

Inhibitory effects of hydrolyzed oat bran proteins on human LDL oxidation and their bile acid binding capacity

By

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Abstract

Free radicals attack to biological molecules can initiate their oxidation and contribute to the development of several chronic diseases. Oxidation of low density lipoprotein, as well as a higher concentration of cholesterol, have been implicated in the development of atherosclerotic lesions and increased risks of cardiovascular diseases. Strategies to decrease the risks then include scavenging of free radicals and the reduction of cholesterol (e.g. binding of bile acids). In this regard, the aim of this project was; 1) to produce and characterize various hydrolyzed proteins from oat brans; 2) determine their antioxidant, metal binding and bile chelating capacities as well as their ability to inhibit low density lipoprotein oxidation (LDL). Medium oat bran samples were treated with two cell wall polysaccharide degrading enzymes (cellulase and viscozyme) to generate two protein isolates named CPI and VPI respectively. Ten hydrolysates were subsequently prepared by treating CPI or VPI with five proteases protamex, alcalase, flavourzyme, pepsin and pepsin+pancreatin. The two highest degrees of hydrolysis (26.4 ± 0.5 and $22.0 \pm 0.1\%$) were obtained under simulated gastrointestinal digestion with pepsin and pancreatin. Intrinsic absorbance showed that CPI-alcalase and CPI-pepsin/pancreatin had a higher content of aromatic amino acids. In the antioxidant oxidant assays, VPI-pepsin better scavenged ROO[•] radicals (496.77 ± 5.83 μ M Trolox Equivalents (TE)/g) while VPI-flavourzyme and VPI-pepsin had better quenching for HO[•] (27.95 ± 1.580 and $45.31 \pm 6.6\%$) and O₂^{-•} radicals, respectively. In the metal chelation assays, most of the samples except Pepsin hydrolysates were able to chelate iron in a dose dependent manner, whereas VPI-protamex exhibited the best copper chelating capacity ($59.83 \pm 1.40\%$). All hydrolysates protected human low-density lipoprotein against copper mediated oxidation by reducing the concentration of hydroperoxides from 158.4 to 74.4 – 97.7 μ M H₂O₂/g of LDL. In the bile acid binding assay, none of the hydrolysates bind to chenodeoxycholic acid while there was a binding of up to 46.3% for taurodeoxycholate and taurocholate. Overall VPI alcalase proteins displayed the highest activity in most assay and consequently separated into eleven fractions (F1–F11) by high performance liquid chromatography (HPLC). The separation increased the ROO[•] quenching of F7 and F11 by 2.3- and 2.5-fold but also their copper chelation by 8 and 22%. There was no change in bile acid binding capacity after separation; meanwhile, there was an enhancing effect on the ability of F4, F7 and F9 to inhibit LDL oxidation. In summary, hydrolyzed oat bran proteins reduced oxidative and oxidation of LDL furthermore, separation can enhance the effects.

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Introduction

The oxidation of molecules, such as lipids, proteins, pigments, and aroma compounds can be triggered by light, oxygen or metal ions. It is a major cause of deleterious quality changes in food products, affecting flavor, color and texture, and also leading to the loss of nutritive value (Bamdad, Wu, & Chen, 2011a). In human, an imbalance caused by overproduction of oxidants exceeding the antioxidant capacity of the organism will lead to oxidative stress, resulting in damage to biomolecules (Adom et al., 2002) (Feng, Du, Li, Wei, & Yao, 2007). Oxidative stress also induces a variety of chronic and degenerative diseases including cancer, cardiovascular disorders, atherosclerosis, diabetes, arthritis, Alzheimer's and Parkinson's disease (Lee, Son, Pratheeshkumar, & Shi, 2012). Atherosclerosis is a major source of morbidity and mortality in the developed world (Stocker, 2004). Free radicals or metals play a causal role in oxidative modification of LDL, which is a critical pro-atherogenic event in the onset of the pathogenesis. At the subendothelial level oxidized LDL is crucial for the formation of atheromatous plaque causing vasoconstriction (Rahman, 2007). It is well established that elevated levels of LDL cholesterol are associated with an increased risk of atherosclerosis (Chandrasekara & Shahidi, 2011). Consequently, the inhibition of LDL peroxidation through supplementation with antioxidants becomes an attractive therapeutic strategy to prevent or delay the development of atherosclerosis in human. Many studies have been devoted to the inhibition of lipid peroxidation in LDL using antioxidants. Recent attention has been focused on antioxidants from natural resources (L. Zhu, Jie, Tang, & Xiong, 2008) such as food protein hydrolysates (Marambe, Shand, & Wanasundara, 2008). Anti-oxidative peptides can be released from their protein precursors by *in vitro* proteolytic processes or by gastrointestinal digestion. During the past decade, the antioxidant activity of protein hydrolysates has been reported from both plant and animal sources (Moure, Domínguez, & Parajó, 2006). Previous works have elucidated the antioxidant activity of some hydrolyzed oat proteins but their potential to prevent LDL oxidation has not been investigated.

An alternative approach to inhibit LDL oxidation relies on the reduction of blood cholesterol. This also results in a critical component in the prevention and treatment of cardiovascular diseases (Tiengo, Motta, & Netto, 2011). Many reports have demonstrated that some molecules, such as peptides may influence bile acids (BAs) binding which are major metabolites of cholesterol and facilitate their elimination in the feces through the formation of micelles that solubilize the

cholesterol in the bile. Within the intestinal lumen, BAs interact with lipases and assist the lipolysis and absorption of fats (Kongo-Dia-Moukala, Zhang, & Irakoze, 2011). Normally, they are entirely reabsorbed in the ileum and then transported to the liver via the enterohepatic circulation by different mechanisms (Lin, Tsai, Hung, & Pan, 2010a). The binding of BAs, (i.e. increasing fecal excretion), has been hypothesized as a possible mechanism by which dietary fiber lowers cholesterol (Tiengo et al., 2011). Since the BAs pool is limited, a higher excretion of bile acids requires an increase of their hepatic synthesis from blood cholesterol (Lin, Tsai, Hung, & Pan, 2010b). Food components that bind BAs prevent their reabsorption and stimulate the conversion of blood and liver cholesterol to additional BAs, thereby significantly reducing liver and blood LDL cholesterol levels (Tiengo et al., 2011). In addition to the role of dietary fibers, hydrolyzed food proteins can bind BAs. This study was conducted because there are no reports in the literature for hydrolyzed oat proteins and their BAs chelating activity.

Chapter 1.0- Literature review

1.1. Bioactive compounds

Cereal grains are excellent sources of numerous unique substances including dietary fiber (arabinoxylans, β -glucans, cellulose, lignin and lignans), sterols, tocopherols, tocotrienols, alkylresorcinols, phenolic acids, vitamins and microelements. Although concentrations of these substances in foods are usually small, they have attracted the attention of researchers because of their biological activities and positive impacts on human health (Bartłomiej, Justyna, & Ewa, 2012). Some are considered to be direct free radical scavengers, while others act as cofactors of antioxidant enzymes and metal chelators, which are desirable health benefits that contribute to reduced risk of developing chronic diseases (Singh & Sharma, 2017). These bioactive components are mostly located in the bran of cereal grains, while the germ is rich in unsaturated fatty acids, and its removal is necessary to prevent processes of lipid oxidation (Edge, Jones, & Marquart, 2005).

1.1.2 Role of phytochemicals, consumption of cereals and health outcomes, different classes of compounds in oats.

Cereals are the world's most important food crop (Guerrieri, 2018). They constitute a major part of human nutrition, being an important source of proteins and energy, particularly in developing countries (Smuda, Mohamed, & Karsten, 2018). According to Eurostat statistics, among the cereals cultivated in Europe, wheat is the largest crop, followed by barley, with oats in the third place. In addition, consumption of whole grain cereals has long been considered to be beneficial to human health (Gangopadhyay, Hossain, Rai, & Brunton, 2015). Epidemiological studies have shown that regular intake of whole grains cereals is associated with reduced risks of various types of chronic conditions such as cardiovascular disease, cancers, type 2 diabetes, and obesity (Kadiri, 2017), (R. H. Liu, 2007). For instance a recent cohort study , reported a consistent association between high whole grain intake and lower risk of type 2 diabetes (an 11% and 7% lower risk per whole grain serving (16 g) per day) for all different cereals (wheat, rye, oats) and whole-grain products tested (rye bread, whole-grain bread, and oatmeal/muesli) for men and women (Kyrø et al, 2018). In the (Larsson et al, 2005) population-based cohort study of approximately 60,000 women, the risk association between whole grain intake and colorectal cancer was analyzed. The results indicated that high intake of whole grains (4.5 servings/day) may reduce the risk of colon

cancer in women, when compared with a low intake (<1.5 servings/day). These benefits of cereals have been associated with the existence of phytochemicals present in them (R. H. Liu, 2007). It remains to be determined whether disease reduction is due to a synergistic or additive effect of these nutritional and biologically active compounds (Slavin, Martini, Jacobs, & Marquart, 1999) (Gangopadhyay et al., 2015).

Phytochemicals also play important structural and defence roles in grains (Adom, Sorrells, & Liu, 2003). Whole grains generally contain diverse combinations of phytochemicals depending on the type of cereal, location within the grain and how the grain has been processed. The outer structures of grains, in particular, the pericarp seed coat and aleurone layers, contain much higher levels of phytochemicals, than the germ and endosperm (Belobrajdic & Bird, 2013).

1.1.3 Oats, constituents and biological activities

Different cereals are prevalent in the diets of different communities in the world. Wheat, rice and maize are the leading grains in terms of consumption. Oat remains an important cereal crop in the developing world, and the most popularly cultivated species is *Avena sativa* (Rasane, Jha, Sabikhi, Kumar, & Unnikrishnan, 2013), it is predominantly grown in American and European countries, mainly Russia, Canada and the United States of America (C. Chen et al., 2018). About 14% of the oats produced in Europe are used for human consumption. Oats have been mainly used as animal feed crop, but only in the 19th century it won acceptance as a part of the human diet, mostly consumed as a whole grain (Gangopadhyay et al., 2015). Its use as animal feed has declined steadily owing to emerging use and interest in oats as human health food (Ahmad, Anjum, Zahoor, Nawaz, & Ahmed, 2010). Oat has a well-balanced nutritional composition. It is a good source of carbohydrates and quality protein with good amino acid balance, also contains a high percentage of oat lipids especially unsaturated fatty acid, minerals, vitamins and phytochemicals (Rasane et al., 2013). The amount of oats used for human consumption has increased mainly because of the benefits associated with the dietary fiber β -glucan but also with phytochemicals such as avenanthramides, a unique groups of polyphenols not present in other grains (Rasane et al., 2013). Oat β -glucans (OBG) (Figure 1.1), are the main component of the soluble fiber in oats, consisting of a linear branched chain of D-glucose molecules bonded by mixed β -(1-3) and β -(1-4) linkages (Menon et al., 2016). This polysaccharide is located in oat aleurone and sub-aleurone endosperm cell walls (Decker, Rose, & Stewart, 2014). Research has shown that OBG is responsible for the

reduction of blood glucose and serum cholesterol then, reducing the occurrence of cardiovascular diseases (Tapola, Karvonen, Niskanen, Mikola, & Sarkkinen, 2005) (Davidson et al., 2016). It is also known to possess antitumor and anti-cancer activity (Choromanska et al., 2015), (Choromanska et al., 2017), (Daou & Zhang, 2012). Blood pressure reduction in hypertensive patients has also been associated with these molecules (Streppel, 2005) (Keenan et al., 2002). Although oat consumption has been associated with reduced risk of these chronic conditions, other factors should be considered to demonstrate its beneficial effect. For example, daily ingestion. Clinical trials have demonstrated that 3g per day of OBG are required to lower LDL cholesterol (Mushtaq et al, 2014). Whole grain processing conditions such as extrusion can reduce the viscous nature of OBG thereby reducing their cholesterol lowering benefit (Chutkan et al, 2012). Also the concentration of β -glucan is variable, depending on cultivar, growing, processing conditions, storage and purification protocol, therefore it is necessary to standardize its content on research conditions in order to claim the apparent health correlations (Clemens & Van Klinken, 2014).

Polyphenolic compounds in oats include phenolic acids, flavonoids and a unique group of amides referred to as avenanthramides (AVAs) (Figure 1.1) (Hitayezu, Baakdah, Kinnin, Henderson, & Tsopmo, 2015). AVAs are low molecular weight soluble phenolic compounds. They consist of anthranilic acid derivatives and hydroxycinnamic acid derivatives. They are constitutively expressed in the kernels, appearing in almost all milling fractions, but occur in the highest concentration in the bran and outer layers of the kernel (C.-Y. O. Chen, Milbury, Collins, & Blumberg, 2007). They have been reported to exhibit strong antioxidant activity both *in vitro* and *in vivo* (L. H. Dimberg, et al., 1993) (Lee-Manion et al., 2009) (Bratt et al., 2003) (L. L. Ji, Lay, Chung, Fu, & Peterson, 2003). AVAs might also possess anti-inflammatory and antiatherogenic properties since they inhibit monocyte adhesion to human aortic endothelial cells and inhibit the release of pro-inflammatory cytokines from macrophages (L. Liu, Zubik, Collins, Marko, & Meydani, 2004). They are also involved in controlling the blood pressure, as they produce nitric oxide which dilates the blood vessels (Nie, Wise, Peterson, & Meydani, 2006). Likewise, oat is a source of tocopherols (Figure 1.1), the main ones being α -tocotrienol and α -tocopherol. Oat germ has high levels of tocopherols (a and c isomers), whereas tocotrienols are mainly concentrated in endosperm but, are absent in germ (Peterson, 2001). They act as strong free radical scavengers and have showed lowering cholesterol abilities (Peterson & Qureshi, 1993). As in the case of OBG, despite the fact that AVAs have exhibited bioavailability, antioxidant (Chen et al., 2007), and anti-

inflammatory (Koenig et al., 2014) properties in humans, they also demonstrated poor stability during steaming, autoclaving, and flaking (Boz, 2015). Consequently processing steps need to be taken into account in order to establish effective relationship regarding their health promoting benefits. Similarly they have demonstrated to contribute to the prevention of atherosclerosis in vitro cell culture system (Nie et al., 2006), however clinical trials are needed to confirm this biological activity in humans.

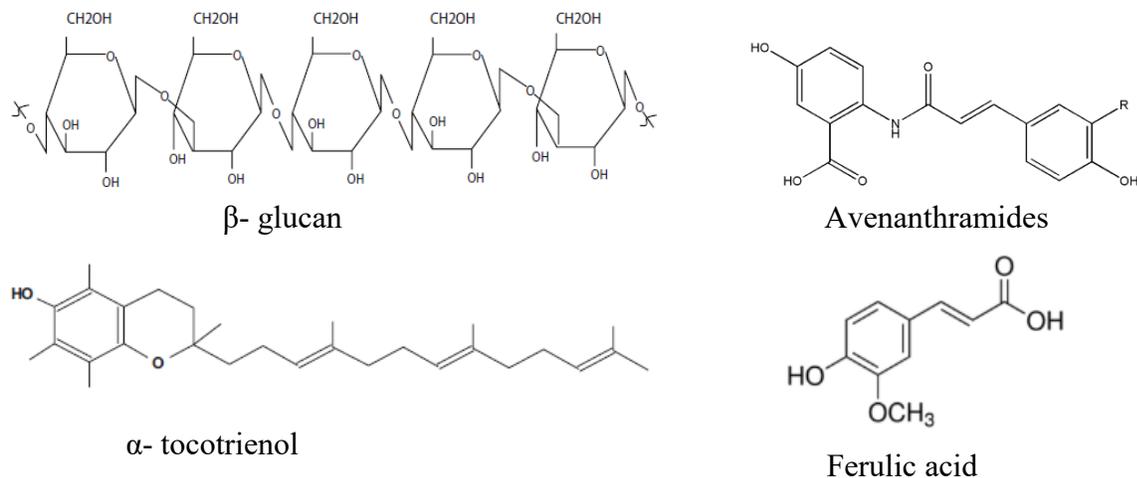


Figure 1.1. - Some phytochemicals found in oats.

The superior nutritional value of oats compared to other cereals have long been recognized since they contain naturally high amounts of valuable nutrients such as proteins (Lásztity, 1998). The proteins have the potential to provide bioactive peptides during processing in the diet (Gangopadhyay et al., 2015). The major group of storage proteins in oats are globulins known as avenalin and make up to 80% total proteins (Cunsolo et al, 2012) whereas prolamins (avenins), account for about 15% (Lásztity, 1998). The consequence of the high-globulin, low-prolamin composition of oat seed explains the better amino acids balance of its proteins (Shotwell et al, 1990). The minor group of proteins, albumins (1–12%) is primarily composed of enzymes. Oats globulin seems to retain considerable native structure even when exposed to prolonged heating (Ma & Harwalkar, 1984). Oats protein isolates have exhibited good emulsifying activity, binding properties (Liu et al., 2009) and foaming capacity (Mohamed et al, 2009). Enzyme-modified protein concentrates from oat bran have also demonstrated good solubility, water retention, foam stability (Prosekov et al., 2018) and emulsification (Guan et al, 2006). Furthermore, it has been reported that oats globulin modified with transglutaminase, improved gelation, water- and fat-

binding capacities (Siu et al, 2002) (Klose & Arendt, 2012). These functional properties imply their suitability for applications in the food industry (Guan et al., 2006).

1.2 Protein hydrolysates

Proteins are important macronutrients that contribute to growth and maintenance of the body. In the case of cereals, functional properties are limited due to poor solubility at the pH range used in foods. It is then common to modify the proteins to enhance not only their functional properties but also to release biologically active peptides that can be used in the formulation of health-promoting foods. The classical, empirical approach for the discovery and production of bioactive protein hydrolysates and peptides involves first identifying a suitable protein source, and then releasing peptide fragments through hydrolysis of peptide bonds, usually by the proteolytic action of enzymes sourced endogenously (autolysis) or exogenously (commercial enzyme preparations), by treatment with acids, or via fermentation (by addition of starter cultures) (Li-Chan, 2015). It is difficult to control the conditions of acid hydrolysis of proteins, and the released peptides might be further broken down; while, fermentation is considered to be less efficient. Nowadays, enzymatic hydrolysis is the predominant way to produce bioactive protein hydrolysates due to its moderate conditions and no damage to amino acids, avoiding extreme environmental conditions required by chemical treatments (Wang & Mejia, 2005). Usually, enzymatic processes avoid side reactions, do not decrease the nutritional value of the protein source, and can improve functional properties because of disruption of tertiary structures. It also increases the concentration of peptides and amino acids with free carboxyl groups that will enhance solubility. Enzyme substrate specificity permits the development of protein hydrolysates with better defined chemical and biological characteristics. A typical enzymatic protein hydrolysate is a mixture of proteases, peptones, peptides, and free amino acids (Liu, Kong, Xiong, & Xia, 2010). The levels and compositions of free amino acids and peptides have been reported to determine the biological activities of the protein hydrolysate. The type of proteases used, and the degree of hydrolysis also affect their biological function (Liu et al., 2010). The resulting crude protein hydrolysate may undergo fractionation processes to yield an enriched bioactive peptide preparation or additional purification steps to isolate single peptides. Following the identification of the sequence of the isolated peptides, bioactivity is validated by testing chemically synthesized pure peptides (Li-Chan, 2015).

1.2.1 Protein hydrolysates health benefits

During the last two decades, there has been a growing interest in the use of protein hydrolysates containing bioactive peptides as agents for maintaining general health and preventing chronic human diseases. Hydrolysates may be produced using a single or a combination of proteases like pepsin, trypsin, chymotrypsin, bromelain, papain, alcalase, neutrase, flavourzyme and many others (Rizzello et al., 2016). As a result of this process, the liberation of fragments generated by different enzymes can produce peptides with a wide range of actions (Tavano, 2013). At present, several enzymatically hydrolysed seed storage proteins have been studied for the presence of bioactive peptides. Examples are provided in Table 1.1.

Table 1.1. - Bioactive hydrolysates obtained by enzymatic hydrolysis of some vegetable food proteins.

Bioactivity	Matrix	Enzymes
Antihypertensive	Sunflower	Pepsin and Pancreatin (Megías et al., 2004)
	Rapeseed	Alcalase (He et al., 2013)
Anti-inflammatory	Wheat	Protease from <i>Aspergillus Oryzae</i> (Sato et al., 2013)
	Soybean	Alcalase (Zhong et al., 2007)
Hypocholesterolemic	Amaranth	Pepsin and trypsin (Soares et al., 2015)
Immunomodulatory	Soybean	Trypsin (Tsuruki et al., 2003)
	Rice	Trypsin (Takahashi et al., 1996)
Anticancer	Rice bran	Alcalase (Kannan et al., 2010)
	Soybean	Thermoase (Kim et al., 2000)
Antioxidant	Rice bran	Pepsin (Adebiyi et al., 2009)
	Hempseed	Pepsin, pancreatin (Girgih et al., 2014)

The reported activities on Table 1.1 have been mainly investigated *in vitro*, reports on the *in vivo* effects of these hydrolysates are quite scarce. The unexpected degradation of ingested peptides by intestinal and/or blood proteases may make it difficult to predict their bioaccessibility *in vivo*. Consequently, results relevant to study their bioavailability as part of the assessment of their

biological activities. This could be done by evaluating transmembrane mobility or apparent permeability (Matsui, 2018).

1.2.2 Oat protein hydrolysates

Health-promoting effects of cereals like oats and barley are usually associated with their content of fibers and phenolic compounds, but peptides released from their proteins are as well beneficial (Ratnasari, Walters, & Tsopmo, 2017). Although the quality of oats proteins is relatively high compared to those from other cereals, their solubility is low, and many processes have used to break the proteins into smaller polypeptides. So far literature has reported that oat protein hydrolysates treated with thermolysin and alcalase & trypsin, have demonstrated an ACE-inhibitory effect and antioxidant activities respectively (Cheung, Nakayama, Hsu, Samaranyaka, & Li-Chan, 2009)(Vanvi & Tsopmo, 2016). Additionally, oat bran protein hydrolysates, treated with proteases such as protamex, and pepsin have shown biological activities such as metal chelating activities and radical scavenging properties (Baakdah & Tsopmo, 2016) (Tsopmo, Gao, & Baakdah, 2014a). Oat bran protein hydrolysates pre-treated with cell wall degrading enzymes have resulted in a protein content increase and antioxidant properties (Jodayree, Smith, & Tsopmo, 2012). Nevertheless, no research exists on their potential to prevent LDL oxidation or to bind bile acids.

1.3 Oxidative stress.

Oxygen is often referred to as the Janus gas, as it has both positive benefits and potentially damaging side-effects for biological systems. Oxygen can participate in high-energy electron transfers, and hence support the generation of large amounts of adenosine-5-triphosphate (ATP) through oxidative phosphorylation. This is necessary to permit the evolution of complex multicellular organisms, but also renders it liable to attack any biological molecule, be it a protein, lipid or DNA when oxygen is partially reduced and converted into various reactive oxygen species (ROS). A complex antioxidant defence system has evolved that generally reduces the destructive effects of ROS attack by maintaining the redox balance. On occasions, however, this balance can be perturbed, leading to oxidative stress which plays a central role in the pathophysiology of many different disorders and diseases (Niki, 2016). The concept of a pro-oxidant-antioxidant balance is central to an understanding of oxidative stress for several reasons: it emphasizes that the disturbance may be caused through changes on either side of the equilibrium, highlights the

homeostatic concentrations of ROS, and draws attention to the fact that there will be a graded response to oxidative stress. Hence, minor disturbances in the balance are likely to lead to homeostatic adaptations in response to changes in the immediate environment, whereas more major perturbations may lead to irreparable damage and cell death. There are many potential sources of ROS, and the relative contributions of these will depend on the environmental circumstances prevailing. As the reactions of ROS are often diffusion-limited, the effects on cell function depend to a large extent on the biomolecules in the immediate vicinity (Burton & Jauniaux, 2011a).

1.3.1 Reactive oxygen species

The term ‘reactive oxygen species’ is applied to both free radicals and their non-radical oxygen intermediates (Burton & Jauniaux, 2011b). A free radical is a chemical species that has an odd number of electrons. In the context of oxidative stress, the radicals are small molecules/ions that are reactive with small activation energies and possess short lifetimes. The small size makes it possible for many of them to penetrate cell membranes. The free radicals can be considered as a subset of reactive oxygen or nitrogen species; Figure 1.2 shows those generated in biological systems. A major part of reactive oxygen species originates as by-products of the aerobic metabolism in the mitochondria (Jensen, 2003).

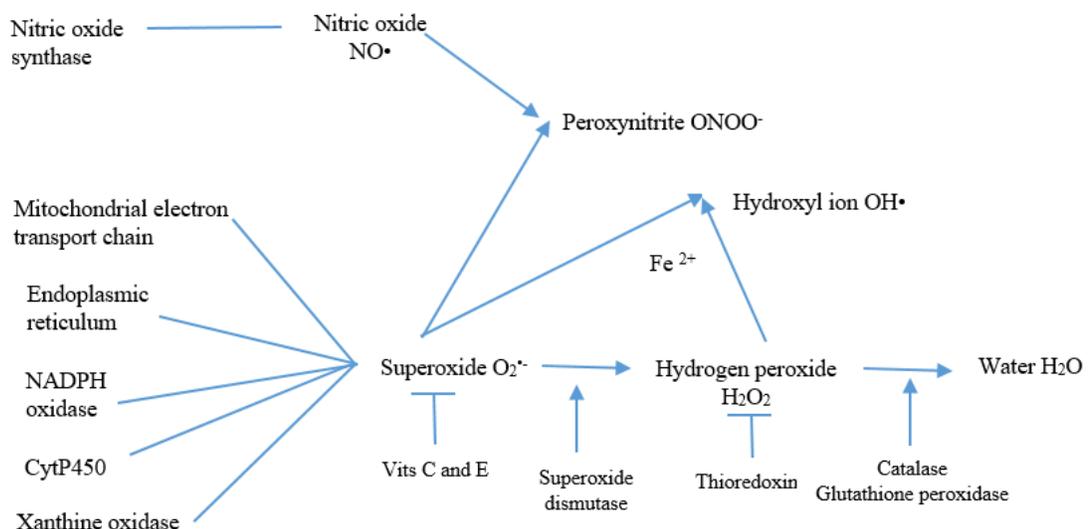
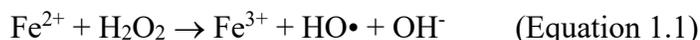


Figure 1.2. - The principal reactive oxygen species, their potential origins and detoxification pathways. NADPH, nicotinamide adenine dinucleotide phosphate.

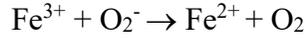
Under physiological conditions, the most common oxygen free radical is the superoxide anion radical ($O_2^{\bullet-}$) produced mainly in mitochondria. Superoxide anion radical is considered the primary ROS and can generate secondary ROS by interacting with other molecules (directly or through enzyme- or metal-catalyzed processes) (Buonocore, Perrone, & Tataranno, 2010).

1.3.2 Hydroxyl radical

The hydroxyl radical has a high oxidant power that makes it a very dangerous radical, probably the most reactive one (Pastor, Weinstein, Jamison, & Brenowitz, 2000). Thus, when produced in vivo, HO^{\bullet} reacts close to its site of the formation with extremely high rate constants. $O_2^{\bullet-}$ and H_2O_2 are less reactive oxidants than HO^{\bullet} , but they have a longer lifetime which allows them to react with molecules in locations far from the site where the free radical was produced (Martinez, 1995). HO^{\bullet} has been reported as the most powerful oxidizing radical that can interact at the site of its generation with most organic and inorganic molecules. These reactions involve hydrogen abstraction, addition, and electron transfer (Kohen & Nyska, 2002b). Due to their extreme reactivity, hydroxyl radicals are virtually eliminated at the same site of formation because they could interact with the first molecule they meet (Buonocore et al., 2010). Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is likely to be the transition metal catalyzed decomposition of superoxide and hydrogen peroxide. Transition metals in human disease such as iron and copper play a key role in the production of hydroxyl radicals in vivo (Stohs & Bagchi, 1995). Hydrogen peroxide can react with iron (II) but also copper (II), chromium (III, V, and IV), cobalt (II), nickel (II, and V) to generate HO^{\bullet} radicals via the Fenton-type reaction, but their abilities to generate free radicals differ (Equation 1.1).



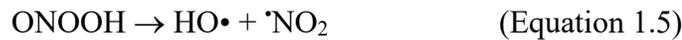
Also, superoxide and hydrogen peroxide can react together directly to produce the hydroxyl radical, but the rate constant for this reaction in aqueous solution is virtually zero. However, if transition metal ions are present a reaction sequence is established that can proceed at a rapid rate (Equation 1.2) (Young & Woodside, 2001):



Net result



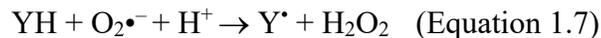
The net result of the reaction sequence illustrated above is known as the Haber-Weiss reaction. Although most iron and copper in the body are sequestered in forms that are not available to catalyze this reaction sequence, it is still of importance as a mechanism for the formation of the hydroxyl radical *in vivo*. Hydroxyl radical formation has also been postulated to occur via interaction between the superoxide radical and nitric oxide, a vasodilator radical produced by several cell types (Equation 1.3, 1.4 and 1.5) (Cheng, Jen, & Tsai, 2002).



A variety of mechanisms have been reported for its generation: ionizing radiation causes decomposition of water, resulting in the formation of HO• and hydrogen atoms, and photolytic decomposition of alkyl hydroperoxides (Buonocore et al., 2010).

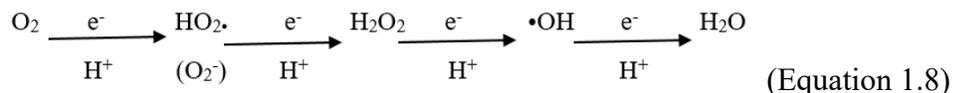
1.3.3 Superoxide radical

Superoxide ($\text{O}_2^{\bullet-}$) is produced by the addition of a single electron to oxygen, and several mechanisms exist by which superoxide can be produced *in vivo* (Young & Woodside, 2001). It is considered the ‘primary’ ROS. It can interact with other molecules to generate ‘secondary’ ROS and can be both a reducing and oxidizing factor; generally a molecular oxygen produced by reducing reaction (Equation 1.6) whereas a hydrogen peroxide is generated by oxidizing reaction (Equation 1.7) (Valko, Morris, Cronin, 2005):



In mammalian cells, a bit fraction of total oxygen is utilized for superoxide creation, and the most important route of superoxide creation is the mitochondrial respiratory chain and the heme oxidation (Buonocore et al., 2010). Mitochondria are known to play a key role in maintaining the

bioenergetic status of multiple basic cellular processes. It generates energy using 4 electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anions (Equation 1.8) (Bal & Kasprzak, 2002).



As a precursor of ROS formation, it can form hydrogen peroxide, hydroxyl radical, and singlet oxygen, these have the potential of reacting with biological macromolecules and thereby inducing tissue damage (Halliwell & Gutteridge, 1985). The biological toxicity of superoxide is due to its capacity to inactivate iron-sulfur cluster containing enzymes, which are critical in a wide variety of metabolic pathways, thereby liberating free iron in the cell, which can undergo Fenton-chemistry and generate the highly reactive hydroxyl radical (Gülçin, Huyut, Elmastaş, & Aboul-Enein, 2010). It is also deployed by the immune system to kill invading microorganisms. Superoxide radicals play an important role in the peroxidation of unsaturated fatty acids and possibly other susceptible substances (Bamdad, Wu, & Chen, 2011b)

1.3.4 Human antioxidant defence system

An extensive range of antioxidant defences, both endogenous and exogenous (e.g. obtained as a part of a diet or as dietary supplements), are present to protect cellular components from free-radical induced damage (Halliwell, B Gutteridge, 1989). Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well-being. However, under conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions. The defence system can be divided into three main groups: antioxidant enzymes, chain-breaking antioxidants, and transition metal binding proteins Figure 1.3 (Young & Woodside, 2001) (Halliwell & Gutteridge 1989).

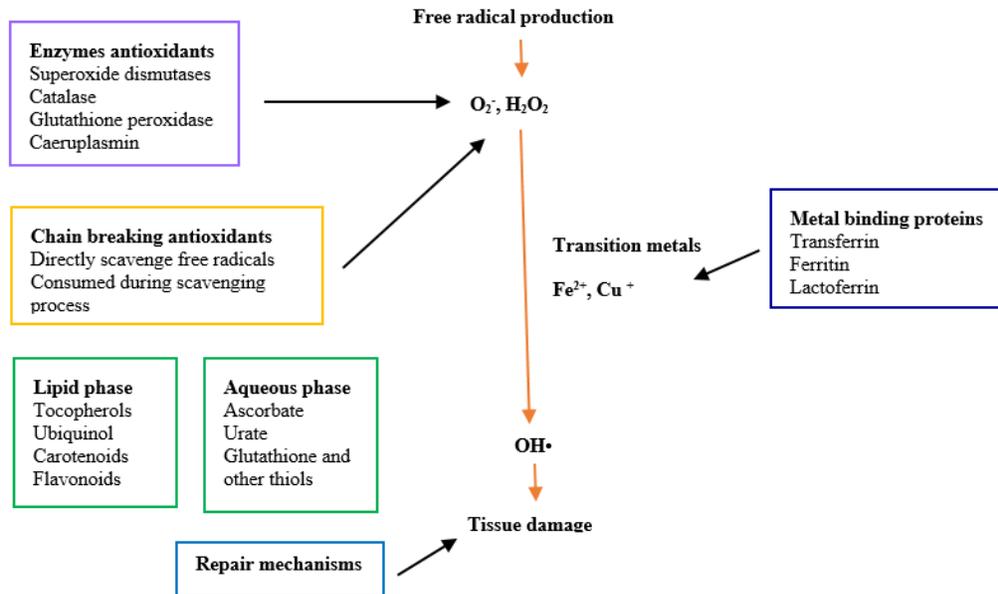
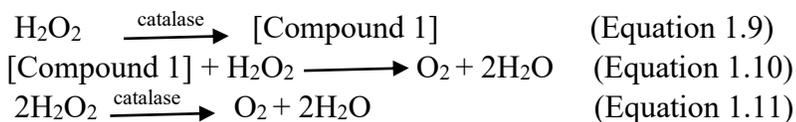


Figure 1.3. - Antioxidant mechanisms. Antioxidant enzymes catalyze the breakdown of free radical species, usually in the intracellular environment. Transition metal binding proteins prevent the interaction of transition metals such as iron and copper with hydrogen peroxide and superoxide producing highly reactive hydroxyl radicals.

1.3.4.1 Antioxidant enzymes

Catalase (EC1.11.1.6) was the first antioxidant enzyme to be characterized (Martinez, 1995). Present in the peroxisome of aerobic cells, it is expressed in the majority of the cells, organs, and tissues and at elevated concentrations, in the liver and erythrocyte (Martinez, 1995). This enzyme efficiently promotes the two-stage conversion of hydrogen peroxide to water and molecular oxygen (fig x) (Kirkman, Galianoe, & Gaetanie, 1987). It also has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Mates, Perez-Gomez, & Nunez de Castro, 1999). Catalase consists of four protein subunits, each containing a haem group and a molecule of NADPH. Each subunit contains ferric ions of the haem group that undergo oxidation following interaction with the first molecule of H_2O_2 to produce Fe^{4+} in a structure called compound 1 (Lardinois, 1995)(Equation 1.9). A second molecule of H_2O_2 serves as an electron donor and results in the destruction of the two H_2O_2 molecules involved to produce an oxygen molecule (Equation 1.10 and 1.11) (Kohen & Nyska, 2002a).



Superoxide dismutase (SOD), (EC 1.15.1.1) is an intracellular enzymatic antioxidant that catalyses the dismutation of the highly reactive superoxide anion to O₂ to the less reactive species H₂O₂ (Equation 1.12) (Landis & Tower 2005). Hydrogen Peroxide must then be removed by CAT or GPX (Brigelius-Flohe, 1999).



SODs contain metal in their active site to act as a cofactor for their function. The mechanism of O₂^{•-} detoxification is by successive oxidation and reduction of the transition metal ion at the active site in a Ping-Pong type mechanism (Meier et al, 1998). In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Limón-Pacheco & Gonsebatt, 2009). CuZnSOD has two protein subunits, each containing a catalytically active copper and zinc atom bridged by a histamine residue and play a major role in the first line of antioxidant defence (Battistoni et al., 1998). Mn-SOD is a homotetramer and contains one manganese atom per subunit, and it cycles from Mn (III) to Mn (II), and back to Mn (III) during the two-step dismutation of superoxide. EC-SOD is a secretory, tetrameric, copper- and zinc-containing glycoprotein synthesized by only a few cell types, including fibroblasts and endothelial cells (Khalid Rahman, 2007)(Mates et al., 1999).

Glutathione peroxidase (GPX EC 1.11.1.19) is a selenium-containing peroxidase (Tappel, 1978). GPX catalyzes the reduction of hydroperoxides (which might be hydrogen peroxide or another species such as a lipid hydroperoxides) using the tripeptide glutathione (GSH) present in high concentrations in cells, thereby protecting mammalian cells against oxidative damage (Equation 1.13). In fact, glutathione metabolism is one of the most essential antioxidative defence mechanisms (Esterbauer, Gebicki, Puhl, & Jürgens, 1992) (Sigalov & Stern, 1998).



There are four different Se-dependent glutathione peroxidases present in humans, and these are known to add two electrons to reduce peroxides by forming selenols (Se-OH), and the antioxidant properties of these seleno-enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction (Chaudiere, J Ferrari-Iliou, 1999). Within cells, the highest concentrations are found in liver although glutathione peroxidase is widely distributed in almost all tissues. The

predominant subcellular distribution is in the cytosol and mitochondria, suggesting that glutathione peroxidase is the main scavenger of hydrogen peroxide in these subcellular compartments (Holben, D Smith, 1999). Although GPX shares the substrate, H₂O₂, with CAT, it alone can react effectively with lipid and other organic hydroperoxides, being the major source of protection against low levels of oxidant stress (Mates et al., 1999).

Glutathione reductase is a flavine nucleotide-dependent enzyme with a tissue distribution similar to that of glutathione peroxidase. The enzyme reduces oxidized glutathione utilizing NADPH generated by various systems (Equation 1.14) (Martinez-Calueya, 1995). The ratio of reduced to oxidised glutathione is usually kept very high as a result of the activity of the enzyme glutathione reductase (Gibson, DD Hawrylko, J McCay, 1985).



1.3.5 Antioxidant compounds

These are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts (B Halliwell, 1995). In general, the charge associated with the presence of an unpaired electron becomes dissociated over to the scavenger and the resulting product will not readily accept an electron from or donate an electron to another molecule, preventing the further propagation of the chain reaction. Such antioxidants can be conveniently divided into aqueous phase and lipid phase antioxidants.

Lipid phase chain-breaking antioxidants scavenge radicals in membranes and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E; however some other molecules such as carotenoids, flavonoids, and Ubiquinol-10 are included in this category (Young & Woodside, 2001). Vitamin E is a fat-soluble vitamin occurring in nature in eight different forms. In humans, α -tocopherol is the most active form (Hensley et al., 2004). The main function of Vitamin E is to protect against lipid peroxidation of cell membranes and stop the radical chain by forming a low-reactivity derivative unable to attack lipid substrates (Pryor, 2000). During the antioxidant reaction, α -tocopherol is converted to an α -tocopherol radical by the donation of a labile hydrogen to a lipid or lipid peroxy radical, and the α -tocopherol radical, can therefore, be reduced to the original α -tocopherol form by ascorbic acid (Kojo, 2004). Thus, vitamin E accomplishes its role in membrane preservation against free radical damage promoted by low-density lipoproteins. It has been assessed that by the intake of

high-dose vitamin E supplements, an inhibition of proatherogenic processes can be achieved. Vitamin E contributes to stabilizing atherosclerotic plaque (Díaz, Frei, Vita, & Keaney, 1997) (Devaraj & Jialal, 1998).

Aqueous phase chain-breaking antioxidants directly scavenge radicals present in the aqueous compartment. Qualitatively, the most important antioxidant of this type is vitamin C (ascorbate), yet some other antioxidants are present in plasma in high concentrations, these include uric acid, protein-bound thiol groups, and albumin. Ascorbic acid has been shown to scavenge superoxide, hydrogen peroxide, hydroxyl radical, hypochlorous acid, aqueous peroxy radicals, and singlet oxygen but also reactive nitrogenated species, by forming semi-dehydroascorbic acid and therefore, prevents the oxidative decay of essential biomolecules (Du, Cullen, & Buettner, 2012). Although ascorbic acid is not a direct scavenger of lipophilic radicals, it participates in regeneration α -tocopherol from α -tocopheryl radicals in membranes and lipoproteins (Carr, A Frei, 1999) (Kojo, 2004), and also raises intracellular glutathione levels thus playing an important role in protein thiol group protection against oxidation (Naziroglu, M Butterworth, 2005).

1.3.6 Important physiological functions that involve free radicals or their derivatives

Although an imbalance of free radicals have deleterious consequences they also exist in biological cells and tissues at low concentrations. When they are maintained at this moderate level, free radicals play several beneficial roles for the organism (Pizzino et al., 2017). For instance phagocytes kill invading pathogens and defend the host through generation of antimicrobial oxidants such as $O_2^{\bullet-}$ or its derived oxidants during the respiratory burst or enhanced oxygen consumption. Within $O_2^{\bullet-}$ and H_2O_2 in the presence of released iron could form hydroxyl radical also a potent antimicrobial oxidant (Kalyanaraman, 2013). Free radicals are also involved in a number of cellular signaling pathways. Probably, the most well-known free radical acting as a signaling molecule is nitric oxide (NO). It is an important cell-to-cell messenger required for a proper blood flow modulation, involved in thrombosis, and is crucial for the normal neural activity. NO is also involved in nonspecific host defense, required to eliminate intracellular pathogens and tumor cells (Pacher, Beckman, & Liaudet, 2018). Important physiological functions that involve free radicals or their derivatives also include intracellular response to stressors (Allen & Tresini, 2000)(Allen & Tresini, 2000), regulation of mitochondrial function (Bushell et al., 2002), expression of stress proteins/ antioxidant levels, activation of immune related receptors (Chandel

et al, 1996), help to combat invading pathogens (Jurg, 2011), control of ventilation (Droege, 2002), sperm capacitation, acrosome reaction, motility activation among other functions (Lamirande & Flaherty, 2008).

1.4 Evidence of oxidative stress and cardiovascular diseases

Cardiovascular diseases (CVD) are a group of disorders of the heart and blood vessels that include coronary heart disease, cerebrovascular disease, peripheral artery disease, aortic atherosclerosis, rheumatic heart disease, congenital heart disease, and/or aneurysm. The progress of endothelial dysfunction and inflammation of the blood vessel causes an atherosclerotic lesion in the arteries preventing blood flow to the heart or brain, which further causes stroke and myocardial infarction (Ganesan, Sukalingam, & Xu, 2018). The most common reason for this is a build-up of fatty deposits on the inner walls of the blood vessels that supply the heart or brain (World Health Organization, 2017). CVDs are the leading cause of morbidity and mortality in the world (World Health Organization, 2017b). CVD risk is mediated via complex interactions of independent and synergistic risk factors. The primary division of cardiovascular risk factors comprises two categories: modifiable and non-modifiable factors. Age, sex, and race/ethnicity, positive family history of cardiovascular diseases and genetic markers are not modifiable, whereas other risk factors such as dyslipidemia, hypertension, hyperglycemia, obesity, tobacco use, low consumption of fruits and vegetables, can be reduced by lifestyle modification including diet (Kulczyński et al, 2017)(Dieter & Tuttle, 2017) (Ofori & Odia, 2016). The main step in the pathogenesis of CVDs is the endothelial damage in which the underlying cell layers expose to harmful inflammatory process that ultimately leads to the formation of lesions. Cellular oxidative stress is the prime pathogenic factor for CVDs (Heitzer, 2001). In pathophysiological conditions, the production of ROS exceeds the natural antioxidant defence of the cells, causing the active ROS to attack and producing cellular alterations (Cai & Harrison, 2000)(Vaziri, 2008). Oxidative stress and inflammation cause injury to cells including endothelium in regard to this topic. Endothelial dysfunction, in turn, promotes a pro-inflammatory environment as evidenced by increased endothelial expression of adhesion molecules and the imbalance of arachidonic acid metabolites (Ng, Kamisah, Faizah, & Jaarin, 2012) and chemoattractant molecules (Savoia et al., 2011). The most straightforward method for conclusively demonstrate a role for oxidative stress in CVD progression is to show elevated oxidative stress levels in tissues or fluids from diseased patients. Among the numerous biomarkers

probed are a variety of lipid oxidation products and ROS-producing enzymes including myeloperoxidase (MPO) and NADPH oxidase (NOX). One of the most widely utilized biomarkers of tissue oxidative stress is the F2-isoprostanes, products of free radical oxidation of arachidonic acid that can be measured in both urine and plasma. Elevated F2-isoprostanes have been detected in atherosclerotic lesions (Pratico et al., 1997), in the plasma of patients with coronary artery disease (Vassalle, Botto, Andreassi, Berti, & Biagini, 2003) in pericardial fluids during heart failure (Mallat et al., 1998) and in the urine of patients experiencing ischemia/reperfusion injury (Reilly et al., 1997). The presence of ROS has been strongly associated with the incidence of CVD, hence dietary components such as proteins and peptides with antioxidant activity have received particular attention because of their potential role in modulating oxidative stress (Khalid Rahman, 2007).

1.5 Lipid Oxidation

Lipids are a major component of food and important structural and functional constituents of cells in biological systems. They are prone to oxidation through various pathways giving rise to quality deterioration in food and negative effects on the integrity of biological systems (Shahidi & Zhong, 2010). In living organisms, lipids may undergo oxidation during normal aerobic metabolism or upon exposure to oxidizing agents or in the presence of enzymes such as lipoxygenases. Unsaturated fatty acids in membrane phospholipids and cholesterol, especially low-density lipoprotein LDL-cholesterol, are the major target substrates of oxidation *in vivo*, causing irreversible cellular and tissue damage (Shahidi & Zhong, 2015). It is widely accepted that lipid oxidation occurs via a free radical mechanism or chain reaction mechanism which proceeds through three stages initiation, propagation, and termination (Figure 1.4).

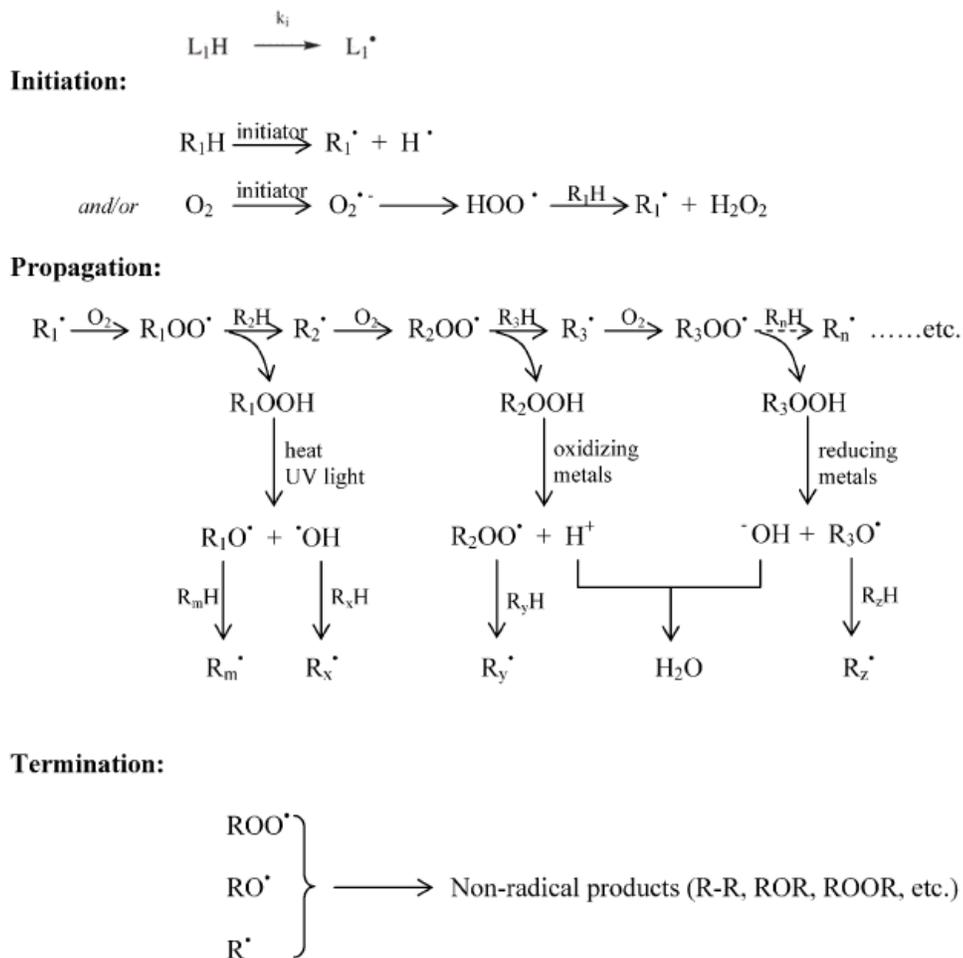
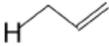
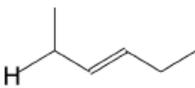
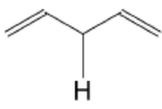


Figure 1.4. - Lipid oxidation pathways.

Oxidation normally proceeds very slowly at the initial stage until it reaches a sudden increase after an induction period. Initiators or catalysts are required to start the lipid oxidation process by removing an electron from either the lipid or oxygen. The most common initiators are metals (cobalt, iron, copper, manganese, magnesium, and vanadium), light (visible, UV), heat, enzymes, metalloproteins, ozone (Bailey, 2005). The initiation process is quite complex and not yet fully understood; however, it is believed to involve removing of a hydrogen atom in the lipid molecule. The loss of hydrogen atom (allylic hydrogen) takes place most readily at the carbon next to the double bond in the olefinic fatty acids, due to lower C-H bond energy (Table 1.2) (Shahidi & Zhong, 2010).

Table 1.2. - The dissociation energy of hydrogen in various compounds

Compounds	ΔE (kcal/mol)
	103
	100
	85
	77
	65
H-OO-R	90

During propagation, the alkyl radical of unsaturated lipid-containing labile hydrogen reacts very rapidly with oxygen to form peroxy radicals. Lipid hydroperoxides are produced as primary products of oxidation (Fig. 1.4). They are unstable and break down to a wide range of secondary oxidation products, including aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds. Alkoxy ($\text{RO}\bullet$), peroxy ($\text{ROO}\bullet$), hydroxyl ($\bullet\text{OH}$) and new lipid radicals ($\text{R}\bullet$) are generated from decomposition of hydroperoxides and further, participate in the chain reaction of free radicals. The type of secondary oxidation products produced depends on the composition of the hydroperoxides and pattern of oxidative cleavage.

1.5.1 Atherosclerosis and LDL oxidation

Atherosclerosis is a complex and relatively slow-progressing disease. It is also the major source of morbidity and mortality in the developed world (Stocker, 2004). It is now widely accepted that elevated plasma levels of LDL cholesterol (LDLC) is the major pathogenic factor in the development of this condition (Miller, Y.I., Choi, S.-H., Fang, L., Tsimikas, 2010). It is characterized by the accumulation of cholesterol deposits in macrophages in large and medium-sized arteries (Hansson, 2009). This deposition leads to a proliferation of certain cell types within the arterial wall that gradually impinge on the vessel lumen and impede blood flow. This process may be quite insidious lasting for decades until an atherosclerotic lesion, through physical forces from blood flow, becomes disrupted and deep arterial wall components are exposed to flowing blood, leading to thrombosis and compromised oxygen supply to target organs such as the heart

and brain. Over the past 150 years, there have been numerous efforts to explain the complex events associated with the development of atherosclerosis, one of the existent theories is the oxidative modification hypothesis, which proposes that atherogenesis is initiated by oxidation of the lipids in low-density lipoprotein (LDL), also termed lipid peroxidation (Díaz, Frei, Vita, & Keane, 1997a). This fact has been demonstrated by numerous in vitro and animal studies, which have shown that LDL oxidation is an important initial event of atherosclerosis (Qin et al., 2011). LDL oxidation proceeds by multiple mechanisms induced by different oxidants (Nikki Etsuo, 2011). Metals such as iron and copper play a role through the production of HO• radicals while enzymes such as myeloperoxidase, lipoxygenase, NADPH oxidase, nitric oxide synthases and cytochrome p450 are also contributors of ROS. (Etsuo, 2011). Various biomarkers of lipid peroxidation have been developed and applied to biological samples. Some representative biomarkers of lipid peroxidation are summarized in Table 1.3 (Etsuo, 2014).

Table 1.3. - Biomarkers of lipid peroxidation

Biomarker	Remarks / methods of measurement
Hydroperoxides	Primary product of lipid peroxidation, not stable/LC–UV, CL, FL, MS; DPPP
Hydroxides	Reduced form of hydroperoxides, HODE and HETE/LC–UV, MS; GC–MS; EIA
Isoprostanes	Free radical mediated oxidation product of arachidonic acid/ LC–MS; GC–MS; EIA, RIA
Neuroprostanes	Free radical mediated oxidation product of DHA/LC–MS; GC–MS
TBARS, MDA	Thiobarbituric acid reactive substances measuring MDA and possibly others/spectrophotometry, HPLC
Conjugated diene	1,3-Diene of hydroperoxides and hydroxides/UV 234 nm
Ethane, pentane	Fragment product of hydroperoxides in exhaled gas/GC
Aldehydes, Ketones	Secondary products from hydroperoxides /LC;DNPH–UV/vis; EIA; RIA
LysoPC	Hydrolysis of PC by phospholipase A2/TLC, LC–MS/MS
7-Hydroxycholesterol	Reduction of 7-hydroperoxycholesterol, enzymatic oxidation/ GC–MS
7- Ketocholesterol	Free radical oxidation of cholesterol/GC–MS
Oxidized LDL	Oxidatively modified LDL by multiple oxidants/EIA, RIA
LPO-modified proteins	Proteins modified by aldehydes/LC–MS; EIA; RIA
Lipofuscin	Fluorescence

CL, chemiluminescence; DNPH, 2,4-dinitrophenylhydrazine; DPPP, diphenylpyrenylphosphine; EIA, enzyme immunoassay; FL, fluorescence; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; GC, gas chromatography; LC, liquid chromatography; LPO, lipidperoxidation; Lyso PC, lysophosphatidylcholine; MS, mass spectrometry; RIA, radio immunoassay; TLC, thin layer chromatography; UV/V, ultraviolet/visible spectrophotometry.

According to the oxidative-modification hypothesis, LDL initially accumulates in the extracellular subendothelial space of arteries and, through the action of resident vascular cells, is mildly oxidized to a form known as minimally modified LDL (Fig. 1.5). (Navab, M Berliner, JA Watson, 1996). This minimally modified LDL induces local vascular cells to produce monocyte chemoattractant protein 1 and granulocyte and macrophage colony-stimulating factors, which stimulate monocyte recruitment and differentiation to macrophages in arterial walls (Parhami et al., 1993). The accumulating monocytes and macrophages then stimulate further peroxidation of LDL. The products of this reaction make the protein component of LDL (apolipoprotein B-100) more negatively charged (Díaz, Frei, Vita, & Keaney, 1997b). By virtue of its increased negative charge, this completely oxidized LDL is recognized by scavenger receptors on macrophages and internalized to form so-called foam cells (Henriksen, Mahoney, & Steinberg, 1981). Oxidized LDL is cytotoxic to vascular cells, thereby promoting the release of lipids and lysosomal enzymes into the intimal extracellular space and enhancing the progression of atherosclerotic lesions (Schwartz, Valente, Sprague, Kelley, Nerem, 1991).

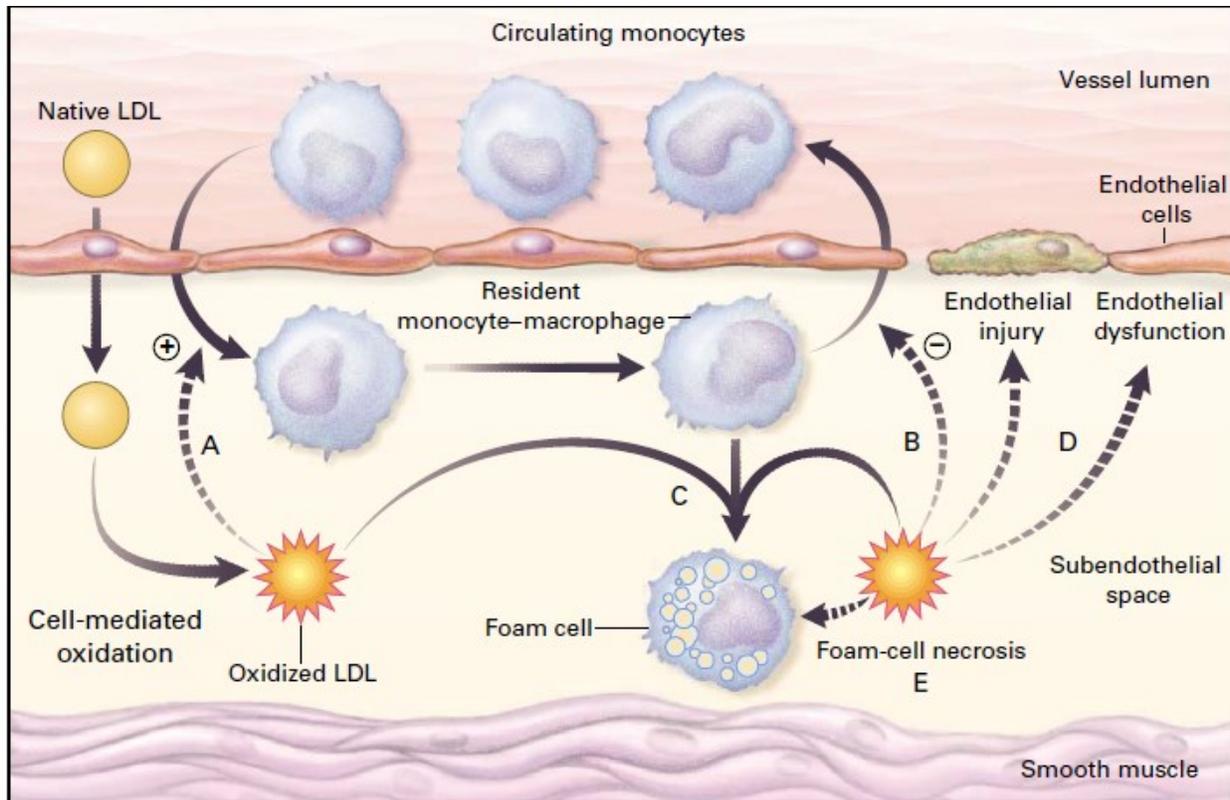


Figure 1.5. - Early Events in Atherogenesis. Native LDL becomes trapped in the subendothelial space, where it can be oxidized by resident vascular cells such as smooth muscle cells, endothelial cells, and macrophages. Oxidized LDL stimulates (plus sign) monocyte chemotaxis (A) and inhibits (minus sign) monocyte egress from the vascular wall (B). Monocytes differentiate into macrophages that internalize oxidized LDL, leading to the foam-cell formation (C). Oxidized LDL also causes endothelial dysfunction and injury (D), as well as foam-cell necrosis (E), resulting in the release of lysosomal enzymes and necrotic debris. Broken arrows indicate adverse effects of oxidized LDL. Adapted from (Díaz et al., 1997).

Research have also shown in vitro that oxidized LDL is toxic to a variety of cell types. Several soluble polar sterols formed during oxidation have been implicated in this cytotoxicity (Avogaro, Bon, & Cazzolato, 1988). The link between the oxidation of LDL and atherogenesis provides a rationale for the beneficial effect of antioxidants on the incidence of coronary artery disease (Ryu, 2000). Literature have reported that some protein hydrolysates such as defatted peanut kernels (Hwanget al., 2010) ,chickpea (Torres-Fuentes et al., 2014) ,amaranth (García Fillería & Tironi, 2017), wheat bran (Wang, et al., 2011), and casein (Wang et al., 2016) have demonstrated inhibitory effect on LDL oxidation, nevertheless, there is no research done so far on oat bran protein hydrolysates to assess this activity.

1.6 Bile Acids

Bile acids are 24 carbon atoms end products of cholesterol metabolism and constitute a major part of the bile (Barrasa, Olmo, Lizarbe, & Turnay, 2013). They are key molecules involved in digestion whose main physiological role is to facilitate the absorption and emulsion of dietary lipids and fat-soluble vitamins in the gut, and for maintaining cholesterol homeostasis in the body (Mertens, Kalsbeek, Soeters, & Eggink, 2017). The emulsification capability of bile acids is attributable to their amphipathic structure, which is a consequence of the hydroxyl groups being oriented toward one side of the steroid nucleus (Agellon, 2013). At the intestinal level, bile acids modulate pancreatic enzyme secretion and cholecystokinin release, and they are potent antimicrobial agents that prevent bacterial overgrowth in the small bowel. Primary bile acids are synthesized in the liver from cholesterol through a cascade of reactions catalyzed by enzymes located at the cytosol, microsomes, mitochondria, and peroxisomes. The modification of the sterol nucleus of cholesterol precedes the oxidative cleavage of its side chain; it begins with the hydroxylation of cholesterol at C-7, catalyzed by cholesterol 7 α -hydroxylase, the rate-limiting enzyme of the pathway. In humans, the two primary bile acids, cholic (CA) and chenodeoxycholic (CDCA), are synthesized through this pathway (Hylemon et al., 2009) (Mertens et al., 2017). After their synthesis, CA and CDCA are conjugated with glycine or taurine in the liver (Figure 1.6), stored in the gallbladder and then released into the intestinal tract. During the intestinal transit, these molecules are mainly absorbed in the ileum, but a small fraction continues its transit into the large bowel where they undergo modifications by intestinal anaerobic bacteria. This biotransformation in the human colon involves mainly deconjugation and oxidation/ epimerization of hydroxyl groups at C-3, C-7 and C-12 as well as dehydroxylation at position C-7. Bacterial dehydratases remove the hydroxyl group at C-7 from CA and CDCA yielding the secondary deoxycholic (DCA) and lithocholic (LCA) bile acid (Barrasa et al., 2013). The structures of some of the most abundant bile acids are shown in Figure 1.6. Secondary bile acids can be reabsorbed in the distal intestine and transported back to the liver.

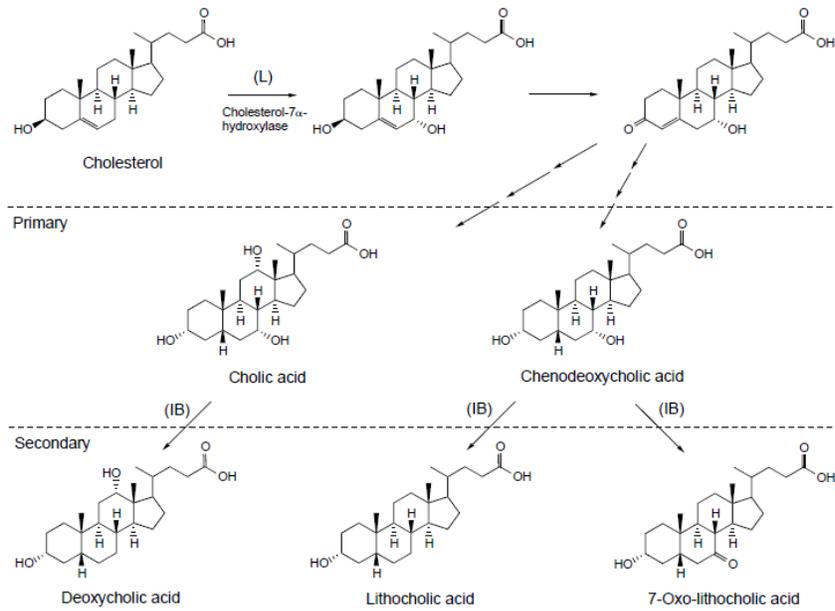


Figure 1.6. - Bile acid metabolism – production of primary and secondary bile acids occurs in the liver (L) and by intestinal bacteria (IB).

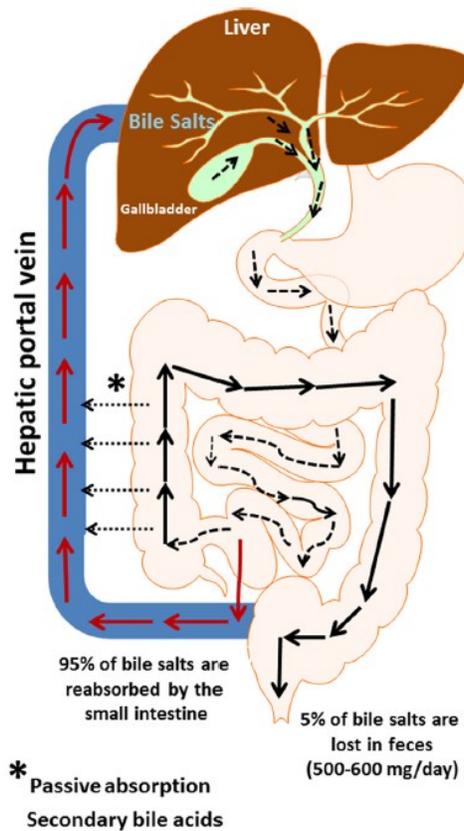


Figure 1.7. - Enterohepatic circulation of bile acids.

More recently it became apparent that bile acids, as a group of molecules, possess a much wider range of biological activities than initially recognized. They function as hormones or nutrient signaling molecules that help to regulate glucose, lipid, lipoprotein, and energy metabolism as well as inflammatory responses (Zhou & Hylemon, 2014). The synthesis and excretion of bile acids is the major pathway in the control and elimination of cholesterol (Cohen, 1999). It has been demonstrated that compounds such as oat β -glucans (Othman, Moghadasian, & Jones, 2011) control cholesterol by binding to bile acids thereby their elimination through the feces. There are other molecules that can control cholesterol through similar mechanisms, some of which are hydrolyzed proteins and peptides from foods (J. Wang, Shimada, Kato, Kusada, & Nagaoka, 2015) (Siow, Choi, & Gan, 2016a). As already mentioned, primary bile acids are synthesized from cholesterol in the liver, and cholesterol 7- α -hydroxylase is considered to catalyze the rate-limiting step in the biosynthesis process (Cohen, 1999). Hepatic bile acid synthesis is controlled in the liver through negative and positive feedback mechanisms (Reihner, Angelin, & Rudling, 1990)(Andersson, Ellegard, & Andersson, 2002). Bile acids recirculating to the liver regulate their own synthesis by influencing the activity of this enzyme (Reihner, Bjorkhem, Angelin, Ewerth, & Einarsson, 1989). Bile acids binding prevent their reabsorption, and promote their fecal excretion (J. Wang et al., 2015). The increased fecal removal of bile acids reduces the amount of bile acids present in the liver. This fecal effect promotes bile acid synthesis from cholesterol through 7- α -hydroxylase activation (Cohen, 1999)(Nwachukwu, Devassy, Aluko, & Jones, 2015). The consequences of increased bile acid excretion are stimulated cholesterol uptake from the circulation followed by a decreased serum cholesterol concentration (Reihner et al., 1990) (Miettinen, 1979).

1.6.1 Protein hydrolysates with bile acid binding properties

Several enzymatically hydrolyzed proteins have been studied for their bile acid binding capacity. These hydrolysates include those from amaranth seed (Tiengo et al., 2011), flaxseed (Marambe et al., 2008), buckwheat (Y. Ma & Xiong, 2009), rapeseed (Yoshie-Stark, Wada, & Wäsche, 2008), corn (Kongo-Dia-Moukala et al., 2011), lupin (Yoshie-Stark & Wäsche, 2004), and lentil (Barbana, Boucher, & Boye, 2011). Nevertheless, to date, there is no research report on oat bran protein hydrolysates and its bile acid chelating ability. The binding power of protein hydrolysates has been explained by favorable structural changes that occur after treatments with proteases (Howard & Udenigwe, 2013c). The degree of hydrophobicity is one of the factors (Kongo-Dia-Moukala et al., 2011). Due to favourable structural chemistry, some protein hydrolysates have demonstrated substantial bile acid-binding activity, which is partly dependent on hydrophobicity of the amino acidic residues of the peptides (Kongo-dia-moukala, Zhang, & Irakoze, 2011). Bile acids are amphipathic with hydrophobic skeletal structure and polar functionality and can thus bind other hydrophobic amino acids via weak hydrophobic forces (Porez, Prawitt, Gross, & Staels, 2012). Thus food protein hydrolysates that contain high amounts of hydrophobic residues have demonstrated high bile acid-binding capacity, due to the interaction of the amino acidic residues with the hydrophobic core of the bile acid (Howard & Udenigwe, 2013a). The charge of the released peptides is also believed to be important because of possible interaction with the anionic carboxylic acid group of bile acids. Moreover, the sequence and arrangement of the peptide molecules in space can also influence the binding. Additionally, the extent of binding of hydrolysates depends on the type of bile acids, because they possess different functional groups, such as a variable number of hydroxyl groups that may influence their association with sequestrants as well as conjugation with taurine or serine (Howard & Udenigwe, 2013d). This has been demonstrated with different protein hydrolysates that exhibited differential binding of different bile acid types although the structural requirements for activity and actual molecular interactions are yet to be elucidated (Barbana et al., 2011) (Yoshie-Stark et al., 2008) (Y. Ma & Xiong, 2009). Most studies that have evaluated the bile acid-binding properties of protein hydrolysates and peptides are based on isolated *in vitro* assays; thus further research is needed in order to estimate possible *in vivo* functions considering expected interactions of the peptides with the food matrix and physiological biomolecules (Howard & Udenigwe, 2013d).

Hypothesis

Digested oat bran proteins with antioxidant activity will inhibit LDL oxidation and bind bile acids in vitro. Further separation will provide novel bioactive peptides.

Research Objectives

- a) Extract proteins from oat brans and generate their hydrolysates by digestion with different proteases (protamex, alcalase, flavourzyme, pepsin and pancreatin).
- b) Determine antioxidant activities of digested proteins by radical scavenging assays
- c) Evaluate inhibition of LDL oxidation of oat bran protein hydrolysates
- d) Assess bile acid chelating capacity of digested proteins
- e) Separate the most active hydrolysate by HPLC in order to evaluate their fractions antioxidant ability

Chapter 2.0- Antioxidant properties of bran protein hydrolysates, and their inhibition of low-density lipoprotein and bile binding capacity

2.1. Abstract

The aim of this study was to characterize oat bran protein hydrolysates and to evaluate their activities through antioxidant, bile acid binding, and prevention of LDL oxidation. Two protein isolates CPI and VPI were extracted from oat bran with the aid of polysaccharides degrading enzymes cellulase and viscozyme, respectively. Ten hydrolysates were subsequently prepared by treating CPI or VPI with five proteases protamex, alcalase, flavourzyme, pepsin and pepsin+pancreatin. The highest degree of hydrolysis (26.4 ± 0.5 and $22.0 \pm 0.1\%$) was obtained under simulated gastrointestinal digestion with pepsin and pancreatin. Intrinsic absorbance showed that CPI-alcalase and CPI-pepsin/pancreatin had a higher content of aromatic amino acids. In the antioxidant assays, VPI-pepsin better scavenged ROO[•] radicals (496.77 ± 5.83 μ M Trolox Equivalents (TE)/g) while VPI-flavourzyme and VPI-pepsin quenched HO[•] and O₂^{•-} radicals by 27.95 ± 1.58 and $45.31 \pm 6.6\%$, respectively. In the metal chelation assays, most of the samples except Pepsin hydrolysates were able to chelate iron in a dose-dependent manner, whereas VPI-protamex exhibited the best copper chelating capacity ($59.83 \pm 1.40\%$). All hydrolysates protected human low-density lipoprotein against copper mediated oxidation by reducing the concentration of hydroperoxides from 158.4 to 74.4 – 97.7 μ M H₂O₂/g of LDL. In the bile acid binding assay, none of the hydrolysates bind to chenodeoxycholic acid while there was a binding of up to 46.3% for taurodeoxycholate and taurocholate.

2.2 Introduction

The oxidation of molecules, such as lipids, proteins, pigments, and aroma compounds can be triggered by light, oxygen or metal ions. It is a major cause of deleterious quality changes in food products, affecting flavor, color and texture, and also leading to the loss of nutritive value (Bamdad et al., 2011b). In human, when the homeostasis between the formation of oxidants and their reduction is disrupted, the accumulation of oxidants will damage biological molecules thereby, altering their structure and function. This process known as oxidative stress causes chain reactions and lead to patho-physiological events and chronic conditions such as atherosclerosis (Sheih, Wu, & Fang, 2009) (Y. Zhang et al., 2011). Free radical-induced or metal ion-dependent oxidations of low-density lipoprotein (LDL) cholesterol is an important step in developing atherosclerotic lesions that lead to coronary heart diseases (Chandrasekara & Shahidi 2011). Consequently, the inhibition of LDL peroxidation through supplementation of diets with antioxidants becomes an attractive therapeutic strategy to prevent or delay the development of atherosclerosis and related diseases in human. A large number of studies have been devoted to the inhibition of lipid peroxidation in LDL using antioxidants. Natural antioxidants derived from food proteins (e.g. hydrolysates, peptides) have attracted special attention because of their safety and broad distribution properties (B. Wang et al., 2016). Previous works have elucidated antioxidant activity of some hydrolyzed oat proteins. Gelatin hydrolysate partially (39.2%) inhibited the oxidation of LDL cholesterol (Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2013a) while there was a complete inhibition by hydrolyzed peanut proteins (Hwang, Shyu, Wang, & Hsu, 2010). Other ways to prevent LDL oxidation is to reduce the amount in the circulation can be achieved using molecules that bind bile acids and prevent their reabsorption and conversion to cholesterol in the liver (Kongo-Dia-Moukala et al., 2011). One group of molecules is dietary fibre and graduating other compounds such as protein hydrolysate. Radical scavenging, inhibition of linoleic acid oxidation and iron chelating properties of hydrolyzed oat proteins have studies (Vanvi & Tsopmo, 2016) (Tsopmo, Gao, & Baakdah, 2014b); however their potential in other model systems is needed. Furthermore, there is no report in the literature regarding the ability of oat bran protein hydrolysates to chelate bile acids. The objective of the present study was to extract proteins from oat brans, digest them with various proteases, characterize the digests and determine their biological activity (antioxidant, bile acid binding, and inhibition of LDL oxidation).

2.3 Material and Methods

Medium oat bran flour (112-001) was supplied by Richardson Milling, MB, Canada. Carbohydrases viscozyme L 100 Fungal Beta Glucanase (FBG)/g and cellulase, 700 Endo-Glucanase Units (EGU)/g from *Trichoderma reesei* as well the different proteases, Pepsin and pancreatin from porcine gastric mucosa; flavourzyme (500 Leucine Aminopeptidase units/g) from *Aspergillus oryzae*, alcalase (2.4 units/g) from *Bacillus licheniformis*; protamex (1.5 amylolytic unit of Nagase/g) from *Bacillus sp.* were obtained from Sigma Aldrich, Oakville, ON, Canada) Reagents and chemicals such as ninhydrin (1,2,3-indantrione monohydrate), copper sulfate pentahydrate $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Folin & Ciocalteu's phenol reagent (2N), sodium tartrate, albumin from bovine serum (BSA), Pyrogallol, Iron (II) chloride, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (Ferrozine), ammonium iron (II) sulfate hexahydrate, Xylenol orange and 1,10-phenanthroline, were also from Sigma Aldrich. Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), L-glutathione reduced (GSH), hydrogen peroxide, pyrocatechol violet, fluorescein and butylated hydroxytoluene were purchased from Fischer Scientific (Nepean, ON, Canada). AAPH (2, 2'-Azobis (2-amidinopropane) was supplied by Wako Chemicals (Richmond, VA, USA). EDTA free Low-Density Lipoprotein (LDL) from human plasma was supplied by Lee Biosolutions, Inc (MO, USA) while chenodeoxycholic acid (CDCA), taurodeoxycholate (TDC) and taurocholate (TC) were from Cayman Chemical (MI, USA). Finally, the total bile assay kit was purchased from CellBio Labs Inc (San Diego, USA). All spectrophotometric and fluorometric measurements were performed on the BioTek® Epoch™ UV-Vis and Biotek FLx 800 microplate reader, respectively, both controlled by Gen5™ data analysis software. Incubations were done on a MaxQ™ 5000 shaker model (Fisher Scientific, Nepean, ON).

2.3.1 Proteins extraction and hydrolysis

Fifty grams of defatted medium oat bran flour was mixed with water (1:10 ratio) and blended to obtain homogeneous slurries. The pH was adjusted to 4.5 by addition of NaOH (1M) followed by addition of Viscozyme (3FBG/g) or Cellulase (20 EGU/g) based on concentrations from a previous work (Vanvi & Tsopmo, 2016) (Jodayree et al., 2012). Polysaccharides were degraded by incubation at 45°C, 150 rpm for 1.5 h. The pH was adjusted to 9.2 with NaOH 1M and further incubated for 1.5 h to solubilize the proteins. Samples were cooled and centrifuged at 2500×g, 4°C for 20 min to remove insoluble residue. Proteins in supernatants were collected after centrifugation

at the isoelectric point (pH 4.5) for 40 min at 4°C and 10000×g for 40 min. They were labelled as cellulase (CPI) and viscozyme (VPI) protein isolates and then freeze-dried. The hydrolysis was performed with pepsin, pepsin+pancreatin, flavourzyme, alcalase, and protamex to generate 10 hydrolysates. In each case, 1g of freeze-dried protein isolate (CPI or VPI) was added to 15 mL of deionized water. The pH of the solution was adjusted to the optimum value of each protease using 1M HCl or 1M NaHCO₃. Each enzyme was added at ration 4% w/w (E:S) after which the sample was incubated at recommended temperature an time; 37 °C and 1 h (pepsin), 50 °C and 3 h (flavourzyme), 60 °C and 3 h (alcalase, protamex) (Vanvi & Tsopmo, 2016) (Laohakunjit, Kerdchoechuen, Kaprasob, & Matta, 2017) (Xie, Huang, Xu, & Jin, 2008a)(Alemán et al., 2011). The simulated gastrointestinal digestion was performed with incubation with pepsin (1 h) followed by addition of pancreatin and further incubation for 2 h at 37 °C (Tounkara, Bashari, Le, & Shi, 2014). At the end of the hydrolysis process, enzymes were inactivated by heating at 90°C for 10 min in a water bath. The hydrolysates were collected after centrifugation and freeze drying.

2.3.2 Free amino acids determination

The degree of hydrolysis of protein hydrolysates was determined by measuring their free amino acid contents. The analysis was performed according to literature (Panasiuk, Amarowicz, Kostyra, & Sijtsma, 1998). All protein hydrolysates and L-serine (standards) solutions were prepared in phosphate buffer (pH 7.4, 75 mM). Standards concentrations were 80, 90, 100, 120, and 140 µg/mL while hydrolysates were made 1 mg/mL. A solution of ninhydrin (0.5%) in water was also freshly prepared before the assay. For the analysis, 0.5 mL of sample, standard or blank (buffer) was transferred to microcentrifuge tube followed by the addition of 0.5 mL of ninhydrin reagent. Tubes were incubated in a boiling water bath (100 °C) for 30 min after which, and they were cooled and diluted at a 1:5 ratio with water. Two hundred microliters of each tube were transferred in triplicate into a clear microplate. Absorbance was read at 570 nm on the Epoch Biotech microplate reader. Free amino acid contents were expressed as the percentage of the hydrolysate.

2.3.3 Determination of protein content

Oat bran protein isolates and their hydrolysates were assayed in order to determine soluble protein contents using Lowry assay (Markwell, Haas, Bieber, & Tolbert, 1978). Reagent A contained 2 Na₂CO₃, 0.4% NaOH, 0.16% sodium tartrate and 1% sodium dodecyl sulfate (SDS) in water; reagent B was made of 4% CuSO₄ in water while reagent C was freshly made by mixing reagents

A and B at a 100 to 1 ratio. The 2N Folin-Ciocalteu phenol reagent diluted 1:1 with water prior to use. Standard concentrations (0, 10, 20, 40, 60, 80, and 100 $\mu\text{g/ml}$) of bovine serum albumin and samples (80 $\mu\text{g/mL}$) were made in 0.5% SDS. Two hundred microliters of each standard concentration or samples were transferred to 1.5 mL vials, mixed with 600 μL of reagent C and incubated at room temperature for 25 min. Sixty microliters of diluted Folin–Ciocalteu phenol reagent were added and vortexed immediately to avoid color localization. Tubes were incubated at room temperature for 45 min followed by absorbance reading at 660nm using the Epoch Biotech microplate reader. The calibration curve constructed with BSA was used to determine the protein content of the samples.

2.3.4 Spectroscopic analysis

Spectroscopic analyses were performed to intrinsic absorbance or aromatic amino acids contents (UV-Spectroscopic) and functional groups (Fourier-transform infrared (FT-IR) spectroscopy) present in the hydrolyzed oat protein samples. Samples of UV (1 mg/mL in 75 mM phosphate buffer pH 7.4) were centrifuged at 10000 $\times g$ for 5 min to remove the undissolved solid matter before the absorbance reading at 280 nm. The spectrum of phosphate buffer was used as the blank. In the FTIR spectra were obtained using MB Series Arid-Zone™ Spectrometer (ABB, Thunder Bay, ON, CA). Sample (1.0 mg) was mixed with 400 mg of KBr and transformed into a pellet before reading. Data analysis was performed using GRAMST™ 32 Software (Thermo Fischer Scientific, Mississauga, ON, CA). The instrument used was Bomem model MB100, 4 cm^{-1} resolution, scan range 4000 to 600 cm^{-1} , which included DTGS detector (Deuterated TriGlycine Sulfate detector), 1 scan summarized.

2.3.5 Radical scavenging assays

Three assays that measured different radical species were used to determine the quenching ability of hydrolysates. Samples and reagents for oxygen radical absorbance capacity (ORAC) and hydroxyl radical ($\text{HO}\bullet$) assays were dissolved in potassium phosphate buffer (75 mM at pH 7.4) while they were dissolved in Tris-HCl buffer (50 mM, pH 8.3) for the superoxide anion radical ($\text{O}_2\bullet^-$) scavenging assay. In the ORAC assay, analysis was carried out by adding in the following order to black microplates 120 μL of fluorescein (0.08 μM) to all wells, 20 μL of buffer solution (blank), 20 μL of standard Trolox (6.25 – 100 μM), 20 μL of control glutathione (0.1 mg/mL), or 20 μL of hydrolysate (0.1 mg/mL). The plate was sealed with a transparent film and incubated for

20 min at 37°C in a fluorescence reader model FLx800 (excitation wavelength 485/20 nm, emission wavelength 528/20 nm). Freshly prepared AAPH (60 µL, 140 mM) was then added to all wells (blanks, samples, standards and control) followed by kinetic measured every minute for 1 h and ORAC values calculated using the area under the curve and expressed Trolox equivalents per gram of hydrolysate.

In the HO• assay, 50 µL of hydrolysate (1 mg/mL) or buffer (blank and control) were added to a 96-well clear microplate followed by the addition of 50 µL of 1,10-phenanthroline (3mM) and 50 µL of 3 mM aqueous Ferrous (II) and finally 50 µL of 0.03% aqueous hydrogen peroxide (sample and control) or water (blank) were added. The microplate was sealed and incubated at 37 °C for 1 h. Absorbance was measured at 536 nm using and the HO• scavenging activity was calculated as the percentage using the following formula $[(A_s - A_c)/(A_0 - A_c)] \times 100$. Where: A_s : absorbance of the sample; A_c : absorbance of the control; A_0 : absorbance of the blank. The O₂^{•-} scavenging activity was determined by adding 160 µL of hydrolysate (1 mg/mL) or buffer (control) to clear 96-well microplate followed by 70 µL of pyrogallol (2 mM in 10 mM HCl). The reaction rate ($\Delta A/\text{min}$) was recorded over 4 min at 420 nm was used to the O₂^{•-} activity:

$$\text{O}_2^{\bullet-} \text{ scavenging activity (\%)} = \{[(\Delta A/\text{min})_{\text{CTL}} - (\Delta A/\text{min})_{\text{SPL}}]/(\Delta A/\text{min})_{\text{CTL}}\} \times 100$$

2.3.6 Ferrous (II) and copper (II) chelating assays

Hydrolysates for Fe (II) ions chelating assay were made in water at 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL. The analysis was then performed by adding to 1.5 mL vials 400 µL of diluted hydrolysate or water (control) and then 50 µL of FeCl₂ aqueous solution (0.4 mM). Vials were mixed and allowed to stand for 5 min before addition of 400 µL of aqueous Ferrozine (2 mM). The absorbance after 10 min was recorded at 562 nm to calculate the ferrous ion chelating activity with the formula $(1 - \text{absorbance sample}/\text{absorbance control}) \times 100\%$. Copper chelating properties of hydrolysates was determined according to literature (Santo et al. 2017). Sodium acetate buffer 50 mM pH 6.0 was used to dissolve hydrolysates (1 mg/mL) and Pyrocatechol violet (PV, 4 mM) while Cu(II) (0.1 mg/mL) was made in water. Thirty microliters of hydrolysate were added to clear 96-wells plate and diluted with 170 µL of the buffer, while or 200 µL of the buffer was used in control wells. Cu(II) solution (30 µL) was added to all wells, mixed let to interact for 5 min after which 10 µL of PV was added. The absorbance was read at 632 nm after 10 min standing at room

temperature. The percentage of formation of Cu(II)-PV complex was calculated as followed: $[(\text{Abs sample}) - \text{Abs control} / \text{Abs Control}] \times 100$

2.3.7 Bile acid binding capacity assay

Bile acid capacity assay was performed in order to determine lowering cholesterol potential of oat bran protein hydrolysates. The method was carried out according to the total bile assay kit supplied by CellBio Labs Inc. Sodium phosphate buffer 50 mM pH 7.0 was prepared in order to dissolve samples and bile acids. Bile acids tested were chenodeoxycholic acid (CDCA), taurodeoxycholate (sodium salt) (TDC) and taurocholate (sodium salt) (TC). Their stock concentrations in buffer were 12.7 mM (CDCA), 9.58 mM (TDC) and, 9.3 TC 9.3 MM because these values provided acceptable absorbance readings when mixed as indicated below. Stocks concentrations of hydrolysates were 2mg/mL. In the initial step, 300 μ L of CDCA, TDC, or TC were added to 300 μ L of oat each hydrolysate. The buffer was used in controls. Tubes were mixed incubated (37 °C, 2 h, 120 RPM) and centrifuged at 16,000g for 5 min. The analysis was then performed using the total bile assay kit supplied by CellBio Labs Inc according to the instructions provided. In brief, 20 μ L of sample or standard were transferred into a 96 well microplate followed 75 μ L of assay reagent A, incubation (37°C, 5 min), then 25 μ L of assay reagent B and further incubation for 30 min. Bile acid chelating ability was expressed as optical density.

2.3.8. Conjugated dienes and lipid peroxidation determination in low-density lipoprotein

The oxidation of low-density lipoprotein (LDL) was evaluated by the quantifying conjugated dienes and hydroperoxides. Phosphate Buffer saline (PBS 10 mM, pH 7.4 containing 0.15 M NaCl) was used to dissolve oat bran hydrolysates (1 mg/mL) and EDTA-free LDL (0.05 mg/mL) while Cu(II) (100 μ M) was prepared in water and used for both tests. The conjugated dienes assay was modified from the work of (Wang et al., 2016). In a microplate, twenty microliters of hydrolysates or buffer (positive and negative controls) were added to wells followed by 160 μ L of LDL. The microplate was incubated at 37°C for 15 min then 20 μ L of Cu(II) were added to samples and positive control while the buffer was added to the negative control. Blank samples (20 μ L of hydrolysate + 160 μ L buffer + 20 μ L Cu(II)) were included in the analysis. The absorbance was measured immediately at 234 nm after addition of Cu(II) and again after 24 h incubation at 37 °C. Differences (Δ ABS) between the initial and final (i.e. 24 h) absorbance were used to calculate the percentage inhibition of the formation of conjugated dienes (CD) by the hydrolysates:

$$\text{CD inhibition (\%)} = (\Delta\text{ABS}_{\text{CTL}^+} - \Delta\text{ABS}_{\text{SPL}}) / (\Delta\text{ABS}_{\text{CTL}^+} - \Delta\text{ABS}_{\text{CTL}^-}) \times 100$$

Lipid hydroperoxides determination was based on their reaction with iron and subsequent complex formation (Gay, Collins, & Gebicki, 1999). The Ferrous oxide xylenol orange-2 (FOX-2) reagent was obtained by mixing one part of solution A made by dissolving 9.5 mg of ferrous ammonium sulfate and 7.5 mg of xylenol orange in 10 mL of H₂SO₄ (250 mM) with 9 parts of solution B (1 mg/mL mg of beta-hydroxytoluene in methanol). One hundred microliters of each hydrolysate or buffer (positive and negative control) were added to each tube followed by 800 µL of LDL. Sample blanks (100 µL hydrolysate + 800 µL PBS) were also included. Tubes incubated at 37 °C for 15 min, then 100 µL of Cu (II) were added to samples, sample blanks and positive control while 100 µL of water was added to the negative control. Immediately after, an aliquot of 150 µL from the tube was removed to measure initial hydroperoxide concentrations. The remaining quantities were incubated at 37 °C for 24 h after which hydroperoxides were quantified again. The measurement was performed by first mixing 150 µL from reaction (0 and 24 h) with 850 µL of FOX-2 reagent followed by 30 min incubated (37 °C) to form the ferrous oxide-xylenol orange complex. The quantification was then performed at 560 nm based on a standard curve constructed using hydrogen peroxide (5, 10, 15, 20, 40, 80, 160 and 200 µM).

2.3.9 Statistical Analysis

Triplicate determinations were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (SAS® Software 9.1, SAS on Demand, 9.4, 2017) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values, and significant differences were set at $P < 0.05$.

2.4 Results and discussion

2.4.1 Determination of protein and free amino acids contents of hydrolysates

Proteins were extracted from oats brans treated with viscozyme or cellulase because previous works have shown that they can degrade polysaccharides and enhance the extraction of proteins and phytochemicals from foods (Jodayree et al. 2012). The soluble protein content of the extracted samples their hydrolysates it was then quantified to compare the effect of carbohydrases and proteases on polypeptide bonds. Figure 2.1A displays the results. The isolate from bran treated with viscozyme (VPI) showed $86.3 \pm 3.3\%$ protein content while cellulase treated brans (CPI) yielded a lower content ($51.4 \pm 2.4\%$). After hydrolysis of VPI with proteases, the protein content decreased ($51.5 - 71.1\%$) in four of the five hydrolysates. This is common because, in addition to polypeptides the digests should contain salts and non-polypeptide molecules such as buffer components, polysaccharides or reducing sugars (Jodayree et al., 2012). These compounds may interfere with antioxidant activities of the hydrolysates through side reactions during proteolysis. In the case of proteins extracted with cellulase (CPI), hydrolysis overall had minimal effects on the concentrations of polypeptides. This is likely because CPI had protein content compared to VPI. Pepsin hydrolysates had the highest content of polypeptides while flavourzyme hydrolysates had the lowest (c.a. 51%) independently of the extraction method. Flavourzyme with both endo- and exo-peptidase activities was shown to produce low-molecular-weight peptide fragments from canola proteins (Cumby et al, 2008) . Similar effects on oat bran proteins might low polypeptide content in this work. Hydrolysates produced under simulated gastrointestinal digestion considerably lower polypeptides contents because pancreatin used in the intestinal phase also possess both endo- and exo-peptidase activities. Other works have used viscozyme, a multi-enzyme complex containing a wide range of carbohydrases (including arabanase, cellulase, β -glucanase, hemicellulase, and xylanase) and cellulase that catalyzes the breakdown of cellulose to enhance the extraction of proteins from foods (Guan & Yao, 2008). In this work, viscozyme was more efficient in the extraction of proteins from oat brans that cellulase because of the above difference in substrate-specificity and this is consistent with precious works on oats (Jodayree et al., 2012) and rice brans (S. Tang, Hettiarachchy, Eswaranandam, & Crandall, 2003).

The extent of proteins hydrolyzed was determined by measuring the free amino acid (FFA) content of hydrolysates. Ninhydrin, a powerful oxidizing agent, decarboxylates alpha-amino acids and yields a bluish purple product measured at 570 nm (Katoch, 2011). FFA content of hydrolysates is a good index of their degree of the hydrolysis of proteins. Pep/Pan hydrolysates had the highest

content of FFA (22.0 ± 0.1 and 26.4 ± 0.5) followed by flavourzyme hydrolysates (15.63 ± 0.12 and $17.94 \pm 0.23\%$) (Figure 2.1B). The explanation might lie to the two-step process of produce VPI-Pep/Pan and CPI-Pep/Pan but also the fact both pancreatin and flavourzyme possess endo- and exo-peptidase activities. This is correlated with the data from the Lowry assay (Figure 2.1A) displaying lower protein content for these hydrolysates. In contrast pepsin hydrolysates with higher protein contents showed lower FFA indicating they might have larger polypeptides. Hydrolysates with lowest FFA contents were VPI-Pro and CPI-Pro treated sample (8.10 ± 0.06 and $9.51 \pm 0.043\%$ respectively). An increase in the degree of hydrolysis indicates that peptide bonds were cleaved providing amino acids and small peptides during hydrolysis (Thamnarathip, et al. 2016) thus, lower FFA content of protamex hydrolysates demonstrated its less effective proteolysis. There are literature data proteases differences to break down proteins (Malomo & Aluko, 2016) (Seung et al, 2014). Overall, the degree of hydrolysis values of proteins is dependent on the source of proteins, sequences, enzyme type, and hydrolysis conditions.

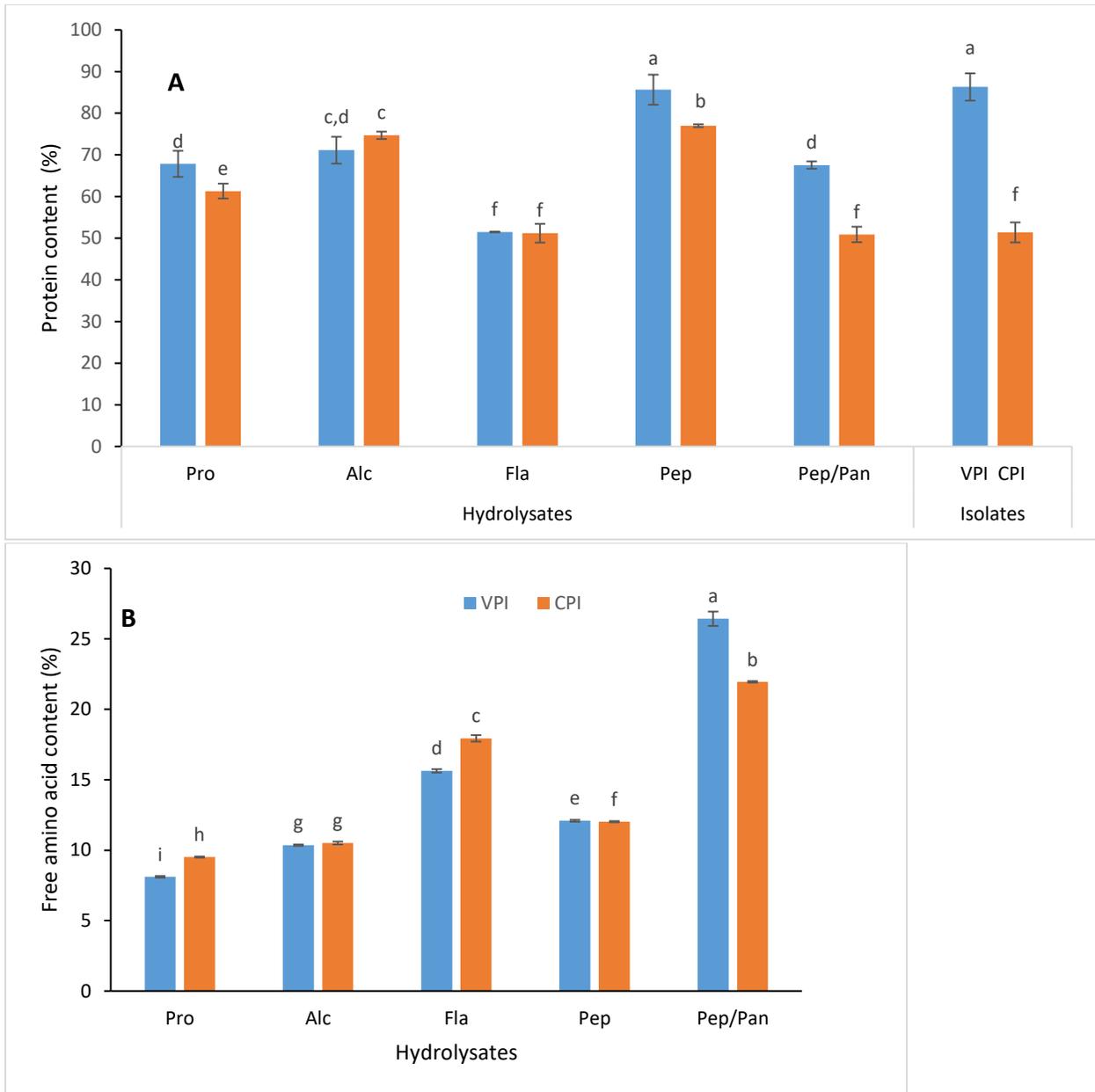


Figure 2. 1.- Proteins (A) and free amino acids (B) contents oat bran proteins extracted with viscozyme (VPI) and cellulase (CPI). Proteins were hydrolyzed with protamex (Pro), alcalase (Alc), flavourzyme (Fla), pepsin (Pep), and pepsin/pancreatin (Pep/Pan). Letters indicate significant differences between group means ($p < 0.05$).

2.4.2 Spectroscopic characteristics of hydrolysates

The hydrolyzed proteins were characterized using Fourier transform infrared (FTIR) and ultraviolet (UV) spectroscopies. FT-IR spectra (Figure 2.2A) showed major absorption bands in hydrolysates at 3408 cm^{-1} characteristics of the stretching of O-H and N-H (amide A band) functional groups proteins, hydrolyzed proteins and peptides as reported in other studies (Kanbargi, Sonawane, & Arya, 2017) (Zheng et al., 2015). Bands at 1653 cm^{-1} assigned to the stretching of C=O (amide I band) and those 1401 cm^{-1} assigned the bending of N-H and stretching of C-N bonds (amide II band) have been reported to be typical of peptide bonds (-NH-C=O-) (Abdelhedi et al., 2017) (Hou et al., 2018). Bands at 2960 cm^{-1} region were due to C-H stretching vibrations of methyl ($-\text{CH}_3$) and methylene ($-\text{CH}_2-$) functional groups (López-Barón et al., 2018)(Zheng et al., 2015), while those at $1074\text{-}1115\text{ cm}^{-1}$ referred to C-H bending and C-C/C=O stretching (Yu et al., 2018). Wavelength maxima of amide I, II and A bands can be different as a result of differences in secondary structures of polypeptides; there is, however, no differences in spectra of samples analyzed in this work. Proteins were hydrolyzed with various proteases and although, polypeptides of various size were generated their length was enough to cause significant changes in stretching and bending of functional groups that would have translated in the shift of wavelength maxima.

UV absorption spectra were also obtained to assess the presence of aromatic amino acid (AAA) residues which constitute the chromophores that absorb in the near UV region. The analysis of spectra (Figure 2.2B) showed that hydrolysates could be grouped into three based on the intensity of the absorbance. In the higher end, there are CPI-Alc and CPI-Pep/Pan hydrolysates while the lower end contained VPI-Pep, CPI-Pep and CPI-Pro. The other five hydrolysates fell into the middle region. In proteins, hydrolyzed proteins and peptides, UV absorption is due to the combined effect of tyrosine, tryptophan, and phenylalanine which often display maximum absorption at approximately 278, 285, and 260 nm, respectively (Ding et al., 2018) (Pal & Suresh, 2017b). The difference in UV peak intensities of hydrolysates then resulted from the exposure of the chromophores upon hydrolysis to proteolysis. The effect of pepsin and flavourzyme although different was independent of the carbohydrase used to extract proteins. In contrast, digestion under simulated gastrointestinal condition (Pep/Pan) and with alcalase greatly expose aromatic amino acids (i.e. high UV peak intensities) for proteins extracted with cellulase (CPI) compared to those extracted with viscozyme (VPI). A study by Silvestre et al. reported that the intensity UV peaks

of casein hydrolysates varied based on both the degree of hydrolysis and the type of protease used for hydrolysis (Silvestre, Dauphin, & Hamon, 1993).

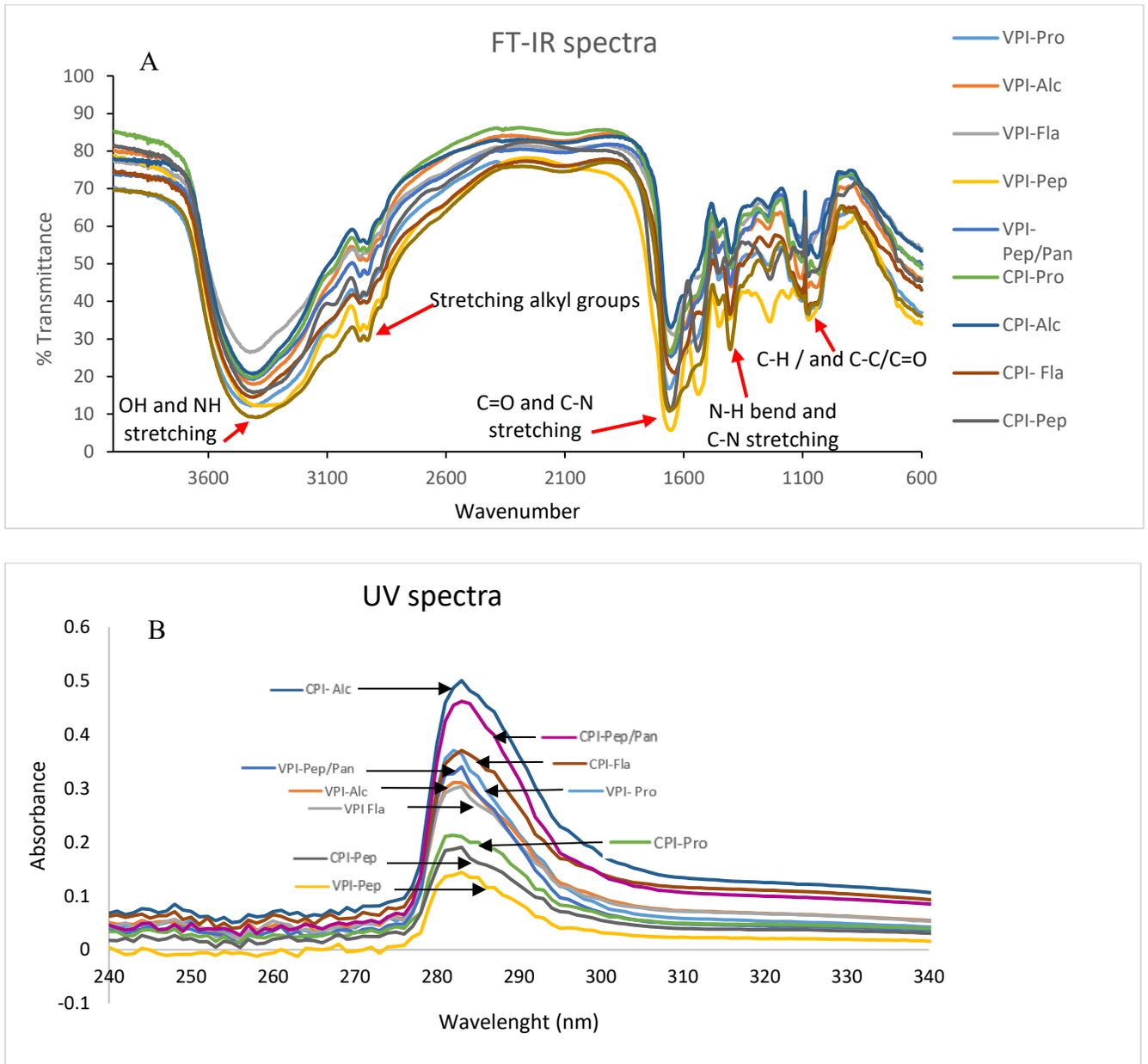


Figure 2. 2. - Oat bran protein hydrolysates FTIR spectra (A) and UV absorption spectra (B). Two cell wall polysaccharides enzymes were mainly used viscozyme and cellulase to generate isolates (VPI &CPI). Proteases used for protein hydrolysis include protamex (Pro), alcalase (Alc), flavourzyme (Fla), pepsin (Pep), and pepsin/pancreatin (Pep/Pan). Spectra were recorded over wavelengths 4000-500 cm⁻¹ for IR. Absorbance was read at the wavelength range of 200–400 nm for UV.

2.4.3. Radical scavenging activities of hydrolysates

The ability of hydrolysates to act as antioxidants was evaluated based on their potential to quench three common radical species which are peroxy ($\text{ROO}\cdot$), superoxide anion ($\text{O}_2\cdot^-$) and hydroxyl ($\text{HO}\cdot$) radicals.

2.4.3.1 Oxygen radical absorbance capacity (ORAC) assay

The $\text{ROO}\cdot$ radical scavenging capacity was determined using the oxygen radical absorbance capacity (ORAC) assay which measures both inhibition time and degree of inhibition. Data (Figure 2.3A) showed that in general, all hydrolysates possessed good $\text{ROO}\cdot$ scavenging power. VPI-Alc, VPI-Pep, and VPI-Pep/Pan demonstrated the highest $\text{ROO}\cdot$ scavenging capacity (496 ± 13 , 497 ± 6 and 479 ± 5 $\mu\text{M TE/g}$) followed CPI-Alc, CPI-Fla and CPI-Pep, (466 ± 12 , 461 ± 9 and 463 ± 3 $\mu\text{MTE/g}$ respectively). The remaining hydrolysates displayed ORAC values in the range of 397-498 $\mu\text{M TE/g}$. There appears to be no relationship between aromatic amino acids as VPI hydrolysates with low and medium UV peak intensities had the highest activities in the ORAC test. Similarly, no correlation was found between ORAC values and free amino acid contents of hydrolysates. Some previous works have found correlations between the length of hydrolysis (i.e. the degree of hydrolysis) using single protease (Di Pierro, O'Keeffe, Poyarkov, Lomolino, & Fitzgerald, 2014) meanwhile, in this work the variable was protease and not time. Peptides are known to display different antioxidant activities depending on their molecular sizes, amino acid compositions and sequences (Chi et al. 2015). It was then likely that these factors played a more important role in $\text{ROO}\cdot$ scavenging activities than both either degree of hydrolysis or the presence of aromatic amino acids. Other residues such as methionine, cysteine and histidine not characterized by FTIR and UV spectroscopy might have contributed as well to ORAC values. This study agrees with previous works on oat bran protein hydrolysates treated with alcalase (Jodayree et al. 2012) and pepsin (Vanvi & Tsopmo, 2016) where they have shown an improved ORAC activity when treated with these proteases.

2.4.3.2 Superoxide anion and hydroxyl radical scavenging activities

The scavenging of $\text{O}_2\cdot^-$ radicals was measured based on the autoxidation of pyrogallol while that of $\text{HO}\cdot$ radicals was based on the reaction between hydrogen peroxide and iron (D. X. Jin, Liu, Zheng, Wang, & He, 2016). They are often measured together with $\text{ROO}\cdot$ because they oxidize food and biological molecules via different pathways (Magnani, Gaydou, & Hubaud, 2000).

Hydrolysates scavenged $O_2^{\bullet-}$ radicals 25.0 ± 3.1 to $45.31\pm 6.6\%$ (Figure 2.3B). The biggest difference was observed for hydrolysates produced with pepsin/pancreatin. When the proteins were extracted with cellulase, treated with pepsin/pancreatin (CPI-Pep/Pan), the $O_2^{\bullet-}$ scavenging power $40.6\pm 3.2\%$ was 1.6-fold higher compared the hydrolysate of proteins extracted with viscozyme (VPI-Pep/Pan, $25.0\pm 3.1\%$), radicals. Cellulase and viscozyme caused different structural changes to proteins that resulted in the difference in extraction. Although VPI-Pep/Pan had higher free amino content this wasn't the factor influencing its $O_2^{\bullet-}$ activity. In contrast, the content aromatic amino acids (Figure 2.2B) appeared to be an important factor to explain the difference between $O_2^{\bullet-}$ activities of VPI-Pep/Pan and CPI-Pep/Pan. There was little difference between the other pairs of hydrolysates. Other factors such charge might play a role as $O_2^{\bullet-}$ carries a negative charge but a correlation with hydrophobicity has been found (Li et al, 2008). In the present work, $O_2^{\bullet-}$ quenching activities of oat protein hydrolysates are within the range of those reported for corn protein hydrolysates (26-53%) (D. X. Jin et al., 2016), but higher than barley hordein (15-32%) (Bamdad et al., 2011b) while they are lower than some wheat germ protein (0-75.40%)(Cheng et al, 2006) and soy protein (24.7-85.6%) (Moure et al., 2006) hydrolysates.

Hydroxyl radical (HO^{\bullet}) is the most oxidizing of the three tested oxidants and react with lipid proteins, nucleotides and sugars. The control of its formation is then probably one of the most effective defence mechanisms of living organisms against various diseases (Je et al. 2007). The HO^{\bullet} scavenging capacity of oat proteins ranged from 5.6 ± 0.94 to $28.0\pm 1.6\%$ (Figure 2.3C). Notably, VPI-Fla had by far the most potent HO^{\bullet} scavenging activity about 2-fold that of other hydrolysates. It was followed by VPI-Alc (14.0 ± 1.6) and similarly active CPI-Fla and CPI-Pro (12.74 ± 0.82 and 11.82 ± 1.11). Differences in the HO^{\bullet} scavenging activities between hydrolysates might be associated protease specificity. Amongst the proteases, flavourzyme is the only one with endo- and exo-proteinase and might have produced peptides with proper sequences to quench HO^{\bullet} radicals. In a previous work, the high content of aromatic amino acids correlated with high HO^{\bullet} radical scavenging activities of yam bean seed hydrolyzed proteins (Ajibola, Fashakin, Fagbemi, & Aluko, 2011) but this does not appear to be the case hydrolysates prepared in this work likely because the sequences of peptides from both studies are different. The HO^{\bullet} of hydrolysates from this study are within the range of other cereals hydrolyzed proteins like barley and corn (Xia, Bamdad, Gänzle, & Chen, 2012) (D. X. Jin et al., 2016).

Radical scavenging capacities ($\text{ROO}\cdot$, $\text{O}_2\cdot^-$, $\text{HO}\cdot$) of oat bran protein hydrolysates could also be due to Maillard reactions products (MRP) such as reductones and Schiff bases (Wang, Bao, & Chen, 2013) (Mohan, Udechukwu, Rajendran, & Udenigwe, 2015). Oat bran digested samples are complex mixtures which include not only peptides but also sugars. Although proteolysis was not conducted at extreme conditions, it was extensive and performed at optimum conditions of the proteases for a prolonged duration (2–4 h). This can encourage side events such as Maillard reaction (MR), leading to changes in the structure of the generated peptides (Van Lancker, Adams, & De Kimpe, 2011). On protein hydrolysates the non-enzymatic glycation reaction has been showed to occur due to heat treatment, and pH conditions during hydrolysis. The generated MR have demonstrated antioxidant activities produced by various mechanisms including breaking radical chains, scavenging free radicals, donating electrons and chelating transitional metals (W.-G. Jin et al., 2018).

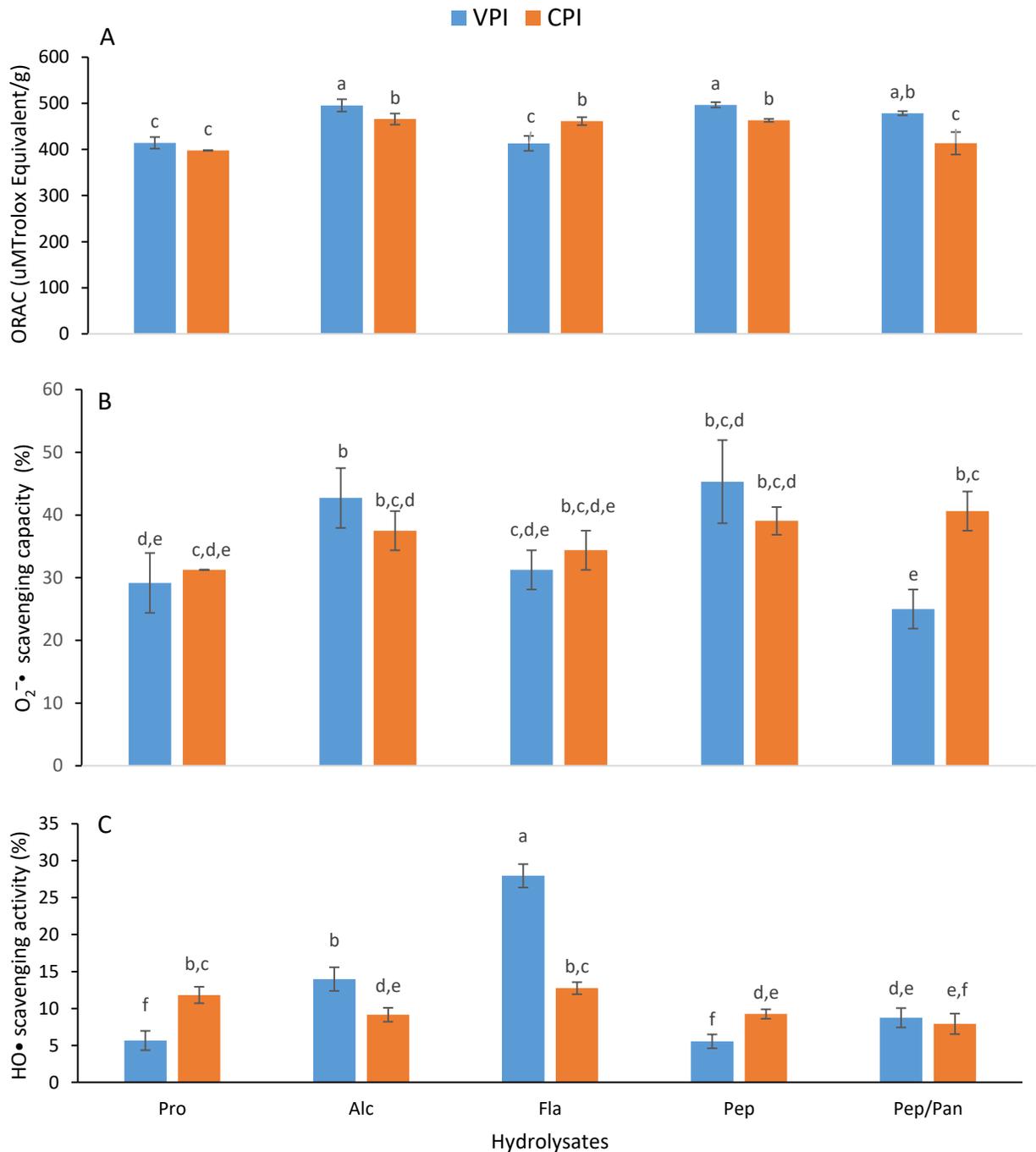


Figure 2. 3. - Antioxidant activities of hydrolyzed proteins. Oxygen radical absorbance activity (A), superoxide radical anion (O₂^{•-}) activity (B), hydroxyl radical (HO•) activity (C). Proteins extracted with viscozyme (VPI) or cellulase (CPI) were hydrolyzed with protamex (Pro), alcalase (Alc), flavourzyme (Fla), pepsin (Pep), pepsin/pancreatin (Pep/Pan). Means with different letters were significantly different in the Duncan test (p<0.05).

2.4.4 Iron and copper chelating activities

Transition metal ions including Fe^{2+} and Cu^{2+} are involved in many oxidation reactions *in vivo* and model systems (Xie, Huang, Xu, & Jin, 2008b). It is essential to identify molecules that can chelate them and potentially improve their absorption or prevent their prooxidant effect by decreasing the concentration of free ions available to participate in the Fenton reaction (i.e. production of $\text{HO}\cdot$ radicals). The Fe^{2+} and Cu^{2+} chelating capacity of hydrolysates was determined based on the reduction of the absorbance of ferrozine- Fe^{2+} and Pyrocatechol violet- Cu^{2+} complexes respectively (K. Zhu, Zhou, & Qian, 2006)(Sánchez-Vioque et al., 2012). Data (Figure 2.4) showed that hydrolysates at the exception of pepsin ones had good chelating activities for both Fe^{2+} and Cu^{2+} ions. In the Fe^{2+} assay, the chelating activities of eight of the hydrolysates increased in a concentration (0.01-1.0 mg/mL) dependent manner to reach similar maxima ($p > 0.05$) between $75.6\pm 0.4\%$ (CPI-Pep/Pan) to $79.9\pm 0.6\%$ (CPI-Alc) (Figure 2.4A). Meanwhile, pepsin CPI and VPI hydrolysates only reach maxima of about 10% at 0.1 mg/mL that were relatively constant up to 0.5 mg/mL before dropping to zero. It is possible that because pepsin hydrolysates reached maximum chelation earlier, higher concentrations led to aggregation and hence the non-detection of iron chelates. The copper chelating activity was performed at 1 mg/mL due to sample limitations. Hydrolysates chelated Cu^{2+} by 25.3 ± 3.1 to $59.8\pm 1.4\%$ (Figure 2.4B). In general, VPI hydrolysates had higher Cu^{2+} chelation activities compared to CPI hydrolysates. Pepsin hydrolysates better chelated Cu^{2+} ions ($25.3\pm 3.1\%$ for CPI-Pep and $53.8\pm 1.0\%$ for VPI-Pep) compared to about 10% for both in the Fe^{2+} test. Similar functional groups are involved in the chelation of divalent ions such as iron and copper. Amino acids that contain such functional groups are ions are mainly aspartic acid, glutamic acid and histidine but cysteine and methionine can participate as well (Cian et al., 2016) (Phongthai et al., 2018). Although these amino acids are present in oat proteins (Baakdah & Tsopmo, 2016), their specific location in in polypeptides differed from one hydrolysate to the other because different proteases were used in this study for hydrolysis. The difference in chelating activities for the same hydrolysate toward Fe^{2+} and Cu^{2+} ions might be because different reagents were used in the assays but more likely because there was some difference in the affinity of the functional groups of the released peptides. Results reported in this study are lower to those exhibited by hempseed (C. H. Tang, Wang, & Yang, 2009) for Fe^{2+} but similar to the common bean in the case of Cu^{2+} (Segura-campos et al., 2013).

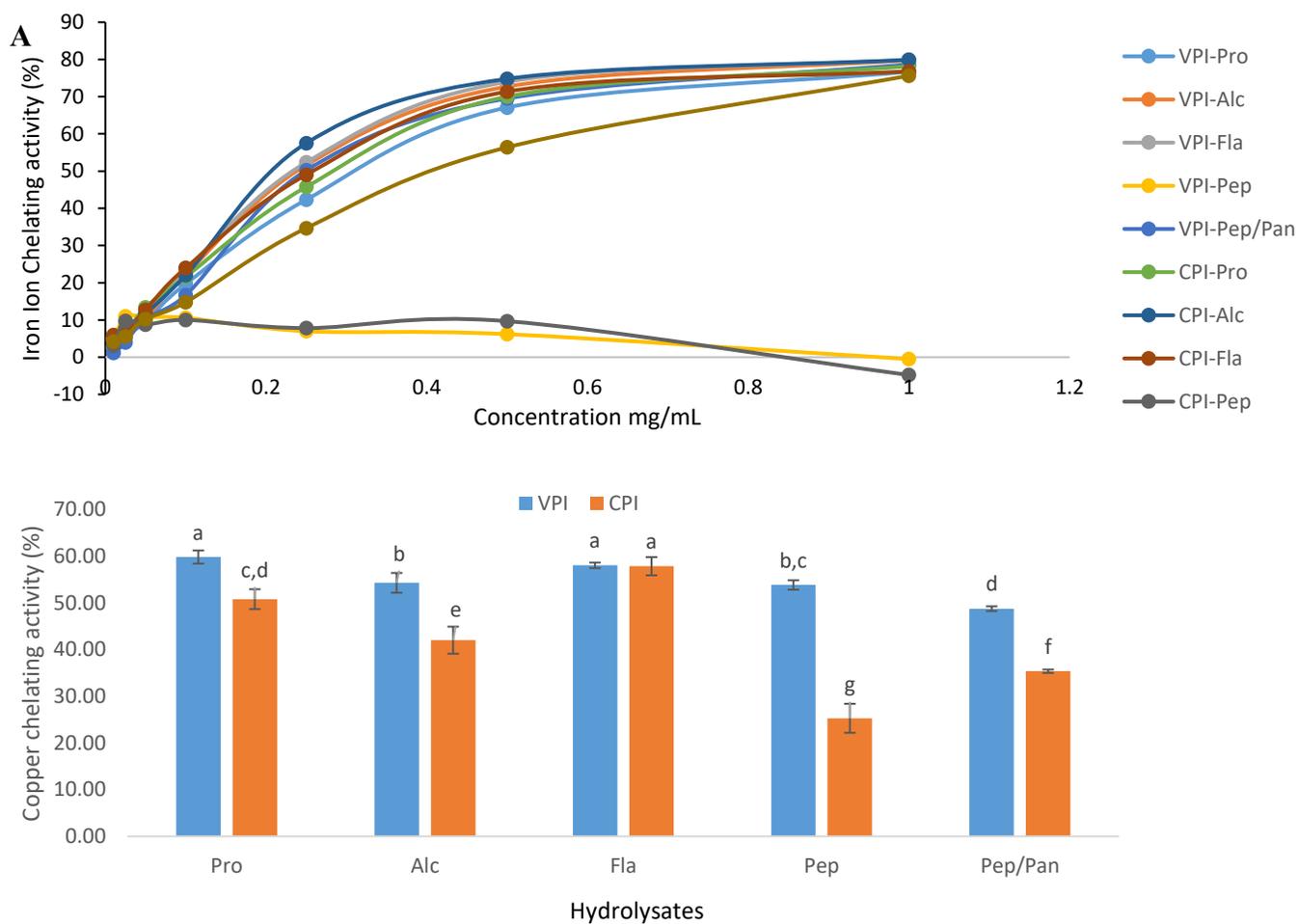


Figure 2.4. - Iron chelating activity (%) (A) and copper chelating activity (%) (B) of hydrolyzed oat bran proteins treated with viscozyme (VPI) and cellulase (CPI). Proteases used for protein hydrolysis include protamex (Pro), alcalase (Alc), flavourzyme (Fla), pepsin (Pep), pancreatin (Pan). Serial dilutions of each sample were assayed (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1 mg/mL) (A). Samples concentration was 1mg/mL. Letters indicate significant differences between means ($p < 0.05$) (B).

2.4.5 Inhibition of low-density lipoprotein oxidation

Conjugated dienes and lipid hydroperoxides determinations were used to assess the protective effect of hydrolysates against copper-induced oxidation of low-density lipoprotein (LDL) cholesterol. Inhibitors of LDL oxidation are important to preserve its biological function and also because oxidized LDL increases the risks of developing cardiovascular diseases (Barry Halliwell, 2009). In addition to protein and cholesterol, LDL contains polyunsaturated fatty acids which are prone to oxidation (Teissedre, Frankel, Waterhouse, Peleg, & German, 1996). In this study, oxidation EDTA-free human LDL was induced by copper followed by determination of conjugated dienes (CD) and lipid hydroperoxides (LHP). Results are displayed in Figure 2.5 A and B respectively. Among all treatments, CPI-Pro, CPI-Alc, and CPI-Pep showed the better capacity to inhibit CD. CPI hydrolysates activity might be related to the presence of aromatic amino acids (Figure 2.2B), as some of them displayed the highest UV absorbance. Several studies have shown that not only aromatic residues but also other amino acids such as histidine, methionine, leucine, glycine or proline in peptide chain can enhance their antioxidant activities (Kittiphattanabawon et al., 2013) (Park et al., 2001) (B. Li, Chen, Wang, Ji, & Wu, 2007). In addition, hydrolysates that exhibited the lowest amount of lipid hydroperoxides (LHP) were VPI-Fla ($83.70 \pm 4.04 \mu\text{M H}_2\text{O}_2/\text{g}$) and VPI-Pep/Pan ($74.37 \pm 6.56 \mu\text{M H}_2\text{O}_2/\text{g}$) followed by CPI-Pep, VPI-Pro and VPI-Pep (87.70 ± 5.66 , 90.03 ± 3.06 and $91.03 \pm 2.89 \mu\text{M H}_2\text{O}_2/\text{g}$ respectively). For some of these samples, data correlates with their high copper chelating capacity as displayed in Figure 2.4B, Cu^{2+} binding has been described as another antioxidant factor delaying LDL oxidation (Ambigaipalan & Shahidi, 2017) (Ambigaipalan & Shahidi, 2015), (Wang et al., 2011). Other mechanisms reported to prevent LDL oxidation by hydrolysates include lipid radical scavenging, hydrogen donation (Brand-Williams, Cuvelier, & Berset, 1995), and protein binding (Liyana-Pathirana & Shahidi, 2006) (Naidu & Thippeswamy, 2002). Data found in the literature regarding CD and LHP cannot be compared to present data due to differences in determination techniques.

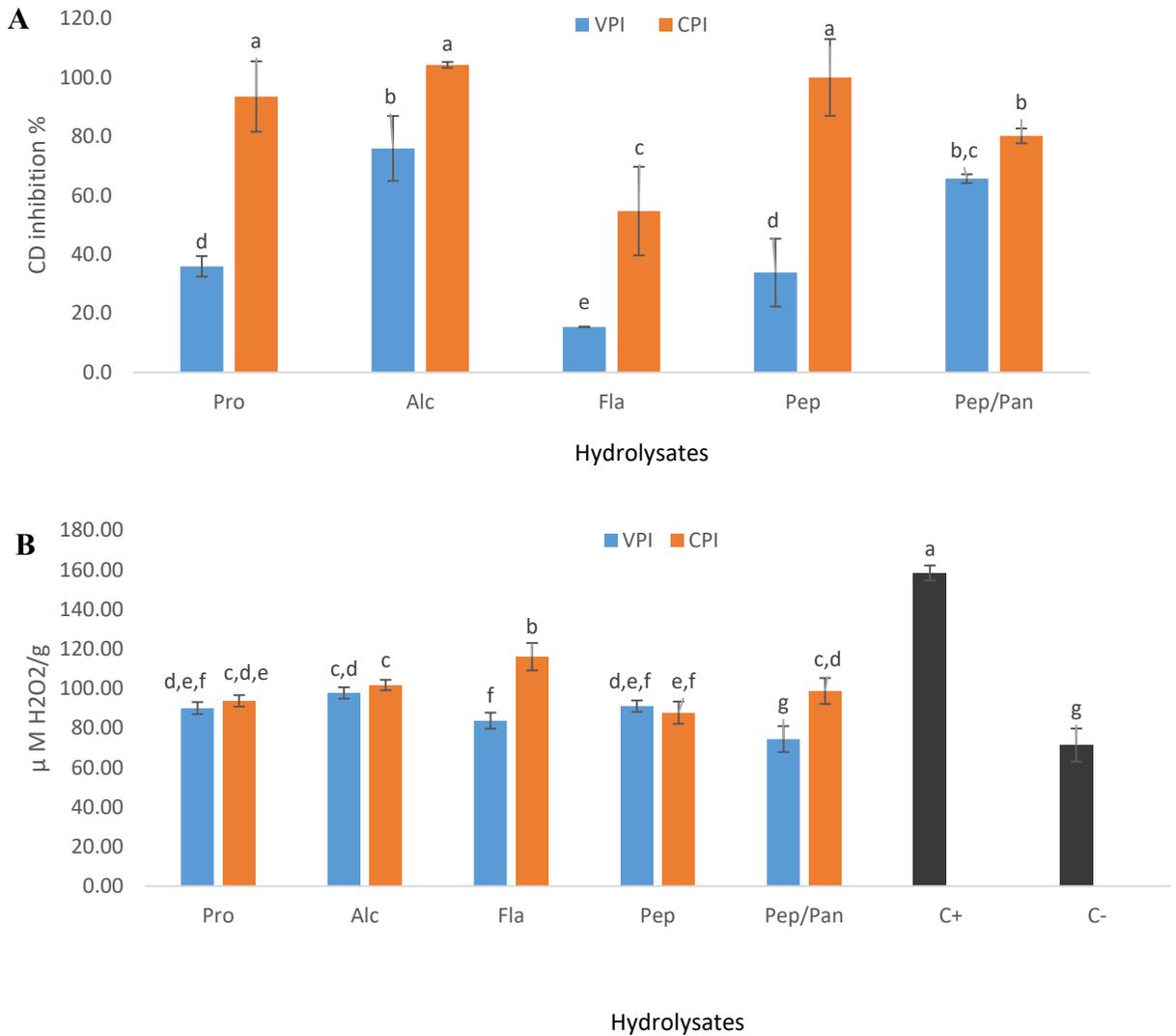


Figure 2.5. - The effects of oat bran protein hydrolysates on conjugated dienes (A) and lipid hydroperoxides (B) produced from LDL oxidation. Samples were treated with viscozyme (VPI) or cellulase (CPI). Proteases used for protein hydrolysis include protamex (Pro), alcalase (Alc), flavourzyme (Fla), pepsin (Pep), pancreatin (Pan). Letters indicate significant differences between means ($p < 0.05$).

2.4.6 Bile acid binding capacity

Bile acids are amphiphilic steroid derivatives that interact with a variety of compounds within the intestine, including proteins, lipids and cations. In addition to molecules that prevent LDL oxidation, those that bind bile acids are important in the reduction of cardiovascular diseases due to their role in the control of circulating cholesterol. In this context, bile acids play an essential role in the digestion and absorption of fat as well as fat-soluble components. They also form micelles to capture cholesterol and accelerate cholesterol absorption (Siow, Choi, & Gan, 2016b). In vitro bile acid binding is used as a measure of the hypocholesterolemic potential of foods since this mechanism of action reduces absorption of cholesterol (Barbana et al., 2011). The binding of three common bile acids, Chenodeoxycholic acid (CDCA), Taurodeoxycholate (TDC) and taurocholate (TC) were assayed to assess the chelating capacity of oat bran protein hydrolysates. Figure 2.6 displays bile acid binding results. None of the hydrolysates exhibited significant binding of CDCA ($p > 0.05$) (Figure 2.6A). On the other hand, seven of the ten hydrolysates showed significant binding ($p < 0.05$) toward TDC (Figure 2.6B). Amongst them, VPI-Pep/Pan, CPI-Fla, CPI-Pro, and CPI-Alc had the highest TDC binding. The three hydrolysates that did not bind TDC were VPI-Fla, CPI-Pep, and CPI-Pep/Pan. As shown in Figure 2.6C, CPI-Fla was the only hydrolysate that did not bind TC. It is worth to mention that VPI hydrolysates showed a better binding capacity to TC. Results showed no correlation between hydrolysates bile acids chelating capacity and their radical scavenging activity, since all display different antioxidant behaviour. In the same manner, FAA content did not associate to bile acids binding activity results, this agrees with other authors who found the same lack of attribution to the degree of hydrolysis (Kongo-Dia-Moukala et al., 2011). Alternatively, hydrolysates that showed activity towards TDC also displayed the ability to prevent CD formation to a certain extent, yet this might not be a critical factor to explain their chelating capacity. Binding effect of hydrolysates has been related to the influence of the nature of the peptide fractions released (Espejo-Carpio et al., 2016). Iwami et al., 1986 showed a correlation between the hydrophobicity of a protein hydrolysate and its chelating capacity towards bile acids (Iwami et al., 1986). Secondary bile acids such as TDC are more hydrophobic, which promotes hydrophobic interactions with side groups of proteins and peptide fractions (Ma & Xiong, 2009). In this regard, it should be noted that some of the enzymes such as pepsin, pancreatin, flavourzyme, and protamex that generated active binding hydrolysates for TDC, preferably cleave peptide bonds containing hydrophobic residues. Although VPI-Fla, CPI-

Pep and CPI-Pep/Pan were also generated using proteases that cleaved off hydrophobic amino acids, they showed poor TDC binding. This might have been caused due differences on peptide sequences and exposure of residues that would not have allowed chelating activity (Ma & Xiong, 2009). Bile acids chelation prevents their recirculation resulting in hepatic clearance of cholesterol. Furthermore, the capacity of oat bran protein hydrolysates to bind secondary bile acids results interesting since they have also been implicated in intestinal inflammation (Yoshie-Stark & Wäsche, 2004).

Although oat bran protein hydrolysates demonstrated bile acid binding capacity *in vitro*, further studies will be important to validate the bile acid chelating properties *in vivo*. This would allow to assess their bioavailability for their use in the formulation of functional foods. The gastrointestinal (GI) tract is known as a major barrier for oral administration of bioactive peptides, since the digestive enzymes and extreme pH conditions might impact the structure and bioactivity of the peptides (Ao & Li, 2013) . In addition, during GI digestion, the peptides are constantly exposed to various food-derived oxidants and toxics, resulting in changes to their functionalities (You, Zhao, Regenstein, & Ren, 2010). Hence, understanding the absorbance efficiency of bioactive peptides may provide valuable insight into their *in vivo* effects before utilizing them as functional ingredients suited for the prevention of certain human illnesses (Zhang et al., 2018).

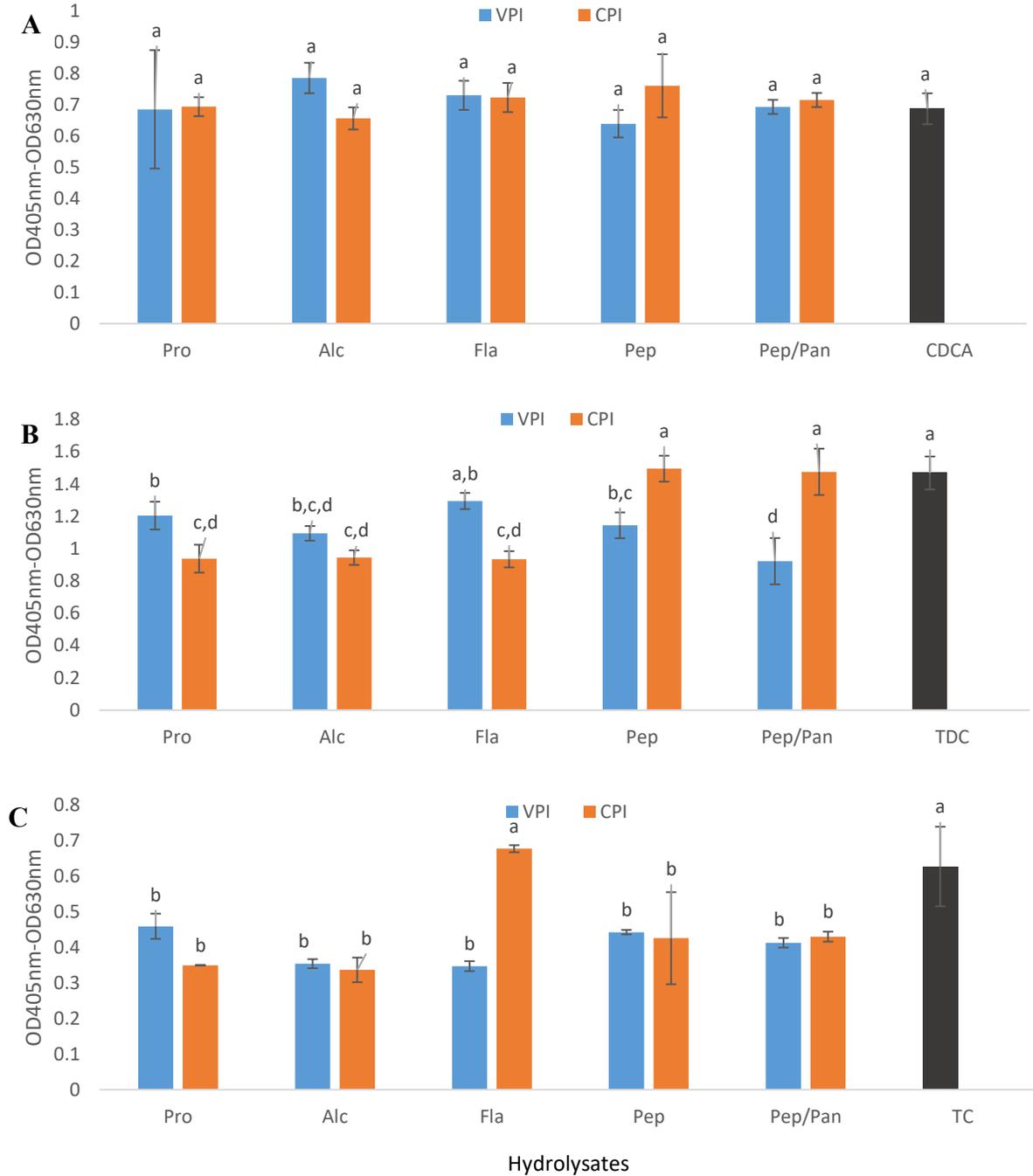


Figure 2. 6. - Bile acid binding capacity of oat bran protein hydrolysates for A) Chenodeoxycholic acid (CDCA), B) Taurodeoxycholate (TDC), and C) Taurocholate (TC). Proteases used for protein hydrolysis include protamex (Pro), alcalase (Alc), flavourzyme (Fla), pepsin (Pep) and pancreatin (Pan). Different letters indicate significant differences between means ($p < 0.05$).

2.5. Conclusion

Results from this study showed that extracted oat bran proteins with viscozyme resulted in greater protein yields compared to the use of cellulase. Meanwhile, this did not necessarily translate into greater activities of hydrolysates from viscozyme extracted proteins. Hydrolysates scavenged peroxy and superoxide anion radicals better than hydroxyl radicals. Hydrolysates prevented the oxidation of low-density lipoprotein partly because of the ability to chelation copper ions. They also bind two bile acids (Taurodeoxycholate and Taurocholate), and this makes suitable for further investigation of cholesterol lowered functional ingredients.

Chapter 3.0- Chromatographic separation of alcalase digested oat bran proteins fractions: Antioxidant, inhibition of human low-density lipoprotein, and bile acid binding capacity.

3.1 Abstract

The aim of this research was to study the influence of RP-HPLC separation of oat bran protein isolates digested with alcalase on their antioxidant and bile acid binding ability. Hydrolysates produced with alcalase showed the highest peroxy radical scavenging activity ($495.49 \pm 13.40 \mu\text{MTE/g}$) and were able to bind Taurodeoxycholate (TDC) and Taurocholate (TC). Therefore, it was subsequently fractionated into eleven fractions (F1–F11) by high-performance liquid chromatography (HPLC). Their abilities to scavenge $\text{ROO}\cdot$ radicals, chelate Cu^{2+} , bind bile acids and prevent LDL oxidation were determined. F7 and F11 showed the highest oxygen radical absorbance capacity values (1218.32 ± 25.3 and $1144.12 \pm 1.95 \mu\text{M Trolox Equivalents (TE)/g}$) compared to alcalase hydrolysate sample and control. Antioxidant capacity was also assessed through copper binding and lipid hydroperoxides assay, F6 and F10 demonstrated the best Cu^{2+} chelating activity by ($78.64 \pm 0.73\%$ and $74.54 \pm 6.51\%$), while F1 generated the lowest lipid hydroperoxides (LHP) amount by $51.00 \pm 5.14 \mu\text{M H}_2\text{O}_2/\text{g}$. In the bile acid chelating assay, peptide fractions showed the best binding capacity than alcalase hydrolysate towards Taurodeoxycholate and Taurocholate.

3.2 Introduction

Free radicals are unavoidable metabolic byproducts that are the result of oxidative metabolism, which may attack macromolecules such as proteins and DNA, leading to serious diseases including hypertension, coronary heart disease, atherosclerosis and cancer (Ji et al., 2014)(Moskovitz et al., 2002). Oxidation of low-density lipoprotein (LDL) cholesterol by free radicals or metal ions is a critical proatherogenic mechanism (Kittiphattanabawon et al, 2013b). The use of various kind of antioxidant compounds may inhibit LDL modification (Wang et al, 2011). Recently, hydrolysates and peptides derived from plant proteins have exhibited diverse activities including antioxidant actions (Hartmann & Meisel, 2007). These have gained increased recognition for their potential benefits in treating and/or preventing the onset of diseases (Udenigwe & Aluko, 2012). Antioxidant peptides in foods are generally released only after enzymatic hydrolysis (Li et al., 2008) or fermentation in the presence of microorganisms (García et al, 2013). Fractionation of oat

bran protein hydrolysates have found antioxidant peptides (Vanvi & Tsopmo, 2016); nonetheless no work has been done to determine their capacity to inhibit LDL oxidation.

Prevention of LDL oxidation might also be accomplished through chelation of bile acids. Food compounds capable of binding bile acids promote their depletion and stimulates liver cholesterol conversion to produce additional bile acids, thus decreasing liver and serum LDL cholesterol on bloodstream (Ma & Xiong, 2009). Protein hydrolysates have been found capable of chelating bile acids. Antioxidant activities such as peroxy, superoxide anion, hydroxyl radical scavenging activities (Vanvi & Tsopmo, 2016), and metal chelating capacity (Baakdah & Tsopmo, 2016) have been reported on peptides resulting from the hydrophobic separation of oat hydrolysates; however, the ability to bind bile acids has not been investigated.

The objectives of this study were: a) to fractionate oat bran protein isolate digested with alcalase by RP-HPLC and b) to evaluate antioxidant, bile acid chelating ability and inhibition of LDL oxidation activities of the fractions.

3.3 Materials and methods

Sodium hydroxide (NaOH), copper sulfate pentahydrate $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), L-Glutathione reduced (GSH), 1,10-phenanthroline, Ammonium iron(II) sulfate hexahydrate $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, Hydrogen peroxide H_2O_2 , Trizma hydrochloride, Pyrogallol, Iron (II) chloride, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt (Ferrozine), CH_3COONa Sodium acetate, Pyrocatechol violet, Xylenol orange, Butylated hydroxytoluene, and hexane were purchased from Sigma Aldrich Ltd (Oakville, ON, Canada). AAPH (2,2'-Azobis(2-amidinopropane) was supplied by Wako Chemicals (Richmond, VA, USA). Fluorescein, potassium phosphate mono- and di-basic, and Tris base were obtained from Fischer Scientific Co. (Nepean, ON, Canada). Sulfuric acid was supplied by VWR International (Mississauga, Ontario). EDTA free Low-Density Lipoprotein (LDL) from human plasma was supplied from Lee Biosolutions, Inc (MO, USA). Chenodeoxycholic acid (CDCA), taurodeoxycholate (TDC) and taurocholate (TC) were supplied from Cayman Chemical (MI, USA). Total bile assay kit was supplied from CellBio Labs Inc (San Diego, USA). High-purity water was produced in the laboratory by an Alpha-Q system (Millipore, Marlborough, MA). Spectrophotometric and fluorometric measurements were performed on the BioTek® Epoch™ UVVis and Biotek FLx 800 microplate reader, respectively, both controlled by

Gen5™ data analysis software. Incubations were done on a MaxQ™ 5000 shaker model (Fisher Scientific, Nepean, ON).

3.3.1. Oat bran alcalase hydrolysates: Separation by RP-HPLC

Alcalase digested bran proteins with the most peroxy radical scavenging activity were selected for HPLC separation. The system included a 1525 binary pump, 2998 photodiode array detector (set at 280 nm), 2707 autosampler maintained at 8°C, and fraction collector III from Waters (Montreal, QC, Canada). The freeze-dried oat bran protein hydrolysate was dissolved in 0.1% acetic acid in water (100mg/mL) and filtered through 0.45 µm nylon membrane. The fractionation was carried out on a semi-preparative column Waters Prep XBridge BEH C18, 130 ° A, 10 µm, 19 × 150 mm, and the injection volume was 2 mL. A linear gradient (5 to 90% B) of 0.1% acetic acid in methanol (B) over 45min was used for separation at a flow rate of 4mL/min. Eluates were collected and pooled into eleven fractions according to chromatogram: : 0–6 min (F1), 6–7 min (F2), 7–8min (F3), 8–11 min (F4), 11–13min (F5), 13–18 min (F6), 18– 20 min (F7), 20–28 min (F8), 28-30min (F9), 30-37min (F10),and 37-42min (F11). The solvent in each fraction was removed under vacuum at 45°C using a Büchi Rotavapor ®R-215, reconstituted in water, freeze-dried, and stored at –20°C.

3. 3.2. Oxygen Radical Absorbance capacity of HPLC fractions

ORAC ability was determined for HPLC fractions resulted from VPI-Alc separation. Briefly a potassium phosphate buffer (0.075 M at pH 7.4) was used to prepare all reagents, standards, samples (0.1 & 0.2 mg/mL) and control (GSH 0.1mg/mL and 0.2mg/mL). Five concentrations of Trolox were used (6.25µM -100µM) to construct a calibration curve. The analysis was carried out by adding 120µL fluorescein (8.21×10^{-2} µM) to all wells, followed by 20µL of buffer solution (blank), or standard, or control, or hydrolysate. The microplate was incubated for 20 min at 37°C in a fluorescence reader model FLx800 (Bio-Tek Instruments, Inc., Winooski, VT). 60µL of AAPH (140 mM) was added to all wells. Kinetic data were obtained every minute for 1 h. ORAC values were calculated using the area under the curve and expressed Trolox equivalents.

3.3.3 Copper chelating assay of HPLC fractions

Briefly 30µl of peptides fraction (0.5 and 1.0 mg/mL) in Sodium acetate buffer (50mM pH 6.0) or EDTA-Na₂ in water (0.25 and 0.5 mg/mL) (standard) were added followed by 170 µl (sample and standard) or 200 µl (sample blank and control) of buffer. Then 60µl of aqueous CuSO₄·5H₂O (100 mg/L) (sample, standard and control) or water (blank) were added. The reagent was let to react for two minutes, followed by the addition of PV (8.5µl, 4mM) in acetate buffer. The microplate was

incubated for 10 minutes at 25 °C/ 120 RPM. Absorbance was read at 632 nm using Epoch Biotech microplate reader. The percentage of formation of the Cu²⁺-PV complex was calculated as:

$$(\%) = [(Abs\ sample) - Abs\ control / Abs\ Control] * 100$$

3.3.4 Bile Acid Binding Capacity Assay for HPLC fractions

The method was carried out according to the total bile assay kit supplied by CellBio Labs Inc. 300µL Chenodeoxycholic acid (CDCA) (12.7 mM), taurodeoxycholate (sodium salt) (TDC) (9.58 mM) or taurocholate (sodium salt) (TC) (9.3 mM) in (Sodium phosphate buffer 50 mM pH 7.0) were added to 300µL of each sample fraction in buffer (2mg/mL). The buffer was used in controls. Tubes were mixed, incubated (37 °C, 2 h, 120 RPM) and centrifuged at 16,000g for 5 min. The analysis was then performed using the total bile assay kit according to the instructions provided. Briefly, 20µL of sample or standard were transferred into a 96 well microplate followed by 75µL of assay reagent A, incubated (37°C, 5 min), then 25 µL of assay reagent B and further incubation for 30 min. Bile acid chelating ability was expressed as optical density

3.3.5 Lipid hydroperoxides quantification (FOX assay) for HPLC fractions

Lipid hydroperoxides determination was based on their reaction with xylenol orange in the presence of ferrous ions (Kin et al. 2014). 100µL of peptide fraction (0.5 and 1.0 mg/mL) in PBS (10mM, pH 7.4, 0.15 M NaCl), or buffer (positive and negative control) were added to each tube followed by 800µL of LDL in buffer (0.05 mg/mL). Sample blanks (100µL hydrolysate + 800µL PBS) were also included. Tubes incubated at 37 °C for 15 min, then 100 µL of aqueous CuSO₄·5H₂O (100 µM) were added to samples, sample blanks, and positive control while 100 µL of water was added to the negative control. An aliquot of 150µL from the tube was removed to measure initial hydroperoxide concentrations. The remaining quantities were incubated at 37 °C for 24 h after which hydroperoxides were quantified again. The measurement was performed by first mixing 150 µL from reaction (0 and 24 h) with 850 µL of FOX-2 reagent followed by 30 min incubated (37 °C) to form a ferrous oxide-xylenol orange complex. The quantification was then performed at 560 nm based on a standard curve constructed using hydrogen peroxide (5, 10, 15, 20, 40, 80, 160 and 200µM).

3.3.6 Statistical Analysis

Triplicate determinations were used to obtain mean values and standard deviations. Statistical analysis was performed with SAS (SAS® Software 9.1, SAS OnDemand, 9.4, 2017) using one-way ANOVA. Duncan's multiple-range test was carried out to compare the mean values for samples and significant differences taken at P < 0.05.

3.4 Results and Discussion

3.4.1 Fractionation of hydrolyzed proteins by HPLC

RP-HPLC fractionation of oat bran protein hydrolysate digested with alcalase was performed to enhance their bioactivities, previous investigations have demonstrated that column fractionation improves antioxidant activities on peptide fractions (Girgih, Udenigwe, & Aluko, 2013). RP-HPLC separates peptides based on their differences in hydrophobic properties. As displayed in Figure 3.1, alcalase hydrolysate was pooled into eleven fractions (F1-F11). F1 was eluted first since it was weakly bound to the column; thus, it has the least net hydrophobicity of fractions. On the other hand, F11 eluted last since it was strongly bound to the column; therefore it exhibited strongest net hydrophobic properties in comparison to the earlier eluting fractions (Pownall, Udenigwe, & Aluko, 2010). This elution behavior could be related to the predominant amino acid composition of each fraction (Ajibola et al, 2013). Researchers have shown that on an RP-HPLC separation, fractions that elute first have the least content of hydrophobic amino acids (HAA) such as Leu, Tyr and aromatic amino acids (AAA), but the highest content of positively and negatively charged amino acids.

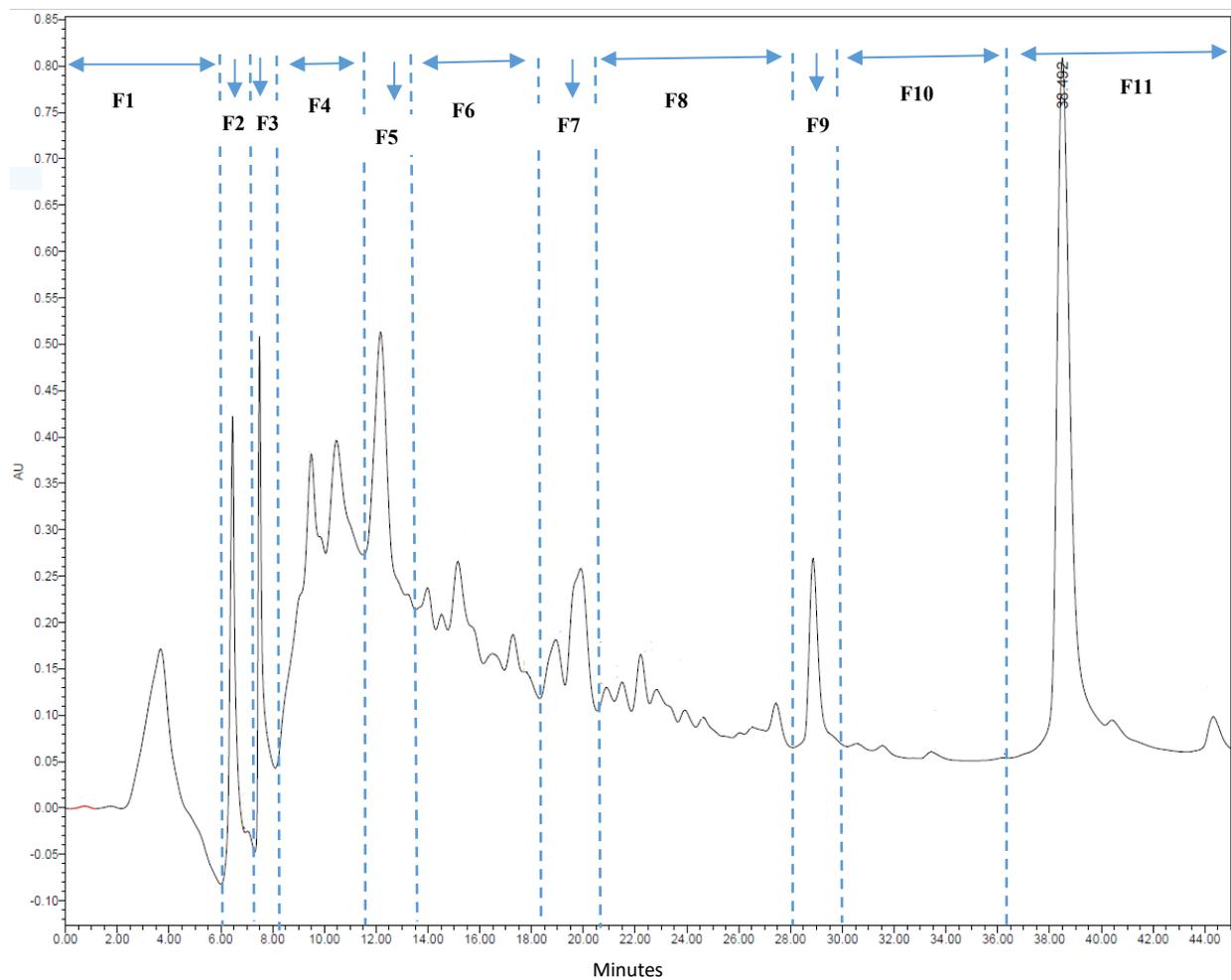


Figure 3. 1. - HPLC chromatogram of hydrolyzed oat bran proteins (Alc-H) on a C18 column. Eleven fractions (F1–F11) were collected as indicated.

3.4.2 Oxygen Radical Absorbance (ORAC) capacity assay

ORAC assay has been widely used to evaluate the antioxidant activity of food protein/peptides (Power et al., 2013). Figure 3.2 displays the results. As shown in Figure 3.2 ORAC values increased with elution from F5 onwards. F7 (1218.32 ± 25.35 μM Trolox Equivalents (TE)/g), followed by F11 (1144.12 ± 1.95 μM (TE)/g) and F5 (1092.74 ± 12.92 μM (TE)/g) exhibited the highest peroxy radical scavenging capacity. In contrast, F1, F2 and F3 presented the lowest ORAC values compared to control and the hydrolysate. This might be related to their hydrophobicity, and since from F5 to F11 eluted with a higher proportion on the non-polar solvent, it is likely to have an increase of hydrophobic amino acids which might be responsible for their antioxidant activity (Vanvi & Tsopmo, 2016). Eight of the fractions presented significantly higher quenching ability than oat bran protein hydrolysate, agreeing with observations of other studies that reported an improved ROO • radicals scavenging capacity by hydrophobic separation (Silva et al, 2017) (Rajapakse et al, 2005) (Girgih et al., 2013)(Vanvi & Tsopmo, 2016). Peptides attributes such as molecular weight have demonstrated to influence the antioxidant ability of fractionated hydrolysates, although this was not determined for this study (Guo et al, 2009). Smaller peptides possess stronger radical quenching properties compared to larger peptides. (Samaranayaka & Li-Chan, 2011) (Silva et al., 2017). This may be due to increased ability to interact and donate electrons to the free radical when compared to bigger peptides that may have reduced interaction capacity (Onuh, et al, 2014). The amino acid composition has also been associated with peroxy radical quenching activity on peptides fractions (Silva et al., 2017). The presence of hydrophobic (Ala, Val, Leu, or Ile) (Guo et al., 2009) (Sarmadi & Ismail, 2010), acidic (Asp and Glu) (Saiga, Tanabe, & Nishimura, 2003), aromatic (Tyr, Trp, Phe, His)(Huang et al, 2005) sulphur containing (Met and Cys) and other amino acids (Gly, Lys, Ala, and Pro) has been recognized as responsible of this property (Rajapakse et al, 2005)(Li et al., 2008)(Chen et al, 1996). In this work, ORAC values are higher than the most active fraction reported for pepsin oat bran (Vanvi & Tsopmo, 2016), but lower than hempseed (Girgih et al, 2013b), and flaxseed hydrolysates (Silva et al., 2017).

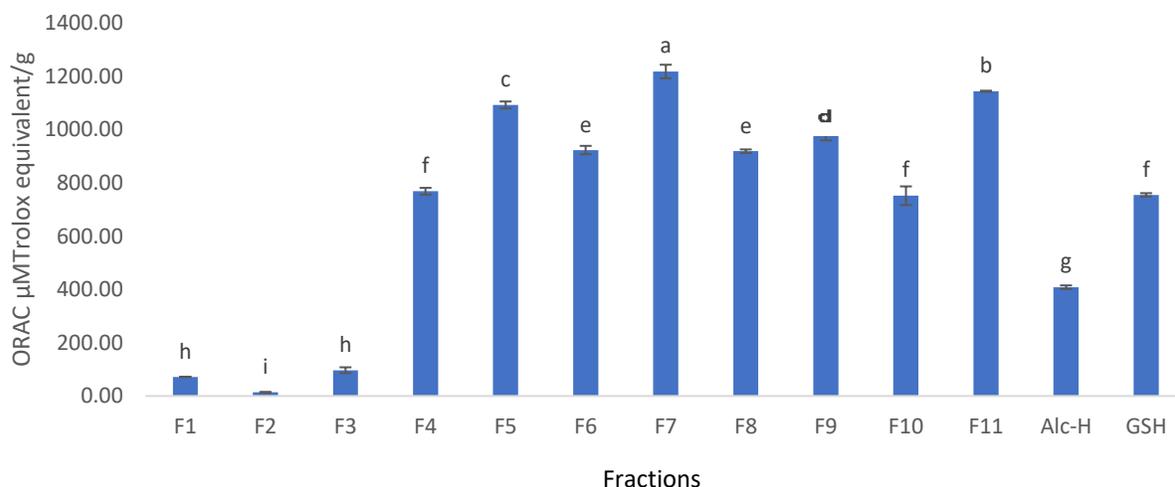


Figure 3. 2. - Oxygen radical absorbance capacity (ORAC) values of oat bran hydrolysate digested with alcalase (Alc-H) and RP-HPLC separated fractions compared to reduced glutathione (GSH). Letters indicate significant differences between means ($p < 0.05$).

3.4.3 Copper chelating assay for peptides fractions

Copper is an essential trace element (Gaetke & Chow, 2003), it can also have a pro-oxidant activity similar to that of iron. Thus, Cu^{2+} can induce oxidative damage to DNA and low-density lipoprotein (LDL) (Burkitt, 2001). Copper-chelating peptides in the diet might prevent LDL oxidation which has been involved in atherogenesis (Megías et al., 2007). Figure 3.3 shows the ability of the peptide fractions and the alcalase hydrolysate to bind this metal. F6 ($78.64 \pm 0.73\%$) along with F10 ($74.54 \pm 6.51\%$) exhibited highest copper chelating capacity when compared with alcalase hydrolysate. On the other hand, F9 ($44.56 \pm 1.36\%$) and F3 ($47.47 \pm 0.59\%$) showed significantly lower activity when compared to alcalase digested sample and the rest of the fractions. F4-F6 and F10-F11 enhanced their antioxidant activity through fractionation by demonstrating a better Cu^{2+} binding ability. There is no direct association between their metal binding activity and hydrophobicity of the fractions. For some of them such as F5 and F11, these values correlated with high $\text{ROO}\cdot$ quenching capacities, although it does not seem to be a determinant cause to bind this Cu^{2+} . The antioxidant effect of the copper-chelating peptides has been shown to be affected by the presence of amino acid residues and short peptide size; however, none of these attributes were evaluated in the present work (Torres-Fuentes et al, 2011b) (Megías et al., 2007). His activity has been largely related to its imidazole ring while (Selamassakul et al, 2018) (Zhang et al, 2011) aromatic amino acids can easily donate protons to electron- deficient

radicals maintaining their stability via resonance structures(Sarmadi & Ismail, 2010) (Carrasco-Castilla et al., 2012). Moreover, it is also believed that acidic and/or basic amino acids play important roles in the chelation of metal ions by their side chains (Suetsuna et al, 2004). Peptide fractions obtained in this study displayed a higher value than chickpea (Torres-Fuentes et al., 2011), and brown rice (Selamassakul et al., 2018).

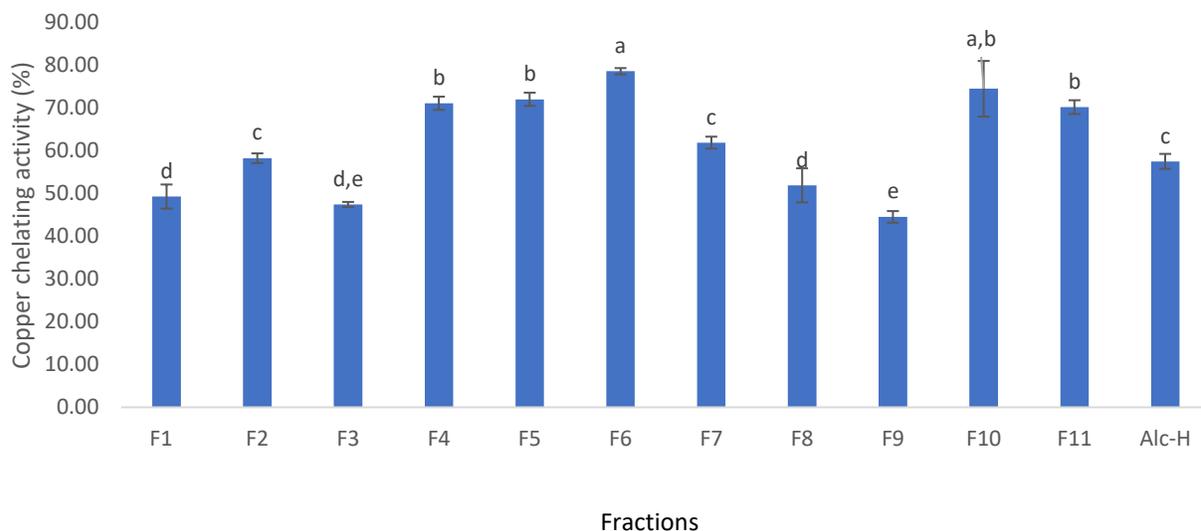


Figure 3. 3. - Copper chelating activity (%) of Alcalase hydrolysate (Alc-H) and its HPLC fractions (F1-F11). Samples concentration was 1mg/mL. Letters indicate significant differences between means ($p < 0.05$).

3.4.4 Lipid hydroperoxides determination for peptides fractions

Antioxidant activity of the unfractionated alcalase hydrolysate and their peptide fractions were also assessed through lipid hydroperoxides (LHP) quantification as presented in Figure 3.4. F1 generated the lowest amount of LHP, followed by F4 and F9 (51.00 ± 5.14 , 77.06 ± 1.29 , and 80.85 ± 4.50 $\mu\text{M H}_2\text{O}_2/\text{g}$ respectively) when compared to alcalase hydrolysate (110.55 ± 1.93 $\mu\text{M H}_2\text{O}_2/\text{g}$). F6, F10 and F11 exhibited highest LHP generation (122.82 ± 5.33 , 118.73 ± 1.93 and 124.03 ± 0.00 $\mu\text{M H}_2\text{O}_2/\text{g}$ respectively). Overall, peptide fractions and alcalase digested sample generated LHP to a lower extent than positive control (185.55 ± 5.14 $\mu\text{M H}_2\text{O}_2/\text{g}$). No correlation is observed with peptide fractions capacity to quench peroxy radicals nor net hydrophobic character. On the other hand, among all the fractions only F4 displayed high copper chelating ability and decreased LHP quantity. Metal binding has been considered as a mechanism to delay lipid peroxidation (Torres-Fuentes et al., 2014). Literature has shown that several factors such as exposition of antioxidant amino acids due to specific cleavage of enzymes (Tamm et al., 2015), peptide composition and degree of hydrolysis have been associated to their antioxidant activity. Furthermore, peptides rich in hydrophobic amino acids have also been related to LHP quenching capacity (Jamdar et al., 2010). For instance (Yahia et al., 2017) associated the high degree of hydrolysis to the generation of more hydrophobic amino acids enhancing hydrolysates free radical scavenging capacity. Among the possible mechanisms of action, amino acids could act as free radical scavengers by donating a hydrogen atom, to inhibit LDL peroxidation (Zhao, et al., 2012). In this study fractionation of alcalase hydrolysate improved antioxidant capacity just for some of the fractions (F1, F3-F4, F7 and F9). Further tests such as hydrophobicity or amino acid composition would be useful for their better description. This determination has not been reported for peptide fractions; however, research on protein hydrolysates such as chickpea (Yahia et al., 2017) amaranth (García Fillería & Tironi, 2015), and rice dreg (Zhao et al., 2012a) has been done.

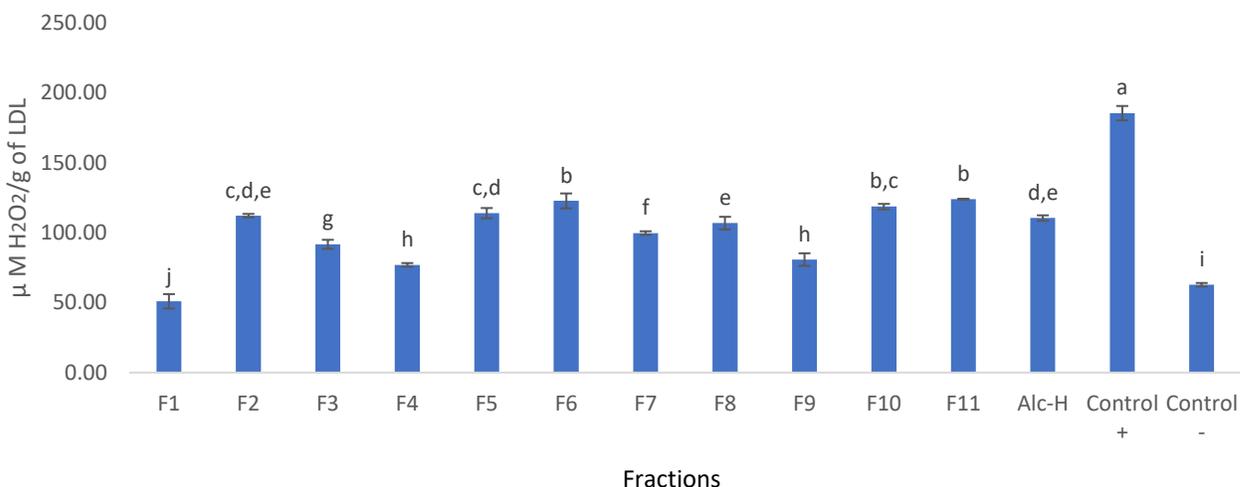


Figure 3. 4. - The effects of Alcalase hydrolysate (Alc-H) and its HPLC fractions (F1-F11) on lipid hydroperoxides produced from LDL oxidation. Letters indicate significant differences between means ($p < 0.05$).

3.4.5 Bile Acid Binding Capacity for peptides fractions

Three bile acids were assayed to test the hypocholesterolemic activity of alcalase hydrolysate and their RP-HPLC fractions. As displayed in figure 3.5A all of the fractions including Alc-H were able to bind CDCA to the same extent compared to the control, which might imply that for this specific bile acid, the activity was not improved through fractionation. For Taurodeoxycholate (Figure 3.5 B) and Taurocholate (Figure 3.5 C) peptide fractions chelating capacity was better as compared to the alcalase digested sample, suggesting that unlike CDCA, hydrophobic separation (RP-HPLC) enhanced their chelating ability. Primary bile acids chelation such as CDCA is relevant since it leads to BAs decrease in enterohepatic circulation deriving in a hypocholesterolemic effect (Howard & Udenigwe, 2013b). On the other hand, the capacity of peptides fractions to bind sodium TDC observed in this study is especially interesting. TDC is a secondary bile acid produced by bacteria in the intestine which remotion results advantageous given the fact that secondary BAs are considered intrinsic promoters of colon carcinogenesis and intestinal inflammation (Yoshie-Stark & Wäsche, 2004)(Kahlon, Chapman, & Smith, 2007).

No direct correlation is observed between bile acid binding activity and peroxy radical scavenging capacity nor copper chelating ability on fractions. Capacity to chelate bile acids has not been evaluated on peptide fractions, nonetheless, it has been quantified on protein hydrolysates. The different affinity to bile acids has been related to different peptides properties such as small size,

structure and hydrophobicity, even though none of these were assessed in the present work, they might still help to explain peptide fractions behaviour (Pérez-Gálvez et al, 2015). Several authors have associated the hydrophobic nature of the peptides residue to the specificity of the protease, in this specific case, hydrolysates were digested with alcalase, which selectively cleaves off hydrophobic amino acids, thus increasing their content (Espejo-Carpio et al., 2016) (Higaki et al., 2006). These kinds of residues strongly bind bile acids due to hydrophobic reactions, since hydrophobic amino acids exhibit a strong interaction with lipids (cholesterol, bile acids, others sterols and others lipids) (Ma & Xiong, 2009). Furthermore molecular weight (Espejo-Carpio et al., 2016) (Pérez-Gálvez et al., 2015), release of specific active peptide sequences as well as the exposure of certain amino acid side chain groups have been implicated in bile acid binding activities (Ma & Xiong, 2009). The degree of hydrolysis is another factor that has been discussed. Some reports agree there is a positive influence, whereas some others state that it is not related (Yoshie-Stark et al., 2008) (Kongo-Dia-Moukala et al., 2011). In this regard, future studies are needed to determine attributes responsible for peptide fractions activity. According to literature, lentil (Barbana et al., 2011) and soybean hydrolysates have reported a higher binding to TC than peptide fractions in this work but lower than lupin (Yoshie-Stark & Wäsche, 2004), and flaxseed digested samples (Marambe et al., 2008). The excretion of bile acids after their binding to sequestrants could play a key role in protection against both coronary heart disease and colon cancer (Barbana et al., 2011). Peptide fractions showed *in vitro* bile acid binding activity; nonetheless, future research is needed to characterize and validate their properties as possible functional ingredients. One of the biggest challenges when developing nutraceuticals and functional foods is their bioaccessibility (Yuwen, Qin, Chunxin, & Qingrong, 2015). Peptides may also face drawbacks such as their susceptibility to enzymatic degradation, rapid kidney clearance and in some extreme cases, peptides can cause an immunogenic response. Therefore these aspects need to be addressed to ensure they are stable and efficient *in vivo* (Erak, Bellmann-Sickert, Els-Heindl, & Beck-Sickinger, 2018).

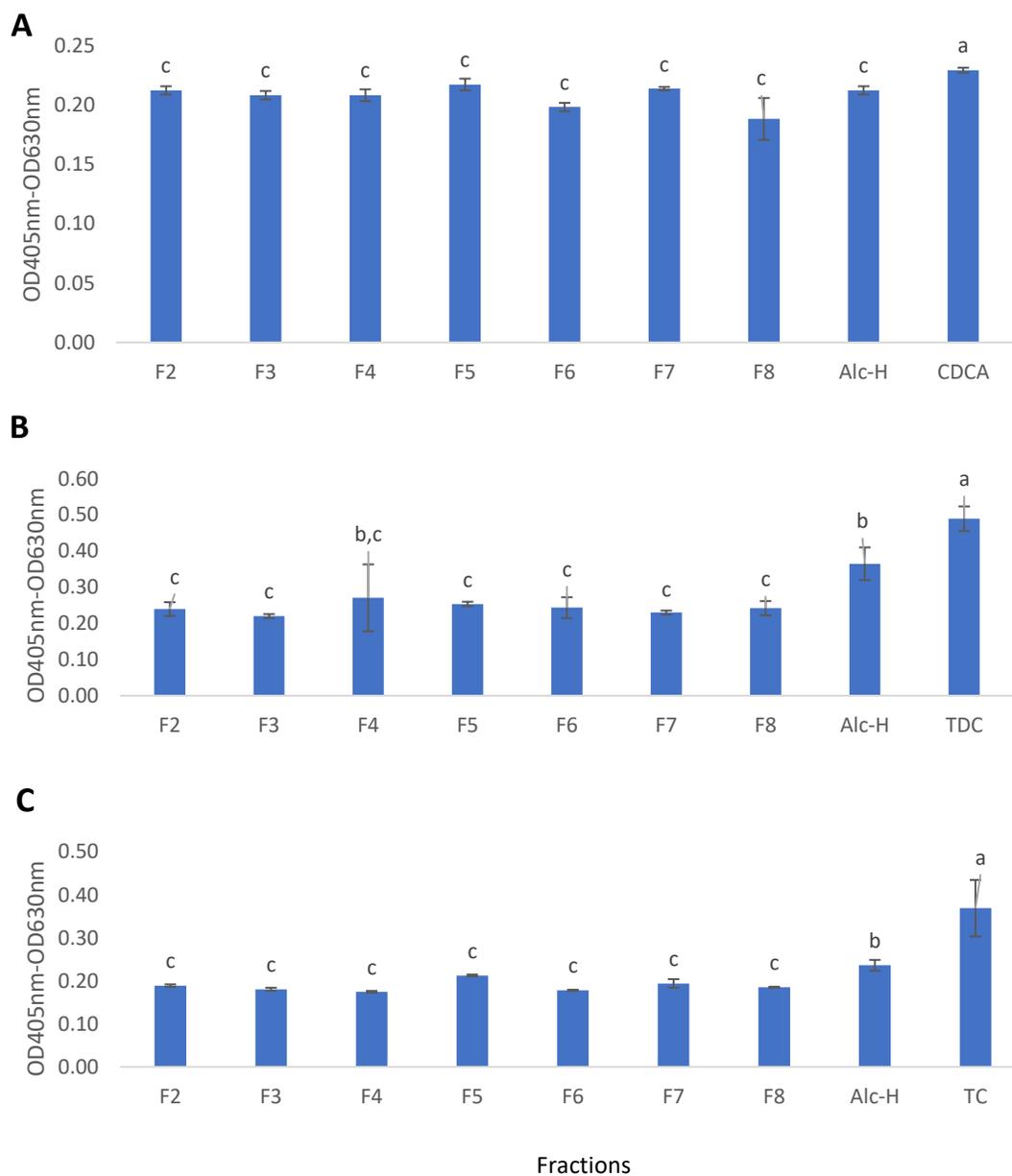


Figure 3.5. - The bile acid binding capacity of Alcalase hydrolysate (Alc-H) and its HPLC fractions (F1-F11) for A) Chenodeoxycholic acid (CDCA), B) Taurodeoxycholate (TDCA), and C) Taurocholate (TC). Letters indicate significant differences between means ($p < 0.05$).

3.5 Conclusion

This study was the first to determine and evaluate the inhibition of LDL oxidation and bile acid binding capacity on HPLC-fractions of oat bran protein hydrolysates digested with alcalase. The present work found that at least one fraction was able to decrease lipid hydroperoxides formation and chelate two different bile acids more effectively than unfractionated hydrolysate. In addition, results also indicates that RP-HPLC separation enhanced peroxy radical scavenging, copper chelating and bile acid binding capacity of the peptide fractions when compared to alcalase digested sample. This is interesting because the decrease of lipid hydroperoxides along with Cu^{2+} binding and bile acids are factors that play an important role in the prevention of LDL oxidation, which may trigger conditions such as atherosclerosis. Future studies are needed to identify novel peptides by tandem mass spectrometry.

Chapter 4.0-General conclusion and future work

4.1 Conclusion

The present study showed that oat bran proteins hydrolysates possessed the capacity to scavenge peroxy radical to a better extent than superoxide or hydroxyl radical. It was also found that hydrolysates were able to inhibit conjugated dienes and lower the amount of lipid hydroperoxides, hence exerting a protective effect against LDL oxidation. Furthermore, they also presented significant binding towards Taurodeoxycholate and Taurocholate. Therefore they may be applied as potential ingredients for functional foods to prevent the development of conditions related to LDL oxidation or hypercholesterolemia such as cardiovascular diseases.

Chromatographic fractionation of oat bran protein hydrolysates digested with alcalase improved their capacity not only to scavenge peroxy radicals but also to chelate copper and bile acids, which may again indicate their use in the food industry as nutraceuticals to delay oxidation or reduce cholesterol.

4.2 Future work

In further studies, chemical assays to determine molecular weight or hydrophobicity will be used to clarify hydrolysates behaviour related to their antioxidant bioactivities. In addition, for a better understanding of the mechanisms involving the prevention of LDL oxidation and bile acid binding capacity, it would be interesting to test *in vivo* models.

On the other hand, sequencing the most active fractions obtained through RP-HPLC separation of alcalase hydrolysate will be helpful. Peptides characterization through mass spectrometry will allow defining responsible amino acids of enhanced activities.

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