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A new chromatographic procedure for separation of typical λ-type molecular species from commercial λ-carrageenan

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A commercial λ-carrageenan preparation was dissolved in 3·0 M ammonium sulphate and loaded onto a Sepharose CL-4B column equilibrated with the same solvent. Fractionation was carried out by stepwise elution with decreasing concentrations of ammonium sulphate at a low temperature. Each fraction obtained was analysed for sulphur content, 3,6-anhydro-D-galactose content, and molecular weight (Mw). The Mw of all the fractions was lower than the Mw of the starting material. The species with the highest sulphur content and lowest 3,6-anhydro-D-galactose content, a composition closest to that of an ideal structure, was recovered in the 1·5 M fraction.

INTRODUCTION

The authors have developed a new method for fractionating sulphated glycosaminoglycans. Solutions prepared in concentrated ammonium sulphate solution are fractionated by using a cross-linked gel such as Sepharose CL-4B. Low temperatures and elution with stepwise decreasing concentrations of ammonium sulphate are employed. This method was based on the difference in solubility of sulphated glycosaminoglycans in concentrated ammonium sulphate solutions (Uchiyama *et al.*, 1985). An interesting relationship was found between the molecular size and composition of disaccharides for both chondroitin sulphate C (ChS-C) and chondroitin sulphate A (ChS-A). The larger the molecular size, the more 6-sulphate disaccharide units in ChS-C; also, the larger the molecular size, the more the 4-sulphate disaccharide units in ChS-A (Ogamo *et al.*, 1987, 1990).

λ-Carrageenan is a polysaccharide obtained from red algae such as *Chondrus* and *Gigartina*. It has inflammatory and anti-viral activities and is used as a thickening agent or stabiliser in foods. Chemically, it is an extensively sulphated galactan consisting of D-galactose with alternate β-1,4 and α-1,3 linkage. It also contains some 3,6-anhydro-D-galactose which is indicative of the presence of other polysaccharide species sulphated to lower degrees.

In the present report, the above separation method, which is based on the difference in solubility of sulphated polysaccharide in high concentrations of ammonium sulphate, was used to examine the heterogeneity of a commercially available λ-carrageenan preparation. The low molecular weight (depolymerised) typical λ-species was obtained in the 1·5 M ammonium sulphate fraction.

EXPERIMENTAL

Materials

λ-Carrageenan (lot no. 96F-0441), κ-carrageenan (lot no. 115F-0665), and β-D-fructose (lot no. 58F-0008) were obtained from Sigma (St Louis, MO, USA). Sepharose CL-4B and Sepharose CL-2B were products of Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Standard molecular size pullulan was purchased from Shodex Standard P-82 (Showa Denko Co., Tokyo, Japan).

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Analytical methods

3,6-Anhydro-D-galactose content was determined by the method of Yaphe and Arsenault (1965), and sulphur content by that of Dodgson and Price (1962). The reduced viscosity of polysaccharide solutions in 0·2 M phosphate buffer, pH 7·3, was measured using a Ubbelohde viscometer (K: 0·0043, 3–12 cS) at 37 ± 0·05°C. The molecular size of λ-carrageenan and each of its fractions were determined by chromatography using a calibration curve obtained from the standard molecular size pullulan. Gel permeation high-performance liquid chromatography (GP-HPLC) was performed using a chromatography apparatus equipped with a liquid delivery pump and autosampler (SP8800, 8780, Spectra-Physics Co., San Jose, CA, USA), a differential refractometer (ERC-7521, ERMA INC, Tokyo, Japan), and using two columns (7·8 × 300 mm) packed with TSK-gel G-5000 PW-XL and TSK-gel G-4000 PW-XL (TOSOH Co., Tokyo, Japan). Each sample was eluted at 60°C with 0·1 M Na_2SO_4 solution at a flow rate of 1 ml/min. The infrared (IR) spectra was recorded with JASCO A202 (Japan Spectroscopic Co. Ltd, Tokyo, Japan) using samples pre-prepared with potassioum bromide.

Fractionation of commercial λ-carrageenan on Sepharose CL-4B in ammonium sulphate solution at 4°C

A solution of commercial λ-carrageenan (300 mg) in 3·0 M ammonium sulphate (150 ml) was applied to a column (2·5 × 21 cm) of Sepharose CL-4B equilibrated with the same solvent. The elution of the column was performed stepwise at a flow rate of 60 ml/h with 3·0 M (600 ml), 2·5 M (600 ml), 2·0 M (600 ml), 1·5 M (600 ml) $(NH_4)_2SO_4$ solution and distilled water (600 ml) at 4°C. The eluate was collected in 14·5-ml fractions, each of which was analysed for hexose content by the phenol–sulphuric acid method. The fractions (3·0 M fraction, 87 ml; 2·5 M fraction, 102 ml; 2·0 M fraction, 261 ml; 1·5 M fraction, 145 ml; water fraction, 160 ml) were separately dialysed against distilled water (6 × 20 litres) for 3 days at room temperature, evaporated *in vacuo* to a small volume and re-dialysed against distilled water (6 × 20 litres) for 2 days. After pH adjustment to 6·5–7·0 with 0·01–0·1 M KOH, each fraction was filtered through DISMIC-25cs (0·45 μm) (ADVANTIC TOYO Co., Tokyo, Japan), evaporated *in vacuo* to a small volume, and freeze-dried. The yield of each fraction isolated was 42·8 mg for the 3·0 M fraction, 5·1 mg for the 2·5 M fraction, 10·8 mg for the 2·0 M fraction, 85·7 mg for the 1·5 M fraction, and 94·3 mg for the water fraction. The total recovery was 79·6%.

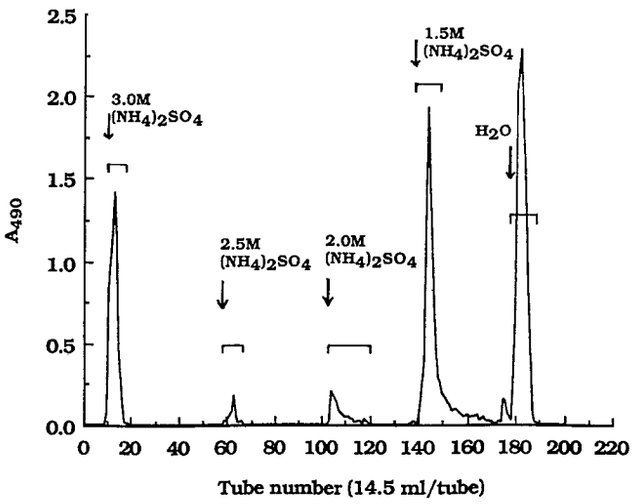

Fig. 1. Fractionation of commercial λ-carrageenan on Sepharose CL-4B in the presence of ammonium sulphate at 4°C. The commercial λ-carrageenan (300 mg) was chromatographed on a Sepharose CL-4B column (2·5 × 21 cm) by using stepwise elution with ammonium sulphate (3·0 to 1·5 M and water) at 4°C. Fractions (14·5 ml) were analysed for hexose content (200 μl sample). Each of the pooled fractions indicated by the square bracket was subjected to isolation.

Fractionation of commercial λ-carrageenan on Sepharose CL-2B gel in 0·2 M NaCl at 20°C

A solution of commercial λ-carrageenan (20 mg) in 0·2 M NaCl (10 ml) was applied to a Sepharose CL-2B Column (2·64 × 90 cm) prepared in 0·2 M NaCl. The column was eluted with the same solvent at a flow rate of 38 ml/h at 20°C. The eluate was collected in 6-ml fractions, and each fraction was analysed for hexose. The elution diagram based on A_{490} was divided into three fractions of equal peak area (fractions 1–3). Each of the fractions was dialysed against distilled water (6 × 20 litres) for 3 days at room temperature, evaporated *in vacuo* to about 20 ml, and re-dialysed against distilled water (6 × 20 litres) for 2 days. After pH adjustment to 6·5–7·0 with 0·01–0·1 M KOH, each fraction was filtered with DISMIC-25cs (0·45 μm), evaporated *in vacuo* to a small volume, and freeze-dried.

RESULTS AND DISCUSSION

Commercial λ-carrageenan was applied onto a Sepharose CL-4B column and eluted by stepwise elution of 3·0 M, 2·5 M, 2·0 M, 1·5 M ammonium sulphate and water at 4°C (Fig. 1). The composition of the five fractions obtained are compared with those of the starting material (λ-carrageenan) in Table 1. The highest sulphur content was found in the 1·5 M fraction, (2·9 mol bound sulphate per disaccharide). The content of 3,6-anhydro-D-galactose tended to decrease with decreasing concentration of ammonium sulphate in the fraction, and was lowest in the 1·5 M fraction. The water fraction was an exception to this and had a large amount of 3,6-anhydro-D-galactose. Analysis of the IR spectrum of each fraction revealed that the 1·5 M fraction had no

Table 1. Composition, viscosity and molecular weight, of λ-carrageenan fractions

λ-Carrageenan fraction	S mol per disaccharide	3,6-Anhydro-D-galactose (%)	Reduced viscosity (dl/g)	Mw ($\times 10^4$)	Distribution of fraction (%)
Commercial λ-carrageenan	2·6	4·7	11·0	125	100
Sepharose CL-4B in 3·0 M $(NH_4)_2SO_4$ → Water					
3·0 M fraction	2·1	6·4	3·8	37	17·9
2·5 M fraction	2·0	5·0	ND[a]	27	2·1
2·0 M fraction	1·9	1·9	ND	14	4·5
1·5 M fraction	2·9	1·4	4·2	41	35·9
Water fraction	2·7	6·2	5·2	66	39·5
Sepharose CL-2B in 0·2 NaCl					
Fraction 1	2·7	1·9	17·5	135	32·5
Fraction 2	2·6	4·5	7·8	103	34·1
Fraction 3	2·1	6·6	2·5	31	33·1

[a]ND—not determined.

absorption peak at 928 cm^{-1} due to 3,6-anhydro-D-galactose. This peak is observed in the spectra of the 3·0 M and water fractions (Fig. 2) indicating that this 1·5 M fraction was composed of a typical λ-species.

Table 1 shows that the reduced viscosities of the ammonium sulphate fractions, and the molecular weights obtained by GPC were clearly lower than those of the starting λ-carrageenan. To confirm this, λ-carrageenan was dissolved in 3·0 M ammonium sulphate, and left to stand at 4°C and 20°C for 5 and 48 h, respectively. It was then recovered by dialysis, filtration, and freeze-drying as previously described for the individual fractions (recovery 92–96%), and the Mw was determined by GP-HPLC. Irrespective of temperature (4 and 20°C), the Mw decreased with time (5 and 48 h) (Table 2). Heat treatment (80°C, 5 min) or ultrasonic treatment (45 kHz, 60 min) or acetate buffer treatment (pH 5·3, the same pH as 3·0 M ammonium sulphate solution) did not cause the apparent Mw reduction found, with 3·0 M ammonium sulphate treatment (data not shown). Furthermore, when λ-carrageenan (commercial preparation) was treated with Chelex chelating resin to completely exclude Ca^{2+} ions, no change to low-molecular-weight compounds occurred (data not shown). This suggests that the effect of ammonium sulphate was not to dissociate but to depolymerise the carrageenan molecule.

λ-Carrageenan was separated into equal amounts of three fractions with different Mw values by conventional gel filtration chromatography (Fig. 3). The data obtained on these fractions is shown in Table 1 and compared the those five fractions obtained by the method using ammonium sulphate. The content of 3,6-anhydro-D-galactose in the high-molecular-weight fraction prepared by conventional gel filtration was lower than that in the low-molecular-weight fraction, and the sulphur content in the high-molecular-weight fraction tended to be higher than that in the low-molecular-weight fraction. When the two methods were compared, the 1·5 M fraction of the ammonium sulphate fraction contained a smaller amount of 3,6-anhydro-D-galactose than fraction 1 obtained by gel filtration method, and the largest amount of sulphur content 2·9 mol which was the highest value obtained. These findings indicated that the ammonium sulphate fractionation (1·5 M fraction) was more efficient as a chromatographic procedure for preparing typical λ-species than gel filtration fractionation. That is, in the chromatography presented here, the commercial preparation of λ-carrageenan is considered to have changed to a molecular species with a low degree of association in the 3·0 M ammonium sulphate solution, and to have been fractionated depending on the concentration of ammonium sulphate based on the solubility difference as shown by the sulphated glycosaminoglycan. This fractionation was carried out at a low temperature (4°C).

When the fractionation using ammonium sulphate was carried out at room temperature (20°C) as opposed to 4°C, the distribution of material in each fraction was 4·8, 0, 11·4, 41·2, and 42·6% for the 3·0, 2·5, 2·0, 1·5 M and water fractions, respectively. That is, the percentages eluted in the 3·0 and 2·5 M fractions were lower, and the

Table 2. Molecular weights of commercial λ-carrageenan and λ-carrageenan treated 3·0 M $(NH_4)_2SO_4$ at 4 or 20°C

Sample	Mw ($\times 10^4$)
Commercial λ-carrageenan	130
treated with 3·0 M $(NH_4)_2SO_4$	
4°C, 5 h	72
20°C, 5 h	78
4°C, 48 h	34
20°C, 48 h	36

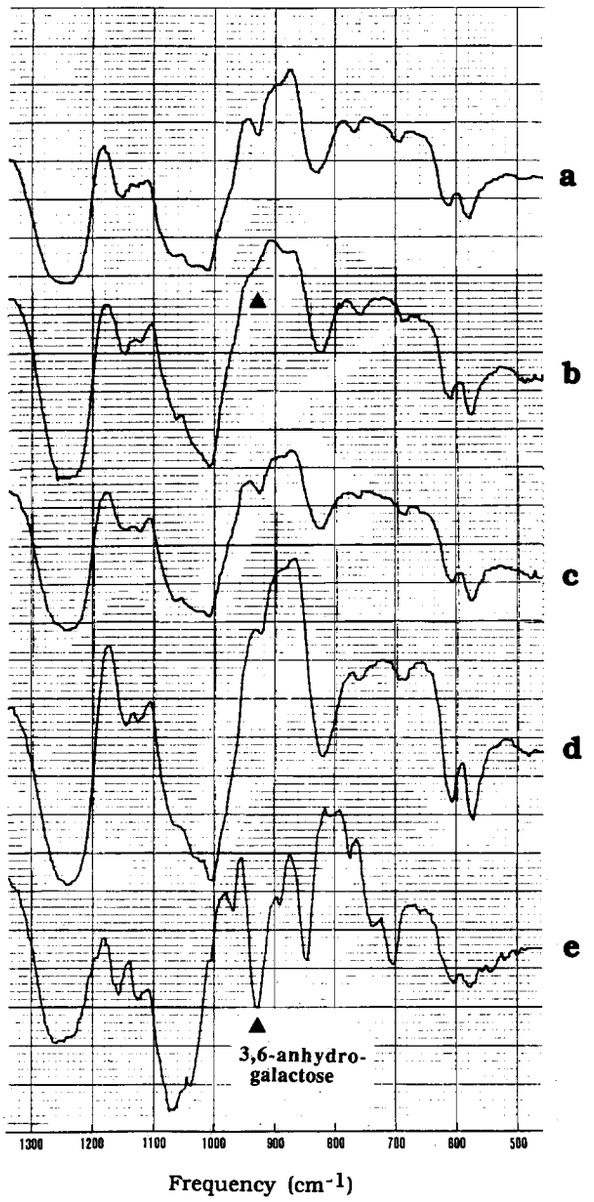

Fig. 2. Infrared spectra of λ-carrageenan fractions separated on Sepharose CL-4B/$(NH_4)_2SO_4$, and of commercial λ- and κ-carrageenans: (a) 3·0 M $(NH_4)_2SO_4$ fraction; (b) 1·5 M $(NH_4)_2SO_4$ fraction; (c) H_2O fraction; (d) λ-carrageenan and (e) κ-carrageenan.

percentages eluted in the 2·0, 1·5 and water fractions were higher than the values obtained at the lower temperature, showing an apparent tendency for the polysaccharide to be retained more by the column at the higher temperature. Analysis of each fraction obtained at 20°C revealed that the sulphur content was the largest in the 1·5 M fraction being 3·0 mol per disaccharide, and the 3,6-anhydro-D-galactose content, i.e. 2·3%, was also the lowest of these fractions. This indicated that the 1·5 M fraction consisted of the typical λ-species as was the case in the fractionation obtained at a low temperature. However, the content of 3,6-anhydro-D-galactose was higher than 1·4% as was determined in the fraction obtained at the low temperature. Therefore, fractionation at a low temperature is considered to be more effective for isolation and purification of typical λ-type polysaccharide species from natural crude λ-carrageenan fractions or commercial λ-carrageenan preparations.

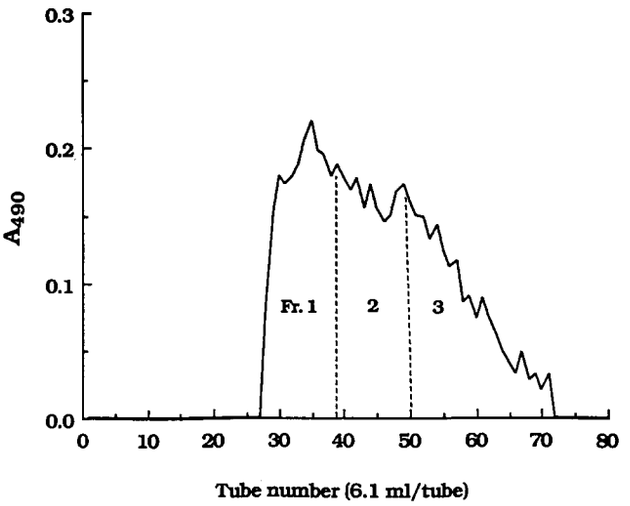

Fig. 3. Fractionation of commercial λ-carrageenan on Sepharose CL-2B in 0·2 M sodium chloride at 20°C. The commercial λ-carrageenan (20 mg) was chromatographed on a Sepharose CL-2B column (2·64 × 90 cm) with 0·2 M sodium chloride at 20°C. Fractions (6·1 ml) were analysed for hexose content (250 μl sample). The elution diagram was divided into three fractions with the same peak area (fractions 1–3).

CONCLUSION

A new procedure of fractionating λ-carrageenan is proposed. This involves chromatography using a Sepharose CL-4B gel in a high concentration ammonium sulphate solution at a low temperature. Fractionation by a stepwise elution of decreasing concentration of ammonium sulphate resulted in the concentration of dissociated typical λ-species in the 1·5 M ammonium sulphate fraction.

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