Antioxidant Activity of Hempseed Protein-Derived Peptides Obtained by Hydrolysis with Proteinase K

By

Bilen Gebrekidan

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences

University of Manitoba

Winnipeg, Manitoba

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Enzymatic hydrolysates from hempseed protein were studied *in vitro* for their antioxidant activities. Four hempseed protein hydrolysates were prepared using different concentrations (1, 2, 3, and 4% by weight of protein in the hempseed protein isolate) of the enzyme Proteinase K, followed by ultrafiltration to fractionate low molecular weight peptides. The effect of enzyme concentration and fraction size was studied with respect to indices of antioxidant activity. The current study showed no relationship between antioxidant activity and the concentration of Proteinase K used. The hydrolysis process, followed by ultrafiltration, led to an increased antioxidant activity at <1KDa and 1-3KDa. Low molecular weight peptides were shown to contribute to the antioxidant property of hemp seed protein. In addition, bioactive peptides can be liberated from the hydrolysis of hempseed protein using a 1% concentration of Proteinase K. The results show that the protein components of hempseed meal yield peptide fractions can be exploited as potential food sources of antioxidant agents.

DEDICATION

To the loving memory of my late Grandmother Timinte Gebrekidan, and late Grandfather Ghilagaber Weldegergis.

ACKNOWLEDGMENTS

This research project would not have been possible without the support of many people. I am greatly appreciative for the love and support that I received from my family and friends during my Master's program. Above all, I could not have accomplished anything without the grace of God.

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LIST OF ABBREVIATIONS

ROS: Reactive Oxygen Species

BHT: Butylated Hydroxytoluene

DPPH: 1, 1-diphenyl-2-picrylhdrazyl

HPH: Hempseed Protein Hydrolysate

ACE: Angiotensin I-converting enzyme

RAAS: renin-angiotensin-aldosterone system

AT I: Angiotensin I

AT II: Angiotensin II

MW: molecular weight

GSH: Glutathione

HPI: Hemp protein isolate

HPH: Hemp protein hydrolysate

FAA: Free amino acid

SOD: Superoxide dismutase

NADPH: Nicotinamide adenine dinucleotide phosphate

MPO: Myeloperoxidase

HPO: Hypochlorus acid

$ONOO^{\bullet}$: Peroxynitrite

CHAPTER ONE

1.0 General Introduction

The demand for functional foods and nutraceuticals is increasing due to a growing consumer's understanding of the relationship between food and health, rising healthcare costs, and the issues related to drug safety. The magnitude of disease associated with diet is estimated to be approximately 40% to 50% for cardiovascular disorders (including associated risk factors such as hypertension and diabetes), while 35% to 50% of all cancers are directly related to dietetic factors (Agriculture and Agri-Food Canada, 2007). Functional foods and nutraceuticals have the potential to reduce healthcare costs and improve the health status of Canadians, while simultaneously supporting economic growth in rural Canadian communities (Agriculture and Agri-Food Canada, 2008). Diet and Nutrition are important factors in the promotion and maintenance of good health throughout one's entire life course. With respect to functional foods and nutraceuticals, the nutritional, functional and biological properties of proteins have been widely acknowledged and documented. Dietary proteins are the source of biologically active peptides that can be used to formulate functional foods and health promoting agents. These peptides are inactive within the sequence of the parent protein but can be released when hydrolyzed by digestive enzymes, microbial enzymes or during food processing (Korhonen et al., 2003). Some health benefits have been attributed to antimicrobial, antihypertensive, antioxidant and immunomodulatory properties of food-derived peptides (Bernardini et al., 2012, Chen at al., 2010, Wiesner et al., 2010, You et al., 2011).

Presently, animal sources such as milk and egg proteins and plant proteins such as soybean and chickpeas are considered potential sources of bioactive peptides.

Bioactive peptides offer potential as a source of derived antioxidants, a major current focus of functional food and nutraceutical development. Oxidative stress is one of the significant factors linked to the initiation or progression of several chronic diseases, including inflammatory bowel disease, chronic kidney disease, coronary heart disease, and cancer (Kunwar et al., 2011). Recently, several studies have shown that peptides with antioxidant properties are released from food sources such as milk, eggs, soy protein, and chickpeas (Amadou et al., 2011, Bernardini et al., 2012, Chen at al., 2010, Haiwei, 2010, You et al., 2011, Yust et al., 2011). One approach to the prevention of diseases associated with oxidative stress is through minimizing oxidative damage with the help of antioxidants (Kunwar et al., 2011). Antioxidants protect the body from direct reactive oxygen species (ROS) attacks and free radical-mediated oxidative reaction. Therefore, antioxidants play a major role in protecting the body against oxidative stress and preventing the progression of diseases (Kunwar et al., 2011). Thus, there is a need to develop antioxidants from natural sources such as dietary proteins that could delay or prevent the onset and progression of chronic diseases which could also be nutritionally beneficial as a source of essential amino acids.

Hemp is a widely cultivated plant of industrial importance as a source of food, fiber and medicine. Hemp is emerging as a significant source of revenue

from agricultural crops in Canada's economy. Hempseed is recognized to be a good source of protein, consisting of 25% protein (House et al., 2010). Due to its high protein content and rich amino acid profile, the value-added use of hempseed can be expanded by utilizing hempseed-derived bioactive peptides in functional foods to provide multiple health benefits during oxidative stress. This will ultimately create new markets for locally-produced hempseed both domestically and abroad. Thus, to expand the use of the valuable hempseed proteins or its hydrolysates as food material, there is a need to explore the bioactive properties of enzymatically-prepared hempseed hydrolysates with respect to their potential use as source of antioxidants. Additional data will contribute to the value-added use of hempseed that will ultimately benefit Canada's economy.

The aims of the research included the production of hempseed protein hydrolysates, fractionation of the hydrolysates using membrane ultrafiltration to generate peptides of different molecular weights, and evaluation of their antioxidant properties *in vitro*. The specific objectives of this study were (i) to determine the optimum concentration of Proteinase K that is required to liberate maximum amounts of bioactive peptide sequences present in hempseed protein and (ii) to determine the effect of peptide size on antioxidant activities.

CHAPTER TWO

2.0. Literature Review

2.1. Biological activity of food protein derived peptides

The nutritional, functional and biological properties of dietary protein are widely acknowledged. The functional property of proteins is associated with their physiochemical properties, while the nutritional property is related to their supply of amino acids. Protein quality relates to the amino acid content of the protein, as well as the physiological utilization of specific amino acids during digestion and absorption. Proteins are important for the survival of animals and humans. Recently, extensive research has shown the potential role of bioactive peptides derived from food as physiologically active components. These biologically active peptides may be utilized as components in functional foods intended to yield health benefits.

Bioactive peptides are food- derived components that provide physiological benefits in the body in addition to their nutritional benefits (Korhonen et al., 2003). These bioactive peptides are inactive within the parent protein sequence (Korhonen et al., 2003, Moller et al., 2008). However, bioactive peptides are released from the parent protein sequence upon hydrolysis by digestive enzymes *in vitro* and *in vivo*, or by microbial enzymes (Korhonen et al., 2003, Moller et al., 2008). In addition, these peptides with bioactive property may also be released during food processing (Korhonen et al., 2003, Moller et al., 2008, Phelan et al., 2011). Once these peptides are released, they may exert physiological benefits to the human body (Phelan et al., 2011). The physiological

function of the bioactive peptide is dependent on the amino acid composition and sequence (Phelan et al., 2011, Sampath Kumar et al., 2011). Bioactive peptides can exist as 2–20 amino acid residues (Ryan et al., 2011, Phelan et al., 2011). The peptides exert several physiological effects when absorbed through the intestine, move across the gut epithelium and reach the target cells through the circulatory system in adequate concentrations (Herregods et al., 2011, Moller et al., 2008, Nagpal et al., 2010, Ryan et al., 2011).

2.1.1 Antihypertensive protein and peptides

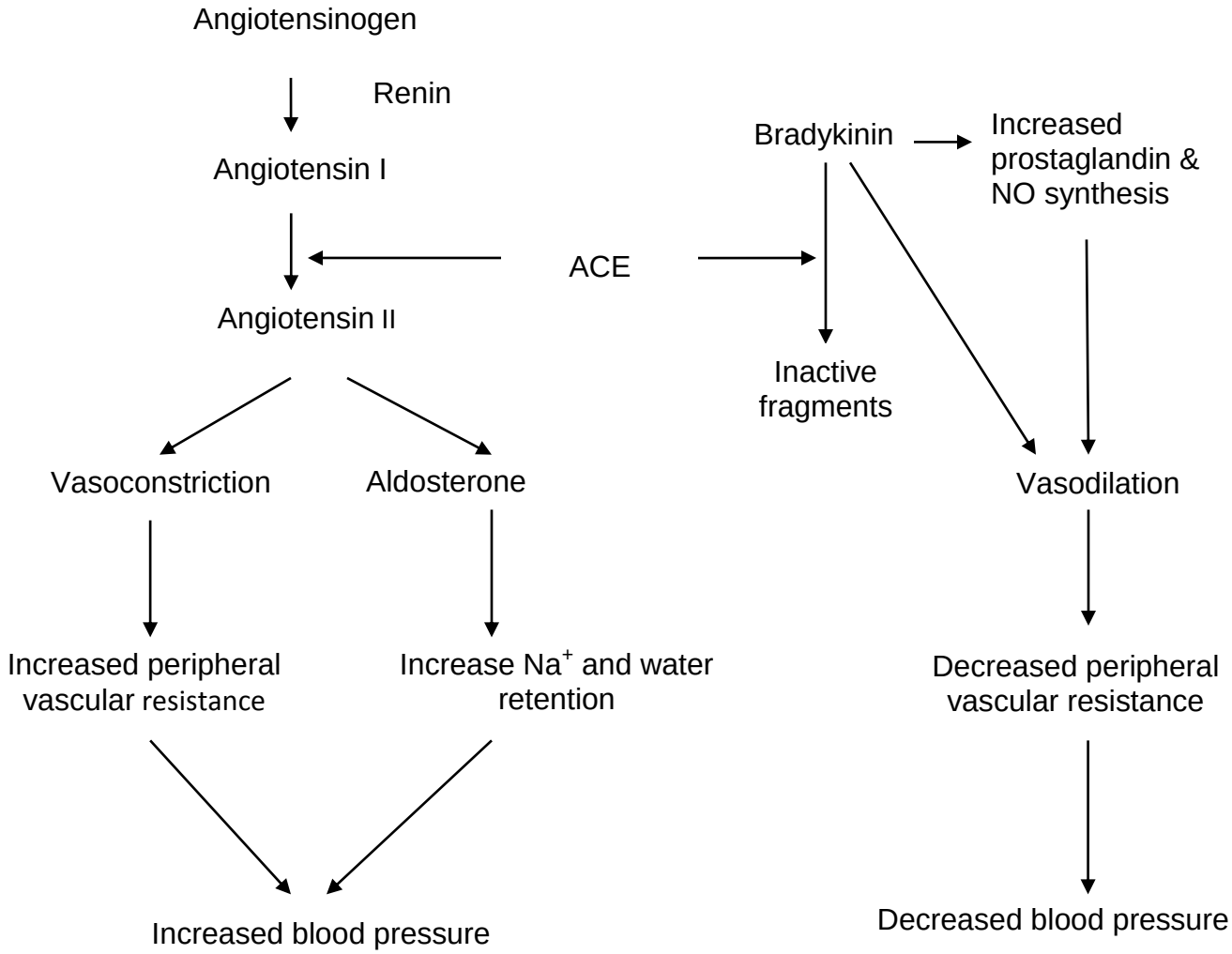
Some food proteins have been identified as a source of Angiotensin I-converting enzyme (ACE) inhibitory peptides (Table 2.1). Cardiovascular disease is caused by disorders of the heart and blood vessels, and includes coronary heart disease, cerebrovascular disease, hypertension, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure (WHO 2012). High blood pressure is one of the major risk factors for cardiovascular disease, a leading cause of death in the developed world (Erdmann et al., 2008). The renin-angiotensin-aldosterone system (RAAS) plays a major role in the regulation of blood pressure and normal heart function (Figure 2.1). Renin is an aspartyl protease that catalyzes the conversion of angiotensinogen to angiotensin I (AT I) (Erdmann et al., 2007, Nagpal et al., 2010, Phelan et al 2011). Subsequently, ACE (a peptidyldipeptide hydrolase, EC 3.4.15.1) catalyzes the conversion of AT I to angiotensin II (AT II) leading to constriction of the blood vessels, hence an increase in blood pressure (Erdmann et al., 2007, Nagpal et al., 2010, Phelan et al 2011). ACE also inactivates the activity of the potent vasodilator, bradykinin

and subsequently results in an increase in blood pressure (Erdmann et al., 2007). Thus, the inhibition of ACE is a crucial target for antihypertensive activity. As a treatment for hypertension, antihypertensive drugs such as Captoptril have been developed to block or suppress the formation of AT II, hence reducing blood pressure. However there are safety concerns over the use of antihypertensive drugs on prolonged administration (Kunwar et al., 2011). Several side effects have been associated with antihypertensive drugs, including coughing, taste disturbances and skin rashes (Wijesekara et al., 2010). Recently, ACE inhibiting peptides have been recognized to be effective in the prevention and treatment of hypertension (Erdmann et al., 2007)

ACE is an exopeptidase that cleaves dipeptides from the C-terminal of peptide substrate. Binding to ACE is mainly affected by the C-terminal tripeptides sequence of the substrate. Though the mechanism is not well known, previous studies have shown the presence of aromatic, hydrophobic and positively charged amino acid residues at the C-terminus has a major influence in the ACE-inhibitory potency of the peptides. Hydrophobic residues tryptophan, tyrosine, phenylalanine and proline were shown to be the most potent ACE inhibitory peptides (Nagpal et al., 2010, Phelan et al., 2011). These inhibitory peptides may bind to the active site of ACE enzyme, thus blocking the binding site of ACE to cleave AT I and subsequently preventing the production of the vasoconstrictor AT II (Nagpal et al., 2010, Phelan et al., 2011, You et al., 2011). Alternatively, these peptides may bind to ACE enzyme, causing a change in the protein

confirmation, preventing the binding of Ang I to the enzyme active site (Phelan et al., 2011).

Figure 2.1. Renin-angiotensin system



Adapted from: Phelan et al (2011)

Table 2.1. Antihypertensive peptides derived from various animal and plant protein

Source	Protease	Bioactivity	Reference
Chickpea (kabuli and desi) hydrolysate Yellow pea (Golden) hydro lysate	Alcalase, flavourzyme, and papain	ACE inhibitory activity in vitro. ACE inhibitory activity is dependent on the type of enzyme used	Barbana et al (2010)
Eggs	Thermolysin and alcalase	ACE inhibitory activity	You et al (2011)
Pork Meat	RPR, KAPVA and PTPVP oral administration to spontaneously hypertensive rats	RPR, KAPVA and PTPVP ACE inhibitory activity RPR with the greatest in vivo activity	Escudero et al (2012)
Bovine (B. taurus) brisket muscle (Pectoralis profundus)	Papa	Brisket sarcoplasmic protein extracts and its 3 kDa filtrates displayed ACE-I inhibitory activity.	Roberta et al (2012)
Gelatin hydrolysate	Thermolysin	ACE inhibitory activity Resistance toward gastrointestinal and mucosal enzymes in vitro Blood pressure lowering effect in vivo AG, AGP, VGP, PY, QY, DY and IY or LY or HO-PY as ACE inhibitory peptides.	Herregods et al (2011)

2.1.2 Immunomodulatory proteins and peptides

Immunomodulation refers to a process associated with the regulation of the immune system, through either stimulation or suppression. Studies have shown fermented milk that contains bioactive peptides has the potential to provide specific health benefits such as the enhancement of the performance of the innate immune system through macrophage activation (Sütas et al., 1996, Tellez et al., 2010). Immunomodulatory food-derived peptides enhance the immune system through the production of lymphocytes, regulation of the phagocytic activities of macrophages, antibody synthesis and cytokine expression (Tellez et al., 2010). Milk-derived peptides have been shown to have positive effects on the cells of the immune system in two ways: 1) Increasing the activity of lymphocytes and stimulation of proliferation, or 2) prevention of lymphocyte activity and decreasing proliferation (Sütas et al., 1996, Tellez et al., 2010). Peptide fractions from fermented milk enhanced immune activity by stimulating macrophages to produce more cytokines, such as IL-6, TNF-α and IL1-b, and nitric oxide (NO) (Qian et al., 2010). In addition, Tellez and colleagues (2010) reported an in vitro immunomodulatory effect of fermented milk by promoting cytokines and nitric oxide production by macrophages and stimulating phagocytic activity. In addition, enzymatically-prepared soybean peptides enhanced immunomodulatory activities by preventing the alopecia induced by cancer chemotherapy. In addition, soy protein hydrolysates obtained with Alcalase and insoluble soy protein exhibited strong immunomodulating activity related to the proliferation of murine splenic lymphocytes and the phagocytic

effect of peritoneal macrophages. Kong et al (2008) reported that soy peptides of lower molecular weight and with a higher content of positive charges enhanced immunomodulating activity.

2.1.3 Anti-inflammatory proteins and peptides

Inflammation is an important biological mechanism that protects the human body and mammals from harmful factors such as pathogens, toxic chemicals and physical injury that may have negative impacts on normal physiological function (Kim et al., 2010). However, chronic inflammation can lead to certain diseases such as arthritis, hepatitis, gastritis, periodontal disease, colitis, atherosclerosis, pneumonia, and neuroinflammatory diseases (Kim et al., 2010, Vidanarachchi et al., 2012). Peptide-mediated anti-inflammatory properties include the modulation of inflammation, binding of toxins, and neutralization of bacteria and fungi (Feng et al., 2010). Studies have documented the anti-inflammatory properties of several food derived peptides, including soybean, milk and pea seed through the inhibition of the generation of proinflammatory responses in macrophages. Nadiaye et al. (2012) reported that enzymatic protein hydrolysates from yellow field pea seeds inhibited the production of pro-inflammatory cytokines TNF-a and IL-6 which are associated with the progression of inflammatory diseases.

2.1.4 Antioxidative proteins and peptides

Reactive oxygen species (ROS) are implicated as being a significant cause of the initiation and/or progression of several chronic diseases, such as

ageing-associated diseases, inflammatory bowel disease, chronic kidney disease, coronary heart disease, atherosclerosis, and cancer (Butterfield et al., 2011, Cadenas et al., 2000, Jadhav et al., 1996, Kruidenier, 2003, Kunwar et al., 2011, Marnett, 2000, Oberg et al., 2004, Sakanaki et al., 2005, Shan et al., 2012, Uchida, 2000, Yu et al., 2004). Antioxidants are important to the human body as they may provide a defense against ROS (Ryan et al., 2011). The dietary consumption of antioxidants may provide aid to endogenous antioxidants in the defence against oxidative stress (Kunwar et al., 2011). Recent studies have shown peptides with antioxidant property are released from food sources, including cow's milk (Kumar et al., 2011), eggs (Chen et al., 2011), soy protein (Amadou et al., 2011), fish (Bougaterf et al., 2009, Najafian et al., 2011), wheat (Koo et al., 2011), marine rotifer (Byun et al., 2009), chickpeas (Yust et al., 2011) and African yam bean (Ajibola et al., 2011).

The exact mechanisms behind the antioxidant effects of food-derived peptides are not fully understood. However, the antioxidant properties of food-derived peptides have been postulated to be a function of the cooperative ability of metal ion chelation, free radical scavenging and singlet oxygen quenching (Chen et al., 2011, Erdmann et al., 2008). The antioxidant function of food protein hydrolysates is dependent on the degree of hydrolysis, the type of enzyme used to generate the peptides, the structural properties of the generated peptides, molecular size, hydrophobicity, and amino acid composition (Chen et al., 2011, Kumar et al., 2011, Najafian et al., 2012).

The amino acid composition of the peptide determines its antioxidant potency. Thus, a peptide rich in amino acids with antioxidant potential will have a strong antioxidant activity. For example, the presence of high amounts of histidine in a peptide can confer antioxidant capacity (Ajiboli et al., 2011, Amadou et al., 2011). The radical-quenching activities of histidine-containing peptides are due to the ability of the imidazole group to donate hydrogen atom, trap lipid peroxyradicals, and chelate metal ions (Chen et al., 1994, Chen et al., 2011, Erdamnn et al., 2008). In addition, aromatic and positively charged amino acid residues in the peptide are important in contributing to the radical-scavenging capacity of peptides through their donation of hydrogen to reactive oxygen species (Ajibola et al., 2011). Furthermore, there is evidence that some aromatic amino acid residues of peptides can enhance the antioxidant activity through their ability to chelate pro-oxidant metal ions and scavenge free radicals. The presence of tryptophan, phenylalanine, glycine and glutamine has been shown to enhance the antioxidant activity of a peptide due to the ability of these amino acids to quench radicals (Chen et al., 2011). For example, tryptophan and phenylalanine have the ability to donate hydrogen to a radical, a characteristic associated to their indolic and phenolic group, respectively (Chen et al., 2011). Additionally, methionine and cysteine have been reported to contribute to the antioxidant activity of a peptide (Chen et al., 2008), through their ability to donate their sulfur hydrogen to radicals. In addition, enhanced cysteine availability contributes to the synthesis of the potent intracellular antioxidant, glutathione (Miesel et al., 2005).

Hydrophobic amino acids also contribute to the antioxidant potency of peptides. Hydrophobic amino acids increase the solubility of peptides leading to an increased interaction between antioxidant peptides and the targets within a hydrophobic atmosphere (Chen et al., 1998, Erdamann et al., 2008). Furthermore, evidence supports the position that the function and antioxidant activity of peptides is largely dependent on the degree of hydrolysis and the type of enzyme used to generate the peptides (Kumar et al., 2011). Upon hydrolysis, smaller peptides and free amino acids are produced, and this is dependent on the specificity of the protease employed and the hydrolysis time (Chen et al., 2011, Erdmann et al., 2008). In addition, the molecular weight (MW) of the peptides is known to influence their antioxidant activity. Short peptides with a MW ranging from 500 to 1500 Da have been reported to possess potent antioxidant activity when compared to higher molecular weight peptides (Amadou et al., 2011, Ajiboli et al 2011, Haiwei, 2010).

2.2 Free Radicals

Reactive oxygen species (ROS) are implicated as one of the significant causes for the initiation or progression of several chronic diseases. Free radicals in the body can contribute to the progression of diseases such as ageing-associated diseases, inflammatory bowel disease, chronic kidney disease, coronary heart disease, atherosclerosis, and cancer (Butterfield et al., 2011, Cadenas et al., 2000, Jadhav et al., 1996, Kruidenier, 2003, Kunwar et al., 2011,

Marnett, 2000, Oberg et al., 2004, Sakanaki et al., 2005, Shan et al., 2012, Uchida, 2000, Yu et al., 2004). ROS, at low concentration have essential physiological functions including; gene expression, cellular growth and defense against infection, proliferation, migration, and metastasis (Kunwar et al., 2011, Shan et al., 2012).

Reactive oxygen species (ROS) are used to characterize a group of oxidants, which are either free radicals or molecular species that have the potential of producing free radicals (Kunwar et al., 2011). The human body is continuously exposed to radicals produced during essential metabolic functions in the body as well as radicals from external sources. During aerobic metabolism, the oxygen produced can be converted to a toxic radical, superoxide ($O_2^{\bullet -}$) anion, through the addition of a single electron (Figure 2.2). Superoxide ($O_2^{\bullet -}$) a cellular by-product, is considered the primary reactive oxygen species, which leads to the formation of other harmful ROS (Kunwar et al., 2011). The majority intracellular ROS produced are $O_2^{\bullet -}$ radicals and $NO^{\bullet}$ radicals (Kunwar et al., 2011). The radical species are converted to hydrogen peroxide (H_2O_2), which goes through Fenton reaction to produce $^{\bullet}OH$ (Kunwar et al., 2011).

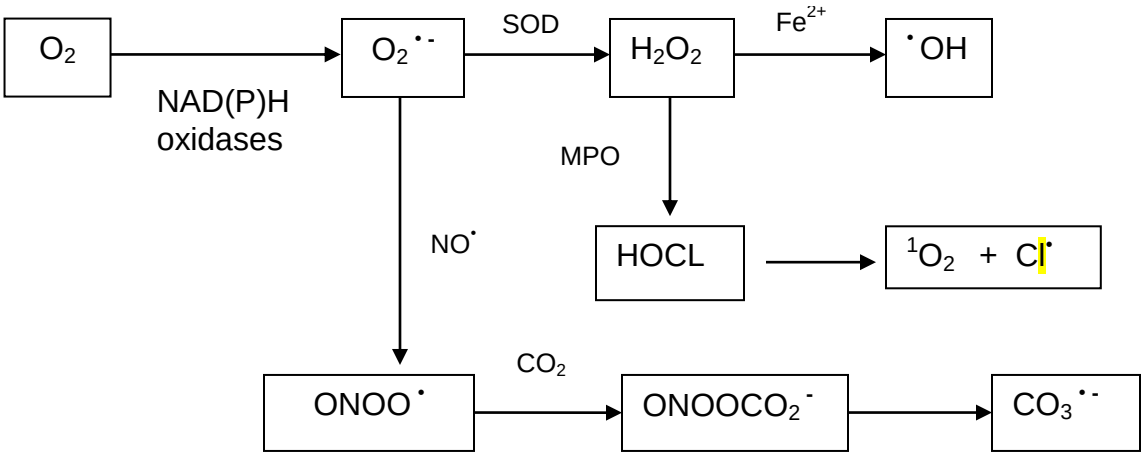
When excessive ROS are released within a biological system it leads to their accumulation causing oxidative stress in the cells (Kunwar et al., 2011). Oxidative stress is caused by the imbalance between the rate of free radical production and the rate at which they are removed by antioxidants (Kunwar et al., 2011, Shan et al., 2012). Oxidative stress maybe caused through infections, as well as exposure to pollutants, UV light, ionizing radiation, increase in ROS level

(Kunwar et al., 2011). Thus, oxidative stress initiates damage to lipids and proteins, and increase the progression of disease. An increased oxidative stress causes a decrease in the mitochondrial performance, which causes the decrease in ATP and necrotic cell death (Shan et al., 2012). The endogenous defences cannot reduce the excess ROS (Kunwar et al., 2011, Shan et al., 2012). The biological system has a defence system to protect the body against ROS mediated oxidative damage. This includes antioxidant defenses. An antioxidant is a molecule that exists at low concentrations and significantly inhibits oxidation of other compounds (Kunwar et al., 2011). Antioxidants have the ability to scavenge reactive oxygen and nitrogen species through the inhibition of radical chain reactions or the formation of radicals (Kunwar et al., 2011). Antioxidants can donate electrons, therefore stabilizing oxidative compound. An antioxidant may function through prevention, interception or repairing process (Kunwar et al., 2011). There are two groups of antioxidant systems; enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants, such as superoxide dismutase, glutathione peroxidase and catalase are present in the body to prevent damage from ROS by catalyzing their conversion to a stable compound. The enzyme superoxide dismutase catalyses the conversion of Superoxide to hydrogen peroxide, which is a long lived molecule that is readily diffusible through cell membranes. Hydrogen peroxide is then catalyzed by the enzymes catalase and Glutathione Peroxidase to oxygen and water, and this allows the survival of the cells. In addition, low molecular weight molecules including glutathione (GSH), α-tocopherol, ascorbate, bilirubin are found inside the cell and

provide secondary defense against free radicals (Kunwar et al., 2011). These antioxidants can scavenge the ROS or inhibit the progression of ROS through chelating of transition metal ions. For example, GSH Glutathione (GSH) is a tripeptide that prevents damage by ROS. Glutathione is synthesized in the body from the amino acids L-cysteine, L-glutamic acid, and glycine. The sulfhydryl (thiol) group of cysteine donates a proton to other unstable molecules, such as reactive oxygen species, thereby inhibiting the progression of ROS.

In addition, non-enzymatic antioxidants such as vitamin E, vitamin C and the carotenoids from dietary sources are important in the protection against ROS damage. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate are added to food products to prevent lipid oxidation (Kunwar et al., 2011, Wanita et al., 1996) and prevent deterioration. However, there are safety concerns over the use of such synthetic antioxidants (Byun et al., 2009).

Figure 2.2. The pathway of free radical formation



Adapted from: Kunwar et al., 2011

2.3 Disease and oxidative stress

ROS mediated oxidization results in the modification of DNA bases, changes in the DNA template, loss of purines, disrupts the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system, and affecting cell division as well as mediating the activation of carcinogens (Kerher, 2000, Kunwar et al., 2011). Thus, leading to the development of cancer and disruption in cell functions (Kerher, 2000).

Lipids provide structural and functional properties in membranes and damage to the lipids may cause cell death. Due to the presence of high concentration of unsaturated fatty acids in membrane lipid components, cellular membranes are easily oxidized by ROS (Kunwar et al., 2011). When membrane lipids are disrupted with ROS, it leads to the formation lipid per oxidation, resulting in formation of lipid hydroperoxide. Lipid hydroperoxide can decompose to various compounds such as malonaldehyde, 4-hydroxy nonenal or form cyclic endoperox-ide, isoprotans, and hydrocarbons. ROS-mediated lipid oxidation may disrupt the membrane fluidity and the formation of lipid-protein bilayers, thereby affecting the functioning of the cell (Kerher, 2000, Kunwar et al., 2011,).

ROS-mediated oxidization of protein results in changes in their tertiary structure, proteolytic degradation, protein-protein cross linkages and destruction of the protein (Kunwar et al., 2011). Protein oxidation results in the formation of aldehydes and ketones (Kunwar et al., 2011). ROS-mediated protein oxidation leads to the formation of reactive products such as protein hydroperoxides, which

may further lead to the production of radicals upon reacting with transition metal ions (Kerher, 2000). Most oxidized proteins are functionally inactive and are rapidly removed from the biological system. However, when oxidized protein accumulates in the biological system they add to the damage associated with aging, diabetes, atherosclerosis and neurodegenerative diseases (Kerher, 2000).

2.4. Food protein derived peptides and antioxidant activity

The neutralization of free radicals is one of the most important approach by which bioactive peptides inhibits oxidative reactions. The formation of radicals involves several mechanisms and the free radicals can be stabilized through different mechanisms. Thus, multiple methods are endorsed for evaluating the antioxidant property of natural compounds. The antioxidant properties of food-derived peptides have been attributed to their ability to scavenge free radicals, act as hydrogen donor, transition metal chelating activity as well as ferric reducing power (Amadou et al., 2011, Udenigwe et al., 2012). Various methods have been used to study the antioxidant property of several food-derived antioxidants. In the present study, the antioxidant activity of Hempseed protein Hydrolysate and its fractionates were determined using several antioxidant evaluation systems including DPPH Scavenging activity, Metal ions chelating activity, Ferric reducing power, Hydroxyl scavenging activity and Superoxide scavenging activity. All antioxidant analyses were compared to reduced L-glutathione (GSH) as a standard.

2.4.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH is a stable radical of organic nitrogen, with a free electron, and is widely used to study the antioxidant activity of natural compounds (Molyneux, 2004; Monica et al., 1999). The DPPH radical is used to measure the ability of natural compounds to donate electrons or hydrogen to form a more stable compound. The DPPH radical is oil soluble and is stable in methanol and can act as oxidizing substrate as well as the reaction indicator molecule. The DPPH assay method is easy, fast and important to measure the activity of antioxidants compounds at maximum absorbance of 515–520 nm (Monica et al., 1999). Antioxidants donate hydrogen to radicals to form a stable molecule, and inhibit the formation of other toxic radicals. When the DPPH radical reacts with hydrogen donor or free radical scavengers, it become reduced causing change in the purple color and decrease in absorption strength (Molyneux P., 2004, Monica et al., 1999). Thus, the decrease in absorbance represents DPPH-scavenging activity. When the DPPH radicals accept an electron from antioxidants, they become a stable product. A low absorbance at 517 nm indicates a strong DPPH scavenging activity. The DPPH assay had been used to evaluate antioxidant activity of various natural compounds

2.4.2 Ferric Reducing Power Activity

The ferric reducing power is used to measure the ability of compound to donate an electron to a free radical compound to form a more stable compound.

The oxidation or reduction of radicals to ions inhibits the radical chains, and the reducing power indicates the ability of compounds to achieve redox state (Prior et al., 2005). Ferrous ions are responsible for the Haber-Weiss reaction leading to the formation of toxic hydroxyl radical which causes damages to cells (James K., 2000). Transition metal ions may catalyze Haber–Weiss reaction; however the iron-catalyzed Haber–Weiss reaction, through the Fenton pathway, is considered to be the major mechanism by which the highly toxic hydroxyl radical is generated in the biological systems (James K., 2000). The Haber-Weiss reaction generates hydroxyl radicals (•OH) from hydrogen peroxide (H_2O_2) and superoxide ($\bullet O_2^-$) in the biological system in a two step reaction. The first step involves reduction of ferric ion to ferrous followed by the second step, Fenton reaction (Prior et al., 2005).

Step 1

$$FE^{3+} + O^{\cdot}_2 \longrightarrow Fe^{2+} + O_2$$

Step 2

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

Studies have shown that antioxidant activity and reducing power are directly related. The FRAP mechanism is involved in the electron transfer reaction, and thus in coalition with other antioxidant assay methods, it can be used to study the different antioxidant mechanisms (Prior et al., 2005). The FRAP assay evaluates the reducing ability based upon the ferric ion (Prior et al.,

2005). The reducing power assay, which is a simple and fast method, can be used to evaluate the antioxidant activity of proteins and peptides. Upon the reduction of ferric Fe^{3+} -ferricyanide complex to the ferrous (Fe II) form, the color of the solution changes from yellow to blue with an absorption maximum of 700nm. Thus, high absorbance indicates strong reducing power.

2.4.3 Superoxide scavenging activity

Superoxide radical is associated in many pathological conditions such as inflammatory tissue damage in ischaemia-reperfusion, arthritis, gout, and gastric ulceration (Paduraru et al, 2008). Superoxide radical has a low reactivity and does not directly initiate lipid oxidation. However, during Fenton reaction, Superoxide can form toxic radicals such as hydrogen peroxide and hydroxyl radical. During aerobic metabolism, oxygen produced can be converted to a toxic radical, superoxide radical ($O_2^{\bullet -}$) radicals anion through the addition of a single electron. Superoxide ($O_2^{\bullet -}$) radicals, a cellular by-product of aerobic metabolism is considered the primary reactive oxygen species, which leads to the formation of other harmful reactive oxygen species (ROS). The enzyme superoxide dismutase catalyses the conversion of Superoxide to hydrogen peroxide, which is a long lived molecule that is readily diffusible through cell membranes. Thus, the scavenging of Superoxide radical thorough the use of antioxidants peptides may prevent ROS generation and therefore inhibit oxidative damage to biological cells.

2.4.4 Metal chelating activity

One strategy to prevent ROS generation is through chelating of transition metal ions. Metal ion catalysts are the most important initiators of lipid oxidation in foods and biological systems. Transition metal ions, such as Fe^{2+} and Cu^{2+} catalyze the formation and progression of reactive oxygen species which causes biological damage (Paduraru et al, 2008, Prior et al., 2005). In addition, Fe^{2+} can catalyze the Haber-Weiss reaction to form toxic hydroxyl radicals. Thus, the chelating of transition metal ions thorough the use of antioxidants peptides may prevent the oxidation reaction associated with redox active metal catalysis.

2.4.5 Hydroxyl radical scavenging activity of food derived peptides

One of the most effective defense against disease associated with hydroxyl radical is through its inhibition. Hydroxyl radical is the most reactive species and is involved in lipid peroxidation and can damage proteins, DNA, polyunsaturated fatty acids, nucleic acids and may lead to the development of cancer and several diseases (Kehrer 2000, Paduraru et al, 2008). Hydroxyl radical can disrupt purine and pyrimidine bases and 2-deoxyribose, leading to mutation which is a significant factor in the cause of carcinogenesis, neurodegenerative and cardiovascular diseases (Kehrer 2000, Paduraru et al, 2008). Hydroxyl is formed from superoxide anion and hydrogen peroxide, in the

presence of metal ions. Thus, the scavenging of Hydroxyl radical thorough the use of antioxidants peptides may prevent oxidative damage to biological cells.

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

Table 2.2 Antioxidant peptides derived from various animal and plant protein

Food Source	Treatment	Function	Reference
H. discus hannai Ino foot muscle and P. yessoensis adductor muscle Hydrolysate	Papain and neutral protease respectively	Hydrolysates exhibited DPPH, hydroxyl radicals scavenging abilities, reducing power, and ferrous ion chelating capacity.	Zhou et al (2012)
Bovine Brisket Sarcoplasmic proteins	Papain	Antioxidant activity was observed fractions Hydrolysate, 10-kDa- and 3-kDa analyzed using the DPPH, FRAP and Fe2+ chelating ability assays.	Bernardini et al (2012)
Egg white protein hydrolysate	Papain	Identified amino acid sequences Tyr-Leu-Gly-Ala-Lys and Gly–Gly-Leu-Glu-Pro-Ile-Asn-Phe-Gln. The antioxidant activity of egg white protein hydrolysates was influenced by the time of hydrolysis and the type of enzyme used for hydrolysis.	Chen et al (2011)
Eggs	Thermolysin and alcalase	Antioxidant activities correlated with the proportion of low molecular weight peptides under 500 Da.	You et al (2011)
Fermented soy meal Protein Hydrolysate	*Lactobacillus plantarum* LP6	The fraction with the higher antioxidant activities observed was attributed to its molecular weight distribution of peptide between 370 to 1500 Da peptides size, high percentages of antioxidative amino acid residues present, free amino acids and hydrophobicity.	Amadou et al (2011)
African Yam Bean Seed	Alcalase	<1 kDa peptides exhibited significantly better ferric reducing power, diphenyl-1-picryhydradzyl (DPPH) and hydroxyl radical scavenging activities when compared to peptide fractions of higher molecular weights.	Ajibola et al (2011)

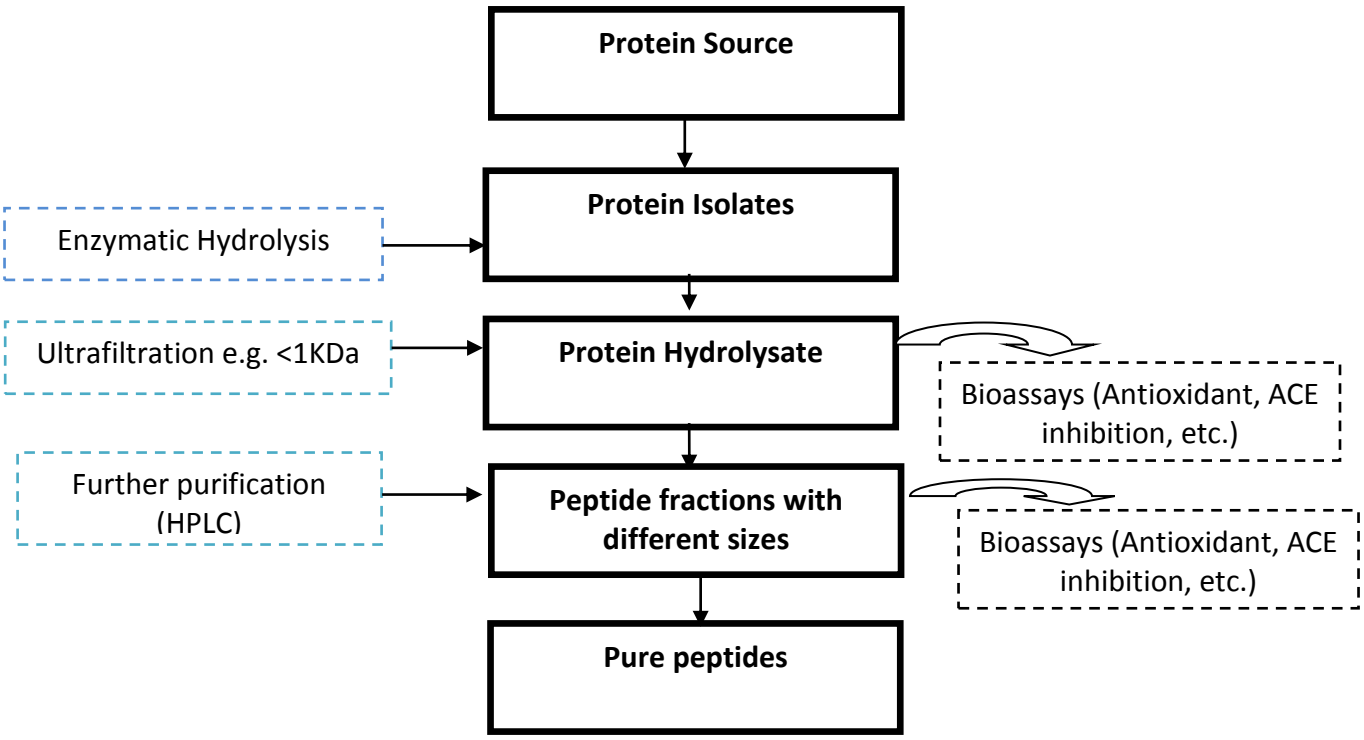
Black Soybean	Alkali protease neutral protease .	The fraction with strongest fraction showed high hydrophobic amino acids (HAA) content and hydrophobicity. The molecular weight distribution was between 100 to 1,000 Da. The antioxidant activity is the amino acid composition, the content of HAA and the molecular mass.	Haiwei (2010)
Chickpeas	Alcalase and flavourzyme	Chickpea hydrolysate showed better antioxidant activity in reducing power and DPPH scavenging effect when compared to chickpea protein isolate.	Yust et al (2011)

2.5 Production of bioactive peptides

Bioactive peptides may be encrypted in the amino acid sequence of a parent protein. The peptides are inactive within the sequence of the parent protein but can be released when hydrolyzed by digestive enzymes, microbial enzymes or during food processing. Several methods of producing bioactive peptides include solvent extraction, enzymatic hydrolysis and microbial fermentation of food proteins. Enzymatic hydrolysis of protein is the most commonly used method (Najafian et al., 2011, Ryan et al., 2011). One of the shortcomings with other methods is the presence of chemical residue in the finished product. In addition, low efficiency and air pollution are associated with the solvent extraction method (Najafian et al., 2011). The use of enzymatic hydrolysis technique is more specific, and thus allows the control of the degree of hydrolysis (Vijai et al., 2010).

The hydrolysis of food proteins can be achieved with enzymes from animal source (pancreatin), plant source (papain) or bacterial and fungal proteases to release defined peptides. After hydrolysis, the peptides are put through membrane ultrafiltration (figure 2.3). Ultrafiltration is a separation process using membranes with determined pore sizes. This process removes high molecular-weight substances and concentrates peptides of defined molecular weight ranges, helping to obtain low molecular weight peptides. Furthermore, reverse phase HPLC on a hydrophobic column matrix can be used to fractionate peptides based on their hydrophobic properties (Najafian et al., 2011, Vijai et al., 2010).

Figure 2.3. Procedure for the production of bioactive peptides from food proteins



Adapted from: Najafian et al., (2011): Ryan et al., (2011)

2.6. Enzymatic Protein Hydrolysis

Protein hydrolysates are formed during the hydrolysis of proteins using different methods. Several methods maybe used in the hydrolysis of proteins 1) hydrolysis using digestive enzymes 2) proteases from plant or bacterial 3) microbial fermentation (Ryan et al., 2011) to produce biological active peptides. Enzymatic hydrolysis of food proteins is the most commonly used method for producing bioactive peptides. Enzymatic hydrolysis of food protein may be accomplished using one enzyme or a combination of several enzymes. There are many factors that affect the hydrolysis process including time, temperature, pH, enzyme/substrate ratio and these factors must be adjusted to achieve optimized enzyme activity (Kamau et al., 2010, Vijai et al. 2010). The type of enzyme used in enzymatic protein hydrolysis is very important because it dictates the cleavage patterns of the peptide bonds (Chen et al., 2011). Protein hydrolysates contain different side chains depending on the enzyme used and the peptides may possess specific physiological roles in Humans (Vijai et al., 2010). Various enzymes have been used to obtain bioactive peptides from food sources including pepsin, pancreatin, protease S, M or P, trypsin, chymotrypsin, thermolysin, papain and alcalase. The enzyme used in this work to hydrolyze hemp proteins into peptides that can potentially act as ingredients in the formulation of functional foods and nutraceuticals is Proteinase K. To the best of our knowledge, there is no information in the literature regarding the identification of antioxidative agents from enzymatically prepared hempseed protein hydrolysate using Proteinase K. In the current study, protein hydrolysis was

initiated by adding Proteinase K at a ratio of 1-4% by weight of protein in the hempseed protein isolate. The 1% concentration of Proteinase K was used to initiate minimum hydrolysis and above 4% concentration of Proteinase K , the proteinase will become saturated by the substrate (Aluko, 2009). Proteinase K is a stable serine protease with broad substrate specificity. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic (isoleucine, valine, leucine, alanine, proline) and aromatic (phenylalanine, tryptophan, tyrosine) amino acids; and the hydrophobic amino acids of the proteins (Roche Product Catalogue, 1996-2011). Thus, due to its broad substrate specificity, it is noteworthy that this enzyme can be used to hydrolyse hempseed protein to release bioactive peptides with antioxidant property.

2.7. Hemp seed protein and amino acid profile

Hemp, also known as *Cannabis sativa L*., is one of the ancient cultivated plants, and has been used for the past 10,000 years as a source of food, medicine and fibre (Agriculture and Rural Development, 2011). Hemp seed is becoming an important commodity to the Canadian economy. Currently, in Canada, the main producers of industrial hemp are the provinces of Manitoba and Saskatchewan (Agriculture and Agri-food Canada, 2007). Cannabis, a diverse plant species, has more than 500 different varieties (Canadian Hemp Trade Alliance, 2012). Although industrial hemp and marijuana belong to the same species of *Cannabis sativa L*., there is a difference between the two of them. Industrial hemp contains very low levels of tetrahydrocannabinol (THC), which is the main psychoactive compound present in marijuana. Due to the low

content of THC, consumption of hemp seed products does not yield psychoactive effects. Thus, unlike marijuana, commercial hemp became legal in Canada in 1998, following a 60-year ban. Since this legalization, hemp seed is becoming an important agricultural commodity to the Canadian economy and to Canadian farmers. In Canada, hemp production is regulated by Health Canada. Hemp producers and manufacturers must have licenses from Health Canada. The regulation requires that cultivated hemp plants must contain less than 0.3% THC. Hemp types currently being cultivated include Alyssa, Anka, CRS–1, CFX–1, CFX–2, Delores and Finola (Canadian Hemp Trade Alliance, 2012).

Due to its numerous nutritional benefits, many new food products containing hemp seed and hemp seed oil are available in the market. Currently, a number of hemp processors have established themselves in Manitoba, including Hemp oil Canada, Manitoba Harvest and Hempco. Hemp seed products include the shelled seed (hempseed nut), hemp seed nut butter, cold-pressed hemp seed oil, hempseed flour and protein powders. Hempseed is considered to be a significant source of protein, consisting of 24% protein by weight (Table 2.3; House et al., 2010). Hempseed has high quality storage proteins, edestin and albumin, which are easily digested (Da Patro et al., 2011). Hemp seed protein contains appreciable amount of all essential amino acids necessary for the human body, limited only by its lysine content (House et al., 2010). The amino acid profile of hempseed protein, relative to other high quality proteins, is given in Table 2.4. Hempseed protein has high amount of arginine, glutamic acid, methionine and cystine, and histidine (Callaway 2004, House et al., 2010). Based

on the FAO/ WHO reference protein for children 2-5 years of age, lysine is the first limiting amino acid in hemp protein (House et al., 2010).

Hempseed contains 30% oil (House et al., 2010), with a fatty acid profile of 81% PUFAs consisting of 59.6% linoleic acid (ω-6), 3.4% γ-linolenic (ω-3), and 18% α-linolenic (ω-6) (Da Patro et al., 2011). Hence, hempseed is a good source of the essential fatty acids linoleic acid (omega-6) and alpha-linolenic acid (omega-3). Moreover, the omega-6 to omega-3 ratio in hempseed oil ranges between 2:1 and 3:1, which is found to be optimal for human health (Da Patro et al., 2011).

Table 2.3. Nutritional content (%) of hempseed

	Whole seed	Seed meal
Oil	30	10
Protein	24	40.7
Ash	4.8	6.7
Gross Energy Value (MJ/Kg)	24.2	20.4
Acid Detergent fiber	23	21.5
Neutral Detergent Fiber.	32	30.5

Adapted form: House et al. (2012)

Table 2.4. Amino acid content (%) of some protein sources compared to hempseed

Amino acid	Soybean	Egg white	Hempseed
Alanine	1.39	0.83	1.28
Arginine	2.14	0.68	3.10
Aspartic acid	3.62	1.23	2.78
Cystine	0.54	0.29	0.41
Glutamic acid	5.89	1.67	4.57
Glycine	1.29	0.50	1.14
Histidine	0.76	0.28	0.71
Isoleucine	1.62	0.74	0.98
Leucine	2.58	1.08	1.72
Lysine	1.73	0.74	1.03
Methionine	0.53	0.47	0.58
Phenylalanine	1.78	0.76	1.17
Proline	1.65	0.5	1.15
Serine	1.54	0.92	1.27
Threonine	1.35	0.58	0.88
Tryptophan	0.42	0.2	0.2
Tyrosine	1.14	0.46	0.86
Valine	1.60	0.98	1.28

Individual amino acid values for each food are given in grams per 100 g.
Adapted from: Callaway (2004)

2.8. Biological activities of hempseed products

Peptides derived from hempseed by means of enzymatic hydrolysis have been shown to exert several biological benefits. The enzymatic hydrolysis of hempseed, using a combination of pepsin and pancreatin, produced purified peptides (molecular weight of 1, 1–3, 3–5, and 5–10 kDa) which exhibited *in vitro* antioxidant activities (Girgih et al., 2011). The membrane fraction of <1 kDa, 3-5kDa and 5–10 kDa exhibited DPPH scavenging activity of 24%, 22% and 18% respectively at 1mg/ml when compared to reduced GSH with DPPH scavenging activity of 28.2% (Girgih et al., 2011). Furthermore, Girgih observed Fe^{2+} chelating activity of HPH with chelating capacity of 72% as compared to membrane fraction of 3-5kDa and 5–10 kDa which showed 29% and 38.5% Fe^{2+} chelating activity respectively. Additionally, Wang et al., (2009) studied the antioxidant property of peptides derived from hemp protein following hydrolysis by Neutrase. Hemp protein hydrolysates with the highest DPPH radical scavenging ability exhibited IC_{50} value of 2.3–2.4 mg/mL observed at 240 min, and Fe^{2+} chelating ability with IC_{50} value of 1.7–1.8 mg/mL observed at 60–180 min. Wang et al., (2009) also observed a decrease in IC_{50} value of the hydrolysates to scavenge DPPH radical with increasing hydrolysis time from 60 (IC_{50}= 3.3 mg/mL) to 240 min (IC_{50}= 2.3 mg/mL). Thus, Wang and colleagues (2009) concluded that increased enzymatic hydrolysis of Hemp protein with Neutrase released more hydrophobic peptides that have the ability to scavenge DPPH radical. In addition, it was reported that hydrolysates with a higher

hydrophobic amino acid content possessed higher DPPH radical scavenging and Fe^{2+} chelating abilities (Wang et al., 2009). Furthermore, a positive correlation between DPPH radical scavenging ability and surface hydrophobicity of the hydrolysates with correlation coefficient of 0.973 was reported (Wang et al., 2009). Chuan et al. (2009) studied the antioxidant properties of hemp protein hydrolysates, derived using a multi-enzyme approach, and reported DPPH scavenging activity and reducing power associated with the hydrolysates. Chuan et al. (2009) concluded that the resultant antioxidant activity depends on the nature of the enzyme used. Chuan and colleagues (2009) observed DPPH radical scavenging ability of hemp protein hydrolysates were dependent on the type of proteases used and the enzymatic hydrolysis time. For example, hydrolysates obtained using protomax for 2 h hydrolysis time, exhibited the strongest radical scavenging ability (IC_{50} = 2.8 mg/mL), when compared to hydrolysates obtained from pepsin and trypsin with the same hydrolysis time. (IC_{50} = 5.5–5.7 mg/mL). In addition, Increase in hydrolysis time from 2h to 4 h with Neutrase, increased the DPPH radical scavenging ability (IC_{50}= 3.3-2.3 mg/mL). In contrast, increase in hydrolysis time from 2h to 4h using flavourzyme decreased DPPH scavenging activity (IC_{50}3.5-3.8), pepsin (IC_{50}5.5-6.3) and protamex (IC_{50}2.8-3.3). Enzymatically prepared hempseed protein hydrolysates obtained by Alcalase, followed by gel filtration and reversed-phase high performance liquid chromatography, resulted in two purified peptide fractions with antioxidant activity (Rong et al., 2010). The purified peptides, at a concentration of 10 µg/ml, showed defensive effects against cell death and oxidative apoptosis

in hydrogen peroxide-induced cell apoptosis in a rat pheochromocytoma line PC12 cell system (Rong et al., 2010).

Antihypertensive effects of Hempseed Protein hydrolysate (HPH) obtained with pepsin and pancreatin hydrolysis have also been reported. In a study by Girgih et al (2011), HPH showed *in vitro* inhibition of angiotensin I-converting enzyme (IC_{50} values at 0.67 mg/mL and renin activities (IC_{50} values of 0.81). In addition, Girgih et al (2011) reported at 200 mg/kg body weight oral administration of HPH to spontaneously hypertensive rats showed a decrease in systolic blood pressure of -20mmHg and -30 mmHg after 4 and 8 h respectively.

CHAPTER THREE

3.0. Materials and Methods

3.1. Materials

Defatted hempseed protein powder (HPP) or hempseed meal was obtained from Manitoba Harvest Fresh Hemp Foods Ltd (Winnipeg, MB, Canada). The enzyme Proteinase K, recombinant, PCR grade Lyophilizate (from Pichia pastoris), was purchased from Roche Diagnostic GmbH (Indianapolis, USA). The ultrafiltration membranes of 1 kDa and 3 kDa molecular weight cut off (MWCO) were purchased from Fisher Scientific (Oakville, ON, Canada). Glutathione, pyrogallol (1,2,3-trihydroxybenzene), Triton X-100, 2,2-diphenyl-1-1-picrylhdrazyl (DPPH), hydrogen peroxide, ethylenediaminetetraacetic acid (EDTA), ferrous sulphate, potassium ferricyanide, trochloroacetic acid (TCA), ferrous chloride, 1,10-phenanthroline, 3-(2-pyridyl)-5, 6-diphenyl-1,2,4-triazine-4,4-disulfide acid sodium salt (ferrozine) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all other chemicals reagents were of analytical grade.

3.2. Preparation of hempseed protein isolate

Hemp protein isolate (HPI) was prepared from defatted HPP as described by Tang et al. (2006), with slight modifications. The HPP was dispersed in deionized water to obtain (1:20, w/v) slurry. The mixture was adjusted to pH 10.0 using 2 M NaOH and the temperature was maintained at 37°C while stirring with magnetic stirrer for 2 hrs. The resultant dispersion after 2 hrs was centrifuged for 60 minutes at 7000xg and at 4 degree Celsius. The supernatant was collected and

filtered using cheese cloth and the pellet were discarded. The pH of the filtered supernatant was adjusted to 5.0 by gradually adding 2 M HCL to precipitate the protein. The precipitated protein was centrifuged for 20 minutes at 10,000xg at 10^{o}C. The resulting precipitate was dispersed in distilled water while the supernatant was discarded. The dispersion was adjusted to pH 7.0 using 2 M NaOH and then freeze-dried to obtain the protein isolate. The HPI was then decolourized with acetone to remove any polyphenol compounds that may contribute to the antioxidant functions and was freeze dried. A volume of 50ml of acetone was added to 5g of HPI and mixed in the fume hood for 2 hrs. The acetone was poured out and another 50 ml of acetone was added to the HPI and mixed for 1hr in the fume hood. After 1 hr, the acetone was poured out, and the HPI was spread to dry in the fume hood overnight. The protein concentration of the decolourized HPI was determined using the modified Lowry method (Markwell et al., 1978). Peptide sample (10 mg/ml) was mixed thoroughly in water . Similarly, the standard, bovine serum albumin (10mg/ml) was also dissolved and mixed thoroughly in water. Reagent C was prepared by mixing 100 parts of Reagent A consisting 2% Na_2CO_3, 1% sodium dodecyl sulfate, 0.4% sodium hydroxide and 0.16% sodium tartrate with 1 part of Reagent B consisting of 4% $CuSO_4 5H_2O$ dissolved in distilled water. Reagent C (3ml) was added to 1 ml of peptide samples and BSA. The mixed solutions were incubated at room temperature for 1 hour. Next, 0.3 ml of Folin Ciocalteu phenol (1 part Folin Ciocalteu reagent with 1 part distilled water) was added to protein samples, mixed vigorously and was incubated for 45 minutes at room temperature. The

absorbance was measured at 66 nm spectrophotometrically. A standard curve using bovine serum albumin (BSA) and peptide samples was prepared with peptide concentrations (10-100 µg/ml). The standard curve was plotted as absorbance (y) against protein concentration (x) of BSA.

3.3. Enzymatic hydrolysis of hempseed protein isolate

Hemp protein was dispersed in distilled water to obtain 5% (w/v) protein slurry. The slurry was heated to 37°C and the pH was adjusted to 8.0 with 1M NaOH. Protein hydrolysis was initiated by adding Proteinase K at a ratio of 1-4% by weight of protein in the hempseed protein isolate. Four protein hydrolysates were prepared using 1%, 2%, 3%, or 4% of proteinase K. The temperature (37°C) and pH (8.0) were maintained constant for 4 h by gradually adding 1 M Na_2CO_3 solution and using a pHstat instrument (Metrohm Titrando, Herisau, Switzerland). After 4 h of hydrolysis, the reaction was terminated by heat treatment by inserting the reaction beaker in a boiling water bath for 15 minutes. Thereafter, the hydrolysates were cooled to room temperature and centrifuged at 7,000xg for 30 min at 4°C. The resulting hemp protein hydrolysates were freeze-dried and stored at -20°C. Four separate hemp protein hydrolysates were obtained and the weight of each resulting hydrolysate was recorded. The protein concentration of the hydrolysate was determined using the modified Lowry method (Markwell et al., 1978) as described above. Each hydrolysate was evaluated for antioxidant activity as described below.

3.4. Fractionation of hemp protein hydrolysates

The hemp protein hydrolysate obtained from 1% proteinase K was separated using membrane ultrafiltration into Low Molecular Weight (LMW) fractions using an Amicon stirred ultrafiltration cell (Millipore Corporation, Bedford, MA, USA). First, the hydrolysate was passed through ultrafiltration membranes with MWCO of <1 kDa, then the retentate was collected and passed through a 3 kDa membrane to obtain peptides of sizes 1-3 kDa. The resulting permeates (<1 kDa and 1-3 kDa) were lyophilized. The peptide fractions of 1 kDa and 1-3 kDa were stored at -20^{o}C. The protein concentration of the peptide fractionates (1 kDa and 1- 3 kDa) was determined using the modified Lowry protein assay method (Markwell et al., 1978), and were evaluated for antioxidant activity as described below.

3.5. Evaluation of antioxidant properties

3.5.1. DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay described by Hou et al (2001) and Ajibola et al (2011) was used to evaluate the antioxidant activity of the HPH using 96- well clear flat bottom plate. Peptide samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to obtain a final concentration of 100 µM. Peptide samples of 100 µL and 100 µl of DPPH solution were mixed to obtain a final assay concentration of 1 mg/ml, and the resulting solution was incubated in the

dark for 30 min at room temperature. A blank control (consisting DPPH and sodium phosphate buffer) and a standard (consisting of glutathione and phosphate buffer and DPPH solution) to obtain a final assay concentration of 1 mg/ml were prepared. The absorbance was read at 517 nm using a spectrophotometre and the scavenging activity of the peptide samples was calculated and compared to the activity of glutathione. A low absorbance at 517 nm shows a strong DPPH scavenging activity.

The DPPH radical scavenging activity of the peptide samples and glutathione was calculated as a percentage using the following equation:

DPPH Radical Scavenging Activity (%) =

$$\frac{(\text{Absorbance}_{517\text{ nm Control}} - \text{Absorbance}_{517\text{ nm Sample}})}{\text{Absorbance}_{517\text{ nm Control}}} \times 100\%$$

3.5.2. Chelation of metal ions

The metal chelation activity was based on the method by Xie et al (2008) with slight modification. One ml of sample solution of peptides or glutathione was mixed with 0.05 ml of an iron dichloride solution (2 mM/L), 1.85 ml double distilled water and 0.1 ml of 5 mM/L FerroZine solution [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4'-disulfonic acid sodium salt] solution was added and mixed vigorously. As a control sample, 1 mL of doubled distilled water was used instead of peptide samples. The mixtures were incubated at room temperature for 10 minutes. Following a reaction time of 10 min, an aliquot of 200 µL was taken and transferred into a 96- well clear flat bottom plate and the absorbance

measured spectrophotometrically at 562 nm. The concentration of peptide needed to inhibit 50% of the metal chelation activity was expressed as the effective concentration (EC_{50}). A low absorbance represented a high ferrous ion chelating ability for the test samples.

The chelating ability of the peptides was calculated as a percentage using the following equation:

$$\text{Metal Chelating effect (\%)}: \frac{(\text{Absorbance}_{562\text{ nm Sample}} - \text{Absorbance}_{562\text{ nm Control}})}{\text{Absorbance}_{562\text{ nm Control}}} \times 100\%$$

3.5.3. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured using the method developed by de Avellar et al. (2004), with slight modification. Peptide samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.4), 3 mM of 1,10-phenanthroline was dissolved in 0.1 M sodium phosphate buffer (pH 7.4), $FeSO_4$ (3 mM) was dissolved in distilled water, and 0.01% hydrogen peroxide was dissolved in distilled water. To start the Fenton reaction, solutions were transferred into a clear, flat bottom 96-well plate in the following order: 1) 50 µL of peptide samples (final assay concentration of 1 mg/mL); 2) 50 µL of 1,10-phenanthroline; 3) 50 µL of $FeSO_4$; and 4) 50 µL of hydrogen peroxide. The mixtures were incubated at 37^0C for 1 h with shaking. Blank (1,10-phenanthroline, $FeSO_4$ (3 mM)) and control (1,10-phenanthroline, $FeSO_4$ (3 mM) , and hydrogen peroxide) mixtures were also prepared. Using a spectrophotometer set at a wavelength of 536 nm, the absorbance was

measured at 10 min intervals for a period of 1h. The hydroxyl radical scavenging activity as a percentage was calculated using the following equation:

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

3.5.4. Superoxide radical scavenging assay

The superoxide radical scavenging activity of the hempseed peptides was evaluated using the method of Udenigwe et al. (2009). A volume of 80 µL of peptide fraction (final concentration of 1mg/ml) or glutathione were mixed with 80 µL of 50 mM Tris-HCl buffer (pH 8.3), containing 1 mM EDTA, directly into a 96 well plate, followed by the addition of 40 µL of 1mM pyrogallol dissolved in 10 mM HCl. Absorbance was measured at 420 nm for four minutes at room temperature spectrophotometically. Tris-HCl buffer was used as a control.. The superoxide scavenging activity of the peptides was calculated as a percentage using the following equation:

$$\text{Superoxide Scavenging Activity (\%)} = \frac{(\Delta\ A/\text{minute control} - \Delta\ A/\text{minute sample})}{\Delta\ A/\text{minute control}} \times 100$$

3.5.5. Ferric reducing power assay

The method described by Zhang et al. (2008) and Theodore et al. (2008), with slight modifications, was used to evaluate the reducing power of the hempseed peptides. Peptide solutions (250 µL) or glutathione (prepared in 250 µL phosphate buffer), with final concentration 1 mg/ml, were mixed with 250 µL phosphate buffer (pH 6.6, 0.2 M) and 250 µL 1% potassium ferricyanide solution.

As a control, water was substituted in place of the peptide samples. The solutions were mixed and heated to a temperature of 50^{o}C for 20 minutes. Thereafter, 250 µL of 10% trichloroacetic acid was added to the mixture. Then, 250 µL of the resulting solution was mixed with 50 µL of 0.1% ferric chloride and 200 µL distilled water. After 10 minute incubation at room temperature, the solution was centrifuged at 1000xg. Finally, an aliquot (250 µL) of the supernatant was put into a clear bottom 96-well plate and the absorbance of was measured at 700 nm.

3.6. Data and statistical analysis

The results are expressed as means ± standard deviation of triplicate determinations. In addition, the samples were analyzed at a final concentration of 1mg/ml. All antioxidant analyses were compared to reduced glutathione (GTH) as a standard. Statistical analysis was done using SAS (Statistical Analysis Software 9.3) using one-way ANOVA. Duncan's multiple-range test was used to compare means. Bars with different letters are significantly different at $p<0.05$.

CHAPTER 4

4.0. Results

4.1 Hempseed protein isolates and protein content

The hempseed protein isolates (HPI) had an initial light green colour. After being subjected to hydrolysis with proteinase K and freeze drying, the hydrolysate appeared creamy brown in colour. The HPH obtained with 1% concentration Proteinase K was further subjected to fractionation using ultrafiltration to obtain low molecular weight peptides of <1KDa and 1-3KDa.

4.2 Enzymatic hydrolysis of hemp seed isolates

The percent yield of HPH was determined as the ratio of peptide weight of the lyophilized HPH to the protein weight of the native HPI, on a dry weight basis. The peptide contents of the HPH samples, as obtained through the use of different concentrations of proteinase K (1%, 2%, 3% and 4% by weight of protein in the hempseed protein isolate), are given in table 4.1. The protein yields of the HPH treatments were 63%, 78%, 79%, and 85% at 1%, 2%, 3% and 4% concentrations of Proteinase K, respectively. The HPH obtained with 4% Proteinase K was significantly higher ($P < 0.05$) than the value (63%) of the HPH obtained with 1% Proteinase K (Table 4.1).

Table 4.1: Protein yield of HPH as affected by the concentration of Proteinase K used during hydrolysis

% Proteinase K	Percent of protein yield (%)
1%	$63.4^{b} \pm 2.17$
2%	$78.3^{a} \pm 1.13$
3%	$79.7^{a} \pm 0.09$
4%	$85.0^{a} \pm 0.12$

Results represent the mean of triplicate samples, (means ± standard deviation) with different letters having mean values that are significantly different at $p<0.05$.

4.3 Peptide fractionation and protein yield

The HPH was fractionated using ultrafiltration to obtain low molecular weight peptides of <1KDa and 1KDa-3KDa. The percent protein yields of the ultrafiltration membrane fractions are shown in Table 4.2. The protein yield of the 1KDa and 3KDa peptide fractions was calculated as the ratio of the peptide content of the peptide permeates to the peptide content of the HPH as determined by modified Lowery method. The ultrafiltration process that produced the 1kDa and 1-3 kDa peptides had protein yields of 47% and 30% respectively (Table 4.2).

Table 4.2: Protein yield (%), as determined by the Lowry method, of HPH fractions subjected to ultrafiltration using different molecular weight cut-offs (<1kDa and 1kDa-3kDa).

Peptide fractions	Protein yield (%)
<1 kDa	46^{a}± 0.94
1-3 kDa	30^{b}± 1.27
Retentate	21^{c}± 0.54

Results represent the mean of triplicate samples, (means ± standard deviation) with different letters having mean values that are significantly different at $p<0.05$.

4.4. Antioxidant activity evaluation of hempseed protein hydrolysates (HPH) derived by varying the concentration of Proteinase K

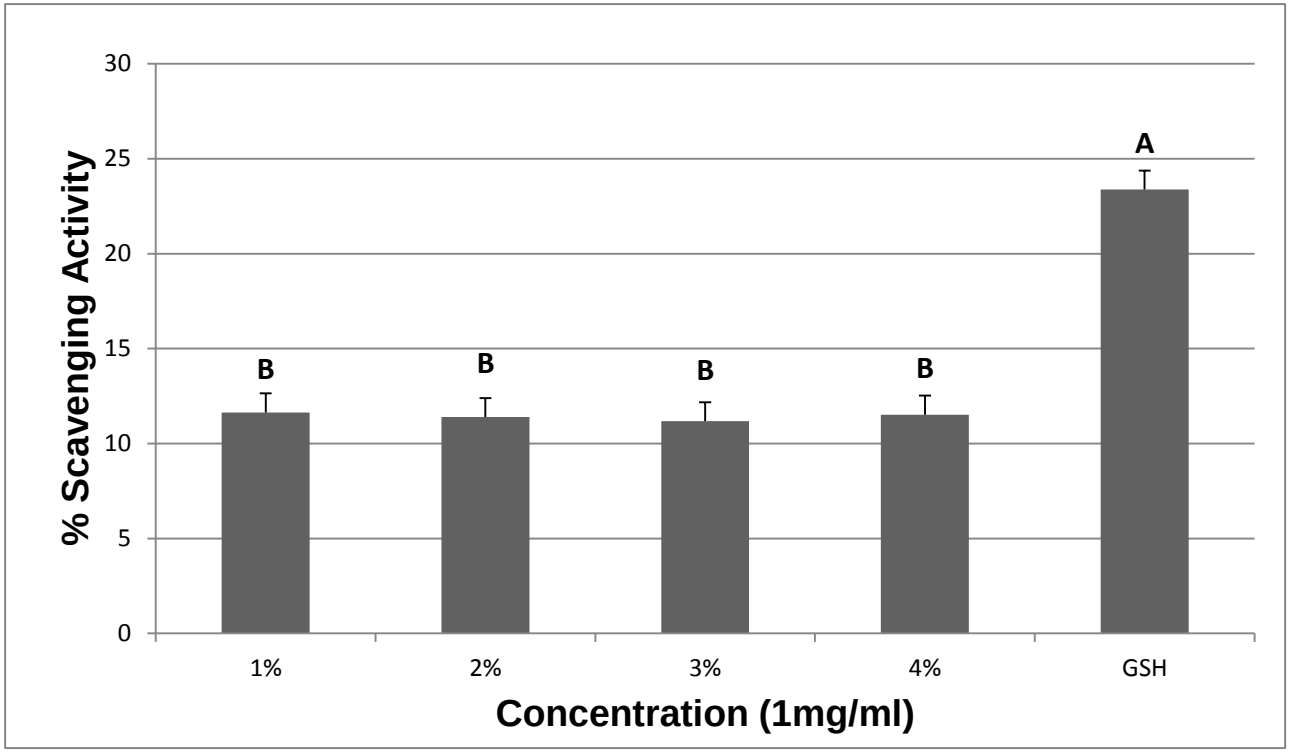
Various methods have been used to study the antioxidant properties of foods. Antioxidant evaluation systems were used to determine the antioxidant activity of the hempseed protein hydrolysate, including DPPH scavenging activity, chelation of metal activity ions, ferric reducing power, hydroxyl scavenging activity, and superoxide scavenging activity. All antioxidant analyses were compared to reduced glutathione (GSH) as a standard.

4.4. DPPH scavenging activity of HPH

The DPPH radical scavenging activities of HPH (result of HPI hydrolysis with different concentration of Proteinase K, 1%, 2%, 3% and 4%) are shown in Figure 4.1. Glutathione showed the strongest DPPH radical scavenging activity

with a value of 23.38% when compared to those of HPH. All the HPH samples that were tested exhibited moderate DPPH radical scavenging activity, with mean values of 11.64, 11.39, 11.18, and 11.52% for the 1, 2, 3, and 4% Proteinase K hydrolysate fractions, respectively. Thus, there was no significant difference ($p>0.05$) in DPPH radical scavenging activity between the hydrolysates.

Figure 4.1. DPPH radical scavenging activity (%) of HPH fractions derived from incubations employing varying concentrations of Proteinase K

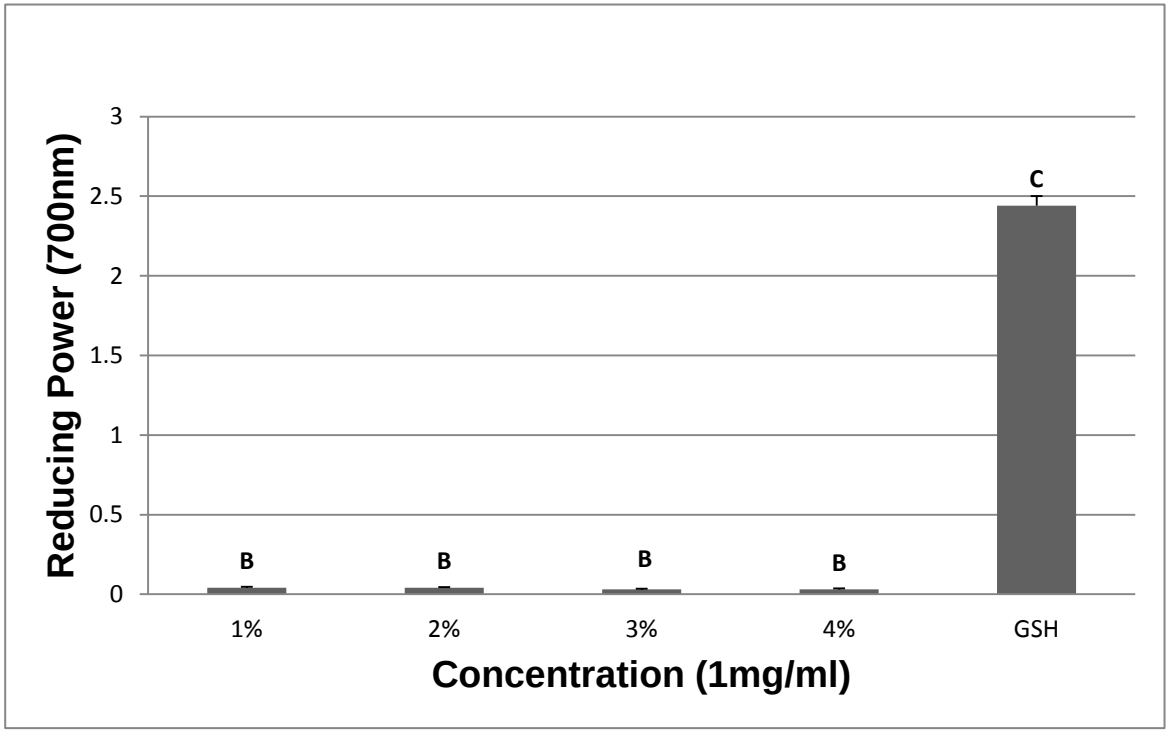


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.4.2. Ferric reducing antioxidant power (FRAP) activity of HPH

The ferric reducing antioxidant power values of HPH fractions obtained through the use of different concentrations of Proteinase K are shown in Figure 4.2. The HPH samples showed low reducing power values of 0.03-0.04 with no significant difference ($p>0.05$) among them. GSH exhibited strong reducing power (2.44) when compared to HPH ($p<0.05$). In addition, the current study shows no relationship between the reducing power of HPH and the concentration of Proteinase K.

Figure 4.2.Ferric reducing antioxidant power (FRAP) of HPH fractions derived from incubations employing varying concentrations of Proteinase K

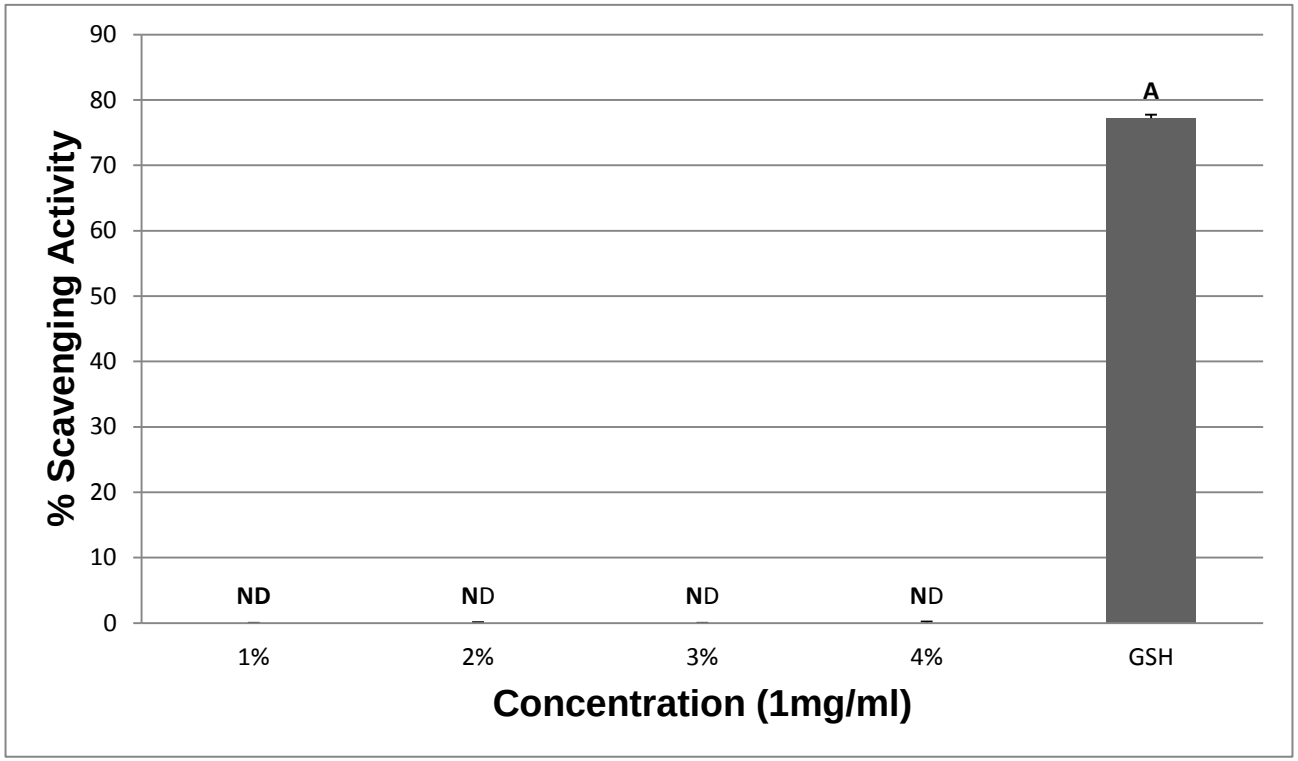


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.4.3. Hydroxyl scavenging activity of HPH

The hydroxyl scavenging activities of HPH and glutathione are shown in Figure 4.3. The HPH samples did not show hydroxyl scavenging activity. Glutathione exhibited strong hydroxyl scavenging activity, with a value of ~75%. Thus, the result shows no relationship between the Hydroxyl scavenging activity and the concentration of Proteinase K.

Figure 4.3. Hydroxyl scavenging activity of HPH fractions derived from incubations employing varying concentrations of Proteinase K



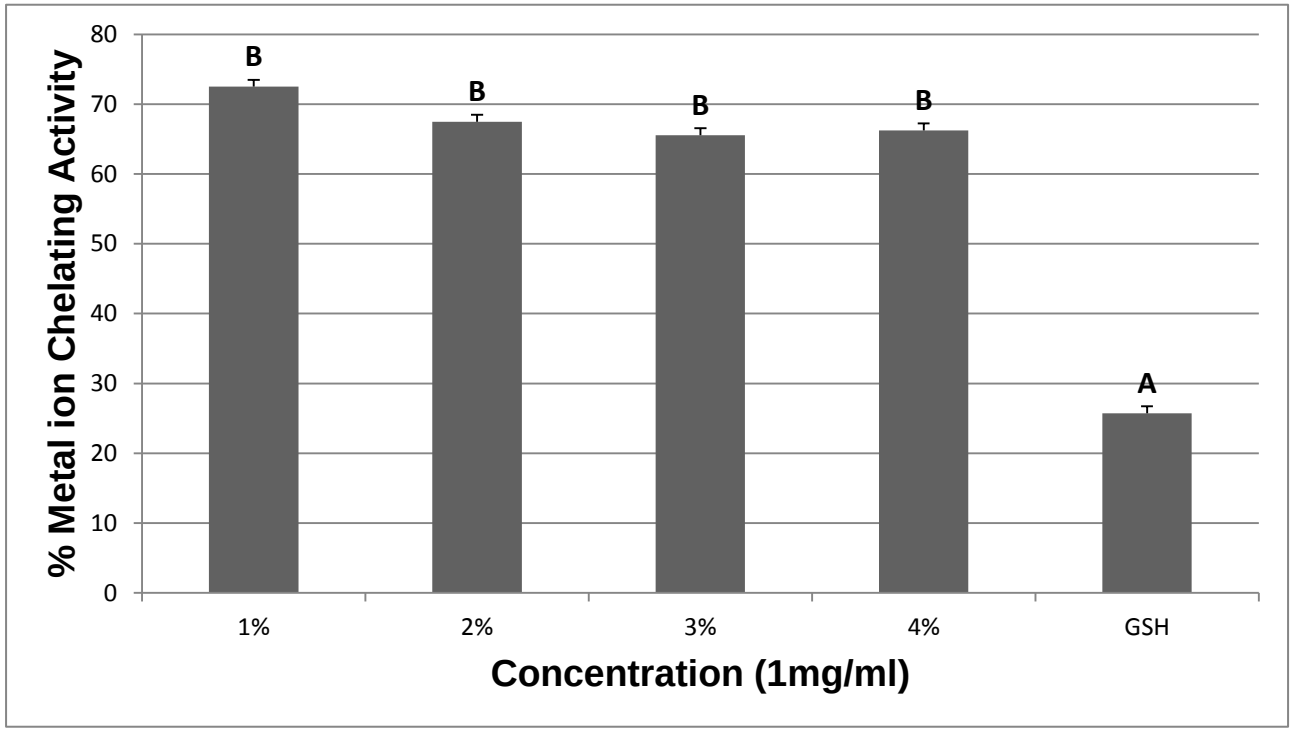
Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

ND = no activity determined

4.4.4. Metal chelation of HPH

The metal chelation abilities of HPH and glutathione are shown in figure 4.4. The different HPH samples showed significantly stronger metal chelating activity (Fe^{2+}) in comparison to glutathione. The HPH samples with 1%, 2%, 3% and 4% concentration of proteinase K exhibited similar metal chelating ability, with values of 72%, 67%, 65%, 66% respectively, and there were no significant differences among the hydrolysates ($P>0.05$). Glutathione showed the weakest metal chelation ability at 25.74% ($p<0.05$).

Figure 4.4. Metal chelating activity of HPH fractions derived from incubations employing varying concentrations of Proteinase K

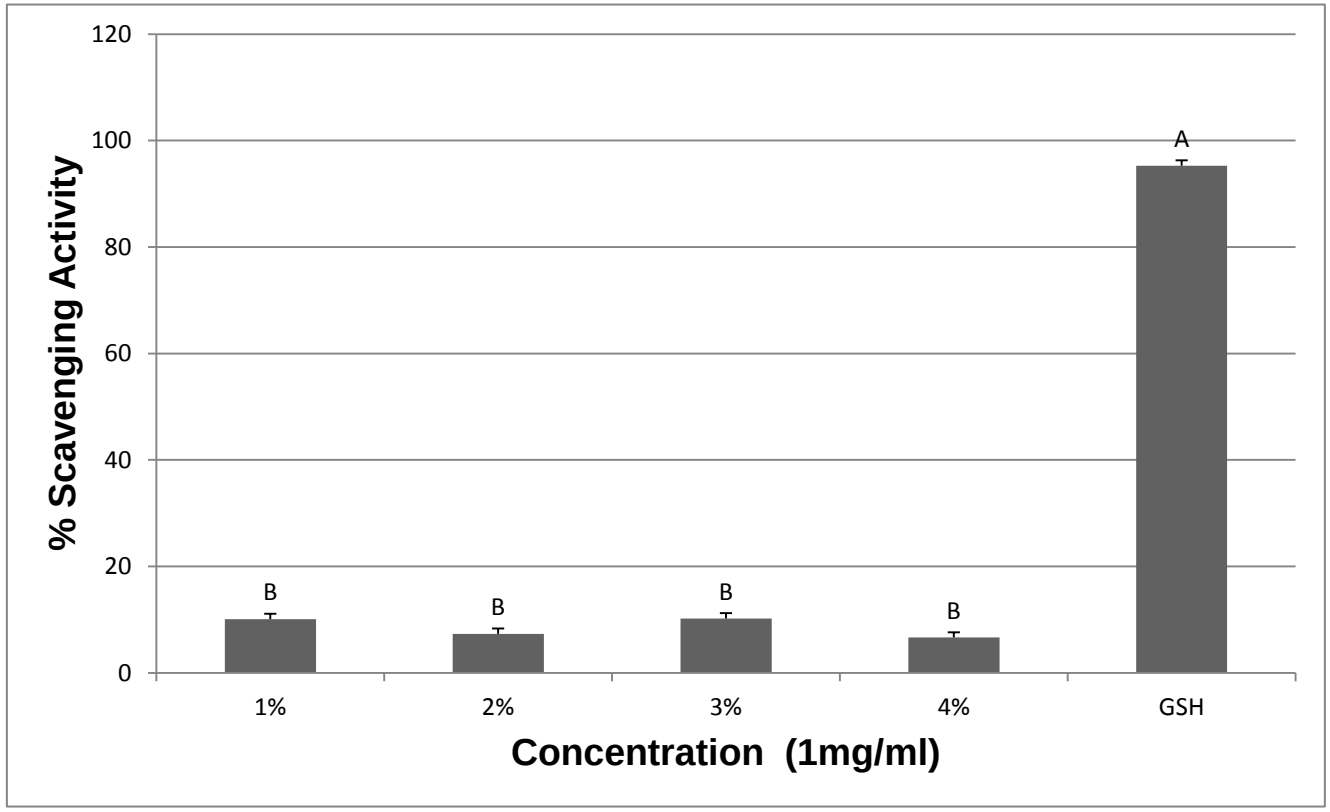


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.4.5. Superoxide radical scavenging activity of HPH

Figure 4.5 shows the superoxide radical scavenging activity of HPH obtained using different concentrations of Proteinase K (1-4% by weight of protein in the HPI) and glutathione at 1 mg/ml. The HPH produced with 1, 2, 3 or 4% Proteinase K displayed moderate superoxide scavenging activity, with values of 10.07, 7.31, 10.19 and 6.62% respectively ($p>0.05$). The HPH exhibited weaker superoxide scavenging activity in comparison to glutathione. The glutathione exhibited a strong superoxide scavenging activity, with a value of 95.26%. There was no relationship in superoxide scavenging activity and the concentration of Proteinase K.

Figure 4.5. Superoxide radical scavenging activity (%) of HPH fractions derived from incubations employing varying concentrations of Proteinase K



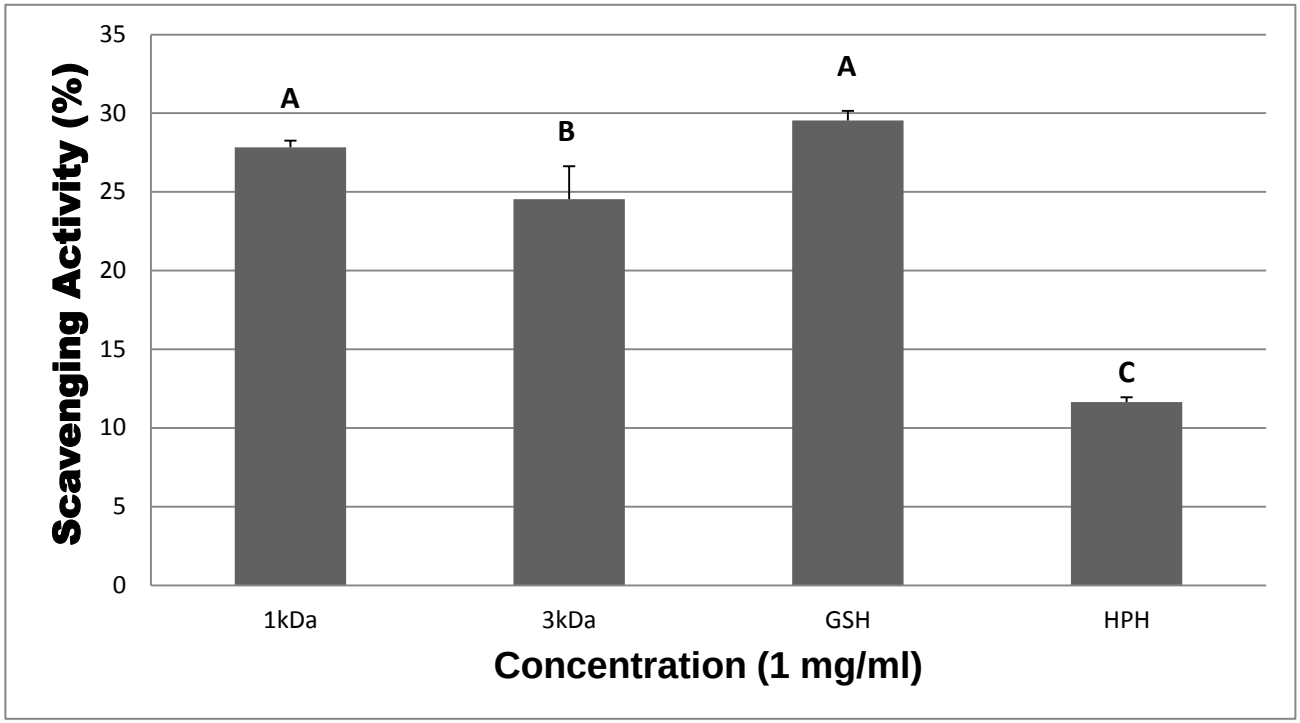
Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.5. Antioxidant activity of low molecular weight fractions of hempseed protein hydrolysates (HPH)

4.5.1. DPPH scavenging activity of low molecular weight HPH fractions

HPH was subjected to ultrafiltration to fractionate low molecular weight peptides of 1-3 kDa and <1 kDa peptide fraction. Figure 4.6. shows the DPPH scavenging activity of <1 kDa and 1-3 kDa peptide fractions compared to glutathione. The 1KDa fraction showed the strongest DPPH scavenging activity at 27.84% when compared to 1-3 kDa fraction at 24.54% ($p<0.05$). Thus, the peptide fraction with the lowest molecular weight at <1 kDa fraction showed the strongest DPPH scavenging activity when compared with the higher molecular weight of 1-3 kDa peptides and HPH. The GSH and the <1 kDa fraction showed the strongest DPPH scavenging activity at 29.54% and 27.84% ($p>0.05$) respectively with no significant difference between the two ($p>0.05$). In addition, the fractionated peptides of <1 kDa and 1-3 kDa possessed stronger DPPH scavenging activity when compared to HPH obtained with 1-4% concentration of Proteinase K ($p < 0.05$).

Figure 4.6. DPPH radical scavenging activity (%) of low molecular weight HPH fractions derived by ultrafiltration.

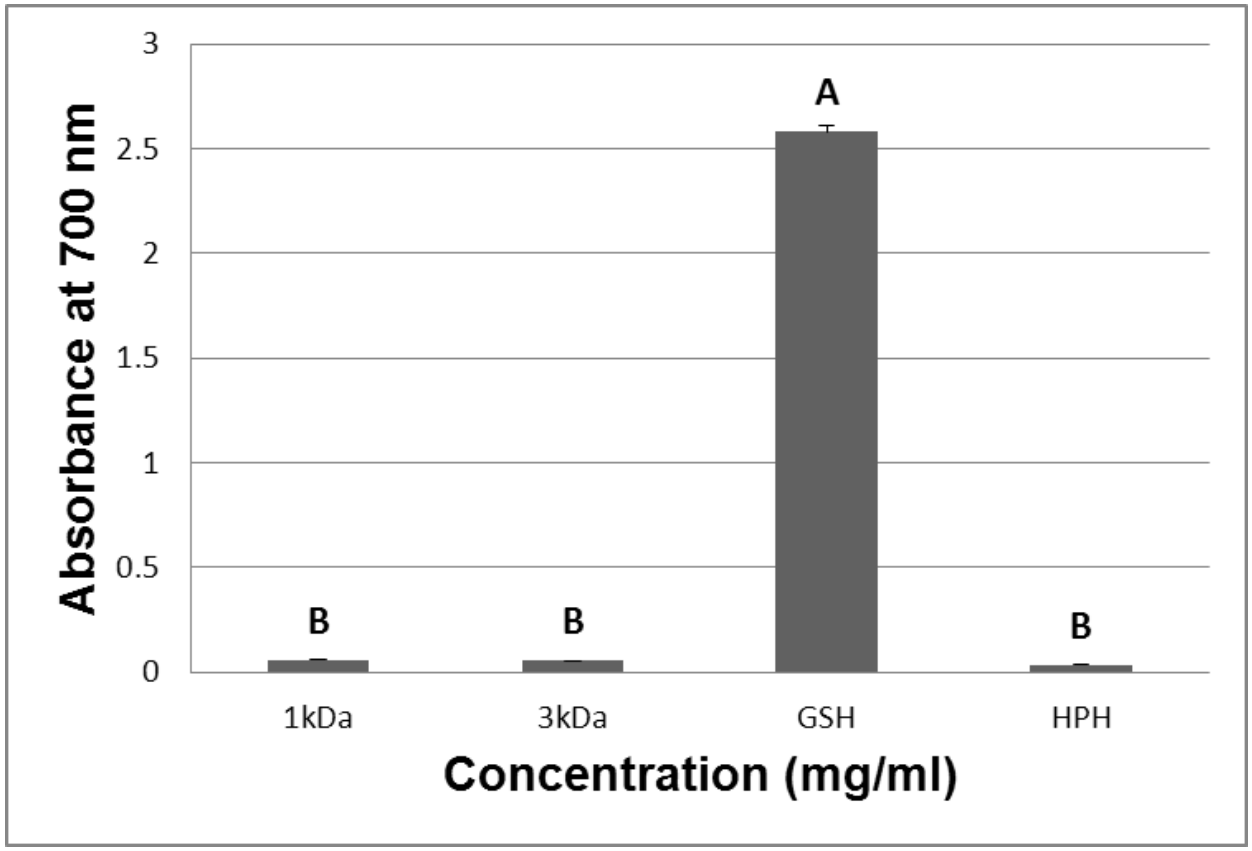


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$

4.5.2. Ferric reducing antioxidant power (FRAP) of low molecular weight HPH fractions

Figure 4.7. shows the ferric reducing antioxidant power of <1 kDa and 1-3 kDa HPH fractions, as compared to glutathione. The peptide fractions of <1 kDa and 1-3 kDa showed weak reducing power, with values of 0.058 and 0.055 respectively, with no significant difference observed between the fractions ($p>0.05$). In addition, there was no significant difference between <1 kDa, 1-3 kDa and the HPH obtained with 1% concentration Proteinase K in ferric reducing power. This result indicates no relation between molecular weight peptides at <1 kDa and 1-3 kDa and the Fe^{2+} reducing power.

Figure 4.7. Ferric reducing antioxidant power (FRAP) of low molecular weight HPH fractions derived by ultrafiltration

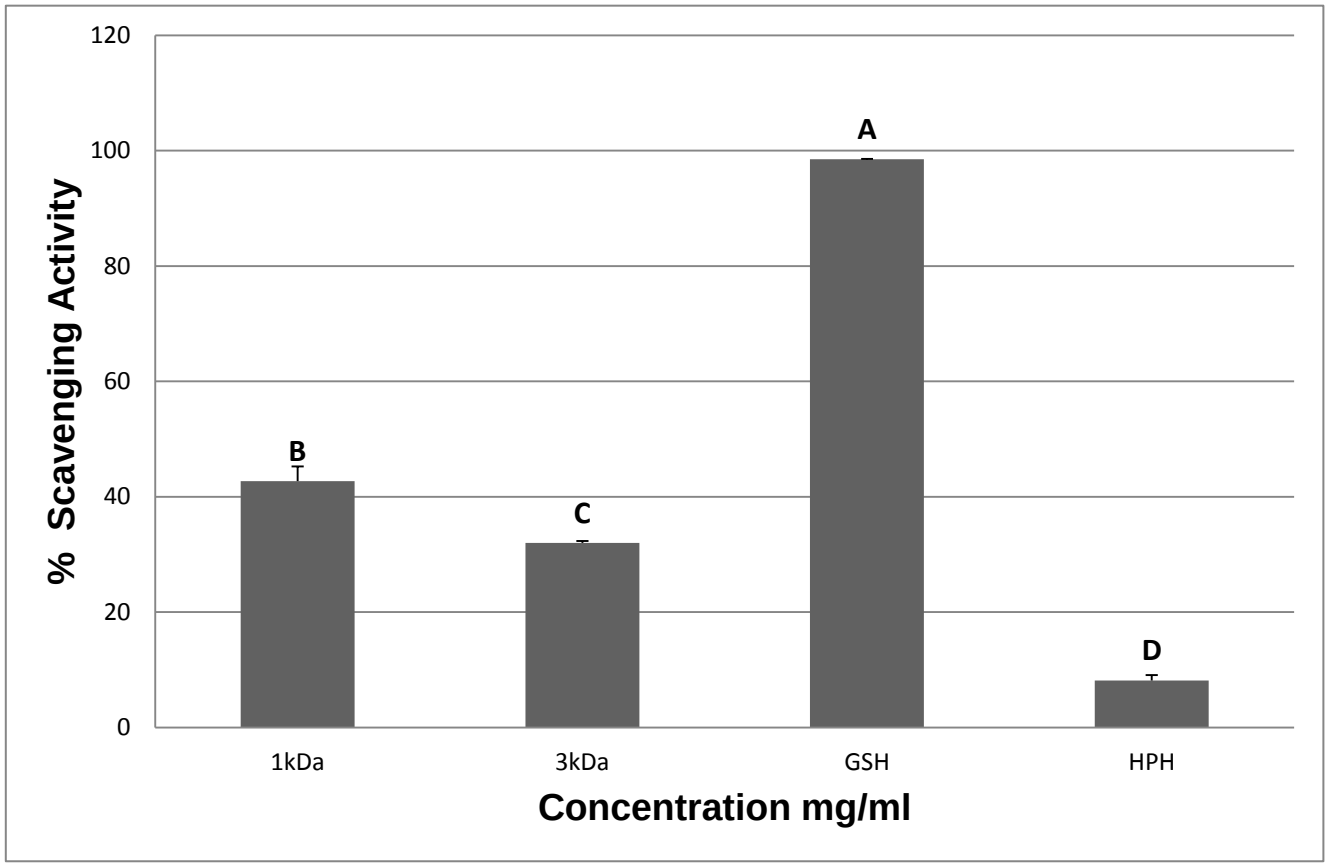


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.5.3. Superoxide radical scavenging activity of low molecular weight HPH fractions

The results of the superoxide radical scavenging activity of low molecular weight HPH fractions, at <1 kDa and 1-3 kDa are given in Fig. 4.8. The <1 kDa fractions showed stronger superoxide scavenging activity, with a value of 42.72%, in comparison to the 1-3 kDa fractions, which yielded values of 31.97% ($p<0.05$). The GSH superoxide radical scavenging activity was equal to ~98%, significantly greater than either the <1 kDa and 1-3 kDa low molecular weight HPH fractions. In comparison to the non-fractionated HPH sample, both low molecular weight HPH fractions showed significantly stronger superoxide scavenging activity ($p<0.05$). Thus, the current study indicates a positive relation between superoxide scavenging activity and low molecular weight.

Figure 4.8. Superoxide radical scavenging activity of low molecular weight HPH fractions derived by ultrafiltration

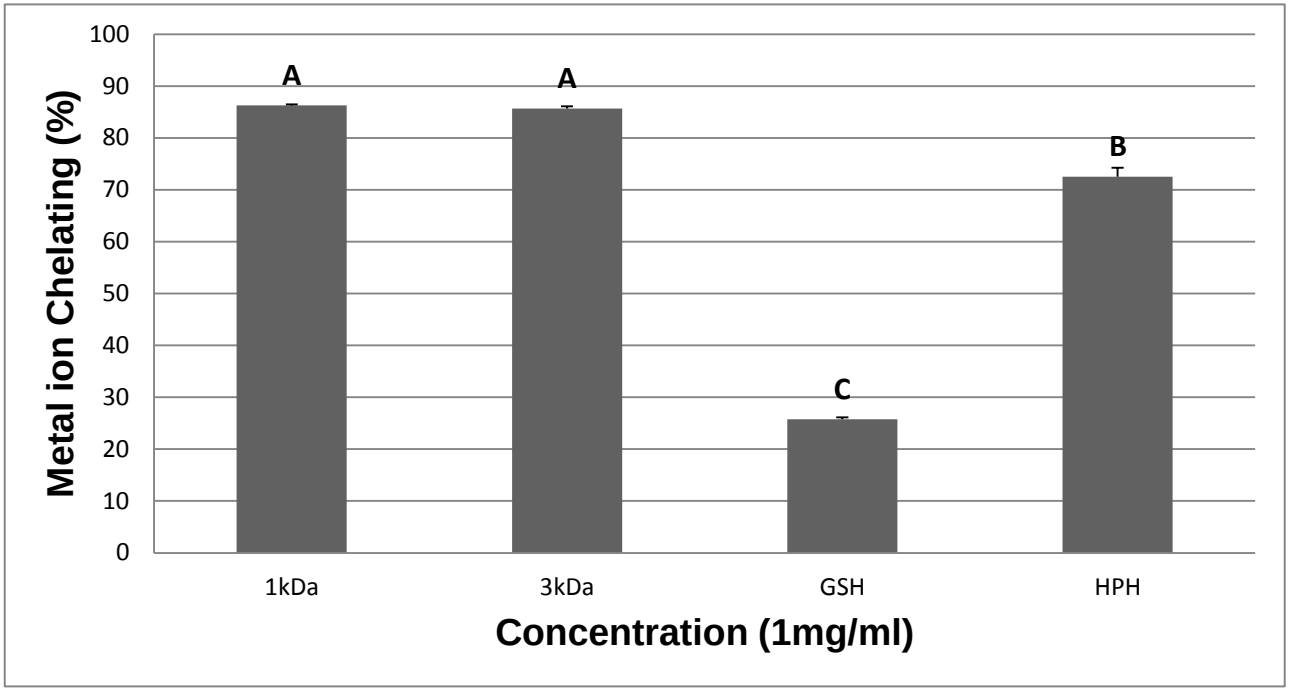


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.5.4. Metal chelation activity of low molecular weight HPH fractions

The metal chelating abilities of the ultrafiltration fractions of HPH and glutathione are given in Figure 4.9. The 1 kDa and 1-3 kDa low molecular weight peptide fractions possessed similar metal chelating activity of 86.3% and 85.65%, respectively ($p>0.5\%$). The low molecular weight peptide fractions at 1 kDa and 1-3 kDa exhibited stronger metal chelating activity (Fe^{2+}) in comparison to glutathione (~25%). In addition, the current study showed lower molecular weight peptides of 1 kDa and 1-3 kDa possessed stronger metal chelating abilities when compared to unfractionated hydrolysate.

Figure 4.9. Metal chelation activity of low molecular weight HPH fractions derived by ultrafiltration

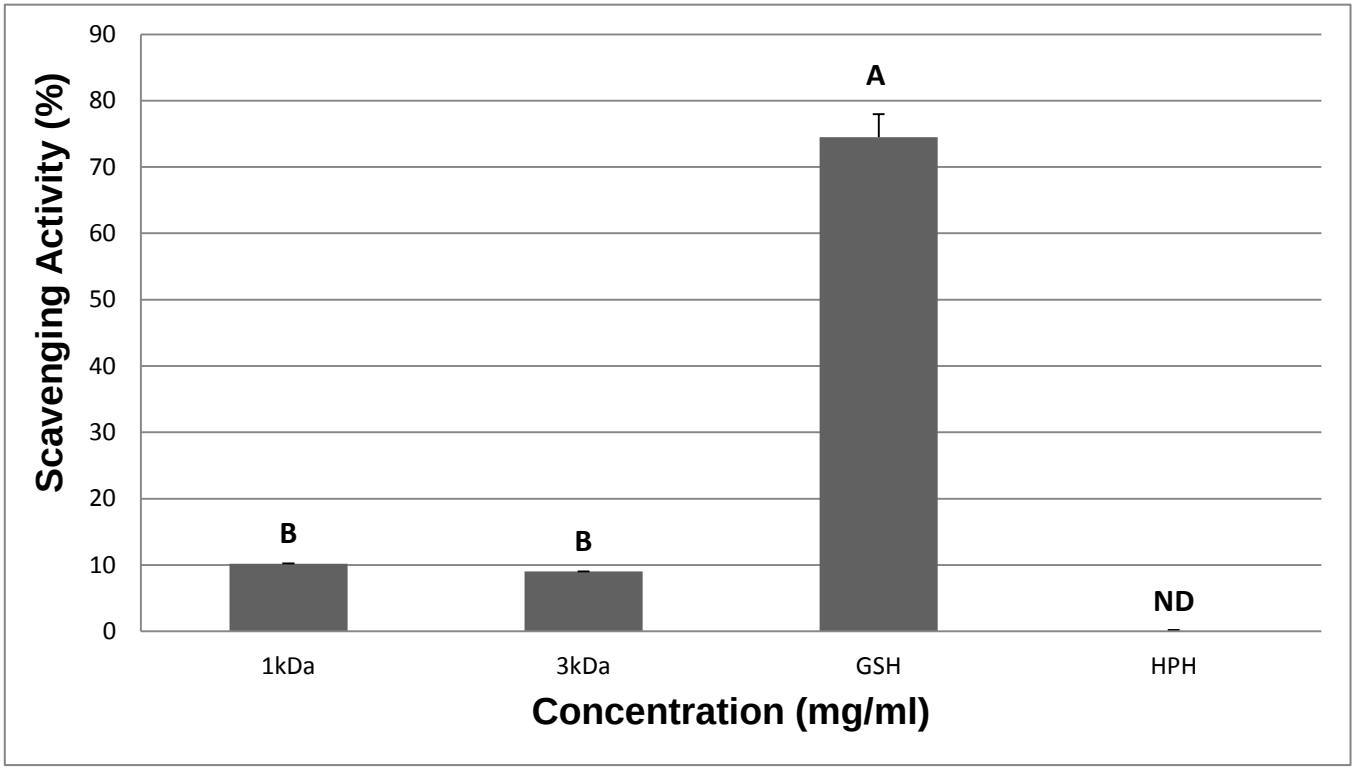


Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$.

4.5.5. Hydroxyl scavenging activity of low molecular weight HPH fractions

The hydroxyl scavenging activity of the low molecular weight HPH fractions and glutathione are shown in Figure 4.10. Upon fractionation of the HPH, increases in hydroxyl scavenging activities were observed. The HPH fractions of <1 kDa and 1-3 kDa exhibited some hydroxyl scavenging activity with a value of 10.21% and 9% ($p>0.05$) respectively. The result indicates, no significant difference between the <1KDa and 1-3KDa peptide fractionates in their Hydroxyl scavenging properties. In addition, HPH samples obtained with 1-4% Proteinase k showed no hydroxyl scavenging activity. Thus, the results indicate low molecular weight peptide fraction at <1KDa and 1-3KDa have stronger hydroxyl scavenging activity compared to its hydrolysate.

Figure 4.10. Hydroxyl scavenging activity of low molecular weight HPH fractions derived by ultrafiltration



Each bar represents the mean of triplicate measurements (mean ± standard deviation) with different letters indicating mean values that are significantly different at $p<0.05$
ND = no activity determined

CHAPTER 5

5.0. Discussion

The biological system has a number of antioxidants such as enzymes (e.g. superoxide dismutase, glutathione peroxidase, and catalase), large molecules (e.g. albumin, ceruloplasmin, ferritin), small molecules (ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, polyphenols) as well as hormones (estrogen, angiotensin, melatonin, etc.) that are used to protect the human body against oxidative stress (Prior et al., 2005). These antioxidants within biological systems have the ability to quench radicals through multiple mechanisms or through a single mechanism and protect the body from for oxidative stress (Prior et al., 2005). There are several free radicals and oxidant molecules such as $O2^{\bullet -}$, $^{1}O_2$, $HO^{\bullet}$, $NO^{\bullet}$, $ONOO^{-}$, HOCl, and $ROO^{\bullet}$ which possess different chemical and physical properties (Prior et al., 2005). Because of the fact that multiple reaction mechanisms are involved, more than one antioxidant assay is required to study the antioxidant property of a compound (Prior et al., 2005). Thus, to study a full profile of antioxidant capacity and evaluate the effectiveness against various reactive oxygen species such as $O_2^{\bullet -}$ and $HO^{\bullet}$, tests using different assays are required to (Prior et al., 2005). In the current study, several antioxidant assays were used including DPPH scavenging activity, metal ion chelating, hydroxyl scavenging activity, superoxide scavenging activity and ferric reducing power to study the antioxidant property of hempseed protein. Each antioxidant assay

addresses different components, but together they can characterize the overall antioxidant capacity of hempseed protein hydrolysate.

Hemp, also known as “industrial hemp,” is grown as an agricultural crop and has multipurpose. Hemp can be used as source of fiber. Hemp fibers are used in fabrics and textiles, paper, carpeting, home furnishings, as well insulation materials. Furthermore, the interior stalk (hurd) is used as animal feeding and low-quality papers and more. Moreover, hempseed and oilcake are used in foods and beverages, as well as food protein source (Johnson, 2012). Oil from the crushed hemp seed is used as a constituent in body-care products as well as nutritional supplements (Canadian Hemp Trade Alliance, 2012). Hemp seed is also used for industrial oils, cosmetics and personal care, and pharmaceutical drugs (Johnson, 2012). Food products containing hempseed and its oil available in the market include; hemp sauce, hemp milk, hemp protein powder, hemp seed butter as well as pasta, frozen desserts and snack products (Canadian Hemp Trade Alliance, 2012, Johnson, 2012). Canada regulation requires, products derived from hemp grain should have less than 10 parts per million (ppm) for THC residues (Johnson, 2012). At the industrial level, hempseed, containing 33-35% oil, goes under industrial hempseed oil extraction to extract the oil. The resulting defatted hempseed meal or seed cake contains mainly protein (30-50%)(Johnson, 2012). The hempseed meal was processed further to produce hemp protein isolate as discussed in the methods section.

Due to its nutritional value, hempseed and hempseed derivatives are emerging as a growing market (Canadian Hemp Trade Alliance, 2012). In 2010,

Canadian farmers harvested more than 26,000 acres of hemp (Canadian Hemp Trade Alliance, 2012). Canadian farmers achieved net profits of $200 to $250 per acre (Agricultural marketing resources canter, 2011). In addition, companies in the hemp industry have gained a 20 to 40 percent increase of business in Canada (Agricultural Marketing Resources Center, 2011). The estimated retail sales of Canadian-derived hemp seed products are $20 to $40 million annually (Canadian Hemp Trade Alliance, 2012). Canada exported 876 MT of hemp in 2007, which was valued at $3.5 million Canadian where 60 percent of Canada's hemp was exported to the United States (Canadian Hemp Trade Alliance, 2012). To increase the value of defatted hempseed meal generated as a by-product of the oilseed processing industry, this study explored the bioactive properties of enzymatically prepared hempseed protein hydrolysates with respect to their potential uses as source of antioxidant components.

The antioxidant activity of food derived protein is linked to the properties of the amino acids/peptides (Chen et al., 2011). Enzymatic hydrolysis is one of the methods for the effective release of antioxidant peptides from protein sources and this may yield enhancement in antioxidant activity (Chen et al., 2011, Erdmann et al., 2008). During hydrolysis, a variety of smaller peptides and free amino acids are generated, depending on the enzyme specificity and the hydrolysis time (Chen et al., 2011, Erdmann et al., 2008). To date, there hasn’t been any work reported regarding the antioxidant capacity of hydrolyzed hempseed proteins generated by Proteinase K. Furthermore, to the best of our

knowledge, there is no literature report on the effects of Proteinase K enzyme concentration with respect to the indices of antioxidant activity.

High yields and effectiveness of the bioactive peptides are important considerations in determining the cost benefits in the production of food-derived peptides (Udenigwe et al, 2011) and the development of functional foods. Protein yields and antioxidant capacity of the bioactive peptides generated from hemp protein hydrolysate via the digestion of isolated hempseed proteins using different concentration Proteinase K were investigated in the current study.

The protein content of the HPI was 100% and upon hydrolysis with different concentration of proteinase K (1-4% by weight of protein in the HPI), the protein content of the resulting hydrolysates ranged between 85%-100% (dry matter basis). The percent yield of HPH was determined as the ratio of peptide weight of lyophilized HPH to the protein content of HPI. As expected, the percentage yield value of (85%) of Hemp protein with 4% proteinase K was significantly higher ($P < 0.05$) than the yield value (60%) of Hemp hydrolysate with 1% of proteinase K. The results indicate increased protein yield with increasing concentration of proteinase K from 1 to 4%. The increase in protein yield with increasing enzyme concentration observed could be due to increased interaction of enzyme and peptide bonds. Interestingly, in previous study, Girgih et al., (2011) reported hempseed protein hydrolysates obtained with pepsin resulted in protein yield of 65.7% while sequential application of pepsin and pancreatin hydrolysis resulted in protein yield of 86.7%. The protein yield (65.7%) obtained with 2hrs hydrolysis with pepsin (4% w/v, protein basis) (Girgih et al.,

2011) was higher than the current findings of 60% obtained with 4hrs hydrolysis with Proteinase K (1% w/v, protein basis). The difference in the protein yield could be due to the difference in enzyme concentration as well as the type of enzyme used in the current study. The enzyme Proteinase K applied in the current study exhibits broad substrate specificity. Proteinase K has no cleavage specificity. It cleaves proteins between amino acids X and Y (X-↓-Y), when X = an aliphatic, aromatic, or hydrophobic amino acid, and Y = any amino acid (Roche catalogue, 1996-2011). However, pepsin is an endopeptidase that cleaves peptide bonds at specific sites, while pancreatin is a combination of endopeptidase and exopeptidases (Abraham et al., 2011). Furthermore, the protein percentage yield (85%) with 4% Proteinase K in the current study is similar to the protein yield obtained with pepsin-pancreatin complex (86.7%) reported by Girgih et al., (2011). Thus, the current study demonstrates that Proteinase K can be used for hydrolysis to obtain the optimum yield of protein.

There are several factors that may affect the antioxidant activity of food derived peptides such as the enzyme specificity, degree of hydrolysis, molecular size, hydrophobicity, and amino acid composition (Chen et al., 2011, Erdmann et al., 2008). For example, during hydrolysis bioactive sequences of hempseed protein are released, and the specific activity is dependent upon the proteases used or the peptide bonds cleaved (Chen et al., 2010, Erdmann et al., 2008). Different enzymes have different cleavage sites within proteins resulting in the release of different peptide sequences (Chen et al., 2010). The hydrolysis process releases active peptides that have the ability to interact with free

radicals, thus converting them to more stable products and inhibiting the progression of the radical chain reaction (Yust et al., 2011). The use of Proteinase K in concentrations between 1 and 4% resulted in the production of peptide sequences with similar DPPH, hydroxyl and superoxide radical scavenging, metal chelating activity, and FRAP activity. This could be attributed to either the enzyme being saturated with the substrate or the occurrence of enzyme inhibition by the derived peptides (Zhou et al., 2012). Interestingly, the protein yield was highest in the HPH obtained with 4% Proteinase K and lowest in the HPH obtained with 1% Proteinase K. However, the antioxidant activity of both hydrolysates was similar. This result indicates that the antioxidant peptides present in the hydrolysates are similar.

Zhou et al., (2012) observed that while the antioxidant activities of the hydrolysate of abalone foot muscle (HAFM) increased with increasing incubation time during the hydrolysis process for up to 180 min, the antioxidant activities of the hydrolysate of scallop adductor muscle (HSAM) increased at first and peaked after 25–30 min of hydrolysis, and decreased gradually (Zhou et al., 2012). Furthermore, Zhou et al., (2012) reported that the duration of hydrolysis may result directly in increased solvent exposure of residue side chain groups, which facilitates the reactions between peptides and free radicals, ROS and transitional metal ions, leading to increased antioxidant activity. In contrast, other studies have shown that extensive hydrolysis could reduce the antioxidant activity of hydrolysates (Saiga et al., 2003). Extensive hydrolysis of protein may lead to the production of a high concentration of free amino acids (Zhou et al., 2012). Thus,

increasing the concentration of Proteinase K to levels of more than 4% may lead to an extensive hydrolysis of the protein, resulting in the formation of free amino acids (FAA) (Zhou et al., 2012, Saiga et al., 2003). Saiga et al., (2003) reported peptides are stronger antioxidants than amino acids due to their increased free radical scavenging activity and chelation of transition metals.

Proteins and their hydrolysates exhibit antioxidant capacity due to their ability to inhibit reactive oxygen species (ROS), quench free radicals, chelate transition metals, as well as donate electron or hydrogen (Chen et al., 2010, Erdmann et al., 2008, You et al., 2011, Zhou et al., 2012). The DPPH radical is used to measure the ability of natural compounds to donate electrons or hydrogen to form a more stable compound. In the current study, hemp protein hydrolysate showed moderate DPPH scavenging activity. DPPH radical scavenging is a single electron transfer reaction, thus the result indicates HPH contain peptides with amino acids that have the ability to transfer electrons to the DPPH radical. In addition, the chelating of transition metal ions through the use of antioxidants peptides may prevent the oxidation reaction associated with redox active metal catalysis. Hemp protein hydrolysate showed strong metal chelating activity. The results indicate that HPH has strong antioxidant ability through the chelating of transition metals Fe^{2+} and therefore inhibit the progression of the Haber-Weiss reaction. In addition, HPH also showed superoxide scavenging activity. However, the results demonstrate weak hydroxyl scavenging activity and FRAP. This finding indicates hemp protein hydrolysate has the ability to

scavenge superoxide and DPPH radicals, as well as chelate transition metal ions.

Studies have shown that hydrolysates with a stronger DPPH radical scavenging activity were correlated with the presence of a high content of hydrophobic amino acids. Wang (2009) examined the antioxidant property of hemp protein hydrolysates achieved using the enzyme Neutrase. They reported that the hydrolysate with the stronger DPPH radical scavenging activity contained a high percentage of hydrophobic amino acids. In addition, DPPH radical scavenging ability of hydrolysates depends on the type of proteases and on the length of hydrolysis time. Interestingly, at hydrolysis time of 2 h, the hydrolysates obtained by protamex had the highest radical scavenging ability (IC50 2.8 mg/mL), while that with pepsin and trypsin was the lowest (IC50 = 5.5–5.7 mg/mL) (Wang et al., 2009). In addition, increase in hydrolysis (4 h) resulted in to various influences on the DPPH radical scavenging ability (relative to 2 h), depending on the type of enzyme used. When neutrase and trypsin were applied, the radical scavenging ability increased when the hydrolysis time was increased from 2 to 4 h, while that for flavourzyme, pepsin and protamex decreased (Wang et al., 2009). The previous results demonstrate that hydrolysates with high antioxidant activities could be obtained by selecting the right type of proteases and the optimal period of hydrolysis time. In the current study, the optimum hydrolysis time of 4h was applied. Furthermore, Proteinase K has a wide broad substrate, and therefore it is possible that it applied a more extensive hydrolysis of peptide bonds compared to enzymes that cleaves at specific sites.

In contrast to Wang (2009), egg protein hydrolysates obtained with pepsin and pancreatin or thermolysin and alcalase showed no correlation between DPPH assay and the amino acid composition (You et al., 2012). Yust and colleagues (2011) reported that chickpea hydrolysates exhibited higher DPPH scavenging activity to chickpea isolates. Thus, the hydrolysis of chickpea with the enzyme alcalase resulted in up to a 50% increase in DPPH scavenging activity (Yust et al., 2011). However, Yust and colleagues (2011) reported no significant differences in amino acid composition between chickpea protein isolate and chickpea hydrolysate. The DPPH radical scavenging activity (11.64%) of the hydrolysate in the current study was higher than what was observed by Girgih et al., (2010), who studied DPPH radical scavenging activity of hempseed protein hydrolysate using the enzymes pepsin and pancreatin. Girgih et al., (2010) reported 4% DPPH scavenging activity HPH that at 1 mg/ml. The difference in the findings could be due to the difference in the enzymes used in the two studies. In the current study, Proteinase K was used to hydrolyze hemp seed isolate and, this could have resulted in the release of peptides with strong ability to scavenge DPPH radical. Pepsin, an endopeptidase, cleaves at specific sites while pancreatin is a mixture of endopeptidase and exopeptidases (Girgih et al., 2011). In addition, Girgih 2010, achieved protein hydrolysis with pepsin (4% w/v, protein basis) for two hours, and pancreatin (4% w/v, protein basis) for 4 hour. Consequently, the difference in hydrolysis time and protease used could have resulted in the different DPPH scavenging activity.

Studies have shown that an increase in the hydrophobic amino acid present in peptides contribute to higher radical scavenging activities when compared with peptide fractions of lower hydrophobic amino acid content (Ajiobla et al., 2010, Chen et al., 2010). In a study by Ajiobla and colleagues (2010), the DPPH scavenging activity of African yam bean seed (Sphenostylis stenocarpa) protein hydrolysate was reported to be ~35% which is higher than the current study (11%). Hydrolysates of bovine brisket sarcoplasmic proteins obtained by papain were reported to have higher DPPH scavenging activity than the current study. Bernardini and colleagues (2012) reported hydrolysates of bovine brisket sarcoplasmic proteins exhibited 18.68% DPPH scavenging activity at a concentration of 1 mg/ml. The differences in results between the studies may be attributed to differences in the type of protein and the hydrolysis pattern of protease.

The relationship between hydrophobic amino acid and the reducing power of hydrolysates have been previously reported (Bernardini et al., 2012). The reducing power assay is used to evaluate the ability of compound to donate electron or hydrogen to free radical thereby converting the radical to stable substances (Chen et al., 2011). In the current study, the HPH samples showed low reducing power. In contrast, Wang et al., (2009) reported HPH obtained with neutrase exhibited reducing power values of 0.28-0.32 as compared to the current study (0.03-0.04). In addition, Girgih (2010) found that hempseed protein hydrolysates obtained with pepsin and pancreatin showed reducing power values of 0.06-0.13, which were higher than the present study. Yust et al., (2011)

reported the chickpea hydrolysate possess stronger reducing power activity than the parent chickpea isolates.

Protein hydrolysate with strong superoxide radical scavenging activity was reported to have higher percentage hydrophobic amino acid (Chen et al., 2011, Li et al., 2008). In the current study, the HPH hydrolysate exhibited superoxide radical scavenging activity. However, the superoxide scavenging activity of HPH in the current study is lower than that of African yam bean hydrolysate which exhibited superoxide scavenging activity of 31.3% (Ajibola et al., 2011). In addition, the hydrolysate from egg white protein displayed superoxide scavenging activity with value ranging between 20-50% (Chen et al., 2012). Furthermore, Chen et al (2011) observed that egg white protein hydrolysate displayed concentration dependent antioxidant activity. For example, at concentrations of 5mg/ml, 10mg/ml, 20mg/ml and 40mg/ml, the hydrolysate exhibited superoxide scavenging activity with values of 27.78%, 20.13%, 34.87% and 50% respectively (Chen et al., 2011). However, in the current study the concentration used was lower (1mg/ml) compared to the study by Chen et al. (2011).

The HPH hydrolysate showed a strong metal chelating activity with a value of 66-72% at a concentration of 1mg/ml. Similarly, it was previously identified that hydrolysate from African yam bean exhibited strong metal chelating activities in vitro (Ajibola et al., 2011). Hydrolysates generated with the enzyme alcalase from African yam bean seed protein isolate displayed metal ion chelating capacity of~65% at a concentration of 1mg/ml. The metal chelating

property was attributed to the presence of carboxyl and amino group in the side chains of the acidic (glutamic acid and aspartic acid) and basic amino acids (Lysine, Histidine and Arginine) (Ajibola et al., 2011). Similarly, Girgih et al. (2010) observed strong metal chelating ability of hempseed protein hydrolysate obtained with a combination of pepsin and pancreatin at a value of 72% at a concentration of 1mg/ml. The antioxidant potential of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain has also been reported. The hydrolysate showed significant Fe^{2+} chelating ability at a concentration of 5 mg/ml with a value of 78.68±3.63% chelating activity (Bernardini et al., 2012). It was proposed that alanine and leucine amino acid residues, as well as glycine, may have been contributing factors to the metal chelating and antioxidant activities of the hydrolysates (Bernardini et al., 2012).

In the current study, the HPH exhibited both DPPH scavenging activity and Fe^{2+} chelating activity. Furthermore, hydrolysate with strong antioxidant activity was produced through the hydrolysis of hemp protein isolate with Neutrase. The hydrolysate was reported to exhibit DPPH radical scavenging and Fe^{2+} chelating abilities (Wang et al., 2009). In addition, Wang 2009 reported a positive correlation between the DPPH radical scavenging and Fe^{2+} chelating abilities and the amino acid composition and hydrophobicity of the hydrolysates. For example, the peptide profiles of the hydrolysates with higher hydrophobic amino acids possessed stronger DPPH radical scavenging and Fe^{2+} chelating abilities (Wang et al., 2009). Thus, the observed results could be attributed to the presence of high number of amino acids in the HPH with the ability to scavenge

DPPH radical and chelate Fe^{2+}. The finding from the current study indicates that hemp protein hydrolysates have the ability to donate electron/hydrogen to radicals, and therefore convert them into stable compounds leading to the inhibition of ROS formation.

Hydroxyl radical is the most biological reactive radical and is involved in lipid peroxidation and can cause damage to proteins, DNA, polyunsaturated fatty acids, nucleic acids and may lead to the progression of chronic disease such as cancer and several diseases (Prior, 2005). Studies have shown hydrophobic amino acids may contribute to the higher hydroxyl radical scavenging activity (Pownall et al., 2010). The HPH hydrolysate exhibited weak hydroxyl scavenging activity, whereas Glutathione exhibited strong hydroxyl scavenging activity with a value ~75%, which may be attributed to the presence of sulfur containing amino acids. In contrast to the current study, Ajibola et al., (2011) observed hydroxyl scavenging activity of African Yam bean. The hydrolysate from African yam bean was reported to have 20% hydroxyl scavenging activity. In addition, egg white hydrolysate derived from different proteases (papain, trypsin, flavourzyme, alcalase and neutrase) was also reported to have hydroxyl scavenging activity. Chen and colleagues (2011) reported that egg white protein hydrolysate exhibited enzyme dependent DPPH radical, superoxide anion and hydroxyl radicals scavenging activities. Hydroxyl scavenging activity of fermented soy protein meal hydrolysate by *Lactobacillus plantarum LP6* has also been reported. Amadou et al., 2012, reported the fraction with the highest antioxidant activity possessed 72% hydroxyl radical scavenging ability at concentration of 5 mg/ml.

This strong antioxidant activity was attributed to the low molecular weight peptide, and the hydrophobicity present in the soy protein hydrolysate. However, the concentration in the current study was lower (1mg/ml) compared to (5mg/ml) applied in the study by Amadou et al., (2012).

Recent studies have shown that protein hydrolysates and its fractions have antioxidant property. In addition, low molecular weight peptides have stronger antioxidant property when compared to the hydrolysate (Chen et al., 2011, Erdmann et al., 2008). Thus, the HPH was subjected to fractionation using membrane ultrafiltration to obtain low molecular weight peptides of <1kDa and 1-3kDa. These peptide fractions were evaluated for antioxidant activity using several antioxidant assays and the protein yields of <1kDa and 1-3 kDa peptide fractions were determined.

The percent yields of the ultrafiltration membrane fractions were calculated as the ratio of peptide content of a lyophilized peptide permeate to the peptide content of the HPH. The 1kDa and 3 kDa peptides had protein content of 59% and 73% respectively with protein yield of 47% and 30%. This result indicates that low molecular weight peptides are more concentrated (47%) when compared to the peptides with size 1-3 kDa (30%). Thus, a high concentration of small peptides in the HPH may have a high application for in vivo bioavailability, leading to an increased health benefit. Short-chain peptides are desired due to the reported evidence that these peptides are able to resist gastrointestinal digestion and can be absorbed intact into blood circulation and reach target cell (Moller et al., 2008).

The fraction peptides <1 kDa and 1-3 kDa exhibited strong DPPH scavenging activity, metal chelating, superoxide and hydroxial scavenging activity, and moderate FRAP activity. The hydrolysis process followed by ultrafiltration led to an increased antioxidant activity of hemp protein hydrolysate, which may be attributed to either an increased concentration of the active peptides with DPPH scavenging ability or due to an increase in the interaction between smaller peptides and free radicals. Thus, the DPPH radical scavenging, metal chelating activity, FRAP, superoxide radical and hydroxyl scavenging activity increased with low molecular weight. The methods of DPPH and FRAP are electron transfer reaction methods. Out of the five methods, the highest antioxidant activity was obtained with the metal chelating ability assay. Moreover, the scavenging activity of the 1 kDa and 3 kDa against DPPH and superoxide radicals was more effective than hydroxial radical. This result indicates the smaller weight peptides are more potent antioxidant activity when compared to HPH. The present study demonstrated that HPH possesses hydrogen/electron donating ability, which could make it interact with free radicals and terminate the radical chain reaction or prevent the formation of radical. Thus, hempseed protein hydrolysates and fractions possess potential as a food source for antioxidant.

In the present study, the DPPH scavenging of hemp protein fractions (1mg/ml) within a MW range of <1.0 kDa and 1–3 kDa were 27.84% and 24.54% respectively, whereas the HPH exhibited 11% scavenging activity. The lower molecular peptide fraction <1 kDa exhibited the strongest DPPH scavenging

activity. These results indicate DPPH scavenging is dependent on the molecular weight of the peptide. Similar findings were reported with peptide fractionation from zein hydrolysates (10mg/ml), at higher concentration than the current study. Tang et al., (2010) reported, at 10mg/ml zein hydrolysate exhibited DPPH radical scavenging activity at ~30% and ~21% for <1 kDa and 1-3 kDa respectively. The authors postulated that DPPH radical scavenging activity is dependent on the size of the peptides.

In addition, similar antioxidant activity of hempseed fractionates were reported by Girgih et al. (2010) where low molecular weight peptide fractions possessed stronger DPPH scavenging activities compared to high molecular weight peptides. Girgih et al. (2010) reported, HPH exhibited weaker scavenging of DPPH (4%) activity when compared to 5–10 kDa peptide fraction (18.7%). In addition <1 kDa peptide fraction (24.2%) exhibited the most potent antioxidant activity (Girgih et al. 2010). Similarly, in the current study, the 1 kDa peptide fraction exhibited the most potent DPPH scavenging activity (27.84%). Interestingly, the current study observed higher DPPH scavenging activity when compared to peptide fraction (24.2%) obtained with pepsin and pancreatin (Girgih et al. 2010). Different enzymes have different cleavage sites to the proteins leading to different peptide profiles. The difference in antioxidant activity between the hydrolysates produced by the enzymes is due to the presence of different peptides generated by different enzymes (You et al., 2011). Thus, the current result indicates Proteinase K is effective in releasing potent peptides from Hempseed protein.

The current study is in agreement with You et al. (2011), who observed positive correlations between egg protein hydrolysates with the molecular mass (< 500 Da) and DPPH scavenging activity. This correlation between molecular weight and peptides size were attributed due to an increase interaction between the smaller peptides and free radicals (You at al., 2011). Similarly, lower molecular weight peptides from African yam bean at <1 KDa exhibited stronger DPPH radical scavenging activities when compared with the high molecular weight at 3–5 kDa and 5–10 kDa peptides (Ajibola et al., 2010) and this property was attributed to the high percentage of hydrophobic amino acids and aromatic amino acids present in the 1 kDa peptide fraction. However, the DPPH radical scavenging reported from African yam bean hydrolysate was ~40% and ~38% for 1 kDa and 1-5 KDa (Ajibola et al., 2010), respectively which is higher than the current study. Ajibola et al. (2010) reported that the hydrophobic amino acid and aromatic amino acid contents in 1 kDa peptide fraction were higher compared to African yam bean protein isolate and African yam bean protein hydrolysate. Previous reports have reported that the presence of hydrophobic amino acids contribute to the hydrophobicity of the peptides, thereby increasing their solubility in lipids and therefore, enhance their antioxidative activity (Kim et al., 2007, Zhu et al., 2006). Furthermore, aromatic amino acid and bulky side groups have been reported to increase radical scavenging activities of peptides. Histidine is considered one of the amino acids with the highest antioxidant activities, and its antioxidant ability is attributable to the chelating and lipid radical-trapping ability of the imidazole ring. The antioxidant activity of aromatic

amino acids tyrosine and phenylalanine has been attributed to their capacity to act as radical scavengers. Furthermore, the antioxidant activity of tyrosine is believed to be associated with the special ability of phenolic groups to act as hydrogen donors, thereby stabilizing free radicals (Bernardini et al., 2012, Guo et al., 2009, Je at al., 2005, Ren et al., Nam et al., 2008,).

Bernardini and colleagues (2012) reported bovine brisket sarcoplasmic proteins at 10 kDa and 3kDa fractions showed 15.07% and 13.38% DPPH scavenging activity respectively. The DPPH scavenging activity was attributed to the high amount of alanine, leucine and glycine residues within the 10 kDa and 3 kDa peptide fractions (Bernardini et al., 2012). However, the reported DPPH scavenging activity is lower than the current study for 1 kDa (27.84) and 3 kDa (24%) at 1mg/ml. In addition, Amadou et al., (2011) studied the antioxidant properties of fermented soy protein meal hydrolysate by Lactobacillus plantarum LP6. The fraction with the most antioxidant activity exhibited 59.43% DPPH radical scavenging ability at 5.0 mg/ml, which was higher than that of HPH observed in the current study. Thus, the DPPH scavenging activity was attributed to the ability of the peptide fractions to donate an electron to a radical compound and therefore stabilizing the radical compound.

The peptide fractions <1 kDa and 1 kDa-3 kDa showed weak reducing power with values of 0.058 and 0.055, respectively. However, Abjiola et al., 2011 reported low molecular weight peptides possessed stronger reducing power when compared with large molecular weight fractions in African yam bean. The 1 kDa fraction had significantly higher reducing power when compared to those of

APH as well as the 1–3, 3–5, 5–10 kDa peptide fractions. In contrast, the 10 kDa and 3 kDa bovine brisket sarcoplasmic hydrolysate produced by papain exhibited antioxidant activity. In addition, there was no significant differences between the hydrolysates and fractionates in antioxidant activity (Bernardini et al., 2012). The hydrolysates from black soybean were found to possess reducing power. The strongest antioxidant fraction of soybean protein contained glutathione, arginine, phenylalanine, lysine, leucine, alanine (Haiwei at al., 2010). Thus, the strongest antioxidant fraction of black soybean hydrolysate was attributed to the amino acid composition, the content of hydrophobic amino acid and the molecular weight of the resulting peptide (Haiwei at al., 2010).

Superoxide is highly toxic species and potential precursors of highly reactive species, such as hydroxyl radical, and the study of the scavenging of superoxide radical is essential. In the current study, the 1 kDa fraction possessed a higher superoxide scavenging activity of 42.72% in comparison to the 1-3 kDa fractions, which were 31.97%. In addition, the low molecular weight at <1 kDa and 1-3 kDa showed significantly stronger superoxide scavenging activity when compared to the hydrolysate. Thus, the result demonstrates positive relationship between superoxide scavenging activity and low molecular weight. In addition, HPH fractions are good antioxidant and have free radical scavenging activity and this fraction can be a potential source of natural antioxidant. A similar finding was reported of African bean peptide fractions that exhibited superoxide scavenging activity at <3 kDa with a value of 43%. Interestingly, Ajibola and colleagues (2011) reported the superoxide scavenging activity was not affected by the

presence of a high amount of hydrophobic amino acid. In previous study, peptide fraction from zein hydrolysate at (<1,1-3, 1-3, 3-5, 5-10, and >10 kDa) displayed antioxidant activity (Tang et al., 2010). The superoxide scavenging activity increased with decreasing molecular weight of the peptides.

Transition metal ions, such as Fe^{2+} and Cu^{2+} catalyze the formation and progression of reactive oxygen species which causes biological damage (Prior et al., 2005). In addition, Fe^{2+} can catalyze the Haber-Weiss reaction to form toxic hydroxyl radicals. In the current study, the <1 kDa and 1- 3 kDa peptide fractions from HPH exhibited strong Fe^{2+} chelating ability of 86.3% and 85.65% respectively. Thus, the result demonstrates HPH peptide fractions are very effective Fe^{2+} chelators. The results provide evidence that HPH and low molecular weight HPH fractions have antioxidant ability through the capturing of transition metals Fe^{2+} and therefore inhibit the progression of the Haber-Weiss reaction. The current study observed higher metal chelating activity in comparison to peptide fractions from African yam bean seed (APH) seed with ~70% Fe^{2+} chelating ability (Ajibola et al., 2011). Interestingly, HPH fraction at <1 and 1–3 kDa were reported to have low chelating activity with value between 15.7 to 20.2% as compared to the current study. The current results indicate HPH obtained with proteinase K is more effective in producing peptide with metal chelating property compared to HPH obtained with enzymes pepsin and pancreatin. Ajibola and colleagues (2011) proposed the high percentage of glutamic acid and glutamine, aspartic acid and asparagine, glycine, leucine, lysine, alanine and phenylalanine amino acids present in the APH and the

membrane fraction contributed to the potent metal ion chelating activity. Furthermore, the 10 kDa and 3 kDa peptide fractions obtained from bovine brisket sarcoplasmic proteins hydrolyzed with papain showed strong metal ion chelating ability of 82.42% and 76.84% respectively (Bernardini et al., 2012). In addition Bernardini et al., (2012) reported the highest Fe^{2+} chelating activity was observed in 10 kDa peptide fractions and the lowest activity in the 3 kDa peptide fractions. For example, 10 kDa and 3 kDa peptide fractions obtained from bovine brisket sarcoplasmic contained in their sequences several alanine and leucine amino acid residues. These findings are in agreement with Ajibola and colleagues (2011) study. Thus, the antioxidant activity of peptides is primarily related to the antioxidant activities of the amino acids present in the sequence.

HPH fractions were evaluated for •OH scavenging activity to better understand their antioxidant properties. •OH is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron (Prior, 2005). Thus, one defense strategy towards oxidative stress is through the scavenging of hydroxyl radical. Upon fractionation of the HPH, an increase in hydroxyl scavenging activity was observed. The HPH fraction of <1 kDa and 1-3 kDa exhibited some hydroxyl scavenging activity with a value of 10.21% and 9% ($p>0.05$) respectively. Thus, the result indicates that the fractionation process of HPH concentrated the active peptides, which lead to an increase in hydroxyl scavenging activity of the peptide. The results demonstrate that the amount of hydroxyl radical scavenging peptides was low in the HPH. Thus, since some degree of hydroxyl scavenging

was observed, peptides from hemp protein may have the potential to be developed as hydroxyl radical scavengers to provide multiple health benefits during oxidative stress. Several studies have shown a good correlation between short peptides with radical scavenging activity of protein hydrolysates and peptides. Abjiola et al., 2011 reported <1 kDa peptides obtained with hydrolysis of African yam bean with alcalase possessed significantly stronger hydroxyl radical scavenging activity of 28.21% when compared to a larger molecular weight at 1–3 kDa, 3–5 kDa, 5–10 kDa and its hydrolysate. The potent hydroxyl radical scavenging property of the <1 kDa peptides was attributed to small peptide size, and the presence of high amount of hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine and aromatic amino acids (phenylalanine, tryptophan and tyrosine) (Abjiola et al., 2011). In addition, peptides derived from Black Soy bean were reported to possess antioxidant activity at concentration of 3 mg/ml. Haiwei (2010) identified peptide fraction from black soy bean with the most potent hydroxyl scavenging activity. Interestingly, the fraction with the strongest antioxidant activity displayed 78.23% inhibition of hydroxyl radical which was attributed to the amino acid composition and the molecular weight. However, the observed hydroxyl radical scavenging (78%) was at higher concentration (3mg/ml) as compared to the current study (1mg/ml). The fraction with most potent antioxidant activity contained high amount of hydrophobic amino acids or hydrophobicity (Haiwei, 2010). Moreover, molecular weight of the fraction with

the most potent antioxidant peptide fraction of black soy bean was reported between 100 to 1,000 Da (Haiwei, 2010).

Overall, antioxidant activity was observed in HPH and its peptide fractions. The HPH showed DPPH radical scavenging activity, metal chelating activity, and superoxide radical scavenging activity. The scavenging activity of HPH against DPPH radical was more effective than superoxide anion radical and hydroxyl radical. In addition the metal chelating activity of HPH and its fractions were stronger than FRAP. Furthermore, fractionation of HPH using membrane ultrafiltration to obtain low molecular weight peptides of <1kDa and 1-3kDa produced peptides with enhanced DPPH radical scavenging activity, metal ion chelating, superoxide radical and hydroxyl scavenging activity. The peptides fractions exhibited stronger scavenging activity against DPPH radical compared to superoxide anion radical and hydroxyl radical scavenging activity. In addition, the HPH fraction (1 KDa and 1-3 kDa) exhibited higher DPPH radical scavenging activity, superoxide radical and hydroxyl radical scavenging activity as well as metal chelating activity when compared to HPH. This study shows due to the rich amino acids content of hempseed proteins, peptides released by Proteinase K activity possessed antioxidant activity. The antioxidant activity of hemp protein hydrolysates and peptide fractions were evaluated using several antioxidant assays. However, in this study the relationships between amino acid profile of fractionated peptides and the antioxidant activities were not determined. Thus further study is required to investigate the profile of amino acids present in the hemp protein fractions.

CHAPTER 6

6.0. Summary and Conclusions

Several antioxidant evaluation systems were used to evaluate the antioxidant activity of peptides derived from Canadian grown hempseed. This study demonstrated the relationship between enzyme concentration and antioxidant activity, as well as the effect of peptide sizes and antioxidant activities against reactive oxygen species.

The present study determined the optimum concentration that is required to liberate maximum amounts of bioactive peptide sequences present in hempseed proteins using 1%.Proteinase K Due to the rich amino acids of hempseed proteins, peptides released by Proteinase K activity possessed antioxidant activity. The current study also demonstrated that Proteinase K released bioactive peptides during hydrolysis of hempseed proteins and this peptides possessed antioxidant properties, *in vitro*. In addition the size of the peptides determined the degree of potency against reactive oxygen species.

Upon hydrolysis of HPI using Proteinase K, the HPH exhibited several antioxidant activities through various mechanisms including DPPH scavenging activity, metal chelating activity, and superoxide radical scavenging activity. The HPH showed weak reducing power, and hydroxyl scavenging activity. In addition, the current study showed no relationship between the antioxidant activity and the concentration of Proteinase K (1-4% by weight of protein in the hempseed

protein isolate). Thus, all the HPH exhibited similar antioxidant activity. Therefore, significant amount of bioactive peptides sequences can be liberated from hydrolysis of hempseed protein at a low concentration (1%) of Proteinase K, thus providing a cost effective benefit in the production of food-derived peptides.

The present study demonstrated that hydrolysis process followed by ultrafiltration led to an increased DPPH scavenging activity, superoxide scavenging activity, metal chelating and hydroxyl scavenging activity at LMW of <1KDa and 1-3KDa. The LMW of <1KDa peptide fraction showed the most potent antioxidant activity.

The current study demonstrated that hempseed proteins can be enzymatically hydrolyzed to produce low molecular peptides at <1KDa and 1-3KDa with potent antioxidant property. Therefore, peptides derived from hempseed can be used as ingredients to formulate functional foods with antioxidant activities, leading to the enhancement of the physiological activity of the products. This additional data will contribute to the value-added use of hempseed that will ultimately benefit Canada’s economy.

CHAPTER 7

7.0. Future Research

Peptides from Hempseed protein showed antioxidant activity through different antioxidant mechanisms. Future research efforts should be directed towards determining the amino acid analysis to determine the relationships between amino acid contents of fractionated peptides and antioxidant activities. Furthermore, more research is needed on purification, isolation and identification of antioxidant peptides and the mechanism of their antioxidant activities.

In addition, to study the relationship between peptide charges and hydrophobic properties reverse-phase high performance liquid chromatography (HPLC) can be used to fractionate peptides based on their hydrophobic properties. Furthermore, peptide fractions of particular net charges can be obtained by chromatography using selective ion-exchange columns.

The ability of bioactive peptides to exert a physiological effect *in vivo* is dependent on the bioavailability of the peptide. Thus, evaluation of *in vivo* health-promoting effects, and bioavailability in human subjects should be determined. The absorption and transport of the peptides should be studied in cell culture models, in human intestinal epithelial cells, with peptides administrated at physiological dose. Thus, animal models would be essential to study the physiological effect in vivo.

CHAPTER 8

8.0. References

1. Agriculture and Agrifood Canada. Functional Foods and Nutraceuticals. 2008. http://www4.agr.gc.ca (accessed online September 15, 2009).

2. Agriculture and Agri-Food Canada (2007). Canada's Industrial Hemp. Industry. 2007. http://www4.agr.gc.ca/AAFC-AAC/display-afficher.do?id=1174595656066&lang=eng (accessed online September 15, 2009)

3. Agricultural Marketing Resources Center. Industrial Hemp profile.; 2011 http://www.agmrc.org/commodities__products/fiber/industrial-hemp-profile/ (accessed online June 15, 2012).

4. Ajibola CF, Fashakin JB, Fagemi TN, Aluko RE. 2011. Effect of Peptide Size on Antioxidant Properties of African Yam Bean Seed (Sphenostylis stenocarpa) Protein Hydrolysate Fractions. International Journal of Molecular science 12:6685-6702.

5. Aluko RE. (2009). Personal communication.

6. Amadou I, Le G-W, Shi Y-H, Jin S. 2011. Reducing, Radical Scavenging, and Chelation Properties of Fermented Soy Protein Meal Hydrolysate by Lactobacillus plantarum LP6. International Journal of Food Properties 14:654-665.

7. Barbana C, Boye JI. 2010. Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. Food Research International 43:1642-1649.

8. Bernardinia RD, Mullena AM, Boltonb D, Kerry J, O'Neillc E, Maria Hayesd M. 2012. Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. Meat Science 90:226-235

9. Butterfield DA, Sultana R. Methionine-35 of Aβ (1–42): Importance for Oxidative Stress in Alzheimer Disease (2011; April 14; Epub ahead of print). Journal of Amino Acids Volume 2011 (2011), [doi:10.4061/2011/198430].

10. Cadenas E, Davies KJA. 2000. Mitochondrial free radical generation, oxidative stress, and ageing. Free radical Biology and Medicine 29:222-230.

11. Callaway JC. 2004. Hempseed as a nutritional resource. An Overview. Euphytical 140:65-72.

12. Canadian Hemp Trade Alliance, 2012. About the Canadian Hemp Trade Alliance. http://www.hemptrade.ca/index.php (accessed online July 30, 2012).

13. Chay BP, Mine Y, Juneja LR, Okubo T, Gauthier SF, Pouliot Y. 2011. Comparative Composition and Antioxidant Activity of Peptide Fractions Obtained by Ultrafiltration of Egg Yolk Protein Enzymatic Hydrolysates. Membranes 1:149-16.

14. Chen C, Chi Y-J, Zhao M-Y, Lei L. (2011; Oct 2; Epub ahead of print]). Purification and identification of antioxidant peptides from egg white protein hydrolysate. [DOI 10.1007/s00726-011-1102-0].

15. Chen HM, Muramoto K, Yamauchi F, Nokihara K. 1996. Antioxidant activity of designed peptides based on the antioxidant peptide isolated from digests of a soybean protein. Journal of Agriculture Food Chemistry 44:2619–23.

16. De Avellar IGJ, Magalhaes MM, Silva AB, Souza LL, Leitao AC, Hermes-Lima M. 2004. Reevaluating the role of 1, 10-phenanthroline in oxidative reactions involving ferrous ions and DNA damage. Biachimica Biophys Acta 1675: 46–53.

17. Erdmann K, Cheung BWY, Schroder H. 2008. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. Journal of Nutritional Biochemistry 19:643–54.

18. Erdmann K, Grosser N, Schipporeit K, Schroder H. 2006. The ACE inhibitory dipeptide Met-Tyr diminishes free radical formation in human endothelial cells via induction of hemeoxygenase-1 and ferritin. Journal of Nutrition 136:2148–52.

19. Gao, D.; Cao, Y.; Li, H. 2010. Antioxidant activity of peptide fractions derived from cottonseed protein hydrolysate. Journal of the Science of Food and Agriculture 90:1855–1860.

20. Girgih AT, Udenigwe CC, Aluko, RE. 2010. In vitro antioxidant properties of hemp seed protein hydrolysate fractions. Journal America oil Chemists' Society 88: 381–389.

21. Girgih AT, Udenigwe CC, Li H, Adebiyi AP, Aluko RE. 2011. Kinetics of Enzyme Inhibition and Antihypertensive Effects of Hemp Seed (Cannabis sativa L.) Protein Hydrolysates. Journal America oil Chemists' Society 88:1767–1774.

22. Guo, H, Kouzuma Y, Yonekura M. 2009. Structures and properties of antioxidative peptides derived from royal jelly protein. Journal of Food Chemistry 113:238–245.

23. Haiwei R. 2010. Antioxidant and free radical-scavenging activities of black soybean peptides (BSP). International Journal of Agricultural and Biological Engineering 3:64-68.

24. Herregods G, Camp JV, Ghesquiere NMB, Gevaert K, Vercruysse L, Dierckx S, Quanten E, Smagghe G. 2011. Angiotensin I-Converting Enzyme Inhibitory Activity of Gelatin Hydrolysates and Identification of Bioactive Peptides. Journal of Agricultural and Food Chemistry 59:552–558.

25. House JD, Neufeld J, Leson G. 2010. Evaluating the quality of protein from hemp seed (Cannabis sativa L.) products through the use of the protein digestibility-corrected amino acid score method. Journal of Food chemistry 58:11801–11807.

26. Johnson R. 2012. Hemp as an Agricultural Commodity. Congressional Research Service. 7-5700. http://www.fas.org/sgp/crs/misc/RL32725.pdf (accessed online July 25, 2012)

27. Kang K-H, Qian Z-J, Ryu B, Karadeniz F, Kim D, Kim S-K (2012 Mar 19: Epub ahead of print). Antioxidant Peptides from Protein Hydrolysate of Microalgae Navicula incerta and their Protective Effects in Hepg2/CYP2E1 Cells Induced by Ethanol. [DOI: 10.1002/ptr.4603].

28. Kehrer JP. 2000. The Haber–Weiss reaction and mechanisms of toxicity. Toxicology 149:43–50.

29. Kim S-K, Wijesekara I. 2010. Development and biological activities of marine-derived bioactive peptides: a review. Journal Functional Foods 2:1–9.

30. Korhonen H, Pihlanto A. 2003. Food-derived bioactive peptides-opportunities for designing future foods. Current Pharmaceutical Design 9:1297–1308.

31. Korhonen H, Pihlanto A. 2006. Bioactive peptides: production and functionality. International Dairy Journal 16:945–60.

32. Kong XZ, Guo MM, Hua Y, Cao D, Zhang CM. 2008. Enzymatic preparation of immunomodulating hydrolysates from soy proteins. Bioresource Technology 99:8873–8879.

33. Koo SH, Bae Y, Lee S, Lee D, Hu B-SH, Lee HG (2011. Epub ahead of print). Evaluation of wheat gluten hydrolysates as taste-active compounds with antioxidant activity [DOI 10.1007/s13197-011-0515-9].

34. Kunwar A, Priyadarsini KI. 2011. Free radicals, oxidative stress and importance of antioxidants in human health. Journal Medical and Allied Sciences 1:53-60.

35. Lee, J.; Koo, N.; Min, D.B. 2004. Reactive oxygen species, aging, and antioxidant nutraceuticals. Comprehensive Review Food Science and Food Safety 3:21–33.

36. Locatelli M, Ginder R, Travaglia F, Coisson J-D, Maurizio Rinaldi M, Marco Arlorio M. 2009. Study of the DPPH Scavenging activity: Development of free software for the correct interpretation of data. Journal of Food chemistry 114: 889-897.

37. Markwell MAC, Haas SM, Biebar LL, Tolbert NE.1978. A modification of the Lowry procedure to simplify protein determination in membrane and in protein samples. Analytical Biochemistry 87:206–211.

38. Marnett L. 2000. Oxyradicals and DNA damage. Carcinogenesis 21:36-370.

39. Molyneux P. 2004. The use of stable free radical diphenylpicryl (DPPH) for estimating antioxidant activity. Journal of Science Technology 26:211-219.

40. Moller NP, Scholz-Ahrens KE, Roos N, Schrezenmeir J. 2008. Bioactive peptides and proteins from foods: indication for health effects. European Journal of Nutrition 47:171-182.

41. Nagpal R, Behare P, Rana R, Kumar A, Kumar M, Arora S, Morotta F, Jaing S, Yadav H. 2011. Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. The Royal Society of Chemistry 18:18-27.

42. Ndiaye F, Vuong T, Duarte J, Aluko RE, Matar C. 2012. Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein

hydrolysate from yellow field pea seeds. European Journal of Nutrition 51:29–37.

43. Najafian L, A.S. Babji A.S. 2012. A review of fish-derived antioxidant and antimicrobial peptides: Their production, assessment, and applications. Peptides, 33:178–185.

44. Pihlanto A. 2006. Antioxidative peptides derived from milk proteins. International Journal of Dairy Technology 16:1306–14.

45. Pihlanto A, Akkanen S, Korhonen HJ. (2008). ACE-inhibitory and antioxidant properties of potato (Solanum tuberosum). Food Chem. 109, 104-112.

46. Powell T. 2009. Effects of Molecular Charge and Hydrophobicity on the Antioxidative properties of Pea (Pisum sativum L.) Protein Hydrolysate Fractions. A Thesis/Practicum, University of Manitoba.

47. Pownall TL, Udenigwe, CC, Aluko R.E. (2010). Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. Journal of Agricultural and Food Chemistry 58:4712–4718

48. Pownall TL, Udenigwe CC, Aluko RE. 2011. Effects of cationic property on the in vitro antioxidant activities of pea protein hydrolysate fractions. Food Research International 44:1069–74.

49. Porto CD, Decorti D, Tubaro F. 2012. Fatty acid composition and oxidation stability of hemp (Cannabis sativa L.) seed oil extracted by supercritical carbon dioxide. Industrial Crops and Products 36:401–404.

50. Qian B, Xing M, Cui L, Deng Y, Xu Y, Huang M, Zhang S. 2011. Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with Lactobacillus delbrueckii ssp. bulgaricus LB340. The Journal of Dairy Research 72:7972-7.

51. Rajapakse N, Mendis E, Byun HG, Kim SK. (2005). Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. Journal of Nutritional Biochemistry 16:562–569.

52. Roche Applied Science. Product Catalog Proteinase K, recombinant, PCR Grade Lyophilizate from *Pichia pastoris http://www.rocheapplied-science.com/proddata/gpip/3_6_14_8_1_7.html*

53. Ryan JT, Ross RP, Bolton D, Fitzgerald GF, Stanton C. 2011. Bioactive Peptides from Muscle Sources: Meat and Fish. Nutrients 3:765-791.

54. Saiga A, Tanabe S, Nishimura T. 2003. Antioxidant Activity of Peptides Obtained from Porcine Myofibrillar Proteins by Protease Treatment. Journal of Agricultural and Food Chemistry 51:3661–3667.

55. Seseko, S, Kaneko Y. 1985. Cough associated with the use of captopril. Archives of Internal Medicine, 145, 1524–1529. Staessen, J. A., Li, Y., & Richart, T. (2006). Oral renin inhibitors.Lancet, 368, 1449–1456.

56. Tang C-H, Ten Z., Wang X-S, Yang X-Q. 2006. Physiochemical and Functional Properties of Hemp (*Cannabis Sativa L.*) Protein Isolate. Journal of Agriculture and Food Chemistry 54*:*8945-8950.

57. Tang X, He Z, Dai Y, Xiong YL, Xie M, Chen J. 2010. Peptide fractionation and free Radical Scavenging Activity of Zien Hydrolysate. Journal of Agricultural and Food Chemistry 58:587-593.

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

59. Takahashi M, Moriguchi S, Yoshikawa M, Sasaki R. 1994. Isolation and characterization of oryzatensin: a novel bioactive peptide with ileum-contracting and immunomodulating activities derived from rice albumin. International Journal of Biochemistry and Molecular Biology 33:1151–8.

60. Theodore AE, Raghavan S, Kristinsson HG. 2008. Antioxidative activity of protein hydrolysates prepared from alkaline-aided channel catfish protein isolates. Journal of Agricultural and Food Chemistry 56:7459–7466.

61. Uchida, K. (2000). Role of reactive aldehyde in cardiovascular diseases. Free radical biology and Medicine 28:1685-1696.

62. Udenigwe CC, Lu Y.-L., Han CH, Hou W.C., Aluko RE. 2009. Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. Food Chemistry 116:277–284.

63. Udenigwe CC, Aluko RE. 2011. Chemometric analysis of the amino acid requirements of antioxidant food protein hydrolysates. International Journal of Molecular Science 12:3148-3161.

64. Vermeirssen V, Van Camp J, Verstraete W. 2004. Bioavailability of angiotensin I converting enzyme inhibitory peptides. British Journal of Nutrition 92:357–66.

65. Wang X-S, Tang C-H, Chen L, Xiao-Quan Yang X-Q. 2009. Characterization and antioxidant properties of Hemp protein hydrolysate obtained with neutrase. Food Technology and Biotechnology 47:428-434.

66. Wiesner J, Vilcinskas A. 2010. Antimicrobial peptides. The ancient arm of the human immune system. Virulence 1:440-464.

67. Xie Z, Huang J, Xu X. & Jin Z. 2008. Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate. Food Chemistry 111:370-376.

68. Yang R, Zhang Z, Pei X, Han X,Wang J,Wang L, Long Z, Shen X, Li Y. 2009. Immunomodulatory effects of marine oligopeptide preparation from Chum Salmon (Oncorhynchus keta) in mice. Journal of Food Chemistry 113:464–70.

69. You S-J, Wu J. 2011. Angiotensin-I Converting Enzyme Inhibitory and Antioxidant Activities of Egg Protein Hydrolysates Produced with Gastrointestinal and Non gastrointestinal Enzymes. Journal of Food Science 76:801-807.

70. Yu LL, Zhou KK , Parry J. 2004. Antioxidant properties of cold-pressed black caraway, carrot, cranberry, and hemp seed oil. Journal of Agricultural and Food Chemistry 91:723–729.

71. Yust MM, Millán-Linares MC, Alcaide-Hidalgo JM, Millán F, Pedroche J. 2011. Hypocholesterolaemic and antioxidant activities of chickpea (Cicer arietinum L.) protein hydrolysates. Journal of Science of Food and Agriculture 92:1994-2001.

72. Zhang SB, Wang Z, Xu SY. 2008. Antioxidant and Antithrombotic Activities of Rapeseed Peptides. Journal of the American Oil Chemists' Society 85:521–527.

73. Zhu L, Chen J, Tang X, Xiong Y.L. 2008. Reducing, radical scavenging, and chelation properties of *in vitro* digests of alcalase treated zein hydrolysate. Journal of Agriculture Food Chemistry 56:2714–2721.