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HIGHLY METHYLATED AGARS WITH A HIGH GEL-MELTING POINT FROM THE RED SEAWEED, *GRACILARIA EUCHEUMOIDES**

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Key Word Index—*Gracilaria eucheumoides*; Gracilariaceae; Rhodophyta; agar; methylated agarose; high gel-melting point agar; NMR.

Abstract—Highly methylated agars were isolated from the red seaweed, *Gracilaria eucheumoides*, harvested in Japan. One of the obtained agars formed a thermo-reversible gel with a hi, ıelting point up to 121° and was indicated to consist of a regularly repeating structure, →3 6-*O*-methyl-β-D-Ga' ' →4) 3,6-anhydro-2-*O*-methyl-α-L-Gal 1→.

INTRODUCTION

Agars are known as water-soluble, gel-forming polysaccharides extracted from agarophytes, such as red algae belonging to the orders, Gelidiales and Ceramiales, and the genus *Gracilaria* of the order Gigartinales [1]. Agarose, the main constituent essential for the gelling property of agars, consists of an alternating repeat of (1→3)-linked β-D-galactose and (1→4)-linked 3,6-anhydro-α-L-galactose residues [2]. The polysaccharides in agars consist of this agarose backbone that is more or less modified in various manners, such as by *O*-methylation, *O*-sulphation and pyruvate ketal formation, depending on source. Such modifications affect physical properties of agars, e.g. 6-*O*-methylation at (1→3)-linked galactose residues causes a slight elevation of gel-melting point, as reported in a comparative studies of agars from various sources [3] and artificially methylated agars [4]. However, all agars known so far are extractable with water below 100°, even if such *O*-methylation takes place and the agar gels melt below 100°. Although this instability of the gel at high temperatures is a characteristic property of agars, it may cause limitations for end-uses.

Amongst a variety of agarophytes, a polysaccharide from the red seaweed, *Gracilaria eucheumoides*, harvested in China has been reported by Ji *et al.* [5] as a highly methylated agarose, with (1→4)-linked units which are virtually completely replaced by 2-*O*-methyl-3,6-anhydrogalactose residues. The present paper describes the isolation and structural investigation of agars from a different source of the same species harvested in Japan. We found that the obtained agars were a new category of agarose-type polysaccharide in view of their structure and gelling properties.

RESULTS AND DISCUSSION

Gracilaria eucheumoides harvested in Japan was extracted exhaustively with water at 100° to afford a gel-forming polysaccharide, PS1. Sediment remaining after the first extraction was extracted with water at 121° using an autoclave to afford a polysaccharide, PS2, forming a thermo-reversible, firm gel, which did not melt below 100°, but melted again at 121°. The sediment which remained after the second extraction appeared as a gelatinous substance highly swollen with water. This substance was recovered as PS3, because it seemed to be distinguished from less swollen cell wall constituents, such as cellulose, which occurs in a closely related alga, *G. crassissima* [6] and cannot be removed by exhaustive extraction.

Major constituents of PS1 were 6-*O*-methyl-D-Gal, D-Gal, 2-*O*-methyl-3,6-anhydro-L-Gal and 3,6-anhydro-L-Gal (Table 1) on the basis of hydrolysis and methanolysis studies; smaller amounts of 3-*O*-methyl-D-Gal, 2-*O*-methyl-L-Gal, D-Xyl and D-Glc were also identified. Total amounts of all the D-Gal and its derivatives were almost the same as those of L-isomers, in agreement with an agarose-type composition. On partial methanolysis, PS1 afforded derivatives of 4-*O*-(6′-*O*-methyl-β-D-galactopyranosyl)-2-*O*-methyl-3,6-anhydro-L-galactose (2,6′-dimethylagarobiose), 2-methylagarobiose and 6′-methylagarobiose, and smaller amounts of agarobiose, which were identified by GC and GC-MS as their corresponding TMSi derivatives. Thus, PS1 is most likely to be

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constructed from an agarose-type backbone in which D-Gal and 3,6-anhydro-L-Gal residues were partly substituted with 6-*O*-methyl-D-Gal and 2-*O*-methyl-3,6-anhydro-L-Gal residues, respectively (Fig. 1a).

PS2 consisted of essentially equal amounts of 6-*O*-methyl-D-Gal and 2-*O*-methyl-3,6-anhydro-L-Gal, together with trace amounts of D-Gal, 3,6-anhydro-L-Gal and D-Glc (Table 1). PS2 was devoid of sulphate groups within the analytical limits of detection. Methylation of PS2, followed by total hydrolysis, yielded 2,4,6-tri-*O*-methylgalactose arising from (1 → 3)-linked 6-*O*-methyl-D-Gal residues. Partial methanolysis of PS2 afforded 2,6′-dimethylagarobiose derivatives, which indicates a repeating disaccharide structure, as shown in Fig. 1b. This was supported by the ^{13}C NMR spectrum of PS2, the most conspicuous 12 signals being assigned as given in Table 2. The C-6 signals were assigned using DEPT135 pulse sequences [7], with which secondary carbon atoms afford negative peaks. Chemical shift values for each signal agreed with reported spectral data for (1 → 3)-linked 6-*O*-methyl-β-D-Gal residues and (1 → 4)-linked 2-*O*-methyl-3,6-anhydro-α-L-Gal residues in partially methylated agars [5, 8, 9]. The smaller amount of Glc residues are likely to arise from (1 → 4)-linked glucan chains, because 2,3,6-tri-*O*-methylglucose was also identified after methylation. A (1 → 4)-linked glucan has been found in an agar from the closely related *G. tenuistipitata* [10] as floridean glycogen on the basis of ^{13}C NMR measurements. The glucan chain in PS2, however, would be of different origin from cellulose, because water-soluble floridean glycogen would have been extracted at 100° to be obtained in the PS1 fraction. Although it is still not certain whether the small amount of glucose chain links covalently to the main chain, or occurs as a contaminant, PS2 can best be described as an idealized 2,6′-dimethyl agarose.

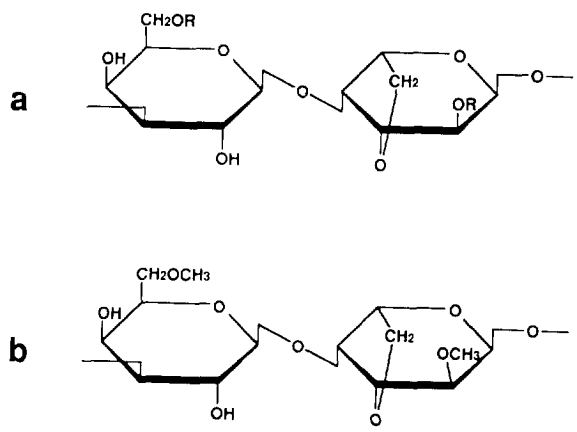

Fig. 1. Covalent structures of polysaccharides from *Gracilaria eucheumoides*. (a) Main moiety of PS1, R = CH_3, H. (b) Idealized structure of PS2 and PS3 (except for glucan moiety).

Due to its insolubility, it was difficult to estimate the composition of PS3 by traditional methods for methanolysis using methanolic HCl. To overcome this, PS3 was methanolysed after dissolving in 72% H_2SO_4, addition of methanolic 72% H_2SO_4 and subsequent addition of MeOH containing anhydrous $CaSO_4$ as desiccant, as described by Roberts *et al.* [11]. PS3 was thus shown to contain the same constituents as PS2 except for a much higher glucose content (Table 1). Though 3,6-anhydride residues apparently degraded under the conditions used, the amount of 2-*O*-methyl-3,6-anhydrogalactose residues is likely to be almost the same as that of 6-*O*-methylgalactose residues, because these component sugars were detected in almost the same proportions as in methanolysate of PS2 performed under identical conditions.

Table 1. Compositions* of polysaccharides from *Gracilaria eucheumoides*

	D-Gal	6-Me-D-Gal†	L-Gal	L-AGal‡	2-Me-L-AGal	D-Glc	Sulphate
PS1	24	76	7	22	66	Trace	6
PS2	6	94	Trace	5	90	5	0
PS3	0	100	0	0	100	67	ND§

* Expressed as molar ratio. Total of D-Gal derivatives taken as 100.
† 6-*O*-methyl-D-Gal.
‡ AGal, 3,6-anhydrogalactose.
§ ND, Not determined.

Table 2. ^{13}C NMR chemical shift values for PS2* from *Gracilaria eucheumoides*

	C-1	C-2	C-3	C-4	C-5	C-6	-OMe
G†	102.20	69.62	82.15	68.60	73.10	71.39	58.51
A‡	98.24	78.33	78.04	77.22	75.27	69.02	58.58

* For structure of PS2, see Fig. 1b.
† (→ 3)6-*O*-Me-β-D-Gal(1 →).
‡ (1 → 4)3,6-anhydro-2-*O*-Me-L-Gal(1 →).

PS3 appeared to be sulphate-free on the basis of its IR spectrum, where bands at 1240–1260 cm^{-1} and 810–850 cm^{-1} characteristic of sulphate ester groups were not observed. Although the structure of PS3 could not be further elucidated in detail due to its insolubility, the results obtained suggest a 2,6′-dimethyl agarose structure homologous to PS2, except for a rather larger amount of Glc residues.

Although it is not clear whether the Glc residues in PS2 and PS3 have arisen from contaminants, such as cellulose, or not, the Glc residues are unlikely to participate in the gelling properties of these polysaccharides. A Glc-free PS2 obtained by a treatment with cellulase made a gel with a melting point higher than 100°. Furthermore, a cellulase-treated PS3, from which no Glc residue was detected on hydrolysis, was still insoluble after extraction at 121°.

When compared with usual agars, PS2 is remarkable in view of its exceptionally high gel-melting point, which would enable new applications by overcoming the limit of normal agars. PS2 and PS3 would also be good models for examining the relation between the regularly repeating structure and the unique gelling property.

EXPERIMENTAL

Materials and general methods. The red seaweed, *G. eucheumoides*, was harvested at Ishigaki Island, Okinawa Prefecture, Japan. Authentic specimens of dimethylacetals of 4-*O*-(β-D-galactopyranosyl)-3,6-anhydro-L-galactose (agarobiose), 6′-methylagarobiose, 2-methylagarobiose and 2,6′-dimethylagarobiose were prepd from commercial agar, partially 6-methylated agar from commercial sources, polysaccharide from *Rhodomela larix* [12] and polysaccharide from *Laurencia undulata* [13], respectively. Cellulase Onozuka RS was a product of Yakult Co. Sulphate content was assayed by the method of ref. [14] after hydrolysis of polysaccharide with HCl. FID-GC was carried using a fused silica WCOT columns of *a*, OV-1 bonded (0.3 mm × 25 m, GL Science Co.) operated at 180°; column *b*, PEG 20 M bonded (0.3 mm × 25 m, at 200°, GL Science Co.); column *c*, CP-Sil 88 (0.25 m × 25 m, at 205°, Chrompack Co.). N_2 was used as carrier gas at a flow rate of 1 ml min^{-1} with a split ratio of 20:1. GC-MS was carried out under the same chromatographic conditions used for GC except for use of He as carrier gas. Absolute configurations of monosaccharides were assigned by GC-MS as TMSi derivative of L-2-octyl glycoside (column *a* at 210°) as described elsewhere [15–17]. ^{13}C NMR at 50.3 MHz were recorded at 80°. Chemical shift values are expressed relative to int. MeOH (49.3 ppm). The DEPT135 pulse sequence [7] was supplied by the manufacturer. IR were recorded in KBr discs.

Extraction of polysaccharides. Dried seaweed (20 g) was ground and extracted with H_2O (500 ml) at 100° for 1 hr. After filtration through nylon cloth, the extract was allowed to gel at room temp. The gel-forming polysaccharide was purified by freezing–thawing × 2 and finally dehydrated with Me_2CO and dried to give PS1 (1.5 g). The sediment from the 100° extraction was then extracted for 1 hr at 121° in an autoclave. The extract was allowed to gel and treated similarly to afford PS2 (5.5 g). The sediment from the second extraction was washed with hot H_2O, dehydrated with Me_2CO and then dried to afford PS3 (3.7 g).

Compositional analysis of PS1. PS1 was methanolysed with 1 M HCl/MeOH for 16 hr at 70°. After neutralization with $AgCO_3$, the methanolysate was concd to dryness, trimethylsilylated [18] and then analysed by GC and GC-MS (column *a*) to detect the components shown in Table 1. For quantitative analysis of the polysaccharide containing acid-labile 3,6-anhydride, another portion of PS1 was partially hydrolysed with 20 mM H_2SO_4 for 2 hr at 100°, neutralized with $BaCO_3$ and then the hydrolysate was treated with $NaBH_4$ for 2 hr at room temp. in order to reduce the liberated reducing end from 3,6-anhydrides. The soln was treated with a Amberlite IR 120 (H^+ form) and the resulting H_3BO_3 removed by co-distillation with MeOH. The reduced partial hydrolysate was then hydrolysed with 1 M H_2SO_4 for 16 hr at 100°, neutralized with $BaCO_3$, the hydrolysate converted into the corresponding alditol acetates [19] and analysed by GC and GC-MS (columns *b* and *c*) using mannitol as int. standard. The results obtained are summarized in Table 1.

Partial methanolysis of PS1. PS1 was methanolysed with 0.1 M HCl in MeOH for 2 hr at 70°. Under these conditions, 3,6-anhydrogalactosyl linkages are cleaved to yield predominantly dimethylacetal groups [20]. The soln was neutralized with $AgCO_3$ and the methanolysate concd to dryness, trimethylsilylated and then analysed by GC and GC-MS (column *a* at 250°). TMSi derivatives of the dimethylacetals of 2,6′-methylagarobiose, 2-methylagarobiose, 6′-methylagarobiose and agarobiose were identified based on their R_t values and MS.

Compositional analysis of PS2. PS2 was dissolved in H_2O at 121° and cooled to 100°. To the soln was added an equal vol. of 40 mM H_2SO_4, the mixt. heated for 2 hr at 100°, neutralized with $BaCO_3$, reduced with $NaBH_4$ and treated in the same way as PS1 to obtain a reduced partial hydrolysate. This was then further hydrolysed as described for PS1 and the final hydrolysate analysed as alditol acetates by GC and GC-MS (columns *b* and *c*) as already described.

Partial methanolysis of PS2. PS2 was partially methanolysed as already described. On GC and GC-MS as its TMSi derivative, 2,6′-dimethylagarobiose dimethylacetal was identified.

Methylation analysis of PS2. PS2 was methylated twice using the methods described in refs [21, 22] using powdered NaOH and MeI in Me_2SO_4. Methylated PS2 was hydrolysed with H_2SO_4 in two steps as already described in the compositional analysis of PS2. The hydrolysate was analysed by GC and GC-MS (columns *b* and *c*) as partially methylated alditol acetates [23] to detect 1,5-di-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol, 1,5-di-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol and a smaller amount of 1,5-di-*O*-acetyl-2,3,6-tri-*O*-methylglucitol.

NMR measurement of PS2. PS2 was dissolved 3% (w/v) in D_2O containing MeOH as int. standard at 121°. To avoid gelation, the soln was kept at 80° before measurements.

Compositional analysis of PS3. Due to its insolubility in MeOH or H_2O, PS3 was methanolysed by the method of ref. [11]. The sample was dissolved to 72% H_2SO_4 at 4°, stood for 30 min and to the soln was added an equal vol. of methanolic 72% H_2SO_4 and the soln allowed to stand for a further 30 min. After addition of MeOH to adjust to the acid concn to 1 M, the mixt. was heated with $CaSO_4$ (Drierite) for 16 hr at 70°, neutralized with $BaCO_3$ and then the resulting $BaSO_4$ and excess $BaCO_3$ removed by filtration. The methanolysate soln was evapd to dryness, the syrup trimethylsilylated and analysed by GC and GC-MS to detect TMSi derivatives of 3,6-anhydro-2-*O*-methylgalactose dimethylacetal, methyl 6-*O*-methylgalactosides and methyl glucosides. Amounts of each component were calculated from peak area measurements. From this analysis, the content of 3,6-anhydride derivatives was apparently underestimated. To compensate for the decomposition of acid-labile 3,6-anhydrides, PS2 was methanolysed and analysed under the identical conditions; 66% of 3,6-anhydride derivatives appeared to be decomposed by comparison with the result obtained from the compositional analysis of PS2.

Cellulase digestion of PS2 and PS3. To the polysaccharide (100 mg) swollen in 10 ml of H_2O (pH 6.7) was added cellulase (Onozuka RS) soln (10 mg ml^{-1}) and the mixt. incubated for 24 hr at room temp. The polysaccharide gel was washed × 3 with H_2O and dehydrated with Me_2CO to afford cellulase-treated polysaccharides. These were hydrolysed and analysed by GC as already described. From both polysaccharides, no Glc was detected.

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