Food Chemistry 141 (2013) 946–953

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Structural characterization and immunostimulatory activity of a novel protein-bound polysaccharide produced by *Hirsutella sinensis* Liu, Guo, Yu & Zeng

Liang He [a], Pengfei Ji [a], Junwen Cheng [b], Yanbin Wang [b], Hua Qian [b], Weiqi Li [a], Xingguo Gong [a,*], Ziyu Wang [c]

[a] Institute of Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310027, China
[b] Key Laboratory of Biological and Chemical Utilization of Zhejiang Forest Resources, Institute of Biological Technology, Zhejiang Forestry Academy, Hangzhou 310023, China
[c] Department of Forest Biomaterials, College of Natural Resources, North Carolina State University, Raleigh, NC 27695-8005, USA

ARTICLE INFO

Article history:
Received 25 January 2013
Received in revised form 7 March 2013
Accepted 15 April 2013
Available online 25 April 2013

Keywords:
Hirsutella sinensis Liu, Guo, Yu & Zeng
Protein-bound polysaccharide
Structure
Immunostimulatory activity
Cytokines

ABSTRACT

HS002-II, a novel protein-bound polysaccharide with 44 kDa molecular weight, was fractionated from submerged cultures of *Hirsutella sinensis* Liu, Guo, Yu & Zeng by DEAE-Sepharose and Sephacryl S200 chromatography. Based on the results of infrared spectroscopy, high performance liquid chromatography, methylation, amino acid analysis, NMR spectroscopy and atomic force microscopy, the polysaccharide moieties of HS002-II mainly contained a long backbone of (1 → 3)-linked α-D-ribofuranosyl units (1 → 4)-linked α-D-xylopyranosyl units and (1 → 4)-linked β-D-glucopyranosyl units, which was substituted at C-6. The two branches were β-D-mannopyranosyl residues and β-D-galactopyranosyl residues terminated with α-L-arabinopyranosyl residues, respectively. HS002-II consisted of 57.9% polysaccharide and 42.1% protein with the existence of N-type carbohydrate–protein linkage. In terms of the pro-inflammatory cytokines assay (NO, TNF-α, IL-1β and NF-κB) using murine macrophages cell line (RAW264.7), HS002-II exhibited significant immunomodulatory activity by stimulating the IκB-NF-κB pathway.

1. Introduction

Natural medicinal mushrooms have been widely utilised for more than two thousand years as an edible and medical resource. As wild and natural fungi is rare and expensive, culture of the fungus in submerged fermentation to produce mycelium or broth in large quantities, has proved to be a promising way to meet the needs of human consumption and to reduce the pressure on natural resources of the species, which is in danger (Cui et al., 2007). More and more published data has highlighted that some exopolysaccharides or polysaccharide-protein complexes, secreted from mycelial biomass and culture broth, exhibited significant immunomodulatory effects, activation of lymphocytes, proliferative inhibition, radical scavenging and obvious anti-fungal activities (Fu, Tian, Cai, Liu, & Li, 2007; Lee, Cho, & Hong, 2009; Lin et al., 2008; Peng & Zhang, 2003).

Cordyceps sinensis or *Ophiocordyceps sinensis* is an entomogenous fungus belonging to *Ascomycota*, *Sordariomycetes*, *Hypocreales*, *Clavicipitaceae (Lindau) O.E. Erikss., Cordyceps (Fr.) Link* (Dong, Xie, Wang, Zhan, & Yao, 2009). It is popularly referred to as the Chinese Caterpillar Fungus or 'Dong Chong Xia Cao' (summer-plant, winter-worm) in Chinese. Most of studies have reported on the active polysaccharides or polysaccharide-peptide complexes in mycelium and liquid medium of *Cordyceps* fungi, which have been used to treat a wide range of conditions, especially the immunostimulation and antitumor activities (Koh, Yu, Suh, Choi, & Ahn, 2002; Wang et al., 2011). On the basis of morphological and molecular evidence, *Hirsutella sinensis* Liu et al. is currently considered as the correct anamorph of *C. sinensis* (Jiang & Yao, 2002). Some recent studies on chemical compositions and biological activities have been carried out on *H. sinensis*. A Chinese patent strain-RCEF0273 of *H. sinensis* has been identified to have similar chemicals, which can be regarded as the substitute of natural fruiting body (Yoon, Yu, Shin, & Suh, 2008). Several studies in China have indicated that cultured *H. sinensis* could be an effective treatment in chemotherapy-induced leukocytopenia, also as a radiation mitigator (Xun et al., 2008) or immunomodulator (Fu et al., 2012). However, to the best of our knowledge, little research has been made to investigate the structure and medicinal properties of exopolysaccharides isolated from the culture broth of *H. sinensis*.

Macrophages play a crucial role in the immune system by immunodefence, immunomonitoring and immunomodulation. It is reported that activated macrophages can defend against pathogen invasion by secreting pro-inflammatory cytokines, and

* Corresponding author. Tel.: +86 571 88206549; fax: +86 571 87798206.
E-mail address: kite006@126.com (X. Gong).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.04.053

releasing some inflammatory molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β or nitric oxide (Han et al., 2003). For these cellular events, the immune function would be realised via various recognition of receptors on the surface of macrophages, which then trigger several different signaling pathways including tyrosine kinases, protein kinase C, phosphoinositide-3-kinase (PI3 K)/Akt, mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) (Hwang et al., 2011). Therefore, an understanding of the signaling pathway of the exopolysaccharide from *H. sinensis* should contribute to its therapy mechanisms. Furthermore, such a specific immunostimulating property is closely associated with certain characteristic structures, due to molecular recognition between receptors and their cognate ligands in life sciences (Hinterdorfer & Dufrêne, 2006).

The main aim of this work was to elucidate the characterization of highly purified soluble proteo-heteroglycans, and evaluate their immunomodulatory effect in macrophage cell culture on cytokine release, which could help in further understanding the relationship between the structural properties and biological activities.

2. Materials and methods

2.1. Materials

H. sinensis Liu, Guo, Yu & Zeng HS002 was kindly provided by Professor Guo Lan-ying, Institute of Microbiology, Chinese Academy of Sciences. D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-galactose, D-xylose, L-arabinose, D-fucose, Lipopolysaccharide (LPS) and 3-(4,5-)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bovine serum albumin (BSA), Medium RPMI-1640, Trizol™ Reagent, IL-1β, NF-κB, TNF-α, iNOS and GAPDH were purchased from Gibco Invitrogen Co. Inhibitory κB-α (IκB-α), Nuclear factor-κB p65 were supplied by Bioworld Technology Co. (MN, USA). All other reagents were of analytical grade and purchased from local chemical suppliers in China.

2.2. Microorganism and growth medium

HS002 was maintained on potato dextrose agar (PDA) supplemented with 10 g/l wheat bran and 10 g/l silkworm pupa powder at 4 °C. The strain was first incubated on the same medium as used for the stock, at 18 °C for 40 d in a petridish, and was then transferred to 250 ml Erlenmeyer flasks. All batch fermentations were carried out in Erlenmeyer flasks (250 ml) with different volumes of fermentation medium (glucose 20 g, peptone 5 g, silkworm pupa powder 10 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g and distilled water 1000 ml). The flasks, after autoclaving at 121 °C for 25 min and cooling to 18 °C, were inoculated with mycelia from 30-day-old cultures.

2.3. Isolation and purification of exopolysaccharides

The supernatant (500 ml, 1 mg/ml) was collected by centrifugation at 3800*g* for 10 min and concentrated *in vacuo*. A combination method of papain enzymolysis and Sevag method were used for deproteinating the concentrated solution. The crude exopolysaccharide fraction was obtained through precipitation with 4 volumes of ethanol and desiccation *in vacuo*. The precipitate was redissolved in distilled water and applied to DEAE-Sepharose FF anion exchange chromatography column (2.6 × 30 cm, Pharmacia), eluting at a flow rate of 2 ml/min successively with distilled water and a gradient of 0–0.5 M NaCl (He, Wu, Cheng, Li, & Lu, 2010). According to the total carbohydrate content quantified by the phenol–sulfuric acid method, the yielded fractions were combined. The second fraction was further purified on a Sephacryl S200 column (2.6 × 100 cm, Pharmacia Co.). The column was eluted with 0.1 M NaCl at a flow rate of 24 ml/h. The second fraction was collected, dialyzed and lyophilized to obtain a white, purified *H. sinensis* polysaccharide (named HS002-II).

2.4. Homogeneity and molecular weight determination

The homogeneity and molecular weight of HS002-II were identified by high-performance gel permeation chromatography (HPGPC) with a Waters HPLC apparatus (UK6 injector and 510 HPLC pump, Waters, Milford, MA) equipped with a TSK-GEL G3000SWXL column (300 × 7.8 mm), a Waters 2410 RI detector and UV detector connected in series with a Millennium32 workstation. Detailed experimental conditions were as follows: concentration of HS002-II, 1 mg/ml, column and RI detector temperature, 35 °C (column temperature auto-control system); injection volume, 20 μl; mobile phase, 0.1 M sodium nitrate; flow rate, 0.6 ml/min; run time, 30 min, and integral pattern, force baseline to peak. Different weight-average molecular weights of standard dextrans, T-2000, T-500, T-70, T-40 and T-10, were prepared as 0.1% (w/v) solutions and 20 μl of solutions was injected in each run. A calibration curve was prepared from the known M_W Dextran T system standards.

2.5. Physicochemical analysis of HS002-II

The monosaccharide components of HS002-II were analysed by reverse-phase HPLC according to PMP (1-phenyl-3-methyl-5-pyrazolone) derivatization procedures with some modification (Lv et al., 2009). Briefly, 11 standard monosaccharides or hydrolyzed sample were dissolved in 0.3 M NaOH (75 μl) and a 0.5 M PMP (50 μl) solution before the derivatization. Then the mixture was neutralised by 75 μl of 0.3 M HCl solution and filtered through 0.22 μm membrane (Millipore, MA, USA). 10 μl of the resulting solution was injected into the RP-C_{18} column. The wavelength for UV detection was 245 nm. Elution was carried out at a flow rate of 1.0 ml/min at 25 °C. The mobile phase was a mixture of 0.05 M KH_2PO_4 (pH 10) – acetonitrile (83:17). Sugar identification was achieved by comparison with reference sugars (rhamnose, ribose, arabinose, xylose, mannose, galactose, glucose, fucose, galacturonic acid, glucuronic acid and N-acetyl-D-galactosamine).

The protein content was measured by the Folin-phenol Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) and amino acids in protein-bound polysaccharide were determined with Hitachi L-8900 amino acid analyzer using an acid hydrolysis and ninhydrin procedure (Kim, Park, Nam, Lee, & Lee, 2003). Linkages in the structure of protein-bound polysaccharide were detected by using UV scanning spectra with and without alkali treatment according to β-elimination reaction method (Hounsell, 1994).

2.6. Methylation analysis

Methylation of HS002-II was carried out by the method of Ciucanu and Kerek (1984) with minor modification. In brief, 5 mg of the sample was placed in a vacuum oven at 40 °C overnight in the presence of phosphorus pentoxide. Then it was dissolved in 2 ml of anhyd DMSO and sonicated completely. Afterwards, 0.6 ml NaOH–DMSO solution under nitrogen was added to the mixture, which was sonicated for at least 30 min. The derivatization was triggered by loading 1 ml of cold CH_3I dropwise until it was fully cooled. The resulting solution was allowed to react for 30 min in the ultrasonic bath and kept for more 30 min. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated sample