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An α-glucan isolated from root of *Isatis Indigotica*, its structure and adjuvant activity

Junjie Shan · Guohui Sun · Jinwei Ren · Ting Zhu · Peiyuan Jia · Wensheng Qu · Qian Li · Junhua Wu · Hao Ma · Song Wen · Yuxia Wang

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Abstract The root of *Isatis indigotica* is a traditional Chinese herbal medicine. An α-glucan (IIP-A-1) was firstly isolated from the roots. In this study we elucidated the chemical structure of IIP-A-1 and determined its adjuvant activity by co-immunizing mice with H1N1 influenza virus split and recombinant hepatitis B surface antigen (HBsAg), respectively. The polysaccharide was pretreated with periodate oxidation, Smith degradation and methylation in order to analyze its structure using GC, HPGPC, FT-IR, NMR and GC-MS. The adjuvant effect was evaluated by determining the antibody titers of serum against H1N1 influenza and HBsAg using ELISA. The proliferation and TNF-α secretion of macrophages administrated with different dose of IIP-A-1 were measured *in vitro*. The results of this study revealed that IIP-A-1 was an α-glucan with the molecular weight of 3,600 Da. The backbone was α-(1→4)-D-glucan with (1→6) branch chain. The α-glucan could significantly enhance the immune response of mice immunized with H1N1 influenza or HBsAg *in vivo* and exert good dose-dependent effects on the proliferation and the TNF-α secretion of macrophages *in vitro*. These results supported that IIP-A-1 was expected to be an efficacious adjuvant candidate for prophylactic and therapeutic vaccines.

Keywords *Isatis Indigotica* · α-glucan · Adjuvant · Vaccine · H1N1 · HBsAg

Junjie Shan and Guohui Sun contributed equally to this work.

J. Shan · T. Zhu · P. Jia · W. Qu · Q. Li · J. Wu · H. Ma · S. Wen · Y. Wang (✉)
Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China
e-mail: wangyuxia1962@hotmail.com

J. Shan
e-mail: shanjunjie001@126.com

T. Zhu
e-mail: 137519419@qq.com

P. Jia
e-mail: peiyuan3@163.com

W. Qu
e-mail: quws2000@sina.com

Q. Li
e-mail: liqian504722@126.com

J. Wu
e-mail: wujunhua1971@163.com

H. Ma
e-mail: majordan@163.com

S. Wen
e-mail: wensong520@163.com

G. Sun
Department of Gastroenterology, Chinese PLA Postgraduate Medical School, Beijing 100853, China
e-mail: sungh301xhk@163.com

J. Ren
State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, China
e-mail: renjw@im.ac.cn

Abbreviations

AAALAC	Accreditation of Laboratory Animal Care International
CMC	1-cyclohexyl–3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate
EI-MS	electron-impact mass spectrometry
ELISA	enzyme linked immunosorbent assays
FBS	fetal bovine serum
FMDV	foot-and-mouth disease virus
GC	gas chromatography
GLC	gas–liquid chromatography and electron-impact mass spectrometry
HBsAg	hepatitis B virus surface antigen
HPGPC	high performance gel permeation chromatography
NF-κB	activating nuclear factor κB
NK	natural killer
OD	optical density
PRRS	porcine reproductive and respiratory syndrome
TFA	trifluoroacetic acid
TMB	tetramethyl benzidine
TNF-α	tumor necrosis factor α.

Introduction

Glucans are natural polysaccharides comprised of D-glucopyranosyl units and are found in a wide variety of organisms, such as cereal, plant, algae, bacteria, fungi and yeast. Glucans could be broadly classified as α-or β-linked according to the type of intra-chain linkage of the polymers. β-glucans are heterogeneous glucose polymers, consisting of a backbone of β (1→3)-linked-D-glucans with β (1→6) linked side chains. In recent years, a number of studies indicated that β-glucans showed the immuno-regulatory effects [1, 2, 3, 4].

However, compared with the intensive studies of β-glucan, fewer works on the biological activity of α-glucan have been reported. Raveendran isolated a (1–4)-α-D-glucan (RR1) from *Tinospora cordifolia*. Its molecular weight was 5.5×10^5 Da. It could activate natural killer (NK) cells, T cells, B cells and the complements in the alternate pathway [5]. Schepetkin and Mark [6] isolated a glycogen-like α-glucan from *Lentinula edodes* mycelia. This α-glucan significantly stimulated phagocytosis in RAW264.7 cells and enhanced the cytokine production. Another α-glucan was isolated from *Cnidium officinale* with the molecular weight of 1.3×10^4 Da. Its structure was a high-branched α-(1–4)-D-glucan with both of 3, 4-and 4, 6-branching points. The glucan showed a significant reticuloendothelial system-potentiating activity [7]. He *et al*. isolated an α-(1–4)-D-glucan with α-(1–6)-D-glucan at the C-6 position (ASLP) from *Arca subcrenata* Lischke. ASLP could stimulate the proliferation of mouse spleen lymphocyte [8]. An α-(1–4)-D-glucan (MP-I) was isolated from *Mytilus coruscus*, which molecular weight was 1.35×10^6 Da. MP-I could protect the animals to avoid the acute liver damage by decreasing the activities of serum alanine aminotransferase, serum aspartate aminotransferase, hepatic malondialdehyde aldehydes levels and by increasing the hepatic total superoxide dismutase activity [9]. Huang *et al*. [10] isolated α-(1–4)-D-glucan from *Panacis Japonici*. This α-glucan showed potent immunopotentiating activity to inhibit S-180 tumor cell growth in mice by increasing the amounts of white blood cell count and lymphocytes and by inducing apoptosis of tumor cells.

The root of *Isatis indigotica* is a traditional Chinese herbal medicine. In China, the water-extract from *Isatis indigotica* is widely used in clinical practices for the treatment of influenza, epidemic hepatitis and epidemic encephalitis B. Chen *et al*. [11] investigated the adjuvant activity of the water-extract co-injected with foot-and-mouth disease virus (FMDV) DNA vaccine in mice. The results exhibited that this extract significantly increased T cell proliferation and serum antibody response. Zhang *et al*. [12] studied the effects of total polysaccharide of *Isatis indigotica* on antibody production and T cell subpopulations in piglets immunized with porcine reproductive and respiratory syndrome (PRRS) live virus vaccine. The total polysaccharides improved the antibody titer after co-injection with PRRS vaccine intramuscularly for 7 days.

In this paper, we isolated an α-glucan from root of *Isatis indigotica* and firstly investigated its chemical structure and adjuvant effect. Its adjuvant activity was analyzed by co-immunized with H1N1 influenza antigen and hepatitis B virus protein (HBsAg) respectively. The studies will provide more insight into the potential use of the α-glucan as an adjuvant in the disease prevention in human beings or animals.

Materials and methods

Plant, cell and animals

The dried root of *Isatis indigotica* produced in Anhui province was purchased in Beijing Tong-Ren-Tang Pharmacy in China. The root was authenticated by professor Yi-min Zhao. A voucher specimen (number 50) was preserved in the Laboratory of Natural Products Chemistry of Beijing Institute of Pharmacology and Toxicology.

Mouse alveolar macrophage cells (MH-S) were presented from Prof. Xiaolin Jiao in China. Balb/C mice (18–20 g) were supplied by the Animal Center of the Academy of Military Medical Sciences (SCXK JUN 2007–004) in Beijing. They were housed in a controlled environment (21±2 °C; 55±5 % of humidity; 12 h dark/light cycle). Food and water were given *ad libitum*. All the animal experiments were carried out in Animal Center of Academy of Military Medical

Sciences, in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the Center, which is in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Reagents

m-hydroxydiphenyl and 1,1,3,3-tetramethylurea were purchased from Aldrich. A set dextrans of different molecular weight and 1-cyclohexyl–3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMC) were obtained from Sigma. Sodium borohydride was from INC Biomedicines. DEAE-cellulose was purchased from Shanghai Reagent Company. Sephadex G-100 and G-75 were purchased from Pharmacia. H1N1 influenza A vaccine (split virion, inactivated) was produced from Sinovac Biotech Ltd (China), the influenza virus strain A, which was recommend by WHO (2009) and grown in embryonated egg. Hepatitis B virus protein was produced by Dalian Hiss Bio-pharm Ltd (China). Horseradish peroxidase-conjugated affinipure rabbit anti-goat IgG and Horseradish peroxidase-conjugated affinipure goat anti-mouse IgG were produced by Gackson. Bovine serum albumin was produced by Beijing Yuanheng Shengma Biotech Research Institute. ELISA kit of TNF-α was produced from Science Biosource Ltd (China). Limulus amebocyte lysate reagent was produced from Chinese Horseshoe Crob Reagent Co.Ltd.

Isolation and purification an α-glucan

The dried root of *Isatis indigotica* (1 kg) was soaked with water (15 L) at 50 °C for 4 h. After filtration and centrifugation, the residue was re-extracted under the same condition. The supernatant was mixed together and concentrated to 1.0 L. 3.0 L of 95 % ethanol was added into the concentrated solution to precipitate total polysaccharides for 72 h. The precipitate was dissolved and dialyzed with distilled water, and then lyophilized to obtain the total polysaccharide (named IIP).

IIP (1.0 g) was fractionated on a DEAE-cellulose column (Φ7.0 cm×30 cm), and was eluted with distilled water, 0.25 and 0.50 M $NaHCO_3$ sequentially at a flow rate of 1.0 mL/min. IIP was isolated into three sugar fractions (determined by the phenol-sulphuric acid method), and were named IIP-A, IIP-B and IIP-C. IIP-A (100 mg) was loaded on Sephadex G-100 gel column (Φ2.0 cm×120 cm), eluted with 0.1 M NaCl at a flow rate of 0.3 mL/min. The major fraction that contained sugar was collected and then applied on Sephadex G-75 column (Φ2.0 cm×120 cm) to get a homogeneous polysaccharide (named IIP-A-1, 60 mg). There was no protein contamination in IIP-A-1, because no absorption was observed at 280 nm and no positive result obtained by ninhydrin color reaction. There was no endotoxin in IIP-A-1, which was detected by limulus amebocyte lysate reagent [13].

Analysis of homogeneity and molecular weight

The homogeneity and molecular weight of IIP-A-1 were determined by high performance gel permeation chromatography (HPGPC, Waters Delta 600, equipped with a TSK-gel G-3000 swxl column, column temperature 35 °C, refractive index detector) and capillary electrophoresis (CE, Beckman P/ACE MDQ, equipped with an uncoated fused silica capillary tube). In HPGPC, a sample solution (20 μL) was injected and eluted with 0.1 M Na_2SO_4 at 0.5 mL/min as the mobile phase. The standard curve was established using a series of dextrans with different molecular weight as the standards. In CE, the purity of IIP-A-1 was identified with borax-sodium hydroxide buffer (pH=10.0) at 25 KV with DAD detection (λ=200 nm).

Analysis of monosaccharide compositions and absolute configuration

IIP-A-1 (5 mg) was hydrolyzed with 3 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. TFA was removed at reduced pressure with methanol. The hydrolyzate was dissolved in 2.0 mL water and carbonyl-reduced with $NaBH_4$ (80 mg) for 4 h at room temperature. The mixture was then transformed into the corresponding alditol acetates with 2 mL pyridine and 2 mL acetic anhydride at 90 °C for 1.5 h. The alditol acetates were analyzed by gas chromatography (GC, equipped with an HP-5 capillary column and flame-ionization detector). As references, seven monosaccharides (rhamnose, fucose, arabinose, xylose, mannose, glucose, and galactose) were converted to their alditol acetates and also analyzed by GC.

The absolute configuration was determinate with Gerwig method [14]. IIP-A-1 (2.0 mg) was hydrolyzed with 2 M TFA at 120 °C for 2 h., and then the acid was removed with methanol. 0.5 mL 0.625 M HCl in R-(+)-2-butanol was added and heated at 80 °C for 16 h. The reactants were then evaporated and TMS-derivatives were prepared with *N,O-bis* (trimethylsilyl) trifluroacetamide (BSTFA). The products were analyzed by GLC.

FT-IR analysis

IIP-A-1 (2 mg) was ground with KBr powder and pressed into pellets for FT-IR measurement (Bruker Vertex 70) in the frequency range of 4,000~500 cm^{-1}.

NMR spectroscopy

IIP-A-1(20 mg) was dried using P_2O_5 in vacuum for 2 days, then exchanged with deuterium by lyophilizing with D_2O for

three times. Using a Bruker Avance III 500 spectrometer, ^{1}H, ^{13}C, COSY, HMQC and HMBC NMR spectra of IIP-A-1 were recorded in D_2O at 25 °C. ^{1}H NMR spectrum of IIP-A-1 was recorded by suppressing the HOD signal (fixed at δ4.75 ppm), ^{13}C NMR spectrum of the polysaccharide was recorded using DMSO as internal standard (fixing at δ39.39 ppm).

Periodate oxidation-Smith degradation

IIP-A-1(20 mg) was dissolved in distilled water (5 mL) and dispersed using a blender. 15 mL of 30 mM $NaIO_4$ was added and the solution was kept in the dark at 4 °C. At 12 h intervals, 0.1 mL of the solution was withdrawn, diluted to 25 mL with distilled water and read in a spectrophotometer at 223 nm. Complete oxidation was finished after 120 h which was identified with a stable absorbance value at 223 nm. The periodated product was dialyzed against distilled water for 72 h. The non-dialysate was concentrated and reduced with sodium borohydride (80 mg) for 12 h at room temperature. After reduction, the solution was adjusted to pH=5.0 by adding 25 % acetic acid and dialyzed against distilled water for 48 h. The non-dialysate was lyophilized to obtain a product. The product was hydrolyzed with 2 M TFA (10 mL) at 120 °C for 2 h. GC analyzed the polyalcohol product as the alditol acetate using the same methods as the monosaccharide analysis.

Methylation analysis

Methylation of IIP-A-1 was carried out twice using the method of Hakomori as described [15]. IIP-A-1 (20 mg) was loaded into a bottle and dried overnight with P_2O_5 in vacuo. 4 mL dimethyl sulphoxide was added into the bottle and the air in the bottle was removed out with N_2. The mixture was completely dissolved by ultrasonication. 200 mg dry power of sodium hydroxide was added in the mixture and stirred before 1 mL tetramethyl-urea was dropwised with a dried syringe. The solution was stirred for 1 h at room temperature, and cooled in an ice bath before 2 mL cold methyl iodide was added into the bottle slowly the mixed solution was then stirred for 2 h. To the excess methyl iodide was removed under an air flow, and 4 mL distilled water was added to the mixture. The methylated polysaccharide was extracted with chloroform for three times. The extract was evaporated to remove chloroform, and the residue was dried in vacuum resulting to obtain a yellow solid. The production was examined by IR spectrometry. Complete methylation was confirmed by the lack of a hydroxyl peak. The methylated polysaccharide was subjected to 5 mL formic acid, sealed and kept at 100 °C for 3 h. The remained formic acid was removed by evaporation. The polysaccharide was hydrolyzed in 2 M TFA at 120 °C for 2 h. The remained TFA was evaporated with methanol. The mixture was added 3 mL of distilled water, reduced with sodium borohydride (80 mg) for 3 h. The mixture was neutralized with 25 % acetic acid and dried in vacuum at 70 °C for 2 h. The dry product was acetylated with 2 mL pyridine and 2 mL acetic anhydride at 90 °C for 2 h. The alditol acetates were analyzed by combined gas–liquid chromatography and electron-impact mass spectrometry (GLC: Agilent 6,890 N, EI-MS: Waters) on a DB-5 column (30 m×0.25 mm) using a temperature gradient of 150–260 °C at 5 °C/min.

Immunization with H1N1 virus split or recombinant HBsAg protein

Three groups of mice (BALB/c, 18–20 g, 6–7 weeks, 5 mice in each group) were immunized intramuscularly in the tibialis muscle with 0.1 mL H1N1 influenza antigen (3 μg/mouse) mixed with IIP-A-1 (0.2 mg/mouse), or with H1N1 antigen alone (3 μg/mouse), or with saline solution (for the control group). The mice received the booster 4 weeks after the first immunization. After the second immunization for 2 weeks, serum samples of immunized mice were collected for measurement of anti-H1N1 antibody and its isotype titers.

Four groups of mice (Balb/C, 18–20 g, 6–7 weeks, 5 mice in each group) were firstly intramuscularly injected in the tibialis muscle with 0.1 mL HBsAg (2 μg/mouse) mixed with IIP-A-1 (0.15 mg/mouse), aluminum hydroxide (0.2 mg/mouse) or saline respectively. The mice treated with saline served only as normal control. The mice received the booster 4 weeks after the first immunization. After the second immunization for 2 weeks, serum samples of immunized mice were collected for measurement of HBsAg-specific antibody titers.

Measurement of antigen-specific antibody and isotype titrations

The antibody titration in the sera of immunized mice was determined by enzyme linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 200 ng/well antigen (2.0 μg/mL) in 0.05 M carbonate buffer (pH=9.6) overnight at 4 °C. The plate was washed three times with washing buffer (PBST, PBS containing 0.1 % Tween 20, pH=7.4) and blocked with 5 % defatted milk in PBS for 60 min at 37 °C. After being washed three times, aliquots of serum samples (100 μL) were diluted with PBST and added to each well and incubated at 37 °C for 1 h. After incubation, 100 μL of peroxidase-conjugated goat anti-mouse IgG was added. Then, the plate was washed five times and 100 μL of tetramethyl benzidine (TMB) substrate solution (10 mL 0.2 mg/mL TMB, 50 μL 30 % H_2O_2 and 10 mL 0.1 M citrate-0.2 M phosphate buffer, pH=5.2) was added to each well, and incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 50 μL of 2 M H_2SO_4. The optical density (OD) of the colorized product

was measured at 450 nm on the microplate reader (Thermo Scientific Varioskanflash).

The levels of antigen specific antibody, IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA were analyzed by ELISA using the ISO2-1 KT following the instruction of the manufacturer. In brief, 96-well plates were coated with 100 μl antigen (2 μg/mL) for 24 h at 4 °C. The plates were washed three times with PBST, and blocked with 5 % defatted milk in PBS for 1 h at 37 °C. After three times washings, 100 μL of sera sample (diluted 1:400) or 0.5 % FCS/PBS as control were added to triplicate wells. The plates were then incubated for 1 h at 37 °C, followed by three times of washing. Aliquots of 100 μl of goat anti-mouse isotype antibody (diluted 1:1,000 with 0.5 % FCS/PBS) were added to each plate and further incubated for 1 h at 37 °C. After incubation, 100 μl of peroxidase-conjugated rabbit anti-goat IgG (diluted 1:1,000 with 0.5 % FCS/PBS) was added and incubated for 1 h at 37 °C. Then, the plate was washed five times and 100 μL of TMB substrate solution was added to each well, and incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 50 μL of 2 M H_2SO_4. The optical density (OD) of the colorized product was measured at 450 nm on the microplate reader.

Proliferation and TNF-α production assay

MH-S macrophage cells were passage cultured in essential medium (DMEM) containing 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. MH-S cells were inoculated into a 96-hole culture plate at 2.5×10^5/well with saline, 10, 50 or 100 μg/mL IIP-A-1. Cells were then incubated under 5 % CO_2 at 37 °C for 48 h. After incubation, the absorbance in each well was measured at 570 nm by method of MTT assay for cell proliferation. The supernatant was collected and TNF-α concentration was determined using ELISA method.

Statistical analysis

The data were expressed as mean ± standard deviation (S.D.) and examined for their statistical significance of difference with SPSS 13.0 and one way ANOVA. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Isolation and purification of IIP-A-1

The roots of *Isatis indigotica* were extracted with water and precipitated with ethanol to produce the total polysaccharides (IIP, yield 0.88 %). IIP (1.0 g) was isolated with DEAE-cellulose column to obtain three fractions which were IIP-A (580 mg), IIP-B (141 mg) and IIP-C (52 mg). The elution curve of DEAE-cellulose column was shown in Fig. 1. IIP-A (100 mg) was further purified with Sephadex G-75 columns to obtain a homogeneous polysaccharide IIP-A-1 (71 mg).

Physicochemical properties and structural feature of IIP-A-1

IIP-A-1 appeared as a white powder. Both HPGPC and CE profiles demonstrated that IIP-A-1 appeared as a single and symmetrically sharp peak (Fig. 1). The molecular weight of the polysaccharide was estimated 3,600 Da from a calibration curve prepared with standard dextrans. IIP-A-1 was hydrolyzed with 2 M TFA. GC analysis of alditol acetates of IIP-A-1 showed the presence of glucose only. The absolute configuration of the glucose was D configuration. The FT-IR spectrum of IIP-A-1 revealed a typical major broad peak at 3391.6 cm^{-1} assigned to the hydroxyl stretching vibration. The band of 2929.7 cm^{-1} is due to C-H stretching vibration. The absorption at 933.1 cm^{-1} and 846.7 cm^{-1} indicated its α-configuration [9, 13]. There was no absorption at 890 cm^{-1} for the β-configuration. IIP-A-1 contained no endotoxin determined with limulus amebocyte lysate reagent.

In the periodate oxidation, IIP-A-1 consumpted 0.05 mmol $NaIO_4$ and generated trace formic acid, suggesting the existence of the trace amount of monosaccharides, which were 1→linked or (1→6)-linked. The periodate-oxidized products were fully hydrolyzed and analyzed by GC analysis. The results showed that the alditol acetates of polyalcohols could be oxidized to produce only alditol acetates of erythritol, suggested the (1→4)-linkages might be the main structure of the α-glycan.

NMR spectroscopy was used to complete the structure characterization of IIP-A-1(Fig. 2 and Fig. 3). In the ^{1}H NMR spectrum (Fig. 2A), the signals appeared in the anomeric region at δ5.35, δ5.31 and δ4.92 ppm, which were assigned as (1→4)-α-D-Glc*p*, (1→6)-α-D-Glc*p* and Glc*p* (1→. In the ^{13}C NMR spectrum (Fig. 2 B), the anomeric carbon region for the (1→4)-D-Glc*p* were assigned at δ101.45 ppm, δ100.10 ppm was assigned to Glc*p* (1→, and δ 97.29 ppm was assigned to (1→6)-D-Glc*p*. All the chemical shifts of IIP-A-1 in the ^{1}H NMR and ^{13}C NMR spectra are summarized in Table 1, which are assigned on the basis of the correlation of the HSQC and COSY (Fig. 3). These assignments of ^{1}H NMR and ^{13}C NMR were in good agree with published values [2, 16, 17].

Methylation analysis of IIP-A-1 by GLC-MS revealed the four partially methylated alditol acetates at a relative molar ration of 1.0 : 18.5 : 0.9 : 0.16 corresponding to the peak areas (Table 2) . The 2, 3, 4, 6-tetra-O-methylglucitol acetates (1.0) has terminal non-reducing glucose, 2, 3, 6-tri-*O*-methylglucitol acetates (18.5) has (1→4)-glucosyl residues, 2, 3, 4- tri-*O*-methylglucitol acetates (0.9) has (1→6)-glucosyl residues, and 2, 3-di-*O*-methylglucitol acetates (0.16) has (1→4,6)-glucosyl residues.

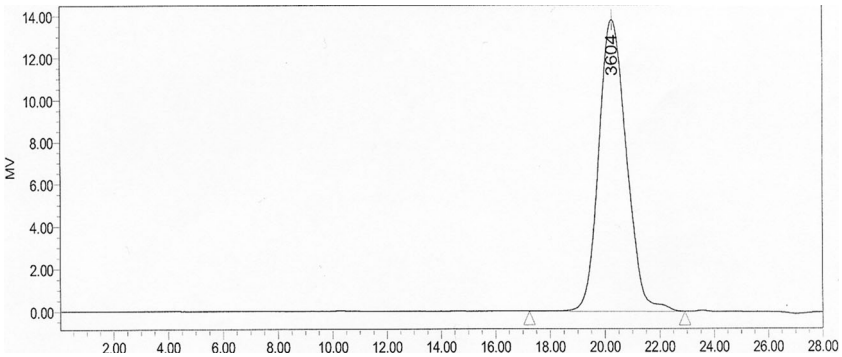
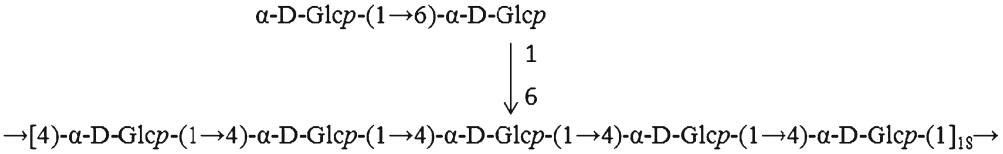


Fig. 1 HPGPC chromatography of IIP-A-1 on a TSK-Gel-3000 swxl column, eluted with 0.1 M Na_2SO_4

Taken all analysis, it could conclude that IIP-A-1 has a backbone chain of eighteen (1→4)-glucosyl residues with one (1→6)-glucosyl residue and one terminal glucose. The possible structure of the polysaccharide was



Effect of IIP-A-1 on the H1N1 antigen-specific antibody response

The H1N1 antigen-specific total IgG, IgM, IgG1, IgG2a, IgG2b and IgA antibody levels in the serum were measured 2 weeks after the first and second immunization using ELISA and the results were shown in Fig. 4. After first immunization for 2 weeks, H1N1 antigen alone induced low levels of H1N1 antigen-specific IgG antibody, and the main isotype of antibody was IgM. However, addition of IIP-A-1 to H1N1 antigen

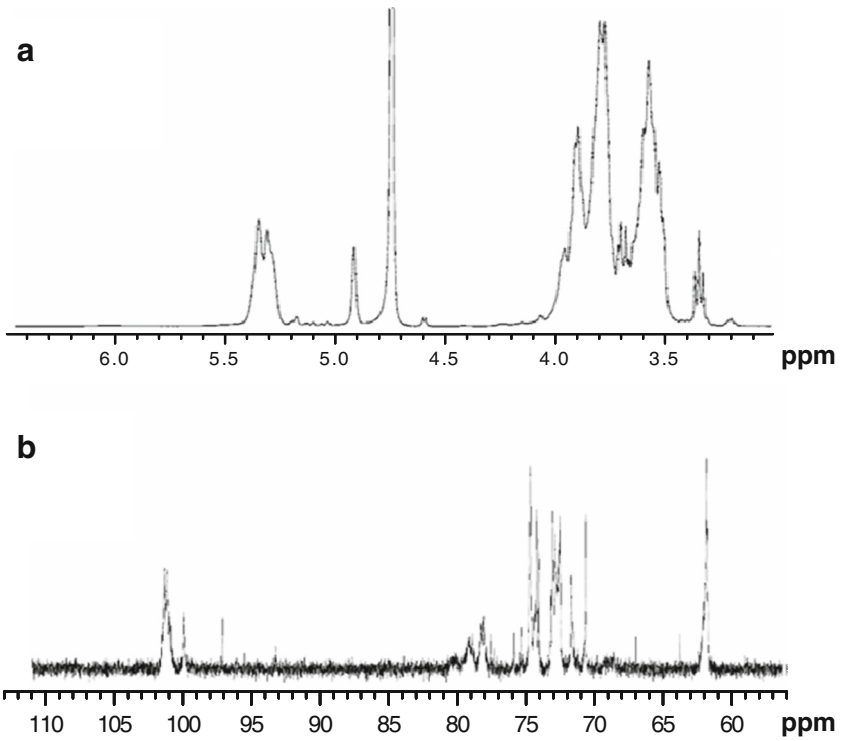


Fig. 2 **a** ^{1}H NMRspectrum of IIP-A-1. **b** ^{13}C NMRspectrum of IIP-A-1

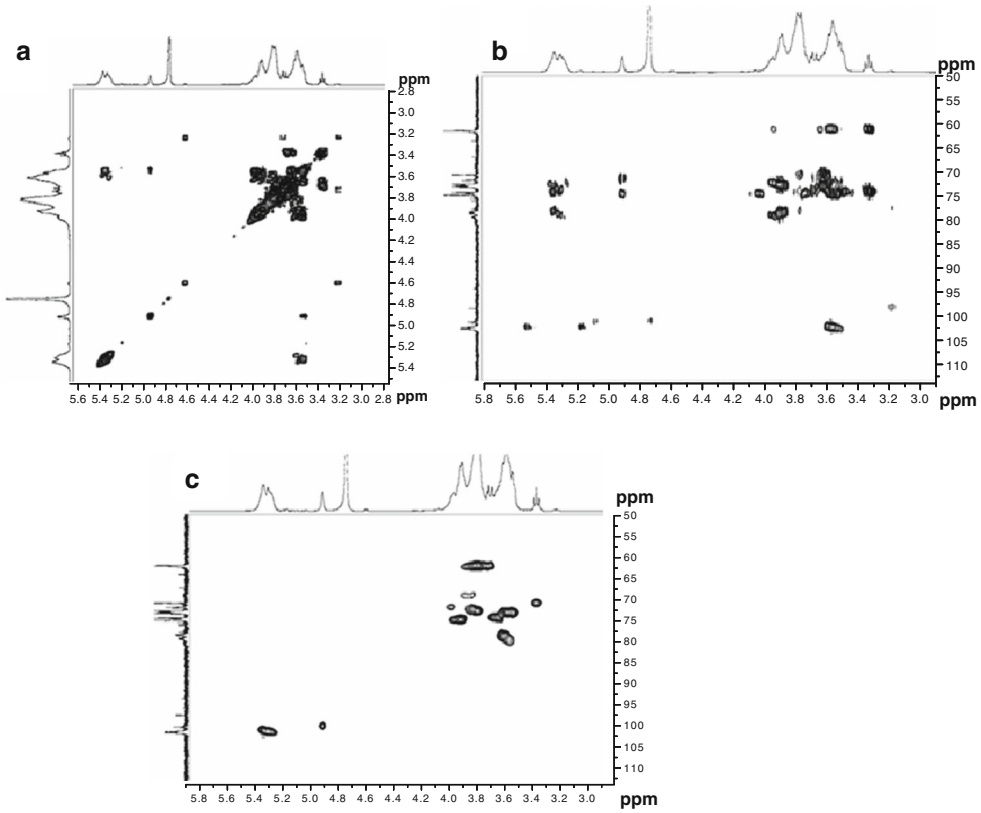


Fig. 3 **a** Two dimensional COSY NMR spectrum of IIP-A-1. **b** Two dimensional HMBC NMR spectrum of IIP-A-1. **c** Two dimensional HSQC NMR spectrum of IIP-A-1

resulted in a significant increase in total IgG antibody titers ($p<0.05$), and IgG1, IgG2a, IgG2b and IgG3 markedly increased ($p<0.05$). After second immunization for 2 weeks, H1N1 antigen alone induced high levels of H1N1 antigen-specific antibodies of IgG, IgM, IgG1, IgG2a, IgG2b and IgG3 compared to saline control ($p<0.01$), and H1N1 antigen plus IIP-A-1 increased more markedly antigen-specific total IgG titer ($p<0.001$) and isotype antibody titers of IgG2b, IgG3 and IgA ($p<0.01$).

Effect of IIP-A-1 on antibody response to HBsAg antigen

The adjuvant effect of IIP-A-1 on anti-HBsAg antibody response in mice was evaluated by determining serum antibody titers by ELISA. Anti-HBsAg antibodies in sera of immunized mice were detected at 2 weeks after first and second immunization. The results were shown in Fig. 5. After first immunization for 2 weeks, anti-HBsAg antibody titers in antigen alone group were lower than that in mice receiving antigen plus IIP-A-1 or alum ($p<0.05$). After boosting immunization for 2 weeks, anti-HBsAg IgG level in mice immunized HBsAg alone increased compared with saline control ($p<0.001$), but anti-HBsAg IgG level was significantly higher in mice immunized HBsAg plus IIP-A-1 or alum than HBsAg alone ($p<0.001$, $p<0.01$). IIP-A-1 exerted stronger adjuvant activity than alum ($p<0.01$).

Effect of IIP-A-1 on proliferation and TNF-α production in macrophages

MH-s cells (2.5×10^5 cells/well) were incubated with IIP-A-1 at concentrations of 10, 50 and 100 μg/mL for 24 h. Cell proliferation was measured by MTT method and TNF-α level was measured by ELISA kit. The result was shown in Table 3. The data illustrated that IIP-A-1 (50 and 100 μg/mL) treatments could well stimulate the proliferation and TNF-α secretion of macrophage versus those of the control group ($P<0.05$, $P<0.01$).

Table. 1 Chemical shift values of the different proton and carbon resonances were deduced from the interpretation of the one dimensional ^{1}H NMR and ^{13}C NMR and the two-dimensional COSY, HMBC and HMQC spectra

Sugar residues	$\delta^{13}C/^1H$ (ppm)					
	1	2	3	4	5	6
α-Glc*p*-(1→	101.1/5.35	71.8/3.38	73.3/3.92	70.8/3.59	70.8/3.91	61.9/3.73
→4)-α-Glc*p*-(1→	101.5/5.31	72.6/3.54	74.8/3.97	79.0/3.62	72.6/3.79	62.1/3.69
→6)-α-Glc*p*-(1→	100.1/4.92	73.1/3.66	72.8/3.81	78.3/3.92	73.3/3.97	67.2/4.08

Table. 2 GLC-MS analysis of methylated products of IIP-A-1

Peaks	Methyl positions	Molar ratio	Fragments (m/z)	Linkages
1	2,3,4,6-Me_4-Glc	1.00	71,87,101,117,129,161,189,202	Glc*p*-(1→
2	2,3,6-Me_3-Glc	18.51	87,99,101,113,117,129,161,173,233	→4)-Glc*p*-(1→
3	2,3,4-Me_3-Glc	0.91	87,101,117,129,161,189,233	→6)-Glc*p*-(1→
4	2,3-Me_2-Glc	0.16	71,87,117,129,189,233,261	→4,6)-Glc*p*-(1→

Discussion

At present, β-glucan is the most known powerful immune stimulant and a very powerful antagonist to many tumors among natural polysaccharides [18, 19]. As vaccine adjuvant, Le *et al.* [20] examined the adjuvant effect of β-glucan (SBG) produced by the black yeast *Aureobasidium pollulans* strain AF0-202. The results showed the H5N1and H5N2 vaccines with SBG initiated significantly higher immune responses in chickens, muscovy ducks and Vietnamese ducks. A β-(1→3)-glucans from yeast (*Saccharomyces cerevisae*) was also used as adjuvant in a Vibrio damsel vaccine for turbot. The vaccine with β-(1→3)-glucans could significantly increase humoral response ($p<0.01$) in turbot after injection for 35 days [21]. A synthetic β-(1→6)-branched β-(1→3) glucohexaose (β-glu6) was immunized with HBsAg antigen intraperitoneally in mice. The titer of HBsAg-specific antibody was greatly enhanced by the use of β-glu6. The β-glu6 could also significantly increase the amount of IL-4-producing cells in response to HBsAg, while it had no effect on the amount of IFN-γ-producing lymphocytes [22]. Further studies found that the *β*-glu6 promoted the recruitment and maturation of dendritic cells, enhanced the activation of CD8+ and $CD4^+$ T cells and

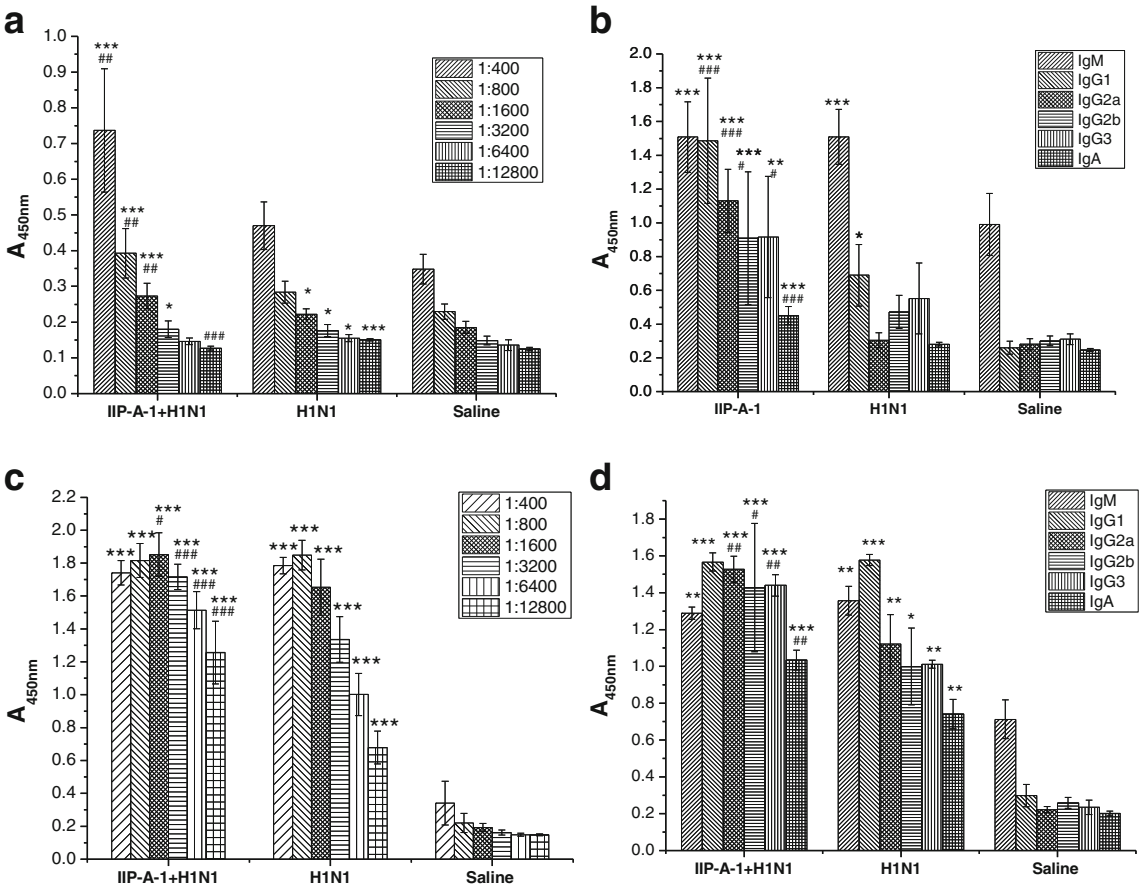


Fig. 4 Co-administration of IIP-A-1 adjuvant with H1N1 influenza vaccine enhances humoral responses. Female Balb/C mice were immunized intramuscularly two times interval of 28 days with 3 μg H1N1 antigen alone or with 100 μg IIP-A-1 each mouse. Blood samples were collected 2 weeks after immunization and total IgG and isotypes titers were measured by ELISA. (**a**) Serum specific-H1N1 total IgG titers at the 14^{th} day after first vaccination. (**b**) Serum specific-H1N1 isotype antibody at the 14^{th} day after first vaccination. (**c**) Serum specific-H1N1 total IgG titers at the 14^{th} day after second vaccination. (**b**) Serum specific-H1N1 isotype antibody at the 14^{th} day after second vaccination. The values are presented as mean ± S.D. ($n=5$). Compared with saline group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; Compared with H1N1 antigen alone group, $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$

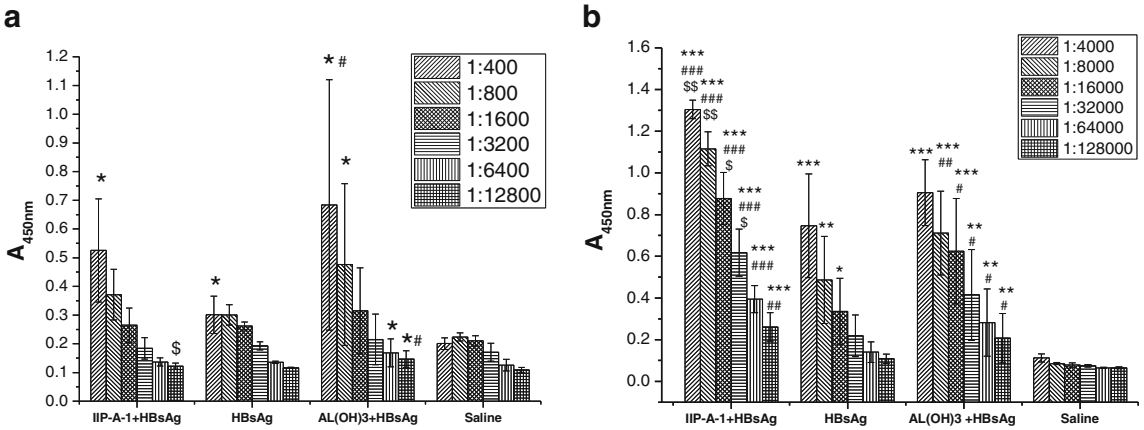


Fig. 5 Co-administration of IIP-A-1 adjuvant with HBsAg antigen enhances humoral responses. Female Balb/C mice were immunized intramuscularly two times interval of 28 days with 2 μg HBsAg antigen alone or with 150 μg IIP-A-1 each mouse. Blood samples were collected 2 weeks after immunization and total IgG titers were measured by ELISA. (**a**) Serum specific-HBsAg total IgG titers at the 14th day after first vaccination. (**b**) Serum specific-HBsAg total IgG titers at the 14th day after second vaccination. The values are presented as mean ± S.D. (n=5). Compared with saline group, * p<0.05, ** p<0.01, *** p<0.001; Compared with HBsAg antigen alone group, # p<0.05, ## p<0.01, ### p<0.001; Compared with alum adjuvant group, $ p<0.05, $$ p<0.01, $$$ p<0.001

increased the amount of specific $CD8^+$/IFN-γ^+ T cells in lymphoid and non-lymphoid tissues in mice immunized by pB144. Immunization with pB144 and β-glu6 increased the IgG and IgG2a antibody titers against HBsAg. These results demonstrate that β-glu6 can enhance the virus-specific CTL and Th1 responses induced by DNA vaccine [23]. Lai *et al.* [24] isolated a (1→3)-β-glucan from *Ganoderma lucidum* and studied its adjuvant activity for tetanus toxoid in mice. *In vivo*, the β-glucan treated mice showed an increase in the number of dendritic cells as well as the cells of CD4, CD8, regulatory T, B, plasma, NK, and NKT in the spleen and also elevated the levels of multiple cytokines and chemokines in the blood of mice.

Even though a few papers described that β-glucans had the adjuvant activities, few studies reported α-glucan might be used as vaccine adjuvant in human and animal. Rydell *et al.* [25] investigated the use of polyacryl starch microparticles as adjuvant for oral vaccination against diphtheria in mice. The starch microparticles induced a strong systemic immune response and induced a strong diphtheria toxin-neutralizing antibody response. In our research, we isolated an (1→4)-α-glucan (IIP-A-1) with molecular mass 3,600 Da, and firstly investigated its adjuvant activity with H1N1influenza and HBsAg in mice. Our result showed that IIP-A-1 enhanced an effective immune response when co-immunized mice with H1N1influenza and HBsAg, respectively. It could significantly increase the total IgG, IgG2a, IgG2b, IgG3 and IgA antibody titers in sera. The mechanism of the α-glucan as vaccine adjuvant has not been well elucidated. Some researchers have given some clues for the study of adjuvant mechanism of IIP-A-1.

Macrophages and dendritic cells are the most efficient professional antigen-presenting cells in pathogen recognition and play a pivotal role in the control of the immune response. Raveendran *et al.* [5] isolated an (1→4)-α-D-glucan from *Tinospora cordifolia* with a molecular mass of 550 kDa. This polysaccharide activated lymphocytes *in vitro* and elicited the synthesis of interleukin (IL)-1β, IL-6, IL-12, IL-18, interferon (IFN-γ) and tumor necrosis factor (TNF)-α at 100 μg/mL concentration, while it did not induce the production of IL-2, IL-4, IL-10 and IFN-α. The cytokine profile clearly demonstrated that RR1 mediated immunity through the Th1 pathway. Raveendran further studied the mechanism of RR1 on macrophages activation. He found RR1 induced TNF-α synthesis in macrophages and activated NF-κB. RR1-induced NF-κB activation occurred through TLR6 signaling as evidenced by the synthesis of IL-8 in TLR6-transfected HEK293 cells. Another α-glucan from *Pseudallescheria boydii* cell wall stimulated macrophages and dendritic cells, induced cytokine secretion, and involved toll-like receptor 2 (TLR2), CD14, and MyD88 [26]. In our study, we also found IIP-A-1 could active the MH-S macrophages and increased the production of TNF-α in a dose-dependent manner.

Dinadayala *et al.* isolated an α-(1→4)-glucan with α-(1→6)-glucosyl linkages from *Mycobacterium tuberculosis* which molecular mass estimated between 65 and 87 kDa [17]. The α-glucan induces monocytes to differentiate into DC with the

Table. 3 The proliferation and the production of TNF-α in MH-S macrophage stimulated by IIP-A-1

Samples	Concentrations (μg/ml)	OD_{540nm}	Proliferation rate (%)	TNF-α (pg/ml)
Saline		1.336±0.151		110.18
IIP-A-1	10	1.565±0.261	17.1	260.68
	50	1.649±0.040*	23.4	390.31
	100	1.956±0.086**	46.4	576.25

Compared with saline group, * p<0.05, ** p<0.01

same altered phenotype and functional behavior of DC derived from *Mycobacterium tuberculosis* -infected monocytes [27]. Geutsen *et al*. found that the α-glucan is a novel ligand for the C-type lectin DC-SIGN (dendritic cell-specific ICAM-3-grabbing nonintegrin). The recognition of α-glucans by DC-SIGN is a general feature and that the interaction is mediated by internal glucosyl residues [28].

Based on the studies mentioned above, we predict that IIP-A-1 might be one of main active components as a vaccine adjuvant and this function is related to the stimulation of macrophages and dendritic cells.

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Conflict of interest The authors declare no conflict of interest.

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