Regulation of Nitric Oxide Synthase Expression by Structure Modified Arabinoxylans from Wheat Flour in Cultured Human Monocytes

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Abstract

The immunomodulatory activity of arabinoxylans (AXs) extracts from cereal sources have been reported to impart health benefits in terms of immune enhancement. In this study, further to the various enzymatic extraction conditions on extraction yield and structural modification of AXs from the wheat flour, nitric oxide (NO) secretion and inducible nitric oxide synthase (iNOS) expression induced by enzyme extracted AXs and water extracted AXs in a human monocyte cell cultures U937 were compared. The results of *in vitro* testing indicate that AXs treatments not only enhanced NO production, but also iNOS levels in U937 cells ($P < 0.05$) compared to untreated cells. The increase in NO secretion seems correspondingly related to iNOS concentration in cultured cells. Moreover,

the enzyme-treated AXs with a much higher proportion of low Mw AXs (1-10KDa) and high A/X ratio (0.83) induced significantly higher ($P < 0.05$) iNOS expression (132.2 ± 11.9 μg/ml) than water-extracted AXs treatment (104.3 ± 4.6μg/ml). In conclusion, an enzymatic method has been developed to efficiently extract AXs with low Mw and high A/X and with a high yield (81.25%) of the molecular structures which had a critical influence on the regulation of iNOS expression and NO production in a cultured human monocytic cell line.

Keywords: Arabinoxylans; wheat flour; Molecular structures; Immunomodulatory activity; Nitric oxide; Inducible nitric oxide synthase..

1. Introduction

Arabinoxylans (AXs) are an important group of hemicelluloses found in the outer-layer and endosperm cell walls of cereals (Izydorczyk & Biliaderis, 1995; Saeed et al.,2011; Vries et al., 1999). Generally AXs are composed of a backbone of β-1, 4 linked D-xylopyranosyl residues with L-arabinofuranose subunits. As a dietary fibre, AXs have been shown to stimulate immune activities in a number of *in vitro*, *in vivo* and

clinical trials (Ghoneum and Matsuura 2004; Zhou et al., 2010; Li, et al., 2015) and may reduce the risk of infection or cancer (Zhang et al., 2015). It has been proposed that dietary fibres including AXs, exert their immunomodulation benefits possibly through two mechanisms: (i) interaction with colonic epithelial cells and innate immune cells (such as macrophages) which can mediate cytokine production leading to an improved immune response, (ii) take up by microfold cells, macrophages and dendritic cells from the intestines and transport to lymph nodes, thus distributingAXs around the body (Mendis et al., 2016; Samuelsen et al., 2011). Several studies have suggested that AXs may be considered a potential bioactive food supplement with immunity improvement properties (Ghoneum and Matsuura 2004; Zhou et al., 2010; Li et al., 2015). However, the role of AXs in certain specific immune responses such as the expression of nitric oxide synthases in NO secretion path way still need further investigation.

Nitric oxide (NO) is a versatile signalling molecule of the immune system produced by various immunological cells,dendritic cell, NK cell, mast cells and phagocytic cells. (Bogdan 2000; Forstermann and Sessa 2012). From previous studies,

55 the immune functions of NO have been found to involve antimicrobial and anti-tumour
56 activities *in vitro* and *in vivo* (Bogdan 2000; Lechner et al. 2005; Nathan 1992; Pervin
57 et al. 2001). Inducible NO synthases (iNOS) are critical in generating NO from L-
58 arginine. iNOS can be expressed in immune cells following stimulation by microbial
59 polysaccharides such as lipopolysaccharides (LPS) and immune cytokines (Bogdan,
60 2000). Interestingly, recent studies have shown that AXs enhance NO secretion in
61 murine and human macrophage cell lines (Ghoneum and Matsuura 2004; Li et al., 2015)
62 and also demonstrated that the molecular structure such as molecular weight (Mw) and
63 substitution degree (A/X) of cereal AXs may affect their immunomodulatory activities
64 (Li et al., 2015; Zhang et al., 2016). However, it remains unclear if AXs with different
65 structures have any function to induce the iNOS expression in NO pathways.
66 Therefore, the objectives of the current study included to develop an effective and
67 efficient extraction and modification processes capable of producing high yields of
68 structurally modified AXs from wheat flour. The sugar compositions and Mw of the
69 extracted and modified AXs were determined using high performance liquid
70 chromatography (HPLC). Subsequently, the relationship between molecular structures

and NO production and iNOS expression in cultured human macrophages were investigated.

2. Materials and Methods

2.1 Materials

The sample of dried pentosan fraction (moisture content 5.9%) was kindly provided by Henan Lianhua Monosodium Glutamate Group Co., Ltd (Xiangchen, Henan, China). Pentopan Mono BG (2500 U/g), an endoxylanase product (EC 3.1.2.8, family 11 of glycosyl hydrolases) from *Thermomyces lanuginosus* (donor)/*Aspergillus oryzae* (host) was kindly supplied by Novozyme (Bagsvaerd, Denmark). 1,4-α-D-Glucan-glucanohydrolase (α-Amylase heat stable, ≥500 U/ml) from *Bacillus licheniformis* and Proteinase (≥500 U/ml) from *Aspergillus melleus* were purchased from Sigma-Aldrich (Gillingham, UK). D-(+)-xylose for AXs determination was purchased from Acros Organics (Loughborough, UK). D-glucose, D-xylose, D-arabinose, and D-galactose for determination of monosaccharide composition were purchased from Sigma–Aldrich (Gillingham, UK).

U937 cell line was purchased from the Public Health England Culture Collections. LPS (Lipopolysaccharides of *E. coli* serotype O111:B4,) used as a positive control in the *in vitro* experiments was purchased from Sigma-Aldrich, (Gillingham, UK). The medium of RPMI-1640 with L-Glutamine was purchased from Lonza (Verviers, Belgium). Foetal bovine serum and penicillin-streptomycin (10,000 units penicillin and 10mg streptomycin/ml) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human iNOS enzyme (116µg/ml, Code: TP311819) was purchased from Cambridge Bioscience, UK. Primary antibody, monoclonal iNOS Antibody (4E5) was purchased from Novus Biologicals (Cambridge, UK). Secondary antibody, the Rabbit anti-mouse Immunoglobulins/HRP (code: P0260) was purchased from Dako (Glostrup, Denmark).

Other chemicals including analytical grade acids, alkaline and organic solvents were purchased from Sigma Aldrich (Gillingham, UK). .

2.2 Methods

2.2.1 Extraction of AXs

The AX extraction process was developed from the method of Li et al. (2013).

103 The Pentosan fraction of wheat flour was ground to a 0.5mm particle size using an
104 Ultra Centrifugal Mill ZM 200 (RETSCH Ltd. United Kingdom). 30g of milled
105 pentosan sample was mixed with 200g of distilled water using a hand blender
106 (800W, WSB800U) for 45s. For the water extraction process, the pentosan-water
107 mixture was incubated in a shaking water bath at 40°C for 2h followed by
108 centrifugation (6000g, 20min), and the supernatant (containing extracted AX) was
109 recovered for ethanol precipitation. In the enzymatic extraction process, the
110 effects of three individual extraction conditions: concentration of endoxylanase
111 (50, 100, 200, 300, 400ppm w/w), extraction time (2h, 3h, 4h) and temperature
112 (20°C, 30°C and 40°C) on AX extraction yield and structures were studied.
113 The AXs extraction process was developed from the method of Li et al.
114 (2015). The 150ml extraction supernatant was added to 300µl Termamyl α-
115 Amylase at 90°C to allow starch hydrolysis to occur. After 1h, the solution was
116 cooled rapidly and adjusted to pH 7. Then, 100µl proteinase (1mg/ml, ≥3units/mg)
117 was added to the solution and the mixture incubated at 60°C for 1h to remove
118 protein from the supernatant and thenhe solution was placed in a boiling water

bath for 10min to deactivate the enzymes and cooled to the room temperature. After centrifugation at 6000g for 20min, the supernatant was collected and ethanol was added into the supernatant to a ratio of 70% of ethanol/supernatant (v/v).. 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.. Then, the residue was placed in 20ml acetone and washed for 1min. After that, the residue was dried in an oven at 45°C overnight. The dried residue (dried AXs sample) was milled using an analytical mill (IKA A11 Basic, Guangzhou, China, 50/60Hz, 160W).

2.2.2 Determination of AXs extraction yields

A standard curve for the determination of xylose was constructed using the method described by Douglas (1980). This curve was used for the determination of xylose content of raw material and extracted AX supernatants. The xylose content was then used to calculate the AX content of samples based on their A/X ratio as determined by mono-sugar composition analysis. The specific AX content is defined as the sum of the monosaccharide content, arabinose and xylose, times

135 0.88 to correct for hydration water (Swennen, Courtin, Lindemans, et al., 2006).
136 Thus, the equation was: AX%=Xylose%×(1+A/X)×0.88. AX extraction
137 yield=AX%×weight of supernatant (g)÷weight of raw material(g)×100.

138 ***2.2.3 Determination of monosaccharide compositions of isolated AXs***

139 The monosaccharide compositions of AX samples were analysed using a
140 method developed from that of Li et al. (2015). 1 ml of H_2SO_4 (1M) was added to
141 20mg of dried AX sample, which was hydrolysed at 100°C for 2h in a glycerin
142 bath. The hydrolysed samples were transferred to a volumetric flask and diluted
143 20 fold (1mg/ml) using HPLC grade water. The pH of diluted solution was adjusted
144 to 6.5-7.2 with 1M NaOH. The solution was then filtered through a 0.45μm nylon
145 membrane and transferred to a 1ml glass shell vial for HPLC analysis. In the HPLC
146 analysis, the mobile phase was HPLC grade water at isocratic elution which
147 wasachieved using a Shimadzu LC-10ADvp Pump. Samples were analysed on
148 SUPELCOGEL Pb (5cm×4.6mm) and Phenomenex ThermaSphere TS-130 columns
149 combined with a JASCO RI-2031 Refractive index (RI) Detector. All analyses were
150 conducted in triplicate.

2.2.4 Molecular structure characterisation of extracted AXs

Size exclusion high-pressure liquid chromatography (SE-HPLC) with a refractive index (RI) detector was used to determine the molecular weights and size distribution of extracted AXs samples using methods as described by Li et al. (2013). The average degree of polymerisation (avDP) was calculated by dividing the apparent peak molecular mass by the molecular mass of anhydropentose sugars (Courtin et al., 2008).

The determination of the molecular weight of AX samples was carried out using SE-HPLC. Mobile phase (Buffer) consisted of 17g $NaNO_3$ and 0.65g NaN_3, dissolved in 2L HPLC grade water. Eight pullulan standards (Shodex, Shanghai, China) with molecular weights in the range of 5,000-800,000Da were used to construct a standard curve. The AXs samples were dissolved in buffer to make 2mg/ml solutions and left overnight under gentle stirring. Then, they were filtered through a 0.45μm nylon membrane and transferred to SE-HPLC analysis.

Mw distribution of the AXs were analysed by columns of BioSep-SEC-S 4000 and BioSep-SEC-S 3000 (Phenomenex, Macclesfield, UK) combined with JASCO RI-

2031 RI Detector (Jasco Corporation, Tokyo, Japan). The two columns were connected in series (BioSep-SEC-S 4000 first) were used to improve the peak shape of AXs samples. Running time was 60min for each sample and the flow rate was 0.6 ml/min. All analyses were conducted in duplicate.

2.2.5 in vitro testing

2.2.5.1 Cell culture

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

2.2.5.2 Polysaccharides medium preparation

The extracted AX samples and LPS were dissolved in the RPMI-1640 medium with 5% FBS overnight at room temperature to allow the sample fully hydrated. Then, they were sterilised using 0.45μm sterile filters and were diluted into a series of concentrations (1, 5, 10, 50, 500 and 1000μg/ml) for cell culture testing.

The samples were stored at 4°C in sterile Falcon tubes (Fisher Scientific, UK).

2.2.5.3 Cell viability and growth analysis

The growth and viability of U937 cells with and without treatment of AX samples and LPS were assessed by cell count and trypan blue uptake. The U937 cells (viability ≥90%) were centrifuged at 1000g for 10min and suspended 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 into each well of a 96-well microplate. The AXs and LPS mediums prepared with three concentrations (50, 500 and 1000µg/ml) were pre-warmed to 37°C and then 100µl of medium, AXs medium and LPS medium was added to six wells of containing cells and mixed thoroughly. After 24h incubation, 40µl of cell fluid was pipetted from each well into a tube, and 40µl of trypan blue (Sigma-Aldrich, UK) was added. The number of cells stained (nonviable cells) and unstained (viable cells) with trypan blue were counted using a TC10 automated cell counter (Bio-Rad, UK). The viability of cells was calculated as a percentage by dividing the number of viable cells against to total number cells (total number of viable and nonviable cells).

199 **2.2.5.4 NO assay**

200 Griess' reagent was freshly prepared for the NO assay according to the
201 methods of Dawson and Dawson (1995) and Griess (1879). Griess' reagent is
202 made up by two components. Component A is 37.5mmol/L sulphanilamide with
203 deionized water in 6.5mol/L HCl by 1:1 (v/v). Component B is N-1-
204 napthylethylenediamine dihydrochloride (NEED) in deionized water at
205 12.5mmol/L.

206 50µl of culture medium of U937 cells was pipetted into each well of a 96-well
207 microplate and 50µl of each AX and LPS sample was added into appropriate wells
208 and it was mixed thoroughly. The 50µl RPMI-1640 with 5% FBS medium was
209 added to wells containing 50µl live cells as an untreated control. The microplates
210 were incubated (37°C, 5% CO_2) for 24 hours. Then, 50µl of Component A of Griess'
211 reagent was added to each well for 10 minutes at room temperature. 50µl of
212 Component B of Griess' reagent was then added to each well. After mixing,it was
213 incubated at 4°C for 20 minutes. The absorbance of each well was measured at
214 540nm using a microplate reader (Synergy HTX Multi-Mode Reader, Biotek, UK).

A nitrite standard reference curve was used to determine the concentration of nitrite in each experimental wells, which were constructed using sodium nitrite (Sigma Aldrich, UK) diluted in RPMI-1640 medium at a range of concentrations (0, 0.1, 1.0, 10, 25, 50, 80 and 100μM).

2.2.5.5 iNOS expression analysis-Dot blot assay

The dot blot method was used to determine the concentration of iNOS expressed by the cell lysates (Bloch et al., 1999). U937 cells were treated respectively with the AXs and LPS samples (50μg/ml) for 24h. Then, 5μl of treated cell samples were individually added to 500μl lysis buffer samples (1:100 dilution). Human iNOS enzyme was diluted in iNOS protein standard buffer to give 1:2, 1:4, 1:6, 1:8 and 1:10 standards. The cell lysis samples and standards were set out as 5μl drops on the nitrocellulose membrane (NC 45 nitrocellulose membrane, Serva Electrophoresis GmbH, Heidelberg, Germany) 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 transferred to a solution of the primary antibody. The blot was shacked at 60rpm at 4°C overnight (≤20h).shaking.

231 Following incubation the primary antibody solution was decanted and the
232 membrane was washed using TBS buffer. Secondary antibody solution was added
233 to the blot and the membrane was shaken at 60rpm at room temperature for 1h.
234 The secondary antibody solution was removed and the membrane was washed
235 using TBS buffer. Then, the chemiluminescent detection agent was added
236 (Biological Industries (BI), Lichfield, UK). Then, the membrane was placed in a
237 G:Box (Chemi HR16, Syngene, Cambridge, UK) for the images of membrane to be
238 captured and processed. The Image J software (National Institute of Health, USA)
239 was used to quantitatively determine the levels of iNOS. The dot blot assay of iNOS
240 was repeated three times in separate experiments.

241 ***2.2.6 Statistics***

242 The assays, unless otherwise stated, were performed in triplicate and the
243 means were compared using one-way ANOVA. A value of $p \leq 0.05$ was chosen as the
244 criterion of statistical significance. The data were expressed as means ± standard
245 error.

3. Results and Discussions

3.1 AXs extraction from pentosan fraction of wheat flour

In the present study, the AX content of pentosan fraction, which was separated from wheat flour through high pressure disintegrated process was determined as 15.79±0.46% (dry basis). As table 1 shows, compared with water extraction, the AXs yield significantly increased from 7.54% to 12.83% ($p<0.05$) with increasing endoxylanase (P-BG) concentration, 0 to 400 ppm. Particularly, in the concentrations range of 50 ppm and 200 ppm, the significant increases in extraction yield is positively corresponding to the increase of endoxylanse concentration ($p<0.05$). Higher concentrations from 200ppm to 400ppm did not change the yield significantly further ($P>0.05$), indicating that the optimum combination for practical use in the extraction process could be based on a concentration range from 200ppm to 400ppm. In addition, the temperature of 30°C-40°C showed a higher AX extraction yield using associated conditions of 200ppm endoxylanase, 2h and pH 4.5 than other temperatures used in this study. Moreover, under the conditions:400 ppm endoxylanse, at 40°C for 2h, AXs

262 extraction from the pentosan fraction reached the highest recovery, which is 81.25%
263 of the total AXs content.

264 The major reason for the increased yield achieved using the
265 endoxylansetreatment is that this enzyme is able to attack the xylan backbone,
266 cleaving internal β-(1,4)-linkages and a portion of water un-extractable AXs
267 (WUAXs) will be rendered soluble and extractable (Andersson et al., 2003; Li et al.,
268 2013; Swennen, Courtin, & Delcour, 2006). This means that some WUAXs of
269 cell wall in pentosan fraction was released into solution, which resulted in an
270 increase in AXs extraction yield. According to the theory of enzyme kinetics, with
271 a certain substrate concentration, the enzyme is merely a catalyst which given
272 sufficient time could convert the substrate to the maximum extent (Berg et al.,
273 2002). However, considering the efficiency of the AX extraction process, the
274 extraction time selected in this experiment was 2h as this would be appropriate
275 for use in an industrial production system. The results indicated extraction
276 temperature and endoxylanase concentration demonstrated significant effects on
277 AX extraction yield.

3.2 Structural characterisation of AXs

3.2.1 Monosaccharide compositions and branch degree analysis of AXs

Table 2 shows that the enzyme extracted AXs samples (E-WEAXs) have a higher A/X ratio than water extracted AXs (WEAXs), and the increase of A/X ratio of E-WEAXs linearly associated with the concentration of endoxylanase from 0ppm to 400ppm (R^2=0.958). In fact, the A/X ratio represents the degree of branching of AXs, which is an indicator of the relative proportions of the substituted residues in xylan chains (Izydorczyk & Biliaderis, 2007). Therefore, the results indicate that AXs extracted from the pentosan fraction using the enzymatic treatments contain more substituted xylose residues.

3.2.2 Molecular characterisation of AXs

The overall Mw distribution of AXs ranged from 159Da to 794KDa ($1\times10^{2.2}$-$10^{5.9}$Da) and it was divided into four ranges (Table 2). The WEAX are mainly (78.52%) wiht the larger Mw range 10KDa to 794KDa (ranges 1 and 2) whereas E-WEAXs are mainly (81-89%) with the smaller Mw range 1KDa to 100KDa (ranges 2 and 3). As shown in Fig. 1, the Mw distribution curve of the E-WEAXs

294 contains two main peaks: the major one, Mw of 12.22KDa (log_{10}Mw≈4.1), and the
295 lesser, Mw of 3.72KDa (log_{10}Mw≈3.6), for which the average degree of polymer
296 (avDP) are 93 and 28 respectively. However, the WEAX sample comprises mainly
297 one peak, 501.19KDa (log_{10}Mw≈5.7), for which the avDP is 3797. In addition, Table
298 2 shows that as the concentration of endoxylanase increased from 50ppm to
299 400ppm, the proportion of the E-WEAX in the small Mw ranges 3 and 4 (0.16KDa
300 to 10KDa) increased from 39.19% to 54.36%, meanwhile the graph of Mw
301 distribution (Fig. 1) shows that the Mw peak at around 3.72KDa (log_{10}Mw≈3.6) as
302 progressing enlarged with the concentration increase of endoxylanse According
303 the previous studies, endoxylanase attacked the β-1, 4 linked D-xylopyranosyl
304 backbone and break down xylan chains, thus, reducing the molecular weight of
305 AXs during enzymatic extraction (Courtin & Delcour, 2001; Izydorczyk & Biliaderis,
306 2007). Zhang et al. (2014) indicated that the Mw of AXs varies depending on the
307 extraction and treatment methods used. Endoxylanase treatment appears to be
308 one of the most effective methods for modifying AXs with a relatively low Mw
309 distribution.

3.3 Immunomodulatory activity of AXs with various molecular structures

In order to further investigate the possible relationship between immunomodulatory activity and molecular structure, WEAX and E-WEAX (400ppm) with significantly different Mw distributions and monosaccharide compositions were selected for the *in vitro* studies.

3.3.1 Effects of AXs on growth and viability of U937 cells

As shown in Table 3, the total cell counts of U937 macrophages treated with AXs (50, 500, 1000μg/ml) are not significantly different compared to control (untreated cells) ($p>0.05$). Therefore, WEAX and E-WEAX did not present a stimulatory effect on the growth of U937 cells over the period of 24h. In addition, the viability of cells with AX treatments was typically 90%, which is similar to the control cells, suggesting that AX samples also have no significant effect on U937 cell survival over the test period of 24h.

However, the total counts of U937 cells were reduced significantly after treatment with 500 and 1000μg/ml LPS compared with untreated control cultures ($p<0.05$), showng inhibitory effect on cell growth, but it did not appear at

326 relatively low concentrations (50μg/ml) of LPS treatments. In addition, the
327 viability of cells with LPS treatments did not show a significant difference
328 compared with control cells ($p>0.05$), suggesting that LPS (50-1000μg/ml) has no
329 effect on U937 cell survival over 24h. This suggests that high concentrations of LPS
330 may have an inhibitory effect on U937 cell growth. Previous studies have indicated
331 that LPS inhibits and blocks macrophage proliferation and it depended on the
332 incubation time and dosage (Muller-Decker et al., 2005; Vadiveloo et al., 1996;
333 Vairo et al., 1992). The inhibitory effect of LPS on cell proliferation was proposed
334 to be tightly regulated through a complex network of cytokines. For example,
335 Vadiveloo et al. (2001) found that bacterial LPS had an inhibitory effect on cell
336 proliferation in mouse marrow-derived macrophages. They found LPS inhibited
337 the expression of cyclin D1, which is an essential protein for proliferation in many
338 cell types.

339 Botanical polysaccharides extracted from plants have received considerable
340 attention in bioscience due to their wide immunomodulatory activities and low
341 toxicity (Schepetkin & Quinn, 2006). Compared with LPS, the present study

342 indicated that the AX extracts have no inhibitory effects on the viability and cell
343 growth of the human U937 macrophage, even at 1000μg/ml.

344 ***3.3.2 Effects of AXs on NO production by U937 cells***

345 Table 4 shows the NO production by U937 cells after treatment with WEAX, E-
346 WEAX and LPS over the concentration range from 1 to 500μg/ml. both AXs
347 extracts significantly elevated NO production by U937 cells after 24h incubation
348 period compared with the untreated control ($p<0.05$). Furthermore, the amount
349 of NO production significantly increased with the treatment of 10μg/ml of E-
350 WEAX compared to with lower concentrations ($p<0.05$). The highest amount of
351 NO released by the E-WEAX treatment was 67.77μM at 50μg/ml. However, there
352 was a significant decrease in NO secretion following E-WEAX treatment at
353 500μg/ml ($p<0.05$) compared to that at 50μg/ml. Unlike E-WEAX treatment, NO
354 secretion increase by WEAX treatment appeared to be significant till the
355 concentration reached 500μg/ml ($p<0.1$), suggesting the peak amount of NO
356 released by the WEAX treatment may not have been reached. Hence, these results
357 indicate that there is a possibly optimal dose of E-WEAX for NO production in the

358 range 10-50μg/ml whilst WEAX has a significantly different optimum dose above
359 500μg/ml. In addition, in the concentration range from 10 to 50μg/ml, NO
360 response of WEAX is much more modest than that produced by similar
361 concentrations of E-WEAX ($p<0.05$). These comparisons show that there are
362 obvious differences between E-WEAX and WEAX treatments in relation to NO
363 stimulation, Again E-WEAX showed stronger immunomodulatory activity than
364 WEAX in the present assay.

365 Secondly, LPS, used as a positive control, significantly stimulated NO secretion
366 at concentrations of 1 to 50μg/ml (Table 4) compared to the untreated control,
367 which is similar to E-WEAX. However, at 500μg/ml of LPS, the amount of NO
368 produced by the U937 cells significantly decreased ($p<0.05$) compared to lower
369 concentrations of LPS, reflecting the substantial inhibitory effect on cell growth
370 and viability at this concentration (Table 3). Compared with AXs, the NO produced
371 following LPS treatment is consistently higher at the concentration range of 1 to
372 50μg/ml.

373 It is obvious that there is a relationship between Mw and structure of AXs and

374 their immunomodulatory activity. As shown, the main structural differences
375 between these two AX samples was in the low Mw range of 1-10KDa. The E-WEAX
376 contains a higher AX portion of AX with lower avDP in this small Mw range
377 compared with WEAX. In addition, E-WEAX presented a higher A/X ratio (0.83)
378 compared to WEAX (0.48). This means that the xylan chains of E-WEAX have more
379 arabinose substitutes. Thus, the large difference in NO stimulatory activity
380 between the two AX samples may be associated with the difference in the low 1-
381 10kDa Mw fraction and the higher A/X ratio.

382 ***3.3.3 Effects of AX treatments on iNOS expression by U937 cells***

383 In order to obtain a better understanding of AX modulation of NO production,
384 the effect of WEAX and E-WEAX on iNOS levels was determined using *in vitro*
385 testing. As shown in Fig. 2A, the effect of AXs treatment on iNOS expression of
386 U937 cells was detected by using dot blot. WEAX and E-WEAX significantly
387 elevated the level of iNOS expression by U937 cells after a 24h incubation period
388 compared with the control ($p<0.05$). From the result of the densitometry analysis
389 shown in Fig. 2B, E-WEAX and WEAX resulted in a 2.6 and 2.1 fold increase in iNOS

390 concentration from U937 cell lysates respectively compared with the control. In
391 addition, the amount of iNOS following treatment with E-WEAX was significantly
392 higher than with WEAX ($p<0.05$). LPS was used as a positive control producing a
393 significant increase in iNOS expression compared to control ($p<0.05$).

394 It is obvious that the stimulatory effect of AXs on iNOS induction is highly
395 correlated with their stimulatory activity on NO production. Therefore, the
396 increased NO production by AXs treatment was possibly due to induced levels of
397 iNOS by U937 cells. The LPS (positive control) also showed a high stimulatory
398 activity on iNOS levels in U937 cell lysates in the testing. This is in agreement with
399 the previous reports that the expression of iNOS in macrophage is induced by
400 cytokines (such as IFN-γ and TNF-α) and microbial polysaccharides (such as LPS),
401 which affect the conversion of L-arginine to citrulline by cationic amino acid
402 transporters and the expression of both iNOS mRNA and protein (Bogdan, 2000).
403 More recently a study found that polysaccharides from D. officinale were able to
404 increase iNOS expression and NO production in RAW 264.7 cells. They indicated
405 that the stimulatory ability of D. officinale polysaccharides on iNOS expression was

406 associated with the disruption of IκBα/NF-κB complexes, leading to the activation
407 of NF-κB (H. Cai et al., 2015; H. L. Cai et al., 2012). Based on these previous reports,
408 it is reasonable to propose that AXs may stimulate NO production in U937 cells
409 through the iNOS pathway. Moreover, the dot blot experiment showed that E-
410 WEAX (50μg/ml) had a higher stimulatory effect on iNOS levels in U937 cell
411 lysates compared to the effect of WEAX at the same concentration. The difference
412 in stimulatory effect of E-WEAX and WEAX on iNOS induction overlaps with their
413 significantly different stimulatory activity on NO production. Hence, AXs with
414 different Mw distributions and branch degree may result in different stimulatory
415 effects on the iNOS expression pathway in U937 cells and thus affecting theon NO
416 production.

417 **4. Conclusions**

418 In this study, an enzymatic method has been developed to efficiently extract
419 high yields of AXs with a high proportion of low MW material and a high degree of
420 branching from wheat flour pentosan. The analysis of the relationship between the
421 molecular structures and the immunomodulatory activity of AX samples in *in vitro*

testing suggests differences in the stimulatory effect on NO secretion are closely associated with the enzyme modified AXs which have a much higher proportion of lower Mw AXs and higher A/X ratio than the non-enzyme treated AXs. Furthermore, the effects of AXs on iNOS levels of human macrophage cells positively related to the increase in NO secretion, which suggest a pathway by which AXs modulate NO production in human macrophage cells. This is an exciting area for future research, the findings of which may elucidate the precise mechanism through which AXs modulate immune responses.

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566 **Table 1.** The AXs extraction yield (dry basis) under different conditions of
567 enzymatic treatment

Treatment conditions		Extraction yields of AXs	AX content of Raw material
Treatment Temperature	20°C	11.12±0.39% [a]	15.79±0.46%
	30°C	12.97±0.34% [b]	
	40°C	12.73±0.53% [b]	
Treatment Time	2 h	12.72±0.54% [b]	
	3 h	12.21±0.34% [b]	
	4 h	12.44±0.34% [b]	
Endoxylanase Concentration	50ppm	10.31±0.26% [a]	
	100ppm	10.71±0.55% [a]	
	200ppm	12.70±0.55% [b]	
	300ppm	12.75±0.40% [b]	
	400ppm	12.83±0.35% [b]	
Control	Water extraction	7.54±0.47% [c]	

568 *(20℃-40℃) means extracted AXs using the different enzyme treatment temperatures and other*
569 *extraction conditions were set up as same (pH4.5, 2h, 200ppm endoxylanase); (2h-4h) means extracted*
570 *AXs using the different enzyme treatment time and other extraction conditions were set up as same*
571 *(pH4.5, 40℃, 200ppm endoxylanase); (50-400ppm) means extracted AXs using enzymatic treatment at*
572 *various concentrations at pH 4.5, 40℃ for 2h. The control indicates water extractable AX (WEAX)*
573 *without enzymatic treatment. The extraction yields are presented as mean + SD and experiments were*
574 *conducted in triplicate. The mean values with different letters (a, b and c) indicate significant differences*
575 *($p<0.05$) in AX extraction yield for each sample amongst all the treatments. The p-values were calculated*
576 *by Student's t-test using Excel.*

577 **Table 2.** The molecular structures of AX samples under different conditions of enzyme treatments

Treatment conditions		Monosaccharides compositions of AXs [a]					Mw distributions of AXs [c]			
		Ara(%)	Xyl(%)	Glu(%)	Gal(%)	A/X [b]	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	Water extraction	26.13±1.01	53.96 ±1.49	10.62±2.04	9.29 ±1.26	0.48	46.46%	32.06%	19.11%	2.37%
Enzyme	50ppm	28.15±1.34	49.65±1.22	11.92±0.95	10.28±1.32	0.57	15.27%	45.54%	35.42%	3.77%
Concentration	100ppm	29.93±0.69	47.92±2.02	11.42±0.83	10.74±1.23	0.62	9.26%	40.29%	46.72%	3.72%
	200ppm	26.77±1.23	37.13±0.76	20.3±0.86	15.81±0.32	0.72	7.17%	40.62%	46.82%	5.39%
	300ppm	27.11±0.76	35.37±2.92	21.32±1.03	16.2±1.43	0.77	6.47%	40.09%	48.88%	4.56%
	400ppm	28.74±1.77	34.51±0.34	21.85±2.22	14.9±2.32	0.83	5.75%	39.89%	49.51%	4.85%
Treatment	2h	26.88±0.83	37.21±0.48	20.3±0.63	15.61±0.78	0.72	7.33%	40.18%	47.38%	5.11%
Time	3h	27.52±0.34	38.22±1.32	19.84±0.63	14.42±0.79	0.72	7.48%	40.30%	47.04%	5.18%
	4h	26.72±0.54	37.81±0.86	20.01±0.89	15.46±1.46	0.71	7.13%	40.45%	46.93%	5.49%
Treatment	20°C	27.11±1.82	39.04±1.64	18.47±1.33	15.38±0.49	0.70	7.15%	40.52%	46.87%	5.46%
Temperature	30°C	28.15±1.67	39.19±0.76	18.01±2.55	14.65±0.64	0.72	7.05%	40.69%	46.79%	5.47%
	40°C	26.67±1.05	37.22±0.35	20.34±0.50	15.77±0.44	0.72	7.23%	40.45%	46.75%	5.57%

578 *a: The proportion of each monosaccharide in AX sample is presented as mean + SD and all experiments were conducted in triplicate. b: A/X means the composition ratio of*

579 *arabinose to xylose. c: The proportion of Mw in different range were analysed using the LC Data Analysis (SHIMADZU Corporation).*

580 **Table 3.** Effects of AXs and LPS on the growth and viability of U937 cells

Sample	Concentration of AXs and LPS (μg/ml)						Untreated Control	
	50		500		1000			
	Total count	Viability	Total count	Viability	Total count	Viability	Total count	Viability
LPS	1.18±0.029	92.63%	0.98±0.03*	92.33%	0.91±0.024*	88.15%	1.17±0.022	90.90%
E-WEAX	1.21±0.021	90.60%	1.22±0.046	90.09%	1.23±0.032	90.70%		
WEAX	1.25±0.026	90.72%	1.23±0.016	90.48%	1.24±0.037	89.60%		

581 *The total count* ($\times 10^6$) *indicated the count of total (viable and unviable) U937 cells after various*
582 *treatments for 24h; the viability was calculated by viable cell count/total cell account; the total cell count*
583 *and viability after AXs and LPS treatments were compared with the control (untreated by AXs or LPS)*
584 *using the one way ANOVA; The symbol * indicated the p-value < 0.05; The total cell counts are presented*
585 *as mean + SEM of six copies samples from experiment.*

586 **Table 4.** NO production by U937 cell under the 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 *	

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