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# THE AGAR-TYPE POLYSACCHARIDE FROM THE RED ALGA *Ceramium rubrum**

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ABSTRACT

Aqueous extraction of the red alga *C. rubrum* gave a galactan sulphate and, possibly, a separate glucan and xylan. The galactan sulphate has an alternating structure of the agar-type with D-galactose or 6-*O*-methyl-D-galactose as one alternating unit, and L-galactose, 3,6-anhydro-L-galactose, and their respective 2-methyl ethers as the other unit. Sulphate hemi-ester groups are present on position 6 of both D- and L-galactose residues, with smaller amounts on positions 2 and 4 of, probably, D-galactose residues. The polysaccharide differs from others previously examined in that most of the L-galactose residues are non-sulphated.

## INTRODUCTION

The galactan sulphates present in most red algae can be considered as variants of either of two basic structures, agar or carrageenan. In the agar-type of polysaccharide, as idealised in agarose, 3-linked β-D-galactopyranose residues alternate with 4-linked 3,6-anhydro-α-L-galactopyranose residues in a repeating structure[1]. In practice, this ideal structure is frequently masked[2], in that D-galactose residues may be 6-*O*-methylated, or may carry sulphate hemi-ester groups at positions 2, 4, or 6. Similarly, the anhydro sugar can be replaced by its biological precursor, L-galactose 6-sulphate[2], and in addition, either of these residues may carry 2-*O*-methyl groups[3,4]. The genus *Ceramium* has species which are occasionally used as sources of agar, and a preliminary examination of the agar from *Ceramium boydenii* has been reported[5]. In addition to D-galactose and 3,6-anhydro-L-galactose, this polysaccharide contained 12% of 6-*O*-methyl-D-galactose. We have now examined the water-soluble polysaccharides of the European species, *Ceramium rubrum*.

## RESULTS AND DISCUSSION

Extraction of the dried alga with hot water gave a mucilage in 11% yield (Found: N, 1.46%). On being cooled, a 1% aqueous solution of the extracted material

*Dedicated to the memory of Professor Edward J. Bourne.

formed a very soft gel. Acid hydrolysis of a portion showed that the major monosaccharide constituents were galactose, glucose, xylose, 6-*O*-methylgalactose, and traces of other sugars, including 2-*O*-methylgalactose. In addition, the hydrolysates contained (t.l.c.) a furan derivative known to be produced by acid degradation of 3,6-anhydrogalactose. Of these products of hydrolysis, all but glucose and xylose could be expected from an agar-type polysaccharide. It is also known that many red algae produce separate glucans (floridean starch) and xylans[6], both of which are water soluble. Several attempts were made to separate the galactan sulphate from glucan and xylan, but none were completely successful. Gel filtration of an aqueous solution of the mucilage through Biogel P-200 gave a broad peak (*A*) followed by a smaller peak (*B*). On hydrolysis, *B* gave glucose only, thus proving that a separate glucan was present. The leading fractions of peak *A* were separated from the remainder of the peak and both portions were hydrolysed. The leading fractions gave galactose, 6-*O*-methylgalactose, and traces of glucose, but no xylose was detected. The remainder of the peak contained all the sugars present in the original mucilage. The absence of xylose from the early fractions again suggests that xylose is present in a separate polysaccharide. However, due to the difficulty in isolating quantities of these polysaccharides, subsequent studies were carried out on the original mucilage.

Fractionation and characterisation of the sugars present in a hydrolysate of the mucilage showed that the galactose was a mixture of D and L forms in the ratio 1.2:1, and that 6-*O*-methylgalactose, glucose, and xylose were all D forms. The small quantity of 2-*O*-methylgalactose precluded all but chromatographic identification. Oxidative hydrolysis[7] with dilute sulphuric acid and bromine led to the characterisation of 3,6-anhydro-L-galactose and 3,6-anhydro-2-*O*-methyl-L-galactose as their respective aldonic acids. The quantitative composition of the galactan sulphate portion of the mucilage is given in Table I.

TABLE I

COMPOSITION OF GALACTAN SULPHATE

| *Component* | *Molar ratio* |
|---|---|
| D-Galactose | 1.60 |
| 6-*O*-Methyl-D-galactose | 1.19 |
| L-Galactose | 1.34 |
| 3,6-Anhydro-L-galactose[a] | 1.64 |
| 2-*O*-Methyl-L-galactose | 0.01 |
| Sulphate | 0.70 |

[a]Includes the 2-methyl ether.

The pattern of galactose derivatives in the mucilage resembles those from *Laurencia pinnatifida*[3] and *Polysiphonia lanosa*[4]. The last two differ from one another and from related polysaccharides in the distribution of ester sulphate groups, and the pattern of sulphation in *C. rubrum* was therefore examined. Of significance is the

relatively low content of ester sulphate (2.53%) compared with 15.4 and 21.6%, respectively, for the two former algae. It has been shown previously[3,4,8] that, in algal galactans of this type, any L galactose residue (and its 2-methyl ether) invariably carries a 6-sulphate group and is thus capable of conversion into the 3,6-anhydro derivative either chemically by treatment with alkali[9], or biologically by the appropriate enzyme[10]. In the mucilage of *C. rubrum*, there is not enough sulphate to esterify all of the L-galactose, implying that some of these residues are not sulphated (see later). Partial, acid hydrolysis of the mucilage and separation of the acidic from the neutral fragments led to the isolation of galactose monosulphates and oligosaccharide sulphates. Preparative paper electrophoresis in borate buffer separated the galactose monosulphates into two bands. The major band was identified by chromatographic and electrophoretic mobility as galactose 6-sulphate; the minor band was shown by paper chromatography and methylation analysis to be a mixture of galactose 2- and 4-sulphates, but these compounds were not further characterised. The galactose 6-sulphate was characterised by hydrolysis to galactose only, and by periodate oxidation (see Experimental). Its optical rotation, however, indicated that it was a mixture of D and L forms in the ratio 1.8:1, which was confirmed by hydrolysis to galactose with a D to L ratio of 1.6:1. From the sulphate content and the relative amounts of sugar sulphates isolated, it is estimated that only one L-galactose residue in about five is sulphated at position 6. In the past, one of the criteria used to indicate the purity of an agarose preparation has been a low sulphate content, as this usually implies a high content of 3,6-anhydrogalactose and hence of agarose. With agar fractions from *Ceramium* sp., this may no longer be a valid criterion, as fractions with little sulphate may still have appreciable amounts of L-galactose instead of 3,6-anhydro-L-galactose.

That the galactan sulphate of *C. boydenii* is an agar has been demonstrated previously by the isolation, as their dimethyl acetals, of the disaccharides agarobiose (3,6-anhydro-4-*O*-$\beta$-D-galactopyranosyl-L-galactose) and $6^2$-*O*-methylagarobiose from partial methanolysates of the polysaccharide[1]. The galactan sulphate from *C. rubrum* was also confirmed as an agar-type by two methods. Mild, oxidative hydrolysis[7] of the polysaccharide gave a mixture of mono- and di-saccharides as their respective aldonic acids. In the disaccharide mixture, the molar ratio for galactose plus 6-*O*-methylgalactose to 3,6-anhydrogalactonic acid was 0.98:1, thus indicating a mixture of the aldonic acids of agarobiose and $6^2$-*O*-methylagarobiose. In the second test, it was shown that an agarase preparation from the bacterium *Cytophaga* sp., which is known to depolymerise agar and related polysaccharides[11], caused extensive degradation of the polysaccharide with production of oligosaccharides similar (t.l.c.) to those produced by porphyran.

## CONCLUSION

The galactan sulphate of *C. rubrum* belongs to the agar-type in which the two basic units, D-galactose and 3,6-anhydro-L-galactose, alternate in a chain. Like other

polysaccharides of this type, it has some of the D-galactose residues 6-*O*-methylated, and the anhydro sugar is to some extent replaced by L-galactose 6-sulphate, 2-*O*-methyl-L-galactose, and 3,6-anhydro-2-*O*-methyl-L-galactose. However, in this polysaccharide, a proportion of the anhydro sugar is replaced by non-sulphated L-galactose residues, only about one in five of these units being 6-sulphated. In this respect, the *Ceramium* polysaccharide differs from others examined previously[3,4,8], in that the latter polysaccharides have virtually complete 6-sulphation of their L-galactose residues. The remainder of the sulphate is distributed on the D-galactose, mainly at position 6 as in *Polysiphonia lanosa*[4], but with smaller amounts at position 2 (as in *Laurencia pinnatifida*[3]) and position 4 (as in porphyran[8,12]).

## EXPERIMENTAL

*General methods.* — The methods of paper chromatography (p.c.), t.l.c., and paper electrophoresis in neutral and in borate buffer have been described[3,4]. Quantitative analysis of sugar mixtures by g.l.c. of glycitol acetates, and the analysis of hexoses in solution with the phenol–sulphuric acid reagent, of sulphate by flame photometry, and of total 3,6-anhydrogalactose in solution have also been described[3,4].

*Extraction of polysaccharide.* — The alga was collected in Anglesey in the summer of 1967. It was sorted, air-dried, and crushed to a coarse powder. The powder (150 g) was stirred at 95° with water (5 l) for 24 h, the pH being maintained at 6. The cooled mixture was centrifuged, and the supernatant solution was concentrated at 40° to 1 l and poured into acetone (1.5 l). The precipitate was collected by centrifugation, washed with ethanol and with ether, and dried. The residual weed was re-extracted with water (2 l) at 95°, and the polysaccharide recovered as described above. The combined polysaccharides were dissolved in warm water (3 l), the solution was dialysed against tap water for 3 days, and the polysaccharide was reprecipitated by addition of ethanol (2 vol.). The recovered polysaccharide was dried at 60° over calcium chloride; yield, 15.5 g (Found N, 1.46%).

A solution of the polysaccharide (20 mg) in 0.75M sulphuric acid (5 ml) was heated at 100° for 3 h, cooled, neutralised with barium hydroxide, and filtered. The filtrate was concentrated, and examined by p.c. and by t.l.c. on cellulose plates.

*Gel filtration of polysaccharide.* — The polysaccharide (0.1 g) in M sodium chloride (5 ml) was absorbed on a column (60 × 2 cm) of Biogel P-200, previously equilibrated in M sodium chloride. Elution with this solvent gave a broad peak (40–360 ml), followed by a small peak (510–610 ml), when the eluate was tested with the phenol–sulphuric acid reagent. The early fractions of the broad peak (40–60 ml) were combined as fraction *1*; the remainder of this peak was fraction *2*, and the smaller peak was fraction *3*. Each fraction was concentrated, and dialysed to remove salt, and the polysaccharide was recovered as described above. Acid hydrolysis (as described above) showed that fraction *1* contained galactose and 6-*O*-methylgalactose together with a trace of glucose, fraction *2* contained all the sugars present in the original polysaccharide, and fraction *3* gave glucose only.

*Characterisation of the sugars.* — A solution of the polysaccharide (5 g) in 0.75M sulphuric acid (500 ml) was heated at 100° for 3 h, cooled, neutralised with barium carbonate and barium hydroxide, filtered, and concentrated to a syrup. The sugars in a portion (250 mg) of the syrup were fractionated by preparative p.c., using butan-1-ol–pyridine–benzene–water (5:3:1:3).

*Galactose.* The syrup (90 mg) had $[\alpha]_D^{20}$ +8.2° (water), indicating a DL mixture in the ratio 1.19:1. The derived phenylosazone had m.p. 184–186°, $[\alpha]_D^{20}$ ~0° (pyridine); lit.[13] m.p. 185–187°.

*6-O-Methyl-D-galactose.* The syrup (15 mg) had $[\alpha]_D^{20}$ +74.8°; lit.[14] $[\alpha]_D$ +77.3°. The derived phenylosazone had m.p. 193–195°; lit.[3] m.p. 195–196°.

*D-Glucose.* The syrup (50 mg) had $[\alpha]_D^{20}$ +51° (water), and the derived phenylosazone had m.p. 204–206°; lit.[15] m.p. 205°.

*D-Xylose.* The syrup (28 mg) had $[\alpha]_D^{20}$ +16.7° (water); lit.[15] $[\alpha]_D$ +19°. The phenylosazone had m.p. 159–162°; lit.[15] m.p. 161°.

*2-O-Methylgalactose.* Insufficient material was isolated for complete characterisation. It migrated with the authentic compound on paper electrophoresis in borate buffer ($M_G$ 0.45), and gave a characteristic orange colour with the *p*-anisidine hydrochloride spray reagent. G.l.c. of the alditol acetate mixture derived from the hydrolysate (see below) also gave a minor peak having the retention time of 2-*O*-methyl-D-galactitol penta-acetate.

*3,6-Anhydro-L-galactose.* Oxidative hydrolysis[7] of the polysaccharide (5 g), with fractionation of the acidic products by preparative t.l.c. on cellulose plates (40 × 10 cm), gave syrupy 3,6-anhydro-L-galactonic acid (40 mg), with $R_F$ 0.56 on cellulose plates in butan-1-ol–ethanol–water (2:1:1), and $[\alpha]_D^{20}$ −32.4° (water); lit.[7] for D form, $[\alpha]_D$ +33°. The derived methyl 3,6-anhydro-2,4,5-tri-*O*-*p*-nitrobenzoyl L-galactonate had m.p. 185°; lit.[7] for D form, m.p. 187–191°.

*3,6-Anhydro-2-O-methyl-L-galactose.* This was also obtained from the oxidative hydrolysate. The syrup (15 mg) had $[\alpha]_D^{20}$ −65°; lit.[16] $[\alpha]_D$ −70.3°; $R_F$ 0.69 on the cellulose plate in the foregoing solvent system. The methyl ester had m.p. 87°; lit.[16] m.p. 90–91°.

*Quantitative determination of sugars.* — With the exception of the anhydro sugars, the sugars in a hydrolysate, prepared as above, were determined[3] as their alditol acetates by g.l.c.

*Isolation of monosaccharide sulphates.* — A solution of polysaccharide (50 g) in 0.25M sulphuric acid (2 l) was heated at 100° for 2 h, neutralised with barium carbonate, filtered, and concentrated to 100 ml. Addition of ethanol (200 ml) precipitated some material of high molecular weight, which was removed, and the solution was concentrated to 10 ml. The product was percolated through a column (60 × 2.5 cm) of DEAE-Sephadex A-50 (chloride form), and neutral sugars were eluted with water. Sugar sulphates were then eluted with 0.2M sodium chloride, the relevant fractions being combined, concentrated, and desalted on a column of charcoal–Celite[4]; inorganic salts were eluted with water, and sugar sulphates with 10% ethanol. Paper electrophoresis in neutral buffer was used to separate mono-

saccharide sulphates (210 mg) from oligosaccharide sulphates. P.c. of the former in butan-1-ol–acetic acid–water (4:2:1) revealed the presence of galactose 6-sulphate, a zone having the $R_F$ value of galactose 2- or 4-sulphate, and traces of a uronic acid. Preparative paper electrophoresis in 0.1M borate buffer (pH 10) separated the 6-sulphate from the 2- or 4-sulphate, and the recovered sulphates were desalted by preparative p.c. in the foregoing solvent system.

*Galactose 6-sulphate.* This was isolated as the sodium salt (27 mg) in chromatographically and electrophoretically homogeneous form with $[\alpha]_D^{20}$ +14.0° (water), indicating a DL mixture (ratio 1.8:1.0). A portion (15 mg) was hydrolysed with 0.75M sulphuric acid at 100° for 3 h and the monosaccharide was recovered as described above. The product was shown to be galactose by p.c. in three solvent systems, and it had $[\alpha]_D^{20}$ +17.2° (water), indicating a DL ratio of 1.56:1.0.

A further portion of the sugar sulphate was oxidised in 15mM sodium metaperiodate[17]; it reduced 3.44 mol of periodate per mol (30 min), increasing to 3.72 mol (24 h), a pattern typical of galactose 6-sulphate[3,4]. Analysis for sulphate on another portion of the sugar sulphate showed that galactose and sulphate were present in a molar ratio of 1.0:0.98.

*Galactose 2- and 4-sulphates.* The sample (5 mg) was analysed by p.c., and contained components having $R_F$ values in two solvent systems identical with those of galactose 2- and 4-sulphates. In order to distinguish between these two sulphates, the remainder of the sample was methylated with methyl iodide and silver oxide in *N,N*-dimethylformamide[18], and the methylated product was methanolysed and analysed by g.l.c.[19]. Peaks having the retention times of methyl glycosides of 2,3,6-tri-*O*-methylgalactose and 3,4,6-tri-*O*-methylgalactose were detected, indicating that the sugar sulphate was a mixture of galactose 2- and 4-sulphates.

*Isolation of disaccharides after oxidative hydrolysis.* — A solution of the polysaccharide (5 g) in 0.25M sulphuric acid and bromine (1 ml) was maintained at 60° for 23 h and then cooled. Excess of bromine was removed by aeration, and the solution was neutralised with barium carbonate and filtered. Cations were removed with ZeoKarb-225($H^+$) resin, and the solution was concentrated. The components of the residual syrup were fractionated by preparative t.l.c. (cellulose; butan-1-ol–ethanol–water, 2:1:1), the disaccharide band only being recovered (195 mg). Complete hydrolysis of a portion with M sulphuric acid at 100° for 3 h gave (t.l.c.) galactose (3+), 6-*O*-methylgalactose (+), and 3,6-anhydrogalactonic acid (3+). A portion of the disaccharide (10 mg) was methylated, as described above, the fully methylated derivative was methanolysed, and the product examined by g.l.c.[19]. Only one peak, with $R_T$ 1.8, was detected, corresponding to the methyl glycosides of 2,3,4,6-tetra-*O*-methylgalactose, thus indicating that both galactose and 6-*O*-methylgalactose were at the non-reducing end.

The disaccharide (4 mg) was dissolved in water (10 ml), and a portion of this solution was analysed for galactose + 6-*O*-methylgalactose with the phenol–sulphuric acid reagent. There were 40.6 μmol of total hexose present. Titration of another portion with 1.7 mM sodium hydroxide showed that 41.6 μmol of acid were present.

This confirms that the compound isolated was a disaccharide mixture containing the aldonic acids derived from agarobiose and $6^2$-*O*-methylagarobiose.

*Treatment of the polysaccharide with an agarase.* — The enzyme preparation, which is specific for agar-type polysaccharides, has been described previously[11]. The polysaccharide (30 mg) and enzyme (1.5 units) in 0.1M phosphate buffer (pH 7.0; 10 ml) were incubated at 37° for 5 days under a layer of toluene. Ethanol (20 ml) was then added, the small amount of precipitated material was removed, and the solution was concentrated to a syrup. Samples of agarose and porphyran were similarly treated with enzyme. The products were examined by t.l.c., on cellulose, using double development with butan-1-ol–pyridine–water (2:1:1) and spraying with the Seliwanoff spray reagent[20]. Components with migration rates relative to galactose ($R_{Gal}$) of 1.21 (trace), 1.01 (3+), and 0.72 (2+) were detected from the polysaccharide, compared with 1.30 (+) and 0.80 (3+) from agarose, and 1.7 (trace), 1.30 (+), 1.05 (3+), and 0.80 (3+) from porphyran. The product with $R_{Gal}$ 1.2–1.3 has been identified previously as neoagarobiose, and that with 0.7–0.8 as neoagarotetraose[11].

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