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STRUCTURAL STUDIES ON THE EXTRACELLULAR POLYSACCHARIDE OF THE RED ALGA, *Porphyridium cruentum**

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ABSTRACT

Partial acid hydrolyzates of the extracellular polysaccharide from *Porphyridium cruentum* yield three disaccharides and two uronic acids. These constitute all of the uronic acid in the polymer. The novel disaccharides are 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactose, 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose, and 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-glucose. The polyanion of high molecular weight contains D- and L-galactose, xylose, D-glucose, D-glucuronic acid and 2-*O*-methyl-D-glucuronic acid, and sulfate in molar ratio (relative to D-glucose) of 2.12:2.42:1.00:1.22:2.61. Preliminary periodate-oxidation studies suggest that the hexose and uronic acids are joined to other residues by (1→3) glycosidic linkages. About one-half of the xylose residues are (1→3)-linked.

INTRODUCTION

Porphyridium cruentum is a unicellular red alga, whose normal habitat consists of numerous single cells embedded in a gelatinous, polysaccharide matrix. In liquid, aerated cultures, however, the colonial form tends to break down, and the polysaccharide becomes dispersed throughout the medium, yielding a rather viscous culture-medium. As *Porphyridium* lacks a typical cell-wall, it was of interest to examine this polysaccharide to determine whether it resembles cell walls of red algae, storage polysaccharides, or mucilages[1].

RESULTS AND DISCUSSION

General structure and composition. — The extracellular polysaccharide is composed of 36% hexose, 30% pentose, 8.5% uronic acid, 9% sulfate, and 1.5% amino acids[2,3]. When the water content (13.9%) of the polymer is considered, the total mass may be accounted for[2]. The molar ratios of components relative to glucose are: D- and L-galactose:xylose:D-glucose:uronic acid:sulfate = 2.12:2.42:1.00:1.22:2.61.

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About 15% of the galactose and all of the glucose have the D configuration. The carbohydrate composition and sulfate content are similar to those reported for the extracellular polysaccharides of *Porphyridium aeruginium*[4] and *Porphyridium cruentum* (by independent investigators)[5]. The polymer appears to be of high molecular weight, as it has an intrinsic viscosity of 20.9 dl per g and displays two components[2] in an analytical centrifuge, having sedimentation coefficients of 12.6 and 18.8 S.

Periodate oxidation of the sulfated and desulfated polysaccharide indicated that the sugar residues of both materials are about equally susceptible to periodate oxidation[2]. About 50% of the xylose residues were decomposed by oxidation (sulfated control, 1.06 μmole per mg and oxidized 0.64 μmole per mg; desulfated control, 1.30 μmole per mg and oxidized 0.62 μmole per mg). Glucose, galactose, and uronic acid residues were not affected by periodate oxidation. These results suggested a polymer having about one-half of the xylose residues, and all of the galactose, glucose, and uronic acid residues, engaged in 1→3 linkages. Our findings for xylose and uronic acid correlate with another report[5], but our results for other residues are not in agreement. Further study will be necessary to explain these differences.

Desulfation of the extracellular polysaccharide by dilute acid[6] or by solvolysis[7] changes the carbohydrate composition of the polymer and may afford information on its gross structure. The ratios of components for sulfated and desulfated polymer are, respectively, uronic acid: galactose:glucose: xylose (μmole per mg) equal to 0.46:0.74:0.56:1.06 and 0.46:0.66:0.48:1.30. Although the galactose and uronic acid contents remain relatively unchanged, the content of xylose and glucose is increased. This result may suggest that the xylose, galactose, uronic acid, and most of the glucose residues exist in the "main" portion of the molecule, whereas a portion of the glucose is located in short side-chains or at the termini. Transmission electron-microscopy of the polysaccharide has shown that it is mostly linear[2].

Analysis of aldobiouronic and free uronic acids in the acid hydrolyzates established the presence of glucuronic acid, which accounted for 90% of the total uronic acid. The novel uronic acid, 2-*O*-methyl-D-glucuronic acid, constitutes the remaining portion[3].

Characterization of uronic acid-containing components of partial acid hydrolyzates. — Isolation and separation of uronic acid and glycosiduronic acids of partial acid hydrolyzates was performed by ion-exchange chromatography[8] on Dowex-1 resin. The four uronic acid-containing peaks, labeled I–IV in order of elution, are illustrated in Fig. 1. Peak components I and II were eluted in the region of standard aldobiuronic acids, and peak components III and IV were found in the region of standard monosaccharides. As Table I illustrates, all of the uronic acid in the polymer was recovered in these four peaks.

Peak IV was identified as D-glucuronic acid. It co-eluted with an authentic standard by column chromatography on Dowex-1 resin. D-Glucuronic acid and D-glucuronolactone, and Peak IV components, migrated identically on paper chromatography in solvent A and on paper electrophoresis in 0.06M borate buffer

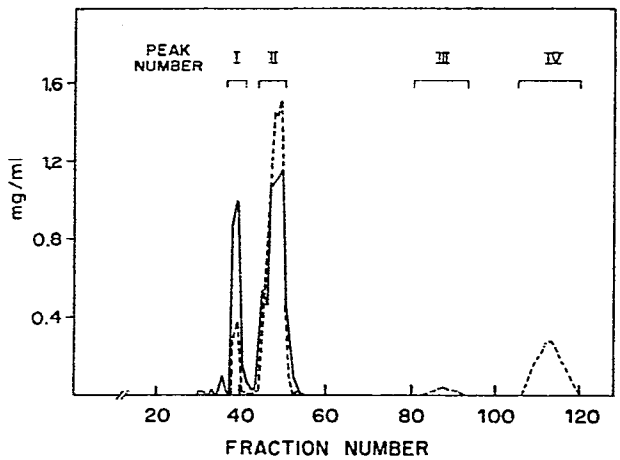

Fig. 1. Fractionation of free hexuronic acids and hexuronic acid fragments on Dowex-1 resin.

Uronic acids and uronic acid-containing fragments were derived from the polysaccharide by acid hydrolysis. Conditions for ion-exchange chromatography are described in the experimental section. Fraction volumes were 1.35 ml. Peaks were pooled as indicated, evaporated and further analyzed by the colorimetric methods described in the experimental section. For the elution profiles, the solid line (——) indicates hexose values and the dotted line (. . .) denotes uronic acid values. The position of elution for D-glucuronic acid is fraction 113, for D-galacturonic acid, fraction 62, and for L-iduronic acid, about fraction 98.

TABLE I

FRACTIONATION OF PARTIAL ACID-HYDROLYSIS FRAGMENTS ON DOWEX-1 RESIN[a]

Sample	*Total hexose*[b]	*Total uronic*[b] *acid*	*Mole ratio hexose: uronate*	*% Uronate recovery*
Unhydrolyzed	233.3	38.6		100
Peak I	11.8	4.3	2.8	11
Peak II	28.1	27.0	1.0	70
Peak III	0.0	0.38		1
Peak IV	0.0	7.9		20

[a]Techniques detailed in experimental section. [b]Values expressed as μmoles per 100 mg of polysaccharide.

(pH 10). The $[\alpha]_D^{25}$ value was +30° in water for Peak IV; this is lower than the literature value of +36.3° for D-glucuronic acid, but we suggest that Peak IV is a mixture of the free uronic acid and its lactone, which has[9] $[\alpha]_D^{25}$ +18.6°. In naurally occurring polysaccharides, only the D form of glucuronic acid has been reported[9,10].

Peak III has been shown to be 2-*O*-methyl-D-glucuronic acid[3]. When the methylated uronic acid appeared in a disaccharide (Table I, Peak I), the uronic acid value was about 66% lower than the value predicted from the hexose content. On this basis, the total amount of 2-*O*-methyl-D-glucuronic acid in Peak I and Peak III was calculated to be 28% of the total uronic acid and 2.5% by weight of the total mass of the polysaccharide.

The Peak II component, containing 70% of the total uronic acid, was the major, uronic acid-containing component of the acid hydrolyzate. It was homogeneous by paper chromatography in Solvent A (R_{Gal} 0.25). It migrated close to 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (R_{Gal} 0.22) and was clearly separated from 4-*O*-(α-D-glucopyranosyluronic acid)-D-galactose and 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

Peak II component had a molar ratio of hexose to uronic acid of 1.0 (Table I). Paper chromatography of an acid hydrolyzate of the Peak II component (M hydrochloric acid, 3 h, 100°) revealed the monosaccharides galactose and glucuronic acid. (Traces of glucose and xylose were noted in acid hydrolyzates of Peak II by quantitative column-chromatography.) Similar results were obtained by quantitative column-chromatography of an acid hydrolyzate (M hydrochloric acid, 4 h, 100°), where a molar ratio of galactose (8.19 μmole per 100 mg) to glucuronic acid (9.21 μmole per 100 mg) of 0.9 was found; about 30% of the theoretical yield of each monosaccharide was recovered from the hydrolyzate. These results indicated that Peak II was composed of glucuronic acid and galactose.

Further evidence that the uronic acid in Peak II was glucuronic acid was provided by chromatography of an acid hydrolyzate of the products of reduction. Only the monosaccharides glucose and galactose were present. The disaccharide could be completely hydrolyzed by acid after reduction, indicating that only glucuronic acid and galactose were present in the untreated material.

The saccharide sequence of (glucosyluronic acid)-galactose of the Peak II component was established by reduction with borohydride. Under conditions[11] whereby the reducing terminal moiety would be reduced to the corresponding alditol, the hexose in Peak II was completely decomposed, but the uronic acid was unchanged (Table II). This result suggested that the hexose moiety was located at the reducing terminus.

The position of the glucosidic linkage in Peak II was determined by examining the products of controlled oxidation by lead tetraacetate[12]. This procedure quantitatively converted the galactose moiety into pentose (Table II), and indicated a (1→3)-linked hexose at the reducing terminal.

The Peak II disaccharide was not cleaved by β-D-glucosiduronase under conditions that caused complete cleavage of 3-*O*-(β-D-glucopyranosyl-uronic acid)-D-galactose[13].

The uronic acid component of the Peak II disaccharide was isolated from acid hydrolyzates and found to be very similar to a standard sample of D-glucuronic acid, by specific rotation, by mobility in several chromatographic systems, and by co-chromatography on an automated column of Dowex-1 resin. The configuration of the galactose moiety in Peak II was examined by performing the D-galactose oxidase[14] assay on the neutral-sugar fraction isolated from an acid hydrolyzate of Peak II. As activity toward D-galactose oxidase was absent from this fraction, the galactose was considered to have the L configuration (Table II).

TABLE II

STRUCTURAL STUDIES OF ACID-HYDROLYSIS FRAGMENTS[a]

Sample	*Hexose: reducing power Mole ratio*	*Lead tetraacetate oxidation*		*Borohydride reduction*		*Periodate oxidation*	D-*Galactose oxidase*[b]
		% Pentose formed	*% Uronate change*	*% Hexose recovery*	*% Uronate recovery*	*% Uronate decomposed*	*% Galactose decomposed*
Peak IA (IB)[c]	0.94 (0.90)	101 (96)	0 (0)	3 (7)	97 (100)	(100)	94 (100)
Peak II	1.00	106	0	0	100	98	<1

[a]Techniques described in detail in experimental section. [b]Assays were performed on the neutral-sugar portion of acid hydrolyzates (M HCl, 5 h, 100°). Hexose was determined by the anthrone method; quantitative column-chromatography of borate complexes was used to determine the amounts of glucose and galactose. [c]Analyses of fraction containing Peak IB in parentheses.

These results suggested that the Peak II component was the novel disaccharide, 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactose.

The Peak I component migrated as one spot on paper electropheresis in 0.06M borate buffer, pH 10 but was resolved into two components on paper chromatography in Solvent A. The major, faster-moving component, called Peak IA, which comprised about 90% of the hexose in Peak I, was eluted from a paper chromatogram and subjected to further analysis. The slower-migrating, minor component (designated Peak IC) was not investigated.

The Peak IA component had a molar ratio of hexose to reducing sugar of nearly 1 (Table II), suggesting that it was a disaccharide. The molar ratio of hexose to uronic acid was 2.8 (Table II). Column chromatography of an acid hydrolyzate of Peak IA revealed galactose (3.02 μmole per 100 mg) as the only neutral sugar.

The uronic acid identified in Peak III as 2-*O*-methyl-D-glucuronic acid was also found in acid hydrolyzates of Peak I components. This was determined by paper and thin-layer chromatography in various solvents, by gas–liquid chromatography[3], and, by co-chromatography with the Peak IV component on a Dowex-1 column[2]. Acid hydrolysis of Peak IA component following esterification and reduction yielded a compound having properties identical to the standard compound, 2-*O*-methyl-D-glucose. Digestion with D-galactose oxidase of the neutral-sugar fraction of an acid hydrolyzate of Peak IA showed that almost all of the galactose had the D configuration (Table II).

The order of saccharide units in Peak IA was established as glycosyluronic acid galactose by borohydride reduction. The reduction procedure decomposed the hexose, whereas the uronic acid value was unchanged (Table II). Controlled oxidation by lead tetraacetate established that the uronic acid was linked to O-3 of the hexose moiety. As shown in Table II, the hexose was converted into a pentose, indicating a (1→3)-linked hexose moiety at the reducing terminus of the disaccharide. The α-anomeric configuration of the glycosidic linkage was suggested by the absence of cleavage by β-D-glucosiduronase.

These results suggested that Peak IA was the novel disaccharide, 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose.

A second disaccharide, 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-glucose, was also found in Peak IA. The component of Peak I was isolated conventionally from a partial acid hydrolyzate of a different preparation of the polysaccharide. This peak was subjected to gel filtration (Sephadex G-10). Two poorly resolved fractions were obtained. Paper chromatography in solvent A of the front-migrating portion of the first fraction revealed a mixture of the Peak IA disaccharide and a minor component (Peak IC). The more-retarded fraction from gel-filtration chromatography migrated as a single band in the position of the Peak IA disaccharide, by paper chromatography in Solvent A. This fraction has been designated Peak IB.

The Peak IB component, comprising 36% of the hexose in Peak I from this polysaccharide preparation, contained glucose and galactose in a about 1:2 proportion by quantitative column chromatography of the neutral-sugar fraction of an acid

hydrolyzate (1.2 μmole of galactose and 0.5 μmole of glucose per 100 mg of polysaccharide). The molar ratio of hexose to uronic acid was 2.8 for the Peak IB component (Table I), but, as already noted, the uronic acid may give a spuriously low color-yield in the carbazole reaction.

The uronic acid was identical to that of the Peak III component, 2-*O*-methyl-D-glucuronic acid, by the same criteria already detailed for the Peak IA component. The neutral sugars both had the D-configuration, as all of the galactose and glucose present in the neutral-sugar fraction of acid hydrolyzates were decomposed by D-galactose oxidase and D-glucose oxidase[15] treatments, respectively.

Evidence that Peak IB contained two aldobiuronic acids may be seen in Table II. After reduction with borohydride, the hexose of Peak IB was nearly all decomposed, whereas the uronic acid value was unchanged (Table II). Both glucose and galactose were located at the reducing terminus.

Oxidation of the Peak IB component by lead tetraacetate suggested that both the glucose and galactose moieties were joined to the uronic acid at their respective O-3 positions (Table II). Digestion by β-D-glucosiduronase did not cleave either of these aldobiuronic acids, suggesting that both had the α-D anomeric configuration.

These findings supported the presence of two disaccharides in Peak IB, namely 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-galactose and 3-*O*-(2-*O*-methyl-α-D-glucopyranosyluronic acid)-D-glucose.

The three aldobiouronic acids described here appear to be novel[9,16]. 3-*O*-(α-D-Glucopyranosyluronic acid)-D-galactose has been isolated from an acid hydrolysate of ketha gum[16].

In the two aldobiouronic acids containing galactose, D-glucuronic acid was linked only to L-galactose, whereas 2-*O*-methyl-D-glucuronic acid was linked to D-galactose. The configuration of the galactose (D or L) might contribute to the specificity of the enzyme(s) synthesizing 2-*O*-methyl-D-glucuronic acid, as there is some evidence suggesting that biosynthesis of 4-*O*-methyl-D-glucuronic acid occurs at the oligosaccharide or polymer level[17].

EXPERIMENTAL

Materials and methods. — All chemicals were reagent grade. Dowex resins were obtained from BioRad Laboratories (Richmond, Ca.). The enzyme β-D-glucosiduronase Type B-3 lot no. 78B-6470 (bovine liver) was obtained from Sigma Chemical Co. The Galactostat (D-galactose oxidase) lot no. 2JA and Glucostat (D-glucose oxidase) lot. no. 74A-041 kits were purchased from Worthington Biochemicals.

Most monosaccharides were obtained from commercial sources. L-Iduronic acid was the gift of Dr. E. L. Stern. L-Idose was prepared from 1,6-anhydro-β-L-idopyranose tetraacetate (gift of Dr. J. A. Cifonelli) by acid hydrolysis. 4-*O*-Methyl-D-glucuronic acid was prepared by acid hydrolysis of mesquite gum (gift of Dr. G. O. Aspinall) and separated by column chromatography on Dowex-1 resin[10]. 3-*O*-Methyl-D-glucose was a gift of the late Dr. M. L. Wolfrom.

Oligosaccharide standards were obtained from the following sources: 6-*O*-β-D-glucuronosyl-D-galactose was a gift of Dr. M. Heidelberger; 4-*O*-α-D-glucuronosyl-D-galactose was a gift of Dr. P. A. J. Gorin. Capsular polysaccharide of *Escherichia coli* K12 was used to prepare 3-*O*-(β-D-glucosyluronic acid)-D-galactose by the method of Rodén and Markovitz[18].

Determinations for hexose[19], pentose[20,21], uronic acid[22–26], reducing power[27], and sulfate[28] were performed by standard methods. D-Galactose oxidase[14] and D-glucose oxidase[15] assays were conducted on acid hydrolyzates of polysaccharide or oligosaccharides and on the neutral-sugar components isolated from the hydrolyzates by ion-exchange chromatography. Aniline phthalate[29] and silver dip reagents[30] were used to locate reducing sugars on chromatograms.

Isolation and characterization of components. — *Porphyridium cruentum* (Ag.) Naegh (Indiana Culture Collection Number 161) was maintained in a chemically defined medium[31] under sterile conditions and purified by a procedure including cetylpyridinium chloride precipitations[2].

Acid hydrolysis for neutral sugar analysis was performed in M hydrochloric acid for 3 h at 100° in a sealed tube. For preparation of oligosaccharides, solutions of 0.5–1% polysaccharide were hydrolyzed in M hydrochloric acid or 0.5M sulfuric acid for 2 h at 100° in sealed tubes. Further hydrolyses of the isolated oligosaccharides were conducted at concentrations of less than 1% carbohydrate in M hydrochloric acid for 4 or 5 h at 100° in sealed tubes. Descending paper-chromatography was performed in 18:3:1:4, (v/v) ethyl acetate–acetic acid–formic acid–water (Solvent A) and 5:5:3:1 (v/v) ethyl acetate–pyridine–acetic acid–water (Solvent B). Acid-washed Whatman 3 MM paper was used for preparative chromatography.

Neutral sugars were separated and quantitated as their borate complexes by the Technicon Sugar Chromatography System. The neutral-sugar values derived from acid hydrolyzates per mg of polysaccharide were 62 μg of glucose, 118 μg of galactose and 130 μg of xylose.

The uronic acids and glycosiduronic acids derived from partial and hydrolyzates were separated and analyzed by the method of Fransson *et al.*[8]. Samples were applied to a column (0.63 × 138 cm) of Dowex 1-X8 (−400 mesh) resin and the components were eluted with 0.3M formic acid at a flow rate of 27 ml/h, and 2.0-ml fractions were collected. After lyophilization, each pooled fraction was passed through a column (1 × 142 cm) of Sephadex G-10 superfine gel or a column (1.5 × 238 cm) of Sephadex G-15

Treatment with borohydride was conducted according to Rodén and Armand[11] in a total volume of 0.45 ml. Oxidation of oligosaccharides by lead tetraacetate was performed according to Charlson and Perlin[12]. Esterification and reduction were carried out according to Cifonelli *et al.*[11]; the procedure was repeated, and periodate oxidation of polysaccharide was performed according to Scott[32]. Desulfation was conducted by the method of Kantor and Schubert[6]. The yield of desulfated polysaccharide was 50% by weight.

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