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Antioxidant and immunological activities of polysaccharides from *Gentiana scabra* Bunge roots

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ABSTRACT

Two polysaccharide fractions, GSP I-a and GSP II-b, were isolated from *Gentiana scabra* Bunge roots. Both GSP I-a and GSP II-b comprised seven monosaccharides: fructose, mannose, rhamnose, galacturonic acid, glucose, galactose, and fucose. Ultraviolet and infrared analyses show that GSP I-a and GSP II-b are proteoglycans. In vitro evaluation of the antioxidant activity suggests that GSP I-a and GSP II-b scavenge 1,1-diphenyl-2-picrylhydrazyl radicals. However, the scavenging activity of the latter is stronger than that of the former. GSP I-a and GSP II-b have relatively low reducing powers and scavenging activities toward superoxide anions and hydroxyls. GSP I-a and GSP II-b significantly increase lymphocyte proliferation when lipopolysaccharide is used as a mitogen for lymphocytes, but only GSP I-a can significantly increase lymphocyte proliferation within the test-dosage range when concanavalin A is used as a mitogen.

1. Introduction

The genus *Gentiana* of the family Gentianaceae comprises over 400 species that are widely distributed in alpine habitats of temperate regions of Asia, Europe, and America. Some species also thrive in Northwest Africa, Eastern Australia, and New Zealand (Georgieva, Handjieva, Popov, & Evstatieva, 2005). *Gentiana scabra* Bunge roots, commonly known as "Longdan" in Chinese herbal medicine, have been used to treat inflammation, anorexia, indigestion, and gastric infections. Modern pharmacological research indicated that *Gentiana scabra* Bunge roots protect the liver, inhibit liver dysfunction, and promote gastric acid secretion, which make them a popular ingredient in Chinese herbal medicine and health products (Kim et al., 2009).

Current research on *G. scabra* Bunge mainly focuses on its primary functional component, gentiopicroside (Chang-Liao, Chien, Lin, & Tsai, 2012; Lian et al., 2010; Wang et al., 2007). However, research on its other components, such as polysaccharides, is relatively insufficient. In our previous studies, we extracted polysaccharides from *G. scabra* Bunge roots and optimized the extraction conditions through response surface methodology (Wang, Wang, Zhang, & Wang, 2014). In the present study, polysaccharides (GSP) from *G. scabra* Bunge roots were further purified through chromatography on a diethylaminoethyl (DEAE)–cellulose anion-exchange column and a Sepharose CL-6B column. The characteristics, immunomodulatory activity, and antioxidant activity of the GSP fractions were also investigated. The results of this work contribute to the use of GSP in the food and pharmaceutical industries.

2. Materials and methods

2.1. Materials and chemicals

G. scabra Bunge roots were collected from Qingyuan (Fushun, Liaoning Province, China). Bovine serum albumin (BSA), ascorbic acid, pyrogallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiazolyl blue tetrazolium bromide (MTT), concanavalin A (ConA), and lipopolysaccharide (LPS) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Roswell Park Memorial Institute 1640 (RPMI-1640) was purchased from Gibco Invitrogen Co. Fetal calf serum was purchased from Hangzhou Sijiqing Biotech Co., Ltd. (Hangzhou, China). Unless otherwise stated, all chemicals used were analytical grade.

2.2. GSP extraction

G. scabra Bunge roots were homogenized, treated with ethanol, and extracted thrice using boiling water for 2 h. All water extracts were combined, filtered, concentrated, and treated with three volumes of ethanol. Crude GSP was then obtained through centrifugation (3000 × *g*, 20 min).

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Crude GSP was dissolved in distilled water and deproteinized using Sevag reagent. After the removal of Sevag reagent, the aqueous fraction was freeze-thawed and centrifuged until no insoluble substance was visible. The solutions were placed on a DEAE–cellulose (Amersham Biosciences, Sweden) column (3 cm × 45 cm) equilibrated with NaCl (0, 0.5, and 1.0 mol/L). The eluted sample was collected and further purified through gel filtration chromatography on a Sepharose CL-6B (Amersham Biosciences, Sweden) column (2.5 cm × 90 cm) with distilled water. The different fractions were dialyzed against distilled water and lyophilized.

2.3. Polysaccharide assay and protein assay

Polysaccharide content was determined through the phenol–sulfuric acid method using D-glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1958). The Bradford method was used to determine the protein content of the polysaccharides using BSA as the standard (Bradford, 1976).

2.4. Analysis of monosaccharide composition

The monosaccharide composition of the polysaccharides was determined through gas chromatography (GC). The polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (120 °C, 3 h). After hydrolysis, the neutral monosaccharides were successively reduced with $NaBH_4$ and acetylated with 1:1 pyridine acetic anhydride at 90 °C for 1 h. The alditol acetates were analyzed through GC on a Shimadzu GC-14C instrument equipped with an Rtx 2330 column (30 m × 0.32 mm × 0.2 μm). The column temperature was maintained at 170 °C for 2 min, increased to 240 °C at a rate of 8 °C/min for 1 min, and then increased to 265 °C at a rate of 8 °C/min for 20 min. Uronic acid content was determined using the m-hydroxybiphenyl colourimetric procedure with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

2.5. Infrared (IR) spectral analysis

The IR spectra of the polysaccharides were determined using a PerkinElmer Spectrum GX (USA). The purified polysaccharides were mixed with KBr powder and pressed into pellets for FTIR measurement within the frequency range of 4000 cm^{-1} to 400 cm^{-1}.

2.6. Antioxidant activity test in vitro

2.6.1. Superoxide radical scavenging assay

Approximately 4.5 mL of 50 mM Tris–HCl buffer (pH 8.2) was mixed with 4.2 mL of deionized water and then incubated at 25 °C for 20 min. After incubation, 1 mL of the polysaccharide solution and 0.4 mL of pyrogallic acid were added, and the mixture was immediately shaken. The reaction was terminated by adding 8 mM HCl after incubation at 25 °C for 5 min. The absorbance was measured at 320 nm using ascorbic acid as the positive control (Marklund & Marklund, 1974). The superoxide radical scavenging capability was calculated using the following formula:

$$\text{Scavenging rate}\,(\%) = \left[\frac{A_c - A_s}{A_c}\right] \times 100 \quad (1)$$

where A_c is the blank absorbance and A_s is the polysaccharide or ascorbic acid absorbance.

2.6.2. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was measured following the method described by Winterbourn and Sutton (1984). The reaction mixture contained 1 mL of 0.15 M phosphate buffer (pH 7.4), 1 mL of 40 μg/mL safranin, 1 mL of 0.945 mM EDTA-Fe(II), 1 mL of 3% (v/v) H_2O_2, and 0.5 mL of PNMP solution. After incubating at 37 °C for 30 min, the absorbance was measured at 560 nm using ascorbic acid as the positive control. The polysaccharide or ascorbic acid EC_{50} value (mg/L) is the effective concentration at which 50% of the hydroxyl radicals are scavenged. The hydroxyl radical scavenging activity is expressed as follows:

$$\text{Scavenging rate}\,(\%) = \left[\frac{A_c - A_s}{A_c}\right] \times 100 \quad (2)$$

where A_c is the blank absorbance and A_s is the polysaccharide or ascorbic acid absorbance.

2.6.3. DPPH scavenging assay

DPPH radical scavenging activity was determined according to the methods described by Shimada, Fujikawa, and Yahara (1992) with slight modifications. The reaction mixture contained 2 mL of DPPH (0.1 μM in 95% ethanol) and 2 mL of the polysaccharide solution. The mixture was incubated at 25 °C for 15 min, and the absorbance of the mixture was determined at 517 nm using ascorbic acid as a positive control. The polysaccharide EC_{50} value (mg/L) is defined as the effective concentration at which 50% of the DPPH radicals are scavenged. The polysaccharide antioxidant activity was evaluated according to the following formula:

$$\text{Scavenging rate}\,(\%) = \left(\frac{A_c - A_s}{A_c}\right) \times 100\% \quad (3)$$

where A_s is the polysaccharide or ascorbic acid absorbance and A_c is the DPPH solution absorbance.

2.6.4. Determination of the polysaccharide reducing power

The polysaccharide reducing power was evaluated according to the method described Deng et al. (2011). The reaction mixtures contained 2.5 mL of phosphate buffer (pH 6.6, 0.2 M), 2.5 mL of potassium ferricyanide (1%, w/v), and polysaccharide solution. After incubating the mixture at 50 °C for 20 min, 2.5 mL of trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction, and the mixture was centrifuged at 1200 × *g* for 10 min. Approximately 2.5 mL of the supernatant was collected and mixed with 2.5 mL of deionized water and 0.5 mL of $FeCl_3$ (0.1%, w/v). After incubating at room temperature for 15 min, the absorbance was measured at 700 nm using ascorbic acid as a positive control.

2.7. Splenocyte activation test

Cell proliferation was assessed using the MTT-based colorimetric assay. Male BALB/c mice (8 weeks to 12 weeks old) were sacrificed by cervical dislocation, and their spleens were aseptically removed. Spleen cells were collected by gently placing the organ in RPMI-1640 medium under aseptic conditions, followed by centrifugation at 3000 × *g* for 10 min at room temperature. Red blood cells were removed by washing twice in hemolytic Gey's solution. The cells were then resuspended in RPMI-1640 complete medium. The cell concentration was adjusted to 2×10^6 cells/mL, and the cell suspension was plated on a 96-well culture plate with or without ConA (5.0 μg/mL) or LPS (20.0 μg/mL). Samples (at 50, 100, 200, 400, and 800 μg/mL concentrations) were added to each cell. After incubation for 72 h at 37 °C in a humidified 5% CO_2 incubator, each well was pulsed with MTT. The plate was incubated for another 4 h. After aspirating the supernatant from the wells, 100 μL of dimethylsulfoxide was added to the wells to dissolve the formazan crystals. The absorbance of each well was read at 570 nm using a microplate reader (Model 680, Bio-Rad Co., USA).

2.8. Statistical analysis

The data were presented as mean ± SEM. Statistical analyses were performed using student's *t*-test and one way analysis of variance. All computations were done by employing the statistical software (SPSS, version 13.0).

3. Results and discussion

3.1. GSP purification

GSP was prepared by hot water extraction, centrifugation, ethanol precipitation, and deproteinization. GSP solution was loaded into a DEAE–cellulose column, which was then equilibrated with NaCl (0, 0.5, and 1.0 mol/L). Two independent elution peaks (GSP I and GSP II, Fig. 1a) were detected by the phenol–sulfuric acid assay, and both fractions showed UV absorption at 280 nm. The two fractions were then collected, dialyzed, concentrated, and loaded into a Sepharose CL-6B column. Each fraction produced three elution peaks (Figs. 1b and c). GSP I-a and GSP II-b, which were mainly elution peaks, were collected for the next analysis.

3.2. GSP I-a and GSP II-b investigation

The monosaccharide composition, protein content, and uronic acid content of GSP I-a and GSP II-b are presented in Table 1. GSP I-a and GSP II-b comprised seven monosaccharides: fructose, mannose, rhamnose, galacturonic acid, glucose, galactose, fucose. GSP I-a and GSP II-b contained proteins.

The IR spectra of GSP I-a and GSP II-b are shown in Fig. 2. The main absorption characteristics of the polysaccharide structures were related to the O—H stretching between 3500 and 3000 cm^{-1}, as well as the C—H stretching between 3000 and 2800 cm^{-1}. The absorption peak at 1745 and 1755 cm^{-1} was due to the existence of —CHO or —COOH. The strong absorption peak at 1611 and 1618 cm^{-1} showed the existence of $-NH^{3+}$ and $-NH_2$, which indirectly illustrated that the polysaccharides contained proteins. The absorbance at 1017 and 1101 cm^{-1} showed that the monosaccharides of both polysaccharides existed as pyranoside.

The absorption at 840 cm^{-1} suggested that α-glycosidic bonds were present in GSP I-a and GSP II-b.

3.3. Antioxidant activity assay

The superoxide radical is a highly toxic substance that is generated by numerous biological and photochemical reactions (Banerjee, Dasgupta, & De, 2005). As shown in Fig. 3a, the superoxide radical-scavenging rate of Vc was directly proportional to its concentration, and the EC_{50} value of Vc was 406 mg/L. Conversely, the superoxide radical-scavenging rates of GSP I-a and GSP II-b were not higher than 50%. Thus, their EC_{50} values were not determined within the experimental range used.

The hydroxyl radical, more likely produced in vivo, is the most reactive and poisonous free radical in organisms because it nonspecifically oxidizes all types of biological macromolecules. It is therefore commonly used to evaluate the effectiveness of antioxidants (Xiong, Li, Huang, Lu, & Hou, 2011). As shown in Fig. 3b, the maximum hydroxyl radical-scavenging activities of GSP I-a and GSP II-b were only approximately 20% within the experimental range used, whereas that of Vc reached 92% when the concentration was 750 mg/L. The EC_{50} values of polysaccharides were not determined within the experimental range of the scavenging assays for superoxide anions, and the EC_{50} value of Vc was 354 mg/L.

DPPH radical is one of the few stable radical sources widely used to test the electron donation and free radical-scavenging abilities of antioxidants (Wang et al., 2010). Fig. 3c shows the scavenging effects of GSP I-a and GSP II-b on DPPH radicals. Unlike the results of scavenging assays for superoxide anions and hydroxyl radical, GSP II-b showed evident scavenging activity toward DPPH radicals in a concentration-dependent manner within the relatively low concentration range of 0 mg/L–100 mg/L. The scavenging activities

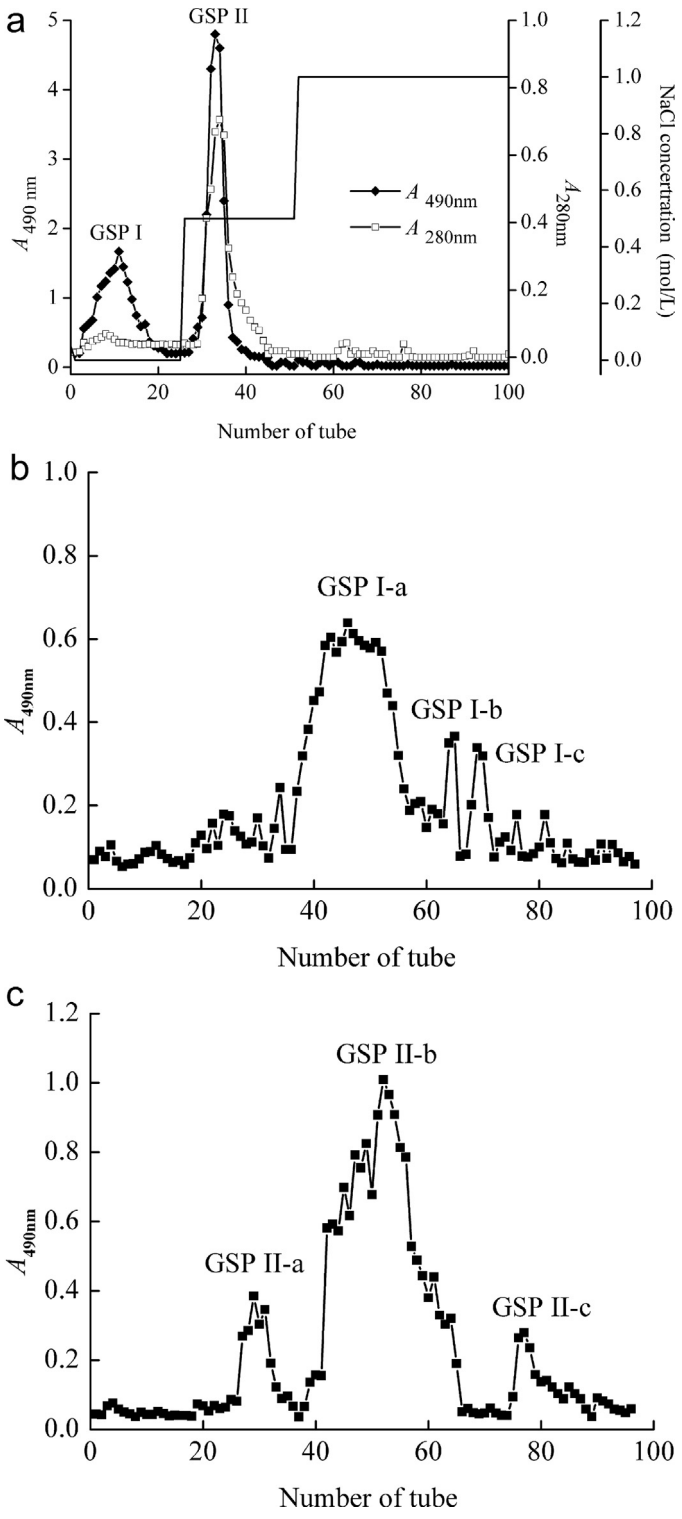

Fig. 1. Elution curve of GSP on DEAE-Cellulose DE-52 column (a) and elution curves of polysaccharides fractions (GSP I and GSP II) on Sepharose CL-6B column (b and c).

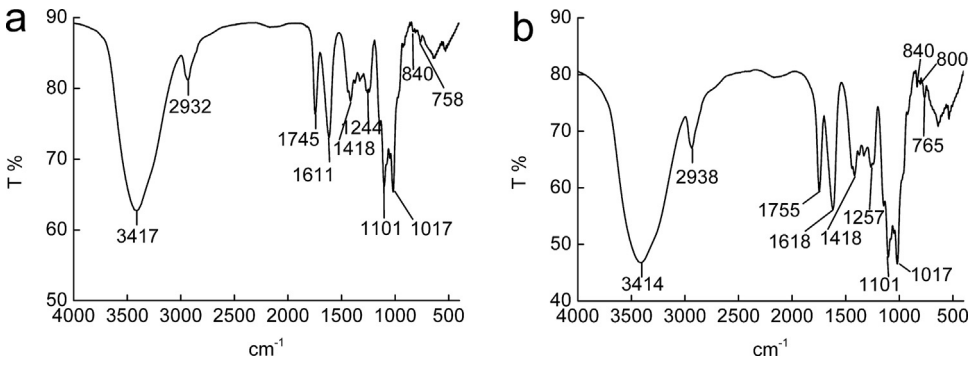

Fig. 2. IR spectrum of GSP I-a (a) and GSP II-b (b).

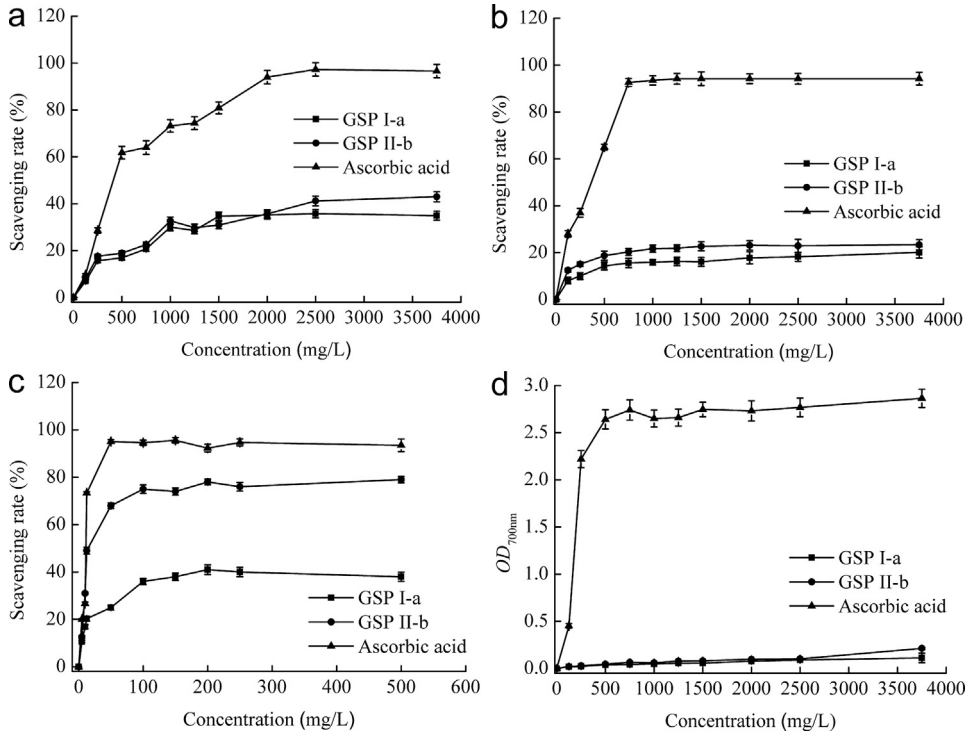

Fig. 3. Antioxidant activity assay of GSP I-a and GSP II-b. (a) Scavenging effects on superoxide anion radicals. (b) Scavenging effects on hydroxyl radicals. (c) Scavenging effects on DPPH. (d) Reducing power.

increased but not significantly at concentrations beyond 50 mg/L and reached the maximum scavenging rate of 79% at 200 mg/L concentration. The EC_{50} values of GSP II-b and Vc were similar and near 12.5 mg/L. The EC_{50} values of GSP I-a were not determined within the experimental range used.

The reducing capacity of a compound may significantly indicate its potential antioxidant activity (Kallithraka, Bakker, & Clifford, 2001). Fig. 3d shows the reducing powers of GSP I-a and GSP II-b. The reducing capacity of GSP I-a and GSP II-b was considerably lower than that of Vc.

3.4. Activity of lymphocyte proliferation

GSP I-a and GSP II-b were subjected to immune tests to evaluate their effects on lymphocyte proliferation. As shown in Fig. 4, both GSP I-a and GSP II-b significantly increased lymphocyte proliferation, and the activity of GSP II-b was stronger than that of GSP I-a within the test dosage range. Only GSP II-b significantly increased lymphocyte proliferation when ConA was used as a mitogen (Fig. 5). However, both GSP I-a and GSP II-b significantly increased lymphocyte proliferation when LPS was used as a mitogen (Fig. 6).

Table 1
Monosaccharide composition and protein content for GSP I-a and GSP II-b from *Gentiana scabra* Bunge.

Fragments	Monosaccharide composition (molar ratios)							Protein content (%)	Uronic acids content (%)
	Fru	Man	Rha	GalUA	Glc	Gal	Fuc		
GSP I-a	2.2	71.9	1	14	73.8	3	8.3	1.54	8.47
GSP II-b	2.7	64.3	1	7.2	57.3	3.2	7	6.36	5.23

Fru, fructose; Man, mannose; Rha, rhamnose; GalUA, galacturonic acid; Glc, glucose; Gal, galactose; Fuc, fucose.

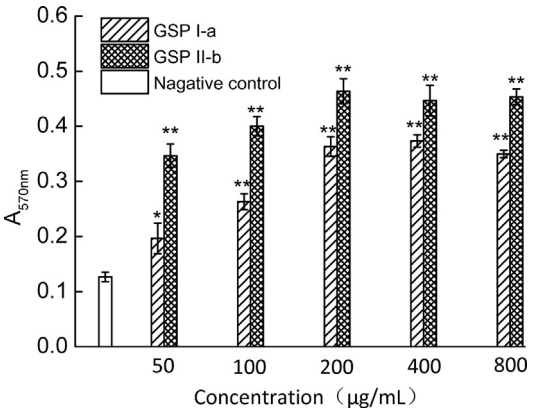

Fig. 4. Effect of GSP I-a and GSP II-b on splenocyte proliferation. Proliferation activities were expressed as the absorption at 570 nm. Values are mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. negative control.

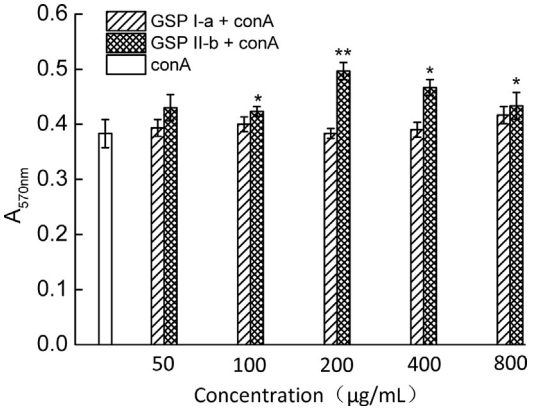

Fig. 5. Effect of GSP I-a and GSP II-b on ConA-induced splenocyte proliferation activities. Proliferation activities were expressed as the absorption at 570 nm. Values are mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ConA.

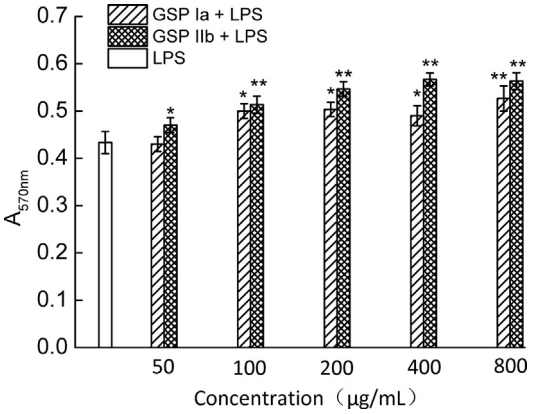

Fig. 6. Effect of GSP I-a and GSP II-b on LPS-induced splenocyte proliferation activities. Proliferation activities were expressed as the absorption at 570 nm. Values are mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS.

4. Conclusion

The polysaccharides from *G. scabra* Bunge roots were fractionated through DEAE–celluose and Sepharose CL-6B column chromatography. Two main components, GSP I-a and GSP II-b, were subsequently purified. Their basic physicochemical properties were investigated, and the results showed that both GSP I-a and GSP II-b were proteoglycans. The antioxidant activity assay showed that both GSP I-a and GSP II-b can scavenge DPPH radicals. The scavenging activity of the latter was stronger than that of the former. GSP I-a and GSP II-b demonstrated relatively low reducing power and scavenging activity toward superoxide anions and hydroxyls. Both also significantly increased lymphocyte proliferation, particularly when LPS was used as a mitogen. In-depth research on the polysaccharide structure and lymphocyte proliferation is currently in progress in our laboratory. Further characterization and applications of GSP in functional medicine are therefore expected.

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