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Studies on Fungal Polysaccharides. XX.[1] Galactomannan of *Cordyceps sinensis*[2]

Toshio Miyazaki, Naoko Oikawa, and Haruki Yamada

Department of Microbial Chemistry, Tokyo College of Pharmacy[3]

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Water-soluble polysaccharide from ascocarps of *Cordyceps sinensis* was purified by ethanol fractionation and gel filtration. Purified polysaccharide, CS-I, $[\alpha]_D$ −45.3° (c=1, H_2O), gave D-galactose (phenylosazone, mp 185—187°) and D-mannose (phenylhydrazone, mp 199—200°) in a molar ratio of 1:1 by acid hydrolysis. From the results of periodate oxidation, Smith-type degradation, methylation analysis, partial acid hydrolysis, and ^{13}C-NMR spectrometry, it is concluded that CS-I is a highly branched galactomannan which consists of mannan core and galactosyl oligomer containing branches. The mannan core mainly contains (1→2)-α-linked-D-mannopyranosyl residues, and the branches contain (1→3), (1→5), and (1→6)-linked-D-galactofuranosyl, (1→4)-linked-D-galactopyranosyl residues. The non-reducing ends are D-galactofuranose and D-mannopyranose, and the branching points are D-mannopyranosyl residues.

Keywords——larva of Lepidoptera; galactofuranose; *Penicillium chrysogenum*; Ascomycetes; gas–liquid chromatography–mass spectrometry; C-13 magnetic resonance spectra; PMR spectra

A parasitic fungus on a larva of Lepidoptera, *Cordyceps sinensis*, belonging to Ascomycetes has been used as a Chinese medicine (冬虫夏草) for eternal youth. A water-soluble mycelial polysaccharide was investigated as a part of structural studies on polysaccharides in fungal crude drugs.

The water-soluble polysaccharide was obtained from the ascocarps of *C. sinensis* by hot water extraction and ethanol fractionation. The crude polysaccharide was digested with Pronase, and treated by the Sevag's method.[4] Purification was carried out by ethanol precipitation and gel filtration on a column of Sephadex G-100. Purified polysaccharide, CS–I, which was revealed homogeneous by paper electrophoresis, showed the sugar content of 92.5%, and nitrogen content of 1.21%. Acidic hydrolysis of CS–I gave D-galactose (phenylosazone, mp 185—187°)[5] and D-mannose (phenylhydrazone, mp 199—201°[5]) in a molar ratio of 1:1 (by the procedure of Dubois, *et al.*).[6] Optical rotation of CS–I was $[\alpha]_D$ −45.3° (H_2O). In the infrared (IR) spectrum, absorption maximum at 840 cm^{-1} suggested the presence of α-glycosidic linkage.[7] In the proton magnetic resonance (PMR) spectrum of CS–I, the signals of anomeric protons based on the α-linkage[8] at 5.0, 5.12 (d, J=3 Hz), and 5.28 ppm (d, J=3 Hz) were observed. The signal of 5.28 ppm was assigned as the anomeric proton due to α-(1→2) linked mannose residue.

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Table I. Relative Retention Times and Prominent Peaks (*m/e*) in the GLC–MS Spectra of Methylated Alditol Acetates Derived from CS–I

Peak O-Acetyl-O-methyl alditol	Relative retention time		*m/e*															
	Observed	Literature	43	45	59	71	87	89	99	101	113	117	129	145	161	189	205	233	261
1,5-Di-OAc-2,3,4,6-tetra-OMe-mannitol	1.0	1.0	+	+		+	+			+		+	+	+	+		+		
1,4-Di-OAc-2,3,5,6-tetra-OMe-galactitol	1.1	1.1	+	+	+			+		+		+					+		
1,2,5-Tri-OAc-3,4,6-tri-OMe-mannitol	1.82	1.82	+	+			+		+	+			+		+	+			
1,3,4-Tri-OAc-2,5,6-tri-OMe-galactitol[a]	1.95	1.95																	
1,4,5-Tri-OAc-2,3,6-tri-OMe-galactitol	2.23	2.22	+	+			+		+	+	+	+						+	
1,4,6-Tri-OAc-2,3,5-tri-OMe-galactitol	2.78	2.76	+							+		+							
1,2,3,5-Tetra-OAc-4,6-di-OMe-mannitol	2.96	2.92	+	+			+			+			+		+				+
1,2,5,6-Tetra-OAc-3,4-di-OMe-mannitol	4.36	4.36	+				+		+				+			+			

a) *m/e* could not determined.

In order to decide the mode of linkage, the fully methylated CS–I was converted to alditol acetates as described in the previous paper,[9] and the products were analyzed by gas–liquid chromatography–mass spectrum (GLC–MS). As shown in Table I, following residues were identified by their relative retention time[10] and *m/e* values[11]: 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-mannitol; 1,4-di-O-acetyl-2,3,5,6-tetra-O-methyl-galactitol; 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-mannitol; 1,3,4-tri-O-acetyl-2,5,6-tri-O-methyl-galactitol; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-galactitol; 1,4,6-tri-O-acetyl-2,3,5-tri-O-methyl-galactitol; 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl-mannitol; 1,2,5,6-tetra-O-acetyl-3,4-di-O-methyl-mannitol, and molar ratio of these derivatives was 1.0: 5.5: 6.8: 0.7: 5.5: 1.5: 3.4: 2.3. These analytical data suggest that 1) non-reducing ends are galactofuranosyl and mannopyranosyl residues, 2) branching points are 1,2,3- and 1,2,6-linked mannopyranosyl residues, and 3) the core and branches are consisted of 1,2-linked mannopyranosyl, 1,3-linked galactofuranosyl, 1,4-linked galactopyranosyl or 1,5-linked galactofuranosyl and 1,6-linked galactofuranosyl residues.

On the other hand, results of periodate oxidation, consumption of 1.32 mol of periodate[12] and formation of 0.4 mol of formic acid[13] per anhydro hexose unit, and of Smith-type degradation[14,15] (molar ratio of the main products was mannose: glycerol: threitol=0.22: 3.5: 1.3) were consistent with that of methylation analysis. That is, above results suggested that at least the presence of 1,2- and 1,3-linked mannopyranosyl (source of glycerol and mannose), and 1,4-linked galactopyranosyl or 1,5- and 1,6-linked galactofuranosyl residues (source of threitol).

Partial acid hydrolysis of CS–I was successively carried out as shown in Chart 1. Galactose (main), galactobiose, galactotriose and small amount of unidentified oligosaccharide were

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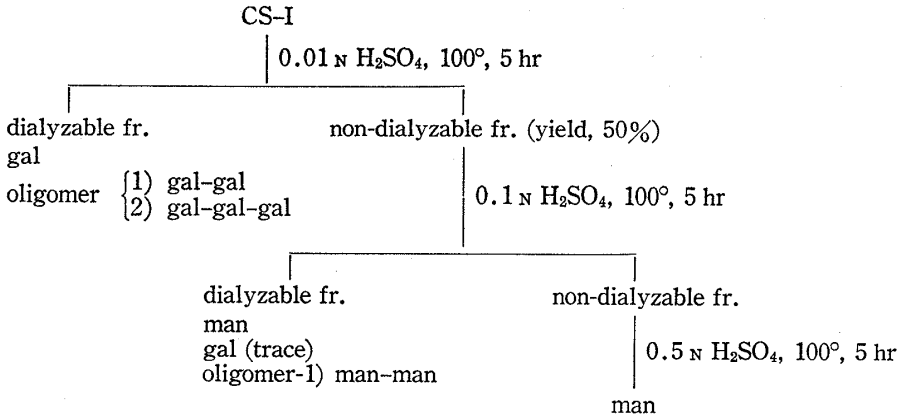

Chart 1. Partial Acid Hydrolysis of CS-I

detected from the dialyzable fraction, after the mild acid hydrolysis of CS–I was carried out using 0.01 N of H_2SO_4, at 100°, for 5 hr. Approximately 50% of CS–I remained as the non-dialyzable fraction. When the non-dialyzable fraction was hydrolyzed with 0.1 N H_2SO_4, mannose, mannobiose and trace of galactose were liberated. Finally, the weak acid-resistant, non-dialyzable fragment gave only mannose by the hydrolysis using 0.5 N H_2SO_4 as described above. These results suggest that acid-labile galactofuranosyl residues exist in the branch part, and mannopyranosyl residues form the core part.

In ^{13}C-NMR spectra of CS–I and galactomannan[16] of *Penicillium chrysogenum*, the signals at 107.12 ppm (CS–I) and 109.12 ppm (P.c) were observed, which assigned to anomeric carbons due to β-galactofuranosyl residue.[17]

On the basis of these results, it is postulated that the molecular form of CS–I resembles a "comb" having a mannan core, galactose-oligomer containing branches, and galactofuranosyl non-reducing ends.

Previously, several kinds of galactomannan were isolated from higher plants[18] and fungi.[19–21] Chemical structures of these glycans were similar, and they have a mannan core and branching galactosyl residue as the common units. However, the galactosyl branches of CS–I are much longer than other. It is interesting that a new type galactomannan was isolated from the mycelia of a parasitic fungus on a larva.

Experimental

Isolation of Water-soluble Polysaccharide——The ascocarps of *C. sinensis* (59.2 g, separated from the crude drug) were defatted with 3 volumes of ethanol–ether (1: 1) extraction at room temperature, and then extracted with H_2O at 100° for 1 hr. After filtration, the residue was extracted with hot H_2O, and this procedure was repeated 12 times. The combined supernatant was dialyzed against running water for 2 days, and the internal solution of Visking cellulose tubing was concentrated *in vacuo*. To the concentrate, 3 volumes of ethanol were added, and the precipitate that appeared was collected by centrifugation, washed successively with ethanol, acetone, and ether, and dried *in vacuo*. The yielded precipitate (4.6 g) was dissolved in 70 ml of phosphate buffer (0.1 M, pH 8.0) and was incubated with Pronase (0.2 g, Kaken kagaku Co., Tokyo) at 38°, overnight. The incubation mixture was dialyzed against running water for 2 days, and the internal solution was shaken vigorously for 0.5 hr with $CHCl_3$–BuOH (4: 1). The mixture was centrifuged to collect the aqueous layer, and the same procedure was repeated until a gelatinous substance was no longer formed in the mixture. After centrifugation, the supernatant was concentrated to a small volume under reduced pressure below 40°, and 4 volumes of ethanol were added to this concentrate. The precipitate was

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collected by centrifugation, washed with ethanol, acetone, and ether, and dried *in vacuo*. The same procedure was repeated 3 times. The solution of deprotenized crude polysaccharide was added to equal volume of ethanol, and after removal of a small amount of precipitate, 4 volumes of ethanol were further added to the supernatant. The precipitate was collected as described above. (CS-O, yield, 0.46 g, corresponding to 0.77% of the ascocarps).

Purification of CS-O by Gel Filtration——A buffer solution of CS-O (0.2 g) was applied to a column of Sephadex G-100 (1.5×90 cm) using phosphate buffer (0.02 M, pH 7.2) at the flow rate of 6 ml/hr. An aliquot of 0.05 ml of each fraction (3 ml) was added with 0.95 ml of H_2O and 3 ml of 0.2% anthrone reagent,[22] and the optical density was read at 625 nm on a colorimeter. The main fraction (Tube No. 18—25) was dialyzed against running water for 2 days, and then against distilled water for 1 day. The concentrated internal solution was added to 4 volumes of ethanol, collected precipitate by centrifugation was washed with ethanol, acetone, and ether, and then dried *in vacuo* (CS-I, yield 0.34% of the ascocarps). Small amount of another fraction was obtained, but could not be examined in detail.

Paper Electrophoresis of CS-I——Paper electrophoresis of CS-I was carried out using 1% sodium borate solution (pH 9.2) or 0.1 M acetate buffer (pH 4.5) on Toyo Roshi No. 51 filter paper under 1 mA/cm, 2 hr. Single spot by periodate–Schiff reagent[23] which moved to the anode was detected.

Component Sugar of CS-I——Component sugar of CS-I was determined as follows: 5.0 mg of CS-I in 2 ml of 1 N H_2SO_4 in a sealed tube was heated in a boiling water bath for 5 hr, and the mixture was treated as described in our previous paper.[24] Galactose and mannose were identified in a molar ratio of 1:1 (by the procedure of Dubois, *et al.*,[6] and by GLC). GLC was carried out with Shimadzu GC-6A unit, equipped with flame ionization detector, using a 200×0.3 cm glass column packed with 3% ECNSS-M on Gas Chrom Q (100—200 mesh); column temperature, 180°; N_2 flow rate, 60 ml/min.

Identification of D-galactose and D-mannose: A part of the acid hydrolyzate was made to react with phenylhydrazine by usual method.[5] Colorless crystals of D-mannose phenylhydrazone, mp 199—200°,[5] and yellow crystals of D-galactose phenylosazone, mp 185—187°[5] were obtained. Melting points were undepressed on admixture with the authentic samples, respectively.

Properties of CS-I——Sugar content of CS-I was 92.5% (as galactose: mannose, 1:1, by the procedure of Dubois, *et al.*[6] and nitrogen content was 1.21% (by elementary analysis). CS-I showed $[\alpha]_D$ −45.3° (c=1, H_2O). IR ν_{max}^{KBr} cm^{-1}: 840 (α-glycosidic linkage), PMR δ ppm (D_2O): 5.0 (s), 5.12 (d, J=3 Hz) and 5.28 (d, J=3 Hz). ^{13}C Nuclear magnetic resonance spectra of CS-I and galactomannan[16] of *Penicillium chrysogenum* were measured at 25.1 MHz with JEOL PS-100 spectrometer in D_2O. Chemical shifts were measured using acetone as an external standard, and were expressed relating to TMS. All protons were decoupled. The spectra were determined after multiple scanning using a time-averaging device.

Methylation Analysis of CS-I——CS-I was fully methylated by the Hakomori method[25] and hydrolyzed with 90% HCOOH at 100° for 10 hr, and then with 0.5 N H_2SO_4 at 100°, for 5 hr. Methylated sugars were converted into alditol acetates and analyzed by GLC–MS[11] with Hitachi RMU-7L mass spectrometer and 3% OV-225 column. The result was shown in Table I.

Periodate Oxidation of CS-I——CS-I (12.5 mg) was oxidized in 25 ml of 0.076 M $NaIO_4$ at room temperature in a dark. Sodium periodate consumption and HCOOH formation were determined by the procedure of Malaprade[12] and Whistler,[13] respectively. The number of moles of $NaIO_4$ consumed per anhydrohexose unit was as follows: 0.23 (1 hr), 0.78 (3 hr), 0.89 (6 hr), 1.01 (9 hr), 1.12 (24 hr), 1.29 (48 hr), 1.32 (72 hr). The corresponding number of moles of HCOOH produced was 0.06 (1 hr), 0.17 (3 hr), 0.20 (6 hr), 0.18 (9 hr), 0.29 (24 hr), 0.33 (48 hr), 0.40 (72 hr).

Smith-type Degradation[14] of CS-I——Smith-type degradation of CS-I (25.0 mg) was carried out after CS-I was oxidized for 48 hr as described above. The oxidized CS-I was reduced with $NaBH_4$ and hydrolyzed with 0.5 N H_2SO_4 at 100°, for 5 hr. The hydrolyzate was neutralized, reduced with $NaBH_4$, and acetylated with acetic anhydride–pyridine (1:1, v/v) as described in our previous report.[15] GLC analysis of the alditol acetates was carried out described above (column temperature: 170° and 194°). Mannose, glycerol, and threitol were detected in the molar ratio of 0.22:3.5:1.3, and small amount of arabinose was also detected.

Partial Acid Hydrolysis of CS-I——CS-I (10.0 mg) was hydrolyzed with 0.01 N H_2SO_4 at 100° for 5 hr and the hydrolyzate was dialyzed against distilled water (500 ml) for 15 hr. The external solution of the hydrolyzate was evaporated to about 30 ml *in vacuo*. After neutralization with $BaCO_3$, and filtration, it and the hydrolyzate was dialyzed against distilled water (500 ml) for 15 hr. The external solution of the hydrolyzate was evaporated to about 30 ml *in vacuo*. After neutralization with $BaCO_3$, and filtration, it was converted to a syrup. Paper chromatographic examination of the syrup using solvent systems A and B,[24] showed the liberation of galactose (main) and oligisaccharides. Major oligosaccharides I and II were extracted from the corresponding area of the paper chromatogram with H_2O, and were further hydrolyzed

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with 0.5 N H_2SO_4 at 100°, for 5 hr. Galactose was detected only from both hydrolyzates. Positions of the paper chromatogram suggested that oligosaccharides I and II should be galactobiose and galactotriose, respectively. The internal solution of the hydrolyzate was concentrated to dryness and weighed (5.0 mg). A part of the non-dialyzable fragment was hydrolyzed with 0.1 N H_2SO_4 at 100° for 5 hr and treated with the same procedure as described above. Mannose, mannobiose and trace of galactose were detected from the dialyzable fraction. Final hydrolysis of the 0.1 N H_2SO_4-treated non-dialyzable fragment with 0.5 N H_2SO_4, 100°, for 5 hr gave mannose only.

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