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STRUCTURAL STUDIES ON THE SOLUBLE POLYSACCHARIDE FROM *Iridaea membranacea**

CHRISTIAN M. IBAÑEZ AND BETTY MATSUHIRO

Departamento de Química, Facultad de Ciencia, Universidad de Santiago, Casilla 5659, Santiago 2 (Chile)

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ABSTRACT

Aqueous extraction of the red alga *Iridaea membranacea* gave a high-sulfated polysaccharide. The structure of the polysaccharide has been investigated by hydrolysis, methanolysis, and fractionation. It resembles carrageenan in composition, but fractionation with KCl, and methylation analysis of two homogeneous fractions, showed that it is a complex mixture of sulfated D-galactans.

INTRODUCTION

The genus *Iridaea* comprises 25 species[2], some of which are used as sources of carrageenans. Few polysaccharides from species of *Iridaea* have been studied in detail[3–5]. The water-soluble polysaccharide of *Iridaea membranacea* has been examined as part of a program intended to gather information on the composition of phycocolloids from red seaweeds found on the Chilean coast.

RESULTS AND DISCUSSION

The polysaccharide from *I. membranacea* was extracted with hot water. By paper chromatography and g.l.c. analysis of the derived alditol acetates, the hydrolyzate was shown to contain the acid-stable monosaccharides galactose (98%), glucose (0.8%), xylose (0.3%), and 6-*O*-methylgalactose (0.9%). Although 6-*O*-methylgalactose is a common sugar in agar-type polysaccharides, it has seldom been identified in carrageenans[6,7].

The infrared spectrum of the whole polysaccharide exhibits the characteristic, broad absorption band for ester sulfate at 1240 cm^{-1}, and a broad band at 850–800 cm^{-1}, indicating the presence of more than one type of ester sulfate. In addition, it showed a peak at 930 cm^{-1} which has previously been assigned[8] to the presence of 3,6-anhydrogalactosyl residues in algal polysaccharides.

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TABLE I

COMPOSITION AND MOLAR RATIO FOR THE POLYSACCHARIDE, AND FRACTIONS THEREOF, OF *I. membranacea*

Fraction	*Concentration (M) of KCl*	*Yield (%)*	*3,6-AnGal (%)*	*Sulfate as $NaSO_3$ (%)*	*Molar ratios of Gal:3,6-AnGal·sulfate*
Whole PS		34.3	11.4	37.29	1.00:0.32:1.47
1 Precipitate at	0.0625	11.8	5 3	12.94	1 00:0.10:0.37
2 Precipitate at	0.25	18.0	15.6	25.27	1.00:0.46:1.04
3 Soluble at	0.25	48.5	12.8	31.48	1.00:0.43:1.48
2-A Precipitate at	0.125	3.6[a]	20.6	7.27	1.00:0.40·0.19
2-B Precipitate at	0.25	11.4[a]	13.8	11.28	1.00:0.33:0.37
2-C Soluble at	0.25	52.8[a]	9 1	36.05	1 00:0 18:1.15
3-A Precipitate at	0.4	8.6[b]	18.5	15.29	1.00:0.58:0.76
3-B Precipitate at	0.7	13.2[b]	10.5	30.54	1.00:0.29:1.20
3-C Precipitate at	1.2	26.7[b]	7.2	30.03	1.00:0 17:1.03
3-D Soluble at	1.2	7.1[b]	4.4	36.19	1 00:0.08:0.97
Alkali-treated whole PS			16.4	30.27	1.00:0.43:1.12
Alkali-treated Fraction 3			17.3	15.80	1.00:0.53:0.67

[a]Over Fraction 2. [b]Over Fraction 3.

From the methanolyzate of the whole polysaccharide, 3,6-anhydro-D-galactose dimethyl acetal, methyl α-D-galactopyranoside and carrabiose dimethyl acetal were isolated and characterized. These results indicated the presence of (1→3)-linked D-galactopyranose and (1→4)-linked 3,6-anhydro-D-galactopyranose residues in the polysaccharide.

The molar composition of the whole polysaccharide and of the fractions are summarized in Table I. The molar ratios 1.00:0.32:1.47 of galactose:3,6-anhydro-galactose:sulfate for the whole polysaccharide lie within the values reported for sulfated galactans from other *Iridaea* species. Polysaccharides obtained from *Iridaea* species have been classified either as carrageenans, having a low content of κ-carrageenan, or as iridophycans[3,4].

As was first shown by Smith and Cook[9], carrageenan can be separated by 125mM KCl into two fractions of different chemical composition. The terms λ- and κ-carrageenans have been used for the soluble and insoluble fractions, respectively. According to Pernas *et al.*[10], the model based on λ- and κ-carrageenan is an over-simplification. The carrageenans cannot consist of a mixture of only two components, but rather of a series of polymers of different chemical composition, and with different solubility in potassium chloride solution. Fractional precipitation, with potassium chloride solutions, of the whole polysaccharide from *I. membranacea* confirmed its heterogeneity. Three fractions were obtained, and were studied by poly(acrylamide) gel-electrophoresis; the results are shown in Fig. 1. Fraction 1 was found to be homogeneous, and to have an unexpectedly low content of 3,6-anhydro sugar; corroborating this result, the infrared spectrum did not show a peak at 930 cm^{-1}.

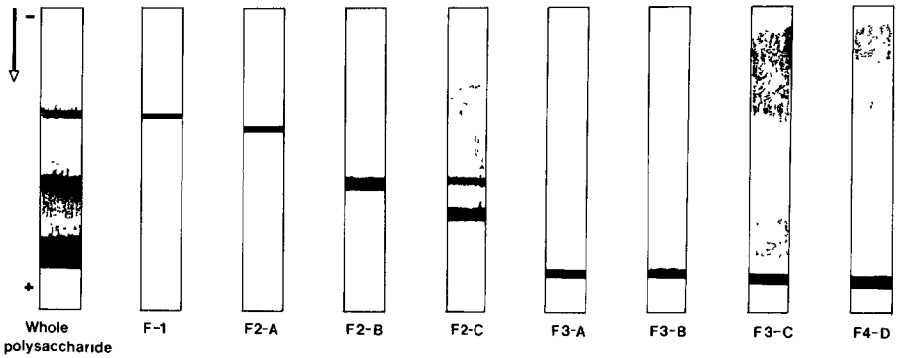

Fig. 1. Gel electrophoresis of carrageenans from *I. membranacea.*

The infrared spectrum of Fraction 2 showed at 930 cm^{-1} a band corresponding to the 3,6-anhydrogalactose residues and another, at 850 cm^{-1}, which could be assigned to a sulfo group linked to an axial, secondary hydroxyl group[11]. This fraction proved to be inhomogeneous, and was separated into three fractions by treatment with KCl solutions. It was found that Fraction 2-A and Fraction 2-B were homogeneous, whereas Fraction 2-C was not. Fraction 3 was separated into four subfractions by treatment with 0.4, 0.7, and 1.2M KCl. Gel electrophoresis showed that only Fraction 3-A, precipitated at 0.4M KCl, and Fraction 3-B, precipitated at 0.7M KCl, were homogeneous. These two fractions differ in their 3,6-anhydrogalactose and sulfate contents. The i.r. spectrum of Fraction 3-A showed a sharp band at 850 cm^{-1}, which could be indicative of the presence of an axial, sulfated secondary hydroxyl group, unlike Fraction 3-B which showed a broad band between 840–800 cm^{-1}, suggesting that sulfate groups of more than one type were present. After methylation analysis, these two fractions afforded the results shown in Table II. It may be seen that, for Fraction 3-A, the high proportion of 2,6-di-*O*-methylgalactitol is quite remarkable. Crystalline 2,6-di-*O*-methyl-D-galactose has been obtained from methylated carrageenans[12,13], indicating the presence of D-galactose 4-sulfate residues linked at O-3, one of the repeating units of κ-carrageenan. The same derivative is the major component of the methylation mixture of Fraction 3-B, but it is found in lower proportions compared to Fraction 3-A. The proportion of 2,3,4,6-tetra-*O*-methylgalactitol from methylated Fraction 3-A lies between the values reported for some carrageenans[14,15]. According to Matulewicz and Cerezo[15], its presence implies the existence of ramification in the polysaccharide. The finding of major proportions of this methylated derivative for Fraction 3-B might be indicative of a highly branched structure. Unfortunately, it proved impossible to carry out measurements of the molecular weight of the fully methylated, sulfated galactans in order to test this assumption. The presence of 2,4,6-tri-*O*-methylgalactitol residues showed that both fractions contain non-sulfated galactose units linked through O-3, although in low proportions. In this respect, these fractions resemble the polysaccharides obtained from *Furcellaria fastigiata*[16].

TABLE II

METHYLATION ANALYSIS OF FRACTIONS OF *Iridaea membranacea* POLYSACCHARIDE

Methylated sugars[a] *(as alditol acetates)*	*Mol %*[b]	
	I	*II*
2,3,4,6-Gal[c]	5.6	26.5
2,4,6-Gal[c]	2.1	2.4
2,6-Gal[c]	64.2	35.6
2,4-Gal	4.0	6.8
6-Gal	7.6	7.9
2-Gal	2.6	3.1
3-Gal + 1,2,3,4,5,6-hexa-*O*-acetyl-Gal	13.5	17.1

[a]2,3,4,6-Gal = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, *etc.* [b]Values are corrected by use of the effective carbon-response factors given by Albersheim *et al.* [31]. I = Fraction 3-A, II = Fraction 3-B. [c]Identified by g.l.c.–m.s., using column *H*.

The pattern of monomethylated galactose units is quite similar in both fractions; they might derive from disulfated residues or monosulfated branching points. The results of methylation of Fractions 3-A and 3-B must be treated with some caution, because of uncertainty as to the extent of methylation, and the possibility of partial demethylation during the subsequent hydrolysis. Nevertheless, methylation analysis and spectroscopic evidence (band at 850 cm^{-1} for axial, secondary sulfate) suggests that Fraction 3-A is mainly composed of (1→3)-linked D-galactose, sulfated at C-4. On the other hand, Fraction 3-B resembles λ-carrageenan in some respects. It is highly sulfated, having about 1.2 sulfate per galactose residue, and it contains a small proportion of 3,6-anhydro-D-galactose, but it is distinct from λ-carrageenan in that it contains (1→3)-linked residues of D-galactose sulfated at C-4 rather than at C-2. Furthermore, methylation analysis showed features unlike any reported before for sulfated galactans of the λ-carrageenan type. At present, no structure can be proposed for this fraction.

The whole polysaccharide and Fraction 3 were treated with alkali; the results are shown in Table I. According to Rees[17], the formation of 3,6-anhydrogalactose takes place by alkali-catalyzed elimination of the sulfuric half-ester. The decrease in the sulfate content should equal the increase in that of 3,6-anhydrogalactose. In the alkali treatment of λ-carrageenan, Rees found the release of 1.2 mol of sulfate per mol of 3,6-anhydrogalactose formed. On the other hand, Smidsrød *et al.* [18] reported an apparent loss of sulfate lower than that expected in the alkali treatment of carrageenans, but no explanation has yet been proposed.

In the case of the whole polysaccharide, a sulfate loss of 7% concomitant with an increase of 5% in the content of 3,6-anhydrogalactose was observed. Alkali treatment of Fraction 3 increases the content of 3,6-anhydrogalactose by 0.03 mol, whereas the sulfate content decreases by 0.15 mol. These results are similar to those previously reported for Fraction 3 of the iridean from *I. laminarioides*[3]. It

seems that both fractions contain the same proportion of galactose 6-sulfate units, and similar proportions of sulfate groups at other positions of the monosaccharide units. The latter might suffer hydrolysis under alkaline treatment; this would explain why the loss of sulfate is higher than that expected for the elimination and for formation of the 3,6-anhydro ring.

It may be concluded that the soluble polysaccharide from *Iridaea membranacea* is a complex mixture of sulfated D-galactans. Its chemical properties resemble those of the polysaccharides described for *I. ciliata*[3] and *I. laminarioides*[4].

EXPERIMENTAL

Material and methods. — The seaweed was collected in Cocholhue, Concepción Bay, in the month of December. Evaporations were conducted *in vacuo,* the bath temperature being kept below 50°. Optical rotations were measured with a Perkin-Elmer 241 spectropolarimeter. Analytical and preparative paper-chromatography were respectively performed on Whatman No. 1 and No. 3 papers. Analytical thin-layer chromatography was performed on Merck aluminum sheets precoated with silica gel 60; zones were detected by charring with 25% sulfuric acid. The solvent systems used were: (*A*) 2:1:1 (v/v) 1-butanol–ethanol–water, (*B*) 8:2:1 (v/v) ethyl acetate–pyridine–water, (*C*) 3:3:1 (v/v) ethyl acetate–acetic acid–water, (*D*) 4:1:2 (v/v) 1-butanol–ethanol–water, (*E*) 3:1 (v/v) petroleum ether (40–60°)–ethyl acetate, and (*F*) 4:3 (v/v) cyclohexane–acetone.

The g.l.c. analyses were performed with a Varian 3700 gas chromatograph equipped with a flame-ionization detector, and having dual stainless-steel columns (2.0 m × 2.0 mm i.d.) packed with (*G*) 3% of SP-2340, (*H*) 3% of ECNSS-M, and (*I*) 2% of OV-101 on Chromosorb W. G.l.c.–mass spectrometry was performed on a 1440 gas chromatograph–Varian-Mat CH-7 spectrometer interfaced to a Varian-Mat Data System 166 using glass columns (1.80 m × 2.0 mm i.d.). The carrier gas was helium at 12 mL/min. Mass spectra were obtained by electron impact at 70 eV at a source temperature of 200°.

^{1}H-N.m.r. spectra (60 and 100 MHz) were recorded, and integrated, with Varian 360-A and Varian XL-100 spectrometers. The percentage of 3,6-anhydrogalactose was determined according to the procedure described earlier[19]. Microanalyses for sulfate were performed by Dr. B. B. de Deferrari (University of Buenos Aires), according to the method reported by Schöniger[20]. The content of galactose was calculated by subtracting the anhydrogalactose content from the total hexose content according to the anthrone method of Yaphe[21], and taking into consideration the minor monosaccharide contents.

Gels obtained by precipitation with potassium chloride were dissolved in sodium acetate solution (5%) and dialyzed against the same solution first, and against water afterwards. Poly(acrylamide) gel-electrophoresis was conducted according to the technique reported by Usov and Arkhipova[22], staining with Toluidine Blue, and destaining with 5% acetic acid in water.

Extraction of the polysaccharide. — The ground, dried seaweed (170 g) was stirred with water (3 L) for 1 h at 80°. The mixture was filtered through muslin, and the extraction process was repeated thrice. The filtrate was centrifuged, and, after dialysis against distilled water, the supernatant liquor was concentrated to a thin syrup, and this poured into ethanol (3.5 vol.), giving a fibrous precipitate (58.5 g).

Hydrolysis of the polysaccharide. — The hydrolysis was performed on the polysaccharide (2.5 g) with 0.5M H_2SO_4; paper chromatography (systems *A*, *B*, and *C*) showed the presence of galactose, glucose, and xylose and/or 6-*O*-methylgalactose. Preparative paper-chromatography (system *B*) afforded a syrup which crystallized from 4:1 ethanol–water. Recrystallization from the same solvent gave D-galactose; m.p. 166–167°, $[\alpha]_D^{20}$ +82.0° (*c* 2.15, water); lit.[23] m.p. 167°, $[\alpha]_D$ +80.2° (water). A small portion of the hydrolyzate was reduced, and the products acetylated according to the procedure reported by Wolfrom and Thompson[24]. The resulting syrup was analyzed by g.l.c. (column *G*), which showed the presence of penta-*O*-acetyl-6-*O*-methylgalactitol (0.9%), penta-*O*-acetylxylitol (0.3%), hexa-*O*-acetylglucitol (0.8%), and hexa-*O*-acetylgalactitol (98%). An aliquot of the syrup crystallized from ethanol, giving hexa-*O*-acetylgalactitol, m.p. 168°; lit.[25] m.p. 168°.

Methanolysis. — Methanolysis was performed on the polysaccharide (10.0 g) according to the procedure reported by Araki and Hirase[26]. The resulting syrup was chromatographed on a cellulose column (70 × 5 cm) with system *D*, and 10-mL fractions were collected. Fractions were examined by paper chromatography, and combined into three main fractions.

Fraction *A*: On evaporation, a syrup (0.080 g) was obtained, R_F 0.67 (system *D*); $[\alpha]_D^{20}$ +32.7° (*c* 0.73, water); lit.[26] for 3,6-anhydro-D-galactose dimethyl acetal (syrup), $[\alpha]_D$ +36.5° (water), R_F 0.67 (system *D*). An aliquot was acetylated, and the product analyzed by g.l.c. (column *H*). It gave only one component, which co-chromatographed with an authentic sample of 2,4,5-tri-*O*-acetyl-3,6-anhydro-D-galactose dimethyl acetal.

Fraction *B*: On evaporation, a syrup (0.940 g) was obtained which was purified by preparative paper-chromatography (system *D*). A small sample was acetylated with acetic anhydride and pyridine, affording methyl 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside; m.p. 86–87°, $[\alpha]_D^{20}$ +132.0° (*c* 0.9, chloroform); lit.[27] m.p. 86–87°, $[\alpha]_D$ +132.5° (chloroform).

Fraction *C*: The syrup (0.320 g) obtained by evaporation was purified by preparative paper-chromatography (system *D*), and acetylated. The resulting syrup was purified by preparative thin-layer chromatography (system *F*), and crystallized from methanol, giving hexa-*O*-acetylcarrabiose dimethyl acetal; m.p. 151–152°, $[\alpha]_D^{20}$ −18.0° (*c* 2.0, benzene); lit.[28] m.p. 153–154°, $[\alpha]_D$ −15.5° (benzene). Its ^{1}H-n.m.r. spectrum was in full agreement with published data[29] for hexa-*O*-acetylcarrabiose dimethyl acetal.

Fractionation of the polysaccharide. — A solution of the polysaccharide (20 g) in water (4 L) was fractionated with potassium chloride according to the

procedure reported by Pernas *et al.*[10]; three fractions were obtained. Homogeneity of fractions was checked by poly(acrylamide) gel electrophoresis; the results are shown in Fig. 1. Fractions 2 and 3 were refractionated with solutions of increasing concentration of KCl.

Methylation analysis. — *(a) Fraction 3-A.* The methylation was performed with dimethyl sulfate and sodium hydroxide according to the procedure reported by Cerezo[30]. Three methylations, each lasting 5 days, were performed. A small portion of the material was hydrolyzed with 0.75M H_2SO_4; the resulting syrup showed only traces of galactose in paper chromatography (system *A*); OMe, 16.9%. Further methylation did not change the methoxyl content. The methylated polysaccharide was treated with 2M trifluoroacetic acid for 16 h at 95°. The acid was removed by repeated evaporation with addition of water, and the resulting syrup was reduced with sodium borohydride, and the products acetylated with acetic anhydride–pyridine. The mixture of partially methylated alditol acetates was studied by g.l.c., using columns *G* and *H*; compounds were identified by co-chromatography with corresponding authentic samples; the results are listed in Table II, column I.

(b) Fraction 3-B. The methylation and g.l.c. analysis were conducted as in (*a*); OMe: 11.2%; the results are listed in Table II, column II.

Alkaline treatment. — Samples of the whole polysaccharide and of Fraction 3 were separately treated with sodium hydroxide solution according to the procedure reported by Rees[17].

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