Note

Chemical heterogeneity of the agar from *Gelidium amansii*

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Agars have been found to be mainly composed of two neutral sugar residues, D-galactopyranose and 3,6-anhydro-L-galactopyranose[1]. Smaller proportions of acidic components, such as sulfate[2], pyruvic acid[3], and uronic acid[4] residues have also been confirmed to be present in agars. Separation of agar into two fractions, one rich in neutral components and the other rich in acidic ones, has so far been achieved by acetylation followed by extraction with chloroform[5], precipitation with cetylpyridinium chloride[6], and precipitation with polyethylene glycol[7]. The former fraction, named agarose, has been shown to be a linear, nearly homogeneous molecule composed of alternating residues of the aforementioned two sugars[8]. However, the homogeneity and the structure of the latter fraction (agaropectin) has not been investigated in any detail except to show that pyruvic acid is bonded to D-galactopyranose residues[9]. It is very questionable whether the agar consists of only two polysaccharides having clearly different structures.

In order to obtain information on this point, the agar from *Gelidium amansii*, one of the most typical agarophytes found on the coastline of Japan, has been fractionated more completely by anion-exchange chromatography. First it was separated into four fractions (Fractions 1 to 4), each of which constitute approximately one fourth of the whole agar mixture. The compositions and yields of these four fractions are shown in Table I. The data indicate that the differences in proportions of the neutral sugars are very small, and the molar ratio of anhydrogalactose to galactose is approximately unity through each of the fractions. This observation suggests that a common structure having a rather homogeneous arrangement, possibly consisting of alternating residues of the two neutral sugars, is present in all agar fractions. By contrast, the proportions of the three acidic residues are remarkably different among the fractions, and it is noteworthy that the proportions of sulfate and uronic acid residues become gradually higher in the order of elution, whereas the proportion of pyruvic acid residue is highest in Fraction 3. Fraction 1 appears to be an essentially neutral polysaccharide, and Fraction 2 is intermediate in composition between Fractions 1 and 3 or 1 and 4. The yields of agarose and agaropectin from the same species of algae, as separated by extraction with chloroform, have been reported to be about 60 and 40%, respectively[8]. Comparing the yields and the compositions of the agar fractions separated by these two methods, agarose by the old method appears

to be a mixture of at least the Fractions 1 and 2 obtained in the present method, and agaropectin is a mixture of the Fractions 3 and 4.

TABLE I

COMPOSITION AND YIELD OF THE AGAR FRACTIONS FROM *Gelidium amansii*

Component	*Content* (%)				
	Agar	*Agar fractions*			
		1	2	3	4
Galactose[a]	47.1	51.0	45.6	43.0	42.8
3,6-Anhydrogalactose[a]	40.5	43.9	45.6	38.5	36.8
Uronic acid[b]	0.64	nil	0.09	0.30	1.39
Sulfate[c]	1.42	nil	0.77	0.94	2.36
Pyruvic acid[d]	2.28	0.16	0.71	5.98	1.05
Yield (%)		16	17	22	20

[a]Expressed as glycosyl residues. [b]Expressed as D-glucopyranosyluronic acid residues. [c]Expressed as SO_3. [d]Expressed as the free acid.

In order to clarify whether the fractions separated were homogeneous or not, Fractions 2 and 3 were subjected to gradient-elution chromatography. Fraction 2 showed a rather broad, but distinctly heterogeneous elution pattern, as revealed by the content of anhydrogalactose. Fraction 3 also showed a heterogeneous, and at the same time more sharply divided pattern, as shown in Fig. 1. The content of pyruvic

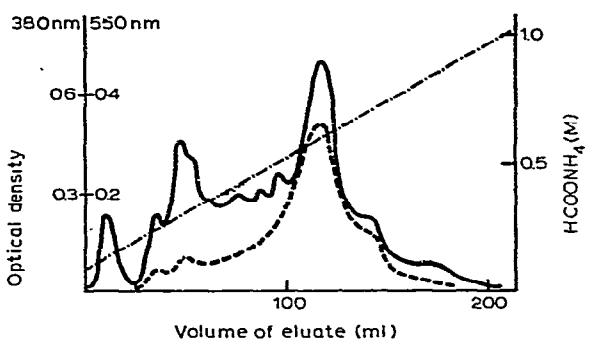

Fig. 1. Gradient-elution chromatography of the agar Fraction 3 from *Gelidium amansii* on a Dowex-1 X2 column. Content of pyruvic acid residue (———). Content of anhydrogalactose residue (———). Eluent concentration (–·–·–·–·–).

acid residues in the eluate was also determined, and is plotted against the elution volume in Fig. 1; this value also indicated heterogeneity in the agar, but the pattern was markedly different from the one obtained from the anhydrogalactose content. Fig. 1 thus indicates that elevation of the eluent concentration increases the content of pyruvic acid residues almost linearly, suggesting that Fraction 3 has marked chemical

heterogeneity. The maximum value of the molar ratio of pyruvic acid to anhydrogalactose residues is 0.45, indicating the presence in Fraction 3 of a polysaccharide containing on an average one pyruvic acid residue per tetrasaccharide unit.

All of the results given above accord with the conclusion that the agar from *Gelidium amansii* consists of a family of polydisperse polysaccharides that have a similar macromolecular structure, with continuously variable proportions of acidic substituents such as sulfate, pyruvic acid, and uronic acid residues. It is therefore necessary to reconsider the nomenclature of agar. The designation agarose may be retained for an essentially neutral polysaccharide consisting of repeated 4-*O*-β-D-galactopyranosyl-3,6-anhydro-L-galactopyranose residues, or agaran may be used instead. However, the designation agaropectin should be discontinued since its original meaning has now become ambiguous. Similar examples of a family of polysaccharides showing homogeneity by some criteria and heterogeneity by others are becoming increasingly common in the field of plant cell-walls and mucilages, for example, in pectic substances[10,11], the water-soluble arabinogalactan from *Codium fragile*[12], and carrageenan from some species[13]. Chemical heterogeneity of these polysaccharides in the cell-wall matrix will presumably include species- and tissue-specific characteristics. More-detailed investigations of these polysaccharides, particularly during cell differentiation and development, will provide an important clue to elucidation of their biological functions.

EXPERIMENTAL

Agar was extracted with hot water from *Gelidium amansii* harvested on the coast of the Izu Peninsula, and was purified by repeated precipitation with ethanol after a freezing and thawing procedure.

Fractionation of agar was performed by an ion-exchange chromatography on a column of Dowex-1 X2 (formate form, 200 to 400 mesh) at 80°. Agar solution (0.25%) in hot water (400 ml) was placed on the hot water-jacketed column (3.4 × 33 cm). The column was washed with water (600 ml), and the combined effluent and washings were concentrated and dialyzed against running water. The non-dialyzed polysaccharide (Fraction 1) was precipitated by addition of ethanol (4 vols.) in the presence of small proportion of sodium acetate, and was washed with acetone, and dried.

The polysaccharides retained by the column were eluted successively with 0.1M (700 ml) and M (900 ml) ammonium formate and 2.0M (1100 ml) ammonium salicylate solutions at pH 7.0 (Fractions 2, 3, and 4 respectively). The flow rates were 300 to 500 ml per h. The polysaccharides were isolated from the eluates by concentration, dialysis, and precipitation with ethanol, in the same manner as used for Fraction 1.

Rechromatography of Fractions 2 and 3 was performed on an analytical scale by using a smaller column (0.9 × 32 cm) for 0.1 to 0.25% sample solutions (3 ml). Concentrations of eluents (ammonium formate solutions) were raised through gradients from zero to 0.1M and 0.1 to M, respectively, during elution. The total

volume of eluents were 300 ml and every 3 ml of eluate was collected. The flow rate was 50 ml per h.

3,6-Anhydrogalactose was determined by the resorcinol method of Yaphe[14]. The content of galactose was calculated by subtracting the anhydrogalactose content from total hexose content as determined by the orcinol method of Hewitt[15]. Pyruvic acid was determined by the dinitrophenylhydrazine method of Katsuki *et al.*[16] after hydrolysis in 0.1M HCl for 4 h at 100°. Uronic acid was estimated as follows. Agar (10 to 15 mg) was partially hydrolyzed in 0.5M sulfuric acid for 2 h at 100°, neutralized with barium hydroxide, and centrifuged. The supernatant was passed through the columns (0.7 × 6.0 cm) of Dowex-50 X8 (H^+form, 200 to 400 mesh) and Dowex-1 X8 (acetate form, 200 to 400 mesh) successively. Uronic acid-containing oligosaccharides retained in the latter column were eluted with M sodium acetate (pH 4.6, 20 ml) and uronic acid in the eluate was determined by the carbazole–borate method of Bitter and Muir[17]. Galactose and anhydrogalactose had been known to be slightly colored by the method, and the contribution of these sugars in the eluate to the optical density was corrected after estimating them separately by the orcinol and the resorcinol methods. Sulfate was estimated by the barium chloranilate method of Wainer and Koch[18] after hydrolysis in M hydrochloric acid for 5 h at 100°.

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