Carbohydrate Research, 66 (1978) 103–111

STRUCTURAL STUDIES OF A CARBOHYDRATE-CONTAINING POLYMER PRESENT IN THE MUCILAGE TUBES OF THE DIATOM *Berkeleya rutilans** (TRENT.) GRUN.**

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(Received January 11th, 1978; accepted for publication, February 27th, 1978)

ABSTRACT

The sulphated, carbohydrate-containing polymer present in the mucilage tube surrounding colonies of the marine diatom *Berkeleya rutilans* (Trent.) Grun. is probably a glycoprotein. The carbohydrate fraction consists of mannose, mannuronic acid, xylose, and a 3,6-anhydrohexose tentatively identified as 3,6-anhydroglucose. Mannose and mannuronic acid are (1→3)-linked, and each is probably present at branch points with branches on C-2, and also as end-groups. Xylose is (1→2)- or (1→4)-linked and present as end-groups. 3,6-Anhydroglucose is (1→4/5)-linked in the polymer. Sulphate is located on either C-2 or C-4 in mannose and/or mannuronic acid. Hydrolysis studies indicate that the new reducing end-groups consist mainly of 3,6-anhydroglucose. The protein moiety has an amino acid composition similar to that of glycoproteins isolated from land plants.

INTRODUCTION

The marine diatom *Berkeleya rutilans* (Trent.) Grun. [syn. *Amphipleura rutilans* (Trent.) Cleve] grows in colonies surrounded by mucilage tubes. Lewin[1] extracted the mucilage from the tubes represented by his fraction R.2. The resulting material contained mainly xylose and mannose, traces of rhamnose, and two unidentified sugars. Protein and sulphate were also present. Further chemical studies were not carried out. We now report on structural studies of the extractable, carbohydrate-containing polymer from the mucilage tubes of *B. rutilans*.

*Synonym: *Amphipleura rutilans* (Trent.) Cleve.
**Dedicated to Dr. Elizabeth Percival.

RESULTS AND DISCUSSION

The marine diatom *Berkeleya rutilans* grows in colonies surrounded by mucilage tubes. Preliminary studies showed that these tubes partly consist of a carbohydrate-containing polymer. Isolation and purification of the polymer gave 1 g of polymer from 2 g of fibrous tubes. The purified material contained 72% of carbohydrate[2] (determined as glucose), 7.5% of protein[3], and 22.5% of sulphate[4] (as SO_3Na). The content of uronic acid[4–6] was 20–22%, and of 3,6-anhydrohexose[7] 17–20% (as 3,6-anhydrogalactose), each as a percentage of the total carbohydrate present. The amino acid composition is given in Table I. The optical rotation was not measured, as the polymer was difficult to dissolve completely after freeze-drying. The polymer moved as a single, anionic compound when subjected to free-boundary electrophoresis, with the mobility of 1.5 cm^2/sV at five different pH-values in the range 2–10. Complete hydrolysis (monitored by p.c.) in various solvent systems gave mannose, mannuronic acid, xylose, and an unknown sugar. The monomers were separated and isolated on a preparative scale by means of ion-exchange chromatography and chromatography on a carbon–Celite column. The chromatographic identifications of xylose, mannose, and mannuronic acid were confirmed by various other methods (see Experimental). The identification of the supposed 3,6-anhydrohexose proved to be more difficult. The derived alditol acetate had the same g.l.c. retention time (column *B*) as the tetra-acetates of 3,6-anhydrogalactitol, 3,6-anhydroglucitol, 3,6-anhydromannitol, and 3,6-anhydrotalitol. The electron-impact (e.i.) mass spectra were also identical, apart from minor changes in the intensities (Table II). The fragments having *m/e* 259 and 272 are probably M−73 and M−60, thus indicating a molecular weight of 332. A chemical-ionisation (c.i.) mass spectrum (Table II) contained peaks at *m/e* 333 (M+1) and 273 (M+1−60), showing that the molecular weight of the alditol acetate is 332. The c.i. mass spectra of the tetra-acetates of 3,6-anhydrogalactitol, 3,6-anhydroglucitol, 3,6-anhydromannitol, and 3,6-anhydrotalitol gave the same mass fragments, but with significant differences in intensities (Table II). McNeil and Albersheim[8] recently proposed that c.i. mass spectrometry can be used to distinguish between, for example, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol and -galactitol, due to clear differences in the intensities of the mass fragments obtained. They showed how this method can be used to characterize unidentified, methylated hexitol acetates. If the theory proposed is applicable to other compounds, the results obtained show that the 3,6-anhydrohexose is not the galactose, mannose, or talose derivatives, but probably 3,6-anhydroglucose.

It was impossible to determine accurately the ratio of the sugars present in the polymer, because of difficulties in identifying the 3,6-anhydrohexose. The relative ratios have been calculated on the basis of the results obtained for the various experiments. The approximate composition is xylose (0.2), mannose (3), mannuronic acid (1), and 3,6-anhydroglucose (1).

An i.r. spectrum of the polymer before desulphation showed bands at 1240 cm^{-1} (S=O stretching vibration) and 850 cm^{-1} (characteristic of sulphate on an axial

TABLE I

RELATIVE PROPORTIONS OF THE AMINO ACIDS PRESENT IN THE ISOLATED POLYMER

Aspartic acid	13.3	Methionine	0.5
Threonine	7.8	Isoleucine	5.2
Serine	6.4	Leucine	7.8
Glutamic acid	11.6	Tyrosine	4.8
Proline	3.8	Phenylalanine	6.7
Glycine	8.2	Lysine	4.9
Alanine	7.0	Histidine	0.4
Valine	7.2	Arginine	4.3

TABLE II

MASS FRAGMENTATION OF 3,6-ANHYDROHEXITOL TETRA-ACETATES

Compound	*Mass fragments (relative intensities)*		
3,6-Anhydrohexitol tetra-acetate	E.i.-m.s.:	43 (100), 69 (9), 73 (3), 85 (10), 97 (16), 103 (4), 110 (12), 115 (10), 128 (7), 139 (7), 145 (4), 157 (7), 170 (29), 187 (6), 212 (11), 230 (11), 230 (1.5), 259 (3), and 272 (1.5)	
		M+1	*M+1−60*
3,6-Anhydrohexitol tetra-acetate	C.i.-m.s.:	333 (30%)	273 (100%)
3,6-Anhydro-D-galactitol tetra-acetate	C.i.-m.s.:	333 (7.7%)	273 (100%)
3,6-Anhydro-D-glucitol tetra-acetate	C.i.-m.s.:	333 (36%)	273 (100%)
3,6-Anhydro-D-mannitol tetra-acetate	C.i.-m.s.:	333 (100%)	273 (62%)
3,6-Anhydro-D-talitol tetra-acetate	C.i.-m.s.:	333 (17%)	273 (100%)

hydroxyl group[9]). These bands disappeared after desulphation with 0.08M methanolic hydrogen chloride.

Since no decrease in sulphate relative to carbohydrate took place on treatment of the polymer with alkali in the presence of potassium borohydride, it is concluded that the sulphate groups are not located on a sugar carbon-atom having a vicinal, free, *trans*-hydroxyl group[10], or on either C-3 or C-6 of mannose residues where HO-6 or HO-3 is unsubstituted. This inference is confirmed, as no increase in 3,6-anhydrohexose relative to carbohydrate was observed during this process[11]. The content of uronic acid relative to carbohydrate was diminished during this treatment, which may indicate that some of the carboxyl groups are esterified.

Attempted methylation of the native polymer was unsuccessful. This result is well known for polysaccharides containing both sulphate and uronic acid. Complete methylation of the desulphated polymer also proved to be impossible. The product obtained after desulphation and reduction, which consisted of xylose, mannose, and 3,6-anhydroglucose, was easy to methylate by the modified Hakomori method[12,13].

After hydrolysis and conversion of the partly methylated monosaccharides into the corresponding alditol acetates, the mixture was analysed by g.l.c.–m.s. (both e.i.- and c.i.-m.s.). The results are given in Table III and confirm the identity of previously known products. The main part of the mannose is (1→3)-linked, with some present as branch points linked through both C-2 and C-3. Some mannose is present as non-reducing end-groups. As the mannose derivatives partly represent the mannuronic acid present in the original polymer, mannuronic acid is also (1→3)-linked. The possible presence of mannuronic acid at branch points or as end-groups cannot be excluded; (1→3)-linked mannuronic acid has not been found in polysaccharides previously. Xylose is present as non-reducing end-groups and as (1→2)- or (1→4)-linked units. The unknown compound ($T = 2.13$) gave, on e.i.-m.s., a fragment having *m/e* 117 that indicated *O*-methyl on C-2. C.i.-m.s. gave an ion having *m/e* 305 (M+1), corresponding to the molecular weight of a 3,6-anhydro-2-*O*-methylhexitol triacetate. The formation of the ions (M+1−32) and (M+1−60) confirms the molecular weight. The compound is (1→4)- or (1→5)-linked. The method does not distinguish between these types of linkages, but, from a model compound, a (1→4) linkage appears the more probable.

TABLE III

DATA FOR THE PRODUCTS OBTAINED AFTER METHYLATION OF THE POLYMER

Alditol acetate corresponding to	T[a]	*Relative peak area*	*Primary fragments in e.i.-m.s.*	*Fragments obtained by c.i.-m.s. (rel. peak intensity)*		
				M+1	*M+1−32*	*M+1−60*
2,3,4-Tri-*O*-methylxylose	0.62	0.1	117, 161	No results	—	—
2,3,4,6-Tetra-*O*-methylmannose	1.00	0.2	45, 117, 161, 205	323 (4%)	291 (6)	263 (100)
2,3- or 3,4-Di-*O*-methylxylose	1.21	0.2	117, 189	307 (16.7%)	275 (1)	247 (100)
2,4,6-Tri-*O*-methylmannose	1.83	2.0	45, 171, 161, 233	351 (3.4%)	319 (6.8)	291 (100)
3,6-Anhydro-2-*O*-methylhexose	2.13	1	117, 231	305 (100%)	273 (5)	245 (100)
4,6-Di-*O*-methylmannose	2.68	1	45, 161, 261	379 (15%)	347 (15)	319 (100)

[a]Retention value relative to 2,3,4,6-tetra-*O*-methyl-D-glucitol 1,5-diacetate.

The methylation results, coupled with the results from the i.r. spectrum, indicate that the sulphate groups in the native polymer are located at C-2 of mannose/mannuronic acid residues in the $^4C_1$ conformation, or at C-4 of these residues in the $^1C_4$ conformation.

TABLE IV

HYDROLYSIS OF THE POLYMER WITH 0.05M SULPHURIC ACID

Time (h)	*Carbohydrate content*[a]	*3,6-Anhydro-hexose*[b]	*Reducing end-groups*[c]	*D.p.*	*After reduction*			
					Carbohydrate content[a]	*D.p.*	*3,6-Anhydro-hexose*[b]	*3,6-Anhydro-hexose as % of original*
0	0.62	0.12	0.04	17.2	0.60	26	0.12	100
0.5	0.61	0.12	0.13	4.7	0.60	3.2	0.07	62
1	0.61	0.12	0.15	4.0	0.38	2.6	0.06	52
2	0.60	0.12	0.18	3.4	0.30	2.0	0.04	35
3	0.62	0.12	0.19	3.3	0.30	1.9	0.035	29
4	0.62	0.12	0.20	3.0	0.27	1.8	0.03	26

[a]Determined by the phenol–sulphuric acid method[1]. [b]Determined by the method of Yaphe[6]. [c]Determined by the method of Nelson[21].

Hydrolysis of the polymer was effected with 0.05M sulphuric acid at 100°. The degree of polymerisation (d.p.) was determined at time intervals by measuring the amount of end-groups formed and the carbohydrate content. The results given in Table IV show a rapid, initial decrease of d.p., but very little change after hydrolysis for 1 h. This result indicates that one type of linkage in the polymer is more susceptible to hydrolysis than the others. The content of 3,6-anhydrohexose was measured before and after reduction of the samples withdrawn; a considerable decrease in the content of 3,6-anhydrohexose after reduction was observed during the first hour, whereas the decrease was less during the rest of the experiment. These results indicate that 3,6-anhydrohexose is responsible for the new, reducing end-groups formed mainly during the first period of hydrolysis. The linkage between 3,6-anhydrohexose and other sugar residues seems to be more susceptible to hydrolysis than linkages not involving 3,6-anhydrohexose.

The sulphated polymer isolated from the "skin" surrounding colonies of the diatom *Berkeleya rutilans* contains both carbohydrate and protein, and is concluded to be a glycoprotein, as free-boundary electrophoresis at various pH-values gave one peak only. The amino acid composition (Table I) of the protein fraction is fairly similar to that obtained for glycoproteins isolated in this laboratory from various land plants (*Cannabis sativa*[14], *Urtica dioica*, *Opuntia ficus-indica*, and *Papaver somniferum*). The carbohydrate fraction consists of xylose, mannose, mannuronic acid, and 3,6-anhydrohexose (tentatively identified as 3,6-anhydroglucose), a sugar composition not found previously in Nature. The nature of the carbohydrate–protein linkage and the identity of the 3,6-anhydrohexose are being further investigated.

The insolubility of this polymer in the native state in sea water may be due to its low solubility in the presence of calcium ions, as demonstrated. It may also be due to the presence of 3,6-anhydrohexose, which is more hydrophobic than normal aldoses, and to the presence of ester groups on some of the uronic acid residues.

EXPERIMENTAL

Concentrations were carried out under diminished pressure below 50° on a rotary evaporator. Dialysis was performed with magnetic stirring against distilled water unless otherwise stated; toluene was added to prevent microbial growth.

Amino acids were analysed on a BioCal B C200 automatic amino-acid analyser after hydrolysis of the sample (10 mg) with 6M hydrochloric acid (2 ml) for 20 h at 110° under nitrogen.

Carbohydrate content was determined by the phenol–sulphuric acid method[2], with D-glucose as the standard. Uronic acid was determined by the cetylpyridinium chloride (c.p.c.) method[4], the method described by Blumenkrantz and Asboe-Hansen[5], and the modified carbazole method[6], with D-mannuronic acid as the standard. The content of 3,6-anhydrohexose was determined by the method of Yaphe[7], with 3,6-anhydro-D-galactose diethyl dithioacetal as the standard. Sulphate was determined by the c.p.c. method[4]. Protein was determined by the method of

Lowry *et al.*[3], with bovine serum albumin as the standard. Free-boundary electrophoresis was performed as described by Myklestad *et al.*[15]. I.r. spectra (potassium bromide discs) were recorded on a Beckman IR-20 spectrophotometer. Unless otherwise stated, hydrolysis was carried out in 90% formic acid[16].

P.c. was performed on Whatman No. 1 paper with *A* pyridine–ethyl acetate–acetic acid–water (5:5:1:3) or *B* pyridine–ethyl acetate–water (11:40:6), and detection with aniline oxalate. G.l.c. of alditol acetates was performed on a Varian 1400 gas chromatograph equipped with a flame-ionisation detector, nitrogen as carrier gas, and the following columns: *A*, 1.5% of silicone XE-60 plus 1.5% of EG succinate on AW-Chromosorb W (100–200 mesh) (200 × 0.25 cm) with temperature-programming (170→210°, 1°/per min); *B*, 3% of OV-225 on Varaport 30 (200 × 0.25 cm) under isothermal conditions (200°; 180° for methylated alditol acetates); and *C*, 3% of ECNSS-M on Chromosorb W (100–200 mesh) (200 × 0.25 cm) at 180°.

For e.i.-m.s., column *B* was coupled[16] to a Varian CH-7 mass spectrometer. For c.i.-m.s., the column was coupled to a Micromass 12F spectrometer; isobutane was used as the ionizing gas. The pressure in the ion source was 0.2 Torr, and the temperature was 180°.

Investigation of the polymer. — The starting material, consisting of the marine diatom *Berkeleya rutilans* (Trent.) Grun. (single cells) enclosed in mucilage tubes, was collected and identified by Dr. Grethe Rytter Hasle, Institute for Marine Biology and Limnology, Blindern, Oslo, at Viksfjorden, near Larvik, Norway.

(*a*) *Isolation.* The total material obtained was stirred with sea water for 1 h. The suspension, consisting of fibrous material (mainly the mucilage tubes) and finely divided material (mainly diatoms), was filtered through a perforated disc, and the fibrous material retained by the disc was treated several times as described above, and then dehydrated by washing with ethanol.

The fibrous material (2 g) was suspended in water and dialysed first against 0.1M EDTA (pH 7), and then against distilled water. The contents of the dialysis bag were centrifuged, and the supernatant solution was retained (extract 1). The slimy residue was extracted twice with water at 100° for several hours (extracts 2 and 3). The extracts were combined, calcium chloride was added to 0.01M, and the polymer was precipitated with 1 vol. of ethanol. Reprecipitation with 1 vol. of ethanol did not occur before the calcium chloride concentration reached 0.01M (weight, 1 g).

(*b*) *Alkali treatment.* To a 0.2% aqueous solution of the polymer was added 0.1% potassium borohydride and potassium hydroxide, to give a M concentration. The solution was heated at 100° for 1 h, made neutral with acetic acid, and dialysed, and the ratio between the contents of 3,6-anhydrohexose and total carbohydrate was determined before and after this treatment. No change in the ratio took place. The uronic acid content was 10.6% of the total carbohydrate.

(*c*) *Desulphation and reduction.* A suspension of the polymer (50 mg) in 0.08M hydrochloric acid in methanol was shaken at room temperature for 24 h. The insoluble material was collected by centrifugation and washed with methanol and ether. The methyl esters of the uronic acid formed during this procedure were reduced with

sodium borohydride. The polymer (32 mg) obtained after dialysis contained no uronic acid or sulphate.

(*d*) *Hydrolysis.* The polymer (300 mg) was hydrolysed with 0.5M sulphuric acid (30 ml) at 100° for 4 h. The hydrolysate was made neutral with barium carbonate, filtered, and concentrated, and the syrupy residue (190 mg) was fractionated on Dowex 1 x8 (AcO^-) resin. The neutral fraction (109 mg) was eluted with water, and the acidic fraction (30.8 mg) with acetic acid. The neutral sugars were fractionated on a column of carbon–Celite (1:1) by elution, successively, with water (mannose and xylose), 2% of ethanol in water [main part (27 mg) of the 3,6-anhydrohexose], and 5% of ethanol in water. The mannose and xylose had the same mobilities as authentic samples in p.c. in various solvents, and their alditol acetates had the same retention values in g.l.c. as those of the authentic compounds.

The lactone, obtained by treatment of the uronic acid with hydrochloric acid, co-chromatographed (p.c., solvent *B*) with mannuronolactone (R_{GLC} 4.7). On electrophoresis in Ca^{2+}/Na^+ buffer[18], the uronic acid had the same mobility as mannuronic acid. When chromatographed on Dowex 1 x8 (AcO^-) resin by using gradient elution, the uronic acid was eluted at the same concentration of acetic acid as mannuronic acid[19].

The 3,6-anhydrohexose gave a characteristic reaction[7] and had R_{GLC} 0.8 (solvent *A*). The derived alditol acetate had T_{XYL} 0.93 and 1.00 in g.l.c. on columns *B* and *C*, respectively. Mass-spectral data (e.i. and c.i.) are recorded in Table II.

3,6-Anhydro-D-mannitol[20], 3,6-anhydro-D-glucose[22], and 3,6-anhydro-D-talose[22] were prepared by procedures described in the literature. 3,6-Anhydro-D-galactitol tetra-acetate was prepared from 3,6-anhydro-D-galactose diethyl dithioacetal (kindly provided by Dr. W. Yaphe, McGill University, Montreal).

The compounds were converted into the corresponding alditol acetates, and analysed by g.l.c.–m.s. (Table II).

(d) *Methylation of the desulphated, reduced polymer.* Methylation was performed by the Hakomori method[12] with modifications[13]. The derived, partly methylated alditol acetates were analysed by g.l.c.–m.s. (Table III).

(e) *Hydrolysis of the polymer with sulphuric acid.* The polymer (30 mg) and 0.05M sulphuric acid (30 ml) were heated at 100° for 4 h. Samples were removed at intervals and analysed for carbohydrate[2], 3,6-anhydrohexose[7], and reducing end-groups[21]. Aliquots were also reduced with sodium borohydride, and the contents of carbohydrate and 3,6-anhydrohexose were measured after reduction. The results are given in Table IV.

ACKNOWLEDGMENTS

The authors are indebted to the Institute of Clinical Biochemistry, Rikshospitalet, Oslo, for permission to use the g.l.c.–m.s. (e.i.) facilities, and to Dr. Truls Grönneberg, Norwegian Agricultural University, Ås, for carrying out the g.l.c–m.s. (c.i.) experiments.

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