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^{13}C NMR STUDY OF HETEROGENEITY IN THE CARRAGEENAN SYSTEM FROM *RISSOELLA VERRUCULOSA*

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Key Word Index—*Rissoella verruculosa*; Rissoellaceae; seaweeds; ^{13}C NMR; omega, kappa, beta carrageenan.

Abstract—The coexistence of polymers of the omega and kappa type, in the carrageenan system of *Rissoella verruculosa*, led us to investigate the possible precursors of these structures. Isolation of a third component, beta carrageenan, is reported. Fractionation by D.E.A.E. chromatography shows that this later polymer always coexists with omega but is never found in the fraction containing kappa. The signification of this coexistence between these different carrageenans is discussed.

INTRODUCTION

In a previous study we reported the structure of a new type of carrageenan from the red seaweed *Rissoella verruculosa*. It was designated omega carrageenan [1]. Classification on the basis of the sulphation pattern of the 3 linked-beta-D-galactopyranosyl residue (2–4) indicates that this carrageenan constitute the new family, defined here as the omega family (Fig. 1). This polymer and another carrageenan, designated as kappa [5] are the main water soluble molecules extracted from *R. verruculosa*. Omega carrageenan could be isolated by potassium chloride fractionation and alkali treatment as a soluble polymer and separated from an insoluble kappa–omega mixture. Gravimetric measurement of the isolated omega and comparison of the intensity of peaks for each type of polymer in the ^{13}C NMR spectrum from the mixed structure show that omega carrageenan is predominant over kappa.

The purpose of this study was to look for precursors for both omega and kappa structures in the carrageenan extracted from *R. verruculosa*. It is generally admitted that, in the formation of most carrageenans, the 3,6 anhydrogalactose unit derives from a 6-sulphated galactose precursor (Fig. 1). Such a precursor for kappa from other red algae was shown to be mu carrageenan, a highly sulphated cold extractable polymer [6]. We could not find any carrageenan of this type in the cold extract from *R. verruculosa* (unpublished results). DEAE Sephadex fractionation was used to separate omega from the omega–kappa mixed structure. We report the results from this separation and the isolation by this technique of a beta structure, the third component in the carrageenan system from *R. verruculosa*.

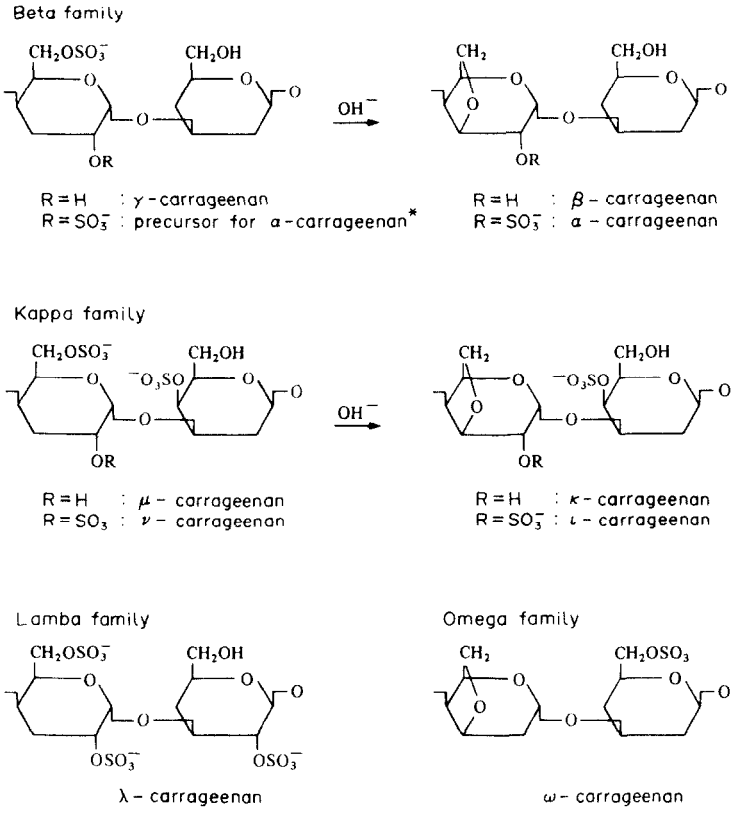
RESULTS

Use of kappa carrageenase to detect kappa carrageenan in hybrid polymer

Hydrolysis of 140 mg of sample R42K2alk (see Experimental) by kappa carrageenase, allows, after running twice on a P2 column, the separation of 9 mg of disaccharide with no contamination by any tetrasaccharide, as seen on TLC. The ^{13}C NMR spectrum of the disaccharide (Fig. 2) was identical to that obtained by Rochas for the DP = 1 oligomer of kappa carrageenan [7].

DEAE fractionation of total carrageenan

No polysaccharidic fraction were obtained on elution by water or with sodium chloride below ionic strength 0.2 M. The quantities of eluted polysaccharides obtained sequentially in the 0.5, 1, 2 and 3 M sodium chloride fractions and their sulphate content are shown in Table 1. It was necessary to boil the DEAE gel for three min to remove polysaccharidic material at an ionic strength above 2 M sodium chloride. In a parallel experiment, verification was made that the DEAE A 25 gel itself did not yield any polysaccharide when eluted under the same conditions. Insolubility of the carrageenan at this ionic strength may be the reason for its retention on the column at 40°. The ^{13}C NMR spectra for the different fractions are shown in Fig. 3. The spectra of fractions 0.5 and 1 M sodium chloride contain 12 peaks assigned to the repeat unit of omega carrageenan [1] and additional peaks with chemical shifts that have been assigned to beta carrageenan [4]. Quantitative estimation of the size of different peaks relative to both structures show that beta carrageenan prevails over omega in the 0.5 M fraction, whereas an inverse ratio is observed in the 1 M fraction. NMR studies of fractions 2 and 3 M show that the former contains pure omega carrageenan, the latter both omega and kappa structures. Detection of kappa with kappa carrageenase confirm the presence of this polymer in the 3 M fraction. Traces of oligosaccharides were detected in the 2 M fraction after hydrolysis with kappa carrageenase. It shows that kappa carrageenan might be present in the 2 M fraction, although in quantities too small to be detected by NMR spectroscopy. Sulphate content increases with ionic strength in the fractions 0.5 to 2 M and then decreases in the 3 M fraction.



*This carrageenan was designated as delta carrageenan [13] and to a structure referred to as deviant iota [14]

Fig. 1. Repeating disaccharides structures of carrageenan and desulphation of the 6-sulphated galactose units into 3,6-anhydrogalactose.

DISCUSSION

No highly sulphated carrageenans, similar to those described as precursors for major carrageenans, could be isolated by the different fractionation procedures used in this study. DEAE fractionation enables the isolation of beta carrageenan, a third representative of this type of molecule, in addition to omega and kappa previously described in the extract from *R. verruculosa*. Whether this beta structure could be a precursor for both omega and kappa structures should be considered. The presence of hybrid gamma, beta, kappa carrageenan in *Eucheuma gelatinae* was demonstrated by Yaphe who suggested that beta could be the precursor for kappa [4]. One approach to the biosynthesis of these polymers is the observation that a carrageenan structure is often bound to its precursor within the same molecule. We were unable to separate beta from omega carrageenan by DEAE fractionation. No beta could be found in the fraction eluted by solutions with ionic strength lower than 0.5 M sodium chloride. One can therefore assume that this neutral polymer is bound to the charged omega carrageenan either by covalent linkage or through electrostatic associations. This suggest the probability of beta and omega carrageenan as being part of the same biosynthetic filiation. The sulphate content of the DEAE fractions increase with ionic strength of elution between

Table 1. Amount of material obtained at different ionic strength by DEAE fractionation of the carrageenan sample R42 from *R. verruculosa*

Fraction (M)	% of total carrageenan before fractionation	% SO_4^*
0.5	6.5	15.0
1.0	16.5	17.0
2.0	14.5	28.1
3.0	48.0	17.9

* Percent relative to total carbohydrate in the fraction.

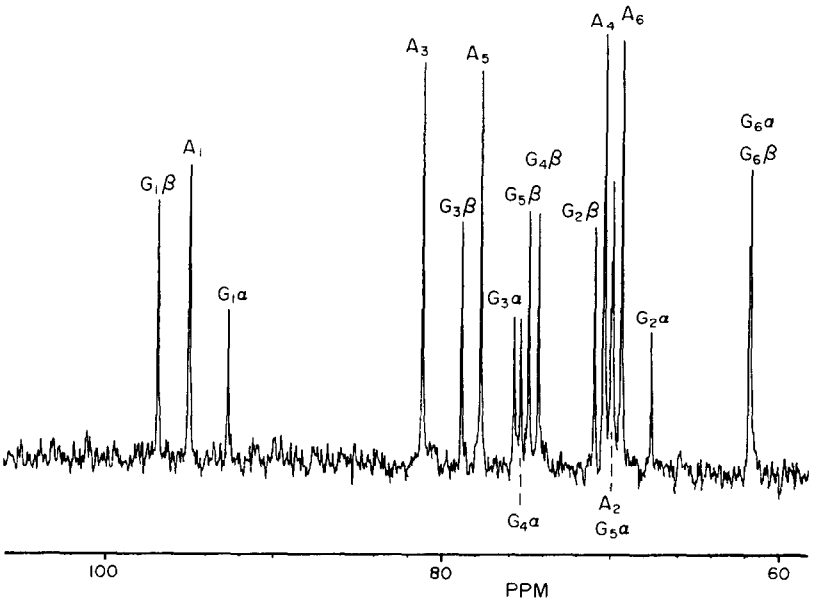


Fig. 2. ^{13}C NMR spectrum of the disaccharide obtained after kappa carrageenase hydrolysis of fraction R42 K2 alk from the carrageenan of *R. verruculosa*. G and A refer to galactose (alpha and beta) and 3,6-anhydro-galactose units, respectively.

0.5 and 2 M sodium chloride, and reflects the decrease in beta content. The sulphation level in fraction 3 M, containing kappa and omega carrageenan, is lower than that in fraction 2 M, containing almost exclusively omega. These structures contain only one sulphate group per disaccharide unit. This would give a 25% theoretical value for sulphate, close to that obtained in fraction 2 M. Desulphation of the polymer in fraction 3 M cannot be detected by NMR and cannot play a significant role in explaining the low sulphate values observed in this fraction. Kappa carrageenan never coexisted with beta in the fractions obtained from DEAE chromatography. The possibility that kappa would be derived from beta is therefore very unlikely.However, the quantity of the polymer precursor to kappa may be very small which could explain why they could not be detected by NMR analysis.

EXPERIMENTAL

Extraction. Sample R42 of *R. verruculosa* (Bert.) J. Ag. was obtained from a tank cultivation experiment as previously described [1]. The carrageenan was prepared by pptn with cetyltrimethylammonium bromide [8], and fractionated by a procedure based on gelation in 0.3 M KCl. The carrageenan samples used in this study were total carrageenan (referred to as R42) and the alkali modified 0.3 M KCl insoluble fraction from R42 (referred to as R42 K2 alk).

DEAE Sephadex A-25 fractionation. The gel was prepared in the Cl form by washing with 0.5 M HCl, 0.5 M NaOH, and 0.5 M HCl and finally rinsed extensively with H_2O. A soln of carrageenan (130 mg) in H_2O (30 ml) was added to 50 ml of gel in a sintered glass funnel. The following concns (M) of NaCl were used sequentially to elute the polysaccharide fractions: 0, 0.2, 0.5, 1.0, 2.0 and 3.0. The temp. of the eluant was maintained at 50°. Appropriate fractions were combined, dialysed and freeze-dried.

Hydrolysis of carrageenan by kappa carrageenase. Carrageenan (0.25% w/v) was dissolved in 0.05 M Na-Pi buffer pH 7.5 and hydrolysed for 24 hr at 30° by excess kappa carrageenase obtained from the bacterium *Pseudomonas carrageenovora* provided by Dr Yaphe. Kappa carrageenan oligomers were desalted and isolated by running the hydrolysate twice on a Biogel P2 column (200–400 mesh, Bio Rad Laboratories). Degradation was monitored by TLC and by the increase in the quantities of reducing sugars.

^{13}C NMR. Proton decoupled ^{13}C NMR spectra (100.62 MHz) were recorded at 80° using a 5 mm dual probe. Chemical shifts (ppm) were measured relative to internal dimethyl sulphoxide (39.6 ppm) and converted into values relative to external TMS.

Analytical measurements. Total carbohydrate was measured by the phenol–H_2SO_4 method using galactose as a standard [9]. Sulphate was determined by a turbidimetric method [10] and reducing sugars by the method of refs [11, 12] using galactose as standard.

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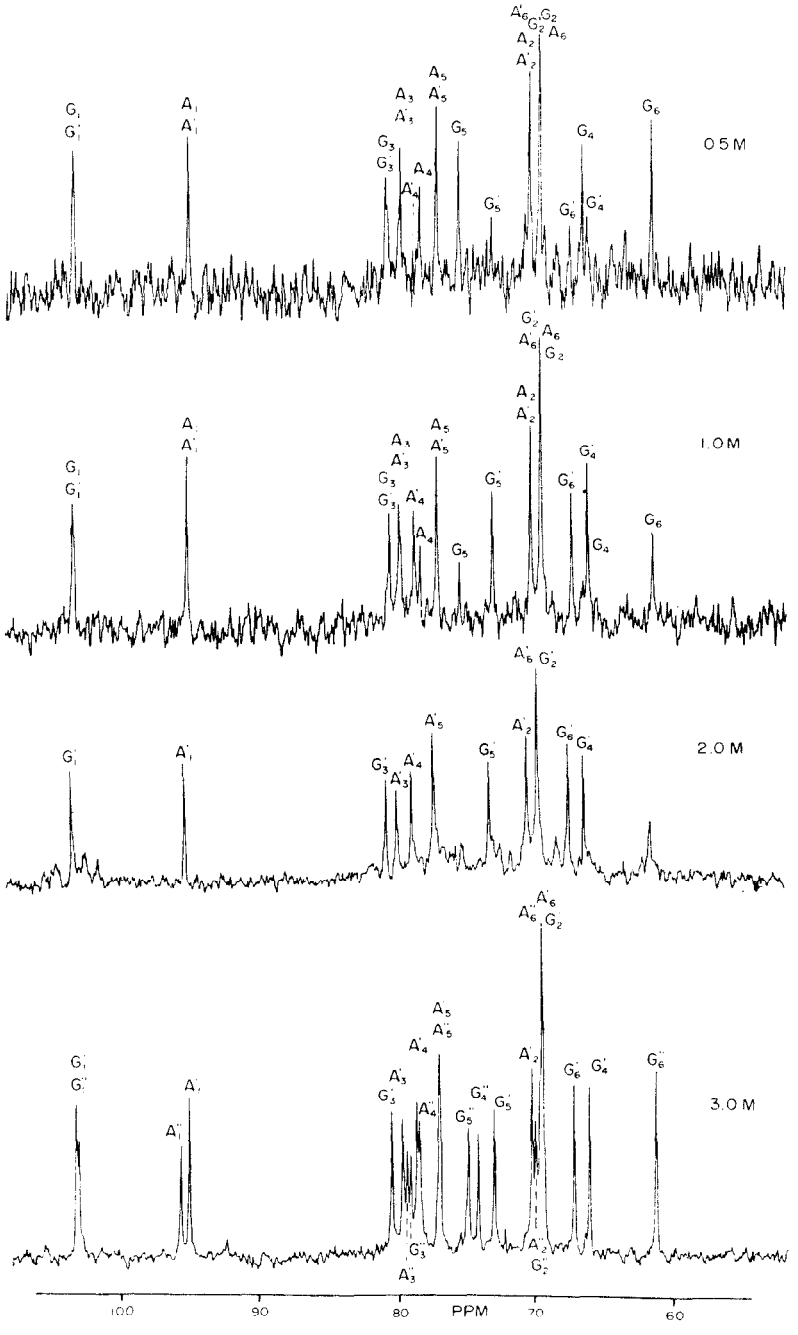

Fig. 3. ^{13}C NMR spectra of fractions obtained at different ionic strength by DEAE fractionation of carrageenan sample R42 from *R. verruculosa*. The following symbols refer to the galactose and 3,6-anhydrogalactose repeat units of beta (A, G), omega (A′, G′) and kappa (A″, G″) carrageenans, respectively.

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