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STRUCTURAL ANALYSIS OF THE ALKALI-SOLUBLE β-D-GLUCAN FROM THE FRUIT BODY OF *Grifola frondosa*

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ABSTRACT

The polysaccharide was extracted from the fruit body of *G. frondosa* with 10% sodium hydroxide. It was further fractionated, to afford *F7* (precipitated by neutralization of the extract) and *F8* (unprecipitated). *F7* and *F8* were found to be homogeneous β-D-glucans, and they had $[\alpha]_D$ +10° and +3.3° (*c* 0.3, 4% sodium hydroxide), respectively. Their molecular weights were determined by gel filtration to be 980,000 (for *F7*) and 830,000 (*F8*). From methylation study, Smith degradation, and enzymic hydrolysis, it was concluded that *F7* and *F8* are polysaccharides composed of a backbone of β-(1→3)-linked D-glucosyl residues and possessed of a single β-D-glucopyranosyl group joined to O-6 of almost every third D-glucopyranosyl residue of the backbone. However, the degree of branching of *F7* is slightly smaller than that of *F8*.

INTRODUCTION

The fruit body of *G. frondosa* has been used from antiquity as an edible fungus in Japan. Today, it is artificially cultivated for the market. As the fruit body was found to contain a polysaccharide similar to the β-(1→3)(1→6)-D-glucan which is known[1] to be an antitumor polysaccharide against Sarcoma 180, we attempted to extract the polysaccharide effectively from the fruit body and to study its chemical structure. Previously, we had reported[2] a study of the polysaccharide extracted from the fruit body with 10% zinc chloride. In continuation thereof, we now describe, for an alkali-soluble β-D-glucan present in the fruit body, a structural outline which was deduced from methylation study, Smith degradation, and enzymic hydrolysis.

RESULTS AND DISCUSSION

The defatted fruit-body was successively extracted with water, 10% zinc

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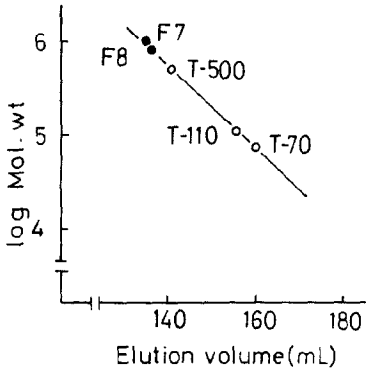

Fig. 1. Determination of the molecular weight of *F7* and *F8*.

chloride, and 2% sodium hydroxide. Each extraction was repeated until the supernatant liquor showed a negative 1-naphthol reaction (Molisch test). The residual material was then extracted with 10% sodium hydroxide for 1 h at 4°, and the extraction was repeated until the supernatant liquor showed a negative Molisch test. The Molisch-positive supernatant liquors were combined, and the base neutralized with acid, to precipitate a crude polysaccharide (*F7*). Another crude polysaccharide (*F8*) was then recovered from the supernatant liquor. *F7* and *F8* were individually purified through the copper–polysaccharide complex (yield, on the basis of the crude material, 54% for *F7* and 68% for *F8*). On complete hydrolysis with acid, both purified *F7* and *F8* gave D-glucose, together with small amounts of D-mannose and D-xylose. Their i.r. spectra showed an absorption near 890 cm^{-1}, characteristic of the β-D-glucosidic linkage.

F7 and *F8* were subjected to gel filtration with Sepharose CL-2B. Their elution profiles each showed a single peak, and the molecular weights were determined to be 980,000 (for *F7*) and 830,000 (for *F8*) from the elution volume (see Fig. 1).

F7 and *F8* were methylated by the method of Hakomori[3], and each methylation product was hydrolyzed with acid. The respective hydrolyzate was analyzed by g.l.c. and g.l.c.–m.s. of the derived alditol acetates. Three peaks that appeared in the chromatogram were identified as the corresponding alditols from 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucose by their mass spectra. From the areas under the peaks (which were obtained by g.l.c. analysis), the molar ratios of these compounds were determined to be 1.00:2.00:1.06 for *F7*, and 1.00:1.80:0.98 for *F8*. These results indicated that both *F7* and *F8* contain mainly (1→3) linkages, and have branching at O-6 of the (1→3)-linked D-glucopyranosyl residues.

The D-glucosidic linkages assigned were also supported by the results of periodate oxidation and Smith degradation. Each sample was oxidized with 0.01M sodium metaperiodate at 4° (see Fig. 2 for *F7*). After complete oxidation [periodate consumption, 0.48 (for *F7*) and 0.56 mol (for *F8*), and formic acid production,

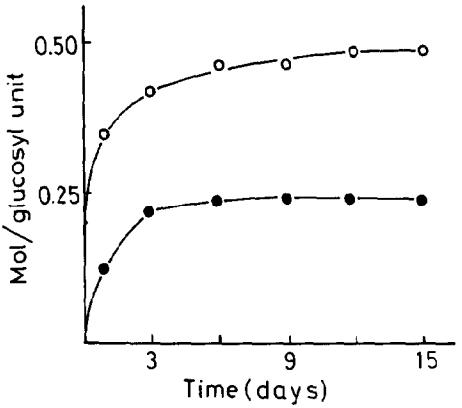

Fig. 2. Periodate oxidation of *F7* (○○○, periodate consumed; ●●●, formic acid produced).

0.22 (for *F7*) and 0.25 mol (for *F8*) per D-glucosyl residue], the oxidized *F7* and *F8* were reduced with sodium borohydride, to yield the corresponding polyalcohols. A portion of the resulting polyalcohol was completely hydrolyzed with acid. G.l.c. of the hydrolysis products as the corresponding alditol acetates indicated the presence of glycerol and D-glucose in the ratios of 1.00:3.30 for *F7* and 1.00:3.20 for *F8*. Smith degradation of the polyalcohols afforded a dialyzable product, l.c. analysis (at ~8 MPa) of which showed that it was glycerol. Nonhydrolyzed material (*F7-1* and *F8-1*, respectively) was resistant to further oxidation.

F7, *F7-1*, *F8*, and *F8-1* were treated with exo-(1→3)-β-D-glucanase; D-glucose and gentiobiose were liberated from *F7* and *F8*, and only D-glucose from *F7-1* and *F8-1*. When *F7* and *F8* were completely hydrolyzed by the enzyme, the reducing powers were 69 and 71% (of the total sugar), respectively. The ratios of D-glucose and gentiobiose liberated were 2.5:1 for *F7* and 2.0:1 for *F8*.

These findings indicate that the branching of *F7* and *F8* occurs at O-6 of almost every third D-glucopyranosyl residue of the β-(1→3)-linked backbone, and that each side chain is composed of only one D-glucopyranosyl group. However, it seems that the degree of branching of *F7* is somewhat smaller than that of *F8*. The structures of *F7* and *F8* are very similar to that observed for a polysaccharide (*F4*) extracted with aqueous zinc chloride from this fruit body[2], although the molecular weights of *F7* and *F8* are very much larger than that of *F4*.

F7 and *F8* were insoluble in water, but soluble in 4% sodium hydroxide. When the alkaline solutions were made neutral with acid, *F7* was precipitated, but *F8* was not. Although, from the present results, the factors that cause *F7* to be insoluble in neutral medium are not clear, the degree of branching could be related to the solubility.

EXPERIMENTAL

General methods. — All evaporations were conducted under diminished pressure at 30–40°. Total sugars were estimated by the phenol–sulfuric acid method[4]. Reducing sugars were determined by the Somogyi–Nelson method[5]. Specific rotations were determined at 20° with a Union PM-201 polarimeter. Infrared spectra were recorded with a JASCO IRA-1 infrared spectrometer. Gas–liquid chromatography (g.l.c.) was performed in a Shimadzu GC-7A apparatus fitted with a flame-ionization detector. (*1*) The glass column (0.3 × 200 cm) was packed with 3% of Silar 10C on Uniport B (60–100 mesh), and programmed from 160 to 260° at 4°/min, with a gas flow-rate of 50 mL of nitrogen per min. (*2*) The glass column (0.3 × 100 cm) was packed with 3% of OV-1 on Chromosorb W (80–100 mesh), and programmed from 160 to 220° at 4°/min, with a gas flow-rate of 50 mL of nitrogen per min. Peak areas were measured with a Shimadzu C-R1A Chromatopac. Gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.) was conducted with a Hitachi model M-52 apparatus equipped with a glass column (0.3 × 100 cm) packed with 2% of OV-1 on Chromosorb W (80–100 mesh), and programmed from 160 to 220° at 5°/min, and mass spectra were recorded at an ionizing potential of 20 eV. Liquid chromatography (l.c.) at 7.84 MPa was performed in a JASCO TWINCLE apparatus equipped with a Shodex RI SE-11 differential refractometer as the detector. The stainless-steel column (0.46 × 25 cm) was packed with Lichrosorb-NH_2, and operated with a flow-rate of 2.0 mL of 70:3 acetonitrile–water per min. Peak areas were measured with a Shimadzu ITG-4A digital integrator.

Preparation of F7 and F8. — The defatted fruit-body (100 g) was successively extracted with water, hot water, 10% aqueous zinc chloride[2], and 2% sodium hydroxide solution. The residual material was then extracted with 10% sodium hydroxide for 1 h at 4°. The extraction was repeated until the supernatant liquor showed a negative 1-naphthol reaction (Molisch test). The Molisch-positive supernatant liquors were combined, and the base was neutralized with 3M hydrochloric acid under cooling. The precipitate deposited was collected by centrifugation, de-ionized by dialysis, and lyophilized (crude *F7*; yield 5.8 g). A polysaccharide which was not precipitated by neutralization was recovered from the supernatant liquor by lyophilization after de-ionization by dialysis (crude *F8*; yield 3.7 g).

Because the sugar content of each fraction was relatively low, each was purified through the copper complex. The crude *F7* and *F8* were individually dissolved in 100 parts of 4% sodium hydroxide, and to this solution was added 7% aqueous copper sulfate dropwise, with stirring. The resulting copper complex was collected by centrifugation, and the polysaccharide regenerated by the usual method (yield, on the basis of the crude material; 54% for *F7*, and 68% for *F8*). The specific rotations of purified *F7* and *F8* (*c* 0.3, 4% sodium hydroxide) were +10° and +3.3°, respectively. Both *F7* and *F8* gave, on complete hydrolysis with acid, D-glucose, together with small quantities of D-mannose and D-xylose, analyzed as the corresponding alditol acetates by g.l.c. (condition *1*).

TABLE I

PRODUCTS OBTAINED BY HYDROLYSIS OF METHYLATED *F7* AND *F8*, AND DERIVATIZATION

Alditol acetate corresponding to	*Relative peak-area*		*Primary mass fragments*
	F7	*F8*	
2,3,4,6-Tetra-*O*-methyl-D-glucose	**1.00**	**1.00**	205, 161, 117, 45
2,4,6-Tri-*O*-methyl-D-glucose	2.00	1.80	233, 161, 117, 45
2,4-Di-*O*-methyl-D-glucose	1.06	0.98	233, 189, 117

Gel filtration. — *F7* and *F8* (3 mg each) were dissolved in 0.5M sodium hydroxide (0.5 mL), and the respective solutions applied to a column (1.95 × 95 cm) of Sepharose CL-2B. The column was equilibrated, and eluted, with 0.2M sodium hydroxide (0.25 mL/min), and the effluent was collected in 1-mL fractions. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method. The column was calibrated with the following dextrans: T-500 (mol. wt. 487,000), T-110 (105,000), and T-70 (70,000), which are products of Pharmacia Fine Chemicals. From the elution volume (see Fig. 1), the molecular weight was determined to be 980,000 for *F7*, and 830,000 for *F8*.

Methylation analysis. — *F7* and *F8* (5 mg each) were methylated by the Hakomori procedure. The reaction mixture was dialyzed against running water for 3 days, and the nondialyzable fraction was dried. The methylation procedure was repeated until the product showed no absorption for free hydroxyl group in its i.r. spectrum. The methylated polysaccharide was hydrolyzed with 90% formic acid for 3 h at 100°, and then with 0.25M sulfuric acid for 15 h at 100°. The acid was neutralized with barium carbonate, and the neutral hydrolyzate was evaporated to dryness. The sugars thus obtained were converted into their alditol acetates[6] for g.l.c. analysis (condition *2*) and g.l.c.–m.s. analysis. The results are summarized in Table I.

Periodate oxidation and Smith degradation. — *F7* and *F8* (500 mg each) were oxidized with 0.01M sodium metaperiodate (200 mL) at 4° in the dark. At intervals, the periodate consumption was determined by the Fleury–Lange method[7], and, after reduction of the excess of periodate with ethylene glycol, the formic acid liberated was titrated with 0.01M sodium hydroxide. *F7* consumed 0.48 mol of periodate and released 0.22 mol of formic acid per D-glucosyl unit, and, for *F8*, 0.56 and 0.25 mol, respectively. After completion of the oxidation, the oxidized polysaccharide was dialyzed against running water for 24 h. The nondialyzable fraction was reduced with sodium borohydride (200 mg) for 24 h at room temperature, with continuous stirring. After decomposition of the excess of borohydride by addition of M acetic acid, the reaction mixture was dialyzed against running water for 2 days. A white powder of the polyalcohol was obtained from the nondialyzable fraction by lyophilization; yield, 290 mg for *F7*, and 210 mg for *F8*.

A portion (10 mg) of each polyalcohol was heated with 90% formic acid (1

TABLE II

THE RATIO OF D-GLUCOSE TO GENTIOBIOSE RELEASED FROM *F7* AND *F8* BY THE ENZYMIC HYDROLYSIS

Complete enzymic digestion	*Molar ratio*	
	D-*Glucose*	*Gentiobiose*
F7	2.5	1.0
F8	2.0	1.0

mL) for 4 h at 100°, and then with 0.25M sulfuric acid (1 mL) for 15 h at 100°. After neutralization of the acid with barium carbonate, the sugars were reduced with sodium borohydride, and analyzed by ~8-MPa l.c. D-Glucitol and glycerol were detected in the molar ratios of 3.30:1.00 for *F7*, and 3.20:1.00 for *F8*.

Another portion (50 mg) of each polyalcohol was subjected to hydrolysis with 0.25M sulfuric acid (50 mL) for 24 h at room temperature. The reaction mixture, after neutralization, was added to an equal volume of ethanol, to yield a precipitate (*F7-1* and *F8-1*, respectively). When the supernatant liquor was de-ionized with Amberlite IRA-410 (OH^-) resin, and subjected to l.c. analysis, only glycerol was detected. On the other hand, the precipitate (*F7-1* and *F8-1*) gave only 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol on methylation analysis, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol was not detected.

Enzymic hydrolysis. — *F7* and *F8* (100 mg of each) were incubated with exo-(1→3)-β-D-glucanase (EC 3.2.1.6)[8] in 200 mL of 0.05M acetate buffer, pH 4.8, at 37°, and the reducing power of the reaction mixture was monitored at intervals. The reducing power increased with the incubation time, and reached a maximum value of 69% (for *F7*) and 71% (for *F8*) (of the total sugar). After inactivation of the enzyme by heating for 10 min at 100°, each incubation mixture was analyzed by ~8-MPa l.c. D-Glucose and gentiobiose were detected in the ratio shown in Table II. *F7-1* and *F8-1* were similarly digested with the enzyme, and the digestion mixtures were analyzed at intervals by l.c. Only D-glucose was detected during the enzymic hydrolysis of both *F7-1* and *F8-1*.

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