Structure-function properties of hemp seed proteins and protein-derived acetylcholinesterase-inhibitory peptides

by

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Abstract

Hemp seed proteins (HSP) were investigated for physicochemical and functional properties in model food systems. In addition, the HSP were enzymatically digested and the released peptides investigated as potential therapeutic agents. Membrane isolated HSP (mHPC) were the most soluble with >60% solubility at pH 3-9 when compared to a maximum of 27% for isoelectric pH-precipitated proteins (iHPI). However, iHPI formed emulsions with smaller oil droplet sizes (<1 µm) while mHPI formed bigger oil droplets. The iHPI was subjected to enzymatic hydrolysis using different concentrations (1-4%) of six proteases (*pepsin, pancreatin, flavourzyme, thermoase, papain* and *alcalase*) to produce various HSP hydrolysates (HPHs). HPHs had strong *in vitro* inhibitions of angiotensin converting enzyme (ACE) and renin activities, the two main enzyme systems involved in hypertension. Oral administration of the HPHs to spontaneously hypertensive rats led to fast and persistent reductions in systolic blood pressure. The HPHs also inhibited *in vitro* activities of acetylcholinesterase (AChE), a serine hydrolase whose excessive activities lead to inadequate level of the cholinergic neurotransmitter, acetylcholine (ACh). Inadequate ACh level in the brain has been linked to neurodegenerative diseases such as dementia and Alzheimer's disease (AD); therefore, AChE inhibition is a therapeutic target. The 1% pepsin HPH was the most active with up to 54% AChE inhibition at 10 µg/mL peptide concentration. The 1% pepsin HPH (dominated by <1 kDa) was subjected to reverse-phase HPLC peptide purification coupled with tandem mass spectrometry, which led to identification of several peptide sequences. Some of the peptides inhibited activities of both animal and human AChE forms with LYV being the most potent against human AChE (IC_{50} = 7 µg/ml). Thus the LYV peptide may serve as a useful template for the development of future potent AChE-inhibitory peptidomimetics. In conclusion, several novel AChE-inhibitory peptides were

discovered and their amino acid sequences elucidated for the first time. Results from this work identified HSP products that could serve as functional ingredients in the food industry. The work also produced and confirmed the *in vitro* AChE-inhibitory activities of several new peptide sequences that may serve as therapeutic agents for AD management.

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Before his departure from this world, he tried his best as a father; LATE (MR.) S. Malomo, GOD rest his soul (Amen). My sweet mother; MRS. G.E. Malomo, you always give me the motherly advice, prayer and needs. May God bless you abundantly to reap the seeds of motherhood.

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"He, who started the work in my life, will surely finish it." I therefore give praises, honour, adoration and glory to my creator, beginning, end, benefactor and my hope. He is my "I AM THAT I AM." The ever trusting and ever supporting GOD!

Malomo, S.A.

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Dedication

To the glory of Awesome and Ever-merciful GOD!

To those that put in all their efforts and supports to seeing me reaching higher heights in life.

FOREWORD

This report is written using the manuscript format. It is composed of six manuscripts (studies) after the general introduction and literature review chapters. The manuscripts are all prepared according to the appropriate journals' specifications and guidelines. For instance, study 1 is written according to styles of *Journal of Food Science*; study 2 in *Food Hydrocolloids* journal format; study 3 in *Innovative Food Science and Emerging Technologies* journal format; study 4 in *Nutrients* journal format; study 5 in *Journal of the American Oil Chemists' Society* format and study 6 in *Journal of Functional Foods* format. Manuscripts 1 and 2 have been published while manuscripts 3, 4, 5 and 6 are being prepared for submission into the above listed appropriate journals. All the studies (manuscripts) are concisely linked together by transition statements at the end of each study for proper flow of study designs and objectives accomplishment. The list of references cited for the general introduction and literature review parts is done according to the *Food Hydrocolloids* journal format. The last chapter of this report therefore, provides a general summary and conclusion of the study, limitations involved and future directions of the study.

Table of Contents

Chapter Five

Chapter Six

Chapter Seven

Chapter Nine

Chapter Ten

Chapter Eleven

List of Tables

List of Figures

LIST OF COPYRIGHT MATERIALS USED IN THE PREPARATION OF THE THESIS

1. Malomo, S. A., He, R., & Aluko, R. E. (2014). Structural and functional properties of hemp seed protein products. *Journal of Food Science,* 79 (8), C1512-1521. Copyright © 2014, with permission from John Wiley & Sons.

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

List of Abbreviations

ACE - Angiotensin converting enzyme

ACh – Acetylcholine

AChE – Acetylcholinesterase

ALB – Albumin

BP - Blood pressure

CD – Circular dichroism

DPPH - 1,1- diphenyl-2-picrylhydrazyl

FI - fluorescence intensity

GLB – Globulin

HPLC – High Performance Liquid Chromatography

HPI - Hemp protein isolate

HPH - Hemp protein hydrolysate

IPD – *In vitro* Protein Digestibility

LGC – Least gelation concentration

ME - Mercaptoethanol

OHC – Oil holding capacity

PS - Protein solubility

SDS - Sodium dodecyl sulfate

S_o - Surface hydrophobicity

THC - Tetrahydrocannibinol

WHC - Water holding capacity

CHAPTER ONE

1. General Introduction

The structure and function of proteins have always been critical indicators that determine the various physiological processes and functions occurring in any biological system (Barac et al., 2014). Scientific investigations into the dynamics of proteins centred on structure and function determinations in order to give a better understanding of their potential uses in foods and biological systems (MuneMune et al., 2014; Tan et al., 2014). For instance, protein activities' research works have brought extensive insight into various biological processes such as protein folding, enzyme functions and target identification of ligand binding to the receptor. Other processes include interaction of proteins with molecules such as the protein itself, lipids (oils), and water. All these processes aid the use of proteins as basic and bioactive ingredients in food and human health applications (Rasool et al., 2013).

As of today, many simple and easily-adopted methods available for protein isolation include, but are not limited to salt extraction, traditional isoelectric precipitation, and enzyme pre-digestion coupled with membrane filtration to remove non-protein materials. The analytical methods involved in protein dynamics studies related to food and human health applications are directed towards investigating physicochemical and functional properties in relation to environmental factors (pH, heat and concentration) (Barac et al., 2014). Apart from native proteins, research works have also been geared towards determining bioactivity potentials of low molecular weight polypeptide chains (peptides) of proteins in the management of several human diseases (Udenigwe & Mohan, 2014).

Several physiological imbalances and malfunctioning of body homeostasis have been the causative background of aggravated human chronic disease conditions such as Alzheimer's

disease (AD) and cardiovascular diseases (CVD) like hypertension (Health Canada, 2014). Various reports (Kumar & Chowdhury, 2014; Iannello et al., 2014; Saravanaraman et al., 2014; Ghribia et al., 2014) have shown the acetylcholinesterase (AChE) enzyme as an important target in research aimed at developing therapeutic tools against a wide range of human metabolic disorders. This is because AChE hydrolyzes the neurotransmitter acetylcholine (ACh) at cholinergic synapses, with a higher catalytic efficiency than other known enzymes. The dysfunction of cholinergic neurotransmission in the brain, the formation and growth of brain amyloid lesions, including the senility-promoting plaques have been widely reported as contributing factors to a Alzheimer's disease (AD) pathogenesis (Willcox et al., 2014). As of today the current available therapy for AD consists of the administration of AChE inhibitors due to their clinical efficacy in prolonging the half-life of ACh (Kumar & Chowdhury, 2014).

Recently, there is awareness in the food industry and in preventive health care activities with respect to the development of natural nutraceuticals and functional foods ingredients from plant materials due to their high biodegradability rate, which results in little or no toxicity (residual effects or negative side effects). An example of such plant materials is the industrial hemp (*Cannabis sativa* L.), which was traditionally grown for its high fibre content. However, the production of fibre from hemp leaves behind the hemp seed, which is a rich source of high quality oil (30%) and proteins (25%) (Radocaj et al., 2014). Therefore, recent efforts at hemp commercialization have actually focused on the seed because the high quality oil and proteins can serve as ingredients to formulate functional foods and nutraceuticals. This has led to a substantial growth in the hemp seed processing industry and an increase in the economic value of the crop.

In Canada, hemp seed processing primarily involves cold-pressing to expel the oil, which leaves behind a high protein (30-50%) residue. The increased utilization of hemp seed for edible oil production has led to abundant amounts of protein-rich meal, which has been converted into various forms of powdered protein products. These protein products are sold as ingredients for the formulation of foods such as plant-based protein shakes, hemp milk (vanilla or chocolate flavoured) and energy bars (House et al., 2010). Current commercial hemp seed protein (HSP) products are mainly in the form of protein concentrates that have less than 70% protein content but with high fibre levels (Lu et al., 2010). The high fibre and phytate contents of these HSP products reduce protein functionality and limit their use in the manufacture of novel food products (Teh et al., 2014). Reports have shown HSP to contain 65% globulin (edestin) and 33% albumin; edestin is composed of six identical subunits with the acidic subunit (AS) linked by one disulphide bond to a basic subunit (BS) (Teh et al., 2014; Teh et al., 2013; Park et al., 2012). In the plant kingdom, these two high-quality storage HSP have been reported to be readily digestible, and are rich in all essential amino acids with exceptionally high arginine and glutamic acid contents (Park et al., 2012).

HSP can be efficiently obtained from defatted hemp seed meal (HSM) by alkali solubilization followed by acid precipitation (Girgih et al., 2014a). The poor food functionality of current hemp seed protein products is due primarily to two reasons, first is the high fibre and phytate content in the concentrates and second is the poor solubility of the protein isolate, which is due to the use of harsh alkali extraction, acid-induced protein precipitation and cross-linking of proteins by phytate molecules (Teh et al., 2014). Therefore, in order to produce novel protein isolates with improved food functionalities, a method that involved fibre and phytate digestion followed by removal of non-protein materials through membrane ultrafiltration was used. The

membrane ultrafiltration obviates the need for the use of undesirable harsh protein precipitation protocols while ensuring high protein yield with minimal denaturation and a potential for strong functional performance in food formulations. This fibre and phytate digestion method had been previously used to obtain high protein, yield and quality soy (Wang et al., 2014), flaxseed (Udenigwe et al., 2009) and rice bran (Wang et al., 1999) protein isolates. However, pre-digestion with carbohydrases and phytases plus coupling with membrane ultrafiltration has not been reported for hemp seed protein isolate production.

The functional properties of the enzyme pre-digested and membrane-produced protein isolates that was determined in the present study include protein solubility (important in the formulation of clear beverages), emulsification (important for the manufacture of oil-in-water emulsions like salad dressings and soups), foaming (important in formulation of desserts such as meringues), and gelation (critical for formulation of semi-solid to solid foods). In addition, the work also studied the relationships between physicochemical properties (polypeptide composition, hydrophobicity, secondary & tertiary structures) and functional properties in order to gain fundamental understanding of the structural factors of the proteins that enhance their functional properties. The obtained protein isolate was then subjected to *in vitro* enzymatic digestion using different proteases to yield AChE-inhibitory and antihypertensive peptides. The active protein hydrolysates were then separated and purified by column chromatography in order to identify peptide amino acid sequences.

1.1. Hypotheses

In order to obtain basic knowledge existing between the structure and function of a protein in the biological system, the following hypotheses were proposed:

1) Enzyme and ultrafiltration membrane-assisted removal of non-protein materials will lead to the production of a hemp seed protein isolate with higher protein content than the isoelectric-precipitated equivalent.

2) The membrane protein isolate will have higher contents of native structural conformations and produce higher level of food functionality when compared to protein materials produced by isoelectric precipitation.

3) Optimized *in vitro* enzymatic hydrolysis of hemp seed protein isolate will lead to the release of multifunctional peptides that possess acetylcholinesterase inhibitory and antihypertensive activities.

1.2. Objectives

This study aimed to address some unanswered questions related to the effects of structure-functions properties of hemp seed protein products in food and human health applications *in vitro* and *in vivo* through the following main objectives:

1) Compare the physicochemical and functional properties of hemp seed albumin and globulin protein fractions,

2) Optimize cellulase and phytase degradation of hemp seed fibre and phytate, respectively, into low molecular weight products that can be removed by membrane ultrafiltration to produce HSP isolate with high protein content and food functionality,

3) Determine the *in vitro* digestibility, physicochemical and functional properties of laboratory-prepared HSP isolates in comparison with commercial hemp seed protein products,

4) Modify the HSP isolate using various enzymes (*alcalase, thermoase, trypsin, papain, chymotrypsin, pepsin*) to produce acetylcholinesterase-inhibitory and antihypertensive peptides, and

5) Purify and determine the amino acid sequence of acetylcholinesterase-inhibitory peptides present in active protein hydrolysates followed by a study of their structure-function properties.

1.3. Justification

Bioactive peptides with low serine-histidine ratio have the potentials to be used as potent agents for fast and effective managing of cognitive malfunction in the body system. Natural peptide products do not have negative side effects associated with synthetic drugs and the hemp seed peptides could serve as alternative anti-AChE agents. Moreover, peptides are more effective nutrition agents when compared to free amino acids. This is because unlike free serine and histidine molecules, low serine or histidine containing peptides have the advantage of being able to penetrate cells directly without the need for transporters. The use of this type of novel peptides is anchored on the fact that AChE is a serine hydrolase with specificity for serine and histidine; so the low ratio of these amino acids would make it unavailable for the enzyme or totally block the active site of action of the enzyme. Therefore, optimized feeding of low serine/histidine containing peptides could provide relief from Alzheimer's disease without the use of drugs. In addition to cognitive decline, hypertension is also a recognized health problem in the elederly population. Thus, the development of multifunctional peptides that can act simultaneously against cognitive decline and hypertension could provide innovative means of managing chronic diseases. More importantly such multifunctional peptides could reduce health care costs

associated with therapeutic treatment since one agent (as against the current practice of multiple agents) will be effective against more than one disease condition.

Conversion of protein constituents into clinical nutrition tools as well as functional and nutraceutical peptides are excellent ways of increasing the economic value of hemp seed. Moreover, different studies have revealed a strong market demand for bioactive peptides and thus, this research work would provide hemp seed peptides that satisfy part of this demand.

1.4. Significance

The significance of this study is based on the growing economic importance of hemp seed protein in Manitoba and Canada at large. For instance, there are currently two hemp seed processing companies (Hemp Oil Canada, St. Agathe; and Manitoba Harvest, Winnipeg) in Manitoba that produce various products including protein concentrates. Thus, this study is expected to increase value-added utilization of the products from these companies which, could lead to improved economic power of individuals (farmers, processors, suppliers and traders), provinces (Manitoba and environs) and Canada as a whole. Also critical to the choice is the fact that HSPs contain the highest level (~11% when compared to <6% for most other food proteins) of arginine (a precursor of the vasodilatory nitric oxide) among foods and as such, it could be used as a rich source of cardiovascular health-promoting amino acid. Finally, the outcome of this work may provide hemp seed peptides for use as ingredients to formulate therapeutic foods for AD prevention and treatment. Peptides could provide these health benefits without the negative side effects such as failure of cholinergic synaptic transmission, deterioration of neuromuscular junctions, flaccid muscle paralysis, and central nervous system seizures that are associated with use of AD drugs.

CHAPTER TWO

2. Literature Review

2.1. Why hemp?

Industrial hemp (*Cannabis sativa* L.) is one of dozens of plant species that represent at least 22 genera and contain <0.1% Δ-9-tetrahydrocannabinol (THC), a known phytochemical drug component in most *Cannabis* species. This particular species has been disruptively selected for bast (phloem) fibre in the stem, multipurpose oil in the achenes, and an intoxicating resin secreted by epidermal glands (Small et al., 2003). It is industrially grown primary for utilization in fibre production; however, the seeds recovered as by-products are then further processed for high quality oil and protein-rich meal (Radocaj et al., 2014). Despite the prohibition on its cultivation in many countries due to the presence of THC, this important crop still serves as an excellent source of food, fibre, dietary oil and medicine (Lu et al., 2010).

In Canada, industrial hemp has gained ground as an important agricultural commodity and now supports the operations of the two Manitoba hemp seed processing companies (The Hemp Oil Canada, St. Agathe; and Manitoba Harvest, Winnipeg). Besides its edible and high quality oil, the fibre-rich hemp plant had been widely used for paper and clothing productions in Canada (Lu et al., 2010). The most important hemp products in the health food market are whole hemp seed, hulled hemp seed, hemp seed oil and hemp protein meal. These hemp seed food products contain very low THC levels that cannot produce intoxication when ingested (Karimi & Hayatghaibi, 2005). In addition to the high quality oil, the legumin protein fraction (edestin) is specifically found in the hemp seed alone but the albumin fraction is very similar to those found in high-proteinous egg white, hence their higher protein quality when compared to other plant albumins (Callaway et al., 2002). Besides, unlike soybean proteins, the HSP is reportedly devoid

8

of the trypsin inhibitors (the albumin-like compounds that can reduce protein digestion) and flatulence-causing oligosaccharides (Karimi & Hayatghaibi, 2005).

2.2. The hemp seed protein

The major storage protein fractions in hemp seed are 2S albumin and 12S globulin. The 12S globulin – (edestin) is a legumin with a similar tertiary structure to 7S vicilins. These two storage protein fractions are secretory proteins synthesized with a signal peptide, which ensure their folded conformations within the lumen of the endoplasmic reticulum. They are also characterized by heterogeneity in molecular weights and charges (Callaway et al., 2002). The albumin fraction has a relatively high solubility in water whereas the globulin fraction is water-insoluble but can be solubilized mostly in dilute salt solution at pH values above or below the isoelectric points. The higher number of hydrophobic associations between these storage proteins helps to stabilize their interactions with other food system compounds (Haard, 1985). The HSP has been proven to contain all the nutritionally-essential amino acids, especially the highest level of arginine among plant proteins, consisting of up to 11% of protein weight when compared to <6% for most other food proteins (Yin et al., 2008).

The HSP has been utilized to produce bioactive compounds with desirable health benefits. Such beneficial effects include as an antioxidant (Girgih et al., 2014a), oxidative apoptosis protective agent (Chakrabarti et al., 2014), antihypertensive (Girgih et al., 2014b), hydrogen peroxide-induced apoptosis protective agent (Lu et al., 2010) and industrial basic functional ingredients (Teh et al., 2014; Teh et al., 2013; Yin et al., 2009; Yin et al., 2008).

2.3. Isolation methods employed for the production of plant protein isolates

The preparation methods (isolation/extraction) for most plant protein isolates are believed to contribute to the physicochemical, digestibility, functional and nutritional properties of

different protein isolate fractions. The protein extraction methods and conditions, downstream processing of extracted proteins (purification and drying), pH, temperature and ionic strength of the food system, have been the important factors that affect functional properties in food systems (Vioque et al., 2000). These intrinsic (structure and size) and environmental (protein separation/production method, pH, ionic strength) factors can vary or determine the dynamic utilization of plant proteins in various food industries. For instance, high oil and water binding proteins could find desirable applications in the food processing industries dealings in meats, sausages, breads, and cakes. Proteins with very high emulsifying and foaming capacity would find useful applications in salad dressing, sausages, bologna, soups, confectionery, frozen desserts and cakes (He et al., 2014b; Vioque et al., 2000). The different isolation methods that have been used to produce plant protein isolates are highlighted below.

2.3.1. Water- and salt-soluble protein isolation

The salt extraction protein isolation method is based on the ionic strength of the solution to dissolve and fractionate the two major storage proteins found in hemp seed - the 12S globulin and the 2S albumin (Park et al., 2012). The high glycoprotein (up to 45%) present in albumin fractions enhances protein-water interactions and thus aids its significant solubility in water. In contrast, the low glycoprotein (3.9%) present in globulin fraction might as well contribute to its water insolubility (Mundi & Aluko, 2012). The dissociation of subunits in these storage proteins might be affected by appropriate conditions like solvent, pH and the ionic strength (Haard, 1985). The salt extraction method has been previously used to isolate proteins from plant sources like canola and flaxseed (Karaca et al., 2011), buckwheat (Choi et al., 2007), kidney bean (Mundi & Aluko, 2012), *Ginkgo biloba* bean (Deng et al., 2011) and African locust bean (Lawal et al., 2005).

Dialysis of the salt extract against water followed by centrifugation produces a globulin-rich precipitate and an albumin-rich supernatant (Mundi & Aluko, 2012). Generally, the homologous storage globular HSP (edestin) has a molar mass of 300 kDa and its quaternary structure is composed of six subunits (Park et al., 2012). Albumin, which has a low molecular mass of 12–14 kDa is the other protein fraction of interest in most oilseeds. This typical rigid 2S protein fraction (albumin) is well stabilized by inter-chain and intra-chain disulfide bridges with 40–45% helix structure (Park et al., 2012). Since most native proteins have poor functional properties, different studies have shown that structural modification may be used to increase protein functionality of albumin and globulin (Tang et al., 2011; Bora, 2002). For example, chemical modification such as acylation was used to influence the surface functionality of rapeseed albumin and globulin fractions (Gerbanowski et al., 1999).

2.3.2. Isoelectric protein precipitation

The most common and simple method of protein isolation involves the use of alkaline solubilisation followed by acid precipitation at the isoelectric point (pI), for instance between pH 4.0 and 6.0 for seed proteins. At pH values above the pI (alkaline environment), the protein will be solubilized with or without heat application while at lower pH values (acidic environment) that coincide with the pI, the solubilized protein becomes insoluble, is precipitated out and recovered as a precipitate after centrifugation. In some cases, the protein isolates generated from this method may have high protein contents; however, protein recovery is incomplete, which might be due to lack of adequate solubilisation or loss in the supernatant during centrifugation of the precipitated proteins (Chavan et al., 2001). Moreover, all the proteins may not have the same pI, hence selective precipitation at a single pH value results in low protein recovery.

Another limitation of this method, besides the poor functional properties, is that the isoelectric-produced protein isolates are composed of higher amounts of ash generated during the acid-base neutralization procedure (Chavan et al., 2001; Tang et al., 2011; Yin et al., 2008, 2009).

2.3.3. Other methods for improving the functional status of protein isolates

In order to improve the status of the plant protein isolates as functional ingredients, several scientific methods have been put into use, which had invariably positively or negatively affected their functional and physicochemical properties. For example, succinylation of lentil seed globulin isolate significantly changed interfacial adsorption kinetics leading to improved foam capacity and emulsion stability (Bora, 2002). High pressure, high heat and combination of both methods were used to improve the physicochemical and functional properties of peanut, soy and rapeseed protein isolates, respectively (He et al., 2014b; Li et al., 2011a; He et al., 2014a). Higher solubility pea, chickpea and lentil protein isolates were obtained from ultrafiltration-based protein isolation (Boye et al., 2010).

The probable positive or negative effects resulted from types of isolation method to obtain plant protein isolates on its functional properties are described below. For instance, peanut roasting during production of peanut protein isolate was found to reduce all the functional properties whereas a reverse effect was reported when a fermentation method was used (Yu et al., 2007). Significantly higher differences in the functional properties, particularly emulsifying capacity, foaming capacity and oil binding, were observed for peanut protein isolates obtained by spray drying and vacuum oven drying (Yu et al., 2007). Other methods of improving the structure-function properties of plant protein isolates have involved the use of glycation on kidney bean vicilin (Tang et al., 2011); micellization on amaranth protein isolates (Cordero-de-

los-Santos et al., 2005) and limited enzyme hydrolysis on lentil protein isolate (Avramenko et al., 2013). The limited enzymatic hydrolysis method by trypsin was used to modify the functional properties of hemp protein isolates (Yin et al., 2008). The enzymatic hydrolysis improved only the protein solubility but led to decreased water and oil holding capacities as well as reduced foaming and emulsion properties. The improved protein solubility resulted from the transformation of insoluble protein components to soluble protein aggregates due to exposure of ionizable amino and carboxyl groups of the protein during the limited enzymatic protein hydrolysis. Meanwhile, the higher soluble aggregates in the hydrolysates were found to inhibit surface viscoelastic membrane formation, caused oil droplets coalescence and led to decreased emulsion capacity of the hydrolysates (Gbogouri et al., 2004). The shorter-chain lengths and low molecular weight sizes of the limited enzymatic hydrolysis products (hydrolysates) might account for the decreased foaming potentials (Yin et al., 2008).

The succinylation and acetylation (or acylation) methods were effectively employed to improve or modify the structure-function properties of hemp seed protein isolates (Yin et al., 2009). The succinylation and acetylation methods gradually increased the proteins' solubility, emulsion capacity (but decreased stability) and produced more compact structural conformation of the proteins. The limited hydrolysis of rapeseed protein isolates led to improved functional properties such as increased emulsification (Vioque et al., 2000). The limited proteolysis study showed a positive relationship between the degree of hydrolysis and functional properties of a protein. The hydrolysis process may have led to protein unfolding and enhanced exposure of hydrophobic amino acid residues, which increased protein interactions with the oil and hence better emulsification ability. Although, the limited enzymatic hydrolysis method of protein isolation helped to expose the hydrophobic amino acid residues which are buried in the protein

core (mostly inaccessible), the resultant hydrolyzed proteins could be bitter due to presence of low-molecular weight hydrophobic amino acid-rich peptides (Gong et al., 2015).

Hence, the limitations in most of these modification methods suggest the need to develop more efficient protein isolation protocols such as those that involve enzymatic pre-digestion of carbohydrates and phytates, which is then coupled to membrane ultrafiltration. Thus, the enzymatic digestion and membrane-based removal of non-protein compounds might be a good opportunity to obtain protein isolates with improved structure-function properties.

2.3.4. Enzymatic digestion of non-protein materials coupled with membrane ultrafiltration

Some plant proteins, especially hemp seeds have higher proportions of carbohydrates (mainly fibres, and soluble sugars) and phytate (antinutrient), which limit the yield and functional properties of protein isolates. These fibrous materials and phytate could be subjected to digestion or degradation by several types of carbohydrases like cellulase, hemicellulase, xylanase, amyloglucosidase and phytase. The reason for using a combination of carbohydrases rather than single entity is due to the differences in the sites (bonds) of their cleavage actions. For instance, cellulase breaks down the $\beta(1\rightarrow4)$-linkage bond of the glucose structure (cellulose) while hemicellulase and xylanase will hydrolyze the $\beta(1\rightarrow4)$-linkage bond of the xylose (hemicellulose and xylans) structure (Haard, 1985). Therefore, combined use of these carbohydrases enhances the possibility that most of the fibres will be hydrolyzed and can be separated from proteins. Use of the pre-digestion by these carbohydrases and phytase enzymes followed by the removal of the digested products by the membrane ultrafiltration is novel and has not been widely reported for plant protein isolate production.

Although, some recent works have reported the successful application of this novel method to produce high quality protein isolates, there is no report for HSP isolate production.

For instance, Wang et al. (2014) reported a protein isolate with higher protein contents (91-93%) and better *in vitro* digestibility when phytase was used to pre-process soybean flour. In the same vein, the works of Udenigwe et al. (2009) have shown that pre-digestion of the fibre in defatted flaxseed meal (known for its higher glucan and gum contents) with cellulase resulted in a flaxseed protein isolate having 23% protein content higher than the protein isolated from untreated flaxseed meal. Wang et al. (1999) produced a high protein content (92%), yield (75%) and better functional (foam capacity) protein isolates from the combination of xylanase and phytase pre-digestion of rice bran. Another study reported the use of microfiltration membranes processes without enzyme pre-digestion to obtain soy protein isolate of improved protein yield, solubility, foaming and emulsifying properties (Chove et al., 2007).

2.4. Structure-function of protein and protein isolates

The utilization of proteins as foods or food ingredients is dependent on its structural and functional properties. These properties (the important determinants of quality protein) can be modified and improved by any processing to enhance its utilization in the food and human health applications (He et al., 2014a). Hence, the various structural and functional characteristics of plant proteins that are essential for modification by appropriate processing (treatments) methods are discussed below.

2.4.1. Polypeptide composition and profiles

The protein polypeptide composition and profiles can be analyzed in the laboratory using the gel electrophoresis method. Polyacrylamide gel electrophoresis (PAGE) is used for separating proteins ranging in size from 5 to 2,000 kDa due to the uniform pore size provided by the polyacrylamide gel (Schägger, 2006). The protein samples are usually analyzed by sodium dodecyl sulfate PAGE (SDS-PAGE) and by native-PAGE. The polypeptide profiles of the non-

denatured protein in their natural forms are determined by native-PAGE while the denatured forms are analyzed by SDS-PAGE (Niepmann & Zheng, 2006). Gel electrophoresis is a common method used to study mostly the size or length of the protein molecules. The main principle behind gel electrophoresis is that proteins travel through the gel towards the positive pole and away from the negative pole of the electrophoresis machine (Arndt et al., 2012). In the native-PAGE analysis, the protein charges and mobility are dependent on the primary amino acid sequence of the protein and the attained pH during the electrophoresis procedures (Arndt et al., 2012).

Prior to the SDS-PAGE analysis, the proteins undergo denaturation in the presence of a detergent such as sodium dodecyl sulfate (SDS) that coats the proteins to make them negatively charged (Berg et al., 2002). Once denatured, the proteins form long rod-like structures instead of their spiral complex tertiary shapes and move across the gel strictly based on molecular size because they have the same SDS:protein ratio and therefore, have similar net charge (Niepmann & Zheng, 2006). The charge to mass ratio of the protein is very important in this type of analysis; therefore, the resulting denatured proteins have an overall negative charge while the relative protein size is dependent on the amount of bound SDS (Berg et al., 2002). Since the SDS binds to the same amount of proteins, the polypeptides will have similar negative charge density and differ only in mass (or size).

Comparing the molecular sizes of the protein bands formed on the resultant gel, the smaller the molecular size of the protein compounds, the further it would travel towards the anode due to faster passage through the gel. Thus, the larger molecular size polypeptides would move slowly towards the anode and can be separated from the faster moving smaller polypeptides. Typical polyacrylamide gel density (degree of cross-linking) used for proteins is 4-

25% either as homogenous or density gradient type. In order to fully resolve very small proteins sizes and peptides, homogenous high density (usually ≥20%) should be used (Schägger, 2006; Berg et al., 2002). Besides molecular size and polypeptide composition determination, PAGE is also used to determine protein purity after isolation and extraction.

2.4.2. Protein hydrophobicity

Research works that investigate extensive protein conformational studies involves determination of intrinsic and surface hydrophobicity, which are mainly based on the fluorescence properties of tyrosine, phenylalanine and tryptophan (Szabo, 2000). It is noteworthy that most intrinsic fluorescence studies involve determination of tyrosine and tryptophan emissions due to their high molar absorptivity, although tryptophan has always been the most dominant (Schmid, 1989). Fluorescence spectroscopy has been used in various protein research works to study structural hydrophobicity due to its high sensitivity level, rapid data acquisition, wide dynamic range and convenient instrumentation (Szabo, 2000). Cheftel et al. (1985) reported that hydrophobic interactions of native protein structures are solution-dependent. This is because hydrophobic side chain amino acid residues that are buried in the protein interior need to be exposed to the surface in order to measure hydrophobicity. Therefore, the solution properties (pH, salt, temperature) will determine position of the aromatic amino acids and dictate the magnitude of measured hydrophobicity. The distribution of polar and hydrophobic groups of amino acid side chains has been suggested to determine protein solubility since these two groups always have opposite interactions in solution (He et al., 2013).

Surface hydrophobicity, which is measured by ANS-binding experiments, can be coupled with modifications that produce conformational changes and alter surface functional groups in the protein (Matsudomi et al., 1985). Such modifications, which could be by deamidation-

induced and acid-induced denaturations were found to increase protein surface hydrophobicity. For example, a previous study reported great improvement (good adsorption kinetics in the interface and decreased molecular size) on the surface behaviours of soybean glycinin after mild acidic treatments (Wagner & Gueguen, 1995).

2.4.3. Protein structural arrangement and conformation

Protein structural conformations are supported by the carbon-bonded sulfhydryl, thioethers and carbonyls functional groups, which help to govern all the functional and biological activities (Marsh & Teichmann, 2013). These activities are dependent on the primary structure (amino acid sequence), secondary structure (α-helices and β-sheets stabilized largely by hydrogen bonds), tertiary structure (3-D organization of secondary structures via disulphide bonds) and quaternary structure (assembly of multiple folded or coiled protein subunits) of the protein (Forman-Kay & Mittag, 2013). Therefore, any alteration in the secondary, tertiary and quaternary structures of the protein could lead to denaturation, a resultant effect that takes place when hydrogen, ionic or hydrophobic bonds are disrupted due to changes in temperature, pH, interfacial area and/or presence of organic compounds (Nooren & Thornton, 2003). Proteins are linear polymeric compounds composed of well-defined amino acid sequences that become folded in required specific conformations for functional and biological activities (Rodger & Ismail, 2000). The various intramolecular hydrogen bonding arrangements and relative orientations within the environment defines a protein's planar and rigid, but rotational, structure.

The common features associated with chiral secondary (α-helix, β-sheet, β-turns and unordered) and tertiary protein structures have been studied using circular dichroism (CD) techniques. In protein chemistry, CD has been used extensively to give useful information about the protein structure (structural conformation), the stability of the designed protein fragments, as

well as the extent and rate of structural changes that occurred within the protein structure (Kelly & Price, 2000). During extraction, isolation, digestion and characterisation, the structural integrity of proteins can become altered; therefore CD is an extremely useful technique for assessing protein conformational changes or alterations (Rodger & Ismail, 2000). CD technology is based on the differential absorption of left and right circularly polarised radiation by chromophores of the protein samples when placed in either intrinsic chirality or chiral environments. This would give an empirical gauge of the structural arrangement and conformation of the protein (Rankin et al., 1998).

The peptide bond absorption in the protein chromophores gives rise to the CD signals at the far ultraviolet (UV) region of 240-180 nm to represent the contents of regular secondary structural features (Cheftel et al., 1985). The other spectrum in the near UV region at 320-250 nm reflects the aromatic amino acid side chain environments to give information about the tertiary structure of the protein (Kelly & Price, 2000).

2.4.4. Behaviour of protein isolates in solution

The behaviour of plant proteins in solution has been used as an arbitrary means of classifying storage proteins into different fractions. Protein molecule solubilization is a simultaneous process that involves wetting, swelling, solvation, and dissolution within the solution. The critical determinant of protein functionality in food processing and applications has always been solubility in an aqueous solution (Karaca et al., 2011; Chove et al., 2007). Protein solubility results from the intermolecular repulsion caused by modification (e.g. succinylation) process of the protein' charges and thereby ionize the interior non-polar groups of a protein (Bora, 2002). The modification disrupts native protein structure through polypeptide chain unfolding and subsequently exposure of buried functional groups. Several factors have been

documented to affect protein solubility, among which are, ionic strength, pH, food matrix medium and temperature. Another critical factor is the isoelectric point of the protein which is the pH at which the protein molecule carries no net electrical charge (Tsumura et al., 2005). The protein molecule derives its overall charges from the different positive, negative, neutral or polar amino acids that make up its complex structure. Thus, the protein will possess a net positive and negative charge at a pH below and above its isoelectric points, respectively. The amphoteric nature (containing both acidic and basic functional groups) of the protein, causes precipitation at the pH corresponding to the isoelectric point where solubility is minimum (Tsumura et al., 2005).

Additionally, the variety or species of plant protein sources may also have great importance in determining solubility and hence, quality functional properties of the final isolates or any other products from the protein (Barac et al., 2014). While reports have been made on use of SDS-PAGE to profile protein molecular weights, the isoelectric focusing (IEF) has been a tool or technique used to relate the functional properties (solubility) of a protein to the charge carried by individual polypeptide chains (Chove et al., 2007). Apart from the effect of isoelectric point in determining the protein solubility, the environment pH is also an important determinant (Achouri et al., 2005). Protein solubility is one of the major functional properties that have been known to be pH-dependent and this had been observed and reported for different protein sources and fractions. For example, the minimum solubility of some proteins have been reported to be at pH 4.5 for glycated soy 11S globulin (Achouri et al., 2005), and pH 4.8 for kidney bean globulin (Mundi & Aluko. 2012).

2.4.5. The water, oil and gelation properties of protein in the food system

The indices of protein interactions with water and oil as well as protein' gelation attributes in the food systems have been termed as water-holding capacity, oil-holding capacity and least gelation concentration of the protein. The water holding capacity is a quantitative indication of the amount of retained water (i.e. entrapped water) within a protein matrix under certain defined conditions (Chou & Morr, 1979). For example, functional food protein ingredients are customarily produced in a dehydrated form with the understanding that once present in the food system, the polypeptides will absorb and hold water for proper processing of such food products (Chavan et al., 2001). This is because the functional properties (reflected in the foaming and emulsifying capacity) of any protein isolate are closely related to the moisture content and water activity of the dried isolates. The protein isolates interact with water in the food system at different levels such as structural, monolayer, un-freezable, hydrophobic, imbibition/capillary and hydrodynamic hydration water (Chou & Morr, 1979).

The water holding capacity of protein isolates is also affected by the protein molecular conformation changes brought about by formation of new polar-polar or hydrophilic-hydrophobic interaction pairs of the protein (Adebowale et al., 2011). The poor water holding capacity of some proteins could possibly result from their low hydrophilic properties as determined by the proportion of hydrophilic to hydrophobic amino acids (Nosenko et al., 2014). For example, rapeseed proteins (Nosenko et al., 2014) have abundant levels of hydrophobic amino acids and therefore, possessed lower water holding capacities than the soy proteins. Water holding capacity is also affected by the protein isolate extraction method as shown by the higher capacity obtained for *bambara* groundnut, soybean (Adebowale et al., 2011) and chickpea (Paredes-Lopez et al., 1991) protein isolates prepared by micellization when compared to

isoelectric precipitated isolates. The differences could be due to greater exposure of polar groups and ability to form hydrogen bonds with water in the micellar protein structures, whereas the isoelectric precipitation method caused the protein aggregation to produce a structure that have limited interactions with water (Stone et al., 2014).

On the other hand, oil holding capacity is related to emulsifying ability, another functional property that deals with hydrophobicity. The method of extraction, protein species and sources determine the oil holding capacity of proteins, which is also dependent on protein surface properties. For example, the oil holding capacity of spring rapeseed protein isolate was ~30% higher than that of winter rapeseed protein isolate (Nosenko et al., 2014). More so, the salt extraction and micellization methods produced pea protein isolates with higher oil holding capacity than the isoelectric precipitated proteins (Paredes-Lopez et al., 1991). Besides, another study reported higher oil holding capacity for micellized Bambara nut protein isolates than the isoelectric pH-precipitated ones (Adebowale et al., 2011). While the protein with higher amounts of hydrophilic groups near the surface will hold onto more water, the protein with higher amounts of hydrophobic groups near the surface holds more oil. The increased non-exposure of protein hydrophobic amino acids to the surface when present in the aqueous solution also influences oil holding capacity. The oil holding capacity has influence on the use of proteins as potential functional ingredients in foods such as high-fat bakery products, doughnuts and emulsion-type foods (Liu et al., 2013).

Protein gelation results from aggregation through limited protein swelling and as a result of the formation of disulfide bonds. Gelation also arises from heating that increased protein-protein interactions thereby causing a tighter gel structure (Chou & Morr, 1979). The protein network is needed to form a gel; therefore, a proper balance between the attractive and repulsive

forces on the respective polypeptide chains has to be present. The dependency of gel formation on protein concentration is depicted when a higher protein concentration was used to form a rigid gel structure from denatured globular proteins. The attractive forces produced an insoluble protein precipitate while the rich disulfide cross-linkages led to an irreversible gel formation. The interaction between protein and water plays an important role in gel formation once the protein molecules are arranged in the appropriate (unfolded) molecular conformation (Okezie & Bello, 1988). This interaction properly activates the protein molecules into an unfolded conformation, which enhances protein-protein interactions that lead to three-dimensional gel network formation (Okezie & Bello, 1988).

2.4.6. Behaviour of proteins at the air-water interface

Protein-rich meals from industrial oil extraction processes have been utilized as valuable raw materials in the production of highly-functional ingredients for health application and novel food formulations (Hojilla-Evangelista et al., 2013). Therefore, the behaviour of proteins at interfaces, for instance at the air-water interface has been a special interest for every food processor due to the much valued foam formation (Hojila-Evangelista et al., 2014). Foams essentially constitute the group of dispersed air bubbles in continuous aqueous, semi-aqueous or solid phases of a food system (Ptaszek, 2013; Balerin et al., 2007). It is noteworthy that the presence of air determines density of the phase structure, the mechanical properties and conditions of the continuous phase while increasing spreadability, homogeneity appearance and distribution uniformity of the foam (Ptaszek et al., 2015; Żmudziński et al., 2014).

Good foam is characterized by protein molecular flexibility, structural disorder and metastability; thus once formed, it is thermodynamically stable over a period of time (Ptaszek et al., 2015; Żmudziński, et al., 2014). Foam disintegration or collapse can be halted by modifying

the interface through surface tension reduction and essentially opening the protein molecule to activate its hydrophilic and hydrophobic groups. The presence of long polysaccharide chains (sugars) might influence the foaming mechanism of the protein molecules (for example, albumin) thereby leading to increase in foam formation and behavioural hydrocolloids as previously observed (Mundi & Aluko, 2012). The foaming capacity of any protein is the ability, under certain conditions (i.e. concentration, pH, temperature) to form a foam, while foam stability indicates how well such protein can retain the foam volume over a desirable period of time (Barac et al., 2010). The foaming properties of protein isolates were reported to be influenced by protein concentration, pH, high pressure, thermal treatment, foam formation procedure, nature and behavior at interfaces (denaturation, protein–protein interactions) as well as their interactions with other food ingredients (Stone et al., 2014). At high pH values (alkaline), very low foam stability is obtained as a result of increased net charge-induced weak protein-protein interactions, which reduce the ability of the protein to form strong interfacial membranes at the air-water interface (Tan et al., 2011). The methods of extraction and drying (spray, freeze and drum drying) also have influence on the foaming properties of the protein isolates.

The differences in the foaming capacity and foam stability of plant protein isolates as affected by the extraction method have been fully documented by several authors (Hojila-Evangelista et al., 2014; Aluko et al., 2005; Pedroche et al., 2004). For example, salt solution-extracted and spray-dried pea protein isolates produced higher foams than the isoelectric precipitated proteins (Stone et al., 2014). However, foam stability is influenced by various factors like the protein adsorption at the water-air interface, the surface rheological properties, diffusion of the air out and into foam cells, size distributions of the cells, liquid surface tension, external pressure and temperature (Hojila-Evangelista et al., 2014). Several plant protein isolates

have been studied in the past for their foaming property, which is one of the desirable protein functional properties in food processing industries such as milk and dairies, breweries and confectioneries (Hojilla-Evangelista et al., 2013; Mundi & Aluko, 2012; Barac et al., 2010; Aluko et al., 2005; Pedroche et al., 2004).

2.4.7. Behaviour of proteins at the oil-water interface

Normally, interactions between water and oils (lipids) are not possible in food systems; therefore, the ability of proteins to act as the intermediary agent that facilitates mixing of the two surfaces (water and oil) is defined as the emulsifying capacity (Vioque et al., 2000). This is very important in the food processing industry and human health applications because the capacity of the hydrophobic residues of the protein to interact with oil while the hydrophilic parts interact with water would help to form stable emulsions and enhanced formed-oil-water-phases of most formulated foods. The surface properties of proteins, which include amino acid composition and structural conformation, have extensively aided applications in food processing industries for coatings, films and emulsions.

Besides their structures, the functional properties of proteins are dependent on their interactions with water and lipids (Barac et al., 2012). Various detailed and comprehensive investigations have been done on the emulsification properties of the major storage hemp seed proteins (12S globulin and 2S albumin). For example, the high level of disulfide bridge-linked polypeptide chains found in the hemp seed globulin protein fractions may contribute to the formation of stable emulsions.The suitability of any protein isolate as an emulsifier is dependent on the rate at which the polypeptides diffuse into the interface and their structural conformation as influenced by interfacial tension (surface denaturation). This is achieved through a relative low molecular weight, balanced amino acid composition (charged, polar and non-polar residues),

solubility, well-developed surface hydrophobicity, and a relatively stable conformation (Barac et al., 2012).

A positive correlation between the surface hydrophobicity, surface tension, and emulsifying activity index of the protein has been reported (Wagner & Gueguen, 1999; Matsudomi et al., 1985). For example, the emulsion forming property of proteins is dependent on a substantial decrease in the interfacial tension as a result of protein adsorption at the oil-water interface (Lqari et al., 2002). The energy barriers (electrostatic, structural and mechanical) from the interfacial layer that oppose destabilization processes determine the emulsion-forming ability of protein (Lqari et al., 2002).

2.5. Enzymatic protein hydrolysis to produce bioactive peptides

The concept of bioactive foods was actualized to provide natural foods (functional foods), food ingredients or dietary supplements (nutraceuticals) that demonstrate specific health or medical benefits towards the prevention, management and treatment of diseases beyond basic nutritional functions (Sharma & Singh, 2010). Broadly, it is believed that complex food constituents (like plant proteins) can be hydrolyzed by natural gastrointestinal tract or microbial enzymes and their digestion products (bioactive peptides) used as treatment or preventive agents against various human metabolic disorders (Sharma & Singh, 2010). The *in vitro* protein digestibility of plant proteins determines their level of hydrolysis (digestion) by proteinases to produce these bioactive peptides (Marambe et al., 2013). Thus, environmental conditions like pH, temperature, time and concentration are the major factors that contribute to the successful *in vitro* protein digestion (Moyano et al., 2014).

Another factor is the specificity of the digestive proteases as well as that of the targeted enzymes that are responsible for pathogenesis of the chronic diseases. This specificity (whether

open or folded conformation) determines their activity and interactions with the substrates (plant proteins) and bioactive peptides (in the case of disease-targeted enzymes) in a competitive or non-competitive manner. Therefore, enzymatic protein digestion involves breaking down the long polypeptide chains into shorter chains that could fit into the specific active site of the disease-related metabolic enzymes. The peptides may also bind to non-active sites on the target disease-causing enzymes through hydrophobic or electrostatic interactions. This is the basis for using bioactive peptides to inactivate disease-related metabolic enzymes during management of chronic diseases (Udenigwe & Mohan, 2014).

Although, the clinical (human intervention) trial issues still remain unclear for some products, different scientific studies have confirmed the production of bioactive peptides from plant proteins through *in vitro* digestion processes. The multi-functionality and potential health benefits of protein-derived bioactive peptides in the management of human diseases include anti-inflammatory, cardioprotective, radioprotective, antihyperglycemic, antitumor, antigenotoxic, antidiabetic, prevention of atherosclerosis and dyslipidemia treatment (Ferreira et al., 2010). The bioactivity of food plant protein-derived peptides is dependent on the degree of hydrolysis (Onuh et al., 2013), protein solubility (He at al., 2013), protein content (Mundi & Aluko, 2014), hydrophobicity (He at al., 2013), structural conformation (He at al., 2013) and amino acid arrangement (Girgih et al., 2014b).

2.6. Targeting the renin angiotensin system (RAS) for improved human health

The dual effects of excessive angiotensin I-converting enzyme (ACE) and renin activities in preventing adequate vasodilation coupled with elevated vasoconstriction (a hypertension-causing factor) have been the major causes of high blood pressure (BP) in human cardiovascular health. Inhibition of ACE and renin activities has been the main mechanism responsible for the

health benefits of several antihypertensive agents, especially synthesized drugs (Ibrahim, 2006). The common pharmaceutical drugs such as captopril, lisinopril, enalapril, ramparil, and zestril have been used for partial or total control of hypertension. However, the use of some of these drugs is associated with some negative side effects such as erectile dysfunction, dry cough, angioedemas and diarrhea (Hernandez-Ledesma et al., 2011). Another shortcoming of these drugs is that they function mainly as ACE inhibitors, which causes elevated plasma renin concentration through increased renal secretion (Pihlanto & Mäkinen, 2013).

Meanwhile, in order to regulate RAS more efficiently, direct renin-inhibition could potentially prevent the occurrence of elevated BP because renin is the first enzyme in the RAS pathway and it converts angiotensinogen to angiotensin-I, the latter being an ACE substrate (Udenigwe & Aluko, 2012). Inhibition of renin reaction would provide a more effective blockade of the RAS and concurrently lead to normal physiological blood flow conditions. The search for effective antihypertensive agents has triggered research in the areas of plant protein-derived bioactive peptides, specifically peptides that have dual inhibitory activities against renin and ACE (Lopez-Barrios et al., 2014; Udenigwe & Aluko, 2012). Single or dual-acting bioactive peptides with higher hydrophobicity and different amino acid residues have been produced, purified by RP-HPLC and found to possess strong RAS regulating ability (Fitzgerald et al., 2014; Mundi & Aluko, 2014; Yu et al., 2013; Siow & Gan, 2013; Harnedy & FitzGerald, 2013; Barbana & Boye, 2011; Chel-Guerrero et al., 2012; Barbana & Boye, 2010; Udenigwe et al., 2009; Li et al., 2005).

Besides functioning as *in vitro* RAS regulators, some of these purified plant protein-derived bioactive peptides have also shown antihypertensive effects during animal and human intervention trials. For example, the *trypsin*-produced tripeptide (HQG) and hexapeptide

(IVGRPR) caused a decrease in systolic BP of spontaneously hypertensive rats (Li et al., 2004). Likewise, the *thermoase*-produced dipeptides (VY, IY, FY and IW) had very strong BP-lowering effects in SHRs (Sato et al., 2008). More so, Li et al. (2011b) reported the efficacy of pea protein hydrolysates in reducing elevated BP in humans when given at a dose of 3g/day in order to substantiate the efficacy of plant protein-derived peptides in human chronic diseases intervention.

2.6.1. Mechanism of actions of RAS inhibitors

The mechanisms of action of ACE and renin in the RAS pathway have been fully reviewed and reported (Udenigwe & Aluko, 2012). The main mechanism involves the first and rate-limiting step activation of the catalytic enzyme, renin to convert angiotensinogen into angiotensin-I. Once formed, the angiotensin-I is then converted to angiotensin–II by ACE, which cleaves a dipeptide residue from angiotensin-I C-terminus. The resultant angiotensin-II has been described as a powerful vasoconstrictor that binds to tissue receptors, which causes blood vessel contractions. In some instances, ACE degrades bradykinin, a vasodilatory peptide that is involved in nitric oxide production. Although, this sequence of renin- and ACE-catalyzed reactions constitute the normal physiological BP-regulating events in the human body, the excessive production of angiotensin-II leads to severe blood vessel contractions (without adequate relaxation) and is a causative factor for hypertension (Lopez-Barrios et al., 2014). Therefore, inhibition of ACE and renin activities can lead to RAS down-regulation, which contributes to BP reduction and is a major means by which antihypertensive agents exert their health benefits.

Previous works have shown that some peptides inhibit ACE or renin activity through mixed inhibition mode of action, which means either binding to both the enzyme active catalytic

site or non-active site residues (Aluko et al., 2015; Girgih et al., 2014b; Li et al., 2004; Sato et al., 2002). For instance, when the bioactive peptides (inhibitor) bind to the targeted enzymes (ACE and renin) at the active site, the catalytic site is altered and no substrate can bind to it but the inhibitor can be displaced at high substrate concentrations. This is known as competitive inhibition process in which the peptides have a close structural resemblance (amino acid sequence, hydrophobicity, or hydrophilicity) to the enzyme substrate (Sato et al., 2002). In this case, it competes with the substrate molecules for the active site of the targeted enzyme and the peptides remain bound to the enzyme and exclude substrate molecules from the active site of the enzymes while it remains attached (Sharma, 2012; Sharma & Singh, 2010).

In contrast, the binding to the non-active site residues of the targeted enzymes (otherwise known as non-competitive inhibition) involves peptides that have no structural resemblance to the enzyme's substrates, which then binds to enzyme at a region other than the active site. The enzyme-peptide interaction leads to a change in the structural conformation of the enzyme protein such that the active site configuration is modified to prevent adequate fit (or entry) of substrates. This process renders a proportion of the targeted enzyme molecules inactive and as a result, the effective targeted enzyme activity is decreased (Sharma, 2012).

Moreover, the peptides can bind to the enzyme-substrate complex and change the structural configuration of the substrates in such a way that it will not be able to bind to the active sites of the targeted enzymes. This process is known as uncompetitive inhibition, in which the peptides (inhibitors) is neither binding to the active sites nor non-active residues of the targeted enzymes (ACE and renin) but rather bind to the enzyme-substrate complex to limit catalysis rate (Udenigwe & Aluko, 2012). The following plant protein-derived bioactive peptides *viz: Ser-Val-Tyr-Thr* (Girgih et al., 2014b); *Leu-Trp; Ile-Tyr* (Sato et al., 2002) and *Ile-Trp; Phe-*

Tyr (Sato et al., 2002) have been found and reported to possess competitive, non-competitive and uncompetitive inhibition, respectively against both ACE and renin activities.

2.6.2. Protein-derived bioactive peptides as RAS inhibitors in cardiovascular health

Proteases were found to produce low molecular weight compounds (peptides) from the polypeptide chains of complex plant proteins. The bioactivities of a large pool of peptides from plant proteins through *in vitro* and *in vivo* studies, have been exploited and reported (Fitzgerald et al., 2014; Zhang et al., 2014; Doyen et al., 2014; Alashi et al., 2014; Girgih et al., 2014a, b, c; Mundi & Aluko, 2014; Siow & Gan, 2013; He at al., 2013). These bioactive peptides were purified, identified and tested for their capacity to attenuate RAS activity and lower elevated systolic blood pressure (SBP) of the spontaneous hypertensive rats (SHR) under various conditions.

Natural bioactive peptides have the unique property of acting in a focused manner (targeted cure) as well as on the whole body (multi-targeted) whereas the synthesized pharmaceutical drugs act mostly on only one biochemical reaction (Fitzgerald et al., 2014). Moreover, plant protein-derived bioactive peptides are devoid of negative side effects that are associated with synthetic compounds (Udenigwe & Mohan, 2014). Table 1 below shows the various bioactive peptides identified from plant proteins, which are found to effectively reduce the high BP *in vivo* after a very short period of time. The findings (Table 1) also reveal that the inhibitory activities of these peptides are dependent on the sources of protein, amino acid sequences and doses of administration to the rat models. The very shorter chain dipeptides possess faster SBP-lowering effects within short period of time than longer chain peptides. Bioactive peptides possess higher efficacy due to their faster rate of absorption and bioavailability in the gastrointestinal tract because of the availability of peptide transporters and

Table 1- Plant protein-derived bioactive peptides effectively used to reduce high BP in spontaneously hypertensive rats models

Plant protein sources	Identified peptide sequences	Dose/ Administration route	Response/ Inference (ΔSBP mmHg)	References
Sweet potato tuber	ITP, IIP, GQY	500 mg/kg hydrolysate; Oral	-30 after 8 h	Ishiguro et al., 2012
Rapeseed protein	VW, IY	7.5 mg/kg; Oral	-9.8 to -10.8 after 2 h	Marczak et al., 2003
Rice protein	TQVY	50 mg/kg; Oral	-40 after 6 h	Li et al., 2007
Corn gluten meal protein	AT	30 mg/kg; Oral	-9.5 after 2 h	Yang et al., 2007
Flaxseed protein	RW, SVR	200 mg/kg; Oral	-17.9 after 2 h	Udenigwe et al., 2012
Mung bean protein	KDYRL, VTPALR	600 mg/kg hydrolysate; Oral	-30.8 after 6 h	Li et al., 2006
Wheat bran	LQP, IQP, VY	10 mg/ml; Oral	-45 after 2 h	Nogata et al., 2011
Hemp seed protein	WVYY	30 mg/kg; Oral	-34 after 2 h	Girgih et al., 2014b
Rapeseed protein	GHS	30 mg/kg; Oral	-17.29 after 6 h	He et al., 2013
Pea seed protein	LTFPG, IFENLQN	30 mg/kg; Oral	-37 after 2 h and 4 h, respectively	Aluko et al., 2015

ability to diffuse into cells based on chain hydrophobicity.

The use of plant protein-derived bioactive peptides in human clinical trials is still under serious investigation and critical consideration in order to avoid any unethical application of these bioactive compounds. Although, a past study has substantiated the efficacy of plant protein-derived peptides in human chronic diseases intervention to reduce elevated-BP when the human volunteers were given a 3 g/day-dose of pea protein hydrolysate (Li et al., 2011). Yet, various concerns are still being generated to ascertain the purity and safety of these plant protein-derived bioactive peptides in human clinical trials; therefore, these concerns need to be looked into before the peptides can be adopted for human therapeutic uses. The low molecular weight peptides are absorbed faster than the unhydrolyzed proteins that require digestion in the gastrointestinal tract. Thus the biological effects of unhydrolyzed proteins are invariably delayed when compared to peptides as therapeutic tools for chronic disease management (He et al., 2013; Alashi et al., 2014).

2.7. Acetylcholinesterase (AChE) in human health

The cholinesterase is the generic name given to the enzymes; Acetylcholinesterase (AChE, E.C.3.1.1.7) and butyrylcholinesterase (BChE, E.C.3.1.1.8), which are found as part of the human nervous/neurological systems (Kozurkova et al., 2011). While AChE hydrolyses acetylcholine (ACh) that is mainly associated with nerves and muscles and typically found on the synapsies, BChE, a plasma cholinesterase or pseudocholinesterase, hydrolyses butyrylcholine (BCh). Although not naturally found in the body, BCh is a known synthetic compound used to distinguish between AChE and BChE (Chen et al., 2015; Pohanka, 2013).

BChE is a non-specific cholinesterase enzyme that binds nerve agent in the bloodstream before it can exert effects in the nervous system (Chen et al., 2015; Brus et al., 2014). BChE is

synthesized by the liver and found in large concentrations in serum (Delfino et al., 2009). The assay of plasma-BChE activity can be used as a pathological liver function test (Pohanka, 2013). The major differences between these cholinesterases are their respective preferences for substrates and degradation half-life. The substrates and half-life of AChE are ACh and 2 days while that of BChE are BCh and approximately 8–16 hours (Huang et al., 2007; Wenthold et al., 1974).

Besides its role as an effective modulator of the central and peripheral nervous systems, the ubiquitous process-regulatory nature of AChE such as in cell growth, locomotion or apoptosis has given it a priority over BChE during recent investigations (Tumiatti et al., 2010; Camps et al., 2010). Therefore, the emphasis in this thesis work is on AChE activities and its inhibition by hemp seed protein-derived peptides. AChE (a serine hydrolase) has long been known to play a very crucial physiological role in maintaining the major neurotransmitter (ACh) in the body by rapidly clearing free ACh from the synapse (Kumar & Chowdhury, 2014). ACh is released by presynaptic cholinergic terminals and is the main neurotransmitter involved in cholinergic neurotransmission through activation of nicotinic and/or muscarinic receptors to modify postsynaptic cell functions. Normally, AChE rapidly and efficiently degrades ACh into acetate and choline, thereby terminating its signaling action (Kolář et al., 2010). AChE is a membrane-bound glycoprotein, which is present in cells in the form of a monomer or an oligomer; however, this is dependent on the type of cells and the enzyme variant (Kolář et al., 2010).

During neurotransmission, ACh is released from the ACh receptor when its concentration in the synaptic cleft is very low, so that a cholinergic neuron can receive another impulse. The release of ACh can lead to accumulation of ACh in the synaptic cleft and then results in impeded

neurotransmission. Meanwhile, AChE was found to possess a very high catalytic activity to help hydrolyse the excess ACh into free choline and acetate. For instance, each molecule could degrade about 25,000 molecules of ACh per second which approaches the limit allowed by diffusion of the substrate (Quinn, 1987). As old age or body imbalance cases arise, the rate of ACh being released is very low but AChE continues to hydrolyze the very little ACh meant for neurotransmission. This is invariably leads to halting of nerve pulse, cholinergic dysfunction and other major symptoms observed in the pathogenesis of chronic AD. Based on this occurrence at the onset or prior to onset of AD, the effective inhibition of AChE with agents that are tolerable, flexible and cost-effective is therefore, needed to manage this chronic disease (Consumer Reports, 2012, Accessed April 2015).

The following are the compounds that function as commercial reversible AChE inhibitors, which are mostly likely found applicable for therapeutic uses in cognitive health, viz: *carbamates, physostigmine, neostigmine, pyridostigmine, ambenonium, rivastigmine, galantamine, demecarium, donepezil, tacrine (also known as tetrahydroaminoacridine (THA), edrophonium, ladostigil, ungeremine*, and *lactucopicrin* etc (Alzheimer's Disease Medications Fact Sheet, Accessed January, 2015; Consumer Reports, 2012, Accessed April, 2015). Their clinical use has been reported to be effective in treating myasthenia gravis (orally) and reversing neuromuscular block (intravenously) (Consumer Reports, 2012). In actual fact, this current report contains the approved drugs by the United State Food and Drug Administration (US-FDA) for Alzheimer's diseases (Table 2) among all these listed drugs (inhibitors). These FDA approved drugs are mostly synthetic or inorganic in nature and their common adverse effects have limited their practical therapeutic use in AD patients.

Based on these negative side effects and recent health awareness of the natural and organic materials, the use of plant protein-derived bioactive peptides have now developed and if successful, may be commercialized for use as drug alternatives for human cognitive health promotion. AChE action also causes decreases in choline acetyltransferase (ChAT) level, which is the rate-limiting enzyme during ACh synthesis (Lane et al., 2006). Apart from the typical role of AChE in the termination of cholinergic neurotransmission, AChE has also been observed in non-neuronal tissues (erythrocytes, megakaryocytes etc.) but without significant catalytic functions (Sperling et al., 2008). The expression of AChE under neurodegenerative disease conditions has been reported to undergo changes at different levels during proliferation, neurite growth, apoptosis and differentiation (Sperling et al., 2008).

Equation 1 summarizes the catalytic action of the AChE on the neurotransmitter ACh as follows:

Acetylcholine　　　　　　　　　　　　　　　　　**Acetate**　　　　　**Choline**

(Eqn 1)
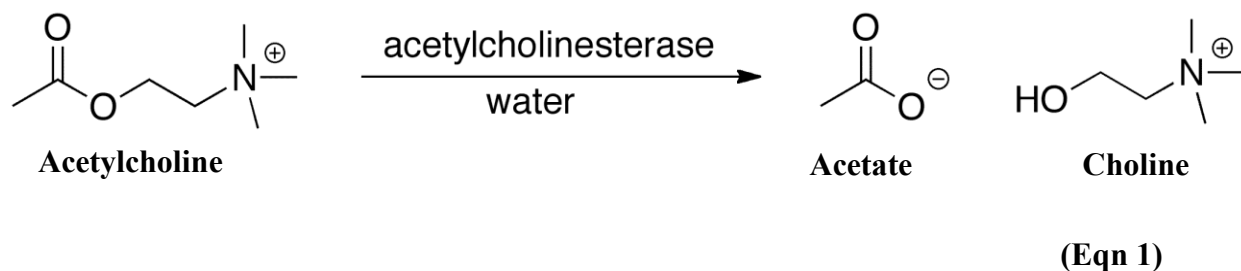
2.7.1. Mechanism of actions of acetylcholinesterase inhibitors

Currently, the precise mechanisms of potent AChE inhibitors have not been fully elucidated, although the ability of the plant protein-derived bioactive peptides to exhibit mixed inhibition mode of action (as described under the RAS inhibition section), can serve as a potential mechanism of action for AChE inhibitors. For instance, the bioactive peptides (inhibitor) can competitively bind to the targeted enzyme (AChE) at the catalytic site to alter its

36

Table 2- FDA approved drugs for Alzheimer's diseases*

Drug (Brand)	Class and Indication	Mechanism of Action	Common Adverse Effects
Donepezil (Aricept)	Cholinesterase inhibitor prescribed to treat symptoms of mild-to-moderate and moderate-to-severe AD	Prevents the breakdown of ACh in the brain.	Nausea, vomiting, diarrhea
Galantamine (Razadyne)	Cholinesterase inhibitor prescribed to treat symptoms of mild-to-moderate AD	Prevents the breakdown of ACh and stimulates nicotinic receptors to release more ACh in the brain.	Nausea, vomiting, diarrhea, loss of appetite, weight loss
Memantine (Namenda)	NMDA antagonist prescribed to treat symptoms of moderate-to-severe AD	Blocks the toxic effects associated with excess glutamate and regulates glutamate activation	Dizziness, headache, constipation, confusion
Rivastigmine (Exelon)	Cholinesterase inhibitor prescribed to treat symptoms of mild-to-moderate AD.	Prevents the breakdown of ACh and BCh in the brain.	Nausea, vomiting, diarrhea, loss of appetite, weight loss, muscle weakness

*AD - Alzheimer's disease; NMDA – N-methyl-D-aspartate. Table adapted from Alzheimer's Disease Medications Fact Sheet (2015) (National Institute on Aging) with open access permission.

active site (*Ser-His-Glu*) and hence, hinder the attack/bind on the substrate (ACh). There could be non-competitive and uncompetitive inhibition cases, when the bioactive peptides (having no amino acid sequence to bind to the active sites of the AChE) attached to the targeted enzyme at the non-active residues. In both cases, there exists a change in the structural conformation of the targeted enzyme molecule and substrate, respectively thereby decreasing or terminating AChE activity (Saravanaraman et al., 2014; Singh et al., 2013).

However, there is a previous report that AChE can terminate cholinergic nerve transmission by inactivating ACh (Katzung, 2001). This enzyme, which is found both on the post-synaptic membrane of cholinergic synapses, and in other tissues e.g. red blood cells, binds to and hydrolyzes ACh to acetate and choline (as illustrated in equation 1 above). Therefore, the use of AChE inhibitors prevent excessive hydrolysis and increases ACh concentration at the synaptic cleft, which enhances proper nerve impulse functioning. Brain AChE has been the major therapeutic target of AChE inhibitors as a treatment strategy for AD. The dysfunction of cholinergic neurotransmission in the brain, the formation and growth of brain amyloid lesions and the senile plaques, are widely believed to be contributing factors to a crucial event in the pathogenesis of AD (Kumar & Chowdhury, 2014).

AD, a leading cause of dementia in developed countries, has been reported as a progressive, degenerative disease characterized by memory loss, language deterioration, poor judgment and impaired visuospatial skills, among others (Darvesh et al., 2003). Besides cognitive improvement, AChE inhibitors with low cytotoxicity could be widely used as multifunctional agents for neuroprotective effects, such as antioxidants to suppress the increased oxidative stress resulting from free radical damage to cellular functions (Fernández-Bachiller et al., 2010). Although interactions of AChE inhibitors with other apoptotic molecules during

apoptosis are not completely clear, their actions have been suggested to improve AD therapy. For instance, recent studies have reported the beneficial apoptotic capacity of AChE inhibitors (Ye et al., 2010; Toiber et al., 2008). This action of AChE helps to potentially down regulate p53 and Bcl-2 family protein expressions during ischemia/reperfusion-induced apoptosis and also blocks the overexpression of the cell death-induction synaptic variant, *N*-AChE-S.

Recently, available therapies that have been selectively designed for human health in the form of commercial AChE-inhibitory drugs include *Donepezil, Galantamine, Rivastigmine,* among others. The essence of administering these AChE-inhibitory drugs is as a result of their clinical efficacy in prolonging the half-life of ACh in the cognitive system (Darvesh et al., 2003). Meanwhile, the dietary plant polyphenols, a broad class of naturally occurring and potent compounds (also regarded as antioxidants), have been found to provide neuroprotection against AD (Wang et al., 2006; Ramassamy, 2006; Dai et al., 2006; Arendash et al., 2006; Rezai-Zadeh et al., 2005; Yang et al., 2005; Lindsay et al., 2002; Lim et al., 2001). For instance, plant polyphenols such as quercetin, catechin, curcumin and resveratrol have shown decreased prevalence rate of AD when consumed from their dietary sources such as red wine, curry, green tea, coffee, fruit and vegetable juices among others (Dai et al., 2006; Ringman et al., 2005; Lindsay et al., 2002; Chandra et al., 2001; Orgogozo et al., 1997).

The areas of ethnopharmaceutical research in AD management indicate promising potential of *Berberis vulgaris* as source of new compounds for the AChE inhibition when its polyphenolic-hexane fraction showed active inhibition (64%; IC_{50} 68 μg/ml) activities (Kolar et al., 2010). The neurotrophic effects of plant polyphenols (IC_{50} 16-73 μg/ml) to improve cognition in AD patients was demonstrated and reported when several plant species used traditionally exhibited a high *in vitro* AChE inhibition (Vinutha et al., 2007). It is noteworthy

that human clinical studies reported the polyphenolic extract, EGb 761 from *Ginkgo biloba*, to produce neuroprotective and cognition-enhancing effects by maintaining the neurotransmitter, ACh in the cognitively-affected adult patients (Birks & Grimley, 2009; Gold et al., 2002). For instance, the polyphenolic extract provided modest cognitive benefits in patients with mild-to-severe AD when 120 mg/day of the polyphenolic extract was orally administered (Le Bars et al., 2002; 2000; 1997). Besides, the mild-to-moderate AD patients reportedly performed significantly better (cognition, behaviour, daily living activities) than the placebo-treated group after consumption of the polyphenolic extracts (Napryeyenko & Borzenko, 2007).

2.7.2 Protein-derived bioactive peptides as acetylcholinesterase inhibitors in cognitive health

The major limitation to the utilization of the synthetic drugs that have been described earlier lies with the fact that they are all characterized by common negative side effects like nausea, vomiting, muscle cramps, decreased heart rate (bradycardia), decreased appetite and weight loss as well as increased gastric acid production (Ghribia et al., 2014). However, recent approaches employed to address this critical problem of negative side effects of these synthetic drugs are based on the use of plants products and their secondary metabolites (Ghribia et al., 2014; Iannello et al., 2014; Kumar & Chowdhury, 2014; Zare-Zardini et al., 2013). The emerging innovation and nutritional knowledge in food and food products to improve the human health result from the concept of functional food (i.e. the relationship between food and health). Hence, the conceptual fact about functional foods in consumer's health is presumed to help in overcoming all these negative side effects associated with synthetic drugs in cognitive health (Peressini & Sensidoni, 2009).

However, there has been scanty report on the use of plant protein hydrolysates and peptides as AChE inhibitors. Naturally occurring bioactive peptides (hydrolysates) from plant proteins are attractive alternatives because they are not known to exhibit negative side effects like synthetic drugs (Zhang, Mu, & Sun, 2014; Barbana & Boye, 2010). As previously indicated, peptide-dependent AChE inhibition can be achieved either through competitive (active site blockage) or non-competitive (attachment to non-active site) actions (Saravanaraman et al., 2014; Singh et al., 2013).

2.8. Separation, fractionation, identification and purification of multifunctional bioactive peptides

The most widely used technique for multifunctional bioactive peptides separation, fractionation, identification and purification is the high or ultra-performance liquid chromatography (HPLC and UPLC respectively). This process can also be employed for the structural characterization and amino acid sequencing of these bioactive peptides by coupling the HPLC with tandem mass spectrometry (Singh et al., 2014). The amino acid sequencing identification is done by consulting updated and accurate peptide banks of significant MS/MS data using search engines such as MASCOT, SEQUEST, X!TANDEM and/or OMSSA (Craig & Beavis, 2004, Geer et al., 2004).

The accurate peptide database is updated with lists of precursor proteins in their active forms as well as their known post translational modifications. Such databases used in peptide sequences identification include, but are not limited to, UniProtKB, BioPep, PepBank, EROP-Moscow, BioPD, PeptideDB, Peptidome and APD (Panchaud et al., 2012). The bioactive peptides from different plant proteins have been compiled and annotated in various peptide databases, which provide information on their activities (Panchaud et al., 2012).

The *de novo* sequencing method can also be an important tool to derive the potential sequences of the bioactive peptides with high level of confidence for proteins whose primary structures are not yet available (Seidler et al., 2010; Picotti et al., 2008). This method involves the use of reliable computer-based interpretation of a product ion spectrum from potential sequences or sequence tags of a particular parent ion. For instance, excellent commercial software such as PEAKS Studio, with detailed modules for dynamic procedures like *de novo* search, database search, PTM search and quantification, has been a handy tool for peptide *de novo* sequence identification (Seidler et al., 2010). The utilization of tandem MS system for *de novo* peptide sequencing through the purity of the peptide ions being selected for fragmentation, is influenced mainly by the performances of both the MS/MS and the connected liquid chromatography system (Schlosser & Lehmann, 2002).

Prior to the use of HPLC-techniques, another separation or fractionation method, ultrafiltration membrane system (UMS), could be used to separate or fractionate the peptides based on their molecular weight. For example, the UMS-screening separation of soy protein hydrolysate produced the potential anticancer, antioxidative, antiviral and immuno-modulatory bioactive hydrolysates (Roblet et al., 2012) for human health applications. Some of the equipments attached to HPLC for further peptide identification or purification are: electrospray ionization (ESI), matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-mass spectrometry (specially designed for peptide profiles generation) and tandem mass spectrometry detection (LC–MS/MS) (specially designed for peptide sequencing and identification) (Singh et al., 2014).

Various multifunctional bioactive peptides have been separated, fractionated, identified and purified for application in the management and improvement of several diseases that affect

human health. It is noteworthy that RAS-inhibitory bioactive peptides from different plant proteins, have been isolated, purified and identified for hypertension management and general human cardiovascular health (Udenigwe & Mohan, 2014). For instance, a strong (IC_{50} 6.25 µg/ml) ACE-inhibitory tripeptide (Gly-Pro-Pro) was purified and identified from buckwheat protein hydrolysates using protein sequencing and electrospray-LC–MS system (Ma et al., 2006). Likewise, a low molecular weight (0.4 kDa) antioxidant tetrapeptide (Ala-Asp-Ala-Phe) was obtained (using RP-HPLC-ESI-MS) from walnut protein hydrolysates (Chen et al., 2012). Moreover, a strong (IC_{50} 14 µg/ml) adipogenesis-inhibitory tripeptide (Ile-Gln-Asn) was purified and identified from black soybean protein hydrolysates (Kim et al., 2007). The bioactive tetrapeptide (Trp-Val-Tyr-Tyr) and pentapeptide (Pro-Ser-Leu-Pro-Ala) were both identified by LC-MS/MS from hemp seed protein hydrolysates and shown to exert maximum systolic blood pressure reduction of -34 and -40 mmHg, respectively, after oral administration to SHRs (Girgih et al., 2014).

Despite the abundance of information on the purification and identification of RAS-inhibitory and antihypertensive bioactive peptides from plant proteins, there exists little or no information on AChE-inhibitory peptides from plant proteins. Therefore, additional efforts are needed to fractionate, purify and identify plant protein-derived AChE-inhibitory peptides, which could be used for future management of AD and other human cognitive diseases.

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CHAPTER THREE

3. Study on the structural and functional properties of hemp seed protein products

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Short version: Structure-function of hemp seed proteins

ABSTRACT: The effects of pH and protein concentration on some structural and functional properties of hemp seed protein isolate (HPI, 84.15% protein content) and defatted hemp seed protein meal (HPM, 44.32% protein content) were determined. The HPI had minimum protein solubility (PS) at pH 4.0, which increased as pH was decreased or increased. In contrast, the HPM had minimum PS at pH 3.0, which increased at higher pH values. Gel electrophoresis showed that some of the high molecular weight proteins (>45 kDa) present in HPM were not well extracted by the alkali and were absent or present in low ratio in the HPI polypeptide profile. The amino acid composition showed that the isolation process increased the *Arg/Lys* ratio of HPI (5.52%) when compared to HPM (3.35%). Intrinsic fluorescence and circular dichroism data indicate that the HPI proteins had a well-defined structure at pH 3.0, which was lost as pH value increased. The differences in structural conformation of HPI at different pH values were reflected as better foaming capacity at pH 3.0 when compared to pH 5.0, 7.0 and 9.0. At 10 and 25 mg/mL protein concentrations, emulsions formed by the HPM had smaller oil droplet sizes (higher quality), when compared to the HPI-formed emulsions. In contrast at 50 mg/mL protein concentration, the HPI-formed emulsions had smaller oil droplet sizes (except at pH 3.0). We conclude that the functional properties of hemp seed protein products are dependent on structural conformations as well as protein concentration and pH.

Keywords: circular dichroism, emulsion, hemp seed, intrinsic fluorescence, protein functionality

Practical Application

Hemp seed is a popular oil seed crop grown in Canada and globally for its oil and protein products. Hemp seed proteins (HSP), which account for 25% of its composition, have been widely studied in detail, but their structural and functional properties at varying processing

conditions are not well understood. This work provides information on the structural conformation and functional performance in model systems of HSP under varying pH conditions that cover the food processing range. The data presented could enhance manipulation of environmental pH conditions for the use of HSP as novel food ingredients.

3.1 Introduction

The structure–function relationships of proteins are known to be dependent on environmental factors such as pH, ionic strength, and temperature, which are commonly encountered in food systems at various stages including food preparation, processing, storage and consumption. Therefore, these environmental factors can be manipulated in order to enhance the functional and nutritional properties of foods or for the development of novel food products. Due to their lower cost in comparison to animal proteins, plant proteins have been well studied as a means of producing cheap food ingredients. For example, some previous works have studied the physicochemical and functional properties of legume flours, protein isolates and their 7S globulins in order to determine their potential functionality in foods (Tan and others 2014; Mune Mune and others 2014; Du and others 2014). Besides functional properties, plant proteins have also been studied for their physicochemical characteristics such as amino acid composition, hydrophobicity, and polypeptide composition (Mundi and Aluko 2012; 2013; Tan and others 2014). Hemp (*Cannabis sativa* L.) originated from Central Asia (Girgih and others 2011) and has been commonly used to produce fibres (Yin and others 2008) for ropes and fabrics. However, some authors (Callaway and others 2005; Svennerstedt and Svensson 2006) have reported hemp seeds as useful materials for animal feed production due to its high levels of edible oil. In Canada, various commercial hemp seed-derived products are available such as snacks, defatted

meal, protein shakes, hemp milk (vanilla or chocolate flavoured), energy bars and oils (House and others 2010; Radocaj and others 2014). The composition of hemp seed protein (HSP) as an excellent natural source of highly digestible amino acids when compared to other protein sources such as borage meal, canola meal and heated canola meal has been documented (Wang and others 2008). The by-product obtained after hemp seed processing into edible oil is called hemp seed protein meal (HPM), which has been reported to be nutritionally similar to other protein sources (Gibb and others 2005). HPM also contains all the essential amino acids in nutritionally sufficient amounts for infants or children as recommended by FAO/WHO (Tang and others 2006).

HPM can be further refined through alkali extraction followed by isoelectric protein precipitation to obtain hemp seed protein isolate (HPI), which also serves as a good source of essential amino acids for human nutrition (Tang and others 2006). While the potential of HSP as a good source of nutritious protein has been shown, there is limited information on potential use as ingredients to formulate high quality foods. In order to enhance value-added utilization of HSP in foods, knowledge of structural properties under various environmental conditions is needed. This is because the structural conformation of proteins is known to be a determinant of their behaviour in food systems; therefore, availability of such information could enhance manufacture of high quality HSP-containing food products through manipulation of various environmental factors. To the best of our knowledge, there is scanty information on the structural and functional properties of HSP, especially under varying pH conditions. Therefore, the aim of this work was to investigate the physiochemical and functional properties of HSP contained in HPI in comparison with HPM. The results obtained could enhance the value of HPI as a food

ingredient since current commercial HSP products are mainly in the form of protein concentrates that have less than 70% protein content.

3.2 Materials and Methods

3.2.1 Materials

Defatted HPM was purchased from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada) and stored at -20 ^{o}C until used for protein extraction. Other analytical grade chemicals and reagents were procured from Fisher Scientific (Oakville, ON, Canada).

3.2.2 Preparation of Hemp Seed Protein Isolates

HPI was produced from HPM according to the method described by Tang and others (2006) with slight modifications. HPM was dispersed in deionized water (1:20, w/v) and the dispersion was adjusted to pH 10.0 using 2 M NaOH to solubilize the proteins while stirring at 37 ^{o}C for 2 h; this was followed by centrifugation (7000 x g, 60 min at 4 ^{o}C). The precipitate was discarded and the supernatant filtered with cheese-cloth, adjusted to pH 5.0 with 2 M HCl to precipitate the proteins and thereafter centrifuged (7000 x g, 60 min at 4 ^{o}C). The resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to obtain the HPI. Protein concentrations of the HPI and HPM were determined using the modified Lowry method (Markwell and others 1978).

3.2.3 Amino acid composition analysis

The amino acid profiles of the hemp seed protein fractions were determined using the HPLC Pico-Tag system according to the method previously described after samples were digested with 6 M HCl for 24 h (Bidlingmeyer and others 1984). The cysteine and methionine contents were

determined after performic acid oxidation (Gehrke and others 1985) and the tryptophan content was determined after alkaline hydrolysis (Landry and Delhaye 1992).

3.2.4 Protein solubility (PS)

PS of HPM and HPI was determined according to the method described by Adebiyi and Aluko (2011) with slight modifications. Briefly, 10 mg of sample (protein weight basis) was dispersed in 1 mL of 0.1 M phosphate buffer solutions (at pH 3.0-9.0) to obtain a 0.1% (w/v) concentration and the resulting mixture was vortexed for 2 min and centrifuged at 10,000 x g for 20 min. Protein content of the supernatant was determined using the modified Lowry method (Markwell and others 1978). Total protein content was determined by dissolving the protein samples in 0.1 M NaOH solution. Protein solubility (PS) was expressed as percentage ratio of supernatant protein content to the total protein content.

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

The freeze-dried HPI and meal were subjected to SDS-PAGE (reducing and non-reducing) according to the method of Aluko and McIntosh (2004) with minor modifications. The protein samples were each dispersed (10 mg/mL) in Tris/HCl buffer, pH 8.0 containing 10% (w/v) SDS only (non-reducing buffer) or SDS + 10% (v/v) β-mercaptoethanol (β-ME), followed by heating at 95 ^{o}C for 10 min, cooled and centrifuged (10000xg, 15 min). After centrifugation, 1 µl of supernatant was loaded onto 8-25% gradient gels and electrophoresis was performed with Phastsystem Separation and Development units according to the manufacturer's instructions (GE Healthcare, Montréal, PQ, Canada). A mixture of protein standards (14.4-116 kDa) was used as the molecular weight marker and the gels were stained with Coomassie brilliant blue.

3.2.6 Intrinsic fluorescence emission

The method as described by Li and Aluko (2006) was used to record intrinsic fluorescence spectra on a JASCO FP-6300 spectrofluorimeter (JASCO, Tokyo, Japan) at 25 $^{\circ}$C using a 1 cm path length cuvette. Protein stock solution was prepared by dispersing 10 mg in 1 mL of 0.1 M sodium phosphate buffer, followed by centrifugation and determination of protein content of the supernatant. The supernatant was then diluted to 0.002% protein content (w/v) and fluorescence spectra recorded at excitation wavelengths of 275 (tyrosine and tryptophan) with emission recorded from 280 to 500 nm. Emissions of the buffer blanks were subtracted from those of the respective samples to obtain fluorescence spectra of the sample.

3.2.7 Measurements of circular dichroism (CD) spectra

CD spectra of samples were measured at 25 $^{\circ}$C in a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using the spectral range of 190-240 nm (far-UV) for secondary structure determinations according to the method described by Omoni and Aluko (2006) with minor modifications. Protein stock solutions were prepared as described above (intrinsic fluorescence) using 10 mM phosphate buffer that contained appropriate NaCl concentration followed by centrifugation at 10000xg for 30 min; the supernatant was then diluted to the concentration required for CD structural analysis. Secondary structure was determined using a cuvette with pathlength of 0.05 cm containing 2 mg/ml protein solution. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of respective buffer spectra. Since the samples contain protein mixtures, the obtained spectra reflect the sum of individual conformation of the various polypeptide components.

3.2.8 Least gelation concentration (LGC)

LGC was determined according to the method of Adebiyi and Aluko (2011) by suspending the samples in water at different concentrations (2-20%, w/v, protein weight basis). The mixture was

vortexed, placed in a water bath at 95 $^\circ$C for 1 h, cooled rapidly under tap water and left in the refrigerator (4 $^\circ$C) for 14 h. The sample concentration at which the gel did not slip when the tube was inverted was taken as the LGC.

3.2.9 Foam capacity (FC)

FC was determined according to the method described by Adebiyi and Aluko (2011) using slurries that were prepared as 20, 40, or 60 mg/mL (protein weight basis) sample dispersions in 50 mL graduated centrifuge tubes containing 0.1 M phosphate buffer, pH 3.0, 5.0, 7.0, and 9.0. Sample slurry was homogenized at 20,000x*g* for 1 min using a 20 mm foaming shaft on the polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The capacity of the continuous phase to include air (FC) was determined as follows (Eqn 2) using the mean of three measurements;

Foam Capacity (FC) = Vol. after homogenization – Vol. before homogenization   x   100

                                Vol. before homogenization   **(Eqn 2)**

The ability to retain air for a certain period of time (foam stability, FS) was calculated by measuring the foam volume after storage at room temperature for 30 min and expressed as percentage of the original foam volume.

3.2.10 Water and oil holding capacity (WHC and OHC)

The WHC and OHC were determined using the method of Adebiyi and Aluko (2011) with slight modifications. Protein sample (1g) was dispersed in 10 mL distilled water (or pure canola oil) in a 15 mL pre-weighed centrifuge tube. The dispersions were vortexed for 1 min, allowed to stand for 30 min and then centrifuged at 7, 000 x g for 25 min at room temperature. The supernatant was decanted, excess water (or oil) in the upper phase drained for 15 min and tube containing the

protein residue was weighed again to determine amount of water or oil retained per gram of sample.

3.2.11 Emulsion formation and oil droplet size measurement

Oil-in-water emulsion was prepared according to the method described by Adebiyi and Aluko (2011) with slight modifications. Protein slurries of 10, 25, or 50 mg/mL concentrations were each separately prepared in 0.1 M phosphate buffer pH 3.0, 5.0, 7.0, or 9.0 followed by addition of 1 mL of pure canola oil. The oil/water mixture was homogenized at 20,000 rpm for 2 min, using the 20 mm non-foaming shaft on a Polytron PT 3100 homogenizer. The oil droplet size ($d_{3,2}$) of the emulsions was determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as dispersant. Under constant shearing, emulsion sample taken from the emulsified layers of the samples was added to about 100 mL of water contained in the small volume wet sample dispersion unit (Hydro 2000S) attached to the instrument until the required level of obscuration is attained. The instrument was set to automatically measure the oil droplet size of each emulsion in triplicate and each sample was prepared in triplicate; the results were used as indicators of emulsifying capacity (EC). Emulsions were kept at room temperature for 30 min without agitation and the oil droplet size distribution and mean particle diameter were measured again to assess emulsion stability (ES) as in Eqn 3.

Emulsion Stability (ES) =   Oil droplet size at 0 min ($d_{3,2}$) x 100

   Oil droplet size after 30 min ($d_{3,2}$)   **(Eqn 3)**

3.2.12 Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA.

Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at $p < 0.05$.

3.3 Results and Discussions

3.3.1 SDS-PAGE

The SDS-PAGE profiles of HPM and HPI in the absence of β-ME as presented in Fig. 1A and B showed the presence of three (23, 34 & 47 kDa) and two (23 & 34 kDa) main polypeptide bands, respectively. Both HPM and HPI also contained a minor band with <14 kDa in size; the intensity of this band increased after treatment of HPM (but not HPI) with β-ME. This minor protein is likely to be the albumins as previously suggested by Tang and others (2006). The HPM was characterized by highly stained but diffused bands that were >66 kDa in size, suggesting reduced solubility in the electrophoresis buffer. Addition of β-ME resulted in the 34 kDa band splitting into 2 closely migrating bands (34 & 36 kDa), suggesting the presence of disulfide bonds in the native polypeptide chain. The presence of 20 and 33 kDa protein bands (identified as components of the main edestin protein fraction) in hemp seed protein products, results that are similar to data presented in the current study have also been previously reported (Tang and others 2006; Wang and others 2008). Intensity of the 47 kDa band was reduced in the presence of β-ME while a heavier polypeptide was detected at ~64 kDa, which probably came from a higher molecular weight disulfide-bonded protein. Previous reports (Tang and others 2006; Wang and others 2008) have also showed the presence of a similar 48 kDa polypeptide in hemp seed proteins. Thus, under reducing conditions, the HPM had three major and two minor polypeptide bands while the HPI had two major and one minor band. The results suggest that the HPI did not contain many disulfide bonds since there was no substantial increase in the number

Fig 1- SDS-PAGE of hemp seed protein meal (HPM) and isolate (HPI) under non-reducing (A)

and reducing (B) conditions
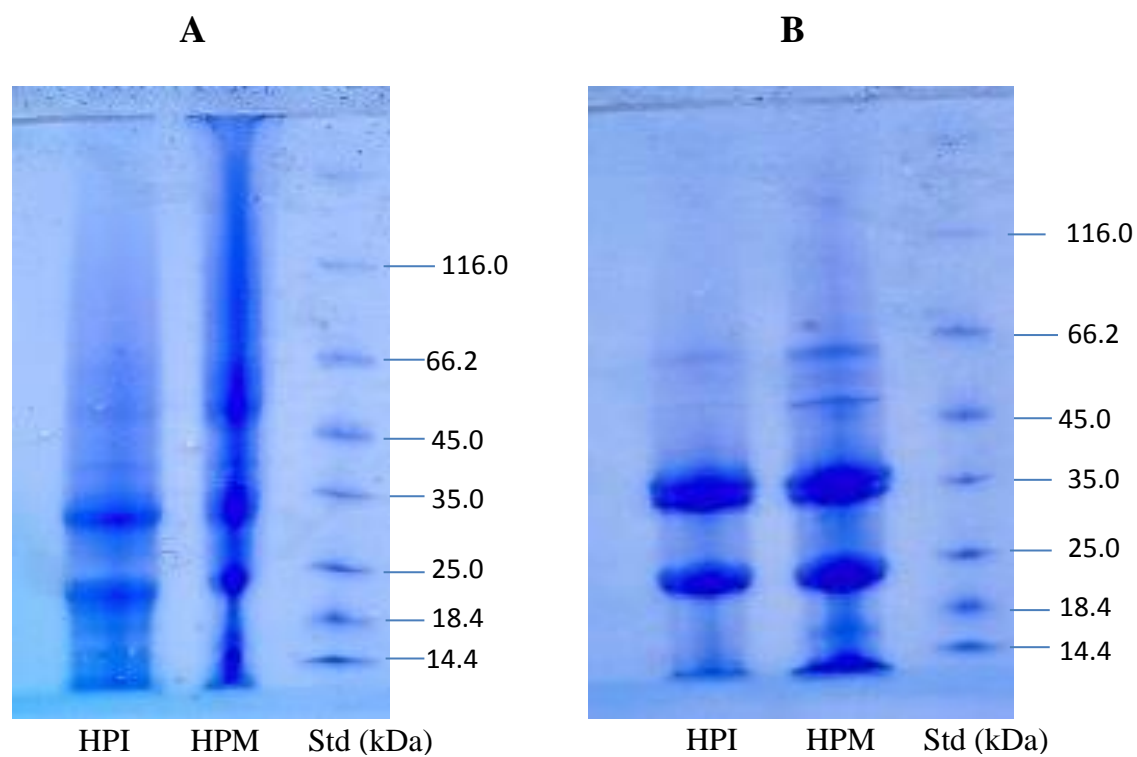
of polypeptide bands when mercaptoethanol was present in the buffer. The proteins found in both HPI and HPM electrophoretic profiles were in the same molecular weight range with 7S vicilin (41 kDa) and phytohemagglutinins (16 to 30 kDa) reported for pinto bean protein isolates (Tan and others 2014).

3.3.2 Protein contents, yields, solubility and amino acid composition

The protein content obtained for HPI (84.15%) is significantly (P<0.05) higher than the corresponding value (44.32%) for HPM. The protein content for HPI in the present study is higher than those previously reported by Mwasaru and others (1999) for pigeon pea isoelectric-precipitated isolates (82.4%), pigeon pea micellization-precipitated isolates (82.8%) and cowpea micellization-precipitated isolates (83.2%) but lower than the cowpea isoelectric-precipitated isolates (89.5%) and the value (90.5%) that was reported by Wang and others (2008) for HPI. The differences in protein content may be due to differences in extraction materials as well as the method of protein precipitation. The protein content obtained for HPM in the present study is in the 31.0-53.3% range reported by House and others (2010) and higher than the previously reported value of 30.7% by Silversides and Lefrancois (2005). Meanwhile, Tang and others (2006) reported a higher protein value (50%) for HPM, which is similar to the values claimed by some commercial producers of HPM (House and others 2010). The yield obtained for the isolates based on protein weight and gross weight was 37.90% and 16.80% respectively.

The HPI was fairly insoluble across the pH 3-9 range with a 25% PS at pH 3.0 and minimal values (0.5-1%) at pH 4.0-5.0, which represents the isoelectric point range for hemp seed proteins (Fig. 2). PS gradually increased as pH was raised from 5 to 9 as shown in Fig. 2. The results are similar to the report of Tang and others (2006) who showed increased PS at pH values >pI. The low PS of hemp seed proteins is believed to be due to *edestin* (major protein)

aggregation at pH below 7.0 and their dissociation at higher pH values (Friedman and Brandon 2001). There is linear proportionality between PS of HPM and pH with minimum PS at pH 3.0 (Fig. 2), and then increased to pH 9.0. Overall, with the exception of pH 3.0, HPM had higher PS values than HPI at all the pH values used in this work. The higher PS of HPM suggests the proteins may be in a more native state than the polypeptides present in HPI. Thus, the results indicate that the isoelectric precipitation method used during protein isolation may have contributed to increased aggregation and hence reduced PS of the HPI when compared to HPM. However, the presence of higher level of non-protein materials in HPM could have also modified protein structure (e.g. protein-sugar interactions) to enhance observed PS. Generally, the PS values obtained in this work are lower than those reported by Tang and others (2006), which probably reflects the seed processing history. The higher PS obtained by Tang and others (2006) reflects the gentle seed processing history (supercritical fluid extraction at <40 $^{\circ}$C), which would have reduced excessive protein denaturation. In contrast, the protein products used in this work were obtained from seeds that have undergone high level of force (cold press) to extract the oil; hence it is more likely that the polypeptides have undergone severe protein-protein interactions, which may be responsible for the observed low PS.

The amino acid composition data (Table 3) showed that the isolation process did not cause any major changes in the content of most amino acids. However, there was a slight increase in the *Arg* content of HPI (15.06%) when compared to its HPM (13.20%). Moreover, the *Arg/Lys* ratio was higher for HPI (5.52) when compared to the HPM (3.35). A higher ratio of *Arg/Lys* in the diet has been shown to produce hypocholesterolemic effects and could be of benefit to improving cardiovascular health (Giroux and others 1999). In this work, the results showed that the HPI may have better positive effects on the cardiovascular system when

Table 3- Percentage amino acid composition of hemp seed protein meal (HPM) and protein isolate (HPI)

Samples	Amino acids (%)																		ARG/LYS ratio
	ASP	THR	SER	GLU	PRO	GLY	ALA	CYS	VAL	MET	ILE	LEU	TYR	PHE	HIS	LYS	ARG	TRP	
HPI	11.31	3.42	5.58	19.10	4.30	4.31	3.81	1.25	4.66	1.92	3.63	6.46	3.61	4.72	3.15	2.73	15.06	1.00	5.52
HPM	10.78	3.75	5.65	18.45	4.37	4.67	4.19	1.52	4.69	2.28	3.59	6.87	3.04	4.68	3.26	3.94	13.20	1.06	3.35

Fig 2- Protein solubility profile of hemp seed protein products at different pH values
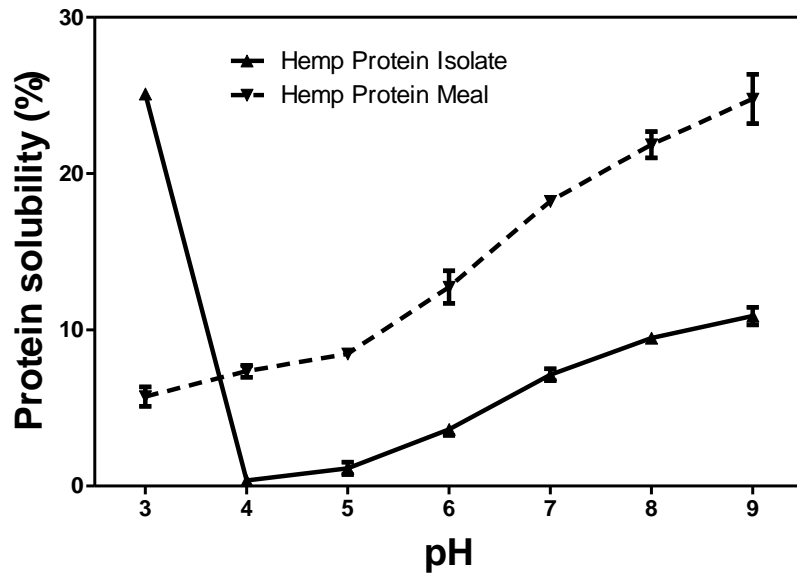
compared to the HPM. The observed *Arg/Lys* ratios obtained in this work are higher than the 2.31 and 1.4 reported for HPI and soybean protein isolate, respectively (Tang and others 2006). The differences between *Arg/Lys* values obtained in this work for the Canadian hemp seed proteins and the value reported by Tang and others (2006) for the Chinese HPI may be due to variations in genetic and environment factors. Our current values are also higher than the <1 values reported for kidney bean proteins (Mundi and Aluko 2012; 2013), or the 0.44 value reported for casein and 2.53-2.65 values for rice proteins (Yang and others 2012).

3.3.3 Intrinsic fluorescence emission

The conformational changes of HPM and HPI at different pH values as measured by intrinsic emission fluorescence spectroscopy technique are shown in Fig. 3. The HPI exhibited a maximum emission wavelength (λmax) of 371 nm at pH 3.0 showing a characteristic fluorescence profile of tryptophan residues that are mostly exposed within a hydrophilic environment. The tryptophan peak was red-shifted to 380 nm in the HPM when compared to the HPI, suggesting a greater open protein structure in HPM. There was also a minor peak at 313 nm, which is typical of tyrosine fluorescence (Fig. 3A) also exposed to a hydrophilic environment. However, the 313 nm peak was absent in the HPM, which indicates a structural conformation whereby the tyrosine residues are shielded by other groups or too close to tryptophan at pH 3.0. The maximum fluorescence intensity (Fmax) was higher for the HPI, which suggests shorter distances between the tryptophan residues and hence the presence of more globular protein structures when compared to the HPM. Thus, the results suggest higher degree of protein-protein interactions for HPI when compared to the HPM. At pH 5.0, the Fmax for HPI decreased to an almost zero value while that of HPM remained virtually unchanged from the pH 3.0 values (Fig. 4). Since the λmax and Fmax values for the tryptophan peak did not

change for HPM from pH 3.0 to 5.0, it means there was no change in the location or environment of the tryptophan residues as a result of the reduced acidity. However, the tyrosine fluorescence at 313 nm was observed also for HPM at pH 5.0 (Fig. 3B), which is an indication of translocation of these residues into a more conserved protein structure and within a hydrophobic environment, when compared to their location at pH 3.0. The Fmax for HPI tyrosine residues at pH 5.0 was about 300% higher than the value obtained at pH 3.0, which suggests increased interactions between the residues. Thus it seems that at pH 5.0, the HPI tryptophan residues became highly shielded from light while the tyrosine residues became more exposed to light source when compared to the structural conformation at pH 3.0.

At pH 7.0 and 9.0, there were slight increases in the Fmax values obtained for both tyrosine and tryptophan peaks, which indicate structural rearrangements that enhanced tyrosine-tyrosine and tryptophan-tryptophan interactions. It is possible that at pH 7.0 and 9.0 where the environment is more hydrophilic, the aromatic groups are moved away from the surface and towards the more hydrophobic core, which led to enhanced emission intensity. This is supported by the fact that there were slight blue shifts in λmax, especially for the HPI at pH 7.0 (376 nm) and 9.0 (368 nm). For the HPM, λmax values of 378 and 375 nm were obtained at pH 7.0 and 9.0, respectively (Fig. 3). The observed blue shifts in λmax at high pH values in the present study in similar to a previous report on quinoa protein isolates (Abugoch and others 2008). The red shift in λmax values and reduced Fmax observed from pH 3.0 to 5.0 for HPI reflect increased exposure of the aromatic residues to the hydrophilic environment and is in agreement with a past report (Naseem and others 2004) on the relationship between isoelectric point and conformational changes in proteins.

Fig 3- Intrinsic fluorescence intensity (arbitrary units) of hemp seed protein products at different pH values
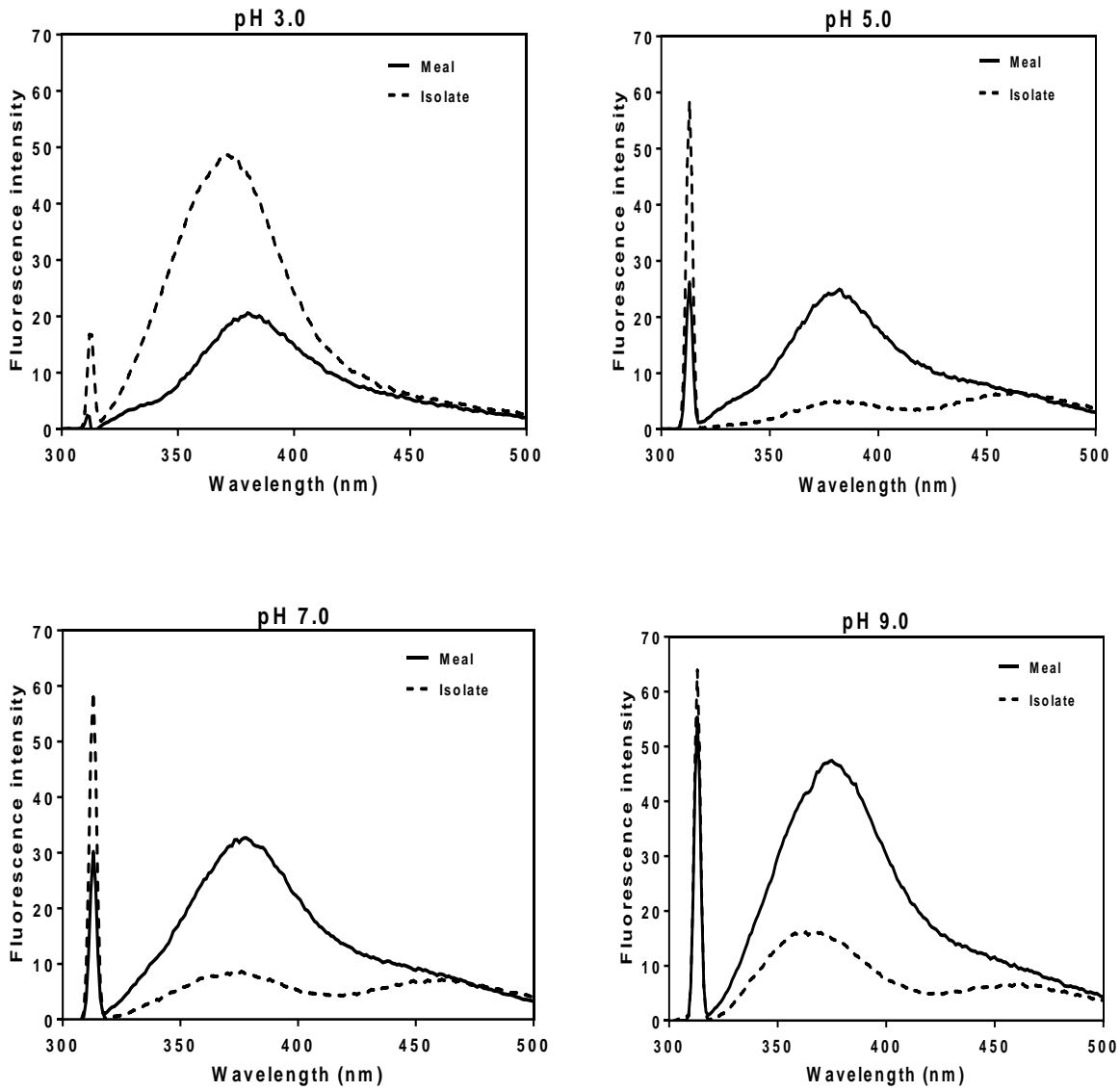
3.3.4 Secondary structure conformations

The effect of pH on CD spectra obtained for secondary structures of HPM and HPI is shown in Fig. 4. Due to reduced protein solubility, CD determination was not possible at pH 5.0 since required level of soluble proteins could not be obtained. At pH 3.0, HPI showed a strong secondary structure dominated mostly by the α-helix conformation as evident in the intense ellipticity between 200 and 220 nm. The HPM had similar ellipticity pattern but was less intense (less α-helix structure) than that of the HPI. However, the ellipticity at 195 nm was more intense for the HPM, which indicates presence of more β-sheet structure when compared to the HPI. The presence of a higher level of β-sheet structure in HPM indicates a more open conformation when compared to HPI. The far-UV CD results are consistent with the fluorescence intensity data that showed reduced Fmax and higher λmax (indication of high degree of exposure of aromatic groups to the hydrophilic environment) exhibited at pH 3.0. When the environment was changed to pH 7.0, the HPM had a very high increase in ellipticity values between 200-220 nm, indicating presence of more α-helix structure than at pH 3.0 (Fig. 4). In contrast, the HPI had less α-helix structure as indicated by the substantial decreases in ellipticity values between 200-220 nm, indicating a more loose protein conformation than present at pH 3.0. The far-UV CD data at pH 7.0 also support the fluorescence emission data (Fig. 3) that showed a lower Fmax value and a red shift in λmax (compared to pH 3.0), which are indications of increased interactions with the hydrophilic environment. The CD ellipticity patterns obtained for HPI and HPM at pH 9.0 were similar to those obtained at pH 7.0, though with reduced intensity, which suggests that increased charge at alkaline pH 9.0 may have produced greater protein-protein electrostatic repulsions and hence a more disorganized structure when compared to pH 7.0.

Fig 4- Far-UV Circular dichroism spectra of hemp seed protein products at different pH values
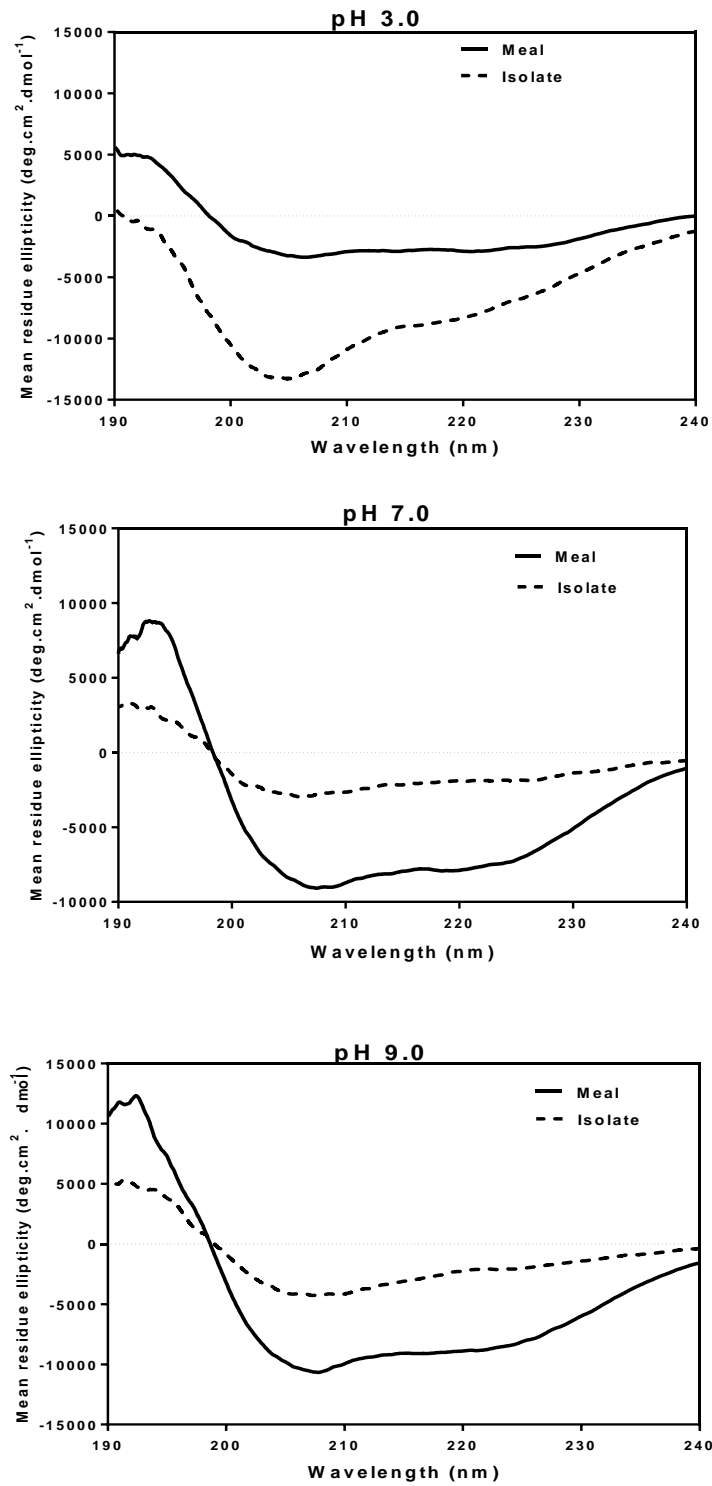
3.3.5 Water holding capacity, oil holding capacity, and least gelation concentration

The ability of any protein isolate to interact with water is dependent on its water-binding properties, which has been reported to be a function of several parameters that include size, shape, and conformational characteristics (Chavan and others 2001). Also important are the hydrophilic-hydrophobic balance of amino acids in the protein molecules, presence of lipids, physicochemical environment (pH, ionic strength) and solubility with reference to polar amino groups of protein molecules, which are the primary sites of protein-water interactions. The WHC of HPI was significantly lower than that of HPM ($P<0.05$), but in contrast, the OHC of HPI was significantly higher (Table 4). The result for WHC of HPI was significantly higher than the previously reported values of 2.7 g/100g (Yin and others 2008) and 3.4 g/100g (Tang and others 2006) for HPI and 3.87 g/100g for Lima bean seed protein isolate (Chel-Guerrero and others 2002). The present values for OHC are higher than the previously reported values of 3.0 g/100g (Yin and others 2008) and 5.3 g/100g (Tang and others 2006) for HPI. Chickpea protein isolate with 1.7 g/100g (Paredes-Lopez and others 1991) and sesame seed protein isolate with 1.5 g/100g (Khalid and others 2003) also had lower OHC when compared to the HPI. The relatively higher WHC and OHC values obtained for HPI in comparison to other plant protein isolates may be due to differences in preparation methods or source of materials (including history of seed treatment) but indicates a loose protein structure with exposure of hydrophilic and hydrophilic groups. This is because OHC has been reported (Abugoch and others 2008) to reflect ability of protein hydrophobic groups to interact with lipids.

The capacity of protein to act as a good gelling agent that provides a 3-dimensional structural matrix for the manufacture of solid or semi-solid foods can be estimated using the LGC method.

Table 4- Water holding capacity (WHC), oil holding capacity (OHC) and least gelation concentration (LGC) of hemp seed protein products

Samples	WHC (g/g)	OHC (g/g)	LGC (%)
Meal	12.32 ± 0.03^{a}	12.54 ± 0.08^{b}	12.00 ± 0.00^{a}
Isolate	12.01 ± 0.08^{b}	13.70 ± 0.29^{a}	22.00 ± 0.00^{b}

Mean values±standard deviation of replicate analysis; means followed by different superscript in each column indicates significant differences at $P<0.05$.

The gelation capacity of HPM (12%) in this study was significantly better (*P*<0.05) when compared to HPI (22%) but is similar to the value reported for lupin protein concentrate (Lqari and others 2002). The LGC value for HPM is also better than the previously reported value of 14% for soybean protein isolate (Okezie and Bello 1988). The poor gel-forming ability of HPI may be due to a high level of protein aggregation during isoelectric protein precipitation, which reduced the flexibility required for network formation. In contrast, the HPM did not undergo isoelectric protein precipitation and probably contained less number of aggregated proteins, hence the higher LGC value.

3.3.6 Foam Capacity and Foam Stability

Besides possessing flexible protein surfactant molecules, a good foaming protein must be rapidly adsorbed at the air–water interface and rearranged to form a cohesive viscoelastic film via intermolecular interactions (Yin and others 2008; Aluko and Yada 1997). At 20 mg/ml protein concentration, the FC of HPM and HPI was similar across various pH values with the exception of pH 3.0 where the HPI had significantly (P<0.05) higher value (Fig. 5). Protein solubility and hydrophobicity/hydrophilicity ratio of samples can also influence some functional properties (Damodaran 1996). Therefore, the high FC at pH 3.0 for the HPI may be due to its superior solubility property when compared to other pH values. The results are also consistent with the higher intrinsic fluorescence intensity and better secondary structure conformation obtained for HPI at pH 3.0. The fluorescence intensity data indicates greater exposure of aromatic groups, which could have enhanced protein-protein interactions to form stronger interfacial membranes and hence higher FC at pH 3.0. The results also showed that the FC of the HPM was consistent with the solubility properties across the pH range studied. Increase in protein concentration from 20 to 60 mg/ml led to reductions in FC at pH 3.0 whereas there were increases at pH 5.0 to 9.0.

Fig. 5- Foam capacity of hemp seed protein products at different pH values: A, 20 mg/mL; B, 40 mg/mL; and C, 60 mg/mL protein concentrations. (*Bars that contain different letters are significantly different at p<0.05)
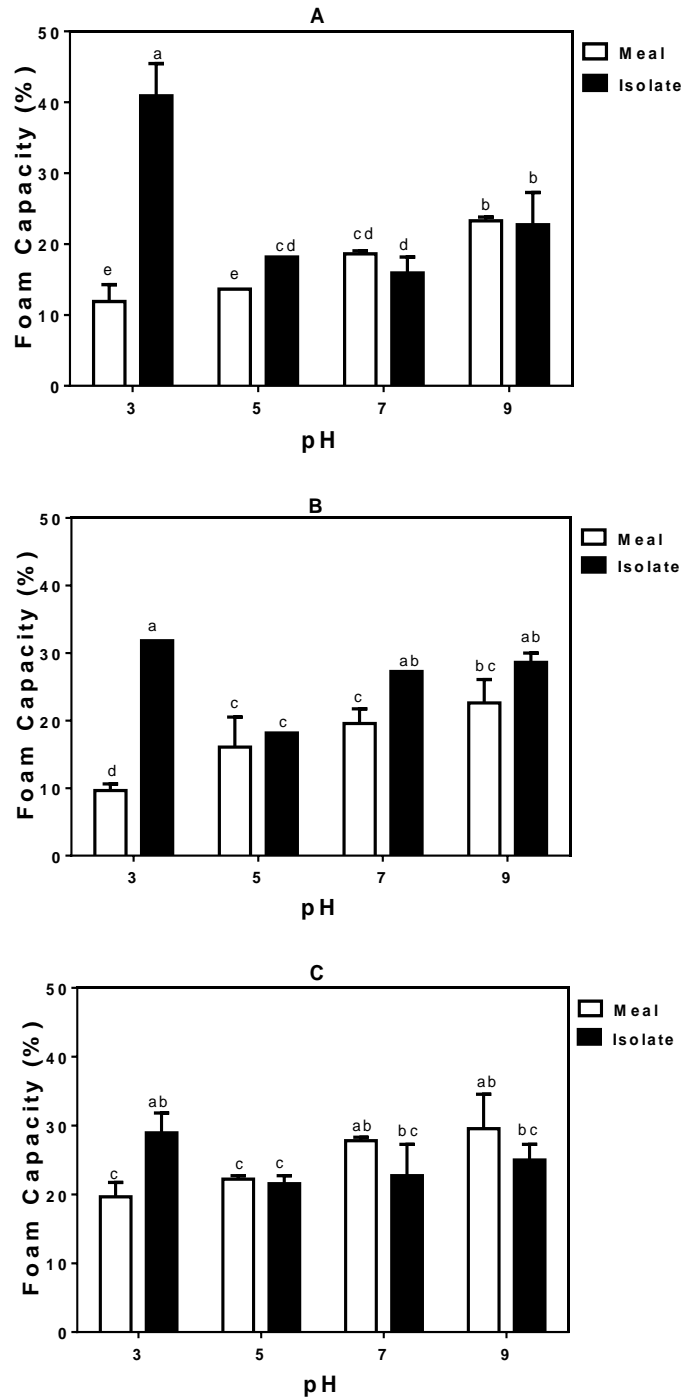
Fig 6- Foam stability of hemp seed protein products at different pH values: A, 20 mg/mL; B, 40 mg/mL; and C, 60 mg/mL protein concentrations (*Bars with different letters are significantly different at p<0.05)
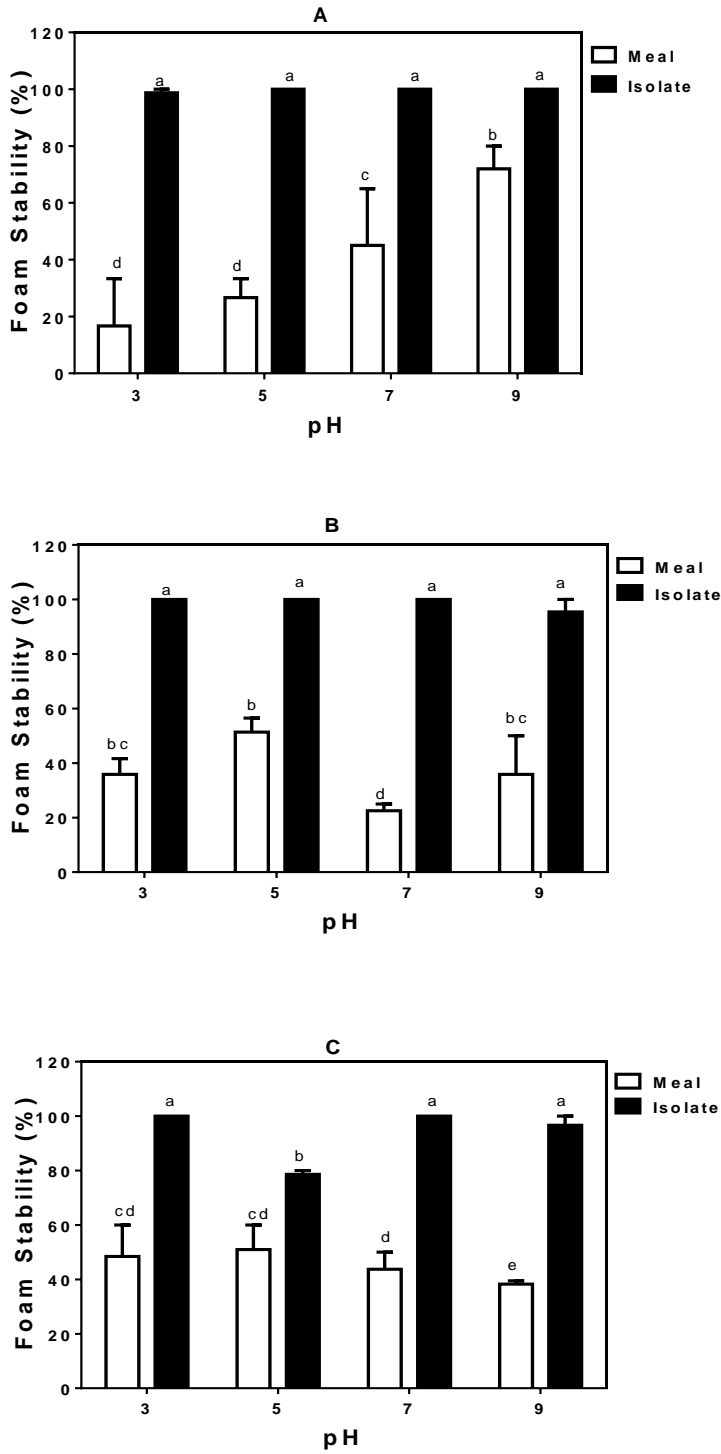
The results suggest that at pH 3.0, a dilute protein concentration provided greater protein flexibility and hence higher FC; increased concentration probably led to a crowded solution that reduced protein flexibility. In contrast, at pH 7.0 and 9.0 there was an increased charge density which could have minimized the crowding effect and hence FC increased with increased protein concentration. Only slight changes in FC were observed when protein concentration was increased at pH 5.0 (Fig. 5). The foams formed by HPI were general more stable at all the different pH values and protein concentrations with no significant differences (P>0.05) between them (Fig. 6). All the HPI foams had significantly higher FS values when compared to the HPM-formed foams. The results agree with a previous work, which showed that samples with higher protein contents formed more stable foams than samples with lower protein contents (Aluko and others 2009). This is because proteins are able to form viscoelastic interfacial membranes through protein-protein interactions and enhance resistance of air bubbles to destabilization.

3.3.7 Emulsion capacity and Emulsion stability

The EC recorded as surface diameter (µm) of the HPM and HPI emulsion oil droplets ($d_{3,2}$) at different pH and protein concentrations are shown in Figure 7. At a low protein concentration of 10 mg/ml, the HPM formed significantly (P<0.05) better emulsions (lower $d_{3,2}$ values) at pH 3.0 and 5.0 when compared to pH 7.0 and 9.0 or all the HPI emulsions. The higher HPM emulsion forming ability may be due to the presence of a higher level of non-protein materials such as polysaccharides that could enhance emulsification, especially at low protein concentrations. In contrast, the HPI has less non-protein materials and coupled with the rigid molecules from the isoelectric protein precipitation produced emulsions with slightly higher $d_{3,2}$ values at 10 and 25 mg/ml protein concentrations. However at a higher protein concentration of 50 mg/ml, the HPI produced emulsions with significantly lower $d_{3,2}$ values at all the tested pH value with the

exception of pH 3.0. Thus, the results suggest that higher protein concentration may be able to compensate for the poor emulsification property of a protein. The exception at pH 3.0 can be attributed to the increased solubility (Fig. 2) and hence higher net protein charge coupled with protein crowding, which may have limited the interfacial properties as a result of increased protein-protein repulsions. In contrast, the decreased PS at pH 7.0 and 9.0 indicates weaker intensity of the protein-protein repulsion forces and hence higher ability to form stronger interfacial protein membranes, which led to better emulsion formation than at pH 3.0 where the forces are stronger. The values presented for the 50 mg/ml protein concentration is in contrast to a previous report (Tang and others 2006) that showed lack of a relationship between EC and PS. This is because at pH 3.0 where higher PS value was recorded for HPI in this work, there was a significantly (P<0.05) lower emulsification ability (higher $d_{3,2}$). However, the higher emulsifying ability (lower $d_{3,2}$) of HPI at a high protein concentration of 50 mg/ml (pH 5.0, 7.0 and 9.0) is consistent with a previous report (Aluko and others 2009) that showed enhanced emulsion ability when sample protein concentration was increased. ES increases when there is strong protein-protein interactions at the oil-water interface of the protein samples, which prevents oil droplet coalescence. At 10 mg/ml protein concentration, all the emulsions were very stable (~100% ES) with the exception of HPI emulsion at pH 5.0, which had only 50% stability (Fig. 8). As protein concentration was increased to 25 and 50 mg/ml, all the emulsions had similar ES values. Therefore, the overall data seem to indicate that the HPM and HPI formed emulsions that were stabilized by formation of strong interfacial membranes that reduced the rate of oil droplet coalescence.

Fig. 7- Oil droplet sizes of emulsions formed by hemp seed protein products at different pH values: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations (*Bars that contain different letters are significantly different at p<0.05)

Fig 8- Emulsion stability formed by hemp seed protein products at different pH values: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations (*Bars that contain different letters are significantly different at p<0.05)
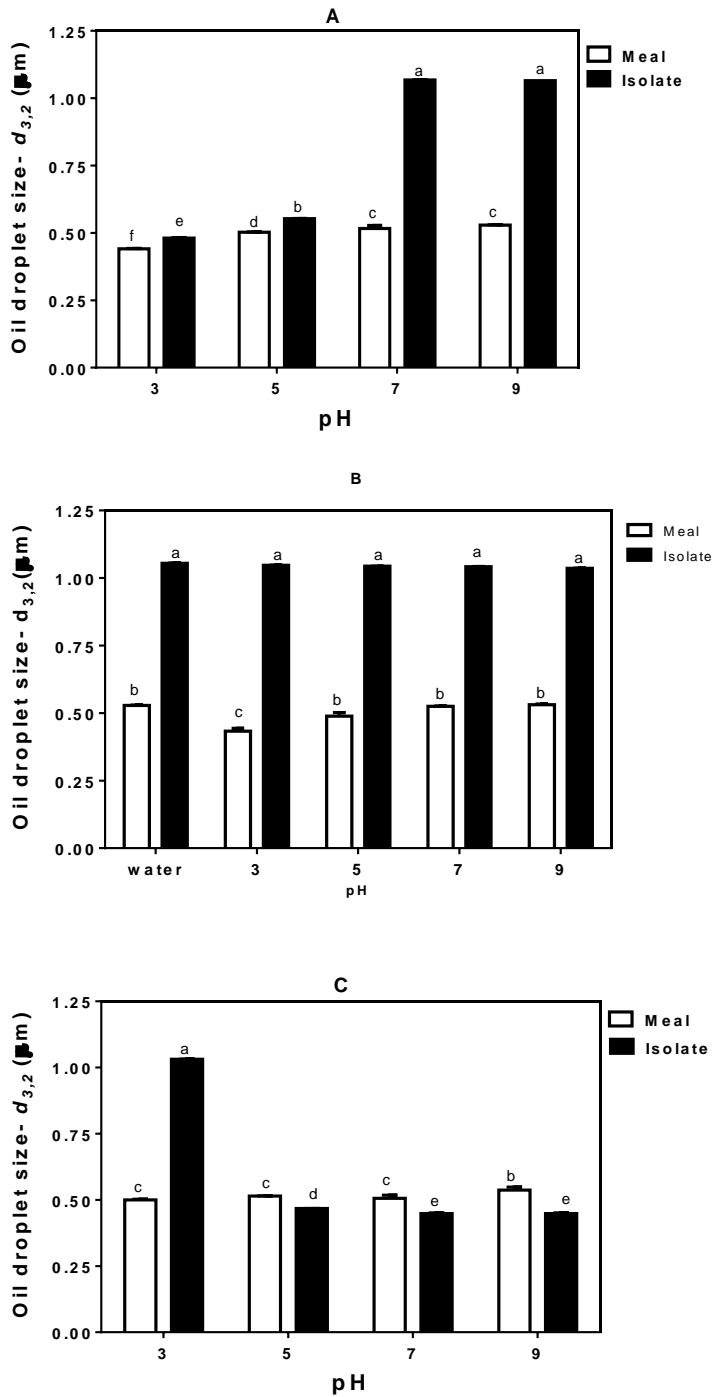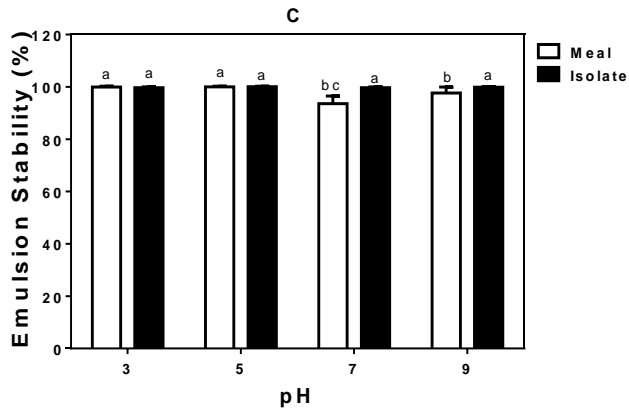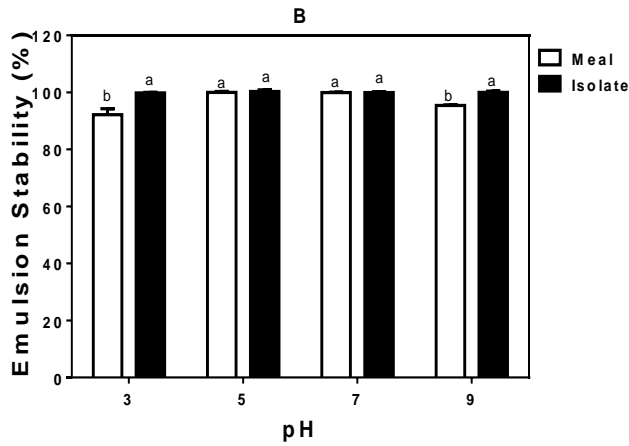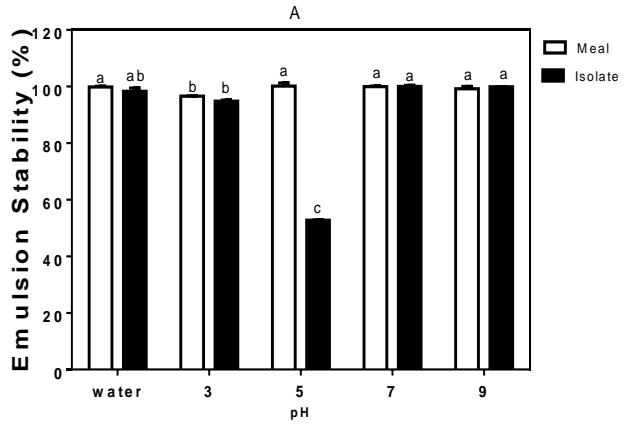
3.4 Conclusion

The findings from this study have shown that, besides the nutritional role of hemp seed protein, there is the potential for use as a basic functional ingredient in the food industry. The results showed that manipulation of pH could be used to increase FC, which is dependent on PS. The oil droplet sizes of some of the emulsions were very small (< 0.5 µm) and comparable to values previously reported for milk and soybean protein emulsifiers. The high ES is an indication of the potential use of the hemp seed proteins to form food emulsions that can maintain stable oil droplet size during short-term storage. The results obtained at pH 3.0 for HPI suggest that the mechanisms responsible for foam formation are probably different from those involved in emulsion formation. Intrinsic fluorescence and CD data revealed that proteins in HPM and HPI exist in different conformational states when evaluated at various pH values. The results suggest that the effect of pH on protein functionality is dependent of protein concentration. The results confirm that protein functionality is highly dependent on structural conformation, which can be modulated through changes in pH of the environment. Moreso, since hemp proteins are not listed as priority allergens which require labelling, therefore, both HSP products could serve as alternative protein ingredients to the priority allergens (e.g. soybean and pea proteins) that require labelling.

97

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Author contributions

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3.5 Statement transfer between the study 1 and study 2

The isoelectric precipitation method produces protein isolates that consist of different fractions through the alkaline solubilisation and acid precipitation. This isoelectric precipitation method of protein isolation leads to limited functionality because of poor structural conformations and partial protein denaturation. The limited functionality arises from protein rigidity and hydrophobic interactions that cause protein aggregation within the harsh acid/alkaline environments used for during extraction. Besides, some of the non-protein seed components (starch, soluble sugars, fibre, and phytate) are also extracted and could thus affects the solubility (and other functionalities) of the resultant protein isolates. Therefore, the next chapter focuses on the use of conventional dialysis method to separate pure and distinct protein fractions (albumin and globulin) from the hemp seed defatted flour. The two protein fractions also have different solubility profiles, hence the conformational properties are expected to differ from each and when compared with the protein isolates. This is because the albumin fraction is highly soluble in water while the pure globulin fraction is only soluble in salt solution. In contrast the protein isolates used in the preceding chapter are soluble mainly in dilute alkaline solutions.

CHAPTER FOUR

4. Comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions

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ABSTRACT

The aim of this work was to determine the structural and functional characteristics of two major hemp seed proteins, the water-soluble albumin (ALB) and the salt-soluble globulin (GLB). 0.5 M NaCl extract of hemp seed protein flour was dialyzed against water to obtain the two fractions: ALB in the water phase and GLB in the insoluble precipitate. The protein fractions were subjected to structural (gel electrophoresis, intrinsic fluorescence and circular dichroism) and functional (protein solubility, foaming, and emulsion) tests. Amino acid composition data showed the presence of higher contents of aromatic and hydrophobic residues in GLB. Gel electrophoresis indicated that the ALB has less disulfide bonds and hence a more open (flexible) structure; this was confirmed by the intrinsic fluorescence and circular dichroism data showing greater exposure of tyrosine residues when compared to GLB. ALB had significantly ($p<0.05$) higher protein solubility and foaming capacity than the GLB at all the pH or sample concentration values but emulsion forming ability was similar for both protein fractions. Differences in emulsion stability were observed mostly at 10 mg/mL sample concentration; these differences were minimized at 25 mg/mL and eliminated at 50 mg/mL. We conclude that the ALB fraction will serve as excellent ingredient for food foam formulation while the GLB may be slightly more useful than the ALB in the formulation of food emulsions.

Keywords: hemp seed; albumin, globulin, functional properties; intrinsic fluorescence, circular dichroism

4.1. Introduction

The amount and structure of proteins present in a food system have been reported to play an important role in making such ingredients useful for novel food formulations (Mundi & Aluko, 2013; Yin et al., 2011). Barac, Pesic, Stanojevic, Kostic and Bivolarevic (2014) studied and reported the influence of the functional properties of a food or its components (in addition to its nutritional properties) in enhancing utilization for novel food product development. This functionality is achieved through the protein's activity (in free or structure changing state) as observed during interactions with other food components (Tang, Sun, & Foegeding, 2011). An example is the tendency of hydrophobic portions of a protein to interact with lipids and lipid soluble compounds (Karaca, Low, & Nickerson, 2011). Critical efforts have been aimed at effective utilization of inexpensive plant proteins as food hydrocolloids for nutritional and functional purposes under different pH conditions. Proteins from plants like canola (Tan, Mailer, Blanchard, & Agboola, 2014), peanut (He, Liu, et al., 2014), kidney bean (Mundi & Aluko, 2012, 2013), lentils (Avramenko, Low, & Nickerson, 2013), pinto bean (Tan, Ngoh, & Gan, 2014), adzuki, pea and soy bean (Barac et al., 2014), amaranth (Shevkani, Singh, Rana, & Kaur, 2014) and cowpea (Oduro-yeboah, Onwulata, Tortoe, & Thomas-Gahring, 2014) have been examined for their structural and functional properties in model food systems.

Food-grade hemp seed is a new but widely cultivated plant of industrial importance and requires detailed functional characterization of component proteins. This is very important because availability of non-drug varieties of hemp seed possessing low δ-9-tetrahydrocannabinol (THC) contents (Lu et al., 2010) has increased industrial utilization for food product manufacture. The legal use of the seeds from low-THC plants as consumables food for humans has been reported in Canada and other North American countries due to its considerable amounts

of over 30% oil and about 25% easily digestible proteins (Teh & Birch, 2013). The uniqueness of hemp seed protein (HSP) has been reported (Park, Seo, & Lee, 2012) to contain 65% globulin called edestin and 33% albumin; edestin is composed of six identical subunits having an acidic subunit (AS) and a basic subunit (BS) linked by one disulfide bond. Previous works have reported the functional (Tang, Ten, Wang, & Yang, 2006; Teh, Bekhit, Carne, & Birch, 2013; Yin et al., 2008) or bioactive (Girgih, He, Malomo, & Aluko, 2014; Girgih, Udenigwe, & Aluko, 2011; Wang, Tang, Chen, & Yang, 2009) properties of HSP isolates and hydrolysates but there is scanty information on the structural and functional properties of the seed globulin and albumin fractions. Therefore, this study was aimed at determining the structural and functional properties of albumin (ALB) and globulin (GLB) fractions of HSP. Specifically, pH-dependent changes in protein solubility, foaming and emulsifying properties were studied in relative to protein conformational changes. The information from this work may provide opportunities for industrial application of HSP as a useful food ingredient and a suitable alternative source of functional proteins to traditional ingredients.

4.2. Materials and Methods

4.2.1. Materials

Defatted hemp seed protein meal (HPM) containing ~40% (w/w) protein content, the by-product obtained from commercial oil extraction was purchased from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada) and stored at -20 ^{o}C until used for protein extraction. Spectra/Por dialysis membrane with 6-8 kDa molecular weight cut-off (MWCO), gel electrophoresis protein markers as well as other analytical grade reagents were purchased from Fisher Scientific (Oakville, ON, Canada).

4.2.2. Protein fraction extraction and isolation

The method of Aluko (2004), with slight modifications was employed in the extraction and fractionation of HPM protein into ALB and GLB. The HPM was extracted (1:10 w/v) with 0.5 M NaCl solution for 1 h at 24 ± 2 ^{o}C under continuous stirring followed by centrifugation (7000 g, 60 min at 4 ^{o}C) and the supernatant clarified with Whatman No 1 filter while the precipitate was discarded. The supernatant was dialyzed against water at 4 ^{o}C for 5 days using the 6-8 kDa MWCO dialysis tubing (with dialysis water changed 3 times daily). The content of the dialysis bag was then centrifuged (7000 g, 60 min at 4 ^{o}C) and the supernatant collected as the albumin protein fraction. The precipitate was washed with 100 ml of distilled water, centrifuged again under similar conditions as above and the precipitate collected as the globulin protein fraction. The two protein fractions were individually freeze-dried followed by storage at -20 ^{o}C until required for subsequent analyses. Protein concentration of the fractions was determined using the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978).

4.2.3. Amino acid composition

The amino acid profiles of ALB and GLB were determined using the HPLC Pico-Tag system according to the method previously described after samples were digested with 6 M HCl for 24 h (Bidlingmeyer, Cohen, & Tarvin, 1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke, Wall, Absheer, Kaiser, & Zumwalt, 1985) while the tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992).

4.2.4. Protein solubility (PS)

PS of ALB and GLB was determined according to the method described by Adebiyi and Aluko (2011) with slight modifications. Briefly, 10 mg of sample was dispersed in 1 ml of 0.1 M phosphate buffer solutions (pH 3.0-9.0) and the resulting mixture was vortexed for 2 min and

centrifuged at 10000 g for 20 min. Protein content of the supernatant was determined using the modified Lowry method (Markwell et al., 1978). Total protein content was determined by dissolving the protein samples in 0.1 M NaOH solution. PS was expressed as percentage ratio of supernatant protein content to the total protein content.

4.2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The freeze-dried ALB and GLB were subjected to SDS-PAGE according to the method of Aluko and McIntosh (2004) with minor modifications. The protein samples were each dispersed (10 mg/mL) in Tris/HCl buffer, pH 8.0 containing 10% (w/v) SDS only (non-reducing buffer) or SDS + 10% (v/v) β-mercaptoethanol (β-ME) as the reducing buffer followed by heating at 95 ^{o}C for 10 min, cooled and centrifuged (10000 g, 15 min). After centrifugation, 1 μl of supernatant was loaded onto 8-25% gradient gels and electrophoresis was performed with Phastsystem Separation and Development units according to the manufacturer's instructions (GE Healthcare, Montréal, PQ, Canada). A mixture of protein standards (14.4-116 kDa) was used as the molecular weight marker and the gels were stained with Coomassie brilliant blue.

4.2.6. Intrinsic fluorescence emission

The method described by Li and Aluko (2006) was used to record intrinsic fluorescence spectra on the JASCO FP-6300 spectrofluorimeter (JASCO, Tokyo, Japan) at 25 ^{o}C using a 1 cm path length cuvette. Protein stock solution (10 mg/ml) was diluted with 0.1 M sodium phosphate buffer to 0.002% (w/v) and fluorescence spectra recorded at excitation wavelengths of 275 nm (tyrosine and tryptophan) with emission recorded from 280 to 450 nm. Emissions of the buffer blanks were subtracted from those of the respective samples to obtain fluorescence spectra of the sample.

4.2.7. Measurements of circular dichroism (CD) spectra

CD spectra of samples was measured at 25 $^{\circ}$C in a J-810 spectropolarimeter (JASCO, Tokyo, Japan) using the spectral range of 190-240 nm (far-UV) for secondary structure determinations and 250-320 nm (near-UV) for tertiary structure according to the method described by Omoni & Aluko (2006). Protein stock solutions were diluted to required concentration in 10 mM phosphate and the secondary structure determined using a cuvette with pathlength of 0.05 cm containing 2 mg/ml protein solution while the tertiary structure was measured in a 0.1 cm cuvette containing 4 mg/ml protein concentration. All the CD spectra were obtained as the average of three consecutive scans with automatic subtraction of the buffer spectra.

4.2.8. Foam capacity (FC)

FC was determined according to the method described by Adebiyi and Aluko (2011) using slurry that were prepared as 20, 40, or 60 mg/mL (protein weight basis) sample dispersions in 50 mL graduated centrifuge tubes containing 0.1 M phosphate buffer, pH 3.0, 5.0, 7.0, and 9.0. Sample slurry was homogenized at 20,000 rpm for 1 min using a 20 mm foaming shaft on the polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The capacity of the continuous phase to include air (FC) was determined as follows (Eqn 4) using the mean of three measurements;

Foam Capacity (FC) = Vol. after homogenization – Vol. before homogenization   x 100

Vol. before homogenization   **(Eqn 4)**

The ability to retain air for a certain period of time (foam stability, FS) was calculated by measuring the foam volume after storage at room temperature for 30 min and expressed as percentage of the original foam volume.

4.2.9. Emulsion formation and oil droplet size measurement

Oil-in-water emulsion was prepared according to the method described by Adebiyi and Aluko (2011), with slight modifications. Protein slurries of 10, 25, or 50 mg/mL concentrations were prepared using 5 mL of 0.1 M phosphate buffer pH 3.0, 5.0, 7.0, or 9.0 followed by addition of 1 mL of pure canola oil. The oil/aqueous mixture was homogenized at 20,000 rpm for 2 min, using the 20 mm non-foaming shaft on a Polytron PT 3100 homogenizer. The oil droplet size ($d_{3,2}$) of the emulsions was determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as dispersant. Under constant shearing, emulsion sample taken from the emulsified layers of the samples was added to about 100 mL of water contained in the small volume wet sample dispersion unit (Hydro 2000S) attached to the instrument until the required level of obscuration is attained. The instrument was set to automatically measure the oil droplet size of each emulsion in triplicate and each sample was prepared in triplicate. Emulsion formed was kept at room temperature for 30 min without agitation and the particle size distribution and mean particle diameter was measured again to assess emulsion stability (ES), which was calculated as the percentage ratio of oil droplet size at time zero to oil droplet size measured at 30 min (Eqn 5).

Emulsion Stability (ES) =   Particle size at 0 min ($d_{3,2}$) x 100

                            Particle size at 30 min ($d_{3,2}$)   **(Eqn 5)**

4.2.10 Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way

ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at $p < 0.05$.

4.3. Results and discussion

4.3.1. Amino acid composition

The amino acid composition data show that in general the amino acid pattern of the ALB was comparable with that of GLB with only some slight differences (Table 5). For example, the GLB has a higher content of sulfur-containing amino acids, especially methionine when compared to ALB. Legume seed proteins are generally known to be poor in sulfur-containing amino acids but our results show that the GLB fraction could be a better source of this amino acid if used separately. The GLB fraction was also higher in hydrophobic and aromatic amino acids, which could contribute to increased protein-protein interactions between the polypeptide chains. The content of branched chain amino acids (BCAA) was higher in the GLB (11.84%) and suggests a nutritionally superior protein in comparison to the ALB (8.97). This is because BCAAs are recognized as important fuels for muscle metabolism and could reduce the muscle wasting symptom associated with liver disease. Arginine to lysine ratio was higher in GLB (4.37) when compared to ALB (1.74), which also adds to the nutritional superiority of the GLB fraction. This is due to the presumed cardiovascular disease risk-lowering effect of high arginine to lysine ratio (Giroux, Kurowska, Freeman, & Carroll, 1999). The cold extraction method employed in the industry to defat the oil seeds has a great influence on the properties of the isolated fractions by preventing the degradation or destruction of these heat labile molecules present in the oil seeds. The protein concentration of the recovered isolated fractions were higher than that of the starting

Table 5

Percentage amino acid composition of hemp seed albumin (ALB) and globulin (GLB) fractions

HSP fractions	Amino acids																			
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp	AAA[1]	HAA[2]
ALB	7.93	4.63	5.12	20.37	3.82	8.26	3.91	3.20	2.90	1.74	2.02	4.05	2.02	1.32	3.68	7.37	12.82	0.16	3.50	17.82
GLB	9.47	2.60	5.73	21.48	3.87	4.10	2.84	3.32	3.41	4.07	2.86	5.57	3.41	3.27	3.87	3.69	16.12	0.34	7.02	22.07

[1]Aromatic amino acids: Tyr, Phe, Trp; [2]Hydrophobic amino acids: Ala, Cys, Val, Met, Ile, Leu

material (HPM, 40%), for instance, the protein concentration of ALB is 80% and that of GLB IS 100%. In contrast to their protein concentration, the protein yield of ALB (6.33%) is relatively higher than that of GLB (6.07%). The low protein yields are a reflection of the harsh seed processing condition (mechanical press) to extract edible oil. The applied strong mechanical force could have caused severe protein-protein interactions and hence low solubility during extraction.

4.3.2. SDS-PAGE

The non-reducing SDS-PAGE profiles (Fig. 9A) showed that ALB and GLB had seven and three major polypeptides, respectively. For the ALB, the estimated molecular weights ranged from 10 to 42 kDa while those for the globulin were estimated to be between 6 and 35 kDa. Addition of β-ME did not have any substantial effect on the ALB polypeptide profile whereas three intense bands with estimated sizes of 21, 34 and 45 kDa were present in the GLB (Fig. 9B). The results suggest that the GLB protein had more disulfide bridges that link polypeptides of different molecular sizes while the ALB had less. The presence of more disulfide bridges in the GLB protein may also contribute to the poor SDS solubility and hence reduced polypeptide band intensity as evident in Fig. 9A. By breaking the disulfide bonds, the β-ME converts the less soluble proteins into smaller size polypeptides with increased solubility in the SDS buffer and hence the higher band intensity obtained for GLB in Fig 9B. However, overall, the electrophoresis separations indicate similar sizes (10-50 kDa) of polypeptides present in both protein fractions.

4.3.3. Fluorescence emission spectra

Fig10 shows that the ALB protein exhibited a typical tyrosine-tyrosine interaction with a major peak at a maximum emission wavelength (λ max) of 314 nm showing a characteristic

Fig 9. SDS-PAGE of hemp seed protein albumin (ALB) and globulin (GLB) under non-reducing (A) and reducing (B) conditions
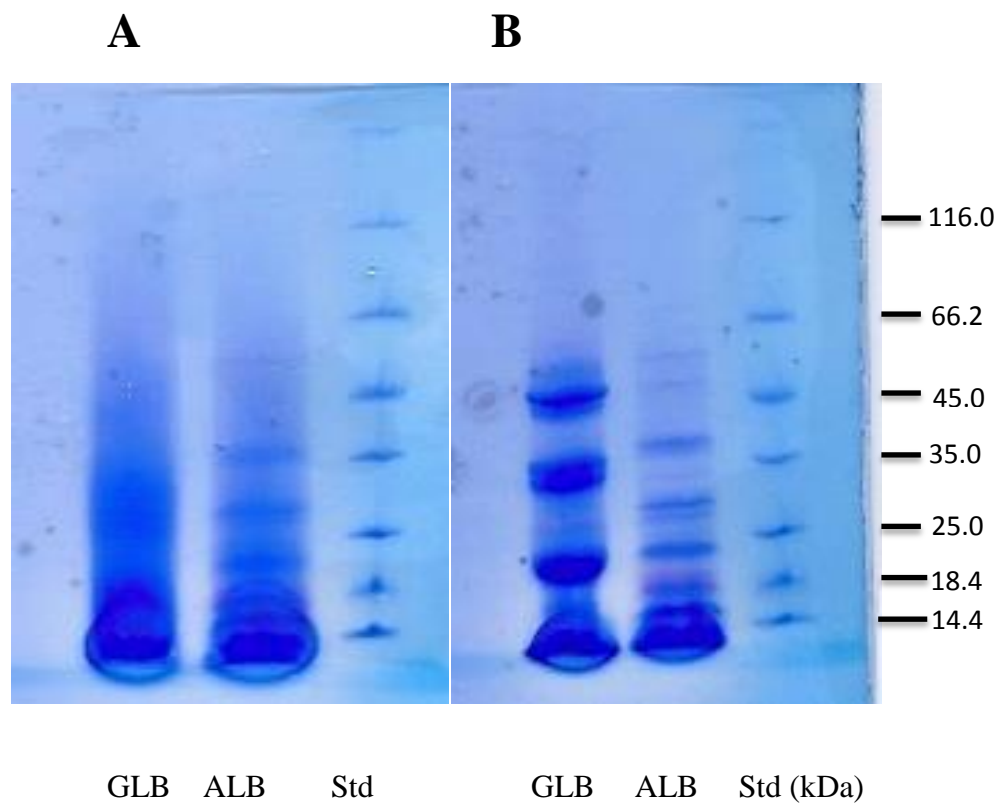
Fig. 10. Intrinsic fluorescence intensity (arbitrary units) of hemp seed protein albumin (ALB) and globulin (GLB) at different pH values
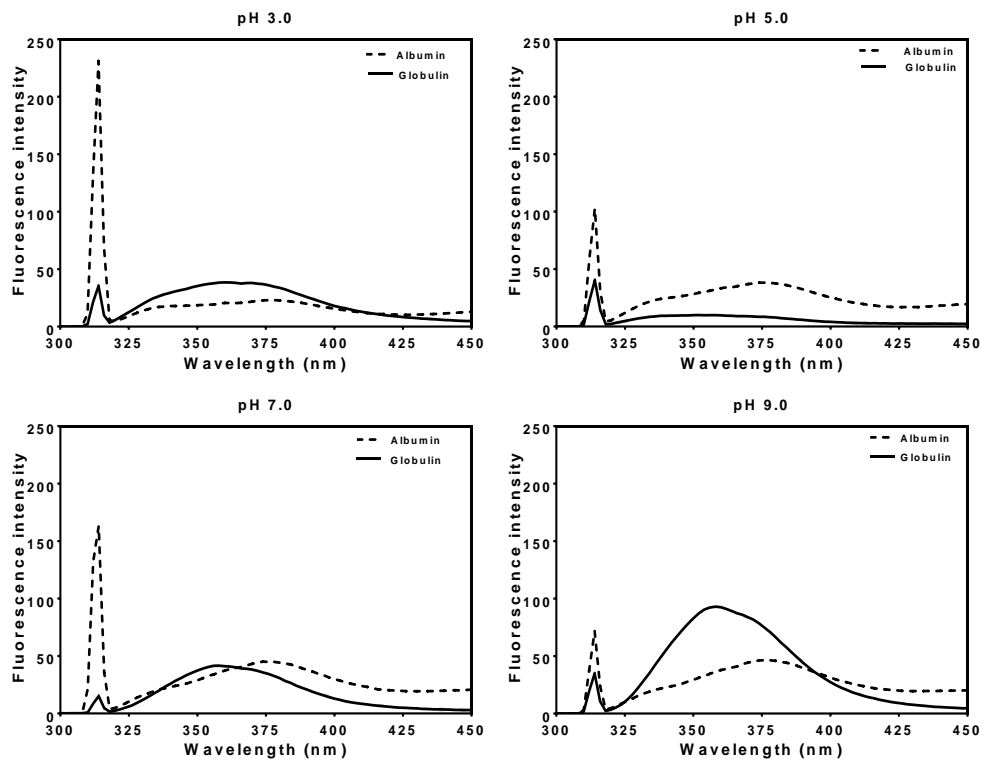
fluorescence profile of tyrosine residues that are more and strongly exposed within a hydrophobic environment (Yin et al., 2011). In contrast, the tyrosine residues in GLB were either highly exposed in a hydrophilic environment or there was increased tyrosine-tryptophan energy transfer as a result of intense protein-protein interactions. The latter effect seems more likely judging from Table 5 that showed higher contents of aromatic and hydrophobic amino acids in GLB, which could promote greater protein-protein interactions when compared to the ALB with less amounts of these amino acids. The λmax of tyrosine residues obtained from this work (314 nm) is comparable to the 318 nm value reported by Yin et al. (2011) but higher than the 303 nm reported by Mundi and Aluko (2013) for kidney bean globulins. In general, the tyrosine environment in GLB did not respond to pH changes when compared to the ALB where fluorescence intensity (FI) was less at pH 5.0 and 9.0. At pH 3.0 and 7.0, tryptophan fluorescence (350-360 nm) indicates the residues were located in a polar environment, which probably contributed to quenching and hence low and broad FI maximum. At pH 5.0, which is near the isoelectric point of hemp seed proteins, tryptophan fluorescence was almost zero. This indicates an almost complete shielding of the residues from excitation light; this result is consistent with the fact that proteins experience intense protein-protein aggregation near the isoelectric point. However, as the pH shifts away from the isoelectric point, the introduction of net charges reduces protein-protein interactions, which contributes to increased exposure of tryptophan residues and hence the observed higher FI especially at pH 9.0. The effect of increased net pH is more apparent in the GLB at pH 9.0 where the tryptophan FI indicates a more conserved structure within a hydrophobic environment, hence the blue shift in the λmax when compared to the ALB protein. This means that the increased charge at pH 9.0 led to a rearrangement in the GLB protein whereby the tryptophan residues were moved away from the

hydrophilic surface into a more hydrophobic interior and the higher FI is consistent with a higher content of this residue (Table 5) when compared to the ALB. Thus the FI data indicates that the GLB protein conformation was more globular in nature at pH 9.0 than at lower pH values. However, the reduced tyrosine FI could also reflect greater closer proximity to tryptophan, which increased tyrosine-tryptophan energy transfer. The values obtained from this work support the report of Yin et al. (2011), which showed that the λmax of tryptophan residues experienced significant shifts as buffer acidity reduced from pH 3.0 to 9.0; this confirms that the micro-environment of tryptophan residues can be manipulated by environmental conditions. Overall, the λmax value obtained for GLB (355 nm) is similar to the previously reported values of 354 and 360 nm for α-lactalbumin (Stanciuc, Aprodu, Rapeanu, & Bahrim, 2013; Stanciuc, Rapeanu, Bahrim, & Aprodu, 2012) and kidney bean vicilin (Mundi & Aluko, 2013), respectively.

4.3.4. CD spectra

In this work, CD determination was not possible at pH 5.0 due to reduced protein solubility which made required level of soluble proteins difficult to obtain. Far-UV spectra showed that at pH 3.0 and 7.0, the ALB had more secondary structure conformation than GLB; however, the conformation at pH 9.0 was similar for both proteins (Fig. 11). The secondary structure was dominated mostly by the α-helix conformation as evident by the double minimum ellipticity observed around 200-210 nm and 222 nm. At pH 3.0 and 7.0, the ALB showed stronger ellipticity, suggesting the presence of a higher degree of α-helix conformation than the GLB. At pH 9.0, there was an increased ellipticity for the GLB when compared to the pH 3.0 and 7.0 spectra, which suggests increased structural compactness (Kelly, Jess, & Price, 2005). The pH 9.0 GLB CD spectra is supported by the intrinsic fluorescence data, which showed increased FI at pH 9.0 and suggests translocation of the tryptophan residues away from the hydrophilic

Fig. 11. Far-UV circular dichroism spectra of hemp seed protein albumin (ALB) and globulin (GLB) at different pH values
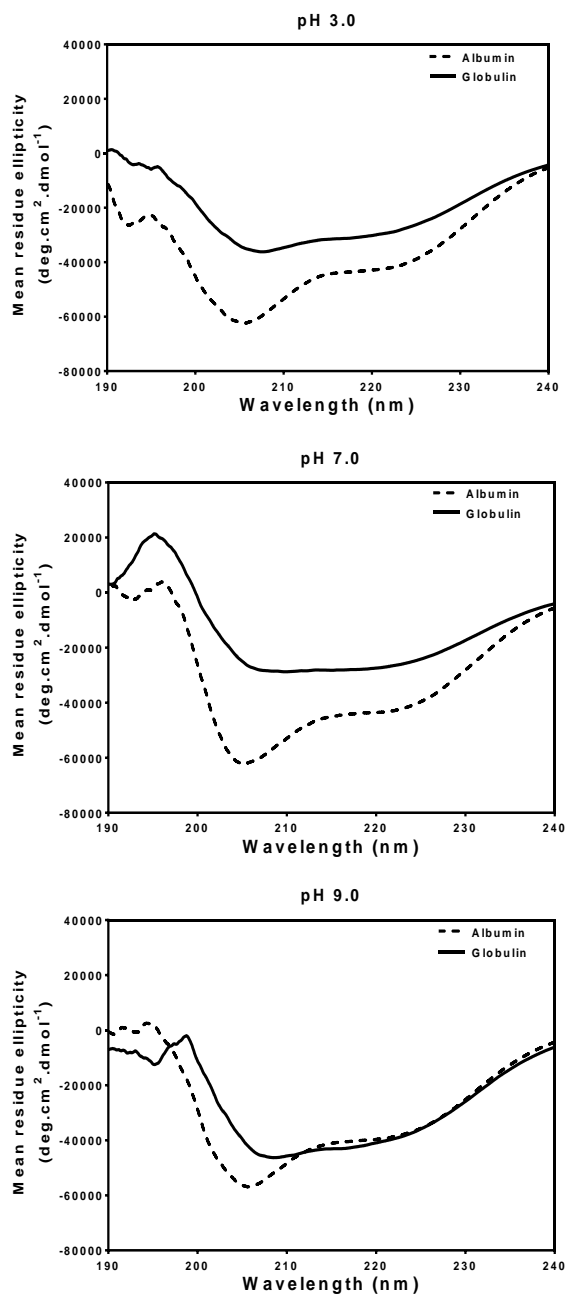
exterior into the hydrophobic interior. The results differ from the work of Mundi and Aluko (2013) who reported that kidney bean globulins showed no defined secondary structure at pH 3.0. Our results also differ from the report of Yin et al. (2011) who showed that kidney bean phaseolin had ellipticity minimum mostly at 216-218 nm, which indicates a predominance of β-sheet structure. However, the present results are consistent with previous reports (Choi & Ma, 2007; Mundi & Aluko, 2013; Yin et al., 2011) that protein secondary structure conformation can be altered as a result of pH-dependent variations in electrostatic interactions. Besides, data from Table 6 confirmed the higher degree of α-helix conformation in ALB than the GLB at pH 3.0-9.0. In contrast, the β-sheet contents in GLB were higher than that of ALB. Decrease in α-helix contents and an increase of β-sheet structure contents at different pH, were also observed between the heat-treated rapeseed protein isolates (He, He, Chao, Ju, & Aluko et al., 2014). This supports the fact that processing conditions like pH and heat can cause alteration in the protein secondary structure conformation. The unordered protein structure contents present in ALB were higher than that of GLB (Table 6) and this might have an effect on their PS. For instance, the higher contents of unordered protein in ALB might suggests a decreased structural compactness in ALB which encourages its higher PS, while lower contents of unordered protein in GLB causes its increased structural compactness, which could have contributed to the observed lower PS as shown in Fig. 13.

The near-UV CD spectra show that the shape and magnitude of both protein fractions were substantially affected by varying the pH conditions (Fig. 12). For instance, at pH 3.0, the ALB had ellipticity minimum at 276 nm, which is consistent with tyrosine residues within a hydrophobic environment; in contrast the GLB showed almost no ellipticity, which indicates residues in a hydrophilic environment. The results are consistent with intrinsic fluorescence data

Table 6

Secondary structure composition of the hemp seed albumin (ALB) and globulin (GLB) fractions

	pH 3		pH 7		pH 9	
	ALB	**GLB**	**ALB**	**GLB**	**ALB**	**GLB**
α-helix (%)	5.93 ± 0.02^c	4.45 ± 0.02^f	7.60 ± 0.02^a	4.83 ± 0.02^e	7.25 ± 0.02^b	5.40 ± 0.02^d
β-strand (%)	16.85 ± 0.05^c	18.83 ± 0.06^a	14.73 ± 0.03^e	17.83 ± 0.05^b	15.45 ± 0.04^d	17.88 ± 0.06^b
β-turns (%)	13.05 ± 0.01^e	13.45 ± 0.00^c	13.45 ± 0.00^c	13.35 ± 0.00^d	13.75 ± 0.00^a	13.55 ± 0.00^b
Unordered (%)	41.45 ± 0.01^b	40.10 ± 0.01^e	41.90 ± 0.00^a	41.25 ± 0.01^c	40.90 ± 0.00^d	40.00 ± 0.02^f

Results are presented as mean ± standard deviation. For each row, mean values that contain different letters are significantly different at $p < 0.05$

Fig. 12. Near-UV circular dichroism spectra of hemp seed protein albumin (ALB) and globulin (GLB) at different pH values
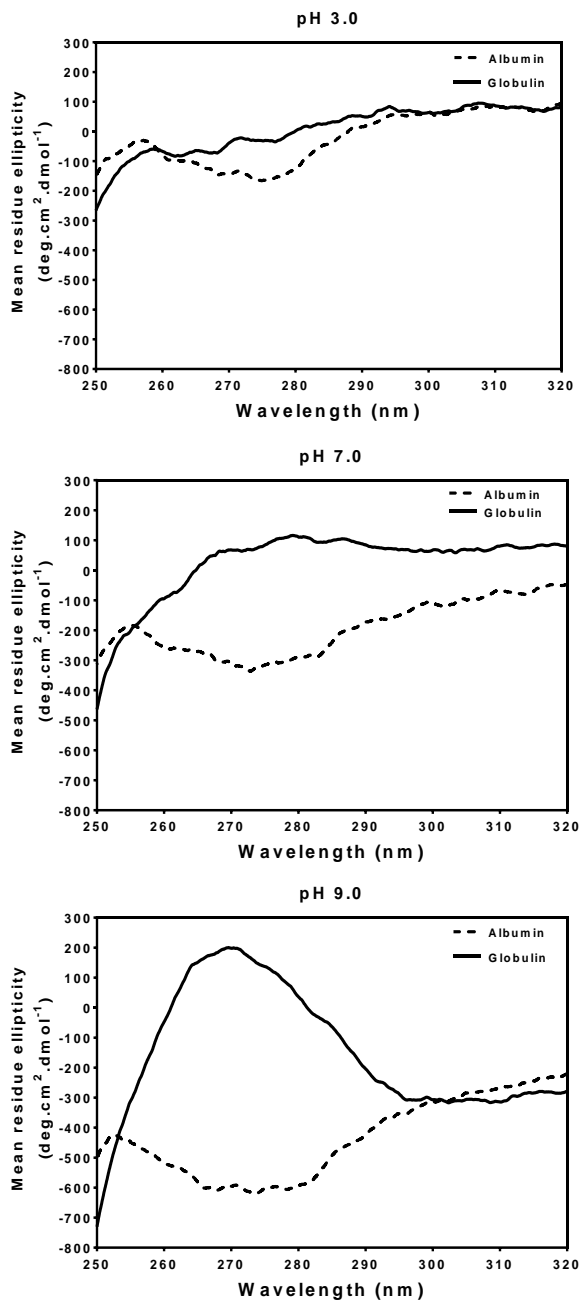
(Fig. 10) that showed tyrosine fluorescence was dominant in the ALB but almost absent in the GLB. At pH 7.0, there was an increased GLB and ALB ellipticity when compared to pH 3.0, which may be due to transfer of the aromatic amino acid residues into the more hydrophobic interior as the exterior becomes more hydrophilic, which also conforms with the intrinsic fluorescence data. This is reflected in an even greater ellipiticity values at pH 9.0 for both proteins and a more prominent tyrosine peak for GLB, which are also consistent with the intrinsic fluorescence data showing increased tryptophan emission. The results are supported by the report of Kelly et al. (2005) who indicated that the actual shape and magnitude of the near-UV CD spectrum of a protein is dependent on various factors like the number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability) and their spatial disposition in the protein.

4.3.5. Protein solubility

The effective utilization of proteins in various novel food applications is highly dependent on solubility (Karaca et al., 2011). Protein solubility occurs as a result of intermolecular repulsion caused by electrostatic interactions which ionizes the interior non-polar groups of a protein, disrupts its native structure through polypeptide chain unfolding and subsequently leads to exposure of buried functional groups (Bora, 2002). While ALB had minimum PS at pH 3.0, the minimum PS for GLB was observed at pH 5.0 (Fig. 13). The PS pattern for GLB is consistent with previous observations showing that legume globulins have minimum solubility at pH values near the isoelectric point; solubility then increases as pH values move away from the isoelectric point (Mundi & Aluko, 2012; Sai-Ut, Ketnawa, & Rawdkuen, 2009; Ragab, Babiker, & Eltinay, 2004; Yin et al., 2010). This solubility pattern is due to increased protein-protein interactions at the isoelectric point, but which gradually reduces in

Fig. 13. Protein solubility profile of hemp seed protein albumin and globulin at different pH values
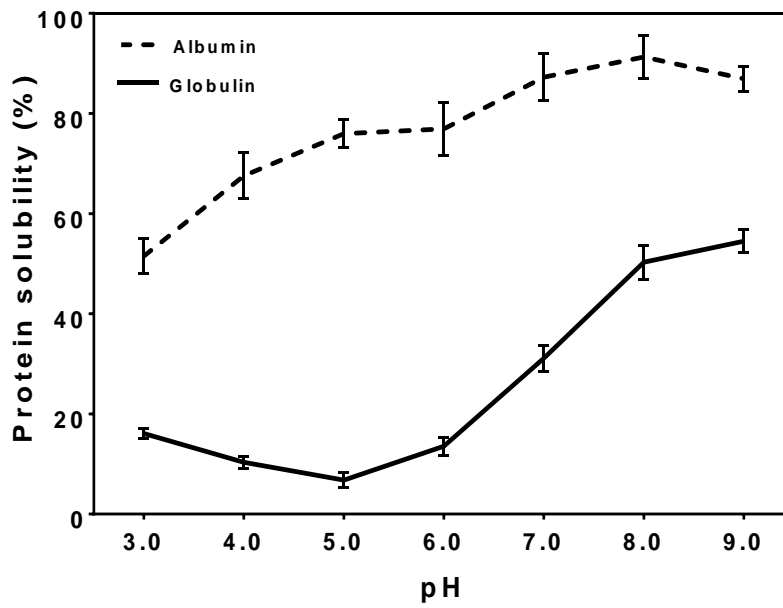
strength as net protein charge increases at higher or lower pH values. The higher PS of the ALB in this study is consistent with the reduced level of aromatic and hydrophobic amino acids or a high isoelectric point when compared to the GLB. The PS data is consistent with the fact that the tertiary structure of ALB was fairly consistent across pH values and did not experience drastic changes unlike the trends observed for GLB.

4.3.6. Foaming capacity and foam stability

FC values ranged between 30 and 100%, which varied according to changes in pH values (Fig. 14). Overall, the ALB had significantly ($p<0.05$) higher FC than the GLB when compared at all the pH and sample concentration values. The higher FC of the ALB is consistent with the observed higher PS and suggests that greater interactions with the aqueous phase enhance ability of the protein molecules to encapsulate air particles. This is because interactions with the hydrophilic aqueous phase will enhance protein unfolding and hence better foam forming ability (Sai-Ut et al., 2009). In contrast, the reduced PS of GLB limits interactions with the aqueous phase, protein conformation experiences limited unfolding and ability to properly encapsulation air particles will become limited. Thus, the higher contents of aromatic and hydrophobic amino acids probably contributed to the limited interactions of the GLB molecules with the aqueous phase in contrast to the ALB that contains less amounts of these amino acid groups. Therefore, the lower FC of GLB could be attributed to non-flexibility arising from high hydrophobicity and globular nature of the protein structure (as evident in Fig. 10), which limited its capacity to diffuse (unfold) at the air–water interface to encapsulate air bubbles. The results differ from the data reported in a previous work that showed similar FC values for ALB and GLB fractions of African locust bean seed (Lawal, Adebowale, Ogunsanwo, Sosanwo, & Bankole, 2005). Unlike a previous report (Mundi & Aluko, 2012), there was no effect of sample protein concentration (20-

125

Fig. 14. Foam capacity of hemp seed albumin and globulin in water and at different pH values: A, 20 mg/mL; B, 40 mg/mL; and C, 60 mg/mL protein concentrations (*Bars that contain different letters are significantly different at p<0.05)
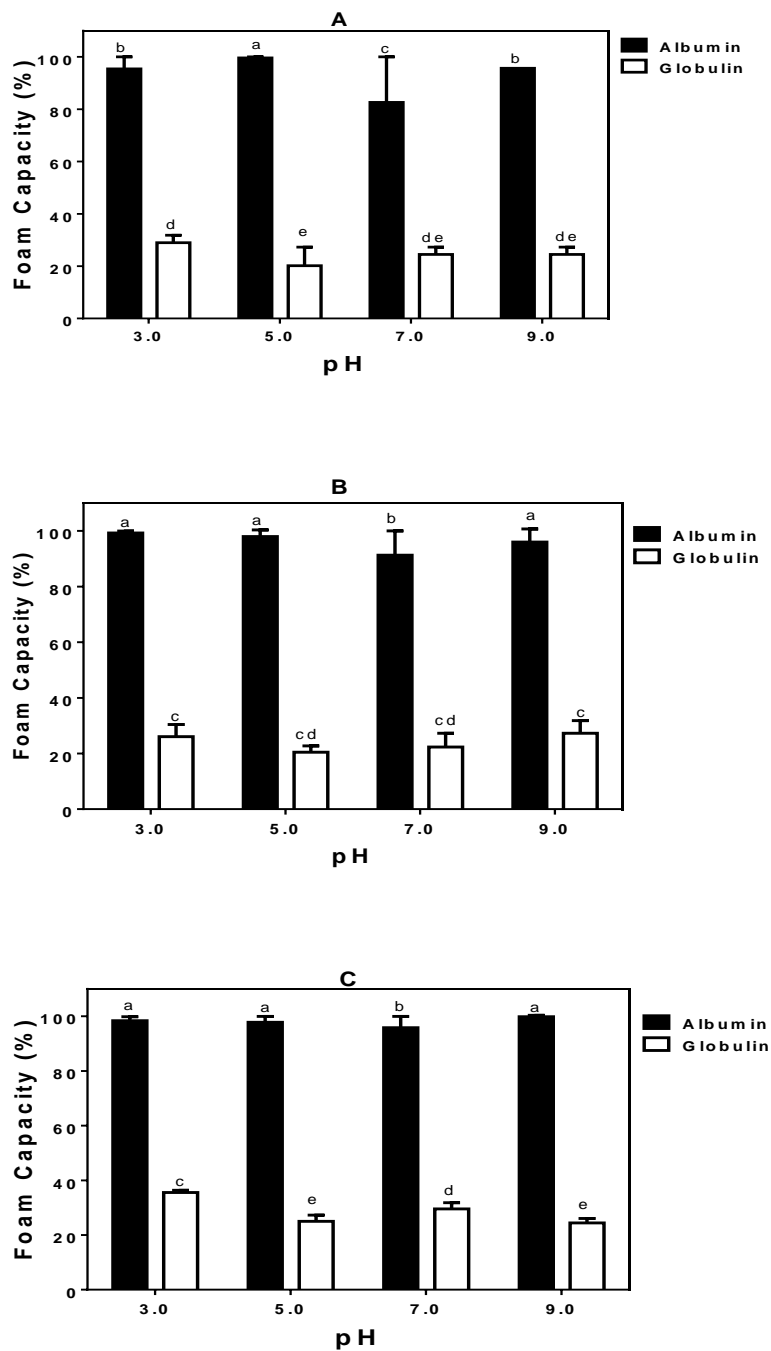
Fig. 15. Foam stability of hemp seed albumin and globulin in water and at different pH values: A, 20 mg/mL; B, 40 mg/mL; and C, 60 mg/mL protein concentrations (*Bars that contain different letters are significantly different at p<0.05)
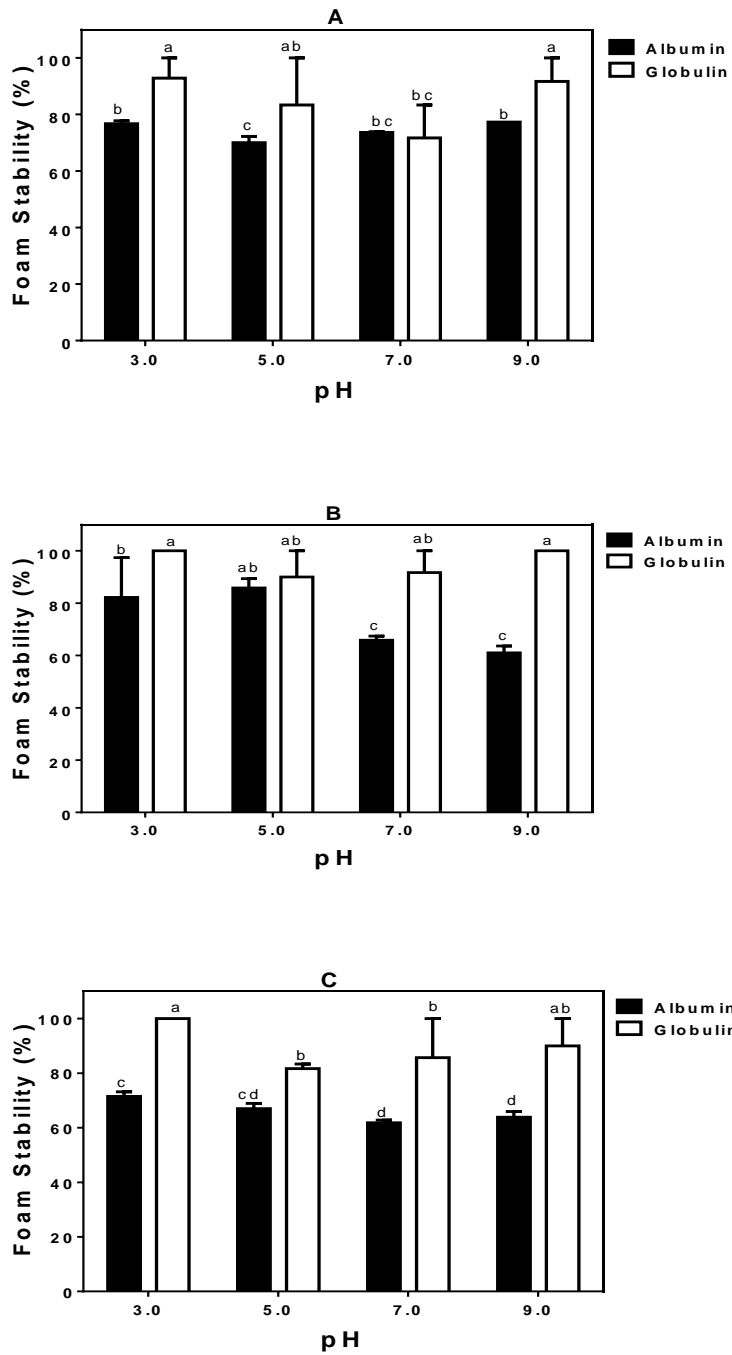
60 mg/mL) on FC of ALB and GLB. pH effect was noticeable mostly for ALB, which showed least FC at pH 7.0 for all the sample concentrations and is an indication that the balance of protein conformation and net charge probably limited air encapsulation ability.

Fig. 15 shows that GLB had higher FS than ALB when compared at most of the pH and sample concentration values. The higher FS properties of GLB may be attributed to higher contents of hydrophobic amino acids, which enhance strong protein-protein interactions (as evident in its conformational and structural changes in Figs. 11 and 12) and formation of a strong interfacial membrane at the air–water interface. This is in contrast to the weaker interfacial membranes formed by the less hydrophobic ALB. Thus the GLB interfacial membrane is able to better withstand air bubble coalescence than the ALB-stabilized foams. In contrast, the GLB fraction of African locust bean seed was reported to form less stable foams than the ALB (Lawal et al., 2005).

4.3.7. Emulsion capacity (EC) and stability (ES)

There was no observed pH-dependent significant difference (p>0.05) in the emulsion oil droplet size measured at 10 mg/mL between the two protein fractions (Fig. 16A). When protein concentration was increased to 25 mg/mL, there was a significant reduction in the GLB emulsion oil droplet size (Fig. 16B), which indicates increased emulsifying ability when compared to the other pH values. The results are consistent with the FI data (Fig. 10), which showed that the GLB had most intense protein-protein interactions that may have shielded the aromatic groups (least FI intensity) at pH 5.0. The increased protein-protein interactions at pH 5.0 would have enhanced better interfacial membrane formation to produce smaller oil droplet sizes. At 50 mg/mL protein concentration, the emulsion oil droplet sizes were also similar except that the ALB emulsions had higher values at pH 7.0 and 9.0 (Fig. 16C). The increased oil droplet sizes of ALB emulsions

Fig. 16. Oil droplet sizes of emulsions formed by hemp seed albumin and globulin in water and at different pH values: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations (*Bars that contain different letters are significantly different at p<0.05)
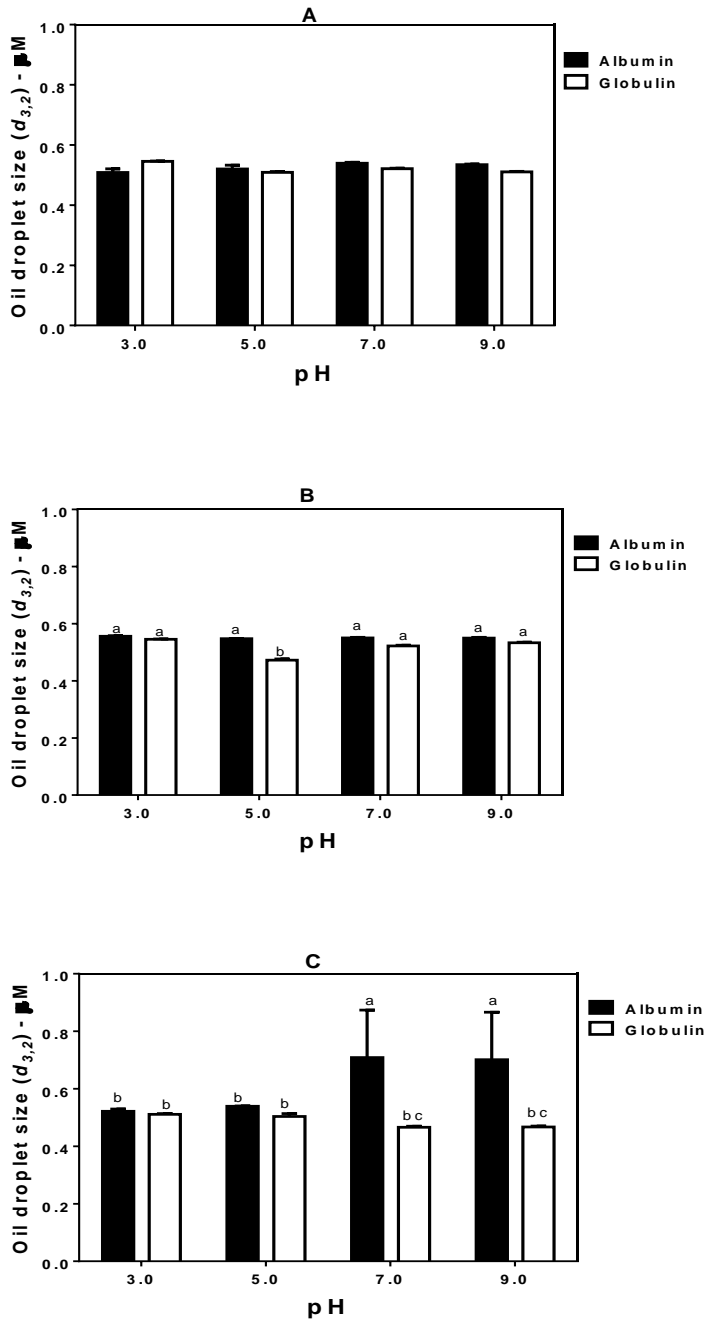
Fig. 17. Emulsion stability formed by hemp seed albumin and globulin in water and at different pH values: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations (*Bars that contain different letters are significantly different at p<0.05)
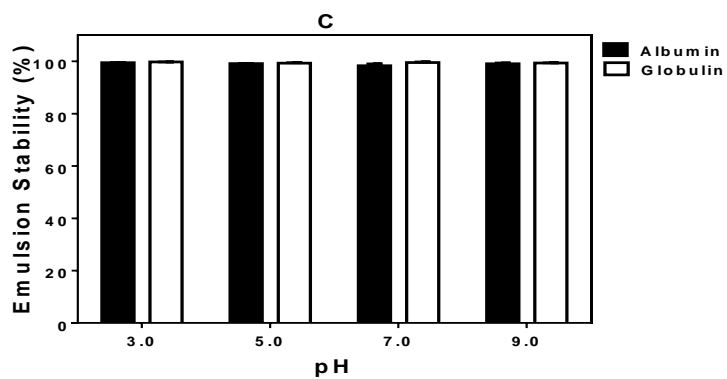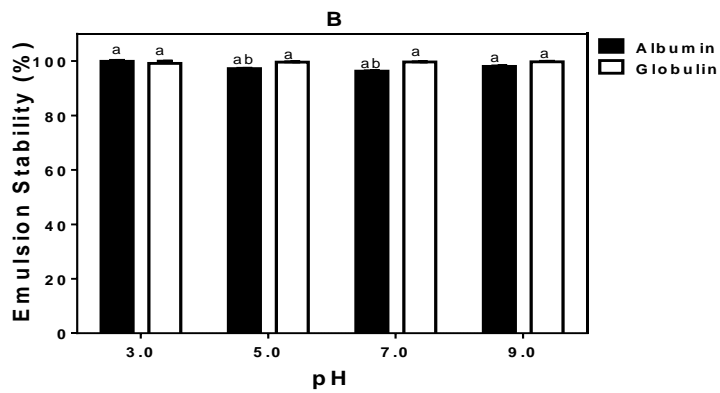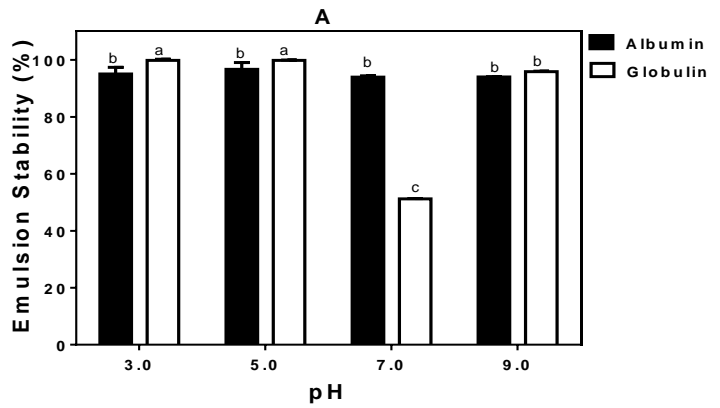
may be attributed to higher net charge effects, which would have reduced ability of the protein molecules to form strong interfacial membranes (due to increased protein-protein repulsions) at neutral and alkaline pH values. The increased protein concentration would have also contributed to the reduced emulsifying ability of ALB at pH 7.0 and 9.0 since there will be more charged protein molecules in solution when compared to lower protein concentrations. Overall, both protein fractions formed emulsions with small particle sizes similar to those reported for dairy whey protein isolate (Taherian, Britten, Sabik, & Fustier, 2011) and indicate better emulsifiers when compared to other plant proteins (Aluko, Mofolasayo, & Watts, 2009; Li, Kong, Zhang, & Hua, 2011).

The results presented in Fig 17 showed that differences in ES was mostly dependent on protein concentration; there were less differences as sample concentration increased from 10 to 50 mg/mL. At 10 mg/mL, the GLB emulsions were the most stable at pH 3.0 and 5.0 but there were no differences between the ALB emulsions (Fig. 17A). The low ES of the GLB emulsion at pH 7.0 indicates formation of very weak interfacial membrane, which was less resistant coalescence when compared to the other pH values. At 25 and 50 mg/mL, the GLB emulsions had slightly higher stability but there were no significant differences. Therefore, it is possible that the effects of differences in molecular structure were minimized by the higher protein contents used to form the emulsions. The increased protein concentrations (25 and 50 mg/mL) would have contributed to formation of thicker interfacial membranes and hence higher ES when compared to the 10 mg/mL emulsions. The results are in contrast to previous reports (Deng et al., 2011; Chavan, McKenzie, & Shahidi, 2001) that demonstrated reduced ES at low pH values due to increased interactions between the emulsified droplets as a result of decreased net charge on the proteins.

4.4. Conclusion

The differences in functionalities of hemp seed ALB and GLB protein fractions may be attributed to variations in amino acid composition and pH-dependent changes in structural conformation. Polypeptide composition indicates the GLB has more disulfide bonded proteins, which produced a rigid structure with reduced exposure of aromatic amino acids when compared to the ALB. The higher level of hydrophobic amino acids and the rigid conformational structure in GLB contributed to decreased PS and FC, which confirms the dependence of these functional properties flexibility and ability of proteins to interact with the aqueous phase. In contrast, emulsion-forming ability was not dependent on PS, which suggests that different mechanisms are involved in foam and emulsion formations. Both protein fractions are potentially good ingredients for the manufacture of food emulsions but only the ALB may find role in the manufacture of food foams.

Acknowledgement

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4.5 Statement transfer between the study 2 and study 3

The conventional dialysis process used in the preceding chapter allowed the study of pure protein fractions but production yield coupled with the isolation method (dialysis) may not be compatible with practical commercialization. Therefore, the next chapter deals with conversion of a waste by-product from hemp seed processing industry into protein isolates using processes that could be readily scaled-up for commercialization. The product will have high solubility properties and better functionalities when compared with protein isoelectric pH-precipitated protein isolates and isolated protein fractions. The starting raw material is a non-functional hemp seed meal residue, which is characterized by low protein content, high phytate and high fibre. The hemp seed meal residue is normally dumped in landfills or used as a source of fertilizer on farms. Therefore, a method was developed to remove some of the fibre and phytate components of the meal residue in order to leave behind a product with higher protein content and improved functionality in foods. This involved the use of an enzyme (carbohydrase and phytase) pre-digestion method to breakdown the fibres (sugars) and phytate into low molecular weight (<10 kDa) digests, which are then removed through membrane filtration process. The modified protein is expected to have entirely different functionality (even when added in small amounts to food products) when compared with parent protein (meal) and isolates from the other methods of isolation. It is believed that isolate from this enzyme pre-digestion cum membrane filtration method can be used in novel food processing and add value to the hemp processing industry.

5. Conversion of a low protein hemp seed meal into a functional protein concentrate through enzymatic digestion of fibre coupled with membrane ultrafiltration

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Abstract

Industrial hemp seed protein meal (HPM) containing ~37% protein content was digested with carbohydrases and a phytase. The digest was then passed through a 10 kDa ultrafiltration membrane system and the retentate freeze-dried as the membrane hemp seed protein concentrate (mHPC), which had 74% protein content. Functional properties of the mHPC was compared with a commercial hemp seed protein concentrate (cHPC, 70% protein) and a traditional protein isolate produced by isoelectric pH precipitation (iHPI, 84% protein). Four main polypeptides (14, 24, 34, and 47 kDa) were most apparent under non-reducing gel electrophoresis, but the mHPC has less intensity of the 24 and 34 kDa bands. There was reduced intensity of the 47 kDa band in the presence of mercaptoethanol, which indicates the protein contains disulfide bonds. The mHPC had the highest contents of essential amino acids as well as branched-chain amino acids among the hemp seed products. Protein digestibility (89%) was significantly ($P<0.05$) higher than the ~85% obtained for the HPM, iHPI, and cHPC. The mHPC also had significantly ($P<0.05$) higher protein solubility than other hemp products in the pH 3-9 range with a minimum of 76% at pH 4.0. In contrast the HPM and iHPI had <25% maximum protein solubility with minimum of 7.5% and 0.35% solubility, respectively. Foaming capacity was highest for mHPC (55-98%) when compared to 10-70% for the other protein products. However, emulsions formed by mHPC and cHPC had bigger oil droplet sizes (4.5-15.5 µm) when compared with <1 µm for HPM and iHPI emulsions. The excellent and improved functional properties for mHPC suggest that the mHPC could be used for the nutritional fortification of low protein foods and drinks (for example fruit juices).

Keywords: hemp seed; protein solubility and digestibility; protein isolate; membrane ultrafiltration; foaming; emulsion

5.1. Introduction

The increased utilization of hemp plant for various industrial purposes has led to abundant amounts of protein-rich seed residue, which can be converted into various forms of powdered protein products. In Canada, hemp seed processing primarily involves the cold-pressing method to expel oil and leave behind a high protein (30-50%) residue/meal (Malomo et al., 2014). The hemp seed protein (HSP) products have been shown as promising raw materials for the production of peptides with potential human health applications (Girgih et al., 2014a, 2014b). However, the hemp seed protein materials are poorly utilised in applications with added value. For instance, the current commercial HSP products are mainly in the form of protein concentrates that have less than 70% protein content and contain high levels of fibre and phytate (Lu et al., 2010). The high fibre and phytate contents of these HSP products have been reported to cause protein functionality reduction, which limits their use as new food ingredients (Yin et al., 2009).

Moreover, current HSP isolates are generally prepared by alkaline extraction, which is then followed by isoelectric precipitation and is the most commonly used traditional procedure for plant protein isolate production (Malomo et al., 2014). The harsh conditions used in the traditional procedure have negative effects on protein functionalities, especially protein solubility and foaming properties (Liu et al., 2013). The poor food functionality of current HSP products can also be attributed to the high phytate content, which can cause protein cross-linking and reduce protein solubility. Therefore, in order to produce novel protein isolates with improved food functionalities, a method that involves fibre and phytate digestion followed by removal of non-protein materials by membrane filtration was developed. The use of membrane ultrafiltration obviates the need for harsh protein precipitation protocols while ensuring high yield of proteins

with minimal conformational denaturation. Recent studies have confirmed the successful application of this novel method of protein isolate production with high quality properties. For instance, Wang et al. (2014) reported a soy protein isolate with higher protein contents (91-93%) and better *in vitro* digestibility from phytase-assisted processing method. A better functional (foam capacity) protein isolates with high protein content (92%) and yield (75%) was produced from rice bran using a combination of xylanase and phytase pre-digestion process (Wang et al., 1999). Another study (Udenigwe et al., 2009) had shown that pre-digestion of the fibre with cellulase resulted in up to 23% higher protein content of flaxseed protein isolate. However, pre-digestion with carbohdrases and phytases coupled to membrane ultrafiltration is novel and has not been previously reported for HSP isolate production.

During industrial hemp seed processing, the defatted meal is usually milled and passed through several sieves to obtain high-value protein products. The milling and sieving process leaves behind a high-fibre waste product that is normally dumped in farms as a nitrogen source or simply discarded in landfills. Therefore, this study aimed to convert this high-fibre but low protein industrial side-stream of hemp seed processing into a high protein product through enzymatic digestion of the main non-protein materials (fibre and phytate) followed by removal of the low molecular weight digests by membrane ultrafiltration. The membrane protein isolate was then compared with the traditional protein isolate from isoelectric pH precipitation and a commercial hemp seed protein concentrate to determine protein digestibility and potential functionality in food systems.

5.2. Materials and Methods

5.2.1. Hemp seed protein products and chemical reagents

Hemp seed protein meal (HPM) a 37% protein content (~9% residual oil) product of the hemp seed oil processing industry was kindly provided by Hemp Oil Canada (St. Agathe, Manitoba, Canada). To produce HPM, the hemp seeds are mechanically pressed to remove the highly valued oil. The defatted hemp seed cake is then milled in a classifier milling system to the desired particle size and sifted through various screens to remove seed coat and other coarse materials. Hemp Pro70, a commercial hemp seed protein concentrate (cHPC) produced through proprietary methods was purchased from Manitoba Harvest Hemp Foods (Winnipeg, MB, Canada). All enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

5.2.2. Preparation of hemp seed protein concentrates (mHPC) by ultra- and diafiltration

mHPC was produced from HPM according to the method previously described (Udenigwe et al., 2009) with slight modifications. Approx. 5% (w/v) slurry of HMR was adjusted to the following conditions: 4.5% cellulase (*Aspergillus niger*; E.C. 3.2.1.4; ~0.8 U/mg) (w/w), 0.75% hemicellulase (*Aspergillus niger*; 647-004-00-4; 1.5 U/mg solid) (w/w), 0.75% xylanase (*Thermomyces lanuginosus*; ≥2500 U/g) (w/w), and 0.5% phytase (wheat; 0.01-0.04 U/mg solid) (w/w) at 37 $^\circ$C and pH 5.0. The digestion was allowed to proceed for 4 h, cooled to room temperature and then subjected to membrane ultrafiltration/diafiltration processing using a 10 kDa molecular weight cut-off (MWCO) membrane. The digested fibre and phytate fragments were removed in the permeate while the target proteins remained in the retentate. Three rounds of ultrafiltration/diafiltration were used in order to ensure that most of the digested non-protein materials were removed, after which the retentate was freeze-dried as the mHPC. Protein concentration of the mHPC was determined using the modified Lowry method (Markwell et al., 1978).

5.2.3. Preparation of traditional (isoelectric precipitated) hemp seed protein isolate (iHPI)

iHPI was produced from HPM according to the previously described method of Malomo et al. (2014) with slight modifications. HPM was dispersed in deionized water (1:20, w/v) and the dispersion adjusted to pH 8.0 using 2 M NaOH to solubilize the proteins while stirring at 37 $^{\circ}$C for 2 h; this was followed by centrifugation (7000 x g, 60 min at 4 $^{\circ}$C). The precipitate was discarded and the supernatant filtered with cheese-cloth; adjusted to pH 5.0 with 2 M HCl to precipitate the proteins and thereafter centrifuged (7000 x g, 60 min at 4 $^{\circ}$C). The resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to obtain the iHPI. Protein concentration of the iHPI was determined using the modified Lowry method (Markwell et al., 1978).

5.2.4. Amino acid composition

The amino acid profiles of HSP products were determined using the HPLC Pico-Tag system according to the method previously described after samples were digested with 6 M HCl for 24 h (Bidlingmeyer et al., 1984). The cysteine and methionine contents were determined after performic acid oxidation (Gehrke et al., 1985) and the tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye 1992).

5.2.5. In vitro protein digestibility method

The *in vitro* protein digestibility of the HSP products was carried out according to a previously described method (Hsu et al., 1977) with slight modifications using an enzyme system consisting of trypsin (bovine pancreas; E.C. 3.4.21.4; 1000 BAEE U/mg) and chymotrypsin (bovine pancreas; E.C. 3.4.21.1). A 10 mL aliquot of aqueous protein suspension (6.25 mg protein/mL) in double distilled water was adjusted to pH 8.0 with 0.1 M NaOH while stirring at 37 °C. The enzyme solution (containing 1.6 mg trypsin and 3.1 mg chymotrypsin/mL) was maintained in an

ice bath and 1 mL of the solution was then added to the protein suspension. The pH drop was recorded automatically over a 10 min period using a Metrohm 842 Titrando system (Mississauga, ON, Canada). The % protein digestibility of each protein sample was calculated using the regression equation predicted by Hsu et al. (1977) as follows:

% Protein digestibility $(Y) = 210.46 - 18.10X_f$

Where X_f is the final pH value of each sample after a 10 min digestion. **(Eqn 6)**

5.2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The HSP products were subjected to SDS-PAGE (reducing and non-reducing) according to the method of Aluko & McIntosh (2004) with some modifications. The protein samples were each dispersed (10 mg/mL) in Tris/HCl buffer, pH 8.0 containing 10% (w/v) SDS only (non-reducing buffer) or SDS + 10% (v/v) β-mercaptoethanol (reducing buffer), followed by heating at 95 ^{o}C for 10 min, cooled and centrifuged (10000 x g, 15 min). After centrifugation, 1 µL of supernatant was loaded onto 8-25% gradient gels and electrophoresis performed with Phastsystem Separation and Development units according to the manufacturer's instructions (GE Healthcare, Montréal, PQ, Canada). A mixture of protein standards (14.4-116 kDa) was used as the molecular weight marker and the gels were stained with Coomassie brilliant blue.

5.2.7. Protein solubility (PS)

PS of HSP products was determined according to the method described by Malomo et al. (2014) with slight modifications. Briefly, 10 mg of sample was dispersed in 1 mL of 0.1 M phosphate buffer solutions (pH 3.0-9.0) and the resulting mixture was vortexed for 2 min and centrifuged at 10,000 x g for 20 min. Protein content of the supernatant was determined using the modified Lowry method (Markwell et al., 1978). Total protein content was determined by dissolving the

protein samples in 0.1 M NaOH solution. PS was expressed as percentage ratio of supernatant protein content to the total protein content.

5.2.8. Water and oil holding capacity (WHC and OHC)

The WHC and OHC were determined using the method of Malomo et al. (2014) with slight modifications. Protein sample (1 g) was dispersed in 10 mL of distilled water (or pure canola oil) in a 15 mL pre-weighed centrifuge tube. The dispersions were vortexed for 1 min, allowed to stand for 30 min and then centrifuged at 7, 000 x g for 25 min at room temperature. The supernatant was decanted, excess water (or oil) in the upper phase drained for 15 min and tube containing the protein residue was weighed again to determine amount of water or oil retained per gram of sample.

5.2.9. Foaming capacity (FC)

FC was determined according to the method of Malomo et al. (2014) using slurries that were prepared as 20, 40, or 60 mg/mL (protein weight basis) sample dispersions in 50 mL graduated centrifuge tubes containing 0.1 M sodium phosphate buffer, pH 3.0, 5.0, 7.0, and 9.0. Sample slurry was homogenized at 20,000 x g for 1 min using a 20 mm foaming shaft on the polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The foam volume was recorded as the FC. The ability to retain air for a certain period of time (foam stability, FS) was calculated by measuring the foam volume after storage at room temperature for 30 min and expressed as percentage of the original foam volume.

5.2.10. Emulsion formation and oil droplet size measurement

Oil-in-water emulsions were prepared as previously described (Malomo et al., 2014) but with slight modifications. Slurries of 10, 25 and 50 mg protein/mL concentrations were prepared in 0.1 M sodium phosphate buffer at pH 3.0, 5.0, 7.0, or 9.0 and 1 mL of pure canola oil added. The

oil/water mixture was homogenized at 20,000 rpm for 2 min with a 20 mm non-foaming shaft on the Polytron PT 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The emulsion oil droplet size ($d_{3,2}$) was determined using a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K.) with distilled water as dispersant. Under constant shearing, an emulsion sample was taken from the emulsified layer and added to about 100 mL of water contained in the small volume wet sample dispersion unit (Hydro 2000S) until the required level of obscuration was attained. The instrument setting automatically measured the oil droplet size of each emulsion in triplicate while each sample was prepared in triplicate. Emulsions formed were kept at room temperature for 30 min without agitation and the particle size distributions and mean particle diameters were measured again to assess emulsion stability (ES), which was calculated as the percentage ratio of oil droplet size at time zero to oil droplet size measured at 30 min.

$$\text{Emulsion Stability (ES)} = \frac{\text{Particle size at 0 min } (d_{3,2})}{\text{Particle size at 30 min } (d_{3,2})} \times 100 \qquad \textbf{(Eqn 7)}$$

5.2.11. Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at $p < 0.05$.

5.3. Results and discussions

5.3.1. Amino acid composition

There seemed to be no notable differences in the amino acid composition data of all the HSP products before and after protein isolation process, with the exception that the mHPI had slight increases in valine content but decreased arginine (Table 7). However, the essential amino acid

Table 7

Percentage amino acid composition of hemp seed protein products*

Amino acid	HPM	mHPC	iHPI	cHPC
Asx	10.78	11.17	11.31	10.70
Thr	3.75	3.77	3.42	3.48
Ser	5.65	5.55	5.58	5.49
Glu	18.45	17.53	19.10	18.92
Pro	4.37	4.98	4.30	4.54
Gly	4.67	4.86	4.31	4.22
Ala	4.19	4.25	3.81	4.22
Cys	1.52	1.62	1.25	1.46
Val	4.69	5.34	4.66	4.78
Met	2.28	1.94	1.92	2.04
Ile	3.59	3.81	3.63	3.67
Leu	6.87	6.88	6.46	6.62
Tyr	3.04	2.79	3.61	3.32
Phe	4.68	4.74	4.72	4.49
His	3.26	3.48	3.15	3.05
Lys	3.94	4.21	2.73	3.75
Arg	13.20	11.98	15.06	14.29
Trp	1.06	1.05	1.00	0.99
EAA	34.12	35.22	31.69	32.87
BCAA	15.15	16.03	14.75	15.07
AAA	8.78	8.58	9.33	8.80
BCAA/AAA	1.73	1.87	1.58	1.71
Lys/Arg	0.30	0.35	0.18	0.26

*Essential amino acids (EAA), branched chain amino acids (BCAA), Aromatic amino acids (AAA). HPM; hemp seed protein meal; mHPC, membrane ultrafiltration hemp seed protein concentrate; iHPI, isoelectric pH-precipitated hemp seed protein isolate; cHPC, commercial hemp seed protein concentrate

(EAA) and branched-chain amino acid (BCAA) contents of mHPI (35.22% and 16.03%, respectively) were higher than those of the other products. The BCAA values obtained in this study are higher than the 12.40-13.00% reported for flaxseed protein products but lower than 17.00% of soy protein (Oomah, 2001). The higher BCAA/AAA ratio in mHPC could contribute to better muscle metabolism and maintenance of protein homeostasis (Herman et al., 2010; Sun et al., 2011). However, the BCAA/AAA ratios obtained in this work are lower than the values reported for *Brassica carinata* protein hydrolysates (Pedroche et al., 2006) as well as flaxseed high-molecular weight 12S protein and soy protein (Oomah, 2001).But the high Lys/Arg ratio reduces potential contributions to improved cardiovascular health. This is because arginine is a precursor for the vasodilatory nitric oxide; therefore, higher arginine levels are desirable for a healthy cardiovascular system. On the other hand, the *Lys/Arg* ratios obtained in this work (mHPC, 0.35) are lower than the 0.37-1.00 and 0.88 reported for flaxseed protein products and soy proteins (Oomah, 2001), respectively. The results suggest that the hemp seed proteins may have better impact than flaxseed protein fractions and soy protein in improving cardiovascular health.

5.3.2. SDS-PAGE

The non-reducing SDS-PAGE profiles revealed that the 47 kDa band contains the main polypeptide in the HSP products (Fig. 18A). Other polypeptides with lower band intensity are apparent in the 14, 24, and 34 kDa bands. However, the 24 and 35 kDa bands seem to be absent in the mHPC. The similarity in the polypeptide composition of cHPC, iHPI and HPM indicates that the processing methods used for the protein products extracted similar proteins from HMR. In contrast the mHPC had a slightly different polypeptide composition, which suggests that the membrane ultrafiltration protocol led to retention of higher ratios of the 14 and 47 kDa

Fig. 18. Polypeptide composition of hemp seed protein products under non-reducing (A) and reducing (B) sodium dodecyl sulfate polyacrylamide gel electrophoresis conditions: cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated hemp seed protein isolate; mHPC, membrane ultrafiltration hemp seed concentrate; HPM, hemp protein meal.
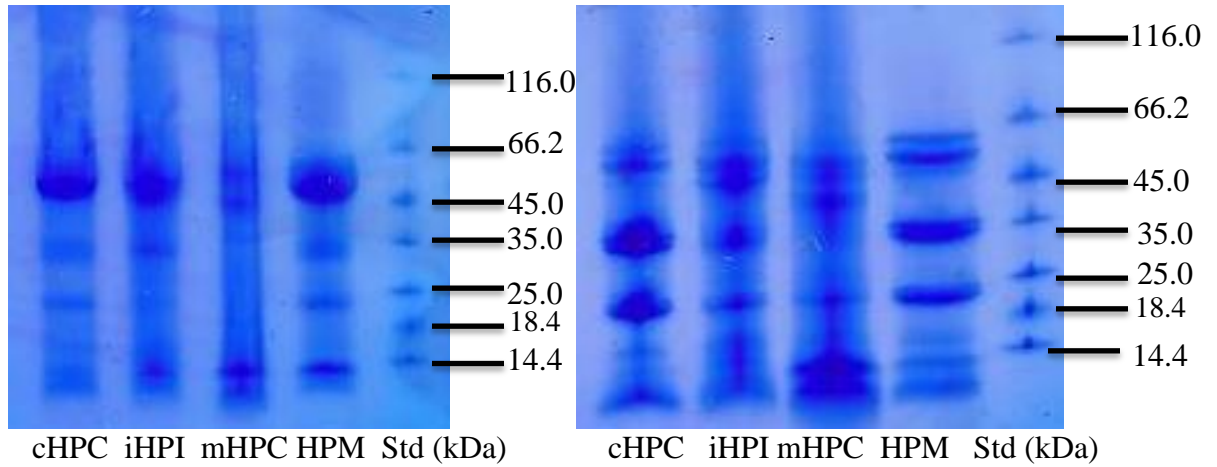
polypeptides and hence less intensity of the 24 and 34 kDa proteins. The results are consistent with our previous report which showed the presence of these main polypeptides in other types of HSP products under non-reducing SDS-PAGE (Malomo et al., 2014). However, unlike the present work where the 47 kDa was the main polypeptide, our previous work showed that the 24 and 34 kDa polypeptides were the major proteins in a previously reported protein isolate (Malomo et al., 2014). The difference could be because the protein isolate used by Malomo et al. (2014) was prepared from a different defatted meal (50% protein content) and not the seed waste product used for the present work. However, under the reducing conditions (presence of mercaptoethanol), the 14 and 47 kDa bands were dissociated into lower molecular weight polypeptides, which suggest the presence of disulfide bonds (Fig. 18B). Thus the mHPC lane showed more visible polypeptide bands under reducing SDS-PAGE than non-reducing. For the cHPC, iHPI and HPM, there were increased band intensities for the 24 and 34 kDa polypeptides under reducing conditions, which may have been due to dissociation of disulfide bonds in the higher molecular weight proteins. The results are also consistent with reports of Tang et al. (2006) and Wang et al. (2008) who showed presence of a 48 kDa protein in hemp seed protein isolates.

5.3.3. Protein contents and Protein solubility (PS)

The mHPC (74% protein, wet weight basis) and iHPI (84% protein, wet weight basis) were successfully obtained from the starting material, HPM (~37% protein) in this study after employing the enzyme pre-digestion coupled with membrane ultrafiltration and isoelectric precipitation methods, respectively. The results indicate that the enzyme cocktail used was able to degrade most of the non-protein materials in the original waste product to produce a 200% protein enriched product when compared with HPM. The mHPC also had slightly higher protein

Fig. 19. Protein solubility profile of hemp seed protein products at different pH values: cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated protein isolate; mHPC, membrane ultrafiltration protein concentrate; HPM, hemp seed meal residue.
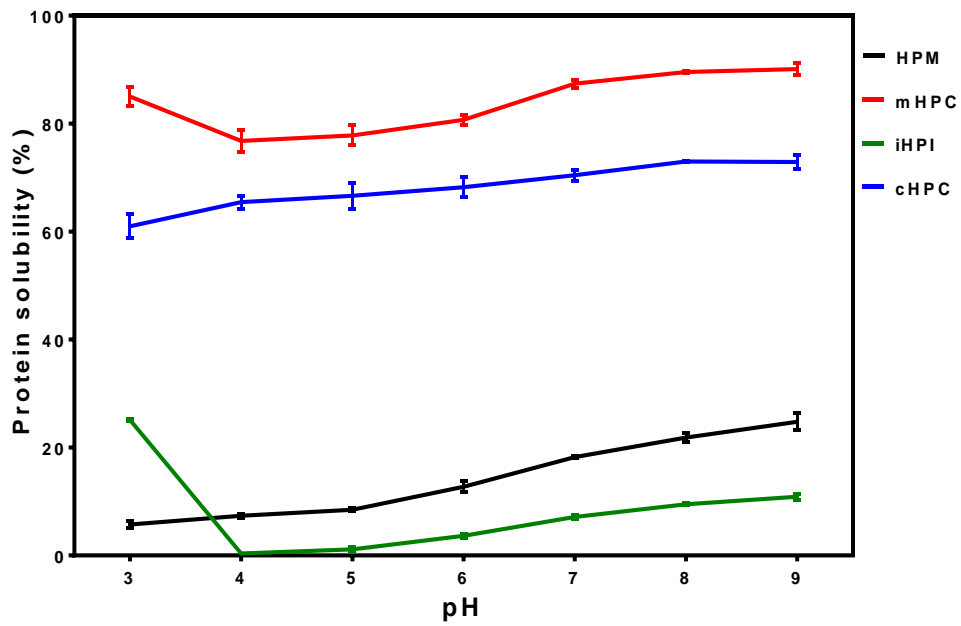
content (74%) than the 70% for commercial product (cHPC). Teh et al. (2014) obtained hemp (alkali soluble, 95%; acid soluble, 91% protein) and canola (alkali soluble, 93% acid soluble, 94% protein) protein isolates from their starting materials, hemp (33% protein) and canola (37% protein) seed cakes, respectively. These findings indicate that the type and sources of raw materials as well as methods used for protein product preparation are the major determinants of protein contents of the resultant protein isolates.

The PS of any protein at various pH levels has been a useful indicator in determining potential incorporation and performance in the food systems (Mohamed et al., 2009; Adebowale et al., 2007). Fig. 19 shows that the mHPC and cHPC had >60% PS across the pH 3-9 range but the former had higher values. In contrast the iHPI and HPM had very poor PS (<25% at pH 3-9); the iHPI was virtually insoluble at pH 4-5, which is the isoelectric point range for these proteins. However, the poor solubility of iHPI could have been caused by intense protein-protein interactions that led to aggregation and precipitation during the isoelectric pH protein isolation protocol (Adebowale et al., 2007). As expected HPM proteins were also very insoluble because crosslinking by phytate may have reduced ability to properly interact with water. Similarly, the HPM contained high levels of insoluble fibre that reduced protein-water interactions. In contrast, the removal of some of the fibre and phytate provided beneficial effects in terms of protein-water interactions.

The high PS of the mHPC could have also been due to the absence of protein aggregation that is common to iHPI. Thus the mHPC would have experienced less protein denaturation (such as increased exposure of hydrophobic groups), which ensures better water interactions by the native polypeptide chains. The high PS of the cHPC suggests that the proprietary method used for protein isolation did not involve isoelectric pH-induced precipitation. The PS reported for

154

mHPI (>75%) in this study is in the range of those reported for thermally-improved *adzuki* (28-79%), soy (49-82%) and pea (40-82%) protein isolates in the same pH (3-9) range (Barac et al., 2015). The high PS observed for mHPC (>75%) in the pH 3-5 range indicates potential use in the formulation of acidic foods such as protein-enriched beverages (Adebowale et al., 2007).

5.3.4. In vitro Protein digestibility (IPD)

The trypsin-chymotrypsin combination has been previously used and reported to simulate gastrointestinal enzymatic processes that occurred in the normal human digestion of food proteins (Pihlanto & Markinen, 2013). During protein digestion, peptide bond hydrolysis releases H+, which causes pH to decrease; faster decreases represent higher rates of digestion and can be used as an index of protein digestibility. Fig. 20 shows that the pH of mHPC decreased rapidly from 8.0 to 7.15 about 25 sec after addition of trypsin-chymotrypsin cocktail; in contrast the iHPI and cHPC digests were each at pH 7.45 and 7.43, respectively. After the first 25 sec, the mHPC digest experienced slow but continuous pH decreases until 475 sec and were then stable till 600 sec for a final pH 6.7 value. Meanwhile, other products experienced pH stability at 225 sec and final pH 6.91-6.93 values. The faster rate of mHPC digestion is consistent with easier protease accessibility to peptide bonds because of the reduced levels of non-protein materials (Adebowale et al., 2007). Based on Fig. 20, the calculated IPD of mHPC was significantly ($p<0.05$) higher than similar values for iHPI and cHPC (Table 8). The higher IPD of mHPC can be attributed to the pre-digestion reduction in polysaccharides and phytate. This is because fibres have been reported to have negative effect on IPD as a result of non-specific interactions between proteins and polysaccharides constituents of foods (Marambe et al., 2013).

The IPD obtained for mHPC in this study is higher than the 68, 71, 85, and 84% reported for flaxseed (Marambe et al., 2013), *pinto* bean, soybean (Tan et al., 2014) protein isolates and

Fig. 20. Protein digestion progress measured as time-dependent decreases in pH: cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated protein isolate; mHPC, membrane ultrafiltration protein concentrate; HPM, hemp seed protein meal.
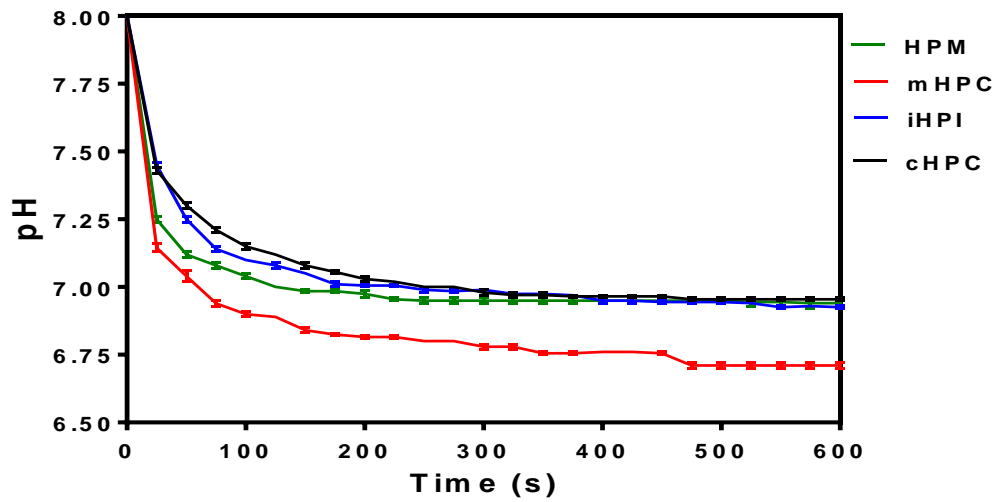
soy protein concentrate (Mohamed et al., 2009), respectively. However, the mHPC has lower IPD when compared to the 91-95% reported for *Mucuna* bean protein isolates (Adebowale et al., 2007) and 96% for chickpea protein isolate (Sanchez-Vioque et al., 1999).

5.3.5. Water and oil holding capacity (WHC and OHC)

The importance of WHC of any protein is centered on the hydrophilic-hydrophobic balance of its amino acids. The polar amino groups, which are the primary sites for protein-water interactions, contributed greatly to this hydrophilic-hydrophobic balance (Malomo et al., 2014; Chavan et al., 2001; Chou & Morr, 1979). Likewise, the OHC has been interestingly known to reflect the interaction ability of protein hydrophobic groups with lipids (Abugoch et al., 2008; Liu et al., 2013). The presence of lipids, physicochemical environment (pH, ionic strength) and solubility are some of the factors that could contribute to the WHC and OHC of any protein molecules in the food system (Malomo et al., 2014). Table 8 indicates that mHPC has a significantly ($p<0.05$) higher WHC (13.18 g/g) when compared to iHPI (12.01 g/g) and cHPC (12.05 g/g). WHC is considered to be an important factor in maintaining flavour and texture of foods (Liu et al., 2013; Mohamed et al., 2009); thus, mHPC seem to be of higher quality as a food ingredient in this aspect. The present WHC results are higher than the 8.7, 8.2, 8.5, 7.8, 3.99 and 3.55 g/g reported for alkali-soluble hemp, acid-soluble hemp, alkali-soluble canola, acid-soluble canola (Teh et al., 2014), defatted wheat germ (Liu et al., 2013) and commercial soy (Zhu et al., 2010) protein isolates, respectively. Results of the WHC test seem to suggest a positive association with the PS data, which may be due to the fact that both tests depend on protein-water interactions. mHPC had a similar OHC as iHPI but the values are significantly ($p<0.05$) higher than those obtained for cHPI and HPM.

Table 8

Water holding capacity (WHC) and oil holding capacity (OHC) of hemp seed protein products*

Samples	WHC (g/g)	OHC (g/g)	% Protein digestibility
HPM	12.32 ± 0.03^b	12.54 ± 0.08^b	84.85 ± 0.51^b
mHPC	13.18 ± 0.06^a	13.76 ± 0.19^a	89.00 ± 0.26^a
iHPI	12.01 ± 0.08^c	13.70 ± 0.29^a	85.12 ± 0.13^b
cHPC	12.05 ± 0.04^c	12.58 ± 0.06^b	84.58 ± 0.13^b

* cHPC, commercial hemp seed protein concentrate; iHPI, Isoelectric pH-precipitated protein isolate; mHPC, membrane ultrafiltration protein concentrate; HPM, hemp seed protein meal

Results are presented as mean ± standard deviation. For each column, mean values that contain different letters are significantly different at $p<0.05$

The higher OHC of mHPC and iHPI is probably a reflection of their higher protein contents, since interactions with the lipid phase is enhanced more by the protein molecules and less by the insoluble polysaccharides. The results indicate a high potential for mHPC usage as a functional ingredient in high-fat foods, such as bakery products and emulsion-type foods. The OHC values obtained in this study are higher than the 8.0, 7.5, 7.5, 7.0, 2.98, 2.43, 1.80 and 1.43 g/g reported for alkali-soluble hemp, acid-soluble hemp, alkali-soluble canola, acid-soluble canola (Teh et al., 2014), defatted wheat germ (Liu et al., 2013), commercial soy (Zhu et al., 2010), *pinto* bean (Tan et al., 2014) protein isolates and optimized cowpea concentrates (Mune Mune et al., 2014), respectively. The enzymatic pre-digestion and removal of some of the non-protein components during mHPC preparation may have contributed to a loose protein structure which enhanced interactions of its hydrophilic and hydrophilic groups with the environment.

5.3.6. Foaming capacity (FC) and foam stability (FS)

Foaming properties are important aspects of protein utilization in foods but are highly influenced by environmental factors. Factors such as protein concentration, PS and hydrophobicity/hydrophilicity ratio can also influence foaming properties (Malomo et al., 2014; Chel-Guerrero et al., 2002; Damodaran, 1996). At all pH values and protein concentrations, the mHPI formed significantly (p<0.05) higher foam volumes (FC) than iHPI and cHPC (Fig 21). The higher FC of mHPC indicates a more flexible structure that reflects minimal level of protein aggregation and is also consistent with the observed high solubility. In general, the cHPC had significantly (P<0.05) higher FC than the iHPI and HPM, especially at pH 7.0 and 9.0, which may also be due to the higher solubility of cHPC. This is because increased PS enhances protein-water interactions, which can unfold protein structure for better air encapsulation to give higher FC as previously demonstrated for other hemp seed proteins (Malomo & Aluko, 2015).

Fig. 21. pH-dependent foaming capacity of hemp seed protein products: A, 20 mg/mL; B, 40 mg/mL; and C, 60 mg/mL protein concentrations. cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated protein isolate; mHPC, membrane ultrafiltration protein concentrate; HPM, hemp seed protein meal. (*Bars with different letters are significantly different at p<0.05)

Fig. 22. pH-dependent foam stability of hemp seed protein products: A, 20 mg/mL; B, 40 mg/mL; and C, 60 mg/mL protein concentrations. cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated protein isolate; mHPC, membrane ultraf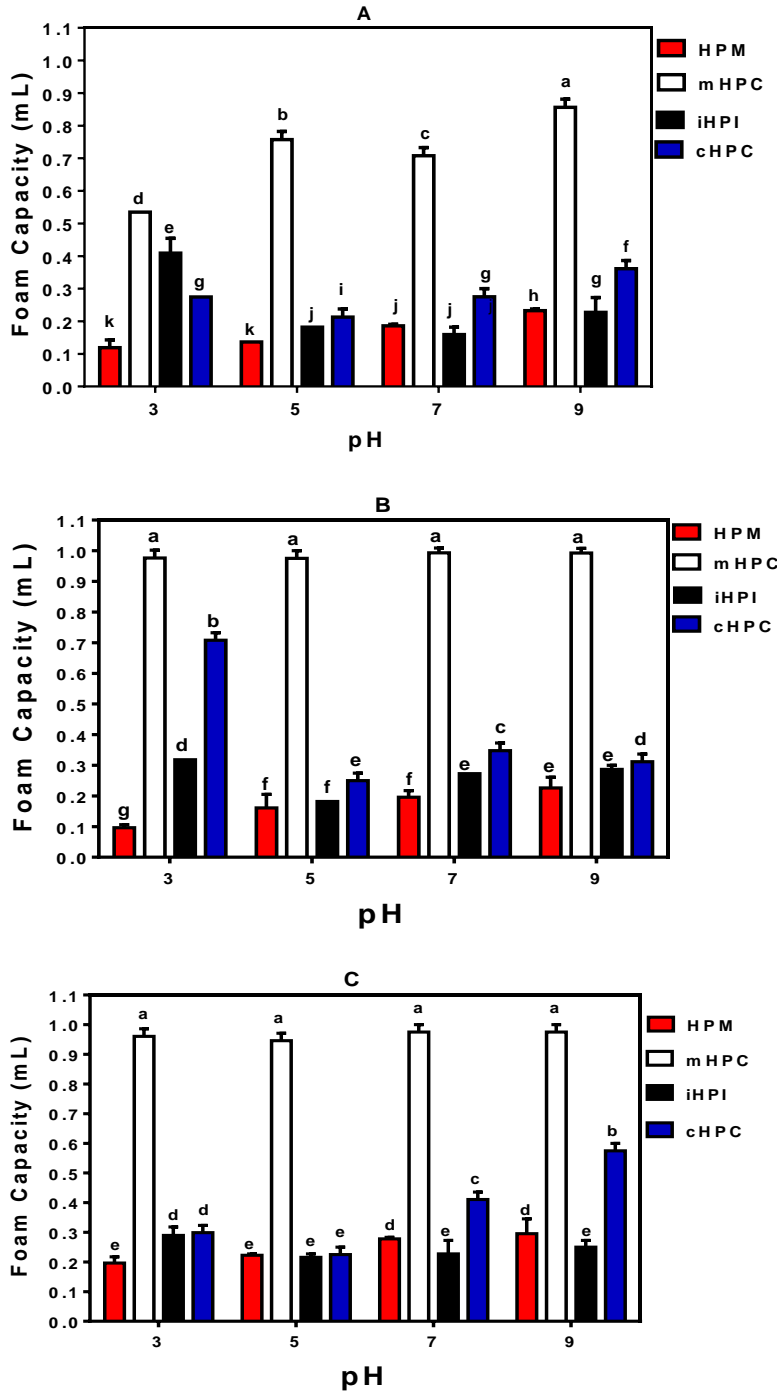iltration protein concentrate; HPM, hemp seed protein meal. (*Bars with different letters are significantly different at p<0.05)
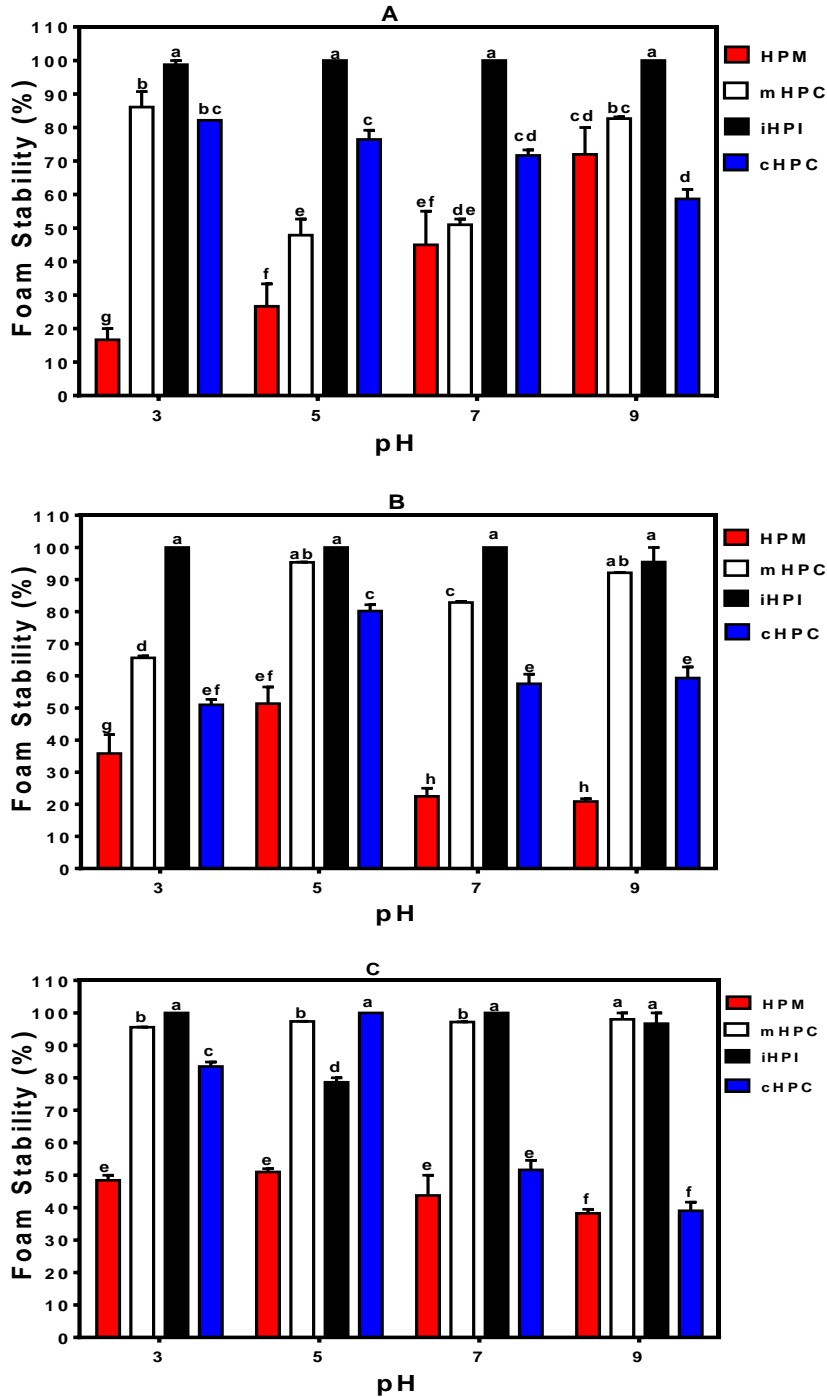
In contrast, the lower PS of iHPI and HPM reduces protein-water interactions, which is unfavourable for foam formation, hence the lower FC values. FC was slightly increased when protein concentration increased from 20 to 40 mg/mL but this was noticeable mostly for the mHPI. The higher FC of mHPC at 40 mg/mL suggests that the additional protein molecules were used to form more foam particles. Increasing the concentration from 40 to 60 mg/mL did not have any substantial effect on FC, except at pH 9.0 where the cHPC showed a higher value. The results agree with a previous report indicating increased protein concentration could favour higher FC (Mundi & Aluko, 2012).

At all the protein concentrations, FS of iHPI were significantly ($p<0.05$) higher than the other foams samples (Fig. 22). The higher FS of iHPI may be because of formation of thick or strong interfacial membranes, which are favoured by the aggregated nature of this protein isolate. Formation of such a cohesive protein membrane will reduce foam drainage and assist in maintaining a stable foam product. FS increased as mHPC concentration was increased to 60 mg/mL, which could be due to formation of stronger interfacial membranes as more protein molecules become available to participate in membrane formation. FS values of cHPC and HPM were lower than those of iHPI and mHPC at all the pH values and protein concentrations. The results suggest that the lower protein contents in cHPC and HPM may have limited formation of strong interfacial membranes and hence foam drainage is more than those of iHPI and mHPC. This is consistent with a previous work which showed that pea flour products with higher protein contents tended to form more stable foams than those with lower protein (Aluko et al., 2009).

5.3.7. Emulsion capacity (EC) and stability (ES)

The emulsifying activity of any protein lies in its potential to interact with and stabilize an oil-water mixture to prevent phase separation. Therefore, exposure of hydrophobic amino acid

residues would enhance formation of good emulsions because of the propensity to interact with the hydrophobic lipid phase. Good emulsifiers will form emulsions with smaller oil droplet sizes (larger surface area) than poor emulsifiers. Fig. 23 shows that the HPM and iHPI formed excellent emulsions with oil droplet sizes that are <1 µm. In contrast the mHPC and cHPC emulsion-forming abilities were much poorer with oil droplet sizes in the 6-15 µm range. Thus, the iHPI interacted better with the oil phase probably as a result of protein denaturation that exposed the hydrophobic amino acids. The excellent emulsion-forming ability of the HPM was unexpected but could be due to the high level of insoluble polysaccharides that have better interactions with oil rather than water. The results are consistent with the PS data, which suggests the presence of more hydrophilic surfaces in mHPC and cHPC but higher levels of hydrophobic surfaces in iHPI and HPM. For mHPC and cHPC, the oil droplet size decreased as protein concentration or pH was increased. The results suggest better formation of interfacial membranes with availability of higher number of protein molecules. The better emulsion formation at alkaline pH values suggests increased ability of the proteins to unfold, which is a prerequisite for oil encapsulation. Protein molecules will acquire more negative charges at higher pH values, which would enhance protein unfolding and enable better formation of emulsified oil droplets.

The oil droplet sizes obtained in this work are lower (i.e. better emulsions) than the 18-30 µm reported for kidney bean proteins (Mundi & Aluko, 2012) and the 18-85 µm reported for canola and soy proteins (Tan et al., 2014) at the same pH (3-9) range. The oil droplet sizes for HPM and iHPI are very similar to values reported for a different hemp seed meal and protein isolate (Malomo et al., 2014) as well as the albumin and globulin fractions (Malomo & Aluko, 2015). However, the emulsions formed by mHPC and cHPC have bigger oil droplet sizes

Fig. 23. pH-dependent emulsion-forming ability of hemp seed protein products: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations. cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated protein isolate; mHPC, membrane ultrafiltration protein concentrate; HPM, hemp seed protein meal. (*Bars with different letters are significantly different at p<0.05)
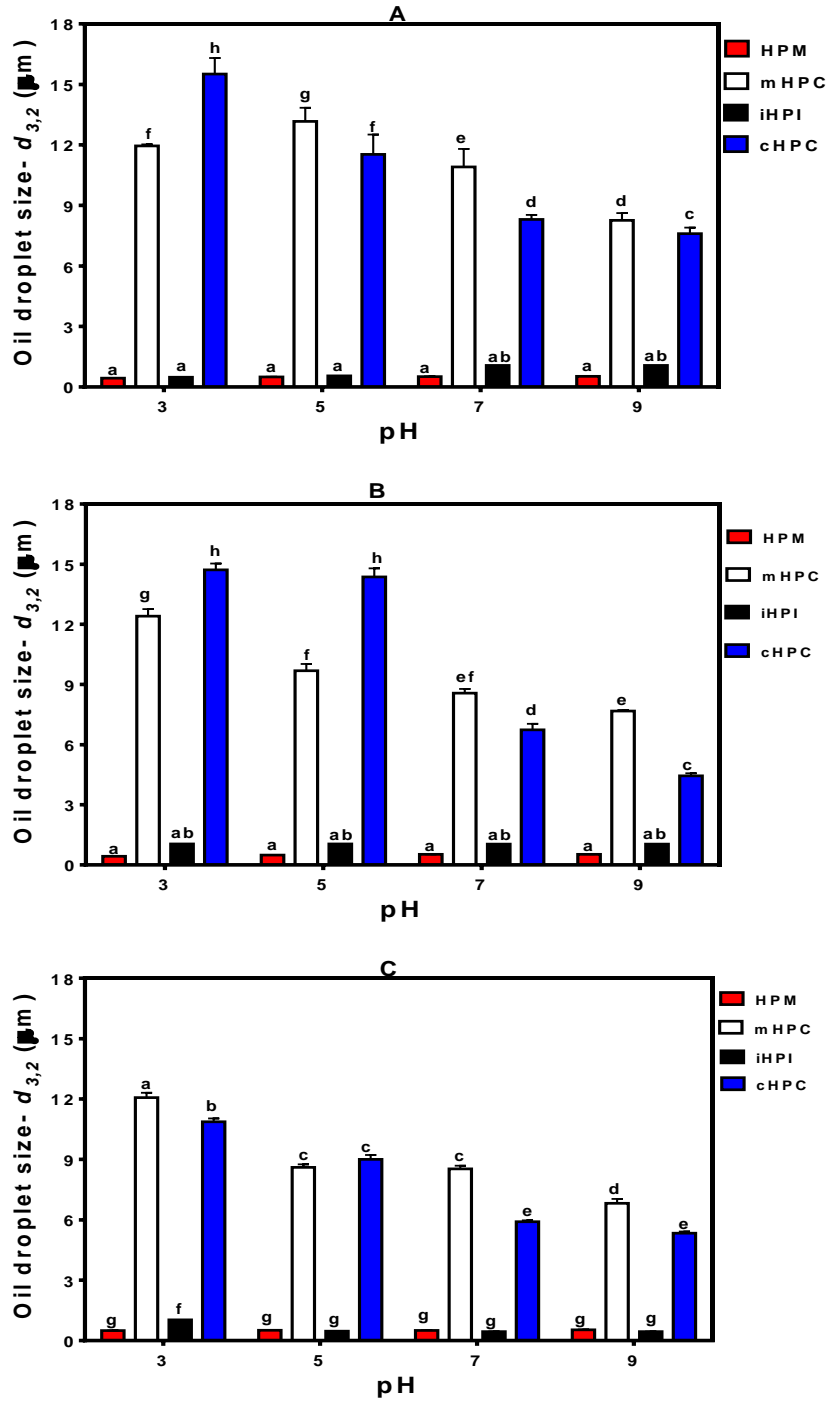
Fig. 24. pH-dependent emulsion stability of hemp seed protein products: A, 10 mg/mL; B, 25 mg/mL; and C, 50 mg/mL protein concentrations. cHPC, commercial hemp seed protein concentrate; iHPI, isoelectric pH-precipitated protein isolate; mHPC, membrane ultrafiltration protein concentrate; HPM, hemp seed protein meal. (*Bars with different letters are significantly different at p<0.05)
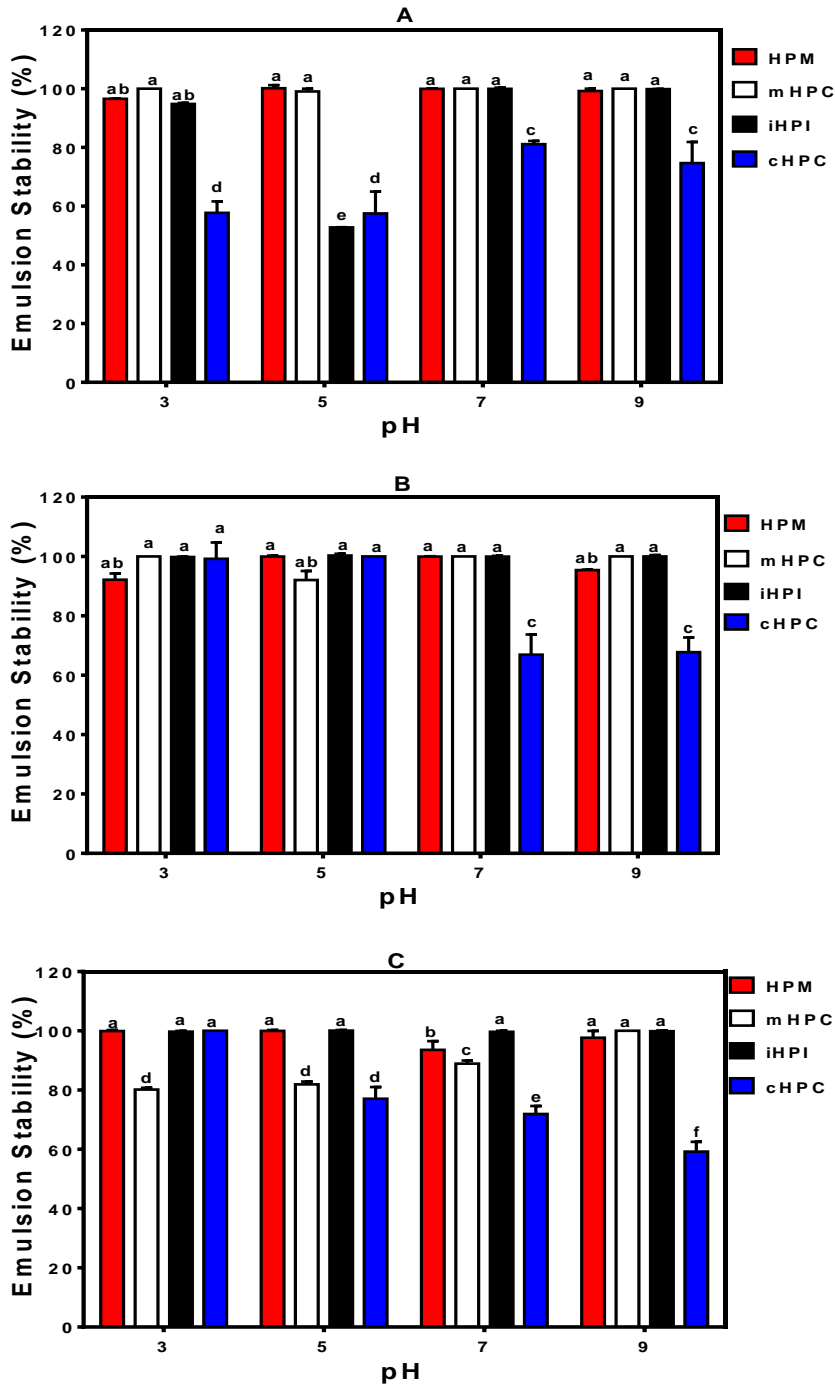
(i.e. poorer emulsions) than those of the previously reported hemp seed protein products (Malomo et al., 2014) and protein fractions (Malomo & Aluko, 2015). These differences suggest that the type of raw material used for protein product preparation is an important determinant of protein functionality. Contrary to the oil droplet size results, the data presented in Fig 24 show that most of the emulsions were stable with values >50%. The mHPI emulsions were particularly stable at 10 and 25 mg/mL protein concentrations, which suggest formation of strong interfacial membranes to reduce oil droplet coalescence. The decreased ES of mHPC at 50 mg/mL could be due to protein crowding, which limits the level of protein-protein interactions required to form strong interfacial membranes. The 100% ES presented in this study is higher than the 50% value reported by Teh et al. (2014) for alkali- and acid-soluble hemp protein isolates. However, a similar 100% ES has been reported for alkali- and acid-soluble canola protein isolates (Teh et al., 2014). The high ES produced by mHPC may be due to its molecular flexibility, which enhances formation of strong interfacial membranes that resist oil droplet coalescence. This is because formation of a stable emulsion is dependent on the ability of proteins to adequately balance their molecular size, charge, surface hydrophobicity and molecular flexibility (Wang et al., 2014; Liu et al., 2013; Avramenko et al., 2013; Zhang et al., 2009).

5.4. Conclusions

The potential uses of protein isolates are largely dependent on their physicochemical and functional properties with great influence from protein digestibility. A low protein industrial hemp seed meal (37% protein content) was successfully converted into a functional protein concentrate (mHPC) with 74% protein content after digestion and removal of some of the non-protein materials. The high protein solubility over the pH 3-9 range, excellent foaming properties and high protein digestibility most probably indicate that most of the mHPC polypeptides were

present mostly in their native forms with little or no denaturation. The mHPC solubility in the pH range is highly desirable for protein fortification of acidic foods and beverages, such as fruit juices. The high level of essential amino acids and branched-chain amino acids suggests that the mHPC could be used for the fortification of products with low protein content. However, the native conformation of mHPC did not favour formation of good emulsions, which indicates lack of exposure of hydrophobic groups and hence poor interactions with the oil phase. In contrast, the original low protein waste material (HPM) and the isoelectric-precipitated protein isolate (iHPI) had excellent emulsifying ability probably due to the presence of polysaccharides and denatured protein molecules with exposed hydrophobic groups, respectively.

Acknowledgement

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5.5 Statement transfer between the study 3 and study 4

The isolated hemp seed protein products in the first 3 chapters are made of complex structures (amino acid composition and three dimensional structures) with different subunits linked together by the disulphide bonds. Thus, polypeptide chains were the principal structural elements present in the protein molecules. In order to further exploit value-added utilization, the complex proteins need to be broken down through enzymatic digestions to produce small-size peptides that could be used as bioactive peptides. Globally, human health is being shaped by rapid environmental changes (age, stress, disease, urbanization, globalized unhealthy lifestyles) that cause oxidative stress and homeostatic imbalance in the body. Ultimately, these negative changes lead to the development of chronic diseases, one of which is hypertension. High blood pressure is a recognized risk factor for the development of various cardiovascular diseases, which are currently managed with synthetic compounds (drugs). However, the use of several antihypertensive drugs is associated with negative side effects that reduce compliance with physician's prescribed dosage. This has led to increased consumer demand for natural antihypertensive agents that will have reduced or nil negative side effects. This chapter deals with the enzymatic modifications of hemp seed proteins using selected proteases to cleave the specific peptide bonds to produce distinct peptide fractions that could be used as antihypertensive agents. The hemp seed bioactive peptides work as modulators of the renin-angiotensin system, which is the main controller of mammalian blood pressure.

CHAPTER SIX

6. Study on the structural and antihypertensive properties of enzymatic hemp seed protein hydrolysates

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Abstract

The aim of this work was to produce antihypertensive protein hydrolysates through different enzymatic hydrolysis (2% pepsin, 4% pepsin, 1% alcalase, 2% alcalase, 2% papain, and 2% pepsin+pancreatin) of hemp seed proteins (HSP). The hemp seed protein hydrolysates (HPHs) were tested for *in vitro* inhibitions of renin and angiotensin converting enzyme (ACE), two of the enzymes that regulate human blood pressure. The HPHs were then administered orally (200 mg/kg body weight) to spontaneously hypertensive rats and systolic blood pressure (SBP)-lowering effects measured over a 24 h period. Size exclusion chromatography showed mainly a 300-9560 Da peptide size range for the HPHs while amino acid composition data had the 2% pepsin HPH with the lowest cysteine content. Fluorescence spectroscopy revealed higher fluorescence intensities for the peptides when compared to the unhydrolyzed hemp seed protein. Overall, the 1% alcalase HPH was the most effective ($p<0.05$) SBP-reducing agent (-32.5 $\pm$ 0.7 mmHg after 4 h) while the pepsin HPHs produced longer-lasting effects (-23.0 $\pm$ 1.4 mmHg after 24 h). We conclude that an optimized combination of the fast-acting HPH (1% alcalase) with the longer-lasting HPHs (2% and 4% pepsin) could provide daily effective SBP reductions.

Keywords: hemp seed; protein hydrolysates; renin; angiotensin converting enzyme; degree of hydrolysis; fluorescence intensity; systolic blood pressure; spontaneously hypertensive rats

6.1 Introduction

Increased rate of high blood pressure (BP) at a certain age in life has led to critical hypertensive conditions in most nations and is responsible for ~45-51% of total global deaths [1]. BP is said to be under normal and adequate control when the systolic and diastolic values are ≤140 and ≤90 mm Hg, respectively [1]. Occurrence of elevated BP is a known risk factor for the development of several cardiovascular diseases like, coronary heart disease, heart failure, stroke, peripheral arterial disease, and renal failure [2]. Antihypertensive drug therapy (a key aspect of hypertension management), which is currently being employed to screen, treat and control high BP in order to reduce the incidence of hypertension, has posed a significant healthcare burden on several nations [3]. For instance, in order to control this public health burden, the USA incurred a direct and indirect cost of about US$93.5 billion in 2010 for hypertension awareness, treatment and control [3]. Likewise, an increasingly significant health, policy and financing challenges have been recorded in some countries, which inevitably create unsustainable pharmaceutical costs for government [4].

The renin–angiotensin aldosterone system (RAAS) is the primary physiological pathway that has been described for BP regulation and management [5]. To control BP, renin is synthesized in the kidneys and then released into the blood circulatory system where it cleaves the N-terminal region of angiotensinogen to release a decapeptide, angiotensin (AT)-I [6]. AT-I further circulates in the blood until its C-terminal dipeptide residue is cleaved by angiotensin I-converting enzyme (ACE) to form an octapeptide AT-II (a potent vasoconstrictor). The pharmaceutical industry has historically exploited ACE inhibition to produce commercial antihypertensive drugs like captopril, enalapril, and lisinopril [7]. Meanwhile, a comparative reduction in elevated BP that could lead to treatment for hypertension other than ACE inhibition

has been suggested for research [8]. Such alternative antihypertensive compounds target renin and bioactive peptides from plant proteins have been shown to have renin- and ACE-inhibitory properties [9-15]. The need to reduce negative side effects (nausea, vomiting, dry cough) of antihypertensive therapy has spurred research into alternative natural sources of effective compounds such as food protein-derived peptides. Thus there are various reports that have shown bioactive peptides as potential antihypertensive agents [9-14,16-24].

Hemp seed protein (HSP), which is obtained from the industrial production of edible oil, has been shown to be a suitable raw material for antihypertensive peptide production [12,14]. However, the previous works have focused solely on the use of simulated gastrointestinal digestion as a tool to produce blood pressure-reducing peptides from hemp seed proteins. Therefore, there is the need to expand hemp seed antihypertensive research scope by examining the potential use of other commercially available enzymes as suitable proteolytic agents. This is because past studies have demonstrated the possibility of generating antihypertensive peptides with varying potencies simply by using different enzymes and enzyme:substrate ratios [9,17,22,25,26]. This work aimed to determine the structural properties and BP-lowering effects of different hemp seed protein hydrolysates (HPHs) produced using four enzymes and different enzyme:substrate ratios. Specifically, the HPHs were tested for *in vitro* inhibitions of renin and ACE activities, which were then related to observed BP-lowering effects after oral administration to spontaneously hypertensive rats.

6.2. Materials and methods

6.2.1. Hemp seed products and chemical reagents

Hemp seed protein meal (HPM, 37% protein content) produced as a by-product of the hemp seed oil processing industry was a gift from Hemp Oil Canada (St. Agathe, Manitoba,

Canada). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA) while other enzymes such as pepsin (porcine gastric mucosa; E.C. 3.4.23.1; ≥250 U/mg), pancreatin (porcine pancreas; 647-014-00-9), papain (papaya latex; E.C. 3.4.22.2), alcalase (*Bacillus licheniformis*; E.C. 3.4.21.62), and ACE (rabbit lung; E.C. 3.4.15.1; ≥250 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

6.2.2. Preparation of hemp seed protein isolates (HPI)

HPI was produced from HPM according to the method previously described [12] with slight modifications. HPM was dispersed in a glass beaker that contained deionized water (1:20, w/v) and the dispersion was adjusted to pH 8.0 using 2 M NaOH to solubilize the proteins while stirring at 37°C for 2 h; this was followed by centrifugation (7000 x g, 60 min at 4°C). The precipitate was discarded and the supernatant filtered with cheese-cloth, adjusted to pH 5.0 with 2 M HCl to precipitate the proteins and thereafter centrifuged (7000 x g, 60 min at 4°C). The resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to obtain the HPI. Protein concentration of the HPI was determined using the modified Lowry method [27].

6.2.3. Preparation of enzymatic hemp seed protein hydrolysates (HPHs)

Hydrolysis of the HPI was conducted using each of the following enzyme and reaction conditions as previously reported [9] with slight modifications: alcalase (50°C, pH 8.0, 4 h); papain (65°C, pH 6, 4 h); and PP (pepsin at 37°C, pH 2.0, 2 h followed by pancreatin at 37°C, pH 7.5, 4 h). HPI (5%, w/v, protein weight basis) was suspended in deionized water in a glass beaker equipped with a stirrer, heated to the appropriate temperature and adjusted to the appropriate pH value prior to addition of the proteolytic enzymes. Proteases were added to the HPI slurry based

on the optimized enzyme to substrate ratio (E/S) *viz*: 1:100 (alcalase), 2:100 (pepsin, alcalase, papain, pepsin+pancreatin (PP), and 4:100 (pepsin), based on HPI protein content. During digestion, the required pH was maintained constant by addition of NaOH after which the enzymes were inactivated by adjusting to pH 4.0 with 2 M HCl followed by immersing the reaction vessel in boiling water bath for 10 min. The undigested proteins were precipitated by centrifugation (8000 x *g*, 60 min at 4°C) after cooling and the supernatant (contains target peptides) was freeze dried as the HPH, which was then stored at -20°C until needed for further analysis. The protein contents of the freeze dried HPH was determined using the modified Lowry method [27].

6.2.4. Determination of degree of hydrolysis

The percent degree of hydrolysis (DH) of HPHs was determined according to the trinitrobenzene sulfonic acid method as previously described [28]. Briefly, HPI was digested under vacuum with 6 M HCl for 24 h and the digest was used to determine total amino groups as L-leucine equivalent. The DH was calculated as percentage ratio of the leucine equivalent of HPHs to that of HPI.

6.2.5. Amino acid composition analysis

The amino acid profiles of HPI and HPHs were determined using the HPLC S4300 Amino Acid Analyzer, (Sykam Mfd Co., Eresing, Bavaria, Germany) according to the method previously described [29] after samples were digested with 6 M HCl for 24 h. The cysteine and methionine contents were determined after performic acid oxidation [30] and the tryptophan content was determined after alkaline hydrolysis [31].

6.2.6. Analysis of molecular weight distribution

Molecular weight (MW) distribution of HPH peptides was determined by size exclusion chromatography (SEC) as previously described [9] with slight modifications, using an AKTA FPLC system (GE Healthcare, Montreal, PQ) equipped with a Superdex Peptide 10/300 GL column (10 x 300 mm), and UV detector (λ= 214 nm). An aliquot (100 µl) of the sample (5 mg/mL in 50 mM phosphate buffer, pH 7.0 containing 0.15 M NaCl) was loaded onto the column and elution was performed at room temperature using the phosphate buffer at a flow rate of 0.5 mL/min. The column was calibrated with Cytochrome C (12,384 Da), Aprotinin (6512 Da), Vitamin B_{12} (1855 Da), and Glycine (75 Da) as the standard proteins. Peptide sizes of the samples were estimated from a plot of log MW versus elution volume of the standard proteins.

6.2.7. Intrinsic fluorescence

Fluorescence intensity spectra of protein and peptide samples were obtained using a previously described method [32] on a JASCO FP-6300 spectrofluorimeter (JASCO, Tokyo, Japan) at 25 °C in a 1-cm path length cuvette. The sample stock solution was prepared as 10 mg/mL in 0.1 M sodium phosphate buffer, pH 7.0; this was followed by centrifugation and determination of protein content of the supernatant. After diluting the supernatant to 0.002% protein content (w/v), the fluorescence spectra was recorded at 280 nm excitation wavelengths and 300 to 500 nm emission. The final fluorescence emission spectrums of each sample were obtained after subtraction of the buffer emission spectrum.

6.2.8. ACE inhibition assay

The ability of HPHs to inhibit *in vitro* ACE activity was measured according to a spectrophotometric method using synthetic N-[3-(2-Furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as the substrate (Sigma-Aldrich, St. Louis, MO, USA) [33]. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50mM Tris-HCl buffer containing 0.3 M NaCl, pH 7.5) was

mixed with 20 µL of ACE (20 mU final reaction activity) and 200 µL sample dissolved in same buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature using Varian Cary 50-UV/Visible spectrophotometer (Varian Inc., Belrose, NSW, Australia). The buffer was used instead of sample solutions in the blank experiment. The concentration of sample that inhibited ACE activity by 50% (IC_{50}) was calculated from a non-linear regression plot of percentage ACE activity versus sample concentrations. ACE activity was expressed as the rate of reaction ($\Delta A/min$) and inhibitory activity was calculated as:

$$\text{ACE inhibition (\%)} = \left[\frac{1 - \Delta A \cdot min^{-1}_{(sample)}}{\Delta A \cdot min^{-1}_{(blank)}} \right] \times 100 \qquad \textbf{(Eqn 8)}$$

where $\Delta A \cdot min^{-1}_{(sample)}$ and $\Delta A \cdot min^{-1}_{(blank)}$ represent ACE activity in the presence and absence of the HPHs, respectively.

6.2.9. Renin inhibition assay

In vitro inhibition of human recombinant renin activity by HPHs was conducted using the Renin Inhibitor Screening Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the method previously described [9]. Prior to the assay, renin buffer was diluted in 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with the assay buffer before use and pre-warmed to 37°C prior to initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA, USA) maintained at 37°C. Before the reaction, (i) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water were added to the background wells; (ii) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli-Q water were added to the blank wells; and (iii) 20 µL substrate, 150 µL assay buffer, and 10 µL sample were added to the inhibitor wells. The reaction was initiated by adding 10 µL renin to the blank and sample wells. The microplate was shaken for 10 sec to mix, incubated at 37°C for 15

min, and the fluorescence intensity (FI) was recorded using excitation and emission wavelengths of 340 and 490 nm, respectively. The concentration of sample that inhibited renin activity by 50% (IC_{50}) was calculated from a non-linear regression plot of percentage renin activity versus peptide concentrations. The percentage renin inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = \frac{[1 - FI_{sample)}]}{FI_{(control)}} \quad x \quad 100 \qquad \textbf{(Eqn 9)}$$

6.2.10. BP-lowering effect of peptides in Spontaneously Hypertensive Rats (SHRs)

Animal experiments were carried out following the Canadian Council on Animal Care Ethics guidelines with a protocol approved by the University of Manitoba Animal Protocol and Management Review Committee. The 30-week old male SHRs (Charles River Laboratories, Montreal, QC, Canada) 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 hr 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: HPH (test sample), captopril (positive control) and phosphate buffered saline (PBS, pH 7.4) as the negative control. HPHs (each at 200 mg/kg bw) and captopril (10 mg/kg bw) were dissolved in 1 mL PBS and administered to the SHRs by oral gavage followed by measurement of systolic blood pressure (SBP) at 2, 4, 6, 8, and 24 h using the tail-cuff method in slightly anesthetized rats as previously described [12]. Prior to sample administration, the baseline (time-zero) SBP was determined. The change in SBP (ΔSBP, mmHg) was determined by subtracting the baseline data from the data obtained at different time points. Oral gavage was used to ensure all rats received the same amount of the HPHs while the 200 mg/kg rat bw translates to 32.43 mg/kg bw (~2 g/day for 60 kg) in an adult human being [34].

6.2.11. Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at $p<0.05$.

6.3. Results

6.3.1. Amino acid composition of HPI and HPHs

Table 9 shows that the protein isolate and hydrolyzed samples had very similar amino acid composition, which is consistent with the hydrolyzed samples being derived from same protein starting material. However, the 2% pepsin hydrolysate had very low cysteine content (0.12%) in comparison to the 1.2-1.43% for the other samples. The 2% papain HPH tended to have the highest proline content.

6.3.2. DH and size exclusion chromatography analysis of HPHs

Extent of hydrolysis can be estimated from the DH values, which can be used an indication of peptide chain length; higher and lower values indicate mean shorter and longer lengths, respectively. As expected, the PP digest had significantly ($p < 0.05$) higher DH (28.16 + 0.34%) than the other enzyme digests (Figure 25), probably due to the exo- and endo-proteinase activities of the enzyme preparations. Similar to our results, a flavourzyme protein hydrolysate was shown to exhibit the highest DH, which was attributed to the presence of endo- and exo-proteinase activities in this enzyme [35]. The peptide sizes were estimated by SEC and the results showed distribution into four major peaks with approx. MW ranging from 300 to 9560 Da (Figure 26). These peptide peaks were most noticeable for the 2% pepsin HPH; increasing the enzyme concentration to 4% led to reduced intensities of the bigger peptide peaks (2775 and

183

Table 9: Percentage amino acid compositions of hemp seed protein isolate (HPI) and enzymatic protein hydrolysates

Amino acid	HPI	2% pepsin	4% pepsin	1% alcalase	2% alcalase	2% papain	2% pepsin+ pancreatin
Asx	11.31	11.36	11.37	11.51	11.55	11.18	10.74
Thr	3.42	2.92	2.85	2.89	2.78	2.93	2.82
Ser	5.58	5.98	5.95	6.10	6.09	6.00	5.84
Glu	19.10	19.36	19.28	19.63	19.64	19.56	19.06
Pro	4.30	4.94	4.74	4.83	4.75	5.23	5.17
Gly	4.31	3.82	3.84	3.73	3.70	3.63	3.56
Ala	3.81	4.79	4.76	4.53	4.60	4.75	4.48
Cys	1.25	0.12	1.34	1.20	1.22	1.31	1.43
Val	4.66	4.60	4.33	4.25	4.20	4.36	4.71
Met	1.92	2.26	2.18	2.28	2.24	2.34	2.21
Ile	3.63	3.17	3.04	2.95	2.90	3.03	3.33
Leu	6.46	6.64	6.48	6.38	6.39	6.35	6.60
Tyr	3.61	3.49	3.39	3.39	3.38	3.28	3.62
Phe	4.72	4.65	4.52	4.45	4.48	4.44	4.80
His	3.15	3.09	3.13	3.12	3.13	3.02	3.00
Lys	2.73	3.19	3.32	2.96	3.03	3.35	3.33
Arg	15.06	14.63	14.47	14.77	14.80	14.24	14.21
Trp	1.00	1.01	1.01	1.05	1.11	1.00	1.08

Fig. 25. Degree of hydrolysis of different enzymatic hemp protein hydrolysates
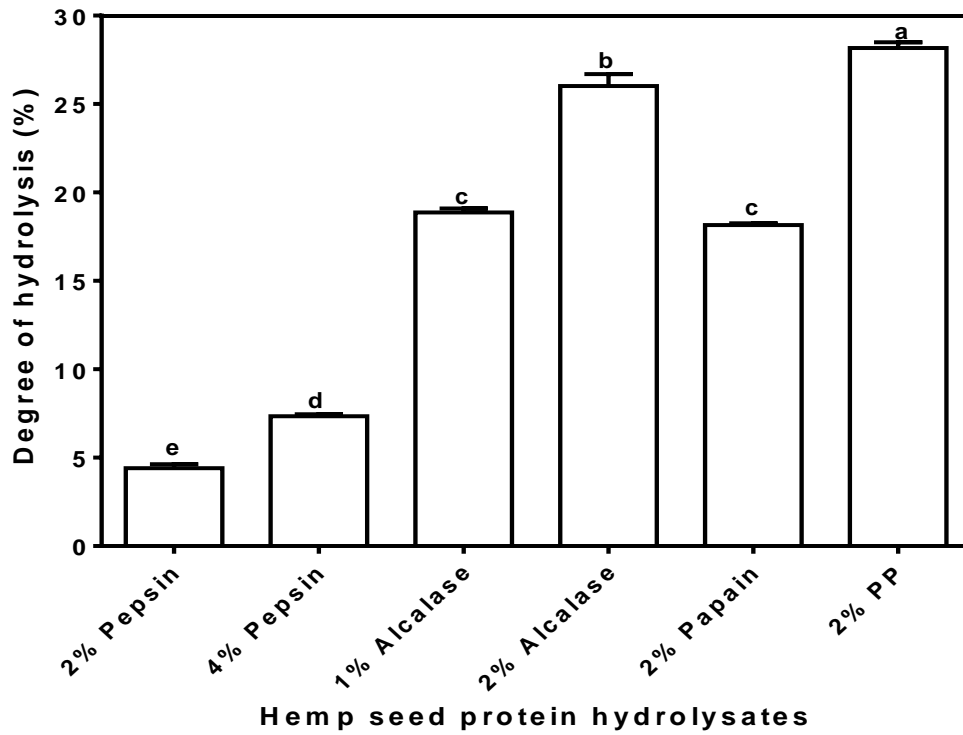
Note: PP = pepsin+pancreatin

*Bars that contain different letters are significantly different at p<0.05

Fig 26- Gel-permeation chromatograms of different enzymatic hemp seed protein hydrolysates at different concentration after passage through a Superdex Peptide 10/300 GL column. Column was calibrated with Cytochrome C (12,384 Da), Aprotinin (6512 Da), Vitamin B$_{12}$ (1855 Da), and Glycine (75 Da).
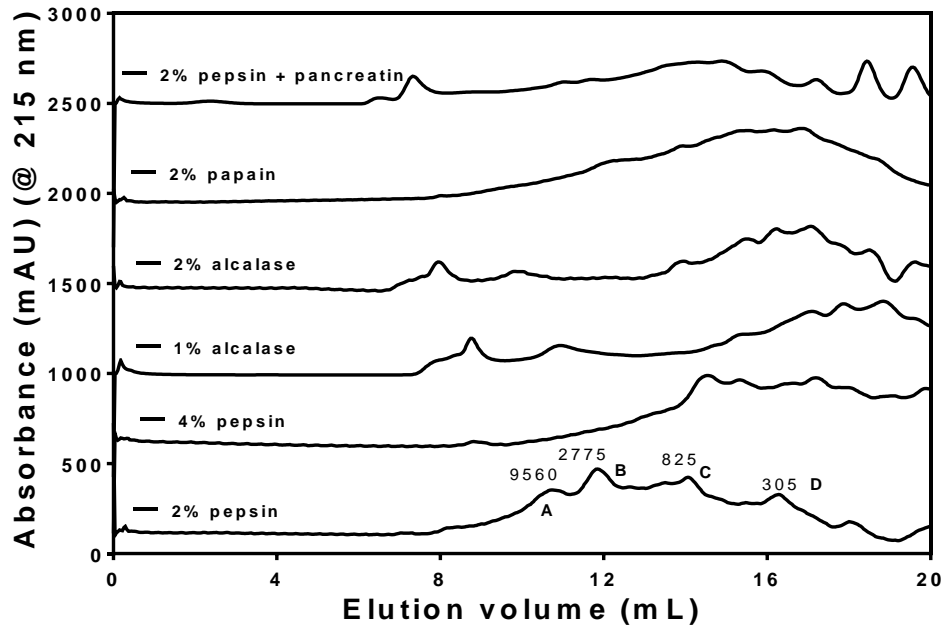
9560 Da). The alcalase and PP HPHs contained a minor peak with size >9560 Da (8-9 mL elution volume), which indicates a polypeptide that is resistant to digestion by these enzymes. Overall, the data show that the HPI was highly susceptible to proteolysis and most of the liberated peptides were <9650 Da in size.

6.3.3. Intrinsic fluorescence properties

Figure 27 shows two main peaks at 317 nm and 378 nm, which represent tyrosine and phenylalanine, respectively in a hydrophilic environment because the observed wavelengths are longer than the normal 303 nm and 350 nm, respectively. There was also a tryptophan shoulder peak at 338 nm, which suggests some tryptophan molecules in a more hydrophobic environment but with reduced fluorescence intensity. All the protein hydrolysates had higher fluorescence intensity (FI) values than the undigested hemp seed protein isolate. The tyrosine and tryptophan peaks were each more intense for the 1% alcalase hydrolysate than the 2% alcalase hydrolysate. The 2% and 4% pepsin hydrolysates showed the most intense tryptophan 378 nm peak while the 2% alcalase had the most intense tyrosine 317 nm peak.

6.3.4. In vitro inhibition of ACE and renin activities of HPHs

The 2% Papain HPH had a significantly ($p < 0.05$) higher ACE-inhibitory activity than the other HPHs when tested at 0.1 mg/mL (Figure 28). The highest ACE-inhibition value (48%) recorded in this current study is lower than the 52-80% reported for several alcalase-treated *Parkia speciosa* seed protein hydrolysates [24] at a similar peptide concentration. However, the 48% ACE inhibition obtained for the 2% papain HPH is similar to the 50% reported for various sweet potato protein digests at 0.13-0.15 mg/mL peptide concentrations [36]. The percent ACE-inhibitory activities were directly reflected in the IC_{50} values, which were lowest (most active) for 2% papain and highest (least active) for 2% PP (Figure 29). The IC_{50} values obtained in this

Fig 27. Intrinsic fluorescence properties of different enzymatic hemp protein hydrolysates
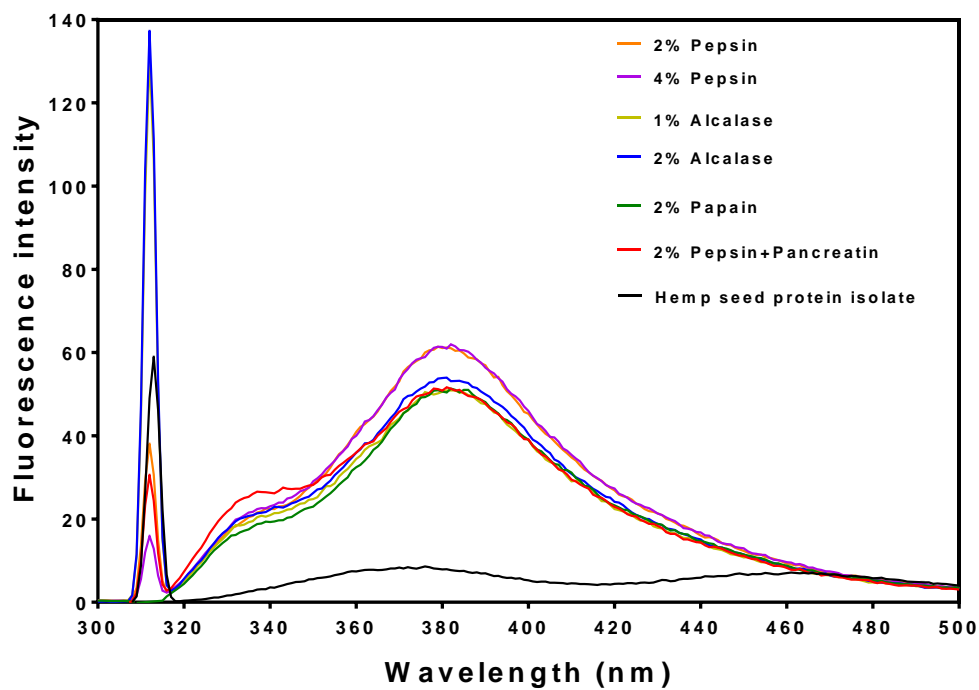
**Fig 28**- Inhibition of renin and angiotensin converting enzyme (ACE) by enzymatic hemp protein hydrolysates
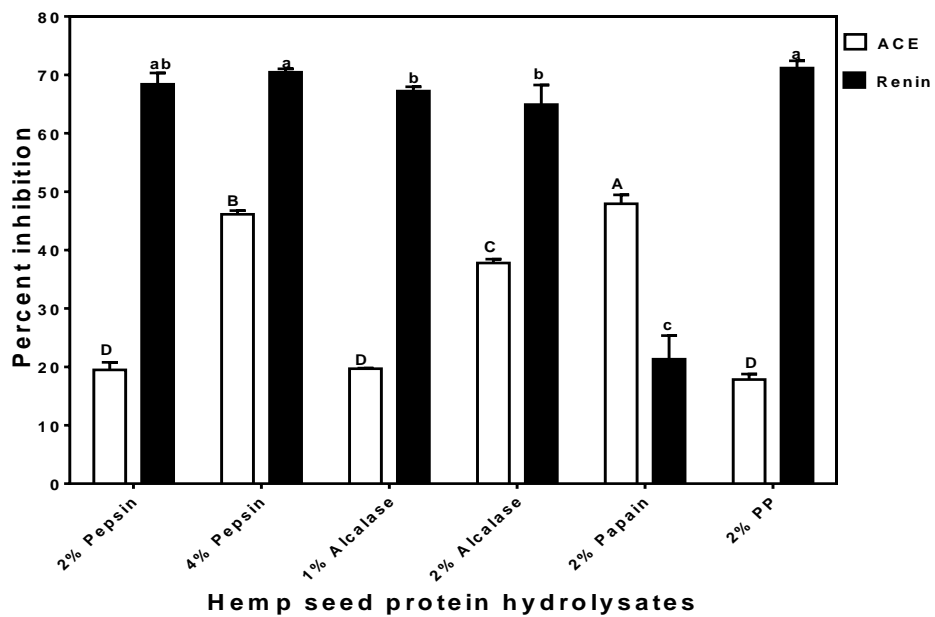
Note: PP = pepsin+pancreatin

*Bars that contain different letters are significantly different at $p<0.05$

Fig 29- Peptide concentrations that inhibited 50% activity (IC_{50}) of renin and angiotensin converting enzyme (ACE)
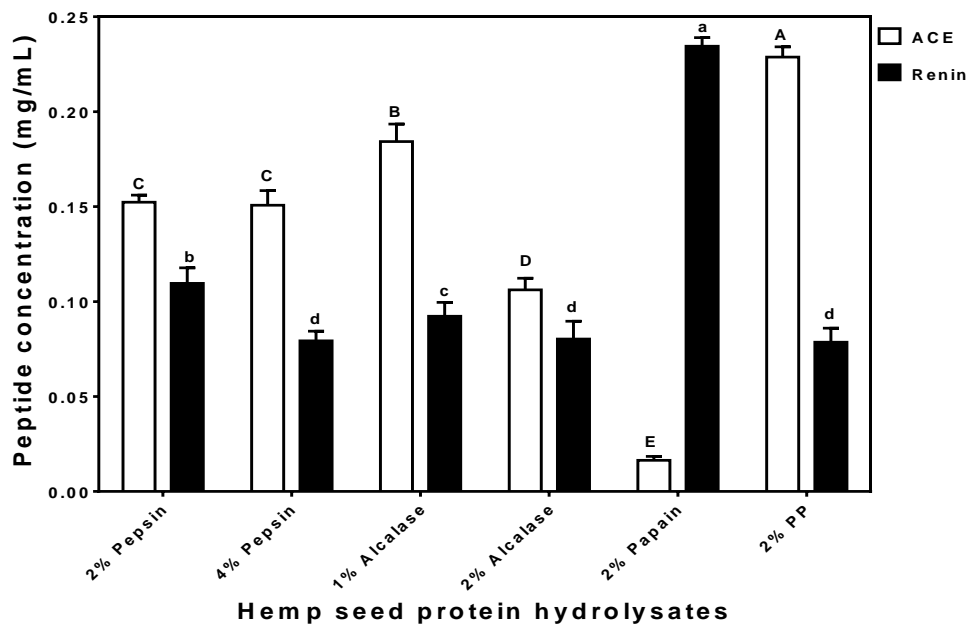
Note: PP = pepsin+pancreatin

*Bars that contain different letters are significantly different at $p < 0.05$

work (0.016 to 0.228 mg/mL) are lower and reflect higher ACE-inhibitory potency when compared to the 0.158-1.083 mg/mL single enzyme catalyzed sweet potato protein hydrolysates [36]. The HPH IC_{50} values are also lower than previously reported values for protein hydrolysates from *Adzuki* bean albumin, globulin, or glutelins protein fractions [37] and canary seed [38].

Figure 28 shows that with the exception of the 2% papain, the HPHs had very high (>50%) renin-inhibitory activities and renin inhibition was higher than ACE inhibition. The noticeable higher renin-inhibitory activities in these HPHs suggest the presence of peptides that interact strongly with renin. The renin inhibition values reported in this study (with the exception of 2% papain) are higher than those (<50%) reported for macroalgae protein hydrolysates [39,40], which were performed at a higher (1 mg/mL) peptide concentration. The current results are different from our previous works that showed hemp seed [12] and canola [22] protein hydrolysates inhibit ACE activity more than renin activity. However, He et al. [10] showed than two HPLC fractions of rapeseed protein hydrolysate had higher renin inhibition than ACE inhibition. As shown in Figure 29, renin-inhibitory IC_{50} values also followed percent inhibitory values whereby the 2% PP, 2% alcalase and 4% pepsin HPHs had the lowest value (0.079 mg/mL) while the 2% papain had the highest (0.23 mg/mL). The renin-inhibitory IC_{50} values obtained in this work are lower than previously reported values (>0.8 mg/mL) for flaxseed [41] and hemp seed [12] protein hydrolysates.

6.3.5. In vivo reduction of blood pressure by HPHs

Single oral administration (200 mg/kg bw) of the HPHs to SHRs resulted in varying but significant ($p < 0.05$) changes in SBP up to the 24 h period when compared to the negative control (Figure 30). The 1% alcalase was the most active with -32.5 $\pm$ 0.7 mmHg after 4 h, a

Fig 30- Time-dependent changes in systolic blood pressure (SBP) of spontaneously hypertensive rats (SHRs) after oral administration of different enzymatic hemp protein hydrolysates (HPHs) and hemp protein isolate (HPI). (*Bars that contain different letters are significantly different at p<0.05)
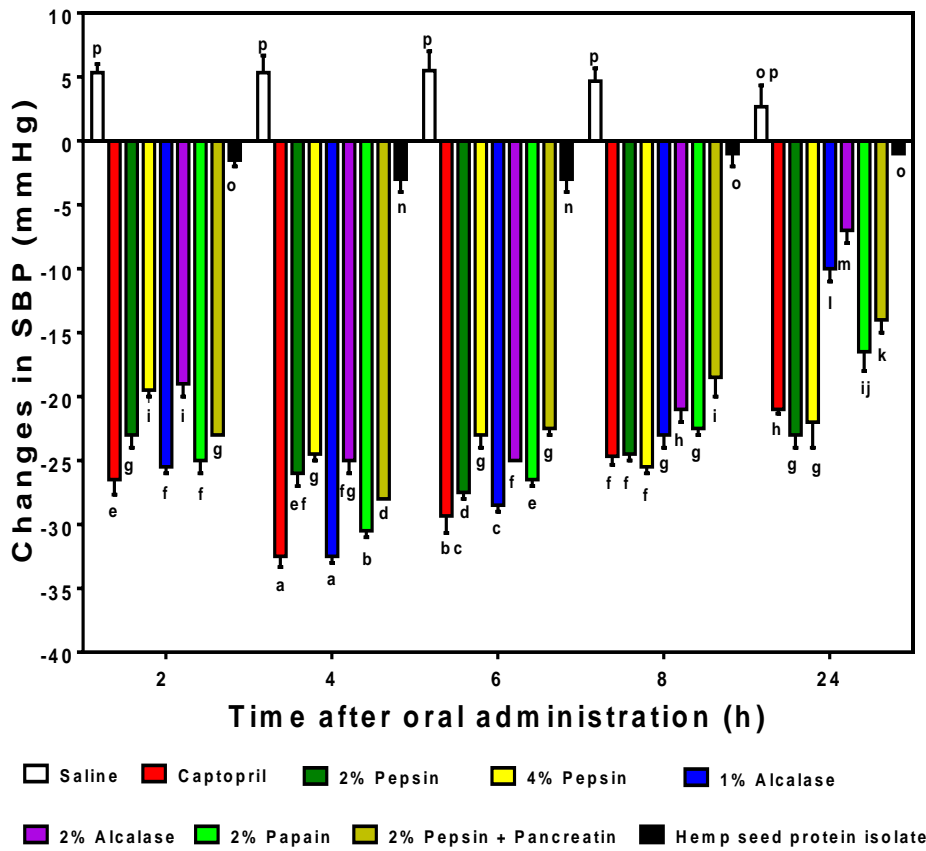
result that is similar to that of the lower dose (10 mg/kg bw) drug (captopril). The 2% pepsin had significantly ($p < 0.05$) lower reductions after 2-6 h than the 4% pepsin, which is similar to the trend for the alcalase HPHs. However, both the 2% and 4% pepsin HPHs produced the longest-lasting effects with -23.0 ± 1.4 mmHg after 24 h of oral administration. The 1% and 2% alcalase HPHs had least persistence effects with 24 h values of -10.0 ± 1.4 mmHg and -7.0 ± 1.4 mmHg, respectively. Interestingly, the SBP-reducing effects did not have a strict direct relationship with observed *in vitro* ACE and renin inhibitions. However, the 2% papain with the highest ACE-inhibitory activity still produced highly significant ($p < 0.05$) SBP reductions, especially after 4 h (-30.5 ± 0.7 mmHg). But the 2% alcalase which had low ACE and renin inhibition IC_{50} values also produced less SBP reductions when compared to other hydrolysates with higher *in vitro* inhibitory values.

6.4. Discussion

Apart from elucidating the arrangement of amino acids on a peptide chain, knowledge of the levels of amino acids present may also provide information on structural basis for observed hydrolysate inhibitory activities. Only the cysteine level tended to differ among the hydrolysates but the reason for the low cysteine level in 2% pepsin HPH is unclear. However, we can infer that at the 2% pepsin concentration, native protein structural restrictions may have limited proteolysis in areas that contained rigid disulfide bonds. At a higher 4% pepsin concentration, this restriction was probably overcome with availability of more enzyme molecules, hence the cysteine content increased to a comparable level with other samples. The significantly higher DH value for the PP HPH may be attributed to the endo- and exo-peptidase activities of pancreatin, which enhances protein digestion through hydrolysis of more peptide bonds when compared to an enzyme with only endopeptidase activity. Moreover, predigestion with pepsin could have

enhanced subsequent digestion by pancreatin and may have contributed to the high DH. Gonzalez-Garcia et al. [35] have also shown that Flavourzyme, an enzyme with similar endo- and exo-peptidase activities as pancreatin also produced a hydrolysate with highest DH. In contrast, the 2% pepsin digest had significantly lowest DH value of $4.40 \pm 0.22\%$, which may be due to the specificity of the enzyme for mostly peptide bonds formed by aromatic amino acids. Alcalase and papain are non-specific endopeptidases, hence the high DH values when compared to pepsin. DH was higher when enzyme concentration increased, which is consistent with availability of more enzyme molecules for protein digestion.

The peptide sizes were estimated by SEC and the results showed distribution into four major peaks with approx. MW ranging from 300 to 9560 Da. These peptide peaks were most noticeable for the 2% pepsin HPH; increasing the enzyme concentration to 4% led to reduced intensities of the bigger peptide peaks (2775 and 9560 Da). The results support a higher proteolysis level at 4% pepsin, which is consistent with the higher DH when compared to the 2% pepsin. The alcalase and PP HPHs contained a minor peak with size >9560 Da (8-9 mL elution volume), which indicates a polypeptide that is probably resistant to digestion by these enzymes. The presence of minor peaks with size <305 Da (most likely free amino acids) were most intense in the PP, which could have been due to the presence of exoproteases in pancreatin. Overall, the data show that the HPI was highly susceptible to proteolysis and most of the liberated peptides were <9650 Da in size. The interrelationship between the MWs of hydrolysates and their bioactivities in human health application is very crucial in functional foods and nutraceutical formulations. Thus the presence of such low molecular weight peptides enhances the absorption potential and ability of the peptides to have bioactive effects during in vivo tests.

Variations in fluorescence intensity (FI) reflect structural conformation of proteins and peptides. Tyrosine and tryptophan are the main chromophores with emission maxima at 303 and 350 nm, respectively [42]. However, fluorescence intensity decreases and wavelength of maximum emission increases (red shift) upon exposure to aqueous environment because of water-dependent chromophore quenching. In contrast, fluorescence intensity increases and wavelength of maximum emission decreases (blue shift) when the chromophores are moved into a hydrophobic environment. Figure 3 shows two main peaks at 317 nm and 378 nm, which represent tyrosine and phenylalanine, respectively in a hydrophilic environment. There was also a tryptophan shoulder peak at 338 nm, which suggests some tryptophan molecules in a more hydrophobic environment. Thus the tryptophan residues were in two different microenvironments, probably consisting of folded (338 nm) and open linear (378 nm) conformations. The HPI FI was very low, which indicates a denatured protein structure with most of the chromophores exposed to the hydrophilic environment. The higher FI of the peptides indicates close proximity between the tryptophan molecules and between the tyrosine molecules, which indicates some level of peptide-peptide interactions. These interactions could have been facilitated by the shorter and more flexible peptide conformations. The FI data confirms the structural differences between peptides, which are of short-chain amino acid sequence and the proteins with long amino acid sequences.

The better ACE-inhibitory activity of the 2% papain HPH may be due to the slightly higher proline content, when compared to that of the other HPHs. This is because proline has been suggested as a potency-enhancing factor for ACE-inhibitory peptides based on the fact that the first two ACE-inhibitory peptides (VPP and IPP) isolated from fermented milk both contained proline [43]. However, proline content alone was not directly related to ACE-

inhibitory activity of all the HPHs, which suggests that other peptide structural factors (e.g., proline position, presence of aromatic amino acids, or absence of disulfide bonds) may be responsible for the observed results. For example, the 2% pepsin HPH has very low cysteine level while the 2% PP HPH had slightly higher tyrosine level, both of which may have contributed to the poor ACE-inhibitory activities. The ACE-inhibitory levels are lower than some of the previously reported values for enzymatic food protein hydrolysates such as *Parkia speciosa* seed protein hydrolysates [24]. These differences may be due to the use of different protein materials for enzyme hydrolysis, since variations in the primary structure will lead to the liberation of peptides with distinct amino acid composition and sequences.

Unlike ACE, only very few reports [10,11,14,16,38,44] are available for peptide-dependent renin inhibition. A higher renin-inhibition seems to be related to phenylalanine content since this amino acid was highest in the 2% pepsin, 4% pepsin and 2% PP HPHs. However, other structural features (e.g., amino acid arrangement and type) are likely to be involved in determining renin-inhibitory properties of the HPHs. A previous report has suggested the importance of branched-chain amino acids and aromatic amino acids in potentiating renin-inhibitory properties of food protein-derived peptides [45]. Generally, renin inhibitory activity of the hydrolysates was higher than ACE-inhibitory activity, which is different from other reports that showed a reverse trend [9,12,22,46]. Overall, these results confirm previously published literature data that indicate peptide-dependent ACE-inhibitory potency are more likely to be different from that renin inhibition. Since catalytic mechanisms differ between ACE and renin, molecules that have strong inhibition against one enzyme may not necessary have the same inhibition level against the other enzyme.

Single oral administration of the HPHs to SHRs resulted in varying but significant (P<0.05) changes in SBP up to the 24 h period when compared to the negative control (Figure 30). The 1% alcalase was the most active with -32.5 ± 0.7 mmHg after 4 h, a result that is similar to that of the lower dose drug (captopril). The 2% pepsin had significantly (P<0.05) lower reductions than the 4% pepsin after 2-6 h, which is similar to the trend for the alcalase HPHs and indicate higher enzyme concentrations may not necessarily produce more effective antihypertensive peptides. The results are similar to the work of Alashi et al. [22] who also showed that alcalase and pepsin canola protein digests produced the most SBP reductions in SHR also at 200 mg/kg bw dose. He et al. [9] also showed that an alcalase digest of rapeseed was the most effective SBP-reducing agent among several enzymes used. The results suggest that the lower renin-inhibition potency of the 2% papain HPH may have been compensated for by the higher ACE-inhibitory activity, hence strong reductions in SBP. The lack of direct correlation between *in vitro* and *in vivo* activities of the HPHs is consistent with previous reports [9,22]. The maximum SBP reductions obtained in this work are similar to the maximum value obtained for a sweet potato protein hydrolysate, which was administered at a higher 500 mg/kg bw dose [36].

Pistachio [17] and almond [19] protein hydrolysates at 1000 and 800 mg/kg bw doses, respectively also produced lower SBP-reducing effects than the HPHs used in this work. In contrast to the HPHs, the unhydrolyzed HPI was less active (SBP values of -1 to -3 mmHg), which suggests that enzymatic predigestion converted the inactive proteins into bioactive peptides. Even though predigestion with pepsin alone or in combination with pancreatin produced SBP-reducing HPHs, the lack of effect after ingestion of HPI suggests that other *in vivo* factors (buffering capacity and duration of hydrolysis) may have prevented effective digestion and liberation of antihypertensive peptides in the SHR gastrointestinal tract. Similar

works with pea [23] and rapeseed [9] have also shown better and faster SBP-reducing effects of predigested proteins when compared to undigested proteins.

6.5 Conclusion

The present study confirmed the dual *in vitro* ACE and renin inhibition capacity of enzymatic HSP digests. There was no relationship between DH and ACE inhibitory activity of the protein hydrolysates though renin inhibition tended to be inversely related to DH, which suggests longer hemp seed peptide chains may be more potent in vitro renin inhibitors than shorter chains. The DH and *in vitro* ACE or renin inhibition had no relationship with actual SBP-reducing effects in SHR, which suggests peptide amino acid sequence or synergistic effects between different peptides may be the determining factors for the observed antihypertensive potency. The significantly ($P<0.05$) longer-lasting SBP-reducing effects of the pepsin digests, which also had the least DH (longer peptide chains) is an indication of reduced rate of absorption coupled with higher resistance to enzymatic clearance after absorption from the gastrointestinal tract. The alcalase digest had the fastest and most SBP-reduction and may be combined with the papain digest (also highly potent with long-lasting effects) to produce consistent SBP-lowering effect on a 24 h basis. However, a combination of pepsin and alcalase digests may also produce desirable 24 h SBP-reducing effects. Further studies are needed to determine the amino acid sequence of the most active HPH peptides (pepsin, alcalase, and papain) in order to perform structure-function studies. Overall, the results provide critical information on HPHs that may be used as active ingredients to formulate antihypertensive functional foods and nutraceuticals.

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Author Contributions

Sunday Malomo performed all the *in vitro* tests and data analysis; he also wrote the first draft of this manuscript. Sunday Malomo, John Onuh and Abraham Girgih conducted the rat feeding and blood pressure measurements. Dr. Rotimi Aluko is the grant holder for the project; he produced the experimental concept and edited the final manuscript draft.

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6.6 Statement transfer between the study 4 and study 5

In recent years, research findings have made progress toward the development of naturally occurring bioactive multifunctional peptides (or protein hydrolysates). The previous chapter reported the effective lowering of blood pressure by hemp seed protein hydrolysates. In this chapter, enzymatic protein hydrolysis was also used to produce a different set of protein fractions that can modulate activities of one of the agents involved in the nervous system. Acetylcholinesterase (AChE) is a main factor involved in neurological disorders and is therefore, a target for therapeutic agents against dementia and Alzheimer's disease. Since the AChE substrate has a structural resemblance to peptides, enzymatic digestion of hemp seed proteins could produce very active peptide inhibitory agents. Therefore, in this chapter, optimized enzymatic hydrolysis of hemp seed proteins was carried out at different enzyme:substrate concentrations in order to generate a wide range of peptides with varied structural properties. The hemp seed protein hydrolysates were then tested for *in vitro* AChE-inhibitory properties as potential agents for human cognitive health improvement. The most active peptide fractions were examined for peptide sequences to determine type of amino acids that are important for potent AChE-inhibition.

7. Study on the *in vitro* acetylcholinesterase-inhibitory properties of enzymatic hemp seed protein hydrolysates

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Abstract

The aim of this work was to produce acetylcholinesterase (AChE)-inhibitory peptides through enzymatic hydrolysis of hemp seed proteins. Hydrolysis was performed using six different proteases (*pepsin, papain, thermoase, flavourzyme, alcalase* and *pepsin+pancreatin*) at different concentrations (1-4%). Degree of hydrolysis (DH) was directly related to amount of protease used but had no relationship with AChE-inhibitory activity. Amino acid composition results showed that the hemp seed protein hydrolysates (HPHs) had high levels of negatively charged amino acids (39.62-40.18%) as well as arginine. The 1% *pepsin* HPH was the most active AChE inhibitor with ~6 µg/mL IC_{50} value when compared to 8-11.6 µg/mL for the other HPHs. The 1% *pepsin* HPH was dominated by high cysteine-containing peptides though negatively charged and aromatic amino acids were also present. We conclude that formation of disulfide bonds between peptides and AChE polypeptides may constitute an important use as potential inhibitory mechanism. The HPHs could be an important source of agents for maintaining a healthy nervous system.

Key words: hemp seed; protein hydrolysate; peptides; acetylcholinesterase

7.1. Introduction

The serine hydrolase, acetylcholinesterase (AChE), has long been known to play a very crucial physiological role in maintaining the major neurotransmitter, acetylcholine (ACh) in the body by rapidly clearing free ACh from the synapse (Kumar & Chowdhury, 2014). During neurotransmission, ACh is synthesized from choline and acetyl-CoA by the enzyme choline acetyltransferase. ACh is then released from the nerve into the synaptic cleft where it binds to receptors on the post-synaptic membrane thereby relaying the signal from the nerve to the brain (Singh et al., 2013). AChE converts excess ACh into the inactive metabolites, choline and acetate, thus promoting normal brain and muscles functions by preventing over-stimulation of the nervous system. The liberated choline is taken up again by the pre-synaptic nerve and combined with acetyl-CoA to synthetize ACh through the action of choline acetyltransferase (Singh et al., 2013). Thus normal functioning of AChE also ensures proper recycling of choline and acetyl-CoA such that optimal level of ACh is maintained within the nervous system. As aging and dietary changes set in, less ACh is synthesized due to non-regeneration of used neurons and shortage of choline. Meanwhile, AChE continues to hydrolyze the little amounts of ACh; net result is low levels of ACh. This leads to reduced functioning of nerve impulse and inadequate signal transmission to the brain, thus eventually contributing to memory impairment such as in Alzheimer's disease (AD) and dementia (Willcox et al., 2014).

The increasing occurrence of AD has been implicated as a contributing factor to higher global morbidity/ mortality rate (US Burden of Disease Collaborators, 2013). This is because AD is a progressive, degenerative disease which is characterized by memory loss, language deterioration, poor judgment and impaired visuospatial skill. The cholinergic neurotransmission dysfunction in the brain, formation and growth of brain amyloid lesions, and senile plaques, are

widely believed and reported as crucial events in the pathogenesis of AD (Alzheimer's Association, 2013). The various socio-economic factors leading to AD have been suggested to include diet, population aging and lifestyle. For instance, the aging process affects the diet of individuals by partially or totally changing their diet type as well as the quality and quantity of consumed foods (Willcox et al., 2014).

AChE inhibition is an important research in the wide range of associated health implications in humans because of the ability to hydrolyze ACh at cholinergic synapses with high catalytic efficiency than other known enzymes (Saravanaraman et al., 2014; Zare-Zardini et al., 2013). AChE inhibitors are therefore, employed to reduce the rate at which ACh is broken down, thereby increasing brain ACh concentration and combating the loss caused by the death of cholinergic neurons (Hasnat et al., 2013). The approach involves the use of compounds that possess structural similarity to ACh such that they can fit into the enzyme active site, thereby reducing catalysis rate (Saravanaraman et al., 2014; Singh et al., 2013). As of today the current available therapy for AD consists of the administration of synthetic AChE inhibitors due to their clinical efficacy in prolonging the half-life of ACh (Kumar and Chowdhury, 2014). Such synthetic drugs (like tacrine, rivastigmine, galantamine, memantine and donepezil) are characterized by common negative side effects like nausea, vomiting, muscle cramps, decreased heart rate (bradycardia), decreased appetite and weight as well as increased gastric acid production (Ghribia et al., 2014; Iannello et al., 2014; Saravanaraman et al., 2014).

However, many approaches have been recently employed to address this critical problem of negative side effects, some of which includes the uses of natural polyphenolic plant products and their secondary metabolites (Borowiec et al., 2014; Ghribia et al., 2014; Iannello et al., 2014; Kumar & Chowdhury, 2014). Zare-Zardini et al. (2013) recently isolated a 31-mer peptide

(Snakin-Z) from *Ziziphus jujuba* fruits, which inhibited *in vitro* AChE activity. However, to the best of our knowledge, there is scanty information on the potential use of naturally occurring bioactive peptides (hydrolysates) from plant proteins as AChE inhibitors. Food protein hydrolysates contain peptides that consist of peptide bonds, which have structural similarity to the ester bond in ACh. Moreover, several peptides contain positively charged nitrogen atoms just like found in ACh. Therefore, theoretically, small-size peptides could simulate the structural conformation of ACh and enable peptide interaction with the AChE active site. Since peptides are not AChE substrates, such binding to the active site could exclude ACh, slow down enzyme catalysis and potentially enhance physiological levels of ACh during aging.

Food protein-derived hydrolysates have reduced risk of imparting negative side effects in consumers and therefore, could serve as suitable alternatives or complementary treatment to AD drugs. Previous works have demonstrated several in vitro (Girgih et al., 2011a, 2013) and in vivo (Girgih et al., 2011b, 2014a,b) antioxidant and antihypertensive effects of hemp seed protein hydrolysates (HPH). A previous work also showed the oxidative apoptosis protective effect of hemp seed peptides (Lu et al., 2010) but so far there has been no report of AChE inhibition by food protein hydrolysates. Therefore, the aim of this study was to determine, for the first time, the *in vitro* AChE-inhibitory properties of several HPH generated from the action of different enzymes during proteolysis of hemp seed plant proteins. The amino acid composition, peptide size distribution and potential peptide sequences of the most active HPHs were determined.

7.2. Materials and methods

7.2.1. Hemp seed products and chemical reagents

Hemp seed protein meal (HPM) (37% protein content) produced as a by-product of the hemp seed oil processing industry was a gift from Hemp Oil Canada (St. Agathe, Manitoba, Canada).

All enzymes- pepsin (porcine gastric mucosa; E.C. 3.4.23.1; ≥250 U/mg), papain (papaya latex; E.C. 3.4.22.2), alcalase (*Bacillus licheniformis*; E.C. 3.4.21.62), flavourzyme (*Aspergillus oryzae*; ≥500 U/g), pancreatin (porcine pancreas; 647-014-00-9) and *Electrophorus electricus* (electric eel; 1256 U/mg protein) acetylcholinesterase Type VI-S were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thermoase (*Aspergillus* sp.; Protease A "Amano" 2 SD) was a gift from Amano Enzyme Inc (Nishiki, Naka-ku, Nagoya Japan). Dithio-bis-nitrobenzoic acid (DTNB) and acetylthiocholine were also purchased from Sigma-Aldrich. Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

7.2.2. Preparation of hemp seed protein isolate (HPI)

HPI was produced from HPP according to the method of Girgih et al. (2011a) with slight modifications. HPP was dispersed in deionized water (1:20, w/v) and the dispersion was adjusted to pH 8.0 using 2 M NaOH to solubilize the proteins while stirring at 37 ^{o}C for 2 h; this was followed by centrifugation (7000g, 1 h, 4 ^{o}C). The precipitate was discarded and the supernatant filtered with cheese-cloth, adjusted to pH 5.0 with 2 M HCl to precipitate the proteins and then centrifuged (7000g, 1 h, 4 ^{o}C). The resultant precipitate was re-dispersed in deionized water, adjusted to pH 7.0 with 2 M NaOH and freeze-dried to obtain the HPI. Protein concentration of the HPI was determined using the modified Lowry method (Markwell et al., 1978).

7.2.3. Preparation of enzymatic hemp seed protein hydrolysates (HPHs)

Hydrolysis of the HPI was conducted using each of the following enzymes and reaction conditions: alcalase (50 ^{o}C, pH 8.0, 4 h); papain (65 ^{o}C, pH 6.0, 4 h), pepsin (37 ^{o}C, pH 2.0, 2 h), thermoase (50 ^{o}C, pH 8.0, 4 h), flavourzyme (50 ^{o}C, pH 8.0, 4 h) and pepsin+pancreatin (first, 37 ^{o}C, pH 2.0, 2 h; then at the same 37 ^{o}C, pH 7.5, 4 h). HPI (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to the appropriate

temperature and adjusted to the appropriate pH value prior to addition of the proteolytic enzymes. Each protease was added to the HPI slurry at five different enzymes to substrate protein ratios (E/S): 0.5:100, 1:100, 2:100, 3:100, and 4:100. During digestion, the reaction mixture pH was maintained constant by addition of 1 M NaOH. After digestion, the enzymes were inactivated by immersing reaction vessel in hot water bath at 95 ^{o}C for 10 min and allowed to cool down. The undigested proteins were precipitated by centrifugation (8000 g, 60 min at 4 ^{o}C) while the supernatant containing target peptides was freeze dried as the HPH and stored at -20 ^{o}C until needed for further analysis. The protein contents of the freeze dried HPHs were determined using the modified Lowry method (Markwell et al., 1978).

7.2.4. Amino acid composition analysis

The amino acid profiles of HPI and HPHs were determined using the HPLC S4300 Amino Acid Analyzer, (Sykam Mfd Co., Eresing, Bavaria, Germany) according to the method previously described (Bidlingmeyer et al., 1984) after samples were digested with 6 M HCl for 24 hr. The cysteine and methionine contents were determined after performic acid oxidation (Gehrke et al., 1985) and the tryptophan content was determined after alkaline hydrolysis (Landry & Delhaye, 1992).

7.2.5. Determination of degree of hydrolysis (DH)

The percent DH of each HPH was determined according to the trinitrobenzene sulfonic acid (TNBS) method as previously described (Adler-Nissen, 1979). Briefly, HPI was digested under vacuum with 6 M HCl for 24 hr and the digest was used to determine total amino groups as L-leucine equivalent. The DH was calculated as percentage ratio of the leucine equivalent of HPHs to that of HPI.

7.2.6. Analysis of molecular weight distribution

Molecular weight (MW) distribution of HPHs was determined as previously described (He at al., 2013) using an AKTA FPLC system (GE Healthcare, Montreal, PQ) equipped with a Superdex Peptide 10/300 GL column (10 x 300 mm) and UV detector (λ= 214 nm). The column was calibrated with Cytochrome C (12,384 Da), Aprotinin (6512 Da), Vitamin B_{12} (1855 Da), and Glycine (75 Da) as the standard proteins. Peptide sizes of the samples were estimated from a plot of log MW versus elution volume of the standard proteins.

7.2.7. Mass spectrometry analysis of protein hydrolysates

The mass spectrometry analysis of the protein hydrolysates is done according to the previously described method (He et al., 2013) with slight modifications. Briefly, a 10 ng/ µL aliquout of the active impure HPHs, being dissolved in deionized water (containing 0.1% formic acid as solvent A) was subjected to mass spectrometry (MS) scan analysis. The elution and MS scan was monitored with the AB SCIEX QTRAP® 6500 MS System (AB SCIEX Pte. Ltd., Foster City, California, USA) coupled with electrospray ionization source using the following parameters: Ion source, Turbo Spray IonDrive; Curtain Gas, 30.0; IonSpray Voltage, 3.5 kV; Temperature, 150 °C; Ion Source Gas, 30; Declustering Potential, 100; Enterance Potential, 10; Flow rate, 30 µL/min for 3 min in the positive ion mode.

7.2.8. Acetylcholinesterase (AChE) inhibition assays

AChE inhibition assay was performed as previously described (Ellman et al., 1961) with slight modifications. Briefly, the reaction was carried out at room temperature using 0.1 M sodium phosphate buffer (pH 7.5) and the final assay mixture was 200 µL in a 96-well microplate (Costar, Corning, NY, USA). Aliquots of 20 µL HPHs were added to the microplate wells that contained 130 µL of the buffer. DTNB (20 µL) and acetylthiocholine (10 µL) were added to each well. Then, 20 µL of AChE (0.5 U/mL final assay concentration) was added to initiate the

214

reaction and the mixture incubated for 15 min at room temperature. The absorbance and the reaction kinetics were read for 2 min at wavelength of 412 nm using a Synergy H4 microplate reader (Biotek Instruments Inc., Winooski, Vermont, USA). In the uninhibited wells, 20 µL of the buffer was used to replace HPH. In the background wells, 20 µL of the buffer was used to replace AChE while 40 µL of buffer was used to replace both AChE and HPH in the blank wells. The concentration of sample that inhibited 50% AChE activity (IC_{50}) was calculated from a non-linear regression plot of percentage AChE activity versus HPH concentration.

7.2.9. Statistical analysis

Triplicate determinations were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples and significant differences taken at $p < 0.05$.

7.3 Results and Discussion

7.3.1. Degree of hydrolysis (DH) and AChE inhibition

Table 10 shows that the DH was significantly ($p < 0.05$) affected by the type and level of proteases, which is consistent with the fact that these proteases have different specificities and will produce peptides at different rates. The flavourzyme-produced HPHs have the highest ($p < 0.05$) DH values, which can be attributed to the dual endoprotease and exopeptidase activity possessed by the enzyme. In contrast, thermoase and pepsin hydrolysates had the least DH values, which suggest that several peptide bonds within the hemp seed proteins were not accessible or were resistant to the catalytic activities of these two enzymes. As expected, higher DH values were obtained as the amount of protease used was increased, which indicates

increased proteolysis. Protein content of the HPHs ranged from ~81-91%; therefore, AChE assays were performed on protein weight basis.

The data on inhibitory properties of the HPHs show that there was no direct relationship between level of enzyme used and the ability of the protein hydrolysate to reduce AChE activity (Table 10). Unlike the DH data, there was no relationship between amount of enzyme used and the AChE-inhibitory activity of the resultant HPH. Thus, the results suggest that there is no direct relationship between peptide size (inversely related to DH) and AChE-inhibitory activity of the HPHs. It is more likely that AChE-inhibitory activity of the HPHs was determined mostly by the type and sequence of amino acids on the peptide chain. The results show that several of the HPHs have higher AChE-inhibitory activity than the 31 amino acid residue peptide (Snakin-Z) isolated from *Ziziphus jujuba* fruits (Zare-Zardini et al., 2013). At 10 µg/mL, the AChE activity inhibition by Snakin-Z was <20% when compared to several of the HPHs with >20% inhibition at same peptide concentration.

The higher AChE-inhibitory effects of some of the protein hydrolysates may be due to synergistic effects of several peptides when compared to the single Snakin-Z peptide. The highest ($p < 0.05$) AChE inhibition of 54% was by the 1% pepsin hydrolysate, which suggests the presence of peptides that bind strongly to AChE protein. The AChE-inhibitory activities of the HPHs which was up to 54% for the 1% pepsin hydrolysate is similar to the maximum value of 57% that was reported by Hasnat et al. (2013) for a *Ganoderma lucidum* aqueous polyphenolic extract at 2.00 mg/mL. Danis et al. (2014) also reported about 56% AChE inhibition by a methanolic extract of *Rhus coriaria* leaves but at 1.2 mg/mL. While it may not be totally correct to compare polyphenolic activity with that of a protein hydrolysate, it should be noted that the HPHs were tested at 120-200 times less than the natural plant extract concentrations.

Table 10- Degree of hydrolysis (DH) and acetylcholinesterase (AChE)-inhibitory activities of hemp seed protein hydrolysate different concentrations*

Enzyme concentration	DH (%)	AChE inhibition (%)
0.5% pepsin	1.78 ± 0.11^p	17.01 ± 0.79^g
1% pepsin	3.69 ± 0.56^o	53.78 ± 0.11^a
2% pepsin	4.40 ± 0.22^o	N/A
3% pepsin	5.20 ± 0.00^o	41.18 ± 0.25^b
4% pepsin	7.34 ± 0.11^n	N/A
0.5% alcalase	15.77 ± 0.11^k	12.25 ± 0.68^i
1% alcalase	18.87 ± 0.22^j	N/A
2% alcalase	26.02 ± 0.67^i	7.36 ± 0.51^k
3% alcalase	25.54 ± 0.00^i	27.70 ± 0.32^e
4% alcalase	31.90 ± 1.35^g	41.82 ± 0.26^b
0.5% papain	14.10 ± 0.90^l	36.77 ± 1.08^c
1% papain	11.71 ± 0.12^m	N/A
2% papain	18.15 ± 0.11^j	18.18 ± 0.67^f
3% papain	16.32 ± 0.22^k	39.51 ± 1.49^b
4% papain	17.99 ± 0.11^j	18.77 ± 0.07^f
0.5% pepsin + pancreatin	14.89 ± 0.44^l	8.18 ± 0.26^k
1% pepsin + pancreatin	25.70 ± 0.67^i	N/A
2% pepsin + pancreatin	28.16 ± 0.34^i	6.65 ± 0.50^{kl}
3% pepsin +pancreatin	29.83 ± 0.12^h	N/A
4% pepsin + pancreatin	34.52 ± 1.91^f	32.62 ± 0.62^d
0.5% thermoase	1.00 ± 0.00^r	N/A
1% thermoase	0.72 ± 0.41^r	N/A
2% thermoase	1.86 ± 0.00^p	14.29 ± 0.82^h
3% thermoase	1.48 ± 0.09^p	N/A
4% thermoase	7.18 ± 0.79^n	33.26 ± 0.63^d
0.5% flavourzyme	40.00 ± 0.45^e	13.79 ± 0.09^i
1% flavourzyme	43.82 ± 0.90^d	10.71 ± 0.01^j
2% flavourzyme	45.73 ± 0.67^c	0.91 ± 0.28^m
3% flavourzyme	57.33 ± 0.00^a	20.23 ± 0.32^f
4% flavourzyme	51.76 ± 0.67^b	2.73 ± 0.39^l

*10 µg/mL peptide concentration; N/A = No observed activity. Results are presented as mean ± standard deviation. For each column, mean values that contain different letters are significantly different at $p<0.05$

Therefore, on a weight basis, most of the HPHs have higher AChE-inhibitory activity than the *G. lucidum* and *R. coriaria* polyphenolic extracts.

Based on the observed percentage AChE-inhibitory activities, the four HPHs with highest values were used for IC_{50} determination, which enables better comparison with literature values. The values obtained in this study for 1% pepsin (5.95 $\pm$ 0.10 µg/mL), 3% pepsin (8.04 $\pm$ 0.33 µg/mL), 3% papain (8.97 $\pm$ 0.41 µg/mL) and 4% alcalase (11.62 $\pm$ 0.32 µg/mL) are lower than those reported for different AChE-inhibitory phytochemical agents (Fig. 31). For example, the following are typical IC_{50} values found in literature: *Pancratium illyricum* L. alkaloid, 3.5 mg/mL (Iannello et al., 2014); GLBR, 1.01 mg/mL (Hasnat et al., 2013); Snakin-Z peptide, 0.58 mg/mL (Zare-Zardini et al., 2013); *Catharanthus roseus* root alkaloids, 0.78 mg/mL (Pereira et al., 2010); *R. coriaria* leaves, 1.17 mg/mL (Danis et al., 2014); and *Amaryllidaceae* alkaloids, 0.72 mg/mL (Lopez et al., 2002). The low IC_{50} results suggest that the HPHs are potentially excellent sources of highly potent peptides that can be used to inhibit AChE activity.

7.3.2. Analysis of molecular weight distribution

The MW of hydrolysate peptides is an important molecular character in considering potential bioactivities. This is because of the general recognition that low molecular weight peptides have better chances of escaping structural degradation within the gastrointestinal tract and getting absorbed into blood circulation than bigger peptides. Small-size peptides are also more likely to fit into an enzyme active site and produce inhibitory effects than bigger peptides. Therefore, one of the aims of protein hydrolysate production is to ensure the abundance of small-size peptides in order to enhance potency against metabolic targets.

The size exclusion chromatograms of the four most-active *in vitro* AChE-inhibitory HPHs show that the MW ranged from 300-9500 Da (Fig. 32). The 1% pepsin HPH had four

Fig 31. Concentrations of the most active enzymatic hemp seed protein hydrolysates that inhibited 50% acetylcholinesterase activity (IC_{50})
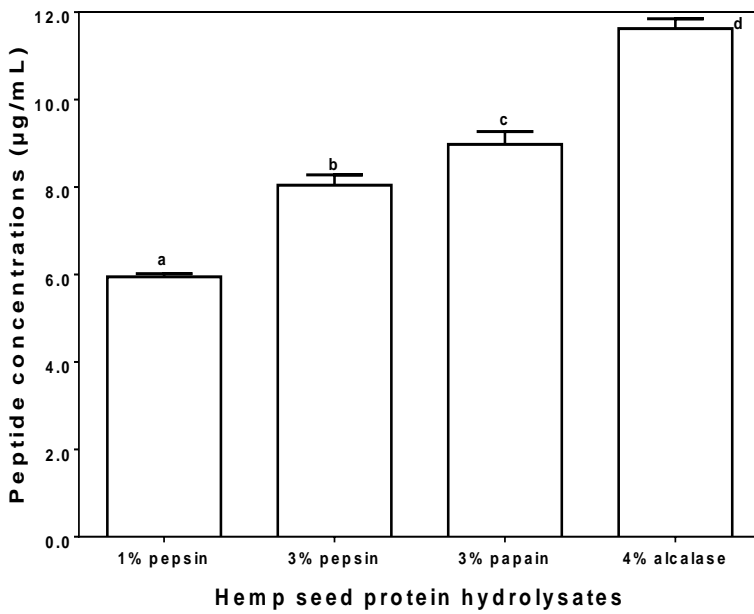
*Bars that contain different letters are significantly different at $p < 0.05$

major peaks (A-D) but the intensity decreased when the concentration was increased to 3% level, which is consistent with the increased DH (more extensive proteolysis) at the higher enzyme level. The 4% alcalase hydrolysate showed lower levels of the 300-9500 Da peaks, which is consistent with having the highest DH among the four protein hydrolysates. Thus, alcalase seems to be more efficient than papain and pepsin in hydrolyzing the hemp seed proteins. However, protein hydrolysate activity is also highly dependent on the type of peptides produced and not just the hydrolysis efficiency. Moreso, the peaks produced by the proteases were different in nature and this could have been the basis for their *in vitro* AChE inhibitory activities. This is supported by the past study (He et al., 2013) which had understandably reported that the structure (size) and activity of peptides is largely dependent on its method of production (enzymes used). The results (<300 Da) obtained in this study for the *alcalase* HPH (even though at 4% level) is in the range with those reported (Alashi et al., 2014) for canola protein hydrolysates (6.47–0.027 kDa) and 2.6-0.14 kDa being reported (He et al., 2013) for rapeseed protein hydrolysates.

It is noted in this study that *alcalase* enzyme produced a higher peak of smaller MW HPHs probably due to its endoprotease activity and its broad specificity for protein digestion. It could also be that HPI was more susceptible to *alcalase*-induced proteolysis as being observed by past studies (Alashi et al., 2014; He et al., 2013) for canola and rapeseed protein hydrolysates respectively.

7.3.3. Amino acid composition and peptide sequences

Table 11 shows that the HPHs are composed of mostly negatively charged amino acids (NCAA), which was followed by hydrophobic amino acids (HAA), while content of aromatic amino acids (AAA) was the least. The peripheral anionic site (PAS) is a unique surface area on

Fig 32. Size-exclusion chromatograms of the most active acetylcholinesterase-inhibitory enzymatic hemp seed protein hydrolysates.
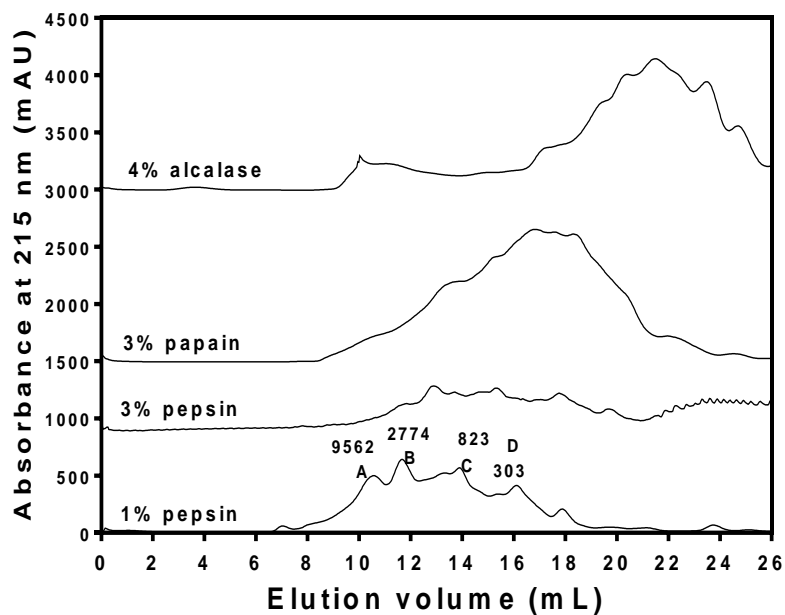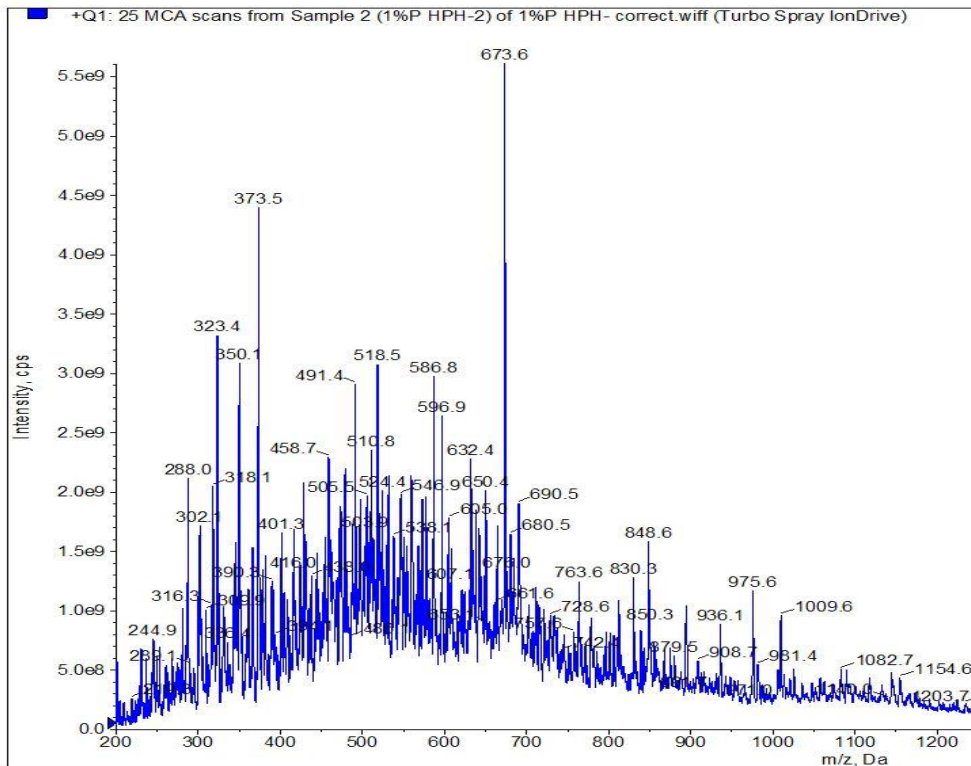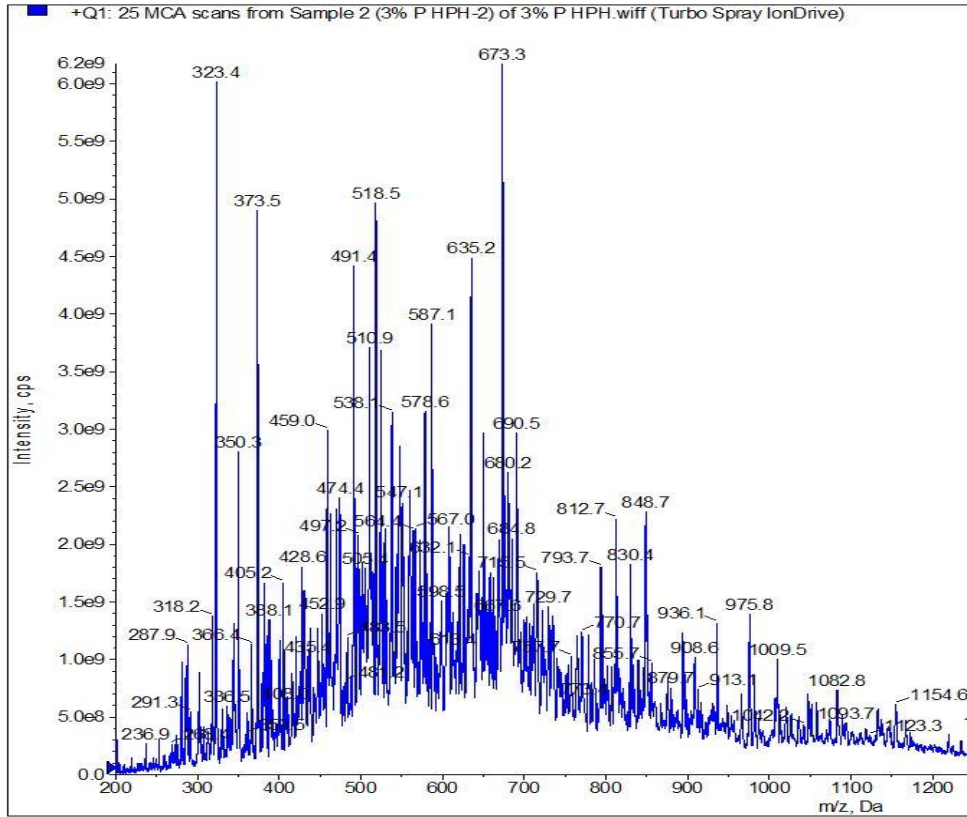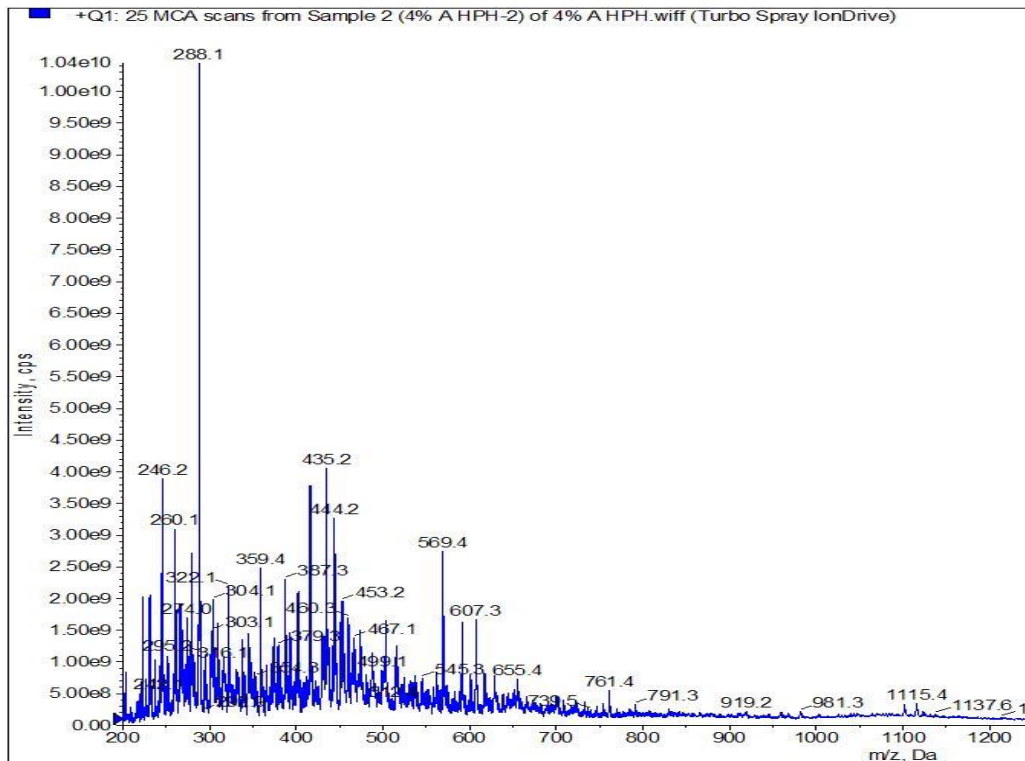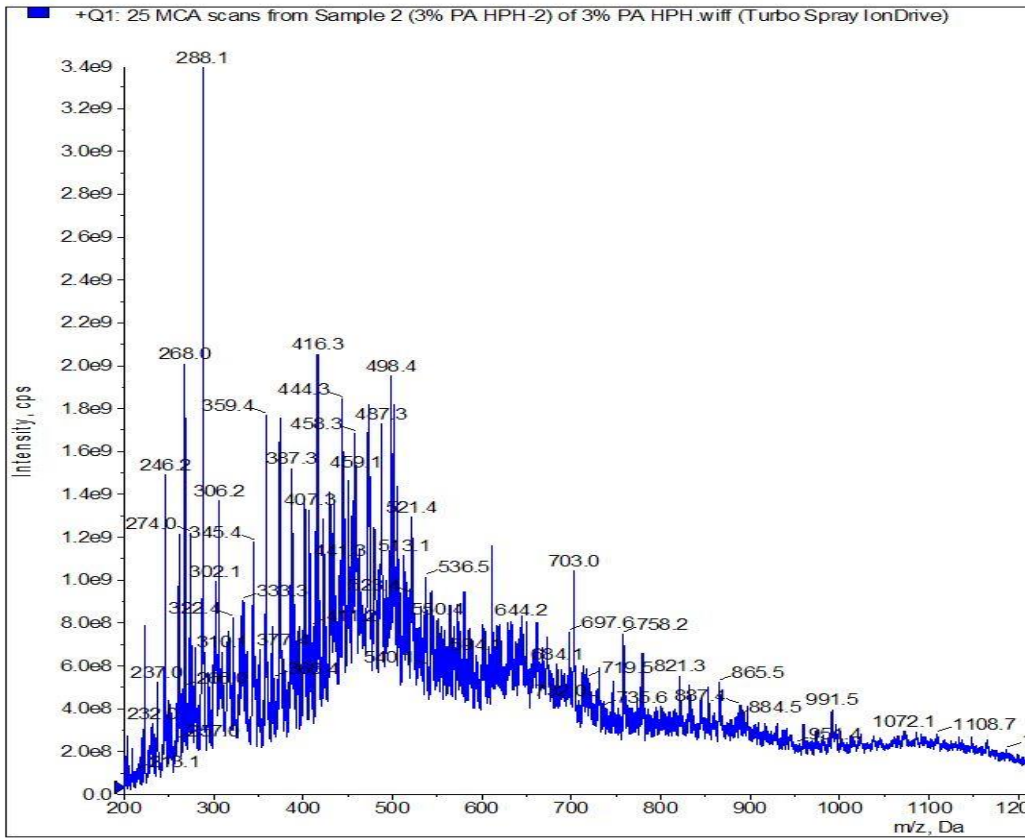
Table 11- Amino acid composition (%) of hemp protein isolate

(HPI) and different enzymatic hemp protein hydrolysates*

Amino acid	HPI	1% pepsin	3% pepsin	3% papain	4% alcalase
Asx	11.31	11.18	11.49	11.04	11.34
Thr	3.42	3.24	3.26	3.31	3.20
Ser	5.58	5.86	5.93	5.89	5.89
Glx	19.10	19.35	19.50	19.68	19.19
Pro	4.30	5.01	5.13	5.23	4.91
Gly	4.31	4.10	4.24	4.13	4.07
Ala	3.81	4.29	4.31	4.27	4.31
Cys	1.25	1.41	1.43	1.33	1.33
Val	4.66	4.32	4.22	4.28	4.15
Met	1.92	2.32	1.60	2.60	2.41
Ile	3.63	3.09	2.98	3.05	2.98
Leu	6.46	6.48	6.41	6.23	6.47
Tyr	3.61	3.44	3.38	3.48	3.61
Phe	4.72	4.55	4.42	4.41	4.63
His	3.15	3.07	3.06	2.92	3.06
Lys	2.73	3.10	3.25	3.41	2.91
Arg	15.06	14.28	14.42	13.78	14.41
Trp	1.00	0.90	0.97	0.97	0.97
NCAA	39.41	39.63	40.18	39.92	39.62
PCAA	20.94	20.45	20.09	20.11	20.38
HAA	26.03	26.92	26.08	26.99	26.56
AAA	9.33	8.89	8.77	8.86	9.21

*NCAA, negatively charged amino acids; PCAA, positively charged amino acids; HAA, hydrophobic amino acids; AAA, aromatic amino acids; Asx, aspartic acid + asparagine; Glx, glutamic acid + glutamine

Fig 33- MS scan of most active AChE-inhibitory HPHs using QTRAP ion drive at positive ESI mode (1%P = 1% pepsin; 3%P = 3% pepsin; 3%PA = 3% papain; 4%A = 4% alcalase)

+Q1: 25 MCA scans from Sample 2 (3% PA HPH-2) of 3% PA HPH.wiff (Turbo Spray IonDrive)

+Q1: 25 MCA scans from Sample 2 (4% A HPH-2) of 4% A HPH.wiff (Turbo Spray IonDrive)

the AChE protein that is believed to be an important binding site for inhibitors (Saravanaraman et al., 2014). Within this binding site, PCAA of inhibitory peptides can form a stable complex with the Trp286 indole ring of the AChE protein, which then prevents entry of substrates to the enzyme active site. Therefore, it is possible that the high arginine contents of the HPHs may have facilitated peptide-AChE interactions, which resulted in the observed inhibitions of enzyme activity.

The most active AChE-inhibitory hydrolysates *viz:* 1% and 3% pepsin, 3% papain and 4% alcalase HPHs were injected to the mass analyzers of a QTRAP ion mass spectrometer (MS) to isolate the targeted peptide precursor ion and several molecular species were obtained as shown in Fig. 33a-d. From the result obtained, the MS scan for 1% and 3% pepsin HPHs are very similar and almost contain the same peptide species of similar molecular masses. For instance, the species with molecular mass of 673.6 Da is very more exposed in both 1% and 3% pepsin HPHs (Fig. 33a-b). The similarity in the result might be from the fact that the same enzyme (pepsin) is used but at different concentration; meanwhile, higher masses are more exposed out in 3% HPH due to its higher enzyme concentration. Likewise, the MS scan obtained for 3% papain and 4% alcalase HPHs are very similar with virtually the same molecular masses. For instance, the species with molecular mass of 288.1 Da is predominantly observed in both their MS scans (Fig. 33c-d). The reason might be connected to the fact that both papain and alcalase have the same optimal condition of activity (pH, 6-8; temperature, 50-65 $^{\circ}$C; time, 4 h).

Meanwhile, some of the species with higher molecular masses (for example, those with masses ≥800 Da) observed in the MS scans of 1% and 3% pepsin HPHs are seemingly diminished in the MS scans of 3% papain and 4% alcalase HPHs. For example, only ≥400 Da are predominant in 3% papain and 4% alcalase HPHs unlike ≥600 Da that are observed in 1% and

3% pepsin HPHs. This might be connected to the differences in time involved for their hydrolytic actions on HSP. For instance, both papain and alcalase use 4 h while pepsin uses 2 h for optimum digestion. The results suggest that disulfide bond formation between peptides and AChE polypeptide may be involved in reducing enzyme catalysis rate. Presumably, the disulfide bond formation could distort AChE conformation such that the substrate does not fit properly into the active site. However, other important amino acid residues include aromatic and negatively charged, which suggest multiple mechanisms of action are involved in the peptide-induced AChE inhibition.

7.4. Conclusion

This study, to our knowledge, is the first to report the *in vitro* AChE inhibition activities of a food protein hydrolysate. The lack of relationship between DH and AChE-inhibitory of the HPHs suggest peptide chain amino acid sequence was more important than peptide chain length as an important structural feature for potency. The results showed the 1% pepsin digest as the most active and the peptides are dominated by cysteine-containing amino acids, which indicate involvement of disulfide bond formation during interactions with AChE polypeptides. The IC_{50} values are lower than values reported for polyphenols and suggest high AChE-inhibitory potency of the peptides within the context of currently available inhibitory natural products. The dominance of low molecular peptides suggests that the HPHs contain potentially bioavailable components. Therefore, the HPHs may constitute important sources of natural AChE-inhibitory agents and provide potential health benefits during metabolic disorders that involve the nervous system. Meanwhile, further work will be performed to determine actual amino acid sequences of peptides present in the most active HPH.

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7.5 Statement transfer between the study 5 and study 6

The preceding evaluated several peptide fractions for AChE-inhibitory properties followed by preliminary identification of probable peptide sequences. However, in order to perform structure-functions, definitive identification of peptide sequences is required. Therefore, this chapter deals with chromatographic separation and purification of peptides present within the most active AChE-inhibitory hemp seed enzymatic digest identified from the preceding chapter. Peptide purification was carried out using reverse-phase (RP) HPLC coupled with *in vitro* assay of AChE-inhibitory properties of fractions. The most active RP-HPLC was rechromatographed to obtain purer peptide fractions that were then subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS) to decipher amino acid sequences. De novo amino acid sequencing was performed based on fragmentation pattern obtained from LC/MS/MS. Sequenced peptides were then synthesized and tested for *in vitro* AChE-inhibitory properties in order to confirm potency and as potential agents against neurological disorders.

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Abstract

This work aimed at identification and testing of the *in vitro* potentials of protein-derived acetylcholinesterase (AChE) inhibitory peptides present in 1% *pepsin*-produced hemp seed protein hydrolysates (HPH). The reverse phase HPLC (RP-HPLC) was used to separate the HPH into eight different fractions (F1-F8), which were analyzed for their *in vitro* AChE-inhibitory properties. After preparative and analytical RP-HPLC separations, the most active fraction was characterized to produce eleven purified peptides and the amino acid sequence determined by tandem mass spectrometry (LC–MS/MS). The study revealed that the RP-HPLC fraction F7 exhibited the strongest ($p<0.05$) *in vitro* AChE-inhibitory properties (98%) while F3 had the least (20%); however, no activity was recorded for F2, F5 and F6. The higher peptide purity of F7 may have contributed to its higher AChE-inhibitory activity (98%; IC_{50}, 5.80 µg/ml) when compared to the unfractionated 1%-HPH (54%; IC_{50}, 6.00 µg/ml), hence F7 was selected for peptide characterization. The *de-novo* sequencing using the LC/MS/MS data led to identification of peptide sequences that were mainly composed of branched-chain and hydrophobic amino acids (V, I, L, Y and F). LYV (IC_{50}, 6.80 µg/ml) was the most potent ($p<0.05$) against AChE (from Eel) activity when compared to LSA (IC_{50}, 21.90 µg/ml), VQFWG (IC_{50}, 17.50 µg/ml) and GGYRS (IC_{50}, 11.60 µg/ml). LYV (IC_{50}, 7.50 µg/ml) was also the most potent ($p<0.05$) inhibitor of human AChE activity. We therefore, conclude that hydrophobic amino acids contribute to the potency of HSP-derived inhibitory peptides, which were very active against both Eel and human AChE.

Key words: hemp protein, acetylcholinesterase, acetylcholine, Alzheimer's disease, hydrolysates, branched chain amino acids, hydrophobicity

8.1. Introduction

The physiological functions of the serine hydrolase, acetylcholinesterase (AChE) is to maintain homeostatic recycling of acetylcholine (ACh), the major neurotransmitter (Kumar & Chowdhury, 2014; Singh et al., 2013). However, the aging process, dietary changes and other body factors (such as stress, disease, sickness, etc) can affect this delicate homeostasis by enhancing excessive AChE activity. High AChE activity levels can cause excessive ACh degradation, which can disrupt neurotransmission and reduce normal signal transmission to the brain. This abberant brain and neurotransmission conditions lead to Alzheimer's disease (AD) pathogenesis (Willcox et al., 2014) that contributes to increased global morbidity/mortality rate (US Burden of Disease Collaborators, 2013). The high catalytic efficiency of the AChE and the importance of its inhibition in AD management has been an important research focus in the development of therapeutic agents (Saravanaraman et al., 2014; Zare-Zardini et al., 2013).

Several synthetic AChE-inhibitory drugs have been developed for use against neurodegenerative diseases, including dementia and AD; however, these drugs are associated with severe negative side effects (Kumar and Chowdhury, 2014; Ghribia et al., 2014; Iannello et al., 2014). Therefore, recent efforts have focused on development of natural AChE-inhibitory alternatives such as plant secondary metabolites, mainly polyphenolic compounds (Ghribia et al., 2014; Iannello et al., 2014; Kumar & Chowdhury, 2014). Zare-Zardini et al. (2013) have also reported isolation of a polypeptide that showed strong in vitro AChE-inhibitory activity. But information on food protein-derived peptides as agents against neurodegenerative diseases remains scanty.

Recent findings have shown that plant protein-derived peptides from various enzymatically hydrolyzed food proteins could emerge as new sources of natural multifunctional

compounds without marked adverse effects (Zhang, Mu, & Sun, 2014). It is noteworthy that several biomedical research works have revealed the efficacy of plant protein-derived peptides as human health-improving agents, but there still exists scanty or no information on their uses in AChE inhibition and AD management.

In a previous study, we found that enzymatic hemp seed protein (HSP) hydrolysates (HPH) possessed significant *in vitro* AChE-inhibitory properties that are superior to various polyphenolic extracts. However, there is paucity of information on the actual amino acid sequence of the hemp seed protein-derived AChE-inhibitory peptides. Information on the amino acid sequence of AChE-inhibitory peptides will enhance structure-function studies and could lead to development of highly potent therapeutic peptides for AD or other neurodegenerative disease management. Therefore, the main aim of this study was to separate, purify and identify the amino acid sequence of *in vitro* AChE-inhibitory peptides from 1% *pepsin*-HPH. The peptide sequences were then synthesized and used to confirm AChE-inhibitory properties.

8.2. Materials and methods

8.2.1 Hemp seed products and chemical reagents

Hemp seed protein meal (HPM) (37% protein content) produced as a by-product of the hemp seed oil processing industry was a gift from Hemp Oil Canada (St. Agathe, Manitoba, Canada). All enzymes- pepsin (porcine gastric mucosa; E.C. 3.4.23.1; ≥250 U/mg), papain (papaya latex; E.C. 3.4.22.2), alcalase (*Bacillus licheniformis*; E.C. 3.4.21.62), *Electrophorus electricus* (electric eel; 1256 U/mg protein) acetylcholinesterase Type VI-S and Human Recombinant acetylcholinesterase (expressed in HEK 293 cells; ≥1000 U/mg protein) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dithio-bis-nitrobenzoic acid (DTNB) and acethylcholine

iodide (AChSI) were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

8.2.2 Preparation of enzymatic hemp seed protein hydrolysates (HPHs)

A 5% (w/v) hemp seed protein isolate (HPI) was obtained according to the previously described method (Malomo et al., 2014) and subjected to hydrolysis using the following enzyme and reaction conditions (*pepsin*; 37$^\text{o}$C, pH 2.0, 2 hr). The pepsin was added to the HPI slurry at an enzyme to substrate ratio (E/S) of 1:100, based on the HPI protein content. Digestion was performed (pH maintained constant by addition of NaOH) after which the enzyme was inactivated by immersing the reaction vessel in boiling water bath at 95$^\text{o}$C for 10 min and allowed to cool down. The undigested proteins were precipitated by centrifugation (8000 x *g*, 60 min at 4$^\text{o}$C) and the supernatant containing target hydrolysates was freeze dried to make 1% pepsin-HPH, which was stored at -20$^\text{o}$C until needed for further analysis. The protein content of the freeze dried HPH was determined using the modified Lowry method (Markwell et al., 1978).

8.2.3 Reverse-phase (RP)-HPLC separation of HPH

The separation of HPH using preparative RP-HPLC was performed on a Varian 940-LC system as previously described (Girgih et al., 2013) with slight modifications. Briefly, freeze-dried HPH was dissolved in double-distilled water (DDW) that contained 0.1% trifluoroacetic acid (TFA) as solvent B at a concentration of 10 mg/ml; a 4 ml volume (filtered through the 0.2 μm membrane discs) was injected onto a 21×250 mm C12 preparative column (Phenomenex Technologies Inc., Torrance, CA, USA). Fractions were eluted from the column at a flow rate of 5 ml/min using a linear gradient of 0-30% solvent A (methanol containing 0.1 % TFA) over 90 min. Elution of peptide fractions was monitored at 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. 1.

The pooled fractions (F1-F8) were freeze-dried (after solvent evaporation under vacuum using the rotary evaporator, maintained at a temperature range between 35 and 45°C) and stored at −20°C until further use. The protein contents of the freeze-dried RP-HPLC fractions were determined using the modified Lowry method (Markwell et al., 1978). Each fraction was tested for AChE-inhibitory activity and the most active subjected to further LC/MS characterization.

8.2.4 LC/MS/MS identification of the purified peptides

The identification of the purified peptide was done according to the previously described method (He et al., 2013b) with slight modifications. A 1 µg/µL aliquot of the most active peptide fraction (F7) was loaded (10 µL) onto an Aeris peptide 2.6 um XB-C18 column (250 x 4.6 mm) (Phenomenex Technologies Inc, Torrance, CA, USA) connected to a Shidmazu UPLC system (Shidmazu Corporation, Nakagyo-ku, Kyoto, Japan) at a flow rate of 0.4 ml/min. The bound peptides were then eluted over 30 min with a gradient elution of 0–30% acetonitrile (containing 0.1% formic acid as solvent B) at a flow rate of 0.35 µL/min. The LC/MS/MS analysis of the peptide was monitored with an AB SCIEX QTRAP® 6500 LC/MS/MS System (AB SCIEX Pte. Ltd., Foster City, California, USA) coupled with electrospray ionization source using the following parameters: Ion source, Turbo Spray IonDrive; Curtain Gas, 30.0; IonSpray Voltage, 3.5 kV; Temperature, 150 °C; Ion Source Gas, 30; Declustering Potential, 100; Enterance Potential, 10; Collision Energy, 50; Collision Energy Spread, 20; in the positive ion mode.

The amino acid sequences of the bioactive peptides were identified using PEAKS® Complete Software for Proteomics (Bioinformatics Solutions Inc., Waterloo, ON, Canada). The

identified peptide sequences obtained were then synthesized (>95% purity) by GenWay Biotech (GenWay Biotech Inc. San Diego, CA, USA).

8.2.5 Acetylcholinesterase (AChE) inhibition assays

AChE inhibition assay was performed as previously described (Ellman, Courtney, Andres, & Featherstone, 1961) with slight modifications. Briefly, the reaction was carried out at room temperature using 0.1 M sodium phosphate buffer (pH 7.5) and the final assay mixture was 200 µl in the 96-well microplates (Costar, Corning, NY). A 20 µl aliquot of peptides (39.06, 78.12 and 156.25 µg/ml) was added to 96-well microplate containing 130 µl of 0.1 M sodium phosphate buffer (pH 7.5). This was followed by addition of 20 µl of DTNB and 10 µl of acetylcholine iodide (AChSI). Then, 20 µl of Electrophorus electrical or human AChE (5 U/ ml) was added to initiate the reaction and the mixture was incubated for 15 min at room temperature. The absorbance and the reaction kinetics were read for 2 min at wavelength of 412 nm using a microplate reader (Biotek Instruments Inc., Winooski, Vermont, USA). In the control wells, 20 µl of 0.1 M sodium phosphate buffer (pH 7.5) was used to replace peptides. In the background wells, 20 µl of 0.1 M sodium phosphate buffer (pH 7.5) was used to replace AChE while 40 µl of 0.1 M sodium phosphate buffer (pH 7.5) was used to replace both AChE and peptides in the blank wells. The concentration of sample that inhibited AChE activity by 50% (IC_{50}) was calculated from a non-linear regression plot of percentage AChE activity versus peptide concentration.

8.2.6 Statistical analysis

Triplicate replications were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA.

Duncan's multiple-range test was carried out to compare the mean values for samples with significant differences taken at p<0.05.

8.3. Results and Discussion

8.3.1 RP-HPLC fractions from 1% pepsin-produced HPH

The reverse phase-HPLC purification of 1% pepsin HPH after 90 min separation produced eight different fractions (F1-F8) of different hydrophobicity as presented in Fig. 34. The RP-HPLC separation of protein-derived peptides is based on hydrophobicity as previously described (Girgih et al., 2014; Girgih et al., 2013). Thus, while the weakly hydrophobic peptides would elute in the earliest period of separation, the strongly hydrophobic peptides will elute at a later time (Girgih et al., 2013). The results in Fig. 34 indicate that F1 and F8 are, respectively the least and most hydrophobic fractions. Similar results (eight different fractions) were obtained from the RP-HPLC separation and purification of a 4% *pepsin + pancreatin* antihypertensive hemp seed protein hydrolysate (Girgih et al., 2013).

### *8.3.2 Inhibition of AChE by different RP-HPLC fractions*

The eight RP-HPLC purified-fractions were screened for their *in vitro* AChE inhibition activities and the results are presented in Fig. 35. Fraction F7 showed an overall significantly (p<0.05) higher AChE-inhibitory effect when compared to the other fractions. For example, F7 and F3 had *in vitro* AChE-inhibitory values of 98% and 20%, respectively while no activities were observed and recorded for fractions 2, 5 and 6. Interestingly, there seemed no significant differences between fractions F1 and F4 (Eel AChE inhibition of 78%) as well as fraction F8 and the unfractionated HPH (AChE inhibition of 54%; IC_{50}, 6.00 µg/ml). Peptide purification has been described (Zhang et al., 2014) as a means to enhance the level of active molecules with potential increase in physiological impact. Therefore, the purified peptides are normally expected

Fig 34- Reverse phase-HPLC separation of 1% pepsin HPH using 30% methanol and 70% water at flow rate of 5 ml/min for 90 mins
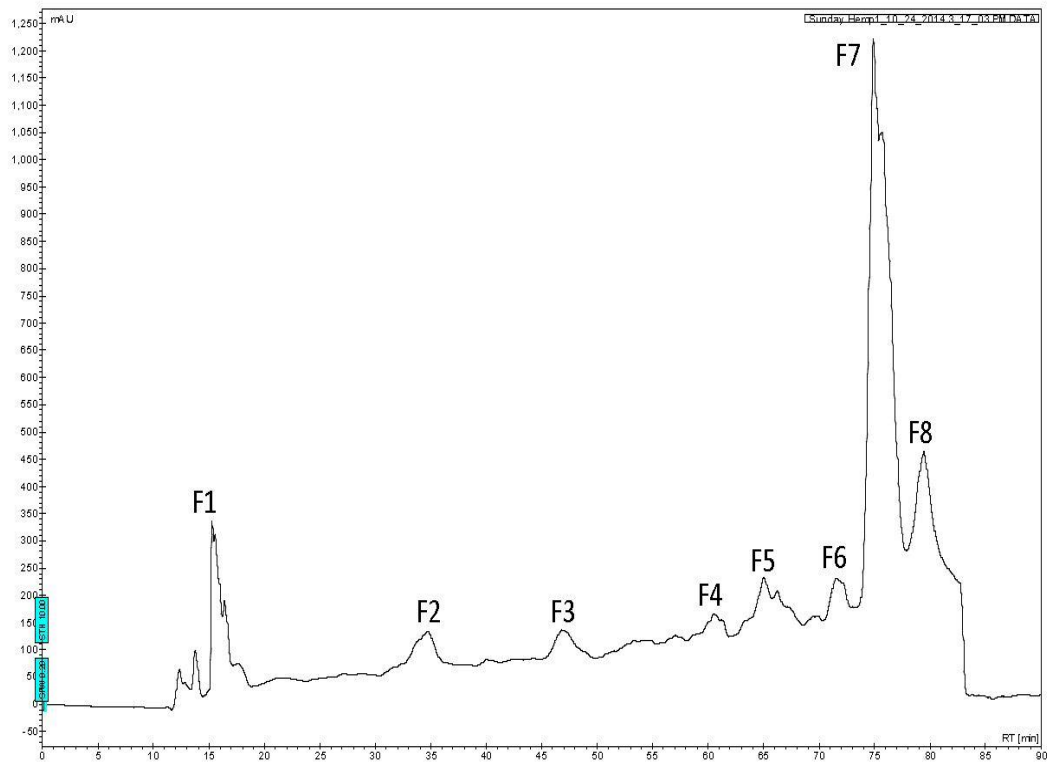
Fig 35- AChE inhibition assay (%) of RP-HPLC separation of 1% pepsin HPH
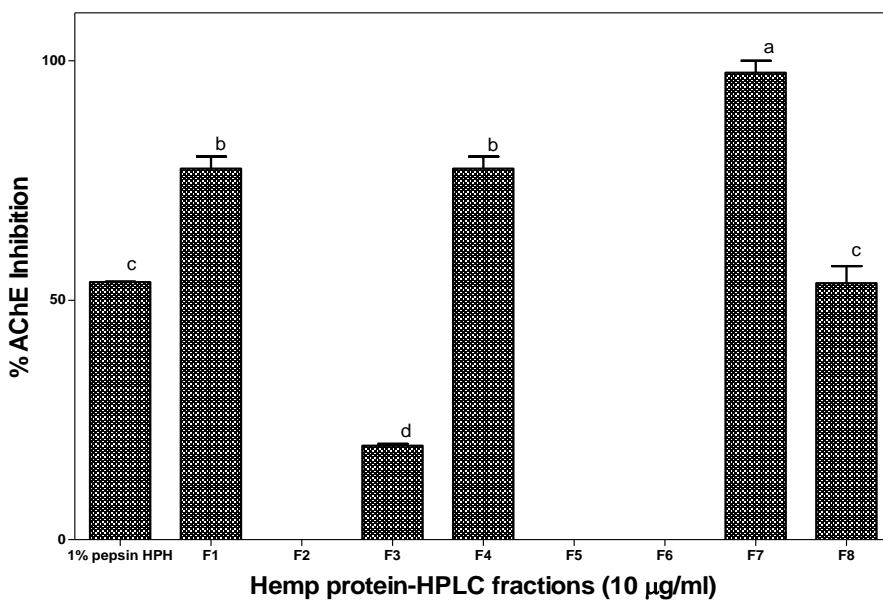
*Bars that contain different letters are significantly different at p<0.05

to possess superior inhibitory properties when compared to the unfractionated hydrolysates (Girgih et al., 2013). Thus, the results from this study (Fig. 35) are consistent with this principle as evident in the significantly higher AChE inhibition by F7 (98%) when compared to the 54% for HPH. In general, the AChE-inhibitory activities of the RP-HPLC purified-fractions in this study, except for fractions F3 (20%) and F8 (54%), are higher than the 57% reported for *Ganoderma lucidum* grown on germinated brown rice (Hasnat et al., 2013). Fraction F7 was then chosen for peptide identification and amino acid sequencing using tandem mass spectrometry based on its higher *in vitro* AChE inhibition activities.

8.3.3 Identification of AChE inhibitory peptides from hemp seed protein hydrolysates
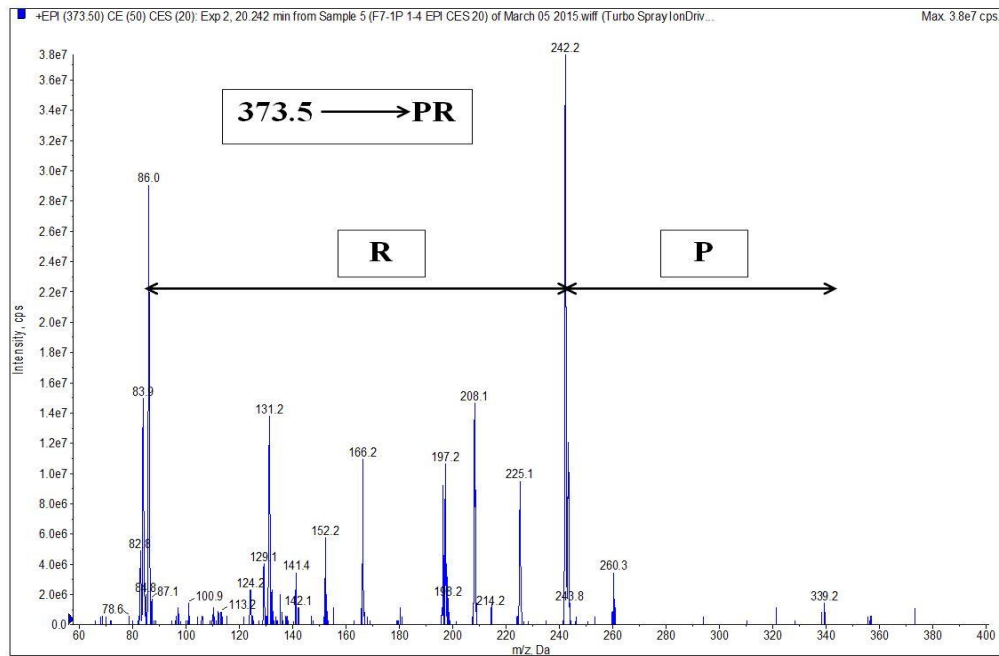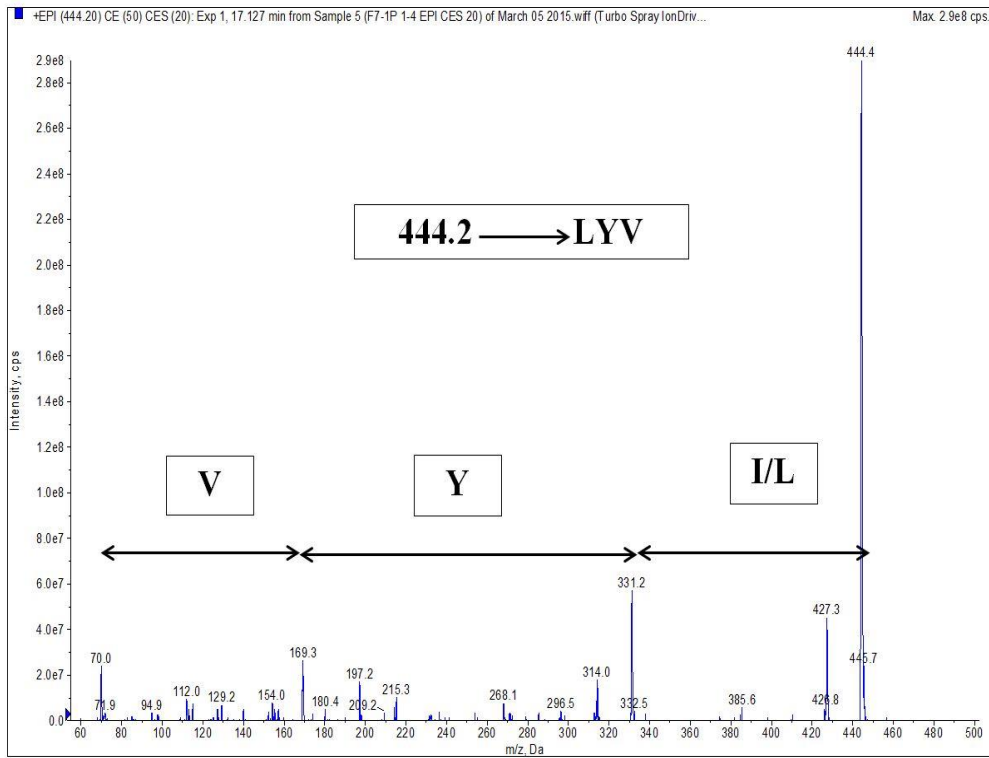
First, for each target amino acid sequence, a precursor/fragment ion relationship is established, which identify each proteotypic peptide at a selected mass-to-charge ratio (m/z) value that are preferentially detectable by MS (Picotti et al., 2008). The F7 was injected to the mass analyzers of a QTRAP ion mass spectrometer to isolate the targeted peptide precursor ion and several molecular species were obtained as shown in Fig. 36. This was then followed by the MS/MS fragmentation to produce the corresponding and diagnostic fragment ions over a period of chromatographic time, which is used for amino acid sequencing. The LC/MS/MS analysis results presented in Fig. 37 (a-h) show the spectrum of eight ions with m/z of 444.20, 373.50, 428.40, 342.10, 401.30, 488.10, 573.10 and 288.10. The amino acid sequences of the identified peptides, as determined by MS/MS spectra were LYV/IYV, PR, VQFWG, PAP, EPS, LSA/ISA, GGYRS, LC/IC, respectively (Fig. 37).
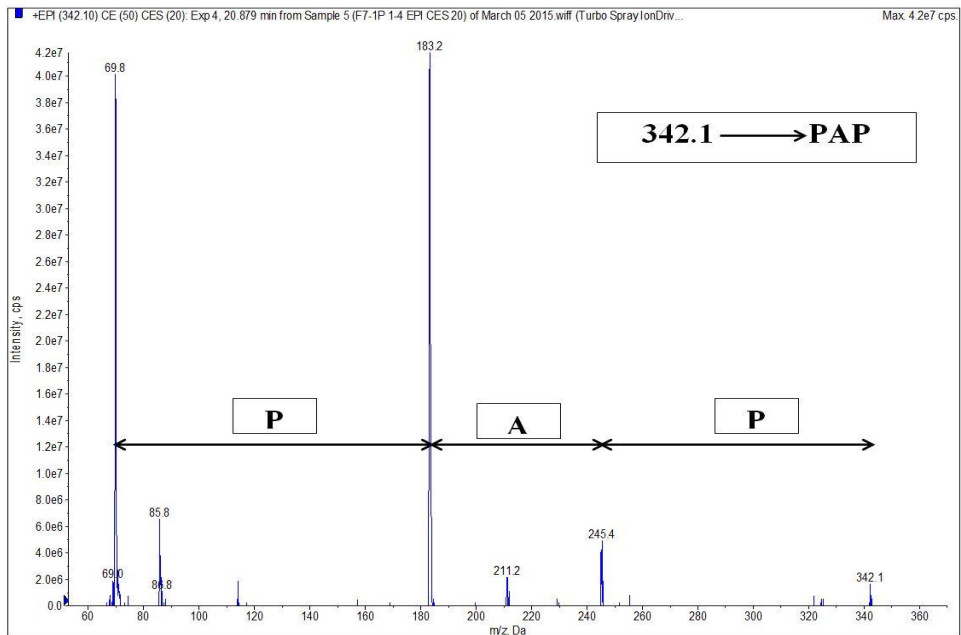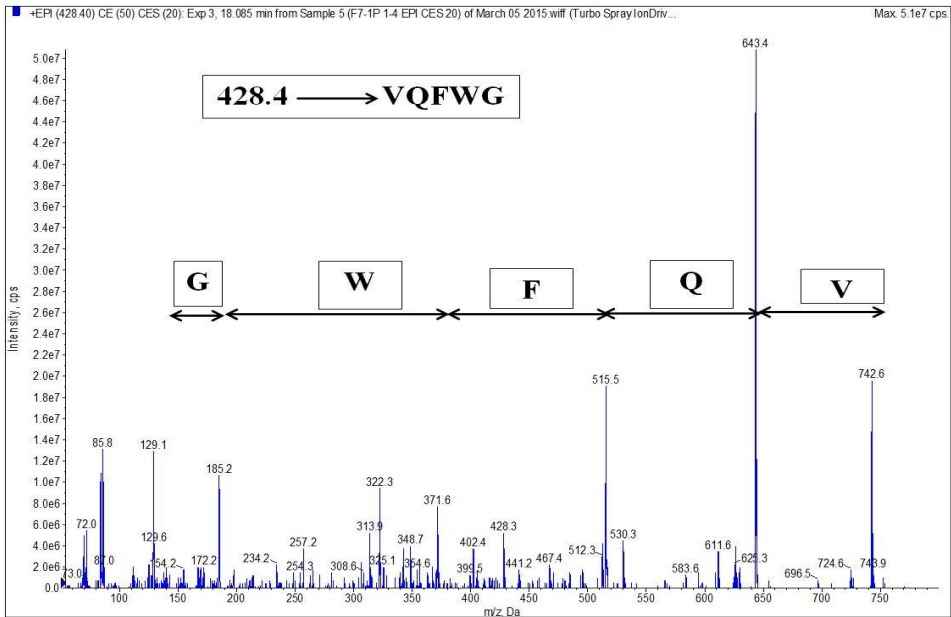
Thus, due to the low yield from analytical column purification, the resultant eleven peptides were then synthesized for use during the *in vitro* studies to test for their inhibitory potentials against the activities of the targeted AChE.
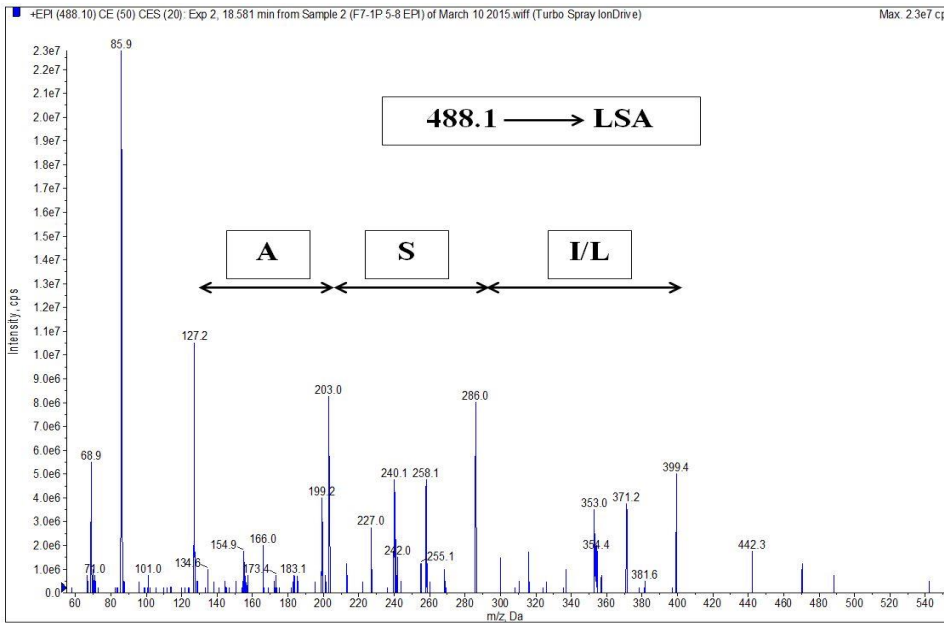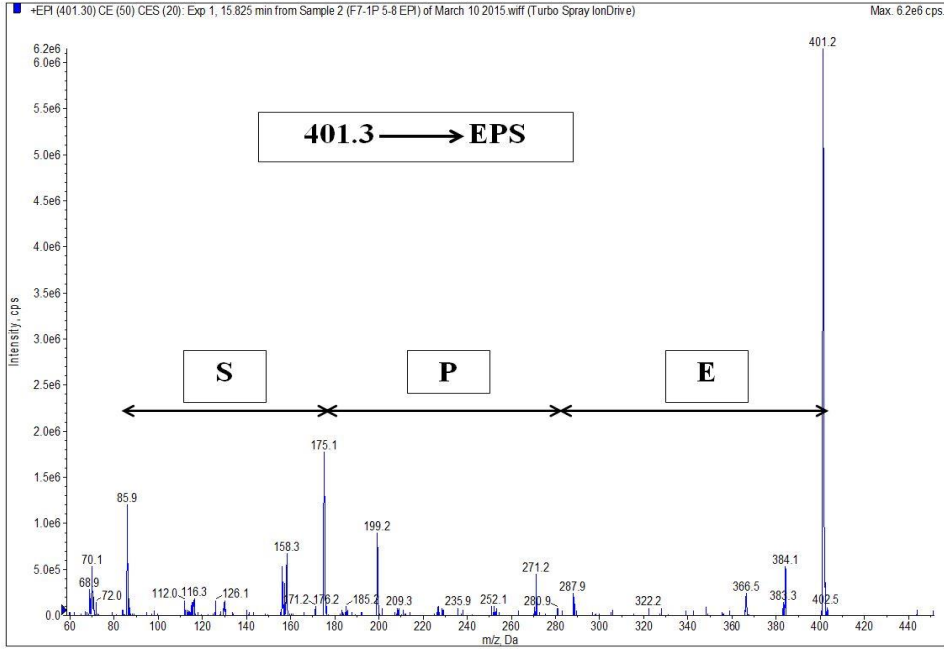
Fig 36- MS scan of RP-HPLC separated fraction F7 using QTRAP ion drive at positive ESI mode
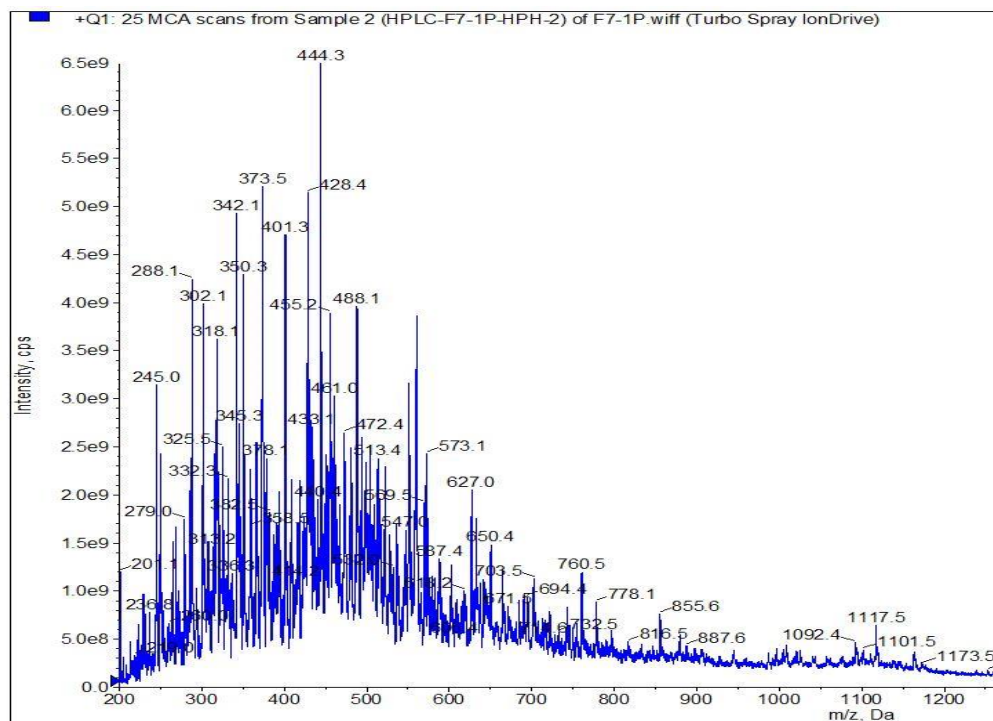
Fig 37- LC/MS/MS of RP-HPLC separated fraction F7 at different *m/z* using QTRAP ion drive at positive ESI mode

+EPI (401.30) CE (50) CES (20): Exp 1, 15.825 min from Sample 2 (F7-1P 5-8 EPI) of March 10 2015.wiff (Turbo Spray IonDrive)   Max. 6.2e6 cps.

401.3 ⟶ EPS

+EPI (488.10) CE (50) CES (20): Exp 2, 18.581 min from Sample 2 (F7-1P 5-8 EPI) of March 10 2015.wiff (Turbo Spray IonDrive)   Max. 2.3e7 cps.

488.1 ⟶ LSA
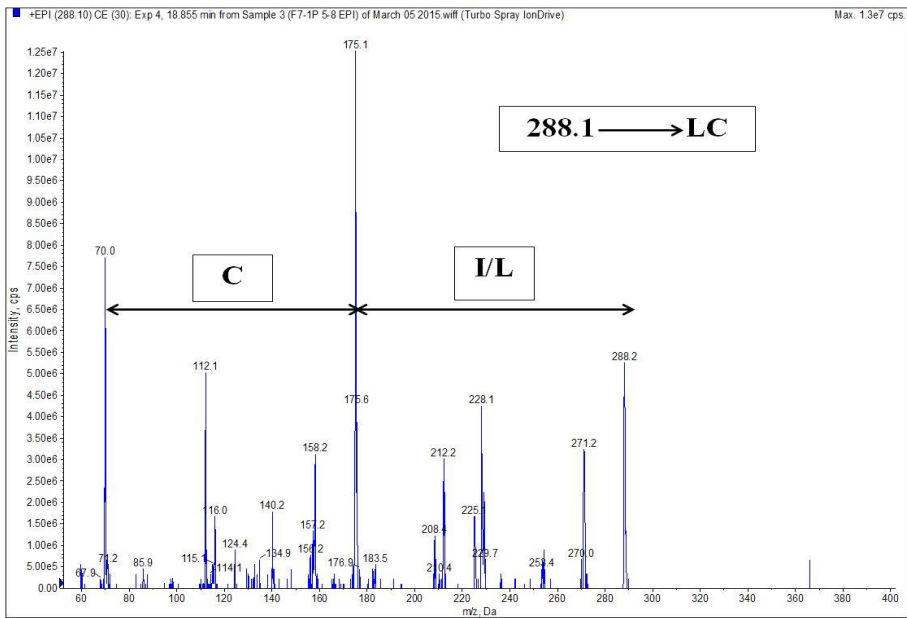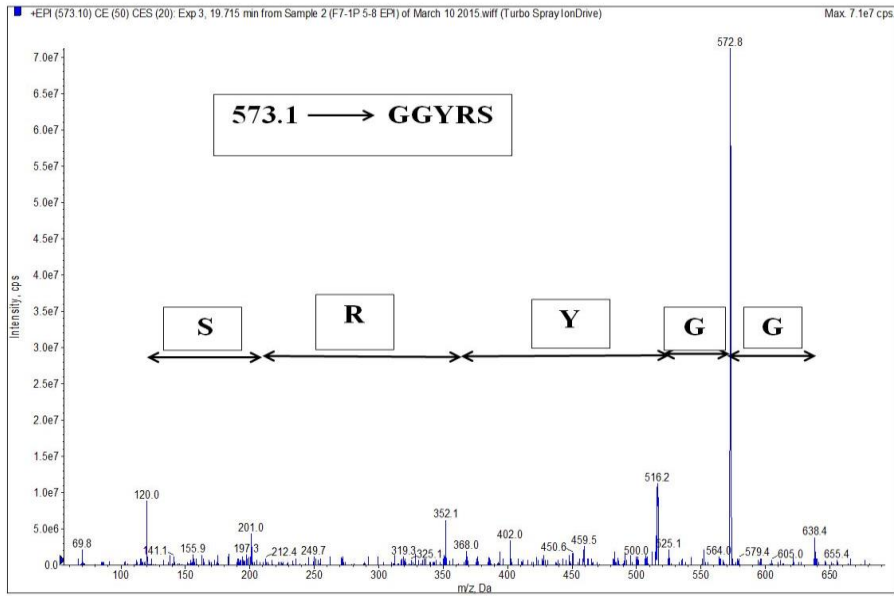
8.3.4 Inhibition of AChE by different hemp protein-derived bioactive peptides

Fig. 38 shows the IC_{50} values of peptides against eel AChE inhibition and indicates LYV (IC_{50}, 6.80 µg/ml) as the most potent (p<0.05) when compared to LSA (IC_{50}, 21.90 µg/ml), VQFWG (IC_{50}, 17.50 µg/ml) and GGYRS (IC_{50}, 11.60 µg/ml). Activities of these peptides are weaker than that of the HPLC-F7 fraction (IC_{50}, 5.80 µg/ml) and the unfractionated 1% pepsinHPH (IC_{50}, 6.00 µg/ml). The two identified dipeptides, LC and IC showed no inhibition against eel AChE activity and moreover, there is no observable significant difference between the inhibitory effects of tripeptide, PAP and the pentapeptide, VQFWG.

Fig. 39 shows the result of the human AChE inhibition by synthesized peptides. LYV (IC_{50}, 7.50 µg/ml) was the most potent (p<0.05) against human AChE activity (Fig. 39) when compared to LSA (IC_{50}, 13.00 µg/ml), VQFWG (IC_{50}, 9.50 µg/ml), GGYRS (IC_{50}, 26.80 µg/ml), HPLC-F7 (IC_{50}, 13.20 µg/ml) and 1% pepsin HPH (IC_{50}, 13.40 µg/ml). Noticeably, the two identified dipeptides, LC and IC showed no inhibition against human AChE activity and moreover, there is no observable significant difference between the human AChE inhibition of tripeptide, ISA and pentapeptide, VQFWG (IC_{50}, 17.50 µg/ml). Besides, there was no observable significant difference between human AChE-inhibitory activities of the tripeptide (LSA) and the HPLC-F7 or 1% pepsin HPH (IC_{50}, 13 µg/ml). The results (Fig. 38 and 39) showed that all the identified tripeptides possessed higher AChE (from both Eel and human) inhibition than the identified pentapeptides.

It is noteworthy that all the eleven identified peptides produced in this study, were of very short chain lengths, which could possibly influence higher rates of absorption from the GIT and potentially increased in vivo AChE inhibition. Another reason for their high inhibitory power might be connected to their observed low molecular weight (<500 Da), which could enhance

Fig 38- Inhibition of AChE (from Eel) by different enzymatic hemp protein-derived peptides at a

50% level of inhibition (IC_{50})
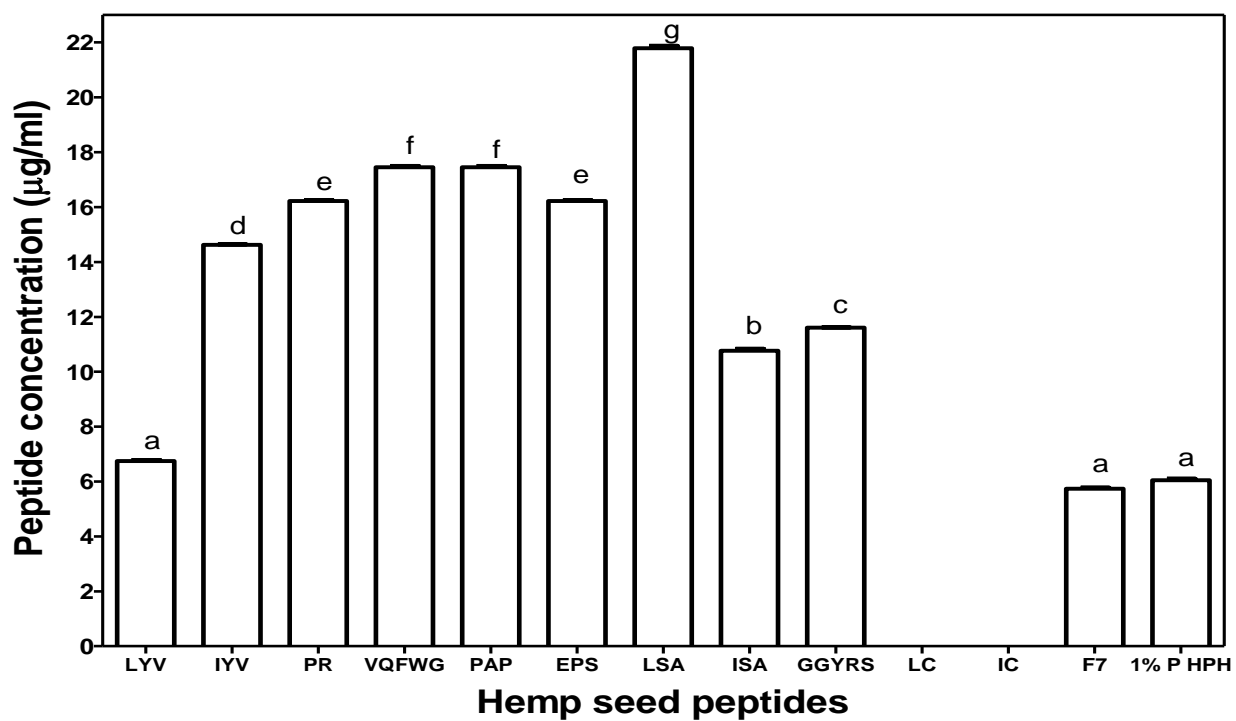
*Bars that contain different letters are significantly different at $p < 0.05$
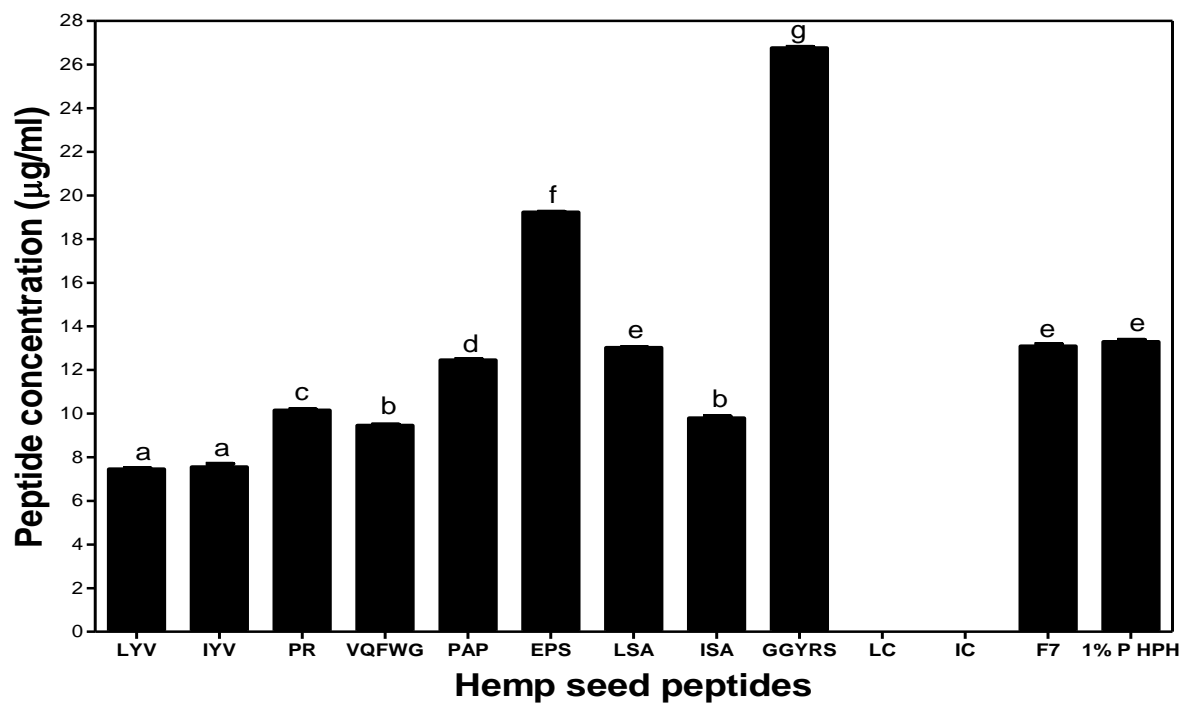
Fig 39- Inhibition of AChE (from human) by different enzymatic hemp protein-derived peptides at a 50% level of inhibition (IC_{50})

*Bars that contain different letters are significantly different at $p < 0.05$

their ability to interact with the enzyme active site. A past study (although produced for cardiovascular-related targeted enzymes) had reported the high inhibition and disease-management power of low molecular weight bioactive peptides from pepsin-produced HPHs (Girgih et al., 2014). About seven out of the eleven identified peptides were mainly composed of branched-chain amino acids (L, V, I) that has been described as a group of amino acids found to enhance the hydrophobic character of bioactive peptides (Aluko et al., 2015). The important role of these branched-chain amino acids to enhance hydrophobicity and inhibitory potentials of bioactive functional components has been also observed in our previous findings. The effect of high hydrophobicity of bioactive peptides lies in their ability to influence strong interactions between peptide and non-polar amino acid residues within the enzyme active site (Aluko et al., 2015; Girgih et al., 2014; Sarmadi & Ismail, 2010).

8.4 Conclusion

The importance of separating and purifying the hemp seed protein-derived bioactive peptides for cognitive human health application had been demonstrated in this study. The higher hydrophobicity of fraction F7 was reflected in the hydrophobic character of the identified peptides. Eleven peptides of very short chain lengths and low molecular weights <500 Da were identified from F7, which were composed mainly of branched chain and hydrophobic amino acids residues that contributed to their AChE-inhibitory potency. The HPLC-separated and purified bioactive peptides possessed higher *in vitro* AChE (from both Eel and human) inhibitory properties (IC$_{50}$, 5.80-26.80 µg/ml). Therefore, for the first time in literature, this work reported the amino acid sequences of food protein-derived peptides that inhibited both Eel and human AChE activities.

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CHAPTER NINE

9. General Summary and Conclusion of the study

The first study reveals a potential use of hemp seed protein products as basic functional ingredients in the food industry besides their nutritional role. The findings show that manipulation of pH could be used to increase foaming ability, which is dependent on protein solubility. The oil droplet sizes of some of the emulsions produced by these proteins were very small and comparable to the well-known and commonly used milk and soybean protein emulsifiers. Although, their highly stable emulsions indicated potential use to form food emulsions that could maintain stability during short-term storage, the findings showed a different mechanism responsible for foam and emulsion formation. Intrinsic fluorescence and CD data from this study revealed that hemp seed proteins existed in different conformational states within different pH environments. The study suggested that the effect of pH on protein functionality is dependent on protein concentration and also confirmed the high dependency of protein functionality on structural conformation as being affected by modulation in pH changes.

The second study gives a concise description of how variations in amino acid composition and pH-dependent changes in structural conformation could differentially affect the functionalities of both hemp seed albumin and globulin protein fractions. The salt-extracted globulin is made up of more disulfide-bonded proteins than the water-soluble albumin. This produces a rigid structure with reduced exposure of aromatic amino acids within the globulins. This study also reported the contribution of higher levels of hydrophobic amino acids and the rigid conformational structure in globulin on its decreased protein solubility and foaming capacity. This is a confirmation of the dependence of these functional properties on protein flexibility and the ability of proteins to interact with the aqueous phase. In contrast, emulsion-

forming ability was reportedly not dependent on protein solubility, suggesting different mechanisms are involved in foam and emulsion formations. However, both protein fractions were observed to be potentially good ingredients for the manufacture of food emulsions with only the albumin playing a superior role in the manufacture of food foams.

The third study proved the potential use of waste products from oil seed processing industries, especially the hemp seed processing industry as well as the resultant effects of the preparation methods of protein isolates on their physicochemical and functional properties. This is because the potential uses of protein isolates are largely dependent on these properties with great influence from protein digestibility. This study revealed the high improvement on the physicochemical and functional properties of enzyme pre-digested and membrane produced-isolates when compared to those from the isoelectric precipitation method. The combination of carbohydrase and phytase treatments led to the production of a 74% protein product, which s twice the 37% protein in the original hemp seed waste product. The significant improvements in the functional properties (protein solubility, foam capacity and emulsion stability) and the excellent *in vitro* protein digestibility reported for this improved protein isolates provides a big advantage over utilization of the acid/alkaline-harsh conditions produced-isolates. The findings also showed the presence of large numbers of disulfide bridge-linked polypeptide chains that is well-known to keep the protein molecules intact against any processing environmental conditions in the membrane produced-isolate.

The fourth study showed the potential of the hemp seed protein derived-peptides as dual *in vitro* ACE and renin inhibitors. The work also demonstrated bioactive effects when these physiologically functional hydrolysates were shown to significantly lower elevated-blood pressure (BP) *in vivo* after oral administration to SHRs. The *in vivo* BP-lowering effects of the

peptides did not correspond directly to their *in vitro* renin-ACE inhibition activities, which suggest other factors may have contributed to peptide-induced antihypertensive effects.

To the best of our knowledge, the fifth study is the first to report the *in vitro* AChE inhibition activities of hemp seed protein derived-peptides obtained from enzymatic hydrolysis of hemp seed proteins. The study showed higher improvement and increase in the protein content and extraction yield of the hydrolysed protein products when compared with its non-hydrolysed forms. The liberation of indigenous bioactive-encepted peptides from the primary structure of plant proteins through *in vitro* enzyme-catalyzed proteolysis (hydrolysis) for disease management is well justified in this study. The study further revealed the lack of relationships between degree of hydrolysis and *in vitro* AChE-inhibition activities. However, the pronounced *in vitro* AChE-inhibitory activities of the hydrolysates in this study was dependent on their high hydrophobic and branched chain amino acid residues as well as the composition and presence of very short chain peptides (e.g. di-, tri- and tetrapeptides).

The sixth study shows the importance of separating and purifying the plant protein-derived AChE-inhibitory peptides. This was revealed from the study when the HPLC-separated and purified bioactive peptides possessed higher *in vitro* AChE-inhibitory properties than the crude and unfractionated hydrolysate. Apart from reporting the probable or suggested amino acid sequences of the bioactive peptide that could be obtained from the most active AChE-inhibitory HPHs, the study confirmed the presence of a very short chain peptide sequence (LYV) with improved *in vitro* AChE-inhibitory properties. The very short chain peptide sequences were composed mainly of branched-chain and hydrophobic-enriched amino acids. Moreover, LYV is a novel peptide with an AChE-inhibitory IC_{50} value of 7 µg/ml that makes it a potential therapeutic tool in human cognitive health applications. Finally, this work also presented other

novel peptide sequences with superior AChE-inhibitory values than previously reported natural products and lays a solid foundation for further studies on the use of bioactive plant protein-derived peptides as agents against neurodegenerative diseases.

Although the antihypertensive activity of 1% pepsin HPH is not shown in this report, but its multifunctionality effects on renin-ACE-inhibition, great reduction of elevated-SBP in SHRs (after 4 h) as well as effective inhibition against AChE activities were observed in this research work. Hence, there is possibility that the identified-bioactive peptides obtained from 1% pepsin HPH might probably inhibit both AChE and RAS enzymes (renin and ACE). This is because most of the identified-bioactive peptides (e.g. PR, LYV, PAP and VQFWG) contain aromatic (tyrosine, phenylalanine and tryptophan), hydrophobic (proline and alanine) and branched-chain (valine and leucine) amino acid residues required for optimal and efficient AChE-inhibition and antihypertensive acitivities. Thus, there seems no notable difference between the bioactive peptides with cardiovascular (renin-ACE inhibition) and cognitive (AChE inhibition) health benefits but their (peptides) metabolic pathway of inhibition of the disease-causative enzymes might be different.

It is therefore, concluded that the improved physicochemical properties of the enzyme pre-digested and membrane-produced protein isolate could make it a suitable functional ingredient in the food processing industries. Potential food applications include formulation of clear beverages, manufacture of oil-in-water emulsions (e.g. salad dressings and soups); formulation of desserts (such as meringues) and meat and dairy products with desired fat/flavour retention. The results also indicate potential use of the hemp seed protein-derived peptides as beneficial multifunctional effects (for example, towards the human cardiovascular and cognitive health applications). The protein hydrolysates may be used as a source of natural AChE-

inhibitory agent and with potential cognitive health benefits applications, especially in the treatment of Alzheimer's disease.

Lastly, this research work has established some of the relationships between physicochemical properties (polypeptide composition, amino acid composition, amino acid sequence), *in vitro* protein digestibility and functional properties of hemp seed proteins and its derived peptides. For instance, the definitive peptide sequences identification exercise reveals that most peptides with aromatic, hydrophobic and branched-chain amino acid residues might possess the ability to improve both functional properties and human health benefits of the HSP.

This type of data is needed for fundamental understanding of the relationships between structural properties of the proteins or peptides and their functions in food systems or human health applications.

CHAPTER TEN

10. Limitations of the study

a. The enzyme pre-digestion and membrane ultrafiltration method of protein isolation was not able to achieve the production of protein isolates with >90% protein content earlier proposed in the course of this research work. The use of more effective carbohydrases and phytases will be required to produce another 10-15% increase in protein content of the membrane isolate.

b. Although, the work reported on the peptide identification and *in vitro* AChE-inhibitory activities of the identified bioactive peptides from the 1% *pepsin*-produced hydrolysates, similar peptide identification was not performed for the most active RAS-modulatory hydrolysates.

c. Currently, there is no report on the human AChE-inhibitory bioactive peptides identified from plant proteins to serve as reference point for our newly identified hemp seed AChE-inhibitory peptides.

d. The AChE inhibition work involved only *in vitro* assays, but tests on animal disease models of dementia or AD are required to confirm potential human health benefits. The peptides will then need to be tested using human intervention trials before the product can be made available to consumers.

11. Future directions of the study

The data from *in vitro* studies alone cannot propagate the application of bioactive hemp seed protein-derived-AChE-inhibitory peptides as therapeutic agents against cognitive health disorders without *in vivo* and human clinical trial studies. Thus, *in vivo* animal and human intervention trials are highly recommended before confirming peptides use in cognitive health. It has been revealed from recent scientific findings that identification of amino acid sequences of plant protein-derived bioactive peptides can pave the way for efficient utilization in developing potent products for human health improvement. Therefore, further studies are required to isolate and determine amino acid sequences of peptides present in the most active antihypertensive HPHs reported in this study. Moreover, there still exist some unresolved cases around the proposed utilization of hemp seed protein products as functional food ingredients in the food industry and human health applications. For instance, the protein content of the enzyme pre-digested coupled with membrane-produced hemp seed protein isolates need further improvement to bring it up to the 85-90% range. Moreover, the exact mechanism of AChE-inhibition by the hemp seed protein-derived peptides needs to be resolved using kinetics of enzyme inhibition studies. These proposed studies would ensure industrial applications of the beneficial features of this plant protein in food product development and human health. Emerging evidences have also revealed the bioactive properties of some compounds obtained during interaction of plant protein hydrolysates with some proteases (plastein aggregates). Therefore, it becomes very imperative to consider emergence or production of such compounds from hemp seed protein hydrolysates and study the beneficial values that these peptide aggregates might contribute to food and human health applications.