Structural Analysis of a Neutral (1→3),(1→4)-*β*-D-Glucan from the Mycelia of *Cordyceps sinensis*

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The extracellular polysaccharide (**1**) extracted from the mycelia of *Cordyceps sinensis* with hot water was fractionated and purified by ion-exchange and gel-filtration chromatography. The structure was investigated using methylation and hydrolysis analysis, periodate oxidation, NMR spectroscopy, and reaction with *β*-D-glucanase, and the results indicated that this D-glucan consisted of a backbone composed of (1→3)-*β*-D-glucosyl residues and carried a single (1→4)-*β*-linked D-glucosyl residue. NMR and IR spectroscopic measurements showed that the sugar residues were *β*-glycosidically linked.

Cordyceps sinensis (Berk.) Sacc. of the family Clavipitaceae, is a fungus endemic to mainland China and is used as a traditional medicine after undergoing alcohol or aqueous extraction. The potential therapeutic applications of *C. sinensis*[1] include the treatment of renal disease[2] and cancer.[3] A number of bioactive constituents from *Cordyceps* species have been reported. These include cordycepin[4,5] and other antibacterial and antitumor adenosine derivatives,[6] ophicordin, an antifungal agent,[7] and L-tryptophan.[8] Recent reports have indicated that *Cordyceps sinensis* contains polysaccharides exhibiting antioxidant activity[9] and nucleosides that inhibit platelet aggregation.[10] There is a strong tradition in Japan from ancient times that Basidiomycetes belonging to the family Polyporaceae are effective against cancer. Ikekawa et al. have reported that a hot-water extract from *Lentinus edodes* inhibits the growth of solid-tumor Sarcoma 180 implanted subcutaneously in mice, and the activity is attributable to (1→3)-*β*-D-glucans.[11,12] Such antitumor glucans have also been obtained from a number of other sources, such as yeasts, fungi, bacteria, and plants.[13] Despite these reports, data on the water-soluble polysaccharides from *C. sinensis* are limited. In this paper we report the structure of a water-soluble polysaccharide (**1**) isolated from the mycelia of *C. sinensis*, which has not been studied previously in detail.

Extraction of the dried mycelia with hot water (100 °C) gave a polysaccharide that upon chromatography using fast-flow DEAE-Sepharose (Pharmacia) gave fraction I (eluted with 0.30−0.15 M NaCl) and that upon chromatography on DEAE-cellulose (Pharmacia) gave fractions II and III (eluted with water). Fraction I had an $[\alpha]_D^{25}$ of +36 (*c* 0.2, water), gave a single peak on gel filtration through Sephadex G-100 (Pharmacia), and was homogeneous under high-voltage paper electrophoresis. Gel filtration of the D-glucan on Sepharose CL-4B indicated a range of molecular weights from 10^4 up to $>10^5$, with a preponderance of material of high molecular weight. Ultracentrifugation analysis indicated the average molecular weight to be 13 620.

Complete acid hydrolysis of the polysaccharide gave only glucose (95 mg), and on Hakomori methylation,[14] followed by conventional methylation analysis, three peaks were detected including the 2,3,4,6-tetra-*O*-methyl, 2,4,6-tri-*O*-methyl, and 2,6-tri-*O*-methyl derivatives in a molar ratio of 1.0:8.0:1.2. Analysis of the methylated sugars was conducted by GLC of their alditol acetates.[15] Therefore, the results indicated a (1→3)-linked backbone with (1→4)-linked branches (Table 1). The glucan consumed 0.27 mol of oxidant per mol of D-glucosyl residue during 12 h, and almost no formic acid was liberated. The periodate-oxidation results confirmed branches of (1→4) linkages in the polysaccharide according to the results of methylation.

Table 1. Molar Ratio of the Hydrolysis Products of Methylated Native Glucans and Smith-Degraded Glucans from *Cordyceps sinensis*

O-methyl-D-glucose	deduced structural unit	molar ratio of glucans	
		native	Smith degraded
2,3,4,6-tetra-	nonreducing end-group (D-glucose)	1.0	1.0
2,4,6-tri-	→ 3)-Glc(1→	8.0	18.0
2,6-di-	→ 3)-Glc(1→ 4 ↑	1.2	

The insoluble glucan was submitted to periodate oxidation, borohydride reduction, and hydrolysis under mild conditions by heating with 0.5 M trifluoroacetic acid at 20 °C for 18 h (Smith degradation).[16,17] Upon Smith degradation of the glucans, 74% of the glucosyl residues survived. This procedure gave a product that could be precipitated with EtOH from an aqueous solution. Methylation of this product gave 2,4,6-tri-*O*-methyl and 2,3,4,6-tetra-*O*-methyl derivatives in a molar ratio of 18:1 (Table 1). These results were in agreement with a (1→3)-linked backbone chain. Detection of only D-glycerol but not D-glucosyl-glycerol in the soluble fraction confirmed the presence of single glucosyl groups as side chains.

The insoluble glucan obtained by Smith degradation of the neutral glucan was hydrolyzed by the exo-(1→3)-*β*-D-glucanase of Basidiomycete QM 806. The elution profile of this digest on Sephadex G-15 showed mainly glucose and a small proportion of intact glucan. Thus, the side chain of the glucan was represented by single (1→4)-linked D-glucosyl groups. The anomeric proton single at *δ* 4.86 in the ^{1}H NMR spectrum confirmed that the sugar residues were *β*-glycosidically linked,[18] which agreed with the presence of an IR band at 876 cm^{-1}.

Graded acid hydrolysis of the glucan gave five fractions, which were characterized conventionally (see Experimental Section). Fractions II−IV were designated as oligomers I−V. These oligomers were separately methylated by the

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Table 2. Structure of the Products of Partial Acid Hydrolysis of the Glucan from *Cordyceps sinensis*

	retention time (min) in column[a]		mole ratio of methylated sugars in graded-hydrolysis product					
O-methyl-D-glucose	1	2	I	II	III	IV	V	linkage indicated
2,3,4,6-tetra-	1.00	1.00	1.0	1.2	1.0	2.3	1.0	Glc(→
2,4,6-tri-	1.95	1.81	1.0	1.9			2.6	→3)-Glc(1→
2,6-di-	2.64	2.47				1.0		→3)-Glc(1→ 4 ↑

[a] Retention times are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on (1) an ECNSS-M column at 170 °C and (2) an OV-225 column at 170 °C.

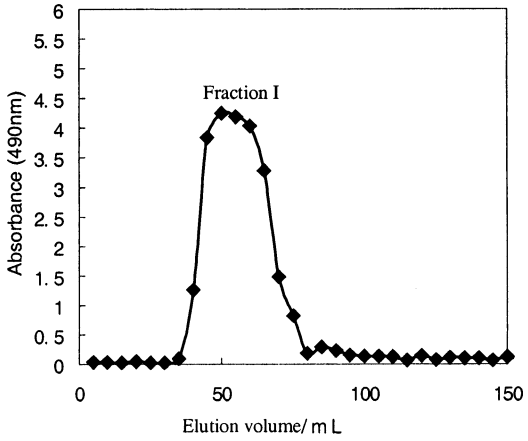

Figure 1. Fraction I from a chromatography column using fast-flow DEAE-Sepharose (eluted with 0.30−0.15 M NaCl).

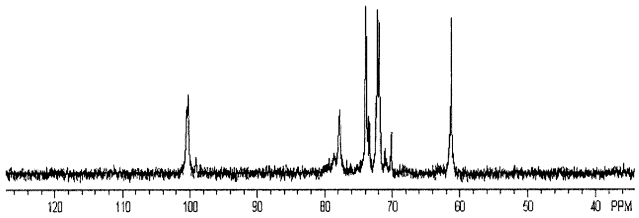

Figure 2. ^{13}C NMR spectrum (125 mHz) of the D-glucan from *Cordyceps sinensis* in D_2O at room temperature.

Hakomori method,[14] and each product was isolated by extraction with chloroform. The extract was washed with distilled water, dried (anhydrous sodium sulfate), and evaporated, and the final product was dried over P_2O_5 in vacuo. These methylated oligomers showed almost no hydroxyl group absorption band at 3600−3300 cm^{-1} in their IR spectra, indicating complete methylation. The methylated oligomers were analyzed[19,20] by the method described for methylation analysis of the glucan. Fractions I−V were shown to have the structures indicated in Table 2.

Confirmation of the high degree of branching of the glucan chain was provided by the ^{13}C NMR spectrum (Figure 2), which showed signals of almost equal intensity at δ 70.15 and 77.84, characteristic of C-4 unsubstituted and substituted glucosyl residues (Figure 2). The other resonances used to confirm the (1→3)-β-linked backbone of the glucan were assigned according to previous results.[21,22] The C-1 signal (a complex resonance due to heavy branching, centered at δ 102.29), the multiplicity of the signals, and the broad C-3 signal, which underwent a low-field shift to δ 86.39 since it was involved in the β-glycosidic linkage, supported the presence of linear (1→3), branched (1→3,1→4), and β-D-glucopyranosyl residues in the glucan. The glucosyl ring carbon atom signals were assigned at δ 73.94 (C-5), 72.21 (C-2), and 61.29 (C-6), respectively.

The results of methylation and hydrolysis analysis, periodate oxidation, NMR spectroscopy, and reaction with β-D-glucanase suggested structure **1** for the D-glucan of *C. sinensis*.

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. UV absorptions were measured on a JASCO V-530 UV/vis spectrophotometer. IR spectra were recorded with an Acculab 10 Beckman instrument with KBr pellets. NMR spectra were recorded on a Bruker 500 instrument. For ^1H NMR spectroscopy at 70 °C, the sample (10 mg) was repeatedly dissolved in D_2O (5 × 5 mL), and the solution lyophilized. The final freeze-dried sample was dissolved in 1 mL of 99.99% D_2O. For ^{13}C NMR spectroscopy at 50 °C, the sample (65 mg/mL) was dissolved in D_2O. GLC was conducted on a Packard Model 419 and a Hewlett-Packard Model 5713 gas chromatograph, each equipped with flame-ionization detector and columns of 1.3% of ECNSS-M on GC-Q (100−200 mesh) and 2.3% of OV-225 on GC-Q (100−200 mesh). GLC-MS was conducted with a Hewlett-Packard 5895 instrument, using a fused-silica capillary column (30 × 25 mm) coated with a 0.2 μm film of OV-1. The ionization potential was 70 eV, and the temperature of the ion source was 220 °C. TLC was done on silica gel 60 (E. Merck), using *n*-butanol−pyridine−water (6:4:3) with detection by anisaldehyde-sulfuric acid and aqueous 2% sulfosalicylic acid.[23] Ascending paper chromatography was performed on Whatman No. 1 and 3 MM paper with the following solvents: A, the upper layer of *n*-BuOH−EtOH−water (4:1:5); B, EtOAc−pyridine−water (8:4:1); and C, *n*-BuOH−pyridine−water (6:4:3). Sugars were detected by aniline hydrogen phthalate[24] and alkaline silver nitrate.[25] Evaporations were conducted under reduced pressure at 60 °C.

Fungal Material. The dried mycelia (1 kg of *Cordyceps sinensis*) used in this study were grown in western mainland China.[26] The material was supplied by Biotechnological Co., Ltd (Lishui, People's Republic of China) and identified by Dr. Shifeng Ni (Department of Botany, Nanjing University). A voucher specimen (DFC 0423) is deposited at the Department of Chemistry, Zhejiang University, People's Republic of China.

Extraction and Isolation. The fungal mycelia (dried, 1 kg) were extracted successively with light petroleum and EtOH (95%) to defat and decolorize and then extracted with hot water (5 L, 100 °C) with constant mechanical stirring at 4 h. The process of extraction was repeated until no further precipitate was obtained. Then the extract was added to three volumes of EtOH (95%), and the extracts were combined, then centrifuged for 30 min at 7200 rpm. The supernatant solution was diluted with EtOH (95%), and the resulting precipitate collected by centrifugation, washed five times with acetone, and dried. A solution of the polysaccharide in distilled water (250 mL) was diluted with EtOH, and the precipitate was collected by centrifugation (7200 rpm, 20 min). This process was repeated several times. A portion (300 mg) of the resulting polysaccharide (10 g) was then eluted from a column (2.6 40 cm) using fast-flow DEAE-Sepharose (Pharmacia), an anion-exchange resin, by 0.30−0.15 M NaCl (600 mL), followed by

passage over DEAE-Cellulose (Pharmacia), an anion-exchange resin using water (600 mL). Fractions (5 mL) were assayed for carbohydrate with phenol−sulfuric acid.[27] Three fractions were obtained, of which I (Figure 1) had $[\alpha]_D^{25}$ +36 (*c* 0.2, water), and constituted 41% of the polysaccharide, and was used in the subsequent studies. Gel filtration in borate buffer (pH 9.5), through a column (1.6 30 cm) of Sephadex G-100 (Phamacia), and high-voltage paper electrophoresis at 2 °C, in the same borate buffer, showed I to be homogeneous.

Determination of Molecular Weight. A solution of the polysaccharide (3 mg) in distilled water (0.5 mL) was applied to a column (2.6 × 80 cm) of Sepharose CL-4B. The column was equilibrated and eluted with distilled water at a fixed flow rate (10 mL/h), and the effluent was collected in 4 mL fractions. The carbohydrate content of each fraction was determined with the anthrone reagent.[28] The column was calibrated with standard dextrans T-200, T-70, T-40, and T-10 (Pharmacia).

Methylation Analysis. The thoroughly dried polysaccharide (12 mg) was methylated three times by the method of Hakomori.[14] The final product was dialyzed, and the solution freeze-dried: yield 7 mg. The product showed no IR absorption for hydroxyl groups. The methylated polysaccharide was treated with 90% aqueous formic acid (5 mL) for 11 h at 100 °C, and the product was isolated and hydrolyzed with 0.5 M sulfuric acid for 14 h at 100 °C. The methylated sugars were converted into their alditol acetates[29] and analyzed by GLC. The results are shown in Table 1.

Acid Hydrolysis. The polysaccharide (50 mg) was hydrolyzed in 0.25 M sulfuric acid (4 mL) for 12 h at 100 °C in a sealed tube, and 15 mg of *myo*-inositol was added as internal standard. The hydrolysate was neutralized with barium carbonate, deionized with Amberlite IR-120 (H^+) resin, and concentrated. TLC (solvents A and B) and paper chromatography (solvents B and C) of the hydrolysate revealed glucose, the identity of which was confirmed by GLC of the derived alditol acetates. The D-configuration was indicated by the $[\alpha]_D^{25}$ value [+46° (*c* 0.2, water)] of the sugar isolated by preparative paper chromatography (solvent C).

Treatment with *β*-D-Glucanase. Exo-(1→3)-*β*-D-glucanase from *Basidiomycetes* sp. QM 806 was prepared according to the method of Huotari et al.[30] Absence of (1→4)-*β*-D-glucanase activity in the preparation was ascertained using a pustulan solution (0.2%) following the usual technique.[31] (1→3)-*β*-D-Glucanase activity was determined by the same method, but with laminaran as the substrate. A solution (5 mL) containing 8 units of (1→3)-*β*-D-glucanase[30] of the polysaccharide (10 mg) in 0.05 M sodium acetate buffer (pH 4.8) was stored at 40 °C for 48 h. Then, the mixture was stirred and dialyzed at 37 °C overnight against the same buffer. The dialyzed solution was heated to 100 °C for 15 min to inactivate the enzyme and then centrifuged, and the supernatant solution was concentrated. The digest (5 mL) was applied to a column (1.6 × 100 cm) of Sephadex G-15 and eluted with distilled water. Fractions (5 mL) were collected, and the sugar content was determined by the phenol−sulfuric acid method. [27]

Periodate Oxidation. The D-glucan (5 mg) was dissolved in water (2.5 mL) and treated with 0.04 M $NaIO_4$ (3.5 mL) and water (3 mL) in the dark at 4 °C. The consumption of the oxidant was monitored spectrophotometrically,[16,17] and the formic acid liberated was estimated by titrating with 0.01 M NaOH, using methyl red as the indicator. The uptake of periodate and the liberation of formic acid became constant after 15 h.

The D-glucan (65 mg) was oxidized with 0.04 M $NaIO_4$ (150 mL) in the dark for 18 h at 4 °C, and the product was isolated and reduced with $NaBH_4$ (650 mg). A part (7 mg) of the product (53 mg, with *myo*-inositol as internal standard) was hydrolyzed with 0.5 M sulfuric acid (5 mL) for 11 h at 100 °C. GLC (Table 2) of the derived alditol acetates indicated that 74% of the glucose was resistant to periodate. The remaining part (46 mg) was treated with 0.5 M sulfuric acid for 15 h at room temperature. The hydrolysate was neutralized with barium carbonate and centrifuged. The supernatant solution was decationized with Amberlite IR-120 (H^+) resin and concentrated. The residue was subjected to a second Smith degradation, and the final solution was added to 450 mL of cold EtOH. The resulting precipitate was collected by centrifugation and dried (yield, 1.6 mg). The precipitate was digested by QM 806 (1→3)-*β*-D-glucanase, as described above.

Graded Acid Hydrolysis. A solution of the glucan (160 mg) in 40% aqueous formic acid (50 mL) was heated for 2.5 h at 100 °C. The formic acid was removed under reduced pressure by co-distillation with water. Paper chromatography (Whatman paper, solvent C) of the hydrolysate gave fractions I−V (Table 2). Each fraction isolated was found to be homogeneous. To establish the sequence of linkages in the oligosaccharide, they were subjected to conventional methylation analysis.[14] The resulting methylated alditol acetates were analyzed[19,32] by GLC (Table 2).

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