J. Pharm. Pharmacol. 1984, 36: 808–813
Received April 2, 1984

Structure-activity in the carrageenans: iota-carrageenan and experimental oedemagenic activity

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The oedemagenic activities of several iota-carrageenans from *Eucheuma spinosum* have been compared using the rat hindpaw model. From the parent ι-carrageenan of weight average molecular weight 541 100 an acid-degraded ι-carrageenan ($\overline{M}_W$ = 20 300) was obtained which on fractionation yielded five ι-carrageenans $\overline{M}_W$ ranging from 73 700 to 4 600. The oedemagenic activity of ι-carrageenan resided in the fraction of $\overline{M}_W$ = 73 700, the parent undegraded high molecular weight ι-carrageenan being no more active and fractions of lower molecular weight being inactive. Fractionation was based on differential solubilities of the barium salts of the fractions in water and aqueous ethanol and was probably determined by aspects of primary structure in addition to molecular weight.

Carrageenan-induced inflammation in the rat hindpaw is characterized by oedema formation (Winter et al 1962) and is of use in the development of anti-inflammatory drugs. For this purpose the carrageenans are often referred to as single substances even though what is used may be an extract of one or more unnamed seaweeds, κ- and λ-carrageenans, respectively the KCl-insoluble and soluble fractions of aqueous extracts of certain Rhodophyceae are also used, but this fractionation by itself does not ensure a single or even constant product. Botanical source and development stage at harvesting can also contribute to the characteristics of the constituents, in addition to inevitable macromolecular polydispersity. Chemical and probably also activity differences can therefore occur among materials used under the carrageenan family name. Apart from quantitative comparisons yielding ranked orders of activity, the nature of difference in oedemagenic activity of different carrageenans is unknown, a fact to be considered alongside incomplete knowledge of mechanism in experimental inflammation.

Chemical differences between specific carrageenans can now be described with some precision (Rees & Welsh 1977) and differences in biological activity between different carrageenans have been found in a number of systems.

Part of a structure-activity study, this paper considers the relationship between oedemagenic activity in the rat hindpaw and the nature of certain ι-carrageenans. ι-Carrageenan has been chosen because it is highly sulphated, and, without removing sulphate, can be gently split into fragments separable according to molecular weight and structure. A variety of fragments of the interrupted repeating carrabiose sequence which constitutes ι-carrageenan is therefore available to probe experimental inflammation.

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MATERIALS AND METHODS

Carrageenans

Undegraded ι-carrageenan was a roller-dried hot aqueous extract of *Euchema spinosum*. Degraded ι-carrageenan was prepared from this extract by treatment with hydrochloric acid in the presence of acetone at room temperature (20 °C) to predetermined viscosity of the resultant degraded ι-carrageenan. The mixture was neutralized with sodium hydroxide to pH 7·2, dialysed against water and freeze-dried (Anderson & Hargreaves U.S. Patent 3,175,942).

Fractionation of degraded ι-carrageenan. Conversion to barium carrageenate was effected by neutralizing the filtrate from a mixture of the degraded ι-carrageenan and Amberlite IR-120(H) resin with saturated barium hydroxide solution to pH 7·2 followed by freeze drying. The barium salt was fractionated by adapting a method described by Ayotte et al (1980) for fractionating heparin and based on the differing solubilities of the fractions as barium salts in aqueous ethanol (ethanol concentration 0–50% v/v). Fractions were recovered by centrifugation at up to 35 000 rev min^{-1}. Conversion of the fractions to water-soluble sodium salts was accomplished using

Amberlite IR-120(H) and precise neutralization of the free acid form of the polysaccharide sulphate to pH 7·2 using 0·1 M and, near the end point, 0·001 M NaOH followed by freeze-drying. Reagents were AR quality.

3,6 anhydro-D-galactose content of the carrageenans was determined by the method given by Craigie & Leigh (1978) using 1 ml sample + 5 ml reagent and standard methyl 3,6 anhydro-D-galactoside.

Infrared spectra were obtained using a silver chloride window to support a film of carrageenan.

Optical rotation was determined at 1% w/v in water using a Bellingham & Stanley Pepol 60 spectropolarimeter, at room temperature.

Inherent viscosity was determined using a suspended level U-tube viscometer. $\eta_{inh} = c^{-1} \ln (t_{soln}/t_{solv})$ where c = g solute in 100 ml solution; t_{soln}, t_{solv} = respectively time(s) of flow of solution and solvent (0·1 M NaCl), at 25 °C.

Sulphur content was determined by Mrs M. Adams, Department of Chemistry, University of Strathclyde (oxygen flask method with barium perchlorate titration).

Molecular weight was determined at room temperature in 0·1 M NaCl using a KMX-6 low angle light scattering photometer; differential index of refraction was found using a Bryce-Phoenix differential refractometer. Weight average molecular weight was calculated by extrapolating the plot of $Kc/R(\theta)$ vs concentration to infinite dilution when the intercept is a function of $\overline{M}_W^{-1}$. $R(\theta)$ is the excess Rayleigh factor; $K = [2\pi^2 n^2/\lambda^4 N](dn/dc)^2(1 + \cos^2 \theta)$; c is concentration, $g.ml^{-1}$; N is Avogadro's number; λ is wavelength in vacuo; dn/dc is the differential index of refraction; θ is the angle of scatter measurement. Equipment, courtesy of Dr R. W. Richards, Chemistry Dept.

Rat paw swelling. Carrageenan oedema was induced in male Wistar rats, 250–350 g, by a single subplantar injection into the left hindpaw of a solution of the carrageenan in 0·9% NaCl (saline) (0·1 ml; 10 mg ml^{-1}). The right paw served as control (0·1 ml saline). Paw swelling was monitored as percentage change in dorso-ventral paw thickness which was measured immediately before, and at hourly intervals after the injection. Percentage increases in paw thickness were calculated for test and control paws. Allowance was made for the non-specific effect of the saline injection by subtracting the value obtained for the control saline-injected paw from that of the test paw at each time of measurement (Al-Haboubi & Zeitlin 1983). A barium chloride oedema control was included using 0·1 ml $2·6 \times 10^{-4}$ M $BaCl_2$ (twice the highest concentration found in any fraction); negligible swelling was observed.

Statistics. Differences between groups were compared using the Mann-Whitney U-test (significance, $P \leq 0·05$). Because sequentially related time-course data are not independent, analysis of paired data for control and test groups is legitimate only under certain restricted conditions. One way of analysing these data is to compare areas under the time-course curve (Lesser et al 1980). Areas under the curve were calculated using a microcomputer implementation of the standard formula for the area of an irregular polygon, and the results (Fig. 2b) are expressed as 'square' units (response·hours).

RESULTS AND DISCUSSION

ι-Carrageenan is an alternating copolymer of D-galactose 4-sulphate and 3,6 anhydro-D-galactose 2-sulphate (carrabiose) together with interrupting, 'kinking', 6-sulphated and 2,6-disulphated galactose residues (Anderson et al 1973). These kinking residues are believed (Rees & Welsh 1977) to control cross-linking and hydration of the chains in which they occur. They could therefore influence behaviour in the present fractionation, the results of which suggest that separation is based not only on molecular weight but, perhaps predominantly, on the possible existence of domains in the molecule that have variations in primary structure which have been exposed by acid degradation, and so facilitate formation of barium salts with differing solubilities in water and aqueous ethanol. The corresponding water-soluble sodium salts, introduced into biological systems, might be expected to have differing capacities for interaction with molecules in-vivo and hence differing activities.

Carrageenan fractions

Data are in Table 1. In barium salt form, fraction 1 is water-insoluble, fractions 2–4 are insoluble in increasing ethanol concentrations and fraction 5 is the remainder. The sodium salts of all fractions are freely soluble in water. Clearly, about 75% of the fractions (2 and 3) have a molecular weight (~18 000) close to that of the parent degraded ι-carrageenan (20 300). Despite the similar molecular weights of fractions 2 and 3 they require different ethanol concentrations for precipitation and have different optical rotations, suggesting structure differences. Of fractions 4 and 5, also of similar molecular weight (~5000), one is soluble, the

Table 1. Chemical data for ι-carrageenans.

ι-Carrageenan	Yield of fraction %*	Ethanol, %, for pptn. of fraction as Ba salt	3,6 Anhydro-galactose, % w/w	Molecular weight, $\bar{M}_w$	η_{inh}, dl.g^{-1}	$[\alpha]_D^{\theta}$	-SO_3Na, %
Undegraded	—	—	16·1	541 100	3·52	+46	30
Degraded	—	—	17·0	20 300	0·27	+43	33
Fraction 1	9·8	0	21·6	73 700	0·71	+47	33
2	53·2	12	19·1	17 800	0·27	+54	34
3	22·6	25	12·7	18 000	0·24	+20	31
4	8·5	50	11·1	5 800	0·11	+15	33
5	6·0	—	15·3	4 600	0·05	+33	37

* Percentage of total yield of all fractions.

other insoluble, in 50% ethanol; these also have different optical rotations and probably also differences in structure. Seemingly, the fractionation has been influenced by both molecular weight and polysaccharide structure. Thus (Table 1) fractions 3 and 4 have least 3,6-anhydrogalactose (on a weight basis) and lowest optical rotations. Optical rotation difference can reflect helix-coil transition (McKinnon et al 1969) and is influenced by molecular shape (Rees et al 1970). Representative ir spectra (Figs 1A–C) are characteristic for ι-carrageenans (Craigie & Leigh 1978) each having a definitive peak at 805–810 cm^{-1} (3,6-anhydrogalactose 2-sulphate) which was least pronounced for fraction 5. Peak absorbance ratio 810/850 decreased for fractions 1–5; that for 810/940 was least for fraction 5. 850 cm^{-1}, 940 cm^{-1} peaks represent axial secondary 4-sulphate and 3,6 anhydrogalactose respectively.

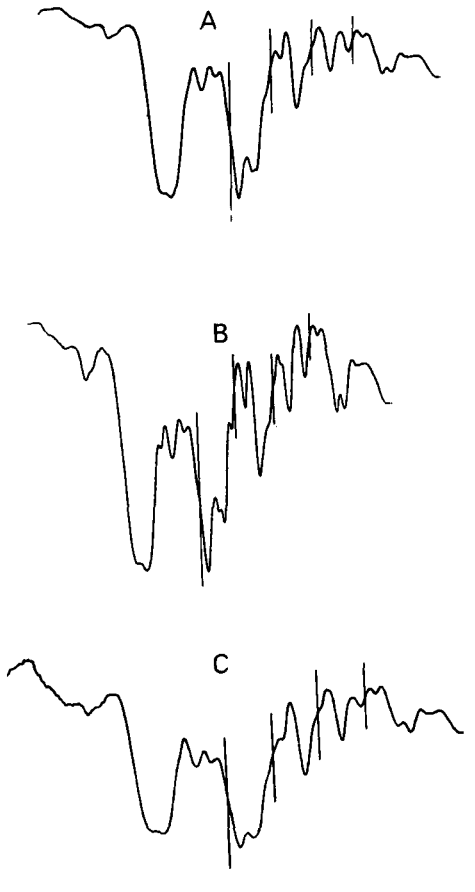

FIG. 1. Infra-red spectra of ι-carrageenans. Vertical lines mark wave numbers (r. to l.): 800, 900, 1000, 1100 cm^{-1} respectively. A, degraded ι-carrageenan; B, fraction 1; C, fraction 5.

Fraction 1 yields an ir spectrum which shows most strongly the characteristics of ι-carrageenans from *E. spinosum* including the as yet unidentified peak at 975 cm^{-1}; also it has most 3,6-anhydrogalactose (on a weight basis), highest molecular weight of the fractions, is that part of the whole molecule apparently most resistant to acid degradation, and appears to carry the oedemagenic activity of ι-carrageenan. Gel permeation chromatography reveals that it has a molecular weight distribution capable of further fractionation. All ι-carrageenans had ≥30%-SO_3Na suggesting that where low 3,6-anhydrogalactose 2-sulphate occurred as indicated by a weak ir peak, the sulphate might occur in such fractions as 6-sulphated or 2,6-disulphated galactose which may (Lawson et al 1973) replace 3,6-anhydrogalactose.

Since the 3,6-anhydrogalactose content of degraded ι-carrageenan is similar to that of undegraded ι-carrageenan, the acid degradation has not significantly lowered the actual content of that sugar. It is the presence of that sugar which facilitates gentle sulphate-conserving acid degradation, possibly with the sacrifice of a small number of units.

Carrageenan oedema of the rat hindpaw (Winter et al 1962)

This has become an inflammation model which is the basis of a widely used assay for anti-inflammatory

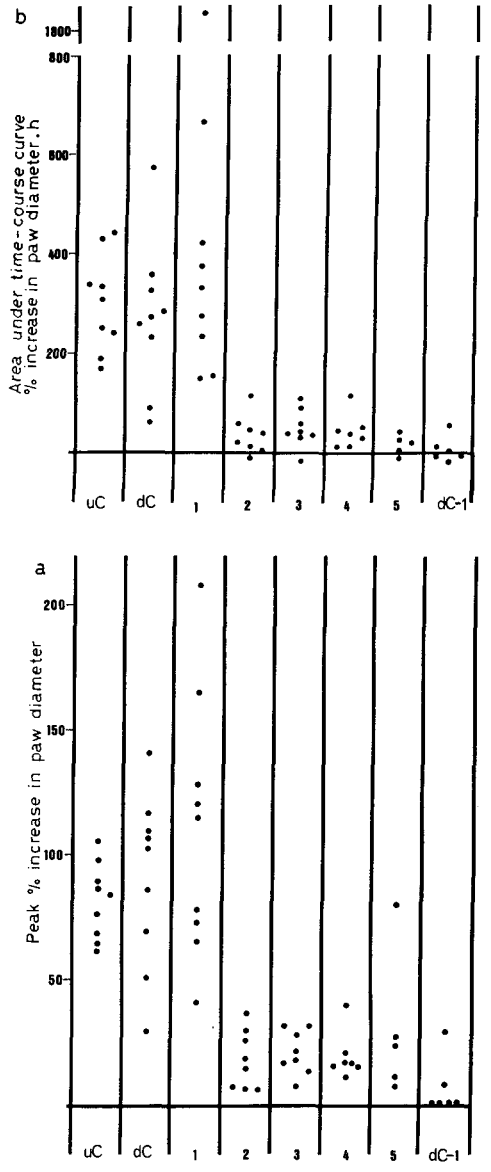

FIG. 2. (a) Oedemagenic activities of ι-carrageenans; peak percentage increase in paw diameter during 6 h after injection. Each point represents one result from one rat. All values have been corrected for non-specific swelling (see methods). uC, dC are undegraded, degraded ι-carrageenans respectively; 1–5 are fractions; dC-1 is degraded ι-carrageenan from which fraction 1 has been removed (residue after isolation of fraction 1, Na salt form). (b) As (a) except that each point represents the area (% increase in paw diameter.h) under the time-course curve of percentage increase in paw diameter for each animal.

drugs. Quantitative differences in activity between κ- and λ-carrageenans have been observed for acute (Atkinson et al 1962) and chronic (McCandless 1965) inflammation. Carrageenan oedema is a monophasic

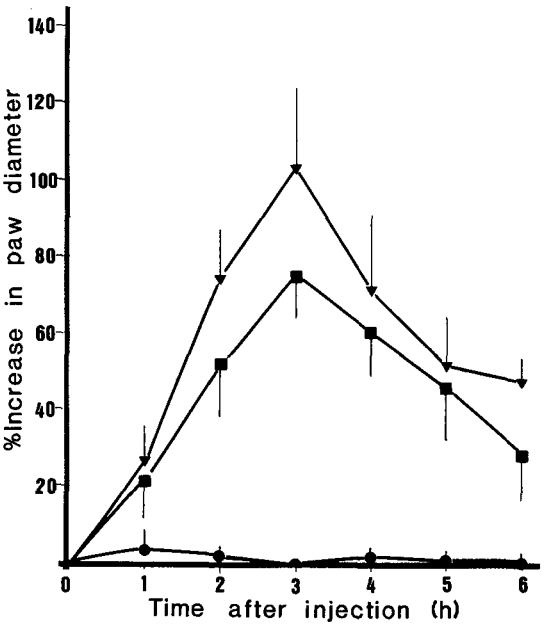

FIG. 3. Oedemagenic activities of ι-carrageenans: time-course curves for ▼, fraction 1; ■, degraded ι-carrageenan; ●, degraded ι-carrageenan from which fraction 1 has been removed (see also legend of Fig. 2a).

response (Van Arman 1979; Al-Haboubi & Zeitlin 1983). Denial (Winter et al 1963; Van Arman et al 1965; Vinegar et al 1969) of a role for histamine in carrageenan oedema formation must now be reconsidered in view of its partial suppression by an H_2 receptor antagonist (Al-Haboubi & Zeitlin 1983). Suppression of the oedema in complement-depleted rats (Di Rosa et al 1971) suggested a mediatory role for local complement activation. Plasma kallikrein has been shown (Van Arman et al 1965; Di Rosa & Sorrentino 1968; Crunkhorne & Meacock 1971; Thomas & Zeitlin 1983) to be involved and may be activated directly, and the complement system indirectly, as a result of Hageman factor activation; this may provide one possible mechanism for carrageenan activation of these systems. Hageman factor may be activated by contact with negatively charged surfaces (Kaplan et al 1982) and centres of strong negative charge are copiously provided by the injected carrageenan. However, molecular conformation, presence of interacting substances and conditions such as ionic strength can determine the effectiveness of the charges in any system.

Structure-activity

Fractions 2 and 3 ($\overline{M}_W \sim 18\,000$) and 4 and 5 ($\overline{M}_W \sim 5000$) had low, inconsistent oedemagenic activity

Table 2. Probabilities of difference in oedemagenic activity of ι-carrageenans (f = fraction).

	Undegraded	Degraded	f1	f2	f3	f4	f5
			a. Area under time-course curve				
Undegraded	—	—	—	—	—	—	—
Degraded	>0·05	—	—	—	—	—	—
Fraction 1	,,	>0·05	—	—	—	—	—
2	<0·01	<0·01	<0·01	—	—	—	—
3	,,	,,	,,	>0·05	—	—	—
4	,,	,,	,,	,,	>0·05	—	—
5	,,	,,	,,	,,	,,	>0·05	—
Degraded minus fr. 1	,,	,,	,,	,,	,,	,,	>0·05
			b. Peak percentage increase in paw diameter				
Undegraded	—	—	—				
Degraded	>0·05	—	—				
Fraction 1	,,	>0·05	—				
2	<0·01	<0·01	<0·01	——— as in a. above ———			
3	,,	,,	,,				
4	,,	,,	,,				
5	<0·05	<0·05	<0·05				
Degraded minus fr. 1	<0·01	<0·01	<0·01				

(Fig. 2); fraction 1 ($\overline{M}_W$ 73 700) had marked activity which was at least as great as that of degraded ι-carrageenan, in which it is present in admixture with the other fractions, and that of undegraded ι-carrageenan in which it is present in covalent linkage with the other fractions. Removal of fraction 1 from degraded ι-carrageenan resulted in loss of activity (Fig. 3 and Table 2). Seemingly then, fraction 1 possesses the oedemagenic activity of ι-carrageenan, determined both as total inflammatory response (area under the time-course curve, Fig. 2b) and as maximal response reached during the first 6 h (Fig 2a). The maximal response was reached in all instances between 3 and 5 h after injection of carrageenan. Thus ι-carrageenans of molecular weight 5000–18 000 were virtually inactive despite containing as much sulphate (≥30%-SO_3Na) as the highly active fraction 1 ($\overline{M}_W$ 73 700) and undegraded and degraded ι-carrageenans ($\overline{M}_W$ 541 100; 20 300 respectively). Thus for a certain minimum sulphate content, molecular weight dependence of oedmagenic activity of ι-carrageenans is not simple.

Were ι-carrageenan simply a carrabiose polymer with regular or even random inclusions of kinking residues there might be greater reason to expect that molecular weight dependence of biological activity would be simple. This is not so; the randomness of the kinking residues cannot be assumed and indeed variations in their distribution have been found for different sulphated polysaccharides (Lawson et al 1973). Since these residues influence hydration and molecular shape they may be expected to influence capacity for interaction with macromolecules in-vivo, hence their distribution in a molecule could influence its biological activity. Size and charge of the carrageenans could determine overall interaction capacity whilst specificity, holding over a range of sizes, could be influenced by these aspects of primary structure. The three active carrageenans have similar optical rotations which could reflect structural similarities.

Molecular shape or conformation could be an important determinant of activity, but little can presently be said about the fractions in this context. However, it is noteworthy that fragments of ι-carrageenan are known (Rees & Welsh 1977) to form helical structures as do the undegraded molecules. Conformational agility is always important because the smallest molecule large enough to adopt a suitable form and having adequate effective charge density will have greatest post-injection mobility and easiest access to interacting sites or molecules in-vivo.

Sulphated polysaccharides

Differing biological activities of high and low molecular weight sulphated polysaccharides have been established for carrageenans (Anderson & Duncan 1965; Anderson & Harthill 1967; Anderson & Soman 1967) and heparins (Laurent et al 1978). For heparins of differing structure and source, differences in activity for similar molecular weights have been observed (Anderson & Harthill 1981), and alternative fractionation (Ayotte et al 1981) revealed a structure dependence of anticoagulant activity to a degree independent of molecular weight. Of course, molecular weight and primary structure cannot be entirely divorced. Haemostasis and its reversal

depend on many factors, and degrees of specificity of interaction could conceivably be deployed over a number of heparins functioning separately in interdependent stages in-vivo; the concept of heparin functioning as a single substance in-vivo is now quite untenable. Correspondingly, enough evidence is now to hand for this other family of sulphated polysaccharides, the carrageenans, amongst which anticoagulant activity is also to be found, to justify a more intensive search for structure-activity relationships in equally complex in-vivo systems where protease cascades could be involved, in order to develop structure-activity relationships for this class of substances. This work provides an example of ι-carrageenans distinguished from each other by possessing, or not possessing, significant biological activity as opposed to merely displaying quantitative differences. Also one of these ι-carrageenans of about one-tenth the molecular weight hitherto used as an oedemagenic stimulus, has been shown to possess the activity of the whole molecule. Clearly the isolation method has ensured possession of regions of primary structure which by themselves or through an effect on molecular shape, ensure interaction with in-vivo molecules responsible for activation of inflammation. Such molecules provide probes for studying inflammation (including colonic ulcerogenesis), haemostasis and other systems in which polyelectrolytes have a profound effect.

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