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Purification, characterization and bioactivity of polysaccharides from *Glossaulax didyma*

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ABSTRACT

In this study, purification, preliminary characterization, immunostimulatory and hepatoprotective activities of polysaccharides from *Glossaulax didyma* (GDPS) were investigated. Firstly, crude GDPS was purified by chromatography of DEAE-52 and Sephadex G-100, resulting in three purified fractions of GDPS-1, GDPS-2 and GDPS-3. The three fractions were mainly composed of glucose with the average molecular weight of 161, 197 and 204 kDa, respectively. GDPS-2 was quite different from GDPS-1 or GDPS-3. It had much higher contents of protein and sulfuric radical. For immunostimulatory activities in vitro, the three fractions could significantly stimulate the proliferation of splenocytes and enhance phagocytic activity of peritoneal macrophages. For hepatoprotective activity in vivo, the administration of GDPS significantly decreased the serum levels of alanine aminotransferase, aspartate aminotransferase and tumor necrosis factor-alpha, inhibited the formation of malondialdehyde and restored the liver activities of superoxide dismutase and glutathione peroxidase in Bacillus Calmette-Guerin/lipopolysaccharide-induced liver injury mice. The results suggested GDPS should be a potent natural polymer with immunostimulatory and hepatoprotective activities.

1. Introduction

Glossaulax didyma, a well-known gastropod mollusk in the family of *Naticidae*, is widely distributed along the Indo-West Pacific region and found under muddy sand beaches (Li, Sun, Wan, & Li, 2008b; Xian, Wang, & Zhang, 2007). This species are carnivores and eat clamshell by the penetration of hole by dissolving the shells with sulfuric acid produced from their metabolites (Li, Li, Jin, Kong, & Yu, 2012). In China, Japan and Korea, *G. didyma* is one of the most commercially important resources due to its high nutritive and economic value (Fujii et al., 2009). Its fresh meat contains high amounts of protein, carbohydrates, essential amino acids and saturated fatty acids and is traditionally used as food supplement in China (Li & Shu, 1996; Liu, Xu, & Wu, 2012; Zheng & Gao, 2002). It has been demonstrated that the fresh meat of *G. didyma* is rich in polysaccharides that may contribute to its biological functions, such as anti-tumor, anti-inflammation and immune-regulation (Yang, Liu, Yu, Han, & Xia, 2012; Zhang, Fan, & Niu, 2003). However, little attention has been devoted to the preparation, characterization, immunostimulatory and hepatoprotective effects of polysaccharides from *G. didyma* (GDPS). Therefore, we report here the preparation, characterization and immunostimulatory and hepatoprotective effects of GDPS. Firstly, crude GDPS was prepared by water extraction and purified by DEAE-52 cellulose and Sephadex G-100 chromatography. The purified factions were then characterized by chemical analysis, gas chromatography (GC), fourier transform-infrared spectroscopy (FT-IR) and high performance liquid chromatography (HPLC). Finally, the immunostimulatory and hepatoprotective effects of GDPS were evaluated by using cell and animal models.

2. Materials and methods

2.1. Materials and reagents

G. didyma was purchased from Huaian Aquatic Product Market (Huaian, China). Arabinose, fucose, galactose, glucose, glucuronic acid, mannose, rhamnose, xylose, DEAE-52 cellulose, Sephadex G-100 and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bacillus Calmette-Guerin (BCG) was obtained from Shanghai Institute of Biological Products (Shanghai, China). Assay kits for protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). RPMI-1640 medium were obtained from

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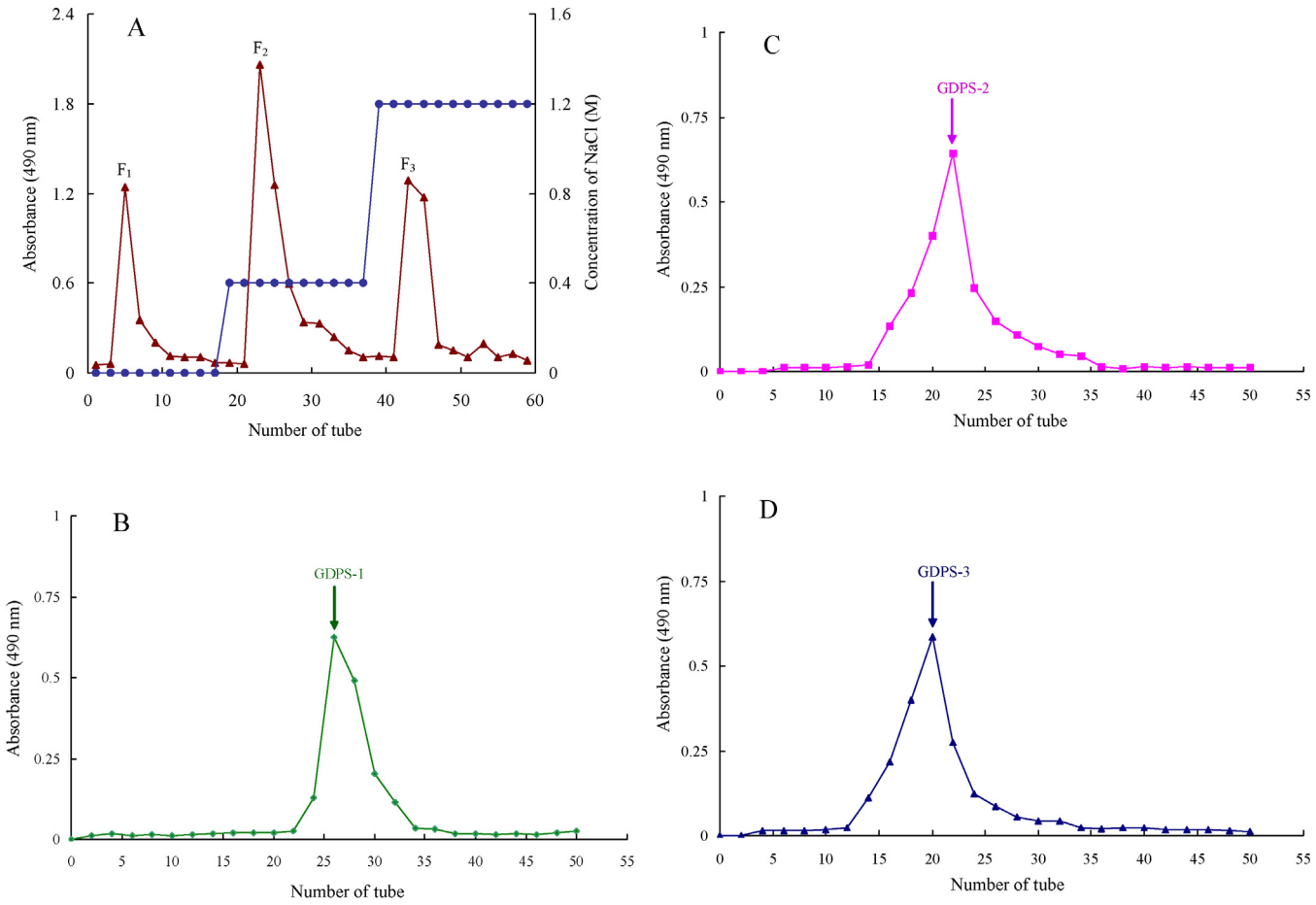

Fig. 1. Stepwise elution curve of crude GDPS on DEAE-52 cellulose chromatography column (A) and elution curve of polysaccharide fractions (F_1, F_2 and F_3) from DEAE-52 cellulose chromatography on size-exclusion chromatography column of Sephadex G-100 (B–D).

Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). An enzyme-linked immunosorbent assay kit (ELISA) for rat tumor necrosis factor-alpha (TNF-α) was purchased from Shanghai Senxiong Biotech Industry Co., Ltd (China). The Kunming mice [SCXK (Guangzhou) 2012-004)] were purchased from the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). All other reagents were of analytical grade.

2.2. Preparation of crude GDPS

The crude GDPS was prepared according to the reported method with some modifications (Jiang, Wang, Liu, Gan, & Zeng, 2011). Briefly, fresh *G. didyma* was collected and washed carefully with cold water. After removing the shells and impurities, the flesh was crushed by a high speed disintegrator and the homogenate was kept in 90% of ethanol (v/v) for two weeks. Then, the collected flesh was air-dried at 50 °C and extracted with distilled water in a ratio of 1:20 (raw material to water, w/v) for 4 h at 85–87 °C for three times. After the treatment, the mixture was centrifuged at 5000 rpm for 20 min, and the insoluble residue was treated again as mentioned above. The supernatants were collected, concentrated to a proper volume by using a vacuum rotary evaporator, deproteinated by the method of Sevag, Lackman, and Smolens (1938) and mixed with three times volume of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 20 min and air-drying at 50 °C to a constant weight, affording the crude GDPS.

2.3. Purification of crude GDPS

The crude GDPS was sequentially purified by chromatography of DEAE-52 and Sephadex G-100 according to the reported methods with slight modifications (Qiao et al., 2009). Briefly, the solution of crude GDPS (3 mL, 15 mg/mL) was applied to a column (2.6 cm × 30 cm) of DEAE-52 cellulose. Then, the column was stepwise eluted with 0, 0.4 and 1.2 M sodium chloride solutions at a flow rate of 60 mL/h. The obtained elute (10 mL/tube) was collected automatically and the carbohydrates were detected by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). As results, three fractions of polysaccharides (Fig. 1) were obtained. Polysaccharides in each fraction were collected, concentrated, dialyzed and further purified through a column (2.6 cm × 60 cm) of Sephadex G-100, respectively, resulting in three purified fractions of GDPS-1, GDPS-2 and GDPS-3. Finally, GDPS-1, GDPS-2 and GDPS-3 were lyophilized for further study.

2.4. Preliminary characterization of GDPS

2.4.1. Determination of molecular weight of GDPS

The molecular weight determination of GDPS purified fractions was performed using a size-exclusion HPLC chromatography instrument (Agilent 1100, USA) equipped with a refractive index detector (RID). All samples (10.0 mg) were dissolved in distilled water (1.0 mL), passed through a 0.45 μm filter and applied to a gel-filtration chromatographic column of TSK-GEL G3000SW$_{xl}$ (7.5 mm × 300 mm, Tosoh Corp., Japan). The column was maintained at a temperature of 25 °C and eluted with 0.1 M Na_2SO_4

solution in PBS buffer (0.01 M, pH 6.8) at a flow rate of 0.8 mL/min. For preparing molecular weight calibration curve, P-5, P-10, P-20, P-100, P-200, P-400 and P-800 (Showa Denko K.K., Japan) were used as the molecular weight references.

2.4.2. FT-IR spectral analysis of GDPS

FT-IR spectrum of GDPS purified fractions was recorded with a Nicolet 6700 FT-IR Spectrometer (Thermo Co., USA) using KBr disks method (Wang et al., 2004). Briefly, samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h, ground with potassium bromide (KBr) powder and then pressed into pellet for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}.

2.4.3. Analysis of chemical compositions of GDPS

The carbohydrate content in GDPS-1, GDPS-2 and GDPS-3 was determined by phenol–sulfuric acid method. The protein content was determined by the method described by Bradford (1976), using bovine serum albumin for the standard curve. The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (Gan, Manaf, Hj, & Latiff, 2010) using D-glucuronic acid as the standard. The content of sulfate radical was determined by the barium chloride-gelatin method using K_2SO_4 as a standard.

The monosaccharide compositions of GDPS-1, GDPS-2 and GDPS-3 were analyzed by gas chromatography (GC). Each sample (5 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid at 120 °C for 2 h. The hydrolyzate was repeatedly co-distilled with methanol to dryness and conventionally converted into aldonitrile acetates. The aldonitrile acetate derivatives of standard monosaccharides were prepared in the same way. Then, all the derivatives were analyzed by an Agilent 7890N GC equipped with flame ionization detector and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The operation conditions of GC were as following: flow rates of N_2, H_2 and air were 25, 30 and 400 mL/min, respectively; the temperatures of oven, detector and inlet were set at 210, 280 and 250 °C, respectively.

2.5. Evaluation of immunostimulatory activity in vitro of GDPS

2.5.1. Assay of splenocyte proliferation

The assay of splenocyte proliferation was performed according to the reported method (Luo et al., 2009). Briefly, Mice were sacrificed by cervical dislocation and sterilized by 70% alcohol submerge. Their spleens were aseptically removed, gently homogenized in cold PBS buffer (0.1 M, pH 7.2) and passed through a 150-mesh stainless steel sieve to obtain a single-cell suspension. After centrifugation, the cell pellets were re-suspended in 3 mL lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA-Na_2, pH 7.2) and gently vibrated for 2–3 min to remove red blood cells. After washing twice with PBS buffer (0.1 M, pH 7.2), the splenocytes were re-suspended in complete RPMI-1640 medium (with 10% new bovine calf serum, 1 mM sodium pyruvate, 100 unit/mL penicillin and 100 mg/L streptomycin) and adjusted to a density of 1×10^7 cells/mL. Then, the cell suspension (50 μL/well) was seeded into in a 96-well plate (Thermo Scientific, USA) and purified polysaccharide with different concentrations (25–100 mg/L) was added giving a final volume of 100 μl. ConA (final concentration of 5 mg/L) and LPS (final concentration of 10 mg/L) were used as positive controls, and complete RPMI-1640 medium was used as a blank control. After incubation at 37 °C in a humidified 5% CO_2 incubator (Thermo Scientific, USA) for 72 h, MTT stock solution (10 μl, 5 mg/mL) was added and the plate was further incubated for 4 h. Then, 100 μl of 10% SDS in 0.01 N hydrochloride solution was added to each well and the plate was kept overnight to fully dissolve the formazan crystals. The absorbance (Abs) at 570 nm was measured in an ELISA reader (Bio-tek, USA). The splenocyte proliferation index was calculated by the following equation:

$$\text{Splenochyte proliferation index} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank control}}}$$

2.5.2. Assay of phagocytosis of peritoneal macrophages

Peritoneal macrophages were obtained according to the methods reported previously (Luo et al., 2009). Mice were injected intraperitoneally with 2 mL of sterile thioglycollate medium to elicit peritoneal macrophages. Three days later, the mice were sacrificed and sterilized by 70% alcohol submerge. Peritoneal macrophages were harvested by peritoneal washing with 10 mL serum-free RPMI 1640 medium and centrifuged at 1500 rpm (4 °C) for 5 min. The cell suspension (2×10^6 cells/mL) was seeded into a 96-well plate (100 μL/well) and incubated at 37 °C in a humidified 5% CO_2 incubator for 3 h. Non-adherent cells were removed by washing twice with PBS buffer (0.1 M, pH 7.2). Then peritoneal macrophages were incubated with purified polysaccharides (100 μL/well) at different concentrations (dissolved by fresh complete RPIM-1640 medium, final concentration of 25–100 mg/L). LPS (final concentration, 10 mg/L) and complete RPMI-1640 medium alone were used as positive and blank control, respectively. After incubation at 37 °C in a humidified 5% CO_2 incubator for 72 h, the supernatant was discarded. Each well with adhered macrophages was filled with 100 μl of neutral red solution at a concentration of 0.075% and incubated for 1 h. Then the supernatant was discarded and the cells in plate were washed with PBS buffer (0.1 M, pH 7.2) twice to remove excess neutral red. The cell lysate (ethanol and 1.0 M acetic acid at the ratio of 1:1, 100 μL/well) was added and the plates were kept overnight at room temperature. Finally, the Abs at 540 nm of each well was measured by an ELISA plate reader. Phagocytosis index was calculated by the following equation:

$$\text{Phagocytosis index} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank control}}}$$

2.6. Evaluation of hepatoprotective effects of GDPS

2.6.1. Animal grouping and experimental design

The hepatoprotective effects of crude GDPS on immunological liver injury in mice were evaluated according to the reported method with some modifications (Guo, Sun, He, Yu, & Du, 2009). Briefly, the mice (8-weeks-old, 20 ± 2 g) were housed under standard environmental conditions (22 ± 0.5 °C, 55 ± 5% humidity and a 12 h light/12 h dark cycle) and maintained with free access to standard laboratory pellet diet and water. All procedures involving animals throughout the experiments were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals. After a 7-day acclimation period, these mice were randomly divided into six groups (ten for each) including normal control group, model control group, silymarin group (positive control) and GDPS group. On the first day of experiment, 2.5 mg of BCG in 0.2 mL saline per mouse was injected via lateral tail vein, with the exception of the normal control group, which received saline alone. Mice in GDPS groups were fed with GDPS in three different doses (200, 400 and 600 mg/kg BW per day, respectively) by gastric gavage. Mice in positive control group were given silymarin at the dose of 50 mg/kg BW per day for 15 days prior to the LPS treatment. Normal and model groups simultaneously received the same amount of physiological saline.

2.6.2. Biochemical assay

Eight hours after the last drugs treatment, the mice from the BCG/LPS model, silymarin-treated and GDPS-treated groups were

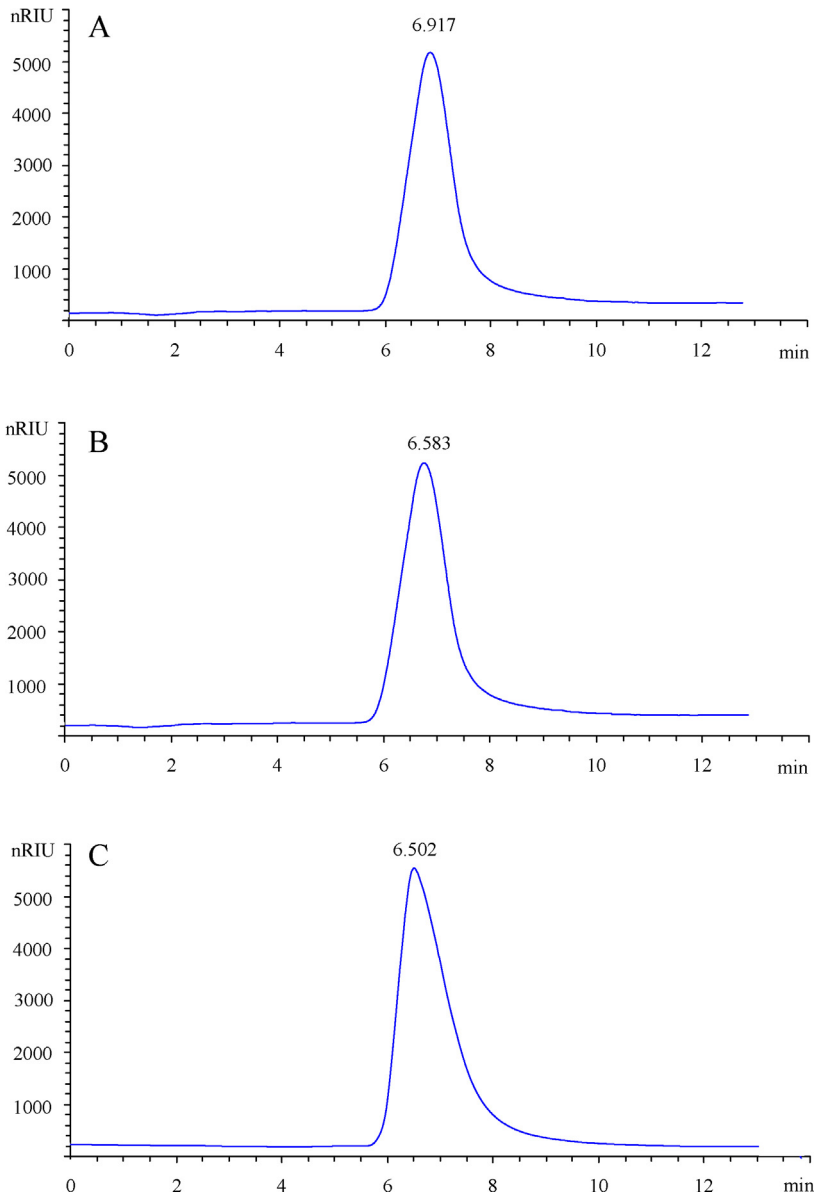

Fig. 2. HPLC profiles of GDPS-1 (A), GDPS-2 (B) and GDPS-3 (C).

treated with 7.5 μg of LPS in 0.2 mL saline per mouse (normal mice received saline alone) via lateral tail vein injection. Mice were then fasted for 16 h before they were sacrificed. Blood samples were collected immediately and centrifuged at 3000 rpm at 4 °C for 10 min to afford the serums. The liver was excised and homogenized immediately in 0.1 g/mL wet weight of ice-cold physiological saline. The suspension was centrifuged and the supernatant was collected for further analysis. All above treatments were done at 4 °C.

Protein content, activities SOD and GSH-Px, levels of MDA, ALT and AST were determined by using commercially available kits according to the instructions. In brief, the protein content was measured according to the Bradford method using bovine serum albumin as the standard. Activities of SOD and GSH-Px were determined according to the methods of xanthine oxidase–xanthine reaction system and reduced glutathione (GSH)-H_2O_2 reaction system, respectively. These enzymatic activities were expressed as unit per milligram of protein (U/mg protein). MDA level was measured by the TBARS method and expressed as nmol per milligram of protein (nmol/mg protein). In addition, both ALT and AST were measured using the ketoglutarate reaction and expressed as units per liter (IU/L). Serum TNF-α was determined by ELISA according the manufacturer's protocol and expressed in nanograms per milliliter (ng/mL).

2.7. Statistical analysis

Data were expressed as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Duncan's multiple range tests. *P*-values of less than 0.05 were regarded as significant.

3. Results and discussion

3.1. Preparation of polysaccharide fractions

The GDPS was prepared from *G. didyma* by hot water extraction, ethanol precipitation and vacuum freeze-drying. The overall yield of GDPS was 4.9% based on the dried flesh used. The crude GDPS was firstly separated through a DEAE-52 anion-exchange column. As a result, three independent elution peaks (F_1, F_2 and F_3) as shown in

Table 1
The average molecular weight and chemical compositions of GDPS-1, GDPS-2 and GDPS-3.

Sample	Molecular weight (kDa)	Carbohydrate (%)	Protein (%)	Uronic acid (%)	Sulfuric radical (%)	Monosaccharide composition (%)		
						Rhamnose	Xylose	Glucose
GDPS-1	161	92.1	2.9	2.2	1.9	1.6	0.1	98.3
GDPS-2	197	83.7	5.1	4.6	4.4	68.5	–[a]	31.5
GDPS-3	204	80.9	4.8	6.7	2.5	–	–	100

[a] Not detected.

Fig. 1A were obtained. Polysaccharides in each fraction were collected, concentrated, dialyzed and loaded onto a Sephadex G-100 column, respectively. The column was eluted with deioned water, and the resulting elute was collected. As shown in Fig. 1B, each fraction generated one single elution peak, affording GDPS-1, GDPS-2 and GDPS-3, respectively.

3.2. Molecular weight of polysaccharide fractions

For determination of the homogeneity and molecular weight of GDPS-1, GDPS-2 and GDPS-3, an Agilent 1100 HPLC system was used. As shown in Fig. 2, the HPLC profile of each purified fraction had a single peak, which indicated that they were homogeneous polysaccharides. In addition, the average molecular weights of GDPS-1, GDPS-2 and GDPS-3 were 161, 197 and 204 kDa, respectively.

3.3. FT-IR spectroscopy of polysaccharide fractions

FT-IR spectroscopy of GDPS-1, GDPS-2 and GDPS-3 are shown in Fig. 3. A strong and broad absorption peak at 3433 cm^{-1} for O—H stretching vibrations, a peak at 2936 cm^{-1} for C—H stretching vibrations, and a strong extensive absorption in the region of 900–1200 cm^{-1} for coupled C—O and C—C stretching and C—OH bending vibrations were observed in GDPS-1, GDPS-2 and GDPS-3, indicating the characteristic absorptions of polysaccharides (Liu et al., 2008). The absorption peak at 1241 cm^{-1} and 1154 cm^{-1} was assigned to the asymmetric stretching vibrations of SO, an evidence of sulfate ester, confirming directly that GDPS-1 and GDPS-2 were sulfated polysaccharides (Qiao et al., 2009). The bands in the region of 1635 and 1420 cm^{-1}, characteristic of a carboxylic group, indicated the possible presence of uronic acids in GDPS-2 and GDPS-3. Finally, there was an absorption peak at about 850 cm^{-1} in the FT-IR spectrum of GDPS-3 indicating the presence of α-type glycosidic linkages in GDPS-1 (Li, Sun, Wan, & Li, 2008a; Li et al., 2008b). The glycosidic linkage type of GDPS-1 was similar to that of polysaccharide from *Hyriopsis cumingii* (Qiao et al., 2009).

3.4. Chemical compositions of polysaccharide fractions

As shown in Table 1, the content of total sugar for GDPS-1, GDPS-2 and GDPS-3 were 92.1%, 83.7% and 80.9%, respectively. The content of sulfate for GDPS-2 was relatively higher than that of GDPS-1 and GDPS-3. The content of protein for GDPS-1, GDPS-2 and GDPS-3 was 2.9%, 5.1% and 4.8%, respectively. The content of uronic acid for GDPS-2 and GDPS-3 was 4.6% and 6.7%, which was relatively high than that of GDPS-1 (2.2%). In addition, GDPS-1 was composed of rhamnose, xylose and glucose in a molar percent of 1.6, 0.1 and 98.3%, respectively. GDPS-2 was composed of rhamnose and glucose in a molar percent of 68.5 and 31.5%, respectively. GDPS-3 was composed of glucose. Arabinose, fucose, mannose and galactose were not found in GDPS-1, GDPS-2 or GDPS-3.

3.5. Immunostimulatory activity in vitro of GDPS

3.5.1. Effect of GDPS on splenocytes proliferation

The immunologic action of polysaccharides may begin with splenocytes proliferation, which is related to immunity improvement of T-lymphocyte or B-lymphocyte (Wang et al., 2012). As shown in Fig. 4A, the stimulating indexes of GDPS-1, GDPS-2 and GDPS-3 were all exceed 1.0, indicating GDPS-1, GDPS-2 and GDPS-3 could promote the splenocytes proliferation. In addition, the

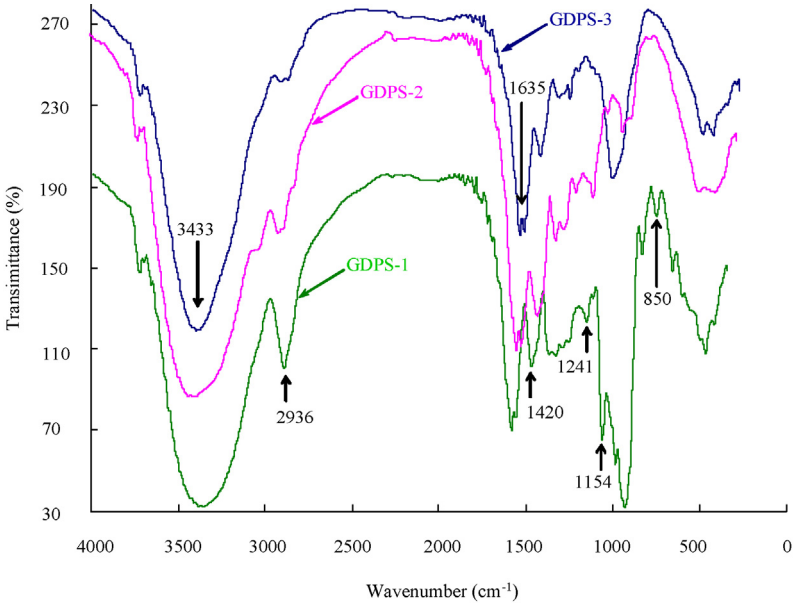

Fig. 3. FT-IR spectra of GDPS.

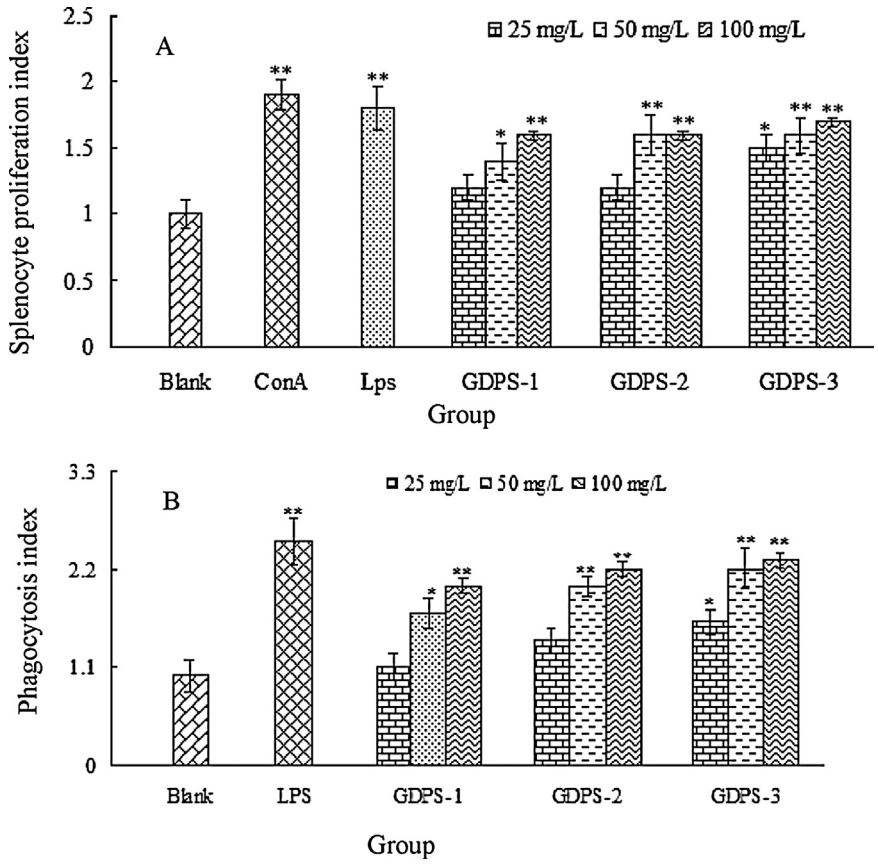

Fig. 4. Effects of GDPS on splenocytes proliferation index (A) and phagocytosis index of peritoneal macrophages (B) in vitro. $^{*}P<0.05$, $^{**}P<0.01$ vs. blank control.

promoting effects significantly increased ($P<0.05$) with the increase of sample concentration ranging from 50 to 100 mg/L. GDPS-3 presented the strongest promoting effect and its stimulating index at 100 mg/L was similar to that of LPS at 10 mg/L. The results indicated that GDPS had directly mitogen effect on mouse splenocytes and could stimulate proliferation of spleen lymphocytes.

3.5.2. *Effect of GDPS on phagocytic activity of peritoneal macrophages*

Activated macrophage not only participates in both specific immune reactions and non-specific immune reactions, but also is the "bridge cell" of these two kinds of immune reactions (Chen et al., 2008). One of the most distinguished features of macrophage activation would be an increase in phagocytic activity. It is well known that macrophages can phagocytose some dyes such as neutral red and malachite green in vitro. Therefore, in the present study we carried out the neutral red phagocytosis assay to evaluate the effect of GDPS on phagocytic activity of peritoneal macrophages. As shown in Fig. 4B, phagocytosis indices of GDPS-1, GDPS-2 and GDPS-3 all exceeded 1.0 and increased in a dose-dependent manner, indicating that all the three fractions had abilities to prime macrophages for an enhanced phagocytic activity. Especially, the phagocytic activity was obviously stimulated by GDPS-3 in a dose-dependent manner ($P<0.05$), and its stimulating index at high concentration was comparable to that stimulated by the positive control (LPS).

3.6. *Hepatoprotective effect in vivo of GDPS*

Hepatic damage, both acute and chronic, is a common pathology worldwide (Sun, Wei, Gui, Wu, & Wang, 2008). In spite of tremendous strides in the modern medicine, there are still only a few drugs available for the treatment of liver disorders (Bhandarkar & Khan, 2004; Huang & Wu, 2005). There is a worldwide trend to go back to natural products origin that are in use for the treatment of liver ailments (Rajesh & Latha, 2004). It has been demonstrated that the fresh meat of *G. didyma* is rich in polysaccharides that may contribute to its biological functions, such as anti-tumor, anti-inflammation and immune-regulation. However, little attention has been devoted to the hepatoprotective effect of GDPS. In the present study, we investigated the hepatoprotective effect in vivo of GDPS on liver injury in mice. It is known that BCG priming and LPS challenge in mice can cause massive liver injury, which consists of priming and eliciting phases. BCG priming induces mononuclear cell infiltration into the liver lobules and granuloma formation. The subsequent LPS injection elicits acute and massive hepatic injury, with a host release of reactive oxygen species, nitric oxide (Guler et al., 2004). Silymarin is an antioxidant flavonoid complex derived from milk thistle (*Silybum marianum*). It has long been used in the treatment of liver diseases and has been proved to have a protective effect against experimental hepatotoxicity (Hagymási, Kocsis, Lugasi, Fehér, & Blázovics, 2002). In this study, therefore, mice treated with BCG/LPS were used as hepatotoxicity animal model and silymarin was used as positive control medicine.

Hepatocellular necrosis leads to elevation of the serum marker enzymes, which are released from the liver into blood. The increased levels of ALT and AST are conventional indicators of liver injury (Achliya, Wadodkar, & Dorle, 2004). The effects of drugs on serum AST and ALT levels are shown in Table 2. Apparently, significant increases in ALT and AST levels ($P<0.05$) were observed in serums of the model control group compared with those of the normal control group, indicating that the hepatotoxicity mice model was established in the present study. Moreover, the

Table 2
Effects of GDPS administration on serum ALT, AST and TNF-α in BCG/LPS-injured mice.

Group	ALT (Units/L)	AST (Units/L)	TNF-α (ng/mL)
I (normal control group)	32 ± 11^{e}	91 ± 25^{c}	1.65 ± 0.34^{d}
II (model control group)	137 ± 38^{a}	153 ± 28^{a}	5.31 ± 0.88^{a}
III (positive control group)	41 ± 15^{d}	101 ± 17^{bc}	3.11 ± 0.76^{c}
IV (GDPS treatment group, 200 mg/kg)	122 ± 37^{a}	134 ± 28^{a}	5.15 ± 0.53^{a}
V (GDPS treatment group, 400 mg/kg)	85 ± 20^{b}	112 ± 30^{b}	4.09 ± 0.82^{b}
VI (GDPS treatment group, 600 mg/kg)	66 ± 18^{c}	108 ± 22^{b}	3.94 ± 0.75^{b}

Data were presented as mean ± SD (n = 10) by one-way ANOVA followed by the Duncan's multiple-range tests, and values not sharing a common superscript letter (a–e) denotes significant difference (P < 0.05).

Table 3
Effects of GDPS administration on the activities of SOD, GSH-Px and level of MDA in livers of BCG/LPS-injured mice.

Group	SOD (U/mg protein)	GSH-Px (U/mg protein)	MDA (nmol/mg protein)
I (normal control group)	257 ± 41^{a}	488 ± 59^{a}	2.3 ± 0.25^{c}
II (model control group)	109 ± 22^{e}	223 ± 51^{d}	4.7 ± 0.33^{a}
III (positive control group)	208 ± 33^{b}	409 ± 56^{b}	2.9 ± 0.27^{b}
IV (GDPS treatment group, 200 mg/kg)	124 ± 27^{d}	341 ± 78^{c}	3.1 ± 0.26^{b}
V (GDPS treatment group, 400 mg/kg)	145 ± 25^{c}	382 ± 49^{c}	2.7 ± 0.41^{b}
VI (GDPS treatment group, 600 mg/kg)	169 ± 21^{c}	420 ± 88^{b}	2.5 ± 0.20^{bc}

Data were presented as mean ± SD (n = 10) by one-way ANOVA followed by the Duncan's multiple-range tests, and values not sharing a common superscript letter (a–d) denotes significant difference (P < 0.05).

elevated levels of serum AST and ALT were significantly reduced (P < 0.05) in the groups pretreated with GDPS (especially at a dose of 600 mg/kg BW). The results demonstrated that the administration of GDPS could decrease the levels of AST and ALT in serums of the BCG/LPS-induced immunological liver injury mice. TNF-α, a multifunctional cytokine, is thought to play a critical role in immune-mediated hepatitis. It is able to induce release of a wide range of pro-inflammatory mediators, including IL-1, IL-6 and adhesive molecules, which play an important role in triggering inflammation and consequent liver damages (Nagakawa et al., 1990; Nakazato, Oku, Yamane, Tsuruta, & Suzuki, 2002). As shown in Table 2, the level of TNF-α in serum was significantly increased in BCG/LPS-treated mice when compared with those of normal control group, indicating considerable inflammatory response in model group mice. On the other site, the administration of GDPS markedly decreased the level of serum TNF-α compared to the model control group. The results suggested that inhibition of TNF-α production might be one of the means by which GDPS attenuated BCG/LPS-induced liver injury in mice.

Oxidative stress is well known as an important mediator in the BCG/LPS-induced immunological liver injury model. It occurs when there is excessive free radical production and/or low antioxidant defense. Over-production of free radicals can initiate reactive oxygen species-mediated cascade causing hepatocyte death, and leading to acute hepatic damage. The major antioxidant enzymes, including SOD and GSH-Px are regarded as the primary defense system against the reactive oxygen species (Inal, Kanbak, & Sunal, 2001). To confirm the effect of GDPS on oxidative stress in immunological liver injury, we examined the oxidative stress parameters, such as SOD and GSH-Px. The results in the present study showed that the activities of SOD and GSH-Px were dramatically decreased in mice by the administration of BCG/LPS. However, pretreatment with GDPS could improve markedly the activities of SOD and GSH-Px in livers of BCG/LPS-treated mice (Table 3). The results suggested that the antioxidative system in liver tended to be normalized by the protective action of GDPS.

MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acid (Vaca, Wilhelm, & Harms-Ringdahl, 1988). It is an indicator of lipid peroxidation. And lower MDA level suggests that there is less lipid peroxidation and weaker oxidant stress (Bagchi, Bagchi, Hassoun, & Stohs, 1995). In the present study, BCG/LPS-induced toxicity caused an increase in the liver tissue MDA level as compared to the normal control group. Pretreatment with GDPS significantly reversed these changes. The administration of GDPS caused a significant decrease in MDA level as compared to the model control group (Table 3). The results suggested that GDPS had a significant protective effect against BCG/LPS-induced hepatotoxicity in mice. The present results are consistent with the previous reports that some polysaccharides are effective in protecting BCG/LPS-induced liver injury (Li, Fang, & Zhang, 2007; Song et al., 2008).

According to the results stated above and some other literatures, it is believed that the hepatoprotective effect of polysaccharide is related to its immunostimulatory activity, which is dependent on the sugar composition, molecular weight and glycosidic linkage of polysaccharides (Wang et al., 2013). The immunological activity of polysaccharide is reported to be positively correlated with its molecular weight (Leung et al., 2004). The high uronic acid content has been proved to be beneficial for immunostimulatory activity of polysaccharides (Xu, Yao, Sun, & Wu, 2009). In the present study, we found that GDPS had high uronic content with high molecular weight and possessed high immunostimulatory activity. Thus it seemed that the chemical and structural properties of GDPS might contribute to its immunostimulatory activity, and then be related to its hepatoprotective effects. However, the immunostimulatory mechanisms of polysaccharides were complex and have not been fully characterized. Therefore, the structure–activity relationship of GDPS requires further and deep investigation.

4. Conclusion

In the present study, crude GDPS was prepared by hot water extraction and further fractionated by DEAE-52 cellulose chromatography and Sephadex G-100 chromatography into three purified fractions of GDPS-1, GDPS-2 and GDPS-3. The three fractions were mainly composed of glucose with the average molecular weight of 161, 197 and 204 kDa, respectively. The assay of the immunostimulatory activities in vitro demonstrated that GDPS-1, GDPS-2 or GDPS-3 could significantly stimulate the proliferation of splenocytes and enhance phagocytic activity of peritoneal macrophages. The assay of the hepatoprotective activity in vitro revealed that the administration of crude GDPS significantly decreased the serum levels of ALT, AST and TNF-α, inhibited the

formation of MDA and restored the liver activities of SOD and GSH-Px in BCG/LPS-induced immunological liver injury mice. Therefore, GDPS should be a potent natural polymer with immunostimulatory and hepatoprotective activities. Further work on its structure and structure-activity relationship is in progress.

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