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Water-soluble Sulfated Polysaccharides from the Red Seaweed *Chaetangium fastigiatum*. Analysis of the System and the Structures of the α-D-(1→3)-Linked Mannans

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SUMMARY

The water-soluble polysaccharides from Chaetangium fastigiatum *were fractionated with cetrimide. The complexed material was subjected to fractional solubilization in solutions of increasing sodium chloride concentration and seven fractions were separated and analyzed. Two of the fractions were subjected to methylation and desulfation–methylation analyses. The results indicate that this seaweed contains a system of sulfated polysaccharides consisting in part of a galactan and an α-D-(1→3)-linked mannan, 2- and 6-sulfated, and having single stubs of β-(1→2)-linked D-xylose. Composition dispersity of the mannan is produced by variation of the amount and disposition of the sulfate groups and of the content of the xylose side-chains.*

INTRODUCTION

The major water-soluble polysaccharides of the Rhodophyceae are galactan sulfates (Percival & McDowell, 1981). Nevertheless, there are red seaweeds which synthesize, as their main water-soluble polysaccharides, a 'mixed linkage' homoxylan type polymer (Percival & McDowell, 1981). These xylans are usually found together with sulfated polysaccharides which have been reported to contain galactose and/or

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mannose as their main sugars (Nunn *et al.*, 1973; Usov *et al.*, 1974; Usov *et al.*, 1975*a*; Percival, 1979).

The red seaweed *Chaetangium fastigiatum* contains a water-soluble β-D-(1→3)-, β-D-(1→4)-'mixed linkage' homoxylan (Cerezo *et al.*, 1971; Cerezo, 1972). We now report on the isolation of a system of water-soluble sulfated polysaccharides consisting in part of a galactan and a family of α-D-(1→3)-linked mannans, 2- and 6-sulfated, and carrying single β-(1→2)-linked D-xylopyranosyl side-chains.

EXPERIMENTAL

Materials

Chaetangium fastigiatum was collected near Puerto Deseado in Southern Patagonia, Argentina, and was dried in the open under strong winds.

Separation and fractionation of the sulfated polysaccharides

The extraction of the water-soluble polysaccharides has been described elsewhere (Cerezo *et al.*, 1971).

To a solution of the polysaccharides (4·95 g) in water (500 ml) a 10% (w/v) aqueous solution of cetrimide (50 ml) was added slowly with stirring, with the stirring continuing overnight. The complexes were removed by centrifugation and suspended in water (500 ml). Finely divided sodium chloride was added, with constant stirring, up to a 0·5 M concentration and the stirring was continued overnight. The precipitate was centrifuged off and the supernatant was extracted with 1-pentanol (3 × 100 ml), dialyzed, concentrated, and freeze-dried. The precipitate was subjected to successive similar procedures so that the concentration of sodium chloride was increased by 0·5–1·0 M each time. The upper limit of the sodium chloride concentration was 4·0 M and the residual precipitate was suspended in water and the suspension was dialyzed and freeze-dried. Ranges of sodium chloride concentrations, yields, and analyses of the seven fractions obtained are given in Table 1.

The supernatant from the cetrimide precipitation was retreated with cetrimide but no further complex formation occurred. The solution was dialyzed, concentrated, and freeze-dried giving 0·98 g (19·8% of the total water-soluble polysaccharides) of the previously worked xylan (Cerezo *et al.*, 1971; Cerezo, 1972).

Subfractionation of the sulfated polysaccharides with ethanol

Fractions (50–200 mg) were dissolved in water (0·2% w/v) and ethanol was added stepwise, in small aliquots (2–10 ml). The precipitates were

TABLE 1

Yields, Analyses, and Optical Rotations of the Fractions Obtained Through Redissolution in Sodium Chloride of the Cetrimide Salts, Sulfated Polysaccharides and Two Desulfated Derivatives

Fraction	*Range of redissolution (M NaCl)*	*Yield*[a] *(%)*	*Sulfate (% SO_3Na)*	*Protein (%)*	$[\alpha]_D$[b] *(°)*	*Sugar composition*						*Mannose:Sulfate molar ratio*
						Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	
						(mol%)						
1	0–0·5	22·9	12·9	16·0	−30·9 (−35·5)	3·0	2·6	18·6	12·1	54·4	9·3	0·4
2	0·5–1·0	11·8	19·2	5·4	+16·2 (+20·1)	2·6	2·5	23·7	57·1	11·9	2·2	1·5
3	1·0–1·5	22·9	23·4	—	+23·7 (+30·9)	—	1·7	23·2	68·4	6·7	—	1·5
4	1·5–2·0	11·6	27·6	—	+27·8 (+38·4)	—	1·9	10·6	77·8	9·8	—	1·3
5	2·0–3·0	13·9	27·3	—	+39·5 (+54·4)	—	2·4	2·2	82·8	12·6	—	1·4
6	3·0–4·0	6·6	23·4	3·0	ins[c]	—	3·1	6·5	73·9	16·4	—	1·5
7	4·0[d]	10·3	14·0	14·6	ins[c]	1·1	4·4	10·3	17·0	64·5	2·5	0·6
3d[e]		65·9[f]	4·2	—	+69·1 (+72·1)	—	—	22·9	67·3	9·7	—	10·1
5d[e]		69·9[f]	11·0	—	n.d.[g]	—	—	5·1	94·9	—	—	4·9

[a] Yields for fractions 1–7 are given as percentages of the recovered (38·0% of the water-soluble polysaccharides).

[b] Optical rotations were determined in 0·1 M sodium chloride. Figures in parentheses indicate values calculated for the non-sulfated derivatives, on the basis that the sulfate groups do not contribute to the rotation (Harris & Turvey, 1970).

[c] Fractions 6 and 7 were insoluble in 0·1 M and 1·0 M sodium chloride and 10% sodium hydroxide.

[d] Insoluble in 4·0 M sodium chloride.

[e] 3d and 5d are the desulfated derivatives of fractions 3 and 5.

[f] Yield from desulfation treatment.

[g] n.d. = not determined.

removed by centrifugation, redissolved in water, and freeze-dried. Ranges of ethanol concentrations, yields, and analyses are given in Table 2 and Table 3.

General and analytical methods

Sulfate (expressed as SO_3Na) was analyzed by the method of Wagner (1957) after removing sodium cations, and by the turbidimetric method of Dodgson & Price (1962). Nitrogen was determined by the method of Dumas & Pregl (1958) and the protein content was calculated by multiplying the nitrogen content by 6·25.

The optical rotation was measured at room temperature in a Perkin-Elmer 141 polarimeter (sodium D-line) using 0·12–0·34% solutions of polysaccharide in 0·1 M or 1·0 M sodium chloride. The infrared spectra were recorded with a Perkin-Elmer 710B infrared spectrophotometer using a polysaccharide film (Matulewicz & Cerezo, 1980).

Hydrolyses of polysaccharides were carried out in sealed tubes with 2 M trifluoroacetic acid for 16 h at 95°C and the sugar mixtures were derivatized for analysis by g.l.c. and g.l.c.–m.s.

G.l.c. was performed on a glass column (0·2 × 180 cm) packed with 3% ECNSS-M on Gas-Chrom Q (100–120 mesh). A Hewlett-Packard 5830A Gas Chromatograph with a flame ionization detector and a

TABLE 2
Yields, Analyses and Optical Rotations of the Subfractions Obtained from Fraction 3 by Stepwise Addition of Ethanol

Ethanol (% (v/v))	*Yield*[a] *(%)*	*Sulfate (% SO_3Na)*	$[\alpha]_D$[b] *(°)*	*Sugar composition*			
				Ara	*Xyl*	*Man*	*Gal*
				(mol%)			
—[c]	9·3	18·5	+38·6	2·0	22·0	69·9	6·1
63–64	15·8	32·6	+20·2	1·8	23·7	68·1	6·4
66–69	14·9	26·7	+21·1	1·7	24·0	68·6	5·7
69–80	10·4	25·2	+28·8	1·5	23·3	71·8	3·4
80–82	4·1	28·3	n.d.[d]	1·4	23·4	70·1	5·1
82[e]	45·4	21·5	+24·4	1·4	23·8	69·1	5·7

[a] Yields are given as percentages of the recovered (97·0%).
[b] Optical rotations were determined in 0·1 M sodium chloride.
[c] Insoluble in water.
[d] n.d. = not determined.
[e] Soluble in 82% ethanol.

TABLE 3

Yields and Sugar Composition of the Subfractions Obtained from Fraction 5 by Stepwise Addition of Ethanol. Refractionation of the Ethanol-soluble Subfraction with Ethanol and by Gel-permeation Chromatography

Ethanol (% (v/v))	*Yield*[a] *(%)*	*Sugar composition*			
		Ara	*Xyl*	*Man*	*Gal*
			(mol%)		
59–63[b]	25·4	2·2	5·7	84·7	7·4
63–80	5·1	2·1	3·8	88·7	5·3
82[c,e]	69·5	1·9	4·8	86·1	7·1
63–73[d]	17·9	4·6	1·8	88·4	5·2
73–82	17·9	3·9	2·8	86·4	6·8
82[c]	64·2	3·7	7·9	80·4	8·0
1[e]	8·9	—	6·9	42·3	50·8
2	35·2	tr[f]	5·0	95·0	tr
3	15·7	4·2	5·0	87·8	2·9
4	17·5	3·4	5·1	91·5	tr
5	18·7	3·3	3·7	81·2	11·7
6	4·0	—	19·9	58·5	21·6

[a] Yields are given as percentages of the recovered (100·0%).

[b] Subfractionation of fraction 5 by stepwise addition of ethanol. The sulfate content (expressed as SO_3Na) was 24·9%, 31·5%, and 23·2% for the subfractions obtained at 59–63% and 63–80% ethanol concentrations and the subfraction soluble in 82% ethanol, respectively. The optical rotations of the first and third subfractions were +29·7° (0·1 M NaCl) and +31·6° (1·0 M NaCl), respectively.

[c] Soluble in 82% ethanol.

[d] Further fractionation of the ethanol-soluble subfraction.

[e] Gel-permeation chromatography of the ethanol-soluble subfraction.

[f] Percentages lower than 1·0% are considered as traces (tr).

Hewlett-Packard 18850A g.c. terminal was used. Chromatography was carried out at (a) 190°C isothermally for the alditol acetates (Sloneker, 1972) and (b) 170°C isothermally for the methylated alditol acetates (Lindberg, 1972) and methylated aldononitrile acetates (Woolard *et al.*, 1977; Stortz & Cerezo, 1985). The nitrogen flow rate was 25 ml min^{-1}, and the injector and FID temperature was 210°C. Computerized g.l.c.–m.s. (El'kin *et al.*, 1972; Lönngren & Svensson, 1974; Seymour *et al.*, 1975; Stortz *et al.*, 1982) was carried out with a Varian Series 1400 Gas Chromatograph coupled to a Varian MAT CH7A mass spectrometer with a MAT 166 data system. Chromatography was performed on a glass column (0·3 × 120 cm) of 3% ECNSS-M on Gas-Chrom Q

(100–120 mesh) programming from 120°C for 1 min and then at 4°C min^{-1} to 190°C; the helium flow rate was 25 ml min^{-1}. Mass spectra were recorded over a mass range of 40–600 atomic mass units using an ionizing potential of 70 eV.

Gel-permeation chromatography

A solution of polysaccharide (3 mg) in water (1 ml) was applied to a column (1·5 × 54 cm) of Sephadex G-100. The column was eluted with water; fractions (1 ml) were collected and analyzed for carbohydrate content by the phenol–sulfuric acid reaction (Dubois *et al.*, 1956). Fractions corresponding to the same peak were pooled, concentrated, and freeze-dried. These were hydrolyzed and analyzed by g.l.c. of the derived alditol acetates. The void and total volumes of the column were determined using Blue Dextran and mannose, respectively.

Desulfation of the fractions 3 and 5

A portion of polysaccharide (30–113 mg) was suspended in 0·1 M methanolic hydrogen chloride (20–75 ml) and the mixture was stirred for 96–120 h at room temperature. Water (10–37 ml) was added and the mixture was neutralized with aqueous sodium bicarbonate and dialyzed. The resulting solution was concentrated and freeze-dried. Yields: desulfated fraction 3, 65·9% (SO_3Na, 4·2%); desulfated fraction 5, 69·9% (SO_3Na, 11·0%).

Methylation analyses

Fraction 3 (11·7 mg), desulfated fraction 3 (21·7 mg), and desulfated fraction 5 (14·2 mg) were permethylated by the method of Hakomori (1964) as described by Lindberg (1972). The Hakomori procedure was repeated until constant composition. Yields: fraction 3, 11·9 mg; desulfated fraction 3, 22·8 mg; desulfated fraction 5, 10·2 mg.

Fraction 5 (24·4 mg) was methylated by the method of Haworth and further fully methylated according to Hakomori. Yield, 24·2 mg.

The permethylated polysaccharides were hydrolyzed and the partially methylated aldoses were converted into the corresponding acetylated alditols and aldononitriles. The mixtures of alditol acetates and aldononitrile acetates were analyzed by g.l.c. and g.l.c.–m.s. and identified by a combination of g.l.c. retention-times (known standards) and mass spectra.

RESULTS

The sulfated polysaccharides extracted with water from the red seaweed *Chaetangium fastigiatum* were precipitated with cetrimide and the insoluble complexes were subjected to fractional solubilization in solutions of increasing sodium chloride concentration. Seven fractions were separated in this way and for each fraction, the yield, monosaccharide composition, and other properties are given in Table 1. Data from Table 1 suggest that the alga does not contain a single polysaccharide but a system of polydispersed polysaccharides which can be divided into: (A) fractions 2–6 in which the molar ratio mannose:sulfate is constant (1·3–1·5). In fractions 2–5 the percentages of sulfate and mannose and the optical rotations are directly related to the concentration of sodium chloride necessary to dissociate the cetrimide complex. (B) fractions 1 and 7 with similar monosaccharide composition, sulfate percentage, and molar ratio mannose:sulfate (0·4–0·6).

Though the fractions were obtained from their aqueous solutions by freeze-drying, they showed a tendency to insolubilization; for instance fraction 2 and fraction 3 (Table 2) became, with time, partially insoluble in water and a similar behavior was observed with some samples of fraction 1 after one year of being prepared. Moreover, fraction 6 became insoluble even in 10% sodium hydroxide.

Fractions of group B (1 and 7) were reserved for further research while fractions of group A (3 and 5) were examined for homogeneity. Subfractionation of fractions 3 and 5 by stepwise precipitation with ethanol showed that they were homogeneous in sugar composition (Table 2 and Table 3). Gel-permeation chromatography on Sephadex G-100 of the ethanol-soluble subfraction of fraction 5 yielded an incipient composition separation (Table 3). In this chromatography all the molecules entered into the gel and the presence of several peaks and shoulders suggested molecular weight polydispersity. It is noteworthy that this subfraction also became, with time, partially insoluble in water. On the other hand, gel-filtration on Sephadex G-100 of the ethanol-soluble subfraction of fraction 3 showed only one asymmetric peak at the void volume with some material entering into the gel.

The ethanol-soluble subfraction of fraction 5, when submitted again to the stepwise precipitation with ethanol, gave new subfractions at lower ethanol concentrations (Table 3) indicating that the solubility populations were erratic and suggesting that they were not only associated with composition but also with temperature-, time-, and conformation-dependent molecular associations.

Fractions 3 and 5 as well as other components of group A showed a broad absorption band at 850 cm^{-1} in the infrared spectra, indicating the prevalence of secondary axial sulfate groups, but not sufficiently defined to permit differentiation between secondary equatorial (830 cm^{-1}) and primary sulphate groups (820 cm^{-1}) (Rees, 1963). The films were very weak and brittle.

When the optical rotations of the fractions of group A were calculated for the non-sulfated polysaccharides (Table 1) and these values were plotted against the percentages of xylose, a straight line was obtained; extrapolation to zero xylose concentration gave a rotation of +55°. The fact that the rotation is inversely proportional to the xylose content suggests that in all the fractions the xylose units have the same structural significance and that their linkage is of the β-D type.

Fraction 5 was methylated and Table 4 shows the composition of the permethylated mannan. This composition indicates a (1→3)-linked

TABLE 4

Relative Proportions (mol%) of Methylated Sugars Formed by Hydrolysis of the Methylated Fractions 3 and 5 and their Desulfated Methylated Derivatives

Sugar	*Fraction 3*	*Desulfated fraction 3*	*Fraction 5*	*Desulfated fraction 5*
2,3,4-Me_3 Xyl	23·7	22·9	2·1	4·5
2,3,4,6-Me_4 Man	—	1·1	tr[a]	tr
2,4,6-Me_3 Man	5·4	42·0	5·1	67·7
4,6-Me_2 Man	34·3	26·6	45·7	15·0
2,6-Me_2 Man	tr	1·3	tr	tr
6-Me Man	2·7	tr	3·4	tr
2,4-Me_2 Man	11·6	2·7	34·5	6·7
2-Me Man	tr	tr	1·3	1·3
4-Me Man	22·3	3·3	7·8	4·7

[a] Percentages lower than 1·0% are considered as traces (tr).

backbone with ~80% of the mannose units monosulfated on C-2 or C-6, in similar proportions. Minor amounts of single D-xylopyranosyl side-chains were also found.

The mannan was desulfated with 0·1 M methanolic hydrogen chloride and the treatment led to an elimination of 59·7% of the sulfate content, without visible degradation (Table 1). The partially desulfated derivative was submitted to methylation analysis (Table 4). The increase of 2,4,6-

tri-*O*-methylmannose together with the concomitant decrease of 2,4-di-*O*-methyl- and 4,6-di-*O*-methyl-mannose is in agreement with the previously postulated structure. The compositions of the methylated sulfated mannan and its partially desulfated derivative suggest that ~ 10% of the units are 2,4-, 2,6-, and 4,6-disulfated.

Fraction 3 was submitted to methylation analysis and the composition of the permethylated derivative (Table 4) showed a structure similar to that of fraction 5, with the following differences: (a) a considerable increase in the side-chains formed by single stubs of D-xylopyranosyl units, (b) a considerable increase of 4-*O*-methylmannose, suggesting that these units are branching points, and (c) the presence of minor amounts of 2,4-di-*O*-methylmannose.

Fraction 3 was desulfated in similar conditions to fraction 5, with an elimination of 82·0% of the sulfate content, and no degradation was observed (Table 1). Methylation analysis of the desulfated derivative (Table 4) showed, besides the increase of 2,4,6-tri-*O*-methylmannose, a considerable decrease of 4-*O*-methylmannose with only a slight decrease in the 4,6-di-*O*-methylmannose content. These results are consistent with the fact that the xylopyranosyl units are linked to the backbone through C-2 of the 3-linked mannopyranosyl residues.

It is noteworthy that only xylose and mannose were detected after the methylation and desulfation–methylation analyses of fractions 3 and 5.

DISCUSSION

The red seaweed *Chaetangium fastigiatum* contains not only a β-D-(1→3)-, β-D-(1→4)-'mixed linkage' homoxylan (Cerezo *et al.*, 1971; Cerezo, 1972) but also a system of sulfated polysaccharides consisting in part of a galactan and an α-D-(1→3)-linked mannan, 2- and 6-sulfated, and having single stubs of β-(1→2)-linked D-xylose. The composition of the mannan varies in the amount and disposition of the sulfate groups and the content of the D-xylose side-chains.

The analysis of the sulfated polysaccharide fraction from *Chaetangium erinaceum* suggests a similar system (Nunn *et al.*, 1973). The red seaweed *Nemalion vermiculare* synthesizes an α-D-(1→3)-linked mannan with the units monosulfated in the 4- and 6-positions (Usov *et al.*, 1974; Usov *et al.*, 1975*a, b*) and carrying a small proportion of single β-(1→2)-linked D-xylopyranosyl side-chains (Usov & Yarotskii, 1975). An α-D-(1→3)-linked mannan was also found among the components of the complex system of water-soluble polysaccharides from the green seaweed *Urospora penicilliformis* (Bourne *et al.*, 1974).

The optical rotation reported for the mannan from *Urospora penicilliformis* was + 50° (water) (Bourne *et al.*, 1974). This value was in agreement with the rotation of the 3-*O*-α-D-mannopyranosyl-D-mannose (+ 50°, water) (Bourne *et al.*, 1974) suggesting that the mannose residues in the polysaccharide were α-D-(1→3)-linked and that the conformation at each glycosidic linkage corresponded to that of the model disaccharide, i.e. the polysaccharide has a random fluctuating conformation rather than an ordered secondary structure. The lower optical rotations found for fractions 2–5 together with the fact that the rotations are inversely proportional to the xylose contents and that extrapolation to xylose nil gives a rotation of + 55°, are evidence that these fractions have the same structure, namely an α-(1→3)-linked D-mannose backbone, substituted with variable amounts of β-D-xylosyl units, and that in aqueous solution a random coil conformation is produced.

Significant amounts of galactose were found in all the fractions but no galactose derivatives were detected in the methylation and desulfation–methylation analyses of fractions 3 and 5. These results together with the rotations and the analogies with the other mannans found in seaweeds suggest that this sugar is a contaminant, possibly due to the coprecipitation of the galactan present in the same system.

The sulfated mannan from *Nemalion vermiculare* loses its solubility in water and does not dissolve even when heated in 20% sodium hydroxide solution after elimination of the sulfate groups (Usov *et al.*, 1974). On the other hand, the mannan from *Urospora penicilliformis* which contains only 2% of sulfate is reported as being readily soluble in water (Bourne *et al.*, 1974). This is in agreement with the erratic solubility properties shown by the mannans 3 and 5 (Table 2 and Table 3), as well as with the partial or total insolubilization observed with the other mannan fractions.

The insolubilization of polysaccharides implies the formation of packed aggregates which exclude solvation of the polysaccharide by the water molecules. These third-order structures depend on a regular conformation: the α-(1→3)-linked D-mannose backbone may form a zigzag arrangement with a ribbon-like conformation, but this conformation is not stabilized due to the impossibility of forming intramolecular hydrogen bonds and to its poor packing properties (Rees & Scott, 1971; Rees, 1977). Nevertheless, the flexibility of the chain is rather restricted by the axial bond to the glycosidic oxygen (Morris *et al.*, 1978) and by the repulsion of the negative charges of the sulfate groups. On the other hand, the solvation of these charged groups as well as the presence of single stubs of β-(1→2)-linked D-xylose would promote solubilization (Rees & Scott, 1971). As a result of these interactions the chain will be in

a random coil conformation and the insolubilization will be produced in conditions which favour the ribbon-like conformation and the subsequent aggregation (i.e. freeze-drying of the polysaccharide solution).

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