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Antioxidant and immunoregulatory activity of *Ganoderma lucidum* polysaccharide (GLP)

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ABSTRACT

Four polysaccharides (GLP-I, GLP-II, GLP-III and GLP-IV) were obtained from fermented soybean curd residue by *Ganoderma lucidum*, and then purified using anion-exchange DEAE Sephadex A-50. Their structural characterization was conducted by Fourier transform infrared spectroscopy (FTIR), and their monosaccharide compositions were determined. The results demonstrated that the basic structural characterization of four polysaccharides were similar, however, monosaccharide compositions of four kinds of polysaccharides were significant difference. GLP-III and GLP-IV were composed of six kinds of monosaccharide. Nevertheless, GLP-II was composed of three kinds of monosaccharide. Moreover, their antioxidant activities were investigated on the basis of hydroxyl radical, reducing power, DPPH free radical, chelating activity, ABTS radical-scavenging and SOD-like activity. The results showed that four polysaccharides exhibited antioxidant activities in a concentration-dependent manner. Among four polysaccharides, GLP-III and GLP-IV exhibited the higher scavenging effects on hydroxyl radicals, ABTS radical, DPPH free radical, and stronger reducing power and SOD-like activity than GLP-I and GLP-II. In addition, treatment with 40 μg/mL of GLP showed significant stimulation to the macrophage proliferation and higher nitric oxide production. Overall, GLP from fermented SCR could have potential applications in the medical and food industries.

1. Introduction

Oxidation is an essential biological process to many living organisms for the production of energy. However, the uncontrolled production of oxygen-derived free radicals is hostile and damaging to cells. It can also cause a chain reaction resulting to the multiplication of new free radicals. The damage they cause includes interference and manipulation of protein, tissue loosening, genetic damage and the promotion of disease and aging. In order to reduce oxidation damage to the human, many synthetic antioxidants are widely used at present. However, recent researches suggested that synthetic antioxidants were restricted due to their potential hazards to health, such as liver damage and carcinogenesis (Yuan, Zhang, Fan, & Yang, 2008). Thus, it is essential to develop and utilize effective natural antioxidants to protect the human body from free radicals and reduce risk of many diseases such as heart disease, cancer, arthritis and the aging process (Nandita & Rajini, 2004).

Soybean curd reside (SCR), a byproduct of tofu, soymilk or soy protein manufacturing, is treated as an industrial waste with little market value because of its short shelf life. In fact, SCR generally contains protein up to 28.4% (dry basis) with high nutritive quality and a superior protein efficiency ratio, suggesting that it is a potential source of low-cost vegetable protein for human consumption (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004).

Ganoderma lucidum, a medicinal fungus called "Lingzhi" in China and "Reishi" in Japan, is one of the most famous traditional Chinese medicines. In regions of China and other Asian countries, *G. lucidum* has been used as a remedy to promote health and longevity (Shiao, 2003). Modern pharmaceutical research shows that *G. lucidum* polysaccharide (GLP) has several physiological and health effects, including strong antioxidant activities (Xu et al., 2009), immunomodulating activities (Lin et al., 2006), and anti-tumor activities (Paterson, 2006).

The traditional production of GLP is extracted from *G. lucidum* fruiting bodies, however, the time of the incubation is more than 60 days, and the concentration of extracted polysaccharides is below 100 mg/g. GLP was produced from the mycelium using SCR as a growth medium, which could not only greatly decrease the production time of GLP, but also increase the development and utilization of the agricultural waste and alleviate the pressure on the environment (Shi, Yang, Guan, Wang, & Zhang, 2012a, 2012b). To date, *G. lucidum* has been incubated in submerged culture using simple

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potato dextrose agar, however, there are few reports of the culture of *G. lucidum* to yield polysaccharide using agricultural waste in solid state fermentation. The curd polysaccharide of *G. lucidum* has been obtained from fermented SCR and it has been confirmed that the curd polysaccharide showed some antioxidant activity (data not shown), however, the antioxidant activity and immune activity of purified polysaccharide need to be further study.

In the past decades, some natural polysaccharides have been demonstrated to play an important role as free radical scavengers in the prevention of oxidative damage in living organism, which could be explored as novel potential antioxidants (Ge, Duan, Fang, Zhang, & Wang, 2009; Matkowski, Tasarz, & Szypula, 2008). Moreover, previous studies indicated that antioxidant activity of polysaccharides might show the ability to improve the activity of antioxidant enzymes, scavenge free radicals and inhibit lipid peroxidation (Chen et al., 2011). Antioxidant activities of polysaccharide can be affected by many factors including its chemical components, molecular mass, structure, conformation, even the culture conditions of the mycelium, because different culture conditions could supply the different nutrients. Although antioxidant activities of the crude *G. lucidum* polysaccharide (CGLP) were investigated, there was a dearth of information about the physiological properties and structure of CGLP. Therefore, isolation and purification of CGLP were necessary and could to better understand structural characteristics, antioxidant and immunostimulating activity, and the relationship between chemical characteristic and activity of GLP.

The aim of this study was to isolate and purify *G. lucidum* polysaccharide from fermented SCR that was fermented to use SCR as the main substance by *G. lucidum*. Furthermore, the antioxidant (DPPH, ABTS and hydroxyl radical scavenging assay, reducing power and SOD-like activity) and immunomodulation activity (the macrophage cells proliferation and nitric oxide release) were evaluated.

2. Materials and methods

2.1. Chemicals and reagents

SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Ascorbic acid, hydrogen peroxide, chloride ferric, ferrous sulfate, trichloracetic acid, ethylenediaminetetraacetic acid (EDTA), potassium bromide, sodium salicylate and trifluoroacetic acid and were purchased from Wako Pure Chemical, Osaka, Japan. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picry-hydrazyl radical (DPPH) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All other chemical reagents were of analytical grade.

2.2. Preparation, isolation and purification of GLP

The fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The crushed powder was removed the impurities for 24 h with 80% ethanol at room temperature. The extract was discarded and the residue was further extracted with the optimal conditions of ultrasonic assisted extraction (30 min, 80 °C, 80 watt of power with 10 of the water to solid ratio). Then, the extract was filtered and centrifuged at 7500 rpm for 30 min at room temperature. The supernatant was concentrated in a rotary evaporator under reduced pressure at 50 °C and removed free protein layer by the use of method of Sevage. At last, the above extract was subjected to the precipitation with fourfold volumes of ethanol. The curd polysaccharides were collected by centrifugation, washed with ethanol twice, and then freeze-dried. 1 g of crude polysaccharides was re-dissolved in 40 mL distilled water and centrifuged at 4500 rpm for 15 min. The supernatant was further purified using an anion-exchange DEAE Sephadex A-50 column (50 cm × 2.6 cm) equilibrated with distilled water. The column was eluted by distilled water, 0.1 M NaCl, 0.5 M NaCl and 1 M NaCl to yield GLP-I, GLP-II, GLP-III and GLP-IV, respectively. 6 mL/tube of eluent was collected at a flow rate of 0.4 mL/min. The polysaccharide concentration of the eluant in each tube was determined by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Pebers, & Smith, 1956). GLP-I, GLP-II, GLP-III and GLP-IV eluants were combined individually. Each fraction was placed in a regenerated cellulose bag filter (MWCO8000, Spectrum, USA), and dialyzed against 4 °C distilled water for 3 days. The purified fractions were further concentrated by a vacuum rotary evaporator (BC-R203, Shanghai Biochemical Equipment Co., Shanghai, China) at 55 °C. Concentrated fractions were lyophilized to fine powder and stored in a desiccator at room temperature.

2.3. Monosaccharide composition of polysaccharide fractions

The GLP fractions (10 mg), dissolved in 2 M trifluoroacetic acid (TFA, 2 mL), were hydrolyzed at 120 °C for 3 h in a sealed glass tube. The hydrolyzate was repeatedly co-concentrated with methanol to remove the excess acid at 50 °C, and then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg of hydroxylamine hydrochloride and 0.5 mL of pyridine by heating in a water bath for 30 min at 90 °C. After the incubation, the tubes were removed from the heat block, allowed to cool to room temperature, and then 0.5 mL of acetic anhydride was added and mixed thoroughly by vortexing. The tubes were sealed and incubated in a water bath shaker set at 90 °C for 30 min again. After cooling, approximately 0.1 mL of clear supernatant was added to the autosampler vials with inserts for injection into the gas chromatograph on a GCMS-QP2010Plus (SHIMADZU, JAP) instrument equipped with a hydrogen flame ionization detector, using a DB-1 column (30 m × 0.25 mm × 0.25 μm). The following chromatographic conditions were used: high-purity helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature of the injector and detector was 250 °C. An initial column temperature held at 100 °C followed by 10 °C/min to 280 °C. Injections were made in the splitless mode. The temperature of mass spectrometer ion source was 230 °C. 1 μL of the sample was injected into the column with the split ratio of 10:1.

2.4. FT-IR

FT-IR spectrum of the sample was determined using a Fourier transform infrared spectrophotometer (FT/IR 3000, Jusco, Japan). The sample was grounded with spectroscopic grade potassium bromide (KBr) powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range 4000–400 cm^{-1} (Funami et al., 2005).

2.5. Assay for antioxidant activities of the GLP fractions

2.5.1. HO• scavenging activity estimation

HO• scavenging activity was measured according to a literature procedure with a certain modifications (Nicholas & Quinton, 1989). HO• were generated from $FeSO_4$ and H_2O_2, and detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL of $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of the sodium salicylate (20 mM), and 1 mL of the GLP fractions. Ascorbic acid was used as the positive control. After this incubation for 1 h at 37 °C, the absorbance of the hydroxylated salicylate

complex was measured at 562 nm. The percentages of the scavenging effect were calculated as

$$\%HO^{\bullet} \text{ scavenged} = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100 \quad (1)$$

where A_1 was the absorbance of the sample or ascorbic acid, and A_0 was the absorbance of the solvent control, whereas A_2 was the absorbance of the reagent blank without sodium salicylate.

2.5.2. Ferrous metal ions chelating activity assay

Ferrous metal ions chelating activity of the GLP fractions was measured according to a literature procedure with a few modifications (Decker & Welch, 1990). Sample or ethylenediaminetetraacetic acid (EDTA) solution (1 mL) were mixed with 50 μL of ferrous chloride (2 mM) and 0.2 mL of ferrozine (5 mM), shaken vigorously, allowed to stay still for 10 min at room temperature, and the absorbance of the mixture was determined at 562 nm. EDTA was included as the positive control. The ion-chelating activity was calculated as

$$\text{chelating rate } (\%) = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100 \quad (2)$$

where A_0 was the absorbance of the control (without sample) and A_1 was the absorbance in the presence of the sample, A_2 was the absorbance without ferrozine.

2.5.3. DPPH free radical-scavenging assay

DPPH radical-scavenging activities of the GLP fractions was measured according to the method described by Shi et al. (2012a, 2012b).

IC_{50} value (mg extract/mL) was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from the linear regression analysis.

2.5.4. Determination of SOD-like activity

The levels of SOD-like activity in the GLP fractions were measured using the SOD Assay Kit-WST according to the technical manual (Shi et al., 2012a, 2012b).

2.5.5. Reducing power

The reducing power of the GLP fractions was measured according to the method of Yen and Chen (1995) with slight modifications (Yen & Chen, 1995). An aliquot of each sample (1 mL), with different concentrations, was mixed with 1 mL of phosphate buffer (200 mM, pH 6.6) followed by 1 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated for 20 min in a water bath at 50 °C. After this incubation, 1 mL of 1% trichloroacetic acid (TCA) was added, followed by centrifugation at 6000 g for 10 min. The supernatant (2 mL) was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride ($FeCl_3$), then the absorbance was measured at 700 nm against a blank in the spectrophotometer. A higher spectrophotometrical absorbance meaned a higher reducing power activity.

2.5.6. ABTS radical-scavenging activity

ABTS was dissolved in distilled water at a final concentration of 7 mM and mixed with a potassium persulphate solution at a final concentration of 2.45 mM. The reaction mixture was left to settle at room temperature for 12–16 h in the dark before use (Trishna et al., 2011). For each experiment, freshly prepared $ABTS^{\bullet+}$ solution was diluted with methanol (99.7%) to adjust its absorbance to within 0.70 ± 0.02 at 734 nm wavelength. 0.15 mL of various concentrations of the sample was subsequently mixed with 2.85 mL of $ABTS^{\bullet+}$ solution. Finally, the absorbance was measured at 734 nm after the incubation at room temperature for 10 min. The scavenging activity of ABTS free radical was calculated using the following equation:

$$\text{ABTS scavenging activity } (\%) = \frac{(C - D) - (A - B)}{(C - D)} \times 100 \quad (3)$$

where A = absorbance of ABTS solution + sample/standard, B = absorbance of potassium persulphate + sample/standard, C = absorbance of ABTS solution + distilled water/methanol and D = potassium persulphate + distilled water/methanol.

2.6. Cell evaluation

2.6.1. Chemicals and reagents

Minimum Essential Medium Eagle (MEM) medium, Griess reagent and fetal bovine serum (FBS), were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA); and the Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

2.6.2. Cell lines

The murine macrophage cell line, RAW 264.7, was obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in MEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere (ESPEC CO_2 incubator). The cells were cultured for 2–3 days to reach the logarithmic phase and then used for experiments.

2.6.3. Activation assay

The effect of the GLP fractions on the proliferation of RAW 264.7 cells was estimated using the Cell Counting Kit-8 (CCK-8), and the method described by Shi et al. (2012a, 2012b).

2.6.4. Measurement of nitric oxide production

The nitrite accumulation was measured using Griess reagent and used as an indicator of nitric oxide (NO) production in the medium (Shi et al., 2012a, 2012b). $NaNO_2$ was used as a standard to calculate the nitrite concentrations.

2.7. Statistical analysis

All experiments were performed at least in duplicate, and analyses of all samples were run in four replicates and averaged. Statistical analysis involved use of the DPS statistical analysis (DPS, version 13.5) software package. The results were presented as means of three determinations ± SD (standard deviation). Significant differences of each GLP fractions between two means were determined by Duncan multiple-range tests. Means were compared by the least significant difference test at 0.05 significant levels.

3. Results and discussion

3.1. Fractionation of GLP-I–IV

The crude polysaccharide, a water-soluble gray powder, was isolated from fermented SCR by *G. lucidum* using ultrasonic assisted extraction with a yield of 11.55% ± 0.03%. Four fractions consecutively eluted by distilled water, 0.1 M NaCl, 0.5 M NaCl and 1 M NaCl through an anion-exchange column were respectively coded as GLP-I, GLP-II, GLP-III and GLP-IV. As shown in Fig. 1, four fractions of GLP exhibited different peak in the profile of anion-exchange chromatogram implying that four fractions were clearly separated. The tube number of four fractions was 1–20, 50–90, 125–170 and 215–250, respectively. GLP-I was a neutral polysaccharide, and other three eluted by NaCl solution were acidic polysaccharides. The yield of GLP-I–IV, respectively, accounted for 5.95%, 15.69%,

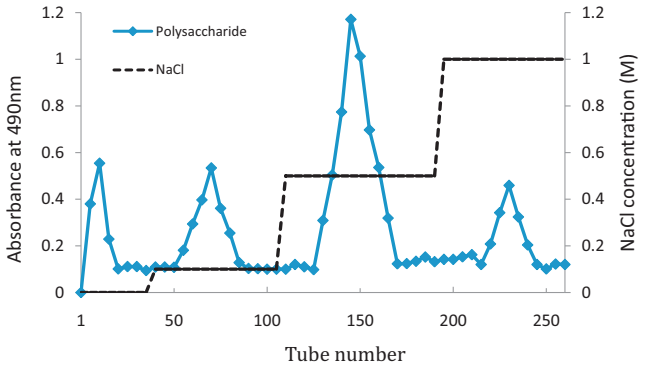

Fig. 1. Anion-exchange chromatogram of *G. lucidum* polysaccharides detected by phenol-sulphuric acid method at 490 nm. Column: DEAE52-cellulose (50 cm × 2.6 cm); flow rate: 0.4 mL/min; fraction volume: 5 mL. GLP I–IV were eluted by distilled water, 0.1 M NaCl, 0.5 M NaCl and 1 M NaCl, respectively.

33.58% and 37.27% of the crude polysaccharide extract, indicating that GLP-III and GLP-IV were the major components of the polysaccharides. In addition, the protein was not detected in all fractions.

3.2. FT-IR spectrum characterization

Carboxylate groups showed two bands: an asymmetrical stretching band near 1638 cm^{-1} and a weaker symmetric stretching band near 1407 cm^{-1} (Rao, 1967). Fig. 2 showed the spectra of polysaccharide samples. The spectra peaks occurring in the range near 3480 cm^{-1} may result from the presence of O—H group. The band at the range 2958 cm^{-1} indicated that —CH_2, H—C═O groups were present. The band at about 1638 cm^{-1} could indicate the presence of C═O groups or C═C groups vibration in structures. Two other bands at 934 and 615 cm^{-1} likely related to sugar cycles were also observed (Fig. 2). The 1200–1000 cm^{-1} region was dominated by sugar ring vibrations overlapping with stretching vibrations of (C—OH) side groups and the (C—O—C) glycosidic bonds vibration (Casu, Scovenna, Cifonelli, & Perlin, 1978).

3.3. Monosaccharide compositions

To further investigate effect of different sources on monosaccharide compositions in GLP-I, GLP-II, GLP-III and GLP-IV, GC–MS analysis were used. As shown in Table 1, GLP-I was composed of arabinose, rhamnose, xylose, mannose and glucose, with the molar ratio of 4.66:1.23:3.14:0.61:1.29. GLP-II was mainly composed of three monosaccharides, i.e. arabinose, xylose and glucose, in the molar ratio of 2.82: 1.33: 0.87. There were no rhamnose and mannose detected in GLP-II. GLP-III was found consist of arabinose, rhamnose, xylose, galactose, mannose and glucose, with the molar ratio of 5.09:0.52:1.07:1.29:0.48:2.76. GLP-IV showed difference monosaccharide composition, with the presence of arabinose, rhamnose, fucose, xylose, mannose and glucose, in the molar ratio of 4.73:0.65:0.72:2.27:0.52:0.92. Furthermore, monosaccharide compositions of GLP-III and GLP-IV were more than those of GLP-I and GLP-II. The diversity of monosaccharide compositions could be the reason that antioxidant and immunostimulating activity of GLP-III and GLP-IV were higher than those of GLP-I and GLP-II.

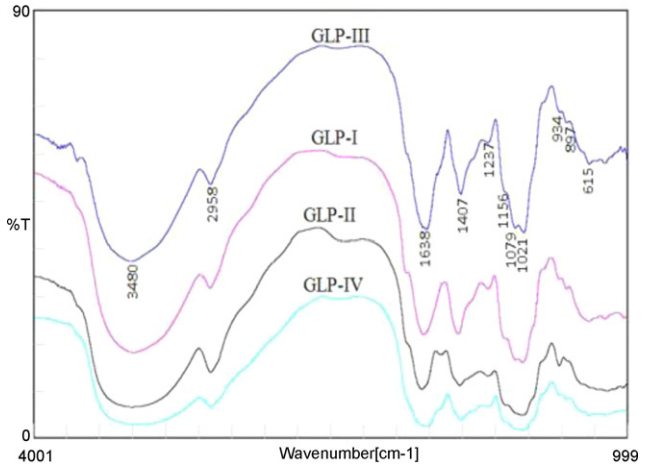

Fig. 2. FT-IR of black *G. lucidum* polysaccharides.

Table 1
The monosaccharide composition of GLP.

Sample	Composition	Molar ratio
GLP-I	Ara, Rha, Xyl, Man, Glu	4.66:1.23:3.14:0.61:1.29
GLP-II	Ara, Xyl, Glu	2.82:1.33:0.87
GLP-III	Ara, Rha, Xyl, Gal, Man, Glu	5.09:0.52:1.07:1.29:0.48:2.76
GLP-IV	Ara, Rha, Fuc, Xyl, Man, Glu	4.73:0.65:0.72:2.27:0.52:0.92

3.4. Antioxidant activities analysis

3.4.1. Scavenging effects on hydroxyl radicals

Hydroxyl radical (•HO) can easily cross cell membranes, readily react with most biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death (Yuan et al., 2008). Thus, removing •HO is important for the protection of living systems. The results of hydroxyl radical scavenging activities of the GLP-I, GLP-II, GLP-III, GLP-IV and ascorbic acid were given in Fig. 3A. Among all samples, the scavenging ability on hydroxyl radical decreased in the following order: GLP-III > GLP-IV > GLP-I > GLP-II. However, the scavenging activities of all samples were weaker than that of ascorbic acid ($P < 0.05$). At a concentration of 10 mg/mL, the scavenging activities were 68.47%, 66.10%, 98.19%, 95.13% and 99.41% for the GLP-I, GLP-II, GLP-III, GLP-IV and ascorbic acid, respectively. The results demonstrated that the polysaccharides from fermented SCR possessed •HO scavenging activities, especially purified fraction of GLP-III showed the strongest activities. The antioxidant mechanism may be due to the supply of hydrogen by polysaccharide, which combines with radicals and forms a stable radical to terminate the radical chain reaction. The other possibility is that polysaccharide can combine with the radical ions which are necessary for radical chain reaction, and the reaction is terminated. However, the exact mechanism underlying the free-radical scavenging activity exerted by polysaccharides is still not fully understood.

3.4.2. Ferrous metal ions chelating activities

Metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals and initiate the radical-mediated oxidative chain reactions in biological or food systems. Ion-chelating agents also may inhibit Fenton reaction and hydroperoxide decomposition. The antioxidant capacities of GLP-I, GLP-II, GLP-III and GLP-IV were shown in Fig. 3B and compared with EDTA as a positive control. The antioxidant capacities of all the samples correlated well with increasing concentration. The antioxidant capacities of purified fractions GLP-III and GLP-IV were significant higher than these of GLP-I and GLP-II. The chelating abilities of GLP-I, GLP-II, GLP-III, GLP-IV and EDTA were 30.48%, 25.18%, 39.76%, 42.00% and 98.87% at the concentration of 10 mg/mL, respectively. These results clearly demonstrated that all the samples possessed antioxidant capacities. The activities of antioxidants have been attributed to various

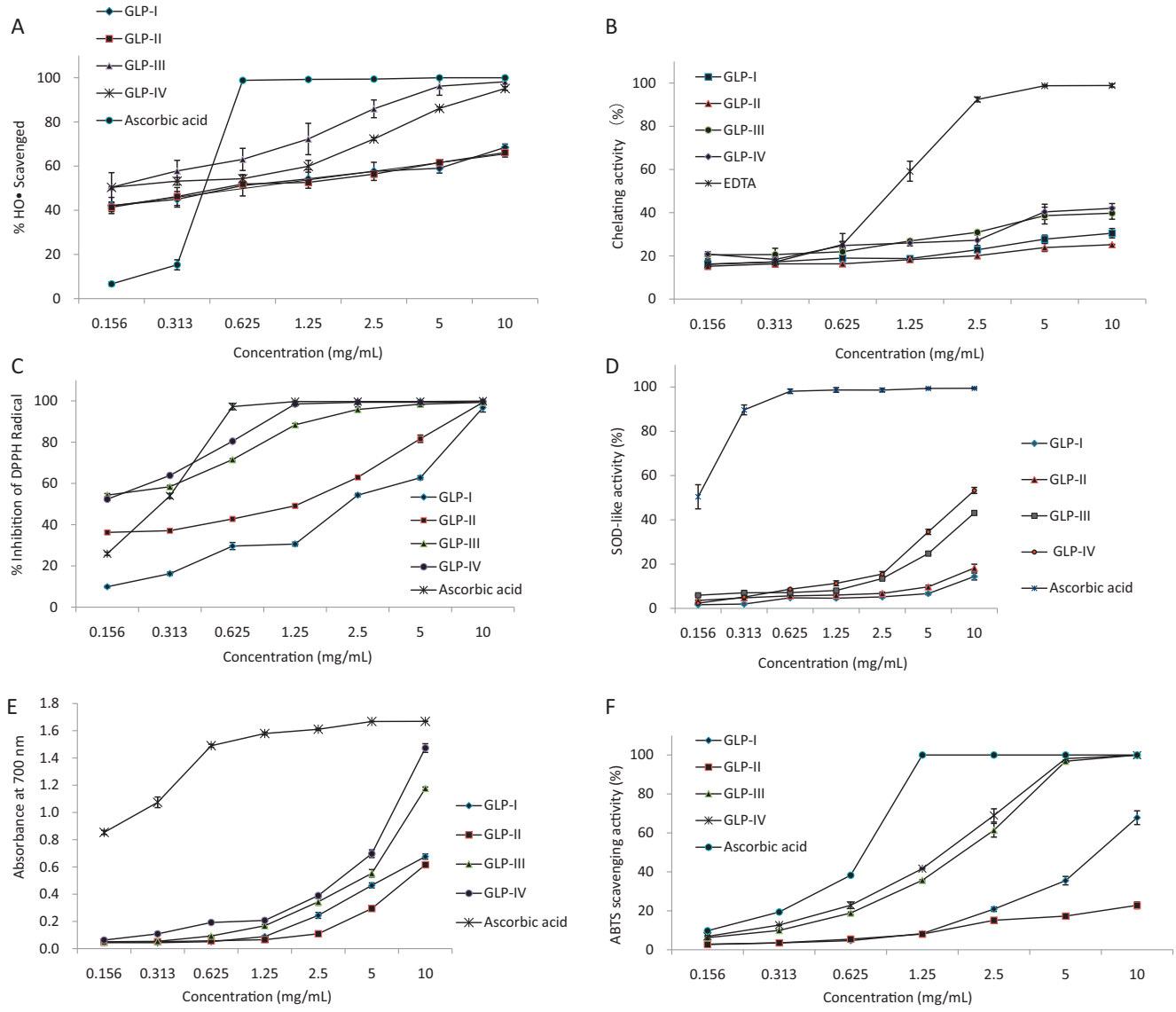

Fig. 3. Antioxidant activities of GLP. Hydroxyl radical scavenging activity (A); chelating activity (B); DPPH radical-scavenging capacity (C); SOD-like activity (D); reducing power (absorbance at 700 nm) (E); and ABTS radical-scavenging capacity (F). All treatments were conducted in triplicate, and mean values were reported. The vertical bars represented the standard deviation of each data point. Ascorbic acid and EDTA were the positive controls.

mechanisms, such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Zou et al., 2008).

3.4.3. Scavenging effects on DPPH radicals

DPPH (1,1-diphenyl-2-picrylhydrazyl) is a free-radical compound that has been widely used to determine the free-radical scavenging ability of various samples. The method of scavenging DPPH• is based on the reduction of DPPH• ethanol solution in the presence of a hydrogen donating antioxidant, resulting in the formation of the non-radical form DPPH-H. DPPH• is a stable free radical and can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Therefore, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH• free radical. As shown in Fig. 3C, the results indicated that, GLP-I, GLP-II, GLP-III and GLP-IV showed obvious scavenging activity on DPPH radical in a concentration-dependent manner. Furthermore, at relatively low concentration range 0.156–1.25 mg/mL, the scavenging activities of GLP-III and GLP-IV increased significantly with increasing concentrations. The scavenging activity of GLP-IV was the strongest, followed by GLP-III, GLP-II and GLP-I, which were lower than that of ascorbic acid. At 1.25 mg/mL, scavenging activities of GLP-I, GLP-II, GLP-III and GLP-IV were 30.62%, 49.13%, 88.38% and 98.48%, respectively. The antioxidant activity of the polysaccharides may be related to monosaccharide component, molecular size, structure and conformation. These monosaccharides in the polysaccharides are reductive agents as they can supply hydrogen, which can combine with radical and form a stable radical to terminate the radical reaction. It has been reported that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds and aromatic amines could reduce and decolourize DPPH• by their hydrogen donating ability (Li, Zhou, & Li, 2007). These results indicate that the GLP-IV might act as electron or hydrogen donator to scavenge DPPH•.

3.4.4. SOD-like activities assay

All living bodies have a complex antioxidant defence system that includes various antioxidant enzymes, such as superoxide dismutase and catalase. A rapid and facile method for the assay of SOD-like

activity, based on the ability to inhibit the auto-oxidation of pyrogallol, is widely used to predict antioxidant capability. Since an SOD standard with a known activity was used in this study, it was possible to calculate the enzyme activity directly from the standard curve. In the present study, the ascorbic acid as a positive control was used to the SOD-like activities assay. As shown in Fig. 3D, although the stronger SOD-like activities (98.08% at 0.625 mg/mL) were observed in ascorbic acid than any other polysaccharides fractions, the SOD-like activities increased with the concentrations of GLP, and at the concentration of 10 mg/mL, SOD-like activities of GLP-I, GLP-II, GLP-III and GLP-IV were 14.36%, 18.22%, 43.12%, and 53.25%, respectively. SOD is an important antioxidant enzyme in vivo and is widely distributed in various biological body, such as animals, plants, microorganisms. SOD has a special physiological activity and primary material to scavenging free radical. Additionally, SOD is direct indicator for aging and death in vivo (Mates, Perez-Gomez, & Nunez de Castro, 1999). SOD-like activities of the extracts of buckwheat sprouts were almost equivalent to that of rutin, isoorientin and orientin. In addition, sulforaphane, present in broccoli sprouts, has been found to lead to marked SOD-like activity (Kazahiro, Akira, Naomi, & Hidenori, 2009). Our results suggested that with their SOD-like activities, GLP from fermented SCR could be a beneficial component that provided comparable biological effect such as those of rutin and sulforphane.

3.4.5. Reducing power assay

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Meir, Kanner, Akiri, & Hadas, 1995). Fig. 3E showed that the reductive potential of GLP-I, GLP-II, GLP-III and GLP-IV increased to 0.68, 0.62, 1.18 and 1.48, respectively as the concentrations increased to 10 mg/mL. However, ascorbic acid showed a stronger reducing power (1.67 at 10 mg/mL) than the polysaccharides examined. The reducing power of GLP-III and GLP-IV increased significantly ($P < 0.01$) with increasing concentration of samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

3.4.6. Scavenging effect on ABTS radical

ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Huang et al., 2008). It should be borne in mind that in vitro activities can only be considered potentially relevant in biological systems and that in vivo activities depend also on bioavailability and biotransformation. The extracts tested presented a similar change in the trend of antioxidant activity. Specific absorbance at 734 nm can be used in both organic and aqueous which was close to that of ascorbic acid. The scavenging ability of GLP-IV was 98.68% at the concentration 5 mg/mL, and which was the strongest among all the others ($P < 0.05$). The ABTS scavenging ability decreased in the order of ascorbic acid > GLP-IV > GLP-III > GLP-I > GLP-II (Fig. 3F). These results indicated that GLP-IV showed a strong scavenging power for ABTS radical and should be explored as potential antioxidants.

Based on the six assays of antioxidant activity, GLP-IV and GLP-III were showed stronger antioxidant activity than GLP-I and GLP-II. This might be due to the difference monosaccharide compositions of GLP fractions.

3.5. Evaluation of macrophage RAW 264.7 cells

3.5.1. Effect of the GLP fractions on macrophage cells proliferation

The macrophage cells proliferation of all GLP-treated groups were significantly stronger than that of blank group ($P < 0.01$) with different dosages of agents, and exhibited remarkable concentration-dependent characteristics (Table 2). The macrophage cells proliferation in GLP-I, GLP- II, GLP-III and GLP-IV-supplemented (2.5–20 μg/mL) groups were lower than that of LPS group ($P < 0.01$). The macrophage cells proliferation in GLP-II group was increased as compared to LPS group ($P < 0.01$) at higher concentration (40 μg/mL) although they were lower than that of LPS group ($P < 0.01$) at lower concentration (2.5–20 μg/mL). However, GLP-II group was no significant difference for the macrophage cells proliferation, compared with GLP-III and GLP-IV at high concentration (40 μg/mL). Some results have also found the similar effect of other mushroom polysaccharide in animal models. Likewise, intravenous injection of lentinan increased the absolute number of monocytes in peripheral blood, as well as number of granulocyte–macrophage progenitor cells in spleen and bone marrow (Schepetkin & Quinn, 2006).

Table 2
Effects of various polysaccharides on the proliferation of the macrophages and the production of the nitric oxide.

Treatment	Concentration (μg/mL)	The proliferation of the macrophages (%)	The production of the nitric oxide (μM)
Control	–	100.43 ± 0.60	6.30 ± 0.34
LPS	1	170.26 ± 2.48	15.02 ± 0.66
GLP-I	2.5	118.79 ± 9.83**,a	10.00 ± 0.73**,a
	5	124.51 ± 10.07**,a	13.24 ± 0.55**,b
	10	141.88 ± 5.98**,b	13.40 ± 0.63**,b
	20	148.38 ± 3.07**,c	14.95 ± 0.59**,c
	40	155.40 ± 5.72**,d	16.23 ± 0.49**,d
GLP-II	2.5	131.77 ± 1.18**,a	13.92 ± 0.19**,a
	5	143.24 ± 2.30**,b	14.16 ± 0.48**,a
	10	160.71 ± 5.46**,c	14.80 ± 0.95**,b
	20	167.81 ± 3.51**,d	15.03 ± 0.77**,b
	40	177.95 ± 9.33**,e	16.07 ± 0.80**,c
GLP-III	2.5	126.04 ± 2.14**,a	11.95 ± 0.68**,a
	5	142.23 ± 6.37**,b	14.59 ± 0.75**,b
	10	147.56 ± 5.93**,b	15.27 ± 0.47**,b
	20	149.55 ± 2.08**,b	16.54 ± 0.55**,c
	40	159.73 ± 4.97**,c	16.39 ± 0.49**,c
GLP-IV	2.5	117.64 ± 2.73**,a	12.03 ± 0.73**,a
	5	124.39 ± 1.62**,b	14.11 ± 0.97**,b
	10	136.29 ± 2.96**,c	15.07 ± 0.66**,b
	20	145.12 ± 3.18**,d	16.37 ± 0.49**,c
	40	161.03 ± 1.13**,e	17.26 ± 0.56**,d

Cells were incubated for 24 h with the indicated concentrations of GLP fractions. Values were mean ± SD of four replicates. Control cells were incubated with medium alone. LPS was the positive control.
Values bearing different letters within columns were significantly different between groups ($P < 0.05$). The same letters suggested differences between groups were not statistically significant ($P > 0.05$).
** $P < 0.01$, significantly different from the control.

3.5.2. The effect of the GLP fractions on nitric oxide production

It was reported that polysaccharides showed a strong immunomodulating activity (Avni, Ernst, Philosoph, & Zor, 2010; Tokunaka et al., 2000). This compound stimulates macrophages to produce pro-inflammatory cytokines and secondary mediators, such as NO. NO is a gaseous molecule synthesized from L-arginine by nitric oxide synthase. It is a highly reactive free radical that can form a number of oxidation products, such as NO_2, NO_2^-, N_2O_3, and *S*-nitrosothiols. NO participates in the physiology and pathophysiology of many systems (Diouf, Stevanovic, & Boutin, 2009). It is an important mediator of the nonspecific host defense against invading microbes and tumors. Thus, NO can be used as a quantitative index of the macrophage activation. The effects of various GLP on NO production in peritoneal macrophage were summarized in Table 2. Macrophages treated by LPS and various GLP produced

larger amounts of NO than that treated by LPS ($P < 0.01$). On concentrations ranging from 2.5 to 40 μg/mL, various GLP significantly promoted NO production in a concentration-dependent manner. When the activities of four of the pure polysaccharides (GLP-I, GLP-II, GLP-III and GLP-IV) were evaluated, the NO production was increased by 212.70%, 234.92%, 242.38% and 239.21% compared to LPS at 1 μg/mL ($P < 0.01$), respectively. GLP-IV showed the highest NO-elevating activity. Various GLP displayed similar cases at higher concentrations. It was worthwhile to note that the activities of GLP-III and GLP-IV were significantly stronger than these of LPS and other GLP fractions when the concentration was up to 20 μg/mL ($P < 0.01$). Thus, GLP-III and GLP-IV were the most potent inducers of NO (relative potency was GLP-IV > GLP-III > GLP-II > GLP-I). Therefore, *G. lucidum* polysaccharide was produced reusing SCR, not only could relieve environmental pollution, but also exhibit a strong antioxidant activities and immunomodulatory activity.

Based on the above results, it could be considered that immunomodulatory activity was related to antioxidant activities. The reasons could be that the strong antioxidant activities (GLP-IV and GLP-III) could scavenge free radical to improve immunity. Therefore, GLP-IV and GLP-III exhibited stronger antioxidant activities, meanwhile immunomodulatory activity, compared with GLP-II and GLP-I.

4. Conclusions

In the present study, four polysaccharides from fermented soybean curd residue by *G. lucidum* were isolated and purified. Preliminary structural characterizations were conducted using FTIR, which results demonstrated that the basic physicochemical property of the four polysaccharides were similar. However, four polysaccharides exhibited conspicuous differences in their monosaccharide compositions, GLP-III and GLP-IV were composed of six monosaccharides, compared with three monosaccharides of GLP-II. Moreover, significant antioxidant activities against DPPH, ABTS, hydroxyl radicals and reducing power were possessed by GLP-III and GLP-IV. In addition, four polysaccharides exhibited remarkable macrophage- activating by the macrophage cells proliferation and NO. Above all, GLP from fermented soybean curd residue by *G. lucidum* could be utilized as antioxidant and immunostimulant for food and pharmaceutical industries. The future challenge is to define the 3D structure of GLP and the structure–function relationship.

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