Regulation of Inducible Nitric Oxide Synthase by Arabinoxylans with Molecular Characterization from Wheat Flour in Cultured Human Monocytes

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Abstract

The immunomodulatory activity of the arabinoxylans (AXs) extracts from cereal sources have been reported to impart health benefits in terms of immune enhancement. This study investigated the effect of enzymatic extraction on extraction yield and structure of AXs from wheat flour pentosan fraction. Under the optimised conditions, the extraction yield of AXs reached up to 81.25%. Furthermore, the study determined whether water-extracted AXs (WEAXs) and enzyme-extracted AXs (E-WEAXs) from wheat flour were able to differentially stimulate nitric oxide (NO) secretion through increased levels of inducible nitric oxide synthase (iNOS) in human U937 monocytes. The results indicated that AXs concomitantly induced ($P < 0.05$) both NO and iNOS productions in U937 monocytes compared to untreated cells. Compared with WEAXs, E-WEAXs resulted in a higher proportion of low Mw (1-10 KDa) AXs (49.51% versus 19.11% in WEAXs), a higher A/X ratio (0.83 versus 0.48 in WEAXs) and a higher yield (12.83 ± 0.35% versus 7.54 ± 0.47% in WEAXs). Moreover, E-WEAXs induced significantly ($P < 0.05$) greater NO and iNOS production per million viable cells (61.8 ± 2.7 μM and 42.41 ± 3.83 *n*g respectively) than WEAXs (51.6 ± 2.6 μM and 33.46 ± 1.48 *n*g respectively). The findings suggest AXs may heighten innate immune activity in the absence of infection or disease

through an iNOS-mediated stimulation of NO production. The immunomodulatory activity of the wheat-derived AXs was enhanced by enzyme treatment, with low Mw and high A/X ratio associated with elevated NO/iNOS levels in human monocytes compared to water-extraction.

Keywords*: arabinoxylans; wheat flour; extraction; molecular structures; immunomodulatory activity; nitric oxide; inducible nitric oxide synthase*

Introduction

Arabinoxylans (AXs) are hemicelluloses found in the outer-layer and endosperm cell walls of cereal grains, such wheat and corn (Izydorczyk and Biliaderis 2007, Fan *et al.* 2016, Li *et al.* 2016). It has been reported that oral administrated dietary fibre including β-glucans and AXs is able come into contact with the mucosal immune system (Mendis *et al.* 2016), in which intestinal epithelial cells together with the immune cells of the Peyer's patches play an important role in regulating immune responses (Smith *et al.* 2011). Previous animal study also showed oral administration of AXs (1.6 – 3.2 mg/day) significantly induced intestinal macrophage phagocytosis, delayed the hypersensitivity reaction and increased spleen lymphocyte proliferation (Zhou *et al.* 2010). Moreover, a human study involving 80 participants showed that consumption of rice-derived AXs as a dietary supplement (3 g/day for 8 weeks) significantly increased interferon gamma (IFN-γ) production in circulating leukocytes of healthy adults (Choi *et al.* 2014), confirming immunomodulatory effects of AXs on human peripheral blood mononuclear cells. Thus, AXs may be considered a bioactive food supplement with immunity improvement properties (Zhang *et al.* 2015). However, the role of AXs in certain specific

53 immune responses, including the expression of nitric oxide (NO) synthases and
54 downstream NO secretion, remains largely undetermined. The immune functions of NO
55 have been found to involve antimicrobial and anti-tumour activities *in vitro* and *in vivo*
56 (Lechner *et al.* 2005, Bogdan 2000). Inducible NO synthase (iNOS) is one isoform in a
57 family of NOS enzymes involved in the generation of NO from L-arginine. Expression of
58 iNOS in immune cells is induced during an immune response following stimulation by
59 microbial polysaccharides such as lipopolysaccharides (LPS) and immune cytokines
60 (Bogdan 2000). Recent studies have shown that AXs enhance NO secretion in both
61 murine and human monocyte/macrophage cell lines (Nagata *et al.* 2001, Ghoneum and
62 Matsuura 2004). However, the precise mechanism through which NO secretion is
63 stimulated by AXs has not been elucidated to date.

64 In addition, the molecular characteristics, such as the molecular weight (Mw) and
65 substitution degree (A/X) of cereal AXs, appear to affect their NO-inducing activities
66 (Zhou *et al.* 2010, Zhang *et al.* 2016). The endo-β-(1,4)-xylanases (EC 3.2.1.8) are
67 commonly involved in cereal AXs isolation and structural modification (Zhang *et al.* 2014).
68 The yields of AX extracts using enzymatic treatment showed generally low. Van Craeyveld

et al. (2010) extracted 32-55% of the wheat bran AX using 3 different endoxylanase treatments, and Escarnot *et al.* (2012) tested various xylanases on the AXs extraction yield of spelt bran and hull and the highest extraction recovery was 68.8% of the total AX of raw materials. The pentosan fraction of wheat flour is a by-product of wheat starch processing, and it has been found to be an AX-enriched source that usually consists of about 12% original AXs content (Li *et al.* 2013). However, the effects of extraction and modification conditions using enzymatic treatment on the yield and molecular structures of AXs from the pentosan of wheat flour have been limited reports. Thus, for the first time, this study investigated whether the iNOS isoform of the NOS enzymes mediates NO production in U937 human monocytes following treatment with AXs from wheat flour. Moreover, the differential influence of the extraction method on the molecular characteristics of AXs and subsequent induction of NO/iNOS levels was investigated.

Materials and Methods

Materials. A 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

85 of glycosyl hydrolases) from *Thermomyces lanuginosus* (donor)/*Aspergillus oryzae*

86 (host), was supplied by Novozyme (Bagsvaerd, Denmark). The U937 cell line was

87 purchased from the Public Health England Culture Collections. LPS (lipopolysaccharides

88 of *Escherichia coli* serotype O111:B4) was purchased from Sigma-Aldrich (Gillingham,

89 UK). RPMI-1640 cell culture medium with L-glutamine was purchased from Lonza

90 (Verviers, Belgium). Human iNOS (116 μg/ml, Code: TP311819) was purchased from

91 Cambridge Bioscience (Cambridge, UK). The primary antibody, monoclonal iNOS

92 antibody (4E5) (1 mg/mL), was purchased from Novus Biologicals (Cambridge, UK). The

93 secondary antibody, the rabbit anti-mouse immunoglobulins/HRP (P0260), was

94 purchased from Dako (Glostrup, Denmark).

95 **Extraction of AXs.** The pentosan fraction of wheat flour was ground to a 0.5 mm

96 particle size using an Ultra Centrifugal Mill ZM 200 (RETSCH Ltd. United Kingdom).

97 Milled pentosan sample (30 g) was mixed with 200ml of distilled water using a hand

98 blender (800W, WSB800U) for 45 s prior to water or enzyme extraction. For the water

99 extraction process, the pentosan-water mixture was incubated in a shaking water bath at

100 40 °C for 2 h followed by centrifugation (6000*g*, 20 min) and collection of the

101 supernatant (containing extracted AX). For the enzymatic extraction process, three
102 extraction conditions (enzyme concentration, extraction time and temperature) were
103 considered according to those previously described for endoxylanase (P-BG) in the
104 preparation of wheat endosperm AXs (Li *et al.* 2013). The effect of endoxylanase
105 concentration (50, 100, 200, 300, 400 ppm, w/w), extraction time (2 h, 3 h, 4 h) and
106 temperature (20 °C, 30 °C and 40 °C) on extraction yield and structure of AXs from the
107 mixed pentosan sample were determined by the enzyme extraction procedure.
108 Following the extraction process, samples were centrifuged (6000*g*, 20 min), and the
109 supernatants were collected. Then the supernatants (150 mL) was added to 300 μl
110 termamyl α-amylase (500 U/ml) at 90 °C to allow starch hydrolysis to occur. After 1 h,
111 the solution was cooled rapidly and adjusted to pH 7. One hundred μl proteinase (1
112 mg/mL, ≥3 units/mg) was added to the solution and the mixture was incubated at 60 °C
113 for 1 h to remove unwanted protein. The sample was placed in a boiling water bath for
114 10 min to deactivate enzymes and then centrifuged at 6000*g* for 20 min. After
115 centrifugation, the supernatants were then precipitated according to the ethanol
116 precipitation process described by Zhang *et al.* (2016). The residue was then placed in

117 an oven overnight at 45 °C to dry and then milled using an analytical mill (IKA A11 Basic,
118 Guangzhou, China, 50/60Hz, 160W).
119 **Determination of AX extraction yields.** The determination method of AXs yield
120 was constructed using the methods described by Zhang *et al.* (2016) to evaluate the
121 xylose content of the raw material and extracted AX supernatants.
122 **Determination of monosaccharide compositions of isolated AXs.**. The
123 composition assessment was performed as previously described by Zhang *et al.* (2016).
124 The mobile phase was HPLC grade water for the HPLC and isocratic elution was achieved
125 using a Shimadzu LC-10ADvp pump. Samples were analysed on SUPELCOGEL Pb (5 cm ×
126 4.6 mm) and Phenomenex ThermaSphere TS-130 columns combined with a JASCO RI-
127 2031 refractive index (RI) detector. All analyses were conducted in triplicate.
128 **Molecular weight distribution characterisation of extracted AXs**. Size exclusion
129 high-pressure liquid chromatography (SE-HPLC) with a RI detector (JASCO RI-2031,
130 Jasco Corporation, Tokyo, Japan) was used to determine the Mw and size distribution of
131 extracted AX samples using methods described by Zhang *et al.* (2016). The average
132 degree of polymerisation (avDP) was value of the apparent peak molecular mass divided

by the molecular mass of anhydropentose sugars (Courtin *et al.* 2008).

Cell culture. Complete cell culture medium was prepared using RPMI-1640 with L-glutamine (Luna, Belgium), 10% foetal bovine serum (FBS) and 2% penicillin-streptomycin (P/S). The human U937 monocyte cell line was grown in complete culture medium under sterile conditions in a 37 °C incubator with 5% CO_2 in air atmosphere, subculturing every 2 days to maintain high cell viability (≥ 90%). For experimental tests, cultured U937 cells were centrifuged at 1000*g* for 10 min and re-suspended in RPMI-1640 medium with L-glutamine and 10% FBS such that the density of cells was set at 1×10^6 viable cells/ml.

Polysaccharide medium preparations. The extracted AX samples and LPS were dissolved in the RPMI-1640 medium with 5% FBS overnight at 4 °C to allow the sample to become fully hydrated prior to sterile filtration through a 0.45 μm sterile filter. The LPS and solubilised AX samples were diluted in RPMI-1640 medium to yield a series of typical concentrations (1, 5, 10, 50, 500 and 1000 μg/ml) (Zhang *et al.* 2016) for the subsequent cell culture testing. The samples were stored at 4 °C prior to use.

Cell viability and growth. Cell growth and viability of U937 cells following

149 treatment with AX samples or LPS were assessed by cell counting and trypan blue
150 uptake. A 100 μL cell suspension at 1×10^6 viable cells/ml was pipetted into each well of
151 a 96-well microplate. The AX and LPS sterile medium preparations at three high
152 concentrations (50, 500 and 1000 μg/ml) were pre-warmed to 37 °C and 100 μL of each
153 concentration was added to six replicate wells of the microplate and mixed thoroughly.
154 After 24 h incubation (Ghoneum and Gollapudi 2003, Zhang *et al.* 2016), 40 μL from
155 each well was mixed with 40 μL of trypan blue (Sigma-Aldrich, UK) for 1 min prior to
156 analysis on a TC10 automated cell counter (Bio-Rad, UK). The number of blue stained
157 (non-viable) cells and unstained (viable) cells in each sample were counted.
158 **NO stimulation**. The NO assay was conducted as previously described by (Zhang *et*
159 *al.* 2016). Fifty μl of U937 cells at $1x10^6$ viable cells/ml was added to a 96-well
160 microplate prior to adding 50 μl of AX or LPS sample to replicate wells. Replicate
161 untreated controls were prepared by adding 50 μl medium with 50 μl U937 cells at
162 $1x10^6$ viable cells/ml to wells. Background levels of nitrite and/or interference from
163 nitrate present in AX samples were internally controlled for within the assay by taking in
164 account the direct activity of AX samples in replicate wells containing 50 μl medium and

50 μl of diluted sterile AX sample in the absence of U937 cells. All experimental samples were evaluated in triplicate with appropriate adjustments for background nitrite/nitrate levels. The microplate was incubated (37 °C, 5% CO_2) for 24 hours before mixing 50µl component A of the Griess' reagent to each well for 10 minutes at room temperature. Griess' reagent was made up with two components. Component A consisted of equal amounts by volume of 37.5 mmol/L sulphanilamide in deionized water and 6.5 mol/L HCl. Component B was 12.5 mmol/L N-1-napthylethylenediamine dihydrochloride (NEED) in deionized water. A further 50µl component B of the Griess' reagent was added to each well, mixing well and then incubating at 4°C for 20 minutes. The absorbance of each well was measured at 540nm using a microplate reader (Synergy HTX Multi-Mode Reader, Biotek, UK).

Determination of iNOS Levels. An immunoblot assay was used to determine the iNOS level in cell lysates according to previously described methodologies (Bloch *et al.* 1999). An equal volume (5mL) of AX treatment, LPS positive control (at 50 μg/mL) or untreated media control (RPMI-1640 with L-glutamine and 5% FBS) was incubated for 24 h with 5mL cultured U937 cells set at 2.0×10^6 viable cells/ml. Samples were

centrifuged (1000*g* for 10 min) and resuspended in the fresh medium at a density of 2×10^6 viable cells /mL. A 9ml aliquot of each sample was centrifuged at 1000*g* for 10 min and the supernatant discarded. The cell pellet was kept on ice and 150 μl ice-cold lysis buffer (0.1 M Tris-HCl and 1 mM ETDA in deionized water; pH 7.8) was added to each cell pellet. The samples underwent a repeated (three times) rapid freeze/thaw process to lyse the cells by placing in a freezer (-80 °C) for 15 min followed by rapid thawing in a 37 °C water bath until just thawed, with vortex mixing for 10 s in between each freeze/thaw cycle. Samples were repeatedly (10 times) placed in a sonication bath (5510E-DTH, 490W, 50/60Hz, Bransonic, Danbury, USA) for 10 s, followed by vortex mixing in between each sonication step. The samples were centrifuged at 10,000*g* for 10 min and supernatants (cell lysates) were collected.

A 5 μl aliquot of each cell lysate was added to 500 μl ice-cold lysis buffer (1:100 dilution). Human iNOS enzyme (116 μg/mL) was diluted in the iNOS standard buffer (10% glycerol, 100mM glycine and 25mM Tris-HCl in deionized water) to provide a set of iNOS standards at 116, 58, 29, 14.5 and 11.6 μg/mL. The cell lysates and iNOS standards were placed as 5 μl immunoblots on NC 45 nitrocellulose membrane (Serva

197 Electrophoresis GmbH, Heidelberg, Germany), and 5 μl BSA solution (1 mg/ml BSA in
198 deionized water) was used as a negative control (absence of iNOS protein). The
199 membrane was incubated in a 1:1000 dilution of monoclonal iNOS primary antibody
200 (4E5) overnight (≤ 20h) at 4°C with shaking at 60rpm.
201 The membrane was washed five times for 5 min using TBS buffer before incubating
202 with the rabbit anti-mouse immunoglobulins/HRP secondary antibody solution (1:1000
203 dilution) at room temperature for 1 h with shaking at 60rpm. The membrane was
204 washed five times for 5 min in TBS buffer and then 1 mL chemiluminescent detection
205 reagent (Biological Industries (BI), Lichfield, UK) was gently distributed across the
206 membrane and left to stand for 1 min in a dark room. Images of the membrane were
207 then captured using a G:Box (Chemi HR16, Syngene, Cambridge, UK) and Image J
208 software (National Institute of Health, USA) was used to quantify the levels of iNOS using
209 the iNOS standards. The iNOS level was determined from three replicate experiments.
210 **Statistical Analysis**. Experiments, unless otherwise stated, were performed in
211 triplicate and analyzed by one-way ANOVA followed by a posthoc Tukey test. A value of p
212 < 0.05 was considered statistical significance in all cases. Data were expressed as mean ±

standard error of the mean (SEM) unless stated otherwise.

Results and Discussion

Extraction of AXs from the pentosan fraction of wheat flour. The original AX content of the wheat flour pentosan was determined as 15.79±0.46% (dry basis). Compared with water extraction (Table 1), the extraction yield of AXs significantly increased ($P < 0.05$) from 7.54% to 12.83% with increasing enzyme (endoxylanase, P-BG) concentration (0 to 400 ppm). In particular, significant increases ($P < 0.05$) in extraction yield positively correlated (r = 0.98) with increased enzyme concentration within the range of 50 ppm to 200 ppm. Higher enzyme concentrations, from 200ppm to 400ppm, did not significantly increase the yield further ($P > 0.05$), indicating that maximum extraction condition was achieved, with no benefit in increasing the enzyme concentration any further beyond this range. The treatment temperature also showed a significant effect on the extraction yield of AXs. Temperatures of 30 °C - 40 °C showed higher AX extraction yield than 20 °C ($P < 0.05$) with an enzyme concentration of 200 ppm, a 2 h incubation and pH 4.5. Moreover, with an enzyme concentration of 400 ppm at 40 °C for 2 h, recovery of AXs from the pentosan fraction reached the highest recovery

level (81.25% of total AX content) identified in the study. The increased extraction yield upon enzyme treatment is likely in large part due to endoxylanases cleaving internal *β*-(1,4)-linkages in the xylan backbone and rendering a portion of water un-extractable AXs (WUAXs) soluble and extractable (Andersson *et al.* 2003). This means that a fraction of the WUAXs in the cell wall of the pentosan will be released into solution, resulting in increased extraction yield.

Monosaccharide compositions and branch degree analysis of AXs. The enzyme extracted AXs samples (E-WEAXs) had a higher A/X ratio of 0.83 (Table S1) than water extracted AXs (WEAXs) of 0.48, with A/X increasing linearly as the enzyme concentration increased from 0 ppm to 400 ppm ($R^2 = 0.958$). The A/X ratio represents the degree of branching of AXs, which is an indicator of the relative proportions of the substituted xylose residues in xylan chains (Izydorczyk and Biliaderis 2007). The degree of substitution plays a key role in the solubility of AXs. Less arabinose substitutes resulted in a lower solubility of AXs in water (Zhang *et al.* 2014), which the reason was the low A/X substitution fraction enhance aggregation of unsubstituted regions of the AXs stabilised by hydrogen bonds, which may result in an increase in viscosity or

245 precipitation of polymer chains (Izydorczyk and Biliaderis 2007). Therefore, the results
246 suggest AXs extracted from the pentosan fraction using the enzymatic treatments
247 contained more substituted xylose residues and higher solubility.

248 **Molecular characterisation of AXs.** The overall Mw distribution of AXs ranged
249 from 159 Da to 794 KDa and it was divided into four ranges (Table S1). Around 79% of
250 WEAXs were mainly in the larger Mw range of 10 KDa to 794 KDa whereas 81-89% of E-
251 WEAXs were in the smaller Mw range of 1 KDa to 100 KDa. The Mw distribution curve of
252 the E-WEAXs (Figure 1) contains two main peaks; the major one with Mw of 12.22 KDa
253 ($\log_{10}$Mw ≈ 4.1, avDP = 93), and the lesser one with Mw of 3.72 KDa ($\log_{10}$Mw ≈ 3.6,
254 avDP = 28). However, the Mw distribution curve for the WEAX sample comprises mainly
255 a single peak with Mw of 501.19 KDa ($\log_{10}$Mw ≈ 5.7, avDP = 3797). In addition, the Mw
256 distribution (Fig. 1) shows the Mw peak at around 3.72 KDa ($\log_{10}$Mw ≈ 3.6) progressing
257 enlarged as the concentration of enzyme increased from 50 ppm to 400 ppm. According
258 to previous studies, endoxylanase attacks the β-1, 4 linked D-xylopyranosyl backbone
259 and breaks down xylan chains, thus reducing the molecular weight of AXs during
260 enzymatic extraction (Izydorczyk and Biliaderis 2007). Zhang *et al.* (2014) showed that

261 the Mw of AXs varies depending on the extraction and treatment methods used.

262 Endoxylanase treatment appeared to be one of the most effective methods for modifying

263 AXs molecular structure, resulting in AXs with low Mw distribution. Furthermore, the

264 Mw has been indicated as a crucial factor influencing the physicochemical properties in

265 solution. Wheat AXs with low Mw exhibited less intrinsic viscosities and weak elastic

266 properties when in solution (Izydorczyk and Biliaderis 2007).

267 **Effects of AXs on the growth and viability of U937 cells.** In order to investigate

268 the possible relationship between the immunomodulatory activity and molecular

269 structure of AXs, WEAX and E-WEAX with significantly different Mw distributions and

270 monosaccharide compositions, were extracted with an enzyme concentration of 400

271 ppm for *in vitro* studies.

272 The total cell counts of U937 macrophages treated with high concentrations of AXs

273 (50, 500, 1000 μg/ml) were not significantly different ($P > 0.05$) compared to the

274 untreated control (Table 2). WEAX and E-WEAX did not induce a stimulatory effect on

275 the growth of U937 cells over a period of 24 h. In addition, the viability of cells following

276 each AX treatment was typically around 90%, which was not significantly different ($P >$

277 0.05) to untreated control cells, suggesting AX samples had no effect on U937 cell
278 survival over a 24 h period. However, the total count of U937 cells was significantly ($P <$
279 0.05) reduced after treatment with 500 and 1000 μg/ml LPS compared with the
280 untreated control (Table 2). This is in concordance with previous studies indicating that
281 LPS inhibits and blocks macrophage proliferation in a dose-dependent manner (Vairo *et*
282 *al.* 1992, Vadiveloo *et al.* 1996, Muller-Decker *et al.* 2005). The inhibitory effect of LPS on
283 cell proliferation is tightly regulated through a complex network of cytokines. For
284 example, Vadiveloo *et al.* (2001) found that bacterial LPS had an inhibitory effect on cell
285 proliferation in mouse marrow-derived macrophages. They found LPS inhibited the
286 expression of cyclin D1, which is an essential protein for proliferation in many cell types.
287 Botanical polysaccharides extracted from plants have received considerable attention in
288 bioscience due to their wide immunomodulatory activities and low toxicity (Schepetkin
289 and Quinn 2006). Compared with LPS, the present study indicated that the AX samples
290 have no inhibitory effects on the viability and growth of human U937 macrophages, even
291 at high concentrations of 1000 μg/ml.
292 **Effects of AXs on NO production by U937 cells.** NO production by U937 cells

293 following treatment with WEAX, E-WEAX or LPS over the concentration range of 1 to
294 500 μg/ml was determined (Table 3). The LPS positive control, significantly stimulated
295 ($P < 0.05$) NO secretion per million viable cells at all concentrations (1 to 500 μg/ml)
296 compared to the untreated control. Although significant changes in NO were not
297 detected across every single successive LPS concentration, overall the NO generated by
298 LPS significantly ($P < 0.05$) increased as LPS concentration increased from 0 to 500
299 μg/ml. All concentrations of WEAX and E-WEAX tested also significantly elevated ($P <$
300 0.05) NO production by U937 cells compared with the untreated control. NO levels per
301 million viable cells significantly increased in a dose-dependent manner as the
302 concentration of E-WEAX increased by 2 or more incremental concentration steps (from
303 1 to 10 μg/ml and 5 to 50 μg/ml), before reaching a maximum of 61.8 ± 2.7 μM following
304 treatment with 50 μg/ml E-WEAX. In contrast, NO stimulation by WEAX in the
305 concentration range of 10 to 50 μg/ml was substantially more modest compared to that
306 produced by similar concentrations of E-WEAX. The highest NO level produced was 53.4
307 μM per million viable cells after treatment with 500 μg/ml WEAX, but this was still
308 significantly increased ($P < 0.05$) in a dose-dependent manner compared to NO levels

309 generated by lower WEAX concentrations in the range 1 to 10 μg/ml. Thus, the optimal
310 dose of E-WEAX for maximum NO production was found to be < 500 μg/ml in this study
311 whereas WEAX probably had an optimum dose somewhere above 500 μg/ml. Although
312 the peak NO secretion for the WEAX treatment may not have been reached in the assay,
313 the findings suggest the maximum NO secretion was being approached and was
314 substantially below that of E-WEAX. These comparisons show that there are obvious
315 differences between E-WEAX and WEAX treatments in relation to NO stimulation, with
316 WEAX generally having weaker NO stimulation than E-WEAX. This is consistent with the
317 NO stimulatory activities of corn bran AXs with low and large Mw in U937 cells (Zhang *et*
318 *al.* 2016).

319 One of main structural differences between the two types of AX samples was in the
320 low Mw range of 1-10 KDa. The E-WEAX contained a higher portion of AX with lower
321 avDP in this small Mw range compared with WEAX. In addition, E-WEAX presented a
322 higher A/X ratio (0.83) compared to WEAX (0.48). Thus, the large difference in NO
323 stimulatory activity between the two AX samples may be associated with the difference
324 in the low 1-10kDa Mw fractions and A/X ratio.

Effects of AX treatments on iNOS levels in U937 cells. In order to obtain a better understanding of AX modulation of NO production, the effect of WEAX and E-WEAX on iNOS levels was determined in human U937 monocytes. Both WEAX and E-WEAX significantly elevated iNOS levels in U937 cells (Figure 2A) after 24h compared with the untreated control ($P < 0.05$), mirroring the elevation detected in NO production. E-WEAX and WEAX resulted in a 2.5 and 2.0 fold increase in iNOS level per million viable U937 cells respectively compared with the untreated control (Figure 2B). In addition, the amount of iNOS following treatment with E-WEAX was significantly higher than with WEAX ($P < 0.05$). Similarly, the LPS positive control significantly increased iNOS levels compared to untreated control ($P < 0.05$). The stimulatory effect of AXs on iNOS was highly correlated with their stimulatory activity on NO production. Thus, the findings suggest the increased NO production by AXs is probably due, at least in part, by elevated levels of iNOS in U937 cells. This is in agreement with the previous reports that show both iNOS mRNA and protein levels in macrophages are induced by cytokines (such as IFN-γ and TNF-α) and microbial polysaccharides such as LPS (Bogdan 2000). More recently a study found that polysaccharides from *Dendrobium officinale* were able to

increase iNOS expression and NO production in RAW 264.7 cells. They indicated that the stimulatory ability of *D. officinale* on iNOS expression was associated with the disruption of IκBα/NF-κB complexes, leading to the activation of NF-κB (Cai *et al.* 2015). Thus, AXs may stimulate NO production in U937 cells through the iNOS pathway. Moreover, the immunoblots showed that E-WEAX (50 μg/mL) had a higher stimulatory effect ($P <$ 0.05) on iNOS level (42.4 ± 3.8 μg/mL per million viable cells) compared to the effect of WEAX (33.5 ± 1.5 μg/mL per million viable cells) at the same concentration. The difference in stimulatory effect between E-WEAX and WEAX on iNOS induction overlaps with their significantly different stimulatory activity on NO production.

The Mw distribution of AX is considered to be a significant determinant of their immune-modulatory activity. Physicochemical properties of AXs in solution including tertiary conformation, solubility, viscosity and elastic properties depend on their molecular weight and degree of branching (Izydorczyk and Biliaderis 2007). For other cereal polysaccharide, such as Beta-glucan, molecular size and solubility have already been confirmed to have a substantial effect on Dectin-1 receptor activation, which is associated with immune-modulating activities of Beta-glucan such as increased level of iNOS, IL-12, TNF-α, IL-1β and

IL-6 (Zhang *et al.* 2015, Liu *et al.* 2015). Recently, wheat AX was reported to activate the Dectin-1 receptor in the HEK-Null1 Dectin-1A and B cell lines (Sahasrabudhe *et al.* 2016). Therefore, it is reasonable to conclude that molecular structure, combined with these physicochemical properties of AX may confer receptor activation that result in different stimulatory effects on iNOS expression in macrophage and subsequent NO production.

4. Conclusion

Dietary intervention of foods and food-derived substances with immune-modulating activities is widely studied and considered a potential way of mediating immune functions to reduce the risk of infection or cancer (Zhang *et al.* 2015, Mendis *et al.* 2016). In this study, an enzymatic method was optimized to efficiently extract high yields (86%) of AXs with a high proportion of low MW (3.72 KDa) material and a high degree of branching (A/X = 0.83) from wheat flour pentosan. AX extracts significantly elevated NO secretion by the U937 cells compared with the untreated control ($P < 0.05$), suggesting the AXs have potential immunomodulation properties for improving immune function and reducing the risk of infection. Analysis of the relationship between the molecular structures and the immunomodulatory activity of AX samples suggests that enzyme-modified AXs, that

have a much higher proportion of lower Mw AXs and higher A/X ratio than the non-enzyme treated AXs, stimulate higher levels of NO production. The iNOS data suggest that stimulation of NO synthesis by AXs is closely mirrored iNOS expression in U937 cells. This is an exciting area for future research, the findings elucidating the precise mechanisms through which the molecular structure of AXs may modulate immunomodulatory activity. Further experimental work is required to identify the AX receptor(s) on macrophages and determine their interaction with iNOS expression.

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Notes

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List of Tables

List of Figures

Table 1. Extraction yield (dry basis) of arabinoxylans (AXs) under different enzymatic extraction conditions

Treatment conditions		Extraction yield of AXs	AX content of Raw material
Treatment Temperature	20 °C	11.12±0.39% [b]	
	30 °C	12.97±0.34% [a]	
	40 °C	12.73±0.53% [a]	
Under pH 4.5, 200 ppm endoxylanase for 2 h in all cases			
Treatment Time	2 h	12.72±0.54% [a]	
	3 h	12.21±0.34% [a]	
	4 h	12.44±0.34% [a]	
Under pH 4.5, 200 ppm endoxylanase at 40 ℃ in all cases			15.79±0.46%
Enzyme Concentration	50 ppm	10.31±0.26% [b]	
	100 ppm	10.71±0.55% [b]	
	200 ppm	12.70±0.55% [a]	
	300 ppm	12.75±0.40% [a]	
	400 ppm	12.83±0.35% [a]	
Under pH 4.5 at 40 ℃ for 2 h in all cases			
Control	Water extraction	7.54±0.47% [c]	

Extracted AXs using the different enzyme concentrations (50 ppm to 400 ppm), treatment temperatures (20 ℃ to 40 ℃) and extraction times (2 h to 4 h) were investigated in terms of yield and AX content. The control indicates water extractable AX (WEAX) that lacked enzymatic treatment. The extraction yields are presented as mean ± standard deviation and experiments were conducted in triplicate. Dissimilar superscripts (^{a,b,c}) highlight significantly different AX extraction yields among the various extraction treatments ($P < 0.05$).

Table 2. Effects of arabinoxylans (AXs) and lipopolysaccharides (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%	0.92±0.05*	90.21%	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%		

The total count ($\times 10^6$ cells) *indicates the number of total (viable and non-viable) U937 cells after various AX treatments for 24 h. The viability (%) was calculated as the viable cell count/total cell count x 100. The total cell count and viability after treatment with AXs or LPS were compared with the untreated control by one way ANOVA, with the symbol * highlighting a significant difference ($P < 0.05$). Total cell counts are presented as mean ± standard error of six replicate samples.*

Table 3. NO production per million viable cells by U937 cells following treatment with water extracted arabinoxylan (WEAX), enzyme extracted arabinoxylan (E-WEAX) or lipopolysaccharides (LPS)

Samples	Concentration of AXs and LPS (μg/ml)					
	0	**1**	**5**	**10**	**50**	**500**
LPS *		**63.0±2.1**	**64.5±0.9**	**64.8±1.9**	**65.7±1.6**	**70.8±0.9**
E-WEAX *		**51.9±3.5 $**	**51.1±2.5 $**	**58.9±2.5 $@**	**61.8±2.7 @**	**51.5±1.5 $**
WEAX *		**48.6±0.9 $**	**48.0±0.4 $**	**48.4±3.3 $**	**51.6±2.6 $**	**53.4±2.7 $**
Control	**43.0±0.4**					

*The NO_2^- concentration (μM), presented as mean ± standard error of triplicate samples, is a measure of NO production by U937 cells. The symbol * indicates significant increase ($P < 0.05$) in NO secretion following AX treatment at **all concentrations tested** compared to the untreated control. The symbol # indicates a significant change in NO secretion ($P < 0.05$) the two indicated concentrations of samples. The symbol @ indicates significant difference ($P < 0.05$) in NO secretion between treatment with E-WEAX and treatment with WEAX at the same concentration. The symbol $ indicates significant ($P < 0.05$) difference in NO secretion between E-WEAX or WEAX treatment and LPS treatment at the same concentration.*

Graphical Abstract

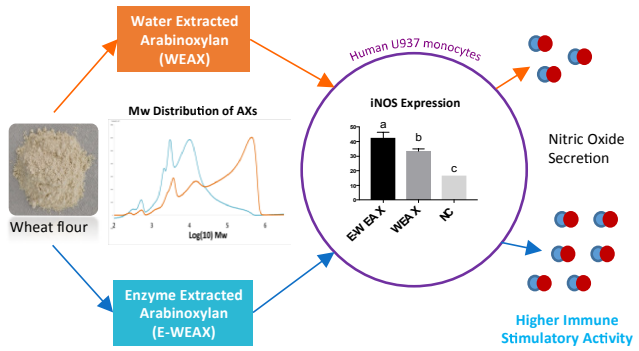


Figure 1

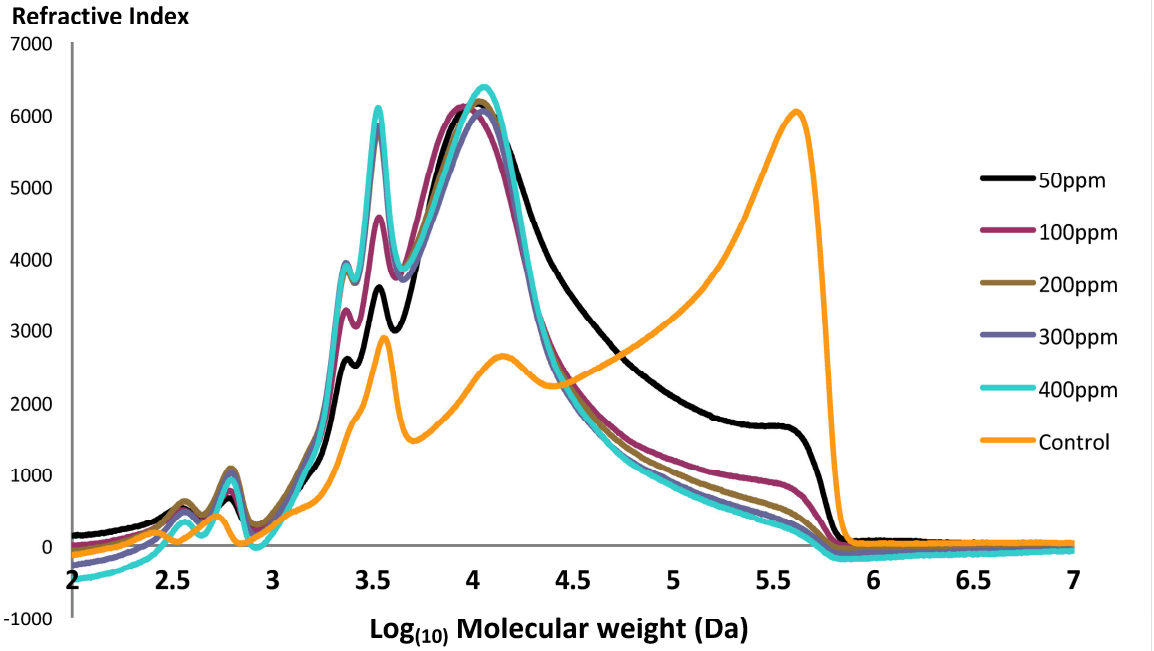
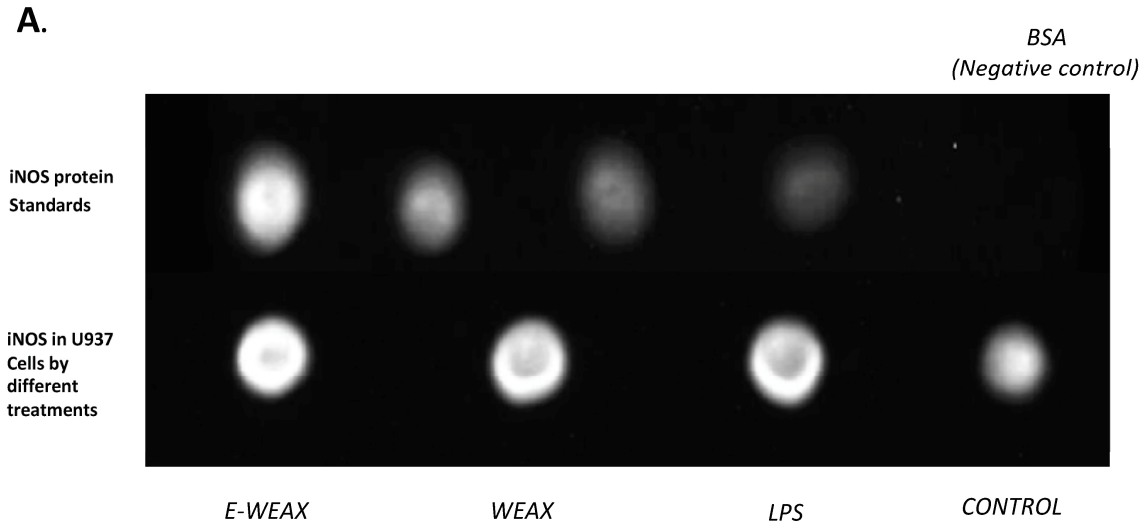
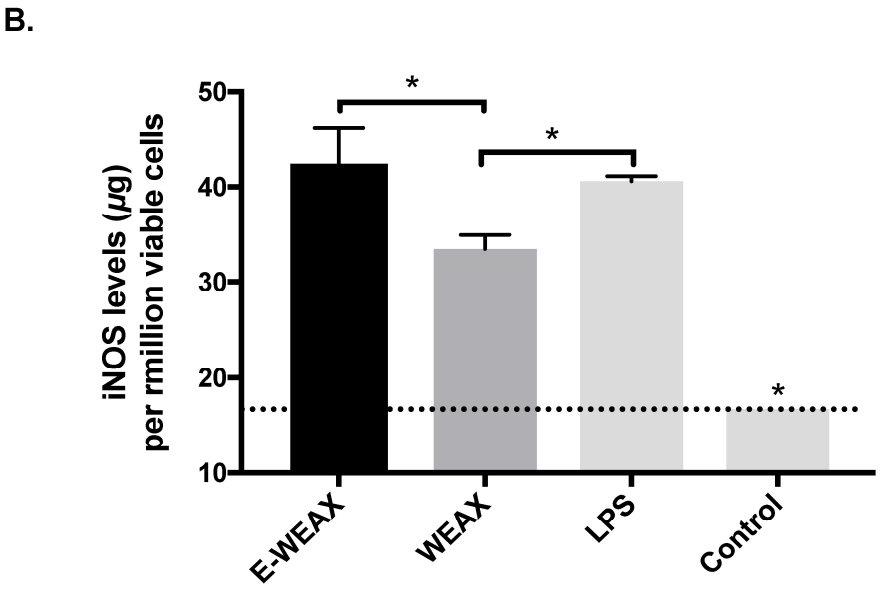


Figure 2


A.
BSA
(Negative control)
iNOS protein
Standards
iNOS in U937
Cells by
different
treatments
E-WEAX
WEAX
LPS
CONTROL


B.
iNOS levels (μg)
per rmillion viable cells
50
40
30
20
10
*
*
*
E-WEAX
WEAX
LPS
Control

583
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Table S1. The structural characteristics of arabinoxylan (AX) samples obtained under different enzyme extraction conditions.

Treatment conditions		Monosaccharides compositions of AXs [a]					Mw distributions of AXs [c]			
		Ara(%)	Xyl(%)	Glc(%)	Gal(%)	A/X [b]	Range 1: 10^5-$10^{5.9}$ Da	Range 2: 10^4-10^5 Da	Range 3: 10^3-10^4 Da	Range 4: $10^{2.2}$-10^3 Da
Control	Water	26.13±1.01	53.96 ±1.49^y	10.62±2.04^x	9.29 ±1.26^x	0.48	46.46%	32.06%	19.11%	2.37%
Enzyme	50 ppm	28.15±1.34	49.65±1.22^y	11.92±0.95^x	10.28±1.32^x	0.57	15.27%	45.54%	35.42%	3.77%
Concentration [d]	100 ppm	29.93±0.69	47.92±2.02^y	11.42±0.83^x	10.74±1.23^x	0.62	9.26%	40.29%	46.72%	3.72%
	200 ppm	26.77±1.23	37.13±0.76^x	20.3±0.86^y	15.81±0.32^y	0.72	7.17%	40.62%	46.82%	5.39%
	300 ppm	27.11±0.76	35.37±2.92^y	21.32±1.03^y	16.2±1.43^y	0.77	6.47%	40.09%	48.88%	4.56%
	400 ppm	28.74±1.77	34.51±0.34^y	21.85±2.22^y	14.9±2.32^y	0.83	5.75%	39.89%	49.51%	4.85%
Treatment	2 h	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 [e]	3 h	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%
	4 h	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 [f]	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%

a: The proportion of each monosaccharide in the AX sample is presented as mean + standard deviation and all experiments were conducted in triplicate. b: A/X represents the composition ratio of arabinose to xylose. c: The distribution (%) of AXs in different Mw ranges were analysed using the LC Data Analysis (SHIMADZU Corporation). d: The temperature range (20 °C to 40 °C) indicates the extraction treatment temperatures, keeping other extraction conditions constant (pH 4.5, 2 h incubation 200 ppm endoxylanase); e: The time range (2 h to 4 h) indicates the different enzyme extraction times, keeping other extraction conditions constant (pH 4.5, 40 °C, 200 ppm endoxylanase); f: The enzyme concentration range (50 to 400 ppm) indicates the different enzyme concentrations during the extraction of AXs, keeping other extraction conditions constant (pH 4.5, 40 °C for 2 h). Dissimilar superscripts ($^{x, y}$) highlight significantly different AX extraction yields among the various extraction treatments ($P < 0.05$).