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Characterisation and immunomodulatory activities of alkaline extracted and enzymatic modified arabinoxylans from corn bran

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Abstract

The relationship between the molecular structures of corn bran arabinoxylans (AXs) and their immune-modulating activities (the structure-activity relationship) was investigated. This study extracted AXs from corn bran using alkaline and developed AXs modification process using three types of endoxylanases from *Thermomyces lanuginosus*, *Neocallimastix patriciarum* and *Penicillium funiculosum.* Furthermore, the non-modified and modified AXs with different molecular structures were investigated with respect to their ability to modulate nitric oxide (NO) production *in vitro* as an indication of their immunomodulatory potential.

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 significantly elevated the level of NO synthesis by human U937 cells ($p<0.05$). The results also indicated there was an obvious dose-response effect for each of AX samples but the effective range over which they work is different. Modified AXs with higher portion of low Mw showed a significant lower optimum dose range than extracted AXs with higher portion of high Mw

(p<0.05). The study suggested that the Mw distribution of AX is a determinant for their optimum dose on NO production by macrophage, thereby affects its immune-modulatory activity.

Keywords: *Arabinoxylan; Corn bran; Molecular weight; Immune-modulatory activity; in vitro*

Introduction

Immune system plays a pivotal role in the protection and maintenance of human health (National Institutes of Health 2003). 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). 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). For instance, 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).

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; W. Li et al. 2015; S. Zhang et al. 2015). 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). More recently, it has been reported by 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 ($\leqslant$25KDa) and higher degree of branching (0.81). This study also suggested that further investigation need 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). The structures of AXs vary in Mw, degree of branching, substitutions and conformation (Izydorczyk and Biliaderis 2007). However, there has been no clear consensus as to which main molecular structural properties are required for AXs to confer their immune-modulating ability. Thus, the mechanisms of the structure-activity relationship of AXs still remain to be studied.

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). Corn bran has also been identified to be a cereal source with a high AXs content (25-30%) and the extraction methods for this material have also been studied (L.W. Doner and Hicks 1997a; L.W. Doner et al. 1998). However, there is limited data exists on corn bran AX's immunomodulatory properties.

Enzyme hydrolysis is an efficient method for modifying the molecular structure of AXs (Z. Zhang et al. 2014). Endoxylanases and cellulases are commonly used to extract and degrade AXs from cereal cell wall tissue. Endo-β-1,4-xylanases (EC 3.2.1.8) are the hemicellulolytic enzymes, which cleave the β-xylosidic bond between two d-xylopyranosyl residues linked in β-(1,4) (Paes et al., 2012; Driss et al., 2013). Endo-β-xylanases (EC 3.2.1.8) are mainly classified into two glycoside hydrolase (GH) families namely GH 10 and GH 11. Endoxylanases from the GH11 family are commonly used to hydrolysis microstructure of AXs because of their higher substrate specificity than GH 10 endoxylanases (Beaugrand et al., 2004;Driss et al., 2013). Three GH family 11 endoxylanases from *Thermomyces lanuginosus*, *Neocallimastix patriciarum* and *Penicillium funiculosum* have been characterised and have been used in different studies for isolation and modification of cereal hemicellulose (Elshafei et al., 1991; Zhou et al., 2010; Li et al., 2015; Malunga and Beta, 2015). However, the effects of these three enzyme modification processes on the molecular structures of the corn bran AXs have not well documented. Thus, this investigation aims to develop extraction and enzyme modification processes for modifying the molecular structures of AXs from corn bran. The monosaccharide compositions, branch degree and Mw distribution 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 *in vitro* are investigated.

1 Materials and Methods

1.1. Materials

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). There were three endo-1,4-β-xylanase products (EC 3.1.2.8) used in the modification of AXs, which were Pentopan Mono BG (P-BG, 2500 u/g) from *Thermomyces lanuginosus* (Novozyme, Bagsvaerd, Denmark), E-XYLNP (1000-1500 u/mg) from *Neocallimastix patriciarum* (Megazyme, Bray, Wicklow Ireland) and Optimash VR (O-VR, 3150 u/g) from *Penicillium funiculosum* (Genencor, Leiden, Netherland). 1,4-α-D-Glucan-glucanohydrolase (a-Amylase heat stable, ⩾500 u/ml) from *Bacillus licheniformis* and Proteinase (⩾500 u/ml) from Aspergillus melleus were purchased from Sigma-Aldrich, Gillingham, United Kingdom.

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). D-glucose, D-xylose, D-arabinose, and D-galactose were purchased from Sigma–Aldrich (Gillingham, UK).

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. LPS (Lipopolysaccharides of *E. coli* serotype O111:B4, Sigma Aldrich, UK) was used as a positive control in the nitric oxide (NO) stimulation assay. 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).

Other chemicals including acids, alkaline and organic solvents were

purchased from Sigma Aldrich, UK and used were of analytical grade.

2.2 Methods

2.2.1 Extraction and purification process of arabinoxylans from corn bran

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 was collected to obtain 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 (1%, 2%, 4% and 8%, w/w) 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.

The ethanol precipitation method was developed from Li et al. (2015). The 600ml AXs-containing supernatants were mixed with 400µl proteinase. 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 precipitate was collected and washed twice with 20ml aliquots of ethanol. Then the precipitate re-suspended in 20ml acetone and washed for 1min. After that, the precipitate was dried in an oven overnight at 45°C to obtain alkaline extracted AX sample (AEAX). The AEAXs were milled using an analytical mill (IKA A11 Basic, Guangzhou, China, 50/60Hz, 160W) and weighed.

2.2.2 Modification process of the extracted arabinoxylans

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 endo-1,4-β-xylanase products (E-XYLNP, P-BG, O-VR) were used to modify the AEAX and the treatment time were 24h and 48h. 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 studies of Malunga and Beta (2015), Li et al. (2015) and the manufacturer instructions. 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. 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 residues were milled using an analytical mill and weighed, which were enzyme modified AEAX samples so that were named as E-AEAXs.

2.2.3 Analysis of extraction yields of arabinoxlyans

A standard curve of xylose was constructed using the method described by S. G. Douglas (1980) for determination of the xylose content of corn bran 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 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:

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

2.2.4 Analysis of sugar compositions of arabinoxylans

The mono-sugar compositions of AX samples were analysed by following a method developed from Li et al. (2015). One ml of 1M H_2SO_4 was added to 20mg dried AX sample. The mixture was hydrolysed for 2 h at 100°C in a glycerin bath. The mixture 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%.

2.2.5 Analysis of molecular weight distributions of arabinoxylans

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 Li et al. (2013) and 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. Eight pullulan standards with molecular weights in the range 5,000-800,000Da were used to construct a standard curve. 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. 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 AXs. 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%.

2.2.6 *in vitro* study

2.2.6.1 Human U937 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 U937 macrophage cell line was grown in complete culture medium using 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.

2.2.6.2 Polysaccharides medium preparation of *in vitro* study

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 (5μg/ml, 50μg/ml, 500μg/ml, 1000μg/ml) for cell culture treatments. The samples were stored at 4°C in sterile Falcon tubes (Fisher Scientific, UK).

2.2.6.3 Toxicity test of corn bran 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 prepared sterile and pre-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).

2.2.6.4 Nitric oxide 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). 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. 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.

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).

2.2.7 Statistics

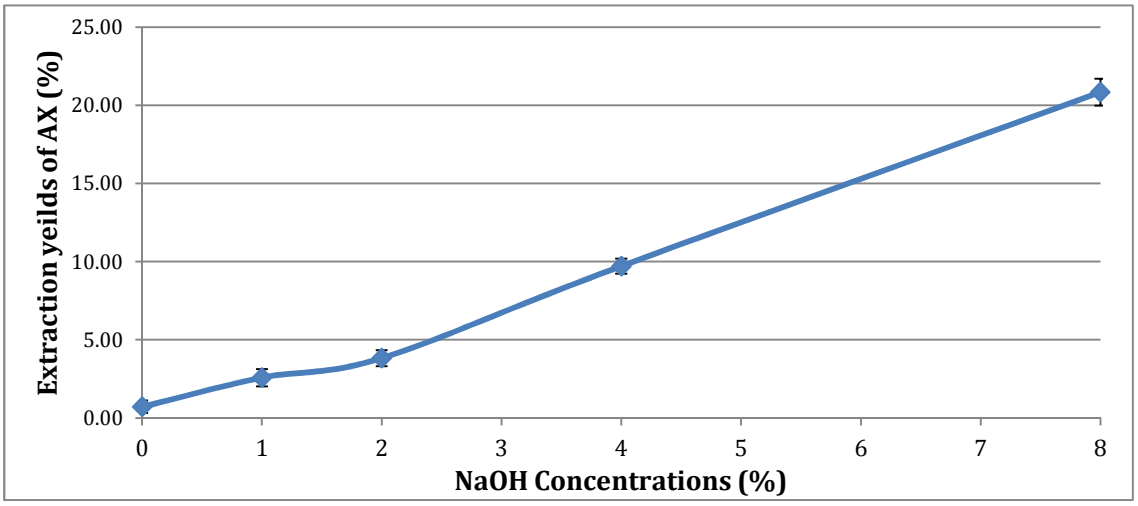
The results, unless otherwise stated, were performed in triplicate and checked using analysis of one-way ANOVA. A value of $p \leqslant 0.05$ was chosen as the criterion of statistical significance. The data were expressed as means $\pm$ standard error (SE).

3. Results and Discussions

3.1 Extraction and modification recovery yield of arabinoxylans

The total AXs content was determined as 25.98±0.31% of corn bran (dry matter basis). 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 concentration on corn bran AXs extraction yields. Figure 1 shows significant effects of the alkaline treatment on AXs extraction yield of corn bran (dry matter basis). Compared to water extraction, 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 1 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.

Following extraction with 8% NaOH, the AEAX sample was modified with three types of endoxylanase treatments, including P-BG, E-XYLNP and O-VR from *Thermomyces lanuginosus*, *Neocallimastix patriciarum* and *Penicillium funiculosum* respectively. As shown in Table 1, high recovery yields (≈88%) of AXs from AEAX sample was achieved from enzyme treatments. In addition, there is no significant difference in the recovery yield of E-AEAX between different endoxylanase treatments and/or different treatment times of the 24h and 48h (p-values>0.05).

Table 1 The recovery yield of E-AEAXs using three types of endoxylanase

Enzyme / Treatment Time	Enzyme treatments		
	P-BG	E-XYLNP	O-VR
24 h	88.02±0.35%	88.01±0.14%	88.00±0.28%
48 h	88.05±0.34%	88.07±0.14%	87.95±0.07%

3.2 Characterisation of the alkaline extracted AXs (AEAX) and the enzyme modified AXs (E-AEAXs) of corn bran

3.2.1 Monosaccharide compositions of the AEAX and the E-AEAXs

As shown in Table 2, 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.

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 2. 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.

Table 2 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.

3.2.3 Effects of various enzyme treatments on molecular weight distributions of extracted arabinxylans

As shown in Table 3, 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 2, the Mw distributions of E-AEAXs showed a small change in Mw range of $1\times10^{4.5}$ to $1\times10^{5.5}$Da. There mainly comprises a peak in large Mw range 1 (501.187KDa; $\log_{10}$Mw≈5.7; DP≈3797). However, AEAX subjected to longer enzymatic treatments 48 hours had a clearly changed Mw (Figure 3). The Mw distribution curve of the E-AEAXs contain two main peaks in small Mw range (3.715KDa； $\log_{10}$Mw≈3.6; DP≈28) and the other peak in large Mw range (around 501.187KDa). 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). 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). 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 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. 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.

Figure 2 The molecular weight distributions of E-AEAXs treated using three different types of enzyme for 24 hours treatment

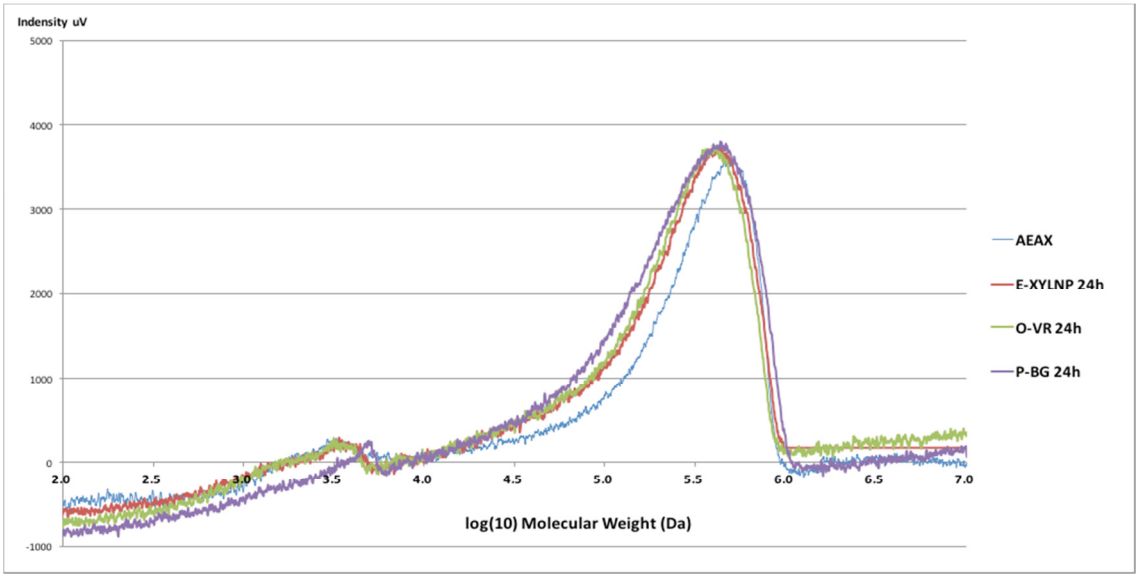
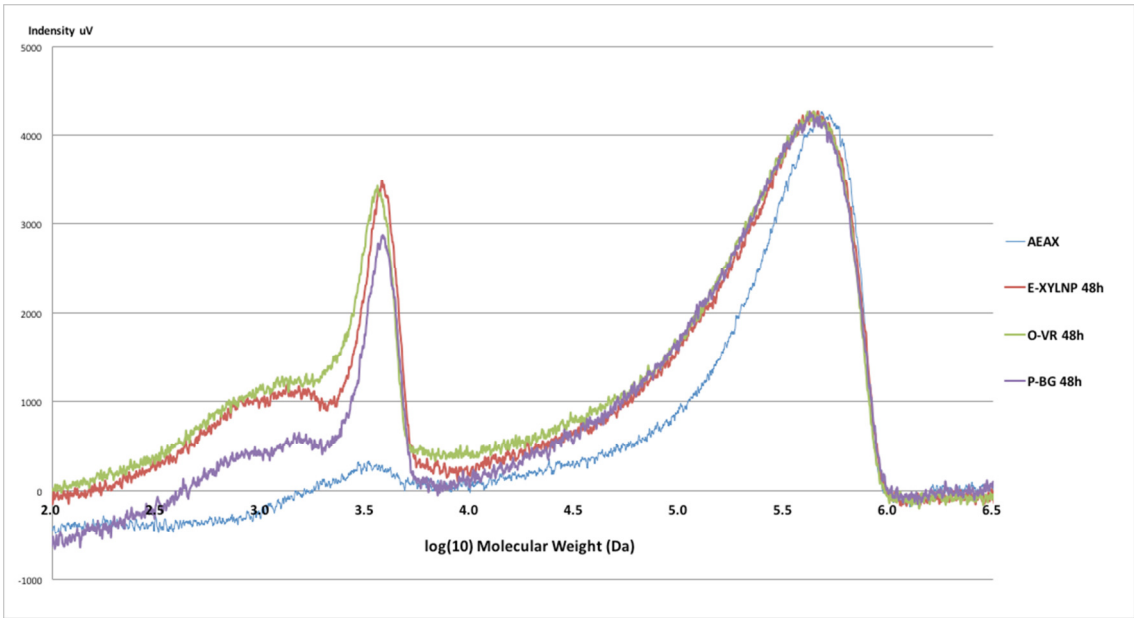


Figure 3 The molecular weight distributions of E-AEAXs treated with 48 h treatment of three types of endoxylanase



According to the results, 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, 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 GH 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 modification 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, 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*. From previous studies, it is important to note that due to the differences in substrate specificities, binding modules and enzyme production technology, different xylanases may have different activity in reducing Mw of xylans (Shoseyov et al., 2006; Driss et al., 2013; Garg et al. 2010).

3.3 Immunomodulatory activity of arabinoxylans

3.3.1 Effects of arabinoxylans treatment on growth and viability of U937 cells

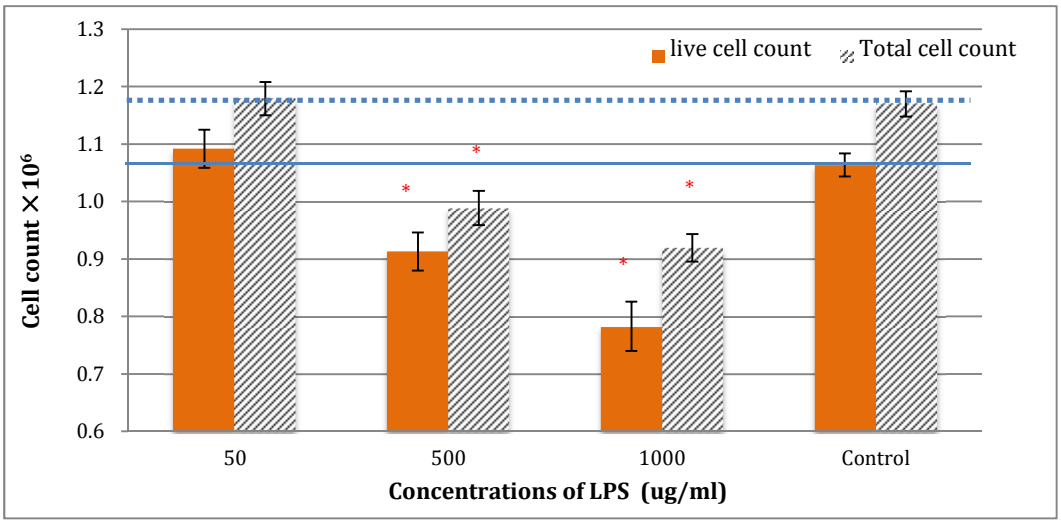
The Trypan blue exclusion assay with cell count was used to assess cell growth and viability of the human macrophage U937 in presence of the AXs and LPS at various concentrations are shown in Figure 4: a-c. Figures 4 b and c demonstrate that the viable and total cell counts for AXs (50, 500, 1000μg/ml) treated U937 macrophages are not significantly different compared to control (untreated cells) (p-values>0.05). Hence, 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 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.

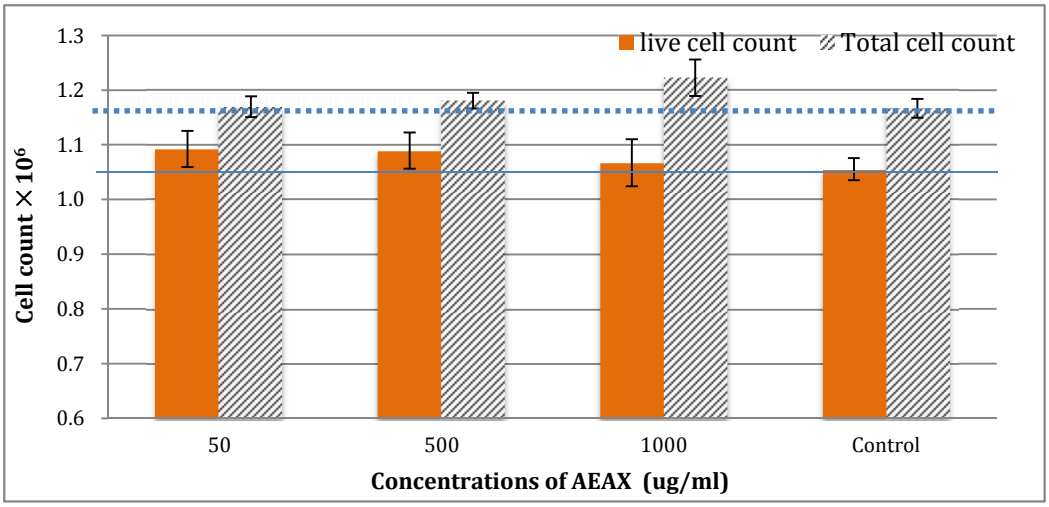
However, as shown in Figure 4 (a), the cell counts of viable and total U937 cells were reduced significantly after treatment with 500 and 1000μg/ml of LPS compared with that of untreated control (p-values<0.01). In contrast, this inhibitory effect on cell growth did not appear at relatively low concentrations (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. Effects of AXs and LPS on the viability of U937 cells

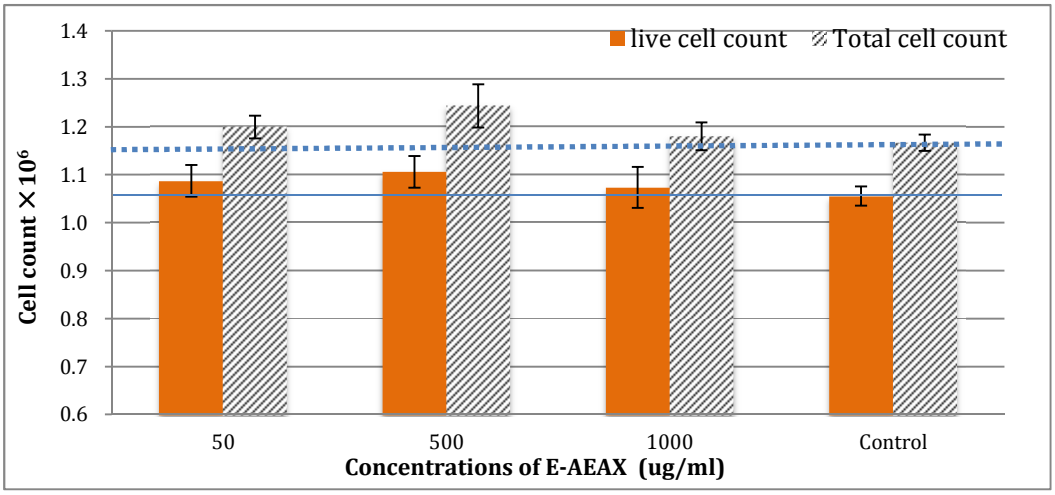
a.



b.



c.



a-e: the U937 cells were treated with the LPS, 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.

3.3.2 Effects of arabinoxylans with different molecular structure on NO production by U937 cells

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. Firstly, over the concentration range from 5 to 1000μg/ml, Table 4 clearly shows that the two AXs samples (AEAX and E-AEAX) 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；Li et al. 2015). Secondly, the LPS was used as a positive control and significantly stimulated NO secretion at all concentrations (Table 4) compared to the untreated control. However, at 500 to 1000μg/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 a).

Table 4 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 * # $	

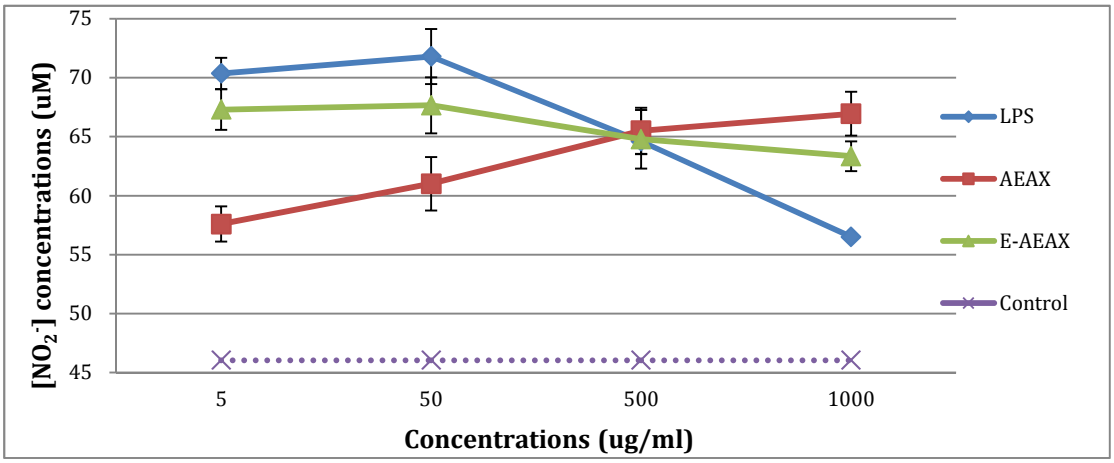
*The NO_2^- concentration (mean + SEM) is an indication of NO production in U937 cells; The symbol * indicates NO production significantly increased ($p<0.05$) compared to the untreated control; The symbol # indicates NO production significantly changed ($p<0.05$) as the sample dosage progressively increased; 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.*

Furthermore, Figure 5 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-AEAX treatments are in the same range 5-50μg/ml whilst the optimum for AEAX treatment should be above 1000μg/ml. AEAX had generally lower NO production response compared to E-AEAX and LPS at concentration below 50μg/ml. Thus, the optimum dose ranges of AEAX and E-AEAX were significant different. According to the Mw distributions of E-AEAX and AEAX in Table 3, 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. In addition, the branch degree (A/X) of E-AEAX and AEAX were the same value of 0.82 and the monosaccharide composition shows no glucose component and a similar proportion of galactose content in these two AX samples. Therefore, when activating NO synthesis by U937 cells, the Mw

distribution of AX is considered a determinant for their optimum dose, thereby affects its immune-modulatory activity.

Corn bran AX with Mw at the range of 0.1KDa to 10KDa appears the most biologically active. 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.

Figure 5 Effect of AEAX, E-AEAX and LPS treatments on NO production by U937 cells



3.3.3 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 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. Conclusions

According to the results form enzyme modification processes of corn bran AXs, treatments of three different endoxylanases appear to modify the Mw distribution of the AX extracts obtained but did not obviously change the ratio of arabinose and xylose observed and other monosaccharide components. In the process of enzyme modification, the enzyme treatment time is a significant factor in the Mw modification of AEAX using the endoxylanase. 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 low Mw range (0.1KDa to 10KDa).

The AX extracts from corn bran 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 *in vitro* study demonstrates that the macrophage stimulatory effects of AXs are significantly influenced by their Mw distributions and concentrations. 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 are closely associated with the modified AXs (E-AEAX) which have a much higher proportion of low Mw AXs than the non-modified AXs (AEAX). Thus, Mw distribution of AX is considered to be a significant determinant of their optimum doses of immune-modulatory activity.

The results of *in vitro* assessments should be useful in further understanding the mechanisms of the structure-activity relationship of AXs. In addition, further study may also need to further investigate the mechanism underlying effect of AX on the NO pathway *in vitro.*

5. Acknowledgements

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