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Antioxidant and immunological activity *in vitro* of polysaccharides from *Gomphidius rutilus* mycelium

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ABSTRACT

Two novel polysaccharide fractions, GRMP1 and GRMP2, were isolated from the mycelium of *Gomphidius rutilus* through submerged fermentation. GRMP1 and GRMP2 had similar average molecular weights (35 and 31 kDa, respectively), and were composed of glucose and xylose at molar ratios of 0.46:1 and 0.63:1, respectively. *In vitro* antioxidant tests showed that GRMP1 and GRMP2 partly scavenged superoxide radical but almost had no scavenging effect on 1,1-diphenyl-2-picrylhydrazyl free radical. The effect of GRMP1 on hydroxyl radicals was stronger than that of GRMP2. Both GRMP1 and GRMP2 had relatively low reducing power and significant lymphocyte proliferation activity. In the presence of concanavalin A or lipopolysaccharide as mitogens for lymphocytes, the lymphocyte proliferation activity increased for GRMP1 but not for GRMP2 within the test dosage range.

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1. Introduction

Mushrooms are appreciated worldwide for their taste and flavour, and are consumed both in fresh and processed forms. They are attracting attention as functional health promoters because of their biochemical composition, *i.e.*, their significant contents of proteins, carbohydrates, lipids, enzymes, minerals, vitamins, and water (Chang, 2008). Various kinds of mushroom polysaccharide have been isolated and found to have wide-ranging bioactivities such as antitumor, antioxidant, anticancer, and immunological properties (Chen et al., 2010; Li et al., 2008; Sun et al., 2008; Yan et al., 2011; Zhang, Cui, Cheung, & Wang, 2007).

Gomphidius rutilus, which belongs to subphylum Basidiomycotina, is a traditional Chinese medicinal and edible fungus often found beneath pine trees. The extraction and antioxidant activity of polysaccharides from the fruiting bodies of *G. rutilus* have been previously studied (Sun et al., 2010). These polysaccharides are found to have high potential antioxidant activity. However, the limited natural resource and difficult artificial cultivation of *G. rutilus* for obtaining fruiting bodies makes large polysaccharide quantities impossible to acquire. Meanwhile, growing mushroom mycelium in a defined medium by submerged fermentation is generally recognised as a rapid alternative method of obtaining fungal biomass with consistent quality. Polysaccharide extraction from mycelium harvested by submerged fermentation instead of fruiting bodies has already been performed (Chen, Wang, Zhang, & Huang, 2012; Lee et al., 2010; Zhu et al., 2012). In a recent study, our group demonstrated that the production of exopolysaccharide from cultured *G. rutilus* exhibits antioxidant activities (Gao et al., 2012). In the current study, polysaccharide from the mycelium of *G. rutilus* (hereafter denoted as GRMP) was purified. Basic biochemical characterisations and bioactivity investigations were also conducted. The results can promote the use of GRMP in food and pharmaceutical industries.

2. Materials and methods

2.1. Chemical

Bovine serum albumin (BSA), dextran (T 130, 80, 50, 20, 10), nitroblue tetrazolium (NBT), methionine (MET), 1,1-diphenyl-2-picrylhydrazyl (DPPH), riboflavin (RF), thiazolyl blue tetrazolium bromide (MTT), concanavalin A (ConA), and lipopolysaccharide (LPS) were purchased from Sigma Chemicals Co. (St. Louis, 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, PR China). Unless otherwise stated, all chemicals used were analytical grade.

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2.2. Organism and extraction preparation

Fresh fruiting bodies of *G. rutilus* were purchased from a local market (Fushun, Liaoning Province, PR China). The strain was isolated from the fruiting body in the laboratory.

G. rutilus was initially grown on potato dextrose agar medium in a Petri dish for 6–7 d. A 10 mm^2 portion of the agar plate culture was sliced with a sterilised cutter and then inoculated into a 500 mL flask containing 200 mL of basal medium. The culture medium (pH 8.0) was composed of 30 g/L sucrose, 3 g/L peptone, 0.5 g/L $MgSO_4$, 1.5 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.03 g/L $ZnSO_4$, and 0.03 g/L $MnSO_4$. The culture medium was cultured in a rotary shaker (180 rpm) at 28 °C for 6 d. The original block of inoculation in the liquid medium was discarded, and the mycelia of *G. rutilus* were obtained by centrifugation (3000 × *g*, 20 min) after cultivation.

The cultured mycelia were washed twice with distilled water, defatted with ethanol, extracted three times with boiling water for 2 h, concentrated, and treated with three volumes of ethanol. Crude GRMP was then obtained by centrifugation (3000 × *g*, 20 min).

GRMP was dissolved in distilled water, freeze thawed, and centrifuged at 4 °C until no insoluble substance was visible. The solutions were applied to a diethylaminoethyl (DEAE)-cellulose (Amersham Biosciences, Sweden) column (3 cm × 45 cm) equilibrated with a linear gradient of NaCl from 0 M to 1.0 M. After adsorption of most of the pigments in the column, the eluted sample was collected and further purified by gel filtration chromatography on a Sepharose CL-6B (Amersham Biosciences, Sweden) column (2.5 cm × 90 cm) with distilled water. The different fractions were dialysed against distilled water and lyophilised.

2.3. Assay of polysaccharide and protein

The polysaccharide content was determined by the phenol–sulphuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1958). Bradford's method with Coomassie brilliant blue R-250 reagent was used to determine the protein content in the polysaccharides using BSA as the standard (Bradford, 1976).

2.4. Investigation of polysaccharide

The average molecular weight of the polysaccharides was determined by high-performance size-exclusion chromatography (HPSEC), which was performed on a Shimadzu system with a TSK-G3000PWXL column (7.8 mm × 30.0 cm) and a Shimadzu RID-10A detector. The eluent was 0.7% Na_2SO_4, and the flow rate was 0.5 mL/min at 40 °C. Molecular mass was estimated using a reference against a calibration curve constructed from a set of standard Dextrans (T 130, 80, 50, 20, 10, Sigma) (Sun et al., 2008).

Gas chromatography (GC) was used for monosaccharide identification. The polysaccharides were hydrolysed with 2 M trifluoroacetic acid (TFA; 120 °C, 3 h). After hydrolysis, the neutral monosaccharides were successively reduced with $NaBH_4$ and acetylated with 1:1 pyridineacetic anhydride at 90 °C for 1 h. The alditole acetates were analysed by GC performed on a Shimudzu GC-14 C instrument equipped with 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 held for 1 min, and increased to 265 °C at a rate of 8 °C/min held for 20 min (Li et al., 2008). Uronic acid content was determined by the m-hydroxybiphenyl colourimetric procedure with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

Fourier transform infrared (FTIR) spectra were obtained in KBr discs on a PerkinElmer Spectrum GX (USA). Sixteen scans were co-added at a resolution of 4 cm^{-1} from 4000 cm^{-1} to 400 cm^{-1}.

2.5. Antioxidant activity test in vitro

Antioxidant activity was determined according to the superoxide anion radical scavenging activity, hydroxyl radical scavenging activity, DPPH radical scavenging activity, and reducing power.

2.5.1. Superoxide radical scavenging assay

The method of Stewar and Beewley (1980), with minor modifications, was used to confirm the scavenging activity of superoxide anion radical. The reaction mixtures used had a final volume of 5 mL and contained the following reagents (final concentrations): 10 mmol/L RF, 75 μmol/L NBT, 100 mmol/L EDTA, 13 mmol/L MET, 50 mM phosphate buffer (pH 7.8), and 0–3750 mg/L polysaccharide samples. The mixtures were left undisturbed at 25 °C for 30 min under a fluorescent lamp and then the absorbance was measured at 560 nm using ascorbic acid (Vc) as a positive control. Inhibition of the ability to scavenge superoxide radical was calculated using the following equation:Scavenging rate (%) = $\left(1 - \frac{A_0}{A_i}\right) \times 100\%$where A_0 is the absorbance of the blank and A_i is the absorbance of GRMP/Vc.

2.5.2. Hydroxyl radical scavenging activity

Tests on the hydroxyl radical scavenging activity were performed by the method of Zhong, Jin, Lai, Lin, and Jiang (2010) with minor modifications. The reaction mixtures contained 1 mL of 9 mmol/L $FeSO_4$, 1 mL of 9 mmol/L salicylic acid-ethanol, 1 mL of polysaccharide sample (0–3750 mg/L), and 1 mL of 9 mmol/L H_2O_2. After incubation at 37 °C for 30 min, the absorbance of the mixture was measured at 510 nm. The ability to scavenge hydroxyl radical was calculated using the following equation:Scavenging rate (%) = $\left(1 - \frac{A_i - A_{i0}}{A_0}\right) \times 100\%$where A_0 is the final absorbance of the blank, A_i is the final absorbance of GRMP/Vc, and A_{i0} is the background value of existing H_2O_2.

2.5.3. DPPH free radical scavenging activity

The scavenging effects of the polysaccharide fractions on DPPH radicals were determined by the method of Deng et al. (2011). A mixture containing 2 mL of polysaccharide samples (0–3750 mg/L) and 2 mL of 0.1 μmol/L DPPH-EtOH was incubated at 25 °C for 15 min. The absorbance of the mixture was then measured at 517 nm using Vc as a positive control. The ability to scavenge DPPH radical was calculated using the following formula:Scavenging rate (%) = $\left(1 - \frac{A_i}{A_0}\right) \times 100\%$where A_0 is the final absorbance of DPPH solution and A_i is the final absorbance of GRMP/Vc.

2.5.4. Determination of the reducing power

The reducing power of the polysaccharide fractions was determined by the method of Deng et al. (2011). The reaction mixture contained 2.5 mL of phosphate buffer (pH 6.8, 0.2 M), 2.5 mL of $K_3Fe(CN)_6$ (1%, w/v), and 2 mL of polysaccharide samples (0–3750 mg/L). After incubation at 50 °C for 20 min, 2.5 mL of TFA (10%, w/v) was added to stop the reaction, and then the mixture was centrifuged at 1200 × *g* for 10 min. After centrifugation, 2.5 mL of the supernatant was mixed with 2.5 mL of deionised water and 0.5 mL of $FeCl_3$ (0.1%, w/v). After incubation at room temperature for 15 min, the absorbance was measured at 700 nm using Vc as a positive control.

2.6. Splenocyte activation test

Cell proliferation was assessed by the MTT-based colourimetric assay. BALB/c mice (male, 8–12 weeks old) were sacrificed by cervical dislocation and their spleens were aseptically removed.

Spleen cells were obtained by gently teasing the organ in RPMI-1640 medium under aseptic condition followed by centrifugation at 3000 × *g* for 10 min at room temperature. The red blood cells were removed by hemolytic Gey's solution. After two washes, the cells were resuspended in RPMI 1640 complete medium. The cell concentration was adjusted to 2×10^6 cell/mL. The cell suspension was plated in a 96-well culture plate with or without ConA (5.0 μg/mL) or LPS (20.0 μg/mL). The samples (concentrations of 50, 100, 200, 400, and 800 μg/mL) 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 further incubated for another 4 h. After aspirating the supernatant from the wells, 100 μL of dimethylsulphoxide was added to dissolve the formazan crystals. The absorbance of each well was read at 570 nm with a microplate reader (Model 680, Bio-Rad Co., USA).

3. Results and discussion

3.1. Purification of crude GRMP

Crude GRMP was prepared from the mycelium of *G. rutilus* by hot-water extraction, centrifugation, and ethanol precipitation. Crude GRMP solution was loaded into a DEAE-cellulose column, which was then equilibrated with a linear gradient of NaCl from 0 M to 1.0 M. Two independent elution peaks (F1 and F2, Fig. 1a) detected by the phenol–sulphuric acid assay were obtained. The two fractions were then collected, dialysed, concentrated, and loaded into a column of Sepharose CL-6B. Each fraction produced one single elution peak (Fig. 1b and c), named GRMP1 and GRMP2.

3.2. Investigation of GRMP1 and GRMP2

GRMP1 and GRMP2 each showed a single symmetrical peak on HPSEC and had estimated average molecular weights of 35 and 31 kDa, respectively. Monosaccharide composition analysis by GC showed that glucose and xylose were the dominant monosaccharide units in both GRMP1 and GRMP2. However, the corresponding molar ratios between GRMP 1 (0.46:1) and GRMP 2 (0.63:1) significantly differed, and uronic acid was not detected.

The IR spectra of GRMP1 and GRMP2 shown in Fig. 2 did not remarkably differ. The main absorption characteristics of the polysaccharide structures were related to O—H stretching between 3500 and 3000 cm^{-1}, as well as C—H stretching between 3000 and 2800 cm^{-1}. The absorbance in 1646 cm^{-1} can be due to water. The absorbance at 1251 and 1258 cm^{-1} was due to the existence of sulphate radical. The absorbances at 1032 cm^{-1} were due to C—O—H and C—O—C, which spanned from 1200 cm^{-1} to 1000 cm^{-1}. The band at about 820 cm^{-1} in Fig. 2a was ascribed to α-anomeric carbon, and the band at 869 cm^{-1} (corresponding to the band at 890–840 cm^{-1} in Fig. 2b) displayed stretching absorption, suggesting that both α- and β-glycosidic bonds were present in GRMP2.

3.3. Antioxidant activities

Antioxidant activities can be demonstrated by various mechanisms and reactions, such as radical scavenging, reductive capacity, prevention of chain initiation, and binding of transition metal ion catalysts, among others (Frankel & Meyer, 2000). In this study, the ability to scavenge superoxidant, hydroxyl, and DPPH radicals, as well as the reducing power of GRMP1 and GRMP2 *in vitro* were evaluated.

Superoxide anions are precursors to active free radicals. Although superoxide anions are not highly reactive, they can produce other reactive oxygen species such as hydroxyl radical and

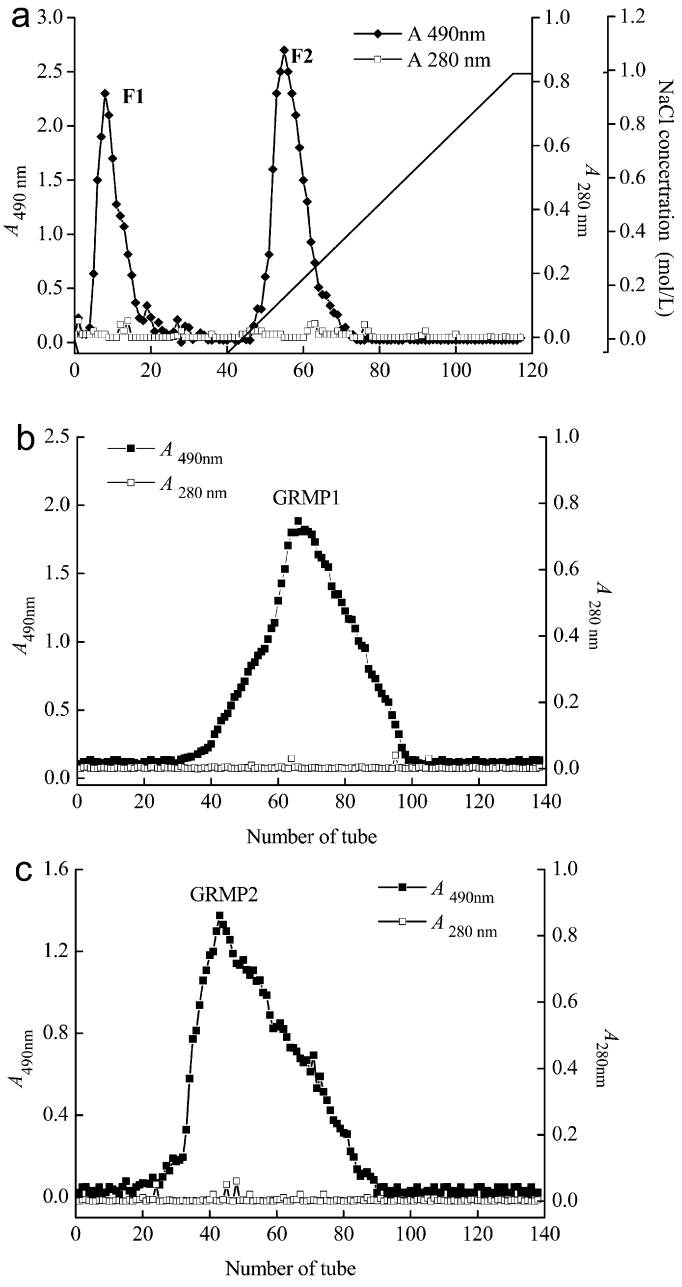

Fig. 1. Elution curve of crude GRMP on DEAE-cellulose DE-52 column (a) and elution curves of polysaccharides fractions (F1and F2) on Sepharose CL-6B column (b and c). $A_{490\ nm}$ and $A_{280\ nm}$ indirectly indicate the polysaccharides content and protein content, respectively.

singlet oxygen, which further inflict damage to biological macromolecules. In Fig. 3a, scavenging effects on superoxide anion radicals of Vc was remarkably higher than those of GRMP1 and GRMP2 within the test dosage range. The scavenging effects of GRMP1 and GRMP2 were weak, and equilibria were reached at 2500 mg/L concentration with the maximum scavenging rates being 29.2% and 39.7%, respectively. Within the test dosage range, the EC_{50} values of GRMP1 and GRMP2 were unconfirmed, whereas the EC_{50} value of Vc was 462.4 ± 5.6 mg/L.

Hydroxyl radical, considered as one of the most reactive oxygen radicals, can easily cross cell membranes, readily react with most biomolecules (such as carbohydrates, proteins, lipids, and DNA) in cells, as well as inflict tissue damage or cause cell death (Yuan, Zhang, Fan, & Yang, 2008). Thus,

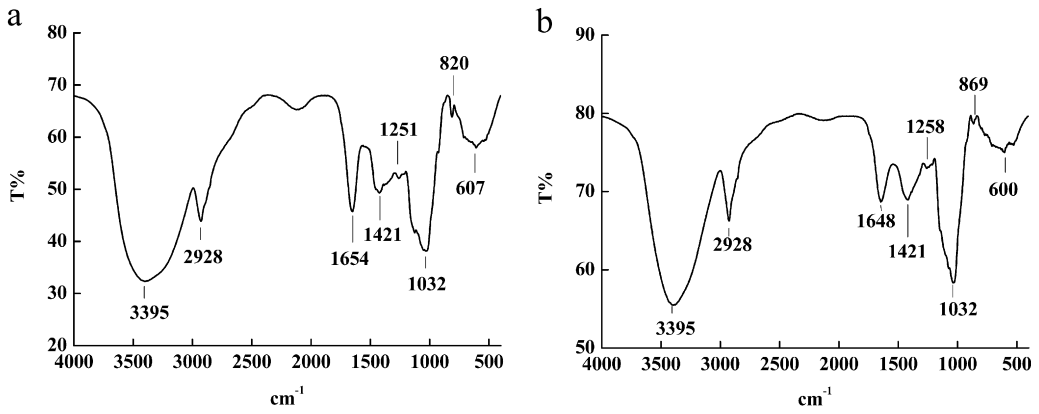

Fig. 2. IR spectrum of GRMP1 (a) and GRMP2 (b).

removing hydroxyl radicals is important for protecting living systems. Fig. 3b shows that both GRMP1 and GRMP2 had hydroxyl radical scavenging abilities, with GRMP1 having a markedly higher ability than GRMP2. The EC_{50} of GRMP1 for hydroxyl radical scavenging activity was 147.2 ± 5.7 mg/L, which significantly differed from the scavenging effect of Vc (379.8 ± 7.2 mg/L). The hydroxyl radical scavenging activity of GRMP2 was weaker, with a maximum scavenging rate of 30.4%, and its EC_{50} cannot be determined within the test dosage range.

DPPH free radical is a stable radical with a maximum absorption at 517 nm and can readily undergo scavenging by an antioxidant. Thus, DPPH free radical is widely used to evaluate the free radical scavenging activities of natural compounds (Leong and Shui, 2002). Fig. 3c shows that both GRMP1 and GRMP2 almost did not affect DPPH radical.

The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity (Kallithraka, Bakker, & Clifford, 2001). In Fig. 3d, although the reducing powers of GRMP1 and GRMP2 gradually increased with increased concentration, the reducing power of polysaccharide was much lower than that of Vc.

Compared with the polysaccharide from fruiting body of *G. rutilus*, antioxidant capacity of GRMP is mainly reflected on the scavenging ability of hydroxyl radical. The other antioxidant

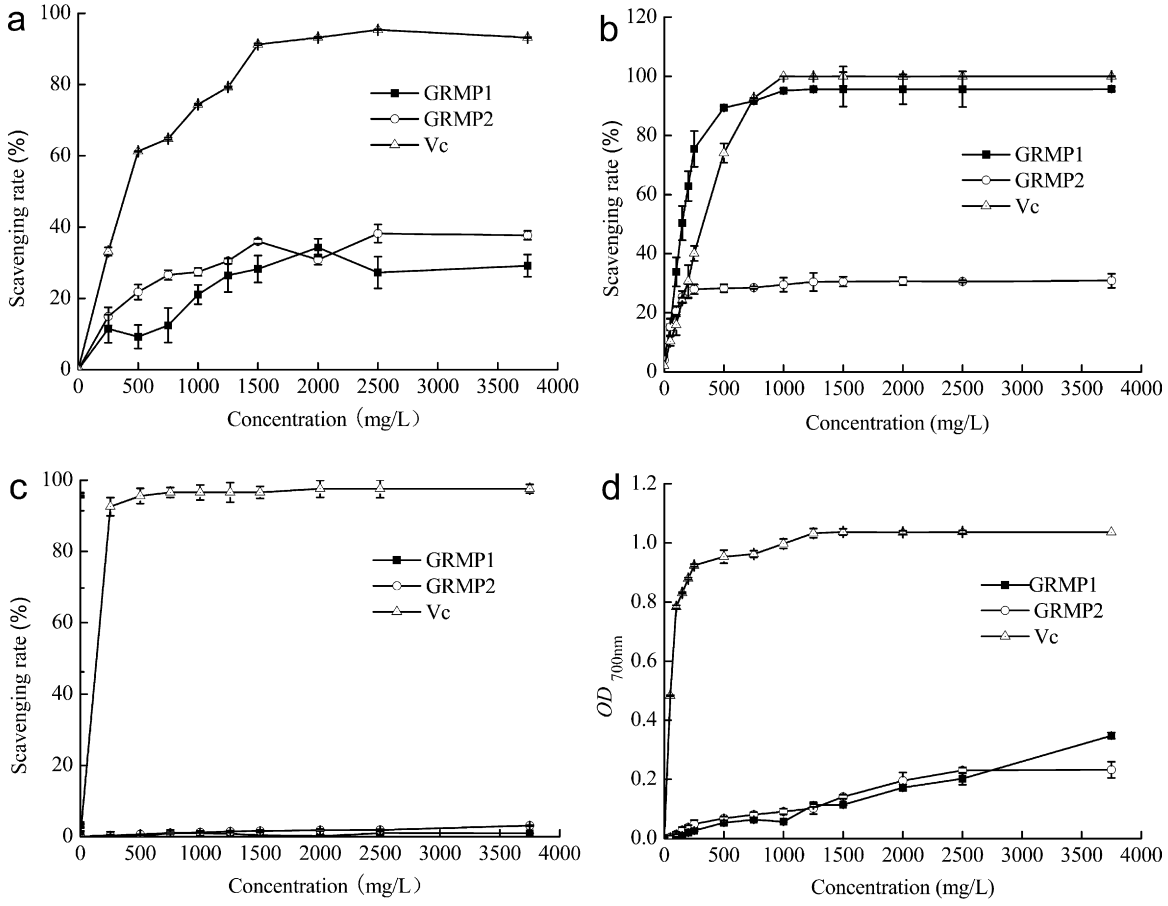

Fig. 3. Antioxidant activity assay of GRMP1 and GRMP2. (a) Scavenging effects on superoxide anion radicals. (b) Scavenging effects on hydroxyl radicals. (c) Scavenging effects on DPPH. (d) Reducing power.

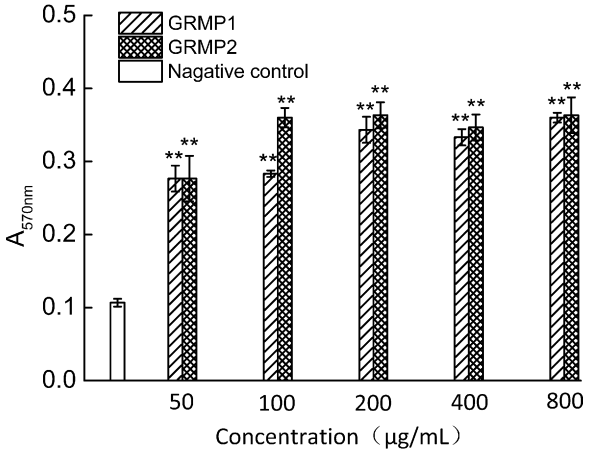

Fig. 4. Effect of GRMP1 and GRMP2 on splenocyte proliferation. Proliferation activities were expressed as the absorption at 570 nm. Values are mean ± SD; $P < 0.05$, $P < 0.01$ *vs.* negative control.

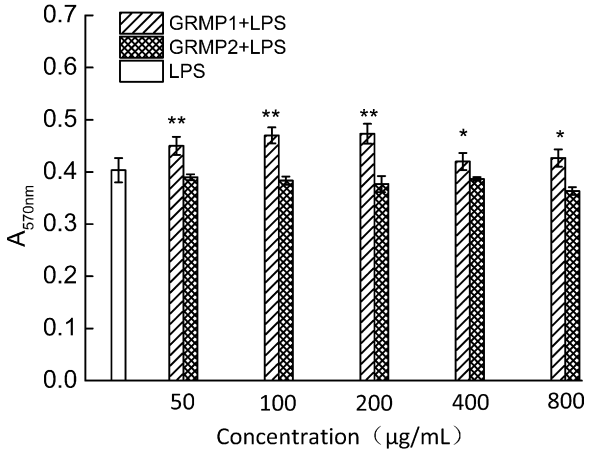

Fig. 6. Effect of GRMP1 and GRMP2 on LPS-induced splenocyte proliferation activities. Proliferation activities were expressed as the absorption at 570 nm. Values are mean ± SD; $P < 0.05$, $P < 0.01$ *vs.* LPS.

capacities of GRMP were significantly weaker than those from fruiting bodies (Sun et al., 2010).

3.4. Activity of lymphocyte proliferation

Many fungal polysaccharides show significant antitumor activity (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Jong & Donovick, 1989). Fungal polysaccharides inhibit tumor by regulating immune system primarily. They can activate T lymphocyte and B lymphocyte to show their effects on the immune system (Lindequist, Niedermeyer, & Jülich, 2005; Zheng, Su, Dai, & Wu, 2005). Lymphocytes proliferation is an indicator of immunoactivation. Lymphocytes induced by ConA *in vitro* may be used as a method to evaluate T lymphocyte activity, while lymphocytes induced by LPS may be used to evaluate B lymphocyte activity (Borchers et al., 1999; Ooi & Liu, 2000). In our research, GRMP1 and GRMP2 were subjected to immune tests to evaluate their effects on lymphocyte proliferation. As shown in Fig. 4, both GRMP1 and GRMP2 significantly increased lymphocyte proliferation ($P < 0.05$). Figs. 5 and 6 show that in the presence of ConA or LPS as mitogens for lymphocytes, the significant increase in lymphocyte proliferation was only observed in GRMP1. The boosting effect of GRMP2 corresponded to the lymphocyte proliferation without Con A or LPS, suggesting that it had no synergy with Con A or LPS within the test dosage range.

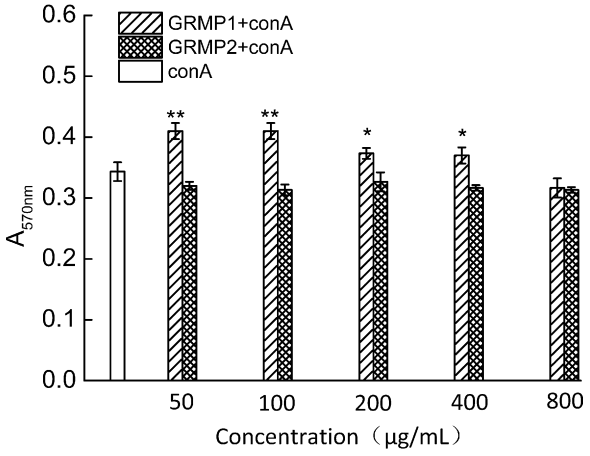

Fig. 5. Effect of GRMP1 and GRMP2 on ConA-induced splenocyte proliferation activities. Proliferation activities were expressed as the absorption at 570 nm. Values are mean ± SD; $P < 0.05$, $P < 0.01$ *vs.* ConA.

4. Conclusion

In the present study, we successfully obtained two purified homogeneous polysaccharide, termed as GRMP1 and GRMP2, by DEAE-Sepharose and Sepharose CL-6B column chromatography. Basic physicochemical properties (average molecular weight, monosaccharide composition, *etc.*) of GRMP1 and GRMP2 were investigated. GRMP1 and GRMP2 have the same monosaccharide composition, however, the proportion of monosaccharide and average molecular weight of polysaccharide are different. The results also showed that both GRMP1 and GRMP2 scavenged superoxide radical with a low total scavenging ability, and that the scavenging ability of GRMP1 to hydroxyl radical was much higher than that of GRMP2. Neither GRMP1 nor GRMP2 well scavenged DPPH radical. Both of GRMP1 and GRMP2 significantly increased lymphocyte proliferation, and the significant increase in lymphocyte proliferation was only observed in GRMP1 when ConA or LPS used as mitogens for lymphocytes. The results suggest that polysaccharides from *G. rutilus* mycelium should be explored as a novel and potential natural antioxidant and immunostimulating agent for use in functional foods or medicine.

It should be pointed out that GRMP1 is much better than GRMP2 for the antioxidant capacity and the synergies with ConA or LPS in lymphocyte proliferation capacity. The differences may be related to polysaccharide structure and should be further clarified. This work is currently under study in our laboratory.

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