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Purification, preliminary characterization and in vitro immunomodulatory activity of tiger lily polysaccharide

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ABSTRACT

A water-soluble polysaccharide (LLPS) from tiger lily was extracted by ultrasonic wave-assisted extraction. The LLPS, which was isolated by alcohol precipitation, was further purified by DEAE Sepharose Fast Flow and Sephadex G-100 chromatography, which resulted in LLPS fractions in LLPS-1, LLPS-2 and LLPS-3, with molecular weights of 350.5, 403.3 and 146.2 kDa, respectively. LLPS-1 and LLPS-2 primarily consisted of glucose and mannose in a molar ratio of nearly 1:2 and 1:1, respectively. In contrast, LLPS-3 was primarily composed of arabinose, galactose, glucose and mannose in a molar ratio of nearly 2:2:2:1. LLPS fractions could stimulate the proliferation of macrophages. The *in vitro* immunomodulatory activity of the fractions was evaluated. The results showed that treatment with 25–400 μg/mL of LLPS fractions could increase phagocytic activity and nitric oxide production of macrophages in a dose-dependent manner.

1. Introduction

Plant-derived polysaccharides have been shown to exhibit a variety of beneficial pharmacological effects via their ability to modulate macrophage immune function (Schepetkin & Quinn, 2006; Zhang et al., 2007). For example, lentinan, a glucan isolated from the fruiting body of *Lentinus edodes*, is known as a biologically active macromolecules (M_w: 5×10^5 Da) with very strong host-mediated anti-cancer activity, via activation of the human immune system (Zhang et al., 2011). The reason why high molecular polysaccharides exert their immunostimulating effects is that they can interact and exchange signals between biomaterials from outside membranes. In addition, most of polysaccharides are relatively nontoxic and do not cause significant side effects (Wasser & Weis, 1999; Zhang et al., 2007).

It is well-known that macrophages exist in almost all tissues of the body and play an important role in immunosurveillance against malignant cells and pathogens (Schepetkin & Quinn, 2006). Depending on the microenvironment, macrophages acquire specialized phenotypic characteristics and exhibit diverse functions. When activated, these cells inhibit the growth of a wide variety of tumor cells and microorganisms (Gordon, 2003; Yeon et al., 2008).

The tiger lily (also called the Yixing lily), *Lilium lancifolium* Thunb., belongs to the genus *Lilium* of the family *Liliaceae* and is primarily distributed and cultivated in the Taihu Lake Basin, Yixing, China. The tiger lily bulb is used to make traditional Chinese medicines and tonics because this bulb possesses wonderful health benefits. The tiger lily bulb not only is a good source of nutrients, including starch, protein and dietary fiber (You et al., 2010), but also contains a variety of bioactive substances, such as polysaccharides (LLPS), saponin and colchicine (Munafo et al., 2010).

Until now, there have been few reports regarding the separation, purification and characterization of polysaccharides from tiger lily bulbs and their immunostimulatory activity. Therefore, in the present study, a water-soluble LLPS was extracted, purified and preliminarily characterized. Moreover, *in vitro* immunomodulatory effects of LLPS fractions on macrophages (RAW 264.7) were also investigated.

2. Materials and methods

2.1. Materials and reagents

Fresh tiger lily bulbs were purchased from Yixing Hubin Lily Sci & Tech Co., (Yixing, China). The murine macrophage cell line RAW 264.7 was purchased from ATCC (Manassas, USA). DEAE Sepharose Fast Flow, Sephadex G-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd.

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(Shanghai, China). Fetal bovine serum (FBS) was obtained from Shanghai Pufei Biotechnology Co., Ltd. (Shanghai, China). DMEM medium, Phosphate buffered saline (PBS), trypsin-EDTA solution, penicillin and streptomycin were purchased from Gibco Co., Ltd. (New York State, Grand Island, USA). The assay kit for nitric oxide (NO) was a product of the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of analytical grade.

2.2. Extraction of polysaccharides

The fresh tiger lily bulbs were cleaned and dried at 55 °C and then pulverized into particles by a disintegrator. The dried powder was treated by refluxing in a Soxhlet apparatus with chloroform–methanol (4:1, v/v) for 8 h to remove fats. The defatted powder was extracted with distilled water at 60 °C under ultrasonic irritation for 4 h, at a ratio of 1:10 (w/v). The ultrasonic pulse sequence was 10 s on and 5 s off. After incubation, the mixture was centrifuged at 10,000 *g* for 15 min at 4 °C. The precipitate was re-extracted as described above, and the supernatants were pooled.

The collected supernatants were concentrated by a rotary evaporator under reduced pressure and deproteinated by the method of Sevag (Sevag et al., 1938). Then, the supernatant was mixed with anhydrous ethanol to a final ethanol concentration of 80% and incubated for 24 h at 4 °C. Finally, the precipitate from centrifugation (10,000 *g* for 20 min at 4 °C) was dissolved in deionized water and dialyzed against water using a membrane (MWCO 5000 Da, Millipore) at 4 °C for 24 h. The retentate was lyophilized to yield crude LLPS. The crude LLPS was subjected to further processing.

2.3. Purification of crude LLPS

The crude LLPS was purified sequentially by chromatography using a DEAE-Sepharose Fast Flow and Sephadex G-100 columns. The crude LLPS solution (20 mg/mL, 5 mL) was applied to a DEAE-Sepharose Fast Flow column (1.6 × 25 cm), which was equilibrated with deionized water, and first washed with deionized water, which was followed by washes with NaCl solutions of increasing ionic strength (0.1, 0.2, 0.4, 0.6 and 1.0 M) at a flow rate of 1 mL/min. The eluate (3 mL/tube) was collected automatically, and the total carbohydrate content was determined using the phenol-sulfuric acid method. Three fractions of were obtained. These three fractions were further fractionated on a Sephadex G-100 column (1.6 × 50 cm) and eluted with deionized water at a flow rate of 0.5 mL/min to obtain LLPS-1, LLPS-2 and LLPS-3, respectively. The relevant fractions were then collected, concentrated, dialyzed (MWCO: 5000 Da) and lyophilized to yield white, purified polysaccharides for further study.

2.4. Molecular weight determination of purified LLPS

Molecular weights of LLPS fractions were determined using high performance gel permeation chromatography (HPGPC). The operation conditions were as follows: a Waters 600 HPLC System (Waters corporation, USA); Ultrahydrogel™ Linear column (300 × 7.8 mm); a 2410 Differential Refractive Index Detector; mobile phase: 0.1 mol/L $NaNO_3$; flow rate: 0.9 mL/min; injection volume: 20 μL; and column temperature: 45 °C. The molecular weights of the LLPS fractions were estimated by referencing a calibration curve, which was created using a set of dextran standards.

2.5. General analysis

The total carbohydrate content in the LLPS was quantified using the phenol-sulfuric acid colorimetric method, with glucose as the standard (Dubois et al., 1956). The uronic acid content was determined using the previously published method (Karamanos et al., 1988). The protein content in LLPS fractions was quantified according to the Bradford method (Bradford, 1976), with bovine serum albumin as the standard.

2.6. Monosaccharide identification

The identification and quantification of the neutral monosaccharide in LLPS fractions were performed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD). The LLPS fractions (10 mg/mL) were hydrolyzed with 2 M sulfuric acid at 100 °C in a sealed tube for 1.5 h. Following hydrolysis, each sample was cooled and subsequently neutralized by the addition of barium carbonate. After centrifugation, the hydrolysate was analyzed using HPAEC-PAD. The hydrolysate was diluted 1000 times with pure water. In total, 25 μL of sample was used for the ionic-chromatography analysis using a Dionex ICS-5000 HPAEC-PAD System (Sunnyvale, CA, USA) with a CarboPAC™ PA20 column (4 mm × 250 mm). The eluents (0.25 M NaOH and 18.2 MΩ Milli-Q water) were mixed *in situ* with a gradient pump. The elution program was as follows: 0–20 min, 0.25 M NaOH 1.6%, water 98.4% and 20–40 min, 0.25 M NaOH 80%, water 20%. The flow rate was 0.5 mL/min. The column temperature was 30 °C.

To assign peaks due to the release of monosaccharide of LLPS, the following compounds were used as standards: fucose, aminoglucose, rhamnose, arabinose, aminogalactose, galactose, glucose, xylose, mannose, and ribose. The HPAEC-PAD chromatogram profile of the standards and hydrolysates of the LLPS fractions is shown in the electronic supplementary material (Figs. S1–S4).

2.7. FTIR spectra of LLPS fractions

FTIR spectra were recorded using a Vector 33 spectrometer (Brucker Company, Germany). The spectra were recorded in a transmittance mode from 4000 to 400 cm^{-1}.

2.8. Determination of immunostimulatory activities in vitro of LLPS fraction

2.8.1. Cell culture

The murine macrophage cell line RAW 264.7 was grown in DMEM medium, which was supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). For passage, the original medium was discarded, and then 1 mL trypsin-EDTA solution (0.25%) was added and incubated for 3–5 min at 37 °C. The suspension was centrifuged (1000 *g*, 5 min), and the cells were mixed with fresh DMEM medium. The culture conditions were a 37 °C saturated humidified atmosphere with 5% CO_2.

2.8.2. Effect of LLPS on the macrophage proliferation

The measurement of macrophage proliferation was determined according to the MTT-based colorimetric method. The culture solution was diluted to a density of 5×10^5 cells/mL with DMEM medium, pipetted into 96-well flat-bottom plate (100 μL/well) and incubated at 37 °C in a 5% CO_2 incubator for 4 h. The DMEM medium was discarded, and the cells were treated with various concentrations of LLPS fractions (25, 50, 100, 200, 400, 500 and 600 μg/mL). The DMEM medium and LPS (10 μg/mL) were used as a blank and positive control, respectively. After incubated for 24 h, media were discarded, and MTT solution (500 μg/mL, 100 μL/well) was added to each well and further incubated for 4 h. Then, media were discarded, and 100 μL DMSO was added. After shaking to dissolve the formazan, the optical density of each well at 570 nm was measured by a microplate reader (Tecan Infinite F200, Switzerland).

2.8.3. Assay of macrophage phagocytosis

The phagocytic ability of macrophages was measured using neutral red uptake (Cheng et al., 2008). Briefly, the culture solution was diluted to a density of 1×10^6 cells/mL, pipetted into 96-well flat-bottom plate and incubated for 4 h. Media were discarded, and the cells were treated with various concentrations of LLPS fractions (25, 50, 100, 200 and 400 μg/mL) for 24 h. Then, 0.075% neutral red solution (100 μL/well) was added and incubated for 1 h. Media were discarded and cells in 96-well plates were washed twice with PBS (pH 7.2–7.4) to remove the neutral red that was not phagocytosed by RAW 264.7 cells. Then, cell lysis buffer (1% glacial acetic acid: ethanol = 1:1, 100 μL/well) was added to lyse cells. After cells were incubated at room temperature for 15 h, the optical density of each well was measured at 540 nm using a microplate reader (Tecan Infinite F200, Switzerland). DMEM medium and LPS (10 μg/mL) were used as the blank and positive control, respectively.

2.8.4. Measurement of nitric oxide (NO) production of macrophages

The culture solution was diluted to a density of 1×10^6 cells/mL, pipetted into 96-well flat-bottom plate and incubated for 4 h. Media were discarded, and various concentrations of LLPS fractions (25, 50, 100, 200 and 400 μg/mL) were put into each well. DMEM medium and LPS (10 μg/mL) were used as a blank and positive control, respectively. After incubation for 48 h, the culture solution was collected and assayed using a NO assay kit according to the manufacturer's instructions (Nanjing Mindit Biochemistry Co., Ltd. Nanjing, China).

2.9. Statistical analysis

The data were presented as the mean ± standard deviations (S. D.), and a one-way analysis of variance (ANOVA) was performed, followed by Duncan's multiple-range tests. $p < 0.05$ were regarded as statistically significant. All statistical analyses were performed using statistical software (SPSS, Version 18.0).

3. Results and discussion

3.1. Extraction, separation and purification of the LLPS

The LLPS was extracted using ultrasonic wave-assisted hot water extraction. The crude LLPS was isolated by alcohol precipitation, and the yield was 9.46% dry weight (DW). The resulting crude LLPS was first purified through a DEAE-Sepharose Fast Flow column (Fig. 1A). Three fractions that were eluted by deionized water, 0.1 M NaCl and 0.2 M NaCl respectively were obtained (Fig. 1A). The fractions were further loaded onto a Sephadex G-100 column and eluted by deionized water, resulting in LLPS fractions LLPS-1, LLPS-2 and LLPS-3, respectively (Fig. 1B–D). All of the fractions were presented as a single peak. The yields of LLPS-1, LLPS-2 and LLPS-3 were 1.66%, 1.71% and 0.28%, respectively. As mentioned above, a combination of DEAE-Sepharose Fast Flow and Sephadex G-100 columns was proven a simple and workable method for the fractionation of the LLPS. The preliminary characterizations of three purified LLPS fractions were carried out.

3.2. Analysis of FTIR spectra of LLPS fractions

Fig. 2 shows the FTIR spectra of LLPS-1, LLPS-2 and LLPS-3. The intense broad peaks at 3384.4, 3351.7 and 3420.3 cm^{-1} were characteristic of hydroxyl groups with stretching vibration, and the peaks at 2884.3, 2885.3 and 2935.7 cm^{-1} were ascribed to weak C—H stretching vibration. The peaks at 1729.6, 1725.8 and

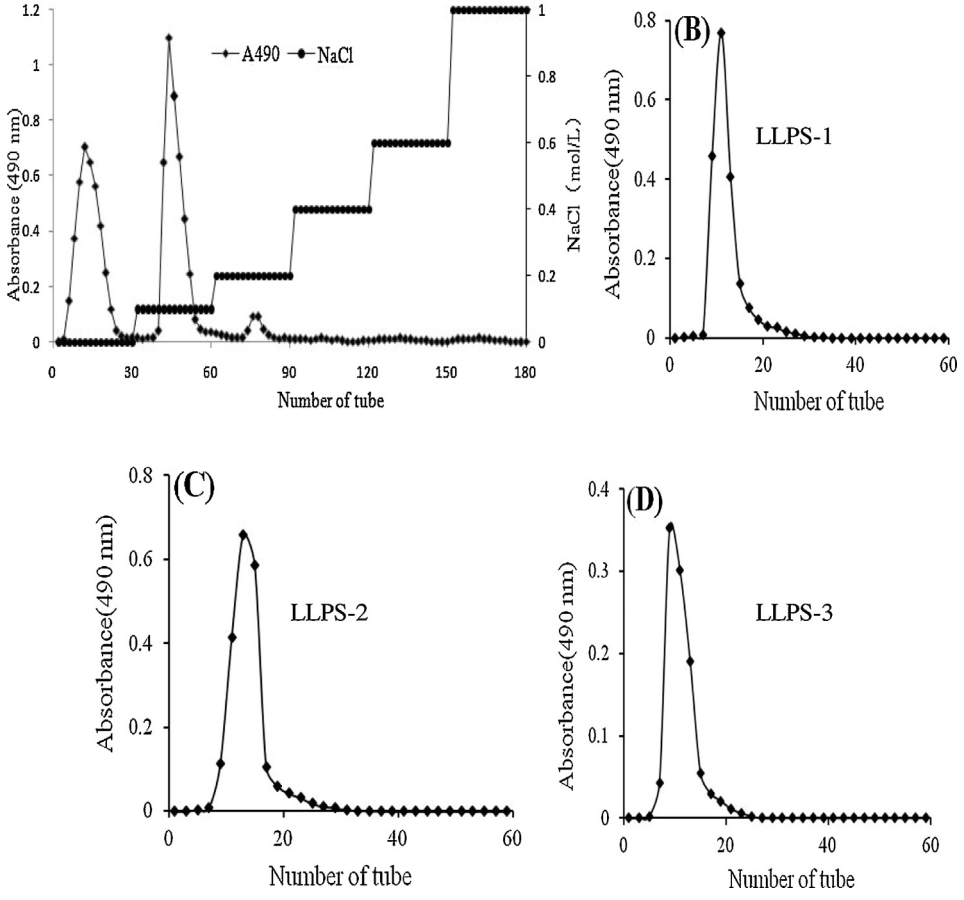

Fig. 1. Stepwise elution curve of crude LLPS on a DEAE-Sepharose Fast Flow column (A) and elution curve of polysaccharides fractions on a Sephadex G-100 column (B–D).

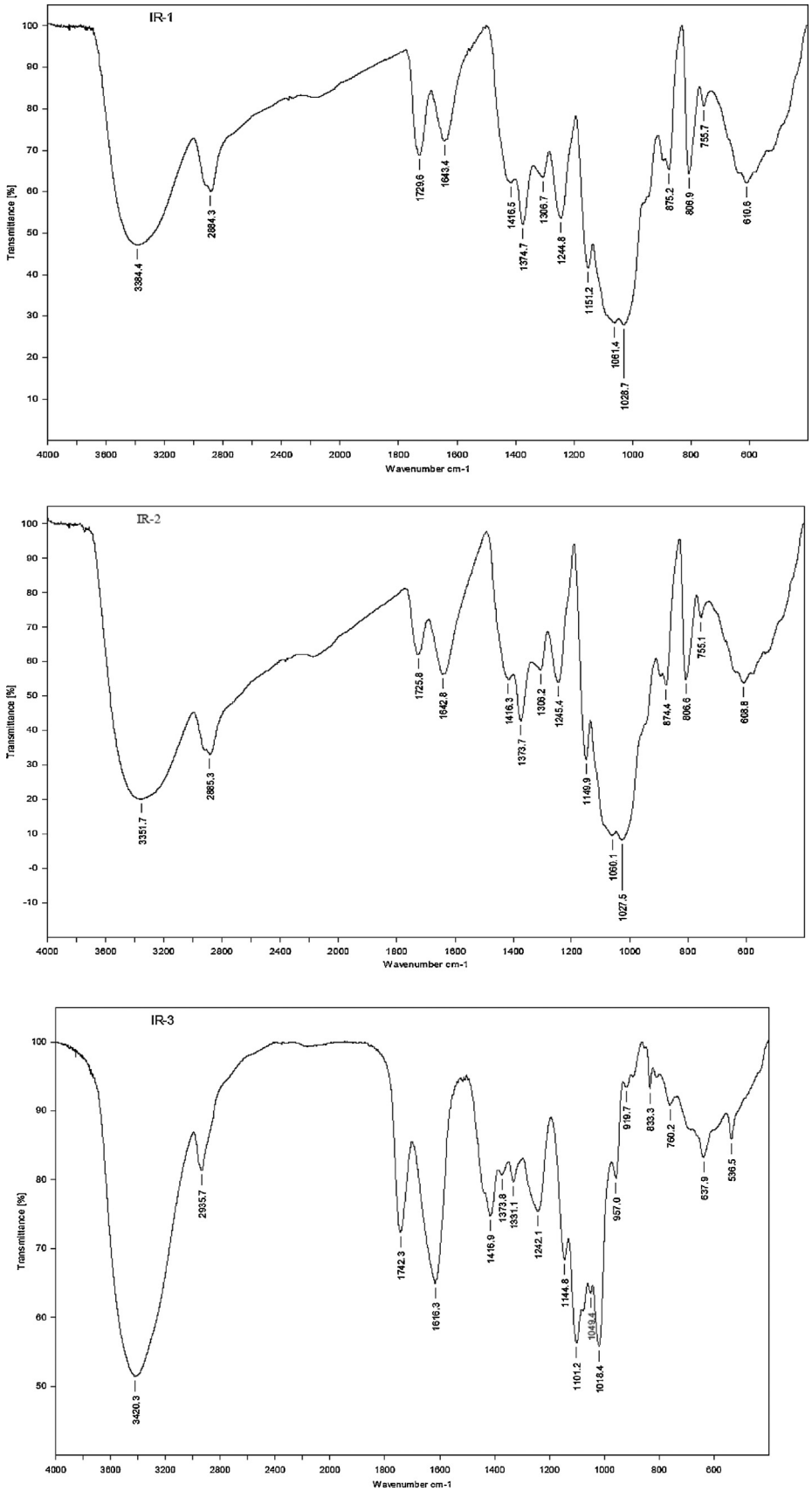

Fig. 2. FT-IR spectra of LLPS fractions (IR-1: LLPS-1; IR-2: LLPS-2; IR-3: LLPS-3).

Table 1
The chemical composition and characterizations of the LLPS fractions.

Index	LLPS-1	LLPS-2	LLPS-3
Total sugar (%)	97.40 ± 0.67	96.19 ± 1.08	79.69 ± 1.31
Protein (%)	n.d.[a]	n.d.	n.d.
Total uronic acid (%)	14.83 ± 1.09	12.19 ± 1.26	37.34 ± 1.05
Molecular weight (kDa)	350.5	403.3	146.2
Composition of neutral monosaccharide (molar ratio)	Glucose: mannose = 2:1; with a trace amount of arabinose and galactose	Glucose: mannose = 1:1; with a minuscule amount of arabinose	Arabinose: galactose: glucose: mannose = 2:2:2:1

[a] n.d.: not detected.

1742.3 cm^{-1} were characteristic of uronic acid. The peak that appeared at approximately 1245 cm^{-1} indicated that sulfate groups were present in the three LLPS fractions. The peaks at 1150 cm^{-1}, 1060 cm^{-1} and 1020 cm^{-1} on the three spectra suggested the presence of C—O and C—C bands, and the peak that appeared at approximately 1060 cm^{-1}, in particular, was due to the vibration of C—O at the C-4 position of a glucose residue (Shingel, 2002).

As shown in the FTIR spectra of LLPS-1 and LLPS-2 (Fig. 2 IR-1 and IR-2), the peak that appeared at approximately 890 cm^{-1} was ascribed to the β-type glycosidic linkage in LLPS-1 and LLPS-2, and the two peaks at 875 and 806 cm^{-1} were attributed to D-glucose and D-mannose in pyranose (Barker et al., 1954). As seen in Fig. 2 IR-3, the absorption peak that appeared at approximately 830 cm^{-1} suggested that α-type linkage was the main glycosidic linkage in LLPS-3, and the peak at 833.3 cm^{-1} was assigned to D-Galactose or D-mannose. The peak at 919.7 cm^{-1} was attributed to α-D-glucose (Barker et al., 1954).

3.3. Chemical composition and molecular weight of polysaccharide fractions

As shown in Table 1, compared with LLPS-1 and LLPS-2, LLPS-3 possessed higher amounts of uronic acid. The monosaccharides of different fractions were analyzed by HPAEC-PAD (for details, see supplementary material: Figs. S1–S4). LLPS-1 and LLPS-2 consisted of glucose and mannose in a molar ratio of 2:1 and 1:1, respectively, with traces of arabinose or galactose (Figs. S2–S3, Table 1), whereas LLPS-3 was primarily composed of arabinose, galactose, glucose and mannose in a molar ratio of 2:2:2:1 (Fig. S4, Table 1).

The average molecular weight of the LLPS fraction was determined using HPGPC. The HPGPC chromatographs of the three fractions are depicted in Figs. S5–S7 (for details, see supplementary material). Each fraction showed a single symmetrical peak, which indicated the chemical component and the molecular weight homogeneity. Based on the calibration with standard dextrans, the average molecular weights of LLPS-1, LLPS-2 and LLPS-3 were estimated to be 350.5, 403.3 and 146.2 kDa, respectively. Subsequently, the LLPS-1–LLPS-3 fractions were subjected to *in vitro* immunostimulatory activity analysis.

3.4. Effect of LLPS on macrophage proliferation

To characterize the effect of LLPS fractions in a macrophage cell model, we first evaluated their effects on the proliferation of murine macrophages (RAW 264.7) (Table 2).

The results indicated that LLPS fractions exhibited a stimulatory effect on the proliferation of the macrophages (Table 2). Notably, in the range of 25–400 μg/mL, three purified fractions stimulated RAW 264.7 cells proliferation in a dose-dependent manner. Although the cell survival rate decreased with increasing concentrations (LLPS-2 > 500 μg/mL; LLPS-1 and LLPS-3 > 400 μg/mL), the RAW 264.7 cell survival rate remained much higher than the controls (Table 2). The concentrations from 25 μg/mL to 400 μg/mL were used in the following experiments.

Macrophages play an essential role in the host defense, including phagocytosis of pathogens and apoptotic cell, and production of cytokines. Stimulating macrophage proliferation is one way to activate macrophages (Schepetkin & Quinn, 2006).

3.5. Effects of the LLPS on macrophage phagocytosis

One of the most distinguished features of activated macrophages is an increase in phagocytosis (Cheng et al., 2008). The phagocytic activity of RAW 264.7 cells was monitored by measuring the amount of neutral red that was internalized in macrophages. The effects of LLPS fractions on RAW 264.7 phagocytosis are depicted in Fig. 3. The neutral red phagocytic assay showed that LLPS fractions significantly and dose-dependently increased the phagocytosis of RAW 264.7 cells. The phagocytic indexes of RAW 264.7 cells under LLPS sample treatments (50–400 μg/mL) all exceed 1.0 (Fig. 3). Compared with the blank control, LLPS fractions could significantly enhance the phagocytosis of macrophages (≥50 μg/mL, $p < 0.01$) as well as LPS action (10 μg/mL, $p < 0.01$) (Fig. 3).

Macrophages can phagocytose aging bacteria, damage cells and necrotic tissues invading the body. Phagocytic capacity is one of the most important indicators of the body's non-specific immunity (Schepetkin & Quinn, 2006). In this study, the results indicated that the LLPS could significantly enhance the phagocytosis of the macrophages, which may be due to the binding of the polysaccharide with a specific receptor on the surface of macrophages (Tai-Nin Chow, Williamson, & Yates, 2005; Schepetkin & Quinn, 2006).

3.6. Effects of the LLPS on NO production in macrophages

NO participates in the physiology and pathophysiology of many systems. NO is an important mediator of the non-specific host defense against invading microbes and tumors (MacMicking et al., 1997). Thus, NO can be used as a quantitative index of macrophage activation (Lorsbach et al., 1993). The results of the stimulatory effect of LLPS fractions on macrophage NO productions are shown in Fig. 4. LLPS fractions could increase NO production in macrophages in a dose-dependent manner. Compared with the untreated

Table 2
Effect of the LLPS on the proliferation of RAW 264.7 macrophages[a].

Dose (μg/mL)	Proliferation rate (%)		
	LLPS-1	LLPS-2	LLPS-3
25	101.5 ± 8.5	102.3 ± 9.4	101.4 ± 9.3
50	107.2 ± 7.6	110.5 ± 6.9	117.8 ± 4.5*
100	111.8 ± 9.2	115.4 ± 6.1	123.9 ± 5.9**
200	119.2 ± 6.5*	121.2 ± 7.7*	130.2 ± 3.6**
400	126.3 ± 7.5**	136.1 ± 6.3**	135.1 ± 7.1**
500	120.1 ± 8.1*	140.2 ± 5.8**	124.2 ± 5.5**
600	117.2 ± 5.4*	130.4 ± 7.7**	119.3 ± 4.7*
Control	100 ± 10.8		
LPS	146.9 ± 7.1**		

[a] After treatment with LLPS fractions (25 ~ 600 μg/mL) or LPS (10 μg/mL) for 24 h, cells were used to test proliferative activity.

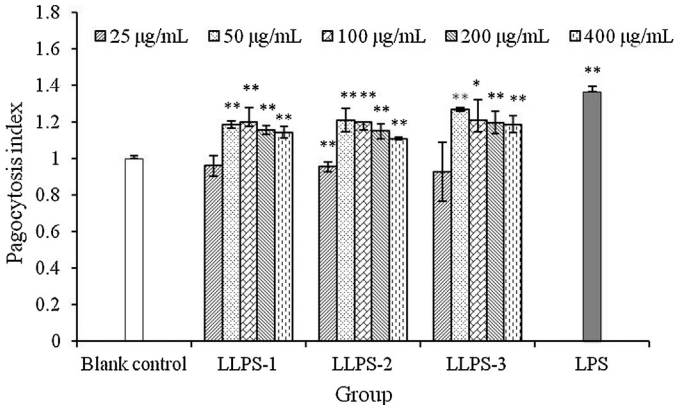

Fig. 3. Effect of the LLPS on the phagocytosis of RAW 264.7 macrophages as determined by a neutral red uptake assay. After treatment with LLPS fractions (25 ~ 400 μg/mL) or LPS (10 μg/mL) for 24 h, the cells were used to test phagocytic activity. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control (0 μg/mL).

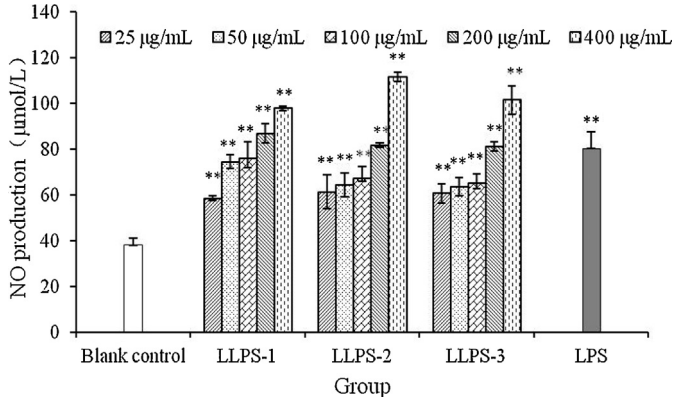

Fig. 4. Effect of the LLPS fractions on NO production in RAW 264.7 cells. Cells were pretreated with LLPS fractions (25 ~ 400 μg/mL) or LPS (10 μg/mL) for 48 h. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control (0 μg/mL).

group, the NO concentration was significantly increased under LLPS fraction treatments (25–400 μg/mL, $p < 0.01$) (Fig. 4). Furthermore, the levels of NO production at 200–400 μg/mL concentrations of the LLPS were comparable to or even greater than that elicited by 10 μg/mL LPS (positive control).

In recent years, NO was found to be a novel signaling molecule and a key mediator of signal transduction in the immune system (MacMicking et al., 1997; Schepetkin & Quinn, 2006). Our results show that LLPS fractions can significantly improve the ability of macrophages to release NO. Most likely, the combination of the polysaccharide and its receptor on the surface of macrophages activated signal transduction pathways in the cells and increased the secretion of cytokine promoters, such as NO, interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), etc. (Moncada et al., 1991; Schepetkin & Quinn, 2006). These immune reactive molecules, as endogenous signals, further induced the production of other cytokines (IL-2, IL-6, IL-8, IL-12, Ig-G) and played a regulatory role in the body's immune response (Peng et al., 1991).

4. Conclusions

A combination of DEAE-Sepharose Fast Flow and Sephadex G-100 columns was proven a simple and workable method for the fractionation of the LLPS. Three LLPS fractions (LLPS-1, LLPS-2 and LLPS-3) were obtained, with molecular weights of 350.5, 403.3 and 146.2 kDa, respectively. LLPS fractions could stimulate the proliferation of macrophages. The LLPS has the potential to be an immunopotentiating agent that could be further developed in the health food industry. It is well-known that the activities of polysaccharides may depend on sugar composition, molecular weight, glycosidic linkage, conformation, degree of branching, and so on. Thus, further studies regarding the chemical structure and mechanism of action of the LLPS are required.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.02.004.

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