STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF HEMP SEED (*CANNABIS SATIVA* L.) PROTEIN-DERIVED ANTIOXIDANT AND ANTIHYPERTENSIVE PEPTIDES

By

Abraham Tartenger Girgih

A Thesis

Submitted to the Faculty of Graduate Studies

of the University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

The aim of this work was to produce enzymatic hemp seed protein hydrolysates (HPH) followed by bioassay guided fractionation to identify antioxidant and antihypertensive peptides. Therefore, simulated gastrointestinal digestion of isolated hemp seed proteins was conducted using consecutive actions of pepsin and pancreatin to produce HPH, which was then separated by membrane ultrafiltration to obtain peptide sizes of <1, 1-3, 3-5, and 5-10 kDa. Evaluation of HPH and its membrane fractions for antioxidant and antihypertensive properties showed that they significantly ($P<0.05$) scavenged radicals, reduced and strongly chelated metal ions as well as inhibited lipid oxidation. During a 24-hr test, the HPH reduced systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR) after oral administration by a maximum of -30 mmHg when compared to -15 mmHg for the membrane fractions.

To reduce production cost, hemp seed protein meal (HPM) was directly hydrolyzed to a protein hydrolysate (HMH) and was shown to also reduce SBP during 4-8 weeks of dietary feeding. The attenuation of SBP correlated to suppressed plasma levels (0.047-0.059 U/mL and 0.040-0.054 µg/mL) of angiotensin converting enzyme (ACE) and renin, respectively, when compared to the control rats (0.123 U/mL and 0.151 µg/mL). A total of 23 peptides were identified to be present in the HPH. WVYY and PSLPA showed superior *in vitro* antioxidant properties, while ACE activity was inhibited by WYT (89%), WVYY (91%) and PSLPA (90%). Renin activity was inhibited by WYT (77%), SVYT (87%) and IPAGV (75%). However, oral administration to SHR showed that the pentapeptides (PSLPA and IPAGV) were more effective SBP-reducing agents (-40 mm Hg) when compared to the tri- (-13 mmHg) and tetrapeptides (-36 mmHg). These results

show for the first time in literature, the bioactive properties of hemp seed peptides and indicate their potential use as ingredients to formulate antioxidant and antihypertensive functional foods and nutraceuticals.

ACKNOWLEDGEMENTS

First and foremost, I would like to sincerely thank God for giving me a unique supervisor in the person of Dr. Rotimi Aluko who has provided me the needed support, adequate funding, excellent supervision and exemplary mentorship throughout the duration of my doctoral studies. I would also like to extend a big thank you to my thesis committee members, Drs. Nancy Ames and Thomas Netticadan for providing useful suggestions and offering their unflinching support throughout the years of my Ph.D program. My deepest appreciation goes to the external examiner, Dr. Elvira de Mejia of the Department of Food Sciences and Human Nutrition, University of Illinois at Urbana Champaign, for accepting the challenge to read and evaluate this thesis despite her tight schedules and overwhelming responsibilities. It is worth acknowledging the financial support that this work received from various sources especially research grants from Natural Sciences and Engineering Research Council of Canada and Advanced Foods and Materials Network of Centres of Excellence as well as scholarships/fellowships from the University of Manitoba, Faculty of Graduate Studies. I sincerely appreciate also the financial support of my state governor, His Excellency, Rt. Hon (Dr) Gabriel Torwua Suswam, the executive governor of Benue State of Nigeria at the early stage of my Ph.D program. While thanking my referees, Professors Daniel Nomishan (Fitchburg State University, MA), Joseph Orban (Southern University at Shreveport, LA) & Paul Ocheje (University of Windsor, ON) for their strong reference letters and recommendations that earned me admission into the University of Manitoba, Winnipeg, Professor Emmanuel Kucha, the Vice Chancellor of the Federal University of Agriculture, Makurdi must also be commended for sustaining my admission for the Ph.D program at U of M, which was

almost revoked due to a mistake made on my transcripts. He followed up the matter ensuring that the correction was effected with an accompanying letter from the Registrar of the University of Agriculture, Makurdi to affirm the authenticity of my results. I am also grateful for the financial assistance received from the Faculty of Human Ecology and the Department of Human Nutritional Sciences. I would also like to thank Drs. Jianping Wu, Harold Aukema and Semone Myrie's Labs for helping out with the sequencing, evaluation of the antioxidant properties of my hemp seed peptides and determining the lipid profile of my rats respectively, without which the thesis would be incomplete. I would like to appreciate all the faculty members who have provided guidance throughout my PhD work: Drs Peter Jones, Michel Aliani, Michael Eskin, Gustaaf Sevenhuysen, Miyoung Suh, Mohammed Moghadasian, Christina Lengyel, Carla Taylor, James Friel, James House, Usher T. Hollander, Peter Eck and Peter Zahradka. Unquantifiable thanks are due to Dr. Aluko's research Lab members and the Department Technicians for their perennial help in the course of my research work. Also deserving commendation is all my brothers, sisters, friends and well wishers who at some point or the other have chipped in a few words of encouragement and have said prayers ceaselessly to support this journey, which has made my graduation a reality. My sincere gratitude goes to my senior brother Mr Augustine Agir Girgih and his late wife, Mrs Juliana Zerena Girgih who layed a sound educational foundation that saw me through my first degree. Thanks to Mr Job and his wife, Elizabeth Girgih whose support earned me the Masters degree. Pastor Philip Akaahan Girgih & family's prayers for success in my educational journey are deeply appreciated. The support and prayers of my Mothers (Monica, Ikyuma and Jato Girgih), Nephews' (Peter Azaagee, James Fila, Shuan and Ageyina Meraba) & families are not

forgotten but highly appreciated. Finally immeasurable appreciation goes to my nuclear family especially my wife Anna and our four boys: Raphael, Michael, Gabriel and Tobit. I wish all who have contributed to this success directly or indirectly God's abundant blessings!

FOREWARD

This thesis was written using the manuscript style and is composed of seven manuscripts which follow immediately after the General Introduction and Literature Review chapters. The manuscripts were written in different journal styles as follows: Manuscripts 1 & 2 (Journal of American Oil Chemists Society) Manuscript 3 (Plant Foods for Human Nutrition), Manuscript 4 (Journal of Functional Foods), Manuscripts 5 & 6 (Food Chemistry), and Manuscript 7 (Food Research International). Manuscripts 1, 2 and 3 have been published, Manuscripts 4, 5, 6 & 7 are currently under internal revision for submission to journals indicated above for peer review. A transition statement at the end of each manuscript links the next chapter for a consistent flow and the format used for list of references cited in the Introduction and Literature Review chapters is according to that of the Journal of Food Chemistry. The last chapter provides an overall summary of the thesis with concluding remarks, novelty of the work, its limitations and future directions of the research project.

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LIST OF ABBREVIATIONS

AAA	Aromatic amino acids
AAPH	2,2'- azobis (2-amidinopropane) dihydrochloride
ACE	Angiotensin converting enzyme
AT-I	Angiotensin I
AT-II	Angiotensin II
BCAA	Branched chain amino acid
BP	Blood pressure
BW	Body weight
CE	Catalytic efficiency of enzyme
CPH	Canola protein hydrolysate
DPPH	2,2-diphenyl-1-picrylhydrazyl
DWB	Dry weight basis
E/S	Enzyme-substrate ratio
FAPGG	*N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine
FRAP	Ferric reducing antioxidant power
FIU	Flourescence Intensity Unit
FPH	Flaxseed protein hydrolysates
GSH	Glutathione
HAA	Hydrophobic amino acids
HMH	Hemp seed protein meal hydrolysate
HMW	High molecular weight
HPLC	High Performance Liquid Chromtography

HPH	Hemp seed protein hydrolysate
HPI	Hemp seed protein isolate
HRSA	Hydroxyl radical scavenging activity
IC_{50}	50% inhibitory concentration of enzyme inhibitor
K_i	Enzyme-inhibitor dissociation constant
K_m	Michaelis constant or enzyme-substrate dissociation constant
K_m', K_m^{app}	Apparent Michaelis constant
KNOS	Kinin-nitric oxide system
LAO	Linoleic acid oxidation
LMW	Low molecular weight
MCA	Metal chelation activity
MW	Molecular weight
MWCO	Molecular weight cut-off
NCAA	Negatively charged amino acids
NO	Nitric oxide
NTRs	Normotensive rats
ORAC	Oxygen radical absorbance capacity
$^{\cdot}OH$	Hydroxyl radical
$O_2^{\cdot -}$	Superoxide radical anion
$ONOO^-$	Peroxynitrite
PCAA	Positively charged amino acids
QSAR	Quantitative structure-activity relationship

RAS	Renin-angiotensin system
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
RP-HPLC	Reverse-Phase High performance liquid chromatography
SBP	Systolic blood pressure
SCAA	Sulphur-containing amino acids
SHRs	Spontaneously hypertensive rats
SRSA	Superoxide radical scavenging activity
$\mathbf{V_{max}}$	Maximum enzyme reaction rate
$\boldsymbol{V_{max}'}$, $\boldsymbol{V_{max}^{app}}$	Maximum apparent enzyme reaction rate

CHAPTER 1

1.1 GENERAL INTRODUCTION

There is a global prevalence of human chronic degenerative diseases such as obesity, diabetes, cancer and cardiovascular diseases (Hernández-Ledesma, Del Mar Contreras & Recio, 2011). These myriad of diseases have been traced to cellular oxidative stress resulting from excessive production of reactive oxygen species (ROS)/free radicals, which overwhelm the body's ability to regulate them (Lobo, Patil, Phatak & Chandra, 2010). The high ROS levels can also cause dysfunction of the immune system, hyper-secretion of vasoactive enzymes into the blood circulatory system, which promotes adverse cardiovascular events and inflammation. ROS/free radicals are very unstable and highly reactive due to their possession of unpaired electrons (Lü, Lin, Yao & Chen, 2010); therefore, they have a high tendency to donate oxygen or electrons to other substances or abstract hydrogen from them and can behave as oxidants or reductants. These reactive species attack and damage macromolecules such as lipids, carbohydrates, DNA, proteins, and cell membranes to cause homeostatic disruption. In addition, this oxidative modification of biomolecules by ROS are involved in physiological and pathophysiological processes such aging, atherosclerosis, inflammation, carcinogenesis and drug toxicity (Dai & Mumper, 2010; Lobo, Patil, Phatak & Chandra, 2010).

The excessive secretion of renin and angiotensin I converting enzyme (ACE) in the renin-angiotensin system (RAS) have been implicated in production of high angiotensin II (AT-II) levels from the inactive angiotensin I (AT-I). High plasma levels of AT-II and ACE leads to severe vasoconstriction (accompanied by inadequate relaxation) and inactivation of the bradykinin vasodilatory properties, respectively, which

results in elevated arterial blood pressure (BP) (Pihlanto & Mäkinen, 2013). These conditions progressively result in a terminal disease state (when left untreated) called hypertension which is one of the leading causes of mortality around the world (Norris & Fitzgerald, 2013). Based on reported statistics by Centre for Disease Control and Prevention (CDC), the estimated costs for treating hypertension and related diseases reached $76.6 billion in the US in 2010. It is thought that prevention through healthy lifestyle choices and engaging in early treatments for individuals with mild hypertensive conditions using natural food sources can significantly reduce global health-care costs. Over the years, pharmaceutical drugs therapy have been used for the treatment and management of these chronic diseases, however, this approach is saddled with safety concerns of the potential for drug toxicity. Prolonged use of certain antihypertensive medications is also associated with negative side effects, which coupled with rising health care costs calls for alternative strategies such as the use of food-derived bioactive compounds that will be safer and affordable. Emerging scientific results from various *in vitro* and *in vivo* studies have supported the use of functional foods and nutraceuticals (FFN) as an effective approach towards the prevention, amelioration, treatment and management of chronic ailments. FFN can also reduce over dependence on drugs through complementary use to lower drug dosage. Though FFN are not as potent as pharmaceutical drugs, they exert their intended physiological/health benefits with minimal to no known side effects. This is because FFN are natural food components that are easily metabolized and do not exert excessive demand on the liver and kidneys.There is currently a search for dietary compounds that will prevent the development of oxidative stress-related and cardiovascular diseases. Recent observational studies and

clinical trials have suggested that increased consumption of proteins particularly from plant sources may act as potent antioxidant and antihypertensive agents that could reduce excessive production of ROS/free radicals. These agents could also suppress over secretion of vasoactive enzymes which might reduce blood pressure and prevent the onset or progression of cardiovascular diseases. Some of these on going FFN research works focus on the production, identification and characterization of bioactive peptides from plant or animal sources with the potential to exert various physiological functions beyond their basic nutritional roles in the provision of nitrogen and essential amino acids (Norris & Fitzgerald, 2013; Pihlanto & Mäkinen, 2013) that will prevent the occurrence or at least help in the management of chronic diseases (Huang, Davidge & Wu, 2013) thus reducing sole reliance on drug therapy. Bioactive peptides are considered specific protein fragments that are inactive within the sequence of the parent protein but become released into active components when cleaved by enzymatic or bacteria hydrolysis. The bioactivity of these peptides depends on several factors including, amino acid composition, sequence/peptide length, enzyme specificity, molecular weight, hydrophobicity, molecular charge and side chain bulkiness features (Pihlanto & Mäkinen, 2013; Ryan, Ross, Bolton, Fitzgerald & Stanton, 2011; Udenigwe & Aluko, 2012). Bioactive peptides usually range in size from 2 to 20 amino acid residues (Ryan, Ross, Bolton, Fitzgerald & Stanton, 2011), although some have been reported to have peptide length of up to 2 to 50 amino acid residues (Hernández-Ledesma, Del Mar Contreras & Recio, 2011) exhibiting different physiological activities, such as antimicrobial, antioxidant, antithrombotic, antihypertensive, immunomodulatory, opioid, anti-inflammatory, antidiabetic, anticancer, prebiotic, mineral-binding, hypocholesterolemic

and antiproliferative activities, among others (Hartmann & Meisel, 2007; Meisel & Fitzgerald, 2003; Sharma, Singh & Rana, 2011) and these bioactivities positively affect the main body systems including, the cardiovascular, digestive, endocrine, immune and nervous systems. Food proteins derived from different plant and animal sources have demonstrated multifunctional physiological activities in (Lu et al., 2010) disease prevention both *in vitro* and *in vivo* conditions. For instance, several studies have isolated bioactive peptides from plant sources including, pea (Li & Aluko, 2010), flax (Udenigwe & Aluko, 2010), sunflower (Ren, Zheng, Liu & Liu, 2010), soy proteins (Park, Lee, Baek & Lee, 2010), rapeseed (Mäkinen, Johannson, Vegarud Gerd, Pihlava & Pihlanto, 2012), hemp seed (Lu et al., 2010), chickpea (Kou, Gao, Zhang, Wang & Wang, 2013), alfalfa (Xie, Huang, Xu & Jin, 2008a), wheat (Thewissen, Pauly, Celus, Brijs & Delcour, 2011), sweet potato (Huang et al., 2012) and animal origin including egg (You, Udenigwe, Aluko & Wu, 2010), fish (Sampath Kumar, Nazeer & Jaiganesh, 2012), bovine lactoferrin (Ruiz-Giménez et al., 2012), tuna (Qian, Je & Kim, 2007), milk (Phelan & Kerins, 2011), shark liver (Huang & Wu, 2010), chicken bone (Cheng et al., 2008), cuttlefish (Balti et al., 2012), porcine skin (Hsu, Tung, Huang & Jao, 2013) and salmon (Ahn, Jeon, Kim & Je, 2012; Girgih, Udenigwe, Hasan, Gill & Aluko, 2013).

Industrial hemp seed (*Cannabis sativa* L.) is an annual dioecious herbaceous plant that has been a source of food, fibre and medicine for thousands of years in Asia, Europe and Africa (Lu et al., 2010). This is a non-drug variety which contains <0.3% of the delta-9-tetrahydrocannabinol (THC), the psychoactive component and are important agricultural commodities in Canada (Callaway, 2004), the second largest world producer of the crop. Industrial hemp seed is a by-product obtained after the commercial utilization

of the crop for fibre and has been reported to possess several physiological/health benefits including acting as potent antioxidant and antihypertensive agents. Hemp seed, after fibre processing, contains 30% oil and 25% protein with appreciable amounts of dietary fibre and minerals. Hemp seed's health benefits have been attributed to their possession of highly digestible proteins containing all the essential amino acids and essential fatty acids (linoleic acid & linolenic acid in the ratio 3:1) in significant amounts approved by health authorities for optimal healthy (Girgih, Udenigwe & Aluko, 2011a; Tang, Wang & Yang, 2009; Wang, Tang, Chen & Yang, 2009; Wang, Tang, Yang & Gao, 2008). Despite their known high nutritional, physiochemical, functional and bioactive properties, hemp seed proteins have not been fully studied to determine the release of bioactive peptides.Therefore, an in-depth study and knowledge of the structure-function properties of hempseed peptides will not only contribute towards the discovery of more potent peptides from other sources but will evolve the design of appropriate processing conditions that can generate and liberate bioactive peptides from their parent proteins. In addition, the elucidated structures could assist pharmaceutical companies to develop potent drugs whereby the identified peptide sequences serve as molecular templates. This is highly relevant because the development of the first pharmaceutically successful antihypertensive drug (captopril) was based on the molecular templates of snake venom peptides (Cushman et al., 1973; Cushman, Cheung, Sabo, & Ondetti, 1977). Therefore, part of this work is dedicated to elucidation of the structural characteristics of antioxidant and antihypertensive hemp seed protein-derived peptides, using *in vitro* and *in vivo* evaluation methods.

1.2 HYPOTHESES

(1) Simulated gastrointestinal tract (GIT) digestion of hemp seed proteins with pepsin and pancreatin will generate low molecular weight peptides that possess multifunctional bioactive properties as antioxidant and antihypertensive agents;

(2) Ultrafiltration membrane fractions derived from hemp seed protein hydrolysate will possess superior bioactive (antioxidant and antihypertensive) properties when compared to those of the unseparated hydrolysate;

(3) Amino acid sequence of active peptides can be obtained by tandem mass spectrometry after repeated fractionation of the protein hydrolysate by HPLC techniques.

(4) Oral administration of hemp seed protein hydrolysates and identified peptide sequences will reduce systolic blood pressure of spontaneously hypertensive rats (SHRs) during long-term or short-term feeding experiments.

1.3 OBJECTIVES

The overall objectives of this research work were to: (a) Use simulated GIT digestion to produce antioxidant and antihypertensive peptides from hemp seed proteins in order to promote the value-added utilization of the defatted hemp seed protein meal; and (b) Identify bioactive antioxidant and antihypertensive peptides present in the hemp seed protein hydrolysates and determine their amino acid sequences. The specific objectives were to:

(1) Determine the *in vitro* antioxidant properties of hemp seed protein hydrolysate and its membrane ultrafiltration fractions;

(2) Determine the antihypertensive properties of hemp seed protein hydrolysate and its membrane ultrafiltration peptide fractions using *in vitro* inhibitions of ACE and renin activities as well as *in vivo* blood pressure-lowering effects in SHRs;

(3) Purify and identify antioxidant and antihypertensive peptides present in hemp seed protein hydrolysate using HPLC and tandem spectrometry methods;

(4) Determine the kinetics of *in vitro* inhibitions of ACE and renin activities by identified peptide sequences followed by measuring their *in vivo* blood pressure-lowering effects in SHRs;

(5) Determine the ability of hemp seed protein hydrolysate to prevent hypertension in growing SHRs and act as therapeutic agents in adult SHRs with established hypertension;

(6) Determine the effects of long-term (4-8 weeks) feeding trials on the antioxidant and antihypertensive capacity of hemp seed protein hydrolysate measured as plasma peroxide levels and lipid profileof SHRs respectively.

It is envisaged that findings from this work will increase the value-added utilization of defatted hemp seed meal and hemp seed protein by-product, which are associated with commercial production of edible hemp seed oil. This is because confirmation of the protein hydrolysate as a potential antihypertensive agent will open up a new line of products with higher economic value than the raw material (defatted hemp seed meal). Through kinetics studies, the mode by which hemp seed peptides exert their bioactivities would be elucidated. Results from this study will also provide useful and fundamental information about the amino acid composition, structural requirements for inhibition of ACE or renin by food protein-derived peptides and their use as ingredients to formulate functional foods and nutraceuticals.

CHAPTER 2

LITERATURE REVIEW

2.1 HEMP SEED PROTEINS AND BIOLOGICAL ACTIVITIES

The two main storage proteins in industrial hemp seed have been identified to be edestin and albumin which are of high-quality because they are easily digested and contain nutritionally substantial amounts of all the essential amino acids. Hemp seed proteins also contain very high levels of glutamic acid and arginine, the latter being a metabolic precursor for the production of nitric oxide (Rodriguez-Leyva & Pierce, 2010). Nitric oxide is a key player in initiating vasodilatory processes in the body and could contribute to blood pressure reduction (Callaway, 2004; De Cobelli et al., 2004). Fig. 1A shows a direct comparison of the amino acid profiles of the gold standard plant and animal proteins from soybean and egg white, respectively to that of hemp seed which revealed that hemp seed protein has an amino acid profile that is comparable to those of these standard proteins. In addition, hemp seed proteins have good amounts of sulfur-containing (methionine and cysteine) and negatively charged amino acids. The nutritional and medicinal properties of hemp seed have long been recognized in Asia, India, Russia and Eastern Europe. Indications from Chinese medicine, anectodal stories coupled with emerging modern animal and human clinical trials strongly suggest that hemp seed has health promoting bioactive properties that enable it to exert various physiological or biological functions essential for improved human health. Fundamental scientific knowledge is required to evaluate some of the hypothetical health benefits associated with bioactive peptide consumption as illustrated in Fig. 1B. Thus, *in vitro* and *in vivo* studies that use different animal models or human intervention trials are required to

ultimately confirm expected health outcomes. These goals can be achieved by isolating the proteins present in hemp seed, and performing simulated GIT digestion to obtain potent bioactive peptides. The released peptides then have a high potential to escape additional structural degradation during digestion and can be absorbed intact into the blood circulatory system from where they are transported to diseased target organs of need. The presence of health promoting bioactive components in hemp seed proteins have promoted its value-added utilization for production of functional foods and nutraceuticals, increased the number of hemp seed products in the market and has boosted the economic value of the crop. Food and nutritional supplement production companies such as Hemp Oil Canada, Nutriva, Manitoba Harvest and Canada Hemp Foods are pioneering processors who also market hemp seed foods and nutraceutical products.

2.2 FOOD PROTEIN-DERIVED BIOACTIVE PEPTIDES

2.2.1 DEFINING BIOACTIVE PEPTIDES

Bioactive peptides are specific food protein-derived fragments that in addition to acting as sources of adequate nutrition by providing nitrogen and amino acids, have numerous potential physiological functions within the body that may ultimately have a positive influence on human health (Harnedy & Fitzgerald, 2011; Sharma, Singh & Rana, 2011). Bioactive peptides have also been defined as having hormone- or drug like activity that eventually modulate physiological functions through binding interactions to specific receptors on target cells and leading to induction of physiological responses (Fitzgerald & Murray, 2006). For food components to be considered as being bioactive, they must satisfy two important criteria: (i) *they should impart a measurable biological*

Figure 2.1A Comparison of the amino acid profiles of the standard plant (soy bean) and animal (egg white) proteins with that of hemp seed

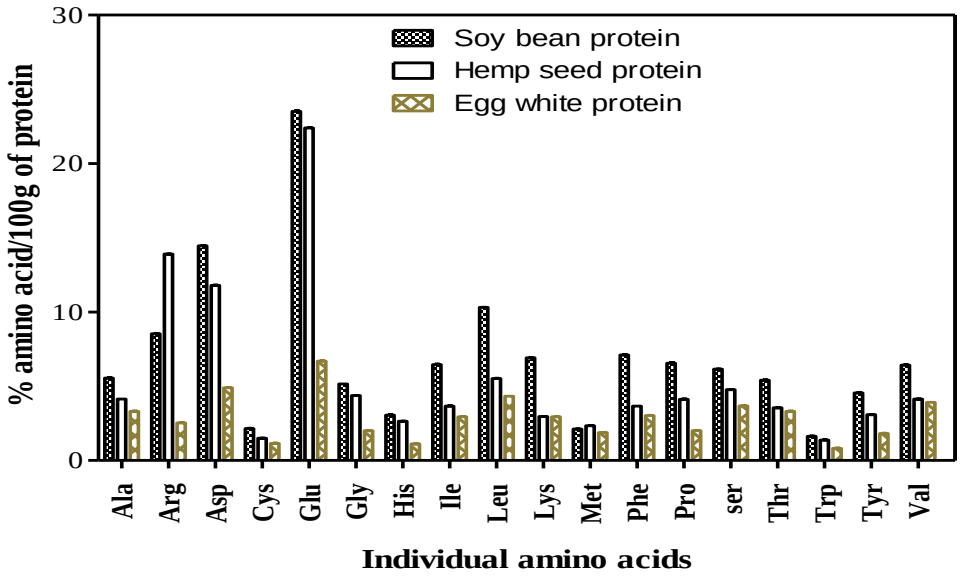
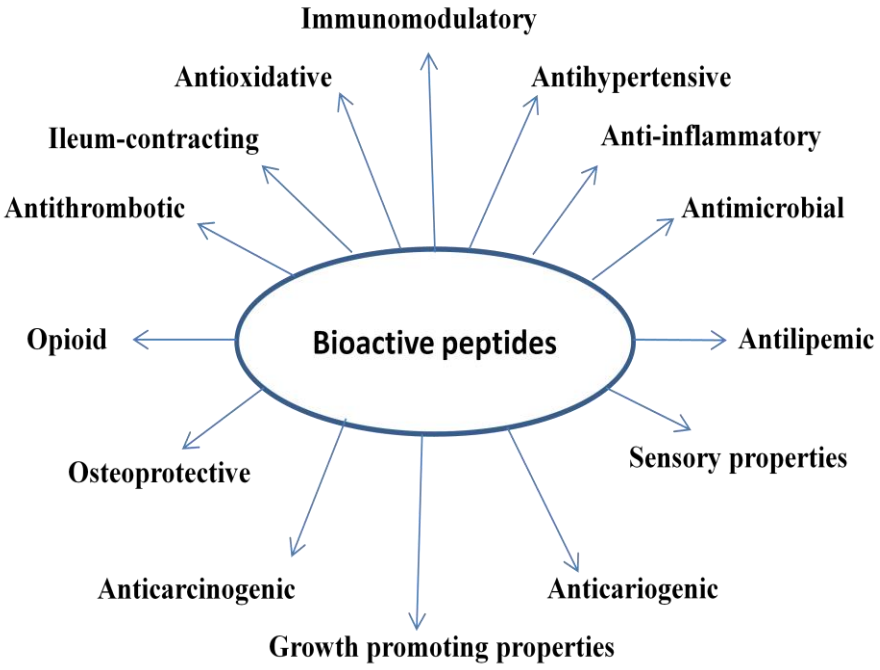


Figure 2.1B An illustration of some of the claimed beneficial effects of bioactive peptides



effect at a physiologically realistic level, and (ii) *the measured bioactivity should have the potential to affect health in a positive way, hence excluding potentially harmful or damaging effects from these definitions* (Möller, Scholz-Ahrens, Roos & Schrezenmeir, 2008).

2.2.2 CATEGORIZING FOOD PROTEIN-DERIVED BIOACTIVE PEPTIDES

It is known that for some peptides, the *in vitro* activity does not always correlate with *in vivo* efficacy. For example, peptides that had potent *in vitro* ACE-inhibitory activity were found to show weak or nil blood pressure-reducing effects when administered to spontaneously hypertensive rats. In order to clarify this discrepancy, ACE-inhibitory peptides were classified into three categories based on their ultimate fate after oral administration (Fujita, Yokoyama, & Yoshikawa, 2000):

a) **True inhibitor-type**: these are peptides whose observed potent *in vitro* activities are not significantly altered by ACE or gastrointestinal tract proteases because they resist cleavage by these enzymes and the inhibitory concentration that reduced enzyme activity by 50% (IC_{50}) values remain unchanged after pre-incubation with these enzymes. Examples of these peptides include IPP & VPP from milk (Turpeinen et al., 2009), IWH, LKP & IKP from bonito (Fujita, Yokoyama & Yoshikawa, 2000) and TF & LY from rapeseed (He et al., 2013).True inhibitor-type peptides usually have very strong *in vitro* ACE-inhibitory IC_{50} values and will exert *in vivo* blood pressure-reducing effects when administered orally to SHRs.

b) **Substrate-type:** these peptides are susceptible to degradation by proteases on pre-incubation with digestive enzymes or ACE. They are hydrolyzed to give peptides with

weaker, or nil activities and have substantially higher IC_{50} values. Examples of substrate-type inhibitors include a heptapeptide, FKGRYYP from chicken with an initial ACE-inhibitory IC_{50} value of 0.55 µM that increased after incubation with proteases to 34 µM (Fujita, Yokoyama & Yoshikawa, 2000). Similarly two octapeptides (FFGRCVSP & ERKIKVYL) from ovalbumin with initial IC_{50} values of 0.4 & 1.2 µM, all increased upon incubation to 4.6 & 6.0 µM respectively, showing a decrease in potency (Fujita, Yokoyama & Yoshikawa, 2000). Substrate-type peptides usually have very strong *in vitro* ACE-inhibitory values but have weak or no blood pressure-reducing effects when administered orally to SHRs.

c) **Prodrug-type inhibitor:** these are peptides that do not have strong ACE-inhibitory effect when measured *in vitro* but produce strong blood pressure-reducing effects when orally administered to SHRs. Thus, it is suggested that the prodrug peptides are converted to true inhibitors by either the action of ACE or GIT proteases. Prodrug peptides are often long-chain peptides which undergo cleavage by proteases during GIT transit or upon interaction with ACE to yield shorter-chain peptides with lower IC_{50} values and long lasting *in vivo* effects after oral administration to SHRs. Examples of prodrug-type peptides are pentapeptides (LKPNM & IWHHT) isolated from bonito which had initial IC_{50} values of 2.4 & 5.8 µM, on incubation however, were degraded to LKP and IW, HHT peptides yielding low IC_{50} values of 0.76, 5.1 & 3.5 µM respectively. These peptides had weak antihypertensive effects *in vitro* until they were subjected to GIT enzyme digestion to produce smaller but active peptides with enhanced physiological activities.

2.2.3. SOURCES OF FOOD-DERIVED BIOACTIVE PEPTIDES

Peptides with bioactive or physiological properties have been isolated from different food sources including from plant and animal materials. The first food-derived bioactive peptide was isolated from milk (Mellander, 1950) who reported that casein phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants. Since then, several peptides have been isolated and identified with diverse bioactivities from animal sources, especially IPP & VPP from milk (Mizushima et al., 2004); ESIINF & IVF from egg (Miguel, Alvarez, López-Fandiño, Alonso & Salaices, 2007; Miguel, López-Fandiño, Ramos & Aleixandre, 2005); IKW & LKP from chicken muscles (Fujita, Yokoyama & Yoshikawa, 2000); VKKVLGNP & KRQKYDI from porcine skeletal muscles (Katayama et al., 2008); VLAQYK & GLSDGEWQ from beef muscles (Jang, Jo, Kang & Lee, 2008; Jang & Lee, 2005) DY & DLTDY from oysters (Shiozaki et al., 2010); GDLGKTTTVSNWSPPKYKDTP & WPEAAELMMEVDP from tuna frame (Lee, Qian & Kim, 2010); GY & VY from salmon (Ono, Hosokawa, Miyashita & Takahashi, 2003); GSTVPERTHPACPDFN & VLSGGTTMYASLYAE from hoki frame (Jung & Kim, 2007; Kim, Je & Kim, 2007); GPL & GPM from Alaska Pollack skin (Byun & Kim, 2001); AFL & YLLP from shrimp (He, Chen, Sun, Zhang & Zhou, 2006) and FDSGPAGVL & NGLEGLK from squid (Mendis, Rajapakse, Byun & Kim, 2005). Plant sources of bioactive peptides are numerous including DLP & DG from soy proteins (Wu & Ding, 2001; Wu & Ding, 2002); LQP & IQP from wheat bran (Nogata, Nagamine & Sekiya, 2011); KF & EF from pea (Li & Aluko, 2010); LY & RALP from rapeseed (He et al., 2013); VF & KY fromWakame (Suetsuna, Maekawa & Chen, 2004); LRP & LSP from maize (Puchalska, Luisa Marina & Concepción García,

2013); KDYRL & VTPALR from mung bean (Li, Shi, Liu & Le, 2006); WNI, LNA, QGR & RW from flaxseed (Marambe, Shand & Wanasundara, 2011; Udenigwe et al., 2012); EVPK & VVGAK from sweet potato (Huang et al., 2011) as well as from mushroom and pumpkin, most of which have multi-functional properties.

2.2.4. PRODUCTION OF BIOACTIVE PEPTIDES

Most of the proteins that occur naturally in unprocessed food materials exert their physiological action either directly or upon enzymatic hydrolysis *in vitro* or *in vivo*. Research of recent years has shown that dietary proteins provide a rich source of biologically active peptides. Basically, bioactive peptides can be produced from precursor proteins where they occur as inactive amino acid sequences but can be released using the following methods: (a) enzymatic hydrolysis by digestive enzymes, (b) fermentation of precursor proteins with proteolytic starter cultures and (c) proteolysis by enzymes derived from microorganisms or plants. In many studies, combinations of (a) and (b) or (a) and (c), have proven effective in generating short chain functional peptides (Korhonen & Pihlanto, 2007). The activity of peptides is based on their inherent amino acid composition and sequence. The size of the active peptide sequences may vary in range from 2 to 20 or even contain more amino acid residues, in some cases, and many of these peptides are known to possess multi-functional properties. Enzymatic hydrolysis is the most commonly employed method for the production of bioactive peptides using gastrointestinal enzymes, usually pepsin and trypsin or combination of proteinases including alcalase, chymotrypsin, pancreatin, and thermolysin. Enzymes from bacterial and fungal sources have also been used to generate large quantities of bioactive peptides from a variety of proteins (Korhonen & Pihlanto, 2006). After enzymatic hydrolysis,

peptides in the resulting hydrolysate could be fractionated, purified and identified. Fractionation is currently achieved by selective membrane ultrafiltration and ion-exchange chromatography, which also serve as peptide enrichment techniques. The use of membrane ultrafiltration produces peptides with specific molecular weight range. To obtain short-chain peptides, membranes with low molecular weight (LMW) cut-offs (1-10 kDa) are used for fractionation of the protein hydrolysate to obtain LMW peptides in the permeate and high molecular weight (HMW) peptides (>10 kDa) in the retentate. The LMW peptides have been reported to be more physiologically active as they are readily absorbed into blood circulation and transported to target organs where they exert their health effects. A series of successive column chromatographic methods can be used to further fractionate and purify peptides in order to enhance identification of amino acid sequences. Some experiments have shown that membrane ultrafiltration and successive chromatographic purifications usually increase potency of the resulting peptides when compared to the crude (unfractionated) protein hydrolysate (Girgih, Udenigwe & Aluko, 2013). In some cases however, the crude hydrolysate has shown superior bioactivities over the peptide fractions which could be attributed to synergistic activities of the crude hydrolysate peptides, which becomes lost upon successive separation (Girgih, Udenigwe & Aluko, 2011a; Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011; Lu et al., 2010). The advantage of crude hydrolysates exhibiting stronger physiological activitities than purified fractions is that it saves costs as expensive purification protocols will no longer be necessary to obtain potent products. On the other hand, identification of the active peptide structures/sequences for the production of nutraceutical supplements would require successive chromatographic purifications to obtain pure peptide substances.

Microbial fermentation is another viable way of producing bioactive peptides. Many industrially utilized starter and non-starter bacteria cultures (*Lactococcus lactis* & *Lactobacillus helveticus*) often used in the manufacture of fermented products are highly proteolytic and can be utilized in the generation of bioactive peptides from different protein sources. Non-conventional production of peptides from natural protein sources besides the use of proteolytic enzymes can also be achieved through the use of recombinant DNA techniques to produce specific peptides or their precursors in microorganisms (Korhonen & Pihlanto, 2006; Kyle, James & Mcpherson, 2012; Liu & Pan, 2010) and by chemical synthesis especially when peptide sequences are already known. In addition, proteolytic enzymes derived from microorganism have also been reported to generate bioactive peptides from proteinaceous raw materials yielding peptides with different health enhancing abilities. Generally, the following factors should be taken into cognizance when engaging in production of bioactive peptides: hydrolysis time, degree of hydrolysis of the proteins, enzyme-substrate ratio, and pretreatment of the protein prior to hydrolysis. For example, thermal treatment of proteins can enhance enzymatic hydrolysis (Inouye, Nakano, Asaoka & Yasukawa, 2009) and may possibly increase the enzyme-protein interactions due to thermally-induced unfolding of the proteins, which makes them more susceptible to enzyme degradation or cleavage to smaller peptides.

2.2.5. MULTIFUNCTIONAL PROPERTIES OF BIOACTIVE PEPTIDES

Food protein-derived peptides have been shown to possess potent multiple biological activities which are largely dependent on their amino acid composition. These peptides are encrypted in the primary structures of plant and animal proteins as inactive amino acid

sequences but are released through food processing, fermentation and enzymatic hydrolysis by *in vitro* or *in vivo* proteolysis (Aluko, 2008; Hartmann & Meisel, 2007). Upon oral administration, bioactive peptides may affect the four major body systems in different ways: namely, the cardiovascular (acting as antioxidants, antihypertensive, antithrombotic, antilipidemic & hypocholesterolemic peptides); digestive (behaving as opioid peptides, antimicrobial, mineral-binding & antiappetizing); immune (acting as antimicrobial, immunomodulatory, cytomodulatory & opioid peptides) and nervous (exhibiting opioid activities) systems (Korhonen & Pihlanto, 2006). The released peptides usually have superior bioactivities compared to their parent unhydrolyzed proteins, implying that breaking of the peptide bonds is essential for enhancement of potent bioactivities of the peptides. For this reason, the potential of distinct dietary peptide sequences to promote human health by reducing the risk of chronic diseases or boosting natural immune protection has aroused increasing scientific and commercial interest over the past decade (Hartmann & Meisel, 2007). Generally, the activity of these peptides against disease targets are less potent than synthetic peptidomimetics and drugs, but the use of bioactive peptides in intervention against human diseases offers several benefits: including safety of the natural product, low health care cost and the additional nutritional source of beneficial or essential amino acids especially for vunerable members of the population with special health needs such as pregnant women and the elderly who are prone to health complications. The multifunctional health benefits of bioactive peptides outlined above in the human body will be discussed in the following section.

2.2.5.1. ANTIOXIDATIVE PEPTIDES

Antioxidants may be defined as compounds that can delay, inhibit, or prevent the

oxidation of oxidizable materials by scavenging free radicals, reducing and chelating catalytic metals thus diminishing oxidative stress conditions. Oxidative stress is an imbalanced state where excessive quantity of ROS or reactive nitrogen species (RNS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite overcome endogenous antioxidant capacity. This leads to ROS/RNS-mediated oxidative degradation of a variety of biomacromolecules such as enzymes, proteins, DNA and lipids. Oxidative stress is important in the development of chronic degenerative diseases including coronary heart disease, cancer and aging (Ames, Shigenaga & Hagen, 1993). ROS and other free radicals are produced in the course of normal physiological activities in the body and are often effectively removed by endogenous antioxidants such as reduced glutathione (GSH), superoxide dismutase or catalase. Under stress or in diseased conditions, however, the body produces excess amount of ROS/RNS/free radicals that overwhelm the capacity of these endogenous enzymes. Subsequently the free radicals accumulate and cause cellular toxicity, which is manifested as damages to DNA, cell membranes, proteins and enzymes. Unchecked, the free radical-mediated cellular damages can progress and develop into chronic diseases such as diabetes, obesity, cancer and adverse cardiovascular events. Apart from toxicity to mammalian cells, ROS/RNS/free radicals could also promote peroxidation of fat containing foods which results in rancidity and off-flavours of processed foods, making them organoleptically objectionable. Food bioactive peptides from different sources have demonstrated the ability to act as antioxidants through donation of electrons or abstraction of hydrogen from these reactive species thereby quenching their destructive tendencies. Oxidation of biomolecules such as proteins and lipids has long been identified as a free radical

mediated process that exerts deleterious impacts on food and biological systems (Elias, Kellerby & Decker, 2008). Therefore, the use of antioxidants is one of the effective methods of prevention against oxidative stress. Shen, Chahal, Majumder, You & Wu (2010) in their search for novel antioxidant peptides from ovotransferrin (a glycoprotein well-known for its iron-binding properties) identified two tetrapeptides (WNIP and GWNI) which showed the very high antioxidant activities. Interestingly, the addition of amino acid residues to either the N or C terminus of the two peptides decreased their antioxidant activities, suggesting that the presence of WNI sequence is responsible for the high antioxidant activities observed.

Lu et al. (2010) investigated the protective effects of purified peptides obtained from alcalase hydrolyzed hemp seed proteins on hydrogen peroxide-induced cell apoptosis in rat pheochromocytoma cell line PC12 cells and found that the two identified peptides (NHAV and HVRETALV) possessed protective effects against cell death and oxidative apoptosis. Recently, several rapeseed hydrolysate peptides were produced using different proteases (alcalase, proteinase-k, pepsin + pancreatin, flavourzyme & thermolysin), which were then screened for antioxidant properties using different evaluation systems. It was found that overall, alcalase and proteinase-k were more efficient proteases in releasing bioactive peptides from rapeseed with potent antioxidant properties compared to combined pepsin + pancreatin, flavourzyme and thermolysin (He, Girgih, Malomo, Ju & Aluko, 2013). Several other natural antioxidant peptides have been produced from soy proteins, sunflower, pea, chickpea, flaxseed, salmon, shark liver, beef, fish skin, milk, chicken bone, etc. Since several diseases have been proposed to be mediated by radical or oxidant species, it is valuable to learn about these antioxidant

compounds that might block, inhibit, or prevent radical-initiated reactions as well as elucidate the mechanisms of of their action (Krinsky, 1992). Knowledge of the various mechanisms by which bioactive peptides are able to achieve their roles as antioxidants in the prevention of oxidative stress related ailments is critical in producing tailored peptide sequences of desired amino acid composition with the potential to scavenge, reduce ROS/RNS/free radicals and chelate transition metals as well as act as lipid peroxidation agents. This knowledge will also help to propose intervention strategies that might suppress or even reverse some of the ravages of oxidant-based diseases in humans.

2.2.5.1.1. Mechanism of Antioxidant Action of Food Bioactive Peptides

Several mechanisms of antioxidant action of food derived bioactive peptides against ROS/RNS and free radicals have been proposed (Dai & Mumper, 2010). These include: (1) scavenging of radical species such as ROS/RNS and free radicals by readily donating hydrogen atoms or electrons to quench their destructive effects on biomolecules, a function which is realized due to the presence of peptide bonds and hydroxyl substituents; (2) suppression of ROS/RNS and free radical formation via inhibition of certain pro-oxidant enzymes and chelating of transition metal ions that are involved in catalyzing free radical production; (3) upregulating the function of the antioxidant enzyme-linked defence mediated by endogenous antioxidants such as reduced glutathione (GSH), ascorbate, superoxide dismutase and catalase (Duthie et al., 2006) or enzyme modulation of cellular physiological and biochemical reactions (Vattem, Ghaedian & Shetty, 2005). Fig. 3 illustrates the initiation of ROS/free radical production, their destructive effects on cellular organelles leading to the development of chronic diseases and the use of bioactive peptides as an intervention strategy. The initiating species for the

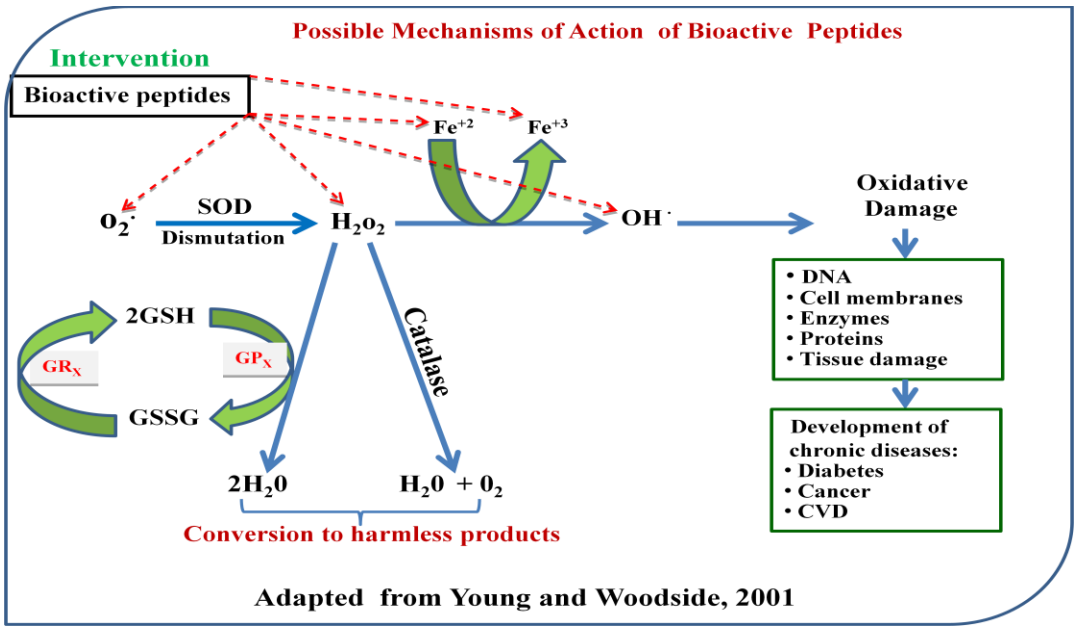
production of ROS/free radicals is superoxide radical which is converted to hydrogen peroxide that could be broken down into harmless metabolites such as water and oxygen in the presence of endogenous antioxidants including superoxide dismutase, catalase, glutathione etc. In disease state, excessive ROS/free radicals are produced and the body's natural mechanism to inactivate them is overwhelmed resulting in the conversion of hydrogen peroxide to the most toxic radical called hydroxyl radical. This reaction is catalyzed by transition metals (Cu^{2+} and Fe^{2+}). If the situation is not attended to, the harmful radicals begin to damage tissues, cell membranes, proteins, enzymes, and DNA, which leads to the progression of chronic diseases such as diabetes, cancer, obesity and can cause adverse cardiovascular events.

2.2.5.2 ANTIHYPERTENSIVE PEPTIDES

Many studies have been conducted to evaluate the antihypertensive potentials of food derived peptides from natural foods of plant and animal origin. Most of the experimental evaluations of the ability of these peptides to prevent or treat hypertension have been carried out *in vitro* but the results do not always translate to *in vivo* efficacy (Hernández-Ledesma, Del Mar Contreras & Recio, 2011; Jauhiainen & Korpela, 2007; Murray & Fitzgerald, 2007). The discrepancy observed in peptide antihypertensive activities during *in vitro* and *in vivo* tests may be attributed to degradation by digestive enzymes during GIT transit. Peptides that are susceptible to GIT enzyme degradation will undergo structural conformation changes that can lead to functional inactivation or reduced absorption level such that they are unable to attain physiologically relevant concentrations in the target organs (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012). The potential antihypertensive effect of a peptide depends on several

factors such as its ability to reach target organs in a structurally intact and active form, resistance to cleavage by digestive proteinases and peptidases, and transportation through the brush border membrane without loss of structural integrity. It is thus difficult to effectively evaluate the relationship between *in vitro* antihypertensive activity and an *in vivo* hypotensive effect; this is further confounded by the utilization of different *in vitro* assays with contrasting conditions. Furthermore, variations during *in vivo* experimental design such as administration by intravenous, subcutaneous or oral administration, and the use of different animal models or hypertensive patients, all contribute to the difficulty in comparing data from different studies (Vermeirssen, Van Camp & Verstraete, 2004).

Figure 2.2 Possible Mechanisms of Action of Bioactive peptides as Antioxidants



Although the blood pressure lowering effect of most bioactive peptides is less than that of pharmaceutical drugs, the negative side effects associated with the long-term use of antihypertensive drugs is causing a gradual shift of attention to natural food protein-derived peptides. Over the past four decades, the natural food protein-derived peptides with potent ACE-inhibitory activity have attracted much attention of researchers

around the world and a large number of these peptides that exhibit various amino acid sequences have been isolated and characterized from enzymatic hydrolysates of foods, such as grass carp fish, oysters, gelatin, egg, milk, whey peptides (Hernández-Ledesma, Del Mar Contreras & Recio, 2011; Kumar, Tandon & Kapila, 2011; Pihlanto, Virtanen & Korhonen, 2010; Rao et al., 2012; Wang et al., 2008b; Zhang, Wang & Xu, 2009). Multifunctional peptides with both ACE- and renin-inhibitory activities have also been identified from plant food sources like peanut, flaxseed, pea, hemp seed, soy sauce and red seaweed (Fitzgerald et al., 2012; Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011; Jamdar et al., 2010; Jimsheena & Gowda, 2010; Li & Aluko, 2010; Rho, Lee, Chung, Kim & Lee, 2009; Udenigwe, Lin, Hou & Aluko, 2009). Identification of antihypertensive agents or bioactive substances that possess both renin and ACE-inhibitory activities has currently become an important research focus since the control of ACE alone does not completely prevent production of Angiotensin II, the vasoconstrictor which is continually produced from an ACE independent pathway catalyzed by chymase. Having a sound knowledge of the mechanism involved in modulating the renin-angiotensin system (RAS) that controls blood pressure is critical for the prevention or treatment of essential hypertension. The regulation of BP involves a complex variety of intertwining metabolic pathways. The most studied BP control pathways with regard to food-derived peptides involve those shown to inhibit ACE and renin enzymes *in vitro*. These enzymes are the main regulators of BP and are both involved in the renin-angiotensin system (RAS), in addition ACE is also involved in the kinin-nitricoxide system (KNOS). Inhibition of ACE and renin in these systems leads to relaxation of the artery walls (vasodilation) and subsequent lowering of BP. Whether this mechanism of

BP lowering is followed strictly *in vivo* or there are other additional pathways involved is not yet fully understood (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012). Studies have shown that di- or tripeptides, especially those with C-terminal hydroxyproline or proline residues, usually resist degradation by digestive enzymes (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012; Pihlanto & Mäkinen, 2013). In addition, short peptides consisting of two or three amino acids are transported and absorbed more rapidly than free amino acids as shown by the detection of ACE-inhibitory tripeptides IPP and VPP in the aorta of SHRs immediately following oral administration of fermented milk (Masuda, Nakamura & Takano, 1996).

2.2.5.2.1 HYPOTENSIVE MECHANISM OF FOOD BIOACTIVE PEPTIDES

Two kinds of hypertension have been identified: primary or essential hypertension whose cause is unknown and originates from within the body in contrast to secondary or inessential hypertension which is believed to be triggered by chronic disease conditions arising from atherosclerosis, kidney malfunction, liver disease, cancer or as a result of side effects from medications. According to recent review by Norris & Fitzgerald (2013), inhibition of ACE enzyme is an excellent physiological target for the treatment of hypertension because of its involvement in the two main systems (RAS & KNOS) that regulate BP in human beings. The RAS seem to be the dominant pressor system for BP control and in this system, the prehormone angiotensinogen produced from the liver is first cleaved by renal renin to decapeptide, angiotensin I (AT-I) which is considered the rate limiting step in RAS. ACE then removes the C-terminal dipeptide (*His-Leu*) to produce angiotensin II (AT-II), a potent vasoconstrictor peptide which directly affects vascular smooth muscles cells of the blood vessels causing them to stiffen. AT-II then

binds to two receptors AT_1 and AT_2 located in the peripheral tissues around the body and within the brain cells; however, it is the AT_1that mediates the vasoconstriction produced by AT-II (Fitzgerald, Murray & Walsh, 2004). ACE also inactivates the vasodilatory peptides (bradykinin & Kallidin), with bradykinin binding to the β-receptor which eventually bring about an increase in the intracellular Ca^{+2} levels. Bradykinin binding to the β-receptor and the up-regulation of Ca^{+2} levels stimulates the enzyme nitric oxide synthase (NOS) to convert L-arginine to nitric oxide (NO), a strong vasodilator. This implies that ACE enzyme activities could cause vasoconstriction (via production of AT-II and indirectly inhibit the production of NO, since it hydrolyses bradykinin (a stimulant for NO production) into inactive fragments (Pihlanto & Mäkinen, 2013). Bioactive peptides with antihypertensive properties can thus be used as an intervention strategy to inhibit ACE activities, which consequentially leads to BP reduction not associated with the side-effects brought about by the use of synthetic drugs.

Studies have shown that prolonged single therapy with ACE-inhibitory antihypertensive agents often results in AT-I accumulation, which can activate the alternate ACE-independent pathway (chymase) and convert AT-I to AT-II resulting in failed treatment of hypertension (He et al., 2013). In addition, the presence of non-RAS enzymes such as tonin and cathepsin is also capable of generating AT-II directly from angiotensinogen, which contributes to the elevated AT-II and aldosterone levels after the patients have been treated with ACE inhibitors (Li et al., 2011; Zaman, Oparil & Calhoun, 2002). It is, therefore important to consider a hypertension treatment that will use dual approach of inhibiting not just ACE but renin also. Moreover, inhibition of renin activities which is the first and rate limiting step in the RAS could modulate significantly

the production of AT-I as substrate for ACE and chymase, thereby down-regulating the BP elevation. Recently, investigators have strongly suggested a combination of ACE and renin therapy to achieve maximal lowering of BP and effective control of hypertension in addition to ensuring organ safety (Harel et al., 2012; He et al., 2013). Figure 2.3A shows the RAS and KNOS with main enzymes responsible for modulation of BP through the use food-derived antihypertensive peptides as intervention agents. Figure 2.3B outlines vasorelaxative peptides/molecules as well as other regulatory pathways of BP control, independent of ACE that may be potential targets for the action of antihypertensive food-derived peptides. The first step employed to determine if a peptide is hypotensive or possess BP lowering effect is to conduct trials with small animal models such as SHRs, which is the accepted model for human essential hypertension. A bioactive peptide can only be said to exhibit 'antihypertensive effect' after a significant decrease in BP is observed during trials with SHRs. There have been many studies carried out in animals to determine whether food-derived ACE-inhibitory peptides have *in vivo* antihypertensive effects. Recently, validation of *in vitro* ACE and renin inhibitions through the antihypertensive effects of food protein-derived peptides were carried out using SHRs (Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011; He et al., 2013; Li & Aluko, 2010) as well as hypertensive human volunteers (Jauhiainen et al., 2010; Li et al., 2011; Yamasue, Morikawa, Mizushima & Tochikubo, 2010). These *in vivo* studies have demonstrated that several ACE-inhibitory peptides or protein hydrolysates significantly reduced BP, either after intravenous or oral administration. However, the studied peptides had little or no effect on the BP of normotensive rats or subjects, which suggests that these peptides work only under hypertensive conditions. Therefore, these antihypertensive peptides

could be used for prevention and initial treatment of mildly hypertensive individuals. These peptides have the advantage as low-cost alternative treatment for hypertension and in addition have not been associated with harmful side effects such as dry cough, diarrhea, skin rashes and angioedema that have been reported for synthetic ACE and renin inhibitors (Jauhiainen & Korpela, 2007).

2.2.5.3 ANTITHROMBOTIC PEPTIDES

These are bioactive peptides with the ability to reduce thrombosis caused by abnormalities in the blood coagulation process. Increased incidence of thrombotic activities is attributed to the presence of high levels of hemeostatic proteins such as fibrinogen, hyperreactive platelets, abnormal fibrinolysis and very high viscosity of the blood. There is a significant amount of molecular similarities between the mechanism involved in milk coagulation and blood clotting. The former involves an interaction between κ-casein with chymosin while the latter is defined by the interaction of fibrinogen with thrombin (Bertina, 1999; Erdmann, Cheung & Schröder, 2008). To correct this abnormally, antithrombotic drugs are commonly used to reduce platelet aggregation while enhancing fibrinolysis but there is always the potential for negative side effects. A previous work showed that a bovine peptide with amino acid sequence MAIPPKKNQDK, which corresponds to position f106-f116 in κ-casein inhibited platelet aggregation and prevented fibrinogen from binding with blood platelets; this inhibitory property was dependent on peptide concentration. Casoplatelins, which are fragments from tryptic hydrolysis of κ-casein were also found to exhibit antithrombotic activity by inhibiting fibrinogen binding to platelets. These bioactive peptides with antithrombotic

Figure 2.3A Mechanism of Blood Presssure Regulation in the Renin-Angiotensin System

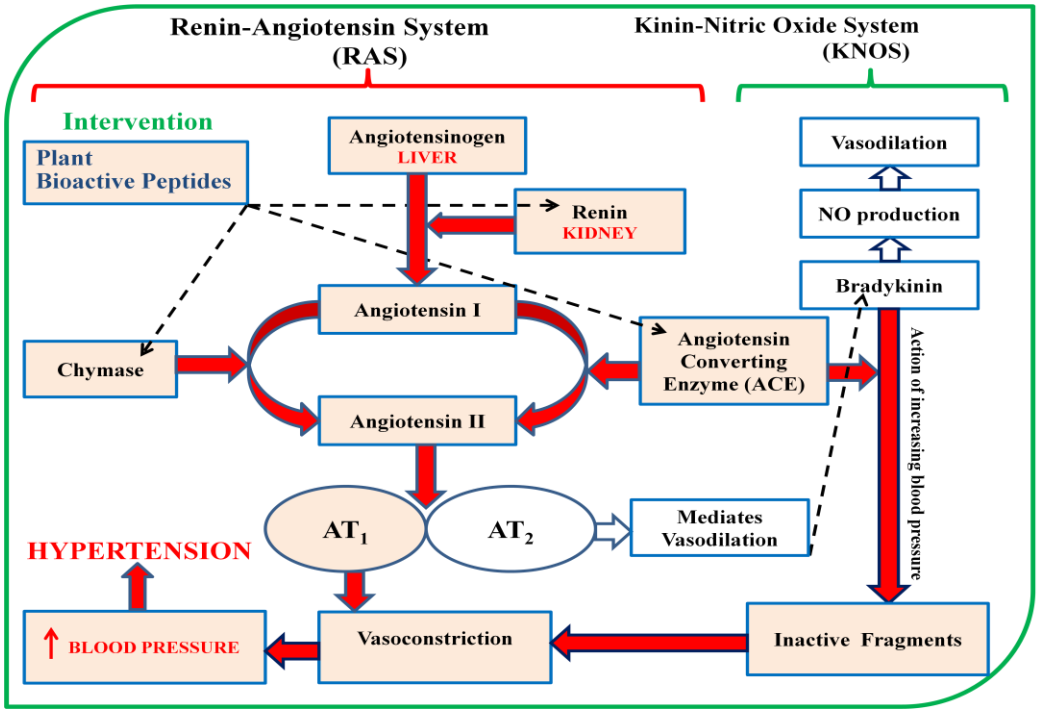
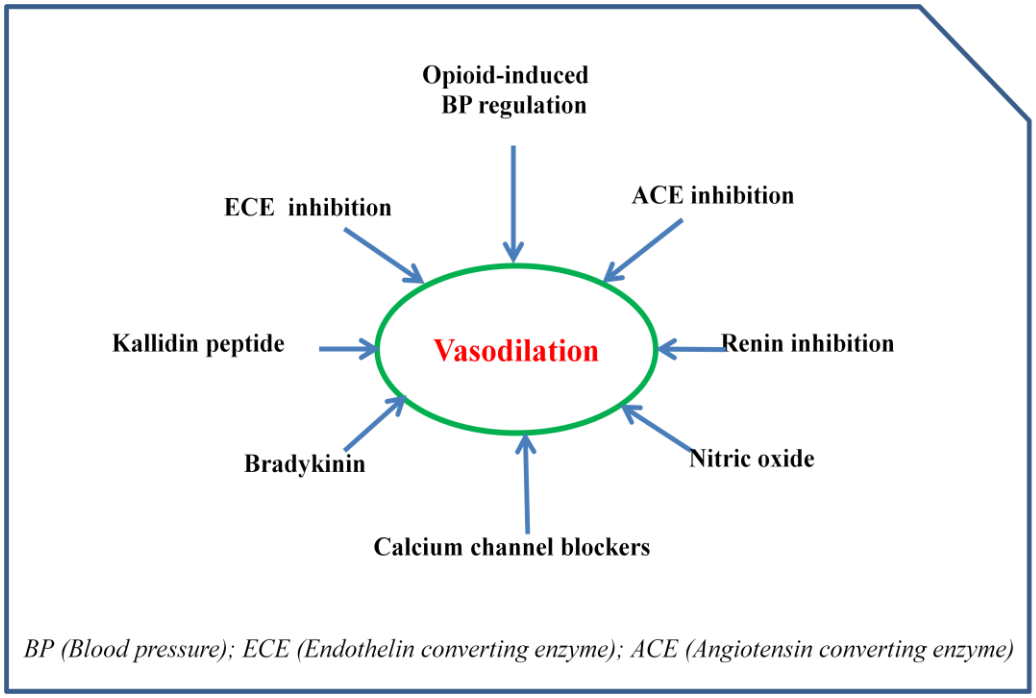


Figure 2.3B Vasorelaxative Enhancing Peptides and Molecules in BP Control



characteristics are released during gastrointestinal digestion and absorbed into blood circulation where they exert their anticoagulatory effects, supporting their *in vivo* efficacy as antithrombotic agents. The potential health beneficial effects of antithrombotic peptides have not yet been fully established, however such peptides have been detected in the blood of infants after breast feeding or consumption of cow milk-based baby formula (Chabance et al., 1995). The presence of Ile, Lys and Asp seem to play a significant role in the efficacy of antithrombotic peptides both during *in vitro* and *in vivo* tests.

2.2.5.4 OPIOID PEPTIDES

These peptides were first discovered towards the end the 1970s and have been reported to play a significant role in the modulation of the nervous system. According to Teschemacher et al. (1997), these peptides are derived mainly from dairy products. Opioid peptides are opioid receptor ligands, which exhibit agonist or antagonist activities and are characteristerized by N-terminal sequences referred to as atypical opioid peptides to distinguish them from the typical endogenous opioid peptides. After absorption into blood circulation, these peptides would travel to the brain and other organs, binding to opiate receptors through which they exert effects similar to opium or morphine which can be reversed by opiate antagonist. Opioid peptides are short sequences of amino acids that simulate the effect of opiates in the brain and have typical lengths ranging from 4-8 amino acids in comparisons to the body's own endogenous opioids such as endorphins that have much longer sequences. The typical natural opioid peptides are produced from three precursor proteins with each protein yielding specific opiate; proopiomelanocortin (endorphins), proenkephalin (enkephalin) and prodynorphin (dynorphins) (Hartmann & Meisel, 2007; Norris & Fitzgerald, 2013). Although the first opioid peptides originated

from milk, other food proteins have also been able to release opioid peptides after enzymatic hydrolysis, for example wheat gluten yielded opioid peptides with very low IC_{50} values in guinea pig ileum (Fukudome & Yoshikawa, 1993), while two δ-selective opioid peptides (rubiscolin-5 and rubiscolin-6) were isolated from spinach with properties of enhancing learning performance, memory improvement in addition to anxiolytic mediated effect in mice (Hirata et al., 2007). In humans, opioid receptors mediating either agonist or antagonist effects are found in the nervous, immune and endocrine systems including the intestinal tract and these receptors may be involved in various regulatory processes in the body including the regulation of blood circulation which often controls high BP (Norris & Fitzgerald, 2013). Nurminen et al. (2000) discovered that after oral administration of α-lactorphin (Tyr-Gly-Leu-Phe), a tetrapeptide to SHRs and normotensive Wister Kyoto rats, it significantly reduced both SBP and DBP in the SHRs but no change was observed in the normotensive rats. The antihypertensive effect of α-lactorphin was considered to be as a result of interaction of this peptide with opioid receptors which mediate production of NO that ultimately caused an endothelium-dependant vasorelaxative effects leading to lowering of BP. Hypotensive effect via opioid receptors have also been reported for casein-derived peptide casoxin D (Tyr-Val-Pro-Phe-Pro-Pro-Phe) which was found to have an endothelium-dependent relaxation in canine mesenteric artery strips (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011). This indicates that opioid peptides can exert their hypotensive effects through oral administration but are required to be absorbed into the blood stream at the brush border membrane before producing their antihypertensive effects (Hernández-Ledesma, Del Mar Contreras & Recio, 2011). In addition, it is possible that opioid-mediated reduction in BP

may be one of the unknown principal mechanisms for modulation of RAS and KNOS systems by antihypertensive peptides. The commonly known structural motif for endogenous and exogenous peptides with opioid activities involves exhibition of a tyrosine residue at N-terminal and the presence of an additional aromatic residue in the third or fourth position away from the N-terminal (Hartmann & Meisel, 2007). The occurrence of proline residue at the second position is critical for three-dimensional orientation of the tyrosine and phenylalanine side chains. The agonistic activity of opioid peptides resembles that of the endogenous ligands, whereas the antagonistic behavior of these peptides exert inhibitory effects similar to naloxone, a potent opiate receptor used as intervention drug in cases of drug overdoses, e.g. heroine overdose (Clare & Swaisgood, 2000). Some opioid peptides e.g casomorphins are involved in regulating gut functions and enhance water and electrolyte absorption, thus acting as antidiarrheal agent (Hartmann & Meisel, 2007; Meisel, 2005).

2.2.5.5 ANTIHYPERLIPIDEMIC AND HYPOCHOLESTEROLEMIC PEPTIDES

Proteolytic hydrolysis of food proteins have been reported to yield peptide sequences with the potential to lower lipid and cholesterol in the body. Food protein sources including buckwheat (Watanabe & Ayugase, 2010), fish (Wergedahl, Gudbrandsen, Røst & Berge, 2009), egg white protein (Manso et al., 2008), sardinelle (Ben Khaled et al., 2012), sesame (Biswas, Dhar & Ghosh, 2010), soy protein (Cho, Juillerat & Lee, 2007; Cho, Juillerat & Lee, 2008; Zhong, Liu, Ma & Shoemaker, 2007) and milk protein (Kirana, Rogers, Bennett, Abeywardena & Patten, 2005a; Kirana, Rogers, Bennett, Abeywardena & Patten, 2005b) have demonstrated various abilities of lowering lipid profile and cholesterol in both animal and human subjects. Soy protein-derived peptide

lipid lowering effect has been the focus of most studies reported and this effect has been partly attributed to the soy 7S globulin (β-conglycinin) component. This soy component has the potential to modulate cholesterol homeostasis by increasing receptor-mediated LDL uptake in Hep G2 cells. Soy protein components also exhibit hypocholesterolemic activities through their ability to bind bile acids and neutral sterols in the intestines, which promotes increased fecal removal of these lipid molecules. Pharmacological studies have also demonstrated that synthetic and natural ACE inhibitors could reduce plasma cholesterol in different animal models (Sugano, Makino & Yanaga, 1996). Significant hypotriglyceridemic activities have been reported in different animal species achieved by the oral administration of hydrolyzed globin. The most potent peptide sequences identified to give maximal hypotriglycerdemic effect were VVYP, VYP and VTL. The hypotriglyceridemic effect of these peptides was associated with decreased intestinal fat absorption as well as an enhanced lipolysis of triglycerides (Kagawa, Matsutaka, Fukuhama, Watanabe & Fujino, 1996). The hypotriglyceridemic activity of hydrolyzed globin peptides has also been demonstrated in humans (Kagawa, Matsutaka, Fukuhama, Fujino & Okuda, 1998). Moreover, a more recent study (Liu, Zhang, Zhang & Liu, 2012) involving protein hydrolysates from *Rhopilema esculentum* has revealed effective modulation of plasma cholesterol concentrations and metabolism of polyunsaturated fatty acids, which resulted in healthy lipid profiles. In addition, these protein hydrolysates may play dual roles of acting as ACE inhibitors while simultaneously exerting their beneficial effect against atherosclerosis by regulating blood lipids to acceptable threshold.

2.2.5.6 ANTIMICROBIAL PEPTIDES

These are peptides which have the ability to inhibit bacterial activities in the body. Antimicrobial peptides have been purified and characterized from different food proteins, particularly milk. The antimicrobial activity of milk is mainly associated with smaller whey proteins, namely lactoferrin which is also found in human milk. Additionally, few other antimicrobial peptides have been identified from α_{S1}-casein & α_{S2}-casein and have the ability to act against diverse Gram-negative and Gram-positive bacteria including *Staphylococcus*, *Salmonella*, *Helicobacter*, *Escherichia* and *Listeria* as well as yeasts and fungi species (Mccann et al., 2006; Rizzello et al., 2005). Antimicrobial lactoferrin can also be produced from various fishes such as sardine, tuna, loach and sole. These proteins have bacteriostatic and bacteriocidial properties linked to iron chelating ability or attachment to bacterial surfaces with high affinity, which disrupts essential membrane functions (Sharma, Singh & Rana, 2011). Lactoferrin's ability to prevent microbial growth is assumed to be due to the iron-binding ability, which deprives microorganisms usage of this essential mineral (Sharma, Singh & Rana, 2011; Simões, Simões & Vieira, 2010). In addition, the presence of α-helix structure and positively charged groups in antimicrobial peptides enables binding with anionic phospholipids-rich membrane components, which triggers cell lysis and increases cell permeability (Adlerova, Bartoskova & Faldyna, 2008). Peptic digestion of bovine lactoferrin was also found to produce a peptide with potent bactericidial effects such that its antimicrobial inhibitory potency was stronger than that of the undigested lactoferrin. Similarly, two peptides were isolated from the N-terminal of lactoferrin which exerted antimicrobial activities against a number of pathogenic and food spoilage microorganisms (Fitzgerald & Murray, 2006).

Antimicrobial peptides have also been identified from many plant sources such as oats, wheat, barley, and pumpkin (García-Olmedo, Molina, Alamillo & Rodriguez-Palenzuéla, 1998; Segura, Moreno, Madueño, Molina & García-Olmedo, 1999).

2.2.5.7 IMMUNOMODULATORY PEPTIDES

These are peptides that can enhance immune system's cellular functions, often measured as lymphocyte proliferation, antibody synthesis, cytokinin regulation and killer cell activity (Fitzgerald & Murray, 2006; Horiguchi, Horiguchi & Suzuki, 2005). Moreover, immunomodulatory peptides might reduce allergic reactions in atopic humans, enhance mucosal immunity in the gastrointestinal tract and could have antitumour effects (Korhonen & Pihlanto, 2003). Immunomodulating peptides have been detected in cow as well as in human milk proteins (Gattegno, Migliore-Samour, Saffar & Jolles, 1988; Migliore-Samour & Jolles, 1988). Two peptides (β-casein, f54-f59 and α-lactalbumin, f51-f53) isolated from human milk protein digests were shown to enhance the phagocytic activity of macrophages in mice and humans, in addition to exerting resistance against certain bacteria in mice (Pérez-Castrillón et al., 2003). Immunomodulatory peptides derived from tryptic hydrolysates of rice and soybean proteins have been found to stimulate superoxide anions and trigger non-specific immune defense systems (Cho et al., 2008). Immune active peptides have also been isolated from bovine caseins (β-casein, f191-f193 and αs_1-casein C-terminal hexapeptide, f194-f199) with stimulatory effects on the microphages resulting in phagocytosis *in vitro* but not *in vivo* conditions in mice (Fiat, Levy-Toledano, Caen & Jollès, 1989). The main physiological mode of action of these peptides is not yet fully known but they principally function to stimulate the proliferation and maturation of immune system cells. Milk derived synthesized peptides

(Tyr-Gly and Tyr-Gly-Gly) had sequences which corresponded with fragments of bovine κ-casein and α-lactalbumin and were shown to enhance proliferation of human peripheral blood lymphocytes. Although most immunomodulatory peptides stimulate the proliferation and maturation of immune cells, however, some like β-casomorphin-7 and β-casokinin-10 are known to suppress stimulation of lymphocyte proliferation in apeptide concentration-dependent manner. Laffineur, Genetet & Leonil (1996) showed that β-casein fermented by lactic acid bacteria and caseins hydrolyzed with *Lactobacillus* and digestive enzymes generated peptide compounds possessing suppressive effects on lymphocyte proliferation. Another very important group of peptides involved in the immune systems stimulation and modulation are called ACE inhibitors. ACE inhibition favours bradykinin activation, known for its mediatory role against acute inflammatory process, hence is able to relax the blood vessels.This leads to stimulation of macrophages, which brings about enhanced lymphocyte migration and an increase in the secretion of lymphokines. The peptides αs_1-casein and β-casein are observed here to play dual roles of both immune stimulatory and ACE inhibitory activities (Paegelow & Werner, 1986). The structure-activity relationship and the mechanism by which food protein-derived peptides exert their immunoregulatory effects are not yet fully understood, however a school of thought holds it that arginine in the N- or C-terminal region of the peptide is an important structural component/entity recognized by specific membrane bound receptors. The commonest structural feature among most immunomodulatory peptides is the presence of arginine in the C-terminal which enhances their stimulatory and proliferatory activities.

2.2.5.8 CYTOMODULATORY PEPTIDES

The progression of chronic disease conditions such as HIV and cancer in individuals with

compromised immune defense system is growing at an alarming rate. The new therapeutic approach to these chronic diseases is the use of macromolecules to modulate the cellular immune system. Cytomodulatory peptides are suitable biologically active macromolecules used for the modulation of body immune cells. They modulate the viability (e.g. proliferation, differentiation and apoptosis) of different cell types, and coupled with the effects of immunomodulatory peptides might help in the control of tumour development (El-Salam & El-Shibiny, 2013); these bioactivities have been supported by cytochemical studies (Hartmann & Meisel, 2007). Some milk-derived peptides, for instance have been shown to trigger apoptosis, especially in malignant cells, whereas normal cells seem to be less susceptible (Meisel & Fitzgerald, 2003). Research has revealed that peptides from Mozzarella cheese whey showed significant antiproliferative effect on CaCo2 cell lines (De Simone et al., 2009). In another study, cytomodulatory peptides were shown to inhibit cancer cell growth while stimulating the activity of immunocompetent cells and neonatal intestinal cells (Meisel & Fitzgerald, 2003). It has been suggested that a combination of the cyto- and immunomodulatory effects of peptides will promote stimulation, proliferation, differentiation, and apoptosis. Such a combination of peptide activity may also improve immune cell functions, antibody synthesis, cytochrome regulations and mucosal immunity, lymphocyte activation, cytokine expression, and non-specific functions of microphages, granulocytes and natural killer cells, thus ensuring competent immune responses against invading microbes or disease progression (Gauthier, Pouliot & Saint-Sauveur, 2006; Huang, Chen, Chen, Hong & Chen, 2010; Meisel, 2004).

2.2.5.9 MINERAL-BINDING PEPTIDES

Mineral-binding peptides are able to provide amino acid sequences with a unique capacity to form complexes with calcium, phosphorus and other minerals in solution at intestinal pH conditions (Sharma, Singh & Rana, 2011). The highly anionic character of these peptides renders them resistant to further proteolytic attack, allowing them to form soluble complexes with calcium and phosphate, while preventing the formation of insoluble calcium phosphate (Berrocal et al., 1989). Milk caseins are known to stabilize calcium and phosphate ions, a reason for which, several phosphopeptides have been identified from enzymatic digest of milk proteins including αs_1-casein comprising fragments (f_{43}-f_{58}, f_{59}-f_{79} & f_{43}-f_{49}), αs_2-casein possessing two fragments (f_1-f_{24} and f_{46}-f_{70}) and β-casein with four fragments (f_1-f_{28}, f_2-f_{28}, f_1-f_{25} & f_{33}-f_{48}) (Berrocal et al., 1989; Gagnaire, Pierre, Molle & Leonil, 1996). During tryptic digestion of casein proteins, caseinophosphopeptides (CPPs) are produced from the N-terminus polar domain which contains clusters of phosphorylated seryl residues (Cross, Huq, Palamara, Perich & Reynolds, 2005; Gagnaire, Pierre, Molle & Leonil, 1996). CPPs can also be formed during cheese ripening due to plasmin and microbial protease activities (Singh, Fox & Healy, 1997). These phosphorylated peptide clusters have been hypothesized to be responsible for the interaction that exist between caseins and calcium phosphate ions that ultimately leads to the formation of casein micelles (Hartmann & Meisel, 2007). The phosphorylated peptides have also been found to retain their ability to stabilize calcium and phosphate ions through the formation of complexes just like the whole casein proteins thus enhancing mineral bioavailability. Literature data of animal and human studies on the effect of CPPs on absorption of calcium in the digestive tract indicates that

CPPs promotes passive calcium transport in the distal small intestine of the rat model system (Erba, Ciappellano & Testolin, 2002); CPPs were detected in the stomach and duodenum of humans following milk ingestion (Chabance et al., 1998). Moreover, the first report of the survival ability of CPPs during passage down to the distal human ileum in intact form was confirmed when CPPs was detected in the ileostomy fluid of human volunteers after consumption of milk (Meisel & Fitzgerald, 2003). The benefits of mineral-binding effects of phosphopeptides was also reported by Cross, Huq & Reynolds (2007) when they demonstrated that phosphopeptides from casein can enhance stability and localization of calcium as well as phosphate ions at the tooth surface thus assisting the promotion of remineralization of worn-out enamel. There are, however some results which contradict the above findings such as the human trials conducted within the frame work of an EU research project, which suggested that CPPs cannot enhance calcium absorption in the gut but may prevent the precipitation of hydroxyl apatite (López-Huertas et al., 2006; Reynolds, 1999; Teucher et al., 2006). The proportion of phosphopeptides interacting with colloidal calcium phosphate correlates with the relative content of phosphoserine residues. Moreover, compositional analyses of phosphopeptide fractions have revealed significant variation in their calcium-binding activities, which may be due to differences in amino acid composition around the phosphorylated domain (Meisel & Frister, 1988).

2.2.6 BIOAVAILABILITY OFINGESTED BIOACTIVE PEPTIDES

The main difficulties involved in directly establishing a relationship between *in vitro* and *in vivo* activities of bioactive peptides is their bioavailability after consumption. It has been found that *in vitro* activities of these peptides do not always correspond or reflect

their actual activities under *in vivo* conditions. The physiological effect of bioactive peptides is measured by their capacity to reach the target organs of need in intact active form and exert health enhancing effects after oral administration, which involves a series of processes that need to be taken into cognizance before arriving their final destination to effect the desired activity (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012). Upon oral administration, bioactive peptides are susceptible to attack by the GIT and brush border peptidases. In addition, prior to absorption through the intestinal barrier, peptides may again be subjected to the action of intracellular peptidases in the transcellular transport, as well as the blood enzymes before reaching the circulatory system and their targeted organs for action (De Leo, Panarese, Gallerani & Ceci, 2009). It is difficult, therefore for ingested peptides, especially those with long sequences not to undergo any form of alteration before eliciting their activity. These concerns have engineered various researches to know the fate of different bioactive peptide sequences in terms of their bioavailability. One of the earliest studies to look at bioavailabilty and intact absorption of dipeptides in the gut lumen was that of Adibi (1971). For instance, protease digestion analysis of the antihypertensive pentapeptide DLTDY from an oyster hydrolysate revealed its susceptibility to the formation of shorter peptide sequences. Among which, DY produced an important reduction of SBP and was considered to be mainly responsible for the observed antihypertensive activity for both the pentapeptide and hydrolysate (Shiozaki et al., 2010). Matsui et al. (2004) also investigated the intact absorption of the dipeptide, VY after oral administration and reported a dose response increase in the plasma levels, which is an indication that exogenous VY could be absorbed intact into blood circulation without structural alteration. Similar experiments

have been conducted with tripetides, IPP and VPP and it was observed that these peptides were transported across the Caco-2 cell monolayer in substantial amounts devoid of structural alteration and their transport was uninhibited by any substrate (Van Der Pijl, Kies, Ten Have, Duchateau & Deutz, 2008).

Absorption of small peptides, especially the di- and tripeptides occurs in the intestinal epithelial cells. Absorption of small peptides is more pronounced at the proximal than the distal small intestine, whereas the absorptive capacity for free amino acids is greater in the distal than in the proximal small intestine. The main known peptide transporters in the small intestines are PEPT1 and PEPT2 collectively called proton-coupled oligopeptide transporters (POTs), which achieve translocation of small peptides/peptidomimics such as di- and tri-peptides across the biological membrane (the mucosal cells) via these H^+ dependent peptide transports. Other transport systems for intact small peptides for paracellular and transcellular routes have been recognized but their significance has not been fully established. In order to design drugs or peptidomimetics targeted at these peptide transporters (PEPT1 & PEPT2), it is essential to take into cognizance their substrate-structural requirements: conformation, size, charge, hydrophobicity and side chain flexibility, which determine the rate and amount of peptides that will pass through the membrane to target sites. PEPT1 is reported to be responsible for the transport of 90% of peptides which reach the small intestines, its activity predominating that of PEPT2.

2.2.7 STRUCTURE-FUNCTION RELATIONSHIP OF BIOACTIVE PEPTIDES

When bioactive substances exhibit potent biological activities against disease targets, it is important to move a step further to determine their chemical or molecular structures in order to relate observed activities to their structures and propose a mechanism of action.

Once the mechanism of action is established, the knowledge will be useful in unraveling the conditions that may favour or increase the potency of these bioactive substances as therapeutic agents for various chronic diseases. Since the discovery of the potent snake venom ACE inhibitors, structure-activity or function correlation studies of ACE-inhibitory peptides have become an important research focus in order to elucidate the structural requirements for inhibition of ACE and other vasoactive enzymes (Cushman, Pluscec & Williams, 1973; Fitzgerald & Meisel, 2000; Ondetti & Cushman, 1982).The pioneering work in the area of relating structure of bioactive substances to their activity or functionality was that put forward by Sneath, who derived amino acid descriptor variables from physiochemical data that was used to develop a quantitative sequence activity model (QSAM) analysis, which assisted in relating structure to activity of chemical compounds and has been applied to a number of peptide-structure activities. However, the approach was criticized for difficulty involved in the interpretation of results because of linear combination problem coupled with exclusion of the conformational state of the peptide in the determination of the final principal properties. A modification of QSAM gave birth to another approach of studying structure and function called quantitative structure-activity relationship (QSAR) modeling, which is currently the most commonly used method. QSAR is a practical and reliable method for studying the relationship between molecular structures of natural therapeutic agents matched to their biological activities. QSAR associated with peptides involves the use of statistical multiple regression analysis such as partial least squares (PLS) projection of latent structure to develop models that relate the molecular structures to the displayed variation in biological activities. Thus, with this approach, bioactivity data of peptides

could be modeled as a function of their molecular structures. Results obtained from these studies could lead to the design and synthesis of a new generation drugs that may be more active than those currently in use, moreover, these drugs may be cheaper antihypertensive agents with no side effects, since these peptides are generated from natural food proteins. Structure correlation of ACE inhibitory peptides (di-, tri- and oligopeptides) have been studied by several authors (Sagardia, Roa-Ureta & Bald, 2013; Udenigwe, Li & Aluko, 2012; Wu, Aluko & Nakai, 2006b) using QSAR with suggested interpretations of structure-activity relationships. It is generally known that peptide sequences with aromatic or hydrophobic amino acid residues or proline at the C-terminus, or amino acids with positively charged functional group at N-terminus are potentially potent inhibitors of ACE. Similarly, it has previously been shown that amino acids with bulky or hydrophobic side chains are preferred for dipeptides whereas, aromatic, positively charged and hydrophobic amino acids are preferred for tripeptides situated at the first, second and third amino acid residues away from the C-terminus (Wu, Aluko & Nakai, 2006a; Wu, Aluko & Nakai, 2006b). The mode of inhibition of ACE by peptides involves competitive, non-competitive and uncompetitive attachment to active site or at points remote to active site resulting in the change of ACE conformation hence its inactivation. Peptides possessing the following features could directly or indirectly promote the inhibition of ACE. For example it has been reported that aliphatic (V, I and A), aromatic (Y and F) as well as basic amino acids such as R residues are preferred to be situated at the penultimate positions for maximal inhibitory activities against ACE, while aromatic (W, Y and F), aliphatic (I, A, L and M) and P residues are preferred at ultimate C-terminal end of the peptide (Pihlanto, Johansson & Mäkinen, 2012; Pihlanto, Virtanen &

Korhonen, 2010). The ACE-inhibitory potency of peptides containing these residues is due in part to the interaction of the residues with the three hydrophobic sub-site located on the ACE active site (Li et al., 2010). Other characteristics of amino acid residues that could impact ACE-inhibitory effects include the positive charge of arginine and lysine when located at the C-terminus (Ndiaye, Vuong, Duarte, Aluko & Matar, 2012). Peptide sequences greater than three amino acids in length need to possess a hydroxyproline derivative residue to efficiently bind ACE at the active site and cause enzyme inhibition. Generally, hydrophobicity of amino acids enhances ACE inhibition while hydrophilicity produces weak or no ACE inhibition because hydrohobicity displays strong affinity for active sub-sites of ACE; in contrast, hydrophilic peptides which are incompatible with the ACE active site and thus are inefficient inhibitors (Wang et al., 2008a)

The antioxidant properties of peptides have been suggested to be related to their composition (sequence), structure and hydrophobic character. The presence of tryptophan, tyrosine, methionine, leucine, and cysteine amino acids are believed to contribute significantly to antioxidant activities. Amino acids with aromatic residues in addition to negatively charged amino acids such as D, E, N and Q are excellent electron donors to electron deficient ROS/RNS/free radicals (Pihlanto, 2006). The imidazole structure of histidine is proposed to be responsible for the efficient metal chelating and lipid peroxyl radical trapping ability of peptides (Rajapakse, Mendis, Jung, Je & Kim, 2005) while SH group in cysteine is known for its ease of associating with other free radicals through sulphur and hydrogen bonds thus making them unavailable to cause cellular damage (Qian, Jung & Kim, 2008). The position occupied by the amino acids in the peptide sequence also plays an important role in determining antioxidant

characteristics. His-His segment in Leu-Leu-Pro-His-His peptide showed a stronger antioxidant activity compared to the Leu-Leu segment while two Tyr amino acids in a tripeptide exhibited more potent antioxidant capacity than two His amino acids in a tripeptide implying that specific amino acid residues situated in particular position in peptide sequence would influence the antioxidant activity (Hernández-Ledesma, Miralles, Amigo, Ramos & Recio, 2005). According to Li, Li, He & Qian (2011), hydrogen bonding, hydrophobicity, hydrophilicity of amino acids next to the C-terminus and the hydrophobicity as well as electronic transfer potential at the N-terminus significantly influences peptide antioxidant properties. The study of structural characteristics is very useful in understanding and predicting the peptide structures responsible for observed activities, which will lead to the development of functional foods with peptides possessing potent antioxidant characteristics. Prior to QSAR modeling, proteins have to be enzymatically hydrolyzed and isolated using bioassay guided fractionation; the peptides are then identified by MS/MS spectrometry and subsequently synthesized for structure-activity relationship.

2.2.8 ANIMAL EXPERIMENTS WITH BIOACTIVE PEPTIDES

After successful *in vitro* screening of bioactive peptides for potential health effects, the next important step involves validation of these observed and measured activities *in vivo* using appropriate animal disease models. Positive results from the animal experiments could further initiate human trials to confirm the claimed physiological effect of the bioactive peptides in diseased and normal human volunteers. The final expected outcome or fate of these bioactive peptides is their utilization for the development of health promoting foods and nutraceuticals. For example, the first step used to determine if a

peptide or any bioactive substance is hypotensive is to conduct animal trial studies using small animals such as SHRs (the accepted and most widely used model for human essential hypertension) with normotensive rats as the positive control. A bioactive substance can thus be said to be antihypertensive if it shows a significant decrease in blood pressure after administration to SHRs. The normotensive Wistar Kyoto rats have also been used to evaluate the effect of bioactive peptides on blood pressure to simulate response to bioactives by human beings with normal blood pressure. In most cases, the normotensive rats do not respond to treatment with antihypertensive peptides, which confirms their usage by humans with normal blood pressure will have no negative effect such as excessive lowering of blood pressure beyond physiologically acceptable levels. Several studies have been conducted using this model of rats to screen potential antihypertensive peptides from different food sources (Cheng et al., 2008; Contreras, Carrón, Montero, Ramos & Recio, 2009; Girgih, Udenigwe, Hasan, Gill & Aluko, 2013; Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011; He et al., 2013; Hiwatashi et al., 2010; Lee, Qian & Kim, 2010; Li & Aluko, 2010; Miguel, Gómez-Ruiz, Recio & Aleixandre, 2010; Quiñones et al., 2010; Staljanssens et al., 2011; Udenigwe et al., 2012). The antihypertensive properties of peptides have recently been reviewed (Norris & Fitzgerald, 2013; Pihlanto & Mäkinen, 2013; Sharma, Singh & Rana, 2011) and two main routes (oral gavage and intravenous administration) are employed for administering these peptides to the rats. In general, it has been found that administering peptides intravenously to rats produces higher decrease in BP than when administered orally, The lower potency of orally administered peptides may be the result of lower bioavailability in the blood stream because of reduced inability to cross the brush border membrane in

an intact form, which causes partial loss of hypotensive potency due to partial hydrolysis in the GIT and by serum enzymes.

The age, degree of hypertension, the type of peptide and dose given all play a role in affecting the ultimate hypotensive outcome. Ile-Pro-Pro (β-casein, f74-76; κ-casein, f108-110) and Val-Pro-Pro (β-casein, f84-86) represent one of the earliest dietary peptides found to have a hypotensive effect in SHRs and used for development of functional food products as well as supplements (Nakamura et al., 2011; Turpeinen et al., 2009). Since then there has been widespread commercialization of antihypertensive food products from different sources, all of which are dependent on the availability of scientific data from animal and human trials that significantly demonstrate BP reduction. There is now in place legislation which governs the approval of health claims in relation to functional foods and potential antihypertensive peptides must go through these regulatory hurdles in order to obtain commercialization approvals that carry a health claim. Different countries have different regulatory agencies for monitoring and approving health claims of natural food products. One of the first to be established was the Japanese FOSHU (food for specified health use) licensing system which was put in place so that foods claiming health benefits must first obtain approval before being allowed commercialization (Norris & Fitzgerald, 2013). The Food and Drug Administration in the USA and the European Commission (EC) with the European Food Safety Authority (EFSA) review evidence of health claims made by food companies all of which aim at ensuring the safety of the consumers.

2.2.9 MARKET POTENTIALS OF BIOACTIVE PEPTIDES

In the last decade, interest for bioactive peptides has significantly grown resulting in the

development of shelf-stable functional food products with different health enhancing effects. This is possible because these compounds can be cheaply produced from food proteins through enzymatic hydrolysis such that the end products from the process are able to escape gastrointestinal tract digestion to reach molecular targets where they are able to exert needed physiological effects. Moreover, bioactive peptides are not limited by dosages like pharmaceutical drugs and have no known side effect concerns, which make them more acceptable by consumers because of their safety. A lot of approved functional foods and nutraceuticals products with different health claim qualifying statements on the product label are now available in supermarkets and drug stores on the open counter for patronage by consumers for various health enhancing benefits. Some bioactive peptides have also found use in the cosmetic industrials to provide certain health benefits to diseased skin. Table 2.1 below summarizes some current commercialized functional foods and nutraceuticals as the market continues to expand with increased consumer knowledge of the importance of a healthy living lifestyle.

2.2.10 POSSIBLE FUTURE HEALTH BENEFITS OF BIOACTIVE PEPTIDES

Research regarding the health benefits of bioactive peptides from both animal and plant sources have focused mainly on oxidative stress related and cardiovascular diseases. More attention needs to be given to producing bioactive peptides tailored for other chronic disease conditions such as Alzheimer's, kidney, liver and cancer-related diseases. These diseases are very difficult to treat or manage using drug therapy but may respond positively to natural bioactive compounds derived from food proteins. To meet the increasing demand for these natural bioactive compounds because of their associated health promoting effects, suitable technological designs must be developed to step-up

production at industrial level so that they are available as needed in food and drug stores. Novel technologies currently in use such as chromatographic and membrane separation techniques seem to be the most appropriate strategies employed for the enrichment of potent/active peptide fractions from the hydrolysates of various food proteins both from plant and animal sources. Production of bioactive peptides from protein enriched materials could be scaled up to industrial level through the utilization of regulated fermentation in bioreactors. Combination of enzyme technology and the use of specific production strains or peptidases obtained from microorganisms would be the likely path to follow in the future. Enzymatic hydrolysis and microbial fermentation provide a natural technology for bioactive peptide production whose potentials has already been demonstrated with dairy products. Bioavailability, safety, stability of the chemical structures of bioactive peptides in different food matrices as well as their ultimate fate in the course of digestion should be critically examined during food formulation that involves incorporation of these peptides. In addition, emerging new technologies such as the micro or nanoencapsulation may offer feasible solutions or at least provide possible answers to improving peptide stability in various food matrices and during digestion (Korhonen, 2009; Korhonen & Pihlanto, 2007). Nanofiltration and improved ultrafiltration techniques are available and are now used industrially to produce ingredients with specific bioactive peptides sequences based on casein or whey protein hydrolysates. These peptide preparations are on a commercial scale being introduced into different consumer products, such as dairy and fruit based drinks, confectionery, chewing gum, pastilles, capsules, salad dressings, smoothies, milk shakes and a host of other domestic consumer goods. At present, marketed products containing active peptide

sequences with specific health benefits include those with stress relieving (antioxidants), satiety, antihypertensive, mineral-binding, anticariogenic, and antiobesity properties. More extensive studies of bioactive peptides in human trials should be intensified to correlate as well as validate *in vitro* and animal results in order to obtain approval of disease reduction claims following administration of the bioactive substances. This is probably the right path towards increasing the utilization of value-added bioactive peptides for various claimed health benefits.

Table 2.1: Commercially available functional foods and nutraceuticals containing bioactive peptides and their claimed health effects: adopted from Korhonen (2009) with permission from Elsevier

Manufacturer	Brand name	Type of product	Bioactive peptide composition	Claimed health effect
Calpis Co., Japan	Calpis	Sour milk	VPP, IPP	Hypotensive
Valio Oy, Finland	Evolus	Ca enriched milk	VPP, IPP derived from β- and k-casein	Hypotensive
Davisco, USA	BioZate	Hydrolyzed whey protein isolate	B-lactoglobulin fragments	Hypotensive
Davisco, USA	BioPURE-GMP	Whey protein isolate	Glycomacropeptide	Prevents dental carries, helps in blood clotting
NIPPON, Japan	Peptide soup	Soup	Bonito-derived peptides	Hypotensive
Campina, Netherlands	C12-Peption ACE	Capsule, bar, beverage	FFVAPFPEVFGK	Regulate BP
Kanebo, Japan	Peptio	Soft	FFVAPFPEVFGK	Regulate BP
Natural Factors, Canada	PeptACE	Capsules	Peptides mixture derived from Bonito	Lowers cholesterol & BP
Kyowa Hakko, Japan	CholesteBlock	Drink powder	Soy peptides bound to Phospholipids	Hypocholesterolemic
Ingredia Nutritional, France	Prodiet F200	Beverage, Chocolate, cookie, dairy product	YLGYLEQLLR	Decrease anxiety, reduce stress

Arla Foods, Denmark	Capolac	Ingredient	CPP	Improves Ca reserves
Suntory, Japan	Tekkotsu Inryou	Soft drink	CPP	Enhancement of mineral absorption
Netherlands	DMV	Peptide WGE 80 GPN	Hydrolysate containing peptide mixtures	Immune response and gut performance
Asahi, Japan	Kotsu Kotsu calcium	Soft drink	CPP	Enhancement of mineral absorption
Borculo Domo Ingrdients (BDI), Netherlands	Vivinal Alpha	Ingredients/hydrolysate	Whey derived peptide	Aids relaxation and sleep
DMV, Netherlands	Glutamine peption WGE80GPA WGE80GPN WGE80GPU	Dry milk hydrolysate	Glutamine-rich peptides	Immunonomodulatory
Living Harvest, Canada	Tempt	Protein powder	Contain all essential amino acids	Nutritional building blocks
Canada Hemp Foods	Organic Hemp Protein Powder	Ingredients	Contain all essential amino acids	Antioxidants

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CHAPTER 3

MANUSCRIPT 1

IN VITRO ANTIOXIDANT PROPERTIES OF HEMPSEED (*CANNABIS SATIVA* L.) PROTEIN HYDROLYSATE FRACTIONS

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3.0 ABSTRACT

Simulated gastrointestinal hydrolysis of hempseed proteins using pepsin and pancreatin followed by membrane ultrafiltration fractionation yielded fractions with peptide sizes of <1, 1-3, 3-5 and 5-10 kDa. Analysis of *in vitro* antioxidant properties showed that the hydrolysate (HPH) exhibited a significantly weaker ($p<0.05$) scavenging of DPPH radicals when compared to the fractionated peptides. Metal chelation activity of the HPH was significantly greater ($p<0.05$) than the activities of fractionated peptides. Fractionation of the HPH led to significant ($p<0.05$) improvements in ferric reducing power, DPPH and hydroxyl radical scavenging radical activities but decreased metal chelation capacity. Peptide fractions with longer chain lengths (3-5 and 5-10 kDa) had better metal chelation and ferric reducing power than the <1and1-3 kDa fractions. HPH and all the peptide fractions significantly inhibited ($p<0.05$) linoleic acid oxidation when compared to the control. Glutathione (GSH) had significantly greater ($p<0.05$) ferric reducing power, and scavenging of hydroxyl and DPPH radicals when compared to HPH and fractionated peptides. In contrast, HPH and peptide fractions >3 kDa had significantly higher ($p<0.05$) metal chelation activity than GSH. The results show the potential use of hempseed protein hydrolysate and peptide fractions of defined size for the treatment of oxidative stress-related diseases.

KEYWORDS: Hempseed protein hydrolysate, Antioxidant properties, Free radical scavenging, Metal ion chelating, Amino acid profile, Linoleic acid, DPPH, Hydroxyl radical, Ferric Reducing Antioxidant Power

3.1 INTRODUCTION

Industrial hemp (*Cannabis sativa* L.), widely cultivated in China and Canada, is a very important plant for food, fiber and medicine. Hempseed, a by-product obtained during the commercial utilization of the plant fiber contains over 30% oil and 25% of high quality protein [1]. Industrial hempseed, which contains a low level (~0.3%) of δ-9-tetrahydrocannabinol (THC) is now legally grown in Canada. Hempseed has been used in the development of numerous products for the cosmetics, therapeutic, functional food and nutraceutical industries [2]. Hempseed is also a rich source of polyunsaturated fatty acids, especially linoleic (ω-6) and α-linolenic (ω-3) acids, and proteins which contains all of the essential amino acids in nutritionally sufficient amounts for FAO/WHO suggested requirements for infants or children [3, 4]. The physicochemical and functional properties of hempseed protein isolate (HPI) have been evaluated and compared with those of soy protein isolate (SPI); HPI had similar or higher levels of essential amino acids than SPI except for lysine. In addition, hempseed protein hydrolysate produced using different proteases have been reported to possess antioxidant properties [5] but the work did not provide information on relationships with amino acid content of peptides. Antioxidant peptides have continued to be of research interest due to the potential contributions to human health and especially for the prevention and treatment of chronic diseases.

Oxidative stress occurs as a result of imbalance between the production of free radicals, reactive oxygen species (ROS) and the scavenging ability of endogenous antioxidants [6]. Excessive production of ROS may damage membranes, proteins, enzymes and DNA resulting in the development of chronic disease conditions [7, 8]. Enzymatic food protein-derived peptides, in comparison to synthetic compounds are

believed to be safer natural antioxidants that can be used as protective agents to help the human body reduce oxidative damage and associated diseases [6]. The antioxidant properties of these hydrolysates largely depend on enzyme specificity, degree of hydrolysis and the nature of the peptides released including molecular weight, amino acid composition and hydrophobicity [9-11]. The antioxidant properties of peptides include their ability to scavenge free radicals, inhibit linoleic acid autoxidation, act as chelating agents of metal ions, or as reducing agents [5]. The presence of certain amino acids like, histidine, tyrosine, methionine, lysine, tryptophan and proline increases the antioxidant potency of most food-derived peptides [12]. To the best of our knowledge, there is lack of information on the relationships of peptide size and amino acid composition with antioxidant activities of hempseed protein-derived hydrolysates. The present work contributes to the growing scientific knowledge on the relationships between amino acid content or peptide size and antioxidant properties of food protein hydrolysates.

Though a previous work [5] had used different enzymes, there is the need to determine the potential liberation of antioxidant peptides during oral consumption of hempseed proteins. Therefore, the objective of this work was to produce a simulated gastrointestinal hempseed protein hydrolysate, fractionate the hydrolysate into peptides of different molecular weights, and evaluate these samples for multifunctional properties using various antioxidant evaluation systems, *in vitro*. Glutathione (GSH) was used for comparison since it is a peptide and because it has physiological relevance as a cellular antioxidant molecule in human tissues.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Defatted hempseed meal, referred to as hempseed protein powder (HPP), was purchased from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada). Pepsin (porcine gastric mucosa, EC 3.4.23.1), Pancreatin (porcine pancrease), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Triton X-100, hydrogen peroxide, ethylenediaminetetraacetic acid (EDTA), ferrous sulphate, potassium ferricyanide, trichloroacetic acid (TCA), ammonium thiocyanate, linoleic acid, ferrous chloride, 1,10-phenanthroline, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphide acid sodium salt (ferrozine), and GSH were purchased from Sigma-Aldrich (St. Louis, MO) while other analytical grade reagents and ultrafiltration membranes (1, 3, 5, and 10 kDa molecular weight cut-off) were obtained from Fisher Scientific (Oakville, ON, Canada).

3.2.2 Methods

3.2.2.1 Preparation of Hempseed Protein Isolates (HPI)

HPI was produced from HPP according to the method described by [3] with slight modifications as follows. Briefly, HPP was dispersed in deionized water (1:20, w/v), and the dispersion was adjusted to pH 10.0 with 2 M NaOH to solubilize the proteins. The resultant dispersion was stirred at 37°C for 2 h followed by centrifugation (7000g at 4 ^{o}C) for 60 min. The pellet was discarded, and the supernatant filtered with cheesecloth and adjusted to pH 5.0 with 2 M HCl to precipitate the proteins. Thereafter, the mixture was centrifuged (7000g at 4 ^{o}C) for 40 min, the resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to produce HPI powder. Protein content of HPI was determined using the modified Lowry method [13].

3.2.2.2 Production of Hempseed Protein Hydrolysate (HPH) and Membrane Fractions

Prior to enzymatic hydrolysis, the green-coloured HPI was decolorized by mixing 1 g with 10 mL of acetone. The mixture was stirred in the fume hood for 3 h and decanted followed by a second and third consecutive extraction of the residue. The resulting HPI was air-dried overnight in the fume hood and used for further studies. The decolorized HPI was enzymatically hydrolyzed according to a previous method [14]. Briefly, 5% (w/v, protein basis) of decolorized HPI slurry was heated to 37 ^{o}C and adjusted to pH 2.0 using 2M HCl. Protein hydrolysis was initiated by the addition of pepsin (4% w/v, protein basis) and the mixture stirred for 2 h. After peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH, pancreatin (4% w/v, protein basis) was added and the mixture was incubated at 37 ^{o}C for 4 h. The enzymatic reaction was terminated by adjusting the mixture to pH 4.0 with 2 M HCl followed by heating to 95 ^{o}C for 15 min to ensure a complete denaturation of residual enzymes. The mixture was centrifuged (7000g at 4 ^{o}C) for 30 min and the resulting supernatant was sequentially passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5, and 10 kDa in an Amicon stirred ultrafiltration cell. The permeate fraction from each MWCO membrane was collected, lyophilized and stored at -20 ^{o}C until needed for further analysis. The protein contents of the freeze-dried HPH fractions were also determined using the modified Lowry method [13]. The above digestion and fractionation protocols were performed in triplicate and the freeze-dried products combined, analyzed for protein content [13] and used for antioxidant assays.

3.2.3 DPPH Radical Scavenging Assay

The scavenging activity of HPH and its fractions against DPPH radical was determined using a previous method [14] with slight modifications for 96-well clear flat bottom plate. Peptide samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final concentration of 100 µM. Peptide samples (100 µL) were mixed with 100 µL of the DPPH solution in the 96-well plate to a final assay concentration of 1 mg/ml and incubated at room temperature in the dark for 30 min. The absorbance values of the control (A_c) and samples (A_s) were measured at 517 nm. The control consisted of sodium phosphate buffer in place of the peptide sample while GSH was used as the positive control. The percent DPPH radical scavenging activity of the samples was determined using the following equation:

$$\text{DPPH Radical Scavenging Activity (\%)} = \left(\frac{A_c - A_s}{A_c} \right) \times 100$$

3.2.4 Chelation of Metal Ions

The metal chelating activity was measured using a modified method of Xie et al. [15]. Peptide sample solution or GSH (final assay concentration of 1 mg/mL) was combined with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL double distilled water in a reaction tube. Ferrozine solution (0.1 mL of 5 mM) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min from which an aliquot of 200µL was removed and added to a clear bottom 96-well plate. A control was also conducted by replacing the sample with 1mL of double distilled water. The absorbance values of control (A_c) and sample (A_s) at 562 nm were measured using a spectrophotometer and the metal chelating activity of the sample was compared to that of

glutathione (GSH). The percentage chelating effect (%) was calculated using the following equation:

$$\text{Metal chelating effect (\%)} = \left(\frac{A_c - A_s}{A_c} \right) \times 100$$

3.2.5 Ferric Reducing Power

The reducing power of peptide samples was measured according to a previous method [16] which was modified as follows: Peptide samples (250 μL) dissolved in 0.2M sodium phosphate buffer at pH 6.6 or double distilled water (control) were mixed with 250μL of buffer and 250μL of 1% potassium ferricyanide solution. Final peptide concentration in the assay mixture was 1 mg/mL. The resulting mixture was heated at 50 ^{o}C and incubated for 20 min. After incubation, 250μL of 10% of aqueous trichloroacetic acid (TCA) was added. Thereafter, 250μL of peptide/TCA mixture was combined with 50μL of 0.1% ferric chloride and 200μL of water and allowed to stand at room temperature for 10 min. The solution was centrifuged at 1000g and 200μL of the supernatant transferred to a clear bottom 96-well plate. The absorbance of the supernatant was measured at 700 nm.

3.2.6 Hydroxyl Radical Scavenging Assay

The Hydroxyl radical scavenging activity was determined based on a previously reported method [17]. Peptide samples and 3 mM of 1,10-phenanthroline were separately dissolved in 0.1 M sodium phosphate buffer (pH 7.4). $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide were both separately dissolved in distilled water. An aliquot (50 μL) of peptide samples (equivalent to a final assay concentration of 1 mg/mL) or buffer (control) was first added to a clear, flat bottom 96-well plate followed by 50 μL of 1, 10-

phenanthroline and then 50 µL of $FeSO_4$. To initiate Fenton reaction in the wells, 50µL of hydrogen peroxide was added to the mixture, covered and incubated at 37^{o}C for 1 h with shaking. The absorbance was measured using a spectrophotometer at 536 nm at 10min intervals for 1 h. The hydroxyl radical scavenging activity was calculated using thereaction rate (ΔA/min) equation below:

$$\text{Hydroxyl radical scavenging activity (\%)} = \left(\frac{(\Delta A/\min)_c - (\Delta A/\min)_s}{(\Delta A/\min)_c} \right) \times 100$$

3.2.7 Inhibition of Linoleic Acid Oxidation

Linoleic acid oxidation was measured using the method described by Li et al. [8]. Peptide samples (final concentration of 1 mg/mL) were dissolved in 1.5 mL of 0.1 M sodium phosphate buffer (pH 7.0) and the mixture added to 1 mL of 50 mM linoleic acid dissolved in 99.5% ethanol. For the control assay, 1.5 mL of buffer was added to the ethanolic linoleic acid solution.The mixtures were kept at 60 ^{o}C in the dark for 7 days. At 24 h intervals, 100µL of the assay solution was mixed with 4.7 mL of 75% aqueous ethanol, 0.1 mL of ammonia thiocyanate (30% w/v) and 0.1 mL of 0.02M ferrous chloride dissolved in 1M HCl. This solution (200µL) was added to a clear bottom 96-well plate and the degree of color development was measured using the spectrophotometer at 500 nm after 3 min incubation at room temperature. An increased absorbance implied an increase in the level of linoleic acid oxidation.

3.2.8 Amino Acid Composition Analysis

The amino acid profiles of samples were determined in duplicate (two separately digested and fractionated samples) using an HPLC system after samples were hydrolyzed with 6 M HCl [18]. The cysteine and methionine contents were determined after

performic acid oxidation [19] and tryptophan content was determined after alkaline hydrolysis [20].

3.2.9 Statistical Analysis

Antioxidant assays were conducted in triplicate and analyzed by one-way analysisof variance (ANOVA). The means were compared by Duncan's multiple range test and significant differences accepted at $p<0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Enzymatic Hydrolysis of HPI and Ultrafiltration

In this study, HPI was decolorized with acetone to remove polyphenolic compounds that could interfere with accurate determination of antioxidant properties of the protein hydrolysates. This is because low molecular weight polyphenols have antioxidant properties, which could confound interpretation of the results. HPI was hydrolyzed consecutively with pepsin and pancreatin followed by sequential fractionation of the hydrolysate by ultrafiltration using 1, 3, 5 and 10 kDa MWCO membranes to produce peptide fractions with varying molecular weights. Pepsin and pancreatin enzymes were used in this study to mimic gastrointestinal protein digestion in human beings. Membrane fractionation resulted in enriched peptides with narrow molecular weight range (<1, 1-3, 3-5, and 5-10 kDa) and increased homogeneity of the peptides, when compared to the original HPH. In previous studies, membrane fractionation of protein hydrolysates resulted in fractions with better bioactive properties than their parent hydrolysates [14, 21]. The HPH had protein content of 90% whereas the <1, 1-3, 3-5 and 5-10 kDa membrane ultrafiltration fractions contained 64, 85, 92, and 90 % protein

contents, respectively. The lower protein content of the <1 kDa permeate suggests that LMW non-protein components such as salts and soluble sugars are present.

3.3.2 Amino Acid Composition

The amino acid composition of HPI, HPH and membrane fractions are shown in Table 3.1. Amino acid content of the HPI was very similar to previously reported data [3] and shows very high levels of arginine. The results indicated that amino acid content of the HPH was very similar to that of the HPI. However, membrane fractionation resulted in higher contents of leucine and phenylalanine in the <1 and 1-3 kDa peptides while the 3-5 and 5-10 peptides had reduced contents. Fractionation also resulted in decreased levels of cysteine in the <1 and 1-3 kDa peptide fractions when compared to the other samples. Valine content was increased in the <1 and 1-3 kDa peptide fractions but remained virtually unchanged in the 3-5 and 5-10 kDa peptides, when compared to the HPI and HPH. Overall, the <1 kDa peptide fraction had the highest concentration of hydrophobic as well as aromatic amino acids except for cysteine and valine where the other samples had higher concentrations.

3.3.3 Antioxidant Activities of HPH and its Fractions

3.3.3.1 Radical Scavenging Activities (RSA)

The DPPH radical is relatively stable and has been widely used to test ability of natural compounds to act as free radical scavengers or hydrogen donors as a means of evaluating their antioxidant potentials [22]. DPPH radicals are stable in methanol and show maximum absorbance at 517 nm. However, in the presence of a proton-donating substance such as an antioxidant, the DPPH radicals are scavenged leading to a reduction

Table 3.1 Percentage amino acid composition of hempseed protein isolate (HPI), hempseed protein hydrolysate (HPH) and membrane ultrafiltration fractions. Results are presented as mean ± standard deviation

AA	HPI	HPH	<1 kDa	1-3 kDa	3-5 kDa	5-10 kDa
ASX	11.81 ± 0.69	11.39 ± 0.03	9.49 ± 0.06	11.70 ± 0.29	12.79 ± 0.47	12.70 ± 0.11
THR	3.54 ± 0.22	3.68 ± 0.29	3.60 ± 0.04	3.77 ± 0.35	4.01 ± 0.19	4.00 ± 0.19
SER	4.78 ± 0.26	4.63 ± 0.09	4.73 ± 0.21	4.79 ± 0.19	4.69 ± 0.07	4.47 ± 0.38
GLX	22.39 ± 2.23	20.06 ± 1.34	15.18 ± 0.96	19.31 ± 1.04	22.71±1.58	22.87 ± 1.02
PRO	4.13 ± 0.54	4.00 ± 0.07	3.19 ± 0.33	4.04 ± 0.56	4.23 ± 0.11	4.89 ± 0.73
GLY	4.38 ± 0.22	4.29 ± 0.23	3.23 ± 0.06	3.93 ± 0.07	4.54 ± 0.20	4.71 ± 0.44
ALA	4.14 ± 0.21	4.47 ± 0.16	4.91 ± 0.06	4.77 ± 0.12	4.30 ± 0.19	4.12 ± 0.09
CYS	1.49 ± 0.04	1.32 ± 0.23	0.29 ± 0.13	0.66 ± 0.01	1.26 ± 0.07	1.58 ± 0.28
VAL	4.14 ± 0.14	4.66 ± 0.02	5.67 ± 0.14	5.26 ± 0.11	4.45 ± 0.19	4.24 ± 0.14
MET	2.36 ± 0.02	1.81 ± 0.39	1.94 ± 0.04	2.03 ± 0.06	1.85 ± 0.14	1.80 ± 0.15
ILE	3.67 ± 0.35	3.84 ± 0.07	4.15 ± 0.13	4.16 ± 0.11	3.98 ± 0.05	3.90 ± 0.04
LEU	5.51 ± 1.23	6.75 ± 0.04	9.91 ± 0.06	7.26 ± 0.10	5.15 ± 0.04	4.82 ± 0.43

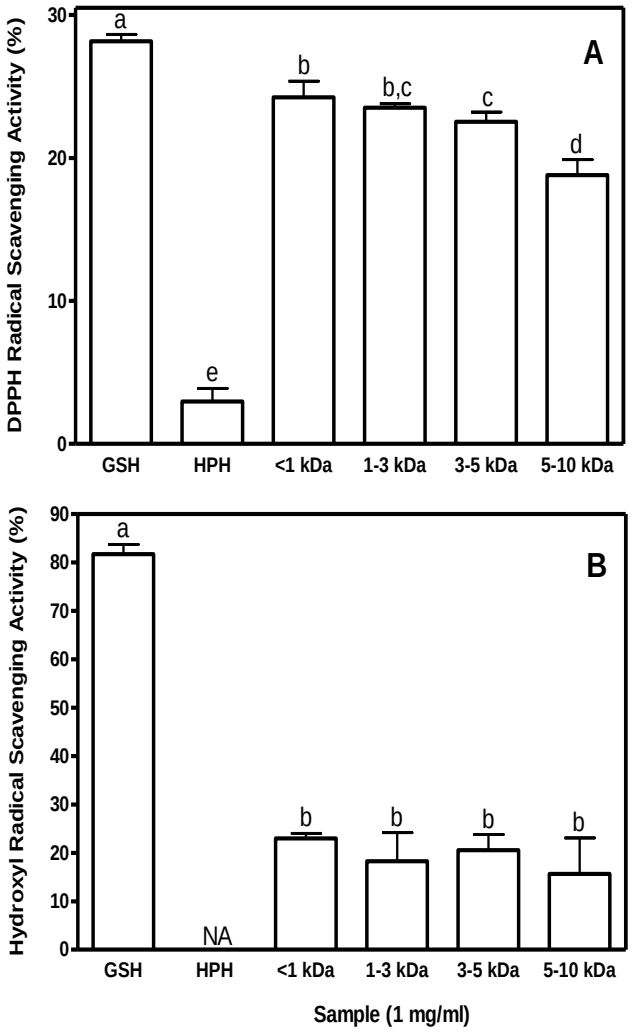
TYR	3.09 ± 0.44	3.45 ± 0.00	4.78 ± 0.02	3.50 ± 0.02	3.06 ± 0.06	3.62 ± 0.96
PHE	3.66 ± 1.10	4.60 ± 0.04	7.68 ± 0.05	5.01 ± 0.14	3.21 ± 0.02	2.85 ± 0.40
HIS	2.65 ± 0.23	2.78 ± 0.03	2.61 ± 0.06	2.47 ± 0.01	2.47 ± 0.03	2.49 ± 0.08
LYS	2.96 ± 0.30	2.97 ± 0.06	3.19 ± 0.11	2.94 ± 0.14	2.56 ± 0.03	2.51 ± 0.07
ARG	13.91 ± 0.55	14.07 ± 0.40	13.87 ± 0.11	12.96 ± 0.13	13.60 ± 0.25	13.31 ± 0.24
TRP	1.39 ± 0.02	1.23 ± 0.16	1.58 ± 0.01	1.44 ± 0.15	1.16 ± 0.12	1.11 ± 0.02
HAA	33.60 ± 2.03	36.13 ± 0.79	44.10 ± 0.84	38.14 ± 0.13	32.64 ± 0.98	32.95 ± 1.11
PCAA	19.51 ± 1.08	19.82 ± 0.36	19.67 ± 0.16	18.37 ± 0.00	18.62 ± 0.19	18.31 ± 0.24
NCAA	34.20 ± 2.92	31.45 ± 1.04	24.67 ± 0.90	31.00 ± 0.75	35.50 ± 1.10	35.56 ± 1.12
AAA	8.14 ± 1.56	9.28 ± 0.12	14.05 ± 0.02	9.95 ± 0.31	7.42 ± 0.19	7.58 ± 0.58

ASX= aspartic acid and asparagine; GLX = glutamic acid and glutamine; Combined total of hydrophobic amino acids (HAA) = alanine, val, Ile, leu, Tyr, phe, Trp, pro, met and cys; Positively charged amino acids (PCAA) = arg,his, lys; Negatively charged amino acids (NCAA) = ASX and GLX; Aromatic amino acids (AAA) = phe, Trp and Tyr.

in absorbance. Fig. 3.1A shows the ability of HPH and its membrane fractions to scavenge DPPH radical. The <1 kDa membrane fraction exhibited the highest RSA of 24.2% and the 5-10 kDa fraction had least activity of 18.7%. The DPPH RSA of the peptide fractions were observed to be dependent on their molecular size, with the smaller (<1kDa and 1-3 kDa) fractions showing higher DPPH scavenging potency than the bigger (3-5 kDa and 5-10 kDa) peptide fractions. This result indicated that low-molecular weight (LMW) peptides possess better RSA than the high-molecular weight (HMW) peptides, which is in agreement with similar findings obtained for quinoa protein hydrolysates fractions [14] but is in contrast with results obtained for flaxseed protein-derived peptide fractions [11] and chickpea peptide fractions [8]. The unfractionated HPH was found to be a poor scavenger of DPPH (~4%) activity when compared to reduced glutathione (28.2%) and the HPH fractions. Previous studies have shown that high DPPH or other radical scavenging activities of protein hydrolysates or peptide fractions are associated with high hydrophobicity [8, 23]. Current results showed that the <1 and 1-3 kDa fractions have the highest contents of hydrophobic amino acids (Table 1), which could explain the higher RSA activities of these two fractions when compared to the HPH and the higher molecular weight fractions (3-5 kDa and 5-10 kDa). In general, the RSA of food protein hydrolysates depend on a variety of factors such as specificity of the proteases used in the protein hydrolysis, size and amino acid composition of the peptides, and the DPPH assay conditions [11]. The RSA of the HPH fractions may be due to the presence or availability of electron donors that reduced the free radicals to more stable inert molecules. In addition, several studies have demonstrated that hydrophobic amino acids act as antioxidants by increasing the solubility of peptides in non-polar

environments thereby facilitating better interaction with free radicals (such as DPPH) and terminating their reactivity [9]. Overall, the RSA of the HPH was lower than those reported for chickpea protein hydrolysates [8].

Figure 3.1 Scavenging activities of hempseed protein hydrolysate (HPH) and its ultrafiltration fractions against DPPH radical (A) and hydroxyl radical (B) compared to reduced glutathione (GSH). Bars (mean ± standard deviation) with different alphabets have mean values that are significantly different at $p<0.05$. NA = no activity detected.

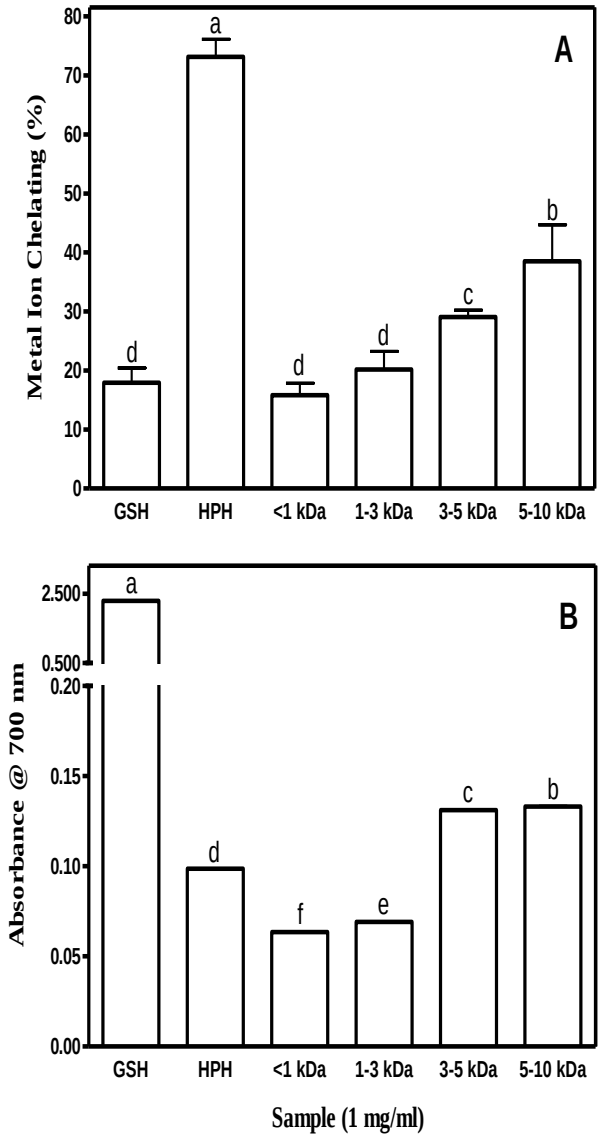


The hydroxyl radical scavenging activity is shown in Fig. 3.1B. The type of free radical system utilized for antioxidant evaluation could influence experimental results; thus, the use of more than one free radical system to investigate radical-scavenging capacities of a selected antioxidant has been recommended [8, 11]. Hydroxyl radical is one of the most reactive ROS, reacting with virtually all cellular macromolecules such as proteins, polyunsaturated fatty acids and nucleic acids to induce severe damages to cells [9] ultimately leading to aging, cancer, diabetes and several other disease conditions [24]. Hydrogen peroxide and superoxide anion can be converted to hydroxyl radicals *in vivo* in the presence of metal ions or may be generated from several other radical systems and leading to oxidative stress. Therefore, the scavenging of hydroxyl radical is an effective defense strategy of the human body against various diseases elicited or propagated by the ROS. The scavenging activities of HPH and its membrane fractions were compared to that of glutathione (Fig. 3.1B).While the HPH showed no activity in scavenging hydroxyl radical, all the HPH membrane fractions exhibited similar and low hydroxyl radical scavenging activities of 15.7-23.7%. These activities were lower than that observed for GSH, which scavenged 81.7% of hydroxyl radical at the same concentration. The hemp seed peptides are poor scavengers of hydroxyl radical when compared to chickpea [8] and wheat germ [22] peptides that showed >30% activity. The results indicate that the ratio of hydroxyl radical scavenging peptides to non-scavenging peptides was very low in the HPH. However, fractionation led to increased segregation of hydroxyl radical scavenging peptides, which was reflected in the higher activity of the membrane fractions.

3.3.3.2 Metal Ion Chelating and Ferric Reducing Activities

Transition metal ions are involved in many *in vivo* oxidation reactions. Ferrous ions (Fe^{2+}) can catalyze Haber-Weiss reaction and induce superoxide anion to form more hazardous hydroxyl radicals. These hydroxyl radicals react rapidly with adjacent biomolecules to cause severe tissue damage. Ferrous ions can also stimulate lipid peroxidation by Fenton reaction, and accelerate peroxidation by decomposing lipid hydroperoxides into hydroxyl and alkoxyl radicals capable of abstracting hydrogen and perpetuating a chain reaction of lipid peroxidation [25, 15]. Chelating agents may serve as secondary antioxidants because they reduce the redox potential, hence stabilizing the oxidized form of the metal ions. Fig. 3.2A shows the Fe^{2+}chelating effects of reduced glutathione, HPH and its membrane fractions. The Fe^{2+} chelating ability was estimated in this study by decrease in the absorbance of ferrozine-Fe^{2+} complex after the addition of the test samples. Clearly, the HPH exhibited the strongest chelating capacity (72%) which could be attributed to the additive effects of all the peptides that are responsible for the activities of the fractions. However, glutathione and the <1 and 1-3 kDa HPH fractions showed low chelating activity ranging from 15.7% to 20.2% compared to the moderate activity exhibited by the 3-5 and 5-10 kDa HPH fractions, which had 29 and 38.5% Fe^{2+} chelating activity, respectively. Thus, the metal ion chelating activities of HPH fractions increased with molecular weight (probably due to additive effects from constituent peptides) and the synergistic effects of the fractions was reflected in the high activity of the unfractionated hydrolysate. Fractions 3-5 and 5-10 kDa also contained the highest levels of negatively-charged amino acids, which could have contributed to increased electrostatic affinity for the positively-charged iron. The present results are similar to

Figure 3.2 Metal chelating effects (A) and ferric reducing power (B) of hempseed protein hydrolysate (HPH) and its fractions compared to reduced glutathione (GSH). Bars (mean ± standard deviation) with different alphabets have mean values that are significantly different at $p<0.05$.



those from a previous work [5], which showed that increased peptide chain length led to higher iron chelating effects. The observed iron chelating property of HPH and its fractions may be beneficial towards the protection of cellular components against oxidative damage. Ferric reducing antioxidant power (FRAP) assay is used to evaluate the ability of natural antioxidants to donate electrons. Some reports have indicated that there is direct correlation between antioxidant activities and reducing power of protein hydrolysate fractions [25, 26]. Fig. 3.2B shows the FRAP of glutathione, HPH and membrane fractions measured at 700 nm. An increase in absorbance indicates better reducing power of the test sample. The HPH and its membrane fractions exhibited low absorbance values of 0.06 to 0.13 compared to GSH which had highest absorbance value of 2.29. This implied that GSH had the highest reducing power when compared to HPH and its membrane fractions. However, the FRAP of the hemp seed hydrolysates is similar to values previously reported for chickpea protein hydrolysates [8]. There was an increase in reducing power of the HPH fractions with increasing peptide size (3-5 and 5-10 kDa fractions were better than <1 and 1-3 kDa fraction), which is an indication of additive effects of active groups within the peptides; long-chain peptides will contain more reducing groups than short-chain peptides. The HPH had significantly higher values than the <1 and 1-3 kDa fractions, which suggest that the presence of 3-5 and 5-10 kDa peptides contributed to the observed HPH activity. The trend showing higher FRAP of the 3-5 and 5-10 kDa fractions are similar to the trend observed for the iron chelating effects of the samples. The presence of amino acids leucine, isoleucine, histidine, methionine, tyrosine, lysine and tryptophan could have contributed to the reducing power activity observed for the protein hydrolysates [27].

3.3.3.3 Inhibition of Linoleic Acid Oxidation

Peroxidation of fatty acids causes deleterious effects in foods by forming complex mixture of secondary metabolites of lipid peroxides. When these foods are further consumed, they can cause some adverse effects including toxicity to mammalian cells [8]. Lipid peroxidation proceeds via radical mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids [28]. The antioxidant activities of GSH, HPH and its membrane fractions against peroxidation of linoleic acid were evaluated and the results obtained after 7 days of incubation. Table 3.2 shows that the HPH and fractionated sampleseffectively inhibited linoleic acid autoxidation at varying degrees similar to the effect of GSH. After the 4^{th} day, GSH and the peptide samples seem to gradually lose their antioxidant effects as evident by slight increases in absorbance from day 5-7. The present results are similar to previous work [22], which showed that wheat germ protein hydrolysate gradually lost its effectiveness against linoleic acid oxidation after 3 days of incubation. Overall, HPH showed the highest activity at day 7 in protecting linoleic acid against peroxidation, which may be due to the additive effects of all the active peptides present in the different fractions.The strong antioxidant effects of the peptide samples may have been due to the high levels of hydrophobic amino acids, which enhanced solubility of the peptides in the lipid phase and thus facilitating better interactions with free radical species [29]. In addition, the presence of histidine (His) in the peptides has also been reported to act against lipid peroxidation because His possess an imidazole ring in its structure, which may be involved in hydrogen donation and lipid radical trapping ability [30]. The observed rapid increase in linoleic acid oxidation in the absence of antioxidants (control) up to the 4^{th} day is similar to previous reports [23, 31].

As the incubation proceeds beyond the 4th day, the linoleic acid is probably depleted and production of reactive oxidation products (e.g., hydroperoxides) becomes limited, which is believed to be responsible for the sharp decrease in absorbance values [31].

3.4 CONCLUSIONS

This study has shown that the HPH and its ultrafiltration fractions have effective *in vitro* antioxidant properties. The ability of the peptides to scavenge hydroxyl and DPPH radicals, reduce ferric metal ions and chelate transition metal ions is indicative of their potential use to manage metabolic disorders that arise from excessive levels of reactive oxygen species. Based on the results from this work, defatted hempseed meal possesses the potential for use as raw material for the production of peptide ingredients that could be used to formulate functional foods and nutraceuticals with multifunctional bioactive properties against various free radicals that may cause oxidative stress related diseases. However, *in vivo* availability, potency and safety must be determined before the products can be used for therapeutic purposes.

Acknowledgments

Funding for this work was provided through a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) and an operating grant from the Advanced Foods and Materials Network of Centres of Excellence, Canada (AFMNet) to Dr. R.E. Aluko. C.C. Udenigwe is a recipient of NSERC Alexander Graham Bell PhD scholarship.

Table 3.2: Lipid oxidation measured in linoleic acid model system for 7 days in the presence of 1 mg/ml glutathione (GSH), hempseed protein hydrolysate (HPH) and peptide fractions. The control contains only linoleic acid and no antioxidant compound. Results are presented as mean ± standard deviation

	Duration (days)						
	1	2	3	4	5	6	7
Control	0.189± 0.001^{d}	0.226± 0.012^{a}	0.287± 0.052^{a}	0.454± 0.05^{a}	0.380± 0.017^{a}	0.260± 0.019^{a}	0.283± 0.005^{a}
GSH	0.181± 0.000^{e}	0.184± 0.002^{b}	0.193± 0.006^{b}	0.194± 0.010^{b}	0.214± 0.011^{b}	0.245± 0.003^{b}	0.252± 0.004^{b}
HPH	0.204± 0.003^{b}	0.148± 0.001^{d}	0.151± 0.013^{c}	0.132± 0.019^{d}	0.152± 0.003^{e}	0.204± 0.006^{c}	0.183± 0.020^{b}
<1 kDa	0.209± 0.001^{a}	0.191± 0.005^{b}	0.178± 0.001^{c}	0.166± 0.017^{c,d}	0.196± 0.024^{b,c}	0.208± 0.013^{c}	0.204± 0.008^{b}
1-3 kDa	0.205± 0.001^{b}	0.173± 0.002^{c}	0.184± 0.002^{c}	0.189± 0.002^{b,c,b}	0.199± 0.027^{b,c}	0.218± 0.010^{c}	0.238± 0.030^{b}
3-5 kDa	0.196± 0.003^{c}	0.168± 0.003^{c}	0.174± 0.001^{c}	0.152± 0.018^{c,d}	0.183± 0.021^{c,d}	0.205± 0.006^{c}	0.234± 0.007^{b}
5-10 kDa	0.178± 0.002^{e}	0.155± 0.003^{d}	0.160± 0.007^{c}	0.153± 0.016^{c,d}	0.162± 0.017^{d,e}	0.204± 0.003^{c}	0.206± 0.031^{b}

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TRANSITION STATEMENT 1

The first step in production of bioactive peptides is to screen the proteins using bioassay guided fractionation through enzymatic hydrolysis and testing for various claimed as well as potential physiological benefits *in vitro* and positive results obtained are indicative of potential biological activity of the protein. The overall objective of this thesis being to produce antioxidant and antihypertensive peptides from hempseed proteins, the pioneering manuscript in part addressed the potential use of hemp seed as a source of antioxidants by producing simulated gastrointestinal digested hempseed protein hydrolysate, fractionated the hydrolysate into peptides of different molecular weights, and evaluated these peptides for multifunctional properties using various antioxidant evaluation systems, *in vitro*. These peptides had effective *in vitro* antioxidant properties with the ability to scavenge hydroxyl and DPPH radicals, reduce ferric, chelate transitions metals and prevent lipid peroxidation an indication of their potential use to manage degenerative disorders arising from damage impacted by excessive production of ROS/free radicals. The second manuscript evaluated the use of hemp seed peptides and ultrafiltration fractions as potential antihypertensive agents through the *in vitro* inhibition of ACE and renin activities, as well as *in vivo* lowering of systolic blood pressure (SBP) in spontaneously hypertensive rats (SHRs). The study also determined the kinetics of renin and ACE inhibition by HPH and its fractions as well as the degree of hydrolysis (DH) and yield in case of commercial utility. It was established that these peptides were LMW peptides of high DH (90%) and yield (89%) with potential antihypertensive properties and peptides could thus be used as potential therapeutic agents for the prevention, amelioration, treatment or management of chronic cardiovascular diseases.

CHAPTER 4

MANUSCRIPT 2

KINETICS OF ENZYME INHIBITION AND ANTIHYPERTENSIVE EFFECTS OF HEMP SEED (*CANNABIS SATIVA* L.) PROTEIN HYDROLYSATES

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4.0 ABSTRACT

The aim of this study was to determine the antihypertensive effects of enzymatic hemp seed protein hydrolysate (HPH) and its peptide fractions. Hemp seed protein isolate was digested by sequential action of pepsin and pancreatin to mimic gastrointestinal digestion in human beings. The resultant HPH was separated by membrane ultrafiltration into peptide fractions with different sizes (<1 and 1-3 kDa). The HPH had significantly higher ($p<0.05$) *in vitro* inhibition of the activities of angiotensin I-converting enzyme (ACE) and renin, the two main enzymes involved in abnormal blood pressure elevation (hypertension). Kinetic studies showed that HPH and peptide fractions inhibited renin and ACE activities in a mixed-type pattern, indicating binding to areas other than the active site. Oral administration of HPH (200 mg/kg body weight) to spontaneously hypertensive rats showed significant reductions ($p<0.05$) of systolic blood pressure (SBP) that reached a maximum of -30 mmHg after 8 h. In contrast, the hypotensive effects of peptide fractions (<1 and 1-3 kDa) had a maximum value of about -15 mmHg after 6-8 h, post oral administration. The results suggest a synergistic antihypertensive effect of peptides present within the HPH; this effect was reduced significantly ($p<0.05$) upon separation into peptide fractions.

4.1 Introduction

Hypertension is a major global health problem generally affecting 20-45% of the population: 15-20% of all adults and nearly 50-60% of elderly people. Hypertension is one of the main controllable risk factors associated with cardiovascular disease events like myocardial infarction, heart failure, and end-stage diabetes [1]. Hypertension is mainly regulated by the renin-angiotensin system (RAS) which generates a variety of regulatory peptides that modulate blood pressure, fluid and electrolyte balance throughout the human body. Renin and angiotensin I-converting enzyme (ACE), a Zn protease, are the key enzymes that control the RAS pathway [2]. In the RAS, renin converts angiotensinogen to angiotensin I (AT-I) which in turn is converted by ACE to angiotensin II (AT-II), a vasoconstrictor. In disease conditions or as a result of genetic and environmental influences, the level of ACE in the body is up-regulated resulting in high levels of AT-II which promotes undesirable rates of blood vessel contraction that leads to the development of high blood pressure and hypertension. ACE is also known to inactivate bradykinin, a potent vasodilator further leading to inability of the blood vessels to relax following contraction. The current treatment for hypertension in human beings has involved the use of synthetic ACE inhibitors, such as captopril, enalapril, alacepril and lisinopril. However, some undesirable side effects like dry cough, diarrhea taste disturbances and skin rashes have been reported, which limit their use in treating hypertension [3, 4]. There is a growing interest in the use of naturally occurring renin and ACE-inhibitory peptides as therapeutic agents for the treatment and management of hypertension and other chronic diseases [4]. Food-derived ACE-inhibitory peptides, though less effective as hypotensive agents *in vitro* when compared to drugs, have no

known side effects and have the potential to offer lower healthcare cost [1]. Furthermore, these peptides are observed to have multifunctional properties, are easily absorbed to target organs [5] and possess inherent high nutritional value that contributes to the overall wellbeing of the individual.

Historically, hemp seed (*Cannabis sativa* L.) cultivation for food utilization has been limited due to the presence of the psychoactive compound called tetrahydrocannabinol (THC). Availability of low THC hemp plant has increased accessibility to the edible seeds, which is a by-product obtained during the commercial utilization of the valuable hemp plant fibre, and is a rich source of high quality oil and protein [6]. The seed contains over 30% oil (composed primarily of linoleic and α-linolenic acids) and about 25% protein; the storage proteins mainly consist of edestin (globulin) and albumin, with a superior essential amino acid profile and high digestibility [7, 8]. The increased utilization of hemp seed for edible oil production has led to abundant amounts of protein-rich meal, which serves as a suitable raw material for production of peptide products. This is because of the presence of high levels of residues, especially arginine and the branched chain amino acids [9], which are desirable components of bioactive peptides. Increasingly, food consumption is considered not only a source of nutrients but also of bioactive compounds such as peptides. Enzymatic hydrolysis could be applied to modify the properties of food proteins but additionally it could impart these proteins with added value, such as potential health effects. Bioactive peptides from natural sources usually contain 3-20 amino acid residues and their activity is based on the amino acid composition and sequence [10]. These peptides which are inactive within the parent protein could be activated by enzymatic hydrolysis to exhibit

various health regulatory effects such as antioxidant properties [11] and antihypertensive activities [2]. Several food protein hydrolysates have been shown to possess *in vitro* inhibition of ACE activity resulting in the lowering of elevated blood pressure in spontaneously hypertensive rats [2] and in hypertensive human beings [12]. However, scanty reports are available for natural food based renin inhibitors. Moreover, it has been hypothesized that the direct inhibition of renin activity provides a more effective control of elevated blood pressure since it is the first and rate limiting step in the RAS. Renin prevents the production of AT-I, which could otherwise be converted to appreciable amounts of AT-II in certain organs through an ACE-independent pathway catalyzed by chymase, a product of masts cells in the connective tissues of the heart and blood vessels [13]. Inhibiting renin alone in the RAS does not completely prevent ACE-catalyzed bradykinin degradation that could also result to vasoconstriction [14]. It has therefore been proposed that a better approach to lowering of elevated blood pressure is to develop natural therapeutic agents that could exhibit multiple effects such as the simultaneous inhibition of renin and ACE activities [13]. Recently, some renin-inhibitory peptides have been reported from the enzymatic hydrolysis of flaxseed and pea proteins [13, 15].

Therefore, the objectives of this study were to determine the antihypertensive properties of hemp seed protein hydrolysate (HPH) and its peptide size-based fractions using the *in vitro* inhibition of ACE and renin activities, as well as *in vivo* lowering of systolic blood pressure (SBP) in spontaneously hypertensive rats (SHRs). We also determined the kinetics of renin and ACE inhibition by HPH and its fractions in order to provide a mechanistic basis for the inhibitory properties.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Defatted hempseed meal, referred to as hempseed protein powder was purchased from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), trinitrobenzene sulfonic acid (TNBS), sodium dodecyl sulfate, N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Other analytical grade reagents and ultrafiltration membranes (1 and 3 kDa molecular weight cut-off) were obtained from Fisher Scientific (Oakville, ON, Canada).

4.2.2 Methods

4.2.2.1 Production of Hempseed Protein Hydrolysate (HPH) and Membrane Fractions

Hemp seed protein isolate (HPI), hydrolysate (HPH) and membrane fractions were prepared according to procedures described in our previous publication [11]. Briefly, HPH was produced by consecutive treatment of HPI with pepsin (2 h) and pancreatin (4 h) followed by centrifugation to recover soluble peptides. The soluble peptides were first passed through 1 kDa membrane and the permeate collected as the <1 kDa sample. The retentate was then passed through a 3 kDa ultrafiltration membrane and the permeate collected as the 1-3 kDa sample. The HPH and membrane permeates were freeze-dried and peptide contents determined by the modified Lowry method [16] using bovine serum albumin as standard. The degree of hydrolysis (DH) of hemp seed protein

hydrolysates was determined according to the trinitrobenzenesulfonic acid (TNBS) method [17]. The yields of hemp seed protein hydrolysates (HPH) were determined for samples hydrolyzed with pepsin alone and in combination with pancreatin. The percent yield of HPH was determined as the ratio of peptide weight of lyophilized HPH to the protein weight of unhydrolyzed HPI. Similarly, percent yields of the ultrafiltration membrane fractions were calculated as the ratio of peptide weight of a lyophilized peptide permeate to the peptide weight of the original HPH.

4.2.2.2 Kinetics of ACE Inhibition

The ability of hemp seed peptide fractions to inhibit activity of ACE *in vitro* was measured in triplicates as previously reported [13]. Briefly, 1 ml of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 µL of ACE (final activity of 20 mU) and 200 µL of hemp seed protein hydrolysate in 50 mM Tris–HCl buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. Tris–HCl buffer was used instead of peptide fraction solutions in the blank experiment. ACE activity was expressed as rate of reaction (ΔA/min) and inhibitory activity was calculated using Eq. below:

$$\text{ACE inhibition (\%)} = [(\Delta A/\min_{(\text{blank})} - \Delta A/\min_{(\text{sample})})/\Delta A/\min_{(\text{blank})}] \times 100$$

where $(\Delta A/\min_{(\text{sample})}$ and $[(\Delta A/\min_{(\text{blank})}$ are ACE activity in the presence and absence of the hemp seed peptide samples, respectively. The concentration of peptide fractions that inhibited ACE activity by 50% (IC_{50}) was calculated for each sample using non-linear regression from a plot of percentage ACE inhibition versus sample concentrations. The kinetics of ACE inhibition was studied with 0.0625, 0.125, 0.25 and 0.5 mM FAPGG. The mode of ACE inhibition was determined from the Lineweaver–Burk plots

while inhibition constant (K_i) was calculated as the X-axis intercept from a plot of the slope of the Lineweaver–Burk lines against peptide concentration [18].

4.2.2.3 Renin Inhibition Assay

In vitro inhibition of the activity of human recombinant renin assay was conducted according to the previously described method [15] using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37 °C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37 °C. Before the reaction, (1) 20 μL of substrate, 160 μL of assay buffer, and 10 μL of Milli-Q water were added to the background wells; (2) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of Milli-Q water were added to the control wells; and (3) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of sample were added to the inhibitor wells. The reaction was initiated by adding 10 μL of renin to the control and sample wells. The microplate was shaken for 10 s to mix and incubated at 37 °C for 15 min, and then, the fluorescence intensity (FI) was recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. The percentage inhibition was calculated as follows:

Inhibition% = [(FI of control well-FI of sample well)/(FI of control well)] X 100

The concentration of protein hydrolysates that inhibited 50% of renin activity was determined and defined as the IC_{50} value. The renin inhibition kinetics studies were conducted using 0.625, 1.25, 2.5, 5 and 10 μM of substrate in the absence and presence of peptides.

4.2.2.4 SHRs and Measurement of Systolic Blood Pressure

Animal experiments were carried out following the Canadian Council on Animal Care ethics guidelines with a protocol approved by the University of Manitoba Animal Protocol and Management Review Committee. SHRs (6 rats/treatment, 20-weeks old, male, 250-300 g of body weight, BW) with tail SBP of over 150 mmHg were purchased from Charles Rivers Laboratories (Montreal, PQ, Canada). SHRs were housed individually in steel cages in a room kept at 25 ^{o}C with a relative humidity of 50% and a 12 h light-dark cycle, and fed a standard laboratory diet (chow) with free access to water. HPH and the membrane permeates (<1 and 3 kDa) were dissolved in 1 mL of saline at a dose of 200 mg/kg BW. Captopril (an antihypertensive drug) was administered at a dose of 3 mg/kg BW as positive control while negative control rats received the same volume of saline solution. Following oral administration by syringe gavage, the effects of samples on SBP were compared to that of captopril. SBP was measured by tail-cuff plethysmography at 2, 4, 6, 8 and 24 h (post oral administration of samples) in mildly anesthetized rats according to the method of Aukema et al [19]. In order to mitigate the SBP depression effect of isofluorane, the gas flow was optimized such that rats became conscious usually within 3-4 min after removal from the chamber, which provided enough time to perform the blood pressure measurement. Rats were first anesthetized in a chamber (maintained at about 40 ^{o}C) with 4% isofluorane for 4 min. They were then removed from the isofluorane chamber and tail-cuff measurement of blood pressure performed in the unconscious state by taking 3 electronic readings.

4.2.2.5 Statistical Analysis

Analyses were conducted in replicates as indicated above and analyzed by one-way

analysis of variance (ANOVA). Data were reported as mean ± standard deviation. Statistical significance of differences was evaluated by Duncan's multiple range test ($P < 0.05$) using the Statistical Analysis Systems software version 9.2 (SAS, Cary, NC, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Yield and DH of HPH and Membrane Permeates

Table 4.1 shows the DH and yield of HPH and membrane permeate fractions. The DH is usually employed as a proteolysis-monitoring parameter to ascertain the extent to which peptide bonds have been broken down to release short chain peptides [20]. The DH value of 39.1% obtained for the peptic hydrolysate (HPH_{pep}) was significantly ($p<0.05$) lower than that of the sequential pepsin-pancreatin (HPH) hydrolysate (90.6%). The higher DH exhibited by the HPH could be due to the different domain of cleavage of bonds associated with each of the enzymes used. While pepsin is an endopeptidase that cleaves at specific sites, pancreatin is a mixture of endo- and exo-peptidases, which enhances a more extensive hydrolysis of peptide bonds. Moreover, the duration of pancreatin hydrolysis was 4 h in contrast to the 2 h period for pepsin; previous reports have shown that the DH increases with increase in time of hydrolysis [21, 22]. The greater efficiency of hydrolysis observed for the combined enzyme usage could also be because pre-digestion with pepsin led to exposure of susceptible peptide bonds, facilitating optimal hydrolysis by pancreatin. Percent yield is an indication of the efficiency of enzyme hydrolysis process because a higher yield of peptides is the expected outcome for increased protein breakdown. The yield also puts into perspective the economic viability of commercializing the protein hydrolysate as an ingredient to

Table 4.1 Percent yield and degree of hydrolysis (DH) of hemp seed protein hydrolysates and membrane ultrafiltration fractions

Sample	Peptide yield (%)	DH (%)
Hydrolysates		
$HPH_{(pep)}$	65.7 ± 3.75	39.1 ± 0.44
HPH	86.7 ± 5.07	90.3 ± 1.15
Peptide fractions		
<1 kDa	41.8 ± 0.67	NA
1-3 kDa	32.8 ± 0.45	NA
Retentate	25.7 ± 0.20	NA

**$HPH_{(pep)}$ = unfractionated hydrolysate from pepsin digestionof hemp seed protein isolate (HPI); HPH = unfractionated hydrolysate from pepsin-pancreatin digestion of HPI;peptide fractions (<1 and 1-3 kDa) were separated from HPH;Retentate is the peptide fraction >3 kDa. NA = not applicable.*

formulate functional foodsand nutraceuticals. Higher percent yield are more beneficial to commercial processing and marketing of new products. As expected, the yield (65.7%) of HPH_{pep} was significantly lower ($p<0.05$) than the value (86.7%) obtained for HPH. The results showed a direct correlation between the extent of hydrolysis and the yield of protein hydrolysate, which is consistent with previously reported works [23, 24]. Size-dependent separation of the HPH by membrane ultrafiltration showed that the low molecular weight peptides (<1 and 1-3 kDa) are present in a higher proportion (74%)

when compared to the peptides with size >3 kDa (26%). The high content of small peptides in the HPH suggests a high potential for *in vivo* bioavailability during oral administration and may contribute to increased physiological efficiency. This is because short-chain peptides have been shown to be usually resistant to gastrointestinal proteolysis and can be absorbed intact into blood circulation whereas long-chain peptides are susceptible to proteolysis [25].

4.3.2 Enzyme Inhibitory Activities of HPH and Membrane Fractions

4.3.2.1 ACE Inhibition

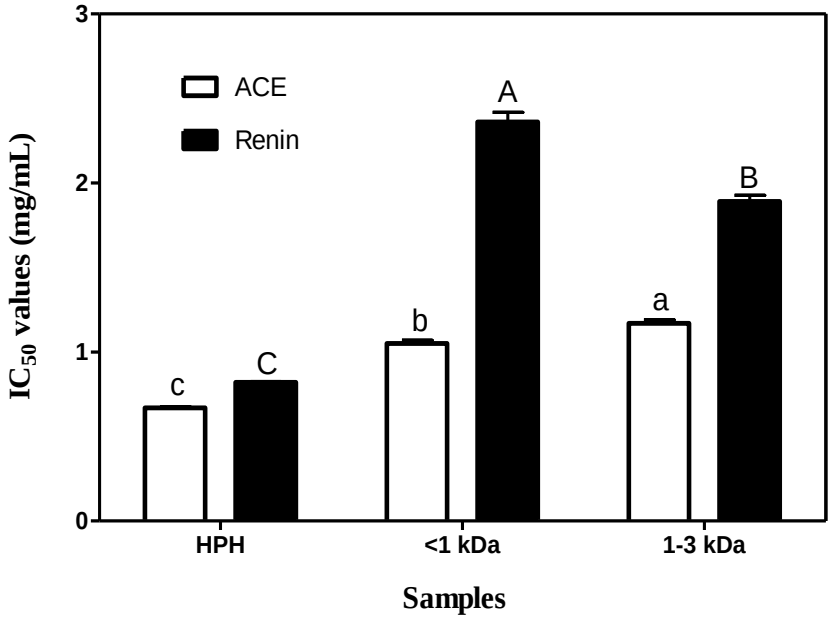
ACE catalyzes two main reactions responsible for the constriction of blood vessels that leads to blood pressure elevation. The IC_{50} values of HPH (0.67 mg/mL) against ACE activity was significantly lower ($p<0.05$) than the values obtained for the membrane fractions (1.05 and 1.17 mg/L, respectively for <1 kDa and 1-3 kDa membrane fractions (Fig. 4.1, open bars). The IC_{50} values obtained in this study are similar to values (1.03-1.06 mg/mL) reported for ACE-inhibitory bovine milk using tryptic hydrolysates [26]. In contrast, flaxseed protein hydrolysate fractions inhibited ACE activity with lower IC_{50} values of 0.024-0.15 mg/mL [13] while fermented products containing mainly β-casein-derived peptides had IC_{50} values from 0.008-0.0112 mg/mL [27]. Fractionation of the HPH by membrane ultrafiltration resulted in peptide fractions with reduced potency against ACE (Fig. 4.1), which is in contrast to results obtained for shrimp protein hydrolysate [24] where the <3 kDa fraction was shown to have higher activity than the original hydrolysate. The <1 kDa peptides had significantly lower ($p<0.05$) IC_{50} value than the 1-3 kDa peptides, which suggests that smaller size peptides may be more effective as ACE inhibitors when compared to the larger size peptides. The

results are similar to reports obtained for cod frame protein hydrolysate [28] and shrimp [24] that showed an increase in ACE-inhibitory activity with decreasing MW of peptide fractions produced by ultrafiltration. The reduced ACE-inhibitory activities of the ultrafiltration membrane fractions suggest that there was a synergistic effect when the peptides are present in the HPH. ACE prefers to bind to substrates or inhibitors containing hydrophobic (aromatic or branched-chain) amino acid residues at each of the three C-terminal positions; many naturally occurring ACE-inhibitory peptides contain Tyr, Phe, Trp, Pro or Lys at the C-terminal especially the di and tripeptides [29]. Pro, Trp and Lys are the most effective in increasing the ACE-inhibitory activity [12,29] while the branched-chain aliphatic amino acids such as Ile, Leu and Val are the most prevalent in highly active peptide inhibitors [2]. Thus, the higher amounts of Phe and Leu in the <1 kDa fraction [11] may have contributed to a higher ACE-inhibitory activity when compared to the 1-3 kDa fraction.

4.3.2.2 Renin Inhibition

Renin inhibitors produce highly selective inhibition of the RAS resulting in improved side-effects profile for therapeutic agents, since renin catalyzes hydrolysis of only one naturally occurring substrate, angiotensinogen [30]. Thus, the search for natural renin inhibitors has become a challenge and most approaches of obtaining some have been focused on peptide modification or synthesis. In this study, the peptide samples exhibited moderate renin inhibitory activities with IC_{50} values of 0.81, 2.52and 1.89 mg/mL for HPH, <1 and 1-3 kDa fractions, respectively (Fig. 4.1, solid bars). Similar to the observed effects on ACE activity, the HPH had significantly lower IC_{50} values when compared to the <3 kDa fractions obtained from membrane separation. The results also

Figure 4.1: Peptide inhibitory concentrations that reduced 50% (IC_{50}) activity of angiotensin converting enzyme (ACE) and renin inhibitions by hemp seed protein-derived peptides. HPH is the protein hydrolysate from pepsin-pancreatin digestion of hemp seed proteins isolate while <1 and 1-3 kDa are the peptide fractions from membrane ultrafiltration of HPH. Bars belonging to the same enzyme but with different alphabets are significantly different at $p<0.05$.



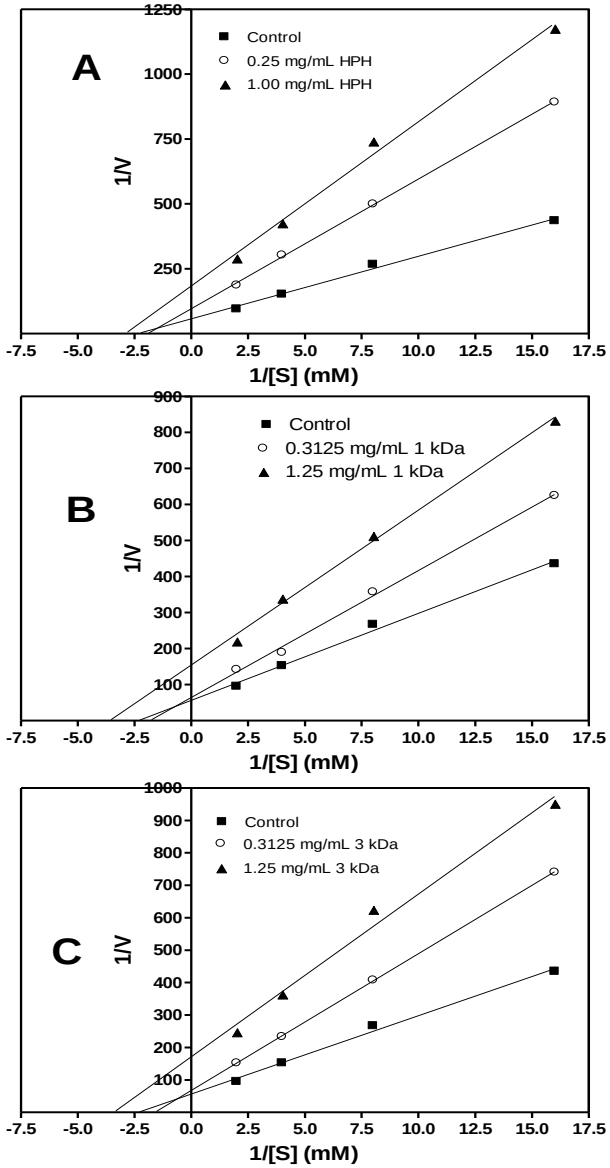
suggest that the peptides act synergistically to produce a more effective renin inhibition when present together in the HPH. Fractionation of the peptides resulted in loss of this synergistic effect probably due to size-dependent partitioning of active peptides into different groups. It was earlier reported that ACE inhibition is more easily achieved when compared to renin inhibition, thus, there are more ACE-inhibitory peptides than food protein derived-peptide inhibitors of renin [13]. Renin inhibition by food protein hydrolysates are rare because of the difficulty associated with the down regulation of the activities of renin both *in vitro* and *in vivo.* Currently, there are only few food protein-

derived peptide inhibitors of renin [13, 15]. Udenigwe et al [13] reported that flaxseed protein hydrolysate fractions moderately inhibited human recombinant renin activity which was also dependent on the type of enzyme used to hydrolyze the proteins. The IC_{50} values for renin inhibition by HPH, <1 and 1-3 kDa fractions are higher than those for ACE inhibition, which is in agreement with the fact that inhibition of ACE is easily achieved compared to renin inhibition.

4.3.3 Kinetics of Enzyme Inhibition

Based on the inhibitory results of the peptides used in this experiment, the mode of inhibition of ACE and renin activities was investigated via kinetic studies in the absence and presence of the hemp seed protein hydrolysate and membrane fractions. Kinetic parameters are vital to interpreting the effectiveness of peptides in eliciting their inhibitory potential against the activities of enzymes. Furthermore, kinetic plots give a rough estimate of the amount of substrate or peptides (inhibitor) required to accelerate the reaction or inhibit the activities of the enzymes which are reflected by the affinity to bind to the active site of the enzyme. K_i is the dissociation constant that defines inhibitor binding ability to the enzyme to form the enzyme-inhibitor complex. Lineweaver-Burk plots of ACE reaction with and without peptide inhibitors at two concentrations are shown in Fig. 4.2A-C. The pattern of inhibition displayed was mixed-type tilting more towards noncompetitive inhibition, which means that the peptide can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound to the enzyme active site or not. This also implies that the peptide binds at a different site from the substrate hence act as ACE inhibitor by forming enzyme-substrate-inhibitor and enzyme-inhibitor complexes, which will reduce

Figure 4.2: Lineweaver-Burk plots of angiotensin I-converting enzyme (ACE) inhibition by different concentrations of: (A) unfractionated protein hydrolysate (HPH) from pepsin-pancreatin digestion of hemp seed protein isolate; (B) <1 kDa and (C) 1-3 kDa peptide fractions (from membrane ultrafiltration of HPH) at varying substrate concentrations (0.0625-0.5 mM). V = the initial rate of reaction (ΔA_{345}nm/min).



efficiency of enzyme catalysis. The $K_i$values of 2.55, 3.96 and 4.74 mg/mL for ACE inhibition by HPH, <1 and 1-3 kDa peptide fractions, respectively, are directly correlated with the *in vitro* ACE-inhibitory effects of the samples. Thus, the HPH binds more effectively to ACE and could explain the higher inhibitory effects when compared to the <1 and 1-3 kDa peptides that have higher values of K_i.

Figures 4.3A-C shows the double reciprocal plots for the inhibition of human recombinant renin by HPH and the 1 and 3 kDa peptide fractions. These peptides all exhibited mixed type non-competitive mode of renin inhibition. The K_i values are directly correlated to IC_{50} values obtained for renin inhibition by HPH and peptide fractions. For instance, HPH had a lower K_i of 2.45mg/mL compared to the <1 and 1-3 kDa with 11.25 and 3.02 mg/mL, respectively, which implies that less amount of HPH is required for enzyme inhibition than the amount of the <1 or 1-3 kDa fractions required to achieve same effects.

4.3.4 Hypotensive Effects of Hemp seed Protein Hydrolysates in SHRs

Figure 4.4 shows the SBP lowering effects of hemp seed protein and peptide samples in SHRs when compared to the antihypertensive drug, captopril. A single oral administration of HPI, HPH, fractions (<1 and 3kDa) and captopril to SHRs revealed that HPH significantly ($p<0.05$) decreased SBP (-20 mmHg) after only 2 hr of oral administration and a maximum effect of -30 mmHg reduction in SBP was achieved after 8 h. By the 4 h mark, the antihypertensive effect of the HPH was already comparable to the effect obtained for captopril and remained similar up to 24 h. In contrast, the hypotensive effects of the <1 and 3 kDa peptide fractions was significantly different ($p<0.05$) from the control rats at 6-8 h, but were significantly lower ($p<0.05$) than the

Figure 4.3: Lineweaver-Burk plots of the inhibition of human recombinant renin by different concentrations of: (A) unfractionated protein hydrolysate (HPH) from pepsin-pancreatin digestion of hemp seed protein isolate; (B) <1 kDa; and (C) 1-3 kDa peptide fractions (from membrane ultrafiltration of HPH) at varying substrate concentrations (0.625-10 µM); V = initial rate of reaction (Change in fluorescence intensity/min).

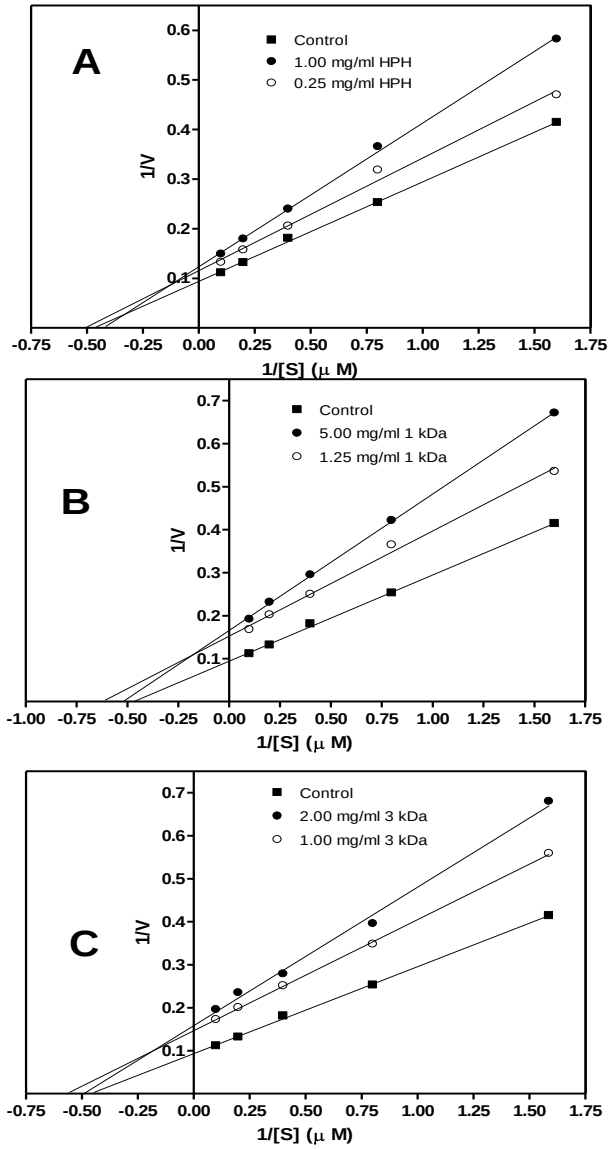
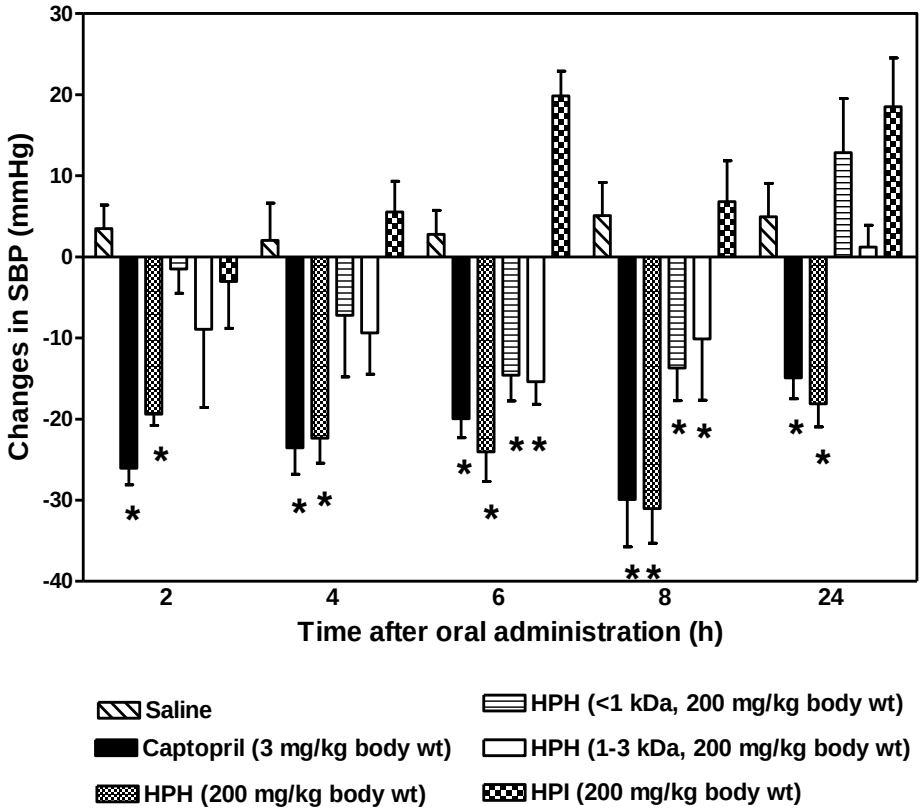


Figure 4.4: Time-dependent changes in systolic blood pressure (SBP) of spontaneously hypertensive rats after oral administration of 200 mg/kg body weight each of hemp seed protein isolate (HPI), unfractionated protein hydrolysate (HPH) from pepsin-pancreatin digestion of HPI and membrane ultrafiltration fractions of HPH (<1 and 1-3 kDa) in comparison to 3 mg/kg body weight of captopril. Bars with asterisks (*) have mean values that are significantly lower ($p<0.05$) than the mean values for saline and HPI bars.



effect of HPH. The results suggest that the HPH is faster acting and more potent than the peptide fractions, which may be indicative of synergistic effects of peptides present in the HPH; this synergy was lost upon peptide fractionation. The degree of hypotensive effect

observed for the HPH and peptide fractions was directly correlated with observed *in vitro* inhibition of ACE and renin activities. This is because the ultrafiltration fractions (<1 and 1-3 kDa) showed milder SBP lowering effects, which is in agreement with their *in vitro* inhibition effects against ACE and renin that were lower than that of the HPH. Moreover, unlike the HPH the hypotensive effects of the <1 and 1-3 kDa peptide fractions was eventually lost by the 24^{th} hr. The HPI and saline solution had negative effects of elevating rather than suppressing SBP in rats, which suggests that it is the *in vitro* hydrolysis of hemp seed proteins that led to production of bioactive peptides. Similar blood pressure reducing effects have been reported by other researchers using hydrolysates from tuna muscle [31], tuna frame [32] and oyster [33]. Results from current study have demonstrated that hemp seed protein hydrolysate can lower SBP in SHRs and could be used as an antihypertensive agent for the prevention or management of hypertension.

4.4 CONCLUSIONS

The HPH and <3 kDa peptide fractions produced from pepsin-pancreatin hydrolysis of hemp seed proteins possessed inhibitory activities towards ACE and renin under *in vitro* conditions. The high yield of HPH coupled with its hypotensive effects in SHRs indicates a potential for commercial utility, especially as an ingredient to formulate antihypertensive agents. Since HPH was more bioactive than the peptide fractions, there will also be no additional cost associated with peptide purification that is normally designed to enhance potency. The positive correlation between *in vitro* and *in vivo* data with respect to enzyme (ACE and renin) inhibition and hypotensive effect supports

efforts aimed at using the less expensive *in vitro* approach to predict the potential bioactive properties of food protein-derived peptides.

Acknowledgements

This work was funded through a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to Dr R.E. Aluko.

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TRANSITION STATEMENT 2

The first two studies established that hemp seed protein hydrolysate (HPH) and ultrafiltration membrane fractions possessed multifunctional potentials as antioxidant and antihypertensive agents, which were revealed from the bioassay guided separation as well as*in vitro* and *in vivo* data. The next step was to further fractionate the HPH using Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) and test for activity enhancement, since it turned out to have the overall most potent antioxidant and antihypertensive properties compared to its ultrafiltration fractions. The third manuscript addressed the third objective of this thesis which was essentially to determine whether separation of HPH using RP-HPLC into several peptide fractions would improve the antioxidant capacity of hemp seed peptide fractions and link the observed functionality to their amino acid profile. It was evident from this study that the RP-HPLC HPH peptide fractions showed superior antioxidant potentials when compared to the unfractionated HPH, which indicates that separation of peptides by HPLC enhances bioactivity. Seven antioxidant evaluation systems were used and all showed significant activities of the RP-HPLC peptide fractions as potential antioxidants, however, the yield of the fractions significantly diminished when compared to the original HPH. This study also opened up an avenue to further purify the hemp seed peptide fractions and identify the sequences that may be responsible for the observed potent antioxidant properties. Thus these peptides could serve as ingredients for formulation of functional foods that would reduce excessive production of ROS/free radicals as a means of attenuating progression of oxidative stress and related metabolic disorders.

CHAPTER 5

MANUSCRIPT 3

REVERSE-PHASE HPLC SEPARATION OF HEMP SEED (*CANNABIS SATIVA* L.) PROTEIN HYDROLYSATE PRODUCED PEPTIDE FRACTIONS WITH ENHANCED ANTIOXIDANT CAPACITY

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5.0 ABSTRACT

Hemp seed protein hydrolysate (HPH) was produced through simulated gastrointestinal tract (GIT) digestion of hemp seed protein isolate followed by partial purification and separation into eight peptide fractions by reverse-phase (RP)-HPLC. The peptide fractions exhibited higher oxygen radical absorbance capacity as well as scavenging of 2,2-diphenyl-1-picrylhydrazyl, superoxide and hydroxyl radicals when compared to HPH. Radical scavenging activities of the fractionated peptides increased as content of hydrophobic amino acids or elution time was increased, with the exception of hydroxyl radical scavenging that showed decreased trend. Glutathione (GSH), HPH and the RP-HPLC peptide fractions possessed low ferric ion reducing ability but all had strong (>60%) metal chelating activities. Inhibition of linoleic acid oxidation by some of the HPH peptide fractions was higher at 1 mg/ml when compared to that observed at 0.1 mg/ml peptide concentration. Peptide separation resulted in higher concentration of some hydrophobic amino acids (especially proline, leucine and isoleucine) in the fractions (mainly F5 and F8) when compared to HPH. The elution time-dependent increased concentrations of the hydrophobic amino acids coupled with decreased levels of positively charged amino acids may have been responsible for the significantly higher (p<0.05) antioxidant properties observed for some of the peptide fractions when compared to the unfractionated HPH. In conclusion, the antioxidant activity of HPH after simulated GIT digestion is mainly influenced by the amino acid composition of some of its peptides.

KEYWORDS: RP-HPLC; Hemp seed; Amino acid composition; Protein hydrolysate; Antioxidant properties

Abbreviations:

AAA – Aromatic amino acids; AAPH – 2,2'- azobis (2-amidinopropane) dihydrochloride; DPPH – 2,2-diphenyl-1-picrylhydrazyl; DRSA – DPPH radical scavenging activity; FRAP – Ferric reducing antioxidant power; GSH – Glutathione; HAA – Hydrophobic amino acid; HPH – Hemp seed protein hydrolysate; HRSA – Hydroxyl radical scavenging activity; MCA – Metal chelation activity; NCAA – Negatively charged amino acid; NO – Nitric oxide; $O_2^{\cdot -}$ - Superoxide radical anion; ORAC – Oxygen radical absorbance capacity; PCAA – Positively charged amino acid; ROS – Reactive oxygen species; RP-HPLC – Reverse-Phase High performance liquid chromatography; SRSA – Superoxide radical scavenging activity

5.1 INTRODUCTION

Endogenous and exogenous reactive oxygen species (ROS) and free radicals are implicated in the etiology and progression of human degenerative diseases. The body possesses natural mechanisms to neutralize the damaging effects of ROS via the use of endogenous antioxidants. However, excessive production of ROS can overwhelm the protective ability of endogenous antioxidants, which can lead to development of oxidative stress [1]. The progression of multifaceted metabolic disorders is linked to increased oxidative stress that can be alleviated by the regular consumption of functional foods and nutraceuticals containing bioactive peptides with antioxidant properties. Consumers are increasingly demanding for natural antioxidants especially from plant sources because of their relative safety when compared to synthetic antioxidants that could have potential adverse effects on human health [2-4]. Therefore, it is important to investigate the antioxidant health effects of hemp seed proteins and its associated

products (e.g. protein hydrolysates) in order to expand their industrial utilization as a protein ingredient for both the local and international markets. Moreover, it is envisaged that conversion of the protein by-product into bioactive peptides could improve the negative image associated with hemp seed plant due to the presence of tetrahydrocannabinol (a psychoactive compound). The antioxidant properties of protein hydrolysates or their purified fractions largely depend on protease specificity, degree of hydrolysis and the nature of released peptides (e.g., molecular weight and amino acid composition). Despite the growing research on the antioxidant properties of plant-based food proteins, there is the need to provide additional information on antioxidant properties of hemp seed peptide fractions that differ in amino acid composition.

Reverse-phase HPLC (RP-HPLC) separation of peptides is based mostly on hydrophobic character; fractions collected at different time periods during elution will differ in type and content of amino acids. Therefore, the objectives of this study were to evaluate the antioxidant potentials of hemp seed protein hydrolysate (HPH) obtained from simulated human gastrointestinal tract (GIT) digestion which were separated into several peptide fractions using RP-HPLC. The measured antioxidant activities were then related to peptide amino acid profiles. Glutathione (GSH), a physiologically relevant antioxidant peptide was used as a control.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Defatted hemp seed meal (Hempseed protein powder, HPP) was obtained from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada). Hemp seed protein isolate (HPI) was obtained from HPP following the method described by (Tang, Wang & Yang,

2009) and was subsequently hydrolyzed to produce HPH as reported by Girgih et al. [6]. Briefly, 5 % (w/v, protein basis) aqueous HPI was digested with pepsin (4 % w/v, protein basis) at pH 2.0 and 37 ^{o}C for 2 h, which was then followed by pancreatin (4 % w/v, protein basis) digestion at pH 7.5 and 37 ^{o}C for 4 h. The reaction was terminated by adjusting the mixture to pH 4.0 followed by heating to 95 ^{o}C for 15 min. The mixture was centrifuged (7,000 x *g* at 4 ^{o}C) for 30 min and the resulting supernatant freeze dried to obtain HPH. The hydrolysis was carried out in triplicates and each of the hydrolysis products was kept as replicates for later analysis.

5.2.1.1 RP-HPLC Separation of HPH

Separation of HPH using preparative RP-HPLC was performed on a Varian 940-LC system according to the method reported by (Pownall, Udenigwe & Aluko, 2010) [7]. Briefly, freeze-dried HPH was dissolved in double-distilled water that contained 0.1 % trifluoroacetic acid (TFA) as solvent A at a concentration of 100 mg/ml, and a volume of 4 ml (sequentially filtered through 0.45 μm and 0.2 μm membrane discs) was injected onto 21 x 250 mm C12 preparative column (Phenomenex Inc., Torrance, CA, USA). Fractions were eluted from the column at a flow rate of 10 ml/min using a linear gradient of 0-100 % solvent B (methanol containing 0.1 % TFA) over 60 min. Elution of peptide fractions was monitored at absorbance of 220 nm. Fractions were collected using an automated fraction collector every 1 min and pooled into eight fractions according to elution time as shown in Fig.5.1. The pooled fractions were freeze-dried (after solvent evaporation under vacuum using the rotary evaporator, maintained at a temperature range of between 35-45 ^{o}C) and stored at -20 ^{o}C until further use.

5.2.1.2 Amino Acid Composition Analysis

Sample replicates were mixed together and amino acid profiles determined using the HPLC system after samples were digested with 6 M HCl [8]. The cysteine and methionine contents were determined after performic acid oxidation [9]and the tryptophan content was determined after alkaline hydrolysis [10].

5.2.1.3 Determination of Oxygen Radical Absorbance Capacity (ORAC)

ORAC values of the hempseed peptide samples were determined following a method reported by You et al. [11] with some modifications. The peptide sample or GSH were each dissolved in phosphate buffer (75 mM, pH 7.4) and then 20 µl mixed (1 mg/ml final concentration) with 120 µl of 300 nM fluorescein in a 96-well microplate followed by incubation at 37 ^{o}C for 15 min. Thereafter, a 50-µl aliquot of 80 mM AAPH solution was added to the mixture and the change in fluorescence was measured at 1 min intervals for 90 minat excitation and emission wavelengths of 485 mM and 528 mM, respectively. Trolox concentrations of 5-80 µM were used to prepare a standard curve; ORAC values were calculated as previously reported [11] and expressed as µM Trolox equivalent per gram of sample.

5.2.1.4 DPPH Radical Scavenging Activity (DRSA)

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging was determined using a previously described method [12] with slight modifications for a 96-well clear flat-bottom plate. An aliquot (100 µl) of peptide samples (in 0.1 M phosphate buffer, pH 7.0, containing 1 %, w/v Triton X-100) was mixed with 100 µl of 100 µM methanolic DPPH solution (final peptide concentration of 1 mg/ml) and incubated at room temperature in

the dark for 30 min. Absorbance of the blank (Ab) and samples (As) was measured at 517 nm. DRSA of the samples was calculated as [(Ab – As)/Ab]×100.

5.2.1.5 Metal (Iron) Chelating Activity (MCA)

The MCA was measured using a modified method [13]. A 1 ml aliquot of peptide sample or GSH at final assay concentration of 1 mg/ml was combined with 0.05 ml of 2 mM $FeCl_2$ and 1.85 ml distilled water. An aliquot (0.1 ml) of 5 mM of ferrozine solution was then added and mixed thoroughly. The mixture was allowed to stand at room temperature for 10 min and 200 µl was transferred into a clear bottom 96-well plate. The blank sample was conducted by replacing peptide sample with 1 ml of distilled water. The absorbance values of both the blank (Ab) and samples (As) were measured at 562 nm using a spectrophotometer. The percentage metal chelating activity (%) was calculated as [(Ab – As)/Ab]×100.

5.2.1.6 Ferric Reducing Antioxidant Power (FRAP)

The FRAP of samples was measured according to a previously reported method [14] with some modifications. Peptide or GSH sample (250 µl) was dissolved in 0.2 M phosphate buffer (pH 6.6) and mixed with 250 µl of same buffer and 250 µl of 1 % potassium ferricyanide solution. The final peptide concentration in the assay mixture was 1 mg/ml and the mixture was heated at 50 ^{o}C and incubated for 20 min. After incubation, 250 µl of 10 % aqueous tricholoroacetic acid (TCA) was added. Thereafter, 250 µl of peptide/TCA mixture was combined with 50 µl of 0.1 % $FeCl_3$ and 200 µl of distilled water and allowed to stand at room temperature for 10 min. The solution was then centrifuged at 1,000 x *g* and 200 µl of the clear supernatant transferred to a clear bottom 96-well plate;

absorbance at 700 nm was determined using an assay mixture that omitted peptide or GSH as the blank.

5.2.1.7 Hydroxyl and Superoxide Radical Scavenging Activities

The hydroxyl and superoxide radical scavenging activities were determined following previously reported method of de Avelar et al. [15] and Li et al. [16], respectively. An aliquot of 50 µl and 80 µl of samples for hydroxyl and superoxide radical scavenging activity assays were respectively added directly to a 96-well plate equivalent to a final concentration of 1 mg/ml in the reaction mixture.

5.2.1.8 Inhibition of Linoleic Acid Oxidation

Linoleic acid oxidation was measured using the method of Li et al. [16]. The peptide sample or GSH (1.5 ml, final concentration of 1 mg/ml in the reaction mixture) was dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0) and the mixture added to 1 ml of 50 mM ethanolic linoleic acid. For the blank assay, 1.5 ml of buffer was added to the ethanolic linoleic acid solution. The mixtures were kept at 60 ^{o}C in the dark for 7 days. At every 24 h interval, 100 µl of the assay solution was withdrawn and mixed with 4.7 ml of 75 % aqueous ethanol, 0.1 ml of ammonium thiocyanate (30 %, w/v) and 0.1 ml of 0.02 M $FeCl_2$ dissolved in 1 M HCl. A 200 µl aliquot of the resultant mixture was added to a 96-well plate and the degree of color development was measured at 500 nm after 3 min incubation at room temperature.

5.2.1.9 Statistical Analysis

The results of the antioxidant assays were conducted on three separately tested samples and analyzed by one-way analysis of variance (ANOVA), assuming equal variances between samples. Data were reported as mean ± standard deviation. Statistical

significance of differences were evaluated by Duncan's multiple range test ($p<0.05$) using the Statistical Analysis Systems Software Version 9.2 (SAS, Cary, NC, USA).

5.3 RESULTS AND DISCUSSION

5.3.1 Amino Acid Composition of RP-HPLC Peptide Fractions

The HPH was fractionated using preparative RP-HPLC and pooled into eight peptide fractions (F1-F8) based on retention time (Fig. 5.1). RP-HPLC separated the HPH peptides based on their differences in hydrophobic characters with the more hydrophilic peptides eluting first while more hydrophobic peptides eluted at longer times. This elution behaviour is reflected as F1 with the least content of hydrophobic amino acids (HAA) and aromatic amino acids (AAA) but highest contents of positively charged (PCAA) and negatively charged (NCAA) amino acids (Table 5.1) was eluted first (imply it was the most hydrophilic fraction with a mixture of positive and negative charges and was more likely to solubilize in aqueous elution solvents). The highest contributors to the observed HAA in the fractionated hemp seed peptides were *Leu* and *Tyr* exhibiting a 3 and 4 fold increases in the F2 when compared to values of these amino acids obtained for HPH. *Trp* and *Phe* contents were increased 2 and 3 folds in F5 and F3, respectively, when compared to the level in HPH. The presence of high amounts of *Trp* and *Phe* in peptides has been suggested as a factor that can enhance metal chelating ability because of the several binding sites on the aromatic rings [7]. Fractionation increased the concentrations of positively charged amino acids (*His*, *Arg* and *Lys*) in F1 by 1.7, 1.9 and 2.2 fold, respectively when compared to HPH. *His* is a strong metal chelator due to the imidazole ring, which can enhance metal chelating activities of peptides [17]. The NCAA contents were pronounced in almost all the fractions with relatively high levels in F1

(42.9 %), F2 (42.8 %) and F4 (40.5 %), which provides a reservoir of electrons that can be donated to neutralize or reduce the toxic effects of ROS [18]. F1 had no measurable or detectable antioxidant effects, probably because of high salt content and is not reported in the following sections. The antioxidant methods used have physiological relevance (e.g., superoxide, hydroxyl, metal chelation, FRAP, linoleic acid oxidation) and all have been widely reported in literature, which allows for comparison with literature values.

Figure 5.1: RP-HPLC separation of hemp seed protein hydrolysate into peptide fractions.

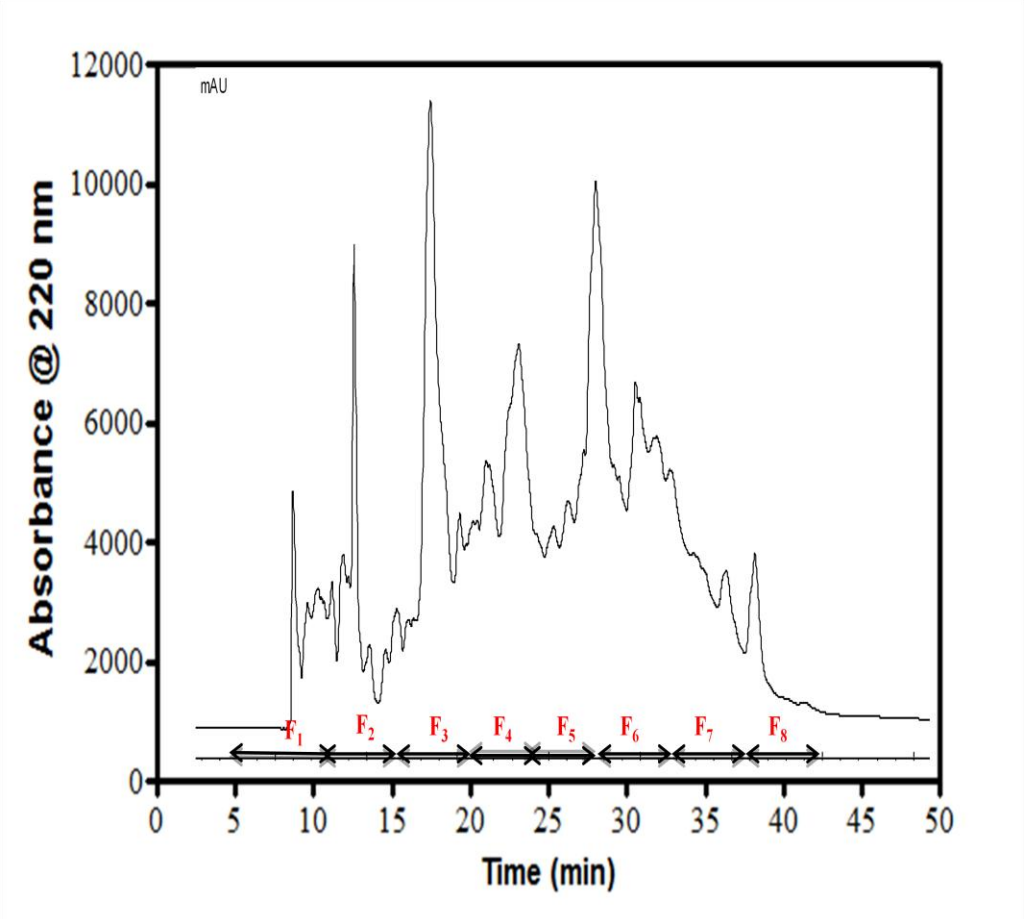


Table 5.1: Percentage amino acid composition of RP-HPLC purified HPH peptide fractions

AA	HPH	F1	F2	F3	F4	F5	F6	F7	F8
ASX	11.39	11.88	8.94	11.36	12.58	13.64	13.40	10.40	12.56
THR	3.68	3.52	4.20	3.46	2.94	2.65	3.82	5.76	4.06
SER	4.63	8.23	5.12	4.91	4.51	4.61	4.24	4.43	4.88
GLX	20.06	19.26	16.34	19.62	22.75	19.56	18.55	13.86	15.17
PRO	4.00	1.83	2.68	3.83	4.76	6.31	7.52	10.44	8.58
GLY	4.29	4.13	4.03	4.04	5.48	5.05	5.21	5.63	4.99
ALA	4.47	5.82	3.09	3.43	3.41	4.66	3.45	2.94	2.90
CYS	1.32	0.21	0.18	0.48	1.25	1.96	2.79	3.62	2.89
VAL	4.66	5.35	4.32	4.32	5.23	5.85	5.21	4.69	5.75
MET	1.81	0.87	1.14	1.26	2.00	2.91	2.26	1.55	1.96
ILE	3.84	0.49	6.15	3.49	3.77	4.71	6.70	7.44	6.60
LEU	6.75	0.61	20.48	5.33	4.83	4.65	6.31	10.81	8.73
TYR	3.45	0.81	13.46	1.80	1.50	1.63	1.13	1.84	2.21
PHE	4.60	0.00	0.11	15.26	6.24	2.97	3.68	4.95	4.49
HIS	2.78	4.76	1.78	2.77	2.40	2.65	2.89	2.91	2.49
LYS	2.97	6.42	1.38	1.93	2.90	2.18	1.88	1.20	1.76
ARG	14.07	26.61	6.92	12.44	12.72	12.05	9.40	5.71	7.87
TRP	1.23	0.01	0.08	0.31	0.87	2.82	1.73	1.89	2.19
HAA	26.85	15.18	38.04	22.14	25.25	31.05	34.24	41.49	37.41
AAA	9.28	0.82	13.65	17.37	8.61	7.42	6.54	8.68	8.89
PCAA	19.82	37.79	10.08	17.14	18.02	16.88	14.17	9.82	12.12
NCAA	39.76	42.89	34.60	39.35	42.78	40.46	40.01	34.45	36.67

HPH AA profile data derived from (Girgih, Udenigwe & Aluko, 2011b).

ASX= *Asp* + *Asn*; GLX = *Glu* + *Gln*. Aliphatic hydrophobic amino acids (HAA) = *Ala, Val, Ileu, Leu, Pro, Met, Cys*; Positively charged amino acids (PCAA) = *Arg, His, Lys*; Negatively charged amino acids (NCAA) = *Asx, Glx, Ser, Thr*; Aromatic amino acids (AAA) = *Phe, Trp, Tyr*.

5.3.2 Oxygen Radical Absorbance Capacity (ORAC) of the Hempseed Peptide Fractions

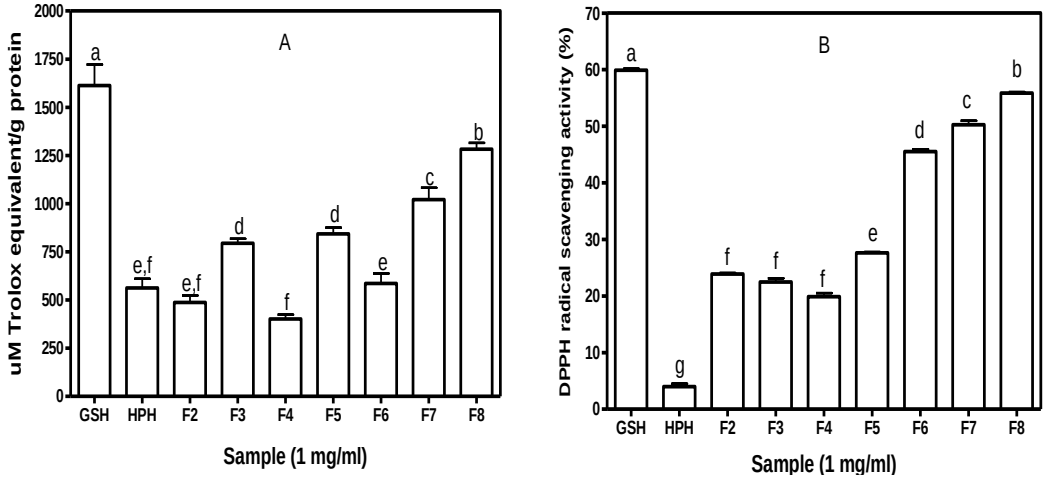
High antioxidant content in foods can be of benefit to human health because they can potentially reduce oxidative stress along with associated cellular damages and degenerative diseases [18]. Fig. 5.2A shows the ORAC values of HPH and its RP-HPLC fractions compared to the standard antioxidant peptide (GSH). ORAC values of test samples and control varied from 402 to 1613 µM TE/g. Among the peptide fractions, F8had a significantly higher (p<0.05) ORAC value (1282µM TE/g) and F4 the lowest (402 µM TE/g) but GSH showed the highest ORAC value of 1613 µM TE/g. The ORAC values of some of the RP-HPLC separated fractions (F3, F5, F7 and F8) were significantly higher (p<0.05) than the value obtained for HPH (Fig. 2A), which suggests that the column fractionation enhanced concentration of potent antioxidant peptides in certain fractions. The ORAC values reported in this work are greater than the maximum of ~81 µM TE/g that was reported for soybean protein hydrolysates [19].

5.3.3 DPPH Radical Scavenging Activity (DRSA)

Fig. 5.2B shows that partial purification of HPH using RP-HPLC led to enhanced DRSA of the fractionated peptides. DRSA of the RP-HPLC fractions ranged from approximately 24 to 56 % while HPH had only 4 %, all of which were significantly lower than that of GSH (60 %). Fraction F8 had the highest (56 %) DRSA among the HPH fractions, but its activity was significantly lower than that of chickpea protein hydrolysate fraction (86 %) obtained by gel filtration on Sephadex G-25 [16] and a peptide fraction isolated from wheat gluten hydrolysate (80-95 %) [20]. The observed differences in the DRSA could be attributed to the differences inthe structural level of protein sources that

were used for peptide production. Additionally, these differences could have arisen from methods used to extract or separate the hydrolysates. As shown in Fig. 5.2B, the fractions, F6-F8 had the strongest DRSA when compared to fractions F2-F5, which may be related to their amino acid profiles. In general, F5-F8 had higher contents of HAA and reduced content of PCAA when compared to HPH and other fractions.

Figure 5.2 (A) Oxygen Radical Absorbance Capacity (ORAC) values and (B) DPPH radical scavenging activity (%) of hemp seed protein hydrolysate (HPH) and RP-HPLC separated HPH fractions compared to reduced glutathione (GSH). Bars with different alphabets are significantly different at $p<0.05$. Values are means (n=3) ± SD.



5.3.4 Superoxide Radical Scavenging Activity (SRSA)

Partial purification of HPH by RP-HPLC led to enhanced SRSA of peptides as shown in Fig. 5.3A. The strongest (56 %) SRSA was displayed by GSH, followed by most hydrophilic F2 (41 %) whereas F7, F8 and HPH did not have any measurable

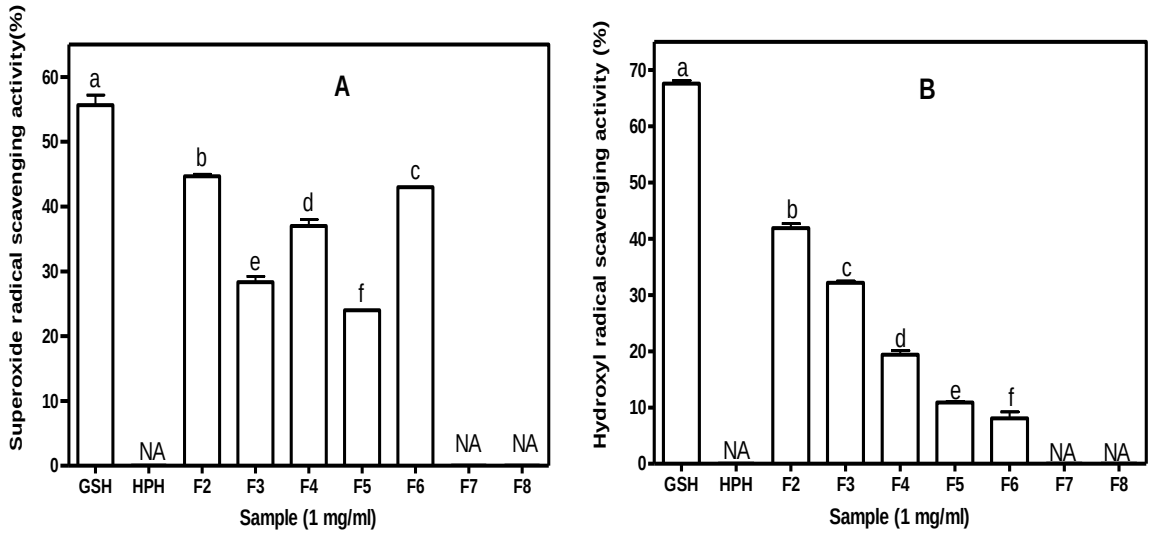
ability. Similar results of SRSA were reported for pea protein hydrolysate (PPH) and its RP-HPLC fractions [7]. The most hydrophobic (F7-F8) fractions showed no detectable activity against SRSA when compared to the most hydrophilic (F2-F6) fractions, which exhibited moderate (28-41 %) SRSA activity. In contrast, previous studies suggested that SRSA was positively related to the content of hydrophobic amino acids present in the fractions [7, 16]. However, a chemometric study has recently suggested that the contents of *Lys*, *Leu* and *Pro* may enhance superoxide scavenging activity of peptides [21], which could have partly contributed to the higher SRSA observed in F2-F6. But the relative high levels of *Lys*, *Leu* and *Pro* in F7 and F8 (lower SRSA) suggest that additional factors contributed to observed SRSA values of the peptide fractions (e.g. high *Cys* or low *Arg*).

5.3 5 Hydroxyl Radical Scavenging Activity (HRSA)

Fig. 5.3B shows that GSH had the significantly highest (68 %) HRSA when compared to the RP-HPLC separated fractions. The HRSA of the RP-HPLC purified fractions ranged from 0-42 % and significantly decreased ($p<0.05$) with increase in elution time of the fractions. The HPH and the most hydrophobic peptide fractions (F7 and F8) did not show any noticeable HRSA. HRSA have been determined for purified peptides from other plant-based sources. For example, at a peptide concentration of 2.0 mg/ml a purified chickpea peptide fraction exhibited over 81 % HRSA [16], which is similar to the values reported for alfalfa leaf protein hydrolysate peptides at 1.2 mg/ml [13]. The HRSA values obtained in the current study are lower than results reported for both chickpea and alfalfa peptides but higher than the values reported for purified peptides isolated from Alaska pollack frame protein hydrolysate (35 %) that was

evaluated by electron spin resonance spectroscopy [22]. The differences in HRSA observed for chickpea, alfalfa and hemp seed HPLC fractions could be due to varying amino acid composition obtained from each protein source.

Figure 5.3 Scavenging activities (%) of hemp seed protein hydrolysate (HPH) and their RP-HPLC separated fractions against (A) Superoxide radical and (B) hydroxyl radical in comparison to reduced glutathione (GSH). Bars with different letters are significantly different at $p<0.05$. NA = no activity. Values are means (n=3) ± SD.



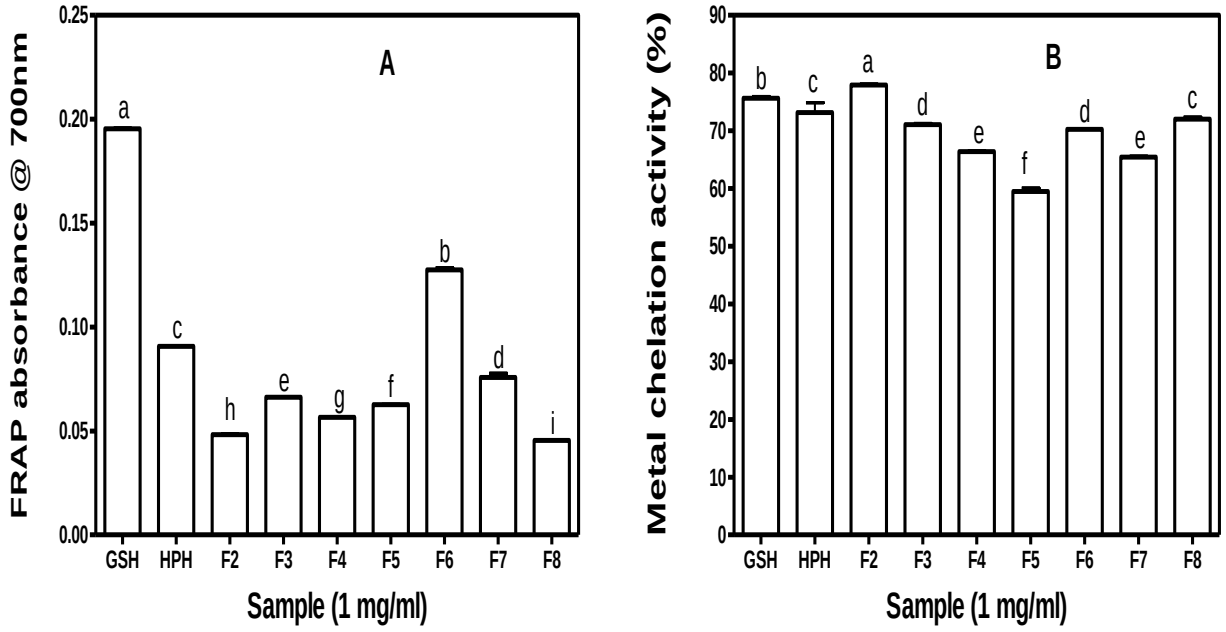
5.3.6 Ferric Reducing Antioxidant Power (FRAP) and Metal (Iron) Chelating Activity (MCA)

Among the purified fractions, F6 had the highest FRAP (13.85 %) while F8 had the least (Fig. 5.4A). All peptide fractions had significantly lower ($p<0.05$) absorption values when compared to that of GSH. Overall, there was no noticeable relationship between FRAP of peptide fractions and elution time, which is in contrast to data previously reported for pea protein peptides, which employed same separation procedure and

equipment (RP-HPLC) [7]. Thus the amino acid compositions of antioxidant peptides in the hemp seed fractions are probably different from those of pea peptides.

Fig. 5.4B shows that all the hempseed peptide fractions exhibited high (59-78 %) MCA with F2 having higher activity (78 %) than GSH (75 %). The MCAs are higher than the reported 4.5-9.0% for pea peptides [7]. With the exception of F2 and F8 fractions, RP-HPLC fractionation led toreduced MCA of peptides when compared to HPH, which agrees with a previous report for pea protein hydrolysate and peptide fractions. These results indicated that HPH fractionation did not substantially improve MCA of the peptide fractions.

Figure 5.4: (A) Ferric reducing antioxidant power and (B) Metal chelating effects of hemp seed protein hydrolysate (HPH) and its RP-HPLC separated fractions in comparison to reduced glutathione (GSH). Bars with different letters are significantly different at $p<0.05$. NA = no activity. Values are means (n=3) ± SD.



5.3.7 Inhibition of Linoleic Acid Oxidation by Hemp Seed Peptides

As shown in Fig. 5.5A-C, the blank reaction had increased absorbance intensity with incubation time and reached a peak on the 4th day followed by a subsequent decline. The decline in absorbance values after the 4th day could be attributed to the decomposition of peroxides and depletion in the amount of linoleic acid available in the system [23].

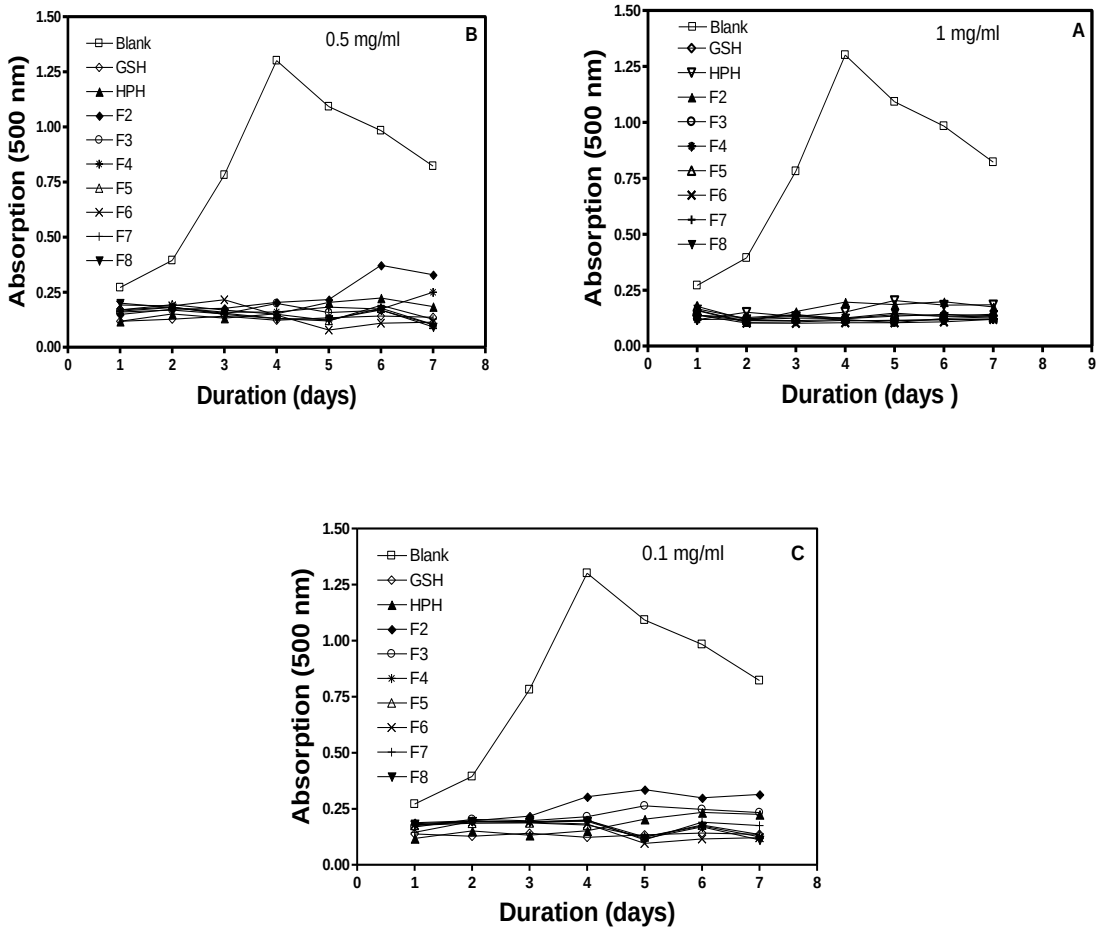
At 1 mg/ml, all the peptide fractions and GSH exhibited the most potent inhibitory activities against linoleic acid oxidation as indicated by their ability to maintain inhibition throughout the 7-day incubation period (Fig. 5.5A). At a lower concentration (0.5 mg/ml), the samples induced strong inhibition of linoleic acid oxidation except for F2, which lost its activity after the 5th day (Fig. 5.5B). At 0.1 mg/ml, some of the peptide samples (F2 and F3) showed similar inhibitory trend but lower activity in protecting against linoleic acid oxidation (Fig. 5.5C).

5.4 CONCLUSIONS

Partially purified HPH peptides obtained using RP-HPLC fractionation demonstrated strong antioxidant properties by scavenging ROS/free radicals, reducing ferric ions, chelating metals and inhibiting linoleic acid oxidation. In this study, although separation of HPH by RP-HPLC improved some of its antioxidant activities (DPPH, hydroxyl and superoxide radical scavenging activities), the crude HPH and the peptide fractions significantly showed strong metal reducing and chelating activities as well an excellent ability to inhibit linoleic acid oxidation. Most of the antioxidant activities were dependent on peptide elution time and/or the level of specific amino acids especially *HAA*, *Lys*, *Leu* and *Pro*. It can thus be concluded that it is a well-recognized fact that the antioxidant activities of peptides are associated with their amino acid composition, or

their hydrophobicity as has been confirmed in this study. Some of the peptides in certain fractions (e.g. F2 and F6) could have an antioxidant activity in the GIT or in the blood stream system if they are able to cross the epithelial cell without being digested by the endopeptidases. F2 and F6 seem to contain the most active peptides because of the relatively high superoxide (F2 and F6), hydroxyl (F2), FRAP (F6), and metal chelation (F2 and F6) antioxidant properties.

Figure 5.5: Time-dependent inhibition of linoleic acid oxidation by HPH and its RP-HPLC separated HPH fractions at different concentrations: A, 1 mg/ml; B, 0.5 mg/ml and C, 0.1 mg/ml in comparison to glutathione (GSH).



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TRANSITION STATEMENT 3

This study was designed based on a challenge from the oil industry that produces hemp seed oil resulting in hemp seed protein meal (HPM) as a by product. The question was whether the direct digestion of HPM with gastrointestinal enzymes without alkaline isolation of the hemp seed proteins would produce peptides with comparable biological effects *in vitro* and *in vivo* as the HPH obtained from the enzymatic hydrolysis of hemp seed protein isolate (HPI). To provide answers to these questions, HPM (residue left behind after pressing out the hemp seed oil) was directly digested with pepsin and pancreatin. The resulting hemp seed meal protein hydrolysate (HMH) was used in animal study experiments to determine the long-term effect of HMH in the attenuation of hypertension in growing SHRs during 8-week feeding period. This was followed by a 4-week secondary study (treatment phase) in SHRs with established hypertension and used normotensive rats for comparison. In both the preventive and treatment phases, the HMH-containing diets showed significant hypotensive effects by lowering SBP and these hydrolysates could be utilized in the development of functional foods and nutraceuticals for prevention of hypertension. The hypotensive effects exhibited by HMH in SHRs were thus comparable to those of HPH, implying there may be no need to go through protein isolation step in the preparation of peptide hydrolysate to enhance bioactivity which will reduce cost of production of these peptides as they will be obtained directly from the meal. The low ACE and renin activities in the plasma of the rats confirmed the *in vitro* inhibitory effects and *in vivo* hypotensive properties of HMH peptides as potential potent antihypertensive agents.

CHAPTER 6

MANUSCRIPT 4

PREVENTIVE AND TREATMENT EFFECTS OF HEMP SEED (*CANNABIS SATIVA* L.) MEAL PROTEIN HYDROLYSATE AGAINST HIGH BLOOD PRESSURE IN SPONTANEOUSLY HYPERTENSIVE RATS

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6.0 Abstract

Purpose This work determined the ability of hemp seed meal protein hydrolysate (HMH)-containing diets to attenuate high blood pressure development in spontaneously hypertensive rats (SHRs). Effects of diets on plasma levels of renin and angiotensin converting enzyme (ACE) in the SHRswere also determined.

Methods Defatted hemp seed protein mealwas hydrolyzed using simulated gastrointestinal tract digestion with pepsin followed by pancreatin and the resulting HMH used as a source of antihypertensive peptides. The HMH was substituted for casein at 0.5 and 1.0% levels and fed to young growing rats for 8 weeks (preventive phase) or adult rats for 4 weeks (treatment phase).

Results Feeding of young growing SHRs with HMH resulted in attenuation of the normal increases in systolic blood pressure (SBP) with an average value of ~120 mmHg when compared to the casein only group of rats (control) with ~158 mm Hg ($p<0.05$). Feeding adult rats (SBP ~145 mmHg) with same diets during a 4-week period led to significant ($p<0.05$) reduction in SBP to ~119 mmHg in comparison to 150 mmHg for the control rats. Plasma ACE activity was significantly ($p<0.05$) suppressed (0.047-0.059U/mL) in rats fed HMH-containing diets when compared to control rats (0.123 U/mL). Plasma renin levelwas also decreased for HMH-fed rats (0.040-0.054 µg/mL) when compared to control rats that were fed only casein (0.151 µg/mL).

Conclusions The results suggest that HMH with strong hypotensive effects in SHRs could be used as a therapeutic agent for both the prevention and treatment of hypertension.

Keywords: Hemp seed meal; Proteinhydrolysate; Spontaneously hypertensive rats; Systolic blood pressure; Plasma ACE activity; Plasma renin activity

6.1 Introduction

Hypertension or high blood pressure defined as systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg forms an important risk factor for the development of cardiovascular diseases [1, 2]. Hypertension is a major public health problem and its global prevalence is increasing at an alarming rate and affecting over 20% of the adult population [3]. Worldwide prevalence is estimated to affect as much as one billion individuals with approximately 7.1 million associated deaths per year [4]. Blood pressure (BP) is regulated by several mechanisms but the most significant and widely studied is the renin–angiotensin–aldosterone system (RAAS). In the RAAS, kidney-secreted renin cleaves angiotensinogen to produce an inactive decapeptide called angiotensin 1 (AT-I). AT-I is then hydrolyzed by angiotensin converting enzyme (ACE) to produce a potent vasoconstrictor octapeptide called angiotensin II (AT-II). ACE also breaks down bradykinin (a vasodilator) to produce inactive fragments leading to increases in arterial blood pressure [5]. Independent of ACE, chymase is an enzyme that also converts AT-I to AT-II, and these combined enzyme actions ultimately are responsible for maintaining normal BP. However, excessive activities of these enzymes could lead to BP elevation that leads to hypertension. Left untreated, hypertension could progress into cardiovascular complications that sometimes result in death. Bradykinin achieves its vasodilation properties by binding to the β-receptor with an eventual increase in Ca^{2+} levels. The binding of bradykinin to β-receptors and the increase in Ca^{2+} level stimulates nitric oxide synthase (NOS) to convert L-arginine to nitric oxide (NO), another

potent vasodilator. Therefore, the hydrolytic action of ACE on bradykinin to produce inactive fragments indirectly inhibits the production of NO.

High BP is routinely treated using a combined therapy of antihypertensive drugs such as captopril, caporal, lisinopril, enalapril, acacelpril, etc [6]. However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, skin rashes or angioneurotic edema which limits their use by some patients such as pregnant women and the elderly who are easily susceptible to health complications. While there are many commercially available synthetic ACE inhibitors, only one known commercial renin inhibitor (Aliskiren) is available for human therapy [7]. Therefore, it has been suggested that research and development to find safer, innovative, and economical ACE and renin inhibitors from food-based sources is necessary for expanding hypertension treatment and prevention strategies [6]. Research has shown that some food proteins possess the ability to release ACE and renin inhibitory peptides after enzymatic hydrolysis or may possess both abilities i.e. they could exhibit multifunctional properties [8]. Such peptides may serve as ingredients for functional foods or nutraceuticals and could be used as alternative or complementary treatment tools for reducing high BP. Therefore, bioactive antihypertensive peptides of food origin are increasingly gaining recognition as alternatives or compliments to synthetic drugs in hypertension therapy. Preliminary *in vitro* studies have shown that industrial hemp seed peptides possess both antioxidant [9-11] and antihypertensive properties [12]. The antioxidant and antihypertensive activities may be due, respectively, to the presence of high levels of negatively charged amino acids for electron donation to reactive oxygen species and arginine for the production of NO, a vasodilating agent. Short-term (24 hr) oral

administration (200 mg/kg body weight) of hemp seed protein hydrolysate (HPH) to spontaneously hypertensive rats (SHRs) was shown to reduce SBP (-30 mmHg after 8 h) and was positively correlated with the *in vitro* ACE and renin inhibitions [12]. Having previously established the ability of a hemp seed hydrolysate to reduce SBP on a short-term basis, the primary objective of this study was to evaluate the ability of a hemp seed hydrolysate to attenuate hypertension (prevention) in growing SHRs during an 8-week feeding experiment. We also determined the BP-lowering effect (treatment) of the hydrolysate in SHRs with established hypertension during a 4-week secondary study while normotensive rats were used for comparison.

6.2. Materials and methods

6.2.1 Materials

Defatted coarse hemp seed protein meal (HPM, 25% protein content) was a gift from Hemp Oil Canada (St. Agathe, Manitoba, Canada). Briefly, the hemp seed is mechanically pressed to extract oil and the resulting product is the defatted hemp seed cake, which is then milled in a Classifier Milling System to the desired particle size. The milled powder is sifted using various screens to obtain products sold as high-value protein powders. The by-product that does not pass through the sieves is the coarse hemp seedmeal, which is normally considered a waste product. Renin enzyme and renin substrate were purchased from Cayman (Cayman Chemical, Ann Arbor, MI). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), captopril, and rabbit lung ACE (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO).

6.2.2 Methods

6.2.2.1 Preparation of hemp seed protein isolate (HPI) and hemp seed meal hydrolysate (HMH)

HPI was prepared according to a previously described protocol [9]. Briefly, the HPM was extracted for 2 h at 37^{o}C with alkaline water (pH 10) followed by centrifugation (7,000*g* for 1 h at 4^{o}C). The supernatant was adjusted to pH 5.0 with 2 M HCl, centrifuged and the precipitate neutralized to pH 7.0 with 2 M NaOH followed by freeze-drying to produce the HPI. The HPI powder was decolorized as previously described using acetone to remove residual fat and phenolics [9]. To prepare HMH, the HPM was directly hydrolyzed sequentially, first with 4% (w/w, HPM protein basis) pepsin (pH 2.0, 37^{o}C, and 2 h) and second with pancreatin (pH 7.5, 37^{o}C, and 4 h) to simulate gastrointestinal tract (GIT) digestion [13]. The digest was centrifuged and the precipitate discarded while the supernatant was lyophilized to produce HMH, which was stored at -20°C until needed for further analysis.

6.2.2.2 Proximate composition

Proximate composition of HPI and HMH were analyzed according to the appropriate standard methods [14]. Crude protein content was determined as nitrogen content multiplied by 6.25.

6.2.2.3 Amino acid composition analysis

The amino acid profiles of the samples were determined using an HPLC pico-tag method, after samples were hydrolyzed with 6 M HCl according to the method of Bidlingmeyer et al. [15]. The cysteine and methionine contents were determined after performic acid

oxidation [16] while the tryptophan content was determined after alkaline hydrolysis [17].

6.2.2.4 Experiment animals and feeding protocols

All rat experiments were performed according to protocols approved by the University of Manitoba Animal Care Protocol and Management Review Committee. The rat feeding experiments were carried out as follows using SHRs (hypertensive) or Wistar Kyoto (WKY) rats (normotensive) purchased from Charles River Laboratories (Montreal, PQ, Canada). In the first feeding experiment, 32 male SHRs (6 weeks old) were housed in the Animal Facility at the Richardson Centre for Functional Foods and Nutraceuticals under a 12 h day and night cycle at 22 °C. The rats were acclimatized by feeding *ad libitum* with a regular chow diet and tap water for two weeks. For feeding trial I, SHRs (now 8 weeks old) were randomly divided into 4 groups (similar average body weight and BP) of 8 rats each that received similar feed but with addition of hydrolyzed (HMH) and unhydrolyzed (HPI) hemp seed products to determine ability of each diet to attenuate high BP development during the rapid growth phase (preventive effect). Table 1 shows composition of the various diets. Baseline SBP was measured at the beginning of the feeding trial using the tail cuff method as previously described [12]. The rats were then fed their respective diets *ad libitum* for 8 weeks during which SBP, feed consumption and body weight were measured weekly. At the end of the 8 weeks of feeding, 4 rats from each group were terminated followed by harvesting and weighing of body organs while blood was collected into heparinised tubes. The blood was centrifuged at 1,500*g* for 10 min to obtain plasma, which was then stored at -80^{o}C until needed for further analysis. All the remaining SHRs were then switched to the regular chow diet for 4 weeks to serve

Table 6.1: Composition of experimental diets showing inclusion levels of HMH and HPI.

Ingredients (g)	Diet group			
	Control	0.5% HMH*	1% HMH*	1% HPI*
Cornstarch……	40.00	40.00	40.00	40.00
Casein……...	20.00	19.5	19.00	19.00
HMH……	0	0.50	1.00	0
HPI……	0	0	0	1.00
Maltodextrin…….	13.20	13.20	13.20	13.20
Sucrose……..	10.00	10.00	10.00	10.00
Soy oil +TBHQ……... .	7.00	7.00	7.00	7.00
Fiber……..	5.00	5.00	5.00	5.00
Min mix…….	3.50	3.50	3.50	3.50
Vit mix……...	1.00	1.00	1.00	1.00
L-cys…….....	0.30	0.30	0.30	0.30

HMH: Hemp seed meal hydrolysate; HPI: Hemp seed protein isolate

as a washout period and allow establishment of hypertension (SBP>140 mmHg). After the 4-week washout period, the rats (now 20 weeks old) were randomized (4 each) to the four diet groups described above to perform feeding trial II. Feeding (*ad libitum*) was conducted for 4 weeks with weekly SBP and body weight measurements to determine ability to reduce high BP in established hypertension (treatment effect). At the end of the 4-week feeding period, all rats were terminated for blood and organ collection as

described above. Feeding trial III involved the use of normotensive WKY rats (20 weeks old), which were randomly assigned to the following 3 protein treatment groups with 6 rats per group. Control diet (20%, w/w casein); 1% HMH diet (19% casein + 1% HMH) or 1% HPI diet (19% casein + 1% HPI). Only one HMH dose was used for trial III because from the initial SHR experiment, the 1% HMH was a more effective dose than the 0.5% HMH in lowering SBP. The normotensive rats (NTRs) were allowed *ad libitum* access to their respective group feeds for 4 weeks during which body weight and SBP were measured weekly. The NTRs were also terminated at the end of 4 weeks with blood and organs collected and processed as indicated for the SHRs.

6.2.2.5 Determination of *in vitro* ACE and renin inhibitory activities of HMH

The ability of HMH to inhibit *in vitro* ACE activity was determined by aspectrophotometric method using FAPGG as substrate [12], while renin inhibition was determined using the fluorescence spectrometry method [18].

6.2.2.6 Determination of plasma ACE activity

ACE activity in the plasma was measured according to the spectrophotometric method using FAPGG as substrate [12] with slight modifications. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 μL plasma or ACE (final enzyme concentrations were 0.0313, 0.0625, 0.125, 0.25, 0.5 U/mL), and 200 μL of 50 mM Tris-HCl buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at 23 ^{o}C, and the result was expressed as $\Delta A \cdot min^{-1}$, which was plotted against ACE enzyme concentration to obtain a standard curve.Plasma ACE activity (U/mL) was obtained by linear regression using the standard curve.

6.2.2.7 Determination of plasma renin concentration

The plasma renin activity (concentration) was measured using the fluorometric method as previously reported [18] but with slight modifications as follows. Prior to the assay, renin buffer was diluted with 50 mM Tris–HCl (pH 8.0) containing 100 mM NaCl. The renin protein stock solution was diluted to different concentrations (4.15, 8.3, 16.5, 33, 66, 132, and 250 μg/mL) with assay buffer. Before the reaction, 20 μL of renin substrate, and 160 μL assay buffer were added to the wells. The reaction was initiated by adding 10 μL plasma or each diluted renin solution to the wells, the microplate was shaken for 10 s to ensure proper mixing and then incubated at 37 ^{o}C for 15 min in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). The fluorescence intensity (FI) was then recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm, and the results expressed as $\Delta FI \cdot min^{-1}$. A stand curve was obtained by using linear regression from a plot of $\Delta FI \cdot min^{-1}$versus renin concentrations. The $\Delta FI \cdot min^{-1}$ obtained for each plasma was used to calculate plasma renin concentration (μg/mL) from the regression equation.

6.2.2.8 Statistical analysis

All *in vitro* data were collected in duplicate or triplicate while *in vivo* data were based on number of rats used for each experiment per group. Data were subjected to one way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p < 0.05$.

6.3. Results and Discussion

6.3.1 Results

6.3.1.1 Proximate and amino acid composition of HMH and HPI

Table 6.2 shows that the major differences in proximate composition were the higher protein content of HPI and higher ash (NaCl) content of HMH. Therefore, the diet protein was formulated by including HPI and HMH based on their respective protein contents such that total protein content (including casein) was 20% for all the diets. Similarly, since the HMH had higher salt content, NaCl was added to the control and HPI diets in amounts that ensured equal concentrations across the diets. Table 6.3 shows the amino acid profiles of hemp seed products (HMH & HPI) used in this work with the major differences being the contents of arginine and tryptophan. Overall, the HMH had substantially higher total contents of hydrophobic (HAA) and aromatic (AAA) amino acids when compared to HPI.

6.3.1.2 HMH inhibition of ACE and renin activities

Prior to using the HMH for in vivoexperiments, its inhibitory effects against ACE and renin enzymes were tested in vitro. Fig.6.1 shows that ACE was inhibited by 70% in contrast to 35%inhibition observed for renin at 1 mg/mL peptide concentration.

6.3.1.3 Antihypertensive effects of HMH

Fig. 2A shows that the normal SBP of growing rats (feeding trial I) was maintained or significantly ($p<0.05$) reduced by hemp seed protein (HPI) or protein hydrolysate (HMH) containing diets when compared to the casein-only diet during an 8-week feeding experiment. All the rats had similar SBP values one week after the feeding experiment

Table 6.2: Proximate compositions of hemp seed protein isolate (HPI) and hemp seed meal hydrolysate (HMH)

Parameter (%)	HPI	HMH
Moisture	4.67 ± 0.01	14.24 ± 0.23
Dry matter	95.33 ± 0.01	85.77 ± 0.23
Crude protein	95.22 ± 0.83	55.19 ± 0.10
Crude fibre	0.295 ± 0.23	0.42 ± 0.40
Fat	0.00 ± 0.00	4.03 ± 0.14
Ash	5.24 ± 0.33	30.31 ± 0.04
Sodium chloride	0.94 ±0.06	22.35 ± 0.43

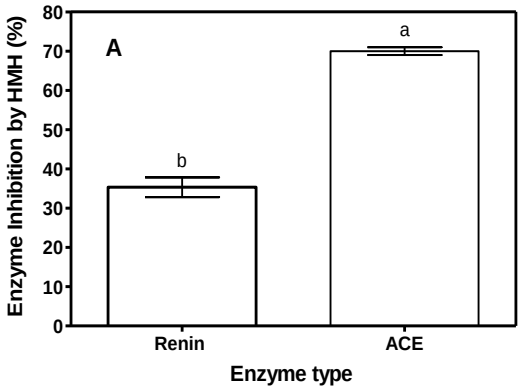
Results are presented as mean ± standard deviation of duplicate determinations.

Table 6.3: Amino acid composition of hemp seed meal hydrolysate (HMH) and hemp seed protein isolate (HPI)

Amino acid	HPI	HMH
ASX	11.81	10.79
THR	3.54	3.70
SER	4.78	5.43
GLX	22.39	18.12
PRO	4.13	4.72
GLY	4.38	4.50
ALA	4.14	3.95
CYS	1.49	1.20
VAL	4.14	5.17
MET	2.36	2.03
ILE	3.67	4.01
LEU	5.51	6.78
TYR	3.09	3.61
PHE	3.66	4.50
HIS	2.65	2.96
LYS	2.96	3.93
ARG	13.91	2.11
TRP	1.39	12.56
BCAA	13.32	15.96
AAA	8.14	20.67
HAA	37.96	53.03
PCAA	19.52	9.00
NCAA	42.52	38.04
SCAA	3.83	3.23

Branched chain amino acids-leucine, isoleucine and valine (BCAA); Aromatic amino acids-phenylalanine, tryptophan, and tyrosine (AAA); Combined total of hydrophobic amino acids-alanine, valine, isoleucine, tyrosine, phenylalanine, tryptophan, proline, methionine, and cysteine (HAA); Positively charged amino acids-arginine, histidine, lysine (PCAA); Negatively charged amino acids-ASX and GLX (NCAA); Sulphur containing amino acids- methionine and cysteine (SCAA).

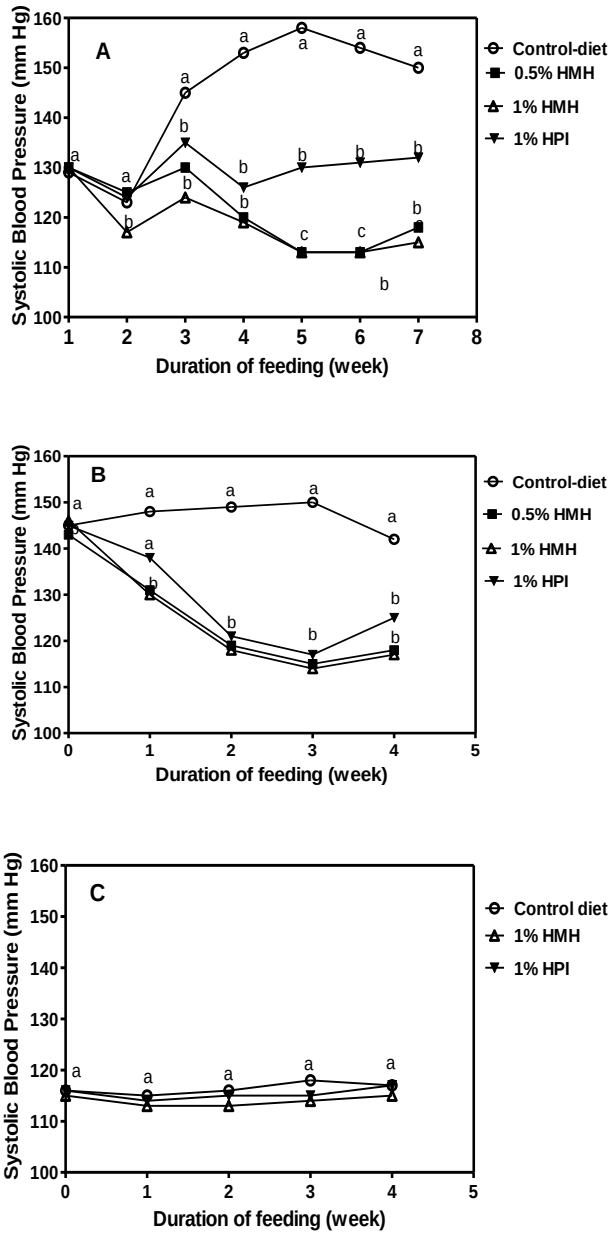
Figure 6.1: *In vitro* ACE- and renin-inhibitory activities of hemp seed meal hydrolysate (HMH) determined at 1 mg/mL peptide concentration. Bars with different letters have mean values that are significantly different ($p< 0.05$).



began. However, by the second week, the rats on diet containing 1% HMH had significantly ($p< 0.05$) reduced SBP when compared to the other diet groups. In the third week, SBP of the casein-only (control) group rose to 156 $\pm$ 1.25 mmHg and continued its increase up to a peak of 158$\pm$ 1.09 mmHg, which correspond to an 18% increase in SBP from week one. However, by the second week, the rats on diet containing 1% HMH had significantly ($p<0.05$) reduced SBP when compared to the other diet groups. The 1% HMH diet lowered SBP to 117 $\pm$2.11 mmHg, which corresponds to a 10% decrease (-17 mmHg) from week one. In weeks 3 and 4, there were no significant ($p>0.05$) differences in SBP-lowering effects of the HPI and HMH-containing diets. However, from week 5-8, the diets containing hydrolyzed hemp seed proteins (HMH) significantly ($p<0.05$) reduced SBP more than the unhydrolyzed hemp seed protein (HPI).

After a 4-week washout period, the remaining rats were then randomized to the four diets and their SBP monitored weekly for 4 weeks (feeding trial II). Fig. 2B shows

Figure 6.2: Effects of casein-only diet or casein diet that contained hemp seed products on the systolic blood pressure (SBP) of (A) young growing spontaneously hypertensive rats (SHRs) from feeding trial I, (B) adult SHRs with established hypertension from feeding trial II, and (C) normotensive rats from feeding trial III. At each time point, differences in letters indicate mean values are significantly different ($p < 0.05$).

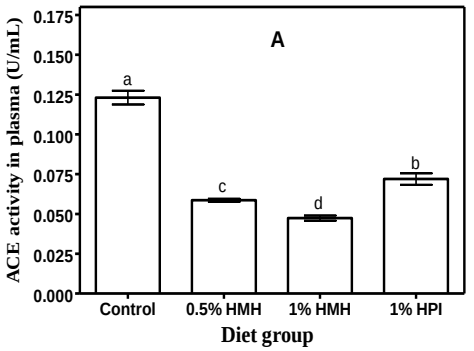


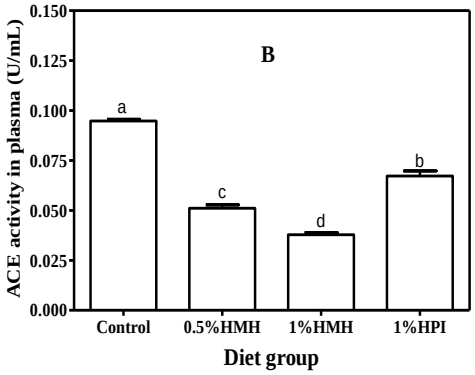
that the hemp seed-containing diets produced the lowest SBP (~119± 2.21 mmHg) by week 3, which is about -31 mmHg less than the value obtained for rats on the casein only diet (150 ±0.13 mmHg). The 1%HPI had similar reduction in SPB (-28 mmHg) as the HMH diets butonly after 2 weeks. Fig. 2C shows that all unlike the data obtained for SHRs, the diets had no significant (p>0.05) effecton SBP of NTRs over a 4-week period (feeding trial III).

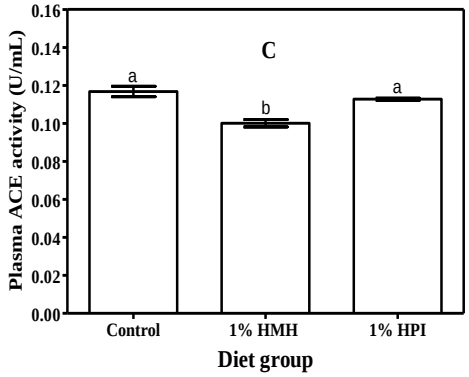
6.3.1.4 Plasma ACE activity in SHRs and NTRs

The plasma ACE activity of SHRs from feeding trials I-III is shown in Figure 3A-C, respectively. Plasma ACE activityin the SHRs was significantly (p<0.05) lower (0.047 and 0.059 U/mL, for 0.5 and 1%, respectively) after the 8-week HMH feeding trial when compared to HPI (0.072 U/mL) and the casein-only (0.123 U/mL) diets (Fig. 3A). In turn, the HPI-containing diet produced significantly (p<0.05) lower SBP and plasma ACE activity when compared to rats fed casein-only diet. After the 8-week study, the rats were placed on regular chow diet for 4 weeks (washout period) during which SBP increased to ~145 mmHg before the rats were then used for the 4-week feeding trial II. A similar pattern of reduced plasma ACE activity as obtained during 8-week feeding period (prevention) was observed after the subsequent 4-week feeding period, which served as an intervention (treatment) to reduce high blood pressure (Fig. 3B). The plasma ACE activityfor the feeding trial II in SHRs group fed 0.5 and 1% HMH were 0.038 and 0.051 U/mL, respectively while values were 0.067 and 0.095 U/mL, respectively for HPI and casein-only rats. The plasma ACE activity inNTRs as shown in Fig. 3C was similar in magnitude for all diet groups, however, value was slightly lower in the 1% HMH-fed rats (0.1 U/mL) in comparison to the 1% HPI (0.112 U/mL) or casein only rats (0.117 U/mL)

Figure 6.3: Effect of long-term feeding of (A) young growing spontaneously hypertensive rats (SHRs) (trial I), (B) adult SHRs with established hypertension (trial II) and (C) normotensive rats (trial III) with casein-only diet or casein diet that contained hemp seed products on plasma ACE activity. Bars with different letters have mean values that are significantly different ($p < 0.05$).Values are means (n=4) ± SD.







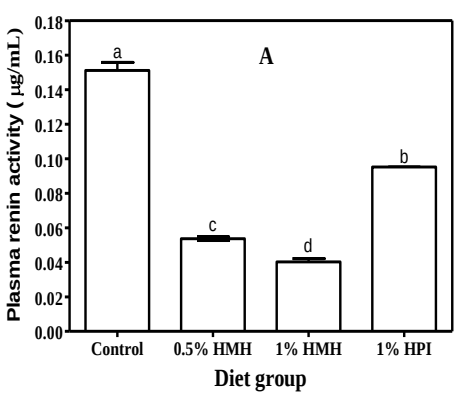
6.3.1.5 Plasma renin level in SHRs and NTRs

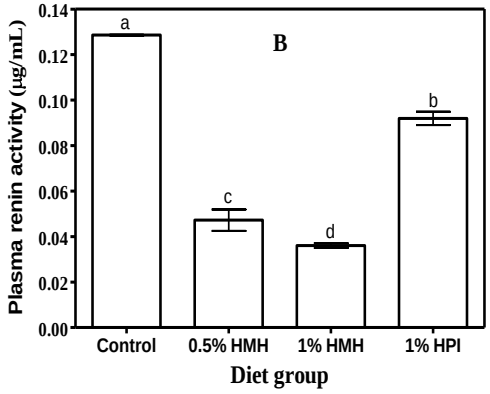
Fig. 6.4A-C shows that the renin level in the plasma of SHRs is directly related to theantihypertensive effects of the diets. In the growing SHRs from feeding trial I (Fig. 4A), 8 weeks of feeding with experimental diets resulted in significant (p<0.05) and dose-dependent suppression of plasma renin level in the HMH rat groups with values of 0.040 and 0.054 µg/mL, for 0.5 and 1% diets, respectively. In contrast, significantly (p< 0.05) higher plasma renin level was obtained for rats that consumed the 1% HPI (0.095 µg/mL) or casein-only (0.151 µg/mL) diets. Similar pattern of results was obtained in feeding trial II during the 4-week post-washout feeding period (Fig. 4B) that tested the treatment potential of hemp seed products. The NTRsfed 1%HMH and HPI (Fig. 6.4C) when compared to casein-control group of rats indicated that the HMH and HPI-treated rats had significantly (p<0.05) lower plasma renin levels (0.065-0.069 µg/mL) when compared to the casein-only rat group with a renin level of 0.079 µg/mL. The results also showed that plasma renin concentration in untreated SHRs (0.151 µg/mL) is significantly (p<0.05) higher than the values obtained for untreated NTRs (0.079 µg/mL), which suggest a direct relationship of renin level with BP. However, just as observed for plasma ACE levels, the changes in plasma renin level did not have any influence on SBP of NTRs

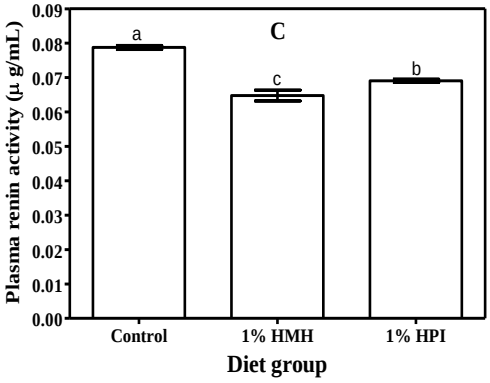
6.3.2 Discussion

The functionality and activity of any peptide under in vitro or in vivo conditions is dependent on the type of proteases and the extent to which the protein is hydrolyzed. Other factors which may influence peptide activity include amino acid composition/sequences and molecular weight [19]. Protein hydrolysates such as HMH

Figure 6.4: Effect of long-term feeding of (A) young growing spontaneously hypertensive rats (SHRs) (trial I), (B) adult SHRs with established hypertension (trial II) and (C) normotensive rats (trial III) with casein-only diet or casein diet that contained hemp seed products on plasma renin level. Bars with different letters have mean values that are significantly different ($p < 0.05$). Values are means (n=4) ± SD.







normally contain high sodium contents due to addition of alkali (NaOH) during digestion to neutralize liberated protons and maintain pH at optimally set value for protease activity. The amino acid composition data suggest that because pepsin and chymotrypsin preferentially hydrolyze peptide bonds that involve aromatic amino acids, most of the bonds hydrolyzed involved tryptophan, hence higher content of tryptophan in HMH. In contrast, it seems that most of the arginine-containing sequences were resistant to protease digestion, hence the low content of arginine in HMH when compared to HPI.High level of hydrophobicity as evident in the high amounts of AAA and HAA can enhance peptide uptake through the cellular lipid bilayer, which could have contributed to the observed enhanced bioactivity of HMH peptides. The arginine (13.91%) and sulfur-containing amino acid (3.85%) contents obtained for HPI in this work are higher than the values of 9.91% and 1.55%, respectively that were reported by Wang et al. [20]. The differences between amino acid composition of HPI used in this work and the HPI reported by Wang et al. [20] could be due to variations in the source of hemp seed meals used to prepare the protein isolate.

Food derived peptides that have shown in vitro ability to modulate the RAS by inhibiting ACE and renin enzymatic activities could be used in animal and subsequently in human clinical interventions experiments against hypertension and associated cardiovascular diseases [21].Therefore, initial screening of protein hydrolysates for their *in vitro* inhibition of enzyme activities could be used to select active samples that can be evaluated for in vivo effects. The greater level of ACE inhibition obtained for HMH agree with previous reportsthat have always shown that it is easier to inhibit *in vitro* ACE activity than renin activity [12, 19]. Previous works have also shown similar [22] or less

[23] values of ACE inhibition by food protein hydrolysates at 1 mg/mL concentration. Reports of renin inhibition by food protein hydrolysates are not very common but a papain protein hydrolysate from macroalgae was shown to have ~42% inhibitory activity [24], which is higher than the ~35% inhibition obtained in this work. Rapeseed protein hydrolysates produced using alcalase, proteinase K, pepsin + pancreatin and thermolysin also had higher renin-inhibitory properties while inhibition by the flavourzyme hydrolysate was similar [25] when compared to values obtained in this work.

SHRs are considered one of the best experimental models for evaluating antihypertensive drugs or food based inhibitors [26].Therefore, the demonstrated better blood pressure-lowering ability of the HMH when compared to HPI suggests contained the former contained peptides that were either more active in nature or had higher absorption characteristics than peptides produced from HPI within the GIT of the rats. The fact that the hemp seed-containing diets maintained or reduced SBP when compared to baseline values during rat growth suggests their potential use in preventing hypertension development. The results obtained in this work are similar to those reported for soybean protein hydrolysate (same diet inclusion values of 0.5 and 1.0%), which was also shown to attenuate hypertension development in SHRs [27]. However, the attenuation effect obtained in this work for the hemp seed diets (SBP $\leq$120 mmHg after 8 weeks) is greater because after 8 weeks, the soybean hydrolysate-fed rats had SBP >120 mmHg. The initial lag time for HPI may be due to the need for extensive enzyme hydrolysis within the GIT to produce bioactive peptides in sufficient quantity whereas the HMH diets already contained pre-digested peptides.

SHRs and NTRs rats are generally compared in terms of their cardiovascular phenotype as models for studying hypertension. These strains have shown some genetic variations in markers of hypertension, observations which suggest that ACE activity in the plasma may be dependent on genetic, environmental, experimental, hormonal and age-related factors [28]. The lower plasma ACE level during attenuation of SBP increase in growing SHRs is consistent with the earlier work of Yang et al. [27]. The ACE values tended to be positively related to SBP changes, which confirm previous works that have shown the plasma level of this enzyme could be used as a hypertension marker in SHRs [29]. The result is further supported by the fact that rats fed the HMH-containing diets had significantly (p<0.05) lower SBP and plasma ACE levels when compared to the rats that consumed the HPI-containing diet.Thus the results further supports the role of high plasma ACE levels in maintaining hypertension conditions and the use of ACE level modulating agents as effective antihypertensive agents.The slightly lower plasma ACE levels observed during feeding trial IIwhen compared to trial I suggest that some of the rats did not regain 100% of enzyme level during the washout period. In contrast to data obtained in this work, a previous report showed no changes in plasma ACE of SHRs after a 30 day feeding period with corn protein-derived peptides [30], which suggests that peptides from different sources may produce antihypertensive effects through different mechanisms.The slight reduction in plasma ACE levelfor the 1% HMH-treated NTRs was not translated to SBP reduction, which is consistent with the fact that thisantihypertensive protein hydrolysate has no blood pressure-reducingeffectunder normotensive conditions.The lack of diet effect on SBP of the NTRs indicates that the blood pressure-reducing effects of HMH observed for SHRs are peculiar to hypertension

conditions and should not cause hypotension if consumed under normal BP conditions. The reduced plasma renin and ACE levels in SHRs could be confirmed as one of the main mechanisms involved in the antihypertensive effects of the HMH and HPI. Inhibition of renin is a very important physiological target for modulation of the RAAS in order to control hypertension because the enzyme catalyzes the rate limiting step in a cascade of reactions that lead to vasocontriction.To the best of our knowledge, this work is the first to showmodulation of plasma renin level in SHRs that have been fed diets containing foodproteinsor peptides.

6.4. Conclusions

Long-term studies of feeding growing SHRs with different levels (0.5 and 1%) ofHMH peptides were used to confirm SBP attenuation, which suggests absorption of bioactive peptides from the diets. The HMH diet but not the HPI diet was able to maintain the SHRs at normal blood pressure. The delayed and reduced antihypertensive effect of unhydrolyzed hemp seed proteins (HPI diet) suggest that pre-digestion in the form of protein hydrolysates can provide a more rapid means of reducing blood pressure during diet intervention. Thus, the HMH could prevent hypertension development in SHRs. In SHRs with established hypertension, diets containing hemp seed peptides or proteins were also effective in reducing blood pressure when compared to a casein only diet. The bioactive products did not change SBP of NTRs, which indicates lack of potency under normal blood pressure conditions. SBP reductions were directly related to plasma levels of ACE and renin, which suggest the *in vivo* mechanism of action. The higher level of plasma renin in SHRs when compared to NTRs confirms the role of this enzyme in maintaining hypertension and that the SHR is a suitable model to study efficacy of

antihypertensive compounds. The results confirm the suitability of HMH as a useful ingredient that can be used to formulate functional foods and nutraceuticals for the prevention and treatment of hypertension. Future experiments will be directed towards purification and identification of active peptide sequences that are responsible for the antihypertensive effects of HMH.

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TRANSITION STATEMENT 4

The aim of the study was to determine the effects of long-term feeding with hemp seed containing diets on the plasma peroxide and lipids levels in spontaneously hypertensive rats (SHRs). The plasma peroxide level is an endogenous marker of lipid oxidation that often resultsfrom oxidative stress-related diseases while plasma lipid level is a risk factor for atherosclerosis which could ultimately lead to hypertension. Long-term feeding with diets containing hemp seed peptides significantly attenuated these risk factors by reducing oxidation of lipids and resulting in low levels of peroxide in the blood while decreasing LDL-C but increasing the triglycerides and HDL-C. Prior to feeding the HMH to SHRs, it was tested for *in vitro* antioxidant properties in which the results showed that it could scavenge DPPH and hydroxyl radicals as well as chelate transition metals such that they are unavailable to catalyze peroxidation reactions. It was found that hemp seed diet significantly ($p<0.05$) increased LDL-C levels in the growing rats, which was decreased in the adult rats. The HDL-C was slightly higher in the growing than in the adult rats with established hypertension. Again this study further demonstrates the ability of hemp seed peptides to act as potent antioxidants and anti-atherosclerotic agents that would reduce oxidative stress and prevent atherosclerotic plaque from building up in blood vessels. The study results are a validation of *in vitro* data that showed that hemp seed peptides are potential antioxidant agents.

CHAPTER 7

MANUSCRIPT 5

EFFECTS OF HEMP SEED (*CANNABIS SATIVA* L.) PROTEIN MEAL HYDROLYSATE AND PROTEIN ISOLATE ON PLASMA LIPID AND PEROXIDE LEVELS DURING LONG TERM FEEDING OF SPONTANEOUSLY HYPERTENSIVE RATS

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7.0 Abstract

The aim of the study was to determine the effects of long-term feeding with hemp seed containing diets on plasma lipids and peroxide level in spontaneously hypertensive rats (SHRs). Defatted hemp seed meal (25% protein content) was hydrolyzed consecutively with pepsin and pancreatin to simulate gastrointestinal tract digestion; the digest was freeze-dried as a hemp seed meal hydrolysate (HMH). HMH was incorporated into rat diet by partial substitution of casein at 0.5 and 1% levels to provide a source of antioxidant peptides. For comparison, an unhydrolyzed hemp seed protein isolate was used at 1% casein substitution level while the control diet contained only casein as the source of protein. Initial *in vitro* antioxidant tests at 1 mg/ml peptide concentration showed that HMH could scavenge DPPH (52%) and hydroxyl radicals (32%) in addition to chelation of metal ions (40%). After 8 weeks of feeding the diets to young (growing) SHRs or 4 weeks of feeding to adult rats, plasma peroxide level (measured as absorbance at 700 nm) was significantly ($p<0.05$) reduced by the HMH (0.41-0.53) and HPI-containing diets (0.51-0.61) when compared to the control diet (0.61-0.74). The hemp seed-containing diets also reduced plasma peroxides when fed to adult normotensive rats over a 4-week period. The HMH diets significantly ($p<0.05$) increased LDL cholesterol in growing rats but not in the adults while HDL cholesterol level was not affected. The results suggest that HMH may be utilized in the formulation of antioxidant food products that can reduce oxidative stress.

7.1 Introduction

Cannabis sativa L. also commonly called industrial hemp seed is historically an important source of food, fibre, dietary oil and medicine (Callaway, 2004); the seed contains about 30% oil and 25% protein. Hemp seed storage proteins consist mainly of globulin (edestin) and albumin (Yin et al., 2008), which have higher digestibility (88-91%) when compared to soy protein (71%) after hydrolysis with pepsin and trypsin enzymes (Wang, Tang, Yang & Gao, 2008). There have been several claims of health benefits of hemp seed proteins and research recently has focused on validating these claims through coordinated laboratory tests and animal studies. The effect of limited or extensive enzymatic protein hydrolysis as a means of improving the functional properties of hemp seed proteins have been reported by different researchers (Tang, Ten, Wang & Yang, 2006; Yin et al., 2008). Proteins from both plant and animal sources including those of hemp seed have been isolated and recognised as essential sources of bioactive peptides capable of exerting various *in vitro* and *in vivo* activities such as antioxidant, antihypertensive, antimicrobial, opioid, antithrombotic, hypocholesterolemic, appetite-reducing, mineral-binding, immunomodulatory and cytomodulatory (Norris & Fitzgerald, 2013; Pihlanto & Mäkinen, 2013; Sharma, Singh & Rana, 2011). Extensive research works have been done on the *in vitro* antioxidant properties of hemp seed peptides with evidence of ability to scavenge toxic free radicals, chelate metal ions, and inhibit linoleic acid oxidation (Girgih, Udenigwe & Aluko, 2011a; Tang, Wang & Yang, 2009).

Oxidative metabolism is essential for the survival of cells and normal physiological functions of a healthy body. In stressful or disease conditions however, over production of free radicals and other reactive oxygen species (ROS) can cause oxidative damages.

Formation of excessive free radicals in the body could overwhelm protective enzymes like superoxide dismutase, catalase and peroxidase to produce destructive and lethal cellular effects (e.g. apoptosis, inflammation) through oxidation of membrane lipids, cellular proteins, DNA, and enzymes (Sharma, Singh & Rana, 2011). These destructive effects lead to a condition called oxidative stress which can be surmounted by the consumption of foods rich in antioxidants (Udenigwe & Aluko, 2010). Functional foods are preferred rather than the use of chemically induced antioxidant defence that may be toxic to mammalian cells after prolonged use. Food-derived antioxidant peptides that contain 2–20 amino acid residues are considered natural and safe antioxidant resources in comparison to synthetic ccompounds such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Sarmadi & Ismail, 2010). BHA, BHT and propyl gallate are added to food products to retard lipid oxidation, a major cause of food quality deterioration that causes rancidity and development of off-flavours (Di Bernardini et al., 2011); however, their use has been limited due safety concerns. Peptides derived from natural sources have potential antioxidant health benefits associated with low molecular weight, low cost, high activity, easy absorption, and little or no negative side effects (Sarmadi & Ismail, 2010). Several successful attempts have generated novel antioxidant peptides from animal sources including blood plasma, shrimp, milk, and egg white (Chen, Chi, Zhao & Lv, 2012; Faithong, Benjakul, Phatcharat & Binsan, 2010; Nongonierma & Fitzgerald, 2013; Sun, Luo, Shen, Li & Yao, 2012). Novel antioxidant peptide products from plant foods such as sunflower, sweet potato, flaxseed and chick pea have also been reported (Huang et al., 2012; Kou, Gao, Zhang, Wang & Wang, 2013; Ren, Zheng, Liu & Liu, 2010; Udenigwe & Aluko, 2010). *In vitro* experiments (Girgih,

Udenigwe & Aluko, 2011a; Lu et al., 2010; Tang, Wang & Yang, 2009) have shown that hemp seed peptides possess antioxidant properties but the *in vivo* effects have not been reported.Therefore, the objectives of this study were to determine the effect of long term (4-8 weeks) feeding with different levels of hemp seed protein meal hydrolysate (HMH) on the plasma peroxide levels and lipid profile of spontaneously hypertensive rats (SHRs) and normotensive rats (NTRs).

7.2 MATERIALS AND METHODS

7.2.1 Materials

Coarse hemp seed powder (25% protein content) produced as a by-product of industrial hemp seed processing industry was a gift from Hemp Oil Canada (St. Agathe, Manitoba, Canada). Briefly, the hemp seed is mechanically pressed to extract oil and the resulting product is the defatted hemp seed cake, which is then milled in a Classifier Milling System to the desired particle size. The milled powder is sifted using various screens to obtain products sold as high-value protein powders. The by-product that does not pass through the sieves is the coarse hemp seedpowder used in this work. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Triton X-100, pyrogallol, ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide, 1,10-phenanthroline, ferroussulfate, linoleic acid, ammonia thiocyanate, and ferrous chloridewere purchased from Sigma-Aldrich (St. Louis, MO, USA), while other analytical-grade reagents were obtained from Fisher Scientific (Oakville,Ontario, Canada).

7.2.1.1 Sample preparation

7.2.1.1.1 Preparation of Hemp seed Protein Isolate (HPI)

The HPI was prepared as reported previously (Girgih, Udenigwe & Aluko, 2011a). Briefly, HPI was produced through alkaline extraction of hemp seed meal (coarse hemp seed powder), which was centrifuged and the supernatant acidified to pH 4.5 (isoelectric point) to precipitate most of the proteins. The acidified supernatant was centrifuged and the resulting precipitate dispersed in deionised water and adjusted to pH 7.0 followed by freeze-drying; the freeze-dried product (HPI) was decolorized by acetone extraction. Protein content of the HPI was determined by modified Lowry method (Markwell, Haas, Biebar, & Tolbert, 1978).

7.2.1.1.2 Preparation of hemp seed meal hydrolysate (HMH)

The hemp seed protein meal (coarse hemp seed powder) was enzymatically hydrolyzed sequentially with pepsin and pancreatin to mimic gastrointestinal (GIT) digestion using a previously reported protocol (Aluko & Monu, 2003). The digest was centrifuged, the precipitate discarded while the supernatant was lyophilized to produce hemp seed meal protein hydrolysate (HMH) which was stored at -20°C until needed for further analysis. Protein content of the HMH was determined by modified Lowry method (Markwell et al., 1978).

7.2.2 Methods

7.2.2.1 *In vitro* Antioxidant Assays

7.2.2.1.1 DPPH Radical Scavenging Assay

The scavenging activity of HMH against DPPH radical was determined using a previously described method (Aluko & Monu, 2003) with slight modifications for a 96-well clear flat-bottom plate. Samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final

concentration of 100 µM. Peptide samples (100 µL) were mixed with 100 µL of the DPPH solution in the 96-well plate to a final assay concentration of 1.0 mg/mL and incubated at room temperature in the dark for 30 min. The absorbance values of the blank (A_b) and samples (A_s) were measured at 517 nm. The blank well contained sodium phosphate buffer instead of the peptide sample. The percent DPPH radical scavenging activity of the samples was determined using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_b - A_s}{A_b} \times 100$$

7.2.2.1.2 Hydroxyl Radical Scavenging Assay

The hydroxyl radical scavenging activity of HMH was determined based on a previously reported method (De Avellar et al., 2004). Peptide samples and 3 mM of 1,10-phenanthroline were separately dissolved in 0.1 M sodium phosphate buffer (pH7.4). $FeSO_4$ (3 mM) and 0.01% hydrogen peroxide were both separately dissolved in distilled water. An aliquot (50 µL) of peptide samples (equivalent to a final assay concentration of 1 mg/mL) or buffer (blank) was first added to a clear, flat bottom 96-well plate followed by 50 µL of 1, 10-phenanthroline and then 50 µL of $FeSO_4$. To initiate the Fenton reaction in the wells, 50 µL of hydrogen peroxide was added to the mixture, covered and incubated at 37^{o}C for 1 h with shaking. The absorbance was measured using a spectrophotometer at 536 nm at10 min intervals for 1 h. The hydroxyl radical scavenging activity was calculated using the reaction rate (ΔA/min) for blank (b) and samples (s) according to the following equation

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{(\Delta A/\text{min})_b - (\Delta A/\text{min})_s}{(\Delta A/\text{min})_b} \times 100$$

7.2.2.1.3 Superoxide Radical Scavenging Assay

The superoxide radical scavenging activity of HMH was determined using a previously reported method (Gao, Yuan, Zhao & Gao, 1998) but with slight modifications. An aliquot of 80 µL (final assay concentration of 1 mg/mL) of sample dissolved in 0.1 M NaOH was mixed with 80 µL of 50 mM Tris–HCl buffer (pH 8.3) containing 1 mM EDTA directly into a clear bottom 96-well plate in the dark. A 40 µL aliquot of 1.5 mM pyrogallol dissolved in 10 mM HCl was then added to each well. Absorbance of sample was measured immediately at 420 nm for 4 min at room temperature. Tris–HCl buffer was used as blank (b) and superoxide scavenging activity of peptides was calculated as follows.

$$\text{Superoxide scavenging activity (\%)} = \frac{(\Delta A/\min)_b - (\Delta A/\min)_s}{(\Delta A/\min)_b} \times 100$$

7.2.2.1.4 Chelation of Metal Ions

The metal (iron) chelating activity was measured using a modified method of Xie, Huang, Xu & Jin (2008a). Peptide sample solution (final assay concentration of 1 mg/mL) was combined with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL double distilled water in a reaction test tube. Ferrozine solution (0.1 mL of 5 mM) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min from which an aliquot of 200 µL was removed and added to a clear bottom 96-well plate. A blank was also conducted by replacing the sample with 1 mL of double distilled water. The absorbance values of blank (A_b) and sample (A_s) at 562 nm were measured using a spectrophotometer. Percentage chelating effect (%) was calculated using the following equation.

$$\text{Metal chelating effect (\%)} = \frac{A_b - A_s}{A_b} \times 100$$

7.2.2.2 Animal Studies

7.2.2.2.1 Long-Term Feeding Protocols

All rat experiments were performed according to protocols approved by the University of Manitoba Animal Care Protocol and Management Review Committee. The rat feeding experiments were carried out as follows using SHR (hypertensive) or Wistar Kyoto (WKY) rats (normotensive)purchased from Charles River Laboratories (Montreal, PQ, Canada). In the first feeding experiment, 32 male SHRs (6 weeks old) were housed in the Animal Facility at the Richardson Centre for Functional Foods and Nutraceuticals under a 12 h day and night cycle at 22°C. The rats were acclimatized by feeding *ad libitum* with a regular chow diet and tap water for two weeks. Thereafter, SHRs (now 8 weeks old) were randomly divided into 4 groups (similar average body weight and SBP) of 8 rats each that received similar feed but with addition of hydrolyzed (HMH) and unhydrolyzed (HPI) hemp seed products to determine ability of each diet to attenuate oxidative stress development during the rapid growth phase (preventive effect). The diets were prepared as follows to contain casein as the main source of protein: 20% (w/w) Casein (control diet); 0.5% (w/w) HMH diet (19.5% casein + 0.5% HMH); 1% (w/w) HMH diet (19% casein + 1% HMH); and 1% (w/w) HPI diet (19% casein + 1% HPI). The rats were then fed their respective diets and tap water *ad libitum* for 8 weeks during which feed consumption and body weight were measured weekly. At the end of the 8 weeks of feeding, 4 rats from each group were terminated followed by harvesting and weighing of body organs while blood wascollected into heparinised tubes. The blood was centrifuged at 1500xg for 10 min to obtain plasma, which was then stored at -80^{o}C until needed for further analysis. All the remaining SHRs were then switched to the regular

chow diet for 4 weeks to serve as a washout period and allow establishment of oxidative stress. To determine potential treatment ability of HMH, the rats (now 20 weeks old) were randomized (4 each) to the four diet groups after the washout period and feeding (*ad libitum*) conducted for 4 weeks with weekly feed consumption and body weight measurements. At the end of the 4-week feeding period, all rats were terminated for blood and organ collection as described above. A third feeding experiment involved the use of normotensive WKY rats (16 weeks old), which were randomly assigned to the following 3 protein treatment groups with 6 rats per group. Control diet (20%, w/w casein); 1% HMH diet (19% casein + 1% HMH) or 1% HPI diet (19% casein + 1% HPI). Only one HMH dose was used in this phase of the study because from the SHR experiments, the 1% HMH was a more effective dose than the 0.5% HMH in lowering plasma peroxide level. The normotensive rats (NTRs) were allowed *ad libitum* access to their respective group feeds and tap water for 4 weeks during which feed consumption and body weight were measured weekly. The NTRs were also terminated at the end of 4 weeks with blood and organs collected and processed as indicated for the SHRs.

7.2.2.2.2 Analysis of Plasma Peroxide Levels

The plasma peroxide levels of rats was measured using a previously reported method (Li, Jiang, Zhang, Mu & Liu, 2008a) with slight modifications as follows. A 1 mL aliquot of rat plasma samples was mixed with 1.5 mL of 0.1 M sodium phosphate buffer, pH 7.0. For the blank assay, 1 mL of buffer was mixed with 1.5 mL of buffer. The sample mixtures and blank were warmed to 60^{o}C and thereafter, 0.1 mL of each was mixed with 4.7 mL of 75% aqueous ethanol, 0.1 mL of ammonium thiocyanate (30% w/v) and 0.1 mL of 0.02 M ferrous chloride dissolved in 1 M HCl. An aliquot (0.2mL) of

this solution mixture was added to a clear bottom 96-well microplate and the degree of color development was measured at 500 nm after 3 min incubation at room temperature. An increased absorbance implied presence of high amounts of peroxides in the plasma sample.

7.2.2.2.3 Plasma Lipid Profile Analysis

Plasma total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) of spontaneously hypertensive rats were determined by automated methods on a Vitros 350 Chemistry Analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). The plasma low-density lipoprotein cholesterol (LDL-C) concentrations were estimated using the Friedewald formula (Friedewald, Levy & Fredrickson, 1972).

7.2.2.3 Statistical Analysis

Except where indicated, in vitro data were collected in triplicate while the *in vivo* data are based on 4 rats/group. Data were subjected to one way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p < 0.05$.

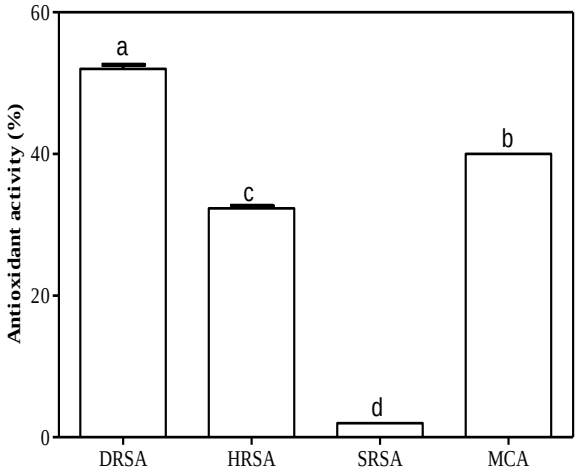
7.3 RESULTS AND DISCUSSION

7.3.1 *In Vitro* Antioxidant Properties of HMH

Fig. 7.1 shows that the HMH had significantly ($p<0.05$) different activities as a free radical scavenger and a metal chelating agent. Scavenging of DPPH radical by HMH was the highest at ~52% when compared to hydroxyl and superoxide radicals. Though DPPH radical is not a physiologically relevant species, the test result can give an initial insight as to whether a sample has the potential to be potent antioxidant against these

toxic free radicals *in vivo*. The HMH scavenged the hydroxyl and superoxide radicals by 32 and 2%, respectively indicating that HMH was a moderate scavenger of hydroxyl but a poor scavenger of superoxide radical. Hydroxyl radicals because of their hyper-reactivity with almost any cellular molecules including proteins, DNA, polyunsaturated fatty acids and cell membranes could cause severe damage to cells that lead to increased oxidative stress and progression of chronic diseases such as CVD, cancer, diabetes and obesity (Young & Woodside, 2001). Therefore, scavenging of hydroxyl radical is an effective defence mechanism employed by the human body against disease causing free radicals. Superoxide radicals are known to be the initiators of the production of other harmful and very reactive free radicals. For example, superoxide and hydrogen peroxide anion can combine to produce hydroxyl radicals (the most active ROS *in vivo*) in the presence of metallic ions (Cu^{2+}& Fe^{2+}) which act as catalysts. The very low (2%) scavenging of superoxide radical by HMH is supported by our previous publication (Girgih, Udenigwe & Aluko, 2013), which also showed that hemp seed protein hydrolysates had very low or zero activity against superoxide radical. The HMH had ~40% metal chelating ability, which measures the capacity of peptides to form complexes with transition metals. Formation of the peptide-metal ion complex can reduce ability of metals to promote or catalyze Haber-Weiss reaction that induce superoxide anions to form more hazardous hydroxyl radicals. Ferrous and copper ions can also stimulate lipid peroxidation via Fenton reaction which accelerate peroxidation by breaking down hydroxyperoxides into hydroxyl and alkoxyl radicals; these radicals then remove hydrogen from surrounding biomolecules to perpetuate a free radical chain reaction of lipid peroxidation (Xie, Huang, Xu & Jin, 2008a). The antioxidant mechanisms are

Figure 7.1: *In vitro* antioxidant and antihypertensive properties of hemp seed meal hydrolysate DRSA = DPPH radical scavenging activity; HRSA = Hydroxyl radical scavenging activity; SRSA = Superoxide radical scavenging activity and MCA = Metal chelating activity. Bars with different alphabets have mean values that are significantly different at p<0.05



diverse and employ different principles which for a given test, the response of antioxidant ability may depend on various factors such as solvent and substrate used, affinity of substrate-antioxidant and the purity of the substrate as well as that of the tested samples (Moure, Domínguez & Parajó, 2006), Therefore, it is recommended that antioxidant activity is better characterised using different assays, based on different mechanisms and using different media (Lu et al., 2010). The high antioxidant capacity of HMH may be due to abundance of certain amino acids such as acidic and aromatic amino acids as we have previously reported (Girgih et al., to be submitted). These peptides are reservoirs of electrons that can be donated toquench ROS and other free radicals in addition to chelation of transition metals.

7.3.2 Effect of Diet on Feed Consumption, Body Weight Changes and Organ Weights

Table 7.1 shows that feed consumption and body weight of growing SHR was affected by diets formulated with or without hemp seed products during an 8-week study period. In comparison to the control (casein only) diet group, rats in the HMH or HPI groups consumed significantly ($p<0.05$) less amount of feed, which could be due to reduced palatability or increased satiating effect of the hemp seed proteins and peptides. Similarly, body weight changes followed same trend over the 8 week feeding period with the control group having a net body weight gain of 116g when compared to 112g for 0.5% HMH, 107g for 1.0% HMH and 101g for 1% HPI. However, only the difference between control and 1% HPI groups was significant ($p<0.05$). The slight reductions in body weight gain during growth of the hemp seed-treated rats may be due to suppression of the renin-angiotensin system, which has been shown to slow body weight gain in the SHR model (Chow, De Gasparo, & Levens, 1997). This is because our previous work showed that the dietary substitution of casein with 0.5% and 1% hemp seed products led to reduced blood pressure and attenuated plasma levels of renin, angiotensin converting enzyme, and angiotensin II (Girgih et al., 2013). These changes in feed consumption and body weight may also be due to the superior amino acid profile of casein; therefore, substitution with a hemp seed protein that has less nutritional value could have been responsible for the reduction in biological value of the feed.

Table 7.1: Feed consumption and body weight of growing (young) spontaneously hypertensive rats fed different diets for 8 weeks*

Feed consumption (g)					**Body weight (g)**			
Week	Control	0.5% HMH	1% HMH	1% HPI	Control	0.5% HMH	1% HMH	1% HPI
1	109±2.98[a]	106±4.65[b]	104±3.66[b]	102±1.04[b]	171±15.0[a]	165±12.6[a]	157±13.8[a]	151±6.60[a]
2	115± 4.26[a]	111±2.39[b]	109±1.98[b]	107±1.06[b]	199±15.2[a]	191±14.2[a]	182±16.1[a]	174±8.40[a]
3	121±4.17[a]	117±3.12[b]	115±2.47[b]	113±2.20[b]	220±14.5[a]	212±14.9[a]	201±14.3[a]	193±8.80[a]
4	127±4.14[a]	123±3.52[b]	121±2.95[b]	118±2.45[b]	239±15.2[a]	231±14.9[a]	220±15.3[a]	210±9.00[a]
5	132±4.02[a]	128±3.91[b]	126±2.97[b]	124±3.00[b]	254±13.9[a]	244±13.9[a]	232±16.9[a]	222±10.8[a]
6	136±4.93[a]	132±3.96[b]	131±3.42[b]	128±3.37[b]	267±14.1[a]	257±13.1[a]	244±25.3[a]	233±12.1[a]
7	141±4.60[a]	136±4.49[b]	135±3.58[b]	134±3.25[b]	275±14.3[a]	264±14.5[a]	253±19.0[a]	241±12.4[a]
8	146±4.13[a]	142±4.63[b]	140±4.13[b]	137±3.11[b]	287±14.1[a]	277±12.9[a]	264±18.5[a]	252±12.3[a]

*HMH: Hemp seed meal hydrolysate; HPI: Hemp seed protein isolate

Table 7.2 compares the feed consumption and body weight changes of grown SHR with those of grown NTR during a 4-week treatment phase. Generally, the NTRs consumed less amount of feed and therefore, had significantly ($p<0.05$) less gain in body weight during the 4-week feeding period when compared to the SHRs. For the SHRs, feed consumption was reduced significantly ($p<0.05$) when hemp seed products were partially substituted for casein in the diet. The results are similar to those obtained above (Table 7.1) during the 8-week feeding trial that involved growing rats. Therefore, it is possible that the hemp seed products contain appetite suppressing components (e.g., peptides) or the feeds were simply less palatable than the casein only feed. However, there was no negative effect on feed consumption when hemp seed products were included in the diet of NTRs. It is difficult to explain the differences in the effects of hemp seed products on feed consumption between the two types of rats but it is possible that the presence of hypertension in the SHRs made them more sensitive to taste when compared to NTRs. In contrast to the results obtained for the growing rats (Table 7.1), the partial dietary substitution of hemp seed products for casein did not have any negative effect on body weight of the adult rats (Table 7.2). The results can be explained by the fact that the rats used in Table 2 are already at the peak of growth; therefore, slight reductions in feed consumption will not have any dramatic effect on body weight gain. For the growing rats (Table 7.1), there is high demand for nutrients to build new tissues and produce rapid weight gains, whereas in already grown-up rats (Table 7.2) most of the diet nutrients will be used for tissue maintenance to prevent body weight loss rather the need for rapid weight gain. The lack of differences in feed consumption by the different dietary groups of NTRs was reflected in the fact that there was also no significant ($p>0.05$) differences

in weight gain. Table 7.3 shows that there was no significant ($p>0.05$) effect of diet on the weights of various body organs such as heart, liver, and kidneys of growing SHRs, adult SHRs and NTRs. Thus, the partial substitution of hemp seed products (peptides and proteins) for casein did not produce any pathological effect on gross weight of the organs. The results suggest that the hemp seed peptide and protein products could be considered safe and produce no abnormality with respect to body organ development. It was however noted that the SHRs group were associated with higher organ weights, which is compatible with their bigger body weights when compared to the NTRs.

7.3.3 Plasma Peroxide Levels in Rats

In biological systems, lipid peroxidation proceeds via a radical-mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids, which initiates a sequenceof reactions that generates aldehydes, ketones and other potentially toxic substances (Winczura, Zdzalik & Tudek, 2012). Peroxide levels in the plasma are markers of the extent of lipid peroxidation at cellular level and could be used to estimate the antioxidant effects of dietary constituents. Results of the plasma peroxide levels in this study are shown in Fig. 7.2A, B & C. In the growing rats (Fig. 7.2A), partial substitution of casein with HMH led to significantly ($p<0.05$) lowest peroxide level with absorbance values ranging from 0.42 ± 0.01-0.47 ± 0.02. In contrast, the HPI-containing diet and control (casein only) group of rats had higher peroxide level values of 0.51 ± 0.00 and 0.60 ± 0.04, respectively. Similar trends with respect to effect of diets were observed for the plasma lipid peroxide values of the adult SHRs (Fig. 7.2B) and NTRs (Fig. 7.2C); the HMH diet groups always had lower absorbance values than the control or HPI groups. The adult rats fed casein only diet had the highest peroxide level (Fig. 7.2B),

which is reflective of their older age when compared to the growing (younger) rats (Fig. 7.2A). The magnitude of plasma peroxide reduction was highest in the adult SHRs rats in comparison to the growing rats (~30%). The fact that the hemp seed products also significantly ($p<0.05$) reduced plasma peroxide level in the NTRs indicates potential benefits in reducing blood lipid oxidation in both disease and normal states.

7.3.4 Plasma Lipid Profile Results for SHRs and NTRs

The levels of plasma total cholesterol (TC), triglycerides (TG), high density lipoproteins (HDL-C) and low density lipoproteins (LDL-C) are presented in Fig. 7.3A, B & C for growing SHRs, adult SHRs and NTRs, respectively. In the growing SHRs group, the plasma TC levels were similar with no statistical significance for all hemp seed protein diets and control. In contrast, the plasma TG level was significantly ($p<0.05$) increased by partial substitution of casein with hemp seed products. Specifically, inclusion of HMH in the diet produced a dose-dependent increase in plasma TG when compared to the casein only diet (Fig. 7.3A). Plasma LDL-C was also significantly ($p<0.05$) increased by the HMH-containing diet. However, the plasma HDL-C was not affected by HMH diets, though the HPI diet caused a significant ($p<0.05$) decrease. The results suggest that hemp seed peptides (HMH) enhanced TG and LDL-C accumulation in the plasma without a negative effect on the beneficial HDL-C during the rapid growth phase of SHRs. In the adult SHRs group with established hypertension, the partial substitution of casein with HMH or HPI produced significant ($p<0.05$) lowering of plasma TC but with no effect on TG, LDL-C and HDL-C (Fig. 7.3B). The TG, LDL-C and the HDL-C levels were slightly decreased in the adult SHRs when compared to their

Table 7.2: Feed consumption and body weight of adult spontaneously hypertensive rats (SHRs) with established hypertension and normotensive rats (NTRs) fed different diets for 4 weeks*

	Feed consumption (g)						
	SHRs				**NTRs**		
Week	Control	0.5% HMH	1% HMH	1% HPI	Control	1% HMH	1% HPI
1	200±6.70[a]	180±15.90[a]	178±5.48[a]	196±14.64[b]	116±2.48[a]	118±2.93[a]	115±1.10[a]
2	205±4.97[a]	184±17.19[b]	174±5.69[b]	200±13.67 [ab]	120±2.73 [ab]	122±2.00[a]	119±1.75[b]
3	208±4.24[a]	188±16.18[b]	170±5.74[b]	204±13.74 [ab]	125±2.71[b]	128±2.14[a]	123±1.52[b]
4	212±3.86[a]	192±16.18[b]	174±5.44[b]	208±13.89[ab]	129±2.25[b]	132±2.23[a]	126±1.21[b]
	Body weight (g)						
1	385±13.1[a]	377±9.82[a]	375±9.93[a]	382±3.33[a]	278±20.6[a]	283±10.7[a]	273±15.5[a]
2	397±7.44[a]	391±13.5[a]	389±10.1[a]	394±4.33[a]	292±17.4[a]	296±11.8[a]	289±18.8[a]
3	405±13.6[a]	400±8.47[a]	398±5.41[a]	403±1.17[a]	304±15.0[a]	305±11.5[a]	300±18.9[a]
4	414±11.3[a]	406±5.09[a]	404±5.46[a]	410±0.92[a]	310±14.3[a]	314±11.5[a]	308±19.3[a]

HMH: Hemp seed meal hydrolysate; HPI: Hemp seed protein isolate

Table 7.3: Organ weights of spontaneously hypertensive rats (SHRs) and normotensive rats (NTRs) fed different diets*

	Young growing SHRs fed for 8 wks				Adult SHRs fed for 4 wks				NTRs fed for 4 wks			
Diet group	Heart (g)	Liver (g)	Right Kidney (g)	Left Kidney (g)	Heart (g)	Liver (g)	Right Kidney (g)	Left Kidney (g)	Heart (g)	Liver (g)	Right Kidney (g)	Left Kidney (g)
Control	1.35 ± 0.13[a]	11.7 ± 2.17[a]	1.10 ± 0.08[a]	1.15 ± 0.13[a]	1.48 ± 0.10[a]	13.4 ± 1.96[a]	1.25 ± 0.06[a]	1.25 ± 0.06[a]	1.12 ± 0.32[a]	9.90 ± 0.40[a]	0.97 ± 0.05[a]	0.93 ± 0.05[a]
0.5% HMH	1.35 ± 0.06[a]	13.7 ± 0.90[a]	1.18 ± 0.05[a]	1.18 ± 0.10[a]	1.55 ± 0.13[a]	13.0 ± 0.31[a]	1.20 ± 0.08[a]	1.18 ± 0.08[a]	DNUN	DNUN	DNUN	DNUN
1% HMH	1.28 ± 0.05[a]	13.1 ± 0.72[a]	1.15 ± 0.06[a]	1.13 ± 0.10[a]	1.60 ± 0.93[a]	13.6 ± 0.13[a]	1.28 ± 0.05[a]	1.30 ± 0.14[a]	1.15 ± 0.23[a]	10.0 ± 0.55[a]	0.95 ± 0.05[a]	0.95 ± 0.05[a]
1% HPI	1.35 ± 0.06[a]	12.8 ± 1.18[a]	1.18 ± 0.05[a]	1.20 ± 0.12[a]	1.53 ± 0.10[a]	13.6 ± 0.73[a]	1.28 ± 0.10[a]	1.23 ± 0.05[a]	1.12 ± 0.10[a]	8.10 ± 3.57[a]	0.92 ± 0.08[a]	0.98 ± 0.12[a]

*HMH: Hemp seed meal hydrolysate; HPI: Hemp seed protein isolate; DNUN: Diet not used for NTRs; Sample mean values in each row with differentalphabets are significantly ($p<0.05$) different.

Figure 7.2: Effect of long term feeding of (A) growing, adult SHRs with established hypertension and (C) adult NTRs with diets containing different levels of hempseed meal hydrolysate (HMH) and hemp seed protein isolate (HPI) on the plasma peroxide level as determined by absorbance at 700 nm. Bars with different alphabets have mean values (n=4 rats ± SD) that are significantly different at $p< 0.05$.

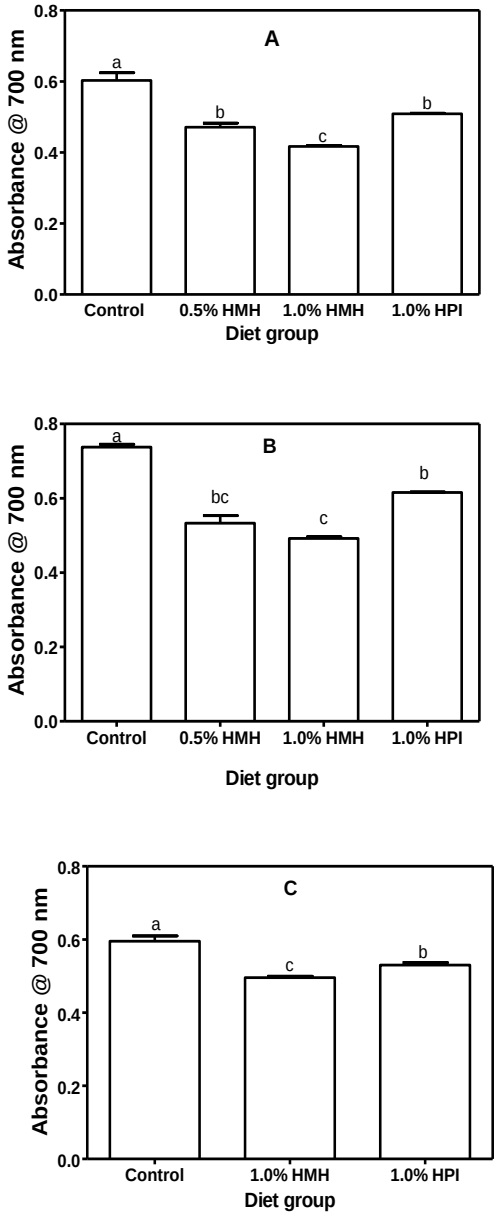
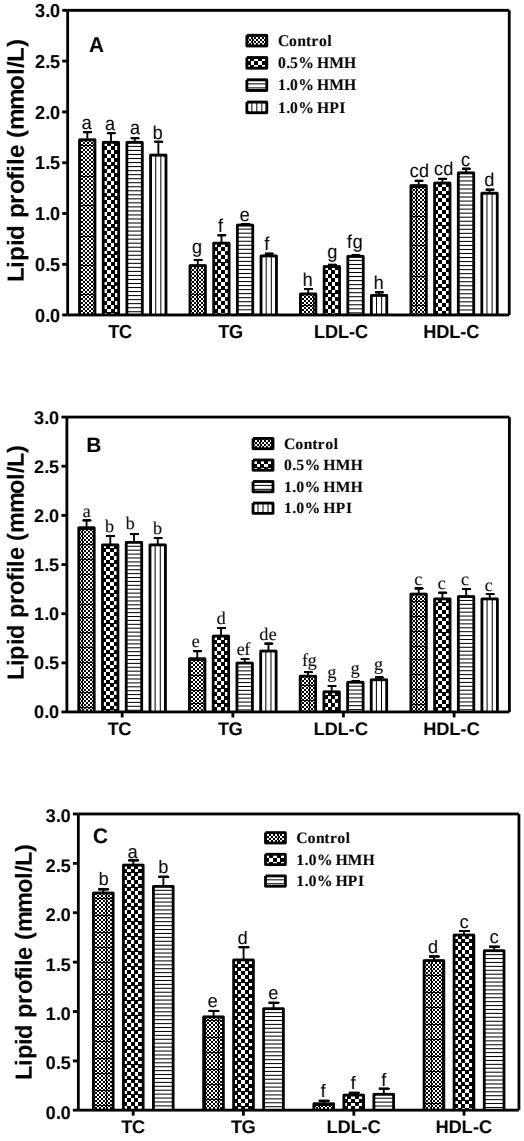


Figure 7.3: Effect of long term feeding on lipid profile of (A) young growing, (B) adult spontaneously hypertensive rats with established hypertension and (C) adult normotensive with different levels of hempseed meal hydrolysate (HMH) and hemp seed protein isolate (HPI) diets. Bars belonging to same lipid type but with different alphabets have mean values (n=4 rats ± SD) that are significantly different at *P*<0.05. TC, total cholesterol; TG, triglycerol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol.



levels observed in growing SHRs. In the NTRs (Fig 7.3C), the TC level was significantly ($p<0.05$) higher (averaging 2.32 mmol/L) than in growing SHRs & adult SHRs (average 1.73 mmol/L) with the 1% HMH group exhibiting the highest TC levels of 2.48 mmol/L. The LDL-C levels were comparably low in NTRs when compared to the SHRs, and vice versa for HDL-C levels. Thus the SHR contains more of the bad cholesterol (LDL-C) and less of the good cholesterol (HDL-C) while the reverse was the case for the NTRs. The lipid profile results trend in this study is similar to reported values by (Boon, Ng, Choo & Mok, 2013) when they evaluated the effect of different palm oil treatments on the blood pressure and lipid profile of SHRs and NTRs. The present work also showed that long term consumption of HMH by SHRs and NTRs decreased total serum cholesterol and triglyceride, and increased high-density lipoprotein cholesterol, with high increases of HDL-C observed more in NTRs than in SHRs. The reverse was the case for LDL-C levels which were more pronounced in the SHRs than the NTRs. Similar results of increasing HDL-C and decreasing LDL-C, TC and TG were reported for SHRs and male Albino rats of Wistar strain, after single and chronic oral administration of *Rhopilema esculentum* protein hydrolysate (RPH) through their antihypertensive and antihyperlipidaemic activities in the rats (Liu, Zhang, Zhang & Liu, 2012). The development and progression of hypertension and hyperlipidaemia are also related to lipid peroxidation damage to tissues caused by increased levels of ROS/free radicals (Yu et al., 2006). The peroxide lowering ability of HMH may thus be attributed to its antioxidant activities. The low plasma peroxide levels obtained in this study support the enhanced antioxidant effect exerted by the HMH peptides. These results indicate that HMH like RPH as well as other food derived peptides may be used in the development

functional foods for the prevention and treatment of oxidative stress, especially peroxide accumulation in the plasma.

7.4 CONCLUSIONS

This work has shown that HMH has the *in vitro* ability to act as an antioxidant by significantly ($p<0.05$) scavenging DPPH and hydroxyl superoxide radicals as well as chelation of metal ions. Incorporation of HMH into rat diet led to reduction in plasma peroxide levels, which confirm antioxidant activity and the down regulation of *in vivo* lipid oxidation. Consumption of diets that contain 0.5 and 1% levels of partial substitution of casein with HMH or HPI led to slight reductions in rat body weight during the rapid growth phase but not in the adult life phase. The hemp seed-containing diets did not cause any noticeable pathological changes in the main body organs of normotensive and hypertensive rats, which suggest that these products are potentially safe for use as health promoting agents. Therefore, HMH could be used to formulate functional foods and nutraceuticals for the therapeutic prevention and treatment of oxidative stress-related metabolic disorders.

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TRANSITION STATEMENT 5

Due to the consistent experimental results with hemp seed peptides showing their ability to act as antioxidant and antihypertensive agents both *in vitro* and *in vivo*, this study further purified the most active RP-HPLC fractions of HPH in order to identify the active components that may be responsible for the observed physiological effects. From the first purification reported earlier, four fractions were selected for second round of purification on the same RP-HPLC but modified injection volume and flow rate. Collected fractions from the second purification were tested for two bioactivities and the most active fractions were used for peptide identification. Using liquid chromatography connected to ESI-MS/MS spectrometer, 23 peptide sequences were identified and chemically synthesized. The synthesized peptides showed varying degrees of bioactivities as antioxidant and antihypertensive agents. Based on these bioactivities, 7 peptides were further selected to evaluate their short-term hypotensive effects in SHRs. The interesting results obtained contrary to observation that the di- and tripeptides are usually stronger bioactive components than longer chain peptides, the tetra- and pentapeptides displayed better of the activities evaluated than the tripeptides. This study identified hemp seed peptides with potential antioxidant and antihypertensive properties, which are useful information that could be utilized for development of more efficient enzyme hydrolysis methods or to enrich the peptide data base. Structure-function relationship studies can further be evaluated to link peptide structure to their observed activities. Additionally, the identified peptides may also find use in the formulation of health foods and nutraceuticals.

CHAPTER 8

MANUSCRIPT 6

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF HEMP SEED (*CANNABIS SATIVA* L.) PROTEIN-DERIVED ANTIOXIDANT AND ANTIHYPERTENSIVE PEPTIDES

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8.0 Abstract

The aim of this study was to identify and test efficacy of antioxidant and antihypertensive peptides present in hemp seed protein hydrolysate (HPH), which was produced through simulated gastrointestinal tract digestion of hemp seed proteins. Consecutive fractionation of HPH by reverse-phase HPLC followed by tandem mass spectrometry analysis of active peaks led to identification of 23 short-chain (≤ 5 amino acids) peptides. At 0.5 mg/mL, WVYY and PSLPA were the most active antioxidant peptides with 67 and 58% DPPH scavenging and metal chelation activity of 94 and 96%, respectively.WVYY and PSLPA showed maximum systolic blood pressure (SBP) reduction in spontaneously hypertensive rats by -34 (2 h) and -40 mmHg (4 h), respectively, after of oral administration of 30 mg/kg body weight dose. WYT (2 h), SVYT (6 h), and IPAGV (4 h) also at 30 mg/kg body weight dose had maximum SBP reductions of -13, -24, and -36 mmHg, respectively.

Keywords: Hemp seed; Angiotensin converting enzyme; Renin; Antihypertensive peptides; Antioxidant peptides; Spontaneously hypertensive rats

8.1 Introduction

Several studies have shown that peptides of plant origin obtained through enzymatic hydrolysis can scavenge free radicals in addition to inhibiting physiologically relevant enzymes and may be effective antioxidant or antihypertensive agents (He, Girgih, Malomo, Ju & Aluko, 2013; Norris & Fitzgerald, 2013; Pihlanto & Mäkinen, 2013; Sharma, Singh & Rana, 2011). These developments coupled with the demonstrated safety of natural peptide sequences have led to an increasing interest in food-derived antioxidant and antihypertensive peptides as potential alternatives or complements for synthetic drugs. Oxidative stress occurs as a result of an imbalance between the productions of reactive oxygen species (ROS)/free radicals and the availability of adequate endogenous antioxidant to remove them as they are generated in the body. Depletion of endogenous antioxidant compounds and/or excessive production of ROS can cause damaging effects to membranes, proteins, enzymes,and DNA resulting in the progression of chronic diseaseconditions (Yongvanit, Pinlaor & Bartsch, 2012). Preventive and treatment strategies to reduce or ameliorate the degree of oxidative stress are key approaches in the management of brain disorders, diabetes, cardiac hypertrophy and cardiovascular diseases (Bains & Hall, 2012; Maulik & Kumar, 2012). Although synthetic antioxidants such as butylated hydroxylanisole, butylated hydroxyltoluene, and propylgallate are commonly used in food systems because of their stronger antioxidant activity when compared to food-derived antioxidants, they must be used under strict regulations due to reported potential health risks arising from possible *in vivo* toxicity (Sarmadi & Ismail, 2010). In addition, higher manufacturing costs and lower efficiency of natural antioxidants such astocopherols, together with the increasing consciousness of consumers

with regard to food additive safety has created a need for identifying alternative natural and probably safer sources of food antioxidants (Pihlanto, 2006). Peptides derived from natural food sources have attracted growing interest because of their potential health benefits associated with low molecular weight, high activity, easy absorption, and little or no negative side effects because of fast clearance from the blood (Sarmadi& Ismail, 2010). In fact, oral administration of 5-fold excess of casein protein-derived peptides to hypertensive and normotensive people over a 4-week period resulted in reduced blood pressure (BP) but without any adverse effect in all the groups (Ishida et al., 2011).

Antioxidant peptides of health significance have been generated from both animal and plant sources and have been used in the production of functional foods and supplements for the management of different health problems. Most of these peptides have been produced using enzymatic hydrolysis, an approach which effectively produces high value-added food-derived peptides and ensures less deleterious effect on the nutritive value and their bioactivity potentials for health enhancement (He, Girgih, Malomo, Ju & Aluko, 2013). Several investigations have demonstrated the production and antioxidant properties of food protein-derived peptides from animal sources like sardinelle, fish, chicken and porcine plasma (Barkia, Bougatef, Khaled & Nasri, 2010; Centenaro, Mellado & Prentice-Hernández, 2011; Liu, Kong, Xiong & Xia, 2010) and from plant origin such as flaxseed, soy proteins, chickpea and cotton seed (Gao, Cao & Li, 2010; Park, Lee, Baek & Lee, 2010; Segev et al., 2010; Udenigwe & Aluko, 2010).

Hypertension claims millions of lives yearly and modification of diet along with lifestyle changes have been recommended as a preventive strategy. The most effective therapeutic approach in the treatment of high BP and associated diseases such as heart

failure and diabetic nephropathy is the inactivation of renin and/or angiotensin I-converting enzyme (ACE). This is because renin and ACE are enzymes that catalyze a sequence of reactions that regulate physiological operation of the renin–angiotensin system (RAS), which is the main pathway, involved in human BP control. Renin converts angiotensinogen to angiotensin I while ACE catalyses the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor; ACE also degrades bradykinin, a potent vasodilator (Zhou et al., 2010). ACE-inhibitory drugs inhibit the formation of angiotensin II and decrease bradykinin catabolism, which enhances systematic dilation of the arteries and veins, hence a decrease in arterial BP and reduced risk for development of congestive heart failure (Ghassem, Arihara & Babji, 2012). Current hypertension therapy involves the use of ACE-inhibitory drugs such as captopril, ramipril, quinapril, benazepril, and lisinopril. Some of the ACE-inhibitory drugs were originally synthesised based on the structure of antihypertensive peptides found in snake venom. However, there is currently only one approved renin-inhibitory drug called aliskiren. These drugs though effective in managing hypertension are associated with side effects such as dry and persistent cough, hyperkalemia, allergic reaction, diarrhea, difficulty in swallowing or breathing, tachycardia, and decrease in the white blood cells (Ghassem, Arihara & Babji, 2012). Therefore, food protein-derived peptides that inhibit renin and/or ACE activities could be used alone or as adjuncts with drugs to reduce BP and ameliorate the negative side effects associated with drugs.

Industrial hemp seed provides quality nutrients, especially amino acids that could be extracted for use as potential therapeutic tools in the management of human chronic diseases. As a result, there is an increase in the number of hemp seed food products,

which are becoming available for public consumption in different countries (Lu et al., 2010). Though previous works have examined antioxidant and antihypertensive properties of hemp seed protein hydrolysates (Girgih, Udenigwe, & Aluko, 2011; Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011), there is paucity of information on the structural identity and functionality of the active peptides. Identification of amino acid sequence of antioxidant and antihypertensive hemp seed peptides could enhance structure-activity relationship studies and provide useful structural templates for development of peptidomimetics with greater potency but lesser negative side effects. Therefore, the main objective of this study was to conduct bioassay guided fractionation of a hemp seed protein hydrolysate (HPH) by reverse-phase HPLC (RP-HPLC) coupled with tandem mass spectrometry to determine amino acid sequence of active antioxidant and antihypertensive (renin and ACE inhibitors) peptides. We then used synthesized peptide sequences to determine *in vitro* activities and BP-lowering effects in spontaneously hypertensive rats (SHRs).

8.2 Materials and methods

8.2.1. Materials

Hempseed protein powder (HPP, 50% protein content) was procured from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB Canada). Pepsin (porcine gastric mucosa, EC 3.4.23.1), pancreatin (porcine pancreas), reduced glutathione (GSH), N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman

Chemicals (Ann Arbor, MI, USA).Other analytical grade reagentswere obtained from Fisher Scientific (Oakville, ON, Canada).

8.2.2. Methods

8.2.2.1 Production of hemp seed protein isolates (HPI)

HPI was produced from HPP according to the method described by Tang, Ten, Wang & Yang (2006) with slight modifications as follows. Briefly, HPP was dispersed in deionized water (1:20, w/v) and the dispersion adjusted to pH 10.0 with 2 M NaOH to solubilize the proteins. The resultant dispersion was stirred at 37^{o}C for 2 h followed by centrifugation (7000*g* at 4^{o}C) for 60 min. The pellet was discarded, the supernatant filtered with cheese cloth and adjusted to pH 5.0 with 2 M HCl to precipitate the proteins. Thereafter, the mixture was again centrifuged (7000*g* at 4^{o}C) for 40 min; the resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to produce HPI powder. Protein content of HPI was determined using the modified Lowry method (Markwell, Haas, Bieber & Tolbert, 1978).

8.2.2.2 Enzymatic hydrolysis of HPI to produce HPH

Prior to enzymatic hydrolysis, HPI was decolorized with acetone as reported by Girgih, Udenigwe & Aluko (2011b) followed by enzymatic hydrolysis according to a previous method (Aluko & Monu, 2003). Briefly, 5% (w/v, protein basis) of decolorized HPI slurry was heated to 37^{o}C and adjusted to pH 2.0 using 2 M HCl. Protein hydrolysis was initiated by the addition of pepsin (4% w/v, protein basis) and the mixture stirred for 2 h. After peptic hydrolysis, the reaction mixture was adjusted to pH 7.5 with 2 M NaOH followed by addition of pancreatin (4% w/v, protein basis) and the mixture was incubated at 37^{o}C for 4 h. The enzymatic reaction was terminated by adjusting the mixture to pH

4.0 with 2 M HCl followed by holding at 95^{o}C for 15 min to ensure a complete inactivation of residual enzyme activity. The mixture was centrifuged (7000*g* at 4^{o}C) for 30 min, the resulting supernatant lyophilized as the HPH and stored at -20 ^{o}C until needed for further experiments. Protein content of HPH was determined using the method of Markwell, Haas, Bieber & Tolbert (1978).

8.2.2.3 RP-HPLC separation of HPH peptides

HPH was first separated by RP-HPLC on aVarian 940-LC system (Agilent Technologies, Santa Clara, CA, USA) as previously described (Girgih, Udenigwe, & Aluko, 2013). In summary, freeze-dried HPH was dissolved in solvent A, which is double-distilled water that contained 0.1% trifluoroacetic acid (TFA) at a concentration of 100 mg/mL, and a volume of 4 mL (sequentially filtered through 0.45 μm and 0.2 μm membrane disks) was injected onto a 21 x 250 mm C12 preparative column (Phenomenex Inc., Torrance, CA, USA). Fractions were eluted from the column at a flow rate of 10 mL/min using a linear gradient of 0-100% solvent B (0.1% TFA in methanol) over 60 min. Elution of peptide fractions was monitored as absorbance at 220 nm. Fractions were collected using an automated fraction collector every 1 min and pooled into eight fractions according to elution time. The pooled fractions (F1-F8) were freeze-dried (after solvent evaporation using the rotary evaporator) and stored at -20^{o}C until required for further analysis. Each of the freeze-dried fractions was assayed for antioxidant (metal chelation and DPPH scavenging) and potential antihypertensive (inhibition of ACE and renin activities) properties using *in vitro* assays.

A second purification step involved further separation of the four (F4, F5, F6 and F7) most active peptide fractions from the first separation. Briefly, the separation was conducted

on the same preparative RP-HPLC column connected to a Varian 940-LC system with an injection volume 2 mLat a concentration of 10 mg/mL and a flow rate of 5 mL/min. The column was eluted with the same solvents A and B using a gradient of 65 to 70% B within 35 min. Two pooled peaks for each of the four fractions (F4-P1, F4-P2; F5-P1, F5-P2; F6-P1, F6-P2; and F7-P1, F7-P2) were collected, solvent evaporated, freeze-dried, and analyzed for *in vitro* antioxidant and potential antihypertensive properties as indicated above for F1-F8. The most abundant and active peptidepeaks (F6-P1 and F6-P2) were then further subjected to UPLC tandem mass spectrometry (LC/MS/MS) analysis for peptide identification.

8.2.2.4 LC/MS/MS analysis

Peptide characterization was performed in a Waters nanoAcquity UPLC system. A 5 µL aliquot of the peptide fractions (F6-P1 and F6-P2) was loaded onto a peptide trap column (180 µm x 20 mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) and was desalted by flushing trap with 2% acetonitrile, 0.1% formic acid in water (solvent A) at a flow rate of 10 µL/min for 2 min. The bound peptides were then eluted onto a nano analytical column (75 µm × 150 mm, Atlantis™ d C18 nanoAcquity™ column, Waters, Milford, MA) and eluted over 45 min with a gradient elution of 1-60% solvent B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.35 µL/min. The column was connected to a q-TOF premier (Waters Corporation, Milford, MA) for electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis in the positive ion mode. Identified peptide sequences were synthesized (>95% purity) by GenWay Biotech (GenWay Biotech Inc. San Diego, CA).

8.2.2.5 DPPH radical scavenging assay

The scavenging activity of peptide samples against DPPH radical was determined using a previously described method (Aluko & Monu, 2003) with slight modifications for a 96-well clear flat-bottom plate. Peptide samples or GSH were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final concentration of 100 µM. Peptide samples (100 µL) were mixed with 100 µL of the DPPH solution in the 96-well plate to a final assay concentration of 0.5 mg/mL and incubated at room temperature in the dark for 30 min. The absorbance values of the blank (A_b) and samples (A_s) were measured at 517 nm. The blank well contained sodium phosphate buffer instead of the peptide sample. The percent DPPH radical scavenging activity of the samples was determined using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_b - A_s}{A_b} \times 100$$

8.2.2.6 Chelation of metal ions

The metal (iron) chelating activity (MCA) of fractions was measured using the method of Xie, Huang, Xu & Jin (2008a), which was modified as follows. Peptide sample or GSH solution (final assay concentration of 0.5 mg/mL) was combined with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL double distilled water in a reaction test tube. Ferrozine solution (0.1 mL of 5 mM) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 10 min from which an aliquot of 200 µL was removed andadded to a clear bottom 96-well plate. A blank was also conducted by replacing the sample with 1 mL of double distilled water. The absorbance values of blank (A_b) and sample (A_s) at 562 nm were measured using a spectrophotometer. Percentage chelating effect was calculated using the following equation.

$$\text{Metal chelating effect (\%)} = \frac{A_b - A_s}{A_b} \times 100$$

8.2.2.7 ACE inhibition assay

The ability of hemp seed peptide fractions to inhibit *in vitro*ACE activity was measured as previously reported (Udenigwe, Lin, Hou & Aluko, 2009). Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 µL of ACE (final activity of 20 mU) and 200 µL of peptide sample in 50 mM Tris–HCl buffer (final peptide concentration of 0.5 mg/mL). The rate of decrease in absorbance at 345 nm of the mixture was recorded for 2 min at room temperature. For the blank experiment, Tris–HCl buffer was used instead of peptide fraction. ACE activity was expressed as rate of reaction (ΔA/min) and inhibitory activity was calculated as follows:

$$\text{ACE inhibition (\%)} = [(\Delta A/\min_{(blank)} - \Delta A/\min_{(sample)}) / \Delta A/\min_{(blank)}] \times 100$$

where $[(\Delta A/\min)_{(sample)}]$ and $[(\Delta A/\min)_{(blank)}]$ are ACE activity in the presence and absence of the hemp seed peptide samples, respectively.

8.2.2.8 Renin inhibition assay

In vitro inhibition of the activity of human recombinant renin assay was conducted according to a previously described method (Li & Aluko, 2010) using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay bufferwas pre-warmed to 37^{o}C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37^{o}C. Before the reaction: (1) 20 µL of substrate, 160 µL of assay buffer, and 10 µL of Milli-Q water were added to the background wells;

(2) 20 µL of substrate, 150 µL of assay buffer, and 10 µL of Milli-Q water were added to the control wells; and (3) 20 µL of substrate, 150 µL of assay buffer, and 10 µL of sample were added to the inhibitor wells (final peptide concentration of 0.5 mg/mL). The reaction was initiated by adding 10 µL of renin to the control and sample wells. The microplate was shaken for 10 s to mix and incubated at 37^{o}C for 15 min, and then, the fluorescence intensity (FI) was recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. The percentage inhibition was calculated as follows:

Inhibition% = [(FI of control well-FI of sample well)/(FI of control well)] X 100

8.2.2.9 BP-lowering effect of peptides in SHRs

Animal experiments were carried out following the Canadian Council on Animal Care Ethics guidelines with a protocol approved bythe University of Manitoba Animal Protocol and Management Review Committee. The 30-week old male SHRs (Charles River Laboratories, Montreal, PQ) with 340-380 g body weight (bw) were kept in the Animal Housing Facility at the Richardson Centre for Functional Foods and Nutraceuticals, under a 12 h day and night cycle at 22 $\pm$ 2 ^{o}C and were fed regular diet and tap water. The rats were divided into three groups with 4 rats per group: peptide, captopril and phosphate buffered saline (PBS, pH 7.4). Peptides (each at 30 mg/kg bw) and captopril (10 mg/kg bw) were dissolved in PBS buffer and administered to the SHRs by oral gavage followed by measurement of systolic blood pressure (SBP) at 2, 4, 6, 8 and 24 husing the tail-cuff method in slightly anesthetized rats as previously described (Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011). Prior to sample administration, the baseline (time-zero) SBP was determined. The change in SBP (ΔSBP, mmHg) was determined by subtracting the baseline from the data obtained at different time points.

8.2.2.10 Statistical analysis

All *in vitro* assays were conducted in triplicate and analyzed by one-way analysis of variance. The mean values were compared using Duncan's multiple range test and significant differences accepted at $p < 0.05$.

8.3. Results and discussion

8.3.1. *In vitro* antioxidant and enzyme-inhibitory properties of HPH peptide fractions

Seven active peptide fractions (F2-F8) were collected in the first round of RP-HPLC fractionation (F1 was inactive) and tested for *in vitro* antioxidant and enzyme inhibition properties (Girgih et al., 2013). Combining the antioxidant and antihypertensive capacities of F2-F8 as already reported by Girgih et al. (2013), fractions F4-F7 were selected for the second round of RP-HPLC purificationbased on their higher gross yield and inhibitory properties. The fractions (F4, F5, F6, and F7) were loaded on the same preparative RP-HPLC used for the first purification at a modified injection volume, flow rate and sample concentration. Two peptide peaks each were isolated as F4-P1, F4-P2, F5-P1, F5-P2, F6-P1, F6-P2 and F7-P1, F7-P2 as shown in Fig. 8.1A-D. The isolated fractions were tested for their *in vitro* antioxidant and enzyme (ACE and renin) inhibitory properties and the results are shown in Fig. 8.1E and F, respectively. Second purification round of the peptide fractions showed DPPH scavenging activities in the 26-57% range (Fig. 8.1E), which is similar to the range observed for F2-F8 after the first round of purification as reported by Girgih et al. (2013). However, MCA was higher and ranged from 82 to 95% in comparison to the 59-78% for F2-F8 obtained after the first purification round. F6 peptide fractions (F6-P1 and F6-P2) showed superior DPPH

scavenging, MCA, renin and ACE inhibition (Fig. 1E and F) when compared to F4, F5 and F7 fractions. Therefore, F6-P1 and F6-P2 were selected for further characterization to identify the inherent amino acid sequences that may have been responsible for the observed antioxidant and enzyme inhibitory properties. Fig. 2 illustrates the typical MS/MS spectra of some of the singly charged ion peaks identified from F6-P1 and F6-P2 fractions. In the absence of information on the primary sequence of hemp seed proteins, a total of 23 peptide sequences were characterized as potential components of the most abundant peaks in F6-P1 and F6-P2; these peptide sequences are summarized in Table 1. Approximately 36% of the 23 peptides identified in this study are composed of five amino acids (pentapeptides) while three and four amino acid residues (tri- and tetra peptides) each constitute 32%. Both the observed and calculated peptide masses (m/z) were not significantly different ($p<0.05$) from each other and ranged from 341.2-630.3 Da. The identified sequences were found to contain about 80% hydrophobic amino acids, a desirable characteristic that enhances antioxidant activity of peptides. Branched-chain and aromatic amino acid contents were 28 and 15%, respectively of the total amino acids in the identified peptide sequences. The progressive antioxidant activities as well as the strong ACE- and renin-inhibitory properties of the identified peptides could be attributable to the presence of substantial amounts of hydrophobic, branched-chain or aromatic amino acid residues such as *Phe*,*Pro*,*Gly*,*Ile*,*Leu*, *Tyr* and *Trp*.This is because a previous work (Sarmadi & Ismail, 2010) had observed that the hydrophobic properties of peptides can enhance their interaction with lipid targets or entry of the peptides into target organs through hydrophobic associations with the cell membrane lipid bilayer, which is favorable to achieving potent antioxidant effects (Sarmadi &Ismail, 2010). Additionally,

previous works (Kobayashi, Yamauchi, Katsuda, Yamaji & Katoh, 2008; Li & Aluko, 2010; Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012) have shown that the presence of C-terminal hydrophobic or aromatic amino acids as well as the availability of groups with negative ionizable functions (*Thr, Glu, Asp, Ser, Met*) positively contributes to higher peptide-induced ACE inhibition. Moreover, the high ACE-inhibitory activities observed for purified hemp seed peptide fractions in this study is also consistent with the fact that branched-chain aliphatic amino acids at the C-terminal as well as the presence of hydrophobic amino acids at the N-terminal contributes to peptide bioactive potency (Huang et al., 2011; Ngo, Vo, Ngo, Wijesekara & Kim, 2012).

8.3.2. *In vitro* antioxidant and enzyme-inhibitory properties of synthesized peptides

The 23 identified hemp seed peptide sequences from the two most active peaks (F6-P1 & F6-P2) of F6 were synthesized and screened for *in vitro* antioxidant and enzyme (ACE and renin) inhibitory properties with results shown in Fig. 3 & 4, respectively. Results of the *in vitro* antioxidant properties revealed that the tetra- and pentapeptides had better antioxidant properties when compared to their tripeptide counterparts (Fig. 3). The pentapeptide (PSLPA) was a weaker scavenger of DPPH radical (58%) than the tetrapeptide (WVYY) with a scavenging ability of 67% (Fig. 3A). WVYY showed better (67%) ability to scavenge DPPH radical when compared to the most active (57%) RP-HPLC peptide fractions (F6-P1, F7-P1, and F7-P2). However, all the peptides had significantly ($p<0.05$) weaker DPPH scavenging activity when compared to GSH (80%), the standard cellular antioxidant peptide. The tetrapeptide (WVYY) and pentapeptide (PSLPA) displayed excellent MCA (94 & 96%, respectively), which was significantly

Figure 8.1: Chromatograms (A-D) from second round of reverse-phase HPLC (RP-HPLC) separation. *In vitro* antioxidant properties and antihypertensive activities of the fractions are shown as in E and F, respectively, at 0.5 mg/mL peptide concentration. For each measured parameter, bar with different alphabets have mean values (n = 3) that are significantly different (p < 0.05).

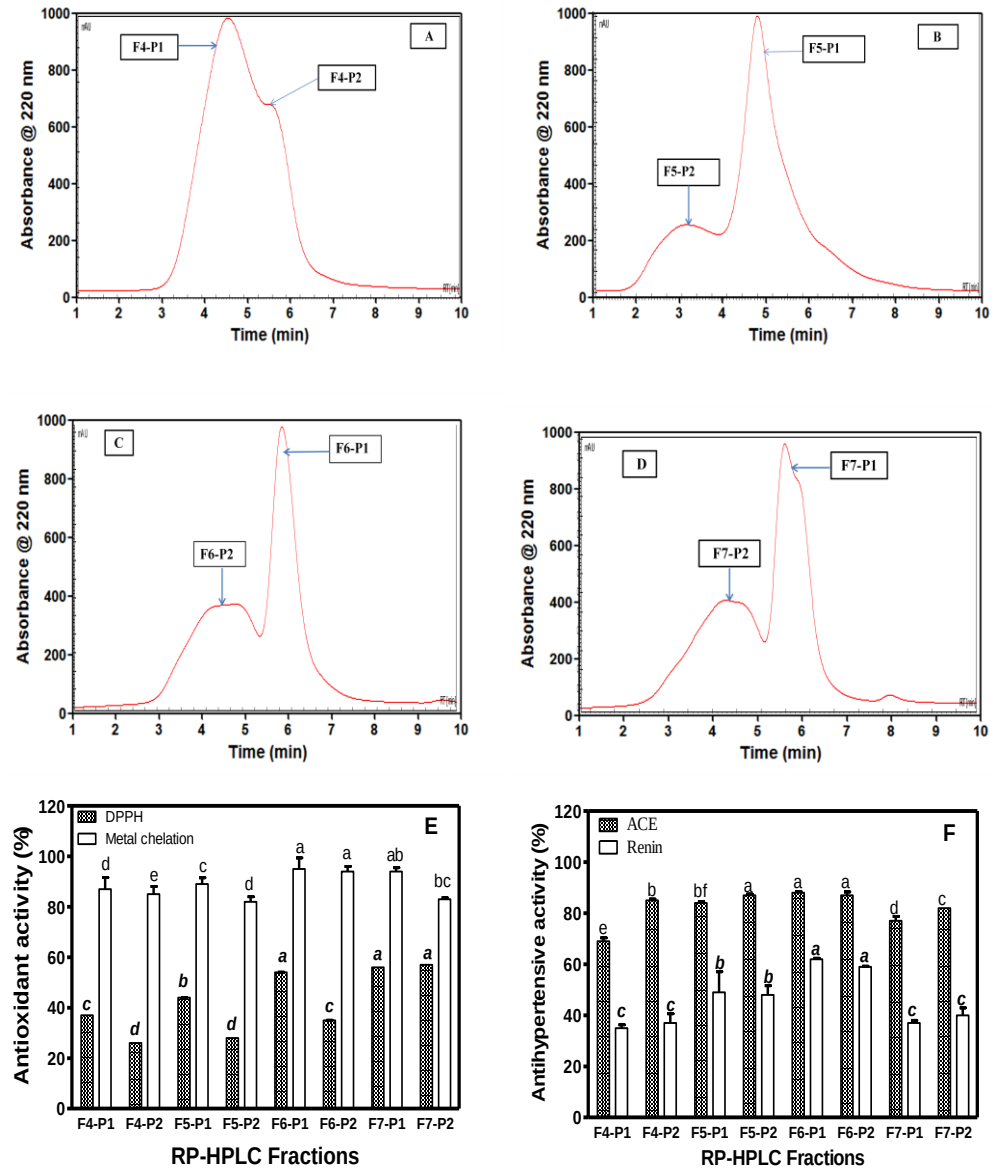
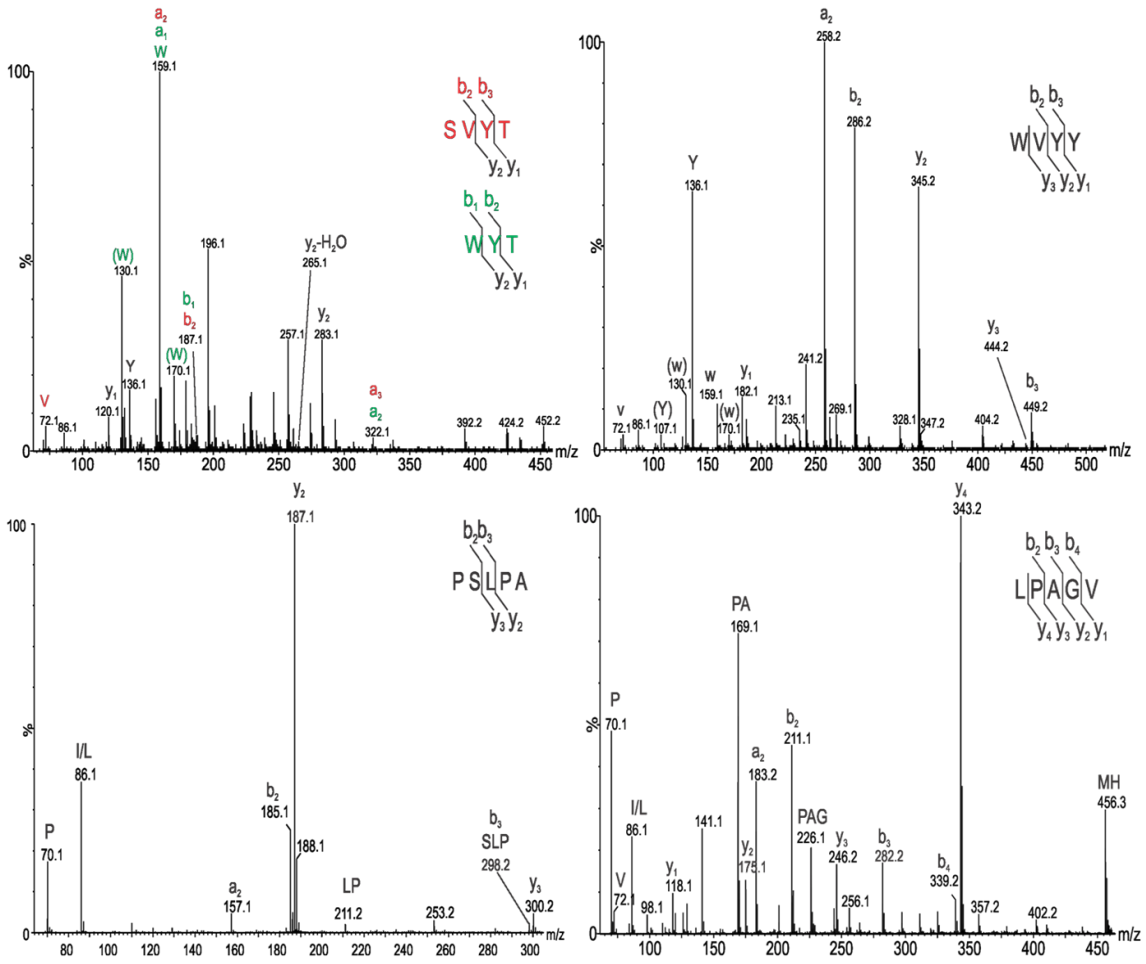


Figure 8.2: Typical MS/MS chromatograms for some of the analyzed peptides with the interpretation of selected MS/MS spectra for the 23 identified peptides sequences in F6-P1 & F6-P2 are summarized in Table 1.

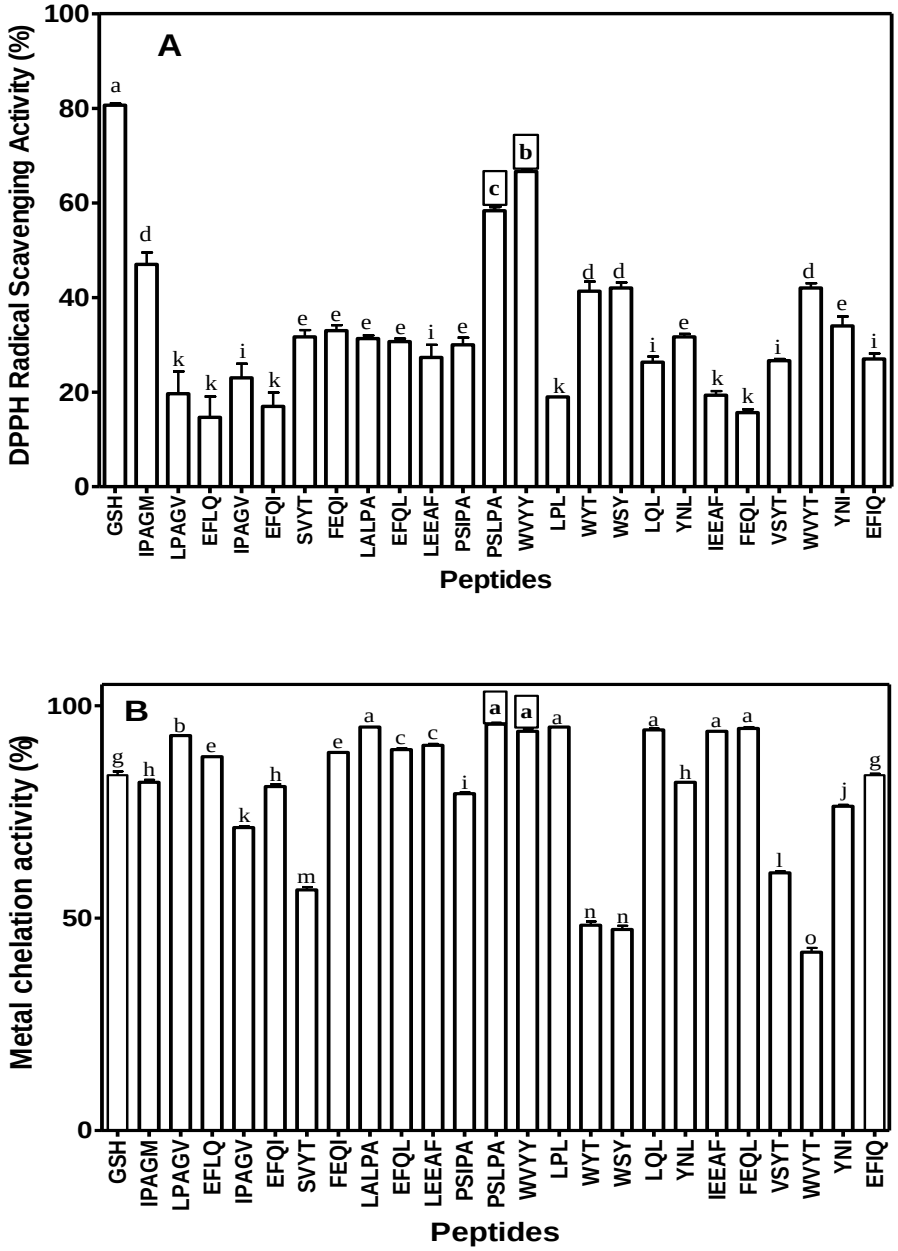


($p<0.05$) higher than that of most of the identified tripeptides that exhibited a moderate to excellent MCA in the 47-95% range (Fig. 3B). Several of the peptides had significantly ($p<0.05$) higher MCA than GSH; therefore, these peptide could have better *in vivo* metal ion chelating properties than GSH depending on bioavailability properties. The peptides, however showed significantly ($p<0.05$) wider variation in its MCA values (47-95%) when compared to the 83-95% range obtained for the RP-HPLC fractions.

Table 8.1: Potential peptides identified from samples F6-P1and F6-P2, their suggested sequences, *m/z* at which the peptides were detected (Obs. *m/z*) and elution time

				F6-P1	F6-P2
Obs. (m/z)	**Z**	**Cal. mass**	**Suggested sequences**	**Time (min)**	**Time (min)**
342.2	1	341.2	LPL	17.3	16.7
373.2	1	372.2	LQL	18.6	17.9
409.2	1	408.2	YNL, YNI	16.8	16.2
455.2	1	454.2	WSY	19.9	19.3
456.3	1	455.3	LPAGV, IPAGV	15.9	15.2
469.2	1	468.2	WYT, VSYT, SVYT	25.6	25.0
484.3	1	483.3	PSLPA, PSIPA, LALPA	19.5	19.1
488.3	1	487.3	IPAGM	-	15.8
536.3	1	535.3	FEQL, FEQI, EFQL, EFLQ,EFQI, EFIQ	18.5	17.7
608.3	1	607.3	LEEAF,IEEAF	20.4	20.4
630.3	1	629.3	WVYY	27.9	27.3

Figure 8.3: (A) *In vitro* screening at 0.5 mg/mL peptide concentration for DPPH radical scavenging and (B) metal chelating activities of synthesized hemp seed peptides. Peptide data are compared with that of reduced glutathione (GSH). Bars with different letters have mean values that are significantly different at $p<0.05$. Values are means (n=3) ± SD.

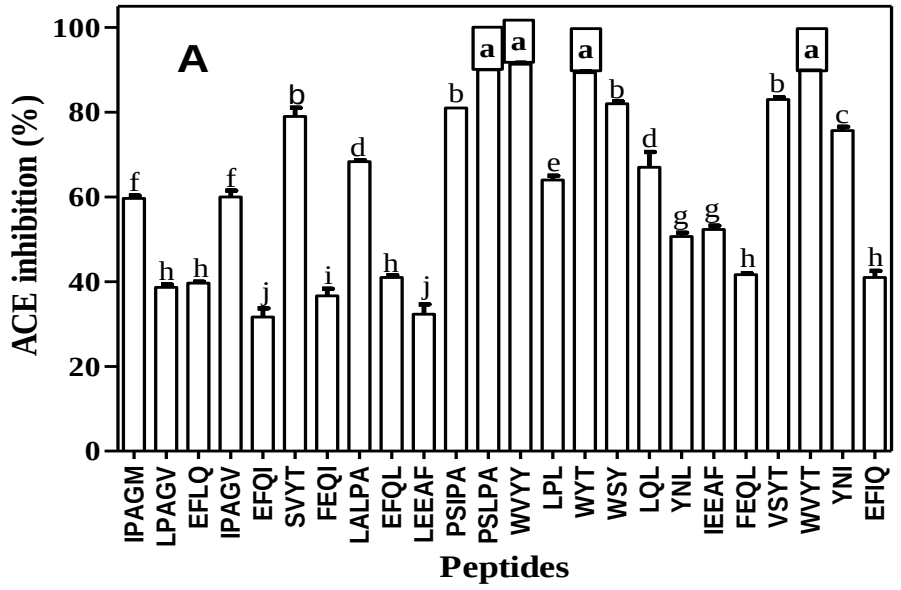


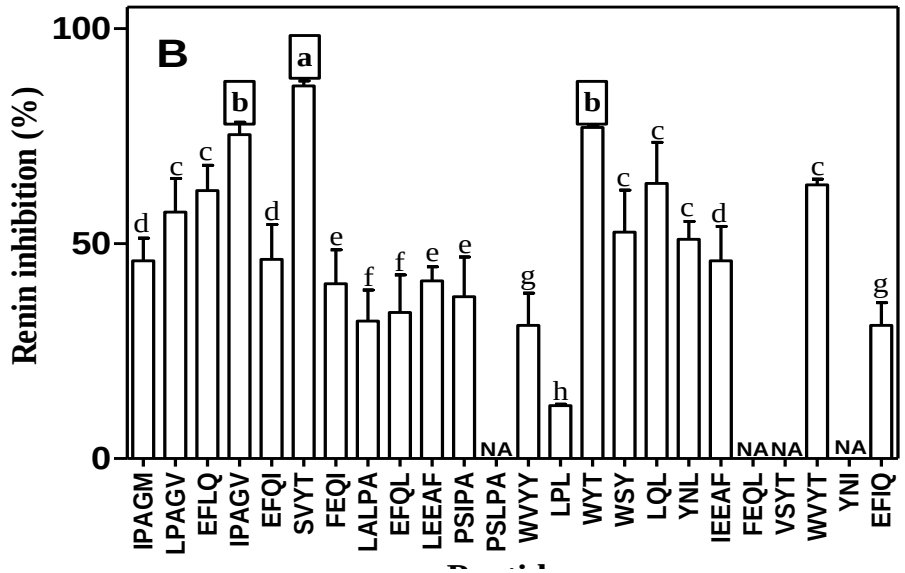
The weak (<50%) MCA of some of the peptides such as WSY and WYT suggests that these peptides may not be responsible or important for the observed MCA potency of F6-P1 and F6-P2 fractions. Similar observations were reported earlier by Ren et al. (2008) indicating that purification process could cause a decrease or increase in antioxidant activity of the peptide fractions after successive purification steps. For example, it has been shown that the "Fraction A" obtained after subjecting hemp seed protein hydrolysate to macroporous adsorption resin separation had the highest DPPH radical scavenging activity of 82.6% when compared to the original hydrolysate and other peptide fractions (Lu et al., 2010). Further separation of "Fraction A" with semi preparative RP-HPLC produced six sub-fractions out of which "Fraction A4" exhibited the highest DPPH scavenging ability of 72.3%. Progressive fractionation of A4 by analytical RP-HPLC resulted in two main peaks (A4a and A4b) with lower DPPH radical scavenging activities of 71.2 and 49.9% respectively, which were lower than the activity of "Fraction A" (Lu et al., 2010). The presence of high amounts (80% in this work) of hydrophobic amino acids (*Val, Pro, Gly, Ile, Tyr, Met, Leu* and *Phe*) as shown in Table 1 is associated with enhanced antioxidative properties especially from naturally occurring protein-derived peptides (Mendis, Rajapakse & Kim, 2005; Rajapakse, Mendis, Jung, Je & Kim, 2005). In addition, enrichment of acidic amino acid and their amides (*Glu, Gln, Asp, and Asn*) in the purified fractions may partly be responsible for the strong antioxidant effects observed due to the presence of excess electrons available to be donatedduring interaction with ROS/free radicals (Udenigwe & Aluko, 2011). The high MCA of the synthesized peptides could enhance their ability to down-regulate the catalytic tendencies of transition metals towards the production of excessive ROS/free

radicals that may cause damage to cellular components leading to progression of degenerative diseases. Thus, we may conclude that the DPPH scavenging and MCA properties of the identified hemp seed peptide sequences are largely influenced by the type of amino acids, length of peptide chain (tetra and penta were more active than tri), and hydrophobicity characteristics.

A screening test was also carried out to determine *in vitro* inhibitions of ACE and renin by the identified peptides as shown in Fig. 4. WYT and SVYT showed the highest dual inhibition of ACE (89 and 79 %, respectively) and renin (77 and 86%, respectively) activities. Both peptides contain bulky aromatic amino acids, which could have contributed to increased potency by enhancing hydrophobic interactions with the enzyme protein. The higher ACE inhibition by SVYT may also be due to the presence of *Val*, a branched-chain amino acid with high affinity for ACE active sub-sites. Some of the peptides such as PSLPA, PSIPA, VSYT, FEQL and YNI had weak or strong ACE-inhibitory activities but lacked detectable inhibition of renin. Overall, ACE inhibition was greater than renin inhibition, which is consistent with previous reports (He, Malomo, et al., 2013; Li & Aluko, 2010; Udenigwe et al., 2012). A previous work has suggested that the more open structure of ACE permits easier access of inhibitors when compared to the more folded structure of renin that could restrict entry of inhibitory molecules (Yuan et al. 2007). Overall, the synthesized tetra- and pentapeptides showed superior ACE-inhibitory activities compared to their tri-peptide counterparts, whereas the tetra-peptides exhibited better renin inhibitory effects than the tri- and pentapeptides.These results are in contrast to traditionally held norm that di- and tripeptides would always have stronger bioactivity than peptide sequences more number of amino acid residues.

Figure 8.4: (A) *In vitro* screening for angiotensin converting enzyme (ACE) and (B) renin inhibitory effects of synthesized hemp seed peptide analogues. Framed capped bars represent peptides that were selected for further assays because of their outstanding activities. Bars with different letters are significantly different at $p<0.05$. Values are means (n=3) ± SD. NA = no activity detected



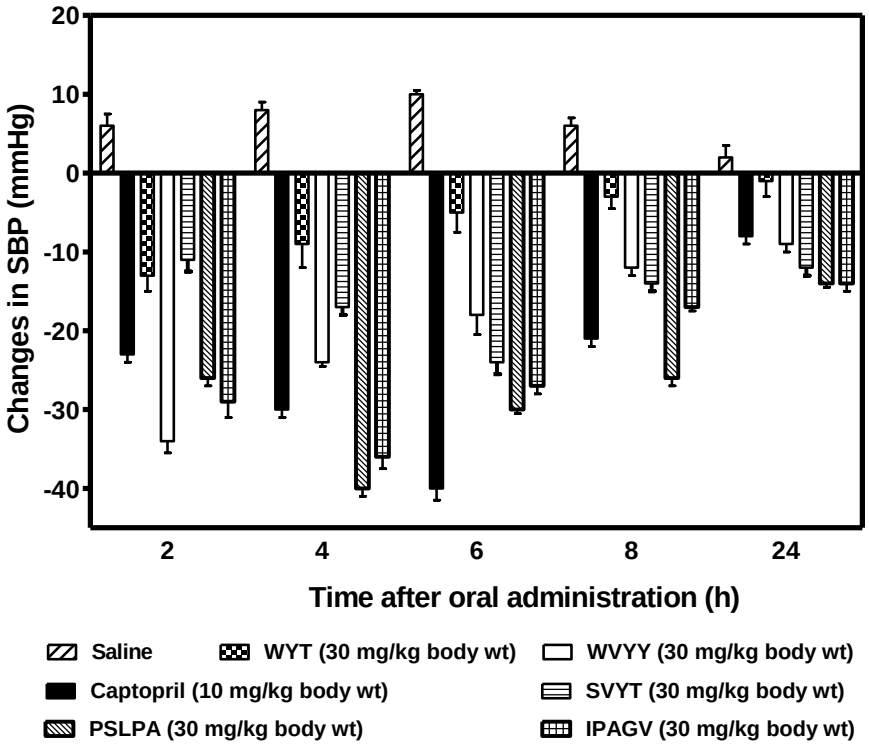


8.3.3. BP-lowering effects of synthetic peptides in SHRs

The selection of synthesized peptides for *in vivo* experiment was based on the dual inhibitory activities of the peptides against ACE and renin, since inhibition of both enzymes simultaneously could provide a more effective strategy to lowering of BP in hypertensive rats. Based on the *in vitro* data, WYT, SVYT, IPAGV were selected as strong dual inhibitors while WVYY & PSLPA were selected for their strong ACE-inhibitory properties but poor renin-inhibitory properties. The BP-reducing effects of these synthetic peptides were evaluated after single oral administration to SHRs in comparison to an antihypertensive drug, captopril as positive control and saline as negative control as shown in Fig. 5. Generally, with the exception of WYT at 8 and 24 hr post administration, all the peptides produced significantly ($p<0.05$) lower BP when compared to the saline (untreated) group. WVYY produced a very rapid decrease (-34 mmHg) in SBP after 2 hr but the effect gradually decreased over the 24 h period. However, the second tetrapeptide, SVYT produced a gradual decrease in SBP that reached a peak of -24 mmHg after 6 h, after which the effect decreased to -12 mmHg by the 24 h.

The two pentapeptides PSLPA and IPAGV were the most effective in lowering SBP with maximum effects of -40 and -36 mmHg, respectively after 4 h of oral administration. Subsequently, the SBP-reducing effects of the pentapeptides also decreased from 6 to 24 h but PSLPA produced lower SBP at the 8 h mark. Though administered at a higher dose, the SBP-lowering effects of the pentapeptides after 4 h was similar to the maximum effect produced by captopril after 6 h. Moreover, the peptides (except WYT and WVYY) maintained significantly ($p<0.05$) lower SBP after 24 h when

Figure 8.5: Time-dependent changes in systolic blood pressure (SBP) of synthesized hemp seed peptides after oral administration to spontaneously hypertensive rats *Significantly ($p<0.05$) different from the saline group.



compared to the antihypertensive drug, captopril. The fast-acting nature of the peptides in reducing SBP 2-4 h after oral administration suggests that the peptides were quickly absorbed from the GIT and transported to target organs of need where they exerted their BP-reducing effects. The peptides with five and four amino acid residues displayed significantly ($p<0.05$) better antihypertensive properties than the tripeptide whose maximum SBP reduction (-13 mmHg) at 2 h after administration was about 50% lower than that of the pentapeptides at the 2 h mark. Therefore, the results (rapid loss of potency) suggest that the tripeptide may be behaving as a substrate-type inhibitor in this study in which ACE or renin may have modified the WYT structure to reduce potency

after absorption (Fujita, Yokoyama, & Yoshikawa, 2000). On the other hand, the tetra- and pentapeptides may either be true or prodrug-type inhibitors (Fujita et al., 2000) in which case they may have passed through the SHRs GIT unaffected by enzyme degradation or they were cleaved to produce more potent peptide sequences that maintained SBP-reducing effects. In this work, the WVYY produced the most rapid decrease in SBP, which suggest faster absorption from the GIT when compared to the longer pentapeptides. It is possible that potency could have been influenced by amino acid composition or positional arrangement of specific amino acids on the C- or N-terminals of the peptide chain. The saline solution showed a negative effect on SBP, causing an elevation rather than suppression throughout the study. In comparison to the original HPH (200 mg/kg bw), which lowered SBP of SHRs by -30 mmHg after 8 h (Girgih, Udenigwe, Li, et al., 2011), three of the current peptides (WVYY, PSLPA and IPAGV) produced better blood pressure reducing effects at a lower dose (30 mg/kg bw) and at a faster rate (<8 h). The fast and better acting property of the pure peptides could be due to increased absorption and absence of inactive peptides that could have diluted the potency when compared to the HPH, which is composed of several active and non-active peptides. The results obtained in this study are significantly ($p<0.05$) higher than those for rapeseed synthetic peptides LY, RALP and TF with SBP lowering effects of approx. -27, -17 and -12 mmHg respectively, at the same oral dose (He et al., 2013). A maximum SBP reduction of -40 mmHg in SHRs was also observed 6 h post-administration when a tetrapeptide (TQVY) isolated from rice protein hydrolysates was orally administered at a dose of 30 mg/kg bw (Li, Qu, Wan & You, 2007), which is similar to the value obtained in this work for pentapeptides after 4 h. A relatively lower

maximal SBP reduction of 9.5 mmHg in SHRs at a dose of 50 mg/kg bw 2 h post-oral administration was reported for a dipeptide AT, which was isolated from hydrolyzed corn gluten meal (Yang, Guanjun, Ping & Liu, 2007). These results indicate that the identified peptide sequences from hemp seed, especially the tetrapeptides WVYY and SVYT as well as the pentapeptides PSLPA and IPAGV represent potential ingredients that can be used to formulate antihypertensive functional foods and nutraceuticals.Howeover, dual inhibition of *in vitro* ACE and renin activities did not translate to better BP-reducing effects of the peptides when compared to peptides that inhibited mostly ACE activity.

8.4. Conclusions

In vitro and *in vivo* studies have shown that hemp seed peptides possess the potential to be used as antioxidant and antihypertensive agents. Identified peptides that showed dual inhibition of ACE and renin (WYT, SVYT, IPAGV) or ACE alone (WVYY & PSLPA) were shown to have substantial BP-reducing ability after oral administration to SHRs. The abundance of hydrophobic, acidic, branched-chain amino acids may have positively contributed to enhancing the antioxidant and antihypertensive potentials of the peptides. The poor SBP-lowering effect of WYT could have been as a result of several factors such as poor binding to target enzymes, rapid structural inactivation in the GIT or within the blood circulatory system or inefficient absorption. In contrast the longer-lasting SBP-lowering effect of SVYT, IPAGV, and PSLPA indicates a more efficient absorption coupled with strong binding to target enzymes and resistance to structural inactivation by GIT or blood circulatory system proteases. Thus, shorter peptide length does not always translate to better physiological properties as some previous works may have suggested. The current work showed no advantage of using peptides with dual *in vitro* inhibition of

ACE and renin activities; however, *in vivo* inhibitory activities may be different and need to be examined in future experiments. However, the results suggest that SVYT, IPAGV, WVYY and PSLPA may be responsible for most of the blood pressure-lowering effects associated with HPH. Several of the peptide sequences reported in this work are new and would contribute to our increasing knowledge of structure-function relationships of food protein-derived peptides. Future work will concentrate on determining the dose response effects of the peptides in animals prior to their use in human intervention trials.

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TRANSITION STATEMENT 6

The seventh manuscript investigated kinetics of enzyme inhibition by the most active synthesized hemp seed peptide sequences by evaluating their *in vitro* inhibitory efficacy against ACE and renin enzymes, the key causative agents of hypertension. One of the striking results of this study was that some of the identified hemp seed peptide sequences exhibited good to excellent renin inhibitory properties (70-89%) which is rare and novel results. The IC_{50} values of the renin inhibitory peptides were also observed to be very low comparable to those of ACE inhibitory peptides in this study. The peptides exhibited mostly competitive and mixed-type inhibition of ACE and renin activities. The synthesized peptides showed low K_i values (high affinity for attachment to the enzyme), which was coupled with low Km and V_{max} implying that the enzymes were effectively inhibited. From evaluating the kinetics parameters it is clear the peptides from hemp seed have the potential to act as effective ACE and renin inhibitors.

CHAPTER 9

MANUSCRIPT 7

KINETICTS OF INHIBITION OF ANGIOTENSIN CONVERTING ENZYME AND RENIN BY HEMP SEED (*CANNABIS SATIVA* L.) PEPTIDES

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9.0 Abstract

The five most active sequences (WVYY, WYT, PSLPA and SVYT & IPAGV) identified from an enzymatic digest of hemp seed proteins were synthesized and used for enzyme inhibition kinetics studies. Results showed that WVYY (IC_{50}, 0.027 mM) was the most potent ($p < 0.05$) against ACE activity and PSLPA (IC_{50}, 0.974 mM) least. However, WYT (IC_{50}, 0.054 mM) was the most potent ($p < 0.05$) against renin activity when compared to SVYT (IC_{50}, 0.063 mM) and IPAGV (IC_{50}, 0.093mM). Kinetics studies showed that the synthetic peptides exhibited mixed-type mode of ACE and renin inhibitions. During ACE inhibition, K_m and V_{max} values were reduced at higher WYT and WVYY concentrations, which was also reflected as reduced catalytic efficiency (CE) of the enzyme. WVYY had lower inhibition constant (K_i) of 0.0307 mg/mL and hence greater affinity for ACE when compared to the 0.8042 mg/mL obtained for WYT. Renin activity was inhibited in a competitive mode at 0.2 µM SVYT concentration but the inhibition was mixed at higher concentration of 0.5 µM. SVYT had lowest K_i value of 0.3936 mg/mL against renin which was also reflected in the ability of this peptide to produce the most decrease in CE, when compared to effects obtained for WYT and IPAGV. The strong affinity of WYT for both ACE and renin could provide useful structural information to design multiple-acting peptides. We conclude that the structural variations contributed to differences in inhibitory effectiveness of the peptides and the information could be useful for the design of new peptides or peptidomimetics with greater renin or ACE inhibition than reported in this work.

Keywords: Hemp seed, bioactive peptides, ACE, renin, IC_{50}, Lineweaver-Burk plot, enzyme inhibition kinetics

9.1 INTRODUCTION

Food protein-derived peptides have been shown to exert a variety of bioactivities during *in vitro* and *in vivo* testings as antioxidant (Chen, Zhao, Zhao, Cong & Bao, 2007; Girgih, Udenigwe, Hasan, Gill & Aluko, 2013; Xie, Huang, Xu & Jin, 2008a), antihypertensive (Martínez-Maqueda, Miralles, Recio & Hernández-Ledesma, 2012; Quiñones et al., 2010; Staljanssens et al., 2011) and immunomodulatory (Choi & Mookherjee, 2012; Regazzo et al., 2010; Seil, Nagant, Dehaye, Vandenbranden & Lensink, 2010) agents. Other works have also produced food protein-derived peptides with antibacterial (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2006; Madureira, Tavares, Gomes, Pintado & Malcata, 2010), anticancer (Bhutia & Maiti, 2008; Hoskin & Ramamoorthy, 2008; Wu et al., 2009), antidiabetic (Billyard, Mcternan & Kumar, 2007; Huang & Wu, 2010; Zhu et al., 2010) and anti-inflammatory (Cifarelli, Trucco & Luppi, 2011; Haidet, Cifarelli, Trucco & Luppi, 2009; Young, Fan & Mine, 2010) properties. These bioactive peptides could be generated from animal and plant sources through enzymatic hydrolysis of the isolated proteins with single or a combination of proteases. In this respect, several studies have produced natural antihypertensive peptides that may be used to prevent or manage hypertension. The use of natural peptide sequences as antihypertensive agents is attractive becausein some patients pharmaceutical drugs have been reported to cause undesirable side effects and health complications such as dry cough, taste disturbances, and skin rashes during prolonged administration (He et al., 2013; Udenigwe, Lin, Hou & Aluko, 2009). Hypertension in human beings is regulated by the renin-angiotensin systems (RAS), which also ensures fluid homeostasis and a malfunctioning of the system could result in the development chronic diseases such

cardiovascular disease, chronic kidney and liver diseases, diabetes and cancer (Crowley & Coffman, 2012; He et al., 2013). Three enzymes named renin, angiotensin I converting enzyme (ACE) and chymase are the key catalysts involved in operation of RAS. The RAS operates as follows (Peach, 1997): renin, an aspartyl protease produced from the kidney cleaves 10 amino acids from the N-terminus of angiotensinogen (a product of the liver) to release the inactive angiotensin I (AT-I). Subsequently, ACE (peptidyldipeptidase A) catalyzes the conversion of AT-I to a pro-hypertensive octapeptide and potent vasoconstrictor angiotensin II (AT-II). ACE also inactivates a vasodilator named bradykinin, which leads to increased vasopressive effects and contributes to blood pressure (BP) elevation (Peach, 1997). Studies have shown that prolong therapy with ACE-inhibitory antihypertensive drugs leads to AT-I accumulation and could activate an alternate pathway involving chymase which is also capable of converting AT-I to AT-II independent of ACE; such a reaction can result in failure of the antihypertensive drug therapy (Borer, 2007). In contrast, there is growing evidence that direct inhibition of renin could offer a better control of elevated BP than ACE because accumulation of AT-I in substantial amounts that could be converted to AT-II in some organs via an ACE-independent pathway catalyzed by chymase is prevented. However, evidence suggests that sole inhibition of renin does not prevent ACE-catalyzed bradykinin degradation, therefore a dual strategy for inhibition of renin and ACE simultaneously is suggested to be the more effective therapy for BP control when compared to single ACE therapy approach (Harel et al., 2012; Udenigwe, Lin, Hou & Aluko, 2009). Currently the main research focus is to purify and identify food based antihypertensive or bioactive substances which would possess dual ability to inhibit renin

and ACE activities that could prevent or treat hypertension. Recently, quite a number of antihypertensive peptides with multi-functional properties capable of inhibiting ACE and renin enzymes *in vitro* and *in vivo* conditions have been produced from various plant and animal food sources. Peptides with dual ability to inhibit ACE and renin have through enzymatic hydrolysis been identified from seaweeds (Fitzgerald et al., 2012), yellow field pea seeds (Li & Aluko, 2010), flaxseed (Udenigwe, Lin, Hou & Aluko, 2009), rapeseed (He et al., 2013) and hemp seed (Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011). Li et al. (2011) also showed that a pea protein hydrolysate reduced renal renin output in a chronic kidney disease rat model after 8 weeks of oral intake through the diet. Our previous work also showed that dietary inclusion of a multifunctional hemp seed protein hydrolysate was effective in the prevention and management of hypertension in hypertensive rats (Girgih et al., to be submitted). Despite the potential bioactive properties as well as health benefits of hemp seed peptides, there is paucity of information in the area of structure-function of blood pressure-lowering peptides as well as enzyme inhibition kinetics studies. Structure-function studies will provide new information on relationships of amino acid composition and sequence with antihypertensive activity, which could enhance method development to produce potent peptides from food proteins. Information from structure-function studies could also enhance development of novel and potent peptidomimetics using known peptide sequences as templates. Kinetic studies will provide useful information to decide the optimal dose of the peptide needed to achieve maximal preventive effects. In addition, the mode of inhibition of ACE and renin by the peptides will provide additional information for structure-function studies. Therefore, the objective of this study was to determine the kinetics of ACE and renin inhibitions by

synthesized forms of previously identified active (antihypertensive) hemp seed peptide sequences.

9.2. Materials and Methods

9.2.1. Materials

N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Peptide sequences (WYT, WVYY, SVYT, PSLPA and IPAGV) were synthesized (>95% purity) by GenWay Biotech (GenWay Biotech Inc. San Diego, CA).

9.2.2 Methods

9.2.2.1 Kinetics of ACE Inhibition

The ability of synthesized hemp seed peptides to inhibit *in vitro* activity of ACE was measured in triplicates as previously reported (Udenigwe, Lin, Hou & Aluko, 2009). Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 µL of ACE (final activity of 20 mU) and 200 µL of peptide in 50 mM Tris–HCl buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. Tris–HCl buffer was used instead of peptide solutions in the blank experiment. ACE activity was expressed as rate of reaction (ΔA/min) and inhibitory activity was calculated using Eq. below:

ACE inhibition (%) = $[(\Delta A/\min_{(blank)} - \Delta A/\min_{(sample)})/\Delta A/\min_{(blank)}]\times 100$

where $(\Delta A/\min_{(sample)}$ and $[(\Delta A/\min_{(blank)}$ are ACE activity in the presence and absence of peptides, respectively. The concentration of each peptide that inhibited ACE activity

by 50% (IC_{50}) was calculated using non-linear regression from a plot of percentage ACE inhibition versus peptide concentration. The kinetics of ACE inhibition was studied with 0.0625, 0.125, 0.25 and 0.5 mM FAPGG. The mode of ACE inhibition was determined from the Lineweaver–Burk plots while inhibition constant (K_i) was calculated as the X-axis intercept from a plot of the slope of the Lineweaver–Burk lines against peptide concentration (Li & Aluko, 2005).

9.2.2.2 Kinetics of Renin Inhibition

In vitro inhibition of human recombinant renin activity was conducted according to the previously described method (Li & Aluko, 2010), using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37^{o}C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37^{o}C. Before the reaction, (1) 20 μL of substrate, 160 μL of assay buffer, and 10 μL of Milli-Q water were added to the background wells; (2) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of Milli-Q water were added to the control wells; and (3) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of peptide samples were added to the inhibitor wells. The reaction was initiated by adding 10 μL of renin to the control and sample wells. The microplate was shaken for 10 s to mix followed by incubation at 37^{o}C for 15 min, and then, the fluorescence intensity (FI) was recorded using excitation and emission wavelengths of 340 nm and 490 nm, respectively. The percentage inhibition was calculated as follows:

Inhibition% = [(FI of control well-FI of sample well)/(FI of control well)] X 100

The peptide concentration that inhibited 50% of renin activity was determined and defined as the IC_{50} value and was calculated by nonlinear regression as performed for ACE. The renin inhibition kinetics studies were conducted using 0.625, 1.25, 2.5, 5 and 10 μM of substrate in the absence and presence of peptides. Lineweaver-Burk plots were used to calculate inhibition constant as described above for ACE.

9.2.2.3 Statistical Analysis

Enzyme inhibition assays were conducted in triplicates and analyzed by one-way analysis of variance. Data are reported as mean ± standard deviation. Statistical significance of differences was evaluated by Duncan's multiple range test ($p<0.05$) using the Statistical Analysis Systems software version 9.2 (SAS, Cary, NC, USA).

9.3. RESULTS AND DISCUSSION

9.3.1. ACE-inhibitory Activities of Hemp Seed Peptides

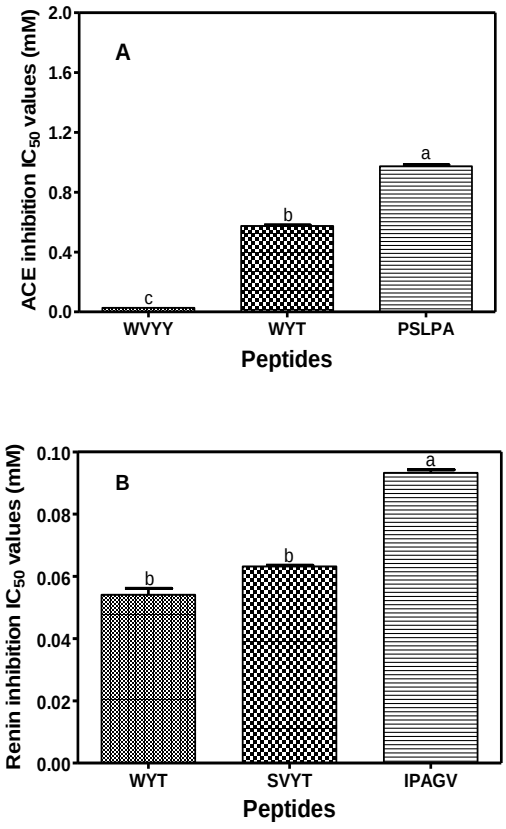
Three synthesized hemp seed peptide sequences, WYT, WVYY, and PSLPA were each tested at an inhibitory final assay concentration of 0.5 mg/mL and showed different ACE-inhibitory activities; their percentage inhibitions were 89, 91and 90% respectively. There were no significant differences ($p>0.05$) between the ACE-inhibitory effects of the tri- (WYT), tetra- (WVYY) and the pentapeptides (PSLPA). The higher inhibitory activities of the tetra- and pentapeptides compared to the tripeptide may be attributed to the presence of increased number of hydrophobic and aromatic amino acid residues. These residues are considered favourable peptide structural features for inhibition of ACE activity through the formation of hydrophobic interactions with ACE protein, which reduce catalytic ability. Fig. 1A shows that the IC_{50} values for the three ACE-inhibitory peptides ranged from 0.027-0.974 mM. WVYY had significantly ($p<0.05$) lowest IC_{50}

value (0.027 mM) and thus with better ACE-inhibitory activity than WYT (0.574 mM) and PSLPA (0.974 mM). Other reports of IC_{50} values are those published by Li & Aluko (2010) in which the synthetic pea dipeptides IR, KF & EF had higher IC_{50} values of 2.25, 7.23 & 2.98 mM, respectively when compared to results obtained in this work. He et al. (2013) also reported similar IC_{50} values for synthesized rapeseed di- and tetrapeptide sequences (LY, TF and RALP) to be 0.107, 0.810 and 0.648 mM respectively with the dipeptide (LY) showing the strongest inhibitory effect against ACE activity. The strong ACE-inhibitory activities exhibited by these plant protein-derived peptides is consistent with earlier reported results which showed that the presence of the C-terminal hydrophobic or aromatic amino acid residues such as *Pro, Phe, Tyr,* and *Trp* positively contribute towards enhancement of ACE-inhibitory activities (Kobayashi, Yamauchi, Katsuda, Yamaji & Katoh, 2008; Wu, Aluko & Nakai, 2006b). Moreover, other studies (Shu et al., 2011; Wang et al., 2011a; Wang et al., 2011b) have shown that the presence of branched-chain aliphatic amino acids (such as *Leu*) at the N-terminal as well as the availability of negative ionizable functions (e.g, in *Tyr*) may also contribute significantly to higher peptide inhibition of ACE activity. The peptides identified from hemp seed proteins, therefore have the potential to inhibit ACE activities in hypertension and could be used for functional foods product development.

9.3.2. Renin Inhibitory Activities of Hemp Seed Peptides

Renin catalyzes the first and rate-limiting step that initiates production of vasocontricting peptides responsible for BP elevation; therefore, down-regulation of its activity may enhance vasodilation. To the best of our knowledge, there is scanty information about the properties and occurrence of renin-inhibitory peptides from natural sources.

Figure 9.1: Inhibitory concentration (IC_{50}) of synthesized hemp seed peptide analogues (WVYY, WYT & PSLPA) and (WYT, SVYT & IPAGV) that reduced 50% of (A) ACE and (B) renin *in vitro* activities respectively. Bars with different letters are significantly different at $p<0.05$. Values are means (n=3) ± SD.



About two decades ago, most studies reporting renin-inhibitory properties mainly involved peptide analogues such as pepstatin, phospholipids and renin antibodies which had poor pharmacokinetic properties such as low bioavailability, high clearance and cost of their large-scale synthesis made them non-attractive as renin inhibitors for clinical use (Yuan, Wu, Aluko & Ye, 2006). Currently, there is only one known commercially available potent non-peptidic renin inhibitor (Aliskerin, IC_{50} = 0.6 nM), thus justifying the on-going research for renin inhibitors from naturally occurring food sources. *In vitro*

screening of the synthesized hemp seed peptides for potential renin inhibitory activities at 0.5 mg/mL peptide final concentration showed that the tetrapeptide (SVYT) had the strongest renin-inhibitory activity (85%), followed by IPAGV with a renin inhibition ability of 75% and WYT showed the least renin inhibitory capacity of 70%. This result is consistent with previous studies which stated that renin inhibitory activity is increased with increases in aliphatic chain length or molecular weight of the inhibitor and that potency is enhanced with increased aliphatic hydrophobicity (Wood et al., 2003; Wu, Aluko & Nakai, 2006b). The IC_{50} values of the synthesized hemp seed peptides identified in this study with potent renin inhibitory properties are shown in Fig. 1B. The tripeptide (WYT) exhibited the strongest inhibitory capacity with the lowest IC_{50} value 0.054 mM compared to the tetrapeptide (SVYT = 0.063 mM) and the pentapeptide (IPAGV = 0.093 mM). IC_{50} values of synthesized hemp seed peptides for renin inhibition in this work are very low compared to those obtain from previous studies (Li & Aluko, 2010) in which the pea dipeptide sequences (IR, KF & EF) had IC_{50} values of 9.20, 17.78 and 22.66 mM respectively. Similarly, higher renin-inhibitory activities were reported for rapeseed peptides RALP (IC_{50} = 0.968 mM), LY (IC_{50} = 1.868 mM) and TF (IC_{50} = 3.061 mM). The potent renin-inhibitory data obtained in this study is in agreement with results from previous work which suggested that good renin inhibitors should consists of all or at least some of the following characteristics: one hydrophobic side chain at the N-terminal and bulky chains at the C-terminal, one hydrogen bond donor and two hydrogen bond acceptors (John et al., 2011; Thangapandian, John, Sakkiah & Lee, 2011; Udenigwe, Li & Aluko, 2012). This is because the hemp seed peptides had most of these qualities as a result of the presence of *Pro, Leu, Tyr Trp, Ile* and *Val*.

9.3.3. Angiotensin I Converting Enzyme Inhibitory Kinetics

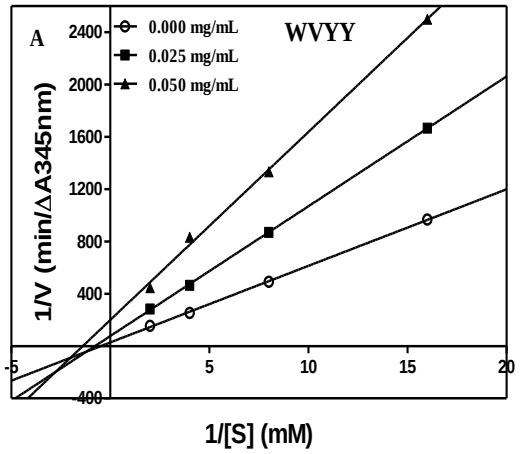
Two peptide sequences (WVYY & WYT) were selected for kinetics studies because they had the lowest IC_{50} values that were <1.0 mM. The Lineweaver-Burk plot was used for the estimation of the modes of ACE inhibition by the hemp seed peptides. The double reciprocal plots of ACE catalysed reactions in the absence and presence of the peptides are shown in Fig. 9.2A & B. ACE inhibition by the most active peptides was investigated using two concentrations, 0.025 and 0.05 mg/mL for WVYY and 0.1 and 0.4 mg/mL for WYT; each peptide concentration was assayed at four substrate levels as shown in Fig. 9.2A & B. From the non-linear regression fit of the kinetics data, the K_m value of ACE activity in the absence of the inhibiting peptide was estimated to be 2.078 mM and decreased dose-dependently in the presence of the WVYY (1.288-0.719 mM) and WYT (1.01-1.018 mM) as shown in Table 9.1. The decreases in K_m values during inhibition suggest that the peptides bind to the enzyme-substrate complex. The V_{max} for uninhibited ACE reaction was 0.0335 $Amin^{-1}$ and this velocity decreased dose-dependently in the presence of peptides (WVYY & WYT), though WVYY seem to be more effective. The results indicate that the activation energy was increased in the

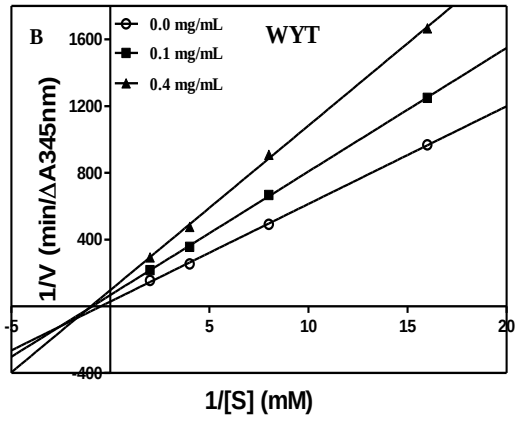
Table 9.1: Kinetics constants of ACE and renin catalyzed reactions in the absence and presence of different concentrations of synthesized hemp seed peptides (ACE: WVYY and WYT) and (Renin:WYT, SVYT and IPAGV) derived from pepsin-pancreatin digestion of hemp seed protein isolate; K_m and K'_{max} are Michaelis constants in the absence and presence of inhibitor, respectively; V_{max} and V'_{max} are maximum reaction velocities in the absence and presence of peptide inhibitor, respectively; CE, catalytic efficiency of ACE; K_i, enzyme-inhibitor dissociation constant.

	ACE-inhibitory peptides (mg/mL)					Renin-inhibitory peptides (mg/mL)						
Catalytic parameter	Control	WVYY		WYT		Control	WYT		SVYT		IPAGV	
		0.025	0.05	0.1	0.4		0.2	0.5	0.2	0.5	0.2	0.8
K_m and $K'_{max\ (mM)}$[a]	2.0781	1.2883	0.7189	1.099	1.0180	0.039	0.017	0.011	0.098	0.052	0.012	0.007
V_{max} and V'_{max}[b]	0.0355	0.0129	0.005	0.0148	0.0103	48.0	9.230	4.61	48.0	17.10	10.76	4.0
CE	0.0171	0.0100	0.0069	0.0135	0.0101	1.2422	0.5577	0.4175	0.4916	0.3258	0.9277	0.6015
K_i (mg/mL)				0.8042			0.6980		0.3936		0.7670	

[a]FAPGG as ACE substrate and (Quiñones et al., 2010)Arg-(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dabcyl)-arg in dimethylsulfoxide (DMSO) as renin substrate;[b]$V_{max,\ A\ min^{-1}}$

Figure 9.2: Lineweaver-Burk plots of angiotensin I-converting enzyme (ACE) inhibition by different concentrations of synthesized hemp seed peptides: (A) tetra- and (B) tri-peptides (WVYY & WYT, respectively) derived from pepsin-pancreatin digestion of hemp seed protein isolate at varying substrate concentrations (0.0625-0.5 mM). V = the initial rate of reaction (ΔA_{345}nm/min).





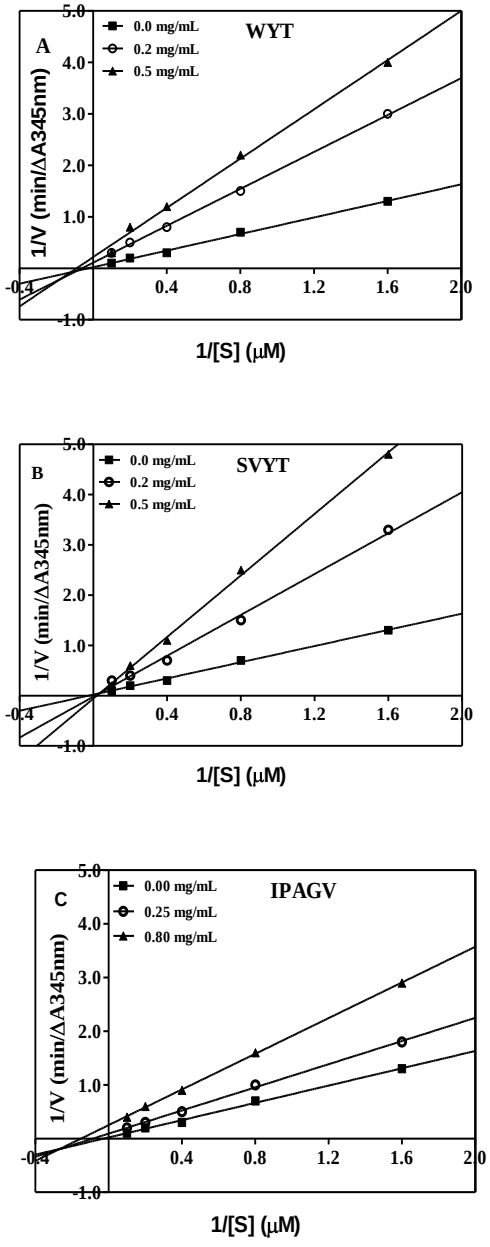
presence of peptides because the rate of enzyme reaction was slowed down. The K_m and V_{max} values obtained in this study are slightly higher than those previously reported for hemp seed protein hydrolysate (HPH) and its ultrafiltration membrane fractions previously (Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011). The catalytic efficiency (CE) of the uninhibited reaction was 0.0171 and decreased from 0.0135 to 0.0069 in the

presence of peptide inhibitors. The results suggest that the peptides have high affinity for binding to the enzyme, which led to reduced catalytic ability. WVYY and WYT exhibited a mixed-type mode of enzyme inhibition implying that the peptides bind to the ACE enzyme at positions other than the active-site in order to reduce catalytic activity. The K_i for the WVYY and WYT are 0.307 and 0.804 mg/mL, respectively, which are lower than the 2.550-4.740 mg/mL values reported for the HPH and its membrane fractions (Girgih, Udenigwe, Li, Adebiyi & Aluko, 2011).

9.3.4. Renin-Inhibitory Kinetics

Fig. 9.3A-C illustrates the double reciprocal plots of renin catalyzed reaction for the estimation of the mode of renin-inhibitory activities by the three synthesized hemp seed peptides, WYT, SVYT & IPAGV. The three peptides were found to display mostly mixed-type mode of renin inhibition. For example WYT and IPAGV reduced the K_m value but also reduced V_{max} while SVYT increased the K_m value but with similar or reduced V_{max} (Table 9.1). The results suggest that the peptides inhibited renin activity by binding to both the active and non-active sites of the enzyme to cause reduction in catalytic activity. Binding to the enzyme protein would have caused conformational changes that limited enzyme catalytic activity, usually by altering the space within the active site and preventing adequate entry of substrate molecules. In contrast, binding to the active site reduces access to substrate molecules, which would also cause reduction in enzyme catalytic activity. Renin inhibition by the peptides was also reflected in the reduced CE as peptide level was increased, indicating reduced ability of the enzyme toconvert the substrate into products. SVYT had the lowest K_i and will bind to renin more tightly than WYT and IPAGV; this could be responsible for the fact that SVYT

Figure 9.3: Lineweaver-Burk plots of the inhibition of human recombinant renin by different concentrations of synthesized hemp seed peptides: (A) tri-, (B) tetra- and (C) penta-peptide (WYT, SVYT and IPAGV, respectively) derived from pepsin-pancreatin digestion of hemp seed protein isolate at varying substrate concentrations (0.625-10 µM); V = initial rate of reaction (Change in fluorescence intensity/min).



produced the least renin CE. The K_m for renin substrate in absence of the inhibitor was determined to be 38.64 µM, which is higher than those from other studies: 1.3µM (Wang, Chung, Holzman & Krafft, 1993), 6.4 µM (Yuan, Wu, Aluko & Ye, 2006), 4.416 µM (Udenigwe, Lin, Hou & Aluko, 2009) and 4.01 µM (Ajibola, Eleyinmi & Aluko, 2011). The difference in K_m values could be attributed to the different sources from which renin enzyme was procured for these studies and the variation in experimental conditions that may exist. There is also the variation in enzyme activity between different batches even when purchased from the same source. It is difficult to make comparison here with other studies because there are no related works that have reported kinetics studies of food based peptides as renin-inhibitory agents. It could be concluded that the synthesized hemp seed peptides have strong renin-inhibitory activities that could make them useful for future structure-function studies.

9.4. CONCLUSIONS

Consistently in the on-going research work, simulated gastrointestinal digestion of hemp seed proteins has produced peptides which have shown *in vitro* and *in vivo* abilities to act as antihypertensive agents. In this particular study, we focused on confirming kinetics characteristics of the synthesized hemp seed peptides and showed that mode of inhibition differed depending on type of enzyme and peptide concentration. ACE activity was inhibited mostly through mixed-type mode whereas renin inhibition was mixed-type and competitive modes. The results indicate that both the ACE and renin active sites were probably accessible to the peptides in addition to peptides being able to bind to other parts of the enzyme, hence the mixed-type mode inhibition. The kinetics data suggest that the tetrapeptides WVYY and SVYT had greater affinity for ACE or renin and were more

active than the tripeptide WYT. However, the fact that WYT showed strong inhibitions of ACE and renin activities suggest potential use as an antihypertensive agent with dual mode of action. These structural differences may be exploited in future studies to design more active antihypertensive agents that work through inhibitions of renin and ACE. Overall, these results suggest that hemp seed peptides could be used to formulate health enhancing foods for the prevention and management of hypertension and other related chronic disease conditions.

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CHAPTER 10

10.1 GENERAL DISCUSSION AND CONCLUSIONS

Pharmaceutical drugs have been the main therapy employed for treatment of most chronic diseases such as diabetes, obesity, cancer hypertension. However, due to the problem of side effects associated with their prolong usage as well as increases in health care cost, there hasbeen increasing interest from consumers and researchers for a safer and cheaper therapy from food origin that could prevent, treat and manage human chronic diseases. Functional foods and nutraceuticals which are foods that provide beyond basic nutritional needs some added physiological/health benefits can play a major role in sustaining the health of the people while reducing health care cost and some of negative effects of pharmaceutical drugs. It has been estimated that consumption of functional foodsin Japan, Europe, United States, Canada and around the world generated market revenue of over $142 billion US in 2010 (Basu et al., 2007). Nutraceuticals and functional foods have been reported to have significant biological actions and their useacross the globe continues to increase due to historical and more recent reports of clinical success through useof these products (Hasler, 2000; Siró, Kápolna, Kápolna & Lugasi, 2008). Interest in functional foods has significantly increased over the course of time due to a number of key factors, including self-care health consciousness, increasing health care costs, advances in technology, changes in food regulation, market opportunity, and new emerging scientific findings linking foods/components to optimal health. This has resulted in a number of new foods in the market place designed to address specific health concerns, particularly as regards to chronic diseases of aging. The

challenge to food technologists and nutritionists will be to develop functional foods and nutraceuticals devoid of undesirable side effects arising from the added peptides, and to retain their stability throughout the shelf life of the product. Therefore testing the efficacy of these peptides in animal model and carrying out controlled trials in humans are mandatory when claiming a health effect for a food. This area of research is still lacking and needs more efforts geared towards it in the near future.

Bioactive peptides are some of the ingredients used for the formulation of functional foods and nutraceuticals and research has shown that these peptides obtained from both animal and plant origin have the potential to prevent and manage chronic ailments when consumed on a regular basis. Bioactive peptides have been shown to possess properties that may be advantageous to reducing oxidative stress associated diseases and improve cardiovascular health. These health effects are achieved by reducing free radical formation as well as the lowering of blood pressure and lipid levels. As part of the search for new sources of bioactive peptides from plant origin, we evaluated the bioactive potentials of defatted hemp seed meal, a by-product obtained after utilization of the vegetative crop parts for fibre and extracting the oil from the seeds.The defatted hemp seed meal was enzymatically hydrolyzed to produce peptide mixtures with structural characteristics that could be applied towards prevention, treatment and management of chronic diseases employing the bioassay-guided fractionation approach, *in vitro* and *in vivo*. This research work has therefore provided the first in-depth report on the physiological potentials of hemp seed peptides as antioxidants and antihypertensive agents. It is considered the foundation research platform upon which further research on hemp seed will be based and industries will rely on these scientific evidenceto develop

new hemp seed products that can enhance expansion of the functional food market. The work has also contributed to the elucidation of the structural characteristics of antioxidant as well as ACE and renin inhibitory food-protein derived peptides from hemp seed for the first time. This information will enrich the bioactive peptide data base and may be useful to chemists and pharmacists engaged in developing novel potent pharmaceutical products for health enhancement. The main findings of this thesis which will be considered the novel accomplishments of this research work are outlined below:

1) Simulated GIT digestion of HPI produced HPH which with its ultrafiltration membrane peptide fractions showed effective *in vitro* antioxidant properties with ability to scavenge DPPH and hydroxyl radicals, reduce ferric metal ions and chelate transition metals ions, an indication of their potential use to prevent or manage disorders arising from over production of ROS/free radicals. This workhas potentiallyestablished the claim that hemp seed proteins contain antioxidative peptide sequences and can be utilized in the development of functional foods thatwill reduce excessive production of ROS/free radicals that may cause oxidative stress related disorders.
2) This work also tested the ability of HPH and its ultrafiltration membrane fractions to act as antihypertensive agents. Results obtained showed that HPHand its fractions significantly ($p<0.05$) inhibited ACE and renin, the causative agents of hypertension. The HPH peptides had low IC_{50} which was indicative of strong enzyme inhibitory effects achieved through mixed-type mode ofinhibition meaning that they could attach to the enzyme protein or to its active site and still inhibit the enzymes' activities. These peptides exhibited high DH implying large

amount of small size peptides were generated during hydrolysis, which could serve as reservoir for peptides with potent physiological activities.The high yield of HPH could enhance profitable commercialization efforts.

3) HPH exhibited the overall best antioxidant and antihypertensive properties; therefore it was further fractionated using RP-HPLC to evaluate the fractions for enhanced bioactivities. Results showed that RP-HPLC fractionation improved the antioxidant properties especially in their ability to scavenging superoxide radical which the crude HPH was unable to accomplish. Fractions also showed enhanced inhibitory activities against ACE and renin enzymes, therefore these are potential candidates for production of nutraceuticals for therapeutic management of hypertension. However, the yield of peptides diminished when compared to the starting material (HPH). Therefore for economic viability to cut down the cost of production, it is advisable to utilize the hydrolysate in the development of health enhancing foods rather than engaging in the RP-HPLC separation exercise which is capital intensive.
4) Hemp seed peptides can potentially be utilized for the production of nutraceuticals peptide sequences with ≤ 5 amino acid residues using LCMS/MS spectrometry and 80% ofthe peptides showed antioxidant and antihypertensive properties. The structural information is critical in linking peptide structure to observed physiological activities. Additionally, the potent *in vitro* activities of the synthesized peptide sequences were validated in SHRs model in which the peptides significantly attenuated BP increases.

5) In order to directly utilize industrial hemp seed by-product without any pre-processing step of the defatted meal to save cost, the meal was directly hydrolyzed with pepsin and pancreatin to mimic GIT digestion and tested for antioxidant and antihypertensive properties. The results showed that peptide generated directly from the meal without alkaline isolation of the proteins exhibited similar antioxidant properties and ACE as well as renin inhibitory effects like the HPI-derived hemp seed protein hydrolysate (HPH). This result showed that direct utilization of hemp seed meal for generation of peptides for the formulation functional foods may not need prior isolation before the enzymatic hydrolysis step which will drastically save cost of production of the peptides. Moreover, when hemp seed peptide diets were fed to SHRs for long periods (4 & 8 wks), they significantly ($p<0.05$) attenuated SBP, reduced plasma peroxide, lipid profile, as well reduced plasma ACE and renin levels.These peptides may thus serve as antiatherosclerotic and antihypertensive agents.
6) The thesis also tested the synthesized peptides for potency and mode of inhibition against the activities of vasoactive enzymes (ACE & renin). Results revealed that the synthesized hemp seed peptides had potent antihypertensive properties and exhibited low ACE and renin IC_{50} values. The synthesized peptides were able to inhibit enzyme activity through binding to the active as well as non-active sites. Structural variations in the peptides were reflected in the antihypertensive effect, which could be used to develop more potent peptides.Confirmation of the dual inhibition of ACE and renin by WYT and other peptides provide useful information on structural properties of potentially multifunctional peptides.

The results obtained in this work will benefit farmers, who will increase production because of the higher demand for industrial hemp seed for various uses. Diversified utilization of hemp seed proteins could lead to new industries springing up that will provide employment and revenue to the government. The expanding hemp seed market will also promote international trade between nations which will ultimately benefit the consumers insearch of health promoting food alternatives to drugs.The results from this work have added a new dimension to the potential health benefits derived from industrial hemp seed components, and could offer the functional foods and nutraceutical industries some new bioactive peptide ingredients.

10.2 Novelty of the thesis findings

There is a general consensus in the literature that to effectively manage hypertension requires inhibition of the two vasoactive enzymes (renin & ACE) mainly responsible for elevation of BP simultaneously. Since the development of ACE inhibitor from the snake venom peptide template, several ACE inhibitor drugs have been produced and some purified from natural plant or animal sources and have been used as therapeutic agents for hypertension. Renin inhibitors are difficult to produce both from natural food and synthetic sources because of their poor bioavailability; hence there is only one known commercially available renin inhibitor (Aliskiren). Our research was able to produce and structurally identify novel peptides from hemp seed with dual functionality as inhibitors of both renin and ACE thereby having the potential to be used as antihypertensive agents that could effectively control BP elevationthus manage hypertension.Structures of these novel peptides could be used as template by pharmaceutical companies to develop potent inhibitors that may provide better control of BP. The following groups of peptides:

tripeptides (YNL, WSY, WVY, WYT & VYT); tretrapeptides (EFLQ, EFQI, EFQL, FEQI, VSYT, WVYY & SVYT) and pentapeptides (IPAGM, IPAGV, LALPA, LEEAF, PSLPA, PSIPA & IEEAF) with unique sequences were purified from hemp seed protein hydrolysate for the first time and showed multifunctional bioactivities including acting as antioxidants as well ACE and renin inhibitors. This implies that these peptides have the potential to prevent oxidative stress related diseases such as inflammation, diabetes, liver, kidney and cancer. In addition, these peptides have shown promise to be potent therapeutic agents for atherosclerosis and hypertension.

10.3 Limitations of this thesis work

A single thesis cannot provide all the answers to all research questions, which was the case with this work. The following areas would have been addressed to obtain holistic results regarding the bioactivity potentials of hemp seed peptides but for time constraint:

1) Due to time constraint, cell culture (*Ex vivo*) experiments and evaluation of the levels of endogenous antioxidant enzymes (Superoxide dismutase, Glutathione & Catalase) in the plasma of rats were not determined to validate the determined *in vitro* antioxidant potentials of hemp seed peptides.

2) Identification of hemp seed peptides was based on the high activity and abundance (% yield) of the most active fraction rather than being based on the activity and purity. There were no sufficient purification steps prior to selecting the most active peak for sequence identification.

3) Markers of kidney malfunction/disease were not tested to determine if the hemp seed protein hydrolysates and peptides caused any negative effects such as organ damage in the experimental rats.

10.4 Future direction

Finally, based on the positive outcomes of this work, it is worth further validating same experiments under *ex-vivo* conditions and carrying out cytotoxicity tests prior to engaging in human trials. This step-by-step validation of different experimental conditions and targets would increase the confidence level of the novel results obtained which may pave way for initiation of clinical trials to support the health claims associated with the consumption of industrial hemp seed proteins. Dose-dependent studies are required on all categories of peptides produced in this study to further determine the dose-relationship and establish the minimum effective dose needed to produce the desired effect when peptides are consumed first in animal experiments and then in humans. The use of quantitative structure activity relationship (QSAR) and DOCKING studies will provide useful information regarding the characteristics of peptides that will effectively inhibit ACE and renin such that future production of peptides will be tailored towards optimizing those qualities. The effect of peptides on genes expression could also be a possible focus in the specialized area called nutrigenomics, i.e., linking nutritional requirements of an individual based on his/her genome characteristics.

10.5 References

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