a). LKPNM, a peptide obtained from dried bonito with a reported IC_{50} value of 2.4 μM, could be hydrolyzed by ACE to produce LKP with a significantly increased ACE inhibitory activity (reported IC_{50}= 0.32 μM). LKP also required lower minimum effective dose and shorter time to reach the maximum reduction of blood pressure than LKPNM (Yokoyama et al., 1992; Fujita & Yoshikawa, 1999). Various ACE inhibitory peptides were also reported from sardine using alkaline protease (Matsufuji, Matsui, Seki, Osajima, Nakashima, & Osajima, 1994). One dipeptide, VY, is able to reduce both systolic and diastolic blood pressure on mild hypertensive volunteers (Kawasaki et al., 2000). Thermolysin digestion of salmon resulted in novel peptides, VW, MW, and IW, with reported IC_{50} values of 2.5, 9.9 and 4.7 μM, respectively. VW and IW were not found in this study but MW was present with a predicted IC_{50} value of 7.6 μM, and it was resistant to GI digestion (Table 4). Our *in silico* study showed 4 potent peptides released from thermolysin digestion with predicted IC_{50} values less than 3 μM, including, VCYNQ (IC_{50}=2.0 μM) from Pollock, IKP (IC_{50}=2.3 μM), LRKKQ (IC_{50}=2.4 μM) and LRCNG (IC_{50}=2.9 μM) from Chum salmon. IKP was also found from pork, beef and chicken and was resistant to GI digestion, indicating its potential bioavailability in vivo.

3.3.6 ACE inhibitory peptides from egg proteins

Three ACE inhibitory peptides, LW (IC_{50}=6.8 μM), ERKIKVYL (IC_{50}=1.2 μM) and FFGRCVSP (IC_{50}=0.4 μM), were characterized from thermolysin digestion of ovalbumin

by Fujita et al. (2000). Miguel et al. (2004) reported a number of peptides from digestion of ovalbumin/egg white using gastrointestinal enzymes. Our *in silico* study released 47 potent peptides and a major proportion of them were resistant to GI digestion (Table 5). Some examples of potent peptides were IRP (IC_{50}=1.9 μM) from high-density lipoprotein (HDL), FW (IC_{50}=3.9 μM) from low-density lipoprotein (LDL) and FYTGG (IC_{50}=3.8 μM) from ovalbumin. Several peptides showed potent inhibitory activity, such as IRWCT (IC_{50}=1.0 μM) from ovotransferrin, but was found to be susceptible to GI hydrolysis. Interestingly, the digestion of thermolysin coupled with pepsin coule produce new potent peptides, such as IRW (IC_{50}=0.6 μM), IQW (IC_{50}=1.4 μM) and LW (IC_{50}=4.4 μM). LW was previously reported to be the only peptides showing in vivo activity in SHR from peptic digestion of ovalbumin (Fujita et al., 2000).

3.4 Conclusion

Proteins from 15 major food commodities were systematically studied as the potential precursors of ACE inhibitory peptides. Our results showed that meat proteins from pork, beef and chicken contain the highest number of potent peptides (IC_{50} < 10 μM), followed by proteins from egg, soybean and canola, whereas proteins from fish (with the exception of salmon) and cereals (oat and barley) contain the least number of peptides. It should be noted that the release of peptides by *in silico* digestion might be different from experimental condition where the release of peptides could be affected by a number of factors including the state of the substrate, temperature, pH and specificity of enzyme. We have proven that predicted peptides could be released with carefully manipulated digestion conditions. Our on-going study is to characterize the presence of these potent peptides in individual hydrolysates, and to confirm their activity using synthetic peptides.

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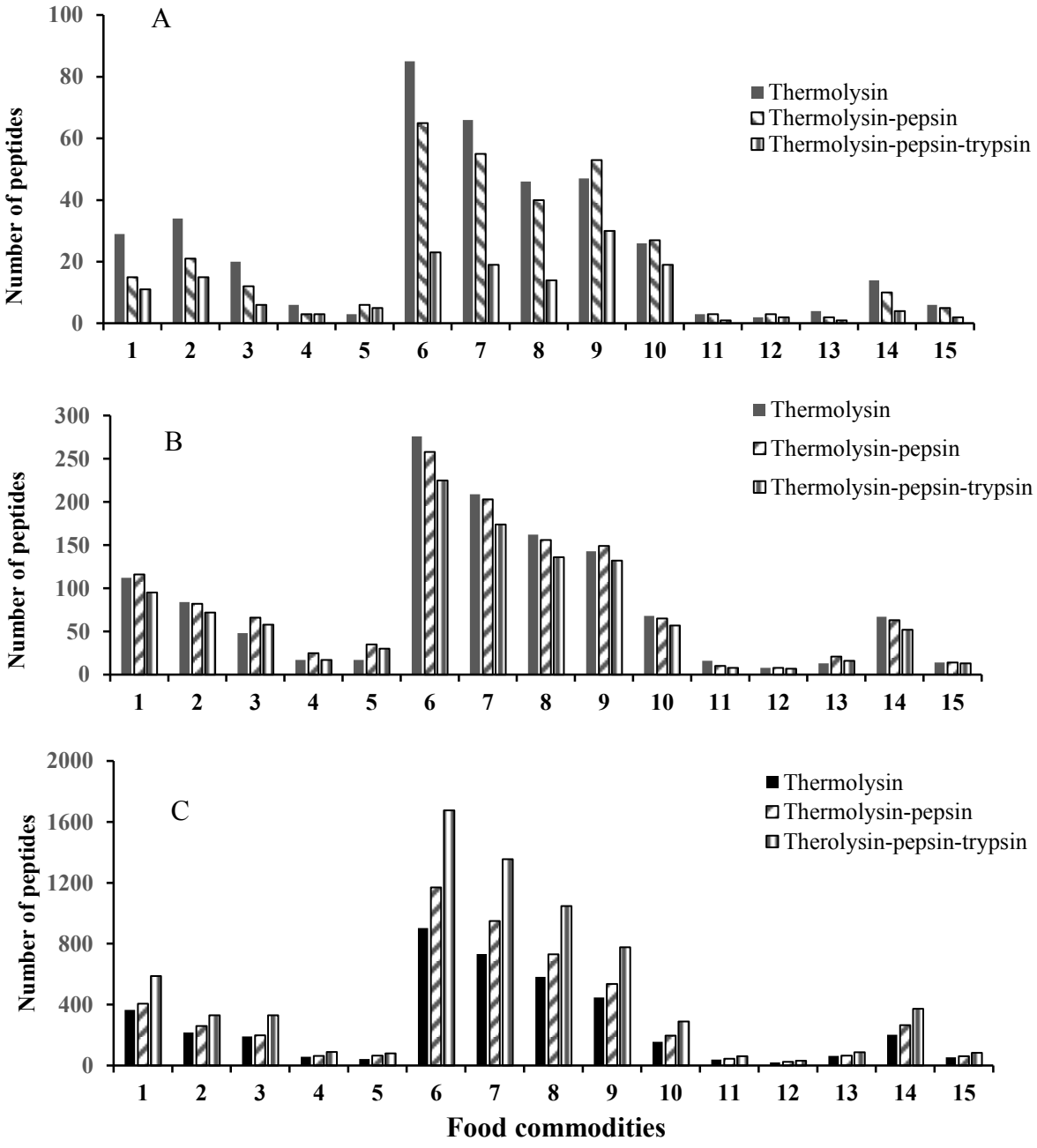

Figure 3.1. Numbers of ACE inhibitory peptides from various food commodities with IC_{50} values (A) <10 μM; (B) 10 <IC_{50}< 50 μM; (C) IC_{50}>50μM. Numerals on X-axis refers to 1, soybean; 2, canola; 3, pea; 4, oat; 5, barley; 6, pork; 7, beef; 8, chicken; 9, egg; 10, milk; 11, Skipjack tuna; 12, Bluefin tuna; 13, Atlantic salmon ; 14, Chum salmon; 15, Alaska Pollock.

Talbe 3.1. Potent Peptides (IC_{50}< 10μM) and their locations from Plant proteins by thermolysin, and their combinations with pepsin and trypsin.

Protein Source	Protein name	Sequence Thermolysin (Location[1])	Sequence Thermolysin-Pepsin (Location[1])	Sequence Thermolysin-pepsin-Trypsin (Location[1])
Soybean	Beta-conglycinin alpha' chain	AFPRS(f592-596), VIP(f541-543)	ARF(f4-f6, **VIP(**f541-543**)**	**VIP**(f541-543)
	Beta-conglycinin Beta chain	AFPGS(f392-396), IYSNN(f248-252), VIP(f341-343)	VRF(f4-6), **VIP**(f341-343)	**VIP**(f341-343)
	Beta-conglycinin alpha chain	AFPGS(f558-562), VIP(f507-509)	ARF(f4-f6), **VIP**(f507-509)	**VIP**(f507-509)
	Glycinin G1	LKSQQ(f466-470), AEFGS(f359-363), LSW(f352-354), LEF(f226-228), AWW(f149-151)		
	Glycinin G2	LW(f342-343), LKSQQ(f456-460), VRN(f237-239), AWW(f146-148)	FREGD(f133-137), **VRN**(f237-239)	
	Glycinin A3B4	LYRNG(f398-402), IRH(f138-140),	YW(f155-156), **IRH**(f138-140),	YW(f155-156),

		VNP(f514-516), LEY(f462-464), ISP(f148-150), VIP(f484-486)	**VNP** (f514-516), **ISP**(f148-150), **VIP**(f484-486)	**VNP**(f514-516), **ISP**(f148-150) **VIP**(f484-486)
	Glycinin G4	LYKNG(f431-435), LRQSQ(f530-534), LNECQ (f29-33), **IRH**(f138-140)	YW(f155-156), **IRH**(f138-140)	YW(f155-156), GCETR (f372-376), ARPSR(f393-397), ISSSK(f24-28)
	Glycinin G3	LRRQQ(f452-456), LSW(f338-340), AYW(f146-148)		
Pea	Legumin A	FW(f149-150), LGGC(f16-19), LRW(f374-376), FRC(f62-64), LVP(f505-507)	RW(f375-376), **FRC**(f62-64), **LVP**(f505-507)	**LVP**(f505-507)
	Legumin A2	FW(f150-151), LGGC(f17-20), LRW(f377-379), FRC(f63-65), LVP(f508-510)	RW(f378-379), **FRC**(f63-65), **LVP**(f508-510)	**LVP**(f508-510)
	Legumin J	VRKGQ(f418-422), LYRNG(f376-380) LRQRQ(f476-480), LHLPS(f81-85),IDPNG (f76-80), LRY(f364-366), LEY(f442-444)	YW(f153-154), **VRKGQ**(f418-422) **IDPNG** (f76-80)	YW(f153-154) **IDPNG**(f76-80)
	Vicinin	AFPGS(f417-421), LSRGQ(f227-231), VIP (f366-368)	ERGDT(f120-124)**,** IKL(f125-127)**, VIP** (f366-368)	SKPHT(f80-84) **VIP**(f366-368)

Barley	B-hordein	VRMPQ(f 154-158), LQPHQ(f 251-255) FRHEA(f 189-193)	FW(f 83-84), **VRMPQ**(f154-158) **LQPHQ** (f 251-255)	FW(f 83-84) **LQPHQ**(f 251-255)
	D-hordein		LQQGG(f 120-124), ATF(f194-196), ATF (f316-318)	LQQGG(f 120-124), ATF (f194-196), ATF(f316-318)
Oat	12S globulin	LRRGQ(f413-417), LCNGS(f18-22), IEPQG(f88-92), LKP(f262-264), IEY(f66-68), FRC(f76-78)	YW(f380-381), **IEPQG**(f88-92), **LKP** (f262-264)	YW(f380-381) **IEPQG**(f88-92) **LKP**(f262-264)
Canola	BNC1	VW(f53-54), FRW(f419-421), VRY(f69-71) AQW(f185-187), LRCSG(f61-65), LHGST (f18-22), LYLPS(f78-82), VVPGC(f102-106)	RW(f420-421), **VVPGC**(f102-106), **VRY** (f69-71), PQW(f362-364)	**VVPGC**(f102-106) PQW(f362-364)
	CRU1	LYLPT(f85-89), VQKGQ(f415-419), VIPQG(f422-426), IEQGG(f80-84), VVPGC (f109-113), IRC(f68-70), FEW (f438-440), LQY (f361-363)	**VQKGQ**(f415-419), **VIPQG**(f422-426) **IEQGG**(f80-84), **VVPGC**(f109-113), **IRC** (f68-70), VEY(f58-60)	**VIPQG**(f422-426) **IEQGG**(f80-84) **VVPGC**(f109-113) VEY(f58-60)
	BNC2	LRCSG(f61-65), LHGST (f18-22), LYLPS (f78-82), VVPGC(f102-106), IESQG(f73-77), FRW (f425-427), VRY(f69-71) AQW(f191-193)	RW(f426-427), **VVPGC**(f102-106) **IESQG**(f73-77), **VRY**(f69-71) PQW(f368-370)	**VVPGC**(f102-106) **IESQG**(f73-77) PQW(f368-370)

CRU4	LRCSG(f60-64), LYLPT (f77-81), IEPQG (f72-76), LEPSQ(f37-41), LQNQQ(f238-242), VVPQG(f379-383), VTPGC(f101-105), LRP(f188-190), FQW(f395-397) AQW(f158-160)	**IEPQG**(f72-76), **LEPSQ**(f37-41), **VVPQG**(f379-383), **VTPGC**(f101-105) **LRP**(f188-190), PQW(f338-340)	**IEPQG**(f72-76), **LEPSQ** (f37-41), **VVPQG**(f379-383), **VTPGC**(f101-105) **LRP**(f188-190), PQW(f338-340)

Note:

The proteins were first in silico digested by thermolysin, and were further in silico digested by pepsin and trypsin. The peptides with bolded letters in the table are resistant to pepsin/trypsin digestion.

[1] represents the location of the peptides sequences in the proteins

Table 3.2. Potent Peptides (IC_{50}< 10μM) and their locations from milk proteins by thermolysin, and their combinations with pepsin and trypsin.

Protein name	Sequence Thermolysin (Location[1])	Sequence Thermolysin-Pepsin(Location[1])	Sequence Thermolysin-pepsin-Trypsin (Location[1])
Alpha S1 casein	LW(f213-214), FYPEL(f160-164)	**LW**(f213-214)	**LW**(f213-214)
Alpha S2 casein	VRY(f219-221), VRN (f59-61) , LQY (f111-113)	**VRY**(f219-221), MKP (f205- 207) **VRN**(f59-61), QKF(f187-189), LNP (f121-123), VIP(f215-217)	MKP(f205-207) LNP(f121-123) VIP(f215-217)
Kappa casein	AKSCQ(f106-110) , IQY (f49-51), AKP (f83-85)	**AKSCQ**(f106-110), **AKP** (f83-85) SRY(f54-56)	**AKP**(f83-85)
Beta casein	**VEP**(f131-133), IHP(f64-66)	**IHP**(f64-66)	VEP(f131-133), **IHP**(f64-66)
β-lactoglobulin	MKC(f1-3)	**MKC**(f1-3)	
Lactoferrin	LRP(f93-95), LEP (f161-163), FRC (f549551) , ADW(f577-579), FKC(f215-217), LKN (f347-349), IIP (f145-147) ITC(f53-55), AGW (f142-144), VTC (f388-390), LVP(f426-428), VRWCT (f25-29), LTWNS(f465-469)	VW(f567-568), GGRPTY (f671-676) VKNDT (f562 -566) , VKETT (f228232) VRW(f25-27), **LRP**(f93-95), LRP(f151-153), ARY (f359-361), **LEP** (f161-163) **IIP** (f145-147), **ITC** (f53-55), **VTC**(f388-390), **LVP**(f426-428)	VW(f567-568), GGRPTY(f671-676) SCHTG(f133-137), **LRP** (f93-95), LRP (f151-153), WQW(f41-43), LEP (f161-163), **IIP** (f145-147), **ITC** (f53-55), **VTC** (f388-390), **LVP** (f426-428)
Alpha-lactalbumin		LKD(f31-33), PEW(f43-45)	PEW(f43-45)

Note: The proteins were first in silico digested by thermolysin, and were further in silico digested by pepsin and trypsin. The peptides with bolded letters in the table are resistant to pepsin/trypsin digestion.

[1] represents the location of the peptides sequences in the proteins

Table 3.3. Potent Peptides (IC_{50}< 10μM) and their locations from livestock proteins by thermolysin, and their combinations with pepsin and trypsin.

Protein Source	Protein name	Sequence Thermolysin (Location [1])	Sequence Thermolysin-Pepsin (Location[1])	Sequence Thermolysin-pepsin-Trypsin (Location[1])
Pig bovine chicken	Actin alpha skeletal	MW(f357-358), VW (f341 342), IWHHT (f87 -91), VHRKC (f372-376), IYEGY (f167-171)	IHETT(f276-280), **VHRKC**(f372-376), RCPET(f258-262)	IHETT(f276-280)
	Actin alpha cardiac	MW(f357-358), VW (f341-342) IWHHT (f87-91), VHRKC (f372-376), IYEGY (f167-171)	IHETT(f276-280), **VHRKC**(f372-376), RCPET(f258-262)	IHETT(f276-280)
Meat (pig)	MHC 2	LW(f440-441), VKHWPW(f828-833), LRCNG (f697-701), VKEDQ (f72-76), LKDTQ (f1654 -1658), AYTQQ (f1311 -1315), LYQKS (f611-615), ARCRG (f794-798), LHEPG (f97-101), MHYGN (f360-364), AYEES (f1491-1495), ITRTQ (f789-793), IKELQ (f1112-1116), LELNQ (f1567 -1571), IKP (f840-842), VRC (f674-676), IQY (f193-195), VKN (f979-981), IHY (f583 -585)	VKHWPW(f828-833), LRCNG(f697-701), VKEDQ(f72-76), QHEAT (f1188-1192), ARCRG(f794-798), SRGKQ (f1306-1310), ITRTQ(f789-793), EEAGG(f1155-1159), IKP(f840-842), VRC(f674-676), VKD(f1794-1796), VNP(f126-128), VEY(f803-805), VKN(f979-981), AKC(f1413-1415)	QHEAT(f1188-1192) EEAGG(f1155-1159) IKP(f840-842) VNP(f126-128) VEY(f803-805)
	MHC1	LW(f440-441), VKHWPW(f828-833), LRCNG (f697-701), VKEDQ (f72-76), LKDTQ (f1654-1658), ARCRG (f794-798), MHYGN (f360-364), AYEES (f1491-1495), ITRTQ (f789-793), IKELQ (f1112-1116), LELNQ(f1567-1571), IKP(f840-842), VRC(f674-676) IQY (f193-195), VKN(f979-981),VKN (f1488-1490), IHY (f583-585), AKC (f1413-1415)	VKHWPW(f828-833), LRCNG(f697-701), VKEDQ(f72-76), QHEAT (f1188-1192), ARCRG(f794-798), SRGKQ (f1306-1310), ITRTQ(f789-793) , EEAGG (f1155-1159), IKP (f840-842), VRC (f674-676), VKD (f1794-1796) VNP (f126-128) , VEY (f803-805) VKN(f979-981) , VKN (f1488-1490), AKC(f1413-1415)	QHEAT(f1188-1192), EEAGG (f1155-1159) IKP(f840-842) VNP(f126-128) VEY(f803-805)

	MHC 4	LW(f440-441), VKHWPW(f826-831), LRCNG (f695-699), AYKRQ (f1879-1883), VKEDQ(f72 -76), LKDTQ (f1652-1656), MHYGN (f360-364), AYEES (f1489-1493), ITRTQ (f787-791), IKELQ (f1110-1114), LELNQ (f1565-1569), IKP (f838 -840), VRC (f672 -674), IQY(f193 -195), IRN (f1642-1644), VKN (f1486-1488), VKN (f977-979), IHY (f583-585), AKC (f1411-1413)	VKHWPW(f826-831), LRCNG (f695-699), VKEDQ(f72-76), QHEAT (f1186-1190), SRGKQ(f1304-1308), ITRTQ(f787-791), EEAGG(f1153-1157), IKP(f838-840), VRC (f672-674), VEF (f801-803), VKD(f1792 -1794), IRN(f1642- 1644), VNP(f126 -128), VKN(f1486-1488), VKN (f977-979), AKC(f1411-1413)	QHEAT(f1186-1190), EEAGG (f1153-1157) IKP(f838-840), VEF (f801-803), VNP (f126 -128)
	MHC7	LRCNG(f693-667), AYKRQ(f1877-1881), VKEDQ(f71 -75), LKDTQ (f1650 -1654), LEFNQ (f1563 -1567), LHREN(f650 -654), AYEES(f1487 -1491), LKELQ(f1108 -1112), IKP(f836 -838), VRC(f670 -672), IGW(f591-593), FNW (f436-438), IQY(f192- 194) ASW (f110 -112), VKN(f824 -826),LNP(f725-727), IHY (f580-582) , LKN (f1484 -1486)	LRCNG(f693-667), VKEDQ(f71-75) QHEAT(f1184-1188), EEAGG(f1151-1155), QKPRN(f564-568), IKP(f836-838), VRC(f670-672), IKD(f1790-1792), INP(f125-127), VKN(f824-826), MEF (f799-801), LNP(f725-727)	QHEAT(f1184-1188), EEAGG (f1151-1155) IKP(f836-838) INP(f125-127) MEF(f799-801) LNP(f725-727)
Meat (bovine)	MHC2	LW(f440-441), VKHWPW(f829-834), LRCNG (f698 -702), VKEDQ(f72 -76), LKDTQ(f1655 -1659), LYQKS(f611 -615), ARCRG(f795 -799), MHYGN (f360-364), AYEES(f1492-1496) IKELQ(f1113-1117), LELNQ(f1568-1572), IKP (f841-843), VRC(f675-677), IQY(f193-195), VKN(f980-982), IHY (f583-585), AKC(f1414-1416)	**VKHWPW**(f829-833),**LRCNG**(f698-702), YRNTQ(f1648-1652), **VKEDQ** (f72-76), QHEAT(f1189-1193), **ARCRG** (f795-799), SRGKQ(f1307-1311), EEAGG(f1156-1160), **IKP** (f841-843), **VRC**(f675-677), VKD (f1795-1797), VNP(f126-128), VEY (f804-806), **VKN**(f980-982) , **AKC** (f1414-1416)	QHEAT(f1189-1193), EEAGG (f1156-1160) **IKP**(f841-843) VNP(f126-128) VEY(f804-806)

	MHC1	LW(f440-441), VKHWPW(f827-832) LRCNG (f696-700), VKEDQ(f72 -76) LKDTQ(f1653 - 1657), ARCRG(f793 -797), MHYGN(f360 - 364), AYEES (f1490 -1494), ITRTQ(f788 -792) IKELQ(f1111 -1115), LELNQ(f1566 -1570), IKP(f839-841), VRC(f673-675) IQY (f193-195), IKN(f1487 -1489) VKN(f978-980), IHY(f583-585), AKC (f1412-1414)	VKHWP W (f827-832), **LRCNG**(f696 - 700), **VKEDQ**(f72 -76)**,** QHEAT (f1187-1191), **ARCRG**(f793-797), YRSTQ (f1646-1650), SRGKQ(f1305-1309), **ITRTQ**(f788-792), EEAGG (f1154-1158), **IKP**(f839-841), **VRC** (f673-675), VKD(f1793 -1795), VNP (f126-128), VEY(f802-804), **IKN** (f1487-1489), **VKN** (f978-980), **AKC**(f1412-1414)	QHEAT(f1187-1191), EEAGG (f1154-1158) **IKP**(f839-841), VNP (f126-128), VEY (f802-804)
	MLC 2	MW(f140-141)	**MW**(f140-141), VKNEE(f63-67)	**MW**(f140-141)
	MLC 1	VKH(f186-188), IEFEQ(f106-110)	IEF(f43-45), IEF(f106 -108), **VKH**(f186-188)	IEF(f43-45), IEF(f106-108)
	MHC 7	LRCNG(f693-697), AYKRQ(f1877-1881), VKEDQ(f71-75), LKDTQ (f1650-1654) LEFNQ(f1563-1567), LHREN(f650-654), AYEES(f1487-1491), LKELQ (f1108-1112), IKP(f836-838), VRC (f670-672), IGW (f591 - 593), FNW(f436 -438), IQY(f192 -194) , ASW(f110-112), VKN (f824-826), LNP(f725 - 727) IHY(f580-582), LKN(f1484-1486)	**LRCNG**(f693-697), **V**KEDQ(f71-75) QHEAT(f1184-1188), EEAGG(f1151-1155), QKPRN(f564-568), **IKP**(f836-838), **VRC** (f670-672), IKD(f1790 - 1792), INP(f125 -127), VKN(f824-826), MEF (f799-801), **LNP**(f725 -727)	**IKP**(f836-838), INP (f125 127), MEF(f799-801), **LNP**(f725-727)
Meat (chicken)	MLC2	MW(f138-139)	**MW**(f138-139) , VKNEE(f61-65)	**MW**(f138-139)
	MLC1	VKH(f186-188)	IEF(f43-45), **VKH**(f186-188)	IEF(f43-45)
	MLC 3	VKH(f144-146)	**VKH**(f144-146)	

MHC 3 embryonic	LW(f440-441), VKHWPW(f829-834), LRCNG (f698 -702), VKEDQ(f72 -76), LKDTQ(f1655-1659), ARCRG(f795-799), MHYGN(f360-364) , AYEES (f1492-1496), ITRTQ(f790-794) , IKELQ(f1113-1117), LELNQ(f1568 -1572), IKP(f841-843), VRC(f675-677) IQY(f193-195), VKN (f980-982), VHY(f583 -585)	**VKHWPW**(f829-834), **LRCNG** (f698-702), **VKEDQ**(f72-76), QHEAT (f1189-1193), **ARCRG** (f795-799), SRGKQ (f1307-1311), **ITRTQ**(f790-794), EEAGG(f1156-1160), **IKP**(f841-843) **,** **VRC**(f675-677), VEF (f804-806) VKD(f179 -1797), VNP(f126-128) **VKN**(f980-982)	QHEAT(f1189-1193) EEAGG (f1156-1160) **IKP** (f841-843) , VEF (f804-806) , VNP(f126 -128)
MHC adult	LW(f440-441), VKHWPW(f827-832) LRCNG (f696-100), VKEDQ(f73-77),LKDTQ (f1653-1657) , ARCRG(f793-797), MHYGN(f360-364) AYEES(f1490-1494) , ITRTQ(f788-792), IKELQ(f1111-1115), LELNQ (f1566-1570), IKP(f839-841), VRC (f673-675) , IQY(f194 -196), VKN (f978-980), AKC(f1412-1414) , VHY(f583-585)	**VKHWPW**(f827-832), **LRCNG** (f696-100), **VKEDQ**(f73-77), QHEAT(f1187-1191),**ARCRG**(f793-797) **, ITRTQ** (f788-792), EEAGG(f1154-1158) **IKP**(f839-841), **VRC** (f673-675) , VKD (f1793-1795), VNP (f127-129), VEY (f802-804), **VKN**(f978 -980) **,** **AKC**(f1412-1414)	QHEAT(f1187-1191), EEAGG (f1154-1158) **IKP**(f839-841), VNP (f127-129) , VEY(f802 -804)
MLC 2B		VKNEE(f60-64)	

Note: The proteins were first in silico digested by thermolysin, and were further in silico digested by pepsin and trypsin. The peptides with bolded letters in the table are resistant to pepsin/trypsin digestion.

[1] represents the location of the peptides sequences in the proteins

Table 3.4. Potent Peptides (IC_{50}< 10μM) and their locations from fish proteins by thermolysin, and their combinations with pepsin and trypsin.

Protein Source	Protein name	Sequence Thermolysin (Location[1])	Sequence Thermolysin-Pepsin (Location[1])	Sequence Thermolysin-pepsin-Trypsin (Location[1])
Fish-tuna	MLC-2	LW(f141-142)	VKNEE(f64-68)	
	MLC1-Bluefin Tuna	VEFEG(f112-116), VKH(f192-194)	VEF(f49-51), VEF(f112-114), **VKH**(f192-194)	VEF(f49-51) VEF(f112-114)
	MLC-1-Skipjack tuna	VEFEG(f112-116), VKH(f192-194)	VEF(f112-114), **VKH**(f192-194)	VEF(f112-114)
Salmon	Calponin 3	FW(f34-35), LQPGS(f65-69)	**LQPGS**(f65-69)	**LQPGS**(f65-69)
	MHC	LW(f442-443), LRKKQ(f1194-1198), LRCNG (f695-699), LKDAQ(f1652-1656), IKELQ (f1110-1114), LELNQ(f1565-1569), IKP(f838-840), VRC(f672-674), LKC(f401-403), IQY (f198-200), LRN(f1645-1647), VKN(f977-979) LYP(f625-627), VHY(f585-587)	**LRCNG**(f695-699), TRGKQ(f1304-1308) QHEAT(f1186-1190), EEAGG(f1153-1157) **IKP**(f838-840), **VRC**(f672-674), VKD (f1792 -1794), VNP(f131-133), **LRN**(f1645-1647), **VKN**(f977-979)	QHEAT(f1186-1190) EEAGG(f1153-1157) **IKP**(f838-840) VNP(f131-133)
	MLC 3	IGYNQ(f38-42), VKH(f155-157)	**VKH**(f155-157)	
Pollock	MLC-2	MW(f141-142)	**MW**(f141-142)VKNEE	**MW**(f141-142)

MLC 1	VCYNQ(f70-74), VDFEG (f108-112) VKH(f188-190)	**VKH**(f188-190), VDF(f108-110)	VDF(f108-110)
MLC 3	VHYEA(f145-149), VKH(f151-153)	**VKH**(f151-153)	

Note: The proteins were first in silico digested by thermolysin, and were further in silico digested by pepsin and trypsin. The peptides with bolded letters in the table are resistant to pepsin/trypsin digestion.

[1] represents the location of the peptides sequences in the proteins

Table 3.5. Potent Peptides (IC_{50}< 10μM) and their locations from egg proteins by thermolysin, and their combinations with pepsin and trypsin.

Protein name	Sequence Thermolysin (Location[1])	Sequence Thermolysin-pepsin (Location[1])	Sequence Thermolysin-pepsin-Trypsin (Location[1])
LDL	FW(f678-679), LRCGG(f767-771) VYHEL(f713-717), VKKTC(f86-90) ICRDL(f387392), LQEDG(f762-766) LYW(f501-503), LYW(f634-636), ISW (f226-228), VDW(f540-542), VLP(f792-794), VDP (f583-585)	**FW**(f678-679), YW(f502-503), YW(f635-636), **VKKTC**(f86-90), GEP(f596-598) **VDP**(583-585)	**FW**(f678-679), YW(f502-503), YW(f635-636), GEP(f596-598), **VDP**(f583-585)
Phosvitin	FW(f192-193), FKP(f194-196)	**FKP**(f194-196)	**FKP**(f194-196)
HDL	IRRGQ(f817-821), VRLPT(f835-839) ARHEI(f207-211), AKDQG(f119-123) LEKTG(f321-325), IGKGG(f596-600) VKELQ(f1040-1044), IRP(f67-69), IKN (f794-796), LVP(f971-973)	**IRRGQ**(f817-821), **ARHEI** (f207-211) **AKDQG**(f119-123), **IGKGG**(f596-600) IKF(f908-910), **IRP**(f67-69), IQF(f1088-1090), IEF(f398-700), INF(f541-543) VRL(f835-837), IHF(f688 -690), IKD(f460-462), VKD(f427-429), **IKN**(f794-796) **LVP**(f971-973)	**IRP**(f67-69) IQF(f1088-1090) IEF(f398-700) INF(f541-543) IHF(f688-690) **LVP**(f971-973)
Lysozyme	AKPEG(f47-51), LSYCG(f52-56), IEP(f87-89)	TRDQQ(f161-165), **AKPEG** (f47-51), **IEP** (f87-89)	**AKPEG**(f47-51) **IEP**(f87-89)
Ovotransferrin	IRWCT(f36-40), VKHTT(f227-231)	**VKHTT**(f227-231), **MRKDQ**(f350-354)	**IQHST**(f559-563), **VRPEK**(f615-619)

	IEWEG(f157-161), MRKDQ(f350-354) IQHST(f559-563), VRPEK(f615-619) LKP(f93-95), LRC(f547-549), ADW (f574-576), AGW(f142-144), VIP(f484-486), LVP(f426-428)	**IQHST**(f559-563), **VRPEK**(f615-619), IRW(f26-28), IQW(f364-366), **LKP**(f93-95), IRD(f623-625), **ADW**(f574-576), **VIP** (f484-486), **LVP**(f426-428)	IQW(f26-28), **LKP**(f364-366), **ADW** (f574-576), **VIP** (f484-486), **LVP**(f426-428)
Ovalbumin	VYLPQ(f125-129 gene x), FYTGG(f125-129 gene y), LQPSS(f162-166 Plakalbumin), IKH(f369-371plakalbumin) IRN(f158-160 Plakalbumin), LEP(f130-132 plakalbumin), LQC (f119-121 plakalbumin), VSP(f384-386 Plakalbumin)	LW(f184-185 Plakalbumin), IW(f184-185 gene Y), MW(f25-26 geneX), **FYTGG** (f125-129 gene y), **LQPSS**(f162-166 Plakalbumin), GRY(f227-229 geneX), GRY (f383-385 gene Y), GRC(f381-383 plakalbumin), **IKH**(f369 371Plakalbumin) IKD (f2-4 geneX), IKD(f158-160 gene y) **IRN**(f158-160Plakalbumin), **LEP**(f130-132 Plakalbumin), **VSP**(f384-386 Plakalbumin)	LW(f184-185 Plakalbumin), IW(f184-185 gene Y), MW(f25-26 geneX), **FYTGG** (f125-129 gene y), ETNGQ (f153-157 gene Y), **LQPSS**(f162-166 Plakalbumin) **LEP**(f130-132 Plakalbumin), **VSP**(f384-386 Plakalbumin)
Ovomucoid		VCNKD(f45-49), LRP(f50-53), GKC(f208-210)	LRP(f50-53)

Note: The proteins were first in silico digested by thermolysin, and were further in silico digested by pepsin and trypsin. The peptides with bolded letters in the table are resistant to pepsin/trypsin digestion.

[1] represents the location of the peptides sequences in the proteins

CHAPTER 4 - LC-MS/MS coupled with QSAR modeling in characterizing of angiotensin I-converting enzyme inhibitory peptides from soybean proteins

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4.1 Introduction

Heart disease is a major health concern in Canada and United States (Lo & Li-Chan, 2005). Hypertension, with systolic blood pressure over 140 mm Hg and/or diastolic blood pressure over 90 mm Hg, is one common type of heart disease. Growing scientific evidence tends to suggest that hypertension is a multifactorial disease, a product of dynamic interactions between genetic, physiological, and environmental elements. A healthy life style, such as increased physical activity, reduced salt intake, weight loss, moderate amount of alcohol consumption, no smoking, together with adoption of Dietary Approach to Stop Hypertension (DASH) diet are recommended as effective ways to keep a healthy blood pressure. The DASH diet highlights fruit, vegetables, low fat dairy products, and other food products low in fat and cholesterol (Zarraga & Schwarz, 2006).

The discovery of angiotensin I- converting enzyme (ACE) (EC 3.4.15.1) was dated back to mid-1950s, when Skeggs and his associates noticed that horse plasma contains an enzyme that converts angiotensin I to angiotensin II (Skeggs, Dorer, Kahn, Lentz, Levin, 1981). However, this matter lay dormant until measuring the conversion of angiotensin I to angiotensin II by bioassay became possible. Right now it is clear that ACE plays a key role in regulating blood pressure. ACE convert angiotensin I, a decapeptide to angiotensin II, a potent vasoconstrictor octapeptide, by cleaving a histidyl-leucine dipeptide from the C-terminal end of angiotensin I. ACE can also inactivate bradykinin, a vasodilating nonapeptide. All these effects eventually result in elevated blood pressure (Erdös, 1975). Therefore, ACE inhibitors reduces the formation level of angiotensin II while increases the level of bradykinin, which eventually lower down the blood pressure (Koike, Ito, Miyamoto & Nishino, 1980). Although synthetic ACE inhibitory drugs are

widely used, they are reported to have side effects such as dry cough, taste disturbances and skin rashes, as well as alterations in serum lipid metabolism (Atkinson & Robertson, 1979; Sesoko & Kaneko, 1985; Israili & Hall, 1992)

There is growing interest in identifying ACE inhibitory peptides from natural food sources. Oshima and coworkers first reported ACE inhibitory peptides produced from gelatin by digestive proteases (Oshima, Shimabukuro & Nagasawa, 1979). Since then, a number of ACE inhibitory peptides have been isolated from different food sources such as dairy protein (FitzGerald, Murray & Walsh., 2004), fish protein (Yokoyama, Chiba & Yoshikawa, 1992;), chicken egg (Fujita, Yokoyama & Yoshikawa, 2000), meat (Vercruysse, Van Camp & Smagghe , 2005) and plant protein (Cheung, Nakayama, Hsu, Samaranayaka & Li-Chan , 2009). Soybean is a traditional food consumed for thousands of years in Asia. In 1999, the U.S. Food and Drug Administration approved a health claim stating "diets low in saturated fat and cholesterol that include 25g of soy protein per day may reduce the risk of heart disease" (FDA, 2012). ACE inhibitory peptides have also been characterised from soy protein hydrolysates (Wu & Ding, 2001; Kodera & Nio, 2006) or fermented products such as fermented soybean paste (Shin, Yu, Park, Chung, Ahn, Nam, Kim & Lee, 2001) and soy milk (Tsai, Lin, Pan & Chen, 2006). Using activity-guided approach as above might not explore the full potential of soybean proteins as a source of ACE inhibitory peptides. Our previous study suggested the importance of understanding the structure and activity relationships of peptides in the characterization of novel peptides with potent activity (Majumder & Wu, 2010). Therefore, the objective of the study was to develop an improved method of characterizing ACE peptides from soybean proteins using LC-MS/MS in combination with QSAR prediction.

4.2 Materials and methods

4.2.1 Materials

Soybean protein was obtained from ADM (Decatur, IL, USA). Thermolysin, angiotensin I-converting enzyme (ACE), Hippuryl-L-histidyl-L-leucine (HHL), hippuric acid (HA), pepsin and trypsin were from Sigma (Oakville, ON, Canada).

4.2.2 Preparation of soybean protein hydrolysate

Soybean protein hydrolysates were prepared with the hydrolysis of thermolysin first, followed by pepsin and trypsin. The hydrolysates were prepared in a jacketed beaker coupled with a Lauda Water Bath (Brinkman, Missisauga, ON, Canada) for careful temperature control and a Titrando (Metrohm, Herisan, Switzerland) for pH control. Soybean protein was dispersed in deionized water (5%, w/v). After heating at 80°C for 10 min with continuous shaking, the sample was cooled down to 55°C and was digested by thermolysin at pH 8 for 3h. Then the pH of the protein slurry was adjusted to 2 by adding 1M HCl to inactivate the enzyme. A portion of the protein hydrolysates was taken out and pepsin was added to the remaining protein hydrolysates for another 3h at 37°C. Another portion of the protein hydrolysates was taken out and the pH was adjusted to 7.6 and trypsin was added for digestion for another 3h at 25°C. After digestion, the enzymatic reaction was terminated by raising the temperature to 95°C for 15min. Hydrolysates were then separated by centrifugation at 10000g for 30min at 4°C, freeze-dried, and used for further analysis .

4.2.3 ACE inhibitory activity assay

ACE inhibitory activity assay was performed according to Wu and co-workers with small modification (Wu, Aluko, & Muir, 2002). In brief, soybean protein hydrolysates were dissolved in 100mM borate buffer, containing 300mM NaCl (pH 8.3). 50μL of 5mM HHL, 20 μL of 2mU of ACE, 10 μL of different concentrations of soybean protein hydrolysates or borate buffer were combined and incubated at 37 °C for 10min in 1.5 mL polyethylene microcentrifuge tubes. ACE was also incubated at 37°C for 10 min before the two solutions were combined and incubated at 37°C in an Eppendorf Thermomixer R (Brinkmann Instruments, New York, USA) with continuous agitation at 450 rpm. The reaction was terminated after 30min of agitation by adding 125 μL of 1 M HCl and the solution filtered through a 0.2 μm nylon syringe filter for reversed-phase (RP)-UPLC analysis (Waters, Miliford, MA, USA) with a Waters C_{18} column (2.1 × 50mm). The column was eluted (0.245 ml / min) with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a gradient of from 5% solvent B to 60% solvent B over 3.5 min, maintained for 0.7min, then decreased to 5% solvent B in 0.8min. External standard HA samples were also prepared and used for calculation of the concentration of HA.

4.2.4 Analysis of peptide sequences by Online RP-UPLC-MS/MS

Three soybean protein hydrolysates including soybean protein digested by thermolysin, soybean protein digested by thermolysin and pepsin, soybean protein digested by thermolysin, pepsin and trypsin were further analyzed by a Waters ACQUITY UPLC system connected online to a Waters Micromass Q-TOF Premier Instrument (Milford, MA, USA). Samples were separated on a Waters Atlantis dC_{18} (75 μm × 150 mm, 3 μm)

UPLC column (Milford, MA, USA), using a two solvent system: (A) 0.1% formic acid in optima LC/MS grade water and (B) 0.1% formic acid in optima LC/MS grade acetonitrile. Samples were dissolved in solvent A; 5 µL of sample was injected to the 5 µm trapping column 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 to 5% A over 1min at a constant flow rate of 0.350 µL/ min. The flow rate was then increased to 0.500 µL/min, hold at 5% A for 2min, increased it to 98% A over 1min, hold for another 27min, and then decreased the flow rate to 0.350 µL/min over 1 min. Ionisation was performed by electrospray ionisation technique (ESI) by nanolockspray ionisation source in positive ion mode (capillary voltage 3.80kV and a source temperature of 100 °C). Peptide mass was detected through Quadrupole Time-of-Flight (Q-TOF) analyser operated in a positive ion MS/MS mode. A MS/MS full-scan was performed for each sample with an acquisition range m/z of 100-1000 Da. The MS/MS data was further processed by manual de novo sequencing.

4.2.5 Protein sequences from NCBI

Soybean protein sequences were obtained from NCBI protein database (http://www.ncbi.nlm.nih.gov/) . Only reviewed protein sequences were chosen.

4.2.6 ACE inhibitory activity prediction and validation

The ACE inhibitory activity of the peptides identified in our study was predicted using the QSAR model reported earlier with small modification (Wu, Aluko, & Nakai, 2006a, 2006b). In our previous study, two QSAR models were built for dipeptides and tripeptides respectively based on 168 dipeptides and 140 tripeptides with potent ACE inhibitory activity reported in previously published works. The prediction of ACE

inhibitory activity was conducted by using the same method as we reported before (Gu, Majumdar, & Wu, 2011). Peptide sequences were converted into X-matrix by using z-scales (Hellberg, Sjostrom, Skagerberg, & Wold, 1987; Hellberg, Eriksson, Jonsson, Lindgren, Sjostrom, Skagerberg, Wold, & Andrews, 1991). The amino acid at the amino terminus was designed as n1, and its properties were described as n1z1, n1z2, and n1z3; the amino acid adjacent to the amino terminus was designed as n2, and its properties were described as n2z1, n2z2, and n2z3, etc. SIMCA-P software version +11 (Umetrics INC., Kinnelon, NJ) was employed. Activity was expressed as IC_{50} values, defined as the concentration of the peptides that can inhibit 50% of the enzyme activity. Five peptides synthesized were validated for their activity using the method above.

4.3 Results and discussion

4.3.1 ACE inhibitory activity of soybean protein hydrolysates.

In this study, thermolysin was used for study because this enzyme is known to generate peptides with preferential cleavage for hydrophobic, especially aromatic amino acid residues, such as Ile, Leu, Phe (Morihara & Tsuzuki, 1970). Peptides containing bulky and aromatic amino acid residues have been known to be important for the potency of peptides (Ariyoshi, 1993; Li, Le, Shi, & Shrestha, 2004; Wu, Aluko, & Nakai , 2006a, Wu, Aluko, & Nakai, 2006b). Pepsin and trypsin were added to test the stability of peptides against gastrointestinal tract enzymes. Soybean protein was hydrolyzed by thermolysin first, followed by pepsin and trypsin. The ACE inhibitory activity of the hydrolysates was tested. Soybean protein digested by thermolysin and pepsin showed highest ACE inhibitory activity, with an IC_{50} value of 51.8 μg/mL, while soybean protein digested by thermolysin showed a close IC_{50} value of 53.6 μg/mL. However, soybean

protein digested by a combination of thermolysin, pepsin and trypsin showed a higher IC_{50} value of 115.6 μg/mL, indicating that further digestion by trypsin can influence the ACE inhibitory activity of soybean protein. In recent years, the ACE inhibitory activity of soy protein with the hydrolysis of different enzymes has been investigated. Chiang and coworkers compared five different enzymes including Alcalase, flavourzyme, trypsin, chymotrypsin and pepsin and found out Alcalase produced the highest ACE inhibitory activity. In optimum condition, the soy protein isolate derived from Alcalase showed an IC_{50} value of 0.67 mg protein/mL and after using 10kDa membrane, the 10kDa permeate obtained showed an IC_{50} value of 0.078mg protein/mL (Chiang, Tsou, Tsai & Tsai, 2006). In another study, soybean protein isolate was subject to in vitro pepsin-pancreatin sequential digestion and an IC_{50} value of 0.28 mg/mL was obtained after digestion (Lo et al, 2005). Our results were comparable with these previously reported data. Different chromatographic fractions generated from soybean protein hydrolysates digested by pepsin and pancreatin showed a range of IC_{50} value between 0.13 mg/mL and 0.93 mg/mL, which led to the speculation that the physiological gastrointestinal digestion can produce different ACE inhibitory peptides (Lo et al., 2005).

4.3.2 General peptide profile of soybean protein hydrolysates from LC-MS/MS results.

Peptides derived from soybean protein with hydrolysis of different combination of enzymes were analyzed by LC-MS/MS (Fig 1). It can be seen from the graph that there are a few peptides with molecular mass between 200 and 350 Da showing highest ion intensity. For instance, 229 was identified by MS/MS as IP or LP, 233 was identified by MS/MS as IT or LT and 279 was identified by MS/MS as FI or FL. Since isoleucine and

Leucine have the same molar mass, it is hard to determine the exact sequence if both of the two sequences present in the soybean protein sequences obtained from NCBI protein database. The graph also showed that although thermolysin produced plenty of peptides, a proportion of peptides were further cleaved by pepsin and trypsin. For instance, IFGM (parent ion mass 467.22 Da) from glycinin A1bB2 subunit and G2 subunit, was further cleaved by pepsin and one product, IF (parent ion mass 279.18 Da) was identified.

4.3.3 ACE inhibitory peptides from soybean protein hydrolysates

Although thermolysin digestion generated abundant peptides, a proportion of them are not resistant to pepsin and trypsin and therefore new peptides may be generated. Therefore, we started to characterize the peptide sequences obtained from the hydrolysates digested by a combination of thermolysin, pepsin and trypsin. The sequences of the peptides digested by thermolysin, pepsin and trypsin with parent ion masses between 200 and 600 Da and ion intensity higher than 15% of the highest ion intensity were analyzed by de novo peptide sequencing. A total of 34 peptide sequences were characterized (Fig 2). There were 12 dipeptides, 10 tripeptides, 7 tetrapeptides, 4 pentapeptides and 1 hexapeptides (Table 1-2). Fig 3 showed the interpretation of representative peptides identified by de novo peptide sequencing. Many of the peptides identified in soybean protein hydrolysates digested by thermolysin, pepsin and trypsin have been predicted previously in the *in silico* digestion, with the exception of a few peptides *such as S*W and FF, which may be due to the complexity of protein 3D structure (Gu, Majumder, & Wu, 2011).

The enzymes contributing to the production of these peptides were subsequently identified (Table 3). In our study, if the peptide sequence doesn't present in the

previously digested sample, then the peptide was determined to be derived from this step of digestion by comparing peptide patterns from the three successive proteolytic digestions. For instance, IIY can be identified in thermolysin-pepsin-trypsin hydrolysate, but can't be found in thermolysin–pepsin hydrolysate; then IIY was determined to be produced by hydrolysis with thermolysin, pepsin and trypsin. In this way, 26 peptide sequences were found from soybean protein hydrolysates digested by thermolysin and 5 sequences were found from protein hydrolysates digested by thermolysin and pepsin, and the remaining 3 sequences were produced only by a combination of thermolysin, pepsin and trypsin.

4.4.4 Predicted IC_{50} value by QSAR model

The IC_{50} value of ACE inhibitory activity of peptides consists of 2-3 amino acid residue was predicted using our previously reported QSAR model (Wu et al. 2006a, 2006b) (Table 4). In the cases that the exact peptide sequence cannot be determined due to presence of Leucine or Isoleucine, all possible sequences were subject to prediction of activity. Therefore, 22 dipeptide sequences and 11 tripeptide sequences were entered into QSAR model. 13 dipeptide sequences were predicted to possess an IC_{50} value less than 100 μM and 10 tripeptide sequences were predicted to show an IC_{50} value less than 20 μM. In addition, there are five tripeptides, IVF, LLF, LNF, LSW, LEF and one dipeptide, LW, with predicted IC_{50} values lower than 10 μM. Among them, LLF, LNF, LSW and LW were produced by soybean protein with hydrolysis of thermolysin and they showed considerable resistance to pepsin and trypsin, while LEF was produced when pepsin was added and IVF was produced by a combination of thermolysin, pepsin and trypsin (Table 3).

A couple of peptides identified from our study had been reported to be potent ACE inhibitory peptides, with reported IC_{50} values close to our predicted values. For example, SF was among 8 dipeptides identified in aqueous extract of garlic. It was then synthesized and its IC_{50} value was determined to be 130.2μM (Suetsuna, 1998), while a predicted IC_{50} value of 122.7 μM was generated by our model. Oral administration of one single dose of SF (200 mg/kg) in spontaneously hypertensive rats (SHR) can bring about maximum decrease of systolic blood pressure within one hour (~ 30 mmHg). FL was isolated before from chum salmon muscle by Ono and coworkers and its IC_{50} value was 13.6 μM, compared to our predicted IC_{50} value of 38.8 μM. The reverse sequence dipeptide LF, however, possessed a much lower ACE inhibitory activity (IC_{50}=383.2 μM) (Ono, Hosokawa, Miyashita & Takahashi, 2006). Adding another amino acid residue to the N-terminal of FL seems to reduce ACE inhibitory activity as AFL, generated from *C. vulgari,* a type of microalgae (Suetsuna & Chen, 2001) and IFL, produced from a fermented soybean food called tofuyo, showed an IC_{50} value of 63.8 μM and 44.8 μM, respectively (Kuba, Tanaka, Tawata, Takeda & Yasuda, 2003). LW was isolated from ovalbumin after peptic digestion. Its IC_{50} value was tested to be 6.8 μM, while a predicted IC_{50} value of 4.4 μM was generated by our model. LW can lower down the systolic blood pressure in SHR by 45 mmHg in SHR after intravenous administration (Fujita, Yokoyama & Yoshikawa, 2000). In all these studies, the ACE inhibitory assay were conducted based on the method of Cushman and Cheung and similar to ours, except that the hippuric acid was extracted by ethyl acetate and the absorbance was measured by spectrophotometer. To our best knowledge, LLF, LSW, LEF, LNF and IVF, with

predicted IC_{50} values lower than 10 μM from soybean protein hydrolysates digested by thermolysin, pepsin and trypsin have not been reported before in the literature.

The relationship between the peptide structure and ACE inhibitory activity has been studied intensively as well. It has been proposed that the active site of ACE cannot accommodate large peptide molecules (Natesh, Schwager, Sturrock, & Acharya, 2003) and most of the ACE inhibitory peptides identified so far consist of 2-12 amino acids (Hernández-Ledesma, Contreras, & Recio, 2011). C-terminal tri-peptide residues have been reported to play a predominant role in competitive binding to the active site of ACE by Cushman and co-workers. They also suggested that the substrates containing a phenylalanine residue in the third position from the C-terminus are bound much more tightly to the enzyme than those having other residues in this position. Yet a penultimate proline residue or a C-terminal glutamic acid residue greatly decreases ACE inhibitory activity of the peptide (Cushman, Pluscec, Williams, Weaver, Sabo, Kocy, Cheung, & Ondetti, 1973). Many highly effective ACE inhibitory peptides reported before contain tyrosine, phenylalanine, tryptophan, and/or proline at the C-terminal. Besides, isoleucine and valine are frequently observed in potent ACE inhibitory peptides. In our previous study, two QSAR models were built for dipeptides and tri-peptides respectively based on 168 dipeptides and 140 tri-peptides with potent ACE inhibitory activity reported in previously published works. The result indicated that for dipeptides, amino acid residues with large bulk chain as well as hydrophobic side chains are preferred, such as phenylalanine, tyrosine, and tryptophan. The structure of the carboxyl terminal of a dipeptide is more relevant to the potency of ACE inhibitory activity than N-terminal. For tripeptides, the most favourable structure is to include an aromatic amino acid residue in

the C-terminal, a positively charged amino acid residue in the middle, and a hydrophobic amino acid residue in the N-terminal (Wu, et al., 2006). Many of the tripeptides identified in our study contains phenylalanine, tryptophan or tyrosine in the C-terminal, and hydrophobic amino acid such as leucine, tryptophan in the N-terminal, which is likely to contribute to the high ACE inhibitory activity predicted. In order to validate our prediction, the predicted 5 tripeptides were synthesized and their ACE inhibitory activity was determined. LSW showed the highest ACE inhibitory activity, with an IC_{50} value of 2.7 μM. IVF (IC_{50}=63.3 μM) and LLF (IC_{50}=63.8 μM) showed similar ACE inhibitory activity, while LNF (IC_{50}=487.4 μM) and LEF (IC_{50}=698.2 μM) showed lower ACE inhibitory activity than we expected.

While a number of food-derived peptides have been proven to be potent ACE inhibitory peptides *in vitro*, establishing a direct relationship between ACE inhibitory activity and antihypertensive activity remains to be difficult. One major reason is that ACE inhibitory peptides must remain active during gastrointestinal digestion and absorption and reach the cardiovascular system. Therefore, pepsin, trypsin, α-chymotrypsin and pancreatin are often used to investigate whether ACE inhibitory peptides resist gastrointestinal digestion. Previous studies have shown that biologically active peptides generated in the diet can be absorbed intact through the intestine and function at the tissue level. Peptides containing two to three amino acid residues can be absorbed intact across the brush border membrane by a specific peptide transport system with the peptide transporter PepT1. However, the potency of the administered peptides decreases as the chain-length increases (Yang, Dantzig, & Pidgeon, 1999; Roberts, Burney, Black, & Zaloga, 1999).

Therefore, in our study both pepsin and trypsin were chosen following thermolysin digestion and research focus was on peptides consists of no more than 6 amino acids.

In conclusion, the potential application of soybean protein as a source of ACE inhibitory peptides was studied with LC-MS/MS analysis and QSAR model. In general, peptides prepared by thermolysin showed resistance to pepsin and trypsin as the ACE inhibitory activity of hydrolysates were not changed significantly and most peptides remained after digestion by pepsin and trypsin. Our study has also revealed a number of potent ACE inhibitory peptides, especially tripeptides with IC_{50} values less than 20 μM, in particular, five tripeptides, IVF, LLF, LNF, LSW, LEF, with predicted IC_{50} values less than 10μM, indicating the potential role of soybean protein in lowering blood pressure. Food grade thermolysin is commercially available therefore it is practical to prepare functional antihypertensive peptides from soy proteins.

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Table 4.1. Dipeptides identified from soybean protein hydrolysates digested by thermolysin, pepsin and trypsin. *

	Observed mass	Sequence	Protein		Observed mass	Sequence	Protein
1	219.15	VT	Glycinin beta-conglycinin	7	261.17	IE LE EI EL	Glycinin beta-conglycinin
2	229.16	IP or LP	Glycinin beta-conglycinin	8	265.17	VF	Glycinin beta-conglycinin
3	233.17	IT or LT	Glycinin beta-conglycinin	9	279.18	FI FL	Glycinin
4	245.19	II IL LI LL	Glycinin beta-conglycinin	10	292.14	SW	Glycinin
5	253.13	SF	Glycinin beta-conglycinin	11	313.16	FF	Glycinin beta-conglycinin
6	253.13	YA	Glycinin	12	318.2	WL or LW	Glycinin

* Since many dipeptides can be found in different subunits of soybean protein and even different location within one protein subunit, the locations of peptides in the protein sequences were not provided.

Table 4.2. Peptides containing 3-6 amino acids identified from soybean protein hydrolysates digested by thermolysin, pepsin and trypsin.

	Observed mass	Sequence	Protein	Location	Accession Number
13	374.2	EIL or IEL	glycinin subunit G7	351-353	NP_001235354.1
			beta-conglycinin alpha prime subunit	488-490	NP_001237316.1
			beta-conglycinin beta-subunit	306-308	NP_001236872.1
14	378.2	IVF	glycinin A5A4B3	500-502	NP_001238008.1
			glycinin subunit G7	469-471	NP_001235354.1
15	392.3	LLF	beta-conglycinin beta-subunit	95-97	NP_001236872.1
			glycinin A2B1a	11-13	NP_001235810.1
			glycinin A1bB2-784	11-13	NP_001236840.1
			glycinin subunit G7	12-14	NP_001235354.1
16	393.2	LNF	beta-conglycinin alpha prime subunit	538-540	NP_001237316.1
			beta-conglycinin alpha-subunit	540-542	NP_001236856.1
			beta-conglycinin beta-subunit	356-358	NP_001236872.1
17	400.2	FSF	glycinA2B1a	470-472	NP_001235810.1
			glycinin A1bB2-784	20-22	NP_001236840.1
18	405.2	LSW	glycinin A1bB2-784	339-341	NP_001236840.1
19	408.3	IIY	glycinin G2	371-373	NP_001235810.1
			glycinin A1bB2-784	368-370	NP_001236840.1
20	408.2	LEF	beta-conglycinin alpha prime subunit	252-254	NP_001237316.1

			beta-conglycinin alpha-subunit	254-256	NP_001236856.1
23	442.2	FYL	glycinin A2B1a	179-181	NP_001235810.1
			glycinin A5A4B3	187-189	NP_001238008.1
			glycinin A1bB2-784	179-181	NP_001236840.1
			glycinin subunit G7	175-177	NP_001235354.1
26	458.2	FEY	glycinin A5A4B3	497-499	NP_001238008.1
21	413.3	LAIP	beta-conglycinin alpha prime subunit	325-328	NP_001237316.1
			beta-conglycinin alpha-subunit	327-330	NP_001236856.1
			beta-conglycinin beta-subunit	148-151	NP_001236872.1
22	431.3	VSII	glycinin A2B1a	160-163	NP_001235810.1
			glycinin A1bB2-784	160-163	NP_001236840.1
24	443.3	VLVI or	beta-conglycinin alpha prime subunit	478-481	NP_001237316.1
			glycinin A5A4B3	146-149	NP_001238008.1
			glycinin precursor	146-149	NP_001236676.1
		LVVI or	glycinin subunit G7	153-156	NP_001235354.1
		IVVL	beta-conglycinin alpha prime subunit	476-479	NP_001237316.1
25	457.4	LVLL or	beta-conglycinin beta subunit	9-12	NP_001236872.1
		IVIL	beta-conglycinin alpha prime subunit	273-276	NP_001237316.1
			beta-conglycinin alpha subunit	478-481	NP_001236856.1

27	479.3	IIYA	glycinin A2B1a	371-374	NP_001235810.1
			glycinin A1bB2	368-371	NP_001236840.1
28	507.3	LDIF	beta-conglycinin alpha subunit	454-457	NP_001236856.1
			beta-conglycinin beta subunit	270-273	NP_001236872.1
29	509.3	FIET	glycinin A1bB2	51-54	NP_001236840.1
			glycininA1bB2-784	416-418	NP_001236840.1
30	514.3	TLPAL	glycinin A5A4B3	416-420	NP_001238008.1
			glycinin A3B4	382-386	NP_001236676.1
31	557.30	IAVPTG	Glycinin A1aBx	142-147	NP_001235827
32	562.27	LDFPA	glycinin A2B1a subunit	337-341	NP_001235810.1
			glycinin A1bB2	334-338	NP_001236840.1
33	581.30	LSAQY	glycinin A5A4B3	425-429	NP_001238008.1
34	610.28	FAPEF	glycinin A2B1a	221-225	NP_001235810.1
			glycinin A1bB2-784	223-237	NP_001236840.1

Table 4.3. Enzymes contributing to the production of peptides identified in soy protein hydrolysates digested by thermolysin, pepsin and trypsin.

Sequence	Enzymes
VT	Thermolysin
IP or LP	Thermolysin
IT or LT	Thermolysin+ pepsin
II IL LI LL	Thermolysin
SF	Thermolysin
YA	Thermolysin
IE LE EI EL	Thermolysin + pepsin
VF	Thermolysin + pepsin +trypsin
FI FL	Thermolysin
SW	Thermolysin
FF	Thermolysin
LW or WL	Thermolysin
EILor IEL	Thermolysin
IVF	Thermolysin + pepsin +trypsin
LLF	Thermolysin
LNF	Thermolysin
FSF	Thermolysin
LSW	Thermolysin
IIY	Thermolysin + pepsin + trypsin
LEF	Thermolysin+ pepsin
LAIP	Thermolysin

VSII	Thermolysin
VLVI or LVVI or IVVL	Thermolysin
LVLL or IVIL	Thermolysin
FYL	Thermolysin
FEY	Thermolysin + pepsin
IIYA	Thermolysin
LDIF	Thermolysin
FIET	Thermolysin
TLPAL	Thermolysin
IAVPTG	Thermolysin
LDFPA	Thermolysin
LSAQY	Thermolysin + pepsin
FAPEF	Thermolysin

Table 4.4. Predicted IC_{50} value for dipeptides and tripeptides identified from soybean protein digested by thermolysin, pepsin and trypsin.

Sequence	Predicted IC_{50}(μM)	Sequence	Predicted IC_{50}(μM)
VT	469.3	YA	339.6
IP	39.2	IE	154.0
LP	39.0	LE	153.3
IT	272.5	EI	470.7
LT	271.3	EL	373.9
II	55.2	VF	21.4
IL	43.9	FI	48.9
LI	55.0	FL	38.8
LL	43.7	SW	44.0
SF	122.7	FF	11.0
LW	4.4	WL	33.02
EIL	114.8	LSW	3.4
IEL	12.8	IIY	10.6
IVF	5.4	LEF	4.6
LLF	6.7	FEY	16.2
LNF	5.2	FYL	34.4
FSF	14.3		

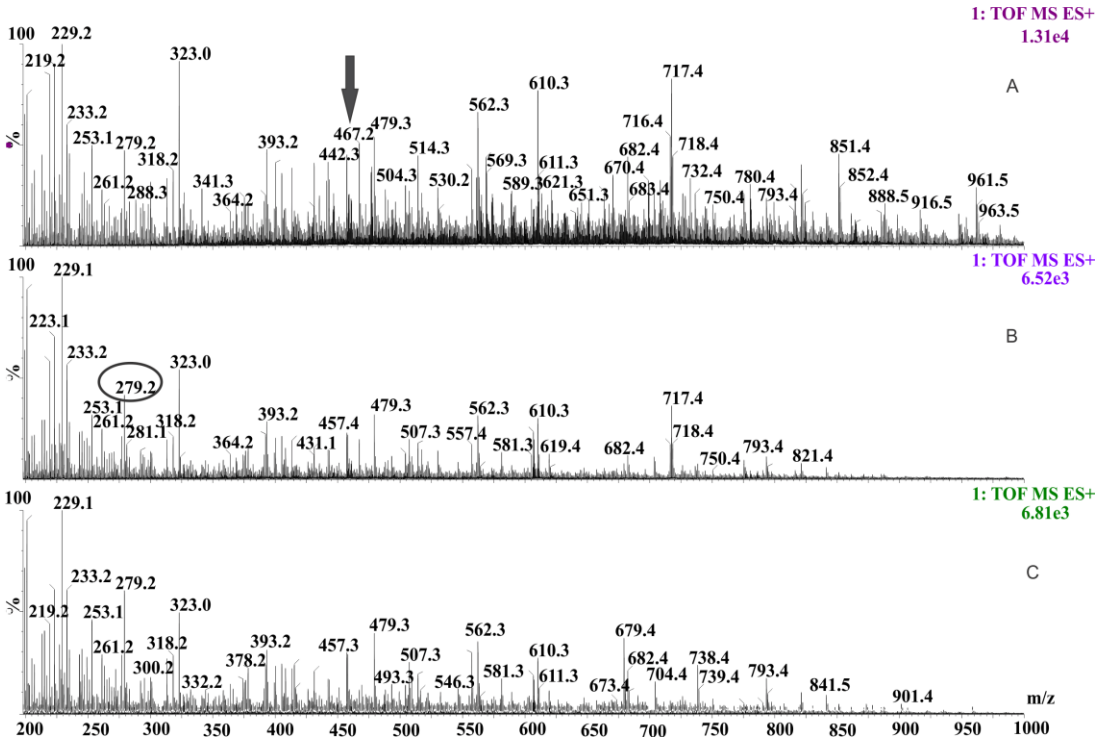


Figure 4.1. Averaged LC-MS spectra of soybean protein hydrolysates. (A) soybean protein digested by thermolysin.(B) soybean protein digested by thermolysin + pepsin. (C) soybean protein digested by thermolysin + pepsin + trypsin.

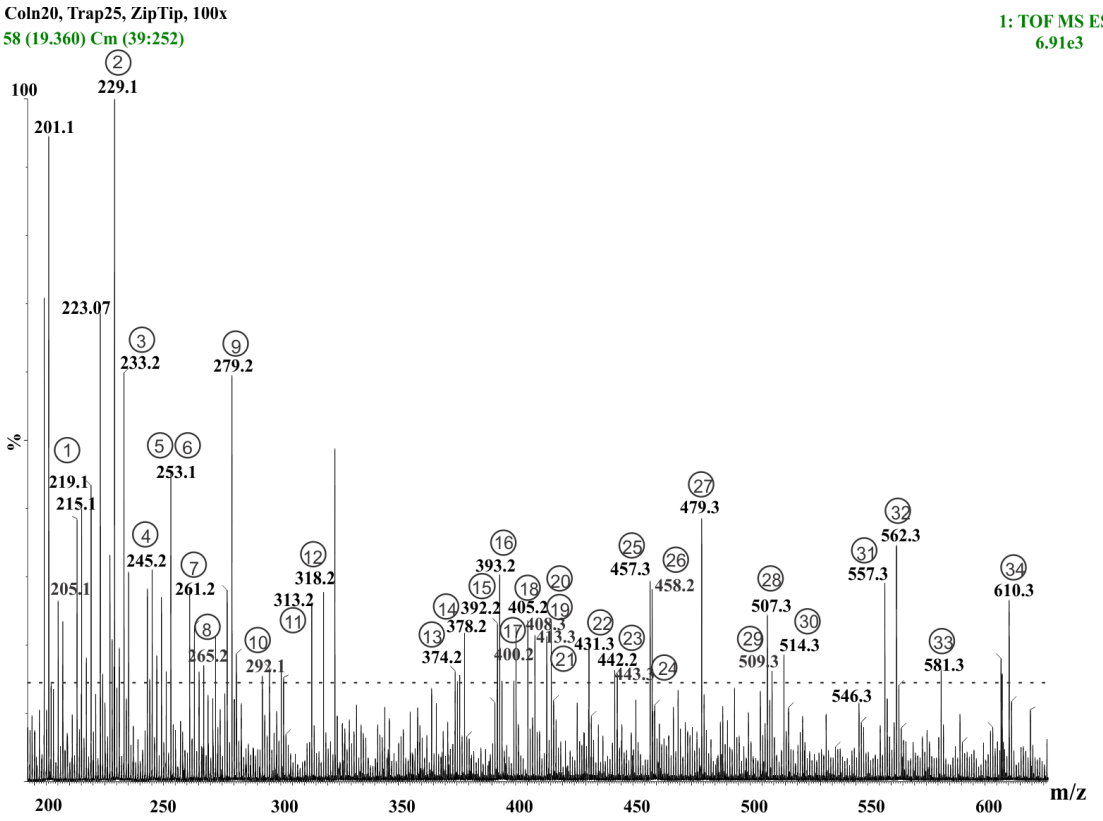
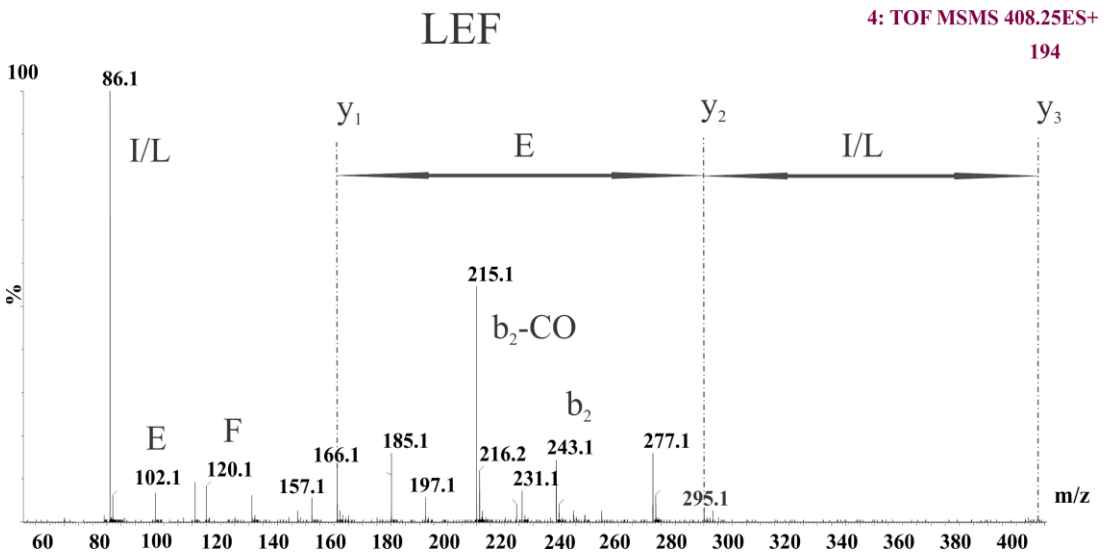
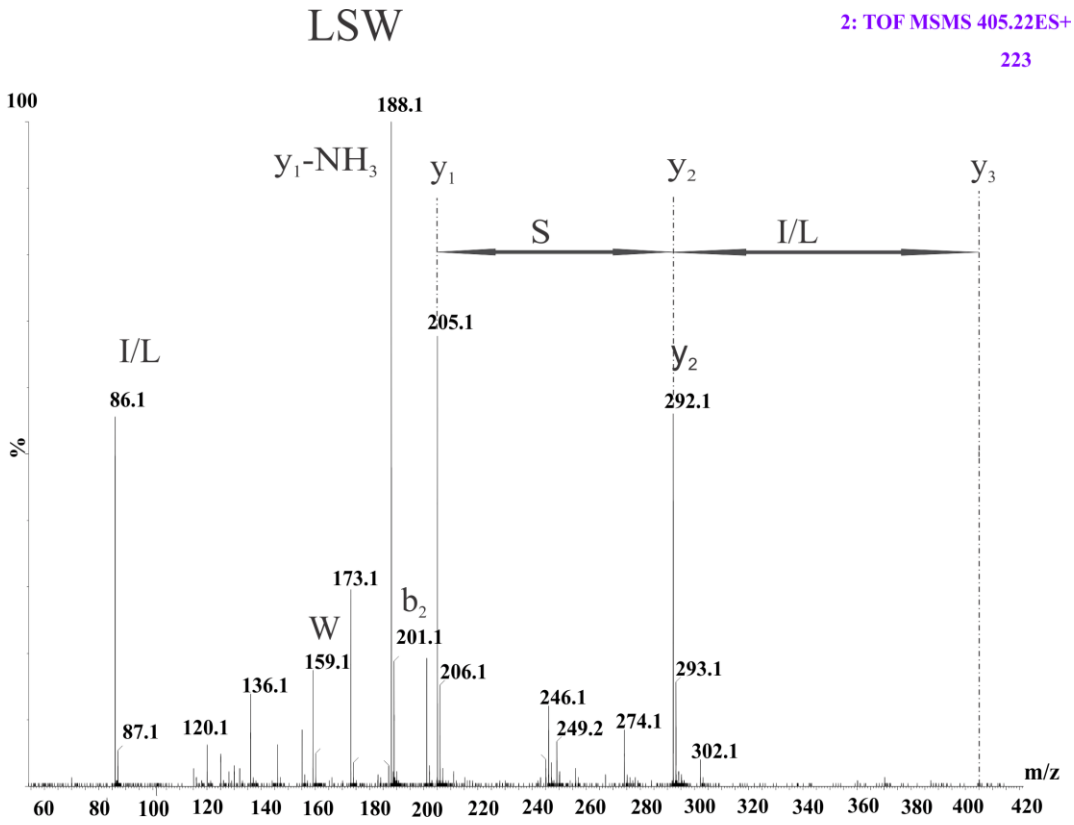


Figure 4.2. LC-MS spectra of identified peptides sequences from soybean protein hydrolysates digested by thermolysin, pepsin and trypsin. The dashed line represents the cut off of ion intensity (15%) for choosing parent ions for peptide sequencing.

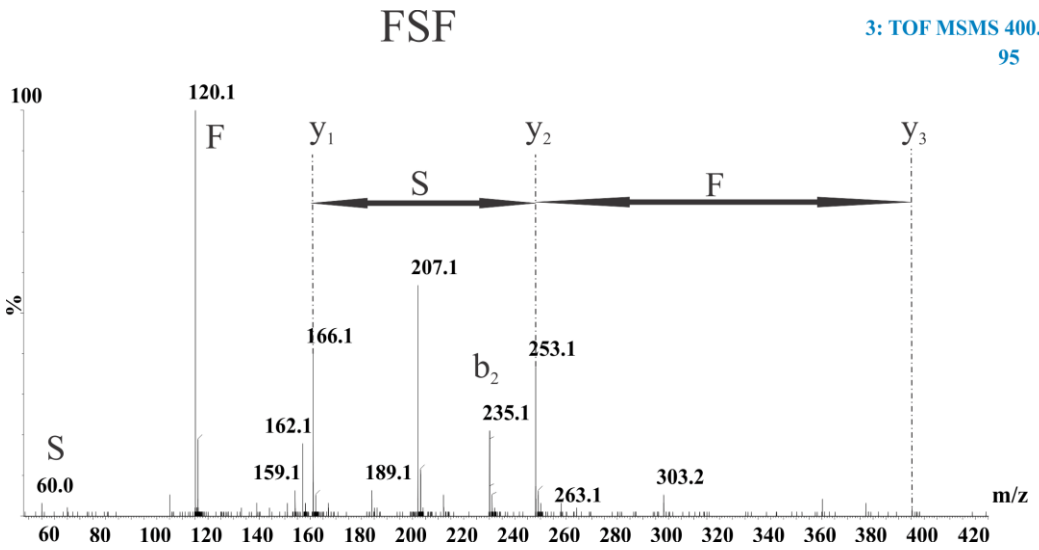
A


LEF
4: TOF MSMS 408.25ES+
194
100
%
86.1
I/L
y1
E
y2
I/L
y3
215.1
b2-CO
b2
E
F
102.1
120.1
157.1
166.1
185.1
197.1
216.2
231.1
243.1
277.1
295.1
m/z
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400

B


LSW
2: TOF MSMS 405.22ES+
223
100
%
188.1
y1-NH3
y1
y2
y3
S
I/L
205.1
I/L
y2
86.1
292.1
173.1
b2
W
201.1
159.1
136.1
206.1
293.1
246.1
87.1
120.1
249.2
274.1
302.1
m/z
60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420

C



D

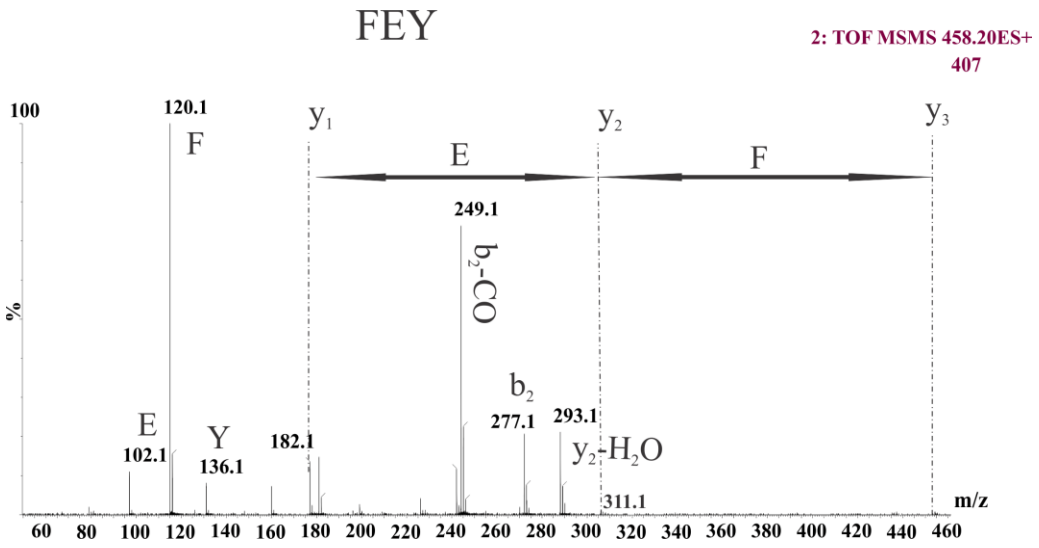


Figure 4.3. Interpretation of LC-MS/MS spectra of four peptides with parent ions shown in Fig 2. Ions at m/z 408.25 (A), 405.22 (B), 400.20 (C) and 458.20 (D).

CHAPTER 5 - Bovine lactoferrin derived ACE inhibitory tripeptide LRP also shows antioxidative and anti-inflammatory activities in endothelial cells

A version of this chapter will be submitted for publication.

5.1 Introduction

Cardiovascular disease is the leading cause of death in the world. According to the report released by World health organization in 2011, an estimated 17.3 million people died from cardiovascular disease in 2008, accounting for 30% of all global deaths, and this number will increase to 23.3 million by 2030 (World Health Organization, 2011). Hypertension is a major contributor to cardiovascular diseases. It has been reported that around one in three people worldwide has elevated blood pressure (World Health Organization, 2012). Hypertension causes serious damage to the functions of a number of organs and enhances the risk of atherosclerosis, heart attacks, kidney and brain damage (Widmaier, Raff & Strang, 2006a).

Hypertension is a multifactorial disease. In recent years, the cooperative role of renin-angiotensin system (RAS), oxidative stress and inflammation in regulating hypertensive response has been well characterized. One example is angiotensin II, a potent vasoconstrictor produced from a series of enzymatic reaction occurred in RAS. Angiotensin II activates NADPH oxidase, the primary source of endothelial superoxide (Zhang et al., 1999; Li & Shah, 2003), resulting in elevated level of oxidative stress. Angiotensin II is also engaged in initiating inflammatory response such as up-regulating the activity of nuclear factor kappa B (NF-κB), whose translocation is mediated by reactive oxygen species (ROS) (Weber, Erl, Pietsch, Strobel, Ziegler-Heitbrock & Weber, 1994). NF-κB regulates the expression of genes involved in inflammation, oxidative stress and endothelial dysfunction (Kumar, Takada, Boriek & Aggarwal, 2004).

Owing to the complicated pathophysiology, an antihypertensive agent with single property and action barely achieves the goal of controlling the blood pressure (Bangalore & Ley, 2012; Ruschitzka, 2011). Besides, it has been reported that the adverse side effect commonly presenting in antihypertensive drugs such as dry cough and skin rashes leads to the low adherence and persistence with antihypertensive regimens (Israili & Hall, 1992; Sesoko & Kaneko, 1985). Food-derived compounds, especially small peptides have emerged as an alternative to synthetic drugs in the treatment of hypertension (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012; Iwaniak, Minkiewicz & Darewicz, 2014). Bioactive peptides from food material are generally considered to be safe because they are mainly produced from natural resources using food-grade agents and processes (Udenigwe, 2014). In addition, bioactive peptide research may be seen as an effective way to enhance the economic value of food product beyond its nutritional value and to expand its market share (Bigliardi & Galati, 2013).

Bovine lactoferrin has been well characterized for its antimicrobial activity (Jenssen & Hancock, 2009). In recent years, a couple of antihypertensive peptides have also been identified from lactoferrin after enzymatic digestion or fermentation (Ruiz-Giménez et al., 2012; García-Tejedor, Sάnchez-Rivera, Castelló-Ruiz, Recio, Salom & Manzanares, 2014). Thermolysin is known to generate peptides with preferential cleavage for hydrophobic, especially aromatic amino acid residues, such as Ile, Leu, Phe (Morihara & Tsuzuki, 1970), whose structures are suggested to be important for ACE inhibitory activity (Ariyoshi, 1993; Li, Le, Shi, & Shrestha, 2004; Wu, Aluko, & Nakai, 2006a, Wu, Aluko, & Nakai, 2006b). Our QSAR aided *in silico* study revealed the presence of several potent ACE inhibitory peptides from bovine lactoferrin with the hydrolysis of

thermolysin and they were resistant to pepsin and trypsin, which are two important enzymes in gastrointestinal tract (Widmaier, Raff & Strang, 2006b). Therefore, the objectives of this study are to: (a) identify potent ACE inhibitory peptides from bovine lactoferrin hydrolysate prepared by therolysin, pepsin and trypsin, (b) determine their antioxidative and anti-inflammatory activities in endothelial cells.

5.2 Materials and methods

5.2.1 Materials

Lactoferrin from US bovine milk was produced by FrieslandCampina (Delhi,NY,USA). Thermolysin (from *Bacillus thermoproteolyticus rokko*), HPLC grade acetonitrile, HPLC grade trifluoroacetic acid (TFA), sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, angiotensin converting enzyme (from rabbit lung), Hippuryl-L-Histidyl-L-Leucine (HHL), Hippuric acid (HA), pepsin, trypsin, pancreatin, porcine gelatin, dithiothreitol (DTT), triton X-100 and dihydroethidium (DHE) were obtained from Sigma-Aldrich (Oakville, NO, Canada). Dulbecco's phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) were purchased from Gibco/Life Technology (Carlsbad, CA, USA). Tumor necrosis factor-alpha (TNF- α) was purchased from R&D System (Minneapolis, MN, USA). The antibodies of vascular cell adhesion molecule-1(VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). α-tubulin antibody (rabbit polyclonal antibody) was from Abcam (Cambridge, MA, USA). All other chemicals and reagents were of the analytic grade.

All the peptides in the study were synthesized by GenScript (GenScript, Piscataway, NJ, USA) with purity over 98% and verified by LC-MS.

5.2.2 Preparation of bovine lactoferrin hydrolysate

Bovine lactoferrin was dispersed in deionized water (5%, w/v), and was heated at 90 °C for 15 min with continuous shaking. The sample was then cooled down to 55°C and was digested by thermolysin at pH 8 for 3 h. Then the pH of the protein slurry was adjusted to 2 by adding 1M HCl. A portion of the protein hydrolysates was taken out and the sample was digested by pepsin for 3 h at 37°C. Then another portion of the protein hydrolysates was taken out and the pH was adjusted to 7.6. The sample was then digested by trypsin for 3 h at 37°C. After digestion, the enzymatic reaction was terminated by heating the sample at 95°C for 15min. The digest was then centrifuged at 10000g for 25 min. The supernatant was collected, freeze-dried and stored at -20°C for further analysis.

5.2.3 Fractionation of protein hydrolysate

Bovine lactoferrin hydrolysate obtained with the hydrolysis of thermolysin, pepsin and trypsin was subject to ultrafiltration, in which it passed through a 3000Da molecular weight cut-off membrane. This is followed by fractionation using a reverse-phase HPLC column (Xbridge C18, 10×150 mm, 5 μm, Waters Inc., Milford, MA, USA) coupled with a guard column (40×10 mm, Waters Inc., Milford, MA. USA) attached to Waters 600 HPLC system. A two solvent system was used: (A) 0.1% TFA in water and (B) 0.1% TFA in HPLC grade acetonitrile. Each run started with 99% solvent A at 5ml/min for 5 min, followed by a gradient from 99% solvent A to 60% solvent A in 45min, then decrease to 5% solvent A in 5min, and eventually return to 99% solvent A in 5min. The elution was monitored at 220nm. The fractions were collected in 2 min-intervals from 10 min to 42 min (in total 16 fractions). The fractions were freeze-dried and their ACE inhibitory activity was determined. The most potent fraction (fraction 5) was further

purified by another reverse-phase HPLC column (XBridge C18, 3.0×250mm, 5μm, Waters Inc., Milford, MA, USA) with a different solvent system: 50mM sodium phosphate buffer as solvent A and 20mM sodium phosphate buffer in 80% acetonitrile as solvent B. The sample was dissolved in solvent A, and was eluted with a flow rate of 0.5 mL/min. 10 fractions were collected and concentrated by vacuum-rotary evaporator, and the concentration of peptides were determined by modified Lowry's protein assay (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as standard. The ACE inhibitory activity of all 10 fractions were measured and the most potent fractions were further subject to LC-MS/MS analysis for peptide characterization.

5.2.4 Measurement of ACE inhibitory activity

The ACE inhibitory activity was measured according to Wu and coworkers (Wu, Aluko, & Muir, 2002). The protein hydrolysates or HPLC fractions were dissolved in 100 mM potassium phosphate buffer containing 300 mM NaCl (pH 8.3). The reaction mixture contained 50 μL of 5 mM HHL, 20 μL of 2 mU ACE and 10 μL of different concentrations of protein hydrolysates or HPLC fractions or potassium phosphate buffer, all in a 1.5 mL polyethylene microcentrifuge tube and was incubated at 37 °C for 10min. ACE was also incubated at 37°C for 10 min before the two solutions were combined and incubated at 37°C for 30min in an Eppendorf Thermomixer R (Brinkmann Instruments, New York, USA) with continuous agitation at 450 rpm. The reaction was terminated by 125 uL of 1M HCL. The solution was filtered through a 0.2 μm nylon syringe filter for reversed-phase (RP)-UPLC analysis (Waters, Miliford, MA, USA) with a Waters C_{18} column (2.1 × 50 mm). The column was eluted (0.245 mL/min) with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a gradient of

from 5% solvent B to 60% solvent B over 3.5 min, maintained for 0.7min, then decreased to 5% solvent B in 0.8min. External standard HA was used to calculate the concentration of HA.

The identified peptides from lactoferrin were synthesized by Genscript and their ACE inhibitory activity were determined using the same method as mentioned above. The ACE inhibitory activity of peptides was expressed as IC_{50} values, defined as the concentration of peptides that inhibits 50% of ACE activity.

5.2.5 Analysis of peptide sequences by Online RP-UPLC-MS/MS

The HPLC fractions with the highest ACE inhibitory activity were subject to LC-MS/MS analysis on a q-Tof premier mass spectrometer (Waters, Milford, MA) coupled with a nanoAcquity UPLC system (Waters, Milford, MA). The sample was first loaded onto a nano trap column (180 µm x 20 mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) followed by a nano analytical column (75 µm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). A two solvent system was used: (A) 0.1% formic acid in optima LC/MS grade water and (B) 0.1% formic acid in optima LC/MS grade acetonitrile. Samples were dissolved in solvent A. Each run started with 99% solvent A at a flow rate of 10 µL/min for 2 min, 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 to 5% A over 1min at a constant flow rate of 0.350 µL/ min. Ionisation was performed by electrospray ionisation technique (ESI) in positive ion mode (capillary voltage 3.3kV and a source temperature of 100 °C). A MS/MS full-scan was performed for each sample with an acquisition range (m/z) of 100-1000 Da in MS mode and 50-1990 in MS/MS mode. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd.,

Wythenshawe, Manchester, U.K.). Peaks Viewer 5.2 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual de novo sequencing for data analysis.

Bovine lactoferrin sequences were obtained from NCBI protein database (http://www.ncbi.nlm.nih.gov/). Only reviewed protein sequences were chosen.

5.2.6 Simulated gastrointestinal digestion of peptides

The synthesized peptides were dissolved in pH 2 buffer composed of hydrochloric acid and potassium chloride to mimic gastric acid. Each peptide sample was first digested by pepsin (E/S: 2%) at 37°C for 1.5 h. After that the pH was increased to 7.4. Half of each sample was removed and pepsin was inactivated by heating at 95°C for 15 min. The rest of the sample was then digested by pancreatin (E/S: 2%) at 40°C for 3 h, followed by heating at 95°C for 15 min to inactivate pancreatin. The ACE inhibitory activity of the product was determined. The digests were also analyzed by RP-UPLC using a Waters C_{18} column (2.1mm × 100mm, 1.7 μm, Waters, Miliford, MA, USA). The column was eluted (0.2 mL/min) with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a gradient from 0% solvent B to 60% solvent B over 20 min.

5.2.7 Endothelial cell culture

A permanent human cell line EA. hy 926 established by fusing human umbilical vein endothelial cells with the permanent human cell line A549 was obtained from ATCC. Cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2/ 95% air in DMEM medium with phenol red supplemented with 10% fetal bovine serum. Cultured EA.hy926 cell line (passage 7-12) was used in this study. Cells grown in 48-well plates

(1.5×10^4-3.0×10^4/well as seeding density) were treated synthesized peptides for different time periods in different experiments.

5.2.8 Determining the expression of adhesion molecules

The expression of vascular cell adhesion molecule-1(VCAM-1) and intercellular cell adhesion molecule-1(ICAM-1) was determined through western blot technique to study the activities of peptides on endothelial inflammatory response using the method reported before with minor modification (Majumder, Chakrabarti, Davidge & Wu, 2013). Cells were pretreated with peptides for 18h followed by 6h stimulation with TNF-α (10 ng/mL). After cell lysis with boiling hot laemmli's buffer containing 0.2% Triton X-100 and DTT (reducing agent), samples were run in a 9% SDS-PAGE and bands of VCAM-1 and ICAM-1 were detected by corresponding antibodies (rabbit polyclonal antibody for VCAM-1 and mouse monoclonal antibody for ICAM-1). The protein bands were normalized to their corresponding bands of loading control. Goat anti-rabbit and donkey anti-mouse fluorochrome-conjugated secondary antibodies (dilution ratio, 1:10,000) were purchased from Licor (Licor Biosciences, Lincoln, NE, USA). The protein band were detected by a Licor Odyssey Bio-Imager and analyzed by densitometry using corresponding software. The cells with TNF- α stimulation alone were run as control. All data were expressed as fold change over the corresponding untreated control.

5.2.9 Superoxide detection

Endothelial superoxide generation was determined by staining with DHE. Before the experiment, the oxidative stress of cells was induced by deprivation of serum from cell culturing medium. The cells were incubated with phenol red free DMEM medium containing 1% FBS for 1h. Cells were then treated with various synthesized peptides for

2h. Cells without any treatment of peptides were used as control. The peptide treatment was followed by incubation with DHE (10μM as final concentration) for 40min. The cells were washed three times and the fluorescence was detected using an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). For each data point, three images were taken from three randomly chosen fields. For each image, the mean fluorescence intensity (MFI) were obtained from Photoshop software and the number of cells were analyzed by Image J. The mean fluorescence intensity per cell (MFI/cell) was then calculated for each image. The final result was expressed as fold change in MFI/cell over the untreated control.

5.2.10 Statistical analysis

All data presented were the mean value ± SEM of at least four independent experiments using EA.hy926 cell line of different passages (7-12). Data were expressed as fold change over control. One-way analysis of variance (one-way ANOVA) was performed for multiple comparisons. Differences were considered to be significant with a *P* value < 0.05.

5.3. Results and discussion

5.3.1 ACE inhibitory activity of lactoferrin hydrolysate

In this study, bovine lactoferrin was first digested by thermolysin, followed by pepsin and trypsin to test the stability of peptides against gastrointestinal tract enzymes. The lactoferrin hydrolysates digested by thermolysin showed an IC_{50} value of 31.0 ± 1.1 μg/mL. This activity was not affected by pepsin (IC_{50} = 30.5 ± 1.4μg/mL) and trypsin (IC_{50} = 36.9 ± 0.9μg/mL) digestion. In the literature, bovine lactoferrin pepsin hydrolysate showed an IC_{50} value of 0.95 ±0.06 mg/mL (Ruiz-Giménez et al., 2007).

Besides enzymatic hydrolysis, fermentation of lactoferrin using *kluyveromyces marxianus*, followed by RP-HPLC resulted in 11 fractions with IC_{50} values ranging from 49 to 288 μg/mL (García-Tejedor, Sάnchez-Rivera, Castelló-Ruiz, Recio, Salom & Manzanares, 2014). Our study demonstrated the capacity of thermolysin to produce a hydrolysate with potent ACE inhibitory activity.

5.3.2 Purification and identification of ACE inhibitory peptides from lactoferrin hydrolysate

HPLC chromatography of lactoferrin hydrolysate obtained from the digestion by thermolysin, pepsin and trypsin resulted in 16 fractions (Figure 1a). All the fractions were tested for ACE inhibitory activity and fraction 5 showed the highest ACE inhibition of 64.7% at a concentration of 50 μg/mL. This fraction was further fractionated by HPLC chromatography using a different solvent system and 10 fractions were obtained (Figure 1b). The ACE inhibitory activities of all ten fractions were determined at 6.25μg/ml (Peptide concentration was determined by modified Lowry's protein assay) and fractions 1, 6 and 7 showed the highest ACE inhibition, at 60.7%, 72% and 70.3%, respectively. The three fractions were named as F5-1, F5-6 and F5-7 and they were subject to LC-MS/MS analysis for peptide characterization.

5.3.3 ACE inhibitory activity of peptides derived from lactoferrin

A total of 8 peptides were identified from the most potent fractions of lactoferrin hydrolysates and were synthesized afterwards. The ACE inhibitory activity of these peptides were first screened at 0.0625 mg/mL (final concentration) and expressed as inhibitory percentage (Table 1). Among them, LRP showed the maximum inhibition and its IC_{50} value was further determined to be 1.2±0.05 μM. In our *in silico* study, LRP was

also predicted to be released from bovine lactoferrin with the hydrolysis of thermolysin, and was resistant to the digestion by pepsin and trypsin. The IC_{50} value of LRP was predicted to be below 10 μM. In the literature, four peptides (DPYKLRP, PYKLRP, YKLRP and GILRP) containing the sequence of LRP were identified from bovine lactoferrin released by K.*marxianus*. However, their ACE inhibitory activity was less potent than LRP. LRP appeared to be active *in vivo*, as gastric intubation of LRP significantly reduced the systolic blood pressure of spontaneously hypertensive rats (SHR) within 24h after administration (García-Tejedor, et al., 2014). Our study showed for the first time that LRP can be directly produced from lactoferrin by thermolysin digestion.

The potent *in vitro* ACE inhibitory activity of LRP may be attributed to its structure. It has been suggested that the rigid structure of proline may act to lock the carboxyl group into a conformation favorable for interaction with the positively charged residue at the active site of ACE (Cushman, Cheung, Sabo & Ondetti, 1977). Besides proline, study on 140 tri-peptides also found that a positively charged amino acid residue in the middle and a hydrophobic amino acid residue in the N-terminal are preferred for ACE inhibitory activity of tri-peptides (Wu, et al., 2006a).

5.3.4 Stability of peptides against simulated gastrointestinal (GI) digestion

To evaluate the stability of peptides against GI digestion, peptides were subject to pepsin and pancreatin digestion *in vitro* and their digests were analyzed by RP-UPLC. UPLC chromatogram showed that LRP was resistant to simulated GI digestion (Figure 3). ACE inhibitory activity test showed that its inhibition was not affected by simulated GI digestion (Table 1). Although trypsin which presents in pancreatin preferentially cleaves Arg and Lys, the presence of Pro at position P1' (the first C-terminal amino acid at the

cleaved site) blocks the action of the enzyme (Keil, 1992). The stability of LRP in simulated GI digestion is in alignment with its *in vivo* efficacy reported previously (García-Tejedor et al., 2014).

5.3.5 The effects of LRP on VCAM-1 expression and ICAM-1 expression

In order to test the potential anti-inflammatory activity of LRP, two inflammatory markers, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) were used to determine the effect of LRP in response to TNF-α stimulation. VCAM-1 and ICAM-1 are two adhesion molecules essential in inflammatory responses in leukocyte adhesion and cell signal transduction, in which they are up-regulated in endothelial cells in the presence of TNF (Bradley, 2008). Endothelial cell line EA.hy926 was used for *in vitro* cell culture studies. EA.hy926 cell line is established by fusing human umbilical vein endothelial cells with the permanent human cell line A549. The cell line has been screened for human factor VIII-related antigen to confirm its preserved features as endothelial cells (Edgell, McDonald & Graham, 1983) and has been widely used for studying vascular endothelium (Bouïs, Hospers, Meijer, Molema & Mulder, 2001; Drabarek, Dymkowska, Szczepanowska & Zablocki, 2012; Armoza, Haim, Basiri, Wolak & Paran, 2013). Our result showed that the expression of VCAM-1 and ICAM-1were dramatically stimulated by TNF-α while significantly reduced by LRP at a concentration of 50 μM (Figure 4).

The theory that inflammation plays a critical role in the pathogenesis of hypertension has emerged over the last ten years with a wealth of experimental evidence (Crowley, 2014). In the event of inflammation, cytokines are produced to shape the local inflammatory response (Crowley, 2014). One of the most widely studied cytokine is TNF-α, a highly

pro-inflammatory agent, both independently and via its ability to induce expression of other pro-inflammatory cytokines (Zhang, Lin, Yip & Vilcek, 1988; Shalaby, Waage, Aarden & Espevik, 1989). Consequently, pharmacological blockade of TNF-α has appeared as an effective way of treating target organ damage and blood pressure elevation in response to hypertension (Muller et al., 2002; Elmarakby, Quigley, Pollock & Imig, 2006; Venegas-Pont et al., 2010).

5.3.6 The effect of LRP on superoxide generation

In this study, the oxidative stress was induced by 3 hours serum withdrawal. This method has been applied to trigger an elevated level of oxidative stress (Barber, Higginbottom, Mead, Barber & Shaw, 2009), which could be rescued by antioxidants such as tempol (Chen, Zeng, Lawrence, Blackwell & Meyrick, 2006). The cells were stained with DHE, which could be oxidized by superoxide generated from elevated level of oxidative stress. Our result showed that LRP at both 20 and 50 μM significantly reduced the level of superoxide, indicating its beneficial effect in suppressing the generation of superoxide and/or scavenging superoxide after its generation.

Oxidative stress is an important factor involved in the pathophysiology of hypertension. The mechanism through which ROS induces hypertension involves vascular remodeling and endothelial dysfunction. Chronic dysfunction of endothelium due to inactivation of NO by superoxide anion has been regarded as a fundamentally important underlying mechanism in hypertension (Panza, Casino, Kilcoyne & Quyyumi, 1993; Cai, 2000). This is because NO is one of the most important vasodilators in the vascular system (Cohen & Vanhoutte, 1995) while low NO bioavailability facilitates the initiation of inflammation and invasion of vascular wall (Khan, Harrison, Olbrych, Alexander & Medford, 1996;

Radomski, Palmer & Moncada, 1987a; Radomski, Palmer & Moncada, 1987b). The product peroxynitrite generated from the inactivation of NO is a strong oxidant involving in the uncoupling of eNOS and the generation of superoxide (Milstien & Katusic, 1999). Moreover, the dismutation of superoxide catalyzed by superoxide dismutase (SOD) forms H_2O_2 and this is the main supply of H_2O_2 in vascular tissue (Touyz, 2000). H_2O_2 is typically associated with lipid and protein oxidation in the presence of transition metals, eliciting specific responses in different vascular cell types which can be linked to the pathophysiology of hypertension (Rao & Berk, 1992; Kähler et al., 2000; Chen, Vita, Berk & Keaney, 2001). Therefore, regulating the level of superoxide is crucial in maintaining the healthy state of vascular system.

The innate and adaptive immunity interacts with ROS in the development of hypertension. Inflammatory cells such as neutrophils have evolved to elaborate ROS to protect the host from foreign invaders. However, inappropriate activation of these defenses by a hypertensive stimulus could misdirect the inflammatory responses against targeted organs, resulting in significant elevation of local oxidative stress and blood pressure. Conversely, ROS exaggerate inflammatory responses, augmenting blood pressure elevation and inciting cardiovascular control organ damage in the setting of hypertension (Crowley, 2014). Our study demonstrated the ability of LRP in down-regulation of cytokine-induced inflammatory protein expression, as well as suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium. The anti-inflammatory activity and antioxidant activity, in conjunction with ACE inhibitory activity, may further enhance the beneficial actions of LRP, suggesting potential application against hypertension.

5.4 Conclusion

In our study, the potential application of bovine lactoferrin as a source of antihypertensive peptides was investigated. LRP with an IC_{50} value of 1.2 ± 0.05 μM in ACE inhibitory test was characterized from lactoferrin digested by thermolysin-pepsin-trypsin and was resistant to simulated GI digestion. Further test showed that LRP was effective in the down-regulation of cytokine-induced inflammatory protein expression, as well as suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium. These results indicate the potential application of bovine lactoferrin against hypertension.

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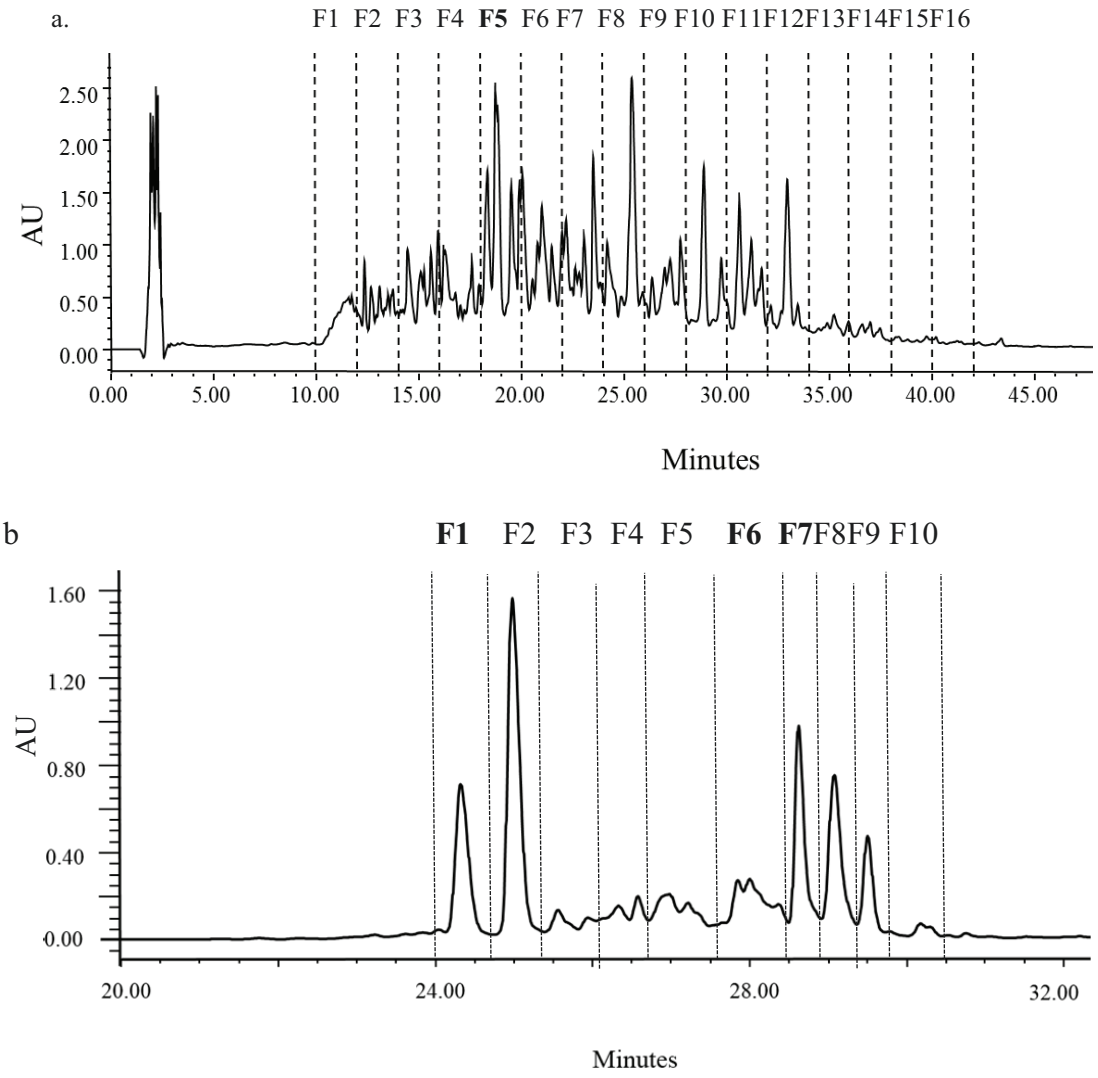

Figure 5.1. Fractionation of ACE inhibitory peptides from bovine lactoferrin hydrolysate. The absorbance was determined at 220nm. (a) RP-HPLC chromatogram of bovine lactoferrin hydrolysate by Xbridge C18 column in the first round of fractionation, 16 fractions were collected and fraction 5 (labeled in bold) showed the highest ACE inhibitory activity. (b) RP-HPLC chromatogram of fraction 5 by Xbridge C18 column in the second round of fractionation, 10 fractions were collected and fractions1, 6 and 7 (labeled in bold) showed the highest ACE inhibitory activity.

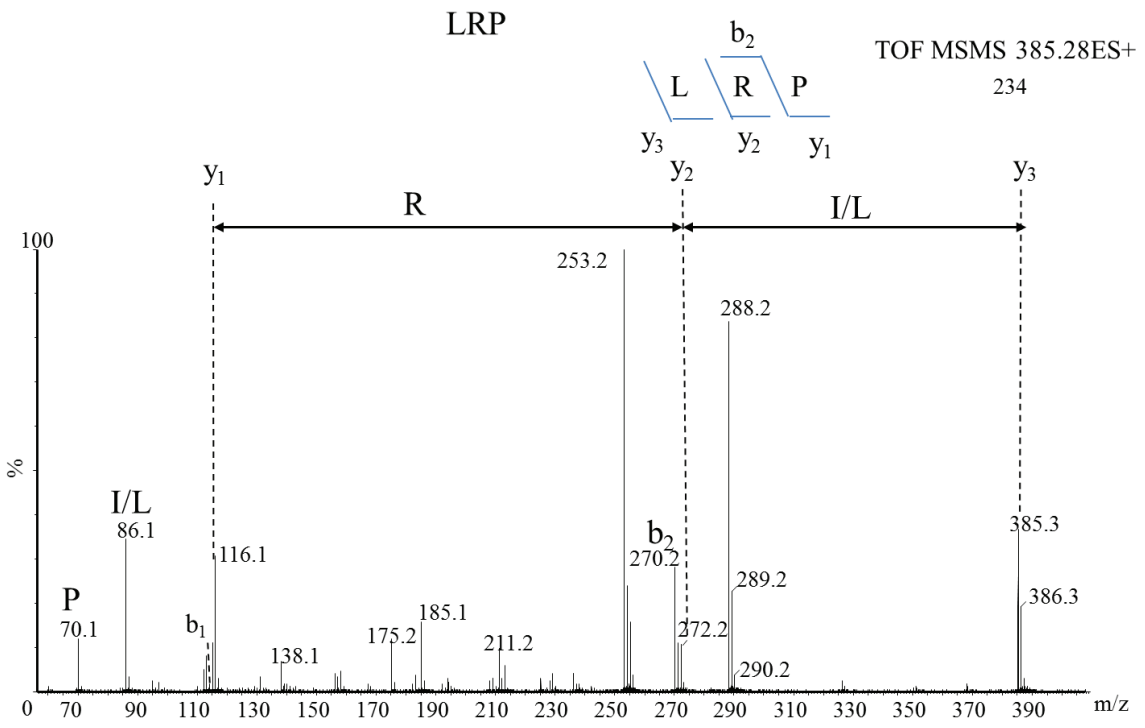

Figure 5.2. Interpretation of LC-MS/MS spectra of peptide LRP. Although isoleucine and leucine have the same molar mass, sequence alignment has confirmed that only LRP presents within the parent protein sequence.

Table 5.1. Peptide screen of 8 peptides identified from bovine lactoferrin. Peptides were tested at 0.0625 mg/mL.

Peptide	Inhibitory percentage[a]	Inhibitory percentage[b]
LRP	98.5	98.5
GEADALN	6.0	7.5
LGAPS	10.4	12.8
ESPQTHY	34.1	13.4
VLAENR	6.9	16.6
LAENR	14.9	7.3
FKDSA	20.7	17.0
PQTHY	37.4	19.8

a. Each peptide was tested before simulated gastrointestinal digestion
b. Each peptide was tested after simulated gastrointestinal digestion

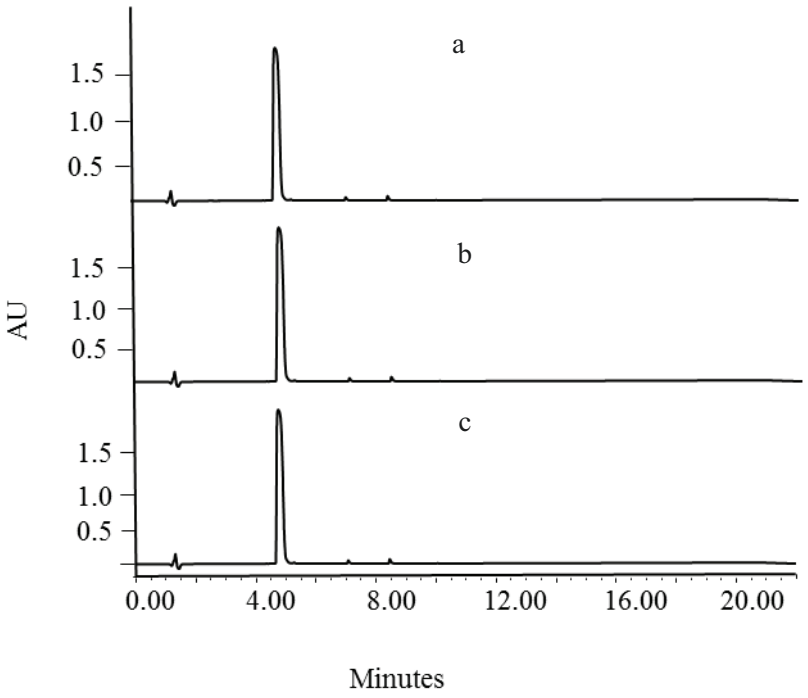

Figure 5.3. RP-UPLC chromatogram of peptide LRP before and after simulated gastrointestinal digestion using Waters C_{18} column (2.1mm × 100mm, 1.7 μm): (a) RP-UPLC chromatogram of peptide LRP before simulated gastrointestinal digestion; (b) RP-UPLC chromatogram of peptide LRP after pepsin digestion; (c) RP-UPLC chromatogram of peptide LRP after pepsin and pancreatin digestion.

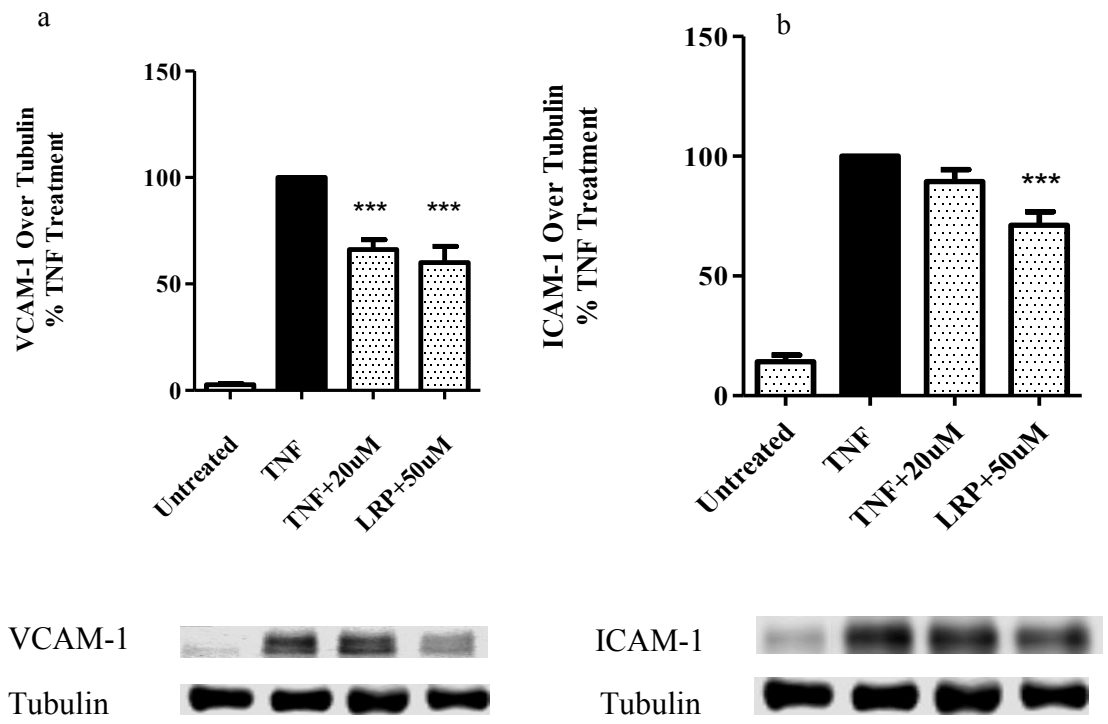

Figure 5.4. Effect of LRP on TNF-induced VCAM-1 and ICAM-1 expression: (a) effect of LRP on TNF-induced VCAM-1 expression; (b) effect of LRP on TNF-induced ICAM-1 expression. Confluent endothelial cells monolayers were pretreated for 18h with LRP at 20μM or 50μM followed by 6 hours of treatment with 10ng/mL TNF-α. VCAM-1 and ICAM-1 protein level were expressed as percentage of TNF-treated alone. Bars represent mean values (mean ± SEM, n=4 independent experiments). $P < 0.001$ were labeled as (***), compared to TNF alone using Dunnett's test.

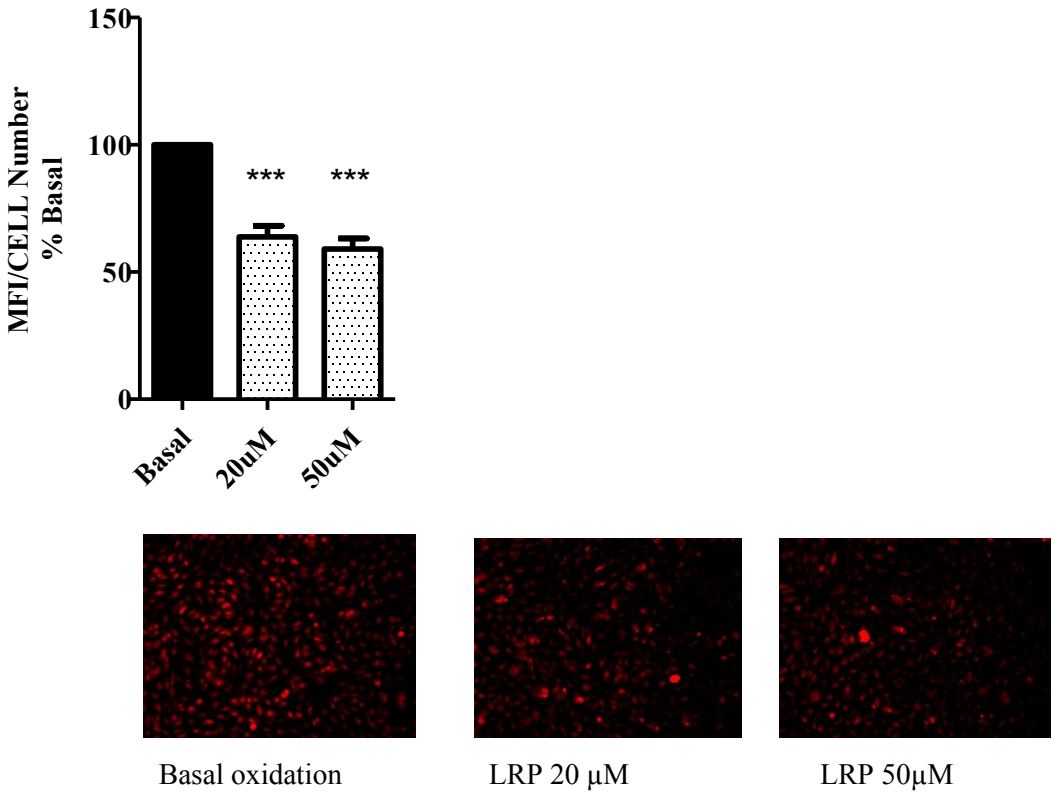

Figure 5.5. Effect of LRP on the level of superoxide in basal oxidation test. Confluent endothelial cells monolayers were pretreated with Q medium containing a reduced level of FBS (1%) and no phenol for 1 hour prior to the treatment with different concentrations of LRP for 2 hours. A representative set of images were shown. Data were calculated as MFI/Cell and were expressed as percentage of basal oxidation group. Bars represent mean values (mean ± SEM, n=4 independent experiments). $P < 0.001$ were labeled as (***) using Dunnett's test, compared to basal oxidation group.

CHAPTER 6 - Spent hen-derived ACE inhibitory peptide IWHHT also shows antioxidative and anti-inflammatory activities in endothelial cells

A version of this chapter will be submitted for publication.

6.1 Introduction

A wealth of scientific evidence has demonstrated that diet and lifestyle are closely correlated to the risk of cardiovascular diseases (Bernstein, Sun, Hu, Stampfer, Manson & Willett, 2010; He et al., 2003; Kris-Etherton, Lichtenstein, Howard & Steinberg, 2004; Stampfer, Hu, Manson, Rimm & Willett, 2000). Thus, functional foods and nutraceuticals have emerged in response to the perception about the relation of food and health (Huang, Davidge & Wu, 2013). Studies have shown that food-derived proteins exert various beneficial actions in experimental models and human bodies once hydrolyzed to peptides or free amino acids. Among them, antihypertensive peptides with angiotensin I converting enzyme (ACE) inhibitory activity have drawn wide and ever-lasting research interest (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012; Patrycja, Alegre & López, 2015). This is mainly owing to the prevalence of hypertension worldwide. Uncontrolled elevation of blood pressure will cause vascular remodeling, decrease the flow of blood and supply of oxygen, lead to heart attack, heart failure, stroke and chronic kidney diseases. Different categories of antihypertensive drugs haven been developed, however, the adverse side effect has significantly affected the persistence and adherence with antihypertensive regimens (Israili & Hall, 1992; Sesoko & Kaneko, 1985). In this context, food-derived proteins have been considered as an alternative in the prevention and treatment of hypertension.

The pathophysiology of hypertension is extremely complicated and involves the interaction among various risk factors. Available evidences have characterized the crucial role of renin-angiotensin system (RAS), oxidative stress and inflammation in regulating hypertensive response. Angiotensin II is a product generated after a series of enzymatic

reaction taking place in RAS. It is a potent vasoconstrictor and facilitates vascular remodeling by stimulating the formation of reactive oxygen species (ROS) and enhancing cell growth, inflammation, fibrosis and constriction, leading to blood pressure elevation (Frank, Eguchi, Yamakawa, Tanaka, Inagami & Motley, 2000; Touyz, He, Deng & Schiffrin, 1999; Touyz, Yao, Viel, Amiri & Schiffrin, 2004). The fact that the action of each risk factor is dependent on the other, indicates that peptides with multi-activities may enhance their antihypertensive effects by interacting with different elements essential in the pathogenesis of hypertension.

Spent hens are old laying hens and are generally regarded as by-products of egg production. The market price of spent hen is low because of its age and relative toughness. Previous studies have identified several antihypertensive peptides from chicken protein (Fujita, Yokoyama &Yoshikawa, 2000; Nakade et al., 2008; Terashima, Baba, Ikemoto, Katayama, Morimoto & Matsumura, 2010). Our computational study has revealed the presence of several potent ACE inhibitory peptides from chicken with the hydrolysis of thermolysin (Gu, Majumder & Wu, 2011). Therefore, the objectives of this study were to: (a) identify potent ACE inhibitory peptides from spent hen hydrolysate prepared by thermolysin, and (b) determine their antioxidative and anti-inflammatory activities in endothelial cells.

6.2 Materials and Methods

6.2. 1 Materials

Spent hens were purchased from local supermarket. Thermolysin (from *Bacillus thermoproteolyticus rokko*), HPLC grade acetonitrile, HPLC grade trifluoroacetic acid (TFA), sodium phosphate monobasic monohydrate, sodium phosphate dibasic

heptahydrate, angiotensin converting enzyme (from rabbit lung), Hippuryl-L-Histidyl-L-Leucine (HHL), Hippuric acid (HA), pepsin, trypsin, pancreatin, porcine gelatin, dithiothreitol (DTT), triton X-100 and dihydroethidium (DHE) were obtained from Sigma-Aldrich (Oakville, NO, Canada). Dulbecco's phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) were purchased from Gibco/Life Technology (Carlsbad, CA, USA). Tumor necrosis factor-alpha (TNF-α) was purchased from R&D System (Minneapolis, MN, USA). The antibodies of vascular cell adhesion molecule-1(VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). α-tubulin antibody (rabbit polyclonal antibody) was from Abcam (Cambridge, MA, USA). All other chemicals and reagents were of the analytic grade.

All the peptides in the study were synthesized by GenScript (GenScript, Piscataway, NJ, USA) with purity over 98% and verified by LC-MS.

6.2.2 Isolation of crude spent hen proteins

Spent hen meat was dissected, ground, and then mixed with water and ice in the ratio of 1:4 (w/v) to make a slurry. The mixture was blended and stirred for 20 min. The insoluble portion was removed by filtering through the sieve, and the pH of the soluble portion was adjusted to 5.0. The slurry was centrifuged at 10000g for 25 min. The precipitate was collected and freeze-dried for further analysis.

6.2.3 Preparation of spent hen protein hydrolysate

The spent hen protein was dispersed in deionized water (5%, w/v), and was heated at 90 °C for 15 min with continuous shaking. The protein slurry was cooled down to 60°C

and the pH was adjusted to 8. The sample was digested by thermolysin (0.5%, w/w) for 3 h. After digestion, the hydrolysis was terminated by raising the temperature to 95°C for 15 min. The digest was then centrifuged at 10000g for 25 min. The supernatant was collected, freeze-dried and stored at -20°C for further analysis.

6.2.4 Purification of protein hydrolysate

Spent hen protein hydrolysate was first subject to ultrafiltration, in which it passed through a 3000 Da molecular weight cut-off membrane. This is followed by fractionation using a reverse-phase HPLC column (Xbridge C18 column, 10×150 mm, 5 μm, Waters Inc., Milford, MA, USA) coupled with a guard column (40×10 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. For each run, 900 μL sample was automatically injected by Waters 2707 autosampler. The elution was performed with a linear gradient from 100% solvent A (0.1% TFA in water) to 5% solvent B (0.1% TFA in acetonitrile) over 5 min, and then to 35% solvent B over 60 min at a flow rate of 5 mL/min, followed by increasing the composition of solvent B to 95% to wash the column. The elution was monitored at 220nm. The fractions were collected in 3 min-interval from 0 min to 65 min (in total 22 fractions). The fractions were freeze-dried and their ACE inhibitory activity were determined. The most potent fractions was further purified by the same column with a different solvent system; 20 mM sodium phosphate buffer with 0.05% TFA as solvent A and 20 mM sodium phosphate buffer in 80% acetonitrile with 0.05% TFA as solvent B. The elution was performed with a linear gradient from 98% solvent A to 90 % solvent A over 5 min, and then to 75% solvent A over 28 min at a flow rate of 3 mL/min. The elution was monitored at 220nm. The

fractions were collected in 2 min-interval from 8 min to 30 min. 11fractions were collected and their ACE inhibitory activity was determined. The most potent fraction was subject to LC-MS/MS analysis for peptide characterization.

6.2.5 Measurement of ACE inhibitory activity

The ACE inhibitory activity was measured according to Wu, Aluko, & Muir (2002). The protein hydrolysates or HPLC fractions were dissolved in 100 mM potassium phosphate buffer containing 300 mM NaCl (pH 8.3). The reaction mixture contained 50 µL of 5 mM HHL, 20 µL of 2 mU ACE and 10 µL of different concentrations of protein hydrolysates or HPLC fractions or potassium phosphate buffer, all in a 1.5 mL polyethylene microcentrifuge tube and was incubated at 37 °C for 10min. ACE was also incubated at 37°C for 10 min before the two solutions were combined and incubated at 37°C for 30min in an Eppendorf Thermomixer R (Brinkmann Instruments, New York, USA) with continuous agitation at 450 rpm. The reaction was terminated by 125 uL of 1M HCL. The solution was filtered through a 0.2 µm nylon syringe filter for reversed-phase (RP)-UPLC analysis (Waters, Miliford, MA, USA) with a Waters C_{18} column (2.1 × 50 mm). The column was eluted (0.245 mL/min) with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a gradient of from 5% solvent B to 60% solvent B over 3.5 min, maintained for 0.7min, then decreased to 5% solvent B in 0.8min. External standard HA was used to calculate the concentration of HA.

The identified peptides from spent hen protein were synthesized by Genscript and their ACE inhibitory activity was determined using the same method as mentioned above. The ACE inhibitory activity of peptides was expressed as IC_{50} values, defined as the concentration of peptides that inhibits 50% of ACE activity.

6.2.6 Analysis of peptide sequences by Online RP-UPLC-MS/MS

The HPLC fractions with the highest ACE inhibitory activity were subject to LC-MS/MS analysis on a q-Tof premier mass spectrometer (Waters, Milford, MA) coupled with a nanoAcquity UPLC system (Waters, Milford, MA). The sample was first loaded onto a nano trap column (180 µm x 20 mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) followed by a nano analytical column (75 µm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). A two solvent system was used: (A) 0.1% formic acid in optima LC/MS grade water and (B) 0.1% formic acid in optima LC/MS grade acetonitrile. Samples were dissolved in solvent A. Each run started with 99% solvent A at a flow rate of 10 µL/min for 2 min, 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 to 5% A over 1min at a constant flow rate of 0.350 µL/ min. Ionisation was performed by electrospray ionisation technique (ESI) in positive ion mode (capillary voltage 3.3kV and a source temperature of 100 °C). A MS/MS full-scan was performed for each sample with an acquisition range (m/z) of 100-1000 Da in MS mode and 50-1990 in MS/MS mode. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Peaks Viewer 5.2 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual de novo sequencing for data analysis.

Spent hen protein sequences were obtained from NCBI protein database (http://www.ncbi.nlm.nih.gov/). Only reviewed protein sequences were chosen.

6.2.7 Simulated gastrointestinal digestion of peptides

The synthesized peptides were dissolved in pH 2 buffer which was composed of hydrochloric acid and potassium chloride to mimic gastric acid. The peptides were first digested by pepsin (E/S: 2%) which was also dissolved in pH 2 buffer at 37°C for 1.5 h. After that the pH was increased to 7.4. Half of the samples were removed and the enzyme was inactivated by increasing the temperature to 95°C and maintaining for 15 min, and they were stored at -20°C for further analysis. The rest of the samples were then digested by pancreatin (E/S: 2%) at 40°C for 3h. After the hydrolysis, the enzyme was inactivated by heating at 95°C for 15 min. The ACE inhibitory activity of the product were determined to compare with the activity of peptides before gastrointestinal digestion. The digests were also analyzed using a Waters C18 column with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a gradient from 0% solvent B to 60% solvent B over 20 min.

6.2.8 Endothelial cell culture

A permanent human cell line EA. hy 926 established by fusing human umbilical vein endothelial cells with the permanent human cell line A549 was obtained from ATCC. Cells were cultured in a humidified atmosphere at 37 °C with 5% CO_2/ 95% air in DMEM medium with phenol red supplemented with 10% fetal bovine serum. Cultured EA.hy926 cell line (passage 7-12) was used in this study. Cells grown in 48-well plates (1.5×10^4-3.0×10^4/well as seeding density) were treated synthesized peptides for different time periods in different experiments.

6.2.9 Determining the expression of adhesion molecules

The expression of vascular cell adhesion molecule-1(VCAM-1) and intercellular cell adhesion molecule-1(ICAM-1) was determined through western blot technique to study the activities of peptides on endothelial inflammatory response using the method reported before with minor modification (Majumder, Chakrabarti, Davidge & Wu, 2013). Cells were pretreated with peptides for 18h followed by 6h stimulation with TNF-α (10 ng/mL). After cell lysis with boiling hot laemmli's buffer containing 0.2% Triton X-100 and DTT as a reducing agent, samples were run in a 9% SDS-PAGE and bands of VCAM-1 and ICAM-1 were detected by corresponding antibodies (rabbit polyclonal antibody for VCAM-1 and mouse monoclonal antibody for ICAM-1). The protein bands were normalized to its corresponding band of loading control. Goat anti-rabbit and donkey anti-mouse fluorochrome-conjugated secondary antibodies (dilution ratio, 1:10,000) were purchased from Licor (Licor Biosciences, Lincoln, NE, USA). The protein band were detected by a Licor Odyssey Bio-Imager and analyzed by densitometry using corresponding software. The cells with TNF- α stimulation alone were run as control. All data were expressed as fold change over the corresponding untreated control.

6.2.10 Superoxide detection

Endothelial superoxide generation was determined by staining with DHE. Before the experiment, the oxidative stress of cells was induced by deprivation of serum from cell culturing medium. The cells were incubated with phenol red free DMEM medium containing 1% FBS for 1h. Cells were then treated with various synthesized peptides for 2h. Cells without any treatment of peptides were used as control. The peptide treatment was followed by incubation with DHE (10μM as final concentration) for 30min. The

cells were washed three times and the fluorescence was detected using an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). For each data point, three images were taken from three randomly chosen fields. For each image, the mean fluorescence intensity (MFI) were obtained from Photoshop software and the number of cells were analyzed by Image J. The mean fluorescence intensity per cell (MFI/cell) was then calculated for each image. The final result was expressed as fold change in MFI/cell over the untreated control.

6.2.11 Statistical analysis

All data presented were the mean value ± SEM of at least four independent experiments using EA.hy926 cell line of different passages (7-12). Data were expressed as fold change over control. One-way analysis of variance (one-way ANOVA) was performed for multiple comparisons. Differences were considered to be significant with a P value < 0.05.

6.3 Results and discussion

6.3.1 ACE inhibitory activity of spent hen protein hydrolysate

The spent hen meat protein hydrolysate digested by thermolysin showed an IC_{50} value of 39.6 ± 2.3μg/mL. This is comparable to the study published by Fujita and coworkers who also use thermolysin to prepare chicken hydrolysate (Fujita, Yokoyama & Yoshikawa, 2000). Besides its meat, other components of chicken have also been reported to possess ACE inhibitory activity. For instance, a study on chicken collagen hydrolysates digested by an Aspergillus species-derived enzyme showed ACE inhibitory activity with an IC_{50} value of 260 μg/mL. This hydrolysate was further subject to ultrafiltration with a molecular-weight cut off of 3000Da and the lower-molecular weight fraction showed

improved ACE inhibitory activity (IC_{50}=130 μg/mL) (Saiga, Iwai, Hayakawa, Takahata, Kitamura, Nishimura & Morimatsu, 2008).

6.3.2 Purification and identification of ACE inhibitory peptides from spent hen protein hydrolysate

The first round of HPLC fractionation of spent hen protein hydrolysates resulted in 22 fractions (Figure 1a). All the fractions were tested for ACE inhibitory activity and fraction 7 showed the highest ACE inhibition of 52.4% at a concentration of 12.5 μg/mL (final concentration). This fraction was further fractionated by HPLC using a different solvent system. The second round of HPLC fractionation resulted in 11 fractions. All the fractions were tested for ACE inhibitory activity and fraction 10 showed the highest ACE inhibition of 44.1% at a concentration of 6.25 μg/mL (final concentration). This fraction is named as F7-10. This fraction was subject to LC-MS/MS analysis for peptide characterization.

6.3.3 ACE inhibitory activity of peptides derived from spent hen

A total of 4 peptides were identified from the most potent fractions of spent hen hydrolysates and were synthesized afterwards. The ACE inhibitory activity of these peptides were first screened at 0.0625mg/mL (final concentration) and expressed as inhibitory percentage (Table 1).

IWHHT (LC-MS/MS interpretation see Figure 2) showed an IC_{50} value of 9.93± 0.65 μM. In our computational study we have successfully predicted the presence of this peptide in chicken hydrolysates digested by thermolysin (Gu, Majumder & Wu, 2011). This peptide has also been identified from dried bonito and it significantly reduced the systolic blood

pressure of spontaneously hypertensive rats (SHR) (Fujita, Yokoyama & Yoshikawa, 2000). Study on the structural-activity relationship of ACE inhibitory peptides containing four to ten amino acid residues has indicated that the tetrapeptide residues from the C-terminal end largely determines the ACE inhibitory activity of long-chain peptides. The quantitative structure-activity relationship (QSAR) model established in the study further suggested that the most influential factors were associated with the fourth amino acid residue from the C-terminal and the most preferred amino acid residue was tryptophan. Whereas for the second position from the C-terminal, preferential amino acid residues were histidine, tryptophan and methonine (Wu, Aluko & Nakai, 2006). This finding agrees well with the structural characteristics of IWHHT.

6.3.4 Stability of peptides against simulated gastrointestinal (GI) digestion

To evaluate the stability of peptides against GI digestion, peptides were subject to pepsin and pancreatin digestion *in vitro* and their digests were analyzed by RP-UPLC and LC-MS. The result showed that IWHHT was not stable against pancreatin digestion and was hydrolyzed by pancreatin to produce a mixture of IW, IWH, HHT, and HT (Figure 3). A peptide screen was conducted to compare the ACE inhibitory activity of IWHHT and the peptides liberated from simulated GI digestion. All the peptides were tested at the same molar concentration and IW showed the highest ACE inhibition. The IC_{50} value of IW was tested to be 2.0 ± 0.06 μM. The production of more potent ACE inhibitory peptide in simulated GI digestion implies the potential application of IWHHT *in vivo*.

6.3.5 The effects of spent hen-derived peptides on VCAM-1 expression and ICAM-1 expression

In order to test the potential anti-inflammatory activity of spent hen-derived peptides, two inflammatory markers, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) were used to determine the effect of peptides in response to TNF-α stimulation (Figure 4). Endothelial cell line EA.hy926 was used for *in vitro* cell culture studies. It has been widely used for studying vascular endothelium (Armoza, Haim, Basiri, Wolak & Paran, 2013; Bouïs, Hospers, Meijer, Molema & Mulder, 2001; Drabarek, Dymkowska, Szczepanowska & Zablocki, 2012). The result showed that the expression of VCAM-1 was dramatically stimulated by TNF-α and was significantly reduced by IWHHT and IWH at 50μM. However, none of the peptide significantly reduced the expression of ICAM-1.

The stimulation of different families of adhesion molecules expressed on the surface of leukocytes and endothelial cells including VCAM-1 and ICAM-1 regulates the process of leukocyte-endothelial cell adhesion, which is a hallmark of the inflammatory process (Granger, & Senchenkova, 2010). This results in the massive production of reactive oxygen species (ROS), proteases and other chemicals that can impair the normal function of vascular system. The production of ROS is mainly by a membrane-associated enzyme NADPH oxidase to generate superoxide, which subsequently generate hydrogen peroxide with the assistance of superoxide dismutase (SOD) (Weiss, 1989; Robinson, 2009). Hydrogen peroxide in turn leads to the generation of hypochlorous acid (HOCL), which is also a potent oxidizing agent (Lau &Baldus, 2006). HOCL activates a variety of leukocyte-derived proteases including elastase, collagenase and gelatinase, which elicit

proteolysis of vascular wall and interstitial matrix (Granger, Grisham & Kvietys, 1994; Weiss, Peppin, Ortiz, Ragsdale &Test, 1985). The activation process presumably takes place in the leukocyte-endothelial cell interface. In addition to cytotoxic activities, various cytokines are produced to shape the local inflammatory response including TNF-α (Elmarakby, Quigley, Pollock & Imig, 2006; Muller et al., 2002), interleukin-1 (IL-1) (Boesen et al., 2008) and interleukin-6(IL-6) (Lee et al., 2006), which contribute to target organ damage and blood pressure elevation.

6.3.6 The effect of spent hen-derived peptides on the generation of superoxide

In the study, the oxidative stress was induced by 3 hours serum withdrawal. This method has been applied to trigger an elevated level of oxidative stress (Barber, Higginbottom, Mead, Barber & Shaw, 2009), which could be rescued by antioxidants (Chen, Zeng, Lawrence, Blackwell & Meyrick, 2006). All the peptides were tested at 50 μM. The result showed that IW, IWHHT and IWH significantly reduced the level of superoxide, indicating their beneficial effect in suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium (Figure 5).

Elevated level of superoxide has a profound implication in hypertension and other cardiovascular disease. This is primarily because superoxide combines with NO to form peroxynitrite (Guzik, West, Black, McDonald, Ratnatunga, Pillai & Channon, 2000). This reaction impairs the normal function of NO such as vasodilation (Cohen & Vanhoutte, 1995), leukocytes adhesion (Radomski, Palmer & Moncada, 1987a) and inhibition of platelets (Radomski, Palmer & Moncada, 1987b). The reaction product peroxynitrite promotes lipid and protein oxidation (Leeuwenburgh et al., 1997). Besides, excessive level of superoxide potentiates the generation of hydrogen peroxide, which is

typically associated with lipid and protein oxidation and elicits specific responses in vascular cells (Kähler et al., 2000; Rao & Berk, 1992). This further augments the damage to cardiovascular control organs and blood pressure elevation.

ROS interacts with the immune system to regulate hypertensive response and targeted-organ injury. As discussed above, activated leukocytes generate massive ROS, deteriorate the normal function of vascular system and potentiate blood pressure elevation. Conversely, elevated level of ROS elicit targeted immune responses that potentiate the development of hypertension. Within the vasculature, ROS initiates an inflammatory cascade by regulating the signaling pathways of several gene transcription factors essential to the immune system (Schröder et al., 2012; Weber, Erl, Pietsch, Strobel, Ziegler-Heitbrock & Weber, 1994) and increasing endothelial permeability (Sima, Stancu & Simionescu, 2009). Our study demonstrated that spent hen-derived IWHHT and its GI digestion products were effective in down-regulation of cytokine-induced inflammatory protein expression, as well as suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium. Given the complicated pathophysiology of hypertension, the multi-activities of IWHHT and its GI digestion products may serve to enhance their beneficial actions against hypertension.

6.4 Conclusions

In our study, the potential application of spent hen as a source of antihypertensive peptides was investigated. IWHHT (IC_{50}=9.93± 0.65 μM in ACE inhibitory test) was obtained from spent hen hydrolysate digested by thermolysin. Simulated GI digestion showed that IWHHT was hydrolyzed by pancreatin to produce a mixture of peptides. Further test demonstrated that IWHHT and its GI digestion products were effective in

down-regulation of cytokine-induced inflammatory protein expression, as well as suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium. These results indicate the potential application of spent hen hydrolysate in lowering blood pressure.

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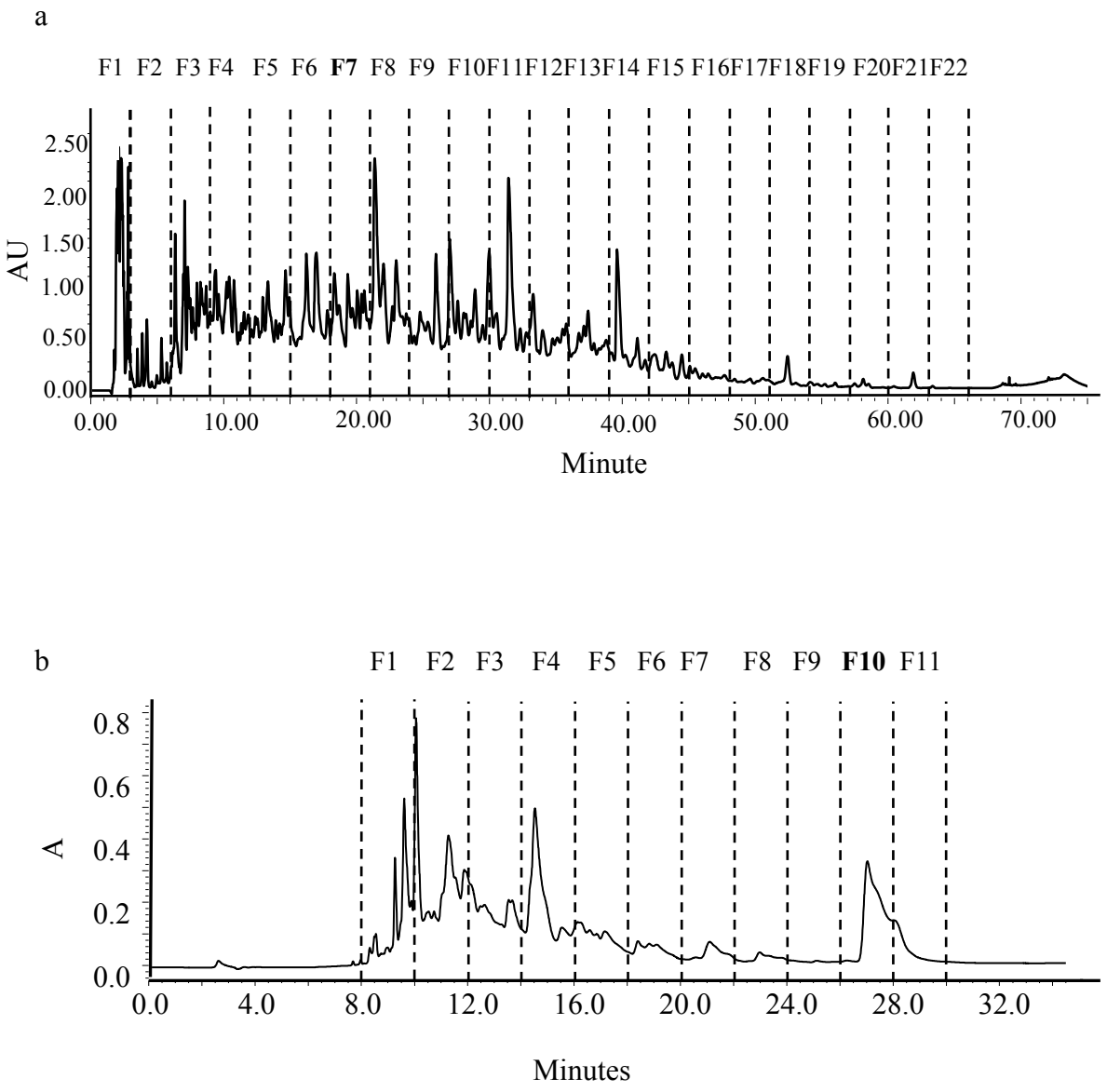

Figure 6.1. Purification of ACE inhibitory peptides from spent hen hydrolysate. (a) RP-HPLC chromatogram of spent hen hydrolysate by Xbridge C18 column in the first round of fractionation, 22 fractions were collected and fraction 7 (labeled in bold) showed the highest ACE inhibitory activity. (b) RP-HPLC chromatogram of fraction 7 by Xbridge C18 column in the second round of fractionation, 11 fractions were collected and fraction10 (labeled in bold) showed the highest ACE inhibitory activity.

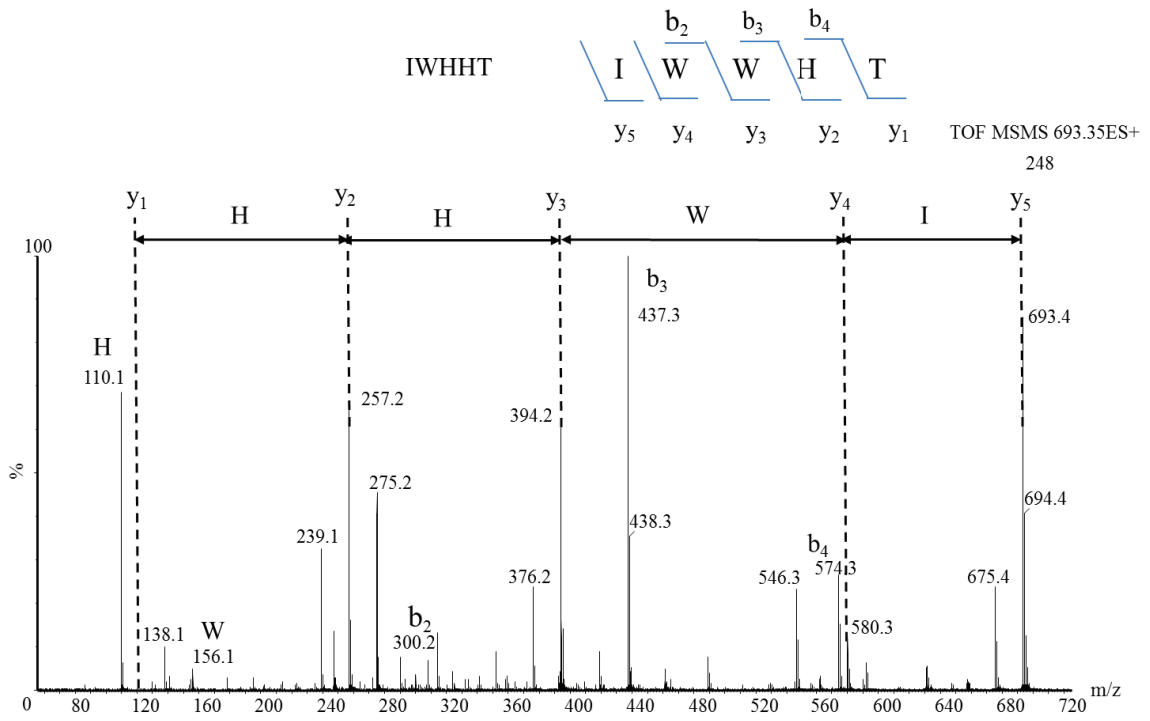

Figure 6.2. Interpretation of LC-MS/MS spectra of peptide IWHHT. Although isoleucine and leucine have the same molar mass, sequence alignment has confirmed that only IWHHT presents within the parent protein sequence.

Table 6.1. Peptide screen of 4 peptides identified from spent hen. Peptides were tested at 0.0625 mg/mL.

Peptide	Inhibitory percentage[a]	Inhibitory percentage[b]
IWHHT	82.2	97.6
LVLHQ	54.0	48.2
FLEEL	5.7	16.5
IINYK	31.9	27.6

a. ACE inhibitory percentage before simulated gastrointestinal digestion
b. ACE inhibitory percentage after simulated gastrointestinal digestion

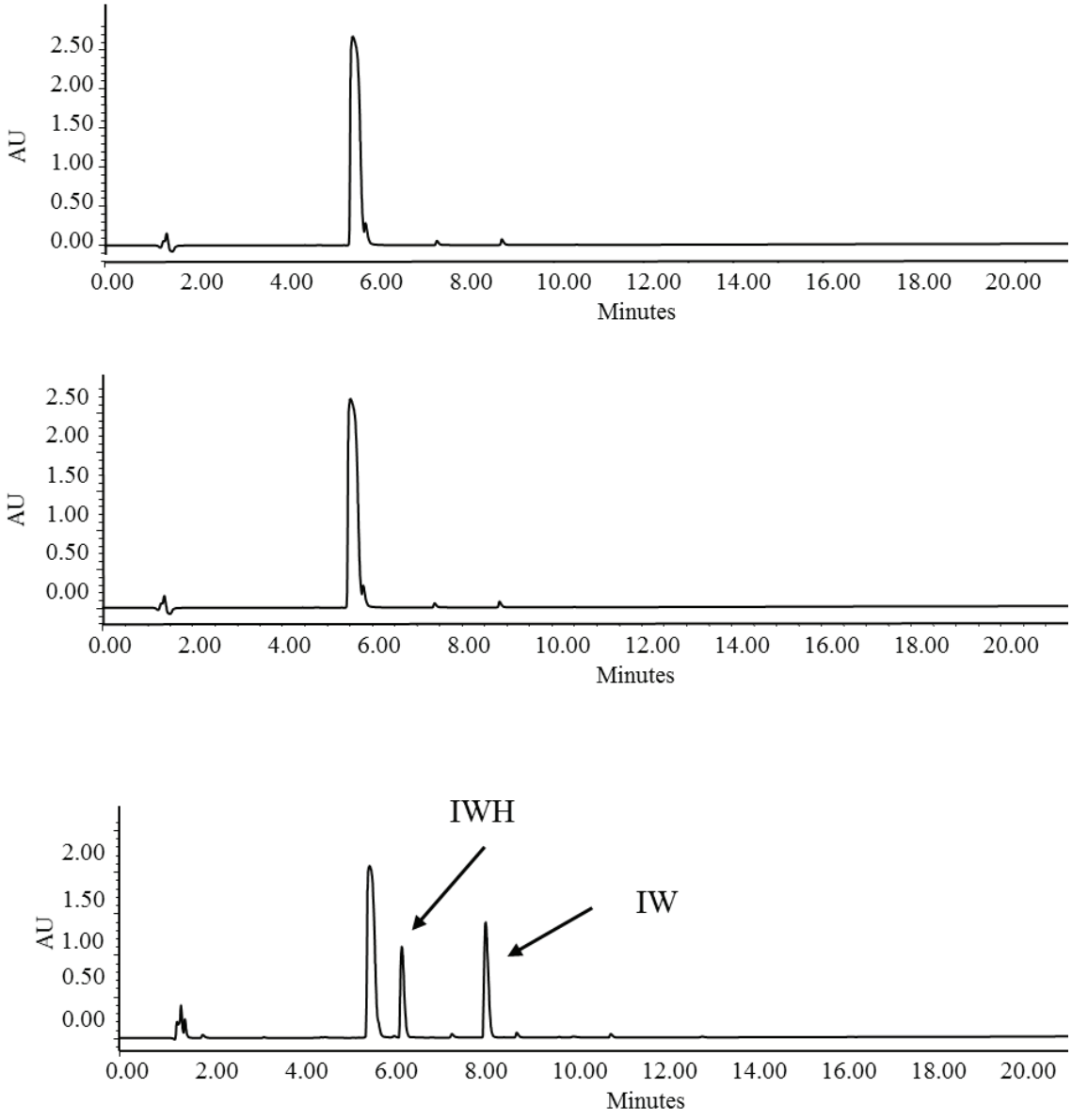

Figure 6.3. Determining the stability of IWHHT against simulated GI digestion. (a) UPLC chromatogram of IWHHT before simulated GI digestion. (b) UPLC chromatogram of IWHHT after digestion by pepsin, peptides sequences were verified by LC-MS. (c) UPLC chromatogram of IWHHT after digestion by pepsin and pancreatin, peptides sequences were verified by LC-MS.

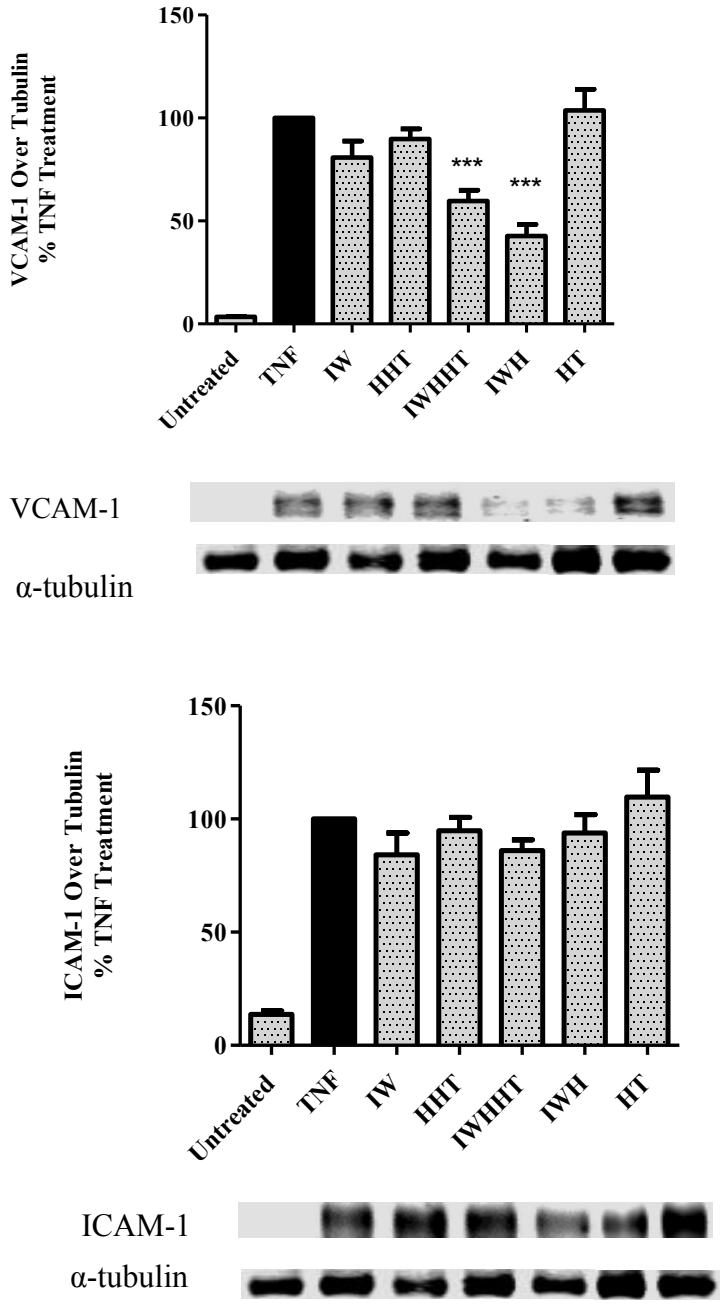

Figure 6.4. Effect of spent hen-derived peptides on TNF-induced VCAM-1 and ICAM-1 expression: (a) effect of peptides on TNF-induced VCAM-1 expression; (b) effect of peptides on TNF-induced ICAM-1 expression. Confluent endothelial cells monolayers were pretreated for 18h with peptides at 50μM followed by 6 hours of treatment with 10ng/mL TNF-α. VCAM-1 and ICAM-1 protein level were expressed as percentage of TNF-α treated alone. Bars represent mean values (mean ± SEM, n=4 independent experiments). $P < 0.001$ were labeled as (***), compared to TNF alone using Dunnett's test.

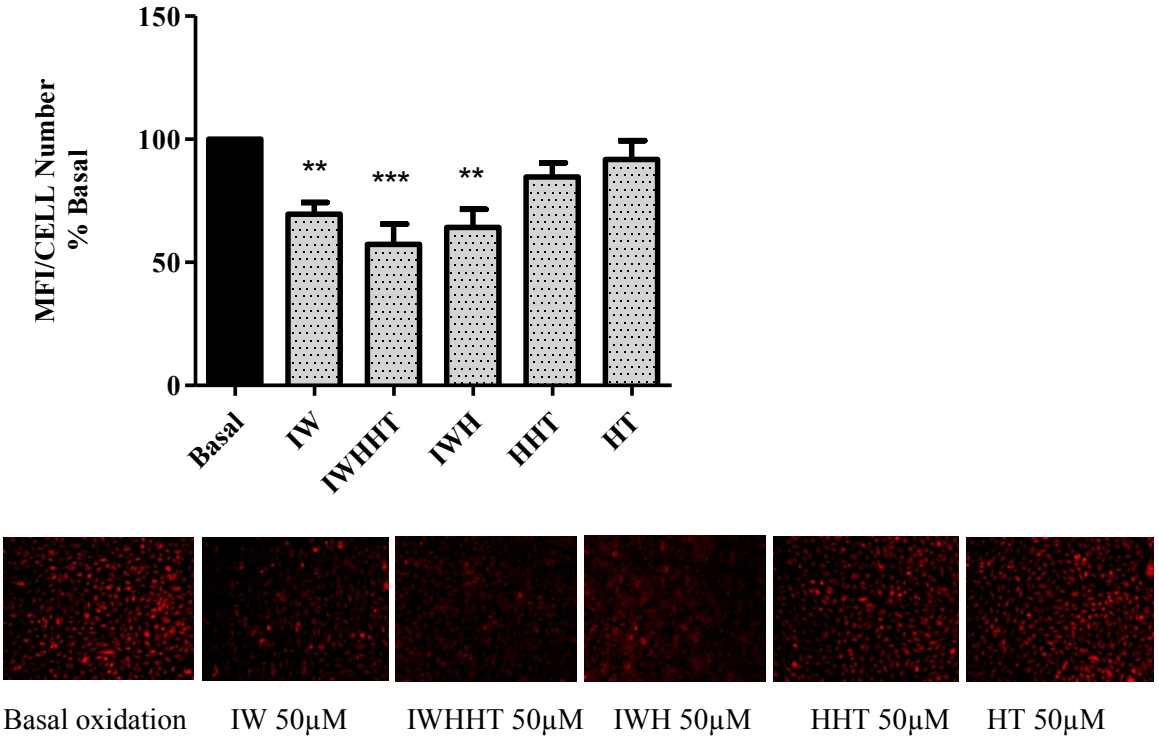

Figure 6.5. Effect of peptides on the level of superoxide in basal oxidation test. Confluent endothelial cells monolayers were pretreated with Q medium containing a reduced level of FBS (1%) and no phenol red for 1 hour prior to the treatment with different peptides at 50µM for 2 hours. A representative set of images were shown. Data were calculated as MFI/Cell and were expressed as percentage of basal oxidation group. Bars represent mean values (mean ± SEM, n=4 independent experiments). $P<0.01$ were labeled as (**), $P<0.001$ were labeled as (***) using Dunnett's test, compared to basal oxidation group.

CHAPTER 7 – General discussion and future directions

7.1 Foundation of present research

Cardiovascular diseases (CVDs) are the leading cause of mortality in the world (WHO, 2011). As a major risk factor of CVDs, hypertension is a multifactorial, polygenic disorder which involves the interplay among various risk factors. A number of scientific evidence has demonstrated that renin-angiotensin system (RAS), oxidative stress and inflammation interact with each other in regulating hypertensive response and targeted organ injury. For instance, angiotensin II is a product generated after a series of enzymatic reaction taking place in RAS. It is a potent vasoconstrictor and facilitates vascular remodeling by stimulating the formation of reactive oxygen species (ROS) and enhancing cell growth, inflammation, fibrosis and constriction, leading to blood pressure elevation (Touyz, He, Deng & Schiffrin, 1999; Frank, Eguchi, Yamakawa, Tanaka, Inagami & Motley, 2000; Touyz, Yao, Viel, Amiri & Schiffrin, 2004). This multidirectional relationship of risk factors involved in the pathophysiology of hypertension implies that antihypertensive agent with single activity can barely achieve the goal of controlling the blood pressure (Bangalore & Ley, 2012; Ruschitzka, 2011). Different categories of antihypertensive drugs are available, however, the long-term adverse side effect has significantly affected the persistence and adherence with antihypertensive regimens (Israili & Hall, 1992; Sesoko & Kaneko, 1985).

Under such circumstances, functional foods or nutraceuticals have emerged as alternative strategies for the prevention and treatment of hypertension. A number of epidemiological studies have pinpointed that diet and lifestyle are closely related to cardiovascular diseases (Stampfer, Hu, Manson, Rimm & Willett, 2000; He, Merchant, Rimm, Rosner,

Stampfer, Willett & Ascherio, 2003; Kris-Etherton, Lichtenstein, Howard & Steinberg, 2004; Bernstein, Sun, Hu, Stampfer, Manson & Willett, 2010). Peptides released from food proteins have shown various biological activities in experimental models (Shahidi & Zhong, 2008). The research on food-derived bioactive peptides will contribute to reducing the health burden worldwide, as well as enhancing the economic value of food product beyond its nutritional value and expanding its market share (Bigliardi & Galati, 2013; Udenigwe, 2014).

The main objectives of the thesis were to identify potent angiotensin-I converting enzyme (ACE) inhibitory peptides from various food proteins and to explore their additional antioxidative and anti-inflammatory activities in endothelial cells. The work presented in this thesis provided a systematic evaluation of various food commodities as potential sources of antihypertensive peptides.

7.2 Key findings of the present research

RAS plays a dominant role in regulating blood pressure and vascular tone in human body (Oparil & Haber, 1974; Zhuo, Ferrao, Zheng & Li, 2013). Renin, released from kidney, converts angiotensinogen to angiotensin I, which is an inactive decapeptide. Angiotensin I is hydrolyzed by ACE to form angiotensin II, a vasoconstrictive octapeptide. Misdirected action of angiotensin II stimulates ROS production and initiates inflammation, leading to blood pressure elevation and end-organ injury. ACE inhibitory peptides have therefore drawn wide research interest. Accumulated information on various ACE inhibitory peptides has greatly advanced our understanding of their structural-activity relationship. Given this background, the first experimental study of this thesis was to systematically evaluate various food proteins as potential sources of ACE

inhibitory peptides. Quantitative structure-activity relationship (QSAR) modeling combined with *in silico* digestion were used to predict the peptide sequences release from enzymatic digestion and their ACE inhibitory activity. 5709 peptides ranging from 2 to 6 amino acid residues were obtained from 15 food commodities with the digestion of thermolysin. Thermolysin is known to generate peptides with preferential cleavage for hydrophobic, especially aromatic amino acid residues, such as Ile, Leu, Phe (Morihara & Tsuzuki, 1970), whose structures are suggested to be important for ACE inhibitory activity (Ariyoshi, 1993; Li, Le, Shi, & Shrestha, 2004; Wu, Aluko, & Nakai, 2006a, Wu, Aluko, & Nakai, 2006b). The stability of these peptides against gastrointestinal digestion was further tested by *in silico* digestion using pepsin and trypsin, which are two major enzymes in gastrointestinal tract. Pork, beef and chicken proteins contain the largest number of potent peptides (predicted IC_{50} < 10 μM), followed by milk, egg, soybean and canola proteins, whereas proteins from fish (with the exception of salmon) and cereals (oat and barley) contain the least number of potent peptides. It should be noted that the number of protein sequences identified from each food commodities varied, which affected the number of potent ACE inhibitory peptides predicted by QSAR modeling. This study demonstrated that proteins from livestock meat, milk, egg, soybean and canola are good sources of ACE inhibitory peptides. The results obtained from the study contribute to establishing the rationale for selecting the enzyme and protein for the identification and characterization of potent ACE inhibitory peptides.

In order to validate our prediction, soybean, lactoferrin and spent hen were selected. Soybean protein hydrolysate digested by thermolysin showed an IC_{50} value of 53.6 μg/mL in ACE inhibitory test. The IC_{50} value decreased slightly to 51.8 μg/mL after

adding pepsin, and increased to 115.6 μg/mL after adding trypsin. The soybean hydrolysate digested by thermolysin, pepsin and trypsin analyzed by LC-MS/MS coupled with QSAR modelling to screen for potent ACE inhibitory peptides. 34 peptide sequences containing 2-6 amino acid residues were identified and five novel tripeptides, IVF, LLF, LNF, LSW, LEF were predicted to possess IC_{50} values lower than 10 μM. Among them, LSW and LEF have been predicted in the computational study (chapter 3). These five peptides were synthesized and LSW (IC_{50}=2.7 μM), IVF (IC_{50}=63.3 μM) and LLF (IC_{50}=63.8 μM) showed potent ACE inhibitory activity. The result indicated that LC-MS/MS analysis coupled with QSAR modeling is an efficient way of screening potent ACE inhibitory peptides (chapter 4). However, using LC-MS/MS may not identify all the peptides presenting in the hydrolysate, especially di- and tripeptides may be lost in the sample pre-treatment beforing running LC-MS/MS.

Bovine lactoferrin has been well characterized for its antimicrobial activity (Jenssen & Hancock, 2009). Our computational study indicated also that bovine lactoferrin can release potent ACE inhibitory peptides with the action of thermolysin. ACE inhibitory test showed that bovine lactoferrin digested by thermolysin showed an IC_{50} value of 31.0±1.1 μg/mL, which was not affected by further pepsin (IC_{50}=30.5 ±1.4 μg/mL) and trypsin (IC_{50}=36.9±0.9 μg/mL) digestion. The protein hydrolysates obtained from the digestion by thermolysin, pepsin and trypsin were subject to reverse phase-HPLC (RP-HPLC) fractionation. 8 peptides were identified from the most potent HPLC fraction by LC-MS/MS analysis and LRP showed the highest ACE inhibitory activity (IC_{50}=1.2 ± 0.05 μM). LRP has been predicted from previous computational study (chapter 5).

Spent hens are old laying hens and are generally regarded as by-products of the egg industry. The market value of spent hen is low because of its age and relative toughness. Thus, the work on exploring potent ACE inhibitory peptides from spent hen will add economic value to this by-product. Spent hen protein digested by thermolysin showed an IC_{50} value of 39.6 ± 2.3 μg/mL in ACE inhibitory test. The hydrolysate was fractionated by RP-HPLC and 4 peptides were identified from the most potent HPLC fraction by LC-MS/MS analysis. Among them, IWHHT (IC_{50}=9.93± 0.65 μM) showed the highest ACE inhibitory activity. IWHHT has been predicted from computational study. Simulated GI digestion indicated that IWHHT was resistant to pepsin digestion but was hydrolyzed by pancreatin to produce a mixture of IW, IWH, HHT and HT. IW showed a higher ACE inhibitory activity than IWHHT (IC_{50}=2.0 ± 0.06 μM) (chapter 6).

Hypertension is a multifactorial disease. RAS, oxidative stress and inflammation are found to interact with each other in a multidirectional relationship to regulate hypertensive response and end-organ injury (Touyz, He, Deng & Schiffrin, 1999; Crowley, 2014). Bovine lactoferrin-derived LRP, spent hen-derived IWHHT and its GI digestion product (IW, IWH, HHT and HT) were further tested for their potential antioxidative and anti-inflammatory activities. Endothelial cell line EA.hy926 was used to as a model system. LRP significantly inhibit tumor necrosis factor-alpha (TNF-α) induced vascular inflammation by reducing the expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) at 50μM (chapter 5). IWHHT and IWH at a concentration of 50μM also significantly reduced the expression of VCAM-1, however, neither of the two peptides significantly reduced the expression of ICAM-1 at the same concentration. TNF-α is one of the widely studied

cytokines produced in response to inflammatory stimuli (Sprague & Khalil, 2009). TNF-α induces the expression of two adhesion molecules, VCAM-1 and ICAM-1 on the surface of endothelial cells. The two adhesion molecules act together with other families of adhesion molecules to facilitate leukocyte-endothelial cells adhesion, a process involving the production and release of ROS, proteases and other cytokines that can impair the normal function of vascular system, leading to blood pressure elevation and targeted organ damage (Granger, & Senchenkova, 2010). In addition, LRP (20 μM and 50 μM), IWHHT (50 μM), IW (50 μM) and IWH (50 μM) significantly reduced the level of superoxide, indicating their beneficial effect in suppressing the generation of superoxide and/or scavenging superoxide in vascular endothelium (chapter 5 and chapter 6). Superoxide elicits specific responses that lead to endothelial dysfunction, lipid and protein oxidation, vasoconstriction and inflammation (Touyz, 2000). Taken together, the ACE inhibitory, antioxidative and anti-inflammatory activities of LRP, IWHHT and its GI digestion product may enhance its antihypertensive effect by interacting with different elements essential in the pathogenesis of hypertension.

In conclusion, various food commodities have been evaluated as potential source of ACE inhibitory peptides. Soybean-derived peptides LSW, IVF and LLF, bovine lactoferrin-derived LRP, spent hen-derived IWHHT have shown potent ACE inhibitory activity. LRP, IWHHT and its GI digestion product also shown antioxidative and anti-inflammatory activities. The multi-activities of food-derived peptides may enhance their action against hypertension. The work presented in this thesis advanced our understanding about bioactivities of food-derived peptides and provided scientific

evidence to support the development and commercialization of functional foods and neutraceuticals.

7.3 Recommendations for future studies

Based on the results of the research presented, recommended further studies are listed below:

1. Further study using endothelial cells is required to understand the mechanism through which these peptides inhibit TNF-α induced inflammation. Since TNF-α induced up-regulation of adhesion molecules is mainly mediated through the transcription factor nuclear factor-κB (NF-κB) (Sprague & Khalil, 2009), whether the peptides have any interaction with this transcription factor needs to be investigated.
2. *In vivo* study using animal model is needed to determine the effect of these peptides in physiological system. Spontaneously hypertensive rat (SHR) could be used to perform this study. SHR is a well-characterized rodent model for hypertension (Trippodo & Frohlich, 1981, Zicha & Kunes, 1999). These animals show elevated level of oxidative stress and increased RAS activity (Liu, 2009; Wu & Juurlink, 2002), which facilitates the *in vivo* test of ACE inhibitory, antioxidative and anti-inflammatory activities of the peptides identified in this thesis. When performing this animal study, normotensive subjects should be included and factors such as age, sex, period of treatment are required to be investigated.
3. Study on the potential interaction between these peptides and other food ingredients is required to determine whether the effects of these peptides will be

affected when incorporated into a food system. Besides, it is important to evaluate the safety of these peptides as well as any potential adverse effects associated with long term use of these peptides.

4. Finally, human clinical trials with both hypertensive subjects and normotensive subjects are essential to determine the ultimate efficacy of these peptides.

The results obtained from this thesis will provide crucial information to justify the further research and application of these peptides for the prevention and management of hypertension.

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