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Note

Determination of the molecular weights of alginates by agarose gel filtration

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The molecular weight of alginic acid has been determined by several techniques such as light scattering, sedimentation, diffusion and viscosity measurements[1–3]. On the other hand, there are few reports on determination of molecular weights by gel filtration chromatography, although chromatography is a much more simple and rapid technique than those mentioned above. In chromatography, it is well known that molecular weights are determined on the basis of the elution volumes of the test samples. However, in the case of acidic substances, the elution volumes are found to be affected by the ionic strength of the eluents employed. Skalka[4] showed that in chromatography on a Sephadex G-200 column, heparin (mol. wt. 6000–20 000) was eluted at the void volume with 0.02 m*M* phosphate buffer solution, and also that as the ionic strength of the buffer increased so did the elution volume. Gelotte[5] demonstrated that acidic amino acids were excluded with pure water from the gel phase of Sephadex G-25. Similar results have been reported for humic and fulvic acids[6,7], D-galacturonic acid oligomers[8], lignins[9] and lignosulphonates[10]. Thibault[8] demonstrated that as the ionic strength, I, of the eluent increased from 0 to 0.075 M so did the partition coefficient (K_{av}) of D-galacturonic acid from 0.05 to 0.46, whereas that of D-galactose remained constant (0.63) under the same conditions. These results indicate that the elution behaviour of acidic substances is different from that of neutral substances in the gel phase, and also that, for the determination of the molecular weights of acidic substances by gel filtration chromatography, eluents with ionic strengths high enough to make K_{av} constant are required.

Recently, we found[11] that the purification of alginic acid (mol. wt. 33 000, by viscosity measurement) can be achieved not by gel filtration on a Sephadex G-100 column (mol. wt. 1000–100 000) but by gel filtration on a Sepharose 4B column (mol. wt. 30 000 – $5 \cdot 10^6$), because on the former column the polysaccharide was eluted at the void volume. Thus, purification of alginic acid has been achieved, but its molecular weight remains to be determined reliably by gel filtration. It is considered that if the molecular weight of alginic acid can be obtained reliably by chromatography, the relationships between its physical and chemical properties and biological activities and molecular weight will be more easily and rapidly investigated.

So, in an attempt to determine the molecular weight of alginic acid reliably, we first examined the effect of the ionic strength of the eluents employed for the chromatography. As a result, although reproducible molecular weights of alginic acid were

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obtained, the values were higher than those obtained by physicochemical methods, which gave almost the same values. So, we examined the relationship between molecular weights obtained by gel filtration chromatography and by viscosity measurements of several purified alginic acids. As a result, we found a linear relationship between the molecular weights obtained by both procedures.

In the present paper, we describe the effect of the ionic strength of eluents employed for gel filtration chromatography, and a relationship between molecular weights obtained by chromatography and by viscosity measurements of purified alginic acids. By use of this relationship, the molecular weight of alginic acid will be more easily obtained from its K_{av} or elution volume in gel filtration chromatography.

EXPERIMENTAL

Alginate sample a, prepared from *Lessonia nigrescens*, was kindly donated by Kimitsu Chemical Industrial Co. (Tokyo, Japan). Samples b and c were prepared as follows: an aqueous solution (0.5%, w/v) of sample a was heated at 100°C for 4 h (sample b) or 8 h (sample c) in an oil-bath, and the by-products produced were then removed by extraction from lyophilized preparation with 70% aqueous ethanol. Alginate sample d was prepared from the fronds of *Sargassum fulvellum* as described previously[11]. Samples e and f were prepared as follows: 300 mg of sample d were partially hydrolized with 100 ml of 0.3 *M* hydrochloric acid at 35°C (sample e) or 40°C (sample f) for 1 h. A half-volume of 95% aqueous ethanol was added to each aqueous solution of the hydrolysates obtained (1.4%, w/v) and the precipitates formed were used in the present investigation. These alginate samples were shown to be homogeneous by electrophoresis, performed on a cellulose acetate paper, Separax (11 cm × 6 cm, Fuji Film) by the following systems: (1) 0.1 *M* pyridine–acetic acid (pH 3.5), 200 V, 15 min; (2) 0.1 *M* hydrochloric acid, 16.5 V, 2.5 h and (3) 0.1 *M* zinc acetate (pH 6.6), 200 V, 1 h.

Preliminary experiments were carried out for alginate sample d on a Sepharose CL-4B column (96.4 cm × 1.2 cm) with pure water, 0.01, 0.05, 0.1, 0.2 and 0.5 *M* sodium chloride as the eluents. On the basis of these experiments, determination of the molecular weights of the above samples was performed by gel filtration chromatography on a Sepharose 2B column (95.5 cm × 1.2 cm) for sample a on a Sepharose CL-4B column (99.7 cm × 1.2 cm) for samples b, c and d and on a Sepharose CL-6B column (97.6 cm × 1.2 cm) for samples e and f with 0.2 *M* sodium chloride as an eluent. Alginate was dissolved in 0.2 *M* sodium chloride (1–2 mg/ml) and the solution applied to each column. The standard pullulan samples used for the determination were as follows: P-20, P-50, P-100, P-200, P-400 and P-800, having molecular weights of 20 800, 46 700, 95 400, 194 000, 338 000 and 758 000. The molecular weights of the alginate samples were estimated from the K_{av} *vs.* log(mol. wt.) curve obtained by using the pullulans; K_{av} was calculated from the elution volume, V_e, by using the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where K_{av}, V_0 and V_t are the partition coefficient, the void volume and total volume of the column, respectively[12]. However, as suitably high-molecular-weight standard samples for the estimation of V_0 were not available, the molecular weight of sample a was estimated by extrapolating its elution volume to that corresponding to a molecular weight of more than 758 000 (mol. wt. of P-800) using the V_e *vs.* log(mol. wt.) curve for the pullulans. Viscosity measurements on the

alginate samples (0.08–4.8 mg/ml) were performed in 0.1 *M* sodium chloride with an Ostwald type viscosimeter (flow time: 159 s for water) at 25.00 ± 0.02°C. The degree of polymerization (DP) of each sample was calculated from the intrinsic viscosity, which was obtained from the measurements, using the formula of Donnan and Rose[3]. The molecular weight was estimated from the DP.

RESULTS AND DISCUSSION

Preliminary experiments were carried out in order to find the ionic strength of the eluent suitable for chromatography of an alginate sample (sample d) on a Sepharose CL-4B column. When pure water was used as the eluent, the K_{av} value of sample d was shown to be 0.09. However, as the ionic strength of the eluent increased, so did K_{av} (0.09 to 0.45) to a constant (0.45) at > 0.05 *M* sodium chloride. Therefore, in order to avoid the effect of the ionic strength of the eluent on K_{av}, we employed 0.2 *M* sodium chloride for the present study.

The respective molecular weights, determined by gel filtration, of alginate samples a, b, c, d, e and f were $18.7 \cdot 10^5$, $7.8 \cdot 10^5$, $4.6 \cdot 10^5$, $2.9 \cdot 10^5$, $1.4 \cdot 10^5$ and $0.6 \cdot 10^5$. On

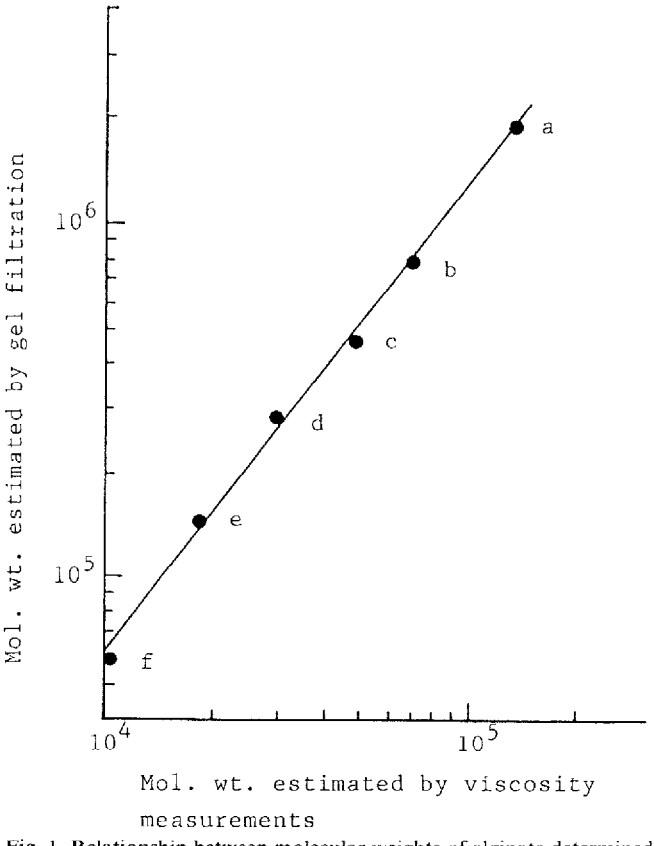

Fig. 1. Relationship between molecular weights of alginate determined by both gel filtration and viscosity measurements. For samples a–f, see text.

the other hand, the molecular weights determined by viscosity measurements were $13.4 \cdot 10^4$, $7.0 \cdot 10^4$, $4.9 \cdot 10^4$, $2.9 \cdot 10^4$, $1.9 \cdot 10^4$ and $1.0 \cdot 10^4$, respectively. These differences may be caused by the use of neutral polysaccharides as molecular weight standards in the chromatography. So we examined the relationship between the molecular weights obtained by the two methods. The results are shown in Fig. 1. The linear relationship found had a correlation coefficient of 0.998.

$$\log B = 1.31 \log A - 0.440$$

where A and B are the molecular weights estimated by viscosity measurements and by gel filtration, respectively. Donnan and Rose[3] determined the intrinsic viscosity, $[\eta]$, and osmotic pressure of alginates in order to estimate their molecular weights and found that $[\eta]$ was proportional to the molecular weight determined by osmotic pressure. Other workers also reported the presence of a linear relationship between $[\eta]$ and the molecular weight of alginate obtained by sedimentation-viscosity measurement[2] or by light scattering[1]. These results indicate that molecular weights determined by the viscosity measurements are reliable. However, viscosity measurements are somewhat troublesome to perform.

In the present study, it was shown that the relationship between molecular weights determined by gel filtration and those obtained by viscosity measurements is linear. Therefore, by using the above equation, reliable molecular weights of alginates can be obtained not only rapidly but also precisely. Polyuronic acids such as pectic acid and polygalacturonic acid may interact with agarose gel in a manner similar to that of alginate. Therefore, the molecular weights obtained by gel filtration may not be precise. If a calibration graph is obtained in the same way as described above, reliable molecular weights of acidic polysaccharides may be also determined by gel filtration chromatography.

REFERENCES

1 O. Smidsrød and A. Haug, *Acta Chem. Scand.*, 22 (1968) 797.
2 W. H. Cook and D. B. Smith, *Can. J. Biochem. Physiol.*, 32 (1954) 227.
3 F. G. Donnan and R. C. Rose, *Can. J. Res., Sect. B*, 28 (1950) 105.
4 M. Skalka, *J. Chromatogr.*, 33 (1968) 456.
5 B. Gelotte, *J. Chromatogr.*, 3 (1960) 330.
6 Y. Saito and S. Hayano, *J. Chromatogr.*, 177 (1979) 390.
7 C. J. Miles and P. L. Brezonik, *J. Chromatogr.*, 259 (1983) 499.
8 J.-F. Thibault, *J. Chromatogr.*, 194 (1980) 315.
9 J. Pellinen and M. Selkinoja-Salonen, *J. Chromatogr.*, 322 (1985) 129.
10 N. G. Lewis and W. Q. Yean, *J. Chromatogr.*, 331 (1985) 419.
11 M. Fujihara, N. Iizima, I. Yamamoto and T. Nagumo, *Carbohydr. Res.*, 125 (1984) 97.
12 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317.