Physicochemical, Functional and *in vitro* Bioactive Properties of Hempseed (*Cannabis sativa*) Protein Isolates and Hydrolysates.

By

GRACE ISINGUZO

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

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Department of Human Nutritional Sciences

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Physicochemical, Functional and *in vitro* Bioactive Properties of Hempseed (*Cannabis sativa*) Protein Isolates and Hydrolysates

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ABSTRACT

Apart from the nutritional importance of proteins, their physicochemical, functional and bioactive properties are dependent on their structure as well as their resultant interactions with other proteins, water and lipids.

We investigated the physicochemical and functional properties of hempseed protein isolate as well the bioactive properties of hemp seed protein hydrolysates (HPH) using antioxidant and antihypertensive assays.

The results show that molecular mass decreased with increase in time of hydrolysis. Alcalase treated HPH had an increase in surface hydrophobicity. Increase in hydrolysis time increased protein solubility and water holding capacity of HPH, while foaming and fat absorption capacity decreased as time of hydrolysis increased.

The <3 kDa permeates of thermolysin hydrolysate had the highest ACE-inhibition activity, while the <5 kDa permeates of alcalase hydrolysates had the highest renin inhibition value.

The various fractions showed low scavenging activity of 1,1–diphenyl-2-picrylhydrazyl, while the superoxide radical scavenging activities were weak to nil. The three various fractions of thermolysin treated hydrolysates displayed the strongest chelating activity. For ferric reducing activities, <5 kDa thermolysin fraction was relatively high when compared to glutathione.

The work concluded that HPH can be used not only as a source of nutrients but also as a functional ingredient in food systems as well as therapeutic agent against chronic diseases such as hypertension and oxidative stress-related disorders.

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LIST OF ABBREVATIONS

THC: Tetrahydrocannibinol

HPI: Hemp protein isolate

HPH: Hemp protein hydrolysate

SDS: Sodium dodecyl sulfate

ME: Mercaptoethanol

BPB: Bromophenol blue

DPPH: 1,1- diphenyl-2-picrylhydrazyl

pI: Isoelecteric point

PS: Protein solubility

FAC: Fat absorption capacity

S_o: Surface hydrophobicity

WHC: Water holding capacity

FRAP: Ferric reducing assay power

ACE: Angiotensin converting enzyme

CHAPTER ONE

GENERAL INTRODUCTION

Research in the area of bioactive peptides has increased immensely in recent years. Bioactive compounds are naturally occurring chemical compounds in plants or animals that possess the ability to exert health benefits in the body. Bioactive peptides are short (2-20 amino acid residues) sequences of amino acids contained within the primary structure of proteins. Bioactive peptides are derived from food proteins through enzymatic hydrolysis and they are able to exert nutritional and physiological effects when ingested into the human body.

Studies have shown that bioactive peptides can be produced from various sources such as animal (Hernadez-Ledesma et al., 2007), marine (Theodore et al., 2008), and plants (Fang et al., 2007). Bioactive peptides as long as they are in their parent proteins remain inactive until they are released by enzymes (proteases) during digestion or food processing (Yoshikawa et al., 2000), though the most common method has been enzymatic hydrolysis. After being released from its parent protein during digestion into a mixture called protein hydrolysate, the bioactive peptides are absorbed from the intestine into the blood circulatory system. These peptides as seen in Table 1 have specific functions and can then exert therapeutic roles such as mineral-binding, immunomodulatory, antimicrobial, antithrombotic, hypocholesterolemic and antihypertensive activities in the body (Rainer and Meisel, 2006).

Table 1. Physiological benefits of bioactive peptides:

Cardiovascular system	Nervous system	Gastrointestinal system	Immune system
Hypocholesterolemic	Opioid	Mineral binding	Immunomodulating
Antioxidative	-Agonist activity	Antimicrobial	
Antithrombotic	-Antagonist activity		
Antihypertensive			

Some peptides are multifunctional and so can carry out more than one physiological activity from the list above (Meisel , 2004). Due to their nutritional benefits, physiological functions as well as therapeutic roles, these bioactive peptides can be used as functional foods and nutraceuticals.

Functional foods and Nutraceuticals

Functional foods and nutraceuticals have the potential to improve health status, reduce healthcare cost as well as support economic growth in rural Canadian communities (Agriculture and Agri-Food Canada, 2009). People are becoming more interested in foods that have not only nutritional benefits, but also disease prevention and health benefits. Functional foods are regular foods that have in addition to their nutritional values, the ability to protect against progression of chronic diseases (Health Canada, 2002). Nutraceuticals on the other hand are isolated and purified substances from food and they are in the form of pills that can be used to prevent development of diseases. The

global demands for functional foods and nutraceuticals have increased in the past years due to increased awareness of their health benefits (Kitts, 1994).

Physiological activities of peptides.

Apart from improvements in functional properties, studies have shown that peptides produced by enzymatic hydrolysis have added physiological properties such as the inhibition of angiotensin converting enzyme (ACE) activity (Kim et al., 2001; Matsui et al., 1993; Wu & Ding, 2001).

Hypertension, also known as high blood pressure, is one of the most common worldwide diseases that affect the human population. It can lead to other serious health problems such as heart disease, stroke, kidney failure, heart failure and diabetes. The main regulator of blood pressure is the renin-angiotensin system (Figure 1), which consists of two key enzymes. First, renin converts angiotensinogen to angiotensin I, which is then acted upon by angiotensin converting enzyme (ACE) to form angiotensin II (a potent vasoconstrictor). ACE also inactivates bradykinin (a potent vasodilator) which leads to an elevation in blood pressure (hypertension). ACE inhibitors, on the other hand, slow ACE activity, thereby reducing blood pressure (Xie, 1990). Therefore, inhibition of ACE is considered to be an important step in the clinical and nutritional treatment of hypertension. Inhibition of renin to prevent the production of angiotensin I is another major step in prevention of hypertension. The secretion of renin by the kidney is due to a decrease in sodium chloride levels or a decrease in arterial blood pressure. Excessive levels of renin and ACE will circulate in the blood system and cause elevated levels of angiotensin II, which leads to hypertension.

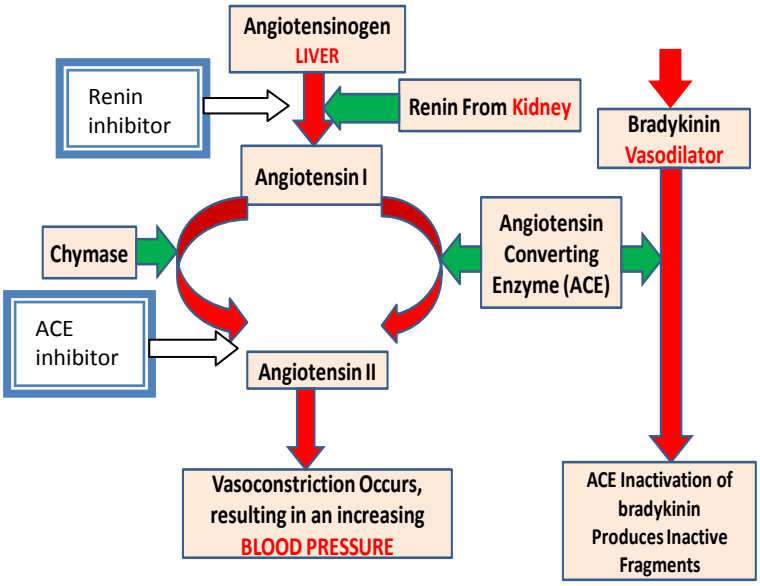


Figure 1. Regulation of blood pressure by the renin-angiotensin system

Functional properties of proteins

Functionality can be defined as properties of food or food components which, apart from its nutritional properties, can affect its utilization (Pour-El, 1979). Studies have shown that food proteins can also be incorporated into food products to improve functional properties. To enhance some functions, these food proteins may be enzymatically hydrolysed. For example, van der Ven et al., (2002) showed that foam formation of casein and whey proteins were improved by enzymatic hydrolysis. Enzymatic hydrolysate of proteins from corn gluten meal had better solubility property when compared to the unhydrolysed proteins (Mannheim and Cheryan, 1992). Functional properties of proteins in food products include fat absorption capacity (FAC), water holding capacity (WHC), emulsification, foam formation as well as gelation. Functional properties of protein can be seen in its interaction with other components of the food system. Interactions of a protein with other components of the food system make it possible to exhibit its functionality. The proteins need to be free, or change its structure in order to be able to move freely in the food system to enhance interactions. Example is the tendency of hydrophobic portions of proteins to interact with lipids and lipid soluble compounds.

Objectives of study:

- Determine the effects of limited proteolysis by alcalase, pepsin or thermolysin enzymes on the physicochemical and functional properties of hemp seed proteins.
- Produce protein hydrolysates through extensive hydrolysis of hemp seed proteins and fractionate the products using ultrafiltration protocols to obtain peptide products that vary in molecular size.

- Determine ability of the ultrafiltration fractions to inhibit activities of ACE and renin.
- Determine the ability of the ultrafiltration fractions to scavenge free radicals and act as antioxidants.

CHAPTER TWO

LITERATURE REVIEW

2.1. Hemp seed plant

Food-grade hemp seed protein products are obtained from the industrial hemp plant *Cannabis sativa* L. Unlike its cousin marijuana, the food grade hemp seed contains only a trace amount of tetrahydrocannabinol (THC), the compound responsible for the psychoactive characteristics of marijuana (Callaway, 2004). For thousands of years, hemp seed has been used as a source of food (raw, cooked or roasted), industrial fibre and medicine (de Padua et al., 1999).

2.2. History of industrial hemp production

Industrial hemp is one of the oldest plants grown for food, fibre and medicine (Yin et al., 2007). It was banned in North America in the late 1930s and was later banned worldwide by the United Nations in 1961. This was due to the high level of THC (6 to over 20%) found in the leaves, flowers and seeds of its cousin Marijuana. The ban was officially lifted in Canada in 1998 so that it is now legal to grow industrial hemp that has THC threshold of 0-0.3%. In 2009 hemp production increased by 72% across Canada. Industrial hemp thrives well in the Prairies, with Manitoba and Saskatchewan provinces being the leading producers in Canada. Canada, France and China are the world leading producers of industrial hemp. There are now several Canadian companies involved in the hemp seed market and they are producing snack foods, hemp meal, milk, oil, oil paints, as well as cosmetic products. One third of Canadian hemp seed production is certified organic. The increase awareness of the use of industrial hemp as a source of food, fibre

and medicine has increased the global demand for the low THC hemp (Oomah et al., 2002). Currently, it is illegal to grow hemp in the United States without a special Drug Enforcement Administration (DEA) permit being issued (Tara, 2003).

2.3. Nutritional value of Hemp seed.

Hemp seed is a very good source of dietary oil, protein and fibre as shown in Table 2.1. Hemp seed contains two main proteins, edestin (globulin) and albumin (Patel et al., 1994), which consists of most of the essential amino acids needed by the human body as shown in Table 2.2 (Callaway, 2004). Hemp seed also contains vitamins and minerals with significant amounts of tocopherols (Kraise et al., 2004). Hemp seed oil contains about 90% unsaturated fatty acids with an abundance of linoleic (18:2 omega -6) and linolenic (18:3 omega -3) acids (Callaway, 2004). The ratio of n6:n3 is between 2:1 and 3:1 which is similar to the ratio found in Mediterranean diets (Kraise et al., 2004; Simopoulos, 2002).

Table 2.1. Typical nutritional content (%) of hemp seed

Nutrients	Content (%)
Oil	35.5
Protein	24.8
Carbohydrates	27.6
Moisture	6.5
Ash	5.6
Total dietary fiber	27.6
Energy(kJ/100g)	22.2

Adapted from: Callaway (2004)

Table 2.2. Typical protein content (%) of hemp seed

Amino Acid	Amount (g/100g) Essential amino acids are indicated by an asterisk(*)
Alanine	1.28
Arginine	3.10
Aspartic acid	2.78
Cystine	0.41
Glutamic acid	4.57
Glycine	1.14
Histidine*	0.71
Isoleucine*	0.98
Leucine*	1.72
Lysine*	1.03
Methionine*	0.58
Phenylalanine*	1.17
Proline	1.15
Serine	1.27
Threonine*	0.88
Tryptophan*	0.20
Tyrosine	0.86
Valine*	1.28

Adapted from: Callaway (2004)

The natural dark oil of hemp seed oil is due to the chlorophyll present in the mature seed, and this is what quickens oxidation of the oil when exposed to light (Callaway, 2004). To avoid conversion of polyunsaturated fatty acids to unhealthy trans-fatty acids and peroxides at higher temperatures, hemp oil and nuts are best used for cold and warm dishes (Leson et al., 1999). The hemp oil should be stored in the refrigerator or freezer. Hemp seed, unlike soy, is devoid of trypsin inhibitors, which block the action of trypsin during digestion, limit protein digestion and subsequent absorption of proteins (Odani & Odani, 1998). Hemp seed is also free of oligosaccharides which can cause stomach upset and flatulence. Hemp seed has fresh nutty flavor and is not known to be an allergen.

Hemp seed is beginning to gain recognition as one of the foods that serves as a functional food due to its nutritional as well as its bioactive properties. Hemp seed has nutritional values that make it a good source of protein - edestin and albumin, and these proteins contain essential amino acids (EAA). Hemp seed also has oil which contains ~84% polyunsaturated fatty acids (PUFAs), particularly linoleic and gamma-linolenic acids. In addition, hemp seed also contains dietary fibre (Callaway, 2004; Oomah et al., 2002). Edestin and albumin are easily digested and can be used as a source of protein nutrition for infants and children as it meets the FAO/WHO suggested requirement for infants and children (Hodson et al., 2001). PUFAs are important in the formation of phospholipids in cellular membranes and brain cells (Hodson et al., 2001). PUFAs are capable of lowering LDL-cholesterol as well as blood pressure (Callow et al., 2002). Hemp seed oil has been topically used in some studies in healing atopic dermatitis (eczema) (Callaway, 2004).

The green colored pigment (polyphenol) of hempseed protein indicates a high amount of chlorophyll content and this may act as a natural detoxifier or antioxidant. The greenish color of hemp seed protein may not be appealing to some people, hence the need for future work to be carried out on decolorizing hemp seed protein in order to increase its appeal.

2.4. Functional properties of hemp seed proteins

Despite the high nutritional qualities, hemp seed has some poor functional properties; especially protein solubility (PS) and emulsifying property when compared to soy protein isolate (SPI) (Tang et al., 2006). These functional limitations could greatly limit the use of hemp seed proteins as ingredients in the food industry. The poor solubility of hemp seed proteins could be attributed to the presence of covalent disulfide bonds between the individual proteins which limit flexibility (Tang et al., 2006). Therefore, modification of hemp seed proteins would be necessary to enhance some functional properties and make it more attractive as an ingredient in the food industry (Tang et al., 2006).

2.5. Enzymatic hydrolysis of proteins and hemp seed proteins

Physical, chemical and enzymatic treatments have been used in the past to modify the functional properties of plant proteins, but enzymatic treatment is usually the most used method. Enzymatic hydrolysis methods are much better because they are milder, easy to control, and the nutrition of the native proteins is retained (Parrado et al., 1993). Enzymatic hydrolysis has, amongst many, the following advantages: it enables the embedded hydrophobic groups to be exposed which improve hydrophobicity of proteins, increases the number of ionizable groups, as well as decreases the molecular weight of

peptides (Panyam & Kilara, 1996). Chemical hydrolysis on the other hand is harsh; there might be production of toxic by-products as well as the partial destruction of essential amino acids. Previous studies indicate that the functional properties of food proteins can be improved through enzymatic hydrolysis (Mannheim & Cheryan, 1992; van der Van et al., 2002). However, functional properties of proteins are influenced by the specificity of the enzymes used, conditions of hydrolysis, as well as the nature of the parent protein (Mahmoud, 1994). Enzymatic hydrolysis is a means by which the functional properties as well as the potential health benefits of proteins can be modified or improved. Qi et al. (1997) were able to show that emulsifying activity index of soy protein isolate that was modified using pancreatin was better than that of soy protein isolate. Solubility of enzymatic hydrolysate of proteins from corn gluten meal was found to be better than the unhydrolysed proteins (Mannheim & Cheryan, 1997). Peptides that are obtained from enzymatic hydrolysis have been shown to exhibit some physiological activities such as antihypertensive activities as well as antioxidant activities. Results from the study carried out by Xueyan et al., (2010) indicated that zein, a byproduct of corn starch processing, when treated enzymatically yielded a protein hydrolysate with antioxidant activities such as scavenging activity against DPPH and superoxide anion (O_2^-). Membrane fraction of the protein hydrolysate of quinoa seed were shown to have the ability to inhibit the activity of ACE (Aluko & Monu, 2003).

Limited enzymatic hydrolysis could be used to improve functionality of hempseed proteins. However, there is scanty information on the use of enzymatic hydrolysis to improve functionality of hempseed proteins. Enzymatic hydrolysis can either be limited or extensive, depending on the degree of hydrolysis (DH) and nature of protein (Guan et

al., 2007; Henning et al., 1997). Guan et al., (2007) showed that enzymatic hydrolysis in a DH-dependent system increased the solubility, water-holding capacity, emulsifying activity and foaming ability of oat bran protein concentrate. Henning et al., (1997) showed that limited hydrolysis resulted in high emulsifying activity of faba bean (a legume), while extensive hydrolysis resulted in lower emulsifying properties. The use of trypsin in limited enzymatic hydrolysis of hemp protein isolate (HPI) resulted in decreases in its surface-related activities such as emulsifying and foaming properties, while increasing the protein solubility (Shou-Wei et al., 2008).

Alcalase is a non-specific proteolytic enzyme that can maintain activity and stability in organic solvents (Chen et al., 1992). Alcalase functions best between 45°C-65°C, with maximum activity at 60°C, above which activity falls rapidly while the optimum pH is between pH7-8. Alcalase, according to Humiski & Aluko (2007) has broader specificities as compared to trypsin as shown by the higher amounts of peptide fractions obtained after alcalase hydrolysis. Thermolysin on the other hand is a bacterial extracellular protease, which unlike majority of proteins that undergo conformational changes, remains stable under 70°C (Antonczak et al., 2000). Thermolysin specifically hydrolyzes peptide bonds on the amino side of bulky hydrophobic residues such as leucine, valine, phenyalanine (Antonczak et al., 2000). Pepsin is most efficient in cleaving peptide bonds between hydrophobic amino acids such as phenyalanine, tryrosine and tryptophan (Dunn, 2001). Pepsin functions best at an acidic environment of pH 1.5-2 (Dunn, 2001). Wang et al. (2008) showed that *in vitro* pepsin digestibility of hemp seed protein isolate (HPI) over time (1 to 120 min) were comparable to that of soy protein isolate.

2.6. Physicochemical properties of proteins.

2.6.1. Surface hydrophobicity S_0.

Many hydrophobic residues are buried in the interior of most native proteins; although some hydrophobic groups may be at the surface. When proteins are denatured, these hydrophobic groups are exposed at the molecular surface (Kato & Nakai, 1980). Heat treatment and hydrolysis affects surface hydrophobicity. Kato et al. (1983) on the other hand observed that emulsifying properties change, depending on the observed S_0 and not on heat. A study by Aluko & Yada, (1993) indicated a relationship between S_0 and other functional properties of proteins.

When the exposed hydrophobic groups of peptides are low, the degree of solubility will be higher due to decreased tendency to aggregate by hydrophobic interactions (Nielsen, 1997). Shimizu et al. (1986) showed that there was a little correlation between surface hydrophobicity and emulsifying activity. Adebiyi et al. (2007) were able to determine that increase in the S_0 of albumin and globulin of high-quality rice bran proteins may be due to the carbohydrate removal which exposed more hydrophobic amino acid residues. Tang et al. (2009) showed that the longer the hydrolysis time for Flavourzyme and Protamax, the higher the S_0. Contrary to this result, Wang et al, (2007) found that the shorter the time for Neutrase hydrolysis, the higher the S_0 value. In principle, there exists a relationship between water solubility and S_0; the higher the S_0, the lower the water solubility and this is due to protein tendency to aggregate by hydrophobic interactions. Wagner et al. (2000) found a trend contrary to this principle, in which the greater the S_0, the greater the solubility. This result was based on the conditions in which the soy protein isolates were prepared.

2.7. Functions of proteins in foods and food products.

2.7.1. Protein solubility (PS)

Solubility is one of the most important functional properties of proteins and it can subsequently affect other functional properties such as emulsification, foaming and gelation (Kinsella, 1976). Solubility is usually the first functional property determined during the testing of protein functional properties (Zayas, 1997). A protein usually has lowest solubility at its isoelectric point. Solubility of protein is dependent on the distribution of hydrophilic and hydrophobic amino acid found on the protein surface (Blow et al., 1969). Proteins with high hydrophobic amino acids on the surface have low solubility (Branden & Yooze, 1991). Proteins generally have low solubility in the acidic pH range and increase solubility at the alkaline pH range.

PS can be improved through enzymatic hydrolysis (Adler-Nissen, 1979), simultaneous addition of glutamate and arginine (charged amino acids) to the buffer (Golovanov et al., 2004), or by succinylation (Allaoua et al., 1998). For example, there was an increase in protein solubility after enzymatic hydrolysis of HPI (Chuan-He et al., 2006) (Shou-Wei, 2004). The increased solubility of whey protein hydrolysates was attributed to the smaller molecular size of the peptides when compared to the unhydrolyzed protein (Turgeon et al., 1992). Denaturation and aggregation have been reported to decrease solubility of most proteins such as whey proteins (Mutilangi, 1995). At low pH, "salting out" due to the addition of NaCl or $CaCl_2$ resulted in poor solubility of sweet potato proteins but as the pH increased, solubility of the sweet potato proteins was significantly improved ($P<0.05$). Allaoua et al. (1998) carried out both enzymatic and chemical modification by succinylation on soy protein isolate and they came to the

conclusion that succinylation enhances PS up to 100% at pH 5.0 and above. Succinylation was able to shift the isoelectric point from pH 5.0 to pH 4.0, which makes the succinylated protein a potential food ingredient for the fortification of acidic beverages.

Initial work done by Chuan-He et al. (2006), had compared HPI to soy isolate with respect to some functional properties such as solubility, emulsion and water holding capacity. In later work done by Shou-Wei et al. (2007), it was discovered that enzymatic hydrolysis with trypsin improved the PS of HPI but led to the marked decrease in emulsifying activity index, foaming capacity, water holding capacity and fat holding capacity. One of the aims of this study was to determine the functional properties of HPI after digestion with each of the following enzymes: alcalase, pepsin and thermolysin.

2.7.2. Fat absorption capacity (FAC)

Oil absorption is very important in food formulation and processing (Kanterewick et al., 1989). It basically involves adding excess oil to a protein powder, mixing thoroughly, centrifuging and then determining the amount of absorbed oil (Kanterewick et al., 1989). Proteins' ability to entrap oil depends on hydrophobicity, degree of hydrolysis, size of protein and the flexibility of the protein network (Tomotake et al., 2002). Decrease in FAC is attributed to enzymatic hydrolysis, thus the hydrophobic groups are buried in the interior, making them unavailable to bind oil (Shoui-Wei, 2008). Raw or unhydrolysed protein isolates have their protein network undisrupted by enzymatic hydrolysis, therefore oil can be entrapped in these networks and this would result in increase in FAC (Wang & Kinsella, 1976). Allaoua et al. (1998) reported that

FAC gradually decreased with increase in succinylation due to the increase in net charge and subsequent decrease in hydrophobicity. Paulson & Tung (1987) had similar results showing decrease in FAC of canola protein isolate as succinylation level increased. Allaoua et al. (1998) also showed that heat treatment at 80°C increased FAC due to unfolding of the protein structure and exposure of hydrophobic groups, which leads to the entrapment of oil. Guan et al. (2007) also had similar result showing that as degree of hydrolysis increased, there was a gradual decrease in FAC of trypsin modified oat bran protein concentrate. This may be due to increased charge and decrease size of hydrolyzed proteins, which would limit interactions with oil.

2.7.3. Water holding capacity (WHC)

The manner in which proteins behave in food systems is related to the degree of interaction with water. Water holding capacity can be defined as the ability of protein to hold water against gravity (Kinsella, 1979). Proteins with amino acids that have a polar side chain can interact with water through dipole-dipole interactions. Due to their insolubility in water, non-polar amino acids have very low interaction with water, while polar amino acids are conducive sites for protein-water interactions. (Chavan et al., 2001). Factors that affect water binding by food proteins include amino acid composition, protein conformation, surface hydrophobicity and temperature. According to Dinakar & Arun (1997), WHC is dependent on the nature of enzyme used in hydrolysis, heat treatment as well as pH during hydrolysis. Most studies show that there is no relationship between WHC and solubility (Ahmedna et al., 1999; Abugoch et al., 2008). Johnson &

Brekke (1983) showed that both acetylation and succinylation resulted in increase in WHC due to the unfolding of protein which increases the protein-water interactions.

The application of WHC in food industries is to maintain moistness and softness of bakery products. WHC is also very important in the meat industry, especially in production of meat analogs, because it affects texture, juiciness and taste. Also WHC of proteins is important in food companies that produce frozen desserts.

2.7.4. Gelation

A gel is a continuous network of macroscopic dimensions immersed in a liquid medium exhibiting no steady-state flow (Zegles et al., 1994). Gelation occurs when proteins form a 3-dimensional network that is resistant to flow under pressure (Boye et al., 2010). Most globular proteins form protein-protein interactions and subsequently gel when they undergo heating. For gelation to take place, proteins must attain a balance between attractive and repulsive forces (Paulson & Tung, 1989). The attractive forces are necessary to form the network and repulsive forces are necessary to prevent it from collapsing. According to Matsumura & Mori (1996), pH, presence of ionic species, enzymes, heating temperature and rate of heating are some factors that affect gel formation. Steps involved in heat induced gelation are protein unfolding, water binding, protein-protein interactions, and water immobilization (Mulvihill & Kinsella, 1987).

When a protein is heated, denaturation takes place and the protein molecules begin to unfold. Unfolding of protein molecule leads to greater interactions with water and a gel network can be formed if there is adequate level of protein-protein interactions. Apart from ionic and hydrophobic interactions that results in cross-linking, disulfide

bonds are also important in gel formation (Kohnhoist & Mangino, 1985). Heat is responsible for the protein to unfold as well as aggregation to take place resulting in gel formation.

Characteristics of the gel to be formed depend on the protein concentration (Severin & Xia, 2004), the type of protein as well as the conditions involved in gel formation (Mulvihill & Kinsella, 1987). When the amount of protein is low, gelation might not occur, but as the protein concentration increases, the tendency of gel formation increases. Enzymatic hydrolysis has different effects on the gelation properties of proteins. These effects may either increase or reduce gelation. These effects could be used as an advantage in food processing (Panyam & Kilara, 1996). For example, casein's ability to be heat-stable and not be denatured to form gel during heating, can be modified in cheese-making using hydrolysis, whereby the casein protein becomes coagulated in the presence of calcium. Poor gelation enables soy-protein hydrolysates to undergo heat processing without changing their flow properties (Panyam & Kilara, 1996).

2.7.5. Emulsifying property

An emulsion is basically a mixture of two or more immiscible liquids (McClements, 1999). Energy input through shaking or homogenizing is needed to initiate emulsion, but after awhile emulsion instability (coalescence) sets in and this leads to phase separation (McClements, 1999). Stabilization of emulsified droplets can be achieved by the addition of molecules that can be partially soluble in both phases. Emulsified droplets can be stabilized by the addition of emulsifiers, and these emulsifiers stabilize emulsion due to their possession of hydrophilic and hydrophobic groups (Hall,

1996). Hydrophobic proteins lower the interfacial tension, giving good correlations with emulsifying activity (Kato & Nakai, 1980). Increase in hydrophobicity can be as a result of enzymatic hydrolysis of proteins which results in the unfolding of the globular structure and this eventually leads to the enhancement in interactions with the oil droplets (Kato et al., 1980). Strong protein-protein interactions at the oil-water interface have been shown to be important and a requirement for emulsion stability (Halling, 1981). Proteins are good emulsifiers due to their amphiphilic nature, and during emulsion formation, they must first reach the oil/water interface and then unfold in order that the hydrophobic end can come into contact with the oil phase (Li-Chan & Nakai 1991). Their amphiphilic nature also causes a pronounced reduction of interfacial pressure and this improves emulsion formation (Kinsella et al., 1985). Previous studies have shown that protein solubility contributes to increase in emulsion formation due to factors such as protein-protein interactions and protein conformation (Kinsella et al 1985); though this might not be the general case (Aluko & McIntosh 2001). Molecules with crosslinks such as disulfide bonds are more rigid and cannot unfold, thereby they cannot form emulsion properly (Tang et al., 2006).

Emulsion stability is the ability of a protein to form an emulsion and after a specific time, and at a given temperature, remains unchanged (Panyam & Kilara, 1996). Ability of protein to aid the formation and stabilization of emulsions is essential for many food applications including mayonnaise and frozen desserts, as well as emulsifiers in soups and sauces.

Jayasena et al. (2010) reported that at isoelectric point (pH 4.5), emulsion activity of Lupin protein isolate was low, which could be due to the minimal solubility at

isoelectric point, and this poor solubility state of the protein affects its ability to orient and act as an effective emulsifier. Soluble proteins increase the rate of oil droplet entrapment and this could eventually lead to improved emulsifying properties (Onweluzo et al., 1995).

2.7.6. Foaming activity and stability

Foams are composed of air droplets surrounded or encapsulated by a thin liquid film. Foams are formed from either whipping or shaking and are thermodynamically unstable (Dickinson & Stainsby., 1989). They are biphasic colloidal mixtures with a continuous liquid and a dispersed gas phase (Panyam & Kilara 1996). For example, egg white and whey proteins have been reported to have good foaming properties. Limited hydrolysis of proteins to produce peptides has been shown to improve foaming properties as shown by enzymatic hydrolysis of whey proteins (Keuhler & Stine 1974). Previous studies have shown that smaller peptides improved foaming property while larger peptides or unhydrolysed proteins are not as suitable for foaming activity to occur (Althouse et al., 1995; Keuhler & Stine, 1994; Aluko et al., 2009). The poor foaming properties of the unhydrolysed proteins and larger peptides are due to hydrophobic interactions or steric hindrance at the interface (Panyam & Kilara 1996), while the smaller peptide size enhances lowering of interfacial tension, which increases foaming abilities. A contrary result by Damodaran (1997) suggested that the larger the molecular size of the protein, the higher the foaming stability. Jayasena et al. (2010) showed results that suggest foaming capacity and foaming stability are dependent on pH; alkaline pH values improved foaming capacity and stability, while acidic pH or isoelectric point

lowers foaming capacity and stability. The improvement of foaming properties in alkaline condition is due to the increase in net charge and molecular flexibility, while in an isoelectric condition foaming properties is poor because the net charge is minimal and this makes the protein less soluble and less flexible, leading to increase in surface tension (Mahajan & Dua, 2002; Khalid et al., 2003). Aluko et al. (2009) showed that foaming capacity and foaming stability are not only dependent on pH, but also on protein concentration. Proteins are believed to be more effective than non-protein components in respect to foaming properties (Aluko et al. 2009), while Snyder & Kwon (1987) suggested that protein concentration is also a determining factor for foam capacity.

It has been suggested by Kinsella (1981) that rapid binding of proteins to the air-water interface increases foaming capacity of proteins, while the foaming stability is enhanced when proteins stay longer at the interface and are able to interact to form strong interfacial membranes around the air particle.

Similar to emulsions, proteins that are used to stabilize foams must reach the interface and then unfold to encapsulate the air particles (Dickinson, 1992). Mechanical disturbance and gravitational drainage can cause the air droplets to collide and rupture, and this will result in the smaller cells merging with the larger cells. Surface viscosity on the other hand will increase the foam stability by limiting kinetic properties of the foam particles, which reduces the propensity of droplet coalescence. Surface viscosity is as a result of the protein at the air-water interface being able to be rigid and also bind water tightly. Surface viscosity also may help to stop water drainage thereby making the film more stable (Hall, 1996). For films to be stable, they should possess sufficient mechanical strength and viscosity to prevent rupture and coalescence. Foaming stability can provide

desirable texture to many aerated foods such as ice-cream, whipped topping, bread, and cake (Dickinson, 1992).

2.8. Antihypertensive property of peptides

Previous works have shown that peptides obtained from extensive hydrolysis of food proteins have potential bioactive properties such as the inhibition of angiotensin converting enzyme activity (ACE) and scavenging of free radicals (Matsui et al., 1993; Kim et al., 2001; Wu & Ding, 2001). Synthetic ACE inhibitors such as Captopril and Enalapril have been used in the medical therapy of hypertension (Dong et al., 2007). However, antihypertensive peptides from food proteins are believed to be safer and have little or no adverse side effects as compared to synthetic ACE inhibitors. (Dong et al., 2007). Hence it is of importance to find and use safe therapeutic peptides from plant sources that have the ability to lower hypertension.

Another critical approach towards lowering and treatment of human hypertension is inhibition of renin activity. Renin is another enzyme that plays a major role in the renin-angiotensin systm (RAS). Renin is a mono-specific enzyme that catalyzes the rate-limiting step that converts angiotensinogen to angiotensin I, which is then acted upon by ACE to produce angiotensin II, a powerful vasoconstrictor. If renin activity is inhibited, the production of angiotensin I could be prevented; however, the inhibition of renin activity does not affect production of ACE (Staessen et al., 2006). According to Yuan et al. (2006) renin has a folded conformation that makes the active site not easily accessible to inhibitors, while ACE has an open conformation that aids accessibility to inhibitors.

This gives an explanation for the greater number of effective ACE inhibitors as shown in previous works, as compared to lesser number of reported effective renin inhibitors.

Reports from Natesh et al. (2003) suggest that renin inhibitors chelate the zinc atom that is responsible for catalyzing the conversion of angiotensinogen into angiotensin I. Yuan et al. (2007) showed that inhibitors such as sodium houttuyfonate analogs modified the enzyme protein structure of ACE such that the substrate (angiotensin I) cannot fit into the active site to initiate catalysis; or in a competitive way, thereby displacing the substrate from the active site. Renin inhibition occurs when non-polar chains of the inhibitory substance interact with the hydrophobic sites of renin, thereby disrupting the conformation that is needed for enzymatic activity to take place (Yuan et al., 2006)

Little work has been done on the inhibition of renin by peptides from enzymatic hydrolysis of food protein hydrolysates. Udenigwe et al. (2009b) investigated the kinetics of the inhibition of renin and ACE by flaxseed protein hydrolysate fractions. Results from this study showed that some of the flaxseed peptide fraction inhibited renin and ACE activities. This gives a better dual prospect in the lowering of blood pressure as compared to other peptides that can inhibit only ACE activity.

2.9. Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive molecules that contain electron-deficient oxygen atom. During the normal physiological and metabolic processes in living things, oxygen is reduced to oxygen derived free radicals such as superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals (Yu, 1994). Generation of ROS

could lead to overwhelming of the antioxidant defense system of the body. Cell proteins, lipids and carbohydrates are usually targets for ROS and this leads to physiological diseases (Campbell & Abdulla, 1995). The reactivity of ROS is due to the presence of unpaired valence electrons and oxidative stress is an accumulation of damages to the cell structure as a result of stress from the environment. Glutathione peroxidases, catalase, superoxide dismutases are enzymes that cells use as defense against ROS, and they are known as endogenous antioxidants (Kaul et al., 1993). Endogenous antioxidants include ascorbic acid, tocopherol (vitamin E) and glutathione (Wolfgang et al., 1997). An antioxidant is a molecule that is capable of slowing or preventing the oxidation of other molecules, thereby stopping the chain reaction that is set off by free radicals. It can also be defined as the body defense system that prevents free radical damage to cells of the body. The mechanisms involved in antioxidant activity include hydrogen and electron donation, metal chelation as well as reducing activity.

ROS are produced during a number of biological processes but mitochondrial respiration is one of the major sources (St. Pierre et al., 2002). Mitochondrial enzymes are known to generate ROS through the leakage of electrons in the electron-transport chain (Cadenas & Davies, 2000), that convert oxygen into superoxide anion (Muhammed et al., 2009). When electrons are unavailable or in limited quantity, ROS generation (especially superoxide) will be decreased. ROS target most proteins in the mitochondria but when they are in short supply, the high oxidative stress results in a lot of pathological conditions such as diabetes mellitus (Thorpe & Baynes,1996), cardiovascular complications (Tain , 2006), aging and hypertension (Chan et al., 2009).

2.10. Antioxidant activities of peptides

Peptides have been shown to have antioxidant activities in *vitro* (Hernandez-Ledesma et al., 2005), which is dependent on the degree of hydrolysis (peptide size, functional and chemical properties), and enzyme specificity (peptide sequence and size) (Tang et al., 2009; Megias et al., 2007). Peptides from food sources have been shown to possess different antioxidant activities depending on the ROS that is to be eliminated. Antioxidant reactions have different reaction mechanisms by which electrons are donated, radicals are scavenged, metals are chelated and reductions of ions are evaluated. Synthetic antioxidants have been shown to have potential health risks to the body while proteins and peptides from food sources could serve as natural antioxidants with less risk to human health (Stich, 1991). Some of these reported food protein-derived antioxidant peptides are shown in Table 2.10

Table 2.10 Proteins from various food sources and their antioxidant properties

Protein source	Antioxidant peptides	Antioxidant activities	Reference
Egg protein	Try-Ala-Glu-Glu-Arg-Try-Pro-IIe-leu	Radical scavenging activity, delay low-density lipoprotein lipid oxidation.	Davalos et al. (2004)
Whey protein	Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile	Radical scavenging activity	Hernandez-Ledesma et al. (2005)
Muscle protein	His-Tyr-Met	DPPH scavenging activity, metal ion chelating activity.	Saiga et al. (2003)
Potato protein	Try-Met-His-Lys-Cys	Ferric reducing activity, radical scavenging activity, Cu^{2+} chelating activity.	Wang & Xiong. (2005)
Maize zein protein	Try-Ala-Leu-Met-Cys-His	DPPH scavenging activity, superoxide radical scavenging activity.	Tang et al. (2010)
Soy protein	Pro-His-His	Ferric reducing activity, radical scavenging activity, peroxynitrite scavenging activity, inhibition of linoleic acid oxidation.	Moure et al. (2003)

The ability of antioxidants to carry out their functions could be based on findings from various studies and suggestions such as the presence of the phenolic ring in tyrosine, phenolic groups present in tryptophan and tyrosine residues (Hernandez-

Ledesma et al., 2005), specificity of proteases (Udenigwe et al., 2009a) molecular weight and hydrophobicity of the peptides (Tang et al., 2010). Peptides with small molecular size or smaller peptide fractions have been shown to have stronger antioxidant activity than larger peptide fractions (Aluko & Monu, 2003; Peng et al., 2009; Bougatef et al., 2009). Previous works also suggest that hydrolysis improves the antioxidant activity of proteins by the release of peptides that have antioxidant potential (Wang et al., 2005; Salami et al., 2010).

Saiga et al. (2003) worked on the effect of pH on the antioxidant activity of porcine myofibrillar protein hydrolysate. The antioxidant activity was examined at pH values of 5.4-7.8, and results showed that suppression of the production of hydroperoxides was better at pH 7.1 than at pH 5.4.

2.10.1. 1,1-diphenyl-2-picrylhdrazyl (DPPH) scavenging activity of bioactive peptides

DPPH is a synthetic free radical that has no physiological relevance but is generally used for evaluating scavenging activity of natural compounds. DPPH is a relatively stable free radical and the assay determines the ability of antioxidants to reduce the DPPH radical, which absorbs at 517 nm by converting the unpaired electrons to paired ones and resulting in decrease in absorption intensity (Verma et al., 2008). DPPH accepts an electron or hydrogen radical when it interacts with an antioxidant. The ability of DPPH radicals to be reduced by an antioxidant can be shown in the decrease in absorbance at 517 nm.

Several works have been done on DPPH scavenging activities. Lei et al. (2009) showed that 3 protein hydrolysate fractions had exceptional high antioxidant activity measured by DPPH scavenging activities. Further results suggested that the 3 kDa hydrolysate fraction obtained from alcalase hydrolysis of soybean proteins had a high antioxidant potential when evaluated with DPPH. Munir et al. (2003) observed that water and ethanol extracts of Fennel seed had strong DPPH scavenging activity, and this was dependent on increased concentration. Pownall and colleagues (2010) identified that pea protein fractions having strong hydrophobic character had the strongest DPPH scavenging activity.

DPPH radical scavenging activity has also been observed for peptides produced from enzymatic hydrolysis of flaxseed proteins. The results showed that peptides fractions exhibited better DPPH activity in acetate buffer than in phosphate and Tris-HCl buffers (Udenigwe et al., 2009a). Similar results showed that pH affects DPPH scavenging activity of peptides (Li et al., 2008).

Li et al. (2008) showed that low molecular weight fraction of chickpea protein hydrolysates had strong DPPH scavenging activity as well as contained high levels of hydrophobic amino acids. The results agrees with the study done by Pownall et al. (2010) that pea protein hydrolysates with the strongest DPPH scavenging activity contained slightly higher amounts of hydrophobic amino acids. Contrary to Li et al. (2008) results that show small molecular weight peptides having better scavenging activity, Udenigwe et al. (2009a) observed that the high molecular weight fractions of flaxseed peptides had a better DPPH scavenging activity. Due to the stability of DPPH radical, it is not similar to the highly reactive peroxly radicals that are involved in physiological systems (Huang

et al., 2005). This makes it apparent that other biologically relevant antioxidant assays should be used further to determine antioxidant properties of peptides that are being evaluated for bioactive properties.

2.10.2. Superoxide scavenging activity of bioactive peptides

When an electron is added to oxygen, a superoxide is formed.

O_2 + electron $\longrightarrow$ $O_2^{\cdot}$

Superoxide is regarded as a weakly reactive radical, but can nevertheless attack a number of biological cells (Key et al., 1994). It is the primary precursor for the formation of other radicals, and hydroxyl radicals are formed as a result of the activities of superoxide radicals. Superoxide is removed from cells by superoxide dismutase (SOD), an important enzyme-based antioxidant defense mechanism mainly found in intracellular cells (McCord & Fridovich, 1969), but can also be found in extracellular cells. Pownall et al. (2010) reported that pea protein hydrolysates had weak superoxide scavenging activity when compared to the fractionated pea protein peptides and glutathione. This is believed to be due to the presence of inactive peptides that dilute the activity of active peptides; fractionation reduced the amount of the inactive peptides. Previous work showed that high superoxide scavenging activity was associated with amino acids that had hydrophobic characteristics (Li et al., 2008). Tang et al. (2010) also demonstrated similar results; low molecular weight peptides of alcalase treated zein hydrolysates having high amount of hydrophobic amino acids exhibited high superoxide scavenging activity.

2.10.3. Metal chelating activity of bioactive peptides.

Metal ions play a vital role in various biological processes that take place in the human body. Though they are trapped and transported into the body through controlled processes, there could be situations where there may be leakage of metal ions into the surrounding environment. Presence of free metal ions could lead to generation of free radicals which would eventually lead to lipid peroxidation and oxidative stress (Aruoma et al., 1989). When iron in the body is not well managed, it could lead to excess accumulation and cause toxicity in body organs especially the liver. Excess of iron in the body subsequently leads to production of free iron radicals that contribute to oxidative stress.

Through the Fenton reaction, highly reactive hydroxyl radical can be generated and metal chelating is very vital to prevent this reaction. The Fenton reaction involves the oxidation of ferrous ion (Fe^{2+}) to ferric (Fe^{3+}) resulting in the production of hydroxyl radical ($OH^{\cdot}$).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Chelation of metal ions would, in the long run, contribute to decreased formation of hydroxyl radical due to unavailability or reduction of the amount of free metal ions that would have been used in the Fenton reaction (Megias et al., 2008).

Saiga et al. (2003) and Pownall et al. (2010) had results that agreed with Dong et al. (2008) that showed a relationship between hydrophobicity and metal chelating activity; increase in hydrophobicity of the hydrolysates resulted in higher metal chelating activity.

2.10.4. Reducing power of bioactive peptides

Bioactive peptides act as reducing agents when they donate electrons to unstable compounds to form more stable ones. The reducing power of bioactive peptides is associated with their antioxidant activity (Huang et al., 2004). The reducing power assay is based on a redox reaction where ferricyanide (Fe^{3+}) complex is reduced to its ferrous (Fe^{2+}) form and simultaneous oxidation of the antioxidant (reductant).

Hemp protein hydrolysates obtained by neutrase was shown to have a significant reducing power (Wang et al., 2009). Results from the reducing power assay activity conducted by Jianping et al. (2010) on Alaska pollack skin did not have as much reducing activity as glutathione. Loach protein hydrolyzed by protamex exhibited increasing reducing power with an increase in degree of hydrolysis, although Loach proteins hydrolyzed by papain had a stronger reducing power (You et al., 2009). It has been reported that apart from peptide and amino acid composition, concentration of the peptides also plays a major role in ferric reducing power (Baohua & Youling, 2006). Munir and colleagues (2003) showed that increase in concentration of fennel seed extracts resulted in increased ferric reducing capacity.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

Defatted hempseed protein powder (HPP) was purchased from Manitoba Harvest Fresh Hemp Foods Limited (Winnipeg, MB, Canada). Alcalase (protease from *Bacillus licheniformis*), thermolysin (from *Bacillus thermoproteolyticus rokko*), pepsin (from porcine gastric mucosa), ACE from rabbit lung (E.C. 3.4.15.1) and N-(3-[2-furyl]acryloyl)-phenylalanylglycyl-glycine (FAPGG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The ultrafiltration membranes of 1, 3, and 5 kDa molecular weight cut-off (MWCO), and other analytical grade reagents were purchased from Fisher Scientific (Oakville, ON, Canada).

3.2. Preparation of hemp protein isolate

Hempseed protein isolate (HPI) was produced from HPP according to the method of Tang et al. (2006), with slight modifications. HPP was dispersed in de-ionized water (1:20, w/v) and the dispersion was adjusted to pH 10.0 using 2 M NaOH while stirring for 2 h at 37°C. The mixture was centrifuged at 10,000xg for 30 min at 10°C, the supernatant adjusted to pH 5.0 by adding 2 M HCl and then centrifuged at 10,000xg for 20 min at 10°C. The resulting precipitate was dispersed in water, adjusted to pH 7.0 using 2 M NaOH and freeze-dried to obtain the protein isolate.

3.3. Determination of protein content

Protein content of the protein isolate, hydrolysates and permeates were determined using the Lowry's method (Lowry et al. 1951) as modified by Markwell et al. (1978)

The hemp protein powder sample (10 mg/ml) and bovine serum albumin (BSA) (10 mg/ml) which is the standard were separately dissolved in water and mixed thoroughly using a magnetic stirrer. An aliquot (1 ml) of the samples and the standard were prepared in 20-100 µg concentration range. A volume of 3 ml of reagent C was added to 1 ml each of the samples and 1 ml each of the BSA, allowed to stand and incubate for 1 h at room temperature. Reagent C consists of reagent A and reagent B. Reagent A consists of 2% Na_2Co_3, 0.4% NaOH, 0.16% Sodium tartrate and 1% Sodium dodecyl sulfate. Reagent B consists of 4% $CuSO_45H_2O$ dissolved in distilled water. After the 1 h incubation, 0.3 ml of Folin Ciocalteu phenol reagent (1 part Folin Ciocalteu reagent with 1 part distilled water) was added to the samples and thoroughly mixed using a vortex. The samples were incubated for 45 min at room temperature. Using a spectrophotometer, the absorbance was measured at 660 nm. Triplicate determinations were used to calculate the protein concentrations.

3.4. Enzymatic hydrolysis of HPI to produce hempseed protein hydrolysates

3.4.1. Limited enzymatic hydrolysis of HPI

Limited enzymatic hydrolysis of HPI was carried out using the method of Aluko & McIntosh (2004). The HPI was dispersed in water (2%, w/v), and was adjusted to pH 9.0 using 1 M NaOH solution for alcalase and thermolysin while pH 2.0 was used for

pepsin digestion. The dispersion was heated to 60°C under continuous stirring on a hotplate equipped with an electronic thermometer. The enzymes (0.5% w/w) were added based on the protein content of the HPI and incubated at constant temperature of 60°C for 2.5 or 10 min. An un-hydrolyzed control for each time was prepared by omitting the enzymes during thermal incubation of the HPI. The reaction mixture was maintained at pH 9.0 using 1 M NaOH solution or pH 2.0 with 1 M HCl. At the end of the incubation period, the hydrolysates were transferred into a boiling water bath for 5 min to inactivate the enzymes. The hydrolysates were cooled to room temperature (22±2 °C) and adjusted to pH 7.0 with 1 M HCl solution (for alcalase and thermolysin digests) or 1 M NaOH (for pepsin digest), freeze-dried and the protein content was determined using the modified Lowry method.

3.4.2. Extensive hydrolysis of hempseed isolate.

Extensive hydrolysis was carried out according to the method of Aluko & Monu (2003). A 5% (w/v, protein basis) slurry of the protein isolate was prepared in distilled water and adjusted to pH 9.0 using 2 M NaOH. The slurry was heated at 50°C and 4% (w/v, protein basis) of alcalase or thermolysin was added under gentle stirring. The digestion was carried out for 4 h at 50°C, while reaction mixture was maintained at pH 9.0 by addition of 2 M NaOH when necessary. After digestion, the enzyme reaction was stopped by adjusting to pH 4.0 using 2 M HCl. The mixture was then cooled to room temperature, centrifuged at 10,000 x g for 15 min and the supernatant was freeze dried. The supernatant was reconstituted with distilled water and was passed through a 1 kDa molecular weight cut-off membrane. The retentate was then passed through a 3 kDa

membrane; similarly retentate from 3 kDa was passed through 5 kDa membrane. Permeates from the membranes were freeze-dried and their protein contents determined by the modified Lowry method.

3.5. Determination of physicochemical properties of hempseed hydrolysate.

3.5.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis was determined using the method by Aluko & McIntosh (2001). The samples were prepared for non-reduced sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by mixing the protein isolate with Tris-HCl buffer solution (pH 8.0) containing 10% SDS and 0.01% Bromophenol blue. The sample was placed in boiling water for 5 min and cooled to room temperature, then centrifuged at 16000 x g for 10 min. An aliquot (1 µL) of the supernatant was then loaded onto the 8-25% gradient gel. Reduced samples were prepared by adding 5% (v/v) 2-mercaptoethanol (ME) to an aliquot of the supernatant from 10% SDS samples, and 1 µL was then loaded onto the gel. Polypeptide separation and staining were carried out using the Phastsystem Separation and Development electrophoresis unit (GE Healthsciences, Montreal).

3.5.2. Surface hydrophobicity (S_0)

S_0 was determined using the method by Kato & Nakai (1980) with some modifications. Protein dispersions of 1 mg/mL were prepared in 0.01 M phosphate buffer (pH 7.0). The solution was stirred for 1 h at room temperature and then centrifuged at 10000xg for 20 min. Lowry modified method was used to determine the protein

concentration in the supernatant. Each supernatant was serially diluted with the same buffer to obtain protein concentrations ranging from 0.35 to 2.8 mg/mL. An aliquot (4 mL) of each diluted sample was added to 40 µL of aniline-8-naphthalene sulfonate (ANS). Fluorescence intensity (FI) was measured at excitation wavelength of 390 nm and emission wavelength of 470 nm. To calculate for the index of S_0, the initial slope of a plot of FI versus protein concentration was calculated.

3.6. Determination of functional properties

3.6.1. Protein solubility (PS)

The method by Shou-Wei et al. (2008) was used and modified as follows. An aqueous solution (1% w/v) of protein sample was stirred for 30 min. With either 0.5 M HCl or 0.5 M NaOH, each solution was adjusted to the desired values (pH 3.0-8.0). The solution was centrifuged at 10000xg for 20 min. Modified Lowry method was used to determine the protein content using bovine serum albumin (BSA) as the standard. Percentage PS was expressed as: (protein content of each sample / total protein content) x 100. All determinations were carried out in triplicates.

3.6.2. Emulsifying properties

Emulsifying activities was determined using the method of Aluko et al. (2001). Five different protein concentrations were used: 50, 75, 125, 250 and 500 mg aqueous dispersion of the protein samples were prepared in 0.01 M sodium phosphate solution, pH 7.0. The emulsion was prepared by adding 1 mL of pure canola oil and 5 mL of 0.01 M sodium phosphate solution of pH 7.0 to the protein hydrolysates, followed by

homogenization for 1 min at 20,000 rpm, repeating the homogenization again after 10 sec interval on a Polytron P T 3100 homogenizer (Kinematica AG, Lucerne, Switzerland). The mean oil droplet size ($d_{3,2}$) and specific surface area (m^2/ml) of the emulsion were determined in a Mastersizer 2000 (Malvern Instruments Ltd., Malvern, U.K) with Milli-Q water as dispersant. The emulsion was prepared in duplicate sample with two Mastersizer measurements for the sample.

3.6.3. Foaming activity and stability

Foaming capacity and foam stability was determined by the method of Fernandez & Macarulla (1997) with minor modifications. Aliquots (10 mL) of protein solutions (1%, w/v) at pH 7.0 in measuring cylinder (25 mL) were homogenized in a high speed Polytron P T 3100 homogenizer (Kinematica AG, Lucerne, Switzerland), at 10,000 rpm for 2 min. Foaming capacity was calculated as the percentage of increase in volume dispersion upon mixing. Foaming stability was an estimation of the percentage of foam remaining after 30 min.

3.6.4. Gelation

Gelation property was determined by the method of Obatolu & Cole (2000) with slight modifications. Aliquots (5 mL) of each protein sample was suspended in distilled water adjusted to pH 7.0 and protein concentrations ranging from 2 to 20% (w/v) with increment of 2%. The test tubes containing the suspensions were heated for 1 h in a boiling water bath and followed by rapid cooling under cold running tap water, after which the tubes were inverted to test for gelation. The test tubes were further cooled at

4ºC for 2 h, and gelation was again tested for the various protein concentrations. Gelation was determined when the sample from the inverted test tube does not fall or slip.

3.6.5. Water holding capacity (WHC)

Water holding capacity (WHC) was determined according to the method by Tomotake et al. (2002) with minor modifications. Each protein sample (1g) was weighed into 25 mL pre-weighed centrifuge tubes. Distilled water (10 ml) was added to each sample in small increments to a series of tubes that were under continuous stirring using a glass rod. After the mixture was thoroughly wet, the samples were centrifuged at 7000 x g for 20 min. After centrifugation the supernatant was decanted and the weight recorded.

WHC (which is grams of water per gram of protein) was calculated as:

$WHC= (W_2-W_1)/W_0$

Where:

W_0 = weight of the dry sample (g)

W_1 = weight of the test tube and dry sample (g)

W_2= weight of the test tube and the paste (g)

Each sample was determined in triplicate (g)

3.6.6. Oil holding capacity or fat absorption capacity (FAC)

Fat absorption capacity was also determined using the method by Tomotake et al. (2002). Each sample (1g) was weighed into 25 mL pre-weighed centrifuge tubes, and then thoroughly mixed with 10 mL of canola oil. The mixture was centrifuged for 20 min

at 7000 x g. After centrifugation, the supernatant was carefully removed and the tubes weighed.

FAC (gram of oil per gram of protein) was calculated as:

$FAC= (F_2-F_1)/F_0$

Where:

F_0 = weight of the dry sample (g)

F_1 =weight of the tube plus the dry sample (g)

F_2 = weight of the tube plus the sediment (g)

Each sample was determined in triplicate

3.7. Determination of bioactive properties

3.7.1. Angiotensin converting enzyme (ACE) inhibition activity

The inhibition of angiotensin converting enzyme was carried out according to the method of Holmquist et al. (1979) as reported by Li et al. (2008). N-(3-[2-furyl]acryloyl)-phenylalanylglycyl-glycine (FAPGG) was used as the substrate. An aliquot (1 ml) of FAPGG (0.5 mM, dissolved in 50 mM Tris-HCl buffer containing 0.3 mM NaCl, pH 7.5) was mixed with 20 µL of ACE (1 U/ml; final activity of 20 mU), 200 µL of sample solutions in the 50 mM Tris-HCl buffer and absorbance readings taken at 345 nm. Cleavage of the Phe-Gly peptide bond results in a decrease in absorbance, and this was recorded every 2 min at room temperature. For the blank, Tris-HCl was used instead of peptide solution.

ACE inhibition was calculated using the equation:

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

Where:

$A \,.\, min^{-1}_{(sample)}$ = Reaction rate in the presence of peptide.

$A \,.\, min^{-1}_{(blank)}$ = Reaction rate in the absence of peptide.

3.7.2. Renin inhibition activity

The ability of peptide samples to inhibit renin activity was measured according to the method of Yuan et al. (2006) using the Renin Inhibitor Screening Assay Kit. Total assay volume of 190 µL contained 10 µM Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Leu-Val-ile-His-Thr-Lys(Dabcyl)-Arg (renin substrate dissolved in dimethyl sulphoxide), human recombinant renin and hempseed peptides in 50 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl. For the blank experiment, Tris-HCl buffer was used instead of the hempseed peptide sample solution. The renin substrate was mixed with the hempseed peptide sample and pre-warmed to 37 °C for 10 min to attain thermal equilibrium. The reaction was initiated by adding the human recombinant renin to the mixture and the increase in fluorescence intensity was monitored for 10 min in a thermostated Spectra Max Gemini Fluoresence Microplate Reader spectrotofluorimeter (Molecular Devices Sunnyvale, CA). The spectrofluorimeter was set at excitation and emission wavelengths of 340 and 490 nm respectively, while excitation and emission bandwidth were set at 5 and 10 nm, respectively. The enzyme activity was expressed as reaction rate, arbitrary fluorescence intensity unit per minute ($FIU.min^{-1}$).

Renin inhibitory activity of the hempseed peptide permeates was calculated using the equation:

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

Where:

FIU . $min^{-1}_{(blank)}$ = Renin reaction rates in the absence of the hempseed peptide

FIU . $min^{-1}_{(sample)}$ = Renin reaction rates in the presence of the hempseed peptide

3.8. Determination of antioxidant activities

3.8.1. DPPH radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical activity was determined according to the modified methods of Hou et al. (2001) and Aluko & Monu (2003). The peptide samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0. To make a concentration of 100 µM DPPH, 4 ml of DPPH was dissolved in 36 ml of methanol. Using a 96-well clear flat bottom plate, peptide samples (100 µL) were mixed with 100 µL of the DPPH solution, blank control consisting of DPPH solution and sodium phosphate buffer as well as a standard consisting of glutathione (1 mg/ml). After standing in the dark for 30 min, the absorbance was read at 517 nm. When the DPPH radical is reduced as a result of accepting an electron from a donating compound, there is loss of color of the solution. The samples, blank as well as the standard were done in triplicates. Glutathione was used as a standard, so the scavenging activities of the peptides were compared to its activity.

The percentage scavenging activity was determined using the equation:

DPPH Radical Scavenging Activity (%) =

$$\frac{\text{(Absorbance 517nm Control – Absorbance 517nm Sample)}}{\text{Absorbance 517nm Control}} \times 100$$

3.8.2. Superoxide scavenging activity

The superoxide scavenging activity methods described by Gao et al. (1998) and Marklund & Marklund (1974) were used. An aliquot (80 µL) of the protein sample or glutathione, both at a concentration of 1 mg/ml were mixed with 80 µL of 50 mM Tris-HCL buffer (pH 8.3) containing 1 mM EDTA directly into a clear bottom 96-well plate in the dark. Pyrogallol (1.5 mM) was dissolved in 10 mM HCl and 40 uL added to each well. For control, Tris-HCl buffer was used. Absorbance was measured at 420 nm for 4 min at room temperature.

Superoxide Scavenging Activity (%) =

$$\frac{\text{(}\Delta\text{ A420 nm/min control- }\Delta\text{ A420 nm/min sample)}}{\Delta\text{ A420 nm/min control}} \times 100\%$$

3.8.3. Metal ion chelating activity

Metal chelating activity of the peptide samples were determined by using the modified method of Xie et al. (2008). Sample peptide solution or glutathione (1 ml) was combined with 0.05 ml of 2 mM iron dichloride solution dissolved in 1.85 ml double distilled water. FerroZine (3-(2- Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt) solution (0.1 ml of 5 mM) was added and mixed vigorously using a vortex machine. The mixture was allowed to stand at room temperature for 10 min. An aliquot (200 uL) was measured out into the clear bottom 96-well plate. A control blank

was run alongside the samples; every other reagent was present except the peptide samples and the absorbance was read at 562 nm. The metal chelating activities of the samples were compared to that of the glutathione.

Metal Chelating Activity was calculated by the equation:

Chelating Activity (%) =

$$\frac{(\text{Absorbance 562 nm Control} - \text{Absorbance 562 nm Sample})}{\text{Absorbance 562 nm Control}} \times 100\%$$

3.8.4. Ferric-reducing absorbance property

The reducing power of the peptide samples was determined according to the modified method of Zhang et al. (2008). The peptide samples and glutathione (250 µL) were dissolved in phosphate buffer followed by addition of 250 µL of phosphate buffer, pH 6.6 and 250 µL of 1% potassium ferricyanide solution. The mixture was thoroughly mixed using a vortex machine and heated at 50°C for 20 min. After incubation, 250 µl of 10% trichloroacetic acid (TCA) (10% in double distilled water) was added followed by 50 µL of 0.1% ferric chloride dissolved in double distilled water and 200 µL of double distilled water. The solution was allowed to stand for 10 min at room temperature, after which it was centrifuged at 1000 x g for 10 min. An aliquot (200 µL) of the supernatant was transferred to a clear bottom 96-well plate and absorbance was measured at 700 nm.

3.9. Data and statistical analysis

All analyses were carried out in triplicates and the results analyzed using ANOVA. Differences between mean values were analyzed using Duncan's multiple range test and significant differences were taken at $p<0.05$. Data are reported as mean ± standard deviation.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1. The protein yield for hemp seed protein powder (HPP)

Extraction of 90.00g of HPP yielded 18.96g of hempseed protein isolate (HPI) with a protein content of 86%, which translates to a percentage protein yield of 21.06%. The HPI had a dark green color and washing of the protein precipitate in acidified water did not reduce the green color, which was due to the presence of chlorophyll.

4.2. Percentage protein content for limited hydrolysis

The percentage protein content for control for 2.5 min and 10 min hydrolysis are 89% and 63% respectively. Protein content after hydrolysis in 2.5 min and 10 min for alcalase HPH, are 78% and 79% respectively, for pepsin HPH are 82% and 74% respectively and for thermolysin HPH are 96% and 80% respectively (Table 4.2.1). The dark green colour of the HPI was still retained after limited hydrolysis.

Table 4.2.1. Percentage protein content of HPH prepared with various enzymes for 2.5 min hydrolysates and 10 min hydrolysates

HPH	**Percentage protein (%)**	
	2.5 min	**10 min**
Alcalase treated HPH	78%	79%
Pepsin treated HPH	82%	74%
Thermolysin treated HPH	96%	80%
Unhydrolyzed (control)	89%	63%

4.3 Physicochemical properties of HPH

4.3.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE profiles of HPI and HPHs (Alcalase treated, pepsin treated and thermolysin treated), in the presence and absence of reducing agent β-mercaptoethanol (2-ME) were able to show various molecular weights (MW), depending on the enzyme used, as well as the time of hydrolysis at both times.

Looking at time difference in Figure 4.3.1, it could be observed that the molecular weight (MW) distribution of the peptides decreased with the increase in time from 2.5 minutes hydrolysis to 10 minutes hydrolysis in the HPI-thermolysin hydrolysate (lane 10), HPI-pepsin hydrolysate (lane 11), and HPI-alcalase hydrolysate (lane 12). The HPI-alcalase hydrolysate (lane 12) experienced significant hydrolysis, as most of the bands were no more visible as compared to the other lanes.

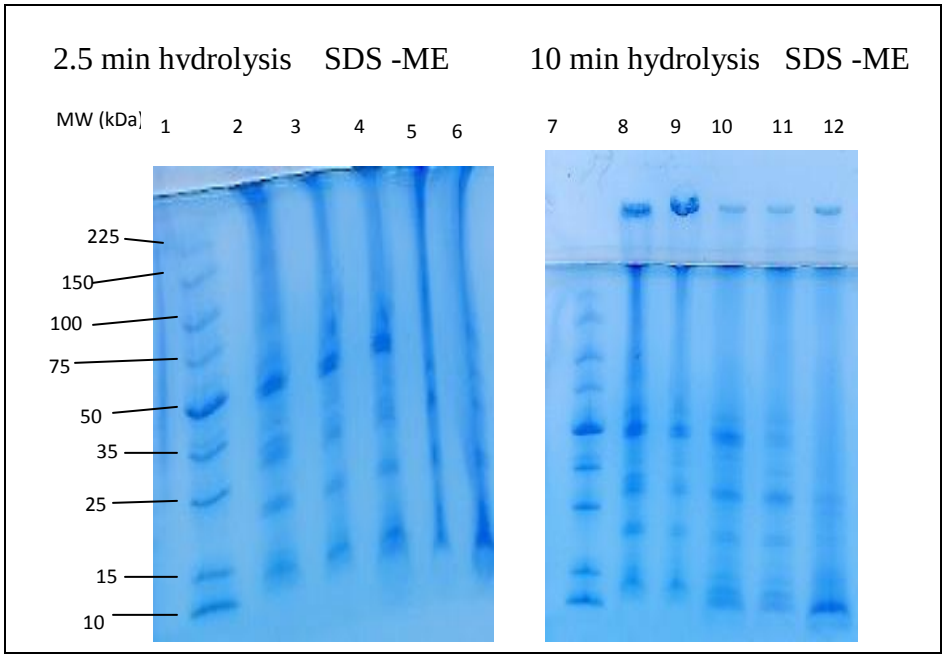


Figure 4.3.1 SDS-PAGE patterns of HPI and HPHs for 2.5 and 10 minutes hydrolysis in the absence of 2-mercaptoethanol. Lanes 1and 7, standard; lanes 2 and 8, HPI; lanes 3 and 9, controls for 2.5min and 10 min respectively; lanes 4 and 10, HPI thermolysin-hydrolysate; lanes 5 and 11, HPI pepsin-hydrolysate; lanes 6 and and 12, HPI alcalase-hydrolysate.

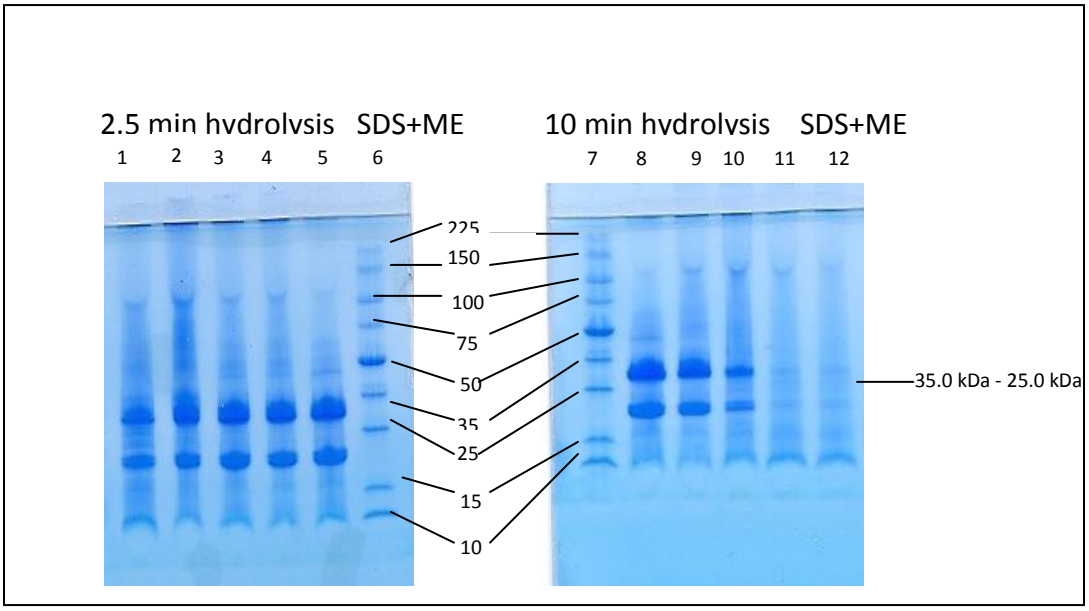


Figure 4.3.1.1 SDS-PAGE patterns of HPI and HPHs for 2.5 and 10 minutes hydrolysis in the presence of 2-mercaptoethanol. Lanes 1 and 8, HPI; lanes 2 and 9, controls for 2.5min and 10 min respectively; lanes 3 and 10, HPI thermolysin-hydrolysate; lanes 4 and 11, HPI pepsin-hydrolysate; lanes 5 and 12, HPI alcalase-hydrolysate; lanes 6 and 7, standards.

In Figure 4.3.1.1 for 10 min hydrolysis, after the addition of 2-mercaptoethanol (ME), it was observed that the protein aggregates (25.0 kDa-35.0 kDa) in the pepsin hydrolysate (lane 11) and the alcalase hydrolysate (lane 12) were reduced, suggesting as did Aluko & McIntosh (2001) that this could indicate the prescence of disulfide bonds in the native protein molecules. The bands in the thermolysin hydrolysate (lane 10) were partially hydrolysed. Alcalase is again able to digest more of the protein bands as compared to the other enzymes. The results agree with the data obtained by Humiski & Aluko (2007), who showed that higher number of peptide fractions obtained by alcalase was due to their broader specificity and higher degree of hydrolysis.

Under non-reduced conditions, the HPI samples were observed to have a high amount of aggregates. This observation agrees with Tang et al. (2006) and Wang et al. (2008) and assumes the poor solubilty of HPI is largely due to the presence of disulfide bonds of edestin. Edestin as reported by Tang et al. (2006) has a molecular weight of about 20.0-33.0 kDa, and it was also observed by Wang et al. (2008). After 10 min hydroysis, the hydrolysates from pepsin and alcalase digestion have less aggregates within the range of 20.0-30.0 kDa, suggesting that the enzyme actions led to size reduction. Upon addition of ME, most of the bands were reduced especially after the 10 min hydrolysis in lanes 11 and 12 (pepsin hydrolysate and alcalase hydrolysate respectively).

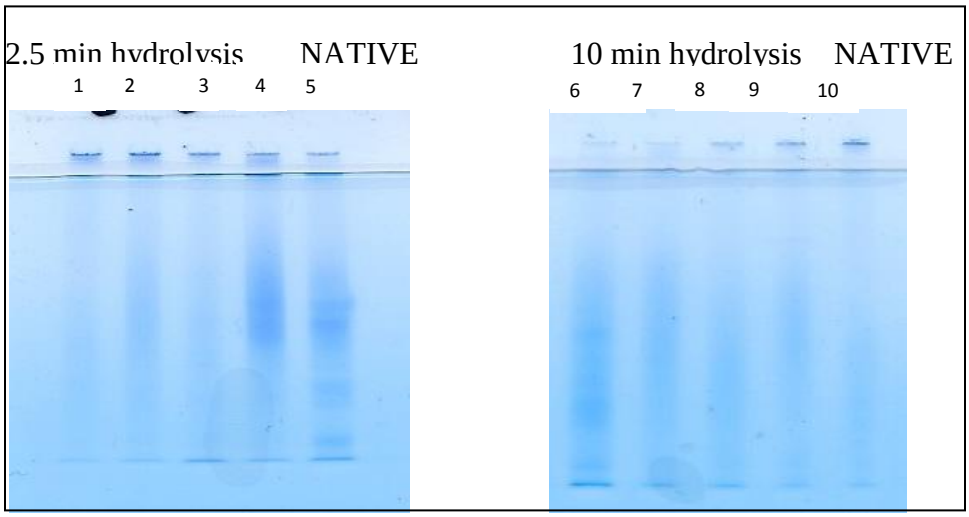


Figure 4.3.1.2.Native Gel Electrophoresis patterns of HPI and HPHs for 2.5 and 10 minutes. Lanes 1 and 10, HPI alcalase-hydrolysate; lanes 2 and 9, HPI pepsin-hydrolysate; lanes 3 and 8, HPI thermolysin-hydrolysate,; lanes 4 and 7, controls for 2.5min and 10 min respectively; lanes 5 and 6, HPI.

Figure 4.3.1.2, represents the Native PAGE for HPI and HPHs for 2.5 minutes and 10 minutes hydrolysis. Typical of native gels, the bands were not distinct because there was no denaturing of protein, but it can still be observed that alcalase (lanes 1 and 10) was able to hydrolyse most of the peptides.

4.4. Surface hydrophobicity (S_0)

The surface hydrophobicity of HPHs at 2.5 min and 10 min are shown in Table 4.4. Results showed that for thermolysin treated HPH and pepsin treated HPH, S_0 decreased with time from 2.5 min to 10 min. This is similar to the results obtained by Wang et al. (2009) which showed that for extensive hydrolysis time (60- 240 min), using Neutrase, there was a decrease in S_0 values. This may be due to the fact that the exposed hydrophobic peptides during extensive hydrolysis could form into soluble aggregates and this could lead to decrease in S_0 (Wang et al., 2009).

Table 4.4 Surface hydrophobicity (S_0 values) for hemp seed protein hydrolysates

	Surface Hydrophobicity (So values)	
HPH	**2.5 Minutes**	**10 Minutes**
Thermolysin-treated HPH	0.4050^{c}	0.0979^{b}
Pepsin-treated HPH	0.9747^{a}	0.0559^{c}
Alcalase-treated HPH	0.7011^{b}	1.3468^{a}

The S_0 for alcalase treated HPH increased from 2.5 min to 10 min; this agrees with the result that Tang et al. (2009) obtained which showed that longer hydrolysis

(4 h) significantly ($P< 0.05$) increased the S_0 of the hemp protein hydrolysates of flavourzyme and protamax. The possible explanation for this occurrence could be that the hydrolysis of alcalase treated HPI took place from the exterior to the interior of the protein molecules, thereby exposing the hydrophobic peptides and subsequently increasing the S_0 of the hydrolysate.

4.5 Functional properties of HPI and HPH

4.5.1 Protein Solubility (PS)

Solubility plays an important role in the functionality of proteins, as it affects other functional properties such as emulsion, gelation and foaming activities (Bora & Neto, 2004). Figure 4.5.1a and Figure 4.5.1b shows the protein solubility profile for HPI and its hydrolysates obtained at various pH values for 2.5 min and 10 min hydrolysis, respectively. The PS for HPI (untreated) was minimal at pH 4.0-5.0. There was a marked increase below pH 4.0 (73 %) as well as an increase (49 %) at above pH 8.0; these results are almost consistent with those reported by Tang et al. (2006).

For the 2.5 min hydrolysates as shown in figure 4.5.1a, enzymatic hydrolysis slightly improved the PS of pepsin treated HPH at pH 7 and thermolysin treated HPH at pH 3 and pH 4. The poor PS could be attributed to the short hydrolysis time (2.5 min) when compared to the 10 min hydrolysis as demonstrated by Shou-Wei et al. (2008). The PS of the hydrolysates was minimal at pH 4.0-6.0, increasing slightly above pH 7.0. The isoelectric point for the 2.5 min hydrolysates was around pH 5.0; this is probably where there are non-ionized forms of amino acids groups (Hassan et al., 2010). This could also

be attributed to protein-protein hydrophobic interaction which results in formation of aggregates, thereby decreasing solubility (Adebiyi et al., 2007).

For the 10 min enzymatic hydrolysis, PS of the HPH was remarkably improved at some pH values (Figure 4.5.1b). The hydrolysates showed different patterns of PS profile compared to PS of HPI. At pH 6.0, the PS of alcalase-HPH, pepsin-HPH and thermolysin-HPH were significantly higher than the HPI ($P<0.05$). Enzymatic hydrolysis over a longer period of time (10 min) improved the solubility of HPH. Protein solubility after 10 min hydrolysis was shown to be better than that of 2.5 min hydrolysis. The longer the time of hydrolysis, the higher the PS value of the hydrolysates. This improvement for the 10 minutes hydrolysates could be attributed to the release of soluble peptides from insoluble aggregates and increased number of exposed ionized amino acids and carboxyl groups (Panyam & Kilara, 1996).

Several studies have shown that most plant proteins have the lowest solubility at their isoelectric points, which is at the acidic pH values, while their solubility increases towards alkaline range (McWatters & Holmes, 1979). Alcalase-HPH from the 10 min hydrolysis had an improved PS value at pH 4.0-5.0 as compared to HPI at the same pH; this could indicate that alcalase-HPH could be used as a potential food ingredient for acidic beverages. This result agrees with Allaoua et al. (1998) who showed that the isoelectric point for soy protein hydrolysate was shifted from pH 5.0 to pH 4.0 when SPI was modified by succinylation. The enhanced solubility observed for the 10 min hydrolysates at pH 8.0 shows that they could be good for food applications.

Thermal heating (60°C) during the preparation of the hydrolysates as well as the termination of the experiment (100^0C) could contribute to aggregation and thermal denaturation (Shou-Wei et al., 2008). This can subsequently as indicated by Naina et al. (1997) lead to increase in molecular weight and decrease in solubility of (soy) protein.

Figure 4.5.1a Protein solubility profiles of HPI and its hydrolysates as a function of pH at 2.5 minutes hydrolysis.

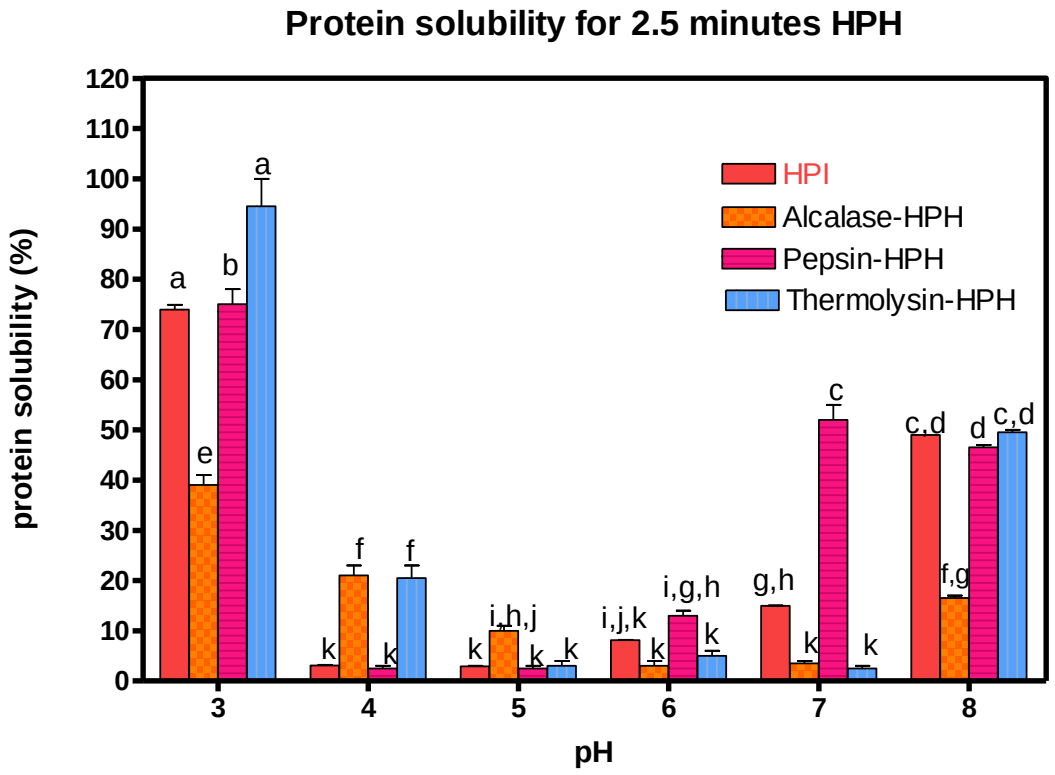
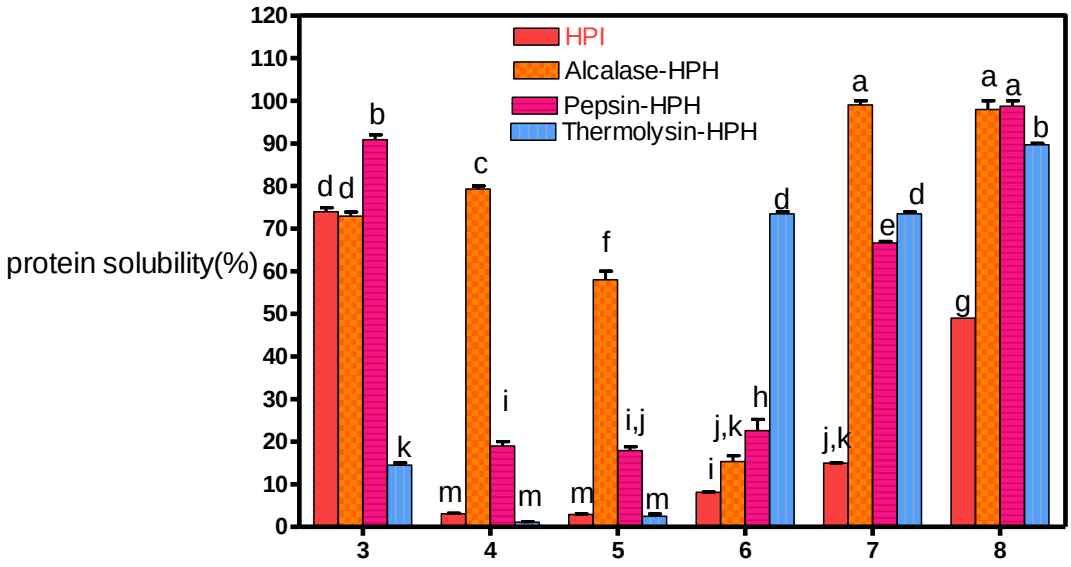


Figure 4.5.1b Protein solubility profiles of HPI and hydrolysates from 10 min enzyme hydrolysis as a function of pH.



4.5.2 Emulsifying properties.

Emulsifying properties are related to the aqueous solubility of proteins (Kinsella, 1976). Many studies have shown a positive correlation between solubility and emulsifying properties (McWatters & Holmes 1979; Lin & Zayas 1987). On the other hand, Tai-Hua et al. (2009) got contrary results to this effect, as they showed that PS and emulsion properties of sweet potato protein did not follow this trend. Various researchers have indicated that plant proteins have the lowest emulsifying properties around their isoelectric points (Aoki et al., 1980; Franzen & Kinsella 1976). Shou-Wei et al. (2008) had results that showed that HPI had low emulsion activity index at pH 5.0 and higher emulsion activity index at pH 7.0-9.0 According to findings by Prima-Hartley et al. (2000), emulsions with smaller droplet size are more stable than emulsions with bigger droplet size. It is of importance to produce an emulsion that is stable as this would be a very useful and important characteristic for use in the food industry. Therefore, the smaller the oil droplet size, the better and more stable the emulsion. According to Aluko et al. (2009), increase in sample concentration resulted in increase in emulsion ability. Comparing the effects of concentration on emulsion capacity (oil droplet size) using different enzymes in Figures 4.5.2a-4.5.2c, it was observed that the control (unhydrolysed) in Figure 4.5.2d had a more narrow range of oil droplet size of 0.1 – 10 µm as compared to the hydrolysed samples (Figures 4.5.2a-c) that had a broader range of 1 – 1000µm. The low oil droplet size of the unhydrolysed hemp protein indicates that hydrolysis had no effect on emulsion capacity. Past studies also suggest that hydrolysis breaks down proteins into smaller, shorter peptide that may not have strong protein-protein interaction that is necessary to form stable films around oil droplets; indicating

that emulsifying properties of proteins are decreased by hydrolysis (van der Ven et al., 2001; Aluko & Monu 2003; Mahajan & Dua, 1998). The effect of concentration was also observed in the various results as shown in Figures 4.5.2a-d. For each hydrolysate, an increase in concentration resulted in a decrease in oil droplet size, which invariably means better emulsions.

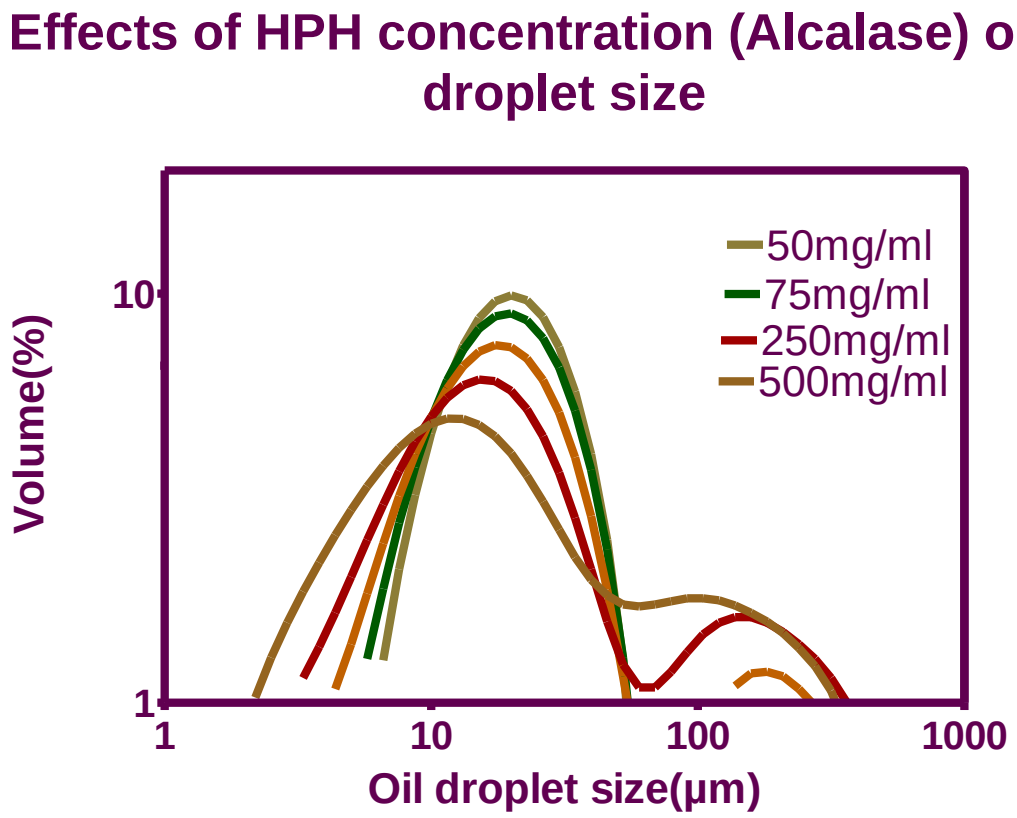


Figure 4.5.2a Effects of protein various concentrations on oil droplet size for alcalase treated hydrolysates

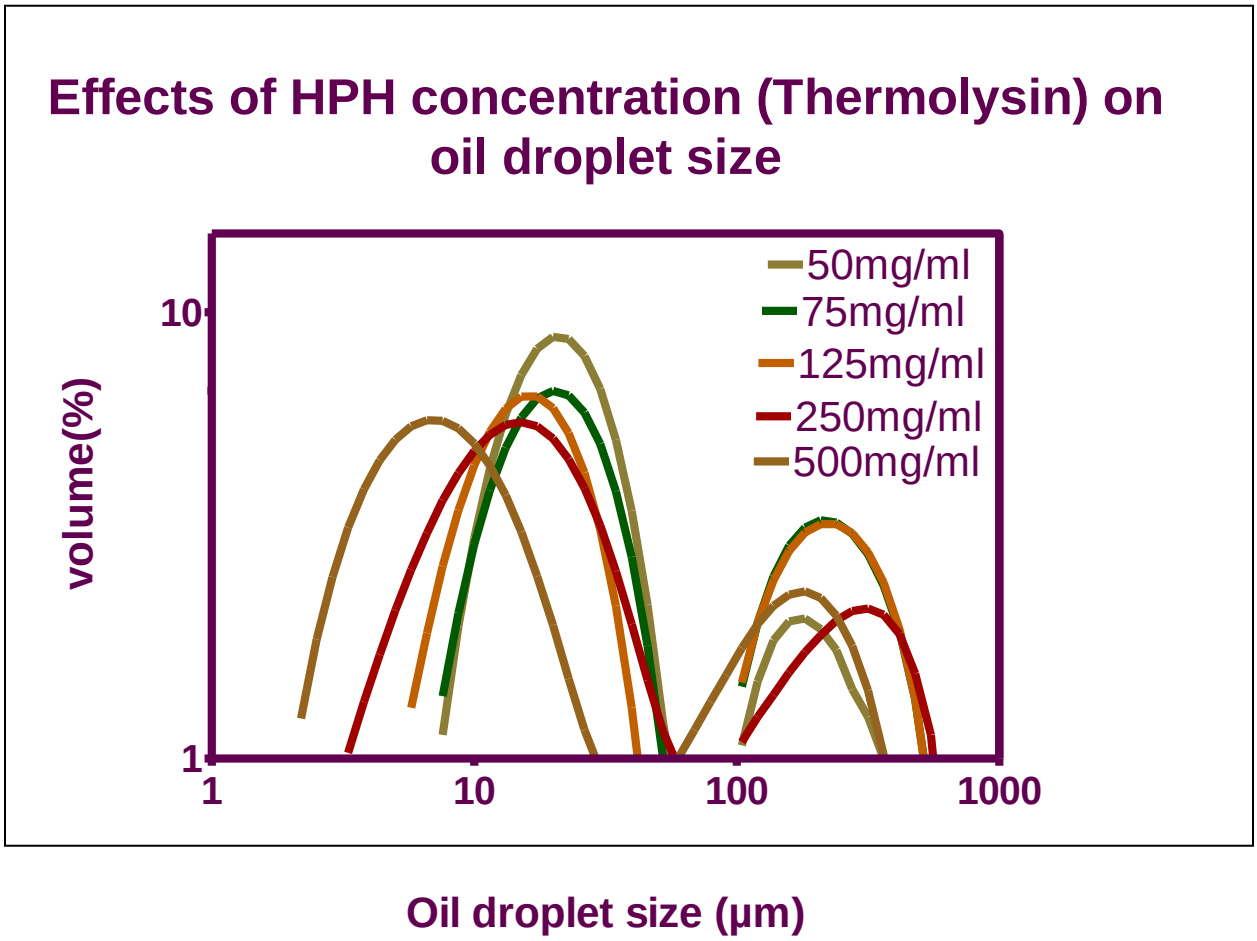


Figure 4.5.2b Effects of various concentrations on oil droplet size for thermolysin treated hydrolysates

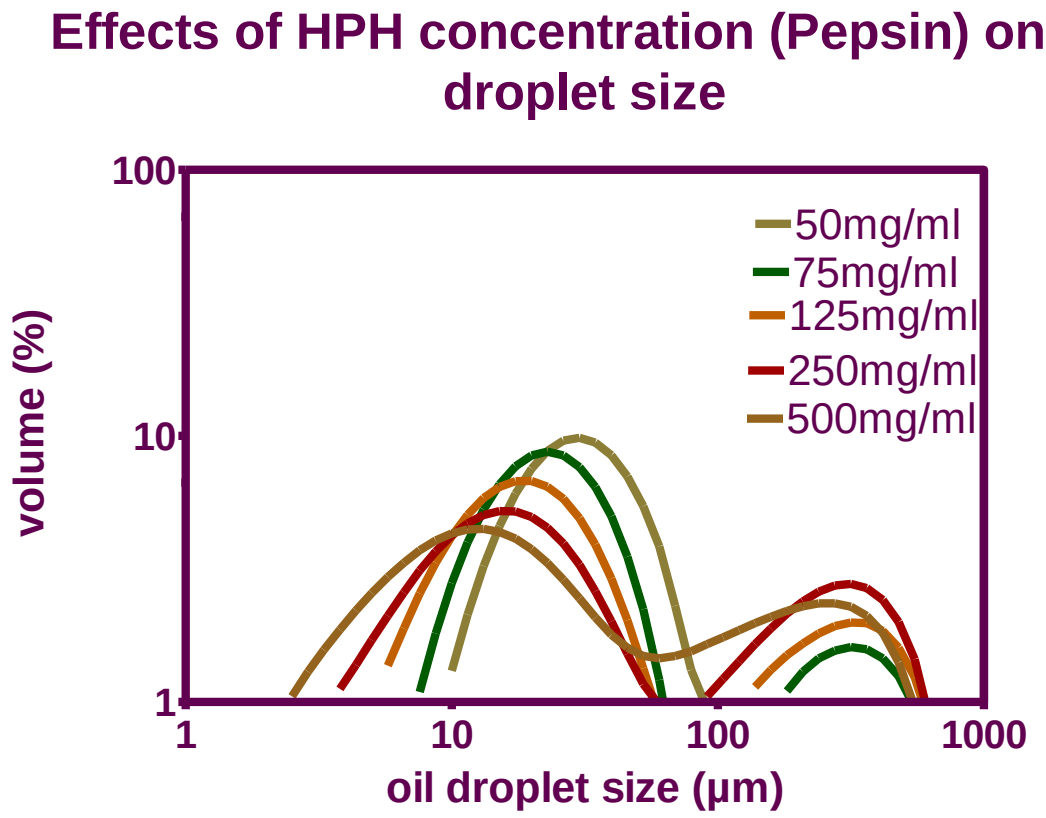


Figure 4.5.2c. Effects of various concentrations on oil droplet size for pepsin treated hydrolysates

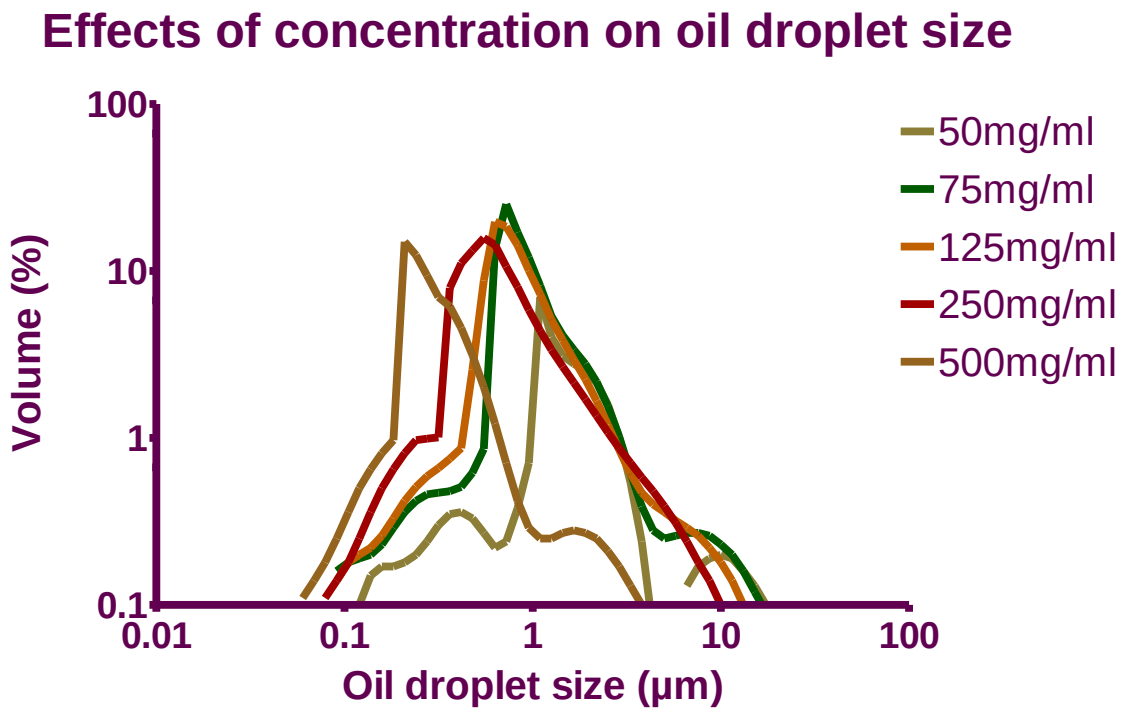


Figure 4.5.2d Effects of various concentrations on oil droplet size for HPI (control)

Table 4.5.2 Mean droplet sizes (D3,2) for concentrations of HPI and its hydrolysates.

Concentration (mg/ml)	Alcalase hydrolysate	Pepsin hydrolysate	Thermolysin hydrolysate	Control
50	14.365	22.950	19.896	23.288
70	13.177	20.203	19.041	17.482
125	12.598	16.580	16.220	14.437
250	12.118	15.289	14.509	11.752
500	9.799	12.225	8.705	5.371

From Table 4.5.2, it can be observed that as the concentration of the various hydrolysates increased, the mean droplet (D3,2) value decreased, thereby resulting to better stability and emulsion.

4.5.3 Foaming properties

Solubility and the ability of proteins to unfold are important factors for effective foaming (Snyder & Kwon, 1987). Proteins have been shown to have good foaming properties when at the air-water interface they are absorbed, rearrange themselves and undergo conformational change, as well as form a cohesive film by intermolecular interactions (Shou-Wei et al., 2008). Proteins are able to achieve this due to their length of peptide chain. This means that enzymatic hydrolysis which results in the breaking down of peptide chains would cause a decrease in foaming capacity and foaming stability (Damodaran, 1997). The results from the 10 min hydrolysis in Table 4.5.3(a) agree with this statement. The hydrolysates from the 10 min hydrolysis had lower foaming properties compared to the HPI (control). As earlier stated, a good foaming agent

(protein) must be able to rapidly diffuse to the air-water interface and then be able to form a strong cohesive film (Kinsella, 1981). HPI had a longer peptide chain length to stabilize the foam, while the hydrolysates have small peptide chains and cannot interact properly at the air-water phase, which results in a reduced protein-protein interaction and subsequent decrease in foam stability. Reduced degree of protein-protein interactions could be due to increased charge density on the peptides. These results agree with previous reports by Perea et al. (1993), Kato et al. (1985), and Lakkis & Villota (1990).

Table 4.5.3a Foaming capacity (FC) and foaming stability (FS) of HPI and its hydrolysates from 10 min enzymatic hydrolysis.

Samples	FC (%)	FS(%)
HPI (control)	260 ± 0.14^{a}	12 ± 0.3^{a}
Alcalase-treated hydrolysate	250 ± 0.06^{b}	4 ± 0.12^{d}
Pepsin-treated hydrolysate	240 ± 0.05c	4.1 ± 0.29^{c}
Thermolysin-treated hydrolysated	240 ± 0.28^{c}	$8.3 \pm .04^{b}$

Data represents means and standard deviations of three replicates.

For the 2.5 min hydrolysis as seen in Table 4.5.3(b), there was no significant difference between HPI (control) and the hydrolysates for foaming capacity, except for the thermolysin treated hydrolysates that had a lower value than the HPI and the other hydrolysates. The results suggest that the 2.5 min duration of enzyme hydrolysis was not enough to break down the native proteins into small peptides when compared to the 10 min hydrolysis. As expected the HPI had significantly higher ($p<0.05$) foam stability than the hydrolysates.

Table 4.5.3b Foaming capacity (FC) and foaming stability (FS) of HPI and its hydrolysates from 2.5 min enzymatic hydrolysis.

Samples	FC (%)	FS (%)
HPI (control)	260 ± 0.07^{a}	7.7 ± 1.05^{a}
Alcalase-treated hydrolysates	260 ± 0.17^{a}	3.8 ± 0.34^{b}
Pepsin-treated hydrolysate	260 ± 0.44^{a}	3.8 ± 0.06^{b}
Thermolysin-treated hydrolysate	250 ± 0.08^{b}	0.00 ± 0.00^{c}

Data represents means and standard deviations of three replicates.

4.5.4 Gelation properties

After rapid cooling, gelation properties of the various hydrolysates from the 10 min hydrolysis had no common pattern as shown in Table 4.5.4a. For the control (HPI), a progression of protein concentrations resulted to gelation as observed in Table 4.5.4a. After 2 h of cooling at 4^{0}C, the same trend was observed for the 10 min hydrolysates and the control (HPI).

The 10 min control (HPI) gelled from 12 %-20 % protein content. Alcalase treated hydrolysate gelled at 14 % to 20% protein content; all the pepsin treated hydrolysates did not form any gel, while hydrolysates from thermolysin gelled at 16 % to 20 % protein content. It could be suggested from these results that some amount of protein is necessary for gelation to take place and this depends also on the enzyme used for hydrolysis. These results also suggest that cooling (both rapid and at 4^0C) increases the gelling ability of proteins, this could be as a result of decrease in mobility of the molecules and thus enhancement of bond formation amongst the molecules (Renkema & van Vliet, 2002).

Table 4.5.4a Gelation properties of HPI and protein hydrolysates from 10 min enzymatic hydrolysis.

Samples	Protein concentration (% w/v)									
	2	4	6	8	10	12	14	16	18	20
HPI (Control)	NG	NG	NG	NG	NG	G	G	G	G	G
Hydrolysates from Alcalase	NG	NG	NG	NG	NG	NG	G	G	G	G
Hydrolysates from pepsin	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Hydrolysates from thermolysin	NG	NG	NG	NG	NG	NG	NG	G	G	G

NG: No gel formed; the sample slipped from the inverted test tube
G: Gel formed; the sample did not fall from the inverted test tube

Table 4.5.4b Gelation properties of HPI and protein hydrolysates from 2.5 min enzymatic hydrolysis.

Samples	Protein concentration (% w/v)									
	2	4	6	8	10	12	14	16	18	20
HPI (Control)	NG	NG	NG	NG	NG	G	G	G	G	G
Hydrolysates from Alcalase	NG	NG	NG	NG	NG	G	G	G	G	G
Hydrolysates from pepsin	NG	NG	NG	NG	NG	G	G	G	G	G
Hydrolysates from thermolysin	NG	NG	NG	NG	NG	G	G	G	G	G

NG: No gel formed; the sample slipped from the inverted test tube,
G: Gel formed; the sample did not fall from the inverted test tube

Results of the gelation properties shows minimum gelation concentration at 12% for the control and hydrolysates as shown in Table 4.5.4b for the 2.5 min hydrolysis . This could be because the higher the concentration, the stronger the gel formation (Sun & Arntfield 2010). Also it is clear that a shorter time of hydrolysis in this case, 2.5 min gave better gelation properties than the longer time of hydrolysis of 10 min. Therefore, the extent of protein fragmentation after 2.5 min hydrolysis did not have a negative effect on ability of the proteins to form gels.

4.5.5 Fat Absorption Capacity (FAC)

A study by Bernardi et al. (1991) suggested that protein networks entrap oil and these networks can be disrupted or broken up by enzymatic hydrolysis thereby decreasing FAC.

As shown in Figure 4.5.5, FAC of the 2.5 min hydrolysates was higher than the 10 min hydrolysates. The 10 min hydrolysate was lower in FAC having undergone further hydrolysis (breaking down of the protein network). It can be suggested that the 2.5 min hydrolysates still have protein networks intact and so exhibited higher FAC than the 10 min hydrolysates. The unhydrolyzed protein was slightly higher than the various 10 min hydrolysates, which may be due to the fact that the protein network was not broken by enzymatic hydrolysis while the 10 min hydrolysates had a lower FAC due to the effect of enzymatic hydrolysis.

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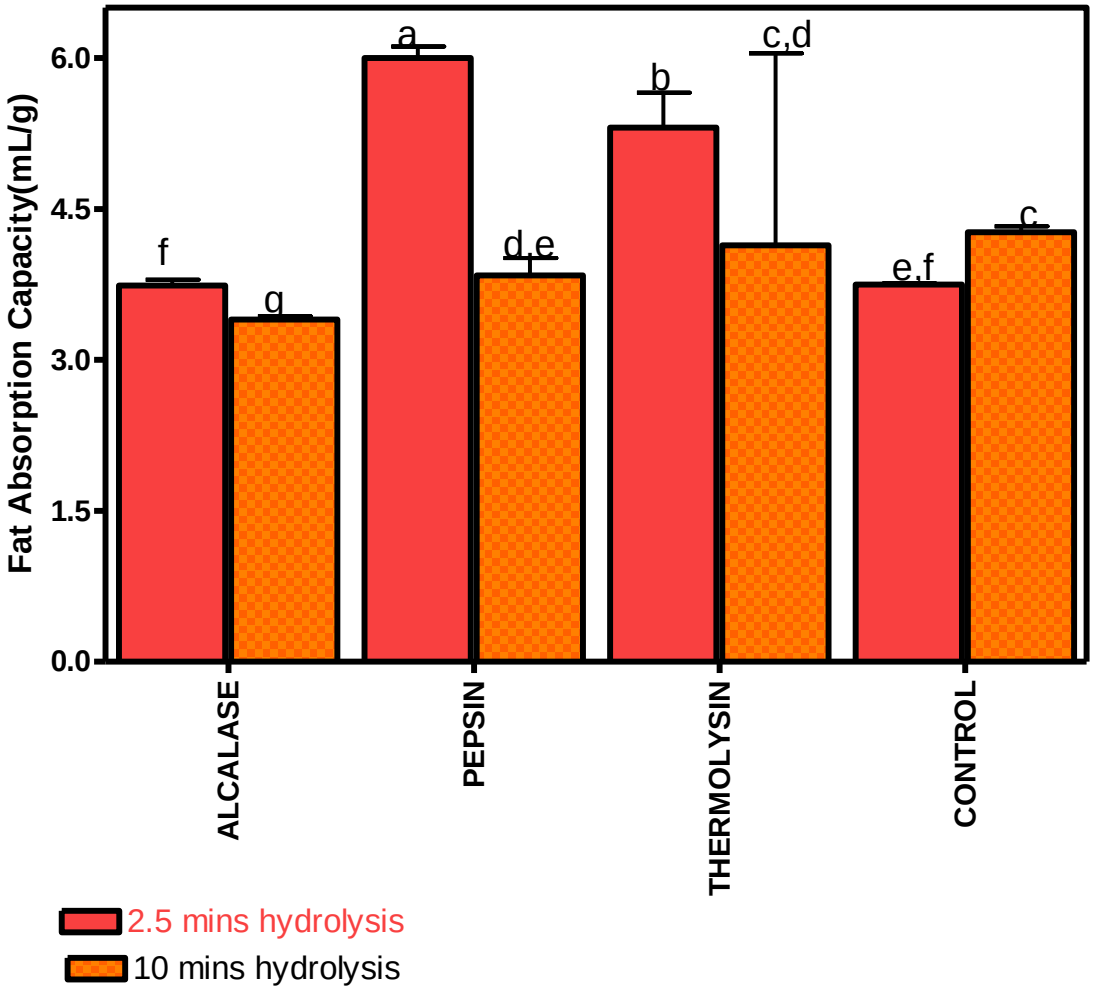
Figure 4.5.5 Fat Absorption Capacity of hemp seed protein isolate and protein hydrolysates



For the 10 min hydrolysis, alcalase treated hydrolysate was lowest in FAC value (3.4), followed by pepsin hydrolysate (3.8) and then thermolysin (4.14). Allaoua et al., (1998) reported that heat treatment (80°C) of soy protein isolate increased FAC. Similar effect was observed in the 10 minutes control samples as they were slightly higher than the other hydrolysates. Zayas (1997) suggested that proteins are responsible for fat absorption, therefore high protein content results in exhibition of high FAC; this is true for the various hydrolysates having high protein content. Thermolysin treated hydrolysates for 2.5 and 10 min hydrolysis have protein content of 96 % and 80 % respectively. Also pepsin treated hydrolysates for 2.5 min and 10 min have 82 % and 74 % protein content respectively. Therefore, thermolysin treated hydrolysates and pepsin treated hydrolysates both have very high FAC due to their high protein content.

4.5.6 Water Holding Capacity (WHC)

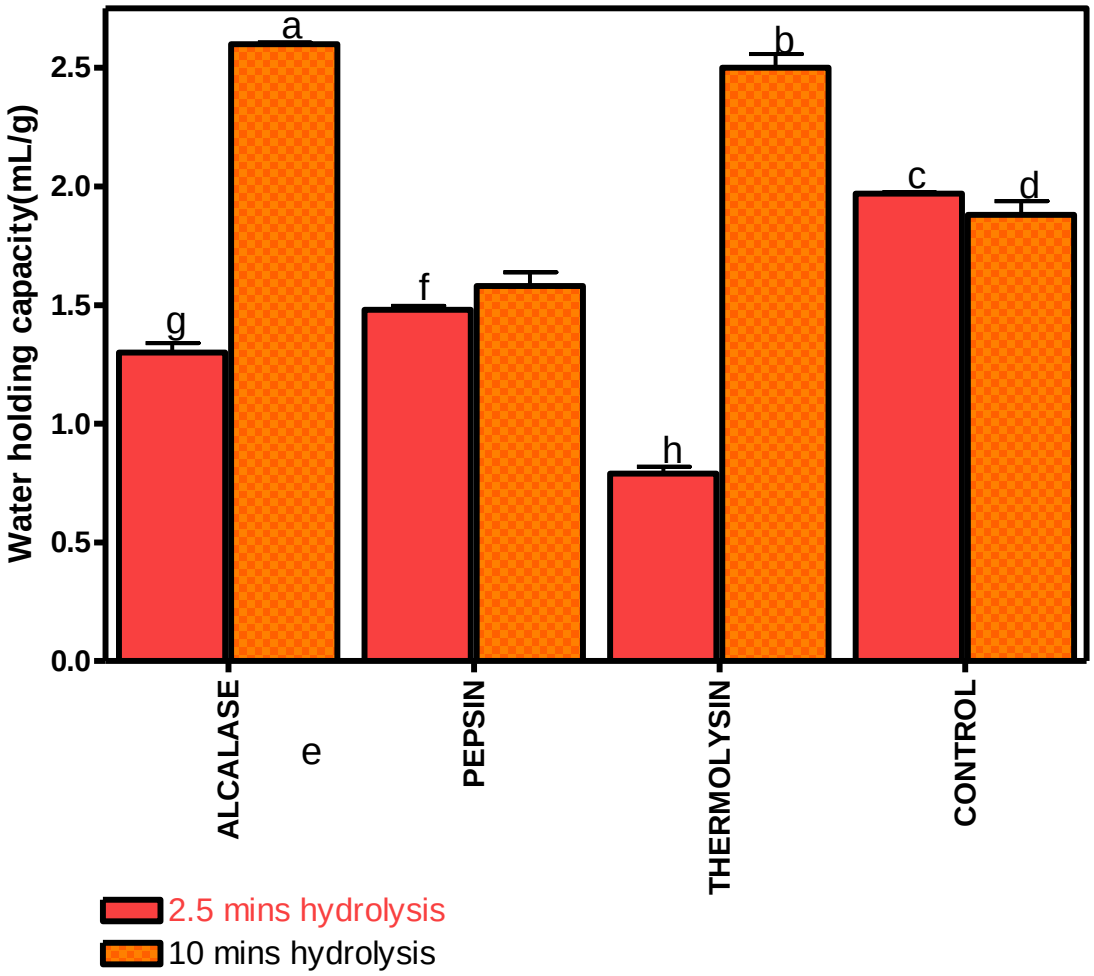
For the 2.5 min hydrolysates, the WHC of HPI (control) was higher than the other hydrolysates (Figure 4.5.6); this according to Shou-Wei et al.(2008) could be due to the presence of hydrophilic groups. Alcalase treated hydrolysates and thermolysin treated hydrolysates of the 2.5 min hydrolysis have significantly lower ($p<0.05$) values when compared to the hydrolysates of the 10 mins hydrolysis, ($p<0.05$). This is probably due to the increased number of exposed charges and residue in the 10 min hydrolysates, which would enhance interaction of the proteins with water molecules.

For the 10 min hydrolysates (Figure 4.5.6), the alcalase treated hydrolysates as well as the thermolysin treated hydrolysates are significantly higher ($p<0.05$) than pepsin treated hydrolysate as well as the control. Thus the pepsin hydrolysate probably had less number

of charges, which is consistent with the fact that it has better enzymatic specificity than alcalase and themolysin. It can be suggested from Figure 4.5.6 that an increase in time for enzymatic hydrolysis from 2.5 to 10 min (using alcalase and thermolysin) favored higher WHC values.

For alcalase HPH and thermolysin HPH, results suggest that there is a relationship between their solubility (at pH 6-pH 7) and WHC. High protein solubility (pH 6-8) resulted in high WHC for alcalase HPH and thermolysin HPH. Contrary to this result, Abugoch et al. (2008) suggested that there is no relationship between the solubility and WHC of quinoa protein isolates; increase in protein solubility did not necessarily result in high WHC. Ahmedna et al. (1999) also showed similar results concluding that protein solubility does not relate to WHC for wheat protein isolate. The higher values of WHC of whole pea flour could be attributed to the presence of fibre, which absorbs water (Agboola et al., 2008). The whole pea flour was milled from whole pea seeds which included the hulls that have a high amount of fibre.

Figure 4.5.6 Water Holding Capacity of hemp seed protein isolate and enzymatic protein hydrolysates.



4.6 Protein content of fractionated HPH peptides

Fractions (1 kDa, 3 kDa, and 5 kDa) obtained by ultrafiltratiom for alcalase HPH and thermolysin HPH had a range of percentage protein content from 73% to 89%, while the control had a percentage protein content of 95% (Table 4.6). All fractions obtained by the ultrafiltration were creamy in colour.

Table 4.6 Percentage protein content for hydrolysates from extensive hydrolysis prepared with various enzymes and different ultra-filtration membranes.

Ultrafiltration membrane	Percentage protein (%)	
	Alcalase	Thermolysin
1kDa	87	78
3kDa	89	73
5kDa	77	86
Control	95	95

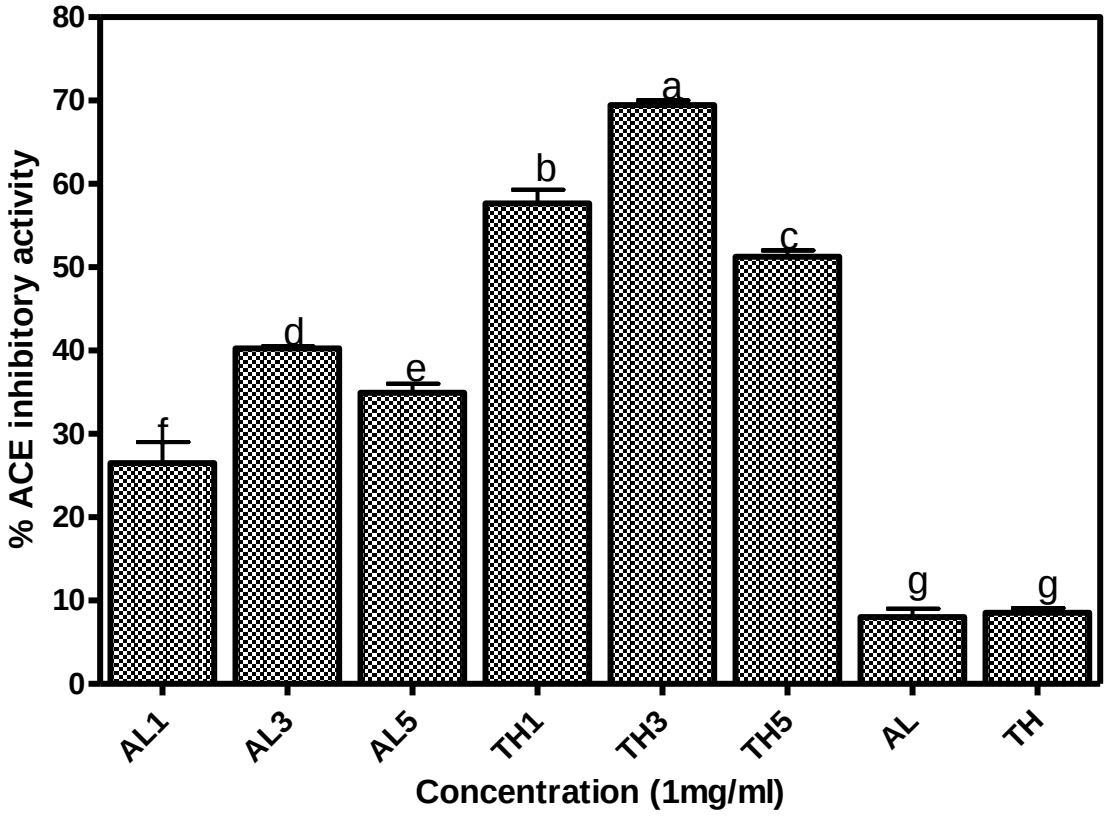
4.6.1 In vitro antihypertensive properties

4.6.1.1 Angiotensin converting enzyme (ACE) inhibition activity of fractionated and unfractionated hemp seed protein hydrolysates

Figure 4.6.1.1 shows the ACE inhibitory activity of HPH and its fractions. Fractions of thermolysin HPH had activity of ACE-inhibition than fractions of alcalase HPH; thus thermolysin was the more effective enzyme in the production of HPH permeates with high degree of ACE-inhibitory activity. Udenigwe et al. (2009a) had similar high results with the low molecular weights peptides from thermolysin flaxseed hydrolysates. Humiski & Aluko. (2007) showed that papain was the best protease in producing pea protein hydrolysates that had high ACE-inhibitory properties. Figure 4.6.1.1, shows that 3 kDa permeates of thermolysin treated HPH had the highest ACE-inhibitory value of 70%.

Past studies have shown that low molecular weight peptides had a high ACE-inhibitory activity (Jeon et al., 1999, Kuba et al., 2005, Aluko and Monu 2003, Udenigwe et al., 2009a). Amongst permeates of alcalase HPH, the 3 kDa fractions also had the highest ACE-inhibitory activity of 40% at 0.61 mg/ml. HPH of alcalase and thermolysin (controls) had very low activity, suggesting that fractionation by ultrafiltration aids in the production of low molecular weight peptides that have improved ACE-inhibitory activity. Parris et al. (2008) showed that enzymatic hydrolysis and fractionation (ultrafiltration) of wet and dry–milled corn proteins resulted in the production of ACE-inhibitory peptides; furthermore they suggested that low molecular weight peptides exhibited ACE inhibition.

Figure 4.6.1.1 Percentage ACE inhibition activity for fractionated and unfractionated hemp seed protein hydrolysates



AL1: 1 kDa alcalase fraction

AL3: 3 kDa alcalase fraction

AL5: 5 kDa alcalase fraction

AL: Alcalase hydroysate

TH1: 1 kDa thermolysin fraction

TH3: 3 kDa thermolysin fraction

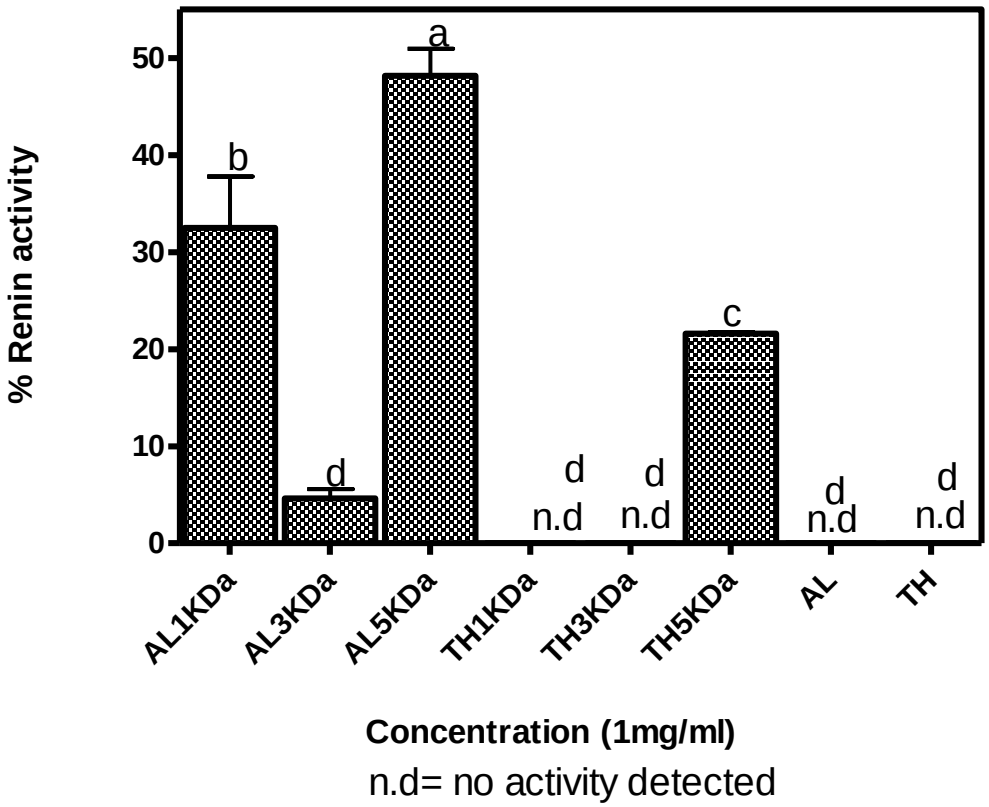
TH5: 5 kDa thermolysin fraction

TH: Thermolysin hydrolysate

4.6.1.2 Renin inhibition activity of hemp seed protein hydrolysates and fractionated peptides

Results from renin inhibition assay (Figure 4.6.1.2) agree with Yuan et al. (2007) that ACE and renin inhibition results do not correspond, because different mechanisms are involved. Compared to the ACE-inhibitory activity in Figure 4.6.1.1, the renin inhibitory activity is lower. The results for renin inhibition assay showed that fractions of alcalase HPH had better activity compared to thermolysin fractions. This was opposite to what was observed in ACE-inhibitory activities. These results agree with the previous work of Udenigwe et al. (2009b). Yuan et al. (2007) indicated that renin has a folded conformation that makes it not easily accessible to inhibitors thereby resulting in less effective inhibitory activities, while ACE has an open confirmation, which makes it more susceptible to inhibition than renin.

Figure 4.6.1.2 Renin inhibition activity of fractionated and unfractionated hemp seed protein hydrolysates



Amongst the 3 permeates of thermolysin HPH, the 5 kDa had a low activity of 21%, while there was no renin activity detected in fractions of 1 kDa and 3 kDa. The 5 kDa fractions of alcalase HPH had the highest renin inhibitory activity of 47% at 19 mg/ml. The renin activity observed in this study can be attributed to moderate unfolding of the renin structure that allowed the inhibitor (fractions of the HPH) access. There was no activity observed in the HPH for both alcalase and thermolysin (controls) suggesting that fractionation (ultra filtration) is necessary to obtain permeates that could exhibit renin activity.

4.7. Antioxidant activities of hemp seed protein hydrolysates and fractionated peptides

Antioxidant activity can be related to amino acid composition, sequence, size and configuration of the peptides (Chen et al., 1998). Glutathione was used in this study as a standard to compare the various antioxidant activities. HPH of alcalase and thermolysin served as the controls.

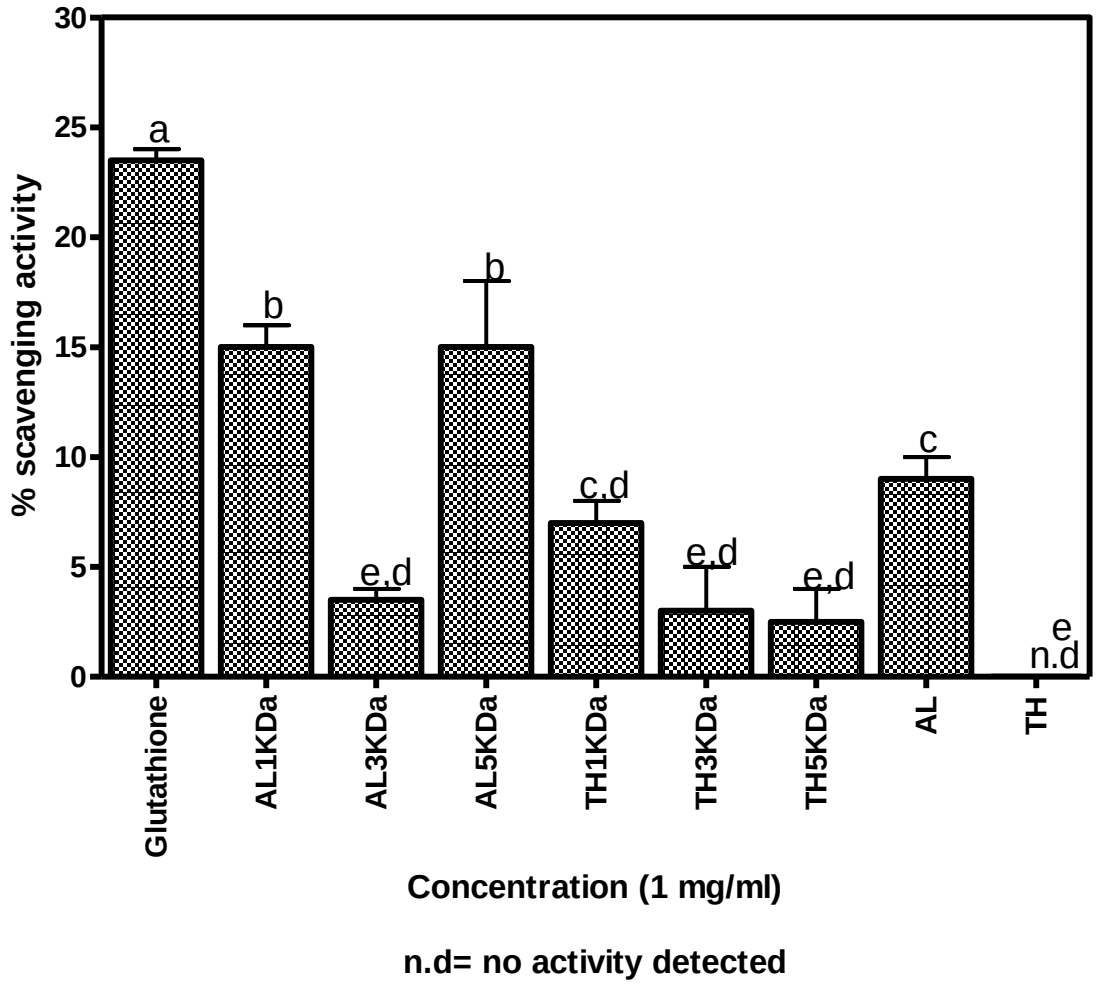
4.7.1 DPPH radical scavenging activity of fractionated and unfractionated hemp seed protein hydrolysates

DPPH is a stable free radical that accepts electron from antioxidants. Pownall et al. (2010) suggested that antioxidant activity of glutathione is attributed to sulfhydryl group of cysteine; therefore cysteine-containing peptides could be considered as good and effective scavengers of DPPH. Figure 4.7.1 shows the DPPH scavenging activity of the various HPH fractions and glutathione as a standard while Alcalase and thermolysin hydrolysates are the controls. The poor DPPH scavenging activity observed by the HPH

fractions could be attributed to the pH condition at which the assay is conducted. Udenigwe et al. (2009a) demonstrated that flaxseed peptide fractions exhibited better DPPH scavenging activity in acetate buffer than in phosphate buffer and Tris HCl buffer. DPPH dependence on pH was also suggested by Lin et al. (2008). At 1 mg/ml, glutathione scavenging activity (23%) against DPPH was significantly higher ($p<0.05$) than the various fractions of HPH. The DPPH activity of the fractions are low (2%-12%) compared to the 30-70% reported by Udenigwe et al. (2009a) and 38-51% by Chang et al. (2007). Humiski & Aluko (2007) also recorded poor DPPH scavenging activity of pea protein hydrolysates (7-11%). Generally, alcalase HPH showed a better DPPH scavenging activity when compared to thermolysin HPH which had low scavenging activity. The peptides present in the thermolysin HPH may have acted antagonistically and could therefore have reduced the DPPH activity. Previous studies have also shown that peptide fractionation increased antioxidant activities of protein hydrolysates. For example, Aluko and Monu (2003) worked on quinoa protein hydrolysates and found that there was no radical scavenging activity by the original hydrolysate until it had been passed through ultrafiltration membrane to obtain fractions with lower molecular weights. Tang et al. (2010) also observed that when the molecular mass of the fractions of protein hydrolysates were smaller (<5 kDa), the DPPH scavenging activities were higher. This trend can be observed in the various fractions except in 3 kDa peptides from alcalase HPH. The various fractions exhibiting traces of DPPH activities might possibly contain some electron donor molecules that would react with unstable DPPH free radicals and make them more stable products.

Contrary to the previous stated results, Udenigwe et al. (2009a) observed that the high molecular mass peptides of flaxseed fractions had a better DPPH scavenging activity. The present results agree with data from other studies which suggested that DPPH scavenging activity of protein hydrolysates may depend on the molecular size of their various peptides (Li et al., 2008; Wang et al., 2007)

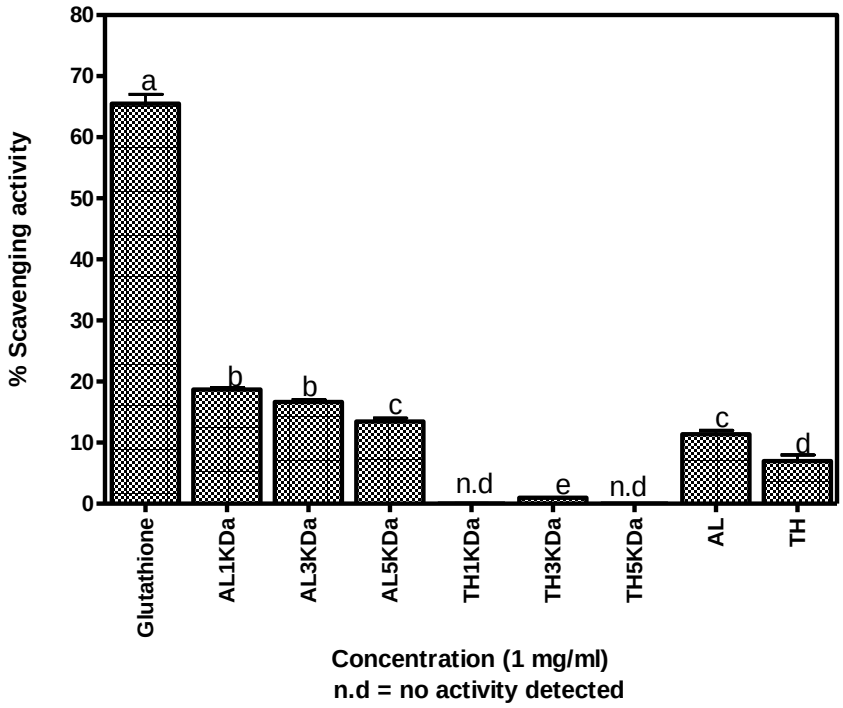
Figure 4.7.1 DPPH scavenging activity (%) of fractionated and unfractionated hemp seed protein hydrolysates



4.7.2. Superoxide scavenging activity of HPH and peptide fractions

As shown in Figure 4.7.2, the peptide fractions demonstrated very poor superoxide scavenging activity. Generally, there was slight decrease in superoxide activity as molecular weight increased. This could mean that the lower molecular weight peptide fractions could have some peptides that could have contributed to superoxide scavenging activity. According to Pownall et al. (2010), those fractions exhibiting superoxide scavenging activity could contain proline which plays an important vital role in superoxide scavenging activity. Results from studies by Tang et al. (2010) as well as Li et al. (2008), showed that lower molecular weight peptide fractions could also contain hydrophobic amino acids that give the superoxide scavenging properties. The alcalase and thermolysin hydrolysates had lower superoxide activity compared to the fractions; with the alcalase higher than the thermolysin hydrolysate. It can be suggested that fractionation plays a vital role in making the hydrophobic amino acids available for superoxide scavenging activity. Udenigwe et al. (2009a) had a contrary result; the higher the molecular weight of the flaxseed peptide fractions, the stronger the superoxide scavenging activity. Glutathione had the strongest activity (67 %) and it could be suggested that cysteine can be a determining factor in scavenging of superoxide radical. From Figure 4.7.2, it can be suggested that alcalase treated HPH, thermolysin treated HPH, as well as their peptide fractions are not efficient superoxide scavengers.

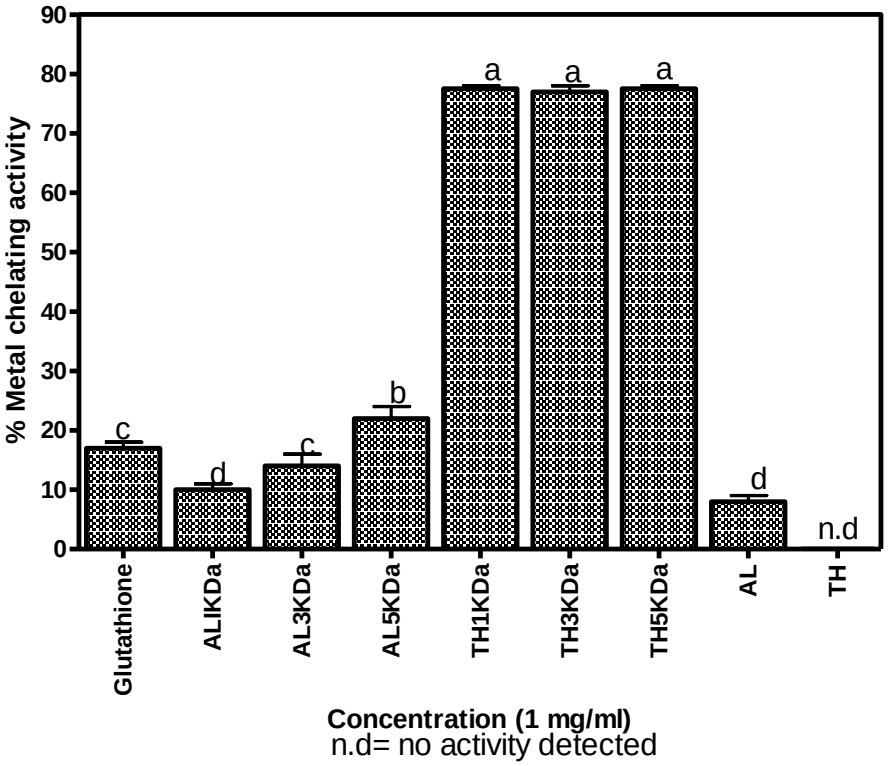
Figure 4.7.2 Superoxide scavenging activity of fractionated and unfractionated hemp seed protein hydrolysates



4.7.3 Metal ion (Fe) chelating activity of HPH and peptide fractions

The chelating of ferrous ions by HPH and the peptide fractions was determined, whereby the free radical metal ions underwent chelation. Foh et al. (2010) worked on fresh minced meat of tilapia fish and showed that alcalase-treated tilapia protein hydrolysates demonstrated a high chelating activity of 82.5% compared to Flavourzyme and Neutrase hydrolysates, which had chelating activity of 75.8% and 77.23%, respectively. Figure 4.7.3 shows an increase in metal chelating activity for fractions of alcalase treated hydrolysates: 1 kDa < 3 kDa < 5 kDa, having values of 11 %, 16 %, 24 % respectively. Permeate fractions of thermolysin treated hydrolysates (1 kDa, 3 kDa, 5 kDa) all had metal chelating activity at 78%. It has been suggested by previous studies that ultrafiltration can result in enrichment of antioxidant peptides (Xie et al., 2008; Kong et al., 2008; Pihlanto et al., 2008). It can then be further suggested from the result of this study that presence of hydrophobic amino acids as well as other peptides that could have antioxidant properties were concentrated during ultrafiltration of the hydrolysates, which enhanced metal chelating activities. Thermolysin can be suggested to be a better alternative to produce hydrolysates that have good chelating activity. Figure 4.7.3 show that glutathione has very low metal chelating activity of 18%. This was also observed by Xie et al. (2008) as well as Pownall et al. (2010) where the glutathione used in their individual studies showed a negligible metal chelating activity. Therefore possession of cysteine by glutathione is not necessary for chelating of metals. The poor performance of the hydrolysates (alcalase and thermolysin), prior to ultrafiltration, could be attributed to low ratio of metal chelating antioxidant peptides when compared to inactive peptides.

Figure 4.7.3 Metal chelating activities (%) of fractionated and unfractionated hemp seed protein hydrolysates.



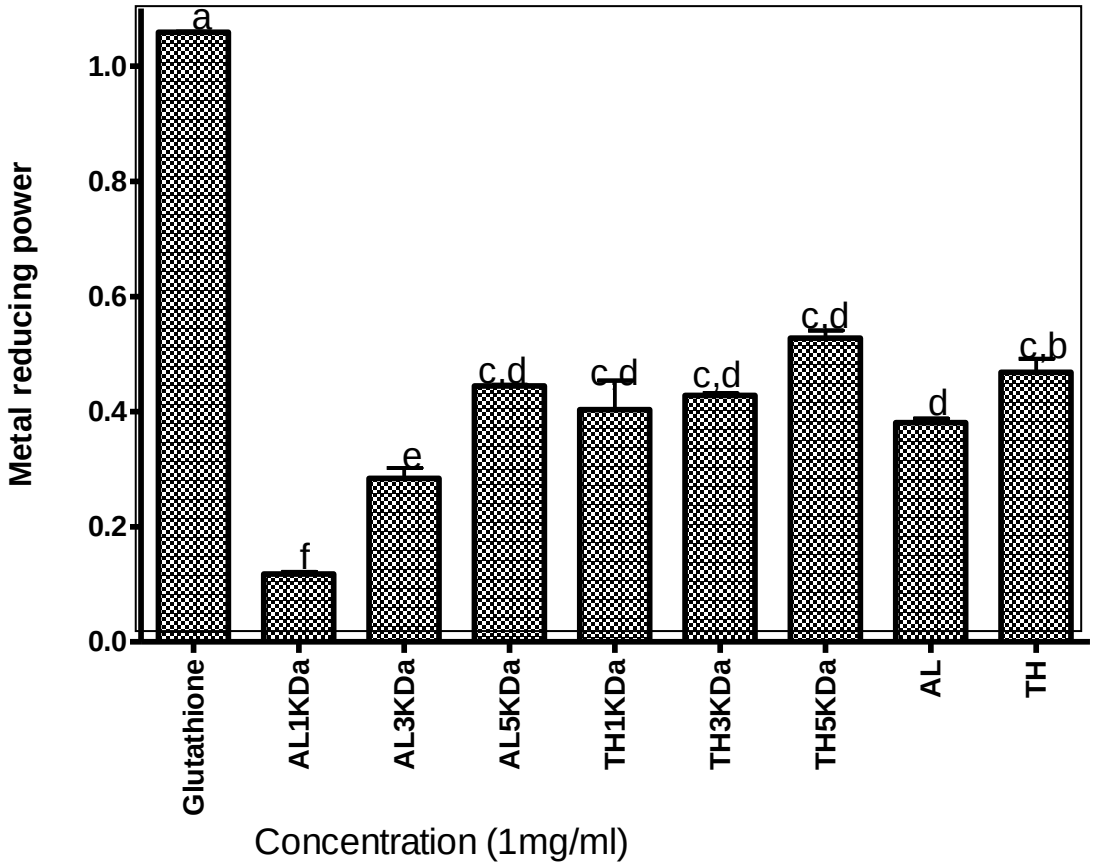
4.7.4 Ferric Reducing Antioxidant Power (FRAP) of HPH and peptide fractions

The reducing power of HPH involves reduction of ferricyanide complex (Fe^{3+}) to ferrous form (Fe^{2+}) at an absorbance of 700 nm. As shown in Figure 4.7.4, glutathione had a significantly higher value of FRAP activity than the hydrolysate fractions as well as the unfractionated hydrolysates. The fractions of alcalase and thermolysin treated hydrolysates exhibited a trend. Thermolysin treated hydrolysates had a higher reducing power than the alcalase treated hydrolysates. The ferric reducing powers of the fractions of alcalase and thermolysin were as follows: 5 kDa > 3 kDa > 1 kDa. Both unfractionated alcalase and thermolysin HPH had FRAP values of 0.4 and 0.47, respectively.

Though the thermolysin hydrolysates (fractionated and unfractionated) had higher FRAP values than the alcalase samples, glutathione had a higher reducing power. Glutathione contains a sulfur-containing cysteine and this is suggested to be responsible for the strong reducing power of glutathione Battin & Brumghim, J.L. (2009).

From Figure 4.7.4, it can be suggested that fractions of thermolysin treated hydrolysates as well as the unfractionated thermolysin HPH may contain some amino acids that could be responsible for the moderate reducing capacity, especially as seen in fractions of thermolysin <3 kDa and <5 kDa having reducing power value of 0.43 and 0.47 respectively. Battin & Brumghim, J.L. (2009) also suggested that the type of amino acid as well as the peptide composition could contribute to ferric reducing activity. This could be used to explain the stronger reducing ability of glutathione; that is the possession of a sulfhydryl group. It can then be suggested that the various fractions of alcalase and thermolysin hydrolysates as well as the HPH had less sulfhydrly group, which gave them lower ferric reducing power compared to glutathione.

Figure 4.7.4 Ferric Reducing power of fractionated and unfractionated hemp seed protein hydrolysates



Baohua & Youling, (2006) showed zein protein hydrolysate have the ability to act as antioxidant and this is dependent on both the concentration and the peptide composition,, while from the result shown in figure 4.7.4, increase in ferric reducing property was dependent on the increase in molecular size of the various peptides fractions. Contrary to the low reducing activity of alcalase treated HPH and thermolysin treated HPH (Figure 4.7.4), Lijuan et al. (2008) showed that FRAP of zein hydrolysates increased 2-fold after it was hydrolyzed by pancreatin. Thus the present results in addition to previous studies suggest that reducing power activity of peptides may be dependent on type of protease used during protein hydrolysis.

Table 4.8 Summary of functional properties of hemp seed protein hydrolysates

Properties	Alcalase	Pepsin	Thermolysin
Protein solubility	Improved	Improved	Improved
Emulsifying property	Decreased	Decreased	Decreased
Foaming property	Decreased	Decreased	Decreased
Gelation property	Improved	Improved	Improved
Fat absorption property	Decreased	Decreased	Decreased
Water holding capacity	Improved	Improved	Improved

Table 4.9 Summary of bioactive properties of hemp seed protein permeates

Peptide sample	Renin		ACE	
	Alcalase	Thermolysin	Alcalase	Thermolysin
HPH	No change	No change	Decreased	Decreased
1 kDa	Improved	No change	Improved	Improved
3 kDa	Slightly improved	No changed	Slightly Improved	Slightly Improved
5kDa	Improved	Slightly Improved	Improved	Improved

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

The present study and research focused on determining the functional properties of hemp seed as well as its antioxidant and antihypertensive properties, using the isolates, hydrolysates as well as peptide fractions.

This work showed that enzymatic hydrolysis improved some functional properties of hemp seed protein isolates. There was an increase in protein solubility after 10 min of hydrolysis. Hemp protein isolates had a higher emulsion capacity (small oil droplet size) than the protein hydrolysates. Hydrolysis did not improve the foaming capacity and foaming stability of hemp protein isolate and hydrolysates. Increase in concentration resulted in the increase in gelation of the isolate and hydrolysates after 2 h of cooling at 4^{o}C. Enzymatic hydrolysis enables the protein network to rupture and this decreased the fat absorption value, especially after 10 min of protein hydrolysis. The 2.5 min compared to the 10 min hydrolysates had a higher FAC value, probably due to less extensive hydrolysis in the former. Water holding capacity was increased for the hydrolysates in the 10 min hydrolysis, which may be due to increased number of charged residues that are able to bind water molecules.

Ultrafiltration membrane produced hemp protein peptides (<3 kDa) that exhibited potential antihypertensive properties by inhibiting ACE activity. Renin activity was inhibited by < 5 kDa permeates but overall renin inhibition was lower when compared to that of ACE inhibition.

This study was able to show that peptide fractions of alcalase and thermolysin had low DPPH inhibitory property as well as metal reducing property while that of

superoxide inhibitory activity was weak to nil. Fractions of thermolysin hydrolysates had good metal chelating activities.

Exploring the potential usefulness of hemp seed in the food industry will make positive contributions to the economic sector of the Canadian prairies. The therapeutic potentials could lead to increased value-added utilization of hemp seed as a crop and provide a boost to the fledging functional foods and nutraceuticals industry.

CHAPTER SIX

FUTURE RESEARCH

Peptide from HPH would be a good source of protein ingredient that could be used in the food processing industry because of its functional properties. The bioactive peptides derived from HPH can also be further used as an antioxidant as well as an antihypertensive agent to formulate functional foods and nutraceuticals. Decolorization can be carried out on HPH to improve its appearance and appeal.

Future work needs to be done to improve some of the poor functional properties of hemp seed proteins by modifications through the use of other proteases and optimization of degree of hydrolysis, concentration, and pH conditions. This modification would lead to production of peptides with better functional properties that would be applicable in the food industry.

In addition, future work should involve investigation and research on the mechanism of the action of ACE and renin inhibiting peptides of hemp seed protein hydrolysates. Such work would lead to a much better understanding of the antihypertensive potential of HPH. Thus peptide purification and amino acid sequencing will be necessary to determine the molecular structure of bioactive peptides, which can lead to additional structure studies.

To the best of our knowledge, scanty work and study has been done on the renin inhibitory property of food protein hydrolysates. Therefore, more work on the therapeutic potential of HPH need to be done.

To date, no animal or human studies have been done on the antihypertensive property of HPH. Future studies using animal and human clinical trials to test physiological efficacy of HPH and peptides will be necessary in order to explore potential commercialization.

CHAPTER SEVEN

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