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Characterization of polysaccharide from longan pulp as the macrophage stimulator

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Abstract

Longan is one of the most popular subtropical fruits in Southeast Asia, because of its attractive flavor and benefits to health. As one of the important active ingredients of longan pulp, polysaccharide LPIIa was obtained by hot water extraction, ion-exchange chromatography and gel filtration chromatography. Its physicochemical characterization and immunostimulatory effects on macrophages were then investigated. Structural analyses indicated that LPIIa was a 44.7-kDa heteropolysaccharide mainly composed of →6)-Glc-(1→, →5)-Ara-(1→, →4)-Man-(1→ and →6)-Gal-(1→. It enhanced macrophage phagocytosis and nitric oxide production in the dose range of 100–400 μg/mL. Moreover, it significantly increased the inducible nitric oxide synthase

activity, tumor necrosis factor-α and interleukin-6 secretion of macrophage at 200 μg/mL. However, these effects were obviously weakened after toll-like receptor 4 (TLR4) or TLR2 was blocked. Likewise, the specific inhibitors of p38 mitogen-activated protein kinase (MAPK), protein kinase C, phosphatidyl inositol 3-kinase, protein tyrosine kinase and nuclear factor κB (NF-κB) selectively depressed the immunostimulatory activities of LPIIa on macrophages. LPIIa stimulated macrophage activation partly *via* TLR4 and TLR2, followed by p38 MAPK- and NF-κB-dependent signaling pathways. The results suggested that LPIIa possessed potent immunomodulatory activity by stimulating macrophage and could be used as an immunotherapeutic adjuvant.

Key words: Longan pulp; Polysaccharide; Structure; Immunostimulatory activity; macrophage.

1. Introduction

Polysaccharides from natural sources are a class of macromolecules which have the potential as immunostimulators with wide clinical applications.[1] Many bioactive polysaccharides have been confirmed that they can activate macrophages, which are key participants in innate immune response. They display enhancing effects on macrophage functions including phagocytosis, nitric oxide (NO) production, inducible NO synthase (iNOS) activity, as well as the secretion of cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-1β and IL-6. Their immunostimulatory activities are thought to be primarily mediated by specific receptors such as TLR4, TLR2, cluster of differentiation (CD) 14, complement receptor (CR) 3, scavenger receptor (SR), mannose receptor (MR) and dectin-1,[2] which are known as pattern recognition receptors that can recognize foreign ligands during initial phases of the immune response.[3] The activated receptors lead to intracellular signaling cascades, which result in transcriptional activation and pro-inflammatory cytokine production.[4] For example, *Acanthopanax senticosus* polysaccharide stimulated the cytokine production of mouse peritoneal macrophage by interacting with TLR2 and TLR4 to lead to the subsequent activation of MAPKs and NF-κB.[5] Fucoidan induced the NO production of macrophage *via* SR, followed by p38 MAPK- and NF-κB-dependent signaling pathways.[6] *Angelica gigas* polysaccharide-induced iNOS expression in mouse peritoneal macrophage was related to the CD14- and CR3-mediated activations of p38 MAPK and NF-κB.[7]

Longan (*Dimocarpus longan* Lour.) is an attractive fruit from *Sapindaceae* family, and is mainly cultivated in subtropical areas including China, Thailand, India and Vietnam.[8] Dried longan pulp has been widely used as a traditional Chinese medicine for health protection importantly due to its immunomodulatory function. The previous works claimed that the bioactive ingredients chiefly contributing to the immunomodulatory effects of longan pulp were polysaccharides,[9-11] in which, LPII might be the main active fraction because of high content and strong immunoenhancing activity.[12] It can effectively stimulate splenic lymphocyte proliferation and macrophage phagocytosis in the dose range of 100–400 μg/mL.[12] However, the molecular mechanism of longan polysaccharide-induced immunoenhancement is still far from clear. This work purposed to evaluate the *in vitro* macrophage activation stimulated by longan polysaccharide and explore the mechanism of action. LPII was fractionated to obtain its sub-fraction LPIIa. The structural characteristic and macrophage immunostimulatory effects of LPIIa were then analyzed. Specially, the potential signaling pathway of macrophage activation stimulated by LPIIa was further investigated.

2. Materials and methods

2.1. Preparation of longan polysaccharide LPIIa

Longan polysaccharide LPII was prepared according to our previous work.[12] Fifty milligrams of LPII were dissolved in 5 mL distilled water, followed by centrifugation at 4,500 rpm for 15 min. The supernatant was collected and injected onto a Sephadex G-100 gel column (60 × 1.5 cm). The column was then eluted with distilled water at a flow rate of 0.2 mL/min. Four milliliters per tube of eluant was continuously collected to determinate polysaccharide concentration by phenol-sulfuric acid method.[13] The eluants were selectively combined according to the elution profile of polysaccharide and concentrated at 55 °C using a vacuum rotary evaporator (RE-2000A, Yarong Biochemistry Instrument Factory, Shanghai, China), followed by vacuum freeze-drying using a lyophilizer (Scientz-12N, Scientz Biotechnology CO., Ningbao, China) to obtain the powdery samples of LPIIa. The molecular uniformity of LPIIa was also identified using a Sephadex G-100 gel column (20 × 1.5 cm), which was eluted with distilled water at a flow rate of 0.1 mL/min.[14] The polysaccharide content of LPIIa was determined by the phenol-sulphuric acid

method [13] and expressed as glucose equivalents, and its protein content was measured by a Bradford protein assay kit (Nanjing Jiancheng Bioengineering Institute, Wuhan, China).

2.2. Structural analysis

Gas chromatography coupled with mass spectrometer (GC-MS) was applied for the determination of monosaccharide composition, and nuclear magnetic resonance (NMR) spectroscopy was used for the determination of glycosidic linkage, according to the reported methods.[12] Methylation analysis of LPIIa was carried out according to the method of Jiang et al.,[15] which was modified based on that of Needs and Sevendran.[16] Fourier transform infrared (FITR) spectrum of LPIIa was scanned in the frequency range of 4000–400 cm^{-1}.[14]

Molecular weight (Mw) of LPIIa was determined by high performance gel permeation chromatography (HPGPC) using a Waters 600E HPLC (Millipore, Milford, MA, USA) equipped with a TSK2GEL G3000SWXL column (300 mm × 7.8 mm, Tosoh, Japan). The column was kept at 35 °C and eluted with NaH_2PO_4-Na_2HPO_4 buffer solution (0.05 mol/L, pH 6.7) containing 0.05% NaN_3 at a flow rate of 0.5 mL/min. LPIIa was dissolved in NaH_2PO_4-Na_2HPO_4 buffer solution and injected onto the column after filtrating through a 0.45 μm filter membrane. Peaks were detected using a differential refractive index detector (Optilab rEX, Wyatt, Santa Barbara, CA, USA). To estimate the M_W of LPIIa, dextran standards with known M_W (7.38×10^2, 5.80×10^3, 1.22×10^4, 2.37×10^4, 4.80×10^4, 1.00×10^5, 1.86×10^5, 3.80×10^5 and 8.35×10^5 Da, APSC, USA) were used for calibration.

2.3. Analysis of macrophage activation

2.3.1. Phagocytosis

RAW264.7 macrophages were provided by Experiment Animal Center of Sun Yat-sen University (Guangzhou, China). The cells were adjusted to the concentration of 5×10^5 cells/mL in DMEM medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco BRL). One hundred microliters per well of cell suspensions were plated in 96-well culture plates and incubated for 3 h (37 °C, 5% CO_2). After washing twice with aseptic phosphate-buffered saline (PBS), the remaining macrophages were incubated with LPIIa (0, 25, 50, 100, 200 or 400 μg/mL) in 100 μL medium for 48 h. Each concentration of LPIIa was designed with six replications. After washing twice with 37 °C PBS, cells in each well were incubated in 100 μL neutral red solution

(0.1%, g/mL) for 4 h. The extracellular neutral red particles were washed away by PBS. To each well was then added 100 μL lysis solutions (the volume ratio of acetic acid to ethanol was 1:1). Finally, the plates were read at 570 nm using a microplate reader (Thermo Labsytems, Helsinki, Finland). The index of macrophage phagocytosis was expressed as absorbance value.

2.3.2. NO production and iNOS activity

Four hundred microliters per well of cell suspensions (5×10^5 cells/mL) were plated in 24-well culture plates and incubated for 3 h (37 °C, 5% CO_2). After washing twice with medium, the remaining macrophages were incubated with stimulant (LPIIa or LPS) in 400 μL medium for 48 h. The final concentration of LPIIa was 0, 25, 50, 100, 200 or 400 μg/mL, and that of LPS was 5 μg/mL. Each concentration had four replications. The medium was then sucked into a 1.5 mL Eppendorf tube containing 20 μL $ZnSO_4$ aqueous solution (300 mg/mL), followed by centrifugation at 5,000 rpm for 10 min. One hundred microliters of supernatant and 100 μL Griess reagent (containing 1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2% phosphoric acid) were mixed in 96-well culture plates with three replications. The plates were measured at 492 nm using a microplate reader.[17] The NO production of macrophage was calculated according to the standard curve established by sodium nitrite and expressed as sodium nitrite equivalents (μmol/mL).

Moreover, after washing thrice with 37 °C PBS, the remaining macrophages were resuspended in 4 °C PBS. Suspension containing 1×10^6 cells was collected in a 1.5 mL Eppendorf tube. The cells were isolated by centrifugation at 1,000 rpm for 5 min, followed by dissociation with 50 μL cell lysis buffers at 4 °C for 20 min. The mixture was centrifuged at 12,000 rpm for 10 min to obtain supernatant.[18] The protein content and iNOS activity of the supernatant were respectively measured using a bicinchoninic acid assay kit and a NOS assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to their instructions of the manufacturer. The index of macrophage iNOS activity (U/μg) was calculated as the ratio of enzyme activity (U/mL) to protein concentration (μg/mL).

2.3.3. Cytokine secretion

Macrophages were plated, stimulated and incubated according to the processes described in

section 2.3.2. The culture supernatants were collected for the measurement of cytokine concentration (IL-1β, IL-6 and TNF-α) using ELISA assay kits (Neobioscience Technology Co., Shenzhen, China) according to their instructions.

2. 4. Analysis of endotoxin contamination

LPIIa (200 μg/mL) and LPS (5 μg/mL) were respectively pre-incubated with or without polymyxin B (PMB, 1000 units/mL, Sigma) for 1 h at 4 °C,[19, 20] and were then used to stimulate macrophages. Their effects on phagocytosis and NO production were analyzed.

2. 5. Analysis of signaling pathway

Four hundred microliters per well of cell suspensions (5×10^5 cells/mL) were plated in 24-well culture plates and incubated for 3 h (37 °C, 5% CO_2). After washing twice with medium, the remaining macrophages were incubated with antibody or inhibitor in 200 μL medium at 4 °C for 1 h. The concentrations of anti-TLR4/MD2, anti-TLR2 and anti-CD11b antibodies (MT510, 6C2 and M1/70, eBioscience, San Diego, CA, USA) were all 20 μg/mL;[20, 21] the concentration of mannose was 10 μg/mL;[22] the concentrations of PD98059, curcumin, SB203580, calphostin C, wortmannin, AG490 and pyrrolidine dithiocarbamate (PDTC) (Enzo, London, UK) were respectively 50, 10, 30, 100, 50, 50 and 30 μg/mL.[6, 22-24] Two hundred microliters of medium-adjusted stimulant (400 μg/mL LPIIa or 10 μg/mL LPS) was then added in and incubated for 48 h. Finally, the NO production, iNOS activity and cytokine secretion of macrophage were analyzed.

2.6. 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 longan polysaccharide LPIIa

As seen in the gel filtration chromatogram of longan polysaccharide LPII (Fig. 1), its molecular weight distribution was relatively wide. The first polysaccharide peak, which represented its main

fraction, was isolated and named as LPIIa. However, the polysaccharide profile of LPIIa was still not symmetrical, indicating its molecular weight was inhomogeneous. In addition, the peak values of polysaccharide and protein appeared at the same elution volume, implying LPIIa was a polysaccharide-protein complex. One hundred micrograms of LPII were dissolved and injected onto the gel column in twice to obtain 72.17 mg LPIIa. The polysaccharide content of LPIIa was (95.23±0.81)%, and the protein content was (3.81±0.22)%.

3.2. Structural characteristics of LPIIa

3.2.1. Molecular weight and composition

According to the calibration curve established by dextran standards, the M_W of LPIIa was calculated to be 44.7 kDa. LPIIa was inhomogeneous, and had the polydispersion index of 1.49. As shown in Table 1, it was mainly composed of glucose, arabinose, mannose and galactose with the molar ratio of 7.55 : 1.45 : 1.22 : 1.00. Methylation analysis indicated that →6)-Glc-(1→, →5)-Ara-(1→, →4)-Man-(1→ and →6)-Gal-(1→ were the major linking types for LPIIa. The molar percentages of Ara-(1→ and Gal-(1→ were relatively little, indicating the low branching structure of LPIIa.

3.2.2. FTIR and NMR spectra

The FTIR spectrum of LPIIa displayed the band characteristics of polysaccharide, including the stretching vibration of hydroxyl group at 3429.8 cm^{-1}, the stretching vibration of alkyl group at 2928.2 cm^{-1}, the stretching vibrations of carbonyl group at 1648.1, 1420.3 and 1363.8 cm^{-1}, the bending vibration of alkyl group at 1458.6 cm^{-1}, the bending vibration of carbonyl group at 1277.8 cm^{-1}, and the bending vibration of hydroxyl group at 1015.9 cm^{-1}. The absorption peaks at 918.9, 847.7 and 764.1 cm^{-1} were respectively identified as the antisymmetrical ring vibration of D-glucopyranose ring, the C-H bending vibration of α-type glycosidic linkage, the symmetrical ring vibration of D-glucopyranose ring. It was implied that the absorption peaks in fingerprint region belonged to the group vibrations of α-D-Glcp.[25]

As seen in Fig.2, the ^1H signal at 4.70 ppm belonged to HOD in D_2O solvent. The signal occurred in ^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, the signals identified at 97.79, 71.48, 73.48, 69.64, 70.27

and 65.68 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 LPIIa.[26-28]

3.3. Immunostimulatory activities of LPIIa on macrophage

3.3.1. Effects of LPIIa on phagocytosis and NO production

The effects of LPIIa on RAW264.7 macrophage activation were evaluated by phagocytosis against neutral red and NO production. As shown in Fig. 3(I), LPIIa-stimulated macrophage phagocytosis was significantly strengthened with the increasing doses from 25 to 100 μg/mL ($p < 0.05$), but further strengthening was not found at 200 and 400 μg/mL. Macrophage NO production was significantly enhanced by 100–400 μg/mL LPIIa ($p < 0.05$). LPIIa (100–400 μg/mL) showed strong effects on phagocytosis and NO production comparable to LPS (5 μg/mL) ($p > 0.05$).

3.3.2. Effects of PMB on the immunostimulatory activities of LPIIa

As seen in Fig. 3(II), both LPIIa (200 μg/mL) and LPS (5 μg/mL) significantly enhanced the phagocytosis and NO production of macrophage compared with the control ($p < 0.05$). The effects of LPIIa were not depressed by the treatment of PMB ($p > 0.05$), but those of LPS were remarkably weakened ($p < 0.05$).

3.3.3. Effects of receptor blockers on the immunostimulatory activities of LPIIa

Clone MT510, clone 6C2, clone M1⁄70 and mannose were respectively used as the receptor blocker of TLR4⁄MD2, TLR2, CD11b and MR to disturb the immunostimulatory activity of LPIIa on macrophages, and their blocking effects were shown in Table 2. All the receptor blockers did not significantly affect macrophage functions including NO production, iNOS activity, TNF-α, IL-6 and IL-1β secretion ($p > 0.05$), but might weaken the enhancements of LPIIa and LPS on these functions. Macrophage NO production could be significantly promoted by LPIIa or LPS compared with the black control ($p < 0.05$). Both LPIIa- and LPS-stimulated NO productions were decreased by clone MT510 or clone 6C2, even were significantly lower than their blocking controls ($p < 0.05$). In addition, clone M1⁄70 and mannose could also weaken LPIIa-stimulated NO production ($p < 0.05$). LPIIa and LPS could both enhance the iNOS activity of macrophage compared with the black control ($p < 0.05$). After TLR4MD2 or TLR2 was specifically blocked, LPIIa- and

LPS-stimulated iNOS activity were significantly depressed ($p < 0.05$). In clone MT510-treated group, LPIIa-stimulated iNOS activity was weaker than the blocking control ($p < 0.05$). In addition, clone M1/70 obviously weakened LPIIa-stimulated iNOS activity ($p < 0.05$).

Both LPIIa and LPS significantly promoted the TNF-α secretion of macrophage compared with the black control ($p < 0.05$). Their activities showed no statistical difference ($p > 0.05$), and were weakened by the blocking of all receptors but only the blocking of TLR4/MD2 was significant ($p < 0.05$) . The IL-6 production of macrophage could be enhanced by LPIIa ($p < 0.05$), as well as LPS which showed a stronger effect ($p < 0.05$). Moreover, LPIIa could not stimulate macrophage IL-1β secretion as LPS, Their stimulatory effects on IL-6 and IL-1β secretion were both decreased by the blocking of TLR4/MD2 or TLR2 ($p < 0.05$) .

3.3.4. Effects of inhibitors on the immunostimulatory activities of LPIIa

As the specific inhibitors of MEK1/2, SAPK/JNK, p38MAPK, PKC, PI3K and NF-κB, PD98059, curcumin, SB203580, calphostin C, wortmannin, AG490 and PDTC were respectively investigated on their effects on LPIIa-induced macrophage activation, as shown in Table 3. The immunostimulatory effects of LPIIa and LPS on the NO production, iNOS activity, cytokine secretion of macrophage were consistent with the results presented in Table2. All the inhibitors did not display inhibitory effect on macrophage NO production. However, calphostin C, wortmannin and AG490 obviously inhibited LPIIa- or LPS-induced NO production ($p < 0.05$). Compared with the control group, only PDTC significantly inhibited unstimulated and LPIIa-stimulated iNOS activity ($p < 0.05$), and all the inhibitors markedly decreased LPS-induced iNOS activity except AG490 ($p < 0.05$). The effects of LPIIa and LPS on NO production and iNOS activity showed no significant difference in all the groups ($p > 0.05$).

PD98059, curcumin, SB203580, wortmannin and AG490 obviously depressed the TNF-α secretion of macrophage ($p < 0.05$). All the inhibitors significantly inhibited LPIIa-induced TNF-α secretion except calphostin C ($p < 0.05$), while curcumin, SB203580, calphostin C, wortmannin and AG490 significantly depressed LPS-induced TNF-α secretion ($p < 0.05$). The IL-6 secretion of macrophage was remarkably decreased after being treated with PD98059, curcumin, calphostin C or AG490 ($p < 0.05$). Both SB203580 and wortmannin had no depressive effect on unstimulated

IL-6 secretion ($p > 0.05$), but significantly inhibited LPIIa-stimulated IL-6 secretion ($p < 0.05$). All the inhibitors could weaken the significant immunostimulation of LPS on macrophage IL-6 secretion ($p < 0.05$). Macrophage IL-1β secretion could be significantly depressed by every inhibitor except PD98059 ($p < 0.05$). Likewise, the inhibitors all weakened LPS-induced IL-1β secretion ($p < 0.05$). LPIIa showed no immunostimulating effect on the IL-1β secretion of macrophage ($p > 0.05$).

4. Discussion

LPIIa was a 44.7-kDa heteropolysaccharide mainly composed of →6)-Glc-(1→, →5)-Ara-(1→, →4)-Man-(1→ and →6)-Gal-(1→. LPIIa had generally the same types of glycosidic linkage as those isolated from longan pericarp and seed, but with different molar percentages. [15, 29] LPIIa could effectively enhance the phagocytosis and NO production of macrophage. To rule out the immunostimulatory activity of LPIIa due to endotoxin contamination, PMB as a specific inhibitor of LPS was used to identify endotoxin-dependent phagocytosis and NO production.[19, 20] As seen in Fig. 3(II), PMB could remarkably weaken LPS- but not LPIIa-induced immunostimulation, indicating the endotoxin contamination in LPIIa was negligible.

In this work, clone MT510, clone 6C2, clone M1/70 and mannose, which could respectively combine with TLR4/MD2, TLR2, CD11b and MR, were used to confirm the receptors which participated in LPIIa- and LPS-induced macrophage activation.[20-22] Based on the results from Table 2, it could be summed up that the blocking of TLR4/MD2 and TLR2 both significantly depressed LPIIa- and LPS-induced macrophage activation. Previous researches indicated that the immunostimulating effects of LPS on macrophages were mainly mediated by TLR4 and TLR2 [5, 30]. The conclusion was accordant with what we found. Further, it could be deduced that the signal transduction of LPIIa-stimulated macrophage activation was mostly triggered by the interaction between polysaccharide and TLRs (TLR4 and TLR2). In addition, CD11b and MR might secondarily participate in the signaling pathway of LPIIa for regulating NO production and iNOS activity. Likewise, polysaccharides isolated from *Acanthopanax koreanum*,[30] *Acanthopanax senticosus* [5] and *Ganoderma lucidum* [31] all stimulated immune cell activation through TLR4 and TLR2. Mammalian TLRs play prominent roles in the direct activation of host defense mechanisms.

Activated TLRs induce innate immune response which involves the productions of direct antimicrobial effector molecules such as NO, and enhances adaptive immune response by promoting the secretions of IL-1β, IL-6, IL-12 and TNF-α that augment both cell-mediated and humoral immune responses.[5] The direct immunostimulating activities of LPIIa on the NO production and cytokine secretion of macrophage were confirmed. The results indicated that LPIIa had a prospect of medical application in infectious disease and cancer.

The activations of MAPKs, PKC, PI3-K PTK and NF-κB in immune cell are mostly involved in LPS- and botanical polysaccharide-induced signal transduction.[4, 32, 33] Therefore, their specific inhibitors, including PD98059, curcumin, SB203580, calphostin C, wortmannin, AG490 and PDTC, have been widely used to confirm the intracellular factors which participated in the signaling pathway of polysaccharide-stimulated cell activation. [6, 7, 23, 24, 34, 35] According to the results from Table 3, SB203580, calphostin C, wortmannin, AG490 and PDTC all inhibited LPIIa- and LPS-induced macrophage activation, implying p38MAPK, PKC, PI3K, PTK and NF-κB were involved in the signal transductions stimulated by LPIIa and LPS. It was suggested that the signaling pathways triggered by LPIIa in macrophage were TLR4/TLR2→PTK→PKC/PI3-K→p38MAPK and TLR4/TLR2→NF-κB, [2, 4] which were similar to those of *Acanthopanax senticosus* polysaccharide.[5] In comparison, polysaccharides from *Carthamus tinctorius* [36] and *Polyporus umbellatus* [21, 37] induced the activation of macrophage *via* TLR4 but not TLR2. The structures of polysaccharide ligands recognized by TLR4 and TLR2 might be different. Besides, PD98059 could weaken the iNOS activity and IL-1β secretion of LPS-stimulated macrophage. The MAPK-dependent signal pathway of LPS might be more complicated than that of LPIIa.

Based on specific molecular structure, polysaccharide can be recognized by macrophage receptors following with a series of immune responses. The structural differences may result in different affinities for receptors. The polysaccharides isolated from *Opuntia polyacantha* [38], *Juniperus scopolorum*,[39, 40] *Aloe vera* L.,[40] *Tanacetum vulgare* L. [41] and *Artemisia tripartite* [42] all could effectively stimulate macrophage activation, and their effects exhibited positive correlations with their molecular weights. The potent macrophage stimulatory effect of high molecular weight polysaccharides may involve in their highly repetitive structures, which can cross-link receptors or other membrane targets in a multivalent fashion.[40] LPIIa shows a relatively

small molecular weight compared with the active polysaccharides reported in reviews,[4, 25] its activity may be importantly related to the flexible chain with low branching structure.[43] Less side chain branches can be beneficial to form a proper fold of polysaccharide, which is important for macrophage receptor recognition.[44, 45] In addition, specific structure region also plays a key role in macrophage activation. The immunostimulating activity of pectic polysaccharide from the *Lemna minor* L. disappeared after the cleavage of the regions of a linear 1,4-α-D-galactopyranosyluronan.[46] Nergard et al. reported that the arabinogalactan side chains of a rhamnogalacturonan core were important for the immunomodulatory activity of polysaccharides from *Veronia kotschyana* roots.[47] The rhamnogalacturonan II-like region containing 2-keto-3-deoxyoctulosonic acid, which was known to be a component of LPS, might be the specific structure cross-linked with TLR4 and TLR2.[47-49] However, the active structure region of LPIIa, which is related to the immunostimulatory effects on macrophage, needs to be further investigated.

5. Conclusions

The immunostimulatory mechanism of macrophage activation of longan polysaccharide was first investigated in the present work. LPIIa isolated by ion-exchange chromatography combined with gel filtration chromatography was a 44.7-kDa heteropolysaccharide mainly composed of →6)-Glc-(1→, →5)-Ara-(1→, →4)-Man-(1→ and →6)-Gal-(1→. LPIIa showed significantly immunostimulatory effects on the NO production, iNOS activity, TNF-α and IL-6 secretion of macrophage *in vitro*. Its immunostimulatory signal might be mediated by TLR4 and TLR2, followed by the activations of p38MAPK and NF-κB pathway. The further investigations will focus on the interaction between LPIIa and receptor, the transduction of intracellular signal and the expression of activation-related gene.

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Table 1 The molar percentages of monosaccharides and glycosidic linkages in LPIIa.

Composition	Glycosidic linkage	Main fragments (m/e)	Molar percentage
glucose			67.28±0.18
	→6)-Glc-(1→	43,87,99,101,117,129,161,189	64.02±0.24
	→4)-Glc-(1→	43,45,87,99,101,113,117, 233	3.22±0.02
arabinose			12.92±0.25
	→5)-Ara-(1→	43,45,71,87,101,129,161	10.10±0.33
	Ara-(1→	43,45,71,87,101,117,129,145,171	2.84±0.11
mannose			10.84±0.03
	→4)-Man-(1→	43,87,101,117,129,161,233	10.87±0.20
galactose			8.91±0.13
	→6)-Gal-(1→	43,87,99,101,117,129,161,189	7.03±0.09
	Gal-(1→	43,45,87,101,117,129,161,205	1.85±0.04

Table 2 Effects of LPIIa and LPS on receptor-blocked macrophage involving in NO production, iNOS activity and cytokine secretion.

Receptor (Blocker)	Stimulant	Evaluation index				
		NO production (μmol/mL)	iNOS activity (U/μg)	TNF-α concentration (pg/mL)	IL-6 concentration (pg/mL)	IL-1β concentration (pg/mL)
Control	-	21.8±0.6 a	0.083±0.009 a	217±9 a	10.0±1.7 a	21.4±0.6 a
	LPIIa	23.2±0.5 b	0.117±0.009 b	720±17 b	22.2±1.6 b	22.9±1.8 a
	LPS	23.4±0.9 b	0.147±0.021 c	742±43 b	35.4±3.4 c	27.3±2.6 b
TLR4/MD2 (MT510)	-	21.2±0.5 b	0.083±0.010 b	204±23 a	9.44±2.1 a	19.0±1.2 a
	LPIIa	19.8±0.2 a*	0.066±0.002 a*	634±28 b*	9.17±0.8 a*	19.8±1.7 a*
	LPS	20.0±0.3 a*	0.077±0.011 ab*	653±6 b*	15.5±1.7 a*	21.7±1.8 a*
TLR2 (6C2)	-	21.5±0.4 b	0.080±0.011 a	220±25 a	12.2±3.2 a	20.2±2.4 a
	LPIIa	19.7±0.3 a*	0.083±0.004 a*	675±22 b	15.6±2.4 a*	18.1±1.3 a*
	LPS	19.6±0.5 a*	0.077±0.006 a*	706±22 b	22.0±3.3 b*	22.8±2.4 a*
CD11b (M1/70)	-	22.3±0.6 a	0.081±0.004 a	218±13 a	11.1±2.1 a	20.0±1.0 a
	LPIIa	22.3±0.3 a*	0.083±0.004 a*	699±17 b	22.7±1.8 b	22.0±1.1 a
	LPS	22.8±0.6 a	0.157±0.009 b	717±37 b	32.5±3.9 c	24.7±1.1 b
MR (Mannose)	-	21.7±0.4 a	0.082±0.006 a	197±13 a	11.0±1.4 a	22.8±1.8 a
	LPIIa	22.6±0.3 b*	0.115±0.009 b	704±19 b	22.7±1.0 b	22.2±0.9 a
	LPS	23.1±0.6 b	0.146±0.015 c	728±18 b	30.9±3.6 c	25.3±1.5 a

Data from same receptor-treated group marked with different letters had significant difference ($p < 0.05$), and marked with same letter had no statistical differences ($p > 0.05$). Data from same stimulant-treated group marked with '*' was significantly different from its corresponding control ($p < 0.05$).

Table 3 Effects of LPIIa and LPS on inhibitor-treated macrophage involving in NO production, iNOS activity and cytokine secretion.

Inhibitor (Object)	Stimulant	Evaluation index				
		NO production (μmol/mL)	iNOS activity (U/μg)	TNF-α concentration (pg/mL)	IL-6 concentration (pg/mL)	IL-1β concentration (pg/mL)
Control	-	17.1±0.2 a	0.067±0.001 a	168±7 a	13.1±1.3 a	25.1±2.4 a
	LPIIa	18.2±0.6 b	0.097±0.006 b	667±11 b	17.4±2.0 b	23.8±1.1 a
	LPS	18.4±0.4 b	0.112±0.017 b	679±21 b	22.7±2.1 c	33.4±2.0 b
PD98059 (MEK1/2)	-	17.5±0.6 a	0.072±0.004 a	108±8 a*	9.8±1.4 a*	23.8±2.3 a
	LPIIa	18.8±0.4 b	0.079±0.013 ab	589±20 b*	11.3±1.7 a*	26.1±2.2 ab
	LPS	18.5±0.6 b	0.093±0.012 b*	655±3 c	15.4±2.2 b*	29.1±2.0 b*
Curcumin (SAPK/JNK)	-	17.3±0.9 a	0.070±0.007 a	104±18 a*	7.5±0.8 a*	21.0±1.7 a*
	LPIIa	17.8±0.4 a	0.089±0.015 a	571±18 b*	10.0±1.4 b*	25.2±2.2 a
	LPS	18.4±0.6 a	0.084±0.002 a*	550±6 b*	13.1±1.3 c*	24.5±2.5 a*
SB203580 (p38 MAPK)	-	17.5±0.4 a	0.079±0.010 a	62±11 a*	11.3±2.0 a	19.3±1.2 a*
	LPIIa	18.6±0.3 b	0.084±0.009 a	274±18 b*	9.4±1.8 a*	22.0±3.2a
	LPS	18.8±0.3 b	0.093±0.009 a*	274±6 b*	11.1±2.1 a*	23.5±1.8a*
Calphostin C (PKC)	-	16.7±0.4 a	0.054±0.011 a	170±7 a	8.8±1.7 a*	18.7±1.0 a*
	LPIIa	16.7±0.4 a*	0.083±0.019 b	651±19 b	9.0±2.1 a*	20.3±1.0 a*
	LPS	16.9±0.6 a*	0.087±0.009 b*	613±46 b*	11.0±1.8 a*	23.8±1.8 b*
Wortmannin (PI3K)	-	17.2±1.0 a	0.069±0.018 a	125±21 a*	14.7±1.7 b	21.1±0.4 a*
	LPIIa	17.1±0.8 a*	0.089±0.006 b	609±23 b*	9.8±1.4 a*	22.5±1.4 a
	LPS	17.6±0.2 a*	0.089±0.003 b*	596±28 b*	12.8±1.7 b*	26.7±1.0 b*
AG490 (PTK)	-	17.6±1.0 a	0.070±0.010 a	57±3 a*	9.0±2.6 a*	21.4±2.0 a*
	LPIIa	17.0±0.6 a*	0.086±0.016 ab	546±18 b*	9.8±1.0 a*	20.2±2.2 a*
	LPS	17.3±0.6 a*	0.095±0.009 b	580±32 b*	11.5±1.3 a*	25.9±2.6 b*
PDTC (NF-κB)	-	17.1±0.7 a	0.041±0.002 a*	173±5 a	12.9±1.3 a	20.5±0.7 a*
	LPIIa	17.7±0.3 a	0.071±0.010 b*	630±9 b*	14.3±1.8 a	20.5±0.9 a*
	LPS	18.0±0.4 a	0.077±0.015 b*	633±19 b	19.4±1.7 b*	25.2±2.3 b*

Data from same inhibitor-treated group marked with different letters had significant difference ($p < 0.05$), and marked with same letter had no statistical differences ($p > 0.05$). Data from same stimulant-treated group marked with '*' was significantly different from its corresponding control ($p < 0.05$).

Figure caption

Fig. 1 Sephadex G-100 gel column chromatograms of longan polysaccharide LPII and its fraction LPIIa. Polysaccharide was detected by phenol-sulphuric acid method at 490 nm, and protein was detected by UV spectroscopy method at 280 nm.

Fig. 2 NMR spectrums of longan polysaccharide LPIIa. The one above was ^{1}H NMR spectrum, and another one below was ^{12}C NMR spectrum.

Fig. 3 Effects of longan polysaccharide LPIIa on the phagocytosis and NO production of RAW264.7 macrophage. The effects related to doses were shown in figure (I), and the effects of 200 μg/mL LPIIa and 5μg/mL LPS affected by PMB were shown in figure (II). The significance of difference ($p < 0.05$) in phagocytosis among the groups was identified by different lowercase letters, and that in NO production among the groups was identified by different capital letters.

Figure 1

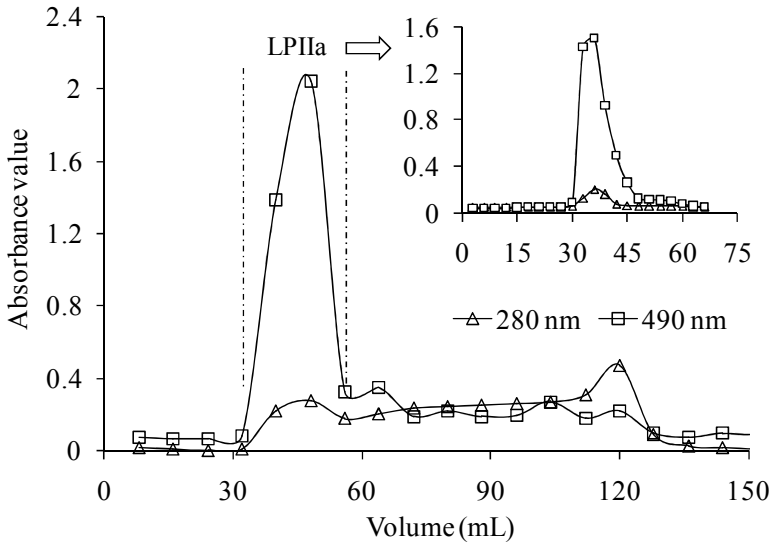

Figure 2

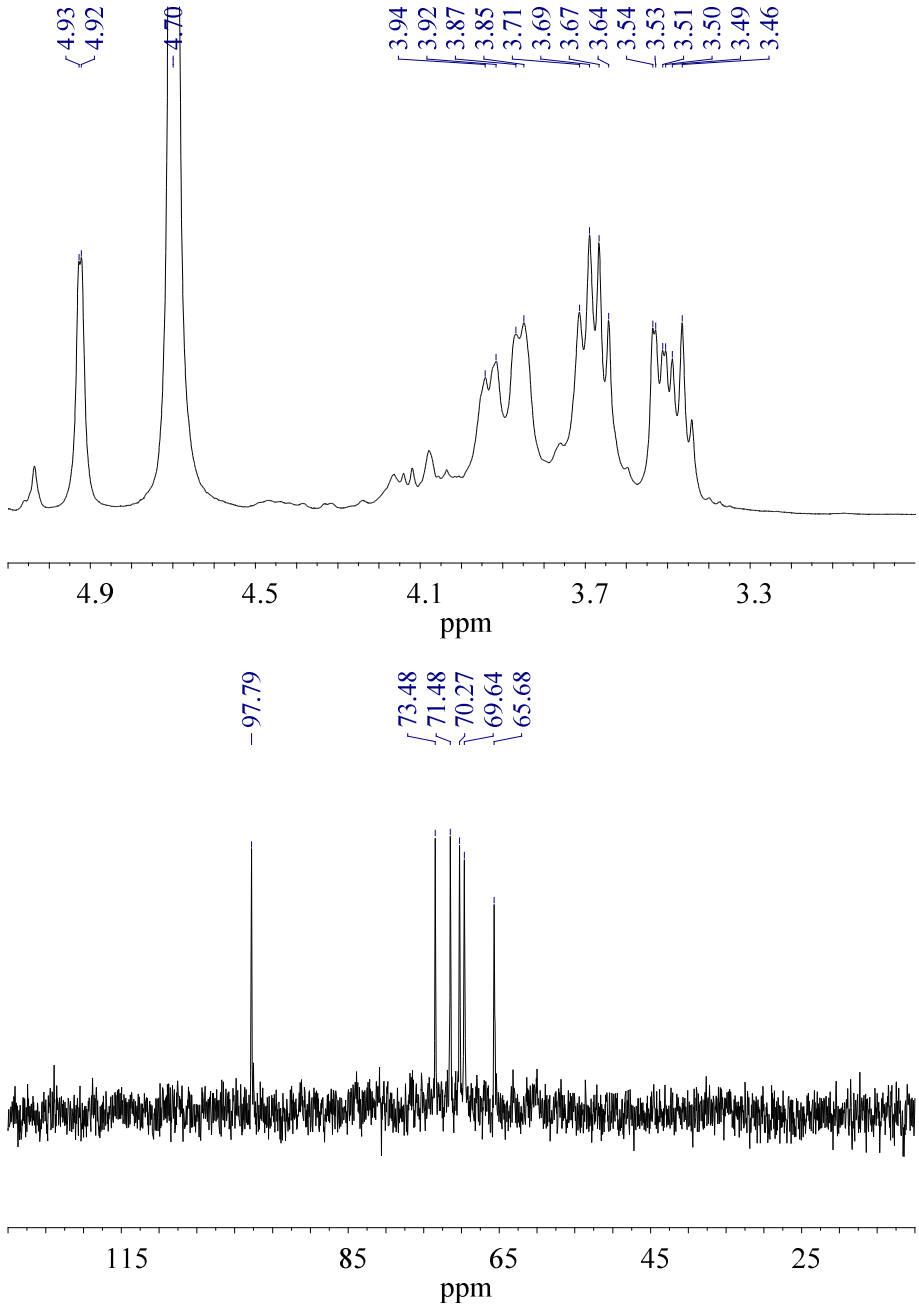

Figure 3