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THE AGAR-TYPE POLYSACCHARIDE FROM THE RED ALGA *Gracilaria secundata**

DONALD J. BRASCH, CHAW T. CHUAH, AND LAURENCE D. MELTON
University of Otago, Dunedin (New Zealand)
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ABSTRACT

Aqueous extraction of the red alga *Gracilaria secundata* gives a low-sulfated, agar-type polysaccharide which gels strongly. The structure of the polysaccharide has been investigated by methylation, partial hydrolysis of the permethylated agar, enzymic oxidation, and ^{13}C-n.m.r. spectroscopy. The agar is mainly composed of the familiar (1→3)-linked β-D-galactosyl residues and (1→4)-linked 3,6-anhydro-α-L-galactosyl residues, but important variations occur. The distribution of 6-*O*-methyl-D-galactosyl residues is considered to be in "blocks".

INTRODUCTION

Polysaccharides from *Rhodophyceae* have been studied by many workers[2], because their rheological[3] and immunological properties[4] give them considerable industrial potential. Agar-type polysaccharides of various gel strengths have been reported[5] from *Gracilaria compressa*, *G. debilis*, *G. foliifera*, *G. domingensis*, *G. damaecornis*, *G. ferox*, and *G. verrucosa*. An early survey[6] found that New Zealand *Gracilaria* appeared to contain agar, and the commercial processing[7] of red seaweeds to give agar is now a well established industry in New Zealand. *Gracilaria secundata*, as well as *Pterocladia* species, are used for producing material presumed (from their gelling properties) to be agar, although the structures of the polysaccharides of these algae have not been studied until recently. New developments include proposals to grow *G. secundata* in sewage-treatment ponds[8], and to farm it on the sheltered mudflats of northern New Zealand harbours.

The purpose of the present investigation was to determine the structure of the polysaccharide isolated from *G. secundata*. The current study is part of a program of investigation of the polysaccharides from New Zealand seaweeds.

RESULTS AND DISCUSSION

Extraction of air-dried *Gracilaria secundata* with hot water yielded a viscous

*Marine Algal Polysaccharides, Part 2. For Part 1, see ref. 1.

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TABLE I

COMPOSITION OF GALACTAN SULFATE

Constituent	*Percentage*[a]
Total galactose	38.1
6-*O*-Methyl-D-galactose	15.3
Sulfate (SO_4^{2-})[b]	2.9
3,6-Anhydro-L-galactose	36.1
Pyruvic acid	0.0
Nitrogen	trace

[a]All monosaccharide residues expressed as mass percentages of total hydrolyzate. [b]Based on sulfur analyses.

solution which gelled on cooling. Three successive extractions gave a total polysaccharide yield of 28%. The first extract was purified by freeze–thawing six times to give, on freeze-drying, a polysaccharide in 8% yield. All further work was conducted on the first extract. Attempts to fractionate this polysaccharide further by column chromatography using DEAE-Sephadex A-50 (Cl^-)[9] and Dowex-1 X-2 (formate)[10] were unsuccessful. Acid hydrolysis of the polysaccharide gave galactose, 6-*O*-methylgalactose and 5-(hydroxymethyl)-2-furaldehyde. The last component was separated from the hydrolyzate by preparative paper-chromatography, and identified by mass spectrometry. It is presumed to be a decomposition product of acid-labile, 3,6-anhydrogalactosyl residues in the polysaccharide[11].

The composition of the polysaccharide is given in Table I. Pyruvic acid residues, which have been found[5b] in traces in polysaccharides isolated from other *Gracilaria* species, were not detected. The analyses are comparable with those found for the agars of *Gelidium cartilaginium*[12] and *Gracilaria verrucosa*. Assuming that all of the sulfate groups are on L-galactosyl residues, and allowing for a small percentage of unsubstituted L-galactosyl residues, the mole ratio of D : L residues in the polysaccharide is 1.05 : 1.00.

Alkali treatment of the *Gracilaria secundata* polysaccharide gave a small increase (1.5 mass %) in 3,6-anhydrogalactose content, and an exactly equivalent decrease in sulfate, suggesting that approximately one-third of the total sulfate present is attached either to C-3 or C-6 of the galactosyl residues. The remaining ester sulfate groups (1.98% of the polysaccharide) are stable towards alkali, and the methylation data, reported later, suggest that these are mainly attached to C-4, as 2,6-di-*O*-methylhexose derivatives were obtained in low yields.

Oxidation of the polysaccharide with chromium trioxide[13], and analysis of the polysaccharide hydrolyzate by using the enzyme D-galactose oxidase[14], showed that 98% of the galactosyl residues in the polysaccharide have the β-D configuration. The ^{13}C-n.m.r. spectrum of the polysaccharide, a complete assignment of which has already been reported[15], is consistent with this, and shows that the 6-*O*-methyl-

TABLE II

G.L.C. ANALYSIS OF THE METHYLATED ALDITOL ACETATES FROM THE PARTIAL AND COMPLETE HYDROLYSIS OF METHYLATED GALACTAN SULFATE

Component separated as alditol acetate	*90% Formic acid (1 h)*		*90% Formic acid (1 h) + 16 h in 0.13M sulfuric acid*			
	Mol	*Mole %*	*Mol*	*Mole %*	*Mol corrected*	*Corrected mole %*
2,3,4,6-Tetra-*O*-methyl-D-galactose	0.0009	0.8	0.002	0.6	0.002	0.3
3,6-Anhydro-2-*O*-methyl-L-galactose	0.0752	63.4	0.037	10.7	0.296	49.0
2,4,6-Tri-*O*-methyl-D-galactose	0.0425	35.8	0.296	85.8	0.296	49.0
2,3,6-Tri-*O*-methyl-L-galactose	trace	—	0.006	1.7	0.006	1.0
2,6-Di-*O*-methyl-D-galactose	trace	—	0.004	1.2	0.004	0.7

galactosyl residues also have the β-D configuration, whereas the 3,6-anhydrogalactosyl residues are α-L.

Complete methylation of the *Gracilaria secundata* polysaccharide was achieved by using a single Hakomori methylation[16]. The methylated polymer was partially hydrolyzed by heating in 90% formic acid for 1 h at 100°, and completely hydrolyzed by heating at 100° with 90% formic acid followed by 0.13M sulfuric acid. The results of g.l.c. analysis of the alditol acetates of the sugars in the hydrolyzates are given in Table II. The mole % values in Table II were calculated directly from the g.l.c. analyses, and it is noteworthy that the hydrolysis with formic acid alone gives a much greater proportion of 3,6-anhydro-2-*O*-methyl-L-galactose than hydrolysis with formic acid followed by sulfuric acid. This result is similar to that obtained[1] from the permethylated porphyran from *Porphyra columbina*, and is discussed later.

Because 3,6-anhydro-2-*O*-methyl-L-galactose is degraded during the acid hydrolyses, the mole % values in Table II were corrected by taking the mole % of the 3,6-anhydrogalactose in the original, unmethylated polysaccharide as 41.6 (deduced from Table I), and calculating the mole % of 3,6-anhydro-2-*O*-methyl-L-galactose from this. All of the mole % values were then corrected accordingly, and the corrected values are also given in Table II.

The large proportion of 2,4,6-tri-*O*-methyl-D-galactose in the hydrolyzate of the methylated polysaccharide proves that part of the D-galactosyl or 6-*O*-methyl-D-galactosyl residues, or of both, in the original polysaccharide is linked through O-3. It also suggests that there is little or no sulfate group present at C-2 of a galactosyl residue.

The 2,3,6-tri-*O*-methyl-L-galactose is a minor component of the hydrolyzate,

showing that a small proportion of unsubstituted, (1→4)-linked L-galactosyl residues is present. The value obtained from the methylation analysis is in reasonable agreement with that found by the D-galactose oxidase method.

Another minor component was shown to be 2,6-di-*O*-methylgalactose. A possible source could be a (1→3)-linked D-galactosyl residue bearing a sulfate group on C-4, which is consistent with the fact that all of the sulfate groups are not removed on alkali treatment. The 2,6-di-*O*-methylgalactose also occurs in the hydrolyzate of the methylated, alkali-treated polysaccharide. Other possibilities are that the 2,6-di-*O*-methylgalactose originated from undermethylated 3-linked galactosyl residues, or from a position of branching, although no proof of branching in agarose-type polysaccharides has thus far been found.

The 1,5-diacetate of 2,3,4,6-tetra-*O*-methylgalactitol was found in all of the g.l.c. analyses, and it originates from terminal (nonreducing) galactosyl groups. The corrected mole % from the complete hydrolysis is 0.3, suggesting that the number-average degree of polymerization of the methylated galactan may be in the vicinity of 300. This figure assumes that all of the nonreducing, terminal groups in the methylated polysaccharide are 2,3,4,6-tetra-*O*-methyl-D-galactosyl, and that no branching occurs.

It was surprising that no 2,3-di-*O*-methyl-L-galactose was present in the hydrolyzate of the methylated polysaccharide. Its absence suggests that those residues bearing sulfate groups, which are readily converted into 3,6-anhydrogalactosyl residues by alkali, are attached to O-3, rather than to O-6, of (1→4)-linked L-galactosyl residues. The 2,6-di-*O*-methyl-L-galactose which would then be expected in the hydrolyzate may not have been separated from the 2,6-di-*O*-methyl-D-galactose, and so was, perhaps, not detected for this reason.

The alkali-treated polysaccharide was also subjected to methylation analysis, the results of which are given in Table III. The main effect of the alkali treatment is probably the removal of some sulfate groups, as already discussed, but the analytical methods are not sufficiently precise to detect the small differences between the two methylated polysaccharides. However the marked increase in the yield of 2,3,4,6-

TABLE III

G.L.C. ANALYSIS OF METHYLATED, ALKALI-TREATED GALACTAN SULFATE

Component separated as alditol acetate	*Mol*	*Mole %*	*Mol corrected*	*Corrected mole %*
2,3,4,6-Tetra-*O*-methyl-D-galactose	0.0029	1.3	0.0029	0.8
3,6-Anhydro-2-*O*-methyl-L-galactose	0.0196	9.1	0.1854	48.6
2,4,6-Tri-*O*-methyl-D-galactose	0.1854	85.9	0.1854	48.6
2,3,6-Tri-*O*-methyl-L-galactose	0.0037	1.7	0.0037	1.0
2,6-Di-*O*-methyl-D-galactose	0.0041	1.9	0.0041	1.1

TABLE IV

PARTIAL HYDROLYSIS OF METHYLATED GALACTAN SULFATE

Time of hydrolysis (and hydrolyst)	*Molar ratio of anhydro residue: 2,4,6-tri-O-methylgalactose*	*Polysaccharide hydrolyzed (%)*
1 h (90% formic acid)	1.77:1.00	37.7
1 h (90% formic acid) + 3 h (0.13M H_2SO_4)	1.00:1.25	38.6
1 h (90% formic acid) + 8 h (0.13M H_2SO_4)	1.00:1.57	76.3
1 h (90% formic acid) + 16 h (0.13M H_2SO_4)	1.00:7.74	100.0

tetra-*O*-methyl-D-galactose shows that depolymerization had also occurred during the alkali treatment.

As already mentioned, partial hydrolysis of the permethylated polysaccharide with 90% formic acid, alone, gave a much higher yield of 3,6-anhydro-2-*O*-methyl-L-galactose, relative to that of 2,4,6-tri-*O*-methyl-D-galactose, than would be expected from the analytical data in Table I. To study this effect further, a series of hydrolyses was performed in which the first step was, in all cases, hydrolysis by heating in 90% formic acid for 1 h at 100°, but this was followed by heating in 0.13M sulfuric acid at 100° for various times. The results, which are given in Table IV, show that, during the early stage of the hydrolysis of the methylated polysaccharide, more 3,6-anhydro-2-*O*-methyl-L-galactose is liberated than 2,4,6-tri-*O*-methyl-D-galactose. It is known[17] that 3,6-anhydrogalactopyranosyl linkages are more labile to acid hydrolysis than hexopyranosyl linkages, and therefore, a linear chain of 3-linked β-D-galactosyl residues alternating regularly with (1→4)-linked 3,6-anhydro-α-L-galactosyl residues should never yield greater amounts of 3,6-anhydro-2-*O*-methyl-L-galactose than of 2,4,6-tri-*O*-methyl-D-galactose. It therefore appears possible that the agarose-like polysaccharide from *Gracilaria secundata* may contain some adjacent, 3,6-anhydro-galactosyl residues. A similar possibility has already been noted[1] for a galactan sulfate isolated from *Porphyra columbina*.

As shown in Table IV, the partial hydrolysis with formic acid, alone, hydrolyzed only 38% of the methylated *Gracilaria secundata* polysaccharide to monosaccharide. Thus, for a ratio of 3,6-anhydro-2-*O*-methylgalactose to 2,4,6-tri-*O*-methylgalactose of 1.8:1.0, the adjacent 3,6-anhydro-L-galactosyl residues would constitute at least 10% of the original polysaccharide molecule.

Presumably, the reason for the relatively high yield of 3,6-anhydro-2-*O*-methyl-L-galactose derivative is that hydrolysis of the methylated polysaccharide with 90% formic acid produces formic esters of 2-*O*-methyl-L-galactose which would be expected to be fairly stable in formic acid solution. The sodium borohydride reduction that was used in the preparation of alditol acetates for g.l.c. analyses would then reduce formic esters directly to alditols, without going through the unstable, free-monosaccharide step. We have thus far been unable to isolate formic esters of

either 3,6-anhydro-2-*O*-methyl-L-galactose or 2,4,6-tri-*O*-D-methyl-galactose from the mixture obtained on hydrolysis with formic acid. Hence, a possible explanation of the results of hydrolysis with formic acid lies in the relative rates of reduction of formic esters of 3,6-anhydro-2-*O*-methyl-L-galactose and of 2,4,6-tri-*O*-methyl-D-galactose, rather than in the presence of adjacent, 3,6-anhydro-2-*O*-methyl-L-galactosyl residues in the methylated polysaccharide, or in other structural irregularities.

In previous investigations of the structure of agar-type polysaccharides containing 6-*O*-methyl-D-galactosyl residues, the position of these residues in the polysaccharide has been ignored, or it has been assumed that their distribution is random rather than in blocks. Because the degree of methylation directly influences the gelling properties of agar-type molecules, any information on the distribution of the 6-*O*-methyl-D-galactosyl units would be useful. To study this matter, the *Gracilaria secundata* polysaccharide was treated with sodium *p*-toluenesulfinate[18], and the resulting 6-sulfone derivative subjected to methylsulfinyl anion, a strong base. The reaction mixture was then dialyzed, and the non-dialyzable material hydrolyzed. Subsequent g.l.c. analysis of the alditol acetates from the sugars in this hydrolyzate showed that 57% of the D-galactosyl residues in the original polysaccharide had been removed by the treatment, although a substantial proportion of undialyzable polymer remained. If it is assumed that the D-galactosyl and 6-*O*-methyl-D-galactosyl residues are distributed randomly between 3,6-anhydro-L-galactosyl residues along the polysaccharide chain, and if the sulfone derivatization was also random, a 57% reaction would be sufficient to degrade the agar completely to low-molecular-weight, dialyzable oligosaccharides.

The isolation of the polymeric product can, therefore, best be explained if the 6-*O*-methyl-D-galactosyl residues occur in groups, alternating with 3,6-anhydro-L-galactosyl residues. Further evidence supporting this conclusion was obtained by oxidizing the original polysaccharide with D-galactose oxidase, followed by treatment with strong base, and dialysis. In this case, only 12% of the galactosyl residues originally present in the polysaccharide remained in the non-dialyzable fraction. An attempt was made to quantitate the D-galactose oxidase-catalyzed oxidation of the polysaccharide by an enthalpimetric method involving the hydrogen peroxide released, by using the following reaction.

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

The reaction is accompanied by a large change in enthalpy[19]. Quantitative results could not be obtained, but the experiment did confirm that D-galactose oxidase reacts with the unhydrolyzed agarose.

EXPERIMENTAL

Isolation of polysaccharide. — The alga *Gracilaria secundata* was obtained from Davis Gelatine, Ltd., Christchurch, New Zealand. The seaweed (100 g, dry weight)

was washed in cold, distilled water (3 L) for 3 h, filtered, and the solid extracted with boiling water (3 L) for 4 h. The seaweed residues were removed from the hot solution on a cloth filter, and the filtrate was kept for 16 h at −10°. The frozen extract was then thawed at room temperature, and filtered, and the solid dehydrated with ethanol, and dried *in vacuo* at 40°, to give the polysaccharide (14% yield). This polysaccharide was redissolved in boiling water, and the freeze–thaw operation was repeated six times. The final polysaccharide was obtained from this treatment in an 8% overall yield.

Materials and methods. — These were identical to those described[1] in Part 1.

Acetylation of the polysaccharide. — The polysaccharide (0.5 g) was dissolved in formamide (100 mL), dry pyridine (50 mL) and acetic anhydride (50 mL) were added, and the mixture was shaken continuously for 16 h. Undissolved material was removed by centrifugation, the supernatant liquor dialyzed against distilled water for 2 days, and the product (0.8 g) isolated by freeze-drying. Two further acetylations gave a polysaccharide acetate which was finally isolated by precipitation from a solution in acetone by using diethyl ether. The i.r. spectrum showed no hydroxyl absorption at 3600–3400 cm^{-1}.

Oxidation of the peracetylated agarose. — Fully acetylated polysaccharide (32 mg) and *myo*-inositol hexaacetate (4.1 mg) were dissolved in glacial acetic acid (2 mL). Finely powdered chromium trioxide (150 mg) was added, and the mixture was agitated ultrasonically for 2 h at 53°. The mixture was poured into water (10 mL), and the water phase extracted with dichloromethane (3 ×). This material was hydrolyzed with 0.25M aqueous sulfuric acid for 16 h at 100°, followed by reduction of the sugars, acetylation of the alditols, and analysis of the acetates by g.l.c. Another portion of the fully acetylated polysaccharide (22.8 mg) and *myo*-inositol hexaacetate was hydrolyzed, and the sugars converted into the alditol acetates for g.l.c. analysis. A comparison of the two analyses showed which sugar residues had been oxidized.

Methylation of the polysaccharide. — The Hakomori method[16] was used as described previously[1]. Complete methylation, shown by the absence of hydroxyl absorption at 3600–3400 cm^{-1} in the i.r. spectrum, was achieved in a single treatment.

Treatment of enzyme-oxidized polysaccharide with base. — Polysaccharide (100 mg) was dissolved in 0.1M potassium phosphate buffer (150 mL). D-Galactose oxidase (EC 1.1.39; Type V from *Dactylium dendroides*, 530 units) and catalase (25 mg) were added, and the mixture was incubated for 3 d at 32°. (Both enzymes were purchased from Sigma, and used without purification.) Ethanol was added to precipitate the polysaccharide, the mixture dialyzed, and the precipitated polysaccharide recovered by centrifugation (40 mg). Part of the precipitate and a known amount of *myo*-inositol, as the internal standard, were hydrolyzed, and the alditol acetates from the sugars in the hydrolyzate were prepared for g.l.c. analysis as previously described[1].

Another portion of the precipitate (35 mg) was dissolved in dimethyl sulfoxide (10 mL), and the solution transferred with a syringe into a solution of dimsyl anion, the latter having been prepared as for the Hakomori methylation procedure[16]. The

mixture was agitated for 1 h in an ultrasonic bath, and kept overnight under a constant flow of dry nitrogen. The mixture was then poured into distilled water (70 mL), made neutral with glacial acetic acid, and dialyzed in running tap water for 3 days. The solution was evaporated to dryness, and a portion of this base-treated, enzyme-oxidized polysaccharide was hydrolyzed, and the sugars in the hydrolyzate converted into alditol acetates for g.l.c. analysis.

Treatment of the p-*tolylsulfone of the polysaccharide with base.* — Sodium *p*-toluenesulfinate was prepared by the method of Field and Clark[20]. The primary *p*-tolylsulfone of the polysaccharide was prepared by the method of Baker and Whistler[18], and the base treatment used was that already described for the base treatment of the enzyme-oxidized polysaccharide.

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