A New Method for Fractionation of Agar

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Agar, gel-forming polysaccharide from several species of red seaweeds, has ever been separated into two fractions, one rich in neutral components and the other rich in acidic ones which are named agarose and agarpectin respectively. The separation has been carried out by acetylation followed by extraction with chloroform,¹⁾ precipitation with cetylpyridinium chloride²⁾ or precipitation with polyethylene glycol.³⁾ The purified agarose fractions separated by repetition of these methods usually still contain small amounts of acidic components such as sulfate residue which give the polysaccharides some undesirable properties for certain application.

The purpose of the present note is to show that agar is separable into four fractions by a chromatographic method and one of the fractions thus separated does not contain sulfate residue at all.

Agar, in a powder form, was purchased from Nakarai Chemicals, Ltd., Kyoto. Fractionation of agar was performed by an ion-exchange chromatography on a column of Dowex 1×2 (formate form, 200~400 mesh) at 80°C. Agar solution (0.25%) in hot water (200 ml) was applied on the column (2.3×40 cm) furnished with a jacket containing hot water. The column was washed with water (300 ml), and the combined effluent and washings were concentrated and dialysed against

running water. The non-dialysed polysaccharide (Fraction I) was precipitated by addition of ethanol (4 vol.), washed with acetone and dried.

The polysaccharides retained in the column were eluted successively with 0.1 M (350 ml) and 1.0 M (450 ml) ammonium formate, and 2.0 M (550 ml) ammonium salicylate solutions at pH 7.0 (Fractions II, III and IV respectively). Flow rates were 200 to 300 ml/hr. Separations of the polysaccharides from the eluates were carried out in the same manner as made for Fraction I.

3,6-Anhydrogalactose was determined by the resorcinol method of Yaphe.⁴⁾ Galactose content was calculated by subtracting anhydrogalactose content from total hexose content determined by the orcinol method of Hewitt.⁵⁾ Sulfate was determined by the barium chloranilate method of Wainer and Koch⁶⁾ after hydrolysis in 1 N HCl at 100°C for 5 hr.

Fractionation of commercial agar was attempted at first by chromatographic techniques using DEAE-Sephadex and cetylpyridinium chloride-impregnated cellulose columns. However, the acidic fractions of agar were adsorbed on the columns irreversibly at room temperature, probably because of its strong gel-forming property. Elution of them in a high yield was successfully achieved only by the use of Dowex 1 column constantly kept at high temperature of 80°C, and fraction-

1) C. Araki, *J. Chem. Soc. Japan*, **58**, 1338 (1937).

2) S. Hjerten, *Biochim. Biophys. Acta*, **62**, 445 (1962).

3) B. Russell, T. H. Mead and A. Polson, *ibid.*, **36**, 169 (1964).

4) W. Yaphe, *Anal. Chem.*, **32**, 1327 (1960).

5) L. F. Hewitt, *Biochem. J.*, **31**, 360 (1937).

6) A. Wainer and A. L. Koch, *Anal. Biochem.*, **3**, 457 (1962).

ation of whole agar into four fractions was attained by the procedure described above.

While Fractions II and III were eluted with anion of a rather weak acid, formate ion, Fraction IV could not be eluted with any anions of common strong acids such as chloride ion or sulfate ion. Among several anions tested only salicylate ion proved suitable for the elution of this fraction from the column. Yields of Fractions I, II, III, and IV were 52, 26, 10, and 8%, respectively. Column chromatography of the four fractions was repeated separately under the same conditions and they all were confirmed to show a reproducible fractionation pattern.

The contents of 3,6-anhydrogalactose, galactose and sulfate residues in the four agar fractions are shown in Table I. It can be seen that the content of sulfate residue becomes remarkably higher in the order of elution. Fraction I can be regarded as an essentially neutral polysaccharide and since the molar ratio of galactose to anhydrogalactose content is nearly unity, this fraction may be considered as agarose itself which has already been known to be a linear molecule composed of alternating residues of the two neutral sugars.⁷⁾

On the other hand, the polysaccharide which has so far been designated as agaropectin should be now considered to be a mixture of at least three polysaccharides, *e.g.* Fractions II, III and IV. It is noteworthy that among these acidic fractions Fractions II and IV are

characteristic for their high and low content of 3,6-anhydrogalactose residue, respectively.

As an example for comparison of this method with others, crude agarose and agaropectin fractions prepared by precipitating once with polyethylene glycol³⁾ were attempted to fractionate further by the column chromatography. The yields of Fraction I to IV were 58, 27, 7.8 and 0% in the case of agarose fraction and 34, 24, 19 and 16% in the case of agaropectin fraction. It is seen from these results that agarose and agaropectin are heavily contaminated with each other in both the fractions. Separation of agarose (Fraction I) from the agaropectin relatively rich in sulfate content (Fractions III and IV) may probably be attained by repeating the precipitation with polyethylene glycol many times. However, it is generally doubtful whether the contamination with the agaropectin relatively poor in sulfate content (Fraction II) can be completely removed from agarose by the methods of precipitation or extraction which have hitherto been applied. On the contrary, the present method will be very useful particularly for the purpose of preparing the purest sample of agarose from agar mixture.

In order to clarify whether the acidic fractions separated were homogeneous or not, gradient elution chromatography of Fractions II and III were carried out on an analytical scale using a column of smaller size. When the molarity of eluent (ammonium formate) was increased linearly from 0 to 0.1 or 0.1 to 1.0, both the fractions showed to be eluted in some distinctly heterogeneous patterns as revealed by the amount of anhydrogalactose. Consequently, the present method will be also useful for the general fractionation and analysis of agaropectin from certain species of red seaweed. The results obtained by applying this method to the agaropectin from *Gelidium amansii* will be reported elsewhere.

TABLE I. COMPOSITION OF THE AGAR FRACTIONS

Component	Fraction (% ^{a)})			
	I	II	III	IV
3,6-Anhydrogalactose	42.7	51.7	44.4	22.3
Galactose	50.0	40.8	44.2	37.0
Sulfate	nil	0.79	2.9	17.0

^{a)} The contents of two neutral sugars are expressed as glycosyl residues and that of sulfate as SO₃.

7) C. Araki, "Proceedings of the Fifth International Seaweed Symposium," ed. by E. G. Young and J. L. McLachlan, Pergamon Press, London, 1966, p. 3.

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