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Immune-enhancing activity of polysaccharides from *Hibiscus sabdariffa* Linn. via MAPK and NF-kB signaling pathways in RAW264.7 cells

Chun-Yan Shen, Wen-Li Zhang *, Jian-Guo Jiang *

College of Food Science and Technology, South China University of Technology, Guangzhou 510640, China

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ABSTRACT

Four novel heteropolysaccharides (HSP-I, HSP-II, HSP-III, HSP-IV) were isolated from blossoms of *Hibiscus sabdariffa*. Average molecular weights of the four polysaccharides were 8.7×10^3 Da, 1.4×10^4 Da, 4.4×10^4 Da and 1.4×10^5 Da, respectively. Glucuronic acid, rhamnose, mannose, glucose and galactose composed the main components of HSP-II at an approximate molar ratio of 1:9.89:4.64:1.99:7.65. (1 → 3)-linked glycosidic bond formed the main linkage type of HSP-II and its branch linkage types were (1→), (1 → 6), and (1 → 2)-linked glycosidic bonds. All fractions could significantly promote the proliferation of spleen cells induced by Con A and LPS. HSP-II showed greater immune enhancement potential than other fractions based on production of NO in RAW264.7 cells. HSP-II also dramatically increased secretion of TNF-α and IL-6, as well as mRNA expression of iNOS, IL-1β and IL-6 through activating phosphorylation of ERK, JNK, p38 and p65. HSP-II might be a novel immunomodulator by activating macrophages through MAPKs and NF-kB signaling pathway.

1. Introduction

Polysaccharides, a kind of natural macromolecular compound of aldose and (or) ketose linked by glycosidic bonds in branched or unbranched chains, are drawing much more attention owing to its low toxicity, few adverse effects and a variety of biological activities including antioxidant, antiviral, anti-fatigue, anticancer, hypoglycemic, anti-inflammatory and immune-enhancing (Balan, Rozewski, Zdanowski, & Skopinska-Rozewska, 2012; Jin, Zhao, Huang, & Shang, 2014; Joseph, Aravind, George, Varghese, & Sreelekha, 2013; Samavati & Manoochehrizade, 2013; Shen, Jiang, Yang, Wang, & Zhu, 2016; Xu et al., 2012; Zeng, Zhang, & Jia, 2014). Specifically, polysaccharide can exert immune-enhancement effects not only by improving host defense against pathogens, but also modulating adaptive immunity, thereby making it become one of the excellent candidates for screening immune-enhancing agents from natural products (Chen et al., 2010; Chen, Tan, & Chan, 2008; Kim et al., 2012).

Hibiscus sabdariffa Linn. (*H. sabdariffa*), a species in the family of *Malvaceae*, has been widely used as edible and medicinal resources nowadays in China. Moreover, its juice is a popular beverage and a herbal medicine in Thailand and claimed to be a thirst quencher, diuretic, gall stone disperser, antipyretic and cough reliever (Hansawasdi, Kawabata, & Kasai, 2000). Recently, a growing number of evidences and clinical trials proved that *H. sabdariffa* was effective in inhibiting mouse skin tumors and the angiotensin I-converting enzyme (ACE) *in vitro*, protecting liver and cardiovascular system, relaxing vascular smooth muscle, as well as antioxidant, anti-tumor, immunomodulating, antihypertensive, diuretic, lipid-lowering and weight loss (Alarcon-Aguilar et al., 2007; Alarcon-Alonso et al., 2012; Ali, Salih, Mohamed, & Homeida, 1991; Hirunpanich et al., 2006; Kang, Seok, Kim, Eun, & Oh, 2007; Ojeda et al., 2010; Tseng et al., 1997; Worawattananutai, Itharat, & Ruangnoo, 2014). Specifically, Fakeye et al. found that the residual water-soluble fraction and ethyl acetate soluble fraction of the dried calyx of *H. sabdariffa* possessed significant immunomodulatory activities in experimental animals (Fakeye, 2008).

Much attention has been focused on the edible pigment and activity of aqueous or alcohol extract of *H. sabdariffa* previously, however, few researches were involved in its polysaccharide which might be of great importance for its further application in drugs and nutraceuticals. In view of the facts, the present study seeks to extract the polysaccharide from *H. sabdariffa* (HSP) and evaluate its immune-enhancement activity. Furthermore, the structure-activity relationship was investigated and discussed to explain the observed bioactivity.

* Corresponding authors.
E-mail addresses: ZWLemily123@163.com (W.-L. Zhang), jgjiang@scut.edu.cn (J.-G. Jiang).

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2. Materials and methods

2.1. Plant materials

H. sabdariffa, collected from the Yunnan Province of China in October 2013, was identified by the corresponding author and shade dried. Then they were ground into fine powder with a laboratory mill kept in seals polyethylene bags at room temperature for future use.

2.2. Chemicals and reagents

LPS (from Escherichia coli 055: B5) and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Shanghai, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), Trizol Reagent and Penicillin G/streptomycin were purchased from GIBCO (Grand Island, NY). Mouse NO, IL-6 and TNF-α ELISA kits were purchased from Cusabio Biothch CO., Ltd. (Wuhan, China). Antibodies of rabbit GAPDH, rabbit NF-Kb P65, rabbit phospho-P44/42 MAPK (ERK1/2), rabbit phospho-SAPK/JNK and rabbit phospho-P38 were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals and solvents used in this study were of analytical grade.

2.3. Extraction, isolation and purification of HSP

Dried flowers of *H. sabdariffa* were crushed by a laboratory mill and then refluxed with 95% ethanol-water twice (2 h each time) to be defatted and remove unnecessary constituents. Then the residue was separated from the organic solvent by vacuum filtration and dried for future use. 100 g pretreated powder was refluxed with distilled water with a liquid-to-solid ratio of 30:1 three times at 100 °C for 3 h. Thereafter, the water extracts were combined and immediately filtered with a vacuum filtration device. The supernatant was collected and condensed to about 200 mL with a rotary evaporator under vacuum at 60 °C. The concentrated solutions of crude polysaccharides were mixed with equal volume of pretreated D354FD resin in water bath at 50 °C for 3 h and were stirred vigorously in the interval to be decolorized. After decoloration, the solution obtained above was mixed with four times the volume of dehydrated ethanol to obtain a final concentration of about 80% (v/v) and left overnight in a refrigerator at 4 °C to precipitate polysaccharides (Zhang et al., 2015). Then the HSPs were dissolved by distilled water to a concentration of 5 mg/mL, and deproteinized for several times with a quarter volume of Sevage solution which was prepared by mixing chloroform and butyl alcohol in a ratio of 4:1 (v/v) until there was no obvious white precipitate in the interface (Shen, Yang, Jiang, Zheng, & Zhu, 2017). Finally, the deproteinized solution was re-precipitated with four times of dehydrated ethanol as mentioned above thereby resulting in the further refined polysaccharides (HSP-C). The yield of HSP-C was calculated by the equation as follows:

$$\text{Yield } (\%) = \frac{\text{weight of HSP-C (g)}}{\text{weight of dried material (g)}}$$

Anion-exchange chromatography was used for further separation of HSP-C. 600 mg HSP-C was re-dissolved with 20 mL distilled water, centrifuged at 4000 rpm for 10 min, and then loaded onto a DEAE-Sepharose fast flow column. Then four fractions named as HSP-I, HSP-II, HSP-III and HSP-IV, respectively, were collected through an anion-exchange chromatography of DEAE-52 eluted with deionized water and different gradient of NaCl solutions including 0.1 M, 02 M and 0.3 M, respectively. At last, the four fractions were concentrated, dialyzed and lyophilized for further research.

2.4. Structure characterization

2.4.1. Components analysis

The polysaccharide contents of HSP-I, HSP-II, HSP-III and HSP-IV were determined by the method of phenol-sulfuric with some modification (Hu, Liang, & Wu, 2015). Meanwhile, Coomassie brilliant blue G-250 was used to quantify the soluble protein. The amount of protein can be determined based on the absorbance at 595 nm with bovine serum albumin (BSA) as a standard (Pustjens, Schols, Kabel, & Gruppen, 2013). Furthermore, amount of total uronic acid was detected at 520 nm through using solution of sulfuric acid/sodium tetraborate and 3-phenyl phenol (Malagoli et al., 2014).

2.4.2. Infrared spectrum analysis

Fourier transform infrared spectra was recorded on a fourier transform infrared spectrophotometer (Bruker, Ettlingen, Germany) in a frequency range of 4000–400 cm^{-1} to investigate the organic functional groups (You et al., 2013). Briefly, 2 mg of each sample (HSP-I, HSP-II, HSP-III and HSP-IV) was mixed with 200 mg spectroscopic grade potassium bromide (KBr) powder, ground and then pressed into 1 mm pellets for transmission infrared spectroscopy (Feng et al., 2015).

2.4.3. Purity and molecular weight determination

HSP-I, HSP-II, HSP-III and HSP-IV were diluted to 1 mg/mL, and 20 μL of which was injected each time. Sample solution was performed with Waters High Performance Liquid Chromatography (717Plus sample injector, 1525 pump; Waters) that equipped with Waters TSK G-5000 PW × L TSK and G-3000 PW × L gel column, eluted with 0.02 mol/L KH_2PO_4 (pH 6.0) solution at a flow rate of 0.6 mL/min and detected by a Waters 2414 Refractive Index Detector (Zhong, Jin, Lai, Lin, & Jiang, 2010). Dextrans of different molecular weights including 3300, 18,300, 100,000, 164,000, 236,000 and 333,000 Da were used to obtain the calibration curve which was Log MW (molecular weight) of standard dextrans on GPC against their elution volume (EV). Similarly, the molecular weights of HSP-I, HSP-II, HSP-III and HSP-IV were also determined based on the calibration curve under the same condition (Wu, Hu, Huang, & Jiang, 2013).

2.4.4. Monosaccharide composition

10 mg of HSP-II was dissolved with 4 mL of 2 mol/L trifluoroacetic acid (TFA) in a 5 mL of adapter tube. Then the adapter tube was sealed using an alcohol blast burner and kept at 110 °C for 6 h to make complete hydrolysis. The excess TFA was removed by codistillation with methanol under reduced pressure for three times. Then the hydrolyzed polysaccharide and standard monosaccharides (glucuronic acid, rhamnose, fucose, arabinose, xylose, mannose, glucose and galactose) were incubated with 10 mg of hydroxylamine hydrochloride and 1 mL of pyridine at 90 °C for 30 min. During reaction, vigorous vortex was conducted every 5 min. After natural cooling, the samples were mixed with 1 mL of acetic anhydride and reacted for another 30 min at 90 °C. Eventually, the acetate derivative was analyzed via a gas chromatography (GC) system fitted with a DP-1701 capillary column (30 m × 0.25 m × 0.25 μm, 180–220 °C at 2 °C/min, and then 220–250 °C at 5 °C/min).

2.4.5. Periodate oxidation-smith degradation

25 mg of HSP-II was carefully weighed, dissolved with 12.5 mL of distilled water and then reacted with 12.5 mL of $NaIO_4$