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Structural identification and immunostimulating activity of a *Laminaria japonica* polysaccharide

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ABSTRACT

In the present study, a new water-soluble polysaccharide (LJP-11) was obtained from *Laminaria japonica* by anion exchange DEAE-cellulose chromatography and Sephacryl S-500 chromatography. The average molecular weight of this polysaccharide was estimated to be about 2.89×10^6 Da by high performance liquid chromatography system. Gas chromatography showed that LJP-11 was composed of arabinose, mannose and glucose in a molar ratio of 1.0:1.16:6.33. LJP-11 contains a long backbone consisting of (1→4)-β-D-Glc*p*Ac, (1→4)-α-D-Glc*p*, (1→6)-β-D-Glc*p* and (1→3,6)-α-D-Man*p*. The 1-linked β-L-Ara*f* was linked to the C-6 of (1→3)-α-D-Man*p* and the sulfate group was attached to the C-4 of (1→6)-β-D-Glc*p*. Pharmacological tests displayed that LJP-11 can stimulate macrophages to release NO, IL-6, TNF-α and IL-10 as well as the up-regulation of their gene expressions, indicating LJP-11 has beneficial effects on immunostimulation. Moreover, LJP-11 exhibited 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. These results suggested that the activation of MAPK and NF-κB signaling pathways is one of the mechanisms responsible for the immunostimulating activity of LJP-11.

1. Introduction

The innate immune system is the body's strongest and most efficient structure to protect organs from the invasion of pathogens and antigens [1]. However, the dysfunction of immune system can result in a higher susceptibility and vulnerability to bacterial infections, which stand out as the most common cause of diseases [2]. In recent decades, evidences suggested that the modulation of innate immunity has a significant impact on the host's ability to respond to a diverse array of pathogens [3–6]. Up to date, several types of immunomodulators have been developed, including mammalian proteins and chemical synthetic drugs [7]. Although these drugs have beneficial effects on the enhancement of immunity, the side effect of high toxicity is still presented in clinical trials [8,9]. Thus, development of novel immunomodulators to enhance the innate immune response has been considered to be a more efficient and possibly more effective long-term healthcare strategy [10]. It is known that the innate immune system mainly contains macrophages, monocytes, granulocytes and humoral elements [11]. Among these, macrophages have been shown to exert a variety of complex biological functions, such as phagocytosis, surveillance, chemotaxis and destruction of targeted organisms [12], suggesting the activation of macrophages might be a promising strategy to resist diseases. In recent years, polysaccharides extracted from different origins have attracted a great deal of attention in the biomedical area due to their broad spectrum of therapeutic properties and low toxicities [13–15]. Moreover, some natural polysaccharides have been reported to display a variety of beneficial pharmacological effects via modulating macrophage immune functions [16,17].

Laminaria japonica, the most important economic seaweed for edible-medicinal use, has high nutritional values and health functions [18]. In the ancient literatures of traditional Chinese medicine, it has been recorded as an important therapeutic agent for phlegm elimination, detumescence and weight loss for more than 1000 years [18]. Recently, *L. japonica* polysaccharides (LJP) have been reported to exhibit prominent bioactivities of antioxidant, hepatoprotective, antitumor and anti-atherosclerosis [19–22]. It is well accepted that chemical structure is the determined factor

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for polysaccharide bioactivity [23,24]. In our previous studies, we found that the crude water-soluble LJPs were mainly composed of arabinose, mannose, galactose and glucose [18]. Beside, these polysaccharides showed good potential for reduction of serum lipids level and enhancement of serum antioxidant enzyme activities [18]. As far as our literature to be ascertained, there is limited reference that reported the immunostimulating activity and the corresponding chemical structure of *L. japonica* polysaccharides.

For these purposes, an immunostimulating *L. japonica* polysaccharide LJP-11 was isolated and purified from *L. japonica* in the present study. Based on this, the structure features were characterized. To study the possible mechanism responsible for the immunomodulatory action of this polysaccharide, the changes in MAPKs and NF-κB signaling pathways and their downstream molecule gene expressions were further investigated after macrophages were stimulated by LJP-11.

2. Materials and methods

2.1. Materials and chemicals

Laminaria japonica was purchased from Lianjiang county, Fujian province, China. D-mannose (Man), D-glucose (Glc), D-galactose (Gal), D-xylose (Xyl), L-arabinose (Ara), D-rhamnose (Rha), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (MO, USA). Fetal bovine serum (FBS), Dulbecco Modified Eagle 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 IL-6, IL-10 and TNF-α 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 obtained from 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. Extraction and purification of L. japonica polysaccharides (LJP)

The fresh materials of *L. japonica* (100 g) were extracted with 500 mL distilled water at 90 °C for 3 h, followed by a centrifugation at 12,000 rpm for 10 min. After centrifugation, the supernatant was concentrated on a rotary evaporator to 100 mL and then precipitated with ethanol (99.99%) at a final concentration of 40% (v/v) to obtain precipitate polysaccharides. The precipitate was re-dissolved in distilled water and Sevag method was performed to remove proteins [25], giving the crude LJP solution. The crude LJP solution was dialyzed with 3500 Da dialysis bags and freeze-dried to obtain crude LJP. The crude LJP (100 mg) was dissolution in 2 mL double distilled water and fractionated on DEAE-cellulose anion-exchange column with double distilled water, giving the fraction LJP-1. LJP-1 was further purified on a Sephacryl S-500 column (1.0 × 80 cm) with double distilled water in a speed of 0.2 mL/min, giving a homogeneous fraction LJP-11. The total carbohydrate content was determined by the phenol–sulfuric method [26] using glucose as standard. The sulfate content in LJP-11 was analyzed by barium chloride–gelatin method [27].

2.3. Determination of purity and molecular weight

The purity and molecular weight were measured by high performance liquid chromatography system (HPLC, 1260 Infinity, Agilent Technologies) equipped with TSKgel column G4000PW$_{XL}$ (7.8 mm × 30.0 cm) and G5000PW$_{XL}$ (7.8 mm × 30.0 cm) connected in series. The column temperature was fixed at 25 °C. The response time of 1260 refractive index detector was set at 4 s. The eluent was double distilled water at a flow rate of 0.5 mL/min. To obtain the molecular weight, T-series dextrans (T-2000, T-700, T-580, T-110, T-80, T-70, T-40 and T-11) were used as the standard.

2.4. Monosaccharide composition analysis

The dried LJP-11 (5.0 mg) was dissolved in 4.0 mL trifluoroacetic acid (TFA, 2.0 M) and hydrolyzed at 120 °C for 2 h in an ampere tube, followed by washing with methanol to remove TFA on a rotary evaporator. The hydrolyzate and monosaccharide standards (Man, Glc, Gal, Xyl, Ara and Rha) were successively reduced with $NaBH_4$ at room temperature for 4 h and acetylated with acetic anhydride–pyridine (1:1, v/v; 2 mL) at 100 °C for 1 h. According to the literature, the resulting alditol acetates were detected by gas chromatography [28].

2.5. IR and UV spectroscopy

LJP-11 (3.0 mg) was ground with KBr and pressed into a 1 mm pellet. The IR spectrum from 4000 to 400 cm^{-1} was recorded on a Nicolet 67 spectrometer. For UV spectrum, the aqueous solution of LJP-11 at 1.0 mg/mL was scanned with the wavelength from 190 to 400 nm on a UV–vis spectrophotometer.

2.6. Methylation analysis

Desulfation was performed to determine the position of sulfate group in LJP-11 according to reference method [29]. Methylation of desulfated LJP-11 and LJP-11 were carried out according to the method described by Needs and Swlvendran with minor modification [30]. The dried polysaccharides were reacted with powdered NaOH and CH_3I in DMSO. The methylated products were hydrolyzed in TFA at 120 °C for 2 h, followed by reduction with $NaBH_4$ and acetylation with acetic anhydride–pyridine at 100 °C for 1 h. The methylated alditol acetates were separated by the chloroform–water system and finally analyzed by GC–MS to determine the glycosyl linkage compositions in LJP-11 [31]. The partially methylated aditol acetates were identified from the retention time, characteristic iron fragments in MS spectra and quantified from the peak areas in the GC chromatogram. The sulfate position can be determined through comparing the differences in glycosyl linkages between the original polysaccharide and its desulfated sample.

2.7. NMR spectroscopy

One hundred milligrams of LJP-11 were prepared by exchanging lyophilized material twice with D_2O, followed by dissolving in 1.0 mL 99.99% D_2O. NMR spectra (^1H NMR, ^{13}C NMR, HSQC and HMBC) were recorded at 50 °C on a Bruker Avance AV400. Data processing was performed using standard Bruker XWIN-NMR software.

2.8. Cell line and culture

RAW264.7 cell, a murine macrophage cell line, was supplied by Professor Jian Liu (Hefei University of Technology, Hefei, China). The cells were cultured in DMEM supplemented with 10% fetal bovine