EXTRACTION OPTIMIZATION, STRUCTURE MODIFICATION AND IMMUNOMODULATORY ACTIVITY IN VITRO FOR ARABINOXYLANS FROM CEREALS

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EXTRACTION OPTIMIZATION, STRUCTURE MODIFICATION AND IMMUNOMODULATORY ACTIVITY IN VITRO FOR ARABINOXYLANS FROM CEREALS

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Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another academic degree, or any other university or other academic or professional institution.

This project is an original and authentic piece of work by myself. I have full acknowledged and referenced all secondary sources used.

Zhengxiao Zhang

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Academic achievements

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Abstract

BACKGROUD: The industrial cereal brans produced as by-products of milling have been confirmed to be rich sources of arabinoxylans (AXs). The immunomodulatory properties of the extracted AXs from cereals have been reported, which potentially lead to corresponding health benefit in immune enhancement. However, this requires a clearer understanding of the relationship between the molecular structures of AXs and their immune-modulating activities (the structure-activity relationship). It is also considered essential to gain an understanding of the mechanisms of the immune-modulating properties of AXs.

AIMS & DESIGN: The aims of this study were to develop and improve the AXs extraction process from pentosan fraction of wheat flour and corn bran and to examine the molecular structures of extracted and modified AXs and investigate the molecular structure-immunomodulatory activity relationship *in vitro* for AXs. The effects of the different enzymatic extraction conditions (endoxylanase dosage, extraction temperature and time) on AXs extraction yield from the pentosan fraction of wheat flour were investigated by using a Box-Behnken experimental design and response surface methodology. For the AXs extraction process from corn bran, various extraction methods (alkaline, aqueous and enzyme extraction methods) were studied and compared. In addition, the treatments of three varieties of endoxylanases for AX molecular structure modification were studied. Furthermore, the extracted and modified AXs with different molecular structures were investigated with respect to their ability to modulate nitric oxide (NO) production and inducible NO synthase (iNOS) expression *in vitro* as an indication of their immunomodulatory potential.

RESULTS: From the pentosan fraction, the maximum recovery rate of AXs reached 86% of total pentosan AXs (dry matter basis) under the optimum extraction conditions. In contrast to the AXs obtained by aqueous extraction, the molecular weight (Mw) distribution of enzymatically extracted AXs was significantly different being more concentrated in the low Mw range (1KDa to 10KDa). The degree of branching (A/X ratio) increased from 0.48 to 0.83 as the concentration of enzyme increased. From the corn bran, using alkaline treatment, the recovery rate of AX was up to 80% (dry matter basis) of total corn bran AX and the Mw distribution of extracted AXs was in the high Mw range (100KDa to 794KDa). Following enzymatic modification, more than 30% of AXs extracted were reduced to the lower Mw range (0.1KDa to 10KDa). *In vitro* studies showed that the extracted and modified AXs from these two cereal sources significantly elevated the level of NO synthesis and iNOS expression by U937 cells ($p<0.05$), but modified AXs with higher portion of low Mw showed stronger activity than extracted AXs with higher portion of high Mw ($p<0.05$). It was also observed that the stimulatory effect of AXs on NO production by U937 cells was associated with their concentrations and sources. In addition, the investigation on the immune-modulatory activity of AXs extracted from 10 cereal sources showed that the stimulatory effect of AXs on NO production by U937 cells seems to be associated with average molecular weight. More interestingly, it was noted that extracted and modified AXs had a significantly different effect on iNOS expression in U937 cells ($p<0.05$), suggesting that NO synthesis stimulated by AXs *in vitro* is closely mirrored by iNOS expression.

CONCLUSIONS: As results of this study, the extraction process of AXs from corn bran and pentosan fraction of wheat flour was optimised. The conditions for modifying the molecular features of AXs were standardised. The experimental conditions for controlling the Mw distributions produced during the extraction and modification in order to enhance the immune-modulating activities of the AXs. The results of *in vitro* assessments should be useful in further understanding the mechanisms of the structure-activity relationship of AXs.

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List of Abbreviations

AEAXs	Alkaline extractable AXs
AXs	Arabinoxylans
AXOS	Arabinoxylo-oligosaccharides
A/X	The ratio of arabinose to xylose
BSA	Albumin from bovine serum
BRMs	Biological Response Modifiers
DP	Degree of polymerization
E-AEAXs	Enzyme modified AEAXs
E-WEAXs	Enzyme extractable AXs
E-XYLNP	Endo-1,4-β-xylanase of Megazme
FBS	Fetal bovine serum
GAXs	Glucuronoarabinoxylans
GH	Glycoside hydrolase
HPLC	High-pressure liquid chromatography
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
MGN-3	Rice bran arabinoxylan supplement
Mw	Molecular weight
NO	Nitric oxide
O-VR	OPTIMASHTM VR enzyme
P-BG	Pentopan Mono BG enzyme
ppm	Parts per million
P/S	Penicillin-streptomycin
SD	Standard deviation
SEC-HPLC	Size exclusion high-pressure liquid
SEM	Standard error of mean
WEAXs	Water extractable AXs
WUAXs	Water un-extractable AXs

Introduction

Arabinoxylans (AXs) are an important group of hemicelluloses found in the outer-layer and endosperm cell walls of cereals (Izydorczyk and Biliaderis 1995; Saeed et al. 2011; Vries et al. 1999). Hemicelluloses are branched polymers formed by combinations of various monosaccharides and cellulose with a linear backbone composed of glucose subunits linked by β-(1, 4) glucosyl (Xu et al. 2006). In the 1920s, a polysaccharide, named 'Pentosan', with high viscosity, which was found to be composed mainly of xylose and arabinose, was extracted from wheat flour, (Freeman and Gortner 1932 ; Hoffmann and Gortner 1927). Subsequently, polysaccharides with a high AXs content were found in the outer-layers of cereal grains such as wheat, corn, rice, barley, oat, rye, and sorghum, and have been studied extensively over the last few decades (Fincher and Stone 1986; Saeed et al. 2011; Vinkx and Delcour 1996). Since the 1980s, AXs are of interest to cereal chemists, as they have been found to have a significant influence on the quality of bread dough and bread (Courtin and Delcour 1998; W. Li et al. 2013). Furthermore, as a result of their high viscosity and excellent water-holding properties, AXs have been used as food thickening agents and stabilizing agents (Carvajal-Millan et al. 2006; Lapierre et al. 2001; M. P. Yadav et al. 2008). More recently, AXs have been reported to possess various biological activities, such as lowering serum cholesterol, antioxidant activity and post-prandial glycaemic response reduction and immunity enhancement as well as an ability to reduce the risk of

coronary heart disease and applications in weight management systems (Benko et al. 2007; Lu et al. 2000; Lu et al. 2004; Swennen et al. 2006).

Cereal brans were found to be cost effective sources of AXs, which are by-products of cereal processing (Izydorczyk and Biliaderis 2007). As a result of their high Mw and high ferulic acid content AXs readily form covalent/non-covalent linkages between AXs chains and with other components of the cell wall such as proteins, β-glucans, lignin and cellulose, hence a high proportion of AXs cannot be extracted using water (Beaugrand et al. 2004b; Saulnier et al. 2007). Therefore, various methods have been developed for the extraction and purification of AXs from cereal by-products, including alkaline and acid extraction (Höije et al. 2005; Hollmann and Lindhauer 2005; Zhou et al. 2010), enzyme hydrolysis (Beaugrand et al. 2004c; W. Li et al. 2013; Maes et al. 2004), microwave-assisted extraction (Rose and Inglett 2010), ultrasound-assisted extraction (Z. Ebringerova et al. 1998; Hromadkova et al. 1999), steam explosion extraction (Allen et al. 2001), hot compressed water extraction (Dien et al. 2006), twin-screw extrusion extraction (Marechal et al. 2004; Zeitoun et al. 2010), ethanol purification and ammonium sulphate precipitation (Izydorczyk and Biliaderis 2007). It has been found that the extraction yields and molecular characteristics of AXs vary depending upon the extraction and modification methods used (Z. Zhang et al. 2014).

The pentosan fraction of wheat flour is a by-product of wheat starch processing using high-pressure disintegration technology (W. Li 2012). This cereal by-product has been found to be an AX-enriched source and the bioactivities of the AXs have been reported (W. Li 2012; W. Li et al. 2015). However, the effects of extraction and

modification conditions on the yield and molecular structures of AXs from the pentosan of wheat flour have been the subject of only limited reports.

In addition, corn bran has also been identified to be a cereal source with a high AXs content and the extraction methods for this material have also been studied (L.W. Doner and Hicks 1997a; L.W. Doner et al. 1998). However, the effects of enzyme modification processes on the molecular structures of the corn bran AXs have not well documented.

Cereal AXs are of significant importance to human health due to their potential to modulate both the adaptive and innate immune systems(S. Zhang et al. 2015). Previous studies have demonstrated that the modified AXs from rice bran (MGN-3) with a low Mw distribution (30-50 kDa) have immune-modulating activities *in vitro* and *in vivo* studies (Ghoneum 1998a; Ghoneum and Brown 1999; Ghoneum and Matsuura 2004). The AXs extracted from wheat bran via alkaline (large Mw: 350KDa) and enzyme (low Mw: 33KDa) both showed stimulating effects on the immune response *in vivo*. The low Mw AXs showed a higher level of macrophage phagocytosis than the large Mw AXs(Zhou et al. 2010). The structure of AXs vary in Mw, degree of branching and conformation (Izydorczyk and Biliaderis 2007). Thus, their structure-activity relationship may be different. However, there has been no clear consensus as to which main molecular structural properties are required for AXs to confer their immune-modulating ability (S. Zhang et al. 2015). More recently, it has been reported by W. Li et al. (2015) that enzyme extracted AXs from the pentosan of wheat flour have greater immune stimulation activities *in vitro* testing than water extracted AXs. This

difference in bioactivity may associated with the enzyme extracted AX having a higher portion of lower Mw (≤25KDa) and higher degree of branching (0.81). However, further investigation is needed to determine whether it is the difference in Mw distribution or the difference in the degree of branching or a combination of these which produced the relatively high stimulatory activity of the enzyme treated AXs (W. Li et al. 2015). In addition, the mechanisms by which AXs modulate the immune system still remain to be studied.

The research in this thesis aims to develop extraction and modification processes for optimizing the yield and modifying the molecular structures of AXs from the pentosan fractions of wheat flour and corn bran. The sugar compositions and Mw of the extracted and modified AXs were determined using high performance liquid chromatography (HPLC). Subsequently, the relationship between molecular structure and immune-modulating activity of the AXs and the mechanism underlying AXs immune modulatory effects on NO production and iNOS expression *in vitro* are investigated.

Objectives

1. To optimize the enzyme treatment conditions for increasing the extraction yield of AXs from the pentosan fraction of wheat flour and develop the alkaline extraction processes for isolating AXs from corn bran.
2. To modify the molecular structure of AXs from the two cereal sources using enzyme treatments and determine the Mw distributions and monosaccharides compositions of the extracted and modified AXs.
3. To assess the immunomodulatory effects of the extracted and modified AXs with different molecular structures from the pentosan fraction and corn bran, on NO production *in vitro* using the human macrophage cell line U937 and analyse the AXs molecular structure and immune activity relationship.
4. To investigate the effect of the AXs with different molecular structures on iNOS expression and propose a possible mechanism for AXs to modulate NO production *in vitro* by the U937 cells.

Hypotheses

1. AXs treatment can induce iNOS expression *in vitro* by U937 cells.
2. NO synthesis *in vitro* by AXs stimulation is due to the modulation of iNOS expression.
3. The stimulatory effect of AXs treatment on NO production *in vitro* by the U937 cells is related to their Mw distributions and sources.
4. The stimulatory effect of AXs treatment on iNOS expression *in vitro* by the U937 cells is related to their Mw distributions.

CHAPTER 1

Literature Review

1.1 Introduction

The aims of this review are to focus on methods for the extraction and modification of AXs from cereal by-products, also to investigate and compare the effects of these methods on the yields and chemical properties of AXs and to give an overview of the immune modulating properties of cereal AXs.

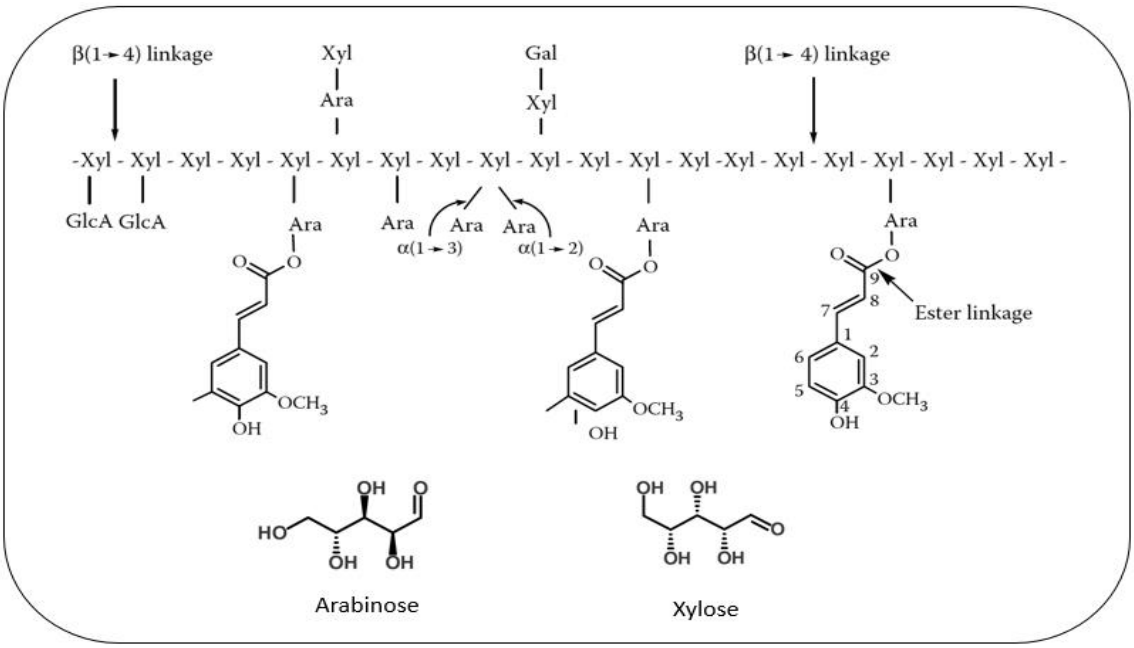
1.2 Arabinoxylans of cereals

1.2.1 Structure

AXs are composed of a backbone of β-1,4 linked D-xylopyranosyl residues. Monomeric α-L-arabinofuranoside can be present at the C (O)-3 and/or the C (O)-2 positions of the xylose moieties (Izydorczyk and Biliaderis 2007). Comparison of molecular structures of AXs from whole cereal grains and cereal by-products, Izydorczyk and Biliaderis (2007) indicated that the AXs from cereal brans from rice, sorghum, finger millet, and maize have more complex side chains (including xylopyranose, galactopyranose, and α-D-glucuronic acid or 4-O-methyl-α-D-glucuronic residues) than those from cereals such as wheat, rye, and barley. The general structure of AXs from cereal bran is shown in Figure 1.1. The AXs can be cross-linked to ferulic acid at the C (O)-5 positions via an ester linkage (Izydorczyk and Biliaderis 1995; Izydorczyk and Biliaderis 2007; Saeed et al. 2011). Ferulic acid side chains can also form

linkages with β-glucan/cellulose/glucose/protein (Andersson and Aman 2000; P. G. Dervilly et al. 2001; Izydorczyk and MacGregor 2000).

Figure 1.1 The general structure of AXs from cereal bran (Izydorczyk and Biliaderis 2007)



The main differences between the various cereal brans are the manner of arabinose residue substitution in the xylan backbone, in the relative proportions and sequence of the various linkages between these two sugars (xylose and arabinose), and in the presence of other substituents (Izydorczyk and Biliaderis 1995). A/X of AXs from wheat endosperm may vary from 0.50 to 0.71 (Cleemput et al. 1993; Izydorczyk et al. 1991; Rattan et al. 1994) but it is usually lower than that found in wheat bran (1.02-1.07) (Brillouet and Joseleau 1987; Shiiba et al. 1993). Rye endosperm AXs are less substituted (0.48-0.55) than the equivalent wheat material. In contrast corn bran usually has A/X in the high range from 0.75 to 1.82 (Ogawa et al. 2005; Rose and Inglett 2010).

1.2.2 Solubility

AXs of cereals and cereal by-products can be divided into water-extractable (WEAXs) and water un-extractable (WUAXs) types(Izydorczyk and Biliaderis 2007). Fengler and Marquardt (1988) reported that rye WUAXs form part of the cell wall and are covalent and non-covalently linked to other AXs and to other cell wall components such as cellulose, lignin or proteins. In contrast to the WUAXs, Mares and Stone (1973) deduced that the WEAXs in wheat are only loosely bound at the cell wall surface. Neukom (1976) indicated that the deficient cross-linking with other components, structural differences or initial enzymatic hydrolysis in the cereal might be the reasons for their water-extractable property.

As summarised in previous studies (Table 1.1), the WEAX component of the total AXs is much lower than the WUAX component in cereals or cereal by-products. Thus, improving the solubility of WUAX has been an important area of research for those wishing to increase AXs extraction yields. Gruppen et al. (1991) reported that the bridges between the AXs molecules and hydrogen and covalent bonds of cell wall matrix were broken when WUAXs were treated with alkali. A large proportion of WUAX molecules were released from the cell wall and became alkali-solution extractable, these are referred to as alkaline-extracted AXs (AEAXs). Courtin and Delcour (2001) discussed whether an enzyme could be used to effect the extraction of AXs from wheat. Treatment of WUAXs with endoxylanases also contributes to the production of enzyme-extracted AXs (E-WEAXs), in which the xylan backbone is degraded. Therefore,

E-WEAXs have reduced molecular weights. However, numerous reports convincingly show that the solubility of AXs depends primarily on the degree of substitution of the AXs (Maes and Delcour 2001; Mandalari et al. 2005). AXs with less arabinose substitutes have lower solubility in water.

Table 1.1 AXs contents of various cereals and cereal by-products (dry basis) (Izydorczyk and Biliaderis 2007)

Raw materials	Tissues	Total AXs (%)	WEAXs (%)	References
Wheat	Whole grain	5.77	0.59	(Hashimoto et al. 1987b)
	Bran	21.4	/	(Courtin and Delcour 2001)
	Bran	25	1	(Hollmann and Lindhauer 2005)
	Bran	19	/	(Bataillon et al. 1998)
	Bran	19.38	0.88	(Hashimoto et al. 1987b)
	Flour	/	0.43	(Ganguli and Turner 2008)
	Flour	1.37-2.06	0.54-0.68	(Izydorczyk et al. 1991)
	Durum wheat	4.07-6.02	0.37-0.56	(Lempereur et al. 1997)
Barley	Whole grain	6.11	0.35	(Hashimoto et al. 1987a)
	Whole grain	3.4-4.1	/	(Izydorczyk and MacGregor 2000)
	Whole grain	/	0.40-0.88	(Oscarsson et al. 1996)
	Whole grain	6.36-8.58	0.379-0.808	(Fleury M. D. 1997)
	Pearled grain	4.45	0.27	(Hashimoto et al. 1987a)
	Pearling	14.14	0.54	(Hashimoto et al. 1987a)
	Pearled flour	/	0.3-1.08	(P. G. Dervilly et al. 2001)
Corn		27.2	/	(M.P. Yadav et al. 2007)
	Bran	29.86	0.28	(Hashimoto et al. 1987a)
	Bran			
Rye		7.6	/	(Bengtsson and Aman 1990)
	Whole grain	8-12.1	2.6-4.1	(Hansen et al. 2003)
	Whole grain	/	1.7	(Figueroa-Espinoza et al. 2004)
	Bran	3.2-3.64	2.2-2.65	(Cyran et al. 2003a)
	Flour			

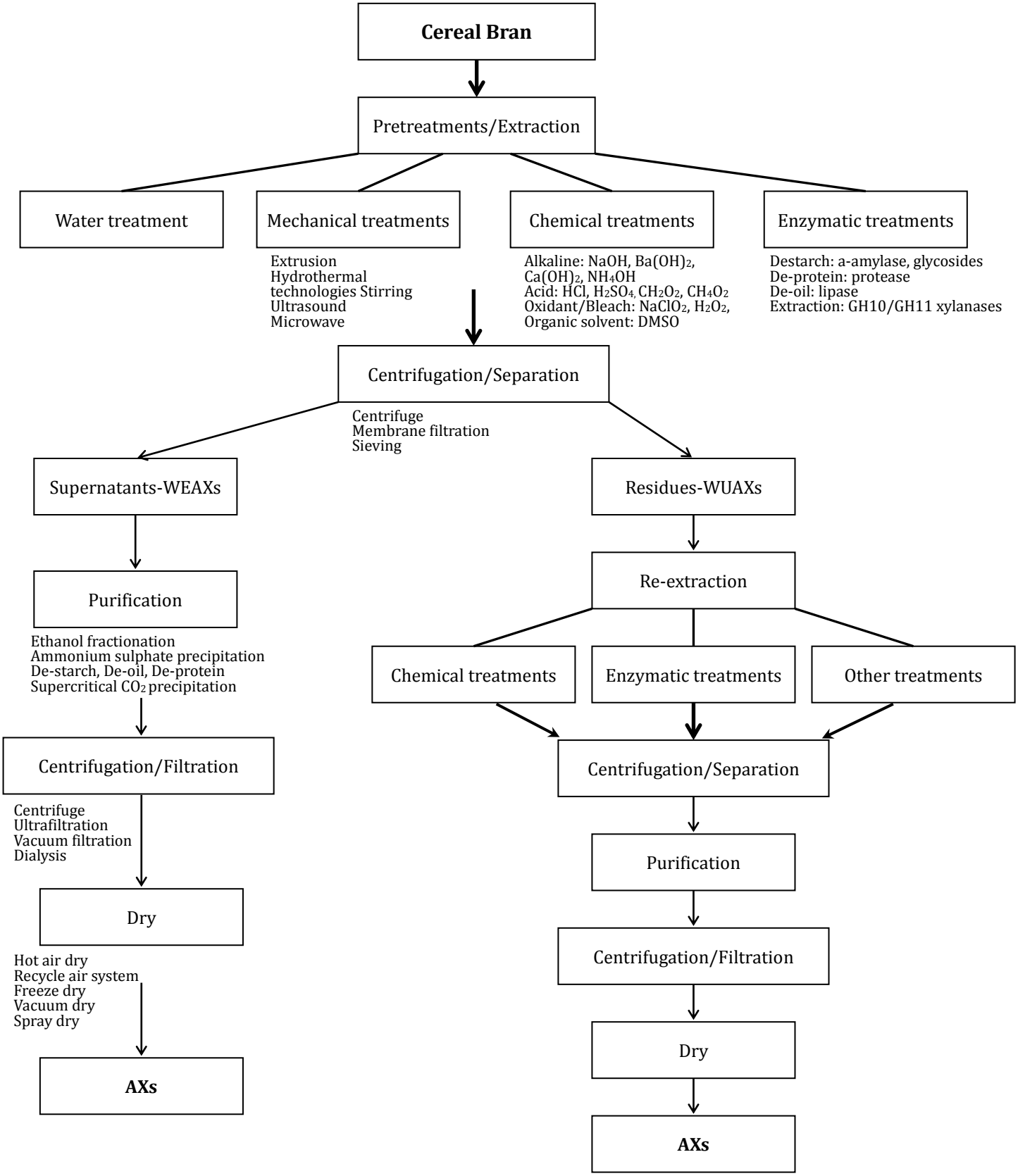
Oats	Whole grain	2.73	0.17	(Hashimoto et al. 1987a)
	Hulls	8.79	0.1	
	Bran	3.5	0.33	(Hashimoto et al. 1987a)
	Pearled grain	3	0.15	(Beaugrand et al. 2004a)
				(Hashimoto et al. 1987a)
Rice	Whole grain	2.64	0.06	(Hashimoto et al. 1987a)
	Hulls	8.36-9.24	0.11-0.11	
	Bran	4.84-5.11	0.35-0.77	(Hashimoto et al. 1987a)
				(Hashimoto et al. 1987a)
Sorghum	Whole grain	1.8	0.08	(Hashimoto et al. 1987a)
	Pearling	5.4	0.35	(Hashimoto et al. 1987a)
Soybean	Hulls	13.1	1.33	(Hashimoto et al. 1987a)

Insolubility of the low A/X substitution fraction has been attributed to an increased aggregation of unsubstituted regions of the AXs stabilised by hydrogen bonds. These interactions may contribute to an increase in viscosity or precipitation of polymer chains (Izydorczyk and Biliaderis 2007). Izydorczyk and MacGregor (2000) reported that there are non-covalent interactions between less substituted AX chains (A/X = 0.18-0.32) and other cellulose such as β-glucans. In the cereal cell wall material, the non-covalent topological associations between β-glucans and AXs could also lead to low water solubility or to low enzyme digestibility of these polymers. Izydorczyk and Biliaderis (1992a) also reported that high Mw AXs of wheat exhibited high intrinsic viscosities when in the form of a slurry and weak elastic properties when in solution.

1.3 Extraction of arabinoxylans

In the intact cell wall of cereal grains, most AXs and other polymers are cross-linked (e.g. diferulic acid bridges between contiguous AXs chains; covalent ester linkage between carboxyl of uronic acids and the hydroxyl groups of AXs) with other wall components to form a structural network, which is not soluble in an aqueous environment (Fincher and Stone 1986; Fry 2004; Gruppen et al. 1992). As a result of these cross-links, a major proportion of the AXs in cereal grains cannot be easily extracted using water and requires stricter treatments such as alkali solutions or enzymatic hydrolysis to remove the AXs from the structural fabric of covalent and non-covalent cross-linking, as well as mechanically assisted treatment. The isolation procedures for extracting AXs from wheat flour, wheat bran and straw have been extensively studied since the 1980s, giving rise to a wide range of extraction and purification methods (Courtin and Delcour 2001; Fincher and Stone 1986; Jacquemin et al. 2012). The most common methods for isolating the AXs from various cereal sources include aqueous, chemical, enzymatic or physically assisted extraction of these polymers. The general extraction process for AXs from cereal bran is shown in Figure 1.2.

Figure 1.2 The flow-chart of the general extraction process for AXs from cereal bran



1.3.1 Water extraction

Water extraction is one of the most common methods for isolating AXs (Izydorczyk & Biliaderis, 2007). Fleury M. D. (1997) reported that AXs were extracted with water from different fractions of two barley cultivars. It was found that the WEAX yield varied depending on barley varieties and sub-fraction. In addition, Cyran et al. (2003b) reported the extraction yields of AEAXs achieved at 4, 40 and 100°C (1:5 w/v) were 1.1-1.4%, 0.17-0.33% and 0.41-0.51% of rye flour respectively. Furthermore, Ganguli and Turner (2008) developed a simplified small-scale process to isolate WEAXs from wheat flour. AX was extracted using water (1:10w/v, 90min, stirring, room temperature) and starch was removed using amyloglucosidase. Subsequently, 65% ethanol was used to purify and precipitate AXs. The extraction yield of WEAX was 0.43% of wheat flour. Based on the previous studies, the water extractability of AXs is lower than that achieved by chemical and enzyme methods. It has been suggested that water combined with relatively gentle conditions (e.g. below 100°C) is not able to break the cross-links between AX and the cell wall matrix (Izydorczyk & Biliaderis, 2007). Therefore, mechanical treatments combined with water extraction such as hydrothermal processing have been studied as means to increase hemicellulose extraction yields (Garrote et al. 1999; Mok and Antal Jr 1992). Hydrothermal technologies use liquid or supercritical water treatments at high temperature (200-600°C) and high pressure (5-40MPa) (A. A. Peterson et al. 2008). Bobleter (1994) reported that hemicellulose is easily isolated from plant material at temperatures

above 180°C. Mok and Antal Jr (1992) reported above 90% hemicellulose could be solubilized from different plants (including sorghum) using hot compressed liquid water 0-15min at 200-230°C. According to Garrote et al. (1999), 65-82% hemicellulose can be extracted from plants under hydrothermal technologies.

1.3.2 Chemical solvent extraction

1.3.2.1 Extraction with alkali solution

Woodman and Evans (1947) noted that as early as 1900, Kellner and Kohler had been using alkaline treatment on cereal bran. They studied the effects of pressure-cooking wheat straw in dilute NaOH solutions and then using water to wash away the alkali. The earliest studies used NaOH to extract polysaccharide components was in order to increase the quality feeds from straw (Beckmann 1921; Jackson 1977).

Recently, research into chemical methods for the extraction of cell wall polysaccharides (including AXs) on a laboratory scale has included a wide variety of solvents including NaOH (X. Zhang et al. 2008b; Zhou et al. 2010), $Ca(OH)_2$ (Ogawa et al. 2005), HCl and NH_4OH (Höije et al. 2005), $Ba(OH)_2$ (Gruppen et al. 1991) and KOH (DuPont and Selvendran 1987).

Alkaline extraction disrupts the hydrogen and covalent bonds and loosens up the cell wall matrix, consequently releasing various polysaccharides from the cell wall (Fincher and Stone 1986). Hydroxyl ions disrupt the hydrogen bonds between cellulose and hemicellulose, and also break ester linkages, and hence solubilise part of the hemicellulose material (Cyran et al. 2004). Alkaline conditions also change the uronic

acid residues to their negatively charged form, which causes repulsion between the different molecules, and as a result improves the extractable of AXs (Southgate 1991).

Ba^{2+} ions are reported to specifically interact with pentose sugars of AXs and preferentially ease their extraction (Fincher and Stone 1986). Gruppen et al. (1991) introduced the use of barium hydroxide as a primary extractant for WUAXs. Barium ions show an enhanced separation of AXs and β-glucans, resulting in the selective extraction of 80% of the wheat flour WUAXs (Gruppen et al. 1991) or 50% of the wheat bran WUAXs (Bergmans et al. 1996).

Alternatively, WUAXs can be extracted by dilute alkaline solutions of hydrogen peroxide, which extracts 69% of the original AXs content in wheat bran (Maes and Delcour 2001). As shown in Table 1.2, X. Zhang et al. (2008b) indicated that when the extraction temperature, time, and the concentrations of sodium hydroxide and hydrogen peroxide were 88°C, 200min, 0.16mol/L and 1.5% respectively, the yield of AXs reached the maximum (21.23% of wheat bran). In addition, alkali concentration (increased from 0.37 M to 1.85 M) and extraction temperature (increased from 40°C to 100°C) have been reported to have a positive effect on the yield of AXs from destarched wheat bran (Table 1.5) (Bataillon et al. 1998; Persson et al. 2009).

Table 1.2 Synthesis of the key literature data on AXs and other polysaccharides extraction from cereal by-products by chemical solution extraction methods

Sources	Extraction	Yield% of AX[a]/GAX[b]/ Hemicellulose[c]	Mw[d] (Da)	A/X	References
Wheat bran (Destarched)	Lab-scale, 0.15M NaOH (0.5% H_2O_2, v/v).	18.5[a]	3.5×10^5-7.2×10^6	0.8	(Zhou et al. 2010)
Wheat bran (Destarched)	Lab-scale, 0.16 mol/ml NaOH(0.5%-1% H_2O_2,v/v).	21.2[a]	/	/	(X. Zhang et al. 2008b)
Wheat bran (destarched)	Lab-scale, delignification (37g $NaClO_2$/100g destarched bran/7.2ml (72%) H_2SO_4/1.5L H_20), Extraction at 40°C, 6 h (2L 0.5M NaOH /destarched bran), purification of microfiltration and atomization.	16[a]			(Bataillon et al. 1998)
Wheat bran (Destarched)	Pilot scale, delignification (30kg $NaClO_2$/81kg destarched bran/1.5L(98%) H_2SO_4/ 900L H_20), Extraction at 40°C, 6 h (35kg NaOH /destarched bran), purification of microfiltration and atomization.	13[a]	/	/	(Bataillon et al. 1998)
Wheat bran	Pilot scale, Purification with boiling 70% ethanol; Extraction with 2% hydrogen peroxide (pH 11, 40°C).	11.7[b]	$1\text{-}1.1\times10^5$	0.8	(Hollmann and Lindhauer 2005)
Wheat straw	Lab-scale, Formic and acetic acids and H_2O (30/60/10, v/v/v) at 85 °C for 4 h, precipitated in 3 volumes of 95% ethanol.	29.6[c]	1.0×10^4	0.04	(Xu et al. 2006)
Wheat straw	Lab-scale, Ethanol and water (60/40, v/v) with 0.1% HCl at 85 °C for 4 h.	8.6[c]	1.84×10^4	0.21	(Xu et al. 2006)
Corn bran (Destarched)	Lab-scale, 33ml(30% H_2O_2) to 1.4L extract, 20ml(50% NaOH) to 1.4L extract, stirring 2 h room temperature.	21-40[c]	/	/	(L.W. Doner et al. 1998)
Barley husks	Lab-scale, HCl and NH_4OH pretreatment; ethanol delignification; NaOH extraction 2h 75°C.	31.1[a*]	4.47×10^4	0.18	(Höije et al. 2005)
Barley husks	Lab-scale, HCl pretreatment (pH 4.0); NaOH extraction.	57.0[a*]	3.59×10^4	0.22	(Höije et al. 2005)

a: AXs extracted yield by raw material dry basis (w/w); a: AXs extracted yield by original AXs content in raw material; b: GAX extracted yield by raw material dry basis (w/w); c: Hemicellulose (consists primarily AXs) extracted yield by raw material dry basis (w/w);d： Mw of extracted material.*

The alkaline treatment also releases some other polymers, for example, lignin from the cell walls of cereal straw, thus decreasing the AXs purity (L.W. Doner and Hicks 1997a; Jackson 1977). It has been shown that alkaline peroxide is an effective reagent for delignification and improving the whiteness of hemicelluloses from cereals (L.W. Doner and Hicks 1997b; Pan et al. 1998). In alkaline media, hydrogen peroxide forms the highly active hydroperoxide anion (HOO^-) (Peng et al. 2012), which oxidise lignin structures by reacting with the hydrophilic (carboxyl) groups, breaking some inter-unit bonds, resulting in the separation of lignin and hemicelluloses (Pan et al. 1998). Sequential alkaline extraction and alkaline hydrogen peroxide bleaching (pH of extraction medium≈11.5, 10g 30% H_2O_2 to 1.4L extract) have been used to prepare corn fibre gum (containing about 87% AXs) in yields ranging from 21 to 40% of destarched corn bran (Doner et al. 1998). Yadav et al. (2008) also used sequential alkaline extraction and alkaline hydrogen peroxide bleaching to extract hemicellulose (containing about 82-86% AXs) achieving yields of 22.2-26.6% from corn fibre (fibre originating from the endosperm portion of corn kernels). L. W. Doner and Johnston (2001) also reported the effects of alkaline hydrogen peroxide treatments on the yields of AXs from corn fibre. It was found that the addition of H_2O_2 enhanced the AXs extraction yield by 8.8% compared to extraction without H_2O_2 in an alkaline environment (pH 11.5 with 50% NaOH, 100°C, 1h).

Bataillon et al. (1998) studied the extraction and purification of AXs from destarched wheat bran at pilot scale. At the pilot conditions (Table 1.2), the yield of purified AXs was 13% of destarched wheat bran equivalent to about 6% of total wheat

bran. The purity of the final AXs was 75%. Hollmann and Lindhauer (2005) studied glucuronoarabinoxylans (GAXs) extraction from wheat bran at the pilot scale (Table 1.2). In this pilot-scale procedure 350g of GAXs (80% purity) were recovered from 3kg of wheat bran. Briefly, wheat bran was milled from the wheat variety Bussard to a particle diameter was 0.5mm. Then the wheat bran was treated with boiling 70% ethanol with stirring. Subsequently, the treated bran was extracted with water to remove WEAX. Then the WEAX-free wheat bran was recovered by sieving (0.4mm) and treated by 2% hydrogen peroxide (pH 11) at 40°C. These procedures took 1 week and required about 30L of 70% ethanol, 40L 2% hydrogen peroxide, 45L H_2O_2. This study shows that GAXs can be extracted at about 80% purity from wheat bran (GAXs yield≈11.7%). The A/X ratio was 0.8, ferulic acid content was below 0.1% and Mw of AXs was 100–110 kDa. Thus the extraction yield of AXs in this pilot was lower than that achieved at laboratory scale studies as compared in Table 1.2.

1.3.2.2 Extraction with organosolv

Treatment with organic acid solution is an alternative extraction method used to obtain AXs from cereals. Xu et al. (2006) compared the effect of various acid treatments including acetic acid, formic acid, methanol, and ethanol combined with 0.1% HCl at 85°C for 4 h on AXs yields from wheat sources. The optimum conditions for high yields of hemicelluloses was found to be a mixture of formic and acetic acids and H_2O (30/60/10, v/v/v), which yielded 29.6% hemicelluloses, on a dry weight basis from wheat straw (Table 1.2). It was found that the extracted hemicellulose contained about

78.1% AXs and the A/X ratio was below 0.1. Xu et al. (2006) indicated that the molecules of hemicelluloses obtained from the organic acids treatment were more linear, mainly because the high acid hydrolysis cleaved the side chains of xylans from the backbone. In contrast, extraction with two organic solvents without acid: methanol and ethanol resulted in a lower yield (5.5% and 8.6% respectively) of the GAXs (Table 1.2). So, organic acid treatment may be a more effective extraction method for high AXs yields compared with using organic solvents. In addition, the study of Xu et al. (2006) used 3 volumes of 95% ethanol to separate lignin and hemicellulose. The results showed over 90% lignin dissolved in the ethanol and was separated from hemicellulose, which is not soluble in the absence of HCl. Thus, this study indicated that the utilization of organic solvents such as ethanol as delignification media, offer the option of removing the lignocellulosic moieties preventing lignin and cellulose contamination of the extracted AXs. However, it is noted that if there is extensive chemical hydrolysis (acid-catalysed hydrolysis) of the cereal bran, a small portion of the AXs might be degraded into very low Mw compounds and dissolve in organic solvent. This may lead to AXs loss during fractionation and hence a decrease in extraction yields.

1.3.3 Enzyme extraction

An alternative extraction method for isolating AXs from bran is enzymatic degradation. Xylanases, key enzymes of carbohydrate metabolism, were isolated from *Bacillus subtilis* in 1986 (Paice et al. 1986). Among the enzymes involved in AXs isolation, the endo-β-(1,4)-xylanases (EC 3.2.1.8) are the most common enzymes

(Beaugrand et al. 2004a; Escarnot et al. 2012; Henrissat 1991). Endo-β-(1,4)-xylanases are from family 11 of the GH classification system (Beaugrand et al. 2004a). Endoxylanases can attack the xylan backbone, cleave internal β-(1,4)-linkages in a random manner and penetrate the cell-wall network, producing a mixture of (un)substituted xylooligosaccharides and a portion of WUAXs will be rendered soluble and extractable (Andersson et al. 2003; Beaugrand et al. 2004c; W. Li et al. 2013; Swennen et al. 2006). The action of xylanases results in the (partial) solubilisation and extraction of WUAXs and the depolymerisation of WEAXs (Courtin and Delcour 2001).

Because of amino acid sequence similarities, most xylanases are categorised into two GH families: GH10 and GH11. GH10 contains xylanases of plant, bacterial, and fungal origin, whilst GH11 contains only xylanases of bacterial and fungal origin. A limited number of xylanases are presently classified as GH5 and GH8 (Henrissat 1991). Some studies have reported that AXs are more effectively solubilized from destarched wheat bran by GH11 xylanases (41-49% of the total AX) than by GH10 xylanases (18-26% of the total AX) (Beaugrand et al. 2004c; Maes et al. 2004) (Table 1.3). The higher efficiency of the former in hydrolysing wheat bran may be explained by the higher selectivity of some GH11 xylanases for insoluble substrates (Maes et al. 2004) and by their greater ability to penetrate the cell wall network (Beaugrand et al. 2004c). The end products of hydrolysis by GH11 xylanases have lower A/X ratios (0.23-0.28) than those produced by GH10 xylanases (0.38-0.43) (Beaugrand et al. 2004c; Maes et al. 2004), indicating that the two enzyme families have distinct patterns of action on destarched wheat bran (Beaugrand et al. 2004c).

AXs extraction yields from various cereal materials using different enzymes and experimental conditions vary considerably. Table 1.3 provides details of the experimental conditions, the enzyme combinations used and the yields achieved. As shown in Table 1.3, the Pentosan mono BG (X. Zhang et al. 2008a), (1-4)-β-endoxylanase (Beaugrand et al. 2004a), crude bacterial xylanase and fungus xylanase (Ogawa et al. 2005) have been used to extract the AXs from wheat bran, pentosan of wheat flour and corn husk. Other enzymes have been used for extraction and purification of AXs for example, α-amylase has been used to remove the starch from corn gum (L.W. Doner et al. 1998). X. Zhang et al. (2008a) optimized the enzymatic (Pentopan mono BG) degradation conditions of AXs of wheat bran were using quadratic regression orthogonal rotary tests. It was concluded from the model that when temperature, time, enzyme concentration and pH value are 60°C, 3h, 0.75% and 5 respectively, the yield is maximal (15.28%) and consistent with the experiment results. In addition, Beaugrand et al. (2004a) concluded that yields of 61-67% AXs of the total carbohydrate content were achieved when destarched bran was treated with xylanase from different wheat cultivars. The A/X ratio (about 0.32) of the soluble AXs fragments is significantly lower than the corresponding value (about 0.6) for total AX in the destarched bran, indicating that xylanases are preferable for solubilising low substituted AXs (Beaugrand et al. 2004a) (Table 1.3). Microscopic analysis of untreated and enzyme-treated wheat bran showed that xylanase treatment causes complete disorganisation of bran aleurone layer (Benamrouche et al. 2002). Xylanase treatment

also releases feruloylated AXs fragments, although diferulic acids are enriched in the insoluble bran material (Beaugrand et al. 2004a).

Comparison of chemical reagents (such as alkali and acid) with enzyme treatment, showed relatively lower AX yields, for example, the extraction rate (12.4%) of AXs from de-starched wheat bran with endoxylanase, (Pentopan Mono BG), was lower than that achieved (18.5%) with alkaline-hydrogen peroxide (Zhou et al. 2010) (Table 1.2 & Table 1.3). Furthermore, compared with the enzyme pre-treatment, the yields of AXs were much higher in the chlorite and HCl pre-treatments: 25.1% and 57% respectively (Höije et al. 2005). Alonso et al. (2003) indicted that obstacles such as the crystalline structure of lignocellulose and the presence of enzyme inhibitors often limit enzyme hydrolysis, resulting in low yield. However, Zhou et al. (2010) stated that the action of alkaline solution is not a friendly environment and may break down the ester bond between ferulic acid side chain and AXs, releasing the ferulic acid which has antioxidant functionality. Enzyme-extracted AX (AXE) showed a higher ferulic acid content and greater immune-enhancing activities than did alkaline extracted AX (AEXA) in an *in vivo* trial (Zhou et al. 2010). It is obvious that the enzymatic method shows environmentally friendly and economic advantages over the chemical method when used in industrial-scale food production.

Table 1.3 Synthesis of the results from key literature data on AXs extraction from cereal by-products by enzymatic extract methods

Sources	Extraction	Yields% of AX[a]	AX Mw (Da)	A/X	References
Wheat bran (Destarched)	Lab-scale,β-(1-4) endoxylanase (10Units/ml).	14.3-18[a]	/	0.32	(Beaugrand et al. 2004a)
Wheat bran (Destarched)	Lab-scale, GH10 xylanase 24 h. GH11 xylanase, 24 h. GH10 + GH11 xylanase, 24 h.	25.5[a*] 49[a*] 50.7[a*]	/ / /	0.43 0.23 0.24	(Beaugrand et al. 2004c)
Wheat bran (Destarched)	Lab-scale, Xylanase (150 units).	12.4[a]	3.25×10^4	0.56	(Zhou et al. 2010)
Wheat bran (Destarched)	Lab-scale, Pentopan Mono BG 0.75%, 60°C, 3 h, pH 5.	15.28[a]	/	/	(X. Zhang et al. 2008a)
Barley husks	Lab-scale, Thermo-stable a-amylase (pH 6, 80°C, 45 min); protease and amyloglucosidase (60°C, 45 min); enzyme inactivate (100°C, 15 min).	25.1[a*]	3.43×10^4	0.37	(Höije et al. 2005)
Rye flour	Lab-scale, 80% EtOH (boiling 30min), centrifugation 30 min. Water extraction, centrifugation 30 min. 250 U (1-3, β-(1-4)-glucan 4-glucano- hydrolase (2 h, 40°C). 20Uβ-glucosidase and 400 U amyloglucosidase (1 h,50°C).	2.1[a]	2.65×10^5	0.52	(P. G. Dervilly et al. 2001)
Rye flour	Lab-scale, Water extraction, centrifugation 30 min.250 U (1-3, 1-4)-β-glucan 4-glucano- hydrolase, 2 h at 40°C. 20U β-glucosidase and 400 U amyloglucosidase, 1 h at 50°C.	1.08[a]	2.88×10^5	0.5	(P. G. Dervilly et al. 2001)
Corn husk	Lab-scale, 0.9%(w/v) $Ca(OH)_2$, $37x10^4$ U of crude bacterial xylanse and $28x10^4$ U of fungus xylanase.	/	5.3×10^4	0.75-0.95	(Ogawa et al. 2005)

a: AXs extracted yield by raw material dry basis (w/w); a: AXs extracted yield by original AXs content in raw material.*

In addition, endoxylanase combined with other enzymes such as cellulase could increase AXs yields in endoxylanase hydrolysis extraction. In a report by Escarnot et al. (2012), several commercial enzyme preparations with mainly endoxylanase activity from *Trichoderma reesei* (Rohalase WL), *Thermomyces lanuginosus* (PentopanMono conc. BG), *Bacillus subtilis* (Belfeed B MP and Grindamyl Powerbake 900), *Humicola insolens* (Ultraflo L), *Aspergillus aculeatus* (Shearzyme 2×), *A. aculeatus* plus *T. reesei* (Shearzyme Plus) were tested and combined with a commercial cellulolytic preparation from *T. reesei* (Celluclast 1.5L). The study indicated that the yields of AXs from spelt bran were improved by the addition of Celluclast to the reaction mixture: without Celluclast the yield was 38.9%-68.6% of the total AX; with Celluclast the yield was 62.6-74.8% of the available AX after 24h. The enhanced yield is a result of the xylanolytic action of celluclast. Van Craeyveld et al. (2010) also reported that a combination of celluclast with the other enzymes greatly increased the yield of glucose and xylose in the soluble extract. The reason might be because the cellulases digest cell wall β-glucans and breakdown the non-covalent topological associations between β-glucans and AXs, which results in more AXs being released. In addition, arabinofuranosidases (remove Araf), feruloyl esterases (remove ferulic/diferulic acids) and acetyl xylan esterase (removes acetyl groups) also alter the intra-chain and inter-chain interactions of AXs leading to increased solubility/extractability (Faulds et al. 2003; Pitkanen et al. 2011; Smaali et al. 2006). The reasons might relate to the presence of Araf/galactose/glucose side chains which can prevent chain-chain interaction, which increases water solubility whereas ferulic/glucuronic acids act to

promote cross-linkages (Izydorczyk and Dexter 2008; Muralikrishna and Rao 2007; Saulnier et al. 2007). In contrast, using two different xylanases in combination did not show an increase in corn bran AXs yields (Escarnot et al. 2012). Beaugrand et al. (2004c) also found that after a 24h treatment, the action of GH11 xylanase caused the release of 49% of the total AX from destarched wheat bran, whereas GH10 xylanase action hydrolysed only half that amount (Table 1.3). A combination of GH11 and GH10 xylanases did not show any synergistic action on wheat bran AXs yields.

1.3.4 Mechanical-chemical extraction

1.3.4.1 Ultrasound treatment

Various mechanical technologies have been studied as pre-treatment processes to improve the efficiency of xylans extraction, for example chemical extraction assisted by ultra-sonication. As shown in Table 1.5, the yield of water soluble xylans was significantly affected by the alkali concentration and extraction temperature in the ultrasound-assisted procedures (Z. Ebringerova et al. 1998; Hromadkova et al. 1999). Ultrasound assisted with NaOH treatment improved the yields of xylans (28%-36%) obtained from corn cobs (Table 1.4 & Table 1.5). In addition, A. Ebringerova and Hromadkova (2002) reported ultrasound used in combination with NaOH (1% and 5% NaOH) can increase (10%-40%) hemicellulose yields and the whole extraction time can be reduced. The ultrasound treatment was able to significantly modify the molecular weight of xylans extracted from corn hull in both a neutral and an alkaline aqueous medium (A. Ebringerova and Hromadkova 1997). These studies show the potential of

ultrasound-assisted extraction processes when applied to the extraction and modification of AXs with high yields from cereal by-products.

Table 1.4 Synthesis of the results from the key literature data on AXs and other polysaccharides extraction from cereal by-products using mechanical-chemical methods

Sources	Extraction	Yields% of AX[a]/ AXOS[b]/ Xylans[c]/ Hemicellulose[d]/ Carbohydrate[e]	Mw[f] (Da)	A/X	References
Wheat bran (Destarched)	Twin-screw extrusion (Clextral BC45) combined with alkaline.	24[d]	3×10^4	/	(Zeitoun et al. 2010)
Wheat bran&straw (Destarched)	Twin-screw extrusion (Clextral BC45) combined with alkaline.	1.2[a]	/	/	(Jacquemin et al. 2012)
Wheat straw	Steam explosion pre-treatment (pilot-scale equipment: 10-Litre reactor, maximum pressure 42kg/cm^2) and Alkaline peroxide treatment.	29-33[d]	$1.43\text{-}1.58\times10^4$	/	(X. F. Sun et al. 2005)
Corn cobs	Ultrasound (Ultragen system PERSON, Slovak Republic, 20kHz) and NaOH 5% (100 W, 10 min, 60°C).	28.8[c]	$6.1\times10^4\text{-}8.82\times10^5$	0.25	(Z. Ebringerova et al. 1998)
Maize bran (Destarched)	Microware (MLS-1200 Mega Microwave Workstation, Sorisole, Italy) and 2M NaOH (180°C, 10 min).	25.8[b]	1.47×10^4	1.82	(Rose and Inglett 2010)
Corn pericarp	Microwave irradiation (MicroSYNTH Labstation: maximum output 1KW, 2.45 GHz; Microwave oven, Milestone Inc. USA) 176.5°C, come-up time 2min, heating time 16min, solid to liquid rate 1/20 (g/ml).	59[c]	/	/	(Yoshida et al. 2010)

a: AXs extracted yield by raw material dry basis (w/w); b: AXOS extracted yield by raw material drybasis (w/w); c: Xylans extracted yield by raw material dry basis (w/w); d: Hemicellulose extracted yield by raw material dry basis (w/w); e: Carbohydrate extracted yield by raw material dry basis (w/w); f: Mw of extracted material.

Table 1.5 Effects of conditions of extraction and modification methods on AXs and other polysaccharides from cereal by-products

Sources	Extraction	Treatment factors	Yields% of AX[a]/ AXOS[b]/ Xylans[c]/ Hemicellulose[d]/ Carbohydrate[e]	Mw[f] (Da)	References
Corn hull	Alkali + ultrasound	Ultrasound time/power (min/W)			(A. Ebringerova and Hromadkova 1997)
		60/100	[c]	8.5×10^4	
		30/100	[c]	1.31×10^5	
		60/80	[c]	1.41×10^5	
Maize bran (Destarched)	Alkali + Microwave	Microwave temperature/time (min)			(Rose and Inglett 2010)
		180/2	7.48[b]	/	
		180/10	25.8[b]	/	
		180/20	23.0[b]	/	
		200/10	10.8[b]	/	
Corn cobs	Alkali + ultrasound	Temperature (5% NaOH, 10 min)			(Hromadkova et al. 1999)
		50°C	30.3[c]	6.1×10^4	
		60 °C	36.1[c]	8.1×10^4	
		70 °C	36.8[c]	8.7×10^4	
Corn cobs	Alkaline+ ultrasound	% NaOH/ultrasound time (min)(60 °C)			(Z. Ebringerova et al. 1998)
		1% NaOH(s.g.1.5) /30 min	29[c]	5.4×10^4	
		5% NaOH(s.g.1.5) /10 min	35[c]	6.1×10^4	
Wheat straw	Alkaline pretreatment	% NaOH (s.g.1.5)(30 min, 190 °C)			(Persson et al. 2009)
		1.0	6.7[a]	/	
		2.0	11.2[a]	/	
		3.0	10.3[a]	/	

		5.0	13.1[a]	/	
Wheat bran (Destarched& Deprotein)	Enzyme pretreatment	Bacillus subtilis endoxylanase			(Maes et al. 2004)
		1U	/	6.2×10^3	
		10U	/	2.4×10^3	
		100U	/	2×10^3	
Wheat bran	Alkaline	Wheat bran/water ration (g/ml) (0.1 mol/L NaOH, 60 °C, 1h)			(X. Zhang et al. 2008b)
		1:10	6[a]	/	
		1:20	6.7[a]	/	
		1:30	7[a]	/	
		Temperature (0.1 mol/L NaOH, Wheat bran/water, w/v, 1:20, 1h)			
		40	2[a]	/	
		80	13[a]	/	
		100	18[a]	/	
		Time (min) (0.1 mol/L NaOH, Wheat bran/water, w/v, 1:20, 60°C)			
		60	4.5[a]	/	
		120	6[a]	/	
		240	7[a]	/	
		Concentration of NaOH (Wheat bran/water ratio 1:20, 60 °C, 1h)			
		0.05	3.5[a]	/	
		0.15	8[a]	/	
		0.20	8.5[a]	/	

a: AXs extracted yield by raw material dry basis (w/w); b: AXOS extracted yield by raw material dry basis (w/w); c: Xylans extracted yield by raw material dry basis (w/w); d: Hemicellulose (consists primarily AXs) extracted yield by raw material dry basis (w/w); e: Carbohydrate extracted yield by raw material dry basis (w/w); f: Mw of extracted material.

1.3.4.2 Microwave treatment

Microwave-assisted treatments have also been studied as a means of improving the extraction efficiency of hemicelluloses extraction (Rose and Inglett 2010). Microwave irradiation is an effective and reliable means of producing soluble feruloylated arabinoxylo-oligosaccharides (AXOS) from corn bran. The advantages of the microwave-assisted method are; shorter extraction times and high heat treatments (Roos et al. 2009). Roos et al. (2009) also suggested that the microwave pretreatment is a good method for forecasting the effects of heat treatments, such as steam pretreatments, on large scale isolation of hemicelluloses from barley husks because this study found the xylan yields (about 9%) using microwave treatment were similar to that using steam treatment. In addition, as shown in table 1.5, the yield of water soluble hemicellulose was dramatically affected by the microwave temperature and time. Maximum yields of about 50% of the original AXs content were obtained with processing conditions of 180°C for 10 min or 200°C for 2 min (Rose and Inglett 2010). Yoshida et al. (2010) reported the optimization of microwave-assisted extraction of polysaccharides from industrial corn starch production waste (Corn pericarp) using response surface methodology with hot water under pressure as the solvent. The optimized conditions were as follows: pressurised water temperature 176.5°C, time to reach the operating temperature 2 min, heating time 16 min and solid to liquid ratio 1/20 (g/mL) respectively. The maximum yield achieved was 70.8% of the total carbohydrates consisting mainly of xylo-oligosaccharides. The carbohydrates were

about 83% in the raw material (Yoshida et al. 2010) therefore, the maximum yield of carbohydrates was about 59% of raw material (Table 1.4).

1.3.4.3 Steam treatment

Steam explosion processing has been tested successfully as a means of extracting AXs from cereal by-products. For this method, cereal materials were pretreated with high-pressure steam, and then the pressure was rapidly released, leading to explosive depolymerisation. This breaks down the lignocellulosic structure and the hemicelluloses are then simply extracted (Cara et al. 2006). X. F. Sun et al. (2005) used a two-stage process based on a steam explosion pretreatment (200°C/15bar for 10-33min or 220°C/22bar for 3-8min), followed by alkaline peroxide post-treatment (2% H_2O_2, 50°C, 5h, pH 11.5) and precipitation in 3 volumes of ethanol. During the steam pre-treatment, the yield of oligo- and monosaccharide was 20.5-28.5% by wheat straw. This two-stage process released 77.0–87.6% of the total original hemicelluloses (mean composition, 38.7%) from wheat straw (Table 1.4). As a pre-treatment for AXs extraction, steam explosion is environmentally friendly and an effective method for breaking down the lignocellulosic structure (Avellar and Glasser 1998).

1.3.4.4 Extrusion and stirred reactor treatment

Twin-screw extrusion and stirred reactor batch extraction have been studied as pretreatment methods combined with alkaline conditions to extract hemicellulose from wheat bran (Zeitoun et al. 2010). Zeitoun et al. (2010) used twin-screw extrusion

combined with alkaline conditions (NaOH/bran = 0.15), thermal (50°C) and mechanical (dry matter flow rate of 13.8 kg/h) treatments to recover the dissolved molecules from the wheat bran by continuous squeezing in a screw press. Ultrafiltration (Mw cut-off of 30KDa) was used to purify the extracts. The extraction yield of purified hemicellulose was 24% in the destarched wheat bran. The xylan content in the extracted hemicellulose was 53% of the organic matter. This study also researched stirred extraction, using a higher liquid/solid ratio of 50:50, combined with alkaline conditions (weight 1:1 alkali/bran), at 40°C for 1h. The yield of hemicellulose was 59% in destarched wheat bran, which contain 76.7% xylans. In this study, twin-screw extrusion shows a lower hemicellulose extraction yield (24%) than was achieved (60%) using the stirred reactor extraction system when both methods were applied to the dry matter of destarched wheat bran. In the later study (Jacquemin et al. 2012), researched a twin-screw extrusion method and obtained AX yields of 1kg from 86kg wheat straw and destarched bran (ratio straw/bran = 6.2:1). Compared with other mechanically assisted methods (Table 1.4), twin-screw extrusion shows relatively lower extraction yields of AXs. However, Zeitoun et al. (2010) and Jacquemin et al. (2012) pointed out that the advantages of the twin-screw extrusion assisted method are shorter residence time and lower water consumption, which indicate that it is possible to develop the procedure as an cost effective method.

Based on the reviews of the various extraction technologies, a comparison of the advantages and disadvantages of each method has been prepared (Table 1.6).

Table 1.6 Comparison of advantages and disadvantages of particular extraction techniques of AXs extraction from various cereal tissues

Type of extraction treatment	Reagents/ techniques	Cereal sources	Advantages	Disadvantages	References
Water extractions	Water	Barley, Rye flour, Wheat flour	No changes to molecules. Indispensable aqueous.	Low AXs extraction yields.	(Izydorczyk and Biliaderis 2007) (Fleury M. D. 1997) (Faurota et al. 1995)
Chemical treatments	NaOH HCl	Wheat bran, Corn bran, Barley husks Wheat straws	Highly efficient treatment. Efficient treatment (slight lower than alkaline extraction).	Break down functional groups (e.g. ferulic acid). Decrease A/X, low branched degree.	(Zhou et al. 2010) (X. Zhang et al. 2008b) (Höije et al. 2005) (Xu et al. 2006)
Enzymatic treatments	Endoxylanese	Wheat bran, Barley husk, Corn husk, Rye flour	Environmental-friendly. No change to functional ingredients (e.g. ferulic acid). Efficient treatment (slight lower than alkaline extraction) Controlled degradation of molecules of AXs.	/	(Zhou et al. 2010) (Maes et al. 2004) (Beaugrand et al. 2004a) (Höije et al. 2005) (P. G. Dervilly et al. 2001) (Ogawa et al. 2005)
Mechanical treatments	Extrusion Steam explosion Ultrasound	Wheat bran & straw Wheat straw Corn bran, hull &	Low water consumption. Highly efficient treatment. Short treatment time. Highly efficient treatment. Low treatment temperature and short	/ High treatment pressure and temperature. Uncontrolled degradation of molecules of	(Zeitoun et al. 2010) (Jacquemin et al. 2012) (X. F. Sun et al. 2005)

	Microwave Supercritical CO_2	cobs, Corn bran & pericarp, Barley Hemicelluloses from DMSO/water mixtures	treatment time. Highly efficient treatment. Short treatment time. Highly efficient separation Low treatment temperature. Short treatment time.	AXs. Uncontrolled degradation of molecules of AXs. High treatment pressure Application limitation: Supercritical CO_2 only applied in xylan separation from organic solvent (e.g. DMSO).	(Josefsson et al. 2002) (Z. Ebringerova et al. 1998) (Hromadkova and Ebringerova 2003) (Lorimer and Mason 1995) (Rose and Inglett 2010) (Roos et al. 2009) (Haimer et al. 2008) (Haimer et al. 2010) (Y.P. de Diego et al. 2006) (Y.P. de Diego et al. 2005)

1.3.5 Environmental impact of AX processing

According to the EU Environmental Impact of Products (EIPRO) study (Tukker et al. 2006), the environmental impacts of food processing were assessed under a range of themes used in Life Cycle Assessment (resource depletion, Global Warming Potential (GWP), ozone layer depletion, human toxicity, ecotoxicity, photochemical oxidation, acidification and eutrophication). Sadhukhan et al. (2014) indicated that the waste-water from hemicellulose organosolv process containing organic solvents, which lead to potential environmental pollution. Long term exposure to solvents and air pollutants can lead to deleterious effects on human health such as respiratory, haematological and thyroid functioning (Uzma et al. 2008). In addition, other chemical treatments such as alkaline extraction process has been recognized one of most efficient methods for AXs isolation, but it will produce waste with high amount of chemicals. The chemical waste such as alkali and acid may lead to environmental pollution and toxic to human (U.S.EPA 2002). Thus, one of challenges for chemical extraction methods of AXs is to reduce the environmental impacts. For instance, in the organosolv process, using the low molecular weight solvents such as acetic acid, formic acid, methanol, and ethanol are relative easy to recover (Sadhukhan et al. 2014). In the alkaline extraction process, neutralization is an effective method dealing with the hazardous waste (Hill 2011). On the other hand, Jacquemin et al. (2012) evaluate the environmental performances of different AX purification methods from wheat straw and bran. According to the Life Cycle Assessment model, the study optimized

ultrafiltration process through reducing water consumption and carbon dioxide emissions. In addition, enzyme technologies in AX extraction can work at low temperature, moderate pH and less chemicals environment. Therefore, enzyme technologies are reasonable considered as environmental-friendly methods for AX processing. Overall, environmental impacts of AX different extraction technologies are still not well documented, which are suggested to investigate in future study.

1.4 Effects of extraction and modification methods on molecular characteristics of arabinoxylans

It is generally agreed that the cereal AXs exhibit structural heterogeneity, which is related both to the methods used for their extraction and modification and to the differences resulting from species variations. The early studies reported the influence of extraction methods and fractional purification on Mw and degree of substitution of extracted AXs.

1.4.1 Chemical solvent treatments

It was reported that alkali through de-esterifying the ferulic acid on the arabinose breakdown the cross linkage, contributed to modification of the molecular structure of AXs (G. Dervilly et al. 2000; Zheng et al. 2011). As shown in Table 1.7, one study compared the AXs extracted by aqueous solutions and different concentrations of NaOH from different hull-less barley milling fractions (bran, shorts and flour) (Zheng et al. 2011). The effects of the processing condition on the type of AX extracted were

demonstrated convincingly using hull-less flour (HBF-AX). AXs extracted from HBF-AX by water had a higher Mw (877,100Da) than those extracted by 0.5mol/L NaOH (758,200Da) and 1.0 mol/L NaOH (604,100Da). This study suggested that NaOH degrades AXs more than water and that the degree of degradation is dependent upon the concentration of NaOH used. Using the same method it has been shown that AXs (HBF-AX) extracted from hull-less flour had the highest molecular weight, whilst those isolated from hull-less shorts AXs (HBS-AX) were lower and those from hull-less bran AXs (HBB-AX) had the lowest molecular weight. The A/X showed the same relationship HBF-AX (0.76), HBS-AX (0.66) and HBB-AX (0.55). This implies that the molecular weight and degree of branching of AXs depend on both the cereal milling fractions and the extraction method used as the source of AXs.

Modification of the alkaline extraction method using different additional chemicals produces AXs of different molecular structures. L.W. Doner et al. (1998) extracted AXs from corn bran using a two alkali method (NaOH and $Ca(OH)_2$) as summarised in Table 1.7. They found that when using NaOH (pH 11.1) as the extract solution, the average Mw of extracted hemicelluloses (mainly AXs) was 3.94×10^5Da, whilst when using $Ca(OH)_2$ (pH 9.8) it was 2.78×10^5Da. When using an equimolar ratio NaOH:$Ca(OH)_2$, the Mw averaged 3.03×10^5Da. The authors suggested that the more extreme alkaline extraction conditions yielded higher Mw of AXs. Thus, the Mw of the AXs extracted may depend upon the pH of the extraction medium used. Saulnier et al. (1995) similarly showed that the Mw of hemicellulose extracted from corn bran with 0.5M NaOH at 30°C for 2h was 2.7×10^5Da (Table 1.7) and that further extraction of the

residual material with 1.5M KOH for 2 h at 100°C produced more hemicellulose material but with the higher average Mw of 3.7×10^5Da.

Table 1.7 Synthesis of the results from the key literature data on effects of extraction and modification methods on molecular characteristics of arabinoxylans

Sources	Extraction/Modification	Mw of AX (Da)	A/X	References
Barley flour	Water treatment 0.5M NaOH 1M NaOH	8.77×10^5 7.582×10^5 6.041×10^5	/ / /	(Zheng et al. 2011)
Corn bran	NaOH, pH 11.1 $Ca(OH)_2$, pH 9.8 NaOH+ $Ca(OH)_2$, (1:1)	3.94×10^5 2.78×10^5 3.03×10^5	/ / /	(L.W. Doner et al. 1998)
Wheat bran	$Ba(OH)_2$ 0.1M trifluoroacetic acid	3.81×10^5 8.0×10^4	0.6 0.24	(Y. Sun et al. 2011)
Spelt hull	NaOH + H_2O_2 Organic acid	$2.2\text{-}2.7\times10^5$ $0.13\text{-}1.13\times10^5$	/ /	(Escarnot et al. 2011)
Wheat bran	*B. subtilis* endoxylanase *A. spergillus aculeatus* endoxylanase	$0.2\text{-}3.09\times10^4$ $1.0\text{-}1.2\times10^3$	/	(Maes et al. 2004)
Wheat	Ethanol fractionation concentration 55%-100% 30%-70%	$7\times10^6\text{-}2.2\times10^6$ $3\times10^6\text{-}4\times10^6$	0.5-0.91 /	(Izydorczyk and Biliaderis 2007)
Wheat flour	$(NH_4)_2SO_4$ precipitation 60%-95%	/	0.58-0.88	(Izydorczyk and Biliaderis 1992b)

In addition to the pH and different alkaline reagents, the treatment time is also a significant factor affecting the Mw of AXs extracted by these treatments. A study of the Mw distribution of AXs extracted from barley straw using different treatments/conditions indicated that, using the same pre-treatment conditions (1% NaOH, 190°C), a longer residence time (range used 10 min to 40 min) results in a lower

Mw distribution (Persson et al. 2009). It was also found that the solution became more acid in pre-treatment. The authors explained that the reason for this could be that the β-glycosidic linkages between the monomer units in the AX molecule are broken in a more acidic environment. Hence, when the pH is higher than 9, extracted AXs will have a higher Mw. Based on these studies, it seems that within a certain range, the concentration of the alkaline solvents used, the treatment time and the pH of the treatment environment will modify the Mw of AXs extracted.

M.P. Yadav et al. (2007) extracted the AXs from defatted and destarched corn kernels. They used alkaline extraction and H_2O_2 bleaching to produce corn fibre gum sample 1 (CFG-1). The alkali-extracted residue was additionally treated by H_2O_2, yielding corn fibre gum sample 2 (CFG-2). The authors concluded that the isolated CFG-2 from each fibre source had a higher Mw than the CFG-1 (332–491kDa compared with 244–348kDa). The authors explained that the higher Mw of corn fibre gum was related to the cell wall matrix because AX retains its structural heterogeneity. Under the alkali treatment, additional H_2O_2 treatment breaks alkali-resistant linkages.

Y. Sun et al. (2011) compared the effects of alkaline and organic acid extraction on the average Mw and A/X of AXs from the bran of Chinese black-grained wheat (Table 1.7). Water unextractable polysaccharide was isolated using saturated barium hydroxide. The AXs isolated had an Mw of 3.81×10^5Da and an A/X of 0.60. In comparison, when wheat bran (150 mg) was hydrolysed with 0.1M trifluoroacetic acid for 1h at 100°C, the Mw of the AXs isolated was 8.0×10^4Da, which was most probably due to some degradation of the molecular backbone. The A/X ratio was 0.24. This

result showed that partial acid hydrolysis of AXs results in the debranching of the xylans with exclusion of arabinose substituents and consequently an associated loss of Mw. Escarnot et al. (2011) reported a similar finding in their study. They found that the alkaline hydrogen peroxide method yielded AXs with a higher Mw in the range 2.2–2.7×10^5Da and higher A/X ratios than achieved with the organic acid methods which produced low Mw AXs in the range 1300–11300Da. Therefore, the degree of branching and the Mw of AXs extracted vary considerably between alkaline and organic acid treatments.

1.4.2 Enzyme hydrolysis

Enzyme hydrolysis is an efficient method for modifying the microstructure of AXs. Endoxylanases and cellulases are commonly used to extract and degrade AXs from cereal cell wall tissue. In addition to the heterogeneous source, the enzyme activity, and the treatment conditions have effects on the characteristics of the extracted material such as Mw and A/X. Adams et al. (2004) used atomic force microscopy to demonstrate that xylanase (the Xyn11A enzyme from *Neocallimastrix patriciarium*) randomly cut along the backbone of AXs molecules. Escarnot et al. (2012) reported the effects of hydrolysis by several commercial enzymes for periods of between 1h and 24h on the macromolecular characteristics of AXs from spelt bran. This study showed that the A/X ratio decreased after treatment for 1h, but that there was almost no further decrease between 4h and 24h. In addition, as the time increased, from 1h to 4h, the Mw of AXs decreased gradually. This result is in agreement with that reported by Maes

et al. (2004) who found that *B. subtilis* endoxylanase released wheat bran AXs with a relatively high Mw (2-31KDa), whereas those released by *Aspergillus aculeatus* endoxylanase had a relatively low Mw (1-1.2KDa) (Table 1.7). Furthermore, the concentration of enzyme has shown effects on Mw of AXs using same enzyme. For example, with concentration of *Bacillus subtilis* endoxylanase increased, the Mw of AXs was from 6.2×10^3Da down to 2×10^3Da (Table 1.5) (Maes et al. 2004). Enzymatic extraction using arabinofuranosidases, feruloyl esterases and acetyl xylan esterase have also produced AXs with different A/X ratios, branching patterns and molecular weights by releasing approximately 50% of the ferulic acid and 30% of Araf side chain (Faulds et al. 2003; Pitkanen et al. 2011).

Compared with alkaline extraction, endoxylanase hydrolysis leads to a reduction in the Mw distribution of AXs. The average Mw of alkaline extracted AXs was 3.5×10^5Da (Zhou et al. 2010) (Table 1.2), which is about 10 times that of enzyme extracted AXs (3.252×10^4Da) (Table 1.3). This may be because endoxylanase works on the 1,4-β-links of the xylan chain, whereas alkali influences the linkage of AX to lignin and cellulose (diferulate bridges and hydrogen bonds) (Iiyama et al. 1994).

1.4.3 Fractional purification

Following chemical, mechanically assisted chemical or enzymatic extraction, the liquor could be further purified and ethanol fractionation was successfully used to purify and isolate AXs from the suspension. The molecular weight and degree of substitution of AXs might be key factors in ethanol fractionation, as lower Mw results

in higher solubility in ethanol–water mixtures. Fractionation of wheat flour WEAXs by ethanol precipitation confirmed varying degrees of substitution of A/X (Swennen et al. 2006). The observation that some WEAXs do not carry ferulic acid residues might be an additional indication, since lack of ferulic acid would prevent these molecules from cross-linking into the cell wall structure. Izydorczyk and Biliaderis (2007) reported that fractionation is based on the differential solubility of wheat AXs with different molecular weights and structures in solutions containing various amounts of ethanol or ammonium sulphate. AXs precipitated by increasing concentrations of ethanol (55%–100%) exhibit an increasing A/X (0.5–0.91) but decreasing Mw (700,000–220,000Da). G. Dervilly et al. (2000) also reported that the A/X and substituted Xylp residues in extracts increased with increasing ethanol concentration. The amounts of ferulic acid were higher in wheat AXs isolated using ethanol at low concentrations. G. Dervilly et al. (2000) showed the Mw characteristics of AXs appeared similar (300,000-400,000Da) among all fractions with different ethanol concentrations (30%-70%). This indicated that the conformation of AXs fitted a semi-flexible pattern, and the degree of substitution of the xylan backbone by arabinose residues has no influence on the conformation of AXs (Dervilly-Pinel et al. 2001). From the above studies, ethanol purification and isolation appears to be a very effective method for fractionating AXs with different A/X ratios and degrees of substitution, however, the Mw modification of AXs during ethanol fractionation requires further study as G. Dervilly et al. (2000) and Izydorczyk and Biliaderis (2007) described.

Ammonium sulphate precipitation has also been used to isolate and purify AXs. Izydorczyk and Biliaderis (1992b) studied fractionation of the water-soluble AXs from wheat flour using a graded ammonium sulphate fractionation technique. Fractionation was achieved in this instance using four levels of saturated NH_4SO_4, 60%, 70%, 80%, and 95% (Table 1.7). With increasing concentration of $(NH_4)_2SO_4$ (60% to 95%), the A/X increased from 0.58 to 0.88 and the proportion of substituted xylose residues in the AXs fractions also increased, however, the isolation yields decreased from 58.8% to 4.4%; in the same experiments the ferulic acid content and the Mw distribution also decreased. This was probably because different molecular sizes have different solubilities in ammonium sulphate. The report indicated that fractional precipitation with NH_4SO_4 is affected by the molecular size and fine structure of the polymer (Izydorczyk and Biliaderis 1992b). Furthermore, Izydorczyk et al. (1998) also established that a concentration of ammonium sulphate greater than 45% was useful in purifying β-glucans from AXs.

Supercritical CO_2 is a compressed anti-solvent process which has been studied in the precipitation of hemicellulose from dimethyl-sulfoxide (DMSO)/water mixtures (Y.P. de Diego et al. 2005; Haimer et al. 2008). It has been found that CO_2 has good miscibility with DMSO at high pressure (Andreatta et al. 2007). DMSO is a solvent commonly applied to dissolve and isolate low-branched AX from plants (A. Ebringerova et al. 1994). Supercritical CO_2 is an anti-solvent injected into hemicellulose solutions causing supersaturation of hemicellulose under supercritical conditions (150bar, 40°C), which can lead to precipitation of hemicellulose from DMSO/water

mixtures (Haimer et al. 2008). It has been reported that supercritical CO_2 is a non-toxic, non-flammable, inert solvent which can easily precipitate hemicelluloses from DMSO/water mixtures (Y.P. de Diego et al. 2006). In the later study, Haimer et al. (2010) found the particle size of xylan can be controlled from 0.1µm to more than 5µm. This technology can be used in the separation, purification and particle size modification of hemicelluloses from DMSO/water mixtures.

1.5 Immunological activities of the cereal arabinoxylans

1.5.1 The human immune system

The immune system rests on two major branches: the innate system and adaptive system. In the early-induced innate response, natural killer cells (NK cells), granulocytes (neutrophils), monocytes and macrophages are triggered to phagocytose bacteria and present intact antigens to B cells and T cells. These early innate responses are fast but non-specific. They sequentially activate the adaptive immune system to release antibodies through B cells and attack foreign molecules by cytotoxic and helper T-cells. When the same antigen attacks the host again, the adaptive immune system can achieve a quicker immune response with the help of memory B cells (Alberts 2008; Voet and Voet 2004).

The immune system in the human body is a network of cells, tissues (groups of similar cells joined to perform the same function) and organs that work together to prevent or limit infection. Infections are primarily caused by foreign microbes such as

bacteria, viruses, parasites and fungi (National Institutes of Health 2003). When the immune system recognizes pathogenic microbes, it responds to attack the invaders and prevent infection. A key article has provided evidence that the immune system can prevent tumours from developing, and thus plays a strong protective role against cancer (Shankaran et al. 2001). In addition, Biological Response Modifiers (BRMs) have been found to be an effective extrinsic tumour-suppressor system, which is a type of treatment that uses substances made from natural organisms to stimulate or suppress the immune system to anti-cancer (Dzivenu et al. 2003). Currently, some dietary fibre-derived BRMs, such as β-1,3-glucan, β-1,6-glucan and α-1,6-mannan, have been discovered that show immune stimulation activity (Brown and Gordon 2003; Rieder et al. 2011; Tzianabos 2002; Volman et al. 2008). AXs have also been suggested to be potent BRMs by modulating both the innate and adaptive immune systems (Ghoneum and Jewett 2000; Ghoneum and Matsuura 2004; S. Zhang et al. 2015).

1.5.2 immune-modulating properties of arabinoxylans

Recently, a review paper has summarized studies undertaken during the past two decades reporting that various cereal AXs show immune-modulating effects on innate system cells (NK cells, macrophage cells, dendritic cells) and adaptive system cells (T and B cells) (S. Zhang et al. 2015). Currently, there is a modified AX with small Mw (<50KDa) from rice bran, also called MGN-3/Biobran, a commercial product of cereal AXs that has been tested for its immune enhancing effects on the immune system by *in vitro* assays and *in vivo* animal/human studies (Cholujova et al. 2009; Ghoneum 1998a;

Ghoneum and Jewett 2000; Ghoneum and Matsuura 2004; Ghoneum and Abedi 2004; Ghoneum et al. 2004; Ghoneum and Gollapudi 2005; Ghoneum et al. 2008). In addition, AXs from various other cereal sources such as wheat bran, corn husk, corn hulls and barley have also demonstrated immune-stimulating abilities *in vitro* and *in vivo* (Cao et al. 2011; Ogawa et al. 2005; Samuelsen et al. 2011; P. Y. Zhang et al. 2004; Zhou et al. 2010). There are also AXs from other cereal sources such as rye, oats and sorghum whose immunological activity has not yet been extensively tested. There is no research paper that has documented in detail the relationship between immune-stimulating ability and cereal source of AX (i.e, by same immunoregulatory function assay using the same cell line to test the effects of various cereal sources on immune response).

1.5.2.1 *in vitro* studies

Previous studies found that Biobran/MGN-3 AXs can induce maturation of immature dendritic cells combined with a mix of two groups of cytokines (1: TNF-α, IL-1β and IL-6; 2: INF-γ with LPS) or via enhancement of co-stimulatory proteins (CD80 and CD86) (Harris et al. 1997; Poindexter et al. 2004). These data demonstrated that Biobran/MGN-3 AXs has the potential to stimulate cytokine-induced dendritic cell maturation.

The immune-modulatory effects of Biobran/MGN-3 AXs on macrophages have also been investigated through 3 assays *in vitro* showing: increased percentage of attachment and phagocytosis of yeasts by U937 (human macrophage cell line); stimulation in the level of TNF and IL6 released by RAW264.7 (murine macrophage cell

line); and enhancement in the level of NO secretion (a mark of cytotoxic activity) by RAW264.7 (Ghoneum and Matsuura 2004). In addition, corn hull AXs also showed immunological function by respiratory burst assay using RAW 264.7 (P. Y. Zhang et al. 2004). The macrophages responded with a significant increase in oxidation of DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) in the presence of corn hull AX compared to the control; and a similar effect was observed in LPS treatment. Nagata et al. (2001) reported that soybean hull hemicelluloses composed of AX stimulate the production of NO and IL1β in murine peritoneal macrophage cells. These findings demonstrate that AXs have the potential to enhance phagocytic function and activation of macrophage.

Ghoneum and Jewett (2000) studied the stimulatory effect of MGN-3 on NK cytotoxic cell activity, using NK cells isolated from the peripheral blood lymphocyte population by testing the MGN-3 function on secretion of TNFα and IFNγ and key cell surface receptors (CD69, CD25 and CD54). The results from this study showed MGN-3 can significantly enhance the production of TNFα and IFNγ in purified NK cells and IL-2 activated NK cells. Furthermore, the NK cell activation marker CD69, together with CD54 (ICAM-1 adhesion molecule) and CD25 (IL-2 receptor), demonstrated up-regulation after MGN-3 treatment in peripheral blood cells. This paper suggested that the mechanism by which MGN-3 stimulated human NK cell cytotoxicity involves the synergistic induction of IFNγ, TNFα and CD54. The immune-modulatory function of AXs using different cell types are summarised in Table 1.8.

Table 1.8 Selected key literature reports on immune-modulating activity of AXs *in vitro*

Sources	Function of AXs	Cell types	References
Rice bran (MGN-3/ Biobran)	NK Cytotoxicity (IFN-γ) ↑	Human peripheral blood Lymphocytes (PBLs)	(Ghoneum 1998b)
	NK Cytotoxicity ↑, IFN-γ secretion ↑, TNF-α secretion↑, CD69 expression↑, CD25 expression ↑	Human peripheral blood Lymphocytes (PBLs)	(Ghoneum and Jewett 2000)
	Anti-tumour cell growth effects CD95 expression ↓, Bcl-2 expression ↓	Leukaemia cell line (HUT78)	(Ghoneum and Gollapudi 2003)
	Phagocytosis ability ↑	Human U937 cell line and murine P-Mφ cells	(Ghoneum and Matsuura 2004)
	TNF-α secretion ↑, IL-6 secretion ↑	Human U937 cell line, murine P-Mφ cells and RAW264.7 cells	
	NO release ↑	Murine P-Mφ cells and RAW264.7 cells	
	↑ Accumulation of chemotherapeutic agent (daunorubicin) in cancer cells	Human breast cancer cell lines (MCF-7 and HCC70 cells)	(Ghoneum et al. 2008)
	Stimulation effect on maturation of dendritic cells	Peripheral blood monocytes	(Cholujova et al. 2009)
Corn hull	DCFH-DA oxidation ↑	Murine macrophage RAW264.7 cell line	(P. Y. Zhang et al. 2004)

1.5.2.2 *in vivo* studies

Ghoneum and Abedi (2004) reported the enhancement of NK cell activity of aged C75BL/6 mice using MGN-3/Biobran AXs. This *in vivo* study indicated that intra-peritoneal (IP) injection of Biobran AXs (desired dosage: 10mg/kg per day) resulted in significantly induced activity of augmented peritoneum NK cells using ^{51}Cr-released assay (35.2 lytic units) at day 2, which remained increased through to day 14 compared to the activity of saline-treated control mice group (5.8 lytic units). On the other hand, orally administered Biobran AXs did not result in a significant change in peritoneum NK cells activity; however, there was a remarkable 200% increase in

splenic NK cells at day 14. The activity of peritoneal NK cells and splenic NK cells was determined by ^{51}Cr-release assay at day 2, 5 and 14 in the presence or absence of Biobran AXs treatments. In addition, a YAC-1 tumour cell line was used as a target cell to determine the peritoneum NK cells' activity measured by N-alpha-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT)-esterase activity. Mice treated by Biobran AXs (100µg/ml) presented a 26% increase in the conjugate formation of peritoneum NK cell to bind to tumour cells compared to control. These experiments showed enhanced activity of NK cells and the potential anti-tumour effects of Biobran AXs *in vivo* (Ghoneum and Abedi 2004). More recently, Badr El-Din et al. (2008) researched the anti-tumour activity of Biobran AXs on Ehrlich carcinoma-bearing mice via IP injection or intra-tumour (IT) injection. The report showed that both injections of Biobran AXs (Desired dosage: 40mg/kg) remarkably suppressed tumour growth during 35 days' treatments (63% reduction in tumour volume and 45% reduction in tumour weight) compared to controls without Biobran AXs injection. Moreover, the immune modulatory abilities of Biobran AXs were revealed by an increase in the percentage of apoptotic cells (76% increase), NK cell activity (2-fold increase), TNF-α (11% increase) and IFNγ (154% increase) expression, as well as inhibition of IL-10 secretion (2-fold decrease) compared to controls. In addition, Corn husks AXs (Mw: 53kDa; A/X: 0.75), whose Mw is similar to MGN/Biobran AX, also demonstrated immune-stimulating function in mice models Ogawa et al. (2005) Orally administered corn husk AX (50mg/kg/day) significantly enhanced IL-2, IFN-γ and showed a slightly increased IL-4 production in spleen cells from mice. These results

were consistent with the previous finding of Ghoneum and Abedi (2004), in which orally administered AX resulted in activity enhancement of spleen immune cells *in vivo*. Furthermore, Ogawa et al. (2005) also reported orally administered corn husk AX reduced tumour growth in early stages (Day 7) in mice which was accompanied by up to 2 fold induction of NK cell activity and TNF-α expression by spleen cells in the presence of ConA and LPS (Ogawa et al. 2005). Thus, these studies suggested that the anti-tumour function of the cereal AX involved its ability to enhance the immune response. Previous *in vitro* studies have also reported the consistent opinion that the MGN-3/Biobran AX seems to exert its anti-tumour ability due to its enhancement effects on TNF-α and IFN-γ in immune cells such as human blood peripheral lymphocytes, human macrophages, and murine macrophage cell lines (Ghoneum and Jewett 2000; Ghoneum and Matsuura 2004). The immune-modulatory function of AXs using animal models are summarised in Table 1.9.

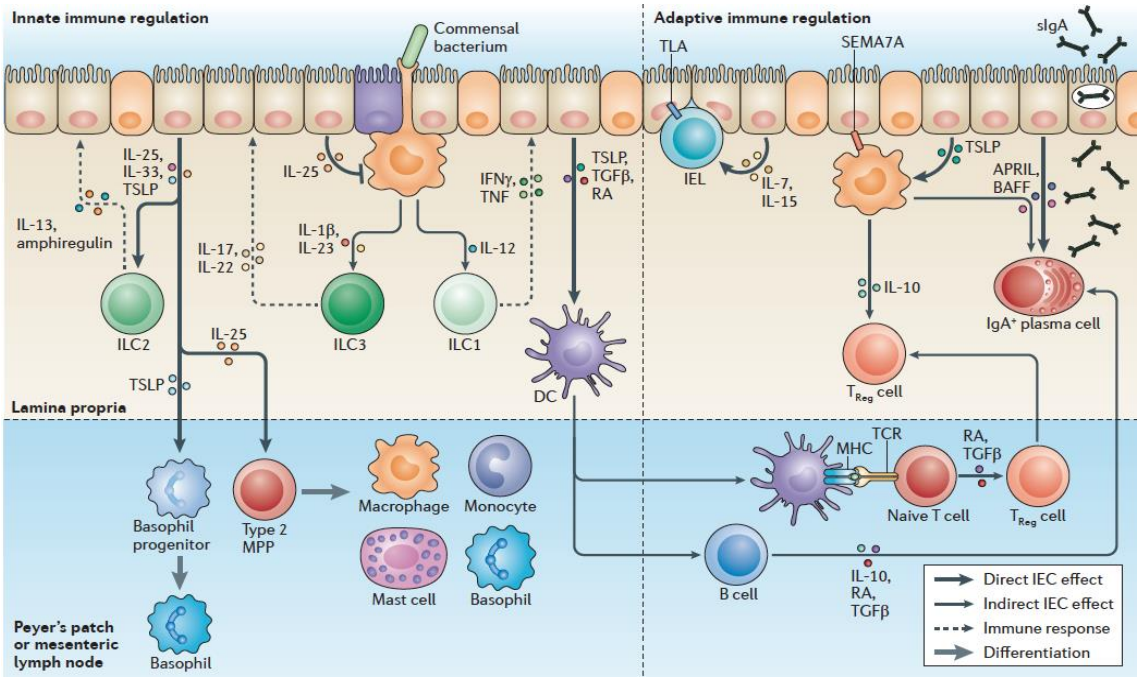
In addition, the oral uptake and processing *in vivo* of oral administered AXs had attracted the recent interest. S. Zhang et al. (2015) thought that if AXs do function in a similar way to other dietary fibre β-glucans, they probably come into contact with the mucosal immune system in the small intestine after oral administration. Figure 1.3 shows the overview of intestine epithelial cells regulating the immune system as documented by L. W. Peterson and Artis (2014). Thus, future studies could try to investigate the effects of AX on small intestine epithelial cells that regulate innate and adaptive immunity. In addition, Geraylou et al. (2012) reported that an enzymatic modified AX material (DP=32, A/X=0.3), arabinoxylan-oligosaccharides (AXOS) can

improve short-chain fatty acid production on fish after two weeks feed, which also stimulated the growth of lactic acid bacteria (LAB) and *Clostridium* sporogenes in the fish gut. This study proposed the enhancement of immune responses may associated with the changes of the gut microbiota communities and the subsequent increase of short-chain fatty acid production *in vivo* (Geraylou et al. 2012). Lactic acid bacteria are found in the intestine of most animals and the beneficial role played by these microorganisms in the humans and other animals, including the effect on the immune system, has been extensively reported (Perdigon et al. 2001). Thus, these previous results suggest that the immunomodulatory effects of AX derived product is probably related to the stimulation of gut bacteria growth such as LAB in the gut, which results in a higher availability of the cell wall components of these microbiota that have immunostimulatory properties. In another study, the other AX derived oligosaccharides, xylo- oligosaccharides (XOS) were reported that also shows regulating innate immunity in the diet of juvenile turbot (Y. Li et al. 2008). Rastall and Gibson (2015) indicated that prebiotic fermentation modulating the immune system towards an anti-inflammatory state. XOS and AXOS are newly discovered candidate prebiotics that have a specific stimulatory effect upon selected growth performance and populations of gut bacteria (Cloetens et al. 2010; Rastall and Gibson 2015). Therefore, these AX derived products have the potential to be prebiotics toward improving gut health via effects on the immune response and gut microbiota populations.

Table 1.9 Selected key literature reports on immune-modulating activity of AXs *in vivo*

Sources	Functions of AXs	Cell types	References
Rice bran (MGN-3/ Biobran)	Chemotherapeutic-induced toxicity ↓	Albino male rats	(Jacoby et al. 2000)
	Protection against cisplatin side effects (body weight loss)	BALB/c female mice	(Endo and Kanbayashi 2003)
	NK cell activity ↑ Anti-tumour effects (conjugate formation ↑)	Peritoneal and bone marrow NK cells from C57BL/6 and C3H mice	(Ghoneum and Abedi 2004)
	Anti-tumour effects Tumour volume ↓, Tumour weight ↓ IFN-γ secretion ↑, TNF-α secretion ↑, IL-10 secretion ↑	Adult female Swiss albino mice	(Badr El-Din et al. 2008)
Wheat bran	Phagocytosis ability ↑	Mice splenic lymphocytes from female BALB/c mice	(Zhou et al. 2010)
Wheat bran	Tumour growth ↓ NK cell activity ↑, macrophage phagocytosis activity ↑, IL-2 secretion↑, DTH reaction ↑, splenocyte proliferation ↑, peripheral leukocyte count ↑, bone-marrow cellularity ↑	K562 and HL-60 cells ICR male mice	(Cao et al. 2011)
Corn husk	Tumour growth ↓, IL-2 secretion ↑, IFN-γ secretion ↑, TNF-αsecretion ↑	Female BALB/c mice	(Ogawa et al. 2005)

Figure 1.3 Overview of Intestinal epithelial cell regulation of innate and adaptive immunity(L. W. Peterson and Artis 2014).



IEC, Intestinal epithelial cell; IL-25, interleukin-25; TSLP, thymic stromal lymphopoietin; type 2 MPP, multipotent progenitor type 2 cells; ILC2s, group 2 innate lymphoid cells; ILC1, innate lymphoid cell subset

1; DCs, dendritic cells; TGFβ, transforming growth factor-β; RA, retinoic acid; TReg, regulatory T cells; SEMA7A, IEC-expressed semaphorin 7A; APRIL, The production of a proliferation-inducing ligand; BAFF, B cell-activating factor; IEL, intra-epithelial lymphocyte; IFNγ, interferon-γ; sIgA, secretory IgA; TCR, T cell receptor; TLA, thymus leukaemia antigen; TNF, tumour necrosis factor.

1.5.2.3 Human studies

Effects of MGN-3/Biobran AXs for immune modulation and anti-cancer have also been investigated in clinical trials. One clinical trial on healthy humans (24 healthy individuals) Ghoneum (1998b) demonstrated that oral administration of Biobran AXs over a period of two months significantly enhanced NK cell activity. There was an approximate 3-fold increase in the binding capacity of NK cells against target cancer cell lines (K562) by Biobran AXs (45mg/kg/day) treatments after one month, which further confirms the potential immune-stimulatory effect of Biobran AXs on NK cell cytotoxicity against tumour cell lines. In addition, the study discovered that Biobran AXs treatments alone did not significantly change the population of NK cells in the blood, which suggested oral administration of Biobran AXs is only responsible for inducing NK cellular immune responses.

There were additional clinical trials that reported the effects of Biobran AXs on humans with cancer. A previous study examined the effect of Biobran AXs on NK cell activity in 32 patients with leukemia, multiple myeloma, breast and prostate cancer (Ghoneum and Brown 1999). Oral administration of Biobran AXs (3g/day) for a month significantly increased NK cell cytotoxicity (up to 10-fold increase) in peripheral blood for all four types of cancer patients. The authors believe that Biobran AXs can possibly

be used in protection of immune-suppression by chemotherapy. These results are encouraging, as MGN-3 is a non-toxic food supplement for human consumption and no hypo-responsiveness has been discovered in patients followed for up to four years (Ghoneum and Brown 1999; Tazawa 2003). Additionally, in a key randomized trial (205 patients with various cancers in late III-IV stages), Biobran AXs combined with chemotherapy improved the long-term (18 months) survival ratio and quality of life (pain, malaise, vomiting and appetite checked by observation and inquiry) of patients compared to the control group (Takahara and Sano 2004). They also determined the NK cell activity by Biobran AXs treatment and found the NK cell activity increased, showing positive correlation with long-term survival ratio. This research suggested NK cell activity can be used as a pathological index of cancer. However, whether the prolonged survival can subsequently produce a better cure ratio remains unknown. More recently, one clinical trial in Vietnam for 3 years (68 patients with hepatocellular carcinoma) documented that oral administration of Biobran AXs (1g/day) enhanced the effects of interventional therapies (IT) for the treatment of liver cancer. In contrast to control subjects, patients treated with Biobran AXs combined with IT treatment showed lower recurrence of the disease, higher survival after the second year, significantly lower alpha-fetoprotein level and a significant decrease in tumour volume (Bang et al. 2010). In conclusion, Biobran AX represents great potential for further investigation in clinical studies where it could be used in conjunction with different cancer therapies to enhance other immune cell (such as B cell, T cell and macrophage) responses and improve treatment efficacy.

1.5.3 Structure-activity relationship of arabinoxylans

Recently, some papers have shown interest in the relationship between the bioactivity of AX and its molecular structural properties, including molecular weight, degree of branching and sugar compositions (Zhou et al. 2010). Zhou et al. (2010) compared immune enhancement ability between the AXs extracted from wheat bran via alkali (large Mw: 350KDa) and enzymes (low Mw: 33KDa), and results showed that AXs had stimulating effects on the immune response *in vivo* but low Mw AXs induced more macrophage phagocytosis than high Mw AXs. The most commonly tested AXs from rice bran (MGN-3/Biobran) are small in size (30-50KDa) with an A/X ratio of 0.5, in which the Mw and A/X are very similar to enzymatic extractable wheat bran AXs (Mw: 33KDa; A/X: 0.55). Both AXs showed high stimulation effects on macrophage activities (Ghoneum and Matsuura 2004; Zhou et al. 2010). Corn husk AXs activated NK cell activity and cytokine production *in vivo* (Ogawa et al. 2005). The corn husk AXs have a similar Mw (53KDa) to Biobran AXs but are more branched (A/X: 0.95). Thus, the Mw of such immune-stimulating AXs may be more important than the degree of branching when activating immune responses. Barley AXs with larger Mw (156kDa) compared to Biobran AXs showed very low immune stimulation activity both *in vivo* and *in vitro*, but with an A/X ratio of 0.49 (very similar to Biobran AXs) (Samuelsen et al. 2011), which again suggests that the degree of branching (A/X) may not have a substantial effect on immune-modulatory activity. Interestingly, in contrast to the barley AXs, wheat bran AXs with larger Mw (352KDa), obtained via alkaline extraction, still demonstrated a

high immune stimulation ability when tested on macrophages (Zhou et al. 2010), which could be explained by the heterogeneity of their structures. From previous studies, the structural properties of AX are not only dependent on the extraction/modification method but also its heterogeneity (Izydorczyk and Biliaderis 2007; Z. Zhang et al. 2014). Although there has been limited well-documented research into the relationship between immune-stimulating ability and various structures and sources of AX, previous studies are encouraging enough to warrant continued investigation into the structure and bioactivity relationship of AXs.

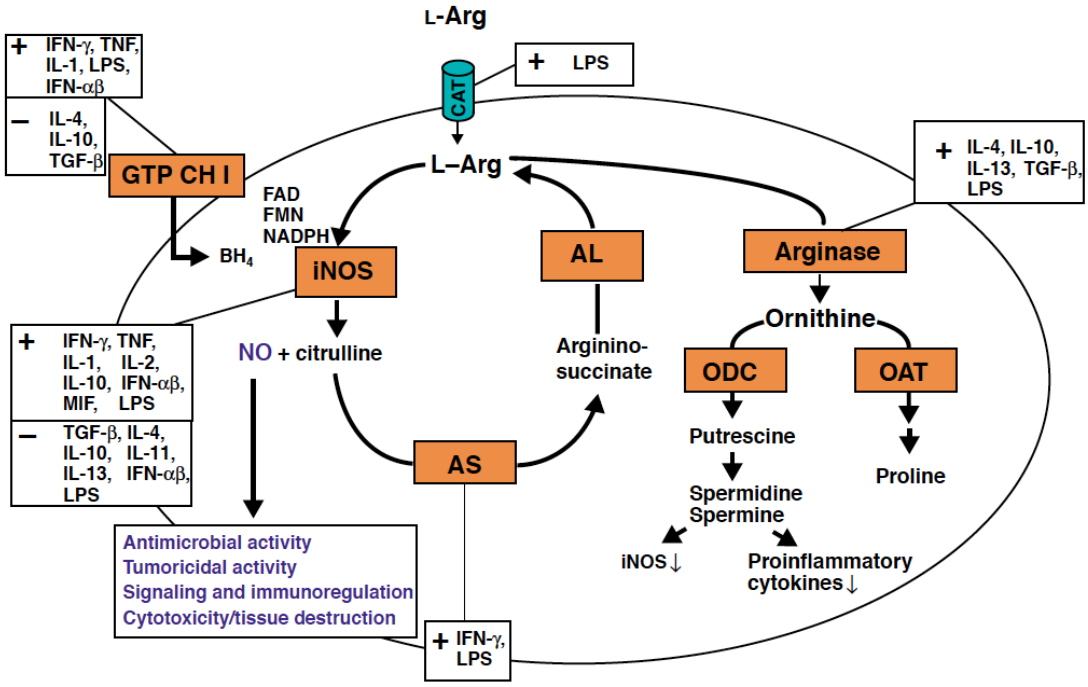
1.5.4 Reaction between biological response modulators and iNOS expression

Nitric oxide (NO) is a versatile signalling molecule of the immune system produced by various cells (dendritic cell, NK cell, mast cells and phagocytic cells including monocytes, macrophages, microglia, Kupffer cells, eosinophils, and neutrophils) from three potential different NO synthases (Inducible NOS (iNOS); Neuronal NOS (nNOS) and/or Endothelial NOS (eNOS) (Bogdan 2000; Forstermann and Sessa 2012). From previous studies, the immune functions of NO have been found to involve antimicrobial (Bogdan 2000; DeGroote and Fang 1999; Nathan and Shiloh 2000) and anti-tumour activities *in vitro* and *in vivo* (Bogdan 2000; Lechner et al. 2005; Nathan 1992; Pervin et al. 2001) .

iNOS is one of three key enzymes generating NO from the amino acid L-arginine. iNOS can be expressed in immune cells like macrophages by BRMs such as LPS and

cytokines (Lechner et al. 2005). For example, Bogdan (2000) has reviewed the pathway of NO production by BRMs and its antimicrobial activity in macrophages (Figure 1.4). Interestingly, some studies discovered that AXs also showed enhancement effects on NO secretion in murine macrophage cells (Ghoneum and Matsuura 2004; Nagata et al. 2001). However, these limited results only indirectly showed the AX might have a similar function to known BRMs like LPS that can induce the iNOS expression via the NO pathway. In any future study, the interaction between AX and NOS expression needs to be directly evidenced (for example, by evaluating protein levels of iNOS via dot-blotting following treatment with AXs *in vitro*).

Figure 1.4 Related pathways and functions of inducible nitric oxide synthase (iNOS) in mouse macrophages (Bogdan 2000)



The activity of iNOS is regulated by cytokines and microbial products (such as LPS), which affect the uptake of L-arginine (L-Arg) by cationic amino acid transporters (CAT), the synthesis of cofactors (such as BH4 by GTP cyclohydrolase I (GTP-CH I)), the expression of iNOS mRNA and protein, the enzymatic recycling of citrulline to arginine and the depletion of arginine by arginase. AL, argininosuccinate lyase; AS, argininosuccinate synthetase; MIF, macrophage migration inhibitory factor; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase.

1.6. Conclusions

Various methods have been developed for extracting AXs from cereal by-product materials, including chemical, enzymatic and mechanical assisted treatments. Amongst these methods, in terms of extraction yields of AXs, alkali treatments and mechanically assisted methods have been proved to be more efficient than other methods when used on a laboratory scale. However, the use of alkali or acid solvent treatment in industrial production may be limited by cost, process safety requirements and adverse environmental effects and by the suitability of equipment (e.g. available centrifuge

speed). The yields of AXs using enzymatic treatment are generally low compared to those achieved using alkali extraction. However these yields can be improved by incorporating a mechanical pre-treatment step into the extraction process. Therefore, a combination of mechanical pre-treatment, or chemical solvent treatment, with enzymes may be an alternative way to increase AXs yields from cereal by-products. The mechanical methods, which have been used to improve the AXs extraction efficiency, include ultrasonic-assisted treatment, microwave-assisted treatment, steam explosion and twin-screw extrusion. Of these four technologies, steam explosion and twin-screw extrusion are more environmentally friendly when applied on a pilot scale.

With the respect to the influence of extraction and modification methods on AXs molecular characteristics, chemical and enzyme hydrolysis both show more significant modifying effects on Mw and A/X of extracted AXs than the various mechanical treatments. The Mw of alkaline extracted AXs appeared to be larger than enzyme extracted AXs from wheat bran. For fractional purification, ethanol and ammonium sulphate have been shown to produce highly pure AXs. The A/X and the relative amount of substituted xylose residues of AXs are affected by the concentration of ethanol or ammonium sulphate used in the extraction process. For ammonium sulphate precipitation increasing the concentration reduced the ferulic acid content and the average Mw. It has also been shown that fractional precipitation with this agent is affected not only by the molecular size but also by the fine structure of the polymer. Overall, according to a number of research studies, the extraction yields and macromolecular characteristics of AXs are significantly influenced by the extraction

methods and modification technologies used. Therefore, the future studies could not only optimize extraction yields but also to improve the environmental friendly impact of AXs extraction and purification processes.

The immune-modulatory function of cereal AXs has been documented using a wide range of *in vitro* and *in vivo* and some clinical trial tests, as a result of which AXs can be considered a potent bioactive food supplement with immunity stimulation applications or used as a BRM having possible synergistic therapeutic effects on cancer. However, there are some limitations in AXs immune-modulatory research literature which have implications for any future study, including: (1) there is still only a limited understanding of receptor and signal pathways of AXs from animal studies and clinical trails; (2) there is a lack of investigations on the interactions between AXs and NOS expression *in vitro*; (3) there is no well-documented relationship between immune-stimulating ability and structural heterogeneity of AXs. In any future study, it will also be necessary to develop the extraction and modification methods in order to produce specific AXs with each of the numerous specific molecular features, which can be used to improve bioactivity of AXs applications.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Pentosan fraction of wheat flour and corn bran

The pentosan fraction of wheat flour is a by-product of wheat starch processing using high-pressure disintegration technology (W. Li 2012). In current study, the sample of dried pentosan fraction (moisture content 5.9%) was kindly provided from Henan Lianhua Monosodium Glutamate Group Co., Ltd. (Xiangchen, China).

Corn bran is a by-product of corn starch processing (M.P. Yadav et al. 2007). In the current study, the sample of dried corn bran (moisture content 4.5%) was kindly provided from Chinese Academy of Agriculture Sciences (Beijing, China).

2.1.2 Chemicals

NaOH (ACS reagent, ≥97.0%, pellets) for AXs extraction was purchased from Sigma-Aldrich (Gillingham, UK). D-(+)-xylose for AXs extraction yield determination was purchased from Acros Organics (Loughborough, UK). Eight pullulan (without side chains) standards of varying molecular weights (5–800KDa) were purchased from Shodex (Shanghai, China). $NaNO_3$ and NaN_3 for the HPLC mobile phase were purchased from Sigma-Aldrich (Gillingham, UK). $NaNO_2$ for nitrite standard determination were purchased from Sigma-Aldrich (Gillingham, UK). RPMI-1640 medium with L-Glutamine purchased from Lonza (Verviers, Belgium). Foetal bovine serum and penicillin-streptomycin (10,000 units penicillin and 10mg streptomycin/ml) were purchased from Sigma-Aldrich (Gillingham, USA).

2.1.3 Enzymes

Table 2.1 shows the main product information for the enzymes used in the extraction and modification AXs from pentosan fraction of wheat flour and corn bran.

Table 2.1 The enzymes were used in extraction and modification of AXs.

Product name	Main enzyme	Organism	Unit	Supplier
Pentopan Mono BG (P-BG)	Endo-1,4-β-xylanase (GH11 family)	*Thermomyces lanuginosus*	2500FXU-W/g	Novozyme, Bagsvaerd, Denmark
Endo-1,4-β-xylanase (E-XYLNP)	Endo-1,4-β-xylanase (GH11 family)	*Neocallimastix patriciarum*	1000- 1500 U/mg	Megazyme, Bray, Wicklow Ireland
OPTIMASH™ VR (O-VR)	Combination of xylanase and cellulase	*Penicillium funiculosum*	3150 CMC-DNS U/g	Genencor International, Leiden, Netherland
α-Amylase	1,4-α-D-Glucan-glucanohydrolase	*Bacillus licheniformis*	≥500 u/ml	Sigma-Aldrich, Gillingham, United Kingdom
Proteinase	Proteinase	*Aspergillus melleus*	≥3u/mg	Sigma-Aldrich, Schnelldorf, Germany

2.1.4 Cell used *in vitro* study

Human macrophage cell line U937 is derived from malignant cells from a pleural effusion from a 37-year-old caucasian male with diffuse histiocytic lymphoma (Sundstrom and Nillon 1976). U937 grows in suspension in conventional culture. U937 cells were purchased from the Public Health England Culture Collections.

2.1.5 Lipopolysaccharide

LPS (Lipopolysaccharides of E. coli serotype O111:B4, Sigma Aldrich, UK) was used

as a positive control in the nitric oxide (NO) stimulation assay and the inducible nitric oxide synthase (iNOS) expression assay.

2.1.6 Arabinoxylan samples

In addition to the AX samples produced in our lab, the other ten AX samples from various plant sources were used the *in vitro* study. These samples were a gift from Eastern Regional Research Centre, United States Department of Agriculture (ERRC, USDA, Wyndmoor, USA). The average molecular weight and A/X ratio of these AX samples has been determined by Eastern Regional Research Centre and the sample detail were given in the chapter 4 (Table 4.5).

2.1.7 Griess' reagent

Griess reagent can be used as a strong surrogate marker and a quantitative indicator of NO production by immune active cells (Bredt and Snyder 1994). Griess reagent is made up by two components. Component A is 37.5mmol/L sulphanilamide with deionized water in 6.5mol/L HCl by 1:1 (v/v). Component B is N-1-napthylethylenediamine dihydrochloride (NEED) in deionized water at 12.5mmol/L. NEED and sulphanilamide were purchased from Sigma Aldrich, UK.

2.2 Methods

2.2.1 Extraction and modification of AXs from pentosan of wheat flour

2.2.1.1 Water extraction procedures and independent experimental design of enzyme extraction

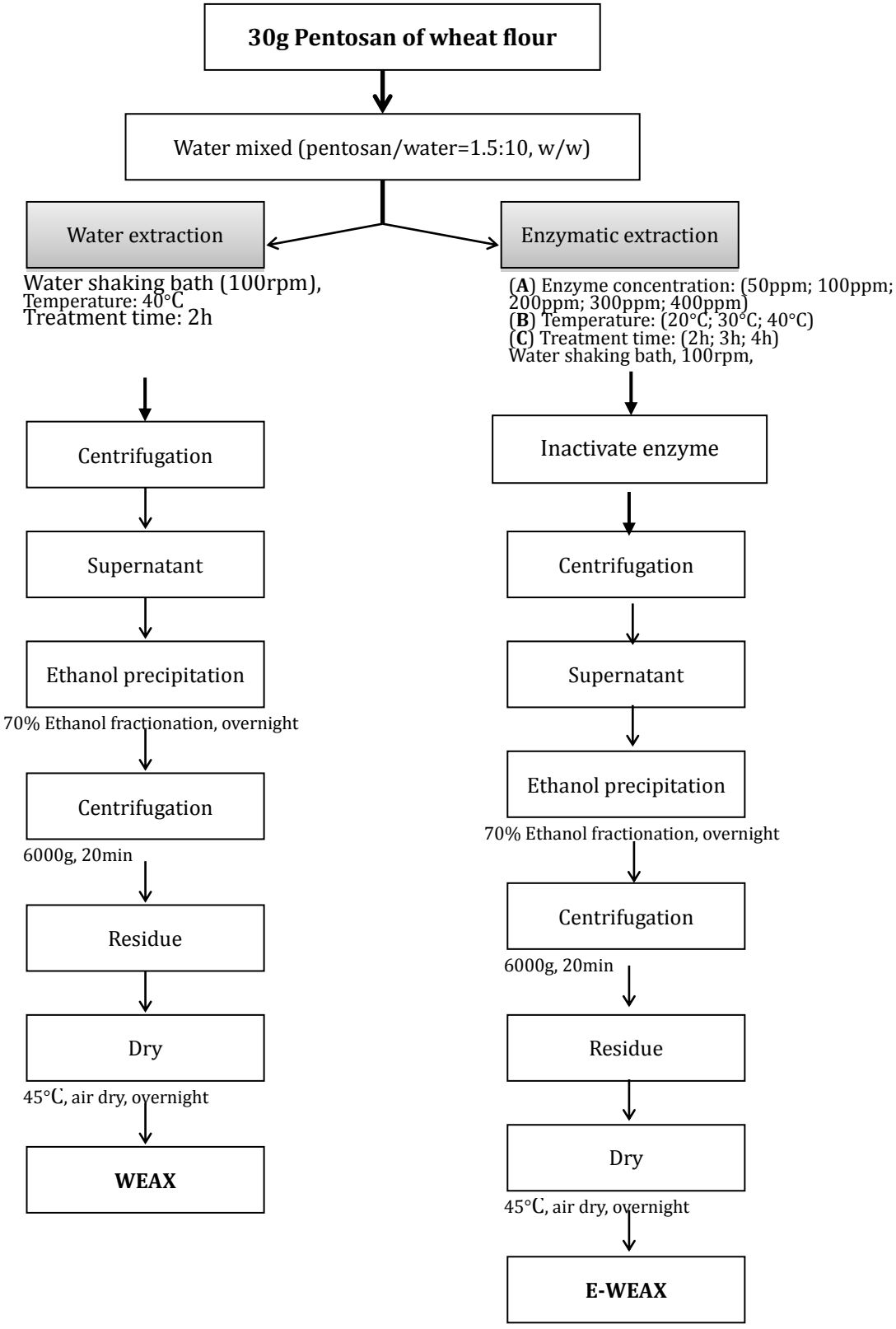
Pentosan fraction of wheat flour sample was ground to a 0.5mm particle using an Ultra Centrifugal Mill ZM 200 (RETSCH Ltd. United Kingdom). The water extraction and enzyme extraction processes of the pentosan fraction AXs (Figure 2.1) were developed from W. Li (2012) and W. Li et al. (2015).

For the extraction process of water extractable AX (WEAX), 30g of milled pentosan was mixed with 200g of distilled water using a hand blender (800W, WSB800U) for 45s. Then the pentosan-water mixture was incubated in a shaking water bath at 40°C for 2h followed by centrifugation (6000g, 20min), and the extraction supernatant (containing extracted AX in supernatant) was recovered and weighed for further ethanol precipitation.

The endoxylanase (P-BG) was added to the pentosan-water mixture to modify AXs during extraction process. In enzymatic extraction experiments, the effects of three individual extraction conditions following by concentration of P-BG concentration, extraction time and temperature, on E-WEAX extraction yield from the pentosan of wheat flour are studied using the independent experiment design (Figure 2.1). A) For the concentration enzyme, five pentosan-water mixtures were prepared, and added

50ppm, 100ppm, 200ppm, 300ppm and 400ppm P-BG to the mixtures respectively. The ppm (parts per million) was used as the units for the enzyme concentration in pentosan-water mixture. i.e. 50ppm of P-BG means added 0.0115g in 230g pentosan-water mixture. The mixtures were placed into a shaking water bath at 40°C for 2h. After enzymatic treatments, the mixtures were boiled for 15 min to inactivate the enzyme. The mixtures were cooled rapidly under cold water followed by centrifugation (6000g, 20min), and the extraction supernatant was recovered and weighed for further ethanol precipitation. B) For extraction temperature, three pentosan-mixtures were prepared, and then added same amount P-BG (200ppm) to these three mixtures. The mixtures were placed into a shaking water bath at different temperatures (20°C, 30°C and 40°C) for same treatment time (2h) respectively. After enzymatic treatments, the mixtures were boiled for 15 min to inactivate the enzyme. The mixtures were cooled rapidly under cold water followed by centrifugation (6000g, 20min), and the extraction supernatant was recovered and weighed for further ethanol precipitation. C) For extraction time, three pentosan-mixtures were prepared, then added same amount P-BG (200ppm) to these three mixtures. The mixtures were placed into a shaking water bath at same temperature (40°C) for different treatment time (2h, 3h and 4h respectively). After enzymatic treatments, the mixtures were boiled for 15 min to inactivate the enzyme. The mixtures were cooled rapidly under cold water followed by centrifugation (6000g, 20min), and the extraction supernatant was recovered for further precipitation.

Figure 2.1 Flow-chart of the extraction process of AX from the pentosan fraction of wheat flour.



2.2.1.2 Optimization of experimental design

In this experiment, the Box–Behnken experimental design was chosen for finding out the relationship between the response functions (extraction yield of AX) and extraction conditions (extraction temperature, time and enzyme concentration). The aim of this design was to optimize the three extraction conditions and to predict the maximum extraction yield of AX from pentosan fraction of wheat flour.

Box–Behnken model is a rotatable second-order design based on three-level incomplete factorial designs (Box et al. 1978; M. C. Douglas 2001; Ferreira et al. 2004). The special arrangement of the Box–Behnken design levels allows the number of design points to increase at the same rate as the number of polynomial coefficients. It means the model is able to make a two-factor factorial design with other factors set at a central value (Souza Anderson et al. 2005). For instance, there are three factors (extraction conditions) in this study; the Box–Behnken model for three factors involves three blocks, in each of which 2 factors are varied through the 4 possible combinations with the level of the third factor set at central value. This design combined with quadratic regression analysis and response surface method were used to identify the effects of three factors on response functions through linear, square and interaction terms analysis using statistical software DOE of Minitab Version 16.0.

For each independent condition, Box–Behnken design requires three equally spaced values, which is coded as -1, 0, +1. The code (0) is the centre point. The aim of this experimental design is to optimize the response functions (extraction yield of AX).

Therefore, according to the results of independent condition experiment, the condition that has relative high extraction yield can be selected as central point (see chapter 3, Table 3.3).

The number of experiment in Box–Behnken design is according to the equation: $N = K^2 + K + c_p$, where (K) is the factor number and (c_p) is the replicate number of the central point (Souza Anderson et al. 2005). Since there are three factors, Box–Behnken experimental design should give a total of 15 experimental runs. However, the analysis performed on the results is more realized and experimental errors are minimized. The number of replicates of experimental design was set at two; thus, the experimental design resulted in 30 experimental runs with 6 replications at central points (see chapter 3, Table 3.4).

The following regression equation was used to predict the maximum response resulted from response surface design (Yoshida et al., 2010):

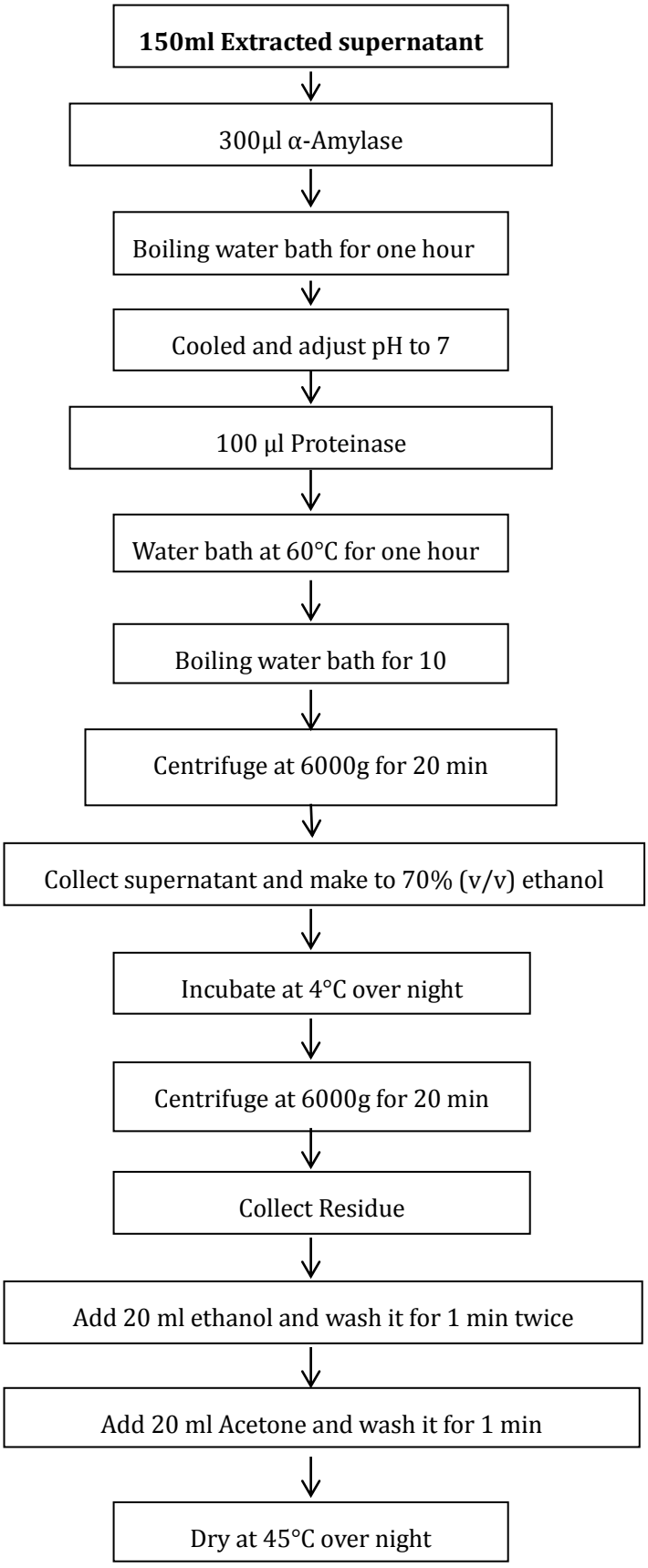
$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum\sum_{i<j} \beta_{ij} x_i x_j$$

Where y is the predicted AX extraction yield response, x_i or x_j is an independent condition, k is the number of factor, β_0 is the regression coefficient of model. β_i is coefficients of linear regression. β_{ii} is coefficients of square regression. β_{ij} is coefficients of interaction. The coefficients are estimated from the experimental results by quadratic regression analysis applying statistical software DOE using Minitab Version 16.0.

2.2.1.3 Ethanol precipitation of wheat pentosan fraction AXs

The precipitation process was adapted from W. Li et al. (2015) shown in Figure 2.2. The 150ml extraction supernatant was added to 300µl Termamyl α-Amylase and placed it in a water bath at 90°C for 1 h to allow starch hydrolysis to occur. After 1h, the solution was cooled rapidly under cold water and adjusted to pH 7. Then, the solution was added to 100µl proteinase (1mg/ml, ≥3units/mg) at 60°C for 1h to remove protein from the supernatant. Then the solution was placed in a boiling water bath for 10 min to deactivate the enzymes. Then the solution was centrifuged at 6000g for 20min. After centrifugation, the supernatant was collected and added to 70% (v/v) ethanol (350ml ethanol). The mixture was kept in the fridge overnight at 4°C. Then the mixture was centrifuged at 600g for 20min. After centrifugation, the residue was collected and washed with 20ml ethanol twice for 1 min. Then the residue was placed in 20ml acetone and washed for 1min. After that, the residue was placed in an oven overnight at 45°C for drying. The dried residue (dried AXs sample) was milled using an analytical mill (IKA A11 Basic, Guangzhou, China, 50/60Hz, 160W).

Figure 2.2 The flow chart of AXs precipitation process



2.2.2 Extraction and modification of AXs from corn bran

2.2.2.1 Extraction of AXs from corn bran

2.2.2.1.1 Extraction of corn bran using water and enzyme

The water extraction and enzyme extraction processes of corn bran AX were similar to that used for the AXs of wheat pentosan fraction. Corn bran (moisture content 4.5%) samples were ground to a 0.5mm particle using an Ultra Centrifugal Mill ZM 200 (RETSCH Ltd. United Kingdom).

For the water extraction process, milled corn bran (30g) was mixed in 200g of distilled water (1.5:10, w/w) using a hand blender (800W, WSB800U) for 45s. Then the corn bran-water mixture was incubated in a shaking water bath at 40°C for 2h followed by centrifugation (6000g, 20min), and the extraction supernatant was recovered and weighed for further precipitation.

For the enzyme extraction process, 200ppm P-BG (0.046g) were added to the corn bran-water mixture (230g). The samples were placed into a shaking water bath at 40°C for 2h. After enzymatic treatment, the mixture was boiled for 15 min to inactivate the enzyme. The mixtures were cooled rapidly under cold water followed by centrifugation (6000g, 20min), and the extraction supernatant was recovered and weighed for further precipitation.

2.2.2.1.2 Extraction of AXs using different concentration of alkaline extraction

Alkaline extraction of AXs from corn bran using a method developed from L.W. Doner and Hicks (1997a) and L.W. Doner et al. (1998). Milled corn bran (300g) was

mixed in 2000g of distilled water (1.5:10, w/w) using a hand blender (800W, WSB800U) for 45s. The pH of the mixture was adjusted to 7.0. 780μl Termamyl α-amylase was added into the mixture, which then treated in a 90°C shaking (100 r/min) water bath for 1h. Then, the mixture was boiled for 15 min to inactivate the enzyme. The mixtures were cooled rapidly under cold water followed by centrifugation (6000g, 20min), and the residue was recovered and placed in an oven overnight at 45°C for drying. Dried residue (251.35g) was collected, which was de-starched corn bran.

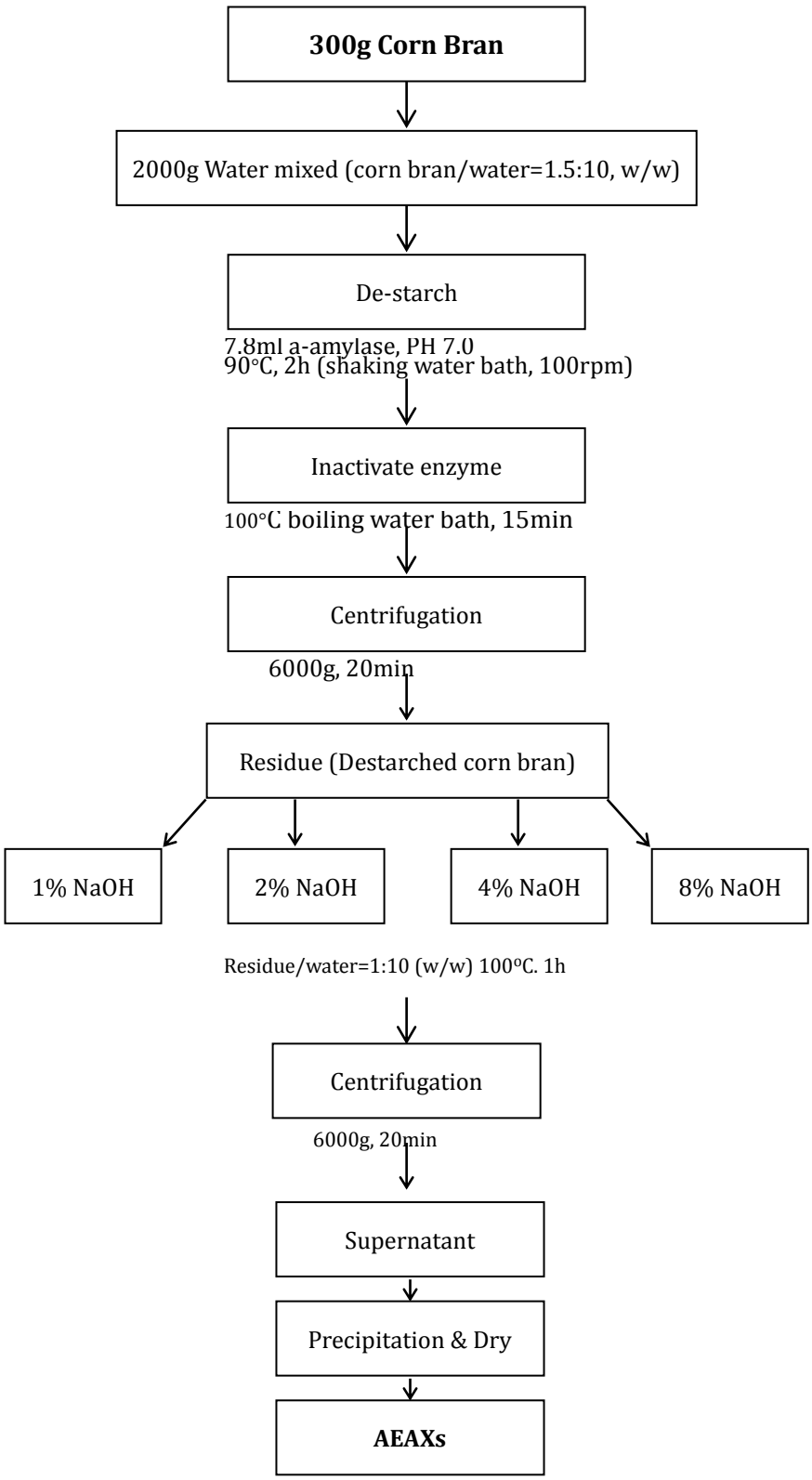
De-starched corn bran samples (60g) was mixed in 600g of distilled water (1:10, w/w) using a hand blender (800W, WSB800U) for 45s. Different amounts of NaOH (0.6g, 1.6g, 3.2g and 4.8g) were added into the mixtures. The mixtures were boiled for 1h with stirring. Then the mixtures were cooled rapidly under cold water followed by centrifugation (6000g, 20min), and then the extraction supernatants were recovered and weighed for further precipitation.

2.2.2.2 Ethanol precipitation of corn bran AXs

The precipitation method was used to obtain corn bran AXs was similar to that used for the AXs of wheat pentosan (the section 2.2.1.3). For the water and enzyme extraction, corn bran AXs samples were precipitated from the supernatants using the same procedures as the AXs of wheat pentosan (the section 2.2.1.3). For the alkaline extraction, the 600ml AXs-containing supernatants were mixed with 400μl proteinase (1mg/ml, ≥3units/mg). This solution was allowed to react at 60°C for 1h to remove protein. Then the solution was placed in boiling water bath for 15min to inactivate the

proteinase. Then, the solution was centrifuged at 6000g for 20min. After centrifugation, the supernatant was collected and mixed with 70% (v/v) ethanol (1.4L). The mixture was kept at 4°C overnight. Then the mixture was centrifuged at 6000g for 20min. After centrifugation, the residue (purified AXs) was collected and washed twice with 20ml aliquots of ethanol. Then the residue re-suspended in 20ml acetone and washed for 1min. After that, the residue was dried in an oven overnight at 45°C. The dried residue (dried AXs sample) was milled using an analytical mill (IKA A11 Basic, Guangzhou, China, 50/60Hz, 160W) and weighed.

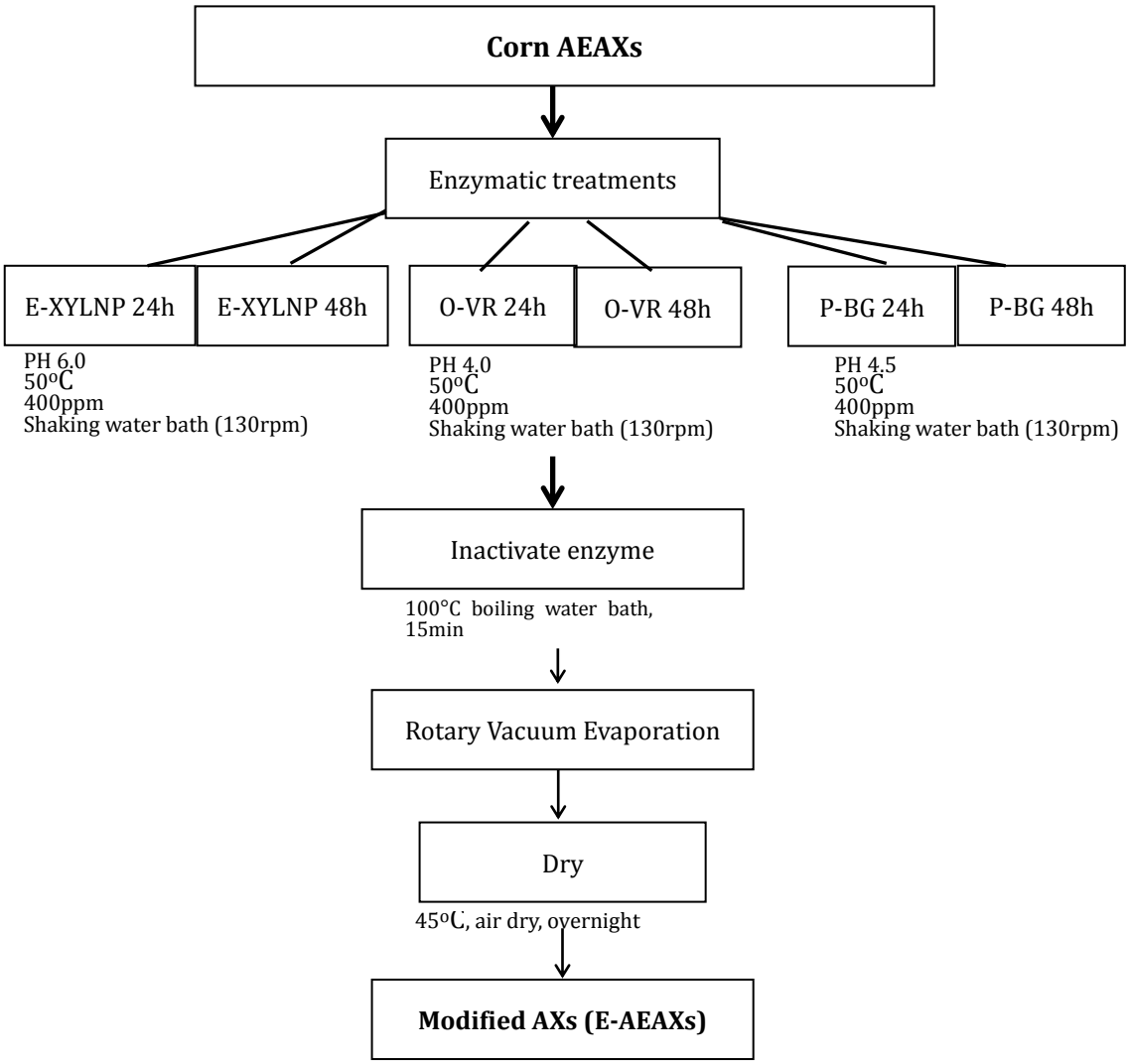
Figure 2.3 The flow-chart of the alkaline extraction process of AX from corn bran



2.2.2.3 AXs modification procedures

3g AEAXs (8% NaOH extracted) were mixed with 72g distilled water so that the ratio of AEAX to mixture was 1/25 (w/w). Three different enzymes (E-XYLNP, P-BG, O-VR) were used to modify the AEAX and the treatment time were 24h and 48h respectively as shown in Figure 2.4. 0.03g of each enzyme (400ppm enzyme of 75g mixture was 0.03g) was added in the mixture. The optimum pH and temperature of enzymes were according to the manufacturers instructions, thus, the conditions of each enzyme treatment were set as pH 6.0, 50°C and 24h/48h for E-XYLNP treatment; pH 4.5, 50°C and 24h/48h for P-BG treatment; pH 4.0, 50°C and 24h/48h for O-VR treatment. After 24h/48h enzymatic treatments, the mixtures were placed in a boiling water bath for 15min to inactivate the enzymes. As shown in Figure 2.4, the modified AXs were collected using a rotary vacuum evaporation procedure. Most of the water in the mixture was evaporated by rotary vacuum evaporation and then the residue was placed in a 45°C oven overnight for drying. The dried residue (modified AXs) was milled using an analytical mill and weighed.

Figure 2.4 The flow-chart of the modification process of AEAX from corn bran



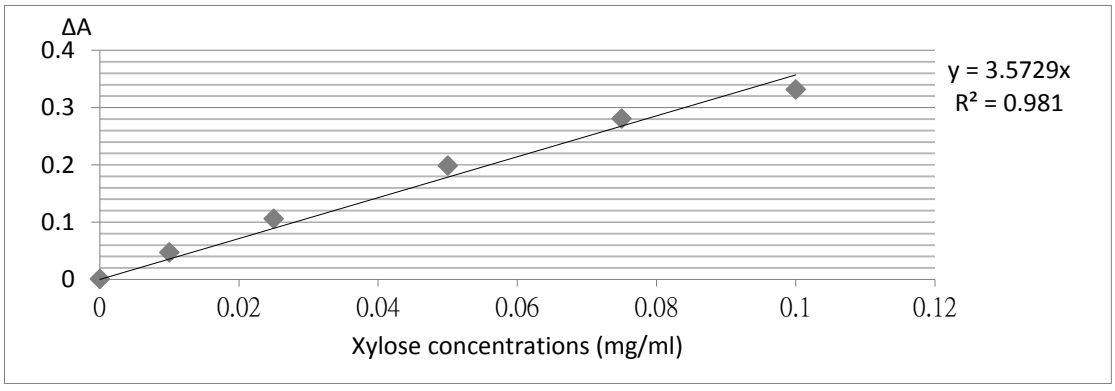
2.2.3 Analysis of extraction yields and compositions of AXs

2.2.3.1 Measurement of extraction yields of AXs

The AX content was determined using the method described by S. G. Douglas (1980).

Standard calibration curve (Figure 2.5): 0.1g D-(+)-xylose was weighed and made up to 100ml in distilled water in a volumetric flask (solution A, 1mg/ml). 10ml of solution A were diluted to 100ml in a volumetric flask, giving 0.01g D-(+)-xylose per 100ml (solution B, 0.1mg/ml). Aliquots of 0.025, 0.5, 1.0, 1.5 and 2.0ml of solution B were made up to 2.0ml with distilled water, and then 10ml of freshly prepared extraction solution was added. The tubes were placed in a vigorously boiling water bath for 25 minutes. Each tube was shaken twice during the heating process. The tubes were then cooled rapidly in cold water and the absorbance measured at 552nm and 510nm. The Spectrophotometer used was a 335907P-04, Genesys, Thermo Electron Corporation.

Figure 2.5 Standard calibration curve of xylose



ΔA*: The difference between absorbance value at 552nm and 510nm.*

Extraction solution for hydrolysing AXs samples were prepared by acetic acid

110ml (glacial), Hydrochloric acid 2ml (concentrated), phloroglucinol 5ml (20% w/v in ethanol), Glucose 1ml (1.75% w/v aqueous). This solution was prepared fresh for every analysis.

Determination of xylose: 2mg cereal samples and 0.5mg extraction supernatants of AXs were weighed into separate stoppered glass tubes and 2ml of water was added followed by 10ml of freshly prepared extraction solution. Each tube was mixed on a vortex for 10-30 seconds. The tubes were covered with foil and placed in a vigorously boiling water bath for 25min. The tubes were cooled rapidly under cold water and the absorbance was measured at 510 and 552nm as soon as possible after cooling. Since there is a gradual loss in colour after cooling, the number of tubes analysed was limited to between 7 and 14 at any one time. All analyses were conducted in triplicate, and values were required to be within a 5% to be considered reproducible.

A standard curve of xylose was constructed for determination of the xylose content of the two raw cereal samples and AXs' extraction supernatants, which, in turn, was used to calculate the content of AX in the samples based on their A/X ratio as tested in mono-sugar composition analysis, which using the equation according to W. Li et al. (2015): AX%=Xylose%×(1+A/X). Then the AX content in extraction supernatant was used to calculate the extraction yield of AX from raw material based on the weight of the supernatant and raw material. Thus, the calculation equation as followed:

$$AX\ \ extraction\ \ yield(\%) = \frac{AX\% \times weight\ \ of\ \ supernantant(g)}{weight\ \ of\ \ raw\ \ material(g)} \times 100$$

2.2.3.2 Measurement of mono-sugar compositions

The mono-sugar compositions of AX samples were analysed by following a method developed from W. Li et al. (2015). Sample preparation: 20mg dried AX sample was weighed out. One ml of 1M H_2SO_4 was added to sample. The sample was hydrolysed for 2 h at 100°C in a glycerin bath. The solution was transfer to volumetric flasks and diluted 20 fold (1mg/ml) using HPLC grade water. The pH value of diluted solution was adjusted to 6.5-7.2 with 1M NaOH. The solution was then filtered through a 0.45μm nylon membrane and transferred separately to 1ml glass shell vials for HPLC analysis. Mobile phase (Buffer) was HPLC water.

The pump was Shimadzu LC-10ADvp Pump. Isocratic elution model was used in the HPLC test. Flow rate of mobile phase was set as 0.5 ml/min. Running time of HPLC was 30min per sample. Detector was JASCO RI-2031 Refractive index (RI) Detector.

Columns were SUPELCOGEL Pb (5cm×4.6mm) and Phenomenex ThermaSphere TS-130. Temperature of treatment was 85°C. All analyses were conducted in triplicate, and p-values were required to be within a 5%.

2.2.3.3 Measurement of molecular weight distribution

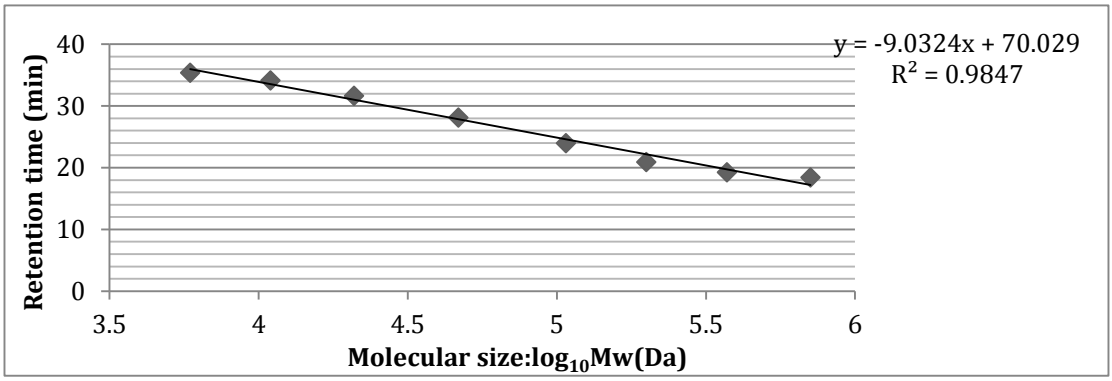
Size exclusion high-pressure liquid chromatography (HPLC-SEC) with a refractive index (RI) detector was used to determine the molecular weights and size distribution of dried AXs samples, which the Mw determination method was according to W. Li et al. (2015).

Mobile phase (Buffer) prepared by 17g $NaNO_3$ and 0.65g NaN_3, which dissolved in

HPLC grade water and transferred to a 2,000ml flask, then topped up with HPLC grade water.

Construction of standard curve: Eight pullulan standards with molecular weights in the range 5,000-800,000 were used to construct a standard curve (Figure 2.6). The standard samples were dissolved in the mobile phase to yield 0.5mg/ml solutions, and left overnight under gentle stirring. They were then filtered through a 0.45µm nylon membrane and transferred to 1ml glass shell vials.

Figure 2.6 Standard curve of molecular weight



The dried AXs samples were dissolved in the mobile phase to make 2mg/ml solutions and left overnight under gentle stirring. Then they were filtered through a 0.45µm nylon membrane and transferred to 1ml glass shell vials for HPLC-SEC analysis.

The pump of Shimadzu LC-10ADvp was used in HPLC-SEC devices. Isocratic elution model was used in the HPLC-SEC test. The detector was JASCO RI-2031 Refractive index (RI) Detector (Jasco Corporation, Tokyo, Japan). Columns of BioSep-SEC-S 4000 and BioSep-SEC-S 3000 (Phenomenex, Macclesfield, UK) were used to determine the Mw distribution of the AX in samples.

Two continuous columns connected in series (start from BioSep-SEC-S 4000) that

were used to improve the peak shape of the AXs samples. Running time was 60min per sample and flow rate was 0.6 ml/min. All analyses were conducted in duplicate, and values were required to be within a 5%.

2.2.4 *in vitro* study of AXs

2.2.4.1 Cell culture

The complete cell culture medium was prepared using the medium of RPMI-1640 with L-glutamine (Lonza, Belgium) with 10% foetal bovine serum (FBS) and 2% penicillin-streptomycin (P/S). The Human macrophage cell line U937 cells were grown in complete culture medium using a sterile tissue culture flasks. The cells were cultured in a cell incubator at 37°C with 5% CO_2 in air atmosphere. The cells were subcultured every 2 days.

2.2.4.2 Polysaccharides medium preparation of *in vitro* study

The E-WEAX sample from the enzymatic treatment (P-BG 400ppm) of the wheat flour pentosan, the WEAX sample from the water extraction of wheat flour pentosan, the E-AEAX sample from the enzymatic modification (O-VR 48h) of corn bran, the AEAX sample from the alkaline extraction (NaOH 8%) of corn bran and LPS were solubilised in a culture medium (the RPMI-1640 medium with 5% FBS) overnight. Then it sterilised using a 0.45µm sterile filter. The completely solubilised samples were diluted to yield a series of concentrations (1µg/ml, 5µg/ml, 10µg/ml, 50µg/ml, 500µg/ml, 1000µg/ml) for cell culture treatments. The samples were stored at 4ºC in sterile Falcon tubes

(Fisher Scientific, UK). In addition to the AX samples produced in our lab, the other ten AX samples from various plant sources were solubilised at 50µg/ml in the medium using the same preparation method as described above.

2.2.4.3 Toxicity test of AXs

Cell growth and viability of U937 cells following treatment with AX samples and LPS were assessed by cell count and trypan blue uptake. Trypan blue is one of the dye exclusion procedures for viable cell counting. This method is based on the principle that viable (live) cells with intact cell membranes are able to exclude trypan blue staining, whereas nonviable (dead) cells can take up trypan blue and are shown to be a blue colour under a microscope (Kim et al. 2005; Palama et al. 2011).

U937 cells were cultured in the complete culture medium until they reached ≥90% viability. Then, the U937 cells were centrifuged (1000g for 10min) and resuspended in RPMI-1640 medium with 10% FBS such that the density of live cells was set at 1×10^6/ml. 100µl cell suspension was pipetted in each well of a 96-well microplate. The AXs and LPS mediums with three high concentrations (50, 500 and 1000µg/ml) were warmed up to 37°C and then 100µl of each concentration was added to six wells of containing cells and mixed thoroughly. The microplates were then placed in an incubator (37°C, 5% CO_2) for 24h. After 24h incubation, 40µl of cell fluid was pipetted from each well into a tube and a similar volume, 40µl, of trypan blue (Sigma-Aldrich, UK) was added to each tube. The account of stained cells (nonviable cells) and not stained cells (viable cells) with trypan blue in each sample were counted using a TC10

automated cell counter (Bio-Rad, UK). The viability of cells was confirmed via number of viable cells by total cells (total number of viable and nonviable cells).

2.2.4.4 NO stimulation assay

This assay is used to evaluate the ability of AX samples and LPS to induce NO production in the cell line U937 using Griess reagent. In the presence of oxygen and water, NO interacts with itself to generate other reactive nitrogen oxide intermediates and ultimately decomposes to form NO_2^- (Dawson and Dawson 1995). The Griess' reagent was used for quantitative determination of NO_2^-, a stable oxidative end product of the antimicrobial effector molecule, nitric oxide (Fiddler 1977). In this study, Griess' reagent was freshly prepared immediately before use in the NO stimulation assay according to the methodology of Dawson and Dawson (1995) and Griess (1879). Various immune cell lines including macrophages have been shown to produce elevated levels of NO as a response to immune-stimulants such as LPS and cytokines (Jeong and Kim 2002). So LPS was used as a positive control.

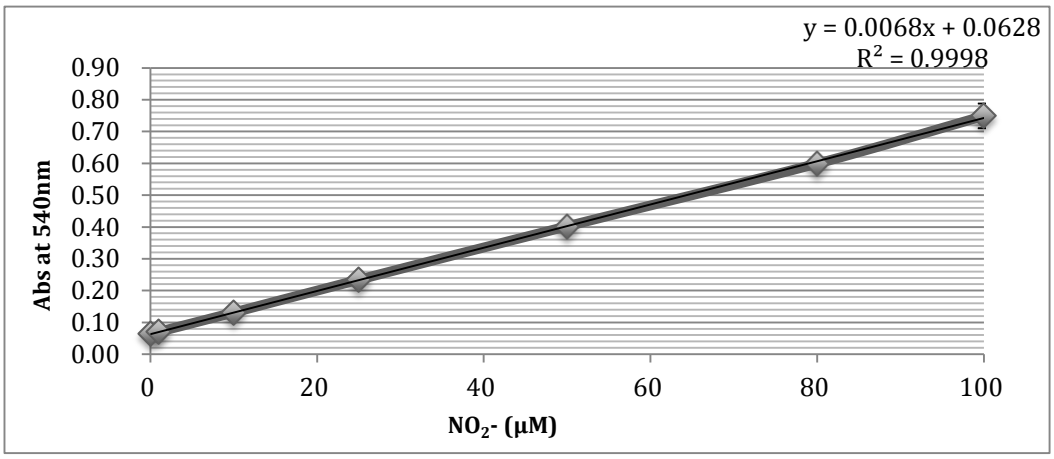
The U937 cells were centrifuged (1000g for 10min) and resuspended in RPMI-1640 with 10% FBS such that the density of live cells was set at 1×10^6/ml. 50µl of cell suspension was pipetted in each well of a 96-well microplate. Polysaccharide samples in RPMI-1640 with 5% FBS were prepared sterile at various concentrations and pre-warmed in a water bath (37°C), and then 50µl of each was added to the appropriate wells of containing cells and mixed thoroughly. The 50µl RPMI-1640 with 5% FBS medium was added to well containing 50µl live cells as a negative control. The LPS

at various concentrations was used as positive control samples. All experimental samples were added to the cells in triplicate wells. After the addition of AXs, LPS and negative control, the microplates were incubated (37°C, 5% CO_2) for 24 hours before NO assessments were made.

A nitrite standard reference curve was used to quantitatively determine the concentration of nitrite production in the experimental wells. Nitrite standards were prepared using sodium nitrite (Sigma Aldrich, UK) diluted in medium (RPMI-1640 with 10% FBS) at a range of concentrations (0, 0.1, 1.0, 10, 25, 50, 80 and 100µM). Then 100µl of each standard was added into each well and incubated (37°C, 5% CO_2) for 24h, alongside the AX sample treatments. All experimental standards were added to the cells in triplicate wells. The nitrite standard reference curve is shown in Figure 2.7.

After 24h incubation, Griess' reagents (Component A and Component B) were warmed up to 37°C before use. 50µl of Component A was added to each well and mixed. The microplate was incubated at room temperature for 10 minutes. 50µl of Component B was then added to each well and mixed and incubated at 4°C for 20 minutes. The absorbance of each well was then measured at 540nm using a microplate reader (Synergy HTX Multi-Mode Reader, Biotek, UK).

Figure 2.7 Nitric standard curve of NO assay



2.2.4.5 iNOS expression assay

iNOS is one of three NO synthases (NOS) which has been found in immune system cells (Bogdan 2000). This experiment evaluated the effect of AX stimulation on iNOS expression in U937 cells. The WEAX (50µg/ml), E-WEAX (50µg/ml) and LPS (50µg/ml) were tested in this assay. The dot blot method was used to determine the concentration of iNOS expressed by monitoring the cell lysates (Bloch et al. 1999).

2.2.4.5.1 Cell treatment

The U937 cells were cultured in RPMI-1640 with 10% FBS until they reached ≥90% viability and the density of live cells was set to 2.0×10^6/ml. 5ml samples (AXs, LPS and negative control mediums) were added to each flask containing 5ml cells. The flasks were then incubated (37°C, 5% CO_2) for 24h. After 24h incubation, samples were centrifuged (1000g for 10min) and resuspended in fresh medium such that the density of live cells was reset to 2×10^6/ml. 9ml samples were centrifuged at 1000g again for 10min and the supernatant was removed. The cell pellet was kept on ice prior to lysis

of the cells.

2.2.4.5.2 Buffer preparation

Cell Lysis buffer: 0.1M Tris-HCl, 1mM ETDA, pH7.8.

Blocking buffer: 1% Bovine Serum Albumin (BSA) in TBS-Tween. 0.25g BSA was dissolved in 25ml TBS-Tween and the pH adjusted to 7.4.

TBS-Tween buffer: 2.422g TRIS base, 16.36g NaCl and 2ml Tween were added to 2 Litres deionized water and the pH adjusted to 7.4.

iNOS protein standard buffer: 10% glycerol, 100mM glycine, 25mM Tris-HCl.

2.2.4.5.3 Cell lysis

150µl lysis buffer was added to each cell pellet and mixed using a vortex mixer for 10s and then placed in a freezer (-80°C) for 15min. The samples were removed from freezer and put into a 37°C water bath until just thawed. The samples were then transferred to ice for 5min. This freeze/thaw process was repeated 3 times. Then, the samples were placed in a sonication water bath (5510E-DTH, 490W, 50/60Hz, Bransonic, Danbury, USA) filled with ice water and subjected to sonication for 10s. The samples were mixed using a vortex and then kept on ice for 10s. This process was repeated 10 times. Following sonication the samples were mixed using a vortex mixer and placed on ice for 5min. Then the samples were centrifuged at 10,000g for 10min. The supernatants (cell lysis samples) were transferred into new tubes and stored at -80°C freezer until the iNOS content could be measured using the Dot blot assay.

2.2.4.5.4 Antibody preparation

Primary antibody preparation: 10µl of iNOS Antibody (4E5) (1mg/ml) was added to

10ml blocking buffer (1:1000 dilution), then the primary antibody preparation was stored at 4°C. Monoclonal iNOS Antibody (4E5) was purchased from Novus Biologicals (Cambridge, UK). iNOS mAb antibody was against human iNOS recombinant protein (Wang et al., 2010).

Secondary antibody preparation: 1.25g skimmed milk powder add to 25ml TBS-Tween (5% milk in TBS-Tween) and pH adjusted to 7.4. 15µl rabbit anti-mouse HRP was added to 15ml 5% milk in TBS-Tween (1:1000 dilution). Then the secondary antibody was stored at 4°C. The Rabbit anti-mouse Immunoglobulins/HRP (code: P0260) was purchased from Dako (Glostrup, Denmark).

2.2.4.5.5 Dot Blot assay

Nitrocellulose membrane (NC 45 nitrocellulose membrane, Serva Electrophoresis GmbH, Heidelberg, Germany) was cut into squares of approximately 40mm x 100mm. A grid was drawn onto the membrane using a pencil to create squares of 20mm x 20mm. The cell lysis samples were removed from the -80°C storage and allowed to stand on ice until nearly thawed. 5µl of cell lysis samples were added to 500µl lysis buffer (1:100 dilution). Human iNOS enzyme (116µg/ml, Code: TP311819, Cambridge Bioscience, UK) was diluted in iNOS protein standard buffer to give 1:20, 1:40, 1:80 and 1:100 standards. The samples and standards were set out as 5µl drops on the nitrocellulose membrane as follows with 5µl of BSA (1mg/ml BSA in deionized water) used as a negative control (no iNOS protein) in the dot blot. The membrane was allowed to dry briefly and then it was transferred to blocking buffer on a shaker for 1h at room temperature, shaking at 60 rpm. The blocking buffer was removed and the

membrane transferred to a solution of the primary antibody (1:1000). The blot was left overnight (≤20h) at 4ºC shaking at 60 rpm.

Following incubation the primary antibody solution was decanted and the membrane was washed five times for 5min using TBS buffer shaking at 110rpm. Sufficient secondary antibody was added to the blot and the membrane was shaken at 60rpm at room temperature for 1h. The secondary antibody solution was then removed and the membrane was washed five times with shaking at 110 rpm using TBS buffer, allowing 5min for each wash. The washed membrane in buffer was then transferred to a dark room. Subsequent steps were carried out under a red darkroom light.

The Chemiluminescence detection kit (Biological Industries (BI), Lichfield, UK) was prepared as follows: 500µl of the component A were added to the component B in a 2ml eppendorf tube according to the manufacturers instructions. The reagents were mixed and left to stand for about 5min. The nitrocellulose membrane was removed from the TBS buffer and dried carefully using tissue to remove excess buffer. Prepared chemiluminescence reagent was pipetted added to the membrane in a drop wise manner until the surface was covered. The covered membrane was allowed to stand for 1min. Then the membrane was dried carefully using tissue to remove excess buffer. The dried membrane was placed in a G:Box (Chemi HR16, Syngene, Cambridge, UK) for the images of membrane to be captured and processed. The Image J software (National Institute of Health, USA) was used to quantitatively determine the levels of iNOS. The dot blot assay of iNOS was repeated by three separate experiments.

2.2.5 Statistics

The results, unless otherwise stated, were checked using analysis of one-way ANOVA followed by Student's t-test. A value of $p \leq 0.05$ was chosen as the criterion of statistical significance. The data were expressed as means±standard deviation (SD).

CHAPTER 3.

Extraction, Modification and Molecular Characterisation of Arabinoxylans from Pentosan Fraction of Wheat Flour and Corn Bran

1

3.1 AXs of pentosan fraction of wheat flour

The pentosan fraction of wheat flour is a by-product of wheat starch processing using high-pressure disintegration technology, and its chemical composition has been shown in a previous report (W. Li et al. 2013), which reported that the pentosan material usually consists of about 53% starch, 8% proteins and 12% AXs. In the present study, the AX content of the pentosan fraction of wheat flour was shown to be 15.79±0.46% (dry basis) using the method described by S. G. Douglas (1980), which is higher than that in pentosan material used in the study of W. Li et al. (2013).

In the first stage, the effect of the extraction conditions, including enzyme (Pentopan mono BG: P-BG) concentration, extraction time and temperature on extraction yield of AX from the pentosan of wheat flour was investigated. Based on the results obtained in the first stage, the enzyme extraction process was optimised using the Box–Behnken design. Furthermore, the molecular weight distribution and degree of substitution of water extracted AX (WEAX) and enzyme-extracted AXs (E-WEAXs) were characterised.

3.1.1 Development of the process for the extraction AXs from the pentosan fraction of wheat flour

3.1.1.1 Effects of enzyme concentrations on AX extraction yield

The impact of various concentrations of endoxylanase (P-BG) on the extraction yield of AXs from the pentosan fraction of wheat flour was studied and the results are

presented in Table 3.1. As this table shown, at a concentration range of 50-400 ppm, the endoxylanase treatments (pH 4.5, 40°C and 2h) significantly increased extraction yield of AXs, compared with that of water extraction (p-values<0.05). The AXs extraction yield significantly increased from 7.54% to 12.83% (p<0.05) with the concentration increased from 0 to 400 ppm. With 400 ppm of P-BG treatment, AXs recovery from the pentosan fraction reached approximately 81.25% of total AXs of the pentosan fraction.

Table 3.1 The AXs extraction yield (dry basis) using different enzyme concentrations treatment

Independent experiment	Extraction yields of AXs (mean±SD)
Control	7.54±0.47% [a]
50ppm	10.31±0.26% [b]
100ppm	10.71±0.55% [b]
200ppm	12.70±0.55% [c]
300ppm	12.75±0.40% [c]
400ppm	12.83±0.35% [c]

The control indicates water extractable AX (WEAX) without enzymatic treatment. 50-400ppm indicates extracted AXs using enzymatic treatment at various concentrations (from 50-400 ppm) at pH4.5, 40℃ for 2h. The mean values with different letters (a, b and c) indicate significant differences (p<0.05) in AX extraction yield for each sample amongst all the treatments. The p-values were calculated by Student's t-test using Excel and experiments were conducted in triplicate.

The major reason for the increased yield achieved using endoxylanase treatment is that these enzymes can attack the xylan backbone, cleave internal β-(1,4)-linkages and a portion of water un-extractable AXs (WUAXs) will be rendered soluble and

extractable (Andersson et al. 2003; Beaugrand et al. 2004c; W. Li et al. 2013; Swennen et al. 2006). This means that a fraction of the WUAXs from the cell wall of the pentosan fraction was released into solution and the apparent extraction yield is increased. According to the theory of enzyme kinetics, with a certain substrate concentration, the enzyme is merely a catalyst which given sufficient time would convert the substrate to the maximum extent (Berg et al. 2002). However, considering the efficiency of the AX extraction process, the extraction time was selected as 2h in this experiment as this would be appropriate for use in industrial production system. The Pentosan (30g pentosan in 200g water) was treated for 2h with the endoxylanase at various concentrations between 50ppm and 400ppm in order to demonstrate effects on AX extraction yield. Concentrations of enzyme between 50ppm and 200ppm showed significant increases in the yield obtained with increasing enzyme concentration ($p<0.05$). Higher concentrations 200ppm to 400ppm did not change the yield obtained significantly ($P>0.05$), indicating that the optimum combination for practical use in the extraction process could be based on a concentration of 200ppm P-BG. Therefore, for the processing optimisation of Box-Behnken experimental design, 200ppm was selected as the centre point in the factor of enzyme concentration and 400ppm is considered as code +1. According to the requirement for three equally spaced values, the value code -1 is 0ppm, which means AXs extraction using the water without enzyme treatment.

3.1.1.2 Effects of extraction times and temperatures on AXs yield

Table 3.2 show that the extraction yield of AXs significantly increased as the extraction temperature was increased from 20°C to 30°C ($p<0.05$) but as the temperature was further increased to 40°C ($p>0.05$) there was no further increase in yield. The temperature of 30°C showed a high AX extraction ability using associated conditions of 200ppm, 2h and pH4.5. Hence the centre point of temperature for the Box-Behnken design was set at 30°C. The values for code +1 and -1 used were 40 and 20 respectively. This range is lower than that reported by X. Zhang et al. (2008a), which was 50°C to 60°C for the endoxylanase P-BG used to extract AXs from wheat bran. Given that the pentosan fraction of wheat flour has a high starch content (approximate 50% of the dried raw material) and starch gelatinization at temperatures higher than 50°C would increase the viscosity of the extraction solution (W. Li et al. 2013), which may have negative effects on the separation of AXs, thus the temperatures were set at less than 40°C.

In addition, there was no obvious difference ($p>0.05$) in extraction yield achieved when the process time was varied between 2 and 4h under same conditions (200ppm P-BG, 40°C, pH4.5) (Table 3.2). This indicated that 2h treatment time with P-BG was sufficient to allow the maximum extraction of AXs from the pentosan fraction of wheat flour. Considering the requirements of the industrial process, the centre point of time is selected at 2h. So the range of extraction time is set from 1h to 3h in the Box-Behnken experimental design.

Table 3.2 The extraction yield of AXs using different treatment conditions

Independent experiment		Extraction yields of AXs [c]
Treatment temperature [a]	20°C	11.12±0.39%
	30°C	12.97±0.34%
	40°C	12.73±0.53%
Treatment time [b]	2 h	12.72±0.54%
	3 h	12.21±0.34%
	4 h	12.44±0.34%

a: (20℃-40℃) means extracted AXs using the different enzyme treatment temperatures and other extraction conditions were set up as same (pH4.5, 2h, 200ppm P-BG) b: (2h-4h) means extracted AXs using the different enzyme treatment time and other extraction conditions were set up as same (pH4.5, 40℃, 200ppm P-BG); c: The extraction yields are presented as mean + SD and experiments were conducted in triplicate.

Based on the results obtained from the independent factor study, the effects of three individual factors on AXs extraction yield from pentosan have been demonstrated. The ranges of these three extraction conditions for optimizing the extraction process have been selected. This data will form the basis for the optimization of the pilot scale experimental design.

3.1.1.3 Optimization of the extraction conditions for AX yield using the Box-Behnken model

3.1.1.3.1 Box-Behnken model analysis

Based on results of the independent factors, the levels of variables, enzyme concentration, extraction temperature and extraction time, were determined for the Box–Behnken design. The values selected are shown in Table 3.3. The enzyme concentrations are in the range of 0-400ppm, extraction times are from 1h to3h and

extraction temperatures are from 20°C to 40°C. The centre point for the concentration of enzyme was set at 200ppm; the extraction temperature was 30°C and the extraction time was 2h. The AXs extraction yields for various extraction conditions are presented in Table 3.4. The details of the regression model analysis are summarized in Table 3.5.

Table 3.3 The level of variables chosen for the Box–Behnken design

Variables	Symbol	Coded variable level		
		High	Centre	Low
		+1	0	-1
Concentration of enzyme (E) (ppm)	X_1	400	200	0
Time of extraction (Ti) (h)	X_2	3	2	1
Temperature of extraction (T) (°C)	X_3	40	30	20

Table 3.4 Box-Behnken model design and experimental results

RunOrder	X_1 (E)	X_2 (Ti)	X_3 (T)	Extraction yield of AX
1	0	0	0	11.85%
2	0	1	−1	9.63%
3	0	−1	1	12.18%
4	−1	−1	0	7.72%
5	1	1	0	12.24%
6	0	−1	−1	8.53%
7	1	0	−1	11.22%
8	0	0	0	11.46%
9	1	0	1	13.15%
10	0	0	0	12.03%
11	1	−1	0	10.69%
12	1	1	0	11.78%
13	0	−1	1	12.49%
14	−1	1	0	7.29%
15	−1	1	0	7.74%
16	−1	0	−1	7.53%
17	−1	0	1	7.77%
18	0	−1	−1	8.60%
19	−1	0	1	7.98%
20	0	1	−1	10.15%
21	1	−1	0	10.90%
22	0	0	0	11.82%
23	1	0	1	12.91%
24	0	1	1	13.06%
25	0	0	0	11.38%
26	−1	0	−1	7.53%
27	0	0	0	11.53%
28	1	0	−1	11.80%
29	−1	−1	0	8.17%
30	0	1	1	12.19%

The model reveals the significance of extraction conditions to AXs yield in quadratic regression analysis (Table 3.5). The statistical relationships between the three individual factors and AXs extraction yield are shown as function curves in Figure 3.1.

The effects of enzyme concentrations on AXs extraction yield are shown in linear and square regression models (p-values<0.001). As Figure 3.2 shows, the AXs extraction yields increased linearly with increasing enzyme concentration from 0 to around 200ppm, which indicates that the P-BG treatment has a strong positive effect on AXs extraction compared to water extraction. From 200ppm to 400ppm, the positive effect of P-BG treatment on AXs extraction yield was generally increasing and the extraction yield reached maximum at the concentration of 367.68ppm. The effect of extraction time on AXs extraction yield is presented as a significant relationship in squarely term ($p<0.05$) (Table 3.5), which indicates the AXs extraction yield increased at the beginning of enzyme extraction, but the increased trend of the AXs yield becomes less with increasing time, and the maximum AX extraction yield was reached at 2.31h (Figure 3.1). The extraction temperature shows a linearly increased relationship with AX extraction yield ($p<0.001$), and the maximum AXs extraction yield was reached at 40°C (Figure 3.1).

In addition, in order to gain a better understanding on the interaction of three conditions, the 3D response surface plots and contour plots were prepared (Figure 3.2). From Figure 3.2A (interaction between enzyme concentration and extraction temperature), the 3D response surface plot appears as a ridge surface. As shown in the contour plot, the darker colour means that the extraction yield of AXs is higher. These two graphs indicate the AXs extraction yield increases as both enzyme concentration and extraction temperature increase, which means the combination of extraction temperature and concentration of enzyme has a synergistic effect on AX extraction

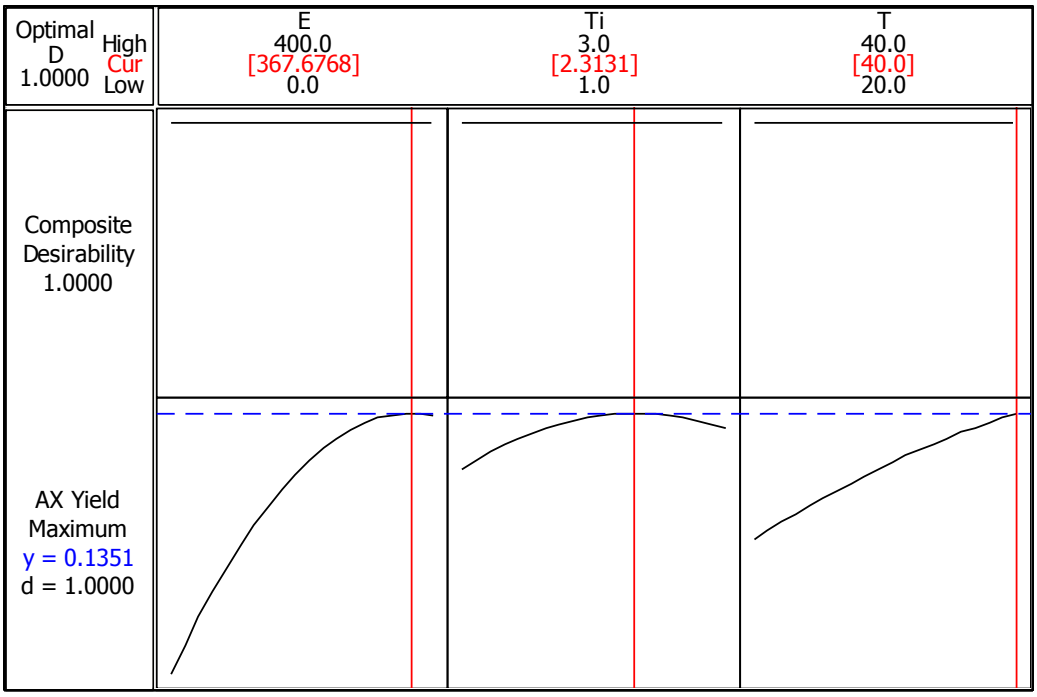
yield. For the enzyme concentration and extraction time (Figure 3.2B), the 3D surface plot also showed a similar ridge surface, and according to the contour plot, the time and concentration of enzyme also shows a synergistic effect for increasing AXs extraction yield. It should be noted that when after the time increased to 1.6h and the concentration of enzyme increased to 230ppm, the interaction between time and concentration of enzyme became weak and the AXs extraction yield had reached a high level. For the interaction relationship between exaction temperature and time, as shown in Figure 3.2C, the 3D surface plot is not presented as a ridge surface. The contour plot indicates that as the extraction time increased from 1h to 2.3h, requirement for extraction temperature decreased from 40°C to 33°C in order to achieve a high AXs yield. However, as the extraction time further increased from 2.3h to 3h, requirement for extraction temperature increased from 33°C to 40°C to achieve a high level of AXs extraction yield. Therefore, the interaction of extraction time and temperature was not a synergistic relationship.

Table 3.5 Analysis of quadratic regression

Source	Degree of freedom	Coefficients [b]	P-value [a]
Regression	9	0.00230545	<0.001**
Linear	3		<0.001**
Enzyme	1	0.000166665	<0.001**
Time	1	0.0315234	0.070
Temperature	1	0.00248606	<0.001**
Square	3		<0.001**
Enzyme*Enzyme	1	-3.72262E-07	<0.001**
Time*Time	1	-0.00622451	0.014*
Temperature*Temperature	1	-2.02903E-05	0.388
Interaction	3		0.120
Enzyme* Time	1	2.05572E-05	0.078
Enzyme* Temperature	1	1.47154E-06	0.198
Temperature*Time	1	-2.57979E-04	0.257
R^2 = 93.24%			

*a: P-value below 0.05 indicate significant factors b: Estimated Regression Coefficients for extraction yield of AX using data in un-coded units (real value of the extraction conditions); *: significant; **： highly significant.*

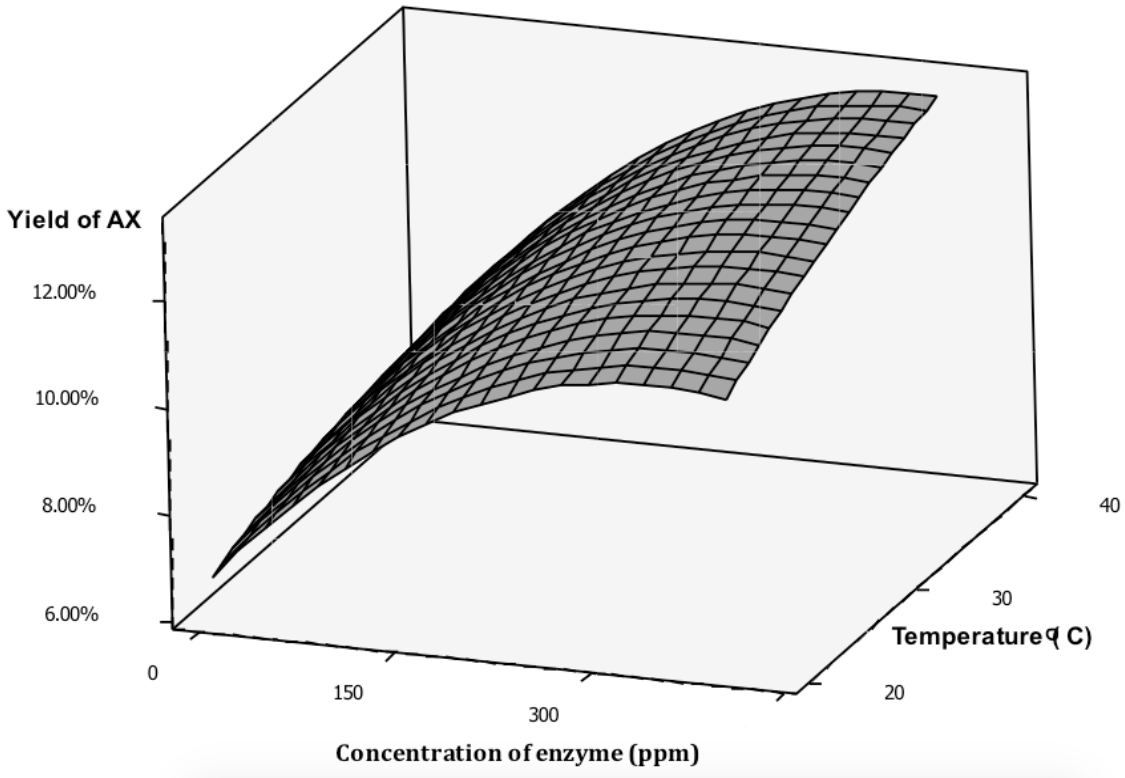
Figure 3.1 Optimization plots for three individual factors

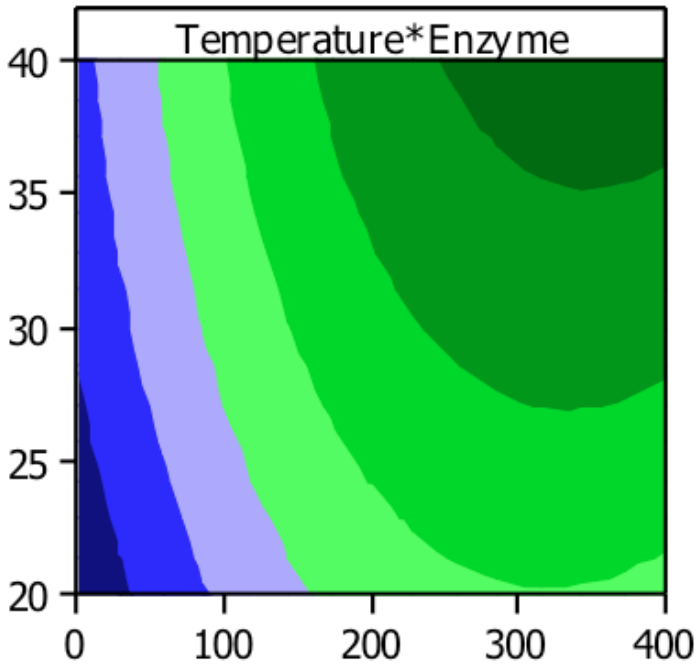


The optimization plots show the optimum value (the number of red colour) of each condition (E: enzyme concentration; Ti: treatment time; T: treatment temperature) and predicted maximum AX yield (13.51%) from pentosan of wheat flour (dry basis). 'Cur' indicates optimal value of each condition. 'D' means composite desirability (D), which evaluates how the settings optimize a set of responses overall. Desirability has a range of zero to one. One represents the ideal case; zero indicates that one or more responses are outside their acceptable limits.

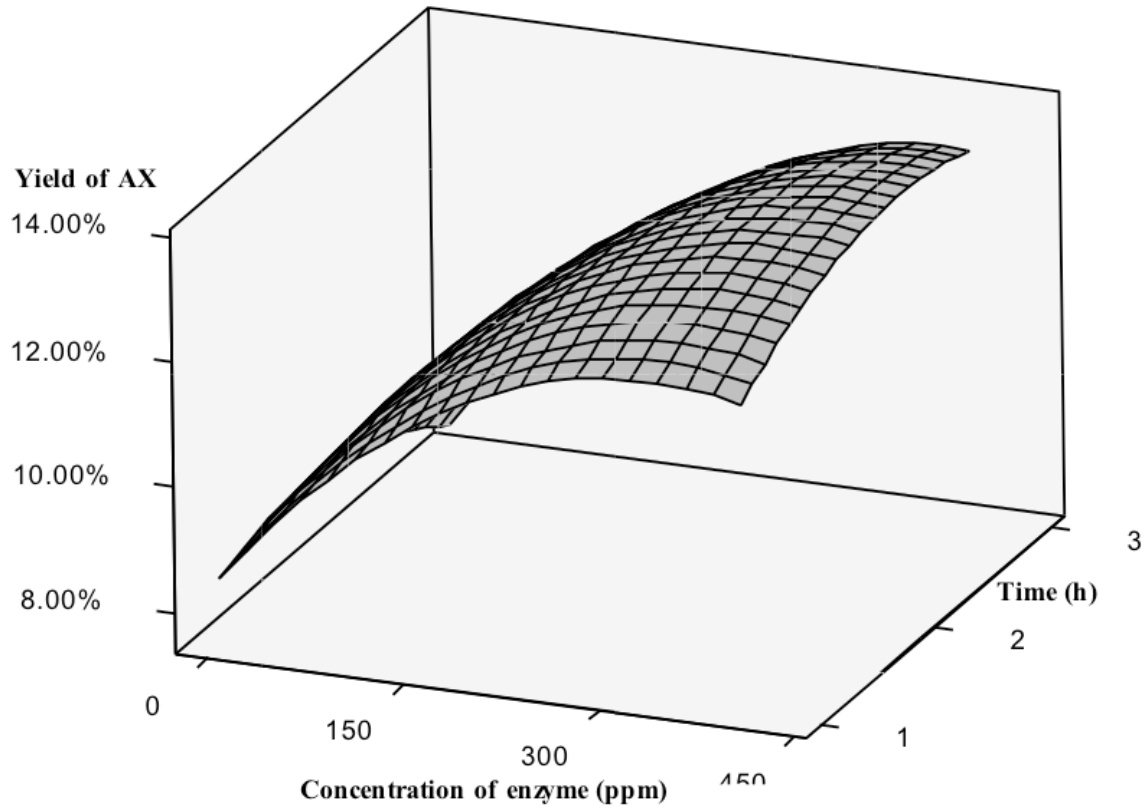
Figure 3.2 Response surface and contour plots

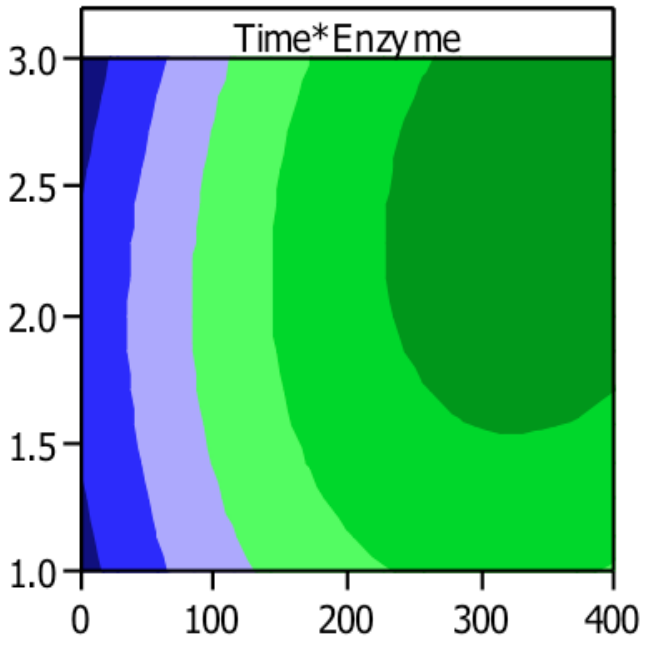
A.



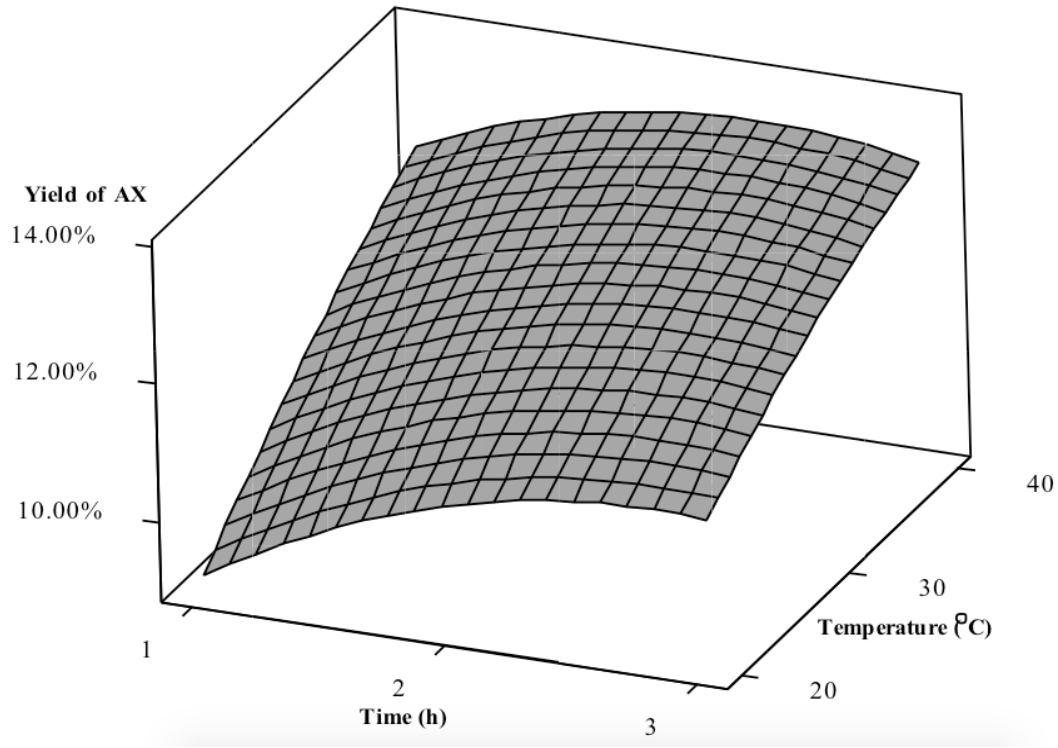


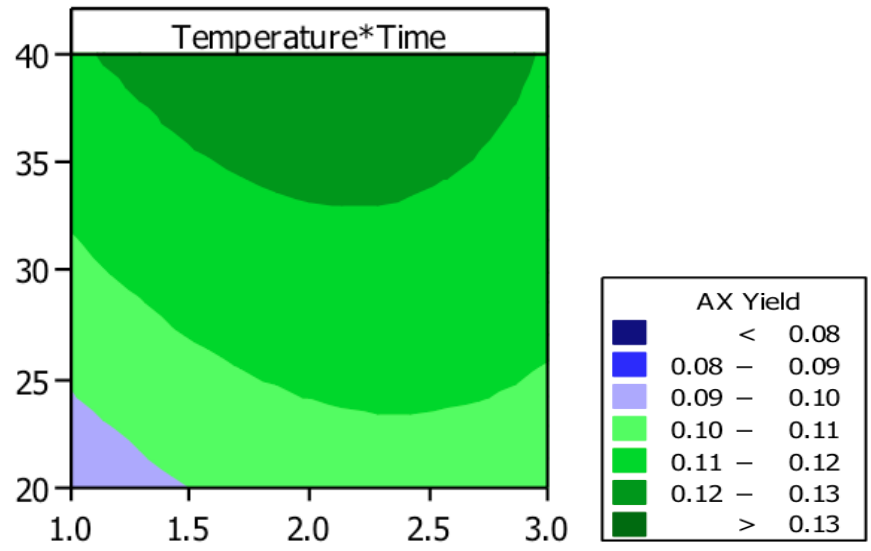
B.


Yield of AX
14.00%
12.00%
10.00%
8.00%
0
150
300
450
Concentration of enzyme (ppm)
1
2
3
Time (h)


Time*Enzyme
3.0
2.5
2.0
1.5
1.0
0
100
200
300
400

C.





3D surface (wireframe) plots are graphs that are used to explore the potential relationship between the three extraction condition factors. The factors were displayed on the x- and y-scales, and the response (AXs extraction yield z-scales) was represented by 3D surface plot. Contour plots displayed the 3-dimensional relationship in two dimensions, with x- and y-factors (condition factors) plotted on the x- and y-scales and response values represented by contours. (A): The interaction of concentration of P-BG and extraction temperature; (B): The interaction of concentration of P-BG and extraction time; (C): The interaction of extraction time and temperature.

3.1.1.5.2 Optimum extraction conditions for the AXs yield

According to the regression equation in section 2.2.1.2 (Chapter 2), the regression equation of this optimization study is summarised below:

Extraction yield of AX = 0.230545 + 0.0166665×E + 3.15234×Ti + 0.248606×T – 3.72262E-05×E^2 – 0.622451×Ti^2 – 0.00202903×T^2 + 0.00205572×E×Ti + 0.000147154E×T – 0.0257979×Ti×T

Here, E is the value of enzyme concentration, T is the value of extraction temperature and Ti is the value of extraction time. The coefficients are given by the variance and regression analysis (Table 3.5). According to the R^2 value, 93.24% of the variability in the responses indicates a good fit for this equation. Based on the response optimization analysis (Figure 3.1), the final optimum conditions for maximum extraction yield are as follows: (367.68ppm, 2.31h, 40.0°C). According to the regression equation, at the optimum point, the predicted maximum AXs extraction yields would reach 13.51%. The composite desirability (D) value is 1 (Figure 3.1), which indicates the three extraction conditions selected can be used to achieve a maximum AXs extraction yield and the prediction would feasible and effective. To validate the optimum conditions of the model, triplicate experiments were carried out at the estimated optimum point and the AXs yield attained was 13.54±0.042% (dry basis), which the experimental result was in good agreement with the predicted extraction yield (13.51%) obtained using the regression equation and it clearly shows this optimization model is sufficiently adequate.

Van Craeyveld et al. (2010) extracted 32-55% of the wheat bran AX using 3 different endoxylanase treatments, and Escarnot et al. (2012) tested various xylanases on the AXs extraction yield of spelt bran and hull and the highest extraction recovery was 68.8% of the total AX of raw materials. In this present study, the maximum AX's recovery yield can be achieved to 85.70% (13.54/15.8%) of total AXs content when using optimum conditions. Thus, the pentosan fraction seems a good source for producing AXs using the enzymatic extraction method. These optimum results would be useful for improving the production efficiency and decreasing the cost of producing AXs from the pentosan of wheat flour.

3.1.2 Characterisation of water and enzyme extracted AXs of pentosan fraction of wheat flour

3.1.2.1 Monosaccharide composition analysis of AXs from pentosan fraction of wheat flour

3.1.2.1.1 Development of a method for analysing monosaccharide compositions

Monosaccharide composition analysis using sulphuric acid hydrolysis has been reported in the previous studies (Escarnot et al. 2011). Therefore, it was decided to test the concentrations of sulphuric acid for the monosaccharide composition test in order to optimize AX hydrolysis conditions in the present study. Four concentrations of sulphuric acid (H_2SO_4) (1M, 1.5M, 2M, 2.5M) were tested for hydrolysing the water extracted AX sample (WEAX) with a hydrolysis time of 2 h at 100°C in a glycerol bath. After H_2SO_4 hydrolysis, the monosaccharide compositions of WEAX samples were

determined on a high-pressure liquid chromatography (HPLC). Table 3.6 shows that four monosaccharides, arabinose (Ara), xylose (Xyl), glucose (Glu) and galactose (Gal), were observed in WEAX when using the 1M and 1.5M H_2SO_4 treatments. However, with stronger H_2SO_4 treatments with concentrations of 2M and 2.5M, the monosaccharide composition of WEAX showed the disappearance of glucose and of glucose and galactose respectively. These substantial changes in glucose and galactose compositions indicate that using the too high a concentration (2M or 2.5M) of H_2SO_4 to hydrolysis WEAX may result in the loss of some monosaccharides (i.e. glucose and/or galactose) thus invalidating the results. Whereas, monosaccharide compositions remain consistent as the concentration of H_2SO_4 increased from 1 to 1.5M (Table 3.6). Statistical analysis (Student's t-test) shows there is no significant difference for Ara(%), Xyl(%), Glu(%) and Gal(%) ($p>0.05$) when using 1M and 1.5M H_2SO_4. This lack of difference suggests that at these H_2SO_4 concentrations there is no loss of monosaccharides due to acid hydrolysis. Additionally, the degree of branching in the AX chain (A/X ratio) was determined as 0.484 and 0.488 with 1M and 1.5M H_2SO_4 treatments respectively. These values are in very good agreement. In conclusion, H_2SO_4 treatment using 1 to 1.5M did not appear to influence the results in contrast to the results obtained with 2 to 2.5M H_2SO_4, which appeared to indicate a loss of monosaccharides due to acid hydrolysis. In the current study, 1M H_2SO_4 was used in AX monosaccharide compositions analysis.

Table 3.6 The monosaccharide compositions of WEAXs using different of concentrations H_2SO_4 hydrolysis

Molar Concentration of H_2SO_4	Monosaccharide compositions of WEAXs [a]				
	Ara(%)	Xyl(%)	Glu(%)	Gal(%)	A/X [c]
1M	26.13±1.01	53.96 ±1.49	10.62±2.04	9.29 ±1.26	0.48
1.5M	26.23±0.83	53.80±0.94	10.18±0.62	9.51±0.32	0.49
2M	26.27±1.90	61.65±1.00	/	12.08±0.90	0.43
2.5M	29.41±3.97	70.59±3.97	/	/	0.42

The proportion of each monosaccharide in AX sample is presented as mean + SD and all experiments were conducted in triplicate. A/X: the ratio of arabinose to xylose, which is used to indicate branching degree of AX.

3.1.2.1.2 Monosaccharide compositions of WEAX and E-WEAXs

Table 3.7 shows the E-WEAX samples have a higher degree of branching than WEAXs, and the A/X increases linearly when the enzyme (P-BG) concentration increases from 0ppm to 400ppm (R^2=0.958, Figure 3.3). In addition, according to Pearson's correlation coefficient analysis, it was also observed that the extraction yield of AXs with P-BG treatment was positively correlated with A/X (r=+0.95). Table 3.8 shows that the extraction temperature and time did not have significant effects on the A/X of E-WEAXs. In addition, comparing WEAX and E-WEAX (200ppm to 400ppm), the glucose (20-22%) and galactose (15-16%) content of the E-WEAXs significantly increased compared to the proportion of glucose (11%) and galactose (9%) in the WEAXs (p-values <0.05) (Table 3.7). These results indicated that the enzyme extracted AX materials might result in a higher proportion of side chains of glucose and galactose consisting compared with water extracted AXs.

The A/X represents the degree of branching of AXs, which is an indicator of the relative proportions of the unsubstituted and substituted xylose residues in AX chains (Izydorczyk & Biliaderis, 2007). As summarised in previous papers, a higher A/X is associated with a higher portion of 2-monosubstituted and disubstituted xylose residues, and a lower portion of 3-monosubstituted and unsubstituted xylose residues(Cyran et al. 2003a; Gruppen et al. 1992; Vinkx and Delcour 1996). As indicated above, the increase in AX yield and A/X may indicate that AXs extracted from the pentosan fraction using the enzymatic treatments may contain more 2-monosubstituted and disubstituted xylose residues.

Table 3.7 The monosaccharide compositions of AX samples under different concentrations of enzyme treatments

Samples	Mono-sugar compositions				
	Ara(%)	Xyl(%)	Glu(%)	Gal(%)	A/X [c]
Control [a]	26.13±1.01	53.96 ±1.49	10.62±2.04	9.29 ±1.26	0.48
50ppm [b]	28.15±1.34	49.65±1.22	11.92±0.95	10.28±1.32	0.57
100ppm	29.93±0.69	47.92±2.02	11.42±0.83	10.74±1.23	0.62
200ppm	26.77±1.23	37.13±0.76	20.3±0.86	15.81±0.32	0.72
300ppm	27.11±0.76	35.37±2.92	21.32±1.03	16.2±1.43	0.77
400ppm	28.74±1.77	34.51±0.34	21.85±2.22	14.9±2.32	0.83

a: the control means water extractable AX (WEAX) without enzymatic treatment; b: (50ppm-400ppm) means extracted AXs using the different concentrations of enzyme and other extraction conditions were set up as same (pH4.5, 40℃ and 2h); c: A/X means the composition ratio of arabinose to xylose. The proportion of each monosaccharide in AX sample is presented as mean + SD and all experiments were conducted in triplicate.

Figure 3.3 The A/X of AXs isolated using different concentrations of enzyme treatments

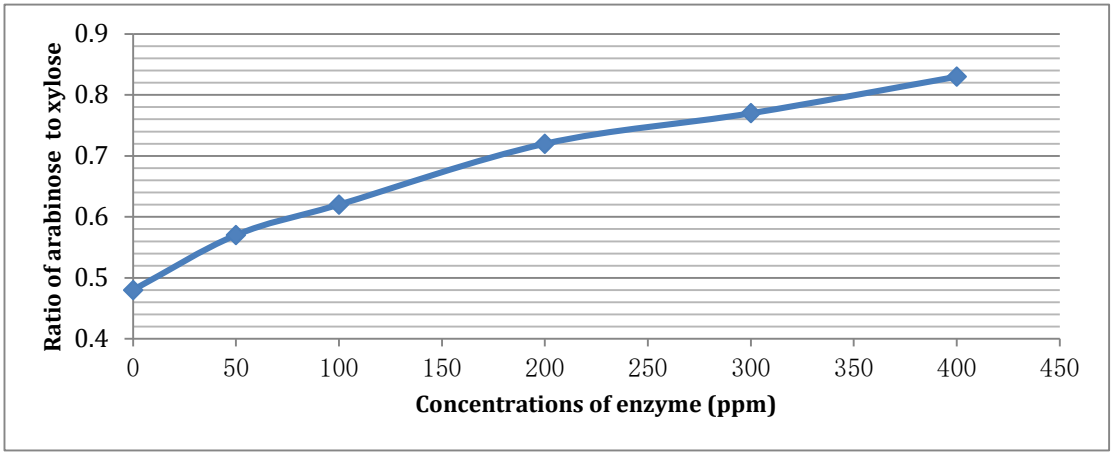


Table 3.8 The monosaccharide compositions of E-WEAX samples using different times and temperatures with 200 ppm P-BG treatment

Samples	Mono-sugar compositions				
	Ara(%)	Xyl(%)	Glu(%)	Gal(%)	A/X [c]
2h [a]	26.88±0.83	37.21±0.48	20.3±0.63	15.61±0.78	0.72
3h	27.52±0.34	38.22±1.32	19.84±0.63	14.42±0.79	0.72
4h	26.72±0.54	37.81±0.86	20.01±0.89	15.46±1.46	0.71
20°C [b]	27.11±1.82	39.04±1.64	18.47±1.33	15.38±0.49	0.70
30°C	28.15±1.67	39.19±0.76	18.01±2.55	14.65±0.64	0.72
40°C	26.67±1.05	37.22±0.35	20.34±0.50	15.77±0.44	0.72

a: (2h-4h) indicate extracted AXs using 200ppm P-BG treatment under different times; b: (20℃-40℃) indicates extracted AXs using 200ppm P-BG treatment at different temperatures; c: A/X means the composition ratio of arabinose to xylose. The proportion of each monosaccharide in AX sample is presented as mean + SD and all experiments were conducted in triplicate.

3.1.2.2 Mw characterisation of WEAXs and E-WEAXs

After separation, the AXs supernatant were purified using the ethanol precipitation method described by W. Li et al. (2015). The Mw distribution of purified

AXs was analysed by size exclusion high-pressure liquid chromatography (HPLC-SEC) and the effects of the enzymatic (P-BG) treatments on the molecular weight (Mw) distributions were compared with that of the water extraction. The overall Mw of AXs ranged from 159Da to 794KDa ($1\times10^{2.2}$-$10^{5.9}$Da) as shown in Figure 7 and it was divided into four ranges (Table 3.9). WEAX portion (78.52%) is mainly in the ranges 1 and 2 (10KDa to 794KDa) whereas E-WEAXs portions (81-89%) are mainly in ranges 2 and 3 (1KDa to 100KDa). The Mw distributions show clear differences depending upon the enzyme treatment. E-WEAXs showed the largest increase in the proportion of AX with small Mw in range 3 compared to that of WEAX (Table 3.9).

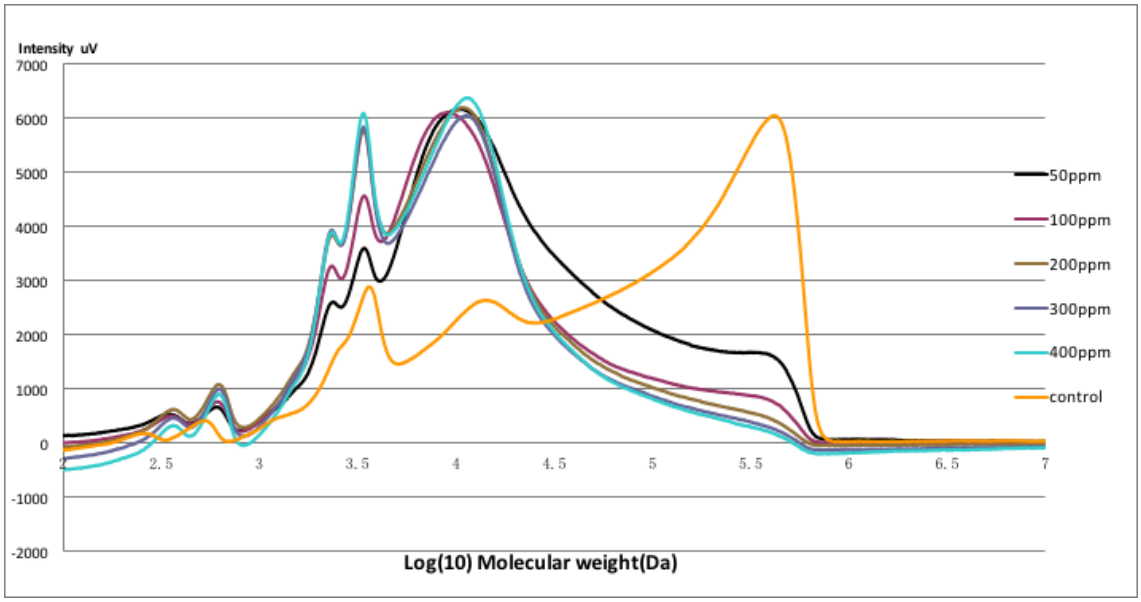
Table 3.9 The proportions of WEAX and E-WEAX in different ranges of molecular weight

Samples	Mw distributions of AXs [e] (%)			
	Range 1: 1×10^{5}-$10^{5.9}$Da	Range 2: 1×10^{4}-10^{5}Da	Range 3: 1×10^{3}-10^{4}Da	Range 4: $1\times10^{2.2}$-10^{3}Da
Control [a]	46.46%	32.06%	19.11%	2.37%
50ppm [b]	15.27%	45.54%	35.42%	3.77%
100ppm	9.26%	40.29%	46.72%	3.72%
200ppm	7.17%	40.62%	46.82%	5.39%
300ppm	6.47%	40.09%	48.88%	4.56%
400ppm	5.75%	39.89%	49.51%	4.85%
20°C [c]	7.33%	40.18%	47.38%	5.11%
30°C	7.48%	40.30%	47.04%	5.18%
40°C	7.13%	40.45%	46.93%	5.49%
2h [d]	7.15%	40.52%	46.87%	5.46%
3h	7.05%	40.69%	46.79%	5.47%
4h	7.23%	40.45%	46.75%	5.57%

a: the control means water extractable AX (WEAX) without enzymatic treatment, b: (50ppm-400ppm)

means extracted AXs using the different concentrations of enzyme and other extraction conditions were set up as same (pH4.5, 40℃ and 2h); c: (20℃-40℃) indicates extracted AXs using 200ppm P-BG treatment at different temperatures; d: (2h-4h) indicate extracted AXs using 200ppm P-BG treatment under different extraction times; e: The proportion of Mw in different range were analysed using the LC Data Analysis (SHIMADZU Corporation) and Microsoft Excel.

Figure 3.4 The molecular weight distribution of enzymatic extraction AXs with different concentrations of enzyme



In contrast to WEAX, with the 400ppm P-BG treatment, the proportion of small Mw of the E-WEAX in range 3 (1KDa to 10KDa) increased from 19.11% to 49.51%, and the proportion of large Mw in ranges 1 (100KDa to 794KDa) decreased from 46.46% to 5.75%. These results show that the treatments of endoxylanase reduce the proportion of high Mw (range 1) of AXs by increasing the proportion of low Mw (range 3). Thus, E-WEAXs has a much lower Mw distribution than WEAX. This finding is consistent with the previous results reported by W. Li et al. (2015) that the endoxylanase modified AX sample contains a larger portion (85.7%) of low Mw AXs (1-25KDa) compared to water extracted AX (49.5%) from wheat flour pentosan. Maes et al. (2004) also observed that

Aspergillus aculeatus endoxylanase released enzymatic-solubilised arabinoxylans with a low Mw distribution (1KDa to 1.2KDa) from wheat bran. It has been explained that endoxylanase attacked the β-1, 4 linked D-xylopyranosyl backbone and break down xylan chains, thus, reducing the molecular weight of AXs during enzymatic extraction (Courtin and Delcour 2001; Izydorczyk and Biliaderis 2007). Z. Zhang et al. (2014) indicated that the Mw of AXs varies depending on the extraction and treatment methods used, the enzymatic treatment appears to be one of the most effective method for modifying AXs with a relative low Mw distributions.

From Table 3.9, it can be seen that as the concentration of enzyme rises from 50ppm to 400ppm, the proportion of the E-WEAX in the low Mw ranges 3 and 4 (159Da to 10KDa) increases from 39.19% to 54.36%, whereas, the proportion of AXs in the high Mw ranges 1 and 2 (10KDa to 794KDa) gradually decreased from 60.81% to 45.64%. The graph of Mw distribution (Figure 3.4) shows that with the concentration of P-BG increased from 50ppm to 400ppm, the Mw peak at around 5.6 ($\log_{10}$Mw=5.6, ≈398KDa, in range 1) generally decreased with the increasing of peak at around 3.6 ($\log_{10}$Mw=3.6, ≈3.7KDa, in range 3). The overall Mw distribution of E-WEAXs in the graph (Figure 3.4) becomes gradually narrowed and more centralized in the low Mw range as the enzyme concentration increases. The major change is that the fractions of AXs with low Mw range (1KDa to 10KDa) increased with a stronger enzyme treatment. This observation is in agreement with the reported by Maes et al. (2004) who found that the average Mw of AXs was reduced with the concentration of *Bacillus subtilis* endoxylanase increased.

In addition, with the same concentration of enzyme treatments (200ppm), there are no obvious changes in the Mw distribution of AXs at different temperatures or during different extraction times (Table 3.9 and Figures 3.5 & 3.6). These results show the temperature (from 20°C to 40°C) and extraction time (from 2h to 4h) have no significant effects on AXs Mw distribution modified when using the 200ppm of P-BG treatment. Therefore, the concentration of enzyme is main factor for modifying AXs Mw distribution. Furthermore, these results are important for developing optimised procedures for extraction of AXs from wheat flour pentosan on an industrial scale. The relatively short extraction time (2h) and low temperature (20°C) may decrease the cost of modify AXs production.

Figure 3.5 The molecular weight distribution of 200ppm enzymatic extraction AXs at different extraction times

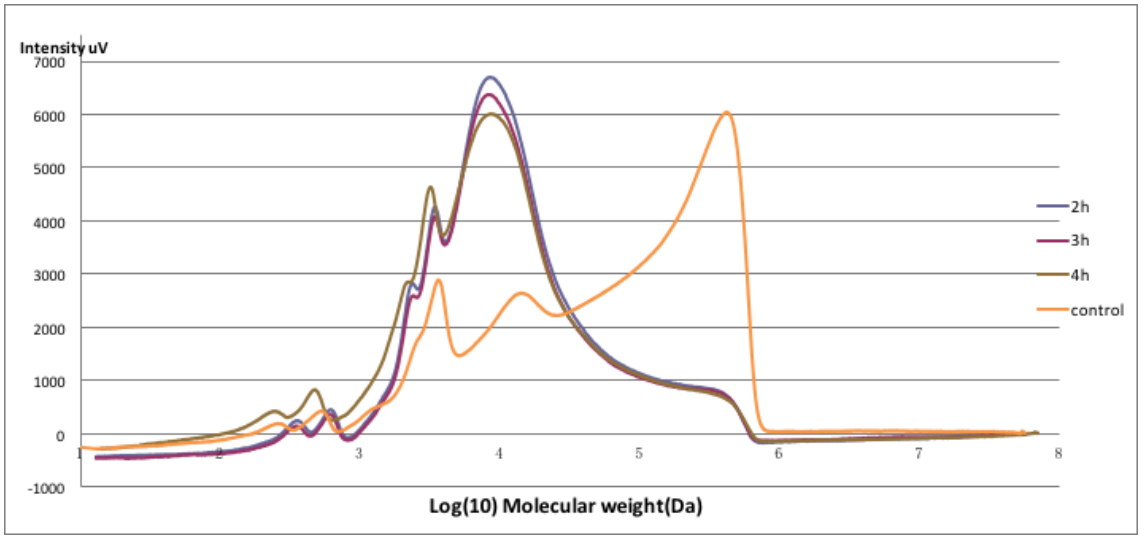
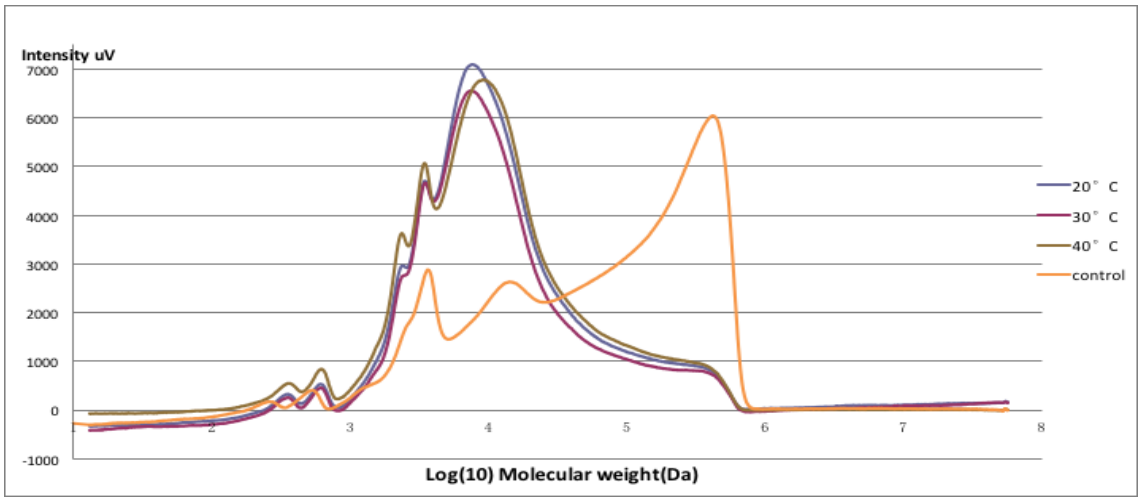


Figure 3.6 The molecular weight distribution of 200ppm enzymatic extraction AXs for different extraction temperatures



3.2 AXs of corn bran

Corn bran is a by-product from the dry and/or wet milling of corn starch and the bran mainly originates from the kernel cell wall fractions (M.P. Yadav et al. 2007). In the present study, the total AXs content was determined as 25.98±0.31% of corn bran (dry matter basis) using the method described by S. G. Douglas (1980) and W. Li et al. (2013). The effects of various extraction methods, including aqueous, enzyme and alkaline extraction, on the yield of AX from corn bran were studied in this section. In addition, the methods for modifying extracted corn AX using various endoxylanases were also developed. Furthermore, the molecular structure of alkaline extracted AX (AEAX) and enzymatic modified AEAXs (E-AEAXs) of corn bran were characterised and compared.

3.2.1 Development of processes for the extraction of AXs from corn bran

3.2.1.1 Comparison of AX extraction yields of corn bran and pentosan of wheat flour using the P-BG and water extraction methods

The extraction methods for the isolation of AXs from corn bran were the water extraction method (40°C, 2h) and the enzymatic extraction method (200pm P-BG, 40°C, 2h, pH4.5). The AXs extraction yields using the two methods were 0.71% and 0.87% respectively (Table 3.10). Table 3.10 also shows the AXs extraction yields of the pentosan fraction of wheat flour compared with the yields from corn bran using the same extraction methods.

Table 3.10 The AXs extract yields (dry matter basis) from the two different cereal sources using the same extraction methods

Sources / Methods	Pentosan fraction of wheat flour (30g)	Corn bran (30g)
Water extraction	7.54±0.47%	0.71±0.87%
Enzymatic extraction (P-BG, 200 ppm)	12.70±0.55%	0.87±0.95%

The extraction yields are presented as mean + SD and experiments were conducted in triplicate.

As Table 3.10 shows, the extraction yield of AX from the pentosan of wheat flour is much higher than that from corn bran in both water extraction and enzymatic treatments. The total AX content in the wheat flour pentosan is about 15.8%. The pentosan fraction AX's recovery rate using the water extraction method was 47.72% of the total AXs. Whereas, the total AX content in the corn bran is about 26.0%. Using

water extraction the recovery rate of corn bran AXs achieved was only 2.73% of the total AXs. This huge difference in water extracted AX yields between the pentosan fraction of wheat flour and corn bran may be due to the differences in the endosperm cell wall structure of wheat and corn bran. The low yield of water extracted AXs from corn bran shows that under relatively gentle conditions (40°C, 2h), water is not able to solubilise AXs by breaking the cross-links between AXs and the cell wall matrix. This result is consistent with previous studies, which found that the water extractable AXs content of corn bran was at 0.28% (Hashimoto et al. 1987a). In contrast, the AXs water extraction yield from the pentosan of wheat flour is much higher than using the same conditions, which suggest that AXs of wheat pentosan fraction are likely deficient in cross-links with other components in the cell wall and may be loosely bound at the cell wall surface in the pentosan of wheat flour.

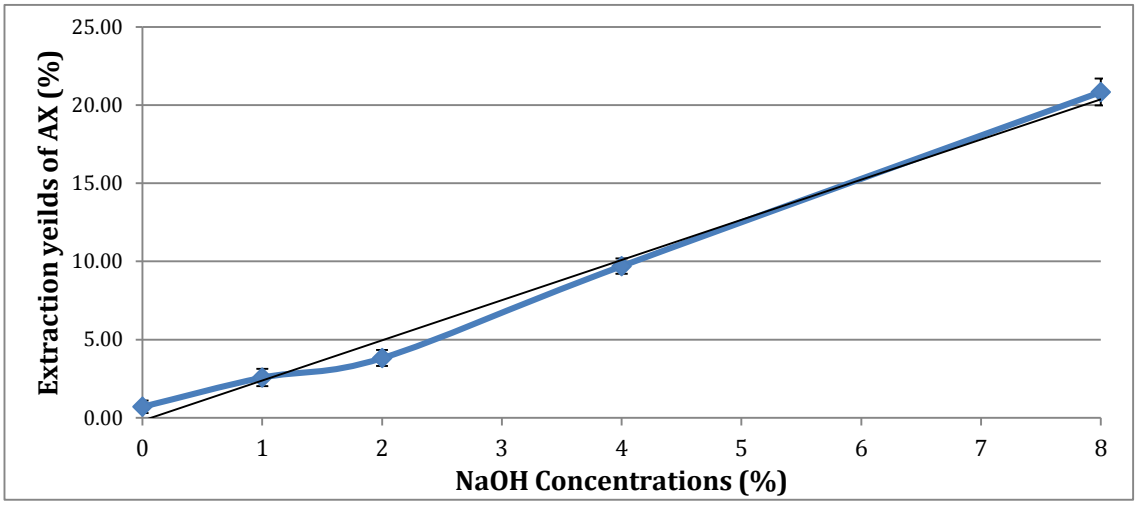
Enzymatic treatment (200ppm, 40°C, 2h) slightly increased the AXs extraction yield from 0.71% to 0.87% (3.35% of total AX content) ($P<0.05$). The enzymatic treatment is able to extract 12.70% from the pentosan fraction of wheat flour including that the AX's recovery rate reached about 80.38% of total AXs. On the one hand, the enzymatic treatment can increase the yield of AX from other cereal sources, such as wheat bran (Courtin and Delcour 2001). The result obtained in the current study is consistent with the previous report that the action of xylanases results in the (partial) solubilisation and extraction of WUAXs and the depolymerisation of WEAXs. Whilst on the other hand, it was found that the treatment of endoxylanase was not able to extract the AXs from corn bran. The reason may be that there is a larger proportion of WUAXs in corn

bran, which form part of the bran and are covalently and non-covalently linked to other AXs and to other cell wall components, such as cellulose, lignin or proteins (Fengler and Marquardt 1988). These chemical linkages cannot be disrupted by endoxylanase treatment under the current conditions. These comparisons show that the AX extractions vary with materials used and depending on the cereal species and fractions used as sources.

3.1.1.2 Effects of alkaline treatment on extraction yield of AXs from corn bran

The alkaline extraction investigated here has been developed from the previous studies of L.W. Doner and Hicks (1997a) and L.W. Doner et al. (1998). The intention being to investigate the effect of NaOH on corn bran AXs extraction yields. Figure 3.7 shows significant effects of the alkaline treatment on AXs extraction yield of corn bran (dry matter basis). Compared to water extraction or enzymatic treatment, alkaline extraction is highly efficient. The AXs extraction yield increased from 2.58% up to 20.84% with increasing NaOH concentration from 1% to 8%. The AX's recovery rate reached about 80% of the total AX using 8% NaOH. The major reasons for the increased AX yield achieved using alkaline treatment can be explained as alkali (OH^-) is more easy to disrupt the hydrogen bonds between AXs and other components compared with water treatment, and may also disrupt some covalent bonds, such as ester linkages, thus, loosening up the cell wall matrix, consequently solubilising part of the AXs materials, including AXs, from the cell wall of corn bran (Fincher and Stone 1986).

Figure 3.7 Effects of NaOH concentrations on AX extraction yields from corn bran (dry matter basis)



The extraction yields are presented as mean with SD bar in the figure. Experiments were conducted in triplicate. NaOH (0%) means water extraction without NaOH treatment.

3.2.1.5 Enzymatic modification of AEAXs of corn bran

Following extraction with 8% NaOH, the molecular modification of alkaline extracted AX (AEAX) was studied with three types of enzyme treatments, including two glycoside hydrolase (GH) 11 xylanases families (P-BG and E-XYLNP) from *Thermomyces lanuginosus* and *Neocallimastix patriciarum* respectively and one combined enzyme product (xylanase and cellulase) (O-VR) from *Penicillium funiculosum*. The enzyme modified AEAXs were named as E-AEAXs. The procedure and conditions for enzyme modification were described in Chapter 2 (section 2.2.2.3). According to the Mw modification results obtained for AXs of pentosan fraction, the concentration of the three enzymes used in the current studies was selected as 400ppm. High recovery yields (>87.95%) of AXs from AEAX sample was achieved from enzyme treatments and there is no significant difference in the recovery yield of E-AEAX between the 24h and

48h treatment times (p-values>0.05). Overall, about 12% of the materials of AEAX sample were not recovered. It mainly due to some AX materials being left in the rotary flask after the rotary vacuum evaporation.

3.2.2 Characterisation of the AEAX and the enzyme modified AEAXs (E-AEAXs) of corn bran

The monosaccharide compositions and Mw distribution of the AEAX (using 8% NaOH) and E-AEAXs were analysed by HPLC and HPLC-SEC and compared in order to understand how the enzyme modification treatments affect the molecular features of AEAXs.

3.2.2.1 Monosaccharide compositions of the AEAX and the E-AEAXs of corn bran

As shown in Table 3.11, the AEAX sample extracted from corn bran using 8% NaOH had an A/X ratio of 0.82, which is consistent with the results from a previous study (L.W. Doner et al. 1998), which reported that the A/X ratio for alkaline extracted AXs of corn bran was 0.82.

Table 3.11 The monosaccharide compositions of the AEAX and E-AEAXs

Samples [a]	Mono-sugar compositions				
	Ara(%)	Xyl(%)	Glu(%)	Gal(%)	A/X
AEAX (8% NaOH)	38.3±0.45	46.97±0.63	/	14.73±0.44	0.82
24 hours enzyme treatment (E-AEAXs 24h) [a]					
E-XYLNP	39.10±1.65	47.1±0.95	/	14.53±0.99	0.83
P-BG	40.04±1.00	45.57±0.92	/	14.50±0.31	0.88
O-VR	39.17±2.12	47.07±1.56	/	13.98±0.48	0.83
48 hours enzyme treatment (E-AEAXs 48h) [a]					

E-XYLNP	41.16±1.32	45.3±0.67	/	14.53±1.03	0.89
P-BG	39.88±0.84	45.21±0.77	/	14.91±0.12	0.88
O-VR	38.84±2.30	47.29±1.56	/	13.87±0.89	0.82

a: (E-XYLNP 48h/24h; P-BG 48h/24h; O-VR 48h/24h) means modified AEAXs using one of those three different enzymes for 48 hours or 24 hours. The proportion of each monosaccharide in AX samples is presented as mean + SD and all experiments were conducted in triplicate.

The monosaccharide compositions of E-AEAXs modified for 24h and 48h by various endoxylanases (E-XYLNP, P-BG and O-VR) are presented in Table 3.11. The difference in A/X ratios (0.82-0.89) between AEAXs and E-AEAXs indicate that there is a higher degree of branching on the β-1,4-D-xylopyranose backbone of the E-AEAXs than that of AEAXs. There appears to be no obvious difference in monosaccharide compositions between AEAX samples and AEAX modified by the three different endoxylanase preparations of the same enzymes. This means that the enzyme treatments have no significant effect on the degree of substitution of AEAX.

3.1.2.2 Mw characterisation of extracted and modified AXs of corn bran

The overall Mw distribution ranges of the AXs are from 0.1KDa to 794KDa. Each molecular weight distribution is divided into four molecular weight ranges (Table 3.12). As shown in Table 3.12, the largest proportion of AEAX (84%) is mainly in range 1 (100KDa to 794KDa). After 24 h treatment with the three enzyme preparations (P-BG 24h; E-XYLNP 24h and O-VR 24h), E-AEAXs portion (17-19%) in the small Mw range 2 (10KDa to 100KDa) slightly increased compared with that of AEAX portion (13.7%) in range 2. E-AEAXs portion (78.1-79.7%) in the large Mw range 1 (100KDa to 794KDa) generally decreased compared with AEAX portion (83.5%) in range 1. As shown in

Figure 3.8, the Mw distributions of E-AEAXs showed a small change in Mw range of $1\times10^{4.5}$ to $1\times10^{5.5}$Da. AEAX subjected to longer enzymatic treatments 48 hours had a changed Mw (Figure 3.9). This longer exposure to the enzymes reduced the proportion of large Mw AX in range 1 by approximately 20-32% and increased the proportion of small Mw AX in ranges 3 and 4 by 17-30% (Table 3.12). Specifically, after 48 hours the treated samples showed reduced values of 63.70% (P-BG), 54.81% (E-XYLNP) and 51.23% (O-VR) compared to the AEAX level of 83.53% in the high Mw range 1 (100KDa to 794KDa), and an increased proportion in the lower Mw ranges from 2.82% to 17.83-24.46% in range 3 and from 0 to 2.19-8.64% in range 4. For the three different enzymes, the sample of E-AEAX using O-VR treatment for 48 h (O-VR 48h) showed the largest increase in the proportion of AX with small Mw ranges 3 and 4 compared to that of AEAX (Table 3.12). Specially, 48h O-VR treatment changed the proportion of large Mw of AEAXs in range 1 (100KDa to 794KDa) from 83.53% to 51.23%, whereas the proportion of low Mw material in ranges 3 and 4 (0.1KDa to 10KDa) increased from 2.82% to 33.1%.

Table 3.12 The proportions of AEAX and E-AEAXS in different ranges of molecular weight

Samples	Mw ranges of AXs [d]			
	Range 1: 1×10^{5}-$10^{5.9}$(Da)	Range 2: 1×10^{4}-10^{5}(Da)	Range 3: 1×10^{3}-10^{4}(Da)	Range 4: 1×10^{2}-10^{3}(Da)
AEAX [a]	83.53%	13.65%	2.82%	/
24 hours [b] (E-AEAXs 24h)				
P-BG	78.08%	19.19%	2.73%	/
E-XYLNP	79.67%	17.53%	2.80%	/
O-VR	79.73%	17.32%	2.95%	/
48 hours [c] (E-AEAXs 48h)				
P-BG	63.70%	16.29%	17.83%	2.19%
E-XYLNP	54.81%	14.39%	23.44%	7.36%
O-VR	51.23%	15.66%	24.46%	8.64%

a: The AEAX samples indicates extracted AXs using 8% NaOH treatment; b: Those three samples means enzyme modified AEAXs (E-AEAXs) using the different types of endoxylanases (P-BG, E-XYLNP and O-VR) for 24 hours; c: Those three samples means enzyme modified AXs (E-AEAXs) using the different types of endoxylanases for 48 hours; d: The percentage of AXs in different Mw range were analysed using the LC Data Analysis (SHIMADZU Corporation) and Microsoft Excel.

Figure 3.8 The molecular weight distributions of E-AEAXs treated using three different types of enzyme for 24 hours treatment

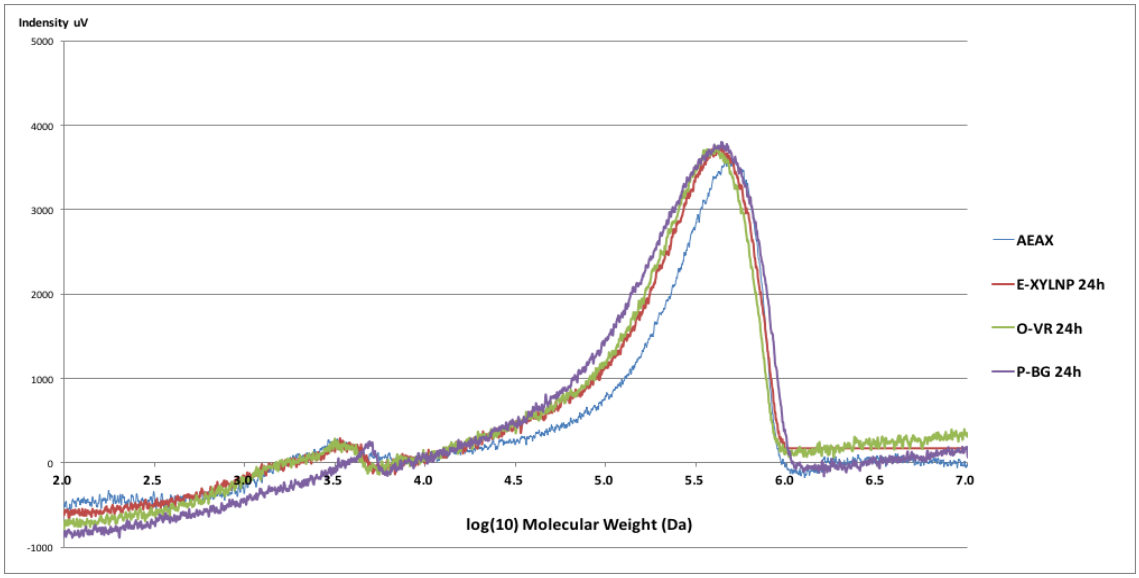
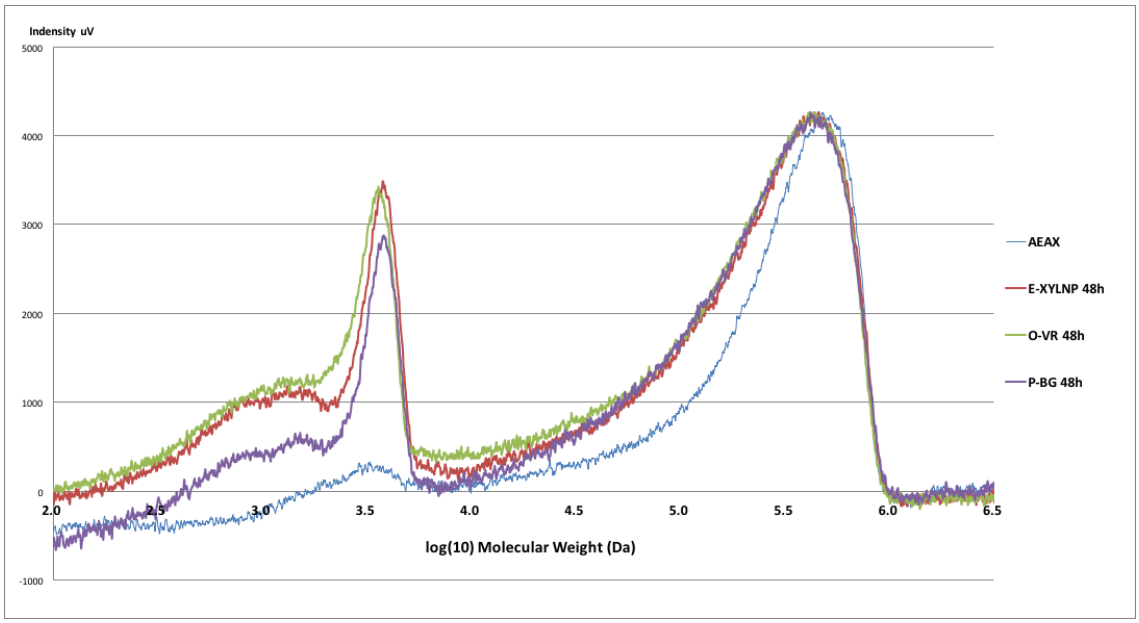


Figure 3.9 The molecular weight distributions of E-AEAXs treated with 48 h treatment of three types of endoxylanase



The effect of enzyme treatment on Mw of AEAX showed an obvious change from 24h to 48h. From Table 3.12, it can be found that as the time of enzyme treatment increased from 24h to 48h, the Mw distribution of E-AEAXs shows a significant change in range 1, 3 and 4. For example, E-AEAX (O-VR 48h) showed a higher proportion (33.1%) in the small Mw ranges 3 and 4 (0.1KDa-10KDa) and with a lower proportion (51.23%) in the large Mw range 1 (100KDa to 794KDa) compared to that of E-AEAX (O-VR 24h) with 2.95% in the small Mw range 1 and 79.73% in the large Mw range 1. These results show that the three types of endoxylanases (P-BG, E-XYLNP and O-VR) with 48h treatment reduced the proportion of high Mw material (range 1) and increased the proportion of low Mw material (ranges 3 and 4) of AEAXs. Therefore, the treatment time is a significant factor in the Mw modification of AEAX using the endoxylanase. AXs have a tendency to form macrostructures in aqueous solutions

through chain aggregation and physical entanglements (Warrand et al. 2005). The molecular size of AX is a key factor for its behaviour in solution. The high Mw AXs may more easily form aggregations and exhibit weakly elastic properties in solution than the low molecular weight fractions (Izydorczyk and Biliaderis 1992a). From Table 3.12, the Mw of AEAX fraction consists largely (>80%) of high molecular weight material (100KDa to 794KDa). Thus, some of AEAXs may form macrostructures that inhibit the AX behaviour in the aqueous solution. Family G/11 endoxylanases have a β-jelly roll structure and are considered to be able to pass through the pores of the xylan network owing to their smaller molecular sizes (Juturu and Wu 2012). When the network of AEAX becomes more tightknit via physical entanglements, this would form a barrier to the endoxylanase，preventing hydrolysis of the xylan chain of the AXs. In this case, a longer treatment time (48h) is necessary to degrade the molecules of entangled AXs. Thus, improving the solubility and reducing the impact of AEAX aggregation should be considered when developing the modification process of AEAXs in future studies. In addition, Shiiba et al. (1993) investigated the effect of endoxylanase on AXs Mw and showed that the reduction in Mw of AXs might depend on the proportion of L-arabinofuranosyl branches. This study compared the hydrolysis effect of endoxylanase on two different AXs from wheat bran with different degrees of branching, and found that the highly branched AX (A/X=1.07, 79% substituted xylose residues) was much more difficult to modify the Mw than the AX with a relative low degree of branching (A/X=0.57, 62% substituted xylose residues). According to Kusakabe et al. (1983), the xylanase is specific for the hydrolysis of xylose chains that

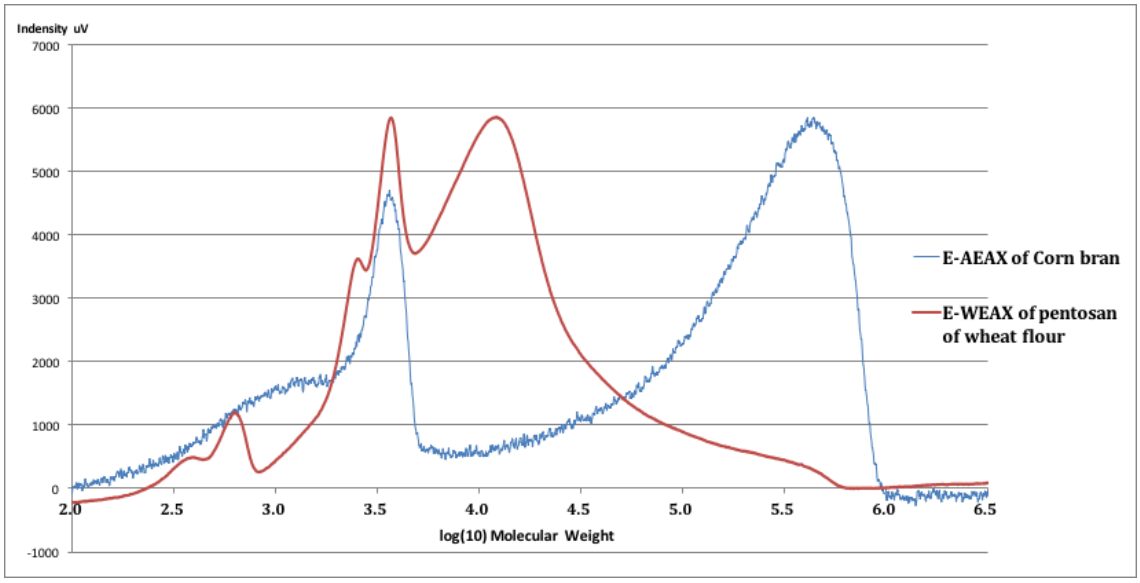
are devoid of branches of L-arabinofuranose residues or 2-O-β-D-xylopyranosyl-L-arabinose. Similarly, Biely et al. (1997) reported that AXs with a low degree of branching are more susceptible to endoxylanase action. In the present study, the A/X ratio of AEAX was 0.82, which is a high degree of branching may explain why the Mw modification process using enzyme treatments was difficult and required a long treatment time.

In addition, it is important to note that due to the differences in substrate specificities and optimal conditions, different xylanases may have different effects in reducing Mw (Garg et al. 2010). Comparison of the abilities of the three types of endoxylanases respectively isolated from *Thermomyces lanuginosus, Neocallimastix patriciarum* and *Penicillium funiculosum* in modifying the Mw of AEAX from corn bran, it was shown that the most effective one is O-VR from *Penicillium funiculosum*, which may be due to the synergistic action of the two enzymes of xylanase/cellulase which resulted in a more efficient hydrolysis of AEAXs.

Figure 3.10 shows that the Mw distribution of enzyme modified AXs samples from the pentosan fraction of wheat flour and corn bran. It can be seen that the two samples are significantly different. The Mw distribution curve of the E-WEAX contains two main peaks in the small Mw range (12.218KDa； $\log_{10}$Mw≈4.1; DP≈93) and middle Mw range (3.715KDa； $\log_{10}$Mw≈3.6; DP≈28). However, the E-AEAX sample mainly comprises a peak in small Mw range (around 3.715KDa) and the other peak in large Mw range (501.187KDa; $\log_{10}$Mw≈5.7; DP≈3797). There was no AX within middle Mw range peak in E-AEAX material (Figure 3.10). As indicated in the discussion above, the

structural heterogeneity of the two sources (pentosan of wheat flour and corn bran) and in variation of extraction processes (enzymatic treatment and alkaline treatment) may lead to the variation in the Mw distributions between E-WEAX and E-AEAX. However, it is interesting to note that the proportion of material in the small Mw range (around 3.715KDa) showed a similarity in E-WEAX and E-AEAX, and that both are about 30% of the total AX material.

Figure 3.10 The comparison of molecular weight distributions between E-AEAXs and E-WEAX



E-AEAX of Corn bran: The samples of E-AEAX was from corn AEAX using 400ppm O-VR treatment for 48 hours; E-WEAX of pentosan of wheat flour: The sample of E-WEAX was from pentosan of wheat flour using 400ppm P-BG treatment for 2 hours.

3.3 Conclusions

The AXs extraction and structure modification methods have been investigated in this chapter. An enzymatic extraction and modification process of AXs from wheat pentosan have been developed. The effects of three individual extraction factors (concentration of endoxylanase (P-BG), extraction temperature and extraction time) on AXs yield have been determined and analysed. Subsequently, the extraction processes with these three extraction factors were optimized for increased AX extraction yield using a Box-Behnken experimental design combined with analysis of quadratic regression and response surface methodology. The optimized conditions for maximum extraction yield were 367.68ppm P-BG, 2.31h extraction time and 40°C extraction temperature. The optimum extraction yield of AX (dry matter basis) from the pentosan of wheat flour was 13.54% and it accounts for 86% of total AXs, which the experimental result was in good agreement with the predicted extraction yield (13.51%) obtained using the regression equation (R^2=93.24%, D-value=1).

In addition, according to molecular structure analysis, it was found that the enzymatic treatments had significant effects on the Mw distribution and the degree of substitution of AXs of the pentosan fraction. In contrast with WEAX, the Mw results of E-WEAXs showed that the enzyme treatments significantly reduced the proportion of large Mw material in the range 100-794KDa of AXs by increasing the proportion of small Mw material in the range 1KDa to 10KDa, and the ratio of arabinose to xylose (A/X) went up from 0.48 to 0.83 with the enzyme treatments.

For the AXs of corn bran, this chapter studied the extraction process for AXs from corn bran using various methods (water, enzyme (P-BG) and alkaline extractions), and investigated modification process for extracted AX using various enzyme treatments that the enzymes produced from *Thermomyces lanuginosus, Neocallimastix patriciarum* and *Penicillium funiculosum*. In contrast to water and P-BG extraction methods, alkaline treatment showed significant effects on the yield of AXs from corn bran. Under alkaline conditions, the maximum yield of AEAXs from corn bran was approximately 21% (dry matter basis). The recovery yield of AEAX of total corn AX was up to 80% (dry basis). In addition, molecular weight analysis found that the enzyme treatments successfully modified the Mw distribution of AEAX. The largest proportion of AEAX (84%) was mainly in the high Mw range (100KDa to 794KDa) and the A/X ratio was 0.82. Following enzyme modification, more than 30% of AEAX was reduced to the low Mw range (0.1KDa to 10KDa), but there was only a very slight change in A/X ratio. It was also noticed that xylanase/cellulase (O-VR) was the most effective of the three enzymes in reducing the Mw of AEAX.

The molecular structures of the extracted AXs and modified AXs from the two by-products of cereal processing have been characterised. The extracted AXs and modified AXs of known structure presented here will be used for the study of the influence of AXs' molecular features on their immune-modulating property.

In addition, the method for the Mw modification of corn AX has been developed and studied in this chapter. However, the method for improving the efficiency of the enzyme treatment on the Mw modification of AEAX needs further investigation.

CHAPTER 4.

Stimulation of Nitric Oxide Production *in vitro* by Arabinoxylan Treatments with Various Molecular Structures

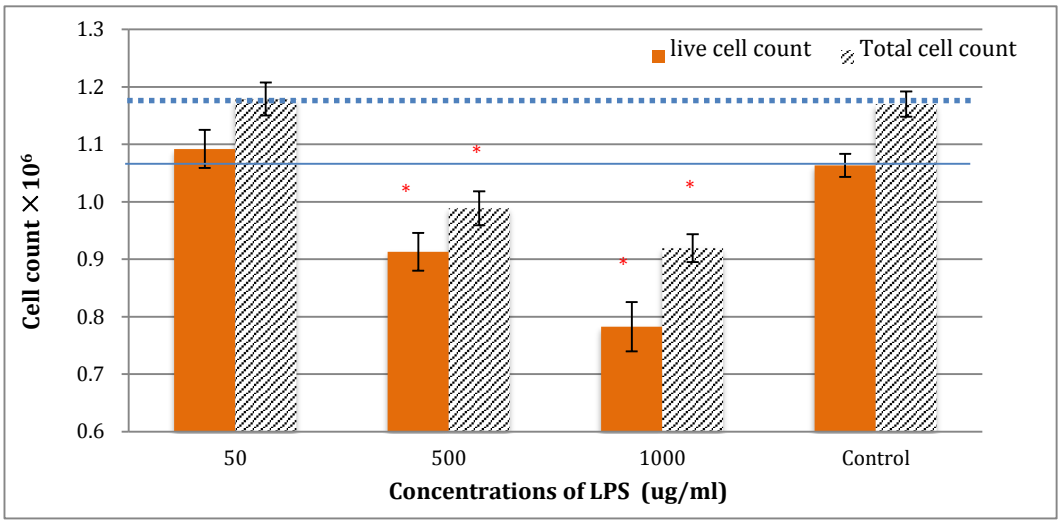
4.1 Effects of AXs and LPS on growth and viability of U937 cells

The Trypan blue exclusion assay with cell count was used to assess cell growth and viability of the human macrophage U937 in presence of the AXs and LPS at various concentrations are shown in Figure 4.1: a-e. Figures 4.1 b-e demonstrate that the viable and total cell counts for AXs (50, 500, 1000µg/ml) treated U937 macrophages are not significantly different compared to control (untreated cells) (p-values>0.05). Hence, WEAX, E-WEAX, AEAX and E-AEAX have no stimulatory effect on the growth of U937 cells over the period of 24h. In addition, the viability of cells with AX treatments was typically over 90%, suggesting that WEAX, E-WEAX, AEAX and E-AEAX also have no effect on U937 cell survival over the period of 24hrs. Polysaccharides extracted from plants have received considerable attention in bioscience due to their wide immune-modulatory activities and low toxicity (Schepetkin and Quinn 2006). The present study indicated that the AX samples have no inhibitory effects on the viability and cell growth of the human U937 macrophage, even at a high concentration of 1000µg/ml.

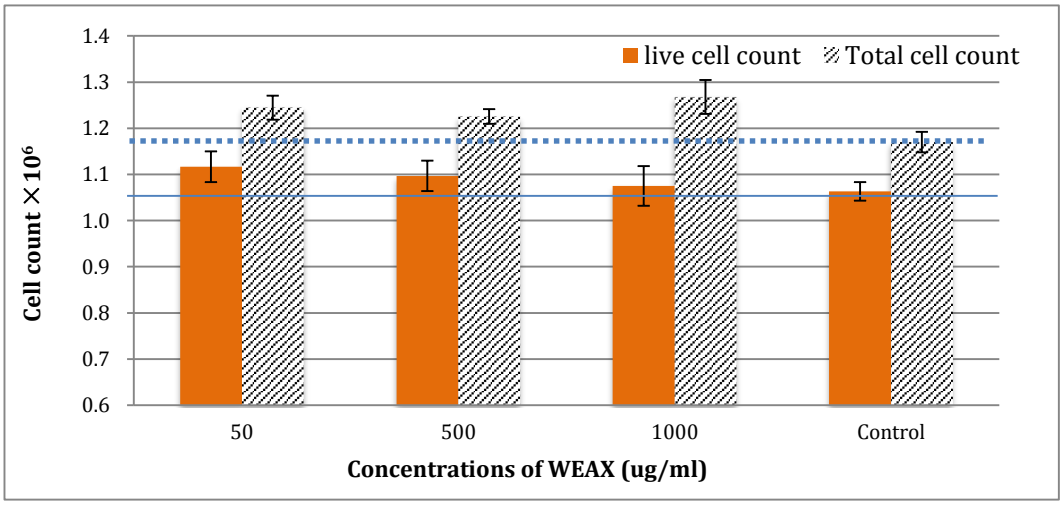
However, as shown in Figure 4.1 (a), the cell counts of viable and total U937 cells were reduced significantly after treatment with 500 and 1000µg/ml of LPS compared with that of untreated control (p-values<0.01). In contrast, this inhibitory effect on cell growth did not appear at relatively low concentrations (e.g. 50µg/ml) of LPS treatments. This suggests that high concentrations of LPS may have an inhibitory effect on U937 cell growth.

Figure 4.1. Effects of AXs and LPS on the viability of U937 cells

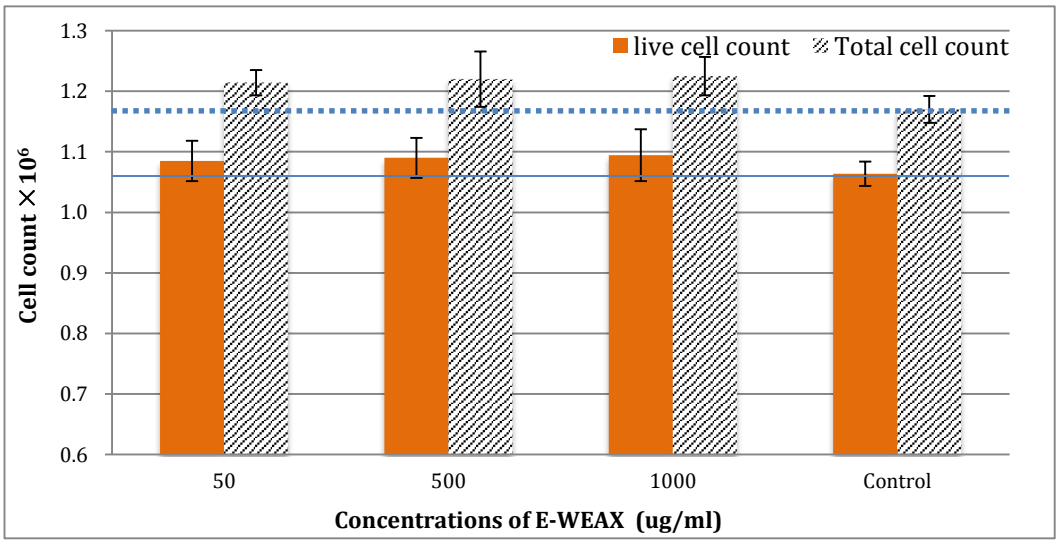
a.



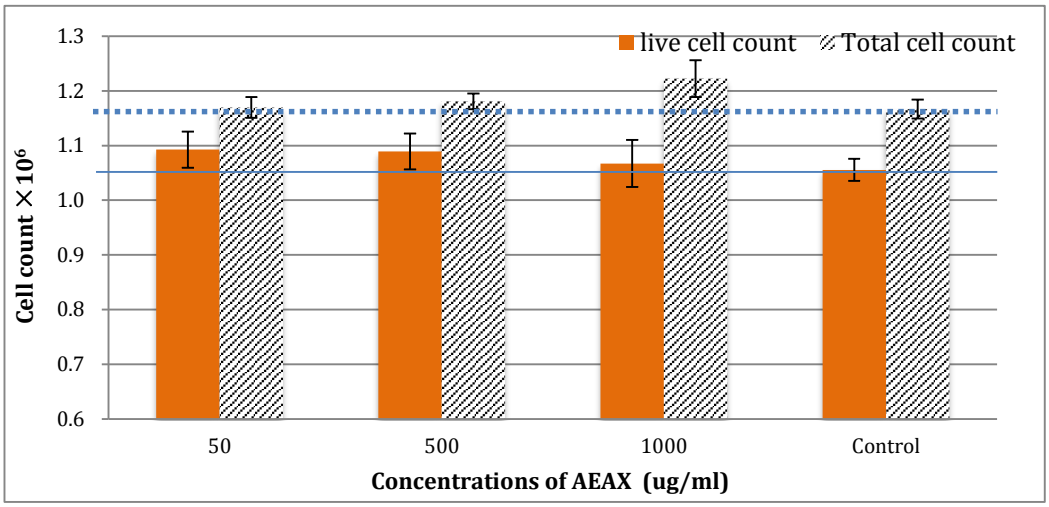
b.



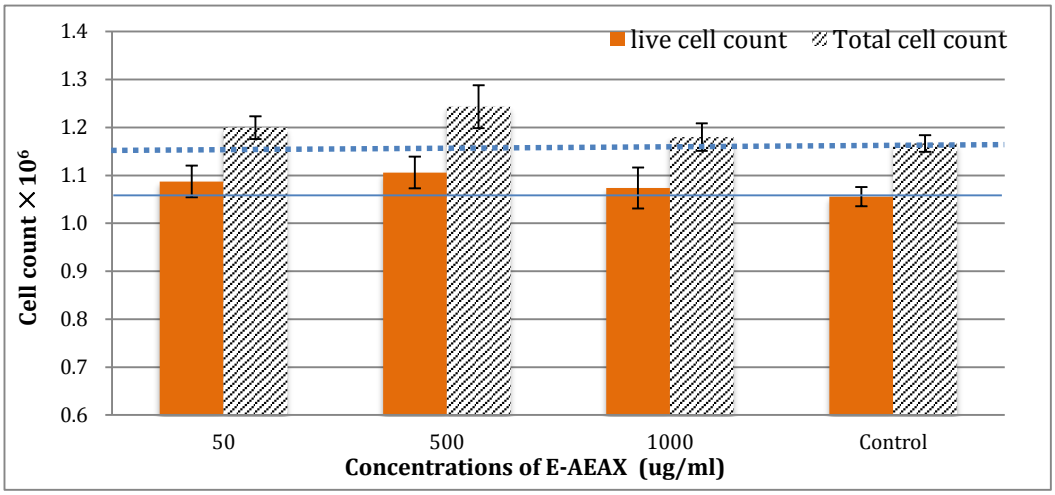
c.



d.



e.



*a-e: the U937 cells were treated with the LPS, WEAX, E-WEAX, AEAX and E-AEAX for 24hrs; The count of viable (live) and total (viable and unviable) cells was confirmed using the trypan blue exclusion method; The solid line '—' indicates the viable cell count of the control and the dotted line '---' indicates the total cell count of the control; The viability: viable cell count/total cell count; the average viable cell count after treatment was compared with the cell count of the control (without AXs or LPS treatment) using the Student's t-test; The symbol *: p-value < 0.01; The cell counts are presented as mean + SEM of six copies samples from experiment.*

According to previous studies, research has indicated that LPS inhibits and blocks macrophage proliferation depending on the incubation time and dosage (Muller-Decker et al. 2005; Vadiveloo et al. 1996; Vairo et al. 1992). The inhibitory effect of LPS on cell proliferation is tightly regulated through a complex network of cytokines. For example, Vadiveloo et al. (2001) found that bacterial LPS had an

inhibitory effect on cell proliferation in mouse marrow-derived macrophages. They found LPS inhibited the expression of cyclin D1, which is an essential protein for proliferation in many cell types. In addition, the viability of cells with LPS treatments was typically over 90%, suggesting that LPS (50-1000μg/ml) has no effect on U937 cell survival over 24hrs.

4.2 Effect of AXs with different Mw from wheat flour pentosan on nitric oxide (NO) production by human macrophage cell line U937

In order to investigate the possible relationship between immune-modulatory activity and molecular structure, WEAX and E-WEAX with different Mw distributions were tested for their ability to induce NO secretion in U937 cells. LPS was used as a positive control as it has been shown to stimulate NO production by various immune cell lines (Bogdan 2000; Cholujova et al. 2009; W. Li et al. 2015).

Table 4.1 shows the NO production by U937 cells after treatment with WEAX, E-WEAX and LPS at various concentrations. Firstly, over the concentration range from 1 to 500μg/ml, the two AXs samples (WEAX and E-WEAX) treatments both significantly elevated NO production by U937 cells after a 24h incubation period compared with the untreated control ($p<0.05$). This result is consistent with previous studies, which found that AXs from various cereal sources were able to stimulate the production of NO in murine and human macrophage cells (Ghoneum and Matsuura 2004; Nagata et al. 2001).

Table 4.1. NO production by U937 cell after 24h treatment of WEAX, E-WEAX and LPS

Samples	Concentration of AXs and LPS (μg/ml)					Untreated Control
	1	5	10	50	500	
LPS	72.87±2.40 *	73.00±1.44 *	73.07±5.50 *	71.79±5.43 *	59.05±2.82 * #	45.72 ±0.47
E-WEAX	48.71±3.73 * $	56.00±0.39 * $	64.54±2.69 * # @	67.77±2.94 * @	56.65±1.62 * #	
WEAX	54.34±1.04 * $	54.00±2.72 * $	53.71±3.62 * $	59.83±2.98 * $	61.84±2.97 *	

*The NO_2^- concentration (mean + SEM) is an indication of NO production in U937 cells. The symbol * indicates NO secretion significantly increased (p<0.05) compared to the untreated control; The symbol # indicates NO secretion significantly changed (p<0.05) as the sample dosage progressively increased; The symbol @ indicates NO secretion with E-WEAX treatment was significantly different than with WEAX at that specific concentration (p-<0.05); The symbol $ indicates NO secretion with E-WEAX or WEAX treatment was significantly different than with LPS treatment at that specific concentration (p<0.05). The p-values were calculated by one-way ANOVA using SPSS.19 and experiments were conducted in triplicate.*

Secondly, the LPS was used as a positive control and significantly stimulated NO secretion at concentrations of 1 to 50μg/ml (Table 4.1) compared to the untreated control. However, at 500μg/ml of LPS, the amount of NO produced by the U937 cells significantly decreased ($p<0.05$) compared to lower concentrations of LPS, mirroring the substantial inhibitory effect on cell growth and viability at this concentration (Figure 4.1a). Compared with AXs, the NO produced following LPS treatment is consistently higher than that produced by WEAX at concentrations of 1 to 50μg/ml ($p<0.05$). Interestingly, there was no significant difference between the amount of NO produced with E-WEAX and LPS treatments at concentrations at and above 10 μg/ml ($p>0.05$), suggesting E-WEAX was equally effective at NO stimulation as LPS at these

concentrations. Furthermore, Table 4.1 shows that the amount of NO production significantly increased at 10μg/ml of E-WEAX treatment compared to the lower concentrations ($p<0.05$). The highest (peak) amount of NO released by the E-WEAX treatment was 67.77μM at 50μg/ml. Then, in a similar manner to LPS, there was a significant decrease in NO secretion following E-WEAX treatment at 500μg/ml compared to that at 50μg/ml ($p<0.05$). However, unlike E-WEAX treatment, there was a general NO secretion increase by WEAX treatment till the concentration was up to 500μg/ml compared lower concentrations 1-10μg/ml treatments ($p<0.1$), suggesting the peak amount of NO released by the WEAX treatment may has not reached yet. Thus, further experiments need to confirm its effective dosage range for NO stimulation. Nonetheless, these results indicate that there is a possibly optimal dose of E-WEAX for NO production in the range 10-50μg/ml whilst WEAX has a different optimum dose above 500μg/ml. In addition, at concentration range from 10 to 50μg/ml, NO response of WEAX is much more modest than that produced by similar concentrations of E-WEAX ($p<0.05$). These comparisons shows there are obvious differences between E-WEAX and WEAX treatment in NO stimulation and WEAX had generally weaker NO stimulation activity than E-WEAX or LPS in the assay.

As shown in Table 4.2, the one of main structural difference between these two AX samples was the Mw distribution. E-WEAX consisted of 89.4% within the molecular weight range 1×10^3-10^5Da. In contrast, the WEAX sample comprised fractions with higher molecular weights, 78.52% in the 1×10^4-$10^{5.9}$Da Mw range. The main difference between these two AX samples was in the low Mw range 3 (1×10^3-10^4Da), which the

E-WEAX containing 49.51% AX portion in this small Mw range compared WEAX containing 19.11% AX portion with this small Mw range (Table 4.2). Thus, the large difference in NO stimulation activity between those two AX samples may be associated with the small Mw proportion of E-WEAX, which is significantly higher than that of WEAX. Li et al. (2015) compared the effect of AXs with different Mw on NO secretion by U937 cells and indicated that the AX with relatively high portion of small Mw (85.7% of AXs Mw≤25KDa) and higher degree of branching of 0.81 had greater stimulatory activity compared with the AX that contained 47% AXs with similar Mw and branching of 0.62. This is consistent with the findings in the present study. In addition, E-WEAX presented a higher A/X ratio (0.83) compared to WEAX (0.48). Thus, the degree of branching of AX is another possible factor affecting the stimulation of NO production by U937 cells in this study. This is discussed further in section 4.3.

Table 4.2 The treatment methods and Mw characteristics of WEAX and E-WEAX in the NO production assay

AX Samples	Extraction methods:	Mw distribution of AXs (%) [a]				Monosaccharide compositions [b]				A/X [c]
	AXs from pentosan of wheat flour	Range 1: 1×10^{5}-$10^{5.9}$Da	Range 2: 1×10^{4}-10^{5}Da	Range 3: 1×10^{3}-10^{4}Da	Range 4: $1\times10^{2.2}$-10^{3}Da	Ara (%)	Xyl (%)	Glu (%)	Gal (%)	
WEAX	Water extraction	46.46%	32.06%	19.11%	2.37%	26.13	53.96	10.62	9.29	0.48
E-WEAX	Enzymatic treatment, (400ppm P-BG)	5.75%	39.89%	49.51%	4.85%	28.74	34.51	21.85	14.9	0.83

a: the data is from Table 3.9. b & c: the data is from Table 3.7.

4.3 Effect of AXs with different Mw from corn bran on the nitric oxide production by human macrophage cell line U937

Corn AEAX and E-AEAX with different Mw distributions were also tested and compared in terms of their ability to induce NO secretion in U937 cells.

Table 4.3 clearly shows that the AX samples (AEAX and E-AEAX) and LPS significantly increased NO production compared to the untreated control at all the concentrations tested ($p<0.05$). These results are consistent with the findings of the NO stimulation experiment reported in section 4.2. The AXs of wheat flour pentosan and corn bran, all significantly ($p<0.05$) stimulated NO secretion in U937 cells. Furthermore, Table 4.3 indicates there is an obvious dose-response effect for each of the three test samples but the effective range over which they work is different. The estimated optimum doses for LPS and E-WEAX treatments are in the same range 5-50μg/ml whilst the optimum for AEAX treatment should be above 1000μg/ml. In addition, AEAX had generally lower NO production response compared to E-AEAX and LPS at concentration below 50μg/ml. These differences between E-AEAX and AEAX treatment in NO response assay are supported by last section as well.

Table 4.3 NO production by U937 cell after 24h treatment of AEAX, E-AEAX and LPS

Samples	Concentrations of AXs and LPS (μg/ml)				Untreated Control
	5	50	500	1000	
LPS	70.35±1.32 *	71.79±2.34 *	64.60±1.09 * #	56.51±0.01 * #	46.05 ±1.95
E-AEAX	67.30±1.72 * @	67.66±2.381 * @	64.78±2.486 *	63.34±1.26 * # $	
AEAX	57.59±1.49 * $	61.01±2.27 * $	65.50±1.949 * #	66.94±1.86 * # $	

*The NO_2^- concentration (mean + SEM) is an indication of NO production in U937 cells; The symbol * indicates NO production significantly increased ($p<0.05$) compared to the untreated control; The symbol # indicates NO production significantly changed ($p<0.05$) as the sample dosage progressively increased; The symbol @ indicates NO production with E-AEAX treatment was significantly different to that with AEAX at that specific concentration ($p-<0.05$); The symbol $ indicates NO production with AEAX or E-AEAX treatment was significantly different to the treatment with LPS at that specific concentration ($p-<0.05$). The p-values were calculated by one-way ANOVA using SPSS.19 and experiments were conducted in triplicate.*

According to the Mw distributions of E-AEAX and AEAX in Table 4.4, 33.1% of E-AEAX had Mw ranging from 158Da to 10KDa (the ranges 3 to 4) whereas AEAX only contained 2.82% AX in this range. Furthermore, as described in section 3.1.2.2 and shown in Figure 3.10 (Chapter 3), it was noted that both E-WEAX and E-AEAX contain a similar proportion of AXs with low Mw in the range 1×10^3-10^4Da. Therefore AX in the low Mw range 1×10^3-10^4Da may include the major active component for increasing the stimulating activity of enzymatic modified AXs compared to non-modified AXs. In addition, the branch degree (A/X) of E-AEAX and AEAX were the same value of 0.82, which is different from the section of this study where there was a notable difference in branching degree between WEAX and E-WEAX. The monosaccharide composition of

E-AEAX and AEAX shows no glucose component and a similar proportion of galactose content in these two AX samples. The AXs degree of branching and side chains of glucose and galactose content may not have a significant effect on triggering AX-induced NO stimulation.

Therefore, when activating NO synthesis by U937 cells, the Mw distribution of AX is considered a possible determinant for their immune-modulatory activity such that AX with Mw at the range of 1KDa to 10KDa appearing to be the most biologically active for wheat flour pentosan fraction and corn bran materials.

Table 4.4. The treatment methods and Mw characteristics of AEAX and E-AEAX in the NO production assay

AX Samples	Extraction methods: AXs from corn bran	Mw distribution of AXs (%) [a]				Monosaccharide compositions [b]				A/X [c]
		Range 1: 1×10^{5}-$10^{5.9}$Da	Range 2: 1×10^{4}-10^{5}Da	Range 3: 1×10^{3}-10^{4}Da	Range 4: $1\times10^{2.2}$-10^{3}Da	Ara (%)	Xyl (%)	Glu (%)	Gal (%)	
AEAX	Alkaline extraction	83.53%	13.65%	2.82%	/	38.3	46.97	/	14.73	0.82
E-AEAX	Enzymatic treatment, (400ppm O-VR, 48h)	51.23%	15.66%	24.46%	8.64%	38.84	47.29	/	13.87	0.82

a: the data is from Table 3.13. b & c: the data is from Table 3.12

4.4 Consequences of AX-induced NO stimulation for human health

NO production by immune cells can be thought of as a signal of immunomodulation, and has been used for assessing the immunomodulatory activity of examined samples in previous studies (Fang et al. 2012; Ghoneum and Matsuura 2004; W. Li et al. 2015). The inhibition of tumour cell growth and/or induction of tumour cell death by activated macrophages was one of major functions of NO in the immune system (Nathan 1992). A number of studies have reported the mechanisms by which macrophage-derived NO can trigger cytostasis or kill tumour cells *in vitro,* such as inhibition of enzymes essential for tumour growth (e.g. enzymes of the respiratory chain, cis-aconitase, ribonucleotide reductase arginase, ornithine decarboxylase); cell-cycle arrest via down regulation of cyclin D1; and induction of tumour cells sensitization for TNF-induced cytotoxicity (Bauer et al. 2001; Nathan 1992; Xie et al. 1996). In addition, NO can form a number of oxidation products such as NO_2, NO_2^- and N_2O_3 because of its highly reactive free radical structure (Cai et al. 2015), which comes into play at macrophage-mediated immune defence against numerous pathogens in infection (Ekman et al. 1999; Rodrigues et al. 2005; Yan et al. 1997). In cases of *Borrelia* and *Leishmania* infection by tick or sandfly, NO has been shown to enhance the killing of the pathogens by host phagocytes. However, tick or sandfly saliva inhibits the NO production, which might increase the initial survival of the transmitted pathogens (Hall and Titus 1995; Kuthejlova et al. 2001). Therefore, stimulation of NO production in the infected host organisms would become essential as protection against the pathogens.

In the present investigation, NO secretion by U937 cells after stimulation with the AXs samples may reflect on their potential to modulate NO-based strategies for tumour treatment and pathogen-mediated immune responses for infection treatment. In addition, NO production by macrophage also was reported that has inmmunoregulatory functions including modulation T cell response, suppression of anti-inflammatory effects and regulation of leukocyte recruitment (Bogdan 2000). Thus, the NO modulation activity of AXs may be able to apply to regulate the immune system response, thereby maintaining human health. Furthermore, the investigation of structure-activity relationship of AX could be useful in improving the immunomodulatory activity of AXs.

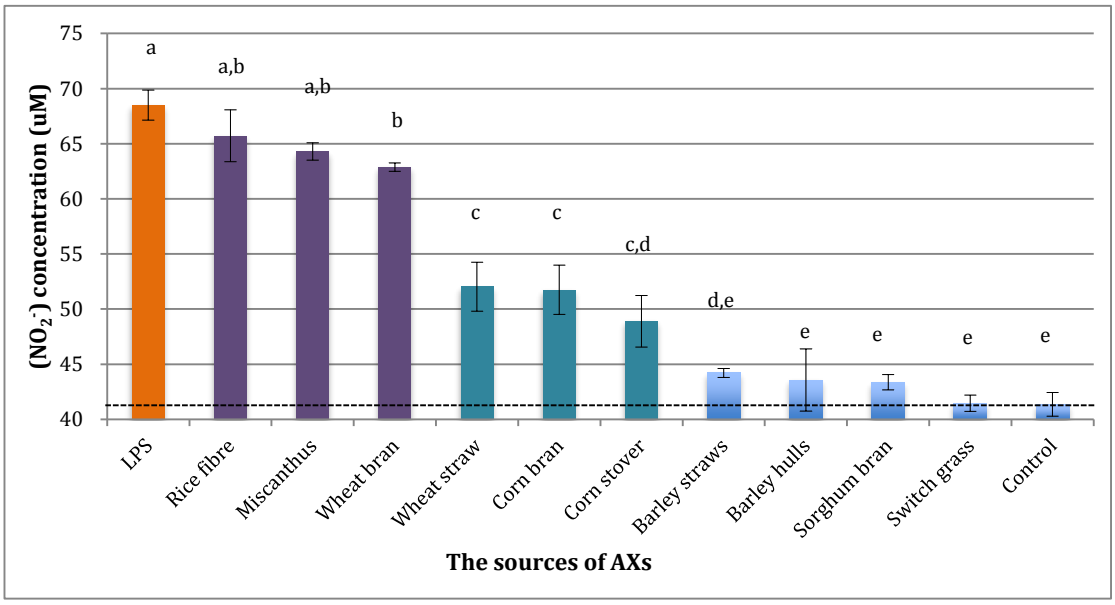
4.5 Effect of various AXs from different plant sources on nitric oxide production by human macrophage cell line U937

In this study, AXs samples extracted from 10 different plant sources have been tested for their stimulatory effect on NO secretion by U937 cells. The average Mw and branch degree (A/X) of these AX samples are shown in Table 4.5. Based on the previous results of two NO experiments (sections 4.2 and 4.3), at the concentration of 50µg/ml there was a significant difference in NO production between the E-WEAX and WEAX treatments and also between the E-AEAX and AEAX treatments. Therefore, in this part of study, a concentration of 50µg/ml was used to compare the stimulation activity of AXs from various plant sources.

Figure 4.2 shows the amount of NO secreted by U937 cells following treatment

with various AXs and LPS at 50μg/ml after a 24h incubation period. LPS was used as a positive control and it still produced the highest stimulation of NO secretion by U937 cells. AXs isolated from rice fibre, miscanthus, wheat bran, wheat straw, corn bran and corn stover significantly elevated the level of NO production by U937 cells compared with the untreated control (p-values≤0.05). As shown in Table 4.5, the average Mw of those 10 AXs ranged from 32.6KDa to 591KDa and the branch degree changed from 0.15 to 0.69. According to Figure 4.2, there are 10 AXs samples, which can be classified into three different groups (with different colours) from high to low in terms of their NO stimulating activity. The first, high activity, group includes AXs from rice fibre, miscanthus and wheat bran; the second, intermediate activity, group includes AXs from wheat straw, corn bran and corn stover; the third, AX samples from barley straw, barley hulls, sorghum bran and switch grass showed no significant effect on NO stimulation compared with the untreated control (p>0.05). It is observed that the NO stimulating activity of AXs may associate with their material sources.

Figure 4.2. NO production by U937 cells treated with AXs from various plant sources



AX samples (50µg/ml) were extracted from 10 different plant sources and the NO_2^- concentration (mean + SEM) was an indication of NO production in U937 cells. The mean values with different letters (a-f) indicate significant differences ($p<0.05$) in NO concentration for each comparison made amongst all the AX treatments. The p-values were calculated by one-way ANOVA using SPSS.19 and experiments were conducted in triplicate. The dotted line '---' indicates the NO production in control cells (treated with culture medium only).

As Table 4.5 shows, in the first group, the AX samples of rice fibre and miscanthus with relative low average Mw (32.6KDa and 69.1KDa) produced the highest stimulation of NO secretion ($p\leq0.05$) compared with other two groups where AX samples had a relatively large average Mw (≥130KDa). However, wheat bran AX with a large average Mw (437KDa) also showed a strong stimulating activity in the first group. In this case, the immune stimulation activity is not related to its an average Mw without consideration of Mw distribution (low molecular component). Further investigation on Mw distribution of wheat bran AXs may reveal that there is low Mw faction of AXs, which have stronger stimulation activity. In addition, the A/X has no significant correlation with AX samples' stimulation activity ($R<0.1$). According to last section studies, from a same cereal source (corn bran or wheat flour pentosan), Mw distribution showed a significant effect on stimulatory activity of AX. When compared AX's NO stimulation activity from various plant sources, this study suggests that the different sources may also determine other properties of AX expected of the Mw structure, which may have an effect on immune-modulatory activity of AX. Therefore, the mechanism causing the effects of different cereal sources on immunomodulating activity of AX *in vitro* warrants further investigation.

Table 4.5. The molecular structure of AXs from various plant sources

Sources	Average Mw (×10^3Da)	A/X
Corn bran	362±3.0	0.57
Corn stover	367±3.0	0.34
Rice fibre	32.6±0.4	0.28
Wheat bran	437±6.0	0.68
Wheat straw	148±2.0	0.36
Switch grass	130±5.0	0.31
Miscanthus	69.1±0.4	0.32
Sorghum bran	324±7.0	0.69
Barley Hulls	197±1.0	0.24
Barley straws	591±9.0	0.15

4.6 Conclusions

Chapter 4 demonstrates that water-extracted AX and enzyme-treated AX (WEAX and E-WEAX) from the pentosan fraction of wheat flour and alkaline-extracted and enzyme-modified AX (AEAX and E-AEAX) from corn bran possess the potential to modulate the immune response as demonstrated by the NO secretion assay in the human macrophage cell line U937. Firstly, all AX samples were tested at high concentrations (up to 1000μg/ml) and had no effect on the viability and growth of U937 cells. Secondly, these four AX samples were tested at various concentrations and they all significantly elevated NO secretion by the U937 cells. In addition, according to the analysis of the relationship between molecular structures and the stimulatory activity of the AX samples, there were significant differences between modified AXs

(E-WEAX and E-AEAX) and non-modified AXs (WEAX and E-WEAX) in relation to their stimulatory effect on NO secretion. These differences are likely to be associated with the modified AXs having a higher proportion of low Mw AXs (1KDa to 10KDa). This study also found that the AX's degree of branching and side chains of glucose and galactose may not have a significant effect on increasing AX stimulating activity. Thus, the NO stimulatory effects of AXs from the same cereal sources are significantly affected by the Mw distributions and concentrations. In addition, this study compared the stimulatory effects of various AXs samples extracted from 10 different plant sources with various average Mw and branch degree on NO secretion by U937 cells. It was observed that the different source may also determine other properties of AX expected the Mw structure. Hence, this study suggests that the stimulatory effect of AXs on NO secretion *in vitro* is closely associated with their Mw distributions, concentrations and sources.

CHAPTER 5.

Stimulation of Inducible Nitric Oxide Synthase Expression in Human Macrophage U937 Cells by Arabinoxylan Treatments with Various Molecular Structures

5.1 Effects of AXs on iNOS expression by U937 cells

Based on the ability of AXs to induce NO release, this study evaluated iNOS enzyme levels by Dot blot analysis to determine if the AXs stimulatory effect on NO production was related to a modulation of iNOS induction. In addition, in order to further study the Mw structure-activity relationship of the AXs in the iNOS assay, the WEAX and E-WEAX from pentosan of wheat flour were selected. According to the results of the NO production assay, at a concentration of 50μg/ml, the samples of WEAX and E-WEAX showed a high stimulatory effect on NO production by U937 cells. Therefore, the concentration of these two AX samples was set at 50μg/ml in the iNOS study.

As Figure 5.1 shows, the effect of AXs on iNOS expression of U937 cells were detected and quantified. WEAX and E-WEAX with concentration of 50μg/ml significantly elevated the level of iNOS expression by U937 cells after a 24h incubation period compared with the control ($p<0.05$). From the result of the densitometry analysis shown in Figure 5.1.B, E-WEAX and WEAX resulted in a 1.1 and 0.75 fold increase in iNOS concentration from U937 cell lysates respectively compared with the control. In addition, the amount of iNOS in U937 cell lysates following treatment with E-WEAX was significantly higher than with WEAX ($p<0.05$). As Figure 5.1.B shows, E-WEAX increased iNOS concentration in cell lysates by 18% compared with WEAX. LPS was used as a positive control and it presented a significant increase in iNOS expression compared to control ($p<0.05$). There was no significant difference between LPS and the

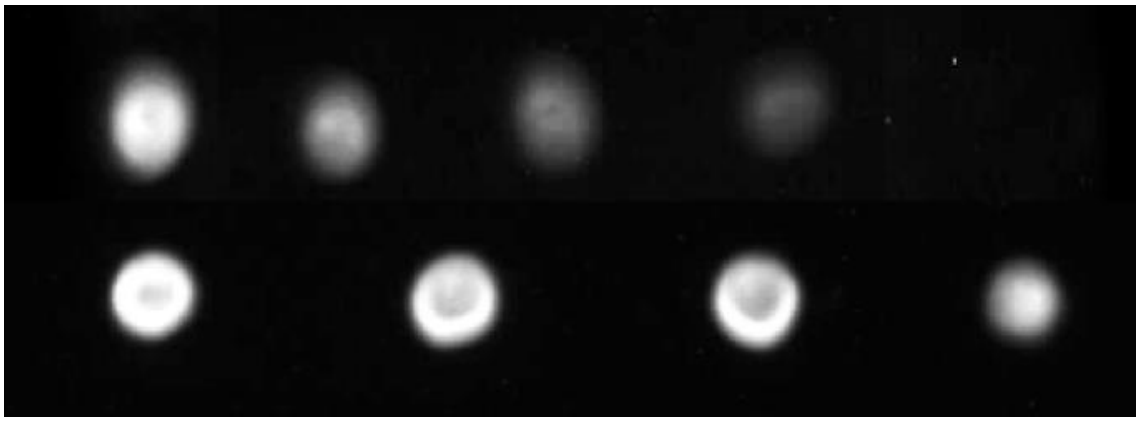
two AX samples in terms of iNOS concentration in U937 cell lysates (p>0.05).

Figure 5.1. Effects of AXs and LPS on iNOS expression by U937 cells

A. *iNOS Standards (Dilution of stock human iNOS enzyme at 116µg/ml)*

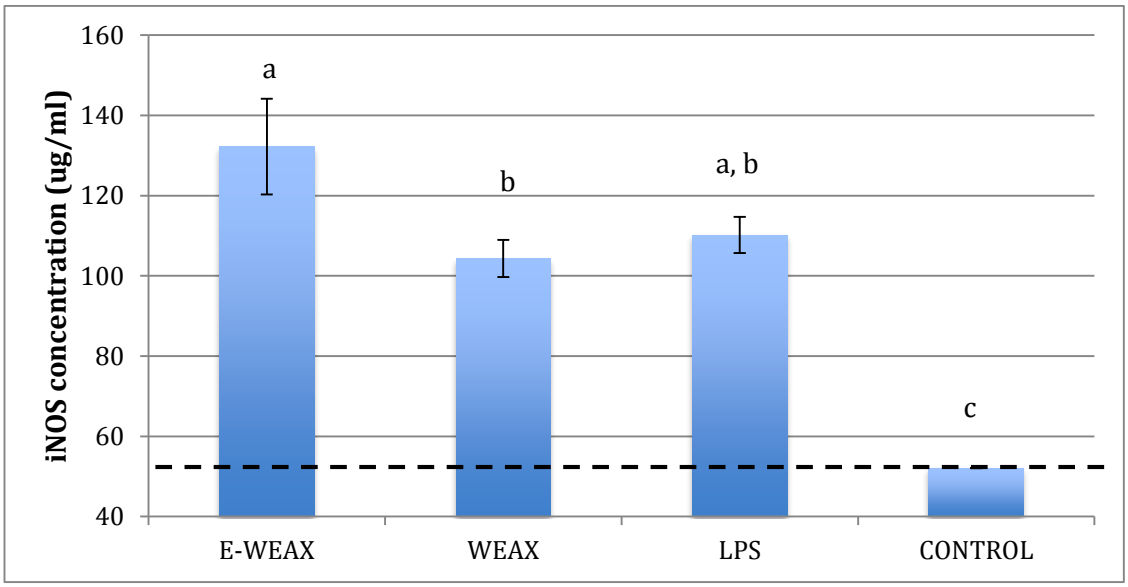
BSA indicates a negative control standard containing BSA but no iNOS protein.

1:2 *1:4* *1:6* *1:8* *1:10* *BSA*



E-WEAX *WEAX* *LPS* *CONTROL*

B.



(A): U937 cells were incubated for 24h with WEAX, E-WEAX and LPS (50µg/ml) treatment separately and iNOS protein of the cells lysates was detected by Dot blot showing the effects of these polysaccharide samples on iNOS expression by the U937 cells. Control means U937 cells were incubated for 24h only using the culture medium. LPS was used as a positive control. The blot shown is representative of three separate experiments. (B): The iNOS levels detected from U937 cells were quantified by densitometry analysis using Image J and using the human iNOS protein (Cambridge Bioscience, UK) as a standard; The mean iNOS concentration (µg/ml) in the cell lysates (mean + SEM of triplicate experiments) was used an

indication of iNOS enzyme levels in U937 cells; The mean values with different letters (a-c) indicate the significance ($p<0.05$) for each comparison among all sample treatments; The dotted line '---' indicates the iNOS level of the control cells.

5.2 Discussions

In order to obtain a clearer understanding of AX modulation of NO production, the effect of WEAX and E-WEAX on iNOS levels was confirmed. Levels of iNOS were significantly increased following stimulation by the AX samples compared to non-stimulated control. The stimulatory effect of AXs on iNOS induction overlaps with their stimulatory activity on NO production. Therefore, the increased NO production by AXs treatment was possibly due to induced levels of iNOS by U937 cells. The LPS (positive control) also showed a high stimulatory activity on iNOS levels in U937 cell lysates. It was reported that the expression of iNOS in macrophage was induced by cytokines (such as IFN-γ and TNF-α) and microbial polysaccharide (such as LPS), which affect the uptake of the conversion of L-arginine to citrulline by cationic amino acid transporters and the expression of both iNOS mRNA and protein(Bogdan 2000). A recent study observed that the NO secreted by iNOS expression through the NF-κB signalling pathway is involved in neuronal migration and regulated by LPS associated with its receptor(Arias-Salvatierra et al. 2011). More recently a study found that polysaccharides of D.officinale were able to increase iNOS expression and NO production in RAW 264.7 cells. They indicated that the stimulatory ability of D.officinale polysaccharide on iNOS expression was associated with the disruption of IκBα-NF-κB complexes, leading to the activation of NF-κB (H. L. Cai et al. 2012; H. Cai et

al. 2015). Based on these previous reports and the fact that AXs stimulated iNOS to similar levels observed by LPS treatment in the present study, it is reasonable to propose that AXs may stimulate NO production in U937 cells through the iNOS pathway, in a similar manner to LPS (NF-κB/iNOS/NO). However, further experiments such as effects of AXs on NF-κB expression are required to confirm this hypothesis and to dissect the precise mechanisms by which AXs modulate NO production in human macrophage.

Moreover, the experiment showed that E-WEAX (50µg/ml) had a higher stimulatory effect on iNOS levels in U937 cell lysates compared to the effect of WEAX at the same concentration. The difference in stimulatory effect of E-WEAX and WEAX on iNOS induction overlaps with their different stimulatory activity on NO production as described in the chapter 4. E-WEAX at 50µg/ml increased NO production in U937 cells by 13% compared with WEAX treatment. Based on previous NO experiments that involved activating NO synthesis in U937 cells, the Mw distribution of AX was considered a possible determinant for their immune-modulatory activity since AXs with Mw at the range of 1KDa to 10KDa appear to have a relative higher stimulatory active. Thus, AXs with different Mw distributions may result in different stimulatory effects on the iNOS expression pathway in U937 cells and thus affecting the NO production. The effect of LPS on iNOS expression by monocytes or monocytically-derived cells through binding with membrane-bond CD14 transfers LPS to toll-like receptor 4 (TLR-4) and works with an obligate accessory protein called MD-2 that initiates intracellular signalling and triggers the induction of iNOS expression (Akashi et al. 2000;

Arias-Salvatierra et al. 2011; Chow et al. 1999; Kitchens and Munford 1998; Medzhitov et al. 1997). According to the mechanism by which LPS induces iNOS expression *in vitro*, the effect of AXs on iNOS expression may be associated with its receptors on U937 cells. Considering the significant difference in iNOS levels and NO production between by E-WEAX and WEAX stimulation, Mw distribution of AXs may be a dominant factor when activating macrophage responses via receptors for AXs. However, AXs receptors in macrophage have not yet been identified, indicating further experimental work is required to investigate this hypothesis.

5.3 Conclusion

The Chapter 5 has demonstrated the differences in stimulatory activities between E-WEAX and WEAX with differing Mw structure on iNOS levels in U937 cells. The results suggest that NO synthesis by AXs is closely mirrored by iNOS expression, and Mw distribution of AXs is a possible determinant affecting their immune-modulatory activity on iNOS levels in U937 cells.

CHAPTER 6.

Conclusions

The immune system in the human body is a network of immune cells, tissues and organs that work together to against attack by pathogens or tumour cells, thereby protecting health (National Institutes of Health 2003). Thus, the immune system plays a pivotal role in the maintenance of human health. However, multiple factors such as genetic predisposition, malnutrition, aging, mental stress or undesirable lifestyles can disturb human immune functions (Gleeson 2005; Hughes 1999; Ibs and Rink 2003; Lomax and Calder 2009; O'Leary 1990; Plat and Mensink 2005; Webster Marketon and Glaser 2008). For instance, delayed type hypersensitivity (DTH), antigen-specific antibody production, the proliferative response of T cells and the relative proportion of T cells decline with aging (Fagnoni et al. 1996; Fagnoni et al. 2000; Mariani et al. 1999; Ravaglia et al. 2000; Sansoni et al. 1993; Stulnig et al. 1995). Many studies have also reported that systemic malnutrition associated with a lack of protein and energy trigger an attenuation in immune functions and results in susceptibility to infection (Brussow et al. 1995; Ledesma et al. 1990; Lotfy et al. 1998; Vasquez-Garibay et al. 2002). Therefore, the ingestion of foods and food-derived substances with immune-modulating activities is widely studied. Further dietary intervention is considered an efficient way of preventing immune functions from declining and reducing the risk of infection or cancer (Canter and Ernst 2004; Cassileth et al. 2009; Kaminogawa and Nanno 2004; Meoni et al. 2013). Some dietary fibre-derived substances, such as β-1,3-glucan, β-1,6-glucan and α-1,6-mannan, have been discovered that show immune stimulation activity (Brown and Gordon 2003; Rieder et al. 2011; Tzianabos 2002; Volman et al. 2008). It has also been proposed that AXs are

potent functional food supplements due to modulating both the innate and adaptive immune systems (Ghoneum and Jewett 2000; Ghoneum and Matsuura 2004; S. Zhang et al. 2015).

This study has shown that the use of enzyme treatments can increase the yields of AXs from a variety of cereal sources. An enzyme extraction process in relation to enzyme mixture, reaction temperature and time have been optimized using a Box-Behnken experimental design combined with analysis of quadratic regression and response surface methodology. The optimum extraction yield of AX (dry matter basis) from the wheat pentosan fraction was 13.54% and this represents an 86% recovery of total AXs. In addition, according to the results form enzyme modification processes of corn bran AXs, enzyme treatments appear to modify the Mw distribution of the AX extracts obtained but did not change the ratio of arabinose and xylose observed. Compared with non-modified AXs, the molecular weights of the modified AXs were obviously reduced from a high Mw range (100KDa to 794KDa) to a lower Mw range (1KDa to 10KDa).

The AX extracts from various cereal sources were tested in a model *in vitro* systems by human macrophage cell line U937. It was observed that the AX extracts significantly elevated NO secretion by the U937 cells compared with the untreated control ($p<0.05$), which indicate potential immunomodulation properties for improving immune function and reducing the risk of infection and cancer. Furthermore, the study investigated the effect of the wheat pentosan AXs on iNOS expression *in vitro* using the dot blot assay, and found that the AXs significantly increased iNOS expression by U937

cells ($p<0.05$). The effect of AX on iNOS levels in human macrophage cells has been confirmed by this study. This provides a clearer understanding of the route by which AXs module NO production. The results suggest that stimulation of NO synthesis by AXs closely mirrored iNOS expression, and the increased NO production by AXs treatment was shown to possibly be due to induced levels of iNOS by U937 cells. Additionally, these tests included the use of LPS as a positive control. The *in vitro* results for the AX extracts and LPS were sufficiently similar to suggest that the AXs might be acting via the same receptor as LPS. This is an exciting area for further research, the findings of which may elucidate the precise mechanism through which AXs modulate immune responses.

In addition, the *in vitro* study demonstrates that the macrophage stimulatory effects of cereal AXs are significantly influenced by their Mw distributions, concentrations and sources. Analysis of the relationship between the molecular structures and the immune stimulatory activity of AX samples in this study suggests differences in the stimulatory effect on NO secretion and iNOS expression are closely associated with the modified AXs (E-WEAX) which have a much higher proportion of low Mw AXs (1KDa to 10KDa) than the non-modified AXs (WEAX). In addition, when comparing NO production by E-AEAX and AEAX isolated from corn bran, it was observed that the degree of branching of the AXs and proportion of glucose and galactose in the AXs chains did not clearly affect the AX's stimulating activity. Thus, Mw distribution of AX is considered to be a significant determinant of their immune-modulatory activity. Molecular structure of AX has already been reported to

have an effect on some of their physicochemical properties in solution including tertiary conformation of AX chain in solution, viscosity of the solution and elastic properties of the solution (Izydorczyk and Biliaderis 2007; S. Zhang et al. 2015). Therefore, it is reasonable to conclude that molecular structure, combined with these physicochemical properties of AX may confer immune-modulating activities. Results of *in vitro* assessments in this study indicate potential underlying mechanisms of the structure-activity relationship of AXs.

Thus, AX extracts have potential as modulators of the immune system. It is now essential to conduct clinical trials to assess their effects in human subject. Confirmation through clinical trials will highlight the beneficial nutritional value of AX-containing foods to human health in terms of enhanced immune responses and reduce the risk of infections and cancer. However, it is also necessary to study methods for including these AX extracts in food products and determining whether processing during food production and preparation influences the immune modulating activity of the AXs.

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Appendixes. Oral presentation abstracts and journal article

Extraction, Modification and Immune-modulating Activities of Cereal Arabinoxylans

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The industrial cereal brans produced as by-products of milling are good sources of arabinoxylans (AXs). The macromolecular characteristics of extracted AXs and the extraction yields achieved, exhibit huge differences as these polysaccharides exhibit great structural heterogeneity. In addition, bioactivities of AXs have been shown in immune enhancement, which potentially lead to corresponding health benefit in food. However, the relationship between the macromolecular of AXs and their immune-modulating activities is still not clear. This paper optimized the extraction, structures modification process and tested immune-modulating activities for AXs from two by-products of cereal processing: pentosan of wheat flour and corn bran.

For the AXs from pentosan, the effects of the different enzymatic extraction conditions (endoxylanase concentration (w/w), extraction temperature and time) were studied by using a Box-Behnken experimental design and response to extraction yield of AXs estimated by the regression model. For the AXs from corn bran, alkaline extraction and enzymatic modification were used. Furthermore, the extracted AXs with different molecular structure have been investigated and compared for their ability to modify immune responses *in vitro* studies.

The regression model shows very good fitting (p-value<0.001) and predicting (value of R^2=93.24%) to this optimization experiment. The optimum extraction yield reached 13.54%(w/w) from pentosan of wheat flour. In contrast with aqueous extraction AXs, the Mw distribution of enzymatic extraction AXs significantly becomes more centralized in low Mw range (1KDa-10KDa) with more enzymatic treatments. The ratios of arabinose to xylose arise from 0.48 to 0.83 as concentration increasing of enzyme. In addition, extraction yield of corn bran AXs can reached to approximately 21% (w/w) using alkaline treatment and the average molecular weights (Mw) of extracted AXs was 501KDa. More than 30% of extracted AXs could be reduced to lower Mw (0.1KDa-10KDa) under further enzymatic modification. Based on results of *In vitro* studies, the extracted AXs were able to significantly promote the level of NO synthesis in U937 cells.

Consequently, through this study, extraction yield and molecular features of AXs could be well controlled in terms of Mw and branch degree for enhancing health benefits.

Keywords: Arabinoxylans; corn bran; wheat flour; extraction; modification; yields; molecular characteristics; immune-activities.

Modification and Extraction of Arabinoxylans from Corn Bran with Enzymatic and Chemical Methods

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The industrial cereal brans produced as by-products of milling are good sources of arabinoxylans (AXs). The recent studies show the AXs have bioactivities in reducing post-prandial blood sugar (Vogel et al., 2012), immune enhancement (Zhou e al., 2010), anti-tumour and antioxidant properties (Cao et al., 2011; Veenashri et al., 2011), prebiotic properties (Grootaert et al., 2009), which potentially lead to corresponding health benefits. However, the relationship between the macromolecular properties of AXs and their bioactivity is still not clear.

This paper describes the optimization of the extraction process for AXs from corn bran using various alkaline methods and the modification of their macromolecular characteristics using three-dosage endoxylanases treatments. The results of these experiments show the maximum extraction yield of AXs can reach to approximately 25% from corn bran under the optimized conditions. In addition, the molecular features of AX can be modified using different extraction and modification routes to produce AXs with average molecular weight of 5.6×10^5 Da and of 2.1×10^3 Da and with the ratio of arabinose to xylose (Ara/Xly) of a range of 0.85 to 2.0. The molecular properties of the extracted AXs have been characterised. The modified AXs of known structure present here will be test materials for the study of the influence of AXs' molecular features on their bioactivity.

Keywords: Arabinoxylans, corn bran, extraction, yields, modification, molecular weight, Ara/Xly

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The Review of Extraction and Modification of Arabinoxylans from Industrial Cereal by-products with Enzymatic and Ultrasound Technology

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The industrial cereal by-products such as corn bran and pentosan fraction are good sources of arabinoxylans (AXs). The recent studies show the extracted and modified AXs have the bioactivities in reducing post-prandial blood sugar, immune-enhancing, anti-tumor and antioxidant properties, which potentially lead to corresponding health benefits for food products.

Therefore, the aims of current project are to optimize the process of extraction and modification of AXs from cereal by-products with enzymatic and ultrasound technology for producing bioactive AXs with the functionalities of reducing post-prandial blood sugar and stimulating immune system. Based on results of single factor extraction experiments, the AXs extraction rate from pantosan fraction increased from 7.54% to 12.83% with the enzymatic technology and increased from 11.12% to 12.73% as the temperature increased from 20°C to 40°C. In the extraction processing, the molecular features of AXs can be modified with enzymatic technology for the improvement of immune-enhancing activity.