Effects of Molecular Charge and Hydrophobicity on the Antioxidative Properties of Pea (*Pisum sativum* L.) Protein Hydrolysate Fractions

By

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A Thesis submitted to the Faculty of Graduate Studies of

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In partial fulfilment of the requirements of the degree of

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ABSTRACT

Reactive oxygen species are implicated to be the basis for a variety of disease conditions, including cardiovascular disease. When produced in excess, reactive oxygen species can have deleterious effects in the biological system; therefore, compounds that can scavenge free radicals could be useful therapeutic agents.

We studied the antioxidant activities of peptides derived from pea protein hydrolysate using seven *in vitro* antioxidant evaluation systems that included the chelation of metal ions and the ability to scavenge reactive oxygen species. Pea protein hydrolysate was separated based on net hydrophobic properties and net cationic charges using reverse phase high performance liquid chromatography and cation exchange chromatography, respectively.

Five fractions with a range of net hydrophobic properties were obtained and screened for antioxidant activities. The fractions with the highest net hydrophobic properties exhibited the strongest scavenging and metal chelating activities except hydrophobic properties did not play a role in the reducing power. The fraction with the most hydrophobic properties also contained the highest concentration of hydrophobic amino acids and the lowest concentration of charged amino acids.

Separation of pea protein hydrolysate based on net cationic charge yielded five fractions with a range of net cationic charges. The fractions with the least net cationic charge displayed the strongest scavenging activities such as superoxide, hydrogen peroxide and 1,1-diphenyl-2-picrylhydrazyl scavenging with the exception of the reducing power. Peptides separated by net cationic charge did not display scavenging activity against the hydroxyl radical and had zero metal chelating activity.

The results indicate that pea protein hydrolysate consists of peptides that possess antioxidant activities. Hydrophobic properties and cationic charges of the peptides were shown to affect the antioxidant activity of pea protein hydrolysate fractions. Separation of pea protein hydrolysate based on hydrophobic properties produced peptide fractions with the most robust antioxidant activities. The results show that the pea protein hydrolysate fractions contain various peptides that have the potential to be used as therapeutic agents against chronic diseases that develop from cell or tissue oxidative damage.

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LIST OF ABBREVIATIONS

ROS: Reactive Oxygen Species

BHT: Butylated Hydroxytoluene

DPPH: 1,1-diphenyl-2-picrylhydrazyl

pI: Isoelectric Point

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

FPLC: Fast Protein Liquid Chromatography

PPI: Pea Protein Isolate

PPH: <3 kDa Pea Protein Hydrolysate

LDL Cholesterol: Low Density Lipoprotein Cholesterol

CHAPTER ONE

GENERAL INTRODUCTION

The functional foods and nutraceuticals industry in Canada generated $2.9 billion in revenue in 2004 according to the Statistics Canada Functional Foods and Nutraceuticals Survey (Palinic, 2005). The demand for functional foods and nutraceuticals is increasing due to the ageing Canadian population and the rising cost of healthcare and pharmaceuticals. It is estimated that Canadians spent $29.8 billion on prescription and non-prescription drugs in 2008 (Canadian Institute for Health Information, 2009). Functional foods and nutraceuticals have the potential to reduce healthcare costs and improve health status of Canadians as well as support economic growth in rural Canadian communities (Agriculture and Agri-Food Canada, 2009). Nutraceuticals are products which are isolated and purified from food sources, therefore, sold in medicinal forms not usually associated with foods and can exert physiological benefits against the progression of chronic diseases (Health Canada, 2002). Functional foods are similar to conventional foods and are generally consumed as part of a usual diet; therefore, functional foods can provide protection against the progression of chronic diseases above and beyond basic nutritional roles (Health Canada, 2002).

Many foods commonly consumed in the Canadian diet have been recognized to contain bioactive compounds which provide health benefits upon isolation and purification. Canadian nutraceutical and functional food companies produce bioactive materials including soluble fibre from oats, barley and pulses, omega-3 fatty acids from fish and flax oil, unsaturated fatty acids from canola oil and proteins from soy (Agriculture and Agri-Food Canada, 2009).

A major focus of the functional foods and nutraceuticals market is antioxidants, as damage caused by reactive oxygen species (ROS) is considered to be the basis for the progression of ageing and a wide variety of diseases, including inflammatory bowel disease (Kruidenier et al., 2003), chronic kidney disease (Oberg et al., 2004), cardiovascular disease, cancer and neurodegenerative diseases (Seifried et al., 2007). In particular, the superoxide anion gives rise to the formation of more harmful ROS (Valko et al., 2007), including the hydroxyl radical ($^{\cdot}OH$) and peroxynitrite ($ONOO^{-}$). Epidemiological data have shown a correlation between the intake of foods with high concentrations of antioxidants and the incidence of cancer, ageing and cardiovascular diseases (Seifried et al., 2007). Antioxidants have the ability to scavenge reactive oxygen and nitrogen species through the inhibition of radical chain reactions or the formation of radicals (Huang & Prior, 2005).

The biological system utilizes antioxidant enzymes (superoxide dismutase, glutathione peroxidase and catalase) to protect membrane lipids and proteins from oxidation (Huang & Prior, 2005). As well, other non-enzymatic antioxidants including dietary sources participate in the protection against damage due to ROS. An endogenous antioxidant synthesized in the biological system is a tripeptide, glutathione, which provides protection against ROS by becoming oxidized to glutathione disulphide (GSSG) or by serving as a cofactor for antioxidant enzymes (Diplock et al., 1998). Glutathione is the major antioxidant in the cytosol, nuclei and mitochondria (Valko et al., 2007).

The body is continuously exposed to ROS produced during essential biological functions including cellular metabolism (Valko et al., 2007) and immune defences (Kobayashi et al., 1998). The human body is also exposed to ROS generated from

exogenous sources including natural and synthetic toxins (Davies, 2000). ROS generated by the biological system are imperative for the maintenance of homoeostasis by protecting the biological system against invading pathogens, for cell signalling and for apoptosis of damaged cells (Seifried et al., 2007). Excessive production of ROS generated within a biological system beyond the amount required to maintain homeostasis will exceed endogenous defences and lead to the initiation of lipid, protein and DNA damage. Oxidation of lipids and proteins in the body may accelerate the progression of disease. Furthermore, oxidation of lipids in food systems can lead to undesirable changes in colour, flavour, texture and produce toxic reaction products (Park et al., 2008). Therefore, there is the need to identify natural antioxidants that could delay or prevent the onset and progression of chronic diseases.

The diet contains a variety of compounds, such as ascorbate, tocopherols, carotenoids and flavonoids, which possess antioxidant activities and scavenge ROS based on their structural properties (Diplock et al., 1998). Ascorbate has been shown to be effective against the superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen (Diplock et al., 1998). However, ascorbate could behave as a pro-oxidant in biological systems, reducing transition metals and therefore increasing their participation in the Fenton reaction (Diplock et al., 1998). Polyphenols display free radical scavenging capacity and metal chelating ability (Soobrattee et al., 2005). The ability of flavonoids to act as antioxidants is thought to be due to the hydroxyl groups in the flavonoid structure (Cao et al., 1997). Although flavonoids are effective antioxidants, their bioavailability is poor as they are rapidly conjugated by phase II detoxification enzymes (Diplock et al., 1998). Synthetic antioxidants are also available, such as butylated hydroxytoluene (BHT),

to increase shelf life and preservation of food products. However, there are concerns regarding the safety of the consumption of synthetic antioxidants (Safer & Al-Nughamish, 1999).

The oxidation of low density lipoprotein (LDL) cholesterol is due to lipid peroxidation initiated by free radicals (Diplock et al., 1998) and oxidation of lipids consists of three stages: initiation, propagation and termination. Polyunsaturated fatty acids are particularly susceptible to lipid oxidation. The superoxide anion, which is produced during cellular metabolism (Valko et al., 2007) and immune defences (Kobayashi et al., 1998), leads to the propagation of other reactive oxygen species and the initiation of damage to biological membranes. Lipid peroxidation occurs by the attack of a reactive oxygen species, such as the hydroxyl radical, that abstracts a hydrogen atom from a methylene group located between two carbon-carbon double bonds (Halliwell & Gutteridge, 1990). The removal of a hydrogen from the methylene group causes the carbon to have an unpaired electron (Halliwell & Chirico, 1993), which can then react with oxygen to form a peroxyl radical (Halliwell & Gutteridge, 1990). The peroxyl radical can then abstract hydrogen from other lipid molecules forming another peroxyl radical and therefore facilitating a chain reaction (Halliwell & Gutteridge, 1990) and the propagation of lipid peroxides.

Antioxidants can act at different stages within the process of lipid oxidation. Antioxidants can prevent the initiation of the chain reaction by scavenging an initiating radical, the hydroxyl radical, chelating metal ions to prevent the formation of the hydroxyl radical, scavenging singlet oxygen (Shahidi, 1997) or interrupting the chain reaction through the donation of hydrogen (Halliwell & Chirico, 1993). Lipid

peroxidation in cell membranes leads to alterations in cell membrane fluidity and increased permeability (Halliwell, 1992) and ultimately contributes to the progression of the development of atherosclerotic plaque.

Peptides could be used in the formulation of functional foods and nutraceuticals to prevent damage related to oxidative stress both in biological and food systems. Although natural antioxidants such as protein hydrolysates display less potent antioxidant activity in comparison to synthetic antioxidants, natural antioxidants can be used at higher concentrations than synthetic antioxidants because of the restricted use of synthetic antioxidants (Li et al., 2008). Also, natural compounds can be used at higher concentrations without the toxic side effects associated with the use of synthetic drugs (Aluko, 2008b).

Peptides have been observed to avoid gastrointestinal digestion, exhibit local effects in the gastrointestinal tract (Erdmann et al., 2008) and be transported across intestinal epithelial cells from the lumen of the gastrointestinal tract. After transport through intestinal epithelial cells, peptides enter blood vessels (Li & Aluko, 2006), where they could elicit bioactivity. As well, it has been suggested that peptides could act locally in the intestinal tract or through receptors and cell signalling (Moller et al., 2008). Furthermore, peptides could be effective *in vivo* as they have been noted to be more efficiently absorbed from the lumen of the gastrointestinal tract in comparison to free amino acids and proteins (Kodera et al., 2006). Extensive research exploring the antioxidant activity of peptides hydrolyzed from food protein has been conducted; however, the structure-function relationship between peptide characteristics and

antioxidant activity has not been fully elucidated (Pihlanto, 2006; Je et al., 2008; Kim et al., 2001).

This study aims to establish the antioxidant activities of peptides derived from enzymatic hydrolysis of proteins from Canadian grown pea seeds on the basis of hydrophobic and cationic properties. Therefore, findings from the present study will contribute to an understanding of the structure-function relationship of antioxidant peptides.

Objectives of Study

- Fractionate pea protein hydrolysate peptides according to their charge and hydrophobic properties
- Determine the antioxidant activities of fractionated peptides using a broad array of biologically relevant assays
- Determine the relationships between peptide charge or hydrophobicity and the antioxidant activities
- Determine the relationships between amino acid contents of fractionated peptides and antioxidant activities

CHAPTER TWO

LITERATURE REVIEW

2.1 Pea protein and amino acid profile

Pea seed is an important commodity to the Canadian economy, and more specifically, to the economy of the Prairie Provinces, Manitoba, Saskatchewan and Alberta (Pulse Canada, 2007). Manitoba is the third largest pea producer in Canada (Agriculture and Agri-Food Canada, 2008) and Canada is the world's largest producer and exporter of pea (Pulse Canada, 2007). Yellow field pea variety accounts for approximately 80% of the peas produced in Canada (Agriculture and Agri-Food Canada, 2008). In 2007-2008, Canada produced 30% of the world's dry pea stock (Agriculture and Agri-Food Canada, 2008).

Pea seed is considered a significant source of protein, consisting of 25% protein by weight (Iqbal et al., 2006). Pea protein has comparable amino acid profile to other commonly consumed legumes (Iqbal et al., 2006). The protein component of pea has been studied in relation to the functional characteristics and physicochemical properties (Shand et al., 2007; Humiski & Aluko, 2007). Due to the high protein content, pea is commonly used as animal feed, however, pea seed contains negligible amounts of sulphur containing amino acids (Wang et al., 2003) and tryptophan. Therefore, is not considered a complete protein. Table 2.1 shows that the predominant amino acids in pea protein are the charged, hydrophilic amino acids including arginine, aspartic acid, glutamic acid, histidine and lysine. In addition, hydrophobic amino acids (leucine and phenylalanine) are present in moderate levels. The major storage proteins of pea seed are globulins, followed by albumins (Wang et al., 2003). Globulins consist of two protein

types, legumin and vicillin that are rich in acidic amino acids (Haydar & Hadziyev, 1973). Pea seeds are widely consumed by both humans and livestock; however, pea protein-based ingredients are not commonly used in food products (Shand et al., 2007).

Table 2.1 Amino acid composition of Canadian field pea seed protein (g/16g of nitrogen)

Amino Acid	Amount (g/16g N)
ASP	12.50
THR	4.40
SER	5.60
GLU	15.60
PRO	4.80
GLY	4.30
ALA	4.10
CYS	1.20
VAL	4.00
MET	1.10
ILE	3.30
LEU	6.50
TYR	2.90
PHE	4.40
HIS	2.30
LYS	6.30
ARG	8.50
TRP	0.80

Adapted from: Wang & Daun (2004)

2.2 Biological activities of pea seed

Peas belong to the pulse family and are widely recognized for the associated health benefits. As well as being considered a significant source of plant protein, field pea is also considered to be a good source of fibre, starch, vitamins and minerals (Wang et al., 2003). Fibre purified from pea has been used in enteral formulas to provide a source of dietary fibre (Marlett et al., 2002). Pea seed also contains phenolic compounds, which are recognized to have antioxidant activity (Xu et al., 2007a, 2007b). Yellow field pea seed (varieties including Eclipse and Golden) have phenolic and condensed tannin content similar to green pea seed and yellow soybeans (Xu et al., 2007b). The phenolic content of yellow pea seed varied dependent on the variety, however, yellow and green pea seed contained less phenolic compounds, condensed tannins and flavonoids in comparison to other commonly consumed legumes (Xu et al., 2007b).

Enzymatic hydrolysis methods have produced peptides derived from pea protein which possess functional activities. Pea peptides rich in positively charged amino acids have been shown to modulate the structure of camodulin *in vitro* (Li & Aluko, 2006). Camodulin is a calcium-binding protein that is important in the signal transduction for metabolic pathways (Li & Aluko, 2006). Peptides hydrolyzed from pea protein using different enzymes have exhibited angiotensin converting enzyme inhibitory activity *in vitro* and weak 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Humiski & Aluko, 2007). As well, proteins isolated from two varieties of peas, *Pisum sativum* var. macrocarpon (Ye & Ng, 2003) and *Pisum sativum* var. *arvense* Poir, (Wang & Ng, 2006), with masses of 31 kDa and 11 kDa, respectively, inhibited fungal growth *in vitro*.

Not only do pea seeds contain beneficial bioactive compounds, there are also undesirable components present, including oligosaccharides and antinutritional factors, such as trypsin inhibitors and phytic acid (Wang et al., 2008). Trypsin inhibitors bind trypsin and decrease enzymatic activity, leading to hypersecretion of trypsin. Phytic acid binds minerals, leading to decreased bioavailability and absorption of essential minerals (Lonnerdal et al., 1989).

2.3 Enzymatic protein hydrolysis

Enzymatic hydrolysis of proteins is one approach to release bioactive peptides and is widely applied to improve functional and nutritional properties of protein sources (Je et al., 2008). Hydrolysis of a protein by different enzymes can lead to the formation of different peptide sequences dependent on the enzyme specificity (Vercruysse et al., 2009). Enzymatic hydrolysis is preferred to chemical hydrolysis because enzymatic hydrolysis is milder and more specific. Each enzyme has different specificities as to where the peptide bond is hydrolyzed (Li-Chan, 2004). Enzymes can also be inactivated once the functional property of the hydrolysate has been reached (Li-Chan, 2004). Furthermore, enzymatic hydrolysis is preferred to chemical hydrolysis as some chemical modifications are not acceptable for human consumption (Li-Chan, 2004).

Thermolysin is a bacterial enzyme used for protein hydrolysis that is thermostable (Antonczak et al., 2000) at 100°C for 30 to 60 minutes (Whitaker, 2004). Thermolysin cleaves peptide bonds from the amino group of hydrophobic amino acids (Antonczak et al., 2000).

2.4 Free radicals

Reactive oxygen species are implicated in the progression of a number of disease conditions including, cardiovascular disease, cancer and neurodegenerative diseases (Seifried et al., 2007). ROS and reactive nitrogen species have been recognized to confer deleterious effects in the biological system and also have beneficial roles essential for the maintenance of homeostasis. The scientific community has recognized that ROS are required for cell signalling pathways, apoptosis and proper functioning of the immune system (Finkel & Holbrook, 2000; Seifried et al., 2007).

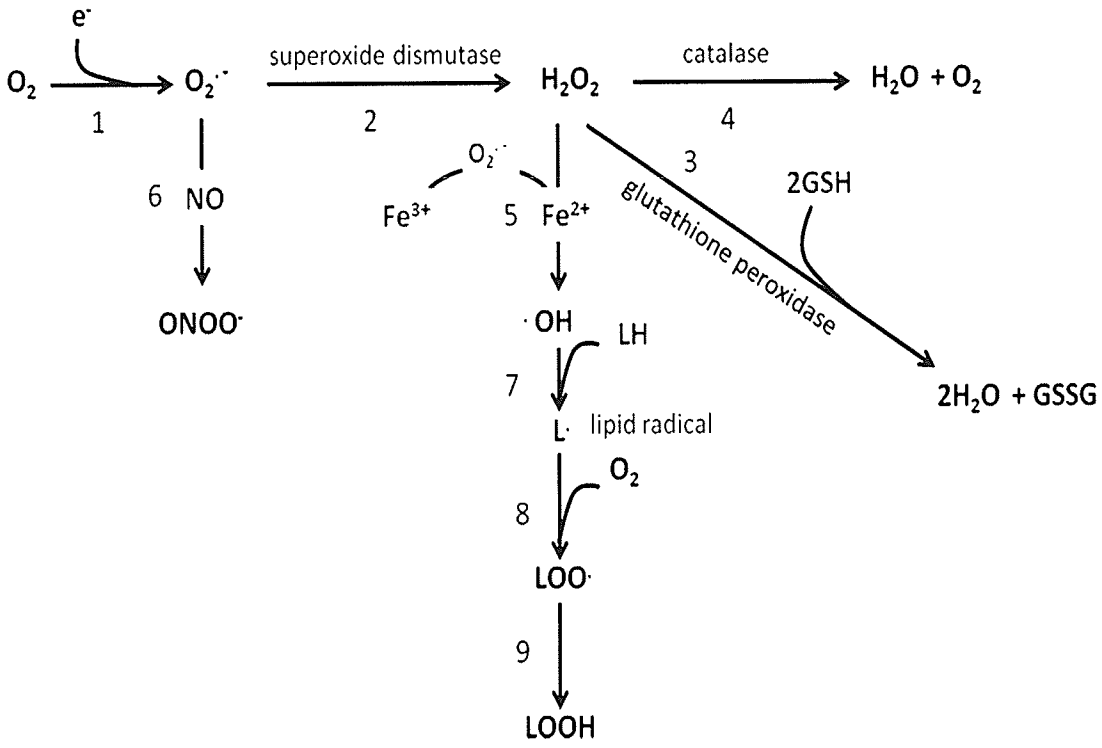
The majority of intracellular ROS produced in the biological system originates from the mitochondria (Finkel & Holbrook, 2000) where electrons leak from the electron transport chain and convert oxygen (O_2) to the superoxide anion ($O_2^{\cdot-}$) (Muhammad et al., 2009) through the addition of a single electron to O_2. Oxygen is considered a radical because it has two unpaired electrons; however, it is poorly reactive (Halliwell, 1992). Superoxide is considered the primary reactive oxygen species (Valko et al., 2007) which leads to the formation of other more harmful ROS as summarized in Figure 2.4 (Reaction 1). Furthermore, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases also contribute to the formation of the extremely unstable radical species, $O_2^{\cdot-}$ (Finkel & Holbrook, 2000), in defence cells, including neutrophils (Kobayashi et al. 1998), which are produced outside the cell by the plasma membrane. In addition to endogenous radicals, the biological system must also protect against radicals generated from synthetic toxins (Davies, 2000).

Superoxide is converted enzymatically to hydrogen peroxide (H_2O_2) by superoxide dismutase (Finkel & Holbrook, 2000) (Reaction 2). Hydrogen peroxide is the

resulting molecule of the addition of two electrons to oxygen (Miller et al., 1990). Hydrogen peroxide is a long-lived molecule and is readily diffusible through cell membranes (Finkel & Holbrook, 2000). As illustrated in Reaction 3 and 4 of Figure 2.4, the resulting hydrogen peroxide can be enzymatically converted to water by catalase and glutathione peroxidase (Elias et al., 2008). However, if hydrogen peroxide is not converted into water, it combines with unbound transition metals, such as iron through the Fenton reaction to form an extremely reactive compound, the hydroxyl radical (Reaction 5). The hydroxyl radical can initiate lipid oxidation, oxidize proteins and damage DNA (Valko et al., 2007). Hydrogen peroxide and superoxide anion do not directly cause DNA damage, unless converted into highly reactive radicals, such as hydroxyl radical (Altman et al., 1995). Upon reaction with DNA, the hydroxyl radical can cause cross links in DNA protein (Altman et al., 1995) and single and double strand breaks (Valko et al., 2007), which may have detrimental biological consequences (Altman et al., 1995). Generally iron within the biological system is bound to storage proteins, however, during increased oxidative stress, superoxide can mobilize iron from the transport proteins allowing iron to participate in the Fenton reaction (Halliwell & Gutteridge, 1990). Hydrogen peroxide can also serve as a precursor for the formation of the highly reactive hypochlorous acid by myeloperoxidase. Nitric oxide is essential for vasodilation and is considered a reactive nitrogen species. Nitric oxide is formed through the oxidation of arginine by nitric oxide synthase (Aruoma, 1998). Nitric oxide can react with the superoxide anion to form peroxynitrite (Reaction 6), which can lead to lipid peroxidation and DNA damage (Vaziri, 2004). Lipid peroxidation occurs by the attack of a reactive oxygen species that abstracts a hydrogen atom from a methylene group of a

polyunsaturated fatty acid (Reaction 7) and can then react with oxygen to form a peroxyl radical (Halliwell & Gutteridge, 1990) (Reaction 8). The peroxyl radical can abstract hydrogen from other lipid molecules forming a lipid peroxide and therefore facilitating the chain reaction (Halliwell & Gutteridge, 1990) (Reaction 9). As well, singlet oxygen can react with membrane lipids to form peroxides.

Figure 2.4: The pathway of reactive oxygen species and lipid oxidation



Adapted from: Vaziri (2004); Valko et al., (2007); Shahidi (1997)

2.5 Antioxidant peptides from various food protein hydrolysates

Bioactive peptides commonly contain 3-20 amino acids per peptide (Korhonen & Pihlanto, 2003) as inactive sequences within large proteins and are released when the parent protein is hydrolyzed by digestive enzymes (*in vitro* and *in vivo*), microbial enzymes or during food processing (Korhonen & Pihlanto, 2003; Kitts & Weiler, 2003; Moller et al., 2008). The biological activity of a peptide is widely recognized to be based on amino acid composition and peptide size (Korhonen & Pihlanto, 2003). Amino acids have been shown to have antioxidant activities *in vitro* (Hernandez-Ledesma et al., 2005) and to increase cellular antioxidant enzymes in cell culture (Katayama & Mine, 2007). In addition, peptides have shown to improve oxidative stability when incorporated into a processed meat food product (Wang & Xiong, 2005).

The function and antioxidant activity of a peptide is dependent on the degree of hydrolysis, specificity of the enzyme and the properties and characteristics associated with the released peptides (Tang et al., 2009a). The degree of hydrolysis determines the peptide size and the functional and chemical properties of peptides (Megias et al., 2007), whereas enzyme specificity determines the peptide sequence and size. Furthermore, antioxidant activities of peptides derived from food sources have been evaluated *in vitro* and have exhibited a range of antioxidant activities, dependent upon the type of ROS the peptide is challenged against.

Elucidating the structure-function relationship of food proteins will help increase their application and use (O'Kane et al., 2005). A wide variety of food sources including animal (Hernandez-Ledesma et al., 2005), plant (Li et al., 2008) and marine (Theodore et al., 2008) sources have been identified to possess bioactive peptides. In particular, pulse

derived peptides generate interest for the production of bioactive peptides because they are more cost effective in comparison to animal proteins (Aluko, 2008b).

Bioactive peptides derived from food sources possess activities beyond the traditional nutrition function and some of them have been shown to possess antihypertensive (Jung et al., 2006), antithrombotic (Zhang et al., 2008) and antioxidative (Je et al., 2008) activities. Food protein derived bioactive peptides have been shown to have strong antioxidant properties, with the ability to scavenge hydroxyl radicals, superoxide anion radicals, hydrogen peroxide and the chelation of metal ions. Each antioxidant evaluation system measures different reaction mechanisms (Xu et al., 2007a), therefore, a diverse variety of evaluation systems must be utilized to fully evaluate the antioxidant profile (Prior et al., 2005) of food-derived peptides.

2.5.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of food derived peptides

DPPH radical is commonly used when evaluating the antioxidant activity of natural compounds (Bougatef et al., 2009) because it is technically simple (Huang et al., 2005). The DPPH radical is a stable nitrogen radical and measures the reducing ability and the hydrogen donation of antioxidants toward the DPPH radical (Prior et al., 2005). DPPH acts both as a radical probe and the oxidant, therefore, the DPPH assay is a non-competitive reaction (Prior et al., 2005).

As indicated in Table 2.5.1, the DPPH radical scavenging evaluation method has been used extensively to evaluate the antioxidant activity of a wide variety of food-derived peptides. Few studies, however, have evaluated the influence of hydrophobic

properties on the DPPH radical scavenging activity. Tang et al. (2009a) found that the scavenging activity for buckwheat protein hydrolysates ranged depending on the degree of hydrolysis. The buckwheat hydrolysate with the strongest DPPH radical scavenging had the highest concentration of hydrophobic amino acids. Humiski & Aluko (2007) also observed that a pea protein hydrolysate with the highest amount of hydrophobic amino acids had the strongest scavenging activity in comparison to other pea protein hydrolysates with less hydrophobic amino acids. A low molecular weight hydrolysate from chickpea protein separated by size exclusion was found to have the strongest DPPH radical scavenging and the highest concentration of hydrophobic amino acids (Li et al., 2008).

Peptides from rapeseed proteins displayed DPPH radical scavenging activity with a dose-dependent increase (Zhang et al., 2008). The fraction of rapeseed peptides which contained the highest concentration of hydrophobic amino acids was found to have the strongest DPPH radical scavenging (Zhang et al., 2007, 2008). The fraction with the strongest activity was rich in leucine, isoleucine, phenylalanine and proline and also contained higher concentrations of tyrosine, tryptophan and cysteine than the crude rapeseed hydrolysate.

Park and colleagues (2008) investigated the DPPH radical scavenging activity of peptides hydrolyzed from soy protein and gluten and identified that anionic peptide fractions had stronger DPPH radical scavenging activity in comparison to cationic fractions. However, the DPPH radical scavenging activity was measured at 10 mg/ml and therefore suggests that peptides from soy and gluten protein are poor antioxidants because of the high concentrations required to observe activity. It was suggested that

anionic peptides were stronger antioxidants than cationic peptides because of the ability to donate a hydrogen atom in order to quench the DPPH radical.

Peptides from porcine collagen had strong DPPH radical scavenging activity dependent on the enzymes used to liberate the peptides, however, similar to Park and colleagues (2008), Li and colleagues (2007) measured the activity at very high concentrations (~11 mg/ml and ~14 mg/ml). Upon consecutive separation, by gel filtration and anion exchange, Li and colleagues (2007) identified a fraction with strong anionic charge that had stronger DPPH radical scavenging activity in comparison to fractions with weak anionic charge. Je and colleagues (2008) also observed an increased DPPH radical scavenging activity in peptides from dark tuna muscle that possessed a strong net anionic charge.

Saiga and colleagues (2003) identified hydrolysates from porcine myofibrillar proteins which moderately scavenged DPPH radical. The antioxidant activity was attributed to the presence of histidine, tyrosine and methionine although these amino acids did not have high concentrations in hydrolysates formed by papain and Actinase E.

DPPH radical scavenging was observed in peptides derived from smooth hound muscle and peptides with a molecular weight of <3.5 kDa had the highest antioxidant activity (Bougatef et al., 2009), which is similar to finding of a hydrolysate from quinoa seed, where peptides <5 kDa displayed stronger DPPH scavenging activity in comparison to high molecular weight peptides (Aluko & Monu, 2003). As well, DPPH scavenging activity from whey peptides was dependent on size (Peng et al., 2009). The fraction with molecular weight from 0.1-2.8 kDa possessed higher DPPH activity (59% scavenging activity) than larger and smaller molecular weight peptide fractions at 1 mg/ml (Peng et

al., 2009). Bougatef et al. (2009) suggested that the hydrolysate of smooth hound muscle contained peptides that could act as electron donors and therefore, stabilize free radicals. The < 3.5 kDa fraction was rich in histidine, methionine, tyrosine, leucine, isoleucine, glycine and arginine, however, the larger fractions were not analyzed for amino acid composition, which would have been beneficial for comparison. Alfalfa leaf hydrolysate (<3 kDa) had strong DPPH radical scavenging activity, reaching 80% at 1.6 mg/ml (Xie et al., 2008).

Generally, DPPH radical scavenging activity appears to be the strongest in smaller molecular weight peptide hydrolysates (<5 kDa), with lower activity observed at larger and smaller molecular weight peptides. Prior and colleagues (2005) suggested that smaller molecules have better access to the radical site and therefore appear to have better radical scavenging activity in this evaluation system. However, there is scarce information relating the hydrophobic properties and charge of peptides to the DPPH radical scavenging activity.

DPPH is a stable radical and does not resemble highly reactive peroxyl radicals involved in lipid oxidation (Prior et al., 2005; Huang et al., 2005), therefore, other antioxidant evaluation systems with biologically relevant ROS must be used to determine the antioxidant activity. DPPH radical scavenging method is commonly used to screen for antioxidant activity because of its simplicity (Huang et al., 2005). Furthermore, it has been suggested that hydrophobic amino acids and anionic amino acids contribute to the DPPH radical scavenging activity although more research needs to be conducted.

Table 2.5.1 DPPH radical scavenging activity of peptides derived from various food sources

Reference	Food Source	Sample Concentration	Separation Method	Results
Park et al (2008)	soy & gluten protein	10 mg/ml	Isoelectric focusing	-anionic fractions had stronger activity in comparison to cationic fractions
Saiga et al (2003)	porcine myofibrillar protein	not indicated	None	-hydrolysates from papain & Actinase E had equal activity; activity was attributed to histidine, tyrosine and methionine
Bougatef et al (2009)	smooth hound muscle	0.5-3 mg/ml	None	-hydrolysates from different enzymes displayed strong activity ranging from 35% to 65% at 1 mg/ml
		0.25-1.5 mg/ml	Size exclusion	-fraction <3.5 kDa had strongest activity; attributed activity to histidine, methionine, tyrosine, leucine, isoleucine, glycine and arginine
Aluko et al (2003)	quinoa seed protein	0.1-1.0 mg/ml	Ultrafiltration	-< 5kDa permeate had higher activity than larger molecular weight permeate (< 10 kDa)
Tang et al (2009b)	hemp protein	IC_{50} from 2.3-6.3 mg/ml	None	-all hydrolysates had activity, but dependent on enzyme used and length of hydrolysis
Peng et al (2009)	whey protein	1 mg/ml	Size exclusion	-scavenging was depended on molecular size; fraction from 0.1 - 2.8 kDa had highest activity
Li et al (2007)	porcine collagen protein	~11 mg/ml & ~14 mg/ml	None	-hydrolysates had activity ranging from 13-87% depended on enzyme used; activity was observed with the highest degree of hydrolysis
		not indicated	Ion exchange	-anionic fraction displayed strongest activity

Hsu et al (2009)	tuna juice protein	not indicated	Size exclusion	-hydrolysate between 0.39-1.4 kDa had strongest activity
Tang et al (2009a)	buckwheat protein	0.05- 1 mg/ml	None	-1 mg/ml scavenging ranged from ~50-75% dependent on degree of hydrolysis; hydrolysate with highest activity had highest amount of hydrophobic amino acids
Chang et al (2007)	porcine haemoglobin	2 mg/ml	None	-hydrolysate with different enzymatic treatments had activity ranging from 39%-52%
Xie et al (2008)	alfalfa leaf protein	0.2-1.6 mg/ml	Ultrafiltration <3 kDa	-<3 kDa hydrolysate had activity reach 80% scavenging at 1.6 mg/ml; hydrolysate -activity was attributed to histidine, tyrosine, methionine and cysteine
Je et al (2008)	tuna dark muscle	3 mg/ml	None	-dependent on enzyme used scavenging ranged from ~10% to ~45%
		Not indicated	Ion exchange	-further purification found that anionic fraction had strongest activity
		Not indicated	RP-HPLC	-further purification of the anionic fraction by RP-HPLC did not show a relationship with activity and hydrophobic properties
Zhang et al (2008)	rapeseed protein	0.025-1.6 mg/ml	Macroporous adsorption resin	-hydrolysates ranged from 20% scavenging to 90% dependent on the crude hydrolysate and the peptide treatment -hydrolysate with stronger activity had a higher amount of hydrophobic amino acids
Chen et al (2007)	peanut protein hydrolysate	0.2 -20 mg/ml	None	increasing activity with increasing peptide concentration; poor activity at low concentrations

Li et al (2008)	chickpea protein hydrolysate	1 mg/ml	Size exclusion	-fraction with lower molecular weight had strongest activity at 86% scavenging -low molecular weight fraction had highest amount of hydrophobic amino acids
Humiski et al (2007)	pea protein hydrolysates	1 mg/ml	None	-hydrolysates had weak activity ranging from 7-11% dependent on enzyme -Flavourzyme hydrolysate had the strongest activity and had the highest amount of hydrophobic amino acids (valine, isoleucine, leucine, tyrosine and phenylalanine

2.5.2 Reducing power of food derived peptides

Reducing capacity of amino acids serves as an indicator of the antioxidant activity and the effectiveness as a reducing agent to donate an electron to a free radical in order to form more stable products (Gimenez et al., 2009) and therefore, inhibit the progression of free radical chain reactions (Prior et al., 2005). The stronger reducing power of a compound indicates a strong ability to donate an electron (Xie et al., 2008). The reducing power method is used because, similar to DPPH radical scavenging assay, it is simple and fast (Prior et al., 2005).

Zhang and colleagues (2008) noted a trend between the hydrophobic amino acids in rapeseed hydrolysates and the efficacy as a reducing agent. Rapeseed hydrolysate with the highest concentration of hydrophobic amino acids displayed the strongest reducing power and the hydrolysate with the lowest concentration of hydrophobic amino acids displayed the weakest activity.

You and colleagues (2009) observed increasing reducing power with an increasing degree of hydrolysis of peptides from loach protein hydrolyzed by Protamex. The amino acid content of aspartic acid, serine, glycine, threonine, alanine, proline, valine, methionine, isoleucine, leucine and phenylalanine increased in concentration with the increasing degree of hydrolysis by Protamex. Loach peptides hydrolyzed by papain had stronger reducing power than peptides from Protamex regardless of the degree of hydrolysis (You et al., 2009). The hydrolysates from Papain contained a higher combined concentration of tyrosine, methionine, histidine, lysine and tryptophan and therefore, the stronger reducing power could be attributed to the specific amino acid and peptide composition (You et al., 2009). Hydrolysates from squid and sole both possessed

reducing activity; however, squid hydrolysates had much stronger reducing power than sole hydrolysates (Gimenez et al., 2009). Squid hydrolysate contained higher amounts of aspartic acid, asparagine, glutamic acid, glutamine, valine, leucine, tyrosine and arginine and lower amounts of glycine, alanine, lysine and proline.

Hydrolysates from smooth hound muscle produced by a variety of enzymes displayed a dose dependent response in the reducing power, although the activity for the hydrolysates was lower than the activity observed for butylated hydroxyanisole (Bougatef et al., 2009). Hydrolysates from porcine haemoglobin displayed reducing power with two-step enzymatic hydrolysis, but the non-hydrolyzed haemogloblin had stronger reducing power (Chang et al., 2007). Hydrolysate (<3 kDa) from alfalfa leaf protein exhibited a dose dependent increase in reducing power, although activity was not as strong as the activity exhibited by glutathione (Xie et al., 2008).

2.5.3 Superoxide scavenging activity of food derived peptides

Superoxide ($O_2^{\cdot-}$) is considered the primary reactive oxygen species, leading to the formation of other radicals (Valko et al., 2007), however, a limited amount of peptides hydrolyzed from food proteins have been challenged with this radical *in vitro*. As well, there is scarce information investigating the association between hydrophobic properties and peptide charge in relation to superoxide radical scavenging. Peng and colleagues (2009) found that whey peptides from 0.1-2.8 kDa sizes had the highest superoxide anion scavenging activity (70% scavenging) than larger and smaller molecular weight peptides at a concentration of 1 mg/ml. Peptides from alfalfa leaf hydrolysate displayed superoxide scavenging activity at concentrations ranging from 0.15-0.9 mg/ml (Xie et

al., 2008). The peptides derived from alfalfa leaf protein had 67% scavenging activity of the superoxide radical at 0.9 mg/ml, whereas glutathione at 0.9 mg/ml had scavenging activity of over 90%. Li and colleagues (2008) also noted an increased superoxide scavenging activity of chickpea peptides with low molecular weight peptides in comparison to larger molecular weight fractions. The low molecular weight fraction of chickpea peptides had the highest amount of hydrophobic amino acids in comparison to the larger molecular weight peptides.

As superoxide is the precursor to other radicals with stronger reactivity, it is important to investigate the ability of compounds to scavenge this particular radical. Scavenging superoxide could interrupt the formation of hydroxyl radical, therefore, decreasing oxidative damage to biological tissues.

2.5.4 Hydrogen peroxide scavenging activity of food derived peptides

Hydrogen peroxide (H_2O_2) is formed by the conversion of superoxide by superoxide dismutase and is a long-lived molecule that is readily diffusible through cell membranes (Finkel & Holbrook, 2000). Hydrogen peroxide can combine with unbound transition metals, such as iron through the Fenton reaction to form an extremely reactive compound, the hydroxyl radical, that can initiate lipid oxidation, oxidize proteins and damage DNA (Valko et al., 2007). Limited work has been performed exploring the hydrogen peroxide scavenging activity of food derived peptides *in vitro*. Guo and colleagues (2009) examined the hydrogen peroxide scavenging activity of peptides synthesized from royal jelly protein. The di and tripeptides examined displayed a range of

scavenging activities against hydrogen peroxide. Three dipeptides with tyrosine in the sequence possessed the strongest activity (Guo et al., 2009).

2.5.5 Metal chelating activity of food derived peptides

The chelation of metal ions is very important to prevent the formation of the highly reactive hydroxyl radical through the Fenton reaction. Saiga and colleagues (2003) identified that hydrolysates from porcine myofibrillar proteins had metal chelation activity and in particular, the hydrolysate derived from Actinase E had higher chelation than the hydrolysate from papain, although the activity was measured at 14 mg/ml and 12.8 mg/ml, respectively. Interestingly, the Actinase E hydrolysate, had half the amount of hydrophobic amino acids than the papain hydrolysate and the other amino acids were comparable between the two hydrolysates. Saiga and colleagues (2003) attributed the difference in metal chelation activity to the differences in peptide structure and chain length and suggested that the chelating activity of metal ions was due to the carboxyl and amino groups in the side chains of anionic and cationic amino acids. Li and colleagues (2007) found metal chelating activity to be the strongest in a hydrolysate with a high degree of hydrolysis, however, the metal chelating activity of a hydrolysate from porcine collagen was also measured at high concentrations (~11 and ~14 mg/ml).

Peptides from silver carp hydrolyzed by Alcalase or Flavourzyme displayed metal chelating activity that increased to greater than 90% chelating activity with increasing time of hydrolysis at 5 mg/ml (Dong et al., 2008). Alcalase hydrolysate possessed stronger chelating activity and contained a higher concentration of hydrophobic amino acids (valine, methionine, isoleucine, tyrosine, phenylalanine and proline) than the

Flavourzyme hydrolysate. However, Megias and colleagues (2008) determined the copper metal chelating activity of peptides hydrolyzed from sunflower proteins with affinity chromatography and further purification with reverse phase high performance liquid chromatography (RP-HPLC). The peptides separated by affinity chromatography were found to have high concentrations of histidine and arginine. Further purification by RP-HPLC produced hydrophilic fractions with chelating activity, whereas the hydrophobic fractions displayed no activity. Peptides from chickpea protein purified by affinity chromatography had stronger copper chelating activity than the whole hydrolysate (Megias et al., 2007). Interestingly, purified peptides from chickpea had higher concentrations of histidine and arginine than the whole hydrolysate, which is similar to the findings of Megias et al. (2008) using sunflower hydrolysate. Upon further purification of chickpea hydrolysate by RP-HPLC, no apparent trend was observed in relation to hydrophobic properties of the peptides and metal chelating activities, although fractions with the strongest chelating activity had the highest content of histidine.

Chang and colleagues (2007) identified hydrolysates from porcine haemoglobin with a range of metal chelating activity with the two-step hydrolysis procedure producing the strongest metal chelation activity. However, the chelating activity was lower than the non-hydrolyzed haemoglobin. Gimenez and colleagues (2009) evaluated the metal chelating activity of sole and squid hydrolysates. Both hydrolysates displayed strong activity with greater than 80% at a low peptide concentration of 0.2 mg/ml and were rich in proline, glycine, alanine and arginine.

Overall, there is limited information regarding the relationship between hydrophobicity and charge of peptides and the metal chelating activity.

2.5.6 Hydroxyl radical scavenging activity of food derived peptides

Hydroxyl radicals (·OH) formed through the combination of hydrogen peroxide and free transition metals can initiate lipid oxidation, oxidize proteins and damage DNA (Valko et al., 2007). Limited information in the current literature exists in the relationship between hydrophobic peptides and charge and the ability to scavenge the hydroxyl radical.

Peptides from rapeseed proteins exhibited dose-dependent increase in hydroxyl radical scavenging ranging from 30%-65% at concentrations ranging from 2-10 mg/ml (Zhang et al., 2008). The peptide fraction that was particularly efficient at scavenging hydroxyl radicals contained less hydrophobic amino acids and more hydrophilic amino acids, including asparagine, glutamate, methionine, lysine, histidine, glycine, alanine, serine and threonine (Zhang et al., 2007). In contrast, Dong et al. (2008) and Li et al. (2008) identified hydrolysates from silver carp and chickpea proteins with strong hydroxyl radical scavenging activities contained the highest concentration of hydrophobic amino acids.

Peptides derived from whey proteins with a molecular weight from 0.1-2.8 kDa had the highest hydroxyl radical scavenging activity than larger peptides and smaller peptides at 1 mg/ml (Peng et al., 2009). Peptides from alfalfa leaf hydrolysate displayed strong (80%) hydroxyl radical scavenging activity at 1.2 mg/ml, whereas glutathione scavenged almost 100% of hydroxyl radicals at 1.2 mg/ml (Xie et al., 2008).

2.5.7 Inhibition of linoleic acid oxidation by food derived peptides

Lipid oxidation is a concern in biological systems and food systems. Oxidation of lipids in food systems can lead to undesirable changes in colour, flavour, texture and produces toxic reaction products (Park et al., 2008). Lipid oxidation is initiated by any ROS that can remove a hydrogen atom from a polyunsaturated fatty acid side chain (Aruoma,1998). Lipid peroxidation contributes to the development of atherosclerosis and ultimately cardiovascular disease (Aruoma, 1998). Oxidized LDL cholesterol is more rapidly engrossed by macrophages in comparison to non oxidized LDL cholesterol molecules, leading to a faster formation of lipid filled foam cells (Witztum & Steinberg, 1991). Oxidized LDL cholesterol and LDL cholesterol are recognized by different receptors and therefore oxidized LDL cholesterol is taken up more rapidly. It is widely suggested that the hydrophobic nature peptides and amino acids increases the accessibility to lipid molecules (Rajapakse et al., 2005; Zhang et al., 2008; Je et al., 2008), therefore, allowing hydrophobic peptides to be effective at preventing lipid oxidation.

Hydrolysate from giant squid muscle, which was composed of primarily of glycine and hydrophobic amino acids with molecular weight <3 kDa displayed strong linoleic acid oxidation inhibition in comparison to larger molecular weight hydrolysates (Rajapakse et al., 2005). Upon sequential purification with cation exchange and RP-HPLC, two active peptides were identified composed primarily of hydrophobic amino acids. It was suggested that the antioxidant activity was due to the molecular weight of the peptides and the hydrophobic amino acids, which have the ability to come into contact with the lipid molecules and donate protons to the lipid radicals.

Je and colleagues (2008) isolated a peptide with strong antioxidant activity from tuna dark muscle. The peptide was rich in hydrophobic amino acids such as leucine, valine, tyrosine and methionine. Peptides from rapeseed hydrolysates displayed a dose-dependent increase in inhibiting lipid oxidation and the strongest activity at 5 mg/ml was observed for the hydrolysate with the highest concentration of hydrophobic amino acids (Zhang et al., 2008). The strong activity of the rapeseed hydrolysate with hydrophobic amino acids was attributed to the ability of the peptides to directly donate protons to lipid-derived radicals. Peptides from silver carp displayed activity protecting against the oxidation of linoleic acid at an unspecified concentration (Dong et al., 2008). Hydrolysates from Alcalase induced protein hydrolysis produced peptides with stronger activity than peptides from Flavourzyme induced protein hydrolysis (Dong et al., 2008). Furthermore, Alcalase hydrolysate contained peptides that were rich in hydrophobic amino acids.

According to Saiga and colleagues (2003), hydrolysates from papain had stronger activity against linoleic acid oxidation than a hydrolysate from Actinase E. The papain hydrolysate had two times more hydrophobic amino acids in comparison to the Actinase E hydrolysate. Upon fractionation by cation exchange, the fraction with the least cationic charge possessed stronger activity in preventing linoleic acid oxidation in comparison to the fraction with the strongest cationic charge. Upon further purification by anion exchange chromatography, the strongest anionic fraction possessed the strongest activity.

Park et al. (2008) identified that cationic fractions from soy protein decreased linoleic acid oxidation in comparison to anionic peptide fractions, however the cationic fractions would be considered poor antioxidants at 7.5 mg/ml. Peptides with weak net

anionic charges from royal jelly protein displayed stronger antioxidant activity in comparison to the fractions with stronger net anionic charges (Guo et al., 2009). Interestingly, peptides containing valine, leucine, isoleucine, alanine, phenylalanine and lysine in the sequence exhibited strong activity against lipid peroxidation (Guo et al., 2009). Porcine collagen hydrolysates inhibited linoleic acid oxidation and stronger activity was noted with a higher degree of hydrolysis, which was also dependent upon the enzyme employed (Li et al., 2007).

It is widely suggested that peptides which exhibit protection against lipid oxidation are rich in hydrophobic amino acids because of the direct proton donation of hydrophobic amino acids (Zhang et al., 2008; Je et al., 2008), however, there is limited information outlining the role of charge on the prevention of lipid oxidation.

2.6 Multifunctional peptides

Many bioactive peptides have more than one biological function and are referred to as multifunctional. For example, peptides hydrolyzed from potatoes and potato processing by-products, as well as an extract from shrimp and quinoa seed have been shown to possess both antioxidant and angiotensin-converting enzyme inhibitory activities *in vitro* (Pihlanto 2008; Benjakul et al., 2009; Aluko & Monu, 2003). The combination of antihypertensive and antioxidant peptides into a multifunctional preparation could be useful in the management of cardiovascular diseases (Vercruysse et al., 2009). As well, peptides from rapeseed protein possessed both antithrombotic and antihypertensive activities (Zhang et al., 2008). Peptides hydrolyzed from yellow pea seed protein by Thermolysin hydrolysis have exhibited antihypertensive activity *in vitro*

and in an animal model (Aluko, personal communication, 2008a). Also, peptides hydrolyzed from yellow pea seed protein using a variety of enzymes have shown moderate angiotensin converting enzyme inhibitory activity and negligible antioxidant activity *in vitro* (Humiski & Aluko, 2007).

ROS have been identified to play a role in the pathogenesis of cardiovascular disease. It has been identified that patients who have chronic kidney disease are exposed to excess levels of oxidative stress and inflammation measured by plasma levels of interleukin 6, F_2-isoprostanes and the plasma protein carbonyl content (Oberg et al., 2004). The increased levels of reactive oxygen species decreases the amount of the vasodilator, nitric oxide, which leads to endothelial dysfunction and the development of hypertension (Vaziri, 2004). Thus, patients with chronic kidney disease are considered to have a high risk for the development, morbidity and mortality of cardiovascular disease (Oberg et al., 2004). As one disease condition can strongly increase the risk of another, there is the need to investigate multifunctional peptides, specifically with a combination of antioxidative activities and antihypertensive activities.

2.7 Antioxidant activity of amino acids

Interestingly, amino acids have been shown to have unique antioxidant activities, dependent on whether it is a free amino acid or whether the amino acid is incorporated into a peptide. *In vitro* antioxidant evaluation using 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) method indicated that aromatic amino acids, tryptophan and tyrosine as well as cysteine presented good antioxidant activity individually (Meucci & Mele, 1997; Hernandez-Ledesma et al, 2005). Interestingly, Meucci & Mele (1997) noted no

antioxidant activity of the charged and neutral amino acids. Hernandez-Ledesma et al. (2005) also reported antioxidant activity in the same amino acids as Meucci & Mele (1997) using the oxygen radical absorbance capacity method and also observed activity for methionine, phenylalanine and histidine. An amino acid may have altered antioxidant activity once it is incorporated into a peptide, as interaction with other amino acids cause an antagonistic or synergistic effect on the overall antioxidant activity of the peptide (Hernandez-Ledesma et al, 2005). Hernandez-Ledesma and colleagues (2005) suggested that the antioxidant activity of tryptophan and tyrosine was due to the indole and phenol ring, respectively, to act as hydrogen donors. As well, it was suggested that cysteine acts as an antioxidant through the donation of the sulphur hydrogen (Hernandez-Ledesma et al, 2005). Taylor and Richardson (1980) also suggested that cysteine acts an antioxidant in a lipid emulsion system because of the sulfhydryl group. Histidine is thought to be an antioxidant because of the imidazole ring (Adebiyi et al., 2009). Furthermore, it must be noted that the antioxidant activity of amino acids is dependent upon the radical it is challenged with as amino acids may behave differently in the presence of different radicals.

Many factors have been shown to play a role in the determination of the antioxidant characteristics of peptides, including peptide chain length and peptide charge, which contribute to the complexity of the structure-function relationship. This study aims to better understand the structure-function relationship of peptides derived from food sources and to understand the implications for human health through the separation of pea seed peptides by RP-HPLC and ion exchange and then subjecting the resultant fractions to an array of *in vitro* antioxidant evaluation systems.

2.8 Disease and oxidative stress

The basis of diseases associated with oxidative damage appears to be the involvement of inflammatory pathways (Finkel & Holbrook, 2000). Oxidative stress and inflammation are interrelated as oxidative stress leads to inflammation and inflammation causes oxidative stress (Vaziri, 2004).

Lipid peroxidation is of clinical significance due to the role in the progression of the development of atherosclerosis. Under normal physiological conditions, there is a minimal amount of unbound iron present in the biological system. Upon absorption of iron, it then is bound to proteins as either transferritin or ferritin. In disease conditions or when there is an increased production of superoxide, it can reduce chelated iron to liberate iron from ferritin because of its small size, therefore making iron available for participation in the Fenton reaction (Thomas et al., 1985). Superoxide is also implicated in lipid oxidation through the conversion to hydrogen peroxide which combines with iron and participates in the Fenton reaction (Thomas et al., 1985).

2.8.1 Cardiovascular Disease

Cardiovascular disease occurs when endothelial cells lining the blood vessels are damaged by hypertension, high levels of LDL cholesterol or other vascular insults, which trigger an inflammatory response as endothelial cells begin to express vascular cell adhesion molecules and bind monocytes and T lymphocytes (Kaperonis et al., 2006). The inflammatory response is triggered by monocytes and macrophages which are attracted to the site of damage (Seifried et al., 2007). The monocytes and macrophages produce superoxide and hydrogen peroxide which cause further damage to the surrounding

endothelial cells (Halliwell & Gutteridge, 1990). Oxidized low density lipoproteins can be absorbed by macrophages efficiently to produce foam cells (Libby, 2008). Endothelial cells and macrophages can oxidize LDL cholesterol to ensure that it is absorbed faster (Halliwell & Gutteridge, 1990) therefore forming foam cells which evolve into complex lesions (Libby, 2008). The immune cells involved in plaque formation produce proinflammatory mediators which promote the adhesion of leukocytes to the endothelium and eventually can lead to the development of an advanced lesion.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Enzymatic hydrolysis of pea protein isolate

Pea protein isolate (80% dry weight basis) was a gift from Nutri-Pea Ltd, Portage La Prairie, Manitoba. Pea protein isolate was exclusively made from a mixture of seeds from the following varieties: Eclipse, Midas and Golden (Nutri-Pea Ltd, personal communication, 2009). Pea protein isolate was dispersed in distilled water to obtain 6.0% (w/v) protein slurry. The slurry was heated to 55°C and adjusted to pH 8.0 using dilute sodium hydroxide solution. Thermolysin was then added to initiate the hydrolysis at a ratio of 0.5% (on the basis of protein weight, w/w). The temperature and pH of the slurry was maintained constant for 3 h. The hydrolysis was stopped by heating the slurry to 95°C for 15 minutes. The hydrolysate was cooled to room temperature and centrifuged at 10,000×*g* for 25 minutes at 4 °C. The clear supernatant was collected and passed through a stirred ultra-filtration cell using a 3,000 Da molecular weight cut-off membrane (Sartorius Co., Germany). The resulting permeate (contains peptides with <3 kDa sizes) was collected, freeze-dried and stored at -20°C for further use.

3.2 Determination of protein content

Protein content of the hydrolysate and hydrolysate fractions was determined by the modified Lowry method (Lowry et al., 1951). First, the peptide sample (10 mg/ml) and a standard (bovine serum albumin) (10 mg/ml) were separately dissolved in water and mixed thoroughly. Next, 100 parts of Reagent A consisting of 2% Na_2CO_3, 1% sodium dodecyl sulfate, 0.4% sodium hydroxide and 0.16% sodium tartrate was combined with 1

part of Reagent B consisting of 4% $CuSO_45H_2O$ dissolved in distilled water to make Reagent C. Reagent C was made fresh for the protein determination of each sample. A standard curve using bovine serum albumin (BSA) and peptide samples were prepared to contain a range of protein concentrations (10-100 μg/ml). Reagent C (3 ml) was added to the 1 ml of peptide samples and BSA and was incubated at room temperature for 1 hour. After incubation, 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 incubated at room temperature for 45 minutes. The absorbance was measured spectrophotometrically at 660 nm. The standard curve was plotted as absorbance (y) against protein concentration (x) of BSA.

3.3 Separation of pea protein hydrolysates

3.3.1 Separation of peptides based on hydrophobicity

Protein hydrolysate (<3kDa permeate) was dissolved in solvent A (0.1% trifluoroacetic acid in double distilled water) at a concentration of 100 mg/ml. Protein sample (2 ml of 100 mg/ml) was loaded using the autosampler into a high performance liquid chromatography (HPLC) system (Varian 940-LC) fitted with a Phenomenex C12 preparative column (250 x 21 mm) at a flow rate of 5 ml/min. The elution gradient was 0% solvent B (0.1% trifluoroacetic acid in methanol) to 100% B over 60 minutes. Solvent B was held at 100% for 10 minutes to ensure all peptides had eluted. The column was equilibrated with 100% of Solvent A at the beginning of each run. The absorbance was measured at 214 nm to monitor the peptide bond and 280 nm to monitor aromatic amino acids. Fractions were collected using an automated fraction collector every 30 seconds

and pooled into five fractions based on hydrophobicity (HF1<HF2<HF3<HF4<HF5). Hydrophobic fraction 1 (HF1) had the least net hydrophobic properties, eluted first and hydrophobic fraction 5 (HF5) had the strongest net hydrophobic properties and eluted last from the column. Fractions were then concentrated by rotoevaporator to reduce the volume, freeze dried and stored at -20°C until further use. Peptide sample and solvents A and B were filtered through 0.2 μM filter discs prior to use in the HPLC.

3.3.2 Separation of peptides based on charge

Protein hydrolysate (<3kDa permeate) was dissolved in 10 mM ammonia acetate (pH 4.0) (solvent A) at a concentration of 250 mg/ml and adjusted to pH 4.0 with acetic acid. An aliquot (2 ml) of the sample was then loaded into a HiLoad 26/10 SP-Sepharose High Performance column connected to a Fast Protein Liquid Chromatography system (FPLC) (GE Healthcare, Montreal, PQ). The column was equilibrated with solvent A (10 mM ammonia acetate, pH 4.0) using 1.5 column volumes and then the peptides were eluted with a gradient of 0-40% solvent B (0.5 M ammonia carbonate, pH 8.8) over 4 column volumes at a flow rate of 10 ml/min. The column was then cleaned with 100% of solvent B using 1 column volume to ensure all compounds have been removed. The absorbance of eluents was monitored at 214 nm, pooled and collected into five fractions, with cationic fraction 1 (CF1) eluting first (least net cationic charge) and cationic fraction 5 (CF5) eluting last, had the strongest net cationic charge. The five fractions were individually desalted using solid phase extraction and dialysis tubing. The desalted fractions were freeze dried and stored at -20°C until further use. Peptide solution and solvents A and B were filtered through 0.2 μM filter discs prior to loading on the FPLC.

3.4 Amino acid composition analysis

Full profile amino acid composition of peptide fractions obtained from the HPLC separation was determined using an amino acid analyzer at the Department of Animal Science, University of Manitoba.

3.5 Antioxidant evaluation systems

3.5.1 DPPH radical scavenging activity

The DPPH radical is not a biologically relevant radical, however, it is widely used to evaluate the antioxidant activity of natural and synthetic compounds in order to test the donating capacity of a compound. The nitrogen centered DPPH radical evaluation system is commonly used because it is a stable radical, the test is simple and fast (Theodore et al., 2008; Xu et al, 2007a) as it has a short incubation period.

The DPPH assay method by Aluko & Monu (2003) and Hou et al. (2001) was used and modified for a 96-well clear flat bottom plate. Reduction of the DPPH radical leads to the loss of colour of the experimental solution (Huang et al., 2005). Peptide samples were dissolved in 0.1 M sodium phosphate buffer, pH 7.0, containing 1% (w/v) Triton X-100. DPPH was dissolved in methanol to a final concentration of 100 μM. A blank control was run with samples consisting of DPPH and sodium phosphate buffer while the standard consisted of glutathione (final concentration of 1 mg/ml) dissolved in phosphate buffer and mixed with DPPH solution. Peptide samples (100 μL) were mixed with 100 μL of the DPPH solution and stood at room temperature in the dark for 30 min. The absorbance was then read at 517 nm. The DPPH radical scavenging activity of all

samples was performed in triplicate. The scavenging activity of the peptide samples was compared to the activity of glutathione.

The percent scavenging activity of the peptide samples was determined 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 Reducing power

The reducing power assay involves the single electron transfer mechanism and measures the ability of antioxidants to reduce the oxidant, Fe^{3+} to Fe^{2+} (Benzie & Szeto, 1999). Strong reducing power of a sample is indicated by an increase in absorbance (Theodore et al., 2008).

The reducing power of peptide samples was measured according to a method by Zhang et al. (2008) and Theodore et al. (2008), with slight modifications. Protein samples (250 µL in phosphate buffer) or double distilled water (control) were mixed with 250 µL phosphate buffer at pH 6.6. (0.2 M) and 250 µL 1% potassium ferricyanide solution dissolved in double distilled water. The solutions were mixed and heated at 50°C for 20 min. After incubation, 250 µL Trichloroacetic Acid (TCA) (10% in double distilled water) was added and 250 µL of incubated protein/TCA solution was combined with 50 µL of 0.1% ferric chloride dissolved in double distilled water and 200 µL of double distilled water. After 10 min of incubation at room temperature, the solution was centrifuged at 1000x*g*. The supernatant was collected and 200 µL of the supernatant was

added to a clear, flat bottom 96-well plate. The absorbance of the supernatant was measured at 700 nm.

3.5.3 Superoxide scavenging activity

The superoxide scavenging method was developed by Gao et al. (1998) and Marklund & Marklund (1974). Protein samples (80 μL, at a final assay concentration of 1 mg/ml) or glutathione were mixed with 80 μL of 50 mM Tris-HCl buffer (pH 8.3) containing 1 mM EDTA directly into a clear bottom 96-well plate in darkness. Then, 40 μL of 1.5 mM pyrogallol dissolved in 10 mM HCl was added to each well. Absorbance was measured immediately at 420 nm for 4 min at room temperature. Tris-HCl buffer was used as a control. The superoxide scavenging activity of the peptides is calculated by the following equation:

$$\text{Superoxide Scavenging Activity (\%)} = \frac{(\Delta A_{420\ nm}/\text{minute control} - \Delta A_{420\ nm}/\text{minute sample})}{\Delta A_{420\ nm}/\text{minute control}} \times 100\%$$

3.5.4 Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity method is based on a method developed by Guo et al. (2009) with modifications. Hydrogen peroxide (60 μL of 0.1 mM in cold double distilled water) was combined with 2.19 ml of 50 mM sodium phosphate buffer (pH 7.0), 300 μL of peptide sample (at 1 mg/ml dissolved in phosphate buffer), 30 μL of 9.7 U/ml peroxidase (in cold phosphate buffer) and 15 μL of 1 mM scopoletin (in methanol). The solution was mixed vigorously and an aliquot of 200 μL was immediately removed and placed in a quartz cuvette. The sample chamber was heated to 37 °C. The

fluorescence intensity change was measured for 60 sec using excitation wavelength of 366 nm and emission wavelength of 460 nm. The slit width for the excitation and emission bands was 2.5 nm. A control blank was run by replacing the sample volume with 300 μL of sodium phosphate buffer. The hydrogen peroxide scavenging activity of the sample was compared to the scavenging activity of glutathione. The percent hydrogen peroxide scavenging activity was calculated using the following equation:

$$\text{Hydrogen Peroxide Scavenging (\%)} = \frac{(\Delta \text{ Intensity}_{\text{Control}} - \Delta \text{ Intensity}_{\text{Sample}})}{\Delta \text{ Intensity}_{\text{Control}}} \times 100\%$$

3.5.5 Chelation of metal ions

The $FeCl_2$-FerroZine complex has a strong absorbance at 562 nm and the decrease in absorbance indicates high ferrous ion chelating activity (Tang et al., 2009b). The metal chelating activity was based on a method which was slightly modified by Xie et al. (2008). Sample solution (1 ml) of peptides or glutathione was combined with 0.05 ml iron dichloride solution (2 mM/L) and 1.85 ml double distilled water. FerroZine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt) solution (0.1 ml, 5 mM/L) was added and mixed vigorously. The mixture stood at room temperature for 10 mins. An aliquot of 200 μL was removed and added to a clear bottom 96-well plate. A control blank was run along side the samples, which replaced the sample volume of 1 ml with double distilled water. The colour change was measured spectrophotometrically at 562 nm. The metal chelating activity of the sample was compared to the chelating activity of glutathione.

The chelating effect of protein is calculated by the following equation:

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

3.5.6 Hydroxyl radical scavenging activity

The hydroxyl radical-scavenging assay was modified based on a method developed by de Avellar et al. (2004) and described by Li et al. (2008). 1,10-phenanthroline (3mM) and peptide samples were separately dissolved in 0.1 M sodium phosphate buffer (pH 7.4). $FeSO_4$ (3mM) and hydrogen peroxide (0.01%) were both separately dissolved in distilled water. Peptide samples (50 µL) were first added to a clear, flat bottom 96-well plate. Then, 50 µL of 1,10-phenanthroline and 50 µL of $FeSO_4$ were added. To initiate the reaction, 50 µL of hydrogen peroxide was added. The reaction mixture was covered and incubated at 37°C for 1 hour with shaking. The absorbance was measured spectrophotometrically at 536 nm. The absorbance was also determined for a blank, which contained 1,10-phenanthroline and $FeSO_4$ and a control, which contained 1, 10-phenanthroline, $FeSO_4$ and hydrogen peroxide. The hydroxyl radical scavenging activity was determined by the following equation:

Hydroxyl radical scavenging activity % =

$$\frac{(\text{Absorbance}_{536\text{ nm Sample}} - \text{Absorbance}_{536\text{ nm Control}})}{(\text{Absorbance}_{536\text{ nm Blank}} - \text{Absorbance}_{536\text{ nm Control}})} \times 100\%$$

3.5.7 Inhibition of linoleic acid oxidation

Linoleic acid oxidation was measured by a method described by Li et al. (2008). Peptide samples (1 mg/ml) were dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0). The mixture was added to 1 ml of linoleic acid (50 mM) dissolved in ethanol (99.5%). This was stored in a glass test tube and kept at 60 °C in darkness for 7 days. The sample solution (100 µl) was mixed with 4.7 ml of 75% aqueous ethanol, 0.1 ml of ammonium thiocyanate (30% w/v) and 0.1 ml of ferrous chloride (0.02 M) dissolved in

HCl (1 M). The experimental solution (200 μL) was added to a clear bottom 96-well plate. The degree of color development was measured spectrophotometrically at 500 nm after 3 minutes incubation at room temperature. The sample solution was monitored for linoleic acid oxidation on a daily basis for one week. An increased absorbance indicates an increase in linoleic acid oxidation.

3.6 Data and statistical analysis

All results are presented as means ± standard deviation. Each analysis was performed in triplicate and sample concentrations were based on the protein content. All fractions were compared to reduced L-glutathione as a standard. All samples were analyzed at a final protein concentration of 1 mg/ml. Statistical analysis was performed with SAS (Statistical Analysis Software 9.1) using one-way ANOVA. Duncan's multiple-range test was carried out to compare means between peptide fractions. Results are considered significant at $p<0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Hydrolysis of pea protein isolate

The pea protein isolate had a creamy, pale yellow colour, similar to what was observed by Shand and colleagues (2007) and after hydrolysis and freeze-drying, the hydrolysate remained pale yellow in colour. Hydrolysates fractionated based on size have been shown to exhibit a range of antioxidant activities. Previous work has found that smaller peptide fractions (<3.5 kDa, <5 kDa, 0.1-2.8 kDa) have shown stronger antioxidant activity than larger peptide fractions (Bougatef et al., 2009; Aluko & Monu 2003; Peng et al., 2009). Therefore, in the present study, the hydrolysate was subjected to ultra-filtration using <3 kDa molecular weight cut off membrane after hydrolysis.

The amino acid composition of the pea protein isolate (PPI) and <3 kDa pea protein hydrolysate (PPH) is presented in Table 4.1. The amino acid composition of PPH was similar to the original PPI. Upon hydrolysis, there was an increase in the concentration of hydrophobic aromatic and aliphatic amino acids except for tryptophan, which decreased in concentration. In particular, there was a marked increase of isoleucine, leucine and phenylalanine. The positively charged amino acids decreased in concentration with hydrolysis. Lysine and arginine both substantially decreased in comparison to histidine, which decreased slightly. It is expected that upon hydrolysis with thermolysin, an increase in hydrophobic amino acids appear in the hydrolysate. Thermolysin hydrolyzes proteins at the amino side of the peptide bond at hydrophobic residues (Antonczak et al., 2000).

Klompong and colleagues (2009) also observed a change in amino acid composition after enzymatic hydrolysis of yellow stripe trevally. The change of amino acid composition was attributed to removal of components that were resistant to hydrolysis (Klompong et al., 2009).

Table 4.1 Percentage of amino acid composition of pea protein isolate (PPI) and <3 kDa pea protein hydrolysate (PPH)

Amino acid	PPI (%)	PPH (%)
ASX	11.81	13.79
THR	3.48	3.6
SER	5.72	6.2
GLX	16.54	13.92
PRO	5.49	5.15
GLY	4.09	3.76
ALA	4.34	5.01
CYS	0.87	0.24
VAL	5.19	5.63
MET	1.12	0.91
ILE	4.73	5.43
LEU	8.79	9.91
TYR	3.78	3.87
PHE	5.49	7.41
HIS	1.74	1.61
LYS	7.35	6.1
ARG	8.6	6.83
TRP	0.83	0.68
HAA	40.63	44.24
PCAA	17.69	14.54
NCAA	28.35	27.71
AAA	10.1	11.96

ASX= aspartic acid and asparagine; GLX = glutamic acid and glutamine

Combined total of hydrophobic amino acids (HAA) = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

Positively charged amino acids (PCAA) = arginine, histidine, lysine

Negatively charged amino acids (NCAA) = ASX and GLX

Aromatic amino acids (AAA) = phenylalanine, tryptophan and tyrosine

4.2 Peptide fractionation

Fractionation of protein hydrolysates resulted in enrichment of the peptides of interest and removed contaminating materials which, therefore, led to an increase in homogeneity of peptides (Aluko, 2004).

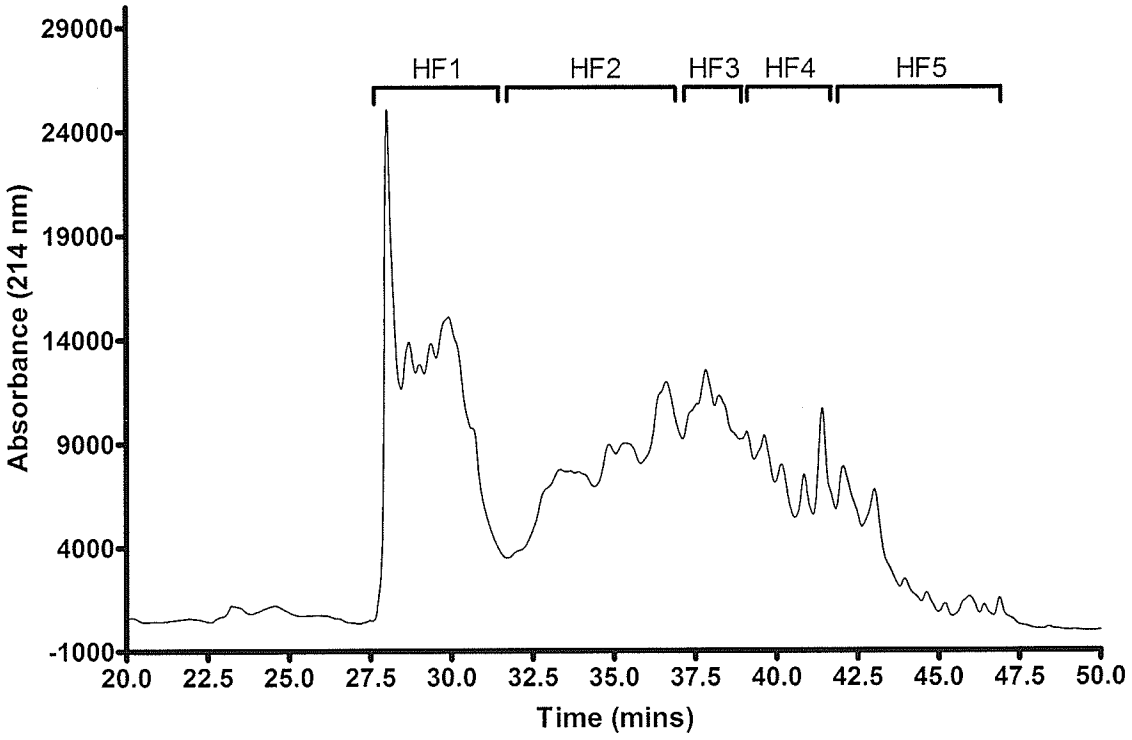
4.2.1 Separation based on hydrophobicity

Reverse-phase high performance liquid chromatography is commonly used to further purify bioactive peptides (Megias et al., 2007). The peptides are separated based on their differences in hydrophobic characteristics and the strength of the stationary phase (Aluko, 2004). The strong hydrophobic characteristics of the stationary phase causes a strong interaction with the peptides and therefore organic solvents are used to elute the bound peptides (Aluko, 2004).

Fractionation of the PPH on a Phenomenex C12 column yielded five peptide fractions (Figure 4.2.1). The C12 column is packed with hydrophobic aliphatic chains; therefore, hydrophobic peptides will bind tightly to the column through hydrophobic interactions, have a longer residence time and will require a higher strength of methanol solvent to detach and elute from the column. In contrast, peptides with less degree of hydrophobicity will bind weakly to the column and can be eluted earlier. Hydrophobic fraction 1, eluted first at 28 minutes with very strong absorbance in comparison to the fractions that eluted later. Hydrophobic fraction 1 (HF1) is considered to have the least net hydrophobic properties than the fractions eluting later since it was bound weakly to the column. Hydrophobic fraction 5 (HF5), was the last fraction to elute and it fully eluted at 47 minutes. Hydrophobic fraction 5 was bound strongly to the column and is

considered to have the strongest net hydrophobic properties in comparison to the other fractions obtained. The length of time the peptides were retained on the column indicates the overall net hydrophobic property of the fraction.

Figure 4.2.1: PPH separation by RP-HPLC



4.2.1.1 Amino acid composition of RP-HPLC fractions

The amino acid composition of the five peptide fractions obtained from the RP-HPLC is presented in Table 4.2.1.1. Similar to what has been found for the amino acid profile for peas (Wang & Daun, 2004), all fractions contained negligible amounts of sulphur-containing amino acids and tryptophan. There are clear trends in the levels of specific amino acids in relation to the net hydrophobic properties of each fraction. Hydrophobic aliphatic amino acids, valine, leucine and isoleucine increased in percentage as the retention time of the fractions increased (HF4 and HF5). Alanine, however, decreased in concentration with increasing net hydrophobic fractions. Interestingly, the percentage of leucine and isoleucine in HF5 (19% and 9%, respectively) more than doubled in comparison to the percentage in fraction 1 (8.7% and 4%, respectively). The hydrophobic aromatic amino acids, tryptophan and phenylalanine also increased from HF1 to HF5. Tryptophan was not present in HF1 and increased to approximately 1.2% in HF5. The percentage of phenylalanine increased four-fold from HF1 (4%) to HF5 (16%). The percentage of tyrosine was the highest in HF3 and was the lowest in HF1 and HF5. Proline, a slightly hydrophobic amino acid increased approximately 3.5 times from 2.3% in HF1 to 8% in HF5. As well, there is a decreasing trend in the percentage of hydrophilic amino acids (serine, lysine, arginine and histidine) from HF1 to HF5. Asparagine and aspartic acid as well as glutamine and glutamic acid decreased in percentage from HF1 (14% and 17%, respectively) to HF5 (11% and 6.6%, respectively).

Table 4.2.1.1: Percentage amino acid composition of peptide fractions separated based on hydrophobic properties

Amino Acid	HF1	HF2	HF3	HF4	HF5
ASX	13.94	10.63	12.59	10.85	11.04
THR	3.89	3.86	3.34	3.11	3.22
SER	6.63	5.71	6.19	4.41	3.82
GLX	17.12	14.78	13.75	12.87	6.64
PRO	2.33	6.47	5.14	5.42	8.05
GLY	3.52	5.00	3.96	4.66	3.26
ALA	5.54	4.30	5.03	3.44	3.62
CYS	0.18	0.39	0.39	0.38	0.29
VAL	5.23	4.45	4.13	5.82	7.68
MET	0.70	1.70	0.87	1.07	0.68
ILE	4.13	4.04	6.71	5.85	9.13
LEU	8.70	6.68	9.95	14.57	19.48
TYR	2.77	5.33	7.15	5.09	2.44
PHE	3.97	7.76	8.73	12.03	16.44
HIS	2.49	3.28	1.90	1.81	0.63
LYS	9.07	7.35	4.26	3.31	1.20
ARG	9.79	8.00	5.15	3.97	1.22
TRP	0.00	0.27	0.74	1.36	1.16
HAA	33.56	41.39	48.85	55.01	68.97
PCAA	21.35	18.63	11.32	9.08	3.05
NCAA	31.06	25.41	26.34	23.72	17.68
AAA	6.74	13.36	16.62	18.48	20.03

ASX= aspartic acid and asparagine; GLX = glutamic acid and glutamine

Combined total of hydrophobic amino acids (HAA) = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

Positively charged amino acids (PCAA) = arginine, histidine, lysine

Negatively charged amino acids (NCAA) = ASX and GLX

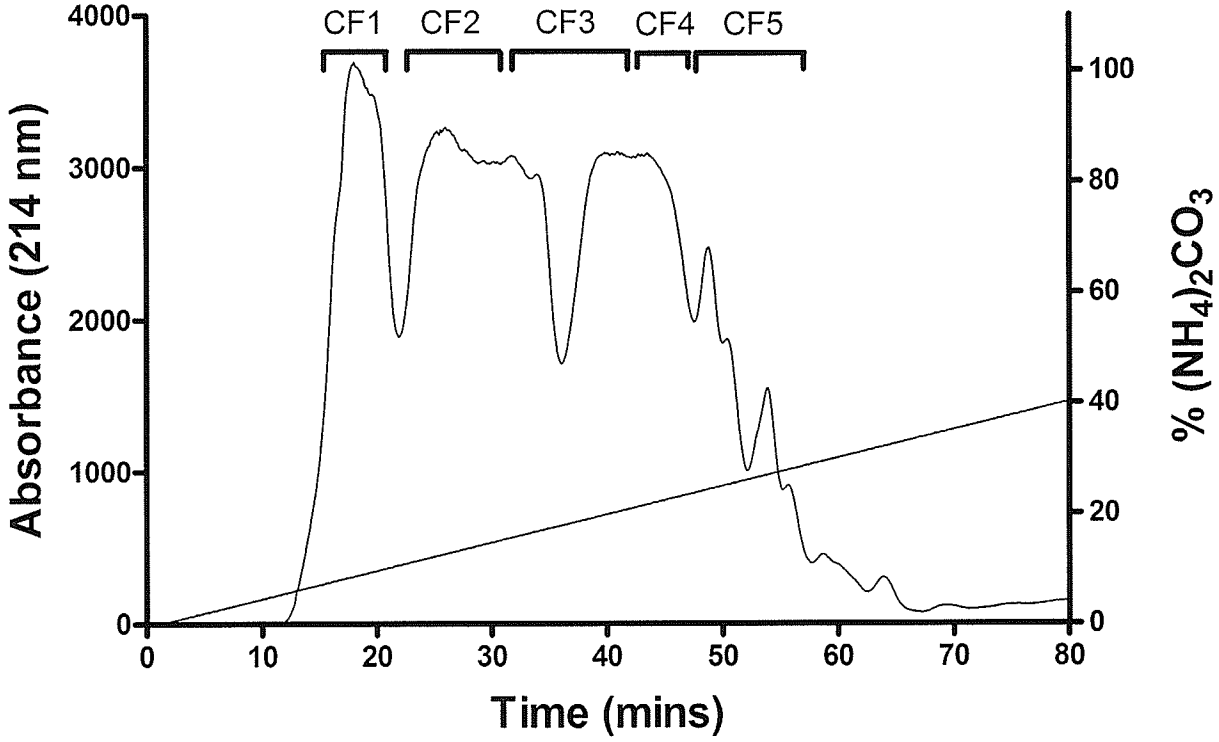
Aromatic amino acids (AAA) = phenylalanine, tryptophan and tyrosine

4.2.2 Separation based on charge

The PPH was fractionated using cation exchange column as indicated in the chromatogram in Figure 4.2.2 with FPLC. The principle of cation chromatography is to separate the pea protein hydrolysate into arbitrary fractions that differ in net positive charges. The net charge of the peptides is dependent on the constituent amino acids and ultimately on the pH of the mobile phase (Aluko, 2004). The cation exchange column is packed with negatively charged polymers, which allows for the positively charged peptides to bind. The unbound peptides are removed from the column by washing with a buffer at a specific pH. The buffer gradient was then altered through increase in pH neutralize the positively charged peptides allowing them to become unbound from the column and elute. As the pH of the mobile phase increases, and approaches the isoelectric point (pI) of the peptides, the peptides lose the overall charge and become unbound from the column. Peptides that have a net weak positive charge eluted early from the column first while the tightly bound peptides were eluted later as the pH increased.

Cationic fraction 1 (CF1), consisted of unbound peptides and weakly bound peptides which eluted first from 12 to 19 minutes, therefore, CF1 consists of peptides with weak cationic charge. Cationic fraction 2 (CF2) eluted from 20 to 31 minutes, cationic fraction 3 (CF3) eluted from 32 to 41 minutes and cationic fraction 4 (CF4) eluted from 42 to 45 minutes. Cationic fraction 5 (CF5) bound strongly with the column and eluted last from 47 to 57 minutes, therefore CF5 is considered to have the strongest net cationic charge in comparison to cationic fractions 1, 2, 3 and 4.

Figure 4.2.2 PPH separation by cation exchange



4.3 Protein content

The protein content for the PPH was 86%. Upon fractionation by RP-HPLC, the protein content of the five hydrophobic fractions ranged from 88% to 100%, as determined by the modified Lowry method (Table 4.3.1). All fractions separated by RP-HPLC were creamy to pale yellow in colour except for hydrophobic fraction 5, which was bright yellow.

Cationic fractions 2, 3, 4 and 5 separated by FPLC were all cream to pale yellow in colour. CF1, the first cationic fraction to elute possessed a very bright yellow/orange colour and contained 62% protein. Cationic fractions 2, 3, 4 and 5 were determined to be 100% protein (Table 4.3.2).

Table 4.3.1: Protein content (%) of fractions separated by hydrophobic properties as determined by the modified Lowry method

Fraction	Percent Protein (%)
HF1	100
HF2	100
HF3	100
HF4	100
HF5	88

Table 4.3.2: Protein content (%) of cation exchange fractions as determined by the modified Lowry method

Fraction	Percent Protein (%)
CF1	62
CF2	100
CF3	100
CF4	100
CF5	100

4.4 Antioxidant activity evaluation

Seven antioxidant evaluation systems were utilized to determine the antioxidant activity of pea protein hydrolysate. The antioxidant activity of the peptides was compared to the antioxidant activity of reduced glutathione. Reduced glutathione was used as a control because it is considered to be a very effective antioxidant, it is biologically relevant and it is abundant in living cells (Meister, 1994).

4.4.1 DPPH scavenging activity

DPPH radical scavenging assay is commonly used to determine the scavenging activity of natural compounds (Bougatef et al., 2009) because it is simple and relatively quick to perform. The DPPH radical scavenging activity indicates the ability to donate electrons or hydrogen therefore converting the radical to a more stable species (Prior et al., 2005).

4.4.1.1 Peptides separated based on hydrophobic properties

The DPPH radical scavenging activity of <3 kDa pea protein hydrolysate (PPH), RP-HPLC fractions and glutathione are shown in Figure 4.4.1.1. The fractions displaying the strongest net hydrophobicity possessed the strongest DPPH radical scavenging activity and scavenging activity appeared to decrease with decreasing net hydrophobicity. Hydrophobic fraction 5 has the strongest activity in comparison to hydrophobic fractions 1, 2 and 3 ($p<0.05$), but has equivalent DPPH scavenging activity as HF4 ($p>0.05$). The radical scavenging activity of HF5 is almost two times stronger than HF2. Hydrophobic fractions 4 and 5 have the same DPPH radical scavenging activity as the PPH, which indicates that these two fractions contribute to the majority of the DPPH scavenging activity of the PPH. HF4 and HF5 are both rich in leucine and phenylalanine, which are hydrophobic amino acids. The correlation between the DPPH radical scavenging and the total amount of hydrophobic amino acids is 0.8312 (Figure 4.4.1.2). Interestingly, these two fractions also contained higher concentrations of valine and tryptophan than hydrophobic fractions 1, 2 and 3 with decreased net hydrophobicity. Glutathione possessed the strongest DPPH radical scavenging activity at 30% ($p<0.05$). Glutathione is recognized to be a potent antioxidant *in vivo* and the activity is attributed to the sulfhydryl group of cysteine, therefore, sulphur containing amino acids could be effective scavengers of the DPPH radical. HF5 contained the lowest content of cysteine, which indicates that a combination of other amino acids, including leucine, phenylalanine, valine and tryptophan can also scavenge the DPPH radical through the donation of electrons.

PPH in the present study had two times stronger activity than what was observed by Humiski & Aluko (2007), who examined DPPH radical scavenging activity of pea protein hydrolysates using several food grade enzymes. At 1 mg/ml, the Flavourzyme hydrolysate produced the strongest DPPH scavenging peptides at 11% (Humiski & Aluko, 2007), which is weaker activity than what was observed for the PPH in the present study obtained by Thermolysin at 20% scavenging. The Flavourzyme hydrolysate contained high concentrations of asparagine, aspartic acid, glutamine, glutamic acid, leucine, lysine and arginine (Humiski & Aluko, 2007). The PPH in the present study, contained higher concentrations of hydrophobic amino acids including alanine, valine, leucine, isoleucine and phenylalanine in comparison to the hydrolysate produced by Flavourzyme (Humiski & Aluko, 2007). The amino acids that had a higher concentration in the PPH in this study using Thermolysin in comparison to the Flavourzyme hydrolysate could be responsible for the stronger DPPH radical scavenging activity.

In previous research, a hydrolysate from smooth hound muscle displayed DPPH radical scavenging activity ranging from 35-65%, dependent on the gastrointestinal protease at 1 mg/ml (Bougatef et al., 2009). The hydrolysate produced by a low molecular weight alkaline protease had the highest DPPH scavenging activity and was separated by gel filtration. The smallest molecular weight gel filtration fraction (<3.5 kDa) exhibited the strongest DPPH scavenging activity at approximately 70% at 1 mg/ml. The fraction was rich in histidine, methionine, tyrosine, leucine, glycine and arginine, which Bougatef et al. (2009) attributed to the antioxidant activity.

DPPH scavenging activity observed for buckwheat hydrolysates ranged from approximately 50% to approximately 75% at a concentration of 1 mg/ml, dependent on

the degree of hydrolysis (Tang et al., 2009a). The buckwheat hydrolysate with the strongest DPPH scavenging activity contained slightly higher amount of hydrophobic amino acids and had the highest surface hydrophobicity compared to hydrolysates with lower DPPH scavenging (Tang et al., 2009a). The findings of Tang et al. (2009a) are in agreement with the results from the current study as the strongest DPPH scavenging activity was seen in fractions with the strongest net hydrophobicity. Li et al. (2008) observed that a low molecular weight fraction of chickpea protein hydrolysate had strong DPPH radical scavenging activity at 86% at a concentration of 1 mg/ml which was attributed to the high concentration of hydrophobic amino acids, including valine, methionine, phenylalanine, isoleucine, leucine as well as the amino acid lysine.

Interestingly, Xie et al. (2008) reported very strong (80%) DPPH radical scavenging activity for glutathione at 0.4 mg/ml, which is much stronger activity than what was observed in the present study where glutathione had 30% DPPH radical scavenging activity at 1 mg/ml and the observations of Udenigwe et al. (2009) who reported approximately 65% DPPH scavenging at 1 mg/ml of glutathione.

DPPH radical scavenging method is a single electron transfer reaction (Huang et al., 2005), therefore, the results suggest that the PPH and the fractions with the strongest net hydrophobic properties contain peptides whose constituent amino acids are effective electron donators to the DPPH radical.

Figure 4.4.1.1: DPPH radical scavenging activity (%) of fractions (HF1-HF5) separated based on hydrophobic properties, PPH and glutathione

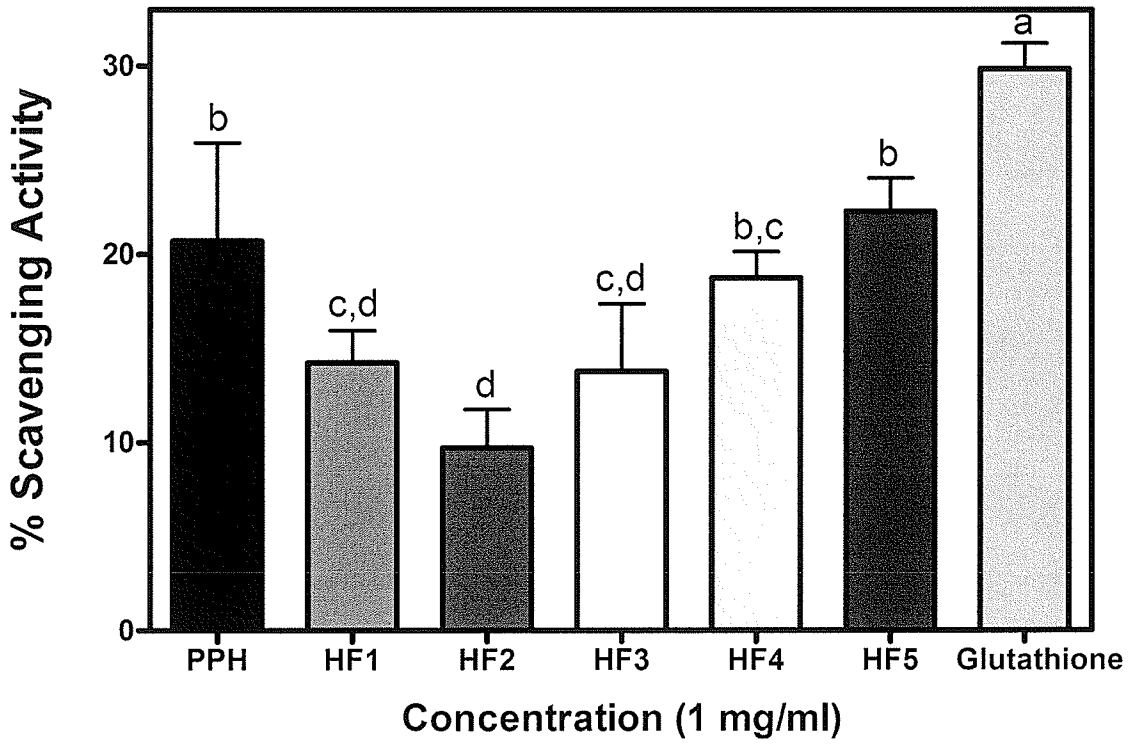
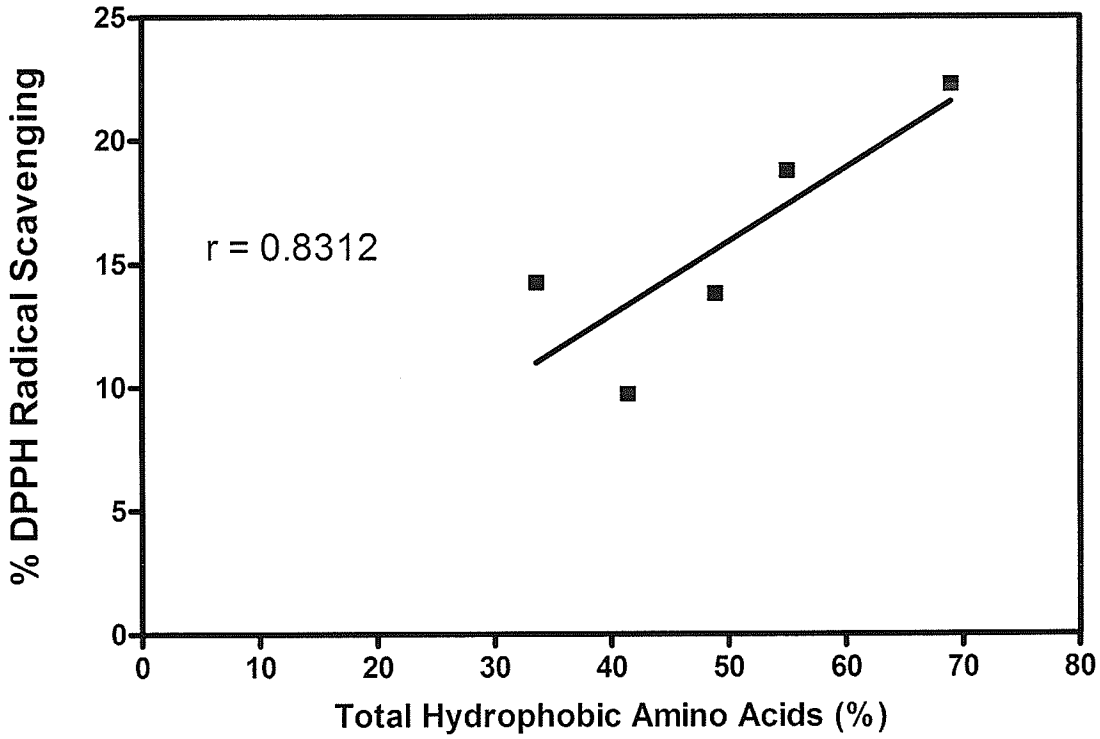


Figure 4.4.1.2: Correlation of DPPH radical scavenging activity (%) and total hydrophobic amino acids (%) of fractions separated by hydrophobic properties



Total hydrophobic amino acids = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

4.4.1.2 Peptides separated based on cationic charge

Peptides separated by cation exchange chromatography displayed a range of activities against the DPPH radical as displayed in Figure 4.4.1.3. Cationic fraction 1, the fraction with the least net cationic charge displayed the strongest DPPH radical scavenging activity at 22% scavenging activity ($p<0.05$) of the five fractions separated based on charge. Interestingly, CF2 did not display DPPH radical scavenging activity. Cationic fractions 3, 4 and 5, which possessed stronger net cationic charge displayed

weak DPPH radical scavenging activity at 5.5%, 8.8% and 4.4%, respectively. CF1 displayed equivalent DPPH scavenging activity as the PPH ($p>0.05$). This suggests that the DPPH scavenging activity of PPH could be primarily due to the peptides concentrated in CF1 and that the peptides responsible for the scavenging activity have weak cationic charges.

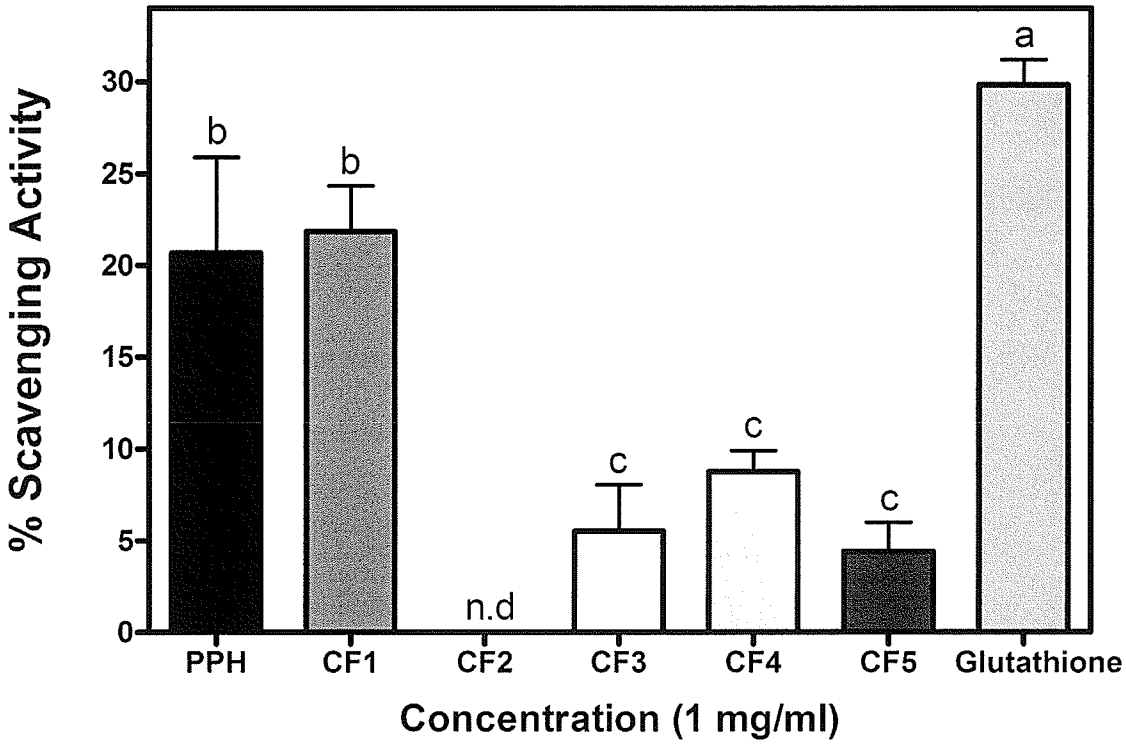
In previous work, Park and colleagues (2008) identified anionic peptides from soy protein and gluten protein which possessed stronger DPPH radical scavenging activity than cationic fractions. Upon sequential separation of porcine skin hydrolysates with gel filtration and anion exchange chromatography, Li et al. (2007) identified that the strongest bound fraction from the anion exchange chromatography had the strongest DPPH radical scavenging activity at an unspecified concentration. Je et al. (2008) identified a hydrolysate from tuna dark muscle produced by pepsin that displayed moderate DPPH radical scavenging activity at approximately 50% at 3 mg/ml. Upon further purification with anion exchange chromatography, the fraction with the strongest net anionic charge displayed the strongest DPPH radical scavenging activity at 50%.

Anionic fractions are suggested to quench DPPH radical through the donation of a hydrogen atom (Park et al., 2008). The present results are supported by the observations of Li et al. (2007) and Park et al. (2008) that the net peptide charge affects the efficacy of the DPPH radical scavenging activity allowing for the donation of hydrogen to neutralize the DPPH radical. The results suggest that high levels of hydrophobic character are more desirable for DPPH scavenging ability of peptides than high levels of charge.

In summary, separation by charge and hydrophobic properties produced peptide fractions with moderate DPPH radical scavenging activity. Overall, fractions separated

based on hydrophobic properties are more effective in scavenging the DPPH radical in comparison to the fraction separated by cationic charge.

Figure 4.4.1.3: DPPH radical scavenging activity (%) of fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



n.d = no activity determined

4.4.2 Reducing power

4.4.2.1 Peptides separated based on hydrophobic properties

The ability of peptides to act as reducing agents through the donation of electrons to form more stable products was measured by the reducing power method. This method measures the ability of peptides to reduce the Fe^{3+} -ferricyanide complex to the ferrous

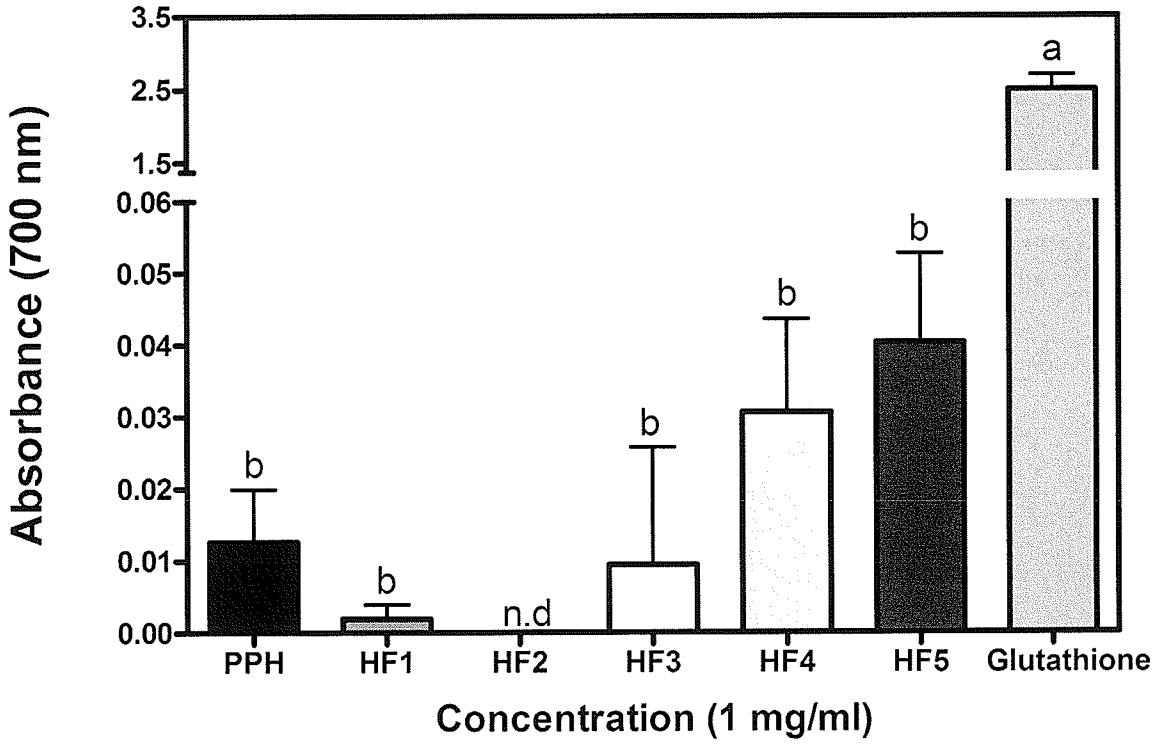
form (Fe^{2+}) (Bougatef et al., 2009). The reducing power is measured spectrophotometrically by evaluating the yellow colour of the test solution to change to a green/blue colour, depending on the reducing power of the peptides. The reducing power of the fractions separated by RP-HPLC is shown in Figure 4.4.2.1. Fractions separated by RP-HPLC and PPH displayed very weak reducing power. HF2 displayed no reducing power activity. The correlation between the reducing power of the fractions separated by hydrophobic properties and the total amount of hydrophobic amino acids is 0.9361 (Figure 4.4.2.2). Glutathione displayed stronger reducing power in comparison to the fractions.

In contrast, it was previously identified that hydrolysates from smooth hound muscle displayed reducing power, which ranged from approximately 0.1-0.4 with absorbance at 700 nm and at a concentration of 1 mg/ml (Bougatef et al., 2009). Gimenez et al. (2009) found that hydrolysates from squid had stronger reducing power than sole hydrolysates. The sole hydrolysate had a higher amount of hydrophobic amino acids, whereas the squid hydrolysate was richer in tyrosine, leucine and valine (Gimenez et al., 2009). Rapeseed hydrolysates, dependent on the fraction displayed good reducing power (Zhang et al., 2008). The rapeseed fractions which displayed the strongest reducing power contained a higher amount of hydrophobic amino acids including leucine, isoleucine, phenylalanine, tyrosine, tryptophan, proline and cysteine (Zhang et al., 2007). These particular amino acids were suggested to be responsible for the reducing power activity (Zhang et al., 2008).

Hydrolysate from alfalfa leaf proteins with a molecular weight <3 kDa possessed moderate reducing power with an absorbance of 0.4, which was lower than that of

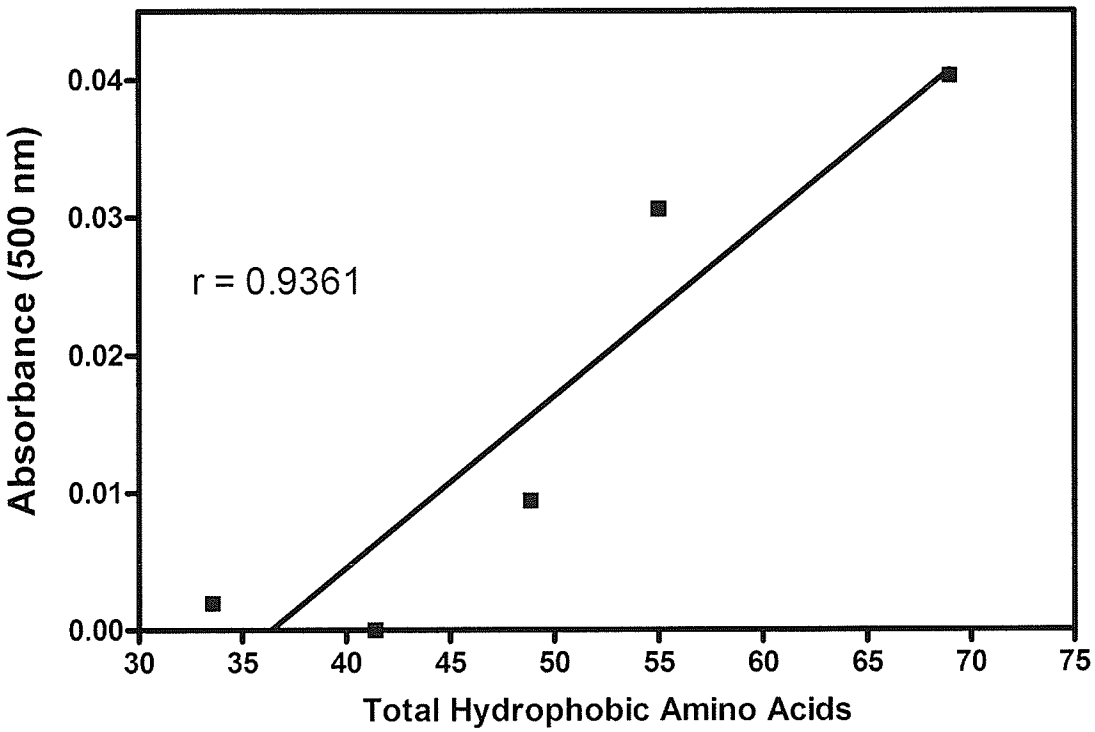
glutathione at 1 mg/ml (Xie et al., 2008). In comparison to PPH, the <3 kDa alfalfa hydrolysate was richer in cysteine, methionine, tyrosine, histidine and tryptophan; however the PPH was richer in leucine and phenylalanine. Xie et al. (2008) suggested the antioxidant activity was due to histidine, tyrosine, methionine and cysteine content of the peptides. PPH contained lower concentrations of the amino acids suggested by Xie et al. (2008) to be responsible for the reducing power; therefore, it appears that the above mentioned amino acids are important for reducing power activity. You and colleagues (2009) observed that loach peptides hydrolyzed by papain had strong reducing power and the hydrolysate contained tyrosine, methionine, histidine, lysine and tryptophan, leading You et al. (2009) to suggest that the reducing power could be attributed to the specific amino acid and peptide composition. As well, in the present study, glutathione displayed very strong reducing power ability which also suggests that the sulfhydryl group of cysteine is an important reducing agent. All fractions and PPH are low in sulphur-containing amino acids, suggesting that cysteine is an effective reducing agent. Based on the findings of the present study, it appears that PPH and fractions separated based on hydrophobicity do not have strong reducing power. Furthermore, hydrophobic properties do not appear to influence the ferric reducing antioxidant power activity in low molecular weight peptides derived from pea protein.

Figure 4.4.2.1: Reducing power displayed by fractions (HF1-HF5) separated by hydrophobic properties, PPH and glutathione



n.d = no activity determined

Figure 4.4.2.2: Correlation between reducing power and total hydrophobic amino acids (%) of fractions separated by hydrophobic properties



Total hydrophobic amino acids = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

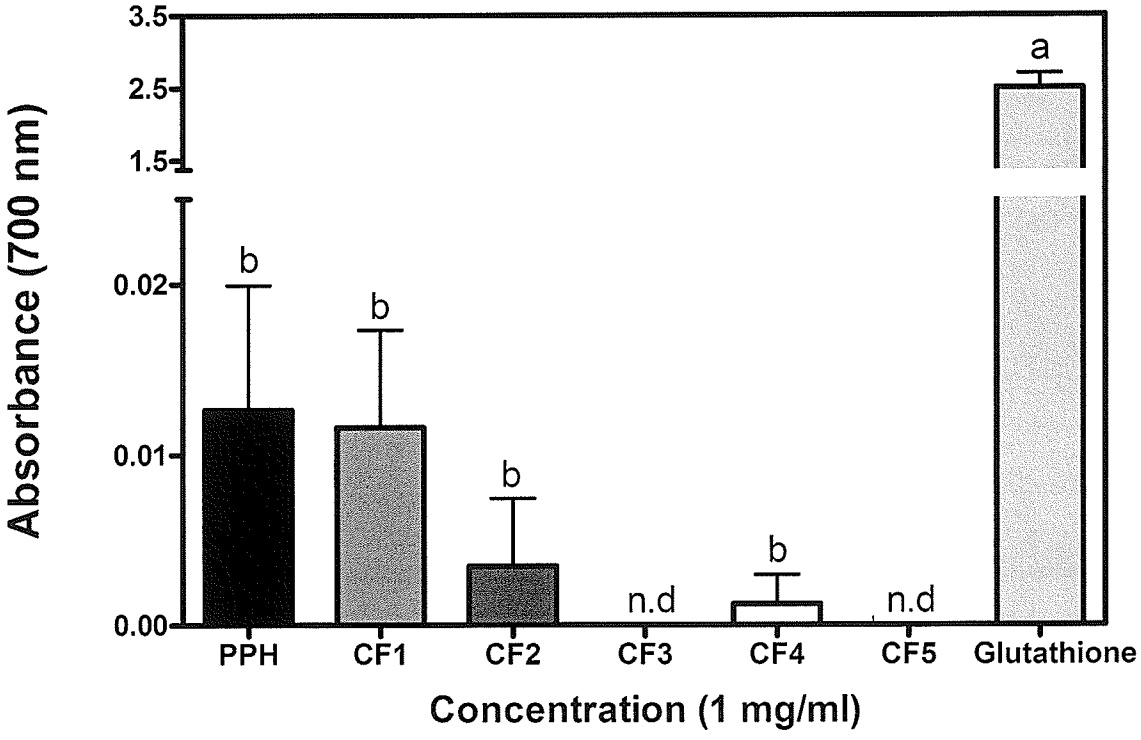
4.4.2.2 Peptides separated based on cationic charge

Reducing power of peptides separated by cation exchange chromatography is shown in Figure 4.4.2.3. Fractions separated based on cation exchange chromatography appear to have very weak reducing power. CF1, CF2 and CF4 as well as the PPH have weak reducing power ($p>0.05$). However, CF3 and CF5, having stronger net cationic charges than the earlier eluted fractions displayed no reducing power. Glutathione has the

strongest reducing power ($p<0.05$), which could be due in part to the reducing power of sulfhydryl group of cysteine. Peptides separated by cationic charge may not be able to reduce ferric iron to ferrous iron and instead maybe repelled from the ferric ion because of the positive charges of the cationic fractions and the ferric ion. It appears that unlike the metal chelating property, charge does not play a significant role in determining the reducing power of peptides from pea protein hydrolysate.

Reducing power displayed by peptides separated based on hydrophobicity and cationic charge was negligible. In comparison to glutathione, fractions separated by both characteristics displayed poor activity.

Figure 4.4.2.3: Reducing power displayed by fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



n.d = no activity determined

4.4.3 Superoxide radical scavenging activity

4.4.3.1 Peptides separated based on hydrophobic properties

Figure 4.4.3.1 shows the percentage of superoxide scavenging radical activity displayed by fractions separated by RP-HPLC, PPH and glutathione at 1 mg/ml. All fractions obtained by RP-HPLC separation displayed moderate superoxide radical scavenging. HF5, the fraction with the strongest net hydrophobicity displayed superoxide scavenging activity at 32%, which has the same activity as HF4 at 28% and HF2 at 28% ($p>0.05$). Hydrophobic fractions 1 and 3 (24.9% and 26.5%, respectively) displayed relatively lower superoxide radical scavenging activity than HF5 ($p<0.05$). The PPH displayed weak superoxide scavenging activity in comparison to the fractionated peptides and glutathione. The peptides present in the PPH may have acted antagonistically and could therefore reduce the overall superoxide radical scavenging activity. Fractionation of peptides is a known method of concentrating peptides (Aluko, 2004) and upon fractionation by RP-HPLC, the active peptides would have become concentrated, leading to stronger superoxide scavenging activity. Interestingly, HF2, HF4 and HF5, with the stronger superoxide scavenging activity contained higher amounts of proline in comparison to the other fractions, which may contribute to the superoxide scavenging activity of these fractions. The correlation between the superoxide scavenging activity of the fractions separated by hydrophobic properties and the total hydrophobic amino acids is 0.9015 ad is shown in Figure 4.4.3.2.

In previous research, a low molecular weight fraction from chickpea protein hydrolysate with strong superoxide radical scavenging activity was observed to have higher concentrations of phenylalanine, isoleucine, leucine and valine in comparison to

other fractions and it was suggested that the superoxide scavenging activity was related to the hydrophobic amino acids (Li et al., 2008). Hydrophobic fractions 4 and 5 in the present study had higher amounts of isoleucine, leucine, valine, phenylalanine and tryptophan in comparison to the PPH, which may contribute to the increased scavenging activity.

Previous research by Xie and colleagues (2008) demonstrated that alfalfa leaf hydrolysates with a molecular weight of <3 kDa had strong superoxide radical scavenging activity at 67% at a concentration of 0.9 mg/ml, which is about 13 times more active than PPH evaluated in the present study. Alfalfa hydrolysate contained higher concentrations of cysteine, methionine, tyrosine, histidine and tryptophan in comparison to the PPH examined in the present study, therefore, these amino acids may strongly contribute to the superoxide radical scavenging activity.

Xie and colleagues (2008) observed strong superoxide radical scavenging activity for glutathione at approximately 95% at a concentration of 0.9 mg/ml in comparison to 90% at 1 mg/ml observed in the present study. Glutathione in the present study possessed stronger activity in comparison to the hydrophobic fractions and PPH, indicating that in addition to some of the above mentioned hydrophobic amino acids, cysteine also contributes to the superoxide radical scavenging.

Figure 4.4.3.1: Superoxide radical scavenging activity (%) of fractions (HF1-HF5) separated by hydrophobic properties, PPH and glutathione

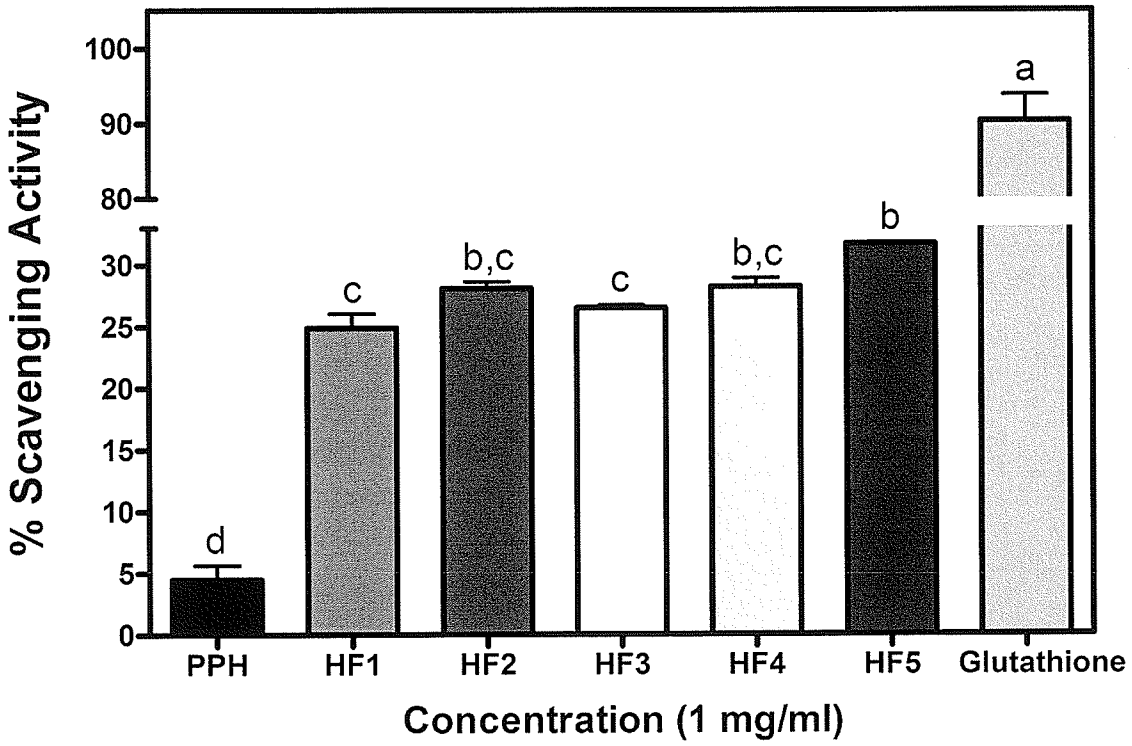
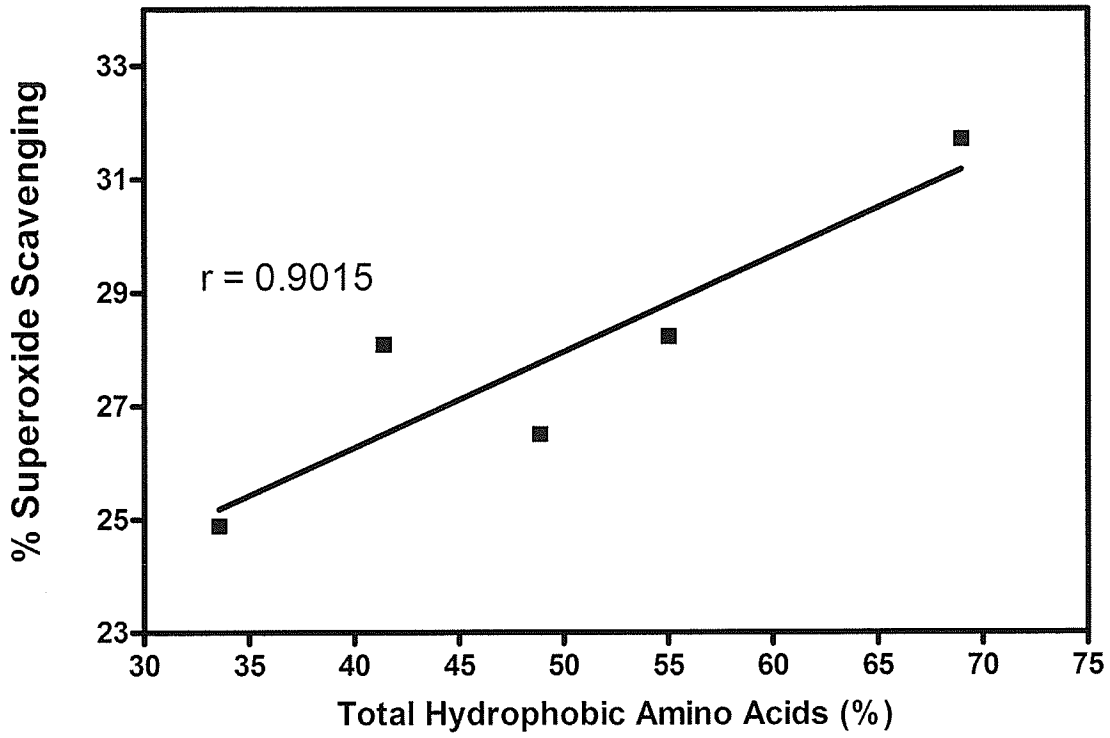


Figure 4.4.3.2: Correlation between superoxide radical scavenging (%) and the total hydrophobic amino acids (%) of fractions separated by hydrophobic properties



Total hydrophobic amino acids = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

4.4.3.2 Peptides separated based on cationic charge

The superoxide scavenging activity of peptides separated based on cationic charge is shown in Figure 4.4.3.3. In comparison to the 5% superoxide radical scavenging activity obtained for the PPH, the fractions separated by cationic charge displayed stronger superoxide radical scavenging activity ranging from 23% to 53% scavenging activity. The least cationic fractions, CF1 and CF2 displayed the strongest superoxide

radical scavenging activity ($p<0.05$) at 46.6% and 53.4%, respectively. The fractions with stronger net cationic charges, CF3, CF4 and CF5, displayed weaker superoxide radical scavenging activity ($p<0.05$) at 23%, 38% and 24%, respectively, in comparison to the fractions with the weakest net cationic charge. The superoxide radical scavenging activity of the fractions separated by cationic charge displayed stronger superoxide radical scavenging activity than the PPH, which was similar to the observed activity for the fractions separated based on hydrophobic properties. It appears that upon fractionation of peptides based on cationic charge, the peptides are concentrated and therefore, exhibit strong superoxide radical scavenging activity. The net cationic charge of peptides influences the activity to scavenging superoxide radicals, with the least cationic net charge peptides displaying the strongest activity.

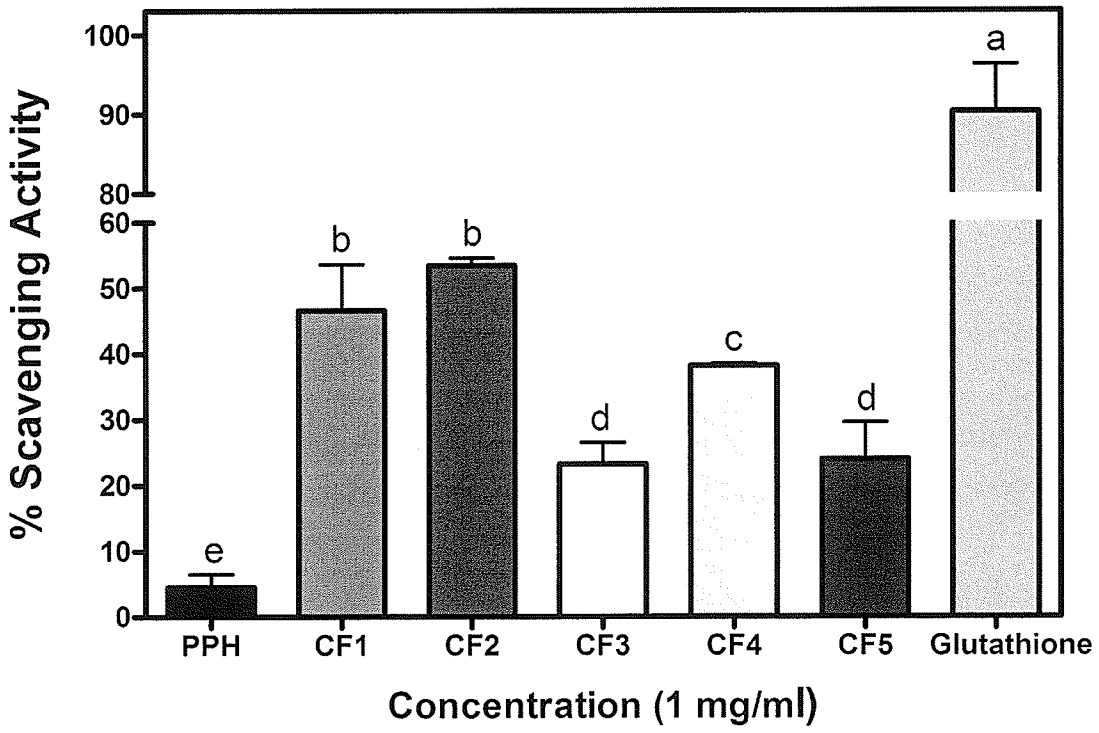
In previous work, Peng et al. (2009) identified a fraction of whey protein hydrolysate with molecular weight from 0.1-2.8 kDa to have strong superoxide radical scavenging activity at 70% measured at a concentration of 1 mg/ml by electron spin resonance. The superoxide radical scavenging activity identified by Peng et al. (2009) was much stronger in comparison to the low activity of PPH observed in the present study. Je et al. (2008) observed that peptides from tune dark muscle had a range of superoxide radical scavenging activities from negligible activity to 25% scavenging activity, dependent on the enzyme used for hydrolysis.

It appears that the net cationic charge of peptides influences the activity to scavenge superoxide radicals, with the least cationic net charge peptides displaying the strongest activity. All fractions displayed potent superoxide scavenging activity in comparison to PPH, although glutathione exhibited the strongest activity. Glutathione

displayed strong superoxide scavenging activity that could be attributed to the presence of cysteine.

Fractions separated based on cationic charge and hydrophobic properties displayed moderate scavenging of the superoxide anion. Overall, fractions separated by cationic charge displayed stronger superoxide radical scavenging and appear to be more effective scavengers of the superoxide anion.

Figure 4.4.3.3: Superoxide radical scavenging activity (%) of fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



4.4.4 Hydrogen peroxide scavenging

4.4.4.1 Peptides separated based on hydrophobic properties

Hydrogen peroxide is not very reactive at low concentrations; however, the combination of hydrogen peroxide with an unbound transition metal (Fe^{2+}) increases the Fenton reaction and leads to oxidative damage (Huang et al., 2005). Figure 4.4.4.1 displays the hydrogen peroxide scavenging activity of the peptides separated by RP-HPLC, PPH and glutathione. Hydrogen peroxide scavenging activity of the peptides separated by based on hydrophobic properties displayed an increasing trend in scavenging activity with an increase in net hydrophobic properties of the fractions. Hydrophobic fraction 1, with the least net hydrophobic property, possessed the weakest hydrogen peroxide scavenging activity at 40% ($p<0.05$). The PPH displayed strong hydrogen peroxide activity, however, the activity was weaker than the scavenging activity of HF5 ($p<0.05$). The fractions with the strongest net hydrophobic properties could be responsible for most of the hydrogen peroxide scavenging activity of the PPH and upon fractionation the peptides could become concentrated displaying more potent activity. There is a strong correlation between the hydrogen peroxide scavenging activity and the total percentage of hydrophobic amino acids of the peptides separated based on hydrophobic properties at 0.9713 (Figure 4.4.4.2). Fractionation of the PPH appears to increase the potency of the fractions with the strongest net hydrophobic properties. Glutathione displayed stronger activity in comparison to the fractions, although the fractions displayed strong activity as well. The activity exhibited by glutathione could be due to the presence of cysteine and the sulfhydryl group, whereas the presence of hydrophobic amino acids (valine, isoleucine, leucine, and phenylalanine) and proline

could be responsible for the activity exhibited by HF5. Hydrophobic properties appear to be important in the ability to scavenge hydrogen peroxide.

Guo and colleagues (2009) identified three tripeptides from royal jelly protein with hydrogen peroxide scavenging activity. All three peptides contained tyrosine which Guo et al. (2009) attributed to the scavenging activity.

Figure 4.4.4.1: Hydrogen peroxide scavenging activity (%) of fractions (HF1-HF5) separated by hydrophobic properties, PPH and glutathione

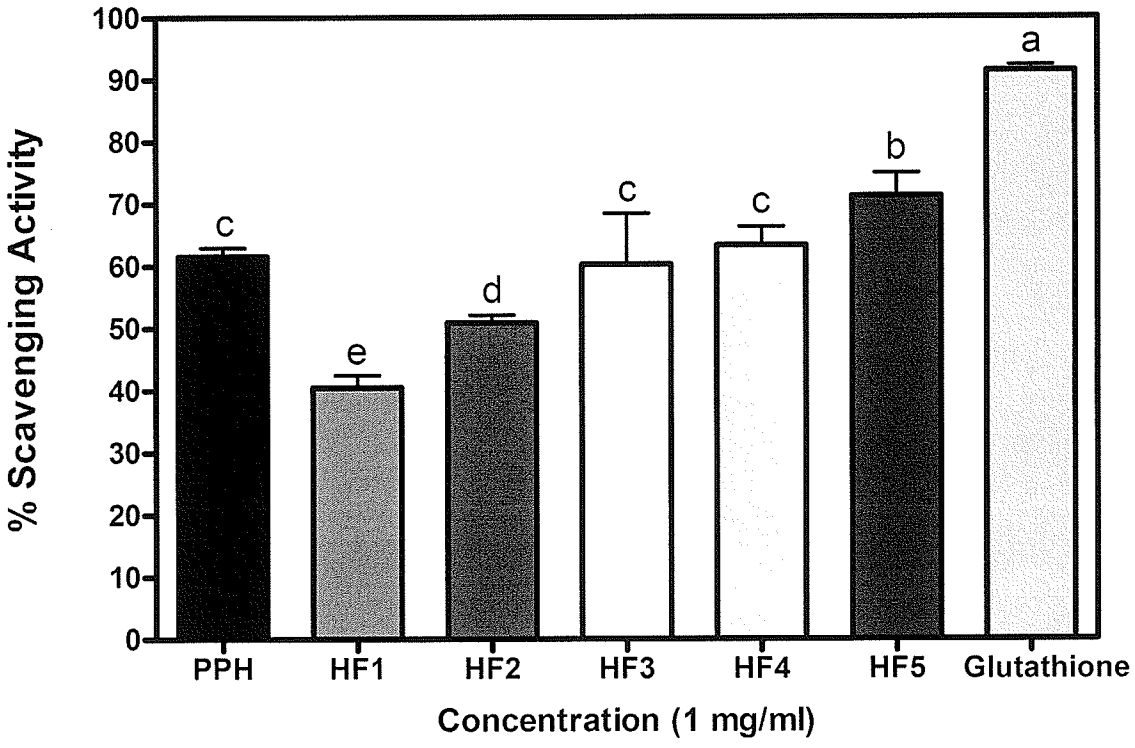
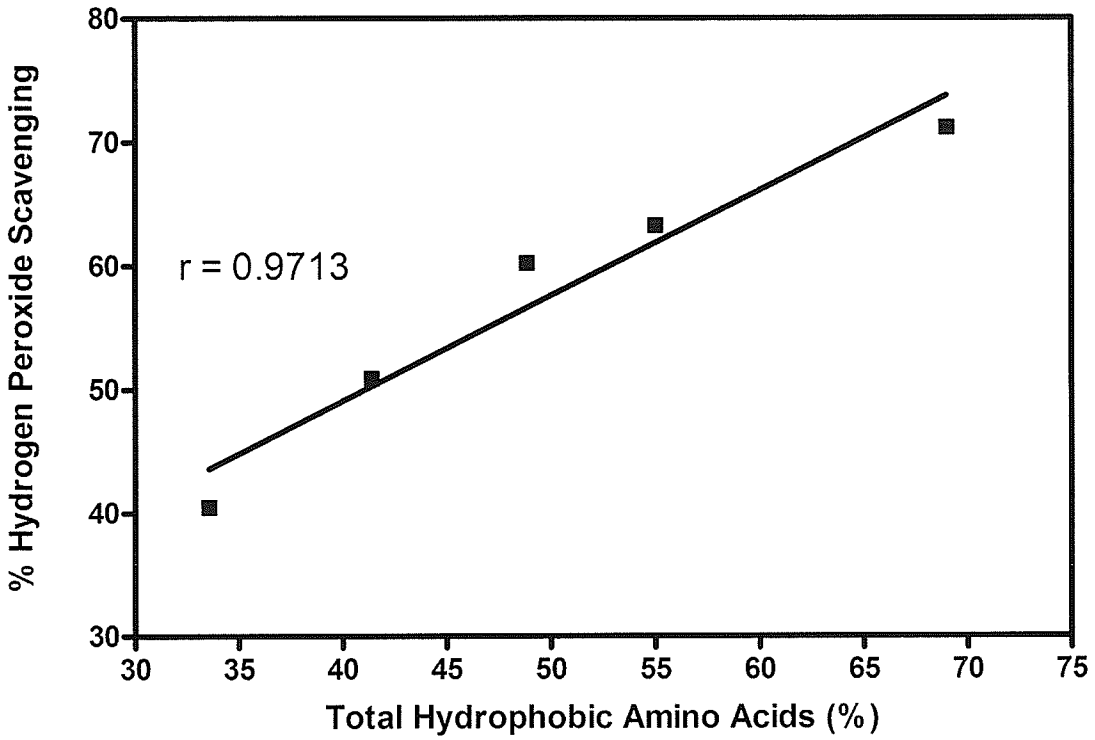


Figure 4.4.4.2: Correlation between hydrogen peroxide scavenging (%) and the total hydrophobic amino acids (%) of fractions separated by hydrophobic properties



Total hydrophobic amino acids = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

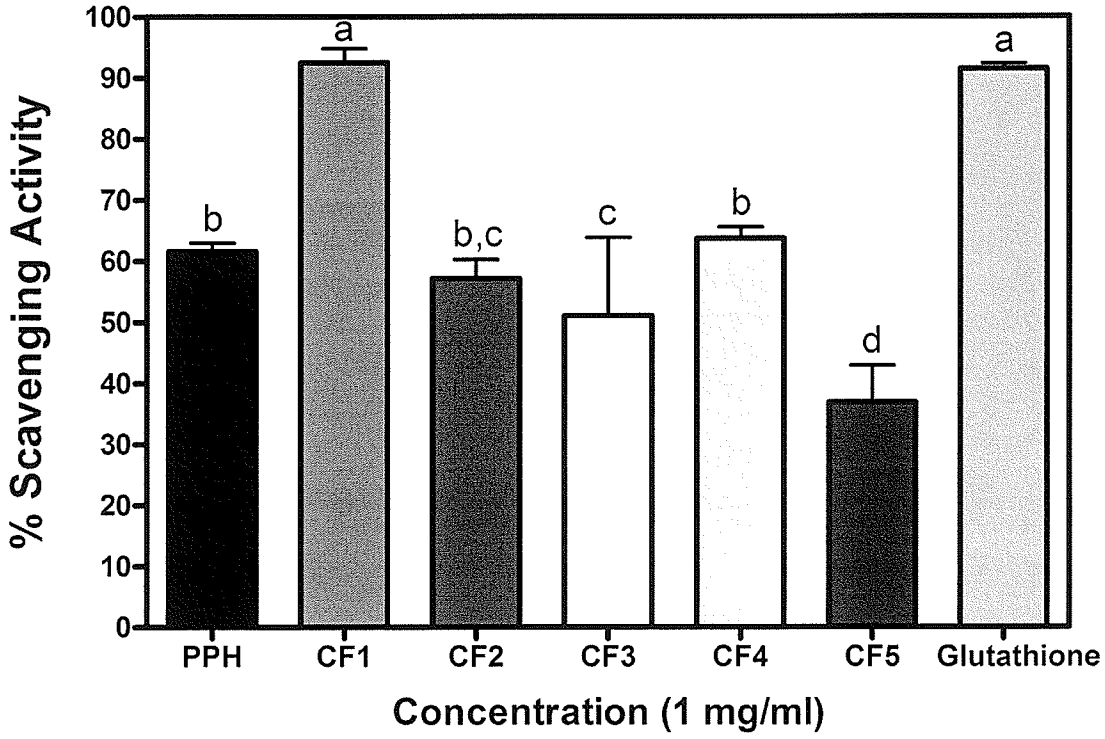
4.4.4.2 Peptides separated based on cationic charge

Figure 4.4.4.3 shows the hydrogen peroxide scavenging activity of fractions separated based on cationic charge. Cationic fraction 1, with the least net cationic charge displayed the strongest activity, similar to the activity exhibited by glutathione ($p>0.05$) at approximately 90%. CF2 and CF4 displayed activities similar to that of PPH ($p>0.05$). CF5, with the strongest cationic charge, displayed the weakest activity at approximately

36% which is less than half the activity displayed by CF1 and glutathione. It appears that separating the amino acids by charge is an effective method to produce a fraction which has activity comparable to glutathione.

Separation of peptides based on hydrophobic properties and cationic charges produce fractions that are effective at scavenging hydrogen peroxide.

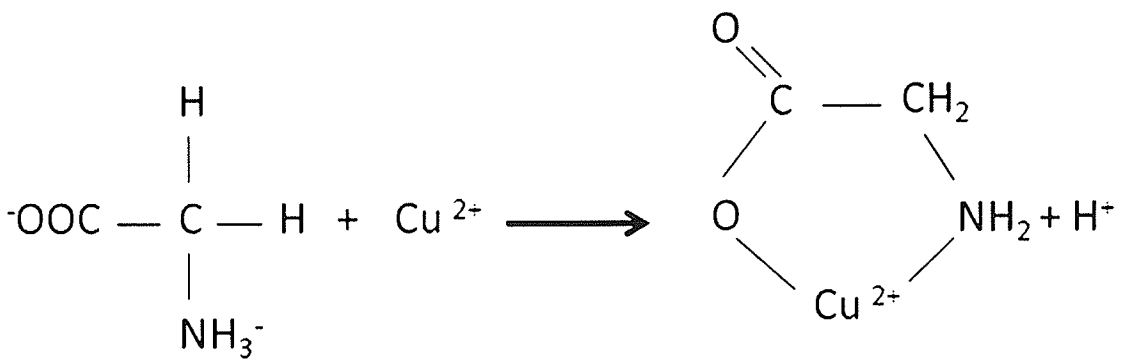
Figure 4.4.4.3: Hydrogen peroxide scavenging activity (%) of fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



4.4.5 Metal chelating activity

FerroZine and Fe (II) combine to form $Fe(II)Fz_3$, a stable, oxidation resistant complex (Berlett et al., 2001). The chelation of metal ions requires two or more atoms on the same molecule which are capable of binding to metal ions (Miller et al., 1990). Miller et al. (1990) suggests that optimal metal chelating involves aliphatic compounds, where a five membered ring is formed, which is composed of the metal ion and two chelating ligands (Figure 4.4.5). Histidine is considered a strong metal chelator due to the presence of an imidazole ring (Megias et al., 2008). Chelating metal ions has clinical implications. A biological system which is overloaded by iron due to hemodialysis will have higher amounts of free iron (Valko et al., 2007). As well, iron can be released from the storage protein, ferritin, during increased oxidative stress or by reducing agents. Superoxide has been noted to mobilize iron from ferritin, therefore, increasing the iron available to participate in the Fenton reaction leading to the production of the highly reactive hydroxyl radical (Halliwell & Gutteridge, 1990). The chelation of metal ions can decrease the amount of free iron available to participate in the Fenton reaction and ultimately decrease the formation of the hydroxyl radical (Halliwell & Gutteridge, 1990). The ability to chelate metal ions is dependent on pH and therefore a net anionic charge of a peptide will lead to an electrostatic attraction to a cationic metal (Elias et al., 2008).

Figure 4.4.5 Chelation of a metal ion



Glycine Copper ion Copper ion chelated by glycine

Adapted from: Miller et al. (1990)

4.4.5.1 Peptides separated based on hydrophobic properties

The ability of the peptides separated by RP-HPLC, PPH and glutathione to chelate metal ions is shown in Figure 4.4.5.1. HF5, the fraction with the strongest net hydrophobicity possessed the strongest (9%) metal chelating activity of the fractions separated by RP-HPLC ($p<0.05$). HF3 and HF4 displayed the same metal chelating activity at 5% and 6%, respectively ($p>0.05$). HF1, HF2 and glutathione did not possess any metal chelating activity. The results indicate that HF3, HF4 and HF5 can disrupt the formation of the $Fe(II)Fz_3$ complex, suggesting that these particular fractions have chelating activity. Similarly, Xie et al. (2008) also observed negligible metal chelating activity of reduced glutathione, therefore indicating that the presence of cysteine in the tripeptide of glutathione is not important for the chelation of metal ions. The PPH chelated 95% of the metal ions indicating that it is a very effective iron chelator ($p<0.05$).

The strong activity displayed by the PPH could be combined or synergistic activities of HF3, HF4 and HF5 as the peptides displayed a synergistic activity in the chelation of metal ions. Subsequent fractionation of the PPH could have separated the metal chelating peptides into different fractions, thereby reducing activity of the fractionated peptides.

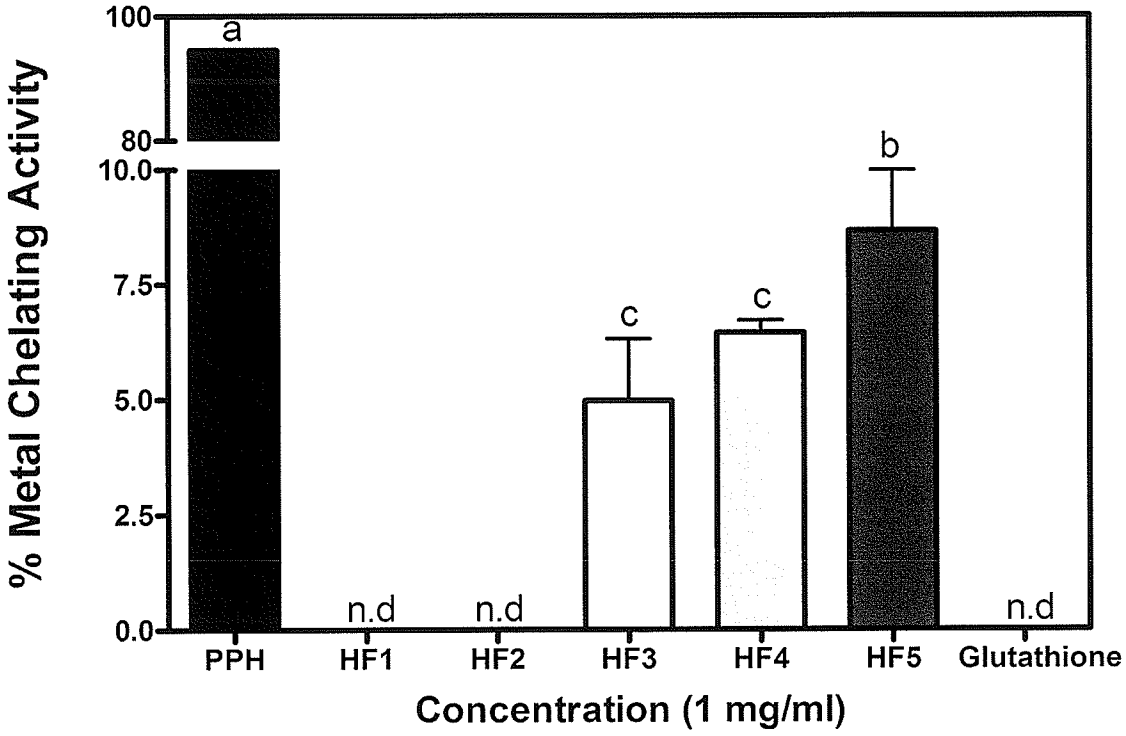
Hydrophobic fractions 3, 4 and 5 all contained higher concentrations of isoleucine, leucine, tyrosine, phenylalanine and tryptophan. HF5 in particular, had the highest percentage of proline, valine, isoleucine, leucine and phenylalanine in comparison to the other fractions. Therefore, the results suggest that the presence of aromatic rings in peptide fractions is a contributory factor to higher metal chelation activities and that the presence of a sulfur containing amino acid does not contribute to metal chelation. The correlation between the metal chelating activities of fractions separated based on hydrophobic properties and the total percentage of hydrophobic amino acids is 0.9498 (Figure 4.4.5.2).

In comparison to the metal chelating activity of hydrolysates derived from porcine collagen and porcine haemoglobin identified by Li et al., (2007) and Chang et al., (2007), the PPH used in the present study appears to have strong metal chelating activity at 95% at 1 mg/ml. Depending on the type of protease treatment of porcine collagen, the chelating activity ranged from 9.5% to 37% at a concentration of 11-13 mg/ml (Li et al., 2007). Two-step hydrolysis of porcine haemoglobin displayed increased metal chelation activity from 50-64% at 5 mg/ml (Chang et al., 2007). Hydrolysate from alfalfa leaf consisting of peptides <3 kDa exhibited strong (65%) metal chelating activities at 0.5 mg/ml (Xie et al., 2008).

Dong et al., (2008) observed hydrolysates from silver carp, produced by different enzymes had strong metal chelating activities reaching 93% at 5 mg/ml, dependent upon the type of enzyme and length of hydrolysis. The hydrolysate with the strongest metal chelating activity had higher concentrations of hydrophobic amino acids (Dong et al., 2008), which is similar to the present results.

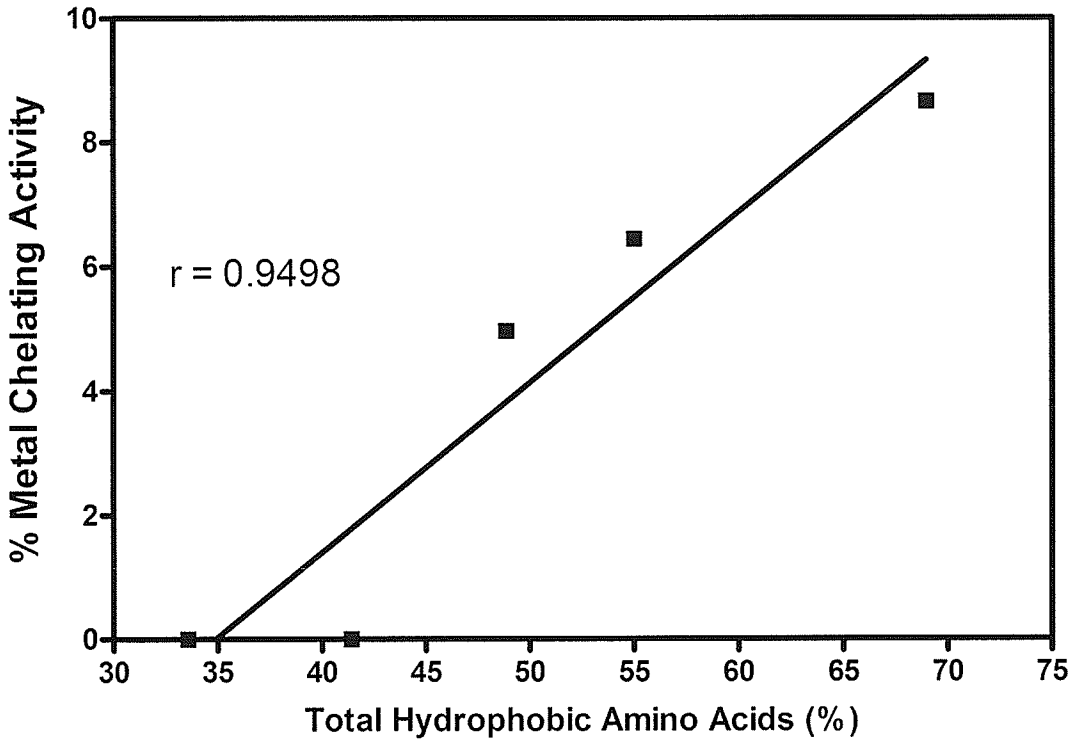
The results from the present study do not agree with the results from Megias et al., (2008) where peptides from sunflower upon affinity chromatography and subsequent RP-HPLC separation produced hydrophobic fractions with no metal chelating activity. However, the least hydrophobic fractions, rich in histidine displayed the strongest metal chelating activity (Megias et al., 2008). Megias et al. (2008) suggested that the imidazole ring of histidine is responsible for the metal-chelating activity. Interestingly, the fractions in the current study with the strongest net hydrophobicity in this study consisted of very small amount of histidine, which suggests that in addition to histidine, other amino acids such as tyrosine and phenylalanine may also be responsible for the metal chelating activity of peptides from pea protein.

Figure 4.4.5.1: Metal chelating activity (%) displayed by fractions (HF1-HF5) separated by hydrophobic properties, PPH and glutathione



n.d = no activity determined

Figure 4.4.5.2: Correlation between metal chelating activity (%) and the total hydrophobic amino acids (%) of fractions separated by hydrophobic properties



Total hydrophobic amino acids = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

4.4.5.2 Peptides separated based on cationic charge

Metal chelating activity of peptides separated based on cationic properties is displayed in Figure 4.4.5.3. Fractions separated by cation exchange did not display iron chelating activity. Similarly, glutathione did not display metal chelating activity. This

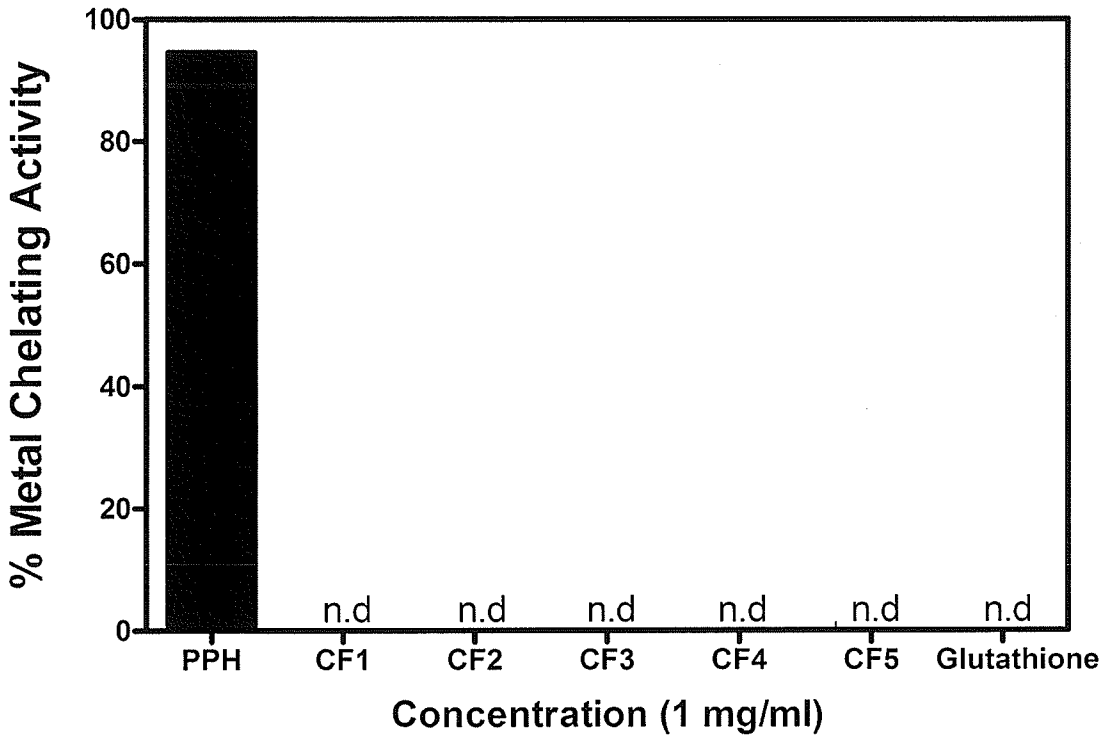
suggests that the peptides in the PPH act in synergy and their separation into charged fractions eliminated the metal chelating properties.

It was suggested by Dong et al., (2008) that an increased amount of peptide cleavages allows for binding to Fe^{2+} because of an increased availability of carboxylic groups and amino groups associated with anionic and cationic amino acids. Although hydrolysates from silver carp, produced by different enzymes had strong metal chelating activities reaching 93% at 5 mg/ml, dependent upon the enzyme and length of hydrolysis, there was equal distribution of charged amino acids between the hydrolysates (Dong et al., 2008).

However, Fe^{2+} is positively charged which would cause positive amino acids to be repelled but will attract mostly peptides that contain negatively charged amino acids. In the current study, the PPH was separated based on cation exchange, therefore leading to fractions that have a range of net cationic charges, with CF1 possessing the least cationic charge and CF5 possessing the most cationic charge. The reason that the cationic peptides do not have metal chelating activity could be because all fractions have some degree of cationic charge which would repel the positively charged Fe^{2+}. The present results confirm that some degree of negative charges are important structural characteristics necessary for metal chelation by peptides.

Separation of peptides based on hydrophobicity produced peptides with metal chelating activity. In comparison, peptides separated by cationic charge did not display metal chelating activity; therefore, separation based on hydrophobic is more effective in producing peptides with the ability to chelate metal ions.

Figure 4.4.5.3: Metal chelating activity (%) displayed by fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



n.d = no activity determined

4.4.6 Hydroxyl radical scavenging

4.4.6.1 Peptides separated based on hydrophobic properties

Figure 4.4.6.1 displays the hydroxyl radical scavenging activity of peptides separated by RP-HPLC, PPH and glutathione. PPH did not display hydroxyl radical scavenging, however, upon fractionation based on RP-HPLC, the hydroxyl radical scavenging significantly increased ($p<0.05$). Hydrophobic fraction 5, the fraction with the strongest net hydrophobic properties displayed the strongest activity inhibiting hydroxyl

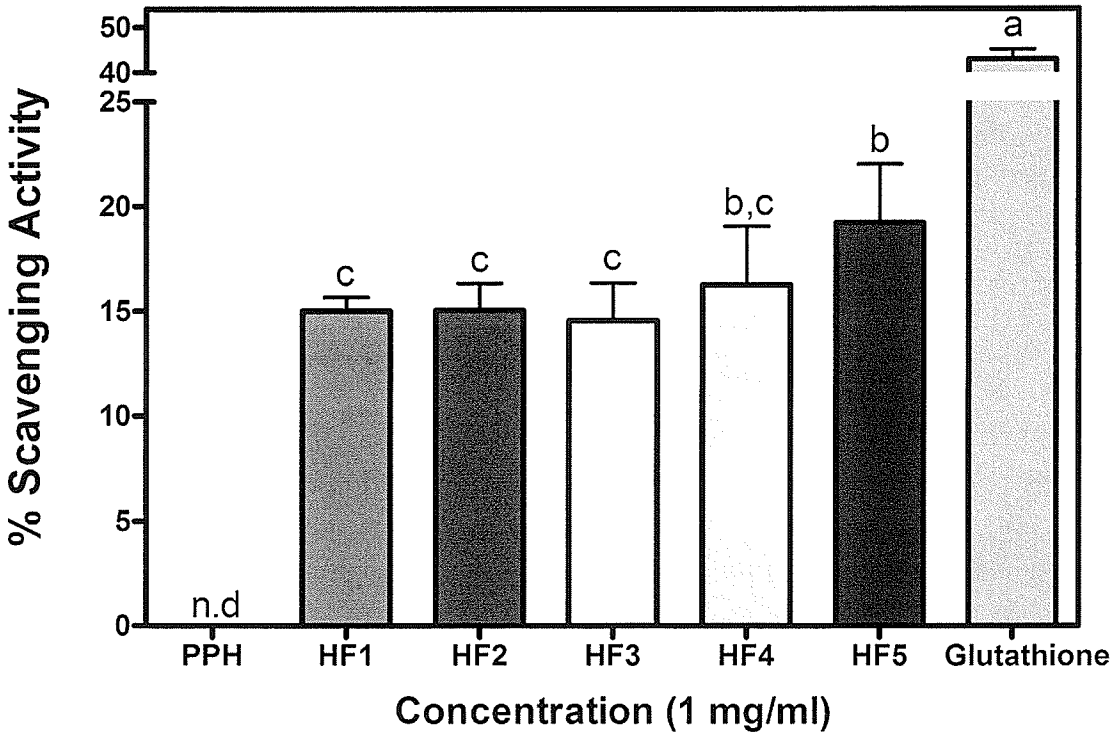
radicals at 17% in comparison to HF1, HF2 and HF3 (14.5%, 15% and 12.6%, respectively) with the least net hydrophobic properties ($p<0.05$). Fractionation of the PPH concentrated the active peptides, which led to increased potency of the active peptides. HF4 and HF5 were both rich in leucine and phenylalanine and also contained higher concentrations of valine and tryptophan in comparison to HF1, HF2 and HF3. The correlation of the fractions separated based on hydrophobic properties in relation to the hydroxyl radical scavenging activity and the total percent of hydrophobic amino acids is 0.8642 (Figure 4.4.6.2). Glutathione possessed the strongest activity at inhibiting hydroxyl radical scavenging at 46% ($p<0.05$) indicating that the presence of sulfur containing amino acids contribute to the hydroxyl radical scavenging.

Dong et al. (2008) observed that an Alcalase hydrolysate from silver carp possessed stronger hydroxyl radical scavenging activity in comparison to a Flavourzyme hydrolysate. The Alcalase hydrolysate ranged in scavenging activity from 35%-60% and the hydrophobic amino acid content of hydrolysates from Alcalase was higher than hydrolysates from Flavourzyme (Dong et al., 2008). In particular, the concentration of valine, methionine, isoleucine, tyrosine, phenylalanine and proline was higher in the Alcalase hydrolysate (Dong et al., 2008). The results published by Dong et al. (2008) are in agreement with the results in the present study as fractions with the strongest hydroxyl radical scavenging contained the highest concentrations of phenylalanine and valine, which could contribute to the hydroxyl radical scavenging. As well, a low molecular weight fraction from chickpea protein hydrolysate with strong hydroxyl radical scavenging activity was also observed to have higher concentrations of hydrophobic

amino acids including, phenylalanine, isoleucine, leucine and valine as well as methionine and lysine in comparison to other fractions evaluated (Li et al., 2008).

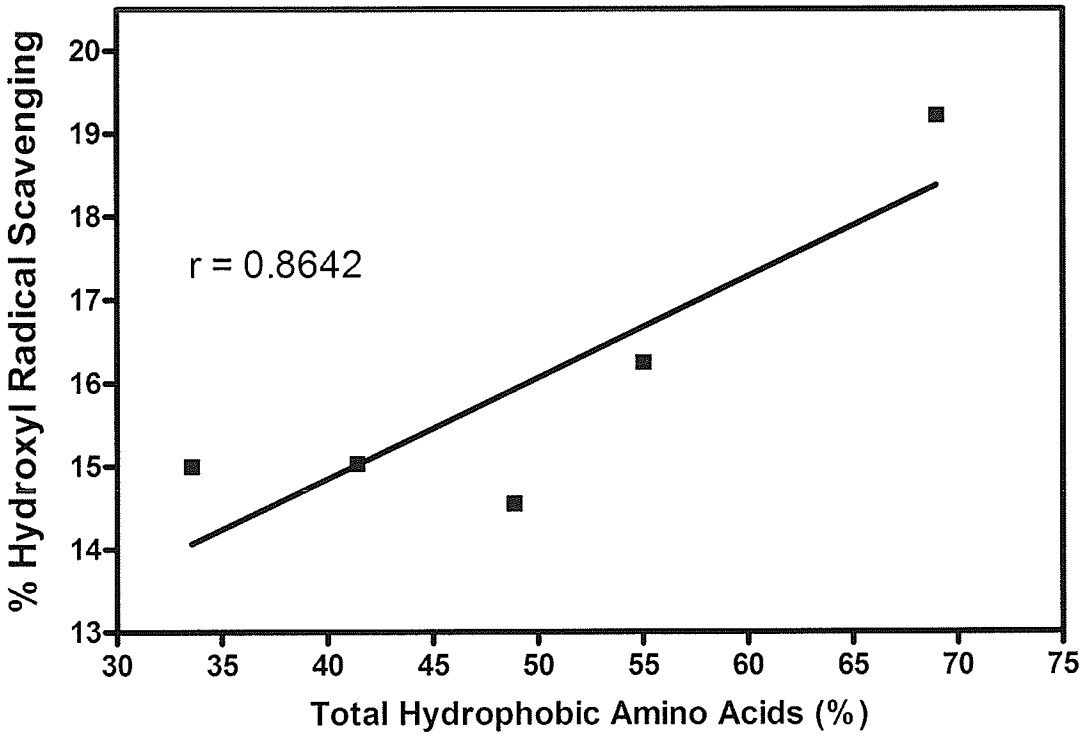
However, the findings of the present study and by Dong et al. (2008) are contrasted by the findings of Zhang et al. (2008). The peptide fraction from rapeseed that was particularly efficient at scavenging hydroxyl radicals contained lower concentrations of hydrophobic amino acids in comparison to a peptide fraction which had poor hydroxyl radical scavenging activity. The active fraction also contained a higher amount of hydrophilic amino acids, including asparagine, glutamate, methionine, lysine, histidine, glycine, alanine, serine and threonine (Zhang et al., 2007).

Figure 4.4.6.1: Hydroxyl radical scavenging activity (%) displayed by fractions (HF1-HF5) separated by hydrophobic properties, PPH and glutathione



n.d = no activity determined

Figure 4.4.6.2: Correlation between hydroxyl radical scavenging activity (%) and the total hydrophobic amino acids (%) of fractions separated by hydrophobic properties



Total hydrophobic amino acids = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine

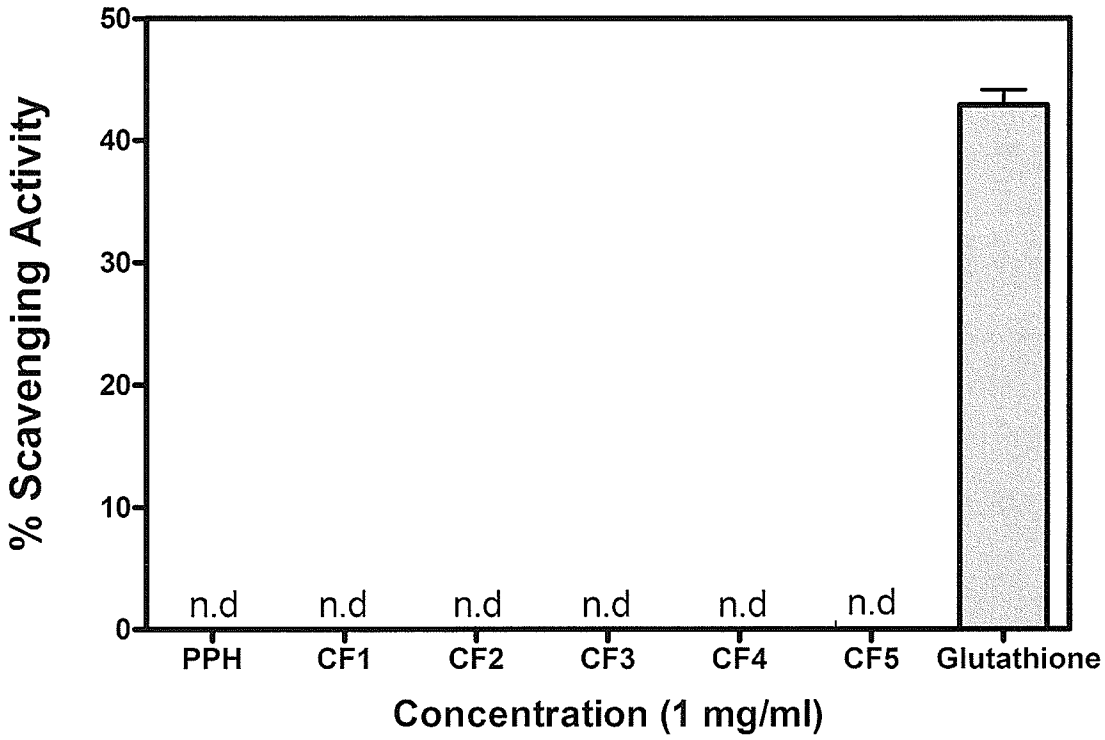
4.4.6.2 Peptides separated based on cationic charge

The hydroxyl radical scavenging activity of peptides separated by cation exchange chromatography, PPH and glutathione is shown in Figure 4.4.6.3. The fractions separated by cation exchange did not exhibit hydroxyl radical scavenging activity. In contrast, glutathione exhibited moderate hydroxyl radical scavenging activity at 46%.

Je and colleagues (2008) identified that tuna dark muscle hydrolysate produced by pepsin produced strong hydroxyl radical scavenging activity over 80% at 3 mg/ml. Upon further purification with anion exchange chromatography, the fraction with the strongest anionic charge displayed the strongest hydroxyl scavenging activity at 50%. However, in the present work, separation of pea protein hydrolysate by cation exchange chromatography did not increase the hydroxyl radical scavenging activity of the peptides. Therefore, the results suggest that anionic character of peptides is more important than cationic character with regards to ability to scavenge hydroxyl radical.

Overall, the separation of PPH by hydrophobic properties was more effective to produce peptides with hydroxyl radical scavenging activity, whereas peptides from cationic exchange were ineffective in the ability to scavenge the hydroxyl radical.

Figure 4.4.6.3: Hydroxyl radical scavenging activity (%) displayed by fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



n.d = no activity determined

4.4.7 Inhibition of linoleic acid oxidation

It has been speculated by a number of researchers that hydrophobic amino acids are strong antioxidants against lipid derived-radicals due to the ability of hydrophobic amino acids to interact with the lipids (Rajapakse et al., 2005; Zhang et al., 2008; Je et al., 2008).

The oxidation of ferrous iron to ferric iron by lipid oxidation products react with ammonium thiocyanate to form a complex of ferric thiocyanate, which results in an

increase in colour formation (Jayaprakasha et al., 2001). In the present study, the level of linoleic acid peroxides in the control, with no peptides increased rapidly and reached the highest concentration by the fourth day, which was similar to the observations of Chen et al. (2007) and Jayaprakasha et al. (2001) where the concentration of linoleic acid peroxides began to decline after the third and fourth day, respectively, of incubation. The rapid decline in absorbance observed in the control is due to the formation of peroxides that have decomposed and, therefore, a negligible amount of peroxides remain in the control solution (Jayaprakasha et al., 2001).

4.4.7.1 Peptides separated based on hydrophobic properties

Figure 4.4.7.1 displays the ability of the fractions separated by hydrophobic characteristics to inhibit linoleic acid oxidation. PPH and all fractions separated based on hydrophobic properties exhibited strong inhibition of linoleic acid oxidation over the duration 7 days. At day 1, HF3 and HF5 had the highest amount of linoleic acid oxidation ($p<0.05$). Hydrophobic fractions 1, 2, 4 and PPH had lower amounts of linoleic acid oxidation than fraction 3 ($p<0.05$), but the same activity as HF5 ($p>0.05$). By day 7, all fractions and the PPH displayed the same ability to inhibit the oxidation of linoleic acid ($p>0.05$).

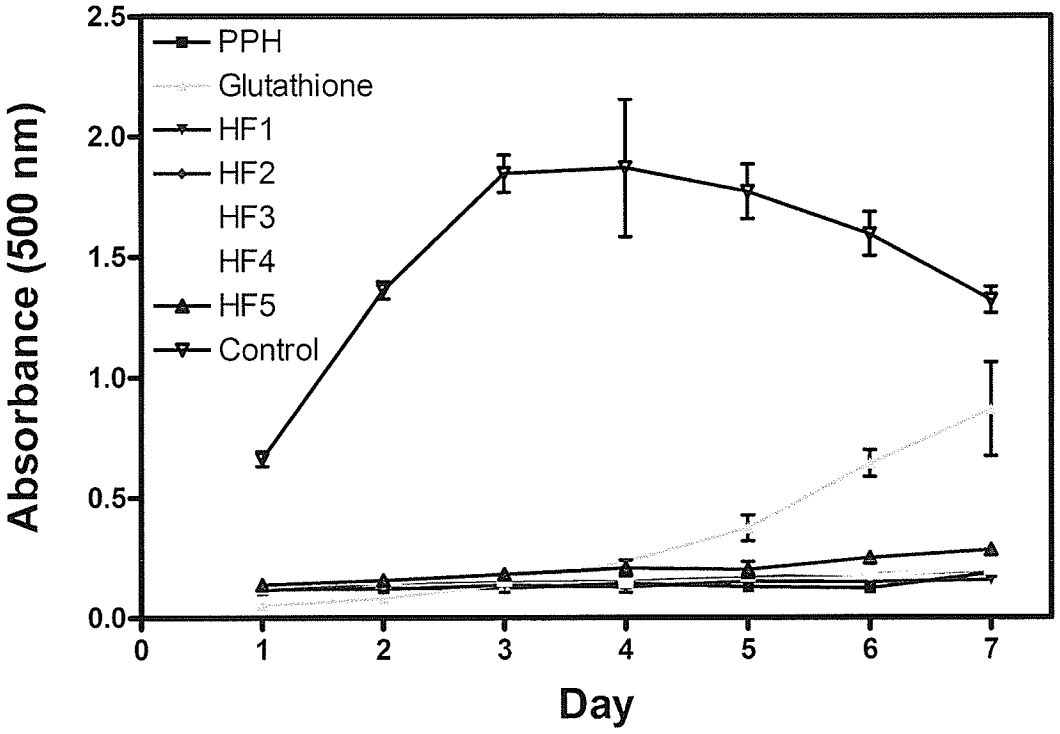
Glutathione initially displayed stronger activity than the pea peptide fractions ($p<0.05$). After day 2, the effectiveness of glutathione against oxidation began to decrease indicated by the increase in absorbance. Glutathione displayed equivalent activity to the peptide fractions and the PPH from day 3 to day 4 ($p>0.05$). From day 5 to day 7, glutathione was significantly less effective in inhibiting linoleic acid oxidation in

comparison to the peptide fractions and PPH ($p<0.05$), as indicated by the increase in absorbance. The decreased ability of glutathione to inhibit lipid oxidation in this test system for a prolonged period of time could be because once glutathione has been oxidized, it forms a disulfide bridge with another glutathione (Halliwell, 2006) and under these test conditions, it cannot be converted back into reduced glutathione and can therefore no longer act as an antioxidant.

Chen et al. (2007) found negligible inhibition of linoleic acid peroxidation at 2 mg/ml in peanut hydrolysates, but observed activity at higher concentrations. Li and colleagues (2008) identified a low molecular weight fraction from chickpea protein which contained a higher amount of hydrophobic amino acids to have the strongest activity in preventing linoleic acid oxidation. Zhang and colleagues (2008) also identified a fraction from rapeseed protein that was effective in inhibiting linoleic acid oxidation. The fraction contained a high amount of hydrophobic amino acids and the activity was in part attributed to the content of phenylalanine, tyrosine, tryptophan, methionine, cysteine, histidine and proline (Zhang et al., 2008).

Separation based on hydrophobic properties appears to be an effective method of producing peptide fractions with strong antioxidant activity against the oxidation of linoleic acid. Over a prolonged period, all fractions were equally effective at inhibiting linoleic acid oxidation.

Figure 4.4.7.1 Inhibition of linoleic acid oxidation by fractions (HF1-HF5) separated by hydrophobic properties, PPH and glutathione



Each data point is the average of triplicate determinations. All samples were measured at a final concentration of 1 mg/ml.

4.4.7.2 Peptides separated based on cationic charge

The inhibition of linoleic acid oxidation by fractions separated by cationic charge is displayed in Figure 4.4.7.2. At day 1, CF1 and CF5, the least net cationic and the most net cationic fractions, respectively, were least effective at inhibiting linoleic acid oxidation in comparison to the other fractions, PPH and glutathione ($p<0.05$). Cationic fractions 2, 3, 4 and glutathione have similar activities ($p>0.05$). By day 7, CF1 and CF5

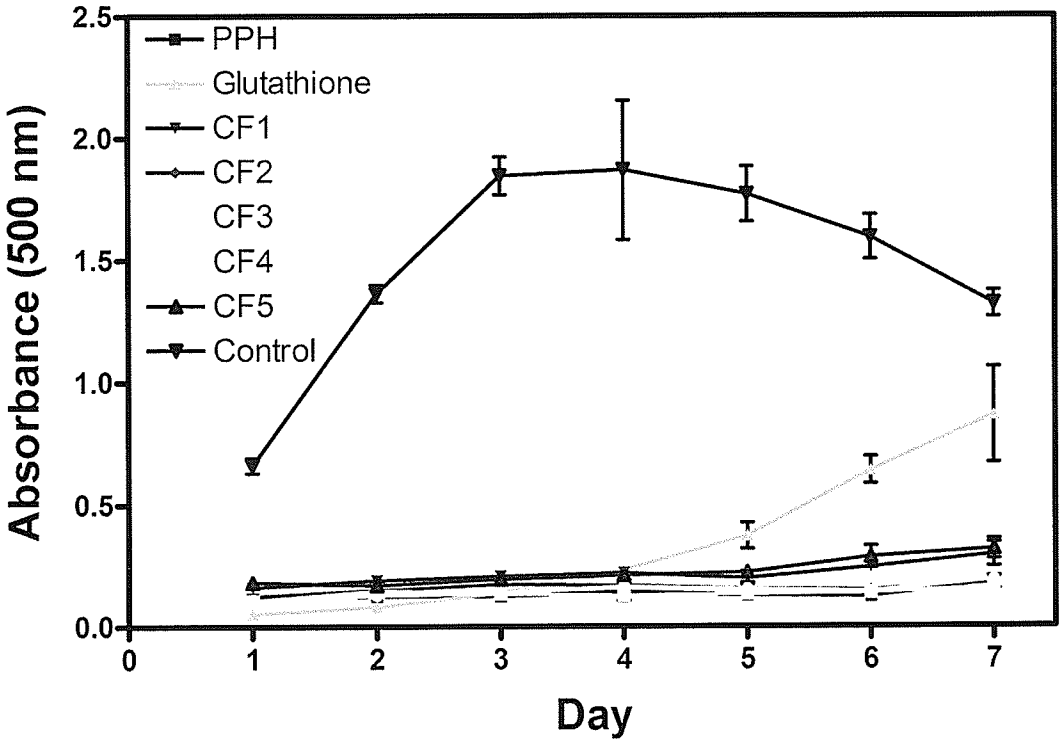
continued to display weaker activity in preventing linoleic acid oxidation. PPH and cationic fractions 2, 3 and 4 at day 7 continued to have stronger activity against linoleic acid oxidation in comparison to CF1 and CF5 and glutathione. Glutathione had poor activity with prolonged incubation period, weaker than the fractions and PPH. The weaker activity of glutathione could be in part due to the absence of compounds capable of reducing the oxidized glutathione in the reaction mixture.

In contrast, Park and colleagues (2008) identified that cationic peptides from soy protein and gluten protein possessed stronger inhibition of linoleic acid oxidation in comparison to anionic peptides. Rajapakse and colleagues (2005) also found that peptides with the strongest net cation charge separated by cation exchange had the strongest activity for inhibiting linoleic acid oxidation. However, Saiga et al. (2003) found that peptides with the least net cationic charge from porcine myofibrillar proteins separated by cation exchange had the stronger activity in protecting against linoleic acid oxidation in comparison to the fractions with the strongest net cationic charge.

It appears that charge does not play a significant role in the ability of peptides from pea protein to protect linoleic acid oxidation although all fractions displayed stronger activity for a prolonged duration in comparison to glutathione.

Overall, separation by hydrophobicity and charge both produced peptide fractions with the ability to effectively inhibit the oxidation of linoleic acid over a period of seven days. The fractions produced by both separation methods generally presented stronger antioxidant activity in comparison to glutathione.

Figure 4.4.7.2 Inhibition of linoleic acid oxidation by fractions (CF1-CF5) separated by cation exchange, PPH and glutathione



Each data point is the average of triplicate determinations. All samples were measured at a final concentration of 1 mg/ml.

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

This study determined the antioxidant activity of peptides derived from Canadian grown pea seeds on the basis of the net hydrophobic and net cationic properties of the peptides. We demonstrated that pea seed protein can be enzymatically hydrolyzed to produce peptides (< 3 kDa) with antioxidant activity. Seven antioxidant evaluation systems were used to determine the antioxidant mechanisms of the peptides including the ability to scavenge free radicals and the metal chelating activity.

Upon fractionation with RP-HPLC, peptides with the strongest net hydrophobic properties exhibited stronger antioxidant activities in comparison to the peptides with the least net hydrophobic properties. Peptides with the strongest net hydrophobic properties displayed scavenging activity and metal chelating ability and poor reducing ability. They contained a higher amount of total hydrophobic amino acids and in particular, contained valine, leucine, isoleucine, phenylalanine and proline.

Separation based on cationic charge produced peptides with a range of net cationic charges. Overall, the fractions with the least net cationic charges appear to be stronger antioxidants in comparison to the fractions with stronger net cationic charges. The former displayed stronger scavenging ability than the latter. However, the range of net cationic charges did not play a role in the ability to chelate metal ions as all fractions did not exhibit activity.

The present study identified that peptides derived from pea protein hydrolysate displayed antioxidant activities through different mechanisms including the chelation of metal ions and the ability to scavenge radicals. Both charge and hydrophobic properties

are important peptide characteristics in the determination of antioxidant activity. Separation of pea protein hydrolysate based on hydrophobic properties produced fractions with more robust antioxidant activities, therefore, separation based on hydrophobic properties produced fractions that are overall more effective antioxidants. The results indicate that pea seed protein hydrolysates could be used as potential ingredients to formulate functional foods and nutraceuticals which would target the population at a risk for higher oxidative stress. Pea seed is an important commodity to the economy of the Canadian Prairie Provinces. The use of the high protein crop in the functional foods and nutraceuticals market would also increase the value of pea seed and stimulate the Canadian economy.

CHAPTER SIX

FUTURE RESEARCH

Peptides from pea proteins have displayed antioxidant activity; therefore, future studies on the isolation and identification of individual peptides are required to fully elucidate the structure-function relationship. Further investigation on the antioxidant activity of the peptides using cellular models needs to be conducted to evaluate the antioxidant activity and the ability of pea peptides to be absorbed by intestinal cells. The absorption and transport of the peptides should be evaluated in cell culture models, primarily human intestinal epithelial cells, with peptides administered at physiological doses. It is also important to determine the bioavailability, stability and the ability of the peptides to reach target systems within the biological system, therefore animal models would be necessary to determine these factors.

Furthermore, the peptides fractionated by cationic charge and hydrophobic properties exhibited a strong ability to inhibit lipid oxidation over a prolonged period of time in comparison to glutathione, therefore, further studies need to evaluate the inhibition of lipid oxidation. To determine if pea protein hydrolysate peptides can improve the ability of glutathione to prevent lipid oxidation over a prolonged period of time, it is suggested that the peptides and glutathione be combined and the ability to prevent lipid oxidation over a prolonged period of time be monitored.

CHAPTER SEVEN

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