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Note

A β-D-glucan from the sclerotia of *Pleurotus tuber-regium* (Fr.) Sing

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Abstract

An alkali-soluble polysaccharide (Hunai polysaccharide, **1**) from the fruit body of *Pleurotus tuber-regium* (Fr.) Sing was shown to be homogeneous by gel permeation chromatography and its molecular weight was $\sim 4.3 \times 10^5$. Complete acid hydrolysis, periodate oxidation, Smith degradation, methylation, FT-IR and ^{13}C NMR analysis, complex formation with Congo Red, indicated that **1** has a β-(1→3)-linked D-glucopyranosyl backbone with a single β-D-glucopyranosyl group at O-6 of every third glucose residue.

Keywords: *Pleurotus tuber-regium*; Polysaccharide, structural studies; (1→3)-β-D-Glucan

1. Introduction

Pleurotus tuber-regium (Fr.) Sing., commonly referred to as *Hunai* in China, is a fungus that belongs to the tricholomataceae basidiomycetes and is found mainly in southern China (e.g., Yunnan Province), Myanmar (Burma), and some other south-eastern Asian countries. In some folk recipes it is used as a tonic and a medicine for the treatment of coughs and asthma. No report related to the chemical composition of the fungus has been found. Many biologically active polysaccharides have been isolated from various fungi [1–8], and some of these polysaccharides, such as schizophyllan and lentinan are used clinically for cancer therapy. The present work is on the isolation and structural study of an alkali-soluble polysaccharide (**1**) from the sclerotia of *P. tuber-regium*, which accounts for 60% of their dry weight.

2. Experimental

Materials.—Sclerotia of *P. tuber-regium* were obtained in Tengcong county, Yunnan province, China; D-glucuronic acid lactone and erythritol from Sigma Chemical Co.; other reagents are analytically pure products from China.

General methods.—Specific rotations were determined at 25 °C with a WZZ-2A automatic polarimeter (Shanghai, China); Ultraviolet spectra were recorded with a Shimazu UV-240 instrument, and infrared spectra were measured on a Nicolet Impact 420 spectrometer (OMNIC 2.1 software); The C, H, N values

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for the polysaccharide were determined by a LECO CHN 600 elemental analyzer; gas chromatography was performed with a Model SC-7 apparatus (Sichuan, China) equipped with a C_{60}-polysiloxane capillary column (13.2 m × 0.25 mm) and a flame ionization detector, and programmed from 170 to 250 °C at 3 °C min^{-1}; gas chromatography–mass spectrometry was on a HP-5988 instrument equipped with the same column as GLC.

Isolation of the polysaccharide.—The powdered sclerotia (80 g) were successively extracted three times with 80% aq EtOH and three times with boiling water (1.2 L). The residue was then suspended in 0.5 M NaOH (1.6 L) containing 0.05% $NaBH_4$ and stirred for 4 h at 4 °C. This was repeated three times. The extracts were combined, deproteinated by the method of Sevag [9], neutralized with AcOH and dialyzed (M_w cut-off 15,000) against running tap water (for 3 days) and then distilled water (for 1 day). The gelatinous dialyzate was successively washed with water, 95% aq EtOH, abs EtOH and acetone, and dried in a vacuum for 24 h at 35 °C (yield 49 g).

Total sugar content.—Total sugar content was determined as anhydroglucose by the modified phenol–H_2SO_4 method [10] using D-glucose as a reference.

Homogeneity and molecular weight.—The homogeneity and molecular weight of **1** (in Me_2SO, 10 mg mL^{-1}) were determined by GPC with a Waters HPLC apparatus equipped with a TSK G-5000 PW column (300 × 7.5 mm), and a 2010 Millennium Workstation was used for the calculation of molecular weight. The polyoxyethylene standards (SE-150, SE-70, SE-8, SE-5, and SE-2) were used for the calibration curve.

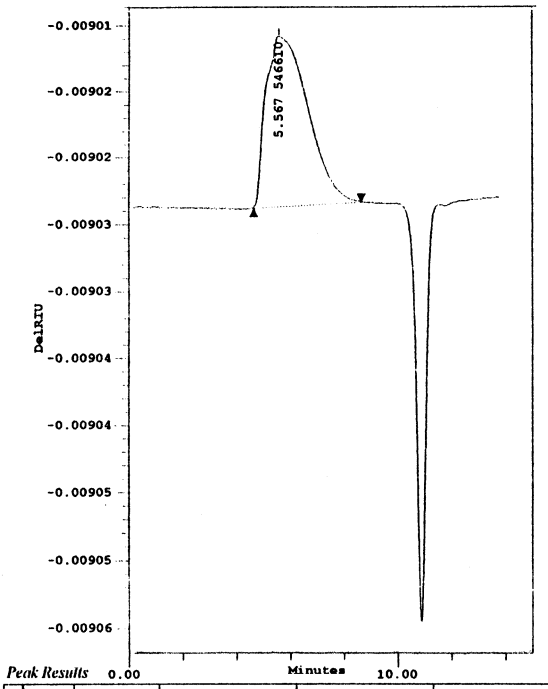
Analysis of components.—Hunai polysaccharide (**1**, 10 mg) was hydrolyzed with 1 M H_2SO_4 (2 mL) in a sealed tube for 15 h at 100 °C. After hydrolysis and neutralization ($BaCO_3$), the filtrate was taken to dryness and alditol acetates of the constituent sugars and of reference standards (D-glucose, D-xylose, D-mannose, D-galactose, D-rhamnose, D-fucose, D-arabinose, and D-Glucuronic acid lactone) were prepared [11] and subjected to GLC analysis.

Periodate oxidation and Smith degradation.—A suspension of **1** (50 mg) in 0.015 M $NaIO_4$ (50 mL) was kept at 10 °C in the dark with stirring. At intervals, the periodate consumption was determined by the Fleury–Lange method [12]. When the oxidation was complete, the excess periodate was decomposed by ethylene glycol and the formic acid liberated was then titrated with 0.01 M NaOH.

The oxidized product was dialyzed against running tap-water (2 days), and then distilled water (1 day). The non-dialyzable residue was filtered off and was dissolved in 1 M aq NH_4OH (20 mL), and then reduced with $NaBH_4$ (0.2 g) for 20 h at 20 °C. The excess $NaBH_4$ was decomposed by addition of 10% AcOH to pH 5.5. The mixture was dialyzed as described and the Hunai polyalcohol (**2**) was obtained as a white powder by filtration. Polyalcohol **2** (10 mg) was hydrolyzed with 88% formic acid (2 mL) for 3 h at 100 °C and then with 1 M H_2SO_4 for 8 h at 100 °C in sealed tubes (formic acid was removed by evaporation in vacuum before treatment with H_2SO_4). After neutralization ($BaCO_3$), the products were converted into their alditol acetates and examined by GLC against the acetates of glucitol, glycerol and erythritol.

Compound **2** (20 mg) was also hydrolyzed with 0.25 M H_2SO_4 (5 mL) for 20 h at room temperature, neutralized with 0.5 M NaOH, and then the solution was dialyzed against deionized water (1.5 L) for 3 days. The filtrate of the non-dialyzable fraction gave 15 mg of material on drying. The dialyzable fraction was deionized on a column of Amberlite IR-118(H^+) and IRA-410(OH^-) resins. Sugars in the concentrated eluant were examined by PC on Xinhua No. 2 filter paper using 10:4:3 EtOAc–pyridine–water and components were located using aniline phthalate.

Permethylation analysis.—Polysaccharide **1** (80 mg) was methylated three times by the method of Hakomori [13]. The mixture was dialyzed against running tap water and distilled water, and the nondialyzable fraction was filtered off, dissolved in $CHCl_3$, and the solution dried (anhyd K_2CO_3) and evaporated to dryness. The product showed no IR absorption for free hydroxyl groups. The perme-



Peak Results 0.00

#	Name	Ret Time (min)	MP (Daltons)	Mn (Daltons)	Mw (Daltons)
1		5.567	546610	194471	431343

Fig. 1. GPC chromatogram of **1** in Me_2SO at 25 °C with Me_2SO as the mobile phase (1 mL min^{-1}). The molecular weight was determined with a 2010 Millennium Workstation and polyoxyethylene as a reference.

thylated **1** was then hydrolyzed with 88% HCO_2H and 0.5 M H_2SO_4 as already described, the acid was neutralized ($BaCO_3$), and the solution was evaporated in a vacuum. The partially methylated sugars thus obtained were converted into their alditol acetates as described for GLC and GLCMS analysis.

Complex-formation of **1** *with Congo Red.*—The complexation of **1** with Congo Red was evaluated from the shift in the maxima absorption of the dye induced by the glucan in NaOH solutions at concentrations between 0 and 0.3 M according to the method of Ogawa et al. [14].

^{13}C NMR spectroscopy.—The spectra were recorded with a Varian-200 spectrometer operating at 50.31 MHz for **1** (30 mg) in 0.5 M NaOD (1 mL) at 25 °C with external Me_2SO-d_6 and 33,920 scans.

3. Results and discussion

An alkali-soluble polysaccharide (Hunai polysaccharide, **1**) was obtained from the sclerotia of *P. tuber-regium* by extraction with 0.5 M NaOH (three times) at 4 °C (yield 60%) on the basis of the dry sclerotia. The total sugar content was determined to be 93.1%.

Polysaccharide **1** is a white powder, $[\alpha]_D$ + 15° (*c* 0.2, 0.1 M NaOH), insoluble in cold or hot water, but soluble in Me_2SO and NaOH solution, with characteristic absorption for polysaccharide at 199 nm, but no absorption at 280 and 260 nm for protein or nucleic acid. Anal. C, 42.21; H, 6.17; N, 0.01.

GPC of **1** in Me_2SO gave a single peak (Fig. 1) and its weight-average molecular weight is $\sim 4.3 \times 10^5$ (with polyoxyethylene as a reference). The IR absorption of **1** at 890 cm^{-1} was indicative of the β configuration, and there was no absorption at 840 cm^{-1} for the α configuration.

Polysaccharide **1** was completely hydrolyzed with 1 M H_2SO_4 for 15 h at 100 °C. Examination of alditol acetates of the product and of the standards by GLC showed only glucose.

Oxidation of **1** with 0.015 M $NaIO_4$ at 10 °C was complete in 16 days; periodate consumed and formic acid liberated were 0.48 and 0.24 moles per mole of glucose residue, respectively. Periodate-oxidized **1** was reduced with borohydride. Hydrolysis of the resultant polyalcohol (**2**) and subsequent acetylation gave acetate derivatives of glycerol and of

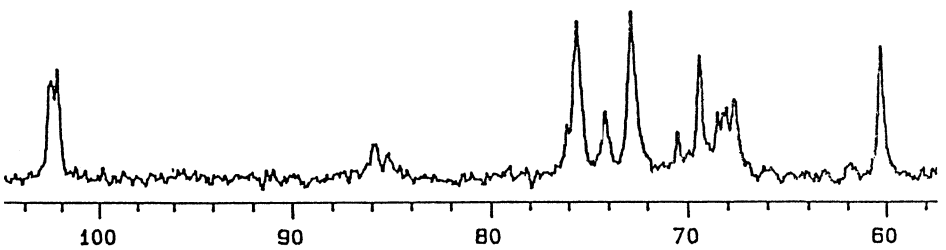


Fig. 2. ^{13}C NMR spectra of **1** in 0.5 M NaOD (30 mg mL^{-1}) at 25 °C with 33,920 accumulations.

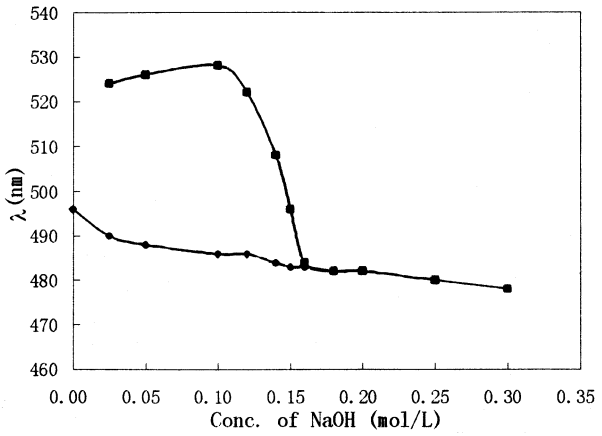


Fig. 3. Change in the absorption maximum of the Congo Red — complex **1** in various concentrations of NaOH solutions: ■ Congo Red + **1**; ◆ Congo Red.

glucitol, in 1:3 molar ratio, as estimated from their peak areas on GLC. After Smith degradation of a sample of **2**, only glycerol was detected on PC. The nonhydrolyzed fraction, recovered in 75% yield (based on **2**), was resistant to further oxidation. The results indicate that **1** contains a β-(1→3)-linked glucosyl backbone with a side-chain on every third glucose residue.

Permethylation was carried out with three Hakomori treatments, and the permethylated material was hydrolyzed, alditol acetates were prepared and examined by GLC and by GLCMS. The molar ratios of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucose were 1:2.06:1.21 (peak areas), indicating that **1** is a β-(1→3)-linked D-glucan with, on average, every third residue carrying a single D-glucose unit on its O-6 position.

For ^{13}C NMR spectroscopy, a solution in 0.5 M NaOD was used to improve the solubility of the glucan and to induce conformational change to give clear signals (sharp and with high intensity) [15–17]. The spectrum is shown in Fig. 2. The β configuration of the D-glucose residues was indicated by the C-1 resonance at 102.6 ppm, and branching at C-6 was confirmed by signals for O-substituted C-6 at 69.4 ppm and for unsubstituted C-6 at 60.3 ppm. The multiple and broad C-3 signal at 85.9 ppm could be ascribed to the presence of (1→3)-linked residues. Signals for other carbons are: 72.8 (C-2), 68.1 (C-4), and 75.6 ppm (C-5). A signal at 74.2 ppm may be ascribed to C-5′ in the branching glucosyl residues. All of these resonances agree with a O-6 branched β-(1→3)-D-glucan structure [6,18,19].

The helical conformation of **1** was indicated by its complex-forming ability with Congo Red [19]. The absorption maximum of Congo Red was shifted markedly to longer wavelength (530 nm) in 0.1 M aq NaOH in the presence of **1**, while at higher concentrations of NaOH (above 0.16 M), the absorption maximum was shifted or only slightly unshifted (Fig. 3). The specific rotation of **1** in 0.1 M aq NaOH was +15° (*c* 0.2), and changed to −2° when the concentration of NaOH solution was raised to 0.5 M, indicating a transition from an ordered conformation to a random coil with increase in the concentration of alkali [20].

Glucans of this type are reported to have antitumoral effects and to display immunological activity [21]. Such bioactivities as antioxidation, antiviral infection, and antitumoral activity of **1** are currently under investigation in our laboratory, and the results will be reported elsewhere.

References

[1] M. Akira, K. Mariko, *Carbohydr. Drug Des.*, (1997) 655–689.
[2] R. Srivastava, D.K. Kulshreshtha, *Phytochemistry*, 28 (1989) 2877–2883.
[3] G. Franz, *Planta Med.*, 55 (1989) 493–497.
[4] N. Ohno, I. Suzuki, T. Sasaki, N. Takasuka, *Carbohydr. Res.*, 47 (1976) 99–104.
[5] Y. Sone, M. Kakuta, A. Misaki, *Agric. Biol. Chem.*, 42 (1978) 417–425.
[6] T. Kiho, M. Sakushima, S. Wang, K. Nagai, S. Ukai, *Chem. Pharm. Bull.*, 39 (1991) 798–800.
[7] C. Gandon, M. Bruneteau, *Carbohydr. Res.*, 313 (1998) 259–263.
[8] Z.J. Pang, Y. Chen, M. Zhou, *Acta Biochim. Biophys. Sin.*, 31 (1999) 284–288.
[9] A.M. Staub, *Methods Carbohydr. Chem.*, 5 (1965) 5–6.
[10] Q. Dong, L.Y. Zheng, J.N. Fang, *Chin. Pharm. J.*, 31 (1996) 551–553.
[11] J.S. Sawardeker, J.H. Sloneker, A.R. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.
[12] P.F. Fleury, J. Lange, *J. Pharm. Chim.*, 17 (1933) 196–208.
[13] S.-I. Hakomori, *J. Biochem.*, 55 (1964) 205–208.
[14] K. Ogawa, J. Tsurugi, T. Watanabe, *Chem. Lett.*, (1972) 689–692.
[15] H. Saitô, T. Ohki, N. Takasuka, T. Sasaki, *Carbohydr. Res.*, 58 (1977) 293–305.

[16] H. Saitô, Y. Yoshioka, N. Uehara, J. Aketagawa, S. Tanaka, Y. Shibata, *Carbohydr. Res.*, 217 (1991) 181–190.
[17] S. Kitamura, T. Hirano, K. Takeo, H. Fukada, K. Takahashi, B.H. Falch, B.T. Stokke, *Biopolymers*, 39 (1996) 407–416.
[18] A. Misaki, M. Kakuta, T. Sasaki, M. Tanaka, H. Miyaji, *Carbohydr. Res.*, 92 (1981) 115–129.
[19] K. Tabata, W. Ito, T. Kojima, S. Kawabata, A. Misaki, *Carbohydr. Res.*, 89 (1981) 121–135.
[20] K. Ogawa, J. Tsurugi, T. Watanabe, *Carbohydr. Res.*, 29 (1973) 397–403.
[21] W.-M. Kulicke, A.I. Lettau, H. Thielking, *Carbohydr. Res.*, 297 (1997) 135–143.