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Studies on isolation and structural features of a polysaccharide from the mycelium of an Chinese edible fungus (Cordyceps sinensis)

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Abstract

The mycelium of an Chinese edible fungus (*Cordyceps sinensis*) was found to contain a D-glucan. Methylation, Smith degradation, acetolysis, NMR spectroscopy (^{1}H, ^{13}C, ^{13}C–^{1}H 2D-COSY) and acid hydrolysis studies were conducted to elucidate its structure. The results indicated that the D-glucan consisted of a backbone composed of (1→4)-D-glucosyl residues and carried a single (1→6)-linked D-glucosyl residue. α-D-glucosidic linkages were present in the polysaccharide according to i.r. and NMR spectra. The D-glucan gave with iodine a faint blue color that had λ_{max} 564 nm, indicating the polysaccharide of α-(1→4)-linkages with short, exterior chains.

Keywords: Polysaccharides; Fungus (Cordyceps sinensis); Mycelium; D-glucan

1. Introduction

The structure of fungal extracellular polymers, which constitute a sheath around hyphae is of particular interest since they play several physiological roles in fungi morphogenesis, in their association with hosts and by their mobilization as food reserve (Krcmar, Cenek, Marie-France, & Jean-Paul, 1999). Several polysaccharides and polysaccharide–protein complexes have been isolated from fungi (mushrooms) and are being used as a source of therapeutic agents (Ooi & Liu, 2000). A number of β-glucans having (1→3)- and (1→6)- linkages (Kitamura et al., 1994; Sasaki & Takasuka, 1976; Wasser & Weis, 1999) and α-glucans (Whistler, Bushway, Singh, Nakahara, & Tokuzen, 1976) with (1→4)-linkages are widely used as antitumor and immunomodulating agents. *Cordyceps sinensis* (Berk.) Sacc. is one of the most valuable medicinal fungi in the Orient, which is an entomopathogenic fungus belonging to the hypocreales of the ascomycetes. It generally exists in two stages: an asexual stage (mitosporic fungi) and a sexual stage. The mitosporic fungi endophytically parasitize dead caterpillars of the moth *Hepilus* spp. Spores of *C. sinensis* germinate inside the caterpillars, filling the caterpillars with hyphae, and produce a stalked fruiting body (sexual stage) (Li, Zeng, Yi, & Huang, 1998; Pu & Li, 1996). It is commonly used in China for over 2000 years to replenish the kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthenia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease (Zhu, Halpern, & Jones, 1998).

The present paper reports the isolation and purification of a D-glucan from the fruit mycelium of an Chinese edible fungus (*C. sinensis*) and the results of structural studies on it. The result of this study introduces *C. sinensis* as a possible valuable source for (1→4), (1→6)-D-glucan which helps to exhibit unique immune stimulating properties (P.K. Raveendran Nair, Rodriguez, Ramachandran, Alamo, Melnick and Escalon, 2004).

2. Materials and methods

2.1. Materials

The edible fungus (*C. sinensis*) was supplied by Biotechnological Co. Ltd in Lishui (China) and authenticated by Dr Omar Ishrud (Tripoli, Libya). This work was carried out with the dried mycelium of *C. sinensis*, which was fed through a mill, and which was not fitted with a screen. After several repetitions of milling, practically all the mycelia were broken into multiple pieces.

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2.2. General

Optical rotations were measured with a Perkin–Elmer 141 polarimeter. i.r. spectra were recorded with an Acculab 10 Beckman instrument with KBr pellets. NMR spectra were recorded on a Bruker 500-instrument. For the ^{1}H NMR spectroscopy at 70 °C, the sample (9 mg) was repeatedly dissolved in D_2O (5×5 mL), and the solution was 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 analyzed on a Packard Model 419 and Hewlett-Packard Model 5713 gas chromatographs each equipped with flame-ionisation detector and columns of 1.3% of ECNSS-M on Gas Chrom 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 ionisation potential was 70 eV and the temperature of the ion source was 220 °C.

2.3. Isolation and fractionation of the polysaccharide

Dried crushed mycelia (1.25 kg) were extracted with ethanol (95 and 85%, respectively) to defat and decolorize, and then extracted with aqueous 75% ethanol overnight. After centrifugation (6700 rpm, 30 min), the residue was dried naturally and then extracted with 0.05 M phosphate buffer (pH 7.0) for 10 h at 80 °C, the process of extraction was repeated. After centrifugation (6700 rpm, 30 min), and the extracting liquids were combined, removed solvents under reduced pressure and dialyzed. The nondialyzable phase was diluted with 95% EtOH, and the resulting precipitate was collected by centrifugation, washed three times with acetone, and dried. The yield of crude polysaccharide fraction was 2.6% of the fresh weight.

The crude polysaccharide fraction was eluted from a column (2.6×60 cm) of DEAE-Cellulose-52 (Pharmacia) with 0.02 M potassium phosphate buffer (400 mL, pH 7.0), followed by 0.10 M NaOH (400 mL). Fractions (5 mL) were assayed for carbohydrate by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Fred, 1956). Two fractions were obtained, of which SCP-I, which had $[\alpha]_D^{25} + 146°$(*c* 0.5, water), was used in the subsequent studies.

2.4. Determination of molecular weight

The molecular weight of SCP-I was determined by a gel-chromatographic technique (Rodriguez & Vanderwieles, 1979). Standard dextrans T-200, T-70, T-40, and T-10 were passed through a Sepharose CL-4B column, and then the elution volumes were plotted against the logarithms of their respective molecular weights. A solution of SCP-I (3 mg) in distilled water (0.5 mL) was applied to the column which was equilibrated and eluted with distilled water at a fixed flow rate (10 mL/h). The elution volume of SCP-I was then plotted in the same graph, and the molecular weight of SCP-I was determined.

2.5. Paper chromatography(PC)

Descending PC were done on Whatmann No. 1 and 3 MM papers, respectively, for analytical and preparative chromatography, all by downward development. The solvents used were: A, 6:4:3 *p*-butanol–pyridine–water; B, 8:5:2 ethyl acetate–pyridine–water, and detection was made with aniline/diphenylamine/phosphoric acid (Bailey & Bourne, 1960).

2.6. Acid hydrolysis of the polysaccharide

The SCP-I (15 mg) was hydrolyzed with 0.5 M sulfuric acid (3.5 mL) for 16 h at 100 °C in a sealed tube. The acid was neutralized with barium carbonate, and the suspension was centrifuged. The supernatant liquor was concentrated to a small volume, and in PC (solvents A, and B) gave only one spot, corresponding to glucose. The SCP-I (3 mg) and inositol (2.5 mg) as an internal standard, were taken in a round-bottom flask, and 2 M CF_3COOH (2.5 mL) was added to it, followed by boiling in a water bath for 20 h. After the hydrolysis was complete, excess acid was removed by co-distillation with distilled water. The hydrolyzate was reduced by $NaBH_4$, followed by acidification with acetic acid. It was then co-distilled with MeOH to remove excess boric acid and dried over P_2O_5. Thereafter, the sugars were treated with pyridine (2 mL) and Ac_2O (1.5 mL) to convert into their alditol acetate. GLC gave a peak corresponding to that of glucose (besides that of inositol).

2.7. Iodine complex of the polysaccharide

The absorption spectrum of the iodine complex was measured under the conditions used by Peat and Co-workers (Peat, Whelan, Hobson, & Thomas, 1954). An aqueous solution (1 mL) containing 1 mg of the polysaccharide was mixed with 0.8 mL of the 0.4% iodine in aqueous potassium iodide. The optical absorption of the solution was measured against a blank containing 0.02% of iodine in 0.2% potassium iodide solution. The absorption maximum was found to be at 564 nm. The effect of salts, viz., $(NH_4)_2SO_4$, $MgSO_4$, $(NH_4)_3PO_4$, and $CaCl_2$, was measured by using the same solution, but containing 2% of the salt.

2.8. Methylation analysis

The SCP-I (10 mg) was performed by the method of Hakomori (Hakomori, 1964). The methylated polysaccharide was treated with 90% aq formic acid (3 mL) for 10 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M trifluoroacetic acid (2 mL) under the same conditions and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$, acetylated with acetic anhydride, and analyzed as the alditol acetates by GLC. The identification of the methylated sugars

Table 1
Molar ratio of the hydrolysis products of methylated native SCP-I

O-Methy-D-glucose	Retention time[a](min)	Retention time[b](min)	Molar ratio of SCP-I native	Linkage indicated
2,3,4,6-Tetra-	1.00	1.00	1.0	Glc(→
2,3,6-Tri-	2.87	2.75	9.6	→4)-Glc(1→
2,3-Di-	5.02	4.91	0.8	→4)-Glc(1→ 6 ↑

[a] Retention times are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on 1.3% ECNSS-M column at 170 °C.
[b] Retention times are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on 2.3% OV-225 column at 170 °C.

was analyzed by GLC–MS (Bjorndal, Lindberg, & Svenndon, 1967; Perret, Bruneteau, Michel, Marais, Joseleau and Ricci, 1992). The results were given in Table 1.

2.9. Acetolysis

Acetolysis of SCP-I (12 mg) was performed according to the literature reported (Dubourdieu, Ribereau, & Fournet, 1981) in 10:10:1 acetic anhydride—acetic acid—H_2SO_4 (11 mL) were stored in sealed tubes at 20 °C for 18, 24, or 36 h. Each mixture was poured on to ice (5 mL) and neutralized to pH 4.8 with sodium hydrogencarbonate. Acetylated sugars were extracted with chloroform (5× 4 mL), and the combined extracts were washed with conc. Aqueous sodium hydrogencarbonate, dried ($CaCl_2$) and concentrated. Each residue was dissolved in acetone (4 mL), and 0.2 M sodium hydroxide (4 mL) was added. After 30 min at 4 °C, the reaction was stopped by adding Dowex 50-X8 (H^+) resin to pH 5. The resin was removed and filtrated, then was concentrated at 50 °C under reduced pressure. The carbohydrates presented in the residue were eluted from a column (1.6×80 cm) of Sephadex G-15 with distilled water. Appropriate fractions were combined and freeze-dried. Polysaccharides excluded from the gel were methylated, and mono- or oligo-saccharides were characterized by GLC of the *O*-trimethylsilyl derivatives (Bayard & Montreuil, 1972).

2.10. Periodate-oxidation and Smith degradation

The SCP-I (15 mg) was mixed with 0.10 M sodium metaperiodate (15 mL) and kept at 4 °C in the dark for 8 days. The oxidation was stopped by addition of 1,2-ethanediol and the solution was dialyzed against distilled water for 2 days. The dialyzed material was reduced with sodium borohydride overnight, neutralized with acetic acid, dialyzed, and partially hydrolyzed in 1 M trifluoracetic acid for 12 h at 50 °C. The undissolved material was removed by centrifugation. The supernatant was neutralized by evaporation of the excess acid and fractionated on a chromatography column of Sephadex G-15 (1.6×80 cm).

3. Results and discussion

We now report the isolation and structural elucidation of a neutral D-glucan from the mycelium of *C. sinensis*. The crude polysaccharide of *C. sinensis* was obtained in yield of 2.6% of fresh weight of the mycelium. The material, defated by ethanol, was released by successive 0.05 M phosphate buffer (pH 7.0) and 0.05 M sodium hydroxide extraction. The alkali extract was fractionated by column chromatography on Sephadex G-100 (Pharmacia). The phosphate buffer-soluble extract was fractionated (Section 2), analysis of the SCP-I fraction (Fig. 1), eluted with 0.02 M potassium phosphate buffer, showed that SCP-I was uniquely homogeneous compound by high-voltage paper electrophoresis. The minor fractions obtained at lower and higher concentration of ethanol were not further studies. The molecular weight of SCP-I was determined by a gel-filtration technique using different carbohydrate markers passing through a Sepharose CL-4B column and found to be 1.84×10^5. The total sugar content of SCP-I was determined by the phenol-sulfuric acid method (Dubois et al., 1956) and was found to be 99.2%. Acid hydrolysis of the polysaccharide yielded only glucose (98.1%), and their optical rotations indicated that they were D-configuration.

The SCP-I gave a blue color with iodine solution (Barker, Bourne, Stacey, & Whiffen, 1954), and the λ_{max} was at 564 nm (E_{max} 0.6). $(NH_4)_3PO_4$, $CaCl_2$, $(NH_4)_2SO_4$, and $MgSO_4$ had little effect on the λ_{max} value, but the E_{max} increased to 0.9. These results indicated the predominance in the polysaccharide of α-(1→4) linkages with short, exterior chains (Archibald, Fleming, Lyddle, Manners, Mercer and Wright, 1961).

The SCP-I showed absorption bands at 3389, 2958, 1639 (br), 927, 842 and 761 cm^{-1} in the i.r. spectrum. The band at 842 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide (Barker et al., 1954). The bands at 842 and 927 cm^{-1} were characteristic of (1→4)-α-glucans. The broad band at 1639 cm^{-1} was due to bound water (Park, 1971). The i.r. spectra, together with the positive specific rotation (which is much smaller than those of amylose and amylopectin), indicated the presence, in the SCP-I, of α-type glycosidic linkages.

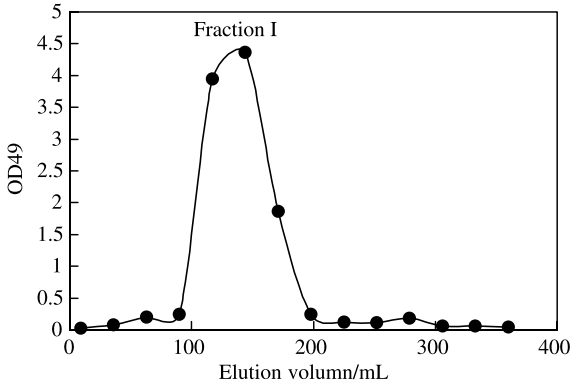

Fig. 1. Isolation and purification of the polysaccharide (Fraction I) on a chromatography column of DEAE-Cellulose-52 (eluted with 0.02 M potassium phosphate buffer, pH 7.0).

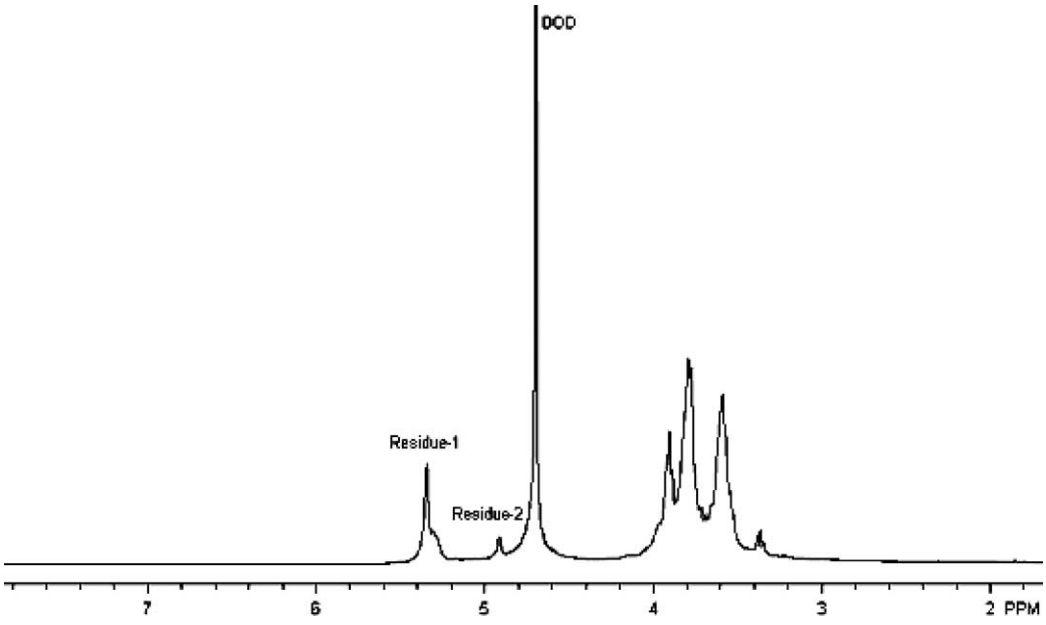

Fig. 2. ^{1}H NMR (500 M) spectrum of SCP-I isolated from an Chinese edible fungus(*Cordyceps sinensis*) recorded in D_2O.

The SCP-I was four times methylated by the Hakomori method (Hakomori, 1964). After hydrolysis reduction and acetylation, GLC of the alditol acetates from fully methylated SCP-I showed three peaks corresponding to 2,3,4,6-tetra-*O*-methyl, 2,3,6-tri-*O*-methyl and 2,3-di-*O*-methyl derivatives in molar ratio of 1.0:9.7:0.9 (Table 1). These results indicated a (1→4)-linked backbone with (1→6)-linked branches. After acetolysis, gel filtration on Sephadex G-50 gave only two fractions (A1 and A2). The highest fraction (A1) of molecular weight fractions was eluted with the void volume, and methylation, hydrolysis, reduction, acetylation, and GLC of derivatives analyzed its structure. The presence of 2,3,6-tetra-*O*-methyl derivatives agreed with a (1→4)-linked oligosaccharides resulting from complete cleavage of the branched chains and probably from some cleavage of (1→4) bonds of the backbone. The second peak (A2) was composed of only glucose monomers, suggesting the presence of single D-glucosyl groups as side chains attached at *O*−6 of some of the main-chain units. The SCP-I 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) (Aspinall & Ferrier, 1957; Dlixon & Lipkin, 1954). The GLC analysis of the methylated smith-degraded polysaccharide showed no peaks corresponding to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl derivatives. Thus, the periodate oxidation and Smith degradation study confirmed the mode of linkages of glucose present in the polysaccharide. Detection of only D-glycerol but not of D-glucosyl glycerol in the soluble fraction confirmed the presence of single glucosyl groups as side chains. The 500-MHz ^{1}H NMR spectrum of SCP-I (Fig. 2) showed two anomeric protons at δ 5.34 and 4.92, which were assigned as (1→4)-α-D-Glc*p* (Residue-1) and (1→6)-α-D-Glc*p* (Residue-2), respectively. These confirmed that the sugar residues were linked α-glycosidically, which agrees with presence of an IR band 842 cm^{-1} (Barker et al., 1954). The chemical shifts from 3.4 to 4.0 ppm were assigned to protons of carbons C-2 to C-6 of glycosidic ring (Chauveau, Talaga,

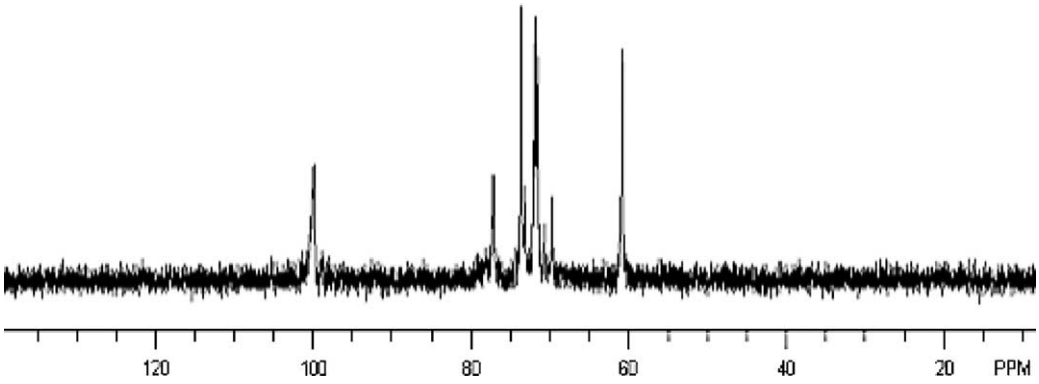

Fig. 3. ^{13}C NMR (125 M) spectra of SCP-I isolated from an Chinese edible fungus (*Cordyceps sinensis*) recorded in D_2O.

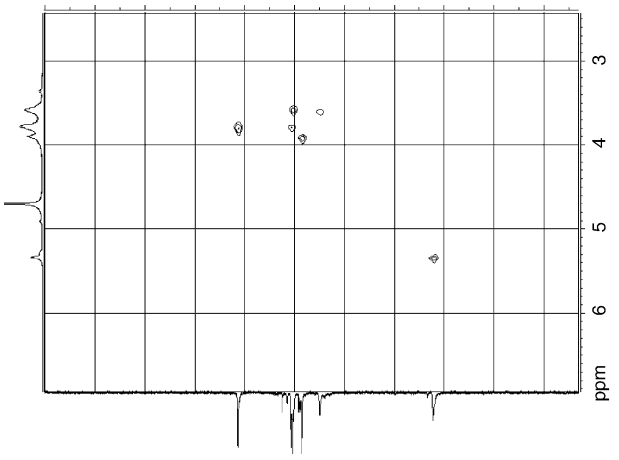

Fig. 4. ^{13}C–^{1}H 2D NMR spectrum of SCP-I isolated from an Chinese edible fungus (*Cordyceps sinensis*).

Wieruszeski, Strecker, & Chavant, 1996). The α configuration of the D-glucosyl groups was clearly evidenced by the presence of two anomeric peaks in the region δ 99.94 and 98.71 ppm from ^{13}C (125 MHz) and 2D-COSY NMR experiments (Figs. 3 and 4). And branchings of C-6 were shown by signals of *O*-substituted C-6 at δ 71.32 and of unsubstituted C-6 at δ 60.97 (Fig. 3). The predominance of the latter, together with the typical signal of *O*-substituted C-4 at δ 78.62 supported the high proportion of α-D-(1→4)-linkages in linear arrangement that was previously demonstrated by chemical analysis (Seymour, Knapp, Nelson & Pfannemuller, 1979). The multiplicity of the signals and the broad C-4 at δ 78.62 could be ascribed to the presence, in the SCP-I, of linear α-D-(1→4), branched α-D-(1→4,1→6), and terminal α-D-glucopyranosyl residues. The glucosyl ring carbon atom signals at δ 71.81, 73.25, 70.68, 60.97 correspond, respectively, to C-2, C-3, C-5, and C-6 of (1→4)-D-Glc*p*. The other signals for (1→6)-D-Glc*p* are C-2 (72.14), C-3 (73.96), C-4 (69.68), and C-5 (72.67). These values were further corroborated from the ^{13}C NMR experiment (Fig. 3, Table 2). Fig. 4 showed the ^{13}C–^{1}H 2D NMR spectrum of the SCP-I. From the correction between C–H, each peak has been assigned as Table 2. C-6 correlate with two protons (Residue-1: δ 3.77, 3.88; Residue-2: δ 3.73, 4.04), which corresponds with results from the nonproton ^{13}C NMR spectrum. C-1, C-2, C-3, C-4, C-5 each correlate with one proton. The other proton signals (H_2–H_5) of SCP-I were not assigned due to overlapping peaks.

Table 2
^{13}C NMR chemical shifts of SCP-I isolated from *Cordyceps sinensis* in D_2O

Sugar residues	Chemical shifts (ppm)[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
SCP-I[b]						
(1→4)-α-D-Glc*p*, residue-1	99.94	71.81	73.25	78.62	70.68	60.97
(1→6)-α-D-Glc*p*, residue-2	98.71	72.14	73.96	69.68	72.67	71.32

[a] In ppm downfield relative to the signal for Me_4Si.
[b] SCP-I fraction obtained by ion-exchange and gel-filtration chromatography.

α-D-Glc*p*

1

↓

6

→4)-α-D-Glc*p*-(1→4)-[α-D-Glc*p*-(1→4)-]$_8$-α-D-Glc*p*-(1→

1

Structure 1.

4. Conclusion

These results of methylation and hydrolysis analysis, acetolysis, Smith degradation and i.r. and NMR spectra suggested structure **1** for the SCP-I of *C. sinensis*. The SCP-I is a D-glucan containing α-(1→4)-linked backbone, branched α-(1→6)-linkage.

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