FUNCTIONAL AND BIOACTIVE PROPERTIES OF FLAXSEED PROTEINS AND PEPTIDES

By

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ABSTRACT

The aim of this thesis was to determine flaxseed protein functionality and confirm the bioactive properties of the enzymatic protein hydrolysates using *in vitro* and *in vivo* methods. Flaxseed albumins and globulins were extracted using NaCl and then separated by membrane dialysis. SDS-PAGE analyses showed that the globulin fraction consisted of polypeptides in the 10-50 kDa range while the albumin fraction mainly contained a 10 kDa polypeptide. Amino acid analysis revealed significantly ($p<0.05$) higher levels of hydrophobic amino acids in the globulin when compared to the albumin, which also corresponded to globulin's higher surface hydrophobicity. A systematic evaluation of methods to determine the protein content of enzymatic protein hydrolysates suggests HPLC amino acid analysis as the most accurate. Subsequent hydrolysis of flaxseed proteins was carried out using thermoase GL-30, a food grade protease, in order to produce flaxseed protein hydrolysates (FPH), which were then fractionated by means of ultrafiltration into peptide fractions ranging in size from <1-10 kDa. When tested for *in vitro* antihypertensive and antioxidant properties, the FPH and its membrane fractions were found to scavenge free radicals, chelate metal ions, and reduce ferric iron, in addition to inhibiting the activities of angiotensin converting enzyme and renin. The flaxseed proteins also reduced systolic blood pressure in spontaneously hypertensive rats after oral administration by up to 37 mmHg within 8 h of administration. Finally, this work demonstrated the capacity of calmodulin-dependent phosphodiesterase (CaMPDE)-inhibitory flaxseed-derived peptides to be transported across the intestinal epithelium, and to influence activity of the target enzyme following absorption. Initial cell culture experiments using Caco-2 cells confirmed cell membrane permeation by the peptides in addition to absence of cytotoxicity. After individual oral administration of six CaMPDE-inhibitory peptides, two were detected in the blood of Wistar

rats up to 90 min while all six peptides each inhibited plasma CaMPDE activity with AGA being the most effective. In addition to the discovery of several novel antioxidant flaxseed peptide sequences, this work demonstrated the antihypertensive properties of flaxseed protein hydrolysates, and provided novel information on the bioavailability and *in vivo* efficacy of flaxseed-derived CaMPDE-inhibitory peptides.

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FOREWORD

The manuscript format was followed in producing this doctoral dissertation. The work consists of five manuscripts bookended by a ***general introduction*** (Chapter 1) and a ***literature review*** (Chapter 2) portion at one extreme, and a ***general discussion and conclusion*** part (Chapter 8) at the other. Each manuscript was prepared according to the style of the journal it is intended to be/ has been published in. Manuscript 1 (Food Chemistry) deals with the physicochemical properties of flaxseed albumin and globulin fractions while manuscript 2 (Food Chemistry) examines the quantification of peptides in food protein hydrolysates. Manuscript 3 (International Journal of Molecular Sciences) describes the *in vitro* and *in vivo* blood pressure-lowering properties of flaxseed protein hydrolysates while manuscript 4 (Journal of the American Oil Chemists' Society) discusses the effects of peptide size and enzyme concentration on antioxidant properties of the hydrolysates. Manuscript 5 (Journal of Agricultural and Food Chemistry) discusses the transepithelial transport, bioavailability and *in vivo* efficacy of calmodulin inhibitory flaxseed protein-derived peptides. A transition statement added at the end of each manuscript chapter links it to the next chapter and provides coherence. Finally, the last chapter (Chapter 8) contains an overall summary of the work, limitations, and possible future directions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

(mean ±standard deviation) with different letters have mean values that are significantly different at $P < 0.05$.

Figure 6.4. Metal chelating effects of flaxseed protein hydrolysates (FPH) and their ultrafiltered peptide fractions compared to that of reduced glutathione (GSH). Bars with different letters are significantly different at $P < 0.05$. Values are means (n=3) ± SD.

Figure 6.5. Ferric reducing antioxidant power (FRAP) of flaxseed protein hydrolysates (FPH) and their membrane fractions compared to that of reduced glutathione (GSH). Bars (mean ± standard deviation) with different letters have mean values that are significantly different at $P < 0.05$. NAD = no activity detected.

Figure 7.1. *In vitro* CaMPDE inhibition: 12 out of 27 peptides screened for *in vitro* CaMPDE inhibitory activity were effective in blocking the enzyme's action to varying degrees as shown. Data presented are mean ± standard deviation. Different letters on the bar charts indicate significant differences ($p < 0.05$) in enzyme inhibition among peptides.

Figure 7.2. Cell viability and cytotoxicity assay. Caco-2 cells were treated with various (2-10 mM) peptide concentrations and the effect of the peptides on cell viability was tested using the CCK-8 reagent. The peptides had no observable toxic effect on the cells but instead stimulated growth in the cell culture.

Figure 7.3. Peptide transport in Caco-2 cells. The transport of various FITC-labelled peptides (AGA, QIAK, KQLSTGC, QQAKQ, AKLMS and RWIQ) in confluent Caco-2 cell line was monitored from 0-24 h by means of fluorescent measurements. Data presented are mean ± standard deviation. For each peptide, different letters on the bar charts indicate significant differences ($p < 0.05$) at different time points.

Figure 7.4. Quantification of peptides present in rat serum at 30, 60 and 90 mins post-gavage using LC/MS/MS. ND = not detected. Data presented are mean ± standard deviation. For each time period, different letters on the bar charts indicate significant differences ($p < 0.05$) between peptides at that time point.

Figure 7.5. Representative chromatograms of peptide samples detected in rat serum. AGA-M-60 = peak for AGA fed to male rats and detected 60 mins post gavage; AKLMS-F-30 = peak for AKLMS administered to female rats and detected 30 mins post gavage; RWIQ-M-90 = peak for RWIQ fed to male rats and detected 90 mins post gavage.

Figure 7.6. Residual plasma CaMPDE activity. Measurement of residual CaMPDE activity in rat plasma as an indication of the inhibitory potential of peptides administered to rats by oral gavage. Data presented are mean ± standard deviation. For each time period, different letters on the bar charts indicate significant differences ($p < 0.05$) between peptides at that time point.

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LIST OF ABBREVIATIONS

AAA	Aromatic amino acids
ABTS	2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACE	Angiotensin-1 converting enzyme
ADME	Absorption, distribution, metabolism and excretion
AIDS	Acquired immune deficiency syndrome
ALA	Alpha-linolenic acid
ALB	Albumin
Ang_{1-7}	Angiotensin 1-7
ANOVA	Analysis of variance
ANS	8-Anilino-1-naphthalenesulfonic acid
AT-I	Angiotensin-I
AT-II	Angiotensin-II
ATCC	American type culture collection
BAPs	Bioactive peptides
BCAA	Branched chain amino acids
BPH	Beef protein hydrolysate
BSA	Bovine serum albumin
Caco-2	Colon adenocarcinoma cells
CaM	Calmodulin (or calcium-modulating protein)
CaMKII	$Ca^{2+/}$calmodulin-dependent protein kinase II
CaMKIIN	Ca^{2+}/calmodulin-dependent protein kinase II inhibitor protein
cAMP	Cyclic adenosine monophosphate

CaMPDE	Calmodulin-dependent phosphodiesterase
CCK-8	Cell counting kit-8
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CPH	Casein protein hydrolysate
CREB	cAMP responsive element binding protein
DASH	Dietary approaches to stop hypertension
DMEM	Dulbecco's modified eagle's medium
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DRSA	DPPH radical scavenging activity
DTT	Dithothreitol
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ES	Emulsion stability
ESI	Electrospray ionization
ExPASy	Expert protein analysis system
FAPGG	*N*-(3-[2-Furyl]acryloyl)-phenylalanylglycylglycine
FBS	Fetal bovine serum
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
FPH	Flaxseed protein hydrolysate
FPI	Flaxseed protein isolate
FPM	Flaxseed protein meal

FRAP	Ferric-reducing antioxidant power
GLB	Globulin
GSH	Reduced glutathione
HAA	Hydrophobic amino acids
HAT	Hydrogen atom transfer
H_4B	Tetrahydrobiopterin
HHL	Hippuryl-l-histidyl-l-leucine
H_2O_2	Hydrogen peroxide
HPH	Hempseed protein hydrolysate
HPI	Hemp protein isolate
HPLC	High performance liquid chromatography
HPLC-API-MRM	High performance liquid chromatography-atmospheric pressure ionisation multiple reaction monitoring
HPM	Hemp protein meal
HRSA	Hydroxyl radical scavenging activity
HT-29	Human colon cancer cell lines
IEC-18	Intestinal epithelial cell line-18
kDa	Kilodalton
LA	Linoleic acid
LC-MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LDL	Low density lipoprotein
MALDI-IMS	Matrix-assisted laser desorption/ionization time-of-flight imaging

	mass spectrometry
MALDI-MS	Matrix-assisted laser desorption/ionization-mass spectrometry
MDCK	Madin-Darby Canine Kidney
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MWCO	Molecular weight cut-off
NCAA	Negatively charged amino acids
PCAA	Positively charged amino acids
PepT1	Peptide transporter-1
NAD	No activity detected
ND	Not detected
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide anion
$\cdot OH$	Hydroxyl radicals
OONO	Peroxynitrite
OPA	Ortho-phthalaldehyde
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PDE	Phosphodiesterase

pI	Isoelectric point
PLN	Phospholamban
POT	Proton-oligopeptide transporter
PPH	Pea protein hydrolysate
PS	Protein solubility
QSAR	Quantitative structure activity relationship
QTRAP®	Quadrupole ion trap
RAAS	Renin-angiotensin-aldosterone system
RAS	Renin-angiotensin system
RLU	Relative luminescence unit
ROS	Reactive oxygen species
RP-HPLC	Reversed phase high performance liquid chromatography
RYRs	Ryanodine receptors
S_o	Surface hydrophobicity
SBP	Systolic blood pressure
SCAA	Sulfur-containing amino acids
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SERCA	Sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase
SET	Single electron transfer
SHRs	Spontaneously hypertensive rats
SPH	Soy protein hydrolysate
SPI	Soy protein isolate

SRSA	Superoxide radical scavenging activity
TCA	Trichloroacetic acid
TEER	Transepithelial electrical resistance
TJs	Tight junctions
TNF-α	Tumour necrosis factor -α
WHO	World health organization
WPH	Whey protein hydrolysate
WST-8	(2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

CHAPTER ONE

1. GENERAL INTRODUCTION

Functional foods are everyday foods to which specific nutrients or bioactive ingredients have been added usually for the purpose of demonstrable health benefits beyond basic nutrition (Agriculture and Agri-Food Canada, 2017; Institute of Food Technologists, 2017). Given the growing consumer interest in disease prevention, increasing global average life expectancy and rising healthcare cost, the global functional food market, which has an average annual growth rate of about 8.5%, has steadily experienced a rapid increase in value and is estimated to exceed USD 305.4 billion by 2020 (Bigliardi & Galati, 2013; Bogue, Collins, & Troy, 2017). Bioactive components can be isolated from functional foods and "generally sold in medicinal forms not usually associated with food" as nutraceuticals (Health Canada, 1998). Unlike functional foods whose beneficial health effects may not be readily felt after ingestion due to poor bioavailability often as a result of complex food matrix interactions (Udenigwe & Fogliano, 2017), nutraceuticals which may be available as pills and therefore more bioaccessible, are by definition "demonstrated to have a physiological benefit or provide protection against chronic disease" (Health Canada, 1998) in the short term. In Canada, the most recent statistics suggests a rapid growth in the functional foods and natural health products sector with about 750 establishments employing over 37,000 workers and reporting a revenue of about $16.4 billion in 2011 (Khamphoune, 2013). Compared to the last publicly available data from Statistics Canada detailing the state of the sector in 2005, these figures represent an enormous increase in market value, job creation and number of businesses established (Palinic, 2007). Similarly, there has been a steady upsurge in demand for the use of plant proteins as ingredients in functional foods

(Ajibola et al., 2016). This growing trend is partly linked to preference for plant-based diets as a result of religious beliefs and the thinking that plant proteins are generally healthier than animal proteins, concern over infected meat and meat products (prion disease), and dietary/lifestyle choices such as veganism (Karaca et al., 2011). In the last three decades, defatted flaxseed meal has emerged as an important source of functional food proteins, additive to functional foods, and nutraceuticals (Marambe & Wanasundara, 2017; Oomah & Mazza, 1993).

Canada is the world's leading producer and exporter of flax (*Linum usitatissimum* L.), a major oilseed crop whose primary purpose of cultivation has gradually evolved from the time it was first grown as a source of fibre in ancient civilizations until the present day (Rowland, 2013; Stefansson, 2013; van Sumere, 1992). Canada grows about 40% of global flaxseed, and together with other major flaxseed producing countries such as Russia, India, China and Kazakhstan, is responsible for >70% of the world's annual flaxseed production (Marambe & Wanasundara, 2017). In the last four years, annual flaxseed cultivation has covered between 850,000 and 1.5 million ha in Canada while annual production has ranged from 548,200 to over 872,000 metric tonnes (Statistics Canada, 2018). The term "flaxseed" is often commonly used to refer to the seeds of flax when used for human consumption while "linseed" is frequently used to describe the seed of flax when intended for feed and industrial applications (Morris, 2007). A member of the Linaceae family, flaxseed's Latin name conveys a sense of its multifunctionality (*usitatissimum* means "most useful"), a point that is borne out by the various uses to which the annual crop's different parts could be put (Marambe & Wanasundara, 2017). For instance, flax was primarily cultivated for textile purposes in ancient Egypt where its stem fibre was used for making linen clothing while the oil crushed from its seed (linseed oil) was mainly a secondary product of linen fibre production (Rowland, 2013). The advent of the Industrial Revolution

altered the focus of flax production and use as the growing demand for paint, linoleum and oilcloths necessitated a redirection of efforts to produce more of its oil for use as industrial drying oil (Rowland, 2013). In more recent times, flax straw has been used to produce fine paper used mainly as cigarette paper as a result of its clean-burning and tasteless attributes, while its fibres have been incorporated into paper money to make it more resistant to abrasion (Rowland, 2013).

The composition of flax varies with genetics, cultivation environment, as well as method of processing and analysis (Daun et al., 2003). Apart from its total dietary fibre content, which has been reported to be up to 28%, the brown Canadian flax contains about 40% fat and up to 25% protein (Morris, 2007; Rowland, 2013). Majority of flaxseed's nutritional and health promoting properties have been traditionally credited to its polyunsaturated fatty acids especially α-linolenic acid (ALA), the essential omega-3 fatty acid and linoleic acid (LA), the essential omega-6 fatty acid (Goyal et al., 2014; Morris, 2007). Given the focus on flaxseed's oil content and fatty acids, the defatted protein-rich meal, which is left over following the extraction of essential oils from the crushed oilseed by (enzyme-aided) pressing or solvent extraction (Anwar et al., 2013; Goyal et al., 2014; Pradhan et al., 2010), has been known to be commonly discarded as an underutilized coproduct. In addition to proteins, defatted flaxseed meal also contains varying quantities of dietary fibre and lignans (Marambe & Wanasundara, 2017).

Several studies have reported the health benefits of defatted flaxseed meal constituents with *in vitro* antidiabetic, antihypertensive, anti-inflammatory, calmodulin-inhibitory and antioxidant properties in particular being attributed to the protein-derived bioactive peptides or BAPs (Doyen et al., 2014; Marambe et al., 2008; Udenigwe & Aluko, 2012a; Udenigwe et al., 2009a). A common assay for determining the potential antihypertensive activity of flaxseed

peptides and protein hydrolysates involves testing their ability to inhibit the *in vitro* activities of angiotensin-1 converting enzyme (ACE) and/or renin, which are two key enzymes in the renin-angiotensin system that controls mammalian blood pressure (Udenigwe et al., 2009b). Similarly, various tests are used for evaluating the *in vitro* antioxidant capacity of protein hydrolysates and peptides including those which test their capacity for scavenging free radicals, chelating metal ions and inhibiting linoleic acid oxidation (Girgih et al., 2013). Since excessive levels of the calcium-binding protein, calmodulin (CaM) could lead to the overexpression of CaM-dependent phosphodiesterase (CaMPDE) and ultimately result in the development of chronic diseases such as cancer, bioactive inhibitors are employed to bind to and minimize or prevent CaM-regulated enzyme reactions that could potentially lead to pathological conditions (Aluko, 2010; Li & Aluko, 2006). In this regard, a previous work has shown flaxseed protein-derived peptides to be potent inhibitors of *in vitro* CaMPDE activity (Udenigwe & Aluko, 2012a). However, there is no report on the bioavailability and *in vivo* CaMPDE-inhibitory ability of these peptides.

Bioactive peptides can be defined as short amino acid sequences, which are usually released from food proteins through enzymatic hydrolysis, gastrointestinal digestion, fermentation or other forms of processing, and which in addition to their regular nutritional benefits, possess the ability to positively influence physiological processes and/or conditions. It has been suggested that the specific bioactivity of BAPs depends on the structural and physicochemical properties of the amino acid residues such as peptide chain length, amino acid primary structure, molecular charge, molecular weight, hydrophobicity, and amino acid composition (Anne Pihlanto & Mäkinen, 2013; Ryan et al., 2011; Udenigwe & Aluko, 2012b). Bioactive peptides have also been described as "cryptic peptides" since they are typically hidden within the parent protein where they remain inactive until their liberation by endogenous or

exogenous enzymes (Udenigwe et al., 2013). BAPs differ from bioactive proteins normally found in foods such as milk-derived immunoglobulins (Kitts & Weiler, 2003) as well as from the bioactive peptide fragments released from precursor proteins by the action of various proteases including signal peptidases and prohormone convertases during the maturation processes of peptidergic hormones and neurotransmitters (Ueki et al., 2007). In addition to the afore-stated *in vitro* beneficial health effects credited to flaxseed protein-derived bioactive peptides, various studies have linked BAPs from both animal and plant sources with multiple health-promoting functions including immunomodulatory, antithrombotic, anti-genotoxic, hypocholesterolemic, antimicrobial, hypolipidemic, opioid, anti-inflammatory and anticancer properties (Bhat et al., 2015; Udenigwe & Aluko, 2012b; Udenigwe et al., 2017). Given the cost and undesirable side effects sometimes experienced with conventional medication (Barton, et al., 2013; Bhat et al., 2015; Girgih et al., 2016), interest in bioactive peptides and other food-derived bioactives such as plant sterols, phenolics, phytoestrogens, bioactive polysaccharides, and organosulfur compounds has grown enormously among researchers and consumers alike in the recent past (Bhat et al., 2015). The synthesis of bioactive peptides using recombinant DNA technology, which represents the most inexpensive technique for producing BAPs on a large scale, has been recently described (Udenigwe et al., 2017).

In recent years, there have been increased efforts to isolate bioactive peptides from underutilized and/or inedible food sources as the continued mining of edible and comparatively more expensive food proteins such as milk for these peptides is unsustainable in the face of growing food insecurity. For instance, peptides with antioxidant, calcium-binding, and blood pressure-lowering properties have been isolated from underutilized sources as diverse as porcine plasma, meat muscle, fish and shellfish wastes, sea urchin, rice dreg, and pumpkin oil cake

proteins (Harnedy & FitzGerald, 2012; Lafarga & Hayes, 2014; Liu et al., 2010; Qin et al., 2011; Ryan et al., 2011; Vaštag et al., 2011; Zhao et al., 2012). In addition to providing alternative and more sustainable sources of functional peptides, the value-added utilization of otherwise low-value foodstuff has been promoted as a strategy for reducing environmental carbon footprint as well as the burden and cost of food waste disposal. For instance, in the poultry industry the disposal of chicken skin, a by-product of chicken meat processing has been both costly and inefficient with detrimental effects for the environment (Feddern et al., 2010). Therefore, it has been demonstrated that enzymatic hydrolysis of chicken skin for the isolation of health-promoting bioactive peptides (Onuh et al., 2013) could be a practical means of valorizing the waste.

In spite of the demonstrated health-promoting properties of food protein hydrolysates and bioactive peptides, challenges to the actualization of their potential health applications and large scale commercialization exist. One of these challenges is the bitter taste of protein hydrolysates and peptides. Although most proteins exhibit no bitterness prior to proteolysis, hydrolysates and peptides are known to develop a range of bitter tastes following digestion by proteases (Aluko, 2017). Amino acids and peptides have been shown to activate about six out of the 25 known members of the human T2R bitter taste receptors (part of the Ca^{2+}-linked G protein coupled receptors) (Kohl et al., 2013). Since the choice of protease used for hydrolysis could greatly influence the bitterness intensity of the digest (Humiski & Aluko, 2007), carefully selecting the enzyme to be used for hydrolysis is critical for minimizing bitter taste. Other strategies for reducing bitterness in hydrolysates and peptides have been studied including additional hydrolytic treatment with an exopeptidase (Cheung et al., 2015), the use of bitterness-masking ingredients such as potassium chloride and sucrose, as well as encapsulating bitter protein

hydrolysates and peptides by means of maltodextrin/cyclodextrin-aided spray drying (Yang et al., 2012).

Reliable and accurate determination of the amount of proteins, bioactive peptides and amino acids in biological samples like foods is critical not only to ensure their quality and safety, but also in order to guarantee the quantification of absolute protein amounts and experimental validity (Moore et al., 2010; Trötschel & Poetsch, 2015). As grimly illustrated by the injury and death of children and pets in the melamine adulteration case that shocked the globe a decade ago (Ingelfinger, 2008), sole reliance on analytical methods which do not discriminate between protein-based nitrogen and non-protein nitrogen could have deleterious consequences not only for the global food industry, but also for public health and international trade (Moore et al., 2010). Commonly used quantification methods such as the Kjeldahl and enhanced Dumas methods have received intense criticism since they quantify proteins and peptides based on the amount of nitrogen contained in the biological samples (Moore et al., 2010). There has been significant growth in the number and sophistication of protein quantification methods developed in the last 40 years, with each method varying from the other in sensitivity, reproducibility, specificity, accuracy, speed, cost and ease of performance among other parameters (Chutipongtanate et al., 2012; Mok et al., 2015; Moore et al., 2010; Sapan et al., 1999). For instance, a recent work reported increased accuracy and a 6-fold decrease in the assay time of a modified ninhydrin test conducted at high temperature in the presence of sodium borohydride (Mok et al., 2015). Given that food proteins and peptides are naturally present in complex food matrices, other components present in the matrix can influence their determination (Moore et al., 2010), thus underlining the unique challenges of quantifying them with accuracy and precision.

The appeal or consumer acceptability of any food is known to be influenced by its sensory attributes (Desai et al., 2013; Font-i-Furnols & Guerrero, 2014; Grigor et al., 2016). For example, functional yogurt containing added whey protein concentrate received the lowest liking scores while flavoured full-fat Greek yogurt was the most preferred in a recent blinded sensory study in which consumers had earlier indicated a purchasing preference for reduced, low or non-fat yogurt (Desai et al., 2013). The functional properties of food proteins such as emulsion-forming, gelation, water holding capacity, solubility and foam capacity have been shown to affect their organoleptic characteristics. For instance, apart from obvious economic implications, the foam capacity of malt-derived proteins can have a considerable influence on overall beer quality (Evans et al., 1999) while water holding capacity is a critical factor in meat quality attributes such as juiciness, tenderness and appearance (Cheng & Sun, 2008). Therefore, the functional and physicochemical properties of proteins, such as flaxseed which could be used as functional food ingredients, warrant careful examination as they could potentially influence the organoleptic qualities of food products and therefore their consumer acceptability and market value.

In order to exert their bioactivity *in vivo*, orally consumed peptides must reach the blood circulatory system in an active and structurally intact form, while withstanding the proteolytic action of intestinal peptidases in the process (Vermeirssen et al., 2004). Several studies have reported the transport of orally administered food-derived bioactive peptides to the blood circulation in both animals and humans with examples being the detection of casein peptides in the plasma of rats (Sánchez-Rivera et al., 2014) and humans (Caira et al., 2016). Although peptides have been linked with various *in vitro* bioactive properties, there is a dearth of similar *in vivo* accounts of beneficial physiological activities (Matsui, 2018). The paucity of *in vivo* studies

corresponding to the beneficial effects observed in *in vitro* studies is not unconnected to the suboptimal absorption, distribution, metabolism and excretion (ADME) properties of peptides, which have been known to contribute to their poor bioavailability (Foltz et al., 2010). Apart from challenges surrounding peptide bioavailability, details of the mechanisms, routes and factors influencing peptide transport are still poorly understood. For instance, although it is known that di- and tripeptides are transported through the H^+-coupled intestinal peptide transporter, PepT1, the transport routes of peptides with more than four amino acid residues remain unclear (Matsui, 2018). Thus, studies which contribute to a greater understanding of the factors influencing the transport and bioavailability of bioactive peptides are needed.

1.1 Hypotheses

I. Specific alterations of the environment surrounding albumin and globulin proteins derived from flaxseed will result in structural and conformational changes, which could affect their functionality in food products.

II. Analysis of the amino acid composition of a food protein hydrolysate will provide the most accurate measurement of its protein content.

III. The enzymatic hydrolysis of flaxseed protein isolate will yield peptides with *in vitro* and *in vivo* antihypertensive properties.

IV. The size of bioactive peptides from flaxseed hydrolysates will influence their *in vitro* antioxidative properties.

V. When administered to rats by oral gavage, flaxseed-derived peptides could be transported across their intestinal epithelia and be detected in plasma.

1.2 Objectives

Based on the afore-stated hypotheses, the objectives of this work were to:

(a). Produce albumin and globulin protein fractions from flaxseed proteins and study their physicochemical and emulsification properties under varying pH conditions.

(b). Employ seven different standard protein measurement methods in determining the protein contents in enzymatic hydrolysates produced from various food proteins.

(c). Determine the ability of enzymatic flaxseed protein hydrolysates and peptides to inhibit the *in vitro* activities of angiotensin-1 converting enzyme (ACE) and renin in addition to reducing the blood pressure of spontaneously hypertensive rats (SHRs).

(d). Use various enzyme concentrations to hydrolyze flaxseed protein isolate, separate the resultant hydrolysates into peptide fractions of different sizes by means of ultrafiltration and determine ability of the peptides to scavenge free radicals and chelate metal ions.

(e). Use Caco-2 cells and Wistar rats as models to study the safety, transport, bioavailability and inhibition of *in vivo* CaMPDE activity of flaxseed-derived peptides.

1.3 References

Agriculture and Agri-Food Canada. (2017). Functional Foods and Natural Health Products. Retrieved from http://www.agr.gc.ca/eng/industry-markets-and-trade/canadian-agri-food-sector-intelligence/functional-foods-and-natural-health-products/?id=1170856376710. Accessed on June 12, 2018.

Ajibola, C. F., Malomo, S. A., Fagbemi, T. N., & Aluko, R. E. (2016). Polypeptide composition and functional properties of African yam bean seed (*Sphenostylis stenocarpa*) albumin, globulin and protein concentrate. *Food Hydrocolloids*, **56**, 189-200.

Aluko, R. E. (2010). Food protein–derived peptides as calmodulin inhibitors. In Y. Mine, & E. Li-Chan (Eds.). *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals,*(pp. 55-65). Ames, Iowa: Wiley-Blackwell.

Aluko, R. E. (2017). Structural characteristics of food protein-derived bitter peptides. In M. Aliani, & M.N.A. Eskin (Eds.). *Bitterness: Perception, Chemistry and Food Processing*, (pp. 105-129). Hoboken, New Jersey: John Wiley & Sons, Inc.

Anwar, F., Zreen, Z., Sultana, B., & Jamil, A. (2013). Enzyme- aided cold pressing of flaxseed (*Linum usitatissimum* L.): enhancement in yield, quality and phenolics of the oil. *Grasas y Aceites,* **64**(5): 463-471.

Barton, S. E., Najita, J. S., Ginsburg, E. S., Leisenring, W. M., Stovall, M., Weathers, R. E., Sklar, C. A., Robison, L. L., & Diller, L. (2013). Infertility, infertility treatment, and achievement of pregnancy in female survivors of childhood cancer: a report from the Childhood Cancer Survivor Study cohort. *Lancet Oncology,* **14**(9): 873-881.

Bhat, Z., Kumar, S., & Bhat, H. (2015). Bioactive peptides of animal origin: a review. *Journal of Food Science and Technology,* **52**(9): 5377-5392.

Bigliardi, B., & Galati, F. (2013). Innovation trends in the food industry: the case of functional foods. *Trends in Food Science & Technology*, **31**(2):118-129.

Bogue, J., Collins, O., & Troy, A. J. (2017). Market analysis and concept development of functional foods. In D. Bagchi, & S. Nair (Eds.). *Developing New Functional Food and Nutraceutical Products*, (pp. 29-45). London, United Kingdom: Elsevier.

Caira, S., Pinto, G., Vitaglione, P., Dal Piaz, F., Ferranti, P., & Addeo, F. (2016). Identification of casein peptides in plasma of subjects after a cheese-enriched diet. *Food Research International*, **84**, 108-112.

Cheng, Q., & Sun, D.-W. (2008). Factors affecting the water holding capacity of red meat products: a review of recent research advances. *Critical Reviews in Food Science and Nutrition,* **48**(2):137-159.

Cheung, L. K. Y., Aluko, R. E., Cliff, M. A., & Li-Chan, E. C. Y. (2015). Effects of exopeptidase treatment on antihypertensive activity and taste attributes of enzymatic whey protein hydrolysates. *Journal of Functional Foods,* **13**, 262-275.

Chutipongtanate, S., Watcharatanyatip, K., Homvises, T., Jaturongkakul, K., & Thongboonkerd, V. (2012). Systematic comparisons of various spectrophotometric and colorimetric methods to measure concentrations of protein, peptide and amino acid: detectable limits, linear dynamic ranges, interferences, practicality and unit costs. *Talanta,* **98**, 123-129.

Daun, J., Barthet, V., Chornick, T., & Duguid, S. (2003). Structure, composition, and variety development of flaxseed. In L.U. Thompson, & S.C. Cunnane (Eds.). *Flaxseed in Human Nutrition*, 2nd edition, (pp. 1-40). Champaign, Illinois: AOCS Press.

Desai, N. T., Shepard, L., & Drake, M. A. (2013). Sensory properties and drivers of liking for Greek yogurts. *Journal of Dairy Science,* **96**(12):7454-7466.

Doyen, A., Udenigwe, C. C., Mitchell, P. L., Marette, A., Aluko, R. E., & Bazinet, L. (2014). Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. *Food Chemistry,* **145**, 66-76.

Evans, D., Sheehan, M. C., & Stewart, D. C. (1999). The impact of malt derived proteins on beer foam quality. Part II: The influence of malt foam-positive proteins and non-starch polysaccharides on beer foam quality. *Journal of the Institute of Brewing*, **105**(3): 171-177.

Feddern, V., Kupski, L., Cipolatti, E. P., Giacobbo, G., Mendes, G. L., Badiale-Furlong, E., & De Souza-Soares, L. A. (2010). Physico-chemical composition, fractionated glycerides and fatty acid profile of chicken skin fat. *European Journal of Lipid Science and Technology,* **112**(11):1277-1284.

Foltz, M., van Der Pijl, P., & Duchateau, G. (2010). Current *in vitro* testing of bioactive peptides is not valuable. *The Journal of Nutrition,* **140**(1):117-118.

Font-i-Furnols, M., & Guerrero, L. (2014). Consumer preference, behavior and perception about meat and meat products: an overview. *Meat Science*, **98**(3): 361-371.

Girgih, A.T., Udenigwe, C.C., & Aluko, R.E. (2013). Reverse-phase HPLC separation of hemp seed (*Cannabis sativa* L.) protein hydrolysate produced peptide fractions with enhanced antioxidant capacity. *Plant Foods for Human Nutrition*, **68**(1):39-46.

Girgih, A. T., Nwachukwu, I. D., Hasan, F. M., Fagbemi, T. N., Malomo, S. A., Gill, T. A., & Aluko, R. E. (2016). Kinetics of *in vitro* enzyme inhibition and blood pressure-lowering effects of salmon (*Salmo salar*) protein hydrolysates in spontaneously hypertensive rats. *Journal of Functional Foods,* **20**, 43-53.

Goyal, A., Sharma, V., Upadhyay, N., Gill, S., & Sihag, M. (2014). Flax and flaxseed oil: an ancient medicine & modern functional food. *Journal of Food Science and Technology,* **51**(9):1633-1653.

Grigor, J. M., Brennan, C. S., Hutchings, S. C., & Rowlands, D. S. (2016). The sensory acceptance of fibre-enriched cereal foods: a meta- analysis. International Journal of Food Science and Technology, **51**(1):3-13.

Harnedy, P. A., & FitzGerald, R. J. (2012). Bioactive peptides from marine processing waste and shellfish: a review. *Journal of Functional Foods,* **4**(1):6-24.

Health Canada. (1998). Nutraceuticals/Functional Foods and Health Claims on Foods. Retrieved from https://www.canada.ca/en/health-canada/services/food-nutrition/food-labelling/health-claims/nutraceuticals-functional-foods-health-claims-foods-policy-paper.html. Accessed on March 13, 2018.

Humiski, L. M., & Aluko, R. E. (2007). Physicochemical and bitterness properties of enzymatic pea protein hydrolysates. *Journal of Food Science,* **72**(8), S605-S611.

Ingelfinger, J. R. (2008). Melamine and the global Implications of food contamination. *The New England Journal of Medicine,* **359**(26):2745-2748.

Institute of Food Technologists. (2017). Functional foods. Retrieved from http://www.ift.org/knowledge-center/focus-areas/food-health-and-nutrition/functional-foods.aspx. Accessed on January 7, 2018.

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. *Food Research International*, **44**(9):2991-2998.

Khamphoune, B. (2013). Results from the 2011 Functional Foods and Natural Health Products Survey. Retrieved from https://www150.statcan.gc.ca/n1/pub/18-001-x/18-001-x2013001-eng.htm. Accessed on January 14, 2018.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design*, **9**(16):1309-1323.

Kohl, S., Behrens, M., Dunkel, A., Hofmann, T., & Meyerhof, W. (2013). Amino acids and peptides activate at least five members of the human bitter taste receptor family. *Journal of Agricultural and Food Chemistry*, **61**(1):53-60.

Lafarga, T., & Hayes, M. (2014). Bioactive peptides from meat muscle and by-products: generation, functionality and application as functional ingredients. *Meat Science*, **98**(2): 227-239.

Li, H., & Aluko, R. E. (2006). Structural modulation of calmodulin and calmodulin-dependent protein kinase II by pea protein hydrolysates. *International Journal of Food Sciences and Nutrition*, **57**(3-4):178-189.

Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry*, **118**(2):403-410.

Marambe, H. K., & Wanasundara, J. P. D. (2017). Protein From Flaxseed (Linum *usitatissimum* L.). In J. P. D. W. S. Nadathur, & L. Scanlin (Eds.). *Sustainable Protein Sources*, (pp. 133-144). Boston, Massachusetts: Elsevier.

Marambe, P. W. M. L. H. K., Shand, P. J., & Wanasundara, J. P. D. (2008). An in-vitro investigation of selected biological activities of hydrolysed flaxseed (*Linum usitatissimum* L.) Proteins. *JAOCS, Journal of the American Oil Chemists' Society*, **85**(12):1155-1164.

Matsui, T. (2018). Are peptides absorbable compounds? *Journal of Agricultural and Food Chemistry*, **66**(2):393-394.

Mok, Y., Arantes, V., & Saddler, J. (2015). A $NaBH_4$ coupled ninhydrin-based assay for the quantification of protein/enzymes during the enzymatic hydrolysis of pretreated lignocellulosic biomass. *Applied Biochemistry and Biotechnology*, **176**(6):1564-1580.

Moore, J. C., Devries, J. W., Lipp, M., Griffiths, J. C., & Abernethy, D. R. (2010). Total protein methods and their potential utility to reduce the risk of food protein adulteration. *Comprehensive Reviews in Food Science and Food Safety*, **9**(4):330-357.

Morris, D.H. (2007). Flax- a health and nutrition primer. 4th edition. Winnipeg, Manitoba: Flax Council of Canada. Retrieved from https://flaxcouncil.ca/resources/nutrition/technical-nutrition-information/flax-a-health-and-nutrition-primer/. Accessed on January 5, 2018.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Research International*, **53**(1): 260-267.

Oomah, B. D., & Mazza, G. (1993). Flaxseed proteins-a review. *Food Chemistry*, **48**(2):109-114.

Palinic, R. (2007). Results from the Functional Foods and Nutraceuticals Survey- 2005. Retrieved from https://www150.statcan.gc.ca/n1/pub/88f0006x/88f0006x2007003-eng.pdf. Accessed on January 14, 2018.

Pihlanto, A., & Mäkinen, S. (2013). Antihypertensive properties of plant protein derived peptides. In B. Hernández-Ledesma, & C.-C. Hsieh (Eds.). *Bioactive Food Peptides in Health and Disease*, (pp. 145-182). Rijeka, Croatia: InTech.

Pradhan, R. C., Meda, V., Rout, P. K., Naik, S., & Dalai, A. K. (2010). Supercritical CO_2 extraction of fatty oil from flaxseed and comparison with screw press expression and solvent extraction processes. *Journal of Food Engineering,* **98**(4):393-397.

Qin, L., Zhu, B.-W., Zhou, D.-Y., Wu, H.-T., Tan, H., Yang, J.-F., Li, D.-M., Dong, X.-P., & Murata, Y. (2011). Preparation and antioxidant activity of enzymatic hydrolysates from purple sea urchin (*Strongylocentrotus nudus*) gonad. *LWT - Food Science and Technology*, **44**(4):1113-1118.

Rowland, G.G. (2013). Flax. Retrieved from https://www.thecanadianencyclopedia.ca/en/article/flax. Accessed on January 3, 2018.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: meat and fish. *Nutrients,* **3**(9):765-791.

Sapan, C. V., Lundblad, R. L., & Price, N. C. (1999). Colorimetric protein assay techniques. *Biotechnology and Applied biochemistry*, **29**(Pt 2):99-108.

Statistics Canada. (2018). Estimated areas, yield, production, average farm price and total farm value of principal field crops, in metric and imperial units. Retrieved from http://www5.statcan.gc.ca/cansim/a26?lang=eng&id=10017. Accessed on January 5, 2018.

Stefansson, B. R. (2013). Oilseed crops. Retrieved from https://www.thecanadianencyclopedia.ca/en/article/oilseed-crops. Accessed on January 3, 2018.

Sánchez-Rivera, L., Ares, I., Miralles, B., Gómez-Ruiz, J. Á., Recio, I., Martínez-Larrañaga, M. R., Anadón, A., & Martínez, M. A. (2014). Bioavailability and kinetics of the antihypertensive casein-derived peptide HLPLP in rats. *Journal of Agricultural and Food Chemistry*, **62**(49):11869-11875.

Trötschel, C., & Poetsch, A. (2015). Current approaches and challenges in targeted absolute quantification of membrane proteins. *Proteomics*, **15**(5-6):915-929.

Udenigwe, C.C., & Aluko, R.E. (2012a). Multifunctional cationic peptide fractions from flaxseed protein hydrolysates. *Plant Foods for Human Nutrition,* **67**(1):1-9.

Udenigwe, C. C., & Aluko, R. E. (2012b). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science*, **77**(1):R11-R24.

Udenigwe, C. C., & Fogliano, V. (2017). Food matrix interaction and bioavailability of bioactive peptides: two faces of the same coin? *Journal of Functional Foods,* **35**, 9-12.

Udenigwe, C. C., Gong, M., & Wu, S. (2013). In silico analysis of the large and small subunits of cereal RuBisCO as precursors of cryptic bioactive peptides. *Process Biochemistry,* **48**(11):1794-1799.

Udenigwe, C. C., Lu, Y. L., Han, C. H., Hou, W. C., & Aluko, R. E. (2009a). Flaxseed protein-derived peptide fractions: antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. *Food Chemistry,* **116**(1):277-284.

Udenigwe, C. C., Lin, Y. S., Hou, W. C., & Aluko, R. E. (2009b). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods,* **1**(2):199-207.

Udenigwe, C. C., Nwachukwu, I. D., & Yada, R. Y. (2017). Advances on the production and application of peptides for promoting human health and food security. In G. V. Barbosa-Cánovas, G. María Pastore, K. Candoğan, I. G. Medina Meza, S. Caetano da Silva Lannes, K. Buckle, R. Y. Yada, & A. Rosenthal (Eds.). *Global Food Security and Wellness*, (pp. 195-219). New York, NY: Springer.

Ueki, N., Someya, K., Matsuo, Y., Wakamatsu, K., & Mukai, H. (2007). Cryptides: functional cryptic peptides hidden in protein structures. *Peptide Science*, **88**(2):190-198.

van Sumere, C. (1992). Retting of flax with special reference to enzyme-retting. In C.F. van Sumere, & H.S.S. Sharma (Eds.). *The Biology and Processing of Flax*, (pp. 153-193). Belfast, Northern Ireland: M Publications.

Vaštag, Ž., Popović, L., Popović, S., Krimer, V., & Peričin, D. (2011). Production of enzymatic hydrolysates with antioxidant and angiotensin-I converting enzyme inhibitory activity from pumpkin oil cake protein isolate. *Food Chemistry*, **124**(4):1316-1321.

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition*, **92**(3):357-366.

Yang, S., Mao, X.-Y., Li, F.-F., Zhang, D., Leng, X.-J., Ren, F.-Z., & Teng, G.-X. (2012). The improving effect of spray- drying encapsulation process on the bitter taste and stability of whey protein hydrolysate. *European Food Research and Technology*, **235**(1):91-97.

Zhao, Q., Xiong, H., Selomulya, C., Chen, X. D., Zhong, H., Wang, S., Sun, W., & Zhou, Q. (2012). Enzymatic hydrolysis of rice dreg protein: effects of enzyme type on the functional properties and antioxidant activities of recovered proteins. *Food Chemistry*, **134**(3):1360-1367.

CHAPTER TWO

LITERATURE REVIEW

2.1 Functional properties of flaxseed albumin and globulin proteins

The functional properties of proteins can play a consequential role in their ultimate use in food product formulations (Amagloh et al., 2013; Nwachukwu & Aluko, 2018; Wouters et al., 2016). These functional attributes including protein emulsion, gelation, solubility, and foaming capacity are often influenced by their structure. For instance, the strategy for the production of cheese or yogurt is predicated on the enzyme-mediated perturbation of casein micelles or modification of their isoelectric point, which can subsequently lead to coagulation or gelation (Huppertz et al., 2018). Also, variations in pH are known to influence the overall charge on ionizable amino acid residues including glutamic acid, histidine, arginine, and lysine, thus effecting changes in electrostatic interactions and possibly modifying the conformation of proteins, which would affect their functionality (Yin et al., 2010). Unlike more popular plant proteins such as those from soy, kidney beans and pea (Aluko et al., 2009; Kinsella, 1979; Liang & Tang, 2013; Shevkani et al., 2015; Were et al., 1997), studies on the structural and functional attributes of proteins from underutilized sources such as flaxseed are limited. Thus, the critical study of structure-dependent functionalities of flaxseed proteins is important given the valuable information that could be derived from such an endeavor.

Although the two main storage proteins (albumin and globulin) found in flaxseed have been previously studied (Ganorkar & Jain, 2013; Marambe & Wanasundara, 2017; Oomah & Mazza, 1993; Sammour et al., 1994), the currently available information on their functional properties can hardly be described as adequate. The predominantly salt soluble 11-12S globulin

fraction is known to have a higher molecular weight (252-298 kDa) and contains about 18.6% nitrogen while the polar 1.6-2S albumin has a comparably lower molecular weight (16-17 kDa) and a nitrogen content of about 17.7% (Karaca et al., 2011; Marambe & Wanasundara, 2017). The albumin fraction is a basic protein and consists of a single polypeptide chain, whereas flaxseed globulin with a 4.75 isoelectric point (pI) is made up of at least five subunits, which are bound by disulfide bonds (Karaca et al., 2011).

2.2 Extraction of flaxseed proteins

The purification of proteins, which typically involves separating the protein of interest from a cluster of proteins or from non-protein components in the cell is an essential step that usually precedes the study of its structure-function properties (Aluko, 2004). To begin the purification process, a protein needs to be extracted from its source using an appropriate solvent (Aluko, 2004). The specific type and duration of the extraction methods and (if applicable) sample pre-treatment protocols adopted are dependent on the nature of the protein source. For instance, non-collagenous materials were removed from jellyfish umbrellas by pre-treating with 0.1 M NaOH for two days prior to collagen protein extraction (Barzideh et al., 2014). Thus, the extraction of proteins from oilseed crops such as flaxseed usually requires defatting the sample as a pre-isolation protocol in order to prevent emulsion formation during protein extraction and minimize the oil content of the extract (Aluko, 2004). Given flaxseed's high mucilage content, removing the mucilage by heat, acid, alkali, or enzyme pretreatment is recommended in order to enhance the protein content of the isolate (Dev & Quensel, 1988; Madhusudhan & Singh, 1983; Wanasundara & Shahidi, 1997).

Various protein extraction methods such as those involving the use of water, aqueous alcohol, buffers and salts have been used to isolate albumin and globulin proteins from different

plant sources. Due to the solubility of albumin proteins in water, they can be isolated by aqueous extraction by mixing with distilled water for about 30-120 min at a low temperature (4 °C) in order to avoid proteolysis (Aluko, 2004). Liu et al (2008) employed the aqueous extraction technique to obtain albumin and globulin from three different chickpea (*Cicer arietinum*) varieties. In each case, defatted chickpea flour was stirred for 60 min in distilled water (pH 7.0) prior to centrifugation. The precipitate containing unwanted insoluble materials could be discarded at this point (Aluko, 2004) or further extracted with water to recover more proteins of interest in the second supernatant (Liu et al., 2008). The combined supernatants were subsequently dialyzed against deionized water to remove any residual salt (Liu et al., 2008). It has been suggested that using a low (<8 kDa) molecular weight cut-off (MWCO) dialysis membrane could minimize the loss of proteins of interest (Aluko, 2004). The supernatant obtained following dialysis could then be centrifuged or filtered to yield albumin as the supernatant or filtrate and globulin as the precipitate or residue (Aluko, 2004; Liu et al., 2008).

In the use of buffers to extract albumin and globulin from food proteins, researchers take advantage of the difference in pI between the protein of interest and the unwanted protein (Aluko, 2004). The solubility of proteins is lowest at their pI since they have no net charge and thus no electrostatic repulsions at that pH, making it possible to exploit the use of a buffer at a pH where difference in solubility exists between the target and unwanted protein. This method was used to isolate a protein fraction containing albumins and globulins from ground chickpea seeds (Chang et al., 2012) . First, the chickpea sample was extracted with NaOH at pH 11.5 for 1 h, before being centrifuged (8,000×g, 10 min). After filtering the resultant supernatant with fine glass wool, the pH of the filtrate was adjusted to 4.5 using HCl, and the isoelectric precipitate containing albumins and globulins was then recovered following another centrifugation step

(Chang et al., 2012). Similarly, a chickpea protein isolate, TpI, containing mainly globulins was obtained after NaOH extraction for 45 min at pH 9.0 and subsequent acid precipitation at pH 4.6 (Papalamprou et al., 2010).

Furthermore, isoelectric precipitation can also be used to differentially precipitate proteins through the use of sequential acid and alkali treatments. Whereas major proteins e.g. casein in liquid food proteins like milk can be isolated by sequential acidification and neutralization of the precipitate to yield caseinate, solid food protein samples need to be first solubilized in an aqueous (water, buffer, salt, etc) solution in order to extract the target protein (Aluko, 2004). Since other contaminating proteins and unwanted non-protein materials are invariably co-extracted with the protein of interest, the extracted mixture has to be fractionated by either filtration or centrifugation followed by pH adjustment (typically by acidification) to the pI of the target protein thus leading to its precipitation (Aluko, 2004).

This described isoelectric precipitation technique was used in the extraction of protein isolate from soy, peanut, and rapeseed protein meals (Chabanon et al., 2007; Chove et al., 2001; Jamdar et al., 2001). In each study, HCl was used to acidify the protein suspension and initiate target protein precipitation following NaOH-induced protein solubilisation and/or cellulose hydrolysis. Following protein precipitation, the precipitate which mainly contains the target protein could be obtained by centrifugation and the resultant protein isolate adjusted to pH 7.0 with dilute alkali to yield the more soluble "proteinate" which can be freeze-dried for subsequent use (Chove et al., 2001; Dev & Quensel, 1988; Udenigwe et al., 2009a). Should an investigator be interested in a protein concentrate rather than the isolate, the precipitation step is excluded and the aqueous extract is instead thoroughly dialyzed against water to remove salts and other impurities before being dried (Aluko, 2004; Sathe et al., 2002).

Proteins can also be isolated based on their solubility in varying concentrations of salts such as sodium chloride and ammonium sulfate (Aluko, 2004). Since protein solubility varies inversely with ionic strength, a salt solution such as that of ammonium sulfate containing various proteins can be adjusted (to a higher ionic strength) to a concentration just below the precipitation point of the target protein thus triggering the precipitation of most of the unwanted proteins while keeping the protein of interest in solution or vice versa (Aluko, 2004). For instance, kidney bean flour was extracted and fractionated into albumin and globulin using this strategy (Mundi & Aluko, 2012). The supernatant obtained from a 0.1 M phosphate buffer solution (pH 8) extraction of the flour was adjusted to 40% ammonium sulfate saturation, after stirring (4 °C, 2 h) and centrifugation (9000 x g, 45 min, 4 °C). The precipitate from a second centrifugation step was discarded while the supernatant was further brought to 80% ammonium sulfate saturation and centrifuged as before. The resultant precipitate was then collected, dispersed in deionized water and dialyzed against water using a 6-8 kDa MWCO dialysis membrane at 4 °C in order to remove residual ammonium sulfate. The contents of the dialysis tubing were centrifuged for the last time (9000×g, 45min at 4 °C), and the supernatant collected as albumin while the precipitate was collected as the globulin fraction (Mundi & Aluko, 2012). Dilute solutions of NaCl can also be used to extract globulin proteins due to their solubility in salt as demonstrated in the use of 0.5 M NaCl to extract and separate proteins from hempseed meal (Malomo & Aluko, 2015). In a protocol comparable to that described for the isolation of albumin and globulin proteins from kidney bean flour, dialysis against water (4 °C) was required to remove NaCl and for the separation of the globulin from the albumin fraction, which was co-extracted by the dilute NaCl solution (Aluko, 2004; Malomo & Aluko, 2015). Salt removal results in globulin precipitation, leaving the water-soluble albumin in solution (Malomo &

Aluko, 2015). Additionally, 7S and 11S globulins were isolated from mungbean (*Vigna radiata* [L.] Wilczek) by stirring the protein flour in 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl (Mendoza et al., 2001). After dialyzing the supernatant against distilled water to remove residual salts, the content of the dialysis bag was centrifuged for 15 min at 13,500 x *g* in order to separate the globulins (precipitate) from the albumins (supernatant).

Following the initial protein extraction step, the protein isolate or concentrate can be made to undergo any of a number of purification steps (Aluko, 2004) depending on the specific objectives of the study, cost and/ or use of the final product. Table 2.1 summarizes the various common protein purification techniques and their underlying principles. Prior to or after any further purification procedure, the protein content of the crude or purified protein isolate/concentrate can be measured using an appropriate protein determination method as described in the next section.

Table 2.1. Purification techniques and related molecular properties of proteins[a]

Technique	Molecular property of protein
Gel filtration (size exclusion chromatography)	Size (effective radius)
Ion exchange	Charge
Hydrophobic interaction and reverse-phase	Hydrophobicity
Affinity	Ligand recognition
Chromato-focusing	Isoelectric point
Foam fractionation	Surface activity

[a] Reproduced from (Aluko, 2004) and used with permission from Woodhead Publishing Ltd.

2.3 Protein determination in hydrolysates and peptides

Following the extraction (and/or purification) of proteins from a plant or animal source, it is essential to correctly determine the protein content of the isolate or concentrate, and ultimately

that of the hydrolysates and/or peptides. Since proteins are heterogeneous polymers, protein content determination could be tedious (Haven & Jorgensen, 2014). Over the last four decades, the number and complexity of protein measurement methods have rapidly increased as a result of efforts by research scientists to develop reliable, quick and accurate assay techniques for determining protein content (Gillette & Carr, 2012; Sapan et al., 1999). These efforts could be in recognition of the limitation of some existing methods such as the far UV-absorption procedure, which is affected by the amino acid composition of the protein sample due to differences in the molar extinction coefficients of the constituent amino acids (Haven & Jorgensen, 2014).

Additionally, the fact that some food components could share similar physicochemical properties further complicates the analysis of proteins (Chang, 2010). For instance, the source of non-protein nitrogen ordinarily present in protein hydrolysates could be one or more of free amino acids, small peptides, nucleic acids, phospholipids, amino sugars, porphyrin, certain vitamins, alkaloids, uric acid, urea, or ammonium ions (Chang, 2010). Thus, protein analytical protocols such as the Dumas method, which measures both organic and inorganic nitrogen lack analytical selectivity and tend to overestimate the protein content of food protein hydrolysates and peptides since they quantify all nitrogen-containing substances present in test samples including nitrates and nitrites (Chang, 2010; Moore et al., 2010). Hence, in measuring the protein content of food protein hydrolysates and peptides, the wide adoption of protein measurement methods, which place a premium on sensitivity, accuracy, reproducibility and the physicochemical properties of the hydrolysate and peptide constituents, is imperative.

2.4 Flaxseed protein-derived bioactive peptides and human health

Food protein hydrolysates and peptides have been linked with various beneficial effects including antihypertensive (Aluko, 2015a), antioxidant (Sila & Bougatef, 2016), calmodulin-

binding (Li & Aluko, 2005; Kizawa, 1997; You et al., 2010), opioid-like (Yang et al., 2001), anxiolytic (Hirata et al., 2007), antidiabetic (Mojica & De Meja, 2016), hypocholesterolemic (Nagaoka et al., 2001), antimicrobial (Sah et al., 2018), anti-thrombotic (Zhang et al., 2008), anti-cancer (Rajendran et al., 2017), anti-inflammatory and immunomodulatory properties (Ndiaye et al., 2012; Zhao et al., 2016) properties. In particular, bioactive peptides from flaxseed proteins have been shown to possess antihypertensive, antioxidant, anti-inflammatory and CaM-inhibitory properties. In one *in vitro* study, flavourzyme-hydrolyzed flaxseed proteins were found to be effective in inhibiting the activity of ACE, a metallopeptidase, and in scavenging hydroxyl radicals but had virtually no capacity for binding bile acids (Marambe et al., 2008). In this study, a maximum ACE-inhibitory activity of 88.29% was achieved with crude protein hydrolysates treated with the enzyme for 12 h while the ~ 22% hydroxyl radical scavenging activity was obtained with hydrolysates digested with flavourzyme for about 45 min. Interestingly, the unhydrolysed protein isolate showed no ACE-inhibitory or $OH^{\bullet}$-scavenging activity, which suggests that the observed bioactivities were solely due to peptides released during proteolysis (Marambe et al., 2008). In other studies, low molecular weight peptides and cationic peptide fractions obtained from enzymatic flaxseed protein hydrolysates following ultrafiltration and ion-exchange chromatography respectively, inhibited the activities of ACE and renin (Udenigwe et al., 2009a). The peptide fractions also scavenged free radicals and inhibited lipopolysaccharide-induced nitric oxide synthesis in RAW 264.7 macrophages (Udenigwe et al., 2009b). Furthermore, cationic peptides obtained following chromatographic separation of alcalase-hydrolysed flaxseed proteins by means of a cation-exchange column were found to bind and inactivate CaM while simultaneously inhibiting the activity of CaM-dependent phosphodiesterase or CaMPDE (Udenigwe & Aluko, 2012a). In fact, this study demonstrated the

multifunctional property of flaxseed bioactive peptides considering the ability of certain cationic peptide fractions to inhibit the activities of ACE, renin and CaMPDE (Udenigwe &Aluko, 2012b). Given the scope of this work, detailed discussions of the health-promoting properties of bioactive hydrolysates and peptides will be limited to their antihypertensive, antioxidant, and CaM-binding properties.

2.4.1 Other sources of bioactive peptides

Functional food protein hydrolysates and peptides have been isolated from animal sources such as eggs, cheese, milk, chicken, beef, shrimp, and oyster (Bhat et al., 2015), as well as from plant sources like rice, barley, oat, maize, wheat, pea, soy, mung bean, chickpea, walnut, sesame, canola, broccoli, spinach and flaxseed (Maestri et al., 2016). Apart from being generally considered the most important source of bioactive peptides partly as a result of the physicochemical and physiological versatility of isolated peptides (Bhat et al., 2015), milk is also the first reported source of food-derived bioactive peptides following the suggestion by Mellander that casein phosphopeptides enhanced vitamin D-independent bone calcification in infants with rickets (Mellander, 1950).

2.5 Hypertension, antihypertensive peptides and the renin-angiotensin system

The term hypertension or high blood pressure is used to describe a medical condition involving protracted elevated blood pressure during which blood is constantly pumped through the blood vessels with excessive force (WHO, 2011). In general, a hypertension diagnosis is made if during a period of sustained elevated arterial blood pressure, a systolic blood pressure (SBP) measurement ≥140 mmHg and/or a diastolic blood pressure reading ≥90 mmHg is obtained (Pickering et al., 2005). Hypertension is the leading risk factor for mortality and

disability globally, affecting about 26% of adults worldwide and contributing to nearly eight million premature deaths every year (Ettehad et al., 2016). High blood pressure is also linked to mild cognitive impairment, cognitive decline and dementia, including Alzheimer's disease (Iadecola, 2014). As a major risk factor for many common chronic disease conditions including stroke, atherosclerosis, chronic renal disease, heart failure, myocardial infarction, vascular dementia, coronary artery disease, and cerebrovascular disease (Picariello et al., 2011; Touyz et al., 2018), hypertension is estimated to affect over 1.5 billion people by 2025 (Liu et al., 2015), and thus represents a growing threat to global public health. According to the World Health Organization, nearly one billion people live with high blood pressure (WHO, 2011). A recent study estimated that in the United States, 34.9% of non-institutionalized adults aged 18 yrs or older had hypertension with the medical condition increasing the total annual medical expenditure of each adult by an additional $2,565 on the average, and costing the country up to USD $198 billion annually (Wang et al., 2017). In Canada where the economy loses more than CAD $22 billion annually in physician services, hospital costs, lost wages and reduced productivity due to heart disease and stroke (Smith, 2009), over 5.3 million people (corresponding to about 17.7 % of the country's population) have high blood pressure (Statistics Canada, 2016). Given the foregoing, it is no surprise that both the prevention and management of high blood pressure have become a public health priority worldwide with governments and researchers committing vast resources and monumental efforts to studies on the development of therapeutic agents and nutritional strategies that could help turn the growing tide of hypertension. Although environmental factors such as sleep quality are associated with hypertension (Liu, et al., 2015), the gains observed with dietary modification such as in DASH (Dietary Approaches to Stop Hypertension) studies suggest that nutritional strategies could be highly effective in

preventing and controlling high blood pressure (Sacks et al., 1999), while underlining the status of diet as the strongest environmental factor affecting blood pressure (Ralston et al., 2011). Given that blood pressure is modulated by the renin-angiotensin system, which is known to be targeted by both pharmacologic agents such as ACE inhibitors and dietary components like food-derived bioactive peptides (Majumder & Wu, 2015), an extensive discussion of that pathway now follows.

2.6 The renin-angiotensin system (RAS)

Although blood pressure can be modulated *via* alternate pathways (Nurminen et al., 2000), the RAS, which is also known as the renin-angiotensin-aldosterone system, (RAAS), plays a central role in blood pressure regulation as well as in physiological fluid and electrolyte balance (Majumder & Wu, 2015). Two key enzymes, renin and ACE are critical in the RAS pathway. The rate-limiting step in the cascade is catalyzed by the aspartyl protease, renin, which converts the zymogen angiotensinogen to angiotensin-I (AT-I) by cleaving the peptide bond between Leu10 and Val11 in the pathway's first reaction (Rao, 2010). AT-I, an inactive decapeptide, is subsequently converted to angiotensin-II (AT-II), a potent vasoconstrictor, by the action of ACE. In the classic RAS, the vasoconstrictive octapeptide AT-II executes its blood pressure-regulating function by binding to two major receptors namely angiotensin type 1 (AT-I) and type 2 (AT-II) receptors (Majumder & Wu, 2015). Although its interaction with these receptors is important for the maintenance of normal blood pressure, the effects of excessive AT-II levels produced in the pathological state could be detrimental. When in excess, the interaction of AT-II with AT-I receptors leads to vasoconstriction in vascular smooth muscle cells and causes adrenal glands to release aldosterone which stimulates sodium and fluid retention, resulting in vascular volume expansion, more constricted vessels and higher blood pressure

(Majumder & Wu, 2015; Rao, 2010; Ryan et al., 2011). Conversely, AT-II receptors found in both vascular smooth muscle and endothelial cells are involved in the release of the vasodilator, nitric oxide (Majumder & Wu, 2015). The vasodilatory nonapeptide, bradykinin, is an inflammatory mediator and ACE substrate that stimulates the release of nitric oxide and vasodilatory prostaglandins with resultant blood pressure lowering and vasoprotective effects (Majumder & Wu, 2015). Thus, bradykinin inactivation by ACE contributes to further constriction of blood vessels and elevated blood pressure (Ryan et al., 2011). Although direct inhibition of renin activity is a more effective strategy for controlling hypertension than ACE activity inhibition because the former blocks the synthesis of AT-I, which can be converted to AT-II *via* an alternative pathway that is independent of ACE but is catalyzed by the enzyme chymase instead (Udenigwe & Aluko, 2012a), renin inhibition does not prevent the hydrolytic conversion of bradykinin to inactive fragments by ACE. Therefore, pleiotropic agents with the ability to simultaneously inhibit the activities of ACE and renin are needed for the effective prevention and management of hypertension (Udenigwe et al., 2009a).

2.7 Mechanism of action of ACE-inhibitory peptides

The prospect of using food-derived peptides as blood pressure-lowering agents has gained increasing interest due to their relatively low cost, biogenic origin, and apparent lack of harmful side effects such as those (including skin rashes, dry cough, angioedema and diarrhoea) reported for synthetic ACE and renin inhibitors (Jauhiainen & Korpela, 2007). An important mode of action of synthetic ACE inhibitors such as fosinopril, enalapril, captopril, and lisinopril, which are regularly employed in hypertension treatment, myocardial infarction and congestive heart failure, is the simultaneous reduction of AT-II synthesis and attenuation of bradykinin breakdown (Erdmann et al., 2008; Iroyukifujita et al., 2000; Ryan et al., 2011). Unlike synthetic

ACE inhibitors which block ACE action by directly binding to the enzyme's active site, ACE-inhibitory peptides work by reacting with the enzyme and making it unavailable to cleave AT-I, and thus preventing the production of AT-II. Many studies such as those highlighted in Tables 2.2 and 2.3 have demonstrated the capacity of food protein-derived hydrolysates and peptides respectively to attenuate elevated blood pressure in rodent models.

Table 2.2. Effects of oral administration of food protein-derived protein hydrolysates on short-term (≤24 h) changes in the systolic blood pressure (SBP) of spontaneously hypertensive rats.[a]

Protein source	Protease(s)	Dose (mg/kg bw)	SBP reduction (mmHg)[b]
Gelatin	Thermolysin	300	−17 after 6 h
Pistachio	Pepsin	1000	−10 after 6 h
Pistachio	Trypsin	1000	−22 after 6 h
Pistachio	Pepsin + Trypsin	1000	−22 after 6 h
Fish	Thermolysin	500	−15 after 4 h
Sesame seed	Thermolysin	1	−30 after 8 h
Canola meal	Pancreatin	200	−15 after 6 h
Canola meal	Pepsin	200	−24 after 4 h
Canola meal	Trypsin	200	−5 after 8 h
Canola meal	Alcalase	200	−34 after 4 h
Canola meal	Chymotrypsin	200	−15 after 6 h
Rapeseed meal	Proteinase K	100	−5 after 8 h
Rapeseed meal	Thermolysin K	100	−9 after 8 h
Rapeseed meal	Alcalase	100	−24 after 8 h
Rapeseed meal	Flavourzyme	100	−17 after 6 h
Rapeseed meal	Pepsin + Pancreatin	100	−20 after 24 h
Almond	Neutrase + N120P	400	−17 after 2 h
Almond	Neutrase + N120P	800	−21 after 2 h
Bamboo shoot	None (aqueous extract)	20	−11 after 4 h
Bamboo shoot	None (aqueous extract)	50	−18 after 4 h
Bamboo shoot	None (aqueous extract)	100	−27 after 5 h
Mung bean	Alcalase	600	−30 after 6 h
Spanish ham	None (aqueous extract)	4.56	−38 after 6 h
Spanish ham	None (aqueous extract)	1.48	−28 after 6 h
Spanish ham	None (aqueous extract)	8.7	−24 after 6 h

Dairy whey	Crude enzyme extract	400	−22 after 4 h
Oyster	Pepsin	20	−16 after 4 h
Mushroom	None (aqueous extract)	600	−48 after 0.5 h
Rapeseed	Pepsin	500	−7 after 4 h
Rapeseed	Alcalase	500	−16 after 4 h
Soybean	Protease D3	50	−25 after 2 h
Soybean	Protease D3	500	−40 after 2 h
Soybean	Protease D3	1000	−50 after 2 h
Jellyfish	Pepsin + Papain	200	−23 after 2 h
Jellyfish	Pepsin + Papain	400	−28 after 4 h
Jellyfish	Pepsin + Papain	800	−31 after 2 h
Shrimp	Pepsin	300	−17 after 4 h
Shrimp	Pepsin	600	−27 after 6 h
Shrimp	Pepsin	900	−35 after 6 h
Bovine casein	A combination of 3 enzymes	5	−20 after 2 h
Bovine casein	A combination of 3 enzymes	10	−28 after 2 h
Bovine casein	A combination of 3 enzymes	100	−42 after 2 h
Bovine casein	Yeast (*Debaryomyces hansenii*)	200	−20 after 2 h
Bovine casein	Yeast (*Kluyveromyces lactis*)	200	−12 after 1 h
Bovine casein	Yeast (*Kluyveromyces marxianus*)	200	−20 after 2 h
Bovine lactoferrin	Yeast (*Debaryomyces hansenii*)	200	−18 after 1 h
Bovine lactoferrin	Yeast (*Kluyveromyces lactis*)	200	−10 after 1 h
Bovine lactoferrin	Yeast (*Kluyveromyces marxianus*)	200	−23 after 1 h
Dairy whey	Alcalase	240	−32 after 8 h
Silk fibroin	Alcalase	100	−11 after 4 h
Silk fibroin	Alcalase	600	−20 after 4 h
Silk fibroin	Alcalase	1200	−42 after 4 h
Hemp seed	Pepsin + Pancreatin	200	−30 after 8 h
Flaxseed	Thermoase	200	−29 after 4 h
Yellow field pea	Thermolysin	200	−19 after 4 h
Flaxseed	Trypsin + Pronase	200	−18 after 2 h
Palmaria palmata	Papain	50	−34 after 2 h

[a]Adapted from (Aluko, 2015a) and used with permission from Annual Reviews.
[b]Some values are estimates obtained from graphs in the source documents.

In addition to the well-known ACE inhibition pattern in the classic renin-angiotensin system, recent reports have suggested that bioactive peptides could lower blood pressure *via* an alternative pathway by upregulating ACE-2, an ACE homologue which counteracts the effects of

elevated ACE levels by cleaving AT-II to Ang_{1-7} (Wu et al., 2017). The oral administration of the egg ovotransferrin-derived tripeptide, IRW, to spontaneously hypertensive rats led to a significant increase in ACE-2 mRNA expression in the rats' mesenteric arteries (Majumder et al., 2015), a result that provides new information on the *in vivo* mechanism of action of antihypertensive peptides (Wu et al., 2017).

In studying the mechanism by which bioactive peptides inhibit ACE activity, double reciprocal plots which delineate the exact type of enzyme inhibition using the Lineweaver-Burk equation have been employed (Girgih et al., 2016; Girgih et al., 2011a; Onuh et al., 2015). For instance, a study of the kinetics of ACE inhibition by chicken skin proteins found that the mode of inhibition by the protein hydrolysates followed a mostly non-competitive or mixed type pattern, (Onuh et al., 2015), thus suggesting that the peptides had no structural similarity to the enzyme's natural substrate and therefore were bound to the enzyme in a region other than its active site, thus altering the enzyme's structural conformation and active site configuration, and effectively resulting in an inactive enzyme-substrate-inhibitor complex (Girgih et al., 2011a). The pronounced effect that slight differences in peptide sequence could have on the interaction between peptides and ACE was illustrated in a well-cited example (Sato et al., 2002) in which single amino acid substitutions altered the ACE inhibition pattern from uncompetitive (IW and FY) to non-competitive (LW and IY).

Although the mechanisms of ACE inhibition by peptides are yet to be entirely elucidated, partly because different food proteins yield ACE-inhibitory peptides with distinct sequences (Li et al., 2004), the interactions (Guo et al., 2017; Wu et al., 2015) of peptide inhibitors with the enzyme protein appear to follow patterns consistent with a previously proposed (Cushman et al., 1982; Ondetti & Cushman, 1982) binding model. For instance, although all three non-competitive

ACE-inhibitory peptides (RYDF, YASGR and GNGSGYVSR), which were recently obtained from the marine worm *Phascolosoma esculenta* (Guo, et al., 2017), formed hydrogen bonds with ACE residues, the binding interactions of the two most active peptides (YASGR and GNGSGYVSR) included bonds with residues located in the enzyme's main active site pockets. These active site pockets (S1, S1′ and S2′) represent the major sites of ACE interaction with ligands (or inhibitors) and are known to contain mainly hydrophobic amino acids (Ondetti & Cushman, 1982). It is known that the C-terminal tripeptide of peptide inhibitors, which strongly influences the interaction of peptides with ACE, can interact with the three subsites in the enzyme's active site (Li et al., 2004; Ondetti & Cushman, 1982). While a variety of peptide ACE inhibitors function as competitive inhibitors (Ryan et al., 2011), indicating their competition with ACE substrate for the enzyme's catalytic site (Li et al., 2004), others such as those purified from hazel nut in a recent study (Liu et al., 2018) function as non-competitive inhibitors. With most competitive ACE-inhibitory peptides containing hydrophobic amino acid residues (Li et al., 2004), the importance of hydrophobic amino acids to ACE inhibition is further underlined by studies suggesting that the crucial last four C-

Table 2.3. Effects of oral administration of food protein-derived peptides on systolic blood pressure (SBP) of spontaneously hypertensive rats[a]

Peptide sequence	Protein source	Protease(s)	Dose (mg/kg bw)	Max. SBP reduction (mmHg)[b]
IHRF	Rice glutelin	Chymotrypsin	5	−18
IHRF	Rice glutelin	Chymotrypsin	15	−39
DPYKLRP	Lactoferrin	Yeast extract	10	−27
PYKLRP	Lactoferrin	Yeast extract	10	−21
YKLRP	Lactoferrin	Yeast extract	10	−20
KLRP	Lactoferrin	Yeast extract	10	−12
LRP	Lactoferrin	Yeast extract	10	−27
GILRP	Lactoferrin	Yeast extract	10	−20

IAK	Casein	Synthesized	4	−21
HLPLP	Casein	Synthesized	7	−24
YAKPVA	Casein	Synthesized	6	−23
HPHPHLSF	Casein	Synthesized	10	−16
KKYNVPQL	Casein	Synthesized	10	−12
LVYPFTGPIPN	Casein	Synthesized	10	−28
WQVLPNAVPAK	Casein	Synthesized	7	−18
VPP	Sour milk	Fermentation	5	−20
IPP	Sour milk	Fermentation	5	−18
MRW	Spinach Rubisco	Pepsin + Pancreatin	30	−20
MRWRD	Spinach Rubisco	Pepsin + Pancreatin	30	−14
IAYKPAG	Spinach Rubisco	Pepsin + Pancreatin	100	−15
DY	Bamboo shoot	None (aqueous extract)	10	−18
AAATP	Spanish ham	None (aqueous extract)	1	−26
VNP	Rice	Alcalase + Trypsin	5	−29
VWP	Rice	Alcalase + Trypsin	5	−38
IRW	Egg ovotransferrin	Thermolysin + Pepsin	3	−10
IRW	Egg ovotransferrin	Thermolysin + Pepsin	15	−22
AVF	Insect	Pepsin + Trypsin + Chymotrypsin	5	−13
VF	Insect	Pepsin + Trypsin + Chymotrypsin	5	−19
GQP	Mushroom	None (aqueous extract)	1	−27
DKVGINYW	Dairy whey	Synthesized	5	−15
DAQSAPLRVY	Dairy whey	Synthesized	5	−10
KGYGGVSLPEW	Dairy whey	Synthesized	5	−20
YFP	Yellowfin sole	Chymotrypsin	10	−22
IY	Rapeseed	Alcalase	7.5	−10
VW	Rapeseed	Alcalase	7.5	−11
RIY	Rapeseed	Alcalase	7.5	−11
VWIS	Rapeseed	Alcalase	12.5	−13
VKKVLGNP	Porcine muscle	Pepsin	10	−24
RPR	Pork meat	Synthesized	1	−33
PTPVP	Pork meat	Synthesized	1	−26
KAPVA	Pork meat	Synthesized	1	−34
KRVIQY	Porcine myosin	Pepsin	10	−23
VKAGF	Porcine myosin	Pepsin	10	−17
MKP	Bovine casein	Alcalase + Trypsin + Protease N	0.1	−35
VEGY	Microalgae	Alcalase	10	−23

YH	Edible seaweed	Synthesized	50	−50
KY	Edible seaweed	Synthesized	50	−45
WY	Edible seaweed	Synthesized	50	−46
IY	Edible seaweed	Synthesized	50	−33
FQ	Buckwheat sprout	Synthesized	0.1	−42
VAE	Buckwheat sprout	Synthesized	0.1	−38
VVG	Buckwheat sprout	Synthesized	0.1	−38
DVWY	Buckwheat sprout	Synthesized	0.1	−59
FDART	Buckwheat sprout	Synthesized	0.1	−32
WTFR	Buckwheat sprout	Synthesized	0.1	−36
MAW	Cuttlefish	Synthesized	10	−13
AHSY	Cuttlefish	Synthesized	10	−14
VYAP	Cuttlefish	Synthesized	10	−22
VIIF	Cuttlefish	Synthesized	10	−19
WYT	Hemp seed	Pepsin + Pancreatin	30	−13
WVYY	Hemp seed	Pepsin + Pancreatin	30	−34
SVYT	Hemp seed	Pepsin + Pancreatin	30	−24
PSLPA	Hemp seed	Pepsin + Pancreatin	30	−40
IPAGV	Hemp seed	Pepsin + Pancreatin	30	−36
LY	Rapeseed	Alcalase	30	−26
TF	Rapeseed	Alcalase	30	−12
RALP	Rapeseed	Alcalase	30	−16
GHS	Rapeseed	Pepsin + Pancreatin	30	−17
WMP	Yellow field pea	Thermolysin	30	−39
ADMFPF	Yellow field pea	Thermolysin	30	−25
IRLIIVLMPILMA	*Palmaria palmata*	Papain	50	−33

[a]Adapted from (Aluko, 2015a) and used with permission from Annual Reviews.
[b]Some values are estimates obtained from graphs in the source documents.

terminal amino acid residues of peptides 4-10 amino acids long (known to be critical for ACE inhibition), usually contain hydrophobic side chains (Wu et al., 2006). For di- and tripeptides, C-terminal hydrophobic (aromatic or branched) side chains including phenylalanine, proline, tyrosine and tryptophan, and N-terminal aliphatic amino acids such as valine, isoleucine and leucine have been shown to be critical for ACE inhibition, which makes such peptides to be potent inhibitors (Li et al., 2004; Wu et al., 2006). Apart from the more effective interaction between such hydrophobic peptides and the hydrophobic residues present in the ACE active site,

other factors that could contribute to their more potent inhibition include hydrogen bond formation and interactions with ACE zinc ions (Erdmann et al., 2008; Lee et al., 2010). In contrast, substrates or competitive inhibitors with C-terminal dicarboxylic amino acids are not known to be as potent since the enzyme has a low affinity for them (Li et al., 2004). In general, hydrophilic peptides are weaker inhibitors of ACE activity since they are not compatible with the kind of interactions essential for inhibition, which typically involve hydrophobic residues located in the enzyme's active site (Erdmann et al., 2008). It is expected that future studies will continue to elucidate critical molecular mechanisms of peptide ACE inhibition using strategies such as RNA sequencing, quantitative structure activity relationship (QSAR) studies, gene functional analysis, computational studies (including molecular docking) and proteomics (Li et al., 2004; Majumder & Wu, 2015).

2.8 Production of bioactive peptides

In order to exert their bioactivity and/or physiological function, bioactive peptides must first be released from their parent proteins where they are "hidden" within such precursor proteins' primary structures (Udenigwe et al., 2013). In general, bioactive peptides are mainly released from food proteins during processing and consumption through gastric digestion, proteolysis by exogenous and endogenous enzymes, or *via* the action of microbial enzymes e.g. by way of fermentation using starter cultures (Udenigwe, 2014). For instance, using porcine pepsin and pancreatin to simulate gastrointestinal digestion, peptides with antioxidant properties were successfully released from cowpea proteins (Quansah et al., 2013). Although enzymatic hydrolysis is the most common method for generating bioactive peptides, they can also be produced using recombinant DNA technology as well as through chemical synthesis (Udenigwe et al., 2017).

Apart from being widely regarded as efficient, quick, safe and low cost, the *in vitro* enzymatic hydrolysis of precursor proteins to release active peptide fragments is also the most commonly used method for isolating cryptic peptides from food proteins (Díaz-Gómez et al., 2017; Ryan et al., 2011). In making the decision to use a particular protease or cocktail of proteases, an investigator must carefully consider the properties of such enzymes including their catalytic mechanism, active site specificity, optimum pH and temperature (Udenigwe et al., 2009a). As an example, the choice of the thermolysin isoform, thermoase GL-30, for the liberation of blood pressure-lowering bioactive peptides (Nwachukwu et al., 2014) was based on its known cleavage specificity for the N-terminal regions of bulky, aromatic and hydrophobic amino acids in order to release branched-chain amino acid-containing peptides, which are known to be highly effective in inhibiting ACE activity (Udenigwe & Aluko, 2010). Active peptide fragments and protein hydrolysates with various bioactive properties including immunomodulatory, antioxidant, anti-inflammatory (Ndiaye et al., 2012), blood pressure-lowering (Chay et al., 2018) and CaMPDE-inhibitory (Udenigwe & Aluko, 2012b) activities have all been obtained from parent proteins *via* enzymatic hydrolysis.

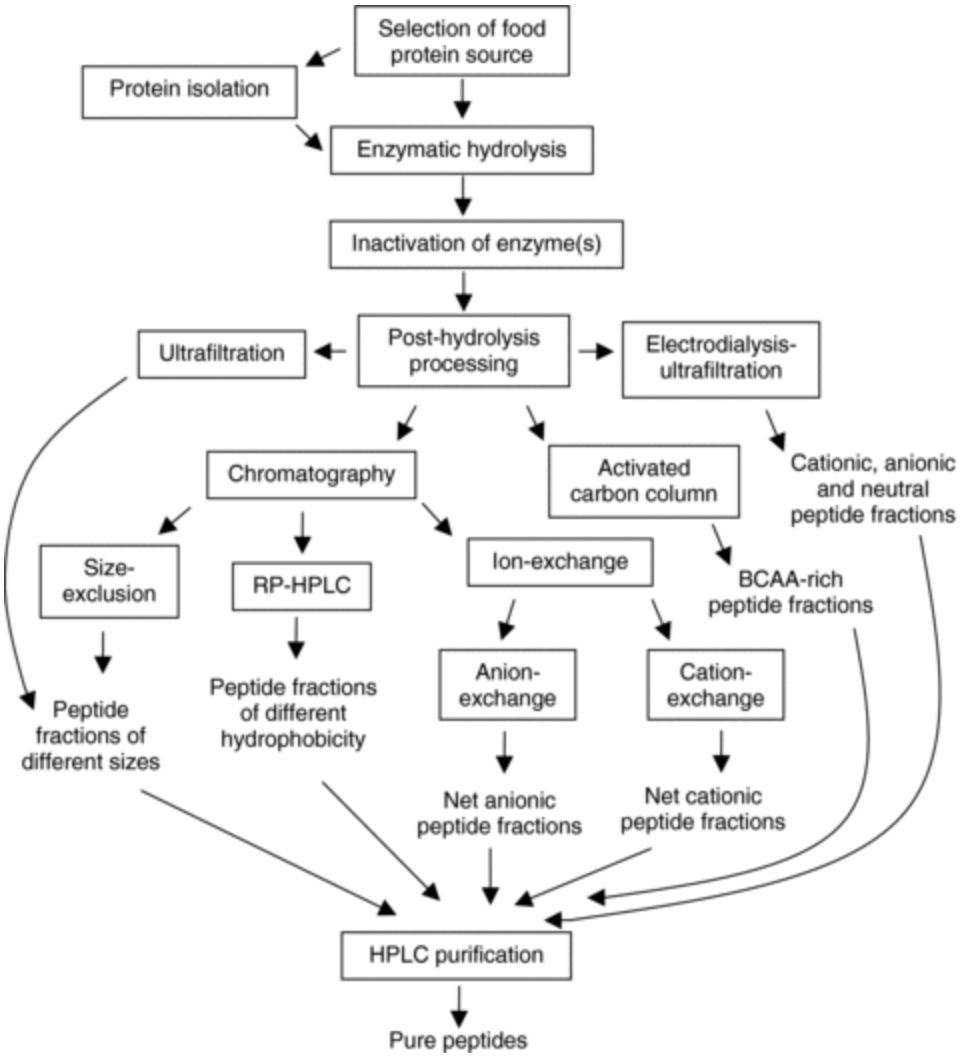

Fig. 2.1. Steps toward the production and processing of food protein-derived bioactive peptides. Reproduced from (Udenigwe & Aluko, 2012a) and used with permission from John Wiley & Sons, Inc.

The chemical properties of such enzyme-derived peptides including their bioactivity are influenced by a number of factors such as degree and duration of hydrolysis, enzyme-substrate ratio, extent of protein denaturation, sample pre-treatment prior to hydrolysis, as well as conditions in the reaction vessel e.g. pH, pressure and temperature (Kilara & Chandan, 2011; Udenigwe & Aluko, 2012a). A clear illustration of the immense enhancement of peptide bioactivity engendered by changes in reaction conditions could be seen in the enzymatic hydrolysis of pinto bean proteins where the peptides obtained under high pressure (up to 200

MPa) were found to possess more effective antioxidant and antihypertensive properties than those produced in the absence of such treatment (Garcia-Mora et al., 2016). It is thought that the increased dissociation of the oligomeric protein chains under high pressure may have led to the exposure of additional cleavage sites to the proteases used in the study (Garcia-Mora, et al., 2016). Usually, fractionation of the crude protein hydrolysate containing bioactive peptides is carried out after enzymatic hydrolysis in order to separate the peptides on the basis of hydrophobicity, size, or net charge thus yielding fractions of a particular chain length or molecular weight and/or fractions with increased concentrations of bioactive peptides of interest (Aluko, 2015a). Although such separation, which could be achieved by means of ion exchange chromatography (Udenigwe et al., 2009a) or ultrafiltration using membranes of various molecular weight cut-offs (Alashi et al., 2014) enables the collection of typically low molecular weight peptides with higher bioactivity in the permeate, and peptides of high molecular weight in the retentate which tend not be to be as biologically active (Aluko, 2015b; Nwachukwu et al., 2014; Tsai et al., 2008; Zhang et al., 2009), fractionation can indeed either increase or decrease peptide bioactivity depending on the protein hydrolysate (Aluko, 2015a; Li et al., 2014).

In a recently reported randomized, placebo-controlled clinical trial, the blood pressure, aldosterone and angiotensin-II levels of pre-hypertensive adults were markedly reduced following eight weeks of supplementation with fermented goat milk (Lu et al., 2018). In another study, bioactive peptides obtained from whey and skim milk fermentation were found to possess antimicrobial, ACE-inhibitory and antioxidant properties (Chandra & Vij, 2018). Bioactive peptides contained in such fermented food proteins are generated through the proteolytic action of microbial enzymes such as those produced by *Lactococcus lactis*, *Lactobacillus plantarum, Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus helveticus*, which are often

present in industrial starter cultures (Chandra & Vij, 2018; Girgih, 2013). When ingested, the bioactive peptides already produced in fermented food proteins by the action of bacterial enzymes could be further digested by gastrointestinal enzymes to release additional bioactive peptides with active sequences different from those in the fermented food product (Möller et al., 2008). Thus, microbial enzymes occupy a special place in the production of bioactive peptides as they could release bioactive peptides from food proteins in the gut (*in vivo*) as well as during food processing (Regazzo, 2010).

A more recent albeit less common means of producing bioactive peptides is through the use of recombinant DNA technology. Using this technique, copious numbers of a single recombinant peptide or several recombinant peptides can be produced within a relatively short period (Perez Espitia et al., 2012). *Escherichia coli* hosts are often used as expression systems for this method of peptide production as was demonstrated in the recombinant production of the chemopreventive peptide lunasin, in which a cellulose binding domain from the anaerobic bacterium *Clostridium thermocellum* was used as a fusion partner protein (Kyle et al., 2012). In addition to prokaryotes, transgenic plants including genetically modified rice and soybean have also been used as expression systems for the recombinant DNA production of bioactive peptides (González-Ortega et al., 2015; Udenigwe et al., 2017). Given its capacity for quickly generating bioactive peptides in abundance, it has been described as the cheapest method for producing food protein-derived peptides on an industrial scale (Li, 2011).

Finally, if the sequence of a peptide of interest is already known, such a peptide can be chemically synthesized under tightly regulated conditions that ensure minimal side reactions and the production of a pure protein fragment with a specific sequence (Perez Espitia et al., 2012; Udenigwe et al., 2017).

2.9 Reactive oxygen species, oxidative stress and antioxidants in human health

An inevitable consequence of everyday metabolic processes is the production of reactive oxygen species (ROS) such as the non-radical hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$) as well as free radicals like superoxide anion ($O_2^{\cdot-}$) or hydroxyl radicals ($^{\cdot}OH$) (Sharma et al., 2012; Udenigwe et al., 2017). ROS are also generated when growth factors and cytokines act to facilitate intracellular signalling (Storz, 2011) from exogenous sources such as diet (Cardoso et al., 2013) as well as during cellular response to xenobiotics and stressful or adverse environmental conditions like metal toxicity, UV-B radiation, extreme temperatures and microbial pathogen attack (Ray et al., 2012; Sharma et al., 2012). For instance, alcohol consumption is known to promote ROS generation (Wu & Cederbaum, 2003) while high fat diets and the accompanying steatosis are often associated with increased ROS production (Cardoso et al., 2013). When produced either in excess or in considerable amounts for prolonged periods, ROS can lead to oxidative stress and overwhelm the body's endogenous antioxidant defence system causing structural and function-related damages to macromolecules like DNA, lipids and proteins, and impairing physiological processes such as membrane fluidity, enzyme activity, ion transport, protein synthesis and protein cross-linking (Sharma et al., 2012; Storz, 2011). The generation of excess ROS in cells has the potential to upset intracellular redox homeostasis and often marks the onset of oxidative stress, thus orchestrating a cascade of mostly aberrant cellular events including cellular and organismal aging, senescence and ultimately, apoptosis (Ray et al., 2012; Storz, 2011). As a result of ROS-mediated damage of biological macromolecules, oxidative stress is often linked with the onset and progression of diseases, conditions and processes like diabetes, atherosclerosis, carcinogenesis, aging, inflammation and chronic neurodegeneration (Lobo et al., 2010; Ray et al., 2012; Wu & Cederbaum, 2003). In fact,

oxidative stress is believed to play key roles in neurological disorders (muscular dystrophy, Alzheimer's disease, Parkinson's disease), inflammatory diseases (glomerulonephritis, arthritis, vasculitis), ischemic diseases (stroke, heart diseases), as well as gastric ulcers, AIDS, emphysema and hemochromatosis among others (Lobo et al., 2010).

The human body is equipped with a robust antioxidant defence system to counteract the effects of ROS produced during normal physiological processes as well as in extreme metabolic events. This system includes a battery of antioxidant enzymes such as catalase, superoxide dismutases and enzymes of the glutathione system (glutathione peroxidases, glutathione reductase, glutathione S-transferases), in addition to non-enzymatic antioxidants like ascorbic acid, vitamin E, glutathione and melatonin (Görlach et al., 2015; Lobo et al., 2010; Storz, 2011). Since sustained oxidative stress can easily overwhelm endogenous antioxidant mechanisms, there have been efforts (Bouayed & Bohn, 2010; Vossen et al., 2011) to use exogenous antioxidants to supplement the protection against ROS offered by the body's natural defence system. For instance, in the food industry, food products have been supplemented with synthetic antioxidants such as propyl gallate, butylated hydroxyanisole, butylated hydroxytoluene and tert-butylhydroquinone in order to enhance their antioxidative potential and as preservatives to curtail oxidation-mediated rancidity (Ni et al., 2000). In addition, investigators have shown considerable epidemiological and clinical evidence for the control of oxidative stress-related diseases using diet-based strategies (Genkinger et al., 2004; Slavin & Lloyd, 2012; Zino et al., 1997). The protection against oxidative stress provided by dietary components typically found in fruits and vegetables such as such as polyphenols, ascorbic acid and carotenoids, as a result of their antioxidant properties is well documented (Crowe et al., 2011; Fiedor & Burda, 2014; Genkinger et al., 2004; Padayatty et al., 2003; Pandey & Rizvi, 2009). In addition to the aforementioned

bioactive food components with antioxidant properties, certain foods also naturally contain peptides with antioxidative properties. These include peptides such as anserine (b-alanyl-L-1-methylhistidine), carnosine (b-alanyl-L-histidine), ophidine (b-alanyl-L-3-methylhistidine) and glutathione (c-Glu-Cys-Gly), which are naturally present in muscle tissues (Samaranayaka & Li-Chan, 2011). Additionally, the histidine-containing dipeptide carnosine has been shown to be very effective in scavenging free radicals, chelating metal ions and inhibiting lipid oxidation (Wu et al., 2005). More recently, bioactive peptides derived from food proteins have received significant research attention as a result of their antioxidant function (Ahmed et al., 2015; Irshad et al., 2015; Mechmeche et al., 2017; Aloğlu & Öner, 2011). These food protein-derived antioxidative peptides are obtained following enzymatic hydrolysis, gastrointestinal digestion, food processing and/or microbial fermentation, and are different from the antioxidant peptides naturally present in foods.

2.10 Antioxidant peptides and hydrolysates from food proteins

Over the years, studies have reported the isolation of antioxidant peptides and proteins from multiple and diverse food protein sources. These include oyster, tuna liver, Amaranth, chickpea, rice endosperm, peanut kernels, soy, whey, algae, alfalfa leaf, grass carp muscle, casein, sardine muscle, egg white albumin, lecithin-free egg yolk, dried bonito, mackerel fillet, salmon, Alaska pollock, yellowfin sole frame, giant squid muscle and sunflower protein. To generate these antioxidant peptides, proteases as varied as pepsin, pancreatin, alcalase, collagenase, flavourzyme, trypsin, chymotrypsin, papain, neutrase, thermolysin, esperase, flavorase and proteinase K have been used (Samaranayaka & Li-Chan, 2011). Table 2.4 contains a selection of specific peptide sequences obtained from various food protein sources and tested for antioxidative property using a number of standard *in vitro* antioxidant assays.

In addition, antioxidative peptides have also been generated following (simulated) gastrointestinal digestion, fermentation or food processing. Peptides with the capacity to exert their antioxidative effects directly in the gut, through gut receptors or at other target sites in the body have been obtained through the gastrointestinal digestion of food proteins using digestive enzymes or microbial enzymes produced by gut microbes (Samaranayaka & Li-Chan, 2011). Following the simulated gastrointestinal digestion of hempseed proteins using pepsin and pancreatin, the resultant crude protein hydrolysates were found to exhibit substantial metal chelation activity (>70%), while there were notable increases in the ferric reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radical scavenging activities of the peptide fractions obtained after ultrafiltration of the hydrolysates (Girgih et al., 2011b). It has also been reported that a novel tetradecapeptide obtained from the simulated gastrointestinal digestion of mussel protein significantly inhibited lipid peroxidation more than two other antioxidants *viz*

Table 2.4. Sources, assays and sequences of selected antioxidant peptides[a]

Source of protein	Assays	Peptide sequence
Chicken egg white	ORAC	AEERYP, DEDTQAMP
Egg white	oxygen radical scavenging, DPPH radical scavenging.	DHTKE, MPDAHL, FFGFN
Rice residue protein	DPPH and ABTS radical scavenging, FRAP-Fe^{3+} reducing assay	RPNYTDA, TSQLLSDQ, TRTGDPFF, NFHPQ
Grass carp (*Ctenopharyngodon idella*) skin	DPPH radical, hydroxyl radical, ABTS radical scavenging, Inhibiting lipid peroxidation	PYSFK, GFGPEL, GGRP
Bluefin leatherjacket skin (*Navodon septentrionalis*)	DPPH, HO, O2´ radical scavenging.	GSGGL, GPGGFI, FIGP
Palm kernel cake proteins	DPPH radical scavenging, Metal chelating ability.	GIFE LPWRPATNVF
Blood clam (*Tegillarca granosa*) muscle	lipid peroxidation, radical scavenging activity.	WPP
Sweet potato	OH radical scavenging.	YYIVS

Croceine croaker (*Pseudosciaena crocea*) muscle	DPPH, superoxide, ABTS and hydroxyl radical scavenging, lipid peroxidation.	WLMSA, VLWEE, MILMR
Spotless smoothhound (*Mustelus griseus*) muscle	Hydroxyl, ABTS, superoxide radical scavenging.	GIISHR, ELLI, KFPE, GFVG, GAA
Bluefin leatherjacket (*Navodon septentrionalis*) heads	DPPH, hydroxyl, ABTS, superoxide radicals scavenging.	WEGPK, GPP, GVPLT
Hemp (*Cannabis sativa* L.) seed	DPPH radicals scavenging.	PSLPA, WVYY
Chickpea protein	DPPH radicals scavenging.	VGDI, DHG
Marine *Sepia brevimana* mantle	DPPH radicals scavenging, lipid peroxidation.	I/LNI/LCCN
Sphyrna lewini muscle	ABTS, DPPH radicals scavenging.	WDR, PYFNK
Tilapia (*Oreochromis niloticus*) gelatin	Hydroxyl radicals scavenging.	LSGYGP
Corn gluten meal	DPPH, ABTS, and hydroxyl radicals scavenging.	LLPF
Oyster (*Saccostrea cucullata*)	DPPH radicals scavenging, Inhibiting human colon carcinoma (HT-29) cell lines.	LANAK, PSLVGRPPVGKLTL, VKVLLEHPVL

[a] Reproduced from (Zou et al., 2016) and used with permission from MDPI.

ascorbic acid and α-tocopherol (Jung et al., 2007). Also, the dipeptide, His-Pro (cyclo), which is produced during the thermal processing of foods such as tuna, noodles, dried shrimp, potted meat and fish sauce has been shown to attenuate oxidative stress-induced neurodegeneration following its absorption through the gastrointestinal tract (Samaranayaka & Li-Chan, 2011). Lastly, fermentation using lactic acid bacteria was credited with releasing potent antioxidant peptides from bovine skim milk (Kim et al., 2017) and cereal flours (Coda et al., 2012).

In the bovine cereal flours fermentation study, a pool of selected lactic acid bacteria containing *Lactobacillus alimentarius* 15M, *Lactobacillus brevis* 14G, *Lactobacillus sanfranciscensis* 7A, and *Lactobacillus hilgardii* 51B among others, was used for the sourdough fermentation of cereal flours including spelt, kamut, wheat and rye in order to obtain peptides with high DDPH radical scavenging and linoleic acid oxidation inhibition activities (Coda et al., 2012).

It has been suggested that the exact contribution of individual amino acid residues to the antioxidative property of a peptide is primarily determined by the nature of the ROS the peptide is exposed to and the reaction medium involved, although the issue of the contribution of such discrete amino acid residues to the antioxidant property of the kind of peptide mixtures typically present in food protein hydrolysates remains unsettled (Aluko, 2012). Since amino acid residue mixtures used in simulating the amino acid composition of antioxidant protein hydrolysates have been reported to not be as potent as peptide antioxidants, it is likely that the synergistic effects of peptide amino acids and their order of arrangement on the peptide chain are more powerful than the individual contributions of similar but free amino acids (Aluko, 2012).

2.11 Mechanisms of action of food protein-derived antioxidant peptides

Although specific mechanisms of action of bioactive peptides are yet to be clearly elucidated, their antioxidant properties have been demonstrated using a collection of *in vitro* and *in vivo* antioxidant assays. Such tests include free radical scavenging assays, inhibition of oxidative semicarbazide-sensitive amine oxidases, and inhibition of ROS-induced oxidation of biomolecules like nucleic acids, proteins and lipids (Udenigwe et al., 2017). Thus, it has been suggested that in general, the mechanisms of action of bioactive peptides could involve one or more of the following: (i) quenching ROS by participating in hydrogen atom transfer (HAT) or single electron transfer (SET) reactions, (ii) enhancing the antioxidative function of components of the endogenous antioxidant defence system including catalase, vitamin C, glutathione and superoxide dismutase, (iii) preventing ROS generation by inhibiting the activity of certain pro-oxidant enzymes, and (iv) chelating metal ions involved in free radical formation (Dai & Mumper, 2010; Girgih, 2013; Huang et al., 2005). Although the notion is widely accepted that peptides are more effective antioxidants than their individual amino acids (since the former

contain electron-donating bonds), certain reports have also suggested that the metal ion chelation, ferric reducing power and free radical scavenging capacity of some free amino acids could be greater than those of their precursor peptides (Erdmann et al., 2008; Kitts & Weiler, 2003; Tang et al., 2009).

A number of other factors including degree of hydrolysis, hydrolysis temperature, hydrolyzing enzyme specificity, peptide chain length, amino acid composition and hydrophobicity are all known to influence the antioxidant bioactivity of peptides and protein hydrolysates (Pihlanto, 2006; Udenigwe et al., 2017). For instance, apart from their contribution to the endogenous antioxidant defence system as a result of being used for synthesis of the principal intracellular antioxidant and redox buffer compound (glutathione), cysteine-containing peptides are also known to be potent antioxidants due to the presence of the thiol functional group in sulfur-containing amino acids (Meisel, 2005). It has been suggested that the electron-rich aromatic rings of tyrosine, tryptophan and phenylalanine residues in peptides can facilitate the chelation of pro-oxidant metal ions by such peptides, with the aromatic ring of phenylalanine in particular being effective in the scavenging of hydroxyl radicals for the formation of more stable *para*-, *meta*- or *ortho*-substituted hydroxylated derivatives thus raising the possibility for a role in the attenuation of post-ischemic dysfunction (Aluko, 2012; Sun et al., 1993). Furthermore, the presence of the imidazole ring in histidine is believed to be linked to its potency in scavenging free radicals and chelating metal ions since the aromatic side chain can take part in HAT and SET reactions (Aluko, 2012). Additionally, the hydrophobicity of non-polar amino acid residues such as histidine, tryptophan and tyrosine is thought to contribute to the antioxidant potential of hydrophobic peptides by increasing the accessibility of such peptides to hydrophobic cellular targets such as the unsaturated fatty acids in biological membranes (Aluko, 2012;

Erdmann et al., 2008). By interacting with cell membranes in biological systems, these hydrophobic peptides could provide *in situ* protection against lipid peroxidation and oxidative stress-induced cellular injury since the polyunsaturated chain of fatty acids within biological membranes are susceptible to ROS-mediated oxidative damage (Aluko, 2012). It is thought that adequate knowledge of the foregoing mechanisms is important for producing peptides designed to contain particular amino acids and with specific sequences, which could be used for controlling, delaying or reversing the course of oxidative stress-induced chronic diseases (Girgih, 2013).

2.12 Oxidative stress and high blood pressure

Although certain mechanisms surrounding the incidence and progression of hypertension are still unclear, there is a large body of work showing that oxidative stress is fundamental to the pathophysiology of elevated blood pressure (González et al., 2014; Harrison & Gongora, 2009; Montezano & Touyz, 2012; Nabha et al., 2005; Ramón et al., 2011). There is ample evidence demonstrating that excess ROS contribute to the development of hypertension and that ROS quenching decreases blood pressure (Loperena & Harrison, 2017; Nabha et al. 2005). In a now well-publicised study, a group of Japanese researchers demonstrated that bolus administration of a modified form of superoxide dismutase acutely reduced blood pressure in hypertensive rodent subjects (Nakazono et al., 1991).

Various investigators have reported that ROS in the kidney, central nervous system (CNS), and the vasculature contribute to the development and maintenance of hypertension (Loperena & Harrison, 2017; Wu & Harrison, 2014). For example, in the brain, the injection of an adenovirus encoding superoxide dismutase into the cerebrospinal fluid in cerebral ventricles led to the attenuation of experimental hypertension induced by either local or systemic AT-II

infusion (Wu & Harrison, 2014). Also, in the proximal tubule of the kidney where sodium transport is stimulated by AT-II and inhibited by the neurotransmitter dopamine, under ROS-induced oxidative stress, the stimulatory effect of AT-II is increased, with the concomitant disruption of dopamine signaling, which leads to increased sodium transport in the proximal tubule (Wu & Harrison, 2014). In fact, it has been reported that stimulation of a dopamine receptor (D_2R) inhibits ROS generation in cells of the proximal tubule and that mice in which one D_2R allele is absent have elevated blood pressure as well as higher ROS levels in the proximal tubule (Cuevas et al., 2012; Wu & Harrison, 2014). Additionally, in the macula densa region of the renal distal tubule, it has been suggested that increased $O_2^{\cdot-}$ levels could inactivate NO resulting in vasoconstriction of the afferent arterioles and ultimately contributing to elevated blood pressure (Liu et al., 2003).

Furthermore, peroxynitrite ($OONO^-$), an important ROS in biological systems, is formed by the diffusion-limited reaction of its progenitor (superoxide, O_2^-) with NO, a critical vasodilator and signalling molecule (Wu & Harrison, 2014). A fascinating illustration of how closely intertwined oxidative stress is with hypertension can be seen in the interaction between arginine, tetrahydrobiopterin (H_4B) and the nitric oxide synthase (NOS) enzymes. By catalyzing the endogenous synthesis of NO in mammalian cells, NOS enzymes play a critical role in cardiovascular function including vascular tone modulation, release of renal renin, blood pressure and the excretion of sodium from the kidneys (Förstermann & Sessa, 2012; Majumder & Wu, 2015). In the absence of H_4B, a vital cofactor of the nitric oxide synthases or their arginine substrate, the enzymes become uncoupled, producing superoxide anion ($O_2^{\cdot-}$) instead of NO (Wu & Harrison, 2014). Additionally, the oxidation of H_4B by oxidants such as $OONO^-$ principally leads to the uncoupling of NOS, a phenomenon, which is associated with the

generation of ROS in diseases such as diabetes, hypertension, atherosclerosis as well as following ischemia and reperfusion injury (Landmesser et al., 2003; Wu & Harrison, 2014). It has been reported that oral intake of H_4B reverses NOS uncoupling in many of these diseases, in addition to slowing the elevation of blood pressure in both animal and human subjects (Porkert et al., 2008; Wu & Harrison, 2014). Additionally, it has been demonstrated that hydrogen peroxide formed from the dismutation of superoxide anion can function as a signalling molecule and promote vasodilation (Wu & Harrison, 2014). However, lipid hydroperoxide and hydrogen peroxide production have also been shown to be higher in hypertensive subjects than in those not diagnosed with hypertension (Baradaran et al., 2014).

Excessive production of superoxide is known to suppress the activity of nitric oxide (NO) and could eventually lead to endothelial dysfunction while higher NO levels can enhance vasodilation and help lower blood pressure. Thus, by scavenging free radicals, antioxidant peptides have the potential to significantly influence NO availability and consequently modulate endothelial function and hypertension (Majumder & Wu, 2015). Given the relationship between oxidative stress and hypertension, a number of studies have examined the role of food protein-derived antioxidant peptides in attenuating elevated blood pressure. Examples include the findings that when treated with the egg-derived tripeptide Ile-Arg-Trp, TNF-α induced oxidative stress was markedly reduced in cultured endothelial cells (Majumder et al., 2013a). The same peptide also reduced oxidative stress in the aorta and kidney of spontaneously hypertensive rats following oral gavage (Majumder et al., 2013b). In addition, the dipeptide Met-Tyr, which was obtained from sardine muscle was found to inhibit ACE activity by suppressing the production of ROS in endothelial cells through the induction of hemeoxygenase-1 and ferritin, the intracellular iron storage protein (Erdmann et al., 2006; Majumder & Wu, 2015).

2.13. Calmodulin (CaM) – an introduction

CaM, the 148-amino acid residue, 16.7 kDa messenger protein is the primary intracellular receptor for calcium (Ca^{2+}) and an important enzyme activator protein (Means, 1994; Tajbakhsh et al., 2018). When produced in excess, CaM could impair normal physiological functions and may eventually result in the development of chronic disease conditions including cardiac hypertrophy, cancer, Alzheimer's disease, and hypertension (Aluko, 2010). Various studies have reported that CaM's activity is inhibited by food-protein derived bioactive peptides (Kizawa, 1997; Kizawa et al., 1995; You et al., 2010). Given CaM's role in some of the body's critical physiological processes including nerve growth, neurotransmission, smooth muscle contraction, cell proliferation, cell differentiation, synaptic plasticity, inflammation, vasodilation and the immune response (Kaleka et al., 2012), the prospect for using bioactive peptides for inhibiting CaM activity could be enormously consequential.

2.14 Modes of action of CaM/CaM-dependent enzymes and their role in human health

The principal mechanism of CaM action is the activation of enzymes such as protein kinases, phosphodiesterases, and nitric oxide synthases, which are involved in the regulation of various metabolic processes in order to influence those processes (Aluko, 2010). For example, the regulation of different isoforms of CaM-dependent nitric oxide synthase (NOS), which catalyze the synthesis of NO is critical in the development of acute and chronic disease conditions such as Huntington's disease, Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, stroke and migraine headaches, among others (Aluko, 2010; Knott & Bossy-Wetzel, 2009). This is because NO is involved in important metabolic functions like vasodilation, neurotransmission and immune response, and thus excessive NO levels could contribute to neurodegeneration as it relates to the development of many diseases of the nervous system (Aluko, 2010; Knott &

Bossy-Wetzel, 2009). Therefore, food protein-derived inhibitors which interfere with the binding and activation of NOS by CaM, could be beneficial in controlling NO synthesis.

Furthermore, the dodecameric holoenzyme, Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII), is a multifunctional enzyme, which is responsible for the phosphorylation of proteins such as cAMP responsive element binding protein (CREB), ryanodine receptors (RYRs), sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and phospholamban (PLN). These proteins are involved in critical and wide-ranging physiological processes and pathological events including cardiac contractility, ischaemic stroke, arrhythmia suppression, cardiomyocyte apoptosis, calcium ion transport, cognitive memory, spermatogenesis and neuronal plasticity (Mustroph et al., 2017; Pellicena & Schulman, 2014; Rostas et al., 2017; Silva et al., 1998; Van Petegem, 2012). Crucially, to become catalytically active, CaMKII needs Ca^{2+} and CaM for a preliminary autophosphorylation step - a reaction that assumes added significance given that if not properly regulated especially during cell proliferation, it could mark the beginning of a cascade of metabolic events ultimately resulting in chronic disease conditions like cardiac hypertrophy and cancer (Aluko, 2010; Pellicena & Schulman, 2014). Over the years, investigators have studied the inhibition of CaMKII with agents such as N-[2-[N-(4-chlorocinnamyl)-N-methylaminomethyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate (KN-93), 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4- phenylpiperazine (KN-62), CaMKIIN, and autocamtide-3 derived inhibitory peptide (AC3-I) among others, as a possible strategy for controlling the incidence of diseases associated with excessive CaM levels (Pellicena & Schulman, 2014). By binding to the CaMKII holoenzyme prior to autophosphorylation, inhibitors such as KN-62 interfere with the ability of Ca^{2+}/CaM to activate the enzyme (Pellicena & Schulman, 2014). Thus, bioactive peptides and

protein hydrolysates which can bind to CaM or Ca^{2+}/CaM have the potential to disrupt the catalytic activity of CaM-dependent enzymes such as CaMKII, and could therefore be useful in the management of chronic diseases linked with increased CaM synthesis (Aluko, 2010; Li & Aluko, 2006).

Similarly, food protein-derived peptides which could inhibit the activity of Ca^{2+}/CaM-dependent phosphodiesterase (CaMPDE or phosphodiesterase I) could prove beneficial in the design of agents for the prevention, control and management of chronic pathologies involving excessive CaM levels (Aluko, 2010). CaMPDE is a member of the 11 PDE families (PDE1-11) and is the only phosphodiesterase family whose activation is Ca^{2+}/CaM-dependent (Wennogle et al., 2017). CaMPDE hydrolyzes the 3' bond of 3'-5'-cyclic nucleotides and could thus degrade cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) (Wennogle et al., 2017). Although cAMP, an important ubiquitous second messenger plays a crucial role in intracellular signal transduction including cellular responses to hormones and neurotransmitters (Sassone-Corsi, 2012), aberrant regulation of the cAMP signalling pathway is known to impair cell proliferation, differentiation and function *via* a mechanism involving G_1-phase cell cycle arrest (Aluko, 2010; Vitale et al., 2009; Zambon et al., 2005). Thus, cAMP has been implicated in the initiation of cancerous tumours (Vitale et al., 2009; Zambon, et al., 2005). Given its ability to catalyze the degradation of cAMP, the overexpression of CaMPDE typically results in a depletion of cAMP levels and could undermine the normal regulation of apoptosis as seen in cancer tumour development (Aluko, 2010). Conversely, inhibiting CaMPDE activity has the potential to upregulate intracellular cAMP synthesis and restore the normal physiological regulation of programmed cell death thus providing a target for cancer therapy (Aluko, 2010). Although certain cAMP-elevating pharmacological agents with the demonstrated capacity to

induce differentiation of neoplastic cells and inhibition of angiogenesis have been reported, these agents are not recommended for use in cancer therapy given their severe cytotoxicity (Vitale et al., 2009). Therefore, CaMPDE-inhibitory peptides such as those obtained from food proteins like flaxseed and egg white lysozyme have the potential to be employed in producing formulations for the prevention and control of cancer and metastasis (Aluko, 2010; Udenigwe & Aluko, 2012b; You et al., 2010).

2.15 CaM-binding peptides from food proteins

A possible therapeutic strategy for preventing and/or controlling the initiation and progression of diseases associated with excessive levels of Ca^{2+}/CaM-dependent enzymes such as CaMPDE is the use of agents with the capacity to chelate Ca^{2+} to limit or prevent the activation of such enzymes (Udenigwe, 2010). However, that strategy is flawed since the involvement of Ca^{2+} in many critical cellular processes suggests that the use of chelating agents would not be sufficiently selective and specific. Thus using CaM-binding agents such as food-derived bioactive peptides and proteins which limit the activity of enzymes like CaMPDE by specifically binding to CaM would be more effective (Udenigwe, 2010). Since CaM is an anionic protein with net negative charge at physiological pH, cationic peptides have a high propensity to interact with and bind to the protein as reported for peptides obtained from α–casein following pepsin proteolysis (Aluko, 2010; Kizawa, 1997). Previous reports from our lab have demonstrated the potential of peptides derived from pea and flaxseed proteins for inhibiting the activities of such enzymes. Cationic peptide fractions obtained from flaxseed proteins following alcalase hydrolysis and cation exchange chromatographic separation were found to inhibit CaMPDE (Udenigwe & Aluko, 2012b). It was observed that peptide-induced CaMPDE inhibition was significantly ($P<0.05$) reduced in the presence of increasing CaM levels thus

suggesting that CaMPDE inhibition occurred as a result of CaM inactivation (Udenigwe & Aluko, 2012b). The cationic peptide fractions bound to and inactivated CaM, thus limiting the formation of the CaM/PDE complex necessary for optimal CaMPDE activity (Udenigwe & Aluko, 2012b). Similarly, cationic peptide fractions from flaxseed were shown to reduce the activities of neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) but using different modes of action (Omoni & Aluko, 2006a; Omoni & Aluko, 2006b). While the peptides inhibited eNOS through a mostly mixed type pattern of enzyme inhibition, they inhibited nNOS through a mainly noncompetitive inhibition pattern. Thus, the results suggest that the peptides inhibited eNOS activity by binding to CaM alone or to the CaM/eNOS complex while the inhibition of nNOS was accomplished by the peptides binding to CaM at a binding site different from the binding site of nNOS and possibly altering the structure of CaM and therefore its capacity to activate nNOS (Omoni & Aluko, 2006a; Omoni & Aluko, 2006b). Another study demonstrating the inhibition of CaMPDE by peptides obtained from egg white lysozyme did not correlate CaMPDE inhibition with an abundance of positively charged amino acids thus suggesting that there could be multiple mechanisms of action for the inhibition of Ca^{2+}/CaM-dependent enzymes by peptides and that the amino acid composition of the peptides could be an important factor (You et al., 2010). Interestingly, while some of the CaMPDE-inhibitory peptides from flaxseed were found to also inhibit the activities of renin and ACE, those from egg white lysozyme possessed mainly antioxidative properties.

2.16 Bioactive peptide transport and bioavailability

In order to provide enduring health-promoting benefits such as blood pressure-lowering, CaMPDE-inhibitory or antioxidative properties in the human body, food-derived bioactive

peptides must maintain their structural integrity during digestion and transport, and remain intact while they are absorbed across the intestinal epithelium in order to reach target organs and systems without losing their bioactivity. Although numerous *in vitro* reports have shown the potential physiological benefits of bioactive peptides, there has been a perennial challenge with replicating those benefits during *in vivo* studies (Matsui, 2018). Part of that challenge is thought to lie with the low metabolic stability and bioavailability of peptides often due to the poor permeability of the peptides across membrane barriers such as those of the intestinal epithelium (Adessi & Soto, 2002; Matsui, 2018). Another formidable challenge facing the efficient transport of peptides through the gastrointestinal tract to the blood circulation is substantial enzymatic degradation to inactive fragments by intestinal and/or blood proteases (Matsui, 2018). It has recently been suggested that peptide-food matrix interactions should form part of the conversation in seeking to understand the gap often observed between *in vitro* bioactivity and *in vivo* efficacy (Udenigwe & Fogliano, 2017).

A number of researchers have reported the detection and quantification of peptides in human and animal plasma, as well as the inhibition of plasma enzyme activity by orally administered bioactive peptides. In one study which examined the effect of aging on peptide absorption, peptides (Gly-Sar, Trp-His, Gly-Sar-Sar, Gly-Sar-Sar-Sar, and Gly-Sar-Sar-Sar-Sar) were orally administered to spontaneously hypertensive rats aged 8 and 40 weeks respectively at a dose of 10 mg/kg body weight (Hanh et al., 2017). Blood samples were subsequently collected from rat tail veins at various time intervals and plasma peptide concentration was determined by TNBS-aided LC-MS. The results indicated that all the peptides were detected in their intact forms in the blood with a higher rate of di/tripeptide absorption reported in the aged SHRs compared to the young rats (Hanh et al., 2017). Furthermore, at a maximum plasma

concentration of 12.2±1.8 nmol/mL and 14.8±1.1 nmol/mL in the young and aged SHRs respectively, Gly-Sar was determined to have the highest absorption rate compared to the rest of the peptides. In another study, investigators also demonstrated the detection of Val-Tyr, an antihypertensive dipeptide, in human plasma 2 h after a single oral administration (Matsui et al., 2002). Compared to the baseline concentration of 159±11 fmol/mL, the concentration of the dipeptide at 2 h was reported to be 1934± 145 fmol/mL plasma (Matsui et al., 2002). Studies on the effect of peptide supplementation on plasma enzyme activity have also shown the capacity of peptides to directly inhibit the activity of enzymes *in vivo* following oral administration. In one study, after oral gavage, whey protein hydrolysate was found to significantly ($p<0.05$) inhibit plasma ACE activity at 240 and 1200 mg/kg body weight but not at 80 mg/kg body weight (Wang et al., 2012). Additionally, at long term oral supplementation doses of 285 and 782 mg/kg body weight per day for 24 days, <3 kDa fractions of a lactoferrin protein hydrolysate significantly ($p<0.01$) reduced plasma ACE activity by up to 42 U/L compared to the negative control (Fernández-Musoles et al., 2013). Together, these studies demonstrate the capacity of food protein-derived peptides to have a direct effect on the activity of enzymes present in the blood following ingestion.

In a study of the bioavailability of HLPLP, an antihypertensive casein-derived pentapeptide, LC-MS analysis using an ultra-high performance liquid chromatography system coupled to a Q-TOF instrument revealed that the peptide was hydrolyzed into two main fragments LPLP and HLPL by plasma peptidases following both oral and intravenous administration in adult male Wistar rats (Sánchez-Rivera, et al., 2014). The chemical modification of food-derived proteins and peptides in order to increase their oral delivery as well as improve their stability to the degradative action of proteolytic and peptidolytic enzymes has

been suggested as a possible solution to their hydrolysis in both the small intestine and blood, and subsequent rapid elimination from the systemic circulation (Adessi & Soto, 2002; Vermeirssen et al., 2004). However, given the cost, this seemingly attractive strategy for increasing peptide stability and bioavailability is virtually impractical especially if the goal is to ultimately provide widely available peptide-based formulations for health promotion.

Although *in vivo* transport studies such as those using rat models offer obvious exclusive advantages such as the ability to monitor peptide distribution and possible biotransformation, *in vitro* experiments provide insights into intestinal peptide absorption mechanisms. Using *in vitro* models such as Caco-2 cells, it has been shown that the transport of oligopeptides across the intestinal epithelium can take place through any of three major mechanisms (Satake et al., 2002). The active transport of di- and tripeptides is mainly carried out by PepT1, a proton-dependent peptide transporter, while the energy-independent passive transport of peptides in the paracellular route is modulated by tight intracellular junctions of the intestinal villi (Brandsch et al., 2008; Miner-Williams et al., 2014). The di- and tripeptides transported by PepT1 are reportedly hydrolyzed with ease to amino acids by cytosolic peptidases present in the epithelial cells leading to the description of the PepT1-mediated active transport as degradative while such breakdown of peptides are not usually associated with the non-degradative paracellular transport route (Patel & Misra, 2011; Shimizu, 2004). The vesicle-mediated transcellular transport form known as transcytosis is the third main mechanism of transepithelial transport and involves endocytotic uptake and basolateral secretion (Shimizu, 2004; Wang & Li, 2017).

2.17 Physiology of the absorption of proteins and peptides

In the gastrointestinal tract, the specialized epithelial membranes which separate each fluid-filled vesicle from the other are essential for tightly regulating the bidirectional flux of substances (Brandsch et al., 2008). The transfer of substances including charged particles like H^+ ions, macromolecules like proteins and small molecules such as bioactive peptides across these specialized epithelial barriers of the gastrointestinal system generally takes place through the transcellular or the paracellular pathway (Patel & Misra, 2011). Whereas the transcellular pathway involves the transport of substances across the cytoplasm through cell membrane channels, carriers, and exchangers, the movement of substances in the paracellular pathway principally occurs through the intercellular spaces between epithelial cells (Tang & Goodenough, 2003).

Tight junctions (TJs), which are found at the apical cell-cell interactions of adjacent epithelial cells function as gatekeepers of the paracellular route, and play a crucial role in regulating the transport of organic and inorganic substances from the intestines to the systemic circulation (Suzuki & Hara, 2004; Tang & Goodenough, 2003). Since the space between these adjacent cells is relatively small (with a radius of 8 Å), often only small peptides are able to pass through, and although the permeability of tight junctions differs considerably from one cell type to another, molecules whose radii are >11-15 Å have been reported to generally fail in permeating TJs (Madara & Dharmsathaphorn, 1985; Patel & Misra, 2011). Thus, it has been reported that the ability of substances to pass through the apical and basolateral membranes in the paracellular pathway by simple diffusion is mainly dependent on their size, whereas size, lipophilicity and charge all play a role in the transport of substances *via* the transcellular route (Brandsch et al., 2008). Nevertheless, given the conformational flexibility of peptides, large

peptides have been known to sometimes permeate TJs (Pauletti et al., 1996). Penetration enhancers are known to greatly improve the movement of peptides through the paracellular route, and enhancers such as zonula occludens toxin, Pz peptide, and chitosan have been used to reversibly open tight junctions between enterocytes (Patel & Misra, 2011). During *in vitro* transport experiments involving Caco-2 monolayers, cytochalasin D, a cell permeable alkaloid produced by moulds and potent inhibitor of actin polymerization, has been used to disrupt TJs in order to study the mechanism of absorption of food-derived bioactive peptides (Ding et al., 2015; Fu et al., 2016; Vij et al., 2016). TJs are also important for maintaining the constitutional integrity of the compositionally distinct apical and basolateral plasma membrane domains by forming a boundary between them (Gumbiner, 1987).

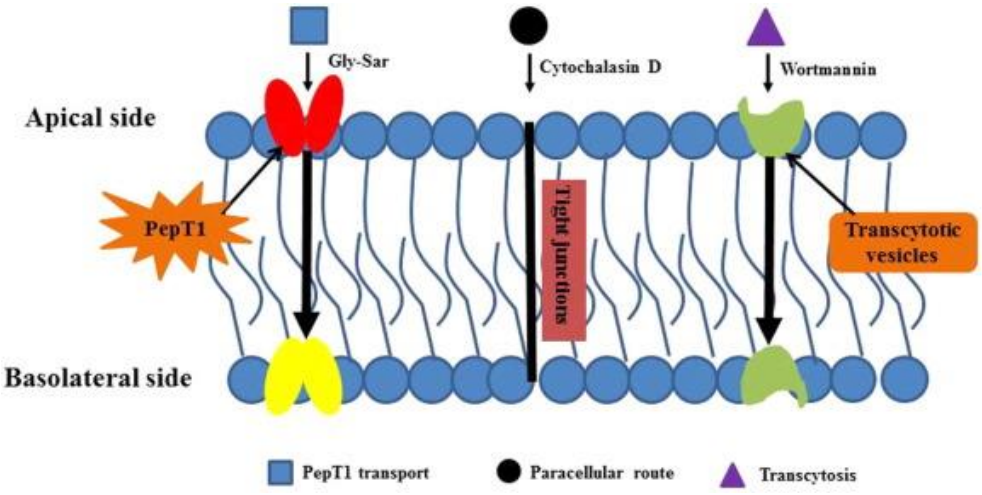

Fig. 2.2.. The three main mechanisms for the transepithelial transport of peptides. Gly-Sar is able to inhibit the PepT1-mediated peptide transport by competitively binding to the proton-coupled membrane transporter. Cytochalasin D can disrupt TJs, thus increasing the transport of peptides *via* the paracellular route. Wortmannin has the capacity to degrade transcytotic vesicles, thus decreasing the transcytosis of peptides. Reproduced from (Wang & Li, 2017) and used with permission from Elsevier.

In the transcellular pathway, substances must traverse the functionally and morphologically distinct cytoplasmic membranes of the apical and basolateral membranes by either active or passive transport (Brandsch et al., 2008). PepT1 (peptide transporter 1), a proton-coupled membrane transporter is one of the most studied active transporters of peptides in the transcellular pathway. This peptide transporter, which in humans consists of 708 amino acid residues, is a member of the POT (proton-oligopeptide transporter) family, and is known to facilitate the transport of small, often hydrolysis-resistant peptides from the lumen, through the intestinal absorptive cells to systemic circulation (Brandsch et al., 2008; Segura-Campos et al., 2011). Although cytosolic peptidases rapidly degrade most small peptides entering cells such as enterocytes, it is now widely accepted that di- and tripeptides are transported through PepT1 (Brandsch et al., 2008; Matsui, 2018). The PepT1 competitive substrate [^{14}C]glycylsarcosine or Gly-Sar is commonly used to study the transport of peptides by the intestinal peptide transporter. In the previously cited study of the egg white protein-derived antihypertensive pentapeptide RVPSL, the lack of a significant ($p<0.05$) reduction in the permeability coefficient of the pentapeptide in the presence of Gly-Sar led investigators to conclude that its transport across Caco-2 cell monolayers was independent of PepT1 (Ding et al., 2015). Conversely, 10 mM Gly-Sar led to a significant decrease ($p<0.05$) in the transport of five peptide fractions obtained from lyophilized casein hydrolysate following fractionation with a SP-Sephadex C-25 cation exchange column thus indicating that PepT1 was involved in their transport (Wang & Li, 2018).

Certain proteins and peptides, which are not able to undergo PepT1-mediated transport as a result of their size are largely translocated across the cell membrane through specialized transcytotic processes involving membrane invagination and endocytosis of vesicles (Brandsch et al., 2008). This transcytotic mechanism of transepithelial transport has been reported for the

transport of large peptides including bradykinin (RPPGFSPFR), a hydrophobic nonapeptide (Shimizu et al., 1997), an antioxidative decapeptide, YWDHNNPQIR (Xu et al., 2017), as well as for a large immunoglobulin G protein fragment (Miner-Williams et al., 2014). During transcytosis, internalized vesicles could carry specific bound ligands (receptor-mediated transcytosis), non-specifically adsorbed ligands (adsorptive transcytosis), or fluids (fluid phase transcytosis) from their sites of entry at the apical face across the cell to the basolateral membrane (Miner-Williams et al., 2014; Shimizu et al., 1997). Upon full engulfment inside the vesicles, proteins and peptides can be processed through various compartments along the vesicular transport pathway including intracellular organelles like lysosomes, endosomes and prelysosomes, and in some cases recycled back to the plasma membrane (Shen et al., 1992). Transcytosis inhibitors such as phenylarsine oxide and wortmannin (an inhibitor of phosphoinositide 3-kinase) have been used to study vesicle-mediated transcytotic transport (Vij et al., 2016). At a concentration of 1 μM, wortmannin significantly ($p<0.05$) inhibited the transepithelial efflux of chemically synthesized bradykinin and the pentapeptide, YPFPG (BCM 5), but not that of VLPVPQK, which suggests transcytosis as the main mechanism of transport of the former but not of the heptapeptide (Vij et al., 2016).

2.18 Physicochemical properties of absorbable bioactive peptides

As has been stated already, the metabolic stability of bioactive peptides (including their transportability across cellular membranes and resistance to the peptidolytic action of gastrointestinal, brush border, serum and intracellular peptidases) is an important indicator or determinant of their absorbability as well as eventual bioavailability and efficacy. Intrinsic properties of peptides such as hydrophobicity, amino acid composition, amino acid sequence, charge, hydrogen bonding potential, and molecular weight could affect their metabolic stability

(Ding et al., 2015; Pauletti et al., 1997; Regazzo et al., 2010; Wang & Li, 2017, 2018). For instance, it has been suggested that peptides containing proline residues are relatively resistant to hydrolysis by digestive enzymes as are peptides containing Pro-Trp bonds (Cardillo et al., 2003; Mizuno et al., 2004). Also, the resistance of casein-derived phosphorylated, anionic peptides to degradation by intestinal peptidases has been linked with their negative charge and phosphate groups (Schlimme & Meisel, 1995). In another study of the transepithelial transport and bioavailability of casein peptides using the Caco-2 cell monolayer as a model, it was found that hydrophilic peptide fractions and those with a net negative charge were mainly transported through PepT1 and paracellular routes (Wang & Li, 2018). Conversely, transcytosis and PepT1-mediated transport were found to be the main mechanisms of transporting hydrophobic and positively charged peptide fractions. Similarly, peptide size has been known to influence transport routes, although the intestinal transport routes of peptides containing more than four amino acid residues have not been completely elucidated (Hong et al., 2016; Matsui, 2018). In a transepithelial transport study using chemically designed peptides, the transport of di- and tripeptides across Caco-2 monolayers was found to be PepT1-mediated while tetra- and pentapeptides were transported *via* the paracellular tight junction route (Hong et al., 2016). Molecular weight is also thought to influence the mode of transepithelial transport as demonstrated in a study of bioactive peptides from milk proteins in which PepT1 was involved in the transport of peptides <500 Da while the paracellular route was the main pathway for transporting peptides with molecular weight 500-1000 Da as well as those of 1300-1600 Da in size (Wang & Li, 2017). Additionally, another study reported that RVPSL, an egg white protein-derived pentapeptide with ACE-inhibitory property, was transported mainly through the paracellular tight junction pathway (Ding et al., 2015). By showing that the transport rate of

RVPSL was significantly ($p<0.001$) increased in the presence of cytochalasin D, a tight junction disruptor, the study suggested that the pentapeptide was transported across the Caco-2 cell monolayers through tight junction-mediated paracellular routes. Conversely, a rapeseed-derived decapeptide YWDHNNPQIR, was found to be transported *via* the intracellular transcytosis pathway, a departure from the usual paracellular transport route of oligopeptides that is possibly connected to its relatively long chain length and hydrophobicity (Xu, et al., 2017).

2.19 Peptide transport methods and models

In the early years of peptide transport work, a number of transport models and techniques including brush border membrane vesicles, dialysis bags, rat everted gut sacs and Ussing chambers, which were first used to study the absorption of drugs and other small molecules, were adopted for use in peptide transport experiments (Lundquist & Artursson, 2016; Wei et al., 2016). In addition to those techniques some of which are still currently used to study peptide transport, the use of cell culture models and animal feeding experiments have also found application in peptide transport experiments (Fu, et al., 2016; Nässl et al., 2011; Sánchez-Rivera, et al., 2014; Vij et al., 2016). In general, the methods for studying peptide transport can be classified into *in vitro*, *ex vivo* (*in situ*) and *in vivo*, with each grouping offering its own unique advantages as subsequently discussed (Lundquist & Artursson, 2016; Wei, et al., 2016).

The ease, convenience and ability to control experimental conditions in *in vitro* techniques involving cell culture models, dialysis bags, and rat gut sacs make them an attractive option for investigating the intestinal absorption of peptides (Lundquist & Artursson, 2016; Wei, et al., 2016). While *in vitro* techniques generally lack the accuracy and true reflection of physiological conditions provided by *in vivo* studies, certain cell culture models in particular enjoy widespread popularity. This is because of their capacity to mimic the intestinal epithelium

and grow rapidly, and as a result mature quickly into confluent monolayers that express various features of differentiated epithelial cells thus providing an important tool for the quick evaluation of intestinal peptide permeability (Balimane & Chong, 2005). Examples of the cell culture models widely used in transport studies include human colon adenocarcinoma cells (Caco-2), MDCK epithelial cells (MDCK), pig kidney epithelial cells (LLC-PK1), rat fetal intestinal epithelial cells (2/4/A1), rat small intestine cell line (IEC-18), and human colon cancer (HT-29) cell lines (Balimane & Chong, 2005).

Among these cell models, the enterocyte-like properties of Caco-2 cell monolayers and their capacity for differentiating into columnar absorptive cells complete with tight junctions, carrier-mediated transport systems and brush borders have contributed to make them the most widely studied and commonly used for investigating the intestinal permeability of small molecules such as peptides (Balimane & Chong, 2005; Wei, et al., 2016). Colorimetric analyses such as the tetrazolium salt-based cell counting kit-8 (CCK-8) test and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT)-based assay have been used to evaluate possible cytotoxic and anti-proliferative effects of bioactive peptides and other substances on Caco-2 cells (Fu, et al., 2016; Masisi et al., 2015; Song et al., 2014). An MTT-based test of the effects on Caco-2 cells of two peptides (VGPV and GPRGF) released following enzymatic hydrolysis of bovine collagen, revealed no cytotoxic effect with the Caco-2 cells maintaining over 95 % viability (Fu, et al., 2016). Thus, in general, cell culture models such as Caco-2 could serve as a tool for the preliminary evaluation of possible peptide toxicity prior to, and while minimizing the risk of lethal injury to animals during, *in vivo* tests. Furthermore, the non-intrusive and non-destructive transepithelial electrical resistance (TEER) test which involves placing two electrodes separated by the cellular monolayer in the upper (apical) and lower (basolateral)

compartment of a cell culture system, is commonly used to evaluate the integrity of Caco-2 cells and monitor tight junction function (Ding et al., 2015; Fu, et al., 2016; Srinivasan et al., 2015; Wang & Li, 2018).

Various studies have demonstrated the successful absorption of peptides through Caco-2 cell monolayers (Ding et al., 2015; Fu, et al., 2016; Vij et al., 2016). After adding 5 mM of the synthetic peptide RVPSL to the apical side of Caco-2 monolayers and incubating for 2 h at 37 °C and 5 % CO_2, 187.46 ± 29.84 μM of the peptide was found to have accumulated on the basolateral surface, thus suggesting that the pentapeptide could be transported intact across the monolayers (Ding et al., 2015). Nevertheless, about 2622.20 μM of the antihypertensive RVPSL was recovered from the apical side post-incubation, thus suggesting that approximately 47.56% of the original concentration of RVPSL that was added to the apical surface had disappeared. Therefore, other than the absorbed portion, about 36.31 % of the initial amount of the pentapeptide introduced to the apical side was possibly degraded to shorter peptides or free amino acids by brush border peptidases (Ding et al., 2015). In another study, 2 h after adding 0.5 mM of the collagen-derived antihypertensive peptides VGPV and GPRGF to the apical side of Caco-2 monolayers, ACE-inhibitory activity was detected on both the apical and basolateral surfaces in addition to no shorter peptide fragment being recovered thus suggesting that passage through the Caco-2 cell membranes did not adversely affect the structural integrity and *in vitro* blood pressure-lowering property of the peptides (Fu, et al., 2016). This is further indication that peptides could be transported intact across Caco-2 cell monolayers without losing their bioactivity.

Ex vivo methods for studying intestinal peptide absorption such as those involving freshly excised tissues mounted in Ussing chambers are highly sensitive experimental systems that

provide a higher level of complexity and organization of gastrointestinal tract barriers than those of cell culture models (Lundquist & Artursson, 2016). In a study of the intestinal absorption of proteins and peptides following simulated pepsin/pancreatin gastrointestinal digestion of a highly hydrolysed casein protein sample (PeptoPro®), a casein protein isolate and a whey protein isolate, jejunal tissues were collected from three pigs and mounted into Ussing chambers (Awati et al., 2009). Following 90 min incubation and after adjusting for endogenous amino acid contribution, quantification of the amino acid content of luminal samples collected every 10 min during the incubation period showed that mean amino acid absorption value for the highly hydrolyzed PeptoPro® sample was significantly ($p<0.05$) higher than that of the casein and whey isolates (Awati, et al., 2009). Although the Ussing chamber represents one of the most advanced tools for small molecule absorption, distribution, metabolism and excretion research, a number of technical and logistical challenges limit its use (Lundquist & Artursson, 2016). Some of these challenges include the need for proximity between the test laboratory and the clinic (site of tissue excision) for quick tissue transfer in order to maintain tissue viability and prevent deterioration, and the strict requirement to ensure that intestinal epithelium from freshly excised tissue is cut from underlying tissues along the submucosa and mounted so that it covers an opening between two contiguous chambers (Lundquist & Artursson, 2016). Other *ex vivo* transepithelial peptide transport methods include intestinal perfusion, intestinal loop and intestinal vascular cannulation (Wei, et al., 2016).

2.20 *In vivo* peptide transport studies

In addition to the afore-stated advantages of *in vivo* peptide transport studies over *in vitro* and *ex vivo* methods, the structural complexity of the intestinal epithelium and its array of specialized heterogeneous cell types including mucous-secreting, endocrine and microfold cells

ensure that the information obtained from *in vivo* studies are more accurate and detailed than those available from the other two methods (Madara, 1991).

For over a century, it was thought that complete hydrolysis to single amino acids and subsequent transfer by specific amino acid transporter-mediated transport systems were prerequisites for the intestinal absorption of dietary protein (Miner-Williams et al., 2014). As has been demonstrated by a number of studies in both animals and humans, small peptides can be absorbed intact from the intestinal lumen to the systemic circulation. For instance, in one placebo-controlled crossover study in which healthy male and female human subjects consumed a yogurt beverage enriched with eight casein-derived ACE-inhibitory peptides including the blood pressure-lowering tripeptide Ile-Pro-Pro, it was shown that the lactotripeptide selectively escaped intestinal degradation to reach the blood circulation intact (Foltz et al., 2007). The emergence of advanced mass spectrometry techniques has also contributed to increased understanding of the fate and stability of peptides absorbed intact across the intestinal epithelium. In a study conducted to evaluate the absorption potential of an anti-atherosclerotic dipeptide Trp-His, following oral gavage in Sprague-Dawley rats, phytic acid-aided matrix-assisted laser desorption/ionization-mass spectrometry (MALDI−MS) imaging analysis revealed details of the distribution of the peptide in the rat intestinal membrane (Tanaka et al., 2015). The data showed that the dipeptide Trp-His was absorbed intact into the systemic circulation due to its selective transport across the rat intestinal epithelium by PepT1. Although results from the preceding *in vitro* Caco-2 transport experiments indicated that Trp-His and another dipeptide with its reverse sequence, His-Trp, had comparable apparent permeability (P_{app}) values, MALDI-MS imaging analysis showed that His-Trp was not absorbed as a result of its degradation by brush border peptidases (Tanaka et al., 2015). A comparable study in which phytic acid was also

used as a matrix additive to enhance the visualization of absorbed peptides, matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-IMS) was used to demonstrate that the blood pressure-lowering dipeptide Val-Tyr was absorbed intact across rat intestinal epithelium prior to its degradation to single amino acids by peptidases located in the microvilli of the small intestine (Hong et al., 2013). A subsequent report suggested that peptide structural design plays a huge role in their interaction with proteases in the gut and blood as shown by the stability of the specifically designed Gly-Sar-Sar to peptidases during transport across rat intestinal membrane (Hong et al., 2016). In another MS technique-based study utilizing HPLC-API-MRM (high performance liquid chromatography-atmospheric pressure ionisation multiple reaction monitoring), 17 small ACE-inhibitory peptides containing two to five amino acid residues in chain length, were detected in plasma collected from human subjects after consuming a peptide-enriched drink, with a limit of detection < 0.01 ng/mL and a limit of quantification ranging from 0.05-0.2 ng/mL (van Platerink et al., 2006).

The fact that peptides are susceptible to enzymatic degradation during *in vivo* intestinal transport (Matsui, 2018) directly leads to the question of whether peptide fragments generated as a result of the peptidolytic action of intestinal or plasma peptidases retain their bioactivity following absorption. In a follow-up to the bioavailability study of a casein-derived pentapeptide discussed earlier in section 2.16, Sánchez-Rivera et al. demonstrated that all three peptide fragments (HLPL, LPLP and HLP) derived from the parent HLPLP following the degradative action of plasma peptidases maintained potent antihypertensive property in spontaneously hypertensive rats and contributed to the inhibition of vascular contraction elicited by angiotensin-I (Sánchez-Rivera et al., 2016).

The sections (Chapters 3-7) that follow describe the original research work carried out to provide answers to identified gaps in the literature. Given the hypothesis that specific alterations of the environment surrounding albumins and globulins derived from flaxseed proteins will result in structural and conformational changes and could thus affect their functionality in food products, albumin and globulin protein fractions were produced from flaxseed proteins and their physicochemical and emulsification properties under varying pH conditions were investigated. Similarly, in order to test the theory that measuring the amino acid composition of a food protein hydrolysate will provide the most accurate determination of its protein content, a range of standard protein measurement methods was employed in determining the protein contents of selected food protein hydrolysates. Subsequently, the capacity of enzymatic flaxseed protein hydrolysates and peptides to inhibit the *in vitro* activities of ACE and renin, and lower blood pressure in SHRs was evaluated in order to determine the *in vitro* and *in vivo* antihypertensive properties of enzyme-hydrolyzed FPI, while the ability of flaxseed peptide fractions of various MWCOs to scavenge free radicals and chelate metal ions was tested so as to determine the influence of bioactive peptide size on *in vitro* peptide antioxidative properties. Finally, the concept that flaxseed-derived peptides administered to animal models by oral gavage could undergo transepithelial transport and be detected in plasma was tested studied using Caco-2 cells and Wistar rats as models.

2.24. References

Adessi, C., & Soto, C. (2002). Converting a peptide into a drug: strategies to improve stability and bioavailability. *Current Medicinal Chemistry*, **9**(9):963-978.

Ahmed, A. S., El-Bassiony, T., Elmalt, L. M., & Ibrahim, H. R. (2015). Identification of potent antioxidant bioactive peptides from goat milk proteins. *Food Research International*, **74**:80-88.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., Malomo, S. A., Girgih, A. T., & Aluko, R. E. (2014). Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. *Food Research International*, **55**:281-287.

Aloğlu, H.Ş., & Öner, Z. (2011). Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt. *Journal of Dairy Science*, **94**(11):5305-5314.

Aluko, R. E. (2004). The extraction and purification of proteins: an introduction. In R. Yada, (Ed.). *Proteins in Food Processing* (pp. 323-351). Cambridge, England: Woodhead Publishing.

Aluko, R. E. (2010). Food protein–derived peptides as calmodulin inhibitors. In Y. Mine, & E. Li-Chan, (Eds.). *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals*, (pp. 55-65). Ames, Iowa: Wiley-Blackwell.

Aluko, R. E. (2012). *Functional Foods and Nutraceuticals*. New York, NY: Springer.

Aluko, R. E. (2015a). Antihypertensive peptides from food proteins. *Annual Review of Food Science and Technology*, **6**:235-262.

Aluko, R. E. (2015b). Structure and function of plant protein-derived antihypertensive peptides. *Current Opinion in Food Science*, **4**:44-50.

Aluko, R. E., Mofolasayo, O. A., & Watts, B. M. (2009). Emulsifying and foaming properties of commercial yellow pea (*Pisum sativum* L.) seed flours. *Journal of Agricultural and Food Chemistry*, **57**(20):9793-9800.

Amagloh, F. K., Mutukumira, A. N., Brough, L., Weber, J. L., Hardacre, A., & Coad, J. (2013). Carbohydrate composition, viscosity, solubility, and sensory acceptance of sweet potato- and maize-based complementary foods. *Food & Nutrition Research*, **57**: 10.3402/fnr.v57i0.18717.

Awati, A., Rutherfurd, S. M., Plugge, W., Reynolds, G. W., Marrant, H., Kies, A. K., & Moughan, P. J. (2009). Ussing chamber results for amino acid absorption of protein hydrolysates in porcine jejunum must be corrected for endogenous protein. *Journal of the Science of Food and Agriculture*, **89**(11):1857-1861.

Balimane, P. V., & Chong, S. (2005). Cell culture-based models for intestinal permeability: a critique. *Drug Discovery Today*, **10**(5):335-343.

Baradaran, A., Nasri, H., & Rafieian-Kopaei, M. (2014). Oxidative stress and hypertension: Possibility of hypertension therapy with antioxidants. In *Journal of Research in Medical Sciences*, **19**(4):358-367.

Barzideh, Z., Latiff, A., Gan, C.-Y., Abedin, M., & Alias, A. (2014). ACE inhibitory and antioxidant activities of collagen hydrolysates from the ribbon jellyfish (*Chrysaora* sp.). *Food Technology and Biotechnology*, **52**(4):495-504.

Bhat, Z., Kumar, S., & Bhat, H. (2015). Bioactive peptides of animal origin: a review. *Journal of Food Science and Technology*, **52**(9):5377-5392.

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants--double-edged swords in cellular redox state health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxidative Medicine and Cellular Longevity*, **3**(4):228-237.

Brandsch, M., Knütter, I., & Bosse-Doenecke, E. (2008). Pharmaceutical and pharmacological importance of peptide transporters. *The Journal of Pharmacy and Pharmacology*, **60**(5): 543-585.

Chabanon, G., Chevalot, I., Framboisier, X., Chenu, S., & Marc, I. (2007). Hydrolysis of rapeseed protein isolates: kinetics, characterization and functional properties of hydrolysates. *Process Biochemistry*, **42**(10):1419-1428.

Chang, S. K. C. (2010). Protein Analysis. In S.S. Nielsen, (Ed.). *Food Analysis*, (pp. 133-146). Boston, MA: Springer US.

Chang, Y.-W., Alli, I., Molina, A.T., Konishi, Y., & Boye, J.I. (2012). Isolation and characterization of chickpea (*Cicer arietinum* L.) seed protein fractions. *Food and Bioprocess Technology*, **5**(2):618-625.

Chove, B.E., Grandison, A.S., & Lewis, M.J. (2001). Emulsifying properties of soy protein isolate fractions obtained by isoelectric precipitation. *Journal of the Science of Food and Agriculture*, **81**(8):759-763.

Cardillo, G., Gentilucci, L., Tolomelli, A., Calienni, M., Qasem, A. R., & Spampinato, S. (2003). Stability against enzymatic hydrolysis of endomorphin-1 analogues containing β-proline. *Organic & Biomolecular Chemistry*, **1**(9):1498-1502.

Cardoso, A. R., Kakimoto, P. A. H. B., & Kowaltowski, A. J. (2013). Diet-sensitive sources of reactive oxygen species in liver mitochondria: role of very long chain acyl-CoA dehydrogenases. *PLoS One*, **8**(10): e77088.

Chandra, P., & Vij, S. (2018). Molecular Characterization and identification of bioactive peptides producing *Lactobacillus* sps. based on 16S rRNA gene sequencing. *Food Biotechnology*, **32**(1), 1-14.

Chay, S. Y., Salleh, A., Sulaiman, N. F., Abidin, N. Z., Hanafi, M. A., Zarei, M., & Saari, N. (2018). Blood-pressure lowering efficacy of winged bean seed hydrolysate in spontaneously hypertensive rats, peptide characterization and a toxicity study in Sprague-Dawley rats. *Food & function*, **9**(3):1657-1671.

Coda, R., Rizzello, C. G., Pinto, D., & Gobbetti, M. (2012). Selected lactic acid bacteria synthesize antioxidant peptides during sourdough fermentation of cereal flours. *Applied and Environmental Microbiology*, **78**(4):1087.

Crowe, F. L., Roddam, A. W., Key, T. J., Appleby, P. N., Overvad, K., Jakobsen, M. U., …Riboli, E. (2011). Fruit and vegetable intake and mortality from ischaemic heart disease: results from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heart study. *European Heart Journal*, **32**(10):1235-1243.

Cuevas, E. S., Zhang, A. Y., Yang, A. Y., Escano, A. C., Asico, A. L., Jones, A. J., … Armando, A. I.. (2012). Role of renal DJ-1 in the pathogenesis of hypertension associated with increased reactive oxygen species production. *Hypertension*, **59**(2):446-452.

Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1982). Development and design of specific inhibitors of angiotensin-converting enzyme. *The American Journal of Cardiology*, **49**(6):1390-1394.

Dai, J., & Mumper, R. (2010). Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. **15**(10):7313-7352).

Dev, D. K., & Quensel, E. (1988). Preparation and functional properties of linseed protein products containing differing levels of mucilage. *Journal of Food Science,* **53**(6):1834-1837.

Díaz-Gómez, J.L., Castorena-Torres, F., Preciado-Ortiz, R.E., & Garcia-Lara, S. (2017). Anti-cancer activity of maize bioactive peptides. *Frontiers in Chemistry,* **5**:44.

Ding, L., Wang, L., Zhang, Y., & Liu, J. (2015). Transport of antihypertensive peptide RVPSL, Ovotransferrin 328- 332, in human intestinal Caco- 2 cell monolayers. *Journal of Agricultural and Food Chemistry,* **63**(37):8143-8150.

Erdmann, K., Cheung, B. W. Y., & Schröder, H. (2008). The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry,* **19**(10): 643-654.

Erdmann, K., Grosser, N., Schipporeit, K., & Schröder, H. (2006). ACE inhibitory dipeptide Met-Tyr diminishes free radical formation in human endothelial cells via induction of heme oxygenase-1 and ferritin. *Journal of Nutrition,* **136**(8):2148-2152.

Ettehad, D., Emdin, C. A., Kiran, A., Anderson, S. G., Callender, T., Emberson, J., Chalmers, J., Rodgers, A., & Rahimi, K. (2016). Blood pressure lowering for prevention of cardiovascular disease and death: a systematic review and meta-analysis. *The Lancet,* **387**(10022):957-967.

Fernández-Musoles, R., Manzanares, P., Burguete, M.C., Alborch, E., & Salom, J.B. (2013). *In vivo* angiotensin I-converting enzyme inhibition by long-term intake of antihypertensive lactoferrin hydrolysate in spontaneously hypertensive rats. *Food Research International*, **54**(1):627-632.

Fiedor, J., & Burda, K. (2014). Potential role of carotenoids as antioxidants in human health and disease. *Nutrients*, **6**(2):466-488.

Foltz, M., Meynen, E. E., Bianco, V., van Platerink, C., Koning, T. M. M. G., & Kloek, J. (2007). Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *Journal of Nutrition,* **137**(4):953-958.

Fu, Y., Young, J. F., Rasmussen, M. K., Dalsgaard, T. K., Lametsch, R., Aluko, R. E., & Therkildsen, M. (2016). Angiotensin I–converting enzyme–inhibitory peptides from bovine collagen: insights into inhibitory mechanism and transepithelial transport. *Food Research International*, **89**(1):373-381.

Förstermann , U., & Sessa, W. C. (2012). Nitric oxide synthases: regulation and function. *European Heart Journal,* **33**(7):829-837.

Ganorkar, P., & Jain, R. (2013). Flaxseed - a nutritional punch. *International Food Research Journal*, **20**(2):519-525.

Garcia-Mora, P., Peñas, E., Frias, J., Zieliński, H., Wiczkowski, W., Zielińska, D., & Martínez-Villaluenga, C. (2016). High-pressure-assisted enzymatic release of peptides and phenolics increases angiotensin converting enzyme I inhibitory and antioxidant activities of pinto bean hydrolysates. *Journal of Agricultural and Food Chemistry,* **64**(8):1730-1740.

Genkinger, J. M., Platz, E. A., Hoffman, S. C., Comstock, G. W., & Helzlsouer, K. J. (2004). Fruit, vegetable, and antioxidant intake and all-cause, cancer, and cardiovascular disease mortality in a community-dwelling population in Washington County, Maryland. *American Journal of Epidemiology,* **160**(12):1223-1233.

Gillette, M. A., & Carr, S. A. (2012). Quantitative analysis of peptides and proteins in biomedicine by targeted mass spectrometry. *Nature Methods,* **10**(1):28-34.

Girgih, A. T. (2013). Structural and Functional Characterization of Hemp Seed (*Cannabis Sativa* L.) Protein-Derived Antioxidant and Antihypertensive Peptides. (Unpublished doctoral dissertation). University of Manitoba, Winnipeg, Canada.

Girgih, A. T., Nwachukwu, I. D., Hasan, F. M., Fagbemi, T. N., Malomo, S. A., Gill, T. A., & Aluko, R. E. (2016). Kinetics of *in vitro* enzyme inhibition and blood pressure-lowering effects of salmon (*Salmo salar*) protein hydrolysates in spontaneously hypertensive rats. *Journal of Functional Foods,* **20**:43-53.

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2011b). *In vitro* antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *Journal of the American Oil Chemists' Society,* **88**(3):381-389.

Girgih, A. T., Udenigwe, C. C., Li, H., Adebiyi, A. P., & Aluko, R. E. (2011a). Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates. *Journal of the American Oil Chemists' Society,* **88**(11):1767-1774.

González, J., Valls, N., Brito, R., & Rodrigo, R. (2014). Essential hypertension and oxidative stress: new insights. *World Journal of Cardiology,* **6**(6):353-366.

González-Ortega, O., López-Limón, A., Morales-Domínguez, J., & Soria-Guerra, R. (2015). Production and purification of recombinant hypocholesterolemic peptides. *Biotechnology Letters,* **37**(1):41-54.

Gumbiner, B. (1987). Structure, biochemistry, and assembly of epithelial tight junctions. *The American Journal of Physiology,* **253**(6 Pt 1):C749-C758.

Guo, M., Chen, X., Wu, Y., Zhang, L., Huang, W., Yuan, Y., Fang, M., Xie, J., & Wei, D. (2017). Angiotensin I-converting enzyme inhibitory peptides from Sipuncula (*Phascolosoma esculenta*): purification, identification, molecular docking and antihypertensive effects on spontaneously hypertensive rats. *Process Biochemistry,* **63**: 84-95.

Görlach, A., Dimova, E. Y., Petry, A., Martínez-Ruiz, A., Hernansanz-Agustín, P., Rolo, A. P., Palmeira, C. M., & Kietzmann, T. (2015). Reactive oxygen species, nutrition, hypoxia and diseases: problems solved? *Redox Biology,* **6**:372-385.

Hanh, V.T., Shen, W., Tanaka, M., Siltari, A., Korpela, R., & Matsui, T. (2017). Effect of aging on the absorption of small peptides in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry,* **65**(29):5935-5943.

Harrison, D. G., & Gongora, M. C. (2009). Oxidative Stress and hypertension. *Medical Clinics of North America,* **93**(3):621-635.

Haven, M. A., & Jorgensen, H. (2014). The challenging measurement of protein in complex biomass-derived samples. *Applied Biochemistry and Biotechnology,* **172**(1):87-101.

Hirata, H., Sonoda, S., Agui, S., Yoshida, M., Ohinata, K., & Yoshikawa, M. (2007). Rubiscolin-6, a delta opioid peptide derived from spinach Rubisco, has anxiolytic effect via activating sigma1 and dopamine D1 receptors. *Peptides,* **28**(10):1998-2003.

Hong, S.-M., Tanaka, M., Koyanagi, R., Shen, W., & Matsui, T. (2016). Structural design of oligopeptides for intestinal transport model. *Journal of Agricultural and Food Chemistry,* **64**(10): 2072-2079.

Hong, S.-M., Tanaka, M., Yoshii, S., Mine, Y., & Matsui, T. (2013). Enhanced visualization of small peptides absorbed in rat small intestine by phytic-acid-aided matrix-assisted laser

desorption/ionization-imaging mass spectrometry. *Analytical chemistry, 85*(21):10033-10039.

Huang, D., Ou, B., & Prior, R. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, **53**(6):1841-1856.

Huppertz, T., Fox, P. F., & Kelly, A. L. (2018). The caseins: structure, stability, and functionality. In R. Yada, (Ed.). *Proteins in Food Processing* (pp. 49-92). Cambridge, England: Woodhead Publishing.

Iadecola, C. (2014). Hypertension and dementia. *Hypertension,* **64**(1):3-5.

Iroyukifujita, H., Eiichiyokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin i-converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science,* **65**(4):564-569.

Irshad, I., Kanekanian, A., Peters, A., & Masud, T. (2015). Antioxidant activity of bioactive peptides derived from bovine casein hydrolysate fractions. *Journal of Food Science and Technology,* **52**(1):231-239.

Jamdar, S.N., Rajalakshmi, V., Pednekar, M.D., Juan, F., Yardi, V., & Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chemistry*, **121**(1):178-184.

Jauhiainen, T., & Korpela, R. (2007). Milk peptides and blood pressure. *The Journal of Nutrition,* **137**(3 Suppl 2):825S-829S.

Jung, W.-K., Qian, Z.-J., Lee, S.-H., Choi, S. Y., Sung, N. J., Byun, H.-G., & Kim, S.-K. (2007). Free radical scavenging activity of a novel antioxidative peptide isolated from *in vitro* gastrointestinal digests of *Mytilus coruscus*. *Journal of Medicinal Food,* **10**(1):197-202.

Kaleka, K. S., Petersen, A. N., Florence, M. A., & Gerges, N. Z. (2012). Pull-down of calmodulin-binding proteins. *Journal of Visualized Experiments,* **59**:3502.

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. *Food Research International,* **44**(9):2991-2998.

Kilara, A., & Chandan, R. C. (2011). Enzyme-modified dairy ingredients. In R.C. Chandan, & A. Kilara, (Eds.). *Dairy Ingredients for Food Processing,* (pp. 317-333). Ames, Iowa: Blackwell Publishing Ltd.

Kim, S. H., Lee, J. Y., Balolong, M. P., Kim, J.-E., Paik, H.-D., & Kang, D.-K. (2017). Identification and characterization of a novel antioxidant peptide from bovine skim milk fermented by *Lactococcus lactis* SL6. *Korean Journal for Food Science of Animal Resources,* **37**(3):402-409.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society,* **56**(3):242-258.

Kitts, D. D., & Weiler, K. (2003). Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Current Pharmaceutical Design,* **9**(16):1309-1323.

Kizawa, K. (1997). Calmodulin binding peptide comprising alpha-casein exorphin sequence. *Journal of Agricultural and Food Chemistry,* **45**(5):1579-1581.

Kizawa, K., Naganuma, K., & Murakami, U. (1995). Calmodulin-binding peptides isolated from alpha-casein peptone. *Journal of Dairy Research,* **62**(4):587-592.

Knott, A. B., & Bossy-Wetzel, E. (2009). Nitric oxide in health and disease of the nervous system. *Antioxidants and Redox Signaling,* **11**(3):541-553.

Kyle, S., James, K. A. R., & McPherson, M. J. (2012). Recombinant production of the therapeutic peptide lunasin. *Microbial Cell Factories,* **11**(1):28.

Landmesser, U., Dikalov, S., & McCann, L. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *Journal of Clinical Investigation,* **111**(8):1201-1209.

Lee, S.-H., Qian, Z.-J., & Kim, S.-K. (2010). A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chemistry*, **118**(1):96-102.

Li, G.-H., Le, G.-W., Shi, Y.-H., & Shrestha, S. (2004). Angiotensin I–converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutrition Research,* **24**(7):469-486.

Li, H., & Aluko, R. E. (2005). Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein-derived peptides. *The Journal of Nutritional Biochemistry*, **16**(11):656-662.

Li, H., & Aluko, R. E. (2006). Structural modulation of calmodulin and calmodulin-dependent protein kinase II by pea protein hydrolysates. *International Journal of Food Sciences and Nutrition,* **57**(3-4):178-189.

Li, P., Jia, J., Fang, M., Zhang, L., Guo, M., Xie, J., … Wei, D. (2014). *In vitro* and *in vivo* ACE inhibitory of pistachio hydrolysates and in silico mechanism of identified peptide binding with ACE. *Process Biochemistry*, **49**(5):898–904.

Li, Y. (2011). Recombinant production of antimicrobial peptides in *Escherichia coli*: a review. *Protein Expression and Purification*, **80**(2):260-267.

Liang, H.-N., & Tang, C.-H. (2013). pH-dependent emulsifying properties of pea [*Pisum sativum*(L.)] proteins. *Food Hydrocolloids,* **33**(2):309-319.

Liu, C., Fang, L., Min, W., Liu, J., & Li, H. (2018). Exploration of the molecular interactions between angiotensin-I-converting enzyme (ACE) and the inhibitory peptides derived from hazelnut (*Corylus heterophylla* Fisch.). *Food Chemistry*, **245**:471-480.

Liu, L. H., Hung, T. V., & Bennett, L. (2008). Extraction and characterization of chickpea (*Cicer arietinum*) albumin and globulin. *Journal of Food Science,* **73**(5):C299-C305.

Liu, R., Ren, Y., Garvin, J., & Carretero, O. (2003). Superoxide enhances tubuloglomerular feedback by constricting the afferent arteriole. *Hypertension,* **42**(3):399-400.

Liu, R.-Q., Qian, Z., Trevathan, E., Chang, J.-J., Zelicoff, A., Hao, Y.-T…Dong, G.-H. (2015). Poor sleep quality associated with high risk of hypertension and elevated blood pressure in China: results from a large population-based study. *Hypertension Research*, **39**(1):54-59.

Liu, W., Pan, H., Zhang, C., Zhao, L., Zhao, R., Zhu, Y., & Pan, W. (2016). Developments in methods for measuring the intestinal absorption of nanoparticle-bound drugs. *International Journal of Molecular Sciences,* **17**(7):1171.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: impact on human health. *Pharmacognosy Reviews,* **4**(8):118-126.

Loperena, R., & Harrison, D. G. (2017). Oxidative stress and hypertensive diseases. *Medical Clinics of North America*, **101**(1):169-193.

Lu, T. M., Chiu, H. F., Lu, Y. Y., Han, Y. C., Shen, Y. C., Venkatakrishnan, K., Golovinskaia, O., & Wang, C. K. (2018). Efficacy of fermented goat milk on blood pressure in prehypertensive adults: a randomized, placebo-controlled, clinical trial. *Journal of Food Biochemistry,* **42**(2):e12474.

Lundquist, P., & Artursson, P. (2016). Oral absorption of peptides and nanoparticles across the human intestine: opportunities, limitations and studies in human tissues. *Advanced Drug Delivery Reviews,* **106**(Pt B):256-276.

Madara, J. L. (1991). Functional morphology of epithelium of the small intestine. In M. Field, & R. A. Frizzell (Eds.). Handbook of Physiology-the gastrointestinal system IV, (pp. 83-120). Hoboken, New Jersey: Wiley-Blackwell.

Madara, J. L., & Dharmsathaphorn, K. (1985). Occluding junction structure-function relationships in a cultured epithelial monolayer. *The Journal of Cell Biology,* **101**(6): 2124-2133.

Madhusudhan, K. T., & Singh, N. (1983). Studies on linseed proteins [*Linum usitatissimum*, India]. *Journal of Agricultural and Food Chemistry*, **31**(5):959-963.

Maestri, E., Marmiroli, M., & Marmiroli, N. (2016). Bioactive peptides in plant-derived foodstuffs. *Journal of Proteomics,* **147**:140-155.

Majumder, K., Chakrabarti, S., Davidge, S. T., & Wu, J. (2013a). Structure and activity study of egg protein ovotransferrin derived peptides (IRW and IQW) on endothelial inflammatory response and oxidative stress. *Journal of Agricultural and Food Chemistry,* **61**(9): 2120-2129.

Majumder, K., Chakrabarti, S., Morton, J. S., Panahi, S., Kaufman, S., Davidge, S. T., & Wu, J. (2013b). Egg-derived tri-peptide IRW exerts antihypertensive effects in spontaneously hypertensive rats. *PLoS One,* **8**(11): e82829.

Majumder, K., Liang, G., Chen, Y., Guan, L. L., Davidge, S. T., & Wu, J. (2015). Egg ovotransferrin derived ACE inhibitory peptide IRW increases ACE2 but decreases proinflammatory genes expression in mesenteric artery of spontaneously hypertensive rats. *Molecular Nutrition and Food Research*, **59**(9): 1735-1744.

Majumder, K., & Wu, J. (2015). Molecular targets of antihypertensive peptides: understanding the mechanisms of action based on the pathophysiology of hypertension. *International Journal of Molecular Sciences,* **16**(1):256-283.

Malomo, S. A., & Aluko, R. E. (2015). A comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions. *Food Hydrocolloids,* **43**:743-752.

Marambe, H. K., & Wanasundara, J. P. D. (2017). Protein from flaxseed (*Linum usitatissimum* L.). In J. P. D. W. S. Nadathur, & L. Scanlin (Eds.). *Sustainable Protein Sources,* (pp. 133-144). Boston, MA: Elsevier Inc.

Marambe, P. W. M. L. H. K., Shand, P. J., & Wanasundara, J. P. D. (2008). An *in-vitro* investigation of selected biological activities of hydrolysed flaxseed (*Linum usitatissimum* L.) proteins. *Journal of the American Oil Chemists' Society,* **85**(12):1155-1164.

Masisi, K., Diehl-Jones, W. L., Gordon, J., Chapman, D., Moghadasian, M. H., & Beta, T. (2015). Carotenoids of aleurone, germ, and endosperm fractions of barley, corn and wheat differentially inhibit oxidative stress. *Journal of Agricultural and Food Chemistry,* **63**(10):2715-2724.

Matsui, T. (2018). Are peptides absorbable compounds? *Journal of Agricultural and Food Chemistry,* **66**(2):393-394.

Matsui, T., Tamaya, K., Seki, E., Osajima, K., Matsumoto, K., & Kawasaki, T. (2002). Val-Tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clinical and Experimental Pharmacology & Physiology*, **29**(3):204-208.

Means, A. R. (1994). Calcium, calmodulin and cell cycle regulation. *FEBS Letters,* **347**(1):1-4.

Mechmeche, M., Kachouri, F., Ksontini, H., & Hamdi, M. (2017). Production of bioactive peptides from tomato seed isolate by *Lactobacillus plantarum* fermentation and enhancement of antioxidant activity. *Food Biotechnology,* **31**(2):94-113.

Meisel, H. (2005). Biochemical properties of peptides encrypted in bovine milk proteins. *Current Medicinal Chemistry,* **12**(16):1905-1919.

Mellander, O. (1950). The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. Some aspects of the pathogenesis of rickets. *Acta Societatis Botanicorum Poloniae,* **55**(5/6):247-257.

Mendoza, E. M., Adachi, M., Bernardo, A. E., & Utsumi, S. (2001). Mungbean [*Vigna radiata* (L.) Wilczek] globulins: purification and characterization. *Journal of Agricultural and Food Chemistry,* **49**(3):1552-1558.

Miner-Williams, W. M., Stevens, B. R., & Moughan, P. J. (2014). Are intact peptides absorbed from the healthy gut in the adult human? *Nutrition Research Reviews,* **27**(2):308-329.

Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T., & Yamamoto, N. (2004). Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease. *Journal of Dairy Science,* **87**(10):3183-3188.

Mojica, L., & De Meja, E. G. (2016). Optimization of enzymatic production of anti-diabetic peptides from black bean (*Phaseolus vulgaris* L.) proteins, their characterization and biological potential. *Food & Function,* **7**(2):713-727.

Montezano, A. C., & Touyz, R. M. (2012). Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. *Annals of Medicine,* **44**(Suppl 1):S2-S16.

Moore, J. C., Devries, J. W., Lipp, M., Griffiths, J. C., & Abernethy, D. R. (2010). Total protein methods and their potential utility to reduce the risk of food protein adulteration. *Comprehensive Reviews in Food Science and Food Safety,* **9**(4):330-357.

Mundi, S., & Aluko, R. E. (2012). Physicochemical and functional properties of kidney bean albumin and globulin protein fractions. *Food Research International,* **48**(1):299-306.

Mustroph, J., Neef, S., & Maier, L. S. (2017). CaMKII as a target for arrhythmia suppression. *Pharmacology & Therapeutics,* **176**:22-31.

Möller, N., Scholz-Ahrens, K., Roos, N., & Schrezenmeir, J. (2008). Bioactive peptides and proteins from foods: indication for health effects. *European Journal of Nutrition,* **47**(4): 171-182.

Nabha, L., Garbern, J.C., Buller, C.L., & Charpie, J.R. (2005). Vascular oxidative stress precedes high blood pressure in spontaneously hypertensive rats. *Clinical and Experimental Hypertension*, **27**(1):71-82.

Nagaoka, S., Futamura, Y., Miwa, K., Awano, T., Yamauchi, K., Kanamaru, Y., Tadashi, K., & Kuwata, T. (2001). Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochemical and Biophysical Research Communications,* **281**(1):11-17.

Nakazono, K., Watanabe, N., Matsuno, K., Sasaki, J., Sato, T., & Inoue, M. (1991). Does superoxide underlie the pathogenesis of hypertension? *Proceedings of the National Academy of Sciences of the United States of America,* **88**(22):10045-10048.

Ndiaye, F., Vuong, T., Duarte, J., Aluko, R., & Matar, C. (2012). Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds. *European Journal of Nutrition,* **51**(1):29-37.

Ni, Y., Wang, L., & Kokot, S. (2000). Voltammetric determination of butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert-butylhydroquinone by use of chemometric approaches. *Analytica Chimica Acta,* **412**(1):185-193.

Nurminen, M., Sipola, M., Kaarto, H., Pihlanto-Leppala, A., Piilola, K., Korpela, R., Tossavainen, O., Korhonen, H., & Vapaatalo, H. (2000). Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats. *Life Sciences,* **66**(16):1535-1543.

Nwachukwu, I. D., & Aluko, R. E. (2018). Physicochemical and emulsification properties of flaxseed (*Linum usitatissimum*) albumin and globulin fractions. *Food Chemistry,* **255**: 216-225.

Nwachukwu, I. D., Girgih, A. T., Malomo, S. A., Onuh, J. O., & Aluko, R. E. (2014). Thermoase-derived flaxseed protein hydrolysates and membrane ultrafiltration peptide fractions have systolic blood pressure-lowering effects in spontaneously hypertensive rats. *International Journal of Molecular Sciences,* **15**(10):18131-18147.

Nässl, A.-M., Rubio-Aliaga, I., Fenselau, H., Marth, M. K., Kottra, G., & Daniel, H. (2011). Amino acid absorption and homeostasis in mice lacking the intestinal peptide transporter PEPT1. *American Journal of Physiology. Gastrointestinal and Liver Physiology,* **301**(1): G128-G137.

Omoni, A. O., & Aluko, R. E. (2006). Effect of cationic flaxseed protein hydrolysate fractions on the *in vitro* structure and activity of calmodulin-dependent endothelial nitric oxide synthase. *Molecular Nutrition and Food Research,* **50**(10):958-966.

Omoni, A. O., & Aluko, R. E. (2006). Mechanism of the inhibition of calmodulin-dependent neuronal nitric oxide synthase by flaxseed protein hydrolysates. *Journal of the American Oil Chemists' Society,* **83**(4):335-340.

Ondetti, M. A., & Cushman, D. W. (1982). Enzymes of the renin-angiotensin system and their inhibitors. *Annual Review of Biochemistry,* **51**:283-308.

Onuh, J. O., Girgih, A. T., Malomo, S. A., Aluko, R. E., & Aliani, M. (2015). Kinetics of *in vitro* renin and angiotensin converting enzyme inhibition by chicken skin protein hydrolysates and their blood pressure lowering effects in spontaneously hypertensive rats. *Journal of Functional Foods,* **14**:133-143.

Oomah, B. D., & Mazza, G. (1993). Flaxseed proteins-a review. *Food Chemistry*, **48**(2):109-114.

Padayatty, S. J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.-H., Chen, S., Corpe, C., Dutta, A., Dutta, S. K., & Levine, M. (2003). Vitamin C as an antioxidant: evaluation of its role in disease prevention. *Journal of the American College of Nutrition*, **22**(1):18-35.

Pandey, K., & Rizvi, S. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity*, **2**(5):270-278.

Papalamprou, E.M., Doxastakis, G.I., & Kiosseoglou, V. (2010). Chickpea protein isolates obtained by wet extraction as emulsifying agents. *Journal of the Science of Food and Agriculture*, **90**(2):304-313.

Patel, G., & Misra, A. (2011). Oral delivery of proteins and peptides: concepts and applications. In A. Misra, (Ed.). *Challenges in Delivery of Therapeutic Genomics and Proteomics*, (pp. 481-529). London: Elsevier.

Pauletti, G.M., Gangwar, S., Knipp, G.T., Nerurkar, M.M., Okumu, F.W., Tamura, K… Borchardt, R.T. (1996). Structural requirements for intestinal absorption of peptide drugs. *Journal of Controlled Release*, **41**(1-2):3-17.

Pauletti, G.M., Okumu, F.W., & Borchardt, R.T. (1997). Effect of size and charge on the passive diffusion of peptides across Caco-2 cell monolayers via the paracellular pathway. *Pharmaceutical Research,* **14**(2):164-168.

Pellicena, P., & Schulman, H. (2014). CaMKII inhibitors: from research tools to therapeutic agents. *Frontiers in Pharmacology*, **5**:21.

Perez Espitia, P. J., de Fátima Ferreira Soares, N., dos Reis Coimbra, J. S., de Andrade, N. J., Souza Cruz, R., & Alves Medeiros, E. A. (2012). Bioactive peptides: synthesis, properties, and applications in the packaging and preservation of food. *Comprehensive Reviews in Food Science and Food Safety*, **11**(2):187-204.

Picariello, C., Lazzeri, C., Attanà, P., Chiostri, M., Gensini, G. F., & Valente, S. (2011). The impact of hypertension on patients with acute coronary syndromes. *International Journal of Hypertension*, **2011**:563657.

Pickering, T. G., Hall, J. E., Appel, L. J., Falkner, B. E., Graves, J., Hill, M. N…Roccella, E. J. (2005). Recommendations for blood pressure measurement in humans and experimental animals. Part 1: Blood pressure measurement in humans. *Circulation*, **111**(5): 697-716.

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal*, **16**(11):1306-1314.

Porkert, M., Sher, S., Reddy, U., Cheema, F., Niessner, C., Kolm, P… Quyyumi, A. A. (2008). Tetrahydrobiopterin: a novel antihypertensive therapy. *Journal of Human Hypertension,* **22**(6):401-407.

Quansah, J., Udenigwe, C., Saalia, F., & Yada, R. (2013). The effect of thermal and ultrasonic treatment on amino acid composition, radical scavenging and reducing potential of hydrolysates obtained from simulated gastrointestinal digestion of cowpea proteins. *Plant Foods for Human Nutrition*, **68**(1):31-38.

Ralston, R. A., Lee, J. H., Truby, H., Palermo, C. E., & Walker, K. Z. (2011). A systematic review and meta-analysis of elevated blood pressure and consumption of dairy foods. *Journal of Human Hypertension,* **26**(1):3-13.

Ramón, R., Jaime, G., & Fabio, P. (2011). The role of oxidative stress in the pathophysiology of hypertension. *Hypertension Research,* **34**(4):431-440.

Rao, M. S. (2010). Inhibition of the renin angiotensin aldosterone system: focus on aliskiren. *The Journal of the Association of Physicians of India,* **58**:102-108.

Ray, P. D., Huang, B.-W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling,* **24**(5):981-990.

Rajendran, S.R.C.K., Ejike, E.C.C.C., Gong, M., Hannah, W., & Udenigwe, C.C. (2017). Preclinical evidence on the anticancer properties of food peptides. *Protein and Peptide Letters,* **24**:(2):126-136.

Regazzo, D. (2010). Bioactive Peptides from Milk Proteins: Focusing on Peptides Displaying Immunomodulatory Activity. (Unpublished doctoral dissertation). Università degli Studi di Padova, Padova, Italy.

Regazzo, D., Mollé, D., Gabai, G., Tomé, D., Dupont, D., Leonil, J., & Boutrou, R. (2010). The (193–209) 17-residues peptide of bovine β-casein is transported through Caco-2 monolayer. *Molecular Nutrition & Food Research*, **54**(10):1428-1435.

Rostas, J. A. P., Spratt, N. J., Dickson, P. W., & Skelding, K. A. (2017). The role of Ca^{2+}-calmodulin stimulated protein kinase II in ischaemic stroke – a potential target for neuroprotective therapies. *Neurochemistry International,* **107**:33-42.

Ryan, J. T., Ross, R. P., Bolton, D., Fitzgerald, G. F., & Stanton, C. (2011). Bioactive peptides from muscle sources: meat and fish. *Nutrients,* **3**(9):765-791.

Sacks, F. M., Moore, T. J., Appel, L. J., Obarzanek, E., Cutler, J.A., Vollmer, W. M…Karanja, N. (1999). A dietary approach to prevent hypertension: a review of the Dietary Approaches to Stop Hypertension (DASH) study. *Clinical Cardiology,* **22**(S3):6-10.

Sah, B. N. P., Vasiljevic, T., McKechnie, S., & Donkor, O. N. (2018). Antioxidative and antibacterial peptides derived from bovine milk proteins. **58**(5):726-740.

Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications. *Journal of Functional Foods,* **3**(4):229-254.

Sammour, R.H., El-Shourbagy, M.N., Abo-Shady, A.M., & Abasery, A.M. (1994). The seed proteins of linseed (*Linum usitatissimum* L.). *Botanical Bulletin of Academia Sinica,* **35**:171-177.

Sapan, C. V., Lundblad, R. L., & Price, N. C. (1999). Colorimetric protein assay techniques. *Biotechnology and Applied Biochemistry,* **29**(Pt 2):99-108.

Sassone-Corsi, P. (2012). The cyclic AMP pathway. *Cold Spring Harbor Perspectives in Biology,* **4**(12):a011148.

Satake, M., Enjoh, M., Nakamura, Y., Takano, T., Kawamura, Y., Arai, S., & Shimizu, M. (2002). Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers. *Bioscience, Biotechnology, and Biochemistry*, **66**(2): 378-384.

Sathe, S. K., Hamaker, B. R., Sze-Tao, K. W. C., & Venkatachalam, M. (2002). Isolation, purification, and biochemical characterization of a novel water soluble protein from Inca peanut (*Plukenetia volubilis* L.). *Journal of Agricultural and Food Chemistry,* **50**(17): 4906-4908.

Sato, M., Hosokawa, T., Yamaguchi, T., Nakano, T., Muramoto, K., Kahara, T…Kobayashi, A. (2002). Angiotensin I-converting enzyme inhibitory peptides derived from wakame (*Undaria pinnatifida*) and their antihypertensive effect in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry,* **50**(21):6245-6252.

Schlimme, E., & Meisel, H. (1995). Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung,* **39**(1):1-20.

Segura-Campos, M., Chel-Guerrero, L., Betancur-Ancona, D., & Hernandez-Escalante, V. M. (2011). Bioavailability of bioactive peptides. *Food Reviews International*, **27**(3):213-226.

Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*, vol. 2012, Article ID 217037, 26 pages.

Shen, W.-C., Wan, J., & Ekrami, H. (1992). (C) Means to enhance penetration: (3) Enhancement of polypeptide and protein absorption by macromolecular carriers via endocytosis and transcytosis. *Advanced Drug Delivery Reviews,* **8**(1):93-113.

Shevkani, K., Singh, N., Kaur, A., & Rana, J. C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: a comparative study. *Food Hydrocolloids,* **43**:679-689.

Shimizu, M. (2004). Food-derived peptides and intestinal functions. *BioFactors (Oxford, England),* **21**(1-4):43-47.

Shimizu, M., Tsunogai, M., & Arai, S. (1997). Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides,* **18**(5):681-687.

Sila, A., & Bougatef, A. (2016). Antioxidant peptides from marine by-products: isolation, identification and application in food systems. A review. *Journal of Functional Foods,* **21**:10-26.

Silva, A. J., Kogan, J. H., Frankland, P. W., & Kida, S. (1998). CREB and memory. *Annual Review of Neuroscience,* **21**:127-148.

Slavin, J.L., & Lloyd, B. (2012). Health Benefits of fruits and vegetables. *Advances in Nutrition*, **3**(4):506-516.

Smith, E.R. (2009). The Canadian heart health strategy and action plan. *Canadian Journal of Cardiology*, **25**(8):451-452.

Song, Y., Guan, R., Lyu, F., Kang, T., Wu, Y., & Chen, X. (2014). *In vitro* cytotoxicity of silver nanoparticles and zinc oxide nanoparticles to human epithelial colorectal adenocarcinoma (Caco-2) cells. *Mutation Research,* **769**:113-118.

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER measurement techniques for *in vitro* barrier model systems. *Journal of Laboratory Automation*, **20**(2):107-126.

Statistics Canada. (2018). High blood pressure, by sex, and by province and territory. Retrieved from https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=1310009609. Accessed on May 4, 2018.

Storz, P. (2011). Forkhead homeobox type O transcription factors in the responses to oxidative stress. *Antioxidants & Redox Signaling,* **14**(4):593-605.

Sun, J.-Z., Kaur, H., Halliwell, B., Li, X.-Y., & Bolli, R. (1993). Use of aromatic hydroxylation of phenylalanine to measure production of hydroxyl radicals after myocardial ischemia *in vivo*. Direct evidence for a pathogenetic role of the hydroxyl radical in myocardial stunning. *Circulation Research,* **73**(3):534-549.

Suzuki, T., & Hara, H. (2004). Various nondigestible saccharides open a paracellular calcium transport pathway with the induction of intracellular calcium signalling in human intestinal Caco-2 cells. *Journal of Nutrition,* **134**(8):1935-1941.

Sánchez-Rivera, L., Ares, I., Miralles, B., Gómez-Ruiz, J. Á., Recio, I., Martínez-Larrañaga, M. R… Martínez, M. A. (2014). Bioavailability and kinetics of the antihypertensive casein-derived peptide HLPLP in rats. *Journal of Agricultural and Food Chemistry, 62*(49): 11869-11875.

Sánchez-Rivera, L., Santos, P. F., Miralles, B., Carrón, R., José Montero, M., & Recio, I. (2016). Peptide fragments from β-casein f(134–138), HLPLP, generated by the action of rat blood plasma peptidases show potent antihypertensive activity. *Food Research International,* **88**(PB):348-353.

Tajbakhsh, A., Pasdar, A., Rezaee, M., Fazeli, M., Soleimanpour, S., Hassanian, S. M... Avan, A. (2018). The current status and perspectives regarding the clinical implication of intracellular calcium in breast cancer. *Journal of Cellular Physiology*, **233**(8):5623-5641.

Tanaka, M., Hong, S. M., Akiyama, S., Hu, Q. Q., & Matsui, T. (2015). Visualized absorption of anti-atherosclerotic dipeptide, Trp-His, in Sprague–Dawley rats by LC-MS and MALDI-MS imaging analyses. *Molecular Nutrition & Food Research,* **59**(8):1541-1549.

Tang, C.-H., Wang, X.-S., & Yang, X.-Q. (2009). Enzymatic hydrolysis of hemp (Cannabis sativa L.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry,* **114**(4):1484-1490.

Tang, V. W., & Goodenough, D. A. (2003). Paracellular ion channel at the tight junction. *Biophysical Journal*, **84**(3):1660-1673.

Touyz, R. M., Alves-Lopes, R., Rios, F. J., Camargo, L. L., Anagnostopoulou, A., Arner, A., & Montezano, A. C. (2018). Vascular smooth muscle contraction in hypertension. *Cardiovascular Research*, **114**(4):529-539.

Tsai, J.S., Chen, J.L., & Pan, B.S. (2008). ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). *Process Biochemistry*, **43**(7):743-747.

Udenigwe, C. C. (2010). Structure-Function Properties of Flaxseed Protein-Derived Multifunctional Peptides. (Unpublished doctoral dissertation). University of Manitoba, Winnipeg, Canada.

Udenigwe, C. C. (2014). Bioinformatics approaches, prospects and challenges of food bioactive peptide research. *Trends in Food Science & Technology*, **36**(2):137-143.

Udenigwe, C. C., & Aluko, R. E. (2010). Antioxidant and angiotensin converting enzyme-inhibitory properties of a flaxseed protein-derived high fischer ratio peptide mixture. *Journal of Agricultural and Food Chemistry*, **58**(8):4762-4768.

Udenigwe, C. C., & Aluko, R. E. (2012a). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science*, **77**(1):R11-R24.

Udenigwe, C. C., & Aluko, R. E. (2012b). Multifunctional cationic peptide fractions from flaxseed protein hydrolysates. *Plant Foods for Human Nutrition*, **67**(1):1-9.

Udenigwe, C. C., & Fogliano, V. (2017). Food matrix interaction and bioavailability of bioactive peptides: two faces of the same coin? *Journal of Functional Foods*, **35**:9-12.

Udenigwe, C. C., Gong, M., & Wu, S. (2013). In silico analysis of the large and small subunits of cereal RuBisCO as precursors of cryptic bioactive peptides. *Process Biochemistry*, **48**(11):1794-1799.

Udenigwe, C. C., Lin, Y.-S., Hou, W.-C., & Aluko, R. E. (2009a). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods*, **1**(2):199-207.

Udenigwe, C. C., Lu, Y. L., Han, C. H., Hou, W. C., & Aluko, R. E. (2009b). Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. *Food Chemistry*, **116**(1):277-284.

Udenigwe, C. C., Nwachukwu, I. D., & Yada, R. Y. (2017). Advances on the production and application of peptides for promoting human health and food security. In G. V. Barbosa-Cánovas, G. María Pastore, K. Candoğan, I. G. Medina Meza, S. Caetano da Silva Lannes, K. Buckle, R. Y. Yada, & A. Rosenthal (Eds.). *Global Food Security and Wellness*, (pp. 195-219). New York, NY: Springer New York.

Van Petegem, F. (2012). Ryanodine receptors: structure and function. *Journal of Biological Chemistry*, **287**(38):1624-31632.

van Platerink, C. J., Janssen, H. G. M., Horsten, R., & Haverkamp, J. (2006). Quantification of ACE inhibiting peptides in human plasma using high performance liquid chromatography-mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, **830**(1):151-157.

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2004). Bioavailability of angiotensin I converting enzyme inhibitory peptides. *British Journal of Nutrition*, **92**(3):357-366.

Vij, R., Reddi, S., Kapila, S., & Kapila, R. (2016). Transepithelial transport of milk derived bioactive peptide VLPVPQK. *Food Chemistry*, **190**:681-688.

Vitale, G., Dicitore, A., Mari, D., & Cavagnini, F. (2009). A new therapeutic strategy against cancer: cAMP elevating drugs and leptin. *Cancer Biology & Therapy*, **8**(12):1191-1193.

Vossen, E., Ntawubizi, M., Raes, K., Smet, K., Huyghebaert, G., Arnouts, S., & De Smet, S. (2011). Effect of dietary antioxidant supplementation on the oxidative status of plasma in broilers. *Journal of Animal Physiology and Animal Nutrition*, **95**(2):198-205.

Wanasundara, P., & Shahidi, F. (1997). Functional properties of acylated flax protein isolates. *Journal of Agricultural and Food Chemistry*, **45**(7):2431-2441.

Wang, B., & Li, B. (2017). Effect of molecular weight on the transepithelial transport and peptidase degradation of casein-derived peptides by using Caco-2 cell model. *Food Chemistry*, **218**:1-8.

Wang, B., & Li, B. (2018). Charge and hydrophobicity of casein peptides influence transepithelial transport and bioavailability. *Food Chemistry*, **245**:646-652.

Wang, G., Zhou, X., Zhuo, X., & Zhang, P. (2017). Annual total medical expenditures associated with hypertension by diabetes status in U.S. adults. *American Journal of Preventive Medicine*, **53**(6S2):S182-S189.

Wang, X., Wang, L., Cheng, X., Zhou, J., Tang, X., & Mao, X.-Y. (2012). Hypertension-attenuating effect of whey protein hydrolysate on spontaneously hypertensive rats. *Food Chemistry*, **134**(1):122-126.

Wennogle, L. P., Hoxie, H., Peng, Y., & Hendrick, J. P. (2017). Phosphodiesterase 1: a unique drug target for degenerative diseases and cognitive dysfunction. In H. Zhang, Y. Xu, & J.M. O'Donnell (Eds.). *Phosphodiesterases: CNS Functions and Diseases*, (pp. 349-384). *Advances in Neurobiology*, vol. 17. Cham, Switzerland: Springer International Publishing AG.

Were, L., Hettiarachchy, N. S., & Kalapathy, U. (1997). Modified soy proteins with improved foaming and water hydration properties. *Journal of Food Science*, **62**(4):821-824.

WHO. (2011). Hypertension fact sheet. Retrieved from www.searo.who.int/entity/noncommunicable_diseases/media/non_communicable_diseases_hypertension_fs.pdf?ua=1. Accessed on May 2, 2018.

Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety*, **15**(4):786-800.

Wu, D., & Cederbaum, A. I. (2003). Alcohol, oxidative stress, and free radical damage. *Alcohol Research & Health*, **27**(4):277-284.

Wu, H. C., Pan, B. S., Chang, C., & Shiau, C. (2005). Low-molecular-weight peptides as related to antioxidant properties of chicken essence. *Journal of Food and Drug Analysis*, **13**(2): 176-183.

Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di- and tripeptides. *Journal of Agricultural and Food Chemistry*, **54**(3):732-738.

Wu, J., Aluko, R. E., & Nakai, S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship modeling of peptides containing 4-10 amino acid residues. *QSAR & Combinatorial Science*, **25**(10): 873-880.

Wu, J., & Harrison, D. G. (2014). Oxidative stress and hypertension. In M. E. Safar, M. F. O'Rourke, & E. D. Frohlich (Eds.). *Blood Pressure and Arterial Wall Mechanics in Cardiovascular Diseases,* (pp. 175-191). London, United Kingdom: Springer-Verlag.

Wu, J., Wang, L., & Udenigwe, C.C. (2017). Revisiting the mechanisms of ACE inhibitory peptides from food proteins. *Trends in Food Science & Technology,* **69**: 214-219.

Wu, Q., Jia, J., Yan, H., Du, J., & Gui, Z. (2015). A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (*Bombyx mori*) protein: biochemical characterization and molecular docking study. *Peptides,* **68**:17-24.

Xu, F., Wang, L., Ju, X., Zhang, J., Yin, S., Shi, J…Yuan, Q. (2017). Transepithelial transport of YWDHNNPQIR and its metabolic fate with cytoprotection against oxidative stress in human intestinal Caco-2 cells. *Journal of Agricultural and Food Chemistry,* **65**(10): 2056-2065.

Yang, S., Yunden, J., Sonoda, S., Doyama, N., Lipkowski, A. W., Kawamura, Y., & Yoshikawa, M. (2001). Rubiscolin, a delta selective opioid peptide derived from plant Rubisco. *FEBS Letters,* **509**(2):213-217.

Yin, S. W., Tang, C. H., Wen, Q. B., Yang, X. Q., & Yuan, D. B. (2010). The relationships between physicochemical properties and conformational features of succinylated and acetylated kidney bean (*Phaseolus vulgaris* L.) protein isolates. *Food Research International,* **43**(3):730-738.

You, S.-J., Udenigwe, C. C., Aluko, R. E., & Wu, J. (2010). Multifunctional peptides from egg white lysozyme. *Food Research International,* **43**(3):848-855.

Zambon, A. C., Zhang, L., Minovitsky, S., Kanter, J. R., Prabhakar, S., Salomonis, N…Taylor, S. S. (2005). Gene expression patterns define key transcriptional events in cell-cycle regulation by cAMP and protein kinase A. *Proceedings of the National Academy of Sciences of the United States of America,* **102**(24):8561-8566.

Zhang, C.H., Cao, W.H., Hong, P.Z., Ji, H.W., Qin, X.M., & He, J.F. (2009). Angiotensin I-converting enzyme inhibitory activity of *Acetes chinensis* peptic hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *International Journal of Food Science and Technology*, **44**(10):2042-2048.

Zhang, S. B., Wang, Z., & Xu, S. Y. (2008). Antioxidant and antithrombotic activities of rapeseed peptides. *Journal of the American Oil Chemists' Society,* **85**(6):521-527.

Zhao, L., Wang, X. Zhang, X.-L., & Xie, Q.-F. (2016). Purification and identification of anti-inflammatory peptides derived from simulated gastrointestinal digests of velvet antler protein (*Cervus elaphus* Linnaeus). *Journal of Food and Drug Analysis*, **24**(2):376-384.

Zino, S., Skeaff, M., Williams, S., & Mann, J. (1997). Randomised controlled trial of effect of fruit and vegetable consumption on plasma concentrations of lipids and antioxidants. *The BMJ,* **314**(7097):1787-1791.

Zou, T., He, T. P., Li, H., Tang, H., & Xia, E. (2016). The structure-activity relationship of the antioxidant peptides from natural proteins. *Molecules,* **21**(1):72.

CHAPTER 3

MANUSCRIPT ONE

PHYSICOCHEMICAL AND EMULSIFICATION PROPERTIES OF FLAXSEED (*LINUM USITATISSIMUM*) ALBUMIN AND GLOBULIN FRACTIONS

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3.0 Abstract

The physicochemical and emulsification characteristics of flaxseed albumin and globulin protein fractions were determined in this study. Flaxseed protein meal was extracted with 0.5 M NaCl, and the extract dialyzed against water followed by centrifugation to obtain the globulin as a water-insoluble precipitate and albumin as the water-soluble albumin. Gel electrophoresis data indicate that the globulin is composed of several polypeptides in the 10-50 kDa range while albumin consisted mainly of the 10 kDa polypeptide accompanied by a minor content of 40 kDa. Amino acid analysis showed significantly ($p < 0.05$) higher levels of hydrophobic amino acids in the globulin, which was consistent with higher surface hydrophobicity when compared to the albumin. All the emulsions had monomodal oil droplet size distribution and wider ranges were directly related to bigger sizes, especially at low (10 mg/mL) protein concentration when compared to the 50 mg/mL.

Keywords: Flaxseed; Globulin; Albumin; SDS-PAGE; Protein solubility; Surface hydrophobicity.

3.1 Introduction

In recent years, there has been a continuous rise in the demand for plant protein use as functional food ingredients in the food industry (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). This is in part because functional properties such as solubility, gelation, emulsion and foaming capacities can contribute to the sensory attributes of a food product (Amagloh, Mutukumira, Brough, Weber, Hardacre, & Coad, 2013; Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016), and thus enormously influence its commercial success. To be a candidate for use in the industrial formulation of novel food products, a plant protein should have desirable physicochemical and functional properties, both of which are typically influenced by structural attributes. For instance, the structural modification of albumin by means of glycation has been shown to result in changes in antioxidant and binding capacities (Rondeau & Bourdon, 2011). Structure-dependent functionalities can also influence the interactions of proteins with other biomolecules as illustrated by the propensity of protein hydrophobic groups to interact with lipids and lipid-soluble compounds (Karaca, Low, & Nickerson, 2011). Alterations of the protein environment e.g. through changes in pH, temperature, ionic strength, etc are known to often result in structure modifications and consequently, in their biological, physicochemical and functional properties (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). For instance, changes in pH have been reported to affect the surface hydrophobicity, solubility, foaming capacity and foam stability of proteins isolated from Australian chia seeds (Timilsena, Adhikari, Barrow, & Adhikari, 2016). The enormous influence of pH change could be seen in the differential solubility of the chia protein isolate which was almost completely soluble at pH 12.0 in stark contrast to its 40% solubility at pH 1.0. In another study, the rheological and gelation properties of myofibrillar proteins were enhanced following incorporation of soy protein isolates

whose processing included pre-treatment at acidic pH for 5 h (Niu, Li, Han, Liu, & Kong, 2017). Since the functionality of food proteins is known to be predicated upon charge distribution, molecular size, and three dimensional structure (among other attributes), and given the influence of pH on the net charge and electrostatic repulsion between protein molecules (Timilsena, Adhikari, Barrow, & Adhikari, 2016), the significance of the current work is obvious.

Flaxseed (*Linum usitatissimum*) was once popular because of its utilization in the industrial production of paint and later as a valuable source of dietary fibres and α-linolenic acid (Hall, Tulbek, & Xu, 2006). But recent works have showed that flaxseed proteins could also be important ingredients suitable for the formulation of health-promoting peptides. For example, our previous works (Nwachukwu, Girgih, Malomo, Onuh, & Aluko, 2014; Udenigwe & Aluko, 2010) documented the growing importance of flaxseed proteins as a source of blood pressure-lowering bioactive peptides. Other studies have also demonstrated the potential of flaxseed as a source of multifunctional proteins/peptides for use as antidiabetic (Doyen et al., 2014), antifungal (Xu, Hall, & Wolf-Hall, 2008) and antioxidant (Marambe, Shand, & Wanasundara, 2013) agents. Although various studies in recent years have examined the structural and functional properties of proteins from inexpensive and underutilized plants including hemp seed (Yin et al., 2008), kidney bean (Shevkani, Singh, Kaur, & Rana, 2015), pinto bean (Tan, Ying-Yuan, & Gan, 2014) and canola (Tan, Mailer, Blanchard, & Agboola, 2014), there is a limited number of such studies on flaxseed proteins. In fact, to the best of our knowledge, there is no literature information on the effects of pH changes on the structural, physicochemical and functional properties of flaxseed albumin and globulin proteins. Thus, given the structural changes (usually mediated by environmental perturbants) that food proteins undergo during

processing, the aim of the current study was to understand the impact of varying pH conditions on the changes in conformation and function of flaxseed albumin and globulin protein fractions.

3.2 Materials and Methods

3.2.1 Materials

Flaxseed protein meal (FPM) defatted by solvent-free cold pressing was donated by Bioriginal Foods and Science Corporation (Saskatoon, SK, Canada) and preserved at –20 °C prior to its use for the extraction of flaxseed proteins. Spectra/Por1 dialysis membrane (Spectrum Labs) with 6-8 kDa molecular weight cut-off (MWCO) and other analytical grade reagents were purchased from Fisher Scientific (Oakville, ON, Canada) or Sigma Chemicals (St. Louis, MO, USA).

3.2.2 Extraction and isolation of protein fractions

Protein extraction from FPM, as well as subsequent fractionation into albumin and globulin proteins was carried out using a modification of previously described protein extraction methods (Aluko, 2004). FPM was mixed with 0.5 M NaCl solution (5% w/v, protein wt basis) and stirred continuously for 1 h at room temperature followed by centrifugation (7000 x *g*, 30 min at 4 °C). The resultant supernatant was filtered using cheesecloth (Fisher Scientific, 6665-18) while the precipitate was discarded. Dialysis of the filtrate against ultrapure (Milli-Q®, Millipore Corporation) water was carried out using a 6-8 kDa MWCO dialysis membrane for 96 h at 4 °C with 12 changes of water. The content of the dialysis membrane tubing was subsequently centrifuged (7000 x *g*, 30 min at 4 °C) and the resultant supernatant was recovered as the albumin fraction. The precipitate was washed with deionized water to remove any contaminating albumin and centrifuged again to collect a precipitate, which was then labelled as the globulin fraction. Both the albumin and globulin fractions were freeze-dried and preserved at –20 °C until

required for further tests. The modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978) was employed in determining protein content of the albumin and globulin fractions.

3.2.3 Proximate composition analysis

The moisture, dry matter, crude protein, and ash contents of the albumin and globulin samples were determined using relevant methods of the Association of Official Analytical Chemists (Horwitz & Latimer, 2005),while their crude fibre and fat contents were analyzed according to the methods of the American Oil Chemists' Society (Mehlenbacher et al., 2009).

3.2.4 Determination of amino acid composition

The amino acid composition of the albumin and globulin proteins was analyzed using an HPLC system with a Pico-Tag column after hydrolyzing the samples with 6 M HCl for 24 h (Bidlingmeyer, Cohen, & Tarvin, 1984). A separate digestion with performic acid was carried out in order to enable the determinations of methionine and cysteine (Gehrke, Wall, & Absheer, 1985), while the tryptophan content was determined following hydrolysis with NaOH (Landry & Delhaye, 1992).

3.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polypeptides present in the freeze-dried albumin and globulin fractions were separated using a previously described SDS-PAGE method (Aluko & McIntosh, 2004). Each protein sample was dispersed to a final concentration of 10 mg/mL in Tris-HCl buffer pH 8.0 that contained SDS (10 %, w/v) for non-reducing condition or SDS plus mercaptoethanol (10, v/v), for reducing condition. The dispersed samples were heated at 95 °C for 10 min, cooled, centrifuged (16000 x *g*, 24 °C, 10 min) and the supernatant saved. A 1 µL aliquot of the supernatant of each sample was then separately loaded onto PhastGel® 10-15 % gradient gels (GE Healthcare, Montreal,

Canada) before initiating the electrophoretic separation of the proteins using a PhastSystem Separation and Development unit (GE Healthcare, Montreal, Canada). The gels were stained with Coomassie Brilliant Blue while a blend of protein standards (Promega) ranging in size from 10 to 225 kDa was used as the molecular weight marker.

3.2.6 Surface hydrophobicity (So)

The method previously described by Wu, Hettiarachchy, and Qi (1998) was used with slight modifications to determine the S_o of albumin and globulin samples. Sample stock solutions were prepared by mixing 10 mg/mL samples dispersions in 0.1 M sodium phosphate buffer (pH 3.0, 5.0, 7.0, or 9.0) for 30 min followed by centrifugation (16000 x *g*, 24 $^{\circ}$C, 30 min) and the supernatant saved. After determining protein contents (Markwell et al., 1978), the stock solution supernatants were serially diluted to final concentrations of 0.005–0.025 % (w/v) in the appropriate corresponding buffer. A 20 µL aliquot of the aromatic fluorescent probe, 8-Anilino-1-naphthalenesulfonic acid (ANS) prepared to a final concentration of 8 mM in 0.1 M sodium phosphate buffer was then added to 4 mL of each serially diluted sample solution. Thereafter, the fluorescence intensity (FI) of each mixture was measured at 390 nm (excitation) and 470 nm (emission) using a FP-6300 spectrofluorimeter (Jasco Inc., Tokyo, Japan). The initial slope of FI against sample concentration plot (calculated by linear regression analysis) was then used as a measure of surface hydrophobicity.

3.2.7 Protein solubility (PS)

The protocol earlier described by Malomo and Aluko (2015) was used to determine the protein solubility of albumin and globulin fractions. Each of the samples was suspended in 0.1 M sodium phosphate buffer (1% w/v, protein basis) at various pH (3.0, 5.0, 7.0, or 9.0), briefly

vortexed, and then centrifuged (10,000 x *g*, 10 min, 25 ^{o}C) before determining the protein content of the supernatant using the modified Lowry method (Markwell et al., 1978). In order to determine total soluble protein content (control), the samples were each dispersed in 0.1 M NaOH (1% w/v, protein basis), vortexed and centrifuged as earlier described before determination of protein content (Markwell et al., 1978). Protein solubility was then calculated as:

$$\frac{protein\ content\ of\ supernatant\ (\frac{mg}{mL})}{total\ protein\ content\ (\frac{mg}{mL})}\ x\ 100\ \%$$

3.2.8 Emulsion formation and determination of oil droplet size

A slight modification of the protocol earlier described by Aluko, Mofolasayo, & Watts (2009) was used to prepare oil-in-water emulsions of the albumin and globulin protein samples. Samples were prepared by mixing 50, 125 or 250 mg (protein weight basis) of each sample with 5 mL of 0.1 M sodium phosphate buffer pH 3.0, 5.0, 7.0, or 9.0 and 1 mL of pure canola oil. Homogenization (20000 rpm for 1 min) of each oil/water mixture was carried out with the aid of a Polytron PT 3100 homogenizer to which a 20 mm non-foaming shaft had been attached. A Mastersizer 2000 particle size analyzer (Malvern Instruments Ltd., Malvern, U.K.) was used to measure mean oil droplet size ($d_{3,2}$) of the emulsions while ultrapure water was used as a dispersant. Samples were carefully transferred (drop-wise) with the aid of a transfer pipet (under steady shearing) into about 100 mL of ultrapure water contained in the small volume wet sample dispersion component (Hydro 2000S) of the particle size analyzer until a sufficient level of obscuration was achieved. After taking triplicate readings, the emulsions were stored at room temperature without agitation for 30 min; the mean particle diameter was recorded again in order to determine the emulsion stability (ES), which was calculated as follows:

$$\frac{initial\ mean\ oil\ droplet\ size\ at\ time\ zero}{mean\ oil\ droplet\ size\ at\ 30\ min}\ x\ 100$$

3.2.9 Statistical analysis

Triplicate sample preparations and measurements were used to obtain values which are reported as mean ± standard deviation. The statistics software SPSS was used to perform one-way ANOVA and values were tested for statistical significance ($p < 0.05$) using Duncan's multiple range test.

3.3 Results and discussion

3.3.1 Proximate composition of flaxseed albumin and globulin proteins

The proximate composition data of the albumin and globulin proteins are shown in Table 3.1A. The significantly ($p < 0.05$) lower moisture content of globulin suggests that it is more likely to maintain its quality than the albumin fraction when both fractions are placed in long term storage. Crude protein content of the globulin fraction was also found to be significantly ($p < 0.05$) higher than that of albumin, consistent with previous studies of lentil and pea proteins, which have also reported higher protein contents for globulin in comparison to albumin (Boye, Zare, & Pletch, 2010). It has been suggested that albumin's lower protein content could be as a result of its higher carbohydrate content since albumins are usually rich in glycoproteins (Ajibola, Malomo, Fagbemi, & Aluko, 2016). The significantly ($p < 0.05$) higher content of non-fibre carbohydrates in the albumin is consistent with the glycoprotein nature of this protein fraction when compared to the globulin. However, the albumin had significantly higher percentage of crude fibre but lower fat content. The higher content of non-fibre carbohydrates coupled with lower fat content in the albumin may have contributed to higher moisture contents since sugars can readily attract water molecules. In contrast, globulin had a higher fat content but

lower content of non-fibre carbohydrates, which may have contributed to the lower water retention during extraction. Since ash is the mineral-rich residue obtained after the burning of samples, the significantly ($p < 0.05$) higher ash content of the albumin fraction suggests it contains a greater quantity of mineral elements when compared to the globulin.

Table 3.1A. Proximate composition of flaxseed albumin and globulin proteins[1]

Parameter	Albumin (%)	Globulin (%)
Moisture content	10.76 ± 0.07^{a}	7.88 ± 0.15^{b}
Crude Protein	54.12 ± 1.99^{a}	62.78 ± 3.97^{b}
Crude Fibre	4.08 ± 0.59^{a}	3.14 ± 0.11^{b}
Fat	1.70 ± 0.15^{a}	7.46 ± 0.08^{b}
Ash	3.89 ± 0.52^{a}	2.38 ± 0.28^{b}
Non-fibre carbohydrates[2]	25.45 ± 0.71^{a}	16.36 ± 4.91^{b}

[1]Values represent mean ± std dev from replicate determinations.
[2]By difference

3.3.2 Amino acid composition

The amino acid compositions of both protein fractions are generally comparable, with the exception of a few major differences (Table 3.1B). For instance, although the methionine content of albumin is about half that of the globulin sample, the former has almost thrice the cysteine content of globulin. Given the role of sulfur-containing amino acids (SCAA) such as cysteine and methionine residues in protecting against oxidative damage (Elias, McClements, & Decker, 2005), the albumin sample appears to have a better potential for use as an antioxidant in food systems. The protein fractions were found to contain high levels of branched-chain amino acids (BCAAs), which may be important for development of antihypertensive agents, given our previous report of the *in vitro* activity modulation of hypertension-inducing enzymes such as

angiotensin converting enzyme and renin by a BCAA-enriched flaxseed protein hydrolysate fraction (Udenigwe & Aluko, 2010). However, the amount of BCAAs in the albumin protein used in this work is lower than that reported for defatted flaxseed meal (Nwachukwu et al., 2014), while the globulin BCAA content is marginally higher. The albumin and globulin fractions had high levels of negatively charged/acidic amino acid residues (including glutamic acid, glutamine, aspartic acid, and asparagine). This is important because asparagine and glutamine from food proteins are known to be stored in tissues where they serve to provide a steady supply of amino groups when required for the body's metabolic processes (Vasconcelos, et al., 2010). Additionally, glutamine is associated with increased liver glycogen synthesis and improved net skeletal muscle protein balance (Franzese, 2000). NCAAs such as glutamic and aspartic acids have excess electrons, which enhance iron reducing properties for the prevention of iron-induced lipid oxidation and could contribute to prevention of oxidative damage to foods (Udenigwe & Aluko, 2011). Therefore, the higher levels of SCAA and NCAA suggest a better antioxidant role in foods for albumin than the globulin. The higher arginine/lysine ratio in the globulin (3.16) compared to the albumin (2.49) could confer a comparative advantage on the former because high Arg/Lys ratios have been linked with lower blood cholesterol levels and enhanced cardiovascular health (Giroux, Kurowska, Freeman, & Carroll, 1999).

Table 3.1B. Percent amino acid composition of flaxseed protein fractions

Amino acid	Albumin	Globulin
Asx	8.53	11.04
Thr	3.00	3.53
Ser	4.94	5.31
Glu	26.60	19.76
Pro	4.04	5.22
Gly	7.74	5.50
Ala	3.54	4.79
Cys	3.08	1.16

Val	4.03	5.16
Met	0.64	1.21
Ile	3.36	4.16
Leu	5.38	6.12
Tyr	1.88	2.68
Phe	3.95	5.60
His	2.12	2.76
Lys	4.47	3.57
Arg	11.13	11.28
Trp	1.57	1.14
AAA	7.41	9.42
BCAA	12.77	15.44
HAA	31.48	37.25
NCAA	35.13	30.81
PCAA	17.72	17.60
SCAA	3.72	2.38

Asx= aspartic acid + asparagine; Glx= glutamic acid + glutamine; AAA= aromatic amino acids; BCAA= branched-chain amino acids; HAA= hydrophobic amino acids; NCAA= negatively charged amino acids; PCAA = positively charged amino acids; SCAA = sulfur-containing amino acids.

3.3.3 SDS-PAGE

In general, the SDS-PAGE separation suggests that the globulin has more polypeptides than the albumin under reducing and non-reducing conditions (Fig. 3.1.). The non-reducing (without β-mercaptoethanol) SDS-PAGE profiles in Fig. 3.1A show a marked difference in the size of polypeptides present in the albumin and globulin samples. While there were major bands corresponding to polypeptides of about 50, 45 and 10 kDa in size, as well as minor bands of approximately 20 and 30 kDa in the globulin sample, only one intense band corresponding to 10 kDa and a minor band corresponding to ~45 kDa could be observed for albumin. The presence of multiple polypeptides may have contributed to higher protein content in the globulin. Under reducing condition, the reduced globulin protein showed intense new polypeptide bands that correspond to 30, 20 kDa in addition to the 10 kDa polypeptide present in the non-reduced gel (Fig. 3.1B). In contrast, the albumin had only the minor 20 kDa polypeptide band in addition to

the main 10 kDa polypeptide. The results suggest that the 10 kDa polypeptide, which is common to both protein fractions, may be devoid of disulfide bonds since there was no reduction in intensity after addition of mercaptoethanol. In contrast, the higher molecular weight polypeptides in globulin (50 and 45 kDa) and albumin (45 kDa) contained disulfide bonds that were broken by the mercaptoethanol to yield smaller polypeptides. The results are different from those reported for hemp seed where the albumin had several polypeptides under non-reducing and reducing conditions (Malomo & Aluko, 2015). However, the presence of new polypeptide bands after addition of mercaptoethanol was evident mainly in the hemp seed globulin but not the albumin.

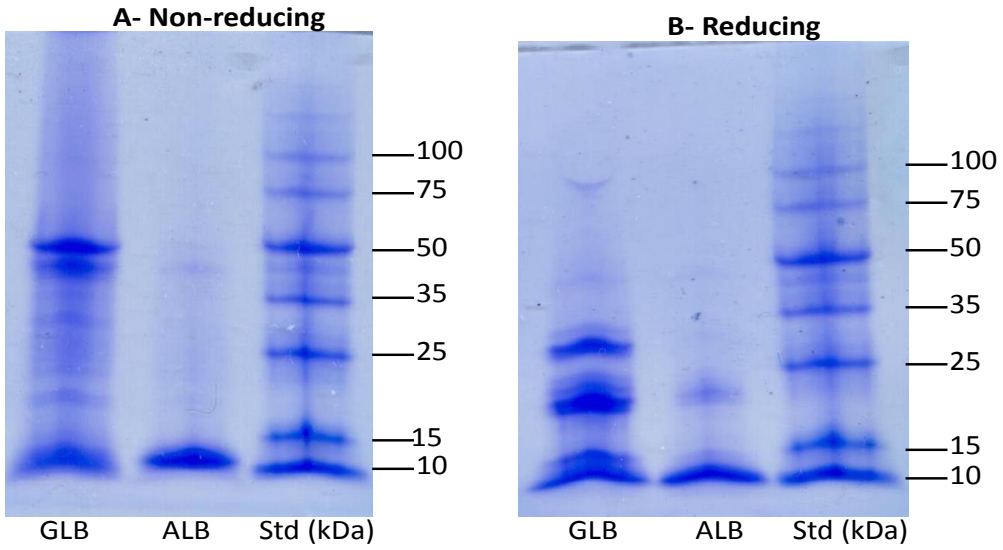


Fig. 3.1. SDS-PAGE profiles of flaxseed albumin (ALB) and globulin (GLB) proteins under (A) non-reducing and (B) reducing conditions.

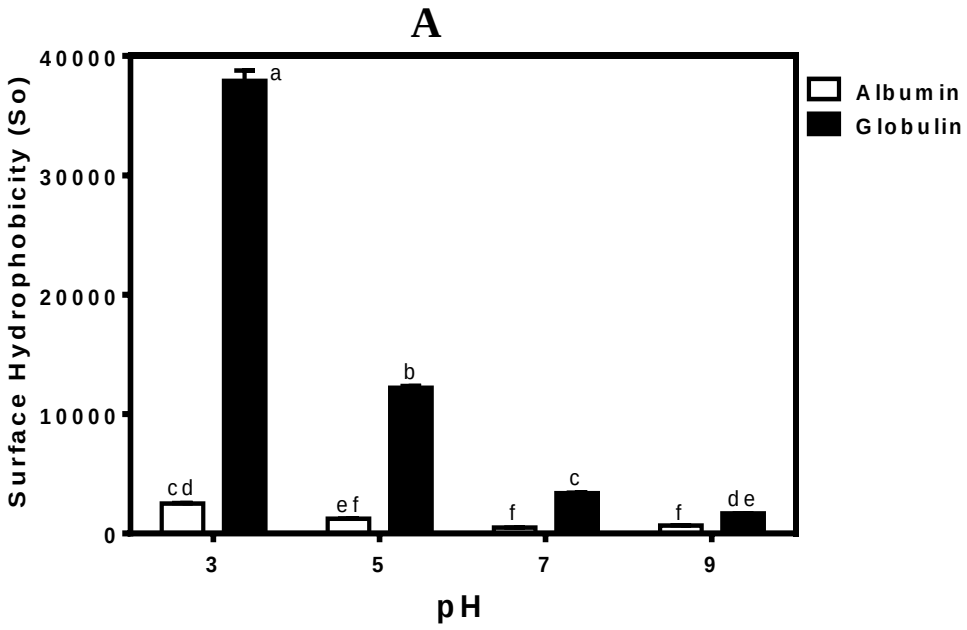
3.3.4 Surface hydrophobicity (So) and protein solubility

Fig. 3.2A shows significantly higher ($p < 0.05$) S_o of the globulin when compared to that of the albumin at each of the four pH conditions (3.0-9.0). The results suggest a protein conformation that exposes more hydrophobic patches in the globulin more than in the albumin. The S_o of both proteins decreased progressively as the pH changed from acidic through neutral to basic, which suggests the influence of protein charge. This is because the ANS probe also has an acidic group

in addition to the main hydrophobic ring. At pH 3, the effect of charge is less since the protein and ANS groups will be protonated and interaction depended mainly on hydrophobicity, hence the higher overall S_o. In contrast, at values above pH 3, there is increased tendency for ionization of acidic groups in the ANS and protein, which will contribute to enhanced repulsion and hence reduced ANS binding (lower S_o). The results obtained in this work are similar to those reported for whey proteins and peptides where ANS hydrophobicity was highest at pH 3 but decreased at pH 5, 7, and 9 (Alizadeh-Pasdar & Li-Chan, 2000). The significantly higher S_o values for globulin is consistent with the presence of higher levels of hydrophobic amino acids and lower levels of negatively-charged amino acids as shown in Table 3.1B. The S_o of polypeptide chains is a measure of the degree to which they adhere to one another in an aqueous environment, and has been shown to greatly affect their structure as well as functional properties (Alizadeh-Pasdar & Li-Chan, 2000). The observed pH-dependent change in S_o illustrates the modification in protein structure and function that occurs following the disruption of hydrogen bonds as a result of changes in alkalinity or acidity of the protein environment.

The results in Fig. 3.2B show marked differences in solubility between the albumin and globulin protein isolates with the albumin being more soluble ($p < 0.05$) over the pH 3-9 range than the globulin. In general, solubility was observed to increase with increasing pH for albumin while the globulin had a minimal solubility at pH 5. The greater solubility seen at higher pH could be as a result of an increase in net protein charge with rising pH, while the observation that globulin was least soluble at pH 5 may be as a result of the proximity to the protein isoelectric point (Wang, Xu, Li, & Zhang, 2015; Wani, Sogi, Singh, Wani, & Shivhare, 2011). The lower contents of hydrophobic and aromatic amino acids in addition to the significantly ($p < 0.05$) higher levels of negatively-charged amino acids and non-fibre carbohydrates may have

contributed to the better solubility properties of albumin. Moreover, the albumin protein is mostly dominated by low molecular weight polypeptides, which would enhance structural flexibility and solubility when compared to the bigger polypeptides present in the globulin (Fig. 3.1.). The results are consistent with previous reports of higher albumin solubility than the globulin fraction of plant proteins (Wang et al., 2015; Wani et al., 2011).



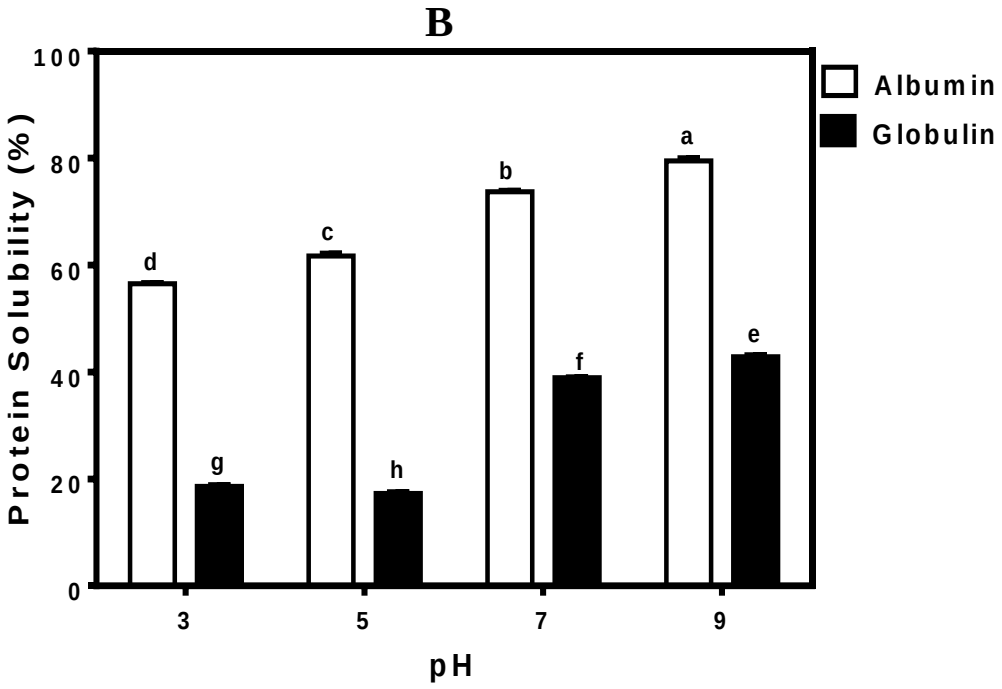


Fig. 3.2. Effect of pH on the (A) surface hydrophobicity and (B) solubility of flaxseed albumin and globulin proteins.

3.3.5 Emulsion formation and stability

The effects of pH and sample concentration on the mean oil droplet size of emulsions formed and stabilized by flaxseed albumin and globulin proteins are shown in Fig. 3.3. Since protein quantity in the continuous phase is an important determinant of interfacial protein membrane formation during emulsion preparation (Romero et al., 2011), different protein concentrations were used to represent low, medium, and high protein levels. In general, increased sample concentration led to improved emulsification capacity at all pH values for both proteins as evidenced by reduced oil droplet size as the sample concentration increased from 10 to 50 mg/mL. The results are consistent with increased protein availability within the continuous phase, which contributes to interfacial membrane formation that is necessary for oil droplet encapsulation. The results are in contrast to the increased emulsion mean oil droplet sizes that

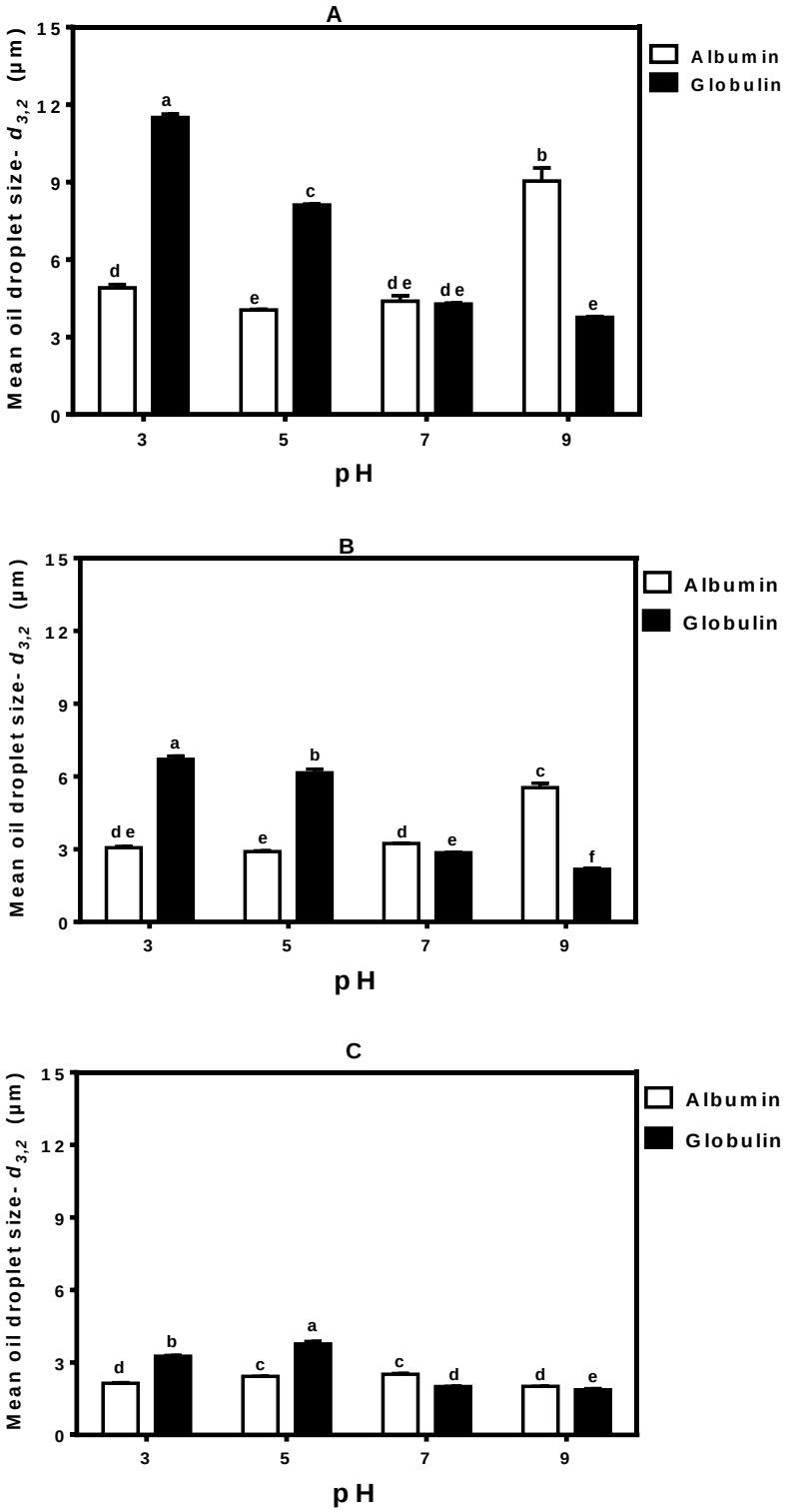
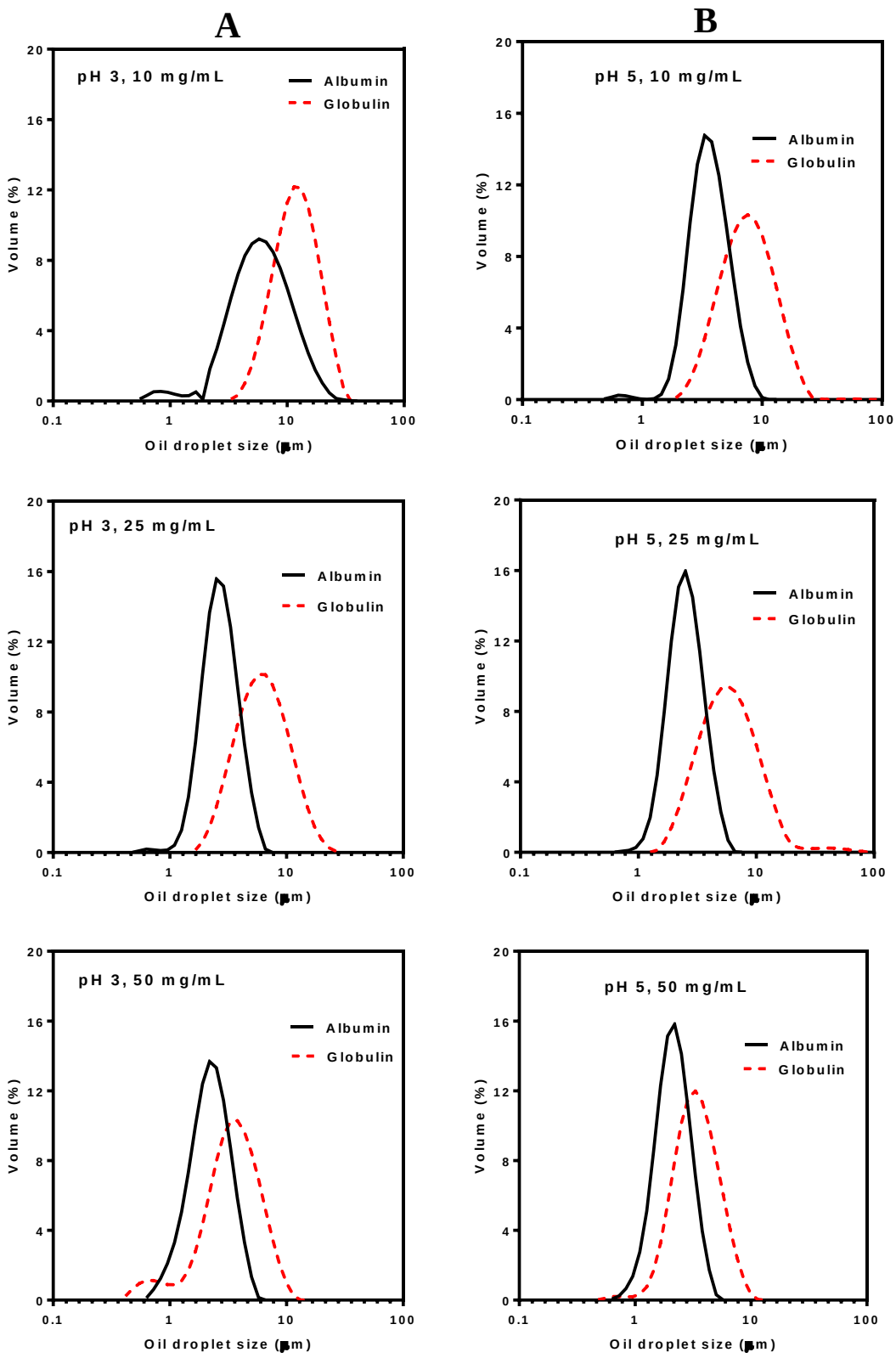


Fig. 3.3. Oil droplet sizes of emulsions formed by flaxseed albumin and globulin proteins at different pH values: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations.

accompanied protein concentration increases for African yam bean globulin (Ajibola et al., 2016). However, with the exception of pH 9, the poorer emulsifying ability (bigger oil droplet sizes) of flaxseed globulin when compared to albumin emulsions are consistent with the African yam bean data. Apart from the 10 mg/mL concentration at pH 7 where neither sample concentration nor pH seemed to have any differential effect on the emulsification ability of the two samples, significant differences in emulsification capacity between the samples were recorded at every other point. At 10 mg/mL, a progressive improvement in the emulsifying ability of globulin with increase in pH was observed, while the albumin was a slightly better emulsifier in neutral and acidic conditions. For the albumin at the 10 mg/mL sample concentration, the most beneficial effect on emulsification was produced at pH 5 and 7, while the globulin was a more effective emulsifier at pH 9.0. In general, the globulin isolate proved to be a better emulsifier at alkaline conditions, while the albumin isolate was found to be a better emulsifier at pH 3 and 5.

Fig. 3.4A-D illustrate the oil droplet size distribution of emulsions formed with albumin and globulin proteins. The size distributions were mainly monomodal, which indicates uniform distribution and better emulsion quality than lentil protein isolate samples which exhibited largely bimodal droplet sizes (Primozic, Duchek, Nickerson, & Ghosh, 2017). At each pH value, the range of oil droplets sizes became narrower and moved towards the lower sizes as the protein concentration increased from 10 mg/mL to 50 mg/mL, which is consistent with the decreases in average oil droplet sizes reported in Fig. 3.3. The narrower size distribution ranges for the albumin emulsions at pH 3 and 5 (Fig. 3.4A and B) are consistent with the lower mean oil droplet sizes reported in Fig. 3.3. At pH 7, the oil droplet


A
B
pH 3, 10 mg/mL
pH 5, 10 mg/mL
pH 3, 25 mg/mL
pH 5, 25 mg/mL
pH 3, 50 mg/mL
pH 5, 50 mg/mL
Albumin
Globulin
Volume (%)
Oil droplet size (μm)
0
4
8
12
16
20
0.1
1
10
100

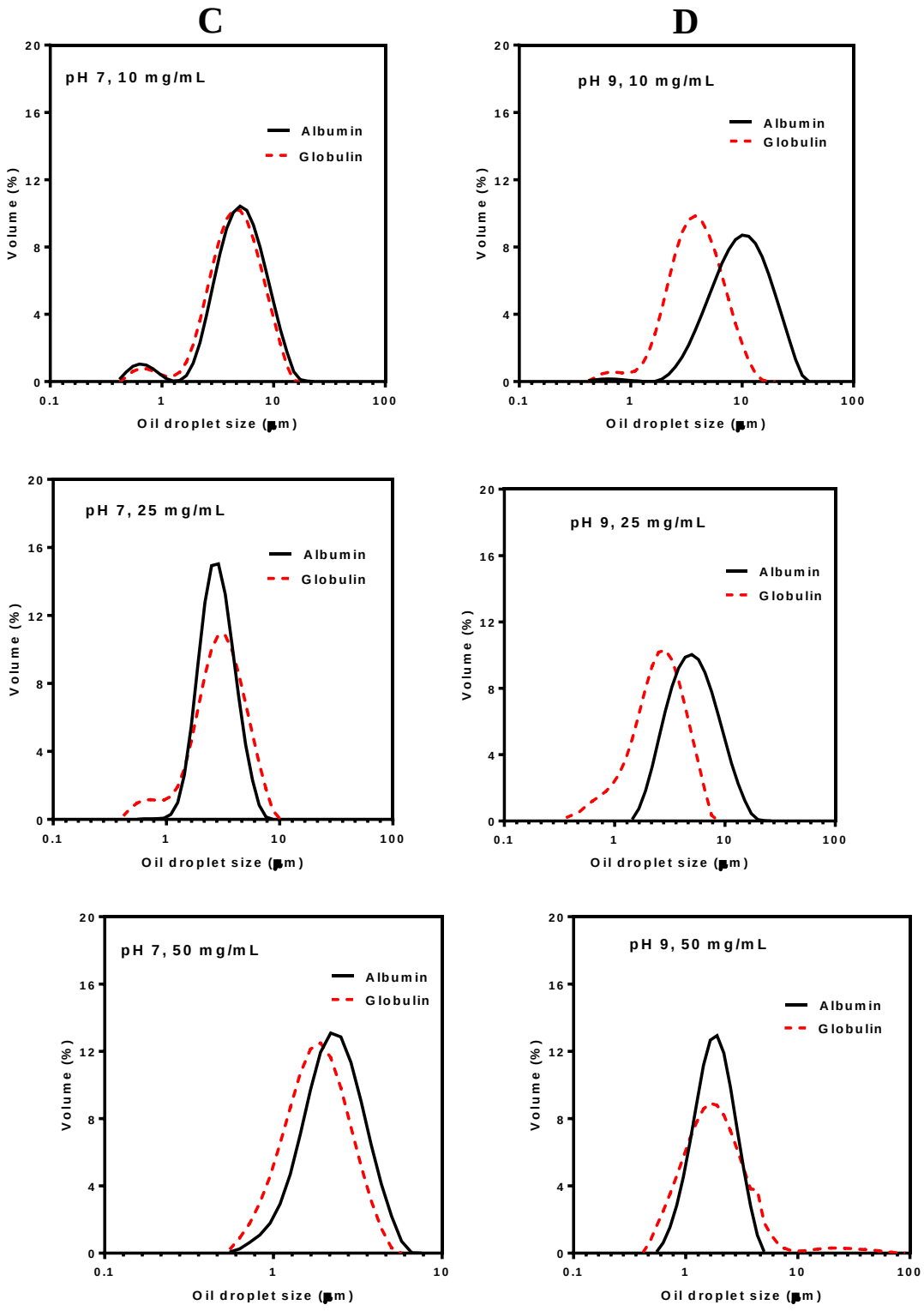


Fig. 3.4. Oil droplet size distribution of emulsions stabilized by flaxseed albumin and globulin proteins at (A) pH 3 and 10, 25 or 50 mg/mL (B) pH 5 and 10, 25 or 50 mg/mL (C) pH 7 and 10, 25 or 50 mg/mL (D) pH 9 and 10, 25 or 50 mg/mL protein concentrations.

size distribution was similar (Fig. 3.4C), which reflects the similarity in mean oil droplet sizes for albumin and globulin emulsions. However at pH 9, the albumin emulsions had wider size distributions for 10 and 25 mg/mL protein concentrations (Fig. 3.4D), which reflect poorer emulsions and consistent with the higher mean oil droplet sizes when compared to the globulin.

The high emulsion stability of the globulin isolate at pH 3, 7, and 9.0 for the 10 and 25 mg/mL sample concentrations was dramatically reversed when protein concentration was increased to 50 mg/mL (Fig. 3.5). The results suggest protein crowding at the 50 mg/mL concentration, which may have led to increased protein charge and viscosity of the continuous phase. The high level of charges would increase protein-protein repulsions while increased viscosity reduces the rate of polypeptide translocation to the oil-water interface, both of which will reduce interfacial membrane strength and cause increased potential for rupture and oil droplet coalescence. Increased protein concentration did not alter the albumin emulsion stability, except at 25 mg/mL and pH 9, which suggest less influence of protein crowding in comparison to the globulin. The reduced negative effect of high protein concentration on albumin emulsion stability may be attributed to the higher protein solubility, which will prevent formation of a viscous continued phase, hence the potential for stronger interfacial membrane formation. The results are different from those reported for hemp seed globulin, which showed no negative effect on emulsion stability when protein concentration was increased to 50 mg/mL (Malomo & Aluko, 2015). The differences may be due to higher solubility of hemp seed globulin at pH 7 and 9, which may have reduced the negative effect of high protein concentration that arises from increased viscosity.

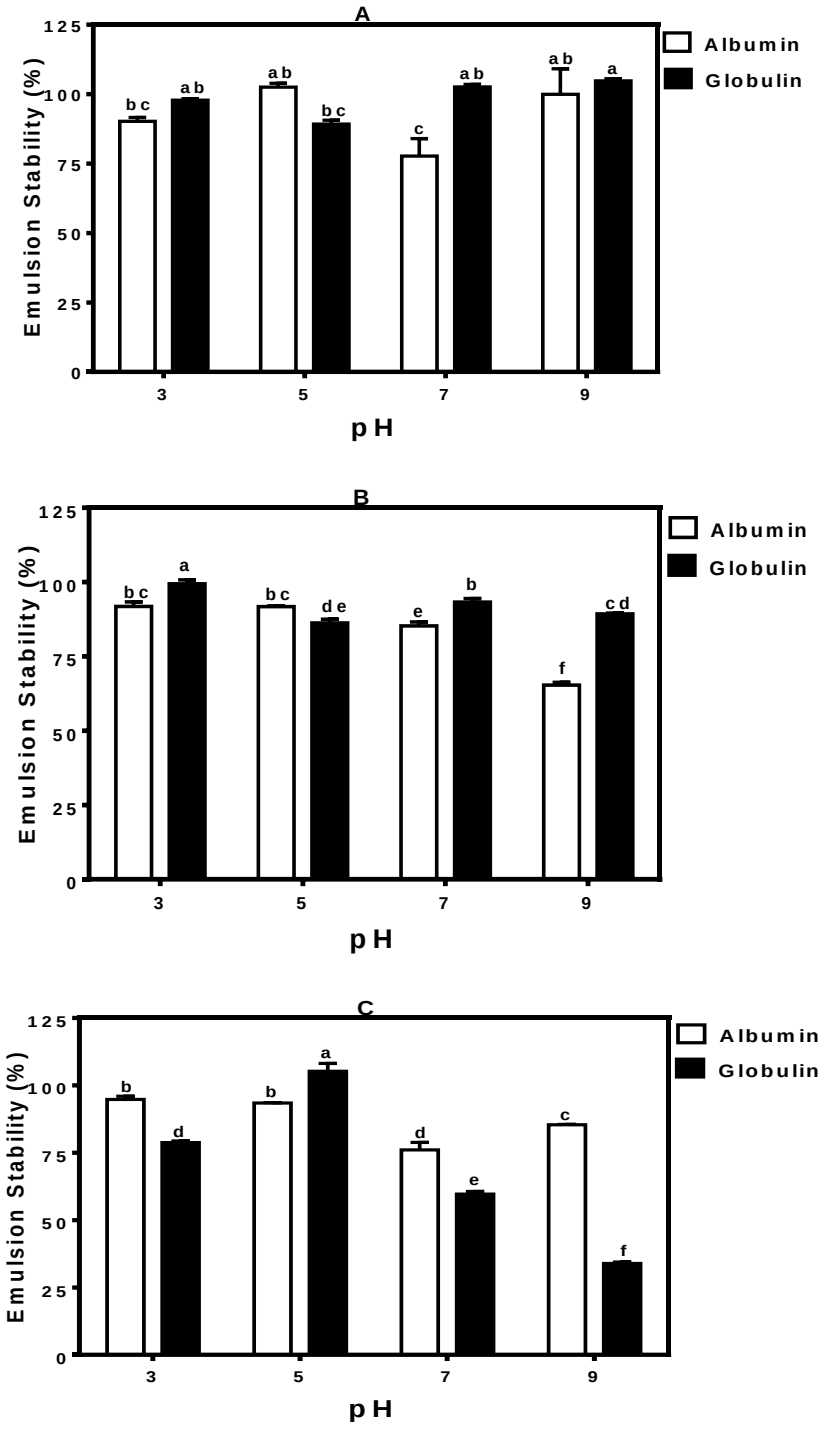


Fig. 3.5. Effect of pH on the emulsifying stability (percent increase in oil droplet size) of flaxseed albumin and globulin proteins at different concentrations: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentration.

Apart from the relatively low stability of the globulin emulsion at 50 mg/mL (pH 7.0 and 9.0), and that of the albumin isolate at 25 mg/mL (pH 9.0), the samples generally demonstrated high emulsion stability (over 80%), thus highlighting their potential for use as ingredients in the formulation of food emulsions where short-term stability is required (Aluko et al., 2009).

3.4 Conclusions

The results from this study suggest that the specific alteration of protein environment during processing or preparation of food products could lead to changes in structural conformation, which will ultimately affect food product functionality. This is because albumin and globulin proteins showed differential functionalities at the same pH. These differences are partly as a result of differences in their amino acid composition and structural conformations. For example, the presence of high molecular weight polypeptides may have contributed to reduced globulin solubility when compared to albumin which had smaller polypeptides and higher solubility. Additionally, protein concentration was also shown to have an effect on functionality as exemplified by the sharp contrast in emulsifying properties at pH 9.0 where high protein concentrations contributed to better emulsion formation but decreased emulsion stability. In general, the results support a conclusion that flaxseed albumin and globulin proteins hold great promise for use as functional food ingredients. Additionally, the use of underutilized and inexpensive plant proteins such as those from flaxseed for the formulation of food products has the potential for increasing their value added utilization and economic value, in addition to contributing to food security.

3.5 Acknowledgements

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3.6 Conflicts of interest

The authors declare no conflict of interest.

3.7 References

Ajibola, C. F., Malomo, S. A., Fagbemi, T. N., & Aluko, R. E. (2016). Polypeptide composition and functional properties of African yam bean seed (*Sphenostylis stenocarpa*) albumin, globulin and protein concentrate. *Food Hydrocolloids, 56*, 189-200.

Alizadeh-Pasdar, N., & Li-Chan, E. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry, 48*, 328-334.

Aluko, R. E. (2004). The extraction and purification of proteins: an introduction. In R. Yada, (Ed.), *Proteins in Food Processing* (pp. 323-351). Cambridge, England: Woodhead Publishing.

Aluko, R. E., & McIntosh, T. (2004). Electrophoretic and functional properties of mustard seed meals and protein concentrates. *Journal of the American Oil Chemists' Society*, *81*, 679-683.

Aluko, R. E., Mofolasayo, O. A., & Watts, B. M. (2009). Emulsifying and foaming properties of commercial yellow pea (*Pisum sativum* L.) seed flours. *Journal of Agricultural and Food Chemistry, 57*, 9793-9800.

Amagloh, F. K., Mutukumira, A. N., Brough, L., Weber, J. L., Hardacre, A., & Coad, J. (2013). Carbohydrate composition, viscosity, solubility, and sensory acceptance of sweet potato- and maize-based complementary foods. *Food & Nutrition Research, 57:* 18717.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography B: Biomedical Sciences and Applications, 336*, 93-104.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*, 414-431.

Doyen, A., Udenigwe, C. C., Mitchell, P. L., Marette, A., Aluko, R. E., & Bazinet, L. (2014). Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. *Food Chemistry, 145*, 66-76.

Elias, R. J., McClements, D. J., & Decker, E. A. (2005). Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase ß-lactoglobulin in oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 53*, 10248-10253.

Franzese, T. A. (2000). Medical nutrition therapy for rheumatic disorders. In L.K. Mahan, & S. Escortt-Stump (Eds.), *Krause's Food, Nutrition & Diet Therapy* (pp. 970-986). Philadelphia, PA: W.B. Saunders.

Gehrke, C. W., Wall Sr, L. L., & Absheer, J. S. (1985). Sample preparation for chromatography of amino acids: acid hydrolysis of proteins. *Journal of the Association of Official Analytical Chemists, 68*, 811-821.

Giroux, I., Kurowska, E. M., Freeman, D. J., & Carroll, K. K. (1999). Addition of arginine but not glycine to lysine plus methionine-enriched diets modulates serum cholesterol and liver phospholipids in rabbits. *Journal of Nutrition, 129*, 1807-1813.

Hall III, C., Tulbek, M. C., & Xu, Y. (2006). Flaxseed. In S. L. Taylor (Ed.), *Advances in Food and Nutrition Research*, vol. 51 (pp. 1-97). Academic Press.

Horwitz, W., & Latimer, G. W. (2005). Official methods of analysis of AOAC International. (18th ed.). Gaithersburg, Md. : AOAC International.

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. *Food Research International, 44*, 2991-2998.

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry, 40*, 776-779.

Malomo, S. A., & Aluko, R. E. (2015). A comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions. *Food Hydrocolloids, 43*, 743-752.

Marambe, H. K., Shand, P. J., & Wanasundara, J. P. D. (2013). *In vitro* digestibility of flaxseed (*Linum usitatissimum* L.) protein: effect of seed mucilage, oil and thermal processing. *International Journal of Food Science and Technology, 48*, 628-635.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry, 87*, 206-210.

Mehlenbacher, V. C., Sallee, E. M., Hopper, T. H., Link, W. E., Walker, R. O., & Firestone, D. (2009). Official methods and recommended practices of the AOCS. *Urbana, Illinois: AOCS Press*.

Niu, H., Li, Y., Han, J., Liu, Q., & Kong, B. (2017). Gelation and rheological properties of myofibrillar proteins influenced by the addition of soybean protein isolates subjected to an acidic pH treatment combined with a mild heating. *Food Hydrocolloids, 70, 269-276*.

Nwachukwu, I. D., Girgih, A. T., Malomo, S. A., Onuh, J. O., & Aluko, R. E. (2014). Thermoase-derived flaxseed protein hydrolysates and membrane ultrafiltration peptide fractions have systolic blood pressure-lowering effects in spontaneously hypertensive rats. *International Journal of Molecular Sciences, 15*, 18131-18147.

Primozic, M., Duchek, A., Nickerson, M., & Ghosh, S. (2017). Effect of lentil proteins isolate concentration on the formation, stability and rheological behavior of oil-in- water nanoemulsions. *Food Chemistry, 237*, 65-74.

Romero, A., Beaumal, V., David-Braind, E., Cordobes, F., Guerrero, A., & Anton, M. (2011). Interfacial and oil/water emulsions characterization of potato protein isolates. *Journal of Agricultural and Food Chemistry, 59*, 9466–9474.

Rondeau, P., & Bourdon, E. (2011). The glycation of albumin: structural and functional impacts. *Biochimie, 93*, 645-658.

Shevkani, K., Singh, N., Kaur, A., & Rana, J. C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: a comparative study. *Food Hydrocolloids, 43*, 679-689.

Tan, E. S., Ying-Yuan, N., & Gan, C. Y. (2014). A comparative study of physicochemical characteristics and functionalities of pinto bean protein isolate (PBPI) against the soybean protein isolate (SPI) after the extraction optimisation. *Food Chemistry, 152*, 447-455.

Tan, S. H., Mailer, R. J., Blanchard, C. L., & Agboola, S. O. (2014). Emulsifying properties of proteins extracted from Australian canola meal. *LWT - Food Science and Technology, 57*, 376-382.

Timilsena, Y. P., Adhikari, R., Barrow, C. J., & Adhikari, B. (2016). Physicochemical and functional properties of protein isolate produced from Australian chia seeds. *Food Chemistry, 212*, 648-656.

Udenigwe, C. C., & Aluko, R. E. (2010). Antioxidant and angiotensin converting enzyme-inhibitory properties of a flaxseed protein-derived high fischer ratio peptide mixture. *Journal of Agricultural and Food Chemistry, 58*, 4762-4768.

Udenigwe, C. C., & Aluko, R. E. (2011). Chemometric Analysis of the Amino Acid Requirements of Antioxidant Food Protein Hydrolysates. *International Journal of Molecular Sciences, 12*, 3148-3161.

Vasconcelos, I. M., Maia, F. M. M., Farias, D. F., Campello, C. C., Carvalho, A. F. U., de Azevedo Moreira, R., & de Oliveira, J. T. A. (2010). Protein fractions, amino acid composition and antinutritional constituents of high-yielding cowpea cultivars. *Journal of Food Composition and Analysis, 23*, 54-60.

Wang, C., Xu, F., Li, D., & Zhang, M. (2015). Physico-chemical and structural properties of four rice bran protein fractions based on the multiple solvent extraction method. *Czech Journal of Food Science, 33*, 283-291.

Wani, A. A., Sogi, D. S., Singh, P., Wani, I. A., & Shivhare, U. S. (2011). Characterisation and functional properties of watermelon (*Citrullus lanatus*) seed proteins. *Journal of the Science of Food and Agriculture, 91*, 113.

Wouters, A. G. B., Rombouts, I., Fierens, E., Brijs, K., & Delcour, J. A. (2016). Relevance of the functional properties of enzymatic plant protein hydrolysates in food systems. *Comprehensive Reviews in Food Science and Food Safety, 15*, 786-800.

Wu, W. U., Hettiarachchy, N. S., & Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. *Journal of the American Oil Chemists' Society, 75*, 845-850.

Xu, Y., Hall III, C., & Wolf-Hall, C. (2008). Antifungal activity stability of flaxseed protein extract using response surface methodology. *Journal of Food Science, 73*, M9-M14.

Yin, S.-W., Tang, C.-H., Cao, J.-S., Hu, E.-K., Wen, Q.-B., & Yang, X.-Q. (2008). Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (*Cannabis sativa* L.) protein isolate. *Food Chemistry, 106*, 1004-1013.

3.8 TRANSITION STATEMENT ONE

The purpose of this thesis is to characterize the functional properties of flaxseed proteins and protein hydrolysates. The functional properties of the two main flaxseed storage proteins (albumins and globulins), which were extracted with the use of dilute NaCl solution were determined in the preceding chapter. Among other findings, the study showed that increasing flaxseed protein levels led to better emulsion formation while decreasing emulsion stability. Additionally, the study found that flaxseed albumins consist of a single 10 kDa polypeptide chain whereas the globulin fraction is made up of multiple polypeptide chains in the 10-50 kDa range. Importantly, the globulin fraction exhibited a higher surface hydrophobicity across all the four pH values tested while the albumin fraction showed better emulsion forming ability. High surface hydrophobicity is known to positively correlate with greater fat binding capacity, and any food protein's capacity to bind and retain fat influences its texture and ability to interact with lipids in emulsions. Thus, this study underlines the significant role that a protein's functional properties could play in its use for food and in food product formulations. Based on the diverse polypeptide composition and amino acid composition, the work also confirms the potential of flaxseed proteins to serve as suitable substrates for the production of bioactive food protein hydrolysates. However, in order to properly characterize flaxseed-derived protein hydrolysates, which contain health-promoting bioactive peptides, accurate information on protein content is required. Since there are different literature reports regarding how to best determine the protein content of hydrolysates, there is the need to first evaluate different methods of protein quantification in a systematic manner prior to characterizing the properties of flaxseed protein hydrolysates. Thus, the next chapter (Manuscript 2) addresses the issue of accurate food protein hydrolysate measurement.

CHAPTER 4

MANUSCRIPT TWO

A SYSTEMATIC EVALUATION OF VARIOUS METHODS FOR QUANTIFYING FOOD PROTEIN HYDROLYSATE PEPTIDES

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4.0 Abstract

This work was carried out to identify accurate methods that could be recommended for the quantification of proteins in food protein hydrolysates. Following hydrolysis with 4% alcalase, the amount of protein in various hydrolysate samples was measured using seven different analytical methods. Although the data obtained using different methods varied, HPLC amino acid analysis with a Pico-Tag column indicated that the highest concentration of amino acids in the protein hydrolysates was present in the casein sample while the lowest amount of protein was found in the sample of hempseed studied. However, the amino acid analysis data was mostly positively correlated with the Dumas and Lowry methods. We conclude that where available, amino acid analysis provides the best estimate of protein content of hydrolysates but the Dumas and Lowry methods can also be recommended as alternatives.

Keywords: protein determination; ninhydrin; Bradford; Lowry; amino acid analysis; protein hydrolysates.

4.1 Introduction

Accurate protein quantification is essential to the study of protein hydrolysates in life science research. In recent years, there has been a growing interest in the use of protein hydrolysates in human and animal nutrition since they represent a source of high quality peptides with nutritional and health-promoting functions including antioxidant, blood pressure-lowering, hypolipidaemic, anticancer, opioid-like and anti-inflammatory properties (Udenigwe & Aluko, 2012; Agyei, Potumarthi & Danquah, 2015; Hou, Wu, Dai, Wang, & Wu, 2017). Additionally, protein hydrolysates have also become increasingly important in the food industry particularly in the formulation of foods for consumers with conditions such as liver disease, food allergies, and phenylketonuria (Wróblewska & Karamać, 2003). In sports nutrition, protein hydrolysates have gained increasing relevance as a result of their ability to modulate skeletal muscle protein anabolism, and are now often used by athletes to enhance recovery and influence body mass control (Wróblewska & Karamać, 2003; Manninen, 2009; Buckley, Thomson, Coates, Howe, DeNichilo, & Rowney, 2010).

A range of methods such as Bradford (Ben Khaled et al., 2014), Kjeldahl (Yousr & Howell, 2015; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017), Lowry (Nwachukwu, Girgih, Malomo, Onuh, & Aluko, 2014; Kose & Oncel, 2015), Dumas (Perreault, Hénaux, Bazinet, & Doyen, 2017), and amino acid analysis (Ghelichi, Sørensen, García-Moreno, Hajfathalian, & Jacobsen, 2017; Hall, Jones, O'Haire & Liceaga, 2017) are commonly used to determine the concentration of protein in hydrolysates. However, almost all of these common protein determination methods are known to be limited in their capacity for accurate measurement of proteins in hydrolysates. For instance, the Kjeldahl and Dumas methods have been linked with issues of poor selectivity while analysis using the Bradford method is unable to detect proteins or

peptides that are less than 3 kDa (Moore, Devries, Lipp, Griffiths, & Abernethy, 2010). Such limitations invariably result in non-uniform data across laboratories and present a real challenge to the use and study of protein hydrolysates.

Based on the foregoing, this study was carried out to measure the protein content in various food protein hydrolysates using a range of commonly used methods in order to determine the most accurate and reliable analytical method. Seven food protein hydrolysates *viz* casein protein hydrolysate (CPH), whey protein hydrolysate (WPH), soy protein hydrolysate (SPH), beef protein hydrolysate (BPH), pea protein hydrolysate (PPH), flaxseed protein hydrolysate (FPH), and hempseed protein hydrolysate (HPH) were used in this study. The protein hydrolysates were obtained using the same protein hydrolysis protocols in order to avoid sample variations that are due to differences in protease and proteolysis parameters.

4.2 Materials and Methods

4.2.1 Materials

Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), copper (II) sulphate pentahydrate, alcalase, cellulase (from *Aspergillus niger*), and β-mercaptoethanol, as well as Bradford, Ninhydrin, o-Phthaldialdehyde (OPA; incomplete) and Folin-Ciocalteau phenol reagents were procured from Sigma-Aldrich (St. Louis, MO, USA). All other analytical grade reagents except where otherwise specified were obtained from Fisher Scientific (Oakville, ON, Canada). Casein and whey protein isolate were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Arla Foods Ingredients Inc. (Basking Ridge, NJ, USA), respectively. Defatted flaxseed protein meal (FPM) and defatted hemp protein meal (HPM) were procured from Bioriginal Foods and Science Corporation (Saskatoon, SK, Canada) and Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada), respectively. Pea protein isolate (Propulse^{TM}) was obtained from Nutri-Pea

Limited (Portage la Prairie, MB, Canada). Soybean seeds and ground beef were purchased from a local grocery store.

4.2.2 Preparation of protein isolates

Flaxseed protein isolate (FPI) was prepared from the FPM using the Dev and Quensel (1988) method as modified by (Udenigwe, Lin, Hou, & Aluko, 2009). Briefly, FPM (5% w/v, protein basis) in deionized water was stirred continuously for 30 min in order to obtain a uniform suspension. The FPM suspension was then adjusted to pH 5.0 using 0.5 M HCl and heated to 37 °C, prior to the addition of cellulase (1%, w/w). The suspension was cooled to 4 °C after 4 h of cellulase-mediated fibre hydrolysis and adjusted to pH 10.0 through the step-wise addition of 0.5 M NaOH. After stirring for 1 h at 25 °C, the alkaline suspension was centrifuged at 15000 x *g* and 4 °C for 30 min. In order to initiate protein precipitation, the supernatant obtained after the centrifugation step was adjusted to pH 4.2 by slowly adding 0.5 M HCl using a transfer pipette. A second round of centrifugation was performed in order to collect the protein precipitate which was then washed thrice with acidified (pH 4.2) deionized water for the purpose of removing non-protein impurities. Finally, the precipitate was suspended in deionized water, adjusted to pH 7.0 and freeze-dried as the FPI.

Hemp protein isolate (HPI) was prepared from HPM according to a slight modification (Girgih, Udenigwe, & Aluko, 2011) of an earlier protocol (Tang, Ten, Wang, & Yang, 2006). In brief, HPM (5% w/v, protein basis) dispersed in deionized water was stirred continuously for 30 min in order to obtain a uniform suspension. To solubilize the proteins, the suspension was adjusted to pH 10.0 using 2 M NaOH, heated to 37 °C and stirred for 2 h. Thereafter, the mixture was centrifuged at 15000 x *g* and 4 °C for 60 min, and the resultant supernatant filtered with cheesecloth, while the pellet was discarded. To initiate protein precipitation, the recovered

supernatant was adjusted to pH 5.0 with 2 M HCl and stirred for 30 mins before being centrifuged (15000 x *g* and 4 °C for 40 min). The collected protein precipitate was re-dispersed in deionized water and adjusted to pH 7.0 with 2 M NaOH before being freeze-dried as the HPI. Soy protein isolate (SPI) was produced following the HPI production protocol with the key differences being that the alkaline extraction step was carried out at pH 8.0 while acid precipitation of the proteins was performed at pH 4.5 according to a previously described method (Molina, Papadopoulou, & Ledward, 2001). The precipitate was re-dispersed in deionized water, adjusted to pH 7.0 and freeze-dried. Production of each protein isolate was performed in triplicate and the freeze-dried isolates were preserved at -20 °C prior to being subjected to enzymatic hydrolysis. The beef protein was obtained by first freeze-drying the ground beef followed by defatting with 20 volumes of acetone. The defatted beef was air-dried at room temperature (~23-24 °C) in a fume hood for 24 h, ground into a powder using a Smartgrind® CBG 100SC coffee grinder (Black & Decker, Baltimore, Maryland, USA) and then stored at -20 °C.

4.2.3 Protein hydrolysis

The alcalase hydrolysate of each protein sample was prepared according to a previously described method (Li & Aluko, 2010). Briefly, hempseed, flaxseed, soy, beef, pea, whey and casein protein isolates were each dispersed in deionized water (5% w/v, protein basis) after which the resultant mixture was continuously stirred, heated to 50 °C and adjusted to pH 9.0 using 1 M NaOH. Alcalase (4% w/w, substrate protein weight basis) was then added to each sample mixture in order to initiate enzymatic hydrolysis. Temperature and pH values were maintained constant throughout the 6 h duration of the reaction by addition of 1 M NaOH when necessary. The reaction was terminated by placing each reaction vessel in a hot water bath (95

°C) and adjusting the reaction mixture to pH 4.0 in order to inactivate the enzyme. Each protein mixture was centrifuged at 15000 x *g* for 30 min and the resultant supernatant adjusted to pH 7.0. The hydrolysis of each protein isolate was performed in triplicate and the freeze-dried hydrolysates were stored at -20 °C prior to being used for further analyses.

4.2.4 Proximate composition analysis

The crude fibre and fat contents of the hydrolysates were determined according to the methods of the American Oil Chemists' Society (Mehlenbacher et al., 2009), while their ash, crude protein, dry matter, and moisture contents were measured using relevant methods of the Association of Official Analytical Chemists (Horwitz & Latimer, 2005). Nitrogen was converted to protein content using the 6.25 conversion factor.

4.2.5 Protein quantification

4.2.5.1 Amino acid analysis

The amino acid composition of each protein hydrolysate was analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) on a Pico-Tag amino acid analysis column after hydrolyzing the samples at 116 °C with 6 M HCl for 24 h (Bidlingmeyer, Cohen, & Tarvin, 1984). The samples were oxidized with performic acid in order to enable measurement of cysteine and methionine residues (Gehrke, Wall, & Absheer, 1985) while the determination of the tryptophan was carried out after alkaline hydrolysis with NaOH (Landry & Delhaye, 1992). The digests were separated on a cation exchange column (4.6 × 150 mm) using a gradient of sodium citrate buffers (pH 3.45 and pH 10.85) at a flow rate of 0.45 mL/min.

4.2.5.2 Flash 2000 N/protein analysis

A Flash 2000 N/Protein Analyzer (Thermo Fisher Scientific, Cambridge, UK) was used to determine the protein/nitrogen content of the samples according to a modification of the standard Dumas combustion method (Immerzeel et al., 2014). In brief, duplicate samples (20 mg) weighed into tin capsules, were introduced into the combustion reactor through the auto-sampler and combined with the amount of oxygen sufficient for complete sample flash combustion. All the nitrogen measured by gas chromatography and detected by the thermal conductivity detector was assumed to be from the proteins in the samples. The appropriate protein-specific conversion factor (Baker 1979, 1982) was applied to the automatically generated data and used in calculating the total amount of protein per sample.

4.2.5.3 Lowry assay

Protein determination using the modified Lowry method was carried out according to the method described by Markwell, Haas, Bieber, & Tolbert (1978). Triplicate aliquots of each hydrolysate sample and BSA standard in a range of concentrations were adjusted to 1 mL with deionized water where necessary, and combined with freshly made Lowry reagent (prepared by mixing one volume of 4% w/v $CUSO_4 \cdot 5H_2O$ with 100 volumes of 2% w/v sodium carbonate containing 1% w/v SDS, 0.4% w/v NaOH and 0.16 % w/v sodium potassium tartrate). The mixtures were incubated for 60 min at room temperature prior to the addition of Folin-Ciocalteau reagent (diluted 1:1 with deionized water). After a further 45 min incubation time, the absorbance at 660 nm was then measured against a water/reagent blank using an Ultrospec 4300 Pro® spectrophotometer (Biochrom, Cambridge, England).

4.2.5.4 O-Phthaldialdehyde (OPA) fluorometric protein assay

Various concentrations (up to 1000 mg/mL) of triplicate aliquots of the hydrolysates and BSA standard were prepared with phosphate buffered saline (pH 7.4). Samples and standards were added to wells in a 96-well microplate and combined with an activated β-mercaptoethanol/OPA reagent mixture as previously described (Held, 2006). Samples were incubated for 2 min at room temperature with moderate shaking before the determination of fluorescence in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA) using excitation and emission wavelengths of 340 and 460 nm, respectively.

4.2.5.5 Bradford assay

Triplicate aliquots of the protein hydrolysate samples and BSA standard were adjusted to 1 mL with water, combined with 10 mL of Bradford reagent and incubated at room temperature for 10 min. The absorbance was then measured at 595 nm against a water/reagent blank (Bradford, 1976) using the Ultrospec spectrophotometer.

4.2.5.6 Ninhydrin assay

Determination of protein in the hydrolysate samples was carried out using a modification of the traditional ninhydrin assay, with BSA as a standard (Haven & Jorgensen, 2014). Briefly, triplicate aliquots (40 µL) were combined with 100 µL of 1 M HCl, thoroughly vortexed and incubated for 16 h at 105 °C. The samples were subsequently cooled to room temperature prior to the addition of 200 µL ninhydrin reagent. Afterwards, the mixture was vortexed, heated at 100 °C for 20 min and once again cooled to room temperature before the addition of 1 mL 50% (v/v) ethanol. After mixing, the samples were briefly centrifuged and the supernatant transferred to a 1 mL cuvette for the measurement of absorbance at 570 nm using the Ultrospec spectrophotometer.

4.2.5.7 Protein measurement by far-UV absorption

Protein determination by far-UV absorption was performed according to a previously described method (Aitken & Learmonth, 2009). Briefly, solutions (0.125-1.0 mg/mL) of each protein hydrolysate sample or standard was diluted with a 0.9 % w/v NaCl solution until the absorbance obtained at 215 nm was <1.5. Further absorbance readings of each sample using an Ultrospec spectrophotometer were measured at 205 and 280 nm and the protein concentration taken as the absorbance at 205 nm (A_{205}) and calculated using the formula:

$A_{205} = 27 + 120 (A_{280}/A_{205})$.

4.2.6 Statistical analysis

Protein hydrolysis for each sample was performed at least thrice and samples combined for protein determination. Except where otherwise indicated, protein determination analyses were conducted in triplicate and the data expressed as mean ± standard deviation, while the statistical significance of difference between treatments was analyzed by one-way analysis of variance with Duncan's multiple range test ($P < 0.05$) using SAS software desktop version 9.2 (Statistical Analysis Systems, Cary, NC, USA). Pearson correlation coefficients were also determined using the SAS software.

4.3 Results and discussion

4.3.1 Proximate composition of protein hydrolysates

Table 4.1 shows data from the proximate composition analysis of the seven food protein hydrolysates (wet weight basis). With the exception of soybean (SPH), protein content was higher in the animal protein hydrolysates (CPH, WPH, BPH) when compared to the plant hydrolysates. The results are difficult to interpret since it has not been determined whether the

nitrogen to protein conversion factor for hydrolysates from different protein sources is the same as used in this work. The high levels of ash in the hydrolysates could be attributed to salt formation during digestion when NaOH is added to neutralize the protons generated by the protease.

4.3.2 Protein measurements by amino acid analysis, nitrogen determination and colorimetry

In Table 4.2, the protein content results from the amino acid analysis are compared with those obtained from nitrogen estimation and colorimetric methods. For the N/protein analysis, which was performed using the Dumas method, the data obtained with the sample-specific conversion factors (Baker 1979, 1982) and those derived with the classical conversion factor of 6.25 are both shown. Apart from the result for the BPH, the data from the sample-specific N/protein analysis (Dumas) method substantially underestimated the proteins in the other protein hydrolysates when compared to the amino acid analysis data. It is possible that the proposed protein-specific factors (Baker 1979, 1982) are actually lower than what should be the "real" conversion factor for each of these proteins (Mæhre, Dalheim, Edvinsen, Elvevoll, & Jensen, 2018). Expectedly, compared to the Dumas values obtained using protein-specific conversion factors, the data obtained from the Dumas method using the traditional 6.25 conversion factor overestimated the protein content of some of the hydrolysates (FPH, HPH, PPH, and SPH), while quite notably underestimating others (casein and whey). The higher protein contents for CPH and WPH could be explained by the higher sample-specific factor of 6.38 used for the determination of proteins in milk, while the lower protein content values obtained for the other four hydrolysates could be attributed to the lower (<6.25) protein-specific factors used in their determination. The conversion factor-dependent nature of these protein content values highlights

the limitation of using such arbitrary values in determining the amount of protein in food protein hydrolysates.

With the exception of BPH and HPH whose Dumas (6.25) values did not differ appreciably from those from the amino acid analysis, the protein contents of the rest of the hydrolysates as measured by the traditional Dumas method were noticeably underestimated. Furthermore, the proximate composition analysis either underestimated (CPH, SPH and WPH) or overestimated (BPH and HPH) the protein contents of all the hydrolysates compared to the values obtained using the amino acid analysis method, except for the FPH and PPH which values are similar to data from the amino acid analysis. Although the proximate composition analysis method also uses the nitrogen-to-protein conversion factor of 6.25, the values obtained for

Table 4.1. Proximate composition of food protein hydrolysates[1,2]

Parameter	CPH (%)	WPH (%)	PPH (%)	FPH (%)	HPH (%)	SPH (%)	BPH (%)
Moisture content	2.35±0.08^{d}	2.68±0.15^{d}	5.34±0.25^{b}	1.80±0.28^{e}	3.77±0.02^{c}	2.68±0.12^{d}	5.74±0.09^{a}
Crude Protein (N x 6.25)	74.92±3.73^{a}	73.90±1.80^{ab}	68.95±2.45^{bc}	69.40±0.03^{b}	63.84±2.51^{c}	73.77±1.82^{ab}	74.96±0.69^{a}
Crude Fibre	2.26±0.04^{ab}	2.55±0.45^{ab}	1.77±0.09^{b}	2.86±0.86^{a}	2.28±0.04^{ab}	1.86±0.01^{b}	1.96±0.16^{ab}
Fat	0.60±0.28^{abc}	0.50±0.14^{bc}	0.60±0.28^{abc}	0.80±0.01^{ab}	0.60±0.28^{abc}	0.20±0.00^{c}	0.99±0.01^{a}
Ash	18.25±0.06^{e}	18.87±0.37^{d}	21.61±0.00^{b}	18.51±0.08^{e}	22.27±0.06^{a}	20.22±0.11^{c}	20.08±0.03^{c}

[1]Data from duplicate determinations expressed as mean ± standard deviation. CPH, casein protein hydrolysate; WPH, whey protein hydrolysate; PPH, pea protein hydrolysate; FPH, flaxseed protein hydrolysate; HPH, hemp seed protein hydrolysate; SPH, soybean protein hydrolysate; BPH, beef protein hydrolysate

[2]Superscript letters (a–e) indicate significant ($P < 0.05$) difference across the same row for a specific parameter.

Table 4.2. Protein content values (%) of food protein hydrolysates based on various protein determination methods[1]

Protein Hydrolysate	Amino Acid Analysis	Dumas[2]	Dumas[3]	Proximate Composition Analysis[4]	Ninhydrin	Lowry	Bradford	OPA
Beef	72.50± 0.05^{d}	73.82±0.43^{b}	73.82±0.43^{b}	79.52±0.80^{a}	147.52± 3.27^{a}	69.73±2.44^{b}	69.00±3.83^{cd}	127.11±8.67^{a}
Casein	85.35±0.44^{a}	78.64±0.30^{a}	77.03±0.29^{a}	76.72±3.88^{ab}	116.77± 3.33^{c}	82.12±1.90^{a}	71.68±2.28^{bc}	51.45±2.45^{b}
Flaxseed	69.06±2.02^{e}	58.80±1.07^{e}	67.94±1.24^{d}	70.67±0.17^{cd}	129.99±8.20^{b}	76.01±3.22^{b}	92.38±1.99^{a}	29.98±1.86^{d}
Hempseed	64.56±1.06^{f}	55.88±0.49^{f}	65.90±0.58^{e}	66.34±2.62^{d}	104.93±3.80^{d}	71.36±0.71^{b}	78.51±1.09^{b}	50.96±0.58^{b}
Pea	71.45±0.84^{de}	62.28±0.26^{d}	70.51±0.29^{c}	72.84±2.79^{bc}	126.37±6.06^{b}	73.24±1.84^{ab}	59.69±2.97^{e}	42.38±1.76^{c}
Soy	76.95±0.42^{c}	68.73±0.06^{c}	75.23±0.06^{ab}	75.80±1.77^{abc}	125.54±3.84^{b}	82.01±0.21^{a}	66.24±2.82^{d}	43.19±1.48^{c}
Whey	82.20±1.81^{b}	75.45±1.44^{b}	73.91±1.41^{b}	75.94±1.73^{abc}	139.93±4.01^{a}	85.50±2.41^{a}	74.65±3.71^{ab}	42.62±1.63^{c}

[1]Values represent mean ± std dev from replicate determinations. Superscript letters (a–f) indicate statistically significant ($P < 0.05$) difference within the same column.
[2]Calculated with sample-specific conversion factor (6.25 for beef, 6.38 for casein and whey, 5.41 for flaxseed, 5.3 for hempseed, 5.52 for pea, and 5.71 for soybean; (Baker 1979, 1982; Jones, 1941)
[3]Calculated with the generic conversion factor of 6.25 generally used in the Dumas method.
[4]Calculated as N x 6.25

virtually all the hydrolysates (apart from SPH) using that method still differed from those obtained using the traditional Dumas method thus emphasizing how the choice of analytical method could influence protein content data. It should be emphasized, however that there is no information on the nitrogen-to-protein conversion factor suitable for protein hydrolysates. Therefore, the conversion factor developed for whole proteins may not apply to protein hydrolysates, hence the need to exercise caution in using this method.

Table 4.2 and Fig. 4.1 also contain the protein content data of the food protein hydrolysates for colorimetric methods such as Bradford, Lowry, ninhydrin and OPA. Since BSA was used as control in each of these assays, the results were normalized to percentage values based on the BSA standard in order to permit even comparison. Overall, the results show that the protein content of the food protein hydrolysates varies with specific protein determination methods. For instance, data from the ninhydrin assay indicate that the BPH sample has the highest amount of protein while the lowest protein content was found in the hempseed hydrolysate. The high protein content of BPH is corroborated by data from the OPA test but the same does not hold true for the other analytical methods. Ninhydrin assay results are known to be influenced by the capacity of ammonia oxidatively deaminated from some amino acids to react with hydrindantin from ninhydrin and form the diketohydrindylidene diketohydrindamine purple dye (Ruhemann's purple), which is measurable at 570 nm (Friedman, 2004). Consequently, non-protein materials that contain or, which upon processing can release ninhydrin-reactive substances could potentially escape detection if used in the adulteration of food proteins in which the ninhydrin test is solely used for quality control (Moore et al., 2010). Similar protein-to-protein variability in dye formation or binding has also been reported in the Bradford assay (Krohn, 2005) and this could explain the noticeable difference observed in the

results where the flaxseed protein hydrolysate was determined to contain the highest amount of proteins.

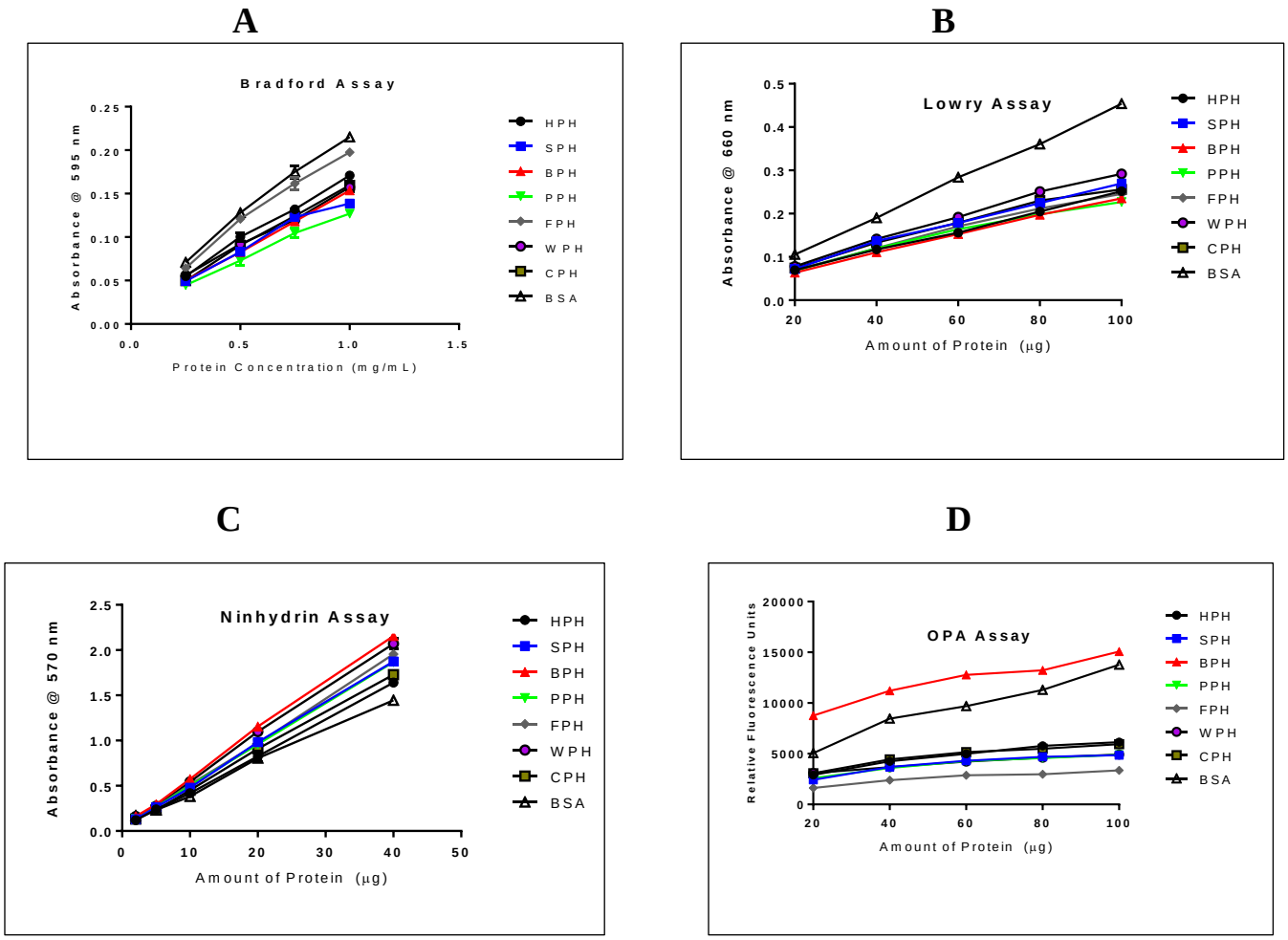

Fig. 4.1. Protein determination using colorimetric methods. CPH , casein protein hydrolysate; WPH, whey protein hydrolysate; SPH, soy protein hydrolysate; BPH, beef protein hydrolysate; PPH, pea protein hydrolysate; FPH, flaxseed protein hydrolysate; HPH, hempseed protein hydrolysate; and BSA, bovine serum albumin (standard).

Electrostatic interactions between basic amino acids such as arginine (present in relatively high amounts in the FPH) and the Coomassie Blue dye used in Bradford assay have been reported to influence the binding of the dye to proteins (Moore et al., 2010). Compared to the other three analytical methods, only the Lowry assay is consistent with the amino acid analytical method in

attributing the three highest protein content values to the whey, casein and soy protein hydrolysates. Nevertheless, the Lowry method itself has been associated with poor specificity since the presence of other compounds (especially polyphenols), which may have been co-extracted with the protein could interfere with colour development (Ponomareva, Golovchenko, Patova, Vanchikova, & Ovodov, 2015).

4.3.3 Protein measurement by far-UV absorption

Fig. 4.2 contains the far-UV protein determination data of the various protein hydrolysates assayed in this study, and shows the SPH having the highest protein content. The determination of protein by far-UV absorption is a quick but not very selective method for protein measurement. Owing to the reliance of this method on the measurement of absorbance at 280 nm, higher protein values would tend to be assigned to proteins containing high amounts of aromatic amino acids or disulfide-bonded cysteine residues (Krohn, 2005) since they exhibit clear UV absorption maxima at that wavelength. As shown in Table 4.3., the soy and casein protein hydrolysates contain the highest amounts of tryptophan, phenylalanine and tyrosine residues compared to the other food protein hydrolysates, while the WPH has the highest content of sulfur-containing amino acids.

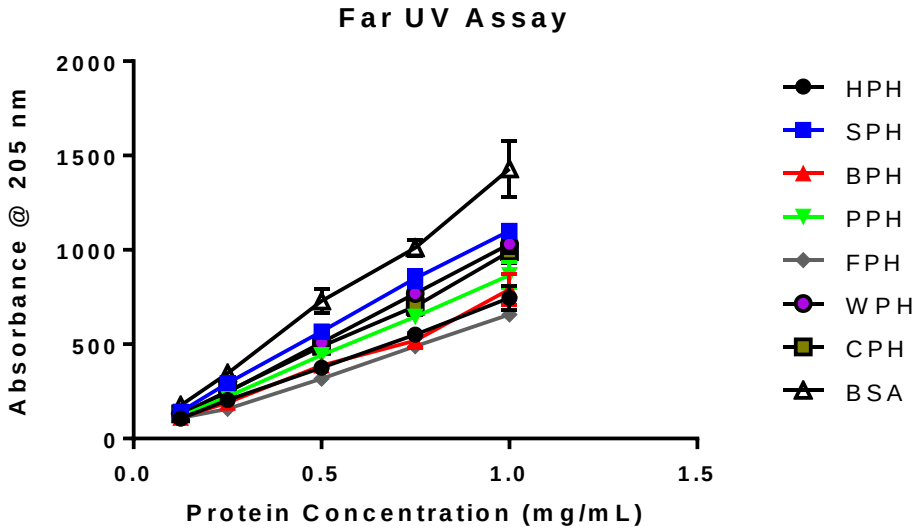

Fig. 4.2. Far-UV absorption measurement for the determination of protein concentration. CPH , casein protein hydrolysate; WPH, whey protein hydrolysate; SPH, soy protein hydrolysate; BPH, beef protein hydrolysate; PPH, pea protein hydrolysate; FPH, flaxseed protein hydrolysate; HPH, hempseed protein hydrolysate; and BSA, bovine serum albumin (standard).

Although the aromatic amino acid content of WPH is not as high as those of CPH and SPH, its content of cysteine residues is the highest among all the hydrolysate samples and may have possibly compensated for the lower amounts of aromatic amino acids, thus contributing to its overall high protein content in this assay. The results underline a central problem of protein determination methods such as far-UV absorption, which depend on the composition and nature of a specific protein. Challenges of using this method for protein determination include the requirement for protein solutions to be clear and colorless as well as the need for sample pre-treatment even for otherwise pure proteins in order to minimize the potential for contamination with compounds that also absorb at 280 nm (Chang, 2010; Moore et al., 2010).

Table 4.3. Percentage amino acid compositions of food protein hydrolysates[1]

Amino Acid	BPH	FPH	SPH	CPH	PPH	HPH	WPH
Asx	7.62	7.57	8.04	6.85	8.87	7.58	9.30
Thr	3.31	2.41	3.15	3.51	2.80	2.06	5.95
Ser	3.26	3.54	4.34	5.09	3.85	3.62	4.48
Glx	11.69	14.63	16.14	18.06	13.44	12.36	14.23
Pro	4.30	3.05	5.51	9.59	3.48	4.40	5.40
Gly	4.02	3.70	2.33	1.38	2.64	2.48	1.03
Ala	4.70	3.39	2.90	2.55	3.13	3.03	4.85
Cys	0.57	0.87	0.65	0.63	0.41	0.76	1.60
Val	3.08	3.35	3.32	4.49	3.05	2.86	4.06
Met	1.19	0.65	0.59	1.41	0.37	0.79	1.10
Ile	3.11	3.08	3.16	3.63	3.21	2.60	4.60
Leu	5.57	4.03	6.15	7.26	5.87	4.05	8.84
Tyr	2.96	2.14	3.62	4.38	2.51	1.97	2.11
Phe	3.02	3.99	4.07	4.14	3.73	2.92	2.33
His	2.75	1.59	2.31	2.43	1.94	1.88	1.64
Lys	6.26	2.09	5.19	6.26	5.59	2.45	7.94
Arg	4.24	8.12	4.72	2.92	5.88	8.14	1.50
Trp	0.87	0.88	0.77	0.78	0.67	0.63	1.26
HAA	29.37	25.42	30.74	38.85	8.87	7.58	9.30
PCAA	9.01	3.68	7.50	8.69	2.80	2.06	5.95
NCAA	19.31	22.19	24.19	24.91	3.85	3.62	4.48
AAA	6.86	7.01	8.46	9.31	13.44	12.36	14.23
SCAA	1.76	1.51	1.24	2.04	3.48	4.40	5.40
BCAA	11.75	10.46	12.63	15.38	2.64	2.48	1.03

[1]CPH, casein protein hydrolysate; WPH, whey protein hydrolysate; PPH, pea protein hydrolysate; FPH, flaxseed protein hydrolysate; HPH, hemp seed protein hydrolysate; SPH, soybean protein hydrolysate; BPH, beef protein hydrolysate. Asx = aspartic acid + asparagine; HAA = hydrophobic amino acid; PCAA = positively charged amino acid; NCAA = negatively charged amino acid; AAA = aromatic amino acid; SCAA = sulfur-containing amino acid; BCAA = branched-chain amino acid.

4.3.4. Correlations between analytical methods

The Pearson correlation coefficient analysis of the protein content values obtained from the various methods is presented in Table 4.4. As shown in the table, protein content values from the amino acid analysis correlated strongly with those obtained from the Dumas-specific (r = 0.90),

Dumas-generic (r = 0.90), Lowry (r = 0.82), far UV (r = 0.72), and proximate composition analysis (r = 0.70) with considerably large effect sizes. Therefore, the Dumas and Lowry methods could also provide reliable estimates of peptide content of protein hydrolysates. In contrast, higher amounts of protein as determined by amino acid analysis correlated with lower protein amounts in the Bradford, ninhydrin and OPA assays. Data from the Bradford method correlated poorly with values from virtually all the other methods with the best Bradford between-group linear correlation occurring with data from the Lowry test. As would be expected, higher protein content values from the Dumas-specific method were correlated with higher protein content values from the Dumas-generic method of protein determination. Compared to the between-group correlation coefficient of all the other analytical methods, the amino acid analysis has the highest number of individual effect (r) sizes ≥ 0.70, and thus has the highest linear correlation of all the methods.

Table 4.4. Pearson correlation coefficient analysis of the various protein determination methods

	Dumas[1]	Dumas[2]	PCA	OPA	Ninhydrin	Lowry	Bradford	AAA	Far UV
Dumas[1]	1								
Dumas[2]	0.94	1							
PCA	0.89	0.89	1						
OPA	0.37	0.25	0.55	1					
Ninhydrin	0.47	0.38	0.72	0.51	1				
Lowry	0.55	0.60	0.31	-0.52	0.05	1			
Bradford	-0.36	-0.49	-0.46	-0.28	-0.11	0.01	1		
AAA	0.90	0.90	0.70	-0.06	0.24	0.82	-0.28	1	
Far UV	0.56	0.72	0.45	-0.23	0.02	0.74	-0.64	0.72	1

[1]Derived from Dumas values calculated with sample-specific conversion factors.
[2]Derived from Dumas values calculated with the generic conversion factor of 6.25.
PCA= proximate composition analysis; AAA = amino acid analysis.

4.4 Conclusions

This study clearly demonstrates the complexity in accurately quantifying proteins in food protein hydrolysates using many of the currently available and widely used analytical methods. For example, some of the methods such as far UV absorption, (modified) Lowry and Bradford are used mainly for the analysis of liquid samples. Methods such as Kjeldahl and Dumas, which are widely used for quantifying proteins in food protein hydrolysates usually measure the amount of total nitrogen in foods, but do not distinguish between non-protein nitrogen and protein-based nitrogen. Although some of the challenges of accurate measurement of protein in hydrolysates could be traced to the specific method used, the unique matrix in which these proteins are found further compounds the problem. The diverse underlying principles of the various food protein analytical methods also add to the challenge as it makes like-for-like comparisons difficult. Apart from amino acid analysis, all the other protein determination methods examined in this study are known to be dogged by issues ranging from problems with generic nitrogen conversion factors to interfering reactions with buffers, making the underestimation or overestimation of their actual protein content a common occurrence. Amino acid analysis is also imperfect since complete hydrolysis of all the peptides is almost impossible during acid hydrolysis. Compounds such as xylose, glucose, cellulose and ethanol are known to be present in hydrolyzed and/or fermented food proteins and have been known to alter the colour intensity in common protein determination assays. Based on the foregoing and on its relative low cost especially when compared to other highly accurate but prohibitively expensive analytical methods such as mass spectrometric protein quantification, amino acid analysis may be the most accurate method for the measurement of proteins in hydrolysates. However, because of the high positive correlation with

amino acid analysis, the Dumas and Lowry methods could also be proposed as suitable for estimating the protein content of hydrolysates.

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4.6 Conflicts of interest

The authors declare no conflict of interest.

4.7 References

Agyei, D., Potumarthi, R., & Danquah, M. K. (2015). Food-derived multifunctional bioactive proteins and peptides. In V. K. Gupta, & M. G. Tuohy (Eds), Biotechnology of bioactive compounds: sources and applications (pp. 483-505). Chichester, UK: John Wiley & Sons, Ltd.

Aitken, A., & Learmonth, M. P. (2009). Protein determination by UV absorption. In J.M. Walker (Ed), The protein protocols handbook (pp. 3-6). Totowa, NJ: Humana Press Inc.

Baker, D. (1979). Report on cereal foods: General Referee Reports. *Journal of the Association of Official Analytical Chemists*, 62, 369-370.

Baker, D. (1982). Report on cereal foods: General Referee Reports. *Journal of the Association of Official Analytical Chemists*, 65, 333-334.

Ben Khaled, H., Ktari, N., Ghorbel-Bellaaj, O., Jridi, M., Lassoued, I., & Nasri, M. (2014). Composition, functional properties and *in vitro* antioxidant activity of protein hydrolysates prepared from sardinelle (*Sardinella aurita*) muscle. *Journal of Food Science and Technology, 51*, 622-633.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography B: Biomedical Sciences and Applications, 336*, 93-104.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein- dye binding. *Analytical Biochemistry, 72*, 248-254.

Buckley, J.D., Thomson, R.L., Coates, A.M., Howe, P.R., DeNichilo, M.O., & Rowney, M.K. (2010). Supplementation with a whey protein hydrolysate enhances recovery of muscle force-generating capacity following eccentric exercise. *Journal of Science and Medicine in Sport*, 13, 178-181.

Chang, S. K. C. (2010). Protein Analysis. In S. Nielsen (Ed), *Food Analysis* (pp. 133-146). Boston, MA: Springer US.

Dev, D. K., & Quensel, E. (1988). Preparation and functional properties of linseed protein products containing differing levels of mucilage. *Journal of Food Science, 53*, 1834-1837.

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of Agricultural and Food Chemistry, 52*, 385-406.

Gehrke, C. W., Wall Sr, L. L., & Absheer, J. S. (1985). Sample preparation for chromatography of amino acids: acid hydrolysis of proteins. *Journal of the Association of Official Analytical Chemists, 68*, 811-821.

Ghelichi, S., Sørensen, A.M., García-Moreno, P.J., Hajfathalian, M., & Jacobsen, C. (2017). Physical and oxidative stability of fish oil-in-water emulsions fortified with enzymatic hydrolysates from common carp (*Cyprinus carpio*) roe. *Food Chemistry*, 237, 1048-1057.

Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2011). *In vitro* antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *Journal of the American Oil Chemists' Society, 88*, 381-389.

Hall III, C., Tulbek, M. C., & Xu, Y. (2006). Flaxseed. In S. L. Taylor (Ed.), *Advances in Food and Nutrition Research*, vol. 51 (pp. 1-97). Elsevier Inc.

Hall, F.G., Jones, O.G., O'Haire, M.E., Liceaga, A.M. (2017). Functional properties of tropical banded cricket (*Gryllodes sigillatus*) protein hydrolysates. *Food Chemistry*, 224, 414-422.

Haven, M. A., & Jorgensen, H. (2014). The challenging measurement of protein in complex biomass- derived samples. *Applied Biochemistry and Biotechnology, 172*, 87-101.

Held, P. (2006). Quantitation of total protein using OPA. *Nature Methods*| *Application Notes.* DOI :10.1038/an1781. Accessed on July 1, 2017.

Horwitz, W., & Latimer, G. W. (2005). Official methods of analysis of AOAC International. (18th ed.). Gaithersburg, Md.: AOAC International.

Hou, Y., Wu, Z., Dai, Z., Wang, G., & Wu, G. (2017). Protein hydrolysates in animal nutrition: industrial production, bioactive peptides, and functional significance. *Journal of Animal Science and Biotechnology*, 8:24.

Immerzeel, P., Falck, P., Galbe, M., Adlercreutz, P., Nordberg Karlsson, E., & Stålbrand, H. (2014). Extraction of water-soluble xylan from wheat bran and utilization of enzymatically produced xylooligosaccharides by Lactobacillus,Bifidobacterium and Weissella spp. *LWT - Food Science and Technology, 56*, 321-327.

Jones, D. B. (1941). *Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins*: US Department of Agriculture, Washington, DC. Available at: http://www.foodcomp.dk/download/Jones_1941%20nitrogen-protein%20conversion%20cir183.pdf. Accessed on June 15, 2017.

Kose, A., & Oncel, S.S. (2015). Properties of microalgal enzymatic protein hydrolysates: biochemical composition, protein distribution and FTIR characteristics. *Biotechnology Reports, 6*, 137-143.

Krohn, R. I. (2005). The colorimetric detection and quantitation of total protein. In R.E. Wrolstad, T.E. Acree, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Schwartz, C.F. Shoemaker, D.M. Smith, & P. Sporns (Eds), Handbook of food analytical chemistry (pp. 77-104). Hoboken, NJ: John Wiley.

Landry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry, 40*, 776-779.

Li, H., & Aluko, R. E. (2010). Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *Journal of Agricultural and Food Chemistry, 58*, 11471-11476.

Manninen, A.H. (2009). Protein hydrolysates in sports nutrition. *Nutrition and Metabolism*, 6:38.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry, 87*, 206-210.

Mehlenbacher, V. C., Sallee, E. M., Hopper, T. H., Link, W. E., Walker, R. O., & Firestone, D. (2009). Official methods and recommended practices of the AOCS. (6th ed.). Urbana, IL: AOCS Press.

Molina, E., Papadopoulou, A., & Ledward, D. A. (2001). Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. *Food Hydrocolloids, 15*, 263-269.

Moore, J. C., Devries, J. W., Lipp, M., Griffiths, J. C., & Abernethy, D. R. (2010). Total protein methods and their potential utility to reduce the risk of food protein adulteration. *Comprehensive Reviews in Food Science and Food Safety, 9*, 330-357.

Mæhre, H. K., Dalheim, L., Edvinsen, G. K., Elvevoll, E. O., & Jensen, I.-J. (2018). Protein determination- method matters. *Foods 2018, 7*, 5.

Neves, A.C., Harnedy, P.A., O'Keeffe, M.B., & FitzGerald, R.J. (2017). Bioactive peptides from Atlantic salmon (*Salmo salar*) with angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory, and antioxidant activities. *Food Chemistry*, 218, 396-405.

Nwachukwu, I.D., Girgih, A.T., Malomo, S.A., Onuh, J.O., & Aluko, R.E. (2014). Thermoase-derived flaxseed protein hydrolysates and membrane ultrafiltration peptide fractions have systolic blood pressure-lowering effects in spontaneously hypertensive rats. *International Journal of Molecular Sciences*, 15, 18131-18147.

Perreault, V., Hénaux, L., Bazinet, L., & Doyen, A. (2017). Pretreatment of flaxseed protein isolate by high hydrostatic pressure: impacts on protein structure, enzymatic hydrolysis and final hydrolysate antioxidant capacities. *Food Chemistry*, 221, 1805-1812.

Ponomareva, S., Golovchenko, V., Patova, O., Vanchikova, E., & Ovodov, Y. (2015). Comparative analysis of the spectrophotometric methods of the protein amount determination in the pectic polysaccharide samples. *Russian Journal of Bioorganic Chemistry, 41*, 133-139.

Tang, C.-H., Ten, Z., Wang, X.-S., & Yang, X.-Q. (2006). Physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate. *Journal of Agricultural and Food Chemistry, 54*, 8945-8950.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science, 77*, R11-R24.

Udenigwe, C. C., Lin, Y. S., Hou, W. C., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*, 199-207.

Wróblewska, B., & Karamać, M. (2003). Analytical methods for estimating protein hydrolysates quality: a review. *Acta Alimentaria, 32*,193-204.

Yousr, M., & Howell, N. (2015). Antioxidant and ACE inhibitory bioactive peptides purified from egg yolk proteins. *International Journal of Molecular Sciences*, 16, 29161-29178.

4.8 TRANSITION STATEMENT TWO

Although a range of methods are commonly used to determine protein concentration, investigators face a myriad of challenges in accurately quantifying proteins in the laboratory. The preceding chapter examined the various methods currently used to measure protein content in food protein hydrolysates and peptides by testing samples from both animal and plant proteins. The enzyme Alcalase was selected for the hydrolysis of the protein samples due to its broad specificity. In addition to the common difficulties associated with protein determination methods in general, the peculiar food matrices in which these proteins naturally occur provide further impetus for recommending accurate methods for quantifying proteins in food-derived hydrolysates and peptides. After employing colorimetric, far UV, Dumas, amino acid analysis and Kjeldahl methods to quantify protein in the test samples, the results indicated that Dumas, Lowry and amino acid analysis were the most positively correlated. Although no single method currently in use can meet the need for accuracy, sensitivity, precision, cost-effectiveness, reproducibility, simplicity and speed (among other criteria for a suitable assay), these three methods were recommended as providing the best estimate of protein content in food protein hydrolysates, based on the results of the Pearson correlation coefficient analysis.

Having established the most reliable methods for protein quantification in food protein hydrolysates, the enzymatic hydrolysis of flaxseed protein isolate was carried out in order to produce hydrolysates which were subsequently tested for antihypertensive properties. Hence, the next chapter (Manuscript 3) examines the potential contribution of flaxseed-derived protein hydrolysates and peptides to the control and management of high blood pressure.

CHAPTER 5

MANUSCRIPT THREE

THERMOASE-DERIVED FLAXSEED PROTEIN HYDROLYSATES AND MEMBRANE ULTRAFILTRATION PEPTIDE FRACTIONS HAVE SYSTOLIC BLOOD PRESSURE-LOWERING EFFECTS IN SPONTANEOUSLY HYPERTENSIVE RATS

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Reprinted from Thermoase-derived flaxseed protein hydrolysates and membrane ultrafiltration peptide fractions have systolic blood pressure-lowering effects in spontaneously hypertensive rats. *International Journal of Molecular Sciences*, 15(10):18131-18147.

5.0 Abstract

Thermoase-digested flaxseed protein hydrolysate (FPH) samples and ultrafiltration membrane-separated peptide fractions were initially evaluated for *in vitro* inhibition of angiotensin I-converting enzyme (ACE) and renin activities. The two most active FPH samples and their corresponding peptide fractions were subsequently tested for *in vivo* antihypertensive activity in spontaneously hypertensive rats (SHR). The FPH produced with 3% thermoase digestion showed the highest ACE- and renin-inhibitory activities. Whereas membrane ultrafiltration resulted in significant ($P < 0.05$) increases in ACE inhibition by the <1 kDa and 1–3 kDa peptides, only a marginal improvement in renin-inhibitory activity was observed for virtually all the samples after membrane ultrafiltration. The FPH samples and membrane fractions were also effective in lowering systolic blood pressure (SBP) in SHR with the largest effect occurring after oral administration (200 mg/kg body weight) of the 1–3 kDa peptide fraction of the 2.5% FPH and the 3–5 kDa fraction of the 3% FPH. Such potent SBP-lowering capacity indicates the potential of flaxseed protein-derived bioactive peptides as ingredients for the formulation of antihypertensive functional foods and nutraceuticals.

Keywords: antihypertensive; hypertension; bioactive peptides; thermoase; flaxseed; angiotensin converting enzyme; renin; spontaneously hypertensive rat; membrane ultrafiltration; systolic blood pressure

5.1 Introduction

Hypertension is not only a major risk factor for cardiac, cerebrovascular and other vascular diseases [1], it is also considered a leading cause of mortality worldwide with over 7 million deaths and 92 million disability-adjusted life years recorded annually [2]. The renin-angiotensin-aldosterone system (RAAS), which is an important signaling pathway credited with the regulation of extracellular fluid volume, arterial pressure, and tissue perfusion has received significant attention from investigators recently because of its potential as an excellent target for blood pressure lowering agents [3–6]. Renin and angiotensin I-converting enzyme (ACE) play key roles in the RAAS pathway; renin is responsible for catalyzing hydrolytic production of the inactive decapeptide angiotensin I (AT-I) from the N-terminus of the plasma protein angiotensinogen in what has been recognized as the initial and rate-determining reaction. ACE subsequently cleaves AT-I to yield the active pressor octapeptide angiotensin II (AT-II), while concomitantly inactivating the potent vasodilator, bradykinin [3,7]. In addition to the adverse effects of the prolonged use of ACE inhibitors as antihypertensive medication, the alternative ACE-independent, chymase-mediated conversion of AT-I to AT-II in certain organs [8] presents further challenges for the management of hypertension. Therefore, safer antihypertensive agents with the capacity to modulate multiple blood regulation pathways are urgently needed. Given the oft-touted safety of bioactive compounds of biogenic origin in contradistinction to chemically synthesized agents (drugs), various investigators [6,9–12] have explored the possibility of developing safer but effective antihypertensive agents from animal and plant food proteins in the last few years.

Flaxseed (linseed or *Linum usitatissimum*) which was once typically used only for the production of oil and paint, and later as a fibre-rich source of the heart healthy α-linolenic acid (an ω-3 fatty

acid) [13], has received significant research attention recently. This is due to the successful investigation of alkali-extracted proteins and enzyme-digested protein hydrolysate samples from its underutilized meal (a by-product of commercial flaxseed oil production) for various salutary benefits including antihypertensive, antidiabetic, antifungal, and antioxidant activities [6,14–16]. Additionally, the high quality flaxseed proteins are rich in branched-chain amino acids (BCAA) [17], which have been demonstrated to be potentially important components of antihypertensive peptides [18]. Therefore, the choice of flaxseed as the source of food proteins used in this work was not only to meet the main objective of producing unique thermoase-derived antihypertensive bioactive peptides from flax but would also increase the value added utilization and economic importance of flaxseed meal. The work also investigated the potential role of defined peptide sizes obtained from membrane ultrafiltration as a determinant of peptide antihypertensive activity using spontaneously hypertensive rats (SHR).

5.2 Results and Discussion

5.2.1 Inhibition of in Vitro ACE and Human Recombinant Renin Activities by Flaxseed Protein Hydrolysates (FPH)

Fig. 5.1 shows the differential inhibition of ACE by flaxseed protein hydrolysates (FPH) samples obtained following digestion with various concentrations of thermoase GL-30. The highest inhibitory value of 87% was recorded for the FPH sample obtained at the 3% thermoase concentration and this level of ACE inhibition was significantly ($P < 0.05$) higher than the values obtained for all the other protein hydrolysate samples. While the increases in enzyme concentration from 0.5% to 1%, and from 1% to 1.5% resulted in statistically significant differences in the percentage of ACE inhibition, neither increasing the enzyme concentration from 1.5% to 2%, nor from 2% to 2.5% produced any difference in ACE inhibition of statistical

significance. The results suggest that increasing enzyme concentration between 0.5% and 1.5% was important for the production of antihypertensive peptides whereas a similar increase in enzyme concentration between 1.5% and 2% yielded negligible or no differences in ACE-inhibiting peptides.

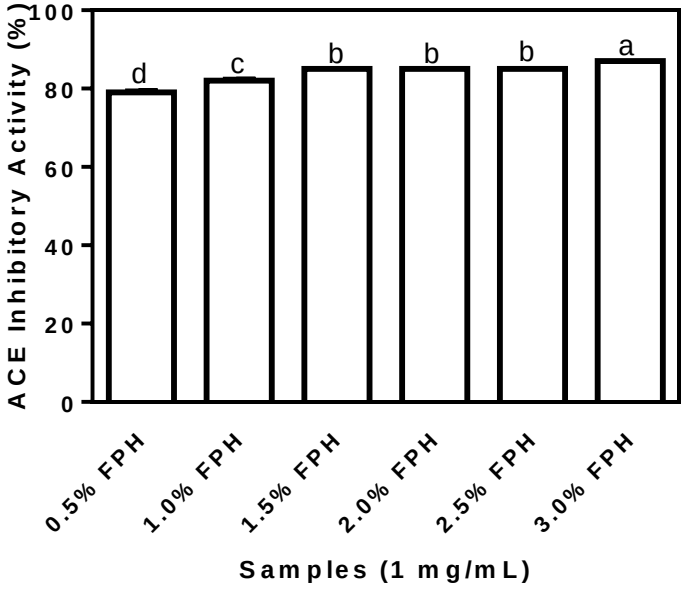

Fig. 5.1. Percentage (mean ± standard error) angiotensin I-converting enzyme- (ACE-) inhibitory activity of flaxseed protein hydrolysates (FPH) digested with thermoase GL-30 (0.5%–3%). Bars with different letters have mean values that are significantly different ($P < 0.05$).

Flaxseed proteins are known to have a high concentration of BCAA [17], which have been shown to be important in the inhibition of ACE activity [18], and thus in potentiating the antihypertensive property of bioactive peptides. Since thermoase GL-30 is an isoform of thermolysin which is known for its specificity in cleaving at the N-terminal regions of aromatic, bulky and hydrophobic amino acid residues and thus releasing BCAA (leucine, isoleucine and valine) [18,19], it was carefully chosen to yield a high proportion of BCAA-enriched hydrolysates. Interestingly, the FPH sample at the 3% enzyme concentration which inhibited ACE activity the most has the highest percentage (15.80%) of BCAA as shown in Table 5.1.

which is comparable to the BCAA content of 16.79% obtained by Udenigwe and Aluko [18] after sequential hydrolysis of flaxseed protein isolate (FPI) with thermolysin and pronase. Given the previously reported capacity of hydrophobic amino acids (HAA) for inhibiting ACE activity [9,20], their relatively high content in all the FPH samples suggests important *in vitro* antihypertensive properties and a potential role in the reduction of elevated blood pressure.

Table 5.1. Percentage amino acid compositions of flaxseed protein meal (FPM), isolate (FPI), and hydrolysates (FPH) samples digested at different thermoase-GL 30 concentrations (0.5%–3%).

AA	FPM	FPI	0.5% FPH	1.0% FPH	1.5% FPH	2.0% FPH	2.5% FPH	3.0% FPH
ASX	9.76	9.39	11.61	11.53	11.01	11.74	11.59	11.41
THR	3.70	3.24	3.50	3.51	3.44	3.55	3.50	3.37
SER	5.05	4.73	5.21	5.19	5.14	5.29	5.19	4.92
GLX	20.92	23.44	22.18	21.47	22.56	21.91	21.59	21.44
PRO	3.95	3.73	4.49	4.43	4.29	4.50	4.31	4.36
GLY	6.14	6.00	5.47	5.37	5.56	5.41	5.34	5.27
ALA	4.59	4.04	4.52	4.56	4.28	4.57	4.51	4.54
CYS	1.80	2.13	1.35	1.27	1.58	1.34	1.35	1.32
VAL	5.17	4.85	5.02	5.18	4.97	5.07	5.02	5.53
MET	1.70	1.77	1.22	1.20	1.28	1.09	1.17	1.14
ILE	4.15	3.80	4.19	4.35	4.11	4.19	4.16	4.61
LEU	5.97	5.62	5.57	5.71	5.63	5.71	5.64	5.66
TYR	2.29	2.35	2.45	2.52	2.51	2.54	2.50	2.45
PHE	4.73	4.82	5.11	5.21	5.11	5.18	5.10	5.19
HIS	2.45	2.33	2.34	2.40	2.30	2.37	2.36	2.34
LYS	4.18	3.81	3.23	3.17	3.29	3.20	3.21	3.19
ARG	10.63	11.59	11.82	11.91	11.32	11.62	11.98	11.83
TRP	1.40	1.45	1.29	1.31	1.39	1.37	1.38	1.34
AAA	8.42	8.62	8.85	9.03	9.01	9.08	8.97	8.98
BCAA	15.29	14.27	14.79	15.23	14.71	14.97	14.81	15.80
HAA	35.75	34.55	35.22	35.72	35.15	35.55	35.11	36.13

ASX = aspartic acid and asparagine; THR = threonine; SER = serine; GLX = glutamic acid and glutamine; PRO = proline; GLY = glycine; ALA = alanine; CYS = cysteine; VAL = valine; MET = methionine; ILE = isoleucine; LEU = leucine; TYR = tyrosine; PHE = phenylalanine; HIS = histidine; LYS = lysine; ARG = arginine; TRP = tryptophan; AAA = aromatic amino acid; BCAA = branched-chain amino acid; HAA = hydrophobic amino acid.

Percentage ACE-inhibitory activity by the six different FPH samples, which ranged from 79% to 87% is comparable to the percentage ACE-inhibitory activities of about 84% and 82%,

respectively, at the same final assay concentration of 1 mg/mL reported for ACE inhibition by thermolysin-digested (pH 8.0, 50 °C, 4 h) rapeseed protein samples [21] and pepsin-digested (pH 3.0, 37 °C, 4 h) canola protein samples [20] using the *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG)-based spectrophotometric method (described in Section 3.2.3.). However, the observed values in this work are significantly ($P < 0.05$) higher than the 70.4% ± 0.4% ACE inhibition obtained using a hippuryl-L-histidyl-L-leucine (HHL)-based chromatographic method, which was reported for thermolysin-digested (pH 8.0, 60 °C, 3 h) rapeseed protein samples [10]. All the FPH samples showed at least 28.0% ± 0.46% renin inhibition (Fig. 5.2.) with the percentages of renin-inhibitory activities of 39% and 40% at the 2.5% and 3% enzyme concentrations, respectively, being significantly ($P < 0.05$) higher than the *in vitro* percentage inhibition ranging from 28% to 32% which was observed at the 0.5%–2% enzyme concentrations. The highest renin-inhibitory activity of 40.0% ± 0.94% which was observed with FPH at the 3% enzyme concentration suggests that just as with ACE inhibition, increasing the concentration of thermoase to 3% resulted in a marked increase in renin inhibition, probably because greater amounts of peptides were liberated from the native protein at higher enzyme concentration. The afore-stated inhibition values of *in vitro* renin activity at the 2.5% and 3% enzyme concentrations are comparable to the values of about 49% and 40%, respectively, which were reported for renin inhibition at the same final assay concentration of 1 mg/mL by trypsin-digested canola proteins [20] and rapeseed protein sequentially digested by 4% pepsin-pancreatin [21]. However, while >2.5 mg/mL of 1% thermolysin-digested flaxseed proteins was reported to accomplish 50% *in vitro* inhibition of human recombinant renin in a previous study [6], the concentration of 1 mg/mL used in the present study resulted in ≈40% renin inhibitory activity at the 2.5% and 3% enzyme concentrations.

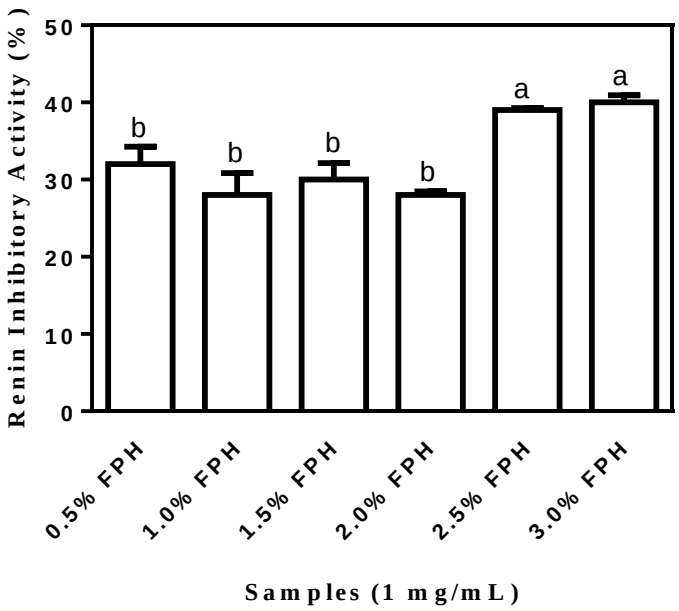

Fig.5.2. Percentage (mean ± standard error) renin-inhibitory activity of flaxseed protein hydrolysates (FPH) after sample hydrolysis with a range (0.5%–3%) of thermoase GL-30 concentrations. Bars with different letters have mean values that are significantly different ($P < 0.05$).

Higher renin-inhibitory activity values of about 45%, 52% and 82%, respectively, have been reported for rapeseed protein hydrolysates produced from 4% thermolysin, 4% proteinase K, and 4% alcalase digestion [21]. However, a 4% alcalase protein hydrolysate of African yam bean was reported to have a renin inhibition value of about 38% which is similar to the results for the 2.5% and 3% thermoase enzyme concentrations in the present study [22]. Furthermore, significantly ($P < 0.05$) lower values than those observed for the 2.5% and 3% thermoase concentrations in this study have been reported for 2% alcalase-hydrolysed chicken thigh skin protein (≈16% inhibition), 3% alcalase-hydrolysed chicken breast protein (≈24%) and 4% alcalase hydrolysed chicken breast protein (≈9%) as well as chicken breast skin protein sequentially hydrolysed with 4% pepsin-pancreatin (≈14%) [12]. As was suggested by Udenigwe *et al.* [6], the considerably lower inhibition of renin by enzyme-derived food protein hydrolysates in comparison to their ACE inhibition could be as a result of the presence of more ACE-inhibitory peptides than similar renin

inhibitors in enzymatically-digested food proteins thus resulting in a relatively easier inhibition of ACE than renin.

The FPH samples obtained at the 2.5% and 3% enzyme concentrations showed the highest inhibition of ACE and renin and were consequently subjected to membrane fractionation as well as used for *in vivo* systolic blood pressure measurements in spontaneously hypertensive rats.

5.2.2 ACE and Renin Inhibition by FPH Membrane Fractions

FPH samples obtained using 2.5% (2.5% FPH) or 3% (2.5% FPH) thermoase concentration were separated by membrane ultrafiltration and the defined peptide size fractions used for in vitro ACE and renin inhibition tests. As shown in Fig. 5.3., membrane ultrafiltration influenced the antihypertensive properties of the <1 kDa FPH fractions at both the 2.5% and 3% enzyme concentrations as evidenced by the considerable increase in ACE inhibition (up to 90%) relative to the inhibition of ACE activity by the unfractionated protein hydrolysate samples (Section 5.2.1). This level of ACE inhibition by the <1 kDa peptide fraction is the highest recorded both for the unfractionated protein hydrolysate and the membrane fractions, and is significantly ($P < 0.05$) higher than the ACE-inhibitory activity of the 1–3 kDa, the 3–5 kDa and the 5–10 kDa fractions. No significant difference was observed in the ACE-inhibitory activity of the <1 kDa peptide fractions obtained from 2.5% FPH (2.5% FPH* < 1 kDa) or 3.0% FPH concentrations (3% FPH* < 1 kDa), which indicates that peptide size at the <1 kDa level played a greater role in ACE inhibition than the amount of enzyme used for proteolysis. For the 3 kDa molecular weight cut-off (MWCO) membrane, peptide fractions from the 3% enzyme-derived hydrolysate (3% FPH* 1–3 kDa) displayed a significantly ($P < 0.05$) higher inhibition of ACE than the corresponding peptide fractions from the 2.5% enzyme hydrolysate (2.5% FPH* 1–3 kDa).

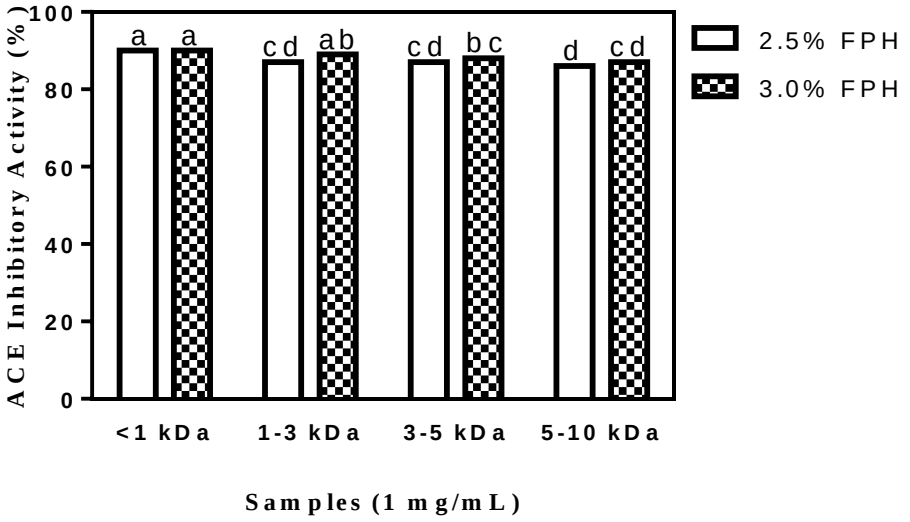

Fig. 5.3. Percentage (mean ± standard error) of ACE-inhibitory activity of flaxseed protein hydrolysate (FPH) membrane ultrafiltration fractions after hydrolysis of isolated flaxseed proteins with 2.5% or 3.0% thermoase GL-30 and passing the hydrolysates through molecular weight cut-offs (MWCOs) of 1, 3 ,5 and 10 kDa. Bars with different letters have mean values that are significantly different ($P < 0.05$).

As the MWCO increased from 3 kDa to 10 kDa, the ability of the fractions to inhibit ACE activity markedly decreased although the amount of enzyme used for digestion remained the same. While increasing MWCO and the correspondingly larger peptide sizes clearly resulted in a decrease in peptide fraction ACE inhibitory potential, interestingly, there was no significant difference between the 5–10 kDa peptide fraction at the 3% enzyme concentration (3% FPH* 5–10 kDa) and those of 1–3 kDa and 3–5 kDa at the 2.5% enzyme concentration (2.5% FPH* 1–3 kDa and 2.5% FPH* 3–5 kDa, respectively), which indicates a lack of influence at high enzyme concentrations.

The ACE inhibition of 90% shown by the two <1 kDa fractions in Fig.5.3 is similar to previously reported results of ≈84% and 88%, respectively, for <1 kDa membrane fractions from 4% thermolysin- and 4% proteinase K-digested rapeseed proteins [21], comparable to results obtained with peptide fractions of the same size from chicken thigh skin protein hydrolysate at 3% alcalase [12] concentration (≈82% inhibition) but significantly ($P < 0.05$) higher than the

≈78% inhibition of <1 kDa membrane fractions obtained following the hydrolysis of chicken breast skin protein with 1% pepsin-pancreatin [12]. The 2.5% FPH* 1–3 KDa peptide fractions from the current work inhibited ACE activity to a degree similar to the 86% inhibition reported for peptide fractions of an identical size from 4% thermolysin-hydrolyzed rapeseed proteins but they were significantly ($P < 0.05$) more potent ACE inhibitors than peptide fractions 1–3 kDa in size from both 3% alcalase-digested chicken thigh skin protein hydrolysate (with ACE inhibitory activity of ≈62%) and chicken breast skin protein hydrolysed using 1% pepsin-pancreatin (≈68% inhibition) [12]. Similarly, at inhibition percentages ranging from 86% to 88%, the 3–5 kDa and 5–10 kDa peptide fractions inhibited ACE activity at levels that are comparable to previously reported values of 82%–85% inhibition for 4% thermolysin-digested rapeseed protein hydrolysate fractions of similar sizes [21] but showed significantly higher inhibitory activity than membrane fractions of identical sizes from chicken protein hydrolysate samples [12]. Altogether, the most potent ACE-inhibitory fractions from this study (2.5% FPH* < 1 kDa and 3% FPH* < 1 kDa) showed a degree of inhibition higher than the maximum of 80% inhibition reported for arginine-rich flaxseed cationic fractions at a final assay concentration of 1 mg/mL [23], which may be due to the presence of more hydrophobic and aromatic amino acid residues in the peptide fractions from the current study [24] as shown in Table 5.1.

As Fig. 5.4. shows, renin activity was moderately inhibited by virtually all the membrane fractions. While the renin-inhibitory activities of the 2.5% FPH* 3–5 kDa, 2.5% FPH* 5–10 kDa and 3% FPH* 5–10 kDa fractions ranged from 37% to 38%, the rest of the membrane fractions accomplished at least 40% *in vitro* inhibition of renin activity. The results for membrane fractions that show <40% renin-inhibitory activity are similar to the 36% and 38% inhibition, respectively, reported at the same final assay concentration for the 3–5 kDa fractions from

flavourzyme-hydrolyzed rapeseed proteins and 1–3 kDa membrane fractions from alcalase-hydrolyzed chicken thigh skin proteins.

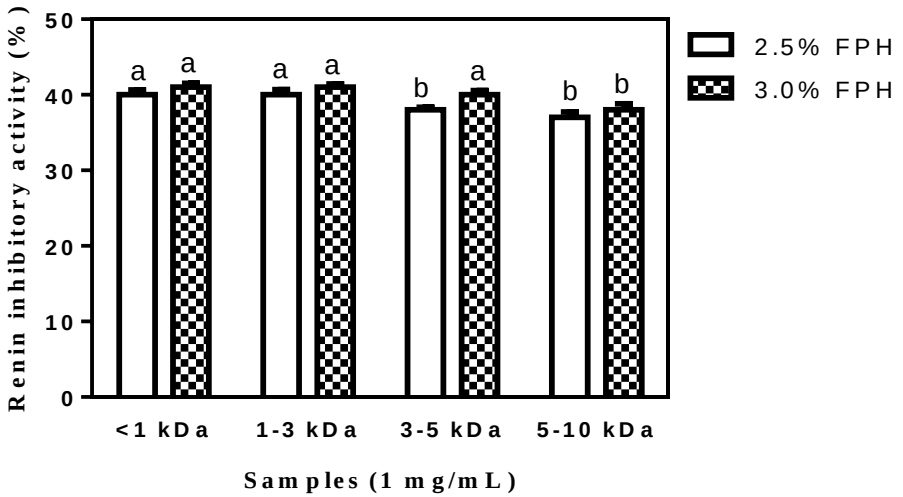

Fig. 5.4. Percentage (mean ± standard error) renin-inhibitory activity of flaxseed protein hydrolysate (FPH) membrane ultrafiltration fractions after hydrolysis of isolated flaxseed proteins with 2.5% or 3.0% thermoase GL-30 and passing the hydrolysates through MWCOs of 1, 3 ,5 and 10 kDa. Bars with different letters have mean values that are significantly different ($P < 0.05$).

Similarly, the >40% renin-inhibitory values are comparable to previous results reported for <1 kDa rapeseed protein hydrolysates and 3–5 kDa chicken thigh skin protein hydrolysates [12,21]. Although no significant difference was observed in the inhibition of renin activity between the <1 kDa and 1–3 kDa fractions at the two different enzyme concentrations used, at 40% renin inhibitory activity, the 3% FPH* 3–5 kDa fraction significantly ($P < 0.05$) inhibited renin activity more than the 38% inhibition observed with the 2.5% FPH* 3–5 kDa fractions. This difference could be attributed to the release of a greater amount of peptides from the native protein structure at the higher enzyme concentration. The results generally suggest higher renin inhibitory activity by the smaller peptides than those larger in size—a trend which has been reported in previous studies [9,21].

Inhibition of renin activity by the 2.5% FPH* < 1 kDa and 3% FPH* < 1 kDa fractions is similar to the 39% and 40% inhibition, respectively, which were previously reported for membrane fractions that contain an identical peptide size obtained from chicken thigh skin proteins digested with 3% alcalase [12] and rapeseed proteins digested with 4% alcalase [21]. The 2.5% FPH* 1–3 kDa and 3% FPH* 1–3 kDa fractions also inhibited renin activity to a degree similar to the 39% renin inhibitory activity accomplished by peptide membrane fractions of an identical size from rapeseed proteins sequentially digested with 4% pepsin and pancreatin [21]. However, the 49% and 57% reported inhibition of renin activity by 1–3 kDa membrane fractions from 4% alcalase- and 4% proteinase K-digested rapeseed proteins respectively are considerably higher than the inhibitory activity shown by fractions of identical size (2.5% FPH* 1–3 kDa and 3% FPH* 1–3 kDa) from the current study [12]. Inhibition of renin activity by the 2.5% FPH* 3–5 kDa and 3% FPH* 3–5 kDa fractions are comparable to the 38% and 42% inhibition respectively reported for fractions of corresponding size obtained from 4% flavourzyme and 4% alcalase hydrolysates of rapeseed [21]. However, the renin-inhibitory activity of 2.5% FPH* 5–10 kDa and 3% FPH* 5–10 kDa fractions was each considerably higher than the 24%, 26% and 23% inhibition respectively accomplished by peptide fractions of the same size from 4% alcalase, 4% pepsin-pancreatin and 4% flavourzyme hydrolysate fractions of rapeseed proteins but substantially lower than the inhibition of 55% and 57% correspondingly shown by 4% proteinase K- and 4% thermolysin-digested rapeseed protein hydrolysate fractions of the same size [21]. A previous work that screened cationic membrane fractions from flaxseed proteins sequentially digested with trypsin and pronase reported a maximum renin inhibition of 44.5% at a peptide final assay concentration of 0.75 mg/mL. In the current work, although the FPH peptide fractions were used at a slightly higher final assay concentration of 1 mg/mL in a

similarly-designed assay, renin-inhibitory values akin to that of the previous work were obtained [23]; the differences may be attributed to variations in the type of amino acid residues present in both fractions [23].

5.2.3 In Vivo Antihypertensive Activity of FPH and FPH Membrane Fractions

As illustrated in Figs. 5.5–5.7, all the FPH and membrane fraction samples administered to the spontaneously hypertensive rats (SHR) significantly ($P < 0.05$) lowered systolic blood pressure (SBP) at all measured time points better than phosphate buffered saline (PBS), thus providing *prima facie* evidence and preliminary confirmation that the test samples do indeed possess *in vivo* antihypertensive effects. The protein hydrolysate which showed the highest SBP reduction (−29 mm Hg after 4 h) was the 3% FPH sample while the unhydrolyzed flaxseed protein isolate (FPI) had the least BP-lowering activity (−4 to −5 mm Hg after 4–6 h) as shown in Fig. 5.5. The SBP-lowering activities of the 2.5% FPH and 3% FPH samples after 2–8 h were comparable to that of captopril (used as a positive control and at a dosage 20 times lower), thus suggesting the potential of the peptide samples to rapidly lower blood pressure on a short term basis. However, the blood pressure-lowering ability of captopril was significantly ($P < 0.05$) better than those of the protein hydrolysates after 24 h of oral administration. Although the hypotensive effect of FPI slightly increased from −2 mm Hg after 2 h to ≈−4 to −5 mm Hg after 4–6 h, the two FPH samples showed significantly ($P < 0.05$) higher SBP decreases than the FPI sample at every time point. The results support the fundamental principle that protein hydrolysates contain more rapidly absorbable peptides than the unhydrolysed protein isolate. Additionally, since proteolysis of native proteins must take place before absorption of any antihypertensive peptides, it is logical to expect weak FPI antihypertensive effect on a short-term basis when compared to FPH samples that contain predigested peptides.

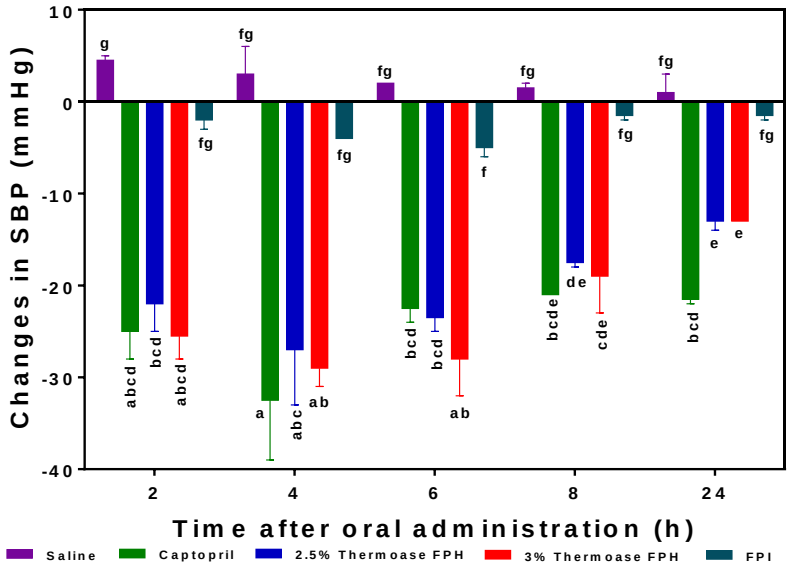

Fig. 5.5. Effects of 2.5% and 3% enzymatic flaxseed protein hydrolysate (FPH), and flaxseed protein isolate (FPI) samples on systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR) after oral gavage. Rats were administered with FPH and FPI using a dose of 200 mg protein/kg rat body weight (BW) while the positive control, captopril was given at 10 mg/kg body weight. Saline was used as negative control. Bars with different letters have mean SBP values that are significantly ($P < 0.05$) different.

The SBP-lowering activity of the two hydrolysate samples were similar but had diminished considerably after 24 h post-oral administration with a maximum value of −13 mmHg, which is still significantly ($P < 0.05$) higher than the maximum effect (−5 mmHg after 6 h) of the unhydrolysed protein (FPI).

In Figs. 5.6 and 5.7, the 2.5% FPH and 3% FPH samples both showed significantly ($P < 0.05$) larger decreases in SBP than some of the corresponding membrane permeates (3–5 kDa and 5–10 kDa) after 2 and 4 h. However, at both the 6 and 8 h marks, the SBP-reducing effect of some of the membrane permeates (1–3 and 3–5 kDa) was significantly ($P < 0.05$) greater than that of their corresponding protein hydrolysates. This could be as a result of the fact that the membrane fractions had longer intrinsic synergistic effects or less antagonistic effects due to the higher level of peptide homogeneity when compared to the high peptide heterogeneity in the

unfractionated protein hydrolysates. Therefore, the protein hydrolysates may be useful ingredients to formulate fast-acting antihypertensive products while the membrane fractions would be suitable for extended-release type of antihypertensive formulations.

Among the peptide fractions from the 2.5% FPH sample (Fig. 5.6.), the hypotensive activities of 2.5% FPH* < 1 kDa and 2.5% FPH* 1–3 kDa tended to be the highest after 2 h indicating their possible ability to lower blood pressure on a short term basis but also suggesting that the larger peptides in the 5 kDa and 10 kDa fractions might not have been as efficiently absorbed as the smaller <1 kDa and 1–3 kDa peptides in such a short amount of time. Alternatively, it is possible that the longer peptides undergo initial hydrolysis to release active fragments that are then subsequently absorbed; this will delay the antihypertensive effect. The significant ($P < 0.05$) increases in the BP-reducing effect of the larger peptides (3–5 kDa and 5–10 kDa) after 4 and 6 h lends credence to this reasoning. Remarkably, all the membrane fractions from both protein hydrolysate samples maintained significant hypotensive effects after 24 h (approximately −8 mm Hg in the least). Contrary to what was observed with the 2.5% FPH fractions, the 3–5 kDa and 5–10 kDa fractions of the 3.5% FPH sample (Fig. 5.7) were very effective in lowering elevated blood pressure in SHR. In fact, unlike in the fractions from the 2.5% FPH sample where 2.5% FPH* 1–3 kDa was responsible for the largest decrease in blood pressure (−34 mm Hg) after 6 h, 3% FPH* 3–5 kDa exerted the highest blood pressure-lowering effect of −37 mm Hg after 8 h among the fractions from the 3% FPH sample. This difference could be as a result of, among other things, the difference in the concentration of peptides liberated by the different enzyme concentrations and additional processing of the peptides in the gastrointestinal tract of the SHR.

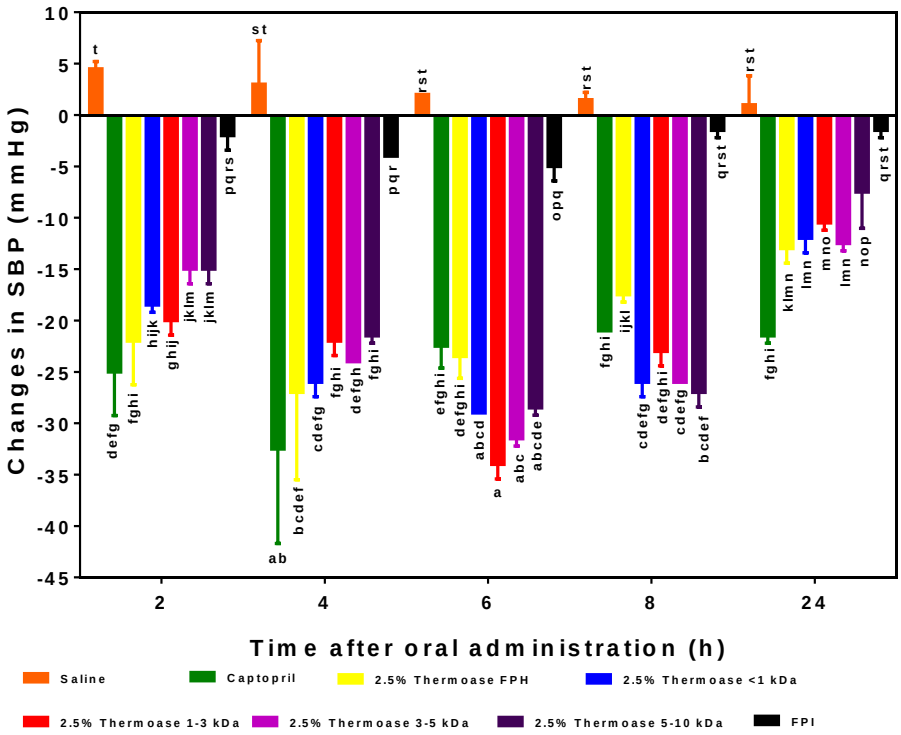

Fig. 5.6. Effects of 2.5% enzymatic flaxseed protein hydrolysate (FPH), membrane ultrafiltration peptide fractions and flaxseed protein isolate (FPI) on systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR) after oral gavage. Rats were administered with FPH, FPH fractions and FPI using a dose of 200 mg protein/kg rat body weight (BW) while the positive control, captopril was given at 10 mg/kg body weight. Saline was used as the negative control. Bars with different letters have mean SBP values that are significantly ($P < 0.05$) different.

Similar results have been obtained with the enzymatic hydrolysate samples of hemp seed protein hydrolysate [9] (after 2 h in comparison to 2.5% FPH at the same time point) and pepsin-digested canola protein hydrolysate after 8 h [20] (compared to 3% FPH after the same number of hours). Although a reduction in SBP of about −15 mm Hg, which is comparable to that of 2.5% FPH after 8 h, was reported with an oral dose of sweet potato protein hydrolysate [25] lower than the 200 mg/kg BW used in this study at the same hour mark, the SBP-lowering effects of the flaxseed protein hydrolysate and membrane fractions used in this study lasted longer (an average of −12 mm Hg after 24 h) when compared to that of the enzyme-digested sweet potato proteins (nearly zero after 24 h).

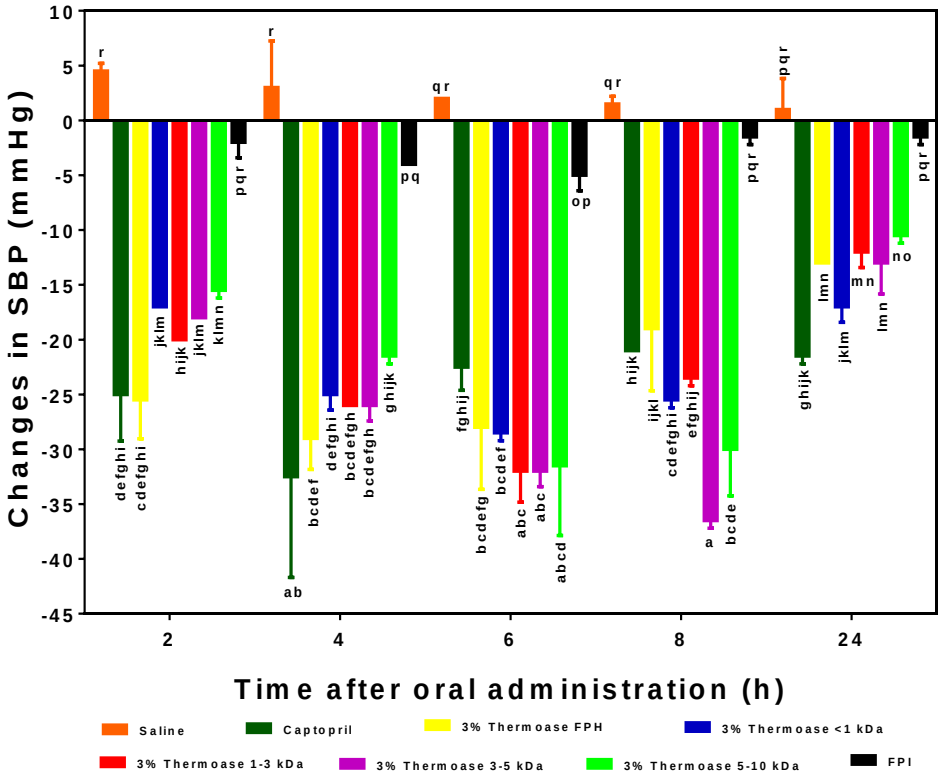

Fig. 5.7. Effects of 3% enzymatic flaxseed protein hydrolysate (FPH), membrane ultrafiltration peptide fractions and flaxseed protein isolate (FPI) samples on systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR) after oral gavage. Rats were administered with FPH, FPH fractions and FPI using a dose of 200 mg protein/kg rat body weight (BW) while the positive control, captopril was given at 10 mg/kg body weight. Saline was used as a blank. Bars with different letters have mean SBP values that are significantly ($P < 0.05$) different.

5.3 Experimental section

5.3.1 Materials

Captopril, cellulase (from *Aspergillus niger*; approximately 0.8 units/mg protein), *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE (from rabbit lung, ≥2.0 units/mg protein) were procured from Sigma-Aldrich (St. Louis, MO, USA). Thermoase GL-30 was from Amano Enzyme Inc. (Nagoya, Japan), while a human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Pulverized, defatted flaxseed meal was obtained from Bioriginal Foods and Science Corporation

(Saskatoon, SK, Canada) while SHR were supplied by Charles River Laboratories (Montreal, PQ, Canada). The ultrafiltration membranes of 1, 3, 5 and 10 kDa MWCO and other analytical grade reagents were procured from Fischer Scientific (Oakville, ON, Canada).

5.3.2 Methods

5.3.2.1 Preparation of Flaxseed Protein Isolate (FPI)

The protein components of defatted flaxseed flour were isolated using an improved modification [6] of the alkali extraction/acid precipitation method [26]. Defatted flaxseed flour (5%, *w*/*v*, dry weight basis) was suspended in ultrapure, deionized water and continuously stirred using a magnetic stirrer until a uniformly dispersed but highly viscous slurry was obtained. The suspension was heated to 37 °C and adjusted to pH 5.0 using 0.5 M HCl. Thereafter, cellulase (1%, *w*/*w*; activity of powder, 1.44 U/mg) was added to the slurry to initiate fibre hydrolysis. After 4 h of reaction, the suspension was cooled to 4 °C and adjusted to pH 10.0 with the addition of 0.5 M NaOH for the alkaline extraction step. The alkaline suspension was mixed for 1 h at room temperature, which was followed by centrifugation at 15,000 g for 30 min; the supernatant was collected and adjusted to pH 4.2 by the gradual step-wise addition of 0.5 M HCl. The supernatant obtained after a second round of centrifugation was then discarded while the precipitated protein curd was washed thrice with acidified distilled water (pH 4.2) before being suspended in a small volume of deionized water. The suspension was subsequently adjusted to pH 7.0 before it was freeze-dried as the FPI.

5.3.2.2 Hydrolysis of Isolated Flaxseed Proteins and Production of Membrane Fractions

After suspending the FPI in deionized water (5%, *w*/*v*, protein basis), the suspension was stirred with a magnetic stirrer, heated to 37 °C and adjusted to pH 8.0 before the addition of thermoase GL-30. Following addition of enzyme to the slurry at the appropriate enzyme-

substrate ratio *E*/*S* (0.5%–3%), the reaction mixture was heated to 37 °C, adjusted to pH 8.0 using 2 M NaOH and stirred for 4 h [6]; temperature and pH were maintained constant during the reaction period. The reaction was terminated when the enzyme was inactivated by immersing the reaction vessel in a boiling water bath for 15 min while the precipitation of undigested proteins was achieved first by using 1 M HCl to adjust the mixture to pH 4.0 followed by centrifugation for 30 min at 15,000 g. The supernatant which contains the peptides of interest was then sequentially passed through ultrafiltration membranes beginning with the MWCO of 1 kDa, and continuing (using the retentate) through those of the 3 kDa, 5 kDa (retentate from 3 kDa) and 10 kDa (retentate from 5 kDa) to obtain permeates with, peptides sizes of <1 kDa, 1–3 kDa, 3–5 kDa and 5–10 kDa, respectively. The permeates were then freeze-dried and stored at −20 °C until used for subsequent tests. The percentage protein content of the FPI, FPH and membrane fractions was determined by a modified Lowry's method using bovine serum albumin as standard [27].

5.3.2.3 ACE-Inhibitory Activity of FPH and Membrane Fractions

The FPH and membrane fractions were assayed for inhibitory activity against ACE as previously described [6, 28]. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was combined with 20 µL ACE (1 U/mL; final activity of 20 mU), and 200 µL of 50 mM Tris–HCl containing 6.1 mg/mL of the appropriate sample (FPH or membrane fraction) for a final peptide concentration of 1 mg/mL. The decreased absorbance at 345 nm, due to ACE-catalyzed cleavage of the Phe-Gly peptide bond of FAPGG, was recorded at regular intervals for 2 min at room temperature using a Varian Cary 50-UV/Visible spectrophotometer (Varian, Victoria, Australia). Tris–HCl buffer was used instead of peptide samples in the blank experiment. All experiments were performed in triplicate. ACE activity was

expressed as rate of disappearance of FAPGG (ΔA/min) and inhibitory activity was calculated as follows:

$$\text{Percentage ACE inhibition} = 1 - [\Delta A \cdot \text{min}^{-1}_{(\text{sample})} / \Delta A \cdot \text{min}^{-1}_{(\text{blank})}] \times 100 \quad (1)$$

Where $\Delta A \cdot \text{min}^{-1}_{(\text{sample})}$ and $\Delta A \cdot \text{min}^{-1}_{(\text{blank})}$ are the reaction rates in the presence and absence of FPH or membrane fractions respectively.

5.3.2.4 Renin-Inhibitory Activity of FPH and Membrane Fractions

The ability of the FPH and membrane fractions to inhibit the activity of human recombinant renin *in vitro* was determined by fluorescence spectrometry using the Renin Inhibitor Screening Assay Kit according to a previously described method [6]. The total assay volume of 190 µL included 19 µL of 10 mg/mL FPH or membrane fraction which had earlier been dissolved in 50 mM Tris–HCl buffer containing 100 mM NaCl (pH 8.0), 10 µM Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-Arg (renin substrate dissolved in dimethyl sulphoxide), and human recombinant renin. Tris–HCl buffer was used instead of the flaxseed peptide solution in the blank experiment while each sample well contained a final peptide concentration of 1 mg/mL. The 96-well plate containing the various thoroughly mixed reaction mixtures was pre-warmed to 37 °C for 15 min to attain thermal equilibrium before monitoring the periodic increases in fluorescence intensity using a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths set at 340 nm and 490 nm, respectively. The percentage inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = [(\text{FIU} \cdot \text{min}^{-1}_{(\text{blank})} - \text{FIU} \cdot \text{min}^{-1}_{(\text{sample})} / \text{FIU} \cdot \text{min}^{-1}_{(\text{blank})}] \times 100 \quad (1)$$

where $\text{FIU} \cdot \text{min}^{-1}_{(\text{blank})}$ and $\text{FIU} \cdot \text{min}^{-1}_{(\text{sample})}$ are the fluorescent intensity in the absence and presence of peptides, respectively.

5.3.2.5 Amino Acid Composition Analysis

The amino acid profiles of FPH samples were determined using an HPLC Pico-Tag method following the digestion of samples with 6 M HCl [29]. The cysteine and methionine contents were determined after oxidation with performic acid [30] while the samples were analyzed for tryptophan content following alkaline hydrolysis [31].

5.3.2.6 Evaluation of FPH and Membrane Fractions for Antihypertensive Activity in Spontaneously Hypertensive Rats (SHR)

All animal experiments were conducted according to protocol F011-015/1/2, which was approved by the University of Manitoba Animal Protocol and Management Review Committee, as prescribed by the guidelines of the Canadian Council on Animal Care Ethics. Male SHR (20-week-old; 250–300 g body weight, BW) with tail SBP of over 150 mmHg were kept at the Animal Care Facility of the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba. Rats were housed individually using steel cages in a room maintained under a 12 h light-dark cycle, temperature of 23 °C (±2 °C) and relative humidity of 50%. The SHR were fed a standard chow diet and tap water *ad libitum* before and after oral gavage. FPI (from Section 3.2.1), 2.5% FPH and 3.0% FPH samples obtained by the digestion of FPI with 2.5% and 3.0% thermoase GL-30, respectively, the membrane fractions (<1 kDa, 1–3 kDa, 3–5 kDa and 5–10 kDa in size) from the ultrafiltration of each of the two FPH samples, as well as captopril were each dissolved in phosphate-buffered saline (PBS), pH 7.2. The samples (each at 200 mg/kg BW), PBS (used as negative control), and captopril (10 mg/kg BW; used as positive control) were administered to the SHR by oral gavage followed by measurement of systolic blood pressure (SBP) by tail-cuff plethysmography (Mouse Rat Tail Cuff Blood Pressure System, IITC Life Science, Woodland Hills, CA, USA) at 2, 4, 6, 8 and 24 h in rats mildly

anesthetized for 4 min with 4% isoflurane [9,32]. The gas flow chamber, maintained at 37 °C, was designed to include positive pressure ventilation in order to sustain independent ventilatory function and attenuate isoflurane-induced depression of spontaneous ventilation in the animals. The entire procedure was optimized to ensure that the SHR regained consciousness within 3–4 min of being taken out of the chamber, thus giving enough time for the measurement of blood pressure while the animals were still chemically restrained, and ensuring negligible or no depression of SBP by the inhalational anesthetic agent. Baseline (time zero) SBP was measured before the administration of samples, and the change in SBP (ΔSBP, mm Hg) was calculated by subtracting the data for the different time points from their respective baseline data.

5.3.3 Statistical Analysis

All assays were conducted in replicates of three and data are reported as mean ± standard deviation. Values were tested for statistical significance of difference using Duncan's multiple range test ($P < 0.05$) with the Statistical Analysis Systems, SAS, software version 9.2 (SAS, Cary, NC, USA).

5.4 Conclusions

The digestion of isolated flaxseed proteins using various concentrations of thermoase yielded SBP-lowering peptides with great potential for use in the formulation of antihypertensive nutraceuticals. The in vivo and in vitro hypotensive effects of the samples were influenced by the concentration of enzyme used for protein hydrolysis as well as the peptide size. Although the samples showed very high ACE-inhibitory activity, their inhibition of renin activity was only moderate. Nevertheless, both the unfractionated hydrolysate and the peptide fractions significantly ($P < 0.05$) lowered SBP in SHR from 2–24 h after oral administration, highlighting their potential to provide fast relief from elevated blood pressure on a short term basis.

Furthermore, the strong SBP-reducing effect of the unfractionated hydrolysate samples also suggests that while membrane purification may increase their potency (as seen with the 2.5% FPH* 1–3 kDa and 3% FPH* 3–5 kDa fractions), subjecting them to further purification could be avoided as a cost-saving measure during formulation of functional foods and nutraceuticals. Overall, the 3% FPH would be preferred as the best blood pressure-reducing agent among all the samples, simply because of the higher potency when compared to 2.5% FPH; the higher potency could enable lower dosage and hence lower cost of the 3% FPH.

5.5 Acknowledgements

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5.6 Author contributions

Rotimi E. Aluko conceived and designed the experiments, provided all materials and reagents used and edited the final draft of the manuscript; the *in vivo* measurement of SBP in SHR was performed by Abraham T. Girgih, Ifeanyi D. Nwachukwu, John O. Onuh and Sunday A. Malomo; Abraham T. Girgih and Ifeanyi D. Nwachukwu screened samples for *in vitro* antihypertensive properties; Ifeanyi D. Nwachukwu analyzed the amino acid composition data and wrote the paper.

5.7. Conflicts of interest

The authors declare no conflict of interest.

5.8 References

1. Lawes, C.M.; Hoorn, S.V.; Rodgers, A. Global burden of blood-pressure-related disease, 2001. *Lancet* **2008**, *371*, 1513–1518.
2. Lim, S.S.; Vos, T.; Flaxman, A.D.; Danaei, G.; Shibuya, K.; Adair-Rohani, H.; AlMazroa, M.A.; Amann, M.; Anderson, H.R.; Andrews, K.G.; *et al.* A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: A systematic analysis for the global burden of disease study 2010. *Lancet* **2012**, *380*, 2224–2260.
3. Atlas, S.A. The renin-angiotensin aldosterone system: Pathophysiological role and pharmacologic inhibition. *J. Manag. Care Pharm*. **2007**, *13*, S9–S20.
4. Chen, J.; Liu, S.; Ye, R.; Cai, G.; Ji, B.; Wu, Y. Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: Purification and characterization. *J. Funct. Food*s **2013**, *5*, 1684–1692.
5. Fengjuan, L.; Takahashi, Y.; Yamaki, K. Inhibitory effect of catechin-related compounds on renin activity. *Biomed. Res*. **2013**, *34*, 167–171.
6. Udenigwe, C.C.; Lin, Y.; Hou, W.; Aluko, R.E. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *J. Funct. Food.* **2009**, *1*, 199–207.
7. Yang, H.Y.T.; Erdös, E.G.; Levin, Y. A dipeptidyl carboxypeptidase that converts angiotensin-I and inactivates bradykinin. *Biochim. Biophys. Acta* **1970**, *214*, 374–376.
8. Segall, L.; Covic, A.; Goldsmith, D.J.A. Direct renin inhibitors: the dawn of a new era, or just a variation on a theme? *Nephrol. Dial. Transpl*. **2007**, *22*, 2435–2439.
9. Girgih, A.T.; Udenigwe, C.C.; Li, H.; Adebiyi, A.P.; Aluko, R.E. Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates. *J. Am. Oil Chem. Soc.* **2011**, *88*, 1767–1774.
10. Mäkinen, S.; Johannson, T.; Vegarud Gerd, E.; Pihlava, J.M.; Pihlanto, A. Angiotensin I-converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. *J. Funct. Foods* **2012**, *4*, 575–583.
11. Martínez-Maqueda, D.; Miralles, B.; Recio, I.; Hernández-Ledesma, B. Antihypertensive peptides from food proteins: A review. *Food. Funct.* **2012**, *3*, 350–361.
12. Onuh, J.O.; Girgih, A.T.; Aluko, R.E.; Aliani, M. Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Res. Int.* **2013**, *53*, 260–267.
13. Hall, C., III; Tulbek, M.C.; Xu, Y. Flaxseed. *Adv. Food Nutr. Res.* **2006**, *51*, 1–97.
14. Doyen, A.; Udenigwe, C.C.; Mitchell, P.L.; Marette, A.; Aluko, R.E.; Bazinet, L. Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions

revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. *Food Chem*. **2014**, *145*, 66–76.

15. Marambe, P.W.M.L.H.K.; Shand, P.J.; Wanasundara, J.P.D. An *in vitro* investigation of selected biological activities of hydrolysed flaxseed (*Linum usitatissimum* L.) proteins. *J. Am. Oil Chem. Soc.* **2008**, *85*, 1155–1164.
16. Xu, Y.; Hall, C., III; Wolf-Hall, C. Antifungal activity stability of flaxseed protein extract using response surface methodology. *J. Food Sci*. **2008**, *73*, M9–M14.
17. Oomah, B.D.; Mazza, G. Functional properties, uses of flaxseed protein. *Inform* **1995**, *6*, 1246–1252.
18. Udenigwe, C.C.; Aluko, R.E. Antioxidant and angiotensin converting enzyme-inhibitory properties of a flaxseed protein-derived high fischer ratio peptide mixture. *J. Agric. Food Chem*. **2010**, *58*, 4762–4768.
19. Keil, B. *Specificity of Proteolysis*; Springer-Verlag: Berlin, Germany, 1992; p. 336.
20. Alashi, A.M.; Blanchard, C.L.; Mailer, R.J.; Agboola, S.O.; Mawson, A.J.; He, R.; Malomo, S.A.; Girgih, A.T.; Aluko, R.E. Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. *Food Res. Int.* **2014**, *55*, 281–287.
21. He, R.; Alashi, A.; Malomo, S.A.; Girgih, A.T.; Chao, D.; Ju, X.; Aluko, R.E. Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chem*. **2013**, *141*, 153–159.
22. Ajibola, C.F.; Fashakin, J.B.; Fagbemi, T.N.; Aluko, R.E. Renin and angiotensin converting enzyme inhibition with antioxidant properties of african yam bean protein hydrolysate and reverse-phase HPLC-separated peptide fractions. *Food Res. Int*. **2013**, *52*, 437–444.
23. Udenigwe, C.C.; Adebiyi, A.P.; Doyen, A.; Li, H.; Bazinet, L.; Aluko, R.E. Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. *Food Chem*. **2012**, *132*, 468–475.
24. Wu, J.; Aluko, R.E.; Nakai, S. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship study of di- and tripeptides. *J. Agric. Food Chem*. **2006**, *54*, 732–738.
25. Ishiguro, K.; Sameshima, Y.; Kume, T.; Ikeda, K.; Matsumoto, J.; Yoshimoto, M. Hypotensive effect of a sweetpotato protein digest in spontaneously hypertensive rats and purification of angiotensin I-converting enzyme inhibitory peptides. *Food Chem.* **2012**, *131*, 774–779.
26. Dev, D.K.; Quensel, E. Preparation and functional properties of linseed protein products containing differing levels of mucilage. *J. Food Sci.* **1988**, *53*, 1834–1837.
27. Markwell, M.A.K.; Haas, S.M.; Bieber, L.L.; Tolbert, N.E. A modification of the lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem*. **1978**, *87*, 206–210.

28. Holmquist, B.; Bunning, P.; Riordan, J.F. A continuous spectrophotometric assay for angiotensin converting enzyme. *Anal. Biochem*. **1979**, *95*, 540–548.
29. Bidlingmeyer, B.A.; Cohen, S.A.; Tarvin, T.L. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr. Biomed. Appl*. **1984**, *336*, 93–104.
30. Gehrke, C.W.; Wall Sr., L.L.; Absheer, J.S. Sample preparation for chromatography of amino acids: Acid hydrolysis of proteins. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 811–821.
31. Landry, J.; Delhaye, S. Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *J. Agric. Food Chem.* **1992**, *40*, 776–779.
32. Aukema, H.M.; Gauthier, J.; Roy, M.; Jia, Y.; Li, H.; Aluko, R.E. Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. *Mol. Nutr. Food Res*. **2011**, *55*, 1044–1051.

5.9. TRANSITION STATEMENT THREE

The preceding chapter investigated the blood pressure lowering properties of flaxseed-derived hydrolysates and bioactive peptides and discussed their potential use in antihypertensive formulations. In the study, the food grade protease, thermoase GL-30, was carefully selected for the production of antihypertensive hydrolysates and bioactive peptide fragments based on its well-known proclivity for releasing potentially antihypertensive peptides. Following hydrolysis, the digest was separated by means of ultrafiltration into peptide fractions of various chain lengths (<1, 1-3, 3-5, and 5-10 kDa). Given the central role of angiotensin-converting-enzyme (ACE) and renin in the blood pressure-regulating renin-angiotensin system, *in vitro* tests demonstrating the inhibitory effect of flaxseed protein hydrolysates against both enzymes represented a preliminary proof of concept. Subsequent *in vivo* tests using spontaneously hypertensive rats showed that the most active flaxseed protein hydrolysates lowered systolic blood pressure by up to 37 mmHg 8 hr post-gavage.

Given the widely recognized relationship between hypertension and oxidative stress (e.g. sustained production of excessive ROS in the central nervous system, kidney and vasculature contributes to hypertension), it was imperative to study the possible antioxidative properties of flaxseed-derived protein hydrolysates and bioactive peptides. In particular, it was crucial to examine the influence of peptide sequence, size and enzyme concentration on the antioxidant potency of the hydrolysates and peptide fragments hence the need for the study (Manuscript 4) reported in the next chapter.

CHAPTER 6

MANUSCRIPT FOUR

ANTIOXIDANT PROPERTIES OF FLAXSEED PROTEIN HYDROLYSATES: INFLUENCE OF HYDROLYTIC ENZYME CONCENTRATION AND PEPTIDE SIZE

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6.0 Abstract

Flaxseed is an emerging source of protein raw materials for bioactive protein hydrolysate production; however, there is insufficient information on the structural properties of its antioxidative peptides. The aim of this work was to determine the peptide composition and relationships to antioxidant properties of flaxseed protein hydrolysates (FPH) obtained using two protease (thermoase) concentrations (2.5 and 3.0%). The FPHs were passed through ultrafiltration membranes to yield fractions with < 1, 1-3, 3-5, and 5-10 kDa peptide sizes, which were also tested for antioxidant properties. Mass spectrometry data yielded several peptide sequences located within the flaxseed conlinin but a lesser number was found in the 3.0% thermoase-FPH. DPPH radical scavenging was lowest (54%) for the 3.0% thermoase-FPH while membrane separation enhanced the values up to 65% for the <1 kDa peptides. Metal chelation and iron reducing ability were also significantly (P < 0.05) enhanced after membrane fractionation. In contrast, the hydroxyl radical scavenging ability was unchanged (2.5% thermoase-FPH) or significantly (P < 0.05) diminished (3.0% thermoase-FPH) after membrane fractionation, except for the 5-10 kDa peptides that had notably (P < 0.05) higher values. We conclude that the < 1 and 1-3 kDa peptides may be useful agents to suppress oxidative stress within living tissues while the 3-5 and 5-10 kDa could be suitable preservatives to enhance food shelf life.

Keywords: Flaxseed; Antioxidant peptides; Peptide sequence; Thermoase; Protein hydrolysates; Membrane ultrafiltration.

6.1 Introduction

The role of plants as natural reservoirs of free radical scavengers such as anthocyanins, carotenoids, flavonoids, dietary glutathione and vitamins is well-studied and long-established (Choi et al., 2002). However, recent reports have shown that short-chain peptides liberated from food proteins by microbial fermentation or enzymatic hydrolysis also possess various antioxidative properties that include free radical scavenging, metal ion chelation, inhibition of lipid peroxidation, and reduction of ferric iron to the ferrous form (Babini, Tagliazucchi, Martini, Più, & Gianotti, 2017; Torres-Fuentes, del Mar Contreras, Recio, Alaiz, & Vioque, 2015). The initial product of enzyme hydrolysis is called a protein hydrolysate because it contains several peptides that differ in chain length, amino acid sequence and bioactive potency (Aluko, 2015). A common practice in refining protein hydrolysates involves the use of membrane ultrafiltration to separate the peptides into different molecular size fractions (Kimatu et al., 2017; Saisavoey, Sangtanoo, Reamtong, & Karnchanatat, 2016). These membrane-separated peptide fractions may or may not have better bioactive properties than the original protein hydrolysate (Kimatu et al., 2017). For example, hemp seed peptide fractions had weaker antihypertensive properties than the protein hydrolysate (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011). In contrast, ultrafiltered soybean peptide fractions had stronger antioxidant properties than the unfractionated hydrolysate (Park, Lee, Baek, & Lee, 2010). Moreover, peptide size is also an important determinant of bioactive potency; smaller peptides (< 1 kDa) from bean protein hydrolysates were shown to have the strongest inhibitory activity against angiotensin converting enzyme than peptides with >1 kDa sizes (Ruiz-Ruiz, Davila-Ortiz, Chel-Guerrero, & Betancur-Ancona, 2013). Similarly, shorter chain (< 3 kDa) soybean protein-derived peptides had better antioxidant properties than longer chain (> 30 kDa) peptides (Park et al., 2010).

Flaxseed (*Linum usitatissimum*), which is also known as linseed, has a long history of use mainly as a source of industrial oil for the paint industry but also as human food (Hall, Tulbek, & Xu, 2006). However, in addition to their nutritional value, recent works have indicated the potential use of flaxseed proteins as sources of bioactive peptides such as angiotensin-converting enzyme inhibitors and free radical scavengers (Marambe, Shand, & Wanasundara, 2008; Marambe, Shand, & Wanasundara, 2011; Udenigwe & Aluko, 2010). The multifunctional nature of flaxseed protein-derived peptides is also evident in studies that have demonstrated potential health-promoting functions such as antimicrobial (Hwang, Chen, Luo, & Chiang, 2016), antihypertensive (Doyen et al., 2014; Nwachukwu, Girgih, Malomo, Onuh, & Aluko, 2014), calmodulin-inhibiting (Udenigwe & Aluko, 2012), anti-inflammatory (Udenigwe, Lu, Han, Hou, & Aluko, 2009), antioxidant (Hwang et al., 2016; Udenigwe, Lu, et al., 2009), and anti-diabetic (Doyen et al., 2014) properties. Thus, flaxseed protein-derived peptides can serve as useful ingredients in the production of functional foods and nutraceuticals targeted at preventing and/or delaying the onset of certain human diseases.

Free radicals such as reactive oxygen species (ROS) include hydrogen peroxide, hydroxyl radicals and superoxide anion radicals that are produced in the course of normal cellular metabolism (Treml & Šmejkal, 2016). These ROS possess the potential to induce oxidative damage to biological macromolecules that include lipids, proteins and nucleic acids (Treml & Šmejkal, 2016). Left unchecked, especially during oxidative stress-mediated depletion of natural cellular antioxidants, such radical-mediated injury could eventually result in chronic degenerative and debilitating diseases like hypertension, atherosclerosis, ageing, cancer, diabetes mellitus, and inflammation-related tissue damage (Chigurupati et al., 2017; Erdmann, Cheung, & Schröder, 2008; Nabha, Garbern, Buller, & Charpie, 2005; Treml & Šmejkal, 2016). For

example, toxic ROS cause a wide range of biological injury to low density lipoprotein (LDL) resulting in increased atherogenicity of oxidized LDL and could also trigger or contribute to the pathological progression of cancer, atherosclerosis, Alzheimer's disease, and diabetes among others (Erdmann et al., 2008; Shibata et al., 2016; Treml & Šmejkal, 2016). In conditions of low levels of cellular antioxidants, supplementation with exogenous (dietary) antioxidants such as peptides could reduce the damaging effects of ROS.

A previous work from our laboratory showed that flaxseed proteins can be hydrolyzed and fractionated into hydrolysates with high contents of branched-chain amino acids (BCAA) (Udenigwe & Aluko, 2010). The BCAA-rich hydrolysate exhibited free radical scavenging activity and inhibited linoleic acid oxidation. Flaxseed protein-derived peptides have also demonstrated inhibitions of calmodulin-dependent phosphodiesterase (Udenigwe & Aluko, 2012) and nitric oxide synthases (Omoni & Aluko, 2006a; Omoni & Aluko, 2006b). In addition, oral administration of flaxseed protein hydrolysates (FPH) to spontaneously hypertensive rats led to blood pressure reductions (Doyen et al., 2014; Nwachukwu et al., 2014). However, there is scant information on the potential use of ultrafiltration to enhance antioxidant properties of flaxseed protein-derived peptides. Moreover, fundamental scientific information on the potential role of amino acid sequence in the antioxidant properties of flaxseed peptides is lacking. In our initial work (Nwachukwu et al., 2014), FPHs were produced using 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% thermoase concentrations followed by determination of antihypertensive properties. We found that the 2.5% and 3.0% peptide fractions possessed the strongest blood pressure-reducing properties. Therefore, these two FPHs and their peptide fractions were chosen for the current work because antioxidants could reduce the risk of oxidative stress-induced hypertension (Nabha et al., 2005). Knowledge of the antioxidant properties of the FPHs could enhance their future use

not only as free radical scavengers in foods or living tissues but as preventive or treatment tools for systemic hypertension. Therefore, the aim of this work was to determine the antioxidant properties of different peptide fractions recovered from ultrafiltration. The work also determined the contributions of amino acid sequence in protein hydrolysates produced at two different enzyme concentrations in order to add to current understanding of the influence of enzyme concentration on the antioxidant properties of such hydrolysates/peptides.

6.2 Materials and Methods

6.2.1 Materials

Defatted flaxseed protein meal (FPM) was obtained from Bioriginal Foods and Science Corporation (Saskatoon, SK, Canada). Reduced glutathione (GSH), cellulase (from *Aspergillus niger*), ethylenediaminetetraacetic acid (EDTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Triton X-100, ferrous sulphate, hydrogen peroxide, potassium ferricyanide, 1,10-phenanthroline, trichloroacetic acid (TCA), ferrous chloride, pyrogallol (1,2,3-trihydroxybenzene), ammonium thiocyanate, and 3-(2-pyridyl)-5, 6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thermoase GL-30 was obtained from Amano Enzyme USA, Co., Ltd (Elgin, IL, USA). The ultrafiltration membranes with 1, 3, 5 or 10 kDa molecular weight cut-offs (MWCO) and other analytical grade reagents were from Fisher Scientific (Oakville, ON, Canada).

6.2.2 Production of Flaxseed Protein Isolate

FPI was prepared from defatted FPM using the original method of Dev and Quensel (1988) as modified by Udenigwe, Lin, Hou, and Aluko (2009). Briefly, FPM (5% w/v, protein basis in 500 mL batches) was dispersed and stirred (300 rpm) on an IKA C-MAG HS 7 hotplate (IKA® Works Inc., Wilmington, NC, USA) continuously for 30 min in deionized water in order to

obtain a uniform suspension. The FPM suspension was first heated to 37 °C and then adjusted to pH 5.0 with 0.5 M HCl, followed by cellulase (1%, w/w) addition for fibre hydrolysis. After 4 h of cellulase reaction, the suspension was cooled to 4 °C and adjusted to pH 10.0 using 0.5 M NaOH. The alkaline suspension was stirred for 1 h at 25 °C followed by centrifugation at 15000 *g* and 4 °C for 30 min. The supernatant was adjusted to pH 4.2 by addition of 0.5 M HCl solution, which led to protein precipitation. The precipitate was collected after a second round of centrifugation was performed as described and washed with acidified (pH 4.2) deionized water to remove non-protein impurities. The washed precipitate was then recovered after another centrifugation round as described, dispersed in deionized water, adjusted to pH 7.0, and finally freeze-dried. The protein isolation procedure was performed in triplicate and the freeze-dried isolates were subsequently combined prior to enzymatic hydrolysis. The yield is about 30% on protein weight basis while the flaxseed meal contains about 36 % protein.

6.2.3 Enzymatic Hydrolysis of FPI and Preparation of Membrane Fractions

FPI was dispersed in deionized water (5 g in 100 mL, protein weight basis) and stirred (300 rpm) on an IKA C-MAG HS 7 hotplate continuously for 30 min to obtain a uniform suspension. The FPI suspension was heated to 37 °C and adjusted to pH 8.0 with 0.5 M NaOH. Thermoase GL-30 was added at 2.5% or 3.0% (based on FPI protein weight) as previously described (Nwachukwu et al., 2014). Following enzyme addition, the protein digestion was performed for 4 h with temperature and pH maintained constant using a thermostat-equipped hotplate and periodic addition of 0.5 M NaOH, respectively. At the end of 4 h, the reaction was terminated by adjusting the mixture to pH 4.0 (precipitates undigested proteins) and heating to 95 °C for 15 min (inactivates thermoase). Thereafter, the suspension was centrifuged (30 min, 15000*g*, 4 °C) and some of the supernatant (approximately 75 mL) was freeze-dried as the flaxseed protein

hydrolysate (FPH). The remaining supernatant was first passed through a 1 kDa MWCO ultrafiltration membrane; permeate was collected as the < 1 kDa peptide fraction. The retentate was passed through a 3 kDa membrane to collect the permeate as 1-3 kDa peptides. Similarly, the 3 kDa retentate was passed through a 5 kDa membrane (permeate collected as the 3-5 kDa peptides), whose retentate was then finally passed through the 10 kDa membrane from which the permeate was collected as the 5-10 kDa peptides. The permeates were then lyophilized and stored at −20 °C until required for further analyses. The protein content (%) of the FPI, FPH and membrane fractions was determined using the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978).

6.2.4 DPPH Radical Scavenging Activity

DRSA of the 2.5 and 3.0% thermoase-FPH samples and their ultrafiltration fractions were determined using a previously described method (Udenigwe, Lu, et al., 2009). The GSH standard and peptide samples were separately dissolved in 0.1 M sodium phosphate buffer (pH 7.0), which contained 1% (w/v) Triton X-100. A 100 µL aliquot of 100 µM DPPH in methanol was added in the dark to 100 µL of each peptide sample or GSH solution in a 96-well clear flat bottom plate to give a final sample assay concentration of 1 mg peptide/mL. The blank control wells contained only sodium phosphate buffer and DPPH. The microplate was incubated in the dark for 30 min at room temperature followed by absorbance measurements at 517 nm for the blank (A_b) and samples (A_s). The DPPH radical scavenging activity (DRSA) of the samples and GSH was calculated using the following equation:

DPPH radical scavenging activity (%) = $[(A_b-A_s)/A_b] \times 100$

6.2.5 Hydroxyl Radical Scavenging Activity

FPHs and ultrafiltration fractions were tested for HRSA according to a previously reported method (de Avellar et al., 2004). The protein samples and GSH were separately dissolved in 0.1 M sodium phosphate buffer (pH 7.4). Thereafter, 50 µL of each sample or GSH (equivalent to a final assay concentration of 1 mg peptide/mL) or buffer (blank) was added to a clear, flat bottom 96-well plate followed by the addition of 50 µL 1,10-phenanthroline (3 mM) and 50 µL $FeSO_4$ (3 mM). The Fenton reaction was initiated by addition of 50 µl 0.01% hydrogen peroxide to each well followed by incubation at 37 °C for 1 h with constant shaking. Absorbance at 536 nm was read at 10 min intervals for 1 h using a spectrophotometer to give $\Delta A/min_b$ and $\Delta A/min_s$ for blank and samples, respectively. The following equation was then used to calculate the HRSA:

$$HRSA\ (\%) = \{[(\Delta A/min)_b - (\Delta A/min)_s]/(\Delta A/min)_b\} \times 100.$$

6.2.6 Superoxide Radical Scavenging Activity

SRSA of the samples was determined using a previously described method (Xie, Huang, Xu, & Jin, 2008). GSH and peptide samples were each separately dissolved in 50 mM Tris–HCl buffer (pH 8.3) containing 1 mM EDTA. Thereafter, 80 µL of sample or GSH (1 mg peptide/mL final assay concentration) was combined with 80 µL of the same buffer in a clear bottom 96-well plate under light protection, while Tris–HCl buffer was used in place of peptide samples for the blank experiment. Subsequently, 40 µL of 1.5 mM pyrogallol (dissolved in 10 mM HCl) was added to each microplate well. The rate of superoxide radical-induced pyrogallol polymerization ($\Delta A/min_s$ and $\Delta A/min_b$ for sample and blank, respectively) was then determined as absorbance increases at 420 nm for 4 min at 25 °C. The SRSA was calculated as follows:

$$SRSA\ (\%) = \{[(\Delta A/min)_b - (\Delta A/min)_s]/(\Delta A/min)_b\} \times 100.$$

6.2.7 Chelation of Metal Ions

The metal ion chelation capacity of peptide samples was evaluated using a previously reported method (Xie et al., 2008), which was slightly modified as follows: each sample or GSH was dissolved in double distilled water to a final assay concentration of 1 mg/mL after which a 1 mL aliquot was added to 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL of double distilled water in a test tube. For the blank, 1 mL of double distilled water was used in place of the peptide samples. A 100 µL aliquot of 5 mM Ferrozine solution was then added to each tube and vortexed. The tubes were incubated at room temperature for 10 min after which a 200 µL aliquot of each mixture was transferred into a clear bottom 96-well plate. Absorbance values of the blank (A_b) and samples (A_s) were determined at 562 nm and percentage metal ion chelating effect calculated as follows:

Metal chelating effect (%) = $[(A_b-A_s)/A_b] \times 100$

6.2.8 Ferric Reducing Antioxidant Power

The iron reducing ability of flaxseed peptides was measured using a previously reported method (Zhang, Wang, & Xu, 2008), which was slightly modified as follows. Briefly, 2.5 mL of each sample or GSH (1 mg peptide/mL final assay concentration in 0.2 M sodium phosphate buffer, pH 6.6) was combined with 2.5 mL of buffer and 2.5 mL of 1% (w/v) potassium ferricyanide. Sodium phosphate buffer was used in place of peptide solution in the blank experiment. Following incubation of the mixtures for 20 min at 50 °C, 2.5 mL of 10% TCA was added to each tube and vortexed. Thereafter, 2.5 mL of the resultant mixture was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride in a test tube and incubated for 10 min at room temperature. Each mixture was then centrifuged (5000 *g*, 5 min, 25 °C) and the supernatant (200 uL) transferred into a clear bottom 96-well plate followed by absorbance measurement at 700 nm.

6.2.9 Mass Spectrometry and Peptide Sequence Determination

MS analysis of the samples was performed using a previously reported protocol (Malomo & Aluko, 2016). Briefly, a 10 ng/µL aliquot of the sample (dissolved in an aqueous solution of 0.1% formic acid) was infused into an Absciex QTRAP® 6500 mass spectrometer (Absciex Ltd., Foster City, CA, USA) coupled with an electrospray ionization (ESI) source. Operating conditions were 3.5 kV ion spray voltage at 150 ^{o}C, and 30 µL/min flow rate for 3 min in the positive ion mode with 2000 m/z scan maximum. To determine peptide sequences, the mass (Da) of each MS peak was entered into the ExPASy Proteomics Server FindPept tool (http://web.expasy.org/findpept/), using the published primary sequence of conlinin as the reference protein. Conlinin is the major globulin protein in flaxseed and it is the only protein for which the primary structure is available. In order to optimize accuracy of the output peptide sequences, mass tolerance was set at $\pm$0.1 Da.

6.2.10 Statistical Analysis

Protein hydrolysis was performed in triplicate and samples combined for ultrafiltration, which was also conducted in three separate fractionations for each enzyme concentration. Permeates from the three separate fractionations were combined, analyzed in triplicate for antioxidant properties and data expressed as the mean ± standard deviation. The statistical significance of difference between treatments was analyzed by one-way analysis of variance with Duncan's multiple range test ($P < 0.05$) using SAS software desktop version 9.2 (Statistical Analysis Systems, Cary, NC, USA).

6.3 Results and discussion

6.3.1 Peptide Composition

Table 6.1 shows some similarities in peptide content between the 2.5 and 3.0% thermoase-FPH

samples, which could reasonably be expected given that both samples were hydrolyzed using the same enzyme. In fact there were 23 peptides common to both FPHs nine of which are dipeptides. Thermoase (a thermolysin isoform) has proteolytic specificity for peptide bonds formed by hydrophobic amino acids, especially branched-chain amino acids (BCAA) (Keil, 1992; Udenigwe & Aluko, 2010); hence, majority of the peptides have *V*, *I*, or *L* at the amino or carboxyl terminals. The results are comparable to our previous work which used thermolysin to hydrolyze flaxseed proteins into a BCAA-rich hydrolysate (Udenigwe & Aluko, 2010). However, the protein hydrolysates were also rich in glutamine (*Q*)-containing peptides, especially at the amino and carboxyl terminals. These polar *Q*-containing peptides could be an important characteristic of these hydrolysates because of the availability of electrons that can be donated to quench toxic free radicals. The presence of polar, charged or hydrophobic amino acids at the N and C terminals is known to contribute to the antioxidant activity of peptides (Torres-Fuentes et al., 2015).

Table 6.1. Amino acid sequence of peptides present in flaxseed protein hydrolysates (FPH) obtained from the action of 2.5% or 3.0% (wt/vol) thermoase concentrations.

Observed Mass (Da)	**Calculated Mass (Da)**	**Matched sequence(s)**		**Location within conlinin**
		2.5% Thermoase	3.0% Thermoase	
219.20	219.13	SL	SL	f6-7
		TV	TV	f27-28
		LS	LS	f104-105
233.20	233.11	-	VD	f20-21
237.00	237.05	-	CD	f98-99
	237.09	-	MS	f5-6
260.20	260.16	QI	QI	f54-55, f142-143
		QL	QL	f103-104
		IQ	IQ	f55-56, f124-

				125, f137-138
		KL	KL	f3-4
		LK	LK	f101-102
		AAV	AAV	f8-10
		AVA	AVA	f9-11
		AIG	AIG	f116-118
274.20	274.18	LAA	-	f7-9
		VR	-	f24-25
276.10	276.12	QE	-	f122-123, f131-132
		EQ	-	f52-53, f89-90
	276.13	AW	-	f167-168
	276.16	ASV	-	f22-24
		EK	-	f70-71
	276.17	RT	-	f25-26, f152-153
288.20	288.20	LR	LR	f61-62
310.20	310.14	-	QY	f92-93
332.20	332.16	-	QGQ	f45-47, f127-129, f161-163
		-	GQQ	f46-49, f128-130, f162-164
		-	QQG	f122-128
332.20	332.18	-	VVD	f19-21
	332.22	-	TVI	f27-29
359.30	359.24	RAI	-	f115-117
365.10	365.16	-	RSC	f62-64
373.30	373.25	-	LAAV	f7-10
379.20	379.13	CEQ	-	f51-53

	379.18	TCR	-	f109-111
		CRT	-	f151-153
394.20	394.20	-	DFL	f59-61
446.30	446.24	-	WIQ	f136-138
	446.26	-	IAKD	f143-146
	446.26	-	AKDL	f144-147
478.30	478.24	LRSC	-	f61-64
	478.27	KLMS	-	f3-6
518.30	518.22	-	QQQD	f56-59
543.30	543.31	AKDLP	-	f144-148
545.40	543.30	GLERA	GLERA	f112-116
		ERAIG	ERAIG	f114-118
		RQEI	RQEI	f121-124
		LAAVAT	LAAVAT	f7-12
576.30	543.34	KQLST	-	f102-106
587.40	587.35	-	IQQAK	f137-141
		-	QAKQI	f139-143
	587.36	-	KVQRG	f71-75
	587.39	-	KQIAK	f141-145
602.30	602.33	QQAKQ	-	f138-142
	602.34	QRWI	-	f134-137
		RWIQ	-	f135-138
629.30	629.39	LIVVDA	-	f17-22
630.40	630.37	-	RGLER	f111-115
656.40	656.40	IAKDLP	-	f143-148
673.40	673.35	LPGQCR	-	f147-152
	673.36	ERAIGQ	-	f114-119
		RQEIQ	-	f121-125
687.50	687.40	-	QIAKDL	f142-147
689.30	689.33	-	TNQGRGG	f33-40
	689.36	-	WEKVQ	f69-73

	689.37	-	TVIIDE	f27-32
716.40	716.33	-	QQQGQQ	f125-130
		-	QQGQQQ	f126-131
	716.34	-	QGRGGGQG	f36-43
		-	RGGGQGGQ	f38-45
	716.37	-	RTQPSQ	f152-157
	716.40	-	EKVQRG	f70-75
	716.42	-	LIVVDAS	f17-23
746.50	746.44	-	LKQLSTG	f101-107
761.40	761.35	-	ETNQGRG	f33-39
763.40	763.40	MSLAAVAT	MSLAAVAT	f5-12
	763.44	LAAVATAF	LAAVATAF	f7-14
		AAVATAFL	AAVATAFL	f8-15
790.40	790.42	-	TTVIIDE	f26-32
834.40	834.40	-	CDDLKQL	f98-104
	834.42	-	TCRGLER	f109-115
	834.44	-	MSLAAVATA	f5-13
843.40	843.47	GLERAIGQ	-	f112-119
876.50	876.49	LMSLAAVAT	-	f4-12
	876.52	LAAVATAFL	-	f7-15
919.50	919.46	-	TVIIDEET	f27-34
		-	TTVIIDEE	f26-33
	919.47	-	DDLKQLST	f99-106
963.40	963.42	QQSCEQQI	QQSCEQQI	f48-55
		QSCEQQIQ	QSCEQQIQ	f49-56
		SCEQQIQQ	SCEQQIQQ	f50-57
967.50	967.44	-	RSHYYNQ	f77-83
	967.47	-	QFMWEKV	f66-72
		-	FMWEKVQ	f67-73
987.60	987.50	AKDLPGQCR	AKDLPGQCR	f144-152
988.50	988.46	TNQGRGGGQGG	-	f34-44

1001.50	1001.44	GGGQGGQGQQQ	-	f39-49
1028.40	1028.50	-	FLRSCQQF	f60-67
1059.60	1059.54	-	VQRGGRSHY	f72-80
1060.50	1060.42	GEQSQYFDS	-	f88-96
	1060.48	ETNQGRGGGQG	-	f33-43
	1060.52	VIIDEETNQ	-	f28-36
		QMRQEIQQ	-	f119-126
		MRQEIQQQ	-	f120-127
	1060.60	IVVDASVRTT	-	f16-27
		VDASVRTTVI	-	f20-29
1072.50	1072.49	-	NQGRGGGQGGQG	f35-46
	1072.50	-	QQQGQQQEV	f125-133
		-	QQGQQQEVQ	f126-134
	1072.55	-	QIAKDLPGQC	f142-151
1157.60	1157.54	-	RGGGQGGQGQQQ	f38-49
	1157.60	-	EVQRWIQQA	f132-140
1163.50	1163.43	GEQSQYFDSC	-	f88-97
	1163.48	QGQQQSCEQQ	-	f45-54
	1163.53	EQQIQQQDF	-	f52-60
1173.50	1173.46	FDSCCDDLKQ	-	f94-103
	1173.54	TNQGRGGGQGGQG	-	f34-46

In this work, we obtained glutamine-rich protein hydrolysates/peptides where the glutamine also appeared at the C terminus. Therefore, it is appropriate to draw comparisons of similarity in antioxidant capacity between the protein hydrolysates from our current work and those reported for chickpea antioxidant protein hydrolysates where hydrophobic and polar amino acids were predominant (Torres-Fuentes et al., 2015). Overall, 75 peptides were detected in the 3.0% thermoase-FPH in comparison to 64 in the 2.5% thermoase-FPH sample, which is consistent with greater proteolysis for a higher enzyme concentration (Ghosh, Prasad, & Saha, 2017).

However, it should be noted that 2000 Da is the maximum peptide size detectable on the mass spectrometer used in this work, which means that peptides with bigger sizes than those presented in Table 6.1. will likely still be present in the hydrolysates. It is also important to note that since conlinin is the only major flaxseed protein whose primary structure is available, the possibility exists that a different conclusion may have been reached if the primary structure of other flaxseed proteins were available.

6.3.2 DPPH Radical Scavenging Activity

The DPPH radical scavenging assay has found wide application in tests evaluating the antioxidant capacity of foods and other biological samples (Aksoy, Kolay, Ağılönü, Aslan, & Kargıoğlu, 2013). The reasons for this include the stability of the nitrogen-containing free radical as well as the rapidity, ease and reliability of the assay (Aksoy et al., 2013; Kedare & Singh, 2011). Unlike most other free radicals, the DPPH molecule does not undergo dimerization because the spare electron is delocalized over the entire molecule. The deep violet color is thus formed in aqueous, methanolic and ethanolic solutions where the synthetic radical has been shown to rarely disintegrate (Kedare & Singh, 2011). As shown in Fig. 6.1., the two flaxseed protein hydrolysates and their ultrafiltration-separated fractions had significantly lower DRSA than GSH (54 - 65% versus 70%, respectively). The 3.0% thermoase-FPH had the lowest DRSA, which was improved by ultrafiltration as shown in Fig. 6.1. The results suggest antagonistic effects within the 3.0% thermoase-FPH, which were then reduced after separation of the peptides into narrower size ranges. For example, the 3.0% thermoase-FPH contains a higher number of peptides with positively (*N, Q*) and negatively-charged amino acids (*D, E*) when compared to the 2.5% thermoase-FPH (Table 6.1.). It is possible that the higher level of these polar amino acid residues led to increased peptide repulsion, which reduced interactions with the DPPH radical

and hence reduced DRSA of the 3.0% thermoase-FPH. It should be noted, however that additional effects from other peptides are possible as not all the peptides could be identified in this work. Separation of the 3.0% thermoase-FPH into peptide fractions would have reduced the repulsive forces, leading to enhanced interactions with DPPH and better DRSA. Our previous work on Australian canola (Alashi et al., 2014) and that of Kimatu et al. (2017) on mushroom demonstrated that separation of peptides produced fractions that scavenged DPPH radicals better than their protein hydrolysates. Separation of the 2.5% thermoase-FPH produced a significantly higher DRSA for the 3-5 kDa peptide fraction alone, suggesting that this fraction has less peptide antagonistic effects (or more synergistic properties) than the FPH. However, all the peptide fractions had similar DRSA, which indicates that chain length may not be an important factor for the DRSA of flaxseed peptides.

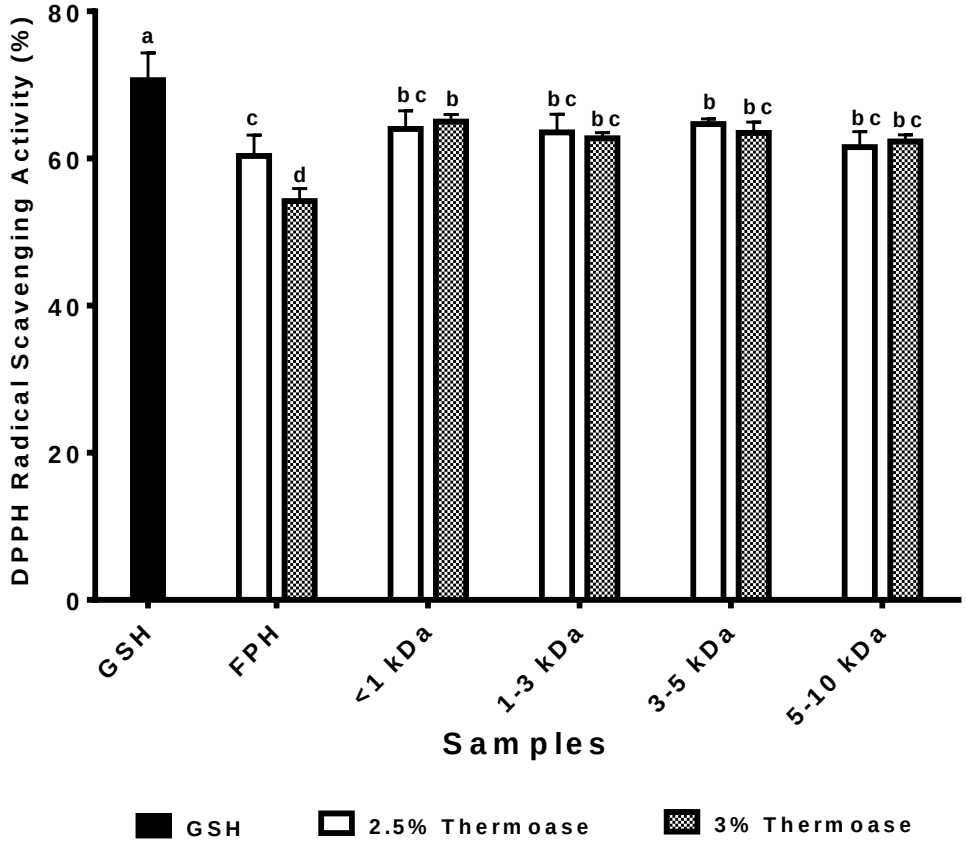

Fig.6.1. DPPH radical scavenging activity (%) of flaxseed protein hydrolysates (FPH) and their ultrafiltered peptide fractions compared to that of reduced glutathione (GSH). Bars with different letters are significantly different at $P < 0.05$. Values are means (n=3) ± SD.

6.3.3 Superoxide Radical Scavenging Activity

Although the superoxide anion radical is not able to directly initiate oxidative degradation of lipids, it is still regarded as highly toxic in biological systems because it is a precursor of other highly reactive ROS such as hydrogen peroxide and hydroxyl radicals (Jamdar, Rajalakshmi, & Sharma, 2012). Compared to GSH, which had over 80% SRSA, the thermoase-digested flaxseed protein hydrolysates and their membrane-separated fractions were generally weak (Fig. 6.2). However, the SRSA for both the 3.0% thermoase FPH-derived < 1 kDa and 1-3 kDa peptide fractions compared favourably with data for similar samples from plant and animal food proteins such as canola (Alashi et al., 2014) and chicken (Onuh, Girgih, Aluko, & Aliani, 2014). No

SRSA was detected for the unfractionated protein hydrolysates and the higher molecular weight peptide fractions (3-5 kDa and 5-10 kDa), which suggests the influence of peptide size. The antagonistic effect, which could have been due to the high content of polar peptides as earlier discussed for the 2.5 and 3.0% thermoase-FPH, may have limited interactions with the superoxide radical (O_2^- also contains excess electrons).

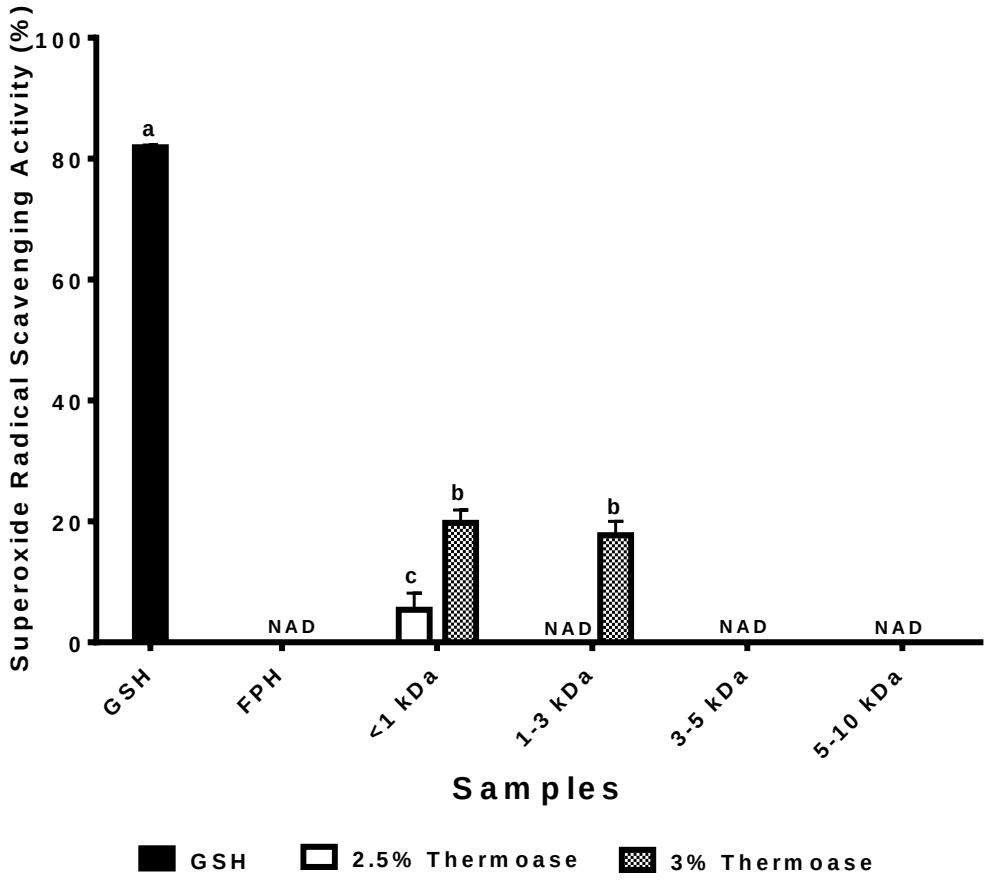

Fig.6.2. Superoxide radical scavenging activity of flaxseed protein hydrolysates (FPH) and their ultrafiltered fractions compared to that of a reduced glutathione (GSH) standard. Bars (mean ±standard deviation) with different letters have mean values that are significantly different at $P < 0.05$. NAD = no activity detected.

A previous work has also shown that peptides with predominant *Q* residues (except VLPPQQQY) had no measureable SRSA (Babini et al., 2017). Separation into smaller peptide sizes such as the < 1 kDa for 2.5% thermoase-FPH or < 1 kDa and 1-3 kDa for 3.0% thermoase-

FPH may have led to reduced repulsions and enhanced interactions with the superoxide anion radical. Increase in peptide size to > 1 kDa for the 2.5% thermoase-FPH fractions and > 3 kDa for 3.0% thermoase-FPH fractions may have increased the repulsive forces due to longer chain length, which then resulted in lack of interaction with the superoxide anion radical. The SRSA of the flaxseed protein hydrolysates and peptide fractions used in this study compares favourably with that of chicken skin protein hydrolysates and membrane fractions (Onuh et al., 2014). However, the SRSA of flavourzyme-hydrolyzed hordein fractions evaluated at a lower concentration (0.5 mg/mL) was better (~30-33%) (Bamdad, Wu, & Chen, 2011) than the values (<18%) obtained for peptides in this work.

6.3.4 Hydroxyl Radical Scavenging Activity

The interaction of Fe^{2+} and H_2O_2 in the Fenton reaction results in production of the extremely volatile and transient hydroxyl radicals. This radical has been implicated in the oxidation of biological macromolecules (e.g. lipids, nucleic acids and proteins) with the potential to trigger or contribute to the development and/or progression of chronic disease conditions (Chigurupati et al., 2017; Shibata et al., 2016; Treml & Šmejkal, 2016). Similar to the DPPH and superoxide radicals, GSH was a better scavenger of hydroxyl radicals than the FPHs and peptide fractions (Fig. 6.3). Peptide size had apparent effect on HRSA because the 5-10 kDa peptide fraction (especially from 3.0% thermoase-FPH) was a much stronger scavenger than the <5 kDa fractions. The notably higher HRSA for 3.0% thermoase-FPH may be due to the greater content of peptides with negatively-charged amino acid residues when compared to the 2.5% thermoase-FPH. The hydroxyl radical has a smaller anionic character, which may explain why the peptides were still able to interact when compared to the bigger superoxide anion. Therefore, peptide-free radical repulsions would have been greater with superoxide radical (hence nil or minimal

scavenging effect) than the hydroxyl radical (low to moderate). It is likely that the electron-donating ability of the peptides was decreased or remained unchanged when the FPHs were fractionated into peptides with <5 kDa sizes. However, the 5-10 kDa peptides were more effective hydroxyl radical scavengers probably due to the longer chain length, which may have increased the level of peptides with positively charged amino acids (*R*, *K*, *H*). These positively-charged residues could have enhanced peptide interactions with the hydroxyl radical as previously suggested (Udenigwe & Aluko, 2011) and hence increased HRSA of the 5-10 kDa fraction.

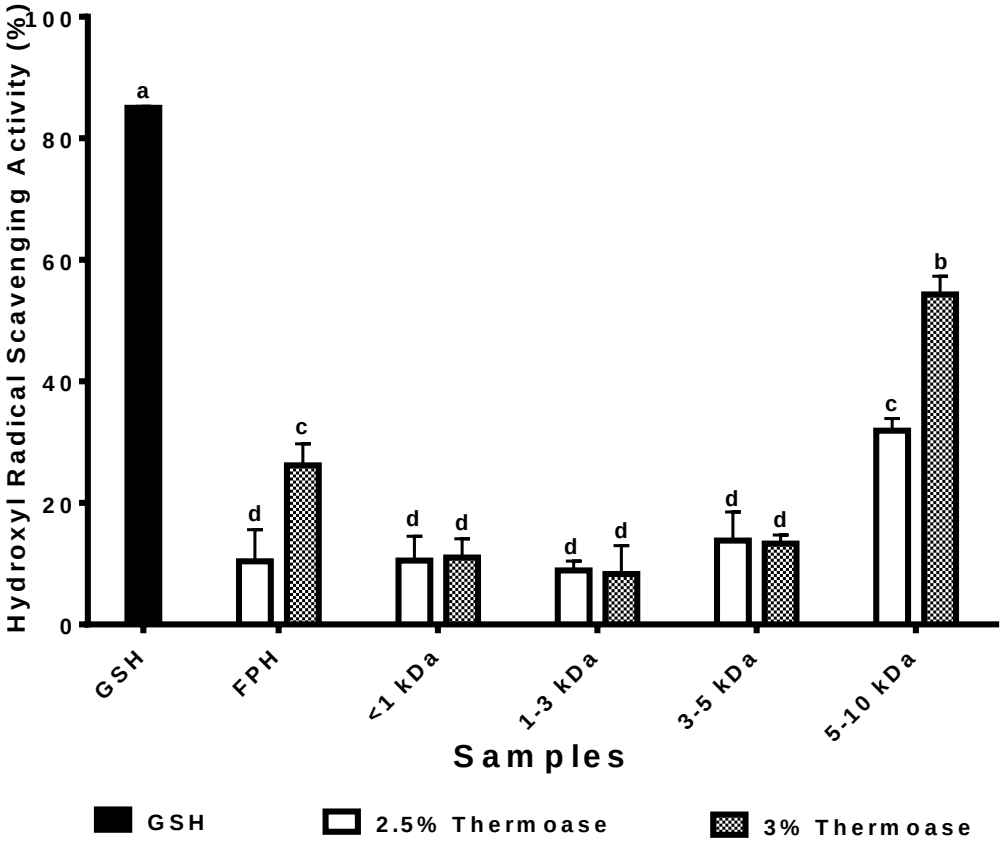

Fig.6.3. Hydroxyl radical Scavenging activity of flaxseed protein hydrolysates (FPH) and their ultrafiltered fractions compared to that of a reduced glutathione (GSH) standard. Bars (mean ±standard deviation) with different letters have mean values that are significantly different at $P < 0.05$.

6.3.5 Chelation of Metal Ions

The flaxseed protein hydrolysates and peptide fractions were very effective metal chelators, with almost all the flaxseed samples sequestering significantly more Fe^{2+} than GSH (Fig. 6.4). In general, the ultrafiltered peptide fractions had stronger metal ion chelating ability than FPH. The relatively poor metal ion chelating ability of GSH is consistent with previous reports (Pownall, Udenigwe, & Aluko, 2010; Xie et al., 2008). The 3.0% thermoase-FPH was a less effective metal chelator than the 2.5% thermoase-FPH, which further supports the role of peptide antagonism in reducing interactions with target molecules.

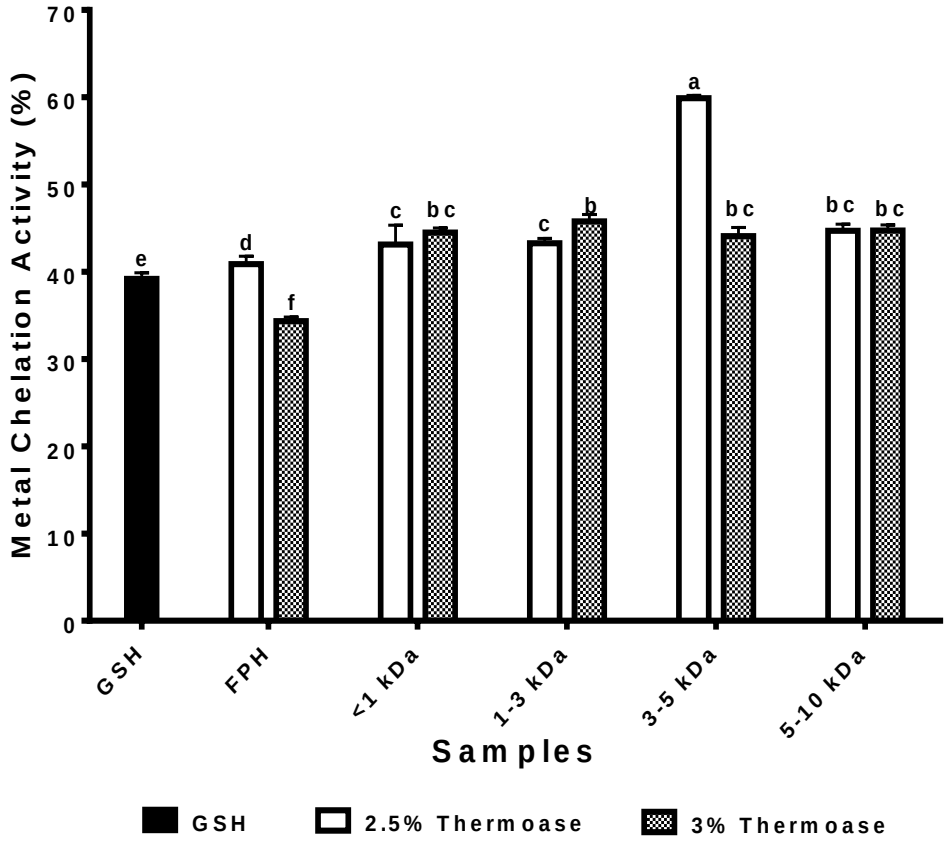

Fig.6.4. Metal chelating effects of flaxseed protein hydrolysates (FPH) and their ultrafiltered peptide fractions compared to that of reduced glutathione (GSH). Bars with different letters are significantly different at $P < 0.05$. Values are means (n=3) ± SD.

Recall in Table 6.1 that the 3.0% thermoase-FPH contains higher numbers of peptides with polar amino acids that would have induced strong peptide-peptide repulsions, which then limited simultaneous binding of several peptides to Fe^{2+} when compared with 2.5% thermoase-FPH. The negative role of electrostatic repulsions is further supported by the significantly increased metal chelating ability after the peptides have been separated into different fractions. Similar data for mushroom peptides also showed higher iron chelation for membrane fractions than the original hydrolysate (Kimatu et al., 2017). For the 3.0% thermoase FPH-derived fractions, peptide size did not affect metal chelating ability given the lack of significant differences, which suggests similar content of polar groups. However, the 2.5% thermoase FPH-derived 3-5 kDa fraction had significantly higher metal chelating ability and may contain a higher level of negative charges than the other peptide sizes.

6.3.6 Ferric-Reducing Antioxidant Power

The FRAP assay was used to measure the capacity of the hydrolysates and peptide fractions to serve as reducing agents by donating electrons to Fe^{3+}-ferricyanide complex, and reducing it to a more stable Fe^{2+} form (Bougatef et al., 2009). Higher absorbance values indicate greater reducing power. GSH had the highest FRAP among all samples (Fig. 6.5). The 3.0% thermoase-FPH had no FRAP, whereas the 2.5% thermoase-FPH displayed a weak value (< 1.0 absorbance). The results are consistent with greater amount of peptides having polar amino acids in the 3.0 % thermoase-FPH. The resultant electrostatic repulsions within the peptides may have prevented adequate (multi-peptide) interactions with the target molecule, hence the lack of a measurable FRAP value. This is based on a previous work which showed that electrostatic repulsions between peptides prevented interactions with a target protein (Noiseux, Gauthier, &

Turgeon, 2002). The use of ultrafiltration enhanced FRAP, which is consistent with reductions in the electrostatic repulsions within the sample.

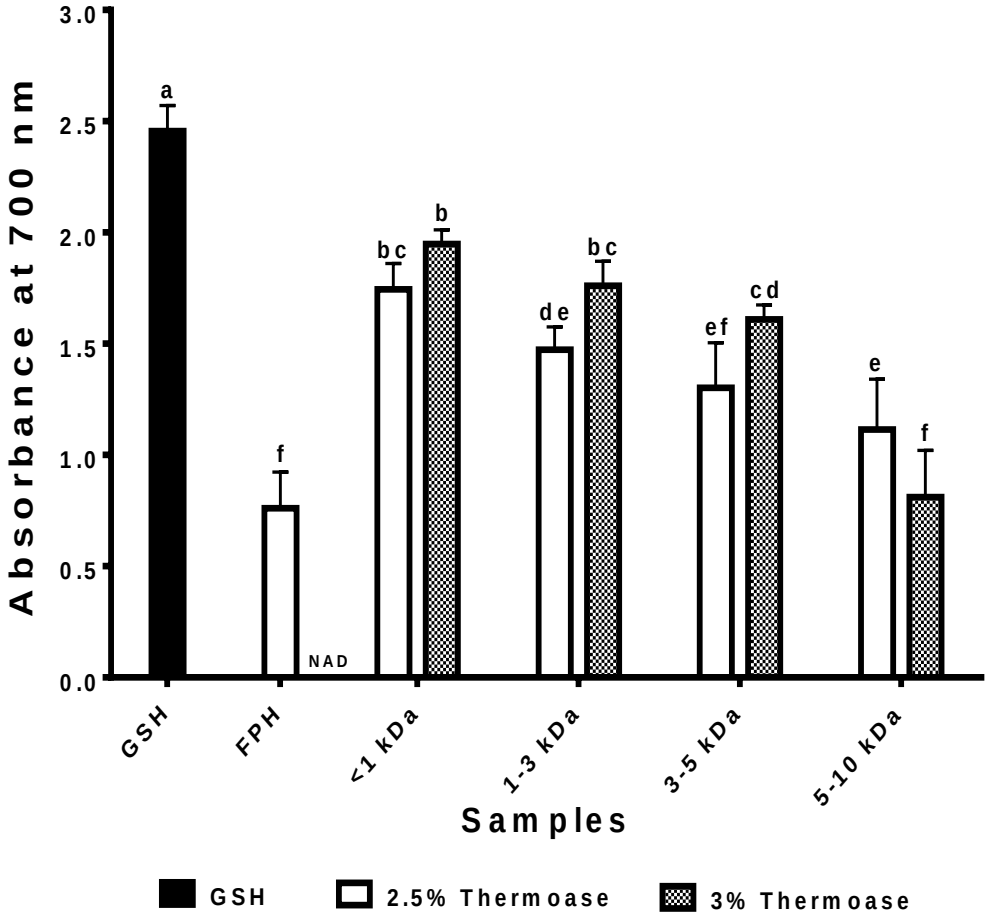

Fig.6.5. Ferric reducing antioxidant power (FRAP) of flaxseed protein hydrolysates (FPH) and their membrane fractions compared to that of reduced glutathione (GSH). Bars (mean ± standard deviation) with different letters have mean values that are significantly different at $P < 0.05$. NAD = no activity detected.

However, FRAP was negatively affected by peptide size, as shown by gradual decreases from <1 kDa to the 5-10 kDa. A comparison of the fractions from the two hydrolysates indicates that the <1, 1-3 and 3-5 kDa fractions from the 3.0% thermoase-FPH had significantly higher FRAP values than the corresponding fractions ultrafiltered from the 2.5% thermoase-FPH. Conversely, the 5-10 kDa fraction from the 2.5% thermoase-FPH had a higher FRAP value than peptides of

the same size from the 3.0% thermoase-FPH. The results indicate that as the peptide size increased, the negative effects of peptide antagonism was initially greater for the 2.5% peptides but at a longer length of 5-10 kDa, the 3.0% peptides experienced the most effect. This finding is similar to previous studies that reported stronger FRAP values for low molecular weight peptides than for high molecular weight peptides (Ajibola, Fashakin, Fagbemi, & Aluko, 2011; Bougatef et al., 2009). In contrast, long-chain mushroom peptides had higher FRAP values than the <1 kDa peptides (Kimatu et al., 2017).

6.4. Conclusions

This study showed that the presence of several peptides containing polar amino acid residues may have negative effects on some antioxidant properties, especially DPPH and superoxide radical scavenging, as well as metal chelation and iron reducing ability. However, this negative effect can be ameliorated through peptide separation into fractions that reduce peptide density within the sample. The effect of peptide size was dependent on sample and type of antioxidant assay, which suggest a role for other peptide structural features such as charge and hydrophobicity apart from chain length. The higher superoxide scavenging and FRAP of the short-chain (<3 kDa) peptides may enhance their utilization as antioxidant agents that can down-regulate oxidative stress within living tissues. This is because of the increased absorption potential for short peptides when compared to long peptides. Similarly, the strong metal chelating ability of the 2.5% thermoase FPH-derived 3-5 kDa fraction may be used to enhance shelf-life of foods by reducing the potential for lipid peroxidation. Confirmation of the potential uses of these peptides will require *in vivo* tests and incorporation into model food systems.

6.5. Acknowledgements

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6.6. Conflicts of interest

The authors declare no conflict of interest.

6.7. References

Ajibola, C. F., Fashakin, J. B., Fagbemi, T. N., & Aluko, R. E. (2011) Effect of peptide size on antioxidant properties of African yam bean seed (*Sphenostylis stenocarpa*) protein hydrolysate fractions. *International Journal of Molecular Sciences*, **12**:6685–6702.

Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z., & Kargıoğlu, M. (2013) Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica*. *Saudi Journal of Biological Sciences*, **20**:235–239.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., … Aluko, R. E. (2014) Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chemistry*, **146**:500-506.

Aluko, R.E. (2015) Antihypertensive peptides from food proteins. *Annual Review of Food Science and Technology*, **6**:235–262.

Babini, E., Tagliazucchi, D., Martini, S., Più, L. D., & Gianotti, A. (2017) LC-ESI-QTOF-MS identification of novel antioxidant peptides obtained by enzymatic and microbial hydrolysis of vegetable proteins. *Food Chemistry*, **228**:186-196.

Bamdad, F., Wu, J., & Chen, L. (2011) Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. *Journal of Cereal Science*, **54**:20-28.

Bougatef, A., Hajji, M., Balti, R., Lassoued, I., Triki-Ellouz, Y., & Nasri, M. (2009) Antioxidant and free radical- scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. *Food Chemistry*, **114**:1198-1205.

Chigurupati, H., Auddy, B., Biyani, M., Chakrabarti, S., Pandit, S., Biswas, T. K., … Stohs, S. J. (2017) Antioxidant and DNA protective effects of NTX, a proprietary glycyrrhizin/D-mannitol product, in association with alcohol consumption: a randomized, placebo-controlled, double-blind, crossover study. *Journal of Functional Foods*, **34**:28-35.

Choi, C. W., Kim, S. C., Hwang, S. S., Choi, B. K., Ahn, H. J., Lee, M. Y., … Kim, S. K. (2002) Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science*, **163**: 1161-1168.

de Avellar, I.G.J., Magalhães, M.M.M., Silva, A.B., Souza, L.L., Leitão, A.C., & Hermes-Lima, M. (2004) Reevaluating the role of 1,10- phenanthroline in oxidative reactions involving ferrous ions and DNA damage. *Biochimica et Biophysica Acta - General Subjects*, **1675**:46-53.

Dev, D.K., & Quensel, E. (1988) Preparation and functional properties of linseed protein products containing differing levels of mucilage. *Journal of Food Science*, **53**:1834-1837.

Doyen, A., Udenigwe, C. C., Mitchell, P. L., Marette, A., Aluko, R. E., & Bazinet, L. (2014) Anti-diabetic and antihypertensive activities of two flaxseed protein hydrolysate fractions revealed following their simultaneous separation by electrodialysis with ultrafiltration membranes. *Food Chemistry*, **145**:66-76.

Erdmann, K., Cheung, B.W.Y., & Schröder, H. (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *The Journal of Nutritional Biochemistry*, **19**:643-654.

Ghosh, B.C., Prasad, L.N., & Saha, N.P. (2017) Enzymatic hydrolysis of whey and its analysis. *Journal of Food Science and Technology*, **54**:1476-1483.

Girgih, A.T., Udenigwe, C.C., Li, H., Adebiyi, A.P., & Aluko, R.E. (2011) Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates. *Journal of The American Oil Chemists' Society*, **88**:1767-1774.

Hall III, C., Tulbek, M.C., & Xu, Y. (2006) Flaxseed. *Advances in Food and Nutrition Research*, **51**:1-97.

Hwang, C., Chen, Y., Luo, C., & Chiang, W. (2016) Antioxidant and antibacterial activities of peptide fractions from flaxseed protein hydrolysed by protease from *Bacillus altitudinis* HK02. *International Journal of Food Science & Technology*, **51**:681-689.

Jamdar, S., Rajalakshmi, V., & Sharma, A. (2012) Antioxidant and ace inhibitory properties of poultry viscera protein hydrolysate and its peptide fractions. *Journal of Food Biochemistry*, **36**:494-501.

Kedare, S., & Singh, R. (2011) Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science & Technology*, **48**:412-422.

Keil, B. (1992) *Specificity of proteolysis* (p. 336). Berlin: Springer-Verlag.

Kimatu, B.M., Zhao, L., Biao, Y., Ma, G., Yang, W., Pei, F., & Hu, Q. (2017) Antioxidant potential of edible mushroom (*Agaricus bisporus*) protein hydrolysates and their ultrafiltration fractions. *Food Chemistry*, **230**:58-67.

Malomo, S.A., & Aluko, R.E. (2016) In vitro acetylcholinesterase-inhibitory properties of enzymatic hemp seed protein hydrolysates. *Journal of The American Oil Chemists' Society*, **93**:411-420.

Marambe, H.K., Shand, P.J., & Wanasundara, J.P.D. (2011) Release of angiotensin I-converting enzyme inhibitory peptides from flaxseed (*Linum usitatissimum* L.) protein under simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry*, **59**:9596-9604.

Marambe, P.W.M.L.H.K., Shand, P.J., & Wanasundara, J.P.D. (2008) An in vitro investigation of selected biological activities of hydrolyzed flaxseed (*Linum usitatissimum* L.) proteins. *Journal of The American Oil Chemists' Society*, **85**:1155-1164.

Markwell, M.A.K., Haas, S.M., Bieber, L.L., & Tolbert, N.E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, **87**: 206-210.

Nabha, L., Garbern, J.C., Buller, C.L., & Charpie, J.R. (2005) Vascular oxidative stress precedes high blood pressure in spontaneously hypertensive rats. *Clinical and Experimental Hypertension*, **27**:71-82.

Noiseux, I., Gauthier, S.F., & Turgeon, S.L. (2002) Interactions between bovine β-Lactoglobulin and peptides under different physicochemical conditions. *Journal of Agricultural and Food Chemistry*, **50**:1587-1592.

Nwachukwu, I.D., Girgih, A.T., Malomo, S.A., Onuh, J.O., & Aluko, R.E. (2014) Thermoase-derived flaxseed protein hydrolysates and membrane ultrafiltration peptide fractions have systolic blood pressure-lowering effects in spontaneously hypertensive rats. *International Journal of Molecular Sciences*, **15**:18131-18147.

Omoni, A., & Aluko, R.E. (2006a) Effect of cationic flaxseed protein hydrolysate fractions on the in vitro structure and activity of calmodulin-dependent endothelial nitric oxide synthase. *Molecular Nutrition & Food Research*, **50**:958-966.

Omoni, A., & Aluko, R.E. (2006b) Mechanism of the inhibition of calmodulin-dependent neuronal nitric oxide synthase by flaxseed protein hydrolysates. *Journal of The American Oil Chemists' Society*, **83**:335-340.

Onuh, J.O., Girgih, A.T., Aluko, R.E., & Aliani, M. (2014) In vitro antioxidant properties of chicken skin enzymatic protein hydrolysates and membrane fractions. *Food Chemistry*, **150**:366-373.

Park, S.Y., Lee, J.-S., Baek, H.-H., & Lee, H.G. (2010) Purification and characterization of antioxidant peptides from soy protein hydrolysate. *Journal of Food Biochemistry*, **34**:120-132.

Pownall, T.L., Udenigwe, C.C., & Aluko, R.E. (2010) Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. *Journal of Agricultural and Food Chemistry*, **58**:4712-4718.

Ruiz-Ruiz, J., Davila-Ortiz, G., Chel-Guerrero, L., & Betancur-Ancona, D. (2013) Angiotensin I-converting enzyme inhibitory and antioxidant peptide fractions from hard-to-cook bean enzymatic hydrolysates. *Journal of Food Biochemistry*, **37:26**-35.

Saisavoey, T., Sangtanoo, P., Reamtong, O., & Karnchanatat, A. (2016) Antioxidant and anti-inflammatory effects of defatted rice bran (*Oryza sativa* L.) protein hydrolysates on RAW 264.7 macrophage cells. *Journal of Food Biochemistry*, **40**:731-740.

Shibata, A., Ishima, Y., Imafuku, T., Chuang, V.T.G., Ouchi, Y., Abe, T., … Maruyama, T. (2016) Human serum albumin hydropersulfide is a potent reactive oxygen species scavenger in oxidative stress conditions such as chronic kidney disease. *Biochemical and Biophysical Research Communications*, **47**:578-583.

Torres-Fuentes, C., del Mar Contreras, M., Recio, I., Alaiz, M., & Vioque, J. (2015) Identification and characterization of antioxidant peptides from chickpea protein hydrolysates. *Food Chemistry*, **180**:194-202.

Treml, J., & Šmejkal, K. (2016) Flavonoids as potent scavengers of hydroxyl radicals. *Comprehensive Reviews in Food Science and Food Safety*, **15**:720-738.

Udenigwe, C.C., & Aluko, R.E. (2010) Antioxidant and angiotensin converting enzyme-inhibitory properties of a flaxseed protein-derived high Fischer ratio peptide mixture. *Journal of Agricultural and Food Chemistry*, **58**:4762-4768.

Udenigwe, C.C., & Aluko, R.E. (2011) Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. *International Journal of Molecular Sciences*, **12**:3148-3161.

Udenigwe, C.C., & Aluko, R.E. (2012) Multifunctional cationic peptide fractions from flaxseed protein hydrolysates. *Plant Foods for Human Nutrition*, **67**:1-9.

Udenigwe, C.C., Lin, Y.-L., Hou, W.-C., & Aluko, R.E. (2009) Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods*, **1**:199-207.

Udenigwe, C.C., Lu, Y.-L., Han, C.-H., Hou, W.-C., & Aluko, R.E. (2009) Flaxseed protein-derived peptide fractions: antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. *Food Chemistry*, **116**: 277-284.

Xie, Z., Huang, J., Xu, X., & Jin, Z. (2008) Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. *Food Chemistry*, **111**:370-376.

Zhang, S., Wang, Z., & Xu, S. (2008) Antioxidant and antithrombotic activities of rapeseed peptides. *Journal of The American Oil Chemists' Society*, **85**:521-527.

6.8. TRANSITION STATEMENT FOUR

The antioxidative properties of flaxseed-derived protein hydrolysates and peptide fractions reported in the last chapter highlight their prospects for use in the design of functional foods and nutraceuticals ingredients, which could be valuable for the control, management and prevention of chronic degenerative and debilitating diseases. The study showed that fractionation using ultrafiltration either diminished or failed to enhance the hydroxyl radical scavenging property of the peptide fractions except that of the 5-10 kDa fraction. Conversely, ultrafiltration led to notable improvements in the metal chelation and ferric iron reduction ability of the samples.

In addition to the already discussed antihypertensive and antioxidative properties of flaxseed protein hydrolysates and peptides, their CaMPDE-inhibitory property is reported in the next chapter. By inhibiting the activity of CaMPDE, bioactive peptides could contribute to the prevention and/or delay of chronic diseases that arise from excessive levels of CaM such as cardiac hypertrophy, cancer and Alzheimer's disease. However, the beneficial bioactive properties of food protein-derived reported in this thesis and elsewhere in literature would ultimately be of no value if peptides prove to be un-absorbable.

There is an ongoing debate on whether peptides are actually absorbed as well as on their capacity to directly affect the activity of target enzymes in the blood. This debate is stoked in part by the gap between demonstrated *in vitro* peptide bioactivity and *in vivo* efficacy. Thus, the work (Manuscript 5) presented in the next chapter is an attempt to provide answers to current questions on transepithelial peptide transport and on the effects of absorbed peptides on a critical metabolic enzyme such as CaMPDE.

CHAPTER 7

MANUSCRIPT FIVE

TRANSPORT, BIOAVAILABILITY, SAFETY AND CALMODULIN-DEPENDENT PHOSPHODIESTERASE-INHIBITORY PROPERTIES OF FLAXSEED-DERIVED BIOACTIVE PEPTIDES

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7.0 Abstract

The aim of this work was to determine bioavailability and *in vivo* calmodulin-dependent phosphodiesterase (CaMPDE)-inhibitory activity of six flaxseed protein-derived peptides (AGA, AKLMS, QIAK, RWIQ, QQAKQ and KQLSTGC) after oral administration to Wistar rats. Initial experiments tested the cytotoxicity and cellular transport potential of the peptides using Caco-2 cells. The cytotoxicity assay indicated that none of the six peptides had an adverse effect on the proliferation and viability of the Caco-2 cells while the transport assay confirmed peptide translocation across the cell membrane. However, only two of the peptides (AGA and RWIQ) were detected in the rat serum up to 90 min post-gavage with traces of RWIQ persisting in serum one week after oral gavage. The six peptides inhibited plasma activity of CaMPDE with AGA (34.63%), QIAK (36.66%) and KQLSTGC (34.21%) being the most effective 30 min after gavage. In contrast, only the AGA maintained a significant plasma CaMPDE activity inhibition (44.35%) after 60 min.

Keywords: *Calmodulin; Phosphodiesterase; Peptide transport; Bioavailability; Caco-2 cells, Cytotoxicity; Flaxseed; Intestinal epithelium.*

7.1 Introduction

As the primary intracellular receptor for calcium (Ca^{2+}), the messenger protein calmodulin (CaM) is essential for a number of physiological processes including cell division, cell growth, cell differentiation and neurotransmission.[1,2] CaM is present in every eukaryotic cell system, and mainly regulates intracellular events by acting as an activator protein for enzymes such as CaM-dependent phosphodiesterases (CaMPDE), nitric oxide synthases (NOS) and protein kinases.[3] For instance, CaM-dependent protein kinase II (CaMKII), which has been described as the most studied member of the family of CaM-dependent multifunctional protein kinases and which plays critical roles in various metabolic reactions including synaptic transmission, is known to require CaM (in addition to Ca^{2+}) for its initial autophosphorylation activation step.[4] Inadequate regulation of the critical autophosphorylation reaction, which could manifest as elevated CaM levels, often results in impaired physiological functions and ultimately leads to pathological conditions including cardiac hypertrophy and cancer.[3] Similarly, the absence of proper calmodulin-related regulatory controls for CaMPDE and CaM-dependent nitric oxide synthases, which are essential for regulating cyclic adenosine monophosphate (cAMP)-induced apoptotic cell death and nitric oxide synthesis, respectively, could ultimately lead to the development of chronic ailments such as cancer, Parkinson's, and Alzheimer's.[3] Since CaM acts by binding to and thus changing the conformation of target proteins, ligands which could bind, and therefore, modify CaM conformation could potentially influence many CaM-mediated cellular reactions, and may be used to design therapeutic agents.[5] Previous studies have identified CaM as a potential target of specific bioactive peptides as well as protein hydrolysates from food sources like casein, wheat, pea and flaxseed.[6-9] For instance, low molecular weight cationic peptide

fractions obtained after ion-exchange chromatography of ultrafiltered pea protein hydrolysates were demonstrated to show a strong affinity for CaM thus resulting in considerable inhibition of CaMKII activity.[9] By inhibiting the activities of CaM-dependent protein kinases, phosphodiesterases and nitric oxide synthases, CaM-binding peptides could be used to control or manage pathological conditions that are associated with anomalous activities of these enzymes. Previous studies have demonstrated the capacity of cationic peptide fractions obtained after ion-exchange chromatographic separation of ultrafiltered alcalase-hydrolyzed flaxseed proteins to inhibit *in vitro* activity of CaMPDE.[10] Based on the potential amino acid sequences of the peptides present in the most active cationic fractions as established using bioinformatics and mass spectrometry tools, 27 peptides were chemically synthesized for use in the present study. The overall aim of this work was to evaluate the *in vivo* CaMPDE-inhibitory activity of orally administered peptides as well as to gain an understanding of their cellular transport and bioavailability. The specific objectives were to: (a) screen the 27 synthesized peptides for *in vitro* CaMPDE-inhibitory activity and select the most active peptide(s) for cell culture and *in vivo* work, (b) evaluate the safety/toxicity of the selected peptides in a cell culture model of the intestinal epithelium, (c) measure the capacity of the selected peptides to traverse the intestinal epithelium using a cell culture model, and (d) determine bioavailability and CaMPDE activity in the blood of rats after oral administration of inhibitory peptides.

7.2 Materials and Methods

7.2.1 Materials

Myokinase from chicken muscle, pyruvate kinase from rabbit muscle, phosphoenolpyruvate, cAMP, 5′-guanosine triphosphate (5´-GTP), dithothreitol (DTT), calmodulin (CaM), as well as phosphodiesterase 1 (PDE1 or CaMPDE) from bovine heart were purchased from Sigma-Aldrich

(St. Louis, MO, USA). Deuterated single amino acid internal standards were obtained from C/D/N Isotopes (Pointe-Claire, Quebec), while LC/MS grade solvents were purchased from Caledon Laboratories (Georgetown, Ontario). The 96-well flat bottom polystyrene microplates were purchased from Corning Incorporated (Ithaca, NY), while TrypLE™ Express cell dissociation reagent was from Life Technologies (Nærum, Denmark). Antibiotics (penicillin and streptomycin) were purchased from Gibco BRL (Burlington, ON, Canada), while the CCK-8 cell proliferation and cytotoxicity kit was from Dojindo Molecular Technologies, Inc. (Rockville, Maryland, USA). Flaxseed-derived protein-derived peptide sequences (>95% purity) were synthesized without labeling or labeled with fluorescein isothiocyanate (FITC) by Genscript Inc. (Piscataway, NJ, USA). The ENLITEN® ATP Assay Bioluminescence Detection Kit containing a luciferin-luciferase reagent was purchased from Promega Corporation (Madison, WI, USA), while multi-well plates with polycarbonate membrane inserts were from Thermo Scientific (Waltham, MA, USA). All other analytical grade reagents except where otherwise indicated were obtained from Fisher Scientific (Oakville, ON, Canada).

7.2.2 Identification of peptide sequences

In an earlier work,[10] we showed the capacity of flaxseed-derived peptides to inhibit *in vitro* CaMPDE activity. Briefly, peptide fractions (<1 kDa) obtained following the ultrafiltration of alcalase-hydrolyzed flaxseed proteins were separated by means of cation exchange chromatography using an SP-Sepharose High Performance XK 50/20 column. A fraction (FII) from the ion exchange chromatographic separation was further separated by RP-HPLC. After determining the molecular weight profile of peptide fractions from the HPLC separation using LC-MS, their potential amino acid sequences were identified using a combination of the MS data and the ExPASy Proteomics Server FindPept tool (Swiss Institute of Bioinformatics). The

primary structures of the 27 peptides synthesized for use in the current work were based on those potential amino acid sequences.

7.2.3 CaMPDE Inhibition Assay

An initial *in vitro* screening test based on the luciferin-luciferase assay[11] was conducted using the unlabeled peptides in order to determine peptides with the highest inhibitory activity against CaMPDE. The reaction mixture was made up of 0.1 mU CaMPDE, 0.05 U CaM, 0.15 mM $CaCl_2$, 1 U myokinase, 1 U pyruvate kinase, 1 mM cAMP, 0.26 mM phosphoenolpyruvate, 25 mM ammonium acetate, 3 mM $MgCl_2$, 17 mM DTT, 0.1 nM guanosine 5′-GTP, 10 % bovine serum albumin and a peptide sample (2 mg/mL final assay concentration) in 300 µL of 50 mM glycylglycine buffer, pH 8.0. For the control assay, the peptide sample was omitted and replaced with buffer. Each reaction mixture was incubated at 37 °C using an Eppendorf Thermomixer R (Eppendorf AG, Hamburg, Germany) for 30 min before transfer to a water bath (95 °C) to terminate the reaction, which was then followed by centrifugation (10,000 x g for 10 min at room 24 °C). Following centrifugation, 10 µL of the supernatant was combined with 100 µL of the luciferin-luciferase reagent in a 1.5 mL microfuge tube. Luminescence was measured as relative luminescence unit (RLU) using a Turner Biosystems $20/20^n$ Luminometer (Fitchbug, WI, USA) in order to quantify the amount of ATP produced in the sample. Heat-inactivated (100 °C for 10 min) CaMPDE was used for the blank and the RLU subtracted from those obtained for the control and peptide-containing samples.

CaMPDE inhibitory activity (%) of the samples was then calculated as:

$$[1–(RLU_S/RLU_C) \times 100]$$

where RLU_C and RLU_S are the net RLU of the control and sample assays (after subtraction of blank RLU), respectively. Each assay was performed in triplicate and the residual enzyme activity was taken as the difference between 100 (uninhibited reaction) and CaMPDE inhibitory activity (%).

7.2.4 Cell culture

Fetal bovine serum (FBS) and L-glutamine (≥99%) were from Gibco (Fisher Scientific, Oakville, ON, Canada), while the Caco-2 (human colorectal adenocarcinoma) cell line was procured from the American Type Culture Collection, ATCC (Manassas, VA, USA). The cells, which were obtained at passage 25, were preserved in liquid nitrogen prior to the study. Subsequently, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were grown in Nunc culture plates (Fisher Scientific, Oakville, ON, Canada), and maintained in a humidified incubator under an atmosphere of 5% CO_2/95% air at 37 °C. The cells were usually sub-cultured after reaching 85% confluence using TrypLE™ Express, and were used for the assays between passage 10 and 15 after recovery from cryopreservation.

7.2.5 Cell Proliferation and Cytotoxicity Assay

The unlabeled peptides were used to test for cytotoxicity against Caco-2 cells as well as their effects on cell proliferation using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocol and the method of Jiehui et al[12], which were slightly modified as follows. Caco-2 cell suspensions (200 µL) were seeded in a 96-well plate and maintained in a humidified incubator as described above for about 48-72 h to reach confluency (a concentration of about 1×10^5/mL). Following cell confluence, media from all wells were aspirated and filter-sterilized peptide solutions (20 µL) were added such that the final peptide concentrations in the

wells ranged from 2-10 mM. PBS was added to control wells in place of the peptides while the blank wells contained PBS only. The plate was then further incubated for 24 h at 37 °C. Thereafter, 20 µL of CCK-8 solution was added to all the wells and the plate was gently swirled before being returned to the incubator for an additional 2 h. With the contents of the wells having settled, the supernatants (200 µL) were transferred to a fresh 96-well plate and the absorbance measured at 450 nm using an Opsys MR 96-well plate reader (Dynex Technologies, Chantilly, VA). Cytotoxicity level (%) was then calculated by comparing absorbance of samples at 450 nm to that of control. The assay was performed in triplicate.

7.2.6 Transport Experiments

Peptide transport across Caco-2 cell membranes was determined using the FITC-labeled peptides according to previously reported methods[13,14] which were slightly modified as follows. Cells (100 µL) were seeded onto Nunc™ polycarbonate cell culture inserts (0.4 µm mean pore size, 0.47 cm^2 culture area, 1 x 10^8 pores/cm^2 pore density; Thermofisher Scientific, Oakville, ON, Canada) containing 0.5 mL and 0.75 mL supplemented DMEM medium, respectively in the apical and basolateral sides in 24-well plates. The integrity of the cell monolayer was measured using an electrical resistance system equipped with a Millicell-ERS-electrode (Millipore, Billerica, MA, USA). The assay was conducted when the transepithelial electrical resistance was greater than 300 Ω cm^2.[14] Peptides dissolved in DMEM medium were added to the apical side prior to the incubation of the plate at 37 °C in 5% CO_2 for 18 and 24 h. At the end of the incubation period, 200 µL of the media on the basolateral side of each insert was transferred to wells in a microplate and the concentration of peptides evaluated in a FLUOstar Omega® microplate reader (BMG Labtech, Germany) by measuring the fluorescence at an excitation and emission wavelength of 495 and 520 nm, respectively.

7.2.7 Animal Feeding Experiment

A total of 24 Wistar rats (5 wks old and 100-200 g body weight) consisting of 12 males and 12 females were purchased from Charles River Laboratories (Montreal, PQ, Canada) and housed individually in steel cages at the Animal Care Facility, Duff Roblin Building, University of Manitoba. The rodents, which were kept under standard conditions (12 h light-dark cycle, 23±2 °C, and relative humidity of 50%), received a regular chow diet and tap water *ad libitum* before and after peptide oral gavage. The animals were allowed to acclimatize for two weeks before commencement of the studies, and measurements of their body weight were taken weekly throughout the duration of the studies using a digital weighing balance. Baseline blood was collected under anesthesia from jugular veins before the rats received one of six chemically synthesized non-FITC labeled flaxseed protein-derived peptides (AGA, AKLMS, QIAK, RWIQ, QQAKQ and KQLSTGC). Each peptide was dissolved in phosphate-buffered saline, PBS (pH 7.2) and administered to six rats (1 mL/rat) by oral gavage at a dose of 200 mg/kg body weight while control rats (6) received only PBS (1 mL/rat). Each group of six rats consisted of 3 males and 3 females. Blood samples were collected in 1 mL Eppendorf tubes from the jugular vein at 30, 60 or 90 min post-gavage using isoflurane[15] as the anesthetic agent. The blood samples were immediately placed on ice to minimize degradation and allowed to clot prior to centrifugation (7000×g, 2 min at 4 °C). The supernatant (serum) and precipitate (blood clot) were subsequently stored at -80 °C until required for further analysis. There was a one week washout period before rats were reused for a different peptide. To determine the activity of residual CaMPDE in the blood, blood clot samples previously stored at -80 °C were gently thawed on ice, combined with appropriate volumes of glycylglycine buffer and vortexed vigorously to solubilize proteins. Each sample tube was then centrifuged (7000×g, 2 min at 4 °C) and the resultant supernatant used as a

source of CaMPDE. The measurement of residual plasma CaMPDE activity was carried out following a protocol similar to that described for the *in vitro* CaMPDE determination method but with 20 µL of the supernatant from the centrifuged blood clot added to the reaction mixture instead of the pure commercial enzyme. Control reactions were performed using 20 µL of pure commercial CaMPDE at final concentrations of 0.0313, 0.0625, 0.125, 0.25, 0.5, 0.75, 1.0 and 1.5 mU. Luminescence values for the control were plotted against the final CaMPDE enzyme concentrations in order to obtain a standard curve. Residual plasma CaMPDE activity was obtained for each absorbance value by linear regression using the standard curve. All animal experiments were conducted according to protocol F13-011/1/2/3, which was approved by the University of Manitoba Animal Ethics Committee.

7.2.8 LC/MS/MS Peptide Quantitation

Peptides present in rat serum were quantified by LC/MS/MS at the Analytical Facility for Bioactive Molecules (The Hospital for Sick Children, Toronto, Canada). Samples (100 µL), pure peptide standards spiked into 100 µL blank rat serum, and deuterated single amino acid internal standards were added to microfuge tubes containing 1 mL methanol. Tubes were vortexed and then centrifuged at 20,000 x g. Supernatants were subsequently transferred to a conical tube and evaporated to dryness under a gentle stream of nitrogen. Afterwards, samples were derivatized with butanol in 3 M HCl for 15 min at 65 °C. Subsequently, samples were dried again and reconstituted in 5:95 water:acetonitrile (v/v) containing 5 mM ammonium formate (pH 3.2) and analyzed by LC/MS/MS. Samples were injected onto a Kinetex 2.6 micron hydrophilic interaction liquid chromatography 50 x 3.0 mm column (Phenomenex, California) on an Agilent 1290 LC system coupled to a Sciex Q-Trap 5500 mass spectrometer. Samples were eluted using a gradient of: (A) 90/10 water/acetonitrile containing 5 mM ammonium formate (pH 3.2), and

(B) 5/95 water/acetonitrile containing 5 mM ammonium formate (pH 3.2) over 9 min. Data was collected and analyzed using Sciex Analyst v1.6.3.

7.2.9 Statistical Analysis

Each assay was conducted at least twice. Where applicable, values are reported as mean ± standard deviation. Statistically significant differences between treatments were determined by one-way analysis of variance (ANOVA) using Duncan's multiple range test ($p < 0.05$) and with the aid of the SAS software desktop version 9.2 (Statistical Analysis Systems, Cary, NC, USA).

7.3 Results and Discussion

7.3.1 In vitro CaMPDE Inhibition

Figure 7.1 shows data from the *in vitro* CaMPDE inhibition screening test conducted using 27 different chemically synthesized peptides whose sequences were originally derived[10] from flaxseed proteins. The aim of the preliminary screening was to select the peptide(s) with the highest inhibitory effect against CaMPDE for use in further (*in vivo*) work. Twelve of the 27 peptides inhibited CaMPDE activity to varying degrees (Figure 7.1.) while 15 peptides had no measurable inhibition (not shown). Due to similarity in CaMPDE inhibitory levels, six of the 12 active peptides namely AGA, AKLMS, QIAK, RWIQ, QQAKQ and KQLSTGC with significantly ($p < 0.05$) highest activities were selected for further work including cell culture (toxicity and membrane transport) and oral gavage to Wistar rats.

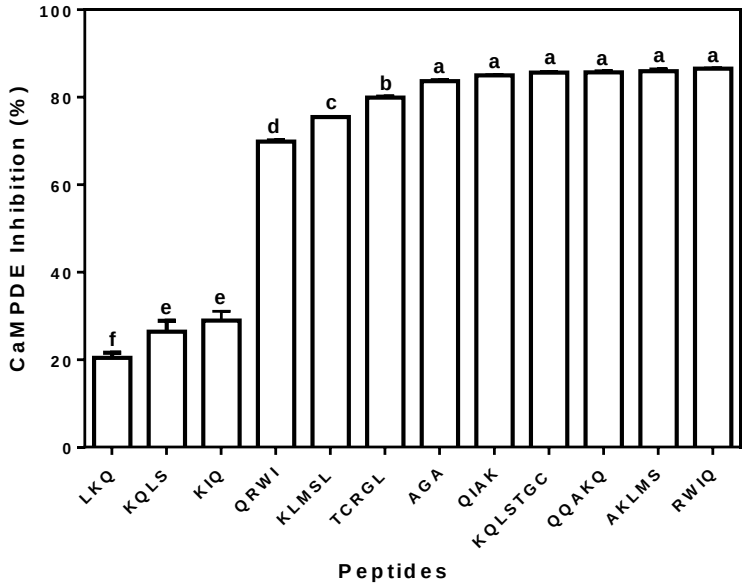


Figure 7.1. *In vitro* CaMPDE inhibition: 12 out of 27 peptides screened for *in vitro* CaMPDE inhibitory activity were effective in blocking the enzyme's action to varying degrees as shown. Data presented are mean ± standard deviation. Different letters on the bar charts indicate significant differences ($p < 0.05$) in enzyme inhibition among peptides.

7.3.2 Effect of bioactive peptides on Caco-2 cell viability

The Caco-2 monolayer has been used extensively for transport studies because of its functional and structural similarity to the human intestinal epithelium.[16] The CCK-8 assay is a very sensitive colorimetric test based on the reduction of the highly soluble tetrazolium salt WST-8 to a yellow product (formazan) by dehydrogenases in cells.[17,18] The absorbance of the formazan dye at 450 nm is proportional to the number of viable cells.[18] In this study, prior to evaluating the transport of peptides across the monolayer, the potential cytotoxic and cell proliferation effects of the peptides were first determined using the CCK-8 assay. Figure 7.2. shows the results of exposing the Caco-2 cells to peptide pretreatment at various concentrations (2-10 mM). The results clearly show that the flaxseed-derived synthetic peptides had no adverse effects on the Caco-2 monolayer and could thus be considered safe. Crucially, pretreating Caco-2 cells with the peptides had no adverse effects on their growth, survival or viability. Usually, a compound or

chemical substance is considered unsafe if it proves to be lethal to half of the organisms it is treated with in a toxicity assay.[16] As evident in Figure 7.2., the two peptides (AGA and RWIQ) apparently stimulated the growth of the Caco-2 cells as did the other four peptides (results not shown). The data for the other four peptides indicate a stimulation of growth beyond the limit of detection of the equipment used for absorbance reading. Although this assay was primarily conducted to assess the safety of the peptides used in this study, the observed growth stimulatory effect of the peptides can be compared to a previous study in which synthetic peptides modulated growth in mammalian cell cultures.[19] In fact, peptones which are mixtures of peptides have been conventionally used as growth supplements in cell culture media, which is consistent with the observed cell growth stimulation in the current report.[20,21]

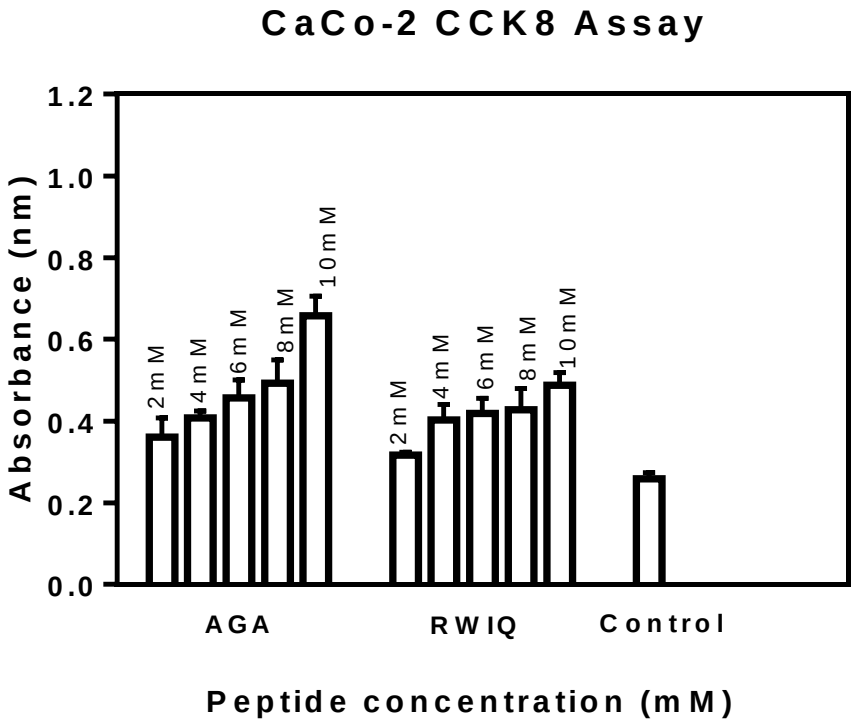


Figure 7.2. Cell viability and cytotoxicity assay. Caco-2 cells were treated with various (2-10 mM) peptide concentrations and the effect of the peptides on cell viability was tested using the CCK-8 reagent. The peptides had no observable toxic effect on the cells but instead stimulated growth in the cell culture.

7.3.3 Peptide Transport Across Caco-2 Cell Monolayer

Results of the transepithelial transport of the peptides across the Caco-2 monolayer as well as for peptides in multi-well plates containing no cells (control) at three different time points are shown in Figure 7.3. As expected, peptides in control wells (no cells) were transported in greater amounts across the polycarbonate inserts with 0.4 µm diameter pores than in the presence of cells. Interestingly, there is a clear effect of transport duration (time) on the amount of peptides that passed through the control inserts into the wells suggesting there is some interaction of the peptides with the polycarbonate membrane.

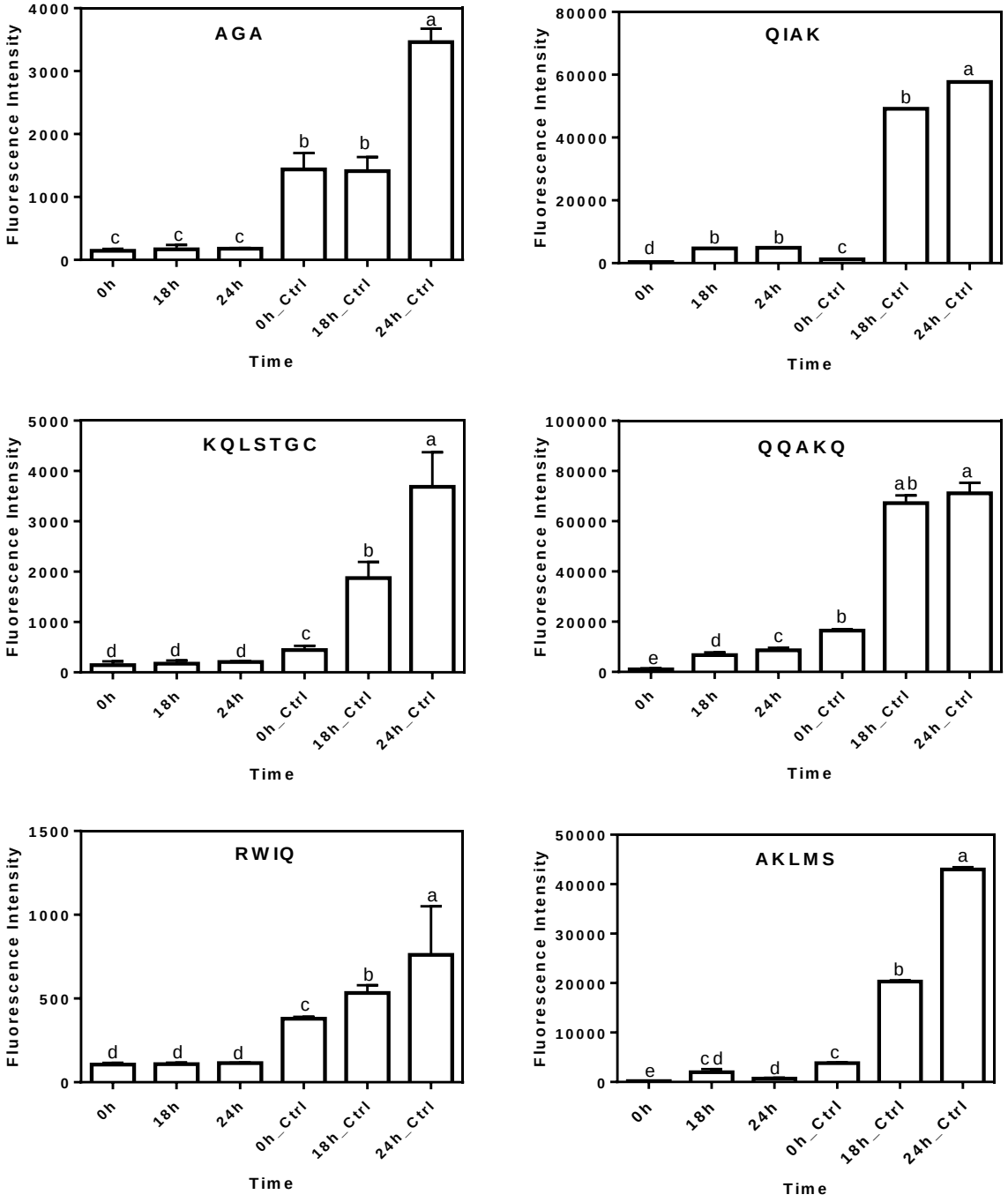


Figure 7.3. Peptide transport in Caco-2 cells. The transport of various FITC-labelled peptides (AGA, QIAK, KQLSTGC, QQAKQ, AKLMS and RWIQ) in confluent Caco-2 cell line was monitored from 0-24 h by means of fluorescent measurements. Data presented are mean ± standard deviation. For each peptide, different letters on the bar charts indicate significant differences ($p < 0.05$) at different time points.

This conclusion is based on the fact that significantly ($p<0.05$) more peptide was detected in the wells after 24 hours when compared to 18 hours (Figure 7.3). With respect to transcellular transport, significantly ($p < 0.05$) higher amounts of QIAK, QQAKQ and AKLMS were detected in the wells beneath the cell monolayer indicating transport had occurred, whereas AGA, KQLSTGC and RWIQ were not detected (Figure 7.3). The formation of an intact monolayer was confirmed by measuring the electrical resistance between the insert and the well for each experiment. In terms of the concentration of peptide transported across the Caco-2 monolayer, QQAKQ was the peptide transported in the highest amount when Caco-2 cells are present in the wells. These data indicate that individual peptides are handled differently by the intestinal epithelium, although it is still essential to establish this *in vivo* since other factors may also influence bioavailability, which is the ability of a peptide to be absorbed. Models such as Caco-2 cells provide an alternative[22] and sometimes complementary tool to *in vivo* transport and bioavailability.

7.3.4 Serum Level of CaMPDE-inhibitory Peptides and in vivo Residual CaMPDE Activity

The data in Figure 7.4 obtained from the LC/MS/MS analysis of the serum samples collected from the rats after oral gavage indicate that only AGA was detected in the blood of the animal models at the three time points (30, 60 and 90 min post-gavage). Two other peptides (AKLMS and RWIQ) were also detected in the rat serum. RWIQ was detected in samples collected from the animal subjects at all the three time points except for the female rats 30 mins post-gavage, while the area ratio ranged from 0.0000475-0.000377. RWIQ was also the only peptide sample detected in the rat serum (0.672 ng/mL) one week post-gavage. For AKLMS, only the blood samples collected from the female rats 30 and 60 mins after oral gavage contained detectable amounts (2.06 ng/mL and 0.675 ng/mL respectively) of the peptide, with the pentapeptide being

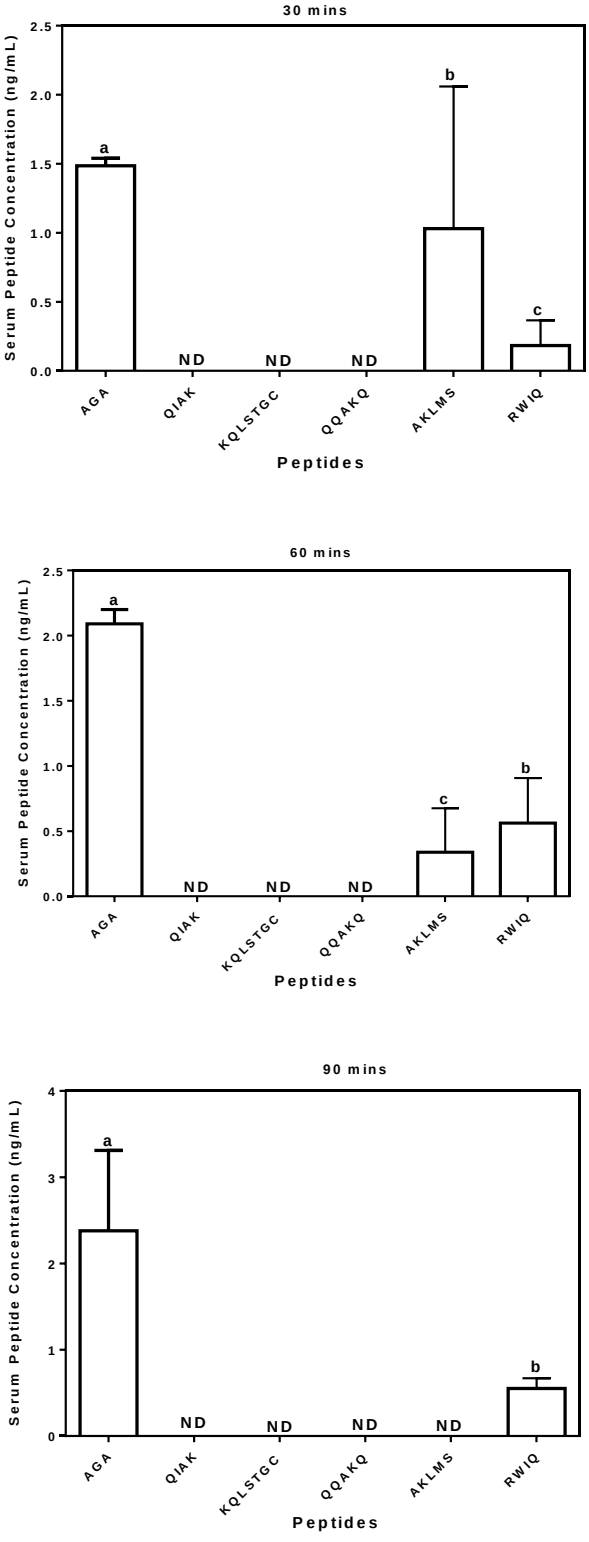
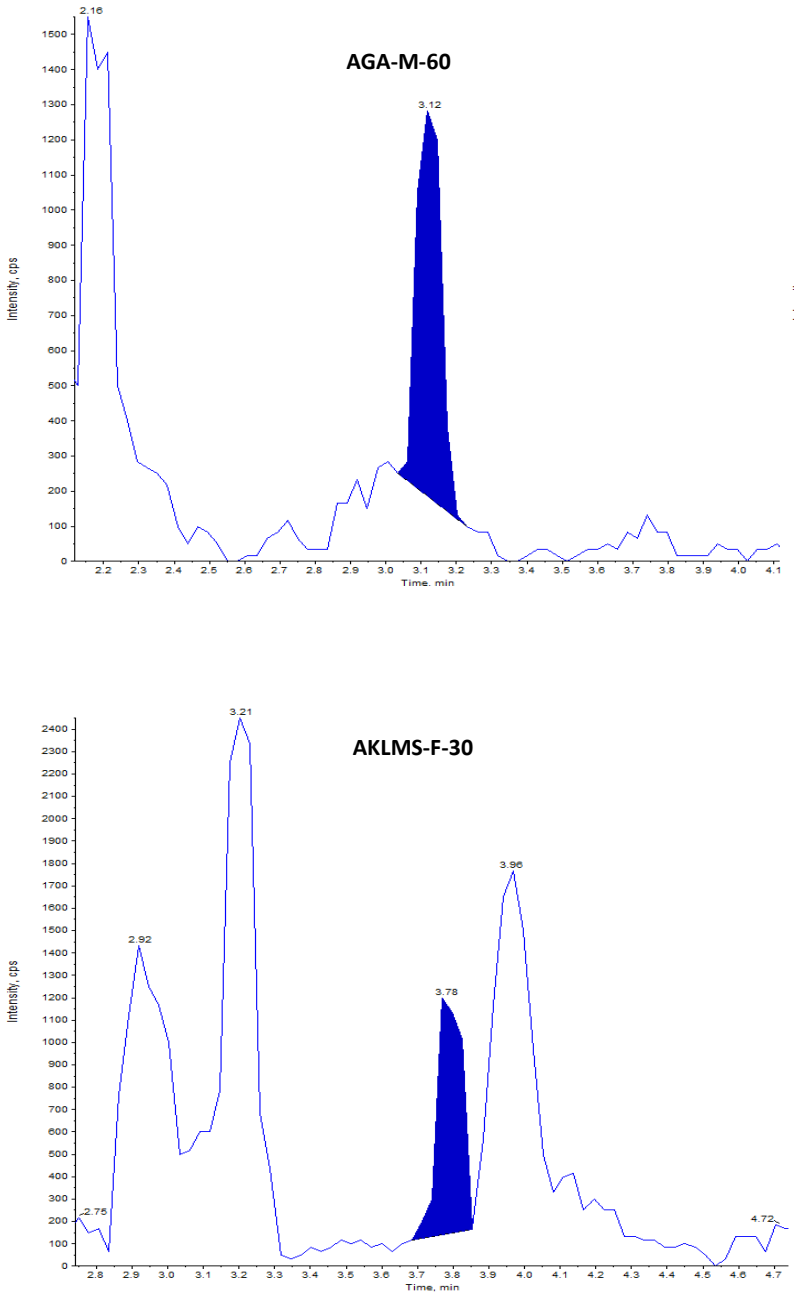


Figure 7.4. Quantification of peptides present in rat serum at 30, 60 and 90 mins post-gavage using LC/MS/MS. ND = not detected. Data presented are mean ± standard deviation. For each time period, different letters on the bar charts indicate significant differences ($p < 0.05$) between peptides at that time point.

absent or below the limit for detection in the other serum samples. Representative chromatograms of the samples detected in rat serum are shown in Figure 7.5. The results are consistent with previous reports that have shown presence of peptide sequences in the blood of humans[23-28] or rats[29,30] after oral ingestion.



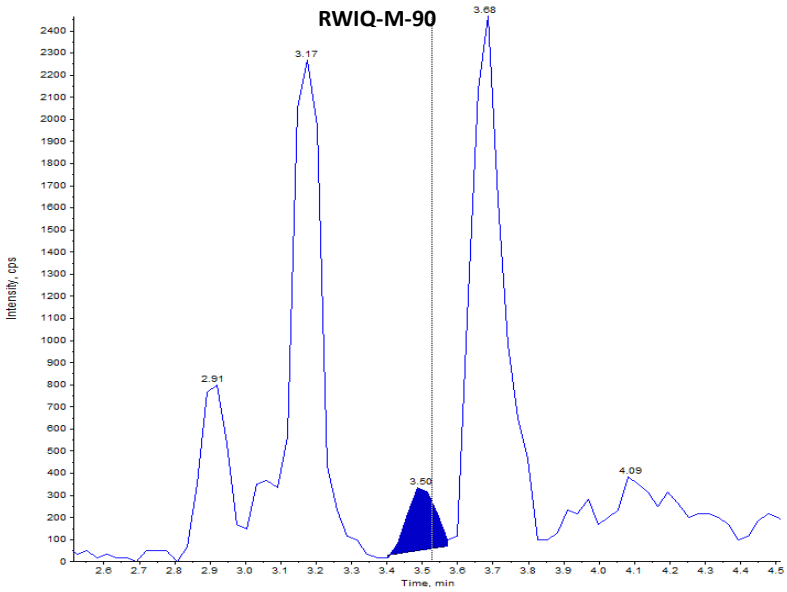


Figure 7.5. Representative chromatograms of peptide samples detected in rat serum. AGA-M-60 = peak for AGA fed to male rats and detected 60 mins post gavage; AKLMS-F-30 = peak for AKLMS administered to female rats and detected 30 mins post gavage; RWIQ-M-90 = peak for RWIQ fed to male rats and detected 90 mins post gavage.

The residual plasma CaMPDE activity in Wistar rats at 30, 60 and 90 min after oral gavage is shown in Figure 7.6. After 30 min, rats receiving the peptides QIAK, KQLSTGC and AGA had significantly lower ($p<0.05$) CaMPDE activity than the rest suggesting that the three peptides were the most effective in inhibiting the enzyme at this time point. However, after 60 min, the tripeptide AGA was the most active inhibitor of plasma CaMPDE activity with a residual enzyme activity of 55.65% which was significantly ($p < 0.05$) lower than the level for the other peptides. Two other peptides namely AKLMS and RWIQ were also noticeably effective in their ability to inhibit the activity of CaMPDE in rat plasma, with residual CaMPDE activities of 70.56 and 69.56 % respectively. In contrast to the earlier time points, the ability of the peptides to inhibit plasma CaMPDE activity was generally weak 90 min post-gavage, with four out of the six peptides having no measurable effect (~100% enzyme activity). Only AGA and RWIQ showed limited inhibition at the 90 min time point with residual enzyme activity of 91 and 96%,

respectively, but were significantly lower than the values obtained for the remaining four peptides.

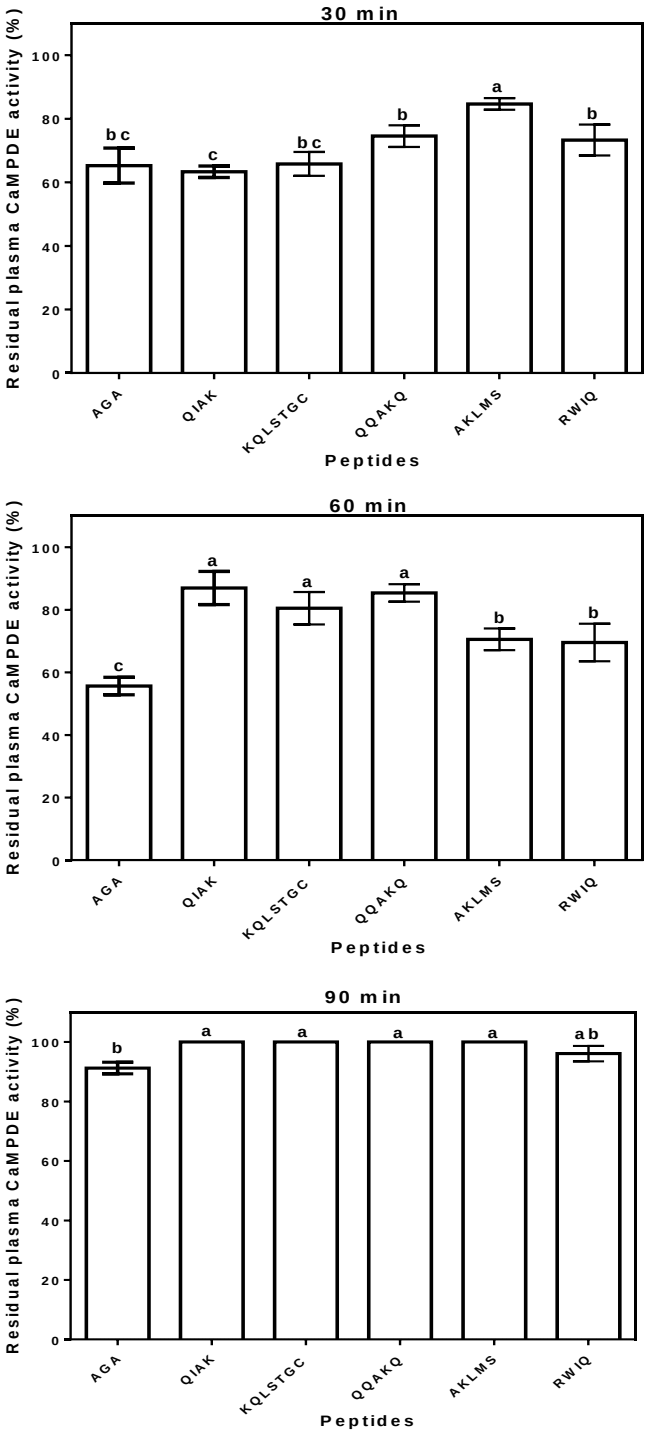


Figure 7.6. Residual plasma CaMPDE activity. Measurement of residual CaMPDE activity in rat plasma as an indication of the inhibitory potential of peptides administered to rats by oral gavage. Data presented are mean ± standard deviation. For each time period, different letters on the bar charts indicate significant differences ($p < 0.05$) between peptides at that time point.

Instructively, the peptides (AGA, AKLMS, and RWIQ) which notably inhibited CaMPDE activity were also invariably those that were detected in rat plasma after LC/MS/MS analysis, thus suggesting that their higher bioavailability contributed to the observed lower residual CaMPDE activity. Although AGA and RWIQ were transported in comparably lesser quantities than QIAK in Caco-2 cells, unlike the latter, they were the only peptides detected in appreciable amounts in rat serum following oral gavage. This is an indication that conditions *in vivo* often differ from those *in vitro* in spite of the presumed similarity of models such as Caco-2 cells to the intestinal epithelium. It is possible that QIAK may have been hydrolyzed into smaller fragments by peptidases present in blood or that it might have been susceptible to first-pass effect.[31] The non-detected peptides may have been present at levels that could not be detected by the method used in this work or they were bound to the CaMPDE protein since some level of enzyme inhibition was observed. However, the hydrolyzed fragments of the non-detected peptides could have contributed to the inhibition of plasma CaMPDE activity. The results are consistent with a recent suggestion that absorption of peptides are determined mainly by their resistance to endogenous peptidases rather than transportability across the cell membrane.[32] Apart from resistance to peptidases, several other factors are also known to influence the transepithelial transport of peptides including their charge, molecular weight and hydrophobicity.[13] Given the various sizes of the peptides used in this study, we conclude that some were resistant to enzyme digestion and transport of peptides across the intestinal epithelium was influenced by their structural and physical properties.

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7.6 Conflict of Interest

The authors declare no conflict of interest

7.7 References

(1) Tajbakhsh, A.; Pasdar, A.; Rezaee, M.; Fazeli, M.; Soleimanpour, S.; Hassanian, S. M… Avan, A. The current status and perspectives regarding the clinical implication of intracellular calcium in breast cancer. *J. Cell Physiol*. **2018**, *233*, 5623-5641.

(2) Means, A. R. Calcium, calmodulin and cell cycle regulation. *FEBS Lett.* **1994**, *347*, 1-4.

(3) Aluko, R. E. Food protein–derived peptides as calmodulin inhibitors. In *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals;* Mine, Y., Li-Chan, E., Jiang, B., Eds.; Wiley-Blackwell: Ames, Iowa, **2010**; pp. 55-65.

(4) Soderling, T.; Stull, J. Structure and regulation of calcium/calmodulin-dependent protein kinases. *Chem. Rev.* **2001**, *101*, 2341-2351.

(5) Li, H.; Aluko, R. E. Structural modulation of calmodulin and calmodulin-dependent protein kinase II by pea protein hydrolysates. *Int. J. Food Sci Nutr.* **2006**, *57*, 178-189.

(6) Kizawa, K. Calmodulin binding peptide comprising alpha- casein exorphin sequence. *J. Agric. Food Chem.* **1997**, *45*, 1579-1581.

(7) Kumrungsee, T.; Akiyama, S.; Guo, J.; Tanaka, M.; Matsui, T. Identification of peptides in wheat germ hydrolysate that demonstrate calmodulin-dependent protein kinase II inhibitory activity. *Food Chem.* **2016,** *213*, 329-335.

(8) Omoni, A. O.; Aluko, R. E. Effect of cationic flaxseed protein hydrolysate fractions on the *in vitro* structure and activity of calmodulin-dependent endothelial nitric oxide synthase. *Mol. Nutr. Food Res.* **2006**, *50*, 958-966.

(9) Li, H.; Aluko, R. E. Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein- derived peptides. *J. Nutr. Biochem.* **2005**, *16*, 656-662.

(10) Udenigwe, C. C.; Aluko, R. E. Multifunctional cationic peptide fractions from flaxseed protein hydrolysates. *Plant Foods Hum. Nutr.* **2012,** *67*, 1-9.

(11) You, S.-J.; Udenigwe, C. C.; Aluko, R. E.; Wu, J. Multifunctional peptides from egg white lysozyme. *Food Res. Int.* **2010,** *43*, 848-855.

(12) Jiehui, Z.; Liuliu, M.; Haihong, X.; Yang, G.; Yingkai, J.; Lun, Z…Shaohui, Z. Immunomodulating effects of casein- derived peptides QEPVL and QEPV on lymphocytes *in vitro* and *in vivo*. *Food Funct.* **2014,** *5*, 2061-2069.

(13) Wang, B.; Li, B. Charge and hydrophobicity of casein peptides influence transepithelial transport and bioavailability. *Food Chem.* **2018,** *245*, 646-652.

(14) Ding, L.; Wang, L.; Zhang, Y.; Liu, J. Transport of antihypertensive peptide RVPSL, ovotransferrin 328-332, in human intestinal Caco- 2 cell monolayers. *J. Agric. Food Chem.* **2015,** *63*, 8143-8150.

(15) Olabiyi, A. A.; Oboh, G.; Adefegha, S. A. Effect of dietary supplementation of tiger nut (*Cyperus esculentus* l.) and walnut (*Tetracarpidium conophorum müll*. Arg.) on sexual behavior, hormonal level, and antioxidant status in male rats. *J. Food Biochem.* **2017,** *41*, e12351.

(16) Masisi, K.; Diehl-Jones, W. L.; Gordon, J.; Chapman, D.; Moghadasian, M. H.; Beta, T. Carotenoids of aleurone, germ, and endosperm fractions of barley, corn and wheat differentially inhibit oxidative stress. *J. Agric. Food Chem.* **2015,** *63*, 2715-2724.

(17) Chang, Y.; Yang, S.-T.; Liu, J.-H.; Dong, E.; Wang, Y.; Cao… Wang, H. *In vitro* toxicity evaluation of graphene oxide on A549 cells. *Toxicol Lett.* **2011,** *200*, 201-210.

(18) Dojindo Molecular Technologies. Cell Counting Kit-8 Technical Manual. URL (www.dojindo.com/store/p/456-Cell-Counting-Kit-8.html) (July 12, 2018).

(19) Franek, F. Peptides modulate growth and productivity of mammalian cell cultures and suppress apoptosis. *BioProcess Int.* **2004,** *2*, 48-52.
(20) Park, S. Y.; Yoon, K. Y. Enzymatic production of a soluble fiber hydrolyzate from Chinese cabbage waste and its health-related properties. *J. Food Biochem.* **2015,** *39*, 274-280.
(21) Mizrahi, A. Primatone RL in mammalian cell culture media. *Biotechnol. Bioeng.* **1977,** *19*, 1557-1561.
(22) Sánchez-Rivera, L.; Martínez-Maqueda, D.; Cruz-Huerta, E.; Miralles, B.; Recio, I. Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. *Food Res. Int.* **2014**, *63*, 170-181.
(23) Foltz, M.; Meynen, E. E.; Bianco, V.; van Platerink, C.; Koning, T. M.; Kloek, J. Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *Journal of Nutrition*, **2007**, *137*, 953–958.
(24) Iwai, K.; Hasegawa, T.; Taguchi, Y.; Morimatsu, F.; Sato, K.; Nakamura, Y.; Higashi, A.; Kido, Y.; Nakabo, Y.; Ohtsuki, K. Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. *J. Agric. Food Chem.* **2005**, *53*, 6531−6536.
(25) Matsui, T.; Tamaya, K.; Seki, E.; Osajima, K.; Matsumoto, K.; Kawasaki, T. Absorption of val-tyr with *in vitro* angiotensin I-converting enzyme inhibitory activity into the circulating blood system of mild hypertensive subjects. *Biol. Pharm. Bull.* **2002**, *25*, 1228-1230.
(26) Ohara, H.; Matsumoto, H.; Ito, K.; Iwai, K.; Sato, K. Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. *J. Agric. Food Chem.* **2007**, 55, 1532−1535.
(27) Shigemura, Y.; Akaba, S.; Kawashima, E.; Park, E-Y.; Nakamura, Y.; Sato, K. (2011). Identification of a novel food-derived collagen peptide, hydroxyprolyl-glycine, in human peripheral blood by pre-column derivatisation with phenyl isothiocyanate. *Food Chem.* **2011**, *129*, 1019−1024.
(28) Shigemura, Y.; Suzuki, A.; Kurokawa, M.; Sato, Y.; Sato, K. Changes in composition and content of food-derived peptide in human blood after daily ingestion of collagen hydrolysate for 4 weeks. *J. Sci. Food Agric.* **2018**, *98*, 1944-1950.
(29) Sontakke, S. B.; Jung, J-H.; Piao, Z.; Chung, H. J. Orally available collagen tripeptide: enzymatic stability, intestinal permability, and absorption of Gly-Pro-Hyp and Pro-Hyp. *J. Agric. Food Chem.* **2016**, *64*, 7127−7133.
(30) Matsui, T.; Imamura, M.; Oka, H.; Osajima, K.; Kimoto, K.; Kawasaki, T.; Matsumoto, K. Tissue distribution of antihypertensive dipeptide, Val-Tyr, after its single oral administration to spontaneously hypertensive rats. *J. Pept. Sci.* **2004**, *10*, 535, 545.
(31) Sánchez-Rivera, L.; Ares, I.; Miralles, B.; Gómez-Ruiz, J. Á.; Recio, I.; Martínez-Larrañaga, M. R… Martínez, M. A. Bioavailability and kinetics of the antihypertensive casein- derived peptide HLPLP in rats. *J. Agric. Food Chem.* **2014,** *62*, 11869-11875.
(32) Matsui, T. Are peptides absorbable compounds? *J. Agric. Food Chem.* **2018**, *66*, 393−394.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

8.1 Conclusions

This thesis work was designed to characterize the functional and bioactive properties of flaxseed proteins and peptides including their physicochemical, antihypertensive and antioxidant functions, as well as their capacity to modify the activities of a key metabolic enzyme such as CaMPDE following transepithelial absorption. The thesis employed a blend of *in vitro* and *in vivo* assays to provide answers to key research questions surrounding the health-promoting properties of flaxseed protein-derived bioactive peptides, their potential for use as ingredients in functional foods and nutraceuticals, and their transport across the intestinal lumen into the systemic circulation. The conclusions from this work are summarized as follows:

A. As reported in the first study (Manuscript 1/Chapter 3), alterations in pH led to changes in functionality in flaxseed albumins and globulins due to the different amino acid composition and structural conformations of the two flaxseed proteins. Specifically, the S_o of the albumin and globulin proteins continuously decreased as the pH changed from acidic to basic. Since changes in surface hydrophobicity are linked to protein conformational changes (Chao et al., 1997), such pH-dependent shifts in S_o exemplify the modifications in protein structure/conformation and function that could be observed following the disruption of hydrogen bonds due to specific alterations in the protein environment, which in this case comprise changes in acidity or alkalinity. Furthermore, structural and conformational changes following pH adjustments and possibly as a result of increased net protein charge resulted in increased solubility of the albumin and globulin proteins. Thus, this study successfully demonstrated the potential use of alterations in acidity or alkalinity of the protein environment to influence the

functionality of flaxseed-containing food protein products. This strategy could be exploited by food quality and food product design units in the food industry in creating food products containing these proteins. In addition to their basic nutritive value, proteins (as a result of their functional properties) are known to influence organoleptic qualities such as taste, mouthfeel, texture and appearance; processing characteristics like foam, gelling and emulsification, as well as storage attributes like palatability and shelf life. Thus, they are considered indispensable ingredients in the formulation of food products. Given the desirable emulsification properties of the studied flaxseed proteins in general, and of the albumin fraction in particular, this study showed that proteins from flaxseed could serve as additives in food products such as salad dressings, desserts (e.g. ice cream) and mayonnaise where excellent emulsions are required. Moreover, by showing a clear effect of protein concentration variation on emulsification properties, the results indicate the flexibility with which these proteins could be used as additives in food products. For instance, since high protein concentration favoured better emulsion formation but led to reduced emulsion stability, flaxseed proteins could be used as additives at low concentrations for food formulations in which long lasting emulsions are desirable, while higher protein concentrations would be ideal for food products in which the intensity of the emulsions formed is more valuable than their stability. Additionally, given the high surface hydrophobicity of flaxseed globulin fraction as shown in this work, it could be a useful additive in ground meat where fat absorption is known to help reduce cooking losses and promote structural cohesiveness. This is because high surface hydrophobicity is positively correlated with increased fat binding capacity (Voutsinas & Nakai, 1983),

and the capacity of a food-derived protein to bind and retain fat influences its texture and ability to interact with lipids in emulsions.

B. Following a systematic assessment of techniques commonly used to measure proteins in hydrolysates and peptides, this work (Chapter 4) recommended the use of three methods (particularly amino acid analysis) for the most reliable and accurate determination of proteins in hydrolysates and peptides. As shown by data from the Pearson correlation coefficient measurement, the result of the amino acid analysis showed the highest number of individual effect (r) sizes indicating the greatest linear correlation of all the methods studied, and thus the most accurate estimate of protein content in food protein hydrolysates. The study employed up to seven analytical methods in evaluating the protein content of a variety of enzymatic hydrolysates selected from both animal and food protein sources thus ensuring the robustness and objectivity of the methods that were ultimately recommended.

   On a wider note, the detection and accurate measurement of the amount of proteins, bioactive peptides and amino acids in foods is of great importance in order to confirm their quality, content and safety, guarantee the correct labelling of products, validate biomarkers in experimental samples, and generally ensure the integrity of research data (Asensio et al., 2008; Moore et al., 2010; Trötschel & Poetsch, 2015). Since methods such as Kjeldahl and Dumas, which are widely used for quantifying food proteins usually measure the amount of total nitrogen in foods, and do not distinguish between non-protein nitrogen and protein-based nitrogen, an unfortunate but inevitable corollary of such absence of analytical selectivity is the adulteration of food products and this often portends grave risks to public health (Moore et al., 2010). To illustrate, about 10 years

ago, the deliberate adulteration of food and feed with melamine, an industrial chemical mainly used as a plastics stabilizer and flame retardant, led to serious illness and death in pets and children (Xin & Stone, 2008). Given that the addition of melamine ($C_3H_6N_6$) to foods raises their nitrogen content and thus their apparent protein content, the contaminated products slipped through the regular nitrogen-based analytical and quality control procedures without detection (Ingelfinger, 2008). Thus, as suggested by the results from this study, a protein determination method like amino acid analysis, which is based only on a food protein's amino acid content and which directly measures the protein content in food hydrolysates rather than the indirect estimation of protein content by converting nitrogen content to protein, offers the most accurate and reliable protein determination procedure for hydrolysates and peptides. By recommending this method of protein measurement, this work has the potential to make the comparison of protein content-based data obtained by researchers working in this area of food science, nutrition and protein chemistry easier and more meaningful.

C. The capacity of flaxseed-derived protein hydrolysates and their ultrafiltration fractions to act as antihypertensive agents was also evaluated in this work. Precisely, the use of thermoase GL-30 for FPI hydrolysis contributed to the release of antihypertensive BCCA-rich (up to 15.80 %) and hydrophobic (up to 36.13 %) amino acids. As reported in Chapter 5, flaxseed protein hydrolysates obtained using various concentrations of the protease thermoase GL-30 and their ultrafiltration fractions inhibited the *in vitro* activities of renin and ACE, the two principal enzymes of the renin-angiotensin system. In particular, the two most active hydrolysate samples inhibited the activity of ACE by at least 85% while their inhibition of renin activity was approximately 40%. The separation

of the hydrolysates into fractions of varying sizes (ranging from <1 - 10 kDa) was effective in enhancing their inhibitory potency. In particular, fractions containing low molecular weight peptides (<1-3 kDa) were very potent in inhibiting the *in vitro* activities of ACE and renin. When administered to spontaneously hypertensive rats, the crude hydrolysates and the peptide fractions showed strong antihypertensive properties with the most effective sample being the 3-5 kDa fraction (obtained using 3% thermoase concentration), which reduced blood pressure by up to 37 mmHg within 8 hrs. In this work, we demonstrated for the first time, the blood pressure-lowering properties of functional food proteins hydrolyzed from flaxseed by the enzyme thermoase. This work is highly significant because thermoase is one of the cheapest proteases available for industrial use; hence the developed method can be readily scaled up to a very cost-effective pilot plant or commercial manufacturing process to produce an affordable antihypertensive flaxseed protein hydrolysate. The inhibition of ACE and renin by up to 40% and 90%, respectively by the most active protein hydrolysates and peptide fractions is demonstrable proof that the flaxseed-derived proteins could modulate multiple blood pressure regulation pathways. Also, given that the most active samples reduced blood pressure by up to 37 mm Hg only 8 hrs post-gavage, they hold great promise for use in the formulation of fast-acting antihypertensive nutraceuticals.

D. The capacity of flaxseed-derived protein hydrolysates and peptide fractions to scavenge free radicals, chelate metal ions and reduce ferric iron to the more stable ferrous form was successfully demonstrated in this work, thus establishing that thermoase could liberate potent antioxidative hydrolysates and peptide sequences from flaxseed proteins. Furthermore, the antioxidative activity of flaxseed protein hydrolysates and membrane

fractions was found to be a factor of peptide size and the concentration of thermoase used for hydrolysis. In particular, low molecular weight peptides (<1 kDa and 1-3 kDa) were more effective in reducing the Fe^{3+}-ferricyanide complex to the more stable ferrous form than high molecular weight peptides (5-10 kDa), thus illustrating the effect of peptide size on antioxidative activity. It is important to note that although several active peptide sequences were revealed by mass spectrometry to be located within flaxseed conlinin, additional potent peptide sequences could also have been found in the other major flaxseed proteins if their primary structure was available like that of conlinin. In general, the data from this portion of the project (Manuscript 4) support the conclusion that flaxseed proteins could serve as active components of functional foods and nutraceuticals designed to prevent, control or delay the onset of oxidative stress-related disease conditions.

E. In Chapter 7, we used a combination of *in vitro* biochemical assays, cell culture experiments and *in vivo* animal work to show for the first time that bioactive peptides derived from flaxseed protein sequences could successfully undergo transepithelial transport and be detected in the blood in their intact forms. Crucially, three (AGA, AKLMS and RWIQ) of the six flaxseed-derived peptides orally administered to rats were detected in their plasma at various time periods post-gavage using LC/MS analysis, thus underlining the capacity of the peptides to be transported across the intestinal epithelium. The capacity of flaxseed-derived synthetic peptides to inhibit the activity of Ca^{2+}/calmodulin-dependent phosphodiesterase was demonstrated using an *in vitro* bioluminescence-based assay. The six most active (out of a total of 27) peptides were tested for cytotoxicity using the CCK-8 assay and were found to have no adverse effect

on cell viability and proliferation. Results from *in vitro* transport studies using Caco-2 cells showed that the peptides could be transported across the intestinal epithelium (since the Caco-2 monolayer is a model of the human intestinal epithelium). Data from the LC/MS analysis of blood samples collected following oral administration of the peptides to Wistar rats showed that some of the peptides could withstand the degradative action of peptidases and were able to influence activity of the target enzyme in the blood, in addition to being detected in the blood in their intact forms, 30-90 min post-gavage. Of particular interest is the detection of RWIQ in blood samples one week after oral gavage. Thus, this work provides evidence supporting the idea that orally ingested bioactive peptides are absorbable and could directly influence physiological functions *in vivo*.

As a general note, by demonstrating the beneficial bioactive properties of flaxseed proteins, this work has the potential to increase the fiscal value of flaxseed which is an economically important crop in Canada.

8.2 Limitations of the Study

Although this thesis work used a variety of tools including multidisciplinary *in vitro* and *in vivo* experimental protocols to provide answers to the important questions the project set out to answer, as is common in research, not every possible experimental ground was covered. As a result of financial and time constraints, the following limitations exist in this work:

I. In addition to the extraction of undigested flaxseed protein meal using 0.5 M NaCl, the use of cellulase to digest an additional sample of the meal, (for the purpose of fibre hydrolysis) prior to NaCl extraction, would have provided additional information on the functional properties of the albumins and globulins.

II. Since the protein isolation protocol included the use of dilute acid and alkali, the flaxseed protein hydrolysates and peptide fractions should have been tested for any toxic effects prior to administering them to spontaneously hypertensive rats.

III. The use of inhibitors such as cytochalasin D, Gly-Sar, wortmannin, etc would have provided more information on the mechanism of transport of peptides used in Caco-2 cell transport studies.

8.3 Future Directions

In this work, we demonstrated the beneficial effects of flaxseed-derived protein hydrolysates and bioactive peptides including their blood pressure-lowering, antioxidative and CaMPDE-inhibitory properties. Having obtained *in vitro* and *in vivo* (animal model) data supporting the idea that flaxseed proteins could act as health-promoting agents, the logical next step for this work would be the replication of these properties in human clinical trials.

Although food protein-derived hydrolysates and bioactive peptides are produced from natural food sources, which have been regularly consumed for ages by humans without adverse effects, it is vital to conduct safety/toxicity tests prior to the commencement of human clinical trials in order to allay all possible peptide safety concerns (Sarmadi & Ismail, 2010). Such tests would not only be relevant to human clinical trials conducted to test the validity of the beneficial health-promoting properties already observed in this work, but also to peptide transport studies, given the reported hemolytic and membranolytic properties linked with certain cell-penetrating peptides (Li-Chan, 2015).

Additionally, given our expectation that a successful human clinical trial outcome would engender and expedite efforts for the addition of flaxseed-derived proteins to functional foods and nutraceuticals, it is imperative to investigate strategies for masking the bitter taste of

flaxseed protein hydrolysates and peptides. Enzymatic protein hydrolysates are known to be notoriously bitter thus impairing their sensory attributes and limiting their consumer acceptability (Aluko, 2017; Zhang et al., 2018). Proteins such as Thaumatin and Monellin enjoy tremendous commercial success partly as a result of their sweet taste attributes, thus effective strategies for masking the inherent bitter taste of flaxseed protein hydrolysates e.g. enzymatic exopeptidase treatment and spray-drying encapsulation, would greatly improve their prospects for use in food product formulations (Li-Chan, 2015; Luyten et al., 2004).

Finally, it would be useful to include an examination of the accumulation of peptides in tissues such as the kidney, heart and lung in future studies of the transport of flaxseed-derived peptides in order to obtain more accurate information on peptide-induced functions like vasorelaxation (Matsui, 2018; Matsui et al., 2004).

8.4. References

Aluko, R. E. (2017). Structural characteristics of food protein-derived bitter peptides. In M. Aliani, & M.N.A. Eskin (Eds.). *Bitterness: Perception, Chemistry and Food Processing*, (pp. 105-129). Hoboken, New Jersey: John Wiley & Sons, Inc.

Asensio, L., Gonzalez, I., Garcia, T., & Martin, R. (2008). Determination of food authenticity by enzyme- linked immunosorbent assay (ELISA). *Food Control,* **19**(1):1-8.

Chao, C-C., Ma, Y-S., & Stadtman, E. R. (1997). Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. *Proceedings of the National Academy of Sciences of the United States of America*, **94**(7): 2969-2974.

Ingelfinger, J. R. (2008). Melamine and the global implications of food contamination. *The New England Journal of Medicine,* **359**(26):2745-2748.

Li-Chan, E. C. (2015). Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science,* **1**(1):28-37.

Luyten, H., Vereijken, J., & Buecking, M. (2004). Using proteins as additives in foods: an introduction. In R.Yada (Ed.). *Proteins in Food Processing* (pp. 421–441). Cambridge, England: Woodhead Publishing.

Matsui, T. (2018). Are peptides absorbable compounds? *Journal of Agricultural and Food Chemistry,* **66**(2):393-394.

Matsui, T., Imamura, M., Oka, H., Osajima, K., Kimoto, K. I., Kawasaki, T., & Matsumoto, K. (2004). Tissue distribution of antihypertensive dipeptide, Val-Tyr, after its single oral administration to spontaneously hypertensive rats. *Journal of Peptide Science,* **10**(9): 535-545.

Moore, J. C., Devries, J. W., Lipp, M., Griffiths, J. C., & Abernethy, D. R. (2010). Total protein methods and their potential utility to reduce the risk of food protein adulteration. *Comprehensive Reviews in Food Science and Food Safety*, **9**(4):330-357.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides,* **31**(10):1949-1956.

Trötschel, C., & Poetsch, A. (2015). Current approaches and challenges in targeted absolute quantification of membrane proteins. *Proteomics,* **15**(5-6):915-929.

Voutsinas, L. P., & Nakai, S. (1983). A simple turbidimetric method for determining the fat binding capacity of proteins. *Journal of Agricultural and Food Chemistry*, **31**(1):58-63.

Xin, H., & Stone, R. (2008). Tainted milk scandal. Chinese probe unmasks high-tech adulteration with melamine. *Science*, **322**(5906):1310-1311.

Zhang, C., Alashi, A. M., Singh, N., Liu, K., Chelikani, P., & Aluko, R. E. (2018). Beef protein-derived peptides as bitter taste receptor T2R4 blockers. *Journal of Agricultural and Food Chemistry*, **66**(19):4902-4912.