J. Microbiol. Biotechnol. (1999), **9**(2), 163–167

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of an Acidic Polysaccharide from Fruiting Bodies of *Lyophyllum shimeji*

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Received: December 30, 1998

Abstract An acidic polysaccharide H-III was extracted from fruiting bodies of *Lyophyllum shimeji* with hot water. Acid hydrolysis and gas chromatography analysis showed that the polysaccharide was almost exclusively composed of glucose with a very small amount of mannose and galactose. Uronic acid of 8.36% was also detected in H-III. Its molecular weight was estimated to be 1×10^6 Da. By ^{13}C-NMR analysis, some repeating units of disaccharide were detected in the polymer H-III. The polysaccharide showed a strong mitogenic activity in a dose-dependent manner.

Key words: *Lyophyllum shimeji*, polysaccharide, mitogenic activity

Recently, much attention has been paid to Basidiomycetes for screening new biologically active molecules. Thus, many biologically active polysaccharides have been purified from fruiting bodies and the mycelia of various mushrooms and from the culture filtrate and have been characterized [13, 16, 18]. Polysaccharides from mushroom have been reported to show various kinds of biological activities including immunostimulating activity [7, 11, 12, 14, 17].

L. shimeji is an edible mushroom belonging to Agaricales Tricholomataceae. The mushroom is a good source of lysine and vitamin B1 [Kikkoman Co. Ltd. 1998. *In* Glossary of Japanese Foods and Related Items. S5. (http://www.kikkoman.co.jp/world/cookbook/glossary/S5.htm)]. However, the composition of the mushroom has not yet been studied in terms of polysaccharides. We extracted an acidic polysaccharide from the fruiting bodies of *L. shimeji* with hot water and characterized it.

Materials and Methods

Extraction, Fractionation, and Purification of Water-Soluble Polysaccharide

The cultivated *L. shimeji* was dried and ground with a blender. Milled mushroom (1.2 kg) was extracted 4 times with 10 volumes of 80% methanol at room temperature, to remove the low molecular weight compounds. The residue was extracted 3 times with hot water (3 l) at 100°C for 5 h. The extracts were combined and centrifuged at 10,000×g for 20 min. The polysaccharide was precipitated with 4 volumes of ethanol from the supernatant and dialyzed against distilled water. After freeze-drying the solution in the dialysis tube, fraction H-I (11 g) was obtained. H-I was dissolved in 5 mM phosphate buffer, pH 7.4, and loaded on a DEAE-cellulose (Cl^-) (Merk Art. 3201) column (6×15 cm).

General Analytical Methods

Total sugar content was measured by the phenol-sulfuric acid method [6]. Protein content was measured by Bradford's method [3] with Bio-Rad protein assay reagent (California, U.S.A.) using bovine serum albumin as a standard. Colorimetric measurement of uronic acid was done by the meta-hyroxydiphenyl reagent [1].

Determination of Molecular Weight

The molecular weight of H-III was estimated by a calibration curve made by HPLC on TSK-GMPW column (30 cm×7.5 mm i. d.) (Tosoh, Japan) with 0.1 M NaCl as an eluant. The eluate was monitored by a refractometric detector (Tosoh, Japan). Pullulans (Showa Denko, Japan) were used as a standard.

Monosaccharide Composition of Polysaccharide

The polysaccharide H-III (1 mg) was hydrolyzed with 1 ml of 2 M trifluoroacetic acid (TFA) for 2 h at 121°C in a screw-capped tube. Acid was removed by repeated evaporation. The hydrolyzate was analyzed by gas chromatography as alditol acetates. Gas chromatography was done with a Varian gas chromatography model GC 3400 fitted with flame-ionization detector (Varian, U.S.A.), using SP-2330 fused silica capillary column (30 m×0.32 mm i. d., 0.2 m film thickness) (Supelco, U.S.A.). The temperature program used was: 200°C for the first 2 min then 4°C/min up to 250°C, which was then held for 10 min.

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^{13}C Nuclear Magnetic Resonance Spectroscopy

The spectrum of H-III (40 mg) was recorded on a JEOL GSX 270 FT NMR spectroscopy (Jeol, Japan) at ambient temperature.

Assay of Mitogenic Activity

The lymphocytes obtained from a mouse spleen were put into a 96-well microplate (200 μl/well) at 1×10^6 cells/ml. Polysaccharide H-III and laminarin (Sigma, U.S.A.), which were dissolved in 0.85% NaCl, were added into the lymphocyte culture at the final concentrations of 1, 10, and 100 μg/ml. Cultures were pulsed with ^{3}H-thymidine (1 μCi/well) for a final 18 h of the 72 h incubation. The lymphocytes were harvested and the incorporation of the ^{3}H-thymidine was measured by a scintillation counter. The mitogenic activity was evaluated by the amount of ^{3}H-thymidine incorporated into the cultured lymphocytes. All experiments were done in triplicate and data are expressed as counts per min (cpm).

Results and Discussion

Fraction H-I was extracted from the fruiting bodies of *L. shimeji* with hot water (Fig. 1). When the crude polysaccharide was subjected to the DEAE-cellulose ion exchange chromatography, the polysaccharide was fractionated into neutral and acidic polysaccharides. Figure 2 shows the elution profile of the crude polysaccharide on ion exchange chromatography. The polysaccharide eluted with 5 mM sodium phosphate buffer, pH 7.4, was designated as the neutral polysaccharide (H-II). After elution of the unbound component with the buffer, the retained component was eluted with 1 M NaCl in the buffer and was designated as the acidic polysaccharide (H-III) (Fig. 2).

Further structural characterization was carried out with polysaccharide H-III, because H-III, but not H-II, showed strong mitogenic activity. The acidic polysaccharide showed almost a single symmetrical peak on the TSK-GMPW column of HPLC (Fig. 3), which indicated the homogeneity of H-III. The molecular weight of H-III was estimated to be about 1×10^6. Kiho *et al.* [10] reported that an acidic polysaccharide (TAP) was isolated with hot water from the fruiting body of *Tremella aurantia* belonging to Tremellaceae, and the molecular weight of TAP was found to be 1.5×10^6. Cho *et al.* [5] reported that acidic polysaccharides with a molecular weight of $1\text{-}3\times10^4$ was obtained from *Fomitella fraxinea*, which belongs to Polyporaceae. The molecular weights of schizophyllan and lentinan were reported to be 4.3×10^6 and 10^6, respectively [4, 20]. Thus, the molecular weight of polysaccharides obtained from mushrooms might be dependent on many factors such as the organism used, extraction solvent, and temperature.

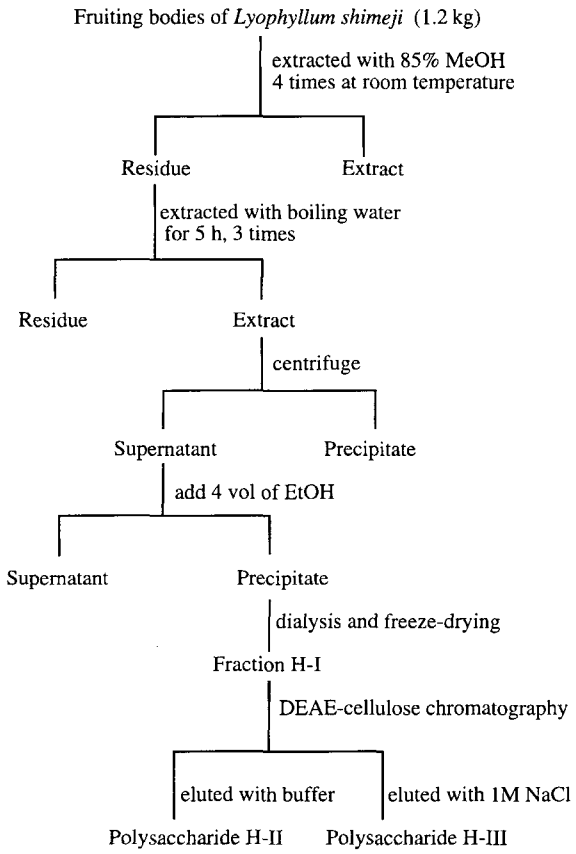

Fig. 1. Preparation scheme of polysaccharides from fruiting bodies of *Lyophyllum shimeji*; MeOH, methanol; EtOH, ethanol.

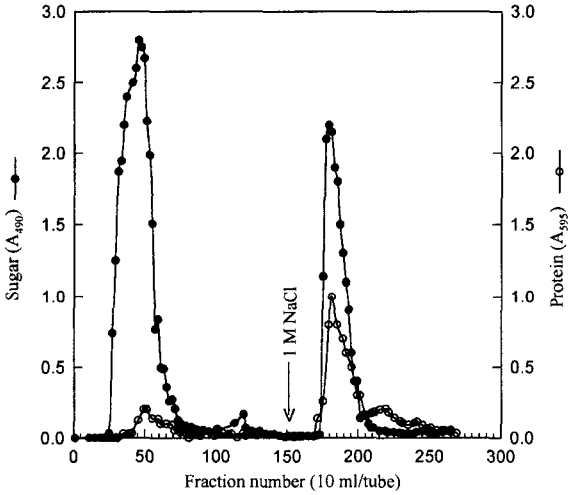

Fig. 2. Fractionation of H-I by DEAE-cellulose column chromatography.

A DEAE-cellulose column (6×25 cm) was equilibrated with 5 mM sodium phosphate buffer, pH 7.4. The solution of fraction H-I was added to the column. The column was washed with the same buffer to elute the unbound polysaccharide (H-II) and the retained polysaccharide (H-III) was eluted with 1 M NaCl in the same buffer. Sugar and protein were measured by the phenol-sulfuric acid method and the Bradford's method, respectively.

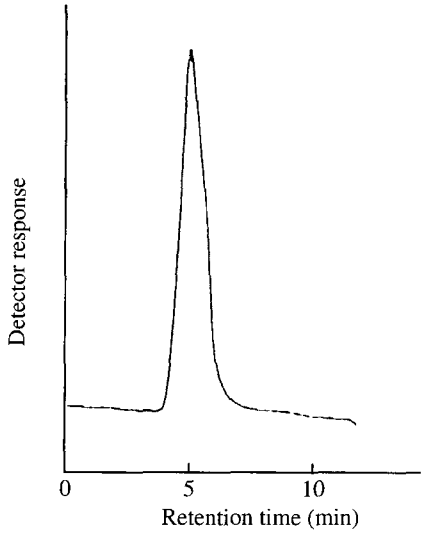

Fig. 3. Elution profile of polysaccharide H-III on TSK-GMPW column by HPLC.
Polysaccharide H-III was eluted with 0.1 M NaCl at a flow rate of 1.0 ml/min. The eluate was monitored by a refractometric detector.

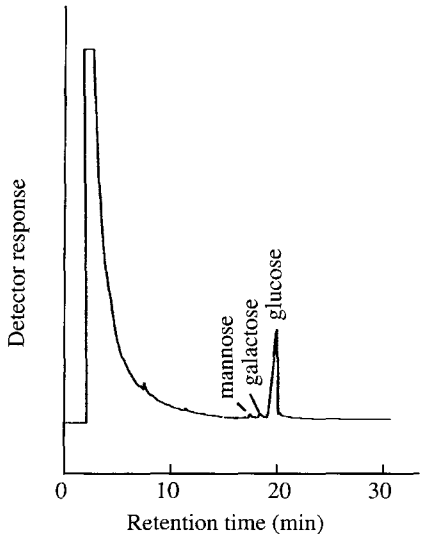

Fig. 4. Gas chromatogram of sugar alditol acetate from polysaccharide H-III.
The hydrolysate of the polysaccharide H-III was derivatized as the sugar alditol acetate. The derivative was analyzed by Varian gas chromatography fitted with a SP-2330 fused silica capillary column. A flame-ionization detector was used.

Polysaccharide H-III consisted of sugar and protein. Protein was measured as 1.23% in this fraction by Bradford's assay [3]. GC analysis showed glucose as the main monosaccharide (96.3%) with small proportions of galactose (2.2%) and mannose (1.5%) (Fig. 4). The uronic acid of 8.36% was also detected by the meta-hydroxydiphenyl reagent, thus the acidic property of H-III was thought to be mainly attributed to the acidic sugar. It was reported that an acidic polysaccharide of galactose and galacturonic acid was isolated from leaves of *Panax ginseng* [19]. It was also reported that an acidic polysaccharide, which was composed of mannose and glucuronic acid, was isolated from the body of *Tremella fuciformis* Berk [8].

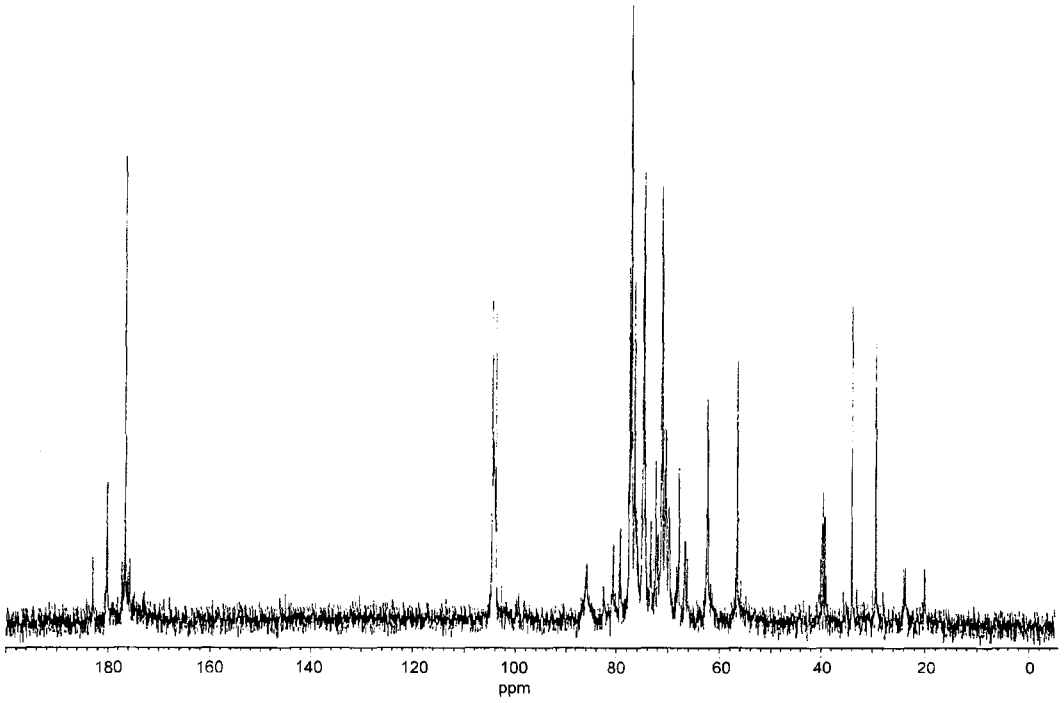

Fig. 5. ^{13}C NMR spectrum of polysaccharide H-III in D_2O at 25°C.

However, those polysaccharides were obtained from plants. Many acidic polysaccharides isolated from Basidiomycetes consisted of more than four sugars such as glucose, galactose, mannose, xylose, fucose, glucuronic acid, and galacturonic acid. Although H-III from *L. shimeji* has four different monosaccharides, the main sugar of H-III was glucose and uronic acid.

The structure of H-III was partially characterized by ^{13}C-NMR spectroscopy. The ^{13}C NMR spectrum (Fig. 5) showed signals for two anomeric carbons at 104 and 103.8 ppm, indicating that H-III may have repeating units of disaccharide. The spectrum also showed one carbonyl carbon at 176.5 ppm, which was assigned to C-6 of uronic acid. A signal of 56.5 ppm indicated the presence of an O-methyl group as carboxylic acid methyl esters. The signal of 61.8 ppm was assigned to the unsubstituted C-6 of glucose [2, 9]. Additionally, a substituted C-3 (86.3 ppm) signal was detected in the spectrum. The signal of 71 ppm might be assigned to the substituted C-6. The signals at about 180 ppm, 29.5 ppm, and 34.0 ppm may result from a peptide of H-III. The DMSO of an internal standard gave a signal of 39.9 ppm. The detailed structure and linkage type between monosaccharides in the repeating units of disaccharide remains to be elucidated.

H-III showed the mitogenic activity in a dose-dependent manner (Fig. 6). H-III treatment of murine lymphocytes caused about a 10-fold increase in ^{3}H-thymidine incorporation compared to a control. However laminarin did not show the activity even at a concentration of 100 μg/ml. Ohtani *et al.* [15] reported that an acidic polysaccharide composed mainly of rhamnose and glucuronic acid showed mitogenic activity. It has been reported that the antitumor activity of polysaccharides such as schizophyllan was mediated through immunopotentiation of the host [19].

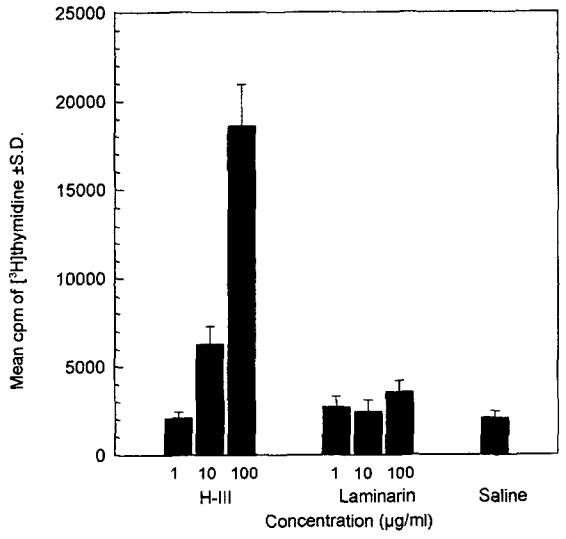

Fig. 6. The mitogenic activity of polysaccharide H-III.
The murine lymphocytes were incubated with polysaccharide solution for 72 h. The culture was pulsed with ^{3}H-thymidine and the incorporated thymidine was measured by a scintillation counter. All experiments were done in triplicate.

In general, the relationship between polysaccharide structure and its mitogenic activity is not yet clear. In case of glucan, however, the molecular weight seems to be an important factor for mitogenic activity. The structure of schizophyllan is similar to laminarin but its molecular weight is 1×10^{6} Da [19]. The molecular weight of laminarin is about 1×10^{4} Da. The acidic polysaccharides from *Fomitella fraxinea* also require a higher molecular weight for the activity [5].

In this study, we described that an acidic polysaccharide extracted from fruiting bodies of *L. shimeji* was composed of mainly glucose and uronic acid and the polysaccharide showed a strong mitogenic activity.

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