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

39 clinical trials (Ghoneum and Matsuura 2004; Zhou et al., 2010; Li, et al., 2015) and
40 may reduce the risk of infection or cancer (Zhang et al., 2015). It has been proposed
41 that dietary fibres including AXs, exert their immunomodulation benefits possibly
42 through two mechanisms: (i) interaction with colonic epithelial cells and innate immune
43 cells (such as macrophages) which can mediate cytokine production leading to an
44 improved immune response, (ii) take up by microfold cells, macrophages and dendritic
45 cells from the intestines and transport to lymph nodes, thus distributingAXs around the
46 body (Mendis et al., 2016; Samuelsen et al., 2011). Several studies have suggested that
47 AXs may be considered a potential bioactive food supplement with immunity
48 improvement properties (Ghoneum and Matsuura 2004; Zhou et al., 2010; Li et al.,
49 2015). However, the role of AXs in certain specific immune responses such as the
50 expression of nitric oxide synthases in NO secretion path way still need further
51 investigation.
52 Nitric oxide (NO) is a versatile signalling molecule of the immune system
53 produced by various immunological cells,dendritic cell, NK cell, mast cells and
54 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

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

73 ## 2. Materials and Methods

74 ### *2.1 Materials*

75 The sample of dried pentosan fraction (moisture content 5.9%) was kindly
76 provided by Henan Lianhua Monosodium Glutamate Group Co., Ltd (Xiangchen,
77 Henan, China). Pentopan Mono BG (2500 U/g), an endoxylanase product (EC
78 3.1.2.8, family 11 of glycosyl hydrolases) from *Thermomyces lanuginosus*
79 (donor)/*Aspergillus oryzae* (host) was kindly supplied by Novozyme (Bagsvaerd,
80 Denmark). 1,4-α-D-Glucan-glucanohydrolase (α-Amylase heat stable, ≥500 U/ml)
81 from *Bacillus licheniformis* and Proteinase (≥500 U/ml) from *Aspergillus melleus*
82 were purchased from Sigma-Aldrich (Gillingham, UK). D-(+)-xylose for AXs
83 determination was purchased from Acros Organics (Loughborough, UK). D-
84 glucose, D-xylose, D-arabinose, and D-galactose for determination of
85 monosaccharide composition were purchased from Sigma–Aldrich (Gillingham,
86 UK).

87 U937 cell line was purchased from the Public Health England Culture

88 Collections. LPS (Lipopolysaccharides of *E. coli* serotype O111:B4,) used as a

89 positive control in the *in vitro* experiments was purchased from Sigma-Aldrich,

90 (Gillingham, UK). The medium of RPMI-1640 with L-Glutamine was purchased

91 from Lonza (Verviers, Belgium). Foetal bovine serum and penicillin-streptomycin

92 (10,000 units penicillin and 10mg streptomycin/ml) were purchased from Sigma-

93 Aldrich (St. Louis, MO, USA). Human iNOS enzyme (116µg/ml, Code: TP311819)

94 was purchased from Cambridge Bioscience, UK. Primary antibody, monoclonal

95 iNOS Antibody (4E5) was purchased from Novus Biologicals (Cambridge, UK).

96 Secondary antibody, the Rabbit anti-mouse Immunoglobulins/HRP (code: P0260)

97 was purchased from Dako (Glostrup, Denmark).

98 Other chemicals including analytical grade acids, alkaline and organic solvents

99 were purchased from Sigma Aldrich (Gillingham, UK). .

100 ***2.2 Methods***

101 ***2.2.1 Extraction of AXs***

102 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

119 bath for 10min to deactivate the enzymes and cooled to the room temperature.

120 After centrifugation at 6000g for 20min, the supernatant was collected and

121 ethanol was added into the supernatant to a ratio of 70% of ethanol/supernatant

122 (v/v).. The mixture was kept in the fridge overnight at 4°C. Then the mixture was

123 centrifuged at 600g for 20min. After centrifugation, the residue was collected and

124 washed with 20ml ethanol twice.. Then, the residue was placed in 20ml acetone

125 and washed for 1min. After that, the residue was dried in an oven at 45°C overnight.

126 The dried residue (dried AXs sample) was milled using an analytical mill (IKA A11

127 Basic, Guangzhou, China, 50/60Hz, 160W).

128 ***2.2.2 Determination of AXs extraction yields***

129 A standard curve for the determination of xylose was constructed using the

130 method described by Douglas (1980). This curve was used for the determination

131 of xylose content of raw material and extracted AX supernatants. The xylose

132 content was then used to calculate the AX content of samples based on their A/X

133 ratio as determined by mono-sugar composition analysis. The specific AX content

134 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.

151 ***2.2.4 Molecular structure characterisation of extracted AXs***

152 Size exclusion high-pressure liquid chromatography (SE-HPLC) with a
153 refractive index (RI) detector was used to determine the molecular weights and
154 size distribution of extracted AXs samples using methods as described by Li et al.
155 (2013). The average degree of polymerisation (avDP) was calculated by dividing
156 the apparent peak molecular mass by the molecular mass of anhydropentose
157 sugars (Courtin et al., 2008).

158 The determination of the molecular weight of AX samples was carried out
159 using SE-HPLC. Mobile phase (Buffer) consisted of 17g $NaNO_3$ and 0.65g NaN_3,
160 dissolved in 2L HPLC grade water. Eight pullulan standards (Shodex, Shanghai,
161 China) with molecular weights in the range of 5,000-800,000Da were used to
162 construct a standard curve. The AXs samples were dissolved in buffer to make
163 2mg/ml solutions and left overnight under gentle stirring. Then, they were filtered
164 through a 0.45μm nylon membrane and transferred to SE-HPLC analysis.

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

167 2031 RI Detector (Jasco Corporation, Tokyo, Japan). The two columns were
168 connected in series (BioSep-SEC-S 4000 first) were used to improve the peak
169 shape of AXs samples. Running time was 60min for each sample and the flow rate
170 was 0.6 ml/min. All analyses were conducted in duplicate.

171 ***2.2.5 in vitro testing***

172 **2.2.5.1 Cell culture**

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

178 **2.2.5.2 Polysaccharides medium preparation**

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

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

184 **2.2.5.3 Cell viability and growth analysis**

185 The growth and viability of U937 cells with and without treatment of AX
186 samples and LPS were assessed by cell count and trypan blue uptake. The U937
187 cells (viability ≥90%) were centrifuged at 1000g for 10min and suspended in
188 RPMI-1640 medium with 10% FBS such that the density of live cells was set at
189 1×10^6/ml. 100µl cell suspension was pipetted into each well of a 96-well
190 microplate. The AXs and LPS mediums prepared with three concentrations (50,
191 500 and 1000µg/ml) were pre-warmed to 37°C and then 100µl of medium, AXs
192 medium and LPS medium was added to six wells of containing cells and mixed
193 thoroughly. After 24h incubation, 40µl of cell fluid was pipetted from each well into
194 a tube, and 40µl of trypan blue (Sigma-Aldrich, UK) was added. The number of
195 cells stained (nonviable cells) and unstained (viable cells) with trypan blue were
196 counted using a TC10 automated cell counter (Bio-Rad, UK). The viability of cells
197 was calculated as a percentage by dividing the number of viable cells against to
198 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).

215 A nitrite standard reference curve was used to determine the concentration of
216 nitrite in each experimental wells, which were constructed using sodium nitrite
217 (Sigma Aldrich, UK) diluted in RPMI-1640 medium at a range of concentrations (0,
218 0.1, 1.0, 10, 25, 50, 80 and 100μM).

219 **2.2.5.5 iNOS expression analysis-Dot blot assay**

220 The dot blot method was used to determine the concentration of iNOS
221 expressed by the cell lysates (Bloch et al., 1999). U937 cells were treated
222 respectively with the AXs and LPS samples (50μg/ml) for 24h. Then, 5μl of treated
223 cell samples were individually added to 500μl lysis buffer samples (1:100 dilution).
224 Human iNOS enzyme was diluted in iNOS protein standard buffer to give 1:2, 1:4,
225 1:6, 1:8 and 1:10 standards. The cell lysis samples and standards were set out as
226 5μl drops on the nitrocellulose membrane (NC 45 nitrocellulose membrane, Serva
227 Electrophoresis GmbH, Heidelberg, Germany) with 5μl of BSA (1mg/ml BSA in
228 deionized water) used as a negative control (no iNOS protein) in the dot blot. The
229 membrane was transferred to a solution of the primary antibody. The blot was
230 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.

278 ## ***3.2 Structural characterisation of AXs***

279 ### ***3.2.1 Monosaccharide compositions and branch degree analysis of AXs***

280 Table 2 shows that the enzyme extracted AXs samples (E-WEAXs) have a
281 higher A/X ratio than water extracted AXs (WEAXs), and the increase of A/X ratio
282 of E-WEAXs linearly associated with the concentration of endoxylanase from
283 0ppm to 400ppm (R^2=0.958). In fact, the A/X ratio represents the degree of
284 branching of AXs, which is an indicator of the relative proportions of the
285 substituted residues in xylan chains (Izydorczyk & Biliaderis, 2007). Therefore,
286 the results indicate that AXs extracted from the pentosan fraction using the
287 enzymatic treatments contain more substituted xylose residues.

288 ### ***3.2.2 Molecular characterisation of AXs***

289 The overall Mw distribution of AXs ranged from 159Da to 794KDa ($1\times10^{2.2}$-
290 $10^{5.9}$Da) and it was divided into four ranges (Table 2). The WEAX are mainly
291 (78.52%) wiht the larger Mw range 10KDa to 794KDa (ranges 1 and 2) whereas
292 E-WEAXs are mainly (81-89%) with the smaller Mw range 1KDa to 100KDa
293 (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.

310 ***3.3 Immunomodulatory activity of AXs with various molecular structures***

311 In order to further investigate the possible relationship between
312 immunomodulatory activity and molecular structure, WEAX and E-WEAX
313 (400ppm) with significantly different Mw distributions and monosaccharide
314 compositions were selected for the *in vitro* studies.

315 ***3.3.1 Effects of AXs on growth and viability of U937 cells***

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

323 However, the total counts of U937 cells were reduced significantly after
324 treatment with 500 and 1000μg/ml LPS compared with untreated control cultures
325 ($p<0.05$), 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*

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

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565 blot assay

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 Concentration	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%
	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 Time	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%
	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 Temperature	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%
	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.*