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

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ABSTRACT

A water-soluble polysaccharide (HCP) with a molecular mass of 6.7×10^5 Da determined by high performance size-exclusion chromatography (HPSEC), was isolated from the fruit bodies of *Sarcodon aspratus* (Berk.) S. Ito., an edible mushroom. HCP was elucidated as a liner glucan with a backbone structure of (1→6)-linked-α-D-glucopyranosyl residues by interpretation of the composition analysis, methylation analysis, periodate oxidation experiment, FT-IR, and NMR spectroscopy. Immunological activity evaluation using H^3-thymidine incorporation method revealed that HCP could significantly stimulate the proliferation of the cultured mice spleen lymphocyte in a dose-dependent manner. HCP might be a potential immunomodulator which can be used against pathogens and tumors in health-care food or medicine. This is the first report on the detailed structure elucidation of the polysaccharide from *S. aspratus*.

1. Introduction

During the last decades, many polysaccharides have been obtained from plants, bacteria, fungus, and marine organisms. As naturally occurring biological constituents, these high molecular weight polymers are highly appreciated for their multipurpose therapeutic properties, *i.e.*, antitumor, immune-modulating, anti-inflammatory, anti-pathogens, and antioxidant activities (Chen, Zhang, Qu, & Xie, 2008; Fu, Tian, Cai, & Li, 2007; Leung, Liu, Koon, & Fung, 2006; Lu, Cheng, Lin, & Chang, 2010; Luo, Xu, Yu, Yang, & Zheng, 2008). Nowadays, the studies on the structural elucidation and bioactivity evaluation of polysaccharides from those uninvestigated organism have aroused a general interest in the fields of chemistry, biology and medicine (Schepetkin & Quinn, 2006; Wu et al., 2006; Zhao et al., 2006.

Sarcodon aspratus (Berk.) S. Ito. is a delicious mushroom distributed in Yunnan province of China. It is a tricholomataceae fungus belonging to basidiomycotina and mainly composed of proteins and polysaccharides as chemicals components (Mou, 2000). In the past years, some papers reported that polysaccharide was the antitumor ingredient of this fungus (Mizuno et al., 2000). However, up to now, there is no documentation on the structure characterization of these polysaccharides, while structure clarification is the foundation of pharmacological mechanism elucidation, therefore, in the course of our seeking of bioactive polysaccharides from natural sources (Wu et al., 2006; Zhao et al., 2006), a bioassay-guided separation was carried on the crude polysaccharides of *S. asparatus*. Five pure fractions were isolated, among which the second biggest isolated fraction in amount HCP showed compared better immunocyte proliferation effect and can be identified as the effective ingredient of the crude polysaccharides of *S. asparatu.* Therefore, in this paper we will describe the detailed isolation, structural elucidation, immunological activity and structure-relationship analysis of this water-soluble polysaccharide.

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 specimen (2006-10-01) was 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 monosaccharide, T-series dextran, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). All other reagents were of grade AR.

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2.2. General methods

UV–Vis absorption spectrum was recorded with a Shimadzu MPS-2000 spectrophotometer. GC was performed on a Agilent 6890 N instrument equipped with a HP-5 column (30 m × 0.25 mm × 0.25 μm) and detected with a flame ionization detector (260 °C), the column temperature was increased from 170–215 °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 μm), and at temperatures programmed from 160 to 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 (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), 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 (3600 *g* for 20 min, at 25 °C), the supernatant was concentrated 10-fold, and precipitated with 95% EtOH (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 × 70.0 cm), and eluted with a 10-step gradient of 0–2 M sodium chloride (1.5 L each step). Guided by the colourimetric total carbohydrate test using the phenol-sulfuric acid method, the 0.4 NaCl eluting fraction was collected, dialyzed, lyophilized, and purified by Sepharose CL-6B (2.6 × 100 cm) and Sephadex G-100 (2.6 × 100 cm) gel-permeation chromatography eluted with water to afford a purified *S. aspratus* polysaccharide (HCP).

2.4. Determination of homogeneity and molecular weight

The homogeneity and molecular weight of HCP were determined by high performance liquid chromatography (HPLC) on an Agilent 1100 system equipped with a TSK-GEL G4000PWXL column and an evaporative light scattering detector (ELSD). 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 2000, 1000, 400, 70, and 10 kDa).

2.5. Composition analysis

The identification and quantification of the monosaccharide of HCP (10 mg) was achieved by GC analysis. HCP (10 mg) was hydrolyzed with 2 M TFA at 100 °C for 2 h (Parikh & Madamwar, 2006). The monosaccharide was conventionally converted into the alditol acetate as described previously (Johnes & Albersheim, 1972; Oades, 1967) and was analyzed by GC. The absolute configuration of the monosaccharide was determined according to the method using (+)-2-butanol described by Gerwig and co-workers (Gerwig, Kamerling, & Vliegenthart, 1979).

2.6. Methylation analysis

HCP (10 mg) was methylated three times according to the literature (Needs & Selvendran, 1993). 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, Shapiro, and Albersheim (1975). The partially methylated alditol acetate was analyzed by GC-MS.

2.7. Partial acid hydrolysis

HCP (100 mg) was hydrolyzed with 0.5 M TFA (100 ml) at 100 °C for 2 h. The products were evaporated to dryness and the residues dialyzed against distilled water for 48 h. The nondialysate residue was purified by a series of Sepharose CL-6B and Sephadex G-100 chromatographies. A pure fraction named HCP-P was isolated as the partial acid degraded product of the native polysaccharide.

2.8. Periodate oxidation

The polysaccharide (100 mg) was oxidized in 0.01 M $NaIO_4$ (200 mL) at 4 °C in the dark (6 days) and monitored spectrophotometrically. The sodium periodate consumption and the formic acid production were calculated according to the change of absorption at 223 nm and the method of 0.01 M NaOH titration respectively.

2.9. NMR measurements

HCP (50 mg) was dried in a 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 AM500 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.

2.10. 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 HCP, HCP-P and LPS (10 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

HCP was obtained from the fruit bodies of *S. aspratus* through a series of DEAE anion exchange cellulose and gel-permeation chromatography. This polysaccharide showed a single and symmetrical peak on HPSEC, indicating its homogeneity. Its molecular weight was determined as 6.7×10^5 Da according to the retention time (Fig. 1). The total sugar content of HCP was determined to be 96.8% by 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.

The strong band at 3421.3 cm^{-1} in the FT-IR spectrum of HCP was attributed to the hydroxyl stretching vibration of the polysaccharide, the one at 2925.7 cm^{-1} to the C–H stretching vibration absorption and the characteristic bands in the region 1000–1100 cm^{-1} suggested a pyranose form of the glucosyl residue in HCP (Luo et al., 2008).