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STRUCTURE OF A GALACTOSAMINOGLYCAN FROM *Cordyceps ophioglossoides*

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ABSTRACT

A water- and alkali-insoluble galactosaminoglycan (CO-N), precipitated with ammonium hydroxide from the culture filtrate of *Cordyceps ophioglossoides*, is composed mainly of 2-amino-2-deoxy-D-galactose (80.5%) together with small proportions of glucose, galactose, and mannose, protein (3.6%), and acetyl groups (1%). CO-N was eluted as a single peak in gel filtration, and the average molecular weight was estimated to be ~50,000. Partial, acid hydrolysis of CO-N gave small CO-N and homologous 2-amino-2-deoxy-D-galacto-oligosaccharides. Small CO-N (mol. wt. ~10,000) was soluble in water and composed only of 2-amino-2-deoxy-D-galactose. The results of methylation analysis, ^{13}C-n.m.r. studies, and enzymic hydrolysis indicated small CO-N to be a (1→4)-linked 2-amino-2-deoxy-α-D-galactopyranan, and the ^{13}C-n.m.r. data indicated the glycosidic linkage in the polygalactosamine moiety of CO-N to be the same as that of small CO-N.

INTRODUCTION

Cordyceps sinensis is a fungus that is parasitic on the larvae of Lepidoptera and has been used as a Chinese medicine for eternal youth. Galactomannan is present as the common constituent in such *Cordyceps sp.* as *C. sinensis*[1] and *C. cicadae*[2] which belong to the *Ascomycetes*. Recently, we reported[3] the isolation and the structural analysis of an extracellular glucan (CO-1) from the culture filtrate of *C. ophioglossoides*. This glucan[3] strongly inhibited the growth of the Sarcoma 180 solid-type tumour. We have now isolated a water- and alkali-insoluble galactosaminoglycan (CO-N) from the same culture filtrate and report on its structure.

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EXPERIMENTAL

Materials. — α-*N*-Acetylgalactosaminidase (2-acetamido-2-deoxy-α-D-galactoside acetamidodeoxygalactohydrolase) from *Charonia lampas* and β-*N*-acetylhexosaminidase (2-acetamido-2-deoxy-β-hexoside acetamidodeoxyhexohydrolase) from Jack-bean were purchased from Seikagaku Kogyo Co., Ltd. (Japan). Bio-Gel P-2 (200–400 mesh) and P-4 (–400 mesh) were obtained from Bio-Rad, and Toyo Pearl HW55 (fine) from Toyo Soda Co., Ltd. (Japan). SPECTRA/POR-6 cellulose dialysis-tubing was purchased from SPECTRUM Medical Industries Inc. (U.S.A.).

General methods. — Optical rotations were determined at 20° with a JASCO DIP-digital polarimeter. T.l.c. was performed on cellulose-coated plastic sheets (Merck) with ethyl acetate–pyridine–acetic acid–water (5:5:1:3), and detection was effected with alkaline silver nitrate[4]. G.l.c. was performed with a Shimadzu GC-6A instrument equipped with a flame-ionisation detector and a glass column (0.3 × 200 cm) of 1% of Silicone OV-225 on Uniport HP, and with nitrogen as carrier gas at 60 mL/min. Neutral sugars and amino sugars were converted[5] into the corresponding alditol acetates and separated on a column of Silicone OV-225 at 130° → 250° (3°/min). G.l.c.–m.s. was performed with a Hitachi M-80 mass spectrometer equipped with a glass column packed with 3% of ECNSS-M on Uniport HP at 204° and operated at an ionisation voltage of 20 eV with an ion-source temperature of 180°. I.r. spectra were recorded with a JASCO A-3 spectrometer. 2-Amino-2-deoxy-D-galactose was determined by using an amino acid analyser (Hitachi 835) after acid hydrolysis (4M HCl, 100°, 22 h), and also by the indole–hydrochloric acid method[6] and the modified method of Elson and Morgan[7] with 2-amino-2-deoxy-D-galactose as the standard. Protein was estimated from the contents of amino acids measured by using the amino acid analyser after hydrolysis (6M HCl, 110°, 22 h) and also by the method of Lowry *et al.*[8] (with bovine serum albumin as the standard). Neutral sugar was determined by the phenol–sulfuric acid method[9] with D-glucose as the standard. Acetyl groups were determined by g.l.c.[10], with acetic acid as the standard, after hydrolysis (2M HCl, 100°, 2 h).

Polysaccharide CO-N. — (*a*) *Purification. C. ophioglossoides* culture was centrifuged to remove mycelium, charcoal was added to the supernatant solution which was then filtered, and ethanol (1 vol.) was added to the filtrate. The resulting precipitate was collected, dried *in vacuo,* resuspended in 0.01M acetic acid to a concentration of 0.5%, sonicated at 4°, and then centrifuged. The supernatant solution was incubated at 63° for 30 min and then centrifuged to remove a white precipitate (glucan)[3], and aqueous 10% ammonium hydroxide was added to pH 9. The resulting precipitate was redissolved in 0.01M acetic acid and reprecipitated with ammonium hydroxide. The precipitate was washed with water to give purified galactosaminoglycan (CO-N; 1 g/L of culture broth).

(*b*) *Gel filtration.* CO-N solution (10 mg/mL in aqueous 2% acetic acid) was applied to a column (2.0 × 97 cm) of Toyo Pearl HW 55 (fine), equilibrated and

eluted with 0.01M acetic acid containing 0.2M sodium chloride at 24 mL/h. The contents of hexose, 2-amino-2-deoxyhexose, and protein of each fraction (3 mL) were monitored by the phenol–sulfuric acid method[9], the indole–hydrochloric acid method[6], and the absorbance at 280 nm, respectively.

(*c*) *Alkaline degradation*[11]. A suspension of CO-N (11 mg) in M sodium hydroxide containing 0.5M sodium borohydride (1.5 mL) was heated at ~100° for 4 h, the pH was then adjusted to 5, and the mixture was centrifuged. The resulting supernatant solution was applied to a column of Toyo Pearl HW 55.

(*d*) *Partial, acid hydrolysis.* A suspension of CO-N (200 mg) in M hydrochloric acid (80 mL) was heated at 100° for 4 h, neutralised with M sodium hydroxide, and dialysed against distilled water for 3 days. The inner solution was collected and lyophilised. A part of this degraded CO-N (small CO-N) was *N*-acetylated with acetic anhydride–saturated aqueous sodium hydrogencarbonate to give small CO-NAc. Gel filtration of small CO-N was performed under the conditions described above. Alternatively, a suspension of CO-N (500 mg) in 3M hydrochloric acid (100 mL) was heated at 100° for 4 h, concentrated, and neutralised, and the product was *N*-acetylated. The *N*-acetylated products were passed through a combined column of AG 50W-X8 and AG 1-X8 resins, and then applied to a column (2.0 × 185 cm) of Bio-Gel P-2 (200–400 mesh). The fraction eluted in the void volume was concentrated and applied to a column (2.0 × 101 cm) of Bio-Gel P-4 (–400 mesh). Each oligosaccharide was rechromatographed on the same column.

Methylation analyses. — Small CO-NAc, and 2-acetamido-2-deoxy-D-galacto-triose and -hexaose, which were obtained in (*d*), were methylated by the Hakomori procedure[12]. The methylated small CO-NAc and 2-acetamido-2-deoxy-D-galacto-oligosaccharides were hydrolysed[13] with 0.25M sulfuric acid in aqueous 93% acetic acid at 100° for 16 h. Each hydrolysate was passed through a column of AG 3-X4A (AcO^-) resin and then converted[4] into the corresponding alditol acetates.

N.m.r. studies. — The ^{13}C-n.m.r. spectra of CO-N, small CO-N, small CO-NAc, 2-acetamido-2-deoxy-D-galactohexaose, and reference substances were obtained for solutions in 50% CD_3COOD–D_2O at 80° at 25 MHz, using a JEOL PS-100/EC-100 spectrometer operated in the Fourier-transform mode with complete proton decoupling. Chemical shifts are expressed in p.p.m. from the signal of sodium 3-(trimethylsilyl)propanoate-d_4 (TSP).

Enzymic hydrolysis. — Solutions of small CO-NAc and 2-acetamido-2-deoxy-D-galactotriose in 50mM citrate buffer (pH 4.0) were incubated at 37° with α-*N*-acetylgalactosaminidase (*C. lampas*) or β-*N*-acetylhexosaminidase (Jack-bean). Released 2-acetamido-2-deoxy-D-galactose was determined by the modified method of Morgan and Elson[14].

RESULTS

Properties of CO-N. — The alkali-insoluble galactosaminoglycan (CO-

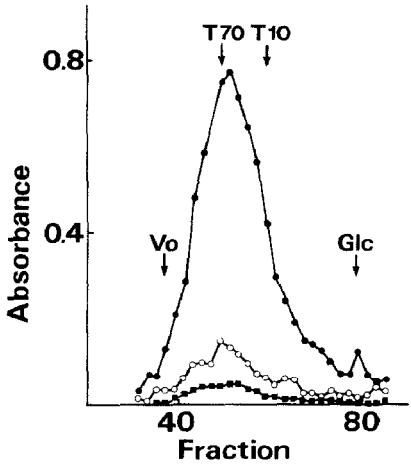

Fig. 1. Gel filtration of CO-N on Toyo Pearl HW 55 (fine): the contents of 2-amino-2-deoxyhexose (—●—, at 492 nm), hexose (—○—, at 490 nm), and protein (—■—, at 280 nm) of each fraction were monitored. T-70 and T-10 refer to dextran standards (mol. wts. 70,000 and 10,000, respectively).

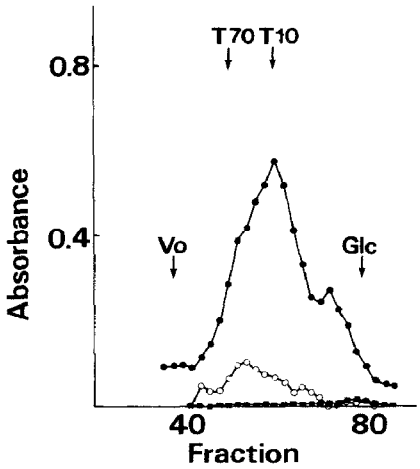

Fig. 2. Gel filtration of the products of alkaline degradation of CO-N on Toyo Pearl HW 55 (fine).

N), precipitated with ammonium hydroxide after removal of glucan (CO-1) from the *C. ophioglossoides* culture fluid, was insoluble in water but soluble in acid. CO-N (*a*) contained 80.5% of 2-amino-2-deoxy-D-galactose, 3.6% of protein (based on amino acid analysis), and 1% of acetyl groups; (*b*) was composed of 2-amino-2-deoxy-D-galactose and small proportions of glucose, galactose, and mannose, which were identified by t.l.c. of an acid hydrolysate and g.l.c. of the alditol acetates prepared after the hydrolysis; (*c*) had $[\alpha]_D^{20}$ +252° (*c* 0.001, 50mM acetic acid); (*d*) showed i.r. absorption at 845 cm^{-1} indicative[15] of α linkages; (*e*) was partially degraded by deamination (5% sodium nitrite in aqueous 20% acetic acid, room temperature, 18 h) with 12% remaining non-dialysable and containing mainly protein and hexose; and (*f*) was absorbed completely on CM-Sepharose.

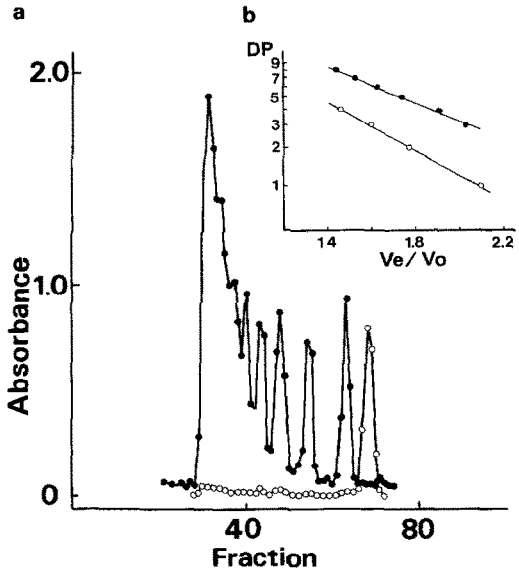

Fig. 3. (a) Fractionation of the *N*-acetylated oligosaccharides derived from CO-N by gel filtration on Bio-Gel P-2 (—●—, 2-amino-2-deoxyhexose at 492 nm; —○—, hexose at 490 nm). The content of 2-amino-2-deoxyhexose was measured[6] after deacetylation (conc. HCl, 100°, 15 min); (*b*) relationship between d.p. and elution volume of the oligosaccharides (—○—, separation on Bio-Gel P-2; —●—, on Bio-Gel P-4).

CO-N was eluted as a single peak (monitoring of amino sugar) in gel filtration on Toyo Pearl HW 55 (fine), and the hexose and protein were also co-eluted with the amino sugar-containing peak (Fig. 1). When CO-N was treated with strong alkali, the elution profile (monitoring of amino sugar) was shifted to the region of lower molecular weight, and the absorbance at 280 nm was decreased on gel filtration (Fig. 2); however, the hexose-containing peak did not coincide with that containing amino sugar, suggesting that the polygalactosamine in CO-N might be linked to protein. The molecular weight of CO-N was estimated by gel filtration to be ~50,000 from the calibration curve obtained using standard dextrans.

Partial, acid hydrolysis of CO-N. — CO-N was treated with M hydrochloric acid at 100° for 4 h, and the non-dialysable portion was recovered and fractionated on Toyo Pearl HW 55. Small CO-N, $[\alpha]_D^{22}$ +324° (*c* 0.005, water), was obtained as a single peak (data not shown). During the hydrolysis, all of the neutral-hexose polymer was hydrolysed to monosaccharides. Small CO-N was soluble in water, contained 96% of 2-amino-2-deoxy-D-galactose, and had a molecular weight of ~10,000 (gel filtration). Treatment of CO-N with 3M hydrochloric acid at 100° for 4 h released oligosaccharides, as shown by gel filtration on Bio-Gel P-2 after *N*-acetylation (Fig. 3a). The fraction eluted in the void volume was further fractionated on Bio-Gel P-4 (data not shown). The 2-acetamido-2-deoxy-D-galacto-oligosaccharides obtained were a homologous series as indicated by the relation between d.p. and elution volume (Fig. 3b).

Methylation analyses. — *N*-Acetylated small CO-N (small CO-NAc), and 2-acetamido-2-deoxy-D-galacto-triose (GalNAc3) and -hexaose (GalNAc6) were methylated, and then hydrolysed with acid, and the products were analysed as the alditol acetates by g.l.c. and g.l.c.–m.s. For GalNAc3, two predominant peaks in g.l.c. were identified as 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl- and 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol, respectively. For small CO-NAc, a major peak of 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol and a minor peak of 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol were detected in g.l.c. GalNAc6 also gave these methylated products. The m.s. fragmentation patterns agreed with these assignments (Table I). The three predominant peaks for reduced GalNAc3 were identified as 1,4-di-*O*-acetyl-2-deoxy-3,5,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol, 1,5-di-*O*-acetyl-2-deoxy-3,4,6-tri-*O*-methyl- and 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol, and 4-*O*-acetyl-2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol as a minor peak. This minor product might be obtained without the 1-*O*-demethylation[16]. The results indicated that GalNAc3, GalNAc6, and small CO-NAc were (1→4)-linked 2-acetamido-2-deoxy-D-galacto-triose, -hexaose, and polysaccharide, respectively.

^{13}C-N.m.r. spectroscopy. — The spectrum of CO-N was obtained for a solution in 50% CD_3COOD–D_2O at 80° (no degradation occurred under these conditions, since, after 18 h, the elution pattern on Toyo Pearl HW 55 did not change), was similar to that of small CO-N in 10% CD_3COOD–D_2O, and contained one signal in the anomeric region at 99.6 p.p.m. (A). The signal of the anomeric carbon atom of small CO-N was shifted 2.4 p.p.m. downfield after *N*-acetylation. The anomeric carbon signal (101.2 p.p.m.) of small CO-NAc corresponded to C-1 of 2-acetamido-2-deoxy-D-galactosyl residues (by comparison with the values of methyl 2-acetamido-2-deoxy-α- and -β-D-galactopyranosides and GalNAc6) as shown in Table II. Signal B (80.0 p.p.m.) for CO-N was assigned to C-4 involved in the glycosidic linkage, because it was downfield by 9 p.p.m. from the signal for C-4 in methyl 2-acetamido-2-deoxy-α-D-galactopyranoside. The signals at 54.7, 69.5, 73.8,

TABLE I

IDENTIFICATION OF PARTIALLY METHYLATED ALDITOL ACETATES FROM GalNAc3, GalNAc6, AND SMALL CO-N

Methylated alditol acetate derivative	T[a]	*Major mass-spectral fragments* (m/z)	*Linkage*
A, 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol	1.0	43, 116, 129, 142, 145, 158, 202, 205	GalNAc-(1→
B, 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol	1.38	43, 116, 129, 142, 158, 202, 233	→4)-GalNAc-(1→

[a]Retention times of the corresponding alditol acetates relative to that of 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol on an ECNSS-M on Uniport HP glass column.

TABLE II

^{13}C-N M R DATA

Sugar	*Chemical shifts (p.p.m.)*								
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*-NHCOCH$_3$*		*O-CH$_3$*
							CH$_3$	*C=O*	
Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside[a]	101.2	52.9	70.8[d]	71.5[d]	73.7	64.3	24.9	177.8	58.1
Methyl-2-acetamido-2-deoxy-β-D-galactopyranoside[a]	105.3	55.1	74.0	70.7	78.0	63.8	24.5	177.9	59.8
GalNAc6[a]	101.2	53.2	69.5	79.2	74.2	62.4	24.7	177.6	
Small CO-NAc[a]	101.2	53.2	69.5	79.1	74.1	62.4	24.7	177.6	
Small CO-N[b]	98.8	53.7	68.7	79.2	73.3	63.0			
CO-N[c]	99.6	54.7	69.5	80.0	73.8	63.9			
	(A)	(F)	(D)	(B)	(C)	(E)			

[a]Solution in D_2O at room temperature. [b]Solution in 10% CD_3COOD–D_2O at room temperature. [c]Solution in 50% CD_3COOD–D_2O at 80°. [d]Assignments may be reversed.

and 63.9 p.p.m. were assigned to C-2,3,5,6, respectively. These assignments accorded with the results of methylation analysis.

Enzymic hydrolysis. — Small CO-NAc and GalNAc3 were treated with α-*N*-acetylgalactosaminidase and β-*N*-acetylhexosaminidase, and the 2-acetamido-2-deoxy-D-galactose released was determined. α-*N*-Acetylgalactosaminidase completely hydrolysed small CO-NAc, whereas β-*N*-acetylhexosaminidase was not effective. For GalNAc3, 65% of the 2-acetamido-2-deoxy-D-galactose was released on treatment with α-*N*-acetylgalactosaminidase, probably because of product inhibition.

DISCUSSION

The galactosaminoglycan (CO-N) from the culture fluid of *C. ophioglossoides* was not sufficiently soluble in methyl sulfoxide and water, so that small CO-NAc and 2-acetamido-2-deoxy-D-galacto-oligosaccharides were used for methylation analysis, ^{13}C-n.m.r. studies, and enzymic hydrolysis. The results of methylation analysis and ^{13}C-n.m.r. spectroscopy indicated that the polygalactosamine part of CO-N contained only (1→4)-linkages. *N*-Acetylated CO-N was resistant to periodate, whereas CO-N was oxidised completely (data not shown). The results of the enzymic hydrolysis and ^{13}C-n.m.r. spectroscopy indicated the glycosidic linkages of small CO-NAc to be α. The ^{13}C resonances of CO-N were similar to those of small CO-NAc, except that of the anomeric carbon. The chemical shift of the signal of the anomeric carbon of CO-N was similar to that of small CO-N rather than that of small CO-NAc. Therefore, CO-N was concluded to be a (1→4)-linked 2-amino-2-deoxy-α-D-galactopyranan which was *N*-acetylated to only a small extent.

Polygalactosamines have been isolated from the culture fluid of *Aspergillus parasiticus*[17], the spherule wall of *Physarum polycephalum*[18], and the cell wall of *A. niger*[19]. The structure of the polygalactosamine moiety in CO-N from *C. ophioglossoides* is similar to those of polygalactosamines from *A. parasiticus*[20] and *P. polycephalum*[21]. Bartnicki-Garcia[22] classified fungi taxonomically on the basis of the cell-wall polysaccharides; *Trichomycetes sp.* were classified as the polygalactosamine–galactan group and *Ascomycetes* as the chitin–glucan group. Our results suggest that polygalactosamine also may be a common constituent of *Ascomycetes* fungi.

Structural analysis of the intact polygalactosamine CO-N is difficult because its solubility is generally very low, but a solution in an acidic solvent was amenable to ^{13}C-n.m.r. spectroscopy. CO-N contained neutral sugar and protein in addition to 2-amino-2-deoxy-D-galactose, and determination of the total structure must await further studies.

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