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Structural characterization and immunostimulatory activity of a novel linear α-(1→6)-D-glucan isolated from *Panax ginseng* C. A. Meyer

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Abstract *Panax ginseng* C. A. Meyer is a well-known plant medicine in the world. Ginseng polysaccharides mainly contain starch-like glucan and pectin. In this paper, a novel glucan WGPA-UH-N1 was purified from ginseng pectin by the treatment of de-esterification and endo-polygalacturonase, followed by the chromatographies on DEAE-Sepharose Fast Flow and Sephadex G-50 column. WGPA-UH-N1 has molecular weight about 17 kDa. WGPA-UH-N1 was determined to be a linear α-(1→6)-D-glucan without side chains by FT-IR, ^{13}C-NMR, ^{1}H-NMR, HMQC and HMBC spectra. It is the first time to isolate a linear α-(1→6)-D-glucan from *Panax ginseng* C. A. Meyer. Immunological activity assays showed that WGPA-UH-N1, although not effective on the phagocytosis of macrophage, could significantly induce lymphocyte proliferation without mitogenic stimuli at 1.0 mg/mL or with LPS at 0.5 mg/mL, also significantly increase NO production at the range of 0.1–1.0 mg/mL in a dose-dependent manner. The immunological activities of WGPA-UH-N1 are different from those of the β-(1→6)-D-glucan (BIWP2) isolated from the fruit bodies of *Bulgaria Inquinans* (Fries).

Keywords Ginseng · Polysaccharide · α-(1→6)-D-glucan · Immunostimulatory activity

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Introduction

Ginseng has been used as a medicinal plant in the world over thousand years. Ginseng polysaccharides have many pharmaceutical activities, such as anti-tumor, anti-oxidant, immunoloregulation, hypoglycemic, anti-fatigue and anti-depression activities [1–6]. Ginseng polysaccharides are composed of neutral glucans and pectin [7]. In recent years, we have started a research program on ginseng polysaccharides in which they could be completely fractionated and the activities of each fraction studied so that their structure-activity relationships could be discussed. In our previous reports, ginseng polysaccharides were fractionated into a series of neutral fractions and pectic fractions. Structural analysis showed that the neutral fractions mainly contained starch-like glucans and the pectic fractions mainly contained arabinogalactan (AG), homogalacturonan (HG) and rhamnogalacturonan I (RG-I) type pectin [8, 9]. For further study of the fine structures of ginseng polysaccharides, the ginseng pectin were treated with pectinase [10]. During fractionating the hydrolysis products, a novel linear α-(1→6)-D-glucan was purified. In this paper, we report the isolation, structural characterization and immunological activity of this glucan.

Materials and methods

Materials

The chromatographic supports DEAE–Sepharose Fast Flow, Sephadex G-75, Sephadex G-50 and Sephadex G-25 were purchased from Sigma–Aldrich. Endo-polygalacturonase (Endo-PG, EC 3.2.1.15 from Aspergillus niger), concanavalin

A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich. All other reagents and chemicals are of analytical grade produced in China.

Isolation and purification of a glucan fraction WGPA-UH-N1

The ginseng polysaccharide WGP, purchased from Hongjiu BioTech Co., Ltd. (Jilin Province, China), was fractionated based on our previous report [8]. Briefly, WGP was applied to DEAE-Cellulose column, eluted with distilled water to give a neutral fraction (WGPN) and then with 0.5 M NaCl to give an acidic fraction (WGPA).

A novel glucan WGPA-UH-N1 was purified as following procedure: WGPA was treated with 0.1 M NaOH at 4 °C for 4 h and the reaction solution was neutralized to pH 4.2 with 10 % glacial acetic acid. To the solution, Endo-PG (2160 units) was added and kept at 50 °C for 2 h. The reaction was terminated by heating at 100 °C for 10 min. The hydrolysates were separated by Sephadex G-25 (4×14 cm) column eluted with distilled water at 2 mL/min to give fraction WGPA-UH (un-hydrolyzed fraction) and WGPA-H (hydrolyzed fraction). WGPA-UH in distilled water was loaded onto a DEAE-Sepharose Fast Flow column (6×20 cm, Cl^-), eluted first with distilled water to give a neutral fraction WGPA-UH-N, and then with NaCl to give acidic fractions. WGPA-UH-N was further fractionated by Sephadex G-50 column eluted with a 0.15 M NaCl solution at 0.15 mL/min. WGPA-UH-N1 was collected nearly to the void elution volume and WGPA-UH-N2 was collected nearly to the total volume.

Analytical methods

The total carbohydrate content was determined by phenol–sulphuric acid method based on literature [11]. All gel permeation and ion exchange chromatographies were monitored by assaying the total carbohydrate content. Starch was detected by I_2-KI assay. Contaminant endotoxin was analyzed by Limulus amebocyte lysate (LAL) assay using an E-TOXATE kit (Sigma, St. Louis, USA) according to the manufacturer's instruction.

The homogeneity and molecular weight were estimated by high performance gel-permeation chromatography (HPGPC)-linked gel permeation column of TSK-gel G-3000 PW_{XL} (7.8×300 mm, TOSOH, Japan), eluted with 0.2 M NaCl at a flow rate of 0.67 mL/min at 35.0±0.1 °C. The gel permeation column was calibrated with standard dextrans (50, 25, 12, 5 and 1 kDa) using linear regression. Sample concentration was 5 mg/mL and injection amount was 20 μL.

The monosaccharide analysis was performed by high performance liquid chromatography (HPLC) as described previously [8]. In brief, the sample (2 mg) was first methanolyzed using anhydrous methanol (0.5 mL) containing 2 M HCl at 80 °C for 16 h. The methanolyzed products were hydrolyzed with 2 M CF_3COOH (0.5 mL) at 120 °C for 1 h. The released monosaccharides were derivatized to be 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives and subsequently analyzed by HPLC on a Shim-pak VP-ODS column (150×4.6 mm i.d.) with a guard column on a Shimadzu HPLC system (LC-10ATvp pump and UV–vis detector) and monitored by UV absorbance at 245 nm.

FT-IR spectrum was obtained on a Nicolet 560 FT-IR spectrometer with DTGS detector in a range of 400–4000 cm^{-1}. The sample was measured as a film on KBr discs.

^1H, ^{13}C, HMBC and HMQC NMR spectra were recorded using a Bruker 5 mm broadband observe probe at 20 °C with a Bruker Avance 600 MHz spectrometer (Germany), operating at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR. The sample (20 mg) was dissolved in D_2O (99.8 %, 0.5 mL), freeze-dried, re-dissolved in D_2O (0.5 mL) and centrifuged to remove the excess sample. All the data were analyzed using standard Bruker software. Chemical shifts were given in ppm, with acetone as an internal chemical shift reference.

Animals

Male ICR mice (6–8 weeks old) were purchased from the Pharmacology Experimental Center of Jilin University (Changchun, China). Animal handling procedures were conducted under National Institutes of Health animal care and were in accordance with the guidelines established by the School of Life Sciences of Northeast Normal University regarding the use and care of laboratory animals. The protocol was approved by the university's committee for animal experiments.

Lymphocyte proliferation assay *in vitro*

Spleens were collected from normal mice under aseptic conditions and minced using forceps through a piece of absorbent gauze in mice lymphocyte separating medium (5 mL). After centrifugation (1500 r/min, 15 min), the supernatant was collected and double diluted to obtain splenocyte pellet. The splenocytes were washed three times in D-Hank's and resuspended in RPMI 1640 complete medium (Gibco). Splenocytes (100 uL/well, 5×10^6/mL) were seeded in a 96-well plate in the presence of ConA (0.005 mg/mL), or LPS (0.01 mg/mL), or medium with WGPA-UH-N1 or BIWP2 (final concentration 0.01, 0.05, 0.1, 0.5 and 1.0 mg/mL), giving a final volume of 200 uL. The plates were incubated at 37 °C, 5 % CO_2 incubator for 48 h, MTT (20 μL, 5 mg/mL) was added to each well and the plate was incubated for another 4 h. Then 20 % SDS (100 μL) was added and the plate was incubated for 20 h. The absorbance at 570 nm was measured using a Bio-Rad (Hercules, CA, USA) microplate reader (model 550).

Macrophage phagocytosis assay *in vitro*

The macrophages were obtained by peritoneal lavage with D-Hank's from the normal mice. After centrifugation, the cells were resuspended in RPMI 1640 complete medium. The cells were placed in a flat-bottomed culture plate and incubated at 37 °C for 4 h in 5 % CO_2 incubator. The supernatant was isolated and the non-adherent cells were washed out, then the mono-layer macrophages were collected.

Phagocytosis of macrophages was measured by neutral red uptake method [12]. In brief, the macrophages were seeded in 96-well plates at a density of 2×10^5 cells/well. The cells were incubated in a medium alone, or medium containing various concentrations (0.01, 0.05, 0.1, 0.5 and 1.0 mg/mL) of WGPA-UH-N1 or BIWP2. LPS (0.01 mg/mL) was used as a positive control. After 24 h incubation in 5 % CO_2 incubator, the supernatant was discarded, 0.075 % of neutral red dye was added to each well (100 μL/well). The plate was incubated for another 3 h and then washed three times with PBS (pH 7.2). Finally, 100 μL of cell lysate (0.1 mol/L acetic acid/ethanol 1:1) was added to each well and the plate was placed at 5 % CO_2 incubator overnight, then absorbance was measured at 540 nm with microplate reader.

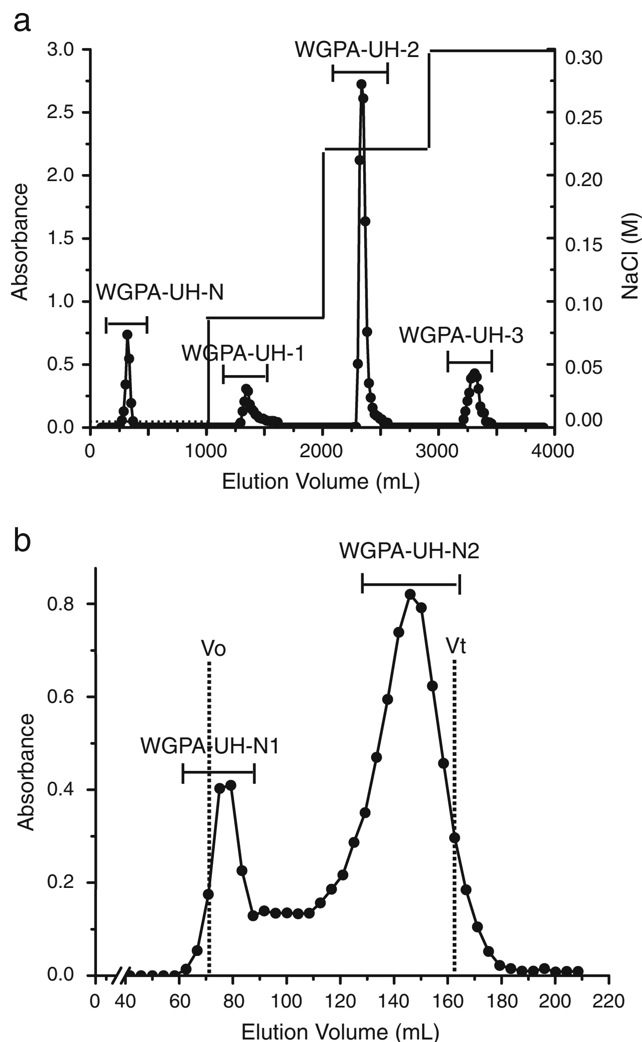

Fig. 1 Elution patterns of **a** WGPA-UH on DEAE–Sepharose Fast Flow column, **b** WGPA-UH-N on Sephadex G-50 column

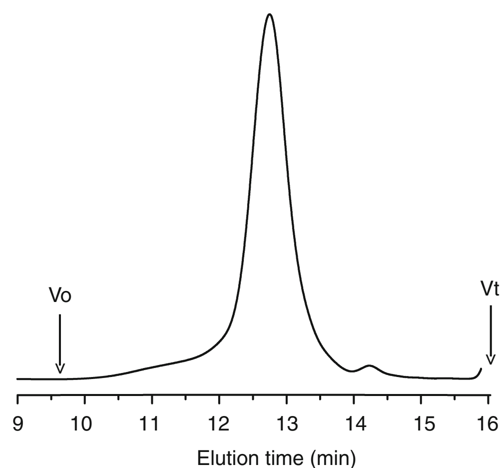

Fig. 2 HPGPC elution profile of WGPA-UH-N1

Nitric oxide (NO) production assay *in vitro*

The peritoneal macrophages (2.0×10^5 cells/well) were plated in 48-well plates and treated with polysaccharides for 48 h as described above. The supernatants were collected and mixed with an equal volume of Griess reagent (1 % sulfanilamide in ddH_2O, 0.1 % naphthylethylenediamine dihydrochloride in 5 % phosphoric acid). After 10 min, absorbance was measured at 540 nm with microplate reader. Sodium nitrite was used to generate a standard curve [13].

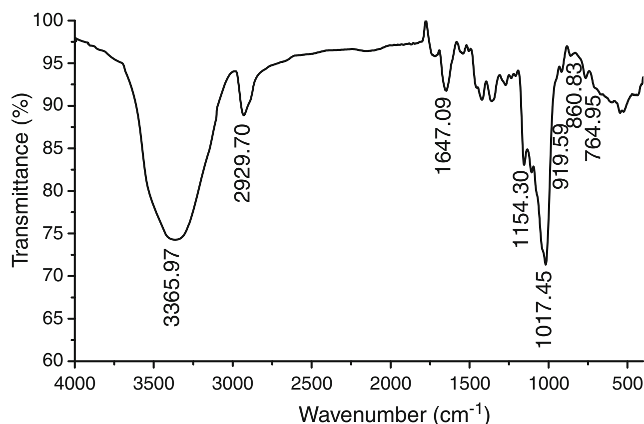

Fig. 3 FT-IR spectrum of WGPA-UH-N1

Table 1 ^{13}C and ^{1}H NMR chemical shifts of WGPA-UH-N1 in D_2O

Glycosidic linkage	Chemical shifts (ppm)						
→6)-α-D-Glc*p*-(1→	C-1	C-2	C-3	C-4	C-5	C-6	
	96.67	70.36	72.36	68.50	69.41	64.51	
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
	4.93	3.52	3.67	3.45	3.85	3.95	3.72

Statistical analyses

Results were expressed as the mean±S.D. of the indicated number of experiments. The data were analyzed for significance using the Student's*t* test. *P*-values of <0.05 and <0.01 were considered statistically significant.

Results and discussion

In our previous work, Water-soluble ginseng polysaccharides (WGP) were fractionated into a starch-like fraction WGPN, a neutral polysaccharide mixture fraction WGPA-N and eight pectic fractions, which included AG, HG and

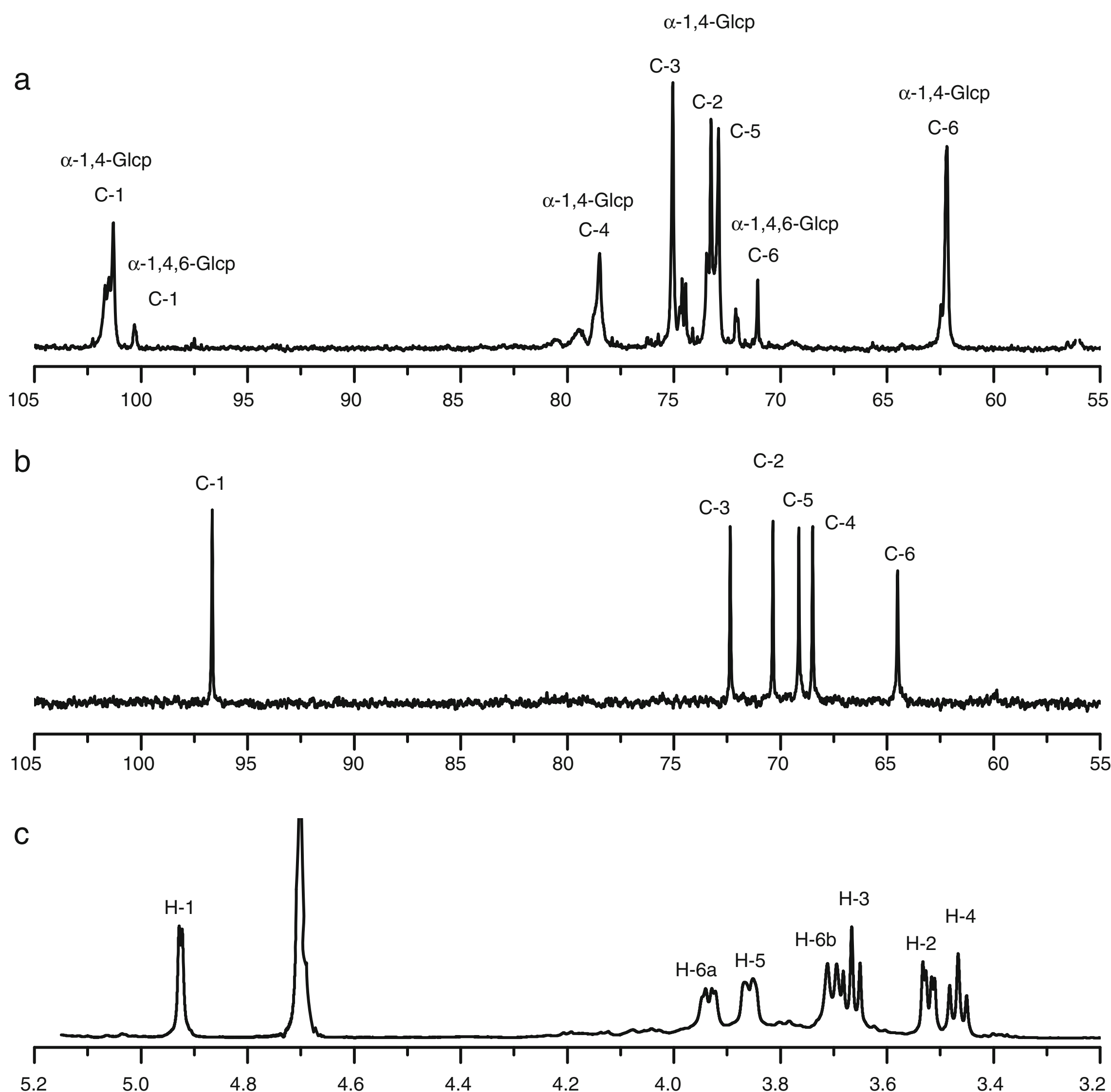

Fig. 4 ^{13}C NMR spectra of **a** WGPN; **b** WGPA-UH-N1 and ^{1}H NMR spectrum of **c** WGPA-UH-N1

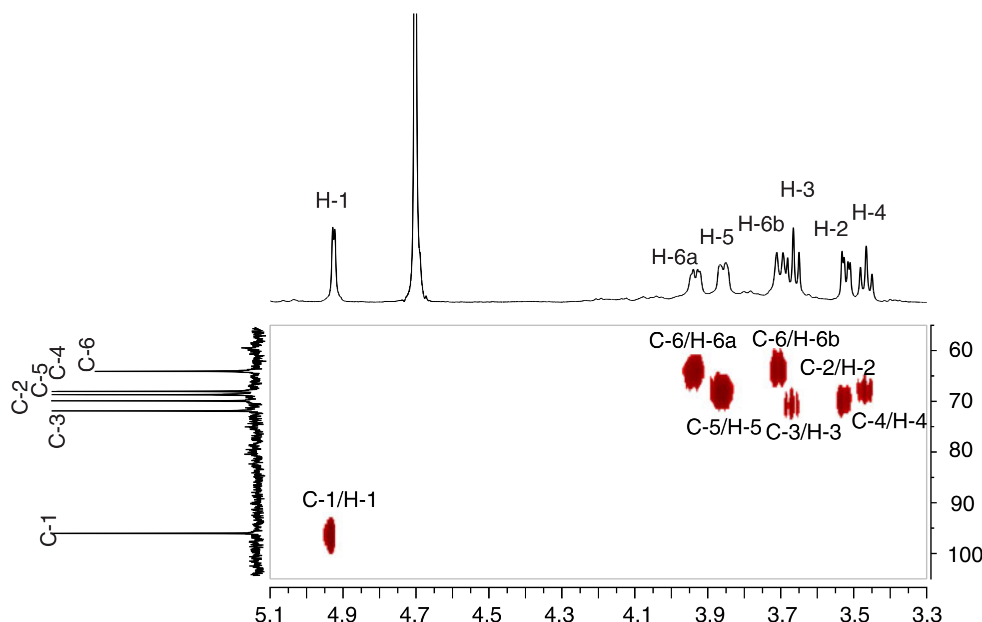

Fig. 5 HMQC spectrum of WGPA-UH-N1. Relevant cross-peaks were labeled

RG-I [8]. In this paper, WGP was fractionated into neutral fraction WGPN and acidic fraction WGPA. Then, WGPA was hydrolyzed by pectinase in order to obtain the structural units of ginseng pectin. WGPA was de-esterified and then hydrolyzed by Endo-PG. The released mono- and oligosaccharides were removed by Sephadex G-25 column. The remaining polymer fraction WGPA-UH (yield 15.8 %) was composed of GalA (25.0 %), Gal (22.6 %), Ara (36.5 %), Rha (7.7 %), GlcA (3.7 %), Glc (2.3 %) and Man (2.3 %). WGPA-UH was further loaded on a DEAE–Sepharose Fast Flow column, eluted with distilled water to give a neutral fraction WGPA-UH-N and then with a stepwise gradient of aqueous solutions of NaCl to give three acidic fractions (Fig. 1a).

WGPA-UH-N was composed of Ara (81.7 %), Glc (7.9 %), Gal (7.1 %) and Man (2.6 %). It gave two peaks on Sephadex G-50 column named as fractions: WGPA-UH-N1 and WGPA-UH-N2, in a ratio of 1:4.5 (w/w) (Fig. 1b).

WGPA-UH-N1 is a glucan, containing 97.5 % of Glc, the yield about 0.2 % in relation to WGPA. It appears as a white powder and is water-soluble. It had a negative response to the I_2-KI assay, indicating that its structure is different from that of the starch-like ginseng polysaccharides. HPGPC showed that it was homogeneous and the molecular weight was estimated to be 17 kDa (Fig. 2).

In the FT-IR spectrum of WGPA-UH-N1 (Fig. 3), absorptions at 919.59 cm^{-1} and 764.95 cm^{-1} were the characteristics

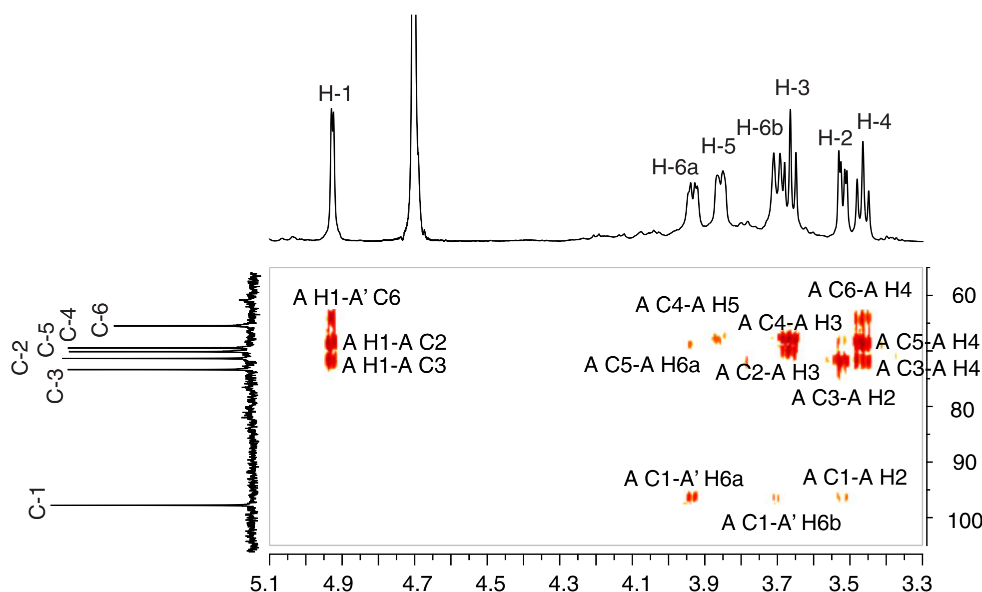

Fig. 6 HMBC spectrum of WGPA-UH-N1. Relevant cross-peaks were labeled

Table 2 The significant connectivities observed in HMBC spectrum for the anomeric carbon/proton of the sugar residue of WGPA-UH-N1

Sugar linkage	Residue(Atom)	δ_C/δ_H(ppm)	Observed connectivities	
			δ_C/δ_H(ppm)	Residue (Atom)
→6)-α-Glc*p*-(1→	A (H-1)	4.93	64.51	A′ (C-6)
			70.36	A (C-2)
			72.36	A (C-3)
	A (C-1)	96.67	3.95	A′ (H-6a)
			3.72	A′ (H-6b)
			3.52	A (H-2)

of D-pyranose-glucan [14]. The characteristic absorption at 860.83 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide [14]. The bands from 1100 cm^{-1} to 1010 cm^{-1} also demonstrated the existence of α-pyranose form of the glucosyl residue. There was no absorption at 890 cm^{-1} for the β-configuration [15, 16]. The FT-IR spectrum indicated the presence of α-glycosidic linkages of glucose in WGPA-UH-N1.

The structure of WGPA-UH-N1 was analyzed by NMR spectra. Based on literatures, HMQC and HMBC correlations, the ^{13}C and ^{1}H NMR signals of WGPA-UH-N1 were assigned (Table 1). In ^{13}C NMR, WGPA-UH-N1 showed six signals, which are consistent with those of α-(1→6)-D-glucan [17, 18], obviously different from those of starch-like polysaccharide fraction WGPN (Fig. 4a). The signal at 96.67 ppm in ^{13}C NMR of WGPA-UH-N1 belongs to C-1, 64.51 ppm to C-6, 72.36, 70.36, 69.41 and 68.50 ppm to C-3, C-2, C-5 and C-4 of α-(1→6)-D-glucan (Fig. 4b), respectively. According to the HMQC spectrum of WGPA-UH-N1 (Fig. 5), the signals at 4.93 and 3.95/3.72 ppm of WGPA-UH-N1 in ^{1}H NMR are typical of H-1 and H-6a/H-6b of α-(1→6)-D-glucan [19], in accordance with the ^{13}C NMR analysis. Therefore, WGPA-UH-N1 was determined to be a linear α-(1→6)-D-glucan.

Long range correlations of ^{13}C-^{1}H were obtained from the HMBC spectrum of WGPA-UH-N1 (Fig. 6). Cross-peaks of proton and carbon of the sugar moiety were examined and both inter- and intra- residual connectivities were observed (Table 2). Two glucose units were considered as A and A′ to explain the HMBC result. The inter-residual cross-peak was observed between H-1 (4.93 ppm) of residue A and C-6 (64.51 ppm) of residue A′ (A H-1, A′ C-6), C-1 (96.67 ppm) of residue A and H-6a (3.95 ppm) and H-6b (3.72 ppm) of residue A′ (A C-1, A′ H-6a; A C-1, A′ H-6b), and *vice versa*. In addition, the intra-residual cross-peaks of H-1 (4.93 ppm) with C-2 (70.36 ppm) and C-3 (72.36 ppm) and of C-1 (96.67 ppm) with H-2 (3.52 ppm) were observed. Further more, these results supported that WGPA-UH-N1 is a linear α-(1→6)-D-glucan. To our knowledge, this is the first time to isolate a linear α-(1→6)-D-glucan, without side chains, from panax ginseng.

The immunological activities of WGPA-UH-N1 were investigated by testing its effects on lymphocyte proliferation, macrophage phagocytosis and nitric oxide (NO) production *in*

Table 3 Effects of WGPA-UH-N1 and BIWP2 on ConA- or LPS-induced lymphocyte proliferation in mouse splenocytes *in vitro*

	Concentration (mg/mL)	Lymphocyte		
		Splenocytes	ConA	LPS
WGPA-UH-N1	0	0.670±0.004	0.821±0.005	0.852±0.013
	0.01	0.628±0.007	0.803±0.018	0.903±0.011
	0.05	0.640±0.007	0.811±0.008	0.834±0.013
	0.1	0.683±0.008	0.844±0.070	0.898±0.014
	0.5	0.679±0.090	0.873±0.003	0.980±0.021*
	1.0	0.918±0.017*	0.826±0.014	1.114±0.049**
BIWP2	0	0.553±0.019	0.861±0.028	0.955±0.026
	0.01	0.670±0.012	0.818±0.019	0.873±0.016
	0.05	0.640±0.006	0.791±0.019	0.854±0.037
	0.1	0.675±0.029	0.877±0.021	0.944±0.009
	0.5	0.713±0.005	0.875±0.009	0.995±0.033
	1.0	0.818±0.032**	1.004±0.008**	1.032±0.013

Proliferation activities were expressed as the absorption at 570 nm. Each value represented the mean±SD from five mice in each group. *$P<0.05$, **$P<0.01$, significantly different from the control

vitro. In order to study the structure-activity relationship, the immunological activities of the linear β-(1→6)-D-glucan BIWP2 (Mw 2.6 kDa), isolated from the fruit bodies of *Bulgaria Inquinans* (Fries) in our previous work [20], was tested for comparison.

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. Spleen lymphocyte proliferation induced by ConA *in vitro* was used as a method to evaluate T lymphocyte activity, while that induced by LPS was used to examine B lymphocyte activity. The effects of WGPA-UH-N1 and BIWP2 on lymphocyte proliferation were not obviously observed at the concentration range of 0.01–0.1 mg/mL (Table 3). But WGPA-UH-N1 could significantly induce lymphocyte proliferation without mitogenic stimuli at 1.0 mg/mL or with LPS at 0.5 and 1.0 mg/mL, and BIWP2 induced lymphocyte proliferation without mitogenic stimuli at 1.0 mg/mL or with ConA at 1.0 mg/mL. To avoid false positive results, contaminant endotoxin was tested by a gel-clot Limulus amoebocytes lysate (LAL) assay using an E-TOXATE kit (Sigma, St. Louis, USA) according to the manufacturer's instruction. The quantities of endotoxin in both glucans were less than 0.015 EU/mg (negative). These results were consistent with an α-(1→6)-D-glucan with a molecular weight of 53.2 kDa, isolated from the roots of *Ipomoea batatas*, and a β-(1→6)-D-glucan with molecular weight 185 kDa, isolated from a somatic hybrid of *Pleurotus florida* and *Volvariella volvacea*, which showed lymphocyte proliferation activity [21, 22]. The differences between the effects of WGPA-UH-N1 and BIWP2 on ConA- or LPS-induced lymphocyte proliferation might be caused by their different structures including their configurations and molecular weights.

The macrophages are considered the pivotal immunocytes of the host defence. Stimulating macrophage phagocytosis is an important way to enhance immunological activity. The effects of WGPA-UH-N1 and BIWP2 on macrophage phagocytosis were tested by phagocytosing neutral red of macrophages, LPS as a positive control. WGPA-UH-N1 was not effective on the phagocytosis of macrophage in the range of test doses, while BIWP2 enhanced phagocytosis of macrophages compared to the control in a dose-dependent manner. At 0.5 mg/mL, BIWP2 significantly increased macrophage phagocytosis to 75.7 % (Fig. 7a).

NO has been identified as one of the major effector molecules involved in the destruction of tumor cells by activated macrophages. The production of NO *in vitro* is assessed by the Griess reaction. WGPA-UH-N1 could stimulate NO production in a dose dependent manner (Fig. 7b). At 0.1 mg/mL, the production of NO is significantly increased, up to 52.2 %, compared to the control. This is similar to the observations that an α-(1→6)-D-glucan isolated from *Pleurotus florida* fruit bodies stimulated NO release [23], and an α-(1→6)-D-glucan (IPS-B2) isolated from cultured *Armillariella*

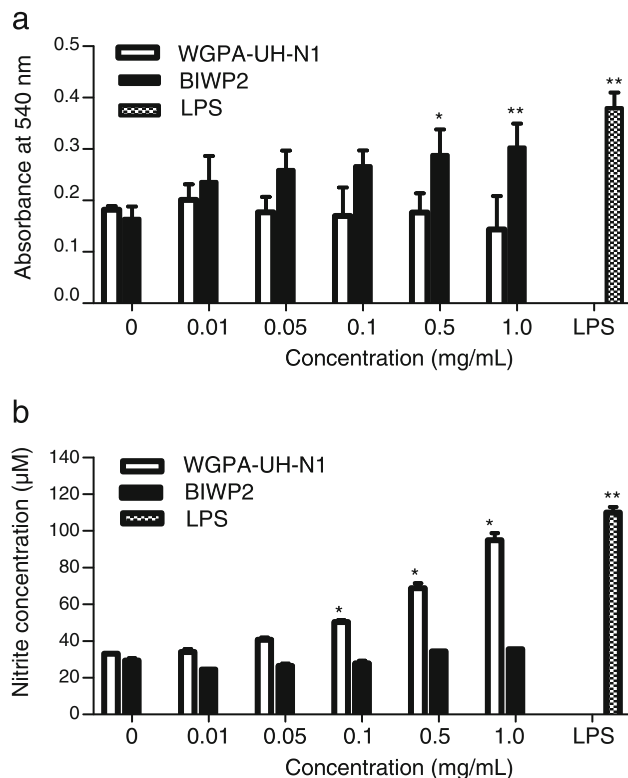

Fig. 7 The effects of WGPA-UH-N1 and BIWP2 on **a** macrophage phagocytosis and **b** NO (as nitrite) production of macrophages *in vitro*. Each value represents the mean±S.D., was measured by an ELISA reader. Significant differences from the negative controls were evaluated using student's *t*-test: *$P<0.05$, **$P<0.01$

tabescens mycelia induced NO production [24]. In contrast, BIWP2 had no effect on the NO production in the range of test doses. However, it has been reported that the β-(1→6)-D-glucan (185 kDa) from a somatic hybrid of *Pleurotus florida* and *Volvariella volvacea* had effects on NO production [22]. This difference might be caused by molecular weight. BIWP2 has lower molecular weight, 2.6 kDa.

Ginseng polysaccharides are active components of ginseng and mainly composed of starch-like glucans and pectin [7, 8]. Ginseng pectin mainly contains HG, RG-I and AG type domains [8]. In literatures, some active glucan fractions have been purified from ginseng, such as hypoglycemic Panaxan A (14 kDa) had a main chain of α-(1→6)-glucan branched at the C-3 [25, 26]; hypoglycemic Panaxan B (1800 kDa) has a main chain of α-(1→6)-glucan branched at the C-3 [27]; a water-soluble radioprotective polysaccharide Ginsan (2000 kDa) was made of α-(1→6)-Glc*p* and β-(2→6)-Fru*f* in a ratio of 5:2 [28]; other two neutral polysaccharides, named GPII (300 kDa) and GPIII (400 kDa), were comprised mainly of (1→6)-, (1→3)- and (1→2)-glycosidic linkages [2]. In this paper, a linear α-(1→6)-D-glucan (17 kDa), WGPA-UH-N1, was purified from the pectinase hydrolyzed products of ginseng pectin. It could not be separated from ginseng polysaccharides by DEAE-cellulose

chromatography because NMR showed that the separated neutral starch-like polysaccharide WGPN did not contain α-(1→6)-D-glucan (Fig. 4). We speculate that the glucan was attached to the pectin molecules by non-covalent through stereochemical encapsulation. After the pectin molecules were hydrolyzed by pectinase, the glucan was released from the interior of pectin as the destroy of the structure of pectin.

The immunological activities of WGPA-UH-N1 and BIWP2 were comparatively studied *in vitro*. The results showed that they have different activities because of their differences in configurations and molecular weights. WGPA-UH-N1 has more effect on B cell proliferation and NO production, while BIWP2 seems to have more effect on T cell proliferation and macrophage phagocytosis.

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