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Polysaccharides in Fungi. XXII.[1] A Water-Soluble Polysaccharide from the Alkaline Extract of the Insect-Body Portion of Chán huā (Fungus: *Cordyceps cicadae*)

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A water-soluble polysaccharide (CI-5N), $[\alpha]_D$ +30° in water, was isolated from the alkaline extract of the insect-body portion of Chán huā (Chinese name) (fungus: *Cordyceps cicadae*). CI-5N was homogeneous on gel filtration and gave a single spot on glass-fiber paper-electrophoresis. It was composed of D-mannose, D-galactose, and D-glucose in the molar ratio of 1.0 : 0.67 : 0.23, and was free from protein. The molecular weight was estimated by gel filtration to be *ca.* 39000. The polysaccharide had affinity for concanavalin A. From the results of methylation analysis, Smith degradation, stepwise hydrolysis, and carbon-13 nuclear magnetic resonance, it was concluded that the polysaccharide has a highly branched structure, and is composed of (1→6)-linked and (1→2)-linked α-D-mannopyranosyl residues, and (1→2)-linked α-D-glucopyranosyl residues. Some of the D-mannopyranosyl residues are present as branching points of (1→2,6)-linkage, and the branches contain short chains having (1→2)-linked β-D-galactofuranosyl residues and single β- and/or α-D-galactofuranosyl residues.

Keywords——polysaccharide; Chán huā; *Cordyceps cicadae*; molecular weight; methylation analysis; ^{13}C-NMR; Con A affinity; polysaccharide structure; galactoglucomannan

Cordyceps cicadae SHING is a fungus parasitic on the larvae of *Cicada flammata* DIST. Both the fruit-body and the insect-body portions are called Chán huā (Chinese name),[2] and have been used as a medicine (*e.g.*, for childhood convulsion or palpitation) and elixir in China. Previously, we reported on a water-soluble galactomannan (C-3)[3] from the fruit-bodies, and two water-soluble galactomannans (CI-P and CI-A)[1] from the insect-body portion, of Chán huā. We have now isolated a new polysaccharide (CI-5N) from the alkaline extract of the insect-body portion. The present paper deals with the purification, characterization, and structural analysis of CI-5N.

The insect-body portion was successively extracted with hot methanol, an aqueous solution containing protease and lysozyme, hot water, and 3% sodium carbonate. The residue was extracted with 1 M sodium hydroxide at room temperature. The final alkaline extract was made neutral, and dialyzed. The soluble fraction in the non-dialyzable solution was precipitated with ethanol, and deproteinized by protease treatment[3] and the Sevag procedure,[4] then purified by anion-exchange column chromatography on diethylaminoethyl (DEAE)-Sephadex A-25. The neutral fraction was separated by gel filtration on Sephacryl S-300, and the solution in the low molecular fraction was lyophilized to afford a polysaccharide, CI-5N, in 0.12% yield; this product showed a symmetrical elution peak in gel filtration of Sephadex G-100 (see Fig. 1), and gave a single spot on glass fiber-paper electrophoresis.

The polysaccharide had $[\alpha]_D^{28}$ +30° (c=0.48, water), and contained no nitrogen (by elementary analysis).[5] CI-5N was composed of D-mannose, D-galactose, and D-glucose in the

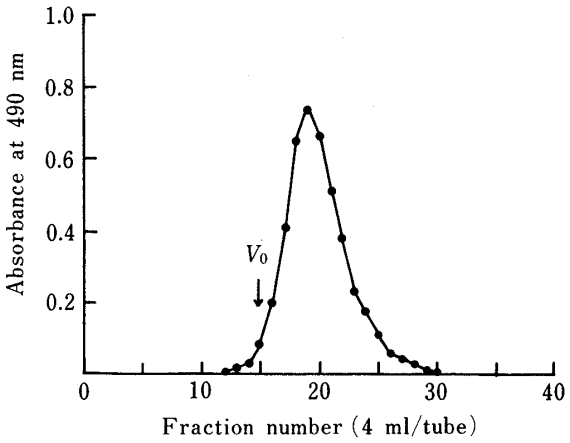

Fig. 1. Chromatogram of CI-5N in 0.1 M NaCl on Sephadex G-100

TABLE I. GLC and GLC-MS Data for the Partially Methylated Alditol Acetates

Methylated sugar (as alditol acetate)	Relative retention times[a]		Primary mass fragments (m/z)	Molar percentage	Mode of linkage
	Column A	Column B			
2,3,4,6-Me_4-Man	1.00	0.98	45, 117, 161, 205	1.5	[Man*p*]1→
2,3,4,6-Me_4-Glc	1.00	1.00	45, 117, 161, 205	1.2	[Glc*p*]1→
2,3,5,6-Me_4-Gal	1.15	1.16	45, 59, 89, 117, 205	16.2	[Gal*f*]1→
3,4,6-Me_3-Glc	2.00	1.80	45, 161, 189	16.6	→2[Glc*p*]1→
3,4,6-Me_3-Man	2.00	1.86	45, 161, 189	27.0	→2[Man*p*]1→
3,5,6-Me_3-Gal[b]	2.28	1.97	45, 59, 89, 189, 205, 305	11.4	→2[Gal*f*]1→
2,3,4-Me_3-Man[c]	2.52	2.24	117, 161, 189, 233	8.0	→6[Man*p*]1→
3,4-Me_2-Man	5.46	4.84	189	18.1	→2,6[Man*p*]1→

a) Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Column A, 3% ECNSS-M; Column B, CP-Sil 88 (capillary column). *b*) It may be an overlapping peak: small proportions of 2,3,6-Me_3-Man. *c*) 2,3,6-Me_3-Gal (m/z: 45, 117, 233).

molar ratio of 1.0:0.67:0.23, as shown by paper partition chromatography (PPC) of the hydrolyzate and gas-liquid chromatography (GLC) of the alditol acetates[6] prepared from the hydrolyzate. The absolute configurations of the component sugars were determined by the method of Leontein *et al.*[7] The molecular weight of CI-5N was estimated to be *ca.* 39000 by gel filtration with the use of standard dextrans on Sephadex G-100, as described in a previous paper.[1]

The polysaccharide was methylated by the method of Hakomori,[8] and the fully methylated polysaccharide, which showed no hydroxyl absorption band in the infrared (IR) spectrum, was hydrolyzed with acid. The partially *O*-methylated sugars were analyzed as the alditol acetate derivatives by GLC and GLC-mass spectrometry (GLC-MS), and identified by comparing their retention times in GLC, and their fragment patterns in MS, with those of authentic samples, or with the values in the literature.[9] The peaks of 3,4,6-tri-*O*-methyl-D-glucitol and 3,4,6-tri-*O*-methyl-D-mannitol acetates overlapped on the column of ECNSS-M, but the two derivatives were resolved on a capillary column of CP-Sil 88. Table I shows the results of the methylation analysis. The results indicated that most of the D-galactosyl residues were present as non-reducing terminal furanosyl and (1→2)-linked furanosyl types, while D-mannose was present mainly as (1→2)-linked, (1→6)-linked, and (1→2,6)-linked D-mannopyranosyl residues, and D-glucose was present mainly as (1→2)-linkages. The results also indicated that some of (1→6)-linked or (1→2)-linked mannopyranosyl residues were

branched at O-2 or O-6.

On oxidation, the polysaccharide consumed 1.34 mol of periodate per hexosyl residue. The periodate-oxidized polysaccharide was reduced with sodium borohydride, and the resulting polyalcohol was hydrolyzed with acid. The hydrolyzate (Smith degradation product) was analyzed by GLC as the alditol acetate derivatives. Large proportions of glycerol and arabinitol acetates, and small proportions of erythritol and threitol acetates were detected, but the derivatives of the component sugars were negligible. The identification of arabinitol acetate confirmed the presence of (1→2)-linked galactofuranosyl residues. The formations of erythritol and threitol acetates also supported the finding of trace amounts of 2,3,6-tri-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-galactose derivatives in the methylation analysis, respectively. The results are approximately consistent with those expected from the methylation analysis.

The polysaccharide was hydrolyzed stepwise with 5 mM sulfuric acid for 5 h at 100 °C, and 50 mM sulfuric acid for 4 h at 100 °C. Only galactose as the free sugar, together with oligosaccharides, was released at the first stage. At the second stage, mannose and galactose in the molar ratio of 1 : 5.4 were detected in the dialyzable fraction. The second non-dialyzable fraction of the acid-degraded polysaccharide was composed of mannose and glucose in the molar ratio of 3.6 : 1.0, and did not contain galactose. The results suggest the presence of a core of mannopyranosyl and glucopyranosyl units, and side chains of galactofuranosyl residues.

The anomeric configurations were assigned by comparing the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of CI-5N with data in the literature.[1,10,11] The two signals at lower field, at 106.7 and 105.0 ppm, probably represent the non-reducing terminal β-D-galactofuranosyl residues, and (1→2)-linked β-D-galactofuranosyl residues, respectively. This assignment was also supported by the *ca.* 2 ppm upfield shift of the (1→2)-linkage signal.[12] The intense signals at 101.4 and 101.2 ppm correspond to C-1 of (1→2)-linked α-D-mannopyranosyl residues and/or non-reducing terminal α-D-galactofuranosyl residues, as previously described.[1] The signal at 101.0 ppm could be attributed to (1→2)-linked α-D-glucopyranosyl residues because the signal was not observed in the galactomannans (CI-P, CI-A, and C-3)[1,3] shown in Table II. The resonances at 100.1 ppm, and at 98.9 and 97.9 ppm, at higher field, would correspond to C-1 of (1→6)-linked α-D-mannopyranosyl residues, and C-1 of (1→2,6)-linked α-D-mannopyranosyl residues, respectively. The configurations of the anomeric carbon atoms of mannopyranosyl and glucopyranosyl residues were supported by the value of $^{1}J_{CH}$ of 173.3—174.5 Hz.

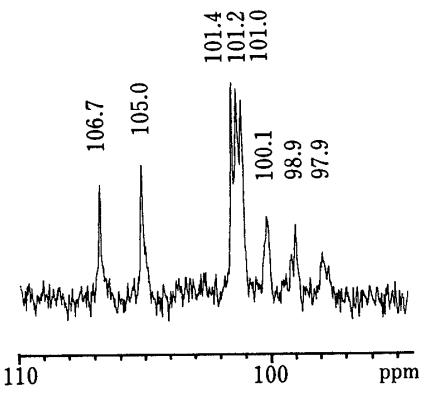

Fig. 2. ^{13}C-NMR Spectrum (Anomeric Region) of CI-5N in D_2O at 70 °C

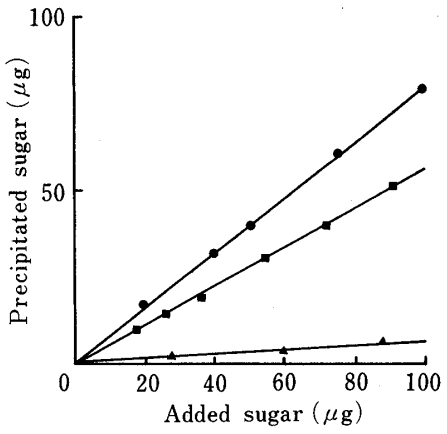

Fig. 3. Quantitative Precipitation Curves of the Polysaccharides (CI-5N, —●—; CI-A, —■—; CI-P, —▲—) with Concanavalin A

TABLE II. Comparison of the Polysaccharides from Chán huā

Mode of linkage	Insect-body			Fruit-body
	CI-5N	CI-P (molar proportion)[a]	CI-A	C-3
[Man*p*]1→	1.5	1.7	5.6	2.5
[Glc*p*]1→	1.2	—	—	—
[Gal*f*]1→	16.2	33.0	22.6	20.6
→2[Glc*p*]1→	16.6	—	—	—
→2[Man*p*]1→	27.0	2.8	22.6	33.1
→2[Gal*f*]1→	11.4	21.5	13.6	17.0
→6[Man*p*]1→	8.0	3.5	7.9	7.6
→2,6[Man*p*]1→	18.1	32.2	26.0	19.1

a) The value is not corrected for the overlapping peak.

Interaction of the polysaccharide (CI-5N) with concanavalin A (Con A) was studied by incubation with a constant amount of Con A in the buffer solution. The quantitative precipitation curves against Con A are shown in Fig. 3. The polysaccharides CI-P and CI-A were minor protein-containing galactomannans from the insect-body portion of Chán huā, as we previously reported.[1] CI-5N showed higher affinity for Con A than CI-P or CI-A. The results were compatible with the methylation analysis data, and the higher proportions of (1→2)-linked α-D-mannopyranosyl and (1→2)-linked α-D-glucopyranosyl residues in CI-5N, suggest that CI-5N has a different fine structure from CI-P and CI-A.

The foregoing data indicate that CI-5N, a highly branched polysaccharide, is composed of a core of (1→6)-linked and (1→2)-linked α-D-mannopyranosyl residues, and (1→2)-linked α-D-glucopyranosyl residues. Some of the D-mannopyranosyl residues are substituted at O-2 or O-6 with short chains of (1→2)-linked β-D-galactofuranosyl residues and single β- and/or α-D-galactofuranosyl residues.

We have reported on the structures of CI-P[1] ($[\alpha]_D$ +15.3°, lower affinity for Con A) and CI-A[1] ($[\alpha]_D$ +10.6°, higher affinity for Con A) from the extract of the insect-body portion with aqueous solution containing protease, and C-3[3] ($[\alpha]_D$ +30°) from the water extract of the fruit-bodies, of Chán huā. These polysaccharides have a core of (1→6)- and/or (1→2)-linked α-D-mannopyranosyl residues and side chains of D-galactofuranosyl residues, but they contained a small amount of protein and the content of glucose as a component sugar was negligible. The molecular weight of CI-5N (39000) is higher than those of CI-P, CI-A (both 25000) and C-3 (27000). This suggests that CI-5N is not a degraded polysaccharide formed from the above polysaccharides (CI-P, CI-A, and C-3) during the alkaline extraction. CI-5N has a highly branched structure, but the branches are fewer than in the above polysaccharides. CI-5N bears most structural resemblance to C-3 among the polysaccharides, in molecular weight, specific rotation, and the proportions of the linkage modes (see Table II). On the other hand, a galactomannan[13] ($[\alpha]_D$ −29.6° in water) from *Cordyceps sinensis* also had a core of α-D-mannopyranosyl residues and D-galactofuranosyl side chains, while the galactofuranosyl chains were in (1→5)-β-D-linkages instead of (1→2)-β-D-linkages. Thus, the present data are useful for chemotaxonomy. Studies on the biological activities of the polysaccharides, such as antitumor activity,[14,15] are in progress.

Experimental

Materials——The dried, crude drug Chán huā was obtained from commercial sources in Hong Kong; its insect-

body was fully invaded and filled with the fungus (*Cordyceps cicadae*). Sephacryl S-300, Sephadex G-100, DEAE-Sephadex A-25, and Con A were purchased from Pharmacia Fine Chemicals. Pronase E (Kaken Kogyo Co., Tokyo) was used as a protease. All chemicals were of analytical grade.

General——Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were recorded with a JASCO A-120 spectrometer. PPC was performed by the triple ascending method, using Toyo Roshi. No. 51A filter-paper and the solvent system of 1-butanol–pyridine–water (6 : 4 : 3). Sugars were detected with alkaline silver nitrate reagent.[16] GLC as alditol acetates was performed in a Shimadzu GC-4CM apparatus equipped with a flame-ionization detector, using a glass column (2 m × 0.3 cm) packed with 3% ECNSS-M on Gaschrom Q (100—120 mesh). Peak areas were measured with a Shimadzu C-R3A or C-R5A Chromatopac. GLC-MS was conducted with a JEOL JMS-D 300 apparatus equipped with a glass column (1 m × 0.2 cm) packed with 3% ECNSS-M as described in a previous paper.[15] The acetylated (+)-2-octyl glycosides were applied to a capillary column fitted to a Shimadzu 8A apparatus equipped with a flame-ionization detector.

Isolation of the Polysaccharide——The dried, pulverized insect-body portion of Chán huā (81 g) was successively extracted with hot methanol (1.5 l) for 20 h, aqueous solution (1.5 l) containing Pronase E (80 mg) for 24 h at 37 °C (3 times), 0.1 M phosphate buffer at pH 6.3 (500 ml) containing lysozyme (80 mg) for 24 h at 37 °C, hot water (700 ml) for 7 h (7 times), and 3% sodium carbonate (1 l) for 20 h at room temperature (5 times). The residue was extracted 8 times with 1 M sodium hydroxide (1 l) for 23 h at room temperature under a nitrogen atmosphere. The alkaline suspension was centrifuged, and the extracts were made neutral with 5 M hydrochloric acid, then dialyzed against distilled water for 5 d. The insoluble materials in the non-dialyzable fraction were removed, and ethanol (1 vol.) was added to the solution. The procedure of ethanol precipitation was repeated twice, and the resulting precipitate was collected by centrifugation, dispersed in water, and lyophilized to give the crude polysaccharide, in 0.79% yield.

The crude polysaccharide was deproteinized by protease (Pronase E) digestion and the Sevag procedure,[4] and further purified by column chromatography on DEAE-Sephadex A-25 (acetate form), as previously described.[3,13] The neutral fraction was separated by gel filtration on a column (87.5 × 2.64 cm) of Sephacryl S-300 (0.1 M NaCl). The low-molecular fraction (frac. No., 28—39; each frac., 8 g) was collected, dialyzed, and lyophilized to afford the purified polysaccharide (CI-5N), in 0.12% yield.

Electrophoresis——Paper electrophoresis was conducted on Whatman GF-81 glass-fiber paper with 0.05 M sodium tetraborate buffer (pH 9.3) for 3 h at 300 V. The spot was detected with 1-naphthol–sulfuric acid reagent.[17] CI-5N showed a single spot at a distance of 9.1 cm (glucose as a reference, 10.8 cm) from the origin.

Gel Filtration and Estimation of Molecular Weight——Gel filtration of CI-5N and standard dextrans on a column of Sephadex G-100 was performed with 0.1 M sodium chloride, and the molecular weight was estimated from a calibration curve constructed by the use of the standard dextrans as described in a previous paper.[1]

Analysis of Component Sugars——The polysaccharide (CI-5N) was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 6 h at 100 °C. The hydrolyzate was evaporated to remove the acid, and analyzed by PPC and by GLC as alditol acetates,[6] as previously described.[1] Determination of the absolute configuration was achieved by GLC on an FS-WCOT capillary column (25 m × 0.25 mm) coated with SP-1000 as described in previous papers[13,18] according to the method of Leontein *et al.*[7] The component sugars (mannose, galactose, and glucose) were identified as being of D-type.

Methylation Analysis——The polysaccharide was methylated 4 times by Hakomori's method, as previously described.[19] The final product showed no hydroxyl absorption band in the IR spectrum. The fully methylated polysaccharide was successively heated with 90% formic acid (1 ml) for 5 h at 100 °C, and with 2 M TFA for 3 h at 100 °C. The hydrolyzate was converted into the alditol acetates, and the resulting, partially *O*-methylated alditol acetates were analyzed by GLC (column temperature, 170 °C; carrier gas (nitrogen), 35 ml/min) and GLC-MS. GLC was further performed on a Shimadzu GC-15A apparatus equipped with a hydrogen flame ionization detector, using an FS-WCOT capillary column (25 m × 0.25 mm) coated with CP-Sil 88 (Chrompack) at 180 °C, at a flow rate (splitter vent) of 92 ml/min of helium, with a split ratio of 1 : 122. The results are shown in Table I.

Periodate Oxidation and Smith Degradation——The polysaccharide (10 mg) was mixed with 10 mM sodium periodate (40 ml) in the dark, with stirring, at 4 °C. Methyl α-D-glucoside was used as a reference. The periodate consumption was estimated by an arsenite method,[20] and the amount per hexosyl residue was 1.34 mol in 6 d. The periodate-oxidized polysaccharide thus obtained was reduced with sodium borohydride, and the resulting polyalcohol was hydrolyzed with 1 M sulfuric acid for 6 h at 100 °C, as previously described.[18] The hydrolyzate (Smith degradation product) was analyzed by GLC as alditol acetates, using a programmed rise temperature of 4 °C/min from 80 to 185 °C. The retention times of glycerol acetate, erythritol acetate, threitol acetate, and arabinitol acetate were 14.8, 24.6, 26.6, and 34.5 min, respectively.

Stepwise Hydrolysis with Acid——The polysaccharide (10 mg) was heated with 5 mM sulfuric acid (5 ml) for 5 h at 100 °C. The resulting solution was dialyzed against distilled water, then the internal solution was treated with 50 mM sulfuric acid (5 ml) for 4 h at 100 °C, and dialyzed. Each dialyzable fraction was analyzed by PPC and GLC, as described above. The second non-dialyzable fraction was successively hydrolyzed with 90% formic acid for 5 h at 100 °C, and which 2 M TFA for 5 h at 100 °C. The hydrolyzate was analyzed in the same manner as above.

^{13}C-NMR Spectroscopy——^{13}C-NMR spectra were recorded with a JEOL GX-270 spectometer operated at

67.7 MHz in the pulsed, Fourier-transform mode with proton decoupling, for a solution in D_2O (43 mg) at 70 °C. The chemical shifts were obtained by the use of tetramethylsilane as an external standard.

Quantitative Precipitation with Con A——Solutions (0.5 ml) of CI-5N at various concentrations were incubated with a solution (0.5 ml) of Con A (1.5 mg) for 24 h at 25 °C, using 50 mM Tris–HCl buffer containing 1 M NaCl (pH 7.0). After incubation, each reaction mixture was centrifuged for 15 min at 3000 rpm. The precipitate was washed twice with the same buffer, then dissolved in 50 mM HCl–KCl, pH 1.8 (2 ml), and the sugar content was determined by the phenol-sulfuric acid method.[21]

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