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Note

Specific fragmentation of carrageenans

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Recent advances in understanding of the fine structure of carrageenans[1] indicated that, even if parts of their molecules could be described in terms of a regular structure, there are also "masking" units[2], "unusual" residues[1], and regions having a different type of regularity, or no regularity at all. Some of these units (such as galactose 6-sulfate or galactose 2,6-disulfate in place of the 3,6-anhydro residues) were recognized a long time ago[2], but others only more recently[1]. All of these structural details are difficult to study in the whole molecule, as they appear in minor proportions or are interspersed in the regular alternating structure, or both. Thus, it is desirable to find a method to fragment the molecules *specifically*,

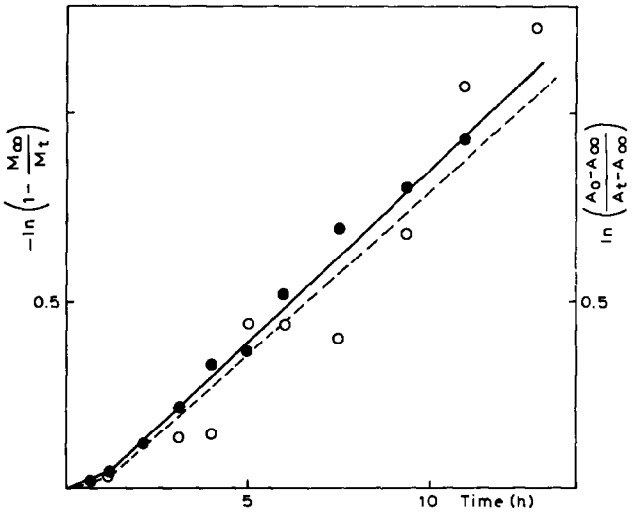

Fig. 1. First-order plot for hydrolysis of the glycosidic (—●—) and 3,6-anhydrogalactosidic (---○---) linkages of the alkali-treated carrageenan (insoluble in 125mM KCl) from *Iridaea undulosa*.

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TABLE I

AUTOHYDROLYSIS OF CARRAGEENANS

Time (h)	*Product*											
	2M KCl-soluble from Iridaea undulosa *(A)*		*Fraction of A soluble in Me_2SO (As, ref. 1)*			*Fraction of A insoluble in Me_2SO (Ai, ref. 1)*		*Alkali-treated from* I. undulosa *insoluble in 125mM KCl (ref. 3)*			*κ-Carrageenan from* Eucheuma cottonii		
	η_{rel}	*Mol. wt.*	η_{rel}	*Mol. wt.*	A^a_{as}	η_{rel}	*Mol. wt.*	η_{rel}	*Mol. wt.*	A^a_{ag}	η_{rel}	*Mol. wt.*	A^a_{ag}
0	5.48	35700	1.92	24300	2.43	3.16	70000	3.34		5.19	12.46	116000	6.3
0.5									68600	3.04			
1	1.57	15900	1.26	18000	2.20			1.16	18500	5.06	1.47	9400	6.6
2	1.27	14300	1.15	10200	2.02	1.24	68000	1.06	6400	2.64	1.09	5100	6.4
3	1.17	11600	1.10	6700	2.70			1.03	3700	4.63	1.05	3050	4.8
4	1.12	9400						1	2650	4.61	1	2240	4.2
4.5			1.08	4800	1.93								
5								1	2400	3.60			
5.5											1	1670	4.9
6	1.09	6500	1.05	4150	2.25			1	1820	3.60			
7						1.02	49000				1	1520	2.8
7.5								1	1480	3.73			
8	1.06	5500											
9			1	3100	3.35						1	1300	3.6
9.5								1	1325	2.95			
11								1	1223	2.23			
12	1.04	3600	1	2550	1.89								

13								1	928	2.00	1	793	4.4
14						1	17700						
15								1	824	4.41			
16			1	2260	1.57								
17											1	912	2.1
18.5								1	768	1.19			
21								1	719	2.13	1	653	3.9
23						1	13300						
23.5			1	1710	3.30								
24								1	779	0.92			
28								1	711	1.82	1	502	0.9
30			1	1650	0.83								
32.5								1	676	3.31			
36			1	1720	0.72	1	11100						
38											1	—	1.6
42								1	787	0.66			
43.5											1	453	—
55											1	398	1.6
59											1	399	0.7
Rate constants	k^b	0.11	0.08			0.09		0.09			0.05		
	$k^c_{3,6\ anh}$		0.08					0.09			0.06		
M[d]		3600	2100			18000		570			550		
Sulfate loss (%)		30	30			25		15 (at 7.5 h)			25		

[a]Absorbance due to linked 3.6-anhydrogalactose (values were normalized). [b]Rate constant obtained from reducing power measurement. [c]Specific rate-constant obtained from the determination of linked 3,6-anhydrogalactose content. [d]Number-average molecular weight calculated for the unit containing one 3.6-anhydrogalactose residue.

producing oligosaccharides in which the "non-regular" parts of the molecules are concentrated, together with others which are formed from the alternating structure (carrabioses). Analysis of the products of fragmentation, as well as isolation of those "non-regular" fragments and their further structural determination, should throw light on the fine structure of carrageenans.

We report the fragmentation of gelling and non-gelling carrageenan molecules through the *specific* autohydrolysis of the 3,6-anhydrogalactosidic linkages, and the fractionation of their fragmentation products.

RESULTS AND DISCUSSION

Solutions (0.3% w/v) of the acid form of the carrageenans (pH 2.45) were heated at 60° for different periods. In all cases, the pH values of the solutions, as well as the other starting conditions, were maintained.

Results of the determinations of the relative viscosity, reducing power, and 3,6-anhydrogalactose content after reduction of the samples with sodium borohydride, in aliquots taken at intervals, are given in Table I, which also shows the number-average molecular weights calculated for the average units containing one 3,6-anhydrogalactose residue, the loss of sulfate after the reaction, and the rate constants of the autohydrolysis calculated on the basis of first-order degradations. Four samples showed rate constants of 0.08–0.11 h^{-1} for the hydrolysis of glycosidic linkages (0.05–0.06 h^{-1} in the case of the κ-carrageenan) (see Table I), and the same values were obtained for the specific rate-constants of hydrolysis of the 3,6-anhydrogalactosidic bond in those products, indicating *that this reaction was the only one occurring during these autohydrolyses* (see Table I). The plots of molecular weight and linked 3,6-anhydrogalactose *vs.* time for alkali-treated carrageenan (from *Iridaea undulosa*[3]) insoluble in 125mM KCl are shown in Fig. 1. The straight lines were calculated by the least-squares method. A lag period of 20–30 min at the beginning of the reactions was observed.

The autohydrolysis cleaved the 3,6-anhydrogalactosidic linkage, but did not degrade the 3,6-anhydrogalatose to levulinic and formic acids, because in all cases the total 3,6-anhydrogalactose content remained constant during the reaction[4,5], and the final products did not show absorption at 285 nm, indicating the absence of 5-(hydroxymethyl)-2-furaldehyde (HMF) derivatives[6]. The ^{13}C-n.m.r. spectra of these products showed signals corresponding to 4-linked 3,6-anhydrogalactose, and, after reduction, those corresponding to 4-linked 3,6-anhydrogalactitol, but not absorptions above 105 p.p.m., where the signals of the aromatic and aldehydic carbon atoms of HMF would appear. Control experiments demonstrated that the results of the resorcinol–hydrochloric acid test after total autohydrolysis and borohydride reduction (see Table I) were due to interferences from galactose and 3,6-anhydrogalactitol.

The products of autohydrolysis were chromatographed on Sephadex G-25, using 0.5M NaCl or water as eluant. The elution patterns were the same in both

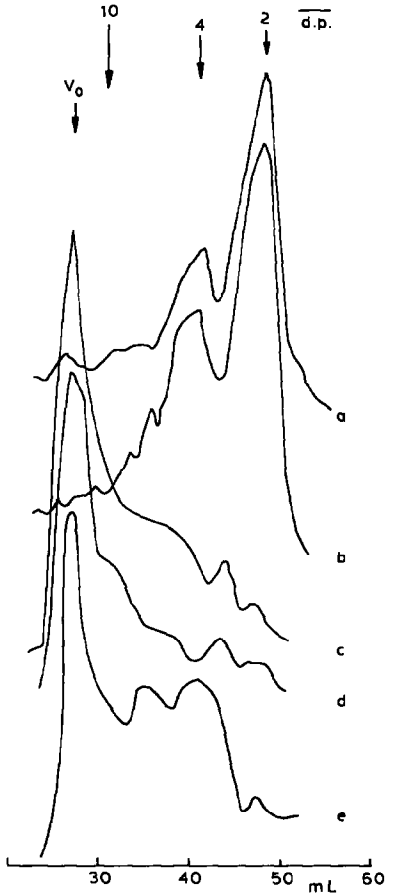

Fig. 2. G.p.c. (Sephadex G-25) of the autohydrolysis products of: (a) κ-carrageenan from *Eucheuma cottonii*; (b) alkali-treated carrageenan (insoluble in 125mM KCl) from *Iridaea undulosa*; (c) 2M KCl-soluble carrageenan from *Iridaea undulosa* (A, ref. 1); (d) Ai, fraction of A insoluble in dimethyl sulfoxide (ref. 1); and (e) As, fraction of A soluble in dimethyl sulfoxide (ref. 1).

cases, and are given in Fig. 2. Isolation of the fraction of d.p. 2, and determination of ^{13}C-n.m.r. spectra before and after reduction with sodium borohydride, suggested that it was composed of carrabiose 4-sulfate.

The study of minor structural details of carrageenans would be improved if the part of the molecules which contain these structures could be isolated. The masked[2] and "unusual"[1] residues are galactose units, whereas the only variation in the 3,6-anhydro unit may be the presence or absence of a sulfate group on C-2. Then, the *specific* cleavage of the 3,6-anhydrogalactosidic linkage would produce the aforementioned fragments. The determination of "consecutive carrabiose content" was based on this principle[2], but the hydrolysis was only selective and the emphasis was put on the analytical determination of carrabiose[2]. The enzymic approach to this problem[7–9] is not desirable as the carrageenases cleave the β-

(1→4)-galactosidic bond and little is known as to their specificity (or that of possible contaminants) against other galactosidic linkages that may appear, in minor proportions, in the carrageenan molecules.

Autohydrolysis is the simplest method for the fragmentation of carrageenan. Painter[10,11] used it unspecifically to produce sulfated monosaccharides. The results obtained in this work show that, if the experimental conditions are carefully chosen, it is possible to cleave *specifically* the 3,6-anhydrogalactosidic linkage, without significant hydrolysis of the galactosidic bond. The presence of a sulfate group on C-2 of a 3,6-anhydrogalactose unit produces differences in the specific rate-constant of *acid* hydrolysis[12] of these linkages. This was not clearly observed in the autohydrolysis, possibly because the higher local concentration of protons around the linkage[13] could counterbalance the protective effect of the sulfate group.

The elution patterns of the gel-permeation chromatography (on Sephadex G-25) of the fragments produced by the specific cleavage of the 3,6-anhydrogalactosidic linkages in different carrageenans are shown in Fig. 2. The same patterns were obtained on using water or sodium chloride solutions, possibly due to the low degree of polymerization of the oligosaccharides fractionated by the Sephadex G-25. The isolation of the smaller fragment showed that the $\overline{d.p.}$ was 2, and the ^{13}C-n.m.r. spectrum suggested that the disaccharide isolated from the κ-carrageenan from *Eucheuma cottonii* was carrabiose 4-sulfate. In no case were monosaccharides or any other molecule of similar size detected. The second peak (Fig. 2a) had $\overline{d.p.}$ 4 and, from the K_d of both fractions, it was possible to calculate that the fragments entering into the gel contained molecules with $\overline{d.p.}$ <10. The elution patterns shown in Fig. 2 are in agreement with the fragmentation of the carrageenan only through the cleavage of the 3,6-anhydrogalactosidic linkage and indicate that the fragments produced can be isolated by simple procedures.

EXPERIMENTAL

The general and analytical methods have been reported[1]. κ-Carrageenan from *Eucheuma cottonii* was purchased from Sigma. The carrageenans from *Iridaea undulosa* were obtained as already reported[1,3]. Gel-permeation chromatography (g.p.c.) was performed in a column (1.2 × 60 cm) of Sephadex G-25, with water or 0.5M NaCl as the eluant. Void and final volumes were determined with Blue Dextran 2000 and galactose, respectively. In the fractions collected, the degree of polymerization was determined by the diminution in absorbance observed in the phenol–sulfuric acid reaction when the fraction was reduced with sodium borohydride[14]. ^{13}C-N.m.r. spectra of solutions in 50% D_2O were recorded with a Varian XL-100 instrument. Absorbance at 285 nm was measured with a Beckman DU spectrophotometer.

For the autohydrolysis, the polysaccharide (0.3 g) was dissolved in water (30 mL), and the solution was passed through a column of Dowex-50 (H^+) resin. The eluate was collected over an ice bath, the column was washed, and the solution was

diluted to 100 mL (pH 2.45). It was heated at 60°, and aliquots were taken at intervals in order to determine the reducing power by the Somogyi's iodometric method[15]. Another aliquot (0.1–0.2 mL) was added to a tube containing an excess of $CaCO_3$; water (1 mL) and sodium borohydride (3 mg) were added, the mixture was kept overnight, and the slurry was centrifuged. 3,6-Anhydrogalactose in the supernatant liquor was determined[16]. The autohydrolysis was stopped by cooling to room temperature, adding barium carbonate (0.5 g) and keeping overnight. The suspension was filtered through sintered glass, and the filtrate was passed through Dowex-50 (Na^+) resin, concentrated, and freeze-dried, giving yields of 60–80%.

The resorcinol–hydrochloric acid test[16] was carried out on control solutions of galactose and 3,6-anhydrogalactitol.

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