Antioxidant activity of oat bran hydrolyzed proteins in vitro and in vivo

by

Sara Jodayree

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Carleton University
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Abstract

Oats contain molecules with well-known health benefits. Its fibers are used in different preparations to improve gastro-intestinal health and to reduce blood cholesterol. Avenanthramides, a group of phenolic antioxidants only present in oats have been demonstrated to be bioavailable in animals and humans. In recent years, proteins from foods have been viewed as not just sources of essential amino acids but also as sources of biologically active peptides (e.g. antioxidant, antihypertensive, antitumor). Antioxidant activities have been reported for hydrolyzed proteins from wheat, rice and corn by-products but not for those from oats. The objective of this thesis was to investigate the antioxidant properties of hydrolyzed oat proteins in three different conditions (in vitro and in vivo). The first part of this thesis focusses on the activity of proteins extracted in the presence of salt and digested with trypsin (TPH) and alcalase (APH). The digests TPH and APH were ultra-filtered using 2kDa, and 10kDa molecular cutoff membranes. The radical scavenging properties were determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance assay (ORAC). It was found that APH 2kDa was the strongest inhibitor (35%) of DPPH radicals and also TPH had a better peroxy radical scavenging activity (ORAC) 434 ± 16 μmol trolox equivalents (TE)/g compared to APH (269 ± 4 μmol TE/g) and TPH >2kDa (345 ± 15 μmol TE/g). In the metal chelating assay, trypsin digest (TPH) better chelated Fe^{2+} ions compared to APH and other fractions. The inhibition of linoleic acid autoxidation, showed that both trypsin and alcalase digests equally lowered the formation of lipid peroxides after five days of incubation. It appeared that trypsin hydrolysate and its $\leq 2\text{kDa}$ fraction possessed the strongest activities and may have application in food products.

The second part of this thesis, demonstrated that pre-treatment of oat bran with four carbohydrases (viscozyme® L, alpha-amylase, amyloglucosidase, and celluclast®) at different concentrations resulted in greater protein extraction up to 82% compared to 54% for control bran not treated with carbohydrases. All protein isolates were hydrolyzed with alcalase and evaluated for antioxidant activities. All brans treated with carbohydrases had higher peroxyl radical scavenging activities compared to the control. The sequences of four potential active peptides were identified by tandem mass spectrometry.

The final part of this thesis research evaluated the potential of oat protein hydrolyzed (OPH) with trypsin (better in vitro antioxidant activity relative to alcalase hydrolysate) to reduce oxidative stress in vivo. Three different concentrations (1, 10, and 100 mg OPH/g diet) were added to the diet of CD-1 male mice. The animals were divided into five groups, normal diet (ND), high fat (HF), and HF containing 1, 10, and 100 mg OPH/g. The study lasted for seven weeks in total, four weeks on normal diet and three weeks on the experimental diet and after mice were sacrificed. Red blood cells, liver, brain, muscle, lung, and heart tissues were collected for analysis. Erythrocytes of mice on 100 mg OPH/g had higher ($p < 0.05$) peroxyl radical scavenging activity compared to HF group. The activity of the antioxidant enzyme superoxide dismutase (SOD) in the liver of mice on HF diet was 13.2% lower compared to the activity of those on the normal diet. Advanced oxidation protein products (AOPP) level in the brain and heart of animals supplemented with 10 mg OPH/g was lowered significantly compared to the rest of the groups. Supplementation with OPH increased ($p < 0.05$) vitamin C level but did not affect vitamin A or E concentrations in the liver. In conclusion, addition of OPH to high

fat diet reduced oxidative stress by either increasing the total antioxidative capacity of erythrocytes, reducing protein oxidation or nitric oxide depending on the organ. From these results, it appears that OPH can be beneficial in conditions associated with oxidative stress.

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List of Publications

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Abbreviations

AAPH	2, 2'-azobis (2-amidino-propane) dihydrochloride
AOPP	Advanced Oxidation Protein Products
APH	Alcalase protein hydrolysate
APH 2kDa	Alcalase protein hydrolysates 2 kDa
Bc	N-(3',4'-dihydroxycinnamoyl)-5-hydroxyanthranilic acid
Bf	N-(4'-hydroxy-3'-methoxycinnamoyl)-5-hydroxyanthranilic acid
Bp	N-(4'-hydroxycinnamoyl)-5-hydroxyanthranilic acid
CAT	Catalase
CD	Celiac disease
EGU	Endo-glucanase units
FOX	Ferrous oxide xylenol
HF	High fat
HPLC	High-performance liquid chromatography
HPH	High-pressure homogenization
MS	Mass spectrometry
NO	Nitrite + nitrate
ORAC	Oxygen radical absorbance capacity
PH	Protein hydrolysate
RNO	Reactive nitrogen species
ROS	Reactive oxygen species
RBC	Red Blood Cell
SOD	Superoxide dismutase

TPH	Trypsin protein hydrolysate
TE	Trolox Equivalents

Chapter 1

Background information

1.1 Introduction

Cereals such as wheat, corn, barley, and oat supply nearly one third of total protein in the human diet in United States and Canada. Proteins from grains have different nutritional properties, and in general, it has been found that those from oat (*Avena sativa*) have higher amino acid score or good nutritive value compared to the ones from other cereals (Jones et al. 1948; Hartwell 1926). Oats are also a good source of fibers, fat and minerals (Arendt and Zannini 2013b), Oat is a caryopsis, tightly covered by hull. Figure 1 shows the anatomy of the kernel caryopsis (Arendt and Zannini 2013b).

Figure 1: Anatomy of the oat kernel (Arendt and Zannini 2013b)

The hull constitutes 30-40% of the total weight, and is mainly composed of cellulose, hemicellulose (Welch 1995), and lesser amount of phenolic compounds and lignin (Arendt and Zannini 2013b). The caryopsis is composed of three main parts, 38-40% bran, 58-60% endosperm, and 3% germ. Bran is rich in vitamins, minerals, phytate, and phytochemicals. The endosperm is the primary storage site for proteins, starches, lipids and β -glucans. The oat germ, from the chemical point of view, contains high levels of protein, and lipids and little amount of starch. The protein bodies contain phytin globoids, small amounts of calcium magnesium, manganese, and iron (Arendt and Zannini 2013a; Arendt and Zannini 2013b) . Figure 2 summarizes the composition of the oat caryopsis.

Figure 2: Composition of oat caryopsis

1.2 Chemical compounds in oats

1.2.1 Polysaccharides or carbohydrates

The main groups of molecules in oats are carbohydrates. They are composed of 40-50% starch, dietary fibers, and β -glucans. Starches are made of amylose and amylopectin while fibers can be divided in two sub-groups based on water solubility. Water-soluble fibers comprise non-starch polysaccharides, gums, pectin, some hemicellulose, arabinoxylan, and β -glucans. Insoluble fibers contain lignin, and cellulose and hemicellulose. Figure 3 summarizes these various major classes of oat carbohydrates.

Figure 3: Division of oat carbohydrate composition.

β -glucans, major group of soluble fibers in oats and other cereals are composed of 70% 1-4 and 30% 1-3 β -D-glucopyranosyl units (Lazaridou and Biliaderis 2007; Arendt and Zannini 2013b)

Figure 4.

Figure 4: Primary structure of mixed linkage of (1→3, 1→4)- β -D-glucans (Nielsen et al. 2008)

1.2.2 Phenol compounds in oats

Phenols possess one or more hydroxylated aromatic ring that contribute to their function. They are widely distributed in the plants and are biosynthesized from a precursor called L-phenylalanine. Series of enzymatic reactions lead to generation of phytochemicals with one or more phenolic rings. Based on the structures and number of phenolic rings these phytochemicals can be classified simple such as phenolic acids (contain one phenolic ring), or polyphenols (two phenolic rings) and tannins (complex phenolic derivatives). Many different phenolic derivatives have been found in oats. The vast majority of polyphenols are located in the outer layers of oat kernel. They are present in free (simple), polymeric and conjugated forms. Phenolic acid are divided into four main different classes: phenolic acids, flavonoids, stilbenes and lignans. Flavonoids are the most studied subgroup and are divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones. Phenolic acids are further divided

in to subclasses, benzoic acid and cinnamic acid. In oat 60-80% of phenols are conjugated with polysaccharides, proteins or lipids. Different groups of polyphenols listed below have been identified in oat.

- Flavonoids: Three major flavones identified in oat flour are apigenin, luteolin, and triclin. Glycosidic derivatives such as 6-C and 8-C-glucoside of apigenin, 3-O-rutinosides of quercetin and kaempferol were identified (figure 5) (Peterson 2001).

Figure 5: Chemical structure of flavonoids identified in oat (David M. Peterson 2001).

- Simple phenolic acids: they are commonly derivative of Cinnamic acids are, Cinnamic acids include *p*-coumaric acid, Caffeic acid, Ferulic acid, Sinapic acid (figure 6) (Bratt et al. 2003).

Figure 6: Chemical structures of cinnamic acid derivatives found in the oats (Bratt et al. 2003)

- Avenanthramides: This is unique group of amides or nitrogen containing constituents found in oats, and they contribute to its the antioxidant activity, Figure 7 shows the chemical structure of the avenanthramides found in oats (Bratt et al. 2003).

Avenanthramides

Figure 7: Structure of avenanthramides, a unique group of polyphenols found in oat (Bratt et al. 2003).

Tocopherols are class of organic compounds that have vitamin E activity. The generic name for the eight tocols is vitamin E, E-vitamins is used to describe them collectively. α -tocopherol, β -tocopherol, gamma-tocopherol delta-tocopherol (Figure 8a) and α -tocotrienol, β -tocotrienol, gamma-tocotrienol, delta-tocotrienol (Figure 8b). They have a similar structure, however, they differ in number and location of methyl groups on the ring structure (Indel et al. 2010). The main tocol found in oat is α -tocotrienol and smaller amount α -tocopherol (Peterson 2001). The germ is source of tocopherols and endosperm is source of tocotrienols.

Figure 8: Structure of tocols. (a) α -tocopherol; (b) and α -tocotrienol (David M *et al.*, 2001).

1.2.3 Proteins

Proteins are made of small subunits called amino acids that are linked together by a covalent bond called peptide bonds (Bernhard 1968). Peptides are classified based on the number of amino acid units in the chain, each unit is amino acid residue. For example when two amino acids are linked together they are called dipeptides, if three amino acids are linked together they are called tri-peptide, four amino acids linked together are called tetra-peptides, 12 amino acids, less than 20 linked together are referred as oligopeptides, and when the chain exceeds several dozen amino acids they are called polypeptide. Proteins are composed of one or more polypeptide chains. Proteins with one polypeptide chain are called monomeric proteins, and

proteins composed of more than one polypeptide is called multimeric proteins. The term homomultimeric is used when the protein is made of one type of polypeptide, and the term heteromultimeric is used when the protein is made of several different polypeptides. Typically, polypeptide chains are made of 100-2000 amino acids.

Oats contain 15-20% of proteins by weight. The embryo contains the higher amount of proteins (over 30%) followed by bran (20%), endosperm (10%) and the hulls (>2%) (Youngs 1972). Globulins are the major oat storage proteins, and are linked with higher nutritional value of oat proteins since they are higher in lysine compared to globulins from other cereals. Prolamins are other storage proteins that are present in higher levels in cereals such as rye (40-50%) and wheat (30-50%), however they only accounted for 10-15% of proteins in oats. Chemical analyses of oat proteins have shown that their amino acid balance is superior to other cereals. Lysine content of oat protein is high (4.2%) however it is below the recommended level of 4.5% (FAO/WHO/UNU 2007). In most of the other cereals, increase of proteins content is relative to increase in prolamins (low in lysine) content, therefore the decline in quality of their proteins. However, in oats increase of the protein is relative to increase of globulin fraction, leading to higher quality. Seed proteins are classified into four different types based on their solubility, globulin, prolamins, albumins, and glutelins (figure 9). Oat storage proteins are made of globulins and prolamins. Usually globulins represent 75% of total seed proteins. Globulins can be identified with three different sedimentation coefficients (3S, 7S, and 12 S). The presence of specific proteins rich in proline and glutamine in other cereal proteins has been described as main triggering in celiac disease (CD), and since oat does not contain them, they are often included in CD diet (Arendt and Zannini 2013a; Arendt and Zannini 2013b).

Figure 9: Classification of main types of seed proteins based on solubility

1.2.4 Other chemicals

Oats contain lipids, vitamins and minerals. Lipids are an excellent source of energy and some unsaturated fatty acids are essential to human nutrition. Minerals in oats are phosphorous, potassium, calcium, magnesium, iron, copper and manganese. Vitamins such as thiamin and pantothenic acid are present in high amounts. Riboflavin and folic acid are also present (Welch 2005). Oats also contain other components such as sterols, saponins and phytic acids (Ryan et al. 2007; Arendt and Zannini 2013b).

1.3 Bioactivity of oat compounds

Consumption of oats and other grains have been associated with reduce risks of chronic diseases like cardiovascular diseases, diabetes, obesity and certain forms of cancer. The observed

beneficial effect can be attributed to individual compounds as well as to the synergetic effect of several molecules.

1.3.1 Activity of dietary fibers

Fibers are non-digestible carbohydrates, meaning resistant to hydrolysis by human digestive enzymes but are fermented by bacteria in the colon. Oat bran is commonly used in breakfast cereals due to its high content of glucans. Consumption of oatmeal and oat bran in glucans have been demonstrated to lower LDL cholesterol. Oat is known to reduce risk of cardiovascular disease and lower cholesterol (Welch 1995). Cholesterol lowering property is due to presence of high amount of soluble fibers (β -glucans) which are considered anti-atherogenic but, can also enhance immune system and protect against infection (Qureshi et al. 1986). The hypocholesterolemic mechanism of fibers is linked to cholesterol absorption, influence on bile acid absorption, production of lipoproteins in liver and removal of lipoproteins in peripheral tissues (Anderson and Gustafson N 1988). Dietary fibers increase the viscosity of the contents in the intestine, leading to slower absorption of cholesterol, another hypothesis is that soluble fibers are fermented in the colon, and produce short chain fatty acids, and these acids are absorbed from the colon, one of these acids is propionate, and this acid inhibits cholesterol synthesis. One more hypothesis is that fibers from oat increase short chain fatty acids, inhibiting the cholesterol synthesis in the peripheral tissues, resulting in lower low density lipoprotein (LDL) receptors, which could increase the rate of LDL clearance (Anderson and Gustafson N 1988).

Many studies have investigated the effect of dietary fibers on the body weight of human and animal models. It has been shown that there is an inverse relationship between consumption of dietary fibers and body weight. A study with 89, 432 healthy European participant, free from

cancer, cardio vascular diseases, diabetes age 20-78 years old, was followed for 6.5 years, showed that there was an inverse relationship between the intake of dietary fibers and the body weight as well as the waist circumference. This study found that higher intake of fibers from cereal and grains was associated with lower body weight and lower waist circumference. However intake of fibers from fruits and vegetable was not associated with body weight change, but the effect on waist circumference was similar to the effect of cereal dietary fibers (Du et al. 2010). In a study where diet of 252 women was measured using 7 day weighted food records, dietary fiber intakes were demonstrated to reduce the body weight and fat independent of physical activity and dietary fat intake (Tucker and Thomas 2009). A study that investigated the body weight and consumption of the whole grain and refined grain of 74, 091 US healthy female nurses free from cancer, cardio vascular disease, age 38-63 year old in years 1984, 1986, 1990, and 1994 through validated food frequency questionnaires concluded that consumption of whole grain dietary fiber is inversely associated with body weight gain (Liu et al. 2003).

Fibers antitumor activity has been attributed in some studies to the presence of phenolic molecules like phytate, and isoflavones present in fiber samples (Cohen 1999). Colorectal cancer is one of the conditions where dietary fibers have been suggested to play a positive role in its prevention. The outer brain layer of these grains contain phenolic polymer such as lignin, which reduces the digestibility of polysaccharides by the digestive enzymes in the colon, making the fecal bulky and making the transit time lower. As well, lignin makes the cell walls hydrophobic; this property increases the cell ability to absorb carcinogens (Harris and Ferguson 2014).

Soluble fibers also slow the increase of blood glucose, which is important in the management of type II diabetes. β -Glucans are also well known for their positive effects on

insulin (Wood 2007). The ingestion of oat products decreases the glycemic index, the insulin response in both diabetics and healthy individuals (Jenkins et al. 1981; Heaton et al. 1988). A study conducted using the soluble fiber psyllium on 12 men, mean age 67.5 year and 8 women mean age 66 year who had type 2 diabetes reported a decreased in glucose absorption, as well as decreased levels of LDL cholesterol, and uric acid in the treatment group and had no effect on the absorption of minerals and vitamins A, E (Sierra et al. 2002). Fiber had been demonstrated to improve blood glucose in both overweight and normal weight women (Behall et al. 2006). Fibers enriched biscuits have been shown to reduce blood glucose, increase serum insulin, and increase high-density lipoprotein cholesterol levels in albino rats of Wister strain that were on the fibers enriched biscuits treatment for two weeks (Erukainure et al. 2013)(Erukainure et al. 2013)(Erukainure et al. 2013)(Erukainure et al. 2013)(Erukainure et al. 2013)(Erukainure et al. 2013)(Erukainure et al. 2013)

1.3.2 Activity of phenolic compounds

Polyphenols are bioactive molecules and are believed to exert their effect mostly through their antioxidant properties. Antioxidant compounds protect the plants cells against free radicals; this protective effect is transferred to human when it is consumed (Pandey and Rizvi 2009). Their addition of antioxidants to foods is common to increase the shelf life through inhibition of polyunsaturated fatty acids oxidation (reduce nutritional value). In vivo antioxidants protect cell membranes from oxidative stress.

Tocopherols, group of lipophilic antioxidant with similar structures but slightly different biological and antioxidant activity (Brindzová et al. 2008). Alpha-tocopherol (Vitamin E) is the most active form. It is fat soluble antioxidant and protects fatty acids in adipose tissue and cell

membrane from oxidation. It also inhibits oxidation of blood lipid; oxidative stress in lungs, skin, eyes, liver, and other organs (Indel et al. 2010). Ferulic acid is one of the major simple phenol in oats. This acid is has shown to have radical scavenging activity that increases with esterification (Kikuzaki et al. 2002). Phytic acid can reduce cell proliferation by increasing the activity of the natural killer cells (Reddy 1999). An illustration of a free radical reacting with phenolic compound is shown in figure 10. Phenols easily oxidize therefore they prevent oxidation of other compounds. The two hydroxyl radical on phenol has a tendency to react with free radical oxygen, to form a semiquinone, then a quinone, and at the end the free radicals are scavenge.

Figure 10: Resonance structures of a phenol after proton donation to a free radical

Avenanthramides are alkaloids that occur only in oats. They are low in molecular weight; phenolic compounds found in the bran at concentrations of about 300 ppm. The three most abundant avenanthramides, are N-(4'-hydroxy-3'-methoxycinnamoyl)-5-hydroxyanthranilic acid (Bf), N-(4'-hydroxycinnamoyl)-5-hydroxyanthranilic acid (Bp), and N-(3',4'-

dihydroxycinnamoyl)-5-hydroxyanthranilic acid (Bc). Their antioxidant capacity has been studied in β -carotene system as well as DPPH system and all three have potent activities. Bc had the higher antioxidant capacity compared to the other two avenanthramides. In addition, Bc and Bf were more active than 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Peterson et al. 2002). A study conducted on human adults who consumed skimmed milk supplemented with avenanthramides 0.5-1 g showed that avenanthramides were bioavailable and increase plasma antioxidant capacity (Chen et al. 2007). In hamsters, avenanthramides act synergistically with vitamin C to protect against LDL oxidation (Chen et al. 2004a). Other than antioxidant activity, they have shown to have anti-inflammatory activity as well (Liu et al. 2004), and inhibited the secretion of proinflammatory cytokines interleukins IL-6 and IL-8 in endothelial cells (Guo et al. 2008). Anti-atherosclerotic activity of avenanthramides has been also reported (Chen et al. 2007).

Bioavailability of each polyphenol is different. Some are absorbed in the intestine and some are absorbed in gastro-intestinal, or other parts of digestive tracts. For example, Flavonoids are absorbed by the small intestine, anthocyanins are absorbed from the stomach (Passamonti et al. 2005). Studies have shown that polyphenols can penetrate in to tissue. Accumulation of polyphenols in the tissue is important because of the biological activity of polyphenols, health benefits of polyphenols depends on both intake and bioavailability.

1.3.3 Activity of proteins/peptides

Protein isolate and protein concentrate has been attracted interest because of their functional properties, such as antioxidant, antihypertensive, and anti-obesity properties. Few studies have investigated the functionality of oat proteins. Only one study investigated the antihypertensive

properties of hydrolysed oat proteins prior to my study (Cheung et al. 2009). Many studies conducted on hydrolysed proteins from other food have been reported to possess various functions. In vitro, cell culture and animal experiments have demonstrated antioxidative (Cheng et al. 2014), cholesterol lowering (Cai et al. 2014) anti-proliferative (Parrado et al. 2006) , and hepatoprotective (Yu et al. 2012) for hydrolyzed proteins from wheat, rice and corn. Proteins extracted from soybean, and treated with proteinase, Alcalase, trypsin, or chymotrypsin resulted in low molecular weight peptides such as Leucine-Leucine-Proline-Histidine-Histidine or Leu-Leu-Pro-His-His, which possessed both antioxidant and antihypertensive effect (Chen et al. 1996; Wu and Ding 2002). Antioxidant peptides have also been obtained from chickpea, white beans, and milk proteins.

The antioxidant activity of proteins depends on the amino acid constitute, for example amino acids that are aromatic donate protons to free radicals, few examples of such amino acids are tyrosine, phenylalanine, and tryptophan as well as sulfur containing amino acids like cysteine. Glutamate, and aspartate are acidic amino acids, and lysine, arginine are basic amino acids. These amino acids scavenge free radicals by metal chelating ions. Histidine amino acid has imidazole ring, and because of this ring, histidine amino acid has metal chelating and free radical scavenging activity. The positioning of amino acids in the protein has an important role in the protein antioxidant activity. Each peptide has different antioxidant activity depending on the proline at the N-terminus as well as C- terminus. Proline at the N-terminus is better in preventing oxidation of linoleic acid compared to the proline at the C-terminus. Peptides that have histidine at the N-terminus are better metal chelating compared to peptides having histidine at the C-terminus. Hydrophobicity has been reported to have a close relationship with antioxidant

activity of peptides as well as proteins, this has been related to the ability to bind and hide unsaturated lipids. Cationic properties of proteins also is related to its antioxidant activity, since lipid oxidation is inhibited by electrostatic repulsion of transition metals away from lipid droplets by the positively charged groups. Other than antioxidant activity, proteins have other functions such as emulsifying activity, for example protein isolate and whey protein are known to have emulsifying activity. Lactoferrin and phosvitin are examples of protein that possess both antioxidant activity as well as anti-microbial activity. Gel formation, flavour binding as well as increase of viscosity are additional functions of proteins. It has been shown that functional and nutritional properties of proteins isolate as well as protein concentrate has been improved by enzymatic hydrolysis.

1.4 Methods for extraction of proteins

Different methods of protein and peptide extractions exist based on the solubility, hydrophobicity, molecular weight, and isoelectric point (IP). They are used to remove interfering compounds such as lipids, nucleic acids, carbohydrates, and pigments. Since proteins are inside the proteins bodies in the cell wall, the first step is to break or disrupt cell walls before extraction and solubilisation.

1.4.1 Cell wall disruption

Mechanical techniques or blenders are commonly used to break the cell wall without denaturing the proteins. Mechanical homogenization and ultra-sonication have been used for grains (rice), dry legumes (lentils, chickpeas)(Boye et al. 2010). High-pressure homogenization (HPH) also has been shown to produce double the amount of proteins compared to atmospheric pressure. HPH can also increase the susceptibility of proteins to enzymes such as alcalase (Barbin et al.

2011). Temperature treatment also in another method used for disruption of cell wall plant, using liquid nitrogen to flash freeze the sample and then homogenizing with a pestle, this method is usually used for tomato and olive but it is not suitable for cereals.

1.4.2 Solubilisation of proteins

The first step is generally to remove fat. Hexane the solvent most commonly used to achieve this (Tzeng et al. 1990; Wu and Muir 2008), however diethyl ether, acetone, 2-propanol, ethanol and 2-methyl pentane have been used (Bader et al. 2011). Defatted samples are then dried at room temperature or under vacuum in an oven prior to solubilisation of proteins. Organic solvents, aqueous solutions, aqueous enzymatic extraction, or subcritical water are used for solubilisation to extract proteins from different food sources. Aqueous alcohols such as ethanol, butanol, or isopropyl alcohol have been used to remove phenolic and oligosaccharides. However, when the functionality of proteins is important, the use of organic solvents should be avoided as they will denature proteins, resulting in loss/reduced functionality. Aqueous solutions are preferred due to their nontoxicity, non-flammability, and non-denaturing properties.

1.4.2.1 Use of salts

The pH and ionic strength (i.e. salt concentration) provide electrostatic repulsion factors that can increase protein extractions (Siong et al. 2011). The use of sodium and calcium salts to extract proteins from cereals and other foods is documented in the literature (Ghaly and Alkoaik 2010; Lestari et al. 2010; Karaca et al. 2011; Nadal et al. 2011). These salts are easily available but the yield of the extracted proteins is generally low due to racemization of amino acids and formation of toxic compounds like lysinoalanine (Sereewatthanawut et al. 2008). The consequence is loss of essential amino acids and decrease of nutritive value. Salt solutions containing the proteins

are often ultra-filtered to purify and concentrate the protein. Then the retentate is diluted with water followed by precipitation of proteins through centrifugation. Compared to alkaline extraction there are less literature for salting method, this could be due to lower protein yield.

1.4.2.2 Alkaline treatment

One of the common ways to extract proteins isolates is alkaline extraction with sodium hydroxide (NaOH) solution, and precipitating it with dilute acid (Aluko and McIntosh 2001; Klockeman et al. 1997). Generally, the defatted sample is stirred or shaken in sodium hydroxide to solubilize the protein, then the sample is centrifuged and the supernatant is adjusted to a specific pH to precipitate the proteins, and again the sample is centrifuged and protein is separated and freeze-dried. Concentrations of the sodium hydroxide, and the pH, that the sample is being adjusted to precipitate the protein varies. It has been found that pH range, 4.5-5.5 is ideal for protein precipitation (Ghodsvali et al. 2005). For different concentrations of NaOH, different pH is needed to precipitate the protein, for example, using 0.1M NaOH, the pH of the supernatant should be adjusted to 4.0 by 0.1 M HCl (Aluko and McIntosh 2001; Aluko et al. 2005), or if using 0.4% NaOH, the pH is adjusted to 3.5 by acetic acid (Klockeman et al. 1997).

1.4.2.3 Pre-treatment with carbohydrases

There are more than 1500 known enzymes and only few are important to food industry and in human nutrition (Bernhard 1968). Cereals are rich source of starch, dietary fibers and other complex polysaccharides. Some of these are linked to proteins making their extraction difficult.

Using enzyme that can break cell wall polysaccharides can enhance proteins extraction in cereals. Carbohydrases such as viscozyme L was used increase protein extraction yield in oat bran from 14.8% to 56.2% (Guan et al. 2007). In another study, treatment of rice brans with viscozyme L, and cellulase also significantly increased protein contents (Ansharullah et al. 1997). Therefore the use of other cell wall degrading polysaccharides will increase protein extraction from oat brans and their bioactivities.

1.5 Protein hydrolysates

Proteases are used to break down proteins into specific peptides and can be evaluated for biological activities in various assays. There are few classes of proteases such as trypsin, chymotrypsin, plasmin, elastase, thrombin, subtilisin, tissue plasminogen activator. It has been demonstrated that in vitro digestion can increase the antioxidant capacity of cereal products. This is because the process can release different molecules than the human gastro-intestinal tract or make increases the solubility of proteins and other complex molecules (Liyana-Pathirana and Shahidi 2006). Gastro-intestinal enzymes such as pepsin, pancreatin, and trypsin are often used. There is a large literature data on the use of alcalase, papain, ficin, proteinase K, or protamex (Ansharullah et al. 1997; Grossman et al. 1980). In certain cases, in vitro digestion under simulated gastrointestinal tract conditions have been used to obtain new bioactive peptides (Pérez-Jiménez and Saura-Calixto 2005). The increase in antioxidant activity of whole cereal grains under in vitro gastrointestinal conditions was explained by higher levels of both hydrolysable phenolic and proteins (Fardet et al. 2008). Antioxidant activity, functional and nutritional properties of hydrolyzed proteins from beans and chickpeas (Arcan and Yemenicioğlu 2007), soy (Chen et al. 1998), and milk (Satué-Gracia et al. 2000) were greatly increased

compared to intact proteins. Protein hydrolysates may have applications in pharmaceuticals, human and animal nutrition (Bueno-Solano et al. 2009).

1.6 Antioxidant peptides, structure and function

The average molecular weights of peptides are generally less than 10kDa while those of proteins are higher. Peptides shorter than 20 amino acids are the ones that often possess physiological benefits. The bioactive peptides are encrypted in protein polypeptide chains and can be released by fermentation with micro-organisms, chemical or enzymatic hydrolyses as described in section 1.5. Upon released, peptides can now exert their physiological function like reduction of blood pressure (Li et al. 2011), neutralization or scavenging of reactive oxygen or nitrogen species, lowering of blood lipids (Jung et al. 2010) or cholesterol (Marques et al. 2015). Peptides can also increase absorption of calcium and other minerals, inhibit the growth of bacteria. Some peptides contain more than one of these activities and are then designated as multifunctional. Mechanistic studies have demonstrated that peptides with antioxidant activities should have one of these three basic structural features (Aluko 2012); 1) the presence of electron donor groups or aromatic ring that can possess several equilibrium forms after donating a proton. The excess electrons on amino acid side chains can stabilize free radicals and the aromatic ring equilibrium formed prevent the oxidised peptide to become a reactive species; 2) the possession of hydrophobic property, which enhances the translocation of active molecules through the lipid cell membrane into the cytoplasm and mitochondria where free radicals are generated; 3) the ability to chelate transition metals or reduce ferric ions (Aluko 2012).

Protein hydrolysates and peptides with some of these features have been produced from foods such as soy, pea, fish, milk casein, whey, and egg. A number of them possess antioxidant

properties (Irshad et al. 2015; Chen et al. 1996; Sakanaka et al. 2004; Davalos et al. 2004; Xu et al. 2007; Aoyama et al. 2000). They have been demonstrated, to scavenge or quench free radicals; inhibit oxidization of macromolecules such as lipids, DNA, and proteins and therefore, have application in the reduction of many chronic diseases associated with oxidative stress (Aluko 2012).

In addition to features mentioned above, the amino acid (AA) composition or the position of AA on the sequence has an influence on the antioxidant activity of peptides (Udenigwe and Aluko 2011). Some of the important AAs are histidine, cysteine, proline, methionine, tyrosine, tryptophan and phenylalanine. The presence of imidazole group in histidine and indole in tryptophan can increase their metal chelating activities, and quenching of superoxide anion and hydroxyl radicals (Grune et al. 2013). This is because both residues can participate in hydrogen atom transfer and single electron reactions resulting in neutralizing the free radicals. The addition of hydrophobic amino acids such as proline, and leucine to the N-terminus of the dipeptide His-His resulted in increased antioxidant activity (Aluko 2012). Electron dense aromatic rings in phenylalanine and tyrosine contribute to their metal chelating property; they can also scavenge hydroxyl radicals thereby forming stable hydroxylated derivatives (Remko et al. 2011).

Sulfhydryl (-SH) group present in cysteine has proton donating ability while sulfur in methionine can be oxidised (instead of biological molecules) to form stable sulfoxides (Cheng et al. 2011).

Acidic amino acids such as glutamic acid and aspartic acid have electron donating ability that contribute to the DPPH scavenging activity, H_2O_2 scavenging and ferric reducing of some food protein hydrolysates (Ajibola et al. 2011). Some peptides containing AA like lysine and leucine have poor hydroxyl radical scavenging or metal chelating properties but good superoxide and

H₂O₂ scavenging effect while proline, phenylalanine and tyrosine have lower superoxide radical scavenging properties (Aluko 2012). Ferric reducing activity of the protein hydrolysates has been shown to be enhanced by the presence of cysteine, methionine, and glutamic acid (Ajibola et al. 2011) while the presence of lysine lowered the activity. DPPH radical scavenging effect of protein hydrolysates may depend on aspartic acid, threonine, valine, and isoleucine but presence of histidine, lysine and arginine have the opposite effect (Aluko 2012). Peptides that can donate cysteine to synthesis of glutathione (GSH) are important for antioxidative activities (Aluko 2012).

1.7 Oxidative stress

Oxidative stress is a term that describes the oxidative damage in a cell, organ, or tissue caused by excess reactive oxygen species (ROS) and nitrogen species (RNS) species. ROS are by product of essential and normal metabolic reactions derived from oxygen species in all aerobic organisms. Excess ROS can damage normal body function or lead to abnormal cell death (Pierce et al. 1991). Many health benefits of oats or its constituents as discussed in section 1.3 are associated with their effects on oxidative stress.

1.7.1 Reactive oxygen species and reactive nitrogen species

Reactive Oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive and are naturally present in the human body. ROS/RNS react with other molecules such as lipids, proteins, nucleic acids, fats, DNA and carbohydrates by either donating an electron or accepting an electron from another molecule (Davis et al. 2010).

Few examples of ROS/RNS are $O_2^{\bullet-}$, $HO^{\bullet}$, $NO^{\bullet}$, $ONOO^-$, $HOCl$, $RO(O)^{\bullet}$, $LO(O)^{\bullet}$, (Prior et al. 2005) and they fall in to two categories, one category is molecules that contain unpaired electrons such as superoxide anion $O_2^{\bullet-}$, hydroxyl ion OH^- , and hydroxyl radical $\bullet OH$ and react directly with biological molecules. The other category is molecules that have the ability to pull electrons from other molecules (H_2O_2 or $HOCl$) and can damage biomolecules through direct interaction or starting a chain reaction where ROS/RNS are transferred from molecule to molecule (Zulueta et al. 2009a).

Superoxide anion ($O_2^{\bullet-}$) is produced from O_2 molecule that acquired an electron (Equation 1).

Hydrogen peroxide is produced via superoxide anion ($O_2^{\bullet-}$) in the presence of antioxidative enzyme Superoxide dismutase (SOD) (Equation 2).

In the presence of high iron concentration hydrogen peroxide (H_2O_2) can be converted to hydroxyl radical ($HO^{\bullet}$) (Equation 3).

Highly reactive peroxynitrite ($ONOO^-$) is also formed via reaction of superoxide anion with nitric oxide (Hazel 2006)(Equation 4).

1.7.2 Reaction of ROS/RNS with biomolecules

Antioxidants inhibit or reduces generation of free radicals, eventually leading to delay or even prevent oxidation reactions, such as lipid oxidation. Lipids are components of cell membrane that are vulnerable to oxidation upon introduction of free radicals, high temperatures, lights, oxygen, and pro-oxidants. In food this oxidation can cause food rancidity, undesirable taste, odors and decrease nutritional value of food. Lipid oxidation products are harmful to mammalian cells, they can effect cell division as well as cell proliferation, and it can result in inflammation. The steps of lipid auto-oxidation are shown below.

Radical initiation

Radical propagation:

Radical termination:

ROS and lipid peroxides can oxidize proteins thereby altering their function. The protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or by-products of oxidative stress (Andersson and Hellstrand 2012; Berlett and Stadtman 1997). There are different types of proteins oxidation modifications. Protein oxidation can result in oxidation of amino acid residues. •OH for example can oxidized tyrosine, phenylalanine, tryptophan, histidine, methionine and cysteine (Berlett and Stadtman 1997). As a consequence of oxidation, hydroperoxides, alcohols and carbonyl compounds are formed (Byrne 2002). Breakage of peptide bond in proteins or protein-protein cross links occurs as a results of oxidation, leading to a secondary effects such as protein folding, protein fragmentation and protein aggregation, causing loss of activity and function in proteins. The steps in protein oxidation are shown below.

As shown in reaction 1, protein oxidation is initiated by OH•, removing H atom from aliphatic amino acid resulting in formation of RCOO• (alkyl peroxide) in presence of O₂, and after formation of alkoxy radical (RCO•), which is then converted to hydroxyl protein derivatives (RCOH). Reaction 2 shows that in absence of O₂, the protein-protein cross-linked derivative occurs and then the carbon-centered radical can react with another carbon-centered radical (Berlett and Stadtman 1997). Protein carbonyl is used as marker of the protein oxidation (Berlett and Stadtman 1997). Increased levels have been seen in Alzheimer's diseases, diabetes, and renal failure. In addition, amino acids that contains sulfur such as cysteine and methionine are more sensitive to react with free radicals (Silva,P,F,N. et al. 2006).

Nucleic acids are sensitive to oxidative damage as well, ROS directly effects DNA, signal transduction, cell proliferation, cell death and intracellular communication. ROS damages DNA by attacking purine and pyrimidine bases and deoxyribose sugars and also by indirect mechanisms. $\text{OH}\cdot$ can cause chemical modification in DNA, through breakage of one and two strands and cross linking with other molecules. Superoxide ($\text{O}_2^{\bullet-}$), nitric oxide (NO) or hydrogen peroxide (H_2O_2) do not react with any of DNA or RNA bases. $\text{OH}\cdot$ reacts with nitrogen bases of nucleic acids or aromatic rings. The main products for the oxidative damage of DNA are 8-hydroxyguanosine (8-OHdG), 8-hydroxyadenine (8-OH-Ade), cytosine glycol, and thymine glycol (oxidative damage to DNA). 8-hydroxydeoxyguanosine (8-OHdG) is one of the well-studied marker of DNA oxidation. Studies have revealed that there is a connection between the oxidation of DNA bases and cancer or inflammatory diseases such as hepatitis (Berlett and Stadtman 1997). Figure 11 shows formation of 8-OHdG by reaction of ROS with DNA.

Figure 11: An illustration of the formation of 8-OHdG, a DNA based oxidation production by free radicals

Oxidative damage by ROS can be measured by its markers, for example protein oxidation can be measured by measurement of thiol groups, ratio of glutathione and oxidized glutathione, or carbonylated proteins. Lipid oxidation can be measured by measurements of malondialdehydes, conjugated dienes, and DNA oxidation can be measured by measurements of (8-OHdG), or 8-OH-Ade. Figure 12 summarized the markers of oxidative stress to proteins, lipids and well as DNA.

Figure 12: Markers of oxidative damage to proteins, lipids and DNA by ROS (Babusikova et al. 2013)

1.8 Human antioxidant defense system

In response to free radicals and oxidative damages body produces natural antioxidant to fight against the pro-oxidants. Human's antioxidant defense system plays a significant role in shielding human's body from oxidative damage. Within human biological system there are general sources of antioxidants: (1) enzymes, such as Superoxide dismutase (SOD, EC 1.15.1.1), glutathione peroxidase (GPx), and catalase (CAT); and (2) non-enzymatic antioxidant such as vitamin C, some vitamin B, Vitamin E and β -carotene (Cheng et al. 2001). SOD converts superoxide anion to hydrogen peroxide then to oxygen. There are three forms of SOD enzymes. Cu/Zn SOD, binds to both copper and zinc, present in cytoplasm. Fe and Mn SOD enzyme that either binds to iron or manganese. Ni SOD binds to nickel. Mn SOD enzyme is present in mitochondria. After SOD enzyme converts superoxide to hydrogen peroxide and oxygen, the hydrogen peroxide is then converted to water by an enzyme name Catalase (CAT, EC 1.11.1.6) to, two H_2O (water) and one oxygen molecule. This enzyme controls the H_2O_2 level in the cells. Glutathione peroxidases (GPXs, EC 1.11.1.9), has peroxidase activity and it protects the organism against oxidative damage. GPx converts H_2O_2 in to water and oxygen. Glutathione transferases (GSTs, EC 2.5.1.18) are involved in the cellular detoxification, also they may reduce ROS to less reactive form.

Vitamins acts as free radical scavengers, by donating an electron to the free radical and they become saturated quickly after donating an electron. Vitamin E are fat soluble antioxidants, and their major function is to protect organism against lipid peroxidation, by donating a hydrogen atom (Pryor 2000). Vitamin C is water soluble antioxidant that works in aqueous environment. Its main function is protect thiol groups of proteins. Vitamin C works with vitamin

E, carotenoids, and enzymatic antioxidants (Uttara et al. 2009). Reduced glutathione (GSH) is a tripeptide and a multifunction antioxidant, it regenerates vitamin C and vitamin E back to their original form. Ubiquinone or Coenzyme Q, also known as (Q10, CoQ10, and CoQ), are present in mitochondria and inhibits lipid peroxidation.

1.9 Oxidative stress and obesity

Obesity means having excess body fat stored in tissues such as adipose tissue and liver (Indel et al. 2010) that result in adverse health effects. Obesity can be a result of intake of high energy food combined with low energy expenditure (Thompson et al.). Excess fat storage causes insulin resistance; insulin resistance can stimulate inflammation (Indel et al. 2010). BMI (body mass index) is an important sign to one's overall health, when BMI is above 30 kg/m²; person is at risk for type 2 diabetes, high blood pressure, heart disease, and other diseases. At least 6 out of 10 diseases leading to death are associated with obesity (Thompson et al.). Obesity is a rising concern facing by develop and non-developing countries. Therefore the health care system will have to deal with diseases associated with obesity. Diabetes, hyperlipidaemia, hypertension, and atherosclerosis are associated with obesity. Obesity is a central component among patients that have metabolic syndrome, however not all of them are obese, even lean people have metabolic syndrome (Ando and Fujita 2009).

Researchers have found that increase in oxidative stress in accumulated fat is linked to the pathogenic mechanism of obesity and obesity associated metabolic syndrome such as inflammation, hypertension, and impaired glucose intake in muscle and fat (Décorde K et al. 2009). In general obesity is associated with reduced antioxidant defenses and/or a strong free radical production. The activity of antioxidant enzymes such as catalase, glutathione peroxidase,

and glutathione reductase was lower in tissues and blood in some obese human and animal studies (Furukawa et al. 2004). Adipocytokines (fat derived hormones) or adipokines, including TNF- α , plasminogen activator inhibitor-1 (PAI-1), resistin, leptin, and adiponectin are dysregulated by oxidative stress in accumulated fat. This plays a role in the pathogenesis of obesity associated metabolic syndrome. Figure 13 is an illustration of increase of ROS in accumulated fat leading to metabolic syndrome. Increases of ROS in accumulate fat via NADPH oxidase and decrease of antioxidant enzymes, causes dysregulation in production of adipocytokines. Metabolic syndrome is associated with induced insulin resistance and ROS encourages insulin resistance (Furukawa et al. 2004).

Figure 13: Illustration of the role of ROS in metabolic syndrome (Furukawa et al. 2004)

1.10 Study hypothesis and objectives

Literature data clearly demonstrated that hydrolysis of food proteins with proteases, generates hydrolysates with antioxidant, anti-inflammatory, anti-hypertensive, anti-obesity or antimicrobial properties. Prior to the start of degree there was only one report of anti-hypertensive properties of digested oat proteins.

1.10.1 Hypotheses

My research hypotheses were:

- a) Proteins extracted from oat under optimised conditions and digested with protease will show strong antioxidant activities in vitro
- b) A hydrolysate with strong antioxidant activity added to high fat diet will decrease oxidative stress in an animal model

1.10.2 Objectives

- i). Optimise extraction conditions of proteins from oat: Use of salts and carbohydrases
- ii). Determine antioxidant properties of hydrolysates produced with various proteases. Determine the sequence of peptides using LC-MS/MS.
- iii). Investigate the effect in vivo of some of the antioxidant hydrolysates on the reduction of oxidative stress in mice models receiving high fat diet.
- iv). Measure in the mice model, body weight, glucose level, respiratory exchange ratio, total antioxidant capacity, activity of antioxidant enzymes, and vitamins.

Chapter 2

Methodology

2.1 Method and Materials

Oat flour and medium oat bran samples were obtained from Can Oat milling (Portage La Prairie, Manitoba MB). Linoleic acid, 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), tocopherol, α -tocopheryl, delta vitamin, α -tocopheryl acetate (vitamin E acetate), gamma vitamin E, retinol, retinyl acetate, retinyl palmitate, acetate ferrozine, ethylenediaminetetraacetic (EDTA), ascorbic acid, Alcalase 2.4L (EC 3.4.21.14), trypsin (3.4.21.4), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), 2,2'- azobis (2-amidinopropane) dihydrochloride (AAPH), mono- and dibasic potassium phosphates, Viscozyme L 100 Fungal Beta Glucanase (FBG)/g, α -amylase 30 units/mg, amyloglucosidase 70 units/mg, celluclast 700 Endo-Glucanase Units (EGU)/g, Alcalase 2.4 Anson Units (AU)/g, Trypsin 13,000-20,000 benzoyl-L-arginine ethyl ester units/mg, chloramine-T, potassium iodide, acetic acid, Tris-HCl, Trichloroacetic acid (TCA), 2,4-Dinitrophenylhydrazine (DNPH), thiourea, Copper(II) sulfate, Sulfuric acid (H_2SO_4), were obtained from Sigma Aldrich (Oakville, ON, Canada). Methanol, ethanol, hexane, ethyl acetate, fluorescein, hydrogen peroxide (H_2O_2), glutathione were purchased from Fisher Scientific Co. (Nepean, ON, Canada). High-purity water was produced in the laboratory by an Alpha-Q system (Millipore, Marlborough, MA). Nitrite/Nitrate assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

2.2 Defatting the oat flour

Oat flour was defatted using hexane in ratio of 1:3 (W:V), the sample was stirred for 1 hour at room temperature, in an incubator shaker (200 rpm) model G25 from New Brunswick (Edison,

NJ, USA), and after the extract was filtered on Watman paper No1. The defatted oat flour was air dried under fume hoods overnight.

2.3 Preparation of protein isolates

Extraction with salt: Defatted oat flour was added to the solution of 1.0 M of NaCl, in the ratio of 1:8 (w:v), the pH was adjusted to 9.5 using 1.0 M NaOH, the slurry was stirred for 30 min at room temperature. The sample was centrifuged at 5000 xg for 25 min at 4°C. The supernatant was collected and the pH of the supernatant was adjusted to 4.0 using 1.0 M HCl. The precipitated protein was washed with 30 mL of pH 4.0 water three times. The precipitates were dispersed in 15 mL of nanopure water, and the pH was adjusted to 7.0 with 0.1 M NaOH (Zhu et al. 2006b). The sample was freeze dried and the protein content was determined using Lowry assay (Markwell et al. 1978). The protein isolates was digested with trypsin and alcalase according to procedure in 2.4

For the animal study the oat protein isolates were digested with trypsin according to section 2.4.

Extraction in the presence of carbohydrases: Defatted medium oat brans (50 g each), was mixed with deionized water at ratio 1:10(w/v). The pH of the slurries was adjusted according to the carbohydrase being used: amylase (pH 6.25), Viscozyme (pH 4.1), Cellulase (pH 5.5), Amyloglucosidase (pH 5.5), and the control sample (no carbohydrase, pH 6.5). Concentrations of the enzymes were as follows: Amylase (60, 2300 unit/ g defatted brain), Celluclase EGU (5, 30, 60 unit/ g defatted brain), Viscozyme FBG (1.5, 3, 15 unit/ g defatted brain), and Amyloglucosidase units (8, 50, 100 unit/ g defatted brain). All the slurries were incubated for 3.5 hours at 200 rpm on New Brunswick incubator shaker, Model G25 (Edison, NJ, USA) , 45 °C. At the end of the incubation, the pH of the slurries were adjusted to 9.5 with 2M NaOH. Samples

were further incubated for 30 min, and were centrifuged at 4°C, 20 min at 2500g. Supernatants were collected, adjusted to pH 4, and were centrifuged at 10,000g, 40 min at 4°C. The precipitates (i.e. protein isolates) were washed with pH 4 water and were centrifuged 30 min at 1100g. Isolates were dissolved in water and the pH was adjusted to 7.0. Protein samples were freeze-dried and protein contents were determined using Lowry method (Markwell et al. 1978). These isolates were digested with alcalase according to procedure in section 2.4.

FBG: Fungal Beta-Glucanase Units; EGU: Endo-Glucanase Units

2.4 Digestion of protein isolate with trypsin and alcalase

2.4.1 Alcalase hydrolysis:

NaCl extracted protein isolates were dissolved in Milli-Q water (300 mg in 20mL), pH was adjusted to 8.0, the alcalase was added at an enzyme substrate ratio of 0.4 Anson Unit (AU)/g of protein. For carbohydrase-treated brans (chapter 4), proteins were solubilized in water 1/10 (w/v) ratios and alcalase was added at an enzyme substrate ratio of 0.4 Anson Unit (AU)/g of protein. Samples were incubated at 50°C for 4hrs, and the hydrolysis was stopped by heating the sample to 90°C for 10 min. Supernatants were collected by centrifugation at 4000g, 15 min and were freeze-dried and were stored at 20°C for further analyses.

2.4.2 Trypsin hydrolysis:

Enzymatic hydrolysis of NaCl extracted proteins was performed at pH 8.0 deionized water (1:10 (w/v)) with trypsin at a substrate/enzyme ratio of 1:50 (w/w). The sample was incubated at 37°C with shaking at 100 rpm for 20 hrs. Samples were heated and supernatants collected as above.

For in vitro assay studies (chapter 3), 300 mg were hydrolyzed. For animal experiments a total of 40.96 g of freeze dried hydrolysates were obtained from 78.46 g of proteins.

Membrane ultra-filtration: To investigate the in vitro antioxidant activity of alcalase (APH) and trypsin (TPH) hydrolysates from NaCl extracted proteins (chapter 3), freeze dried samples were reconstituted in water, and were ultra-filtered on 2 and 10 kDa molecular weight cutoff VivaspinTM 15R membrane from VWR Canada (Mississauga, ON). The samples then were freeze dried and were stored at -20°C.

Amino acid analysis: The amino acid (AA) composition of the freeze dried of trypsin hydrolysate used for animal study (chapter 5) was performed by Advanced Protein Technology Centre at the Hospital for Sick Children, Ontario, Canada. Picomoles of individual amino acids detected were converted to percentage relative to total amino acids content.

2.5 DPPH radical scavenging effect

The scavenging effects of both alcalase protein hydrolysates (APH), trypsin protein hydrolysates (TPH), and the ultra-filtered fractions (2 kDa, 2-10 kDa and >10 kDa) were measured using 60µmol/L DPPH solution freshly prepared in 100% methanol. Sample concentrations of 0.4 and 1 mg/mL in Milli-Q water was reacted with DPPH solution (final concentration 0.08 and 0.2 mg/mL) for 15, 30, 45, and 60 min. The samples were read at 515 nm with spectrophotometer (Gary 300 UV-Visible Spectrophotometer, Varian Inc, Australia) against a blank of 100% methanol. Samples were run in triplicates, and the % Scavenging activity was calculated as follows (Li et al. 2008):

$$\% \text{ Scavenging activity} = (1 - \frac{A_s}{A_{CTL}}) \times 100$$

As is the absorbance at time t and ACTL is the absorbance of control (DPPH) at time zero.

2.6 Oxygen radical absorbance assay of digested oat flour proteins

Peroxyl radical scavenging activity was determined using oxygen radical absorbance capacity (ORAC) assay. The measurement was done on a microplate fluorescence reader model FLx800 (Bio-Tek Instruments, Inc., Winooski, VT), this instrument is equipped with a temperature-controlled incubation chamber and with fluorescence filters (excitation 485/20 nm, emission 528/20 nm). Incubator temperature was set at 37 °C. Potassium phosphate buffer (75 mM/L, pH 7.4) was used to prepare all the reagents, standards, samples, and the control. Trolox was used as a standard and the concentrations prepared were 6.25, 12.5, 25, 50 and 100 μ M. Rutin was used as control. The data were processed with Gen5 software.

For in vitro experiments, PH, TPH and APH were analyzed at 160 and 80 μ g/mL, hydrolysates from carbohydrase treated barns were analyzed at 0.0025 and 0.00125 μ g/mL.

Mice samples: Red blood cells were diluted 1600 times, and plasma samples were diluted 200X. For liver samples concentrations of 0.001mg/ μ L, and for brain samples concentration of 0.004mg/ μ L was used.

The results were expressed as μ mol Trolox Equivalents (TE) and are means $\pm$ SEM (Zulueta et al. 2009b) . All the samples and standards were done in triplicates.

2.7 Measurement of ferrous ion-chelating activity

Protein hydrolysates were prepared in concentrations of 20, 40, 80, 120 and 200 μ g/mL in Milli-Q water, and ultra-filtered fractions (2kDa, 2-10 kDa, and >10 kDa) from trypsin and alcalase digests were prepared. 2 mmol/L of FeCl₂ freshly prepared was added to all the samples. The reactions was initiated by addition of 5 mmol/L aqueous ferrozine solution and the mixture was

shaken vigorously and left at room temperature for 10 min. The absorbance was read at 562 nm by the spectrophotometer. EDTA was used for positive control (Dinis,T,C,P. et al. 1994). The %

Inhibition of ferrozine-Fe²⁺ complex formation was calculated as follows Ferrous ion chelating

$$\text{activity} = \left(1 - \frac{A_1 - \text{ABlk}}{A_0}\right) \times 100$$

A₀ is the absorbance of the control (FeCl₂ and ferrozine)

A₁ is the absorbance in the presence of samples

ABlk is the absorbance of sample blank without FeCl₂

2.8 Inhibition of linoleic acid autoxidation

APH and TPH antioxidative activity were measured in different incubation periods. This measurement was done with linoleic acid model, as follows: 0.8 mg/mL of TPH and APH was prepared in 50 mmol/L phosphate buffer (pH 7.0), this was added to a solution that was made of linoleic acid and 99.5% ethanol in ratio of (30:2500), and the final concentration was 0.4mg/mL. This mixture was incubated at 50°C in dark, in a screw cap. The degree of oxidation was measured by measuring the ferric thiocyanate values (Sakanaka et al. 2004). The reaction solution was then mixed with 75% ethanol, 30% ammonium thiocyanate and 0.02 mol/L ferrous chloride solution in 3.5% HCl in a ratio of 50: 2.35:50:50 respectively. The sample was incubated for 10 min and the absorbance was measured at 500 nm at different intervals during 5 days. α-tocopherol was used as control.

2.9 Hydroxyl radical (HO•) scavenging assay

The alcalase hydrolyzed from carbohydrase treated bran proteins were all analyzed at 1 mg/mL, glutathione, an endogenous antioxidant peptide was used as positive control. All samples were

prepared in 0.75 mmol/L potassium phosphate buffer (pH 7.4). Each sample was mixed with 3 mmol/L 1,10-phenanthroline (1:1 ratio) in phosphate buffer, and 3 mmol/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in water. The reaction was initiated by addition of 0.03% aqueous H_2O_2 , and then was incubated at 37°C for 1 hour with shaking at 200 rpm on a Max-Q 4500 incubator (Fisher Scientific Canada, Nepean, ON). The absorbance of the samples were read at 536 nm using a Cary 50 Bio UV–vis spectrophotometer controlled by CaryWinUV Bio Pack Software (Varian Inc., Mississauga, ON). The absorbance of the blank (without sample and H_2O_2) was also determined. Everything was done in triplicates. The $\text{HO}^\bullet$ scavenging activity was calculated according to the literature (Li et al. 2008). This method was modified from a procedure from the literature (Pownall et al. 2010).

2.10 NanoLC tandem mass spectrometry

The samples were injected in to a 7 cm×200 μm inner diameter trap column, fritted, and packed in-house with 5 cm of 3 μm Magic C18AQ reversed phase packing material (Michrom Bioresources, Auburn, CA) using a pressure vessel constructed in house. The samples were at 1 mg/mL in 2% acetic acid. The trap column was connected in series to a 6 cm×75 μm Picofrit analytical column with a tip opening of 15 μm (New Objective, Woburn, MA), packed with 5 cm of 3 μm Magic C18AQ reversed phase packing material. Peptides were separated using a gradient of 2% B at 0 min, 5% B at 3 min, 10% B at 8 min, 29% B at 70 min, 80% B at 75 min and after the samples were washed for 10 min with 100% B and 5 min of re-equilibration at 2% B, where A and B are 0.1% formic acid/water and 0.1% formic acid/acetonitrile, respectively. Peptides were eluted from the trap and analytical columns at a flow rate of ~250 nL/min, ionized by nanoelectrospray ionization (ESI) and analyzed using a QSTAR XL QqTOF mass spectrometer

(AB Sciex, Concord, ON) operating in information dependent acquisition mode over a mass range of 400 to 1500 m/z. For fragmentation ions with a charge state of 2⁺ and 3⁺ were chosen. The analysis was done on the most intense peaks in the spectrum, one 1-s, two 2-s, and two 3-s tandem mass spectrometric. Masses are sequenced two times before adding them to an exclusion list for 90s.

MS/MS data were determined using Mascot (Matrix Science Ltd, Boston, MA). Mass tolerances were set to ± 100 ppm and ± 0.2 Da for the peptide and fragment ion spectra, respectively.

Peptides identified by Mascot were manually examined and verified for accuracy. Peptide sequences were matched to the published sequences of all proteins from the National Center for Biotechnology Information non-redundant database (NCBI nr). ; One missed cleavage was considered; no modification was selected.

2.11 Animals and diet

The current study protocol was approved by the Carleton University's Animal Care Committee (Protocol number P10-4) and followed guidelines of the Canadian Council on Animal Care.

Fifty two CD-1 male mice were obtained from Charles River Laboratories in Saint Constant, Quebec, 8 weeks old, 20-25 g. Each mice had its own cage, and were kept at room temperature 22°C with 20% relative humidity and 12/12 hr light/dark cycle. Lights were on at 7:00am, during the study. OPH or Oat protein isolated digested with trypsin was prepared according to section 2.3. The animals were fed the normal chow diet in pelletized form for 4 weeks. After this four weeks, each mice was assigned to one of five groups, each group had 10 mice (n=10). The five different groups are: Group 1 is High fat diet (HF); Group 2 is HF + 1mg of oat protein hydrolysate (OPH)/g of HF; Group 3 is HF + 10 mg OPH/g of HF; Group 4 is HF + 100 mg

OPH/g of HF and Group 5 is normal diet (ND) or regular mouse chow. The group division is summarized in figure 14. The OPH was blended with the high fat diet in the mixer. The animals received this diet for three weeks *ad libitum*. Animals had free access to food and water. The normal diet was 2014 Teklad Global 2.9 Kcal/g and the HF diet was 5.1 Kcal/g. These diets were purchased from Teklad Harlan (Indianapolis, IN). The diet composition is summarized in table 1. The high fat diet composition is summarized in table 2, and table 3 shows the vitamin mix in the high fat diet. The high fat diet was based on the AIN-93-VX diet (Reeves 1997).

Body weight and food intake were recorded daily, and the caloric intake calculated based on food consumed. At the end of the three weeks, 30 animals (n=6), were rapidly decapitated. The tissues were rapidly frozen with liquid nitrogen. Twenty animals (n=4) were housed in metabolic chambers (TSE systems, Chesterfield, MO, USA) for 48 hours. Animals had free access to food and water. The locomotor activity was determined using multi-dimensional infrared light beam system, and the respiratory exchange rate (RER) was calculated based on the oxygen consumption, and production of carbon dioxide. Blood samples were collected into heparin-coated tubes following decapitation. Then, all blood samples were centrifuged at 1000 ×g for 15 min at 4 °C to obtain plasma and red blood cells. Glucose level was determined using Accucheck® glucose meter. Liver, lung, brain, muscle and heart samples were removed, rapidly frozen. All tissues were rapidly frozen in liquid nitrogen and stored at -80 °C until analysis. The procedure was standardized for all tissues to avoid hemoglobin oxidation and alteration of enzyme activities. For analysis the tissue samples were homogenized at concentration of 0.1mg/mL with 10 mM phosphate buffer pH 7.4.

Content	HF	HF + 1 mg OPH /g food	HF + 10 mg OPH/g food	HF + 100 mg OPH/g food	Normal Diet
% Protein	23.5	23.5	23.5	23.5	14.0
% Fat	34.3	34.3	34.3	34.3	4
Energy kCal/g	5.100	5.104	5.140	5.500	2.9
Protein hydrolysate	0 mg/g food	1 mg/g food	10 mg/g food	100 mg/g food	0 mg/g food

Table 1: Summary of the composition of normal, high fat (HF) and experimental (HF + hydrolysate) diets. Normal and high fat diets were purchased from Teklad Harlan (Indianapolis, IN)

Content	g/Kg
Casein	265.0
L-Cystine	4.0
Maltodextrin	160.0
Sucrose	90.0
Lard	310.0
Soybean Oil	30.0
Cellulose	65.5
Mineral Mix, AIN-93G-MX (94046)	48.0
Calcium Phosphate, dibasic	3.4
Vitamin Mix, AIN-93-VX (94047)	21.0
Choline Bitartrate	3.0
Blue Food Color	0.1

Table 2: Composition of high fat diet

Formula	g/Kg
Niacin	3.0
Calcium Pantothenate	1.6
Pyridoxine HCl	0.7
Thiamin HCl	0.6
Riboflavin	0.6
Folic Acid	0.2
Biotin	0.02
Vitamin B12 (0.1% in mannitol)	2.5
Vitamin E, DL-alpha tocopheryl acetate (500 IU/g)	15.0
Vitamin A Palmitate (500,000 IU/g)	0.8
Vitamin D3, cholecalciferol (500,000 IU/g)	0.2
Vitamin K1, phylloquinone	0.075
Sucrose, fine ground	974.705

Table 3: Concentration of vitamins in the high fat diet

Figure 14: Different groups of CD1 mice. Group1 is High fat diet (HF); Group 2, is HF + 1mg of oat protein hydrolysate (OPH)/g of HF; Group 3, is HF + 10 mg OPH/g of HF; Group 4, HF + 100 mg OPH/g of HF and Group 5, regular mouse chow or normal diet (ND).

2.12 Total thiols

Total thiols were measured using Ellman's method, this method is based on a colorimetric reaction between the sulfhydryl groups with DTNB (5,5-dithiobis-2 nitro benzoic acid) forming a yellow compound, detected at 412 nm (Avinash et al. 2009). Tissue samples were homogenized with phosphate buffer pH 7.4 (1:10 w/v) and centrifuged at 5000g while plasma was diluted 10 times with buffer prior to measurement as reported in literature (Lushchak and Bagnyukova 2006; Ellman 1959).

2.13 Ferrous oxide xylenol (FOX) orange assay

Peroxides were analyzed by ferrous oxide xylenol (FOX) orange assay on blood and liver samples according to a previously published method (Smith et al. 2002). Briefly, FOX reagent was made by adding one volume of a mixture of xylenol orange (1.0 mM) and ammonium ferrous sulfate (2.5 mM) in 250 mM H₂SO₄ to nine volumes of 4.4 mM BHT prepared in methanol. Eight concentrations of H₂O₂ standards (5-200 µM) were made from 30% H₂O₂ stock solution. FOX reagent was mixed with all samples and standards (in ratio of 1:140:60), followed by incubation for 30 min at room temperature and were read at absorbance of 560 nm. The peroxide concentrations were calculated based on the calibration curve and expressed as nano moles of H₂O₂ per gram of wet weight.

2.14 Determination of superoxide dismutase and catalase activities

Liver tissues extracts for enzyme activity were prepared by homogenization (1:9 (w/v)) in potassium phosphate buffer (10 mM pH 7.4) and centrifugation at 2400g for 10 min. Red blood cells (RBC) and liver samples were aliquoted into 100 µL microtubes and stored in -80 °C until analysis.

Protein content of the liver and RBCs were determined using a modified Lowry's method (Markwell et al. 1978).

Superoxide dismutase (SOD) activity was measured based on the rate of reduction of nitroblue tetrazolium (2.24 mM) to formazan by xanthine oxidase (13.2 U/mL) at 560 nm (Spitz et al. 1989). Eight different concentrations of each sample (2 to 500 mg protein/mL) were used. SOD activities were calculated and expressed as units per milligram of protein. Catalase (CAT) activity was performed according to the method described by Aebi (Aebi H, 1984). The activity

was based on the decay of a 30 mM H_2O_2 at 240 nm and expressed as k/mg protein where k is the first order reaction constant.

2.15 Determination of advanced oxidation protein products (AOPPs)

The brain, heart and liver tissues were homogenized in 0.1M Tris-HCl and centrifuged at 2400xg for 10 min, and the supernatant was collected. Determination of advanced oxidation protein products (AOPPs) in plasma, brain, heart, liver was based on spectrophotometric detection according to Witko-Sarsat et al. Tissue samples were prepared at concentration of 0.01 $\mu\text{g}/\text{mL}$ with 20mM phosphate buffer, pH 7.4, and plasma samples were diluted 40 times with phosphate buffer. Chloramine-T was used as standard, and was prepared in concentrations, 100, 80, 60, 40, 20, 10, 5, 0 μM . Phosphate buffered saline was used as blank. 1.16 mol L⁻¹ potassium iodide was added to all samples, standards and blank in 1:20 ratio. The plate was incubated at room temperature for 5 minutes, and 20 μL of acetic acid were added making the final volume of the assay medium to 230 μL , absorbance was measured immediately at 340 nm. Concentration of AOPPs was expressed as $\mu\text{mol L}^{-1}$ of chloramine-T equivalents.

2.16 Nitrite/Nitrate assay

The Heart and Brain tissues were homogenized in 0.1M Tris-HCl and centrifuged at 2400xg for 10 min. The liver tissues were homogenized 1:9 (w/v) in potassium phosphate buffer (10 mM pH 7.4) and centrifuged at 2400xg for 10 min. The supernatant was collected and formation of NO (nitrite +Nitrate) was detected in tissues using a colorimetric assay kit from Cayman Chemical Company, Ann Arbor, MI, USA). Concentrations were calculated by using nitrite standards, and the results were expressed as $\mu\text{mol/g}$ of tissue.

2.17 Vitamin C

Vitamin C was measured in liver, brain, and lung. L-ascorbic was used as standard, and were prepared in concentrations 0, 3.125, 6.25, 12.5, 25, 50 and 100 $\mu\text{g/mL}$. Hundred mg of the tissue was homogenized in 400 μL of 0.01 M phosphate buffer pH 7.0. Five hundred μL of the 10% TCA was added to the samples and were centrifuged at 3500xg for 20 minutes. Samples and standard were prepared in triplicates. Two hundred fifty μL of the supernatant was mixed with 100 μL of DTC solution (0.75 g of DNPH, 0.1 g thiourea and 0.0125 g CuSO_4 in 9N H_2SO_4). Samples and standards were incubated at 37 °C for 3 hours. After samples and standards were mixed with 750 μL of ice cold 65% H_2SO_4 , and were incubated for 30 minutes at room temperature. Samples and standards were read with microplate reader at 520 nm absorbance and data were analyzed using Gen5™ 2.0 software.

2.18 Determination of vitamin A and E in liver, lung and brain by HPLC

The reagents used are, methanol 100% HPLC grade, ethanol 100% HPLC grade, ethanol 95%, α -tocopherol, α -tocopheryl acetate, delta vitamin, gamma vitamin E, retinol, retinyl acetate, retinyl palmitate, ethyl acetate. The HPLC system consisted of a binary pump (Waters model 1525), an auto sampler (Waters model 2707), a column (Waters Spherisorb ODS 250 x 4.6mm, 5 μm) and a Photodiode Array Detector set at 292 nm (Waters model 2998). The mobile phase used was 100% methanol at a flow rate of 1mL/min. The column was maintained at room temperature and the samples compartment was kept at 8°C. 20 μL of each sample was injected in triplicate and run for 35 minutes. Data were processed and analyzed using the Waters Empower 3 software. Stock solution were prepared in ethanol. Retinyl acetate 20 $\mu\text{g/mL}$: it was used as internal

standard, Retinol (vitamin A): 100 µg/mL, Retinyl palmitate (vitamin A palmitate): 100 µg/mL, α-tocopherol (vitamin E): 200 µg/mL, α-tocopheryl acetate (vitamin E acetate): 200 µg/mL, Gamma vitamin E: 200 µg/mL, Delta vitamin E: 200 µg/mL. Each vitamin standard was run in the HPLC to find the retention time.

Tissue preparation: To 100-200 mg of tissue was added to 0.7 mL of ethanol - water (1:1) and homogenized with a polytron (7mm homogenizer). A 50 µl of a 20 µg/ml retinyl acetate was added. The sample was centrifuged for 1 min. In order to extract the vitamins, 0.7 mL of hexanes was added and the vial was vortex for 1 min, and after the sample was centrifuged at 2400 rpm for 10 minutes. The supernatant was separated, this step was repeated two times, and the supernatants were mixed and were dried with nitrogen gas at 35°C. The residue was reconstituted with 500 µl ethanol: ethyl acetate (3:1) and filtered through a 45 µm filter into an HPLC vial insert for analysis

Statistical evaluation

All data are expressed as mean ± standard error (SE). Comparisons between the groups was done using SPSS. Statistical differences were evaluated by a one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). $P < 0.05$ was considered significantly different between the groups. Scatter plots from Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) were used to obtain correlations between amounts of OPH added to high fat diet and activities.

Chapter 3

Antioxidant activity of digested oat protein hydrolysates extracted in the presence of salts

Introduction

In addition to their nutritional properties, food proteins have now been recognized as sources of peptides that can positively affect the functioning of the human body through various modes of action. Hydrolyzed proteins and peptides from cereals such as wheat, corn and rice have been evaluated for their ability to reduce oxidative stress. Radical scavenging, metal chelating and inhibition of lipid peroxide formation have been reported for digested proteins from wheat germ (Cheng et al. 2006; Suetsuna and Chen 2002), while those from rice reduced atherosclerotic lesions in apoE-deficient mice through up-regulation of antioxidant enzymes (Burris et al. 2010). Pretreatment of mice with corn protein hydrolysates prevented lipopolysaccharide induced immunological and oxidative liver injury (Guo et al. 2009).

Oat (*Avena sativa*), is a popular cereal that has been associated with many health benefits including lower serum LDL-cholesterol in overweight males (Reyna-Villasamil et al. 2007) and healthy adults (Reyna-Villasamil et al. 2007) after consumption of bread and fruits enriched in oat beta-glucans. Other oat molecules such as avenanthramides have been shown to inhibit the secretion of proinflammatory cytokines interleukins IL-6 and IL-8 in endothelial cells (Guo et al. 2008). Oat also has good quality proteins because of higher content of the limiting amino acid lysine (Victor et al. 1977). The antioxidant properties of their peptides has not been investigated. The objective of this chapter is therefore to determine the radical scavenging, metal chelating and inhibition of lipid peroxide formation by hydrolyzed oat proteins and their ultra-filtered fractions.

3.1 Results and Discussion

Proteins were extracted with NaCl as described in section 2.2 and were then digested with trypsin (TPH) and alcalase (APH) to produce trypsin (TPH) and alcalase protein hydrolysates, respectively (see section 2.3). The hydrolysates were then ultra-filtered into ≤ 2 , $2 - 10$ and ≥ 10 -kDa fractions. Antioxidant activity of the each fraction was performed using DPPH, linoleic acid, ferrous ion-chelating, and oxygen radical absorbance capacity (ORAC) assays. The overall experimental design is summarized in figure 15.

Figure 15: Summary of experiment procedures of work done in chapter 3

3.2 Scavenging activity of hydrolysates and fractions

DPPH is a radical often used as a probe to test a molecule ability to scavenge free radicals and therefore its antioxidant activity. This probe was used to measure the ability of APH, TPH and their ultra-filtered fractions to act as antioxidants. The results are shown in Figure 16. The most active fraction was the Alcalase fraction 2kDa (APH 2kDa) with inhibition of 33 and 35 % at 15 and 45 min, respectively. TPH 2kDa inhibition was lower 20 and 32 % at 15 and 45 min. However at 60 min these two fractions had similar inhibition activity. Non-fractionated APH and TPH samples had similar activity ($p>0.05$) during the entire incubation period. The lowest scavenging activity belonged to TPH>10 kDa.

The scavenging activity was also determined using ORAC (Table 4), an assay that measure the scavenging activity of molecules against peroxy radical and commonly used to determine antioxidant activity of food components (Tsopmo et al. 2009; Dávalos et al. 2004). The ORAC values were expressed as $\mu\text{mol/L}$ trolox equivalents per gram of sample. The ORAC value for TPH was 434 ± 16 , and that of APH was 269 ± 4 , these values were significantly different. The ORAC values for the ultra-filtered fractions, for TPH>2 kDa was 345 ± 15 .

Figure 16: DPPH radical scavenging of APH, TPH and their ultra-filtered fractions, <2 kDa, 2-10kDa, and >10kDa.

Sample	ORAC (μ M Trolox/g of sample)
TPH	434 $\pm$ 16a
TPH< 2kDa	270 $\pm$ 8b
TPH> 2kDa	345 $\pm$ 15c
APH	269 $\pm$ 4b
APH< 2kDa	149 $\pm$ 7d
APH> 2kDa	241 $\pm$ 9e

Table 4: ORAC values of APH, TPH and their ultra-filtered fractions. Statistical differences were evaluated by a one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Difference were analyzed using Fisher LSD test, $P < 0.05$ was considered significantly different and data are means $\pm$ standard error.

The amino acid tyrosine and tryptophan both contain aromatic ring that can form a stable radical intermediates after donation of a proton to break the radical chain reactions (Arcan and Yemenicioğlu 2007). Methionine, is an amino acid that can prevent radical reactions and its potency is believed to be superior to that cysteine, histidine and phenylalanine (Dávalos et al. 2004). The position of the amino acid and as well as the length of the peptides has a role in its antioxidant activity, this could be the reason differences in radical scavenging power of the different peptide fractions. Free amino acids present in <2kDa fractions certainly contributed to its activity. Unfortunately, it was not possible to determine the percentage or identity free amino acids present in less than 2 kDa fractions.

3.3 Ferrous ion-chelating activity

The chelating properties of the APH, TPH and fractions were measured by monitoring the formation of the complex between ferrozine and Fe^{2+} ions at 500 nm (figure 17). When the formation of the complex Ferrozine/ Fe^{2+} is disturbed, it means the compound has the chelating properties and this will result in the decrease of the red color and estimation of the metal chelating activity (Yamaguchi et al. 2000). TPH 2kDa had 16.8% chelating effect at 20 $\mu\text{g/mL}$, TPH2-10kDa had 21.6% chelating effect and TPH had 11.5% chelating effect. At lower concentrations, ultra-filtered fractions 2 and 2-10kDa of TPH had higher chelating effect compared to the whole TPH but at higher concentrations (43.8% at 200 $\mu\text{g/L}$) TPH was more active than its fractions. APH>10kDa was the most active at low concentration (20 $\mu\text{g/mL}$) 16.9%, and APH activity was 31.8% at higher concentration (200 $\mu\text{g/mL}$). In summary, Trypsin hydrolysate had a better chelating properties compared to Alcalase at similar concentrations. This could be due the presence of amino acid like histidine, which is known to chelate metals or trap lipid radicals (Je et al. 2004; Rajapakse et al. 2005; Je et al. 2005).

Figure 17: Ferrous ion-chelating activity of the, TPH, APH, and their ultra-filtered fractions.

TPH: trypsin digest; TPH 2kDa: trypsin digest < 2 kDa; TPH 2-10 kDa: trypsin digest 2 to 10 kDa; TPH: trypsin digest > 10 kDa. Each value is expressed as mean $\pm$ SD (n = 3)

3.4 Linoleic acid autoxidation protection by oat protein hydrolysates

The antioxidant activity of the TPH and APH were measured in the linoleic acid emulsion system, using α -tocopherol as a reference (figure 18). The formation of lipid peroxides was inhibited by addition of APH and TPH at a final concentration of 0.4 mg/mL from day 1 to day 5. From these results it can be concluded that APH and TPH contain peptides that can inhibit formation of lipid hydroperoxides in food products. Purified peptides from enzymatic hydrolysis (Rival et al. 2001; Saiga et al. 2003), as well as protein hydrolysates from other foods (Zhu et al. 2006a; Li et al. 2008) has been shown to inhibit the autoxidation of linoleic acid. Increased antioxidant activity of protein hydrolysates and peptides can be due to increase in

hydrophobicity, which increases their interaction with lipids (Saiga et al. 2003). The amino acid histidine, at the N-terminus of the peptide has been shown to increase the antioxidant activity of peptides in the linoleic acid emulsion assay.

Figure 18: Reduction of lipid peroxides in a linoleic acid emulsion system of APH, TPH, Control and vitamin E (α -tocopherol), the effects of both APH and TPH were significantly different compared to control ($P < 0.01$).

3.5 Conclusion

In conclusion, APH 2kDa had higher DPPH radical scavenging compared to TPH 2kDa. APH and TPH had similar DPPH radical scavenging. ORAC value for TPH was higher than APH. Ferrous ion chelating assay showed that ultra-filtered fractions had higher chelating activity at lower concentration, and TPH had higher chelating activity at 200µg/mL. Linoleic acid autoxidation of both APH and TPH was compared to vitamin E, the inhibition was similar to antioxidant vitamin E, and the inhibition activity was higher in days 4, and 5.

Chapter 4

**Investigation of the effect of pre-treatment of
oat brans with different carbohydrases on the
extraction of protein, and on antioxidant
capacity of hydrolyzed proteins**

Introduction

The extraction of proteins from foods and the use of proteases to hydrolyze them may prevent allergenicity and liberate biological active peptides. Common methods to extract proteins from cereals are often performed under salts or alkaline conditions (Cluskey et al. 1973; Ma 1985). However, in crops such as cereals extracting proteins from bran is difficult under alkaline conditions because they are linked to fibers and because of high viscosity of slurries that make proteins precipitation difficult. In addition, the formation of lysinoalanine can occur and thereby reduce the nutritional properties (Wang et al. 1999). Pretreatment, utilizing carbohydrases, to extract proteins from plants at neutral and basic pH has been shown to increase protein yields (Ansharullah et al. 1997; Grossman et al. 1980). This is because enzymes disintegrate the cell wall matrix; hydrolyze the plant cell wall polysaccharide matrix. In a previous study, some carbohydrases were effectively shown to increase protein extraction yields from rice brans (Guan and Yao 2008). The use of carbohydrases in other cereals has not been investigated.

In this chapter, the effectiveness of extraction of proteins from medium oat bran using amyloglucosidase, amylase, viscozyme, and celluclast, and investigating the antioxidative activity of the protein isolate as well as alcalase hydrolysates are covered. The use of tandem mass spectrometry for identification of peptides is also presented. Figure 19 shows an illustration of the overall work done in this part of my research work.

Figure 19: Schematic representation of experimental procedures of work done in chapter 4

Results and discussion

4.1 Protein extraction in the presence of carbohydrases

Alkaline protein extraction is associated with low protein yield, even though the protein extracted using this method has high nutritional content (Wang et al. 1999). This could be due to the presence of high amounts of fiber as well as presence of the membrane bound glycoproteins making the slurry viscous and in this case, proteins are less likely to precipitate at their isoelectric points. In order to compare the effectiveness of the protein extraction from medium oat bran enzymes amyloglucosidase, amylase, viscozyme, and celluclast were used. Three different concentration of each enzyme was investigated, except for amylase (only two).

The results are summarized in table 5. The control sample had no enzyme added to it and it had 54% protein content. This value was similar to the content of proteins extracted using celluclast 5-60 EGU/g. The samples that were treated with amyloglucosidase 8 units/g had 82% protein content. Increasing the concentration of amyloglucosidase from 50 to 100 units/g decreased the protein content. This could be explained by the presence of higher amount of polysaccharides in the sample. Brans that were treated with alpha-Amylase 60 and 2300 units/g and viscozyme at concentrations of 3 and 15 FBG/g had similar protein content, about 70%. In a previous study, different concentrations of viscozyme under different conditions was used to pretreat oat bran before protein extraction, the best environment was found to be pH 4.6, incubation time of 2.8 hr, temperature 44°C, and viscozyme 3 FBG/g. Under these conditions, the protein content of the supernatant was 56% (Guan and Yao 2008). In this thesis, it was found that pretreating the bran with viscozyme 3 FBG/g increased the yield to 68%. This difference could be due to that the protein content in our study was evaluated in protein isolates (after centrifugation at 10,000 g of the supernatant) rather than in the supernatant (Guan and Yao 2008). The protein content of isolate is always higher than protein content of the concentrate (supernatant). The precipitation at isoelectric points removes the interfering non-protein molecules. One study reported that a combination of phytase and xylanase for extraction of proteins from rice bran at pH 5.0, yielded 92.0% of proteins (Wang et al. 1999). The concentrations of the enzymes, α -amylase, celluclast, and viscozyme used for work performed as part of this thesis were based of those used for extraction of proteins from heat stabilized defatted rice bran (Tang et al. 2003).

Enzyme	pH	Concentration (unit/ g defatted bran)	protein content (%) of isolates	ORAC (μM TE/g isolate)
No enzyme	6.5	0	54	53 ± 12^a
Amylase, units	6.5	60	70	117 ± 13^c
		2300	72	165 ± 16^e
Celluclast, EGU	5.5	5	53	73 ± 13^a
		30	53	$95 \pm 12^{b,c}$
		60	52	89 ± 6^b
Viscozyme, FBG	4.5	1.5	42	65 ± 12^a
		3	68	106 ± 8^c
		15	70	$111 \pm 12^{c,d}$
Amyloglucosidase, units	5.5	8	82	125 ± 3^d
		50	57	86 ± 4^b
		100	47	79 ± 8^b

Table 5: Effect of different concentrations of Amylase, celluclast, viscozyme, and amyloglucosidase on protein content; antioxidant activity (ORAC value μM TE/g sample) of protein isolates. Statistical differences were evaluated by a one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). $P < 0.05$ was considered significantly different between the groups using the Fisher LSD test (mean $\pm$ standard error).

4.2 Antioxidant activities of hydrolysates from bran treated with carbohydrases

4.2.1 Oxygen radical absorbance capacity (ORAC) assay

ORAC values of protein isolates and alcalase digested proteins are displayed in Table 5. The ORAC value for the sample that was not treated with an enzyme was $53 \pm 12 \mu\text{M}$ TE/g. Brans treated with viscozyme had ORAC values between 65-111 μM TE/g, and this increase was associated with increase of protein contents. For amyloglucosidase treated brans, increasing the concentrations from 8 units to 100 units/g of defatted bran resulted in decreasing ORAC value

from 125 ± 3 to 79 ± 8 $\mu\text{M TE/g}$. This can be explained by the fact that increasing the concentration of this enzyme resulted in greater degradation of polysaccharides and that may have co-precipitated with proteins. Figure 20 shows the peroxyl radical scavenging of different protein samples hydrolyzed with alcalase. The highest activity belonged to viscozyme and celluclast. α -amylase 2300 units/g had the lowest activity (150 ± 27 $\mu\text{M TE/g}$), and the control had the activity of 243 ± 31 $\mu\text{M TE/g}$. The peroxyl radical scavenging activity of proteins hydrolyzed with alcalase was higher than the non-digested ones. This is because hydrolysis released specific peptides or amino acids with strong antioxidant properties. In fact, the sequence of peptide identified here contains amino acids like tyrosine, histidine and phenylalanine which are good electron donors.

It is also possible that Tryptophan or cysteine are present in the hydrolysates, since they are powerful peroxyl radical scavenger, and they contributed to the ORAC values. Methionine contains sulfur that can be oxidized and therefore prevent or reduce oxidative damage to foods and components (Ngo et al. 2010). The length of the peptide as well as the position of the amino acids contributes to the antioxidant activity (Davalos et al. 2004; Sheih et al. 2009). The difference in antioxidant activity observed in the protein hydrolysates is due to some of these factors.

Figure 20: ORAC value for protein isolates extracted in brans pretreated with different concentrations of carbohydrases followed by hydrolysis with alcalase. V: viscozyme, C: celluclast, a-A: alpha-amylase, AG: amyloglucosidase. Values are mean±SD (n=3). Different letters indicate significant difference between groups ($P < 0.05$).

4.2.2 Hydroxyl radical scavenging activity

Hydrogen peroxide and superoxide can react and form hydroxyl radical ($\text{HO}\bullet$) which is a highly reactive species. In the presence of metal ions such as iron and copper, $\text{HO}\bullet$ reacts with DNA, proteins, lipids and polypeptides (Lee et al. 2004). The formation of hydroxycyclo-hexadienyl radicals can occur by reaction of aromatic compounds with $\text{HO}\bullet$, this intermediate can further react with oxygen to give peroxyl radicals or decomposing to phenoxyl-type radicals by water elimination (Lee et al. 2004). The $\text{HO}\bullet$ scavenging activity of the, control as well as the samples

treated with different enzyme is shown in figure 21, and are expressed as % inhibition. The lowest percentage inhibition belonged to hydrolysates from brans treated with celluclast. Brans treated with 5 EGU/g of celluclast had the % inhibition of, this value is lower than of the control which is 17.5 ± 0.8 . The samples that were treated with amyloglucosidase and viscozyme, all three concentrations had higher percentage inhibition compared to the control. Comparing these results with ORAC, which measures the peroxyl radical scavenging activity; there were differences in activities of the samples. This may be because the $\text{HO}^\bullet$ radical scavenging activity is dependent on the reducing power while the peroxyl radical assay is dependent on electron or proton donating groups (Rice-Evans et al. 1995).

Figure 21: $\text{HO}^\bullet$ scavenging activity of protein hydrolysates from control bran treated with different concentration of carbohydrases. Statistical differences were evaluated by a one-way ANOVA (Fisher LSD test) using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). $P < 0.05$ was considered significantly different between the groups (mean $\pm$ standard error).

Asn-Gly-Leu-Glu-Gly-Leu-Lys is a hepta-peptide from giant squid muscle that has shown to possess antioxidant activity (Rajapakse et al. 2005). Hydrophobic peptides from soybean were shown to possess antioxidant activity (Chen et al. 1995). Several hydrophobic amino acids have been found in oat proteins, these amino acids are Leu, Val, and Ala, and they are known to contribute to radical scavenging properties. These amino acids can facilitate the interaction of peptides with linoleic acid and protect it from oxidation (Rajapakse et al. 2005). The amino acids in the alcalase peptide and the proper sequencing of these amino acids can be the reason for the antioxidant activity of this peptide (Chen et al. 1995; Suetsuna et al. 2000).

4.3 Peptide identification

The QSTAR® QqTOF hybrid mass spectrometer, is the instrument used in this research to analyze the alcalase hydrolysates, from viscozyme and control. This machine can identify biological and functional peptides. The oat bran Alcalase hydrolysates' tandem mass spectrometry was conducted on multiply charged ions over 80 min. An example of ion count chromatogram is shown in figure 22.

Figure 22: Ion count chromatogram of oat bran Alcalase hydrolysates. The peak for the peptide OAP-1 is shown at retention time 56.11 min

Figure 23A shows the average mass spectrum of peptide eluted between 55.5 and 55.7 min. The mass spectrometer was set automatically to perform MS/MS analysis of multiple charged (2^+ and 3^+) only. This is because there is greater probability that multiple charged peaks would belong to peptides. Figure 23B shows an example of a group of peaks that belong to a double charged peptide. This is evidenced by the difference of 0.5 Da between two consecutive isotopic peaks visible at m/z 630.34, 630.85, and 631.34. This peak was then automatically selected for MS/MS analysis. b- and y-ions are most common fragments observed on MS/MS spectra, b-ions are those that extend from amino terminus while y-ions are those that extend from carboxy terminus.

Figure 23: Time of flight MS scan of alcalase digested control oat bran protein at retention time 55.6 min. A: Mass spectrum of peptides eluted between 55.5 and 55.7 min. B: Expansion showing a double charged peak (OAP-1) that automatically submitted for MS/MS analysis.

Figure 24, shows the MS/MS spectrum of OAP-1. b-ions were the most important for its identification. The peak at m/z 1015.50 (b9) resulted from $[M-Ile-Leu]^+$. Other fragments are b8 $[b9-Tyr]^+$, and b7 $[b8-Val]^+$. The sequence Tyr-His-Asn-Ala-Pro-Gly-Leu-Val-Tyr-Ile-Leu or YHNAPGLVYIL was identified and named OAP-1. It is derived from 12S globulin peptide fragment 74-84. MS/MS data from other multiple charged peaks at m/z 692.36 (OAP-2) 582.34 (OAP-3) and 762.43 (OAP-4) were elucidated in the same manner. Their sequences are DVNNNANQLEPR or Asp-Val-Asn-Asn-Asn-Ala-Asn-Gln-Leu-Glu-Pro-Arg (OAP-2) derived from 12S globulin fragment 160-171; GQTVFNDRLRQGQLL or Gly-Gln-Thr-Val-Phe-Asn-Asp-Arg-Leu-Arg-Gln-Gly-Gln-Leu-Leu (OAP-3) from 12S globulin peptide fragment 376–392 and VVNNNGQTVFNDRLRQGQLL or Val-Val-Asn-Asn-Asn-Gly-Gln-Thr-Val-Phe-Asn-Asp-Arg-Leu-Arg-Gln-Gly-Gln-Leu-Leu (OAP-4) from 12S globulin peptide fragment 371–390. All of these peptides were present in the control sample; however, the peptide OAP-2 was only present in the samples that were treated with 15 FBU/g viscozyme. MS/MS analysis of multiple charge peaks has been used to identify peptides in protein hydrolysates (Drogaris et al. 2009; Yang et al. 2007) and biological fluids (Cirulli et al. 2008). Globulin was one of the major proteins in the bran concentrate as analyzed by polyacrylamide gel electrophoresis (Guan et al. 2007). It is also possible that in the protein isolate prepared during this study, globulin was the major protein as all four peptides identified are its fragments. However, it is also possible that 12S globulin derived peptides easily formed multiple charged ions compared to peptides from other oat bran proteins. More separation of the hydrolysates will allow us to identify more peptides from different proteins. From the literature, peptides derived from 11S globulin, 12S

globulin and avenin-3 are potential antihypertensive peptides (Cheung et al. 2009; Gu et al. 2011).

Figure 24: Time of flight (MS/MS) of peptide OAP-1 (m/z 630.34) identified in control, oat bran alcalase protein hydrolysates.

4.4 Possible contribution of identified peptides to antioxidant activities

OAP-1 peptide (Tyr-His-Asn-Ala-Pro-Gly-Leu-Val-Tyr-Ile-Leu) contains tyrosine which is known to be important for metal chelating and hydroxyl radical activities but can also lower superoxide radical scavenging property (Gülçin 2007). It also contains histidine, which is known to increase the metal chelating activity and scavenge radicals because its imidazole group can transfer a hydrogen atom and donate a single electron (Grune et al. 2013). All four peptides

contain asparagine that can increase DPPH radical activities and leucine that may be important in superoxide anion radical scavenging activities (Chen et al. 1996). At the same time OAP-1, and OAP-2 (Asp-Val-Asn-Asn-Asn-Ala-Asn-Gln-Leu-Glu-Pro-Arg) contain proline (Pro) that lowers superoxide anion activity (Udenigwe and Aluko 2011). OAP-2, 3 (Gly-Gln-Thr-Val-Phe-Asn-Asp-Arg-Leu-Arg-Gln-Gly-Gln-Leu-Leu) and 4 (Val-Val-Asn-Asn-Asn-Gly-Gln-Thr-Val-Phe-Asn-Asp-Arg-Leu-Arg-Gln-Gly-Gln-Leu-Leu) contain aspartic acid (Asp) that has electron donating and metal chelating abilities of hydrolysates (Aluko 2012). Phenylalanine (Phe) residues present in OAP-3 and 4 may have contributed to the antioxidant activities of different oat bran protein hydrolysates (Aluko 2012; Grune et al. 2013). It is not possible to determine or predict the contribution of identified peptides to the activities of oat bran protein hydrolysates. This is because quantification was not possible.

Chapter 5

**Investigation of the potential of trypsin
digested oat bran proteins to reduce
oxidative stress in a mice model
receiving high fat diet**

Introduction

Obesity or excessive body weight known to be result from the intake of high fat or high calorie diet, has been associated with many chronic diseases such as inflammation, increased blood pressure, glucose intolerance, heart disease, diabetes and dyslipidemia (Takahashi et al. 1999). High amounts of ROS have been demonstrated in obesity (Furukawa et al. 2004). The damage from ROS has been related to obesity and some of its complications (West 2000). At normal level, some ROS are involved in signaling pathway through reversible oxidation of proteins such as seen in protein kinases or transcription factors (Poli et al. 2004). However, at higher levels ROS cause irreversible oxidation of proteins, DNA and lipids (Valko et al. 2007). In order to fight the ROS, cells contains enzymatic antioxidants. Enzymes, such as SOD, CAT, GPx and non-enzymatic antioxidants such as GSH, vitamin E, vitamin C (Halliwell 1989). The activity of these enzymes has been shown to be low in the plasma of obese human as well as animals (Furukawa et al. 2004). Some studies have reported that the addition of phytochemicals to high fat diets reduced oxidative stress and ROS (Feillet-Coudray et al. 2009). The use of proteins from plants as well as their hydrolytic products such as peptides have biological activities that can be beneficial in the treatment or prevention of some chronic diseases caused by oxidative stress (Wang and De Mejia 2005). Protein hydrolysates derived from pea has been shown for example to reduce blood pressure in humans and hypertensive rats (Li et al. 2011b). It has been demonstrated that, the oat phenolic lowered the risk of the cardiovascular disease through reduction of both plasma cholesterol and oxidation of low-density lipoprotein in humans and animals (Chen et al. 2004). Digested oat proteins have antioxidant properties in vitro (Jodayree et al. 2012), however their effects in vivo have not been investigated. As part of this thesis, hydrolyzed proteins were used for animal experiments because of their better scavenging

activities determined in Chapter 3. This chapter then covers supplementation of high fat diet with oat protein hydrolysates and the effect on oxidative stress markers in CD1-mice. Specifically the following markers were determined

- Body weight, glucose level, respiratory exchange ratio
- Total antioxidant capacity of blood and tissues
- Antioxidant enzymes activities of blood and tissues
- Protein oxidation
- Oxidants like nitrates/nitriles, hydrogen peroxide
- Vitamins.

Figure 25: Illustration of work done in this chapter.

Results and discussion

5.1 Composition of digested oat proteins

The amino acid (AA) composition of the oat protein hydrolysates (OPH) digested by trypsin was determined to contain aspartic acid/asparagine (8.3%), glutamine/glutamic acid (21.1%), serine (7.3%), glycine (9.0%), histidine (2.1%), arginine (7.6%), threonine (4.2%), alanine (6.2%), proline (5.3%), tyrosine (3.3%), valine (4.9%), methionine (0.9%), cysteine (0.4%), isoleucine (4.0%), leucine (6.9%), phenylalanine (5.0%), and lysine (3.4%). This was determined by acid hydrolysis and pre-column derivatization with phenylisothiocyanate on reversed phase HPLC.

5.2 Effect of OPH on red blood cells

Oxygen radical absorbance capacity ORAC, total sulfhydryl (-SH) groups and peroxide levels were used to determine the oxidative status of erythrocytes or red blood cells (RBC). As mentioned in other sections ORAC is accepted as a standard procedure to measure the total antioxidant capacity of foods, dietary supplements, or biological samples (Prior et al. 2007; Ninfali et al. 2009). It measures the oxidative degradation of the fluorescent molecule (fluorescein), after it has been mixed with 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) a free radical generator. The free radical damages the fluorescence molecule, resulting in the loss of fluorescence. Antioxidants will protect the fluorescence molecule from free radicals or from oxidative degradation. The decomposition of fluorescein is measured and used to calculate the total antioxidant capacity or ORAC value. Due to high content of lipids, the presence of transition metals such iron and copper, and the oxygen supply, RBCs are vulnerable to oxidation (Delmas-Beauvieux et al. 1995). Oxidative damage of RBCs membrane mostly

involves lipid peroxidation, and changes its fluidity. This which could be the reason of membrane, membrane-bound enzyme and receptor malfunctioning and this has been proposed as a general mechanism leading to Red blood cells (RBC) hemolysis involved and cell injury. In this thesis red blood cells of mice in group 4 (high fat diet + 100 OPH mg/food) had the highest ORAC value (123.27 ± 11.09 mM TE/mL) meaning that the addition of 100 mg protein hydrolysates /g high fat food increased their scavenging capacity. The value was 26% higher compared to the activity (96.47 ± 6.62 mM TE/mL) of mice in high fat diet (figure 26). This suggests a possible interaction of the protein hydrolysates within cell membranes, which generally serve as a target for lipid peroxidation (Ferrali et al. 1997). There was no difference between the ND (91.0 ± 6.7 mM TE/mL) and HF diet (96.5 ± 6.6 mM TE/mL). Mice in group 3 (HF + 10 mg OPH/g) had slightly lower ORAC value relative to ND and HF groups but this was not significantly different. The higher value in group 4 (HF + 100 mg OPH/g) could be due to higher concentration of thiol moieties present in RBC as discussed below, or possibly OPH increased the synthesis of thiol containing proteins. Cysteine and methionine are sulfur containing amino acids found in OPH and their combined percentage in OPH is 1.3%. There was no correlation between the peroxy radical scavenging activity and the OPH concentration.

Figure 26: Radical scavenging activity (ORAC) of red blood cells of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Ellman assay was used to determine the total thiol. Total thiol includes free amino thiols such as glutathione (GSH), cysteine, homocysteine and protein bound thiol (Malgorzata et al. 2004). Free thiol concentrations will provide an indication on the reduction capacity of the fluid or biological sample. GSH concentration in liver for example has been associated its detoxification potential. Thiol groups (-SH) play an important role in antioxidant reactions and regulation of

electron transport, preserving the correct structure of the proteins (Rokutan et al. 1994). Measurement of the total sulfhydryl groups showed that ND, HF and group 3 (HF + 10 mg OPH/g) had similar total thiol concentration. The total thiol concentration of group 4 (HF + 100 mg OPH/g) was significantly higher than group 1 (Figure 27). There was a correlation between the total thiol and the three concentration of OPH added to the diet ($R^2=0.99$).

Figure 27: Total sulfhydryl groups in RBC of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P<0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Ferrous ion oxidation xylene orange (FOX) assay was used to determine the peroxyl level (total peroxides (LOOH + H₂O₂+ROOH)). FOX assay estimates H₂O₂ concentration (Wolff 1994) . FOX method is based upon oxidation of reagent Fe²⁺ to Fe³⁺ by sample oxidizing agents, Fe³⁺ binds with reagent xylene orange (XO) to give a color complex having absorption maximum at 560 nm (Banerjee et al. 2003; Banerjee et al. 2002). Data from the FOX assay on plasma showed that HF groups had higher peroxide level compared to the ND. Addition of 1 mg OPH/g diet slightly decreased (p > 0.05) the peroxide level, while addition of 100 mg OPH/g increased its amount (p < 0.05) relative to HF diet only (Figure 28).

Figure 28: Levels of peroxides determined by FOX assay in the plasma of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with P < 0.05 were considered significantly different between the groups using the LSD test (mean ± standard error).

The ORAC level in the plasma was investigated and it was found that there was no difference between different groups (Table 6). Difference in scavenging power of RBC and plasma could be due to presence of different proteins and small molecules in plasma. Peptides that contain thiol have been shown to have radical scavenging properties, and in this study thiol levels was increased in the RBC samples. In a study where rats were on HF diets supplemented with polyphenols, an increase of oxidation products was found in plasma proteins but not in lipid or antioxidant enzymes (Feillet-Coudraya et al. 2009) . Increase levels of lipid hydroperoxides (LOOH), has been seen in the adipose tissue of obese mice, but not in the skeletal muscle and aorta (Furukawa et al. 2004).

	HF	HF+1mg	HF+10mg	HF+100mg	ND
Final weight (g)	44.3±1.7 ^a	45.1±1.5 ^a	46.8±1.8 ^a	46.3±1.0 ^a	40.2±1.0 ^b
Food intake (kCal/day)	24.1±0.7 ^a	25.5±1.4 ^a	25.4±0.7 ^a	26.7±1.2 ^a	20.8±0.7 ^b
Locomotor activity (Counts)	1227±184 ^a	1112±173 ^a	964±144 ^a	1370±138 ^a	1099±188 ^a
Plasma ORAC (μM TE/mL)	106.7±6.8 ^a	102.0±5.3 ^a	108.8±11.8 ^a	98.8±8.2 ^a	98.4±6.0 ^a
Liver ORAC (mM TE/g)	3.59±0.25 ^a	4.16±0.32 ^a	3.96±0.35 ^a	3.99±0.34 ^a	3.88±0.30 ^a

Table 6: Blood glucose, final weight, food intake, locomotor activity and ORAC value in plasma and liver of CD-1 mice in different groups, RBC: red blood cells; ORAC: oxygen radical absorbance capacity assay; TE: Trolox equivalents; All values are means ± SEM (n = 10, except RER where n = 4). Values in the same row with different superscript symbols are significantly different, using LSD test (P < 0.05).

SOD, GSH-px and CAT have been recognized and used in the evaluation of oxidative stress in animal model with hypertension, hyperlipidemia, obesity, and diabetes (Saiki et al. 2007). SOD, is an antioxidant enzyme, converts superoxide radicals $O_2^\bullet$ to H_2O_2 , which will then be converted to H_2O based on the activity of GSH-px and catalase, thereby protecting the body from oxygen toxicity. From the current results in the red blood cells, after 21 days on experimental diets SOD activity of mice in group 3 (HF + 10mg OPH/g of HF) was lower compared to activity of those in group 2 (HF + 1mg OPH/g of HF). It was found that in RBC, SOD level of HF fed mice was lower compare to ND, however supplementation with 100 mg OPH/g (group 4)

brought the activity similar to ND group (Figure 29A). This was in agreement with literature data indicating that HF diet cause oxidative stress. The increase of SOD activity in HF+100 mg OPH/g is likely to detoxify excess $O_2^{\bullet-}$ radicals to lesser reactive H_2O_2 (Hodgson and Fridovich 1975), after conversion of $O_2^{\bullet-}$ in to H_2O_2 by SOD, CAT and GSH-Px converts H_2O_2 to H_2O . Catalase (CAT) activity was determined in red blood cells and it was found that the CAT activity in the RBC samples of mice in groups 3 (HF + 10mg OPH/g of HF), 4 (HF + 100 mg OPH/g of HF) and ND were similar, but lower than the activity in HF group, and group 2 (HF + 1mg OPH/g of HF) (Figure 29B). One reason could be gradual increase in hydrogen peroxide concentrations (Furukawa et al. 2004). There was a correlation $R^2=0.99$ between increasing amount of OPH and CAT activity; however this correlation was not seen in the SOD activity. A decrease in CAT activity also has been seen in rats that were in hyper and hypo-energetic diet in combination with the antioxidant molecule lycopene (Moreira et al. 2005).

Figure 29: SOD and CAT level in the RBC of CD-1 mice. Group 1 is high fat diet (HF); Group 2 is HF + 1mg of oat protein hydrolysate (OPH)/g of HF; Group 3 is HF + 10mg OPH/g of HF; Group 4 is HF + 100mg OPH/g of HF and group 5 is regular mouse chow or normal diet (ND). Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

5.3 Effect of OPH on liver

Increase intake of HF diet has been shown to result in accumulation of fat or ROS production in the liver and this can compromise its ability to regulate body energy homeostasis (Dobrian et al. 2001). An increase of ROS will cause inflammation that could lead to non-alcoholic liver disease (Milagro et al. 2006). It is therefore of interest to investigate if the addition of OPH to HF diet will affect oxidative stress markers of fats in this organ. Results from CD-1 mice in the current study showed OPH addition had no effect on several markers including total antioxidant capacity (i.e. ORAC), total sulfhydryl groups and peroxides (table 6 and figure 30). Regarding liver peroxides, there are conflicting data in the literature as some studies have reported increased levels in rats fed HF diet (Milagro et al. 2006) while others found no change (Furukawa et al. 2004). In rats on high energy diets supplemented with an antioxidant tomato powder, a decrease in concentration of malondialdehyde, an end product of lipid peroxidation was obtained but no change was observed in CAT activity in rat livers (Moreira et al. 2005).

The effect of OPH on the activity of two antioxidant enzymes SOD and CAT was investigated. SOD activity on mice on HF diet alone was 13.2% lower than that of those on ND diet confirming the fact that HF increased oxidative stress in the liver (Demori et al. 2006). Addition of OPH 100 mg/g to HF diet brought SOD activity to 53.0 ± 1.9 units/g protein and this was similar ($p > 0.05$) to ND level (57.3 ± 2.5 units/mg protein) (Figure 31A). The activity of SOD (45.3 ± 2.3 units/ mg of protein) for mice on HF + 1mg OPH/g was similar to HF diet only group. However, for reasons non yet clarified, the addition of 10 mg OPH/g lowered ($p < 0.05$) the activity of SOD more than any other group. In a previous study, melon juice extract SOD data are comparable to those of a previous study where a melon juice extract added to HF diet

fed rats increased their liver Mn-SOD activity to similar level of those rats on the standard diet however, the same study reported that the Cu-Zn SOD activity was lower in the group of standard diet compared to those on HF diet and melon extract did not affect SOD activity (Dcord et al. 2010). CAT activities in the liver for all the groups were similar except HF + 1mg OPH/g group that had higher activity (Figure 31B). There were slight increases in peroxide values in HF + 10 mg OPH/g and HF + 100 mg OPH/g groups relative to HF + 1mg OPH/g which although not significant may explained the higher activity of CAT for mice in HF + 1mg OPH/g (figure 30). There was no correlation in SOD activity and the amount of OPH added to the HF diet, however CAT activity decreased with a correlation of $R^2=0.83$. Gallic acid has shown to reduce body weight and liver oxidative stress in rats on HF diet by increasing glutathione peroxidase, this enzyme detoxifies peroxides (Hsu and Yen 2007). There was no difference in SOD or CAT activities in the liver of rats in HF high sucrose diet supplemented with polyphenols (Feillet-Coudraya et al. 2009) while bran xylooligosaccharides increased liver SOD activity of rats on HF diet (Wang et al. 2011), Soy protein isolates added diet of obese rats reduced liver oxidative stress by increasing the activity of enzymes involved in lipid metabolism (Aoyama et al. 2000). Different concentrations of the antioxidant molecule chicoric acid showed no effect on the weight organs like heart, lung and brain however, it increased SOD and CAT levels in the liver of mice on HF diet supplemented with chicoric acid (60 mg/kg/d) and decreased malondialdehyde (MDA) level, a marker for lipid peroxidation (Xiao et al. 2013). In CD-1 mice used in the present study no difference was found between CAT activities of HF only and ND diet although in other models CAT activity has been found to be lower in HF diet group (Demori et al. 2006). One reason may be that CD-1 mice were on the experimental diet for three weeks compared to four weeks ((Demori et al. 2006).

Figure 30: Peroxide level ($\mu\text{M H}_2\text{O}_2/\text{g}$) from FOX assay in the liver of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Figure 31: SOD and CAT activity in the liver tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error). Group 1 is High fat diet (HF); Group 2 is HF + 1mg of oat protein hydrolysate (OPH)/g of HF; Group 3 is HF + 10mg OPH/g of HF; Group 4 is HF + 100 mg OPH/g of HF and group 5 is regular mouse chow or normal diet (ND).

5.4 Measurement of Body Weight, Blood Glucose and Respiratory Exchange Ratio

As it was expected, the average body weight of mice that were on HF diet was higher compared to those on ND, however, supplementation of OPH to HF diet had no influence on the body weight (table 6). Looking at the energy intake, animals consuming HF and HF+OPH had higher energy intake (kcal/day) compared to CD-1 mice on ND. Figure 32 shows changes in mice weight over the three weeks on experimental and ND diets

Figure 32: Body weight of the CD-1 mice, during the baseline diet as well as 21 days that animals were on the experimental diet. Group 1 is high fat diet, group 2 is high fat diet +1mg OPH/ g food, group 3 is high fat diet +10mg OPH/ g, group 4 is high fat diet + 100 mg OPH/ g, and group 5 is normal diet.

The ratio of carbohydrate oxidation to lipid oxidation assuming (protein oxidation is negligible) is the respiratory exchange ratio (RER). It is also defined as the volume of CO₂ expired (VCO₂) over the volume of O₂ inspired (VO₂) is called Respiratory exchange ratio (Martin et al. 2006).

$$\text{RER} = \text{VCO}_2 / \text{VO}_2$$

The volume VCO₂ or VO₂ is derived from the number of moles of oxygen consumed and carbon dioxide exhaled during the production of energy from the two main groups of energy yielding molecules. When the energy is mainly produced from carbohydrates, the equation and RER values are as followed:

$$\text{RER} = \text{VCO}_2 / \text{VO}_2 = 6 \text{ CO}_2 / 6\text{O}_2 = 1.0$$

In the case when most energy is from the oxidation fatty acids, the equation and RER are:

$$\text{RER} = \text{VCO}_2 / \text{VO}_2 = 16 \text{ CO}_2 / 23\text{O}_2 = 0.7$$

In many cases, energy is produced from a combination of fats and carbohydrates. It is therefore not uncommon to find RER values between 0.7 and 1.0. In this study the RER of the twenty animals (n = 4) in metabolic chambers (TSE systems, Chesterfield, MO, USA) were measured to see if OPH contribute to changes in body metabolism. Mice on HF diet had an RER value of 0.81±0.01 and this was significantly lower compared values on mice in HF + 10 mg and HF + 100 mg OPH/g suggesting that OPH prevented metabolic change that was produced by consuming a diet rich in fat. This change was not due to locomotor activity of the CD-1 mice

(Figure 33). It is unclear whether the increase RER is beneficiary since in some cases increase RER activity could be due to increase in body weight (Seidell et al. 1992). In this study CD-1 mice were on chronic high fat diet that would change the metabolism to utilize the main substrate of the diet (i.e. fats). The change was partly prevented when the HF diet was supplemented with OPH.

Figure 33: Respiratory exchange ratio of the animals. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Glucose is a source of energy for body's cells, and is transported via blood stream from the intestines or liver to the body's cells, and it is made available for cell absorption via the hormone

called insulin. Normal glucose level for humans is 5-8 mmol/L (Del Guerra et al. 2009). According to literature, high fat diet increases the blood glucose compared to the normal diet (de Melo C et al. 2010). In this study, group 3 (HF + 10mg OPH/g of HF) blood glucose was significantly higher than blood glucose for animals in the other groups ($P < 0.05$) diet (Figure 34). The blood glucose was measured in non-fasted mice, at the last day of the experiment. Given the results from RER, this could be due to OPH induced changes in metabolism that increased the utilization of carbohydrates, by easing release of glucose, as well since these mice were not fasted overnight, the change in blood glucose could be due to nutrients consumed recently.

Figure 34: Effect of the OPH on glucose of the mice in mM/L. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

5.5 Advanced oxidation protein products (AOPPs) level in the brain, plasma, heart, and the liver tissue of CD-1 mice.

Advanced oxidation protein products (AOPPs), are terminal products of protein exposure to free radicals and therefore markers of degree of protein damage in oxidative stress (Witko-Sarsat et al. 1996). Studies have shown that AOPP are involved in renal complications (Furuya et al. 2009) and cardiovascular diseases (Marsche et al. 2009) . Increased levels of AOPP have also been associated with chronic kidney disease (Witko-Sarsat et al. 1998). In the present study, AOPP levels were significantly lower in brain tissue of mice in group 3 (HF + 10mg OPH/g of HF diet) compared to those in group 1 (HFD), group 2 (HF + 1mg OPH/g of HF), group 4 (HF + 100 mg OPH/g of HF), and normal diet group (figure 35).

Figure 35: AOPP level in the brain of CD-1 mice in $\mu\text{mol/mg}$ protein. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Surprisingly, in plasma, AOPP level of mice on normal diet was higher (133 $\mu\text{mol}/\text{mg}$ tissue) compared to HF diet (159.87 $\mu\text{mol}/\text{mg}$). It then appeared that HF diet lowered the oxidation of plasma proteins by 16.8%. The AOPP level was further lowered in animals in group 4 mice (HF + 100 mg OPH/g of HF diet) to 78.23 $\mu\text{mol}/\text{mg}$, this value is 41.2% reduction than group 1 (HFD) (figure 36).

Figure 36: AOPP level in the plasma of CD-1 mice, in $\mu\text{mol}/\text{mg}$ tissue. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Heart AOPP levels were significantly lower in group 3 (HF + 10mg OPH/g of HF) compare to group 1 (HFD) (figure 37). AOPP level was high in HF diet compare to the normal diet, showing that HF diet increases the AOPP level in the heart, however addition of the OPH in 1mg OPH/g of HF decreases the AOPP level from 167.78 $\mu\text{mol/ mg tissue}$ to 146.51 $\mu\text{mol/ mg tissue}$, about 12.8% and addition of 10 mg of OPH/g of HF further lowers the AOPP level to 95.58 $\mu\text{mol/ mg tissue}$, the AOPP level decreased by about 43% however addition of more OPH to the HF diet group 4 (100 mg OPH/ g of HF) increased the AOPP level to 143.45 $\mu\text{mol/ mg tissue}$.

AOPP level in the heart of the mice on HF, as well as the AOPP level in the brain of the mice was slightly higher in the HF diet than the ND (figure 35, 37).

AOPP level in liver was measured however the results were not significantly different (table 7). In a study where they used a melon juice extract, that is rich in superoxide dismutase to see if it will prevent obesity induced by high fat diet in hamsters, The AOPP level in the liver of hamster was measured and it was found that AOPP level in the animals on the HF diet had higher AOPP level compared to the standard diet, and administration of the melon juice extract decreased the AOPP level close to the standard diet animals. The reason for the difference in our result and this research paper is that the animals in the research paper were on the experimental diet for 12 weeks; however, our animals were on the diet for 3 weeks (Décorde et al. 2010).

Group		AOPP $\mu\text{mol/ mg tissue}$
1	High fat diet	174.38 $\pm$ 16.60 a
2	High fat diet + 1mg OPH/g food	215.61 $\pm$ 33.11 a
3	High fat diet + 10mg OPH/g food	243.94 $\pm$ 34.89 a
4	High fat diet + 100 OPH mg/g food	231.55 $\pm$ 27.19 a
5	Normal diet	204.10 $\pm$ 32.80 a

Table 7: AOPP level in the liver of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Figure 37: AOPP level in heart of CD-1 mice, in $\mu\text{mol}/\text{mg}$ tissue. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

5.6 NO (nitrite + nitrate) level in brain, liver, and the heart tissue.

NO is a messenger molecule that functions as a neurotransmitter, and other processes, it is synthesized by NO synthase (NOS) from L-arginine. Impaired production of NO is associated with diseases such as vascular functions. Neurodegenerative disorders, diabetes mellitus,

cerebral infarctions are associated with over production of NO. Reduced levels of NO also are associated with endothelial dysfunction (Mori et al. 2006). In this study the NO (nitrite + nitrate) in $\mu\text{mol/g}$ tissue was measured using an assay kit. It was found that brain NO levels were significantly higher in group 1 (HFD) compare to rest of the groups. Addition of 1 mg OPH/ g of HF decreased the NO level from 135.95 $\mu\text{mol/ g}$ tissue to 91.66 $\mu\text{mol/g}$ tissue, which is about 32% lower. The NO level is lower in group 3 (HF + 10mg OPH/g of HF) by 37% compared to the group 1 (HFD). The NO level is increased in group 4 (HF + 100 mg OPH/g of HF), to 113.15 $\mu\text{mol/g}$ tissue, however this value is still lower than 135.95 $\mu\text{mol/g}$ tissue in group 1 (HFD). NO level in ND groups were 94.15 $\mu\text{mol/g}$ tissue, and this value is similar to group 2 (HF + 1mg OPH/g of HF), and 3 (HF + 10mg OPH/g of HF) NO level which is 91.66 $\mu\text{mol/g}$ tissue, and 85.51 $\mu\text{mol/g}$ tissue. From this results it can concluded that the NO levels are increased in HF diets compare to ND, however addition of OPH in 1mg OPH/ g of HF diet, and 10 mg OPH/ g of HF diet decreases the NO level similar to the ND (figure 38).

Figure 38: NO (nitrite + nitrate) level in the brain tissue of CD-1 mice in $\mu\text{mol/g}$ tissue.

Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Liver NO levels show that animals in group 3 (HF + 10mg OPH/g of HF) have higher NO levels compared to the rest of the groups. ND group animals have the lowest NO level, which is $43.01 \mu\text{mol/g}$ tissue, and animals on HF diet have NO level of 68.87 which is significantly higher than the animals on ND diet. Addition of 100 mg of OPH/g HF showed no difference compared to ND (figure 39).

Figure 39: NO (nitrite + nitrate) level in the liver tissue of CD-1 mice in $\mu\text{mol/g}$ tissue. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Heart NO levels, as well as nitrite levels in the heart was measured and it was found that there was no significant difference between the different groups in both NO level as well as nitrite level in the heart tissue (Table 8).

Group		NO (nitrite + nitrate) ($\mu\text{mol/g}$ tissue)	Nitrate $\mu\text{mol/ mg}$ tissue
1	High fat diet	42.63 $\pm$ 6.40 a	4.04 $\pm$ 5.56 a
2	High fat diet + 1mg OPH/g food	64.95 $\pm$ 20.93 a	4.01 $\pm$ 3.86 a
3	High fat diet + 10mg OPH/g food	43.00 $\pm$ 13.01 a	3.50 $\pm$ 8.16 a
4	High fat diet + 100 mg OPH/g food	44.47 $\pm$ 9.80 a	1.62 $\pm$ 4.38 a
5	Normal diet	65.76 $\pm$ 18.27 a	4.16 $\pm$ 2.88 a

Table 8: Nitrite+ nitrate $\mu\text{mol/g}$ tissue, and Nitrate $\mu\text{mol/ mg}$ tissue in the heart tissue. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

5.7 Effect of OPH on oxidative stress markers and vitamins A, E, and C levels in the brain tissue

Vitamins A, C, and E are essential nutrients that can also play a role in oxidative stress. In a previous study, the effect of vitamin A, E, C and E+C (15 mg/kg of body weight) on brain oxidative stress was investigated in rats exposed to restraint stress (Zaidi and Banu 2004). It was found that restraint stress resulted in a decrease of GSH concentration and activities of SOD, GST and catalase and that both pre-stress and post-stress vitamin treatments (alone or combined) resulted in alteration of these parameters towards their control values (Zaidi and Banu 2004). In vivo biological anti-oxidative properties of OPH in brain or other organs can therefore preserve the amount of these vitamins.

Oxygen radical absorbance capacity (ORAC), peroxides levels, total thiols, vitamins, A, E, and C levels in the brain tissues were not significantly different between the groups ($P < 0.05$) (table 9, 10).

Group		FOX $\mu\text{M H}_2\text{O}_2$ /mg of sample	Total GSH (μM cysteine/ mL)	ORAC Value Trolox μM $\mu\text{L/g}$
1	High fat diet	13.24 $\pm$ 3.04 a	5.30 $\pm$ 0.42 a	10.16 $\pm$ 1.16 a
2	High fat diet + 1mg OPH/g food	14.67 $\pm$ 2.52 a	5.05 $\pm$ 0.29 a	9.82 $\pm$ 0.80 a
3	High fat diet + 10mg OPH/g food	13.36 $\pm$ 1.76 a	4.61 $\pm$ 0.24 a	9.47 $\pm$ 1.02 a
4	High fat diet + 100 mg OPH/g food	12.62 $\pm$ 2.23 a	5.07 $\pm$ 0.45 a	9.76 $\pm$ 1.32 a
5	Normal diet	13.05 $\pm$ 1.53 a	5.14 $\pm$ 0.33 a	10.58 $\pm$ 0.91 a

Table 9: Peroxide level, total GSH and the ORAC value in the brain tissue of CD-1 mice.

Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Group		Vitamin A µg/g of tissue	Vitamin E µg/g of tissue	Vitamin C µg/g of tissue
1	High fat diet	6.27±0.34a	6.35 ± 0.28a	1101±94.20a
2	High fat diet + 1mg OPH/g food	5.72±0.48a	5.36 ±0.30a	1282±135.58a
3	High fat diet + 10mg OPH/g food	6.55±0.48a	5.41 ± 0.25a	1307±135.30a
4	High fat diet + 100 mg OPH/g food	6.01±0.62a	5.79 ±0.40a	1370±70.07a
5	Normal diet	6.16±0.45a	5.38 ±0.29a	1199.19±102.41a

Table 10: Vitamin A, E, and C µg/ g of tissue, in brain tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean± standard error).

5.8 Vitamin A, Vitamin A palmitate, Retinol equivalent level, and vitamins C, E level in the liver tissue of CD-1 mice

Vitamin A level in the animal liver was measured and it showed that animals on group 5 (ND) had the highest vitamin A compared to the rest of the groups. Animals in group 1 or the HF group had slightly higher vitamin A compared to the animal that the OPH was added to their diet, but this finding was not significantly different (Figure 40).

Figure 40: Vitamin A ($\mu\text{g/g}$ of tissue) level in the liver tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Vitamin A palmitate in the liver samples showed that group 5 (ND) animals had the highest vitamin A palmitate, compared to the rest of the groups, and HF diet lowered the vitamin A palmitate level in the liver samples by 14% compared to the ND, and addition of the 1mg OPH/g of food lowered the vitamin A palmitate level from 619.11 $\mu\text{g/g}$ of tissue to 445.53 $\mu\text{g/g}$ of tissue, which is 28% lower compared to the HF diet and, addition of 10 mg OPH/g of HF food and 100 mg OPH/g of HF food had similar vitamin A palmitate to the HF group (Figure 41).

Figure 41: Vitamin A palmitate level in the mice liver. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Measurement of retinol equivalent level in the liver samples showed that ND had the highest retinol level in the liver, and HF diet lowered the retinol level from 478.6 $\mu\text{g/g}$ of tissue to 371.99 $\mu\text{g/g}$ of tissue or by 22%. Addition of OPH had no effect in the retinol level in the liver tissue (Figure 42).

Figure 42: Retinyl equivalent levels in the liver. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

In my study, vitamin C measurement in the liver tissue showed that both group 1 (HFD) and group 5 (ND) had similar vitamin C level in the liver tissue; and addition of the OPH to the HF diet increased the vitamin C level in the liver tissue. Group 5 (ND) animals had vitamin C level of 484.57 ± 16.96 µg/g tissue, and group 1 (HFD) had vitamin C level of 466.89 ± 20.38 µg/g tissue. Addition of the OPH in 1 mg OPH/ g of HF food, 10 mg OPH/g of HF food, and 100 mg OPH/g of HF food increased the vitamin C level to 646.2 ± 27.06 µg/g tissue, 627.47 ± 26.86 µg/g tissue, and 623.07 ± 30.06 µg/g tissue respectively. Figure 44 represents the vitamin C level in the liver tissue. Since supplementation of OPH increased the vitamin C levels in the liver tissue, it

can be concluded that some peptides in OPH with radical scavenging activities may be bioavailable and this resulted in preserve of vitamin C. Alternatively, OPH may have been further digested into antioxidant amino acids that was absorbed and produced the observed results.

Figure 43: This figure represents the vitamin C level in the liver tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Vitamin E protects membranes from free radicals, and it turns in to a vitamin E radical. There is evidence that vitamin E and Ascorbate interact together, and Ascorbate regenerates vitamin E

from vitamin E radical. In this study, vitamin E level in the liver tissue was measured and it was that group 5 (ND) animals had the lowest vitamin E in their liver tissue. Animals in group 1 (HFD) had vitamin E level of 57.3 ± 8.42 $\mu\text{g/g}$ of tissue and group 5 (ND) animals had vitamin E level of 28.33 ± 8.5 $\mu\text{g/g}$ of tissue. Group 5 (ND) had 50% lower vitamin E in their liver compared to group 1 (HFD). Addition of OPH to the animal diet increased the vitamin E level in the animal liver, however the increased compared to the HF diet was not significant, but compare to the ND diet it was significant. Figure 45 represent the animal vitamin E level in the liver tissue. Since the vitamin E level was higher in all groups that were on high fat diet, it can be concluded that the high fat diet contains vitamin E, and this can be the reason for the increase of the vitamin E, in group 1, 2, 3, and 4. The high fat diet was based on AIN-93 formulation and this diet contains 15.0 g/kg of vitamin E.

Figure 44: Vitamin E level in the liver tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

5.9 Vitamin A, vitamin E, retinol equivalents and vitamin C levels in the lung tissue of CD-1 mice

Vitamin A, vitamin E, retinol equivalents and vitamin C levels in the lung tissue was measured and it was found that there was no difference between the different groups (table 11). However vitamin A palmitate level in the lung tissue showed that group 1 (HFD) animals had the highest vitamin A palmitate level in the lung tissue. Addition of the 1 mg of OPH/ g of HF food lowered the vitamin A palmitate level from 61.99 $\mu\text{g/g}$ of tissue to 28.61 $\mu\text{g/g}$ of tissue, and addition of 10 mg of OPH/g of HF food increased the vitamin A palmitate level similar to the HF group. However, addition of the 100 mg OPH/ g of HF food decreased the Vitamin A palmitate level to 31.78 $\mu\text{g/g}$ of tissue. Vitamin A palmitate level in animals on ND, was slightly lowered than HF groups, but this decrease was not significantly different (figure 43).

Group		Vitamin E $\mu\text{g/g}$ of tissue	Vitamin A $\mu\text{g/g}$ of tissue	Retinol equivalent $\mu\text{g/g}$ of tissue	Vitamin C $\mu\text{g/g}$ of tissue
1	High fat diet	13.05 $\pm$ 2.18a	21.82 $\pm$ 21.82a	52.96 $\pm$ 8.28a	850.65 $\pm$ 53.16a
2	High fat diet + 1mg/g food	27.62 $\pm$ 10.72a	20.35 $\pm$ 3.46a	33.42 $\pm$ 5.46a	774.49 $\pm$ 44.01a
3	High fat diet + 10mg/g food	11.84 $\pm$ 2.19a	28.09 $\pm$ 4.02a	50.17 $\pm$ 7.74a	841.92 $\pm$ 54.08a
4	High fat diet + 100 mg/g food	14.83 $\pm$ 4.46a	22.12 $\pm$ 2.40a	39.46 $\pm$ 5.50a	812.34 $\pm$ 33.58a
5	Normal diet	10.26 $\pm$ 1.35a	30.34 $\pm$ 5.26a	62.96 $\pm$ 10.94a	779.49 $\pm$ 33.78a

Table 11: Vitamin A palmitate, Retinol equivalent, vitamins E, and C $\mu\text{g/g}$ of tissue, in lung tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

Figure 45: Vitamin A palmitate level in the lung tissue of CD-1 mice. Statistical differences were evaluated by one-way ANOVA using SPSS 11.0 for windows 7 (SPSS Inc., Chicago). Values with $P < 0.05$ were considered significantly different between the groups using the LSD test (mean $\pm$ standard error).

5.10 Further discussion of the effect of OPH supplementations

OPH was added to the HF diet of CD-1 mice in three different concentrations. The advantage of addition of hydrolyzed protein versus addition of non-hydrolyzed protein, was that hydrolyzed proteins or OPH had higher antioxidant activity relative to non-hydrolyzed proteins, as it was discussed in chapter 3. Concentrations of OPH used are within the range of those in other studies where animals were fed high fat diets (Nadeem et al. 2012; Décordé et al. 2010; Khalid and Siddiqui 2012; Li et al. 2011a). Hydrolyzed proteins consumed can be absorbed more quickly

and exert better biological function than intact proteins which must first be cleaved by digestive proteases. The HF diet used in this study contained 23.5% proteins and adding 100 mg OPH/ g of OPH brings the proteins content to 25.9%. This is still in the range of 10-30% proteins recommended by Health Canada (Trumbo et al. 2002). Proteins and their hydrolysates are used in nutritional products like granola bars and in beverages. Some granola bars contain 11 g of proteins.

OPH that prevented the formation of lipid peroxides and scavenged radicals in the in-vitro experiments was added to CD-1 mice fed high fat diet. OPH supplementation had no effect on bodyweight despite the fact that the higher level provided additional 10% energy in the form of proteins. Most rodents generally tend to become obese on high-fat diets; however, the response to weight gain can vary. Mice strains such as C57Bl6 or AKR are more susceptible to obesity when fed high-fat diets compared to CD-1 mice (Rossmeisl et al. 2003). If the study was conducted for more than three weeks, differences would have been observed in weight after supplementation with OPH. Contrary to weight, addition of OPH to HF diet did increase the respiratory exchange ratio (RER). The increase was not due to an increase in mobility as there was not a change in locomotor activity, but can be attributed to the fact that mice on OPH used more carbohydrates or the extra protein from OPH to produce energy although protein oxidation is generally considered negligible.

The majority of oxidative stress markers were measured mainly in blood and liver but a few were quantified in brain and heart samples. In red blood cells, it was found that, 100 mg OPH/g increased the total antioxidant capacity (ORAC) and the activity of SOD enzyme. Superoxide

($O_2^{\bullet-}$) which is very reactive is converted by SOD to lesser reactive H_2O_2 which can then be converted into water. The amount of H_2O_2 in plasma was higher ($p < 0.05$) but surprisingly the activity of CAT that can catalyze its conversion to water was lower. Glutathione peroxidase (GPx), another enzyme that can detoxify H_2O_2 was not determined in this study; however, the elevated amount of thiol group found in blood may be useful for the detoxification of H_2O_2 and for the increase of total antioxidant capacity. The higher concentration of H_2O_2 did not lead to protein oxidation as the concentration of advanced oxidation products of proteins was reduced in mice that received 100 mg OPH/g.

The vast majority of oxidative markers were measure in liver because as a main detoxifying organ, it could be prone to oxidative damage. No OPH concentration tested affected total antioxidant capacity, hydrogen peroxides, protein oxidation products, nitric oxide, vitamins A or E levels. The only notable change was in the vitamin C. All three concentrations of OPH added to the HF diet increased liver vitamin C ($p < 0.05$) content in a non-dose dependent manner. Vitamin C had other functions, apart from preventing oxidative damage. It is for example important for growth synthesis of collagen, a protein used in skin, cartilage, tendons, ligaments, and blood vessels (Boyera et al. 1998). It is also involved in the hydroxylation of proline residues in different proteins (Brody 1999). Absorbed peptides from OPH and the extra amino acids provided may have contributed to the increase of vitamin C.

Chapter 6

Conclusion and Future work

Conclusion

APH, TPH, and their ultra-filtered fraction were shown to have different ferrous ion-chelating and radical scavenging activities. The highest radical scavenging activity on DPPH was demonstrated by APH 2kDa, at 15, 45, and 30 min. Better ferrous ion-chelating properties were demonstrated by the 200 g/mL TPH, 43.8% compared to APH which had a 31.8% of ferrous ion-chelating properties. Linoleic acid autooxidation formation was equally inhibited by both TPH and APH. From this finding, it can be concluded that by enzymatic digestion of oat flour, peptides that have radical scavenging properties can be produced, and it can be used in food products to prevent oxidative rancidity, as well as increasing the nutritional values. Therefore the investigation of the structure, activity, and their mechanistic would be beneficial to study.

Results from the extraction of protein using different cell wall carbohydrase degrading enzyme with different concentrations, showed increase protein content except the samples treated with celluclast. The ORAC or the peroxy radical scavenging activity was significantly higher for at least one concentration of the enzymes compared to the sample that were not treated with enzyme (Control). The sample that was treated with viscozyme followed by Alcalase, had both higher peroxy radical scavenging as well as hydroxyl radical scavenging activity. Mass spectrometry helped us to identify four different novel peptides from the control and the sample that was treated with viscozyme. From all these results in this study, it can be concluded that both enzymes viscozyme and amyloglucosidase, can be used in the preparation of protein isolates and hydrolysates with improved antioxidative properties. The biological activities such as anti-oxidant, and anti-inflammatory properties of these isolated peptides should be evaluated in the future studies, as well as fractionation of the viscozyme pretreated digests and the control can be helpful in identifying the peptides with potential biological functions.

In this study, results from mice consumed HF diet in combination of OPH, showed that the radical scavenging activity in RBC increased due to synthesis of thiol containing peptides. Also SOD activity has shown to be increased in RBC as well as slight increase in CAT and SOD activity in the liver has been observed. Since there was opposing results regarding concentration of OPH for example 1 and 100 mg/OPH/g compared to 10 mg OPH/g, further study is needed to find the optimum concentration.

AOPP level, was lowered significantly after addition of OPH to the HF diet in the brain and the heart tissues, NO level was significantly lowered in the brain tissue after addition of the OPH, and vitamin C level was increased significantly after addition of the OPH.

Future work

In this study, the antioxidant capacity of the oat proteins digested with alcalase as well as trypsin was examined. Hydrolysates with proteins were the most active and then subsequently tested in vivo in CD-1 mice fed high fat diets. There are to be no dose dependent and also some contradictory data between the three concentration tested and this could be because of the short duration of the study. Futures studies can therefore:

- 1) Be performed for 6 weeks instead of 3 weeks (animal will gain more weight) and measure liver weight, enzymes involved in lipid metabolism in addition to antioxidant enzymes
- 2) Use other models like AKR can be used because of their ability to gain weight more rapidly
- 3) Determine the sequence of all peptides present in OPH and well as its free amino acid content.

In chapter 4, different carbohydrases were used to break down cell wall polysaccharides and increase protein extraction and antioxidant activities of proteins hydrolyzed with alcalase.

Tandem mass spectrometry was used to identify the sequence of peptides. Preliminary data allow

the identification of 4 peptides from analysis of double charged peaks only. Future investigation can

- a) Tested the antioxidant activity of the four identified peptides and determine their contribution to the activity of the hydrolysates
- b) Investigate the bioavailability of the four peptides and their ability to prevent oxidative stress in cultured intestinal cells
- c) Identify peptides in other samples including those that are single charged

Chapter 7

References

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