Acid Hydrolysis of κ- and ι-Carrageenan in the Disordered and Ordered Conformations: Characterization of Partially Hydrolyzed Samples and Single-Stranded Oligomers Released from the Ordered Structures

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ABSTRACT: κ- and ι-carrageenan that were partially hydrolyzed in dilute acid while being in the ordered conformation (induced by LiI or LiCl, respectively) gave rise to bimodal molecular weight distributions (MWDs) as shown by size-exclusion chromatography (SEC) and gel filtration. Optical rotation measurements showed that the high molecular weight (MW) fraction remained conformationally ordered, whereas the low-MW fraction was in the disordered state. The relative amount of the low-MW fraction increased with degradation time. By inducing the disordered conformation (in 0.01 M LiCl) the weight average molecular weight (M_w) of the partially hydrolyzed samples decreased 1.5−3 times. These results are interpreted in favor of a double-stranded ordered conformation. Partial hydrolysis of this structure yields a metastable, partially double-stranded structure containing chain breaks that are "unexposed" due to partial overlap of the individual chain fragments. When the degree of polymerization (DP) of such fragments decrease below the critical value (DP_c) for duplex stabilization the fragments are released and appear as a separate low-MW fraction in the SEC chromatograms. DP_c was estimated to be approximately 100 residues in both cases. After induction of the disordered conformation in partially hydrolyzed samples the molecular weight distribution remained bimodal. For κ-carrageenan this is attributed to the higher rate of hydrolysis in the disordered fragments than in the parent ordered chains, which also leads to a rapid accumulation of dialyzable oligomers (DP 1−4). For ι-carrageenan, where the rate of hydrolysis ideally is independent of the conformational state, the bimodality is tentatively attributed to the presence of κ-units (partially formed by desulfation), which are hydrolyzed more rapidly in the disordered state than in the ordered state.

Introduction

Polysaccharides are generally susceptible to chemical, biological, and mechanical degradation. Several factors influence the degradation rate, including the conformational state.[1−4] It is well-known that the introduction of ordered, multiple-stranded conformations in xanthan and scleroglucan enhances the stability when expressed in terms of changes in physical properties such as molecular weight (M_w) or intrinsic viscosity.[1−6] Multiple-stranded polymers can apparently tolerate cleavages of glycosidic linkages without the same decrease in the molecular weight as single-stranded polymers[1,2,6,7] because many of chain breaks remain hidden within a "metastable" structure that remains multiple-stranded because of the stabilizing effect of overlapping fragments.[7,8] The term "metastable" reflects the fact that these structures do not correspond to the global free energy minimum, a state which is only obtained by a redistribution of fragments to obtain perfectly matched structures without hidden chain breaks.[8] A total break in the multiple-stranded structure only occurs when chain breaks in adjacent chains are located within a distance along the chains which is smaller than the critical degree of polymerization (DP_c) that is needed for stabilizing a multiple-stranded structure.[9] When the depolymerization becomes extensive, typically after a 10-fold decrease in molecular weight, extensive disintegration of the multiple-stranded structure occurs, and the decrease in molecular weight actually occurs faster than for single-stranded chains, even if the rate of bond cleavage is the same.[7]

The concept of a critical DP which (on average) is required for a fragment to remain associated within a multiple-stranded structure has another important implication: Fragments where DP < DP_c will dissociate from the parent structure and appear as a separate, low-MW fraction.[7] The molecular weight distribution (MWD) of partially degraded polymers will therefore reflect whether the degradation is carried out in a single- or multiple-stranded state. In the former case a monomodal distribution is expected, whereas a bimodal distribution is expected in the latter case. This may readily be investigated by size exclusion chromatography (SEC). SEC profiles of randomly degraded and conformationally disordered κ-carrageenan,[10,11] as well as other single-stranded polymers,[12,13] have revealed the expected monomodal molecular weight distribution. In contrast, random depolymerization of double-stranded xanthan resulted in the gradual appearance of a second, low-MW fraction which eluted closer to the salt-peak.[2,14] For xanthan this peak was indeed shown to correspond to conformationally disordered (single-stranded) chains.[2] Similar data have also been obtained for triple-stranded scleroglucan.[15,16]

The existence of metastable states in partially depolymerized, multiple-stranded polymers, i.e. structures consisting of several fragments connected by overlapping chain ends, may be studied by performing a denaturation/renaturation cycle. Such a treatment should, at least at low polymer concentration, allow a

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redistribution of fragments to form shorter and more perfectly matched structures without internal chain breaks.[8] A shift in the molecular weight distribution will thus occur, and the magnitude of the shift (or decrease in M_w) is strongly dependent upon the degree of chain scission (α) and DP_c.[8] For partially hydrolyzed, double-stranded xanthan, a 7−9 times decrease in M_w was obtained as a result of a thermal denaturation/renaturation cycle.[8]

When polysaccharides carrying side chains or substituents are subjected to depolymerization, changes in the structure and distribution of side chains (or substituents) may also take place.[2,4,14] Such structural changes may in turn influence both the conformational properties and rate of bond cleavage in the main chain. For this reason it becomes essential to monitor the changes in the primary structure upon degradation, and further determine the effect of such changes on the behavior of the polysaccharide chains.

κ-carrageenan is a red algal polysaccharide. The idealized structure consists of alternating residues of 3-linked β-D-galactose (G), substituted with a sulfate hemiester in the C4-position, and 4-linked 3,6-anhydro-α-D-galactose (A). In practice, κ-carrageenan contains a certain amount of structural irregularities, in particular ι-type segments, where the A-unit contains an additional sulfate linked in the 2-position. The polysaccharide can exist in at least two different conformations in solution, a random coil (disordered state) and an ordered state, in addition to the gel state promoted by high polymer concentration and gelling cations such as K^+. A special approach to induce the ordered conformation in κ-carrageenan is by adding iodide salts, because iodide has the ability to stabilize the ordered conformation without further aggregation or gel formation.[17] Iodide is probably subjected to specific binding to the hydrophobic interior of κ-carrageenan helices (2 I^- ions per helical turn).[18,19] This also leads to a mass increase of 11% for fully saturated ordered chains.

The strandedness associated with the ordered conformation of κ-carrageenan is still a matter of debate. The early work of Rees and co-workers,[20−22] which was based on light scattering, X-ray diffraction, and differential scanning calorimetry, led to the conclusion that the ordered structures are double-stranded helices. Later, several authors have supported these findings.[23−25] A 50% decrease in M_w when going from the ordered to the disordered state was observed by static light scattering and SEC−MALLS (multiangle laser light scattering) studies.[23,26] Other reports, including early work from our laboratory,[18,27] conclude that the ordered structures are single-stranded helices, partially based on the observation that M_w remained unchanged upon disordering.[17,28] In a recent study[29] we used SEC−LALLS to investigate changes in M_w in fractions of κ- and ι-carrageenan with quite narrow molecular weight distributions. Measurements were performed across a temperature interval where the conformational transition occurred. For κ-carrageenan (in 0.2 M LiI) we obtained an approximate doubling in M_w upon cooling below T_m (the transition temperature). We also observed an increase in the apparent polydispersity $(M_w/M_n)_{LALLS}$. Both observations were interpreted in terms of a double-stranded structure for κ-carrageenan.

Factors that may contribute to the varying conclusions regarding the strandedness may include differences in concentration regimes and experimental approaches used when inducing the ordered conformations, as well as differences in molecular weight and polydispersity (commercial carrageenans have very broad molecular weight distributions). Further, the content and distribution of ι-segments may differ between different preparations and contribute to differences in physical properties.

In a previous article[3] we showed that induction of the ordered conformation in κ-carrageenan (by cooling below T_m in 0.2 M LiI) led to a 200-fold decrease in the rate of acid hydrolysis when the degradation was monitored in terms of the decrease in specific viscosity (η_{sp}). In contrast, only a 10-fold decrease in the rate of bond cleavage was observed. These findings, as well as the strong upward curvature in plots of c/η_{sp} vs degradation time,[1] have been taken in support of a multiple-stranded structure for κ-carrageenan in 0.2 M LiI.

ι-carrageenan is built up by alternating residues of 3-linked β-D-galactose (G), substituted with a sulfate hemiester in the C4-position, and 4-linked 3,6-anhydro-α-D-galactose (A) sulfated in the C2-position. As for κ-carrageenan, a salt- and temperature-dependent conformational transition may take place. The strandedness of the ordered conformation in the sol state is also controversial. Rees and co-workers proposed that the ordered structures were double-stranded helices,[20−22,30] which has been supported later by other authors.[32−32] A 2-fold decrease of M_w concomitant with the order → disorder conformational transition has been reported on the basis of static light scattering and SEC−MALLS (multiangle laser light scattering) studies.[22,26,30−33] Other reports conclude that the ordered structures are single-stranded helices, in some cases based on the observation of unchanged M_w upon disordering.[27,34−35] In contrast to κ-carrageenan in iodide-salts, ι-carrageenan has a tendency for aggregation even at low concentration and ionic strength. This causes problems in achieving reliable light scattering data, especially for the ordered conformation. Reversible aggregation may typically be observed in the Zimm plots in form of upward curvatures at very low concentrations.[26,35] Extrapolation of such light scattering data to zero concentration resulted in the same M_w in the ordered and disordered state.[35] It has therefore been argued that ι-carrageenan duplexes or higher aggregates of ordered single helices dissolve into single-stranded coils at low concentration,[26,35] and a mixture of double-stranded and single-stranded molecules may further exist.[26] In a recent SEC−LALLS analysis[29] of fractionated samples of ι-carrageenan with reduced polydispersities we obtained an approximate doubling of M_w and an increase in $(M_w/M_n)_{LALLS}$ upon cooling below T_m for a sample with M_w of 65 000 (above T_m). This was interpreted in terms of a double-stranded structure in the ordered state. For a sample with M_w of 170 000 (above T_m) the increase in M_w by cooling below T_m was much larger and depended on the polymer concentration, which was interpreted in terms of the formation of double-stranded species, followed by concentration-dependent aggregation.

In the present work we investigate MWDs of a series of partially hydrolyzed samples of κ- and ι-carrageenan by SEC−LALLS (low-angle laser light scattering). The degradation is performed in both the ordered and the disordered conformations. The partially hydrolyzed samples are further dialyzed against 0.01 M LiCl to induce the disordered conformation and are reanalyzed by SEC−LALLS. Finally, a partially hydrolyzed sample

is subjected to an order → disorder → order cycle to reveal possible interchain rearrangements.

Experimental Section

Samples. κ-carrageenan from *Eucheuma cottonii* was obtained from Sigma (lot no. 120H0502). The sample was dissolved (4 mg/mL) in Milli-Q water and stirred overnight. The sample was prehydrolyzed (0.01 M HCl, 100 °C, 5 min.) to avoid a tendency for gelation at high concentrations (at low temperatures). Following neutralization, the sample was dialyzed extensively against 0.1 M LiCl and then Milli-Q water at ambient temperature to remove excess salt. LiI was added as a 1.5 M solution to a final concentration of 0.2 M to a heated (conformationally disordered) solution of carrageenan. The sample was then slowly cooled to ambient temperature to induce the ordered conformation and subsequently filtered through a 0.8 μm membrane filter (Millipore-AA).

ι-carrageenan from *Eucheuma spinosa* was obtained from Sigma (lot no. 27F0373). The sample was prepared as described above for κ-carrageenan, except that LiCl was used instead of LiI to induce the ordered conformation.

The purity of the samples has previously[3] been determined by NMR. The κ-sample contains 10% of ι-type segments, whereas the ι-carrageenan contains 6% of κ-type segments.

κ-carrageenan oligomers were provided by Grampian Enzymes (deca- and dodecamer) and Sigma (dimer and tetramer).

Analyses. Optical-rotation experiments, reducing end group analysis, and ^{1}H NMR were performed as described earlier.[3] The presence of sulfate was analyzed by ion chromatography (Dionex 2010i). The concentration of polysaccharide was determined on a DOC analyzer (Dohrman, DC-190).

Partial Acid Hydrolysis. κ-carrageenan (2 g/L) in 0.2 M LiI was transferred to an acid-washed glass bottle, and concentrated HCl was added to a final concentration of 0.1 M. The flask was kept in a thermostated water bath at 37 °C. Samples were taken at regular intervals (0−360 h) and neutralized with LiOH. Each sample was divided in two parts. One part was dialyzed against 0.2 M LiI to preserve the ordered conformation. The second part was dialyzed against 0.01 M LiCl to induce the disordered conformation. The concentrations before and after (final) dialysis were assessed by carbon analysis as described above. A separate experiment with hydrolysis in the disordered state (0.1 M HCl, 0.01 M LiCl, 51.5 °C) was performed similar to that in the ordered state, except that no prehydrolysis was performed.

Partial acid hydrolysis of ι-carrageenan was performed as described above, except that 0.2 M LiCl was used instead of 0.2 M LiI.

Molecular Weight Distributions. Samples (1.3−1.7 g/L) were analyzed by size exclusion chromatography (SEC) (columns TSK G4000PWXL and G3000PWXL, serially connected) combined with low-angle laser light scattering (LALLS) (KMX-6, Chromatix) as previously described.[2] Values for the refractive index increments (dn/dc) were 0.122 mL/g for κ-carrageenan and 0.105 mL/g for ι-carrageenan, respectively.[29] For κ-carrageenan in the ordered state, the former value includes the contribution from bound iodide to the polymer concentration.[29] Samples in the ordered state were analyzed at 25 °C with 0.2 M LiI as eluent (0.2 M LiCl for ι-carrageenan), while samples in the disordered state were analyzed at 40 °C with 0.01 M LiCl as eluent.

Gel Filtration. κ- and ι-carrageenan samples that had been hydrolyzed for 80 h were further fractionated on a Sepharose CL-6B column (2.6 × 80 cm, Pharmacia Biotech) with 0.2 M LiI (κ) or 0.2 M LiCl (ι) as the eluent. Fractions (see Results) were collected and concentrated by evaporation (maximum ionic strength of 0.4 M after evaporation) and finally dialyzed.

Denaturation/Renaturation Cycle. Samples that had been hydrolyzed for 80 h in the presence of 0.2 M LiI (κ) and 0.2 M LiCl (ι) were dialyzed against Milli-Q water at ambient temperature to induce the disordered (denatured) state. The samples were then dialyzed against 0.2 M LiI (κ) or 0.2 M LiCl (ι) to reconstitute the ordered state. Finally, the untreated and the renatured samples were analyzed by SEC−LALLS as described above.

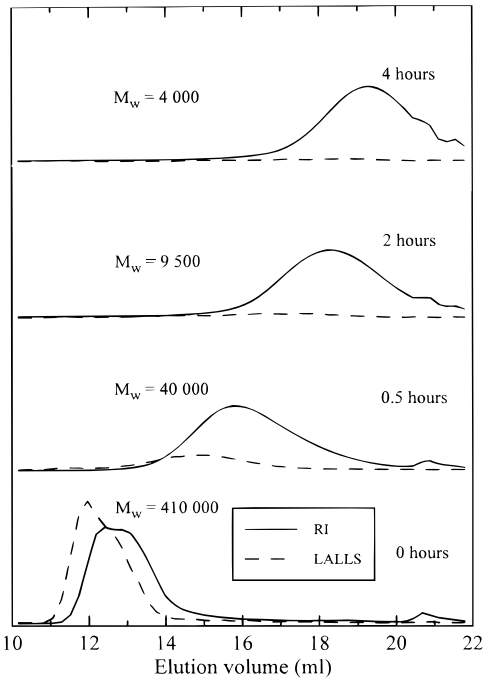

Figure 1. Elution profiles (RI and LALLS detectors) of partially hydrolyzed κ-carrageenan. The hydrolysis was performed in the disordered state.

Monte Carlo Simulations. The depolymerization was simulated adopting the previously described method for simulating the degradation of double- and triple-stranded polymers by random bond scissions in the individual chains.[7] An ensemble with initially 600 duplex chains (DP = 2000) was used. The hydrolysis was assumed to occur exclusively at the 3,6-anhydrogalactose linkage (A-unit).[36] Hence, the end product in the degradation will be the G−A dimer unit. A ratio of 10 between the rate constant for acid hydrolysis of glycosidic linkages in the disordered and ordered conformation[3] was implemented in the case of κ-carrageenan. The MWD in the disordered conformation was simply calculated by neglecting all interstrand interactions following the depolymerization in the ordered conformation. This corresponds to the experiment where the MWD was determined in the disordered state of the sample depolymerized in the ordered conformation.

Results and Discussion

κ-Carrageenan. Hydrolysis in the Disordered State. Acid hydrolysis of conformationally disordered κ-carrageenan resulted as expected in a series of monomodal SEC-profiles (Figure 1). Similar results have been obtained by others, both for κ-carrageenan[10,11] and for other single-stranded polysaccharides.[12,13] The calculated molecular weights are included in Figure 1.

Acid hydrolysis also led to significant desulfation. After 30 h, 18% of the sulfate had been hydrolyzed. A *pseudo* first-order rate constant of $(6.6 \pm 0.5) \times 10^{-3}$ h^{-1} was obtained for the desulfation. In comparison, reducing end-group analyses yielded a rate constant of $(31 \pm 2) \times 10^{-3}$ h^{-1} for cleavage of glycosidic linkages. No influence of the desulfation on the rate of cleavage of glycosidic linkages was observed. This situation differs from that of ι-carrageenan (see below).

Hydrolysis in the Ordered State. A prehydrolyzed sample of κ-carrageenan (see Experimental Section) was further hydrolyzed (at 38 °C) in 0.1 M HCl in the presence of 0.2 M LiI. Under such conditions the high molecular weight κ-carrageenan is conformationally

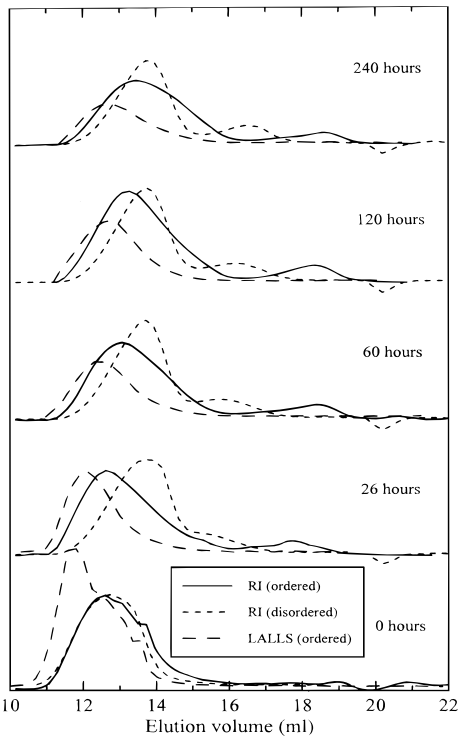

Figure 2. Elution profiles of partially hydrolyzed κ-carrageenan. The hydrolysis was performed in the ordered state, and SEC−LALLS was first performed under conditions preserving the ordered conformation (0.2 M LiI). The disordered conformation was then induced by dialysis against 0.01 M LiCl and the samples were reanalyzed by SEC−LALLS using the same solvent (LALLS signal omitted for clarity).

ordered, which results in a 10-fold decrease in the rate of hydrolysis of glycosidic linkages (value 2.2×10^{-4} h^{-1}) as compared to the disordered state (at the same temperature).[3] We were unable to quantify the rate of desulfation by sulfate analysis because the excess of iodide ions interfered in the analysis. NMR analysis of the sample analyzed for 80 h suggested a decrease in content of ι-segments from 10% to 6% (with large uncertainties), which corresponds to a rate constant on the order of 6×10^{-3} h^{-1}. Despite the uncertainty this estimate suggests that conformational ordering does not stabilize the sulfate groups toward hydrolysis. However, more precise studies are required to reach conclusive data.

Samples were taken during the degradation and divided into two parts. One part was neutralized (with LiOH) and subsequently dialyzed against 0.2 M LiI to preserve the ordered conformation. It may be noted that the removal of HCl leads to a decrease in ionic strength from 0.3 to 0.2 M whereas the corresponding decrease in T_m is only 2−3 °C.[1] Since all measurements and procedures took place well above or well below T_m, the influence of changing the ionic strength is in this case considered to be negligible. The second part of the sample was dialyzed against 0.01 M LiCl, thereby inducing the disordered conformation. The two parts were subsequently analyzed by SEC−LALLS in 0.2 M LiI and 0.01 M LiCl, respectively. Elution profiles (light scattering and RI detectors) are shown in Figure 2.

The undegraded (but prehydrolyzed) sample gave rise to the usual monomodal SEC profile. With increasing degradation time a second (minor) peak appeared close to the salt peak, yielding a bimodal MWD similar to that observed for partially hydrolyzed xanthan.[2] In contrast to xanthan, the minor peak only corresponded to a part of the low-MW fraction. The remaining part consisted of dialyzable fragments, i.e., tetramers and smaller[37] (up to 38% of the total mass after 360 h), which was quantified by a simple mass balance (Table 1).

The main peak for samples hydrolyzed in the ordered conformation moved toward higher elution volumes with increasing degradation time. At the same time the total intensity of the LALLS signal appeared to decrease, reflecting a decrease in the molecular weight. The area of the minor peak increased initially (Table 2) and remained constant at 10−15% throughout the degradation. The peak also moved slightly toward higher elution volume with increasing degradation time.

The next objective was to assign the conformational properties of the molecules corresponding to the high-MW and low-MW fractions. A sample that had been hydrolyzed for 80 h was subjected to gel filtration on Sepahrose Cl-6B with 0.2 M LiI as eluent. As in analytical SEC, gel filtration provided separation of the high-MW and the low-MW fractions (Figure 3). Each of the two fractions were isolated and subsequently analyzed by optical rotation. Results are given in Figure 4. The high-MW fraction displayed the same conformational transition in 0.2 M LiI as for an undegraded sample.[3] The transition temperature (T_m) was 47 °C, which is well above the degradation temperature at 37 °C. These molecules are consequently conformationally ordered during the degradation. The low-MW fraction did not give a conformational transition in the temperature range 10−70 °C (Figure 4), and the low specific rotation was essentially the same as that for κ-carrageenan in the disordered state.[3,17,22,38] The evolution of a bimodal MWD upon depolymerization of ordered chains, where the low-MW fraction consists of disordered chains, is a characteristic feature of multistranded polymers.[7] Our depolymerization data on κ-carrageenan in 0.2 M LiI may therefore be accounted for by a multistranded structure much in the same way as for double-stranded xanthan.[2]

Although the low-MW fraction consisted of up to 80% dialyzable oligomers (Table 2), it also contained considerably larger oligomers. End-group analysis showed that DP_n of the *nondialyzable* fraction was about 14.

The high content of dialyzable oligomers in the low-MW fraction seem to be an effect which may entirely be attributed to the increased rate of hydrolysis occurring in this fraction due to the disordered conformation.

The chemical composition of the high- and low-MW fractions were determined by ^{1}H NMR (data not shown). Despite the complexity of the spectrum of the low-MW fraction (many unassigned peaks tentatively attributed to chain ends), it appeared that the contents of ι-segments were the same in both fractions (~6%). This rules out the possibility that the bimodal molecular weight distributions arise from preferential hydrolysis of regions containing chemical inhomogeneities. It also suggests that ι-type segments are not confined to any particular region of κ-carrageenan chains but appear to be rather uniformly distributed, although we cannot conclude that the distribution is perfectly random.

The partially hydrolyzed samples where the disordered conformation had been induced (following the hydrolysis) also exhibited bimodal MWDs (Figure 2). This appears to conflict the criterion of random depolymerization. It reflects, however, the 10-fold increase in the rate constant of bond cleavage which occurs upon

Table 1. Molecular Weights (M_w) and Mass Distributions of Partially Hydrolyzed κ-Carrageenan in the Ordered and Disordered Conformations, where the Samples Were Hydrolyzed in 0.1 M HCl at 37 °C in the Presence of 0.2 M LiI (in the Ordered State)

	ordered				disordered				
hydrolysis time (h)	$M_{w,O}$ ($\times 10^{-3}$)	high-MW (%)	low-MW (%) nondialyzable	low-MW (%) dialyzable	$M_{w,D}$ ($\times 10^{-3}$)	high-MW (%)	low-MW (%) nondialyzable	low-MW (%) dialyzable	$M_{w,O}/M_{w,D}$
0	260	100	0	0	110	100	0	0	2.4
10	190	98	2	0	71	96	1	3	2.7
26	160	84	10	6	54	80	12	8	2.9
52	120	75	14	11	52	76	16	8	2.4
60	110	75	13	12	50	74	16	10	2.2
80	110	70	15	15	48	74	16	10	2.2
100	96	71	11	18	46	72	15	13	2.1
120	110	69	10	21	40	68	14	18	2.8
179	80	66	10	24	41	65	15	20	1.9
243	71	64	9	27	37	64	14	22	1.9
360	49	54	8	38	27	54	13	33	1.8

Table 2. Molecular Weights (M_w) and Mass Distributions of Partially Hydrolyzed ι-Carrageenan in the Ordered and Disordered Conformations, where the Samples Were Hydrolyzed in 0.1 M HCl at 37 °C in the Presence of 0.2 M LiCl (in the Ordered State)

	ordered				disordered				
hydrolysis time (h)	$M_{w,O}$ ($\times 10^{-3}$)	high-MW (%)	low-MW (%) nondialyzable	low-MW (%) dialyzable	$M_{w,D}$ ($\times 10^{-3}$)	high-MW (%)	low-MW (%) nondialyzable	low-MW (%) dialyzable	$M_{w,O}/M_{w,O}$
0	590	100	0	0	250	100	0	0	2.4
10	420	93	4	3	180	88	6	6	2.3
26	270	91	9	0	110	89	10	1	2.5
52	180	79	17	4	81	80	15	5	2.2
60	170	74	20	6	65	78	17	5	2.6
80	110	68	25	7	51	70	23	7	2.2
100	81	57	31	12	41	60	30	10	2.0
120	57	49	37	14	33	51	39	10	1.7
179	31	18	53	29	20	17	65	18	1.6
243	5.2	2	49	49		2	68	30	
360		1	24	75		>1	49	51	

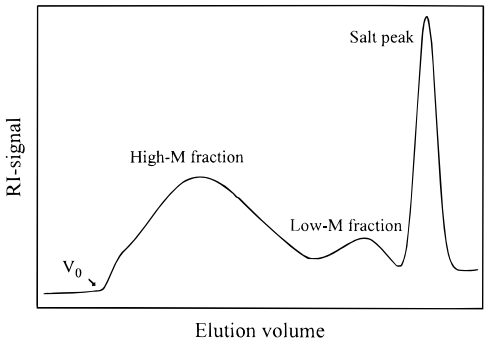

Figure 3. Gel filtration on Sepharose CL-6B of partially hydrolyzed κ-carrageenan (80 h) using 0.2 M LiI as eluent. The void (V_0) and total volumes (salt peak) are indicated.

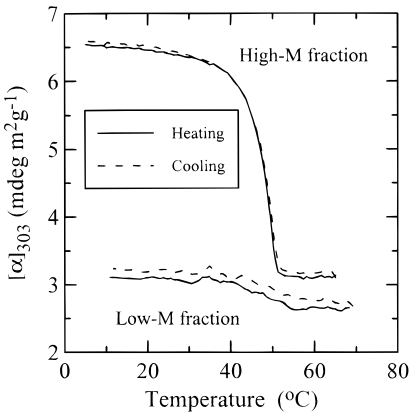

Figure 4. Specific optical rotation ($[\alpha]_{303}$) as a function of temperature for the high-MW and low-MW fractions isolated from partially hydrolyzed κ-carrageenan (80 h).

going from the ordered to the disordered state.[3] This means that the conformationally disordered, single-stranded fragments, once released from the ordered structures, will be degraded considerably faster than the remaining ordered chains.

Although the elution curves remained bimodal after inducing the disordered conformation, the major peak shifted toward higher elution volumes whereas the minor peak (low-MW fraction) shifted in the opposite direction. The former is attributed to a disintegration of duplex structures resulting in a decrease in molecular weight. This is corroborated by a decrease in the LALLS-signal (not shown in Figure 2), and the calculated molecular weights (see below). The shift in elution volume of the minor peak is attributed to the lowered ionic strength (0.2→0.01 M), giving more expanded molecules caused by the electrostatic repulsion of negatively charged sulfate-groups.

Estimation of DP_c. The elution volume corresponding to DP_c in Figure 2 should be in the range near the local minimum between the peaks corresponding to the high- and low-MW fractions, that is, around 16.5 mL. A very rough estimate of the molecular weight may be obtained by extrapolation of the plot of log M vs elution volume (Figure 6, see below for discussion) to lower elution volumes, yielding a molecular weight of approximately 10 000. The corresponding value of DP_c is in the order of 45 (hexose residues), although with large uncertainties. A comparison with the elution volume of κ-oligomers up to DP = 12 (not shown) showed that more than 50% of the low-M peak had a DP above 12, suggesting that $DP_c \gg 12$.

DP_c can be independently estimated by analyzing the relative amount of single-stranded fragments (C_{single}/

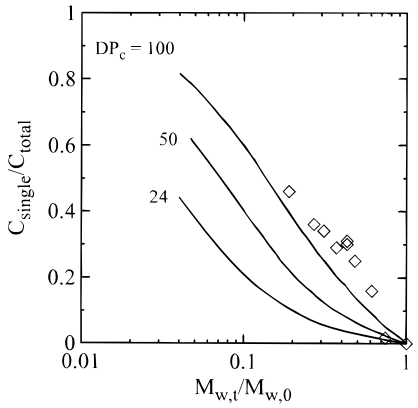

Figure 5. Relative amount of disordered, single-stranded fragments (C_{single}/C_{total}) as a function of the degree of depolymerization ($M_w/M_{w,0}$). The results are based on the experimental data (◇) and from the Monte Carlo simulation with DP$_c$ values of 100, 50, and 24.

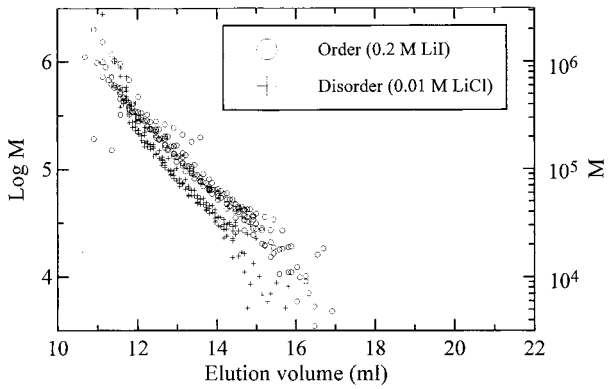

Figure 6. Calibration curves (log M vs V) for undegraded and partially hydrolyzed samples of κ-carrageenan in the ordered state (0.2 M LiI) and for the corresponding samples after inducing the disordered state (0.01 M LiCl).

C_{total}) as a function of the degree of depolymerization (in this case expressed as the ratio between the molecular weight at a given degradation time and for the undegraded sample ($M_w/M_{w,0}$)) and comparing experimental data with those obtained by a Monte Carlo simulation of the degradation of a double-stranded polymer with various values of DP$_c$.[2] The results are shown in Figure 5. The experimental data indicate that DP$_c$ for κ-carrageenan is slightly above 100 residues, which is much larger than the value deduced from the SEC experiments.

Calorimetry studies have suggested that the apparent number of residues participating in the cooperative conformational transition (n_{app}) is 66 ± 6 in 0.1M $N(CH_3)_4I$.[38] DP$_c$ is assumed to be smaller than n_{app}, but the lowered ionic strength used in the determination of n_{app} may account for this difference. In addition, DP$_c$ was determined at 38 °C, only about 10 °C above T_m, which further can explain the large DP$_c$. The relatively high DP$_c$ of κ-carrageenan in 0.2 M LiI is in the same order as that measured for the triple-stranded polysaccharide scleroglucan ($M_{w,min} \sim 40\,000$),[39] whereas DP$_c$ for double-stranded xanthan generally is much smaller.

Molecular Weights of Partially Hydrolyzed Samples. The use of a light scattering detector in combination with a concentration sensitive detector (in this case a refractive index detector) allowed direct determination of the molecular weights and molecular weight distributions. We included the amount of bound iodide (11%) in the concentration term (c_p) in the ordered state, both in the determination of $(dn/dc_p)_\mu$ and in the calculation of M_w (proportional to $(dn/dc_p)_\mu^{-2}\, c_p^{-1}$). M_w values thus include the 11% contribution to the molar mass from bound iodide.

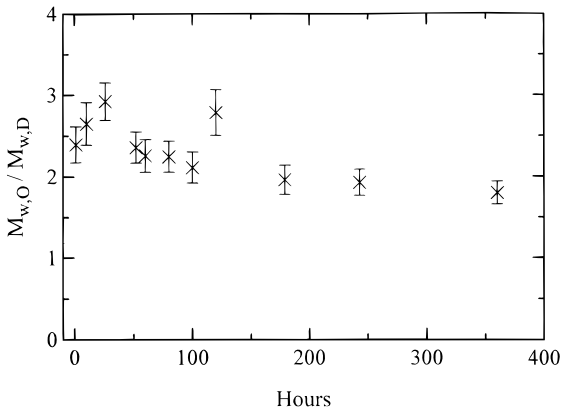

Figure 7. Ratio ($M_{w,O}/M_{w,D}$) between the molecular weights of partially hydrolyzed samples of κ-carrageenan before and after inducing the disordered conformations as a function of the hydrolysis time.

Plots of log M vs the elution volume (V), commonly referred to as calibration curves, are given in Figure 6. Identical (overlapping) calibration curves were obtained irrespective of the hydrolysis time, indicating that the partially hydrolyzed κ-carrageenans are homologous in terms of molecular shape or hydrodynamic volumes. The calibration curves were linear over a M_w range of 1.5 orders of magnitude. This range is much larger than the calibration plots published by Viebke et al.,[26] suggesting a poorer separation in the latter case.

The calibration curves for the ordered state are shifted toward higher elution volumes (or higher molecular weights) as compared to the disordered state. This difference is attributed to both an increase in molecular weight in the ordered state (see below) and the electrostatic expansion of the disordered molecules due to the lowered ionic strength.

It may be noted that that no sign of aggregation in the form of upward curvatures in the calibration curves at low elution volumes can be observed for the ordered state, while some aggregation possibly occurs in the disordered state. However, the amount of aggregated species is insignificant (undetectable on the RI detector) and their influence on the estimation of M_w is negligible.

Table 1 gives the weight average molecular weights (M_w) calculated on the basis of the elution profiles (Figure 2) and the corresponding calibration curves (Figure 6). Results are given both for the ordered state and after having induced the disordered state. These values cover the entire MWD, which means that the loss of mass in the dialysis step has been taken into account. The values are the averages from three injections of each sample, and the experimental error for the calculations of M_w is in the range of 5−10%.

By comparing the molecular weight of a sample in the ordered state ($M_{w,O}$) with that in the disordered state ($M_{w,D}$), additional information about the strandedness of the ordered conformation may be obtained. The results presented in Figure 7 show that $M_{w,O}$ is 2−3 times larger than $M_{w,D}$, indeed suggesting that κ-carrageenan is multiple-stranded in its ordered conformation. After a maximum value of approximately 2.9 (after

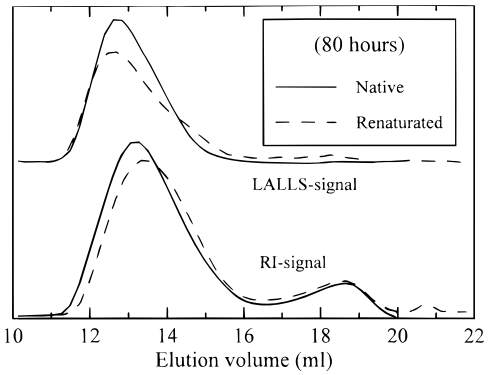

Figure 8. SEC- and LALLS-profiles of the untreated (ordered state) and the renatured sample of a partially hydrolyzed sample of κ-carrageenan (80 h).

26 h) $M_{w,O}/M_{w,D}$ decreases with increasing degradation time, but remains well above 1.0 for all samples.

Denaturation/Renaturation of a Partially Hydrolyzed Sample. In the initial stages of the degradation of double-stranded molecules, internal "hidden" cleavages stabilized by overlapping chains can in principle exist. A denaturation/renaturation cycle may reveal such metastable structures by the formation of more perfectly matched duplexes upon renaturation.[8] The corresponding decrease in M_w depends on the degree of chain scission (α) as well as DP_c.[8] For double-stranded xanthan a 7−9-fold decrease in M_w was observed at low α-values (<0.005).

A denaturation/renaturation cycle was performed by dialysis (0.2 M LiI → 0.01 M LiCl → 0.2 M LiI, ambient temperature) on a partially hydrolyzed κ-carrageenan sample (80 h). The untreated (ordered) and renatured samples were both analyzed by SEC−LALLS (Figure 8). The main peak in the SEC profile moves slightly toward higher elution volume after renaturation. The corresponding ratio between the molecular weight of the untreated and the renatured sample ($M_{w,O}/M_{w,R}$) was 1.3. This ratio is much smaller than for double-stranded xanthan at the same degree of depolymerization. This is primarily attributed to the about 20-fold larger DP_c for κ-carrageenan as compared to that of xanthan.[2] With a large DP_c the number of metastable duplex motifs within a duplex containing hidden chain breaks (at the same α) will be smaller than for a small DP_c. Nevertheless, the results obtained from the denaturation/renaturation experiments supports the assumption that the ordered conformation of κ-carrageenan is double-stranded.

***ι*-Carrageenan. Hydrolysis in the Disordered State.** ι-carrageenan was hydrolyzed in its disordered conformation (0.1 M HCl, 0.01 M LiCl, 51.5 °C). As for κ-carrageenan, the partially hydrolyzed samples gave the expected monomodal elution profiles as shown in Figure 9. The calculated molecular weights are included in the figure.

The extent of desulfation during the acid hydrolysis was measured, and a *pseudo* first-order rate constant of $(4.0 \pm 0.4) \times 10^{-3}$ h^{-1} was determined. In comparison the rate of cleavage of glycosidic linkages was $(3.6 \pm 0.2) \times 10^{-3}$ h^{-1}. The hydrolysis of sulfate will give rise to changes in the chemical composition by increasing the number of κ-segments (see below). This will subsequently influence on bond cleavage for glycosidic linkages, as κ-segments are hydrolyzed faster than ι-segments in the disordered state.[3]

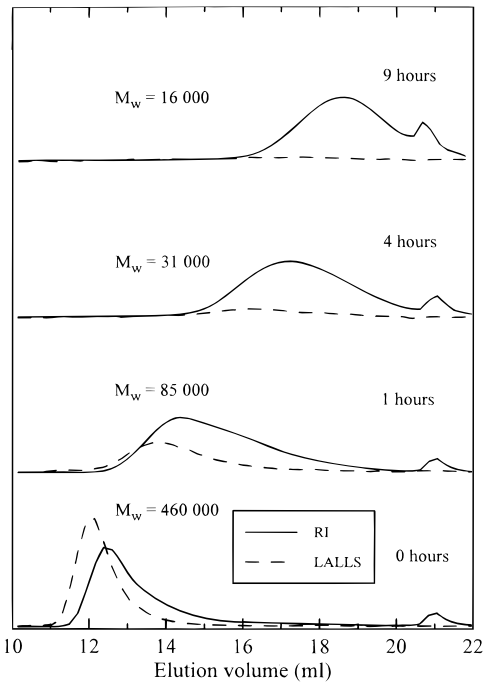

Figure 9. Elution profiles (RI and LALLS detectors) of partially hydrolyzed ι-carrageenan. The hydrolysis was performed in the disordered state.

Degradation in the Ordered State. A prehydrolyzed sample of ι-carrageenan (see Experimental Section) was further hydrolyzed (at 38 °C) in 0.1 M HCl in the presence of 0.2 M LiCl. Samples were taken during the degradation, neutralized, and divided into two parts. One part was preserved in the ordered state (0.2 M LiCl), and for the other part the disordered state (dialyzed against 0.01 M LiCl) was induced, as described for κ-carrageenan. The samples were analyzed by SEC−LALLS in 0.2 and 0.01 M LiCl, respectively. The elution profiles are shown in Figure 10. Only the RI signals are shown for the samples in the disordered state.

The results are analogous to those for κ-carrageenan. The undegraded sample gave rise to a monomodal elution profile, whereas partially hydrolyzed samples displayed bimodal profiles. The peak close to the total volume only represents a part of the total low-*M* fraction. The remaining part, which consists of dialyzable oligomers (up to 75% of the total mass after 360 h), was quantified by a mass balance (Table 2).

The degradation appeared to proceed faster than for κ-carrageenan at comparable ionic strength and temperature, as the peak corresponding to the high-MW fraction was displaced faster toward larger elution volumes than for κ-carrageenan. This is not unexpected since the rate constant for the cleavage of glycosidic linkages is about 1.6 times higher at 37 °C.[3] In addition, the total light scattering intensity decreased more rapidly, and all the high-MW material appeared to be degraded at long degradation times. This can be seen from the SEC−LALLS profile of the 240 h sample and from Table 2. Correspondingly, the area of the peak corresponding to the low-MW fraction increased more rapidly than for κ-carrageenan.

A partially hydrolyzed (80 h) ι-carrageenan sample was fractionated by preparative gel filtration while preserving the ordered conformation (0.2 M LiCl) (Figure 11). The high- and low-MW fractions were analyzed

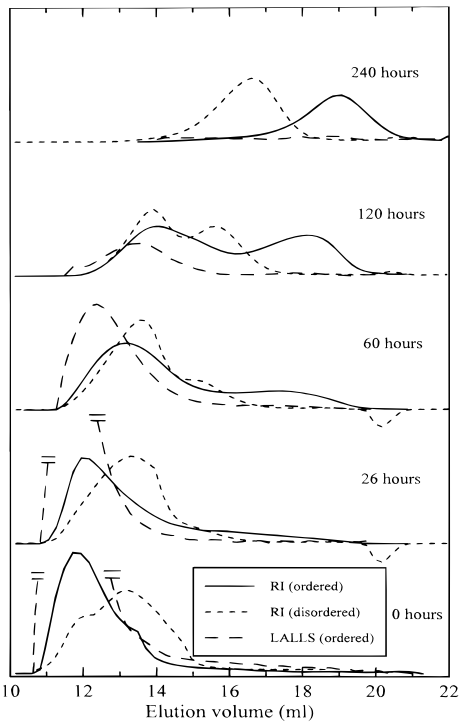

Figure 10. Elution profiles of partially hydrolyzed ι-carrageenan. The hydrolysis was performed in the ordered state, and SEC−LALLS was first performed under conditions preserving the ordered conformation (0.2 M LiCl). The disordered conformation was then induced by dialysis against 0.01 M LiCl and the samples were reanalyzed by SEC−LALLS using the same solvent (LALLS signal omitted for clarity).

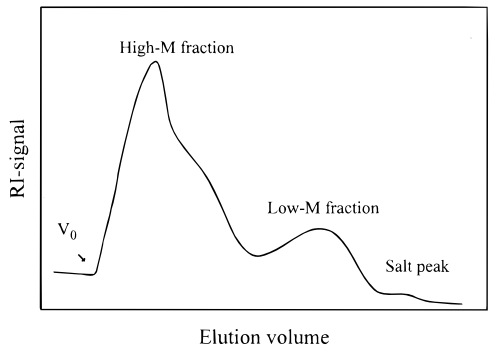

Figure 11. Gel filtration on Sepharose CL-6B of partially hydrolyzed ι-carrageenan sample (80 h) using 0.2 M LiCl as eluent. The void (V_0) and total volumes (salt peak) are indicated.

by optical rotation (Figure 12). The high-MW fraction displayed a conformational transition with transition temperature (T_m) at 42 °C, close to T_m for the undegraded sample.[3] At the hydrolysis temperature (37 °C), the chains were therefore nearly fully ordered. The low-MW fraction did not show any conformational transition and displayed a low specific rotation at all temperatures, corresponding to ι-carrageenan in the disordered state.[3,22,34] As for κ-carrageenan, the low-MW fraction consisted of up to 75% dialyzable oligomers (Table 2).

The chemical compositions for the high- and low-MW fractions were determined by ^{1}H NMR. The two fractions had a content of κ-units of 15% compared to only 6% for the undegraded sample. This difference is attributed to the hydrolysis of sulfate. A comparison of the rate constants for desulfation of κ- and ι-carrageenan suggests that there is no preferential hydrolysis of the sulfate at C-2 in the 3,6-anhydrogalactose unit.

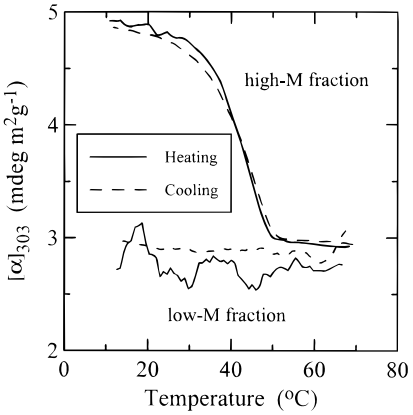

Figure 12. Specific optical rotation ($[\alpha]_{303}$) as a function of temperature for the high-MW and low-MW fraction of a partially hydrolyzed ι-carrageenan sample (80 h).

This has been suggested earlier by Rees,[40] who claimed that the sulfate groups in ι-carrageenan are hydrolyzed at the same rate as they both are in the axial position.

The partially hydrolyzed samples were dialyzed at low ionic strength (0.01 M LiCl) to induce the disordered conformation, and subsequently analyzed by SEC−LALLS in the same solvent. The results (only RI signals) are shown in Figure 10. The observed bimodal MWDs are, contrary to κ-carrageenan, not expected since there is normally no difference in the rate of bond cleavage between the disordered and ordered state.[3] In this case the degradation should be random across the entire distribution of chains, and the MWD obtained in the disordered, single-stranded state should follow the Kuhn-distribution, irrespective of the nature of the ordered conformation where the degradation took place.

The origin of the bimodality is not clear but may tentatively be ascribed to the hydrolysis of sulfate, yielding more κ-type segments in the ι-carrageenan molecules. In the ordered state, the degradation rate of κ-units is about half of that for ι-carrageenan[3] and will not affect the degradation of the ordered chains. In the disordered state κ-units are hydrolyzed 5 times faster than ι-units.[3] Hence, the chains corresponding to the low-MW fraction will, following their release from the ordered structures, have a higher overall degradation rate than the remaining ordered molecules and will subsequently give bimodal MWDs.

Estimation of DP_c. A very rough estimate of DP_c was obtained directly from the SEC-profile (Figure 10) and the corresponding calibration curves (Figure 14) as described for κ-carrageenan, suggesting a DP_c in the range of 20−50 sugar residues. Alternatively, the comparison of experimental results for the relative amount of single-stranded fragments as a function of $M_{w,t}/M_{w,0}$ to data from a Monte Carlo simulation of the degradation of double-stranded ι-carrageenan (the presence of 6% κ-units was implemented, in addition to the relative rate constants given in Table 3) (Figure 13) suggest that DP_c correspond to about 100 hexose residues.

Molecular Weights of Partially Hydrolyzed Samples. The calibration curves obtained from the SEC−LALLS experiments are given in Figure 14. As for κ-carrageenan the calibration curves of partially hydrolyzed ι-samples are independent of the hydrolysis time, indicating that the molecules belong to a homologous series in terms of the hydrodynamic volumes. A tendency for aggregation can be observed as upward

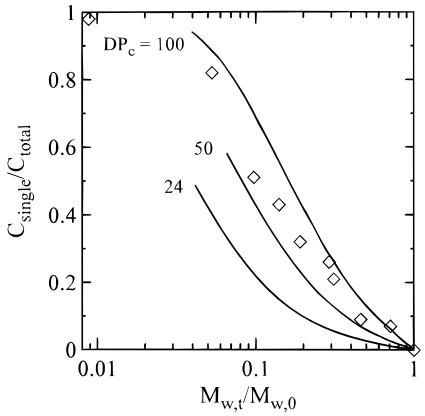

Figure 13. Relative amount of disordered, single-stranded fragments (C_{single}/C_{total}) as a function of the degree of depolymerization ($M_w/M_{w,0}$). Symbols: Experimental data (◇) and data from Monte Carlo simulations (—) with DP$_c$ values of 100, 50, and 24. The presence of 6% κ-units in the ι-carrageenan was implemented in the simulation.

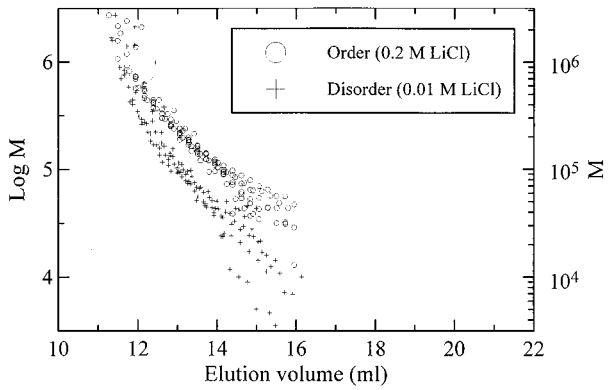

Figure 14. Calibration curves (log M vs V) for partially hydrolyzed samples of ι-carrageenan in the ordered state (0.2 M LiCl) (○) and for the corresponding sample after inducing the disordered state (0.01 M LiCl) (+).

Table 3. Relative Rate Constants[3] for Acid Hydrolysis of Glycosidic Linkages in κ- and ι-Carrageenan in Different Conformational States (Implemented in the Monte Carlo Simulations)

	κ, disordered	ι, disordered
κ, ordered	10	2
ι, ordered	5	1

curvatures of the calibration curves at low elution volumes. The calibration curves for ι-carrageenan in the ordered state are also shifted toward higher elution volumes, due to a change in molecular weight (see below) as well as the electrostatically driven expansion of the disordered molecules at lowered ionic strength.

The calculated molecular weights obtained on the basis of the elution profiles and calibration curves (Figure 14) are given in Table 2. The presence of dialyzable oligomers has been accounted for in the estimation of M_w. For the most extensively hydrolyzed samples there were no detectable LALLS signals, and the molecular weights could therefore not be calculated.

The ratios between the weight average molecular weight in the ordered state and the corresponding value for the disordered state for different degradation times are given in Figure 15. The results show that the ratio ($M_{w,O}/M_{w,D}$) is well above 1.0 for all samples, with a maximum value of 2.4 ± 0.2 for the undegraded sample. These observations suggest that ι-carrageenan is double-stranded in the ordered state. At prolonged degradation, the ratio decreases. This is expected for a random depolymerization of a multiple-stranded polymer as shown for κ-carrageenan since, ultimately, all the molecules become smaller than DP$_c$ and cannot form multiple-stranded structures.[7]

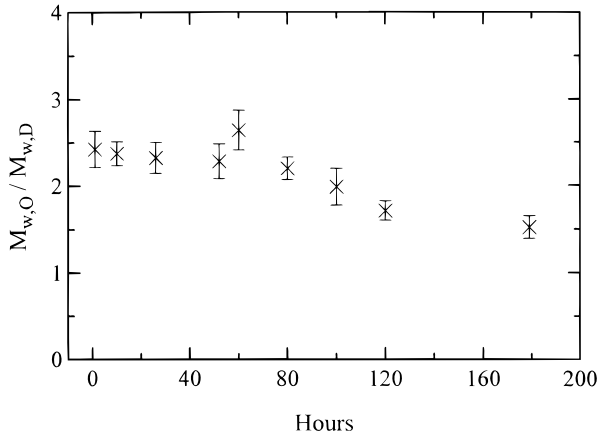

Figure 15. Ratio ($M_{w,O}/M_{w,D}$) between the molecular weights of partially hydrolyzed samples of ι-carrageenan before reaction and after inducing the disordered conformations.

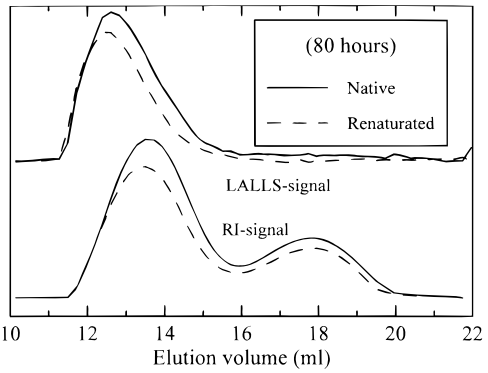

Figure 16. SEC- and LALLS-profiles of the untreated and the renatured sample of a partially hydrolyzed sample of ι-carrageenan (80 h).

Denaturation/Renaturation cycle. Figure 16 shows the SEC and LALLS elution profiles of the untreated and renatured sample of a partially hydrolyzed (80 h) ι-carrageenan. Except for minor differences in the sample concentrations, the chromatograms obtained before and after the renaturation appear to be nearly identical. It is thus unlikely that the high-MW fraction contains "hidden" cleavages, since no rearrangements may be observed. This may tentatively be attributed to chain rearrangements occurring in the hydrolysis step (at 37 °C). The high value of DP$_c$ further limits the number of possible "hidden" cleavages, as such cleavages only are allowed for regions of chain overlaps that exceed DP$_c$.

Conclusions. Partial acid hydrolysis of conformationally ordered κ- and ι-carrageenan gave, in contrast to disordered chains, rise to bimodal molecular weight distributions. Optical rotation studies demonstrated that the high-MW fraction in both cases remained conformationally ordered, whereas the low-MW fraction indeed was disordered. The molecular weight distributions were also strongly influenced by the differences in the rates of cleavage between κ- and ι-type segments and between the ordered and disordered conformations. By transferring of the partially hydrolyzed carrageenans

from the ordered to the disordered conformations, an approximate 2-fold decrease in M_w was obtained. All results thus point toward the conclusion that conformationally ordered κ- and ι-carrageenan are predominantly double-stranded.

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