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ANALYSIS OF CARRAGEENAN FROM *Hypnea musciformis* BY USING κ- AND ι-CARRAGEENANASES AND ^{13}C-N.M.R. SPECTROSCOPY*

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ABSTRACT

Carrageenan extracted from *Hypnea musciformis* was analyzed by using κ- and ι-carrageenanases and ^{13}C-n.m.r. spectroscopy. κ-Carrageenanase caused extensive degradation of the polysaccharide, with the production of low-molecular-weight oligosaccharides of κ-carrageenan. Limited degradation of the polymer occurred with ι-carrageenanase, with the virtual absence of low-molecular-weight oligosaccharides. The native carrageenan had a ^{13}C-n.m.r. spectrum characteristic for κ-carrageenan, but, after treatment with κ-carrageenanase, resonance peaks for ι-carrageenan were observed in the spectrum of the enzyme-resistant fraction. The carrageenan from *H. musciformis* is a hybrid, consisting mainly of κ-type repeating units, with minor proportions of ι-type repeating units and other components.

INTRODUCTION

Carrageenans are water-extractable, cell-wall polysaccharides found in certain members of the Rhodophyta. They are composed of a backbone structure of alternating α-D-(1→3)- and β-D-(1→4)-linked D-galactosyl residues[1]. Modification of this fundamental, repeating disaccharide unit, by the addition of ester sulfate or anhydride formation of the 4-linked residue, is the basis for classification of these polysaccharides[2–4]. Carrageenans are classified in two families, based on the sulfation pattern of the 3-linked D-galactosyl residue. The κ-family is composed of carrageenans in which the 3-linked residue is sulfated at C-4 (κ,ι,μ,ν), whereas, in the λ-family, the 3-linked residue is sulfated at C-2 (λ,ξ,π). κ- and ι-Carrageenans (see Fig. 1) contain 3,6-anhydro-D-galactose, which enables the formation of thermo-

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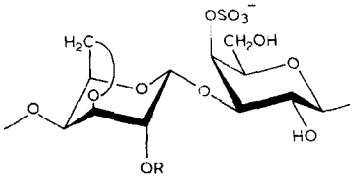

Fig. 1. Disaccharide structures of κ- and ι-carrageenans: R = H, κ-carrageenan, R = SO_3^-, ι-carrageenan.

reversible gels; however, the absence of the anhydride in the other types of carrageenan leads to the formation of viscous solutions that do not gel.

Analysis of carrageenans from different algal sources has revealed the hybrid nature of these polymers, and homopolymers containing only one type of repeating disaccharide may not exist in Nature[2,3,5,6]. The use of bacterial enzymes, specific for the repeating disaccharides of κ- and ι-carrageenan, had greatly facilitated these studies[5]. The enzymic removal of the major type of repeating disaccharide provides a means of enriching for the minor components present in carrageenans, often at too low a concentration to be detected by spectroscopic techniques alone.

The carrageenan from *Hypnea musciformis* had previously been reported as being a κ-carrageenan[7–9]; however, based on sulfate determinations, and infrared spectroscopy, the presence of other components is implicated[8,9]. The purpose of the present work was to analyze the carrageenan from *Hypnea musciformis* by using κ- and ι-carrageenanases, and ^{13}C-n.m.r. spectroscopy.

EXPERIMENTAL

Carrageenan preparation. — *H. musciformis* was collected from St. Lawrence Bay, Barbados, West Indies. Algal fronds were rinsed with water and air-dried. Carrageenan was extracted by the method of Craigie and Leigh[10]. Dry, depigmented algal powder was first extracted with 50mM NaCl for 24 h at 22° (cold extract; not analyzed). The algal residue remaining was extracted with 0.5M $NaHCO_3$ for 2 h at 90°. Following filtration, carrageenan was precipitated with cetylpyridinium chloride (CPC). The CPC-precipitated material was washed, exchanged for the sodium salt, washed, lyophilized, and weighed. The CPC-precipitated material was, by KCl precipitation, fractionated into a KCl-soluble component and a KCl-insoluble component as described[10].

Enzyme preparation. — κ-Carrageenanase was prepared from the cell-free medium of *Pseudomonas carrageenovora* grown in κ-carrageenan from *Eucheuma cottonii* (Marine Colloids, U.S.A.) by the method of McLean and Williamson[11]. A purified ι-carrageenanase was prepared from the cell-free medium of a Gram-negative marine bacterium[5], grown in ι-carrageenan from *Eucheuma spinosum* (CECA, France). Both enzyme preparations gave single protein bands in SDS-poly(acrylamide) gel electrophoresis.

Carrageenan analysis. — For viscosity and reducing sugar assays, carrageenan extracted from *H. musciformis* was dissolved in 0.1M sodium phosphate buffer, pH 7.5, at a concentration of 0.15%.

Viscosity assay. — Carrageenan solution (4 mL) and buffer (0.9 mL) were added to an Ostwald viscometer, and equilibrated at 30°. The reaction was initiated by the addition of diluted enzyme (100 μL), and flow rates were measured until no further change was detected.

Reducing sugar assay. — Carrageenan solution (4 mL) and buffer (0.8 mL) were equilibrated at 30°, and the reaction was initiated by the addition of the diluted enzyme (200 μL). The increase in reducing sugar was determined by ferricyanide reduction[12], using D-galactose as the standard.

Gel chromatography. — The molecular-weight-distribution pattern of native and of enzyme-hydrolyzed carrageenan was determined on Sepharose CL-4B (Pharmacia, Sweden) at 50°. CPC-precipitated carrageenan (2 mg) was dissolved in 50 mM sodium phosphate buffer, pH 7.5, and exhaustively hydrolyzed by the addition of enzyme at 24-h intervals. After incubation for 72 h at 30°, the solutions were lyophilized. The samples were dissolved in distilled water (0.5 mL), and applied to a column (K16/40, Pharmacia, Sweden) with a gel height of 37 cm, equilibrated in 5 mM sodium phosphate buffer, 0.2M NaCl, pH 7.0, and eluted with the same buffer. Fractions were assayed for carbohydrate by the phenol–sulfuric acid method[13], with D-galactose as the standard.

Preparation of enzyme-resistant fraction (ERF). — KCl-insoluble carrageenan (500 mg) was completely hydrolyzed as just described, and the enzyme-resistant fraction was precipitated with ethanol (2 vol.), pelleted by centrifugation, dissolved in distilled water, and the solution dialyzed and lyophilized, and the product weighed.

^{13}C-N.m.r. spectroscopy. — ^{1}H-Decoupled, ^{13}C-n.m.r. spectra were recorded with a Bruker BZH 400/50 spectrometer at 100.62 MHz. Samples (80 mg) were dissolved in D_2O (2 mL) and spectra recorded, at 80°, at a spectral width of 1.2 to 1.5 kHz. Chemical shifts (p.p.m.) were measured relative to internal dimethyl sulfoxide, and converted into values relative to external tetramethylsilane.

RESULTS

Enzymic analysis. — The CPC-precipitated carrageenan from *H. musciformis* constituted 30% of the dry weight of the depigmented algae. Analysis of this material with κ- and ι-carrageenanases by diminution in viscosity and increase in reducing sugar (R.S.) is shown in Fig. 2. κ-Carrageenanase caused a rapid decrease in specific viscosity, accompanied by a rapid increase in reducing sugar. ι-Carrageenanase caused a decrease in viscosity and an increase in reducing sugar, but not to the same extent as with κ-carrageenanase. The CPC-precipitated carrageenan was enriched for κ-carrageenan by KCl precipitation, with 90% being insoluble in 0.3M KCl. Analysis of the KCl-insoluble material with κ- and ι-car-

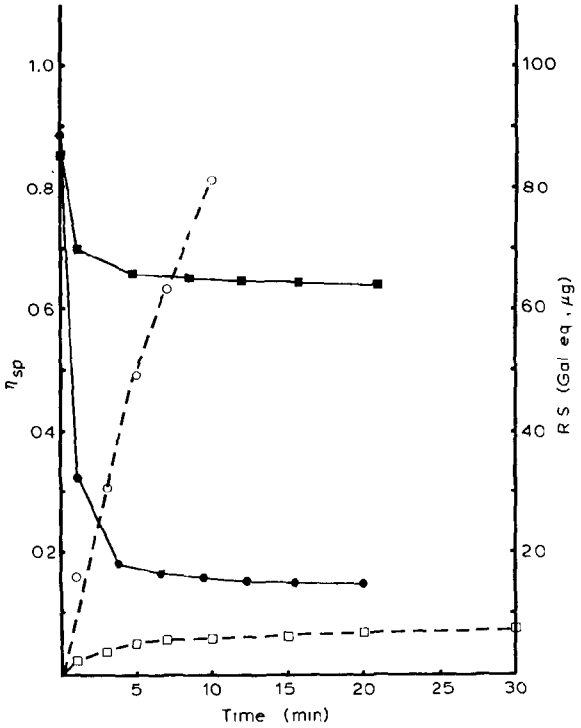

Fig. 2. Enzymic analysis of CPC-precipitated carrageenan. [Effect on viscosity by κ-carrageenanase (●), and ι-carrageenanase (■). Effect on reducing sugar (R.S.) by κ-carrageenanase (○), and ι-carrageenanase (□).]

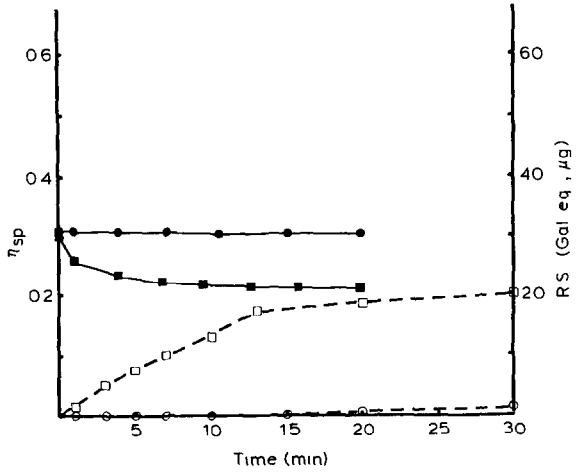

Fig. 3. Enzymic analysis of enzyme-resistant fraction (ERF). (Legend as in Fig. 2).

rageenanases showed essentially the same results as the total, CPC-precipitated material. When the KCl-soluble component was analyzed, diminution in viscosity and increase in reducing sugar were not observed with either κ- or ι-carrageenanase. The KCl-insoluble carrageenan was exhaustively hydrolyzed with κ-carrageenanase, with 19% (ERF) being insoluble in ethanol (2 vol.). Further treatment of the ERF with κ-carrageenanase did not alter the viscosity, or produce an increase in reducing sugar (see Fig. 3). Treatment with ι-carrageenanase resulted in both a decrease in viscosity and an increase in reducing sugar.

Molecular-weight distribution. — Fractionation of native (undigested), CPC-precipitated carrageenan on Sepharose CL-4B at 50° indicated polydispersity, with most of the material being eluted in fractions 30–40, immediately after the void volume (see Fig. 4). Treatment with κ-carrageenanase resulted in the production of low-molecular-weight oligosaccharides (fractions 74–80), principally the di- and tetra-saccharides of κ-carrageenan. A small proportion of the polymer was resistant to κ-carrageenanase, and was eluted over the entire, molecular-weight range of the column. Treatment with ι-carrageenanase caused only limited degradation of the native carrageenan, producing a low concentration of low-molecular-weight material (fractions 74–80). The di- and tetra-saccharides of ι-carrageenan could not be detected in the hydrolyzate. Simultaneous treatment of the carrageenan with κ- and ι-carrageenanase did not significantly alter the elution pattern, as compared to the action of κ-carrageenanase alone.

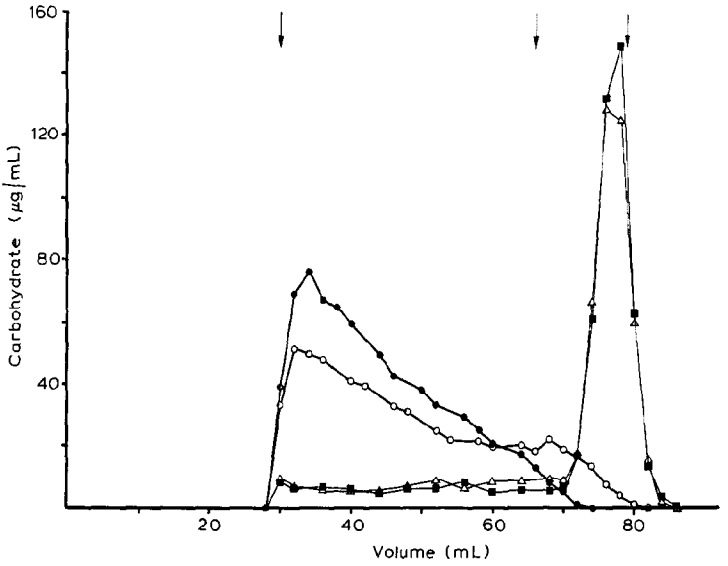

Fig. 4. Molecular-weight-distribution pattern of CPC-precipitated carrageenan. Undegraded (native) polymer (●); κ-carrageenanase hydrolyzate (△); ι-carrageenanase hydrolyzate (○); hydrolyzate after treatment with both enzymes (■). Arrows indicate the elution positions of Dextran 2000 (fraction 30) (mol. wt. 2×10^6), Dextran 10 (fraction 66) (mol. wt. 10^4), and galactose (fraction 79).

	C-1	C-2	C-3	C-4	C-5	C-6
Galactose (G)	102 5	70.0	79.1	74 2	74.8	61.4
Anhydro–galactose (A)	95 4	69 9	79.2	78 3	76 9	69 4

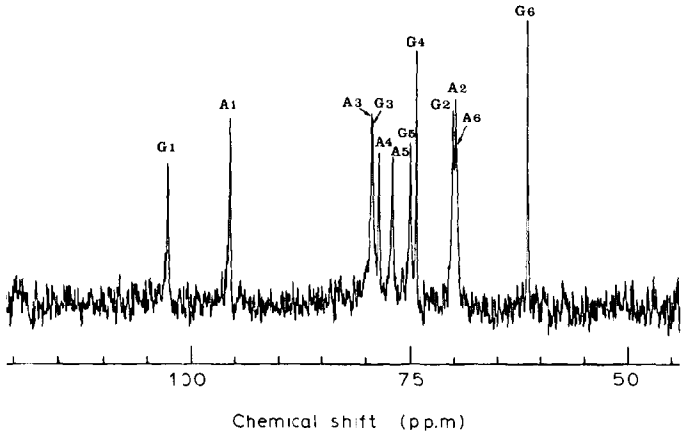

Fig. 5. ^{13}C-N.m.r. spectrum of CPC-precipitated carrageenan.

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra of CPC-precipitated carrageenan (see Fig. 5) and of the KCl-insoluble fraction showed the 12 carbon signals for the repeating disaccharide of κ-carrageenan. After complete digestion of the KCl-insoluble material with κ-carrageenanase, and analysis of the ERF by ^{13}C-n.m.r. spectroscopy (see Fig. 6), the resonance peaks for the repeating disaccharide of ι-carrageenan were observed: C-1 of 3,6-anhydrogalactose 2-sulfate at 92.2 p.p.m., and C-4 of galactose 4-sulfate (adjacent to 3,6-anhydrogalactose 2-sul-

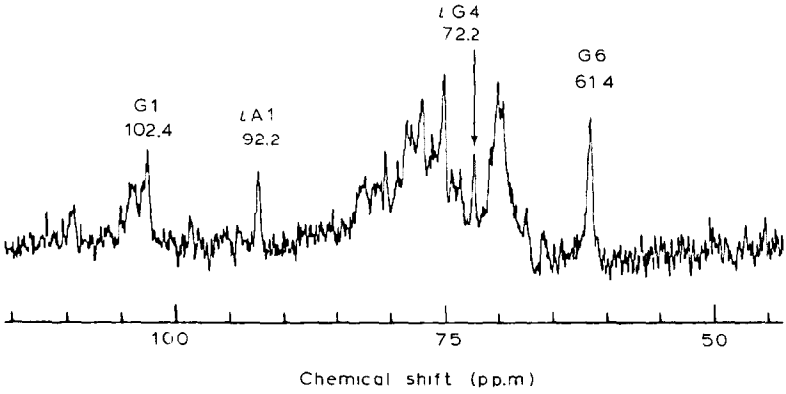

Fig. 6. ^{13}C-N.m.r spectrum of ERF after complete degradation with κ-carrageenanase.

fate) at 72.2 p.p.m. The absence of a peak at 95.4 p.p.m. (C-1 of 3,6-anhydrogalactose in κ-carrageenan) indicated removal of virtually all of the κ-carrageenan component. The spectrum also contained several resonance peaks not attributed to C atoms of ι-carrageenan.

DISCUSSION

The carrageenan from *H. musciformis* was initially identified as a κ-carrageenan, because degradation with κ-carrageenanase proceeded at the same rate as with commercial κ-carrageenan[7]. The infrared spectrum also indicated that the major component was κ-carrageenan[8]; however, there was a minor peak at 805 cm^{-1}, characteristic of ι-carrageenan repeating units. The present results using κ- and ι-carrageenanase, clearly demonstrate the presence of ι-carrageenan. ι-Carrageenanase lessened the viscosity, produced a limited proportion of reducing sugar, and affected the molecular-weight-distribution pattern on Sepharose CL-4B. Because no ethanol-soluble, low-molecular-weight oligosaccharides were observed after complete degradation with ι-carrageenanase, the data suggest that ι-carrageenan is present as a minor component, organized in short sequences within the polymer. The inability of either enzyme to attack KCl-soluble material suggests the presence of components other than κ- and ι-carrageenan. The presence of other components in the CPC-precipitated material was also suggested by the molecular-weight-distribution properties after enzymic hydrolysis. After complete degradation by both κ- and ι-carrageenanase, some material was resistant to the enzymes, and was eluted in the high-molecular-weight range of the column.

The importance of specific removal of major repeating-units by enzymic degradation is clearly an advantage in fine structural analysis of these polysaccharides. The ERF produced after complete degradation with κ-carrageenanase demonstrated the presence of ι-carrageenan repeating units. The ^{13}C-n.m.r. spectrum (see Fig. 6) of the ERF contained two new peaks, at 92.2 and 72.2 p.p.m. These two resonance signals are respectively due to C-1 of the 4-linked residue (92.2 p.p.m.) and C-4 of the 3-linked residue (72.2 p.p.m.) of ι-carrageenan[4,6,14] (see Fig. 1). These peaks are not observed in the spectrum of the starting material (see Fig. 5), as the material responsible is present at low concentrations and gives signals below the resolving power of the instrument. The ^{13}C-n.m.r. spectrum of the ERF also indicates the presence of other components. Several minor signals are observed in the regions of the spectrum that have been attributed to precursor-type carrageenans of the μ or ν type[15], and desulfated, κ-carrageenan-type repeating units may also be present.

Previous analysis of the carrageenan from *H. musciformis* showed that the amount of ester sulfate was lower than the predicted value for κ-carrageenan[8,9]. The demonstration of the presence of ι-carrageenan implies that sulfate values should be higher than those found in κ-carrageenan alone. In the present work, several fractions of polysaccharide were obtained that failed to be precipitated with

CPC, indicating a very low charge-density; however, these were not analyzed. This "anomaly" reflects the need for more-rigorous techniques for the fractionation and analysis of carrageenans. The use of enzymes specific for the κ- and ι-carrageenan fractions has facilitated analysis of the minor components, which are often present at concentrations too low to be detected by spectrometric techniques alone. The use of these enzymes also lessens the need for tedious, chemical analysis. Enzymic and ^{13}C-n.m.r.-spectral analyses of the carrageenan from *H. musciformis* indicated a hybrid polymer composed primarily of κ-carrageenan, with minor components of ι-carrageenan and, possibly, other carrageenan-type components. Analysis by this technique of all carrageenans produced by algae (thus far studied) has revealed the hybrid nature of these polymers. This is an important consideration that must be accounted for when studying the physical properties of carrageenans.

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