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# Antioxidant and immunoregulatory activity of different polysaccharide fractions from tuber of *Ophiopogon japonicus*

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ABSTRACT

Four sulfated heteropolysaccharide fractions (OJP-1, OJP-2, OJP-3 and OJP-4) were isolated and purified from the tuber of *Ophiopogon japonicus* by DEAE-Sepharose Fast Flow and Sepharose 6 Fast Flow column chromatography. OJP-1, the least sulfated product, was sulfated synthetically to OJP-1S. Their chemical–physical properties were determined by chemical methods, GC, FT-IR spectroscopy, and HPLC. The antioxidant and immunomodulation activities of these fractions were also investigated. The content of hexuronic acid and sulfate were decreased in the order OJP-4 > OJP-3 > OJP-2 > OJP-1 and OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1, respectively. In comparison with OJP-1, other polysaccharides showed stronger 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and hydroxyl radical scavenging activity. Five polysaccharides also exhibited remarkable macrophage-activating capability by the promotion of phagocytic capacity, energy metabolism rate, NO and interleukin-1 production. It was significantly different between five polysaccharides ($P < 0.01$) and decreased in the order OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1. Taken together, our results suggested that hexuronic acid and sulfate were effective indicators of antioxidant and immunomodulation activity of polysaccharide.



## 1. Introduction

Polysaccharides obtained from different natural sources and their derivatives represent a structurally diverse class of macromolecules. These compounds are well known to possess many important biological activities, especially the antioxidant and immunomodulation activity such as scavenging free radical, inhibiting lipid oxidation, promoting natural killer cells (NK) cytotoxicity, activating macrophages and potentiating interleukins (Dalmo & Boqwald, 2008; Song, Zhang, Zhang, & Wang, 2010; Wang et al., 2010; Yang, Zhao, & Lv, 2008; Zhu & Lin, 2006). Bioactivity of polysaccharides mainly depends on several structural parameters including sugar composition, molecular weight, type of glycosidic bond of the main chain, and degree of sulfation (Lu, Wang, Hu, Huang, & Wang, 2008; Wang et al., 2010). In view of their safety and potent application in functional food and medicine, more and more research has been focused on the isolation, purification, and structure-bioactivity of polysaccharide from plenty of plants, animals and microorganisms (Forabosco et al., 2006; Xiong & Jin, 2007).

The tuber of *Ophiopogon japonicus* (Thunb.) Ker-Gawl, widely distributed in Southeast Asia, is also an important traditional Chinese herbal medicine. It is potent in treating a wide range of disorders such as thrombosis, myocardial ischemia, arrhythmias, respiratory disease and hyperglycemia (Li, Zhu, Qi, Qin, & Yu, 2010) because it has a wide variety of bioactive components including saponins, polysaccharide and homoisoflavonoidal compounds (Anh, Sung, Porzel, Franke, & Wessjohann, 2003; Chen et al., 2011). It is reported that polysaccharide extracted from tuber of *O. japonicus* exhibited many biological activities such as immunostimulation, anti-ischemia, and hypoglycemic. However, the recorded polysaccharide was only crude extract which might contain saponins, lectins and homoisoflavonoids, resulting in inaccurate examination of these bioactivities for their prominent corresponding activities. In recent years, some polysaccharide fractions were obtained from tuber of *O. japonicus* with modern isolation-purification technologies. Two kinds of sulfated polysaccharides were isolated from it by SephadexG-100 column chromatography, with the molecular weight of them being 27,064 Da and 48,651 Da, respectively (She & Shi, 2003). One polysaccharide was obtained with DEAE-cellulose column and Sephadex G-100 column from it (Chen et al., 2011). However, there is a dearth of information about the antioxidant and immunoregulatory activity of purified polysaccharides from tuber of *O. japonicus*.

The aim of this study was to better understand structural characteristics, antioxidant and immunostimulating activity, and the relationship between chemical characteristic and activity of polysaccharides from the tuber of *O. japonicus*. They were isolated and purified simultaneously by dialysis and DEAE-Sepharose Fast Flow anion-exchange chromatography and Sepharose 6 Fast

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Flow gel chromatography. Furthermore, we evaluated in vitro the physical–chemical properties, antioxidant (DPPH and hydroxyl radical scavenging assay) and immunomodulation activity (phagocytic activity, energy metabolism rate, nitric oxide release and interleukin-1 production).

## 2. Materials and methods

### 2.1. Materials and chemicals

Fresh tuber of *O. japonicus* was collected in Mianyang known as *Sichuan Ophiopogon japonicus*. Monosaccharide standard (rhamnose, fucose, galactose, mannose, xylose, and arabinose), galacturonic acid standard, 3-tert-butyl-4-hydroxyanisole (BHA), 2,2-diphenyl-1-picry-hydrazyl radical (DPPH), m-hydroxydiphenyl, ConA and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-Sepharose Fast Flow and Sepharose 6 Fast Flow were obtained from Phamacia. RPMI-1640 Medium and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and phenazine methosulfate were purchased from Amresco. All the reagents were of analytical grade.

### 2.2. Extraction, isolation and purification of polysaccharide

Crushed tuber *O. japonicus* was firstly recirculated with 85% alcohol for removing pigment, polyphenols, and monosaccharide, and the treatment was repeated twice. The recirculated residues were stripped of alcohol, prior to be extracted by water at 90 °C for 4 h with constant stirring. The extract was deproteinated with five percent trichloracetic acid (TCA) at −20 °C for 15 min. The obtained solution was filtered through filter cloth in vacuum. Filtrates and washings were centrifuged with 4000 × *g* for 15 min. The upper solution was adjusted to 7.0 with 0.5 M NaOH, concentrated in vacuum, precipitated by four volumes of anhydrous ethanol for 10 h at 4 °C. The later precipitate (crude polysaccharide) was obtained by centrifugalization (4000 × *g* for 15 min).

Crude polysaccharide was dissolved in 50 mM sodium acetate starting buffer (pH 5.5) and membrane filtered (0.45 μm). Then the filtrate was loaded onto a chromatographic column (2.5 cm × 40 cm) and eluted with 50 mM sodium acetate starting buffer at a flow rate of 2.5 ml/min and 5.0 ml eluent per tube. Thereafter, the column was eluted successively with mixed buffer combined with starting buffer and starting buffer containing 2 M NaCl in a linear gradient manner. Total polysaccharide and peptide were determined by Phenol–$H_2SO_4$ (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) read at 490 nm as well as recording absorbance at 220 nm, respectively. Various fractions were concentrated, dialyzed against deionized water, and finally lyophilized separately.

The polysaccharide fractions were further subjected to gel filtration on Sepharose 6 Fast Flow column (1.6 cm × 70 cm), respectively. The eluent was deionized water and the eluting rate was 0.1 ml/min. The polysaccharide and peptide were monitored with methods mentioned above. Various polysaccharide fractions were pooled, concentrated and lyophilized, respectively.

### 2.3. General analysis of polysaccharide

The homogeneity and relevant molecular mass of purified polysaccharide were recorded on high-performance liquid chromatography instrument equipped with a Waters 2410 Refractive Index Detector (Waters Co., USA). The chromatographic column conditions: chromatographic column, Ultrahydrogel™ Linear 300 mm × 7.8 mm id × 2; mobile phase, 0.1 M sodium nitrate; flow rate, 0.9 ml/min; column temperature, 45 °C. The column was calibrated with authentic dextrans (Xiong & Jin, 2007). IR spectra of various polysaccharides were recorded on a Nicolet Nexus FT-IR spectrometer (Nicolet Instrument Co. USA) using the KBr-disk method in the range 4000–400 $cm^{-1}$. Assay of hexuronic acid by modified m-hydroxybiphenyl-$H_2SO_4$ (Xiong, Li, Wu, & Wei, 2009) and sulfate by barium chloride method (Doggson & Price, 1962) with galacturonic acid and potassium sulfate as standards, respectively. Gas chromatography (GC) analysis of neutral monosaccharide in polysaccharide was performed on a Varian 450 GC instrument (Varian Instruments, Co., USA) with flame ionization detector and the sample preparation was carried out as follows: Polysaccharide was hydrolyzed with 4 M trifluoroacetic (TFA) at 100 °C for 6 h in a sealed glass tube. After removing TFA by vacuum concentration, the residue sample was mixed with 0.5 ml of anhydrous pyridine, 10 mg of hydroxylamine hydrochloride and 4 mg of myo-inositol. The mixture obtained was heated at 90 °C for 30 min, and cooled to room temperature. Then 0.5 ml of acetic anhydride was added. It was again heated at 90 °C for 30 min. After cooling, it was directly used for GC analysis of the aldononitrile peracetates (Seymour, Chen, & Bishop, 1979). GC conditions were as follows: Varian WCOT FUSED SILICA CP SIL 8CB column (30 m × 0.32 mm × 0.25 μm), $N_2$ (1 ml/min), temperature gradient: 130 °C (5 min)–240 °C (5 min) with a slope of 2 °C/min. The temperature of injector and detector were 280 °C and 300 °C, respectively.

### 2.4. Sulfation of fraction OJP-1

The sulfation of purified fraction OJP-1 was prepared using dry pyridine and chlorosulfonic acid as previously described (Chen, Yu, Yang, & Pan, 2010; Chen, Wang, et al., 2010; Xiong & Jin, 2007). OJP-1 (300 mg) was suspended in 20 ml of dry N,N-dimethylformamide at room temperature with stirring for 45 min, and the pyridine–$PSO_3$ complex was added dropwise. After 2 h at 70 °C, 20 ml ice water was added to stop the reaction, and immediately neutralized by 4 M NaOH. The obtained product was precipitated with three volumes of cool anhydrous ethanol, and then collected by centrifugation. The sulfonated polysaccharide (OJP-1S) obtained was isolated by extensive dialysis against deionized water and lyophilized.

### 2.5. Free radical scavenging activity of polysaccharide

#### 2.5.1. DPPH radical scavenging activity of polysaccharide

The sample solution (1 ml) was added to screw-capped tube containing 1.5 ml of DPPH alcohol solution. The mixture was vortexed and left to stand for 30 min, prior to be measured at 517 nm using UNICO 2102 spectrophotometer (UNICO Instruments Co. Ltd., Shanghai, China). Deionized water (1 ml) in place of test article was used as blank control (Chawla, Chander, & Sharma, 2009). Ascorbic acid and BHA were used for positive comparison. The scavenging ability was calculated as follows: DPPH radical scavenging rate (%) = $100 \times (A_0 - A_a)/A_0$, where $A_0$ is absorbance of blank control, $A_a$ is absorbance value of sample and DPPH. The $IC_{50}$ value (mg test article/ml) is the effective concentration at which the DPPH radical was scavenged by 50%.

#### 2.5.2. Hydroxyl radical scavenging activity of polysaccharide

Hydroxyl radical (•OH) can be generated via well-known Fenton reaction ($Fe^2 + H_2O_2 \rightarrow Fe^3 + {}^{\bullet}OH + OH^-$). Salicylic acid captures •OH to form 2,3-dihydroxyl benzoic acid, which had maximum absorbance at 510 nm. When a free-radical scavenger is present, it may compete with salicylic acid and decrease the generation of 2,3-dihydroxyl benzoic acid, suggesting higher hydroxyl radical scavenging activity (Wu et al., 2008). Briefly, 2 ml deionized water

(blank) or tested samples were added to screw-capped tubes containing 0.35 ml of 8.8 mM $H_2O_2$ and 0.35 ml of 6 mM $FeSO_4$. The mixture was thoroughly shaken and set for 10 min. Subsequently, salicylic acid was added to this mixture and shaken vigorously. After incubation for 10 min, the reduction of the •OH was recorded by reading the absorbance at 510 nm. The •OH scavenging activity was calculated according to the following equation: •OH scavenging rate (%) = $100 \times (A_0 - A_m)/A_0$ where $A_0$ is absorbance value of blank, $A_m$ is absorbance value of sample. The calculation of $IC_{50}$ was the same as DPPH radical.

### 2.6. Immunomodulation activity of various polysaccharides

#### 2.6.1. Animal, cell isolation and culture

Male Kunming strain mice (7–8 weeks old, 19 ± 0.5 g body weight) were obtained from the Experimental Animal Institute of Sichuan Academy of Medical Science. Each mouse was intraperitoneally injected with 5% soluble starch three days in advance before experiments and housed in plastic cages with free access to water and food at 25 °C.

Macrophages were prepared from mice as described earlier (Li, Nie, Yang, Qiu, & Xie, 2011; Yang, Zhao, Li, Wang, & Lv, 2008) with some modifications. Briefly, peritoneal fluid was harvested from sterile peritoneal cavities of male mice by injecting intraperitoneally 5 ml ice-cold sterile phosphate buffered saline (PBS, pH 7.4. It was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4{\cdot}2H_2O$ and 0.24 g $KH_2PO_4$ in 800 ml of distilled water, adjusting to 7.4 using hydrochloric acid or sodium hydroxide, and topping up to 1000 ml. The obtained solution was membrane filtered (0.25 μm) and stored at 4 °C for use). After centrifugation at 1650 g for 10 min, the cell pellets were suspended with Roswell Park Memorial Institute 1640 (RPMI-1640) cell culture media supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and cultivated in a 96-well plate at a density of $5 \times 10^5$ cells/ml, and allowed to adhere for 3 h at 37 °C in a humidified 5% $CO_2$ incubator. Then the cultures were washed twice with PBS to remove nonadherent cells prior to the addition of fresh RPMI-1640 (1 ml) containing 10% FBS. The viability of the adherent cells was assessed by trypan blue exclusion test. All experiments were divided into three treatments including blank (PBS), positive control lipopolysaccharide (LPS, 2 μg/ml) and polysaccharides with different concentrations (100, 200, 400 μg/ml).

#### 2.6.2. Assay of phagocytic activity

The effect of polysaccharide on the phagocytic activity of macrophage was determined using neutral red assay as reported before (Yi et al., 2010) with some modifications. In brief, 100 μl of macrophage ($5 \times 10^5$ cells/ml) was preincubated for 3 h in a 96-well plate before removal of nonadherent cells with PBS. RPMI-1640 cell culture media supplemented with 10% FBS and tested sample were added to each well and incubated for an additional 48 h. 100 μl of 1% neutral red physiologic saline solution was then added. After incubation for 3 h, supernatant was poured and washed 3 times with PBS prior to the addition cytolysate (glacial acetic acid:alcohol, 1:1, 100 μl). The absorbance at 540 nm was recorded on Multiskan Spectrum microplate spectrophotometer (Thermo Scientific, America) after cytolysis.

#### 2.6.3. Energy metabolism of macrophages

The effect of polysaccharide on the energy metabolism of macrophages was determined using MTT assay as reported before (Lv, Wang, Han, LV, & Qin, 2010) with some modifications. It is based on the capacity of the mitochondrial enzyme (succinate-dehydrogenase) of viable cells to transform the MTT tetrazolium salt into a blue colored product (MTT-formazan compound) which is correlated positively with functional status and energy metabolism of living cells present (Saravanan et al., 2003). The higher the absorbance at 540 nm indicates the higher the formation capacity of MTT-formazan compound, which is positively correlatable with energy metabolism and vigor of macrophage. In brief, macrophage ($5 \times 10^5$ cells/ml, 100 μl) was preincubated for 44 h in a 96-well plate and 20 μl of MTT (5 mg/ml PBS) was added. After cultivation for 4 h, supernatant was removed and 100 μl of dimethyl sulfoxide (DMSO) was added in each well. The color generated was determined by measuring the absorbance at 540 nm on microplate spectrophotometer.

#### 2.6.4. Measurement of NO production

Nitric oxide (NO) production was assayed by measuring the nitrite concentration in the supernatant of cultured macrophages using the Griess reagent (Lee, Cho, & Hong, 2009; Lee et al., 2010; Liu et al., 2005). Tested sample was added in well with marcophages ($5 \times 10^5$ cells/ml), respectively and the mixtures were incubated for 48 h. The culture fluid was centrifuged with 5000 rpm for 10 min. 100 μl of supernatant and isochoric Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) were combined and shaken slightly for 5 min at room temperature. The nitrite concentration was determined at 570 nm on microplate spectrophotometer using $NaNO_2$ as standard.

#### 2.6.5. Determination of interleukin-1

Interleukin-1 (IL-1), secreted by various rhagiocrine cells including macrophage and vascular endothelial cell, plays important roles in immunologic response such as stimulating the proliferation and differentiation of T lymphocyte. Thus, the activity of IL-1 can be indirectly evaluated according to the proliferation effective of thymocyte lymphocyte which again be measured by MTT colorimetry (Lin, 1999).

This experiment utilized IL-1 inductor (LPS or various polysaccharides) to stimulate macrophage for producing IL-1 in vitro, which coordinated concanavalin A (ConA) to stimulate mitochysis reaction of thymocyte. The mitochysis intensity of thymocyte was used for determinating the activity of IL-1 (Lin, 1999).

Induction of IL-1: Tested sample was added to cell culture plate containing macrophage suspension at a density of $5 \times 10^5$ cell/ml, respectively and cultivated for 48 h. The supernatants were collected and freezed for determinating IL-1 activity.

Preparation of thymocyte: Thymocyte suspension was obtained from thymus isolated aseptically by grinding in RPMI-1640 cell culture media contained 10% FBS. It was filtered through 8-layer pre-moistened sterile absorbent gauze. The filtrate was centrifuged at 2000 rpm for 10 min. The purified thymocyte was harvested by discarding supernatant and washing repeatedly sediments with PBS. It was then adjusted to $1 \times 10^7$ cell/ml with RPMI-1640 and stimulated 3 days by 2 μg/ml of ConA.

Determination of IL-1 activity: Thymocytes were washed 3 times with PBS for eliminating remnant ConA. Then, 100 μl of thymocyte ($5 \times 10^5$ ml$^{-1}$) and 100 μl of supernatants (IL-1 inducer) were added to cell culture plate and cultivated 67 h at 25 °C before MTT (50 μg/well) was added. Cultures were centrifuged at $1650 \times g$ for 10 min. Precipitate was added in DMSO and the absorbance of it was measured at 492 nm on microplate spectrophotometer. The higher absorbance suggested the higher production of IL-1.

### 2.7. Statistics

Samples were assayed in triplicate and results are given as averages ± SD. Significant differences between two means were determined by LSD multiple-range tests. Means were compared by the least significant difference test at 0.05 significant levels.

**Table 1**
Physical–chemical property of various polysaccharides.

| Polysaccharide | Iodine–potassium iodide reaction | Hexuronic acid (%) | Molecular weight (kDa) | Neutral monosaccharide composition | Sulfate content (%) |
|---|---|---|---|---|---|
| OJP-1 | – | – | 2.74 | Mannose, glucose | 1.11 |
| OJP-2 | – | 11.21 | 124.30 | Rhamnose, xylose, arabinose, glucose | 4.08 |
| OJP-3 | – | 10.79 | 324.65 | Arabinose, glucose | 9.42 |
| OJP-4 | – | 15.31 | 6.75 | Rhamnose, glucose | 15.19 |

## 3. Results and discussion

### 3.1. Isolation, purification and characterization of polysaccharide

The crude polysaccharide, a water-soluble gray powder, was isolated from tuber of *O. japonicus* using hot water with a yield of 30.36% ± 0.87%. Crude polysaccharide was separated by anion-exchange chromatography on a DEAE-Sepharose fast flow column and four fractions (designated as OJP-1, OJP-2, OJP-3 and OJP-4) were obtained (Fig. 1). Four polysaccharide components were separately collected and further purified by Sepharose 6 Fast Flow gel chromatographic column to obtain symmetrical peak, indicating that they were homogeneous polysaccharide (data not shown). The main physical–chemical properties of various fractions are shown in Table 1. The negative reaction of four polysaccharides with iodine–potassium iodide exhibited that they were not plant starch. Fraction OJP-1 did not contain hexuronic acid and the content of hexuronic acid of other fractions (OJP-2, OJP-3 and OJP-4) were 11.21%, 10.79%, 15.31%, respectively. The content of sulfate decreased in the order OJP-4 > OJP-3 > OJP-2 > OJP-1. The molecular weight of them (OJP-1, OJP-2, OJP-3 and OJP-4) were 2735, 124,300, 324,652 and 6746 Da, respectively. OJP-1 mainly consisted of mannose and glucose. The second fraction OJP-2, eluted at the beginning of the salt gradient, was mostly composed of rhamnose, xylose, arabinose and glucose with smaller amount of arabinose residues. OJP-3 mainly consisted of arabinose and glucose. OJP-4 mainly consisted of glucose with a little of rhamnose. Obviously, all polysaccharide contained glucose. What is more, OJP-4 and OJP-3 contained a little of peptide, belonging to carbohydrate conjugate. These data in this work have not been reported previously by other researches. Two sulfated polysaccharides from root of *O. japonicus* were composed of glucose as reported in the work of She and Shi (2003). Chen et al. (2011) found it contained glucose, arabinose and galactose. Interestingly, mannose and rhamnose were found to be in the tubers of *O. japonicas* in this experiment.

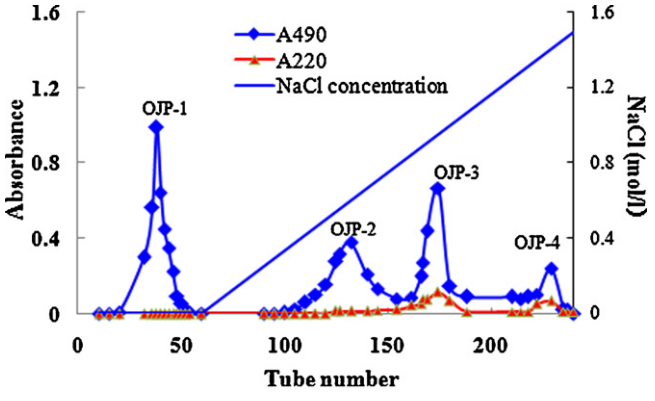


**Fig. 1.** Elution pattern of crude polysaccharide on DEAE-Sepharose Fast Flow anion-exchange chromatography column (2.5 cm × 40 cm). It was eluted with 50 mM sodium acetate starting buffer at a flow rate of 2.5 ml/min and 5.0 ml eluent per tube. Thereafter, the column was eluted successively with mixed buffer combined with starting buffer and starting buffer containing 2 M NaCl in a linear gradient manner. Total polysaccharide and peptides were determined by reading absorbance at 490 nm and 220 nm, respectively. OJP-1, OJP-2, OJP-3 and OJP-4 were the purified polysaccharide fraction from *Ophiopogon japonicas*, in which N was sulfated to obtain OJP-1S.

### 3.2. IR spectroscopy

The IR spectra of various polysaccharides are shown in Fig. 2, which display a broadly stretched intense peak at 3600–3200 $cm^{-1}$ characteristic of hydroxyl groups stretching vibration and a weak signal at around 2930 $cm^{-1}$ characteristic of C–H antisymmetrical stretching vibrations. The peaks at 1000–1200 $cm^{-1}$ suggested the presence of C–O–C and C–O–H link bonds (Xu et al., 2009). The weak peaks at around 850 and 1255 $cm^{-1}$ of four polysaccharides were derived from the bending vibration of C–O–S of the sulfate in axial position and stretching vibration of S–O of sulfate, respectively, indicating that all of them contain a small quantity of sulfate group. The intense peaks at 1250 $cm^{-1}$, 920 $cm^{-1}$, and 820 $cm^{-1}$ in OJP-1S exhibited characteristics of higher sulfate group, the content of it was 40.05% by the analysis of barium chloride method. The bands of 887 $cm^{-1}$ and 815 $cm^{-1}$ in fraction OJP-1 were the characteristic of mannose. The absorption peaks around 1415 $cm^{-1}$ of polysaccharide fractions (OJP-2, OJP-3 and OJP-4) were due to carboxylate groups symmetric stretching (Lai, Wen, Li, Wu, & Li, 2010), indicating that they contained uronic acid.

### 3.3. DPPH radical scavenging activity of polysaccharide

DPPH radical was one of the few stable radical sources and widely used to test electron-donating and free radical-scavenging ability of antioxidant. When DPPH encounters a proton-donating substance, the radical would be scavenged and the absorbance at 517 nm is reduced. A lower absorbance indicates higher DPPH radical-scavenging rate (Wang et al., 2010). The scavenging ability of all tested samples on DPPH radicals displayed a significant concentration-dependent increase pattern at tested concentrations (Fig. 3). Ascorbic acid and BHA exhibited higher free radical scavenging activity than those of polysaccharides with various concentrations. $IC_{50}$ of OJP-1, OJP-1S, OJP-2, OJP-3, and

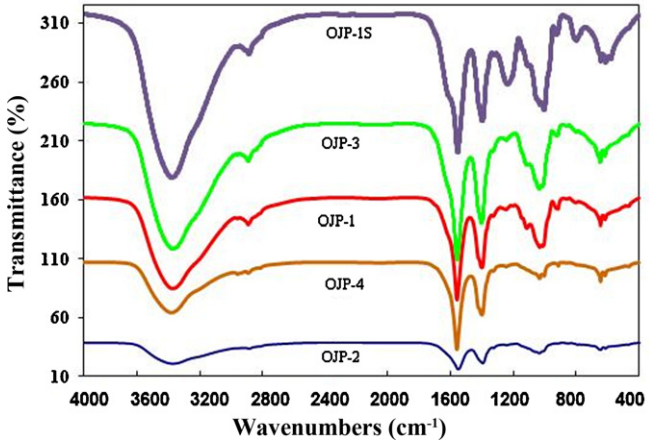


**Fig. 2.** IR spectra of various polysaccharides from *Ophiopogon japonicas*.

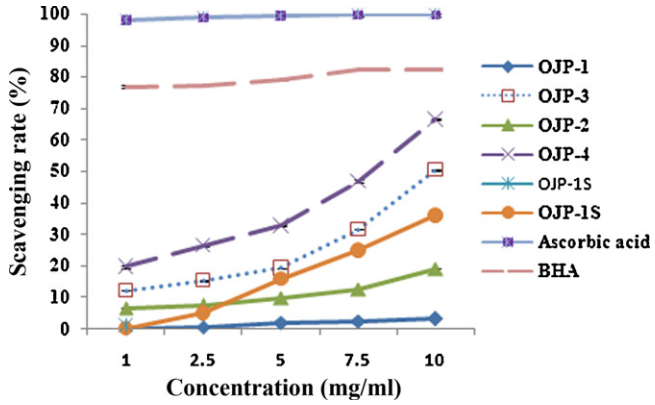


**Fig. 3.** Scavenging activity of various polysaccharides on DPPH radical.

OJP-4 were 145.26, 13.62, 34.21, 11.12, 7.52 mg/ml, respectively, which were significantly lower than ascorbic acid (1.1 μg/ml) and BHA (3.08 μg/ml). Among five polysaccharides, OJP-4 showed the strongest scavenging ability increased significantly with the concentration, while OJP-1 showed the weakest effect. However, OJP-1S was more effective in scavenging DPPH radical and significantly stronger than that of natural fraction OJP-1 ($P < 0.01$) though it exhibited lower activity as compared to three polysaccharides that contained hexuronic acid. These results demonstrated that the contents of hexuronic acid and sulfate group were important factors which contribute to the promotion of DPPH radical scavenging activity of polysaccharide. The sulfated polysaccharide exhibited higher activated capacity of the hydrogen atom of the anomeric carbon owing to the presence of $-OSO_3H$ groups and the change of its spatial configuration, which led to stronger hydrogen atom-donating capacity and DPPH radical scavenging activity (Wang et al., 2010). All these results indicated that the stronger scavenging activity of OJP-1S and OJP-4 on DPPH radical may be partially due to the higher content of hexuronic acid and sulfate group.

### 3.4. Hydroxyl radical scavenging activity of polysaccharide

The hydroxyl radical, more likely to be produced in vivo, is considered to be the most reactive and poisonous free radical in organisms because it can nonspecifically oxidize all classes of biological macromolecules including lipids, proteins, and nucleic acids (Özyürek, Bektaşoğlu, Güçlü, & Apak, 2008). Therefore, it is used extensively as the free radical to evaluate effectiveness of antioxidants. As shown in Fig. 4, all the samples, except OJP-1 and OJP-1S, exhibited obvious scavenging activity on hydroxyl radical

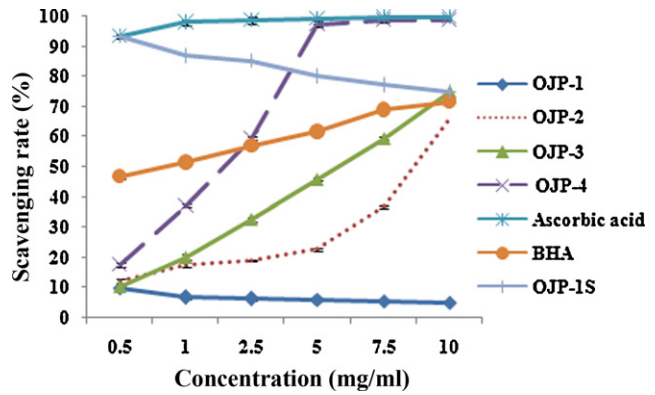


**Fig. 4.** Scavenging activity of various polysaccharides on hydroxyl radical.

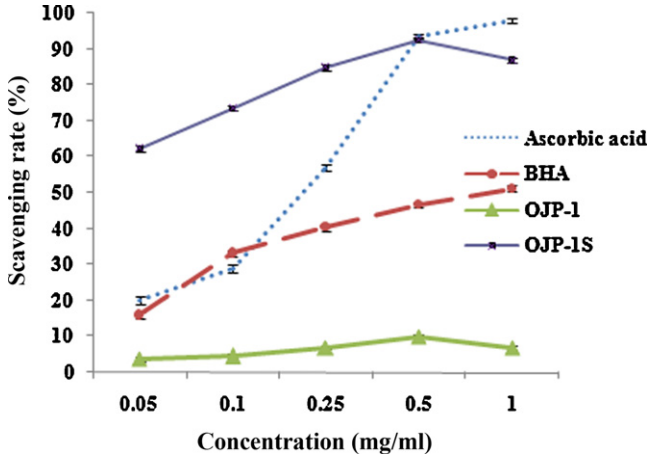


**Fig. 5.** Scavenging activity of OJP-1 and OJP-2 on hydroxyl radical at lower concentration.

in a concentration-dependent pattern at all concentrations. OJP-1 and OJP-1S with lower concentration (0.05–1 mg/ml) still showed a dose-dependent increase in hydroxyl radical-scavenging activity (Fig. 5). Especially, at the concentration of 0.5 mg/ml, OJP-1 and OJP-1S exhibited the highest scavenging rate on hydroxyl radical that were 10.15% and 93.64% respectively, and in concentration over 0.5 mg/ml, the scavenging ability of them decreased with concentration increasing (Figs. 4 and 5). Moreover, the antioxidant activity of OJP-1S was much stronger than that of ascorbic acid and BHA at the dosage range of 0.05–0.30 mg/ml. In particular, it exceeded 90% and was close to ascorbic acid when concentration was from 0.30 to 0.50 mg/ml. It was important to note that the hydroxyl radical scavenging ability of OJP-1 was always less than 50% when the concentration is from 0.05 to 10 mg/ml, indicating that it had little antioxidant activity. The $IC_{50}$ of OJP-1S, OJP-4, OJP-3, and OJP-2 were 0.04, 2.07, 5.89, 8.65 mg/ml, respectively. Thus, the hydroxyl radical scavenging ability decreased in the order of OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1, the same with the order of the sulfate and hexuronic acid content. OJP-4 was much weaker than ascorbic acid at the dosage range of 0.5–5 mg/ml, but with continuously increasing concentration, the scavenging effect of it was rapidly close to ascorbic acid and exceeded BHA. This was in accordance with the results of Qi et al. (2005), who found polysaccharide with higher sulfate content exhibited stronger hydroxyl radical scavenging activity, and considered that this may be attributed to its higher reducing power and chelating ability on ferrous ion. The production of $^{\bullet}OH$ is dependent on the content of $Fe^{2+}$ and $H_2O_2$ according to the Fenton reaction. It was widely reported that the scavenging activity of hydroxyl radical of antioxidant was due to its inhibition of hydroxyl radical generation by chelating ions such as $Fe^{2+}$ and $Cu^{2+}$ (Wang et al., 2010) and the reaction of reductones in it with precursors of peroxide which thus prevented peroxide formation (Qi et al., 2005; Zhang, Lu, Fu, Wang, & Zhang, 2011). The outstanding antioxidant activities of OJP-4 and OJP-1S may be attributed to the higher content of carboxy groups and sulfate groups which can reduce the generation of hydroxyl radicals by chelating $Fe^{2+}$ generated by the reaction of $Fe^{2+}$ with $H_2O_2$, and promote their reducing power. Therefore, the content of sulfate and hexuronic acid may be used as indicators of scavenging activity on hydroxyl radical of polysaccharide. In addition, other factors including monosaccharide constituent, molecular weight, and protein content may affect the chelating properties of polysaccharides, and also influence their antioxidant activities. For example, Tsiapali et al. (2001) stated that the free radical-scavenging activity was partially related to monosaccharide constituent. Higher antioxidant activities from Bryopsis plumosa were found when the molecular

**Table 2**
Effects of various polysaccharides on phagocytic activity and energy metabolism of macrophage.

| Treatment | Concentration (μg/ml) | Phagocytic activity ($A_{540}$) | Energy metabolism ($A_{540}$) |
|---|---|---|---|
| Control | – | $0.148 \pm 0.003^{A}$ | $0.262 \pm 0.004^{A}$ |
| LPS | 2 | $0.759 \pm 0.002^{L}$ | $0.566 \pm 0.002^{F}$ |
| OJP-1 | 100 | $0.271 \pm 0.004^{B}$ | $0.455 \pm 0.002^{B}$ |
| | 200 | $0.528 \pm 0.002^{E}$ | $0.467 \pm 0.002^{C}$ |
| | 400 | $0.547 \pm 0.002^{F}$ | $0.487 \pm 0.002^{D}$ |
| OJP-2 | 100 | $0.371 \pm 0.003^{C}$ | $0.523 \pm 0.002^{E}$ |
| | 200 | $0.657 \pm 0.004^{I}$ | $0.604 \pm 0.002^{H}$ |
| | 400 | $0.747 \pm 0.003^{K}$ | $0.676 \pm 0.004^{K}$ |
| OJP-3 | 100 | $0.386 \pm 0.002^{D}$ | $0.577 \pm 0.003^{G}$ |
| | 200 | $0.679 \pm 0.002^{J}$ | $0.685 \pm 0.003^{L}$ |
| | 400 | $0.790 \pm 0.002^{N}$ | $0.718 \pm 0.001^{N}$ |
| OJP-4 | 100 | $0.576 \pm 0.003^{G}$ | $0.683 \pm 0.002^{L}$ |
| | 200 | $0.785 \pm 0.003^{M}$ | $0.703 \pm 0.003^{M}$ |
| | 400 | $0.862 \pm 0.001^{N}$ | $0.755 \pm 0.004^{O}$ |
| OJP-1S | 100 | $0.626 \pm 0.002^{H}$ | $0.660 \pm 0.003^{I}$ |
| | 200 | $0.864 \pm 0.002^{N}$ | $0.670 \pm 0.004^{J}$ |
| | 400 | $0.872 \pm 0.003^{O}$ | $0.784 \pm 0.002^{P}$ |

*Note*: Values bearing different letters within columns are significantly different between groups by LSD ($P < 0.01$). The same letters suggested differences between groups were not statistically significant ($P > 0.05$).

weight increased (Song et al., 2010). In contrast, a relatively low molecular weight and high protein content appeared to increase the antioxidant activity of polysaccharide from green tea (Chen, Zhang, Qu, & Xie, 2008). Interestingly, the antioxidant activity of OJP-1 was the weakest although the molecular weight of it was the lowest. Thus, the antioxidant activities of the polysaccharide were not a function of a single factor but a combination of several factors. The exact mechanism underlying the DPPH radical and hydroxyl radical scavenging activity exerted by polysaccharides needs to be further investigated.

### 3.5. Macrophage activation by polysaccharides

Activated macrophages play a critical role in innate immune response and tissue repair such as phagocytosis of pathogens and apoptotic cells, production of cytokines, and presentation of foreign antigens (Xie et al., 2008). Immunostimulation itself is regarded as one of the important strategies to improve the defense mechanism of people including cancer patients (Lee et al., 2010). One of the most distinguished features of macrophage activation would be an increase in phagocytic activity (Yi et al., 2010; Zhao, Dong, Chen, & Hua, 2010). Phagocytic activity of polysaccharide was examined by the uptake of neutral red (0.1%) in this experiment. The significant differences of various polysaccharides on phagocytic activity of peritoneal macrophage were observed in this work (Table 2). Higher absorbance at 540 nm indicates stronger phagocytic activity of sample. The phagocytic activities of all the polysaccharide-treated groups were significantly stronger than that of blank group ($P < 0.01$) after mice peritoneal macrophages received treatment with different dosages of agents, and exhibited remarkable concentration-dependent characteristics. The phagocytic activities in OJP-1 and OJP-2-supplemented (100–400 μg/ml) groups were significantly lower than that of LPS group ($P < 0.01$). The phagocytic activities in OJP-3 and OJP-4 groups were significantly increased as compared to LPS group ($P < 0.01$) at higher concentration (OJP-3, 400 μg/ml; OJP-4, 200–400 μg/ml) although they were lower than that of LPS group ($P < 0.01$) at lower concentration (OJP-3, 100–200 μg/ml; OJP-4, 100 μg/ml). It is important to note that the activated ability of fraction OJP-1 on macrophage was remarkably increased after sulfation by pyridine and chlorosulfonic acid in the range of 100–400 μg/ml ($P < 0.01$), indicating that the sulfate in the polysaccharide plays an important role in stimulating phagocytosis of peritoneal macrophage. In general, the order of stimulating activity of various polysaccharides was OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1, further indicating that the contents of carboxyl group and sulfate group in the polysaccharide played a key role in immunoregulating activity.

For further exploring the macrophage-activating potential of various polysaccharides, the concentration dependence of the different polysaccharide fractions on energy metabolism of macrophage was determined using MTT methods. The macrophage activation in response to polysaccharide at different concentrations is read after 48 h of culture. As shown in Table 2, there was significant increase in energy metabolism rate ($A_{540}$) when peritoneal macrophages were incubated with LPS and different concentrations of five polysaccharides in comparison with blank group ($P < 0.01$). A dose-dependent enhancement of energy metabolism rate of macrophages treated with 100–400 μg/ml doses of various polysaccharides was also discovered, which is similar to phagocytic activity. Especially, at the concentration of 400 μg/ml, energy metabolism rate of macrophages in OJP-4 and OJP-1S groups were significantly higher than that of LPS group ($P < 0.01$). Thus, the results of both phagocytic activity and energy metabolism rate of peritoneal macrophage indicated that various polysaccharides, especially fractions (OJP-3, OJP-4 and OJP-1S), can activate macrophage and enhance its energy metabolism rate, suggesting that they are potent activators of macrophage.

### 3.6. The effects of various polysaccharides on NO release and IL-1 production

Activated macrophages play a key role in the innate and adaptive immune responses by secreting secondary compounds such as cytokines, interleukin-1 beta, NO, and other inflammatory mediators (Lee et al., 2010). These secondary compounds are again harmful to cancer cells, which are important indicators of antitumor activity of macrophages. For further investigation of mechanism of immunoregulatory activity polysaccharide from *O. japonicas*, the concentration dependence of the different polysaccharide fractions on NO and IL-1 production from macrophages was investigated. NO produced from the macrophages was determined in terms of nitrite by using the Griess method, because NO is known to be converted to nitrite soon after its production (Granger, Taintor, Boockvar, & Hibbs, 1996). The effects of various polysaccharides on NO production in peritoneal macrophage were summarized in Table 3. Macrophages treated by LPS and various polysaccharides produced larger amounts of NO than that treated by PBS ($P < 0.01$). On concentrations ranging from 100 to 400 μg/ml, various polysaccharides significantly promoted NO production in a concentration-dependent manner. When the activities of five of the pure polysaccharides (OJP-1, OJP-2, OJP-3, OJP-4 and OJP-1S) were evaluated, the NO production was increased by 17.78%, 84.92, 141.93%, 190.43%, 221.15% compared to PBS at 100 μg/ml ($P < 0.01$), respectively. OJP-1S showed the highest NO-elevating activity. Various polysaccharides displayed similar cases at higher concentrations. It was worthwhile to note that the activity of OJP-4 and OJP-1S was significantly stronger than that of LPS and other polysaccharide fractions when the concentration was up to 400 μg/ml ($P < 0.01$). Thus, OJP-1S and OJP-4 were the most potent inducers of NO (relative potency was OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1).

IL-1 was involved in many kinds of immune responses such as promoting proliferation and differentiation of T and B lymphocyte, enhancing killing activity of NK cell and facilitating gene expression of immune molecules. Previous data also exhibited that IL-1 possessed initiation, magnification and up-regulation

**Table 3**
Effects of various polysaccharides on NO and IL-1 production.

| Treatment | Concentration (μg/ml) | NO (μmol/l) | $A_{492}$ |
|---|---|---|---|
| Control | – | $10.35 \pm 0.71^{A}$ | $0.099 \pm 0.004^{A}$ |
| LPS | 2 | $59.70 \pm 0.67^{O}$ | $0.197 \pm 0.003^{J}$ |
| OJP-1 | 100 | $12.19 \pm 0.73^{B}$ | $0.111 \pm 0.008^{B}$ |
| | 200 | $18.34 \pm 0.91^{C}$ | $0.128 \pm 0.007^{C}$ |
| | 400 | $20.91 \pm 0.30^{E}$ | $0.130 \pm 0.004^{C}$ |
| OJP-2 | 100 | $19.14 \pm 0.42^{D}$ | $0.137 \pm 0.005^{D}$ |
| | 200 | $32.51 \pm 0.46^{H}$ | $0.154 \pm 0.004^{F}$ |
| | 400 | $42.71 \pm 0.44^{J}$ | $0.161 \pm 0.002^{G}$ |
| OJP-3 | 100 | $25.04 \pm 0.37^{F}$ | $0.149 \pm 0.003^{E}$ |
| | 200 | $44.92 \pm 0.46^{K}$ | $0.176 \pm 0.004^{H}$ |
| | 400 | $51.15 \pm 0.37^{N}$ | $0.186 \pm 0.003^{I}$ |
| OJP-4 | 100 | $30.06 \pm 0.89^{G}$ | $0.161 \pm 0.003^{G}$ |
| | 200 | $50.22 \pm 0.54^{M}$ | $0.195 \pm 0.002^{J}$ |
| | 400 | $65.24 \pm 0.54^{P}$ | $0.231 \pm 0.005^{L}$ |
| OJP-1S | 100 | $33.24 \pm 0.23^{I}$ | $0.175 \pm 0.010^{H}$ |
| | 200 | $45.29 \pm 0.48^{L}$ | $0.211 \pm 0.002^{K}$ |
| | 400 | $67.86 \pm 0.32^{Q}$ | $0.240 \pm 0.003^{M}$ |

*Note*: Values bearing different letters within columns are significantly different between groups by LSD ($P < 0.01$). The same letters suggested differences between groups were not statistically significant ($P > 0.05$).

activities in immune reaction, and inhibition effects on the growth of tumor cell (Voronov et al., 1999). In the absence of any treatment, macrophages generated very low levels of IL-1 (vbg). Whereas, a dose-dependent enhancement of IL-1 production was observed in macrophages treated with various concentration of polysaccharides (100–400 μg/ml), with OJP-4 and OJP-1S being the most active fractions. Thus, the individual polysaccharide indicated a similar pattern with respect to their ability to induce NO and IL-1 production by macrophages. These results further proved that various polysaccharide fractions from tuber of *O. japonicas* would regulate immune response such as anti-infection, anti-tumor, and promoting proliferation and differentiation of lymphocyte.

Overall, five polysaccharides (OJP-1, OJP-2, OJP-3, OJP-4, OJP-1S) possessed strong capacities of activating macrophage, promoting nitric oxide release and IL-1 production. They were significantly different among five polysaccharides ($P < 0.01$) and decreased in the order OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1, which suggested that sulfate and hexuronic acid played important roles in immunoregulatory activity. Wang et al. (2010) reported that sulfated modification of polysaccharide from *Lycium barbarum* could enhance its immune-enhancing activity, which was a positive relativity with the degree of substitution of sulfated polysaccharide. Many other studies also found that sulfated polysaccharide could improve immunoregulatory and antitumor activity (Ma, Guo, Wang, Hua, & Shen, 2010; Wijesekaraa, Pangestutia, & Kim, 2011; Ye, Wang, Zhou, Liu, & Zeng, 2008). Macrophage activation by polysaccharides also depends on the structure such as molecular weight, monosaccharide constituent, and the content of hexuronic acid and sulfate. Lee et al. (2010) found that a small molecular mass polysaccharide with a random coil conformation of the 1,6-branched-β-heteromannan was a potent murine macrophage stimulator. In this study, the highest immunostimulating activity OJP-4 and OJP-1S may be due to their lower molecular weight and larger amounts of hexuronic acid and sulfate. However, Chen et al. (2010) compared the immunoregulatory activity of nature polysaccharide and its derivatives (sulfated polysaccharide and desulfated polysaccharide), and found that the immunoregulatory activity of the natural polysaccharide was stronger than that of sulfated polysaccharide while desulfated polysaccharide was the least. The antiviral activity of modified heparin was mainly dependent on both the content of sulfate and stereochemical structure change before and after sulfated modification (Rider et al., 1994). Although the content of sulfate in polysaccharide was related to its immunomodulating activities, it did not suggest that the higher content of it would possess stronger activity. Some polysaccharide loses its immunomodulating activities after sulfated modification, which also may be due to change of its stereochemical structure. Therefore, the content and position of carboxy group and sulfate, spatial structure, monosaccharide composition and molecular weight of polysaccharide need to be well characterized for elucidating their mechanism of immunoregulatory activity in the future.

## 4. Conclusions

In this study, four sulfated polysaccharide fractions with different contents of hexuronic acid were successfully isolated and purified from the tuber of *O. japonicus* by dialysis and DEAE-Sepharose Fast Flow anion-exchange chromatography and Sepharose 6 Fast Flow gel chromatography. In order to evaluate the bioactivity mechanism of polysaccharide, the first fraction OJP-1 without uronic acid was sulfated. The molecular weight, contents of sulfate and hexuronic acid, and monosaccharide constituent in various polysaccharides were remarkably different. OJP-1 had little DPPH and hydroxyl radical scavenging activity. The antioxidant activity was remarkably increased when OJP-1 was sulfated. Polysaccharides (OJP-1S, OJP-2, OJP-3, and OJP-4) showed moderate DPPH radical scavenging activity ($IC_{50}$: 13.62, 34.21, 11.12, 7.52 mg/ml) and stronger hydroxyl radical scavenging activity ($IC_{50}$: 0.04, 8.65, 5.89, 2.07 mg/ml). In addition, five polysaccharides exhibited remarkable macrophage-activating by the promotion of phagocytosis, energy metabolism rate, NO and interleukin-1 production. The macrophage-activating capacities were significantly different among five polysaccharides ($P < 0.01$) and decreased in the order OJP-1S > OJP-4 > OJP-3 > OJP-2 > OJP-1. Therefore, the antioxidant and immunoregulatory activity of polysaccharide chiefly depends on its structural characteristic including molecular weight, contents of sulfate and hexuronic acid, monosaccharide constituent, and not a function of a single factor but a combination of several factors. Above all, polysaccharides from the tuber of *O. japonicus* can be utilized as antioxidant and immunostimulant for food and pharmaceutical industries.

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