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Characterisation of kappa- and iota-carrageenan coils and helices by MALLS/GPC

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Iota- and kappa-carrageenans were degraded by ultrasonication to obtain samples of different molecular weights. The coil and the helix conformations of the degraded and non-degraded samples were investigated by multi-angle laser light scattering (MALLS) coupled to gel permeation chromatography (GPC), and the weight-average molecular weight, M_w, as well as the z-average radii of gyration, $R_{g,z}$, was obtained for all samples in both conformations. The conformational state was controlled by temperature and/or electrolyte content. All measurements were done under non-aggregating conditions, and an approximate doubling of the weight-average molecular weight was seen for both iota- and kappa-carrageenan helices over the entire molecular weight range studied, implying a double-helix formation. Zimm plots for non-degraded kappa-carrageenan coils and helices confirmed the approximate doubling of the molecular weight for the helix conformation.

INTRODUCTION

Carrageenans are linear sulfated polysaccharides extracted from many species of red seaweeds (Rees *et al.*, 1982; Painter, 1983). The backbone is based on a repeating disaccharide sequence of β-D-galactopyranose residues linked glycosidally through position 1 and 3, and α-galactopyranose residues linked glycosidally through position 1 and 4 (Fig. 1). The carrageenans are well known for their gel forming properties, and are used extensively in the food industry as gelling agents.

Normally, the gelation of a carrageenan solution is induced by cooling a hot solution containing certain gel-inducing cations. The gel formation involves a conformational transition of the carrageenan molecule according to the following scheme.

$$\text{coil} \rightarrow \text{helix} \rightarrow \text{gel}. \qquad (1)$$

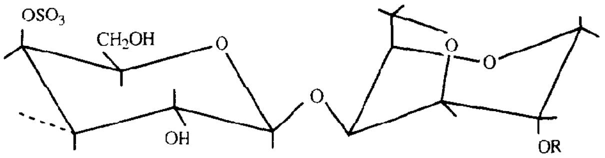

Fig. 1. Primary structure of carrageenans, R=H and SO_3^- for kappa- and iota-carrageenan, respectively.

While the detailed mechanism of gelation is still under debate, the majority of researchers agree that helix formation is required. Under gelling conditions, the helix formation thus has to lead to an association between the polymer strands, but there must also be a branching, so that an infinite three-dimensional network is formed.

The network formation of a helix-forming polymer may occur on two different levels (Piculell *et al.*, 1994; Viebke *et al.*, 1994). In the case of a multiple helix formation, association and branching could occur on the helical level, through incomplete formation of multiple helices such that each network strand joins in helices with two or more other strands (Higgs & Ball, 1989). This is the currently accepted model for gelatin gels (Djabourov *et al.*, 1988). The other possibility is that the network is formed on the superhelical level, by the aggregation of fully developed helices. This type of mechanism has been suggested (Miller *et al.*, 1978; Shukla, 1992) for the gelation of the synthetic polypeptide poly(γ-benzyl α, L-glutamate). Note that the cold-set gelation of poly(benzyl glutamate) does not involve a coil–helix transition; the molecule is always in the helical form, both in solution and in the gel.

Both types of mechanisms have been suggested for the gelation of carrageenans (Rees *et al.*, 1969; Rees & Welsh, 1977; Smidsrød, 1980; Rochas & Rinaudo, 1984), and more complex mechanisms, involving both branching at the (double) helical level and superhelical

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aggregation (Morris *et al.*, 1980), have also been proposed. In a recent study by our group (Viebke *et al.*, 1994), it was concluded that there is no significant formation on the helical level for kappa-carrageenan, but that the network formation essentially occurs on the superhelical level, by helix aggregation. These conclusions were based on both experimental evidence and on theoretical analyses of the coil–helix transition. The experiments utilised the important finding (Grasdalen & Smidsrød, 1981) that kappa-carrageenan helices, under certain salt conditions, may be stable in solution, without forming gels. It was shown that the transition between the solution and gel states of kappa-carrageenan could be induced isothermally and reversibly, by dialysis against different salts, under conditions where the molecules were always helical. The theoretical analysis (assuming double helix formation) indicated that the coil-to-helix transition was so co-operative that the occurrence of two separate helical regions on the same polymer (a requirement for network formation) was very unlikely.

The issue of the mechanism of gelation of the carrageenans is complicated by the seemingly contradictory evidence regarding the very nature of the carrageenan helix. Many studies by a variety of techniques, such as light scattering (Jones *et al.*, 1973; Norton *et al.*, 1983*a*; Austen *et al.*, 1985), osmometry (Jones *et al.*, 1973) and stopped flow polarimetry (Norton *et al.*, 1978, 1983*b*; Austen *et al.*, 1985, 1988) support the original proposal (Rees *et al.*, 1969) of a bimolecular helix. Nevertheless, there is also strong evidence of the existence of monomolecular species under certain (dilute) conditions, at temperatures and salt contents where the helical conformation is normally stable. The earliest evidence in support of this came from studies by osmometry and light scattering from the Trondheim group (Smidsrød *et al.*, 1980; Grasdalen & Smidsrød, 1981; Smidsrød & Grasdalen, 1984). The more recent extensive studies by the Leuven group clearly confirm these observations. Slootmaekers *et al.*, (1988) made an extensive study of the polyelectrolyte behaviour of kappa-carrageenan in NaI and NaCl as a function of the ionic strength. The study was done both under coil and helix conditions, and no doubling of the molecular weight could be seen for the helix. A conformational transition was still indicated however, since the observed radii of gyration were higher under conditions favouring the helical state. Recently, Vanneste (1993) studied the static light scattering of iota-carrageenan in dilute (0·05–0·04 g/l) solutions. She used both temperature (25 or 45°C) and added salt (0·02–0·09 M NaCl, NaI or LiCl) to control the conformational state of iota-carrageenan, and the light scattering measurements were made using both multi-angle and low-angle techniques. A most significant finding of Vanneste was that the reduced scattering intensity of iota-carrageenan, under conditions favouring helical conformation, went through a minimum at a polymer concentration around 0·2 g/l. This corresponds to a decrease in the apparent molecular weight with decreasing concentration. The limiting value of the scattering intensity was found to correspond to a molecular weight of a single chain of iota-carrageenan. Extrapolations from concentrations above the minimum in the scattering intensity, however, were found to lead to higher (up to a factor of 2) molecular weights.

Very recently, the finding of cyclic and hairpin-like structures in electron micrographs of iota-carrageenan under helical conditions have provided a possible clue to the conflicting results regarding the number of molecules in the carrageenan helix (Stokke *et al.*, 1993). Indeed, these structures are compatible with a local double-helical conformation generated intramolecularly, in an antiparallel (hairpin) or a parallel (cyclic structure) fashion. The intramolecular helices should be favoured for long chains, and at low concentrations. Therefore, it seems important to study the helix formation not only of intact carrageenans, but for degraded materials with a range of molecular weights. This is one of the main objectives of the present study, where static light scattering measurements are performed on samples of different degrees of degradation, obtained by ultrasonic treatment for varying lengths of time. Both iota- and kappa-carrageenan are studied, and in both conformations. Most of the analyses are performed using multi-angle laser light scattering (MALLS) coupled with gel permeation chromatography (GPC). The chromatographic separation provides an additional important test of the molecular dispersity of the sample and eliminates errors due to the possible presence of small fractions of highly aggregated molecules. (It should be remembered that non-negligible amounts of structural impurities are found in most carrageenan preparations (Bellion *et al.*, 1981; Rochas & Heyraud, 1981; Piculell *et al.*, 1989; Piculell & Rochas, 1990).) Previously published GPC analyses of carrageenan, with or without light scattering detection, have been restricted to carrageenans in the coil state (Ekström *et al.*, 1983; Ekström, 1985; Lecacheux *et al.*, 1985; Slootmaekers *et al.*, 1991).

From the MALLS/GPC analysis in this study we invariably find, for all degrees of degradation of both types of carrageenan, that the molecular weight of the helical state is very close to twice that of the coil state. The implications of these findings, and the findings of previous investigators, are discussed at the end of the paper.

MATERIALS AND METHODS

Samples

Kappa- (from *Euchema cottonii*) and iota- (from *Euchema spinosum*) carrageenan were gifts from Sanofi Bio-Industries. Dextran standards were supplied by Pharmacia (Uppsala, Sweden) and Polymer Standard

Service (Mainz, Germany). The carrageenan samples were converted to the pure sodium forms by pouring a hot carrageenan solution through an ion-exchange column at elevated temperatures. The depolymerisation of the carrageenans was carried out by ultrasonication, using a 600 W, 20 kHz, high-intensity ultrasonic processor (model VC50, Sonics & Materials, Inc., Danbury, USA). For iota-carrageenan, the sample treated by sonication was a 20 ml, 0·5% (w/w) solution in 50 mM NaI. Fractions were taken out at different times for immediate analysis by light scattering. The kappa-carrageenan samples were sonicated in 20 ml batches of 0·8% salt-free solutions and then freeze-dried. Freeze-dried carrageenan samples were dissolved in the appropriate electrolyte solution by stirring and were heated for at least 30 min at 80°C to obtain a true solution. The sonication process leaves an impurity in the samples which were probably eroded particles from the piezoelectric material. This was removed by filtering before injecting the samples into the GPC system.

Measurement procedures

Light scattering was measured at 632·8 nm on a Dawn F MALLS photometer (Wyatt Technology, Santa Barbara, CA, USA) equipped with a 5 mW He–Ne linearly polarised laser. Pure toluene with a known Rayleigh ratio was used to calibrate the system. For the sample measurements, the instrument was either coupled to a GPC system for on-line analysis or used in a batch-wise mode, to obtain classical Zimm plots. The amount of substance injected into the GPC system was 0·15–0·35 mg, dissolved in 50 μl of solvent. The larger injected amount was required for the lower molecular weights, owing to their low scattering intensity. Solutions to be measured in the batch-wise mode were first kept at 70°C in a water bath, and were then filtered (with a peristaltic pump) through a 0·22 μm millipore filter into the Dawn F flow cell, which was kept at the desired measurement temperature. A steady reading of the light scattering was obtained in less than 20 min, and all solutions were allowed to equilibrate for at least this length of time, prior to measurement. The intensity of scattered light was measured at 15 different angles, from 21 to 160°. The software used was ASTRA and EASI for GPC/MALLS analysis and AURORA for batch analysis, all supplied by Wyatt Tech.

The GPC system consisted of a Pharmacia pump P-500 and an auto-injector ACT-100, both controlled by a liquid chromatography controller LCC-500, and a set of two Waters ultrahydrogel columns ('linear' and '250'), coupled in series. A Wyatt/Optilab 903 interferometric refractometer, working at the same wavelength as the light-scattering laser, was used as a mass-sensitive detector. The columns, the RI-detector and the LS detector could be thermally regulated from 25 to 80°C. The coil-to-helix transition was measured by optical rotation at 435 nm on a Jasco DIP-360 polarimeter in a jacketed cell with a 5 cm pathlength.

Evaluation of light scattering data

The scattering of light from a macromolecular solution depends on the molecular weight and the radius of gyration of the macromolecule according to the following equation (Kratochvil, 1987):

$$Kc/R(\theta) = 1/[M_w P(\theta)] + 2A_2 c. \tag{2}$$

Here c is the concentration of the macromolecule, M_w is its weight-average molecule weight and A_2 is the second virial coefficient. The excess Rayleigh ratio, $R(\theta)$, which is a function of the intensity measured at different angles, is given by the instrument. The radius of gyration enters the equation through the particle scattering function, $P(\theta)$, which is a function of the size and shape of the molecule. $P(\theta)$ approaches 1 as θ approaches zero. K is an optical constant given by

$$K = 2\pi^2 n_0^2 (dn/dc)^2 \lambda_0^{-4} N_A^{-1}, \tag{3}$$

where n_0 is the refractive index of the solvent at the incident wavelength (λ_0), dn/dc is the refractive index increment, and N_A is Avogrado's constant.

The molecular weight and the radius of gyration may be obtained from a Debye plot of $R(\theta)/Kc$ vs $\sin^2(\theta/2)$, through an extrapolation to zero concentration and zero angle. In extrapolating the scattering data to zero angle, a first order fit was used of data from at least eight scattering angles. The calculations require data, obtained independently, on dn/dc and A_2. The second virial coefficient was obtained from Vanneste (1993) in the case of iota-carrageenan. For kappa-carrageenan it was obtained from a Zimm plot. The values are shown in Table 1. The value of A_2 did not have a large impact on the results obtained.

The refractive index increment should be measured at constant chemical potential of the salt in the binary salt/water solvent. Since the carrageenans are polyelectrolytes, this means that a dialysis equilibrium should be established between the carrageenan solution and the solvent (Huglin, 1972). However, the accuracy we

Table 1. Parameters used in the analysis of light scattering data for carrageenans

Sample	dn/dc (ml/g)	$A_{2\,\mathrm{helix}}\ 10^3$ (ml mol/g^2)	$A_{2\,\mathrm{coil}}\ 10^3$ (ml mol/g^2)
Iota-carrageenan	0·127 ± 0·003	0·89[a]	4·45[b]
Kappa-carrageenan	0·140 ± 0·004	2·36	3·10

[a] From Vanneste, K. (1993). Table III, p. 33 for 0·09 M NaI at 25°C.

[b] From Vanneste, K. (1993). Table II, p. 30 for 0·09 M NaI at 45°C.

obtained via this method was poor, mainly because of difficulties in controlling the exact carrageenan concentration in the dialysed solutions. The scatter in the data within every concentration series was high, and different attempts gave different values of the refractive index increment (between 0·1 and 0·2 ml/g). Therefore, we used d*n*/d*c* measured in pure water (Table 1), which gave a good reproducibility. The temperature and molecular weight dependence for the refractive index increment is assumed to be negligible (Kratochvil, 1987). In as much as the refractive index increment is insensitive to the conformational state of the carrageenan molecule, any error in d*n*/d*c* will not affect the ratio of the molecular weights for the helix and coil states. We note in this context that there is a large spread in the literature data on d*n*/d*c* for carrageenans. The values range between 0·095 and 0·150 ml/g for kappa-carrageenan (Slootmaekers *et al.*, 1988; Semenova *et al.*, 1988; Lecacheux *et al.*, 1985; Vreeman *et al.*, 1980) and between 0·113 and 0·154 ml/g for iota-carrageenan (Lecacheux *et al.*, 1985; Norton *et al.*, 1983*a*; Jones *et al.*, 1973; Vanneste, 1993).

RESULTS

The accuracy of the GPC/MALLS data depends to a large extent on the chromatographic conditions. The separation on the columns must be good and no adsorption to the packing material should occur. Therefore, the MALLS/GPC system was first tested with well-characterised dextran standards, covering a range of M_w from 4×10^4 to 5×10^5. The results from these measurements were in good agreement (within 1–2%) with the values provided by the suppliers. The *z*-average radii of gyration, $R_{g,z}$, were also obtained in the measurements. For the highest dextran fraction ($M_w = 4{\cdot}8 \times 10^5$), we obtained $R_{g,z} = 20$–25 nm, which is comparable with the value of 18·9 nm reported by Beri *et al.* (1993). For the lower dextran fractions, the radii of gyration were too small to be accurately determined. The adsorption of dextran on the columns was also found to be negligible. This could be estimated by comparing the injected mass with the mass obtained from the RI signal. Thus, the chromatographic system performed well with dextran.

For the carrageenans we used salt and temperature conditions to control the conformational state, and to ensure that no further aggregation would occur in the helical state. The conformational state was checked by optical rotation on lowering the temperature, yielding, as usual, a quite well-defined temperature of onset of helix formation T_O, but a more gradual levelling off of the optical rotation at low temperatures to values corresponding to full helix conversion. For kappa-carrageenan, it is well established that iodide salts stabilise the helical conformation, but prevent further helix aggregation (Grasdalen & Smidsrød, 1981; Slootmaekers *et al.*, 1988). Consequently, we chose 100 mM sodium iodide at 25°C for the analysis of kappa-carrageenan helices. Under these salt conditions, we found $T_O = 38$°C, and at 25°C, we estimated the helical content to be at least 80%.The kappa-carrageenan coils were analysed at the same temperature (25°C) and ionic strength (100 mM), but in sodium nitrate, where T_O was 17°C. In contrast to kappa-carrageenan, the conformational transition of iota-carrageenan is largely insensitive to the identity of the (monovalent) salt (Austen *et al.*, 1985; Piculell *et al.*, 1987). Vanneste (1993) reports, however, that iodide ions impede helix aggregation also for iota-carrageenan. Therefore, we chose to analyse iota-carrageenan in the presence of sodium iodide; 50 mM at 37°C ($T_O = 32$°C) for the coil state and 100 mM at 25°C ($T_O = 46$°C) for the helix conformation. Again, the degree of helix conversion under the latter conditions was estimated to be at least 80%.

Under these conditions, good chromatograms were obtained for both the coil and the helix conformers of the carrageenans, as exemplified in Fig. 2a (coil) and b (helix) for non-degraded iota-carrageenan. The peaks look as expected for a GPC experiment. The volume difference between the peak maxima of the RI and the light scattering signals (the latter represented by the 90° signal) is due to polydispersity; the RI detector is only

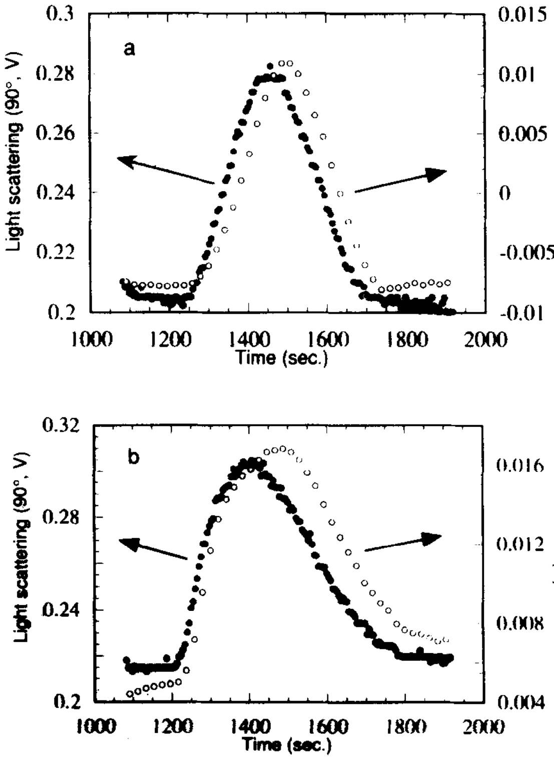

Fig. 2. Chromatograms for: (a) non-degraded iota-carrageenan coils 50 mM NaI, 37°C; and (b) helices (100 mM NaI, 25°C). Filled and open circles denote light scattering (90°) and RI signals, respectively.

mass sensitive, whereas the light scattering signal is also sensitive to molecular size. The two peaks should overlap only for monodisperse samples. There was no sign of aggregation in the two investigated systems under the conditions of operation. This should have been noticed at the high molecular weight end of the peaks or at the void volume for the column.

The separation within a peak can be checked by a plot of the logarithm of the molecular weight vs the elution time (volume). Figure 3 shows such plots for the various iota-carrageenan samples in the helix conformation. Only the linear range is shown; at high and low elution volumes the noise was too large, owing to a low concentration of polymer. Experimental data points (every 20th) are shown for the three samples with the highest molecular weights. The curves for the various samples do not overlap due to limited column resolution (dispersion effects). This means that there is a non-negligible polydispersity in each sample fraction eluted from the columns. However, it has been shown from computer simulations (Kotaka, 1977) and experimental results obtained with polystyrene in tetrahydrofuran (He *et al.*, 1982) that M_w values, averaged over the whole sample, are unaffected by column dispersion.

The adsorption onto the columns was found to be higher for the carrageenans than for the dextrans. For the coils the adsorption was 5–10% and for the helices somewhat higher, about 15–20%. These estimates will, of course, depend on the accuracy of d*n*/d*c*. Assuming no adsorption to the columns, we could calculate refractive index increments from the RI detector peak. The values thus obtained, from 0·110 to 0·130 ml/g, were close to some of the values in the literature (Semenova *et al.*, 1988; Lecacheux *et al.*, 1985; Vreeman *et al.*, 1980; Jones *et al.*, 1973). To conclude, the GPC/MALLS measurements on carrageenans were reproducible, and no abnormal features were seen in the chromatograms.

The average (over the entire sample) value of M_w and $R_{g,z}$ for each of the carrageenan samples is given in Table 2. In Fig. 4a and b the changes in M_w with sonication time are followed for iota- and kappa-carrageenan, respectively. The depolymerisation is clearly seen. The fastest change in M_w occurs at the beginning of the sonication and a slower degradation is seen after about 50–100 min, when the molecular weight is below 1×10^5. As can be seen in Fig. 4a and b, M_w is systematically larger for the helix states. This implies that the coil–helix transition involves an association between polymer strands. The extent of this association is shown in Fig. 5 for both iota- and kappa-carrageenan. Here the ratios between the weight-average molecular weights of the helix and the coil states are plotted vs the molecular weight of the coil state. Clearly, there is an approximate doubling of the weight-average molecular weights for both iota- and kappa-carrageenan over the entire investigated molecular weight range.

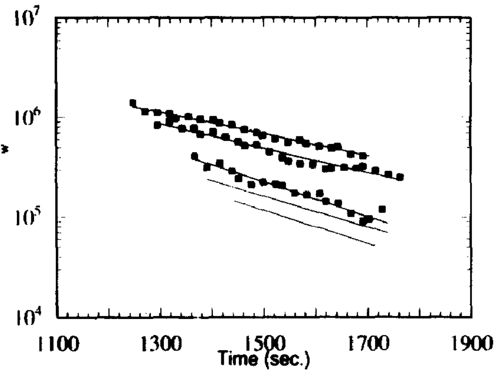

Fig. 3. Logarithm of weight-average molecular weight (M_w) vs elution time within the peaks for iota-carrageenan samples in helix conformation (100 mM NaI, 25°C). Experimental data points are shown for the three highest average molecular weights (see text).

A check of the results obtained was provided by full Zimm plot analyses for the non-degraded kappa-carrageenan sample in the presence of 0·2 M sodium iodide, where $T_Q = 55°C$. Figure 6 shows the plots, yielding $M_w = 296{,}000$ and $R_{g,z} = 62$ nm for the coil state at

Table 2. Molecular parameters for non-degraded and sonicated carrageenans from GPC/MALLS

Sample	Sonication (min)	$M_{w,\text{helix}}$	$M_{w,\text{coil}}$	$R_{g,z,\text{helix}}$/nm	$R_{g,z,\text{coil}}$/nm
Iota-carrageenan	0	738 000	332 000	60	47
	10	466 000	203 000	56	38
	60	190 000	82 000	38	19
	120	121 000	70 000	34	–
	180	89 000	36 000	28	–
Kappa-carrageenan	0	468 000	324 000	60	48
	30	270 000	164 000	51	34
	60	192 000	109 000	45	29
	120	158 000	72 000	39	31
	240	120 000	51 000	32	26

Each value of M_w, $R_{g,z}$ is an average from three independent measurements.
Conditions of measurements: 25°C, 100 mM NaI (iota-carrageenan helix and kappa-carrageenan helix); 37°C, 50 mM NaI (iota-carrageenan coil); 25°C, 100 mM $NaNO_3$ (kappa-carrageenan coil).

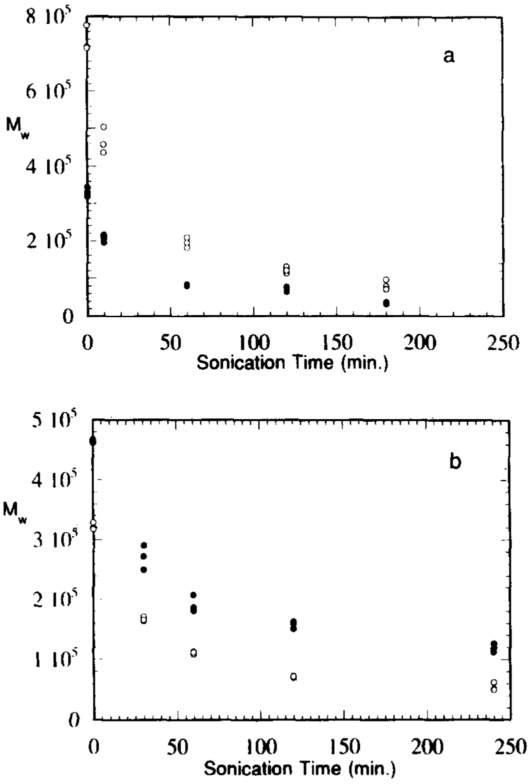

Fig. 4. Weight-average molecular weight vs sonication time for (a) iota-carrageenan and (b) kappa-carrageenan coils (filled circles) and helices (open circles). Three independent runs were made on each sample, all data are shown in the figure.

60°C, and $M_w = 707{,}000$ and $R_{g,z} = 104$ nm for the helix state at 25°C. The same sample solutions were used in both measurements, and the solutions were always filtered hot into the MALLS flow cell, as described in the experimental section. According to this protocol, the coil-to-helix transitions of the helical solutions occurred in the flow cell, as a result of cooling, prior to the measurements. Compared with the GPC/MALLS data in Table 2, M_w for the helix is higher, and so are the $R_{g,z}$ values for both conformations. Nevertheless, the ratio $M_{w,\text{helix}}/M_{w,\text{coil}}$ from the Zimm plots ($= 2{\cdot}4$) clearly falls within the range of the ratios shown in Fig. 5.

The radii of gyration of the various samples, as obtained from the MALLS/GPC experiments (Table 2), are shown in Fig. 7 as are plots of log $R_{g,z}$ vs log M_w. The data, which include both iota- and kappa-carrageenan states, show the expected trend, i.e. an increase in $R_{g,z}$ with increasing molecular weight. According to theory, the slope of such a plot should be equal to 1 for a rod, 0·5–0·6 for a random coil and 0·33 for a sphere (Richards, 1980). However, the values that we obtained vary between 0·3 and 0·4 and are obviously too low.

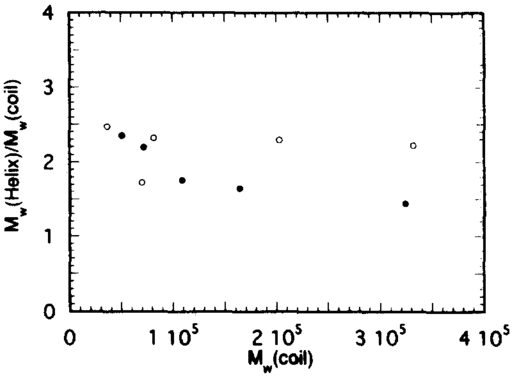

Fig. 5. Ratios of the weight-average molecular weights of the helix and coil states (from Table 2) plotted vs the weight-average molecular weight for the coil conformation for iota-carrageenan (open circles) and kappa-carrageenan (filled circles).

One contribution to the low exponents is a systematic underestimation of $R_{g,z}$ for the higher molecular weights, due to the non-linearity of the particle scattering function. In Fig. 8 we have calculated data points for the scattering from an unperturbed random coil with $R_{g,z} = 59{\cdot}5$ nm, using the relevant particle scattering function derived from the Debye theory (Kratochvil, 1987),

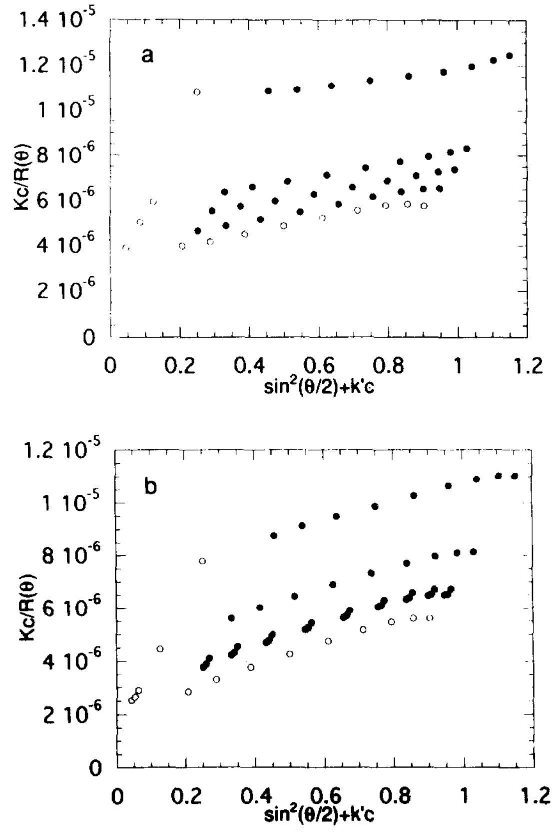

Fig. 6. Zimm plots for: (a) kappa-carrageenan coils (0·2 M NaI, 60°C) and (b) helices (0·2 M NaI, 25°C). Solid circles are experiment data from a concentration range of 0·52–2·44 mg/ml (coil) or 0·43–2·44 mg/ml (helices) and a scattering angle range of 44°–135°. Open circles are extrapolations to zero concentration or angle. k' is the scaling factor.

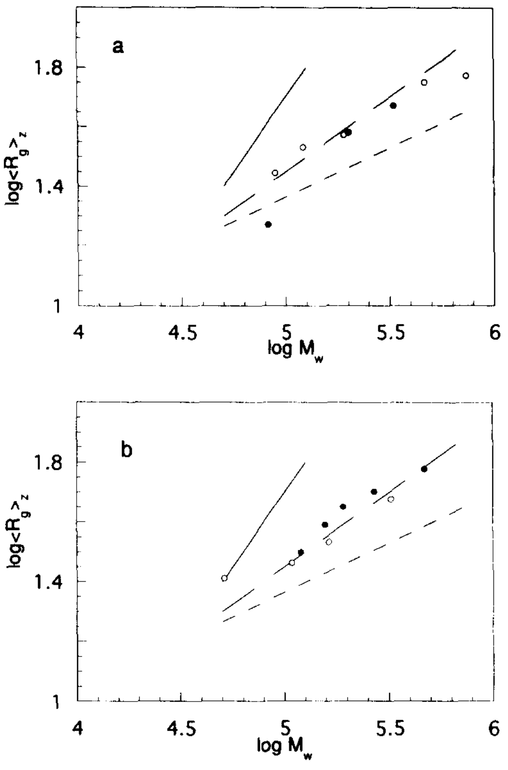

Fig. 7. Logarithm of the z-average radius of gyration vs the logarithm of the weight-average molecular weight for: (a) iota-carrageenan and (b) kappa-carrageenan coils (filled circles) or helices (open circles). Lines show slopes of 1 (——), 0·5 (— —), and 0·33 (- -).

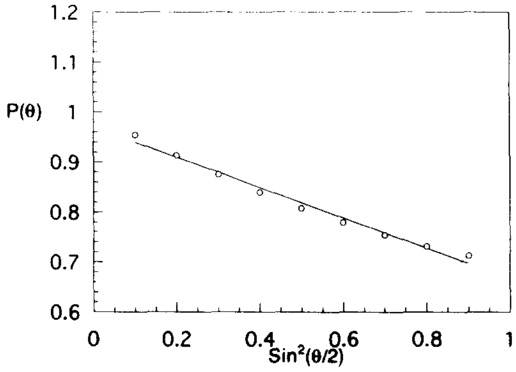

Fig. 8. Comparison (see text) between the exact Debye theory (open circles) for $R_{g,z} = 59{\cdot}5$ nm and a linear fit to the points, which gives $R_{g,z} = 50{\cdot}5$ nm.

$$P(\theta) = (2/x)[e^{-x} - (1 - x)], \qquad (4)$$

where $x = [16\pi^2 \sin^2(\theta/2) < R_g >^2 / \lambda^2]$. The data points correspond to the typical conditions of our MALLS experiment, with $\lambda = 632{\cdot}8$ nm and with θ varying in the range 35–150°. In the analysis of the GPC/MALLS data, we employed a linear fit to the data points, using only the linear term in the expansion

$$P(\theta) = (x/3) + (x^2/12) - \ldots. \qquad (5)$$

If the same procedure is applied to the data in Fig. 8, a value of $R_{g,z} = 50{\cdot}5$ nm is obtained corresponding to an underestimate of the radius of gyration by 20%. (As is evident from Fig. 8, the intercept in the plot, corresponding to M_w, is also underestimated, but only by a few per cent.) Indeed, we found that a second order fit of the experimental data yielded consistently higher values of $R_{g,z}$, especially for the higher molecular weights. (The largest relative discrepancy was found for the helix of undegraded iota-carrageenan, where $R_{g,z}$ from the second order fit was larger by 40%.) These results, and the example in Fig. 8, might be taken to indicate that the second order fit gives a more reliable estimate of the molecular parameters. However, the spread in the data at low angles was not negligible, and it was not obvious that more reliable values would be obtained in this fashion.

The problem with the non-linearity of the scattering function is reduced for smaller molecular weights, but here, on the other hand, the signal-to-noise ratio in the experiment is poorer. Our conclusion is, therefore, that the uncertainty in the $R_{g,z}$ values of Table 2 may be quite large, although the values should be of the correct order of magnitude. The molecular weights, on the other hand, are more reliable; they depend on the intercept of the scattering function, rather than on the slope, and are therefore less sensitive both to uncertainties in the data and to the choice of scattering function.

DISCUSSION

Comparisons with previous results

Our results regarding the molecular weights of undegraded kappa- and iota-carrageenans in the random coil configuration, generated either by altering the salt conditions or by increasing the temperature, are quite similar to those found previously for similar samples by the Leuven group (Slootmaekers *et al.*, 1988; Vanneste, 1993). The $R_{g,z}$ values of the latter laboratory are consistently larger (by about 50%) than the values we obtained in the GPC/MALLS experiment, but the difference is not surprising in view of the uncertainties in our analysis, as discussed above. For the degraded samples, our radii of gyration are comparable with those obtained by Norton *et al.* (1983*b*) for iota-carrageenan.

In contrast, our results regarding the helical conformation differ from those obtained for non-degraded kappa-carrageenan by Slootmaekers *et al.* (1988). The latter group did not obtain any doubling of the molecular weight on conformational ordering. At present, we are unable to explain the discrepancy between their results and ours. There are no obvious reasons to suspect important differences in the two samples investigated. Both were obtained from *Euchema cottonii* and had closely similar molecular weights. As regards the experimental procedure, we note that the carrageenan concentrations used in the Zimm plot in Fig. 6b are slightly higher than those of Slootmaekers *et al.* (less than 0·8 g/l). We obtained, however, virtually the same molecular weight (690,000) for the helix as found from Fig. 6b, in an independent Zimm plot analysis using more dilute solutions (0·225–1·234 g/l). A further difference in the procedures lies in the way the helix formation was induced. In the present study, the helices were always obtained by cooling a sample at constant salt, whereas Slootmaekers *et al.*, added helix-inducing salt, at constant temperature, to a solution of kappa-carrageenan. It is not likely that this difference can explain the observed difference in the aggregation number of the helix.

To further compare our results and methods with those of the Leuven laboratory in particular, we also tried to reproduce the findings regarding iota-carrageenan from the same laboratory (Vanneste, 1993). In a batch-wise experiment covering a concentration range of 0·07–0·8 g/l, we did indeed find a minimum in the reduced scattering intensity of non-degraded iota-carrageenan in 0·1 and 0·2 M NaI. Interestingly, this minimum occurred at a higher carrageenan concentration in our experiments (about 0·4 g/l) compared with the experiments of Vanneste. Most importantly, however, our results also point to the existence of a monomolecular species at low concentrations.

The nature of carrageenan helices

The results presented above show that, under a wide range of conditions, a doubling of the molecular weight on helix formation is found for both iota and kappa carrageenan. These findings add to earlier evidence (Jones *et al.*, 1973; Norton *et al.*, 1978, 1983*a*, 1983*b*; Austen *et al.*, 1985, 1988) that clearly indicates a preferred bimolecular stoichiometry of helical carrageenans. On the other hand, we could also reproduce the results of Vanneste, finding a monomolecular structure for iota-carrageenan at sufficient dilution. Although we did not find such a species for kappa-carrageenan, we have found no reason to distrust the findings of previous investigators (Smidsrød & Grasdalen, 1984; Slootmaekers *et al.*, 1988) in this respect. Our conclusion at this stage is, therefore, that both mono- and bimolecular species of iota- and kappa-carrageenan can be observed under conditions normally favouring the helical structure. We will now discuss the possible nature of these single- and bimolecular species, keeping in mind that the structures we propose should be compatible also with other well-established experimental observations.

The simplest explanation of the bimolecular stoichiometry is in terms of a double helix. The structure of this species in solution has not been determined, so it could, in principle, correspond to any of the 'helical dimer' structures discussed in the literature (Paoletti *et al.*, 1984, 1985). However, unless compelling evidence suggests otherwise, the most probable candidate must be the double-helical structure found by X-ray diffraction on ordered hydrated fibres (Anderson *et al.*, 1969; Arnott *et al.*, 1974; Millane *et al.*, 1988). According to the latest analyses (Millane *et al.*, 1988), coaxial parallel (or, for kappa carrageenan, also antiparallel) structures are compatible with such diffractograms, whereas non-coaxial side-by-side dimers are not allowed.

Much less information is available about the monomolecular species formed at high dilution. The first possibility to be considered is that the double helix separates into two disordered cells at high dilution. Independent evidence of conformational ordering is difficult to obtain at these very low concentrations but this possibility must not be dismissed lightly. For instance, the optical rotation results presented by Vanneste (1993) for iota-carrageenan were obtained at 0·4 g/l. Although this is a very low concentration for optical rotation measurements, it is still far above the concentration range where, according to her reduced scattering intensities, the monomolecular structure dominates. Slootmaekers *et al.* (1988) give no information on the concentrations used in their study of the optical rotation of kappa-carrageenan. However, their data on the radii of gyration indicate that the structure at infinite dilution, under helix-inducing conditions of salt and temperature, is indeed different from a random coil.

Another long-standing candidate for a monomolecular species is an extended single helical structure (Paoletti *et al.*, 1984, 1985; Rochas & Landry, 1987), with dimensions corresponding to one of the strands of the proposed double helix. One of us has argued in detail against this particular structure elsewhere (Nilsson & Piculell, 1989). It was noted then that the only type of analysis that supports this particular monomolecular structure (a combination of calorimetric evidence and electrostatic analysis; Paoletti *et al.*, 1984, 1985; Rochas & Landry, 1987) neglects enthalpic and electrostatic contributions from the specific ion interactions that contribute to the stability of the helix. In addition, it was shown (Nilsson & Piculell, 1989) that different results could be obtained depending on the electrostatic model used, and a later study (Nilsson & Piculell, 1991) has shown that a thermodynamic/electrostatic analysis, which assumes a double helix conformation and includes specific ion binding, is indeed in agreement with calorimetric data. Thus, we maintain that no firm evidence has been presented that points to the existence of the extended single helix. This does not prove, of course, that this structure does not exist. There are, however, severe fundamental difficulties with this particular structure (Nilsson & Piculell, 1989): first, it does not display any short-range inter-residue interactions which may explain the stability of a conformation with long-range order. Second, it provides no mechanism for an unfavourable helix initiation step, as is required in order to explain the observed co-operativity of the coil–helix transition (Poland & Scheraga, 1970).

Other candidates for intramolecular ordered structures are available, which do not suffer from the deficiencies of the extended single helix. In particular, the possibility of cyclic or hairpin-like structures, caused by a chain aligning with itself in a locally double-helical structure, is well understood and documented in other multi-helical systems (Poland & Scherage, 1970; Cantor & Schimmel, 1980; Stokke *et al.*, 1993). Since molecular shapes suggesting such structures have indeed been found for very dilute preparations of iota-carrageenan by electron microscopy (Stokke *et al.*, 1993), we should regard these structures as the most likely candidates for the monomolecular species found in dilute solutions of carrageenans under conditions favouring helix formation.

CONCLUSIONS

In this study we have investigated the molecular weight dependence of the coil to helix transition for kappa- and iota-carrageenan under non-aggregating conditions. Analysis by MALLS/GPC confirmed that, by suitable choice of temperature and added salt, it is possible to obtain solutions of non-aggregated helices of both carrageenan types. In both cases, a preferred bimolecular stoichiometry of the helical molecules is found, regardless of the extent of degradation of the carrageenans. These findings support a double-stranded bimolecular structure of the carrageenan helix. A bimolecular structure for kappa-carrageenan was also found from a Zimm analysis of a non-degraded sample. The latter finding is in conflict with earlier investigations, where only monomolecular species were found, under conditions normally favouring helix formation. A monomolecular conformation was, however, confirmed for iota-carrageenan under helix-stabilising conditions, but at high dilution. Generally, the observations of monomolecular structures seem restricted to highly dilute solutions, but the possible role of other factors, such as sample history and molecular weight, are not yet clear. Independent results from electron microscopy, as well as arguments based on coil–double helix transition theory, suggest that the monomolecular helices might be cyclic or hairpin-like, locally double-helical structures. We note, however, that although an intramolecular helix formation should be favoured for high polymers, we detected no dependence of helix stoichiometry on chain length within the ranges and conditions of the present study.

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