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Note

Polysaccharides of the sea grass *Phyllospadix torreyi*

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The chemistry and biochemistry of apiose have recently been reviewed[1]. Apiose occurs widely in Nature[1], and was first identified by Bell and co-workers[2] as a component sugar in polysaccharide materials. The occurrence of apiose in the polysaccharide isolated from *Phyllospadix* has been reported[1,3]. The isolation of the polysaccharides from *Phyllospadix torreyi*, their fractionation with hexadecyltrimethylammonium bromide, and some structural features of polysaccharide C-1, are described herein.

TABLE I

DATA ON THE POLYSACCHARIDE FRACTIONS[a] FROM *Phyllospadix torreyi* BY SUCCESSIVE USE OF SOLVENTS

Solvent	*Fraction*	*Recovery*	$[\alpha]^{20}_{D}$	*Neutral sugars*[b]						
(g)		*(g)*	*(degrees)*	*Rha*	*Ara*	*Xyl*	*Api*	*Man*	*Gal*	*Glc*
A Hot water	A-1	0.08	+41	4	3	10	3	2	11	4
	A-2	0.06	−10	5	11	10	5	tr	22	1
	A-3	0.02	−20	tr[c]	21	10	5	1	31	15
	A-4	0.24	+ 2	tr	3	10	2	1	8	16
B 5% Ammonium oxalate	B-1	0.03	−19	tr	13	10	21	tr	25	6
	B-2	—	—	—	—	—	—	—	—	—
	B-3	0.01	−35			n.d.[d]				
	B-4	0.11	−33	1	4	10	2	1	7	9
C 7% Sodium hydroxide	C-1	2.20	+63	8	2	10	4	tr	8	tr
	C-2	1.54	+20	4	1	10	5	tr	4	tr
	C-3	0.45	+17	3	1	10	4	tr	5	4
	C-4	4.47	+15	2	2	10	tr	tr	3	3

[a]Polysaccharides fractionated with hexadecyltrimethylammonium bromide. [b]Mole ratio, relative to xylose = 10. [c]tr, trace. [d]n.d., not determined.

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The polysaccharides have been isolated from a defatted sample of *Phyllospadix torreyi* by successive extraction with hot water (polysaccharide A), 5% ammonium oxalate (polysaccharide B), and 7% sodium hydroxide (polysaccharide C). Each polysaccharide was separated into four fractions with hexadecyltrimethylammonium bromide[4]; the analytical data for these fractions are given in Table I. Apiose, arabinose, galactose, glucose, mannose, rhamnose, and xylose were detected in the hydrolyzates of the polysaccharide fractions. Mannose was generally present in traces, and the apiose content varied from 2 (C-4) to 28% (B-1).

Polysaccharide C-1 is electrophoretically homogeneous, and the neutral part of the acid hydrolyzate contained apiose, L-arabinose, D-galactose, L-rhamnose, and D-xylose. Previous studies[1,3] had shown that apiose has the D configuration. Mannose and glucose were also present in the hydrolyzate of polysaccharide C-1 in traces. Esterification of the acid fraction of C-1, and reduction, followed by g.l.c. analysis (alditol acetates), gave a single component having the same retention time as hexa-*O*-acetylmannitol, indicating the presence of mannuronic acid in polysaccharide C-1. A total acid content of 36% was determined by the carbazole colorimetric method[5]. The neutralization equivalent of polysaccharide C-1 was found to be 496 (35.5% of uronic acid). Polysaccharide C-1 therefore contains L-rhamnose, L-arabinose, D-xylose, D-apiose, D-galactose, and mannuronic acid in the molar ratios of 4:1:5:2:4:9.

Methanolysis of the permethyl ether of polysaccharide C-1, and analysis of the methyl glycosides by g.l.c. gave the products listed in Table II. The presence of methyl 2,3,5-tri- and methyl 2,5-di-*O*-methylarabinosides in the methanolyzate indicates that the arabinose residues in polysaccharide C-1 are in the furanose form. These arabinose residues occur either as nonreducing end-groups or as chain-units substituted at O-3. L-Rhamnose occurs in the pyranose form, and is (1→2)-linked in the polysaccharide chain. D-Xylose is present in the polysaccharide as chain-linked residues, and the D-galactose residues occur in equimolar amounts of chain-units and branch-points. Major peaks, having *T* 3.94, 10.7, and 13.9, were present in the

TABLE II

COMPOSITION[a] OF THE METHANOLYZATE OF PERMETHYLATED POLYSACCHARIDE C-1

Component	T[b]	*Molar ratio*
2,3,5-Tri-*O*-methylarabinose	0.59,0.83	1
2,5-Di-*O*-methylarabinose	1.38,3.15	1
3,4-Di-*O*-methylrhamnose	0.95,1.23	6
2,3-Di-*O*-methylxylose	1.85,2.03 2.28	6
2,3,6-Tri-*O*-methylgalactose	3.94,4.87 5.56,6.39	3
2,4-Di-*O*-methylgalactose	25.7,29.6	3

[a] Methyl ethers of apiose and uronic acid not identified. [b] Column *B*.

gas–liquid chromatogram of the methanolyzate of permethylated polysaccharide C-1. The peak at *T* 3.94 was considerably larger than that expected for methyl 2,3,6-tri-*O*-methyl-D-galactoside. These *T*-values are similar to those found[6] for the methyl ester methyl glycosides of 2,3,4-tri- (*T* 2.78, 3.72) and 2,3-di-*O*-methylglucuronic acid (*T* 11.7, 15.8). It is therefore assumed that these unidentified peaks in the methylation analysis of polysaccharide C-1 arise from the methyl ester methyl glycosides of 2,3-di-*O*-methylmannuronic acid (*T* 10.7, 13.9) and 2,3,4-tri-*O*-methylmannuronic acid (*T* 3.94). These sugar acids were present in the methanolyzate of permethylated polysaccharide C-1 in the molar ratio of 2.8:1. G.l.c. peaks having *T* 2.82, 7.64, 9.21, 16.4, and 18.4 were not identified; they probably result from the methyl ethers of apiose, mannose, and glucose.

Oxidation of polysaccharide C-1 with sodium periodate resulted in the uptake of 1.18 molecules of periodate per sugar residue. The oxidation of polysaccharide C-1 resulted in the liberation of 0.57 molecule of formic acid and 0.08 molecule of formaldehyde per sugar residue. The polyalcohol contained arabinose and apiose in the ratio of 1.3:1, indicating that ~80% of the apiose residues were cleaved by periodate.

EXPERIMENTAL

General. — Extractions, effected on ~10% (w/v) suspensions of the sea grass, were repeated until analysis for carbohydrate showed that the extraction was complete. I.r. spectra were recorded with a Beckman Model IR 5A spectrophotometer. Optical rotations were measured with a Perkin–Elmer Model 141 automatic polarimeter. Gas–liquid chromatography (g.l.c.) was performed with an F and M Model 402 chromatograph, with helium as the carrier gas at a flow rate of ~60 mL/min. Glass columns (120 × 0.4 cm) were packed with (*A*) 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh)[7], or (*B*) 14% of ethylene glycol succinate polyester on Chromosorb W (80–100 mesh)[6]. Retention times (*T*) are given relative to those of hexa-*O*-acetylmannitol (alditol acetates) or methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside (methyl glycosides). Electrophoresis was conducted on strips (17 × 2.5 cm) of cellulose acetate in 0.1M ammonium carbonate buffer (pH 8.9), and in 0.05M acetate buffer (pH 5.0), at 20 V/cm. Polysaccharides were detected[8] by spraying with periodate–Rosaniline hydrochloride. Moving free-boundary (Tiselius) electrophoresis was conducted in a Perkin–Elmer Model 38-A apparatus, using 0.05M sodium borate (pH 9.2) at 20 mA and 150 V. Descending, partition chromatography was performed on Whatman No. 1 or 3 MM paper in the following solvent systems: (*A*) 5:3:1:3 1-butanol–pyridine–benzene–water, (*B*) 4:1:5 1-butanol–ethanol–water (upper layer), (*C*) 10:4:3 ethyl acetate–pyridine–water, or (*D*) 18:4:1:3 ethyl acetate–acetic acid–formic acid–water. Components were detected with *p*-anisidine hydrochloride.

Extraction of the polysaccharides from Phyllospadix torreyi. — An air-dried, powdered sample of *Phyllospadix torreyi* (420 g) was steeped in water (2 L) containing acetic acid (400 mL) for 24 h at 20°. The liquid was removed by filtration, and the residue was successively extracted in a Soxhlet apparatus with methanol, 2:1 methanol–chloroform, and 1-butanol saturated with water. An aqueous slurry of the residue was kept for 4 h at 90°, and the supernatant liquor obtained by centrifugation was dialyzed and filtered. The filtrate was concentrated to 100 mL and poured into ethanol (700 mL). The precipitate, polysaccharide A, was dissolved in water and freeze-dried (11.5 g). The residue from the hot-water extraction was extracted with 5% ammonium oxalate for 4 h at 90°. The carbohydrate material in the supernatant liquor, polysaccharide B (6.3 g), was obtained, after dialysis, by the procedure described for polysaccharide A. The plant residue was suspended in water (1.5 L) at 65°, and delignified with glacial acetic acid (400 mL) and sodium chlorite (300 g)[9]. The holocellulose was washed with water until free of acid, and extracted four times with 7% sodium hydroxide. Each extraction was carried out for 16 h at 20° in a nitrogen atmosphere. The pH of the supernatant liquor obtained by centrifugation was adjusted to 5.0 with acetic acid, and the clear solution was dialyzed, concentrated to 300 mL, and poured into ethanol (2 L). The precipitate, polysaccharide C (11.7 g), was dissolved in water and the solution freeze-dried.

Fractionation of polysaccharides A, B, and C with hexadecyltrimethylammonium bromide[4]. — An aqueous solution of polysaccharide C was de-ionized by passage through a column of Rexyn 101 (H^+) resin, and the pH of the eluate was adjusted to 7.0 with sodium hydroxide. The polysaccharide was precipitated with ethanol, dissolved in water, and the solution freeze-dried. Polysaccharide C (11.4 g) was dissolved in water (800 mL), and a solution of hexadecyltrimethylammonium bromide (30 g) in water (300 mL) containing sodium sulfate (1 g) was added. The pH was adjusted to 7.0, and the solution was stirred for 2 h at 35°. The precipitate was recovered by centrifugation, and dissolved in 4M sodium chloride (60 mL), and the clear solution was poured into ethanol (400 mL). The precipitate was dissolved in water, the solution dialyzed, and the product, polysaccharide C-1 (2.2 g), was freeze-dried. The aqueous, supernatant liquor from the precipitation at pH 7 was made 0.01M with respect to sodium borate, and the pH was adjusted to 8.5. The resulting precipitate was dissolved in 4M sodium chloride, and the polymeric material, polysaccharide C-2, was recovered by the method described for polysaccharide C-1. The pH of the supernatant liquor from the precipitation at pH 8.5 was adjusted to 12 with sodium hydroxide; processing of the precipitate gave polysaccharide C-3 (0.45 g). Finally, the supernatant liquor from the precipitation at pH 12 was poured into ethanol (3 L). The resulting precipitate was washed with 80% ethanol, dissolved in water, and the solution dialyzed. The product, polysaccharide C-4 (4.47 g), was recovered by freeze-drying.

Similar treatment of polysaccharide A with hexadecyltrimethylammonium bromide gave fractions A-1, A-2, A-3, and A-4; polysaccharide B afforded polysaccharides B-1, B-2, B-3, and B-4.

The yields and analytical data for the subfractions of polysaccharides A, B, and C are given in Table I.

Electrophoretic analysis of polysaccharide C-1. — The polysaccharide migrated as a single band in electrophoresis on cellulose acetate films. Moving free-boundary electrophoresis gave a single, symmetrical peak.

Composition of polysaccharide C-1. — The polysaccharide (200 mg) in M sulfuric acid (16 mL) was heated for 10 h at 96°. The cooled solution was made neutral (barium carbonate), the suspension was filtered, and the filtrate was de-ionized with Rexyn 101 (H^+) resin, and separated into its neutral and acidic components by passage through a column of Amberlite IR-45 (OAc^-) resin. A portion of the neutral sugars (8 mg) was reduced (sodium borohydride), the products acetylated (acetic anhydride–pyridine), and the acetates analyzed by g.l.c. (column *A*). The rest of the neutral sugars (95 mg) was resolved into the component sugars on Whatman 3MM paper using solvent *A*.

Fraction I (19 mg), a syrup, had $[\alpha]_D^{20}$ +5° (*c* 1.9, water), and the same chromatographic mobility (solvents *A–D*) as L-rhamnose. The derived alditol acetate had the same retention time (*T* 0.22) as penta-*O*-acetyl-L-rhamnitol.

Fraction II (9 mg), a mixture of sugars, had $[\alpha]_D^{20}$ +40° (*c* 0.22, water), and the same chromatographic mobilities (solvent *A*) as L-arabinose and D-apiose. The derived alditol acetates had *T* 0.37, the same as that of penta-*O*-acetyl-L-arabinitol, and *T* 0.51 and 0.83, identical with those obtained for an authentic sample of penta-*O*-acetyl-D-apiitol. The g.l.c. peaks having *T* 0.83 and 0.51 were in the ratio of 2.6:1. Quantitative g.l.c. of the mixture of alditol acetates indicated that this fraction contained arabinose (5 mg) and apiose (4 mg).

Fraction III (15 mg), a syrup, had $[\alpha]_D^{20}$ +14° (*c* 1.16, water), and was chromatographically identical (solvents *A–D*) with D-xylose. The derived alditol acetate had the same retention time (*T* 0.54) as penta-*O*-acetylxylitol.

Fraction IV (10 mg), a syrup, had $[\alpha]_D^{20}$ +76° (*c* 0.82, water), and the same chromatographic mobility (solvents *A–D*) as D-galactose. The derived alditol acetate had the same retention time (*T* 1.16) as hexa-*O*-acetylgalactitol.

The acid material (18 mg) was eluted from the Amberlite IR-45 resin with 5% formic acid. This fraction was treated successively with diazomethane and sodium borohydride. The product was hydrolyzed (M sulfuric acid), the product neutralized (barium carbonate), the sugar reduced (sodium borohydride), and the alcohol acetylated (acetic anhydride–pyridine). G.l.c. showed a single component, having *T* 1.0, identical with the retention time of hexa-*O*-acetyl-D-mannitol.

The neutralization equivalent of polysaccharide C-1 was found to be 496. The uronic acid content (36%) was determined[5] by the carbazole colorimetric method, using D-galacturonic acid as the standard.

The occurrence of 6-deoxyhexose residues in polysaccharide C-1 was verified colorimetrically[10], using L-rhamnose hydrate as the standard. This procedure showed C-1 to contain 15% of rhamnose. A colorimetric analysis[11] of polysaccharide C-1 for total carbohydrate, using a standard sugar mixture comprising L-rhamnose, D-xylose, D-galactose, and D-galacturonic acid in the same molar proportions as they occur in C-1, showed this polysaccharide to have a carbohydrate content >98%.

Methylation analysis of polysaccharide C-1. — The polysaccharide (150 mg) was methylated successively by the Hakomori and Purdie procedures, and the permethyl ether (95 mg), purified by precipitation from a chloroform solution with petroleum ether (b.p. 60–80°), had $[\alpha]_D^{20}$ $-4°$ (*c* 0.5, chloroform), and showed, in its i.r. spectrum, no absorption attributable to OH. Permethylated polysaccharide C-1 was methanolyzed with 4% methanolic hydrogen chloride in a sealed tube for 12 h at 96°. The solution was made neutral (silver carbonate), the suspension filtered, and the filtrate analyzed by g.l.c. (column *B*). The results are summarized in Table II.

Periodate oxidation of polysaccharide C-1. — A sample of C-1 (28.6 mg) was treated with 0.03M sodium periodate (15 mL) at 4–5° in the dark. Aliquots were removed at intervals, and the amount of periodate consumed was determined titrimetrically[12]. The oxidation was complete after 17 h (1.18 molecules of periodate were consumed per sugar residue). The amount of formic acid produced during the oxidation was determined titrimetrically with 0.01M sodium hydroxide[13]; formaldehyde was assayed by the modified Nash procedure[14], using D-mannitol as the standard. The excess of periodate was decomposed with ethylene glycol (1 mL), and the solution was dialyzed. The polyaldehyde was reduced (sodium borohydride), and the reaction was terminated by passage through a column of Rexyn 101 (H^+) resin. The eluate was concentrated to 1 mL, and hydrolyzed. G.l.c. (alditol acetates, column *A*) showed arabinose and apiose in the molar ratio of 1.3:1.

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