

Polysaccharides in Fungi. XXIV.¹⁾ A (1→3)- β -D-Glucan from the Alkaline Extract of the Insect-Body Portion of Chán huā (Fungus: *Cordyceps cicadae*)

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A water-insoluble glucan (CI-6P), $[\alpha]_D^{20} + 7.3^\circ$ ($c = 0.30$, 0.5 M sodium hydroxide), was isolated from the alkaline extract of the insect-body portion of Chán huā (Chinese name) (fungus: *Cordyceps cicadae*), and its molecular weight was estimated by gel filtration to be ca. 21000. From the results of methylation analysis, periodate oxidation, Smith degradation, enzymic hydrolysis, partial acid hydrolysis, and carbon-13 nuclear magnetic resonance spectroscopy, it was concluded that CI-6P was composed of a backbone of β -(1→3)-linked D-glucopyranosyl residues, and side chains of a single, β -(1→6)-linked D-glucopyranosyl group attached, on average, to every 25th residue of the backbone. CI-6P and its carboxymethylated glucan exhibited antitumor activity against sarcoma 180 in mice.

Keywords (1→3)- β -D-glucan; Chán huā; *Cordyceps cicadae*; polysaccharide structure; antitumor activity; ¹³C-NMR; carboxymethylation

Previously, we elucidated the structural features of two water-soluble galactomannans (CI-A and CI-P)²⁾ and a galactoglucomannan (CI-5N)³⁾ from the insect-body portion of Chán huā⁴⁾ (fungus: *Cordyceps cicadae*); these polysaccharides showed different affinities for concanavalin A. We have now isolated a water-insoluble β -D-glucan (CI-6P) from the alkaline extract of the insect-body portion. The present paper deals with the structural features of CI-6P, and with the antitumor activity of CI-6P and its carboxymethylated glucan (CI-6P-CM).

The insect-body portion was successively extracted with hot methanol, aqueous solutions containing protease and lysozyme, hot water, 3% sodium carbonate, and 1 M sodium hydroxide at room temperature as reported previously.³⁾ The residue was then extracted with 1 M sodium hydroxide at 65°C. The alkali-soluble extract was deproteinized by protease treatment and the Sevag procedure, and subjected to ethanol precipitation and dialysis. The water-insoluble material in the non-dialyzable fraction was collected, dispersed in water, and lyophilized to give the polysaccharide (CI-6P). The polysaccharide showed a symmetrical elution pattern in gel filtration on Toyopearl HW-55 with 0.5 M sodium hydroxide, as shown in Fig. 1. CI-6P was composed of D-glucosyl residues, as shown by paper partition chromatography (PPC) of the hydrolyzate and also by gas-liquid chromatography (GLC) of the alditol acetate prepared from the hydrolyzate. The glucan had $[\alpha]_D^{20} + 7.3^\circ$ ($c = 0.30$, 0.5 M sodium hydroxide), and by gel filtration on Toyopearl HW-55 with standard dextrans, its molecular weight was estimated to be ca. 21000 (see Fig. 2).

The glucan was methylated by the method of Hakomori,⁵⁾ and the fully methylated glucan was hydrolyzed with acid. The partially O-methylated sugars were analyzed as the alditol acetate derivatives by GLC and GLC-mass spectrometry (MS), and identified by comparing their retention times with those of authentic samples or the values⁶⁾ in the literature, and from MS analyses. Table I shows the results of GLC and GLC-MS, in which D-glucopyranosyl residues were present mainly in (1→3)-linked form, together with small proportions of non-reducing, terminal residues and (1→3,6)-linked branching residues, and a trace of (1→6)-linked glucose. On periodate oxidation, CI-6P consumed only 0.15 mol of periodate per

glucosyl residue, and the Smith degradation (sequential periodate oxidation, borohydride reduction, and acid hydrolysis) gave 1.0 mol of glycerol and 16.2 mol of glucose. The glycerol must have arisen from the terminal glucosyl residues, and the occurrence of glucose must be due to the presence of (1→3)-linked glucose. The results are in good agreement with the methylation analysis.

The glucan was digested with *exo*-(1→3)- β -D-glucanase (lysing enzymes), and glucose and gentiobiose were identified as the enzymic degradation product by PPC and GLC.

The glucan was partially hydrolyzed with 50% (v/v)

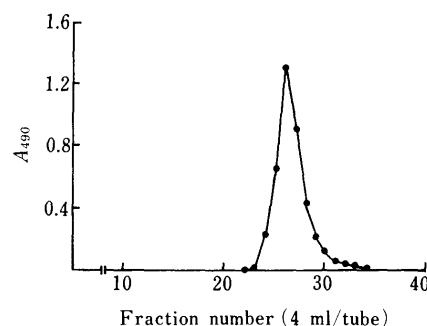


Fig. 1. Chromatogram of CI-6P on Toyopearl HW-55

The column (1.5×98cm) was eluted with 0.5 M NaOH, and carbohydrate was assayed by the phenol-sulfuric acid method.

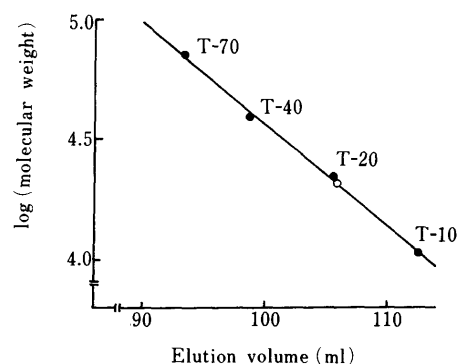


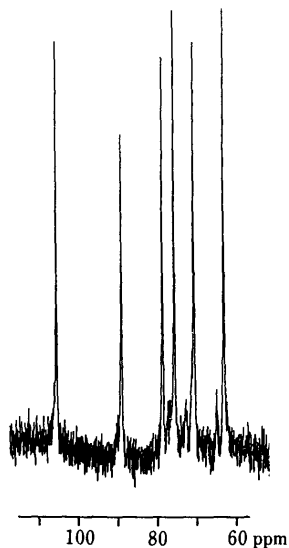
Fig. 2. Determination of Molecular Weight of CI-6P by Gel Filtration on Toyopearl HW-55 with Standard Dextrans

○, CI-6P; ●, standard dextrans (molecular weight) T-70 (70000), T-40 (39500), T-20 (22300), and T-10 (10400).

TABLE I. GLC^{a)} and GLC-MS Data for the Partially Methylated Alditol Acetates

Methylated sugar (as alditol acetate)	Relative retention time ^{b)}	Main mass-fragment (<i>m/z</i>)	Molar ratio	Mode of linkage
2,3,4,6-Me ₄ -Glc	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	0.05	[Glc _p]1 →
2,4,6-Me ₃ -Glc	1.80	43, 45, 87, 101, 117, 129, 161, 233	1.00	→3[Glc _p]1 →
2,3,4-Me ₃ -Glc	2.21	43, 87, 99, 101, 117, 129, 161, 189, 233	0.01	→6[Glc _p]1 →
2,4-Me ₂ -Glc	3.98	43, 87, 117, 129, 189	0.04	→ ⁶ ₃ [Glc _p]1 →

a) Column: 3% OV-225 on Chromosorb W at 185 °C. b) Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Fig. 3. ¹³C-NMR Spectrum of CI-6P in 0.5 M NaOD

sulfuric acid at 4 °C to give laminarabiose and gentiobiose as disaccharides. Identification of laminarabiose and gentiobiose indicates the presence of β-(1→3)-linked glucosyl and β-(1→6)-linked glucosyl residues, respectively. In spite of the small amount of (1→6)-linkages in the glucan, gentiobiose was clearly detected because (1→6)-linkages are more stable than (1→3)-linkages in mineral acids.

CI-6P showed characteristic absorbance at 890 cm⁻¹ in the infrared spectrum. The carbon-13 nuclear magnetic resonance (¹³C-NMR) spectrum of CI-6P is shown in Fig. 3. The resonances at 106.18, 89.67, 79.63, 76.40, 71.43, and 63.92 ppm are assigned to C-1, C-3, C-5, C-2, C-4, and C-6 of (1→3)-linked β-D-glucopyranosyl residues, respectively. The ¹J_{CH} was found by the insensitive nuclei enhanced by polarization transfer (INEPT) method to be 163.3 Hz. The results indicate the presence of β-(1→3)-linked D-glucopyranosyl residues, and the spectrum of CI-6P (less branched (1→3)-linked D-glucan) was similar to that¹⁾ of curdian-type polysaccharide (no branched (1→3)-β-D-glucan), whereas (1→3)-β-D-glucan having more (1→3,6)-branched residues shows a triplet signal⁷⁾ at substituted C-3.

The foregoing data indicate that the glucan CI-6P, isolated from the alkaline extract of Chán huā, has a main chain composed of β-(1→3)-linked D-glucopyranosyl residues, and side chains of a single, β-(1→6)-linked D-glucopyranosyl group attached, on average, to every 25th residue of the main chain. Thus, we elucidated that the insect-body portion of Chán huā contained a water-

TABLE II. Antitumor Activity of the Polysaccharides against Sarcoma 180 Solid Tumor

Sample	Dose (mg/kg/d × 10)	Mean tumor wt. ± S.D. (g)	Inhibition ratio (%)	Complete regression
CI-P	10	2.28 ± 2.50 ^{a)}	74	1/8
	20	5.28 ± 3.02	40	0/8
CI-6P-CM ^{b)}	10	4.42 ± 6.43	50	0/8
	20	2.54 ± 3.90 ^{a)}	71	0/8
Control	—	8.83 ± 8.13	—	0/17

a) Significant difference from the control, *p* < 0.005. b) Carboxymethylated product (DP: 0.736) of CI-6P.

insoluble and alkali-soluble (1→3)-β-D-glucan (CI-6P), in addition to two water-soluble galactomannans (CI-A and CI-P) and a water-soluble galactoglucomannan (CI-5N).

CI-6P has a structural resemblance to various less-branched or unbranched, alkali-soluble, and water-insoluble glucans,⁸⁾ such as pachyman from *Poria cocos*,⁹⁾ curdian from *Alkaligenes faecalis*,⁹⁾ the glucan (G-A) from *Ganoderma japonicum*,¹⁰⁾ and paramylon from *Peranema trichophorum*,¹¹⁾ *Astasis ocellata*,¹²⁾ and *Euglena gracilis*.¹³⁾ However, CI-6P was different from these glucans in the molecular weight and the degree of branching.

The conformation of (1→3)-β-D-glucans has been discussed on the basis of the visible absorption spectra of the complex formed with Congo Red at various concentrations of alkali, as previously reported.¹⁴⁾ The value of the absorption maximum (λ_{\max}) of Congo Red (488 nm) was shifted to longer wavelength (495 nm) in the presence of CI-6P at low alkali concentration (0.1 M sodium hydroxide), but the λ_{\max} was not shifted in high alkaline solution (0.25 M sodium hydroxide). The results suggest that CI-6P had an ordered, triple helical structure in weakly alkaline solution.

Many (1→3)-β-D-glucans have been reported to exhibit antitumor activity against the solid form of sarcoma 180 implanted in mice as previously described,¹⁴⁾ and Sasaki *et al.*¹⁵⁾ have reported that the antitumor activity of the glucans was enhanced by carboxymethylation. The antitumor activities of CI-6P (water-insoluble) and its carboxymethylated glucan (CI-6P-CM) (water-soluble) against sarcoma 180 in mice are listed in Table II. CI-6P at a dose of 10 mg/kg/d and CI-6P-CM at a dose of 20 mg/kg/d showed significant activity, but CI-6P-CM having a degree of substitution (DS) of carboxymethyl groups of 0.736¹⁶⁾ did not show increased the antitumor potency. It has been reported^{15,17)} that the antitumor activity of carboxymethylated (1→3)-β-D-glucans relates to the DS of carboxymethyl groups. The DS and location of carboxymethyl groups on CI-6P-CM may not be best suited for the antitumor

activity. Studies on DS and location of carboxymethyl groups in some (1→3)- β -D-glucans including CI-6P will be reported in the near future.

Experimental

General Gel filtration, determination of the component sugars, methylation analysis, periodate oxidation, Smith degradation, and antitumor activity test were conducted as previously reported.¹⁴⁾ GLC for disaccharides was performed on a Shimadzu GC-4CM apparatus, using a glass column (0.3 × 200 cm) packed with 2% Silicon DC QF-1 on Chromosorb W (80–100 mesh) at 205 °C and with at a flow rate of 60 ml/min of nitrogen. ¹³C-NMR spectra were recorded with a JEOL GX-270 spectrometer operating at room temperature in the Fourier-transform mode with complete proton decoupling for a solution in 0.5 M NaOD (30 mg/0.5 ml), and 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) was used as an external standard.

Isolation and Purification The dried, pulverized insect-body portion of Chán huá (81 g) was successively extracted with hot methanol, aqueous solution containing protease, 0.1 M phosphate buffer containing lysozyme, hot water, 3% sodium carbonate, and 1 M NaOH at room temperature, as previously reported.³⁾ The residue was extracted 3 times with 1 M NaOH (1 l) for 3 h at 65 °C under a nitrogen atmosphere. The alkaline suspension was centrifuged, and the extracts were made neutral with dilute HCl, then dialyzed against distilled water. The insoluble materials in the non-dialyzable fraction were collected by centrifugation, and dissolved in 0.5 M NaOH. The solution was made neutral, deproteinized²⁾ by protease (Actinase E, Kaken Kogyo Co., Tokyo) digestion and the Sevag procedure, and precipitated by addition of 3 volumes of ethanol. The precipitate collected by centrifugation was redissolved in 0.5 M NaOH, and the solution was neutralized, then dialyzed. The insoluble material in the non-dialyzable fraction was collected by centrifugation, washed with water, dispersed in water, and lyophilized to afford the purified polysaccharide (CI-6P), in 1.4% yield.

Enzymic Hydrolysis CI-6P (5.0 mg) was treated with *exo*-(1→3)- β -D-glucanase (lysing enzymes, Sigma, 2.5 mg) in 17 mM McIlvaine buffer, pH 4.90 (20 ml), for 72 h at 38 °C. The reaction mixture was heated for 15 min at 100 °C, and dialyzed against distilled water. The dialyzable solution was concentrated, and passed through small columns of Amberlite CG-120 (H⁺) and Dowex 1 × 8 (OH⁻). A part of the eluate was analyzed by PPC using the solvent system of 1-butanol–pyridine–water (v/v), 6:4:3, as described in a previous paper.¹⁾ A part of the eluate was applied to a column (1.5 × 97 cm) of Bio-gel P-2, and eluted with water, then fractions (1.2 ml each) were collected. Disaccharide fraction (fraction number, 92–98) was reduced with NaBH₄, and the disaccharide-alcohol was trimethylsilylated,³⁾ and analyzed by GLC. The peak at the retention time of 62.8 min was identified as the derivative of gentiobiose.

Partial Acid Hydrolysis CI-6P (4.8 mg) was partially hydrolyzed, with stirring, with 50% (v/v) H₂SO₄ for 16 h at 4 °C and for 1 h at 35 °C, and the reaction mixture was neutralized with BaCO₃, and passed through a column of Amberlite CG-120 (H⁺). The partial acid hydrolyzate was applied to a column of Bio-gel P-2, disaccharide fraction was collected, and the disaccharides were analyzed as disaccharide-alcohol derivatives by

GLC, as already described. The derivatives of laminarabiose and gentiobiose were detected, in the molar ratio of 1.9:1.0, at 40.8 min and 62.8 min, respectively.

Interaction with Congo Red in Aqueous Sodium Hydroxide CI-6P (1 mg/ml) was dissolved in sodium hydroxide solution (0.1 and 0.25 M) containing Congo Red (0.1 mM), and λ_{\max} was measured using a Hitachi 323 spectrometer.

Carboxymethylation The glucan was carboxymethylated according to the method of Sasaki *et al.*¹⁵⁾ CI-6P (262 mg) was suspended in 2-propanol (7 ml) with stirring at room temperature for 30 min. Then a 30% solution of NaOH was slowly added with stirring over a period of 60 min, and vigorous stirring was continued for 90 min. Monochloroacetic acid (315 mg) was added, the mixture was filtered, and the residue was successively washed with methanol–acetic acid (7:3), 80% aqueous methanol, methanol and acetone. The washed residue was dried under reduced pressure, and the carboxymethylated glucan (CI-6P-CM) (284 mg) was obtained by dialysis and lyophilization. The DS value of CI-6P-CM was determined to be 0.736 by the method previously reported.¹⁶⁾

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