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MINOR, PROTEIN-CONTAINING GALACTOMANNANS FROM THE INSECT-BODY PORTION OF THE FUNGAL PREPARATION CHÁN HUĀ (*Cordyceps cicadae*)*

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ABSTRACT

Two minor, water-soluble, protein-containing galactomannans, CI-P ($[\alpha]_D$ +15.3°) and CI-A ($[\alpha]_D$ +10.6°), isolated from the insect-body portion of the fungal preparation Chán huā (*Cordyceps cicadae*) showed single peaks on gel filtration, and one spot on glass-fiber paper electrophoresis. The polymers are composed of D-mannose and D-galactose in the molar ratios of 1:0.85 for CI-P and 1:0.57 for CI-A, together with traces of D-glucose, and small proportions of protein. Although both polysaccharides have a molecular weight of ~25,000, the affinity of CI-A for Con A was stronger than that of CI-P. From the results of methylation analysis, Smith degradation, stepwise hydrolysis, and ^{13}C-n.m.r. spectroscopy, it was concluded that the polysaccharides are highly branched, with structures composed of (1→6)-linked α-D-mannopyranosyl main chains, having most residues substituted at O-2 with single α- or β-D-galactofuranosyl groups, short chains of (1→2)-linked β-D-galactofuranosyl residues, or chains of (1→2)-linked α-D-mannopyranosyl residues. CI-A has fewer and shorter D-galactofuranosyl side-chains and longer (1→2)-linked α-D-mannopyranosyl side-chains than CI-P.

INTRODUCTION

Cordyceps cicadae Shing (Ascomycetes) is a fungus parasitic on the larva of *Cicada flammata* Dist. A preparation comprising the fungal fruit body and the insect body is called Chán huā (the Chinese name). We have already reported on the structure of a water-soluble galactomannan[2] (C-3, $[\alpha]_D$ +30°) from the fruiting bodies of the fungus, and have now isolated two minor, protein-containing galactomannans (CI-P and CI-A), showing different affinities for Con A, from the insect-

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body portion of Chán huā. The present paper deals with the purification, characterization, and structural analysis of these galactomannans, and their interaction with Con A.

RESULTS AND DISCUSSION

In the step of purification of the aqueous extract from the insect-body portion of Chán huā, the polymer in the neutral fraction obtained on DEAE-Sephadex A-25 chromatography was still not homogeneous as judged by electrophoresis. Therefore, the crude polymer was purified by affinity chromatography on Con A–Sepharose 4B. The polymers CI-P and CI-A were obtained from the non-adsorbed and the adsorbed fractions, respectively. Each showed one spot in glass-fiber paper electrophoresis and a homogeneous pattern in gel filtration on Sephadex G-100.

The polymers CI-P and CI-A had $[\alpha]_D$ +15.3° (*c* 0.39, water), and $[\alpha]_D$ +10.6° (*c* 0.37, water), respectively, and their component sugars were D-mannose and D-galactose in the molar ratios of 1:0.85 in CI-P and 1:0.57 in CI-A, together with a trace of D-glucose. The absolute configurations of the sugars were determined by the method of Leontein *et al.*[3]. The molecular weight of both polymers was ~25,000, as estimated by gel chromatography on a column of Sephadex G-100 calibrated with standard dextrans (see Fig. 1).

Each galactomannan was fully methylated by the method of Hakomori[4], and the product was hydrolyzed. The hydrolyzates were analyzed as the alditol acetates

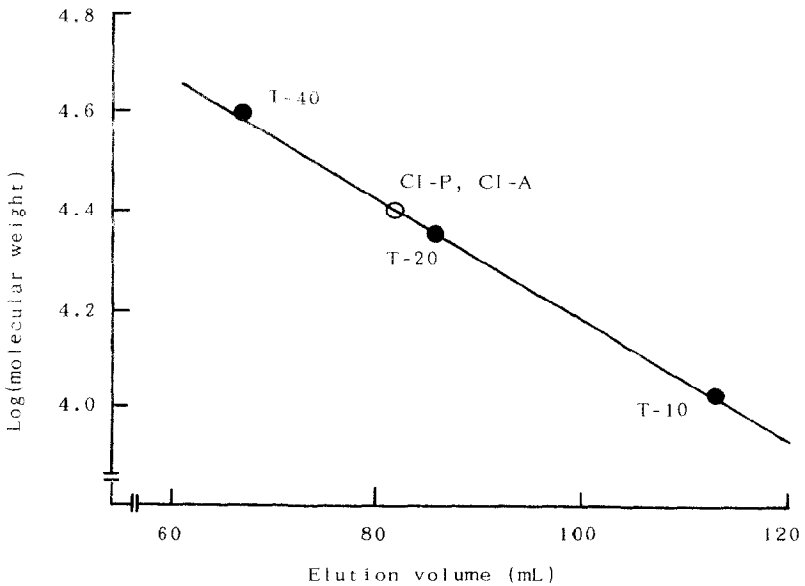


Fig. 1. Determination of the molecular weights of CI-P and CI-A. The molecular weights of the standard dextrans are: T-40, 39,500; T-20, 22,300; and T-10, 10,400.

TABLE I

G.L.C. AND G.L.C.–M.S. DATA FOR THE ALDITOL ACETATES DERIVED FROM THE METHYLATED POLYSACCHARIDES

Methylated sugar (as alditol acetate)	T^a	*Primary mass fragments* (m/z)	*Proportions (mol %)*		*Linkage indicated*
			CI-P	*CI-A*	
2,3,4,6-Me_4-Man	1.00	45, 117, 161, 205	1.7	5.6	Man*p*-(1→
2,3,5,6-Me_4-Gal	1.12	45, 59, 89, 117, 205	33.0	22.6	Gal*f*-(1→
3,4,6-Me_3-Man	1.89	45, 161, 189	2.8	22.6	→2)-Man*p*-(1→
3,5,6-Me_3-Gal	2.17[b]	45, 59, 89, 189, 205, 305	21.5	13.6	→2)-Gal*f*-(1→
2,3,4-Me_3-Man	2.42[c]	117, 161, 189, 233	3.5	7.9	→6)-Man*p*-(1→
3,4-Me_2-Man	5.16	189	32.2	26.0	→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. [b]Unresolved peak including a small proportion of 2,3,6-Me_3-Man. [c]Unresolved peak including a small proportion of 2,3,6-Me_3-Gal

by gas–liquid chromatography (g.l.c.) and g.l.c.–mass spectrometry (g.l.c.–m.s.), and identified as previously described[1]. Table I shows the results of the methylation analysis, in which the peaks having retention times of 2.17 and 2.42 min were found by mass-fragmentation patterns to contain small proportions of 2,3,6-tri-*O*-methylmannitol and 2,3,6-tri-*O*-methylgalactitol derivatives, respectively. Galactosyl residues were prominently detected as 2,3,5,6-tetra-*O*-methyl and 3,5,6-tri-*O*-methyl derivatives, which indicate the presence of nonreducing end-groups and (1→2)-linked residues of the furanosyl type. Mannose was found to be present mainly as nonreducing, (1→2)-linked, (1→6)-linked, and (1→2,6)-linked pyranosyl residues.

Periodate oxidations of CI-P and CI-A were conducted, with monitoring by the Fleury–Lange method[5]. CI-P and CI-A consumed 1.32 mol and 1.42 mol of periodate per hexosyl residue, respectively. The oxidized polysaccharides were treated with sodium borohydride, and the resulting polyalcohols were hydrolyzed with acid. The hydrolyzates were analyzed, by g.l.c., as the alditol acetate derivatives and the trimethylsilyl ethers of oxime derivatives. Glycolaldehyde, glycerol, glyceraldehyde, erythritol (small proportion), threitol (small proportion), and arabinose were revealed in each hydrolyzate. The occurrence of arabinose and glyceraldehyde confirmed the presence of (1→2)-linked galactofuranosyl residues and (1→2)-linked mannopyranosyl residues, respectively.

Polymers CI-P and CI-A were hydrolyzed stepwise with 5mM sulfuric acid for 5 h at 100°, and then with 50mM sulfuric acid for 4 h at 100°. In the first stage, only galactose and galacto-oligosaccharides were detected in the dialyzable fraction. In the second stage, galactose and mannose in the molar ratios of 6:1 for CI-P and 4:1 for CI-A were released as the free sugars. The nondialyzable fractions of the acid-degraded polysaccharides were found by methylation analyses to consist mainly of (1→2)-linked and (1→6)-linked mannopyranosyl residues (1:5 in degraded CI-P

and 1:0.9 in degraded CI-A). On mild hydrolysis, the proportion of (1→6)-linked mannopyranosyl residues increased, and (1→2,6)-linked branching residues disappeared. In the native polysaccharides, the molar ratios of (1→2)-linkage and (1→6)-linkage were 1:1.25 in CI-P, and 1:0.35 in CI-A, respectively. These results suggest that CI-P and CI-A have a core consisting of a (1→6)-linked mannopyranosyl main chain with attached (1→2)-linked mannopyranosyl residues, and side chains of galactofuranosyl residues.

Partial ^{13}C-n.m.r. spectra of CI-P and CI-A are shown in Fig. 2. The signals were assigned by comparing the spectra with data in the literature[2,6,7]. The two lines at lower field, 106.9 and 105.1 p.p.m., probably represent nonreducing (terminal) and (1→2)-linked β-D-galactofuranosyl residues, respectively[8]. The intense signals at 101.6 and 101.5 p.p.m. correspond to 2-*O*-substituted α-D-mannopyranosyl residues or nonreducing α-D-galactofuranosyl residues, or both. As shown by the methylation analysis, the proportion of (1→2)-linked D-mannopyranosyl residues in CI-P is very low, but, in CI-A, is about equal to nonreducing terminal galactofuranosyl residues. Therefore, the bulk of these signals in CI-P are probably due to the nonreducing α-D-galactofuranosyl residues. The resonance at 100.8 p.p.m. is attributed to (1→6)-linked α-D-mannopyranosyl units, and that at 97.8 p.p.m. to (1→2,6)-linked α-D-mannopyranosyl residues. The assignment of the α-anomeric configuration to the mannopyranosyl residues is supported by $J_{C\text{-}1,H\text{-}1}$ values[9] of 178–179 Hz.

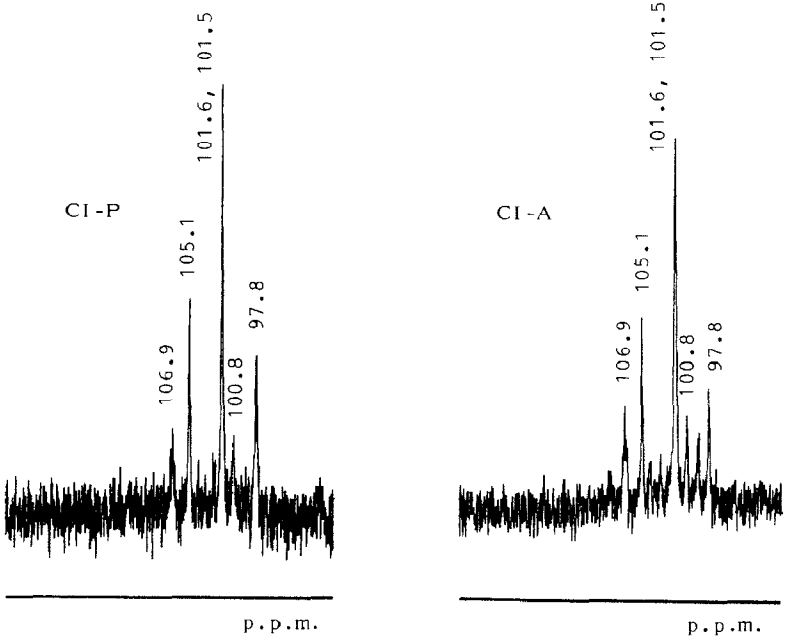


Fig. 2. ^{13}C-N.m.r. spectra (anomeric region) of CI-P and CI-A.

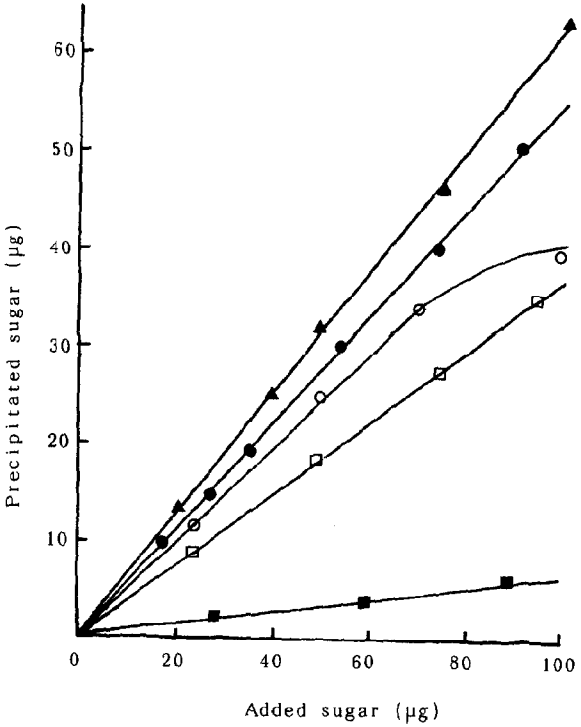
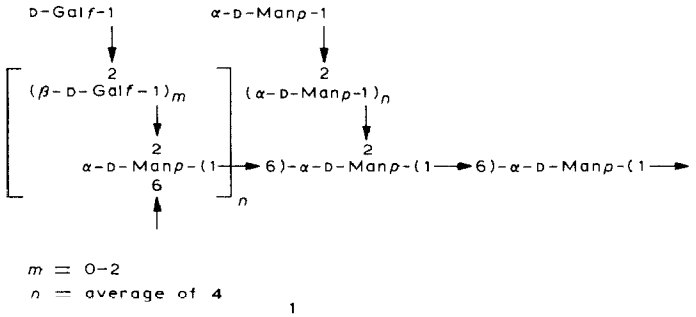


Fig. 3. Quantitative precipitation curves for Con A with CI-P (—■—), degraded CI-P (—□—), CI-A (—●—), degraded CI-A (—○—), and C-3 (—▲—).

CI-A, adsorbed by affinity chromatography, gave one precipitation line on double-diffusion against Con A in agar gel, but CI-P did not show this behavior. The quantitative precipitation curves of CI-P and CI-A against Con A are shown in Fig. 3. The activity of CI-A was much higher than that of CI-P. Acid degradation of CI-P by the stepwise hydrolysis procedure raised its affinity, but the affinity of the acid-degraded CI-A was little different from that of CI-A in lower concentration. The results suggest that the galactofuranosyl side-chains interfere with the affinity for Con A; that is, the side chains mask such critical, lectin-binding residues as (1→2)-linked mannopyranosyl units.

The foregoing data indicate that polysaccharides CI-A and CI-P are highly branched galactomannans, composed of (1→6)-linked α-D-mannopyranosyl main-chains, most of which are substituted at O-2 with short chains of (1→2)-linked α-D-mannopyranosyl units, short chains of (1→2)-linked β-D-galactofuranosyl residues, or single α- and β-D-galactofuranosyl groups. It is suggested that CI-A has the average repeating unit shown in **1**. CI-P has more, and longer, side chains of galactofuranosyl residues, and shorter side chains of (1→2)-linked α-D-mannopyranosyl residues.



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The polymers contained small proportions of protein (nitrogen by elementary analysis 1.96% in CI-P, 1.93% in CI-A), which could not be removed by the Sevag procedure[10], by gel filtration on Sephadex G-100, or by anion-exchange chromatography on DEAE-Sephadex A-25. The amino acid compositions of CI-P and CI-A are shown in Table II, but it is not sure whether the protein moieties are associated with, or linked to, the galactomannans. The large proportions of the hydroxylamino acids serine and threonine in both preparations are noteworthy.

We earlier reported on the structure of a galactomannan (C-3) from fruiting bodies of *C. cicadae*[2] (Ascomycetes). The galactomannans (C-3, CI-P, and CI-A) which we have isolated from the fungus have a core of α-D-mannopyranosyl residues, and D-galactofuranosyl side-chains. C-3 resembles CI-A (adsorbed to Con A) more than it does CI-P. However, CI-P and CI-A have more highly branched

TABLE II

AMINO ACID COMPOSITION OF CI-P AND CI-A

Amino acid	*Proportions (mol %)*	
	CI-P	*CI-A*
Threonine	22.1	24.7
Serine	19.5	20.6
Proline	13.2	13.3
Glycine	11.8	8.7
Alanine	11.4	9.2
Aspartic acid	5.6	4.0
Valine	4.6	6.3
Glutamic acid	3.6	6.4
Lysine	1.9	1.1
Leucine	1.4	1.3
Isoleucine	1.2	1.6
Histidine	1.2	0.9
Arginine	0.9	0.7
Cysteine	0.6	0.3
Tyrosine	0.4	0.9
Phenylalanine	—	0.5

structures than C-3, with CI-P having more nonreducing terminal galactofuranosyl residues. C-3 bound to the Con A-Sepharose 4B, and the affinity for Con A was stronger than those of CI-A and CI-P, as shown in Fig. 3. The affinities had a proportional relationship to the content of (1→2)-linked D-mannopyranosyl residues (C-3, 33.1%; CI-A, 22.6%; and CI-P, 2.8%). A minor, protein-containing galactomannan (CT-4N)[11], $[\alpha]_D$ −29.6°, from *C. sinensis* also has a core of mannopyranosyl residues and galactofuranosyl side-chains, but the side chains consist of (1→5)-linked β-D-galactofuranosyl residues instead of the (1→2)-linkage. The occurrence of D-galactopyranosyl residues in galactomannans has been reported in many fungi[12], but the pyranose type could not be found in CI-P and CI-A. The presence of unusual polysaccharides having the galactofuranosyl residues in *Cordyceps* species may be due to the need to overcome a protection system of the host insect, *e.g., Cicada flammata.* The present data are of interest in connection with a role of α-D-galactofuranosyl residues in polysaccharides, for there are few descriptions of galactomannans having both α-D- and β-D-galactofuranosyl residues. The data also reveal subtle differences in interaction of the polymers with a lectin, and show the variation in structures of the polysaccharides from the fruit-body and the insect-body portions, respectively, of Chán huā.

EXPERIMENTAL

The general experimental methods have been reported previously[11].

Materials. — The dried Chán huā was obtained from commercial sources in Hong Kong. Sephadex G-100, DEAE-Sephadex A-25, Con A–Sepharose 4B, Con A, and standard dextrans (dextran T-40, T-20, and T-10) were purchased from Pharmacia Fine Chemicals. Protease (Pronase E) was purchased from Kaken Kogyo Co., Tokyo.

Isolation of the polysaccharides. — The insect-body portion (87 g) of Chán huā was pulverized, and extracted with hot methanol. The residue was suspended in an aqueous solution (1.5 L), adjusted to pH 8.0, and treated with Pronase E (80 mg) for 24 h at 37°, and the procedure was repeated 3 times. The aqueous extract collected by centrifugation was dialyzed against distilled water. The non-dialyzable fraction was deproteinized by the Sevag procedure[10]. To the aqueous solution was added ethanol (3 vol.), and the resulting precipitate was further purified by column chromatography on DEAE-Sephadex A-25 (acetate) resin. The neutral fraction was dialyzed, and lyophilized, to afford crude polysaccharide in 1.8% yield. A solution of the crude polysaccharide was applied to a column (3 × 17 cm) of Con A–Sepharose 4B, which was first eluted with 0.05M Tris·HCl buffer (pH 7.0), and then with 0.1M methyl α-D-mannopyranoside. The elution was monitored spectrophotometrically at 206 nm, and the fractions were analyzed by the phenol–sulfuric acid method[13]. The non-adsorbed (first) and adsorbed (second) fractions were separately dialyzed and lyophilized, to afford CI-P (yield, 0.7%) and CI-A (yield, 0.7%).

Gel filtration. — The sample (1.8 mg) in 0.1M sodium chloride (0.5 mL) was applied to a column (1.5 × 90 cm) of Sephadex G-100, and eluted with the same solvent. An aliquot of each fraction (4 mL) was analyzed by the phenol–sulfuric acid method[13]. The column was calibrated with standard dextrans as previously described[11].

Electrophoresis. — Glass-fiber paper electrophoresis was conducted on Whatman GF-81 glass-fiber paper (9 × 40 cm) in 0.05M sodium tetraborate buffer, pH 9.3, for 3 h at 300 V. The spot was detected with the 1-naphthol–sulfuric acid reagent[14]. Each polymer showed one spot, at distances of 10.5 cm for CI-P and 12.0 cm for CI-A.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded with a JEOL-FX 270 spectrometer operated at 27° in the Fourier-transform mode with complete proton-decoupling, for solutions in D_2O (~50 mg/mL). Tetramethylsilane was used as an external standard.

Analysis of constituent sugars. — Samples were hydrolyzed with M sulfuric acid for 6 h at 100°, and hydrolyzates were analyzed by paper chromatography (p.c.) and by g.l.c. of the alditol acetates, as previously described[11]. The absolute configurations of component sugars were determined by g.l.c. of the acetylated (+)2-octyl glycosides as previously described[11].

Methylation analysis. — Samples (CI-P, CI-A, degraded CI-P, and degraded CI-A) were methylated 4 times each by the method of Hakomori[4], as previously described[11]. The final methylation products showed no hydroxyl absorption in the i.r. spectrum. Each fully methylated polymer was heated successively with 90% formic acid for 6 h at 100°, and 0.25M sulfuric acid for 6 h at 100°. After neutralization of the acid with barium carbonate, the components of the hydrolyzates were converted into their alditol acetates[15]. The mixtures were then analyzed by g.l.c. (column, 3 mm × 2 m; 3% of ECNSS-M on Gaschrom Q; column temperature, 175°; carrier gas, nitrogen at 40 mL/min), and by g.l.c.–m.s., as previously described[11]. The results are shown in Table I.

Periodate oxidation and Smith degradation. — Each sample (10 mg) was oxidized in the dark with 10mM sodium metaperiodate (30 mL) for 5 days at 4°. Methyl α-D-glucopyranoside was used as a reference substance. The periodate consumption, estimated by an arsenite method[5], was 1.32 mol per hexosyl residue for CI-P, and 1.42 mol for CI-A. A part of the polyalcohols prepared from the periodate-oxidized products by reduction with sodium borohydride was hydrolyzed, and the hydrolyzate was separated by g.l.c. as alditol acetates and as trimethylsilyl derivatives, as previously described[11].

Stepwise hydrolysis with acid. — Each sample (10 mg) was heated with 5mM sulfuric acid for 5 h at 100°. The resulting solutions were dialyzed against distilled water, and the retentates were treated with 50mM sulfuric acid for 4 h at 100°, and again dialyzed. Each dialyzate was analyzed by p.c. and g.l.c., as described previously[11]. The final retentates (non-dialyzable fractions) were studied by methylation analyses.

Agar-gel diffusion and quantitative precipitation against Con A. — Agar-gel diffusion studies were conducted according to the procedure described by Goldstein and Hayes[16]. The quantitative precipitation reaction was performed on samples (CI-P, CI-A, degraded CI-P, degraded CI-A, and C-3) by incubation for 24 h at 25° with Con A (1.5 mg), in 0.05M Tris·HCl buffer containing M NaCl, pH 7.0 (1 mL). After incubation, the reaction mixtures were centrifuged for 15 min at 3000 r.p.m., and the precipitates were washed twice the same buffer. The washed precipitates were each dissolved in 2 mL of 0.05M aqueous HCl–KCl (pH 1.8) and analyzed by the phenol–sulfuric acid method[13].

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