International Journal of Biological Macromolecules 47 (2010) 420–424

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Short communication

Structure elucidation and immunological activity of a novel polysaccharide from the fruit bodies of an edible mushroom, *Sarcodon aspratus* (Berk.) S. Ito.

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ARTICLE INFO

Article history:
Received 7 February 2010
Received in revised form 23 May 2010
Accepted 26 May 2010
Available online 4 June 2010

Keywords:
Sarcodon aspratus (Berk.) S. Ito.
Edible mushroom
Polysaccharide
Glucan
Immunological activity

ABSTRACT

A water-soluble polysaccharide with a molar weight of 4.3×10^5 Da, termed as HBP was isolated from the fruit bodies of an edible mushroom, *Sarcodon aspratus* (Berk.) S. Ito. Composition, methylation analysis, Fourier transform infrared spectroscopy, and nuclear magnetic resonance spectroscopy experiments were conducted to elucidate its structure. The results indicated that HBP is a glucan featuring a backbone of (1→6)-linked-β-D-glucopyranosyl residues, which occasionally branched at *O*-3 position on along the backbone and substituted by the side chains that consisting of (1→3)-linked-β-D-glucopyranosyl, (1→4)-linked-β-D-glucopyranosyl and non-reducing end β-D-glucopyranosyl residues. Immunological activity evaluation by H^3-thymidine incorporation method revealed that HBP can significantly stimulate the proliferation of the cultured mice spleen lymphocyte in a dose-dependent manner, thus, HBP is a promising potential immunomodulator that can be used as healthcare food or medicine against pathogens and tumors.

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1. Introduction

In recent years, polysaccharides from the fruit bodies of mushroom have drawn a great deal of attention in the area of biochemistry and pharmaceutical science due to their broad spectrum of therapeutic properties, including immune-stimulatory, anti-tumor, anti-inflammatory, antifungal, antidiabetic, antioxidant, and free radical scavenging as well [1–4]. Also, some of these natural polymers have been successfully used in clinical oncology to increase the effectiveness of chemotherapeutic preparations and reduce their side effects, such as Lentinan from *Lentinus edodes*, Schizophyllan from *Schizophyllum commune* and krestin from *Coriolus versicolor*. Large amount of experimental and clinical trials demonstrated that these mushroom derived polysaccharides could prolong the survival and improve the quality of life for cancer patients [5]. Therefore, discovering novel structurally and biologically polysaccharides from mushroom, especially those unexploited species has become a hot spot of great interest.

S. aspratus (Berk.) S. Ito. is a delicious edible and medicinal fungus distributed in Yunnan province of China, which is a Tricholomataceae fungus belonging to the Basidiomycetes, and is also called "Huzhangjun" in China because of its tiger palm like appearance. It contains mainly the polysaccharides [6], and exhibited significant antibacterial activities to *Penicillium citrinum* and *Aspergillus niger* for its enzyme degraded aqueous extraction. The polysaccharides from the fruit bodies can significantly elicit the release of tumor necrosis factor-α and nitric oxide of murine macrophages [7,8]. However, to our best knowledge, there is no publication documented about the structure characterization of the polysaccharide up to now. In the course of our seeking bioactive polysaccharides from the natural resources [9,10], the fruit bodies of *S. asparatus* was investigated chemically and biologically. Herein, we describe the isolation, structural elucidation and immunological activity of a water-soluble polysaccharide from the fruit bodies of *S. aspratus*.

2. Experimental

2.1. Materials and chemicals

Dried fruit bodies of *S. aspratus* were collected in Yunnan province of China in September 2006, and identified by professor Peng-Fei Tu. The voucher specimens (2006-10-01) were kept in the herbarium of Peking University Modern Research Centre of Traditional Chinese Medicines. Sepharose CL-6B was purchased from Amersham (Sweden). DEAE-cellulose was purchased from Pharmacia Biotech. Standard monosaccharides, T-series dextrans, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), and

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doi:10.1016/j.ijbiomac.2010.05.021

lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of grade AR.

2.2. General methods

UV–Vis absorption spectrum was recorded with a Shimadzu MPS-2000 spectrophotometer [11]. GC was performed on a Agilent 6890 N instrument equipped with a HP-5 column (30 m × 0.25 mm × 0.25 (m) and detected with a flame ionization detector (260 °C), the column temperature was increased from 170 to 215 °C in a rate of 2 °C/min then hold on 5 min. Gas chromatography mass spectrometry(GC–MS) was measured on a Finnigan Trace (GC–MS) instrument coupled with a DB-5 column (30 m × 0.25 mm × 0.25 (m), and at temperatures programmed from 160–250 °C at 5 °C/min and then hold on 17 min. The FT-IR spectra (KBr pellets) were recorded on SPECORD in a range of 400–4000 cm^{-1}. Total carbohydrate content was determined by the Dubois's method [11], using D-glucose as the standard.

2.3. Extraction and isolation of polysaccharide

The fruit bodies of *S. aspratus* (1.0 kg) were extracted with 95% EtOH (3 L) at 100 °C for 1.5 h to remove lipid. The supernatant was removed, and the residue was extracted with distilled water at 100 °C for 3 times (2 L × 3; 1.5 h each time). After the centrifugation (36 × *g* for 20 min, at 25 °C), the supernatant was concentrated 10-fold, and precipitated with 95% ethanol (1:4, v/v) at 4 °C for 12 h. The precipitate collected by centrifugation was suspended in distilled water to remove the protein by the Sevage method. After that the polysaccharide was exhaustively dialyzed against water for 2 days, the concentrated dialysate was precipitated with 4 volumes of 95% EtOH, followed by washing with absolute ethanol, acetone and ether, respectively to obtain the crude polysaccharide of *S. aspratus*.

A portion of the crude polysaccharides (8 g) dissolved in water (100 mL), was loaded on a DEAE-52 Cellulose chromatography column (5.0 cm × 70.0 cm), and eluted with a 10-step gradient of 0–2 M sodium chloride (1.5 L each step). Guided by the calorimetric total carbohydrate test using the phenol–sulfuric acid method, the 0.2 NaCl eluting fraction was collected, dialyzed, lyophilized, and purified by Sepharose CL-6B (2.6 cm × 100 cm) gel-permeation chromatographies eluted with water to afford a purified polysaccharide (HBP).

2.4. Determination of homogeneity and molecular weight

The homogeneity and molecular weight of HBP were determined by high performance liquid chromatography (HPLC) on an Agilent 1100 system equipped with a Lonpak S-805 column and an evaporative light scattering detector (ELSD) [12,13]. 10 μL of sample solution (1.0 mg/mL) was injected each run, with water as the mobile phase at a flow rate of 1.0 mL/min. The linear regression was calibrated with T-series dextrans standards (Mw 1000, 400, 70, and 10 kDa).

2.5. Composition analysis

The identification and quantification of the monosaccharides of HBP (10 mg) was achieved by GC analysis. HBP (10 mg) was hydrolyzed with 2 M TFA at 100 °C for 2 h [14]. The monosaccharides were conventionally converted into the alditol acetates as described previously [15,16] and were analyzed by GC. The absolute configuration of the monosaccharides were determined according to the method using (+)-2-butanol described by Gerwig et al. [17].

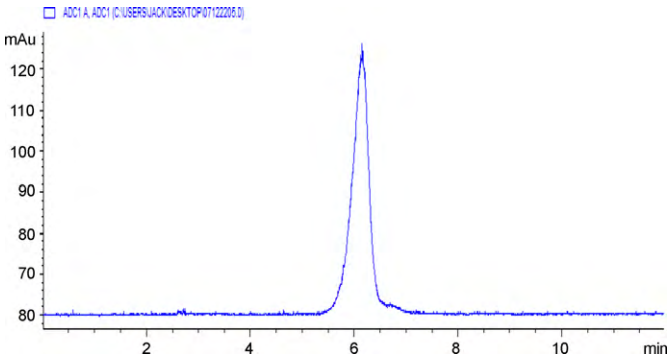

Fig. 1. HPLC profile of HBP.

2.6. Methylation analysis

HBP (10 mg) was methylated three times according to the literature [18]. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, reduced and acetylated as that described by Sweet et al. [19]. The partially methylated alditol acetates were analyzed by (GC–MS).

2.7. NMR studies

HBP (50 mg) was dried in vacuum over P_2O_5 for 72 h, and then exchanged with deuterium by lyophilizing with D_2O for three times. The deuterium exchanged polysaccharide was put in a 5 mm NMR tube and dissolved in 1.0 mL 99.96% D_2O. All 1D and 2D NMR spectra were obtained with a Bruker AM 500 spectrometer with a dual probe in the FT mode at room temperature. TMS was used as external standard for the ^{13}C NMR spectrum, and D_2O was used as internal standard for ^{1}H NMR spectrum.

2.8. Measurement of immunomodulating activity

The Balb/c male mice animals of 6–8 weeks old were purchased from Experimental Animal Laboratory of Peking University Health Science Center, Peking, China, bodyweight 20 ± 2 g.

The mice were sacrificed and their spleens were removed and passed through a sterilized iron sieve to obtain single cell suspension (SCS). The SCS was washed with PBS, and then the red blood cells were lysed with lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA, pH 6.8) for 3 min The spleen cells were washed, and then cultured in U-bottom well plates (2×10^5/well) in a volume of 200 μL per well in the presence of 10, 30, 100 μg/mL HBP and LPS (2.5 μg/mL), control (absence of sample) groups respectively. After a 3-day treatment, DNA synthesis was measured by H^3-thymidine (Du Pont) incorporation (1 μCi/well) in the final 6 h of the cultured period. The data were tested for statistical differences using Single-factor ANOVA, *t*-test.

3. Result and discussion

HBP was obtained from the fruit bodies of *S. aspratus* through a series of DEAE anion exchange cellulose and gel-permeation chromatographies. This polysaccharide showed a single and symmetrical peak on HPSEC, indicating its homogeneity. Its molecular weight was determined as 4.3×10^5 Da according to the retention time (Fig. 1). The total sugar content of HBP was determined to be 95.6% by using the phenol–sulfuric method. GC analysis indicated that it was composed of only glucose, and the absolute configuration test revealed that all monosaccharides in the glucan are of D configuration.

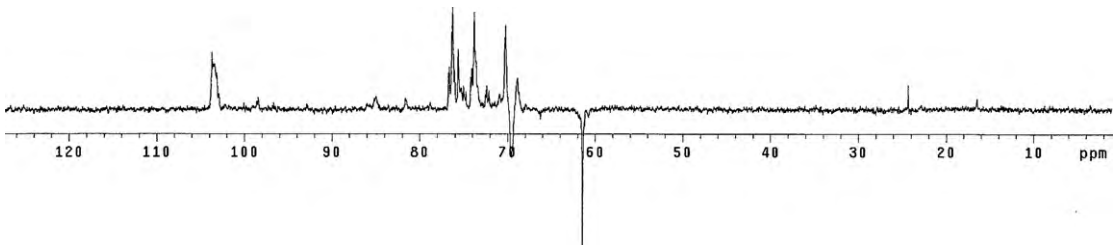

Fig. 2. DEPT spectrum of HBP.

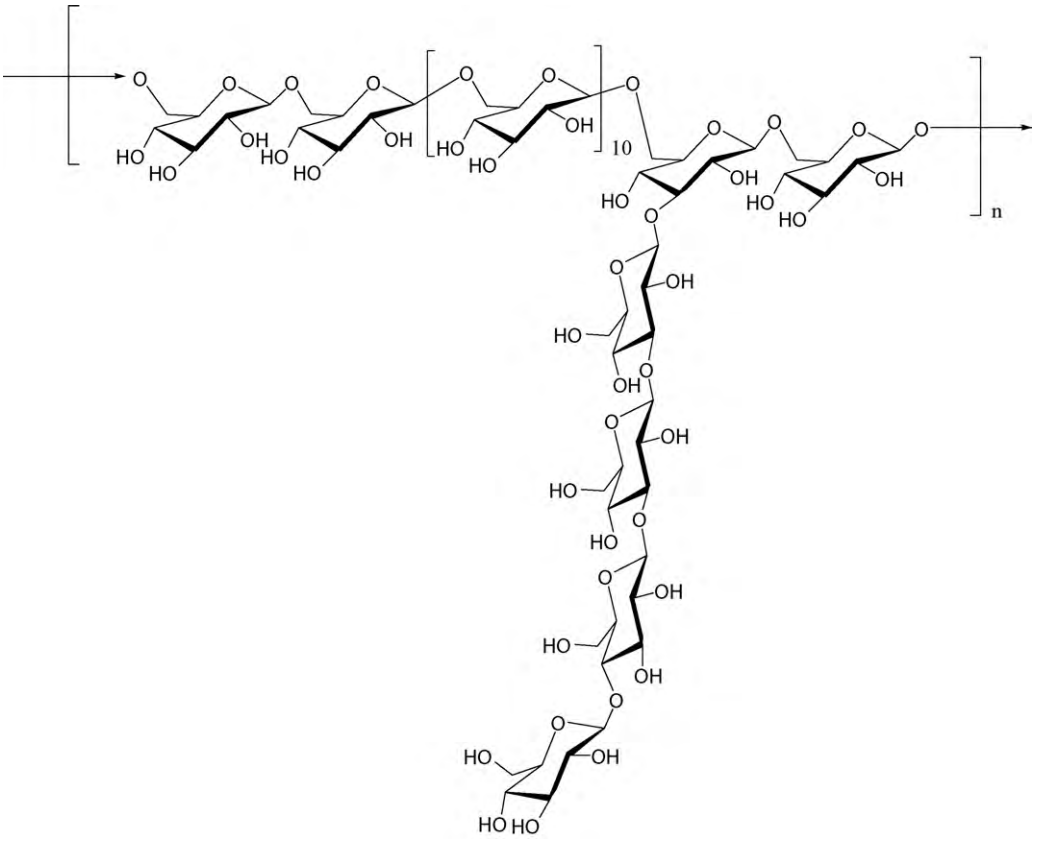

Fig. 3. Putative structure of HBP.

The strong band at 3418.8 cm^{-1} in the FT-IR spectrum of HBP was attributed to the hydroxyl stretching vibration of the polysaccharide, and that at 2923.3 cm^{-1} was due to the C–H stretching vibration absorption. Characteristically, the bands at 1000–1100 cm^{-1} suggested the presence of pyranose form of the glucosyl residue in the HBP [20].

Methylation analysis by GC–MS of HBP mainly produce five partially methylated alditol acetates, 1,5,6-tri-acetyl-2,3,4-tri-*O*-methyl glucitol (67.0%), 1,3,5,6-tetra-acetyl -2,4-di-*O*-methyl glucitol (4.9%), 1,3,5-tri-acetyl-2,4,6-tri-*O*-methyl glucitol (13.8%), 1,4,5-tri-acetyl-2,3,6-tri-*O*-methyl glucitol (4.4%), and 1,5-di-acetyl-2,3,4,6-tetra-*O*-methyl glucitol (9.9%). Based on these results, HBP correspondingly contained five glucosidic linkage forms, *i.e.,* (1→6)-linked glucosyl (Residue A), (1→3, 6)-linked glucosyl (Residue B), (1→3)-linked glucosyl (Residue C), (1→4)-linked glucosyl (Residue D), and non-reducing terminal (Residue E) (Table 1). These results suggested HBP to be a branched heteroglucan.

All the NMR data of HBP was assigned as complete as possible by the 1D and 2D NMR spectra, including ^{1}H, ^{13}C, H–H COSY, DEPT, HSQC, HMBC, NOESY, and TOCSY (Table 2). The signal at δ 4.54, δ 4.52, δ 4.74, δ 4.78, δ 4.53 in the ^{1}H NMR spectrum, were assigned to the anomeric protons of Residues A, B, C, D, E (Rds A–E), respectively, However, only one anomeric carbon signal at around δ 103.7 can be observed in the ^{13}C NMR spectrum, which confirmed that all the residues in HBP are of β configuration, and in its high magnetic field, C-3 signals of Rds B and C were both downshifted to δ 85.0; C-4 signal of Rd D was downshifted to δ 81.0 due to the α effect of glycosylation [21]. Same effect happened to r C-6 of Rds A and B that downshifted to δ 69.6, δ 68.8 respectively. Furthermore, the *O*-6-substitution of the residues foresaid can be further supported by the corresponding converse signals in DEPT spectrum (Fig. 2).

Table 1
GC–MS data for the methylated sugar moieties of HBP.

Residue no.	Methylated sugars	Type of linkage	Molar ratio
A	2,3,4Me3-Glc	1,6-Linked Glcp	13.6
B	2,4-Me2-Glc	1,3,6-Linked Glcp	1.0
C	2,4,6-Me3-Glc	1,3-Linked Glcp	2.8
D	2,3,6-Me3-Glc	1,4-Linked Glcp	0.9
E	2,3,4,6-Me4-Glc	Terminal Glcp	2.0

Table 2
^{1}H NMR and ^{13}C NMR chemical shifts of HBP[a].

Glycosyl residue	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a, H-6b/C-6
→6)-β-D-Glcp-(1→	4.54	3.32	3.73	3.48	3.63	3.90, 4.22
A	103.7	73.7	74.0	70.3	75.0	69.6
→3,6)-β-D-Glcp-(1→	4.52	3.42	3.76	3.54	3.44	3.90,4.22
B	103.7	73.7	85.0	68.7	75.9	68.8
→3)-β-D-Glcp-(1→	4.74	3.35	3.76	3.54	3.74	3.86,3.76
C	103.7	74.0	85.0	68.7	75.4	61.4
→4)-β-D-Glcp-(1→	4.78	3.48	3.50	3.78	3.44	3.92
D	103.7	73.7	75.8	81.0	75.9	61.4
β-D-Glcp-(1	4.53	3.34	3.50	3.63	3.48	3.90,3.73
E	103.7	74.1	76.0	68.9	73.6	61.4

[a] Measured in 500/125 MHz, δ in ppm.

The sequence of glycosyl residues was determined from NOESY studies followed by confirmation with HMBC experiments. Inter-residue NOESY correlations between Rd-A H-1/Rd-B H-6 (a, b), Rd-B H-1/Rd-A H-6 (a, b), Rd-C H-1/Rd-B H-3, Rd-D H-1/Rd-C H-4, Rd-E H-1/Rd-D H-4, together with the inter-residues long range carbon and proton correlations observed in HMBC spectrum, *i.e.*, Rd-A H-1/Rd -B C-6, Rd-B C-1/Rd-A H-6 (a, b), Rd-C C-1/Rd-B H-3, Rd-B C-3/Rd-C H-1, Rd-D H-1/C-4, Rd-C H-4/Rd-D C-1, Rd-D H-4/Rd-E C-1, Rd-E H-1/Rd-D C-4 revealed that Rd-A was attached to the 6 position of Rd-B, Rd-B to the 6 position of Rd-A, Rd-C was linked to the 3 position of Rd-B, Rd-D to the3 position of Rd-C, Rd-D to the 4 position of Rd-C, and the final Rd-E was attached to the 4 position of Rd-D.

In comprehensive composition analysis, methylation analysis and NMR experiments, it is, therefore, can be concluded that HBP is a glucan with a backbone structure of (1→6)-linked-β-D-glucopyranosyl residues, which was subsitituded at *O*-3 position occasionally on alone the backbone residues by the side chains, the branches were composed of (1→3)-linked-β-D-glucopyranosyl residues, (1→4)-linked-β-D- glucopyranosyl and terminated with non-reducing end β-D-glucopyranosyl residues (Fig. 3). This is the first time to report the detailed structure of a polysaccharide obtained from the fruit bodies of *S. aspratus*. Comparing with the branched glucans of (1→3) linked-β-D-glucopyranosyl or (1→6) linked-β-D-glucopyranosyl backbone that isolated from some famous medicinal mushroom such as *Pleurotus florida*, *Hericium erinaceus* and *L. edodes*, HBP has its own structure features, including the median size molar weight, minuet branching degree and the long side chains that is made up of 1,3-linked and 1,4-linked glucose residues, which are the important factors that will directly influence the conformation of a polysaccharides, and eventually influence the *in vivo* biological effect, so HBP can be used as a new structure model to clarify the interaction process of polysaccharides and immune cells. [3–5].

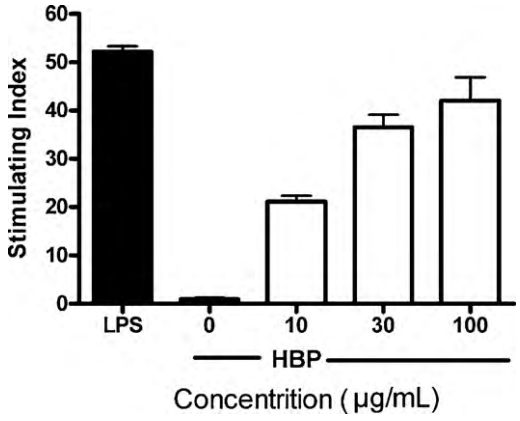

Fig. 4. Immunological activity of HBP.

As a crucial event in the activation cascade of both cellular and humoral immune responses, the lymphocyte proliferation was primarily selected to evaluate the immunological activity for HBP through the mice spleen lymphocyte cell proliferation experiment.

In cultured mice spleen cells, HBP was applied at 10, 30, and 100 μg/mL for 3 days. The DNA synthesis as revealed by the incorporation of [^{3}H] thymidine was determined in the treated spleen cells to quantify the proliferation rate. As shown in Fig. 4, the application of HBP can significantly induce the lymphocyte proliferation in a dose-dependent manner. Thus, HBP plays a key role in increasing the immunological activity, and can be seen as a promissing immunopotentiating agent for use in health-care food or medicine, while its structure–function relationship needs further research.

4. Conclusion

A good water-soluble polysaccharide with a trivial name HBP was isolated and purified from an edible mushroom *S. aspratus*. Its structure was characterized in detail for the first time. HBP exhibited significant effect of lymphocyte proliferation stimulation in a dose-dependent manner. This study provides a powerful foundation for the nutrition and medicine value for the extensive use of *S. aspratus*.

Acknowledgements

The authors thank Prof. Qin Li of Peking University Health Center, Analytical and Testing Center for her great support on the NMR measurement. We also thank Mrs. Hai-Yan Tao for her kind help in GC–MS analysis.

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