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Structural features and immunomodulatory activities of polysaccharides of longan pulp

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ABSTRACT

An aqueous extract of polysaccharides from longan pulp was chromatographed on a DEAE anion-exchange column to yield four fractions (LPI–IV). Immunomodulatory activities of these polysaccharides were also evaluated *in vitro*. The purified products, neutral polysaccharide LPI, polysaccharide–protein complex LPII and acidic polysaccharides LPIII and LPIV, exhibited conspicuous differences in their monosaccharide composition, molecular mass and glycosidic linkages. Except for LPI, the other three significantly stimulated lymphocyte proliferation in the dose range of 100–400 μg/mL compared with the normal control ($P < 0.05$), and might electively stimulate B cells, but not T cells. Furthermore, their stimulations on normal/lipopolysaccharide-induced proliferation and depressions on concanavalin A-induced proliferation could be ordered as LPIII > LPIV > LPII > LPI. All the fractions had the optimal dose of 100 μg/mL on enhancing macrophage phagocytosis. Among them, LPII had the considerable yield and activity for exploiting as a potential immunoadjuvant.

1. Introduction

Polysaccharides are widely distributed in the tissue of plants, microorganisms (fungi and bacteria), algae and animals. They play a critical role in cell–cell communication, cell adhesion and molecular recognition in the immune system and as such are essential biomacromolecules (Dwek, 1996). Although polysaccharides derived from 35 plant species among 22 families have been demonstrated to have immunomodulatory activity (Schepetkin & Quinn, 2006), there are only limited reports about polysaccharides from fruits. Indeed, the stimulating effects of polysaccharides isolated from *Prunus dulcis* (Dourado et al., 2004) and *Ziziphus jujub* (Zhao, Li, Wu, et al., 2006) on lymphocyte proliferation have been detailed. Because most polysaccharides derived from higher plants are relatively nontoxic and do not cause significant side effects compared with immunomodulatory bacterial polysaccharides and synthetic compounds, they are ideal candidates for immunomodulatory, anti-tumor and wound-healing therapy (Lazareva et al., 2002; Ovodov, 1998; Sherenesheva et al., 1998).

Longan (*Dimocarpus longan* Lour.) is a commercially attractive fruit which is widely distributed in subtropical areas. Its pulp has been used as a traditional Chinese medicine to promote blood metabolism, soothe nerves, relieve insomnia and prevent amnesia in folk remedies for a long time (Park et al., 2010; Yang et al., 2008). Early studies suggest that the numerous health benefits of longan pulp may be related to polysaccharides which serve as bioactive ingredients. The immunomodulatory activities of the aqueous extract of longan pulp, such as enhancing macrophage phagocytosis, stimulating splenocyte proliferation and promoting antibody production, have been confirmed by Su et al. (2010) and Zhong, Wang, He, & He (2010), but the potential active component has not been declared. The polysaccharides from longan pulp have been isolated and investigated on molecular mass and monosaccharide composition (Yang et al., 2008). Interestingly, little is known regarding the immunomodulatory properties of longan polysaccharides (LPs) *in vitro*, especially regarding the potential structure–function relationship.

To scan the immunomodulatory LP, we purified different fractions from a crude polysaccharide extract of longan pulp by DEAE-cellulose anion-exchange chromatography, and identified their structural features by gas chromatography coupled with mass spectrometer (GC–MS), reverse phase-high performance liquid chromatography (RP-HPLC), size exclusion chromatography coupled with laser light scattering (SEC–LLS), fourier transform infrared spectrum (FTIR) and nuclear magnetic resonance spectroscopy (NMR). Furthermore, we evaluated their effects on splenic lymphocyte proliferation and macrophage phagocytosis *in vitro*.

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2. Materials and methods

2.1. Plant materials

Fresh fruits of longan (cv. Chu-liang) at the mature stage were provided by Pomology Research Institute of Guangdong Academy of Agricultural Sciences (Guangzhou, China). Fruits were selected based on their uniformity of size and color, and then dried in an oven at 50 °C for a week. The pulp was stripped manually and grinded by a mill (A11 basic, ZKA-WERKE, Germany). The generated powder was packaged and stored at −20 °C.

2.2. Preparation and isolation of longan polysaccharides

An aqueous extract of LPs, which consisted of 90.44% polysaccharides, 4.31% hexuronic acids and 3.20% proteins, was obtained by the methods as described in our previous studies (Yi et al., 2010a,b). It was identified as polysaccharide–protein complex, and its carbohydrate portion was mainly composed of glucose, mannose and arabinose with the molar ratio of 14.16:6.40:1.00.

200 mg crude extract 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 DEAE52-cellulose column (50 cm × 2.6 cm) equilibrated with distilled water. The column was eluted by distilled water for 12.5 h, 0.1 M NaCl for 11 h, 0.2 M NaCl for 8.5 h and 0.3 M NaCl for 8.5 h to yield LPI, LPII, LPIII and LPIV, respectively. 6 mL/tube of eluant 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). The absorbance of protein was measured on a UV-vis spectrophotometer (UV 1800, Shimadzu, Japan) at 280 nm. LPI, LPII, LPIII and LPIV 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. Characterization analysis

2.3.1. GC–MS

A GC–MS method (Huang, Wu, Hu, & Lin, 2006) was applied for the determination of monosaccharide. Briefly, 40 mg sample was hydrolyzed at 100 °C for 6 h in 10 mL of 2 M sulphuric acid. Saturated barium hydrate was added for neutralizing the excess sulphuric acid. The hydrolysate was filtered through 0.2 μm syringe filters (Whatman, England), dried under a stream of N_2, and fully mixed with 5 mL pyridine containing 70 mg hydroxylamine hydrochloride at 90 °C for 60 min. 5 mL acetic anhydride was added into the tube containing reaction mixture at room-temperature. The acetylated hydrolysates in the mixture were extracted by trichloromethane followed by evaporation under a stream of N_2. 1 mL final product was analyzed by GC–MS with a DB-1 column (15 m × 0.2 mm, 0.33 μm, J&W Scientific) following the temperature programme: the initial temperature of column was 100 °C, then increased to 280 °C at a rate of 10 °C/min, and kept for 15 min at 280 °C; injection temperature was 280 °C. This procedure was performed on an Agilent 6890 GC coupled with 5973 MS. The temperature of mass spectrometer ion source was 230 °C. 1 μL sample was injected into the column with the split ratio of 10:1.

2.3.2. RP-HPLC

An online pre-column derivatization coupled with HPLC analysis methods (Zheng et al., 2005) were employed for determination of amino acids in polysaccharide–protein complex (LPII) using ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC-Cl) as derivatives. Briefly, 20 mg LPII was accurately weighed into a screw-capped tube and 6 M hydrochloric acid was added. Tube was sealed and hydrolyzed for 22 h at 110 °C. After hydrolysis, sample was introduced into an online derivatization system. The analysis was performed on Agilent 1100 series apparatus within a Hypersil ODS column (dimensions 4.0 mm × 125 mm and particle size 5 μm) and a programmable fluorospectrophotometer detector. The binary gradient elution was linear over 25 min from 0% to a final composition of 100% mobile B. The column was maintained at 40 °C and the flow rate at 1.0 mL/min. Mobile phase A was sodium phosphate buffer (PB, 10 mmol L^{-1}, pH 7.2) containing 0.5% (φ) tetrahydrofuran, while mobile phase B was PB–methanol–acetonitrile (volume ratio, 50:35:15). OPA derivatives were detected by the programmable fluorometer with excitation (λ_{ex}) and emission (λ_{em}) wavelengths set at 340 and 450 nm, respectively. The FMOC derivatives were detected at λ_{ex} 260 nm and λ_{em} 305 nm, the wavelength change occurred at 20.5 min.

2.3.3. SEC–LLS

The SEC–LLS measurement was performed on a multi-angle laser light photometer (λ = 633 nm, Wyatt-DAWN HELEOS-II, Wyatt Technology Co., USA) combined with a pump (Waters 515 HPLC) equipped with SEC column (Shodex SB-804 connected with Shodex SB-802) at 25 °C. A differential refractive index detector (RI, Wyatt-Optilab rex) was simultaneously connected. The concentration of polysaccharide solution was 3.0–5.0 mg/mL in distilled water, and 200 μL was injected after passing through a 0.2 μm syringe filter (Whatman, England). The isocratic mobile phase was 0.1 M sodium nitrate at a flow rate of 0.5 mL/min. A specific refractive index increments (dn/dc) value of the polysaccharide in distilled water, which was determined by OPTILAB DSP differential refractometer (Wyatt Technology Co., USA) at 633 nm and 25 °C, was 0.147 mL/g. Astra software was utilized for data acquisition and further analysis.

2.3.4. FTIR

FT-IR spectrum of the sample was determined using a Fourier transform infrared spectrophotometer (Nexus 5DXC FT-IR, Thermo Nicolet, America). 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 of 4000–400 cm^{-1} (Funami et al., 2005).

2.3.5. NMR

The freeze-dried polysaccharides were kept over P_2O_5 in vacuum for several days. The deuterium-exchanged polysaccharides (30–40 mg) were put in a 5-mm NMR tube and dissolved in 0.7 mL 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM 400 MHz spectrometer (Bruker, Rheinstetten, Germany, operating frequencies 400.13 MHz for ^1H NMR and 100.61 MHz for ^{13}C NMR) at 30 °C. Chemical shift was expressed in ppm. Tetramethylsilane was used as an internal standard.

2.4. Immunomodulatory activity analysis

2.4.1. Animals and cells

Specific pathogen-free Kunming mice (male, 20.0 ± 2.0 g, certificate number: SCXK-Yue 2006-0015) were purchased from Laboratory Animal Sciences Center of Southern Medical University (Guangdong, China). The mice were bred on a 12-h-dark/12-h-light cycle at 22 ± 2 °C and allowed free access to standard laboratory rodent diet (Laboratory Animal Sciences Center of Southern Medical University, China) and tap water. 8–12 weeks old mouse was sacrificed by cervical dislocation, and its spleen was removed

aseptically and then teased in aseptic phosphate-buffered saline (PBS). The splenic cells were harvested through sterilized meshes (200 meshes) at room temperature. After the red blood cells were removed by hemolytic Gey's solution, the remaining cells were washed twice and resuspended by RPMI 1640 complete medium (Gibco BRL,Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL). The cell concentration was adjusted to 1×10^7 cells/mL. The resident macrophages of mice were harvested by peritoneal lavage, before their cells were cultured in complete medium at the concentration of 2×10^6 cells/mL. The experiment procedures were approved by the laboratory animal committees of Guangdong Province. All the treatments to animals were performed in accordance to the Guide for the Care and Use of Laboratory Animals.

2.4.2. Splenic lymphocyte proliferation

The assay of splenic lymphocyte proliferation was implemented according to the method described by Li, Jiao, et al. (2008) and Wang, Wang, Lin, & Lin (2005). 50 μL/well of splenocyte suspension was plated in a 96-well culture plate with or without mitogen (5.0 μg/mL concanavalin A (ConA) or 5.0 μg/mL lipopolysaccharide (LPS), Sigma, St. Louis, MO, USA). The filter-sterilized samples (final concentration: 0, 50, 100 and 200 μg/mL) were added into the cell well. After incubated for 68 h (37 °C, 5% CO_2), each well was pulsed with 5 mg/mL of 3-[4,5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium bromide (MTT, Sigma). The plate was further incubated for 4 h, and 100 μL/well acidified isopropyl alcohol was then added and kept for 12 h to dissolve formazan crystals. Finally, the plate was analyzed at 570 nm using a microplate reader (Thermo Labsytems, Helsinki, Finland).

2.4.3. Macrophage phagocytosis

The purity of macrophages was tested by adherence (Li, Jiao, et al., 2008). 100 μL/well of macrophage suspension was plated in a 96-well culture plate and incubated for 3 h (37 °C, 5% CO_2). The adherent macrophages were washed twice by complete medium and then incubated with samples (final concentration: 0, 50, 100 and 200 μg/mL) for 24 h. The stimulated cells were washed twice by PBS, and 100 μL neutral red (0.1%, w/v) was used to assess the phagocytosis. The plate was incubated for 4 h. After the removal of unphagocytized neutral red by PBS, 100 μL cell lysate (the volume ratio of acetic acid to ethanol was 1:1) was added in and kept for 12 h. The OD value of each well was read at 570 nm. The phagocytosis index (%) was calculated as: $(OD_S–OD_C)/OD_C \times 100$, in which OD_S represented the OD value of stimulated well while OD_C stands for that of control well (Wang et al., 2005).

2.5. Statistical analysis

The data were expressed as means ± standard deviations. Significance of difference was evaluated with one-way ANOVA, followed by the Student–Newman–Keuls test by SPSS 11.5 software. *P*-value of 0.05 was used as the threshold for significance.

3. Results

3.1. Fractionation of LPI–IV

Four fractions consecutively eluted by distilled water, 0.1 M NaCl, 0.2 M NaCl and 0.3 M NaCl through an anion-exchange column were respectively coded as LPI, LPII, LPIII and LPIV. LPI was a neutral polysaccharide, and other three eluted by NaCl solution were acidic polysaccharides. The yield of LPI–IV respectively accounted for 56.02%, 22.08%, 4.77% and 2.95% of crude polysaccharide extract, while the ratio of their peak areas was 35.02: 12.54: 3.07: 1.86 indicating that LPI and LPII were the major components

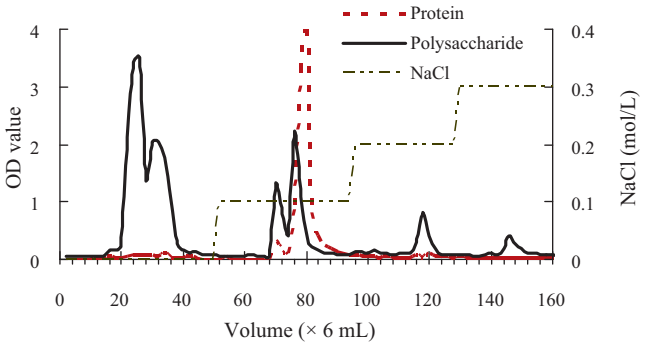

Fig. 1. Anion-exchange chromatogram of longan polysaccharides detected by phenol-sulphuric acid method at 490 nm and UV measurement at 280 nm. Column: DEAE52-cellulose (50 cm × 2.6 cm); flow rate: 0.4 mL/min; fraction volume: 6 mL. LPI–IV were eluted by distilled water, 0.1 mol/L NaCl, 0.2 mol/L NaCl and 0.3 mol/L NaCl, respectively.

of longan polysaccharides (Fig. 1). Both LPI and LPII exhibited two peaks in the profile of anion-exchange chromatogram implying that two sub-fractions in them. In addition, the peaks of polysaccharide coincided with that of protein in LPII suggesting that LPII was a polysaccharide–protein complex. However, protein was not detected in LPI, LPIII and LPIV.

3.2. Structural characteristics of LPI–IV

3.2.1. Chemical composition

Monosaccharide composition showed big differences among LPI–IV as seen in Table 1. LPI was composed of glucose, mannose and arabinose, LPII was composed of glucose, mannose, arabinose, galactose and ribose, LPIII was composed of arabinose, rhamnose, galactose and ribose, and finally LPIV was composed of xylose, ribose, mannose and glucose. With regard to the protein portion of LPII, 15 types of amino acids were detected, and the involved aspartate, glutamic, serine, glycine, threonine, histidine, alanine, arginine, tyrosine, valine, phenylalanine, isoleucine, leucine, lysine and proline (data not shown) accounted for 5.6% (mg/mg) of LPII.

3.2.2. Molecular mass

The profile of SEC chromatogram detected by RI responded to the concentration of polysaccharide. As seen in Fig. 2, all fractions exhibited a main peak indicating one major component. The weight-average molar masses (M_w) of LPI–IV were 14.59, 68.34, 107.4 and 5282 kDa, respectively. Besides, each fraction displayed more than one peak in the chromatogram, and their polydispersion indexes (M_w/M_n) were individually determined to be 2.139, 1.867, 1.121 and 4.935, indicating the inhomogenicity of each fraction.

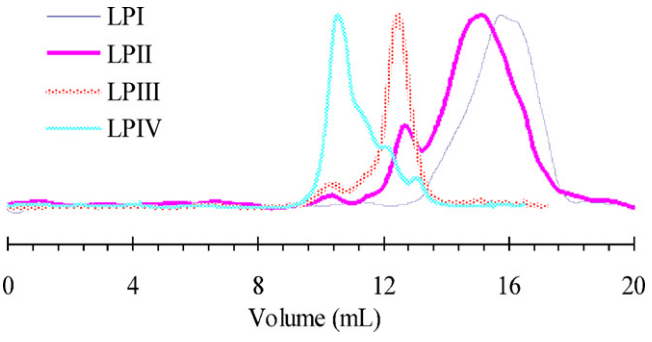

Fig. 2. SEC chromatograms of LPI–IV detected by RI in water at 25 °C.

Table 1
Monosaccharide compositions of LPI–IV.

Monosaccharide	LPI	LPII	LPIII	LPIV	Retention time (min)
Ribose	0.57	1.00	1.00	5.04	7.52
Rhamnose	0.01	0.22	3.21	0.13	7.58
Arabinose	1.00	3.00	4.70	0.89	7.69
Xylose	0.20	0.21	0.56	7.83	7.82
Mannose	9.64	5.85	0.41	2.31	9.59
Glucose	21.84	14.62	0.66	1.00	9.70
Galactose	0.73	1.77	2.18	0.66	9.91

The monosaccharide compositions of LPI–IV were determined by gas chromatograph–mass spectrometer, and the content of monosaccharide was expressed as molar ratio.

3.2.3. FTIR spectrum characterization

The absorptions, functional groups and structural characteristics of FTIR spectra of LPI–IV were summarized in Table 2. All the fractions showed the band characteristics of polysaccharide, including hydroxyl group bands in 3600–3200 cm^{-1} and 1075–1010 cm^{-1}, alkyl group bands at about 2926/1458 cm^{-1} and carboxyl group bands in 1740–1200 cm^{-1} (characteristics of uronic acid) (Zhang, 1994).

3.2.4. NMR spectrum characterization

The ^{1}H and ^{13}C NMR spectra of LPI–III are shown in Fig. 3. NMR spectroscopic analysis indicated that LPI–III were all heteropolysaccharides. There were same glycosyl residues among them due to same ^{1}H and ^{13}C signals. The ^{1}H signal at 4.70 ppm belonged to D_2H. The anomeric ^{1}H signals mainly occurred in the range of 4.91–5.34 ppm, which indicated that the configurations of LPI–III were mostly α form. The ^{13}C signal range of 67–70 ppm confirmed the presence of (1 → 6) glycosidic linkages in LPI–III, while that of 80–83 ppm implied the presence of (1 → 3/4) glycosidic linkages.

3.3. Immunomodulatory activities of LPI–IV

3.3.1. Effects on splenic lymphocyte proliferation

The effects of LPI–IV on normal (without mitogen) and mitogen-induced splenic lymphocyte proliferation were investigated in the dose range of 100–400 μg/mL, as shown in Table 3. Without the actions of LPI–IV, both ConA and LPS could significantly stimulate lymphocyte proliferation compared with that of normal ($P < 0.05$). However, the proliferation was obviously repressed when ConA and LPS acted together ($P < 0.05$). LPI exhibited no stimulation on normal proliferation in 100–400 μg/mL ($P > 0.05$), but showed a dose-dependent suppressive effect on mitogen-induced proliferation. LPII–IV had excellent activities on normal proliferation in 100–400 μg/mL ($P < 0.05$). Their effects gradually decreased with the increase of dose and showed no significant difference between 100 and 200 μg/mL ($P > 0.05$). In ConA-induced proliferation, LPII–IV displayed strong depressant effects (in direct correlation with dose) compared with the ConA control ($P < 0.05$). In LPS-induced proliferation, LPII (100 or 200 μg/mL) and LPIII (100 μg/mL) significantly increased cell proliferation compared with the LPS control ($P < 0.05$). At the same dose of LPII, LPIII or LPIV, the proliferation induced by LPS was significantly higher than that of ConA ($P < 0.05$).

The effects of LPI–IV on splenic lymphocyte proliferation were implemented with group comparison at 100 or 200 μg/mL, as seen in Fig. 4. LPII–IV significantly stimulated normal proliferation compared with the normal control ($P < 0.05$), showed significant depressant effects on ConA-induced proliferation compared with the ConA control ($P < 0.05$), and exhibited significant stimulation on LPS-induced proliferation compared with the LPS control ($P < 0.05$). Their stimulations on normal/LPS-induced proliferation and depressions on ConA-induced proliferation could be ordered as LPIII > LPIV > LPII > LPI.

3.3.2. Effects on macrophage phagocytosis

As seen in Fig. 5a, each fraction had various enhancing effects on macrophage phagocytosis in the dose range of 100–400 μg/mL, and the optimal dose for them all was 100 μg/mL. In addition, the activities of LPI–IV on macrophage phagocytosis were implemented with group comparison at 100 or 200 μg/mL, as seen in Fig. 5b. LPI exhibited the lowest phagocytosis index ($P < 0.05$). At the dose of 100 μg/mL, both LPIII and LPIV showed the best enhancement on macrophage phagocytosis ($P < 0.05$). However, the best effect at 200 μg/mL was stimulated by LPII ($P < 0.05$).

Table 2
FTIR spectrum analysis of functional groups of LPI–IV.

Absorption (cm^{-1})				Functional group	Structural characteristic
LPI	LPII	LPIII	LPIV	(Zhang, 1994)	
3422.2	3421.6	3424.5	3421.4	Hydroxyl group (–OH)	Stretching vibration of O–H
2929.9	2931.5	2931.0	2927.0	Alkyl group ($–CH_2–$)	Stretching vibration of C–H
1647.5	1637.4	1617.2	1617.5	Carbonyl group (–C=O or –CHO)	Stretching vibration of C=O
1458.2	1458.0			Alkyl group ($–CH_2–$ or $–CH_3$)	Bending vibration of C–H
	1406.4	1419.1	1419.0	Carboxyl group (–COOH)	Stretching vibration of C–O
1363.4	1375.0		1363.8	Carboxyl group (–COOH)	Symmetrical stretching vibration of C=O
1208.9			1262.0,1200.2	Carboxyl group (–COOH)	Bending vibration of O–H
1154.4				Ether (–C–O–C–)	Stretching vibration of C–O
1019.5	1018.0	1040.1	1077.3,1035.9	Hydroxyl group (–OH)	Bending vibration of O–H
919.8				D-Glucopyranose ring	Antisymmetrical ring vibration
		894.4		β-D-glucopyranose or β-D-manopyranose	
			872.4	Manopyranose or galactopyranose	Bending vibration of C-H
854.6				α-Type glycosidic linkage	Bending vibration of C–H
		820.0		α-D-galactopyranose	
765.0	763.5	781.4		D-Glucopyranose ring	Symmetrical ring vibration

The FTIR spectra of LPI–IV were determined using a Fourier transform infrared spectrophotometer in the frequency range of 4000–400 cm^{-1}. The informations of functional group and structural characteristic were referred to the book of Zhang (1994).

Table 3
Effects of LPI–IV on splenic lymphocyte proliferation.

Dose (μg/mL)	Mitogen	LPI	LPII	LPIII	LPIV
0	—	0.074 ± 0.005 b	0.089 ± 0.009 b	0.097 ± 0.007 d	0.081 ± 0.007 b
	ConA	0.333 ± 0.008 h	0.317 ± 0.012 j	0.243 ± 0.009 f	0.482 ± 0.010 k
	LPS	0.299 ± 0.009 g	0.274 ± 0.009 h	0.243 ± 0.008 f	0.470 ± 0.013 k
	ConA + LPS	0.056 ± 0.002 a	0.063 ± 0.003 a	0.051 ± 0.003 a	0.061 ± 0.003 a
100	—	0.076 ± 0.007 b	0.165 ± 0.007 e	0.392 ± 0.005 i	0.328 ± 0.009 i
	ConA	0.265 ± 0.004 f	0.194 ± 0.010 f	0.105 ± 0.007 d	0.178 ± 0.005 f
	LPS	0.265 ± 0.008 f	0.296 ± 0.019 i	0.317 ± 0.012 g	0.403 ± 0.017 j
200	—	0.079 ± 0.003 b	0.160 ± 0.007 e	0.388 ± 0.011 i	0.322 ± 0.009 i
	ConA	0.218 ± 0.007 e	0.186 ± 0.009 f	0.085 ± 0.006 c	0.148 ± 0.007 e
	LPS	0.261 ± 0.010 f	0.299 ± 0.011 i	0.249 ± 0.012 f	0.389 ± 0.020 j
300	—	0.080 ± 0.004 b	0.147 ± 0.008 d	0.371 ± 0.006 h	0.307 ± 0.007 h
	ConA	0.189 ± 0.007 d	0.164 ± 0.009 e	0.067 ± 0.006 b	0.126 ± 0.006 d
	LPS	0.264 ± 0.006 f	0.246 ± 0.009 g	0.179 ± 0.008 e	0.322 ± 0.021 c
400	—	0.083 ± 0.005 b	0.130 ± 0.007 c	0.312 ± 0.010 g	0.289 ± 0.008 g
	ConA	0.165 ± 0.006 c	0.156 ± 0.010 de	0.055 ± 0.005 a	0.111 ± 0.005 c
	LPS	0.210 ± 0.008 e	0.190 ± 0.010 f	0.110 ± 0.007 d	0.290 ± 0.012 g

Splenocyte proliferation was measured by the MTT method. Values were expressed as means ± standard deviation ($n = 6$). The statistically significant differences among the groups were evaluated with ANOVA followed by the S–N–K test. Different letters following the data represent significant difference in the same sample at $P < 0.05$.

4. Discussion

One neutral polysaccharide (LPI) and three acidic polysaccharides (LPII–IV) were purified from the crude polysaccharide extract of longan pulp by using an anion-exchange DEAE52-cellulose chromatographic column. Their monosaccharide compositions, molecular masses and structural characteristics were determined differently compared with the report of Yang et al. (2008) due to different longan fruit species and separating condition used. They all had the band characteristics of a polysaccharide (Table 2). Notablely, the absorption peaks of LPI at 919.8, 854.6 and 765.0 cm^{-1} indicated the characteristics of α-D-Glcp (Capek, Matulova, & Combourieu, 2008; Li, Wang, et al., 2008; Shi, Sheng, Yang, & Hu, 2007). The absorption peak of LP III at 894.4 cm^{-1} implied the presence of β-D-glucopyranose or β-D-manopyranose, while the 872.4 cm^{-1} of LPIV might be caused by the bending vibration of C–H of manopyranose or galactopyranose. The absorption

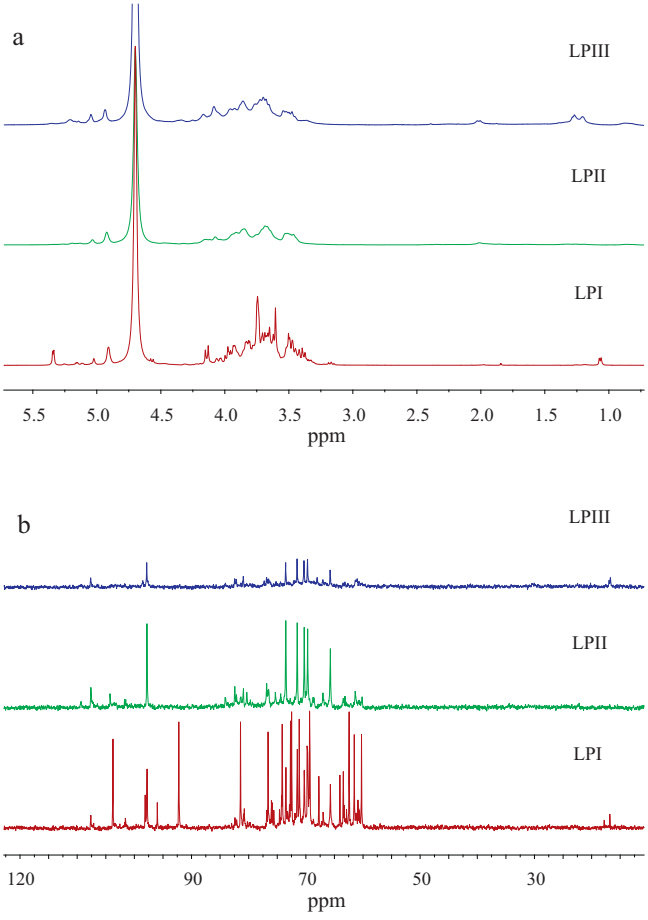

Fig. 3. NMR spectra of LPI–III. (a) ^{1}H NMR spectra, (b) ^{13}C NMR spectra.

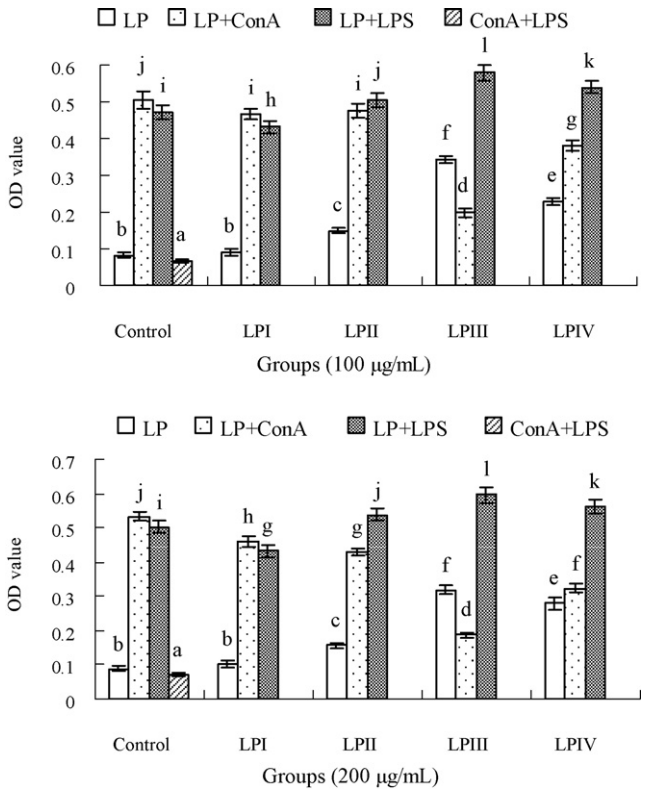

Fig. 4. The splenic lymphocyte proliferations of LPI–IV at 100 or 200 μg/mL. Proliferation activities were measured by the MTT method and expressed as means ± standard deviation ($n = 6$). The statistically significant differences among the groups were evaluated with ANOVA followed by the S–N–K test. Different letters represented the statistical difference at $P < 0.05$.

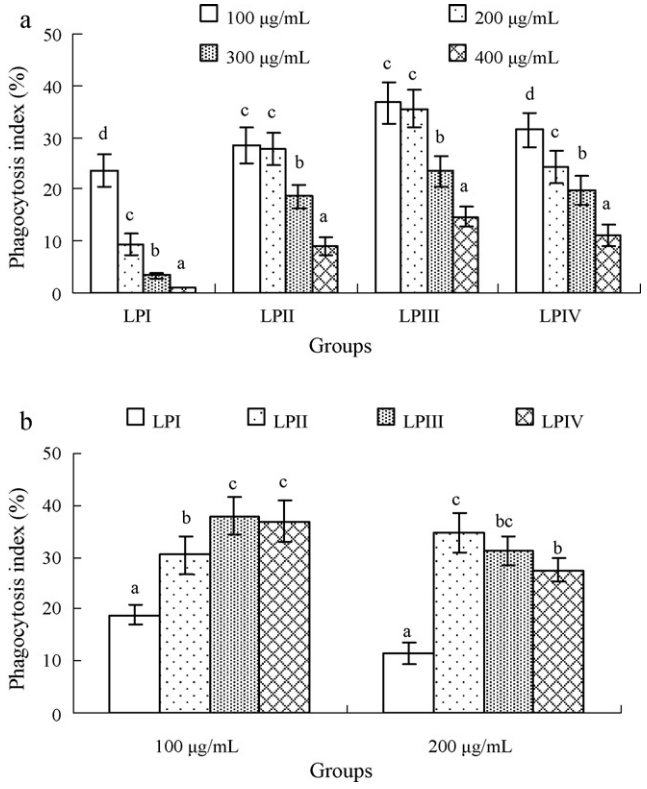

Fig. 5. Effects of LPI–IV on macrophage phagocytosis. (a) The phagocytosis indexes of LPI–IV in the dose range of 100–400 μg/mL. (b) The comparison of phagocytosis indexes of LPI–IV at 100 or 200 μg/mL. The activity was evaluated by the phagocytosis index of neutral red and expressed as means ± standard deviation ($n = 6$). The statistically significant differences among the groups were evaluated with ANOVA followed by the S–N–K test. Different letters marked on the same fraction (a) or the same dose (b) represented the statistical difference at $P < 0.05$.

of α-D-galactopyranose at 820.0 cm^{-1} coincided with the high galactose content of LP III (Zhang, 1994). Furthermore, NMR spectroscopy was used to understand the structural characterization. A same signal of LPI–III, which occurred in the anomeric region of ^1H NMR spectrum at about 4.92 ppm, could be assigned as (1 → 6)-α-D-Glcp. Accordingly in the anomeric region of ^{13}C NMR spectrum, same signals identified at 97.8, 71.5, 73.5, 69.7, 70.3 and 65.7 ppm could be assigned to the C-1, C-2, C-3, C-4, C-5 and C-6 of →6)-α-D-Glcp-(1→ linkage in LPI–III (Li, Wang, et al., 2008; Liu et al., 2007; Zhao, Li, Luo, & Wu, 2006). In addition, the ^1H signal at about 5.03 ppm and the ^{13}C signal at 107.6 ppm indicated the presence of (1 → 5)-α-L-Araf in LPI–III (Brecker et al., 2005; Yang et al., 2009). The →4,6)-α-D-Glcp-(1→ linkage of LPI could be characterized by the signals at 98.1, 71.2, 74.1, 76.6, 69.3 and 67.7 ppm, and the signals at 96.0 and 92.2 ppm might also belong to α-D-Glcp (Cao et al., 2006; Zhang, 1994). The signals at 103.8, 72.5, 72.7, 81.4 and 62.4 ppm in the ^{13}C spectrum of LPI were related to the C-1, C-2, C-3, C-4, C-5 and C-6 of →4)-β-D-Manp-(1→ (Zhang, 1994). The ^1H signals at 4.47 and 4.32 ppm and the ^{13}C signal at 104.3 ppm confirmed the presence of β-D-Galp in LPII (Brecker et al., 2005; Yang et al., 2009). The ^{13}C signals at about 98.5 and 82.3 ppm might be originated from the C-1 and C-4 of →4)-β-D-Rha-(→ (Zhang, 1994). To sum up, LPI mainly consisted of (1 → 6)-α-D-Glcp, (1 → 4)-β-D-Manp and (1 → 5)-α-L-Araf. LPII mainly consisted of (1 → 6)-α-D-Glcp, (1 → 5)-α-D-Araf and β-D-Galp. LP III contained (1 → 4)-β-D-Rhap and (1 → 5)-α-L-Draf.

Spleen lymphocyte proliferation induced by ConA *in vitro* has been used to evaluate T lymphocyte activity, while that induced by LPS has been used to examine B lymphocyte activity (Sun, Liang, Zhang, Tong, & Liu, 2009; Zhang & Huang, 2005; Zhao, Li, Luo, et al., 2006). The present results indicated that LPI had no direct activity on lymphocytes and exhibited depressant effect on mitogen-induced proliferation. Specifically, LPII–IV could selectively enhance the proliferation of B cells as the action of LPS, and showed strong depressant effects on ConA-induced proliferation of T cells (Table 3), indicating their effects of two-ways regulation on lymphocyte proliferation (Li, Jiao, et al., 2008). To date, the effects of polysaccharides on lymphocyte proliferation were variously reported. For example, polysaccharides or polysaccharide–protein complexes from *Armillaria mellea* (Sun et al., 2009), *Polyporus albicans* (Sun et al., 2008), *Aconitum carmichaeli* (Zhao, Li, Luo, et al., 2006) and *Ganoderma lucidum* (Li, Fang, & Zhang, 2007) displayed stimulating effects on ConA- and LPS-induced lymphocyte proliferation. Besides, polysaccharides separated from *Misgurnus anguillicaudatus* (Zhang & Huang, 2005) and *Strongylocentrotus nudus* (Liu et al., 2008) selectively activated T cells and macrophages, but did not affect B cells directly. The splenocyte proliferation stimulated by polysaccharide–protein complex from *Lycium barbarum* was proved to be T cells, but not B cells (Chen, Tan, & Chan, 2008). In addition, polysaccharide or polysaccharide–protein complex isolated from *Phellinus linteus* (Kim et al., 2006), *G. lucidum* (Ye et al., 2011; Zhang, Tang, Zimmerman-Kordmann, Reutter, & Fan, 2002) and *P. dulcis* (Dourado et al., 2004) mainly increased the proliferation of B cells, but not T cells. Polysaccharides might mediate mouse splenic B cell activation by the induction of Blimp-1, a master regulator capable of triggering the changes of a cascade of gene expression during plasmacytic differentiation (Lin et al., 2006).

Immune cells can bind polysaccharides by membrane receptors leading intracellular signaling cascades for immunologic responses. The stimulating activities of polysaccharides triggered by the recognization of cell receptor importantly depend on monosaccharide composition, molecular mass and glycosidic linkage. Kralovec et al. (2007) deemed that bioactive polysaccharides or polysaccharide–protein complexes were mainly composed of galactose, rhamnose and arabinose with the molecular mass more than 100 kDa. Both mannose and molecular mass had positive effects on the immunoregulatory activity of polysaccharide from *Aloe vera* (Leung et al., 2004). Moreover, it was found that the effects of polysaccharides on lymphocytes were closely related to the branch of molecular chain mainly composed of D-mannopyranosyl residues, D-galactopyranosyl residues and D-glucopyranosyl residues (He et al., 2007; Sun & Liu, 2009; Sun et al., 2008; Zhao, Kan, Li, & Chen, 2005; Zhao, Li, Luo, et al., 2006). LPI did not exhibited stimulation on lymphocytes implying that →6)-α-D-Glcp-(1→ and →4)-β-D-Manp-(1→ trivially contributed to the proliferation. Polysaccharides with higher molecular mass usually have more repetitive ligands of cell receptor for enhancing the stimulation by cross-linking between polysaccharide and receptor. Although the molecular mass of LPIV was higher than LPIII, its activation on lymphocytes was weaker. It was suggested that →4)-β-D-Rhap-(→ and →5)-α-L-Araf-(1→ were the important elements for lymphocyte activation. Lo, Jiang, Chao, & Chang (2007) considered that arabinose, mannose, xylose and galactose played an important role in the stimulation of macrophage, but not glucose. Macrophage phagocytosis enhanced by LPI might mainly depend on the mediation of the mannose receptor of the macrophage. Arabinose, galactose and xylose might also act as active units for the effects of LPII–IV on macrophages. Nergard et al. (2005) analyzed the relationship between structure and immunological characteristic of polysaccharide from the roots of *Vernonia kotschyana*, and suggested that the bioactive sites of the polysaccharide on complement fixation and induction of B-cell mitosis might be located in the more peripheral parts of the molecule, the inner core of the 'hairy

region' and the larger enzyme-resistant chains. β-D-GlcpA-(1 → 6)-β-D-Galp-(1 → 6)-D-Galp and β-D-GlcpA-(1 → 6)-D-Galp were the active sites of the polysaccharide from *Bupleurum falcatum* during mitogenic activity of B cell (Sakurai, Matsumoto, Kiyohara, & Yamada, 1999). But these structural units have not been found in LPI–III, the bioactive sites of LPII–IV need to further investigated.

LPI–IV isolated from longan pulp displayed many differences on monosaccharide composition, molecular mass, structural characteristic and immunomodulatory activity. Their stimulations on normal/LPS-induced proliferation and depression on ConA-induced proliferation could be ordered as LPIII > LPIV > LPII > LPI in the dose range of 100–400 μg/mL. In addition, all fractions could best enhance macrophage phagocytosis at 100 μg/mL. However, the further challenges are to define the active units of the polysaccharides and their mechanisms for immunomodulation.

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