STRUCTURE-FUNCTION PROPERTIES OF FLAXSEED PROTEIN-DERIVED

MULTIFUNCTIONAL PEPTIDES

By

Chibuike Chinedu Udenigwe

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Food and Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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**ABSTRACT**

Food protein-derived peptides have increasingly become important sources of ingredients for the formulation of therapeutic products. The main aim of this work was to study the *in vitro* and *in vivo* bioactive properties of structurally diverse group of peptides produced through enzymatic hydrolysis of flaxseed proteins (FP). Hydrolysis of FP with seven proteases followed by fractionation into low-molecular-weight (LMW) cationic fractions yielded multifunctional peptides that inhibited angiotensin converting enzyme (ACE) and renin activities, which are molecular targets for antihypertensive agents. The LMW peptides also exhibited antioxidant properties by scavenging free radicals and inhibiting amine oxidase activity. The peptide fractions showed inhibition of calmodulin-dependent phosphodiesterase, an enzyme that has been implicated in the pathogenesis of several chronic diseases. Moreover, FP hydrolysis with thermolysin and pronase followed by mixing with activated carbon yielded branched-chain amino acids (BCAA)-enriched multifunctional peptide mixture (Fischer ratio of 23.65) with antioxidant properties and *in vitro* ACE inhibition; Fischer ratio of 20.0 is considered minimum for therapeutic purposes. The BCAA-enriched peptide product can be used in clinical nutrition to treat muscle wasting symptoms associated with hepatic diseases. Furthermore, an arginine-rich peptide mixture (31% arginine versus 11% in the original flaxseed protein) was produced by hydrolysis of FP with trypsin and pronase followed by separation using electrodialysis-ultrafiltration. Arginine plays important physiological roles especially as precursor to vasodilator, nitric oxide. The arginine-rich peptide mixture exhibited *in vitro* ACE and renin inhibition and led to decreased systolic blood

pressure (−17.9 and −11.7 mmHg, respectively at 2 and 4 h) after oral administration to spontaneously hypertensive rats. For the first time in the literature, we showed that arginine peptides have superior physiological effects when compared to the amino acid form of arginine. Lastly, quantitative structure-activity relationship studies using partial least squares (PLS) regression yielded two predictive models for renin-inhibiting dipeptides with *z*-scales amino acid descriptors. The PLS models indicated that hydrophobic and bulky side chain-containing amino acids contribute to renin inhibition if present at the amino- and carboxyl-terminal of dipeptides, respectively. Based on this study, Ile-Trp was discovered as potent renin-inhibiting dipeptide, and may serve as a useful template for the development of potent antihypertensive peptidomimetics.

**ACKNOWLEDGEMENTS**

**FOREWARD**

This thesis was prepared using the manuscript format and it is composed of six manuscripts following the General Introduction and Literature Review chapters. The manuscripts were written in different journal styles as follows: Manuscript 1 (Journal of Functional Foods), Manuscript 2 (Food Chemistry), Manuscript 3 (Food Chemistry), Manuscript 4 (Journal of Agricultural and Food Chemistry), Manuscript 5 (Food Chemistry) and Manuscript 6 (Amino Acids). Manuscripts 1, 2 and 4 have been published, Manuscripts 6 is currently under consideration for publication, and Manuscripts 3 and 5 have been prepared for submission to the journals indicated above. The manuscripts were linked by transition statements at the end of each manuscript for coherence. The format for list of references cited for the Introduction and Literature Review chapters follows that of the journal, Food Chemistry. The last chapter provides a general summary of the thesis, concluding remarks and future directions of the project.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAA | Aromatic amino acids |
| ACE | Angiotensin I-converting enzyme |
| AEM | Anion exchange membrane |
| AT-I | Angiotensin I |
| AT-II | Angiotensin II |
| BCAA | Branched-chain amino acids |
| BW | Body weight |
| CaM | Calmodulin |
| CaMPDE | Calmodulin-dependent phosphodiesterase |
| CE | Catalytic efficiency of enzymes |
| CEM | Cation exchange membrane |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DWB | Dry weight basis |
| $EC_{50}$ | 50% Effective concentration |
| EDUF | Electrodialysis-ultrafiltration |
| E/S | Enzyme-substrate ratio |
| ESR | Electron spin resonance |
| FAPGG | *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine |
| FIU | Flourescence Intensity Unit |
| FPI | Flaxseed protein isolate |
| FPH | Flaxseed protein hydrolysates |

| | |
|---|---|
| FPLC | Fast Protein Liquid Chromatography |
| GSH | Glutathione |
| HMW | High molecular weight |
| HPLC | High Performance Liquid Chromatography |
| $IC_{50}$ | 50% Inhibitory concentration |
| $K_i$ | Enzyme-inhibitor dissociation constant |
| $K_m$ | Michaelis constant or enzyme-substrate dissociation constant |
| $K_m'$, $K_m^{app}$ | Apparent Michaelis constant |
| KNOS | Kinin-nitric oxide system |
| LA | Linoleic acid |
| LMW | Low molecular weight |
| LPS | Lipopolysaccharide |
| MW | Molecular weight |
| MWCO | Molecular weight cut-off |
| NO | Nitric oxide |
| $^{\cdot}OH$ | Hydroxyl radical |
| $O_2^{\cdot-}$ | Superoxide radical |
| $ONOO^-$ | Peroxynitrite |
| PDE | Phosphodiesterase |
| PLS | Partial least square projection of latent structure |
| PMB | Polymyxin B |
| QSAR | Quantitative structure-activity relationship |

RAS               Renin-angiotensin system

RAAS              Renin-angiotensin-aldosterone system

ROS               Reactive oxygen species

RLU               Relative Luminescence Unit

SFR               Structure-function relationship

SHR               Spontaneously hypertensive rats

SNP               Sodium nitroprusside

SSAO              Semicarbazide-sensitive amine oxidase

UFM               Ultrafiltration membrane

$V_{max}$              Maximum enzyme reaction rate

$V_{max}'$, $V_{max}^{app}$     Maximum apparent enzyme reaction rate

**CHAPTER ONE**

**GENERAL INTRODUCTION**

The human body is constantly subjected to physiological imbalances and exposure to extrinsic toxic substances that perturb the normal functioning of the system leading to various health conditions, which can be controlled by physiological homeostasis or through the use of therapeutic agents especially in acute and chronic conditions. It is generally established that the nutritive and non-nutritive constituents of human diets can be used to modify the risk of developing or aggravating human disease conditions (Health Canada, 1998). In this regard, functional foods have emerged during the last two decades as adjuvant or alternative to chemotherapy especially in prevention and management of human diseases, and for maintaining optimum human health state. However, the positive effects of functional foods may not be observed in acute health conditions following short-term consumption since the levels of the active ingredients are often below the therapeutic amounts, or not readily biologically available due to complex interactions with polysaccharides and proteins of the food matrix (Manach et al., 2004; Parada & Aguilera, 2007). As a result, bioactive ingredients present in functional foods can be isolated and administered as pills to increase the amount of these molecules that are absorbed into blood circulation and transported to cellular targets. These bioactive ingredients are known as nutraceuticals, which were formally defined by Health Canada (1998) as: *"...products isolated or purified from foods that are generally sold in medicinal forms not usually associated with food. Nutraceuticals are demonstrated to have physiological benefits or provide protection against chronic*

*diseases"*. Nutraceuticals and bioactive compounds-enriched food products have increasingly become subject of various R&D programs, as the health and well-being of consumers gradually became the primary focus of the food industry. The growing market for functional foods and natural health products can be attributed to the consumers' understanding of the relationship between diet and disease, safety of the natural health products and low cost associated with the bioactive molecules compared to chemotherapeutic agents or drugs (Agriculture and Agri-Food Canada, 2010).

In Canada, the functional foods and nutraceuticals sector has experienced substantial growth during the last ten years. According to Statistics Canada, a total of $2.9 billion was reported in annual sales of functional foods and nutraceutical products by 389 firms in Canada during 2003-2004 (Palinic, 2007); this represents a substantial increase compared to the 294 firms earlier reported for 2002 (Tebbens, 2005). In 2007, the number of functional foods and natural health products firms in Canada increased to 689 with annual revenues of $3.7 billion; these firms produced a variety of products for Canada and international markets (Cinnamon, 2009). Many of these firms are recognized worldwide for their bioactive products such as flax and fish-derived omega-3 fatty acids, plant sterols and stanols, soluble fibres, soy proteins, prebiotics and probiotics (Agriculture and Agri-Food Canada, 2010). In Canada and other parts of the world, another growing trend in the functional foods and nutraceuticals sector is the use of food protein-derived peptides for intervention against chronic human disease conditions and for maintenance of general well-being. This approach involves enzymatic hydrolysis or fermentation of food proteins to release bioactive peptide sequences

followed by simple or complex post-hydrolysis processing to isolate the bioactive peptides from the complex mixture of other inactive molecules; bioactivity of the peptides represent physiologically-relevant beneficial properties in the human system beyond normal and adequate nutrition (Hartmann & Meisel, 2007). The food processing steps lead to concentration of the active peptides with the enhancement of the physiological activity of the products, which could also be nutritionally beneficial as a source of essential amino acids. This approach can provide the opportunity for diversification of the use of Canada's major protein-rich agricultural crops, especially low value crop products, beyond basic nutritional purposes.

Flax (*Linum usitatissimum* L.) is a major oilseed crop cultivated commercially in Canada, a leading world producer and exporter of flaxseed (Oomah & Mazza, 1993; Agriculture and Agri-Food Canada, 2007). Most of the nutritional and health benefits attributed to flaxseed are due to its omega-3 fatty acid ($\alpha$-linolenic acid) content, which is extracted from the crushed oilseed by pressing or use of solvent extraction (Hall et al., 2006). The by-product of the flaxseed oil extraction process is known as defatted flaxseed meal, and this contains large amounts of dietary fibre, lignans and proteins. Both flaxseed-derived dietary fibres and lignans possess human health benefits (Hall et al., 2006). In 2006-2007, the world flaxseed meal production was estimated at 1.4 million tonnes, and Canada generated annual revenue of $2.5 million from exporting flaxseed meal (Agriculture and Agri-Food Canada, 2007). Moreover, the Canadian flaxseed from 2009 harvest was reported to contain 22% protein with a 10-year mean value of 23.2% protein (Canadian Grain Commission, 2009). Thus, there is a worldwide

production of about 0.31 million tonnes of flaxseed proteins per annum. This large amount of flaxseed proteins has not been optimally utilized in human food systems, although flaxseed meal has been incorporated into livestock feeds as protein and fibre supplement (Agriculture and Agri-Food Canada, 2007; Bell & Keith, 1993). The use of flaxseed meal as feed supplement, and perhaps as protein source in human, has been hindered due to its constituent anti-nutritional factors especially cyanogenic glycosides (Oomah & Mazza, 1993), but the development of advanced processing techniques could encourage removal of these toxic compounds to enhance value of the oilseed meal. Compared to other food proteins, flaxseed proteins possess rich amino acid profiles with a high amount of branched-chain and cationic amino acids (Oomah & Mazza, 1995; Hall et al., 2006). The abundance of low-value flaxseed meal that is underutilized in human food system underscores the need for conversion of defatted flaxseed meal into value-added products, especially bioactive peptides, for increased human health and economic benefits.

Food protein hydrolysates and purified food-derived peptides have exhibited several *in vitro* and *in vivo* physiologically-relevant activities such as antihypertensive, antioxidant, immunomodulatory, antimicrobial and lipid-lowering activities (Erdmann et al., 2008; Aluko, 2008a, b; Meisel, 2004; Pihlanto, 2006). Plant proteins are preferred over animal proteins as source of bioactive peptides because plant proteins are cheaper and more abundant for large scale peptide production (Aluko, 2008a). The specific bioactivity of food peptides depends primarily on their structural properties such as chain length and physicochemical characteristics of the amino acid residues, e.g.

hydrophobicity, molecular charge and side chain bulkiness (Pripp et al., 2004a). Therefore, in-depth knowledge about the structure-function property of peptides can contribute towards the discovery of more potent peptides and design of appropriate processing conditions to liberate these peptides from their parent proteins. Previous peptide quantitative structure-activity relationship (QSAR) studies have focused mainly on peptide inhibitors of angiotensin I-converting enzyme (ACE), a class of antihypertensive agents, resulting in the discovery of peptides with more potent activities (Pripp et al., 2004b, 2005; Pripp, 2005; Wu et al., 2006a,b; Shu et al., 2009). ACE and renin are key metabolic enzymes of the blood pressure regulating renin-angiotensin system (RAS). Unlike ACE-inhibiting peptides, there is dearth of literature information on the inhibition of renin by food-derived peptides as well as their structural requirements for potency. This information would be beneficial towards antihypertensive therapy because renin catalyzes the rate-limiting step of the RAS pathway, and its inhibition can potentially provide better control over elevated blood pressure than the inhibition of only ACE activity. Moreover, peptides with more than one (multifunctional) bioactive property such as ACE/renin inhibitors and antioxidants can be beneficial in diseases with multiple symptoms such as cardiovascular disease.

In this thesis, it was hypothesized, based on the amino acid profile of flaxseed meal proteins, that controlled enzymatic hydrolysis could generate structurally distinct peptides with physiologically-relevant multifunctional bioactive properties. Specifically, it was hypothesized that:

(1)  Low molecular weight peptides derived from flaxseed proteins would possess multifunctional bioactive properties as antioxidants, anti-inflammatory and antihypertensive agents;

(2)  Flaxseed peptides with net positive charge (cationic peptides) would bind the negatively charged calmodulin (CaM) leading to the inhibition of CaM-dependent phosphodiesterase (CaMPDE), which has been implicated in chronic human diseases;

(3)  Branched-chain amino acid-enriched peptide product could be generated from flaxseed protein through enzymatic treatments and simple cost-effective processing;

(4)  Arginine-enriched low molecular weight peptide product could be generated from flaxseed protein, and this product, when efficiently absorbed, could serve as a precursor of nitric oxide, which possesses physiological vasodilatory effect and could be used to lower blood pressure during hypertension;

(5)  The sequences and structural characteristics of amino acid residues in bioactive peptides determine their *in vitro* renin-inhibitory activities.

Based on these hypotheses, the general objectives of this project were to: a) produce bioactive peptides from flaxseed proteins as means of increasing the value-added utilization of defatted flaxseed meal, which will provide economic benefits to Canada and oilseed processing industry; and b) study the structure-function properties of low molecular weight renin inhibiting peptides. The specific objectives of this project were to:

(1)    Optimize the isolation of flaxseed proteins from defatted flaxseed meal and generate various functional enzymatic hydrolysates from these proteins under controlled conditions;

(2)    Determine kinetics of the inhibition of ACE, renin and CaMPDE by the protein hydrolysates as well as their ability to scavenge free radicals and inhibit nitric oxide production in activated macrophages;

(3)    Fractionate low molecular weight peptides that can simultaneously inhibit ACE, renin and CaMPDE activities from the flaxseed protein hydrolysates using chromatographic techniques;

(4)    Study the structure-function properties of renin inhibiting natural dipeptides by developing QSAR models using the partial least squares (PLS) regression method;

(5)    Design a cheap and efficient process for the production of high Fischer ratio peptide mixture from flaxseed protein isolate and evaluation of the multifunctional bioactive properties of this product;

(6)    Use electrodialysis-ultrafiltration, a novel membrane technology, to separate arginine-rich peptides from flaxseed protein hydrolysates and evaluate the peptide product for *in vitro* (inhibition of ACE and renin) and *in vivo* (antihypertensive activity in spontaneously hypertensive rats, SHR) activities.

Therefore, findings from this study could contribute towards value-added utilization of defatted flaxseed meal, which is an abundant by-product of Canada's oilseed processing industry. Results from this work can also provide fundamental information on the structural requirements for renin inhibition by food peptides, which

can serve as template for the design and synthesis of more potent peptide and peptidomimetic antihypertensive agents.

**CHAPTER TWO**

**LITERATURE REVIEW**

**2.1. Hypertension and the renin-angiotensin system (RAS)**

Hypertension or sustained elevated blood pressure is a medical condition regarded as a controllable risk factor in the development of cardiovascular, cerebrovascular and other vascular diseases such as stroke, myocardial infarction, left ventricular and smooth muscle cell hypertrophy (Kannel, 1996). Although not a disease, hypertension has emerged as a global public health concern that represents a major cause of death worldwide. A World Health Organization (WHO) report estimated that 7.5 million premature deaths worldwide were due to hypertension, leading other risks with a total contribution of 12.8% deaths (WHO, 2009). In 2000, 972 million people worldwide had hypertension and this number was projected to increase to 1.56 billion in 2025 (Kearney et al., 2005). In U.S., about 31% and 25% of adults are hypertensive and pre-hypertensive, respectively (National Centre for Health Statistics, 2008; Lloyd-Jones et al., 2010) and it was estimated that over $76 billion (US) will be spent in 2010 in healthcare services, medication and other costs associated with hypertension (Lloyd-Jones et al., 2010). The Canadian Health Measures Survey completed between 2007 and 2009 reported that 19% of the Canadian adult population have hypertension (Wilkins et al., 2010). Therefore, hypertension has attracted substantial research interests especially towards the discovery of therapeutic agents and lifestyle modification approaches to reduce its prevalence. The latter strategy, also known as dietary approaches to stop hypertension (DASH), has been reported to be successful in lowering

blood pressure in hypertensive patients (Colin et al., 2000) while therapeutic agents are primarily used to target blood pressure-regulating pathways.

Blood pressure is physiologically controlled by the renin-angiotensin system (RAS) and the kinin-nitric oxide system (KNOS) as shown in Figure 2.1. The RAS involves activation of angiotensinogen, a zymogen, by the proteolytic activity of renin, an aspartate protease, which converts it to angiotensin (AT)-I. This reaction is the first and rate-limiting step of the RAS pathway. AT-I is then cleaved at the histidyl residue from the C-terminus by the activity of angiotensin-I converting enzyme (ACE) to produce AT-II. AT-II is a powerful vasoconstrictor that functions by binding to receptors, which are located in tissues all over the body. Interactions of AT-II with receptors will elicit physiological reaction cascades that lead to blood vessel contractions that maintain normal blood pressure. However, in pathological conditions, there is excessive level of AT-II, which causes severe blood vessel contractions and limited relaxation to produce high blood pressure. The KNOS system is involved in the production of bradykinin, which exerts its antihypertensive effects by eliciting reactions that increase intracellular $Ca^{2+}$ concentration leading to activation of nitric oxide synthases (NOS) that produce nitric oxide (NO), a powerful vasodilator. ACE degrades bradykinin, and increased concentration of ACE leads to dual effects, the prevention of vasodilation and activation of vasoconstriction (Figure 2.1). Based on the roles of ACE in the RAS pathway, inhibitors of this enzyme have been used as antihypertensive agents (Ibrahim, 2006). Moreover, direct inhibition of renin can potentially provide better control of elevated blood pressure than ACE inhibition since it prevents the synthesis of AT-I, which can be

converted to AT-II in some tissues via an ACE-independent alternative chymase-catalyzed pathway (Segall *et al.*, 2007). However, inhibition of renin activity does not prevent ACE-catalyzed bradykinin degradation (Staessen *et al.*, 2006). Thus, there is a need to develop therapeutic agents that could provide multiple effects as ACE and renin inhibitors.

**Figure 2.1 The renin-angiotensin system (RAS) showing the molecular targets for antihypertensive agents; AT-I, angiotensin I; AT-II, angiotensin II; ACE, angiotensin I-converting enzyme**
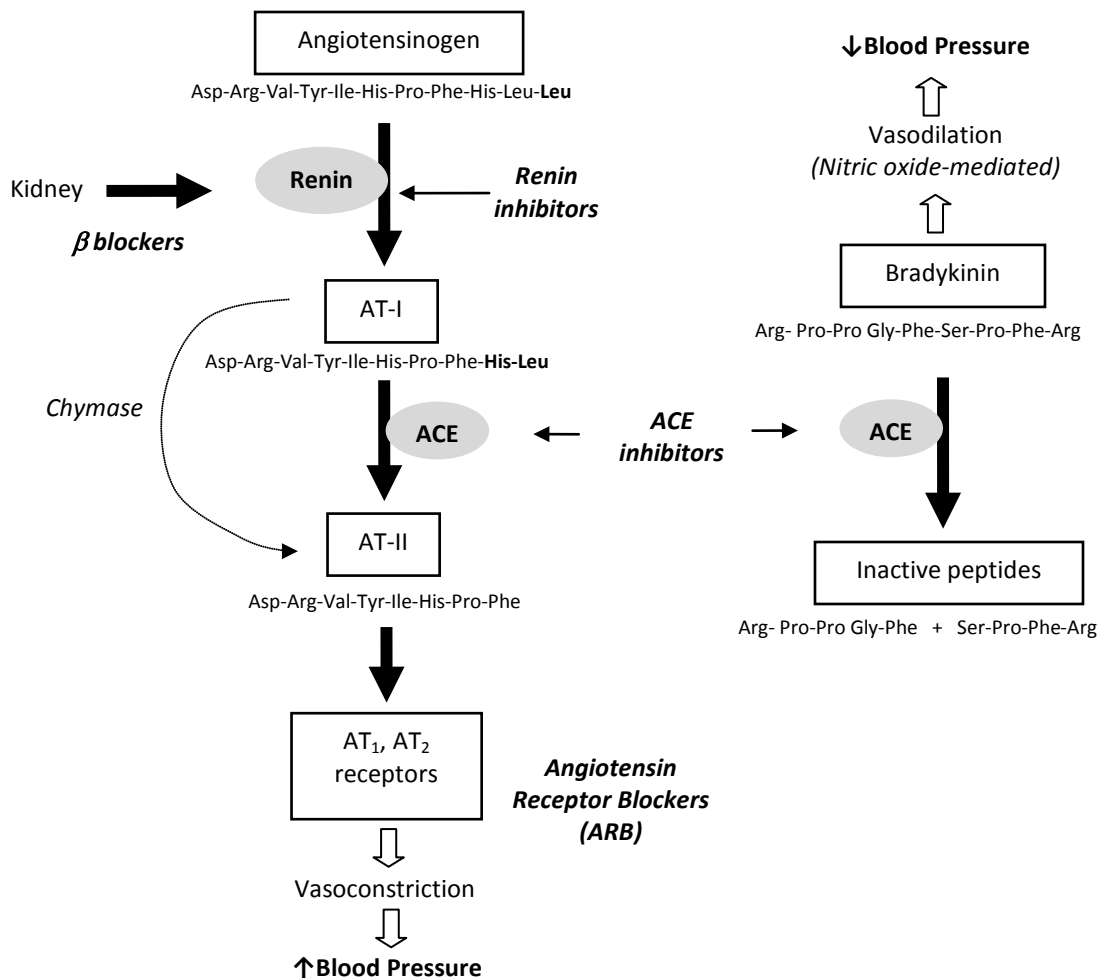
## 2.2. Reactive oxygen species and free radicals in human health conditions

## 2.2.1. Inflammatory responses

In cells, reactive oxygen species (ROS) and free radicals are constantly produced as by-products of cellular processes, secondary messengers or agents of immune response reactions (Halliwell, 1991; Turrens, 2003). The human body is also exposed to exogenous ROS/free radicals from diets and the atmosphere, and to microbial pathogens that can induce overproduction of ROS (Halliwell, 1991). Upon exposure to microorganisms or endotoxins, the immune system becomes activated and phagocytic cells (e.g. macrophages, monocytes and neutrophils) release several pro-inflammatory cytokines leading to activation of NF-κB transcription factor and subsequent up-regulation of the production of enormous amounts of ROS to combat the invading pathogens and protect cells from infection (Dinarello, 2000). Figure 2.2 shows the production of ROS, their involvement in cellular damages and the role of endogenous antioxidant system in maintaining normal homeostasis. Upon binding of endotoxins to surface receptors, phagocytic immune cells produce ROS by activation of the NADPH oxidase complex during phagocytosis (Heyworth et al., 1999). Activated NADPH oxidase catalyzes the reduction of molecular oxygen to superoxide radical ($O_2^{\cdot-}$). The amount of $O_2^{\cdot-}$, also produced due to electron leakage in the mitochondria, can be controlled by superoxide dismutase (SOD), which converts $O_2^{\cdot-}$ to hydrogen peroxide ($H_2O_2$) and oxygen. $H_2O_2$ is in turn detoxified to water and oxygen by catalase or glutathione peroxidase, but can also be converted to highly reactive hydroxyl radical ($^{\cdot}OH$) through the transition metal ion (e.g. $Fe^{2+}$)-mediated Fenton reaction, or to other powerful

oxidants, such as hypochlorous acid and singlet oxygen ($^1O_2$), by the action of myeloperoxidase (Klebanoff, 1999; Turrens, 2003).

**Figure 2.2 Production of ROS/free radicals and oxidative stress-mediated oxidative damage to biological macromolecules**



**SOD**, superoxide dismutase; **GPx**, glutathione peroxidase; **GR**, glutathione reductase
**GSH**, glutathione (reduced); **GSSH**, glutathione (oxidized); iNOS, inducible NOS
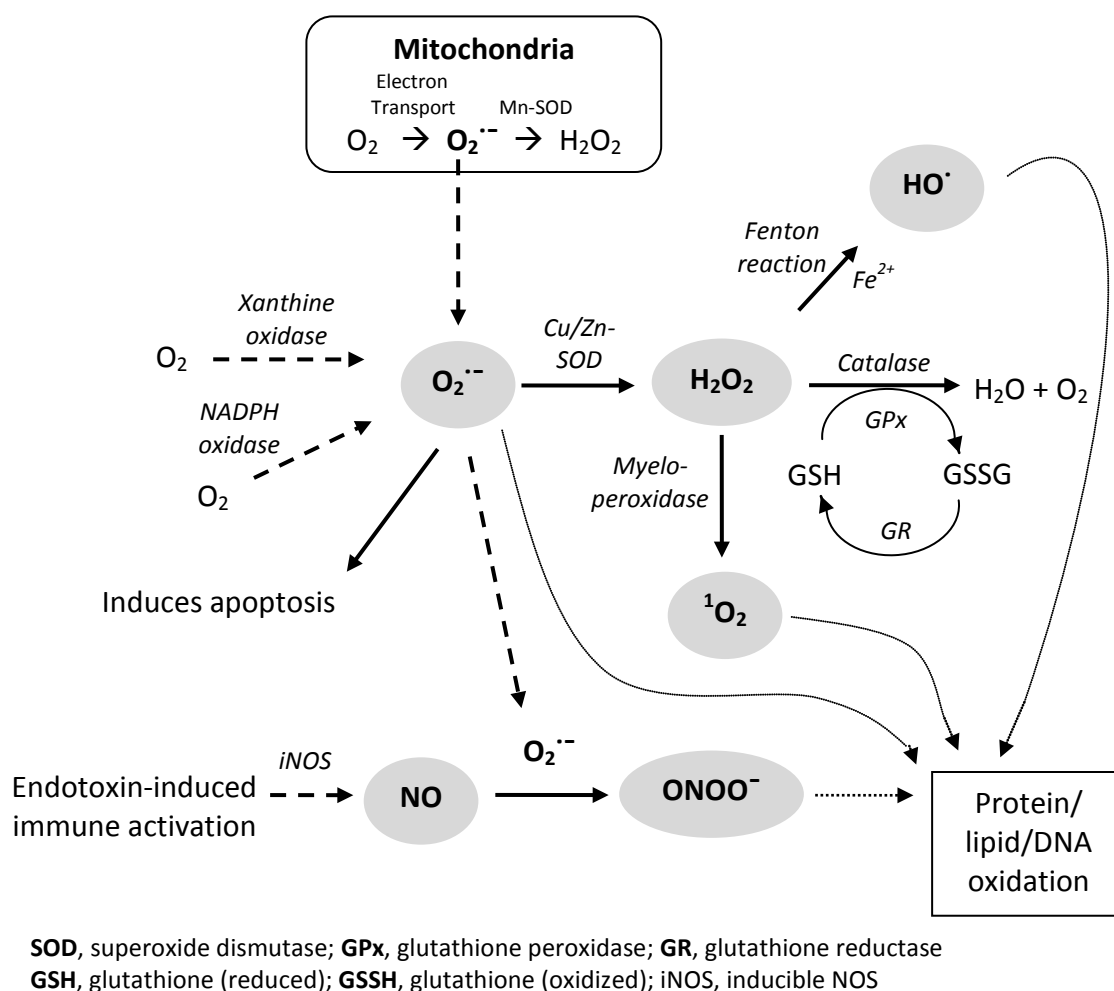
In addition, immune activation involves cytokine-mediated activation of the expression of inducible nitric oxide synthase (iNOS or NOS2), which catalyzes the production of nitric oxide (NO) from arginine and molecular oxygen, and cycloxygenase-

2 (COX-2), which produces proinflammatory prostaglandin $E_2$ from unsaturated fatty acids (Moncada & Higgs, 1993; Pacher et al., 2007). NO is beneficial in signal transduction and vasodilation, but can react with the abundant $O_2^{·-}$ to form highly reactive peroxynitrite ($ONOO^-$) that causes most cellular damages attributed to NO (Pacher et al., 2007); these N-containing oxidants are also referred to as reactive nitrogen species (Turrens, 2003). These oxidative processes are tightly controlled by the endogenous antioxidant system, but when produced in large amounts due to chronic or acute immune activation, ROS can overwhelm the endogenous redox regulatory system leading to cellular oxidative damages such as degradation of membrane lipids (loss of membrane integrity), DNA fragmentation, protein modification (e.g., tyrosine nitration) that causes loss of function and activation of the cysteine proteases (caspases) of the cell death pathway, resulting in apoptosis or programmed cell death (Ames, 1983; Ames et al., 1993; Wiseman & Halliwell, 1996; Pacher et al., 2007). The condition whereby ROS are over-produced in cells is known as "oxidative stress" or "respiratory burst", to reflect the increased cellular oxygen consumption associated with the process. Oxidative stress plays important roles in the aetiology and progression of human physiological and disease conditions (Ames, 1983; Ames et al., 1993; Pacher et al., 2007; Torres et al., 2002; Liu et al., 2002). Several studies have established that ROS/free radicals contribute to initiation, promotion and progression of cancer by causing DNA damage leading to the inactivation of proto-oncogenes or tumour-suppressor genes, and modification of other genes and proteins involved in cell proliferation, differentiation and apoptosis (Wiseman & Halliwell, 1996). In addition, excessive ROS

production could trigger inflammatory disease-related tissue damage (Liu et al., 2002) and pathogenesis of human immunodeficiency virus (HIV)-1 infection (Torre *et al*., 2002). Oxidative stress can be surmounted by the consumption of dietary or drug antioxidants to support the overwhelmed endogenous antioxidant system (Ames et al., 1993; Halliwell, 1996; Wiseman & Halliwell, 1996).

### 2.2.2. Neurodegenerative diseases

ROS and free radicals also play important pathophysiological roles in aetiology and progression of neurodegenerative diseases (Figure 2.3). A number of toxins and pathogens, such as bacteria, fungi and viruses, are capable of invading the brain cells through blood circulation, and elicit cellular immune responses. Of the 3 major resident cells involved in the phagocytosis and clearance of the invading pathogens, the microglia plays a crucial role as macrophages in the central nervous system (CNS). Microglia constitutes about 15% of all the cells of the CNS and serves as immune cells in the defence of brain cells (Rock et al., 2004; Carson et al., 2008). Upon activation, the microglia releases several pro-inflammatory cytokines that initiate the oxidative process. It has been shown that, in the glial cells, TNF-$\alpha$, IL-1$\beta$ and interferon-$\gamma$ are the most potent activators of the expression of iNOS and COX-2 genes (Hunot & Hirsch, 2003), whose protein products catalyze the production of nitric oxide and pro-inflammatory prostaglandins, respectively; iNOS is not normally produced in brain cells (Murphy, 2000).

**Figure 2.3 Production of ROS and free radicals in the brain and their roles in oxidative damages to cellular macromolecules leading to neuroinflammation and neuronal cell death during neurodegenerative diseases**
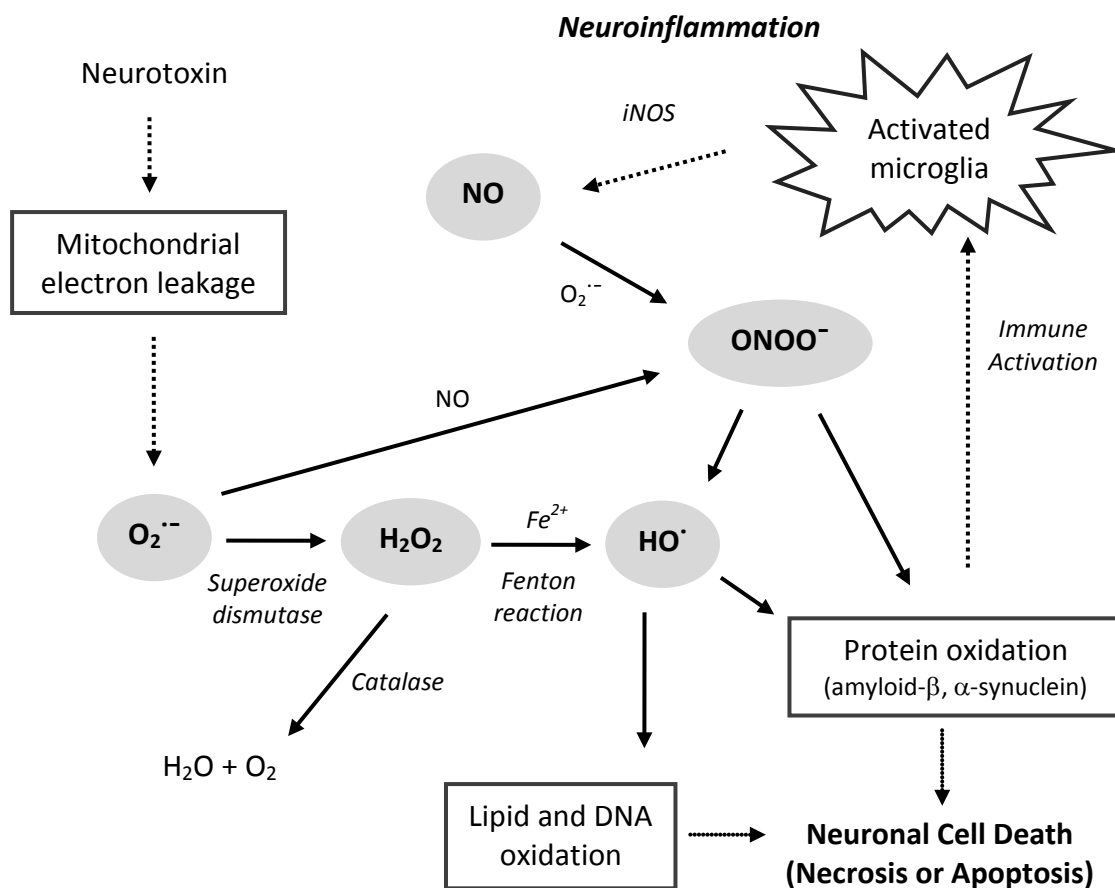


Other enzymes induced by this process in the brain immune cells include NADPH oxidase and myeloperoxidase, leading to excessive production of $O_2^{\cdot-}$ and hypochlorous acid, respectively. These reactive oxygen species are utilized by the macrophage-like cells in attacking the pathogens. Nitric oxide is less damaging by itself but reacts with $O_2^{\cdot-}$, which is also excessively produced by the macrophages due to mitochondrial

electron leakage, to form ONOO⁻ that causes damage to biomacromolecules including DNA in glial cells and peripheral dopaminergic neurons (Hunot & Hirsch, 2003; Pacher et al., 2007). Superoxide radical could also cause direct damage to cells by reacting with polyunsaturated fatty acid components of the biological membrane lipid bi-layer. Endogenous antioxidant superoxide dismutase utilizes $O_2^{\cdot-}$ to produce $H_2O_2$, with concomitant production of $^{\cdot}OH$ through the Fenton reaction. These oxidative processes are tightly regulated by the endogenous antioxidant system but can be exacerbated in chronic and acute infection, and other pathological conditions, leading to neuronal cell damages associated with diseases such as Alzheimer's and Parkinson's (Hirsch & Hunot, 2009). Moreover, the brain cells are particularly susceptible to oxidative damage due to its high oxygen consumption (Halliwell, 2001). Overall, oxidative burst and inflammation in neuronal cells contribute to pathogenesis in neurodegenerative diseases (Ames et al., 1993). Thus, it is important to develop therapeutic agents that can reduce oxidative pathogenesis associated with these diseases by scavenging these reactive species, inhibiting their production, or by decreasing the levels of the pro-inflammatory cytokines released during microglial activation. According to Halliwell (2001), it is important that the reactive species targeted by the therapeutic agent should have a direct relevance in the disease process, and must be present at the site of injury; thus, all the aforementioned ROS play important roles in propagating neuronal cell damages (Hirsch & Hunot, 2009).

### 2.2.3. Oxidative stress, inflammation and hypertension

Hypertension is proinflammatory. Elevated levels of AT-II during hypertension lead to phospholipase C activation followed by increase in intracellular $Ca^{2+}$ concentration (causes smooth muscle (SM) contraction and hypertrophy), and increased SM lipoxygenase activity, which increases oxidation of low density lipoproteins (LDL) (Ross, 1999). The oxidized LDL (LDL-oxd) in the arterial intima is taken up by scavenger receptors of macrophages and accumulation of cholesterol causes macrophage transformation into foam cells (Hansson, 2005). LDL-oxd activates inflammation by triggering endothelial cells to express vascular cell adhesion molecules that bind monocytes and T-lymphocytes; the immune cells attracted to the site also express inflammatory genes (Ross, 1999; Hansson, 2005). Activated macrophages overproduce ROS, which react with NO to decrease its availability in the vascular endothelium leading to increased leukocyte adhesion to the endothelium (Ross, 1999). These processes increase peripheral vascular resistance and blood pressure, which ultimately increases the risk of cardiovascular disease.

### 2.3. Calmodulin (CaM) and CaM-dependent enzymes

Calmodulin (CaM) is a ubiquitous negatively-charged 148-amino acid (16.6 kDa) $Ca^{2+}$-binding protein that is involved in the activation of several important proteins in response to increased intracellular $Ca^{2+}$ concentration (Ikura et al., 1992; Hooks & Means, 2001). Instead of having multiple $Ca^{2+}$-sensing proteins in several cells, CaM has evolved to serve as mediator for detecting $Ca^{2+}$ signals and activating target proteins in

several cells (Lakowski et al., 2007). The activation process involves binding of $Ca^{2+}$-free CaM (apo-CaM) to $Ca^{2+}$, which induces conformational changes that expose a methionine-rich hydrophobic region on CaM structure for binding a short peptide sequence of target proteins with high affinity (Crivici & Ikura, 1995; Schauer-Vukasinovic et al., 1997). Some clinically important enzymes that require $Ca^{2+}$/CaM activation include endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase, cyclic nucleotide phosphodiesterase 1 (CaMPDE), adenosine triphosphatase, phospholipase $A_2$, adenylate cyclase and protein kinase II (Itano et al., 1980). Thus, CaM plays important roles in several cellular processes including cell growth, cell proliferation, neurotransmission and smooth muscle contraction (Cho et al., 1998).

A total of 11 PDE families (PDE1-11) have been identified in human. CaMPDE is the isoform from the PDE1 gene family, which gives three distinct gene products namely PDE1A, PDE1B and PDE1C; they are expressed in various cell types including coronary vascular smooth muscle cells (SMC) where CaMPDE play a role in the regulation of vascular tone (Bender & Beavo, 2006; Kass et al., 2007). CaMPDEs possess two binding sites for $Ca^{2+}$/CaM complex, and complete binding is required for up to 10-fold or more activation of the enzyme (Rybalkin et al., 2003). As shown in Figure 2.4, CaMPDE catalyzes the conversion of cyclic nucleotide monophosphates (PDE1A/B/C for cGMP and PDE1C for cAMP) to nucleotide monophosphates (GMP and AMP), thereby modulating cellular processes mediated by the second messengers (cAMP/cGMP) including neurotransmission, immune cell activity, vasodilation, cell growth and proliferation (Cho et al., 1998). Chronic over-expression of CaMPDE has been found in

atherosclerosis, heart pressure-load stress, heart failure and vascular proliferation (Kass et al., 2007). Separate inhibitors of PDE1A and PDE1C expressions and activity in cultured cells has been reported to decrease PDE1-induced proliferation of human SMC, which is important towards treatment of atherosclerosis, cancer and other responses due to SMC proliferation (Rybalkin et al., 2003; Nagel et al., 2006). Moreover, inhibition of PDE1B expression and activity inhibited proliferation and also induced cAMP-mediated apoptosis in cultured human leukemic cells, which is favourable for cancer chemotherapy since normal blood cells do not express PDE1B (Jiang et al., 1996). In addition, AT-II increases PDE1A1 isoform expressions in SMC through AT-II-mediated increase in cellular $Ca^{2+}$ concentration (Kim et al., 2001). Thus, CaMPDE-induced pathogenesis can also be linked to RAS-mediated hypertension; CaMPDE has been implicated in pulmonary arterial hypertension (Schermuly et al., 2007). PDE1B2 expression and activity are also upregulated during monocyte differentiation, and contributes to pathogenesis during inflammation, which makes CaMPDE a good target for anti-inflammation therapy (Bender et al., 2005).

One approach towards reducing these negative effects of CaMPDE include the use of $Ca^{2+}$ chelating agents to prevent CaM activation thereby decreasing CaMPDE activity; however, specificity will not be achieved since $Ca^{2+}$ is involved in numerous cellular processes. Another approach is the use of CaM-binding agents, which can specifically regulate the activity of only CaM-dependent PDE isoforms and other CaM-dependent enzymes. Thus, CaM-binding compounds can be used as therapeutic agents against diseases induced or exacerbated by increased CaM or CaMPDE expression. Some

known potent chemical inhibitors of CaMPDE include vinpocetine, KS-505a, IC224, SCH51866, W-7 and phenothiazines (Bender & Beavo, 2006). Several natural products and synthetic peptides have also displayed CaM inhibition (Martínez-Luis et al., 2007). Details on CaM-binding natural peptides can be found in Section 2.4.6.

**Figure 2.4 The role of Ca$^{2+}$/calmodulin-dependent phosphodiesterase (CaMPDE) in the regulation of cellular processes**
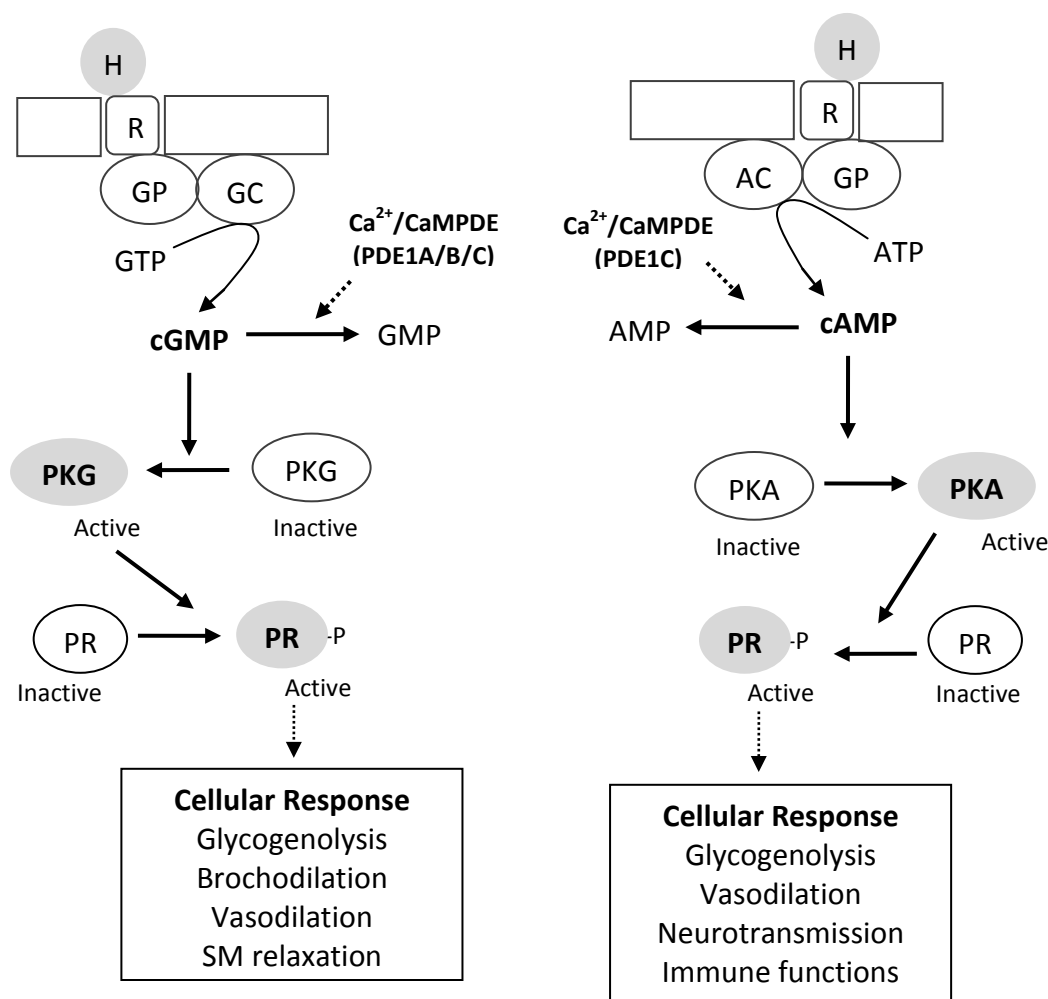


H, hormone; **GP**, G protein; **R**, GP-coupled receptors; **GC**, guanylate cyclase; **AC**, adenylate cyclase; **ATP**, adenosine triphosphate; **GTP**, guanosine triphosphate; **PKG**, cGMP-dependent protein kinase; **PKA**, cAMP-dependent protein kinase; **PR**, target protein; **SM**, smooth muscles

**2.4. Food protein-derived bioactive peptides in human health**

Food protein hydrolysates have been shown to exhibit potent biological activities (Figure 2.5), which are largely due to their constituent peptides. These peptides are encrypted in the primary structure of plant and animal proteins as inactive amino acid sequences but they can be released by fermentation, food processing and enzyme-catalyzed proteolysis *in vitro* or *in vivo* after human consumption (Li et al., 2004; Hartmann & Meisel, 2007; Aluko, 2008b). In most cases, these protein hydrolysates and peptides have demonstrated better bioactivities (Figure 2.5) compared to their parent proteins, and this shows that hydrolysis of peptide bonds is important in liberating the potent peptides. Several factors affect the bioactive properties of the peptides including the enzymes used for hydrolysis, processing conditions and the size of the resulting peptides, which greatly affects their absorption across the enterocytes and bioavailability in target tissues. Generally, the activity of these peptides against molecular disease targets are regarded as lower than synthetic peptidomimetics and drugs, but the use of bioactive peptides in intervention against human diseases offers many advantages including safety of the natural product, low health cost and the additional nutritional benefits of the peptides as source of beneficial amino acids. The current literature contains a vast amount of information on food protein-derived bioactive peptides with physiologically-relevant bioactive properties including ACE-inhibiting/antihypertensive, anticancer or cytomodulatory, antioxidant, opioid-like, hypocholesterolemic, immunomodulatory, antimicrobial and mineral-binding activities (Table 2.1). These peptides are mostly derived from milk and soy proteins, and range in

sizes from di-, tri- and oligopeptides to high molecular weight (HMW) polypeptides (Hartman & Meisel; 2007; Erdmann et al., 2008; Hernández-Ledesma et al., 2009a). Based on these bioactivities, several food-derived peptide products have been developed and commercialized for use as human health promoting agents as shown in Table 2.2. Details on the biological properties of some of the food-derived peptides are discussed in the following sub-sections.

**Figure 2.5 Bioactive properties of food protein-derived peptides relevant to the promotion of human health and disease prevention**
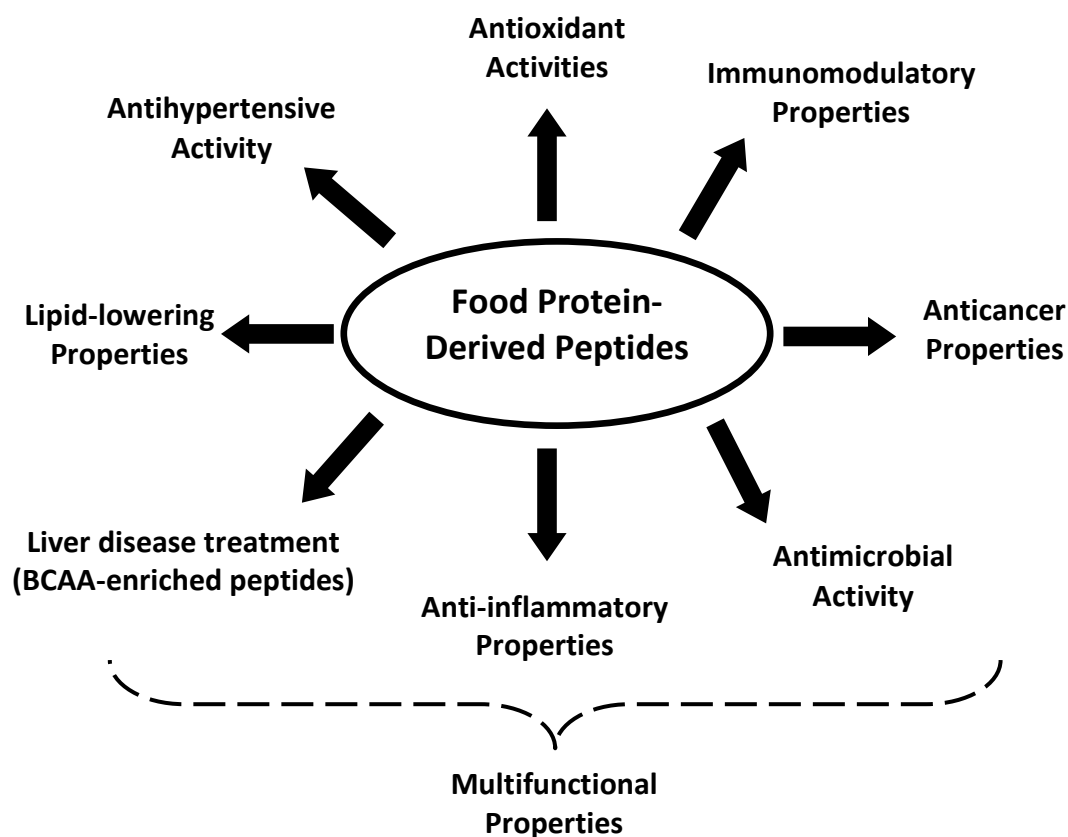
**Table 2.1 Examples of bioactive peptide-based products that are derived from foods[a]**

| Effects | Origin | Encrypting protein(s) | Name/remarks/sequence (in single-letter code) |
|---|---|---|---|
| ACE-inhibitory/hypotensive | Soy | Soy protein | NWGPLV |
| | Fish | Fish muscle protein | LKP, IKP, LRP (derived from sardine, bonito, tuna, squid) |
| | Meat | Meat muscle protein | IKW, LKP |
| | Milk | α-LA, β-LG | Lactokinins (e.g. WLAHK, LRP, LKP) |
| | | α-, β-, κ-CN | Casokinins (e.g. FFVAP, FALPQY, VPP) |
| | Egg | Ovotransferrin | KVREGTTY |
| | | Ovalbumin | Ovokinin (FRADHPPL), Ovokinin (2–7) (KVREGTTY) |
| | Wheat | Wheat gliadin | IAP |
| | Broccoli | Plant protein | YPK |
| Immunomodulatory | Rice | Rice albumin | Oryzatensin (GYPMYPLR) |
| | Egg | Ovalbumin | Peptides not specified |
| | Milk | α-, β-, κ-CN, α-LA | Immunopeptides (e.g. αS1-immunocasokinin) (TTMPLW |
| | Wheat | Wheat gluten | Immunopeptides |
| Cytomodulatory | Milk | α-, β-CN | α-Casomorphin (HIQKED(V)), β-casomorphin-7 (YPFPGPI) |
| Opioid agonist | Milk | Lactoferrin | Lactoferroxins |
| | | κ-CN | Casoxins |
| Antimicrobial | Egg | Ovotransferrin | OTAP-92 (f109–200) |
| | | Lysozyme | Peptides not specified |
| | Milk | Lactoferrin | Lactoferricin |
| Antithrombotic | Milk | κ-CN (glycomacropeptide) | κ-CN (f106–116), casoplatelin |
| Mineral-binding, anticarcinogenic | Milk | α-, β-CN | Caseinophosphopeptides |
| Hypocholesterolemic | Soy | Glycinin | LPYPR |
| | Milk | β-LG | IIAEK |
| | Fish | Sardine muscle | MY |
| | Wheat | Wheat germ protein | Peptides not specified |
| | Milk | α-LA, β-LG | MHIRL, YVEEL, WYSLAMAASDI |

[a]Table adapted from Hartman & Meisel (2007) with permission from Elsevier; CN, casein; LA, lactalbumin; LG, lactoglobulin

**Table 2.2 Examples of commercially available functional foods or food ingredients carrying bioactive peptides[a]**

| Product name | Manufacturer | Type of food | Bioactive peptides | Health claim |
|---|---|---|---|---|
| Calpis AMEEL S (Japan) or Calpico (Europe) | Calpis Co., Japan | Sour milk | VPP, IPP from $\beta$- and $\kappa$-CN | Hypotensive |
| Evolus | Valio, Finland | Fermented milk, calcium-enriched | VPP, IPP from $\beta$- and $\kappa$-CN | Hypotensive |
| BioZate | Davisco, USA | $\beta$-LG hydrolysate | Whey peptides | Hypotensive |
| C12 Peption | DMV, Netherlands | Ingredient | Casein-derived dodecapeptide FFVAPFPEVFGK | Hypotensive |
| Peptide Soup | NIPPON, Japan | Soup | Bonito-derived peptides | Hypotensive |
| Casein DP Peptio Drink | Kanebo, Japan | Soft drink | Casein-derived dodecapeptide FFVAPFPEVFGK | Hypotensive |
| BioPURE-GMP | Davisco, USA | Whey protein hydrolysate | Glycomacropeptide | Anticarcinogenic, antimicrobial, antithrombotic |
| CholesteBlock | Kyowa Hakko, Japan | Drink powder | Soy peptides bound to Phospholipids | Hypocholesterolemic |
| CSPHP ProDiet F200 | Ingredia, France | Milk drink, confectionery | $\alpha$S1-CN (f91–100), YLGYLEQLLR | Reduces stress |
| Capolac | Arla Foods, Denmark | Ingredient | CPP (caseinophosphopeptide) | Helps mineral absorption |
| Tekkotsu Inryou | Suntory, Japan | Soft drink | CPP | |
| Kotsu Kotsu calcium | Asahi, Japan | Soft drink | CPP | |
| CE90CPP | DMV, Netherlands | Ingredient | CPP (20%) | |
| Glutamine Peption WGE80GPA WGE80GPN WGE80GPU | DMV, Netherlands | Dry milk protein hydrolysate | Glutamine-rich peptides | Immunomodulatory |

[a]Table adapted from Hartman & Meisel (2007) with permission from Elsevier; CN, casein; LA, lactalbumin; LG, lactoglobulin

**2.4.1. ACE-inhibitory and antihypertensive peptides**

The pioneering work on naturally occurring ACE-inhibiting antihypertensive peptides from snake (*Bothrops jararaca*) venom (Ferreira et al., 1970; Ondetti et al., 1971; Kato et al., 1971) sprouted several investigations on the use of food protein-derived peptides as antihypertensive agents. Till date, several ACE-inhibiting peptides have been reported from an enormous list of plant and animal food proteins most especially milk (casein and whey), fish, egg and soy proteins (Li et al., 2004; FitzGerald et al., 2004; Aluko et al., 2008a,b; Hartman & Meisel, 2007; Erdmann et al., 2008). Table 2.1 shows some ACE-inhibiting peptide sequences derived from food proteins. The peptide inhibitory concentration that reduced ACE activity by 50% ($IC_{50}$) were reported to be as low as 2, 5 and 9 $\mu M$ for VAP ($\alpha$s1-casein f25–27), $\beta$-casein-derived IPP (f74-76) and VPP (f84-86), respectively (Nakamura e al., 1995; FitzGerald et al., 2004). IPP and VPP are the active ingredients of hypotensive products Calpis AMEEL S and Evolus (Table 2.2), and these tripeptides were reported to reduce systolic blood pressure (SBP) by −28.3 and −32.1 mmHg, respectively in spontaneously hypertensive rats (SHR) (Nakamura et al., 1995). In mildly hypertensive human subjects, Calpis (IPP/VPP) reduced SBP and diastolic blood pressure (DBP) by −14.1 and −6.9 mmHg, after consumption of 95 ml/day of the sour milk product for 8 weeks (Hata et al., 1996). Likewise, whey-derived peptide product, BioZate, exhibited hypotensive activity in hypertensive human volunteers by decreasing SBP (−11 mmHg) and DBP (−7 mmHg) after 6 weeks of consuming 20 g of the product per day (Pins & Keenan, 2002).

The mechanism of ACE inhibition by peptides has been studied using both synthetic and food protein-derived peptides. Using the Lineweaver-Burk plots for inhibition kinetics studies, most peptide ACE inhibitors exerted their activities by competitive mode of inhibition (Sato et al., 2002; Li et al., 2004), characterized by competition of the peptides with ACE substrate for the active (catalytic) sites of the enzyme. Moreover, some peptides have also exhibited non-competitive (e.g. LW and IY) and uncompetitive (e.g. IW and FY) modes of inhibition (Sato et al., 2002). The above example shows that a single amino acid substitution (even with isomers) can greatly influence the nature of interactions between peptides and ACE. Thus, an understanding of the structural basis for potency can lead to the discovery of more potent peptides. The molecular mechanism of ACE inhibition by peptides is still unclear. In a proposed binding model between the inhibitor and the active site of zinc metallopeptidase (ACE belongs to this class of enzyme), three circular cleft subsites or pockets ($S_1$, $S_1'$ and $S_2'$) located at the active site on ACE structure were designated the major sites for interaction of the C-terminal tripeptide residues (Ondetti & Cushman, 1982; Li et al., 2004). These pockets have been reported to contain predominantly hydrophobic amino acids (Ondetti & Cushman, 1982). This implies that food protein-derived peptides containing hydrophobic amino acid residues could effectively bind these hydrophobic pockets where they are held together by hydrophobic interactions. In support of this proposed mechanism, several reports have indicated that most competitive ACE-inhibiting peptides contain hydrophobic amino acids (Sato et al., 2002; Li et al., 2004). QSAR studies have also indicated that, for dipeptides and tripeptides, C-terminal bulky

hydrophobic amino acids (e.g. proline, tryptophan, phenylalanine and tyrosine) and N-terminal aliphatic amino acids (leucine, isoleucine and valine) are necessary structural features for ACE inhibition by peptides (Wu et al., 2006a). Moreover, structure-function studies have also showed that the last four C-terminal amino acid residues in 4-10 amino acid-containing peptides are important determinants for ACE inhibition, and that hydrophobic amino acids mostly occupy these positions in potent peptides (Wu et al., 2006b). Other amino acids, arginine and lysine with positive charge on the $\varepsilon$-amino group, also contribute substantially to ACE inhibition if present at the C-terminal of peptides, possibly by interacting with anionic allosteric binding sites different from the active site of ACE (Vermeirssen et al., 2004). The overall effect of ACE inhibition using food protein-derived peptides is decrease in the amount of circulating AT-II and elevated level of bradykinin with concomitant decrease in elevated blood pressure.

There is consensus that *in vitro* ACE inhibition by peptides does not generally translate into *in vivo* antihypertensive activity, and this is due to concerns about absorption, bioavailability and susceptibility of the peptides to degradation into inactive fragments by physiological proteases and peptidases (Vermeirssen et al., 2004). Thus, in order to use food-derived peptides as enterally potent hypotensive agents, they must show stability against gastrointestinal proteases following oral administration (FitzGerald et al., 2004; Li et al., 2004; Vermeirssen et al., 2004). This ensures that the original peptide sequences that displayed *in vitro* ACE inhibition are conserved and delivered to the cellular sites of action. In addition, peptides must also be absorbed through the enterocytes to the serum without degradation by brush border and serum

peptidases (FitzGerald and Meisel, 2003). As an example, peptides derived from milk proteins such as $\alpha_{s1}$-casein (f23-27) (Maruyama et al., 1987) and $\alpha_{s1}$-casein (f104-109) (Maeno et al., 1996) were reported to possess potent *in vitro* ACE-inhibitory activity, but they showed no effects in decreasing SBP in SHR (FitzGerald et al., 2004). In this regard, it would be desirable to investigate the ACE-inhibitory properties of small peptides such as di-, tri-peptides, and 4 amino acid-containing oligopeptides. Peptides of small sizes have demonstrated significant antihypertensive effects *in vivo*, resistance to peptidolysis and can be absorbed intact into blood circulation to target tissues (Abubakar et al., 1998; Vermeirssen et al., 2004). Moreover, ACE-inhibitory activity of peptides can be enhanced due to gastrointestinal peptidolysis. For example, intravenous injection of IVGRPRHQG did not produce hypotensive activity, but when hydrolyzed with trypsin into IVGRPR and HQG, the products decreased blood pressure in SHR (Li et al., 2004). A practical application of this concept is an antihypertensive "prodrug" Katsuobushi oligopeptide (LKPNM), which is converted by digestive enzymes into the active form, LKP (Hartmann & Meisel, 2007).

### 2.4.2. Antioxidant peptides

Dietary consumption of antioxidants can supplement the endogenous enzymatic and non-enzymatic antioxidant systems against oxidative stress (Fang et al., 2002). Although synthetic food antioxidants have been widely applied in the food industry for food preservation, the use of food-derived peptides has generated interest as both food preservative and health products. There is abundant literature information on several

food protein hydrolysates and peptides with antioxidant properties in various oxidative reaction systems *in vitro* and in cell culture. Plant and animal food protein sources of antioxidant peptides include pea, soy, fish, quinoa, milk casein, whey and egg (Erdmann et al., 2008; Humiski & Aluko, 2007; Pihlanto, 2006; Aluko & Monu, 2003). The antioxidant properties of these peptides include scavenging or quenching of ROS/free radicals and inhibition of ROS-induced oxidation of biological macromolecules such as lipids, proteins and DNA. The radical quenching activities of food antioxidants are due to the ability of the antioxidants to participate in single electron transfer (SET) or hydrogen atom transfer (HAT) reactions (Huang et al., 2005); thus, the abundance of peptidic amino acid residues that can transfer electrons or hydrogen atom to ROS/free radicals at physiological pH can contribute to enhanced antioxidative property. Other mechanisms of antioxidant activity of peptides include transition metal chelating activity and ferric reducing power. It is generally agreed upon than peptides possess better antioxidative activities than their constituent amino acids due to the electron-donating peptide bond, but some studies also indicated that some amino acids can be more active than their parent peptides (Erdmann et al., 2008; Kitts & Weiler, 2003).

Factors that affect the antioxidant activity of food protein hydrolysates include specificity of proteases used for hydrolysis, degree of hydrolysis and the structural properties of the resulting peptides including molecular size, hydrophobicity and amino acid composition (Pihlanto, 2006). The amount of histidine, cysteine, proline, methionine and aromatic amino acids contribute to the antioxidant activity of food peptides. Structure-function studies revealed that histidine residue of peptides can

chelate metal ion, quench active oxygen and scavenge ˙OH (Chen et al., 1996; Chen et al., 1998) and these properties were attributed to its imidazole group, which can participate in HAT and SET reactions (Chan & Decker, 1994). Similar potent antioxidant activity has also been reported for another histidine-containing dipeptide, carnosine (β-Ala-His), derived from muscle cells (Chan et al., 1994). Moreover, the addition of hydrophobic amino acids, proline and leucine, to the N-terminus of a dipeptide His-His resulted in enhanced *in vitro* antioxidative property of the peptides, and these new peptides also displayed synergistic effects when combined with non-peptide antioxidants (Chen et al., 1996; Kitts & Weiler, 2003). Hydrophobic amino acids are important for enhancement of the antioxidant properties of peptides since they increase the accessibility of the antioxidant peptides to hydrophobic cellular targets such as the polyunsaturated chain of fatty acids of biological membranes (Chen et al., 1998; Erdmann et al., 2008). Moreover, the electron-dense aromatic rings of phenylalanine, tyrosine and tryptophan residues of peptides can contribute to the chelating of pro-oxidant metal ions whereas phenylalanine can also scavenge ˙OH radicals to form more stable *para-*, *meta-* or *ortho-*substituted hydroxylated derivatives (Sun et al., 1993). Therefore, the specific contribution of individual amino acid residues to the antioxidant activity of a peptide depends largely on the nature of the ROS/free radical and the reaction medium.

In addition to the direct antioxidant activity due to the sulfhydryl functional group, cysteine residues of peptides can also serve as precursor for the synthesis of glutathione (γ-L-glutamyl-L-cysteinylglycine), a ubiquitous cellular antioxidant tripeptide,

thereby contributing towards regeneration of the physiological antioxidant defence system (Meisel, 2005). Food-derived peptides can also display antioxidant property by induction of gene expression of proteins that protect cellular components from oxidative stress-induced deterioration. In endothelial cells, a dipeptide Met-Tyr derived from sardine muscle protein stimulated the expression of heme oxygenase-1 and ferritin leading to a sustained cellular protection from oxidative stress (Erdmann et al., 2006).

### 2.4.3. Hypolipidemic and hypocholesterolemic peptides

Protease-assisted hydrolysis of food proteins can also release peptide sequences that possess cholesterol and lipid-lowering activities. These food protein sources include soy protein (Nagaoka et al., 1999; Aoyama et al., 2000; Cho et al., 2007), milk protein (Kirana et al., 2005), buckwheat protein (Kayashita et al., 1997), egg white protein (Manso et al., 2008) and fish protein (Wergedahl et al., 2004). However, enzymatic hydrolysis can also lead to reduced lipid-lowering activity of food proteins (Kayashita et al., 1997). Most literatures on lipid-lowering peptides were focused on soy protein hydrolysates and peptides. The hypocholesterolemic and hypolipidemic properties of soy protein hydrolysates reported in animals (Aoyoma et al., 2000) and in humans (Hori et al., 2001) have been partly attributed to the soy 7S globulin (β-conglycinin). The α+α′ subunit of this protein strongly upregulated the expression of LDL-R in cultured hepatocytes leading to an increase in LDL uptake and degradation (Lovati et al., 1998). The peptide region responsible for the activity has been identified from the α′ subunit and sequenced (Lovati et al., 2000). This 24-amino acid peptide that corresponds to

position 127-150 of the α′ subunit displayed potential in modulating cholesterol homeostasis by increasing LDL-R-mediated LDL uptake in Hep G2 cells (Lovati et al., 2000). Moreover, Cho et al. (2008) also identified an octapeptide (FVVNATSN) from the enzymatic digest of soy protein as the most active stimulator of LDL-R transcription in Hep T9A4 human hepatic cells. Thus, proteolytic digestion of the soy protein was important for releasing more active small peptides with improved cardioprotective property. This has also been demonstrated in a study by Mochizuki et al. (2009) that produced bioactive peptides from purified isoflavone-free soy 7S β–conglycinin using bacterial proteases. The resulting 7S-peptides showed hypotriglyceridemic properties by altering gene expressions related to TAG synthesis and also decreased Apo B-100 accumulation in Hep G2 cells partly due to increase in LDL-R mRNA expression (Mochizuki et al., 2009). Apo B-100 is a functional component of VLDL and its degradation reduces VLDL synthesis. These observations supported a previous study which showed that soy β-conglycinin possesses beneficial effects on plasma TAG in humans (Kohno et al., 2006).

In addition to alterations of gene expressions, soy protein hydrolysates and constituent peptides also exhibited hypocholesterolemic activity by binding bile acids and neutral sterols in the intestine leading to increased fecal removal (Cho et al., 2007; Yang et al., 2007). The ability of the soy protein hydrolysates to bind bile acids may depend in part on their insoluble high-MW peptide fraction rich in hydrophobic amino acids (Higaki et al., 2006), as earlier observed for high-MW fraction of the tryptic digest of buckwheat protein (Kayashita et al., 1997). This shows that even though large

bioactive peptides may not be able to cross the intestinal epithelium into circulation to exert their beneficial effects in the liver, they might be useful in cholesterol homeostasis by enhancing fecal removal of bile acids and exogenous cholesterol from the intestine depending on their hydrophobic properties.

## 2.4.4. Anticancer peptides

Peptides with anticancer properties have also been reported from foods. Most of the research investigations on anticancer peptides have been focused on lunasin, a 43 amino acid-containing polypeptide (MW 5.4 kDa) found in soy, barley, rye and wheat (Hernández-Ledesma et al., 2009a; Wang et al., 2008). The anticancer property of lunasin is predominantly against chemical and viral oncogene-induced cancers, and based on the modulation of histone (H) acetylation and deacetylation pathways specifically by inhibiting histone acetyl transferase (HAT). This leads to inhibition of acetylation of H3 and H4, repression of cell cycle progression (arrest at G1/S phase) and apoptosis in cancer cells (Hernández-Ledesma et al., 2009a). Although lunasin showed excellent potential as anticancer agent in cell cultures, its large molecular size raises questions as to its absorption and use as an orally bioavailable therapeutic agent. Dia et al. (2009a) recently reported that 4.5% of lunasin was absorbed in human subjects that consumed lunasin-containing soy protein. They also observed other lunasin-derived peptide sequences in the plasma, which could be attributed to degradation by gastrointestinal proteases and plasma peptidases. Another study reported efficient absorption of lunasin from rye consumption into the liver, kidney and blood, and the

tissue-derived extract retained the anticancer HAT-inhibitory property of the parent molecule (Jeong et al., 2009). It has been suggested that the activity of protease inhibitors present in lunasin-containing whole food contributed to the resistance of lunasin against gastrointestinal digestion as opposed to its synthetic form (Hernández-Ledesma et al., 2009a). Additional studies are needed to understand the mechanism of lunasin activity, absorption kinetics into the blood circulation and cancer cell targets, and application as effective food-derived anticancer nutraceutical.

Other soy protein-derived peptides have also shown promising activities for anticancer therapy. Wang et al. (2008) reported that enzymatic hydrolysates from different soy varieties inhibited the viability of cultured leukemia cells (L1210) with $IC_{50}$ values of 3.5–6.2 mg/ml, which were lower than the activity of lunasin ($IC_{50}$ 78 μg/ml or 13.9 μM). Moreover, a lunasin-containing glutelin fraction of *Amaranthus hypochondriacus* when digested with trypsin induced apoptosis in HeLa cells by 30% and 38% at 1 and 5 μg/ml, respectively (Silva-Sánchez et al., 2008). Soy protein-derived hydrophobic peptide fraction also exhibited cytotoxicity ($IC_{50}$ 0.16 mg/ml) against a macrophage-like murine tumour cell line (P388D1) by arresting cell cycle progression at the G2/M phases (Kim et al., 2000). Thus, enzymatic hydrolysis of proteins can release peptides with anticancer properties. This has been clearly demonstrated in another study where the antiproliferative activity of *A. mantegazzianus* protein hydrolysates was twice the activity of the parent protein (Barrio & Añon, 2010). Based on these studies, animal and clinical human intervention studies are needed to evaluate the safety and physiological anticancer activities of these peptides.

**2.4.5. Immunomodulatory and anti-inflammatory peptides**

Immunomodulation involves suppression or stimulation of human immune functions. Immunomodulatory food peptides act by enhancing the functions of immune system including regulation of cytokine expression, antibody production and ROS-induced immune functions (Hartmann & Meisel, 2007). For example, a tryptic digest of rice protein improved immune function by promoting phagocytosis and increasing superoxide anion production in human polymorphonuclear leukocytes (Takahashi et al., 1994). Egg-derived peptides also showed immunostimulating activities and were used to increase immune functions during cancer immunotherapy (Mine & Kovacs-Nolan, 2006). In human volunteers, consumption of 3 g/day of wheat protein hydrolysate for 6 days increased activity of natural killer cells (Horiguchi et al., 2005). In addition, whey protein-derived peptides can activate cellular immune functions (Gauthier et al., 2006).

The anti-inflammatory properties of food-derived peptides have also been reported mostly in modulating endotoxin-induced production of proinflammatory responses in macrophages. For example, soy lunasin and lunasin-like peptides exhibited anti-inflammatory properties by decreasing ROS production, TNF-$\alpha$, IL-6, IL-1$\beta$, NF-$\kappa$B levels, and downregulating NO/PGE$_2$ synthesis and iNOS/COX-2 expressions in activated macrophages (Dia et al., 2009b; Gonzalez de Mejia & Dia, 2009; Hernández-Ledesma et al., 2009b). The activity of lunasin was due to the suppression of nuclear translocation of p65/p50 subunits of NF-$\kappa$B in RAW264.7 macrophage, which reduces binding of NF-$\kappa$B to target genes with concomitant inhibition of proinflammatory markers gene activation and the gene products, e.g. IL-6, iNOS, COX-2 (Gonzalez de Mejia & Dia, 2009).

## 2.4.6. Calmodulin (CaM)-binding peptides

Considering the roles of CaM in human health conditions (see Section 2.3), CaM-binding agents can be used as multifunctional therapeutic agents. The amino acid sequences of many natural CaM-binding proteins and peptides revealed the presence of repeated positively-charged (cationic) and hydrophobic amino acid residues at the CaM-binding sites (O'Neil & DeGrado, 1990). These structural features are thought to be more important than the specific amino acid sequence in determining affinity of peptides for CaM (Kizawa et al., 1995). The affinity of the cationic residues for the net negatively-charged CaM led to a rationale to use cationic peptides as CaM-binding agents (Itano et al., 1980; Barnett et al., 1983). A number of food protein-derived cationic peptides have been reported to bind calmodulin leading to the inhibition of CaM-dependent enzymes. Earlier works by Kizawa et al. (1995) and Kizawa (1997) reported the isolation of CaM-binding peptides from casein, specifically $\alpha_{s2}$-casein (f164-179, f183-206, f183-207 and f90-109), which inhibited CaMPDE activation with $IC_{50}$ values of 38, 6.9, 1.1 and 1.0 µM, respectively, without any effects on the basal PDE activity. Based on this work, our laboratory has explored other cationic amino acid-rich food protein sources for the production of CaM-binding peptides. Li and Aluko (2005) reported that pea protein-derived cationic peptide fraction inhibited CaM-dependent protein kinase II activity by competitive mode of inhibition. In other studies, two cationic peptide mixtures fractionated from Alcalase-prepared flaxseed protein hydrolysates bound CaM with concomitant inhibition of the activities of eNOS and nNOS (Omoni & Aluko, 2006a,b) via mixed-type and non-competitive modes of inhibition, respectively.

The authors reported that these activities were due to decreased $\alpha$-helix/unfolding of CaM and increased rigidity of the $Ca^{2+}$/CaM complex due to binding of the cationic peptides. Moreover, a recent study have shown that cationic peptide fractions from egg white lysozyme can simultaneously inhibit CaMPDE and also act as antioxidants (You et al., 2010), which makes these peptides good candidates for multiple therapeutic uses (see Section 2.4.8. for details about multifunctional peptides). These cationic peptides can be easily purified from inactive enzymatic food protein hydrolysates due to their unique physicochemical characteristics (Kizawa et al., 1995). Although these peptides have shown potent interaction with CaM and inhibition of CaM-dependent enzymes, there is dearth of information on *in vivo* activity of CaM-binding peptides in ameliorating human health and disease conditions.

## 2.4.7. Branched-chain amino acid-rich peptides and liver diseases

Liver disease conditions are associated with plasma amino acid imbalance especially for those amino acids metabolized in the liver. Thus, there is an observed increase in the plasma levels of aromatic amino acids (AAA: Tyr, Phe and Trp) and Met with a concomitant decrease in the levels of branched-chain amino acids (BCAA: Leu, Ile and Val) (Clemente, 2000). The molar ratio of plasma BCAA to AAA (termed Fischer ratio) has been used as a parameter to measure abnormal amino acid metabolism and imbalance especially in liver disease patients. The amino acid imbalance results in corresponding plasma Fischer ratio of <2.5, <1.5 and <0.8 in liver disease, hepatic coma and profound coma, respectively as opposed to the normal plasma Fischer ratio of 3.5-4

(Fischer et al., 1975). In addition to use as diagnostic parameter during human liver disease, plasma amino acid Fischer ratio has been used for formulation of therapeutic amino acid-enriched food products. As shown in Figure 2.6, branched-chain amino acid products with high Fischer ratio can be used to improve plasma amino acid imbalance during chronic liver disease with concomitant improvement of associated hepatic encephalopathy and muscle loss (Fischer, 1990; Okita et al., 1985; Holecek, 2010). This is due to the fact that BCAAs play pivotal roles in the up-regulation of muscle protein synthesis, inhibition of AAA transport across the blood-brain barrier (to decrease the biogenesis of false neurotransmitters in the brain) and removal of excessive ammonia through glutamine synthesis during chronic liver disease such as hepatic encephalopathy (Clemente, 2000; Holecek, 2010). BCAAs are also available as carbon skeleton for peripheral metabolism in advance liver disease and can also be used to improve muscle mass and strength (Clemente, 2000; Di Pasquale, 2008; Holecek, 2010).

A number of food proteins with high amounts of BCAA and low amounts of AAA have been reported as good sources of high Fischer ratio peptide product, after appropriate enzymatic treatments and post-hydrolysis processing using activated carbon treatment and gel filtration chromatography. Adachi et al. (1993) reported that hydrolysis of casein with thermolysin followed by papain and subsequent passage of the hydrolysate solution (pH 2.5) through activated carbon-packed column yielded a peptide mixture containing 27.5% BCAA with Fischer ratio of 31.6. In addition, a similar approach of activated carbon treatment was used, in addition to ultrafiltration and desalting using ion-exchange resin, to prepare an oligopeptide mixture (Fischer ratio=34.71, MW of 1-

1.3 kDa, 11.6% yield) from corn gluten meal digested with Alcalase and papain (Ma et al., 2008). Other studies on activated carbon treatment of casein (Pedroche et al., 2004) and pearl oyster protein hydrolysates (Zheng et al., 2009) yielded peptide mixtures with Fischer ratios of 30.6 and 24.6, respectively. The differences in the Fischer ratios in these studies depend on the nature of the proteins, the specificity of the proteolytic enzymes in removing AAA, and the efficiency of the activated carbon column filtration and gel chromatography matrices in adsorbing the liberated AAA and AAA-rich peptides.

**Figure 2.6 Targets of branched chain amino acids (BCAA) in the treatment of hepatic diseases. AAA, aromatic amino acids; BBB, blood-brain barrier**
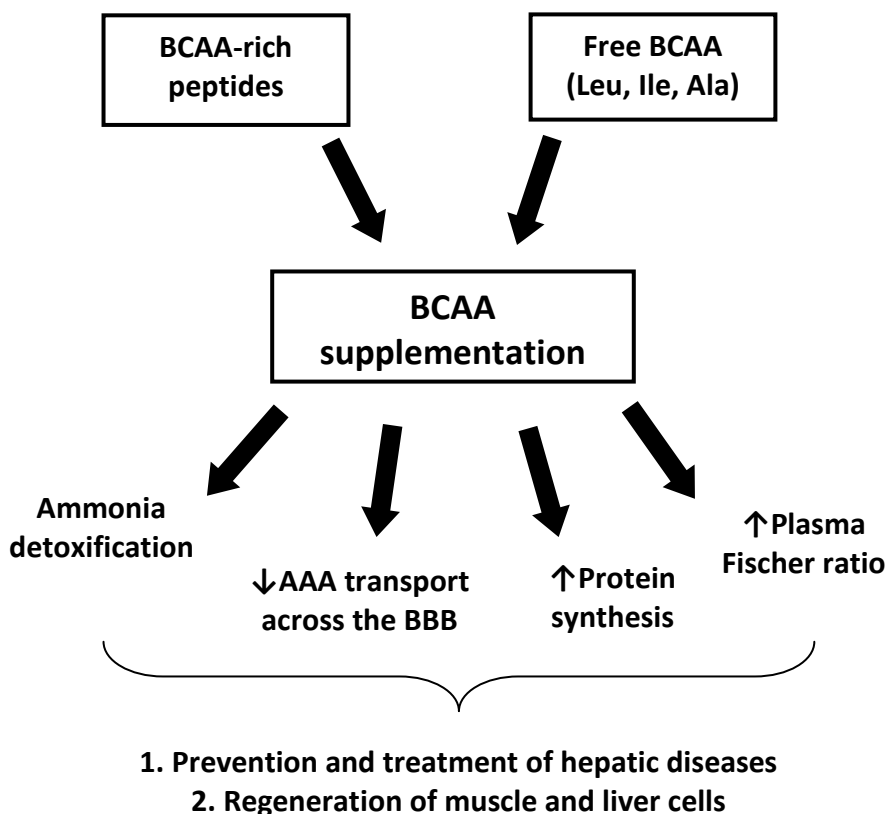
## 2.4.8. Multifunctional peptides

Multifunctional peptides have been discovered from some food proteins and they have been reported to possess more than one significant physiologically-relevant bioactive property. Studies on milk proteins reported that a hexapeptide (TTMPLW) derived from $\alpha_{S1}$-casien (f194-199) by trypsin-catalyzed digestion exhibited both ACE-inhibitory and immunomodulatory activities (Meisel, 2004) while a β-lactoglobulin-derived β-lactorphin (YLLF) inhibited ACE activity and also possessed opioid-like activity (Antila et al., 1991; Mullally et al., 1997). In addition, several other milk-derived peptides such as α-lactorphin (YGLF), α-immunocasokinin (TTMPLW), β-casomorphin-7 (YPFPGPI) and β-casokinin (AVPYPQR) are regarded as multifunctional, some possessing *in vivo* bioactive properties (Meisel, 2004). Moreover, crude chymotryptic α-casein hydrolysates displayed several *in vitro* bioactivities such as ACE and propyl endopeptidase inhibition, antioxidant, $Zn^{2+}$-binding and antibacterial activities (Srinivas & Prakash, 2010). Other partially purified food peptides from quinoa/pea proteins and hen egg white lysozyme have also displayed multifunctionality as ACE-inhibiting antioxidants (Aluko & Monu, 2003; Humiski & Aluko, 2007) and CaMPDE-inhibiting antioxidants (You et al., 2010), respectively. The multiple bioactivities displayed by these peptides can increase their impact towards the amelioration of more than one disease target or multiple symptoms of a disease, such as cardiovascular disease, since many human diseases are interrelated in terms of aetiology and progression. Therefore, the conditions for generating and processing bioactive food protein hydrolyastes can be carefully designed to yield multifunctional peptides with diverse applications.

**2.5. Quantitative Structure-Activity Relationship (QSAR) studies of peptides**

Structure-function relationship (SFR) studies of bioactive compounds involve analysis of structural functionality of the bioactive molecules and relating these characteristics to their pharmacological activity. This approach is known as quantitative structure-activity relationship (QSAR) studies, which is a practically reliable method in chemometrics for SFR studies. In peptide QSAR, potency of bioactive peptides has been found to depend largely on peptide chain length and physicochemical properties such as molecular size, charge, hydrophilicity/hydrophobicity and position of amino acid residues of the peptides (Pripp et al., 2004a). Therefore, the first step in peptide QSAR is to obtain a library of peptides with varying bioactivity (designated as Y variables) and different fully-characterized structural features (X variables) relevant to the bioactivity (Wold et al., 2001). Subsequently, models of the relationship between these variables are obtained using statistical multiple regression analysis (Wold et al., 2001). Partial least squares (PLS) method has been widely used in peptide QSAR for this purpose (Sandberg et al. 1998; Wu et al. 2006a, b). PLS modelling of these data will result in two important parameters: (1) the multiple correlation coefficients ($R^2$) that indicate the model fit, and (2) the cross-validation coefficients ($Q^2_{cv}$) that indicate the predictive power of the model (Hellberg et al., 1987; Wold et al., 2001). $Q^2_{cv}$ can be reliably used for model validation in the absence of external model validation set especially when working with limited number of observations in the training set (Wold et al., 2001); combination of both validation methods gives better results on the predictive ability of PLS models.

Some factors to consider prior to QSAR studies using PLS method include the size of the analogues in peptide datasets, which is determined by the number of positions to be varied on the peptide chain (Hellberg et al., 1987). In addition, peptide structural descriptors need to be carefully chosen to reflect the bioactivity under investigation. The $z$ scale amino acid descriptors are widely used for peptide QSAR studies (Hellberg et al., 1987; Sandberg et al., 1998). The 3-$z$ scale ($z1$, $z2$ and $z3$) have been developed by principal component analysis of 29 physicochemical variables of 20 coded amino acids, and were interpreted to be related to hydrophobicity ($z1$), steric properties or side chain bulk ($z2$) and electronic properties ($z3$) (Hellberg et al., 1987). This approach has received some criticisms because of difficulty in interpretation and the lack of consideration of the peptides' conformations in the derivation of the principal properties (Dunn & Wold, 1995). An extended 5-$z$ scale was subsequently developed to include additional variables ($z4$ and $z5$), which are difficult to clearly interpret and are related to heat of formation, and molecular "hardness" and electrophilicity, respectively (Sandberg et al., 1998). The $z4$ and $z5$ variables contain important information that improved modelling quality (Sandberg et al., 1998; Siebert, 2003; Wu et al., 2006b). Other amino acid descriptors have also been proposed for peptide QSAR. Lin et al. (2008) recently proposed the "V-scale" that used the primary amino acid side chain data that influence interaction with protein receptors including Van der Waal's volume, net charge index and hydrophobic parameter without compression by PCA. They reported that models generated for bitter-tasting dipeptides using these descriptors ($R^2$=0.948 and 0.903, $Q^2_{cv}$=0.921 and 0.860) were more robust than the $z$-scale based model

($R^2$=0.824). However, the descriptors were based on the amino acid side chains only and not the entire peptide sequence, which limits their use for enzyme inhibition modelling where the entire peptide functionality is involved. The PLS method has been used to develop good QSAR models for elastase substrates and neurotensin analogues (Sandberg et al. 1998), functional properties of polypeptides (Strøm et al., 2001; Siebert, 2003) and ACE-inhibiting peptides (Pripp et al., 2004b; Wu et al. 2006a, b) leading  to accurate predictions of potency.

**2.6. Flaxseed**

Flaxseed or linseed (*Linum usitatissimum* L.) is an oilseed that is cultivated in various parts of the world, especially in Canada, China, USA, India and Argentina (Bhatty, 1995; Agriculture and Agri-Food Canada, 2007; Hall et al., 2006). Flaxseed has been used for the production of oil, paint and other industrial products, and has increasingly gained particular interest in the human food system due to its rich nutritional components and medicinal values. Whole flaxseed is rich in nutrients (e.g. oil, dietary fibre and proteins), non-nutrients (e.g. lignans) and anti-nutritional factors (e.g. cyanogenic glycosides and phytic acids) (Oomah & Mazza, 1993, 1995; Bhatty, 1995; Oomah, 2001; Hall et al., 2006). This section discusses the nutritional composition and beneficial aspects of flaxseed.

**2.6.1. Nutritional quality, compositions and health benefits of flaxseed**

Flaxseed can be crushed for human consumption, and the oil components can be extracted by pressing or solvent extraction. In 2009, oil from Canadian-cultivated flaxseed made up 46% of the whole flaxseed and was comprised mainly of $\alpha$-linolenic acid, ALA (58% of oil) with minor levels of linoleic acid (15.6%), oleic acid (16.9%), stearic acid (3.1%) and palmitic acid (4.8%) (Canadian Grain Commission, 2009); this makes flaxseed the best known terrestrial food source of omega-3 ALA (Bhatty, 1995). In addition to ALA, flaxseed also contains high amounts of non-starch polysaccharides (gum and fibre). Flaxseed contains large amounts of proteins ranging from 10.5% to 31% with mean value of 21% (Oomah & Mazza, 1995); these proteins serve primarily as nutrient reservoirs. Canadian annual quality survey showed that flaxseed No. 1 Canada Western contained 22% proteins (%N × 6.25) in 2009 compared to the 1999-2008 mean value of 23.3% (Canadian Grain Commission, 2009). Mean protein contents of 28.9% and 34.1% were also reported for 11 cultivars grown in North Dakota and Canada, respectively (Oomah & Mazza, 1993; Bhatty, 1995). Thus, the total protein content of flaxseed protein varies with cultivars and location/environment (Oomah & Mazza, 1995; Hall et al., 2006). Flaxseed also contains large amounts of lignans, a class of polyphenolic phytoestrogens, and is considered the richest food source of secoisolariciresinol diglucoside (SDG), the major flaxseed lignan (Bloedon & Szapary, 2004; Hall et al., 2006).

The current market for whole and ground flaxseed is primarily focused on the constituent omega-3 ALA, fibres and lignans. These flaxseed components contribute to its multiple positive effects in cardiovascular health, cancer prevention and diabetes

(Oomah, 2001; Bloedon & Szapary, 2004; Hall et al., 2006). Human consumption of flaxseed resulted in reduction of CVD risk factors including decrease in serum total and LDL cholesterol concentrations and markers of inflammation (Bloedon & Szapary, 2004). Flaxseed lignans inhibit oxidative stress and inflammation whereas the insoluble fibre components improve gut health and also possess hypolipidemic effects (Oomah, 2001; Bloedon & Szapary, 2004). However, flaxseed proteins have not been well studied for therapeutic applications. Most studies on flaxseed proteins have focused on characterization of the structures and functional properties of the proteins.

### 2.6.2. Flaxseed proteins

The major storage proteins found in various flaxseed cultivars include a globulin-like salt soluble 11-12S protein with molecular weight (MW) of 252-298 kDa, and an albumin-type water soluble basic 1.6-2S protein with MW of 16-17 kDa (Madhusudhan & Singh, 1983, 1985a, b; Oomah & Mazza, 1995). These proteins were named linin and conlinin, respectively by Vassel and Nesbitt (1945). The 11-12S and the 1.6-2S proteins account for 56-73.4% and 20-42% of the total flaxseed proteins, respectively (Youle & Huang, 1981; Madhusudhan & Singh, 1983; 1985a, b; Marcone et al., 1998). The primary amino acid sequence of flaxseed conlinin has been elucidated and reported to contain 168 or 169 amino acid residues (UniProtKB/TrEMBL accession # Q8LPD4). Protein sequences of other 142-180 amino acid-containing oil-binding flaxseed proteins, known as oleosins, have also been reported from gene transcripts (UniProtKB/TrEMBL accession # A0RZJ4, A0RZJ6, A0RZJ7 and A0RZJ8). Compared to other food proteins such

as soy, maize and milk proteins, the amino acid sequences of flaxseed proteins need to be fully characterized for in-depth understanding of functional properties and food applications of the proteins.

The first report of flaxseed protein isolation was by T.B. Osborn in 1892 during the period that he worked on several other food proteins (Vassel & Nesbitt, 1945). Since the past three decades, several efforts have been made to optimize flaxseed protein isolation methods and characterize the protein for use in the human food system. Approaches used for successful isolation of flaxseed protein from defatted meal include acid precipitation at isoelectric point (p$I$=4.2) after alkaline solubilisation (Dev & Quensel, 1988), buffered salt (NaCl) extraction (Oomah et al., 1994), polyphosphate extraction (Wanasundara & Shahidi, 1996) and micellisation (Krause et al., 2002) with each process yielding isolates that contained high protein levels. Distinctive similarities among these extraction methods include the use of laboratory samples of the desired cultivars of defatted and dehulled flaxseed as starting material, which enhances the protein isolation process. Moreover, protein analyses were based on total nitrogen (N × 6.25), which can overestimate proteins due to reaction of other non-protein N-containing organic compounds. Practically, the oilseed processing industry generates large amounts of defatted flaxseed meal as by-product after oil extraction from crushed whole flaxseed, which contains high amounts of mucilage. Mucilage interferes with protein extraction from flaxseed, but removal of the mucilage from whole flaxseed by soaking in water or enzymatic treatments improved the protein contents of the extract (Madhusudhan & Singh, 1983; Wanasundara & Shahidi, 1997). Table 2.3 shows the

amount of protein (%) in various flaxseed fractions of varying mucilage content and also the differences based on extraction methods.

**Table 2.3 Differences in flaxseed protein contents (%) based on different isolation methods and mucilage contents of the starting materials**

| FRACTION | PROTEIN (%) |
|---|:---:|
| Seed | 19.2 |
| Dehulled seed | 21.8–23.9 |
| Hull | 17.3–20.3 |
| Meal | 22.9-49.0 |
| Laboratory-prepared meal | 43.9 |
| Commercial meal | 34.7 |
| Dehulled meal | 48.9-50.0 |
| PROTEIN EXTRACT | |
| Micelle protein isolate | 93.0 |
| Isoelectric-precipitated protein isolate | 89.0 |
| LMF[a] | 56.4 |
| LMPC[b] | 59.7 |
| HMPC-S[c] | 63.4 |
| HMPC-EC[d] | 65.5 |
| LMPI[e] | 86.6 |
| HMPI[f] | 66.3 |

[a]LMF, low mucilage flour; [b]LMPC, low mucilage protein concentrate; [c]HMPC-S, high mucilage protein concentrate from seed; [d]HMPC-EC, HMPC from expeller cake; [e]LMPI, low mucilage protein isolate; [f]HMPI, high mucilage protein isolate; data adapted from Hall et al., 2006 with permission from Elsevier

Most defatted flaxseed meal produced in Canada are exported to international markets for livestock feed (Agriculture and Agri-Food Canada, 2007). At the moment, based on literature information, little emphasis has been placed on value addition to this industrial by-product and creation of opportunities for local use in Canada, even though flaxseed meal proteins contain rich amino acid profile. As shown in Table 2.4, whole flaxseed, flaxseed meals and isolated proteins are rich sources of glutamic acid/glutamine (Glx), arginine (Arg), branched-chain amino acids (valine, Val and leucine, Leu) and aromatic amino acid (tyrosine, Tyr and phenylalanine, Phe). There is no apparent difference between the amino acid profiles of laboratory-prepared and commercial defatted flaxseed meal (Bhatty, 1995), but the histidine (His) content was reported to be higher in flaxseed flour compared to the whole seed and defatted meal (Table 2.4). Defatting and protein extraction processes generally increased Arg, Leu and cysteine (Cys) compositions (%) compared to the whole seed. Likewise, Arg and Leu contents (%) of hexane extracted protein were also higher than commercial protein extracts. Table 2.5 shows the distribution amino acids in the flaxseed protein subunits. The amounts of Arg in the 1.6 S and 12 S subunits are equal, but tend to be lower in the 2 S proteins. Glx and aspartic acid/asparagine (Asx) are predominant in the 1.6 S and 12 S subunits, respectively, whereas BCAA (Ala+Val) and AAA (Phe+Tyr) are more in the 12 S than the 1.6 S subunits. This information can lead to isolation of a particular flaxseed protein subunit enriched with amino acids of interest for nutritional and therapeutic applications.

**Table 2.4 Amino acid compositions (%) of flaxseed, flaxseed meals and flaxseed protein extracts**

| AMINO ACIDS | Oomah & Mazza, 1995 FLAXSEED[a] | Dev & Quensel, 1986 FLAXSEED FLOUR | Bhatty, 1995 FLAXSEED MEAL LABORATORY SAMPLES[b] | Bhatty, 1995 FLAXSEED MEAL COMMERCIAL SAMPLES[b] | Wanasundara & Shahidi, 1994 FLAXSEED PROTEIN EXTRACT HEXANE EXTRACT | Wanasundara & Shahidi, 1994 FLAXSEED PROTEIN EXTRACT MAW-HE[c] | Wanasundara & Shahidi, 1994 FLAXSEED PROTEIN EXTRACT COMMERCIAL |
|---|---|---|---|---|---|---|---|
| ARG | 9.2 | 10.4 | 11.8 | 11.1 | 11.5 | 11.20 | 9.78 |
| HIS | 2.2 | 5.9 | 2.9 | 3.1 | 2.69 | 2.46 | 2.36 |
| ILE | 4.0 | 4.6 | 5.2 | 5.0 | 4.78 | 4.54 | 4.19 |
| LEU | 5.8 | 6.5 | 6.8 | 7.1 | 6.70 | 6.39 | 5.96 |
| LYS | 4.0 | 6.0 | 4.1 | 4.3 | 4.38 | 4.14 | 3.92 |
| MET | 1.5 | 3.0 | 2.2 | 2.5 | 1.45 | 1.41 | 1.24 |
| CYS | 1.1 | N.D. | 3.8 | 4.3 | 3.29 | 3.39 | 3.16 |
| PHE | 4.6 | 6.5 | 5.3 | 5.3 | 5.13 | 4.91 | 4.63 |
| TYR | 2.3 | 4.6 | 2.9 | 3.1 | 2.21 | 2.12 | 1.98 |
| THR | 3.6 | 3.1 | 4.9 | 5.1 | 3.40 | 3.33 | 3.00 |
| TRP | N.D. | N.D. | 1.8 | 1.7 | 0.46 | 0.46 | 0.25 |
| VAL | 4.6 | 4.9 | 5.6 | 5.6 | 5.75 | 5.64 | 5.02 |
| ALA | 4.4 | 4.3 | 5.4 | 5.5 | 4.81 | 4.64 | 4.61 |
| ASX[d] | 9.3 | 8.3 | 12.5 | 12.4 | 9.18 | 9.16 | 8.03 |
| GLX[e] | 19.6 | 22.8 | 21.3 | 26.4 | 16.70 | 16.36 | 14.45 |
| GLY | 5.8 | 4.9 | 7.0 | 7.1 | 6.44 | 6.26 | 5.64 |
| PRO | 3.5 | 3.0 | 5.2 | 5.5 | 3.64 | 3.65 | 3.32 |
| SER | 4.5 | 4.1 | 5.8 | 5.9 | 4.94 | 4.99 | 4.48 |

[a]Data for NorLin flaxseed variety; [b]mean values (n=6); [c]MAW-HE, methanol-ammonia-water/hexane extracted; [d]ASX, aspartic acid and asparagine; [e]GLX, glutamic acid and glutamine

**Table 2.5 Amino acid composition of the two major flaxseed 11-12 S (linin) and 1.6-2 S (conlinin) protein subunits**

| AMINO ACIDS | Madhusudhan & Singh (1985a) | Youle & Huang (1981) | Madhusudhan & Singh (1985b) |
|---|---|---|---|
| | 1.6 S | 2 S | 12 S |
| ARG | 13.1 | 6.0 | 12.5 |
| HIS | 1.6 | 1.2 | 2.5 |
| ILE | 2.8 | 2.9 | 4.6 |
| LEU | 5.4 | 5.3 | 5.8 |
| LYS | 4.9 | 6.0 | 3.1 |
| MET | 0.8 | 1.9 | 1.7 |
| CYS | 3.5 | 8.2 | 1.4 |
| PHE | 2.4 | 2.2 | 5.9 |
| TYR | 1.4 | 1.5 | 2.3 |
| THR | 2.1 | 3.6 | 3.9 |
| TRP | 2.0 | 0.8 | 1.3 |
| VAL | 2.6 | 3.9 | 5.6 |
| ALA | 1.9 | 5.1 | 4.8 |
| ASX[a] | 5.5 | 6.4 | 11.3 |
| GLX[b] | 35.0 | 23.8 | 19.8 |
| GLY | 8.3 | 13.8 | 4.8 |
| PRO | 3.0 | 1.6 | 4.5 |
| SER | 3.9 | 6.1 | 5.1 |

[a]ASX, aspartic acid and asparagine; [b]GLX, glutamic acid and glutamine

Till date, there is little global interest in promoting the value and use of flaxseed meal proteins despite their rich amino acid profiles. A number of studies have shown that flaxseed proteins possess potential for therapeutic applications. For example, peptides derived from enzymatic hydrolysis of flaxseed proteins inhibited CaM-dependent eNOS/nNOS and ACE activities, and also displayed *in vitro* antioxidant activities (Omoni & Aluko, 2006a, b; Marambe et al., 2008). Moreover, a recent study also reported that a flaxseed protein extract inhibited growth of a wide range of food spoilage fungi (Xu et al., 2008) but this activity could be due to other non-protein components, such as lignans, present in the crude protein extracts. As previously stated in the General Introduction (Chapter 1), it will be worthwhile to investigate the potential human health benefits of flaxseed protein by exploiting the distinct structural features of the predominant amino acids (e.g. Arg and BCAA) in producing peptides with physiologically-relevant bioactive properties.

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**CHAPTER THREE**

**MANUSCRIPT 1**

**KINETICS OF THE INHIBITION OF RENIN AND ANGIOTENSIN I-CONVERTING ENZYME BY FLAXSEED PROTEIN HYDROLYSATE FRACTIONS**

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## 3.0. ABSTRACT

Enzymatic hydrolysates from flaxseed protein were investigated for *in vitro* inhibition of angiotensin I-converting enzyme (ACE) and renin activities. Pepsin, ficin, trypsin, papain, thermolysin, pancreatin and Alcalase were used to hydrolyze flaxseed proteins followed by fractionation using ultrafiltration to isolate low-molecular-weight peptides, and separation of the Alcalase hydrolysate into cationic peptide fractions. Using *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine as substrate, the protein hydrolysates showed a concentration-dependent ACE inhibition (IC$_{50}$, 0.0275–0.151 mg/ml) with thermolysin hydrolysate and Alcalase cationic peptide fraction I (FI) showing the most potent activity. Flaxseed peptide fractions also showed nil or moderate inhibitory activities against human recombinant renin (IC$_{50}$, 1.22–2.81 mg/ml). Kinetics studies showed that the thermolysin hydrolysate and FI exhibited mixed-type pattern of ACE inhibition whereas cationic peptide fraction II inhibited renin in an uncompetitive fashion. These results show that the protein components of flaxseed meal possess peptide amino acid sequences that can be exploited as potential food sources of antihypertensive agents.

## 3.1. INTRODUCTION

Since the past two decades, the renin-angiotensin-aldoresterone system (RAAS) has turned out to be an excellent physiological target for the development of antihypertensive agents. This is due to the fact that RAAS has been reported to play vital roles in the progression of cardiovascular and chronic kidney diseases in human beings (Segall *et al.*, 2007; Staessen *et al.*, 2006). The RAAS pathway comprises a series of reactions that produce molecules capable of regulating blood pressure in humans; renin and angiotensin I-converting enzyme (ACE) are the two key enzymes in this pathway. In the RAAS pathway, renin (an aspartyl protease) catalyzes the initial and rate-limiting step by converting angiotensionogen to angiotensin I (AT-I), a decapeptide. Subsequently, ACE (peptidyldipeptidase A) catalyzes the conversion of AT-I into a potent vasoconstrictor octapeptide, angiotensin II (AT-II), and also degrades bradykinin, a vasodilator (Segall *et al.*, 2007; Yang *et al.*, 1970). ACE inhibitors have found clinical applications in blocking or suppressing the formation of AT-II thereby reducing the occurrence of elevated blood pressure. However, various ACE-inhibitory compounds that have been developed as antihypertensive drugs, on prolonged administration, have shown some undesirable side effects in human such as cough, taste distortion, skin rash and angioedema (Acharya *et al.*, 2003; Seseko & Kaneko, 1985). Thus, it is imperative to discover safer therapeutic compounds for lowering human blood pressure during hypertension, a risk factor in cardiovascular disease and other associated health complications. Moreover, it is thought that the direct inhibition of renin activity provides a better control of elevated blood pressure than ACE inhibition since it

prevents the production of AT-I, which could also be converted to a substantial amount of AT-II in some organs via an ACE-independent pathway catalyzed by chymase; however, renin inhibition does not prevent ACE-catalyzed bradykinin degradation (Segall *et al.*, 2007; Staessen *et al.*, 2006). Thus, there is a need to develop therapeutic agents that could exhibit pleiotropic effects in the inhibition of ACE and renin activities. Several food protein hydrolysates and constituent peptides have been shown to display *in vitro* inhibition of ACE activity (Aluko, 2008) resulting in the lowering of elevated blood pressure in spontaneously hypertensive rats (Aluko, 2008; Wu & Ding, 2001) and in hypertensive humans (FitzGerald *et al.*, 2004; Seppo *et al.*, 2003). Synthetic peptides and peptidomimetic compounds have also been developed and applied parenterally as renin inhibitors in the clinical treatment of hypertension; moreover, Aliskiren has been developed as the first non-peptide oral renin inhibitor (Staessen *et al.*, 2006). To the best of our knowledge, there is no information in the literature regarding the inhibition of renin activity by enzymatically prepared food protein hydrolysates. The health-promoting potential of peptides derived from food proteins has remained a subject of interest to many food scientists as new and underutilized food protein sources are continuously investigated for bioactive properties.

Flaxseed (*Linum usitatissimum*, also known as linseed) has long been used as raw material in the production of oil, paint and other industrial products. In the recent past, flaxseed has emerged as one of the major focuses of the food and health sectors worldwide. The emerging economic importance of flaxseed is primarily due to its constituent lignans, dietary fibre and the Omega-3 fatty acid, α-linolenic acid, which

possess potential to provide cardiovascular and other health benefits in humans (Hall III

*et al.*, 2006). However, the underutilized protein components of flaxseed also have

potential therapeutic properties that remain to be explored. Flaxseed meal is generated

as a by-product of industrial flaxseed oil extraction, and has been widely used as a

protein supplement in the formulation of livestock feed (Bell & Keith, 1993). The world

flaxseed meal production is estimated at 1.1–1.4 million tonnes with Canada, USA and

Argentina making up the largest exporters of this product (Agriculture and Agri-Food

Canada, 2007). It has been estimated that flaxseed and its defatted meal contain high

amounts of proteins, which are comparable in amino acid compositions to other high

quality food proteins like soy protein, with a preponderance of basic and branch-chain

amino acids (Bhatty, 1995; Oomah & Mazza, 1993, 1995). In 2007, the protein contents

of Flaxseed, No. 1 Canada Western ranged from 19.4 to 30.8% (N × 6.25), with a mean

value of 24.3% (Canadian Grain Commission, 2007).

Apart from its nutritional significance in containing most of the amino acid

requirements of humans and animals, the intact flaxseed proteins may possess other

biological activities; thus, it was recently reported that flaxseed protein extract showed

antifungal activity against a wide range of food spoilage fungi (Xu *et al.*, 2008). It was

also recently reported that the crude flaxseed protein hydrolysate prepared using

Flavourzyme inhibited ACE *in vitro* and displayed antioxidant properties in scavenging

hydroxyl radical (Marambe *et al.*, 2008). However, in a bid to improve the value of

defatted flaxseed meal generated as a by-product of the oilseed processing industry,

this study explored the bioactive properties of enzymatically prepared flaxseed protein

hydrolysates with respect to their potential use as source of antihypertensive agents. The objective of this work was to determine the potential ACE and renin inhibitory activities of low-molecular-weight (LMW) flaxseed protein hydrolysates produced from hydrolysis of isolated flaxseed proteins using different food-grade proteases.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Materials

Defatted flaxseed meal was a gift from Bioriginal Foods and Science Corporation (Saskatoon, SK, Canada). Cellulase (from *Aspergillus niger*), Alcalase (protease from *Bacillus licheniformis*), thermolysin (from *Bacillus thermoproteolyticus rokko*), ficin (from fig tree latex), pepsin (from porcine gastric mucosa), trypsin (from bovine pancreas), papain (from papaya latex), pancreatin (from porcine pancreas), ACE from rabbit lung (E.C.3.4.15.1) and *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The ultrafiltration membranes of 1, 3 and 5 kDa molecular weight cut-off (MWCO), the 100 Da MWCO dialysis membrane and other analytical grade reagents were purchased from Fischer Scientific (Oakville, ON, Canada).

### 3.2.2. Isolation of flaxseed proteins

The defatted flaxseed meal cake was pulverized using a Retsch ZM 200 ultracentrifugal mill (Retsch GmbH, Haan, Germany) at 14,000 $min^{-1}$ and the resulting

flour stored in an air-tight container at 4°C until used. The protein components of the meal were isolated according to our newly developed method that significantly (P<0.05) increased protein yield when compared to the traditional method of Dev and Quensel (1988). Defatted flaxseed meal (5%, w/v, dry weight basis) was suspended in deionized water and stirred thoroughly using a magnetic stirrer. The highly viscous suspension was adjusted to pH 5.0 and temperature of 37°C, followed by the addition of cellulase (1%, w/w; activity of powder, 1.44 U/mg) to initiate fibre hydrolysis. After 4 h of reaction, the resulting less viscous suspension was cooled to 4°C followed by alkaline solubilisation (pH 10.0) with 0.5 M NaOH and acid-induced flaxseed protein precipitation (pH 4.2) with 0.5 M HCl according to a previous method (Dev & Quensel, 1988). The resulting protein precipitate was washed thrice with acidified water (pH 4.2), suspended in a small volume of deionized water and the pH adjusted to 7.0 using 0.5 M NaOH. The suspension was freeze-dried and stored at −20°C until used as isolated flaxseed protein.

### 3.2.3. Preparation of flaxseed protein hydrolysates

Hydrolysis of the isolated flaxseed proteins was conducted with pepsin, ficin, trypsin, papain, thermolysin, pancreatin and Alcalase under different conditions using a Metrohm 842 Titrando pH-stat connected to a Metrohm 800 Dosino device (Metrohm AG, Herisau, Switzerland). Flaxseed protein isolate (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to the appropriate temperature and adjusted to the appropriate pH prior to the addition of the proteolytic enzyme; the reaction conditions are shown in Table 1. The enzymes were

added to the slurry at an enzyme-substrate ratio (E/S) of 1:100 (based on the protein content of the protein isolate) separately except for the Alcalase reaction. Digestion was performed at the above conditions for 4 h; pH of the reaction mixture was kept constant by the pH-stat with 2 M NaOH except for the pepsin reaction. Alcalase hydrolysis was carried out as previously reported at an E/S of 1.5:25 (Omoni & Aluko, 2006). After digestion, the enzyme was inactivated by immersing the reaction vessel in boiling water bath (95-100°C) for 15 min and undigested proteins were precipitated by adjusting the pH to 4.0 with 2 M HCl followed by centrifugation at 14,941 × $g$ for 30 min. The supernatant containing target peptides was collected for further fractionation. Protein content of samples was determined by a modified Lowry's method using bovine serum albumin as a standard (Markwell *et al.*, 1978).

### 3.2.4. Fractionation of flaxseed protein hydrolysates

The flaxseed protein hydrolysates (supernatants from section 2.3) were each separated into the LMW fractions using membrane ultrafiltration. The supernatant resulting from protein hydrolysis was passed through an Amicon stirred ultrafiltration cell (Millipore Corporation, Bedford, MA, USA) set-up using a 1 kDa MWCO membrane, and resulting permeate was collected. The flaxseed protein hydrolysate resulting from Alcalase digestion was sequentially passed through ultrafiltration membranes first with MWCO of 1 kDa then the retentate through 3 kDa whose retentate was passed through 5 kDa to separate peptides of sizes <1, 1-3 and 3-5 kDa. The resulting permeates were dialyzed for 48 h against deionized water at 4°C using a 100 Da MWCO dialysis tube, and

dialyzates were freeze-dried and stored at –20°C until used as flaxseed protein hydrolysate fractions in ACE and renin inhibition studies.

### 3.2.5. Preparative ion-exchange liquid chromatography

The cationic peptides in the <1 kDa permeate from the Alcalase prepared flaxseed protein hydrolysate were separated as previously reported (Omoni & Aluko, 2006) with some modifications. Briefly, a solution of the freeze-dried permeate (250 mg/ml in 0.1 M ammonium acetate buffer) was adjusted to pH 7.0 using 2 M ammonium hydroxide. Four ml of the solution was injected onto an SP-Sepharose High Performance XK 50/20 cation exchange chromatography column (column volume, 196.35 ml) coupled to an AKTA Fast Protein Liquid Chromatography system (Amersham Biosciences, Montreal, QC, Canada). Prior to sample injection, the column was pre-equilibrated with 1.5 column volume (CV) of 0.1 M ammonium acetate buffer (pH 7.5). After sample was loaded, the column was washed with 1.5 CV of the 0.1 M ammonium acetate buffer to remove unbound peptides, followed by a gradient elution of the bound peptides using 0–50% 0.5 M ammonium carbonate (pH 8.8) in 0.1 M ammonium acetate buffer at a flow rate of 10 ml/min; eluted peptides were monitored at 214 nm. Two major peaks were observed and fractions within these peaks were pooled and concentrated by vacuum evaporation at 37°C. Thereafter, the concentrated peptide solution was dialyzed for 48 h against deionized water at 4°C using a 100 Da MWCO dialysis tube. The dialyzed solutions were freeze-dried and stored at –20°C until used as cationic peptide fractions I (FI) and II (FII).

### 3.2.6. Determination of ACE inhibition

The ability of the flaxseed peptide fractions to inhibit the activity of ACE *in vitro* was measured according to the spectrophotometric method of Holmquist *et al.* (1979) as reported by Lin *et al.* (2008) using FAPGG as substrate. Briefly, 1 ml of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 mM NaCl, pH 7.5) was mixed with 20 µl of ACE (1 U/ml; final activity of 20 mU) and 200 µl of flaxseed protein hydrolysate in 50 mM Tris-HCl buffer. The decreased absorbance at 345 nm, due to cleavage of the Phe-Gly peptide bond of FAPGG, was recorded at regular intervals for 2 min at room temperature. Tris-HCl buffer was used instead of peptide fraction solutions in the blank experiment. ACE activity was expressed as rate of disappearance of FAPGG ($\Delta A.min^{-1}$) and inhibitory activity was calculated using Eq. (3.1).

$$\text{ACE inhibition (\%)} = 1 - (\Delta A.min^{-1}_{(sample)} / \Delta A.min^{-1}_{(blank)}) \times 100 \qquad (3.1)$$

$\Delta A.min^{-1}_{(sample)}$ and $\Delta A.min^{-1}_{(blank)}$ are the reaction rates in the presence and absence of the peptide fraction, respectively. Dose-dependent ACE inhibition was investigated using at least four different concentrations of peptide fractions. The concentration of peptide fractions that inhibited ACE activity by 50% ($IC_{50}$) was calculated using a non-linear regression from a plot of ACE inhibition versus sample concentrations. All experiments were performed in triplicate.

**3.2.7. Determination of kinetics parameters of ACE inhibition**

The kinetics of ACE-catalyzed conversion of FAPGG to $N$-(3-[2-furyl]acryloyl)-phenylalanine was studied in the absence and presence of three different concentrations of flaxseed protein-derived peptide fractions with 0.0625, 0.1, 0.125, 0.25 and 0.5 mM FAPGG, a range selected to represent concentrations above and below reported $K_m$ for FAPGG (Holmquist $et$ $al.$, 1979; Hou $et$ $al.$, 2003; Lin $et$ $al.$, 2008). The mode of inhibition of ACE was determined from the Lineweaver-Burk plots. Kinetic parameters ($K_m$, $K_m'$, $V_{max}$ and $V_{max}'$) were estimated from non-linear regression fit of the kinetics data to the Michaelis-Menten equation using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). The catalytic efficiency (CE) of ACE in the absence and presence of the peptide fractions was calculated using Eq. (3.2).

$$CE = V_{max}/K_m \text{ or } V_{max}'/K_m' \quad\quad\quad (3.2)$$

$V_{max}$ and $K_m$ are the maximum reaction velocity and Michaelis constant, respectively, whereas $V_{max}'$ and $K_m'$ represent their apparent values in the presence of the inhibitor. The enzyme-inhibitor dissociation constant ($K_i$) was determined as the intercept on the X-axis of a secondary plot of the slope of the Lineweaver Burk plot versus concentrations of inhibiting peptide fractions. All kinetics experiments were performed in duplicate.

### 3.2.8. Renin inhibition assay

*In vitro* inhibition of the activity of human recombinant renin was conducted by fluorescence spectrometry using the Renin Inhibitor Screening Assay Kit and the method of Yuan *et al.* (2006) modified slightly as follows. Briefly, the total assay volume of 190 μl contained 10 μM Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg (renin substrate dissolved in dimethyl sulphoxide), human recombinant renin and flaxseed peptide fraction in 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl. The Tris-HCl buffer was used instead of the flaxseed peptide solution in the blank experiment. The renin substrate and flaxseed peptide sample were mixed and pre-warmed to 37°C for 10 min to attain thermal equilibrium. Thereafter, the reaction was initiated by adding human recombinant renin to the mixture; the increase in fluorescence intensity was monitored for 10 min in a JASCO FP-6300 spectrofluorimeter (Japan Spectroscopic Company, Tokyo, Japan) equipped with a thermostated cell compartment that was maintained at 37°C using a circulatory water bath. The spectrofluorimeter parameters were set as follows: excitation wavelength, 340 nm; emission wavelength, 490 nm; excitation bandwidth, 5 nm; emission bandwidth, 10 nm. The enzyme activity was expressed as reaction rate, arbitrary fluorescence intensity unit per min (FIU.min$^{-1}$). Renin inhibitory activity of the peptide fractions was calculated using Eq. (3.3).

Renin inhibition (%) = [(FIU.min$^{-1}$$_{(blank)}$ − FIU.min$^{-1}$$_{(sample)}$) / FIU.min$^{-1}$$_{(blank)}$] × 100

(3.3)

FIU.min$^{-1}$$_{(blank)}$ and FIU.min$^{-1}$$_{(sample)}$ represent the renin reaction rates in the absence and presence of the flaxseed peptide fraction, respectively. In addition, kinetic studies was conducted for the most active flaxseed peptide fraction using 1.25, 2.5, 5 and 10 μM renin substrate in the absence and presence of the peptide fraction (0.5 and 2 mg protein/ml). This was followed by the determination of mode of inhibition and kinetics parameters of the renin reaction as earlier described in the ACE assay.

### 3.2.9. Statistical analysis

Data are reported as mean ± standard deviation. Statistical significance of differences was evaluated by Student's t-test and Duncan's multiple range test (P<0.05) using the Statistical Analysis Systems (SAS) software (SAS, Cary, NC, USA).

### 3.3. RESULTS AND DISCUSSION

In the recent past, food protein-derived peptides have been reported to display various biological functions towards the management and treatment of human diseases, including antimicrobial activity (Pellegrini, 2003), immunomodulatory activity (Gauthier et al., 2006), antioxidant, antithrombotic, hypolipidemic and hypocholesterolemic activities (Erdmann et al., 2008). In addition, a wide range of food proteins have also afforded highly potent ACE-inhibiting peptides, which have been actively investigated for their ability to lower blood pressure in SHR (Aluko, 2008; Erdmann et al., 2008; Wu & Ding, 2001) and in hypertensive human subjects (FitzGerald et al., 2004; Seppo et al., 2003). These food proteins include zein (Yano et al., 1996), corn gluten (Suh et al.,

2003), milk (FitzGerald *et al.*, 2004; Seppo *et al.*, 2003), soybean, wheat and quinoa (Aluko, 2008), yam dioscorin (Hsu *et al.*, 2002; Lin *et al.*, 2006), bovine skin gelatin (Kim, Byun, Park, & Shahidi, 2001) and canola (Wu *et al.*, 2008). To date, there is only one recent literature regarding ACE-inhibiting hydrolyzed flaxseed proteins generated by Flavourzyme digestion (Marambe *et al.*, 2008). It is noteworthy that these underutilized flaxseed proteins also contain peptide sequences with properties that could be exploited in the development of therapeutic food products.

The potential of food proteins to provide bioactive peptides depends on several factors including the ability of proteases to digest and release the active peptide sequences. Thus, the work reported in this paper investigated the hydrolysates generated from digestion of isolated flaxseed proteins using different proteases for *in vitro* effects on ACE and renin activities; the proteases were used at their optimum reaction conditions. These proteases were considered safe for food applications since they are either part of the human digestive system or have been used extensively in the food industry. Following enzymatic hydrolysis, the <1 kDa fractions of the flaxseed protein hydrolysates were separated using membrane ultrafiltration, and their protein contents ranged from 77.1 to 99.7% after dialysis with protein yields of between 5.4 and 18.5% (Table 3.1). The interest in short-chain peptides is due to reported evidence that these peptides are able to withstand proteolytic degradation by the enzymes of the gastrointestinal tract when orally ingested by humans (Aluko, 2008). Moreover, it is widely accepted that LMW peptides are more potent *in vitro* ACE inhibitors than larger peptides. Partial purification of the 1 kDa ultrafiltration permeate of the Alcalase

hydrolysate by fast protein liquid chromatography using a cation-exchange column yielded two cationic peptide fractions (FI and FII) as previously reported; FII possesses a stronger cationic character than FI (Omoni & Aluko, 2006). FI and FII contained 95.5 and 70.6% proteins, respectively, after desalting by dialysis. These protein hydrolysates and cationic peptide fractions were evaluated for ACE and renin inhibitory activities.

The protease treatments of flaxseed proteins liberated bioactive peptides from the native protein structure, and these peptides inhibited *in vitro* activity of ACE; the presence of the peptide fractions in the assay led to a concentration-dependent loss of ACE activity (Figure 3.1A). The LMW peptides resulting from thermolysin hydrolysis of flaxseed proteins displayed the most potent activity with the inhibition of 20 and 61% ACE activity at 0.00625 and 0.05 mg protein/ml, respectively. This extent of ACE inhibition was also observed for other enzymatic flaxseed protein hydrolysate fractions but at slightly higher concentrations. The $IC_{50}$ values of these protein hydrolysate fractions in ACE inhibition is shown in Figure 3.1B; the thermolysin LMW peptide fraction gave the least $IC_{50}$ value, similar to activity previously reported for crude thermolysin hydrolysates from $\alpha$-zein, $IC_{50}$ = 0.021 mg/ml (Miyoshi *et al.*, 1995) and dried bonito, $IC_{50}$ = 0.029 mg/ml (Fujita *et al.*, 1995) even though these studies used a different assay system to measure ACE activity. The peptides resulting from pepsin and trypsin hydrolyses of flaxseed proteins had similar inhibitory effects on ACE activity. Among all the enzymatic digests investigated, the pancreatin LWM peptide fraction showed the weakest ACE inhibition. These data suggest that ACE inhibition observed in this study could be more associated with the bioactive peptide sequences released

**Table 3.1 Reaction conditions for enzymatic hydrolysis of flaxseed proteins; protein contents and yield of the ultrafiltration permeate**

| Enzyme | Abbreviation | Reaction conditions | | Ultrafiltration permeate | |
|---|---|---|---|---|---|
| | | pH | Temp (°C) | Protein (%)[a] | Yield (%)[b] |
| Pepsin | PepH | 2.0-2.2 | 37 | 77.1 | 8.7 |
| Ficin | FicH | 7.0 | 37 | 94.7 | 7.4 |
| Trypsin | TrypH | 8.0 | 37 | 99.7 | 9.0 |
| Papain | PapH | 6.5 | 37 | 73.4 | 5.4 |
| Thermolysin | ThermoH | 8.0 | 37 | 97.9 | 5.6 |
| Pancreatin | PancH | 8.0 | 40 | 87.6 | 18.5 |

[a]Protein contents of <1 kDa permeates after dialysis using a 100 Da MWCO membrane

[b]Protein basis

during hydrolysis rather than the amount of small peptides, since there was no linear relationship between the protein yield of the LMW peptide fractions and ACE inhibition; moreover, the pancreatin hydrolysate fraction with the highest <1 kDa protein yield gave the lowest ACE inhibition and the reverse was observed for thermolysin hydrolysate. Besides, these levels of ACE inhibition observed for the flaxseed protein digests are comparable to inhibitory activities reported for crude and ultrafiltration-fractionated simulated gastrointestinal digests of pea and whey proteins using similar assay conditions (Vermeirssen *et al.*, 2005). A combination of the protein yield and $IC_{50}$

values reported here showed that the LMW peptides in thermolysin hydrolysate have the best potential for industrial application as ACE inhibitor followed by trypsin, pepsin and ficin digests. When peptides of higher molecular weight (MW) were evaluated, the Alcalase protein hydrolysate containing peptides of MW range of 3-5 kDa showed better ACE inhibition than the 1-3 kDa peptides. Moreover, a previous report showed that when Flavourzyme was used to hydrolyze flaxseed proteins to three different (11.9%, 24.6% and 25.1%) degrees of hydrolysis, the crude hydrolysates showed ACE inhibition with $IC_{50}$ values 0.07 and 0.09 mg protein/ml using hippuryl-histidyl-leucine as substrate (Marambe *et al.*, 2008). Considering that some of the proteases investigated in this study are present in the gastrointestinal tract of humans, it is possible that these bioactive peptides could be released during human consumption of intact flaxseed proteins.

**Figure 3.1 (A) Flaxseed protein-derived peptide fractions exhibit concentration-dependent ACE inhibition; results are expressed as means of ACE activity ± standard deviation of triplicate determinations. (B) 50% inhibitory concentration (IC$_{50}$) values of peptide fractions in ACE inhibition; Each bar represents the mean of triplicate determinations of IC$_{50}$ ± standard deviation; bars with different letters are significantly different at P< 0.05; 1-3AlcH and 3-5AlcH stand for the 1-3 kDa and 3-5 kDa peptides of alcalase hydrolysate, respectively (For other abbreviations, see Table 3.1)**
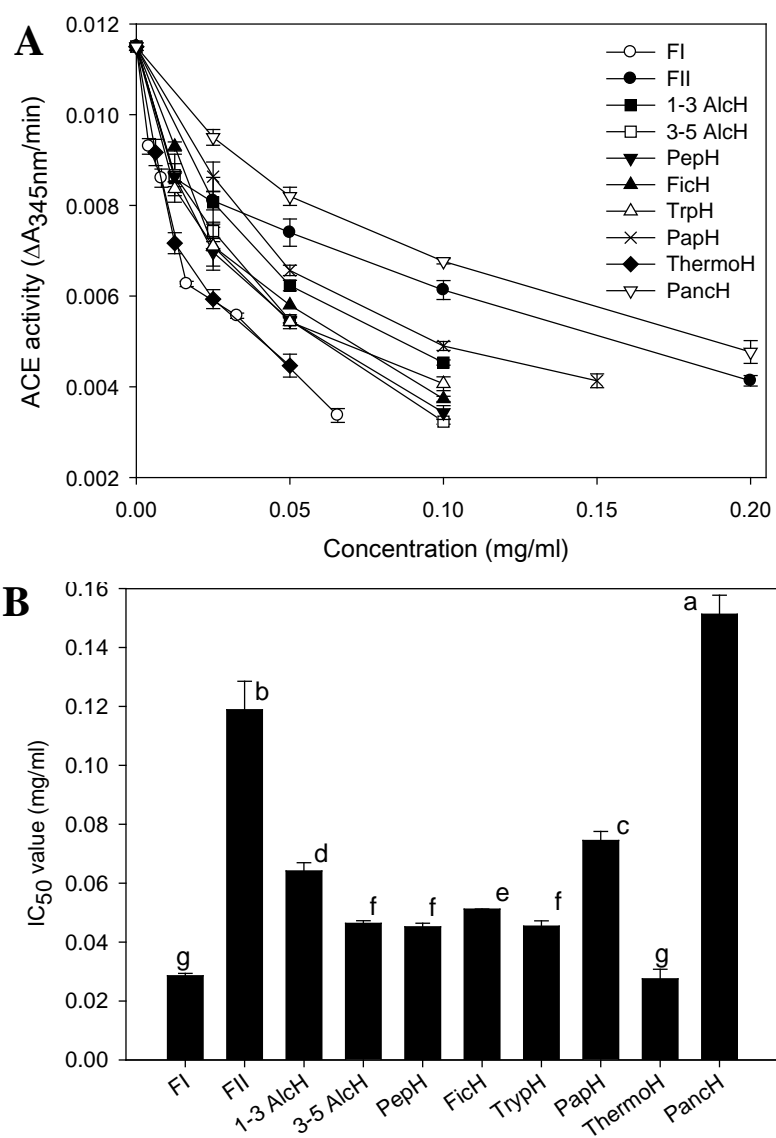
Food protein-derived cationic peptides have also demonstrated *in vitro* ACE inhibition; thus we investigated the ACE-inhibiting potentials of cationic peptides in the flaxseed protein digests. When the <1 kDa Alcalase protein hydrolysate was separated into its cationic peptide fractions, one of the resulting fractions showed a strong ACE-inhibitory activity. Cationic peptide fraction I showed activity ($IC_{50}$ = 0.0288 mg protein/ml) similar to that observed for thermolysin hydrolysates with the inhibition of about 70% ACE activity at 0.065 mg protein/ml whereas cationic peptide fraction II showed weaker activity with the inhibition of 64% ACE activity at 0.2 mg protein/ml ($IC_{50}$ = 0.118 mg protein/ml). Thus, it could be concluded from these data that the ability of these flaxseed protein-derived peptide fractions to inhibit ACE activity *in vitro* is independent of their cationic strength. This observation is contrary to a trend previously reported for ACE-inhibiting cationic peptides derived from pepsin-pancreatin soy protein hydrolysate (Farzamirad & Aluko, 2008). Based on these enzyme inhibitory activities, cationic peptide fraction I and the thermolysin hydrolysate fraction were selected for kinetics studies to elucidate their mode of inhibition of ACE activity. Cationic peptide fraction II was also studied for comparison.

Several studies have reported the mode of inhibition of peptides purified from food-protein hydrolysates. However, there are few studies in the literature that investigated the kinetics of ACE inhibition in the presence of food protein hydrolysates. This information will be necessary to elucidate the synergistic effects of the peptide mixture on ACE reaction kinetics prior to *in vivo* studies especially if the protein

hydrolysates are highly potent ACE inhibitors that do not require further purification. In this study, Lineweaver-Burk plots were used to estimate the modes of ACE inhibition by the flaxseed peptides. Figure 3.2A–C shows the double reciprocal plots of ACE-catalyzed reaction in the absence and presence of flaxseed peptide fractions; ACE inhibition by various concentrations of flaxseed-protein derived peptides was evident across the FAPGG concentrations investigated. Using the non-linear regression fit of the kinetics data, the $K_m$ value of ACE activity in the absence of the inhibiting peptides was determined to be 0.3058 mM FAPGG, which was similar to previously reported values of 0.3 mM (Holmquist *et al.*, 1979) and 0.292 mM FAPGG (Hou *et al.*, 2003). The Alcalase cationic peptide and thermolysin hydrolysate fractions showed mixed-type inhibition of ACE activity characterized by various effects on the catalytic parameters at different peptide concentrations (Table 3.2). These results are similar to the mode of ACE inhibition reported for glutathione and carnosine under similar assay conditions (Hou *et al.*, 2003). This shows that the constituent peptides of the fractions exhibited their activities possibly by binding ACE in both its free and FAPGG-bound forms. This also means that the peptides could bind ACE at other sites different from the FAPGG binding site, which might affect the conformation of the active site resulting in loss of activity. The presence of the Alcalase cationic peptide fraction I in the assay resulted in a two-fold decrease in the affinity of FAPGG for ACE at 0.0164 mg protein/ml, almost the same magnitude as the effect reported for 0.0164 mM glutathione (Hou *et al.*, 2003). Moreover, both the Alcalase cationic peptide fraction II and thermolysin hydrolysate fraction showed a concentration-dependent increase in $K_m$. The effects of these

flaxseed protein hydrolysate fractions on $V_{max}$ did not follow any pattern, and was not concentration-dependent. However, there was an observed linear decrease in the catalytic efficiency of ACE with increase in the concentration of inhibiting peptides; Alcalase cationic peptide fraction I and thermolysin protein hydrolysate showed better effects than fraction II (Table 3.2). These results confirm the ability of the peptides to dose-dependently reduced the efficiency of the reaction regardless of their mode and extent of ACE inhibition. Despite having similar $IC_{50}$ values, the Alcalase cationic peptide fraction I had more affinity for ACE than the thermolysin hydrolysate as evident in its lower $K_i$ value. However, the $K_i$ for cationic peptide fraction II was about twice the value for thermolysin protein hydrolysate even though the latter was more than four times more active in ACE inhibition ($IC_{50}$).

Furthermore, the flaxseed protein-derived peptide fractions moderately inhibited renin activity, and this was also dependent on the proteolytic treatments of the flaxseed proteins; their $IC_{50}$ values are shown in Figure 3.3. The trend of renin inhibition observed for the cationic peptide fraction II and thermolysin hydrolysate fraction was the opposite of their observed ACE-inhibitory activities, with the cationic peptide showing the best activity whereas the thermolysin hydrolysate was weakest. The results suggest that renin inhibition observed in this study was dependent on the cationic character of the peptide fraction because the weaker cationic fraction (FI) did not show any renin-inhibitory activity in this assay. In addition, the <1 kDa peptide fractions from papain and pancreatin hydrolysates, and the high MW Alcalase hydrolysate fractions, did not show renin inhibition under same assay conditions.

**Figure 3.2 Lineweaver-Burk plots of the inhibition of ACE by different concentrations of: (A) cationic peptide fractions I; (B) cationic peptide fractions II; (C) ThermoH, at varying concentrations of FAPGG; _V_ is the initial rate of reaction ($\Delta A_{345nm}$/min)**
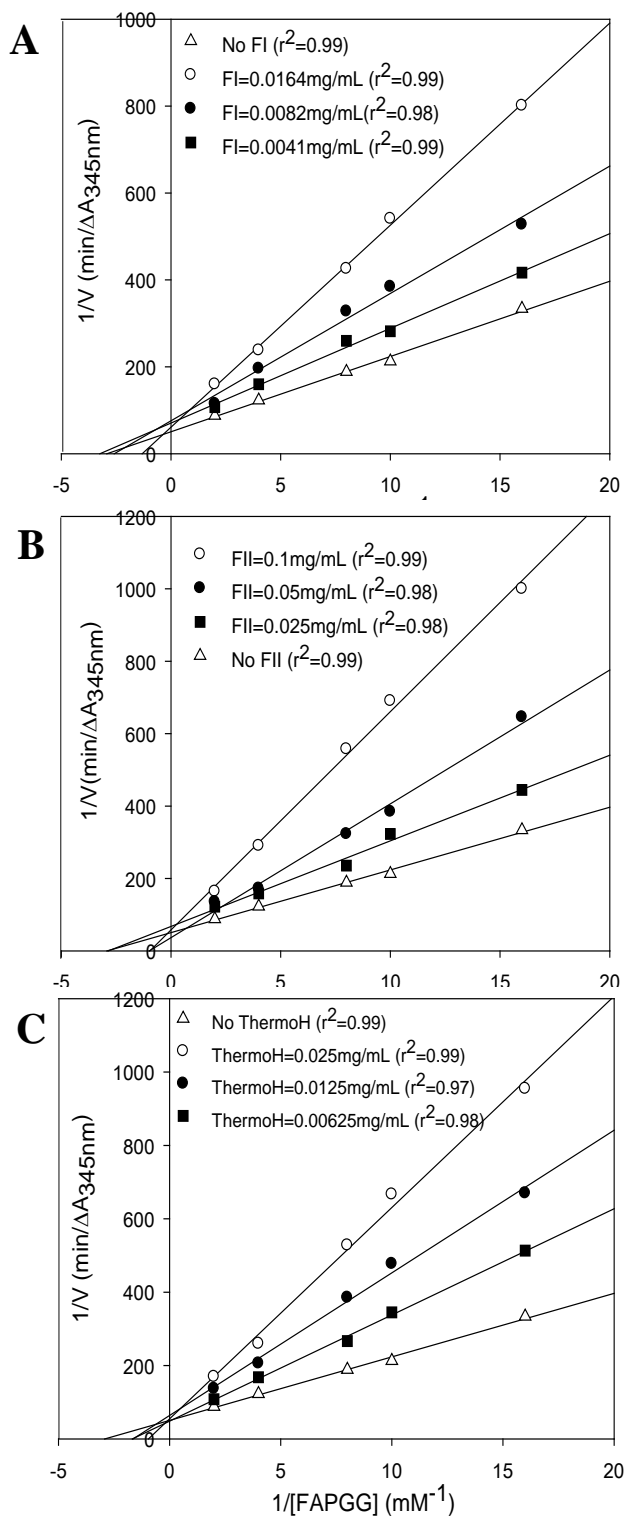
**Table 3.2 Kinetics constants of ACE catalyzed reaction in the absence (control) and presence of different concentrations of Alcalase cationic peptide fractions I and II, and thermolysin hydrolysate (ThermoH); $K_m$ and $K_m'$ are Michaelis constants in the absence and presence of inhibitor, respectively; $V_{max}$, and $V_{max}'$ are maximum reaction velocities in the absence and presence of inhibitor, respectively; CE, catalytic efficiency of ACE; $K_i$, enzyme-inhibitor dissociation constant**

| Catalytic parameter | Control | Fraction I (mg/ml) | | | Fraction II (mg/ml) | | | ThermoH (mg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.0041 | 0.0082 | 0.0164 | 0.0250 | 0.0500 | 0.1000 | 0.0063 | 0.0125 | 0.0250 |
| $K_m$ or $K_m'$ (mM)[a] | 0.3058 | 0.3964 | 0.7799 | 0.6109 | 0.2790 | 0.4052 | 1.764 | 0.5551 | 0.6782 | 0.9444 |
| $V_{max}$ or $V_{max}'$[b] | 0.0183 | 0.0166 | 0.0221 | 0.0140 | 0.0129 | 0.0138 | 0.0276 | 0.0195 | 0.0173 | 0.0172 |
| CE | 0.0599 | 0.0419 | 0.0282 | 0.0229 | 0.0463 | 0.0339 | 0.0156 | 0.0351 | 0.0255 | 0.0182 |
| $K_i$ (mg/ml) | | | 0.0065 | | | 0.0240 | | | 0.0128 | |

[a]FAPGG as substrate

[b]$V_{max}$, $\Delta A.min^{-1}$

The results are consistent with the fact that ACE inhibition is more easily achieved when compared to renin inhibition and this is reflected in the numerous ACE-inhibitory peptides already reported in the literature but almost no food protein-derived peptide inhibitors of renin. Based on this activity, the kinetics of renin inhibition was studied for Alcalase cationic peptide fraction II.

In this study, the $K_m$ value of human recombinant renin in the absence of any inhibitor was determined to be 4.416 µM for the  renin substrate, lower than a value 6.4 µM reported by Yuan *et al*. (2006) and higher than a value 1.3 µM reported by Wang *et al*. (1993). The difference in $K_m$ values could be attributed to the different sources of renin in these studies. The double reciprocal plot (Figure 3.4) showed that the cationic peptide fraction II exhibited an uncompetitive mode of renin inhibition with large decrease in $V_{max}$ from 39.17 FIU/min for the uninhibited reaction to 24.2 and 10.47 FIU/min in the presence of 0.5 and 2 mg/ml inhibitor sample, respectively. The observed decrease in $V_{max}$ was associated with decrease in $K_m$ value to 3.912 and 2.72 µM with 0.5 and 2 mg/ml inhibitor, respectively. Thus, it could be concluded that the cationic peptide fraction II contained peptides that did not bind to the human recombinant renin active site but associated with other regulatory sites on the enzyme-substrate complex. This observation justifies the high IC$_{50}$ value for the cationic peptide fraction since high inhibitor concentration would be required to effect a change in the conformation of the renin active site, depending on its interaction with the inhibitor-binding sites. It was difficult to make comparison since there are no related renin inhibition kinetics studies with enzymatic food protein hydrolysates in the literature.

**Figure 3.3 50% Inhibitory concentration (IC$_{50}$) values of flaxseed protein-derived peptide fractions in inhibiting human recombinant renin activity[1]; n.a, no renin inhibitory activity observed; bars with different letters are significantly different at P<0.05**



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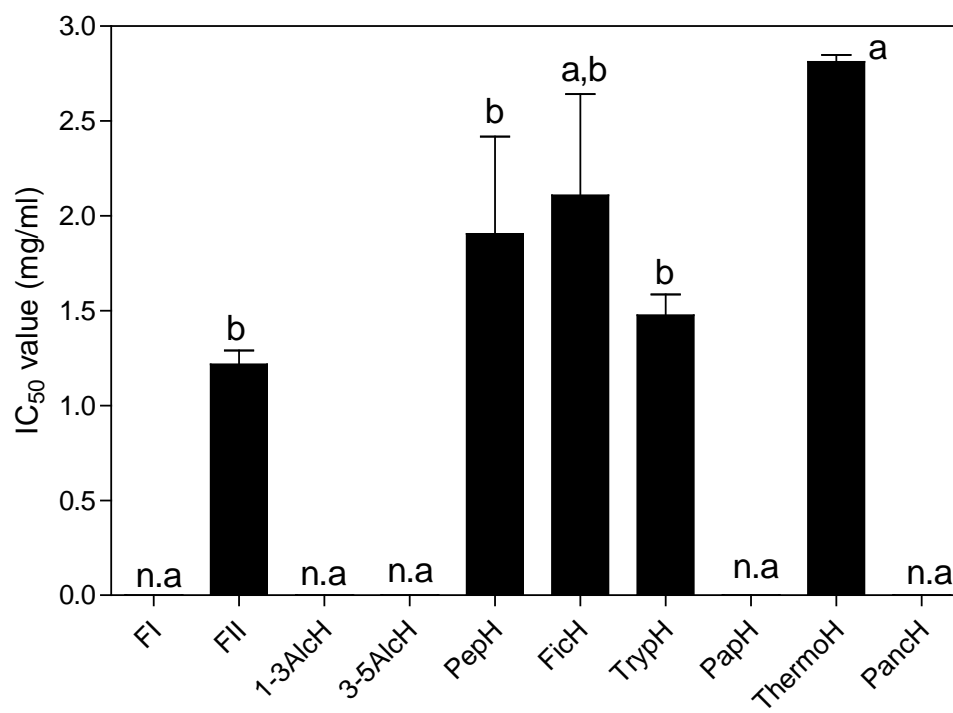
[1] See Figure S3.5 in Appendix A for dose-dependent inhibition of human recombinant renin activity by the flaxseed protein-derived peptide fractions

**Figure 3.4 Lineweaver-Burk plot of the inhibition of human recombinant renin by**

**flaxseed protein-derived cationic peptide fraction II at 0.5 and 2 mg protein/ml**
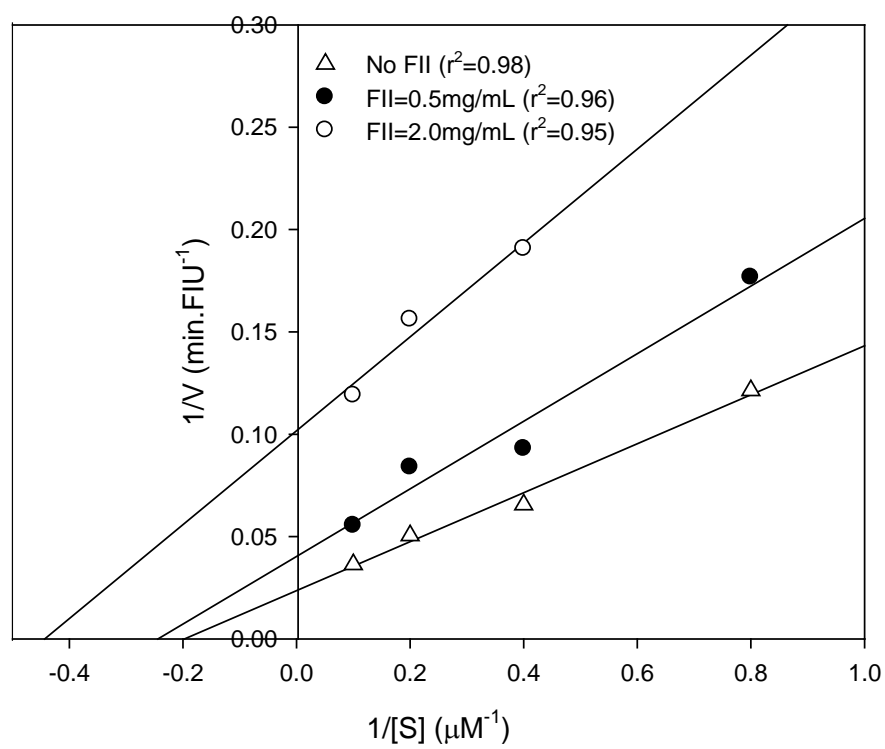


**3.4. CONCLUSIONS**

The results of this study clearly indicate that flaxseed protein hydrolysates possess potential as a food source of antihypertensive agents. The inhibition of the activities of ACE and human recombinant renin observed in this study is dependent on the ability of the different proteases used for hydrolysis to release bioactive peptide sequences from flaxseed proteins rather than the protein yield of low-molecular-weight peptides. The cationic peptide fraction I and thermolysin hydrolysate gave promising results due to their ability to inhibit ACE in a mixed-type inhibition pattern at very low concentrations. In addition, the flaxseed peptide fractions that inhibited both ACE and renin activities possess better prospects, and potentially could provide better *in vivo* lowering of blood pressure when compared to peptides that inhibit ACE alone. Further work is needed to characterize the effects of these flaxseed protein hydrolysate and cationic peptide fractions in lowering of blood pressure, and to identify their constituent bioactive peptides. This will contribute towards increased value-added utilization of flaxseed meal, currently a low-value by-product of the oilseed processing industry.

**3.5. REFERENCES**

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**APPENDIX A: SUPPLEMENTAL INFORMATION**

The rationale for using microbial cellulase (endo-$\beta$(1$\rightarrow$4)-glycosidase) devoid of any proteolytic activity for pre-treatment of the defatted flaxseed meal was to cleave the $\beta$(1$\rightarrow$4) glycosidic bonds of the cellulose and hemi-cellulose of the soluble fibre (mucilage) of defatted flaxseed meal. This treatment reduces viscosity of the aqueous suspension of flaxseed meal and thus enhances protein solubilisation in alkaline solution; the longer the fibre polymer, the more viscous the slurry and the lower the efficiency of the protein extraction process. In theory, soluble cellobiose and shorter polysaccharides are produced due to cellulase activity (the $\beta$(1$\rightarrow$4) bond in cellobiose cannot be hydrolyzed by cellulase) and subsequently removed by washing during protein isolation. The optimization experiments were conducted with 5% defatted flaxseed meal (5 g in 100 ml of distilled water).

In addition to this cellulase hydrolysis method (described in details in Section 3.2.2) for mucilage removal prior to protein isolation, the flaxseed mucilage was also removed by soaking in acidified water. The water was acidified (adjusted to pH 4.2, isoelectric point for flaxseed protein) to prevent solubility and loss of proteins during the extraction process. This experiment was chosen to reduce the cost associated with the use of enzyme in food processing and to enhance protein extractability from flaxseed by the removal of protein-bound water-soluble fibres. The overall aim of the process was to improve the protein yield from the high-mucilage defatted flaxseed meal. Briefly, 5 g of defatted flaxseed meal was dispersed in 100 ml of acidified distilled water (adjusted to pH 4.2 using 0.5 M HCl). The suspension was gently stirred for 1 h

and allowed to settle at room temperature. Thereafter, the suspension was decanted and the resulting meal rinsed with 100 ml acidified water followed by protein isolation by the conventional method of direct alkaline solubilisation and acid-induced protein precipitation, as previously outlined in Section 3.2.2. The protein content of the resulting freeze dried flaxseed protein isolate (FPI) was compared to the flaxseed protein isolates obtained by conventional method and cellulase treatments (2 and 4 h).

The protein contents (%) and dry weight yields (%) of the FPI isolated using different methods are shown in Fig. S3.1. Treatment of defatted flaxseed meal with 1% cellulase for 4 h prior to protein isolation gave a flaxseed protein isolate with the lowest yield (9.36%, dry weight basis) and highest protein content of 87.6% compared to the control with 76.0% protein. This represents a 13.2% increase in the protein content of the FPI. However, the 2-h treatment of flaxseed meal with 1% cellulase gave an FPI with 67.1% protein; thus, longer cellulase pre-treatment duration is required to ensure the breakdown of flaxseed mucilage and subsequent release of the proteins from the complex. On the other hand, pre-treatment of the defatted flaxseed meal by soaking in acidified water resulted in a product with 55.4% protein. This observation could be due to the loss of the proteins in the fibre-protein complex during the water extraction of the mucilage, or due to co-extraction of the acidified water-insoluble fibres during protein isolation. The latter could have also accounted for the higher dry weight yield of the control FPI compared to the FPI from cellulase pre-treatment method. Based on these results, the 4-h cellulase pre-treatment process was adopted for scale-up production of large amount of flaxseed protein isolate.

**Figure S3.1 Protein contents (%) and yield (%, dry weight basis) of flaxseed protein isolate (FPI) from the different isolation methods; Control, conventional method; FPI-1, pre-treatment of the defatted flaxseed meal with 1% cellulase for 2 h; FPI-2, pre-treatment of the defatted flaxseed meal with 1% cellulase for 4 h; FPI-3, soaking of the defatted flaxseed meal in acidified water for 1 h prior to protein isolation**
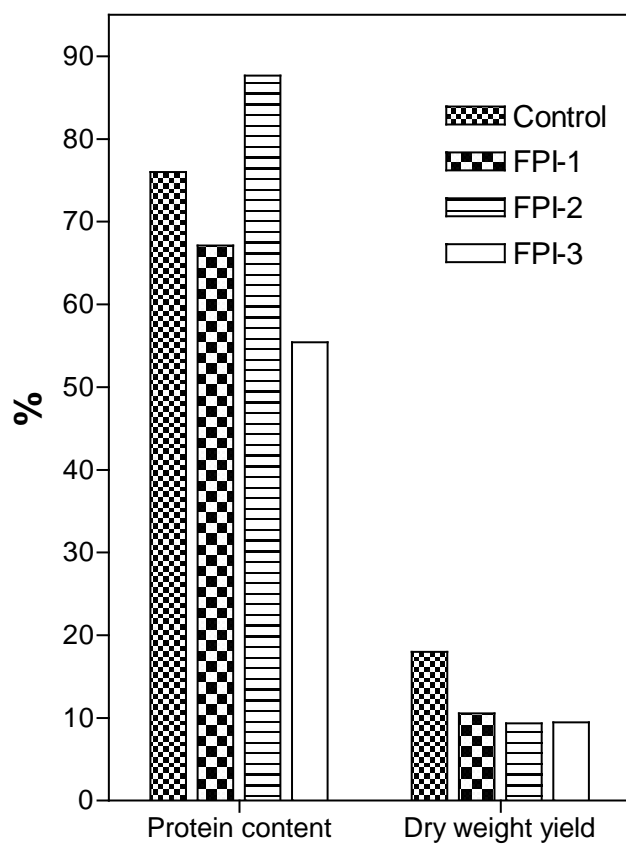
In the scale-up experiment, 75 g of defatted flaxseed meal was suspended in 1500 ml of distilled water followed by the addition of 1% cellulase (w/v); reaction was carried out under the same conditions described in Section 3.2.2. This scale-up process produced an FPI with a dry weight yield of 12.01 ± 1.88 (n=15) and 78.9% protein representing a 22.7% increase in protein content compared to control (61% protein) under similar conditions. Although the protein content of the FPI from the scale-up cellulase pre-treated sample was lower than that from the small-scale process, the magnitude of increase from the control sample was higher when the former process was used. Thus, the high mucilage defatted flaxseed meal was pre-treated with 1% cellulase for 4 h to produce a less viscous suspension that yielded the FPI used for the entire project.

The nutritional quality of the FPI was evaluated by comparing its amino acid composition with those of other food proteins most importantly soy and egg proteins, which are good quality plant and animal proteins, respectively (Table S3.1). According to the FAO/WHO suggested requirements, the essential amino acid composition of egg protein is superior to FPI except for Trp content whereas soy protein is comparable to FPI but for the lower amounts of Leu, Lys and aromatic amino acids in FPI (Table S3.2). Based on these suggestions, it could be observed that FPI satisfies the amino acid nutritional requirements of adults, but not for infants and older children due to the limited amounts of Leu and Lys. These observations support the need for large scale production and commercialization of FPI for use in food systems in countries such as Canada that generate enormous amounts of protein-rich defatted flaxseed meal.

**Table S3.1 Amino acid composition (%) of flaxseed protein isolate (FPI) compared to soy, egg white, pea, rapeseed and wheat proteins**

| AMINO ACIDS | SOY[a] | EGG[a] | PEA[a] | RAPESEED[a] | WHEAT[a] | FPI[b] |
|---|---|---|---|---|---|---|
| ARG | 7.58 | 5.48 | 8.25 | 7.01 | 4.42 | 11.28 |
| HIS | 2.45 | 2.17 | 2.49 | 2.96 | 2.24 | 2.29 |
| ILE | 4.55 | 5.09 | 4.37 | 4.23 | 3.42 | 4.5 |
| LEU | 8.20 | 8.44 | 7.84 | 7.97 | 7.02 | 5.8 |
| LYS | 6.11 | 6.73 | 7.85 | 5.79 | 2.73 | 3.04 |
| MET | 1.19 | 3.69 | 1.13 | 2.00 | 1.69 | 2.02 |
| CYS | 1.15 | 2.71 | 1.54 | 2.50 | 2.28 | 1.36 |
| PHE | 5.42 | 5.98 | 5.10 | 4.31 | 4.99 | 5.47 |
| TYR | 3.89 | 4.24 | 3.58 | 2.87 | 2.98 | 2.52 |
| THR | 3.70 | 4.51 | 4.26 | 4.26 | 3.00 | 3.67 |
| TRP | 1.10 | 1.41 | 0.89 | 1.52 | 1.15 | 1.73 |
| VAL | 4.73 | 6.53 | 5.07 | 5.33 | 4.38 | 5.21 |
| ALA | 4.21 | 5.77 | 4.84 | 4.79 | 3.57 | 4.89 |
| ASX[c] | 11.37 | 10.08 | 11.86 | 7.66 | 5.17 | 11.29 |
| GLX[d] | 19.95 | 13.49 | 16.73 | 20.65 | 31.72 | 19.83 |
| GLY | 4.07 | 3.46 | 4.72 | 5.29 | 4.05 | 5.47 |
| PRO | 5.22 | 3.62 | 4.41 | 6.38 | 10.70 | 5.29 |
| SER | 5.11 | 6.58 | 5.05 | 4.48 | 4.50 | 4.34 |
| EAA[e] | 42.49 | 51.50 | 44.14 | 43.73 | 35.88 | 37.61 |
| NEAA[f] | 57.51 | 48.50 | 55.86 | 56.27 | 64.12 | 62.39 |

[a]Data derived from Sarwar et al., *J. Food Sci*. 1983, *48*, 526-531; [b]data from this work; [c]ASX, aspartic acid+asparagine; [d]GLX, glutamic acid+glutamine; [e]EAA, essential amino acids; [f]NEAA, non-essential amino acids;

**Table S3.2 Essential amino acid composition (mg/g) of flaxseed protein isolate (FPI) from this study and other food proteins compared to FAO/WHO suggested requirements for humans**

| AMINO ACIDS | AMINO ACID COMPOSITION (mg/g)[a] | | | FAO/WHO suggested requirements[a] | | | |
|---|---|---|---|---|---|---|---|
| | EGG | SOY | FPI | 1 y.o. | 2-5 y.o. | 10-12 y.o. | ADULT |
| THR | 45.1 | 37.0 | 36.7 | 43 | 34 | 28 | 9 |
| CYS+MET | 64.0 | 23.4 | 33.8 | 42 | 25 | 22 | 17 |
| VAL | 65.3 | 47.3 | 52.0 | 55 | 35 | 25 | 13 |
| ILE | 50.9 | 45.5 | 45.0 | 46 | 28 | 28 | 13 |
| LEU | 84.4 | 82.0 | 58.0 | 93 | 66 | 44 | 19 |
| TYR+PHE | 102.3 | 93.1 | 79.9 | 72 | 63 | 22 | 19 |
| HIS | 21.7 | 24.5 | 22.9 | 26 | 19 | 19 | 16 |
| LYS | 67.3 | 61.1 | 30.4 | 66 | 58 | 44 | 16 |
| TRP | 14.1 | 11.0 | 17.3 | 17 | 11 | 9 | 5 |

[a]Egg white and soy proteins data derived from Sarwar et al., *J. Food Sci*. 1983, *48*, 526-531; FAO/WHO data adapted from Friedman, *J. Agric*. *Food Chem*. 1996, *44*, 6-29; y.o., year(s) old

**Figure S3.2 Flowchart for flaxseed protein isolation**

Defatted flaxseed meal (5%)

↓

| Mucilage hydrolysis | *1% Cellulase, pH 5.0, 37°C, 4 h*

↓

| Alkaline solubilisation | *0.5 M NaOH, pH 10.0, 30 min, r.t.*

↓

| Centrifugation | *14,941 × g, 4°C, 30 min*

| Discard residue |   | Acid precipitation of supernatant | *0.5 M HCl, pH 4.2*

↓

| Centrifugation | *14,941 × g, 4°C, 15 min*

*3 times with acidified water (pH 4.2)* | Wash precipitate |   | Discard supernatant |

↓

| Freeze dry precipitate | *Adjusted to pH 7.0 prior to freeze drying*

↓

Flaxseed protein isolate

**Figure S3.3 Flowchart for enzymatic hydrolysis of flaxseed protein isolate and post-hydrolysis processing. FPH, flaxseed protein hydrolysates**

Flaxseed protein isolate (5%)

↓

| Enzymatic hydrolysis |

*Alcalase, pepsin, ficin, trypsin, papain, thermolysin, or pancreatin*
*For conditions, see Section 3.2.3*

↓

| Centrifugation |

| Discard residue | | Collect supernatant |

FPH (Alcalase)　　　　　　　　FPH (Other enzymes)

↓　　　　　　　　　　　　　　　↓

*1, 3, 5 kDa MWCO membranes* | Membrane ultrafiltration |　　| Membrane ultrafiltration | *1 kDa MWCO membrane*

↓　　　　　　　　　　　　　　　↓

| Freeze dry permeates |　　| Freeze dry permeates |

<1 kDa AlcH　　1-3 kDa, 3-5 kDa AlcH　　　<1 kDa FPH PepH, FicH, TrypH, PapH, ThermoH, PancH

↓

| Cation exchange chromatography | *See Fig. S3.4 & Section 3.2.5.*

↓

| Freeze dry fractions FI & FII |

↓

| Desalt by dialysis |

↓

| Freeze dry |

↓

Cationic peptide fractions FI & FII

**Figure S3.4 Fast Protein Liquid Chromatography of the <1kDa flaxseed protein hydrolysate ultrafiltration permeate using a cation exchange column. Column was washed with 0.1 M ammonium acetate buffer to remove unbound peptides, followed by a gradient elution of the bound peptides using 0–50% 0.5 M ammonium carbonate (pH 8.8) in 0.1 M ammonium acetate buffer. $F_0$, unbound peptides; FI, cationic fraction I; FII, cationic fraction II**
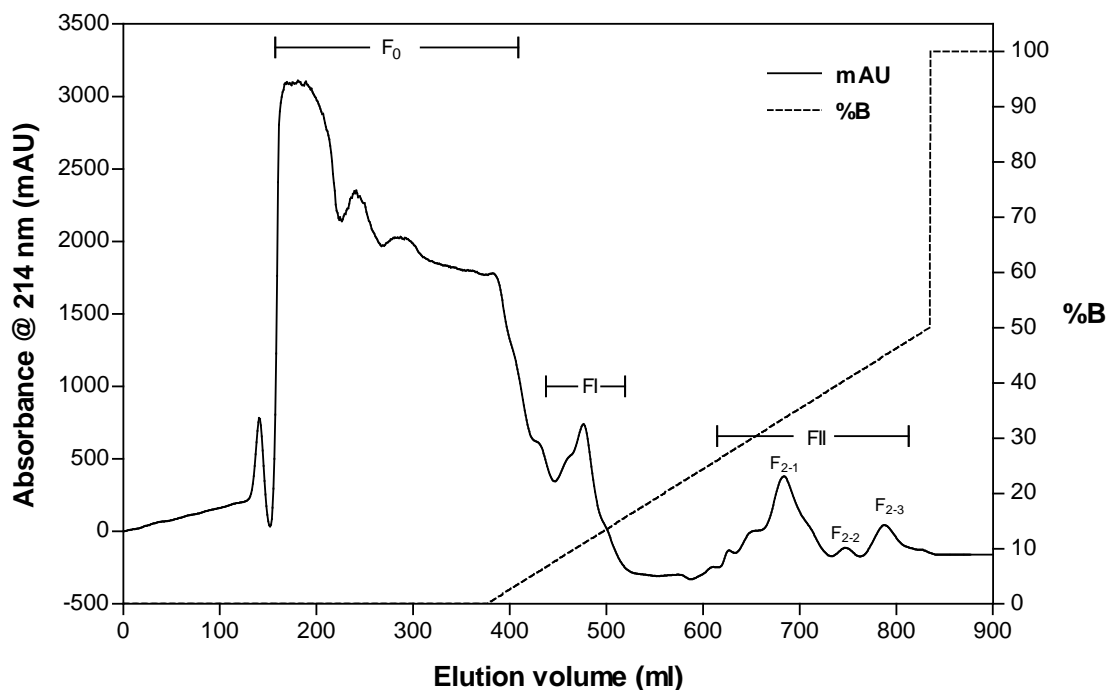


From the chromatogram, the least cationic peptide fraction (FI) eluted from 48 min [6-16% 0.5 M $(NH_4)_2CO_3$ buffer] while the other 3 cationic peptide fractions ($F_{2-1}$, $F_{2-2}$ and $F_{2-3}$) eluted at 69, 76 and 80 min, respectively [25-42% 0.5 M $(NH_4)_2CO_3$ buffer], and were pooled together as fraction FII.

**Figure S3.5 Dose-dependent inhibition of human recombinant renin activity by flaxseed protein-derived peptide fractions; 50% inhibitory concentration (IC$_{50}$) values are shown in Figure 3.3**
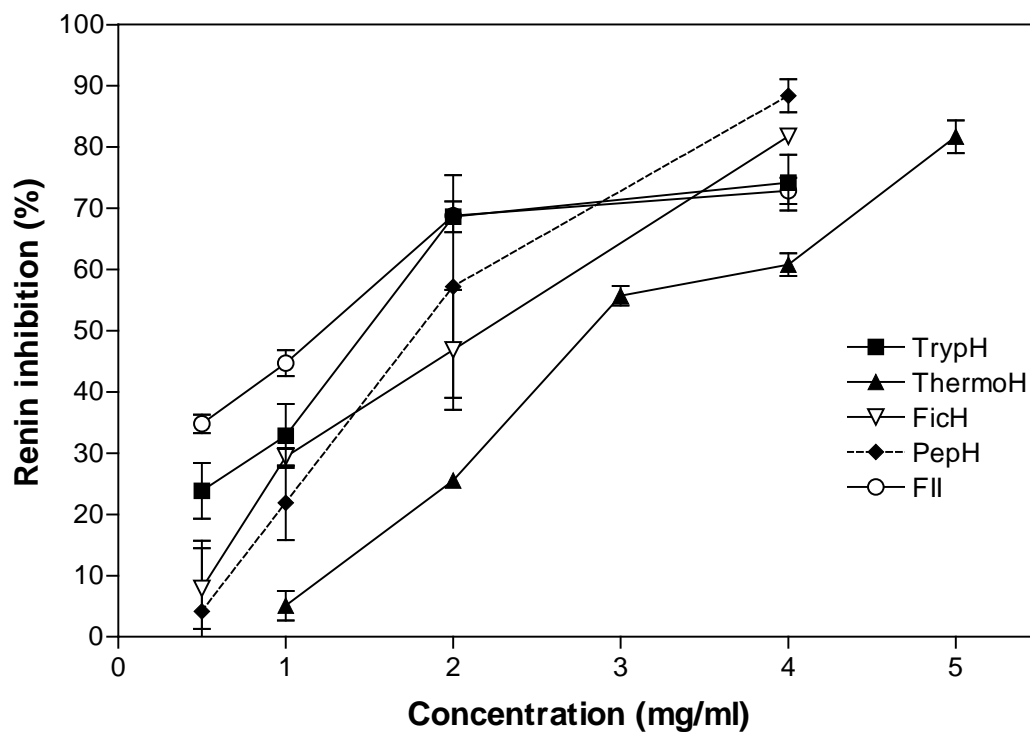
**TRANSITION STATEMENT**

This paper addressed the potential use of flaxseed protein isolated from defatted flaxseed meal as a source of low-MW antihypertensive peptide fractions, which modulated the RAS *in vitro* by inhibiting both ACE and renin activities. In order to maximize their value and utilization in ameliorating human disease conditions, these flaxseed peptide products were also investigated for multifunctional properties as antioxidant and anti-inflammatory agents using *in vitro* and cell culture assays. Details can be found in the next chapter. The ROS systems targeted in this part of the project included $O_2^{\cdot-}$, $\cdot OH$, NO and synthetic DPPH radical, as well as suppression of endotoxin-induced nitric oxide production in murine macrophages and inhibition of semicarbazide-sensitive amine oxidase reaction. It is believed that RAS-modulating peptides that possess these other biological activities can be used as a single agent to target and ameliorate multiple human health and disease conditions, thereby reducing the expenditures needed to develop multiple nutraceuticals for different diseases.

**CHAPTER FOUR**

**MANUSCRIPT 2**

**FLAXSEED PROTEIN-DERIVED PEPTIDE FRACTIONS: ANTIOXIDANT PROPERTIES AND INHIBITION OF LIPOPOLYSACCHARIDE-INDUCED NITRIC OXIDE PRODUCTION IN MURINE MACROPHAGES**

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**4.0. ABSTRACT**

A protein isolate was produced from cellulase-treated defatted flaxseed meal followed by hydrolysis with seven proteases and evaluation of the hydrolysates for antioxidant and anti-inflammatory properties. The flaxseed protein hydrolysates (FPH) were processed by ultrafiltration and ion-exchange chromatography to isolate low molecular weight (LMW) and cationic peptide fractions, respectively. The peptides showed antioxidant properties in scavenging 2,2-diphenyl-1-picrylhydrazyl radical, superoxide anion radical, electron spin resonance-detected hydroxyl radical and nitric oxide. In addition, all peptide fractions inhibited semicarbazide-sensitive amine oxidase activity. Antioxidant activities of these peptides were dependent on the specificity of proteases and size of the resulting peptides. The LMW fractions from pepsin, ficin and papain FPH also inhibited lipopolysaccharide-induced nitric oxide productions in RAW 264.7 macrophages without apparent cytotoxicity; thus, these peptides may act as anti-inflammatory agents. Thus, flaxseed protein hydrolysates may serve as potential ingredients for the formulation of therapeutic products.

**KEYWORDS: Antioxidant; Flaxseed protein hydrolysates; nitric oxide; RAW 264.7 macrophages; Semicarbazide-sensitive amine oxidase**

**4.1. INTRODUCTION**

Reactive oxygen species (ROS) from dietary sources, cellular processes and mitogen-activated immune cells have been shown to be associated with the aetiology and pathogenesis of human physiological and disease conditions due to their roles in the oxidative degradation of biological macromolecules (Ames, Shigena, & Hegen, 1993; Ames, 1983; Pacher, Beckman, & Liaudet, 2007). Epidemiological studies have shown a positive correlation between consumption of fruits and vegetables, which are rich sources of antioxidants, and reduction of risk of some ROS-mediated diseases (Kris-Etherton *et al.*, 2002). The predominant ROS and reactive nitrogen intermediates generated from human physiological processes include hydrogen peroxide ($H_2O_2$), superoxide anion radical ($O_2 \cdot^-$), hydroxyl radical ($\cdot OH$), nitric oxide (NO) and peroxynitrite ($ONOO^-$). NO is a signalling molecule responsible for the regulation of physiological processes including vasodilation and neurotransmission (Pacher *et al.*, 2007). It is synthesized in various cell types from L-arginine and molecular oxygen by a group of enzymes known as nitric oxide synthases (NOS). In macrophages and neutrophils, NO is produced in large amounts by calcium/calmodulin (CaM)-independent inducible NOS (iNOS) in response to pro-inflammatory cytokines and endotoxins. In the cells, NO may react with $O_2 \cdot^-$ to form a more reactive DNA-damaging $ONOO^-$, which could also be converted to $\cdot OH$ (Pacher *et al.*, 2007). These highly reactive intermediates could trigger degenerative cellular processes including inflammation-related tissue damage such as arthritis (Ames *et al.*, 1993; Ames, 1983; Pacher *et al.*, 2007; Torre, Puliese, & Speranza, 2002; Liu, Gao, Wang, Jeohn, Cooper, &

Hong, 2002). In addition, overproduction of NO in macrophages has also been shown to play a crucial role in the pathogenesis of human immunodeficiency virus (HIV)-1 infection (Torre *et al.*, 2002). Therefore, suppression of mitogen-induced cellular nitric oxide production could constitute a step in treatment of inflammatory diseases (Liu *et al.*, 2002) and in suppressing virus-induced pathogenesis in HIV-1 infection (Torre *et al.*, 2002).

Semicarbazide-sensitive amine oxidase (SSAO, E.C.1.4.3.6.) is the name of a group of enzymes ubiquitously distributed in plants and animals. Amongst its several physiological roles, it catalyzes the oxidative deamination of various primary amines to produce their respective aldehydes, ammonia and $H_2O_2$ (Göktürk *et al.*, 2003; Stolen *et al.*, 2004a; Stolen, Yegutkin, Kurkijarvi, Bono, Alitalo, & Jalkanen, 2004b; Magyar, Mészáros, & Mátyus, 2001). Methylamine and aminoacetone are well-known endogenous substrates for SSAO; their deamination products are potential cytotoxic agents and precursors of advanced glycation end-products (AGEs) (Göktürk *et al.*, 2003; Stolen *et al.*, 2004a, 2004b). Plasma level of SSAO has been reported to be elevated in disease conditions such as diabetes mellitus, atherosclerosis, cerebral infarction, liver cirrhosis and congestive heart failure (Göktürk *et al.*, 2003; Stolen *et al.*, 2004a, 2004b; Magyar *et al.*, 2001; Boomsma, De Kam, Tjeerdsma, Van Den Meiracker, & Van Veldhuisen, 2000). The increased serum activity of SSAO has been associated with progression of vascular endothelial damages mediated by AGEs (Stolen *et al.*, 2004b). Based on these findings, it was proposed that inhibition of SSAO could reduce the damages observed in these disease conditions (Göktürk *et al.*, 2003; Magyar *et al.*,

2001); moreover, SSAO inhibitory activities have been reported for natural and synthetic compounds and biomaterials (Lin, Wang, Lu, Wu, & Hou, 2008; Liu, Wu, Liang, & Hou, 2007a).

Flaxseed (*Linum usitatissimum*) is an oilseed found in various parts of the world including Canada, producer of the largest amount of the world export market of flaxseed (Agriculture and Agri-Food Canada, 2007). It has been widely studied for its abundant $\alpha$-linolenic acid, dietary fibre and lignan constituents, which have been reported to possess potential to reduce the risk of cardiovascular disease in human (Dodin *et al.*, 2008). However, the protein components of flaxseed have not been optimally utilized especially in human nutrition. Flaxseed proteins contain high amounts of arginine, lysine and branch-chain amino acids (Oomah & Mazza, 1993; Hall III, Tulbek, & Xu, 2006). Due to their amino acid profiles, there are considerable interests in value-added use of flaxseed proteins isolated from defatted flaxseed meal. Thus, previous studies have reported that cationic peptides from Alcalase-catalyzed flaxseed protein hydrolysate (FPH) bound and inactivated CaM with concomitant inhibition of endothelial and neuronal NOS (Omoni & Aluko, 2006a, 2006b). Moreover, it has also been reported that Flavourzyme-catalyzed FPH inhibited angiotensin-converting enzyme activity, and showed antioxidant properties in scavenging $O_2^{\cdot-}$ (Marambe, Shand, & Wanasundara, 2008). The aim of this study was to determine the antioxidant and anti-inflammatory properties of low molecular weight (LMW) and cationic peptide fractions from flaxseed proteins that have been hydrolyzed with various food-grade and human gastrointestinal tract proteases. The inhibitory activity of the peptides against

lipopolysaccharide (LPS)-induced nitric oxide production in RAW 264.7 macrophages was used as a model to determine the potential anti-inflammatory properties of these flaxseed peptide fractions.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Materials

Defatted flaxseed meal was a gift from Bioriginal Foods and Science Corporation (Saskatoon, SK, Canada). Cellulase (*Aspergillus niger*), Alcalase (*Bacillus licheniformis*), thermolysin (*Bacillus thermoproteolyticus rokko*), ficin (fig tree latex), pepsin (porcine gastric mucosa), trypsin (bovine pancreas), papain (papaya latex), pancreatin (porcine pancreas), pyrogallol (1,2,3-trihydroxybenzene), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), benzylamine, bovine plasma, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrous sulphate, horseradish peroxidase (148 units/mg solid), lipopolysaccharide (LPS), sodium nitroprusside (SNP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), polymyxin B (PMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Other analytical grade reagents were obtained from Fischer Scientific (Oakville, ON, Canada).

### 4.2.2. Production of FPH and cationic peptide fractions

The protein components of the defatted flaxseed meal were isolated using a new method as previously reported (Udenigwe, Lin, Hou, & Aluko, 2009). Briefly, defatted

flaxseed meal (5%, w/v, dry weight basis) was suspended in deionized water and stirred thoroughly using a magnetic stirrer. The highly viscous slurry was adjusted to pH 5.0 and a temperature of 37°C followed by addition of cellulase (1%, w/w; activity of powder, 1.44 U/mg) to initiate fibre hydrolysis. After 4 h of reaction, the resulting suspension was cooled to 4°C followed by alkaline solubilisation (pH 10.0) with 0.5 M NaOH and acid-induced protein precipitation at pH 4.2 with 0.5 M HCl following the method of Dev and Quensel (1988). The resulting protein precipitate was washed thrice with acidified water (pH 4.2), suspended in a small volume of deionized water and the pH adjusted to 7.0 using 0.5 M NaOH. The suspension was freeze-dried and stored at −20°C.

Hydrolysis of the protein fractions of flaxseed meal was conducted under different conditions using a pH-stat instrument (Metrohm AG, Herisau, Switzerland) as reported by Udenigwe *et al.* (in press). Flaxseed protein isolate (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to 37°C and adjusted to the appropriate pH prior to the addition of one of the following enzymes; pepsin (pH 2.0–2.2), ficin (pH 7.0), trypsin (pH 8.0), papain (pH 6.5) or thermolysin (pH 8.0). The pancreatin reaction was conducted at temperature of 40°C and pH 8.0. Each enzyme was added to the suspension at an enzyme-substrate ratio (E/S) of 1:100 (protein basis). Digestion was performed at the above conditions for 4 h; pH of the reaction mixture was kept constant by the pH-stat with 2 M NaOH except for the pepsin reaction. The Alcalase hydrolysis was carried out as previously reported (Omoni & Aluko, 2006a, 2006b). After digestion, the enzyme was inactivated by immersing the reaction vessel in boiling water bath (95-100°C) for 15 min and

undigested proteins were precipitated by adjusting the pH to 4.0 with 2 M HCl followed by centrifugation at 14,941 × $g$ for 30 min. The supernatant containing the peptides was collected for further fractionation. Protein content of samples was determined by a modified Lowry's method (Markwell, Haas, Biebar, & Tolbert, 1978). The supernatant resulting from protein hydrolysis was passed through an Amicon stirred ultrafiltration cell set-up using a 1 kDa molecular weight cut-off (MWCO) membrane, and resulting permeate was collected. The FPH resulting from Alcalase digestion was passed through ultrafiltration membranes with MWCO of 5, 3 and 1 kDa to separate peptides of sizes 3-5, 1-3 and <1 kDa. To remove residual salts, the resulting permeates were dialyzed for 48 h against deionized water at 4°C using a 100 Da MWCO dialysis membrane and the retentates freeze-dried and stored at –20°C until needed.

The cationic peptides in the <1 kDa permeate from the Alcalase FPH were separated as previously reported (Omoni & Aluko, 2006a) with some modifications. A sample solution (4 ml of 250 mg/ml) of the freeze-dried hydrolysate was loaded onto an SP-Sepharose High Performance XK 50/20 cation exchange chromatography column connected to an AKTA Fast Protein Liquid Chromatography system (Amersham-GE Biosciences, Montreal, Canada). The column was pre-equilibrated with 1.5 column volume (CV) of 0.1 M ammonium acetate buffer, pH 7.5. After sample loading, the column was washed with 1.5 CV of 0.1 M ammonium acetate buffer to remove unbound peptides, followed by a gradient elution of the bound peptides using 0–50% 0.5 M ammonium carbonate, pH 8.8, in 0.1 M ammonium acetate buffer at a flow rate of 10 ml/min. The elution of peptides was monitored at 214 nm. Two major peaks (FI and FII)

were observed and fractions within these peaks were pooled and concentrated by vacuum evaporation at 37°C. Thereafter, the concentrated peptide solution was dialyzed for 48 h against deionized water at 4°C. The content of the dialysis bag was freeze-dried and stored at −20°C until needed.

### 4.2.3. *In vitro* antioxidant assays

### 4.2.3.1. DPPH$^{\cdot}$ scavenging assay

The scavenging activity of flaxseed peptide fractions against DPPH$^{\cdot}$ was measured according to a previous method (Hou, Chen, Chen, Lin, Yang, & Lee, 2001), which was slightly modified. Briefly, 160 μl of 100 μM DPPH$^{\cdot}$ in methanol was mixed with 40 μl of flaxseed peptide fraction in a 96-well microplate for 20 min at room temperature under light protection. Thereafter, absorbance of the mixture was measured at 517 nm (*As*). Distilled water was used instead of the peptide fractions in blank experiments (*Ac*) whereas reduced glutathione (GSH) and BSA were used as positive and negative controls, respectively. The scavenging activity of peptide fractions was calculated using Eq. (4.1).

$$\text{Activity} = \left[ \frac{Ac - As}{Ac} \right] \times 100\% \tag{4.1}$$

Concentration-dependence of the scavenging properties of the active peptide fractions against DPPH$^{\cdot}$ was also determined at four concentrations. The concentration of the samples that resulted in scavenging of 50% of DPPH$^{\cdot}$ was calculated by non-linear regression and expressed as half maximal effective concentration (EC$_{50}$). The effect of

buffers and pH on the scavenging activities of the peptide fractions against DPPH⋅ was also investigated. The peptide fraction (60 μl at final concentration near the $EC_{50}$) was mixed with 40 μl of 0.1 M acetate buffers (pH 4.0, 4.5, 5.0, 5.5, 6.0), 0.1 M phosphate buffers (pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) and 0.1 M Tris-HCl buffers (pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0) in a 96-well microplate. DPPH⋅ scavenging assay was performed as described above. The buffer solutions of different pH values were used instead of peptide solutions for control experiments. All assays were performed in triplicate.

### 4.2.3.2. Superoxide radical ($O_2^{\cdot-}$) scavenging assay

In this assay, $O_2^{\cdot-}$ was generated from autoxidation reaction of pyrogallol (Marklund & Marklund, 1974; Gao, Yuan, Zhao, & Gao, 1998). Eighty microliters of flaxseed peptide fraction of various concentrations was mixed with 80 μl of 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA in a 96-well microplate followed by the addition of 40 μl of 1.5 mM pyrogallol in 10 mM HCl. The rate of $O_2^{\cdot-}$-induced polymerization of pyrogallol ($\Delta A$/min $s$) was measured as increase in absorbance at 420 nm for 4 min at room temperature. Tris-HCl buffer was used instead of peptide fraction in blank experiment ($\Delta A$/min $c$) whereas GSH was used as positive control. All assays were performed in triplicate, and the scavenging activity of peptide fractions was calculated using Eq. (4.2).

$$Activity = \left[ \frac{\Delta A/\min c - \Delta A/\min s}{\Delta A/\min c} \right] \times 100\% \qquad (4.2)$$

### 4.2.3.3. Hydroxyl radical ($^{\cdot}$OH) scavenging assay

Flaxseed peptide fractions were assayed for scavenging properties against $^{\cdot}$OH generated from Fenton's reaction using electron spin resonance (ESR) spectroscopy (Lin *et al.*, 2008). Briefly, the reaction mixture (0.5 ml) contained 5 mM DMPO, 0.05 mM FeSO$_4$, 0.25 mM H$_2$O$_2$ and various concentrations of flaxseed peptide fractions. This solution was mixed and transferred to an ESR quartz cell. The cell was placed in the cavity of the ESR spectrometer and the relative intensity of the DMPO-OH spin adduct signal (*INTs*) was measured. Deionized water was used instead of sample solution for blank experiments (*INTc*). All ESR spectra were recorded at the ambient temperature (300 K) on a Bruker EMX-6/1 EPR spectrometer under the following conditions: center field 345.4 ± 5.0 mT; microwave power 8 mW (9.416 GHz); modulation amplitude 5 G; modulation frequency 100 kHz; time constant 0.65 s; scan time 1.5 min. Scavenging activity of peptide fractions against $^{\cdot}$OH was calculated as percentage using Eq. (4.3).

$$\text{Activity} = \left[ \frac{INTc - INTs}{INTc} \right] \times 100\% \qquad (4.3)$$

### 4.2.3.4. Nitric oxide (NO) scavenging assay

NO was generated from SNP and measured as nitrite by the Griess reaction (Fiorentino *et al.*, 2008). The assay mixture contained 5 mM SNP and 0.2 mg/ml flaxseed peptide fractions in 0.4 ml of 0.1 M phosphate buffer (pH 7.4). The assay mixture was incubated at 37°C for 2 h. Thereafter, 0.1 ml of the reaction mixture was withdrawn and

added onto a 96-well microplate followed by the addition of Griess reagent (50 µl of

0.1% N-1-napthylethylenediamine dihydrochloride in water, and 50 µl of 1%

sulphanilamide in 5% phosphoric acid). The mixture was kept in the dark for 10 min at

room temperature followed by measurement of the absorbance at 530 nm ($A_{530nm}$).

Phosphate buffer was used instead of the peptide samples as blank. The scavenging

activity of the samples against NO was expressed as percentage relative to $A_{530nm}$ of the

blank experiment. All assays were performed in triplicate.

## 4.2.4. Determination of SSAO inhibition

SSAO inhibition assay was determined based on previous methods (Szutowicz,

Kobes, & Orsulak, 1984; Lin *et al.*, 2008) that were modified as follows. Bovine plasma

(P-4639 reconstituted in 10 ml of deionized water) was used as a source of SSAO. The

reaction mixture (200 µl) contained the following: 50 µl of 8 mM benzylamine, 50 µl of

200 mM phosphate buffer (pH 7.4), SSAO (2.53 units) and flaxseed peptide sample. The

reaction mixture was incubated at 37°C for 1 h followed by heating at 100°C for 5 min to

inactivate the enzyme and terminate the reaction. After cooling to room temperature

and a brief centrifugation, 40 µl of the reaction mixture was withdrawn and added to a

160 µl solution containing 50 µl of 200 mM phosphate buffer (pH 7.4), 25 µl of 1 mM

ABTS solution, 50 µl of horseradish peroxidase (5 µg/ml) and 35 µl of deionized water in

a 96-well microplate. The rate of release of the reaction product was recorded as

change in absorbance at 420 nm for 1 min ($\Delta A$/min *s*). Deionized water was used instead

of peptide sample solutions as blank ($\Delta A$/min *c*). The percent (%) inhibition of SSAO

activity was calculated using Eq. (2), and the concentration of the samples that resulted in inhibition of 50% of the activity of SSAO expressed as half maximal inhibitory concentration ($IC_{50}$). All assays were performed in triplicate.

### 4.2.5. LPS-induced NO production in RAW 264.7 macrophages

### 4.2.5.1. Cell culture and cell treatment

RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM (GibcoBRL, USA) supplemented with 10% fetal calf serum. The cells were seeded onto a 24-well culture plate at a density of 5 × $10^5$ cells/ml and incubated at 37°C in 5% $CO_2$ for 6 h prior to cell treatment. Thereafter, the cells were treated with only LPS, or LPS and PMB, or LPS and flaxseed peptides, in triplicate, and incubated at 37°C for 24 h.

### 4.2.5.2. Nitrite quantification and cell viability assay

Nitrite formation was used to quantify NO production in the cell cultures. After 24 h incubation, 50 µl were withdrawn from each of the cell cultures and added onto a 96-well microplate followed by measurement of nitrite with Griess reagent as earlier described. Nitrite standard curve was prepared using 0–200 µM sodium nitrite. The % inhibition of NO production was calculated using the LPS-only treatment as 100%. The concentration of active flaxseed peptide fractions that suppressed 50% of NO production in the macrophages was calculated relative to control (no LPS) treatment and expressed as $IC_{50}$. Following the 24 h incubation, viability of the cells in all

treatments was determined by MTT staining. The results were expressed as relative cell viability (%) using the blank (no LPS) treatment as reference (100%).

### 4.2.6. Statistical analysis

All data are reported as mean ± standard deviation of three separate determinations. Statistical significance of differences was evaluated by Student's t-test and Duncan's multiple range test ($p < 0.05$) using the Statistical Analysis Systems software (Statistical Analysis System, Cary, NC, USA).

### 4.3. RESULTS AND DISCUSSION

### 4.3.1. Peptide production and isolation

Despite their nutritionally rich amino acid profile, there is no report of large scale production of isolated flaxseed proteins for human consumption. This could be attributed to interferences of the polysaccharide gums of seed hulls during flaxseed protein isolation, which results in low protein yields (Oomah & Mazza, 1993). Several authors have attempted to improve the yield of isolated flaxseed protein using various methods including removal of the mucilage by de-hulling the flaxseed prior to protein isolation. Recently, Marambe *et al*. (2008) reported that laboratory de-oiling and subsequent removal of flaxseed mucilage using salt solution at 50°C resulted in an isolated flaxseed protein with 80% protein content (N% × 6.25). However, the flaxseed meal utilized in our study was crushed and defatted for industrial applications without removal of the seed hull, and mucilage removal from the meal using dilute salt solution

will solubilize its protein constituents. Thus, we developed a method to optimize the isolated protein yield from flaxseed meal by enzymatic removal of the seed mucilage using cellulase prior to protein isolation by alkaline solubilisation and acid-induced protein precipitation; this method provided an isolated flaxseed protein with 78.9% protein based on the modified Lowry protein assay.

The isolated flaxseed proteins were hydrolyzed using seven proteases to generate potential bioactive peptides followed by fractionation into <1 kDa fractions using membrane ultrafiltration. The Alcalase hydrolysate was also fractionated into three fractions of molecular weight ranges 3-5, 1-3 and <1 kDa. The <1 kDa fraction of Alcalase FPH was further separated by fast protein liquid chromatography on a strong cationic exchange column, and this resulted in isolation of two cationic peptide fractions, FI and FII, with the latter possessing stronger cationic character as previously reported (Omoni & Aluko, 2006a). These peptide fractions were evaluated for antioxidant and potential anti-inflammatory properties. Since antioxidant activity may depend on factors such as the type of radical species involved in the reaction and the functionality of the antioxidant, it is appropriate to evaluate the antioxidant property of a sample using various assays. In this study, the potential antioxidant properties of flaxseed protein-derived peptide fractions were evaluated using free radical-scavenging assays against $DPPH^{\cdot}$, $O_2^{\cdot-}$, $^{\cdot}OH$ and NO, as well as inhibition of SSAO activity.

### 4.3.2. Peptide-induced scavenging of free radicals

DPPH· is a stable N-containing free radical widely used in the primary assessment of antioxidant capacity of food. As shown in Fig. 4.1A, the flaxseed protein-derived peptides possess concentration-dependent scavenging activities against DPPH·; $EC_{50}$ of the active fractions are shown in Table 1. The 1-3 kDa fraction of Alcalase FPH had the most potent activity better than those of the 3-5 kDa fraction and GSH. The papain-hydrolyzed FPH fraction scavenged similar amount of DPPH· as GSH at twice the sample amount. The other peptide fractions and BSA did not scavenge DPPH·. Previous studies have reported the DPPH·-scavenging activities for various enzymatic food protein hydrolysates (Sakanaka & Tachibana, 2006; Wang, Zhao, Zhao, & Jiang, 2007; Li, Jiang, Zhang, Mu, & Liu, 2008a; Cumby, Zhong, Naczk, & Shahidi, 2008; Xie, Huang, Xu, & Jin, 2008; Li, Han, & Chen, 2008b). In this study, it could be observed that the release of DPPH·-scavenging peptides from flaxseed proteins depends in part on the specificity of the proteases used in hydrolysis. Moreover, it was also observed that the high molecular weight (HMW) peptide fractions had better activity in reducing DPPH·. Similarly, other studies have reported that the DPPH scavenging activities of food protein hydrolysates may depend on the size of their constituent peptides (Wang *et al.*, 2007; Li *et al.*, 2008a; Li *et al.*, 2008b; Wu *et al.*, 2003). Since DPPH·-scavenging reaction is a single electron transfer (SET) reaction (Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005), results from this study suggest that the HMW peptide fractions contained more amino acid groups that could readily donate electrons to DPPH· when compared to the smaller peptides.

Furthermore, the DPPH˙ scavenging properties of flaxseed peptide fractions were observed to be dependent on both pH and buffer (Fig. 4.1B). These results show that peptide fractions exhibited better radical scavenging activity in acetate buffer than in phosphate and Tris-HCl buffers; similar DPPH˙-scavenging activity patterns were observed for all peptide samples in the different buffers except for the activity of LMW peptides from papain FPH at pH 5.5 and 6.0 in acetate buffer. Using Tris-HCl buffer, it was observed that the peptide fractions exhibited their best activity at pH 7.0, and this was lost with increase in pH even at 8.0, which is similar to the condition presently used in most DPPH˙ scavenging assays. Furthermore, it was observed that phosphate buffer is not suitable for this assay since the peptide fractions lost up to 35% of their DPPH˙ scavenging properties; the most potent DPPH˙ scavenger in this study showed almost no activity in phosphate buffer at pH 8.0. These results are in agreement with previously reported evidence that pH affects the DPPH˙-scavenging properties of antioxidants (Liu *et al.*, 2007a; Lin *et al.*, 2008). In the SET DPPH˙-scavenging reaction, as suggested by Lin *et al.* (2008) for geraniin, pH conditions might affect ionization potential and electron transfer capacity of peptides, which could explain the different activities observed under different pH. However, due to the stability of DPPH˙, some potential antioxidants may not show scavenging activity within the assay duration (Huang *et al.*, 2005). Moreover, DPPH˙ is not functionally similar to highly reactive peroxyl radicals that cause oxidation of biological macromolecules (Huang *et al.*, 2005; Prior *et al.*, 2005), which raises questions about the physiological relevance of DPPH˙-scavenging antioxidants. Based on

**Figure 4.1 (A) DPPH· scavenging activity of glutathione (GSH), 1-3 kDa (1-3AlcH) and 3-5 kDa Alcalase FPH (3-5AlcH), and <1 kDa papain FPH (PapH). (B) The effects of pH and buffers on the DPPH· scavenging activity of the peptide fractions at concentration near their $EC_{50}$ values; pH 4.0–6.0, 0.1 M acetate buffer; pH 5.5–8.0, 0.1 M phosphate buffer; pH 7.0–10.0, 0.1 M Tris-HCl buffer; Each point represents an average of three determinations**
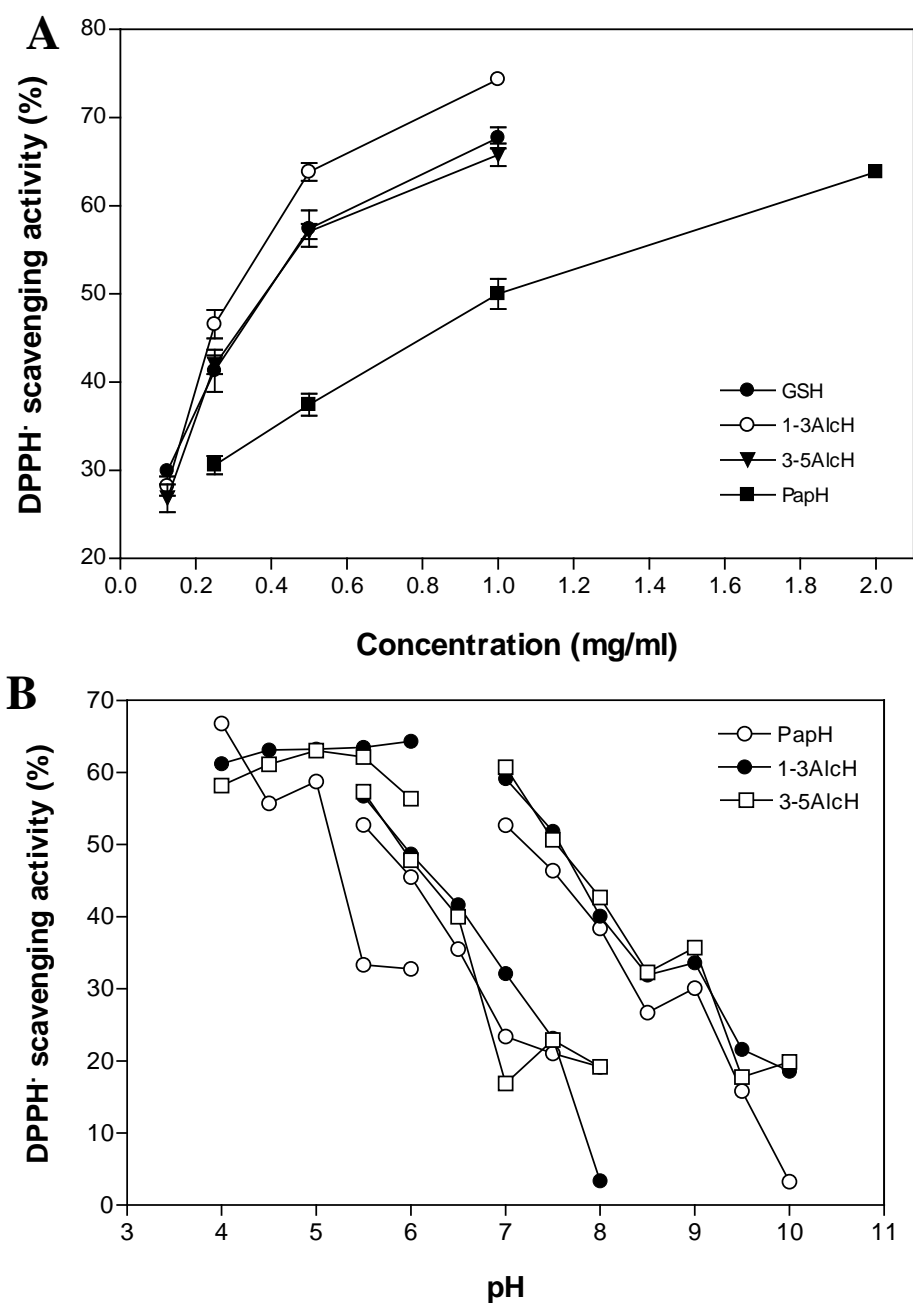
these and other limitations, highly reactive and physiologically-relevant free radicals were used to evaluate antioxidant properties of the flaxseed peptide fractions.

In alkaline solution, $O_2^{\cdot-}$ induces pyrogallol autoxidation leading to the formation of conjugation products that can be detected at 420 nm; reducing compounds inhibit this reaction by acting as $O_2^{\cdot-}$ scavengers; EDTA is used as metal chelator to eliminate the effects of interfering metal ions (Marklund & Marklund, 1974; Gao *et al.*, 1998). The $O_2^{\cdot-}$ generated from autoxidation of pyrogallol was used to evaluate the antioxidant property of the flaxseed peptides, which showed concentration-dependent activity (Fig. 4.2A). As observed in the DPPH$^{\cdot}$ assay, activity of the peptide fractions in scavenging $O_2^{\cdot-}$ differed depending on the protease used in protein hydrolysis and MW ranges of the fractions; the higher MW peptide fractions showed stronger scavenging activity with lower $IC_{50}$ (Table 4.1). Hydrolysis with ficin and papain yielded <1 kDa peptides with moderate and similar $O_2^{\cdot-}$-scavenging activities. Moreover, purification of the <1 kDa fraction of Alcalase FPH using a cation-exchange column yielded cationic peptide fraction II, which also scavenged $O_2^{\cdot-}$. The $O_2^{\cdot-}$-scavenging activities of these peptide fractions were observed to be weaker than the activity of GSH, which completely scavenged $O_2^{\cdot-}$ at 0.5 mg/ml. In comparison, the $O_2^{\cdot-}$-scavenging of Alcalase alfalfa leaf protein hydrolysate (0–0.9 mg/ml) was reported to range from 0–67% (Xie *et al.*, 2008) and that of Alcalase chickpea hydrolysate fractions (2.0 mg/ml) from 35–69% (Li *et al.*, 2008a). In addition, egg yolk proteins hydrolyzed with proteinase from *Bacillus* sp.

**Figure 4.2 (A) Flaxseed peptide fractions and glutathione (GSH) with dose-dependent O$_2$$^{\cdot-}$ scavenging activities; see Table 1 for abbreviations. (B) The scavenging activities of peptide fractions against $^{\cdot}$OH measured by electron-spin resonance spectroscopy**
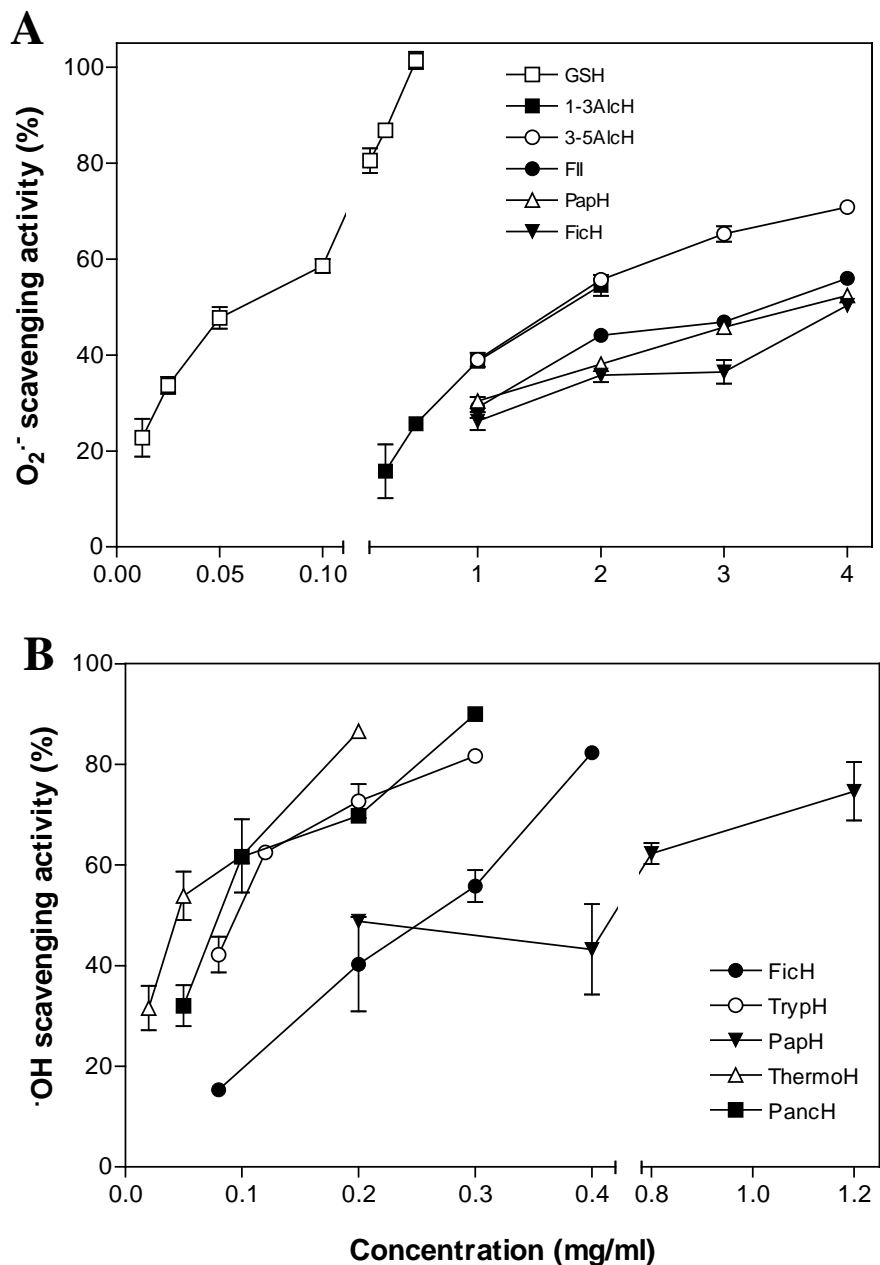
**Table 4.1** EC$_{50}$ (mg protein/ml) values of flaxseed-protein derived peptide fractions in the scavenging of DPPH$^{\cdot}$, O$_2^{\cdot-}$ and $^{\cdot}$OH, and IC$_{50}$ (mg protein/ml) for *in vitro* inhibition of SSAO; results are expressed as mean ± standard deviation

| Sample | Radical Scavenging Activity (EC$_{50}$) | | | SSAO Inhibition (IC$_{50}$) |
|---|---|---|---|---|
| | DPPH$^{\cdot}$ | O$_2^{\cdot-}$ | $^{\cdot}$OH | |
| Glutathione | 0.385 ± 0.022 | 0.059 ± 0.009 | 0.0341 ± 0.0030[d] | – |
| FI | ND[a] | ND | <0.0800 | >0.50 |
| FII | ND | 3.558 ± 0.117 | ND | 0.1321 ± 0.0218 |
| 1-3AlcH[b] | 0.298 ± 0.019 | 1.719 ± 0.114 | ND | 0.0064 ± 0.0003 |
| 3-5AlcH[b] | 0.382 ± 0.012 | 1.660 ± 0.060 | ND | 0.0069 ± 0.0006 |
| PepH[c] | ND | ND | ND | >0.40 |
| FicH[c] | ND | 3.976 ± 0.017 | 0.2566 ± 0.0146 | 0.0926 ± 0.0052 |
| TrypH[c] | ND | ND | 0.09507 ± 0.0052 | 0.0269 ± 0.0019 |
| PapH[c] | 1.02 ± 0.083 | 3.944 ± 0.049 | 0.5053 ± 0.1272 | 0.1537 ± 0.0083 |
| ThermoH[c] | ND | ND | 0.0457 ± 0.0047 | 0.0377 ± 0.0045 |
| PancH[c] | ND | ND | 0.0682 ± 0.0048 | 0.1277 ± 0.0076 |

[a]ND, not determined because of low or nil activity

[b]Ultrafiltration permeate from Alcalase FPH: 1-3 kDa, 1-3AlcH; 3-5 kDa, 3-5AlcH

[c]Ultrafiltration permeate (<1 kDa) from FPH produced with pepsin, PepH; ficin, FicH; trypsin, TrypH; papain, PapH; thermolysin, ThermoH; pancreatin, PancH

[d]Concentration-dependence was lost after 0.04 mg/ml glutathione (GSH)

scavenged about 5–90% $O_2^{\cdot-}$ at 0.0313–0.5% protein (Sakanaka & Tachibana, 2006). In this study, the <1 kDa permeate of trypsin and thermolysin FPH as well as Alcalase cationic peptide fraction I showed meagre antioxidant properties by scavenging only 12.6, 17.5 and 19.4% $O_2^{\cdot-}$, respectively at 2 mg protein/ml whereas both the pepsin and pancreatin treatments yielded LMW peptides that could not scavenge $O_2^{\cdot-}$.

The $^{\cdot}$OH-scavenging property of the flaxseed peptides was evaluated using an assay similar to the physiological system whereby $^{\cdot}$OH was generated by reaction of $Fe^{2+}$ and $H_2O_2$ (Fenton reaction). The $^{\cdot}$OH produced in the reaction was spin-trapped with DMPO, and the intensity of the DMPO-OH adducts measured using ESR spectrometer. A decrease in intensity or disappearance of the DMPO-OH signal is an indication of the $^{\cdot}$OH scavenging activity of antioxidants (Li *et al.*, 2004). It was observed in this study that most of the FPH fractions were capable of reducing the intensity of DMPO-OH signals at various protein concentrations. Fig. 4.2B shows that there was a linear relationship between $^{\cdot}$OH-scavenging activity and the amount of peptide samples in the assay. Unlike the antioxidant properties observed in the DPPH$^{\cdot}$ and $O_2^{\cdot-}$ assays, the LMW flaxseed peptides showed potent $^{\cdot}$OH-scavenging activities except for the pepsin hydrolysate (Table 4.1). The thermolysin, pancreatin and ficin hydrolysates scavenged 86.5, 89.9 and 82.3% $^{\cdot}$OH, respectively at 0.2, 0.3 and 0.4 mg/ml, respectively. The least cationic peptide fraction from Alcalase hydrolysate (FI) also displayed potent $^{\cdot}$OH-scavenging activity with inhibition of 55.6, 76.7 and 81% $^{\cdot}$OH at 0.08, 0.12 and 0.2 mg/ml, whereas the stronger cationic peptides (FII) did not. This observation is inversely correlated with their observed activity in the $O_2^{\cdot-}$-scavenging assay. Moreover, the $^{\cdot}$OH-

scavenging properties of these flaxseed peptide fractions reported in this paper are better than the recently reported activity of crude hydrolysates from Flavourzyme digestion of flaxseed proteins ($IC_{50}$, 1.56–3.06 mg/ml) (Marambe *et al.*, 2008). Previous studies have also reported that, using different spectrophotometric assay systems, the peptide fractions from Alcalase chickpea protein hydrolysate (1.5 mg/ml) scavenged 38% to 81% ˙OH (Li *et al.*, 2008a) whereas treatment of egg yolk protein with *Bacillus* sp proteinase yielded hydrolysates that scavenged 74% of ˙OH at 0.5 mg/ml (Sakanaka & Tachibana, 2006). In addition, a recent study reported that whey protein hydrolysate and its peptide fractions showed moderate antioxidant properties against ˙OH and other free radicals when measured by ESR spectroscopy (Peng, Xiong, & Kong, 2009) similar to the method used in this present study. Contrary to the results in the DPPH˙ and $O_2^{˙-}$ assays, the HMW flaxseed peptide fractions did not show ˙OH-scavenging activity. It should be noted that this assay method has a limitation as it is almost impossible to determine whether the activity of antioxidants is due to scavenging of ˙OH or chelation of $Fe^{2+}$, since most potential antioxidants are also good metal chelators that might decrease ˙OH production (Huang *et al.*, 2005), thus reducing the intensity of the DMPO-OH adduct. However, Peng *et al.* (2009) recently reported that the ˙OH-scavenging activity observed in their study for whey protein hydrolysate fractions could be attributed to the hydrolysates, which they reported to possess poor $Fe^{2+}$ chelating activities.

### 4.3.3. Peptide-induced inhibition of SSAO

The *in vitro* SSAO inhibitory activities of the flaxseed protein-derived peptide fractions are shown in Fig. 4.3; all the peptide samples evaluated in this study exhibited concentration-dependent inhibition of SSAO activity which was also dependent on size of the peptides. The HMW peptides from Alcalase hydrolysate potently inhibited SSAO activity at low concentrations. Moreover, the LMW peptide fractions as well as Alcalase cationic peptide FII also inhibited SSAO activity. The weakest inhibitory activity was observed for the pepsin hydrolysate LWM fraction and the Alcalase cationic peptide FI. The $IC_{50}$ values for the peptide fractions in SSAO inhibition are shown in Table 4.1. To the best of our knowledge, this work is the first to report the activity of enzymatic food protein hydrolysates in inhibition of SSAO activity. Since physiological SSAO reaction generates $H_2O_2$ and reactive aldehydes that play important roles in pathogenesis of disease conditions, its inhibition by these flaxseed peptide fractions may constitute a step in treatment of inflammation and other related diseases.

**Figure 4.3 Inhibition of semicarbazie-sensitive amine oxidase (SSAO) by flaxseed peptide fractions; inhibitory activity of peptides was dependent on size and nature of the peptides; thus, the higher MW peptides showed the best activity**

**4.3.4. Peptide-induced inhibition of *in vitro* and *ex vivo* NO production**

The cellular toxicity of NO has been associated with its reaction derivatives especially $ONOO^-$, which could lead to DNA fragmentation and protein structure modification. Thus, NO scavengers could lower the risk of cellular and tissue damages associated with excessive NO production. Fig. 4.4 shows the *in vitro* scavenging activities of the flaxseed peptide fractions against NO; at 0.2 mg/ml, the peptide fractions showed weak NO scavenging properties. Both the thermolysin and pancreatin FPH fractions gave the strongest activity by scavenging 27.1% and 35.1% NO, respectively. The other peptide fractions scavenged ≤20.3% NO at the same concentration. Moreover, in order to evaluate their anti-inflammatory properties, the flaxseed peptide samples were screened for effects on LPS-induced NO production in macrophages. The treatment of RAW 264.7 macrophages with LPS resulted in increased NO production in the cell culture (Fig. 4.5). The addition of PMB to the cell culture prior to LPS activation resulted in normalized NO production, since PMB binds and inactivates LPS. On treatment of the macrophage cultures with LPS and the various flaxseed peptides, the pepsin, ficin and papain FPH fractions were observed to exhibit concentration-dependent inhibition of LPS-induced NO production in the macrophages with $IC_{50}$ values of 0.250, 0.504 and 0.215 mg protein/ml, respectively (Fig. 4.5A–C). At 1 mg protein/ml, the pepsin and papain FPH fractions completely inhibited the endotoxin-induced NO production to the basal level observed for control and PMB treatments. Moreover, these flaxseed peptide fractions did not show cytotoxicity against the cells within the range of concentrations studied using the MTT staining assay (Fig. 4.5D). It is noteworthy that, despite showing

**Figure 4.4 *In vitro* scavenging activities of 0.2 mg/ml flaxseed protein-derived peptide**

**fractions against nitric oxide (NO) produced from sodium nitroprusside**
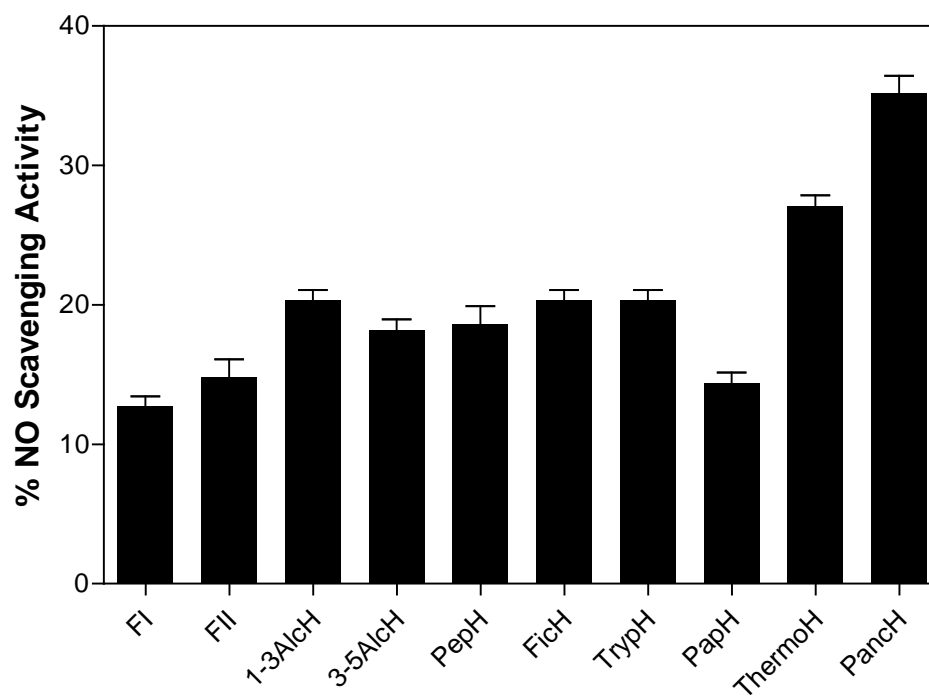
**Figure 4.5 Effects of flaxseed protein-derived (A) PepH, (B) FicH and (C) PapH on LPS-induced nitric oxide (NO) production in RAW 264.7 cells; the cells were treated as follows: no LPS (control); LPS (100 ng/ml) only; LPS (100 ng/ml) plus PMB (50 µg/ml); and LPS (100 ng/ml) plus 0.2–1.0 mg protein/ml flaxseed peptide fraction. PMB was used as positive control. The treated cells were incubated at 37°C and 5% $CO_2$ for 24 h followed by determination of cellular production of NO and cell viability. Each bar represents mean of triplicate determinations; bars with the same letter within each graph are not significantly different at $p$ = 0.05 (D) Viability of RAW 264.7 cells in the presence of the peptide fractions, which showed no toxicity against the growth of the macrophages**

**C**

(Nitrite ($\mu$M) vs. Control, LPS, LPS+PMB, and PapH (mg/ml) + LPS (100 ng/ml) at 0.2, 0.4, 0.6, 0.8, 1.0)

**D**

(Relative cell viability (%) for PepH (mg/ml), FicH (mg/ml), and PapH (mg/ml) at 0.2, 0.4, 0.6, 0.8, 1.0)
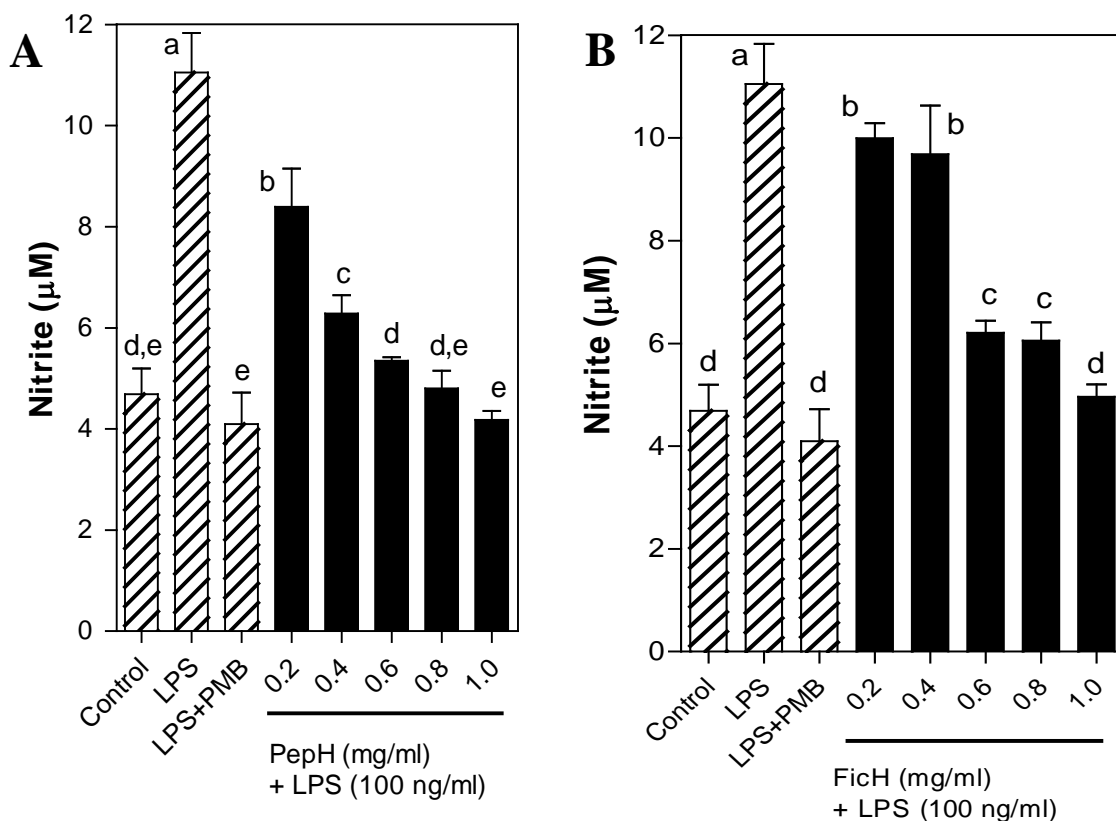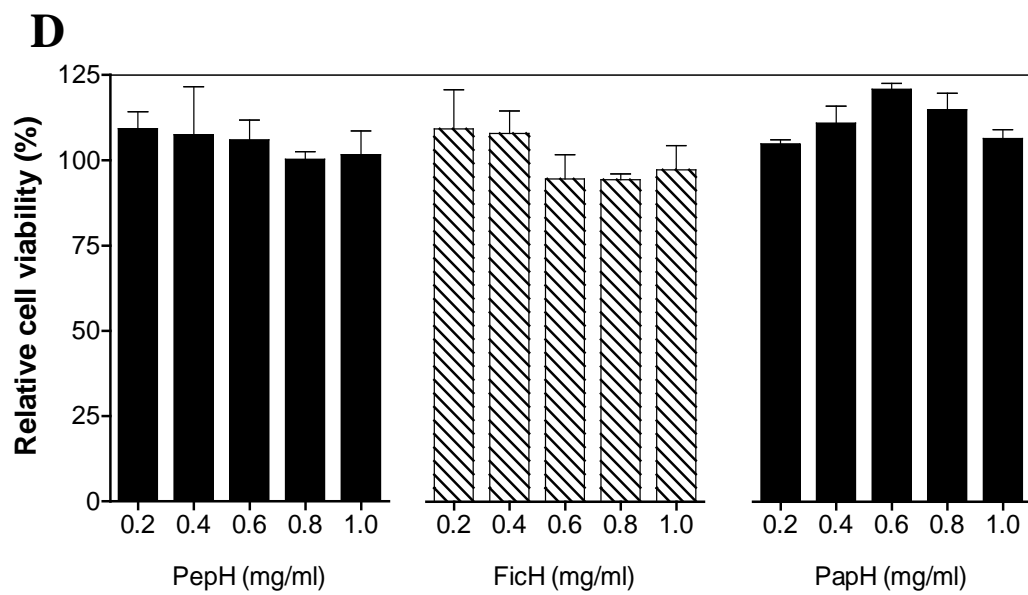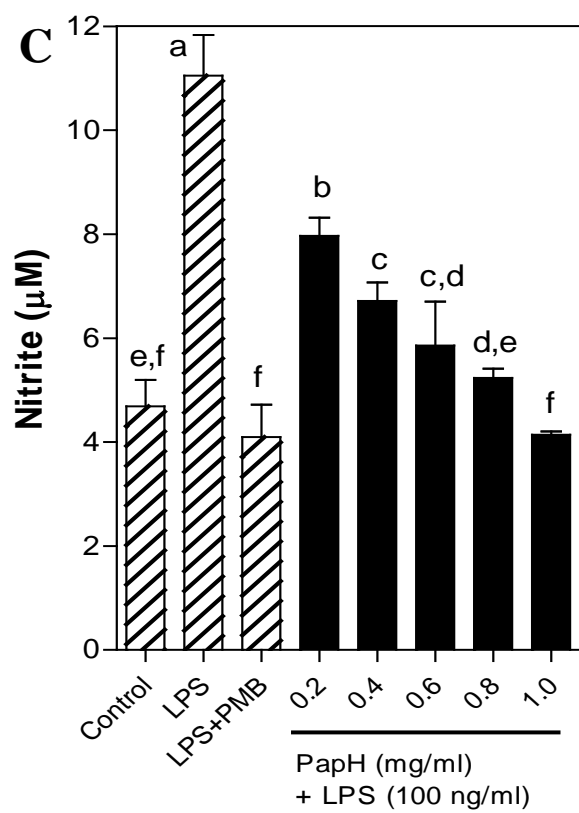
the best *in vitro* NO scavenging activities (Fig. 4.4), the thermolysin and pancreatin FPH fractions did not affect concentrations of NO in the cell cultures when compared to the LPS-only treatment (data not shown)[2]. Thus, the ability of the flaxseed peptides to scavenge NO *in vitro* is not correlated with their inhibitory activities against NO production or their potential NO scavenging properties in the LPS-treated culture. It is possible that the bioactive peptides present in thermolysin and pancreatin FPH were metabolized to inactive fragments by the macrophages. It could also be suggested that the active flaxseed peptide fractions may have altered the macrophage NO synthesis pathway. It has been shown that the activity of potential therapeutic agents in inhibition of NO production could be through inhibition of transcription factor NF-κB activation and subsequent inhibition of iNOS mRNA and protein expressions in macrophages (Chen, Shen, Lee, Hou, Yang, & Lee, 2001; Ho & Lin, 2008; Pan *et al*., 2008). To the best of our knowledge, there is limited information in the literature regarding the effects of enzymatic food protein hydrolysates on endotoxin-induced NO production in cell cultures. A previous study reported that a hydrolyzed casein diet fed to young diabetes-prone BB rats led to restoration of NO production to basal level in interleukin-1β-induced overproduction of NO in the pancreatic islets (Malaisse, Olivares, Laghmich, Ladrière, Sener, & Scott, 2000). Recently, lunasin, a naturally occurring oligopeptide from soybean was reported to possess potential *in vitro* anti-inflammatory activity due to its inhibitory effects on the production of NO and prostaglandin $E_2$ through the inhibition of iNOS and cyclooxygenase-2 protein expressions, respectively, in LPS-

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[2] See Figures S4.2 in Appendix B for data

activated RAW 264.7 macrophages (Dia, Wang, Oh, de Lumen, & Gonzalez de Mejia, 2009). However, a yam storage protein, dioscorin, had been shown to exhibit immunomodulatory effects partly by inducing NO productions in macrophages in the absence of LPS (Liu, Shang, Wang, Hsu, & Hou, 2007). Moreover, it was also observed in this present study that the flaxseed protein-derived peptide fractions did not induce excessive production of NO in the macrophages when LPS was eliminated with PMB (data not shown). Furthermore, all the flaxseed protein-derived peptide fractions in this study were also evaluated for cytotoxicity against two cancer cell lines, HL-60 (human promyelocytic leukemia, ATCC CCL-240) and MCF7 (human breast adenocarcinoma, ATCC HTB-22) using the MTT staining assay. The flaxseed peptide fractions did not show cytotoxicity against these cancer cell lines at 0.2 mg protein/ml after 24 h of incubation (data not shown)[3].

## 4.4. CONCLUSIONS

Enzymatic hydrolysis of flaxseed proteins by various proteases resulted in the release of bioactive peptides with antioxidant and potential anti-inflammatory properties. Bioactive properties of the flaxseed peptide fractions were dependent on catalytic specificity of the proteases as well as the size of peptides in the resulting protein hydrolysate fractions. Papain treatment released multifunctional flaxseed peptides with MW less than 1 kDa that exhibited comparatively moderate biological activities in all the *in vitro* antioxidant assays evaluated in this study. On the other hand,

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[3] See Figures S4.4 and S4.5 in Appendix B for data

pepsin-catalyzed hydrolysis yielded poor free radical-scavenging <1 kDa flaxseed peptides but with excellent restoration of LPS-induced NO production to basal level in macrophages. The other <1 kDa flaxseed peptide fractions showed potent or moderate activities in scavenging free radicals; however, the <1 kDa cationic flaxseed peptide fractions did not exhibit good antioxidant property except the least cationic fraction (FI) that potently scavenged ˙OH. Overall, the HMW peptides (1-3 and 3-5 kDa) showed better antioxidant properties than the LMW peptides except in scavenging ˙OH and inhibition of NO production in macrophages. These bioactive properties could encourage increased value-added utilization of flaxseed meal proteins for the formulation of therapeutic products. Evaluation of the effects of the flaxseed protein-derived peptides in appropriate animal disease models is required to confirm some of the preliminary results obtained in this work.

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**APPENDIX B: SUPPLEMENTAL INFORMATION**

**Figure S4.1 Electron spin resonance (ESR) spectra showing concentration-dependent decrease in the intensity of DMPO-OH adduct spin resonance induced by flaxseed protein-derived (A) cationic peptide fraction FI (0.04–0.20 mg/ml) and (B) FicH (0.08–0.40 mg/ml); this represents the scavenging of hydroxyl radicals (˙OH) by the flaxseed peptides samples. Details of the ˙OH scavenging activity (%) for all the samples are shown in Figure 4.2B and Table 4.1, and discussed in Section 4.3.2**
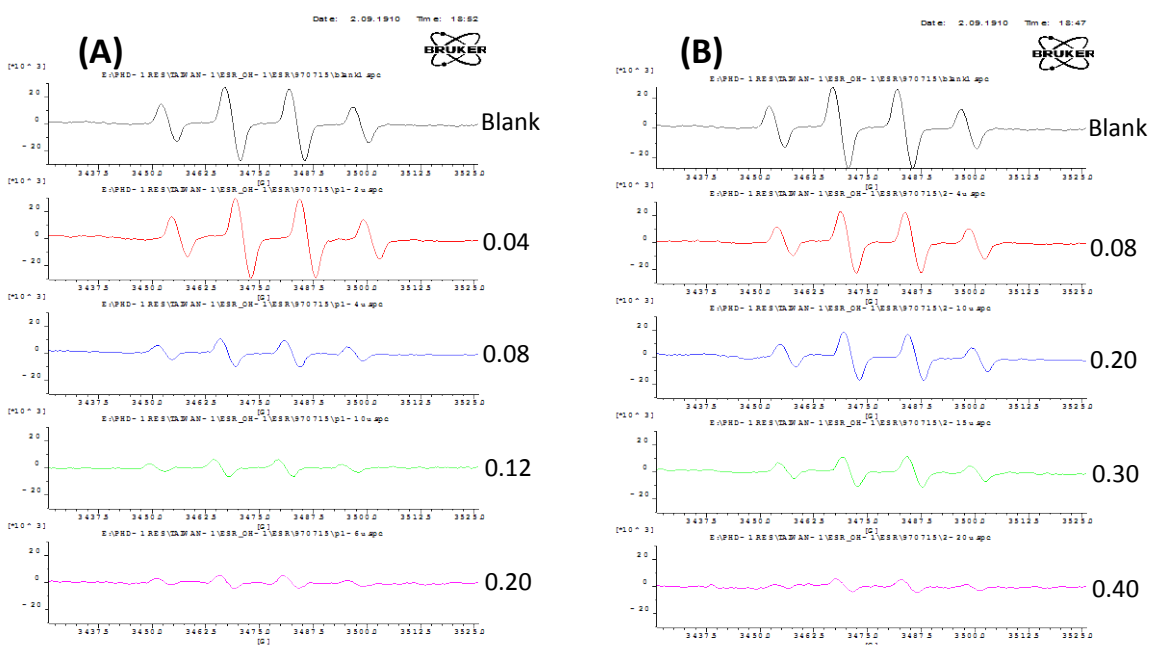
**Figure S4.2 Screening for inhibitory activity of the flaxseed peptide samples (0.1 mg/ml) against nitric oxide production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages; no LPS (control), LPS (100 ng/ml) only, LPS (100 ng/ml) plus polymyxin B (PMB) (50 µg/ml), and LPS (100 ng/ml) plus flaxseed peptide fraction. Bar with different letters are significantly different at P=0.05. The most active samples (PepH, FicH and PapH) were selected for further studies**
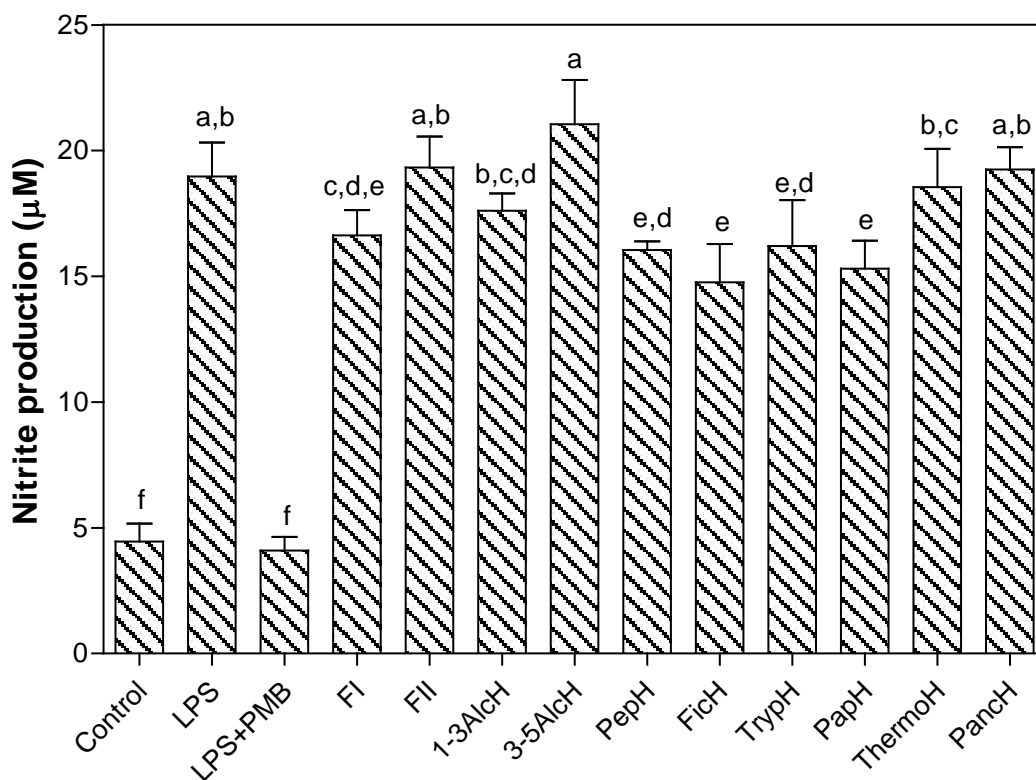
**Figure S4.3 Screening for the induction of nitric oxide production in RAW 264.7 macrophages in the absence of LPS by flaxseed peptide samples; control (no LPS); polymyxin B (PMB, 50 μg/ml); LPS only (100 ng/ml LPS); and 0.1 mg protein/ml flaxseed peptide fraction plus 50 μg/ml PMB. Bar with different letters are significantly different at P=0.05. Compared to LPS only treatment, none of the samples showed substantial induction of nitric oxide production in the absence of LPS**
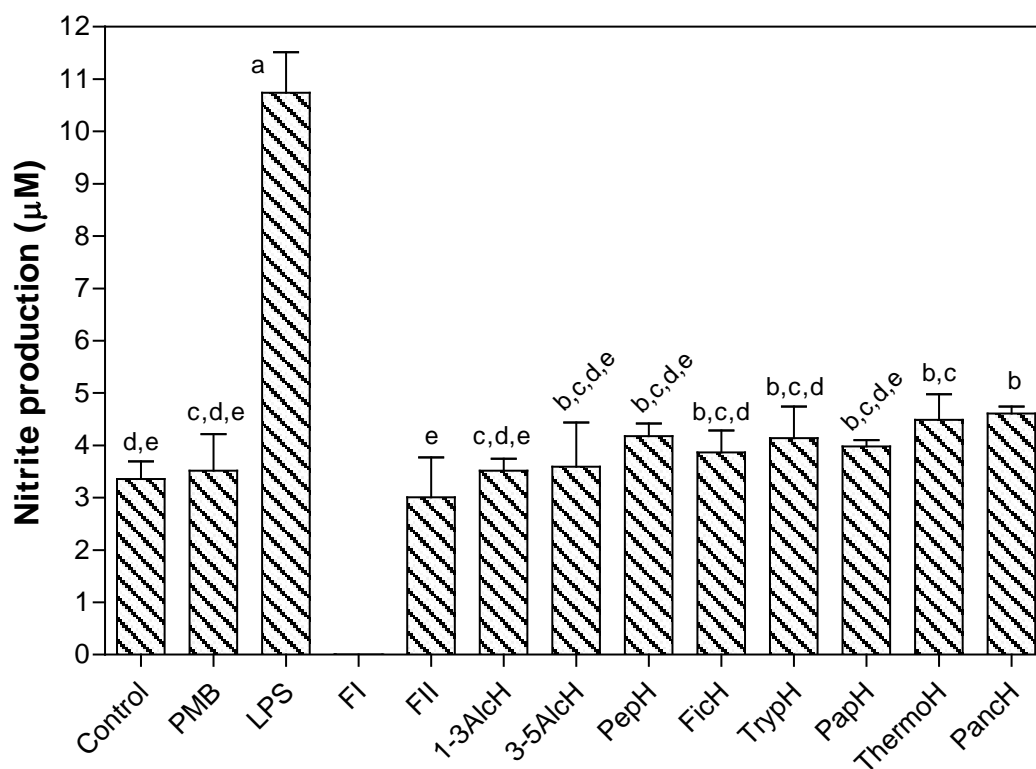
**Figure S4.4 Screening for effects of flaxseed peptide samples against the viability of human breast adenocarcinoma cell line (MCF7). Cell viability was measured using the MTT assay. Bar with different letters are significantly different at P=0.05. The samples did not show any considerable inhibition of MCF7 cell viability at 0.1 mg/ml peptide concentration**
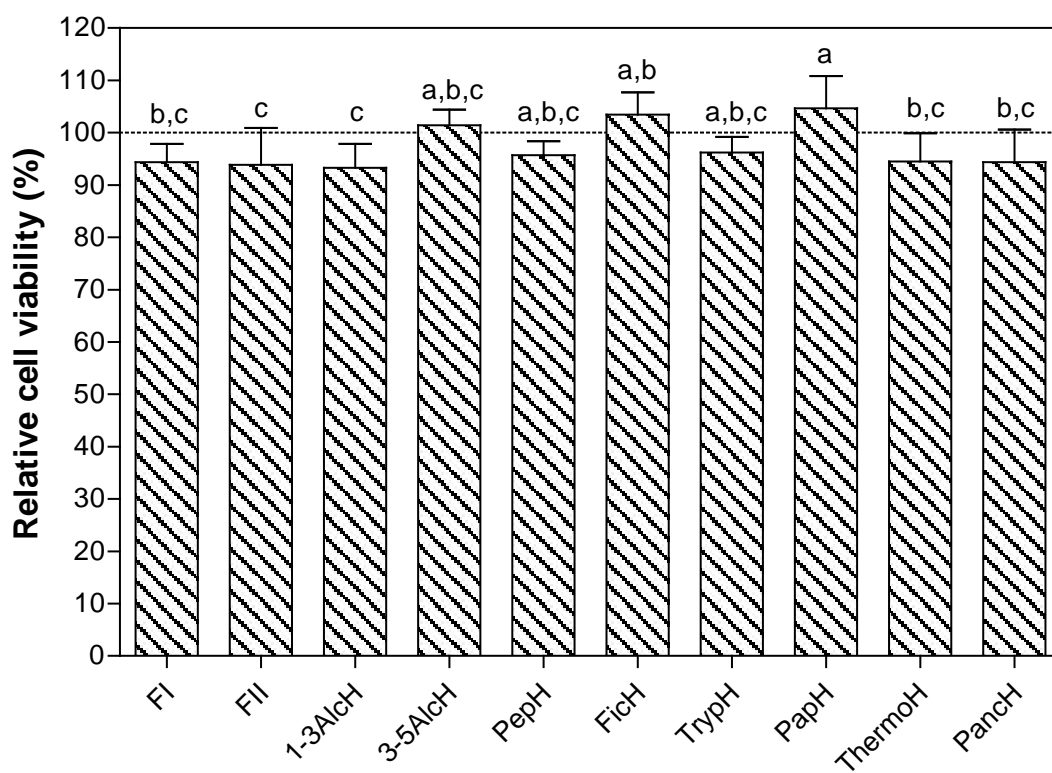
**Figure S4.5 Screening for effects of flaxseed peptide samples against the viability of human promyelocytic leukemia cell line (HL-60). Cell viability was measured using the MTT assay. Bar with different letters are significantly different at P=0.05. The samples did not show any considerable inhibition of HL-60 cell viability at 0.1 mg/ml peptide concentration**
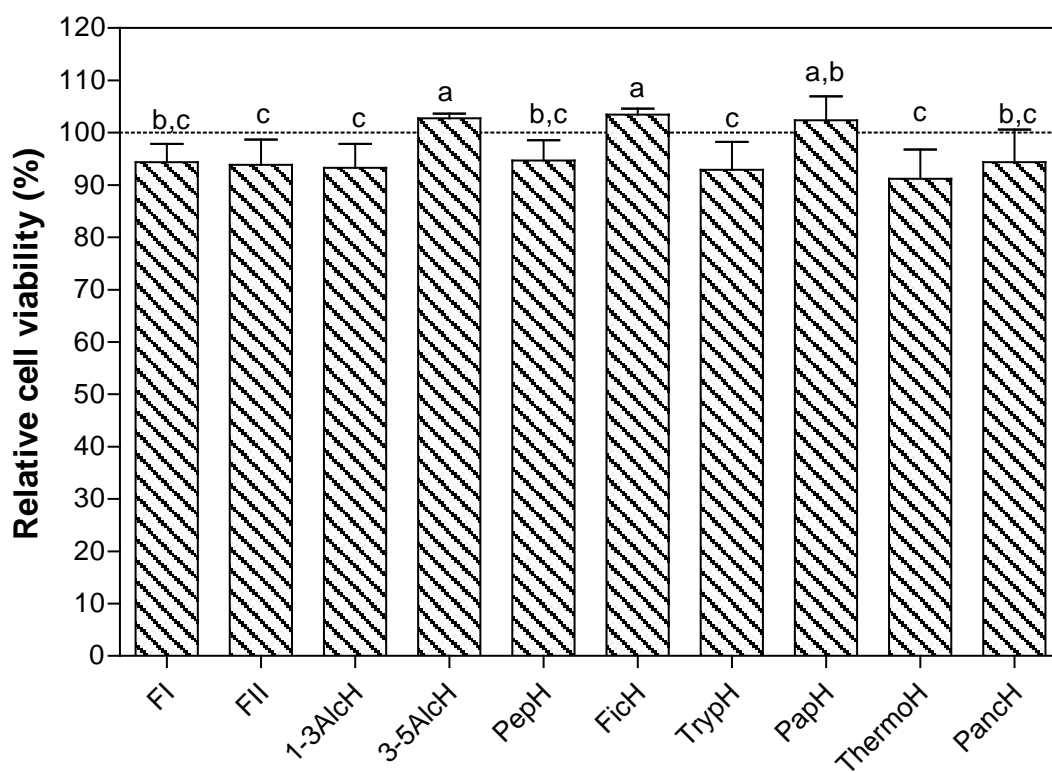
**TRANSITION STATEMENT**

The multifunctional bioactive properties of some of the flaxseed peptides produced in this study have promising application in intervention against multiple human disease conditions especially hypertension and oxidative stress-induced diseases. In order to address specific objectives two and three of this thesis, the ACE and renin-inhibiting flaxseed protein-derived cationic peptide fraction II (FII) was targeted against calmodulin (CaM) to investigate if the positively-charged peptide fraction could bind the negatively-charged CaM with concomitant inhibition of CaM-dependent phosphodiesterase (CaMPDE), which have been implicated in the aetiology and progression of chronic human diseases. The following chapter discusses the kinetics of inhibition of CaMPDE by flaxseed cationic peptides (FI and FII) and fractionation of peptides from FII that could simultaneously inhibit the activities of ACE, renin and CaMPDE. Multifunctional peptides possess an advantage over single-function peptides since they can target multiple symptoms and risk factors of chronic human diseases, thus reducing the cost associated with developing many nutraceutical products.

**CHAPTER FIVE**

**MANUSCRIPT 3**

**MULTIFUNCTIONAL PEPTIDES FROM A CATIONIC FRACTION OF FLAXSEED PROTEIN HYDROLYSATES**

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**5.0. ABSTRACT**

Alcalase hydrolysis of flaxseed protein fractions liberated cationic peptides, which were separated into two major fractions (FI and FII) by chromatography using a cation-exchange column. Due to their cationic property, the peptide fractions bound and inactivated calmodulin with concomitant inhibition of calmodulin-dependent phosphodiesterase (CaMPDE); this activity was substantially reduced with increased CaM concentrations. Enzyme kinetics studies showed competitive inhibition of CaMPDE for FI and FII with enzyme-inhibitor dissociation constants ($K_i$) of 0.0202 and 0.0511 mg/ml, respectively. The cationic FII was further fractionated by reverse phase HPLC to concentrate the active peptides. All the HPLC fractions inhibited CaMPDE, angiotensin converting enzyme and renin activities at 0.1, 0.2 and 1.0 mg/ml, respectively, but the multifunctional activities was more pronounced in HPLC fraction F6. The multifunctional properties of the cationic peptide fractions can increase their use in targeting multiple symptoms of cardiovascular and other diseases, considering the roles of these enzymes in aetiology and progression of chronic human diseases.

## 5.1. INTRODUCTION

Aberrant production of angiotensin converting enzyme (ACE), renin and calmodulin (CaM)-dependent phosphodiesterase (CaMPDE) have been observed in various health conditions, and are related to aetiology and progression of chronic human diseases. ACE and renin are key enzymes of the blood pressure-regulating renin-angiotensin system (RAS) pathway and have been widely targeted with chemical compounds for antihypertensive therapy. CaMPDE is expressed in various cell types including coronary vascular smooth muscle cells (SMC), brain, kidney and heart (Bender & Beavo, 2006; Kass et al., 2007) where it catalyzes the conversion of cyclic nucleotide monophosphates (cGMP and cAMP) to GMP and AMP making it important in regulating cellular processes mediated by the cyclic nucleotides including neurotransmission, immune cell activity, vasodilation, cell growth and proliferation (Cho et al., 1998). Chronic over-expression of CaMPDE has been found in atherosclerosis, heart pressure-load stress, heart failure and vascular proliferation (Kass et al., 2007). CaMPDE inhibition decreased CaMPDE-induced human SMC proliferation and also induced cAMP-mediated apoptosis in cultured human leukemic cells, which are relevant activities toward treatment of atherosclerosis and cancer (Rybalkin et al., 2003; Nagel et al., 2006; Jiang et al., 1996). Moreover, angiotensin II (AT-II) increases CaMPDE expressions in SMC through AT-II-mediated increase in cellular $Ca^{2+}$ concentration (Kim et al., 2001); thus, CaMPDE-induced pathogenesis can also be linked to RAS-mediated hypertension. CaMPDE has also been implicated in pulmonary arterial hypertension (Schermuly et al., 2007) and inflammation since CaMPDE is upregulated during monocyte differentiation

(Bender et al., 2005). Thus, CaM-binding agents can be used as specific CaMPDE inhibitors in modulating the effects of increased expression of the enzyme.

Food protein-derived peptides have generated particular interests as adjuvant or alternative therapeutic agents against chronic human health conditions. This has provided the opportunity for diversification of the use of major protein-rich agricultural crops beyond basic nutritional purposes. In a bid to improve the value of defatted flaxseed meal, a number of bioactive peptide fractions have been reported from enzymatic flaxseed protein hydrolysates and these peptides exhibited physiologically-relevant activities such as antioxidant, anti-inflammatory, ACE and renin-inhibitory activities (Marambe et al., 2008; Udenigwe et al., 2009a,b; Udenigwe & Aluko, 2010). Cationic peptides from flaxseed protein were also reported to bind calmodulin (CaM) with concomitant inhibition of CaM-dependent nitric oxide syntheses (Omoni & Aluko, 2006a, b). Moreover, food-derived peptides with more than one bioactive property can be used to target multiple diseases or a disease with more than one symptom, which will ultimately reduce the cost associated with developing different health promoting products for particular diseases.

Therefore, the objectives of this project were to (1) determine the kinetics of inhibition of CaMPDE by ACE and renin-inhibiting CaM-binding cationic peptide fractions derived from flaxseed protein hydrolysates, and (2) separate the most multifunctional cationic peptide fraction using high performance liquid chromatography (HPLC) to concentrate the constituent multifunctional peptides that can simultaneously inhibit ACE, renin and CaMPDE activities.

**5.2. METHODS**

**5.2.1. Production of flaxseed cationic peptide fraction**

The flaxseed protein-derived cationic peptide fraction was produced as previously reported (Udenigwe et al., 2009a). Briefly, flaxseed protein isolate was hydrolyzed with Alcalase (E/S 1:25) for 4 h followed by centrifugation at 14,941$g$ for 30 min. The supernatant was subjected to nitrogen gas pressure-driven ultrafiltration using a 1000 Da molecular weight cut-off membrane and the resulting permeate was collected and freeze dried. A solution of the permeate (4 ml of 250 mg/ml) was loaded onto an SP-Sepharose High Performance XK 50/20 cation exchange chromatography column connected to an AKTA Fast Protein Liquid Chromatography system (Amersham-GE Biosciences, Montreal, Canada). The sample was eluted from the column by first washing with 1.5 column volume of 0.1 M ammonium acetate buffer (pH 7.5) to remove unbound peptides followed by elution of the bound peptides using a gradient of 0–50% 0.5 M ammonium carbonate (pH 8.8) in 0.1 M ammonium acetate buffer at a flow rate of 10 ml/min; elution was monitored as absorbance at 214 nm. After elution of the bound peptides, two major peaks were observed; fractions within the peak that eluted at a later time were pooled, freeze dried and subsequently used as cationic peptide fraction II (FII).

**5.2.2. CaM-dependent PDE (CaMPDE) inhibition assay**

CaMPDE activity was measured by the luciferin-luciferase technique of Kizawa et al. (1995) and Weiss et al. (1972) as recently reported by You et al. (2010). The reaction

mixture contained 0.05 U CaM, 0.1 mU phosphodiesterase, 0.15 mM $CaCl_2$, 1 U pyruvate kinase, 1 U myokinase, 1 mM cAMP, 25 mM ammonium acetate, 3 mM $MgCl_2$, 0.26 mM phosphoenolpyruvate, 17 mM dithiothreitol, 0.1 nM guanosine 5′-triphosphate, 10% bovine serum albumin and flaxseed peptide sample in 300 µl of 50 mM glycine-glycine buffer (pH 8.0). The assay mixture was incubated at 37°C using an Eppendorf Thermomixer R for 30 min and the reaction was terminated by heating the mixture in a water bath (95-100°C) to inactivate the enzymes, followed by centrifugation at 10,000 × $g$ for 10 min. A portion (10 µl) of the protein-free supernatant was placed in a 1.5 ml microfuge tube followed by the addition of 100 µl of luciferin-luciferase reagent (Promega ENLITEN® ATP Assay Bioluminescence Detection Kit). Thereafter, luminescence was measured as relative luminescence unit (RLU) using a Turner Biosystems 20/20$^n$ Luminometer to quantify ATP produced during assay. Blank assays were conducted for all samples using heat-inactivated (95–100°C) phosphodiesterase whereas control assay did not contain the peptides. CaMPDE inhibitory activity (%) of the active samples was calculated as: $\{[(RLU_c–RLU_s)/ RLU_c] \times 100\}$, where $RLU_c$ and $RLU_s$ are the RLU of the control and sample assays, respectively. Kinetics of the inhibition of CaMPDE was also conducted by varying CaM concentration (0.0832–0.6656 U/ml) at fixed CaMPDE and peptide concentrations. Enzyme inhibition kinetics parameter ($K_m$, $V_{max}$, $K_m^{app}$ and $V_{max}^{app}$) and mode of inhibition were determined by non-linear regression (Michaelis-Menten equation) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Lineweaver-Burk double reciprocal plots were used to represent the kinetics data. The dissociation constant of enzyme-inhibitor complex ($K_i$)

was determined as $\{K_i = [I]/\alpha - 1\}$; $[I]$ = inhibitor concentration, $\alpha = K_m^{app}/K_m$ (Marangoni, 2003); $K_m$ is the dissociation constant of CaM-PDE complex; $V_{max}$ is the reaction rate at saturating CaM concentration; $K_m^{app}$ and $V_{max}^{app}$ represent their apparent values in the presence of the inhibitor.

### 5.2.3. ACE inhibition assay

ACE inhibition assay was conducted as previously described using FAPGG as ACE substrate (Udenigwe et al., 2009a). Briefly, 1 ml of 0.5 mM FAPGG in 50 mM Tris-HCl buffer containing 0.3 mM NaCl (pH 7.5) was mixed with 200 µl of the buffered peptide sample (pH 7.5) followed by the addition of 20 µl of 1 U/ml ACE solution. The enzyme activity was recorded for 2 min at room temperature as rate of decrease in absorbance at 345 nm ($\Delta A.min^{-1}$). The buffer was used instead of peptide fraction solutions in the blank experiment. ACE inhibition (%) was calculated as: $\{1 - (\Delta A.min^{-1}_{(sample)} / \Delta A.min^{-1}_{(blank)}) \times 100\}$, where $\Delta A.min^{-1}_{(sample)}$ and $\Delta A.min^{-1}_{(blank)}$ are the ACE reaction rates in the presence and absence of the peptide samples, respectively.

### 5.2.4. Renin inhibition assay

Renin inhibition assay was conducted by fluorescence spectrometry as previously described (Udenigwe et al., 2009a). Briefly, the total assay volume of 190 µl contained 20 µl of 10 µM renin substrate, 10 µl of human recombinant renin and the peptide sample in 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 8.0). The Tris-HCl buffer was used instead of the peptide solution in the blank experiment. The renin

substrate and peptide samples were mixed and pre-warmed to 37°C for 10 min prior to the assay. Thereafter, the reaction was initiated by adding renin to the mixture. The increase in fluorescence intensity was measured for 10 min in a JASCO FP-6300 spectrofluorimeter (Japan Spectroscopic Company, Tokyo, Japan) with a thermostated cell compartment that was maintained at 37°C using a circulatory water bath. The spectrofluorimeter was set at excitation wavelength of 340 nm, emission wavelength of 490 nm, excitation bandwidth of 5 nm and emission bandwidth of 10 nm. Renin activity was expressed as reaction rate, fluorescence intensity unit per min ($FIU.min^{-1}$) and inhibitory activity (%) of the peptide samples was calculated as: $\{[(FIU.min^{-1}_{(blank)} - FIU.min^{-1}_{(sample)}) / FIU.min^{-1}_{(blank)}] \times 100\}$, where $FIU.min^{-1}_{(blank)}$ and $FIU.min^{-1}_{(sample)}$ are the renin reaction rates in the absence and presence of the peptide samples, respectively.

## 5.2.5. High Performance Liquid Chromatography (HPLC)

The multifunctional cationic peptide fraction II (FII) was fractionated using reverse-phase HPLC (RP-HPLC) technique. An aqueous solution of FII (0.2 ml of 100 mg/ml) was subjected to RP-HPLC on a Phenomenex C12 column (21 mm × 250 mm) connected to a Waters HPLC system equipped with a photodiode array detector. The column was pre-equilibrated with 0.1% trifluoroacetic acid (TFA) in water (solvent A), and both the sample and solvents were filtered with 0.2 μm Fisherbrand general filtration membrane filters prior to fractionation. The sample was eluted from the column at a flow rate of 10 ml/min with a linear gradient of 0-50% methanol that also

contained 0.1% TFA (solvent B) for 45 min, maintained at 100%B for additional 10 min followed by equilibration with 100%A for 10 min. Peptide elution was monitored as absorbance at 214 nm. Fractions were collected using an automated fraction collector every 0.5 min and pooled into 10 fractions of distinct peaks or groups of peaks. The pooled fractions were desolventized in a rotary evaporator while the aqueous residues were freeze dried and stored at -20°C for further use.

## 5.3. RESULTS AND DISCUSSION

Food protein-derived low MW bioactive peptides have gained particular interest for use as therapeutic agents in human disease intervention due to safety of the natural peptide products, low cost and potential efficient absorption through the enterocytes into blood circulation to exert their activities in target tissues. The activities of these peptides depend largely on the peptide sequence, molecular size, and physicochemical properties of the constituent amino acid residues (Pripp et al., 2004). Thus, careful processing of enzymatic food protein hydrolysates can lead to enrichment of particular structural features in peptides, which can exert multifunctional physiological activities depending on the nature of the molecular disease targets. For example, the rich arginine composition of flaxseed proteins was basis for the production of highly cationic peptide fractions that bound and inactivated negatively-charged CaM (Omoni & Aluko, 2006a, b), and exhibited antioxidant activities in addition to inhibition of enzymes of the renin-angiotensin system (Udenigwe et al., 2009a, b). CaM-binding oligopeptides have been identified from insect venom (Martínez-Luis et al., 2007), but the high molecular size

reduces their prospective use as enteral therapeutic agents due to concerns about low levels of absorption and bioavailability. Thus, the current study investigated the bioactive properties of smaller peptides (<1 kDa) in order to increase their potential use in human.

In this study, two cationic amino acids-enriched fractions from flaxseed proteins were targeted against CaM in order to inhibit the activity of CaM-dependent PDE. The cationic peptide fraction FI showed concentration-dependent inhibition of the activity of CaMPDE *in vitro* with maximum inhibition of 41.6% at 0.09 mg/ml peptides (Figure 5.1). Moreover, FII also showed concentration-dependent CaMPDE inhibition resulting in a loss of 25% of the enzyme activity at 0.1 mg/ml. These activities are slightly lower than that of previously reported casein-derived cationic peptide fraction, which inhibited 50% of CaM-activated PDE activity at 0.014 mg/ml (Kizawa et al., 1995). Although FII contained more cationic peptides that could bind and inactive CaM, the CaMPDE-inhibitory activity of FII was lower than FI. This is contrary to results of similar previous investigations on CaM-dependent nitric oxide synthases and protein kinase II, where the more cationic peptide fractions displayed stronger interactions with CaM leading to more potent inhibition of CaM-dependent activities of the enzymes (Li & Aluko, 2005; Omoni & Aluko, 2006a, b). This discrepancy could be due to other peptides and non-peptides that are present in FI and could bind CaM or directly inhibit PDE leading to enhanced potency of the peptide fraction. CaM-binding activities have been reported for various natural products including alkaloids, coumarins, terpenoids and lignans (Rojas et al., 2003; Martínez-Luis et al., 2007). Therefore, the presence of small amounts

of flaxseed lignans in the cationic peptide fraction can contribute to increased CaM-binding activity. In order to verify that the observed CaMPDE inhibition in this study was due to CaM inactivation, different peptide concentrations were targeted against CaMPDE at different CaM concentrations. As shown in Figure 5.2A, the residual

**Figure 5.1 Concentration-dependent inhibition of calmodulin-dependent phosphodiesterase (CaMPDE) by flaxseed-derived cationic fractions FI and FII**
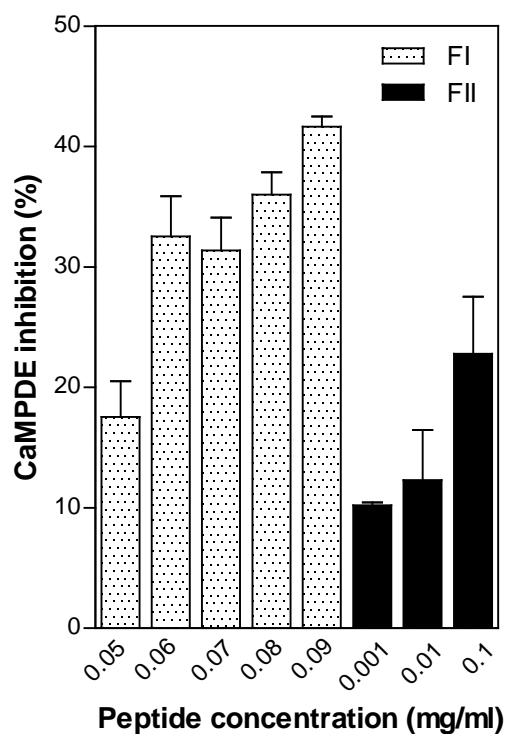


CaMPDE activity was dependent on CaM concentration at three different peptide concentrations; the extent of FI-induced CaMPDE inhibition was significantly ($P<0.05$) reduced by increasing CaM concentration. This trend was also observed for FII where the CaMPDE-inhibiting activity of the peptide fraction at 0.1 mg/ml was overcome by

increasing the substrate (CaM) concentration (Figure 5.2B). This suggests that the flaxseed cationic peptide fractions possibly bound CaM and hindered the formation of CaM-PDE complex since increase in CaM concentration decreased the potency of the peptides in inhibiting CaMPDE. These observations are similar to previous reports on the binding of food-derived cationic peptides to CaM leading to inhibition of other CaM-dependent enzymes that play important roles in aetiology and progression of chronic human diseases (Li & Aluko, 2005; Omoni & Aluko, 2006a, b).

**Figure 5.2 Effects of (A) FI and (B) FII on calmodulin-dependent phosphodiesterase (CaMPDE) activity at different concentrations of CaM**
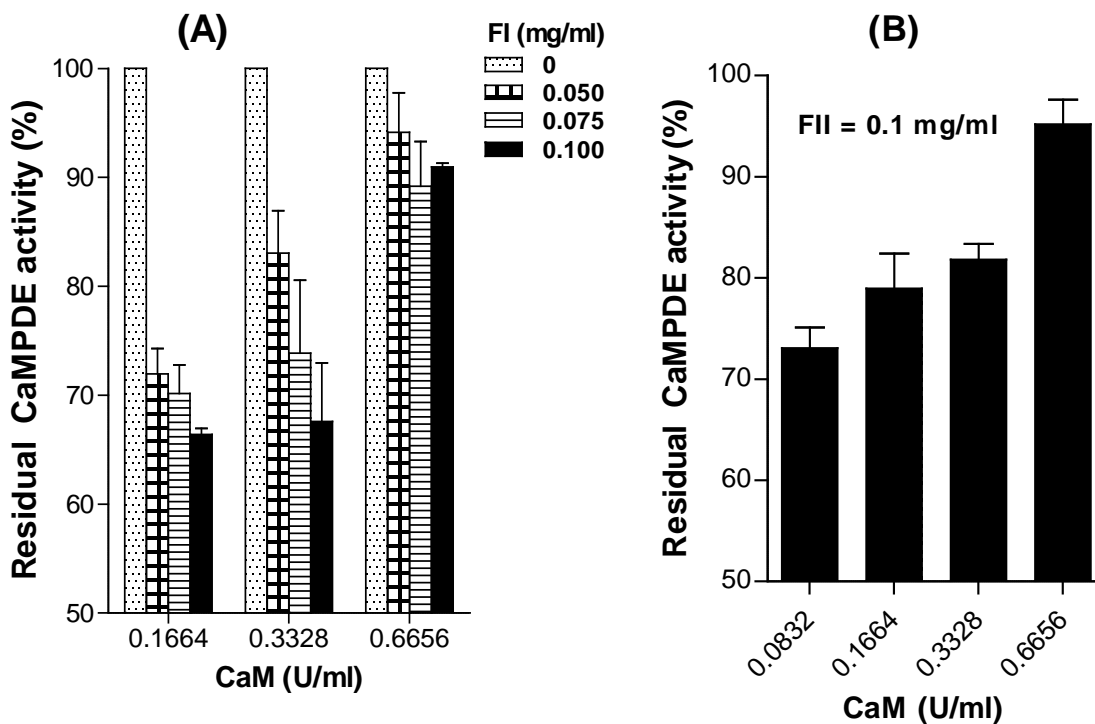
In order to confirm binding mechanisms and mode of CaMPDE inhibition, non-linear Michaelis-Menten equation and Lineweaver-Burk plots were used to analyze the CaMPDE inhibition kinetics data. The kinetics plots (Figure 5.3A,B) showed that, with CaM as substrate and at fixed enzyme activity, cationic FI and FII did not have considerable effects on the $V_{max}$ of CaMPDE ($1.59 \times 10^{-14}$ s.moles$^{-1}$); $V_{max}^{app}$ (FI) = $1.53 \times 10^{-14}$ s.moles$^{-1}$, $V_{max}^{app}$ (FII) = $1.52 \times 10^{-14}$ s.moles$^{-1}$. However, the presence of both peptide fractions caused substantial increase in the $K_m$ for CaM from 0.0184 U/ml for the uninhibited reaction to $K_m^{app}$ of 0.0638 and 0.0544 U/ml at 0.05 and 0.1 mg/ml of FI and FII, respectively. These observations are characteristics of competitive mode of enzyme inhibition, which confirmed that the peptide fractions each bound and inactivated CaM or competed with CaM in the CaM-PDE complex. Based on this mode of inhibition, the CaMPDE-inhibitor dissociation constants ($K_i$) were calculated using the enzyme kinetics parameters and determined to be 0.0202 and 0.0511 mg/ml for FI and FII, respectively. These values represent concentrations of the cationic peptide fractions that occupied half the CaM-binding sites leading to CaMPDE inhibition; thus, FI had higher affinity for CaM than FII due to its lower $K_i$ value. Similar enzyme kinetics studies reported that these flaxseed peptides exhibited mixed-type and non-competitive modes of inhibition of the activities of CaM-dependent endothelial and neuronal nitric oxide syntheses, respectively (Omoni & Aluko, 2006a, b). Thus, the exact behavior and final outcome of the complex formed between CaM-binding cationic peptides and CaM or CaM-enzyme complex depends on nature of the target enzyme.

**Figure 5.3 Lineweaver-Burk plots of the binding of (A) FI and (B) FII to CaM with concomitant inhibition of calmodulin-dependent phosphodiesterase (CaMPDE); $V_{max}$, s.moles$^{-1}$; $K_m$, U/ml; $K_i$, mg/ml**



|  | I = 0 | I = FI | I = FII |
|---|---|---|---|
| $V_{max}(\times 10^{-14})$ | 1.59 | 1.53 | 1.52 |
| $K_m$ | 0.0184 | 0.0638 | 0.0544 |
| $K_i$ | na | 0.0202 | 0.0511 |
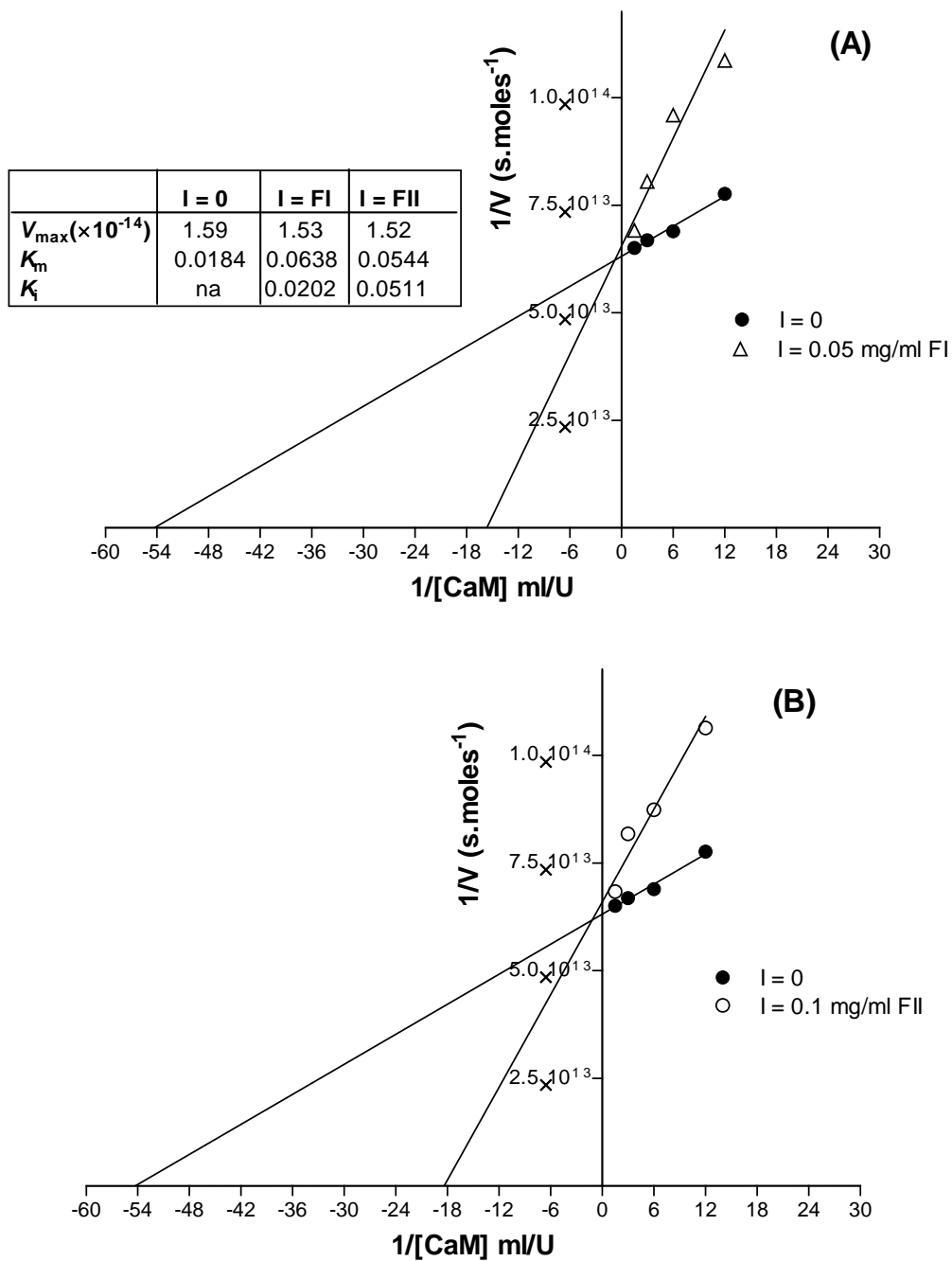
Although FII displayed lower affinity for CaM, it was selected for further fractionation to concentrate the potent multifunctional peptides since it also displayed ACE and renin-inhibitory activities as opposed to FI, which did not inhibit renin activity (Udenigwe et al., 2009a). Peptide fractionation by HPLC using a reverse-phase column yielded 8 peptide fractions designated as FII-2, FII-3, FII-4, FII-5, FII-6, FII-7, FII-8 and FII-9 (Figure 5.4) with recovery yields of 0.84%, 5.79%, 15.53%, 6.73%, 1.74%, 4.15%, 0.87% and 2.10% (dry weight basis), respectively; the rest of the sample was composed of ammonium salts from the FPLC buffers (FII-10) and an oily residue (FII-1). These HPLC peptide fractions (FII-2–FII-9) were screened for multifunctional properties as ACE, renin and CaMPDE inhibitors. Figure 5.5 shows the ACE-inhibitory activities of the HPLC fractions. At 0.05 mg/ml peptides, the HPLC fractions displayed different weak ACE-inhibitory activities (6.0–17.2%) except FII-5 and FII-7, which were inactive at this concentration. Moreover, the activities of these HPLC fractions were lower than that of the parent cationic peptide fraction (FII), which inhibited 31% of ACE activity at that concentration. However, all the HPLC fractions exhibited moderate to strong ACE-inhibitory activities at 0.2 mg/ml of the peptides. Compared to the cationic FII, HPLC fractions FII-6 and FII-8 displayed better ACE inhibition (73.6% and 74.5% respectively) whereas the other fractions, except FII-4, were significantly ($P<0.05$) less active. Thus, FII-6 and FII-8 contained the most potent peptides although the ACE-inhibiting cationic peptides were distributed in all the HPLC fractions. There was no observed relationship between ACE inhibition and hydrophobicity of the cationic peptides, represented by time of elution of the peptide from the C12 reverse-phase HPLC column.

**Figure 5.4 High Performance Liquid Chromatography (HPLC) fractionation of the flaxseed cationic peptide fraction II (FII)**
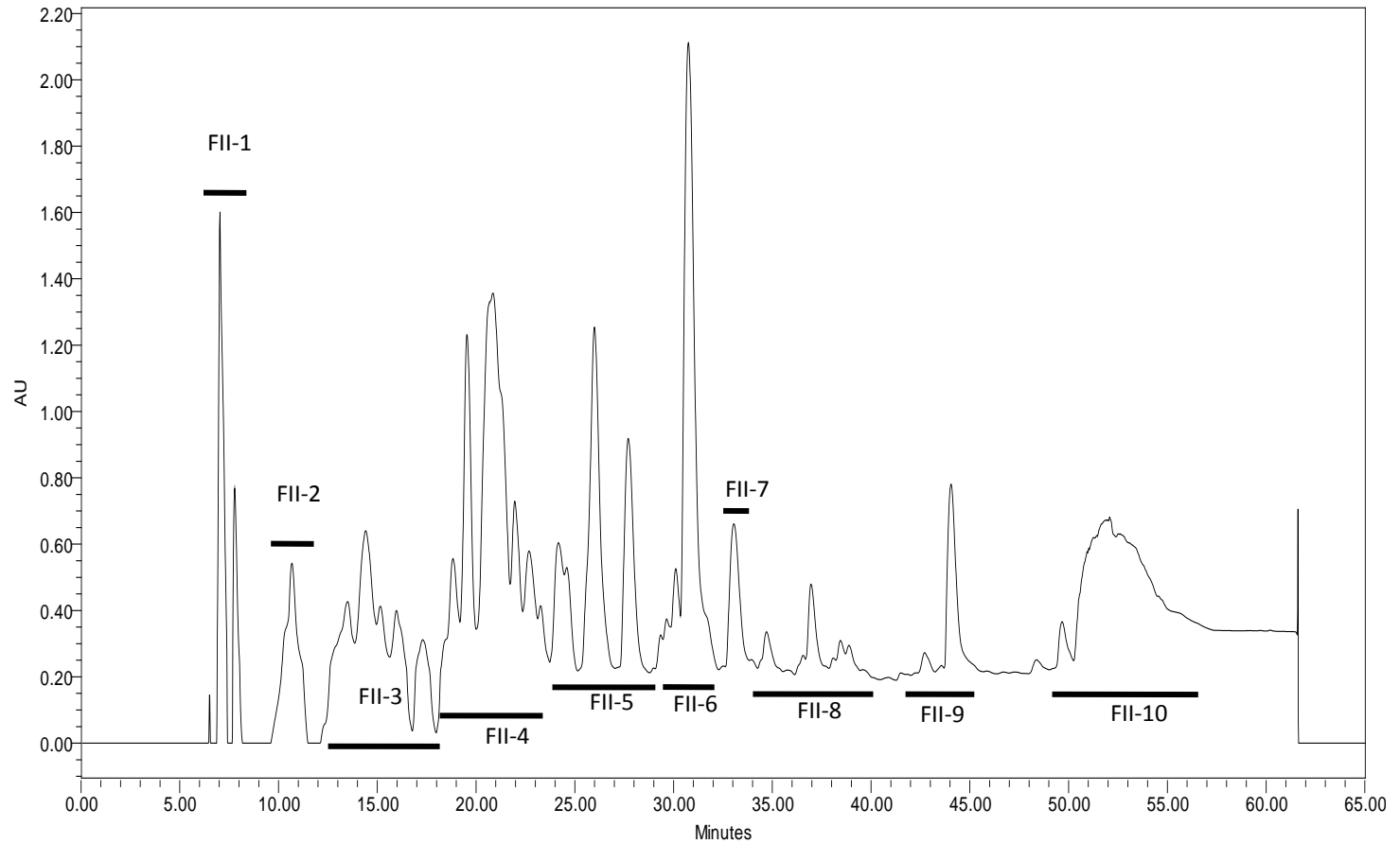
**Figure 5.5 Angiotensin converting enzyme (ACE)-inhibitory activities of the HPLC fractions of flaxseed cationic peptide fraction II (FII) at 0.05 and 0.2 mg/ml**
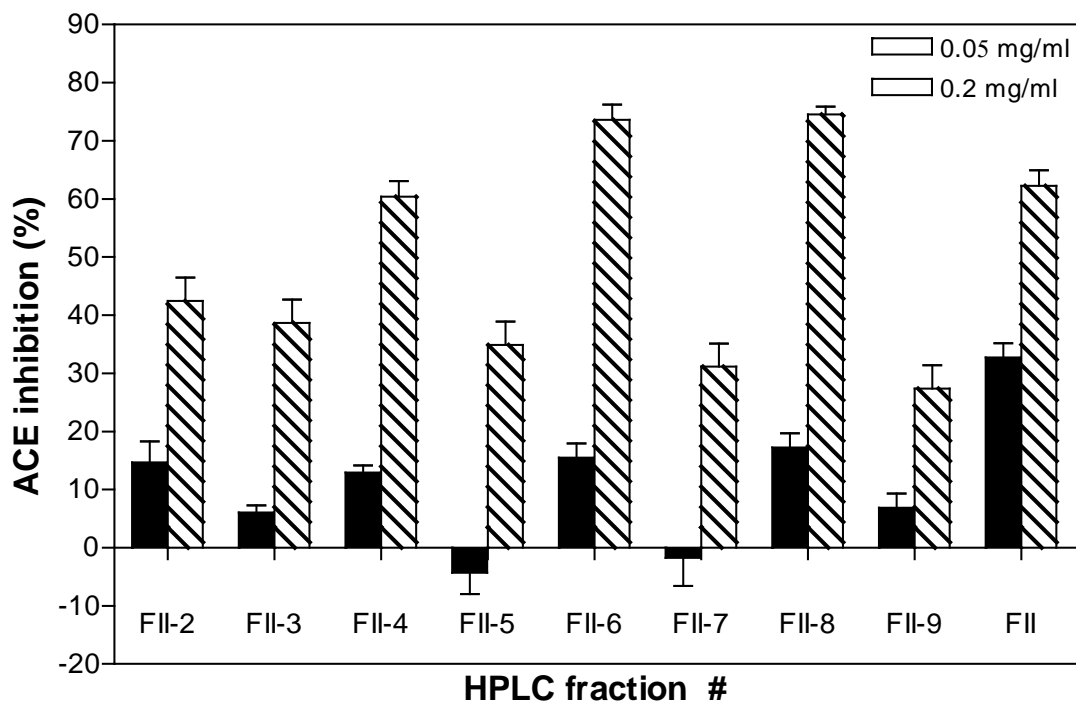


*In vitro* renin inhibition assay showed that all the HPLC fractions exhibited a wide range of renin-inhibitory activities, which were pronounced in FII-9 and FII-2 with 94.6% and 57.2% renin inhibition, respectively, at 1 mg/ml of peptides compared to FII with 44.6% renin inhibition (Figure 5.6). HPLC fractions FII-5 had the weakest renin-inhibitory activity (4.7%) whereas the activities of FII-6 and FII-7 were comparable to that of the cationic FII. In contrast to their inhibitory activities against the enzymes of the RAS pathway, all the HPLC fractions exhibited similar CaMPDE inhibitory activities except FII-3 and FII-7, which were slightly more (27.0%) and less (11.5%) active, respectively,

compared to the activity of FII (18.6%) at 0.1 mg/ml of peptides (Figure 5.7). These activities were slightly less than the activities of similar HPLC fractions of $\alpha$-casein-derived cationic peptides, which inhibited 50% of CaMPDE activity at 0.0034–0.077 mg/ml peptides (Kizawa et al., 1995), and hen egg white lysozyme-derived cationic peptides with up to 43.5% CaMPDE inhibition at 0.1 mg/ml peptides (You et al., 2010). As observed in ACE inhibition, there was no correlation of the hydrophobic properties of the peptides with both renin and CaMPDE inhibition, although a combination of hydrophobicity and cationic properties have been observed in the amino acid sequences of potent CaM-binding peptides (O'Neil & DeGrado, 1990; Kizawa et al., 1995). Thus, the bioactivities of the flaxseed peptide fractions could be specifically due to their cationic characters and amino acid sequences as previously demonstrated in insect venom peptides, which lost their CaMPDE inhibitory activities after removal of their cationic functional groups by acetylation (Martínez-Luis et al., 2007). When the results from the enzyme inhibition assays were compared, HPLC fraction FII-6 showed excellent prospect for further studies due to its multifunctional activity in inhibiting the three enzymes at levels comparable to or better than the activity of the original cationic peptide fraction (FII). Moreover, HPLC fraction FII-9 can be explored for potent renin inhibiting peptides, FII-8 for potent ACE-inhibiting peptides, and FII-3 for potent CaMPDE inhibiting peptides due to their enhanced activities in the corresponding assays compared to the activities of FII.

**Figure 5.6 Renin-inhibitory activities of the HPLC fractions of flaxseed cationic peptide fraction II (FII) at 1 mg/ml**
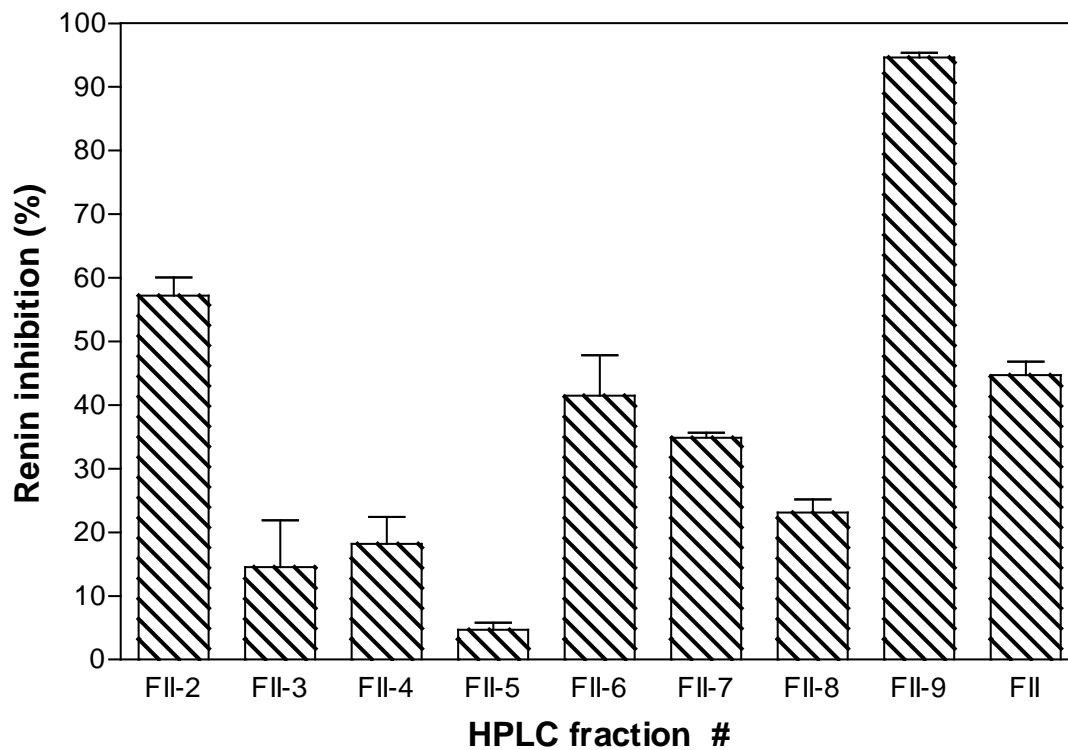
**Figure 5.7 The inhibition of calmodulin-dependent phosphodiesterase (CaMPDE) activity by the HPLC fractions of flaxseed cationic peptide fraction II (FII) at 0.1 mg/ml**
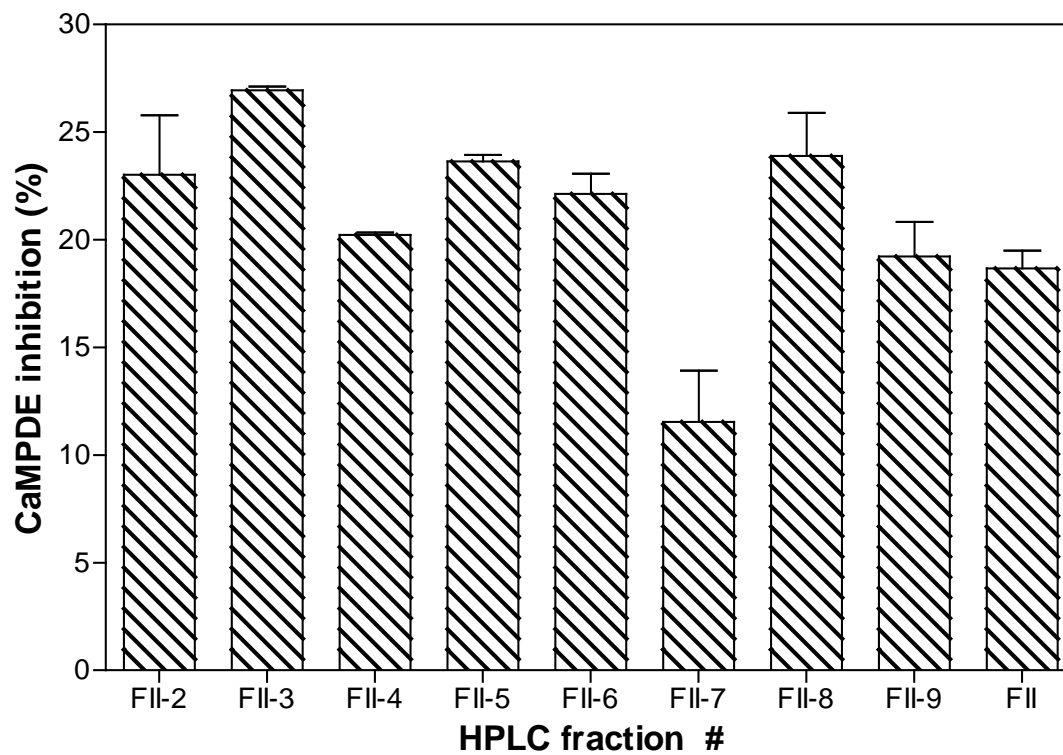
## 5.4. CONCLUSIONS

In conclusion, ACE and renin-inhibiting CaM-binding cationic peptide fractions from flaxseed proteins bound and inactivated CaM leading to competitive inhibition of CaMPDE activity. The multifunctional properties of the cationic peptides were conserved in the HPLC fractions, but specific inhibitory activities against one particular enzyme (ACE, renin or CaMPDE) were substantially enhanced in some of the fractions. The multifunctional nature of these peptide fractions can be used against multiple symptoms of chronic human diseases such as cardiovascular disease, thus reducing the cost associated with developing multiple bioactive products. The identity of the peptides in the most potent multifunctional fraction need to be determined for studying the structure-function properties of the peptides and for design of processing methods that can efficiently generate high yields of the bioactive peptides from the primary sequence of the parent proteins.

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**TRANSITION STATEMENT**

The three preceding chapters addressed the first three objectives of this project by establishing that flaxseed protein-derived peptide fractions and cationic peptides possess multifunctional bioactive properties *in vitro* and in cell culture. To address the fifth objective, a unique approach was used to generate a peptide product containing dipeptides, tripeptides and oligopeptides from flaxseed proteins that could be applied as a source of branched-chain amino acids (BCAA) for restoration of plasma amino acid imbalance during the treatment of liver disease. These BCAA-enriched peptide mixtures are known as high Fischer ratio (molar ratio of BCAA to aromatic amino acids, AAA); they contain high amounts of BCAA and corresponding low amounts of AAA. This part of the project is important because this flaxseed protein-derived BCAA-enriched peptide mixture also possess additional beneficial properties that are relevant to human health sustenance, which also increases the value of the product, and potentially the value and utilization of defatted flaxseed meal.

**CHAPTER SIX**

**MANUSCRIPT 4**

**ANTIOXIDANT AND ANGIOTENSIN CONVERTING ENZYME-INHIBITORY PROPERTIES OF**

**A FLAXSEED PROTEIN-DERIVED HIGH FISCHER RATIO PEPTIDES MIXTURE**

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**6.0. ABSTRACT**

Hydrolysis of flaxseed proteins using thermolysin and pronase followed by treatment with activated carbon yielded a peptide mixture with a Fischer ratio (branched-chain amino acids/aromatic amino acids) of 23.65 and a phenylalanine+tyrosine content of 1.11%. Gel permeation chromatography showed that the flaxseed peptide sample contains mainly low molecular weight peptides (<4 kDa). The high Fischer ratio peptide sample exhibited antioxidant property by scavenging 2,2-diphenyl-1-picrylhydrazyl radical, superoxide radical, hydroxyl radical, and also by protecting linoleic acid from oxidation. In addition, the peptide mixture showed antihypertensive property by inhibiting angiotensin I-converting enzyme in a mixed-type inhibition pattern. Protein hydrolysates with Fischer ratio higher than 20 and phenylalanine+tyrosine content lower than 2% has been used to treat patients with hepatic encephalopathy; thus, this multifunctional flaxseed peptide mixture could be used to formulate food products with multiple human health benefits in liver diseases, oxidative stress and hypertension.

**KEYWORDS: Flaxseed protein; Fischer ratio; Activated carbon; Branched-chain Amino acids; Aromatic amino acids; Antioxidant; Angiotensin converting enzyme**

**6.1. INTRODUCTION**

Acute and chronic liver failure is associated with alterations in protein metabolism, which can lead to loss of muscle protein mass. Plasma amino acid imbalance have been found in patients with liver diseases, and this is characterized by elevated levels of aromatic amino acids (AAA; tyrosine and phenylalanine) and methionine, and decreased levels of branched-chain amino acids (BCAA; leucine, isoleucine and valine) (Clemente, 2000). The ratio of plasma BCAA to AAA is termed Fischer ratio and is used to measure abnormal amino acid metabolism during liver disease, and also in clinical nutrition for the formulation of amino acid products for therapeutic purposes. In human beings, the normal plasma Fischer ratio is 3.5–4; this value decreases to <2.5 in liver diseases, <1.2 in hepatic coma and could drop to below 0.8 in profound coma (Fischer et al., 1975). Clinical nutrition approaches towards treatment of liver diseases involve feeding patients with BCAA-enriched formulae (Fischer et al., 1976; Capollo et al., 1992; Khanna & Gopalan, 2007; Schulze et al., 2008) or protein hydrolysates with Fischer ratio higher than 20 and phenylalanine+tyrosine content lower than 2% (Okita et al., 1985). BCAAs have been shown to reduce muscle wasting and plasma concentrations of AAA with concomitant improvement in encephalopathy (Fischer, 1990). The therapeutic effect of BCAA is due to the fact that they are preferentially taken up by the muscle, inhibit the transport of AAA across the blood-brain barrier, and are available for peripheral metabolism in advanced liver disease (Clemente, 2000). In addition, some athletes also use BCAA as a natural way of improving muscle mass and strength (Di Pasquale, 2008).

A number of studies have reported successful production of high Fischer ratio peptide mixtures by enzymatic hydrolysis of food proteins and subsequent processing using activated carbon and gel filtration chromatography (Adachi et al., 1993; Ma et al., 2008; Pedroche et al., 2004; Pedroche et al., 2006; Zheng et al., 2009). In clinical nutrition, peptides are preferred over individual amino acids due to the fact that short peptides have higher rates of intestinal absorption and the osmotic pressure of peptides is lower than that of corresponding free amino acids (Grimble et al., 1987; Monchi & Rerat, 1993). The recommended criteria for selecting the starting food protein that will yield high Fischer ratio peptide mixture include high amounts of BCAA, low amounts of AAA (Clemente, 2000) and availability of the raw material.

Flaxseed (*Linum usitatissimum*) is a readily available oilseed predominantly produced in the Prairie regions of Canada, and a major dietary source of $\alpha$-linolenic acid, dietary fibres and lignans (Hall III et al., 2006). These flaxseed components have been reported to possess various physiological activities relevant to human health sustenance especially in cardiovascular disease prevention, cancer and diabetes (Hall III et al., 2006). However, the protein components of flaxseed have been scarcely explored for nutritional and therapeutic applications. These proteins constitute a major part of defatted flaxseed meal, which results from industrial flaxseed crushing and oil extraction, and is comparable in nutritional quality and amino acid composition to other high quality proteins, e.g. soy protein (Hall III et al., 2006; Oomah & Mazza, 1993). Moreover, previous studies have reported that enzymatic hydrolysis of flaxseed proteins afforded low molecular weight (MW) peptides that possess antioxidant and anti-

inflammatory properties in cell cultures (Udenigwe et al., 2009a) as well as *in vitro* antihypertensive properties (Udenigwe et al., 2009b). Flaxseed proteins contain high levels of BCAA, with 50, 71 and 56 mg/g of isoleucine, leucine and valine (Oomah & Mazza, 1995), respectively. Thus, through careful choice of enzymes and processing conditions, flaxseed proteins have the potential to be converted into protein hydrolysates enriched with BCAA.

Food protein-derived peptides possess physiological therapeutic roles such as antioxidant and antihypertensive properties. Endogenous and exogenous reactive oxygen species and free radicals are implicated in the initiation and progression of human disease conditions (Ames et al., 1993). The amount of these reactive species is controlled by endogenous antioxidants until it reaches a level when the antioxidants are overwhelmed, a state known as oxidative stress. This condition can be surmounted by the consumption of foods rich in antioxidants. The renin-angiotensin system, which controls blood pressure in human beings, has been targeted for the treatment of hypertension through the inhibition of angiotensin I-converting enzyme (ACE) and renin. ACE inhibitors are thought to possess the ability to lower blood pressure in hypertensive subjects and animals (Aluko, 2008). Food protein hydrolysates and constituent peptides with multifunctional bioactive properties against these diseases targets are desirable for the formulation of food products with human health benefits in multiple physiological and disease conditions. Therefore, a BCAA-enriched product that possesses other bioactive properties could, in addition to the positive effects on liver disease, be

potentially useful as a single agent to provide simultaneous relief from other associated disease symptoms.

The objectives of this project were to produce a high Fischer ratio enzymatic flaxseed protein hydrolysate and determine the ability of the hydrolysate to scavenge free radicals, inhibit *in vitro* oxidative reactions and activities of ACE and renin.

## 6.2. MATERIALS AND METHODS

### 6.2.1. Materials

Thermolysin (from *Bacillus thermoproteolyticus rokko*), Alcalase (from *B. licheniformis*), ficin (from fig tree latex), papain (from papaya latex), activated carbon, pyrogallol (1,2,3-trihydroxybenzene) and *N*-(3-[2-furyl]acryloyl)-phenylalanylglycyl-glycine (FAPGG) were purchased from Sigma Chemicals (St. Louis, MO). Pronase (from *Streptomyces griseus*) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemical reagents were of analytical grade.

### 6.2.2. Enzymatic hydrolysis of flaxseed protein

Flaxseed protein isolate (FPI) was prepared from defatted flaxseed meal as previously described (Udenigwe et al., 2009b). The overall approach involved hydrolysis of flaxseed proteins with thermolysin followed by treating the resulting protein hydrolysate with papain, ficin, alcalase or pronase. FPI (5% w/v) was suspended in distilled water; the slurry was adjusted to pH 7.5 with 1 M $Na_2CO_3$ solution and to a temperature of 37°C. Thereafter, protein hydrolysis was initiated by adding thermolysin

at an E/S ratio of 1:100 (based on protein content). During hydrolysis, the reaction mixture was maintained at pH 7.5 by adding 1 M $Na_2CO_3$ solution using a pH stat instrument (Metrohm Titrando, Herisau, Switzerland). After 5 h, the enzymatic reaction was terminated by adjusting the pH to 4.0 using 1M HCl. The temperature and pH of the reaction mixture was adjusted appropriately and subjected to another hydrolysis using one of these proteases: papain (pH 6.5, 37°C), ficin (pH 7.0, 37°C), alcalase (pH 8.0, 50°C) and pronase (pH 7.4, 40°C). These enzymes were separately added to the resulting hydrolysate for further hydrolysis at E/S ratio of 1:100 (based on protein content); pH was also maintained with 1 M $Na_2CO_3$ solution. After 5 h, the reaction was stopped by adjusting the pH to 4.0 using 1M HCl. The resulting flaxseed protein hydrolysate (FPH) was collected by centrifugation at 15000×g, freeze-dried and stored at −20°C until used. The protein content of the resulting powder was determined by a modified Lowry protein assay method (Markwell et al., 1978).

### 6.2.3. Adsorption of flaxseed protein hydrolysate onto activated carbon-packed column

To separate the BCAA-containing peptides from the AAA-containing peptides, each of the freeze-dried FPH was dissolved in deionized water at 10 mg/ml and the solution was adjusted to pH 2.5 using 0.5 M HCl (Adachi et al., 1993) followed by filtration using Whatman No. 1 filter paper. Thereafter, the resulting acidic filtrate was passed through a column packed with activated carbon (9.5×80mm) and sample was eluted using a single-channel Peristaltic pump model P-1 (GE Healthcare, Montreal, PQ,

Canada). The unbound peptides (BCAA-rich) were collected in the flow-through solution and labelled as FPH-col. The column was regenerated for subsequent fractionation by eluting with absolute ethanol to remove bound peptides (AAA-rich) followed by washing with distilled water. Peptide elution was monitored using a spectrophotometer by measuring absorbance at 220, 260 and 280 nm. The collected peptide solutions were freeze-dried and stored at −20°C until used.

### 6.2.4. Adsorption of flaxseed protein hydrolysate onto activated carbon by mixing

The AAA-containing peptides and free AAA were also removed from the FPH by mixing 10 mg of FPH (adjusted to pH 2.5 using 0.5 M HCl) with various amounts of activated carbon (10, 25, 50, 75, 100 and 150 mg) in 1 ml of deionized water for 10 min at room temperature in a beaker. Thereafter, the mixture was centrifuged at $15000 \times g$ for 30 min and the supernatant (contain BCAA-rich peptides) filtered through a Whatman No.1 filter paper to completely remove traces of activated carbon. The filtrate (FPH-mix) was freeze-dried and stored at −20°C until used. The levels of peptides and AAA in the FPH-mix peptide product were initially estimated using a spectrophotometric absorbance at 220 and 260+280 nm, respectively.

### 6.2.5. Amino acid analysis

An HPLC system was used to determine the amino acid profiles after samples were hydrolyzed with 6 M HCl as previously reported (Bidlingmeyer et al., 1984). The cysteine and methionine contents were determined after performic acid oxidation

(Gehrke et al., 1985) and tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992). The Fischer ratio was calculated as the ratio of BCAA to AAA.

### 6.2.6. Gel permeation chromatography

Molecular weight distribution of the peptides present in the FPH-mix with the highest Fischer ratio was estimated by gel filtration chromatography as previously reported (Humiski & Aluko, 2007) with some modifications. The peptide sample (10 mg/ml) was filtered using a 0.2 μm membrane and 100 μl of the solution was injected onto a Superdex 75 100/300L column connected to an AKTA fast protein liquid chromatography system (GE Healthcare, Montreal, QC, Canada). The column was equilibrated with 1 column volume (23.5 ml) of 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.0) prior to loading the sample. The peptides were eluted from the column using a flow rate of 0.75 ml/min and peaks were detected at 214 nm. The column was calibrated using the following standards: cytochrome c (12.3 kDa), aprotinin (6.5 kDa), pepstatin A (685.9 Da), glutathione (307.33 Da) and glycine (75 Da); blue dextran was used to determine the column void volume. The estimated molecular weights of the eluted peaks were determined from a plot of the elution volume versus the $\log_{10}$ MW of the standards.

### 6.2.7. Antioxidant assays

The ability of the FPH-mix peptide sample with the highest Fischer ratio to scavenge DPPH radical was evaluated as previously reported (Udenigwe et al., 2009a).

Superoxide ($O_2^{\cdot-}$) scavenging assay was also evaluated using the pyrogallol autooxidation method as previously described (Udenigwe et al., 2009a). Briefly, 80 µl of the peptide solution of various concentrations was mixed with 80 µl of 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA in a 96-well microplate followed by the addition of 40 µl of 1.5 mM pyrogallol in 10 mM HCl. The rate of $O_2^{\cdot-}$-induced polymerization pyrogallol ($\Delta A$/min) was measured as increase in absorbance at 420 nm for 4 min at room temperature. Tris-HCl buffer was used to replace the peptide solution in control experiment whereas glutathione was used as positive control. $O_2^{\cdot-}$ scavenging activity (%) was calculated as [($\Delta A$/min $c$ − $\Delta A$/min $s$)/ $\Delta A$/min $c$] × 100, where $c$ and $s$ represent control and sample, respectively.

In addition, $^{\cdot}$OH scavenging activity of the high Fischer ratio FPH-mix sample was assayed based on a method described by Li et al. (2008). Peptide samples (50 µl in 0.1 M sodium phosphate buffer, pH 7.4) were mixed with 50 µl of 3 mM 1,10-phenanthroline (in phosphate buffer) and 50 µl of 3 mM $FeSO_4$ (in water) in a 96-well microplate. Thereafter, 50 µl of 0.01% aqueous $H_2O_2$ was added to initiate the Fenton reaction. The reaction mixture covered and incubated at 37$^{\circ}$C for 1 h and the absorbance measured at 536 nm ($A_{536nm}$ $s$). A control experiment was also conducted by replacing the peptide sample with buffer ($A_{536nm}$ $c$). The $^{\cdot}$OH scavenging activity (%) was calculated as [($A_{536nm}$ $c$ − $A_{536nm}$ $s$)/$A_{536nm}$ $c$] × 100.

The ability of the flaxseed peptide to inhibit linoleic acid (LA) oxidation was also investigated as previously described (Li et al., 2008) with modifications. LA (1 ml of 50 mM dissolved in 99.5% ethanol) was mixed with 0.5 ml of a solution of different

concentrations of the peptide sample (final concentrations of 5, 2.5 and 1.25 mg/ml of

0.1 M phosphate buffer, pH 7.0) or glutathione (final concentration of 1.25 mg/ml). The

mixture was kept at 60$^{\circ}$C in darkness for 6 days. An aliquot of 0.1 ml of the sample

solution was withdrawn after every 24 hr, and mixed with 4.7 ml of 75% aqueous

ethanol, 0.1 ml of ammonium thiocyanate (30% w/v) and 0.1 ml of 20 mM FeCl$_2$

(dissolved in 1 M HCl). The absorbance of the mixture was measured at 500 nm after 3

min incubation at room temperature.

## 6.2.8. ACE and renin inhibition assays

The potential antihypertensive property of the FPH-mix sample with highest

Fischer ratio was determined based on its ability to inhibit the activities of ACE and renin

*in vitro*. At various concentrations of the peptide sample, ACE and renin inhibitory

assays were carried out as previously described using FAPGG as ACE substrate and Arg-

Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg as renin substrate

(*20*). In addition, the kinetics of inhibition of ACE was also studied to determine the

mode of the peptide-induced ACE inhibition. Kinetics parameters ($K_m$ and $V_{max}$ , and

their apparent values in the presence of the peptide sample) were estimated from non-

linear regression fits of the kinetics data to the Michaelis-Menten equation using

GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). The catalytic efficiency

of ACE in the presence and absence of the peptide sample was calculated as $V_{max}/K_m$. All

the kinetics experiments were conducted in duplicate.

**6.2.9. Statistical analysis**

Except where indicated, data were collected in triplicates and subjected to one way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p < 0.05$.

**6.3. RESULTS AND DISCUSSION**

**6.3.1. Enzymatic flaxseed protein hydrolysis**

Fig. 6.1 shows the procedure used in hydrolyzing the flaxseed protein and processing the resulting FPH. The aim of the enzymatic hydrolysis of flaxseed protein in this study was to remove the AAA, and also to generate low-MW peptides for efficient intestinal absorption. Thermolysin was chosen for the first hydrolysis step because of its specificity in cleaving proteins at the N-terminal regions of hydrophobic amino acids, phenylalanine, tyrosine, leucine, isoleucine and valine. In order to release the N-terminal AAA, the resulting protein hydrolysate was subjected to another controlled hydrolysis using other specific proteases, papain and ficin. Papain has a wide specificity but preferentially cleaves the $X_1–X_2$ peptide bond in Phe– $X_1–X_2$, where $X_1$ and $X_2$ are any amino acids, thereby releasing a Phe-containing dipeptide into the solution. A combination of thermolysin and papain has been previously used to produce high Fischer ratio peptide mixtures (Adachi et al., 1991; 1993). Ficin, a papain-like protease, was used as a control since it has a wide specificity in cleaving the carboxyl side of 10 different amino acid residues, including valine and tyrosine. Moreover, the highly non-

specific proteases were used to produce peptides of shorter chain length that could be easily absorbed in the intestine. Alcalase is an endopeptidase whereas pronase possesses both endopeptidase and exopeptidease activity and can generate low molecular weight peptides and free amino acids from native and denatured proteins and large peptides. Thus, pronase can cleave the N-terminal AAA resulting from the thermolysin activity. The protein contents of the FPH were 81% (papain), 85.2% (ficin), 66.3% (alcalase) and 60.7% (pronase) based on Lowry assay. The observed low protein contents of the FPH generated with pronase may be due to their exopeptidase activity, which released free amino acids that were not detected by the Lowry assay.

## 6.3.2. Activated carbon treatments, amino acid profiles and Fischer ratios

The FPH was passed through a tightly packed activated carbon column to remove AAA-containing peptides and recover the BCAA-rich peptides in the flow-through fraction. To improve the elution of the peptides from the column, the flaxseed protein hydrolysates were acidified prior to loading onto the activated carbon column. This treatment was necessary to precipitate any large peptides and residual undigested proteins, which have high adsorption capacity on activated carbon (Adachi et al., 1993) and may block the activated carbon matrix thereby decreasing elution efficiency. The absorbance values of the flow-through sample at 280 and 260 nm, compared to the crude hydrolysate solution, showed that over 90% of AAA were removed from the FPH. However, data from amino acid analysis of the freeze-dried powder showed that the activated carbon column specifically removed 39–57% of AAA from FPH. This resulted in

**Figure 6.1 Flowchart for production of branched-chain amino acids (BCAA)-enriched high Fischer ratio mixture by enzymatic hydrolysis of isolated flaxseed protein and activated carbon treatment**
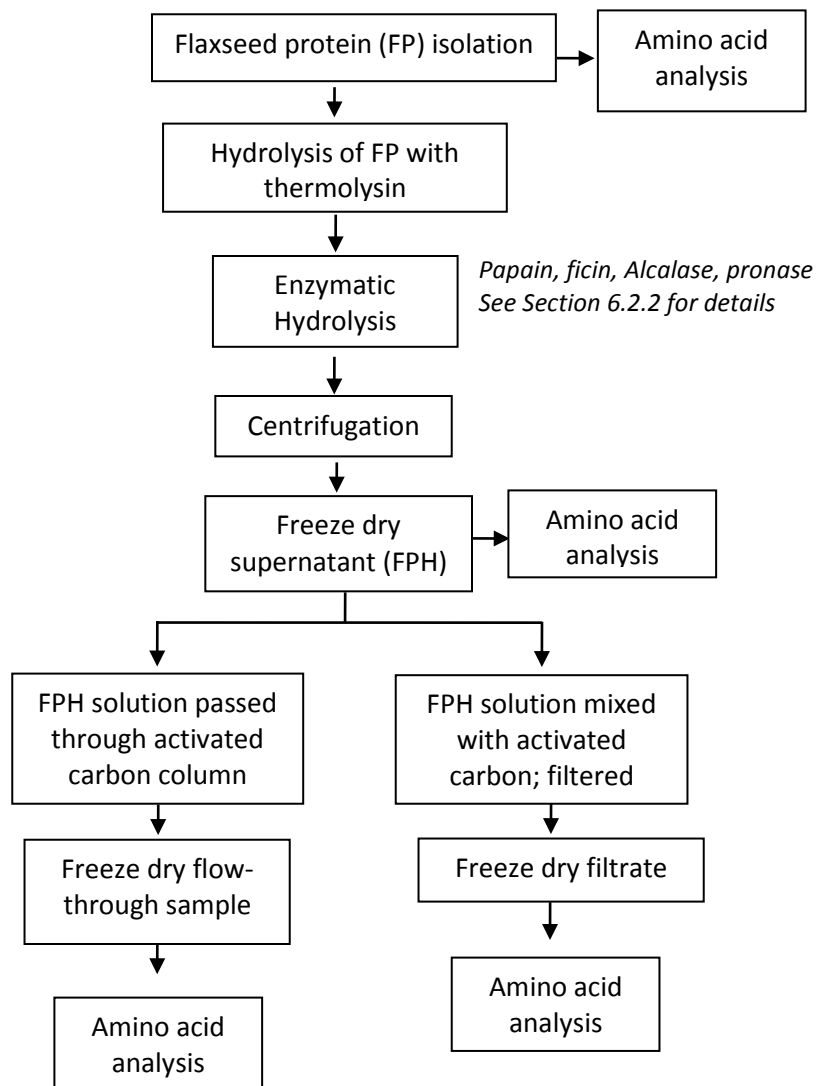
an overall increase in the Fischer ratio of the resulting FPH-col peptide samples when compared with the crude FPH (Table 6.1). However, the Fischer ratios of the FPH-col flow-through samples were low, ranging from 2.5 to 4.04 representing up to 2.3-fold increase relative to the crude FPH. The highest Fischer ratio (4.04) was observed for the hydrolysate product of thermolysin-pronase activity. It could be observed that increase in the Fischer ratio correlated with increase in the cleavage sites of the enzymes, except for alcalase. This showed that the less specific enzymes liberated smaller peptides that passed through the packed activated carbon in the column. The low Fischer ratio obtained in the flow-through samples indicated that some of the BCAA-containing peptides may have been trapped in the activated carbon matrix in the column. The result obtained in this study is contrary to that of a previous study that reported the preparation of protein hydrolysates with high Fischer ratio of 31.6 by enzymatic hydrolysis of casein and passage of the resulting acidified hydrolysates through an activated carbon-packed column (Adachi et al., 1993). Recently, Ma et al. (2008) also used similar activated carbon treatment to prepare a peptide mixture with Fischer ratio of 34.71 from Alcalase-papain hydrolyzed corn gluten meal. Due to the low Fischer ratio of the flaxseed peptide mixture, another approach was adopted to minimize BCAA loss while effectively removing the AAA-containing peptides and free AAA.

Mixing of the FPH with activated carbon resulted in concentration-dependent removal of AAA and decrease in the protein content. The optimized condition (2.5% activated carbon, w/v) that gave the highest peptide yield (unadsorbed peptides as $A_{220nm}$) with the removal of most of the AAA was used for the scale-up experiment. Data

**Table 6.1 Fischer ratios (BCAA/AAA) and Phe+Tyr contents of enzymatic flaxseed protein hydrolysates (FPH) passed through a column packed with activated carbon (FPH-col) or mixed with activated carbon inside a beaker (FPH-mix)[a,4]**

| | FPH Thermolysin | | | |
| --- | --- | --- | --- | --- |
| | Papain | Ficin | Alcalase | Pronase |
| **Fischer ratio** | | | | |
| FPH | 1.84 | 1.60 | 1.67 | 1.72 |
| FPH-col | 3.05 | 2.92 | 2.53 | 4.04 |
| FPH-mix | 2.80 | 6.21 | 4.26 | 23.65 |
| | | | | |
| **Phe+Tyr (%)** | | | | |
| FPH | 8.29 | 8.29 | 8.06 | 8.30 |
| FPH-col | 5.28 | 2.75 | 3.71 | 3.55 |
| FPH-mix | 5.06 | 5.03 | 4.95 | 1.11 |

[a]BCAA = branched-chain amino acids; AAA = aromatic amino acids

from amino acid analysis of the freeze-dried filtrate showed that the activated carbon resulted in the removal of 33–87% of AAA. The activated carbon treated FPH-mix sample from thermolysin-pronase activity had the highest amount of BCAA (29%) and lowest amount of phenylalanine+tyrosine (1.11%) with a Fischer ratio of 23.65, representing 13.7-fold increase relative to the crude FPH and 14.8-fold increase relative to the intact flaxseed protein (Table 6.2). In this sample, mixing with activated carbon

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[4] See Appendix D for protein contents and yield (Table S6.1) of the FPH treated samples and % aromatic amino acid removed as a result of the activated carbon treatments

decreased the AAA composition from 9.78% in the crude FPH to 1.23% with up to 2-fold enrichment of BCAA (Table 6.2). After similar activated carbon treatment, the other FPH-mix from papain, ficin and alcalase treatments had lower Fischer ratio of 2.8–6.2 due to their high AAA contents, although there was an increase in the Fischer ratio when compared with the crude FPH and isolated flaxseed protein. Protein hydrolysates or peptide mixtures of Fischer ratio higher than 20 and phenylalanine+tyrosine value lower than 2% can be used to treat patients with liver diseases (Okita et al., 1985); thus, these results show that the highest Fischer ratio flaxseed peptide mixture from thermolysin-pronase treatment has promising application for use in treating liver diseases in humans. Other recent studies have reported the production of peptide mixtures with Fischer ratios 24.58, 28.3 and 30.6 respectively from pearl oyster meat protein (Zheng et al., 2009), *Brassica carinata* protein (Pedroche et al., 2006) and bovine casein (Pedroche et al., 2004) generated through enzymatic hydrolysis, activated carbon treatment and gel filtration chromatography. Thus, the process reported in this paper represents a cost-effective method of production of high Fischer ratio peptide mixture from flaxseed protein since activated carbon is cheap and can be regenerated, and the process did not require gel filtration chromatography.

**Table 6.2 Percentage amino acid composition of flaxseed protein isolate (FPI), thermolysin-pronase hydrolysate (FPH), FPH passed through a column packed with activated carbon (FPH-col) and FPH mixed with activated carbon in a beaker (FPH-mix)**

| AMINO ACIDS | FPI | FPH | FPH-col | FPH-mix |
|---|---|---|---|---|
| ASX$^a$ | 11.29 | 10.83 | 11.40 | 8.27 |
| THR | 3.67 | 3.60 | 4.40 | 4.36 |
| SER | 4.34 | 4.99 | 6.28 | 5.79 |
| GLX$^b$ | 19.83 | 21.30 | 17.79 | 13.65 |
| PRO | 5.29 | 4.08 | 4.12 | 2.57 |
| GLY | 5.47 | 5.22 | 5.50 | 4.48 |
| ALA | 4.89 | 4.47 | 6.70 | 6.80 |
| CYS | 1.36 | 1.38 | 0.58 | 0.18 |
| VAL | 5.20 | 5.72 | 5.71 | 10.10 |
| MET | 2.02 | 1.49 | 1.10 | 0.76 |
| ILE | 4.50 | 5.14 | 4.35 | 7.56 |
| LEU | 5.80 | 5.93 | 6.61 | 11.34 |
| TYR | 2.52 | 2.78 | 1.19 | 0.28 |
| PHE | 5.47 | 5.52 | 2.36 | 0.83 |
| HIS | 2.29 | 2.23 | 3.12 | 3.27 |
| LYS | 3.04 | 3.03 | 4.98 | 5.97 |
| ARG | 11.28 | 10.79 | 13.23 | 13.68 |
| TRP | 1.73 | 1.48 | 0.57 | 0.12 |
| AAA$^c$ | 9.73 | 9.78 | 4.13 | 1.23 |
| BCAA$^d$ | 15.51 | 16.79 | 16.68 | 29.00 |
| FISCHER RATIO | 1.59 | 1.72 | 4.04 | 23.65 |
| PHE+TYR | 7.99 | 8.30 | 3.55 | 1.11 |

$^a$Asx, aspartic acid+asparagine; $^b$Glx, glutamic acid+glutamine; $^c$AAA: phenylalanine, tyrosine, tryptophan; $^d$BCAA: valine, leucine, isoleucine

### 6.3.3. Estimated peptide MW distribution

The MW of peptides is important in determining their absorption following oral intake. It is believed that low-MW peptides can be efficiently transported across the enterocytes through several peptide transporters. MW estimation using gel permeation chromatography showed that the high Fischer ratio FPH-mix contains peptides of sizes <3.96 kDa (Fig. 6.2). The first peak (1) contains oligopeptides of MW 357–3960 Da whereas peak 2 contains mostly dipeptides, tripeptides and free amino acids as indicated by the MW range of 68–353 Da. The low-MW peptide content of this sample increases its potential for use as nutraceutical in treating liver diseases.

**Figure 6.2 Size-exclusion gel chromatogram of the high Fischer ratio flaxseed protein hydrolysate mixed with activated carbon (FPH-mix)**

| Peak | MW range (Da) |
|------|---------------|
| 1 | 3960 - 357.8 |
| 2 | 353.2 - 68.4 |
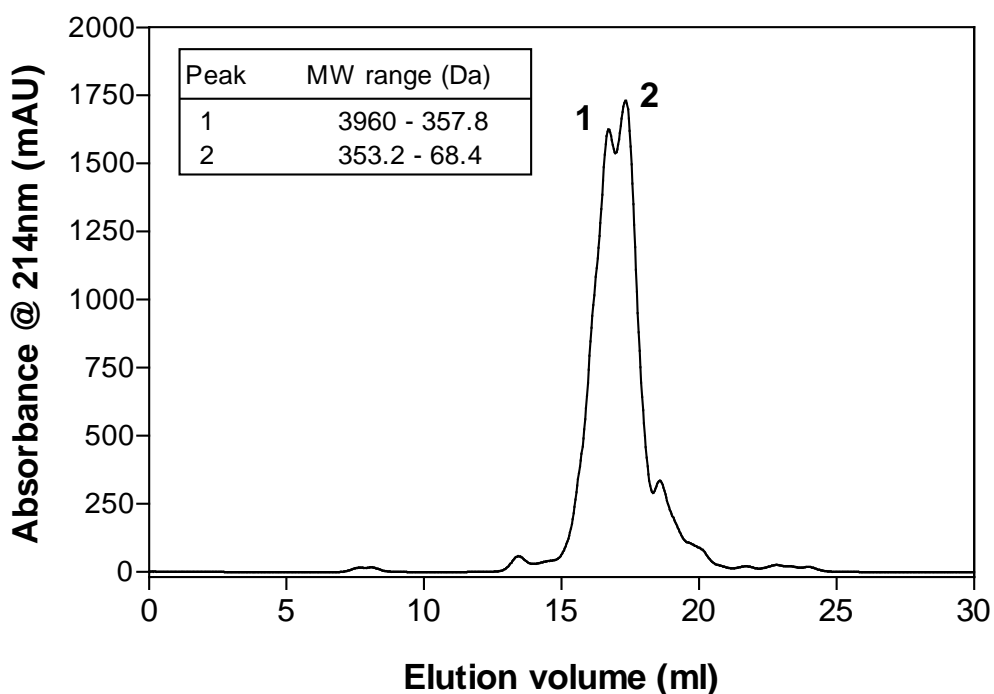
### 6.3.4. Antioxidant activity

DPPH radical is an N-containing radical used for the primary screening of antioxidant capacity of food components. The high Fischer ratio **FPH-mix** showed moderate DPPH radical scavenging activity at 5 mg protein/ml compared to glutathione (Fig. 6.3); dose-dependent assay was not conducted due to the low activity of the peptide sample in scavenging DPPH radical. This activity is weak compared to the DPPH radical scavenging activity previously reported for 1 mg/ml flaxseed protein hydrolysates (Udenigwe et al., 2009a) and 1.6 mg/ml alfalfa leaf protein hydrolysates (Xie et al., 2008), which scavenged more than 60% of DPPH radical. However, the high Fischer ratio **FPH-mix** showed concentration-dependent scavenging of $O_2^{\cdot-}$ produced from pyrogallol autoxidation (Fig. 6.4) with a 50% effective concentration ($EC_{50}$) value of 1.67 ± 0.19 mg protein/ml. At 4 mg protein/ml, the sample scavenged almost 100% of the $O_2^{\cdot-}$ produced in the reaction. In comparison, this antioxidant activity is stronger than the $O_2^{\cdot-}$ scavenging of low-MW flaxseed protein-derived peptides (Udenigwe et al., 2009a), but similar to the activity of three chickpea protein hydrolysate fractions that scavenged 47–69% of $O_2^{\cdot-}$ at 2 mg/ml (Li et al., 2008). Excessive $O_2^{\cdot-}$ produced during oxidative stress, from mitochondrial electron leakage and activated macrophages, is implicated in the oxidative damage of biological macromolecules associated with degenerative diseases in humans. Thus, the high Fischer ratio peptide mixture produced in this work could offer protection to cellular components against these oxidative damages.

**Figure 6.3 DPPH radical scavenging activity of 5 mg protein/ml of the high Fischer ratio FPH-mix compared to glutathione**
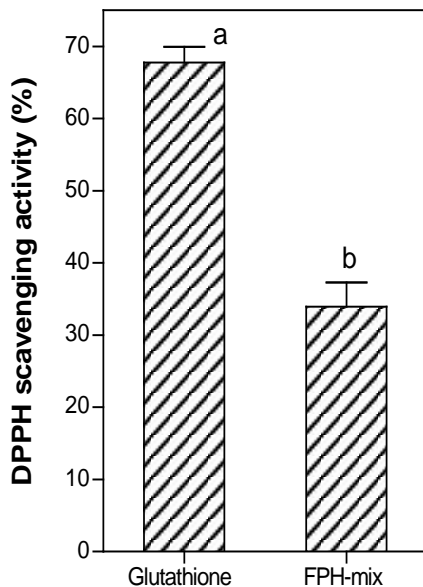


**Figure 6.4 Concentration-dependent scavenging of superoxide radical ($O_2^{\cdot-}$) by high Fischer ratio FPH-mix with effective concentration ($EC_{50}$) value of 1.67 mg protein/ml; bars with different letters are significantly different at $p < 0.05$**
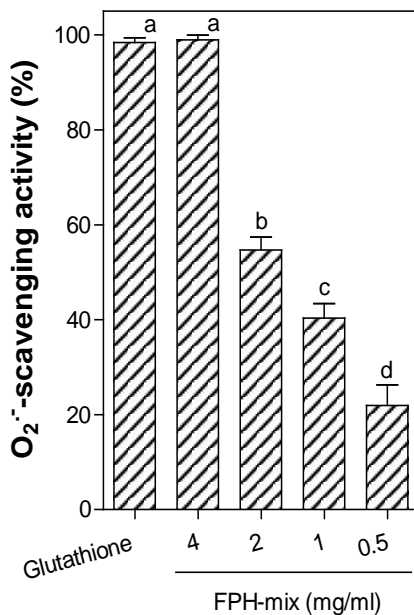
In addition, the flaxseed peptide mixture displayed moderate scavenging of ˙OH in a concentration-dependent fashion with a maximum scavenging of 31.5% at 5 mg protein/ml whereas glutathione showed similar scavenging activity at a lower concentration of 1 mg/ml (Fig. 6.5). This activity is weaker than those previously reported for low-MW peptides from flaxseed protein that scavenged more than 70% of ˙OH at <1.5 mg/ml (Udenigwe et al., 2009a) and chickpea protein hydrolysate fractions, which scavenged 38–81% of ˙OH at 1.5 mg/ml (Li et al., 2008). The moderate ˙OH scavenging activity of *FPH-mix* may be due to the low levels of phenylalanine present in the peptide mixture. This is because phenylalanine possesses strong radical-scavenging property since it can trap ˙OH via hydroxylation of the aromatic ring leading to the formation of stable *ortho-*, *meta-* or *para-*tyrosine (Sun et al., 1993).

**Figure 6.5 Concentration-dependent scavenging of hydroxyl radical (˙OH) by branched-chain amino acid (BCAA)-rich FPH-mix; bars with different letters are significantly different at p<0.05**
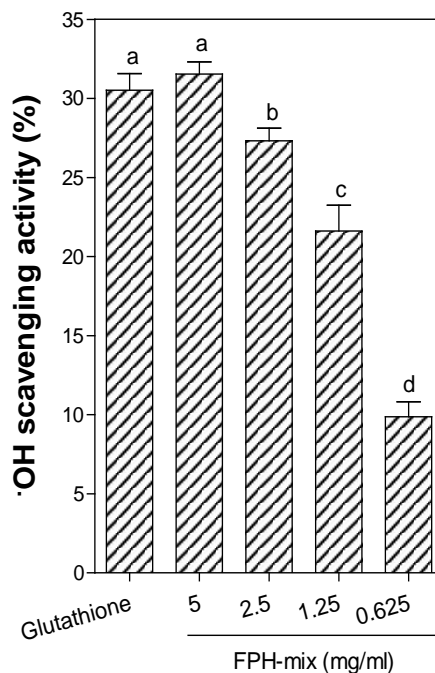
The **FPH-mix** with highest Fischer ratio (BCAA-rich) was found to show strong inhibition of linoleic acid oxidation in an *in vitro* evaluation system. As observed in Fig. 6.6, the increase in $A_{500nm}$ indicates an increase in linoleic acid oxidation, and this occurred during the first three days followed by a gradual decrease during the next three days. On day 1, the BCAA-rich mixture (1.25–5 mg/ml) inhibited approximately 45% of linoleic acid oxidation whereas glutathione (1.25 mg/ml) was over 2-fold more active. As glutathione lost its activity in protecting linoleic acid from oxidation between days 2–6, the BCAA-rich sample maintained antioxidant property at the three concentrations. Glutathione lost its activity with time since its antioxidant function group, the –SH group of cysteine, could not have been regenerated during the assay. In the last two days of the experiment, 5 mg/ml of the BCAA-rich sample displayed the best inhibitory property against linoleic acid oxidation ($p < 0.05$). On the 6th day, 5, 2.5 and 1.25 mg/ml of the peptide mixture concentration-dependently inhibited linoleic acid oxidation by 48%, 37.5% and 36.4%, respectively whereas glutathione completely lost its activity. In contrast, a chickpea protein hydrolysate fraction displayed a stronger activity by inhibiting *in vitro* linoleic acid peroxidation by 81% at 1 mg/ml after 8 days of incubation (Li et al., 2008). The oxidation of polyunsaturated fatty acid components of biological membranes is an important mechanism of cellular damages induced by reactive oxygen species; thus, the BCAA-rich sample from flaxseed protein may offer protection to cellular components by reducing oxidative damages.

**Figure 6.6 Protective effect of different concentrations of the high Fischer ratio FPH-mix and glutathione against linoleic acid oxidation; data points at day 6 with different letters are significantly different at P=0.05**
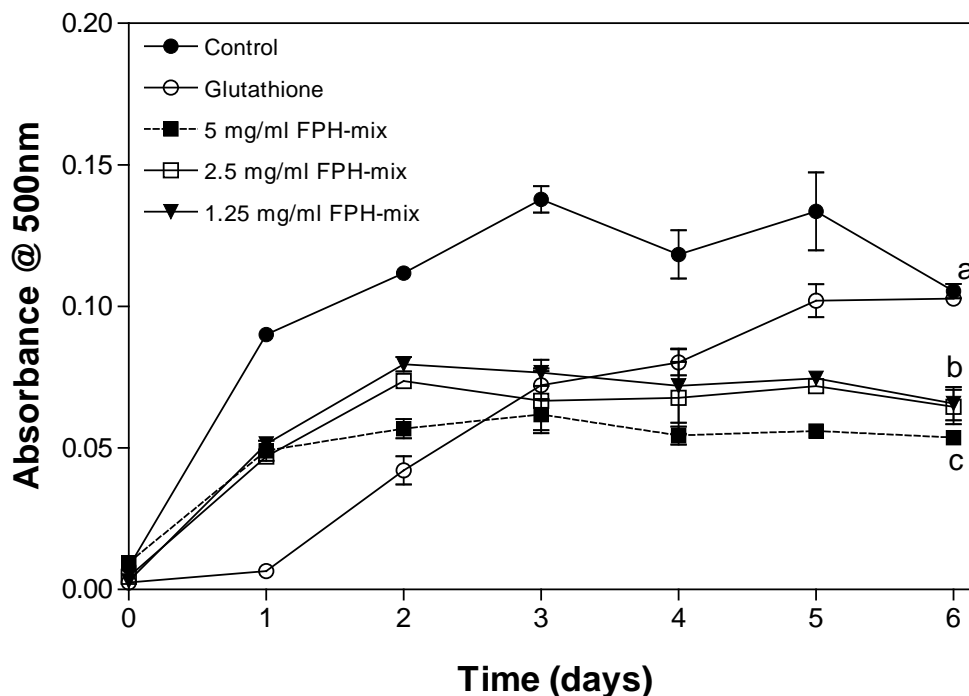


### 6.3.5. Antihypertensive properties

In addition to its antioxidant properties, the BCAA-rich sample also displayed potential antihypertensive property as evident from the concentration-dependent inhibition of ACE activity with up to 70.8% ACE inhibition at 0.4 mg/ml (Fig. 6.7) and 50% inhibitory concentration ($IC_{50}$) value of 0.16 ± 0.0055 mg/ml. This $IC_{50}$ value is comparable to the ACE-inhibitory activity of low-MW FPH from pancreatin hydrolysis ($IC_{50}$ = 0.151 mg/ml), but weaker than the activity observed for <1 kDa thermolysin prepared FPH with $IC_{50}$ value of 0.0275 mg/ml (Udenigwe et al., 2009b) and crude α-zein hydrolysates with $IC_{50}$ value of 0.021 mg/ml (Miyoshi et al., 1995). Considering the

role of hydrophobic amino acids in ACE inhibition (Wu et al., 2006), it could be that the decreased amount of AAA in the BCAA-rich sample contributed to the decreased potency of the peptides in ACE inhibition when compared to the previously reported flaxseed peptide mixtures (Udenigwe et al., 2009b).

Kinetic studies of ACE inhibition using the Lineweaver-Burk plot showed that the BCAA-rich sample inhibited ACE in a mixed-type inhibition pattern (Fig. 6.8). This was characterized by an increase in $K_m$ from 0.3121 mM for the uninhibited reaction to 0.6179 and 0.5860 mM in the presence of 0.1 and 0.4 mg/ml peptides, respectively. The reaction $V_{max}$ also decreased in a concentration-dependent fashion from 0.0112 $\Delta A$ /min for the uninhibited reaction to 0.009416 and 0.004521 $\Delta A$ /min in the presence of 0.1 and 0.4 mg/ml of the inhibitor, respectively. These results are similar to the mode of ACE inhibition reported for low-MW crude and cationic peptide fractions from flaxseed protein (Udenigwe et al., 2009b). This indicates that the constituent peptides in the BCAA-rich sample inhibited ACE activity by binding both the enzyme active and allosteric sites; in other words, the peptides (or amino acids) interacted with ACE in both its free and substrate-bound forms. This shows that ACE inhibition by the BCAA-rich sample cannot be completely overcome by increasing the substrate concentration. The catalytic efficiency of ACE was observed to decrease from 0.0358 to 0.0152 and 0.0077 in the presence of 0.1 and 0.4 mg/ml of the peptides, respectively, which indicates that inhibition of ACE activity by the BCAA-rich sample was concentration-dependent. In contrast to these results, Cinq-Mars et al. (2008) reported that both fractionated and unfractionated Pacific hake fillet hydrolysates exhibited competitive mode of ACE

inhibition using hippuryl-histidyl-leucine as ACE substrate. Information on the kinetics of

ACE inhibition by a peptide mixture could contribute to the elucidation of its mode of

action prior to *in vivo* blood pressure-lowering studies.

**Figure 6.7 Concentration-dependent inhibition of ACE activity by high Fischer ratio**

**FPH-mix with 50% inhibitory concentration (IC$_{50}$) value of 0.16 mg protein/ml**
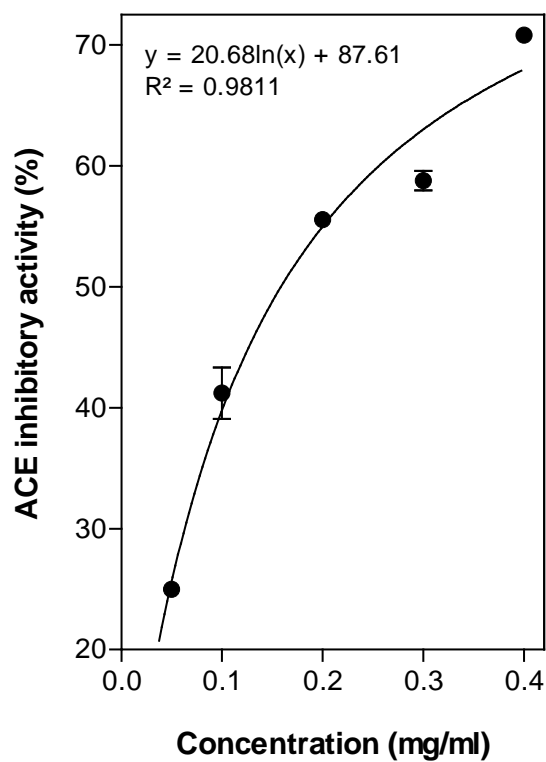
**Figure 6.8 Double reciprocal plot of the inhibition of ACE activity by 0.1 and 0.4 mg/ml of the high Fischer ratio FPH-mix indicating non-competitive mode of inhibition**
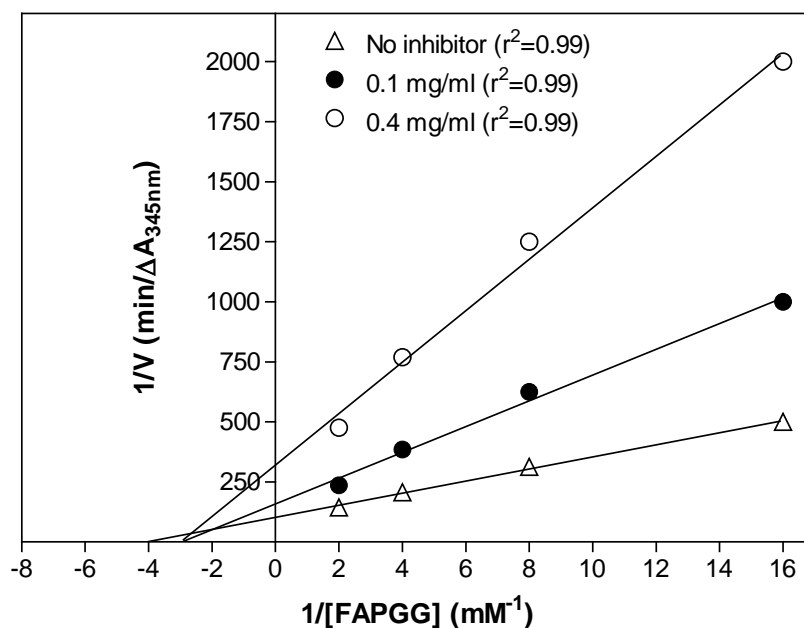


In addition to ACE inhibition, molecules that can inhibit the activity of renin can potentially provide better blood pressure lowering activity since renin controls the rate-limiting step of the renin-angiotensin system. In our present study, the BCAA-rich sample did not show any considerable inhibitory activity against renin; the peptide mixture inhibited 7.1% renin activity at 4 mg/ml. In contrast, a recent study reported that low-MW flaxseed protein-derived peptides moderately inhibited human renin *in vitro* by 50% at concentrations ranging from 1.21 to 2.81 mg/ml (Udenigwe et al., 2009b). The renin-angiotensin system has been widely studied as target for the treatment of hypertension in humans, and a wide range of food proteins have afforded ACE-inhibiting peptides, some with antihypertensive activities in animals and humans.

## 6.4. CONCLUSIONS

In conclusion, treatment of flaxseed protein isolate with thermolysin and pronase followed by mixing with activated carbon afforded a BCAA-rich peptide mixture with high Fischer ratio. The multifunctional bioactive properties of the peptide will potentially enhance the value-added utilization of defatted flaxseed meal by making it an important raw material in the functional foods and nutraceuticals industry. Animal feeding experiments using this peptide preparation need to be conducted in animal model of liver disease to evaluate the *in vivo* effects of the peptides in improving plasma Fischer ratio and reducing muscle wasting.

**Abbreviations Used.** AAA, aromatic amino acids; ACE, angiotensin I-converting enzyme; BCAA, branched-chain amino acids; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FAPGG, *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine; FPI, flaxseed protein isolate; FPH, flaxseed protein hydrolysates; LA, linoleic acid; MW, molecular weight

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**APPENDIX C: SUPPLEMENTAL INFORMATION**

**Figure S6.1 Optimization of flaxseed protein hydrolysate (FPH) adsorption on activated carbon by mixing in a beaker. A solution of FPH was mixed with different amounts of activated carbon (0.0125–0.15 g) for 10 min followed by centrifugation. The absorbance of the supernatant was measured at 260 nm to represent an approximation of the amount of Tyr, which was used to calculate the % adsorbed aromatic amino acids (AAA). Increase in the amount of activated carbon increase the amount (%) of AAA removed from FPH**
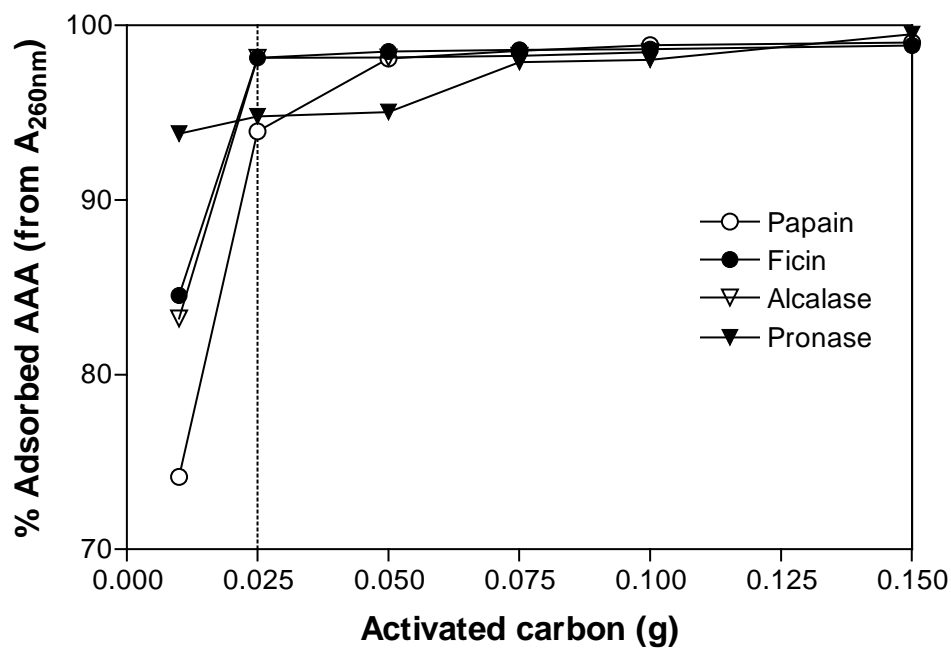
**Figure S6.2 Optimization of flaxseed protein hydrolysate (FPH) adsorption on activated carbon by mixing in a beaker. The absorbance values at 280 nm represent an approximation of the amount of Phe and Trp, and these were also used to calculate the % adsorbed aromatic amino acids (AAA). Increase in the amount of activated carbon increase the amount (%) of AAA removed from FPH**
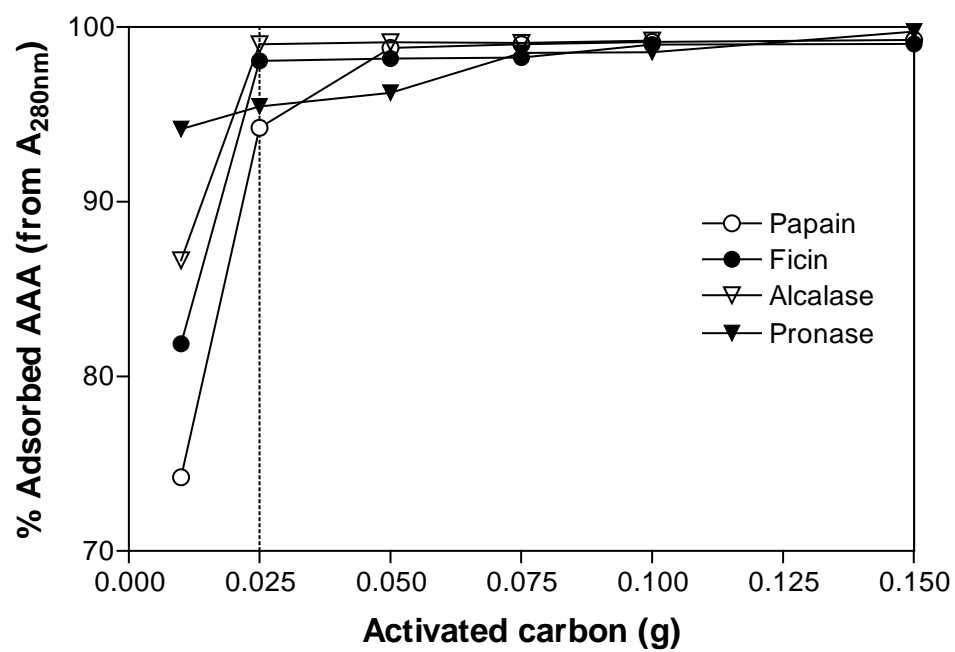
**Figure S6.3 Optimization of flaxseed protein hydrolysate (FPH) adsorption on activated carbon by mixing in a beaker. The absorbance of the supernatant at 220 nm was measured to represent approximate measurements of the FPH sample peptide concentrations. The activated carbon treatment that efficiently removed most of the aromatic amino acids (AAA) with a high protein yield (0.025 g activated carbon) was selected for scale-up experiments**
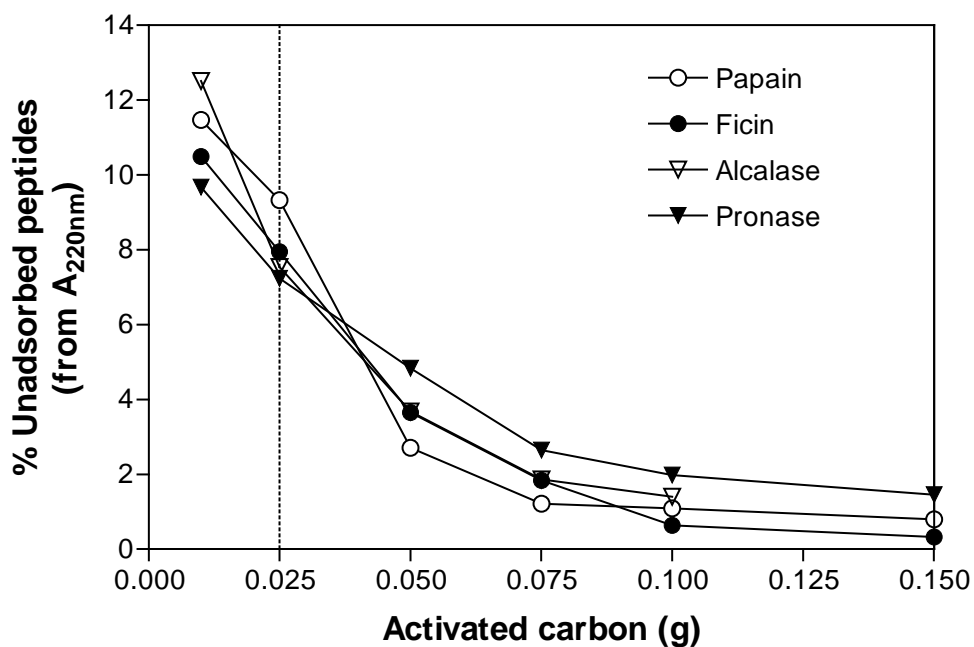
**Figure S6.4 Aromatic amino acids (%) removed from the flaxseed protein hydrolysate (FPH) passed through a column packed with activated carbon (FPH-col) or mixed with activated carbon (FPH-mix) for the different enzyme treatments (based on the complete amino acid profile of the FPH samples). This shows that simple mixing of FPH solution with activated carbon is generally more effective in removing aromatic amino acids (AAA) than passing the FPH through an activated carbon packed column; this could be due to the increased surface area during the former treatment that promotes binding of the AAA to the activated carbon matrix**
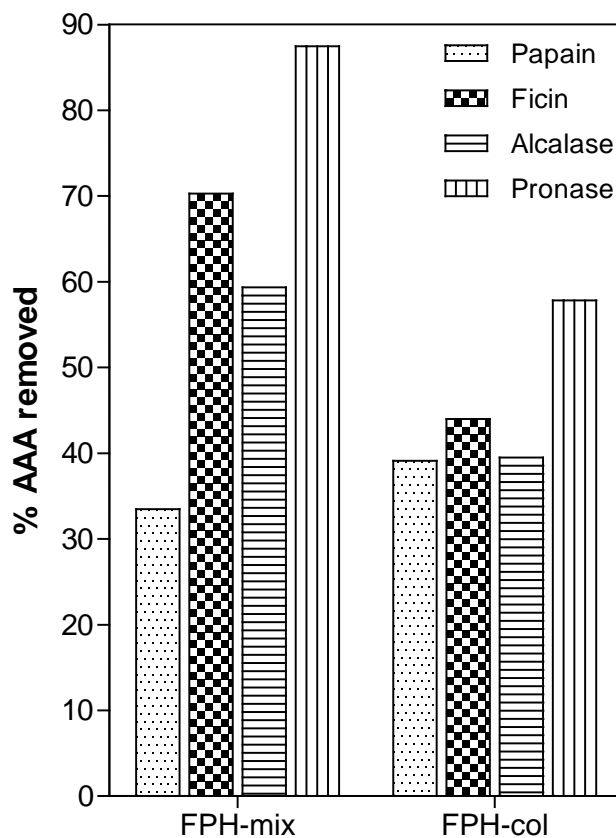
**Table S6.1 Protein contents and yield of flaxseed protein hydrolysate (FPH), FPH passed through a column packed with activated carbon (FPH-col) or mixed with activated carbon in a beaker (FPH-mix) for the different enzymatic treatments**

| | FPH Thermolysin | | | |
|---|---|---|---|---|
| | Papain | Ficin | Alcalase | Pronase |
| **Protein content (%)** | | | | |
| FPH[a] | 81.0 | 85.2 | 66.3 | 60.7 |
| FPH-col[b] | 90.1 | 97.8 | 72.6 | 77.4 |
| FPH-mix[b] | 77.1 | 83.1 | 60.0 | 48.0 |
| | | | | |
| **% Yield (DWB)[c]** | | | | |
| FPH[a] | 90.6 | 96.9 | 88.2 | 96.6 |
| FPH-col[b] | 15.5 | 15.0 | 22.0 | 16.0 |
| FPH-mix[b] | 23.0 | 16.5 | 24.0 | 18.0 |
| | | | | |
| **%Yield (Protein basis)** | | | | |
| FPH[a] | 73.4 | 82.6 | 58.5 | 58.6 |
| FPH-col[b] | 13.9 | 14.7 | 15.9 | 12.4 |
| FPH-mix[b] | 17.7 | 13.7 | 14.4 | 8.6 |

[a]From flaxseed protein isolate; [b]From the corresponding flaxseed protein hydrolysates (FPH); [c]DWB, dry weight basis

**Table S6.2 Amino acid composition (%) of the activated carbon treated FPH samples (FPH-col and FPH-mix)**

| Amino acids | FPH[e] | FPH-mix[e] | FPH-col[e] | FPH[f] | FPH-mix[f] | FPH-col[f] | FPH[g] | FPH-mix[g] | FPH-col[g] |
|---|---|---|---|---|---|---|---|---|---|
| Asx[a] | 10.98 | 10.39 | 11.93 | 11.17 | 11.06 | 11.62 | 11.05 | 10.37 | 11.27 |
| Thr | 3.59 | 4.30 | 3.96 | 3.65 | 3.88 | 4.12 | 3.61 | 4.12 | 3.92 |
| Ser | 5.11 | 6.20 | 6.06 | 5.01 | 5.63 | 5.84 | 5.07 | 6.15 | 6.53 |
| Glx[b] | 21.57 | 17.12 | 20.79 | 21.28 | 19.31 | 19.33 | 21.51 | 20.17 | 21.00 |
| Pro | 4.44 | 4.67 | 4.24 | 4.02 | 4.30 | 4.63 | 3.96 | 3.92 | 4.27 |
| Gly | 5.60 | 5.86 | 6.12 | 5.52 | 5.58 | 5.69 | 5.56 | 5.97 | 6.79 |
| Ala | 4.71 | 6.48 | 5.62 | 4.80 | 5.45 | 6.10 | 4.76 | 5.99 | 6.16 |
| Cys | 1.44 | 0.26 | 0.72 | 1.31 | 0.74 | 0.65 | 1.34 | 0.53 | 0.98 |
| Val | 5.03 | 6.12 | 5.19 | 5.70 | 5.66 | 5.75 | 5.37 | 5.88 | 4.87 |
| Met | 1.29 | 0.99 | 1.34 | 1.23 | 1.06 | 1.30 | 1.18 | 0.91 | 1.25 |
| Ile | 4.47 | 4.73 | 4.45 | 4.77 | 4.97 | 4.81 | 4.52 | 4.41 | 3.96 |
| Leu | 5.92 | 6.78 | 6.01 | 5.99 | 6.08 | 6.09 | 5.97 | 6.13 | 5.68 |
| Tyr | 2.74 | 0.78 | 1.75 | 2.66 | 1.64 | 1.70 | 2.65 | 1.16 | 1.68 |
| Phe | 5.55 | 1.97 | 3.28 | 5.63 | 3.64 | 3.36 | 5.41 | 2.55 | 3.27 |
| His | 2.24 | 3.40 | 2.67 | 2.25 | 2.85 | 2.73 | 2.44 | 3.15 | 2.58 |
| Lys | 3.11 | 5.27 | 3.46 | 3.03 | 4.08 | 4.00 | 3.10 | 4.84 | 3.88 |
| Arg | 10.94 | 14.59 | 12.09 | 11.32 | 13.39 | 11.90 | 11.06 | 13.61 | 11.13 |
| Trp | 1.26 | 0.09 | 0.32 | 0.68 | 0.68 | 0.40 | 1.43 | 0.15 | 0.79 |
| AAA[c] | 9.55 | 2.84 | 5.35 | 8.96 | 5.96 | 5.46 | 9.49 | 3.86 | 5.74 |
| BCAA[d] | 15.42 | 17.63 | 15.65 | 16.46 | 16.71 | 16.65 | 15.85 | 16.43 | 14.51 |
| Fischer ratio | 1.61 | 6.21 | 2.92 | 1.84 | 2.80 | 3.05 | 1.67 | 4.26 | 2.53 |

[a]Asx, aspartic acid+asparagine; [b]Glx, glutamic acid+glutamine; [c]AAA: phenylalanine, tyrosine, tryptophan; [d]BCAA: valine, leucine, isoleucine; [e]Thermolysin-ficin sample; [f]Thermolysin-papain sample; [g]Thermolysin-Alcalase sample

**TRANSITION STATEMENT**

In addition to its rich BCAA content, flaxseed protein also contains a high amount of arginine, a basic/cationic amino acid. Arginine plays several physiological roles especially as a precursor for the synthesis of nitric oxide (NO), a vasodilating gaseous agent previously known as endothelial-derived relaxation factor (EDRF). It is thought that arginine-enriched peptides could serve as vehicles to deliver arginine into the vascular endothelium for increased NO production, and that this could lead to antihypertensive activity and other beneficial physiological activities. Therefore, the sixth objective of this project, discussed in the next chapter, was to use controlled enzymatic hydrolysis to release arginine-rich peptides from flaxseed proteins and to separate these peptides using electrodialysis-ultrafiltration, a novel membrane technology that separates biomolecules based on a combination of molecular size and net molecular charge.

**CHAPTER SEVEN**

**MANUSCRIPT 5**

**SEPARATION OF ARGININE-RICH PEPTIDES FROM FLAXSEED PROTEIN HYDROLYSATES BY ELECTRODIALYSIS-ULTRAFILTRATION: ANGIOTENSIN CONVERTING ENZYME, RENIN-INHIBITORY AND ANTIHYPERTENSIVE ACTIVITIES OF THE PEPTIDES**

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**7.0. ABSTRACT**

Flaxseed protein isolate (FPI) contains high amount of arginine, which plays important physiological roles especially as nitric oxide precursor in the vascular endothelium. In this study, enzymatic hydrolysis of FPI with trypsin and pronase resulted in a hydrolysate (FPH), which was fractionated using electrodialysis-ultrafiltration (EDUF) to concentrate the arginine-rich peptides. The EDUF experiment resulted in migration of anionic (KCl 1) and cationic peptides (KCl 2) to the recovery compartments of the set-up with $1.10 \pm 0.15\%$ and $1.46 \pm 0.09\%$ peptide yields, respectively; more FPH peptides migrated to KCl 2 than KCl 1 and this was confirmed by mass spectrometry scan of the fractions. Compared to FPI and FPH with 11% arginine, about one-third of KCl 2 was composed of arginine. The arginine-rich peptides moderately inhibited angiotensin converting enzyme and renin activities, and decreased systolic blood pressure in spontaneously hypertensive rats. Thus, the multifunctional properties of this peptide product will be relevant in clinical intervention against human health conditions especially hypertension.

## 7.1. INTRODUCTION

Apart from its role as precursor for proline and glutamate synthesis, arginine (2-amino-5-guanidinopentanoic acid), a conditionally essential basic/cationic amino acid, is converted to nitric oxide (NO) and citrulline in the human vascular endothelium by a $Ca^{2+}$/calmodulin-dependent endothelial nitric oxide synthase (Moncada & Higgs, 1993; Marin & Sessa, 2007; Pacher, Beckman & Liaudet, 2007). The NO produced from the L-arginine-NO pathway is a significant factor for decreasing vascular resistance and can be used to treat disease symptoms such as hypertension, vasospasm, cerebral ischemia, preeclampsia, male impotence and bronchial asthma (Moncada & Higgs, 1993, 1995; Marin & Sessa, 2007; Pacher et al., 2007; Coman, Yaplito-Lee & Boneh, 2008; Pasquale, 2008; Heffernan, Fahs, Ranadive, & Patvardhan, 2010). NO can also react with homocysteine, a key indicator for the risk of cardiovascular disease, to form the non-toxic S-nitroso-homocysteine, which also possesses vasodilatory property (Perna, Ingrosso, & De Santo, 2003). In addition, L-arginine is required for hepatic synthesis of creatine which is subsequently transported to the muscle where it is phosphorylated to form phosphocreatine, an important energy source for cellular energy and muscular contraction (Pasquale, 2008). It was demonstrated through several reports that L-arginine plays important roles in enhancing immune system function (Park, Hayes, Garlick, Sewell & Eremin, 1991; Delage et al., 2010), neurotransmission, cell signalling, ammonia detoxification through the urea cycle (Coman et al., 2008), and amelioration of experimental kidney diseases (Ketteler, Border & Noble, 1994). Some products of the arginine metabolism pathways also function in prevention and inhibition of early stages

of certain cancers (Lowell, Parnes & Blackburn, 1990; Delage et al., 2010; Ma et al., 2010). Moreover, human clinical interventions with L-arginine in free amino acid form have resulted in endothelium-dependent vascular dilation in patients with endothelial dysfunction but not in healthy individuals during short and long-term intakes (Heffernan et al., 2010). These physiological effects of arginine have elicited our interest in the development of arginine-enriched nutraceutical peptide products, from low value flaxseed processing by-product, that can be used to increase physiological arginine levels when needed, with concomitant increase in the active endogenous NO levels especially in patients with endothelial dysfunction.

A recent survey reported that Canadian flaxseed from 2009 harvest contained about 22% proteins (N × 6.25) with a 10-year mean of 23.2% (Canadian Grain Commission, 2009). In 2007-2008, the world flaxseed meal production was estimated at 1.4 million tonnes (Agriculture and Agri-Food Canada, 2007); this translates to about 0.31 million tonnes of flaxseed proteins. This large amount of flaxseed proteins produced annually has not been efficiently incorporated into the human food system, but flaxseed meal has been fed to livestock for protein and fibre supplementation (Agriculture and Agri-Food Canada, 2007; Bell & Keith, 1993). Flaxseed protein contains a high amount of arginine (up to 11.2%) (Oomah & Mazza, 1995; Udenigwe & Aluko, 2010), when compared with other food proteins such as soy (7.6%), pea (8.2%), rapeseed (7.0%), wheat (4.4%) and egg white (5.48%) (Sarwar et al., 1983). Therefore, the global flaxseed meal production can generate more than 0.034 million tonnes (34 million kg) of arginine per year. Therefore, flaxseed proteins represent an excellent

source for the production of nutraceutical peptides enriched with arginine. It is preferable to use peptides as a vehicle to deliver arginine into physiological locations as opposed to the use of individual amino acids because small peptides are more efficiently absorbed in the intestine and also due to the fact that osmotic pressures of peptides are lower than those of their individual amino acid constituents (Grimble, Rees & Keohane, 1997; Monchi & Rerat, 1993). Arginine-containing peptides have also been shown to have cell penetrating capacity without the need for cellular carriers (Kosuge, Takeuchi, Nakase, Jones, & Futaki, 2008; Schmidt, Mishra, Lai, & Wong, 2010). Therefore, arginine in the form of peptides can be more readily delivered into cells when compared to the free amino acid form. In addition, peptides may possess other physiologically-relevant bioactive properties due to the peptide bonds, configuration and sequence of amino acids as opposed to single amino acids.

Enzymatic hydrolysis has been extensively used in food processing to generate bioactive peptides, which are often further separated into fractions with enhanced activity. The choice of techniques for separating bioactive peptides from the complex mixture depends on molecular properties of the peptides. Since peptides resulting from extensive enzymatic hydrolysis of proteins often possess a narrow molecular size range, it is imperative to use alternative processing methods especially when the separation is not solely based on size (Firdaous et al., 2009). Electrodialysis-ultrafiltration (EDUF) is a novel membrane technology used in food processing to separate valuable biomolecules from complex mixture based on their electrical charge and molecular size (Bazinet, Amiot, Poulin, Labbé & Tremblay, 2005). This process can be used to separate peptide

mixtures into fractions containing cationic, anionic and neutral peptides, and has been previously applied in fractionation of tryptic digest of β-lactoglobulin (Poulin, Amiot & Bazinet, 2006) and alfalfa white protein (ribulose-1,5-bisphosphate carboxylase) hydrolysates (Firdaous et al., 2009). In both studies, the electroseparation process resulted in the migration of cationic and anionic peptides to the cathode and anode, respectively with the recovery of antihypertensive and angiotensin converting enzyme (ACE)-inhibiting peptides in the cationic compartments. The advantages of the EDUF process include simultaneous separation and concentration of the peptides, and the absence of fouling of ultrafiltration membranes as opposed to the conventional pressure-driven ultrafiltration and nanofiltration processes (Lapointe, Gauthier, Pouliot & Bouchard, 2005; Firdaous et al., 2009).

The objectives of this present study were to (1) use a controlled enzymatic process to hydrolyze flaxseed protein in order to release arginine-containing peptides, (2) use electrodialysis-ultrafiltration to concentrate these arginine-rich peptides into a fraction, (3) characterize the molecular constituents of the peptide fraction, and (4) determine the potential bioactive properties, specifically antihypertensive activity in spontaneously hypertensive rats and *in vitro* inhibition of the activities of ACE and renin, which are two important enzymes of the blood pressure regulating renin-angiotensin system.

## 7.2. MATERIALS AND METHODS

### 7.2.1. Materials

Bovine pancreas trypsin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and pronase from *Streptomyces griseus* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). NaCl and KCl were obtained from Laboratoire MAT (Québec, QC, Canada). The CMX-SB and AMX-SB membranes were purchased from Eurodia (Wissous, France) and the ultrafiltration membranes of 20 kDa molecular weight cut-off (MWCO) purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). All other chemical reagents were of analytical grade.

### 7.2.2. Enzymatic hydrolysis of flaxseed protein

Flaxseed protein hydrolysis was carried out using trypsin and pronase. Previously prepared flaxseed protein isolate (Udenigwe, Lin, Hou & Aluko, 2009) was suspended in distilled water (5% flaxseed protein, w/v) and the slurry was adjusted to pH 7.0 with 0.5 M NaOH and temperature of 37°C. Trypsin was added at an E/S of 1:100 to initiate protein hydrolysis, and the reaction mixture incubated for 2 h. During hydrolysis, the reaction was maintained at pH 7.0 with 0.5 M NaOH using a pH-Stat instrument (Metrohm Titrando, Herisau, Switzerland). The reaction was stopped by adjusting the mixture to pH 4.0 using 0.5 M HCl. Thereafter, the mixture was readjusted to pH 7.4 and temperature of 40°C, followed by the addition of pronase at E/S of 1:100; hydrolysis was continued for additional 2 h and terminated with 0.5 M HCl (pH 4.0). The resulting mixture was cooled to room temperature and centrifuged at 15,000 x g for 1 h. The

supernatant was collected as the flaxseed protein hydrolysate (FPH), adjusted to pH 6.5 using 0.5 M NaOH, freeze dried and stored at –20°C for further processing.

### 7.2.3. Electrodialysis-ultrafiltration (EDUF)

EDUF was used to separate the cationic peptides of flaxseed protein hydrolysate in order to obtain an arginine-enriched fraction. The experiment was conducted using a modified version of a previously described method (Firdaous et al., 2009) as shown in Figure 7.1. The electrodialysis cell had an effective surface area of 200 cm$^2$ and consisted of stacked membranes: CMX-SB (cation-exchange), AMX-SB (anion-exchange) membranes, and two neutral ultrafiltration membranes with MWCO of 20 kDa. The EDUF cell configuration consisted of two electrode rinsing compartments (containing 2 L of 0.34 M NaCl), one feed compartment (containing the 1 L of 0.5% FPH) and two permeate or recovery compartments (each containing 2 L of 0.0026 M KCl) connected to an IONICS Electrodialyzer (IONICS Inc., Watertown, MA, USA). Each compartment was connected to a separate external reservoir and re-circulated through the electrodialysis stack using four pumps at flow rates of 3 L/min for the NaCl pump and 1 L/min for the pumps connected to the feed and recovery compartments. Voltage (6–10V) and current (0.2-0.35A) were supplied to the set-up using a direct current generator. The experimental set-up was equipped with a pH meter model SP20 (Thermo Orion, West Chester, PA, USA) and an YSI conductivity meter model 3252 (Yellow Springs Instrument Co., Yellow Springs, OH, USA) for pH and conductivity measurements, respectively.

**Figure 7.1 Configuration of the electrodialysis cell; anionic (P–) and cationic peptides (P+) migrate to recovery compartments KCl 1 and KCl 2, respectively whereas peptides with zero net charge (P±) remain in the feed compartment; AEM, anion exchange membrane; CEM, cation exchange membrane; UFM, ultrafiltration membrane**
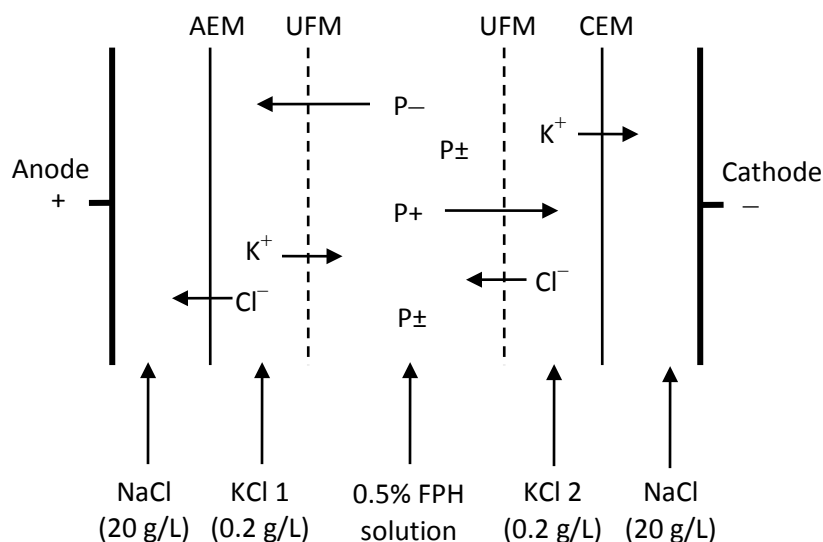


The electrodialysis experimental runs were conducted for 360 min (n=5) in a cold room (4°C). During the experiments, conductivity and pH of the solutions in the recovery and feed compartments were monitored, and about 1 ml aliquots of samples were withdrawn from the recovery compartments to determine peptide migration. After each electroseparation, the electrodialysis cell was thoroughly cleaned for 30 min using an alkaline solution followed by rinsing with distilled water, cleaning with acid solution for another 30 min and thorough rinsing with distilled water to neutral pH.

**7.2.4. Determination of peptide migration during EDUF**

The concentration of flaxseed peptides in the recovery compartments was monitored during EDUF. A 1-ml aliquot was withdrawn from the KCl 1 and KCl 2 compartments at intervals of 0, 30, 60, 120, 180, 240, 300 and 360 min during each electroseparation experiment. Peptide concentration in the solutions was determined in duplicates using the MicroBCA$^{TM}$ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL, USA) following the manufacturer's procedure. The peptide sample (100 μl) was mixed with 100 μl of the assay working reagent in a microplate and the mixture incubated for 2 h at 37°C. Thereafter, the solution was cooled to room temperature and the absorbance measured at 562 nm using a microplate reader. The peptide concentration (μg/ml) was calculated from a calibration curve prepared using the crude FPH as a reference standard sample.

**7.2.5. Amino acid analysis**

Amino acid profiles of the FPH and the flaxseed peptide fractions from EDUF was determined using the HPLC method after samples were hydrolyzed with 6 M HCl as previously reported (Bidlingmeyer, Cohen & Tarvin, 1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke, Wall, Absheer, Kaiser & Zumwalt, 1985) and tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992). The peptide samples were analyzed for amino acid composition by separation on an analytical column packed with a sulphonated polystyrene cation-exchange resin, followed by post-column derivatization with

ninhydrin (2,2-dihydroxy-1,3-indandione) at 135°C using a SYKNM Amino Acid Analyzer S433 system (SYKAM GmbH, Eresing, Germany). The amino acid composition was expressed as percentage of total amino acid content of each sample.

### 7.2.6. Liquid chromatography-mass spectrometry (LC-MS)

Molecular masses of peptides recovered in the KCl 1 and KCl 2 permeates after EDUF and hydrolysate solution were determined by LC-MS using an LC-MSD QUAD Agilent 1100 series system (Agilent Technologies, Palo Alto, CA, USA) equipped with two pumps (bin G1323A), an automatic sample injector (G1329A) and an in-line degasser. Peptides were loaded on a Luna 5 μm Phenomenex $C_{18}$ column (2 × 250 mm) and separated by gradient elution with 0.11% trifluoroacetic acid (TFA) in water (solvent A) and solvent B, which was a mixture of acetonitrile/water/TFA (90/10/0.1% v/v/v), at a flow rate of 0.2 mL/min. A linear gradient of 2–100% solvent B was applied for 115 min; the eluting peptides were detected with DAD G1315A detector at a wavelength of 214 nm (Firdaous et al., 2009). The MS signals were recorded in positive mode using a 90-V fragmentation with a scan range of 300–3000 $m/z$.

### 7.2.7. ACE and renin inhibition assays

The arginine-enriched peptide fraction was also evaluated for modulation of the RAS pathway through the *in vitro* inhibition of ACE and renin activities. The sample was desalted using an SPE column prior to the assays. The ACE and renin inhibition assays were conducted in duplicates as previously reported using *N*-(3-[2-furyl]acryloyl)-

phenylalanylglycyl-glycine (FAPGG) as ACE substrate and a fluorogenic peptide as renin

substrate (Udenigwe et al., 2009). The ACE and renin inhibitory activities of the peptide

sample were reported as percent inhibition at various sample concentrations and also

as the concentration that resulted in the inhibition of 50% enzyme activity ($IC_{50}$),

determined from non-linear regression plot.

**7.2.8. Evaluation of antihypertensive activity in spontaneously hypertensive rats (SHR)**

Animal experiments were carried out following the Canadian Council on Animal

Care ethics guidelines with a protocol approved by the University of Manitoba Animal

Protocol and Management Review Committee. The spontaneously hypertensive rats

(SHR) were kept in the Animal Housing Facility at the Richardson Centre for Functional

Foods and Nutraceuticals, University of Manitoba under a 12-h day and night cycle at

$21^oC$ and were fed a regular chow diet and tap water, *ad libitum*. The SHR were divided

into 6 groups that received the following treatments (dissolved in phosphate buffered

saline, pH 7.2): arginine-enriched flaxseed peptide fraction (n=3), free arginine amino

acid (n=6), FPH (n=3), FPI (n=6), captopril (positive control, n=12), saline (negative

control, n=12). The samples (each at 200 mg/kg body weight, BW) and captopril (3

mg/kg BW) were administered to the SHR by oral gavage followed by measurement of

systolic blood pressure (SBP) at 2, 4, 6, 8 and 24 h by the tail-cuff method in slightly

anesthetised rats. Prior to sample administration, the baseline (time zero) SBP was

determined. In order to mitigate the blood pressure (BP) depression effect of

isofluorane, the gas flow was optimized such that rats became conscious usually within

3-4 min after removal from the chamber, which provided enough time to perform the blood pressure measurement. Rats were first anesthetised in a chamber (maintained at about $40^{\circ}$C) with 4% isofluorane for 4 min. They were then removed from the isofluorane chamber and tail-cuff measurement of blood pressure performed in the unconscious state. The change in SBP ($\Delta$SBP, mmHg) was determined by subtracting the data for the different time points from their respective baseline data.

## 7.2.9. Statistical analysis

The experimental data were collected in replicates as indicated above and subjected to one way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p < 0.05$.

## 7.3. RESULTS AND DISCUSSION

### 7.3.1. Enzymatic hydrolysis of flaxseed protein

The flaxseed protein isolate was hydrolyzed with trypsin followed by pronase at the optimum reaction conditions of the enzymes to release peptides of interest. Trypsin was used for the first reaction because it specifically cleaves the carboxyl-terminal of the peptide bond that contains arginine or lysine leading to the release of several peptides with C-terminal arginine residues. Pronase was then used to generate low molecular weight (LMW) peptides from the resulting trypsin-treated hydrolysate since it possesses both endopeptidase and exopeptidease activities. It is expected that most of the LMW

peptides will contain at least a C-terminal arginine in their sequences. LMW peptides were produced during the process in order to increase the physiological relevance of the product since these peptides can be absorbed intact without gastrointestinal peptidolysis and transported to the cellular locations were activity is needed (Mathews and Adibi 1976; Roberts, Burney, Black, & Zaloga, 1999; Vermeirssen, Van Camp & Verstraete, 2004). After the trypsin-pronase hydrolysis, the resulting freeze-dried hydrolysates had a protein content of 84% and a dry weight yield of 80.9%.

### 7.3.2. Peptide migration during EDUF

The hydrolysate was adjusted to pH 7.0 prior to EDUF since the side (R) group of arginine (p$I$ = 10.76, p$K_{aR}$ = 12.48) and some other amino acids such as lysine (p$I$ = 9.74, p$K_{aR}$ = 10.53) and histidine (p$I$ = 7.59, p$K_{aR}$ = 6.00) will be positively charged at that pH while all the remaining amino acids will be negatively charged or neutral (Damodaran, 1996). This will ensure that peptides containing arginine, depending on their net charges, will migrate to the cathode during EDUF to yield an arginine-rich fraction that may also contain substantial amounts of lysine and tryptophan. During electroseparation of the flaxseed protein hydrolysates, peptide migration into the recovery compartments was monitored indirectly using conductivity and directly by measuring actual peptide concentrations in the compartments. As shown in Figure 7.2, the conductivity of solutions in the recovery compartments (KCl 1 and KCl 2) decreased after every 60 min over the 360-min duration of electroseparation of the peptides. The decrease in conductivity indicates peptide migration into the KCl compartments during

electrodialysis leading to counter-migration of $K^+$ and $Cl^-$ into the NaCl and feed compartments (Figure 7.1), as previously demonstrated in EDUF fractionation of other food protein hydrolysates (Poulin et al., 2006; Firdaous et al., 2009). This counter-migration of the ions in the process was also confirmed by the observed 20.5% increase in the conductivity (from $994.6 \pm 59.3$ to $1198.4 \pm 56.6$ μS/cm) of the FPH solution in the feed compartment after EDUF. The conductivity of the KCl 1 solution decreased by 11%, 16% and 22% at 60, 180 and 360 min, respectively, whereas demineralization of the KCl 2 compartment occurred at a higher rates of 20%, 37% and 55%, at the respective time points. This observation indicated that migration of the peptides to the cathode was over 2 times more than migration of peptides to the anode at pH 7.0, and this information also reflects on the final amounts of peptides in both compartments.

**Figure 7.2 Change in conductivity in KCl 1 (anionic) and KCl 2 (cationic) compartments during electrodialysis (n=5); decrease in the conductivity indicates peptide migration to the electrodes during electrodialysis leading to counter-migration of $K^+$ and $Cl^-$**
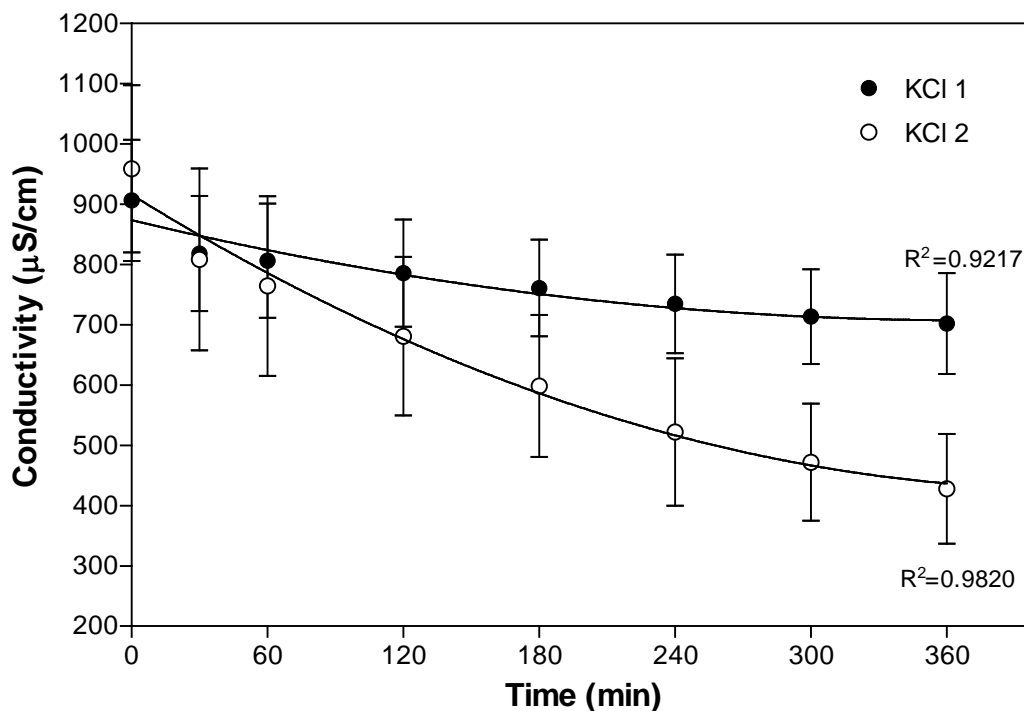


The peptide concentration in the compartments was also directly monitored during EDUF. As shown in Figure 7.3 using crude FPH solution as reference, there was an increase in the peptide concentration ($\mu$g/ml) in the KCl compartments with time. This observation also confirms the migration of flaxseed peptides to the electrodes at pH 7.0, and indicates that applied electrical field was the major force responsible for the separation of the peptides (Bargeman, Houwing, Recio, Koops & van der Horst, 2002; Firdaous et al., 2009). In addition, Figure 7.3 showed that, at the end of the experiment, the peptides that migrated to the cathode (73.2 ± 4.5 $\mu$g/ml) were significantly more in

amount than the peptides that migrated to the anode (55.2 ± 6.7 µg/ml); this supports

the conductivity data, which showed that peptide migration was more to the cathode

than to the anode. This was confirmed by the MS total ion current plot (TIC), shown in

Figure 7.4, which indicated that 13 or more peptides migrated to the KCl 2 compartment

while only 3 peptide peaks were observed in KCl 1. Thus, the FPH contained more net

positively-charged peptides than negatively-charged peptides at approximately pH 7.0.

The peptide distribution in the compartments observed in the TIC clearly showed the

effective selectivity of the EDUF process in separating peptides of similar MW and other

properties (e.g. elution time) but different net molecular charge. Based on the peptide

concentrations, the entire EDUF process (n=5) yielded a total of 551.9 mg and 732.7 mg

of the net anionic (KCl 1) and net cationic (KCl 2) peptides, respectively representing a

total yield of 1.10 ± 0.15% and 1.46 ± 0.09% from the original FPH.

**Figure 7.3 Peptide migrations during electrodialysis of flaxseed protein hydrolysates (n=5) as determined using the microBCA$^{TM}$ assay method; flaxseed protein hydrolysate (FPH) was used as reference in calculating the peptide concentrations**
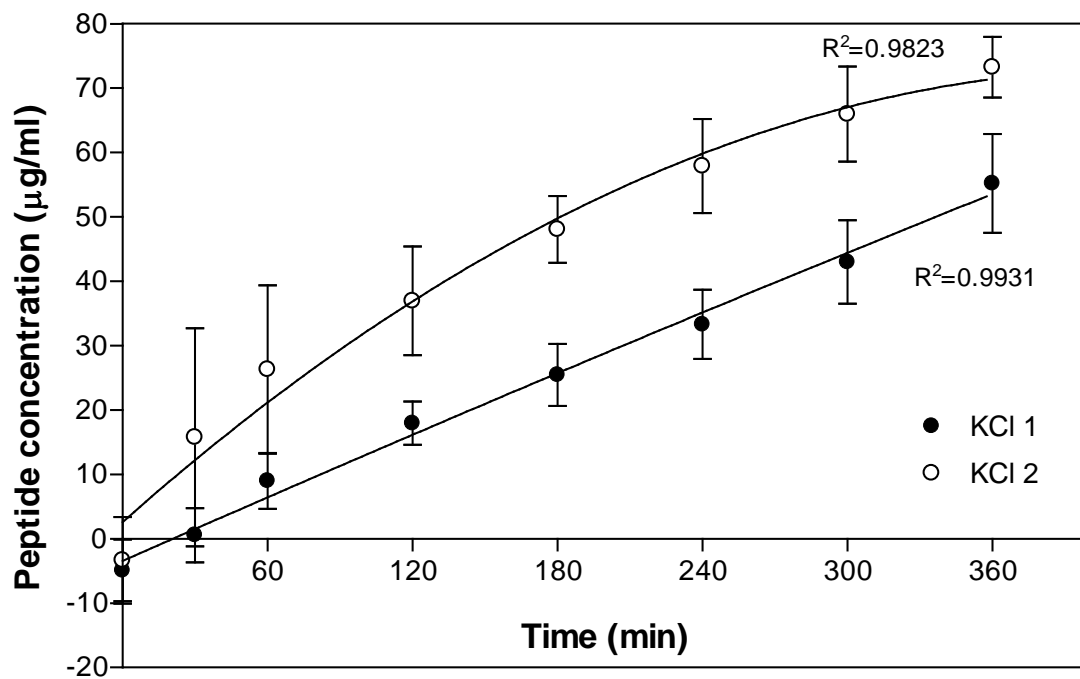
**Figure 7.4 Mass spectrometry total ion current plot (TIC) indicating migration of peptides from feed compartment (containing flaxseed protein hydrolysates, FPH) to the KCl 1 (anionic) and KCl 2 (cationic) compartments during electrodialysis-ultrafiltration**
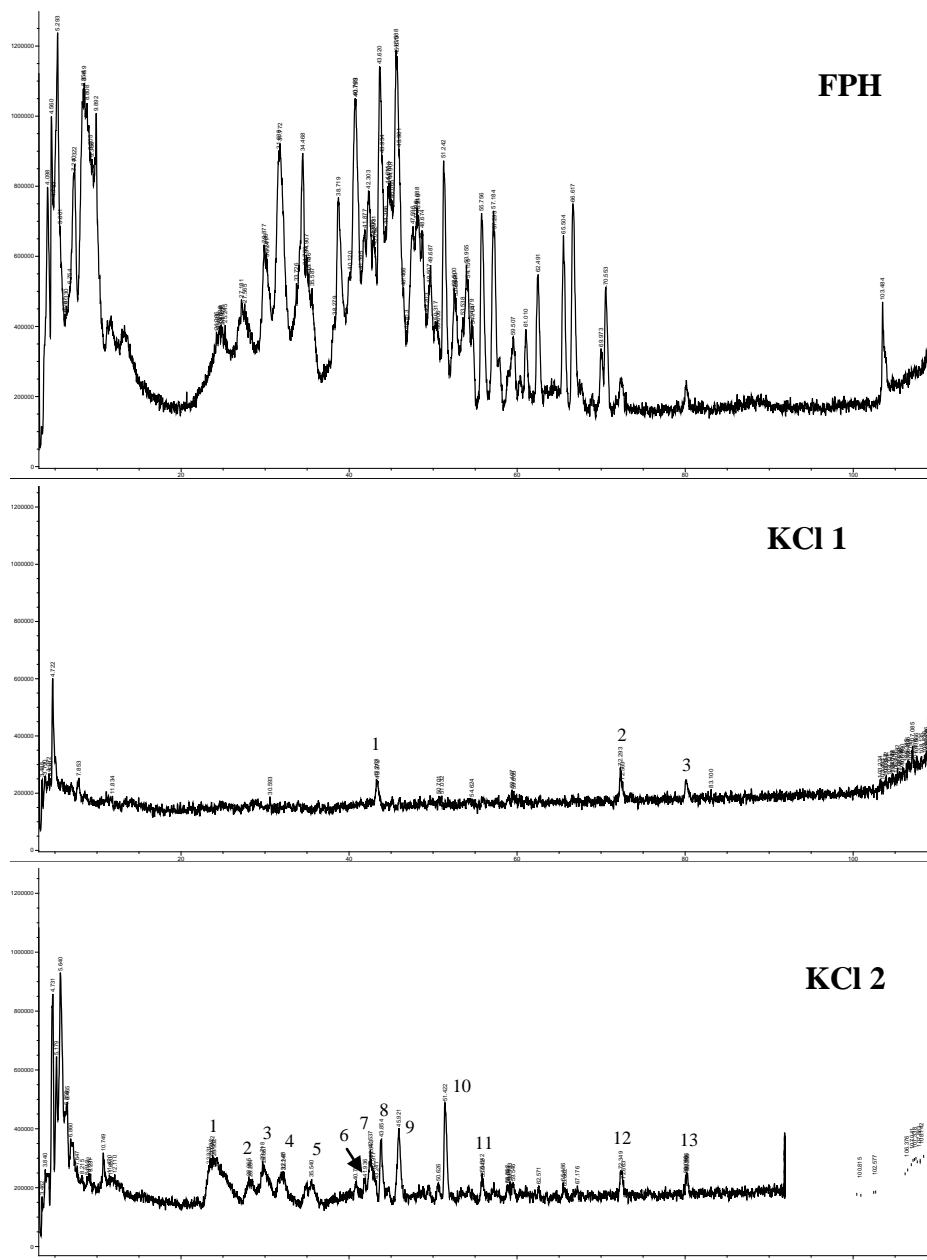
### 7.3.3. Amino acid profiles and peptide identification

The amino acid profiles of flaxseed protein isolate, FPH and the EDUF samples were determined to confirm peptide migration and enrichment of the KCl 2 fraction with peptides containing arginine, which are the peptides of interest in this study. As shown in Table 7.1, about one-third (31.2%) of the freeze-dried KCl 2 sample resulting from EDUF comprised of arginine compared to the original FPH and flaxseed protein isolate, which contained 11.1% and 11.3% of arginine, respectively. This represents about 3-fold increase in the amount of arginine in the samples. On the other hand, the KCl 1 sample contained 2.61% of arginine, which migrated to the anode probably due to the net negative molecular charge of their parent peptides. These results confirmed that EDUF is an efficient process for the separation of arginine-rich peptides from enzymatic flaxseed protein hydrolysates. It could also be observed that the process of enzymatic hydrolysis of flaxseed protein did not affect arginine composition; thus, the arginine-containing peptides were mostly retained in the FPH. Generally, it could be observed that the total amount of positively charged amino acids (arginine, lysine and histidine) was more in the KCl 2 fraction (43.2%) than in the KCl 1 fraction (7.6%) whereas the acidic amino acids (Asx and Glx) were concentrated in the KCl 1 fraction (22%) compared to the KCl 2 fraction (9.6%). However, it was observed that the remaining FPH in the feed compartment after EDUF contained a substantial amount (9.32%) of arginine; thus, the EDUF process used in this study did not separate all the arginine from FPH. This observation could be due to the net zero charge or large size of these arginine-containing peptides, or due to the EDUF conditions used in our study. A previous study

reported that the strength of the electrical field applied and the effective membrane surface area independently affect peptide migration during EDUF (Poulin et al., 2007).

The peaks observed in the TIC of the flaxseed peptide samples were tentatively identified from the MS data using the ExPASy Proteomics Server FindPept tool (Swiss Institute of Bioinformatics) and the primary sequence of flaxseed conlinin, the only major flaxseed storage protein with reported amino acid sequence (UniProtKD/TrEMBL # Q8LPD3). Table 7.2 shows the MW identification and potential sequences of the KCl 1 and KCl 2 peptides; 10 peptides were identified in KCl 2 and 6 peptides in KCl 1 based on the MS data, net charge and calculated isoelectric point ($p$I) of the peptides at pH 7.0. The net charge of the peptides at the experimental pH corresponded to their migration to the electrodes. All of the possible KCl 1 peptides contained arginine in their sequence, except peak 9, while all the KCl 1 peptides contained aspartic acid/glutamic acid, which contributed strongly to their net charge. However, some peptides (peaks # 2, 4, 6, 8 and 10) tentatively identified in KCl 2 possessed zero net charge with $p$I values of 6.1 to 6.8. This observation could possibly be due to fluctuations in pH (values decreased to the $p$I of these peptides, data not shown) observed during EDUF leading to temporary change in net charge and migration of the peptides to the cathode. This explanation is supported by the net negative charge observed for all the potential peptides in KCl 1, which possessed calculated $p$I values of 2.9 to 3.1 (lower than the experimental pH conditions). It is important to note that these peptide sequences are tentative since flaxseed contains another major storage protein, linin, which could have also contributed substantially to the peptide pool.

**Table 7.1 Amino acid compositions (%) of flaxseed protein isolate (FPI), protein hydrolysate (FPH) and peptides from the anode (KCl 1), cathode (KCl 2) and feed (FPH-X) compartments after electrodialysis-ultrafiltration**

| AMINO ACID | FPI[a] | FPH | FPH-X | FPH (KCl 1) | FPH (KCl 2) |
|---|---|---|---|---|---|
| ASX | 11.29 | 11.65 | 11.10 | 8.86 | 5.19 |
| THR | 3.67 | 3.97 | 3.64 | 7.16 | 3.17 |
| SER | 4.34 | 5.08 | 4.34 | 5.23 | 2.88 |
| GLX | 19.83 | 20.12 | 19.87 | 13.18 | 4.41 |
| PRO | 5.29 | 4.15 | 4.07 | 12.05 | 3.54 |
| GLY | 5.47 | 5.65 | 5.98 | 7.27 | 5.03 |
| ALA | 4.89 | 5.00 | 5.19 | 6.70 | 4.41 |
| CYS | 1.36 | 1.15 | 1.17 | 0.80 | 0.12 |
| VAL | 5.20 | 5.57 | 6.47 | 8.18 | 6.14 |
| MET | 2.02 | 1.70 | 1.88 | 0.34 | 0.00 |
| ILE | 4.50 | 4.82 | 5.53 | 4.77 | 5.48 |
| LEU | 5.80 | 6.02 | 6.55 | 6.82 | 6.72 |
| TYR | 2.52 | 2.75 | 3.00 | 3.41 | 2.93 |
| PHE | 5.47 | 5.78 | 6.33 | 7.61 | 6.72 |
| HIS | 2.29 | 2.34 | 2.44 | 3.41 | 2.06 |
| LYS | 3.04 | 3.14 | 3.13 | 1.59 | 9.97 |
| ARG | 11.28 | 11.10 | 9.32 | 2.61 | 31.23 |
| CATIONIC | 14.32 | 16.59 | 14.88 | 7.61 | 43.26 |
| ACIDIC | 31.12 | 31.77 | 30.96 | 22.05 | 9.60 |

[a]Data derived from Udenigwe and Aluko (2010); FPH, flaxseed protein hydrolysate; FPH-X, flaxseed protein hydrolysate remaining in feed compartment after electrodialysis; FPH (KCl 1), peptides that migrated towards the anode (anionic); FPH (KCl 2), peptides that migrated towards the cathode (cationic)

**Table 7.2 Molecular weight identification and potential sequences of peptides that migrated to the KCl 1 (anionic) and KCl 2 (cationic) compartments during electrodialysis-ultrafiltration of flaxseed protein hydrolysates**

| peak# | $R_t$ (min)[a] | obs MW[b] | calc MW[c] | potential sequences | location[d] | net charge[e] | pI |
|---|---|---|---|---|---|---|---|
| KCl 2 | | | | | | | |
| 1 | 23.33 | 360.14 | 359.40 | QGR | f36-38, f82-84 | +1 | 11 |
| | | | 360.19 | RW | f134-135 | +1 | 11 |
| | | | 360.20 | SVR | f23-25 | +1 | 11 |
| 2 | 28.07 | 1287.46 | 1287.59 | GQMRQDIQQQG | f117-127 | 0 | 6.8 |
| 3 | 29.72 | 532.19 | 532.29 | ASVRT | f22-26 | +1 | 11 |
| 4 | 32.14 | 417.16 | 417.16 | RQD | f120-122 | 0 | 6.8 |
| 6 | 41.94 | 755.29 | 755.32 | DYLRSC | f58-63 | 0 | 6.1 |
| | | | 755.39 | ARDLPGQ | f143-149 | 0 | 6.8 |
| 8 | 43.85 | 499.26 | 499.27 | RDLP | f144-147 | 0 | 6.8 |
| 9 | 45.92 | 586.22 | 586.24 | YYNQ | f79-82 | 0 | 5.9 |
| 10 | 51.42 | 556.28 | 556.26 | RDLPG | f144-148 | 0 | 6.8 |
| 12 | 72.35 | 700.40 | 700.39 | RGLERA | f110-115 | +1 | 10.9 |
| 13 | 80.17 | 904.90 | 904.45 | TCRGLERA | f108-115 | +1 | 9 |
| KCl 1 | | | | | | | |
| 1 | 43.26 | 332.09 | 331.40 | VVD | f19-21 | -1 | 3.1 |
| 2 | 72.29 | 322.90 | 323.07 | DSC | f94-96 | -1 | 3.1 |
| | | 340.20 | 339.40 | CCD | f96-98 | -1.1 | 3.1 |
| | | 359.20 | 359.20 | IID | f29-31 | -1 | 3.1 |
| | | 680.30 | 680.26 | QEQDY | f55-59 | -2 | 3 |
| | | 701.40 | 701.40 | IVVDASV | f18-24 | -1 | 3.1 |
| | | 702.34 | | VIIDED | f28-33 | -3 | 2.9 |

[a]retention time on the MS TIC; [b]observed MW; [c]calculated MW; [d]peptide location on the primary sequence of flaxseed 2 S conlinin (storage protein); [e]calculated at pH 7.0

### 7.3.4. Inhibition of ACE and renin activities by the arginine-enriched peptide fraction

Food-derived peptides that can modulate the renin-angiotensin system by inhibiting ACE and renin activities can be used in human clinical interventions against hypertension and associated cardiovascular diseases. In this study, the arginine-rich flaxseed peptide sample (KCl 2) was found to exhibit concentration-dependent moderate inhibitory activity against ACE *in vitro* (Figure 7.5). A maximum of 80% inhibition of ACE activity was observed at 1 mg/ml of the desalted KCl 2, which gave an $IC_{50}$ value of 0.38 ± 0.01 mg/ml. Previous studies on flaxseed protein-derived peptides yielded more active ACE inhibitors especially LMW peptide fraction from thermolysin-catalyzed flaxseed protein hydrolysates with $IC_{50}$ value of 0.0275 mg/ml (Udenigwe et al., 2009). The structural requirements for ACE inhibition include the presence of hydrophobic and bulky amino acids in dipeptides, and C-terminal aromatic amino acid with middle cationic amino acid in tripeptides (Wu, Aluko & Nakai, 2006). Thus, the low amounts of hydrophobic and aromatic amino acids in the KCl 2 sample (Table 7.1) could have contributed to its low ACE-inhibitory activity. The LMW peptides released from flaxseed protein in this study contain arginine located mostly at the C-terminal of the peptides due to the activity of trypsin; the tentative peptide sequences in KCl 2 contain arginine at both terminals and within the sequence, but these arrangements may not have contributed to potency in ACE inhibition.

On the other hand, the arginine-rich flaxseed peptide sample displayed a weak renin-inhibitory activity, which was also observed to be concentration-dependent with a maximum of 44.5% renin inhibition at 7.5 mg/ml of the peptide sample (Figure 7.6). This

activity is weaker than those previously reported for various LMW peptides generated from flaxseed proteins (Udenigwe et al., 2009) and C-terminal arginine-containing dipeptides Ile-Arg (IR), Leu-Arg (LR) and Asn-Arg (NR) (Udenigwe, Li, & Aluko, unpublished – see Chatpter 8). A recent study reported that amino-terminal small hydrophobic amino acids and C-terminal bulky amino acids contribute to enhanced potency of renin inhibiting dipeptides (Udenigwe et al., unpublished – see Chatpter 8). Therefore, it was expected that a C-terminal arginine-containing peptide could potently inhibit renin activity at low concentrations if the N-terminal is occupied by a hydrophobic amino acid.

**Figure 7.5 ACE-inhibitory activity of flaxseed protein-derived arginine-enriched peptide fraction (KCl 2)**



The chart contains:
$y = 23.82\ln(x) + 74.09$
$R^2 = 0.9365$
$IC_{50} = 0.38 \pm 0.01$ mg/ml
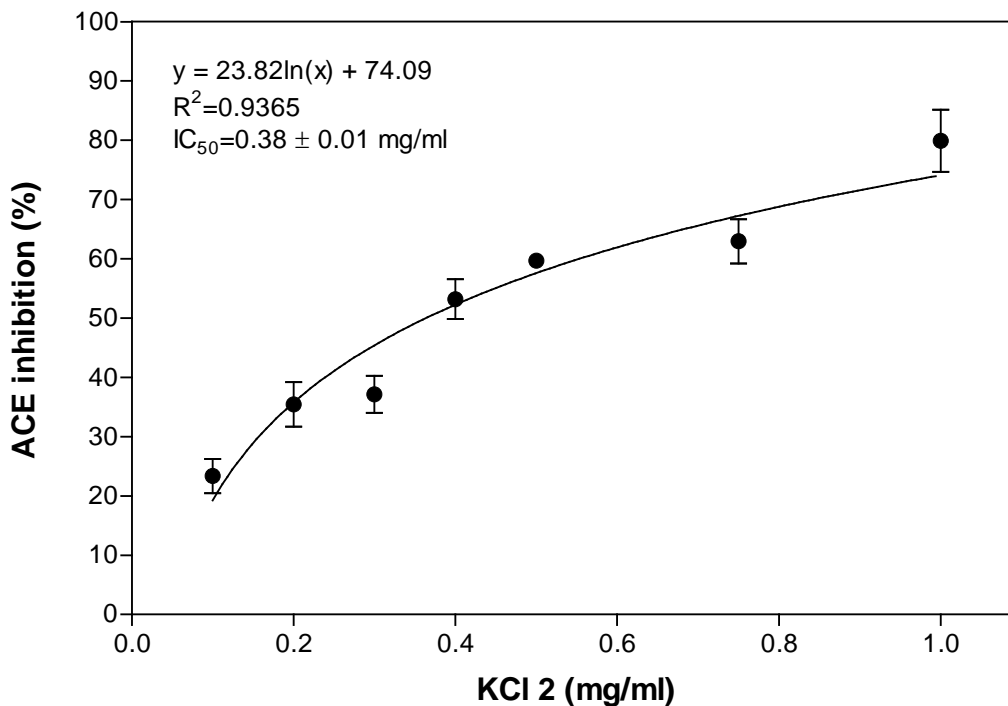
**Figure 7.6 Renin-inhibitory activity of flaxseed protein-derived arginine-enriched peptide fraction (KCl 2)**



$y = 5.8129x - 2.0462$
$R^2 = 0.9837$

*Renin inhibition (%)* vs **KCl 2 (mg/ml)**
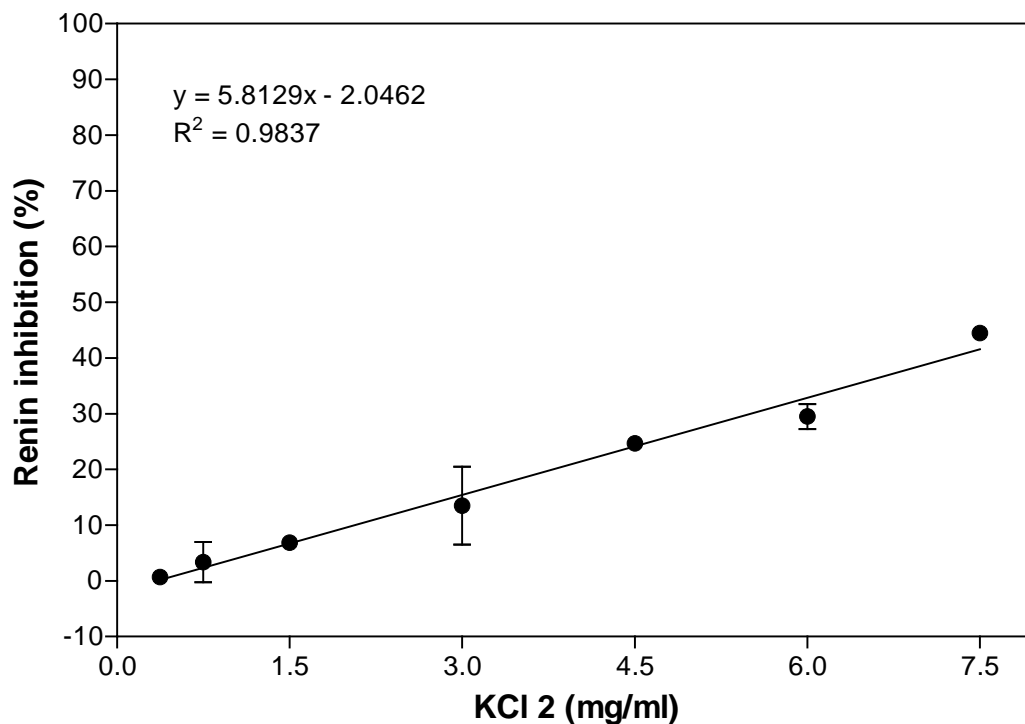
### 7.3.5. Antihypertensive activity of the arginine-rich peptide fraction

Results from the animal study indicated that, at 200 mg/kg BW, the arginine-rich flaxseed peptide fraction decreased SBP by −17.9 and −11.7 mmHg, respectively after 2 and 4 h following oral gavage when compared to the effect of free arginine (ΔSBP of −10.3 and −2.8 mmHg, respectively) (Figure 7.7). The hypotensive activity of the arginine-rich peptides was similar to that of captopril after 2 h, which indicates ability to provide fast relief from hypertension on a short-term basis. The hypotensive effect observed for the arginine-enriched peptide product was greater than those observed for the amino acid form of arginine as well as the flaxseed protein isolate. The results support our

hypothesis that peptides could be a better form of delivering fast therapeutic effects when compared to free amino acids and large-size proteins. The hypotensive effect of the arginine-enriched peptide product was observed to be long-lasting compared to the free amino acid; this indicated a more efficient absorption of the peptides compared to free arginine. The observed activity could be due to the vasodilatory nitric oxide synthesized from the arginine or possibly due to ACE and renin inhibition by the cationic peptides. Moreover, the crude FPH showed similar hypotensive effects to the arginine-enriched peptide product with sustained decrease in SBP during the first 6 h. Therefore, the FPH could be used as a source of the arginine-containing peptides, which would obviate the need for the EDUF process with substantial savings in processing costs. Surprisingly, FPI also exhibited hypotensive activity with a maximum ∆SBP of −18.4 mmHg at 6 h. The slow-acting effect of the FPI was expected since digestion of the inherent large proteins must precede absorption of the bioactive peptides. In contrast, the FPH and arginine-enriched peptide product are already pre-digested, hence absorption proceeded quickly and they provided faster hypotensive effects than the FPI. Detailed short-term and long-term studies are underway in our laboratory to characterize dose-response of these treatments on SBP, circulating AT-II, renin and ACE protein levels and activities in SHR tissues, especially with the arginine-rich peptides, crude FPH and FPI.

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**Figure 7.7 Effects of arginine-rich flaxseed peptides, free arginine, flaxseed protein hydrolysate (FPH) and protein isolate (FPI), each at 200 mg/kg body weight (BW), on systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR); captopril (3 mg/kg BW) and saline were used as positive and blank controls, respectively**
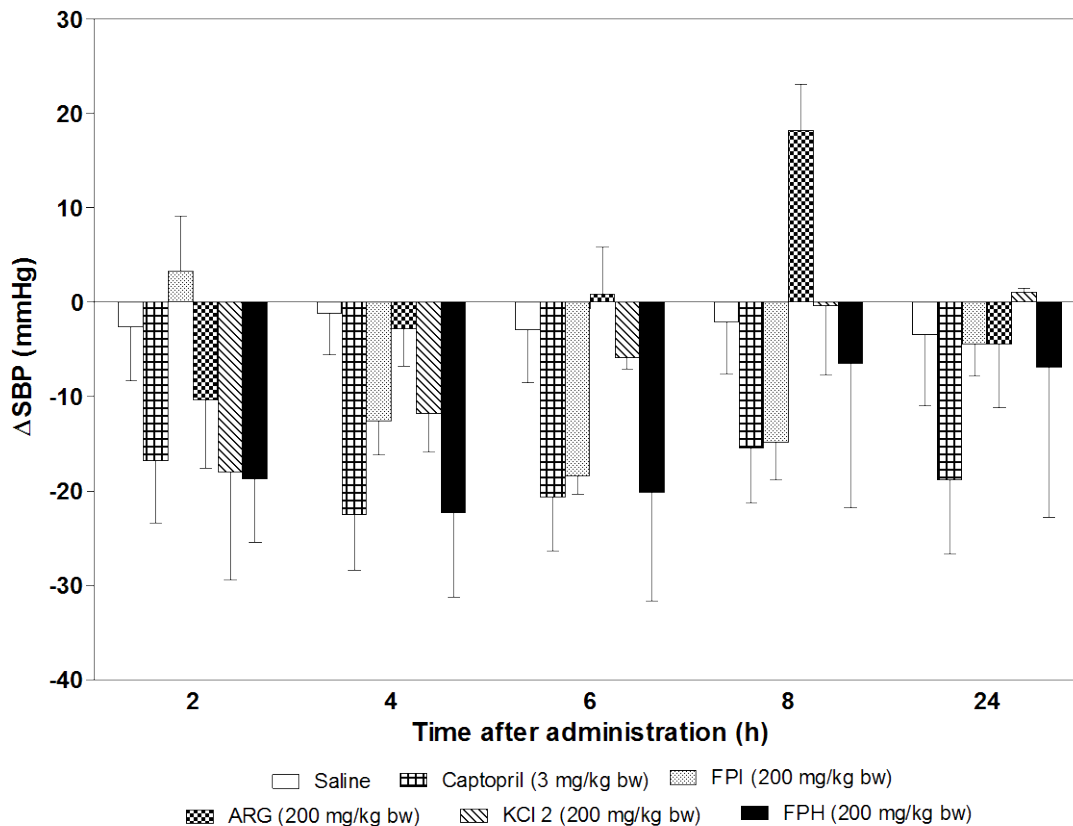


## 7.4. CONCLUSIONS

It can be concluded that flaxseed meal protein is an excellent protein source for the production of arginine-rich peptides by controlled enzymatic hydrolysis, and that combined electrodialysis and ultrafiltration process can be used to concentrate these peptides into a fraction. The low yield obtained for the separated fraction and the high

arginine level in the feed compartment after separation suggest that the efficiency of both the enzymatic hydrolysis and EDUF processes need to be further investigated and improved. However, the EDUF process may not be required since the FPH produced similar hypotensive effects to the cationic peptides. But the peptide product is still a good candidate for further studies because of its beneficial physiological hypotensive activity in SHR and activity in moderately inhibiting the enzymes of the blood pressure-regulating pathway. The fast-acting nature of the FPH and arginine-enriched peptide product could be an advantage over the original protein isolate by providing quick relief from high blood pressure. The hypotensive activities observed for FPI and crude FPH will encourage further studies on the use of these products as therapeutic agents without further processing. These studies will ultimately contribute to value addition to defatted flaxseed meal generated from the oilseed industry.

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**TRANSITION STATEMENT**

The structure of peptides determines their interactions with biological molecular disease targets and invariably influences their physiological activities. This project has so far shown that low-molecular size peptides derived from flaxseed proteins possess prospects as therapeutic agents especially as antihypertensive agents due to their ability to inhibit ACE and renin. Till date, several studies have reported some structural requirements of peptides of various sizes for ACE inhibition, and this has contributed towards the discovery of more potent ACE inhibitors. However, there is scanty information in the literature on the structure-function properties of renin-inhibiting natural peptides. The next chapter reports on the quantitative structure-activity relationship (QSAR) studies on renin-inhibiting food-derived dipeptides. Information derived from this study will be invaluable in the design and synthesis of potent orally bioavailable antihypertensive peptides and peptidomimetics.

**CHAPTER EIGHT**

**MANUSCRIPT 6**

**QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP MODELLING OF RENIN-**

**INHIBITING DIPEPTIDES**

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## 8.0. ABSTRACT

Partial least squares regression method was used to analyze a peptide dataset and construct inhibitory models for renin inhibitory natural dipeptides. The models were computed with the renin inhibitory activity as dependent variable ($Y$) and the peptide structural properties as predictors ($X$); validation was conducted using cross-validation and permutation tests. The amino acid descriptors were based on the *3-* and *5-z* scales of 20 coded amino acids to produce models that explained 71.6% of $Y$ with a 33.8% predictive ability and 75.2% of $Y$ with a predictive power of 50.8%, respectively. In both models, low molecular size amino acids with hydrophobic side chains were preferred at the N-terminus, while amino acids with bulky side chains were preferred at the C-terminus for potency. Based on the *5-z* model, four Trp-containing antihypertensive dipeptides (IW, LW, VW and AW) were predicted as the most potent renin inhibitors. The peptides were synthesized and *in vitro* inhibition assay showed that IW and LW inhibited 70% ($IC_{50}$, 2.3 mM) and 37% renin activity at 3.2 mM, respectively whereas VW and AW were inactive. There was no correlation between the observed renin-inhibitory activities and angiotensin-converting enzyme inhibitory activities of the dipeptides. We concluded that the structural similarities between isoleucine and leucine could have contributed to their distinct inhibitory activity when compared with alanine and valine. Therefore, IW may be a useful template for the development of advanced forms of highly active low molecular size antihypertensive peptides and peptidomimetics.

**KEYWORDS: Renin; Dipeptides; Quantitative structure-activity relationship (QSAR); Partial least squares (PLS); Amino acids descriptors**

**8.1. INTRODUCTION**

The renin-angiotensin system (RAS) has provided key molecular targets for therapeutic agents towards the treatment and management of hypertension, a contributing risk factor to human cardiovascular diseases and a major public health concern especially in the Western world. This is primarily due to the role of the RAS pathway in controlling blood pressure through the production of angiotensin (AT)-II, a potent vasoconstrictor, from AT-I by the action of angiotensin I-converting enzyme (ACE). Consequently, ACE inhibitors have been widely used to control blood pressure during hypertension, and several food protein-derived peptides have shown both ACE-inhibitory and antihypertensive activities (Aluko 2008). Another important molecular target for antihypertension drug discovery is the renin-catalyzed conversion of angiotensinogen to AT-I, which is the rate-limiting step of RAS (Staessen et al. 2006). The inhibition of renin activity can provide better blood pressure control during hypertension than ACE inhibition since it prevents the formation of AT-I, which can be converted to AT-II in some cells via an ACE-independent chymase-catalyzed reaction (Staessen et al. 2006; Segall et al. 2007). Moreover, since angiotensinogen is the only known renin substrate, targeting renin in the RAS pathway ensures high specificity in controlling elevated blood pressure as opposed to ACE inhibition, which can also affect other biochemical pathways leading to toxic effects (Acharya et al. 2003; Staessen et al. 2006). Several synthetic peptides and non-peptides have been reported to possess renin inhibitory activity but their application as orally-active antihypertensive agents has been hindered due to poor pharmacokinetic attributes (Rahuel et al. 2000; Fischer and

Hollenberg 2001; Wood et al. 2006). Till date, aliskiren is the only known clinically-relevant non-peptide renin-inhibiting antihypertensive agent (Sepehrdad et al. 2007); current research efforts are continuously directed towards the discovery of potent non-toxic renin inhibitors. Recent studies have shown that food protein-derived peptides and enzymatic hydrolysates possess the ability to inhibit renin activity *in vitro* (Udenigwe et al. 2009; Li 2010). These research efforts could lead to the discovery of a new generation of cheaper renin inhibitors with no side effects since the peptides were generated from food proteins. Moreover, knowledge of the structure-function property of these food-derived peptides can lead to the design and synthesis of more potent renin inhibiting antihypertensive agents, especially peptidomimetics. To the best of our knowledge, there is scanty information in the literature on the structural requirements for renin inhibiting food-derived peptides that contain only coded amino acids.

Quantitative structure-activity relationship (QSAR) modelling is a practical and reliable method in chemometrics for studying the relationship between molecular structures of therapeutic agents and biological activities. In peptide QSAR, statistical multiple regression analysis such as partial least squares projection of latent structure (PLS) have been widely used to develop models that relate the molecular structures to variation in the biological activities. In other words, bioactivity data can be modelled as a function of molecular structures of peptides (Sandberg et al. 1998; Wu et al. 2006a). Using amino acid *z* scale descriptors, PLS has been successfully applied in developing good models in QSAR study of various bioactive peptides (Hellberg et al. 1987) including models that can explain the structural requirements for ACE-inhibiting peptides of

various chain lengths (Wu et al. 2006a, 2006b). QSAR models for peptides' bitterness property (Wu and Aluko 2007) and for the study of the functional properties of polypeptides (Siebert 2003) have also been reported.

The objectives of this study were to (i) develop QSAR models to explain the structure-activity relationship of a group of natural renin inhibiting dipeptides, (ii) use the models to predict potent renin-inhibiting peptides followed by *in vitro* evaluation of their bioactivity, and (iii) evaluate the ACE-inhibitory activity of the most potent predicted peptides in order to determine if there is a similarity between the structural requirements for ACE and renin inhibition.

## 8.2. METHODS

### 8.2.1. Peptide dataset

The peptide dataset consisted of thirteen renin-inhibiting dipeptides that were originally identified in our lab (Li 2010) from mass spectrometry analysis of an enzymatic pea (*Pisum sativum*) protein hydrolysate (Table 8.1). The renin inhibitory activities (RI, %) were measured at 3.2 mM peptide concentration and were log-transformed prior to modelling. Amino acid sequences of the peptides, RI and log RI are presented in Table 8.1.

**Table 8.1 Dipeptide dataset with *in vitro* renin-inhibitory activity (RI) at 3.2 mM peptide concentration and log RI of the dipeptides**

| No. | Peptide sequence | RI (%) | log RI |
|-----|------------------|--------|--------|
| 1 | IR | 49.11 | 1.69 |
| 2 | LR | 33.97 | 1.53 |
| 3 | NR | 25.36 | 1.40 |
| 4 | KF | 28.78 | 1.46 |
| 5 | EF | 22.36 | 1.35 |
| 6 | QF | 12.04 | 1.08 |
| 7 | RF | 6.35 | 0.80 |
| 8 | SF | 15.9 | 1.20 |
| 9 | YA | 15.13 | 1.18 |
| 10 | FK | 8.92 | 0.95 |
| 11 | FE | 1.81 | 0.25 |
| 12 | FQ | 8.64 | 0.93 |
| 13 | FT | 20.4 | 1.31 |

**8.2.2. Partial least squares (PLS) method**

The peptide QSAR was carried out using PLS analysis as previously reported for other bioactive peptides (Wold et al. 2001; Wu et al. 2006a, 2006b). This approach to peptide QSAR employs the amino acid *3-z* scale and the extended *5-z* scale to describe the structural features of the amino acid components of the dipeptides, and this constitutes the multivariate peptide descriptor (*X*) matrix. Due to the wide range of data for the pea protein-derived dipeptides, bioactivity data was expressed as log RI and this constitutes the dependent variable (*Y*) matrix. In order to ensure consistency in data interpretation, both the *3-z* scale and the *5-z* scale amino acid descriptors used in this study were reported by the same research group (Hellberg et al. 1987; Sandberg et al. 1998), and the same approach was also used for the bioactivity data. The *3-z* scale (*z1*, *z2*, *z3*) of the amino acids were previously calculated by principal component analysis from a matrix that consisted of 29 physicochemical variables of each of the 20 coded amino acids including molecular weight, $^1$H- and $^{13}$C-NMR data, p$K_a$, p*I*, substituent van der Waals volume, and so on. The *z* scales are interpreted to be related to hydrophobicity (*z1*), steric properties or side chain bulk/molecular size (*z2*) and electronic properties (*z3*) (Hellberg et al. 1987) whereas the additional descriptors (*z4* and *z5*) in the *5-z* scale, which are difficult to clearly interpret, are related to heat of formation, and "hardness" and electrophilicity, respectively (Sandberg et al. 1998). The amino acid at the N-terminus of the dipeptides was designated as *n1* and its structural properties were described as *n1z1*, *n1z2* and *n1z3* for the *3-z* scale or *n1z1*, *n1z2*, *n1z3*, *n1z4* and *n1z5* for the *5-z* scale (Wu et al. 2006a, 2006b). Likewise, the amino acid at the

C-terminus was designated as *n2* and its properties were described as *n2z1*, *n2z2* and so on. The relationship between the renin inhibition data (*Y*) and the peptide structural properties (*X*) was modeled by PLS using SIMCA-P software version 11.0.0.0 (Umetrics AB, Umeå, Sweden). All variables were centered and scaled to unit variance to ensure equal contribution in the models. The models were theoretically validated using a combination of cross-validation and permutation tests as previously described (Wold and Eriksson 1995; Wold et al. 2001; Wu et al. 2006a).

### 8.2.3. Peptide synthesis

The most potent predicted peptides (IW, LW, AW and VW) were synthesized by Genscript Corp. (Piscataway, NJ, USA). The purity (95–99%) of each peptide was measured by high performance liquid chromatography (HPLC) and their structures were confirmed by mass spectrometry (Genscript Corp.).

### 8.2.4. Renin inhibition assay

Renin inhibition assay was conducted by fluorescence spectrometry using the Renin Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) as previously described (Udenigwe et al. 2009). Briefly, the assay mixture contained 10 $\mu$M of renin substrate, human recombinant renin and the peptide sample in 50 mM Tris-HCl buffer containing 100 mM NaCl (pH 8.0). The renin substrate and peptide samples were mixed and pre-warmed to 37°C for 10 min prior to the assay followed by the addition of renin to the mixture to initiate the reaction. The increase in fluorescence intensity was

measured for 10 min at 37°C.  The spectrofluorometer was set at excitation wavelength of 340 nm, emission wavelength of 490 nm, excitation bandwidth of 5 nm and emission bandwidth of 10 nm. Renin activity (RA) was expressed as fluorescence intensity unit per min and inhibitory activity (%) of the peptides was calculated as $\{[(RA_{(blank)} - RA_{(sample)})/RA_{(blank)}] \times 100\}$, where $RA_{(blank)}$ and $RA_{(sample)}$ are the renin reaction rates in the absence and presence of the peptide samples, respectively.

### 8.2.5. ACE inhibition assay

The predicted peptides were also evaluated for ACE inhibition in order to investigate if there are similarities in the structural requirements of the peptides for renin and ACE inhibition. The ACE inhibition assay was conducted as previously reported using *N*-(3-[2-furyl]acryloyl)-phenylalanylglycyl-glycine (FAPGG) as ACE substrate (Udenigwe et al. 2009). The activities of the dipeptides were reported as the concentration that resulted in 50% inhibition of ACE activity ($IC_{50}$), which was calculated from non-linear regression analysis of a plot of ACE inhibition (%) versus peptide concentration.

### 8.2.6. Statistical analysis

The enzyme inhibition data were reported as mean ± standard deviation of 2 or 3 replicated assays and all data subjected to one-way analysis of variance. Statistical significance of differences was evaluated by Duncan's multiple range test (P=0.05) using the Statistical Analysis Systems (SAS) software version 9.2 (SAS Institute, Cary, NC, USA).

**8.3. RESULTS AND DISCUSSION**

**8.3.1. QSAR of renin-inhibitory dipeptides by PLS**

Low molecular size peptides, especially di- and tripeptides, with biological properties are excellent therapeutic candidates since they are usually resistant to gastrointestinal proteolysis and can be absorbed intact into blood circulation to sites where physiological activity is needed (Mathews and Adibi 1976; Vermeirssen et al 2004). Thus, peptides containing only two amino acid residues were chosen for this QSAR study in order to increase the physiological relevance of the resulting predicted potent renin-inhibiting peptides. The dipeptides used in the dataset were originally identified from a renin-inhibiting pea protein hydrolysate fraction, and these peptides individually showed varying renin inhibitory activities (Table 8.1). The unique structural properties ($X$) and the wide range of bioactive properties ($Y$) of the peptides were used to develop QSAR models to explain the relationship between $X$ and $Y$, and also to predict novel peptides with enhanced renin-inhibitory activity. The first modelling of the *3-z* scale amino acid descriptors with $Y$ resulted in a three-component PLS model that explained 35.6% of the sum of squares in $Y$-variance with a predictive ability of 10.5% of the dipeptides whereas the *5-z* scale provided a one-component model that could explain 46.2% of $Y$ with a predictive power of 9.53% (derived from cross-validation coefficient, $Q^2_{cv}$). The *t1/u1* (*tu*) PLS scores plots, which shows the relationship between $X$ and $Y$, revealed the presence of two outliers (No. 9 and 13 in Table 8.1) in these models, which were removed from the peptide dataset (Wold et al. 2001). Subsequently, the new peptide dataset was used to construct second models in an

attempt to increase their predictive powers. The summary of these new PLS models are shown in Table 8.2. The second modelling resulted in improved models, (1) a one-component model based on the *3-z* scale descriptors that could explain 71.6% of the *Y* sum of squares (predictive ability of 33.8%) and (2) another one-component PLS model based on the *5-z* scale descriptors that explains 75.2% of the renin inhibition data with 50.8% predictive ability of the peptide dataset. These models are illustrated in Figure 8.1, which shows the relationship between the observed and predicted renin inhibitory activity (log RI) of the peptide dataset. The multiple correlation coefficients ($R^2$) of the models were higher than 0.70 with the *5-z* scale model showing higher value than the *3-z* scale model. Thus, the robustness of the model was substantially improved when the *5-z* scale was used compared to the *3-z* scale, which is in agreement with previous QSAR studies of ACE-inhibiting tetra- and penta-peptides (Wu et al. 2006b) and protein functional properties (Siebert 2003).

One approach used to validate QSAR models is to use part of the dataset as training set in developing the model and the other part as test sets in testing the predictive capability of the model (Hellberg et al. 1987; Wold et al. 2001). This approach was not applied to this present work due to the limited number of observations. Instead, the *z* scale-based PLS models were validated initially by cross-validation during modelling and their predictive power also validated by permutation, where the *Y* data were each randomly permuted a number of times but with unaltered *X*-variable followed by a QSAR modelling of each permutation (Wold and Eriksson 1995). Twenty permutation rounds yielded cumulative $R^2$ ($R^2_{cum}$) and $Q^2_{cv}$ intercept values of 0.181 and

-0.093 for the *3-z* scale, and 0.191 and -0.177 for the *5-z* scale, respectively (Table 8.2). $R^2_{cum}$ and $Q^2_{cv}$ intercepts are measures of model fit and it was suggested that $R^2_{cum}$ intercept < 0.3 and $Q^2_{cv}$ intercept < 0.05 constitute a desirable limit for a valid model (Sandberg et al. 1998; Andersson et al. 1998). Thus, the $R^2_{cum}$ and $Q^2_{cv}$ intercept values observed for these two models indicate that the models can be considered valid and could not have resulted by chance. These models are regarded as good models considering that the modelling was based on random peptide dataset that were not statistically designed.

**Table 8.2 Summary of the PLS models using the amino acid *3-z* scale and *5-z* scale. Multiple correlation coefficient ($R^2$) indicates the sum of squares of *Y* explained and estimate of model fit whereas the cross-validated correlation coefficient ($Q^2_{cv}$) indicates the model's predictive ability**

| Model | N | *A* | $R^2Y$ | $Q^2_{cv}$ | Intercept $R^2_{cum}$ | Intercept $Q^2_{cv}$ | RMSEP |
|-------|---|-----|--------|-----------|--------------|--------------|-------|
| *3-z* scale | 11 | 1 | 0.7161 | 0.3381 | 0.181 | -0.093 | 0.1981 |
| *5-z* scale | 11 | 1 | 0.7524 | 0.5086 | 0.191 | -0.177 | 0.1929 |

N = number of peptides in the dataset; *A* = number of significant components; RMSEP = root mean square error of prediction

**Figure 8.1 Relationship between the observed and the predicted values of log renin inhibition (RI) using the amino acid (a)** *3-z* **scale and (b)** *5-z* **scale**
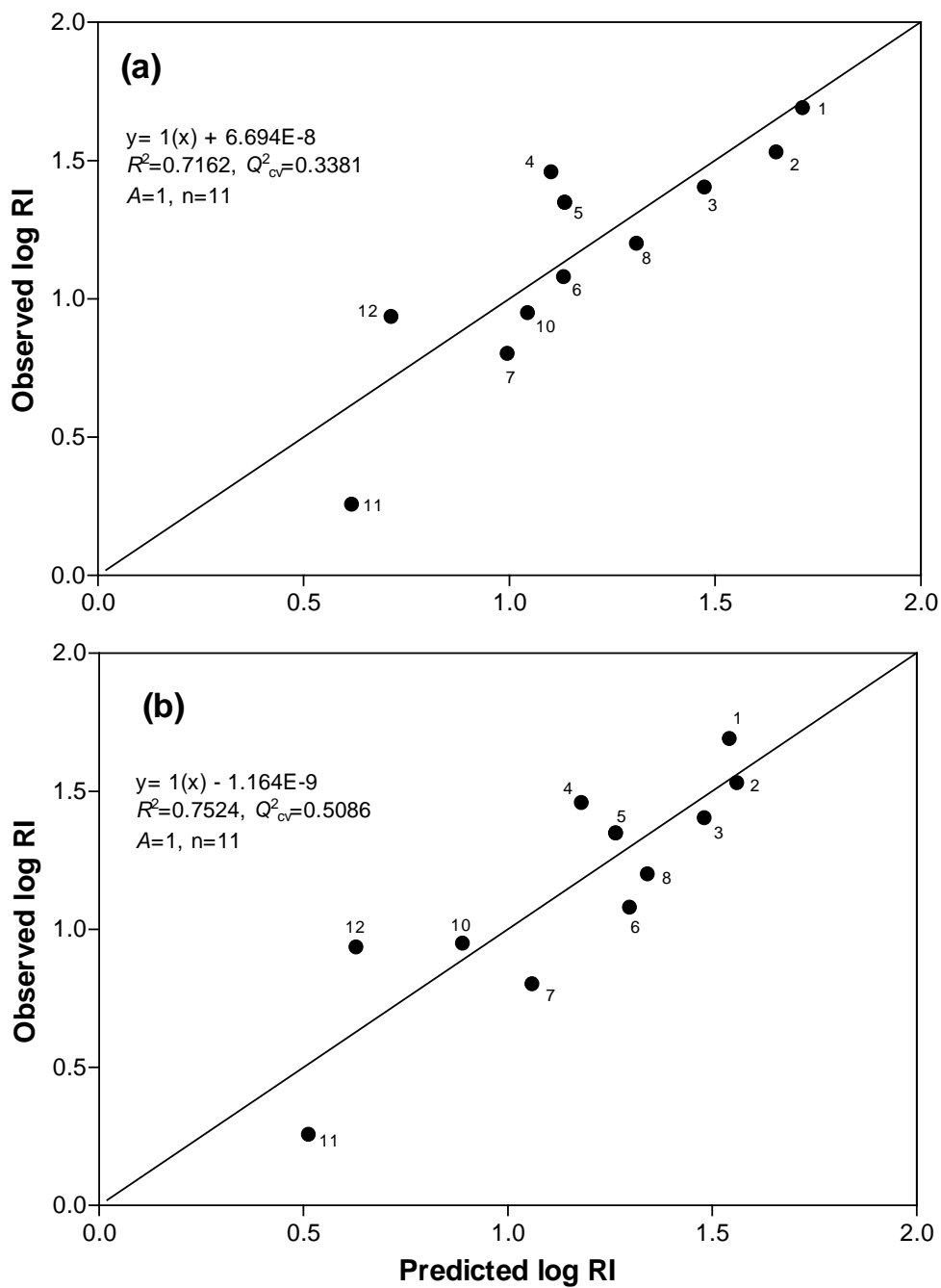
**8.3.2. Peptide prediction**

The models were used to determine structural properties of the dipeptides that contribute substantially to renin inhibition, and were subsequently applied in predicting peptides with putatively enhanced activity. The PLS coefficient plot shows the extent of contribution of the peptide structural descriptor ($X$) to modelling of the bioactive property (Figure 8.2). The contribution of an $X$-variable in the models depends on the coefficient value relative to the origin in the loading space (Wold et al. 2001), that is, the higher the coefficients in both directions, the more the contribution of the $X$-variable in explaining or predicting $Y$, and the sign indicates the direction of the relationship (Sandberg et al. 1998). The coefficient plot of the *3-z* scale model (Figure 8.2a) shows that *n1z1* and *n2z2* are positively correlated to $Y$ whereas *n2z1*, *n1z2*, *n1z3* and *n2z3* are negatively correlated to bioactivity. This pattern was also observed for the *z1-z3* descriptors in the *5-z* scale model, whose coefficient plot also indicated a positive relationship of renin inhibition ($Y$) with *n2z4* and *n1z5*, and inverse correlation with *n1z4* and *n2z5* (Figure 8.2b). In order to determine the importance of the variables in terms of relative contributions of the descriptors ($X$) in modelling both $Y$ and $X$, the Variable Importance for the Projection (VIP) plots were obtained for the models. A VIP value that is greater than one indicates an important $X$ variable with above average contribution while VIP values of less than 0.5 indicate unimportant peptide descriptors; values between 0.5–1 could be important or not depending on the size of the dataset (SIMCA-P 11 Software Analysis Advisor 2005). For this work, $X$ is regarded as important contributor only when its VIP > 1. The VIP plots indicate that *n2z2* and *n1z2* are very

**Figure 8.2 Partial least squares (PLS) regression coefficients of the (a) *3-z* scale and (b) *5-z* scale of dipeptides; the contribution of an *X*-variable in the models depends on the coefficient value relative to the origin**
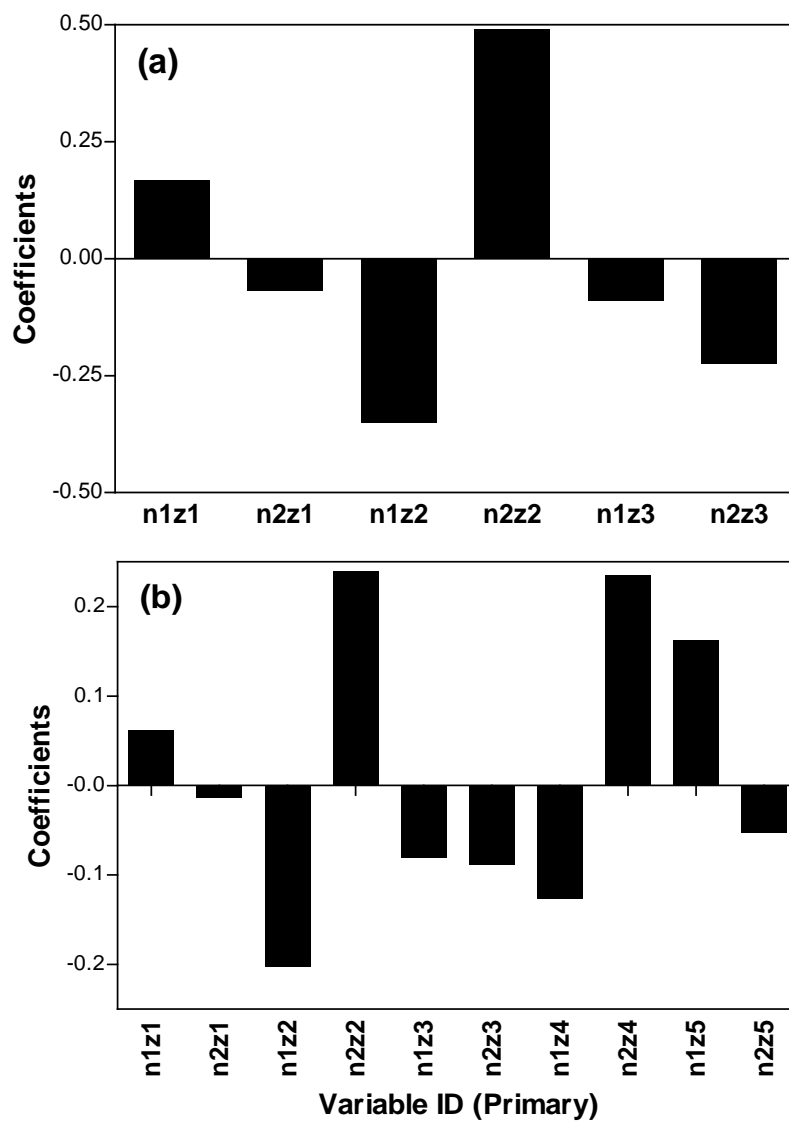
important descriptors in both models, in addition to *n2z4* and *n1z5* contributions to the

*5-z* scale model (Figure 8.3); this also shows the contributions of *z4* and *z5* to model

quality. Based on the coefficient and VIP plots of these PLS models, it could be observed

that steric properties or side chain bulk/molecular size (*z2*) of the amino acid plays the

most important role in determining the potency of the peptides as renin inhibitors

regardless of the location of the amino acid residue on the dipeptide; thus, both *n1* (N-

terminus) and *n2* (C-terminus) of the peptides are equally important. New models that

excluded unimportant *X*-variables with VIP < 0.5 resulted in improved model quality and

predictive abilities of the PLS models especially the *5-z* scale model (N=11, *A*=1,

$R^2Y$=0.777, $Q^2_{cv}$=0.614); *3-z* scale model (N=11, *A*=1, $R^2Y$=0.665, $Q^2_{cv}$=0.446).

According to the old and new models, bulky or high molecular size amino acid

residues decrease the renin inhibitory property of a dipeptide if the amino acid is

located at the N-terminus of the peptide whereas the same amino acids could

substantially increase the potency of the peptide if located at the C-terminus. In

addition, the N-terminus amino acid residue must be highly hydrophobic for increased

renin inhibition by the dipeptide. Based on the PLS regression coefficients (Figure 8.2),

electronic properties of the amino acid residues at both terminal of the dipeptides are

negatively correlated to log RI. Accordingly, these amino acids should be non-polar for

increased potency, even though their contributions are regarded as less important in

the VIP plot (Figure 8.3b). Therefore, in order to observe potent renin inhibitory activity,

these models showed that amino acids Val, Leu, Ile and Ala ($\uparrow$ *z1* $\uparrow$*z5*, $\downarrow$*z2* $\downarrow$*z3*) are

preferred at the C-terminal of dipeptides. Consequently, different combinations of these

**Figure 8.3 Variable Importance for the Projection (VIP) of the (a) *3-z* scale and (b) *5-z* scale models**
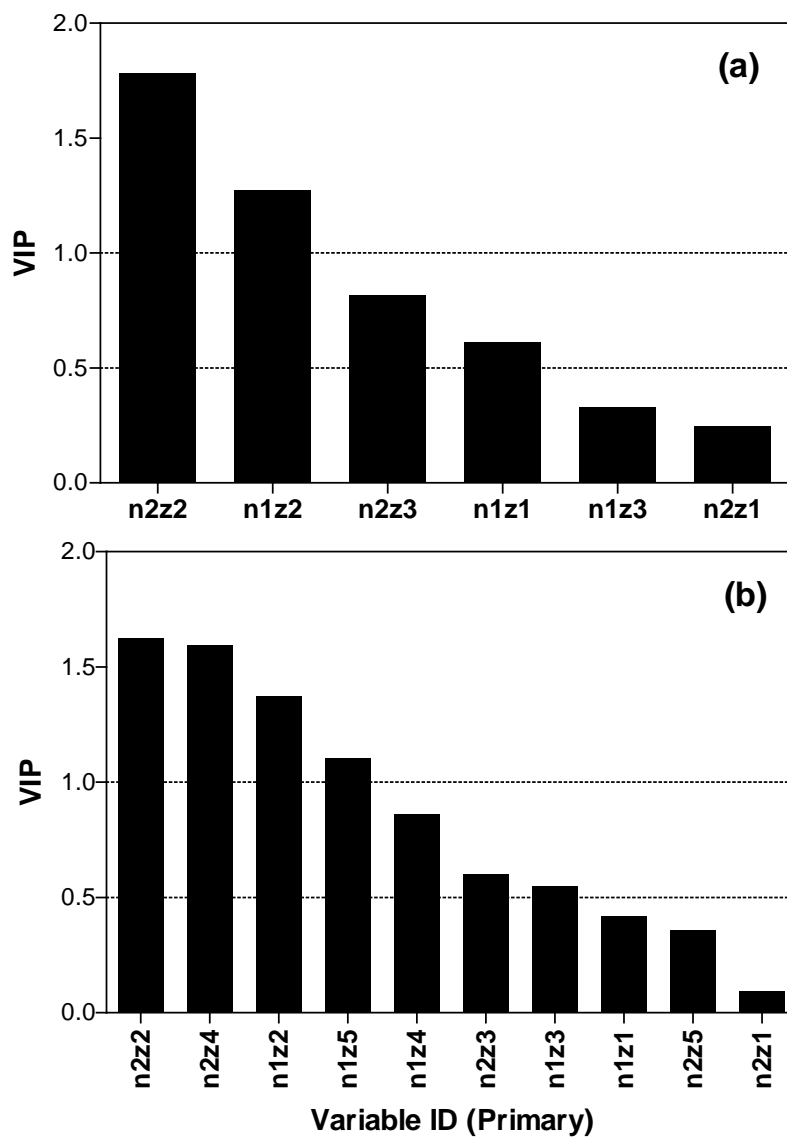
amino acids yielded 12 dipeptides with a range of predicted log RI of 1.17–1.98 for the

*3-z* scale model and 1.33–1.91 for the *5-z* scale model, which translate to 15.0–96.5%

and 21.7–81.0% renin-inhibitory activity, respectively at 3.2 mM peptide concentration.

It was observed that the most potent predicted peptides all possess Trp at the C-

terminus and include VW, AW, IW and LW with predicted renin inhibition of 96.5%,

82.2%, 72.5% and 62.5%, respectively for the *3-z* scale model and 71.1%, 81.2%, 66.2%

and 69.0%, respectively for the *5-z* scale model, at 3.2 mM peptide concentration.

Conversely, peptides with C-terminus Phe showed the weakest predicted renin

inhibition compared to those with C-terminus Trp or Tyr. The most potent Trp-

containing peptides were synthesized for confirmation of the predicted activities and for

testing of the robustness of the models.


### 8.3.3. Renin inhibition by predicted dipeptides

*In vitro* enzyme inhibition assay using human recombinant renin showed that

dipeptides IW and LW inhibited 70% and 37% of renin activity at 3.2 mM peptide

concentration, respectively whereas VW and AW were inactive under the same assay

conditions (Figure 8.4). The activity observed for IW was similar to the values predicted

using the two PLS models but that of LW was lower than the predicted values (Table

8.3). In addition, Figure 8.5 showed that IW displayed a concentration-dependent

inhibition of renin activity with 50% inhibitory concentration ($IC_{50}$) value of 2.3 ± 0.07

mM (0.72 ± 0.02 mg/ml). Although the most potent predicted peptide (IW) was not as

active as high molecular size peptide and non-peptide renin inhibitors (Fischer and

Hollenberg 2001; Rahuel et al. 2000; Staessen et al. 2006; Wood et al. 2003), possible efficient absorption across the enterocytes due to size and resistance to gastrointestinal proteolysis *in vitro* (Enari et al. 2008) could encourage its potential use as oral antihypertensive agent. Moreover, IW was found to be more active than all the dipeptides in the original dataset used to develop the PLS models. Thus, the *z* scale models reported in this study were relevant towards the discovery of a new renin-inhibiting dipeptide. Conversely, the PLS models did not correctly predict the renin inhibitory potential of dipeptides VW and AW, which showed high prediction errors (Table 8.3). It is important to note that the bioactive predicted dipeptides contain branched-chain aliphatic amino acid isomers, Leu or Ile, at the amino end of the peptides contrary to the inactive peptides that possess N-terminal Val or Ala. Consequently, the pattern of renin inhibition by the dipeptides correlated with hydrophobicity of the N-terminal amino acids where Ile>Leu>Val>Ala (*z1* values of -4.44, -4.19, -2.69 and 0.07, respectively). This observation confirms the need for the presence of a highly hydrophobic, low molecular size N-terminal amino acid in renin-inhibiting dipeptides, as suggested by the PLS models. Moreover, since the N-terminal hydrophobic amino acids possess different conformations that affect their interaction with the enzyme, the lack of contribution of peptide conformational state to the derived principal properties (Dunn and Wold 1995) also constitutes a limitation in the predictive power of the *z* scale-based PLS models.

**Figure 8.4** *In vitro* **inhibition of human recombinant renin activity by the predicted dipeptides at a concentration of 3.2 mM; N.A., no renin inhibitory activity; bars with different asterisks have mean values that are significantly different at P=0.05**
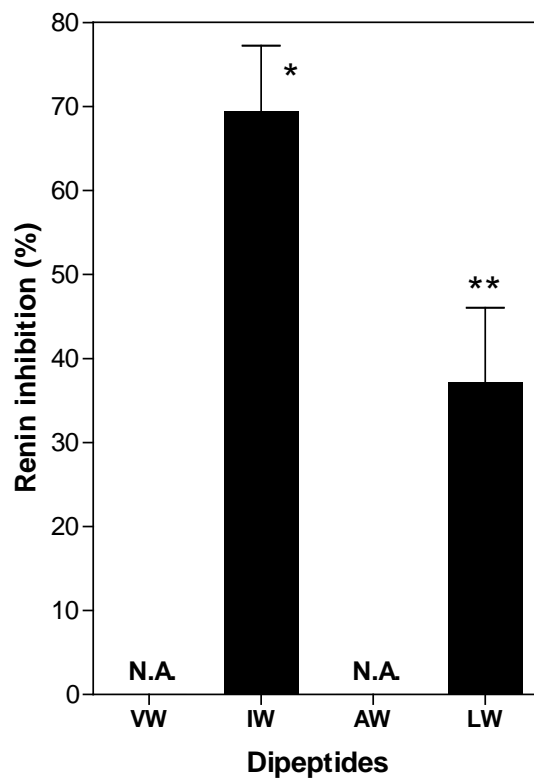
**Figure 8.5 Concentration-dependent *in vitro* inhibition of human recombinant renin by dipeptide IW with $IC_{50}$ value of 2.32 ± 0.07 mM**
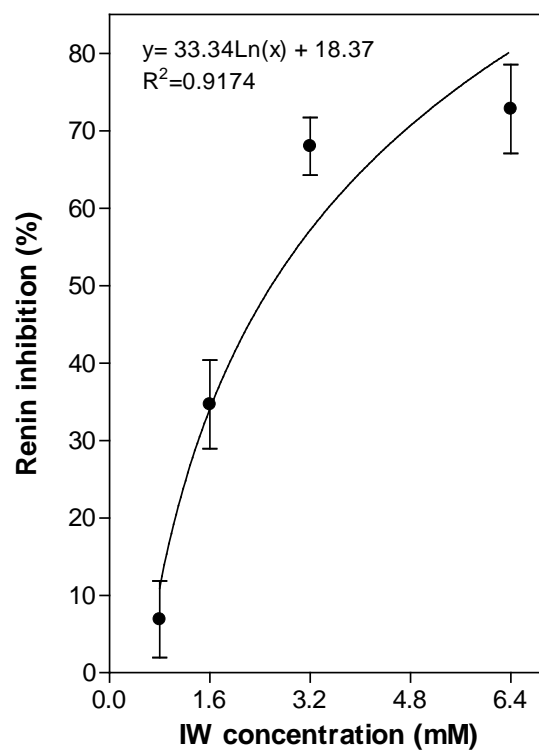
**Table 8.3 Predicted and observed log percentage renin inhibition (RI) at 3.2 mM peptide concentration for the predicted renin-inhibiting dipeptides and the prediction errors**

| | log RI | | | | |
| --- | --- | --- | --- | --- | --- |
| | PREDICTED | | OBSERVED | PREDICTION ERROR | |
| PEPTIDE SEQUENCE | *3-z* scale | *5-z* scale | | *3-z* scale | *5-z* scale |
| IW | 1.86 | 1.82 | 1.84 | -0.02 | 0.02 |
| LW | 1.79 | 1.83 | 1.57 | -0.22 | -0.26 |
| AW | 1.91 | 1.91 | 0.00 | -1.91 | -1.91 |
| VW | 1.98 | 1.85 | 0.00 | -1.98 | -1.85 |

**8.3.4. Relationship between renin and ACE inhibition**

Since there are similarities between the predicted structural requirements for renin inhibition reported in this study and predicted requirements for ACE inhibition by dipeptides (Cheung et al. 1980; Wu et al. 2006a), the renin inhibition data were compared to the ACE-inhibitory activities of the synthesized dipeptides. Literature data on the ACE-inhibitory activities of the four predicted dipeptides were inconsistent; a wide range of log $IC_{50}$ were reported for these peptides (Wu et al. 2006a), which translate to $IC_{50}$ values of 1.4–10.8 μM for VW, 10–18.6 μM for AW, 1.5–12.4 μM for IW and 6.7–50.1 μM for LW. Due to this inconsistency, the dipeptides were evaluated for

ACE inhibition in our laboratory under the same assay conditions. This present study confirmed that the dipeptides inhibited ACE activity in concentration-dependent fashions with $IC_{50}$ values of 4.74 µM for IW, 7.1 µM for VW, 34.8 µM for AW and 38.9 µM for LW. Table 4 shows that this trend of ACE inhibition by the dipeptides is in agreement with a previous report (Sato et al. 2002) and the activity of IW is exactly the same as reported by Ono et al. (2006). Based on these data and the aforementioned literature information, there was no observed relationship between the renin and ACE-inhibitory activities of the dipeptides. Moreover, IW was found to be generally more active than the other Trp-containing dipeptides in inhibiting both enzymes of the RAS pathway. All the predicted dipeptides have been previously shown to lower blood pressure in spontaneously hypertensive rats (Fujita et al. 2000; Sato et al. 2002; Nii et al. 2008). The *in vivo* antihypertensive effect of IW was observed to be more pronounced than the other dipeptides; it reduced systolic blood pressure (SBP) by 6.3% (-14.6 mmHg) and 6.4% (-13.8 mmHg) at 0.1 and 1 mg/kg body weight (BW), respectively after 9 h of administration (Sato et al. 2002), and in another study, by 22 mmHg at 60 mg/kg BW (Fujita et al. 2000). Therapeutic agents with dual effects as ACE and renin inhibitors can provide better blood pressure-lowering effects during hypertension than compounds that inhibit only ACE activity. Thus, the combined moderate renin inhibition and strong ACE inhibition displayed by IW may have contributed to its effects in SHR leading to pronounced blood pressure-lowering activity.

**Table 8.4 ACE-inhibitory activities (IC$_{50}$) of the Tryptophan (W)-containing dipeptides**

| PEPTIDE SEQUENCE | IC$_{50}$ ($\mu$M) | | |
|---|---|---|---|
| | A | B | C |
| IW | 4.74 ± 0.04 | 4.7 | 1.5 |
| LW | 38.92 ± 1.97 | 17.4 | 23.6 |
| AW | 34.86 ± 1.42 | 6.4 | 18.8 |
| VW | 7.08 ± 0.08 | 2.5 | 3.3 |

A, data from the present study using FAPGG as ACE substrate; B, data adapted from Ono et al. (2006); C, data adapted from Sato et al. (2002). B and C used hippuryl-L-histidyl-L-leucine (HHL) as ACE substrate

## 8.4. CONCLUSIONS

This is the first QSAR study towards elucidation of structural requirements for renin inhibition by food protein-derived peptides using the *z* scale amino acid descriptors. Based on these models, we concluded that hydrophobic amino acid residue at the N-terminus (e.g. Ile) and bulky amino acid at the C-terminus (e.g. Trp) contribute to the potency of renin-inhibiting dipeptides. The fact that only IW and LW were effective against renin suggests that the required configuration for inhibition is very specific since these peptides have the same size but are isomers. The high activity of IW suggests that it may serve as a useful template for developing more efficient renin-inhibitory peptides and peptidomimetics.

**ACKNOWLEDGEMENTS**

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**APPENDIX D: SUPPLEMENTAL INFORMATION**

**Table S8.1 Descriptor (*3-z*) scores for the 20 coded amino acids**

| AMINO ACID ID | | $z1$ | $z2$ | $z3$ |
|---|---|---|---|---|
| ALA | A | 0.07 | -1.73 | 0.09 |
| VAL | V | -2.69 | -2.53 | -1.29 |
| LEU | L | -4.19 | -1.03 | -0.98 |
| ILE | I | -4.44 | -1.68 | -1.03 |
| PRO | P | -1.22 | 0.88 | 2.23 |
| PHE | F | -4.92 | 1.3 | 0.45 |
| TRP | W | -4.75 | 3.65 | 0.85 |
| MET | M | -2.49 | -0.27 | -0.41 |
| LYS | K | 2.84 | 1.41 | -3.14 |
| ARG | R | 2.88 | 2.52 | -3.44 |
| HIS | H | 2.41 | 1.74 | 1.11 |
| GLY | G | 2.23 | -5.36 | 0.3 |
| SER | S | 1.96 | -1.63 | 0.57 |
| THR | T | 0.92 | -2.09 | -1.4 |
| CYS | C | 0.71 | -0.97 | 4.13 |
| TYR | Y | -1.39 | 2.32 | 0.01 |
| ASN | N | 3.22 | 1.45 | 0.84 |
| GLN | Q | 2.18 | 0.53 | -1.14 |
| ASP | D | 3.64 | 1.13 | 2.36 |
| GLU | E | 3.08 | 0.39 | -0.07 |

**Table S8.2 Descriptor (*5-z*) scores for the 20 coded amino acids**

| AMINO ACID ID | | $z1$ | $z2$ | $z3$ | $z4$ | $z5$ |
|---|---|---|---|---|---|---|
| ALA | A | 0.24 | -2.32 | 0.6 | -0.14 | 1.3 |
| VAL | V | -2.59 | -2.64 | -1.54 | -0.85 | -0.02 |
| LEU | L | -4.28 | -1.3 | -1.49 | -0.72 | 0.84 |
| ILE | I | -3.89 | -1.73 | -1.71 | -0.84 | 0.26 |
| PRO | P | -1.66 | 0.27 | 1.84 | 0.7 | 2 |
| PHE | F | -4.22 | 1.94 | 1.06 | 0.54 | -0.62 |
| TRP | W | -4.36 | 3.94 | 0.59 | 3.44 | -1.59 |
| MET | M | -2.85 | -0.22 | 0.47 | 1.94 | -0.98 |
| LYS | K | 2.29 | 0.89 | -2.49 | 1.49 | 0.31 |
| ARG | R | 3.52 | 2.5 | -3.5 | 1.99 | -0.17 |
| HIS | H | 2.47 | 1.95 | 0.26 | 3.9 | 0.09 |
| GLY | G | 2.05 | -4.06 | 0.36 | -0.82 | -0.38 |
| SER | S | 2.39 | -1.07 | 1.15 | -1.39 | 0.67 |
| THR | T | 0.75 | -2.18 | -1.12 | -1.46 | -0.4 |
| CYS | C | 0.84 | -1.67 | 3.71 | 0.18 | -2.65 |
| TYR | Y | -2.54 | 2.44 | 0.43 | 0.04 | -1.47 |
| ASN | N | 3.05 | 1.62 | 1.04 | -1.15 | 1.61 |
| GLN | Q | 1.75 | 0.5 | -1.44 | -1.34 | 0.66 |
| ASP | D | 3.98 | 0.93 | 1.93 | -2.46 | 0.75 |
| GLU | E | 3.11 | 0.26 | -0.11 | -3.04 | -0.25 |

**Table S8.3 Structural properties of the dipeptides used to develop the PLS models based on the *3-z* scale; *n1* and *n2* represent the amino acids at the N- and C-terminal of the dipeptides, respectively**

| PEPTIDE ID | *n1z1* | *n2z1* | *n1z2* | *n2z2* | *n1z3* | *n2z3* | log RI |
|:---:|:---:|:---:|:---:|:---:|:---:|:---:|:---:|
| IR | -4.44 | 2.88 | -1.68 | 2.52 | -1.03 | -3.44 | 1.69 |
| LR | -4.19 | 2.88 | -1.03 | 2.52 | -0.98 | -3.44 | 1.53 |
| NR | 3.22 | 2.88 | 1.45 | 2.52 | 0.84 | -3.44 | 1.40 |
| KF | 2.84 | -4.92 | 1.41 | 1.3 | -3.14 | 0.45 | 1.46 |
| EF | 3.08 | -4.92 | 0.39 | 1.3 | -0.07 | 0.45 | 1.35 |
| QF | 2.18 | -4.92 | 0.53 | 1.3 | -1.14 | 0.45 | 1.08 |
| RF | 2.88 | -4.92 | 2.52 | 1.3 | -3.44 | 0.45 | 0.80 |
| SF | 1.96 | -4.92 | -1.63 | 1.3 | 0.57 | 0.45 | 1.20 |
| YA | -1.39 | 0.07 | 2.32 | -1.73 | 0.01 | 0.09 | 1.18 |
| FK | -4.92 | 2.84 | 1.3 | 1.41 | 0.45 | -3.14 | 0.95 |
| FE | -4.92 | 3.08 | 1.3 | 0.39 | 0.45 | -0.07 | 0.25 |
| FQ | -4.92 | 2.18 | 1.3 | 0.53 | 0.45 | -1.14 | 0.93 |
| FT | -4.92 | 0.92 | 1.3 | -2.09 | 0.45 | -1.4 | 1.31 |

**Table S8.4 Structural properties of the dipeptides used to develop the PLS models based on the *5-z* scale**

| PEPTIDE ID | *n1z1* | *n2z1* | *n1z2* | *n2z2* | *n1z3* | *n2z3* | *n1z4* | *n2z4* | *n1z5* | *n2z5* | log RI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IR | -3.89 | 3.52 | -1.73 | 2.5 | -1.71 | -3.5 | -0.84 | 1.99 | 0.26 | -0.17 | 1.69 |
| LR | -4.28 | 3.52 | -1.3 | 2.5 | -1.49 | -3.5 | -0.72 | 1.99 | 0.84 | -0.17 | 1.53 |
| NR | 3.05 | 3.52 | 1.62 | 2.5 | 1.04 | -3.5 | -1.15 | 1.99 | 1.61 | -0.17 | 1.40 |
| KF | 2.29 | -4.22 | 0.89 | 1.94 | -2.49 | 1.06 | 1.49 | 0.54 | 0.31 | -0.62 | 1.46 |
| EF | 3.11 | -4.22 | 0.26 | 1.94 | -0.11 | 1.06 | -3.04 | 0.54 | -0.25 | -0.62 | 1.35 |
| QF | 1.75 | -4.22 | 0.5 | 1.94 | -1.44 | 1.06 | -1.34 | 0.54 | 0.66 | -0.62 | 1.08 |
| RF | 3.52 | -4.22 | 2.5 | 1.94 | -3.5 | 1.06 | 1.99 | 0.54 | -0.17 | -0.62 | 0.80 |
| SF | 2.39 | -4.22 | -1.07 | 1.94 | 1.15 | 1.06 | -1.39 | 0.54 | 0.67 | -0.62 | 1.20 |
| YA | -2.54 | 0.24 | 2.44 | -2.32 | 0.43 | 0.6 | 0.04 | -0.14 | -1.47 | 1.3 | 1.18 |
| FK | -4.22 | 2.29 | 1.94 | 0.89 | 1.06 | -2.49 | 0.54 | 1.49 | -0.62 | 0.31 | 0.95 |
| FE | -4.22 | 3.11 | 1.94 | 0.26 | 1.06 | -0.11 | 0.54 | -3.04 | -0.62 | -0.25 | 0.25 |
| FQ | -4.22 | 1.75 | 1.94 | 0.5 | 1.06 | -1.44 | 0.54 | -1.34 | -0.62 | 0.66 | 0.93 |
| FT | -4.22 | 0.75 | 1.94 | -2.18 | 1.06 | -1.12 | 0.54 | -1.46 | -0.62 | -0.4 | 1.69 |

**Table S8.5 Predicted peptide dataset based on the *3-z* scale descriptor and their predicted renin inhibitory activity (RI, %)**

| PEPTIDE ID | *n1z1* | *n2z1* | *n1z2* | *n2z2* | *n1z3* | *n2z3* | Predicted | |
| | | | | | | | log RI | RI (%) |
|---|---|---|---|---|---|---|---|---|
| **VW** | -2.69 | -4.75 | -2.53 | 3.65 | -1.29 | 0.85 | 1.98 | **96.55** |
| VY | -2.69 | -1.39 | -2.53 | 2.32 | -1.29 | 0.01 | 1.64 | 43.95 |
| VF | -2.69 | -4.92 | -2.53 | 1.3 | -1.29 | 0.45 | 1.37 | 23.35 |
| **LW** | -4.19 | -4.75 | -1.03 | 3.65 | -0.98 | 0.85 | 1.79 | **62.53** |
| LY | -4.19 | -1.39 | -1.03 | 2.32 | -0.98 | 0.01 | 1.45 | 28.47 |
| LF | -4.19 | -4.92 | -1.03 | 1.3 | -0.98 | 0.45 | 1.18 | 15.13 |
| **IW** | -4.44 | -4.75 | -1.68 | 3.65 | -1.03 | 0.85 | 1.86 | **72.50** |
| IY | -4.44 | -1.39 | -1.68 | 2.32 | -1.03 | 0.01 | 1.52 | 33.01 |
| IF | -4.44 | -4.92 | -1.68 | 1.3 | -1.03 | 0.45 | 1.24 | 17.54 |
| **AW** | 0.07 | -4.75 | -1.73 | 3.65 | 0.09 | 0.85 | 1.91 | **82.22** |
| AY | 0.07 | -1.39 | -1.73 | 2.32 | 0.09 | 0.01 | 1.57 | 37.43 |
| AF | 0.07 | -4.92 | -1.73 | 1.3 | 0.09 | 0.45 | 1.29 | 19.89 |

**Table S8.6 Predicted peptide dataset based on the *5-z* scale descriptor and their predicted renin inhibitory activity (RI)**

| PEPTIDE ID | n1*z*1 | n2*z*1 | n1*z*2 | n2*z*2 | n1*z*3 | n2*z*3 | n1*z*4 | n2*z*4 | n1*z*5 | n2*z*5 | Predicted | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | log RI | RI (%) |
| **VW** | -2.59 | -4.36 | -2.64 | 3.94 | -1.54 | 0.59 | -0.85 | 3.44 | -0.02 | -1.59 | 1.85 | **71.09** |
| VY | -2.59 | -2.54 | -2.64 | 2.44 | -1.54 | 0.43 | -0.85 | 0.04 | -0.02 | -1.47 | 1.44 | 28.03 |
| VF | -2.59 | -4.22 | -2.64 | 1.94 | -1.54 | 1.06 | -0.85 | 0.54 | -0.02 | -0.62 | 1.36 | 23.33 |
| **LW** | -4.28 | -4.36 | -1.3 | 3.94 | -1.49 | 0.59 | -0.72 | 3.44 | 0.84 | -1.59 | 1.84 | **69.01** |
| LY | -4.28 | -2.54 | -1.3 | 2.44 | -1.49 | 0.43 | -0.72 | 0.04 | 0.84 | -1.47 | 1.43 | 27.21 |
| LF | -4.28 | -4.22 | -1.3 | 1.94 | -1.49 | 1.06 | -0.72 | 0.54 | 0.84 | -0.62 | 1.35 | 22.65 |
| **IW** | -3.89 | -4.36 | -1.73 | 3.94 | -1.71 | 0.59 | -0.84 | 3.44 | 0.26 | -1.59 | 1.82 | **66.18** |
| IY | -3.89 | -2.54 | -1.73 | 2.44 | -1.71 | 0.43 | -0.84 | 0.04 | 0.26 | -1.47 | 1.41 | 26.09 |
| IF | -3.89 | -4.22 | -1.73 | 1.94 | -1.71 | 1.06 | -0.84 | 0.54 | 0.26 | -0.62 | 1.33 | 21.72 |
| **AW** | 0.24 | -4.36 | -2.32 | 3.94 | 0.6 | 0.59 | -0.14 | 3.44 | 1.3 | -1.59 | 1.91 | **81.20** |
| AY | 0.24 | -2.54 | -2.32 | 2.44 | 0.6 | 0.43 | -0.14 | 0.04 | 1.3 | -1.47 | 1.50 | 32.02 |
| AF | 0.24 | -4.22 | -2.32 | 1.94 | 0.6 | 1.06 | -0.14 | 0.54 | 1.3 | -0.62 | 1.42 | 26.65 |

**Figure S8.1** Concentration-dependent inhibition (%) of angiotensin I-converting enzyme activity by the four predicted dipeptides (a) IW, (b) AW, (c) LW and (d) VW
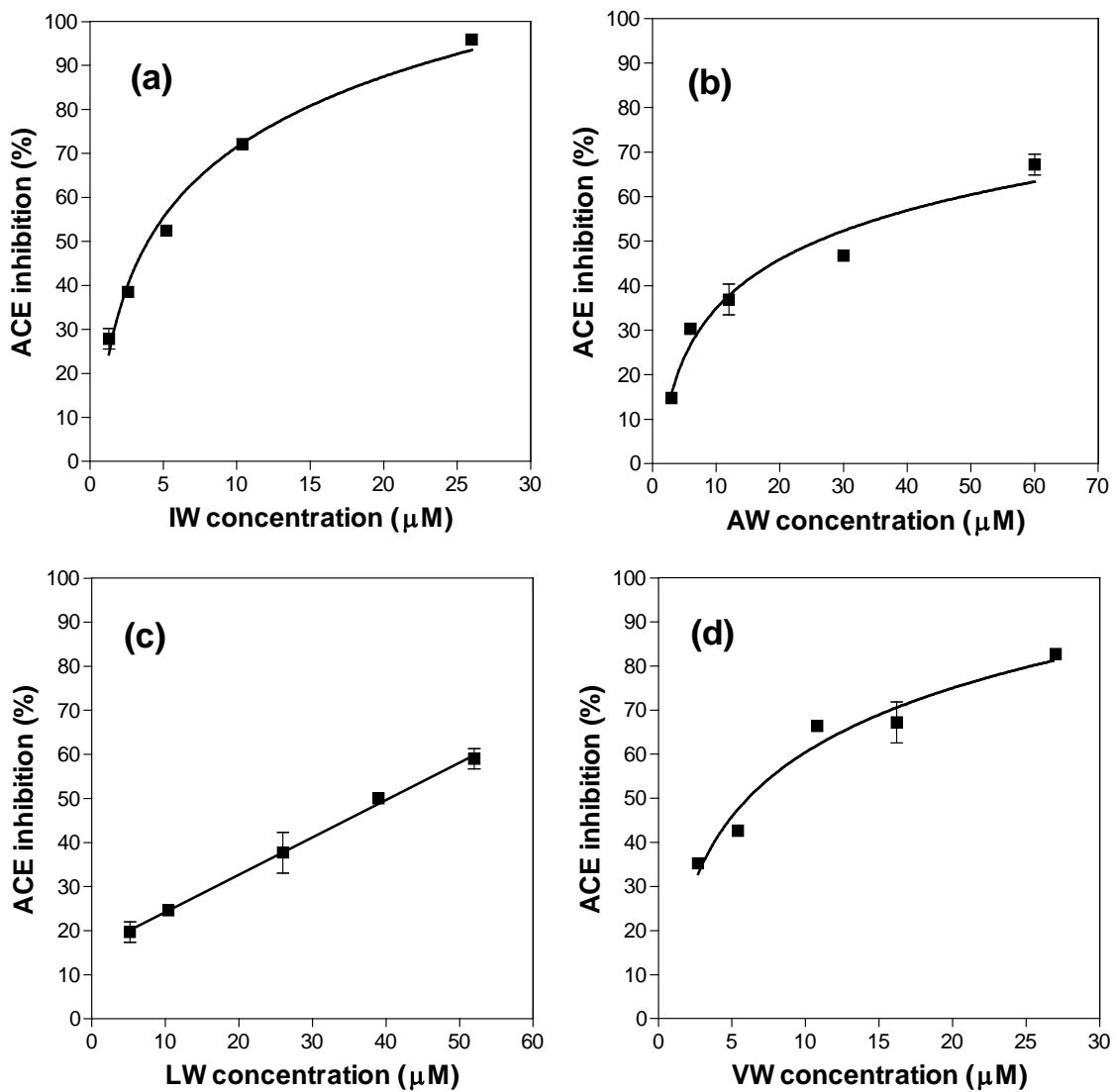
**CHAPTER NINE**

**GENERAL DISCUSSION AND CONCLUSIONS**

In a bid to utilize flaxseed meal proteins in human foods, we took a number approaches to produce peptide mixtures with structural properties that can be applied towards management and treatment of chronic human diseases. Experimental approach included *in vitro* and *in vivo* methods, which provided fundamental as well as applied knowledge relating to the potential use of flaxseed proteins and peptides as functional foods and nutraceuticals. Therefore, our work has provided a potential platform for the comprehensive utilization of flaxseed proteins by the foods and nutrition industries. This work also contributed to the understanding of structural requirements for renin inhibition by food protein-derived dipeptides that may be useful for biologists and chemists interested in developing novel active pharmaceutical ingredients. The key outcomes of this thesis are as follows:

(1) Development of a novel processing method for isolation of flaxseed proteins from highly viscous aqueous suspension of defatted flaxseed meal. This approach involved the use of a food grade cellulolytic enzyme in removing interfering flaxseed mucilage prior to protein isolation by conventional method resulting in a higher yield of isolated flaxseed protein when compared to previously reported methods. This process distinctly utilizes industrially produced high mucilage defatted flaxseed meal by-product as opposed to reported methods for flaxseed protein isolation from laboratory-prepared samples that usually contain less mucilage after

dehulling. Thus, our method confirmed that industrial flaxseed meal protein can be isolated and potentially utilized in the food system.

(2) Controlled hydrolysis of the isolated flaxseed proteins using specific and non-specific proteases, and processing using a combination of membrane ultrafiltration and ion-exchange chromatography to yield LMW peptides with multifunctional bioactive properties. Multifunctional peptides have the advantage of modulating more than one disease-related cellular processes thereby targeting more than one disease condition at a time. The LMW peptides in our studies showed antioxidant activities against various free radicals implicated in pathogenesis of human diseases, suppressed nitric oxide production induced by bacterial lipopolysaccharide in cultured murine macrophages, and also exhibited ACE and renin inhibition *in vitro*. Enzyme inhibition kinetics studies showed, for the first time, that the food peptide fraction inhibited human recombinant renin via an uncompetitive inhibition pattern; the peptides potentially exhibited both active site and allosteric inhibition of human renin. Thus, these flaxseed peptides possess multifunctional properties with relevance to the management and treatment of hypertension and associated cardiovascular disease, including oxidative stress and inflammation-related diseases. Most bioactive peptides are designed to target one disease symptom at a time, thus, the multifunctionality of the flaxseed peptides could potentially reduce the cost associated with the development of multiple bioactive peptides for multiple diseases.

(3) Successful development of a cheap, efficient and industrially-feasible method to enzymatically generate and fractionate BCAA-rich high Fischer ratio peptides from flaxseed proteins. Due to the high amount of BCAA and low amount of AAA, this peptide product can be used in clinical nutrition to increase plasma amino acid Fischer ratio in patients with chronic liver diseases with concomitant improvement of associated hepatic encephalopathy and muscle loss. Unlike previously reported methods, our method involves a simple mixing of peptide products with activated carbon in a container followed by centrifugation. In our opinion, this process is cheaper and less time-consuming than traditionally reported methods that involved various forms of expensive column chromatography.

(4) Successful development of a production process to enzymatically release Arginine (Arg)-containing peptides from flaxseed proteins and separation of these peptides based on their net molecular charge using a novel membrane technology, electrodialysis-ultrafiltration. The resulting peptide product contained high amounts of Arg and decreased systolic blood pressure in spontaneously hypertensive rat either through the vasodilatory activity of nitric oxide produced from Arg, or through the inhibition of ACE and renin activities. This is a novel approach towards managing hypertension using processed food ingredients, different from modulation of RAS or blocking AT-II receptors. To the best of our knowledge, this will be the first report of the production and utilization of food protein-derived arginine-enriched peptide mixture for the management of hypertension. An attractive advantage of the product is the ability to attenuate high blood pressure at

a fast rate (< 2 h) when compared to amino acid form of arginine and the protein isolate. The work also successfully showed for the first time that flaxseed proteins in the unhydrolyzed form can modulate blood pressure, though at a slower rate than the peptide product. Therefore, flaxseed proteins may find utilization in food formulations designed to remedy cardiovascular impairment, especially hypertension. Successful incorporation and adoption of flaxseed proteins in foods could result in significant economic impact specifically on the Prairie Provinces and generally on the Canadian economy.

(5) Moreover, this study was the first to report the structural requirements for renin inhibition by food protein-derived peptides using QSAR. The PLS models reported in this thesis provided a potent dipeptide, Ile-Trp (IW), which can serve as template for the design and synthesis of peptidomimetics with potent renin-inhibitory and *in vivo* antihypertensive properties. One of the significant outcomes is the fact that renin inhibition differs for peptides that have same molecular weight and amino acid sequence but in which the amino acids are geometric isomers.

It could be concluded from this thesis work that flaxseed proteins contain multifunctional bioactive peptide sequences that can be released by enzymatic hydrolysis, and that the structure of dipeptides determine their *in vitro* renin inhibitory and antihypertensive properties. Thus, this project has uniquely contributed knowledge that can enhance value-added utilisation of defatted flaxseed meal generated from Canada's oilseed processing industry, and to the elucidation of structure-function properties of renin-inhibiting natural dipeptides. Since these peptides are generated

from edible food sources, these contributions to research are important in the discovery of a new generation of non-toxic therapeutic agents for clinical intervention against chronic human diseases especially hypertension and cardiovascular disease, renal and inflammatory diseases. The fact that the arginine-rich peptides exhibited blood pressure-lowering activity provides preliminary evidence of the *in vivo* bioavailability of the flaxseed protein-derived peptides especially in the vascular endothelium where activity is required. Moreover, more extensive animal studies are needed to analyze the animal tissues for the presence of intact peptides following oral intake of therapeutic amounts of these flaxseed peptides.

Based on the outcome of this project, future studies include pilot scale isolation of flaxseed proteins using the method reported in this thesis, to investigate the feasibility for food industry production and practical application of the proteins in the human food system. As a follow-up to the short-term antihypertensive studies, animal feeding trials using the flaxseed protein isolates are ongoing in our laboratory to investigate the effects of long-term consumption of the intact flaxseed proteins on SBP in SHR. Moreover, animal studies are also needed to confirm the positive effects of the BCAA-enriched flaxseed peptides during chronic hepatic diseases. Due to low yield of the arginine-enriched peptides, future studies should focus on optimization of the enzymatic hydrolysis process to produce a more potent flaxseed protein hydrolysate, and electrodialysis-ultrafiltration process to increase yield of the final product, as well as long-term animal studies to investigate sustained positive effects of the peptide product on elevated blood pressure. A long-term study to determine the sustained hypotensive

effects of flaxseed protein hydrolysate containing arginine peptides is warranted. Future work should also determine the contributions of arginine-enriched peptides to the nitric oxide content of blood vessels and major organs. It will be important to determine whether arginine-enriched peptides work as hypotensive agents through enhanced nitric oxide production or inhibition of the renin-angiotensin system or both. Finally, peptide chemometrics studies should focus on synthesis of structural variants of IW and evaluation of the renin-inhibitory and antihypertensive activities of these peptidomimetics.

The consumption of flaxseed for human health benefits is increasingly gaining global attention especially in Canada, and this focus is mostly due to the presence of bioactive constituents including omega-3 oil, dietary fibres and polyphenolic lignans. The outcome of this project has added a new dimension to the potential health benefits derived from flaxseed components, and could provide the Canadian consumer with an alternative choice of healthy food products for the prevention and management of many chronic human diseases and health conditions.