Characterization of Cereal Arabinoxylans and their Health Benefits

By

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Dedication

To my parents (Esau and Margret) and my family (Alexandra and Chikondi)

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Abstract

Consumption of whole grain cereals is associated with decreased risk of colorectal cancers and diabetes, but the underlying mechanism still remains unclear. Oxidative damage is involved during initial or developmental stages of colorectal cancers, whereas diabetes is typified by high concentration of plasma glucose. Presence of arabinoxylan (AX) in cereal grain is thought to result in viscous digestion, thereby trapping of potential carcinogens and delaying carbohydrate digestion. The limitation to viscosity hypothesis is that AX may exhibit shear thinning during intestinal peristaltic force. Thus the main objective of this research was to understand the role feruloyl polysaccharides and oligosaccharides play in the maintenance of gut health and digestion and absorption of dietary carbohydrate in order to propose an alternative mechanism through which cereals exerts their perceived health benefits. Investigation into the antioxidant potential of water extractable arabinoxylans from wheat, barley, and corn revealed that AXs are capable of donating both electron and hydrogen atom during redox reactions. Their antioxidant potential against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$) and peroxyl radicals was highly influenced by degree of substitution, total phenolic acids, total phenolic content (TPC), and ferulic acid content. Further ammonium sulfate fractionation of AXs obtained from wheat aleurone and wheat bran indicated that ferulic acid content, degree of xylose substitution, and pattern of substitution are the major determinants their antioxidant properties. Moderate degree of substitution was important as unsubstituted xylose appeared to participate in redox reactions. The presence of ferulic acid was essential such that its removal rendered AX impotent against free radicals. The factors influencing antioxidant capacity of arabinoxylan oligosaccharide (AXOS) were also investigated. The antioxidant capacity of AXOS was highly

influenced by the type of xylanase. The mean antioxidant capacity of *T. viride* treated AXOS was higher than *N. patriciarum* treatment. Xylanases leading to AXOS with higher content of esterified ferulic acid and degree of substitution are desirable for better antioxidant. Investigation into the presence of feruloylated mono/oligosaccharides in maize and wheat showed that only feruloyl arabinose and feruloyl arabinosyl xylose are present. Feruloyl arabinose appeared to be predominant of the two in both maize and wheat. The levels of feruloyl arabinose increase during gastric digestion due to low pH. The amount of resultant mono/oligosaccharide during gastric digestion is dependent on the content of insoluble bound ferulic acid. Feruloylated oligosaccharides were found to inhibit both mammalian intestinal α-glucosidase and glucose transporter 2 (GLUT2) activities. Ferulic acid bound to the oligosaccharides was responsible for their inhibition properties. At equal ferulic acid concentrations the degree of substitution did not affect the inhibition potency of oligosaccharides towards α-glucosidase and glucose transporter 2 (GLUT2). On the other hand, AX polysaccharides did not affect starch hydrolysis to maltose regardless of concentration or type. However, the polysaccharides were capable of significantly inhibiting intestinal α-glucosidase activity (noncompetitively). Evidence from our study suggests that the solubility of AX is important and consequently not all AXs have the same inhibitory effect. AX with higher arabinose to xylose ratio, but less ferulic acid may have high inhibition potential. Thus based on our results, the following mechanism may be involved for AX to exert their perceived health benefits a) water extractable AXs donates electrons or hydrogen atom to neutralize dietary free radicals (implicated in the initiation and/or development of chronic diseases) as they traverse through gastrointestinal tract and b) AXs noncompetitively inhibit intestinal α-glucosidase and/or AX mono-/oligosaccharide inhibit intestinal α-glucosidase and glucose transporter thereby attenuating postprandial blood glucose levels.

CHAPTER 1

General Introduction, Hypothesis and Objectives

1.1 Introduction

Epidemiological studies have shown that consumption of wholegrain cereals protects against chronic diseases (Aune, Chan, Lau, Vieira, Greenwood, Kampman, et al., 2011; Jonnalagadda, Harnack, Liu, McKeown, Seal, Liu, et al., 2011; Slavin, 2004) but the underlying mechanisms remain unclear. Thus governments and health professionals recommend incorporation of wholegrain in the diet (Marquardt, Asp, Richardson, Kamp, Asp, & Miller Jones, 2004; Richardson, 2003). The low intake of wholegrain food products (Devlin, McNulty, Gibney, Thielecke, Smith, & Nugent, 2013; Kyrø, Skeie, Dragsted, Christensen, Overvad, Hallmans, et al., 2012; Mann, Pearce, McKevith, Thielecke, & Seal, 2015; Thane, Jones, Stephen, Seal, & Jebb, 2005) is attributed to its poor sensory scores on taste, appearance and texture when compared to products made with refined flours (Bakke & Vickers, 2007; Kuznesof, Brownlee, Moore, Richardson, Jebb, & Seal, 2012). Thus, there is a need of understanding what factors make wholegrain health with the hope of enriching the refined flours or development of functional foods. The bran and germ fractions lost during milling contain high amounts of dietary fiber and bioactive minerals and compounds such as magnesium, selenium, vitamins, phenolics, alkyresorcinols, and betaine among others (Fardet 2010; Liu 2007).

Research for the past decades has focussed on radical scavenging hypothesis to explain the probable mechanism through which phenolic compounds exert the health benefit (Fardet, 2010; Kanner & Lapidot, 2001; Perron & Brumaghim, 2009). It is reasonable enough as oxidative damage is involved at either initial or developmental stage of most diseases and iron is involved

in the formation of free radicals (Perron & Brumaghim, 2009). However, the reported concentration of dietary phytochemicals circulating in plasma is too small to offer any significant defense against free radicals (Clifford, 2004; Kroon, Clifford, Crozier, Day, Donovan, Manach, et al., 2004). Ferulates are mostly bound to arabinoxylan in cereals (Krygier, Sosulski, & Hogge, 1982). This has led researchers to suggest that the site of action is the gastrointestinal tract where both antioxidants and free radicals are present in considerable high amounts (Clifford, 2004; Holst & Williamson, 2008; Russell & Duthie, 2011). Thus, it is vital to understand whether feruloylated arabinoxylans are capable of exhibiting antioxidant activity.

Dietary arabinoxylans may include both polysaccharides and oligosaccharides. The basic structure of arabinoxylans constitutes a (1-4)-β-D-xylopyranose chain with α-L-arabinofuranose substitutions at O-2 and/or O-3 position. Their structure varies greatly within and among cereal grains or their botanical fractions (Izydorczyk & Biliaderis 1995). Arabinoxylan oligosaccharides (AXOS) are a subject of interest because of their prebiotic and antioxidant properties (Ou & Sun 2014). AXOS reported in literature are a product of enzymatic or mild acid hydrolysis of arabinoxylan polysaccharides. The resultant AXOS are of different degree of polymerization and substitution (Dervilly, Saulnier, Roger, & Thibault, 2000; Swennen, Courtin, Lindemans, & Delcour, 2006). Data on whether these structural differences may affect their antioxidant potential is limited. Thus, part of the work was to isolate and fractionate the AXOS and evaluate their antioxidant potential. Also, limited literature exists on whether AXOS are present naturally in cereals and/or whether AXOS are released during gastric digestion.

Consumption of a diet supplemented with arabinoxylans decreased postprandial glucose levels in both healthy and diabetic subjects with few exceptions (Gemen, de Vries, & Slavin, 2011). The antiglycemic effects of wholegrain have been highly associated with soluble dietary fibers due to

their viscosity enhancing properties. High viscosity is thought to reduce interaction between food and digestive enzymes in the gastrointestinal tract (Dikeman & Fahey Jr 2006). The viscosity effect of arabinoxylans may be offset under intestinal peristaltic force (Dhital, Dolan, Stokes, & Gidley, 2014). Indeed, arabinoxylans have been reported to demonstrate shear thinning properties (Izydorczyk & Biliaderis 1995). Thus the antiglycemic effect of AX may involve a different mechanism beyond viscosity. Therefore, studies to explore inhibition of dietary carbohydrate enzymes by AX are necessary.

Glucose absorption in the intestines involves sodium-dependent glucose transporter 1 (SGLT1) and two passive glucose transporters (GLUT2 and GLUT5) (Burant, Takeda, Brotlaroche, Bell, & Davidson, 1992; Davidson, Hausman, Ifkovits, Buse, Gould, Burant, et al., 1992; Wright, Loo, Panayotovaheiermann, Lostao, Hirayama, Mackenzie, et al., 1994). SGLT1 and GLUT5 are expressed on the apical side for import of glucose and fructose. GLUT2 is expressed on both sides dependent on the concentration of luminal glucose (Kellett & Brot-Laroche 2005). Recently, evidence suggests that flavonoids do inhibit absorption of some nutrient by impairing enterocyte transporters (Kwon, Eck, Chen, Corpe, Lee, Kruhlak, et al., 2007; Ma, Kim, & Han, 2010). Whether phenolic acids, which are abundant in cereals can impair nutrient transporters is not known. At the same time, even if they do, less than 10% of the total phenolic compounds in cereals are free as >90% exists in bound form (Adom & Liu, 2002; Adom, Sorrells, & Liu, 2003; Kim, Tsao, Yang, & Cui, 2006; Liyana-Pathirana & Shahidi, 2006; Ohta, Semboku, Kuchii, Egashira, & Sanada, 1997; Ohta, Yamasaki, Egashira, & Sanada, 1994; Sosulski, Krygier, & Hogge, 1982; Verma, Hucl, & Chibbar, 2008). Thus, it is important to explore the effect of cereal phenolic acids (both free and bound) on intestinal glucose transporters.

Therefore, understanding of the role of arabinoxylans in the maintenance of gut health and assimilation of dietary carbohydrate is important in prevention and management of chronic diseases, especially given that glucose and/or oxidative stress are involved in the onset or development of most chronic disease. The findings of this work provide an extension to the mechanisms through which wholegrain cereals exert their perceived health benefits.

1.2 Research hypothesis

a) Feruloyl oligosaccharides and/or feruloyl arabinoxylan are capable of quenching free radicals, or terminating free radical chain reactions in the gastrointestinal tract by donating electrons/hydrogen atoms.
b) Feruloyl arabinoxylans undergo partial degradation during gastric digestion and release some feruloyl mono/oligosaccharides.
c) Feruloylated arabinoxylans (including feruloyl mono/oligosaccharides) decrease postprandial blood glucose through direct inhibition of carbohydrate digestive enzymes and/or intestinal glucose transporters.

1.3 Research objectives

The main objective is to understand the role feruloyl polysaccharides and oligosaccharides play in cereal health benefits and to propose an alternative mechanism through which cereals exert their perceived health benefits. Specifically, we investigated:

a) The antioxidant capacity of feruloylated arabinoxylans from different cereal grains and factors influencing their antioxidant behavior;
b) The effect of feruloylated arabinoxylans on pancreatic α-amylase and intestinal α-glucosidase;

c) Whether feruloylated arabinoxylan oligosaccharides and available free phenolic compounds inhibit intestinal glucose transporters; and
d) Whether feruloyl arabinoxylans undergo partial degradation in the stomach and release some feruloyl oligosaccharides.

The thesis has been designed following manuscript sandwich format such that chapter 2 gives a literature review. Chapter 3 will provide evidence whether arabinoxylans have antioxidant properties and their contribution toward cereal antioxidant properties. The fourth and fifth chapters look at factors influencing the antioxidant capacity of water soluble arabinoxylans and arabinoxylan oligosaccharides, respectively. The sixth and seventh chapter explores antiglycemic properties of arabinoxylans. The last chapter provides discussion, recommendation and conclusions.

CHAPTER 2

2 Literature Review

Cereal grain arabinoxylans and phenolic compounds are discussed based on the current understanding. Also mechanisms through which cereals exert their perceived health benefits are discussed.

2.1 Feruloylated Arabinoxylans of Cereal Grains

2.1.1 Occurrence and distribution of arabinoxylans in cereals

Arabinoxylans (AXs) are a member of non-starch polysaccharides comprising of pentosans, arabinose and xylose. AXs constitute the second most abundant carbohydrate and were first reported in cereals by Hoffmann and Gortner in 1927 (Izydorczyk & Biliaderis 1995). Arabinoxylan (AX) content varies across and within cereal grains and AX content is also influenced by environmental factors (Rakszegi, Lovegrove, Balla, Láng, Bedő, Veisz, et al., 2014; Shewry, Piironen, Lampi, Edelmann, Kariluoto, Nurmi, Fernandez-Orozco, Andersson, et al., 2010; Shewry, Piironen, Lampi, Edelmann, Kariluoto, Nurmi, Fernandez-Orozco, Ravel, et al., 2010). In general, the amount of arabinoxylans in major cereal grains is as follows in decreasing order - rye (7.6–12%), barley (3.5–6.5%), wheat (5.8%), oats (2.7–3.5%), rice (2.6%) and sorghum (1.8%) (Izydorczyk & Biliaderis, 2007). Data from HEALTHGRAIN project shows that bran contains high amount of AX (13.0, 13.0, 7.3, and 9.6% for wheat, rye, barley and oat respectively) compared to their corresponding flour fractions (1.8, 3.6, 1.9 and 1.8%, respectively) (Table 2.1). Hand dissected wheat (Antoine, Peyron, Mabille, Lapierre, Bouchet, Abecassis, et al., 2003; Barron, Surget, & Rouau, 2007) and barley samples (Izydorczyk, 2010) have shown that the concentration of AX increases when traversing from the endosperm to outer pericarp (Table 2.2).

AXs have significant influence on bread quality despite being a small fraction of cereals. Based on the method of extraction, AX can be classified as water extractable (WEAX) or water un-extractable (WUAX). WEAX make up 0.15 – 1.2% of dry matter content of rice (Rao & Muralikrishna, 2004, 2006); ~1.1 – 1.4% in barley (M. Cyran, Courtin, & Delcour, 2003); 0.5 – 1.5% in wheat and 1.9 – 2.1% in rye (Hartmann, Piber, & Koehler, 2005). WEAX obtained from rye and wheat contains about 600 and 492 µg/kg of ferulic acid, respectively (Hartmann, Piber, & Koehler, 2005). In general, WEAX make up 13 – 33% and 2 – 10% of total AX in whole grain and bran fraction, respectively (Table 2.1).

2.1.2 Chemistry of cereal arabinoxylans

The primary chain of arabinoxylans (AXs) consists of (1-4)-B-D-xylopyranose with α-L-arabinofuranose substitutions at O-2 and/or O-3 position (Lequart, Nuzillard, Kurek, & Debeire, 1999; Perlin, 1951) (Figure 2.1). The monosubstituted xylose at the O-3 position is present in AX of all cereal grains. In contrast, O-2 mXyl substitution is mostly found in barley AX (6 – 16%) (Oscarsson, Andersson, Salomonsson, & Åman, 1996; Storsley, Izydorczyk, You, Biliaderis, & Rossnagel, 2003) but very rare in wheat AX (<1.9 %) (Cleemput, Van Oort, Hessing, Bergmans, Gruppen, Grobe, et al., 1995; Gruppen, Hamer, & Voragen, 1992). Xylose residues may also be substituted with α-(1,2)-glucuronic acid and/or α-(1,2)-4-O-methylglucuronic acid linkages (Ma, Jia, Zhu, Li, Peng, & Sun, 2012). Arabinose residue may be ester linked to ferulic acid at O-5 position (Bunzel, Ralph, & Steinhart, 2005; Smith & Hartley, 1983). Some reports also indicate presence of arabinofuranose residues being substituted with O-2, O-3 and O-5 linkages with other arabinose residue (Izydorczyk & Dexter, 2008). The distribution of arabinose residues along xylan chain is conserved (Izydorczyk & Biliaderis, 1995) but it is not clear whether the positioning of mono or disubstituted residues is random or non-random (Dervilly-Pinel, Tran, & Saulnier, 2004). The biosynthesis mechanism appears to favor disubstitution over monosubstitution.

Different models for arabinoxylan structure have been proposed based on fragments obtained following endoxylanase degradation of the AX polymers and data on prevalence of un-, mono-

and disubstituted xylose residues. The proposed structures of wheat endosperm AX (Figure 2.2), suggest three regions varying in the density of un-, mono- or disubstituted xylose residues (Izydorczyk & Biliaderis, 1994). Region I constituting of high proportions of disubstituted xylose residues may be found "isolated, in pairs or even as three contiguously substituted residues". The second region is characterized by the presence of arabinose linked at O-3 monosubstituted xylose residues, whereas the last region is mostly built from unsubstituted xylose residues. However, another WEAX isolated at 100% ammonium sulfate saturation only exhibited patterns of region I and III. In rye, WEAX were categorized into four groups based on the prevalence of un, mono and disubstituted xylose residues along the xylan backbone (Cyran & Saulnier, 2005). AX I comprised mostly of unsubstituted (58%) and O-3 monosubstituted (38%) xylose with trace amounts of disubstituted xylose (3%). The second group (AX II) had significant amounts of unsubstituted (53%), monosubstituted (14%) and disubstituted (32%) xylose residues, whereas AX III comprised of 34, 9, and 57 % respectively. The last group (AX IV) had 77% of the xylose residues unsubstituted and 14% monosubstitution. The AX I was predominant (57 – 70%) in the isolated WEAX from rye flour or bran. These differences in structure affect the AX resistance to endoxylanase activity as well as their physiochemical properties (Cyran & Saulnier, 2012).

The ratio of arabinose and xylose (A/X) is used to define the degree of substitution of AX. Data from the HEALTHGRAIN project (Table 2.1) indicated that A/X varies greatly within and among cereal grains (0.2 – 1.7) (Andersson, Lampi, Nyström, Piironen, Li, Ward, et al., 2008; Gebruers, Dornez, Boros, Dynkowska, Bedő, Rakszegi, et al., 2008; Nyström, Lampi, Andersson, Kamal-Eldin, Gebruers, Courtin, et al., 2008; Shewry, Piironen, Lampi, Nyström, Li, Rakszegi, et al., 2008). For arabinoxylans extracted from flours, the order of branching appears to be rye>oat>barley>wheat (Ward, Poutanen, Gebruers, Piironen, Lampi, Nyström, et al., 2008). Compared to bran arabinoxylans, wheat flour AXs are less branched, rye flour AXs are more branched and barley flour AXs are equally branched (Table 2.1). However, these mechanically prepared fractions (flour or bran) are usually a blend of different grain fractions. Hand dissected wheat (Antoine, et al., 2003; Barron, Surget, & Rouau, 2007) and barley (Izydorczyk, 2010) fractions suggest that the A/X increases as we transverse from inner to the outermost layers of the grain outer layers (Table 2.2). Pericarp AXs exhibit high substitution and levels of di-ferulic acid, but less ferulic acid. Thus AX from bran is mostly insoluble and poorly accessed by endoxylanase due to ferulic acid bridges between neighboring AX polymers or with other adjacent cell wall

polymers (Izydorczyk, 2010). On the other hand, AX from aleurone is fairly branched with high levels of ferulic acid, but insignificant amounts di-ferulic acid. Consequently, aleurone arabinoxylans are susceptible to endoxylanase degradation. A/X is thought to govern the physiochemical properties of AX. Unsubstituted xylan chains tend to form aggregates via hydrogen bonds, making it less soluble. Thus, it is plausible that highly branched AX tends to be more soluble in water. Indeed, removal of some arabinose residues from AX through arabinofuranosidase decreased the solubility of AX. However, reports of WUAX having a higher A/X ratio than WEAX are common, suggesting that more factors are involved. Presence of ferulic acid crosslink bridges makes extraction of WUAX difficult. Hence saponification with sodium hydroxide solubilizes AX through disruption of ferulic acid crosslink bridges.

2.1.3 Method of extraction of arabinoxylans

Different methods have been described in literature for extraction, purification and quantification of AXs (Cyran, Courtin, & Delcour, 2003; Rao & Muralikrishna, 2004, 2006; Hartmann et al., 2005). In general, flour is deactivated by heat (95-130 °C in 80-90% ethanol under reflux for 60 – 90 minutes). The deactivated flour is mixed with water (1:4 w/v) for 90 minutes at room temperature followed by centrifugation. The supernatant is treated with enzymes (amylase, proteinase and amyloglucosidase) to digest starch and protein. It is eventually followed by centrifugation and dialysis (48 hrs) to eliminate cleaved amino acids and glucose. The supernatant is freeze dried. Alternatively, after enzyme treatment the supernatants are precipitated using ethanol (1:4 v/v) followed by freeze drying. Extraction with ethanol precipitation tends to have high yield, but less AX purity compared to the dialysis method of extraction (Hartmann et al., 2005).

The resultant WEAX can be fractionated by using ion exchange chromatography (Rao & Muralikrishna, 2004; 2006), size exclusion chromatography (Hartmann et al., 2005), gel filtration

or ammonium sulfate precipitation (Cyran, Courtin, & Delcour, 2003) techniques. Feruloylated WEAX are mostly found in high molecular mass fraction (>110, 000) but absent in the low molecular mass (<5,000) (Hartmann 2005). Fractions obtained at 60% and 80 % ammonium sulfate fractionation contain around 95% AX and 0.8% ferulic acid (Cyran, Courtin, & Delcour, 2003).

2.2 Phenolic Compounds in Cereal

2.2.1 Occurrence of phenolic acids in cereal grains

Phenolic compounds are substances containing aromatic rings bearing one or more hydroxyl groups including functional derivatives. They are classified into hydroxycinnamic acids, hydroxybenzoic acids, flavonoids, stillbene, tannins and lignins. Hydroxycinnamic acids, commonly referred to as phenolic acids, include p-coumaric acid, caffeic acid, ferulic acid and sinapic acid. Benzoic acid family includes hydroxybenzoic acid, vanillic acid, syringic acid and gallic acid. Flavonoids consist of flavonone, flavones, flavononol, flavonol, isoflavone, and flavan-3-ols such as catechin (Liu, 2007).

Phenolic compounds found in wheat include hydroxybenzoic acids (p-hydroxybenzoic acid, vanillic acid and syringic acid); hydroxycinnamic acids (p-coumaric acid, ferulic acid and four ferulic acid derivatives); and flavonoid (apigenin). Ferulic acid constitutes the predominant compound among the total phenolics found in wheat followed by syringic and p-hydroxybenzoic acids (Hernandez, Afonso, Rodriguez, & Diaz, 2011; Mattila, Pihlava, & Hellstrom, 2005; Sosulski, Krygier, & Hogge, 1982).

The total phenolic content of wheat grain is approximately 168 - 459 µg/g and is highly localized in the bran fraction (1258-3157 µg/g), and the endosperm having the least (44 - 140 µg/g) (Adom

& Liu, 2002; Adom, Sorrells, & Liu, 2003; Liyana-Pathlrana & Shahidi, 2007; Vaher, Matso, Levandi, Helmja, & Kaljurand, 2010). The total phenolic content and composition varies with cultivar and growing conditions (Abdel-Aal, Hucl, Sosulski, Graf, Gillott, & Pietrzak, 2001; Adom, Sorrells, & Liu, 2003; Beta, Naing, Nam, Mpofu, & Therrien, 2007; Heimler, Vignolini, Isolani, Arfaioli, Ghiselli, & Romani, 2010; Verma, Hucl, & Chibbar, 2008; Zuchowski, Jonczyk, Pecio, & Oleszek, 2011). For example, phenolic acids, flavones, and flavonols were present in all wheat cultivars, but anthocyanins were found only in purple wheat (Liu, Qiu, & Beta, 2010). Similarly, ferulic and p-coumaric acids and the total phenolic acid content may be higher in organic wheat compared to conventional wheat (Zuchowski, Jonczyk, Pecio, & Oleszek, 2011). Furthermore, red wheats may have higher total phenolic content than that of the white wheats (Kim, Tsao, Yang, & Cui, 2006).

Phenolic compounds in wheat exist in free or bound form with a majority (≈90%) being in bound form (Adom & Liu, 2002; Adom, Sorrells, & Liu, 2003; Kim, Tsao, Yang, & Cui, 2006; Liyana-Pathirana & Shahidi, 2006; Sosulski, Krygier, & Hogge, 1982; Verma, Hucl, & Chibbar, 2008). Syringic acid is the principal compound among the free phenolic compounds and contributes about 77.0% of the total free phenolic compound in wheat (Pham Van, Hatcher, & Barker, 2011) whereas ferulic acid is the major compound among the bound phenolic acids as well as total phenolic compounds (Hernandez, Afonso, Rodriguez, & Diaz, 2011; Mattila, Pihlava, & Hellstrom, 2005; Vaher, Matso, Levandi, Helmja, & Kaljurand, 2010; Verma, Hucl, & Chibbar, 2008). Ferulic acid is highest in the aleurone layer (Anson, van den Berg, Havenaar, Bast, & Haenen, 2008; Sosulski, Krygier, & Hogge, 1982) as it is bound to arabinoxylan (Rao & Muralikrishna, 2006; Renger & Steinhart, 2000) whereas p-coumaric acid is highest in bran as it is bound to lignin. Ferulic acid in wheat is found in both monomeric and dimeric forms (diferulic acid) (Renger & Steinhart, 2000;

Saulnier, Crepeau, Lahaye, Thibault, Garcia-Conesa, Kroon, et al., 1999) and the dimers have been shown to have a higher antioxidant capacity (Garcia-Conesa, Plumb, Waldron, Ralph, & Williamson, 1997). Dimeric ferulic acid varies from 4 – 20% of the total ferulic acid depending on the wheat cultivar (Mattila, Pihlava, & Hellstrom, 2005). Dimerization of ferulic acid is essential for cross linking of arabinoxylan polymers (Renger & Steinhart, 2000; Saulnier, et al., 1999).

The differences in the total polyphenol content found in literature are partly influenced by the extraction or hydrolysis method deployed (Barberousse, Roiseux, Robert, Paquot, Deroanne, & Blecker, 2008; Nardini & Ghiselli, 2004; Sosulski, Krygier, & Hogge, 1982). Alkaline hydrolysis results in lower amounts of polyphenols compared to acid hydrolysis (Arranz & Saura Calixto, 2010). The presence of strong antioxidants like ascorbic acid and a metal chelator (ethylenediaminetetraacetic acid) during alkaline hydrolysis improves phenolic acids recovery rate to over 98% (Nardini & Ghiselli, 2004).

2.2.2 Absorption and metabolism of cereal phenolic compounds

The bioavailability data of phenolic acids from cereals are limited and most studies have been done with wine and other beverages. Studies suggest that phenolic compound can be absorbed from the stomach, small intestine and colon, but jejunum is where most are absorbed (Zhao & Moghadasian, 2010). Bound phenolic compounds may be partially hydrolyzed and released in the small intestine as esterase activity have been observed in mammalian intestine (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001a, 2001b). Absorption of phenolic compounds has been demonstrated to be facilitated by Na^+ -dependent mediated transport and may be influenced by intracellular HCO_3^- and/or pH (Wolffram, Weber, Grenacher, & Scharrer, 1995). Absorption of phenolic compounds may be governed by the food matrix with free hydroxycinnamic acids being readily absorbed,

whereas bound phenolic compounds are poorly absorbed in the intestine (Adam, Crespy, Levrat-Verny, Leenhardt, Leuillet, Demigne, et al., 2002). In an ileostomy, only 33% of ingested chlorogenic acid was absorbed, whereas about 95% of the caffeic acid was absorbed in the small intestine (Olthof, Hollman, & Katan, 2001). Chlorogenic acid is purportedly cleaved to caffeic acid and quinic acid by the intestinal microflora and esterase (Plumb, Kroon et al. 1999). Phenolic compounds present in conjugated form with glucose may have a higher absorption rate compared to other dietary forms (Hollman, van Trijp, Buysman, van der Gaag, Mengelers, de Vries, et al., 1997). Approximately 5% only of the total dietary intake of the phenolic compound is absorbed and the rest remains in the lumen (Clifford 2004).

Upon absorption, phenolic compounds are treated as xenobiotic such that they undergo glucuronation/sulfation in the enterocyte and hepatocyte (Milbury, Cao, Prior, & Blumberg, 2002; Nardini, Cirillo, Natella, & Scaccini, 2002; Spencer, Schroeter, Rechner, & Rice-Evans, 2001; Williamson, Plumb, & Garcia-Conesa, 1999). The plasma concentrations of phenolic acids in rats vary greatly upon consumption of a diet rich in phenolic acids or supplements, partly due to poor understanding of the probable metabolite formed (Karakaya, 2004; Scalbert & Williamson, 2000) but largely depending on the food matrix (Adam, et al., 2002). In humans, peak plasma concentration of caffeic acid was observed at 1 hour after taking coffee (Nardini, Cirillo, Natella, & Scaccini, 2002; Natella, Nardini, Giannetti, Dattilo, & Scaccini, 2002). Results from different animal studies suggest that phenolic acids are rapidly excreted in the urine (Adam, et al., 2002; Karakaya, 2004). However, it may take around 5 to 7 hours to reach maximum urinary excretion of ferulic acids in humans (Bourne & Rice-Evans, 1998; Milbury, Cao, Prior, & Blumberg, 2002).

2.3 Glucose Absorption and Metabolism

Dietary carbohydrates are classified into polysaccharide (starch and fiber), oligosaccharide, and simple sugars (mono and disaccharides) (Asp, 1996; Englyst & Hudson, 1996). Monosaccharides (glucose, fructose and galactose) are readily absorbed and disaccharides are hydrolyzed to their constituting monomers by brush border enzymes in the small intestines before being absorbed (Gray, 1975). Digestible polysaccharides and oligosaccharides are broken down to simple sugars by the action of pancreatic amylase. The fate of non-digestible polysaccharide is still not clear, but it is thought they are subjected to microbial fermentation in the large intestine (Roberfroid, 1993).

Facilitative glucose transporters 1-12 (GLUT) and sodium-dependent glucose transporter (SGLT) are responsible for glucose absorption and transport across cell membranes in the body (Uldry & Thorens, 2004a, 2004b; Wright & Turk, 2004). GLUT and SGLT belong to the SLC2 and SLC5 gene family, respectively. SGLT uses energy via sodium-gradient to transport sugars whereas GLUT involves down-hill transport of sugars (facilitated diffusion). GLUT1 transports glucose across the blood - brain barrier whereas GLUT4 facilitate absorption of glucose from the circulatory system to skeletal muscle and GLUT3 is transporting glucose to the brain (Burant & Bell, 1992).

Sugar absorption in the small intestine mainly involves GLUT2, GLUT5 and SGLT1 (Burant, Takeda, Brotlaroche, Bell, & Davidson, 1992; Davidson, et al., 1992; Wright, et al., 1994). The predominant glucose transporter in the intestine is GLUT2 as it is involved in both uptake and export (Kellett & Brot-Laroche, 2005). SGLT1 and GLUT5 are expressed mostly on the apical side of enterocytes, whereas GLUT2 is usually found on the basolateral membrane and a fraction of GLUT2 can be recruited to the apical membrane (Wright, Hirayama, & Loo, 2007). Glucose

and galactose (at low concentration) are absorbed through SGLT1 whereas fructose is absorbed through GLUT5 (Wright, Martin, & Turk, 2003). The sugars are exported from the enterocytes through facilitated diffusion via GLUT2 and GLUT5 (Wright, Martin, & Turk, 2003). In case of high glucose and galactose levels in the lumen, SGLT1 facilitates insertion of GLUT2 on the apical membrane of the enterocyte to aid in glucose or galactose uptake (Kellett & Brot-Laroche, 2005). The molecular basis and functional studies on SGLT1 has been reviewed by Wright et al (2007) and that for GLUT2 by Uldry and Thorens (2004).

The normal blood glucose level is about 4 mM but increases to about 12 mM within 30 minutes after intake of a high carbohydrate diet (Jenkins, Wolever, Taylor, Barker, Fielden, Baldwin, et al., 1981; Wright, Hirayama, & Loo, 2007). High glucose concentration triggers the secretion of insulin hormone which induces hepatocytes in the liver to take up excess glucose for glycogen synthesis (glucose storage) and increase uptake of glucose by muscle cells through activation of GLUT4 (Vaulont et al. 2000). On the other hand, low plasma glucose level results in the release of glucagon hormone which triggers glycogenolysis in the liver in order to maintain glucose supply to the tissues. The release of the two hormones from the pancreas is tightly regulated for glucose homeostasis in the body (Vaulont, Vasseur-Cognet, & Kahn, 2000). Lapse in insulin response or production results in chronic diseases called diabetes (Reaven, Bernstein, Davis, & Olefsky, 1976). Therefore, gradual intestinal glucose absorption or release might be essential in controlling diabetes.

2.4 Whole Grain and Diabetes Management

Consumption of whole grain cereals has been associated with a slow increase in blood glucose level compared to the consumption of refined flours (Foster-Powell, Holt, & Brand-Miller, 2002; Ludwig, 2002) even though some results suggest otherwise (Jenkins, et al., 1981). The mechanism

through which whole grains might reduce rapid increase in blood glucose is still not clear (Ludwig, 2002). Whole grains contain a lot of bioactive compounds that may contribute to antiglycemic properties, particularly its dietary fibers (Gemen, de Vries, & Slavin, 2011) and phenolic compounds (Shobana, Sreerama, & Malleshi, 2009). Phenolic acid extracts inhibited both α-amylase and α-glucosidase activity noncompetitively (Shobana, Sreerama, & Malleshi, 2009). Similarly, consumption of breakfast supplemented with finger millet significantly reduced postprandial blood glucose in non-insulin dependent type 2 diabetes human subjects (Kumari & Sumathi, 2002). Recently, Kwon et al (2010) reported that flavonoids might inhibit the GLUT2 transporter. Thus, it is plausible that antiglycemic properties of whole grain may be derived by inhibition of both digestive enzymes and intestinal glucose absorption. This can be supported by the fact that the glycemic response does not depend on starch digestibility (Eelderink, Moerdijk-Poortvliet, Wang, Schepers, Preston, Boer, et al., 2012). Also, glucose increase in blood was not affected by carbohydrate chain length in healthy individuals (Wahlqvist, Wilmshurst, & Richardson, 1978). However, the antidiabetic properties of whole grain have mostly been attributed to their dietary fiber content as discussed later.

2.5 Arabinoxylans in Diabetes Prevention and Management

Diabetes is a chronic disease epitomized by high circulating plasma glucose. Thus management of postprandial glucose is critical in the prevention and treatment of type 2 diabetes patients. Human intervention studies have shown that consumption of arabinoxylan rich diet attenuates postprandial blood glucose levels in healthy (Lu, Walker, Muir, Mascara, & O'Dea, 2000), impaired glucose tolerance (Garcia, Otto, Reich, Weickert, Steiniger, Machowetz, et al., 2007; Garcia, Steiniger, Reich, Weickert, Harsch, Machowetz, et al., 2006) and diabetic (Hanai, Ikuma, Sato, Iida, Hosoda, Matsushita, et al., 1997; Z. Lu, Walker, Muir, & O'Dea, 2004) subjects. In contrast, Mohlig et al

found no effect on glucose response upon feeding healthy human bread rolls supplemented with AX (Möhlig, Koebnick, Weickert, Lueder, Otto, Steiniger, et al., 2005). Animal studies have also reported mixed results on the effect of arabinoxylan (Hartvigsen, Jeppesen, Lærke, Njabe, Knudsen, & Hermansen, 2013; Vogel, Gallaher, & Bunzel, 2012). Comparison of data from intervention studies is difficult as limited chemical details of AX are provided (Gemen, de Vries, & Slavin, 2011). The underlying mechanism remains unclear, but it is thought that soluble fiber increases lumen viscosity thereby delaying nutrient absorption (Dikeman & Fahey Jr, 2006; Jenkins, Wolever, Leeds, Gassull, Haisman, Dilawari, et al., 1978). Thus Galler and Bunzel (2012) fed rats a modified wheat bran arabinoxylan to study the effect of viscosity. AXs were modified through oxidative gelation to increase their viscosity. The results indicated that rats fed on modified arabinoxylan had improved blood glucose response, but not those fed on native arabinoxylans. Their findings supported the notion that AX affects postprandial blood glucose via viscosity.

The intrinsic viscosity of native arabinoxylans varies greatly and is dependent on asymmetrical conformation, length of xylan backbone, and concentration (Izydorczyk & Biliaderis, 1995). Of these three factors, concentration of AXs seems to influence the viscosity of AX the most (Kale, Yadav, Hicks, & Hanah, 2015; Saulnier, Sado, Branlard, Charmet, & Guillon, 2007). Thus the effect of arabinoxylan on blood glucose is dose dependent. Also, apparent viscosity of the arabinoxylans is dependent on shear stress, such that higher shear result in non-Newtonian fluid behavior (Izydorczyk & Biliaderis, 1995). Recent studies suggest that viscosity effects of AX may be offset by strong intestinal peristalsis (Dhital, Dolan, Stokes, & Gidley, 2014). Amylolysis of starch was performed in the presence of 1 and 2 % AX concentration. A viscosity increase by a factor of 10 and 100 in the presence of 1 and 2 % AX concentration, respectively was observed.

Consequently, digestion rate was reduced by 51 and 63 % times for 1 and 2% AX concentration, respectively. However, digestion rate was only decreased by 29 % when the mixing was introduced regardless of AX concentration. Thus the effect of AX on starch assimilation may go beyond viscosity aspects. The viscosity of digesta is complex and may be influenced by both soluble and insoluble fibers (Takahashi, Oda, Naoi, & Kitamori, 2012). Insoluble fibers have high water holding capacity and absorb lumen water, thereby increasing digesta viscosity. Soluble fibers increase viscosity by virtue of their chemical characteristic. Studies suggest that soluble and insoluble fibers are more effective when present together.

WUAX is entrapped in the cell wall matrix through cross linkages with neighboring polymers which lead to their high water holding capacity. Thus endoxylanase are being used to solubilize WUAX. The resultant AXs are of low molecular weight polymer which may not be as viscous WEAX. Data on the effect of resultant AX oligosaccharide (AXOS) on postprandial blood glucose is limited. However, the AXOS are readily fermented to short chain fatty acid (SCFA) (François, Lescroart, Veraverbeke, Marzorati, Possemiers, Evenepoel, et al., 2012). SCFA may decrease hepatic glucose production and increase glucose uptake in the skeletal muscle by activating AMP activated protein kinase (AMPK) (Hu, Chen, Xu, Ge, & Lin, 2010; Winder & Hardie, 1999).

2.6 Whole Grain and Gut Health

Consumption of fiber from cereal grains is associated with reduced risk of colon and colorectal cancers (CRC) (Aune, Chan, Lau, Vieira, Greenwood, Kampman, et al., 2011) but the underlying mechanisms remain unclear (Zeng, Lazarova, & Bordonaro, 2014). The anticancer properties may be derived from a number of properties including promotion of healthy gut microbiota, substrate for short chain fatty acid production, increase of fecal bulk and viscosity, and binding of potential cancer-causing agents as reviewed by Zeng et al (2014). However, it is possible that cereal fiber

may as well serve as an indicator of consumption of plant food which is rich in bioactive compounds (Ferguson & Harris, 2003). For example, phenolic acid present in cereal grain may offer protection against oxidative damage of the gastrointestinal tract.

Also, studies have shown that populations consuming high fat have high incidences of CRC. In general, animal fats, more especially those from red meat, present a higher risk compared to those from plant source among other exogenous factors (Cross, Leitzmann, Gail, Hollenbeck, Schatzkin, & Sinha, 2007; Giovannucci, Rimm, Stampfer, Colditz, Ascherio, & Willett, 1994). Indeed a meta-analysis study found that high consumption of red meat increases the risk of colorectal and colon cancers (Larsson & Wolk, 2006). Among other plausible theories, fat and heme iron in red meat is thought to be the risk factors (Santarelli, Pierre, & Corpet, 2008). Hemoglobin or myoglobin is known to promote lipid peroxidation (Kanner & Lapidot, 2001). Both hemoglobin and lipid hydroperoxides (LOOH) are capable of inducing DNA oxidation at high concentration (>100 μM) (Angeli, Garcia, Sena, Freitas, Miyamoto, Medeiros, et al., 2011). Synergistic effects of hemoglobin and lipid hydroperoxides on genotoxicity have also been reported (Angeli, Garcia, Sena, Freitas, Miyamoto, Medeiros, et al., 2011; Kanner & Lapidot, 2001). Heme-iron facilitates degradation of LOOH (Carlsen, Møller, & Skibsted, 2005) to peroxyl andalkoxyl radical, which may eventually damage the DNA. Thus the presence of antioxidant may mitigate the development or propagation of CRC associated with high fat or red meat diets.

Whole grain cereals contain phenolic acids, which are present either as free or bound to complex arabinoxylans. Phenolic acids are powerful antioxidant and are present in the gastrointestinal tract in larger quantities upon consumption of whole grain cereal rich diet. However, these phenolic acids were found ineffective against LOOH under simulated gastric digestion (Kanner & Lapidot, 2001). However, the phenolic compounds were only effective in the presence of alcohol. This is

possibly due to the low solubility of phenolic compounds under physiological conditions (Kanner & Lapidot, 2001). The solubility of FA is increased from 0.6 mg/mL (Mota, Queimada, Pinho, & Macedo, 2008) to 10 mg/mL (Fang, Wang, Chang, Hu, Hwang, Fu, et al., 2013) when esterified to arabinose. Consequently, the antioxidant capacity of feruloylated arabinoxylan oligosaccharide is much higher than that of ferulic acid at the same ferulic acid molar concentration (Ohta, Semboku, Kuchii, Egashira, & Sanada, 1997; Ohta, Yamasaki, Egashira, & Sanada, 1994).

Polysaccharides from fruits and mushrooms have been shown to possess the ability to scavenge free radicals in *in vitro* studies (Li, Li, & Zhou, 2007; Yang, Zhao, Shi, Yang, & Jiang, 2008). The exact mechanism on how polysaccharides inhibit free radicals is not well understood. However, from the studies done so far, it seems molecular weight, degree of branching, monosaccharide composition, presence of bound proteins, and heterogeneity of polysaccharides affect the antioxidant activity of polysaccharides (Lo, Chang, Chiu, Tsay, & Jen, 2011). Limited data exist with regard to the antioxidant activity of polysaccharides from cereals. Water-soluble FAXn from rice have been shown to exhibit antioxidant activity *in vitro* (Rao & Muralikrishna, 2006). Despite its predominance, insoluble FAXn may have very low antioxidant activity compared with WEAX (Serpen et al. 2008). Compared to ferulic acid, FAXn demonstrated a higher antioxidant activity (Rao & Muralikrishna, 2006).

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Table 2.1: Arabinoxylan content of flour and bran from different cereal grains

	Whole grain				Bran Fraction			
	Total AX (%)	A/X	WEAX (%)	A/X	Total AX (%)	Total (A/X)	WEAX (%)	(A/X)
Wheat[a]								
Winter wheat	1.90	0.60	0.50	0.50	18.0	0.60	0.40	1.00
Spring wheat	2.00	0.60	0.50	0.50	16.8	0.65	0.40	1.00
Durum wheat	1.95	0.65	0.40	0.55	12.0	0.75	0.40	1.25
Spelt wheat	1.75	0.60	0.35	0.50	12.7	0.50	0.30	1.40
Einkorn wheat	1.95	0.65	0.60	0.55	10.0	0.55	0.55	0.95
Emmer wheat	1.70	0.60	0.25	0.35	8.9	0.55	0.30	1.50
Mean (wheat)	***1.88***	***0.60***	***0.40***	***0.49***	***13.07***	***0.60***	***0.39***	***1.18***
Range (Wheat)	***1.35 – 2.75***	***0.50 – 0.70***	***0.15 – 1.40***	***0.20 – 0.65***	***6.1 – 22.1***	***0.45 – 0.80***	***0.20 – 0.85***	***0.85 – 1.70***
Rye[b]								
Zlote	3.19	0.66	1.18	0.57	12.4	0.54	1.21	0.71
Warko	3.11	0.76	1.15	0.68	12.08	0.58	1.14	0.82
Rekrut	3.98	0.71	1.49	0.62	13.44	0.48	1.44	0.76
Nikita	4.31	0.73	1.43	0.64	14.76	0.5	1.4	0.78
Lovaszpatonai-1	3.68	0.7	1.05	0.58	12.89	0.52	1.1	0.75
Grandrieu	3.62	0.7	1.22	0.62	13.97	0.58	1.04	0.78
Queyras	3.6	0.71	1.1	0.62	14.41	0.52	1.42	0.77
Haute Loire	3.98	0.74	1.17	0.67	14.06	0.56	1.46	0.83
Portugaise-3	3.74	0.72	1.24	0.63	13.41	0.51	1.47	0.75
Portugaise-6	3.19	0.74	1.05	0.67	12.06	0.55	1.06	0.84
Mean (Rye)	***3.64***	***0.72***	***1.21***	***0.63***	***13.35***	***0.53***	***1.27***	***0.78***

Range (Rye)	***3.11-4.31***	***0.66-0.76***	***1.05-1.49***	***0.57-0.68***	***12.06-14.76***	***0.48-0.58***	***1.04-1.47***	***0.71-0.84***
Barley[c]								
Dicktoo	2.16	0.63	0.32	nd	9.03	0.58	0.29	nd
Plaisant	2.13	0.64	0.38	nd	9.84	0.56	0.35	nd
Igri	1.83	0.64	0.24	nd	8.06	0.62	0.25	nd
Rastik	1.4	0.68	0.15	nd	6.05	0.67	0.19	nd
CFL93-149	2.24	0.63	0.24	nd	5.81	0.58	0.22	nd
CFL98-398	2.01	0.62	0.27	nd	8.38	0.54	0.28	nd
CFL98-450	2.01	0.74	0.15	nd	4.84	0.74	0.15	nd
Erhard-Frederichen	1.69	0.7	0.26	nd	7.59	0.55	0.27	nd
Borzymowicki	2.12	0.58	0.2	nd	7.9	0.54	0.24	nd
Morex	1.53	0.66	0.24	nd	7.8	0.62	0.24	nd
Mean (Barley)	***1.91***	***0.65***	***0.25***		***7.53***	***0.6***	***0.25***	
Oats[d]								
Cacko	0.97	nd	0.16	nd	3.83	Nd	0.19	
MV-Pehely	1.26	nd	0.18	nd	13.2	Nd	0.2	
Fengli	1.14	nd	0.18	nd	11.52	Nd	0.21	
Expander	1.05	nd	0.15	nd	8.75	Nd	0.19	
Bajka	1.15	nd	0.18	nd	8.02	Nd	0.21	
Mean (Oat)	***1.11***		***0.17***		***9.06***		***0.2***	

Data for a, b, c, and d was adapted from Gebruers et al., (2008), Nystrom et al., (2008), Andersson et al., (2008) and Shewry et al., (2008), respectively.

Table 2.2: Arabinoxylan distribution in barley and wheat hand dissected botanical fractions

	Barley[a]				Wheat[b]			
	Whole grain	Endosperm	Aleurone	Pericarp	Whole grain	Endosperm	Aleurone	Pericarp
Total AX%	4.69	1.64	25.55	40.1	5.9	1.6	20.8	46.2
A/X	/	0.83	0.61	1.16	0.72	0.84	0.44	1.13
FA (%)	/	0.4	21.5	11.7	0.86	0.05	8.1	3.3
diFA (%)	/	0.05	4.4	8.94	0.15	0.0	0.34	2.4
WEAX (%)	/	22.6	1.34	0.9	/	/	/	/
WEAX (A/X)	/	0.67	0.85	0.89	/	/	/	/
WUAX (A/X)	/	0.95	0.57	1.17	/	/	/	/

[a] Data adapted from Izydorzyck (2010)

[b]Data adapted from Barron et al., (2007) and Antoine et al., (2003)

A/X = arabinose to xylose ratio; FA is ferulic acid; diFA = diferulic acid, WEAX = is water extractable arabinoxylan; WUAX = water unextractable arabinoxylan. / = not reported

Figure 2.1: Schematic presentation of arabinoxylan structural linkages

(a) unsubstituted Xyl_p; (b) monosubstituted Xyl_p at O-2; (c) monosubstituted Xyl_p at O-3 with ferulic acid residue esterified to Ara_f and (d) disubstituted Xyl_p at O-2,3. (Reprinted from Izydorczyk & Dexter, 2008).

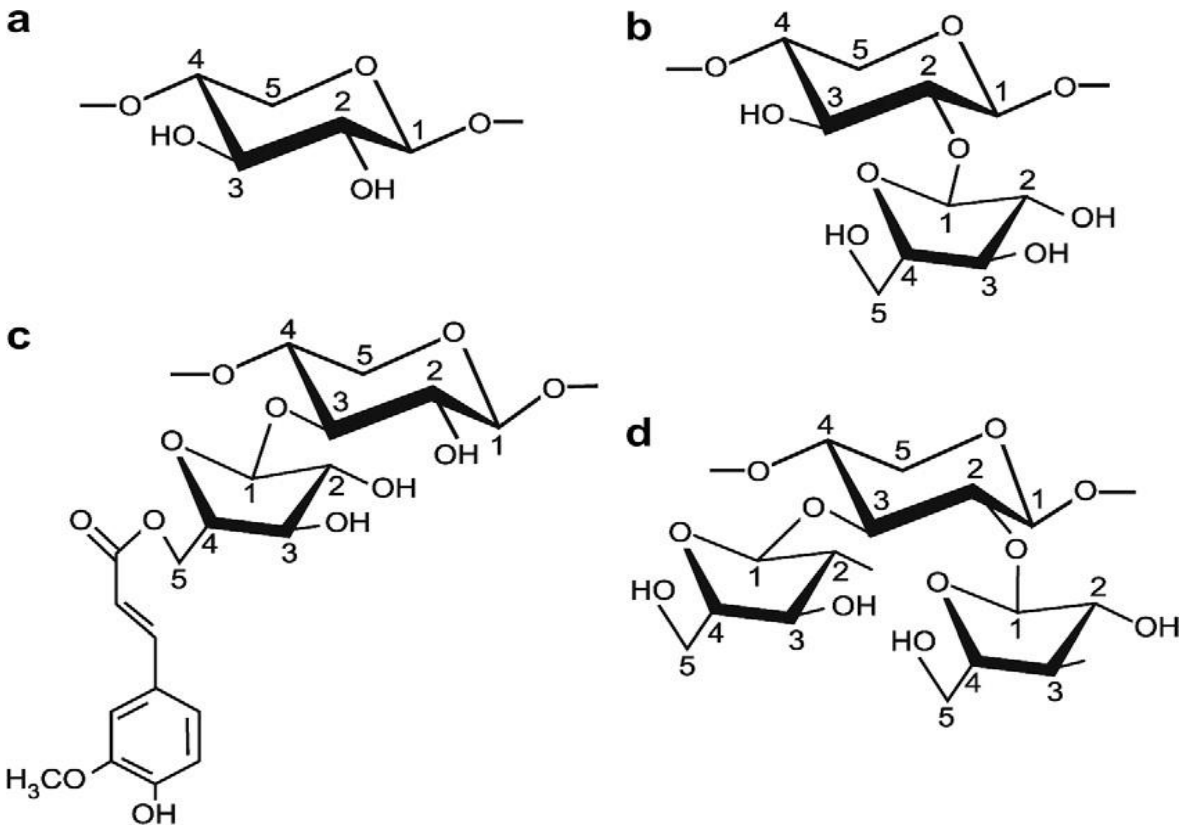
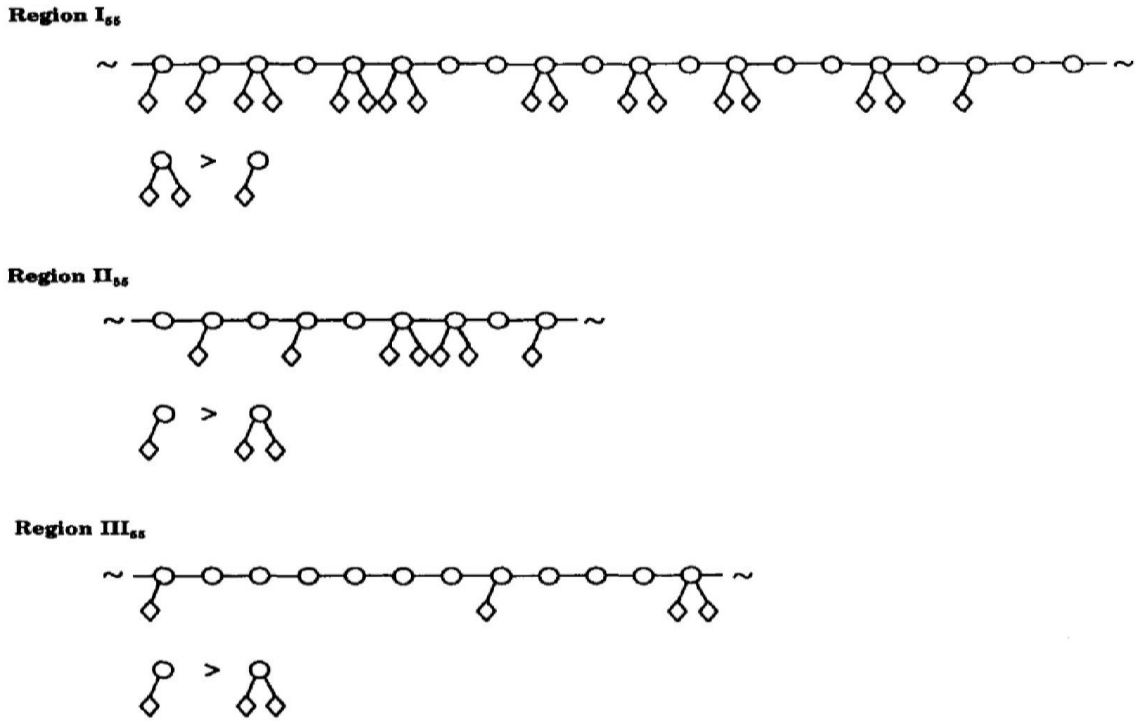


Figure 2.2: Proposed structural models of wheat endosperm arabinoxylans

(Reprinted from Izydorczyk & Biliaderis, 1994)



CHAPTER 3

3 Antioxidant Capacity of Water-Extractable Arabinoxylan from Commercial Barley, Wheat, and Wheat Fractions

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3.1 Abstract

The objective of this research was to analyze the antioxidant capacity directly of water-extractable nonstarch polysaccharides (NSP) and feruloylated arabinoxylans (WEAX) following their characterization. NSP were isolated from barley, wheat, and wheat fractions (germ, bran, and aleurone). WEAX were extracted only from wheat fractions. Antioxidant capacity of NSP measured with the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3ethylbenzothiazoline-6-sulfonic acid (ABTS), and oxygen radical absorbance capacity (ORAC) assays was 24.0–99.0, 40.0–122.0, and 140.0– 286.0μM Trolox equivalents (TE)/g, respectively. The antioxidant capacity of WEAX was 75.7–84.0, 58.0–105.0, and 110.0–235.0μM TE/g for those three assays. DPPH and ABTS were highly correlated to xylose content (R^2 = 0.85), degree of substitution (R^2 = –0.99), total phenolic acids (R^2 = >0.73), total phenolic content (TPC) (R^2 = >0.78), and ferulic acid content (R^2 = >0.86). ORAC was only influenced by TPC (R^2 = 0.63). By taking yield and antioxidant capacity into account, NSP would provide about 0.4– 4.2, 0.6–5.1, and 2.8–12.0μM TE/g of flour of radical scavenging activity as measured by DPPH, ABTS, and ORAC, respectively, compared with WEAX (0.4–1.0, 0.3–1.3, and 0.6–2.8μM TE/g). Our results suggest that NSP or WEAX may play a role in protection against free radicals in a food matrix and likely in the gastrointestinal tract.

3.2 Introduction

Regular consumption of whole grain cereals and cereal dietary fiber including arabinoxylans (AX) and other nonstarch polysaccharides (NSP) has been associated with a lower risk of chronic diseases (Aune, Chan, Lau, Vieira, Greenwood, Kampman, et al., 2011; Jonnalagadda, Harnack, Liu, McKeown, Seal, Liu, et al., 2011; Kaczmarczyk, Miller, & Freund, 2012; Kyrø, Skeie, Loft, Landberg, Christensen, Lund, et al., 2013). A recent study by Damen et al. (2012) demonstrated favorable modulation of intestinal fermentation and overall gastrointestinal properties in healthy humans consuming breads with the in situ–produced AX oligosaccharides. Authors observed an increase in concentration of fecal total short-chain fatty acids mainly attributed to butyrate production as well as suppression of urinary excretion of potentially detrimental phenol and *p*-cresol. The urinary excretion of phenolic compounds was considered a reflection of their generation in the colon as bacterial catabolites of phenylalanine and tyrosine. No direct measurements were made of phenolic compounds associated with the in situ–produced AX oligosaccharides before and after consumption. The copassengers of cereal dietary fiber, which include phenolics such as ferulic acid, may exert additional protective effects. Zhao et al. (2003) recovered 20% of the administered ferulic acid moiety in rat urine and feces when feruloylated AX (FAXn) were administered. The liver is the main site where ferulic acid is metabolized, mainly to the sulfoglucuronide form, whereas absorption occurs along the entire gastrointestinal tract (Zhao & Moghadasian, 2008).

Research in the past decades has focused on the radical scavenging hypothesis to explain the probable mechanism (Fardet, 2010; Perron & Brumaghim, 2009). It is scientifically accepted that oxidative damage is involved at either the initial or the developmental stage of most diseases (Sun, 1990). Antioxidant compounds, if present, may donate hydrogen atoms or electrons to neutralize

harmful free radicals circulating in the body or in a food matrix (Huang, Ou, & Prior, 2005). Cereals contain significant levels of such antioxidants in the form of phenolic compounds, of which 90% are bound to AX (Ishii, 1997; Sosulski, Krygier, & Hogge, 1982) However, only 5–10% of the total phenolic antioxidants consumed are absorbed into the human body (Clifford, 2004) despite the presence of esterase enzymes in the human digestive system (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001). This low absorption has led to suggestions that the site of action might be the gastrointestinal tract, where both free radicals (Kanner & Lapidot, 2001) and bound phenolic compounds (Clifford, 2004; Holst & Williamson, 2008; Russell & Duthie, 2011) are present in high concentrations. Thus, phenolic acids in their bound form should be able to directly scavenge free radicals to mitigate oxidative damage of colonocytes.

FAXn are a major constituent of the cell wall NSP in cereals and can be water extractable (WEAX) or water unextractable. NSP content and chemical composition vary greatly among and within cereal species. The structure of FAXn is very complex and diverse in plant tissues, but the primary chain consists of (1-4)-βD-xylopyranose with α-L-arabinofuranose substitutions at the *O*-2 or *O*-3 positions or both (Lequart, Nuzillard, Kurek, & Debeire, 1999). The xylan chain can also be substituted with α-(1,2)-glucuronic acid or α-(1,2)-4*O*-methylglucuronic acid branches or both (Ma, Jia, Zhu, Li, Peng, & Sun, 2012). The AX may be feruloylated with ferulic acid at the *O*-5 position of arabinose units (Bunzel, Ralph, & Steinhart, 2005). The degree of branching or linearity for FAXn is often defined by the molar ratio of arabinose to xylose (Ar/Xy). The mean Ar/Xy ratio for WEAX varies in wheat fractions. For example, the mean Ar/Xy ratio for WEAX obtained from the endosperm is around 0.55 (Cleemput, Van Oort, Hessing, Bergmans, Gruppen, Grobe, et al., 1995; Dervilly, Saulnier, Roger, & Thibault, 2000; Izydorczyk & Biliaderis, 1994). However, it is

0.35 for the aleurone (Antoine, Peyron, Mabille, Lapierre, Bouchet, Abecassis, et al., 2003) and 0.7 for whole flour (Guttieri, Souza, & Sneller, 2008).

Polysaccharides from fruits and mushrooms have been shown to possess the ability to scavenge free radicals in *in vitro* studies (Li, Li, & Zhou, 2007; Yang, Zhao, Shi, Yang, & Jiang, 2008). The exact mechanism on how polysaccharides inhibit free radicals is not well understood. However, from the studies done so far, it seems molecular weight, degree of branching, monosaccharide composition, presence of bound proteins, and heterogeneity of polysaccharides affect the antioxidant activity of polysaccharides (Lo, Chang, Chiu, Tsay, & Jen, 2011). Limited data exist with regard to the antioxidant activity of polysaccharides from cereals. Water-soluble FAXn from rice have been shown to exhibit antioxidant activity *in vitro* (Rao & Muralikrishna, 2006). Despite its predominance, insoluble FAXn may have very low antioxidant activity compared with WEAX (Serpen, Gökmen, Pellegrini, & Fogliano, 2008). Thus, in the present work, we only considered testing the antioxidant capacity of cereals based on the water-extractable NSP or WEAX to understand their antioxidant potential in the gastrointestinal tract or in the food matrix.

We hypothesized that the existing differences in chemical characteristics of WEAX between cereals or cereal fractions affect their antioxidant properties. Therefore, the aim of this work was to 1) determine the variation in chemical composition of water extractable NSP or WEAX obtained from whole grain cereals (wheat and barley) and commercial wheat fractions (aleurone, bran, and germ) and 2) evaluate the antioxidant capacity of the resultant water-extractable NSP or WEAX with chemical assays.

3.3 Materials and methods

3.3.1 Sample and chemicals

A commercial wheat aleurone (Grainwise wheat aleurone) was a gift from Cargill Limited and Horizon Milling (Wichita, Kansas, U.S.A.). It constitutes 4.5, 15.2, 7.4, and 2.5% lipid, protein, ash, and starch, respectively (Chen, Dunford, & Goad, 2013). Ambassador wheat (soft white wheat), common barley, purple barley, hard red winter wheat bran, soft white wheat bran, and wheat germ were obtained locally from Bulk Barn (Winnipeg, Manitoba, Canada). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein, amyloglucosidase from *Aspergillus niger* (300 units/mL, EC 3.2.1.3), heat-stable α-amylase (EC 3.2.1.1), protease from *Bacillus licheniformis* (EC 3.4.21.62), phenolic acid standards (gallic, protocatechuic, *p*-hydrobenzoic, caffeic, vanillic, syringic, ferulic, *p*-coumaric, and sinapic acids), and sugar standards (D-glucose, D-xylose, D-arabinose, and D-galactose) were obtained from Sigma-Aldrich (Milwaukee, WI, U.S.A.). Endo-1,3(4)-β-glucanase (lichenase) from *B. subtilis* (1,000 units/mL, EC 3.2.1.73), and β-glucosidase from *A. niger* (200 units, EC 3.2.1.21) were purchased from Megazyme (Bray, Ireland). All acids and organic solvents were purchased from Fischer Scientific (Whitby, ON, Canada). All chemicals used were of analytical grade.

3.3.2 Preparation of water extractable NSP

Water-extractable NSP were prepared as described by Dervilly et al. (2000) with minor modifications. Briefly, 50 g of ground cereal sample was boiled under reflux with 200 mL of 95% ethanol at 90°C for 30 min. After it cooled to room temperature, the mixture was filtered through Whatman filter paper (number 41) and dried at 60°C overnight. Deactivated flour (25 g) was

suspended in 200 mL of sodium phosphate buffer (pH 6.8, 20mM), and heat-stable α-amylase (600 μL) was added to hydrolyze the starch. The flour suspension was incubated with continuous shaking at 250 rpm in a water bath (89032-226, VWR International, Radnor, PA, U.S.A.) for 1 h at 90°C. After the mixture cooled to room temperature, the pH was adjusted to 7.5, and protease (600 μL) was added prior to incubation for 1 h at 60°C. Amyloglucosidase (400 μL) was added after adjusting the pH to 4.5. The suspension was incubated at 60°C for 1 h. The enzymes were inactivated by heating the suspension at 90°C for 30 min. After being cooled down to 4°C with an ice bath, the suspension was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was collected. The residue was rinsed twice with 100 mL of distilled water and centrifuged as described before. The supernatants were pooled together and dialyzed for 48 h at 4°C against distilled water (MWCO 12000) before freeze drying (Virtis Genesis 25XL, SP Scientific, Gardiner, NY, U.S.A.) to obtain a water-soluble extract.

The resultant water-soluble extract (4 g) was suspended in 100 mL of sodium phosphate buffer (pH 6.8, 20mM) and incubated for 2 h in a shaking water bath (250 rpm) at a temperature setting of 60°C. After centrifuging at 13,000 × *g* for 20 min at 4°C, the supernatant was treated with 200 μL of heat-stable α-amylase for 1 h at 95°C while shaking continuously at 250 rpm. To digest residual protein, protease (600 μL) was added after adjusting the pH to 7 with 0.5M NaOH prior to incubation of the sample for 1 h at 60°C. The digest was then heated for 20 min at 95°C to inactivate enzymes. Following rapid cooling in an ice bath, the digest was centrifuged at 13,000 × *g* for 20 min at 4°C. Whatman filter paper (number 41) was used to transfer the supernatant from the centrifuge tubes throughout the extraction process. Ethanol (95%, 530 mL) was added to the supernatant, and the suspension was left overnight at 4°C. The suspension was later centrifuged at 13,000 × *g* for 20 min at 4°C. The residue was washed twice with 80% ethanol (50 mL) followed

by 95% ethanol (50 mL) and centrifuged as before. The residue was later washed with acetone (50 mL). Acetone was removed with filtration. The residue was left in a fume hood to evaporate any residual acetone before drying it overnight in an oven set at 45°C. The dried residue was labeled as NSP.

3.3.3 Preparation of WEAX

WEAX were prepared according to the method of Dervilly et al. (2000). Briefly, 1 g of NSP was solubilized in 100 mL of sodium phosphate buffer (pH 6.8, 20mM) and incubated with 50 μL of lichenase for 1 h at 35°C. The solution was then treated with amyloglucosidase (200 μL) and β-glucosidase (25 μL) at pH 4.5 for 1 h at 60°C in a shaking water bath set at 250 rpm. The mixture was heated at 95°C for 20 min to inactivate the enzymes, followed by centrifugation at 13,000 × *g* for 20 min at 4°C. Ethanol (95%) was added to the supernatant to achieve 70% ethanol concentration. The suspension was left overnight at 4°C. The suspension was centrifuged at 13,000 × *g* for 20 min at 4°C. The residue was washed twice with 80% ethanol followed by 95% ethanol and acetone (50 mL of each) as described before. The residue was then solubilized in water prior to freeze drying to obtain WEAX.

3.3.4 Chemical composition of WEAX and NSP

3.3.4.1 *Protein content*

The Bradford protein assay was used to determine protein content of WEAX (Bradford, 1976). Bovine serum albumin (BSA) (0.1 mg/mL) was diluted to obtain seven different concentrations (0– 20 μg/mL). WEAX or NSP (125 μg/mL, 1.2 mL) and diluted BSA solutions (1.2 mL) were transferred to a test tube. Then 300 μL of Bradford assay dye was added to each test tube and the contents mixed thoroughly. After 10 min, the mixture was transferred into a 2 mL cuvette for absorbance reading (Ultrospec 1100 Pro UV/visible spectrophotometer, Biomicron, Cambridge,

U.K.) at 590 nm. The absorbance readings for BSA standards were used to generate a calibration curve.

3.3.4.2 *Monosaccharide composition*

An Englyst and Cummings (1984) method for determining monosaccharide composition was used with modifications. Briefly, 20 mg of WEAX or NSP was solubilized in 1 mL of 12M sulfuric acid at 35°C for 1 h. Water (11 mL) was added and the contents vortexed rapidly. The mixture was heated for 2 h at 110°C. D-Allose (1 mL, 2 mg/mL in water) was added as an internal standard after cooling to room temperature. The hydrolysates were neutralized and alditol acetates prepared as described by Englyst and Cummings (1984). The alditol acetates were injected into an Agilent 6890N gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with a flame ionization detector by using an autosampler (Agilent Technologies 7863 series). A Supelco 2380 column (30 m × 320 μm × 0.2 μm) (Sigma-Aldrich) and helium gas at a flow rate of 0.9 mL/min were used for separation. Alditol acetates (0.2 μL) were injected, and GC conditions were set as follows: injection, column, and detector temperatures of 275, 275, and 250°C, respectively, and a run time of 30 min. Monosaccharides were identified by using retention times of their respective standards and quantified by using the response factor of the internal standard (D-allose). AX content was calculated from the formula (% arabinose + % xylose) × 0.88.

3.3.4.3 *Uronic acid content*

Glucuronic acid content was quantified as described by Englyst and Cummings (1984) with spectrophotometry. A mixture of 300 μL of the hydrolysates, 300 μL of sodium chloride/boric acid solution, and 5 mL of sulfuric acid (concentrated) was incubated at 70°C for 40 min. After the mixture cooled to room temperature, 200 μL of 3,5-dimethylphenol (1 mg/mL in acetic acid) was added, and the tubes were incubated in water at room temperature. The mixture was vortex mixed

and left to stand at room temperature for 10 min before being transferred into a 2 mL cuvette for measurement of absorbance (Ultrospec 1100 Pro, UV/visible spectrophotometer) at 400 and 450 nm. The absorbance difference ($A_{450} - A_{400}$) of different concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.6, and 0.7mM) of glucuronic acid was used to plot the calibration curve for determining the glucuronic acid content.

3.3.4.4 *Phenolic acid content*

Phenolic acids were extracted following the method described by Hartmann et al. (2005). Briefly, 30 mg of WEAX was hydrolyzed with 2M sodium hydroxide (2 mL) for 15 h at 25°C under nitrogen with continuous shaking at 250 rpm. The pH was then adjusted to 1.7 with 6M hydrochloric acid before extracting three times with ethyl acetate (3 mL). A Büchi Rotavapor R-205 device (Büchi Laboratoriums Technik, Flawil, Switzerland) was used to remove ethyl acetate under nitrogen, and 2 mL of methanol/water (50:50) was then added. The extract was passed through a 0.2 μm filter and analyzed with a reverse phase high-performance liquid chromatograph (Alliance 2695, Waters, Milford, MA, U.S.A.) equipped with a Waters 2996 photodiode array detector and a 5 μm RP C18 column (250 × 4.6 mm) (Shim-pack HRC-ODS, Shimadzu, Tokyo, Japan). For analysis, 10 μL of a sample was injected into the liquid chromatograph. The sample and column temperatures were set at 15 and 35°C, respectively. A flow rate of 0.6 mL/min was used. The operating linear gradient consisting of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol) was as reported by Guo and Beta (2013), as follows: 0–11 min, 9–14% B; 11–14 min, 14–15% B; 14–17 min, 15–15% B; 17–24 min, 15– 16.5% B; 24–28 min, 16.5–19% B; 28–30 min, 19–25% B; 30–36 min, 25–26% B; 36–38 min, 26–28% B; 38–41 min, 28–35% B; 41–46 min, 35–40% B; 46–48 min, 40–48% B; 48–53 min, 48– 53% B; 53–70 min, 53–70% B; for a total of 70 min. Phenolic acid monomers were identified by using retention times

of their respective standards at a wavelength of 280 nm. Phenolic acid standards (gallic, protocatechuic, *p*-hydrobenzoic, caffeic, vanillic, syringic, ferulic, *p*-coumaric, and sinapic acids) were also prepared and diluted to different concentrations in methanol/water (50:50) to obtain calibration curves for quantification of individual phenolic acids in the sample.

3.3.4.5 *Total phenolic content (TPC)*

A Folin–Ciocalteu method (Singleton & Rossi, 1965) as modified by Gao et al. (2002) was used to determine the TPC of the alkaline extracts of WEAX and NSP. The alkaline extracts were diluted appropriately with acidified methanol (HCl/methanol/water ratio of 1:80:10). Diluted extracts (0.2 mL) were mixed with 1.5 mL of freshly diluted Folin–Ciocalteu reagent in water (1:9 v/v) in darkness. Sodium carbonate (1.5 mL, 60 g/L in distilled water) was added after a 5 min equilibration, and the mixture was vortexed. The absorbance of the mixture was read at 90 min at 725 nm with a UV/vis spectrophotometer (Ultrospec 1100 Pro) against 50% MeOH as a blank. Ferulic acid at concentrations of 0, 25, 50, 75, 100, 125, 150, 175, and 200 μg/mL was used to obtain a standard curve for determination of TPC. The results were expressed as micrograms of ferulic acid equivalent (FAE) per gram of WEAX or NSP (μg of FAE/g of WEAX or NSP).

3.3.5 Determination of antioxidant activity of WEAX and NSP

3.3.5.1 *Determination of DPPH scavenging activity*

We modified the DPPH scavenging activity method of Sharma and Bhat (2009) to avoid precipitation of DPPH or WEAX. Briefly, the isolated WEAX were dissolved in 1.5 mL of distilled water to obtain concentrations of 0, 267, 400, 533, 667, and 800 μg/mL for optimization of the method. A concentration of 300 μg of WEAX or NSP per milliliter of water was used for antioxidant activity determination. Similarly, Trolox was dissolved in water to make 0, 2.5, 5.0, 7.5, 10.0, and 12.5 μg/mL standard solutions. A 45μM DPPH solution was prepared by first

dissolving 1.8 mg of DPPH in 30 mL of methanol and vortexing until dissolved. Then 20 mL of water was added and vortexed rapidly. The DPPH solution was filtered through Whatman filter paper (number 41) and kept in the dark at room temperature. It was used within 1 h. An aliquot (400 μL) of WEAX or NSP was transferred into a 2 mL centrifuge tube, and 350 μL of methanol was added followed by 750 μL of DPPH solution, resulting in a reaction medium of 53% aqueous methanol. The mixture was vortexed and left in the dark for 35 min. It was later transferred to a 2 mL cuvette after vortexing for absorbance reading at 515 nm. The readings were taken at 40 and 60 min. IC_{50} was determined from the plot of % DPPH quenched, calculated as $\{1 - [(A_{sample} - A_{blank})/(A_{control} - A_{blank})]\}$, against sample or Trolox concentration.

3.3.5.2 *$ABTS^+$ radical scavenging assay*

$ABTS^+$ scavenging activity was determined following a method described by Re et al. (1999) with modifications. $ABTS^+$ radical reagent was produced by allowing 7mM aqueous solution of ABTS (3 mL) to react with 2.45mM potassium persulfate (3 mL) in the dark at room temperature for 12–16 h. $ABTS^+$ reagent (4.0–4.5 mL) was diluted to 250 mL with distilled water to obtain an absorbance of approximately 0.70 at 734 nm. Then 100 μL of WEAX or NSP (0.3 mg/mL) was mixed with 1.7 mL of $ABTS^+$ reagent in a 2 mL amber centrifuge tube. The mixture was vortexed and left in the dark for 30 min. It was later transferred to a 2 mL cuvette after vortexing for absorbance measurement at 734 nm. The % decolorization, calculated as $\{1 - [(A_{sample} - A_{blank})/(A_{control} - A_{blank})]\} \times 100$, was used to determine the free radical scavenging ability of the sample. A calibration curve of % decolorization obtained from different concentrations of Trolox was used to quantify the antioxidant capacity of the sample.

3.3.5.3 *Oxygen radical absorbance capacity (ORAC) assay*

The ORAC assay followed the procedure described by Cao et al. (1993) and modified by Bellido and Beta (2009) with minor modifications. We used a Precision 2000 automated microplate pipetting system (Bio-Tek Instruments, Winooski, VT, U.S.A.) that automatically transferred ORAC reagents into a 96-well flat-bottomed polystyrene microplate (Corning Inc., Corning, NY, U.S.A.) and an FLx800 microplate fluorescence reader (Bio-Tek Instruments) controlled by KC4 3.0 software. Changes in the fluorescence of fluorescein under controlled temperature conditions (37°C) were measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. A sample (0.1 mg/mL in water) of WEAX or NSP was used for analysis, and 0.0, 6.25, 12.5, 25.0, 50.0, and 75.0μM Trolox in water was used to generate a standard calibration curve.

3.3.6 Statistical analysis

All analyses were performed in duplicate, and all statistics were calculated with one-way analysis of variance with JMP 10 statistical software (SAS Institute, Cary, NC, U.S.A.). Sample means were compared with Tukey's HSD method, and significant differences were determined at $P \leq 0.05$. Correlations between parameters were done by Pearson's correlation test and linear regression (coefficient of determination, R^2).

3.4 Results and discussion

3.4.1 Yield and chemical composition

Water-extractable NSP were obtained from common barley, purple barley, wheat, and three wheat fractions (aleurone, germ, and bran), and their yield and protein content are presented in Table 3.1. Water-extractable NSP generally constitute a small fraction of the total NSP in cereals or cereal fractions (Guttieri, Souza, & Sneller, 2008; Ragaee, Guzar, Dhull, & Seetharaman, 2011). Both common and purple barley had the highest amount of water extractable NSP (4–6%) compared

with whole wheat and wheat fractions (≈2%), which had relatively lower levels except for the aleurone fraction (4%). NSP from whole flours were characterized by low protein (<3%) compared with wheat fractions (≈10%).

The monosaccharide compositions of the NSP are presented in Table 3.2. As expected, NSP from both purple and common barley had high glucose content (≈90%) owing to high levels of β-glucan compared with other samples (Kandil, Li, Vasanthan, Bressler, & Tyler, 2011). The distribution of monosaccharides including glucuronic acid was similar between purple and common barley. Barley contains about 5% water-extractable β-glucans (Kandil, Li, Vasanthan, Bressler, & Tyler, 2011), which resulted in high yields of NSP obtained for both barleys. NSP from wheat germ had high galactose content (15%) compared with other samples (<5%), suggesting high levels of arabinogalactans. The molar ratio of arabinose, xylose, and galactose in germ were similar (≈14.0%), which is consistent with the findings reported by Hedin et al. (1994). The distribution of monosaccharides in both red and white wheat bran was similar, including significantly high levels of glucose (40%). The glucose observed in wheat and wheat fraction NSP was likely from residual starch, β-glucans, and cellulose. Thus, further treating of NSP with endo-1,3(4)-β-glucanase and β-glucosidase reduced the glucose content from about 40% to <2% in WEAX. The results confirmed our hypothesis that glucose present in NSP was mostly from starch, β-glucan, and cellulose.

AX content was least in barley samples (10%) and highest in wheat aleurone (52%) in the extracted NSP. Only NSP with AX content greater than 45% (bran and aleurone) were used for extraction of WEAX. The yield of WEAX from aleurone (1.2%) was twofold higher than those of brans from both soft and hard wheat. In general, 50% of the AX in NSP was recovered as WEAX. Our yield of WEAX was in agreement with earlier reports (Kandil, Li, Vasanthan, Bressler, & Tyler, 2011;

Maes & Delcour, 2002; Ordaz-Ortiz & Saulnier, 2005). The total AX in the recovered WEAX were >70%, calculated as AX% = (% arabinose + % xylose) × 0.88. The WEAX also had significant galactose content (6.6%), signifying traces of arabinogalactan. It also contained some trace amounts of glucose and mannose. The galactose content of WEAX was twice the levels of their respective NSP, whereas mannose content was unaltered. The protein content (≈10%) of WEAX did not vary significantly ($P \leq 0.5$) between aleurone and bran.

The degree of substitution (DS) is often defined by the molar ratio Ar/Xy. Higher DS characterizes a highly branched AX. DS for whole grains (wheat, purple barley, and common barley) did not vary significantly (Table 3.2). However, the DS for wheat fractions varied greatly, ranging from 0.50 (aleurone) to 1.15 (germ). Our finding is consistent with the literature, because WEAX from different wheat fractions were reported to have different DS (Antoine, et al., 2003; Cleemput, et al., 1995; Dervilly, Saulnier, Roger, & Thibault, 2000; Guttieri, Souza, & Sneller, 2008; Izydorczyk & Biliaderis, 1994). The DS for the white and red wheat bran did not vary significantly ($P \leq 0.05$). AX from aleurone have been reported to have less substitution compared with those from bran (Antoine, et al., 2003; Barron, Surget, & Rouau, 2007). A similar trend was observed in our study, in which WEAX from aleurone had a lower DS (0.64) compared with that of wheat brans (>0.70). The DS values for WEAX from both bran samples are within the range reported in the literature (Guttieri, Souza, & Sneller, 2008). It was generally observed that WEAX had a higher DS compared with their corresponding NSP.

3.4.2 Phenolic acid composition

The alkaline extracts of the NSP or WEAX were screened for the presence of nine monomeric phenolic acids (gallic, protocatechuic, *p*-hydrobenzoic, caffeic, vanillic, syringic, ferulic, *p*-coumaric, and sinapic acids) as presented in an HPLC chromatogram (Figure 3.1). The phenolic

acid compositions of NSP and WEAX are presented in Table 3.3. Ferulic acid was the predominant phenolic acid, whereas gallic acid was absent in all samples. Ferulic, vanillic, and *p*-hydroxybenzoic acids were present in all NSP. The total phenolic acid (TPA) was higher in NSP compared with the corresponding WEAX product. Barley had the lowest TPA (<850 μg of TPA/g) compared with whole wheat and wheat fractions (germ, aleurone, and bran), possibly because of low AX content. β-Glucans are not considered to be esterified to phenolic acids (Ahluwalia & Fry, 1986). The composition and levels of phenolic acids were not significantly different between common and purple barley. A similar finding was reported by our laboratory that the difference between TPA of soluble dietary fiber obtained from common and purple barley was insignificant (Guo & Beta, 2013). Soft wheat has been reported to have high phenolic acid content compared with hard wheat (Ragaee, Abdel-Aal, & Noaman, 2006; Ragaee, Guzar, Dhull, & Seetharaman, 2011). A similar trend was observed between NSP obtained from soft wheat and hard wheat in our study, even though the difference was not significant (at $P \leq 0.05$). However, phenolic acid composition and levels varied significantly among wheat fractions. Specifically, NSP from germ and aleurone had significantly high amounts of *p*-hydroxybenzoic acid (>300 μg/g) when compared with bran (≈100 μg/g). Vanillic acid was highest in aleurone (250 μg/g) followed by germ (150 μg/g) and lowest in white bran among the wheat fractions. Similar to NSP, ferulic acid was the predominant phenolic acid in WEAX samples. Phenolic acids are mostly bound to AX; hence, we expected higher TPA or ferulic acid in WEAX (because of its high AX content) compared with NSP. On the contrary, TPA content was significantly low in WEAX compared with NSP in this study.

3.4.3 Total phenolic compounds

The TPC of alkaline extracts of NSP or WEAX is presented in Table 3.4. TPC was at least twofold higher in wheat fractions (≈3,000 μg of FAE/g) compared with all whole grain flours. Among the fractions, TPC of aleurone (5,035 μg of FAE/g) was almost twice that of the two brans in our study. In contrast, TPC did not vary among whole grain cereals. Similarly, alkaline extracts of soluble dietary fiber from barley and wheat were reported to have the same TPC (Guo & Beta, 2013). Similar to TPA, it was also observed that TPC of brans was high in NSP compared with their respective WEAX. In contrast, TPC of WEAX from aleurone did not vary significantly from that of its originating NSP

3.4.4 Antioxidant capacity

3.4.4.1 *Antioxidant capacity of NSP and WEAX*

Antioxidants terminate oxidation reactions in a food matrix or cell by donating an electron or a hydrogen atom. Free radicals or oxidation chain reactions occur in different reaction media, hence the need to use different assays to assess antioxidant potency of compounds. In this present study, antioxidant properties of NSP and WEAX were assessed by using DPPH, $ABTS^{+}$, and ORAC assays. The antioxidant capacities of NSP and WEAX are presented in Table 3.5 in μM Trolox equivalent (TE)/g both of NSP or WEAX and of flour. Both DPPH (Blois, 1958; Brand-Williams, Cuvelier, & Berset, 1995) and $ABTS^{+}$ radical (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999) lose color upon being reduced by an antioxidant. The free radical scavenging ability of NSP or WEAX varied significantly ($P < 0.05$) between samples (Table 3.5). NSP from purple barley exhibited 1.6-fold higher antioxidant activity compared with that of common barley and wheat. Among wheat fractions, the antioxidant capacity was highest in NSP or WEAX from aleurone followed by germ and bran. The ORAC assay is based on the principle that antioxidant

compounds will prevent production of peroxyl radicals. ORAC value is a measure of a compound's ability to delay the loss of fluorescence intensity over time (Cao, Alessio, & Cutler, 1993). Unlike DPPH and ABTS results, NSP from wheat germ had the highest ORAC values compared with all samples, followed by that of wheat aleurone (Table 3.5).

When the assays were considered together, it was observed that antioxidant values obtained for WEAX were at least 20% less than their corresponding NSP (Table 3.5). Chen et al. (2008) and Jiang et al. (2013) also reported that crude polysaccharide extracts had a higher antioxidant capacity compared with purified polysaccharides. This observation may signal possible synergy between heterogeneous polysaccharides present in NSP or loss of other antioxidant compounds during purification (Chen, Xie, Nie, Li, & Wang, 2008). In this case, loss of phenolic acid was evident because WEAX had lower TPC and TPA compared with NSP.

Consumption of a high-fiber diet is associated with reduced risk of colorectal cancer (Ben, Sun, Chai, Qian, Xu, & Yuan, 2014; Peters, Sinha, Chatterjee, Subar, Ziegler, Kulldorff, et al., 2003). The current understanding is that dietary fiber protects against colorectal cancers by limiting carcinogens from contacting the intestinal lining (Kaczmarczyk, Miller, & Freund, 2012) and that its fermentation products (short-chain fatty acids) have anticancer properties (Zeng, Lazarova, & Bordonaro, 2014). Some carcinogens found in the gastrointestinal tract are the free radicals generated in the stomach or taken together with meals (Kanner & Lapidot, 2001).

Presence of antioxidants will thus mitigate the toxicity of free radicals. Our observation that NSP and WEAX are capable of scavenging the free radicals is vital, because not all NSP can easily be fermented by gut flora (Crittenden, Karppinen, Ojanen, Tenkanen, Fagerström, Mättö, et al., 2002; Hughes & Kolida, 2007). We therefore factored in the yield of NSP or WEAX to obtain their

contribution to the overall antioxidant capacity of the tested cereal or cereal fraction. Barley had a higher antioxidant capacity compared with wheat bran or wheat because of its high NSP yield despite the low antioxidant activity of its NSP. Different studies that used methanol or alkaline extracts have also reported high antioxidant capacity in barley compared with wheat (Guo & Beta, 2013; Ragaee, Abdel-Aal, & Noaman, 2006; Serpen, Capuano, Fogliano, & Gökmen, 2007). We also observed that there was no significant difference between DPPH or $ABTS^{+}$ antioxidant activity of common barley and purple barley (≈2.3μM TE/g). Similarly, the antioxidant activity of wheat brans did not vary (≈1.6μM TE/g). Aleurone from wheat had the highest antioxidant capacity (around twofold higher than both normal and purple barley) owing to its high NSP yield. Aleurone also had at least twofold higher antioxidant capacity compared with those of soft white and hard red wheat bran based on WEAX. The total antioxidant capacity of cereals based on alkaline extracts in the literature (Guo & Beta, 2013; Okarter, Liu, Sorrells, & Liu, 2010; Ragaee, Abdel-Aal, & Noaman, 2006) is about 1- to 10-fold high compared with our results upon factoring in yield % irrespective of the assay. Considering that only 5–10% of total phenolic compounds are bioavailable, the estimation of antioxidant capacity of cereal based on water extractable NSP has the advantage that it is bioavailable in the gastrointestinal system, where most radicals are formed (Kanner & Lapidot, 2001).

Even though the relevance of test tube antioxidant assays to human health is debatable, they help to understand the possible reaction mechanism of antioxidants. A stepwise regression model was deployed to better understand the main determinant of the antioxidant behavior of WEAX. Ferulic acid and AX contents were the main determinants ($P < 0.05$ and adjusted $R^2 = 0.92$) in the DPPH antioxidant assay. AX, TPC, and Ar/Xy were the main predictors for ABTS and ORAC antioxidant behavior of WEAX or NSP ($P < 0.05$ and adjusted $R^2 = 0.90$).

3.4.4.2 *Role of monosaccharide composition in antioxidant capacity of WEAX*

Monosaccharide composition and glycosyl linkages of polysaccharide may influence their antioxidant properties (Lo, Chang, Chiu, Tsay, & Jen, 2011). We observed that the antioxidant activity of NSP or WEAX was positively associated with the AX content (R^2 = 0.99). Both uronic acid content and molecular weight of WEAX have been reported to influence their antioxidant capacity (Rao & Muralikrishna, 2006). We did not find any association between antioxidant capacity and uronic acid content (R^2 = 0.20). This variation may be attributed to differences in extraction methods. However, among the constituent sugars of AX (arabinose, xylose, and glucuronic acid), only xylose content influenced the antioxidant activity of the WEAX (R^2 = 0.85). Xylose residues in AX are either unsubstituted or are mono- or disubstituted with arabinose residues (Niño-Medina, Carvajal-Millán, Rascon-Chu, Marquez-Escalante, Guerrero, & Salas-Muñoz, 2010). The DS (Ar/Xy ratio) was negatively associated with antioxidant activity (R^2 = –0.99). These results suggest that the order of antioxidant potency of AX is unsubstituted > monosubstituted > disubstituted. The antioxidant activity of WEAX might therefore be attributed to the free hydroxyl group of unsubstituted or monosubstituted xylose residue. Our results corroborate the finding by (Lo, Chang, Chiu, Tsay, & Jen, 2011) that presence of hydrogen from specific monosaccharides may be responsible for antioxidant capacity of polysaccharides. Because xylose forms the backbone of AX, our result does provide one of many possible explanations for the reported association between antioxidant capacity of FAXn and molecular weight (Rao & Muralikrishna, 2006). On the other hand, DPPH scavenging ability was not influenced by xylose content but rather the ratio of ferulic acid to arabinose (R^2 = 0.90). This discrepancy can be explained by differences in reaction mechanisms among the assays.

3.4.4.3 *Role of phenolic acids in antioxidant capacity of WEAX*

Serpen et al. (2007) found that alkaline hydrolysis of wheat bran fiber reduced its antioxidant capacity by over 70%, suggesting that bound phenolic compounds are responsible for the antioxidant activity. We also observed a positive correlation ($R^2 = 0.62$) between TPA and DPPH or $ABTS^+$ antioxidant activity. There was also a positive correlation ($R^2 = 0.60$) between ferulic acid content and the DPPH or $ABTS^+$ antioxidant activity. However, the low correlation coefficient values (R^2) for TPA and ferulic acid can be attributed to the differences in AX content in our NSP samples. We pooled together NSP samples with the same AX content and determined the correlation between their mean DPPH antioxidant activity and TPA or ferulic acid. The correlation between DPPH or $ABTS^+$ antioxidant activity and TPA was improved to $R^2 \geq 0.73$, and that of DPPH or $ABTS^+$ antioxidant activity and ferulic acid was improved to $R^2 \geq 0.90$. The correlation between DPPH antioxidant activity and other phenolic acids was weak ($R^2 \leq 0.5$). Thus, we concluded that TPA and ferulic acid influenced the antioxidant activity of WEAX and NSP.

The antioxidant capacity of alkaline or methanol extracts from cereals in most cases positively correlates with TPC (Guo & Beta, 2013; Shao, Xu, Sun, Bao, & Beta, 2014). The correlation between TPC and antioxidant capacity in these cases is reasonable enough, because the alkaline extract itself is used for determination of antioxidant capacity. However, in our study we determined the antioxidant capacity of the NSP directly. We also observed a positive correlation between DPPH or $ABTS^+$ scavenging ability and TPC of alkaline extract of NSP ($R^2 = 0.72$). Similar to TPA, the correlation was improved to $R^2 = 0.99$ after pooling together NSP with the same AX content. Our results suggest that phenolic acid contributed to the antioxidant activity of NSP. The role of phenolic acids was further confirmed by our observation that WEAX (characterized by lower TPC, TPA, and ferulic acid content) from aleurone and bran had a lower

antioxidant activity compared with the corresponding NSP (characterized by higher TPC, TPA, and ferulic acid content). In contrast, the antioxidant activity based on the ORAC assay was influenced by TPC (R^2 = 0.61) and *p*-hydroxybenzoic acid content (R^2 = 0.63) but not TPA (R^2 = 0.22) nor ferulic acid (R^2 = 0.16).

3.5 Conclusions

In the present work, NSP and WEAX were shown to exhibit antioxidant activity by using DPPH radical, $ABTS^+$ radical, and ORAC antioxidant assays. Our results demonstrate that AX content, TPC, xylose content, and DS influenced NSP and WEAX antioxidant capacity. This result may suggest that NSP can enhance antioxidant capacity in the food matrix and may help in prevention of colorectal cancers by mitigating the toxicity of free radicals. Thus, it can be proposed that the antioxidant capacity of NSP should be used in conjunction with that of free phenolic acid extracts (not bound) to determine the total antioxidant activity of cereal or cereal food products.

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Table 3.1: Yield (% w/w) of water-extractable nonstarch polysaccharide and arabinoxylan and protein content (% w/w) in 1 g of water-extractable nonstarch polysaccharide and arabinoxylan[z]

Extract, Source	Yield (%)	Protein %
Nonstarch polysaccharide Aleurone (wheat)	4.2	10.9 ± 0.5a
Barley	5.8	0.5 ± 0.0c
Germ (wheat)	1.9	2.9 ± 1.2b
Purple barley	4.0	1.1 ± 0.4c
Red bran (wheat)	2.1	11.5 ± 1.4a
Wheat	1.7	1.3 ± 0.5c
White bran (wheat)	2.2	10.0 ± 0.5a
Water-extractable arabinoxylan Aleurone (wheat)	1.2	10.1 ± 1.5a
Red bran (wheat)	0.5	11.3 ± 1.3a
White bran (wheat)	0.6	9.8 ± 0.5a

[z] Values presented as mean ± standard deviation (n = 2). Data in the same column with the same letter are not significantly different at $P \leq 0.05$. Yield is expressed as (% w/w) of water-extractable nonstarch polysaccharide and arabinoxylan per sample on a dry basis.

Table 3.2: Mean monosaccharide composition (as mole %) of water-extractable nonstarch polysaccharide and arabinoxylan[z]

Extract, Source	Arabinose	Xylose	Galactose	Mannose	Glucose	Glucuronic Acid	Arabinoxylan	Ar/Xy
Nonstarch polysaccharide								
Aleurone (wheat)	19.6 ± 0.2	39.5 ± 1.8	3.6 ± 0.2	1.0 ± 0.1	36.2 ± 1.9	1.6 ± 0.2	52.0 ± 1.2	0.50
Barley	5.1 ± 0.8	6.7 ± 0.3	…	…	92.1 ± 5.5	0.1 ± 0.0	6.9 ± 4.8	0.76
Germ (wheat)	14.9 ± 0.4	12.9 ± 0.2	15.4 ± 0.1	…	56.8 ± 0.3	0.2 ± 0.0	24.5 ± 0.1	1.15
Purple barley	5.9 ± 0.1	5.7 ± 0.2	…	…	88.5 ± 0.2	0.4 ± 0.0	10.1 ± 0.1	1.04
Red bran (wheat)	20.5 ± 0.6	35.1 ± 1.3	5.1 ± 0.1	1.4 ± 0.2	38.0 ± 1.7	0.2 ± 0.1	48.9 ± 4.1	0.58
Wheat	12.5 ± 0.2	12.8 ± 0.0	3.6 ± 0.2	…	71.0 ± 0.4	0.4 ± 0.0	22.3 ± 0.2	0.98
White bran (wheat)	19.6 ± 0.5	30.5 ± 1.4	4.4 ± 0.1	1.5 ± 0.2	44.0 ± 2.0	0.1 ± 0.0	48.1 ± 1.0	0.64
Water-extractable arabinoxylan								
Aleurone (wheat)	35.7 ± 0.5	55.5 ± 1.0	6.6 ± 0.2	0.9 ± 0.9	1.4 ± 0.1	1.5 ± 0.1	80.3 ± 0.7	0.64
Red bran (wheat)	35.3 ± 5.9	48.25 ± 4.8	12.6 ± 1.3	1.9 ± 0.3	2.1 ± 0.3	0.2 ± 0.0	73.5 ± 5.2	0.73
White bran (wheat)	40.9 ± 0.9	44.9 ± 0.7	11.0 ± 0.3	1.2 ± 0.1	2.1 ± 0.1	0.2 ± 0.1	75.5 ± 0.7	0.91

Values presented as mean ± standard deviation (n = 2). Ar/Xy = arabinose-to-xylose ratio. Glucuronic acid is presented as % of nonstarch polysaccharide or arabinoxylan. Arabinoxylan was calculated using the formula (% arabinose + % xylose) × 0.88.

Ellipsis (…) indicates not detected

Table 3.3: Phenolic acid content (μg) in 1 g of water-extractable nonstarch polysaccharide (NSP) and arabinoxylan[z]

Extract, Source	GA	PA	CFA	*p*-HBA	*p*-CA	VNA	FA	SIA	SYA	TPA
NSP								59.1 ± 2.1	90.1 ± 3.5	
Aleurone (wheat)	…	…	56.6 ± 2.5	304.4 ± 73.1a	…	249.6 ± 4.5a	3,724.4 ± 23.8a			4,484.2
Barley	…	…	…	26.1 ± 0.6d	70.6 ± 0.0	26.6 ± 0.6c	592.9 ± 6.5g			716.2
Germ (wheat)	…	62.5 ± 13.7a	…	319.8 ± 104.3a	…	149.5 ± 31.5b	1,196.3 ± 72.3e			1,728.1
Purple barley			…	30.6 ± 6.1d	70.4 ± 3.3	75.7 ± 29.7bc	643.6 ± 78.5g			820.3
Red bran (wheat)	…	53.6 ± 9.6ab	…	101.1 ± 39.8bc	…	108.9 ± 17.8b	1,572.4 ± 297.1cd			1,836.0
Wheat	…	…	…	70.2 ± 12.8c	70.0 ± 0.0	87.2 ± 6.5b	825.9 ± 71.3f		74.0 ± 4.5	1,127.7
White bran (wheat)	…	30.8 ± 9.4b	55.9 ± 10.0	91.6 ± 19.0b	36.4 ± 6.7	80.7 ± 12.9b	1,898.2 ± 505.6b	49.8 ± 0.5	70.4 ± 5.5	2,383.0
WEAX										
Aleurone (wheat)	…	…	…	…	…	…	2,143.9 ± 227.0b	77.9 ± 9.2	38.3 ± 3.3	2,260.7
Red bran (wheat)	…	…	…	…	…	81.0 ± 7.4	1,534.1 ± 97.0cd			1,615.1
White bran (wheat)	…	…	…	…	…	204.9 ± 12.7	1,117.1 ± 83.7e			1,322.0

[z] GA = gallic acid; PA = protocatechuic acid; CFA = caffeic acid; *p*-HBA = *p*-hydrobenzoic acid; *p*-CA = *p*-coumaric acid; VNA = vanillic acid; FA = ferulic acid; SIA = sinapic acid; SYA = syringic acid; TPA = total phenolic acid; and WEAX = water-extractable arabinoxylan. Values presented as mean ± standard deviation ($n = 2$). Data in the same column with the same letter are not significantly different at $P \leq 0.05$. Ellipsis (…) indicates not detected.

Table 3.4: Total phenolic content (μg of ferulic acid equivalent) in 1 g of water-extractable nonstarch polysaccharide and arabinoxylan[z]

Extract, Source	Total Phenolic Content
Nonstarch polysaccharide	
Aleurone (wheat)	5,035.0 ± 657.6a
Barley	1,080.0 ± 205.1e
Purple barley	1,585.0 ± 325.3cde
Red bran (wheat)	2,832.5 ± 279.3bc
Wheat	1,580.0 ± 190.9cde
White bran (wheat)	2,767.5 ± 251.0bcd
Water-extractable arabinoxylan	
Aleurone (wheat)	5,300.0 ± 70.7a
Red bran (wheat)	1,524.5 ± 0.7de
White bran (wheat)	1,620.5 ± 17.7d

[z] Values presented as mean ± standard deviation ($n = 2$). Data in the same column with the same letter are not significantly different at $P \leq 0.05$.

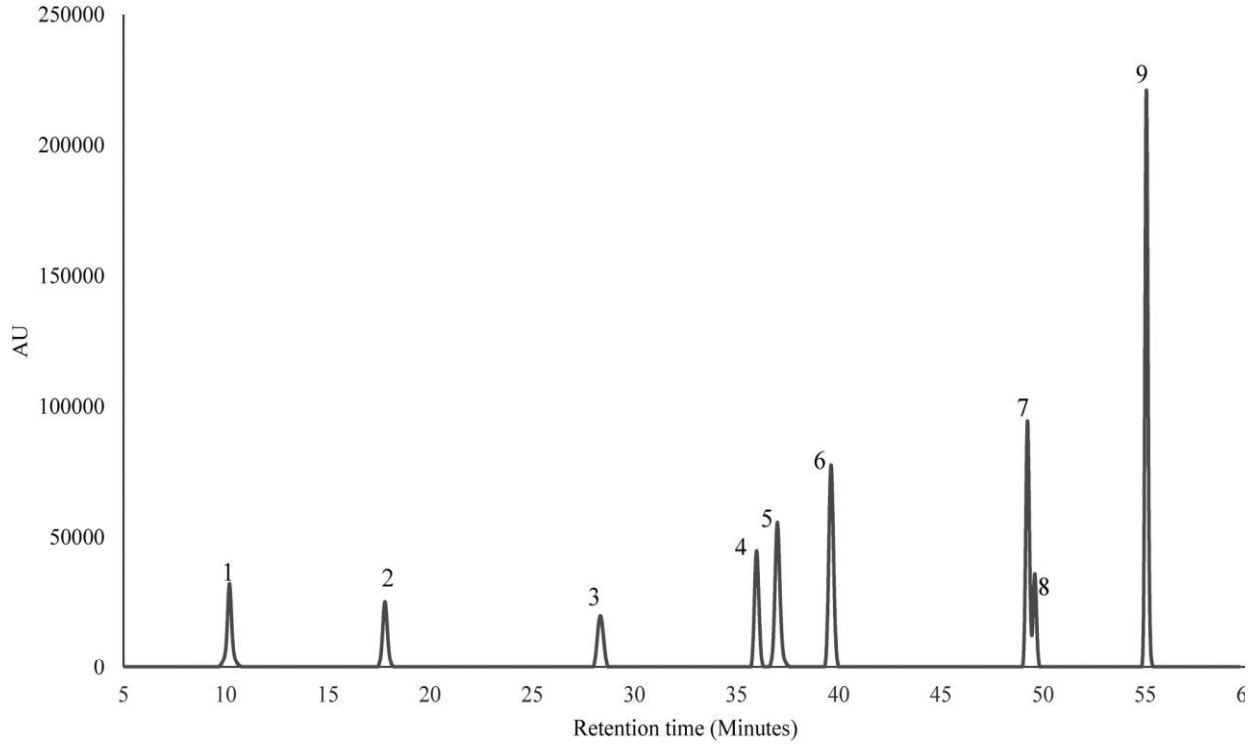
Table 3.5: Antioxidant capacity (μM trolox equivalent [TE]) of 1 g of water-extractable nonstarch polysaccharide (NSP) or water-extractable arabinoxylan (WEAX) or flour[z]

	DPPH μ**M** TE/g		$ABTS^+$ μ**M** TE/g		ORAC μ**M** TE/g	
Source	**NSP**	**Flour**	**NSP**	**Flour**	**NSP**	**Flour**
Aleurone (wheat)	99.1 ± 3.0a	4.2 ± 0.1	121.9 ± 5.7a	5.1 ± 0.2	286.6 ± 1.9c	12.0 ± 0.1
Barley	37.4 ± 1.8e	2.2 ± 0.1	40.0 ± 1.6f	2.3 ± 0.1	140.4 ± 15.6g	8.1 ± 0.1
Corn	59.9 ± 3.7d	0.5 ± 0.0	71.1 ± 6.0d	0.6 ± 0.1	326.9 ± 25.5b	2.9 ± 0.0
Germ (wheat)	81.1 ± 5.2b	1.5 ± 0.1	82.8 ± 8.0cd	1.6 ± 0.1	376.9 ± 22.4ab	7.2 ± 0.1
Purple barley	60.8 ± 3.4d	2.4 ± 0.1	65.7 ± 9.1de	2.6 ± 0.1	246.9 ± 16.8de	9.9 ± 0.1
Red bran (wheat)	69.7 ± 1.1c	1.5 ± 0.0	115.3 ± 2.3ab	2.4 ± 0.0	266.6 ± 7.5d	5.6 ± 0.0
Wheat	24.4 ± 0.5f	0.4 ± 0.0	39.6 ± 1.6f	0.7 ± 0.0	170.1 ± 5.8f	2.9 ± 0.0
White bran (wheat)	87.0 ± 16.1abc	1.9 ± 0.4	108.5 ± 5.3b	2.4 ± 0.4	263 ± 4.8d	5.8 ± 0.4
	WEAX	**Flour**	**WEAX**	**Flour**	**WEAX**	**Flour**
Aleurone (wheat)	84.6 ± 0.3b	1.0 ± 0.0	105.9 ± 3.7bc	1.3 ± 0.0	235.1 ± 0.7e	2.8 ± 0.0
Red bran (wheat)	84.8 ± 2.4b	0.4 ± 0.0	58.4 ± 8.3e	0.3 ± 0.0	110.5 ± 5.8g	0.6 ± 0.0
White bran (wheat)	75.7 ± 8.5bc	0.5 ± 0.1	76.8 ± 22.9cde	0.5 ± 0.1	150.8 ± 36.2fg	0.9 ± 0.1

Values presented as mean ± standard deviation ($n = 2$). Data in the same column with the same letter are not significantly different at $P \leq 0.05$. Antioxidant activity of the flour = yield% × antioxidant capacity of NSP or WEAX. DPPH = 2,2-diphenyl-1-picrylhydrazyl; $ABTS^+$ = 2,2′-azino-bis (3-ethylbenzothiazoline6-sulfonic acid; and ORAC = oxygen radical absorbance capacity

Figure 3.1: HPLC chromatogram indicating the retention times for nine phenolic acid standards.

1 = gallic acid; 2 = protocatechuic acid; 3 = p-hydroxybenzoic acid; 4 = caffeic acid; 5 = vanillic acid; 6 = syringic acid; 7 = ferulic acid; 8 = p-coumaric acid; and 9 = sinapic acid.



CHAPTER 4

4 Effect of Water Extractable Arabinoxylan from Wheat Aleurone and Bran on Lipid Peroxidation and Factors Influencing their Antioxidant Capacity

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4.1 Abstract

Dietary lipid hydroperoxides (LOOH) are implicated in the rise of cancers of the colon. We investigated the effect of water extractable arabinoxylans (WEAX) on lipid peroxidation under simulated gastric conditions. The factors influencing the antioxidant capacity (AOC) of WEAX were also studied. WEAX was isolated from wheat aleurone and wheat bran and fractionated using ammonium sulphate (AS) at 50 and 75% saturation. The concentration of LOOH (121.2 ± 3.8 µM/ g) of grilled chicken breast muscle increased by ~30% under simulated gastric conditions. However, the concentration of LOOH remained unaltered or decreased (≤35%) depending on the type and concentration of WEAX added. The AOC of WEAX based on DPPH, ABTS and ORAC was 28.2 – 147.9, 91.2 – 355.3, and 185.9 – 527.5 µM TE/ g, respectively. Ferulic acid content (R = 0.99) and relative proportions of monosubstituted xylose residues (R = 0.80) but not molecular weight (R=0.20) influenced the AOC of WEAX. The presence of bound ferulic acid is essential for WEAX to exhibit antioxidant properties. Thus, consumption of diets rich in WEAX may be protective against oxidative damage of the gastrointestinal tract.

Key words: Water soluble arabinoxylan, antioxidant capacity, lipid peroxidation, bran, aleurone, feruloylated arabinoxylan

4.2 Introduction

Consumption of fiber from cereal grains is associated with reduced risk of cancers of the colon (Aune, Chan, Lau, Vieira, Greenwood, Kampman, et al., 2011) but the underlying mechanisms remain unclear (Zeng, Lazarova, & Bordonaro, 2014). These anticancer effects of dietary fiber may be derived from a number of possible mechanisms, including promotion of healthy gut microbiota, substrate for increasing short chain fatty acid production, increasing fecal bulk and viscosity, and binding of potential cancer-causing agents (Zeng et al, 2014). Also, studies have shown that populations consuming high fat have high incidences of colorectal and colon cancers (CRC). In general, animal fats, especially from red meat, present a higher risk compared to lipids from plant sources. (Cross, Leitzmann, Gail, Hollenbeck, Schatzkin, & Sinha, 2007; Giovannucci, Rimm, Stampfer, Colditz, Ascherio, & Willett, 1994). Indeed, a meta-analysis study found that high consumption of red meat increases the risk of colorectal and colon cancers (Larsson & Wolk, 2006). Among many exogenous factors, it has been proposed that fat and heme iron in red meat poses a particular risk factor (Santarelli, Pierre, & Corpet, 2008). Hemoglobin or myoglobin is known to promote lipid peroxidation (Kanner & Lapidot, 2001). Both hemoglobin and lipid hydroperoxides (LOOH) are capable of inducing DNA oxidation at high concentration (>100 μM) (Angeli, Garcia, Sena, Freitas, Miyamoto, Medeiros, et al., 2011). Synergistic effects of hemoglobin and lipid hydroperoxides on genotoxicity have also been reported (Angeli, et al., 2011; Kanner & Lapidot, 2001). Heme-iron facilitates degradation of LOOH (Carlsen, Møller, & Skibsted, 2005) to peroxyl and alkoxyl radicals, which may eventually damage the DNA. Thus the presence of antioxidants may mitigate the development or propagation of CRC associated with high fat or red meat diets. Recently, reports have shown that feruloylated arabinoxylan, a major

constituent of cereal dietary fiber, possesses antioxidant properties (Chapter 3; Rao & Muralikrishna, 2006).

Arabinoxylans (AX) constitute the highest proportion of dietary fiber in cereal grains (60 -70%) (Antoine, Peyron, Mabille, Lapierre, Bouchet, Abecassis, et al., 2003) and their content varies with the source or grain fraction. Whole grain wheat contains about 1.3 – 2.7% AX of which 14-30% are water extractable (Gebruers, Dornez, Boros, Fras, Dynkowska, Bedo, et al., 2008). Wheat bran contains 8-18% AX of which 2.2 – 5.5 % are water extractable (WEAX) (Gebruers, et al., 2008). AX content from hand-isolated wheat aleurone and pericarp was 20 and 45 %, respectively (Antoine, et al., 2003). The AX structure consists of (1-4)-β-D-xylopyranose chain with α-L-arabinofuranose residues linked to xyl*p* at O-2 and/or O-3 position (Lequart, Nuzillard, Kurek, & Debeire, 1999). Substitution with α-(1,2)-glucuronic acid and/or α-(1,2)-4-O-methylglucuronic acid linkages have also been reported on the xylan chain (Ma, Jia, Zhu, Li, Peng, & Sun, 2012). Feruloylation may occur at O-5 position of arabinose residues (Bunzel, Ralph, & Steinhart, 2005; Smith & Hartley, 1983). The ratio of arabinose to xylose, pattern of arabinose substitution, degree of feruloylation, and molecular weight of AX vary greatly among and within cereal grains (Izydorczyk & Biliaderis, 1995).

Evidence for the role of water soluble AX in preventing lipid peroxidation under gastric conditions is limited. Hence we isolated WEAX from wheat bran and wheat aleurone to investigate their potency against lipid peroxidation. Moreover, factors affecting the antioxidant capacity of AX are complex owing to the heterogeneity of the AX structure. The antioxidant capacity of WEAX may be dependent on their ferulic acid content, uronic acid content, molecular weight, and degree or pattern of substitution (Chapter 3; Rao & Muralikrishna, 2006). Ferulic acid content did not influence antioxidant capacity of AX extracted from corn (Ayala-Soto, Serna-Saldívar, García-

Lara, & Pérez-Carrillo, 2014). These discrepancies warranted this research to investigate the association of FA content and ability of WEAX to scavenge free radicals.

4.3 Materials and Methods

4.3.1 Sample and chemicals

A commercial wheat aleurone (Grainwise wheat aleurone) was a gift from Cargill Limited and Horizon Milling (Wichita, Kansas, U.S.A.). It constitutes 4.5, 15.2, 7.4, and 2.5% lipid, protein, ash, and starch, respectively (Chen et al. 2013). Hard red winter wheat bran was purchased locally from Bulk Barn (Winnipeg, Manitoba, Canada). Its moisture, ash and protein content were analyzed to be 5.8, 5.3, and 11.1%, respectively. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (trolox), porcine pancreas α-amylase (EC 3.2.1.1, Type I-A), ferulic acid standard, sugar standards (D-glucose, D-xylose, D-arabinose, D-mannose, D-glucouronic acid and D-galactose) and dialysis tubing cellulose (molecular weight cut off (MWCO) 12000), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein, and fuller's earth were bought from Sigma-Aldrich ((Milwaukee, WI, USA). Ammonium sulphate, all acids and organic solvents were bought from Fischer Scientific (Whitby, Ontario, Canada). All chemicals used were of analytical or HPLC grade.

4.3.2 Preparation of water extractable arabinoxylan from wheat bran and aleurone

Wheat bran and wheat aleurone samples (~200 g) were boiled under reflux in 2 L aqueous ethanol (80%, v/v) at 85 °C for 2 hours to inactivate endogenous enzymes. Samples were cooled to room temperature and centrifuged (10 000 g, 4 ^{o}C and 20 minutes). The residue was washed twice with 500 mL 95% ethanol and the pooled supernatants were discarded. The residue was air dried in a fume-hood overnight at room temperature.

WEAX were isolated at 45°C according to the method described by Izydorczyk and Biliaderis (1994) with some modifications. The air dried bran or aleurone (150 g) were mixed with 1.5 L deionised water. The mixture was stirred overnight and centrifuged (12000 g, 4°C) for 20 minutes. The residue was washed twice with deionized water and the supernatant pooled together. The supernatant was boiled at 95°C for 15 minutes to denature soluble proteins. After centrifugation, the supernatant was mixed with acid washed celite (10g /L) for 20 minutes to remove proteins followed by centrifugation. Residual proteins were further removed by mixing the supernatant with Fuller's earth (20 g / L) for 20 minutes. The mixture (celite or Fuller's earth) was stirred slowly using a magnetic stirrer at room temperature to facilitate protein adsorption. The mixture was then centrifuged (12000 g, 4°C) for 20 minutes. The supernatants were mixed overnight with α-amylase (1821 U/L) to remove starch. The sample was heated to 95°C and further purified using celite and Fuller's earth as before. The purified material was fractionated by graded ammonium sulphate (AS) method. Three fractions were obtained at 50, 75 and 100 % AS saturation. The sample was finally dialyzed (12 kDa cut-off membrane) for 48 hours and freeze dried. The material collected at 100 % AS saturation was discarded as it did not contain significant amounts of AX. The materials collected from wheat aleurone were labelled (WA) followed by the concentration of AS at which they were obtained (WA50 or WA75). Similarly, the extracted materials from the wheat bran (WB) were denoted as WB50 or WB75.

4.3.3 Characterization of the water extractable arabinoxylan extracts

Protein content (% N X 6.25) was determined by method 46-30.01 (AACC International 1999). Monosaccharide composition was determined by gas liquid chromatography as described by Izydorczyk et al. (2014). Molecular weight of isolated WEAX was determined using a high-performance size-exclusion chromatography system using a method described by Irakli et al.

(2004). The samples were dissolved in $NaNO_3$ buffer (1.5 mg/mL). A Shodex column (806, 804) and $NaNO_3$ buffer were used at 30 °C. ^1H NMR spectroscopy was performed on a Bruker Avance 500 III HD spectrometer operated at 500.13 MHz and 20 °C. Samples (5 mg) were dissolved in 1 mL D_2O.

4.3.4 Prevention of lipid hydroperoxides formation in simulated gastric digestion

A Kanner and Lapidot (2001) method for generation of lipid peroxide in the stomach was used with modifications. WEAX was dissolved in simulated gastric fluid (SGF). Four concentrations (0, 0.25, 0.5 and 0.75 mg/ mL) of WEAX were used. Grilled lean chicken breast (30 g) was blended in 100 ml SGF (ice cold) using a kitchen blender at maximum speed for 2 minutes. Meat suspension (20 mL) was transferred into four conical flasks with a screw cap (50 ml). The suspension was incubated in a shaking water bath for up to 2 hrs at 37.0°C and 250 rpm. Samples were drawn at 0, 40, 80 and 120 minutes for determination of lipid hydroperoxides. Lipid hydroperoxides were determined by FOX2 assay with some modifications. Samples (1mL) (6 times) were transferred in 30 ml test tube covered in an aluminum foil. Triphenylphosphine (TPP) (1 mL) and 0.5 mL acidified methanol was added to 3 of the 6 test tubes. To the remaining test tubes, 1 ml of methanol was added instead of TPP. The mixture was incubated at room temperature with periodic mixing (vortex) for 30 minutes. FOX 2 reagent (22.5 mL) was added, followed by incubation for 30 minutes at room temperature and darkness. It was later centrifuged for 10 minutes at 15000 x g and 25 °C. The supernatant (2 mL) was used for absorbance measurement (Ultrospec 1100 Pro, UV/Visible Spectrophotometer (Biomicron Ltd, Cambridge, CB4QFJ, England)) at 560 nm. The experiment was repeated 3 times for each sample.

4.3.5 Determination of antioxidant activity using DPPH, ORAC, and ABTS assays

Antioxidant capacity assays for DPPH, $ABTS^{+}$ and ORAC were performed according as described in Chapter 3.

The effect of ferulic acid on the antioxidant activity of WEAX was done using two approaches. In the first approach, samples were saponified in 2M NaOH under nitrogen at 4 °C. After 4 hours, samples were neutralized using 6M HCl. Ethanol (4 volumes) was added to precipitate AXs. After centrifugation, the residue was washed twice with 95% ethanol. The dried residue was used for DPPH, ABTS and ORAC assays as described before. In the second approach, samples (3.0 mg/ mL sodium phosphate buffer (100 mM, pH 6.5)) were treated with endoxylanase (15 units) for 2 hours at 40 °C. The aliquots were used for the DPPH antioxidant assay. No xylanase was added to control samples.

4.3.6 Statistical analysis

All analyses were performed in triplicate (unless indicated otherwise) and all statistics were calculated using one way analysis of variance (ANOVA) on a JMP 10 statistical software (SAS Institute Inc., Cary, NC). Sample means were compared using Tukey HSD method and significant differences determined at $p \leq 0.05$. Correlations between parameters were done using Pearson's correlation test.

4.4 Results and Discussion

4.4.1 Composition of water extractable preparations from wheat bran and wheat aleurone

The combined yield of WEAX material for wheat aleurone (0.77%) was slightly higher compared to wheat bran (0.66%). However, factoring in the AX content in water extractable preparation (Table 4.1), the yield was almost twice in wheat aleurone (0.6%) compared to that of wheat bran

(0.35 %). The AX content was high in aleurone fractions (>70%) and relatively low in wheat bran material. The material from bran contained considerably higher levels of beta glucans reflected by high proportions of glucose. β-glucan analysis revealed that wheat bran isolated WEAX material contained about 14% β-glucans whereas aleurone contained trace amounts.

Fractionation with AS at 50% and 75% saturation resulted into two substantial WEAX material for each sample. WA50 and WB50 constituted 42% and 28%, respectively, of the total recovered material. The arabinose to xylose (Ar/Xy) ratio is the measure of degree of substitution (DS). As expected, the bran WEAX material had higher Ar/Xy ratio compared to the aleurone obtained at the same AS concentration (Table 4.4). The Ar/Xy decreased with increase in AS concentration in both aleurone and bran fractions. Our observed precipitation pattern is similar to that reported for barley malt WEAX (Cyran, Izydorczyk, & MacGregor, 2002). However, our results are in contrast to those observed in WEAX from the wheat endosperm (Izydorczyk & Biliaderis, 1992) and rye (Vinkx, Reynaert, Grobet, & Delcour, 1993). Our results suggest that Ar/Xy ratio alone does not influence the precipitation behavior of AX. For example, WA50 with Ar/Xy of 0.58 precipitated at 50% AS saturation while WB75 with similar Ar/Xy precipitated at 75%.

The phenolic acid content of WEAX material (Table 4.2) was higher for the material collected at 50% AS saturation compared to those at 75%. Also, aleurone material had higher phenolic acid content compared to those from the bran. The phenolic acids present were trans-FA, cis-FA, di-FA, sinapic acid, and p-coumaric acid. Phenolic acids are mostly bound to AX and not to beta-glucans (Ahluwalia & Fry, 1986). As expected, trans-FA was the most abundant in all AX material.

4.4.2 Molecular weight determination

Figure 4.1 shows that the molecular weight distribution varied greatly within and between samples. The chromatogram for WA50 and WA75 (Figure 4.1a) showed one major peak eluting between 25 – 40 minutes, whereas WB50 and WB75 (Figure 4.1b) chromatograms suggest that both contain two molecular populations given the two distinct peaks. The second peak in the chromatograms for WB50 and WB70 could be beta-glucans or proteins which were high in these fractions. The mean molecular weight (Table 4.3) ranged from 468 to 677 kDa with WA75 and WB75 being the highest and lowest, respectively.

4.4.3 ^{1}H NMR analysis

The ^{1}H NMR spectra (Figure 4.2a-d) showed 3 peaks observed in arabinose anomeric proton region (5.10 – 5.45). According to the literature (Roels, Collado, Loosveld, Grobet, & Delcour, 1999), the anomeric protons depicted at the first peak (δ 5.35 ppm) were assigned to (1→ 3) - α-arabinofuranosyl (α-L-Araf*)* substituent linked to a monosubstituted β-D-xylopyranosyl (β-D-xyl*p*) residue. The signals on δ 5.23 and 5.18 ppm originated from anomeric protons of α-L-Araf which are linked to (1→ 3) and (1→ 2) position, respectively, of the same β-D-xyl*p* residue (disubstitution) (Roels, Collado, Loosveld, Grobet, & Delcour, 1999).

Table 4.4 shows the relative percentage of xylose residues of the isolated WEAX that are unsubstituted, monosubstituted and disubstituted. The percentages of un- or di- or monosubstituted β-D-xyl*p* residues in the WEAX were obtained by combining data from gas chromatography and H NMR (Roels, Collado, Loosveld, Grobet, & Delcour, 1999). Our results indicate that most of the xylose units were unsubstituted in all fractions followed by disubstituted β-D-xyl*p* residues. On average WEAX fractions isolated at 50% AS saturation had higher levels of disubstituted xylose but less unsubstituted xylose compared to their respective fractions

obtained at 75% AS saturation. Wheat bran fractions had on average 1.4 times more disubstituted β-D-xyl*p* residues compared to aleurone WEAX at the same AS saturation. However, the percent of xylose being un- or di- or monosubstituted did not influence the precipitation behavior. WA50 and WB75 had the same percentage distribution of un- or di- or monosubstituted xylose as an example. The percentage of disubstituted xylose positively correlated with Ar/Xy ratio (R^2 =0.99). This observation is similar to that reported elsewhere in the literature for wheat AXs (Roels, Collado, Loosveld, Grobet, & Delcour, 1999). The ratio of disubstituted β-D-xyl*p* residues to that of monosubstituted was conserved in aleurone samples (~0.9) and bran samples (1.2) in agreement with other reports for wheat WEAX (Rondeau-Mouro, Ying, Ruellet, & Saulnier, 2011).

4.4.4 Prevention of lipid hydroperoxides formation

Lipid peroxides are a major source of dietary oxidants and purported to be involved in the development or propagation of CRC and heart related chronic illnesses. The concentration of LOOH in grilled chicken breast muscle was 121.2 ± 3.8 μM/ g. The level of LOOH in grilled chicken breast steadily increased under the simulated gastric conditions (Figure 4.3). A linear increase was observed during the first 80 minutes of incubation suggesting a propagation stage. Kanar and Lapidot (2001) described the stomach as "bioreactor" that promotes lipid peroxidation. The presence of myoglobin and oxygen under low pH promotes the propagation of lipid hydroperoxides. Metmyoglobin catalyses hydrolysis of lipid hydroperoxides to free radicals (ferrly myoglobin ($MbFe^{4+}$-OH) or deoxymyglobin ($MbFe^{2+}$)). These free radicals eventually promote further lipid peroxidation. An equilibrium was reached after a ~30% increase in LOOH. In red meats, equilibrium can be attained after 6-fold increase in LOOH due to high levels of hemoglobin (Kanner & Lapidot, 2001). Addition of WEAX material to the grilled chicken breast effectively inhibited lipid peroxidation under gastric conditions (Figure 4.3). Thus the presence of antioxidant

material mitigated lipid peroxidation by donating hydrogen atoms to free radicals. WEAX fractions obtained at 50% AS saturation were more potent inhibitors compared to those precipitating at 75%. Comparatively, WA50 reduced the concentration of lipid hydroperoxides the most and WB75 the least at the same WEAX concentration likely due to the heterogeneous nature of AX polysaccharide.

Antioxidant mechanism against free radical may involve chain- breaking or quenching chain-initiating compounds. Figure 4.3 suggests that WEAX at low concentration (0.25 mg/ mL) are capable of breaking chain reaction (lipid peroxidation). Consequently, the initial concentration of LOOH remained relatively unaltered during the first 60 minutes in the presence of WEAX. LOOH degradation results in cytotoxic compounds (peroxyl or alkoxyl radicals) which are capable of oxidizing DNA (Angeli, et al., 2011; Carlsen, Møller, & Skibsted, 2005). Also, Figure 4.3 shows that WEAX at higher concentration are capable of quenching LOOH. Thus consumption of dietary fiber rich feruloylated AX may be beneficial towards lipid hydroperoxides associated health problems.

4.4.5 Antioxidant capacity of water extractable arabinoxylans

The results on antioxidant capacity of the WEAX material are presented in Table 4.5 in μM Trolox equivalent (TE) /g extract. DPPH assay is a good model for electron transfer, whereas ORAC assay is a good model for hydrogen atom transfer for neutralizing free radicals. Our results show that all WEAX fractions used were effective electron and hydrogen atom donors to neutralize free radicals. We also observed a direct positive correlation ($R \geq 0.98$) between DPPH, ABTS and ORAC assays. Similar to simulated gastric digestion of chicken breast, samples obtained at 50% AS saturation had a higher capacity to scavenge free radicals compared to WEAX material collected at 75% AS saturations. WEAX material from the aleurone had a relatively higher antioxidant capacity than that of wheat bran at the same AS saturation. FA content, degree of substitution, uronic acid content, molecular weight and pattern of substitution in WEAX are

thought to influence the antioxidant behavior of AX (Chapter 3; Rao & Muralikrishna, 2006). Table 4.6 suggests that FA and pattern of substitution may have influenced the antioxidant capacity.

FA positively influenced the antioxidant capacity of the extracts when data sets were considered together (R= 0.98). Evidently enough, WEAX material collected at 50% AS saturation had higher FA content and antioxidant capacity compared to WA75 or WB75. In contrast, WEAX from rice with lower FA had higher antioxidant capacity compared to those with higher FA content (Rao & Muralikrishna, 2006). Moreover, FA did not influence the order of antioxidant capacity of AX extracted from corn (Ayala-Soto, Serna-Saldívar, García-Lara, & Pérez-Carrillo, 2014). Thus the antioxidant capacity of WEAX fractions was assayed after removing their bound FA. The deferuloylated samples did not show any antioxidant activity. The same observation was made when the antioxidant capacity of wheat AX (free of bound FA) from Megazyme was measured. Thus it was evident that the antioxidant capacity of AX was dependent on the presence of bound FA. A positive association was observed between the antioxidant activity and FA content of AX oligosaccharides (Chapter 5). Also, feruloylated AX oligosaccharides have a higher antioxidant capacity compared to FA at the same FA molar concentration (Ohta, Semboku, Kuchii, Egashira, & Sanada, 1997). This is possibly due to the low solubility of phenolic compounds under physiological conditions (Kanner & Lapidot, 2001). The solubility of FA is increased from 0.6 mg/mL (Mota, Queimada, Pinho, & Macedo, 2008) to 10 mg/mL (Fang, Wang, Chang, Hu, Hwang, Fu, et al., 2013) when esterified to arabinose. All the AX material used were water soluble and both ABTS and ORAC antioxidant assays were performed in aqueous medium. Thus, one would expect that AX with high FA to exhibit high antioxidant activity.

However, AX polysaccharides may crosslink in the presence of free radical generating oxidants in a phenomenon called oxidative gelation (Izydorczyk, Biliaderis, & Bushuk, 1990). During the first

stage of oxidative gelation, FAs from adjacent AX are covalently bound to form a 3 dimensional structure (Hoseney & Faubion, 1981; Izydorczyk, Biliaderis, & Bushuk, 1990). The gelation potential of AX may be affected by the density of FA in the AX (Dervilly-Pinel, Rimsten, Saulnier, Andersson, & Åman, 2001). Thus, it is possible that WEAX fraction with high FA content (WA50 and WB50) may be oxidatively coupled with the presence of DPPH or ABTS reagent thereby reducing their potential to scavenge free radicals. However, oxidative gelation was not observed during the experiments possibly due to low levels of free radical generating oxidants.

AX with high molecular weight have been reported to have high antioxidant capacity (Rao & Muralikrishna, 2006). In this study, molecular weight of WEAX material did not influence their antioxidant activity, partly because of heterogeneity of the material used. Therefore, the WEAX material was treated with endoxylanase to reduce their molecular weights in order to study the effect of molecular weight on antioxidant activity. The addition of xylanase doubled the antioxidant activity of WA75 and WB75 but did not change that of WA50 and WB50 (Figure 4.4). This observation suggested that at the same FA concentration, AX with lower molecular weight are likely to have higher antioxidant potential compared to those with higher molecular weight. The increase in antioxidant capacity of WB75 and WA75 may have been caused by improved solubility due to decreased molecular weight.

Presence of mono- or disubstituted xylose offers steric hindrance to intermolecular crosslink formation (Izydorczyk & Biliaderis, 1995). Hence, AX with a higher degree of substitution tends to have higher antioxidant activity at the same FA molar concentration (Chapter 4). The relative % of mono-Xyl$_p$ positively influenced the antioxidant capacity of WEAX material ($R \geq 0.79$). In particular, (1→ 2) - α-L-Ara*f* linked to a monosubstituted β-D-xyl*p* monosubstitution at C-2 was a major determinant ($R \geq 0.90$) compared to monosubstitution at (1→ 3) - α-L-Ara*f* linked to a

monosubstituted β-D-xyl*p* (R ≤0. 54). This is possibly due to the fact that most FA are attached to arabinose residues linked at C-2 of xylose residue (Saulnier, Vigouroux and Thibault, 1995; Smith and Hartely, 1983). A positive association (R = 0.99) was also observed between the total phenolic acids and arabinose residues linked at the C-2 position of xylose. Therefore, the pattern of arabinose substitution may influence the antioxidant activity of AX.

4.5 Conclusion

Our results indicated that WEAX are capable of breaking the lipid peroxidation progression or direct quenching of the lipid hydroperoxides under gastric condition. The antioxidant capacity of WEAX is dependent on FA content. However, the antioxidant capacity of AX may also be influenced by patterns of xylose substitution, and molecular weight of the AX polymer. Thus, consumption of a diet rich in water extractable arabinoxylan may be protective against oxidative damage of the gastrointestinal tract.

4.6 Acknowledgement

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) through the Discovery Grant program. We are also grateful to Cargill Limited for provision of wheat aleurone samples and Ce Zhou, Alison Ser and Pat Kenyon of the Department of Food Science, University of Manitoba for their technical support.

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Table 4.1: Chemical composition of water extractable arabinoxylan from wheat aleurone (WA) and wheat bran (WB)

Water-extractable Fraction	Fraction Yield (%)	Proteins (%w/w)	**Monosaccharide Composition (% Mole of neutral carbohydrates (CHO))**							Total Neutral CHO (%w/w)	AX (%w/w)	β-Glucans (%w/w)
			Ribose	**Arabinose**	**Xylose**	**Mannose**	**Galactose**	**Glucose**	**Ara+Xyl**			
Wheat aleurone												
WA50	0.35	8.7±0.2	0.14±0.00	35.09±0.05	60.24±0.02	0.19±0.00	0.83±0.00	3.50±0.08	95.3	86.3±0.7	74.0	0.4
WA75	0.42	8.6±0.2	0.06±0.01	29.33±0.10	66.65±0.15	0.10±0.00	0.68±0.01	3.17±0.05	95.9	97.2±1.1	83.9	0.7
Wheat bran												
WB50	0.26	16.9±0.3	0.97±0.00	34.30±0.22	40.50±0.09	0.39±0.00	1.65±0.01	22.18±0.12	74.8	61.7±0.6	41.5	12.5
WB75	0.40	11.3±0.3	0.08±0.00	28.70±0.05	51.48±0.15	0.17±0.00	1.13±0.01	18.45±0.20	80.2	92.3±1.4	66.6	15.7

Values presented as mean ± standard deviation (n = 3). CHO: carbohydrates (sum of all monosaccharides), Arabinoxylan (AX): sum of Arabinose (Ar) and Xylose (Xy). WA50 and WA75: WEAX fraction from WA obtained at 50 and 75% ammonium sulphate saturation, respectively. WB50 and WB75: WEAX from WB obtained at 50 and 75% ammonium sulphate saturation, respectively.

Table 4.2: Uronic acid and ferulic acid content (% w/w) of water soluble arabinoxylans from wheat aleurone (WA) and wheat bran (WB)

	Phenolic Acids (mg/g)							Uronic Acid
Water-extractable Fraction	p-Coumaric	Sinapic	cis-Ferulic	trans-Ferulic	**Total Ferulic**	***di-Ferulic***	**Total Phenolics**	**(mg/g)**
Wheat aleurone								
WA50	0.19±0.01	0.72±0.07	1.61±0.04	24.40±0.30	**26.01±0.40**	0.51±0.02	**27.43**	0.40±0.01
WA75	0.05±0.01	0.36±0.01	0.42±0.10	6.11±0.10	**6.53±0.20**	0.26±0.01	**6.94**	0.50±0.01
Wheat bran								
WB50	0.08±0.01	1.41±0.04	1.30±0.01	15.48±0.33	**16.78±0.35**	0.28±0.02	**18.27**	0.80±0.01
WB75	0.02±0.01	0.49±0.05	0.48±0.07	3.86±0.04	**4.34±0.11**	0.10±0.02	**4.85**	1.00±0.01

Values presented as mean ± standard deviation (n = 3). WA50 = AX from WA obtained at 50% ammonium sulphate saturation. WA75= AX from WA obtained at 75% ammonium sulphate saturation. WB50= AX from WB obtained at 50% ammonium sulphate saturation WB75= AX from WB obtained at 75% ammonium sulphate saturation

Table 4.3: Molecular weight distribution of water soluble arabinoxylans material from wheat aleurone (WA) and wheat bran (WB)

Water-extractable Fraction *Wheat aleurone*	**Peak molecular weight M*p***	**Weight average molecular weight M*w***	**Radius of gyration R*w* (nm)**	**Intrinsic Viscosity (mL/g)**	**Relative %**
	Peak (RT 25-40 min)				
WA50	144,000	551,000	44	157	100
WA75	141,000	677,000	48	142	100

Water-extractable Fraction *Wheat bran*	**Peak molecular weight M*p***	**Weight average molecular weight M*w***	**Radius of gyration R*w* (nm)**	**Intrinsic Viscosity (mL/g)**	**Relative %**	**Peak molecular weight M*p***	**Weight average molecular weight M*w***	**Radius of gyration R*w* (nm)**	**Intrinsic Viscosity (mL/g)**	**Relative %**
	Peak I (RT 25-35.7 min)					***Peak II (RT 35.7-40.0 min)***				
WB50	105,000	643,000	39	159	71	52,000	62,000	36	11.7	29
	Peak I (RT 25-35.7 min)					***Peak II (RT 33.5-40.0 min)***				
WB75	86,000	468,000	31	212	51	28,000	30,000	25	34.7	49

WA50 = AX from WA obtained at 50% ammonium sulphate saturation. WA75= AX from WA obtained at 75% ammonium sulphate saturation. WB50= AX from WB obtained at 50% ammonium sulphate saturation WB75= AX from WB obtained at 75% ammonium sulphate saturation

Table 4.4: Relative distribution (%) of un-, mono-, and disubstituted xylose in water soluble arabinoxylans from wheat aleurone (WA) and wheat bran (WB) determined by liquid-state ^{1}H NMR spectroscopy

Water-extractable Fraction	**Ara/Xyl Ratio**	**Unsub- Xyl*p* (%)**	Mono-Xyl*p* at C-2 (%)	Mono-Xyl*p* at C-3 (%)	**Total Mono-Xyl*p* (%)**	**Di-Xyl*p* (%)**
Wheat aleurone						
WA50	0.58	61.0	3.2	16.8	20.1	19.0
WA75	0.44	70.0	0.2	15.9	16.1	14.0
Wheat bran						
WB50	0.85	45.1	1.4	23.4	24.8	30.1
WB75	0.56	63.8	0.1	16.4	16.5	19.8

un-Xyl*p:* unsubstituted xylose residues, mono-Xyl*p:* O-3 monosubstituted xylose residue, di-Xyl*p:* O-2 and O-3 disubstituted xylose residues WA50: arabinoxylan (AX) fraction from WA obtained at 50% ammonium sulphate saturation, WA75: AX from WA obtained at 75% ammonium sulphate saturation, WB50: AX from WB obtained at 50% ammonium sulphate saturation, WB75: AX from WB obtained at 75% ammonium sulphate saturation.

Table 4.5: Antioxidant capacity (µM Trolox equivalent) of 1g of water extractable arabinoxylan (AX) from wheat aleurone (WA) or wheat bran (WB)

	DPPH	$ABTS^{+}$	ORAC
Wheat aleurone			
WA 50	147.85 ± 8.54a	355.26 ± 0.01a	527.47 ± 13.21a
WA 75	49.38 ± 8.86c	157.60 ± 9.33c	255.85 ± 14.37c
Wheat bran			
WB 50	106.29 ± 12.13b	320.40 ± 21.06b	484.91 ± 34.15b
WB 75	28.21 ± 11.16c	91.20 ± 8.96d	185.86 ± 11.06d

Values presented as mean ± standard deviation (n = 6). Data in the same column with the same superscript are not significantly different at $p \leq 0.05$. WA50 and WA75: WEAX fraction from WA obtained at 50 and 75% ammonium sulphate saturation, respectively. WB50 and WB75: WEAX from WB obtained at 50 and 75% ammonium sulphate saturation, respectively.

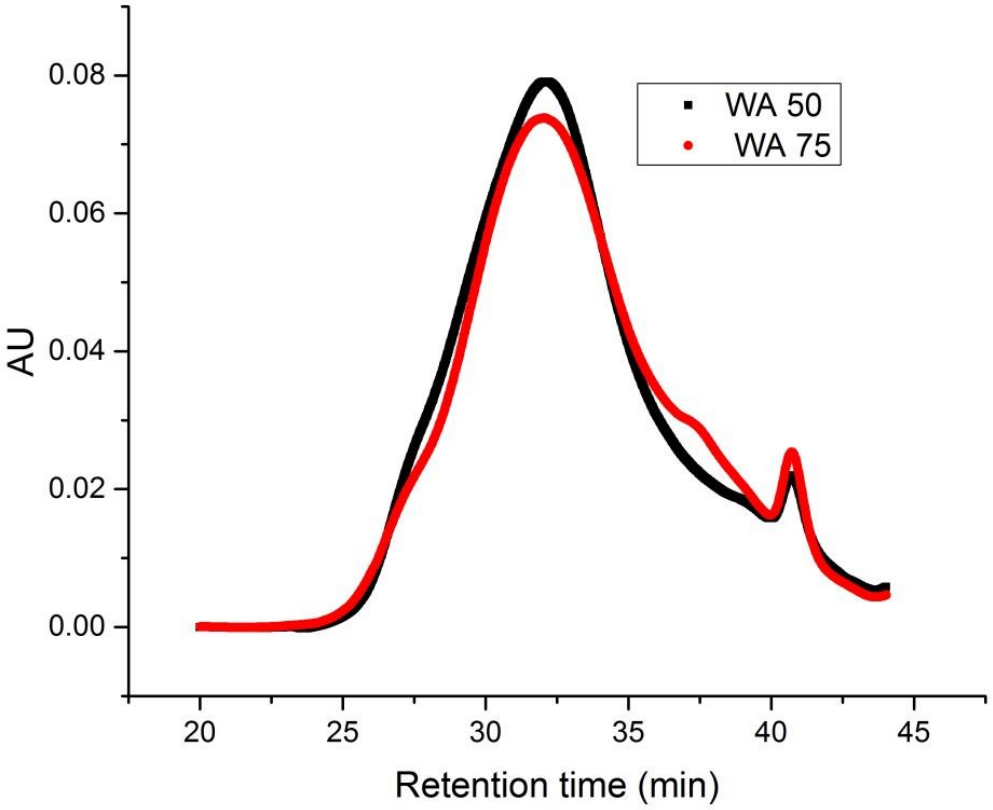
Table 4.6: Correlation coefficient of arabinoxylan antioxidant capacity and its structural properties

	DPPH	$ABTS^{+}$	ORAC
Total phenolic acid	0.996868	0.96392	0.965963
Ferulic acid	0.990216	0.941102	0.942794
Un-Xyl*p*	-0.4775	-0.61216	-0.63506
Mono-Xyl*p* at C-2	0.969067	0.897907	0.900422
Mono-Xyl*p* at C-3	0.357368	0.5282	0.543498
Total Mono-Xyl*p*	0.668889	0.79123	0.8055
Di-Xyl*p*	0.347796	0.484266	0.511684
Molecular weight	0.160053	0.294132	0.259657
Uronic acid	0.289958	0.429984	0.485296

Data represent Pearson correlation coefficient values at $p \leq 0.05$. un-Xyl*p:* unsubstituted xylose residues, mono-Xyl*p:* monosubstituted xylose residue, di-Xyl*p:* O-2 and O-3 disubstituted xylose residues. WA50: AX fraction from WA obtained at 50% ammonium sulphate saturation, WA75: AX from WA obtained at 75% ammonium sulphate saturation, WB50: AX from WB obtained at 50% ammonium sulphate saturation, WB75: AX from WB obtained at 75% ammonium sulphate saturation.

Figure 4.1: Size exclusion chromatograms for water extractable arabinoxylans (WEAX). WA50 and WA75: WEAX fraction from WA obtained at 50 and 75% ammonium sulphate saturation, respectively. WB50 and WB75: WEAX from WB obtained at 50 and 75% ammonium sulphate saturation, respectively.

A



B

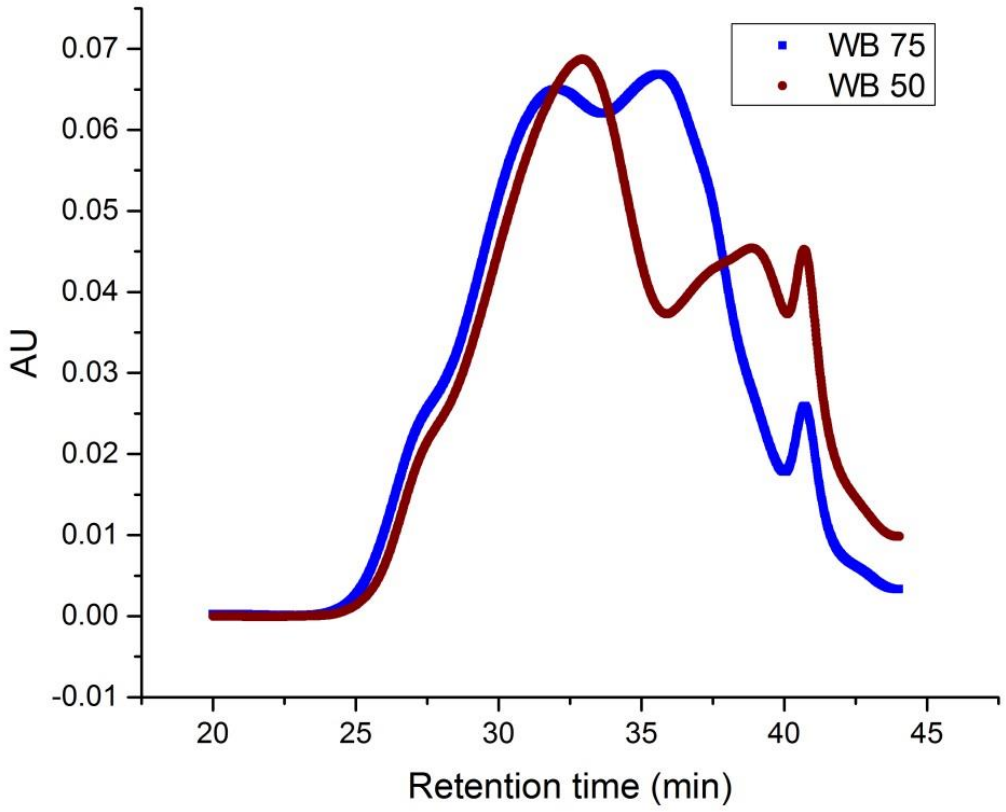
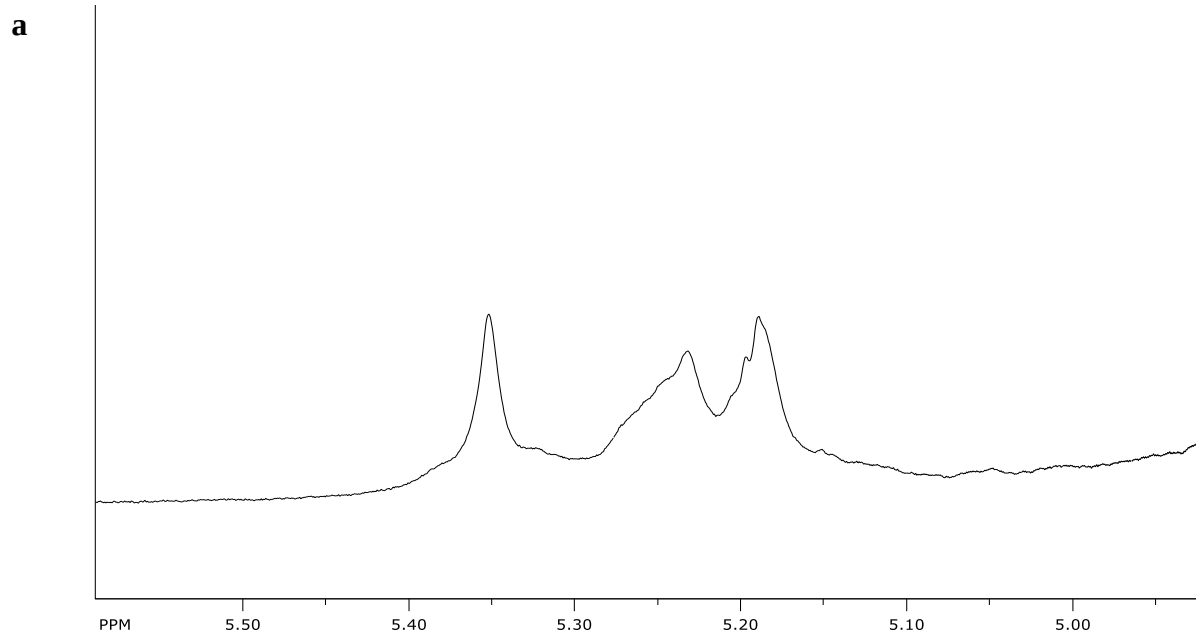
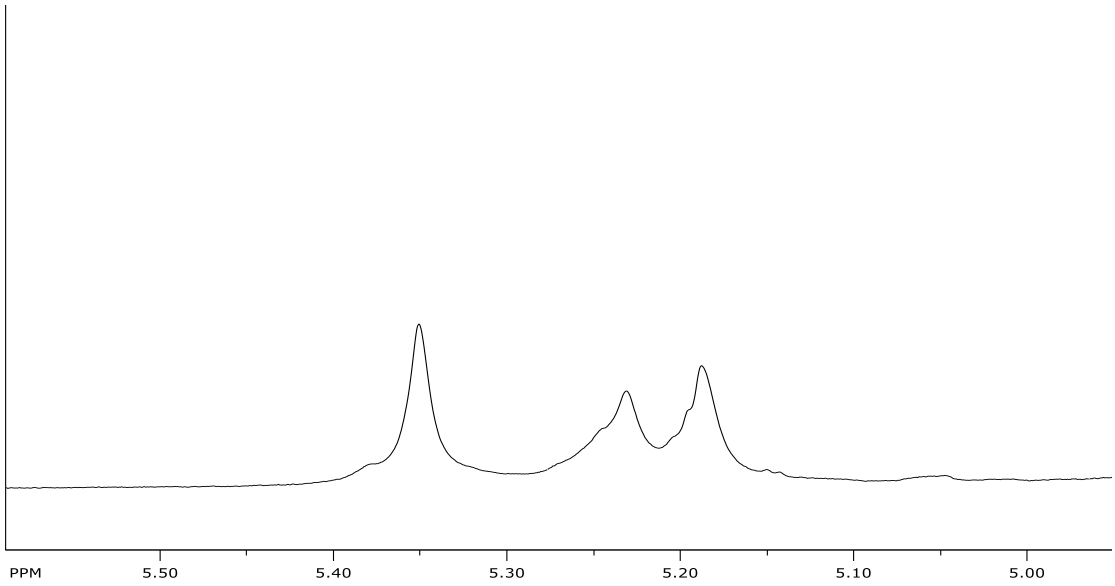

WB 75
WB 50
AU
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
20
25
30
35
40
45
Retention time (min)

Figure 4.2: ^{1}H NMR spectra of water extractable arabinoxylans dissolved in D2O at 20 °C.

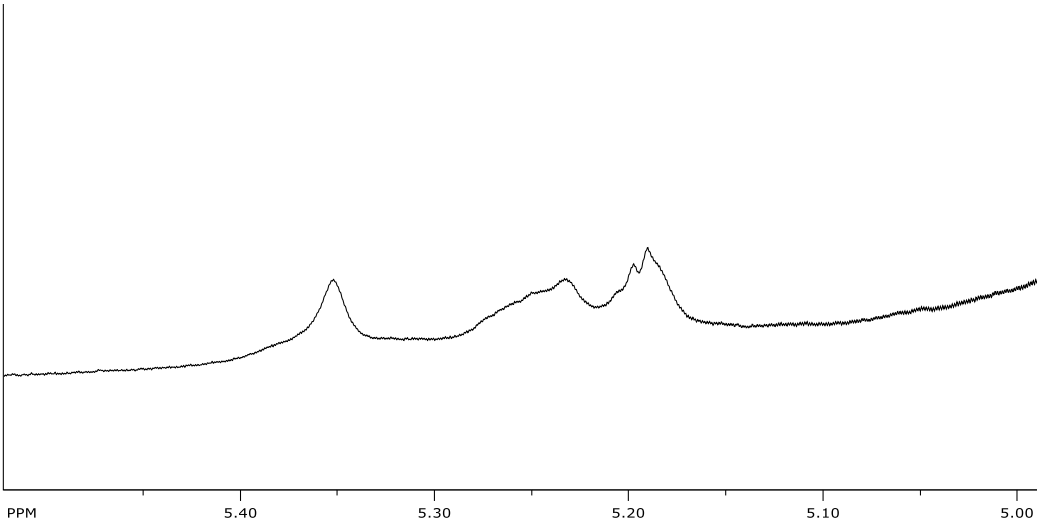
(a) WA50: WEAX fraction from WA obtained at 50% ammonium sulphate saturation, (b) WA75: WEAX from WA obtained at 75% ammonium sulphate saturation, (c) WB50: WEAX from WB obtained at 50% ammonium sulphate saturation, and (d) WB75: WEAX from WB obtained at 75% ammonium sulphate saturation.



b



c



d

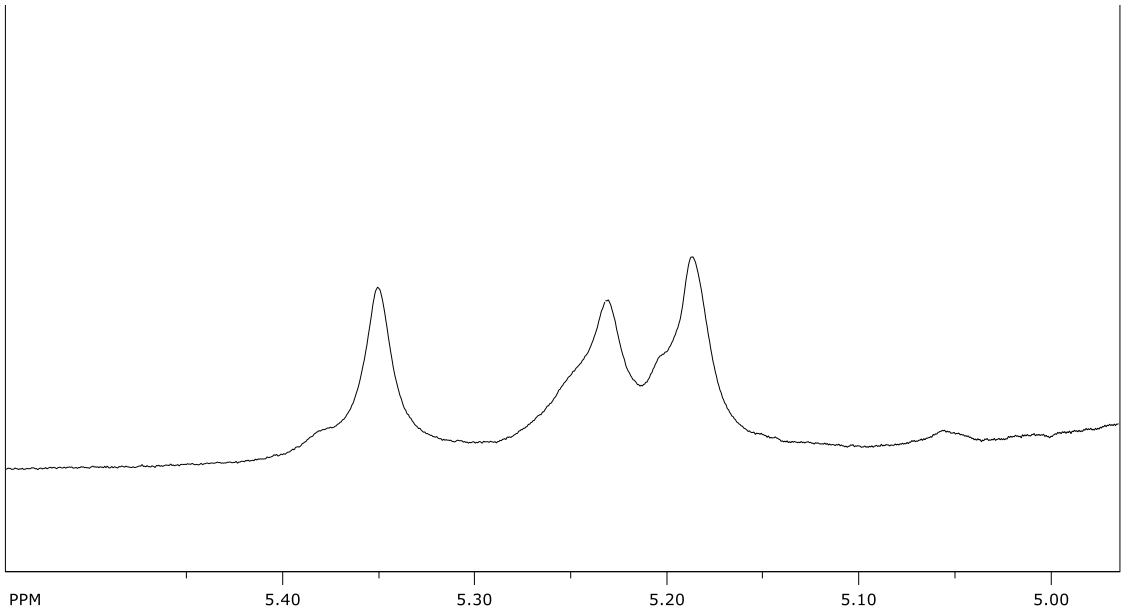
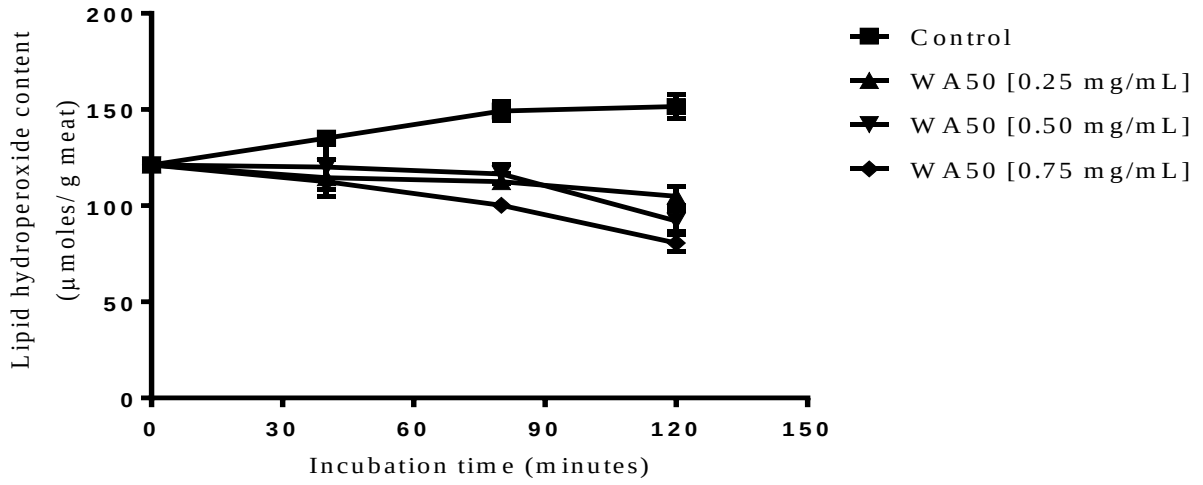
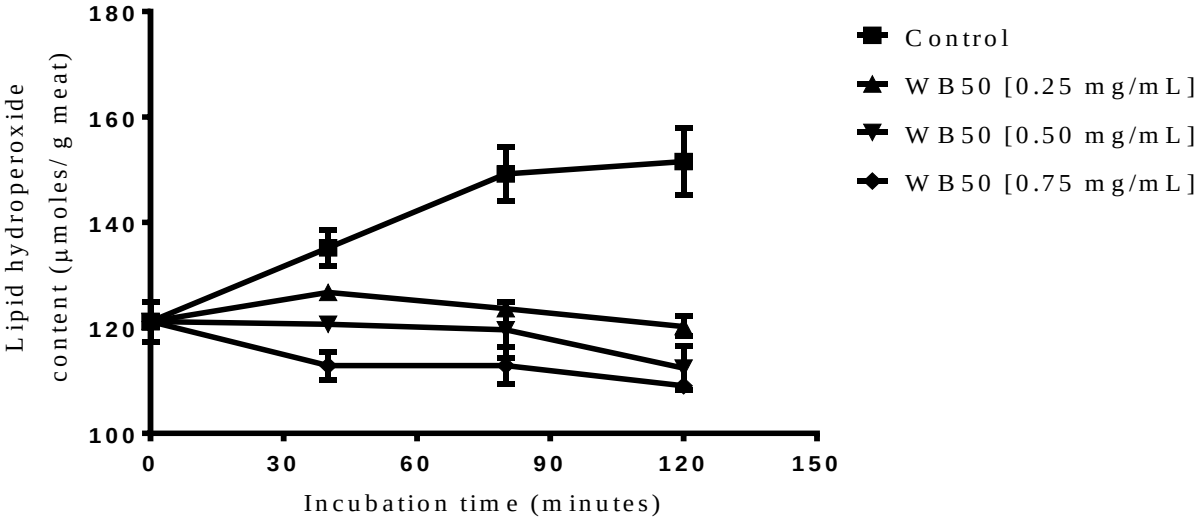


Figure 4.3: Effect of water extractable arabinoxylan (WEAX) on lipid peroxidation under gastric conditions. (a) WA50 and (b) WA75: WEAX fraction from WA obtained at 50 and 75% ammonium sulphate saturation, respectively. (c) WB50 and (d) WB75: WEAX from WB obtained at 50 and 75% ammonium sulphate saturation, respectively.

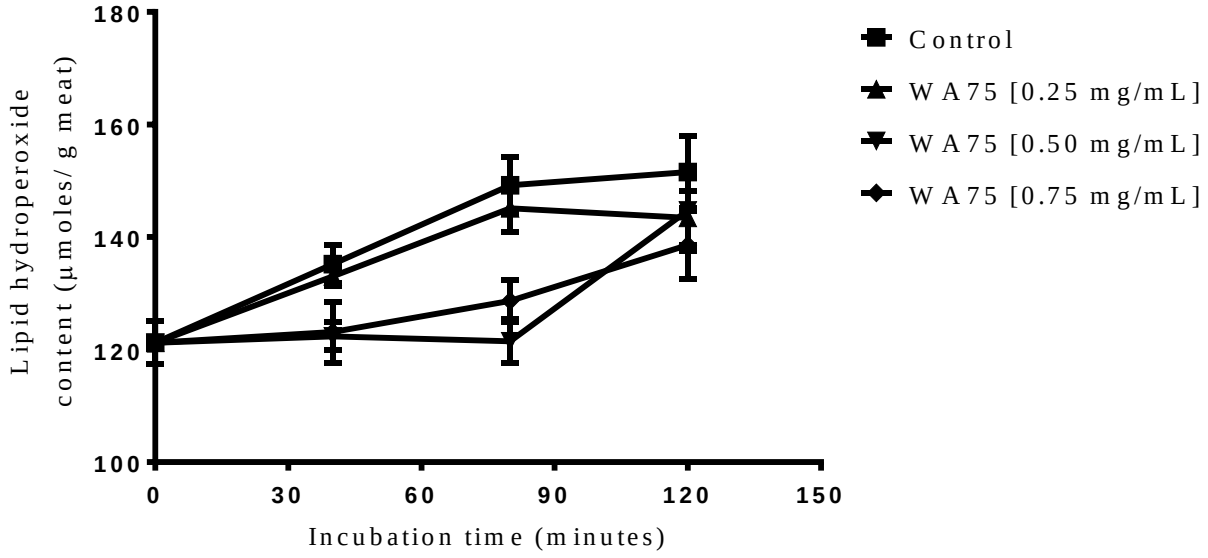
(a)



(b)



(c)


Lipid hydroperoxide content (µmoles/ g meat)
180
160
140
120
100
0
30
60
90
120
150
Incubation time (minutes)
Control
WA75 [0.25 mg/mL]
WA75 [0.50 mg/mL]
WA75 [0.75 mg/mL]

(d)

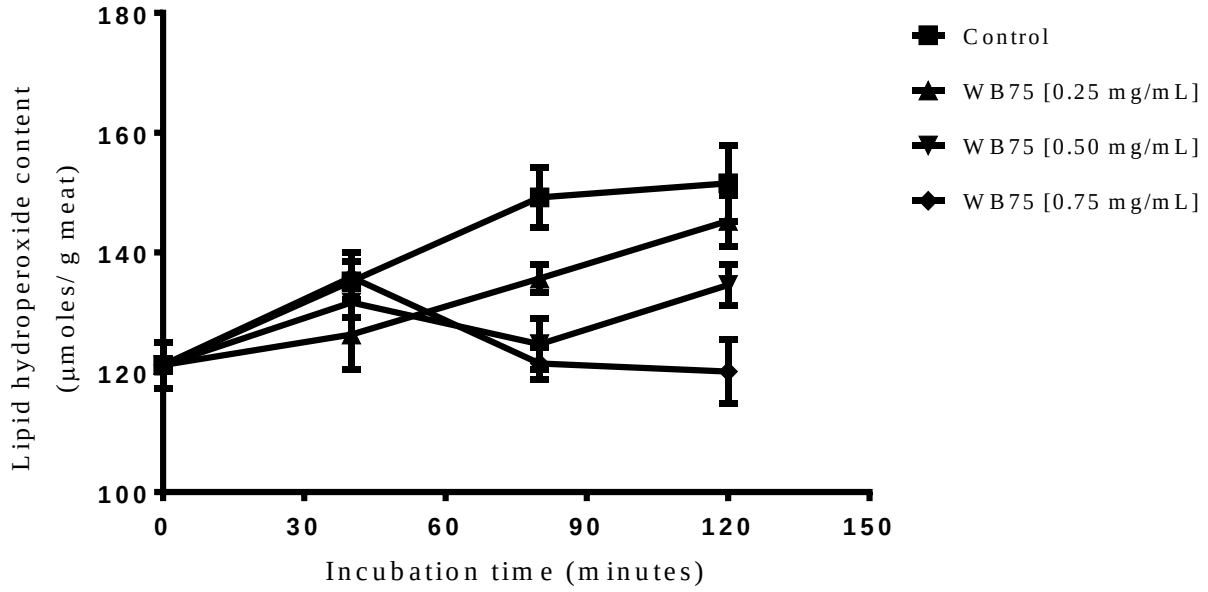
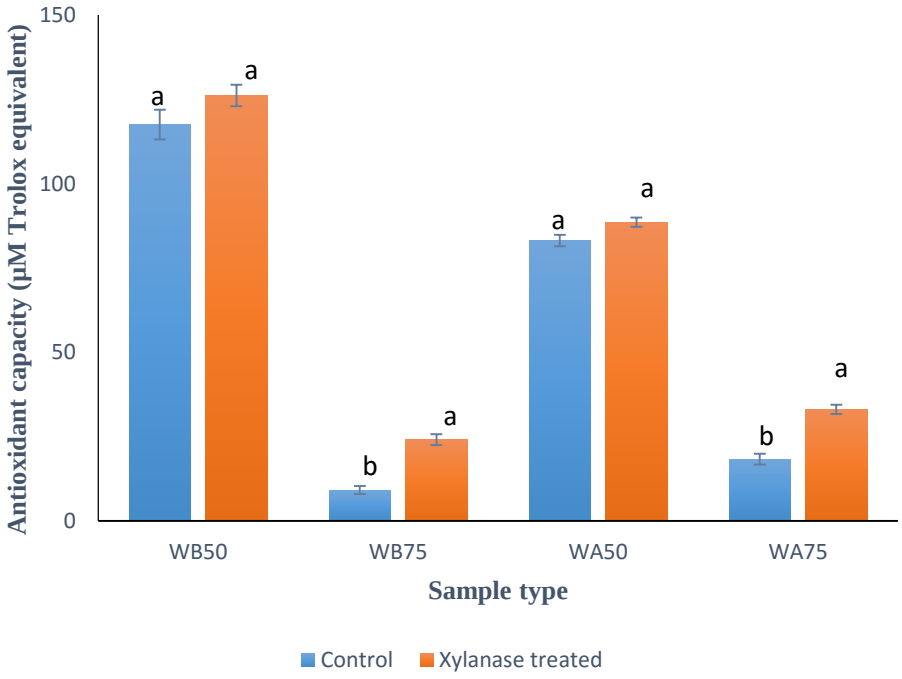

Lipid hydroperoxide content (µmoles/ g meat)
180
160
140
120
100
0
30
60
90
120
150
Incubation time (minutes)
Control
WB75 [0.25 mg/mL]
WB75 [0.50 mg/mL]
WB75 [0.75 mg/mL]

Figure 4.4: Antioxidant capacity of water extractable arabinoxylan (WEAX) following xylanase treatment. WA50: AX fraction from WA obtained at 50% ammonium sulphate saturation, WA75: AX from WA obtained at 75% ammonium sulphate saturation, WB50: AX from WB obtained at 50% ammonium sulphate saturation, WB75: AX from WB obtained at 75% ammonium sulphate saturation.



CHAPTER 5

5 Antioxidant capacity of arabinoxylan oligosaccharide fractions prepared from wheat aleurone using Trichoderma viride or Neocallimastix patriciarum xylanase

Lovemore Nkhata Malunga and Trust Beta

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5.1 Abstract

The effect of xylanase type (*Trichoderma viride* or *Neocallimastix patriciarum*) and graded ethanol fractionation on the antioxidant capacity (AOC) of arabinoxylan oligosaccharides (AXOS) obtained from wheat aleurone was investigated. AXOS yields were higher using *N. patriciarum* (62%) than *T. viride* (44%). The fraction (F100) collected at >80% ethanol concentration constituted 60% of total recovered AXOS. Degree of substitution ranged from 0.20 to 0.60 for ethanol graded fractions. Ferulic acid (FA) esterified to AXOS (8.0 µg/ mg) was 2-fold lower for the *N. patriciarum* treatment. The mean AOC (41.6, 183.1, and 394.9 µM TE/mg) of T. viride treated AXOS was >1.4-fold higher than *N. patriciarum* treatment using DPPH and ABTS and ORAC assays, respectively. Fraction F100 had highest AOC. AOC was influenced by the content of esterified FA ($R^2 = 0.94$). The type of xylanase had a major influence on the AOC of the resultant AXOS rich in FA content.

Keywords: Keywords: Wheat aleurone; Arabinoxylan oligosaccharide; Antioxidant activity; Ferulic acid; Xylanase.

5.2 Introduction

Excess production of free radicals (reactive oxygen/nitrogen species) in the body may result in chronic health diseases as oxidative damage is involved at either initial or developmental stage of these diseases (Sun, 1990). The presence of antioxidant compounds may donate hydrogen atoms or electrons to neutralise free radicals circulating in the body. Thus consumption of foods rich in antioxidants can prevent onset or mitigate progress of such diseases (Fang, Yang, & Wu, 2002). Feruloylated arabinoxylan oligosaccharides (FOS) have been shown to possess antioxidant properties (Ohta, Semboku, Kuchii, Egashira, & Sanada, 1997; Ou et al., 2007; Wang, Sun, Cao, & Wang, 2010; Yuan, Wang, Yao, & Chen, 2005). For example, FOS enhanced the antioxidant capacity in rat plasma, demonstrated stronger antioxidant activity against LDL peroxide system compared to ferulic acid, and inhibited induced DNA damage by hydrogen peroxide (Ohta et al., 1997; Ou et al., 2007; Wang et al., 2010; Yuan et al., 2005). FOS are a product of enzyme or mild acid hydrolysis of arabinoxylan (AX).

The structure of arabinoxylan is very complex and diverse in plant tissues but the primary chain consists of (1–4)-β-D-xylopyranose with a-$_{L}$-arabinofuranose substitutions at O-2 and/or O-3 position (Lequart, Nuzillard, Kurek, & Debeire, 1999). The xylan chain can also be substituted with α-(1,2)-glucuronic acid and/or α-(1,2)-4-O-methylglucuronic acid branches (Ma et al., 2012). The arabinoxylans may be feruloylated with ferulic acid at the O-5 position of the arabinose units (Bunzel, Ralph, & Steinhart, 2005). The degree of substitution (DS) is often defined by the molar ratio of arabinose to xylose and a higher DS signifies a highly branched arabinoxylan (Ma et al., 2012). The distribution of AX in each tissue of wheat grain has been reported by Barron, Surget, and Rouau (2007). Wheat starchy endosperm, aleurone, and pericap contain about 1.5%, 20–30% and 40–45% AX, respectively. The AX found in bran is highly branched and cross-linked as shown

by a high DS (>1.10) and diferulic acid content (0.4%) compared to DS (0.4) and diferulic acid (0.1%) of aleurone AX (Antoine et al., 2003; Barron et al., 2007). AXs with low DS are more susceptible to endoxylanase action (Biely, Vrsanska, Tenkanen, & Kluepfel, 1997). Additionally, wheat aleurone AX has a higher ferulic acid to AX ratio (4.2) than bran AX (1.4) (Antoine et al., 2003; Barron et al., 2007). Thus compared to other grain fractions, aleurone is likely to give high yield of AXOS with low DP and high FOS content. The structures of different FOS from wheat bran, corn bran, and sugar beet have been established (Gruppen et al. 1992; Katapodis et al., 2003; Yuan, Wang, & Yao, 2006). However, data on AXOS obtained from wheat aleurone is limited.

Endoxylanases randomly hydrolyses the (1 → 4)-β-D-xylopyranose glycosidic bonds of arabinoxylans (Dervilly, Saulnier, Roger, & Thibault, 2000). Consequently, the resulting AXOS are a mixture of arabinoxylans of different degrees of polymerisation and substitution (Dervilly et al., 2000; Swennen, Courtin, Lindemans, & Delcour, 2006; Van Craeyveld et al., 2010). The degree of substitution or polymerisation is also affected by the source of endoxylanase used (Beaugrand et al., 2004). Amberlite XAD-2 column is used followed by fractionation on Sephadex LH-20 column to obtain FOS (Saulnier, Vigouroux, & Thibault, 1995; Saulnier et al., 1999). Moreover ultrafiltration or graded ethanol precipitation can be used to obtain fractions with similar degrees of polymerisation and substitution (Swennen, Courtin, Van der Bruggen, Vandecasteele, & Delcour, 2005). The latter has mostly been used to obtain AXOS for prebiotic studies and therefore, limited data exists on their antioxidant properties.

Endo-b-xylanases (EC 3.2.1.8) are mostly available in two glycoside hydrolase (GH) families namely GH 10 and GH 11. In comparison, GH11 family endoxylanase have higher efficiency to penetrate cell walls than GH 10 (Beaugrand et al., 2004). The other advantage of GH11 over GH10 is their high specificity towards arabinoxylan unlike GH10 which also contains catalytic domains

for other cell wall polysaccharide (Beaugrand et al., 2004; Collins, Gerday, & Feller, 2005). In this study, arabinoxylan oligosaccharides were produced from a wheat aleurone-rich fraction using two GH family 11 endoxylanases. Xylanase from *Trichoderma viride* and *Neocallimastix patriciarum* were selected. Both xylanases are well characterised and have been used in different studies for production of AXOS (Black, Hazlewood, Xue, Orpin, & Gilbert, 1994; Sancho, Bartolomé, Gómez-Cordovés, Williamson, & Faulds, 2001; Ujiie, Roy, & Yaguchi, 1991). The obtained AXOS were fractionated by graded ethanol precipitation. Eight fractions were collected for each enzyme treatment and their yield, monomeric composition, FOS content, degree of substitution (DS), and antioxidant capacity were compared.

5.3 Materials and Methods

5.3.1 Materials

A commercial wheat aleurone (Grainwise™ wheat aleurone) was a gift from Cargill Limited from Horizon Milling (Wichita, Kansas, USA). It contains about 4.5%, 15.2%, 7.4% and 2.5% lipid, protein, ash and starch, respectively (Chen, Dunford, & Goad, 2012). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7, 8-tetramethylchoman-2-carboxylic acid (trolox), $2,2^0$-azino-bis(3ethylbenzothiazoline-6-sulphonic acid (ABTS), 2, 2^0-azobis (2-amidinopropane) hydrochloride (AAPH), fluorescein, amyloglucosidase from *Aspergillus niger* (300 units/ml, EC 3.2.1.3), a-amylase heat stable (EC 3.2.1.1), protease from *Bacillus licheniformis* (EC 3.4.21.62), ferulic acid standard, endo-1,4-b-xylanase from *T. viride* (100–300 units/mg protein, EC 3.2.1.8), D-glucose, D-xylose, D-arabinose, and D-galactose were purchased from Sigma–Aldrich (Milwaukee, Wisconsin, USA). Endo-1,4-β-xylanase from *N. patriciarum* (10000 units/ml, EC 3.1.2.8) was purchased from Megazyme (Bray, Wicklow, Ireland). All acids and organic solvents

were obtained from Fischer Scientific (Whitby, Ontario, Canada). All chemicals used were of analytical grade.

5.3.2 Preparation of arabinoxylan oligosaccharides

Arabinoxylan oligosaccharides were prepared as described by Swennen et al. (2006) with modifications. Specifically, 400 g of aleurone flour passed through 0.5 mm sieve was suspended in 4000 ml distilled water and autoclaved at 121 °C for 15 min to deactivate enzymes. After cooling to room temperature, the contents were centrifuged at 10,000g and 4 °C for 20 min. The supernatant was decanted and the residue was dried in an oven at 45 °C for 48 h.

Deactivated aleurone flour (25 g) was suspended in 200 ml phosphate buffer (pH 6.8, 20 mM). Heat stable a-amylase (600 µl) was added and the flour suspension incubated at 90 °C for 1 h under continuous shaking in a water bath (Model 89032226, VWR International, Philadelphia, PA, USA)) at 200 rpm. The pH was adjusted to 7.5 after cooling down, and 600 µl of protease was added prior to incubation at 60 °C for 1 h. Amyloglucosidase (400 µl) was added after adjusting the pH to 4.5 and the contents were incubated at 60 °C for 1 h. The temperature was later adjusted to 90 °C and the suspension incubated for 30 min to deactivate the enzymes. Following centrifugation at 10,000g and 4 °C for 20 min, the residue was rinsed twice with 100 ml of distilled water and centrifuged as above. The residue was dried at 50 °C for 36 h and labelled destarched and deproteinised aleurone flour.

Destarched and deproteinised aleurone flour (16 g) was suspended in 320 ml of acetate buffer (pH 4.5, 50 mM). Endo-1,4-β-xylanase (12.5 µl) from *T. viride* was added prior to incubation at 30 °C for 24 h under continuous shaking in a water bath at 250 rpm. A second set of samples was suspended in sodium phosphate buffer (pH 6.0, 100 mM) followed by addition of 0.5 ml of endo-1,4-β-xylanase from *N. patriciarum* (250 units/ml). The mixture was incubated at 40 °C for 24 h

with continuous shaking. The temperature was later adjusted to 90 °C and the suspension incubated for 20 min to deactivate the enzymes. The contents were finally centrifuged at 10,000g and 4 °C for 20 min. The residue was rinsed twice with 100 ml distilled water and the supernatants were pooled together for freeze drying.

The dried arabinoxylan oligosaccharides were suspended in double distilled water (100 mg/ml) and shaken continuously at 250 rpm in a water bath for 2 h at room temperature. After centrifuging at 10,000g for 20 min at 18 °C, the residue was discarded and supernatant freeze dried to obtain water extractable arabinoxylan oligosaccharide (WE-AXOS) for further analysis.

5.3.3 Fractionation of the arabinoxylan oligosaccharide

WE-AXOS (4 g) was solubilised in 40 ml double distilled water followed by addition of 95% ethanol to attain 20% ethanol concentration. After 24 h at 4 °C, the contents were centrifuged at 10,000g and 4 °C for 20 min. The residue was solubilised in distilled water, freeze dried and labelled F20. The ethanol concentration of the supernatant was adjusted to 30% and left for 24 h at 4 °C. Similarly, after centrifugation at 10,000g and 4 °C for 20 min, the residue was solubilised in distilled water, and freeze dried and labelled F30. The ethanol concentration of the supernatant was increased further by 10–40%. The cycle was repeated to obtain fractions F40, F50, F60, F70, and F80. The supernatant collected after 80% was evaporated to dryness using a rotary evaporator (Buchi rotavapor R – 205, Laboratoriums Technik AG, Flawil, Switzerland) and reconstituted with distilled water for freeze drying to obtain F100.

5.3.4 Determination of monosaccharide composition of arabinoxylan oligosaccharide

The AXOS sample (20 mg) was suspended in 2 M sulphuric acid (3 ml) and hydrolysed at 110 °C for 1 h (Swennen et al., 2006). The suspension was later neutralised with 4 M NaOH and then passed through 0.45 µm filter. Individual monosaccharides were analysed using a Waters

Corporation's Dionex HPLC system (Waters, Milford, MA) equipped with a Dionex Carbopac PA1 column and electrochemical (EC) detector (model ED40) as described by Parsons, Cenkowski, Sorensen, Beta, and Arntfield (2013) using aqueous NaOH (3.5 mM) as eluent at a flow rate of 1.0 mL/min, injection volume of 25 µl, column temperature 36 °C and sample temperature 4 °C. The peak areas of different concentrations (0.01, 0.1, 1.0, 4.0, 7.0, 10.0, 20.0 and 50.0 mM) of D-glucose, D-xylose, D-arabinose, and D-galactose standards were used for the calibration curve to determine the content of corresponding sugars in extract. The peaks were confirmed by spiking individual standards in selected samples.

Glucuronic acid content was quantified as described by Englyst and Cummings (1984) using spectrophotometry. A mixture of 300 µl of the hydrolysate, 300 µl sodium chloride–boric acid solution, and 5 ml sulphuric acid (concentrated) was incubated at 70 °C for 40 min. After cooling to room temperature, 200 µl of 3,5dimethylphenol (1 mg/ml in acetic acid) was added whilst the tubes were incubated in water at room temperature. The mixture was vortex mixed and left to stand at room temperature for 10 min before being transferred into a 2-ml cuvette for measurement of absorbance (Ultrospec 1100 Pro, UV/Visible Spectrophotometer (Biomicron Ltd., Cambridge, CB4QFJ, England)) at 400 and 450 nm. The absorbance difference (A450 -A400) of different concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.6 and 0.7 mM) of glucuronic acid was used to plot the calibration curve for determining the glucuronic acid content.

5.3.5 Estimation of free and esterified ferulic acid content in arabinoxylan oligosaccharide

A spectrophotometric method as described by Saulnier et al. (1995) was used to determine the quantity of free or esterified ferulic acid using molar absorption coefficients (M^{-1} cm^{-1}) of 19,662 (7630) and 23,064 (31,430) for free ferulic acid and esterified ferulic acid at 345 (375) nm, respectively. One milligram of AXOS sample was dissolved in 1 ml distilled water. Later 100 µl

was transferred to 900 µl of glycine–sodium hydroxide buffer (pH 10, 0.04 M) and vortexed. After 5 min, the mixture was transferred into a 2-ml cuvette for reading of absorbance (Ultrospec 1100 Pro, UV/Visible Spectrophotometer (Biomicron Ltd., Cambridge, CB4QFJ, England) at 345 and 375 nm.

5.3.6 Identification of feruloylated arabinoxylan oligosaccharides (FOS) by LC–MS

FOS were identified using LC–MS method as described earlier (Wang et al., 2009) with minor modifications. AXOS were AXOS (100 µg/ml in 50% methanol) was filtered through 0.45 µm and eluted using a reverse phased high performance liquid chromatography (Waters Alliance 2695 instrument (Waters, Milford, MA)) equipped with a Waters 2996 photodiode array detector and 5.0lm Phenomenex C18 column (150 X 4.6 mm). For analysis, 10 µl of a sample was injected into the liquid chromatograph. The operating isocratic gradient consisted of 90% solvent A (0.5% acetic acid in water) and 10% solvent B (0.5% acetic acid in acetonitrile). A flow rate of 1.0 ml/min was used. The sample and column temperatures were set at 15 and 35 °C, respectively. The absorbance was monitored continuously at 325 nm. The elution from the HPLC was introduced into the mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA) using electrospray ionisation (ESI) in negative mode. The MS was set as follows: desolvation temperature 250 °C; source temperature, 120 °C spray voltage; capillary voltage, 1.2 kV; sample cone voltage, 45 V; extraction cone voltage, 4 V; syringe rate 3 µl; cone gas flow, 50 L/h; and desolvation gas flow 900 L/h.

5.3.7 Estimation of antioxidant activity of arabinoxylan oligosaccharide

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity of AXOS was determined by using a method described by Sharma and Bhat (2009) with modifications. An aliquot (50 µl) of AXOS (1 mg/ml water) was transferred into a 2-ml centrifuge tube and 700 µl 50% aqueous methanol was

added followed by 750 µl DPPH (1.8 mg DPPH dissolved in 50 ml 60% aqueous methanol). The mixture was vortexed and left in the dark for 35 min. It was later transferred to a 2-ml cuvette after vortexing for absorbance measurement (Ultrospec 1100 Pro, UV/Visible Spectrophotometer (Biomicron Ltd., Cambridge, CB4 QFJ, England)) at 515 nm. The readings were taken at 40 min and the% DPPH decolourization

((1- [(A_{sample} - A_{blank})/($A_{control}$ - A_{blank})]) * 100) was used to determine the free radical scavenging ability of the sample. A calibration curve of % DPPH decolourization obtained from different concentrations of trolox (6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid) was used to quantify the antioxidant capacity of AXOS.

$ABTS^{+}$ ($2,2^{0}$-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) scavenging activity of AXOS was determined by using a method described earlier (Re et al., 1999) with modifications. ABTS+ radical reagent was produced by allowing 7 mmol/L aqueous solution of ABTS (3 ml) to react with 2.45 mmol/L potassium persulfate (3 ml) in the dark at room temperature for 12–16 h. ABTS+ reagent (4.0–4.5 ml) was diluted to 250 ml with distilled water to obtain an absorbance of approximately 0.70 at 734 nm using UV/Visible spectrophotometer (Ultrospec 1100 Pro, Biomicron Ltd., Cambridge, CB4QFJ, England). AXOS (0.1 ml, 0.3 mg/ml) was mixed with 1.7 ml of ABTS+ reagent in a 2-ml amber centrifuge tube. The mixture was vortexed and left in the dark for 30 min. It was later transferred to a 2 ml cuvette after vortexing for absorbance measurement at 734 nm. The % decolourization ((1 - [(A_{sample} - A_{blank})/ ($A_{control}$ - A_{blank})]) * 100) was used to determine the free radical scavenging ability of the sample. A calibration curve of % decolourization obtain from different concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid) was used to quantify the oxidant capacity of AXOS.

The ORAC assay to measure the antioxidant activity of AXOS was conducted as described by Cao, Alessio, and Cutler (1993) and as modified by Bellido and Beta (2009) with further minor modifications. We used a precision 2000 well automated microplate pipetting system (Bio-Tek Instruments Inc., Winooski, VT, USA) that automatically transferred ORAC reagents into a 96-well flat-bottom polystyrene microplate (Corning Inc., Corning, NY, USA), and an FLx800 microplate fluorescence reader (Bio-Tek Instruments Inc., Winooski, VT, USA) controlled by KC4 3.0 software. Changes in the fluorescence of fluorescein under controlled temperature conditions (37 °C) were measured at excitation wavelength of 485 nm and an emission wavelength of 520 nm. AXOS (0.1 mg/ml water) was used for analysis and 0.0, 6.25, 12.5, 25.0, 50.0, 75.0 μM Trolox in water was used to generated a standard calibration curve.

5.3.8 Statistical analysis

All analyses were performed in duplicate and all statistics were calculated using one way analysis of variance (ANOVA) on a JMP 10 statistical software (SAS Institute Inc., Cary, NC). Sample means were compared using Tukey HSD method and significant differences determined at $p \leq 0.05$. Correlations between FOS and antioxidant capacity were done by Pearson's correlation test.

5.4 Results and Discussion

5.4.1 Arabinoxylan oligosaccharide production

Endo-1,4-β-xylanase from *T. viride* and *N. patriciarum* were used to produce AXOS from wheat aleurone. We found that the yield of AXOS was higher when the later was used giving 61.6 ± 3.5 compared to 44.4 ± 1.1% of deproteinised and destarched flour. The yield corresponds to about 15% and 25%, respectively, of the starting material which is higher than the reported yield of AXOS from wheat bran (6–10%) (Swennen et al., 2006). This increase in AXOS yield compared to bran might be attributed to the fact that aleurone AX is readily solubilised by xylanase due to

its low DS (Biely et al., 1997). The observed difference in AXOS yield between the xylanase treatments in our study suggests that endoxylanase from *N. patriciarum* has higher specificity for aleurone AX compared to that of *T. viride*. Van Craeyveld et al. (2010) also reported that endoxylanase from different sources resulted into different yields.

Endoxylanase randomly hydrolyses xylan chain resulting in AXOS of varying degrees of polymerisation (DP) and substitution (DS) (Biely et al., 1997; Schooneveld-Bergmans, Beldman, & Voragen, 1999). Among other fractionation methods, graded ethanol precipitation has been used to isolate AXOS of similar DP and DS (Swennen et al., 2005, 2006). In our study, 8 fractions of AXOS were collected. The mean yield of resultant fractions ranged from about 2–41%. The highest yield among the fractions was F100 in all treatments (Table 5.1) which is consistent with literature (Swennen et al., 2006). Fractions F40, F50, and F60 generally constituted the lowest yields in all treatments. It was also observed that the mean yield of fractions obtained from *T. viride* treated AXOS were about 20–50% lower compared to those from *N. patriciarum* at the same ethanol concentration.

5.4.2 Monosaccharide composition of arabinoxylan oligosaccharide

The mean monosaccharide composition of AXOS is given in Table 5.2. We found that arabinose and glucuronic acid contents did not differ significantly between the two endoxylanase treatments in our study. On the other hand, xylose in *T. viride* treated samples was 10% lower compared to that of *N. patriciarum* treated samples. Similarly, glucose contamination was 50% higher in *T. viride* treatment when compared to that of *N. patriciarum* (Table 5.2). The total arabinoxylan content and DS (0.24) were not significantly different between the two treatments in our study. Our results for the DS are consistent with literature that assumed that AXOS collected from their bran samples originated from aleurone (Swennen et al., 2006; Van Craeyveld et al., 2010).

According to literature, an increase in ethanol concentration results in arabinoxylans fractions with decreased DP and increased DS (Cleemput et al., 1995; Dervilly et al., 2000; Swennen et al., 2006). Regardless of treatment, DS did not vary significantly for fractions obtained with 50% ethanol concentration or lower but started to increase with further addition of ethanol up to 70% after which a decrease was also noted (Table 5.2). We did not analyse DP of AXOS but we assumed that DP decreased with increase in ethanol concentration in accordance with literature (Swennen et al., 2005).

The arabinoxylan content was highest for fractions obtained after 70% ethanol concentration in both treatments (>80.0%). In general, it was observed that the purity (decrease in glucose contamination) of AXOS fractions increased with increase in ethanol concentration (Table 5.2). Glucose contamination was predominantly observed in fractions F20, F30, F40 and F50. These fractions are associated with high DP in the order F20 > F30 > F40 > F50 (Swennen et al., 2006). The observed traces of glucose originated from beta glucan polymers which precipitated out at low ethanol concentrations. Aleurone contains about 15% beta-glucans (Antoine et al., 2003) which have been reported to co-precipitate with arabinoxylans at low ethanol concentration (Cleemput et al., 1995).

Glucuronic acid content in collected fractions from *N. patriciarum* increased with increase in ethanol concentration up to 60% and started to decrease with further addition of ethanol. In contrast, glucuronic acid content in fractions from *T. viride* treated samples was inconsistent with F50 having the highest and F100 the lowest. Data on the glucuronic acid content on wheat AX-OS is limited for comparison purposes.

The observations in our study are consistent with literature which may imply that DS did not influence precipitation behaviour during ethanol increase up to 50% but DP may have had an

influence (Swennen et al., 2006). Secondly, DS but not DP may have influenced the precipitation behaviour of AXOS collected from 51% to 70%. The precipitation behaviour of fractions F80 and F100 might have been influenced by DP.

5.4.3 Amount of ferulic acid esterified to arabinoxylan oligosaccharide (FOS)

Results on the amount of ferulic acid (FA) esterified to AXOS and free ferulic acid using spectrophotometric method are presented in Table 5.3. The mean concentration (µg/mg AXOS) of ferulic acid esterified AXOS was 2-fold lower in *N. patriciarum* when compared to that of *T. viride*. Esterified ferulic acid represented about 0.8% and 1.5% of the AXOS by weight, respectively. Free ferulic acids were below detection limit in the unfractionated AXOS regardless of treatment.

Esterified FA was about 25–600% higher in fractions obtained from *T. viride* compared to those fractions from *N. patriciarum* treatment at the same ethanol concentrations (Table 5.3). Esterified FA content within each treatment did not change significantly with increase in ethanol concentrations up to 60%. However, esterified FA content was least for F50 (*T. viride* treatment) or F60 and F70 (*N. patriciarum* treatment) but was highest for fractions obtained after 70% ethanol concentration. In contrast, the levels of unbound ferulic acid were very low and their traces were mostly found in fractions obtained from *N. patriciarum* treatment (Table 5.3). The trace levels were mostly observed in fractions F20, F30 and F40 likely due to poor solubility of ferulic acid in water.

5.4.4 Identification of FOS using HPLC

The presence of FOS was verified and identified by LC–MS. The HPLC chromatogram of AXOS (Figure 5.1) showed 4 prominent peaks at retention peaks 5.5, 5.95, 6.83 and 7.98 suggesting four possible types of FOS were present. We performed MS scan using negative electron-spray

ionisation (ESI) mode in order to identify FOS at each peak. Limitations included a mass spectrometer that could only be calibrated up to 1000 daltons and background chemical noise due to the use of unpurified AXOS. However, the negative ion mass spectra for the peak at 5.52 (Figure 5.2a) showed possibility of composite FOS with m/z = 721.1; 456.5; 589.2; and 853.1 were present (in order of intensity). Our data suggested that the predominant FOS was an oligosaccharide with 4 sugars (xylose or arabinose) esterified to a ferulic acid moiety and molecular weight of approximately 722.0 daltons. Other FOS present were those with molecular weight of about 458.0 or 590.0 or 854.0 daltons suggesting the presence of compounds with 2 or 3 or 5 sugars respectively, with a single ferulic acid attached. The mass spectra for other peaks (Figure 2b–d) were identical with only m/z 456.5 being a possible FOS (2 sugars with a single ferulic acid attached). Thus we concluded that the resultant AXOS contained FOS of DP 2, 3, 4 and 5.

5.4.5 Antioxidant capacity of arabinoxylan oligosaccharide

The resultant fractions were examined for their antioxidant properties using both electron transfer and hydrogen atom transfer assays. DPPH and ABTS radical scavenging assays whose reaction mechanism involves transfer of electron by the reducing agent to the DPPH/ABTS radical ($R^{\cdot}$ + ArOH → R^{-} + $ArOH^{+}$) were used (Blois, 1958; Leopoldini, Marino, Russo, & Toscano, 2004). The ORAC assay which measures the ability of AXOS to transfer hydrogen atom mechanism (ROO + AH/PheOH →ROOH + A/Phe-O) was also used. The transfer of the hydrogen atom is highly dependent on the bond dissociation energy of O–H whereas electron transfer is dependent on ionisation potentials (Leopoldini et al., 2004; Rice-Evans, Miller, & Paganga, 1996).

5.4.5.1 *DPPH antioxidant activity of AXOS*

DPPH scavenging assay is based on a measurement of discoloration resulting from a reduction of DPPH free radical by an antioxidant (Blois, 1958; Brand williams, Cuvelier, & Berset, 1995). Our

results (Table 5.4) suggest that the ability to quench DPPH free radical by unfractionated AXOS obtained from *T. viride* treatment was 46% higher compared to that of AXOS from *N. patriciarum* treatment. Similarly AXOS fractions obtained from *T. viride* treatment had their mean antioxidant capacities about 40–430% higher when compared to the corresponding fractions from *N. patriciarum* at the same ethanol concentration ($p \leq 0.05$).

The antioxidant activity for the fractions obtained within the same treatment ranged from 10.0–37.0 and 22–57 µM Trolox equivalent/mg AXOS for *N. patriciarum* and *T. viride* treatment, respectively (Table 5.4). We observed that the ability to quench DPPH radicals for *T. viride* treated fractions decreased with the increase in ethanol concentration up to 50% (F20 > F30 > F40 > F50). This may suggest that the antioxidant capacity is not only influenced by the amount of esterified FA. In this particular case, F20, F30 and F40 (which had the same amount of esterified FA and DS) resulted in different antioxidant capacities. The difference may have been influenced by DP. Rao and Muralikrishna (2006) reported that feruloylated arabinoxylans with higher molecular weight, also had higher antioxidant capacity. It was also noted that fractions obtained after 50% ethanol concentration had their antioxidant activity increased with increase of the ethanol concentration F50 < F60 < F80 < F100 with highest being F70. Our results for fractions F70, F80 and F100 further suggest that when esterified FA levels and DP are about the same, their antioxidant capacity is influenced by DS.

However, the antioxidant capacity of fractions obtained from *N. patriciarum* followed a different trend. Specifically, antioxidant activity of F20 significantly decreased and remained the same for F30 and F40 before further declining upon reaching F50. Despite their similar esterified FA content and DS, DP may have influenced the observed variation as explained earlier. Increasing

ethanol concentration from 50% to 70% did not affect the antioxidant activity but there was a 50% increase from F70 to F80.

Overall, the scavenging ability of AXOS demonstrated a positive correlation between esterified FA content and DPPH ($r = 0.94$, $p \leq 0.05$). The antioxidant capacity of F100, the highest among the collected fractions, was almost 30% higher compared to AXOS before fractionation (WE-AXOS) from the same treatment. The finding illustrates the advantage of graded ethanol fractionation of AXOS.

5.4.5.2 ***ABTS scavenging activity***

ABTS+ scavenging assay is similar to DPPH and measures the ability of an antioxidant compound to reduce ABTS+ radical. ABTS+ radical loses its blue-green colour when reduced to ABTS at 570 nm (Re et al., 1999). Unfractionated AXOS obtained from *T. viride* had 45% higher antioxidant activities compared to those from *N. patriciarum* treatment (Table 5.4). Similarly the mean antioxidant capacity of AXOS fractions obtained from *T. viride* treatment were 15–330% higher compared to the corresponding fractions from *N. patriciarum*. Generally, there was a strong positive correlation between ABTS radical scavenging and esterified FA content ($r = 0.89$, $p \leq 0.05$) when comparing fractions with the same DS. The possible role of DS in ABTS scavenging was inconsistent. Specifically, DS did not influence the ABTS reduction by *T. viride* treated fractions ($r = 0.23$, $p \leq 0.05$). In contrast, fractions from *N. patriciarum* suggested a strong negative correlation ($r = 0.81$, $p \leq 0.05$) between DS and ABTS antioxidant capacity in AXOS. These observations may suggest that ABTS scavenging capacity of AXOS could be influenced by both esterified FA content and DS. ABTS scavenging ability of F100 from *T. viride* treated samples was 25% higher compared to that of AXOS before fractionation (WE-AXOS). However, there was

no significant difference between the antioxidant capacity of unfractionated AXOS and F100 from *N. patriciarum*.

5.4.5.3 ***Oxygen radical scavenging capacity (ORAC)***

The ORAC assay is based on the principle that antioxidant compounds will prevent peroxyl radicals being generated by AAPH from decaying the fluorescence intensity of fluorescein (Cao et al., 1993). Antioxidant capacity is measured as the ability of a compound to delay the loss of fluorescence intensity over time. We compared the mean ORAC values of AXOS from both treatments and the results are presented in Table 5.4. The unfractionated AXOS obtained *from T. viride* treatment had about 70% higher antioxidant activity when compared to that of *N. patriciarum*. Similar to the above antioxidant assays, the antioxidant capacity of *T. viride* fractions was about 40–470% higher than that of *N. patriciarum*. The antioxidant capacity of AXOS fractions was dependent on esterified FA content ($r = 0.84$ and $r = 0.87$, $p \leq 0.05$) in both *T. viride* and *N. patriciarum*, respectively.

The association between esterified FA content and ORAC values was further confirmed by comparing fractions with relatively similar DS (0.24) but different esterified FA content. It was observed that, fractions with higher esterified FA content resulted in high antioxidant activity compared to their corresponding fractions with lower esterified FA content, ($r = 0.94$, $p \leq 0.05$). It was also observed that when DS and esterified FA content are relatively similar (F20, F30, F40 and F50 for *N. patriciarum*), the antioxidant capacity decreased with increase in ethanol concentration. This may suggest that the observed difference in ORAC values was due to DP. Also, we observed that DS had strong negative association with antioxidant capacity of AXOS for *N. patriciarum* treated fractions ($r = 0.67$, $p \leq 0.05$). In contrast, DS did not influence antioxidant activity of AXOS obtained from *T. viride* ($r = 0.07$, $p \leq 0.05$). ORAC values for fraction with

highest antioxidant capacity was not significantly different from that of unfractionated AXOS in both treatments suggesting that purification might not be necessary for antioxidant purposes.

AXOS obtained from *T. viride* and also their fractions had higher antioxidant activities compared to AXOS from *N. patriciarum* at the same ethanol concentration. Even though the relevance of test tube antioxidant assays to human health is debatable, they help to understand the possible reaction mechanism of antioxidants. Presence of ferulic acid (free or conjugated) is essential for the antioxidant activity of arabinoxylans (Rosa, Dufour, Lullien-Pellerin, & Micard, 2013 & Serpen, Capuano, Fogliano, & Gokmen, 2007). Removal of esterified ferulic acid from wheat bran arabinoxylan reduced its antioxidant potency by over 70% (Serpen et al., 2007). In our study content of free ferulic acid did not influence the antioxidant activity of AXOS fractions. AXOS from *N. patriciarum* had lower antioxidant activity despite having high free ferulic acid content compared to AXOS obtained from *T. viride*. In agreement with literature (Hromádková, Paulsen, Polovka, Košťálová, & Ebringerová, 2013; Rao & Muralikrishna, 2006; Serpen et al., 2007), the amount of esterified ferulic acid had strong correlation with antioxidant activity of AXOS (R = 0.94). The association of DS and DP with the antioxidant behaviour of fractionated AXOS also provides insight to the probable reaction mechanism. Monosaccharide composition and glycosyl linkages of polysaccharide may influence their antioxidant properties (Lo, Chang, Chiu, Tsay, & Jen, 2011). In particular, increased glucose and arabinose content has a negative impact on the antioxidant capacity of polysaccharides (Lo et al., 2011). We also observed a negative association between Ar/Xy ratio and antioxidant activity of AXOS. Hydroxyl groups of polysaccharide are capable of donating electrons or hydrogen atom in a redox system (Li, Li, & Zhou, 2007). This may suggest that un-substituted xylose at O-2 or/and O-3 participated in antioxidant reaction by

donating a hydrogen or an electron. Consequently, increased (1–4)-β-D-xylopyranose linkages (increased DP) increases the potency of AXOS against free radicals.

Our data from the antioxidant assays suggest that the bioactive compounds in these AXOS fractions are largely esterified FA using both electron transfer and hydrogen atom transfer as possible antioxidant mechanisms. FOS has been shown already to possess antioxidant properties in other studies (Ohta et al., 1997; Ou et al., 2007; Wang et al., 2010; Yuan et al., 2005). Purification of AXOS using graded ethanol precipitation may have helped to produce FOS fractions of similar DP or DS but did not significantly improve the antioxidant capacity in our study. Fractions with highest antioxidant capacity were slightly higher or not significantly different from that of unfractionated AXOS. Thus choice of xylanase would be critical to ensure production of AXOS fractions (FOS) rich in esterified FA and consequently high antioxidant capacity.

5.5 Conclusion

The two xylanase enzymes applied on the wheat aleurone material resulted in distinct arabinoxylan oligosaccharides with different esterified ferulic acid content and antioxidant capacity. The antioxidant capacity of AXOS fractions was influenced by the content of esterified ferulic acid, Ar/Xy ratio and degree of polymerisation. There was a positive correlation between ORAC, ABTS and DPPH data obtained in this study. The majority of AXOS fractions were collected after adjusting to 80% ethanol concentration which gave the highest esterified FA content and antioxidant capacity compared to other fractions. However, graded ethanol precipitation did not make a significant difference in the antioxidant capacity of AXOS. Thus xylanase, like endo-1,4-β-xylanase from *T. viride* that results in AXOS with high FOS would be recommended for production of AXOS meant for protection against free radicals. However, taking into consideration of yield, *N. patriciarum* endoxylanase can still be used for production of arabinoxylan

oligosaccharide meant for reduction of free radicals in such application where high amounts of AXOS are tolerated. This data will enhance the potential of arabinoxylan oligosaccharide application in food industry.

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Table 5.1: Mean yield of ethanol fractionated oligosaccharide arabinoxylan as % of original, unfractionated arabinoxylan oligosaccharide (w/w).

	T. viride	*N. patriciarum*
F_{20}	2.6 ± 0.6^{aBC}	3.7 ± 0.3^{Ad}
F_{30}	3.5 ± 0.1^{bB}	5.4 ± 0.3^{aC}
F_{40}	1.8 ± 0.0^{bC}	3.3 ± 0.1^{aDE}
F_{50}	2.3 ± 0.5^{aC}	2.7 ± 0.6^{aE}
F_{60}	2.1 ± 0.4^{aC}	2.7 ± 0.7^{aE}
F_{70}	4.3 ± 0.1^{aB}	6.7 ± 1.3^{aC}
F_{80}	4.1 ± 0.4^{bB}	14.4 ± 0.4^{aB}
F_{100}	32.3 ± 2.1^{bA}	41.3 ± 1.1^{aA}
Total recovery	52.8	73.7

Values presented as mean ± standard deviation (n = 2). Data in the same row with the same superscript (small letter) are not significantly different at $p \leq 0.05$. Data in the same column with the same superscript (capital letter) are not significantly different at $p \leq 0.05$. The subscript in proceeding F denotes the percentage of ethanol at arabinoxylan oligosaccharide precipitated.

* Denotes the source of xylanase used to produce the arabinoxylan oligosaccharide. Total recovery is the sum of all fractions.

Table 5.2: Mean monosaccharide composition (as mole %) of oligosaccharide arabinoxylan

	Arabinose		Galactose		Glucose		Xylose		Glucuronic acid		Arabinoxylan		Arabinose/ Xylose	
	N. patriciarum	*T. viride*	*N. patriciarum*	*T. viride*	*N. patriciarum*	*T. viride*	*N. patriciarum*	*T. viride*	*N. patriciarum*	*T. viride*	*N. patriciarum*	*T. viride*	*N. patriciarum*	*T. viride*
**F_0	16.2	16.5	0	0	16.8	8.7	66.8	74.4	0.3	0.4	73.0	80.1	0.24	0.22
F_{20}	14.3	17.0	0.0	0.0	20.2	22.1	65.2	60.4	0.3	0.5	69.9	68.1	0.22	0.28
F_{30}	15.1	12.3	0.0	0.0	17.8	32.3	66.5	54.9	0.6	0.4	71.8	59.2	0.23	0.22
F_{40}	16.6	12.5	0.0	0.0	8.0	32.3	74.5	54.5	0.8	0.8	80.2	58.9	0.22	0.23
F_{50}	16.0	14.3	0.0	3.1	18.4	37.2	64.5	43.9	1.1	1.5	70.9	51.3	0.25	0.33
F_{60}	23.8	27.9	0.0	0.0	8.9	14.6	65.6	56.8	1.6	0.6	78.7	74.6	0.36	0.49
F_{70}	32.9	32.7	0.0	0.0	7.0	9.6	59.4	57.0	0.7	0.6	81.3	79.0	0.55	0.57
F_{80}	29.5	28.3	0.0	0.0	9.5	6.7	60.6	63.9	0.4	1.0	79.3	81.2	0.49	0.44
F_{100}	13.6	13.5	0.0	0.0	3.9	3.2	82.3	83.3	0.3	0.0	84.3	85.2	0.17	0.16

Values presented as mean (n = 2). The subscript in proceeding F denotes the percentage of ethanol at which arabinoxylan oligosaccharide precipitated. ** being raw arabinoxylan oligosaccharide. * denotes the source of xylanase used to produce the arabinoxylan oligosaccharide. Arabinoxylan was calculated using the formula ((% arabinose + % xylose)*0.88).

Table 5.3: Esterified ferulic acid (feruloylated oligosaccharides) and free ferulic acid content (µg) in 1 mg oligosaccharide arabinoxylan

	* *N. patriciarum*	* *T. viride*	* *N. patriciarum*	* *T. viride*
F20	6.3 ± 0.4^{bC}	14.1 ± 1.0^{aCD}	1.2 ± 0.2^{aAB}	Nd
F30	4.9 ± 1.3^{bDE}	13.0 ± 0.8^{aCD}	1.3 ± 0.5^{aAB}	0.6 ± 0.6^{bABC}
F40	6.9 ± 1.2^{bBC}	12.8 ± 0.3^{aD}	1.1 ± 0.2^{aAB}	1.1 ± 0.5^{aABC}
F50	4.4 ± 0.9^{bDE}	8.0 ± 0.1^{aEF}	1.8 ± 0.8^{aA}	0.5 ± 0.4^{bBC}
F60	3.3 ± 0.2^{bE}	11.4 ± 0.8^{aD}	1.4 ± 0.0^{aAB}	Nd
F70	2.8 ± 3.9^{bCD}	18.9 ± 1.3^{aA}	0.4 ± 0.6^{aBC}	Nd
F80	5.2 ± 0.2^{bD}	15.6 ± 0.9^{aBC}	0.9 ± 0.1^{aBC}	Nd
F100	12.2 ± 1.1^{bA}	17.8 ± 0.7^{aA}	Nd	Nd
** F_0	8.0 ± 0.6^{bB}	15.2 ± 0.2^{aC}	0.7 ± 0.1^{aC}	Nd

Values presented as mean ± standard deviation (n = 2). Data in the same column with the same superscript (capital letter) are not significantly different at $p \leq 0.05$. Data in the same row with the same superscript (small letter) within the same variable are not significantly different at $p \leq 0.05$.

* Denotes the source of xylanase used to produce the arabinoxylan oligosaccharide. The subscript in proceeding F denotes the percentage of ethanol at which arabinoxylan oligosaccharide precipitated.

Table 5.4: Antioxidant capacity (µM Trolox equivalent) of 1 mg of oligosaccharide arabinoxylan.

	DPPH antioxidant capacity		$ABTS^+$ antioxidant capacity		ORAC antioxidant capacity	
	* *N. patriciarum*	* *T. viride*	* *N. patriciarum*	* *T. viride*	* *N. patriciarum*	* *T. viride*
F20	$19.2.0 \pm 0.6^{bC}$	36.4 ± 1.6^{aC}	$72.69.4 \pm 7.8^{bC}$	177.1 ± 15.3^{aAB}	190.7 ± 8.4^{bC}	261.0 ± 8.6^{aC}
F30	15.8 ± 0.3^{bCD}	28.4 ± 1.6^{aD}	92.0 ± 6.2^{bB}	130.1 ± 8.9^{aD}	205.9 ± 3.0^{bC}	310.4 ± 5.6^{aB}
F40	16.2 ± 0.3^{aCD}	23.6 ± 2.4^{aE}	98.3 ± 7.8^{bB}	137.4 ± 19.8^{aD}	135.9 ± 2.2^{bD}	257.8 ± 31.3^{aC}
F50	12.2 ± 0.8^{bE}	23.2 ± 2.4^{aE}	113.1 ± 6.6^{aAB}	130.8 ± 14.5^{aD}	100.6 ± 8.7^{aE}	144.8 ± 5.0^{aD}
F60	10.6 ± 0.3^{bE}	36.4 ± 0.8^{aC}	48.5 ± 3.9^{bD}	114.3 ± 31.8^{aDE}	59.0 ± 4.2^{bF}	246.8 ± 9.2^{aC}
F70	10.6 ± 2.0^{bE}	56.4 ± 2.4^{aA}	41.4 ± 2.4^{bD}	178.8 ± 0.0^{aBC}	58.0 ± 0.0^{bF}	337.6 ± 20.0^{aB}
F80	19.2 ± 2.8^{bC}	49.6 ± 1.6^{aB}	67.8 ± 5.3^{bC}	129.9 ± 1.7^{aD}	129.5 ± 3.7^{bD}	397.0 ± 11.2^{aA}
F100	37.4 ± 3.1^{bA}	52.4 ± 0.4^{aAB}	116.9 ± 11.9^{aAB}	230.0 ± 38.0^{aA}	268.3 ± 11.9^{aA}	374.6 ± 60.0^{aA}
** F_0	28.0 ± 3.6^{bB}	41.6 ± 0.8^{aC}	125.4 ± 8.9^{bA}	183.1 ± 13.1^{aAB}	233.3 ± 8.7^{bB}	394.9 ± 38.2^{aA}

Values presented as mean ± standard deviation (n = 2). Data in the same column with the same superscript (capital letter) are not significantly different at $p \leq 0.05$. Data in the same row with the same superscript (small letter) within the same variable are not significantly different at $p \leq 0.05$. *Denotes the source of xylanase used to produce the arabinoxylan oligosaccharide. The subscript in proceeding F denotes the percentage of ethanol at which arabinoxylan oligosaccharide precipitated. ** F0 is unfractionated arabinoxylan oligosaccharide. ‘‘nd’’ means not detected.

Figure 5.1: LC–UV/visible chromatogram of arabinoxylan oligosaccharides

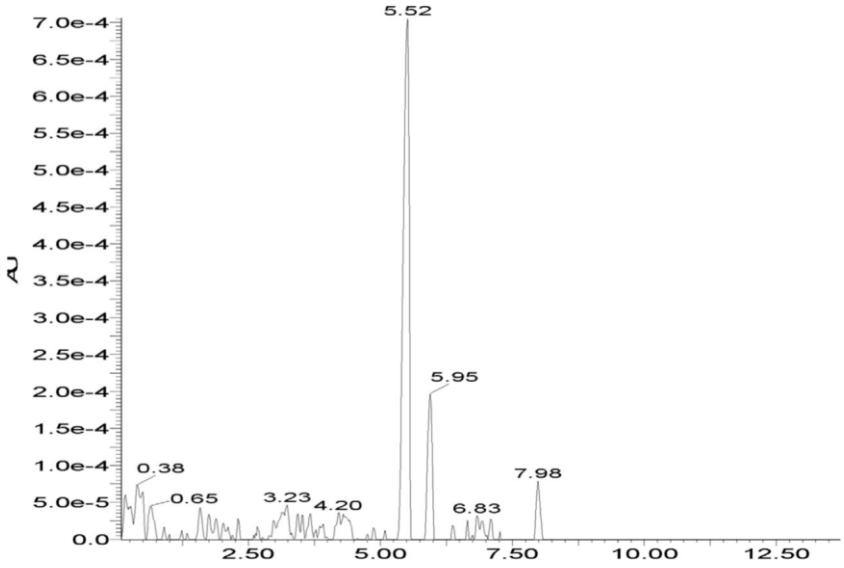
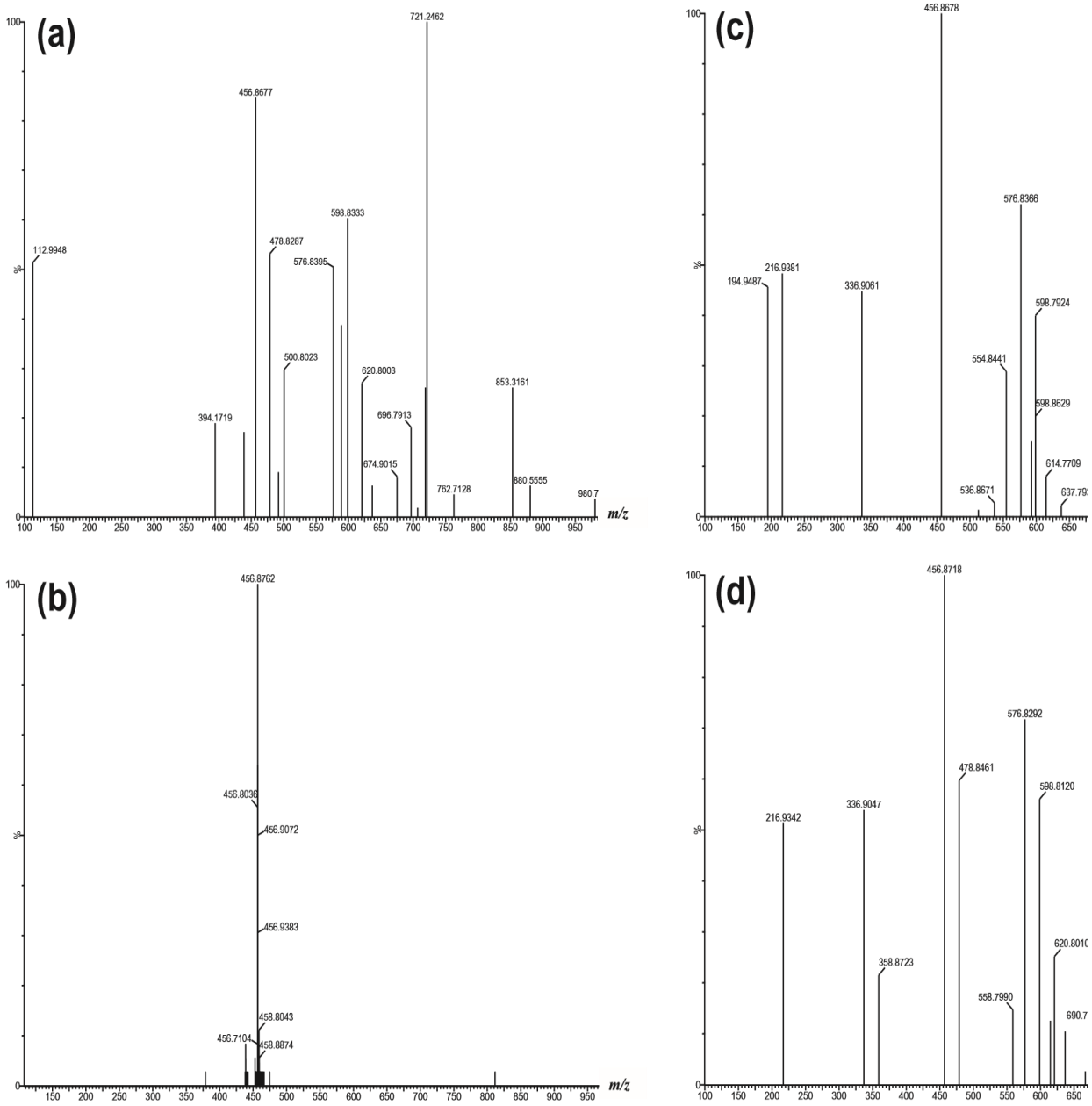


Figure 5.2: Negative ion mass spectra of arabinoxylan oligosaccharides for retention peak (a) 5.5; (b) 5.95; (c) 6.83 and (d) 7.98.



CHAPTER 6

6 Isolation and Identification of Feruloylated Arabinoxylan Mono- and Oligosaccharides from Undigested and Digested Maize and Wheat

(The manuscript has been accepted for publication in Heliyon)

6.1 Abstract

Feruloylated arabinoxylan mono- and oligosaccharides (F-AXOS) are a subject of interest because of their prebiotic and antioxidant properties. We aimed at isolating and identifying F-AXOS from maize, wheat, wheat bran and wheat aleurone using HPLC and LC-MS/MS. Prior to extraction of F-AXOS, samples were subjected to either simulated gastric fluid with enzymes (gastric) or without enzymes (pH) or water (aqueous) at 37 ^{o}C. F-AXOS present in all samples were identified as 5-O-feruloyl-α-L- arabinofuranose and possibly 5-O-feruloyl-α-L-arabinofuranosyl-(1→3)-O-β-D-xylopyranose. Their mean content, measured as esterified ferulic acid (FA), was 2.5 times higher in maize (10.33 ± 2.40 µg/ g) compared to wheat. Digestion under gastric or pH conditions resulted in a two-fold increase in F-AXOS in all samples. The level of F-AXOS produced during gastric or pH condition was positively correlated to the insoluble bound FA content of the sample (R^2 = 0.98). 5-O-feruloyl-α-L- arabinofuranose was the only identifiable F-AXOS released during gastric digestion. Our results suggest feruloyl arabinose is the most abundant form of F-AXOS in maize and wheat.

Key words: Feruloylated arabinoxylan oligosaccharide, feruloyl arabinose, maize, wheat, gastric digestion

6.2 Introduction

Arabinoxylan oligosaccharides (AXOS) are a subject of interest due to their perceived health benefits (Ou, & Sun, 2014). AXOS have been shown to possess prebiotic (Damen, Cloetens, Broekaert, François et al., 2012; Yang, Maldonado-Gómez, Hutkins, & Rose 2014; Yuan, Wang, & Yao, 2005) and antioxidant properties (Katapodis, Vardakou, Kalogeris, Kekos, Macris, & Christakopoulos, 2003; Lin, Ou, & Wen, 2014; Ohta, Semboku, Kuchii, Egashira, & Sanada, 1997; Yuan, Wang, Yao, & Chen, 2005) both *in vivo* and *in vitro* studies. Consumption of 2.4 g AXOS per day for 3 weeks significantly increased bifidobacteria and short chain fatty acid concentrations in healthy individuals (Damen et al., 2012). Moreover, dietary supplementation with 1% (w/w) feruloylated arabinoxylan oligosaccharides (F-AXOS) increased the antioxidant potential of plasma in rats (Wang, Sun, Cao, & Tian, 2009). The F-AXOS antioxidant potential is dependent on ferulic acid content, degree of substitution and polymerization (Chapter 5; Snelders, Olaerts, Dornez, Van de Wiele, Aura, Vanhaecke, Delcour, & Courtin 2014). Thus, F-AXOS obtained from ragi, rice, maize, and wheat brans had significantly different antioxidant potential (Veenashri & Muralikrishna, 2011).

F-AXOS are produced through enzymatic (endoxylanase) or mild acid hydrolysis of arabinoxylan polysaccharides. In general, arabinoxylans contain a linear (1-4)-β-D-xylopyranose chain that is substituted with L-arabinofuranose at O-2 and/or O-3 position (Lequart, Nuzillard, Kurek, & Debeire, 1999). The xylan chain can also be substituted with α-(1,2)-glucuronic acid and/or α-(1,2)-4-O-methylglucuronic acid branches (Ma et al., 2012). The arabinoxylans may be feruloylated with ferulic acid at the O-5 position of the arabinose units (Bunzel, Ralph, & Steinhart, 2005). The length and sugar constituents of feruloylated side chain varies, however, the most

common ones are mono- and disaccharides of arabinose and xylose (Ishii, 1997; Smith & Hartley, 1983; Wende & Fry, 1997).

A method to classify ferulic acid in cereal grain was recently proposed by Vaidyanathan and Bunzel (2012). Among four of those classes was ferulic acid esterified to mono-/ oligosaccharides which is identical to conjugated or soluble bound ferulic acid. Soluble bound or conjugated ferulic acid has been studied for over three decades, however, the nature or type of mono-/ oligosaccharide to which the ferulic acid is bound is not well documented. Feruloyl oligosaccharides reported in literature are usually a product of mild acid (Ohta et al., 1997; Wende & Fry, 1997) or enzyme treatment (Ahluwalia & Fry, 1986).

Arabinoxylans are generally considered as dietary fibers which are expected to transverse the digestive system unaltered chemically unless fermented by the resident microbes in the colon. Arabinose linkages of the arabinoxylan are susceptible to low gastric pH during digestion (Zhang, Zhang, & Whistler, 2003). Thus exposure of cereal grains to low gastric pH may partially release feruloyl arabinose since ferulic acid is bound to O-5 position of the arabinose units. Feruloyl arabinose may be bound to glycoprotein (Obel, Porchia, & Scheller, 2003). It is likely that low gastric pH and/or protein hydrolysis will increase F-AXOS content during gastric digestion. However, data on release of F-AXOS during gastric digestion is limited.

Wheat and maize are among the highly consumed cereal grains in the world. Maize arabinoxylans have higher ferulic acid, di-ferulic acid, tri-ferulic acid (Bunzel, 2010; Jilek & Bunzel, 2013) and degree of substitution (Knudsen, 1997) compared to wheat arabinoxylans. The percentage of unsubstituted, mono-substituted, and di-substituted xylose in maize arabinoxylans may be 24, 52 and 24, respectively (Rumpagaporn et al., 2015). In contrast, water extractable arabinoxylans from

wheat contains about 60 - 65% unsubstituted xylose (Saulnier, Sado, Branlard, Charmet, & Guillon, 2007). Hence maize is considered to have a more complex arabinoxylan structure compared to wheat (Pedersen et al., 2015). Moreover among grain botanical fractions, arabinoxylans from wheat bran have higher degree of substitution and di-ferulic acid compared to that of aleurone (Antoine et al., 2003; Saulnier et al., 2007). The complexity of the structure affects susceptibility of arabinoxylans to microbial fermentation (Pedersen et al., 2015) or hydrolysis by endoxylanase (Biely, Vršanská, Tenkanen, & Kluepfel, 1997).

Therefore, this work aimed at isolating and identifying feruloylated arabinoxylan mono-/ oligosaccharide (F-AXOS) from maize, wheat and wheat aleurone and bran. The effect of gastric digestion on F-AXOS content was also investigated.

6.3 Materials and Methods

6.3.1 Materials

Pepsin from porcine gastric mucosa (P7000-25G), α-amylase from porcine pancreas (A3176-1MU), phenolic acid standards and XAD2 resin were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA). Sodium hydroxide, sodium chloride, sodium sulphate, all acids and organic solvents were obtained from Fischer Scientific (Whitby, Ontario, Canada). All chemicals and solvents used were of analytical or HPLC grade.

6.3.2 Samples

Four whole grain wheat, five whole grain maize, one wheat aleurone and one wheat bran samples were used. One yellow Argentinian flint maize (DASCA) was grown in USA copper belt while orange (MW5021) and white maize samples were grown in a mountainous region (Dedza District) in Malawi. Another sample set of orange (MW5021) and white maize samples were also grown in

a plain region (Ekwendeni, Mzimba District) in Malawi. The Malawi maize samples were all flint type. The four wheat samples (purple wheat, Caledonia, Ambassador, and MSUD8006) grown in Michigan, U.S.A. were all soft wheat varieties. A commercial wheat aleurone (Grainwise™ wheat aleurone) was a gift from Cargill Limited from Horizon Milling (Wichita, Kansas, USA). Red winter wheat bran was purchased locally from Bulk Barn (Winnipeg, Manitoba, Canada). Samples were ground using a laboratory coffee grinder to pass through sieve number 35 (0.5 mm). Samples (Table 6.1) were analyzed for their protein, ash, and moisture content using AOAC methods. Samples were kept in a desiccator at -20 ^{o}C for isolation of feruloylated arabinoxylan mono-/ oligosaccharides. Five subsamples from each grain or bran or aleurone were collected and used during the outlined experiments.

6.3.3 Isolation of feruloylated arabinoxylan mono-/ oligosaccharides from untreated whole grains (maize and wheat), wheat aleurone and wheat bran

The method of Vaidyanathan and Bunzel (2012) was used with modification. Flour samples (20 g) were mixed with 20:80 water: ethanol (250 mL) and boiled under reflux for 30 minutes to deactivate endogenous enzymes. The cooled samples were centrifuged (10 000 g, 4 ^{o}C and 20 minutes) and supernatant collected. The residues were washed twice with 200 mL 80% ethanol and centrifuged to collect the supernatants. Ethanol (80%) is essential to avoid solubilisation of arabinoxylan polysaccharide. The residues were air dried for further experiments and labelled as deactivated ground samples. The pooled supernatants were evaporated to dryness under vacuum using a rotary evaporator (Buchi rotavapor R – 205 (Laboratoriums Technik AG, Flawil, Switzerland)) at 40 ^{o}C. The dried supernatants were reconstituted with 20 ml ultrapure water and filtered through Whatman filter paper No. 41 to remove the gummy residues. The filtrates were kept at -20 ^{o}C for further purification and analysis of F-AXOS.

6.3.4 Extraction of feruloylated arabinoxylan mono-/ oligosaccharides following gastric digestion and pH treatment of deactivated ground whole grains, wheat aleurone and wheat bran

Simulated gastric digestion was done according to Chandrasekara and Shahidi (2012) with modifications. Briefly, deactivated ground samples (5 g) were mixed with 45 mL ultrapure water and 30 mL 0.15M sodium chloride solution. The mixture was incubated at 37 ^{o}C for 10 minutes under continuous shaking in a water bath. Three milliliters of porcine amylase in 5 mg/ mL sodium phosphate buffer (20 mM, pH 6.9, 1 mM calcium chloride) was then added. After 5 minutes incubation, 9.5 mL hydrochloric acid (0.15 M) was added and pH adjusted to 2. Porcine pepsin (3 mL, 40 mg/ mL 20 mM HCl) was also added prior to incubation at 37 ^{o}C for 1 hour. The samples were then boiled at 95^{o}C for 5 minutes to deactivate the enzymes. Incubation of samples at 95^{o}C for 5 minutes was verified in our laboratory not to result in release of F-AXOS. The samples were freeze dried and labeled simulated gastric digesta (SG). Control samples (aqueous treated, AG) were treated similarly to SG except that only ultrapure water was being added at every step. Another group of samples (pH treated, pH) were treated likewise except that only buffers were being added without enzymes.

Feruloylated arabinoxylan mono-/ oligosaccharides (F-AXOS) were extracted thrice from each sample using 80% ethanol. Briefly, ~2.0 g freeze dried samples (AG, SG and pH treated) were mixed with 25 ml 80% ethanol and shaken continuously on a wrist shaker (250 rpm) for an hour at room temperature. The suspension was centrifuged at 10000 g for 10 minutes at room temperature. The supernatants were pooled together and passed through Whatman filter paper (No. 41) to a round bottom evaporating flask. A Buchi rotavapor R – 205 (Laboratoriums Technik AG, Flawil, Switzerland) was used to remove ethanol at 40 ^{o}C and 200 rpm. The samples were

reconstituted in 20 ml ultrapure water and kept at -20 ºC for further purification and analysis of F-AXOS.

6.3.5 Determination of free, mono / oligosaccharide (soluble) or insoluble bound ferulic acid content

A modification of the method reported by Vaidyanathan and Bunzel (2012) was used to estimate esterified ferulic acid content in F-AXOS extracts. Five milliliter of the reconstituted samples was transferred to two 50 ml conical flasks for extraction of free ferulic acid and bound ferulic acid. The reconstituted samples in the first flask were acidified with 6M hydrochloric acid to pH 1.5 and free phenolic acids were extracted thrice using 25 mL ethyl acetate. To the second flask, 4 M sodium hydroxide (5 mL) was added and incubated under nitrogen in the dark for 2 hours. Phenolic acids were extracted thrice using a total of 75 mL ethyl acetate after adjusting pH to 1.5. Ethyl acetate was evaporated under vacuum and samples reconstituted with 2 ml water: methanol (50:50). Phenolic acids were quantified using HPLC method as described by Malunga and Beta, 2015b. The difference in ferulic acid concentration between free phenolic acid (flask 1) and total phenolic acid (flask 2) was considered the amount of ferulic acid esterified to mono-/ oligosaccharides (esterified FA or soluble bound FA). Insoluble bound ferulic acid was determined by first saponifying 200 mg of deactivated ground samples with 5 mL 2M sodium hydroxide under nitrogen at 4 ºC for overnight. The mixture was acidified by 6M hydrochloric acid to pH 1.5 and centrifuged at 10000 g for 10 minutes at 4 ºC. The residue was washed twice with 5 mL ultrapure water and the supernatants pulled together. Ferulic acid was extracted 3 times using 50 mL ethyl acetate. Ethyl acetate was evaporated under vacuum and samples reconstituted in 2 ml water: methanol (50:50) for analysis of ferulic acid. The insoluble bound ferulic acid include both ferulic acid esterified to water soluble and insoluble arabinoxylan.

6.3.6 Identification of feruloylated arabinoxylan oligosaccharides (F-AXOS) using LC-MS

The reconstituted sample from sections 2.3 or 2.4 (10 mL) was further purified on 80 mL XAD2 resin column as described earlier (Saulnier, Vigouroux, & Thibault, 1995). XAD2 column was preconditioned with 1 column volume ethanol (80 mL) followed by 25 mL ultrapure water. After sample application, it was eluted with 1 column volume water to remove sugars, 1 column volume methanol: water (50:50) and 1 column volume methanol. Both free and bound ferulic acids are adsorbed to XAD2 resin (Saulnier et al., 1995). Elution with 50% methanol liberates more F-AXOS than free ferulic acid. Further purification with LH20 or preparative reverse phase HPLC is usually deployed to obtain pure F-AXOS but this was not necessary as identification was carried out on LC-MS. The 50% methanol eluent was collected and evaporated to dryness in a rotary evaporator (40 °C). F-AXOS extracts were reconstituted with 3 mL methanol: water (50:50) and kept at -20 °C. F-AXOS extracts were separated with reverse phase column (5.0-µm Phenomenex C18 column (150 X 4.6 mm)) using high performance liquid chromatograph (Waters Alliance 2695 instrument (Waters, Milford, MA)) system as described by Malunga and Beta (2015b). The F-AXOS were identified by introducing HPLC eluent into the mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA) using electrospray ionization (ESI) in negative mode. The operating conditions were set as follows: desolvation temperature 300 °C; source temperature, 120 °C spray voltage; capillary voltage, 1.2 kV; sample cone voltage, 45 V; extraction cone voltage, 4 V; cone gas flow, 50 L/hr; and desolvation gas flow 900 L/hr. The collision energy for MS/MS was 20 V.

6.3.7 Statistical analysis

All analyses were performed in quintuplicate and all statistics were calculated using one way analysis of variance (ANOVA) on a JMP 10 statistical software (SAS Institute Inc., Cary, NC).

Sample means were compared using Tukey HSD method and significant differences determined at $p \leq 0.05$.

6.4 Results and Discussion

6.4.1 Estimation of free ferulic acid, soluble or insoluble bound ferulic acid content

Ferulic acid is the most abundant in cereal grains and found as free or soluble or insoluble bound. We analyzed free, soluble or insoluble bound ferulic acid content in maize and wheat grain and the results are presented in Table 6.2. Our results suggest that the content of free, soluble or insoluble bound ferulic acid did not vary significantly within flint maize nor within soft wheat samples. The mean total ferulic acid content in maize was twice that found in wheat samples. The majority of the ferulic acid (99%) was insoluble bound in both maize and wheat. Ferulic acid is mostly bound to arabinoxylans of the cell walls. This was reflected in the high content of total ferulic acid in the wheat aleurone (~4000 µg/g) and wheat bran (~2400 µg/g) samples. Our results are within the reported ranges of ferulic acid content in wheat (Li, Shewry, & Ward, 2008) and maize (Lopez-Martinez et al., 2009).

Wheat had higher free ferulic acid (1.5 times) but less esterified or soluble-bound FA (3 times) compared to maize (Table 2). Also the proportion of free ferulic acid to soluble-bound FA in wheat (1:1) is different from that of maize (1:4). Free ferulic acid has low solubility (0.6 mg/ mL) under physiological conditions (Mota, Queimada, Pinho, & Macedo, 2008). However, the solubility of ferulic acid can increase to 10 mg/ mL when it is esterified to arabinose (Fang et al., 2013). Thus having higher concentration of feruloyl mono-/ oligosaccharide may be advantageous on bio-accessibility of ferulic acid. Dedza grown white maize (~13 µg/ g) had highest concentration of esterified FA and yellow maize (DASCA) (~7 µg/ g) had the lowest among the maize cultivars. The mean concentration of esterified FA was not significantly different for maize (white or

orange). Among wheats, the difference in mean concentration of esterified FA in wheat cultivars was not significant ($p < 0.05$). The outer layers (wheat bran or wheat aleurone) had higher content of esterified FA compared to both whole grain maize and wheat. It is well documented that wheat genotypes have wide range of ferulic acid concentration (Li et al., 2008).

6.4.2 Identification of the feruloylated mono- and oligosaccharides

This work aimed at isolating and identifying F-AXOS from maize, wheat and wheat aleurone and bran. XAD2 resins was used to isolate F-AXOS. The LC-MS was used to identify F-AXOS present in our extract. The presence of ferulic acid esterified to sugars enables detection at 325 nm using photodiode array detector. Peaks having a maximum absorbance of 320 – 325 were considered as belonging to F-AXOS. Two peaks (Figure 6.1a) with retention times of 21.05 and 28.52 minutes met this criterion and were labeled F1 and F2, respectively. However, we observed that peak F1 was not present in UV spectra for F-AXOS extracts from digestion or pH treated samples (Figure 6.1b). F2 was the most prevailing compound in our extract judging by its highest peak area (Figure 6.1a).

The negative ESI mass spectra for F1 and F2 showed a compound with m/z 457 (Figure 6.2a) and m/z 325 with some traces of m/z 457 (Figure 6.2b), respectively. Thus compound F1 had a molecular weight of 458 and F2 of 326 Da. The generally accepted structure of arabinoxylan is that ferulic acid is always attached to the O-5 position of the arabinose units (Izydorczyk & Biliaderis, 1995). Our results suggest that the predominant compound in our extract was a feruloyl arabinose (F2). Furthermore, the mass spectra for F1 suggest that our extract contained a disaccharide (xylose or arabinose) esterified to ferulic acid. Feruloyl arabinose (5-O-feruloyl-L-Ara*f*) and feruloyl arabinofuranosyl xylose have been isolated from maize or wheat bran through acid or enzymatic hydrolysis (Lin et al., 2014; Saulnier et al., 1995; Smith & Hartley, 1983).

Compound F1 was fragmented under negative ion mode to confirm the structural identity and the resulting ms/ms spectrum is shown in Figure 6.3a. Fragment ions m/z 397 and 367 indicated cross link cleavage at $^{0,2}A_2$ and $^{2,4}A_2$ of the xylopyranose resulting in loss of $C_2H_4O_2$ and $C_3H_6O_3$, respectively as illustrated in Figure 6.3b. Secondly, fragment ion m/z 325 suggests glycosidic cleavage occurred at $C_{1\alpha}$ and consequently losing a dehydrated xylopyranose ion (m/z 132). Cross ring and glycosidic cleavage is a common phenomenon during negative ion fragmentation of sugars (Wang et al., 2009). Occurrence of cross link cleavage resulting in loss of both $C_2H_4O_2$ and $C_3H_6O_3$ may suggest (1→3) arabinose – xylose linkage. Finally, ferulic acid fragment ion (m/z 193) was observed and typified by its daughter ions m/z 175, 159 and 134 (Sun, Liang, Bin, Li, & Duan, 2007). Thus we concluded that compound F1 was a feruloyl arabinofuranosyl xylose. The fragmentation pattern and literature, F1 is likely to be a 5-O-feruloyl-α-L-arabinofuranosyl-(1→3)-O-β-D-xylopyranose.

We also fragmented the parent ion for compound F2 (m/z 325) in order to confirm our proposed identity (5-O-feruloyl-L-Ara*f*). Figure 6.4 shows the MS/MS spectra of the daughter ions for compound F2. Cross ring cleavage of the sugar moiety was observed giving fragment ions of m/z 265 and m/z 59 (Figure 6.4b). Our data also suggested further dissociation of daughter ion (m/z 265) at sugar-ferulic acid ester linkage resulting in ferulic acid (m/z 193) and a remaining cross ring fragment of arabinose (m/z 71). It appears that ferulic acid was further fragmented as shown by the presence of ions with m/z 175, 149 and 134. Ferulic acid may lose CO_2 from carboxylic acid and/or CH_3 during fragmentation (Sun et al., 2007). Thus we concluded that the compound F4 is in fact 5-O-feruloyl-α-L- arabinofuranose.

Our results suggest that the 5-O-feruloyl-α-L-arabinofuranosyl-(1→3)-O-β-D-xylopyranose as the feruloyl oligosaccharide present in maize or wheat grains but at low concentrations. Although the

concentration of 5-O-feruloyl-α-L-arabinofuranose is relatively high, it does not meet the criterion for oligosaccharides as it only has one sugar moiety. Feruloyl arabinoxylan oligosaccharides are feruloylated sugars with degree of polymerization of 2 – 10. Cereal grains contain endogenous xylanase enzymes that would act on arabinoxylan polysaccharides to produce arabinoxylan mono- / oligosaccharide (Cleemput, Bleukx, Van Oort, Hessing, & Delcour, 1995). However, in this experiment, the samples were heat inactivated with ethanol to ensure that the observed F-AXOS were present naturally in cereal grain.

6.4.3 Effect of gastric digestion on feruloyl mono- and oligosaccharides content

Bio-accessibility of phenolic compounds increases as the food passes through gastro-intestinal tract following hydrolysis of the macronutrients (Chandrasekara & Shahidi, 2012; Gawlik-Dziki, Dziki, Baraniak, & Lin, 2009). Table 6.3 shows the content of F-AXOS (measured as esterified FA) following gastric digestion of wheat and maize sample. Esterified FA were present in all samples following gastric digestion but not in aqueous treated samples. The observed F-AXOS could be due to release of those bound to glycoproteins (Obel et al., 2003) and/or partial acid hydrolysis (Zhang et al., 2003) of feruloylated arabinoxylan. Thus another set of sample were treated with gastric pH conditions at 37 ^{o}C. Our results show no significant difference ($p \leq 0.05$) between the content of esterified FA of pH treated and gastric digested samples (Table 6.3). This does suggest that protein hydrolysis did not affect the esterified FA content but rather the low gastric pH. Arabinose linkages of arabinoxylans are susceptible to gastric pH (Zhang et al., 2003). We used 3 type of flint maize (white, orange and yellow) and 4 soft wheats (ambassador, Caledonia, MSDU and purple) in our study. The structure of arabinoxylans in maize and wheat has been reported to vary greatly in degree of substitution (Knudsen, 1997) and FA cross linkages (Bunzel, 2010). The mean degree of substitution of whole grain maize arabinoxylan is 0.7

(Huisman, Schols, & Voragen, 2000; Knudsen, 1997) and that for wheat is 0.6 (Gebruers et al., 2008; Knudsen, 1997). We observed a ~2 fold increase in esterified FA in all samples regardless of grain type.

The mean concentration of esterified FA produced during simulated gastric digestion of maize (9.5 µg/g) was about 2.5 times that of wheat. The difference in esterified FA concentration was not significant among the maize or wheat samples (Table 6.3). The order of esterified FA content produced was aleurone> bran> maize> wheat suggesting a possible association with insoluble bound ferulic acid in samples. Thus we performed a Pearson correlation analysis to establish the association between the insoluble bound ferulic acid and esterified FA produced during simulated gastric digestion. A strong positive correlation (R^2= 0.98) was observed between the content of insoluble bound ferulic acid and esterified FA. Cereal grains or their fractions with highest insoluble bound ferulic acid are likely to result in high concentration of esterified FA when exposed to gastric conditions.

Also considering that peak F1 was not present in pH or gastric treated samples, our result may suggest that feruloyl arabinose is the F-AXOS released during gastric digestion. Hence we concluded that gastric pH released the feruloyl arabinose from the feruloylated arabinoxylans present in the cereal grains. Also assuming that all the esterified FA originated from F-AXOS, we concluded that F-AXOS may be doubled during gastric digestion.

6.5 Conclusion

In this work, feruloylated arabinoxylan mono- and oligosaccharides were isolated from maize and wheat. These were identified to be 5-O-feruloyl-α-L-arabinofuranosyl-(1→3)-O-β-D-xylopyranose and 5-O-feruloyl-α-L- arabinofuranose using HPLC-MS/MS. F-AXOS content (measured as esterified FA) was significantly higher in maize compared to that of wheat. Gastric

pH conditions resulted in over two-fold increase in F-AXOS content. 5-O-feruloyl-α-L-arabinofuranose was the only identifiable F-AXOS released during gastric digestion.

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6.7 Reference

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Table 6.1: Proximate composition (g/100g) of maize, wheat, wheat aleurone and wheat bran samples prior to aqueous, pH and gastric treatment for extraction of feruloylated oligosaccharides

	Moisture	**Ash**	**Protein**	**Lipid**
Wheat (Caledonia)	12.1 ± 0.3	1.6 ± 0.0	6.0 ± 0.7	2.0 ± 0.1
Wheat (MSDU)	12.3 ± 0.2	1.5 ± 0.0	7.5 ± 0.4	2.3 ± 0.1
Wheat (Ambassador)	12.1 ± 0.1	1.6 ± 0.0	6.9 ± 0.1	1.9 ± 0.2
Wheat (purple)	12.9 ± 0.5	1.7 ± 0.0	6.3 ± 0.1	2.1 ± 0.2
Aleurone (wheat)	8.6 ± 0.2	7.6 ± 0.0	12.4 ± 0.1	*
Red bran (wheat)	5.8 ± 0.0	5.3 ± 0.0	11.1 ± 0.2	*
Maize - white (Ekwendeni)	13.7 ± 0.8	1.4 ± 0.1	8.8 ± 0.4	5.8 ± 0.5
Maize - orange (Ekwendeni)	14.0 ± 1.4	1.3 ± 0.0	9.0 ± 0.4	5.3 ± 0.1
Maize - white (Dedza)	13.5 ± 0.8	1.4 ± 0.1	9.7 ± 0.7	5.6 ± 0.4
Maize - orange (Dedza)	13.3 ± 0.9	1.4 ± 0.2	8.9 ± 0.6	5.4 ± 0.7
Maize (DASCA)	11.5 ± 0.3	1.2 ± 0.0	6.8 ± 1.1	6.1 ± 0.2

Values presented as mean ± standard deviation (n = 5). * means that it was not determined

Table 6.2: Concentration of free ferulic acid, ferulic acid bound to mono-/ oligosaccharide (soluble) and insoluble bound ferulic acid (µg) per g flour of maize, wheat, wheat aleurone and wheat bran samples

	Free	**Mono-/ Oligosaccharide**	**Insoluble bound**
White maize (Ekwendeni)	2.93 ± 0.67^{cd}	9.44 ± 0.59^{b}	1273.45 ± 157.22^{c}
White maize (Dedza)	1.65 ± 0.35^{d}	13.54 ± 1.91^{a}	1582.51 ± 275.32^{c}
Orange maize (Ekwendeni)	3.47 ± 0.50^{c}	10.89 ± 1.31^{ab}	1622.36 ± 272.16^{c}
Orange maize (Dedza)	1.61 ± 0.23^{d}	10.82 ± 1.90^{ab}	1390.45 ± 212.24^{c}
Yellow maize (DASCA)	2.15 ± 0.41^{d}	6.95 ± 0.50^{bc}	1759.42 ± 300.15^{c}
Mean (maize)	**2.36 ± 0.81**	**10.33 ± 2.40**	**1525.64 ± 193.08**
Ambassador wheat	3.06 ± 0.23^{c}	3.91 ± 0.18^{d}	780.09 ± 80.35^{d}
Caledonia wheat	3.13 ± 0.18^{c}	3.38 ± 0.29^{d}	818.18 ± 150.76^{d}
MSDU wheat	3.92 ± 0.27^{c}	3.45 ± 0.21^{d}	773.77 ± 101.52^{d}
Purple wheat	3.81 ± 0.27^{c}	3.05 ± 0.23^{d}	584.82 ± 90.34^{d}
Mean (wheat)	**3.48 ± 0.39**	**3.37 ± 0.35**	**739.21 ± 104.79**
Aleurone (wheat)	6.42 ± 1.23^{ab}	13.86 ± 1.23^{a}	4064.97 ± 123.35^{a}
Red bran (wheat)	9.19 ± 0.93^{a}	15.87 ± 1.79^{a}	2377.03 ± 233.67^{b}

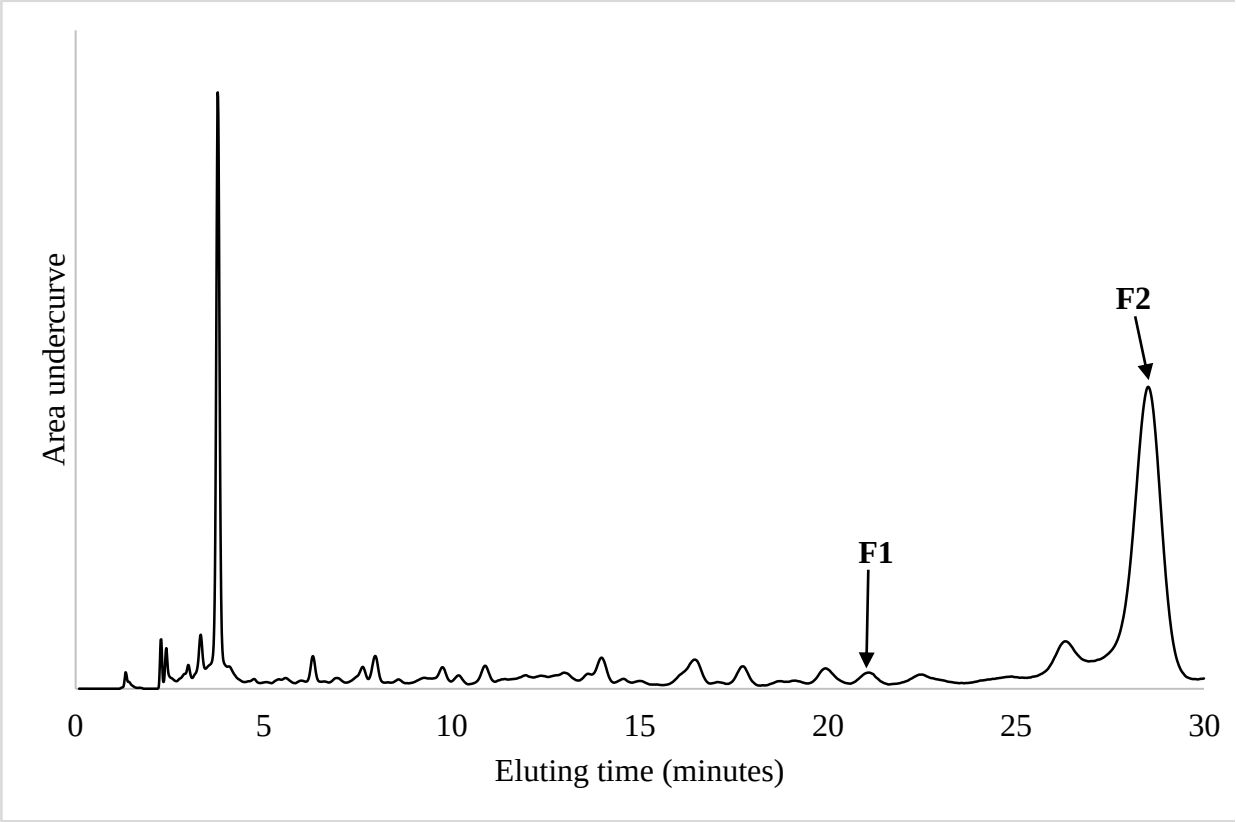
Values presented as mean ± standard deviation (n = 5). Data in the same column with the same superscript are not significantly different at $p \leq 0.05$.

Table 6.3: Concentration of mono-/ oligosaccharide esterified ferulic acid (µg) per g flour of maize, wheat, wheat aleurone and wheat bran samples following aqueous, pH and gastric treatment

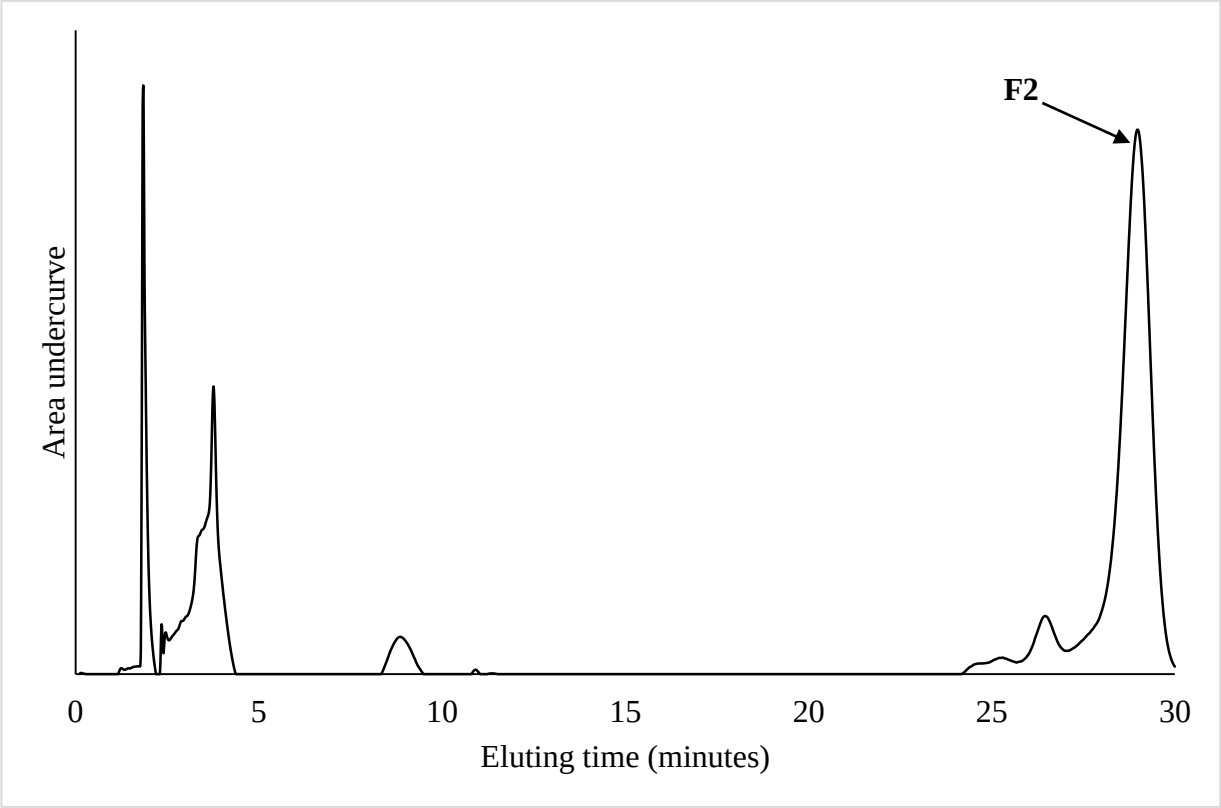
	Esterified ferulic acid (µg /g)*		
Treatment type	Aqueous	pH	Gastric digestion
White maize (Ekwendeni)	nd	8.37 ± 1.89	6.94 ± 1.33
White maize (Dedza)	Nd	10.24 ± 1.83	11.34 ± 1.64
Orange maize (Ekwendeni)	Nd	10.38 ± 1.77	11.57 ± 2.08
Orange maize (Dedza)	Nd	8.49 ± 0.62	11.62 ± 1.89
Yellow maize (DASCA)	nd	10.29 ± 0.90	8.27 ± 1.20
Mean (maize)		**9.55 ± 1.02**	**9.95 ± 2.19**
Ambassador wheat	nd	3.01 ± 0.25	3.58 ± 0.17
Caledonia wheat	nd	4.23 ± 0.15	4.61 ± 0.30
MSDU wheat	nd	3.52 ± 0.18	6.33 ± 0.24
Purple wheat	nd	3.59 ± 0.42	4.65 ± 0.37
Mean (wheat)		**3.59 ± 0.43**	**4.69 ± 1.01**
Aleurone (wheat)	nd	23.51 ± 2.32	17.83 ± 1.25
Red bran (wheat)	nd	13.28 ± 1.37	13.48 ± 1.93

Values presented as mean ± standard deviation (n = 5); nd – not detected.

Figure 6.1: Typical HPLC chromatograph of feruloyl arabinoxylan mono- and oligosaccharide extracts from wheat and maize samples. (a) Untreated sample; and (b) gastric digested sample. Peaks (F1 and F2) with maximum UV absorption spectra of 320 – 325 nm were considered feruloylated arabinoxylan mono-/ oligosaccharide.

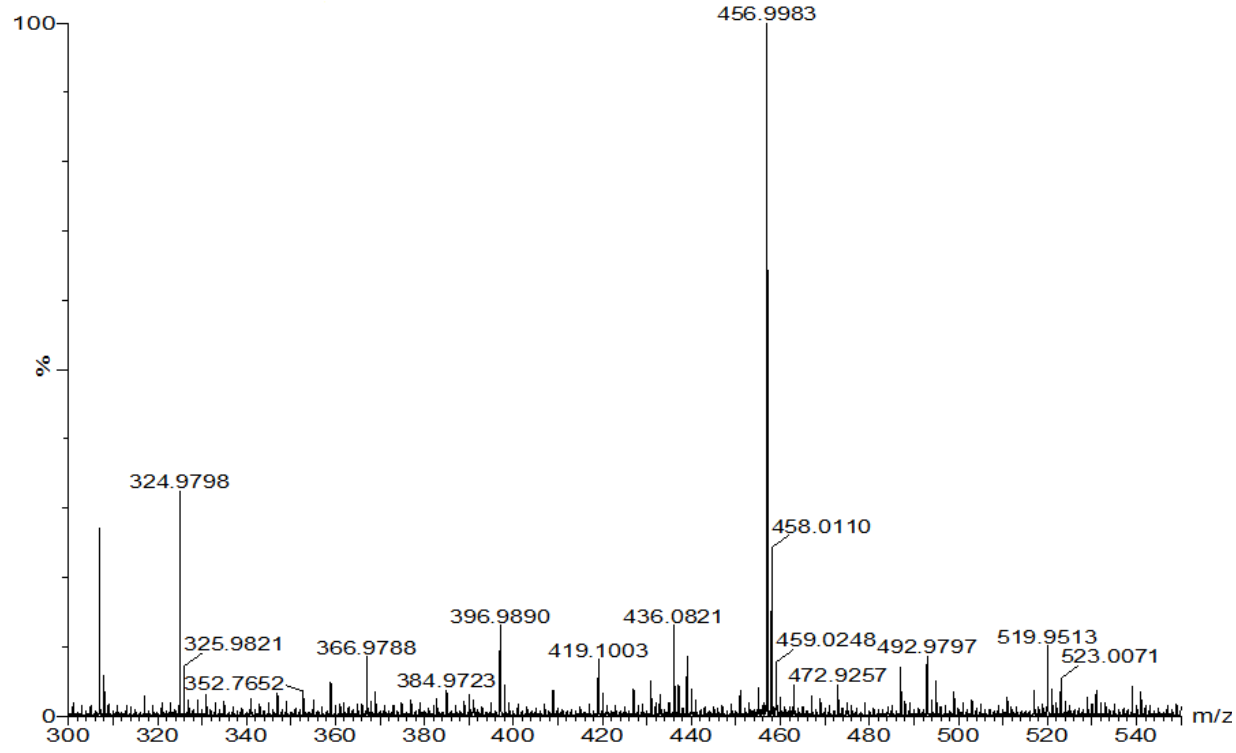


a)

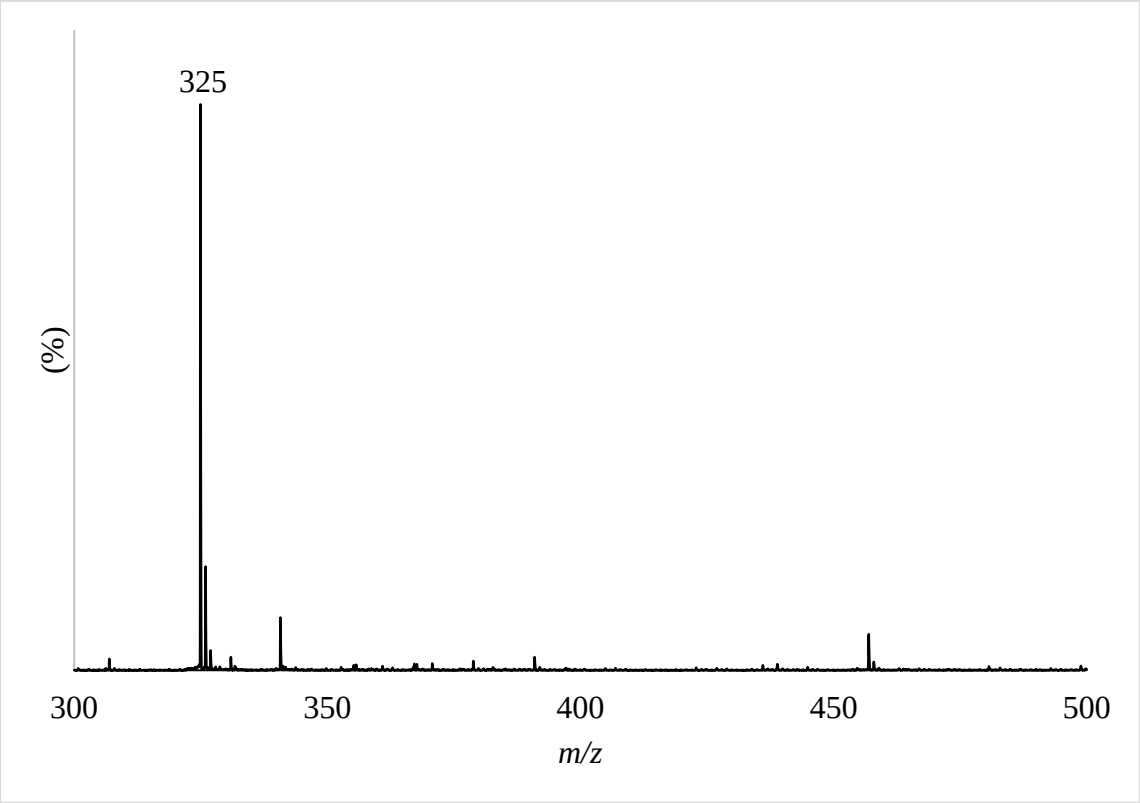


b)

Figure 6.2: Negative ion mass spectra of compound eluting at 21.05 minutes labeled F1, (a); and compound eluting at 28.52, F2, (b).

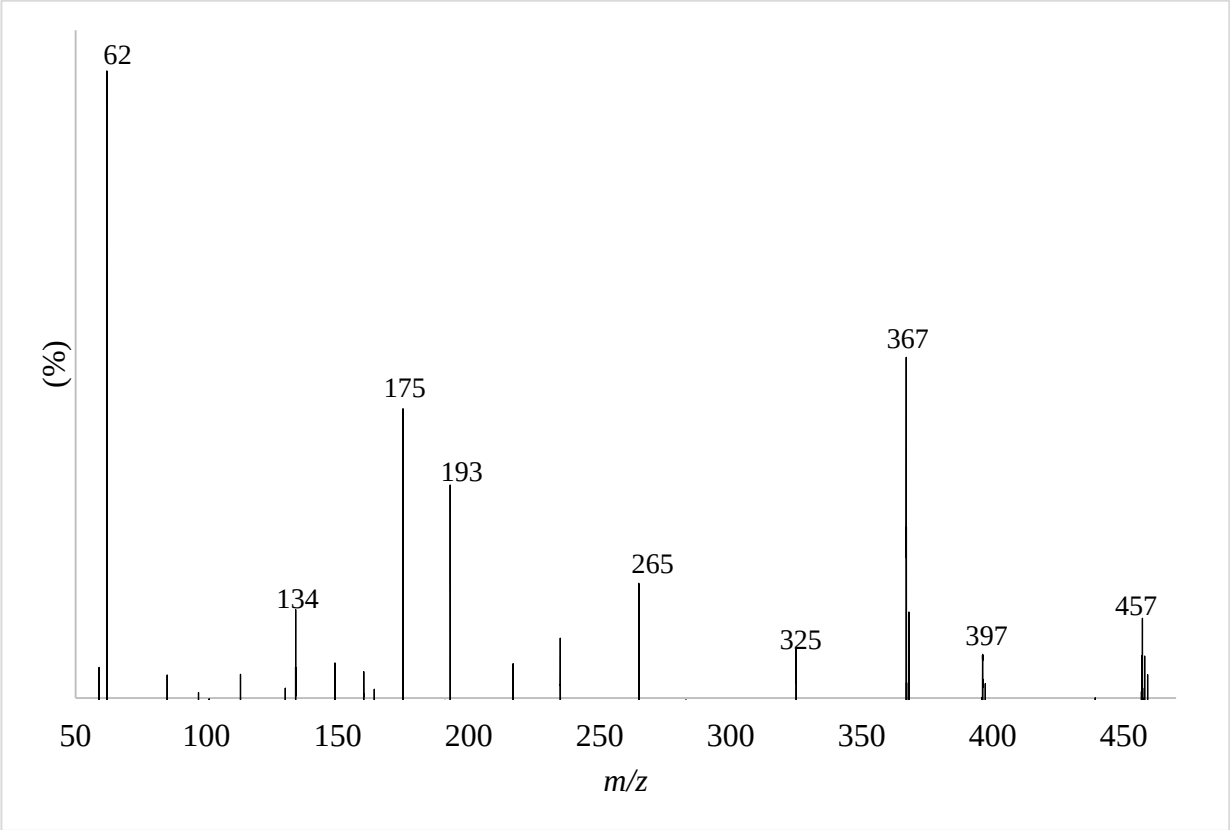


a)

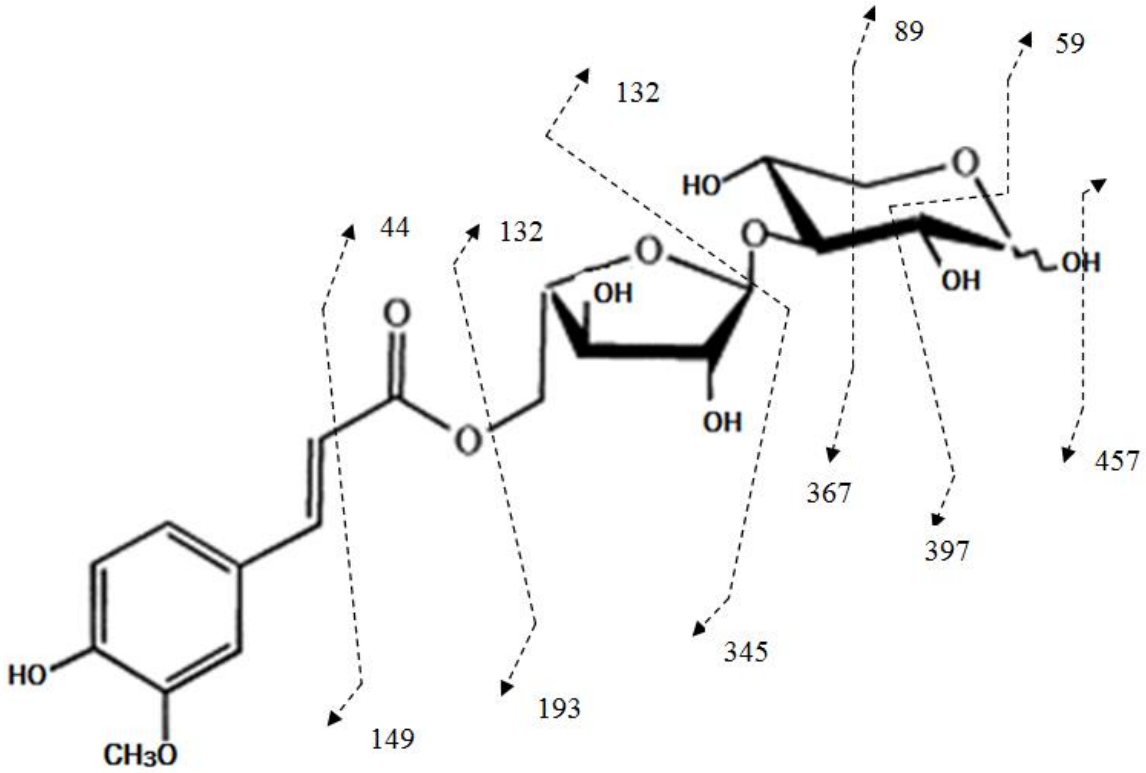


b)

Figure 6.3: (a) MS/MS spectrum of compound eluting at 21.05 minutes labeled F1 (m/z 457); (b) an illustration of fragmentation pattern of compound F1.

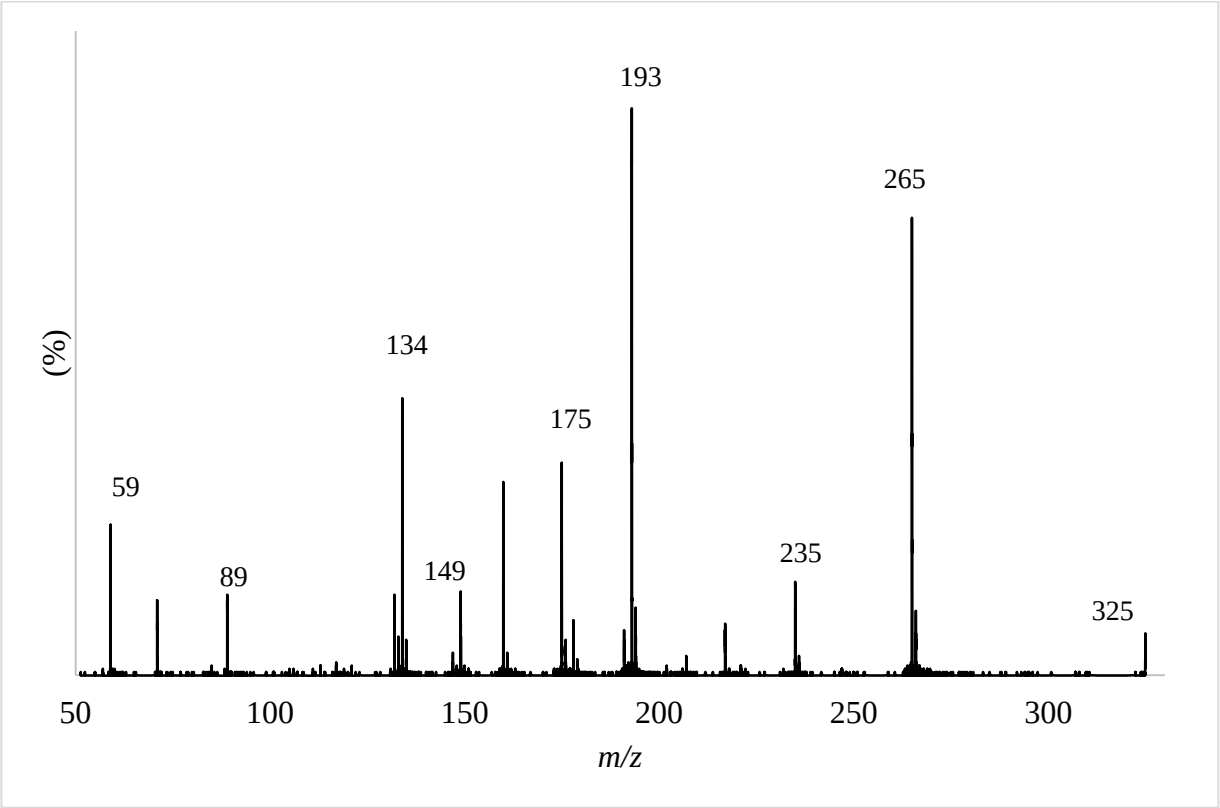


a)

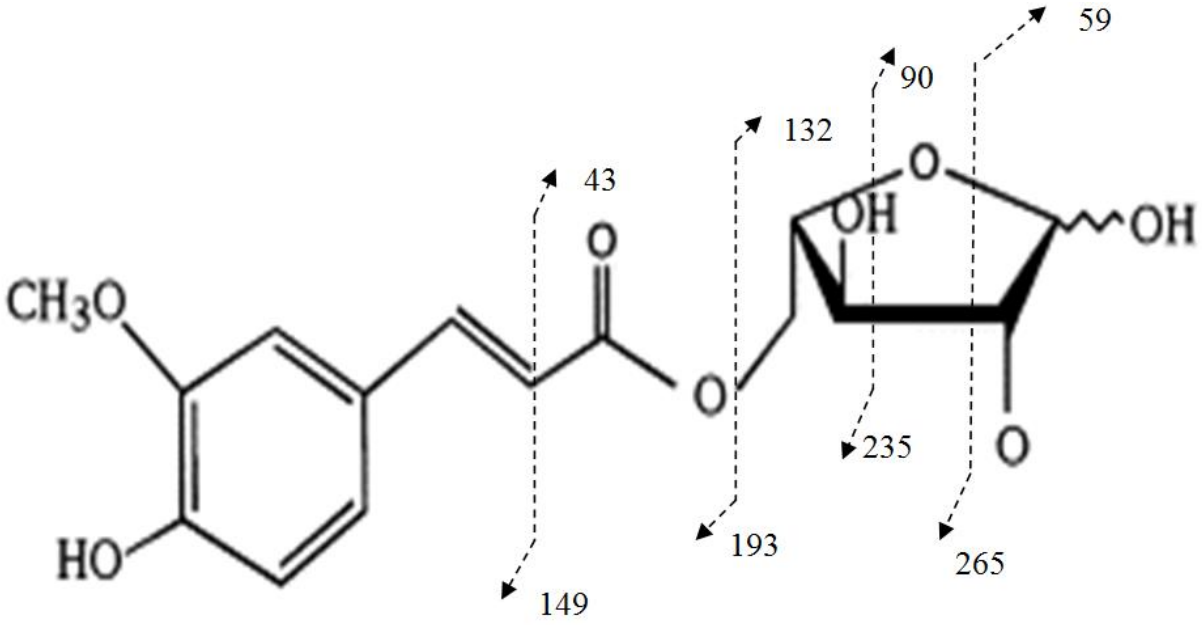

89
59
132
HO
O
O
OH
OH
44
132
O
OH
O
OH
457
367
397
345
193
149
HO
CH3O

b)

Figure 6.4: a) MS/MS spectrum of compound eluting at 28.52 minutes, labeled F2 (m/z 325); (b) an illustration of fragmentation pattern of compound F2.



a)



b)

CHAPTER 7

7 Inhibition of intestinal α-glucosidase and glucose absorption by feruloylated arabinoxylan mono- and oligosaccharides from corn bran and wheat aleurone

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7.1 Abstract

The effect of feruloylated arabinoxylan mono- and oligosaccharides (FAXmo) on mammalian α-glucosidase and glucose transporters was investigated using human Caco-2 cells, rat intestinal acetone powder and *Xenopus laevis* oocytes. The isolated FAXmo from wheat aleurone and corn bran were identified to have degree of polymerization (DP) of 4 and 1, respectively, by HPLC-MS. Both FAXmo extracts were effective inhibitors of sucrase and maltase functions of the α-glucosidase. The IC50 for FAXmo extracts on Caco-2 cells and rat intestinal α-glucosidase was 1.03 – 1.65 mg/mL and 2.6 – 6.5 mg/mL, respectively. Similarly, glucose uptake in Caco-2 cells was inhibited up to 40 %. The inhibitory effect of FAXmo was dependent on their ferulic acid (FA) content (R = 0.95). Sodium independent glucose transporter 2 (GLUT 2) activity was completely inhibited by FAXmo in oocytes injected to express GLUT2. Our results suggest that ferulic acid and feruloylated arabinoxylan mono-/oligosaccharides have potential for use in diabetes management.

Key words: Feruloylated arabinoxylan oligosaccharide, feruloyl arabinose, ferulic acid, α-glucosidase, glucose absorption, diabetes, GLUT2

7.2 Introduction

The burden of diabetes mellitus is expected to increase by 55% in 2035. Globally, the cost of diabetes treatment was over US$ 612 billion in 2014. Diabetes is a chronic disease epitomised by high circulating plasma glucose. Type 1 diabetes is caused by deficiency in insulin excretion by pancreatic beta cells whereas type 2 diabetes is a result of organs insensitivity to insulin. The normal blood glucose level is about 4 mM but increases to about 12 mM within 30 minutes after intake of high carbohydrate diet (Jenkins, Wolever, Taylor, Barker, Fielden, & Gassull, 1981; E. M. Wright, Martín, & Turk, 2003). High glucose concentration triggers secretion of insulin hormone which makes the liver to take up excess glucose for glycogen synthesis (glucose storage) and increase uptake of glucose by muscle cells through activation of GLUT4 (Vaulont, Vasseur-Cognet, & Kahn, 2000). Lapse in insulin response or production results in diabetes (Reaven, Bernstein, Davis, & Olefsky, 1976). Thus management of postprandial glucose is critical in prevention and treatment of type 2 diabetes patients. Decrease in postprandial hyperglycaemia can be attained by limiting intestinal carbohydrate digestion or uptake.

Starch and sucrose are the most common sources of dietary carbohydrates. Starch is digested primarily to maltose and other short chain carbohydrates by salivary and pancreatic amylase (Dona, Pages, Gilbert, & Kuchel, 2010). Human maltase-glucoamylase (MGAM) and sucrase-isomaltase (SI) (Sim, Willemsma, Mohan, Naim, Pinto, & Rose, 2010) are the small intestinal brush border glucosidases responsible for final digestion of dietary carbohydrates prior to their absorption. Sugar absorption in the small intestine mainly involves GLUT2, GLUT5 and SGLT1 transporters (Burant, Takeda, Brot-Laroche, Bell, & Davidson, 1992; Davidson, Hausman, Ifkovits, Buse, Gould, Burant, et al., 1992; E. M. Wright, Loo, Panayotova-Heiermann, Lostao, Hirayama, Mackenzie, et al., 1994). The predominant glucose transporter in the intestine is

GLUT2 as it is involved in both uptake and export (Kellett & Brot-Laroche, 2005). SGLT1 and GLUT5 are expressed mostly on the apical side of enterocyte whereas GLUT2 is usually found on the basolateral membrane (Wright, Hirayama, & Loo, 2007). Glucose and galactose (at low concentration) are absorbed through SGLT1 whereas fructose is absorbed through GLUT5 (Wright, Martín, & Turk, 2003). The sugars are exported from the intestine through passive diffusion via GLUT2 and GLUT5 (Wright et al. 2003). In case of high glucose and galactose levels in the lumen, SGLT1 facilitates insertion of GLUT2 on the apical membrane of the enterocyte to aid in glucose or galactose uptake (Kellett & Brot-Laroche, 2005). Thus reduction of intestinal α-glucosidase activity and/or glucose absorption could effectively prevent or treat type 2 diabetes mellitus.

Consumption of whole grain cereals has been associated with a slow increase in blood glucose level compared to consumption of refined flours (Foster-Powell, Holt, & Brand-Miller, 2002; Ludwig & Eckel, 2002) even though some results suggest otherwise (Jenkins et al. 1981). The mechanism through which whole grains might reduce rapid increase in blood glucose is still not clear (Belobrajdic & Bird, 2013). The presence of soluble dietary fibre is thought to negate starch hydrolysis and glucose absorption through increased viscosity. However, recent studies suggest that viscosity effect may be offset by strong intestinal peristalsis (Dhital, Dolan, Stokes, & Gidley, 2014). On the other hand, polyphenols have been reported to inhibit the activity of pancreatic amylase (Narita & Inouye, 2009), intestinal α-glucosidase (Zhang, Wang, & Dong, 2015) and glucose transporters (Kwon, Eck, Chen, Corpe, Lee, Kruhlak, et al., 2007). Phenolic compounds can bind to proteins thereby affecting their functionality. Data on whether phenolic acids, which are abundant in cereals, can impair α-glucosidase and nutrient transporters is limited. Caffeic acid was reported to have mixed type inhibition potency towards α-amylase (Narita & Inouye, 2009).

FA and its dimers are the most abundant phenolic acids in cereals and are mostly bound to arabinoxylan (Ishii, 1997). FA is concentrated in the outer layer of the cereal grains. Feruloylation happens at O-5 position of arabinose (Bunzel, Ralph, & Steinhart, 2005) substituted at O-2 and/or O-3 position of (1-4)-β-D-xylopyranose chain (Lequart, Nuzillard, Kurek, & Debeire, 1999). Feruloylated arabinose is the simplest feruloylated arabinoxylan present naturally in cereal and their quantity may increase by 2 fold following gastric digestion (Chapter 6). Feruloyl arabinoxylan oligosaccharides (FOS) are also present in cereals (Chapter 6) but are mostly a product of enzymatic or acid hydrolysis of feruloylated arabinoxylan polysaccharides. FOS structures have been established and vary greatly in degree of polymerization and substitution (Gruppen, Hoffmaann, Kormelink, Voragen, Kamerlin, & Vliegenthart, 1992; Katapodis, Vardakou, Kalogeris, Kekos, Macris, & Christakopoulos, 2003; Yuan, Wang, & Yao, 2006). FOS are a subject of interest because of their prebiotic and antioxidant potential (Ou & Sun, 2014). At equal FA concentration, the FOS with higher DP tend to have higher antioxidant capacity (Chapter 5). Thus, we wanted to observe whether differences in DP would similarly affect their inhibition potencies towards intestinal α-glucosidase and glucose transporters. Hence our inclusion of FA, feruloylated arabinose and FOS in our study.

We hypothesise that FA and FAXmo present in whole grain might contribute to decrease in postprandial plasma glucose by inhibiting activities of intestinal α-glucosidase and GLUT2 or SGLT1 transporter. Thus, we investigated the effect of FA, feruloylated arabinose (prepared from corn bran) and FOS (prepared from wheat aleurone) on mammalian intestinal α-glucosidase and intestinal glucose transporter.

7.3 Materials and Methods

7.3.1 Materials

Yellow corn was purchased locally from Bulk Barn (Winnipeg, Manitoba, Canada). A commercial wheat aleurone (Grainwise™ wheat aleurone) was a gift from Cargill Limited's Horizon Milling (Wichita, Kansas, USA). [^{3}H] 2-Deoxyglucose (25.5 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA). Dulbecco's modified Eagle medium (DMEM) (25 mM glucose) was obtained from Biofluids (Rockville, MD, USA) and all other media supplements were from GIBCO Life Technologies (Gaithersburg, MD, USA). Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD, USA). Acarbose, FA standard, sucrose, maltose monohydrate, D-glucose, D-xylose, D-arabinose, and D-galactose were purchased from Sigma-Aldrich (Milwaukee, Wisconsin, USA). All acids and organic solvents were obtained from Fischer Scientific (Whitby, Ontario, Canada). All chemicals used were of analytical grade.

7.3.2 Preparation of feruloylated arabinoxylan mono- and oligosaccharides extracts

Feruloylated arabinose was prepared from corn bran using mild acid hydrolysis (Allerdings, Ralph, Steinhart, & Bunzel, 2006) and feruloylated arabinoxylan oligosaccharides were prepared from wheat aleurone using xylanase treatment (Chapter 5). Specifically, hand dissected corn bran was obtained according to a method by Ndolo and Beta (2009). Both wheat aleurone and corn bran flour were destarched and deproteinised according to a method described in chapter 5. The treated corn bran flour (20 g) was suspended in 200 mL of 50 mM hydrochloric acid and heated at 100 °C for 3 hours in a shaking water bath. The suspension was neutralised with 6 M ammonium hydroxide and centrifuged at 10000 g and 4°C for 10 minutes. The supernatant was freeze dried. Wheat aleurone flour was treated with endo-1,4-β-xylanase from *N. patriciarum* as described in chapter 5.

The samples were reconstituted with 10 mL millique water and further purified on XAD2 column as described by Saulnier et al (1995). XAD2 column was preconditioned with 1 column volume ethanol. After sample loading, it was eluted with 1 column volume water, 1.5 column volume methanol: water (50:50) and 1 column volume methanol. The 50% methanol eluent was collected and evaporated in rotary evaporator (40 °C). The extracts were reconstituted with water and freeze dried.

7.3.3 Chemical composition of feruloylated arabinoxylan mono- and oligosaccharides

Monosaccharide composition, FA content and protein content were analyzed as described previously (Chapter 3). Feruloylated arabinoxylan mono-/ oligosaccharide species were identified by a reverse phased high performance liquid chromatography (Waters Alliance 2695 instrument (Waters, Milford, MA)) system coupled to mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA) as described previously (Chapter 5).

7.3.4 Cell culture

Caco-2E stock cell cultures were maintained in 75-cm^2 plastic flasks and cultured at 37 °C in a 95% air, 5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium containing 15 mM glucose supplemented with 10% heat-inactivated FBS, 0.1 mM non-essential amino acids, and 0.1 mM glutamine. All experiments were carried out on cells of passage number 15 to 25 and media was refreshed 3 hours prior to experiments.

7.3.5 Inhibition of mammalian α-glucosidase activity assays

7.3.5.1 *Inhibition of α-glucosidase activity assay in Caco-2 cells*

Confluent Caco-2 cell grown on 24-well plates were used for α-glucosidase inhibitory activity according to Pan et al. (2003) with modifications. Briefly, 100% confluent cells were rinsed 3 times with PBS. Samples (350 µL) dissolved in 10 mM HEPES buffer (pH 7.4, glucose free, 147.0 mM NaCl, 5.0 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 0.3 mM $MgSO_4$-$7H_2O$, 1.0 mM $MgCl_2$-$6H_2O$, 1.5 mM $CaCl_2$-$2H_2O$) were added followed by 28 mM substrate (maltose or sucrose in HEPES buffer) (350 µL). The cells were incubated at 37 °C for 40 minutes. The substrate solutions were collected and boiled at 95 °C for 10 minutes to deactivate enzyme activity. After centrifugation at 12000 rpm for 5 minutes, the supernatants were collected for glucose analysis using megazyme glucose oxidase/peroxidase (GOPOD) glucose test kit. Cells were lysed with 10 mg CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) in 0.1 M NaOH (1 mL). The supernatant was analysed for protein content using Lowry method. The data obtained was expressed as glucose per µg protein (A). Alpha-glucosidase (sucrase or maltase) inhibition % was calculated as $(1 - [(A_{sample} - A_{blank})/ (A_{control} - A_{blank})])$. IC50 (defined as the sample concentration resulting in 50% inhibition of α-glucosidase activity) was determined from the plot of % α-glucosidase inhibition against sample concentration.

7.3.5.2 *Inhibition assay for rat intestinal α-glucosidase activity*

The α-glucosidase inhibitory method by Oki et al. (1999) was used with modifications. Briefly, rat intestinal acetone powder (500 mg) was mixed with 10 mL sodium phosphate buffer (pH 6.9, 0.1 M) and sonicated in ice bath for 30 seconds (12 times) with 15 seconds break to prevent heat buildup. It was later centrifuged at 10000 g at 4 °C for 10 minutes. The supernatant was collected

and labeled rat intestinal α-glucosidase. Later, 50 µL rat intestinal α-glucosidase was mixed with 100 µL sample or buffer (control) and incubated at 37 °C for 5 minutes. 50 µL of 20 mM sucrose or 10 mM maltose was added and further incubated for 60 minutes (sucrose) or 30 minutes (maltose). The enzyme activity was stopped by heating to 95 °C for 10 minutes. After centrifugation at 10000 g for 10 minutes, the supernatants were collected for glucose analysis using megazyme GODP glucose test kit. Alpha-glucosidase (sucrase or maltase) inhibition % was calculated as $(1 – [(A_{sample} – A_{blank})/ (A_{control} - A_{blank})])$. IC50 value was determined from the plot of % α-glucosidase inhibition against sample concentration. Inhibition of rat intestinal α-glucosidase with acarbose (a known α-glucosidase inhibitor) was also done for comparison purpose. Acarbose concentrations of 1.625, 3.25, 4.9, 6.5, 9.8, and 13 µg/ mL were used instead of sample.

7.3.6 Assay for glucose uptake inhibitory activity in Caco-2 cells

A method as described by Kwon et al. (2007) was used with modifications. Confluent Caco-2 cells grown on 96-well plates were rinsed 3 times with PBS and incubated in pre-incubation buffer (HEPES buffer with 5 mM glucose) for 30 minutes at 37 °C. After decanting, 50 µL of HEPES buffer (pH 7.4, glucose free) containing sample was added followed by 50 µL [^{3}H] 2-deoxyglucose (5 mM in glucose free HEPES buffer). The cells were incubated at room temperature for 15 minutes and transport activity was stopped by adding 100 µL of ice cold preincubation buffer immediately after removal of transport buffer. Pre-incubation buffer was replaced with 60 µL lysis buffer (10 mg CHAPS in 1 mL of 0.1M NaOH) and incubated at room temperature for 2 hours. Aliquot was transferred to a scintillating vial for scintillation spectrometry (45 µL) and protein (10 µL) measurements. The glucose uptake was expressed as counts per minute beta (cpma) per µg protein.

To study the effect of sample on glucose transporter 2 (GLUT 2), glucose uptake studies were done similarly but HEPES buffer without sodium was used instead.

7.3.7 Assay for glucose uptake in GLUT2 injected oocytes

Transport of glucose in oocytes from *Xenopus laevis* were done as described by Kwon et al (2007). Briefly, oocytes were defolliculated by incubating the open ovarian lobes for 60 min at 23 °C in OR-2 buffer without calcium (5mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1 mM Na2HPO4, 100 µg/mL gentamicin, pH 7.8) containing 2 mg/mL collagenase (Sigma). Mature oocytes were isolated and kept at 18 °C in OR-2 buffer containing 1 mM calcium chloride and 1 mM pyruvate. After 24 hours, the oocytes were injected with 36.8 nL cRNA (0.3 µg/µL) coding for GLUT2 or water using a Nanoject II injector (Drummond Scientific, Broomall, PA). The oocytes were incubated for 72 hours with media change every 12 hours.

FAXmo were dissolved in OR-2 containing calcium chloride without pyruvate at concentrations shown in the results section. Ferulic acid was first dissolved in dimethyl sulfoxide (DMSO) and diluted with OR-2 containing calcium chloride without pyruvate such that the final concentration of DMSO is less than 1%. Glucose transport was initiated by adding equal volumes (100 µL) of treatment solutions and 2-[3H] deoxyglucose to 20 oocytes. OR-2 buffer was used as a control. After 30 minutes, transport buffer was aspirated and 1 mL ice cold OR-2 buffer were added to terminate glucose transport. The oocytes were washed four times with ice cold buffer. Each oocyte was put in anti-static pony scintillation vial (Perkinelmer, Canada) containing 200 µL sodium dodecylsulfate (10%). After 10 minutes, 5 mL scintillation cocktail was added prior to reading on scintillation spectrometry (Perkinelmer, Canada). The internalised glucose was expressed as

counts per minute beta (cpma) per oocyte. Oocytes injected with water (instead of GLUT2 cRNA) were used to verify uptake of glucose in absence of GLUT2 cRNA.

7.3.8 Statistical analysis

All cell culture experiments were done in three different generations of cells and data represents mean and standard deviation of at least six results that were similar. Data for rat intestinal α-glucosidase inhibition studies are mean and standard deviation of triplicate analyses. Each data point for glucose uptake in oocytes represents mean of 15 - 20 oocytes. All statistics were calculated using one way analysis of variance (ANOVA) on a JMP 10 statistical software (SAS Institute Inc., Cary, NC). Sample means were compared using Tukey HSD method and significant differences determined at $p \leq 0.05$. Correlations between FAXmo and inhibition capacity were done by Pearson's correlation test.

7.4 Results and Discussion

7.4.1 Isolation and identification of feruloylated arabinoxylan mono- and oligosaccharides

We aimed at obtaining feruloylated arabinoxylan extracts rich in feruloyl arabinose or FOS. FAXmo extracts from corn bran had about 19% protein, 9.85% FA, and 65.9% carbohydrate by weight on dry basis. The monosaccharide constituents in the carbohydrate fraction were arabinose (52.87%), xylose (42.95%), glucose (2.13%), mannose (1.07%) and galactose (0.97%). FAXmo extract from wheat aleurone was characterized to contain 29% protein, 7.2% FA and 59.4% total carbohydrates. The molar percent distribution of the wheat bran FAXmo for arabinose, xylose, glucose, mannose and galactose was 26.15, 65.15, 5.23, 2.12, and 1.36%, respectively. The HPLC chromatograms for corn bran (Figure 7.1a) and wheat aleurone (Figure 7.1b) FAXmo showed that the extracts contained different compounds. The negative ESI mass spectra for

FAXmo in corn and wheat aleurone extract showed a compound with m/z = 325 and m/z = 721, 854, and 985 respectively (Figure 7.2). The results suggest that corn bran FAXmo extract contained feruloyl arabinose (*Mw* = 326) and wheat aleurone extract was a heterogeneous FOS consisting of FA esterified to an arabinoxylan with 4 or 5 sugar moieties with majority being 5. Fragmentation of the deprotonated ion m/z = 325 yielded daughter ions with m/z = 265, 193 and 134 (Figure 7.3a) typifying 5-O-feruloyl-L-Arabinofuranofuranosyl. Feruloyl arabinose was also produced through acid hydrolysis of corn bran (Fang et al., 2013). Fragmentation of compound (m/z = 853) resulted in daughter ions 775, 721, 643, 325 and 265 (Figure 7.3b) which is similar to that reported by Wang et al (2009) extracted from wheat bran. They concluded that the structure was *O*-β-d-xylopyranosyl-(1 → 4)-*O*-[5-*O*-(feruloyl)-α-l-arabinofuranosyl-(1 → 3)]-*O*-β-d-xylo-pyranosyl-(1 → 4)-*O*-β-d-xylopyranosyl-(1 → 4)-d-xylopyranose following NMR analysis. Likewise the fragmentation pattern of compound with m/z = 721 (Figure 7.3c) matched that of *O*-β-d-xylopyranosyl-(1 → 4)-*O*-[5-*O*-(feruloyl)-α-l-arabinofuranosyl-(1 → 3)]-*O*-β-d-xylopyranosyl-(1 → 4)-d-xylo-pyranose (Wang et al. 2009).

7.4.2 Effect of ferulic acid and feruloylated arabinoxylan mono- and oligosaccharide extracts on mammalian α-glucosidase

The data of the effect of FAXmo extract and FA on α-glucosidase are presented as IC50 value (Table 7.1). The results suggest that both FAXmo extracts and FA can inhibit α-glucosidase activity in a dose depended manner. In Caco-2 cells, corn bran FAXmo showed a higher potency towards both sucrase and maltase activity inhibition compared to that of wheat aleurone FAXmo extracts. The inhibitory potency of FAXmo was highly correlated to FA content (R = 0.95). Similarly, at equal FA concentration (0.04 mg/mL), FA, and FAXmo from corn bran and wheat aleurone inhibited sucrase activity by 18.70 ± 5.25, 23.28 ± 4.51% and 19.45 ± 4.85%,

respectively. Also, IC50 values based on FA were not significantly different ($p < 0.05$) for FA (0.09 mg/mL), and FAXmo from corn bran (0.10 mg/mL) and wheat aleurone (0.09 mg/mL). Thus FA suppressed intestinal α-glucosidase activity in both free and bound form. Acarbose (a known α-glucosidase inhibitor) was 75 times more potent compared to FA (Table 7.1b). Acarbose is currently used as drug for type 2 diabetic mellitus. However, FAXmo are consumed in large quantities as part of the dietary fiber.

Phenolic compounds have been reported to non-competitively inhibit α-glucosidase activity (Shobana, Sreerama, & Malleshi, 2009; Zhang, Wang, & Dong, 2015). Phenolic compounds binds to the enzyme complex thereby suppressing its activity (Li, Gao, Shan, Bian, & Zhao, 2009). Activity of the sucrase-isomaltase complex can be partially or completely suppressed depending on the nature of phenolic compound present. Anthocyanins only inhibited maltase activity of the sucrase-isomaltase complex and had no effect on sucrase (Matsui, Ueda, Oki, Sugita, Terahara, & Matsumoto, 2001). The inhibition potency of some polyphenol extracts was much higher for sucrase than that of maltase (Zhang, Wang, & Dong, 2015). In our study, the IC50 values for maltase and sucrase activity were not significantly different ($p < 0.05$) suggesting both were equally suppressed. Both D-xylose (Matsuura & Ichikawa, 1997) and L-arabinose (Seri, Sanai, Matsuo, Kawakubo, Xue, & Inoue, 1996) may inhibit sucrase activity. Thus we anticipated FAXmo extracts to have higher inhibitory effect compared to FA. Our results suggest that at the same FA concentration, arabinose or xylose did not affect the inhibition potency of FA towards α-glucosidase activity in Caco-2 cells. In contrast, esterification to arabinose and/or xylose reduced the inhibition potency of FA by 50% in rat intestine α-glucosidase activity.

7.4.3 Effect of ferulic acid and feruloylated arabinoxylan mono- and oligosaccharide on glucose uptake in Caco-2 cells

We studied the effect of FA and FAXmo on glucose uptake in the presence of sodium. Our results (Figure 7.4) suggest that FA and FAXmo inhibit glucose uptake in Caco-2 cells. On weight basis, FA was the most potent inhibitor followed by feruloyl arabinose rich extract suggesting that FA is the active site in FAXmo. Adjusting for FA content, the % inhibition potential of FA, feruloyl arabinose and FOS on glucose uptake were not significantly different ($p \leq 0.05$). Therefore, we concluded that FA can inhibit glucose uptake whether in free or bound form. Glucose is the predominant end product of dietary carbohydrate digestion. Glucose is absorbed in the small intestine via the sodium glucose linked transporter (SGLT1) and sodium independent glucose transporter 2 (GLUT2) expressed on the apical side of the intestinal epithelial cells.

We also conducted glucose uptake studies in the absence of sodium in order to understand which of the two transporters is being inhibited by FA and FAXmo. In the absence of sodium, glucose uptake was equally inhibited (Figure 7.5) suggesting that FA and FAXmo interfere with GLUT2 activities. GLUT2 is expressed both on the apical and basolateral of the intestinal epithelial cells. On the basolateral side, it functions to export glucose into the circulatory system. FA is well absorbed in the small intestine and consumption of arabinoxylan increases the concentration of plasma FA. Thus inhibition of GLUT2 by FA and FAXmo can be effective in attenuating postprandial hyperglycemia because it will decrease both intestinal glucose import and export. On the other hand, SGLT1 is arguably the chief glucose importer in the intestine when luminal concentrations are low. However, in case of high glucose and galactose levels in the lumen, SGLT1 facilitates insertion of GLUT2 on the apical membrane of the enterocyte to aid in glucose or galactose uptake (Kellett & Brot-Laroche, 2005). It was interesting to note that absence of sodium

did not significantly affect amount of glucose uptake in absence of inhibitors. This observation is consistent with reports suggesting that SGLT1 does not transport 2-deoxyglucose (Kwon et al., 2007). This could explain why the amount of glucose internalized by cells in sodium free buffer were not significantly different compared to when sodium was present during our Caco-2 studies.

Several flavonoids have been reported to inhibit only GLUT2 but not SGLT1 (Kwon et al., 2007). Our results suggest that one of the most efficacious inhibitors, quercetin (78%) is 4 times more potent than FA at the same concentration. A plot of FAXmo's FA concentration against % glucose uptake inhibition (Figure 7.6) provides an insight of inhibition type. At very low concentrations, the % inhibition is proportional to increase in FAXmo concentration. However, as the concentration of FAXmo increases beyond 200 µM FA equivalent the change in inhibition% becomes less noticeable. It appears that the maximum % inhibition attainable is 40% at our experimental conditions. This may suggest a non-competitive inhibition behavior where FA binds to transporter protein on non-active site. Phenolic acid or polyphenols in general have the ability to bind to proteins.

7.4.4 Effect of ferulic acid and ferulic acid sugar esters on glucose uptake in oocytes

In order to verify inhibition potency of FAXmo or FA towards GLUT2, oocytes injected with human GLUT2 cRNA were used to study glucose transport. The glucose uptake by oocytes injected with GLUT2 was 127.92 ± 10.29 cpma/oocyte in absence of inhibitors. However, glucose uptake was zero in the presence of FAXmo or FA at different concentrations (100, 150 and 200 µM ferulic acid equivalent). Our results suggest that glucose uptake was completely blocked in the presence of FA or FAXmo (≥ 100 µM). Thus confirming that FAXmo inhibits GLUT2 as observed in Caco-2 studies. We noted that the % maximum inhibition differed between oocytes

(100%) and Caco-2 (~40%) glucose uptake studies. Glucose uptake studies in Caco-2 cells are usually non-conclusive due to the presence of other glucose transporters (Mahraoui, Rodolosse, Barbat, Dussaulx, Zweibaum, Rousset, et al., 1994). In particular, Caco-2 being cancer cells expresses GLUT1 and GLUT3 (in addition to SGLT1, GLUT2 and GLUT5) to maximize glucose uptake unlike normal intestinal cells (Mahraoui et al., 1994). Therefore, it is probable that GLUT2 contributed to about 40% of total glucose uptake in Caco-2 cells in absence of SGLT1 activity. Thus upon nullifying GLUT2 activity, increase in FAXmo concentration did not increase the percent inhibition of glucose uptake beyond 40% in Caco-2 cells. On the other hand, oocytes are method of choice in establishing specificity of glucose uptake inhibitors because de-folliculated oocytes are devoid of glucose transporters. Thus injection of human GLUT2 cRNA into oocytes guarantees that any absorbed glucose is a consequent of the expressed GLUT2.

Thus we conclude that FA and feruloyl mono-/oligosaccharides may contribute to the anti-hyperglycemic properties of whole grain cereals. Studies on postprandial glucose levels after consumption of whole grain diet have yielded inconsistent data. This could be in part due to intra/ interspecies variation in cereal grains phenolic acid content (Ndolo & Beta, 2014; Nyström, Lampi, Andersson, Kamal-Eldin, Gebruers, Courtin, et al., 2008; Sosulski, Krygier, & Hogge, 1982). On average, corn has 4 times more FA content compared to wheat (Sosulski, Krygier, & Hogge, 1982) despite having approximately the same starch content. Also, FA content within wheat ranges from 162 – 721 µg/g (Li, Shewry, & Ward, 2008). On the other hand, wheat endosperm have been reported to have about 120 µg/g (Ndolo & Beta, 2014). Hence, it is difficult to assess the role of phytochemicals in type 2 diabetes based on epidemiological studies where food frequency questionnaires are being used. Therefore, assuming 2 L gastric volume, we can extrapolate from our data that daily consumption of 77 mg of FA (free or bound to mono-/oligosaccharide) may

effectively suppress postprandial plasma glucose levels. Whether dietary uptake of 77 mg of FA can be achieved depends largely on the type of cereal grain and cultivar. For example, consumption of 100 g whole grain corn or wheat can provide about 80 mg or 23 mg FA (free or bound to mono-/oligosaccharide), respectively (unpublished data from our lab).

On the other hand, low water solubility of FA, 0.6 mg/ mL (Mota, Queimada, Pinho, & Macedo, 2008) may present another challenge towards bio-accessibility under physiological conditions. The solubility of FA can increase to 10 mg/ mL when FA is esterified to arabinose (Fang, Wang, Chang, Hu, Hwang, Fu, et al., 2013). Arabinoxylan oligosaccharides (AXOS) are getting attention for their antioxidant and prebiotic properties. Thus use of AXOS high in feruloyl arabinoxylan mono-/oligosaccharides content might be beneficial in management or prevention of type II diabetes.

7.5 Conclusion

In this work, we have demonstrated for the first time that feruloylated arabinoxylan mono-/oligosaccharide inhibit mammalian intestinal α-glucosidase and glucose transporters. Both sucrase and maltase activities were equally inhibited. GLUT2 was inhibited in the presence of feruloylated arabinoxylan mono-/oligosaccharide. FA moiety of feruloyl arabinoxylan mono-/oligosaccharide was the active site for the observed inhibitory activity. These result may partly explain the anti-hyperglycemic properties of whole grain as both FA and feruloyl arabinoxylan mono-/oligosaccharide are present in significant quantities in cereals.

7.6 Acknowledgement

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7.7 Reference

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Table 7.1: Effect of ferulic acid and feruloylated arabinoxylan mono- and oligosaccharide extracts on α-glucosidase when sucrose or maltose was used as substrate

a) Caco-2 cells α-glucosidase

Extract	**IC50 (mg/ mL)**	
	Sucrose	Maltose
Corn bran FAXmo	1.03 ± 0.04^{b} (0.10)	1.65 ± 0.27^{a} (0.16)
Wheat aleurone FAXmo	1.28 ± 0.05^{a} (0.09)	1.34 ± 0.05^{b} (0.09)
Ferulic acid	0.09 ± 0.01^{c} (0.09)	0.08 ± 0.00^{c} (0.08)

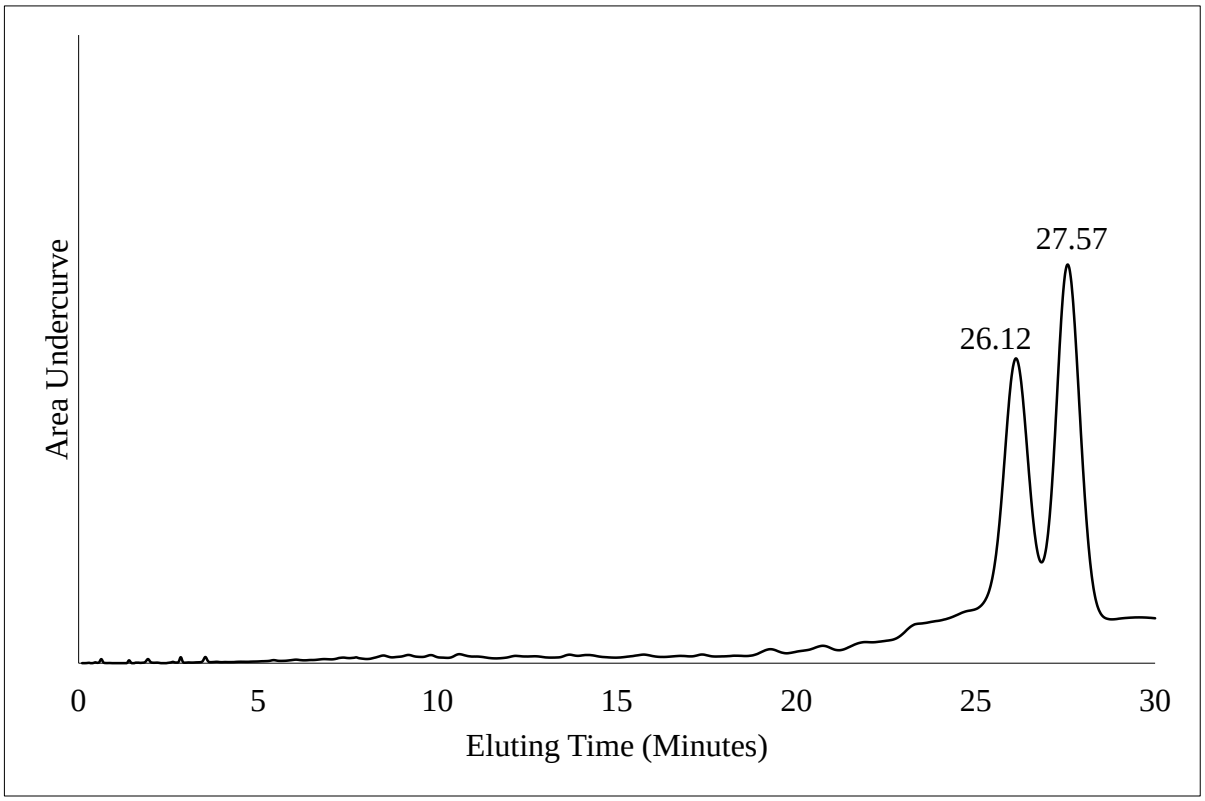
b) ***Rat intestinal α-glucosidase***

Extract	IC50 (mg/ mL)	
	Sucrose	Maltose
Corn bran FAXmo	2.66 ± 0.64^{b} (0.27)	4.8 ± 0.36^{b} (0.48)
Wheat aleurone FAXmo	6.43 ± 0.83^{a} (0.45)	5.08 ± 0.01^{a} (0.35)
Ferulic acid	0.22 ± 0.13^{c} (0.22)	0.22 ± 0.00^{c} (0.22)
Acarbose	0.005 ± 0.00^{d}	0.003 ± 0.00^{d}

Values presented as mean ± standard deviation (n = 3 for rat intestinal α-glucosidase and n = 6 for Caco-2 studies). Data in the same column with the same superscript are not significantly different at $p \leq 0.05$. Data in parenthesis are IC50 values in ferulic acid equivalent (mg/ mL). IC50 value is the sample concentration resulting in 50% inhibition of α-glucosidase activity. FAXmo means feruloylated arabinoxylan mono- and oligosaccharide extract.

Figure 7.1: UV spectra of feruloyl mono- and oligosaccharide arabinoxylans from corn bran (a) and wheat aleurone (b).

a)



b)

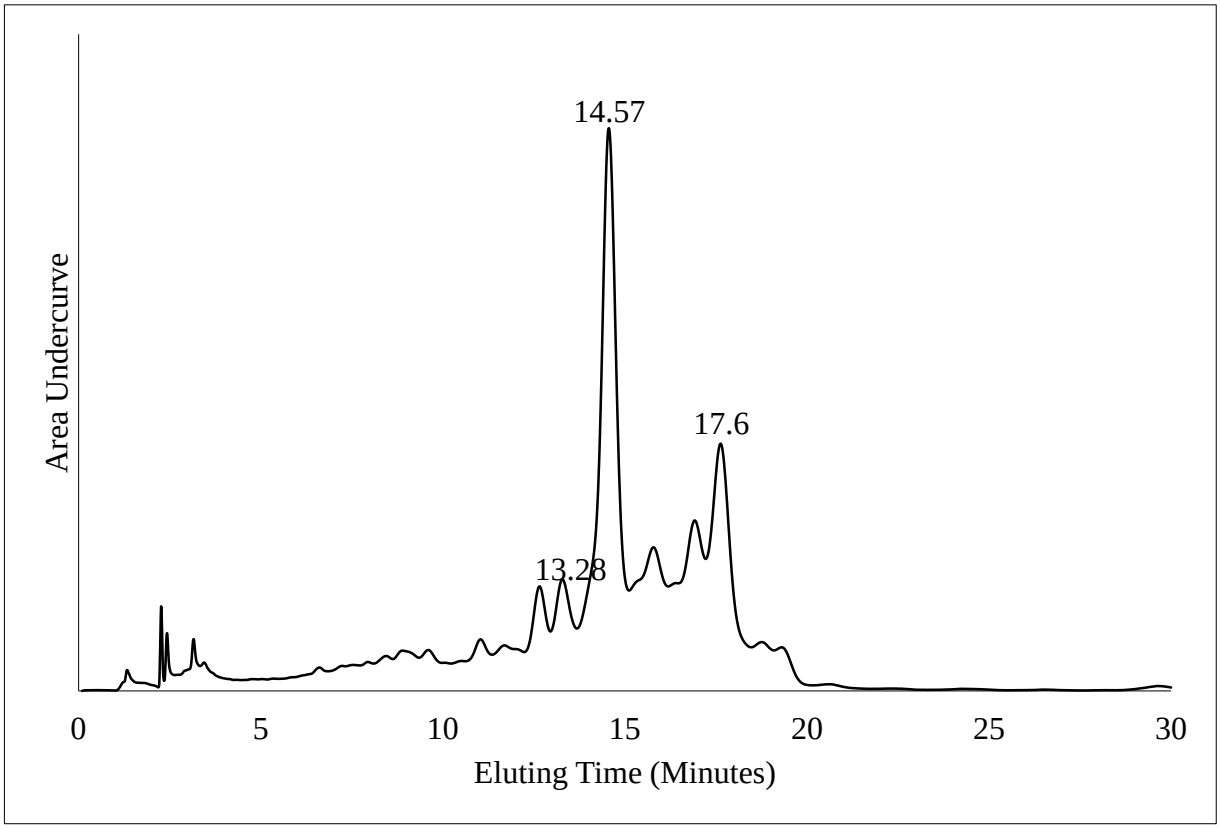
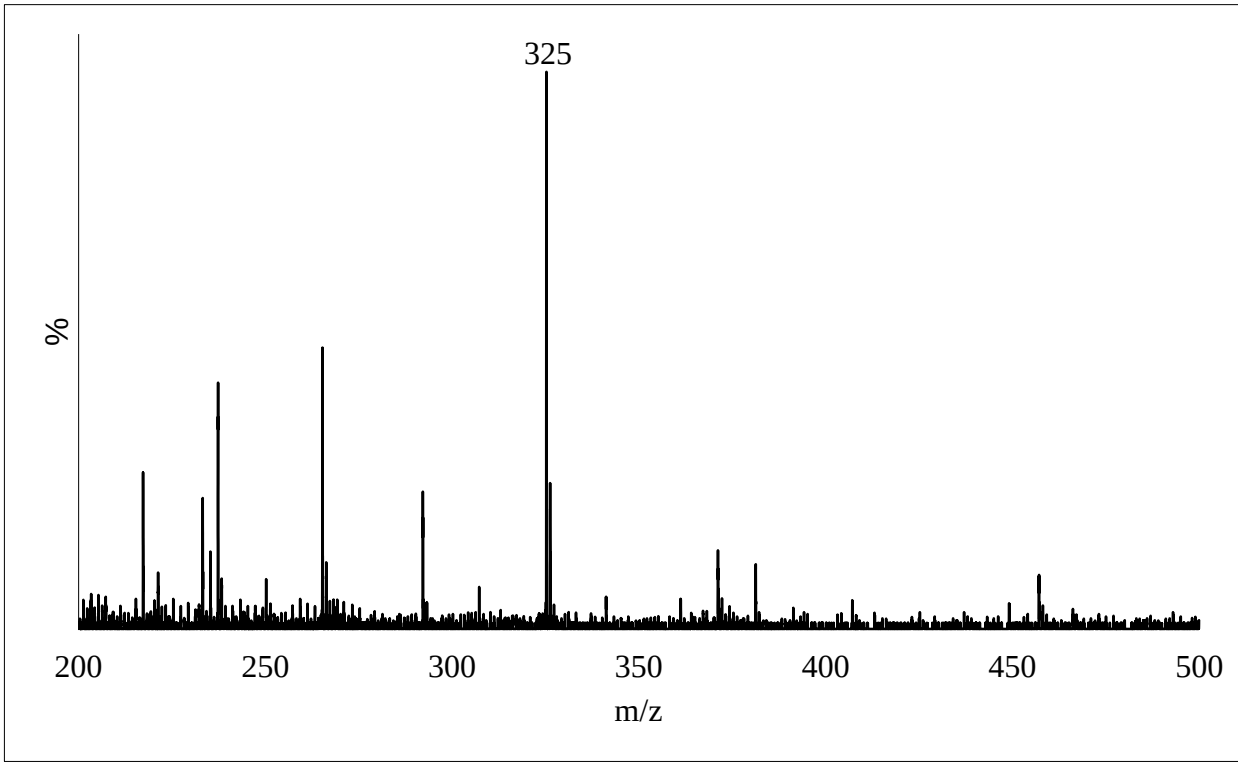

14.57
17.6
13.28
Area Undercurve
0
5
10
15
20
25
30
Eluting Time (Minutes)

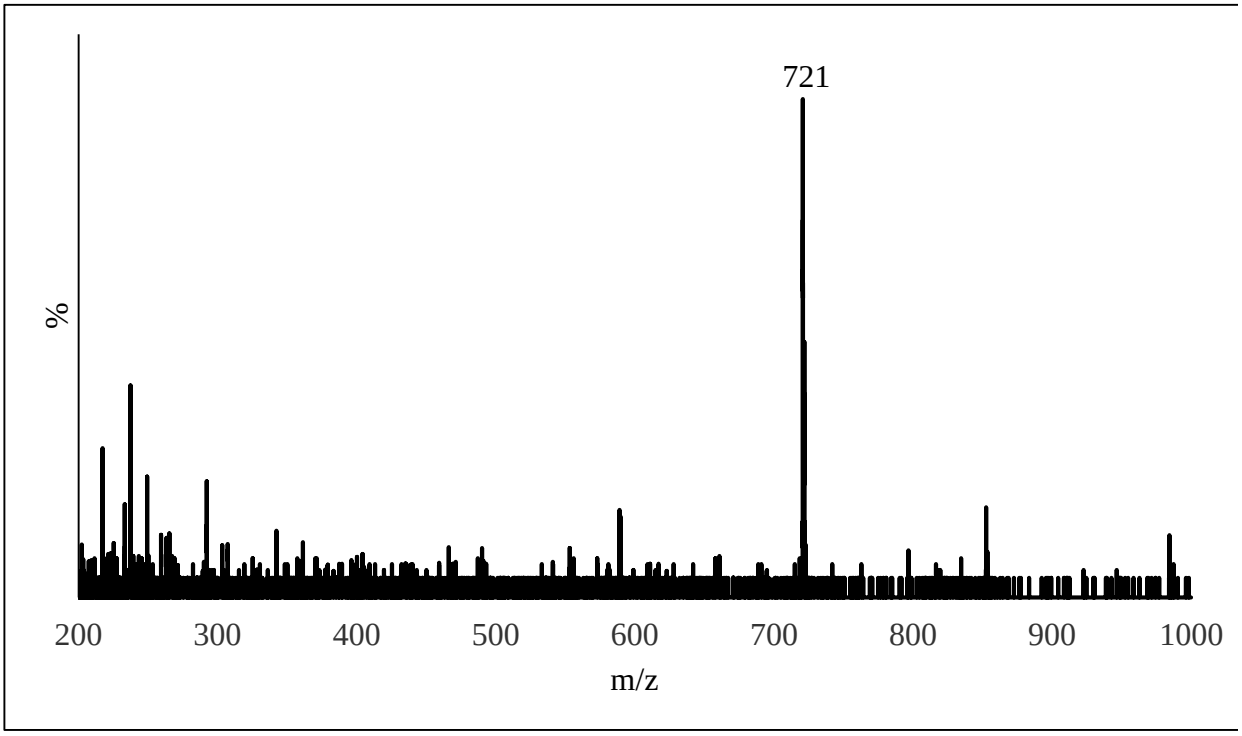
Figure 7.2: Negative ion mass spectra of feruloyl mono/oligosaccharide arabinoxylans $[M-H]^-$ from corn bran (a) and wheat aleurone (b and c).

Compounds eluting at 26.12 and 27.57 minutes in corn bran UV spectra had same molecular weight $[M-H]^-$ of 325 (a). Also compounds eluting at eluting at 13.28 minutes in wheat aleurone UV spectra had m/z = 721 (b) whereas those at 14.57, 15.78, 16.92 and 17.63 minutes had same m/z= 853 (c).

(a)



(b)


721
%
200
300
400
500
600
700
800
900
1000
m/z

(c)

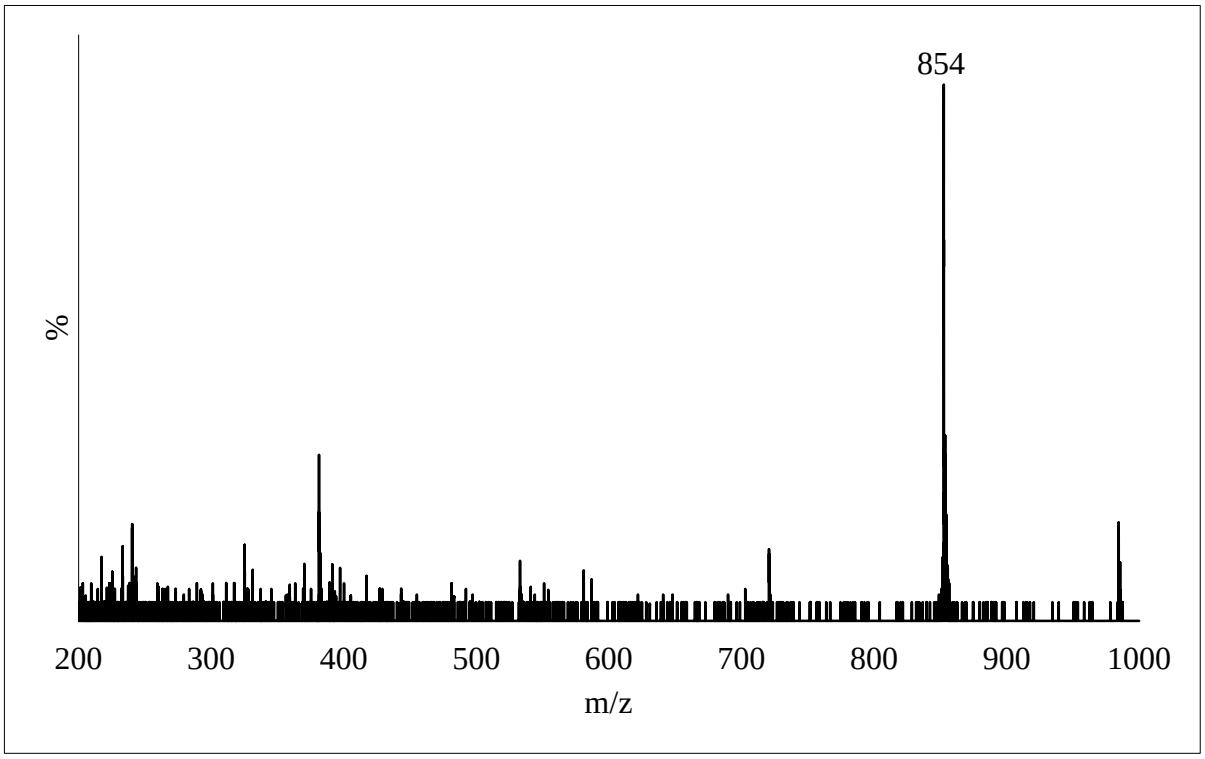
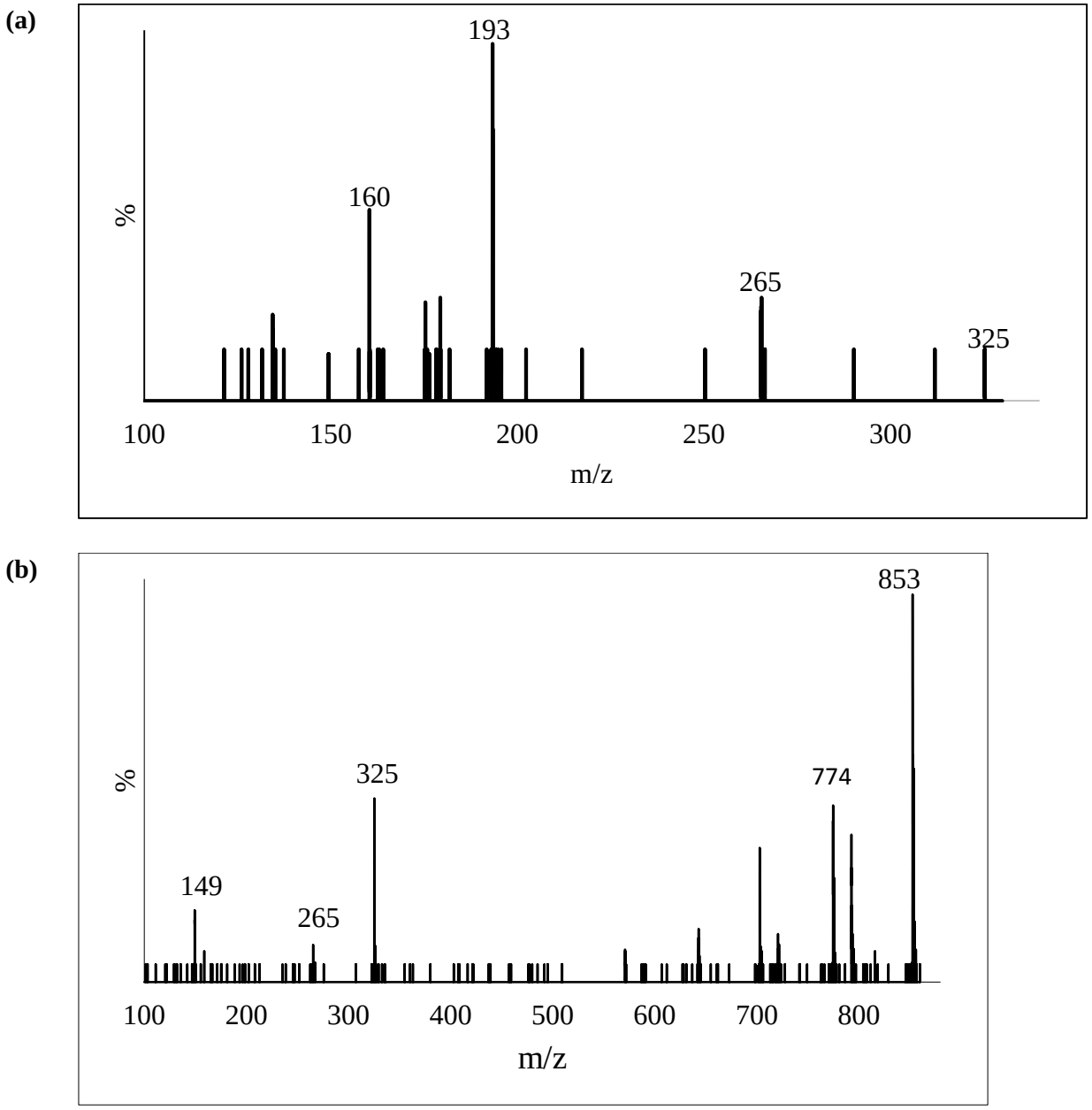

854
%
200
300
400
500
600
700
800
900
1000
m/z

Figure 7.3: MSMS spectra of feruloyl mono- and oligosaccharide arabinoxylans $[M-H]^-$ from corn bran and wheat aleurone (a) *m/z* = 325; (b) *m/z* = 853; and (c) *m/z* = 721



(c)

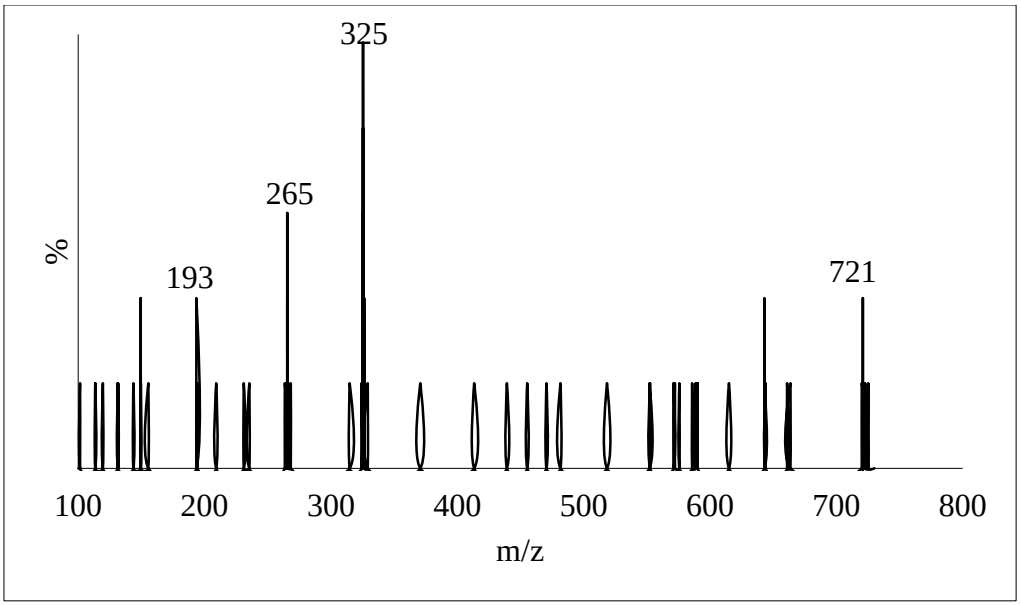
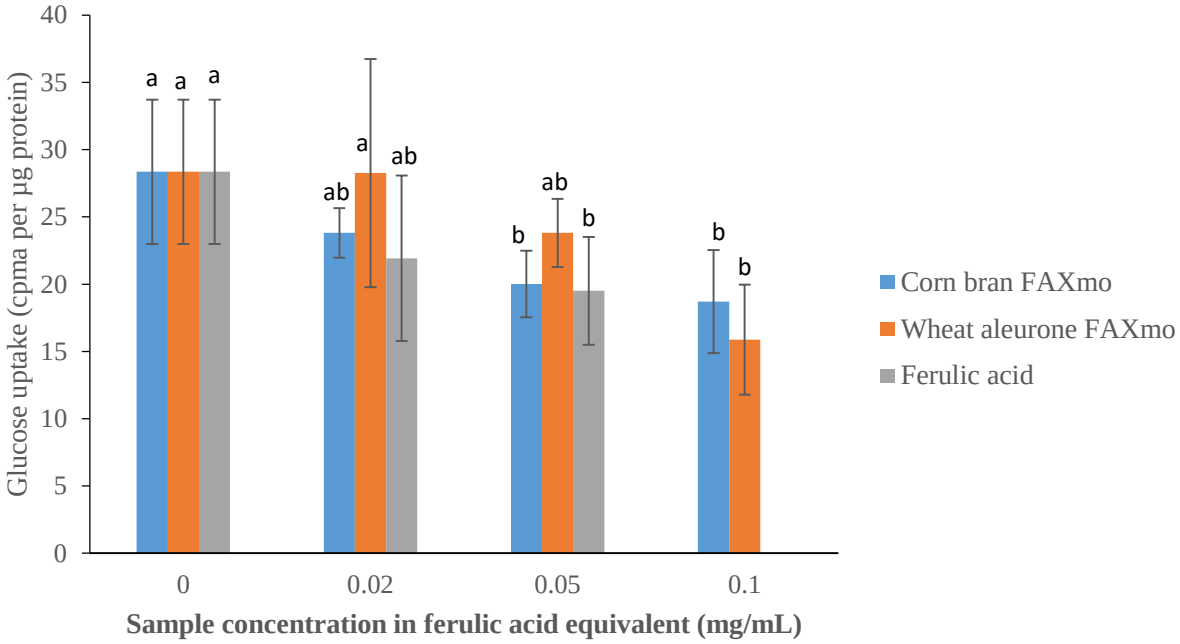
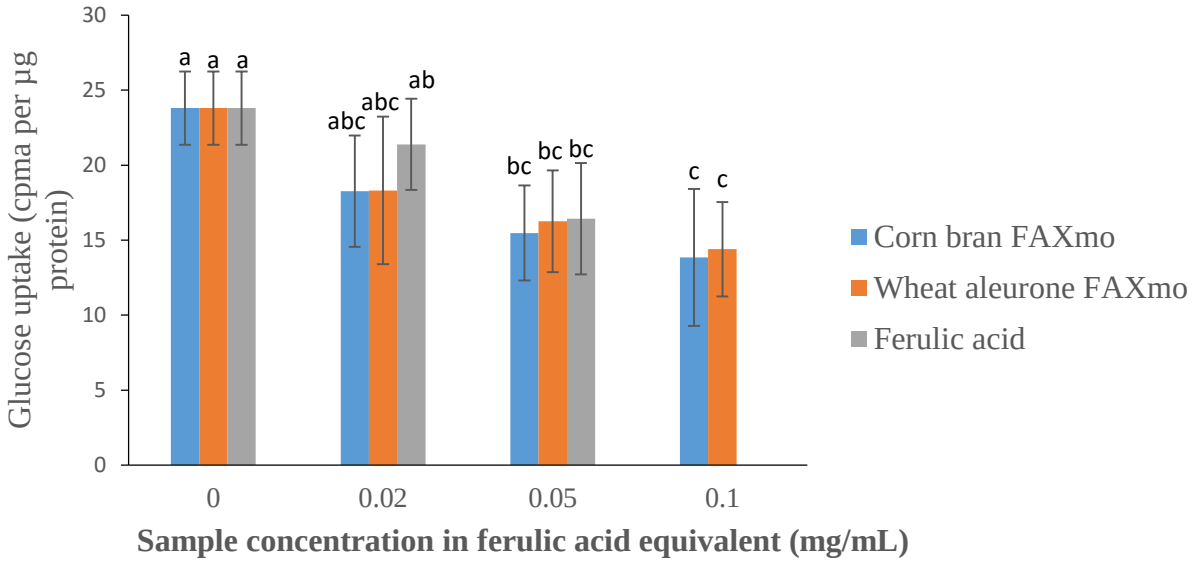


Figure 7.4: Effect of ferulic acid and feruloylated arabinoxylan mono- and oligosaccharides extracts on glucose uptake in Caco-2 cells in sodium plus medium.



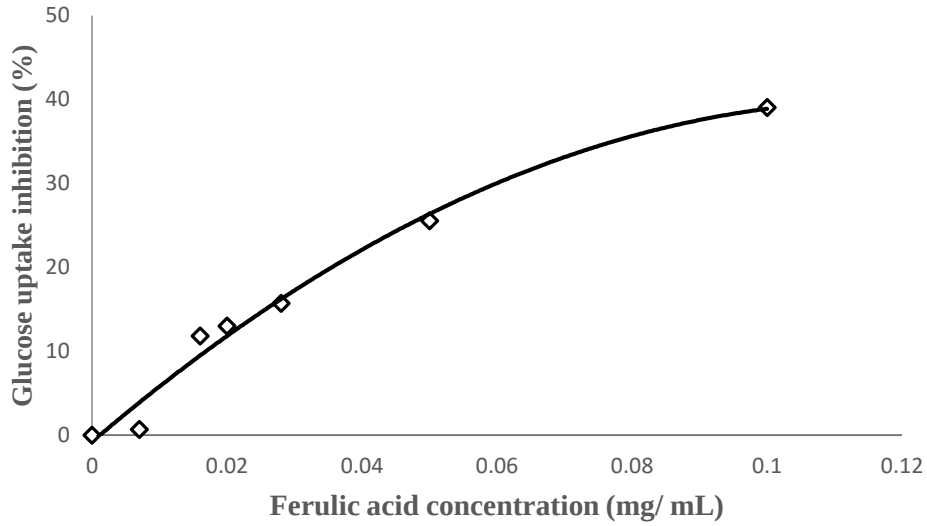
Values presented as mean ± standard deviation (n = 6). Data with the same superscript are not significantly different at p≤0.05. FAXmo means feruloylated arabinoxylan mono- and oligosaccharide extract.

Figure 7.5: Effect of ferulic acid and feruloylated arabinoxylan mono- and oligosaccharides extracts on glucose uptake in Caco-2 cells in sodium free medium



Values presented as mean ± standard deviation (n = 6). Data with the same superscript are not significantly different at p≤0.05. FAXmo means feruloylated arabinoxylan mono- and oligosaccharide extract.

Figure 7.6: Glucose uptake inhibition (%) by ferulic acid in Caco-2 cells



A plot to demonstrate that ferulic acid is the active site for inhibiting glucose absorption in Caco-2 cells. Inhibition percent was calculated from means of glucose uptake in the presence of ferulic acid or ferulic acid sugar esters.

CHAPTER 8

8 Antiglycemic effect of water extractable arabinoxylan from wheat aleurone and bran

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8.1 Abstract

The effects of arabinoxylans (AX) postprandial glucose response have resulted in contrasting data owing to the diversity in AX structures. Four water extractable AX (WEAX) extracts were used to investigate the effect of AX on activities of α-amylase and α-glucosidase, the influence of AX chemical composition on their inhibition potency, and kinetics of enzyme inhibition. α-Amylase activity (727.83 ± 30.0) was not significantly affected by the presence WEAX regardless of type or concentration. WEAX inhibited α-glucosidase activity only when maltose was used as a substrate but not sucrose. The IC50 values of WEAX (4.88 ± 0.3 – 10.14 ± 0.5 mg/ mL) was highly correlated to ferulic acid content (R= -0.89), arabinose to xylose ratio (R = -0.67), and relative proportions of xylose being unsubstituted (R = 0.69), disubstituted (R = -0.63) and monosubstituted (R = -0.76). Lineweaver – Burk plot suggested an uncompetitive enzyme inhibition mode. Thus, our results suggest that antiglycemic properties of WEAX may be derived from direct inhibition of α-glucosidase activity.

Key words: Water soluble arabinoxylan, antiglycemic, α-glucosidase, bran, aleurone, feruloylated arabinoxylan

8.2 Introduction

The prevalence of type 2 diabetes is increasing globally. Diabetes is a chronic disease epitomized by high circulating plasma glucose. Thus management of postprandial glucose is critical in the prevention and treatment of type 2 diabetes patients. Human intervention studies have shown that consumption of arabinoxylan (AX) rich diet attenuates postprandial blood glucose levels in healthy (Lu, Walker, Muir, Mascara, & O'Dea, 2000), impaired glucose tolerance (Garcia, Otto, Reich, Weickert, Steiniger, Machowetz, et al., 2007; Garcia, Steiniger, Reich, Weickert, Harsch, Machowetz, et al., 2006) and diabetic (Hanai, Ikuma, Sato, Iida, Hosoda, Matsushita, et al., 1997; Lu, Walker, Muir, & O'Dea, 2004) subjects. In contrast, Mohlig et al found no effect on glucose response upon feeding healthy human bread rolls supplemented with AX (Möhlig, Koebnick, Weickert, Lueder, Otto, Steiniger, et al., 2005). Animal studies have also reported mixed results on the effect of arabinoxylan (Hartvigsen, Jeppesen, Lærke, Njabe, Knudsen, & Hermansen, 2013; Vogel, Gallaher, & Bunzel, 2012). The underlying mechanisms remain unclear, but it is purported that soluble fibers increases lumen viscosity thereby delaying nutrient absorption (Jenkins, Wolever, Leeds, Gassull, Haisman, Dilawari, et al., 1978). The intrinsic viscosity of arabinoxylans varies greatly and is dependent on asymmetrical conformation, length of xylan backbone, and concentration (Izydorczyk & Biliaderis, 1995). Of these three factors, concentration of AX seems to influence the viscosity of AX the most (Kale, Yadav, Hicks, & Hanah, 2015; Saulnier, Sado, Branlard, Charmet, & Guillon, 2007). Thus the effect of arabinoxylan on blood glucose is dose dependent. The apparent viscosity of the arabinoxylans is also dependent on shear stress, such that higher shear results in non-Newtonian fluid behavior (Izydorczyk & Biliaderis, 1995). Recent studies suggest that viscosity effects of AX may be offset by strong intestinal peristalsis (Dhital, Dolan, Stokes, & Gidley, 2014).

AX structure is complex and heterogeneous but consists of (1-4) -β-D-xylopyranose chain with α-L-arabinofuranose linkages at O-2 and/or O-3 position (Ishii, 1997; Izydorczyk & Biliaderis, 1995). Xylose residues may also be substituted with α-(1,2) -glucuronic acid and/or α-(1,2) -4-O-methylglucuronic acid linkages (Ma, Jia, Zhu, Li, Peng, & Sun, 2012). Arabinose residue may be ester linked to ferulic acid at O-5 position (Bunzel, Ralph, & Steinhart, 2005; Smith & Hartley, 1983). The ratio of arabinose to xylose, pattern of arabinose substitution, degree of feruloylation, and molecular weight vary greatly among and within cereal grains (Izydorczyk & Biliaderis, 1995). Arabinoxylans (AX) constitute the highest proportion of dietary fiber in cereal grains (60 -70%) (Antoine, Peyron, Mabille, Lapierre, Bouchet, Abecassis, et al., 2003) and their content varies with the source or grain fraction. AX accounts for 1.3 – 2.7% w/w of wheat (Gebruers, Dornez, Boros, Fras, Dynkowska, Bedo, et al., 2008). A majority of AX in wheat is water insoluble (70 -86 %) (Gebruers et al., 2008). Wheat aleurone and pericarp contains 20 and 45 % AX, respectively (Antoine et al., 2003).

Dietary carbohydrates are hydrolyzed to the monomeric sugars, glucose or fructose prior to their absorption in the gastrointestinal tract (Dona, Pages, Gilbert, & Kuchel, 2010). Starch is digested primarily to maltose and other short chain carbohydrates by salivary and pancreatic amylase. The resultant disaccharide or oligosaccharide and sucrose are digested to glucose or fructose by the small intestinal brush border glucosidases (maltase-glucoamylase and sucrase-isomaltase (Sim, Willemsma, Mohan, Naim, Pinto, & Rose, 2010). Sugar absorption in the small intestine mainly involves GLUT2, GLUT5 and SGLT1 transporters (Burant, Takeda, Brot-Laroche, Bell, & Davidson, 1992; Wright, Hirayama, & Loo, 2007). Thus a decrease in postprandial hyperglycemia can be attained by limiting intestinal carbohydrate digestion or uptake. Despite the enormous differences in structure of AX, most studies report very little or no detail of composition or

structure of the arabinoxylan used which make it difficult to compare the results (Gemen, de Vries, & Slavin, 2011). Very limited data also exist on the effect of purified water extractable arabinoxylans on carbohydrate digestive enzymes. Thus, in this study, we aimed at investigating a) the effect of AX on activities of α-amylase and α-glucosidase b) the influence of AX chemical composition on their inhibition potency, and c) the kinetics of enzyme inhibition.

8.3 Materials and Methods

8.3.1 Chemicals and reagents

Wheat unmodified starch, maltose, sucrose, acarbose, porcine pancreas α-amylase (EC 3.2.1.1, Type VI-B), amyloglucosidase (EC 3.2.1.3) from aspergillus, and intestinal acetone powders from rat were bought from Sigma-Aldrich ((Milwaukee, WI, USA). Ammonium sulphate, all acids and organic solvents were bought from Fischer Scientific (Whitby, Ontario, Canada). Maltose, sucrose and glucose assay kit (K-MASUG 08/13), wheat and arabinoxylan (medium viscosity) was purchased from Megazyme International Ireland (Bray, Wicklow, Ireland). All chemicals used were of analytical or HPLC grade.

8.3.2 Water extractable arabinoxylan samples

The water extractable arabinoxylans (WEAX) used was obtained from wheat aleurone and wheat bran, followed by ammonium sulphate fractionation as described in chapter 5. The chemical and structural descriptions of WEAX are presented in Table 8.1.

8.3.3 Inhibition assay for α-amylase activity

Wheat starch (300 mg) was suspended in 15 mL sodium phosphate buffer (pH 6.0, 0.1 M) and cooked at 95 °C for 15 minutes (Dhital et al., 2014). WEAX or AX samples (40 mg) were dissolved in 2 mL sodium phosphate buffer (pH 6.9, 0.1 M). Samples were diluted such that the final

concentration in the reaction mixture was 0.5, 0.3, 0.2, and 0.0% (w/v). Equal volumes (200 µL) of starch and WEAX (or control) were mixed and votexed. Starch hydrolysis was initiated by adding 70 µL of porcine pancreatic α-amylase (130 U/ mL) and 40 µL of fungal amyloglucosidase (240 U/ mL). The reaction was stopped after 30 minutes by heating at 95 °C for 5 minutes. The mixture was immediately cooled on ice and centrifuged (Thermo Scientific, Sorvall Legend Micro21, Germany). The supernatants were collected and analyzed for glucose using Megazyme glucose test kit. Human intervention studies have reported that 0.25 – 0.7% AX concentration being effective, hence our choice of the concentration range.

8.3.4 Inhibition assay for rat intestinal α-glucosidase activity

The α-glucosidase inhibitory method by Oki et al. (1999) was used with modifications. Briefly, rat intestinal acetone powder (500 mg) was mixed with 10 mL sodium phosphate buffer (pH 6.9, 0.1 M) and sonicated in ice bath for 30 seconds (12 times) with 15 seconds break to prevent heat buildup. The mixture was later centrifuged at 10000 g at 4 °C for 10 minutes. The supernatant was collected and labeled rat intestinal α-glucosidase. WEAX samples (40 mg) were dissolved in 2 mL sodium phosphate buffer (pH 6.9, 0.1 M). Later, 50 µL rat intestinal α-glucosidase was mixed with 100 µL sample or buffer (control) and incubated at 37 °C for 5 minutes. 50 µL of 20 mM sucrose or 4 mM maltose was added and further incubated for 60 minutes (sucrose) or 30 minutes (maltose). Final sample concentration was 0.5, 0.4, 0.3, 0.2, 0.1 and 0.0% (w/v). The enzyme activity was stopped by heating to 95 °C for 10 minutes. After centrifugation at 10000 g for 10 minutes, the supernatants were collected for glucose analysis using Megazyme GOPOD glucose test kit. Alpha-glucosidase (sucrase or maltase) inhibition % was calculated as $(1 - [(A_{sample} - A_{blank})/ (A_{control} - A_{blank})])$. IC50 value was determined from the plot of % α-glucosidase inhibition against sample concentration. Inhibition of rat intestinal α-glucosidase with acarbose (a known α-

glucosidase inhibitor) was also done for comparison purpose. Acarbose concentrations of 1.625, 3.25, 4.9, 6.5, 9.8, and 13 µg/ mL were used instead of samples.

8.3.5 Statistical analysis

All analyses were performed in sextuplicate (unless indicated otherwise) and all statistics were calculated using one way analysis of variance (ANOVA) on a JMP 10 statistical software (SAS Institute Inc., Cary, NC). Sample means were compared using Tukey HSD method and significant differences determined at $p \leq 0.05$. Correlations between parameters were done using Pearson's correlation test.

8.4 Results

8.4.1 Effect of water extractable arabinoxylan on starch hydrolysis

The effects of WEAX on α-amylase are presented in Figure 8.1. We compared the amount of glucose produced over 30 minutes of incubation with α-amylase in the presence or absence of WEAX. Addition of WEAX decreased the amount of glucose produced compared to control treatment. However, statistical comparisons of treatment groups and control showed that the mean difference was not significant ($p < 0.05$) for WA50, WA75 and WB75 regardless of WEAX concentration. The presence of 0.5% WB50 resulted in a significant decrease in amylolysis compared to control ($p < 0.05$).

8.4.2 Effect of water extractable arabinoxylan on α-glucosidase activity

Table 8.2 shows the effect of WEAX on α-glucosidase activity in the presence of sucrose or maltose as substrate. The data is presented as IC50 which is the concentration of the inhibitor resulting in 50% inhibition of α-glucosidase activity. The IC50 values ranged from 4.88 – 10.14 mg/mL against α-glucosidase activity when maltose was the substrate. However, no inhibition was

observed when sucrose was used as a substrate. The inhibitory potency of WEAX against intestinal maltase is 1000 – 2000 times less compared to acarbose (a positive control).

8.5 Discussion

Dietary glucose mostly originates from starch or sucrose (table sugar). Starch is hydrolyzed to maltose through the action of saliva and pancreatic α-amylase. The resultant maltose together with sucrose is hydrolyzed by intestinal brush border sucrase-isomaltase to their monomeric sugars – glucose and/or fructose. Supplementation of diet with AX has been reported to decrease postprandial glucose, but the underlying mechanisms remain unclear. Thus, in this study, we explored the effect of WEAX on α-amylase and α-glucosidase activity. Our results, suggest that WEAX only affects α-glucosidase activity, but not amylase activity. Our results for alpha amylase activity are in contrast to other reports in the literature (Dhital et al., 2014) possibly due to concentration and type of AX. They performed amylolysis of starch in the presence of 1 and 2 % AX concentration and the AX used was devoid of ferulic acid. We used concentrations of AX (~5 - 10 g) equivalent to that reported to attenuate postprandial blood glucose in human studies (Lu et al., 2000). Amylolysis was only affected in the presence of WB50 at 0.5% possibly due to high levels of beta glucans.

It is widely hypothesized that viscosity may be the cause of the effect of arabinoxylan on postprandial glucose (Gemen et al., 2011). However, in this case, we used a similar concentration of WEAX in both α-amylase and α-glucosidase activity studies and yet only the latter was inhibited. This may imply that the effect of WEAX on α-glucosidase activity may not have been a consequence of viscosity but rather substrate-enzyme-inhibitor interaction. The same is supported by the observation that the sucrose activity of α-glucosidase was not affected by the presence of WEAX in our study. Thus, it is possible that the antiglycemic effect of arabinoxylan may be due

to inhibition of α-glucosidase activity. Even though its potency on α-glucosidase activity is 1000 – 2000 times less compared to acarbose, IC50 concentration is achievable upon consumption recommended dietary requirement of fiber (21 - 38 g/ day).

The structure of AX is complex and heterogeneous in nature and the differences in their fine structure influences AX behavior. Thus we used 4 WEAX samples of varying chemical composition and structure (Table 8.1). The inhibitory potency varied significantly ($p < 0.05$) between the WEAX samples with WA50 being the most potent. WEAX isolated at 50% ammonium sulphate saturation exhibited a higher inhibition capacity compared to their corresponding fractions obtained at 75%. A Pearson correlation analysis (Table 8.3) suggested that ferulic acid content, arabinose to xylose ratio, and pattern of xylose substitution may have influenced the inhibition activity of WEAX. Ferulic acid content of AX was a major determinant ($R = -0.88$) of their inhibition potency. Inhibition of intestinal alpha glucosidase by arabinoxylan mono-/oligosaccharide was also associated with their ferulic acid moiety. WA50 and WB75 had significantly different potency towards α-glucosidase activity despite having similar Ar/Xy, X_u, X_d, and X_m but different ferulic acid content.

Arabinose to xylose ratio is a measure of degree of substitution or branching (DS). A strong negative linear association ($R= -0.67$) was observed between DS and IC50. This could be a consequence of increased solubility of AX due to high DS. Thus highly branched WEAX seems to have a lower IC50 value (high inhibition potency). The same observation is supported by negative association between inhibition potency and relative proportion of unsubstituted xylose residues. The apparent X_d to X_m ratio did not matter, but the extent of xylose substitution. Thus, it is probable that the effect on α-glucosidase activity may emanate from arabinose residues of WEAX. There have been reports on arabinose inhibiting α-glucosidase activity (Seri, Sanai,

Matsuo, Kawakubo, Xue, & Inoue, 1996). Attempts to remove arabinose residues from WEAX using arabinofuranosidase were not successful in order to prove this hypothesis. However, mono-Xylp at C-2 was observed to be a major determinant compared to mono-Xylp at C-3 suggesting that inhibition potency is beyond the mere presence of arabinose residue. There was a strong correlation between % arabinose residues linked to xylose at C-2 and ferulic acid content (R = 0.99). There have been reports suggesting that ferulic acid is attached to O-5 position of (1→ 2) -α-L-Ara*f* linked to a monosubstituted β-D-Xyl*p* (Saulnier, Vigouroux and Thibault, 1995; Smith and Hartely, 1983). Thus, it is possible that the influence of DS could have been derived from that of ferulic acid.

The Lineweaver – Burk plot (Figure 8.2) was used to calculate the apparent maximum velocity (Vmax) and Michaelis– Menton constant (Km) for α-glucosidase activity on maltose in the presence and absence of WEAX. The effect of WEAX on Vmax and Km was analyzed to determine the type of inhibition. The Vmax and Km of α-glucosidase for maltose in absence of WEAX was 17.5 µg glucose per minute and 5.99 mM, respectively. Table 8.4 shows that addition of WEAX decreased both Vmax and Km values which suggest that WEAX inhibited α-glucosidase activity through uncompetitive mode. A typical characteristic of uncompetitive inhibition is that both Vmax and Km decreases in the presence of inhibitors. Thus, it is plausible that WEAX binds to enzyme substrate complex, thereby decreasing both Vmax and Km. Arabinose was also found to inhibit α-glucosidase activity through uncompetitive mode (Seri et al., 1996).

Our results may provide an explanation for the inconsistency observed in literature about the effect of arabinoxylan on postprandial glucose level. Feeding of Zukar diabetic rats with arabinoxylan (ar/xy = 0.9) supplemented bread resulted in a significant decrease in postprandial blood glucose level (Hartvigsen et al., 2013). In contrast, intake of native arabinoxylan (ar/xy = 0.5) had no effect

on blood glucose response (Vogel et al., 2012). Also, supplementation of diets with 6 and 12 g arabinoxylan (ar/xy = 0.66 or 0.8) decreased blood glucose in both healthy and diabetic subjects (Garcia et al., 2007; Lu et al., 2004; Lu et al., 2000). However, arabinoxylan (ar/xy = 0.8) did not attenuate the postprandial glucose response in healthy human adults (Möhlig et al., 2005). The absence of detailed chemical composition and structures makes it difficult to compare the results on the effectiveness of arabinoxylan (Gemen et al., 2011). Thus, even though concentrations of arabinoxylans used may be the same, but its effectiveness would depend on the nature of arabinoxylan used. We have demonstrated that AX obtained at 50% ammonium saturation exhibited twice inhibition potency compared to AX obtained at 75%.

8.6 Conclusion

The results of this study indicated that the antiglycemic effect of arabinoxylans may be derived from inhibiting intestinal α-glucosidase activity but not amylase activity. The potency of water extractable arabinoxylan on α-glucosidase activity was influenced by ferulic acid contents, arabinose to xylose ratio, and pattern of xylose substitution. The findings also suggest that inhibition of α-glucosidase activity occurs through uncompetitive mechanism. Thus, consumption of a diet rich in water extractable arabinoxylan may attenuate the postprandial blood glucose level.

8.7 Acknowledgement

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Table 8.1: Chemical composition and characteristics of water extractable arabinoxylan from wheat aleurone (WA) and wheat bran (WB)

	Wheat aleurone		**Wheat bran**	
	WA50	WA75	WB50	WB75
Total carbohydrate content (% w/w)	76.0 ± 0.6	85.7 ± 1.0	54.6 ± 0.5	81.6 ± 1.3
Protein content (% w/w)	8.7±0.2	8.6±0.2	16.9±0.3	11.3±0.3
Beta glucan content (% w/w)	0.4	0.7	12.5	15.7
Arabinoxylan content (% w/w)	74.0	83.9	41.5	66.6
Arabinose to xylose ratio	0.58	0.44	0.85	0.56
Total ferulic acid content	26.01±0.40	6.53±0.20	16.78±0.35	4.34±0.11
Uronic acid content (%)	0.04 ± 0.0b	0.05 ± 0.0b	0.08 ± 0.0a	0.10 ± 0.0a
Average molecular weight (kDa)	551.0	677.0	643.0	468.0
Pattern of substitution				
Unsub- Xylp (%)	61	70	45.1	63.8
Mono-Xylpat C-2 (%)	3.2	0.2	1.4	0.1
Mono-Xylp at C-3 (%)	16.8	15.9	23.4	16.4
Total Mono-Xylp (%)	20.1	16.1	24.8	16.5
Di-Xylp (%)	19.0	14.0	30.1	19.8

Values presented as mean ± standard deviation (n = 3). Un-Xyl*p:* unsubstituted xylose residues, mono-Xyl*p:* monosubstituted xylose residue, di-Xyl*p:* O-2 and O-3 disubstituted xylose residues WA50 = AX from WA obtained at 50% ammonium sulphate saturation. WA75= AX from WA obtained at 75% ammonium sulphate saturation. WB50= AX from WB obtained at 50% ammonium sulphate saturation WB75= AX from WB obtained at 75% ammonium sulphate saturation.

Table 8.2**:** Effect of feruloylated arabinoxylan on intestinal α-glucosidase when sucrose or maltose was used as substrate

	IC50 (mg/ mL)	
	Maltose	Sucrose
WA50	4.88 ± 0.30^{d} (0.49)	*
WA75	10.14 ± 0.56^{a} (1.01)	*
WB50	5.73 ± 0.19^{c} (0.57)	*
WB75	8.15 ± 0.46^{b} (0.81)	*
Acarbose	0.005 ± 0.00^{e} (~0.0005)	0.003 ± 0.00

Values presented as mean ± standard deviation (n = 6). Data in the same column with the same superscript are not significantly different at $p \leq 0.05$. Data in parenthesis are IC50 values in % w/v. IC50 value is the sample concentration resulting in 50% inhibition of α-glucosidase activity. WA50 = AX from WA obtained at 50% ammonium sulphate saturation. WA75= AX from WA obtained at 75% ammonium sulphate saturation. WB50= AX from WB obtained at 50% ammonium sulphate saturation WB75= AX from WB obtained at 75% ammonium sulphate saturation.

Table 8.3: Correlation coefficient of arabinoxylans' anti-hyperglycemia activity and its structural properties

	α-Glucosidase activity (IC50)
Arabinose to xylose ratio	-0.67
Ferulic acid content	-0.89
Unsub-Xyl*p*	0.69
Mono-Xyl*p* at C-2	-0.86
Mono-Xyl*p* at C-3	-0.51
Total Mono-Xyl*p*	-0.76
Di-Xyl*p*	-0.63
Molecular weight	0.23
Uronic acid	0.36

Data represent Pearson correlation coefficient values at $p \leq 0.05$. un-Xyl*p:* unsubstituted xylose residues, mono-Xyl*p:* monosubstituted xylose residue, di-Xyl*p:* O-2 and O-3 disubstituted xylose residues.

Table 8.4: Inhibition kinetics of water extractable arabinoxylans derived from Lineweaver – Burk plots

	Vmax (µg glucose/minute)	Km (mM maltose)
Control	17.5 ± 0.48^{a}	5.99 ± 0.16^{a}
WA50	12.07 ± 0.22^{c}	4.54 ± 0.08^{c}
WA75	16.73 ± 0.42^{a}	5.07 ± 0.13^{b}
WB50	nd	nd
WB75	14.73 ± 0.33^{b}	4.91 ± 0.11^{b}

Values presented as mean ± standard deviation (n = 3). Data in the same column with the same superscript are not significantly different at $p \leq 0.05$. Vmax = maximum velocity; Km is the Michalelis- Menten constant (substrate concentration required for an enzyme to reach half Vmax). nd means not determined. WA50 = AX from WA obtained at 50% ammonium sulphate saturation. WA75= AX from WA obtained at 75% ammonium sulphate saturation. WB50= AX from WB obtained at 50% ammonium sulphate saturation WB75= AX from WB obtained at 75% ammonium sulphate saturation.

Figure 8.1: Effect of water extractable arabinoxylan on starch hydrolysis.

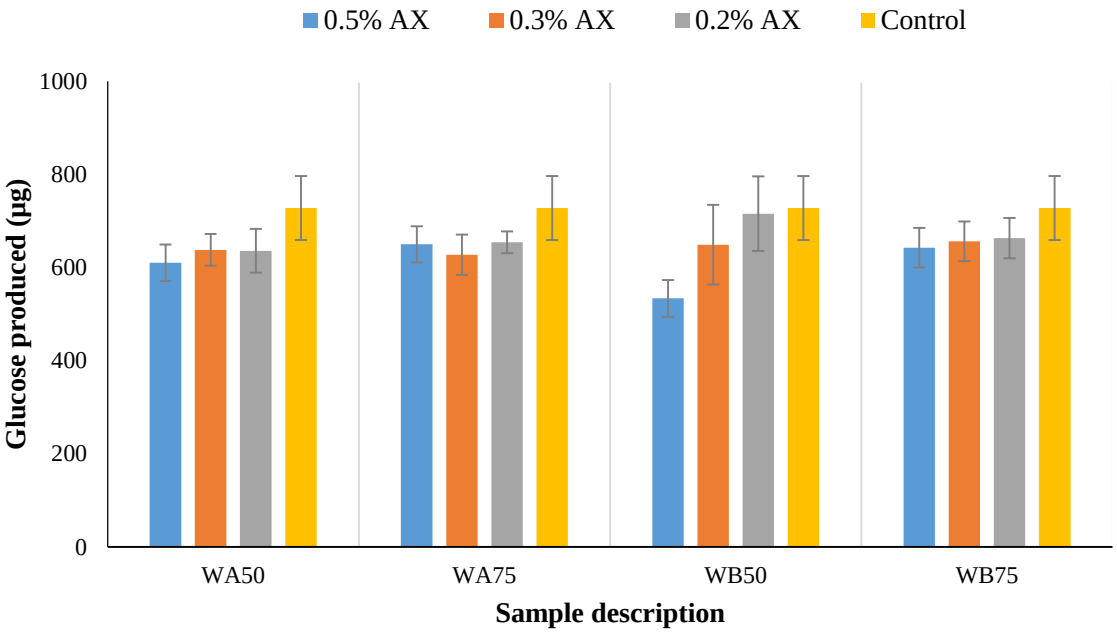
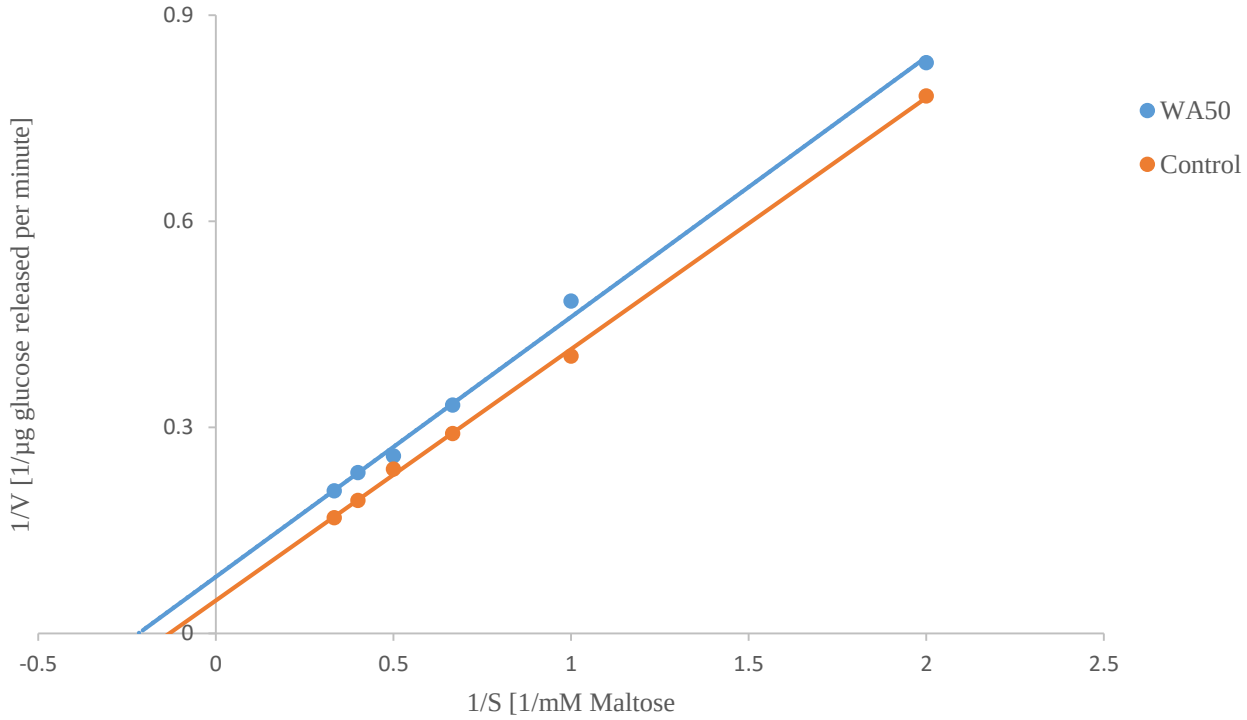


Figure 8.2: Lineweaver-Burk plot of rat intestinal α-glucosidase inhibition by water extractable arabinoxylan



CHAPTER 9

9 General Discussion and Conclusion

9.1 Research summary

Our results add weight to the role of arabinoxylans on prevention of chronic diseases such diabetes (Gemen, de Vries, & Slavin, 2011) and colorectal cancers (Zeng, Lazarova, & Bordonaro, 2014). Consumption of grains has been associated with reducing the risk of colorectal cancers and diabetes, but the underlying mechanism is not clear (Jonnalagadda, Harnack, Liu, McKeown, Seal, Liu, et al., 2011). Even though viscosity (Dikeman & Fahey Jr, 2006) is a plausible explanation, water extractable arabinoxylans may lose their viscosity when exposed to intestinal peristaltic force in a phenomenon referred to as shear thinning (Izydorczyk & Biliaderis, 1995). Western diets are typified by high fat foods with least fiber, which are rich in heme and lipid hydroperoxides (Kanner & Lapidot, 2001). The presence of antioxidants may neutralize heme catalyzed lipid peroxidation or other dietary free radicals in the gastrointestinal tract.

Arabinoxylans possess ferulic acid linked to O-5 position of some arabinofuranosyl residue (Krygier, Sosulski, & Hogge, 1982) which is a potent antioxidant. Most studies on cereal antioxidants are done using liberated phenolic acids on the assumption that they are released and eventually absorbed in the gastrointestinal tract (Karakaya, 2004). Despite the presence of esterase activity in the gastrointestinal tract, only less than 5% of ferulic acid is absorbed. Thus, in this research, we explored whether arabinoxylans (reservoir of ferulic acid) possess antioxidant properties. Our results (Chapter 3 and Chapter 4) suggest that water soluble arabinoxylans are good antioxidants capable of donating both electrons and hydrogen atoms to free radicals. Thus, when present in digesta, AX may neutralize dietary free radicals as they transit through the gastrointestinal tract.

Cereal AX is structurally complex with high heterogeneity index and vary greatly within and among cereal grain in ferulic acid content, arabinose to xylose ratio, uronic acid, and the nature of xylose substitution (Izydorczyk & Biliaderis, 1995). These differences also influence their physiochemical properties. Chapter 3 and 4 established that arabinose to xylose ratio and ferulic acid were the major determinant factors of AX antioxidant potency. AX with higher A/X are supposedly more soluble (Dervilly, Saulnier, Roger, & Thibault, 2000) hence expected to have higher antioxidant activity. Chapter 3 and 5 suggest moderate substitution is important as unsubstituted xylose residues may be involved in the redox reactions. Also ferulic acid is the active electron or a hydrogen donor site of AX. Thus, it is reasonable to expect that AX with higher ferulic acid will have a higher antioxidant capacity. However, our study (Chapter 4) suggests that in as much as ferulic acid is essential, factors leading to the high solubility of AX are important to attain high antioxidant activity. This observation is critical as it may explain why insoluble AX may not exhibit antioxidant properties (Serpen, Capuano, Fogliano, & Gökmen, 2007) as they are mostly crosslinked through ferulic acid bridges (Bunzel, Ralph, & Steinhart, 2005).

Dietary AX may also include oligosaccharide (AXOS) as xylanases are added during milling or bread making processes or AXOS may be added directly as prebiotics (Broekaert, Courtin, Verbeke, Van de Wiele, Verstraete, & Delcour, 2011). Different endoxylanase are being used in the industry resulting in AXOS of varying degrees of substitution, polymerization, and feruloylation. This research (Chapter 3) has demonstrated that the type of xylanase may influence the antioxidant potential of resultant AXOS. Xylanases leading to feruloylated AXOS with higher degree of polymerization would be ideal for maintenance of gut health because of their higher antioxidant potential. This observation would help the industry in selecting endoxylanase to better

human health. Endoxylanase devoid of esterase activity would be of choice in order to retain ferulic acid ester linkages with arabinose.

Also, this research has made contributions by identifying for the first time feruloylated arabinose and feruloylated pentosan disaccharides present in maize and wheat (Chapter 6). This observation may be crucial in our understanding of the assembly feruloylated arabinoxylans. It is not clear whether these side chains are preassembled prior to their addition to xylan chain (Doering, Lathe, & Persson, 2012; Fincher, 2009). It is interesting to note that the degree of polymerization of our isolated arabinoxylans coincides with the reported lengths of arabinoxylan side chains of 1 and 2 (Ishii, 1997; Smith & Hartley, 1983; Wende & Fry, 1997). This observation may suggest (but not conclusive) that the feruloylated arabinoxylan side chains may be preassembled. However, further studies are necessary to test such a hypothesis.

The fate of arabinoxylan in the gastrointestinal tract is poorly understood. It is widely believed that they are partially fermented by resident microbes as they transverse the colon. We further demonstrated that feruloyl arabinose is released during gastric digestion due to a low gastric pH. These observations are important because AXOS have a higher antioxidant capacity compared to ferulic acid (Chapter 5). Phenolic compounds required alcohol to exhibit antioxidant behavior under simulated gastric digestion of turkey muscles (Kanner & Lapidot, 2001). Cereal grains with higher content of bound ferulic acid resulted in a high amount of feruloylated arabinose during gastric digestion.

The antiglycemic activity of AX (Chapter 7 and 8) was studied with emphasis on their ability to directly interact with the enzyme and nutrient transporters. Thus, for the first time we have reported that feruloyl arabinoxylan mono-/oligosaccharides do inhibit intestinal α-glucosidase and glucose transporter 2 (GLUT2). Their potency is dependent on ferulic acid, but not the degree of

polymerization. AX (polysaccharide) only inhibited α-glucosidase activity (noncompetitively) but not starch hydrolysis to oligosaccharides. Evidence from our study suggested that solubility of WEAX is important and consequently not all WEAX have the same inhibitory effect. WEAX with higher ferulic acid content and A/X ratio may have high inhibition potency. Thus, animal and human intervention studies with AX are bound to yield inconsistent results unless such factors are taken into consideration. Unfortunately, most studies do not provide detail on the chemical characteristics of AX used (Gemen, de Vries, & Slavin, 2011). However, it appears that the concentration of soluble AX to achieve 50% inhibition of α-glucosidase activity may be difficult to achieve (based on WEAX % of cereal grains) unless if insoluble AX shares the same inhibitory attributes. Thus, our findings imply that the antiglycemic effect of wholegrain may be derived from a synergy among soluble AX, feruloyl mono-/oligosaccharide and free ferulic acids present in the digesta.

The observed inhibitory effects of feruloylated oligosaccharides against oxidants, α-glucosidase and GLUT2 can aid in the promotion of AXOS utilization by the food industry. Consequently, AXOS production would add value to cereal bran which is usually used as animal feed.

9.2 Limitations and future studies

The main limitation of our research is that we used *in vitro* studies to understand the possible mechanism through which arabinoxylans may exert their perceived anti-hyperglycemia and anti-cancer properties. Thus, it will be interesting to conduct the experiments *in vivo* for further understanding of the role of arabinoxylan in human health.

Secondly, the structure of arabinoxylans is complex and it appears that the existing methods for their characterization still provide limited information to understand their chemical and

physiochemical properties. Thus, studies to elucidate the chemical structure of arabinoxylans are recommended.

Finally, water unextractable arabinoxylans constitute a higher proportion of cereal arabinoxylans but could not be studied as it is difficult to extract them without disturbing their structure matrix. Thus, there is a need to develop a method of extracting water unextractable arabinoxylans. This would help to understand their role or any synergy that may exist in promoting human health.

9.3 Conclusions

Thus from our studies, it appears that arabinoxylans may indeed promote good health as speculated in the epidemiological and human intervention studies. However the mechanism involved should extend beyond viscosity hypothesis. From our research, the following mechanism may be involved for arabinoxylan to exert their perceived health benefits:

a. Arabinoxylans donate electrons or hydrogen atom to neutralize dietary free radicals (implicated in the initiation and/or development of chronic diseases) as they traverse through the gastrointestinal tract.
b. Arabinoxylans noncompetitively inhibit intestinal α-glucosidase and/or arabinoxylan mono-/oligosaccharides inhibit intestinal α-glucosidase and glucose transporter thereby attenuating postprandial blood glucose levels.

The quantity and heterogeneity in arabinoxylan structures present in the digesta have a big influence on the outcome.

9.4 References

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