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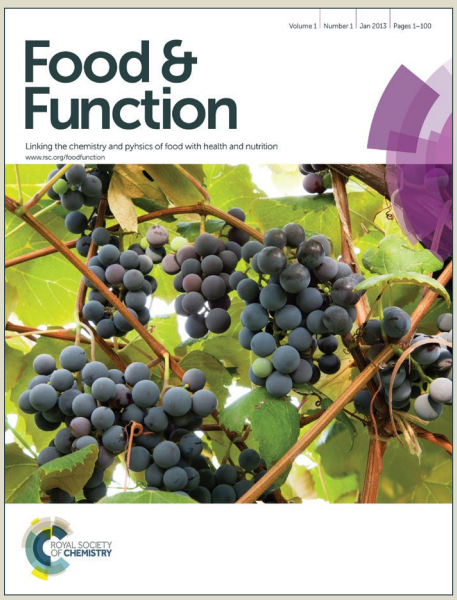

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Adjuvant properties of water extractable arabinoxylans with different structural features from wheat flour against model antigen ovalbumin

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Abstract

Despite the numerous benefits of AX on immune system and gut bacteria, the potential adjuvant activity of WEAX on immune responses has not been adequately investigated. In the present study, three kinds of WEAX with different structure feature were obtained and their adjuvant potential on the specific cellular and humoral immune responses in ovalbumin (OVA) immunized mice were assessed. Our data demonstrated that WEAX had potent effects on innate and acquired immune responses through up-regulating the NK cell activation and promoting Th2 type immune response. Furthermore, this study also elucidated possible relationship between the adjuvant activity of WEAX and the structure. Compared with the other characteristics of the WEAX, we found that the immunomodulatory activity may be related to their content of ferulic acid, and not to the molecular weight.

Key words

Water extractable arabinoxylan (WEAX); Immunomodulatory; Adjuvant; Molecular weight; Ferulic acid

1. Introduction

Vaccination is the most cost-effective approach for the control of infectious diseases. The majority of vaccines require association with adjuvants capable of increasing the potency or stimulating the appropriate immune response [1]. Polysaccharide-based compounds and formulations are potential vaccine adjuvant candidates [2]. Most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effect, which is a major problem with immunomodulatory bacterial polysaccharides and synthetic compounds [3, 4]. Thus, the food polysaccharides are recognized as an effective biological response modifier with low toxicity.

Arabinoxylan (AX) , one of the main components of cereal grain cell walls, consists of backbone chains of β-(1-4)-linked D-xylopyranosyl residues to which α-L-arabinofuranose units are linked as side chains [5]. AXs can be used as a source of fiber, providing the known associated health benefits. AXs extracted from wheat have been shown to exert positive effects on gut epithelial integrity, and enhance fermentation and regulation of intestinal bacteria [6, 7]. Several previous studies in our laboratory showed that AXs inhibited significantly the growth of transplanted tumors in mice and also could be explored as the good source of natural immunomodulator [8, 9].

The general structural description of AXs is said to vary significantly due to the complexity of tissue components within cereal grains and extraction procedures adopted [10, 11]. AXs can be fractionated into two categories based on extraction properties that have a structural and conformational basis: water-extractable (WEAX) and water-unextractable (WUAX) molecules. A large part of WUAX typically remains unaffected during its passage throughout the intestinal tract, causing a bulking effect. Compared to WUAX, WEAX were more effective. WEAX were highlighted as they are helpful in regulating blood cholesterol and acted as prebiotics for microorganisms residing in the gastrointestinal tract [12]. WEAX as soluble dietary fiber can be fermented by gut microbiota to produce physiologically active short chain fatty acids (SCFAs). Immunomodulation of polysaccharides are related to their

variation in structure [13]. Structural features like molecular weight, arabinose-to-xylose ratio (Ara/Xly), substitution pattern of arabinose, and content of feruloyl groups may be crucial to the biological activity of arabinoxylans such as fermentation property and the stimulation of the immune system [14].

Although there is a general consensus on immunomodulatory activities of WEAX isolated from wheat flour, the adjuvant potential of WEAX on the specific cellular and humoral immune responses in ovalbumin (OVA) immunized mice, and the strcture-immunomodulatory activity relation of WEAX remain unclear. In the present study, three kinds of WEAX with different structure feature were isolated from wheat flour by hydrothermal extraction, acid hydrolysis and ethanol precipitation, and their potential adjuvant activity were assessed.

2. Materials and methods

2.1. Materials

Wheat flour was supplied by Beijing Guchuan Wheat Flour Co. (Beijing, China). Thermo-α-amylase (Pentopan Mono) was kindly provided by China branch of Novozyme Co. (Beijing, China). Ovalbumin (OVA), concanavalin A (ConA), lipopolysaccharide (LPS), dimethylsulfoxide (DMSO), MTT (3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), propidium iodide [6], RPMI-1640 medium, and goat anti-mouse IgG, IgG1, IgG2a and IgG2b peroxidase conjugate were purchased from Sigma Chemical Co. (Saint Louis, Missouri, USA). Cytokine (IL-4 and IFN-γ) detecting ELISA kits were from Wuhan Boster Biological Technology Co. Ltd. (Hubei, China). Fetal bovine serum (FBS) was from HyClone Laboratories (Logan, UT, USA). CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, WI, USA).

K562 cell lines were purchased from Institute of Cell Biology, Chinese Academy Sciences (Shanghai, China). They were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with 2 mM L-glutamine (Sigma), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum in an incubator set at

37 °C and 5% CO_2.

2.2. Preparation of WEAXs with Different Molecular Weights

As for extraction of WEAX1, the refined wheat flour passed through 100 mesh sieve was suspended in 10 volume (w/v) of deionized water at room temperature for 1 h under continuous shaking and centrifuged at 10000 × g for 20 min. The supernatant was decanted and incubated with thermo-α-amylase for 90 min at 80 °C then boiled for 10 min to inactivate the enzyme. After cooled to room temperature and centrifuged at 5000 × g for 30 min. The supernatant was concentrated up to 1/5th of the original volume under reduced pressure in a rotary evaporator. The concentrate was precipitated by using ethanol (the final concentration 65%), followed by centrifugation at 5000 × g for 5 min at 4 °C. The sediments were dissolved in deionized water and centrifuged (5000 × g, 30 min, 4 °C). The supernatant was precipitated by ethanol as above and freeze-dried. The obtained fraction was WEAX1. The estimated relative weight average molecular weights (Mw) was about 87.58 kDa for WEAX1.

For extraction of WEAX2, WEAX1 was mixed with 10 volume (w/v) of deionized water, and incubated with 4% Citric acid (0.15 M) at 90 °C for 2 h with continuous stirring. After a cooling to room temperature, the hydrolysis solution was centrifuged at 5000 × g for 30 min, and the suspension was precipitated and purified as above and then freeze-dried. The WEAX2 was obtained. The estimated relative weight average molecular weights (Mw) was about 32.96 kDa for WEAX2.

For extraction of WEAX3, WEAX1 was hydrolyzed by 4% Citric acid (0.30 M) as described above. The pure ethanol was slowly added into the hydrolysis solution with continuous stirring to final concentration of 85%. The insoluble material was removed by centrifugation and purified as above. The estimated relative weight average molecular weights (Mw) was about 6.35 kDa for WEAX3.

2.3. Chemical analysis

All chemical analyses were performed in duplicate. Average molecular weights and molecular weight distributions were determined by high-pressure size-exclusion chromatography [15]. Protein, purified WEAXs fractions were determined by ICC standard methods No. 109/1, 104/1, 105/2 and the AOAC (Association of Analytical Communities) Official Method 996.11 (Starch (Total) in Cereal Products) [16]. Pentosan content was estimated by the orcinol–hydrochloric acid method [17]. The ferulic acid content was measured by HPLC [18].

2.4. Animals

Twenty health adult ICR mice weighing 20.0 ± 2.0 g of either sex were selected for oral acute toxicity test and forty ICR male mice (grade II, 6 weeks old) weighing 18.0 ± 2.0 g were used in this study. Animals were purchased from Vital River Lab Animal Technology Co. (Certificate No. SCXK-2012-0001, Beijing, China). The animals were housed in specific pathogen-free conditions (12-h light/12-h dark, 22 ± 1 °C, 55 ± 5% relative humidity). All mice were allowed to acclimate in the facility for 1 week prior to experiments. All experiments were carried out according to the P.R. China legislation regarding the use and care of laboratory animals and were approved by the Bioethics Committee of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College.

2.5. Acute toxicity test

An acute toxicity test was performed using OECD/OCED guideline 425. The test procedure minimizes the number of animals required to estimate the oral acute toxicity. The test involved 4 groups of 5 male and 5 female ICR mice/group. Animals were fasted (food but not water was withheld overnight) prior to dosing.

The WEAX1, WEAX2 and WEAX3 were respectively administrated in the dose of 5000 mg/kg body weight orally to one animal. This first test animal survived. Then, four other animals were tested sequentially. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days.

Animals were individually observed for mortality and clinical signs, weighed, and their food and water consumption measured throughout the study in line with the OECD guideline.

2.6. Experimental design

ICR mice were randomly divided into five groups with ten mice in each: control group; model group (OVA); orally administered WEAX1 group (OVA-WEAX1); orally administered WEAX2 group (OVA-WEAX2); orally administered WEAX3 group (OVA-WEAX3). The animals were subcutaneously injected twice with 100 μg of OVA with 2-week intervals. One day before the first immunization, the mice had already been orally administered daily for 15 days with WEAX1, WEAX2 or WEAX3 (100 mg/kg bodyweight). Saline-treated animals were included as controls.

2.7. OVA specific IgG titer and the IgG subclasses

Blood samples were collected at 2 weeks after the boost for detection of IgG titers and the IgG subclasses. Serum samples were used to measure OVA-specific IgG titer and isotypes by an indirect double antibody sandwich enzyme-linked immunosorbent assay as described [19]. Briefly, the microtiter plate wells (costar) were coated with 100 μL OVA solution (50, 25, 50 and 50 μg/mL in the carbonate-bicarbonate buffer for IgG, IgG1, IgG2a and IgG2b antibodies, respectively), and incubated overnight at 4℃. After three washes, the wells were blocked with 5% skimmed milk and incubated at 37℃ with 5% CO_2 for 1 h. Followed by three times of washing, 100 μL of serum (diluted serially for IgG titer analysis or diluted 1: 50 in PBS 5% skimmed milk for isotype analysis) was added to each well and incubated at 37℃ with 5% CO_2 for 1 h. The horseradish peroxidase-conjugated antibodies against IgG, IgG1, IgG2a or IgG2b were incubated for 1 h at 37℃ with 5% CO_2. After washing, the peroxidase activity was assayed as following: the substrate solution (10 mg of O-phenylenediamine and 37.5 μL of 30% H_2O_2 in 25 mL of 0.1 M citrate phosphate buffer, pH 5.0) was added to each well, and incubated for 10 min at 37℃ with 5% CO_2. The reaction was stopped by 2 M H_2SO_4. The optical density (OD) was measured in an ELISA reader at

450 nm. Data were expressed as the mean OD value of the samples minus the mean OD value of the blank.

2.8. Splenocytes proliferation assay

Proliferation of spleen cells was measured by MTT incorporation as described [8]. Splenocytes were isolated by gently pressing spleen tissue through nylon mesh filters. Cells were washed twice with RPMI-1640 and cultured in triplicate wells of 96-well flat bottom plates (5×10^6 cells/well) with ConA (5.0 μg/mL) or LPS (7.5 μg/mL) or OVA (10.0 μg/mL). After 44 h incubation at 37°C with 5% CO_2, 20 μL MTT (5 mg/mL) was added into each well for another 4 h incubation, then the MTT medium was removed, and 100 μL cell dissociation solution (20%, wt/vol SDS, 50%, vol/vol DMSO, pH 4.5) was added to each well, and the absorbance was evaluated on a microtiter plate reader (Bio-Tek MQX200) at 570 nm. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for the stimulation-cultures divided by the absorbance value for non-stimulated cultures.

2.9. Assay of NK cell activity

Spleens collected from each mouse were aseptically removed and placed onto a steel mesh of 200 immersed in chilled RPMI-1640 medium in a Petri dish. The spleen was passed through the steel sieve and collected in RPMI-1640 medium. Contaminating erythrocytes in the splenic cells were lysed with double distilled water. Remaining cells (splenic lymphocytes) were washed twice with RPMI-1640 and were cultured in 96-well U-bottom microtiter plate at 3×10^5 cells/100μL in RPMI 1640 complete medium for use as effector cells. The cytotoxic activities of the NK cells were determined using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit as previously described [20]. Briefly, for NK activity, splenic cells in each group were treated with K562 cells at effector: target (E: T) ratios of 50: 1. Cultures of effector and target were incubated for 4 h. Cellular cytotoxicity was determined by lactate dehydrogenase (LDH) release assay by using commercially available kit, NK cell activity was calculated as follows:

$$\%\text{Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

2.10. IFN-γ and IL-4 production by splenocytes in vitro

The single splenocyte suspension was prepared on day 14 after the boost, and the splenocyte (2.5×10^6 cells/mL) were incubated with OVA (final concentration 10 μg/mL) in 96-well culture plates at 37°C in 5% CO_2. After 48 h, the culture supernatant was collected for the detection of IL-4 and IFN-γ levels using commercial ELISA kits according to the manufacturer's instructions.

2.11. Pathological and histological findings

Necropsy with macroscopic postmortem examination was performed on all animals. The full set of tissues were preserved by fixation in 10% buffered formalin solution in line with regulatory guidelines. Full histopathology was performed in the control group and WEAXs groups.

2.12. Statistical analyses

Data were processed with SPSS 17.0 statistics software and results are given as mean ± standard deviation. Multiple means were compared with One-Way ANOVA. After homogeneity of variance test, heterogeneous differences were tested with Dunnett's-t method and the homogeneous one with the LSD-t method. Test standard was α=0.05.

3. Results

3.1. Characterizations of WEAXs

The basic chemical composition of three AXs are given in Table 1. The molecular weights of WEAX2 and WEAX3 were gradually lower than that of WEAX1 by citric acid hydrolysis.

WEAX1 and WEAX3 had the highest and lowest pentosan contents (78.6% and 62.2%, respectively). The main neutral sugars of the three samples were arabinose and xylose. The Ara/Xly radio of WEAX1, WEAX2 and WEAX3 are 0.80, 0.56 and 0.23

respectively. In addition, compared with WEAX1, WEAX3 contained more protein, while WEAX2 contained less ferulic acid.

3.2. Acute toxicity studies

The animals received 5000 mg/kg orally was not found to cause any mortality and non-significant changes were observed in wellness parameters used for evaluation of toxicity during the experimental period (14 days). The mice were physically active. No neurological or behavioral effects could be noted. No mortality was found up to 14 days study indicating that the LD 50 value was more than 5000 mg/kg body weight[21].

3.3. OVA-specific IgG and the IgG isotypes

The OVA-specific IgG and the IgG isotypes titers were measured by an indirect ELISA to evaluate the adjuvant effect of WEAXs on the humoral immune responses. Fig.1 showed that OVA-specific IgG and the IgG isotypes titers were significantly higher in mice orally administered WEAX1 or WEAX3 once a day for 15 days than that in OVA alone group ($p < 0.05$). The findings indicated that WEAX1 and WEAX3 significantly enhanced OVA-specific antibody production in the immunized mice. However, no significant differences were detected between OVA alone and OVA-WEAX2 groups.

3.4. Splenocytes proliferation responses

To detect the effects of WEAXs on splenocyte proliferative responses, the proliferation assay was performed by the MTT method. Mice were orally administered WEAXs for 15 days, and then immunized subcutaneously injected twice with 100 μg of OVA at 2 weeks intervals. Splenocytes were prepared at second week after the boost and cultured with ConA (5.0 μg/ml) or LPS (7.5 μg/ml) or OVA (10 μg/ml) or RPMI-1640 for 48 h. Splenocytes proliferation was measured by the MTT method as described in the work and shown as a stimulation index (SI). The values are represented mean ± SD (n = 10). As shown in Fig.2, SI of the proliferation response to ConA, LPS and OVA were gradually increased in mice administered

WEAX1 or WEAX3 ($p < 0.05$), while mice administered WEAX2 showed no significant difference compared to the OVA group.

3.5. NK cell activity

As shown in Table 3, the activity of NK cells of OVA group was increased compared with the control group. Interestingly, the NK cell activities in the spleens of WEAX1-treated mice and WEAX3-treated mice increased to 63.56% and 64.35% at ratios of 50: 1 (E: T), respectively. The results showed that WEAX1 and WEAX3 significantly enhanced NK cell activity against K562 cells line compared with the control group or OVA group ($p < 0.05$).

3.6. Effects of WEAXs on IL-4 and IFN-γ production

To evaluate whether WEAXs influence IL-4 and IFN-γ production, the concentration of IL-4 and IFN-γ was tested by ELISA. As shown in Table 3, IL-4 and IFN-γ production were significantly higher in the cultures of cells from the mice administered WEAX1 and WEAX3 than that from the OVA group ($p < 0.05$). However, IL-4 and IFN-γ production showed no significantly difference between the cultures of cells from the OVA-WEAX2 and OVA groups.

3.7. Effects of WEAXs on the body weight of mice

To evaluate the safety of WEAXs, the body weight of mice was measured before and after the first immunization and boost. No abnormal behavior and side effects were observed in mice throughout the experiment. Besides, there was no significant difference in the body weight between the mice administered WEAXs and the control group (Table 4) ($p > 0.05$).

3.8. Pathological and histological findings

There was no evidence of treatment-related adverse macroscopic findings in any of the animals at postmortem examination. Each drug group compared with the control had no significant differences in pathological changes. The histopathological of major organs was shown in Supplementary material 1.

4. Discussion

Ideally, adjuvants should promote an appropriate immune response, be biodegradable and not be immunogenic themselves. Adjuvants should also offer excellent safety, tolerability, ease of manufacture and formulation. Such adjuvant promote good humoral and cell-mediated immunity. The development of such adjuvants would benefit from increased knowledge of the molecular mechanisms and factors controlling these responses. In this study, it was demonstrated first the in vivo efficacy of WEAX isolated from wheat flour as an adjuvant for OVA-based vaccines.

The capacity to elicit an effective T- and B-lymphocyte immune response can be shown by the stimulation of lymphocyte proliferation response [3]. It is generally known that LPS stimulates B cells and ConA stimulates T cells. In this study, both WEAX1 and WEAX3 could improve the splenocytes proliferation in the OVA-immunized mice, and increase OVA specific IgG and the IgG subclasses titers, suggesting WEAX can promote the humoral and cellular immune response. Cytokines play a key role on the regulating functions of Th cells. In this study, WEAX improved the IgG1, Ig2a, and IgG2b titer in the OVA immunized mice, besides, up-regulated expression of IL-4 and IFN-γ. Antibody (IgG2a/IgG1) and cytokines (IFN/IL-4) are really demonstrating Th2 biased, suggesting the WEAX promoted Th2 type immune response.

NK cells are important in the defense against tumors and viruses, and represent the major populations of cytotoxic lymphocytes [22]. In this study, both WEAX1 and WEAX3 were found to significantly enhance the killing activity of NK cells from mice splenocytes, suggesting that WEAX could also enhance the specific and non-specific cytolytic activities against autologous cells and viruses.

An ideal adjuvant for vaccine should be safe enough to induce the minimal side effects to prove acceptable for use in healthy individuals. In acute toxic study, the three samples were found to be safe up to 5000 mg/kg orally which are in agreement with other previous studies [23, 24]. In this study, no abnormal behaviors and side effects

were observed in mice throughout the experiment, and there was no significant difference in the body weight between the mice of administered WEAX and the control. In addition, the pathological and histological findings also reveal that oral administration of 100 mg/kg doses in our study were safe or practically non-toxic. It suggests that oral administration of WEAX from wheat flour is safe. The acute toxic test is the preliminary study. In the future, we will continue the safety research by administering the AX through different routes in different species of animals and in human.

The structural properties of AXs determine to a large extent their physicochemical effects and fermentation processes as well as their immunological modulation in different organisms. However, the effect of different structural AXs are less studied as an adjuvant for OVA-based vaccines. Hot water treatments led to one population of WEAX1 with high molecular weight, high Ara/Xyl ratios, and containing large ferulic acid. The results are similar to those for WEAX in recent reports [25]. WEAX2 and WEAX3 fractions were obtained from wheat flour by different concentration citric acid hydrolysis and ethanol precipitation. The estimated relative weight average molecular weights were significantly decreased and reached 32.96 and 6.35 kDa for WEAX2 and WEAX3 suggesting that citruc acid hydrolysis was an effective method of reducing the molecular weight. Arabinose to xylose ratio indicates differences in degree of substitution of the xylan backbone with arabinose residues. The markedly decrease of the Ara/Xyl ratio from WEAX1 to WEAX3 may be mainly due to the release of arabinosyl branch residues by drastic hydrolysis conditions. The similar result was observed in other research [26].

Another significant differences were observed in ferulic acid contents among three AXs. WEAX1 contained more ferulic acid than WEAX2 and WEAX3 (822 μg/g vs. 366 μg/g and 507 μg/g). It is well known that ferulic acid by ester bond are chemically associated with AXs, but these chemical bonds are easily hydrolyzed from AX in acidic or alkaline environment, thus WEAX2 and WEAX3 contained less ferulic acid.

The mechanisms of the immunostimulating effects of wheat AX may be related to its function as a prebiotic [9]. Structurally different wheat-derived AXs have different prebiotic and fermentation properties. Some reports focused on the relationship between functional properties and molecular size of AXs. In vivo and in vitro studies showed that AXs with a lower avDP produced more colonic acetate and butyrate and boosted bifidobacteria concentrations in the cecum than those AXs with higher avDP [27, 28]. However, in our study, WEAX1 with the highest molecular weight and WEAX3 with the lowest molecular weight significantly enhanced immunomodulatory activity compared with the control group or OVA group. While WEAX2 with the middle molecular weight did not show the immunomodulatory activity. Although this point has not agreed with previous reports that AXs exhibit functional properties related to their molecular weight. Our result is consistent with many others findings. A research group reported the effects of AX with longer avDP on the innate immune response were more pronounced [29]. Similarly, another research team [30] also found that AX with a long chain displayed the strongest impact on immune modulation. It indicated that the influence of the molecular weight was apparently limited and possibly overshadowed by that of the other characteristics. Moreover, we observed that the ability to enhance the immunogenicity against the OVA induced increased with the improve in content of ferulic acid (WEAX2 < WEAX3 < WEAX1). We infered that the immunomodulatory activity may be related to their content of ferulic acid. The beneficial effects of AXs are attributed to ferulic acid besides their own molecular structures, which has a slow and continuous release and absorption through the colon to provide important antioxidant protection. There was other report demonstrating that presence of ferulic acid is essential for the antioxidant activity of arabinoxylans [25]. AXs in conjunction with ferulic acid residues not only exerted prebiotic but also antioxidant properties. Some scientists found that feruloyl AXOS more stimulated growth of Bifidobacterium than Xylo-oligosaccharides [31]. Some phenolics could selectively inhibit the growth of several pathogenic bacteria, while the growth of probiotic bacteria was less severely affected or even selectively increased [32, 33]. Those probiotic bacteria, such as Lactobacillus spp. and Bifidobacterium spp. were able to

372 release ferulic acid from AXs or AXOS by producing ferulic acid esterases, and the
373 ferulic acid can help AXs enhance the antioxidant capacity of blood serum and organs
374 in diabetic rats and inhibit oxidative DNA damage in lymphocytes [34, 35]. The
375 synergistic and additive effect of these phenolic acids largely explained the
376 augmentation of immunomodulatory activity with the improve in content of ferulic
377 acid.

378 In conclusion, these data we presented here demonstrated that WEAX extracted from
379 wheat flour by hydrothermal extraction and citric acid hydrolysis had potent effects
380 on innate and acquired immune responses through up-regulating the NK cell
381 activation and promoting Th2 type immune response. Furthermore, there was not a
382 correlation between WEAX molecular weight and immunomodulatory activity.
383 Compared with the other characteristics of the WEAX, we surmised that the
384 immunomodulatory activity may be related to their content of ferulic acid.

385 **5. Acknowledgement：**

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388 **6. Glossary**

389 WEAX: Water Extractable Arabinoxylan

390 Ara/Xyl: arabinose to xylose ratio

391 OVA: Ovalbumin

392 ConA: Concanavalin A

393 LPS: Lipopolysaccharide

394 MTT: 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

395 DMSO: Dimethylsulfoxid

396 NK: Natural Killer

397

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459 **Legends**

460

461 **Figure 1. Effects of WEAXs on OVA-specific IgG in OVA-Immunized mice**
462 **(OD450 value)**

463 Data areexpressed as the means ± SD. Significant differences with Control group
464 were designated as: #p < 0.05 and ##p < 0.01; Significant differences with OVA group
465 were designated as: *p < 0.05 and **p < 0.01

466

467 **Figure 2. Effects of WEAXs on the splenocyte proliferation in OVA-immunized**
468 **mice**

469 Data areexpressed as the means ± SD. Significant differences with Control group
470 were designated as: #p < 0.05 and ##p < 0.01; Significant differences with OVA group
471 were designated as: *p < 0.05 and **p < 0.01

472

473

474 **Supplementary material 1. Histological alteration.**

475 Representative results are shown: Hearts (A); Spleens (B); Livers (C); Lungs (D);
476 Kidneys (E).

477

Table 1 Weight-average molecular weight (Mw), number-average molecular weight (Mn) and polydispersity (Mw/Mn) of WEAXs from wheat flour

	WEAX1	WEAX2	WEAX3
Mw (kDa)	87.58	32.96	6.35
Mn (kDa)	74.92	21.48	5.57
Mw/Mn	1.169	1.535	1.140

Table 2 Structure feature and compositions of WEAXs extracted from wheat flour

	WEAX1	WEAX2	WEAX3
Protein (%)	8.80	7.97	32.0
Pentosan (%)	78.6	78.9	62.2
Arabinose (%)	49.7	33.2	7.50
Xylose (%)	62.1	59.2	32.4
Ara/Xyl	0.80	0.56	0.23
Ferulic acid (μg/g)	822	366	507

Table 3 Effects of oral administration of WEAXs on the activity of NK cell (%), IL-4 and IFN-γ production (pg·mL−1).

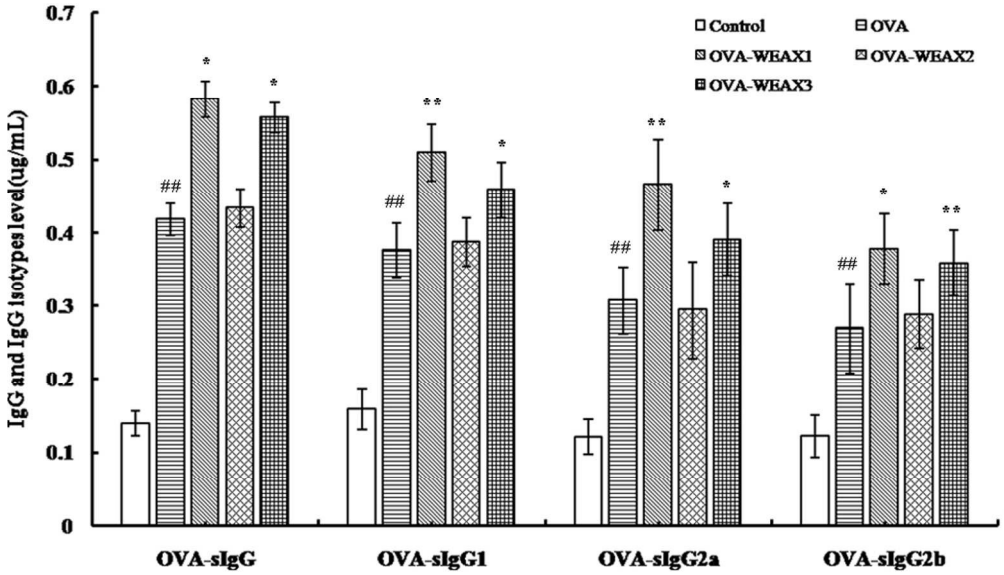
Group	NK cell activity	IL-4	IFN-γ
Control	36.20±6.33	109.62±14.52	441.75±78.14
OVA	46.63±11.14 #	113.34±19.60	460.29±93.54
OVA-WEAX1	63.56±10.40 **	171.68±17.67 **	616.60±132.59 *
OVA-WEAX2	48.21±14.30	123.78±20.14	504.95±120.92
OVA-WEAX3	64.35±14.54 *	153.52±13.63 *	558.32±98.14 *

Significant differences with Control group were designated as: #$p < 0.05$ and ##$p < 0.01$

Significant differences with OVA group were designated as: *$p < 0.05$ and **$p < 0.01$

Table 4 Effects of oral administration of WEAXs on the mean body weight (g)

Group	Before immunization	Before boost	2 weeks after boost
Control	28.0±1.2	30.4±1.0	32.6±1.4
OVA	27.8±1.9	30.7±2.1	31.8±2.8
OVA-WEAX1	26.4±2.0	30.4±2.5	31.0±2.4
OVA-WEAX2	26.9±1.1	29.4±1.0	30.5±1.0
OVA-WEAX3	26.1±1.7	30.4±2.2	32.5±3.0



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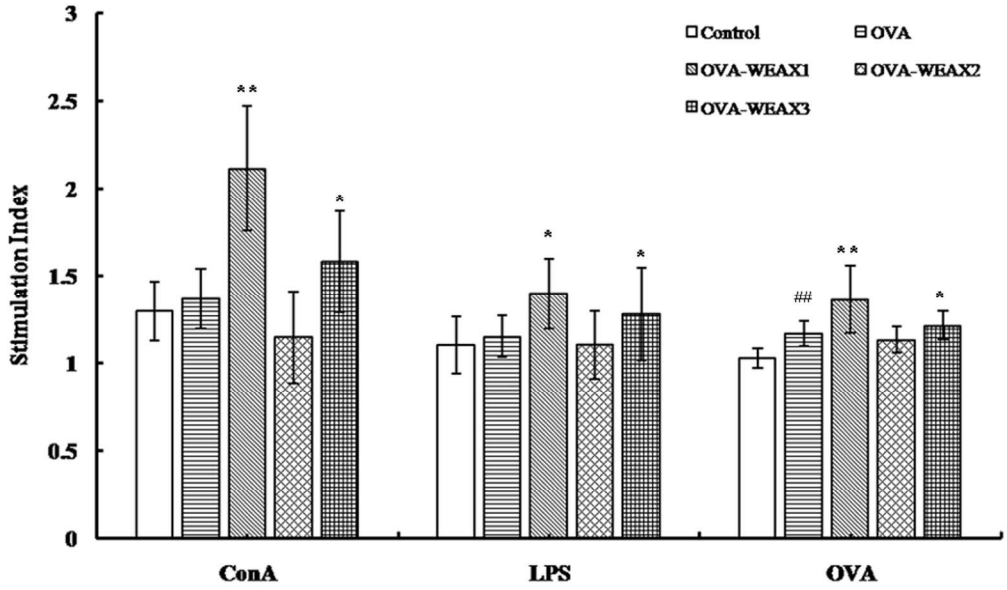

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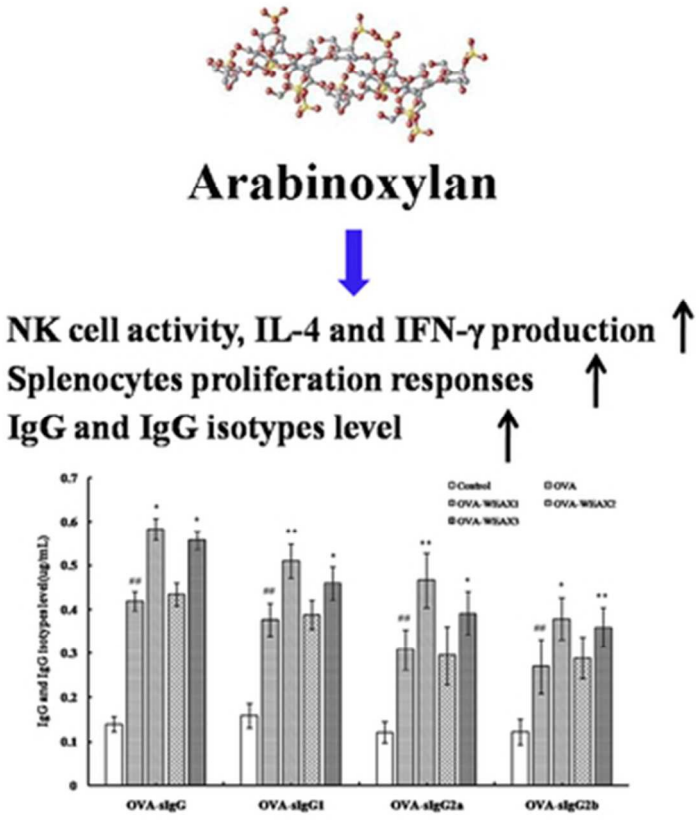

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