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Immunomodulatory activity on macrophage of a purified polysaccharide extracted from *Laminaria japonica*

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ABSTRACT

In this work, a novel water-soluble homogeneous polysaccharide (LJP-31) with a molecular mass of 2.24×10^6 Da was isolated and purified from *Laminaria japonica* using DEAE-cellulose and Sephacryl S500 chromatography. Results showed that LJP-31 mainly consists of arabinose, mannose, glucose and galactose in a molar ratio of 1.0:7.8:6.6:0.8. LJP-31 exhibited significant stimulation on macrophages and enhanced the production of NO, TNF-α, IL-1β, IL-6 and IL-10 as well as the up-regulation of their gene expressions. Western blot analysis suggested that LJP-31 has the positive effects on the translocation of NF-κB p65 from cytoplasm to nucleus and the phosphorylation of IκBα, ERK1/2, JNK1/2 and P38 in macrophages. Flow cytometric and confocal laser-scanning microscopy analysis indicated that toll-like receptor 4 (TLR4) was at least one of the recognition receptors of LJP-31 on the plasma membrane of macrophages. Taken together, LJP-31 may exert its immunostimulating potency via TLR4 activation of MAPK and NF-κB signaling pathways.

1. Introduction

Immunity is defined as the ability of an organism to protest disease by identifying and destroying foreign harmful substances or organisms. The immune system is important for health, which includes tissues, cells, and molecules that mediate resistance to infections (Hooper, Littman, & Macpherson, 2012). The host defense mechanisms consist of innate immunity and adaptive immunity, where the innate immunity is the first line of defense mediated the initial protection against infections. It is known that the innate immune system mainly contains macrophages, monocytes, granulocytes and humoralelements (Chávez-Sánchez et al., 2014). Among these components, macrophages are reported to exhibit a variety of biological functions, such as phagocytosis, surveillance, chemotaxis and destruction of targeted organisms (Gordon & Martinez, 2010), indicating the activation of macrophages might be a hopeful strategy to resist diseases. In recent years, natural polysaccharides have attracted a great deal of attention because of the promise that they show in a variety of applications in protection of human health through modulating the immune system (Zong, Cao, & Wang, 2012; Pangestuti & Kim, 2011; Li, Chen, Wang, Tian, & Zhang, 2009).

Laminaria japonica, a popular marine vegetable, has been used as a popular therapeutic agent for phlegm elimination, detumescence and weight loss for more than one thousand years (Zha et al., 2012). Many studies have recently suggested that polysaccharides were the main active components in *L. japonica*, exhibiting a wide range of pharmacological properties, such as anti-inflammation, anti-virus, anti-coagulant, anti-cancer, anti-oxidant and anti-atherosclerosis (Islam et al., 2013; Makarenkova, Deriabin, L'vov, Zviagintseva, & Besednova, 2009; Zhao, Dong, Wang, Li, Chen, & Li, 2012; Vishchuk, Ermakova, & Zvyagintseva, 2011; Zha et al., 2012). Our previous study showed that the polysaccharides extracted from *L. japonica* are very complicated and consists of various fractions with different molecular weights (Zha et al., 2012). However, as far as our knowledge to be ascertained, limited study was reported to show the immunomodulatory activity, the underlying mechanism and structural features of *L. japonica* polysaccharides.

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For these purposes, we extracted, purified and characterized a water-soluble polysaccharide from *L. japonica* in the present study. Based on this, we further tested the immunomodulatory ability and the underlying mechanism responsible for the bioactivity of this polysaccharide.

2. Materials and methods

2.1. Materials and chemicals

L. japonica was purchased from Lianjiang county, Fujian province, China. D-Galactose (Gal), D-mannose (Man), D-xylose (Xyl), D-glucose (Glc), L-arabinose (Ara), D-rhamnose (Rha), Lipopolysaccharide (LPS), 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), streptomycin and penicillin were obtained from Hyclone Co. (UT, USA). Trizol Reagent and SYBR Green I detection reagents were purchased from Bio-Rad Co. (CA, USA). ELISA kits of TNF-α, IL-1β, IL-6 and IL-10 were obtained from R&D Co., Ltd. (Nanjing, China). NO kit was supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Bovine serum albumin (BSA) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All the primary antibodies were supplied by the Cell Signaling Technology Inc. (MA, USA). The secondary antibody was purchased from Wuhan Boster Co. (Wuhan, China). All other reagents were analytical grade and purchased from local chemical suppliers in China.

2.2. Fractionation of L. japonica polysaccharides

All dried *L. japonica* were pulverized in a grinder to obtain a fine powder. The dried powder was mixed with deionized water at a ratio of 1:60 (w/v), and extracted in a water bath at 90 °C for 2 h. After the extracts were centrifuged at 10,000 rpm for 10 min, the supernatant was combined, concentrated and precipitated with ethanol at a final concentration of 40% (v/v), giving the crude *L. japonica* polysaccharides (LJP). The proteins were removed from the crude LJP using Sevag reagents (Navarini et al., 1999). Then, the crude LJP was fractionated on a anion exchange DEAE-cellulose column (1.6 × 60 cm) with double distilled water (ddH_2O) and NaCl aqueous solution at a final concentration of 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, 1.0 M and 2.0 M, giving the fraction of LJP1, LJP2, LJP3, LJP4, LJP5, LJP6, LJP7, LJP8, LJP9 and LJP10, respectively. The LJP3 was further purified on a Sephacryl S-500 column (1.0 × 80 cm) using ddH_2O as the eluent, giving a homogeneous fraction LJP-31. According to the references, determination of carbohydrate content, protein content, purity and molecular weight of LJP-31 were performed (Shao, Chen, & Sun, 2014; Zha et al., 2012).

2.3. Monosaccharide composition analysis

According to the reference reported by Zha et al. (2012), the monosaccharide composition was determined by a 7890A gas chromatographic system made in Agilent Technologies after LJP-31 was completely hydrolyzed and converted into alditol acetates.

2.4. Infrared spectroscopic analysis

For IR spectroscopy, samples were mixed with KBr, grinded, and pressed into a 1 mm pellets for the analysis. The IR spectra were recorded in the range of 400–4000 cm^{-1} on a Nicolet 67 spectrometer.

2.5. Glycosidic bond composition

The methylation reaction was performed to analyze the glycosidic bond composition of LJP-31 according to our previous reports (Shao, Liu, Chen, Fang, & Sun, 2015).

2.6. Cell line and cell culture

RAW264.7 cell was supplied by Professor Jian Liu (Hefei University of Technology, Hefei, China). The cells were incubated in DMEM medium containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator with atmosphere of 95% air and 5% CO_2.

2.7. Cell viability analysis

The effects of LJP-31 on the cell viability were determined by MTT assay. Briefly, RAW264.7 cells were seeded into 96-well plates at a density of 5×10^4 cells/well and cultured at 37 °C in a humidified incubator with 5% CO_2. After incubation for 4 h, these cells were treated with LJP-31 at final concentrations of 50, 100, 200 and 400 μg/mL for another 20 h. All LJP-31 were dissolved in DMEM medium. At the end of culture, the medium were removed and 5 mg/mL of MTT working solution were added to each well followed by incubation for 4 h at 37 °C. At last, 100 μl of DMSO were added to each well, and the absorbance was recorded at 570 nm on the Bio-Rad model 680 Microplate Reader (PA, USA).

2.8. Determination of NO, TNF-α, IL-1β, IL-6 and IL-10

The RAW264.7 cells were seeded into 96-well plates at a density of 1×10^6 cells/well and cultured at 37 °C in a humidified incubator with 5% CO_2. After incubation for 4 h, 20 μL DMEM medium in the presence or absence of LJP-31 (0, 50, 100 and 200 μg/mL) or LPS (5 μg/mL) were added to different well followed by incubation for another 24 h. The cell treated with LPS was used as the positive control. The cell cultured in DMEM in the absence of LJP-31 and LPS was used as the normal control. At the end of incubation, the conditioned media were collected and stored at −20 °C for analysis. The NO, TNF-α, IL-1β, IL-6 and IL-10 were determined using commercial kits according to the instruction of manufacturers.

2.9. Quantitative RT-PCR analysis

The RAW264.7 cells were cultured according to the description in the section of 2.8. The TRIzol reagent (Takara biotech Co., Ltd., Beijing, China) was employed to extract total RNA from the conditioned cells. The RNA was reverse-transcribed using iScript™ cDNA Synthesis kit according to the manufacturer's instructions. The quantitative PCR was performed in a reaction volume of 20 μL containing 10 μL 2 × iTaq™ Universal SYBR® Green supermix, 0.4 μL of each forward and reverse primer, 1 μL cDNA and 8.2 μL PCR grade sterile water. The thermal cycling condition was fixed as follows: initial denaturation at 94 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 6 s. The cycle threshold (Ct) values were normalized to the expression of GAPDH.

2.10. Western blot analysis

After the RAW 264.7 cells were cultured in a 6-well flat-bottomed tissue culture plate at the density of 5×10^6 cells/well for 4 h, these cells were stimulated with LJP-31 at a final concentration of 0, 50, 100 and 200 μg/mL for 2 h. The cells treated with LPS at the concentration of 5 μg/mL were used as the positive control. At the end of incubation, all conditioned cells were