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Observation of the Molecular Weight Change during the Helix−Coil Transition of κ-Carrageenan Measured by the SEC−LALLS Method

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ABSTRACT: Temperature dependencies of the optical rotation and size exclusion chromatography with low-angle laser light scattering (SEC−LALLS) were performed on the sonicated K^+, κ-carrageenan in KCl salt solutions. The molecular weights were measured at the temperatures of the coil, helix, and coil−helix transition regions. The temperature at each region was determined by the optical rotation experiment. In a solution of low KCl salt concentration, the molecular weights did not change at any of the temperatures investigated. In this case, no hysteresis in the temperature dependence curve of optical rotation was observed. On the other hand, when the salt concentration increased, the molecular weight increased according to the transition from the coil to a helical conformation. In this case, a hysteresis was observed in the optical rotation curve. Moreover, the greater the increase in the KCl concentration, the greater the increase in molecular weight. These results indicate the existence of multiple conformational states for κ-carrageenan that depend upon the solution conditions. That is, κ-carrageenan makes a conformational transition from the coil to a single helical conformation at low KCl salt concentrations. However, κ-carrageenan takes on a helical conformation with a dimerized molecular weight when the salt concentration increases. These dimerized helical molecules further aggregate when the salt concentration increases further.

Introduction

Carrageenans are water soluble polysaccharides extracted from red algae. Their basic structure consists of a repeating unit of 3,6-anhydro-α-D-galactose and β-D-galactose. The characteristic feature of this polymer is that its solution can form a gel when the solution temperature lowers. This phenomenon attracts many researchers and has been investigated for many years. The basic mechanism of the gelation is inferred to be a helix formation when the solution is cooled. However, there has been a great deal of controversy as to the helical conformation of carrageenan. Because the fiber X-ray diffraction experiment has shown that in gels κ- and ι-carrageenans have double helical conformation,[1−3] many works have been published that support the double helical conformation in solution.[4−8] On the other hand, Smidsrod and co-workers have proposed a single helix model for carrageenans in an ordered state.[9−11] Recently, Vanneste et al. have shown, using a low-angle laser light-scattering experiment, that the ι-carrageenan in NaI and NaCl solutions exists in a single helical conformation.[12] In contrast, Hjerde et al. have reported in their recent work the possibility of a double helical state based on their acid hydrolysis experiment, which conflicts with their early claim of the existence of a single helical state.[13] Very recent work of Ciancia et al. showed the difficulty of this problem again, since they obtained different conclusions, which depend on the experimental methods, on the ordered conformation in the presence of iodide.[14] Rochas and Landry have suggested the possibility that an equilibrium exists between the single helix and the helical dimer, depending on the ionic concentration.[15]

The measurement of the molecular weight is the most straightforward way to clarify whether carrageenans exist in a single or a double helical conformation. When the dimerization of a carrageenan occurs, the apparent molecular weight should double. Several static light-scattering experiments of carrageenan solutions have been performed.[16−18] Norton et al. have measured the light scattering of ι-carrageenan in Me_4N^+, Na^+, and K^+ salts solutions and reported a doubling of molecular weight and the occurrence of aggregation at the helix region.[19] Slootmaekers et al. have also performed a static light-scattering measurement of κ-carrageenan in a NaI salt solution and showed that the conformational change of the polymer chain occurs without a noticeable change in molar mass.[16] Recent light-scattering work has been carried out to determine the change in molecular weight of ι-carrageenan in NaI and NaCl salt solutions. In this experiment, low-angle laser light scattering (LALLS) was also used to check the accuracy of the extrapolation in the Zimm plot, and it was concluded that the occurrence of the ordered state of ι-carrageenan at low polymer concentration in both solutions was attributable to the formation of a single helical conformation.[12] Generally, the static light-scattering experiment is difficult because even a small amount of aggregation and/or a microgel gives rise to a strong light scattering, disturbing the accurate observation of the weak light scattering from the helical molecules in solution. The complete removal of these large impurities is difficult because the gel formation occurs simultaneously with or just after the helix formation. In this situation, size exclusion chromatography (SEC) is a powerful tool for obtaining the molec-

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ular weight of helical molecules separately from the large impurities because most of these impurities are removed by the in-line microfilter just before the LALLS cell and/or are eluted earlier than the main portion of the helical molecules. Sworn et al. have applied the SEC method to obtain molecular weights and the molecular weight distribution of carrageenans.[20] The combined method of SEC and LALLS provides an even more useful tool, in that we can obtain absolute values for the molecular weights and the molecular weight distribution. This method has been applied to carrageenans, and detailed procedures have been reported by Lecacheux et al.[21] and Slootmaekers et al.[22] Recently, Ciancia et al.[14] have measured the MALLS/SEC and have reported no doubling of the molecular weight of κ-carrageenan in iodide solution, although their enthalpy experiments do support the existence of a double helical conformation.

In this work, SEC−LALLS with a refractive index monitor (RI) was used to further investigate the molecular weight change in the coil to helix transition of κ-carrageenan. The dependence of the molecular weight of κ-carrageenan on the KCl salt concentration was also investigated in detail. The results show that the helical conformation depends on the KCl salt concentrations. That is, κ-carrageenan takes on a single helical conformation at low concentrations. In contrast, in solutions with high concentrations of salts, KCl, it takes on a dimerized molecular conformation and then aggregates. The above “dimerized molecular conformation”, whether it is double helical or a simple helical dimer conformation, cannot be clarified by this experiment.

Experimental Section

The sample of κ-carrageenan, extracted from *Eucheuma Cottonii*, was supplied by Mitsubisi Rayon, Co. Ltd. (Tokyo). To clearly observe the coil to helix transition, the molecular weight of the sample was lowered by sonication. The sonicated sample was then fractionated with a 0.1 M NaCl/2-propanol system to obtain an appropriate molecular weight with narrow molecular weight distribution samples. The procedures were the same as described before in detail.[23, 24] Two sample fractions were used in this study,hereafter referred to as S-1 ($M_w = 5 \times 10^4$) and S-2 (3.5×10^4). These fractions were rigorously dialyzed against distilled water, converted to the acidic form through an ion-exchange column, and then neutralized with potassium hydroxide to obtain potassium forms of κ-carrageenan. The samples were freeze-dried and stored. Solutions for the SEC−LALLS measurements were prepared by dissolving a dry sample into salt solutions with different KCl salt concentrations. The solutions were then dialyzed for 4 days against KCl solvents with the same salt concentrations. The polymer concentrations were all set at 1×10^{-3} g/cm^3. NaN_3 (200 ppm) was added to the solvent as a bactericide.[21]

Size exclusion chromatography coupled with low-angle laser light scattering (SEC−LALLS, a system of Toyo Soda Co. Ltd., Japan) was used in this experiment. This apparatus has two detectors of LALLS (low-angle laser light scattering, LS-8000) and RI (differential refractive indices, RI-8011), which are attached to a conventional SEC system (Pump, HLC-803D). Two SEC columns of TSKgel GMPW$_{XL}$ (Tosoh Co. Ltd., Japan) were connected in series and used in all experiments. To measure the temperature dependence of SEC−LALLS signals at any desired temperature, SEC columns were dipped in a thermostated water bath, and the light-scattering cell was covered with winding flexible tube in which the thermostated water was circulated. All connecting tubes were also dipped in the thermostated water bath or wound with flexible tube. The whole system was placed in a temperature-controlled room. The temperature of the sample solution was monitored by the direct insertion of a thermistor inside the light-scattering cell. Pullulan (Shodex standard P82, No P-50) with molecular weight $M_w = 4.8 \times 10^4$ was used as a standard sample to calculate instrumental constants k. The flow rate was 1 mL/min, and the injected volume was 0.5 mL. All sample solutions were once heated to 60 °C in order to completely disperse the molecules and then cooled to the desired temperature just prior to injection in a manner similar to that used in the optical rotation measurements in order to prevent the time effect between both measurements of optical rotation and SEC−LALLS.[12] A 0.45 μm in-line microfilter was placed between the columns and the LALLS cell.

The refractive index increment dn/dc of κ-carrageenan was determined by using RM-102 (Union Giken Co. Ltd., Japan) to be 0.127 in a 0.1 M NaCl solution at 25 °C. This value is smaller than the value of 0.149 reported by Slootmaekers et al.,[16] but a little larger than the value of 0.118 obtained by Vreeman et al.[18] Nordmeier[25] has measured the temperature dependence of the dn/dc of dextran and pullulan samples and showed that the values of dn/dc are independent of temperature. Slootmaekers et al.[16] have measured the dn/dc of κ-carrageenan at two different NaCl concentrations and obtained approximately similar values. Therefore, we use the value of 0.127 for all analyses of our data, since the purpose of this work is to compare the relative change in molecular weight at the conformational transition of κ-carrageenan. The optical rotation was measured at 405 nm by using PM-71 (Union Giken Co. Ltd., Japan) using the same experimental conditions described for the SEC−LALLS measurements. The concentrations of the injected samples for SEC−LALLS measurements and optical rotation were set at the same polymer concentration of 1.0×10^{-3} g/cm^3.

Analysis

The signal intensities of refractive index $I^{RI}{}_i$ and low-angle light scattering $I^{LS}{}_i$ of the SEC fraction i eluted at a time from the column are given as

$$I^{RI}{}_i = k_1 c_i \tag{1}$$

$$I^{LS}{}_i = k_2 c_i M_i \tag{2}$$

where k_1 and k_2 are constants and c_i and M_i are the concentration and molecular weight of the polymer molecules of SEC fraction i. By use of the above equations, the molecular weight of fraction i can be obtained from the ratio of the equations of (1) and (2) as follows.

$$\frac{I^{LS}{}_i}{I^{RI}{}_i} = \frac{k_2}{k_1} M_i \tag{3}$$

The instrumental constants $k \equiv (k_2/k_1)$ can be calculated from the following equation.

$$k \equiv \frac{k_2}{k_1} = \left(\frac{k_2}{k_1}\right)_{STD} \frac{(dn/dc)}{(dn/dc)_{STD}} \tag{4}$$

where $(k_2/k_1)_{STD}$ was evaluated from eq 3 by eluting the standard sample solution with known molecular weight.[26] $(dn/dc)_{STD}$ is the refractive index increment of a standard solution, and 0.148 at 633 nm was used in all experiments, as Nordmeier has reported that the values for the refractive index are independent of the molar mass and temperature but do depend upon the wavelength.[25] From the values of c_i and M_i for all SEC fractions, the weight-average molecular weight M_w can be calculated in the usual way.

Results and Discussion

The temperature dependence of the optical rotation $[\alpha]_{405}$ was measured for K$^+$, κ-carrageenan of the S-1

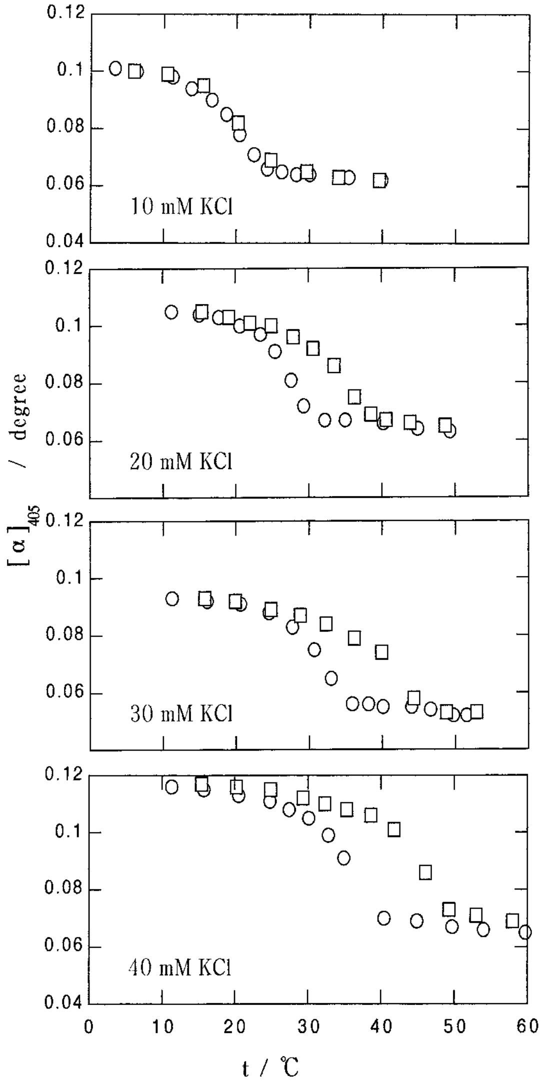

Figure 1. Temperature dependence of the optical rotation $[\alpha]_{405}$ of K^+, κ-carrageenan (S-1 sample) at four KCl salt concentrations: (○) cooling process; (□) heating process.

sample at four different KCl salt concentrations, and the results are shown in Figure 1. The measurements of optical rotation started from the high-temperature point. The samples were then cooled and heated again. The values of optical rotation increase cooperatively with decreasing temperature at all KCl concentrations. This indicates that the coil to helix transition of K^+, κ-carrageenan occurs when the temperature decreases.[27] Rochas and Landry have shown that a maximum of helix state is reached when the ratio $[\alpha]_{\text{helix}}/[\alpha]_{\text{coil}}$ is 1.56.[15] The ratio for our optical rotation values shows 1.6 ± 0.1 in all KCl concentrations. This indicates that the κ-carrageenan takes on a complete helix at low temperatures at all KCl salt concentrations. The transition temperature shifts higher as the salt concentrations increase. With a 10 mM KCl salt solution, the optical rotation curves for both the cooling and heating processes coincide with each other. On the other hand, optical rotation curves measured at higher KCl concentrations show a hysteresis between the cooling and heating curves, which is thought to be evidence of aggregation.[7] Typical temperatures at the coil, helix, and coil−helix transition regions were selected from these figures, and the SEC−LALLS measurements were performed at these temperatures.

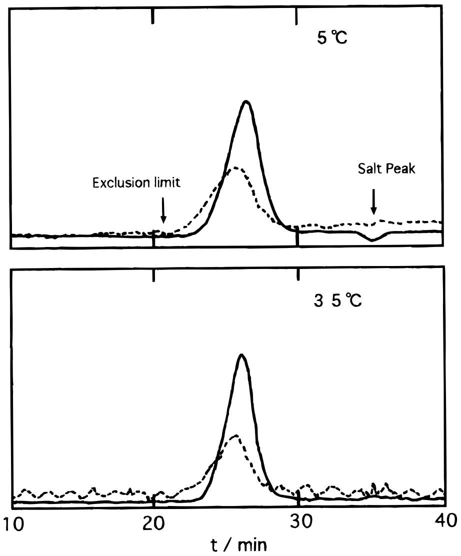

Figure 2. Temperature dependence of the SEC−LALLS signals of K^+, κ-carrageenan (S-1) in a 10 mM KCl salt solution. Solid and dashed lines indicate the RI and LALLS signals, respectively. The intensities of the signals are arbitrary, but the same scale is used at all temperatures for the RI and LALLS signals.

Table 1. Molecular Weights and Their Polydispersities of Sonicated κ-Carrageenan S-1 Sample Measured at Various Temperatures in KCl Solutions, Which Were Obtained by Using the SEC-LALLS Method

KCl (mM)	t (°C)	conformation	$10^5\,k$	$10^{-4}\,M_w$	M_w/M_n
10.0	5.0	h	3.7	6.1	1.2
	10.0	h	3.5	5.3	1.1
	15.0	c → h	3.5	5.2	1.4
	35.0	c	3.4	5.0	1.2
20.0	10.0	h	3.7	11.7	1.2
	10.0	h	3.4	11.9	1.3
	40.0	c	3.9	5.2	1.2
	40.0	c	4.0	4.9	1.2
30.0	10.0	h	3.9	23.8	1.4
	10.0	h	3.9	23.0	1.5
	30.0	c → h	3.7	21.9	4.5
	30.0	c → h	3.8	20.0	3.7
	50.0	c	3.9	5.4	1.2
	50.0	c	3.7	6.1	1.2
40.0	35.0	c → h	3.9	24.3	2.1
	35.0	c → h	4.1	23.8	2.3
	50.0	c	4.7	7.3	1.2
	50.0	c	4.6	7.2	1.1

Figure 2 shows the SEC−LALLS chromatograms of κ-carrageenan in the S-1 sample measured at a 10 mM KCl salt concentration. Only the typical cases of helix (5 °C) and coil (35 °C) states are shown in the figure. The intensity scales of both figures were the same for both the RI and LALLS signals, so that one can easily estimate the molecular weight change by simply comparing the ratio of the intensity of these RI and LALLS signals. The ratios of the intensity of RI and LALLS signals are almost the same in both chromatograms. This immediately indicates that the molecular weight of κ-carrageenan in the 10 mM KCl solution does not change at these temperatures, although the coil to helix transition occurred in this temperature range. The weight-average molecular weights, M_w, for all temperatures are calculated and shown in Table 1. The instrumental constants, k, which were obtained from the standard sample measurement injected just prior to the injection of each sample, are also shown in Table 1. The values are in good agreement with each other at all temperatures. This indicates a good reproducibility for the chromatograms of RI and LALLS signals in this experiment. Although the M_w at 5 °C is a slightly larger value, it is not so large that the carrageenan molecules would be assumed to form a helical dimer. From those results, it can be concluded that the κ-carrageenan of the S-1 sample does not change in molecular weight during the transition from the coil to a helix conformation. This indicates that κ-carrageenan makes the transition from a coil to the single helical conformation in a 10 mM KCl salt solution. The molecular dispersity of M_w/M_n has been calculated, and the values are also shown in Table 1. As Slootmaekers has pointed out,[22] the uncertainty of the M_n value obtained from SEC−LALLS is rather large. Therefore, the values of M_w/M_n in Table 1 should be considered to be a qualitative measure of the polydispersity. The average value of M_w/M_n of κ-carrageenan is approximately 1.2 in both the coil and helix states.

The elution time of the RI signal may also be a measure of the molecular weight and/or hydrodynamic size of the molecules, as it is analyzed in the conventional SEC measurement. As a standard, the elution times of the pullulan samples were measured at each temperature. Those values are nearly identical, with a time of 28.7 min at 35 °C and 28.8 min at 5 °C. On the other hand, the elution time for the carrageenan sample is 25.8 min at 35 °C and 26.3 min at 5 °C. The carrageenan sample at 35 °C eluted slightly faster than that at 5 °C. This indicates that the hydrodynamic volume of the helical state is slightly smaller than that of the coiled state. This may indicate that the length of the carrageenan sample, which is used in this work, is short. This short segmented carrageenan cannot become an entangled shrunk coil but instead becomes a stretched and elongated structure because of the repulsive force between charged sites on the molecule. Our unpublished intrinsic viscosity data for a κ-carrageenan sample with $M_w = 4.5 \times 10^4$ (close to our present sample) indicates that the intrinsic viscosity of the coiled state is slightly larger than that of the helical state. The helical state may have a rather small hydrodynamic volume compared to the coiled state and therefore has a slower elution time than the coiled state. Our present experiment was performed with the same time scale of ORD measurement in order to confirm the coil-to-helix transition. However, it is also very interesting to investigate the kinetic effect of the coil to helix or aggregation processes. In the next work, we plan to investigate a systematic time effect. The areas of the RI peaks of all chromatograms are almost the same. This means that the sample recovery from the SEC column is close to 100%.

Figure 3 shows the SEC−LALLS chromatograms of κ-carrageenan in 20 mM KCl salt solutions at coil and helix temperatures. The calculated molecular weights, M_w, are listed in Table 1. At the coil region, the value of M_w is 5×10^4, which is the same as the M_w obtained in the 10 mM KCl salt solution. However, contrary to the case of 10 mM KCl, the weight-average molecular weight at the helix region is 12×10^4, which is double the value of the coiled region. This can easily be qualitatively confirmed from the height of the LALLS signal in Figure 3, which is twice the size of that of the coiled state. This clearly indicates that the dimerization of κ-carrageenan occurs in this KCl salt concentration. However, it cannot be elucidated only from the SEC−LALLS data whether this dimerization is attributable to the formation of a double helical conformation or a simple dimerization of single strand helixes.

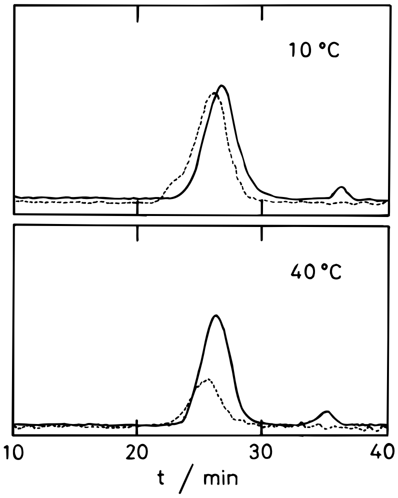

Figure 3. Temperature dependence of the SEC−LALLS signals of K^+, κ-carrageenan (S-1) in a 20 mM KCl salt solution. Notations are the same as in Figure 2.

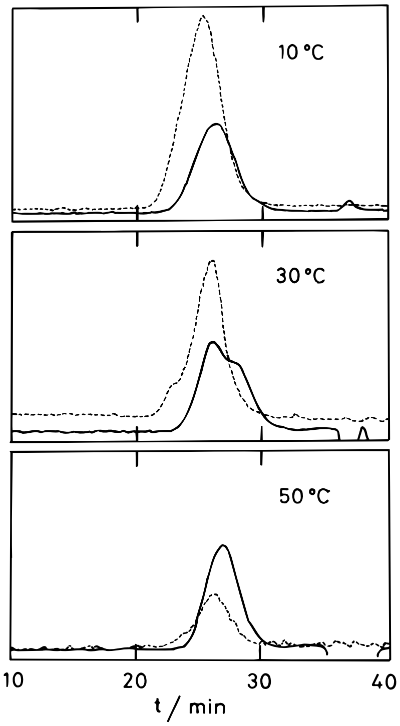

Figure 4. Temperature dependence of the SEC−LALLS signals of K^+, κ-carrageenan (S-1) in a 30 mM KCl salt solution. Notations are the same as in Figure 2.

Figure 4 shows the SEC−LALLS chromatograms of κ-carrageenan in 30 mM KCl salt solutions at the coil, helix, and coil−helix transition temperatures. The calculated values of M_w and M_w/M_n are also listed in Table 1. The RI chromatogram in Figure 4 at the middle region of the coil-to-helix transition shows a shoulder. It is noteworthy that the retention time of the shoulder is higher than that of both the coil and helix states. This indicates the presence of an intermediate state which is more compact than both the coil and helix states. This may imply the existence of a compact intermediate state at the helix−coil transition. The intensity of the LALLS signals strongly increased

Table 2. Molecular Weights and Their Polydispersities of Sonicated κ-Carrageenan S-2 Sample Measured at Various Temperatures in KCl Solutions, Which Were Obtained by Using the SEC-LALLS Method

KCl (mM)	t (°C)	conformation	$10^5\ k$	$10^{-4}\ M_w$	M_w/M_n
10.0	5.0	h	2.8	3.7	1.7
	10.0	h	2.4	3.5	1.3
	15.0	c → h	2.3	3.7	1.4
	35.0	c	2.5	3.6	1.6
	45.0	c	2.4	3.5	1.4
20.0	10.0	h	1.4	7.6	1.5
	25.0	c → h	1.9	4.7	1.9
	40.0	c	2.3	3.4	1.4
	50.0	c	2.1	3.6	1.4
30.0	10.0	h	2.5	11.3	1.4
	20.0	h	2.7	10.2	1.4
	30.0	c → h	2.5	5.4	1.9
	40.0	c	2.2	3.3	1.3
50.0	10.0	h	1.7	21.8	1.8
	20.0	h	1.6	22.5	1.7
	35.0	c → h	1.6	6.3	5.9
	50.0	c	1.5	3.0	1.3

in both the transition region and the helix region. The calculated weight-average molecular weights were approximately 20×10^4 in both regions, which is 4 times larger than that observed for the coiled state. This should be attributed to the aggregation of the κ-carrageenan molecules. The data obtained in the above experiments are summarized in Table 1 with the data from the 40 mM KCl solution.

The same SEC−LALLS measurements were performed on the S-2 sample, and the results are summarized in Table 2. The weight-average molecular weight of the S-2 sample was 3.5×10^4 in the dispersed state. These results are quite similar to those obtained for S-1. In the 10 mM KCl salt solution, no molecular weight change was observed at any of the temperatures observed, which is the same as was seen in the S-1 sample. This again indicates that the S-2 sample of K^+, κ-carrageenan also makes the transition from a coil to the single helical conformation in this KCl salt solution. In the KCl solutions with concentrations above 10 mM, the increment of the molecular weight according to the coil to helix transition can be seen. From the results for both the S-1 and S-2 samples, it can be concluded that the coil-to-single helix transition certainly occurs under our experimental conditions with a 10 mM KCl salt concentration. However, at higher KCl salt concentrations, the occurrence of dimerization and/or aggregation was confirmed. A detailed conformation of the dimerization observed at 20 mM KCl salt solution cannot be elucidated in this work. Therefore, it cannot be confirmed as to whether there exists a double helical conformation or a side-by-side aggregation of a single helical conformation. Although many thermodynamic experiments support the existence of a double helical conformation, further study is needed in order to clarify this point. For example, Rees et al. have investigated this question using optical rotation methods[28,29] and Austen et al. have used a stopped-flow polarimetory technique.[30] Gekko and Kasuya have measured the effects of pressure on the sol−gel transition of carrageenans.[31] Their results were all well explained by the double helical model of carrageenan molecules. Additionally, some authors have asserted that κ-carrageenan helices are stable only as aggregated forms, especially in potassium salt solutions, whereas the ι-carrageenan helix can exist as an isolated helix.[15,32] However, our study shows that κ-carrageenan can also exist as an isolated helix at low KCl salt concentrations. This discrepancy may arise from the difference in the molecular weight of the samples; that is, the works of the previous authors were done with unsegmented κ-carrageenan molecules that have large molecular weights. Our study was done with sonicated molecules with low molecular weights. A short segment of κ-carrageenan molecule may clearly show the intrinsic properties of the helical formation of this molecule. In this study, we showed that the helical states of κ-carrageenan depend on solution conditions.

Conclusion

The results of the SEC−LALLS measurements show that the conformation of the helical state changes depending on the solution conditions. The temperature dependence of the molecular weight measured in a 10 mM KCl salt solution showed that κ-carrageenan exists as a single-strand helix. However, it cannot be determined whether the state of dimerization found in the 20 mM KCl solution is attributable to a double helix conformation or a simple side-by-side aggregation of the single helical conformation. The helical conformation is of interest in connection with the hysteresis found in the optical rotation experiments. When no hysteresis appears, as was the case at 10 mM KCl under our experimental conditions, the conformation of κ-carrageenan is a single-strand helix. On the other hand, when the hysteresis appears, that is, at the 20 mM and higher KCl salt concentrations used in our experiments, the conformation of κ-carrageenan appears to be dimerized and/or an aggregation. Actually, most of the previous experiments, which favor the double helical conformation as the helical form of carrageenan on the basis of thermodynamic properties, were carried out at conditions where the optical rotation showed hysteresis curves.

The remaining uncertainty of our experiment is the dilution that took place during the separation by the SEC column. This has a possibility to shift the concentration dependent helix−coil equilibrium toward the coiled state. However, our preliminary experiments of the optical rotation confirmed that the coil-to-helix transition occurs even at 0.025% solution, which is 4 times lower than the polymer concentration used in this experiment, although the intensity of the optical rotation signal was very small at this concentration. The equilibrium of the helix−coil transition is well-known to depend not directly on the polymer concentration but on the ionic strength of the solution. The ionic strength of the solution can be calculated by using the following equation.[10]

$$I = C_s + 1/2 f C_p \tag{5}$$

where C_s is the equivalent concentration of simple salt, C_p is the polyion concentration, and f is the uncompensated fraction of the polyion charge by the counterions and is equal to unity when the charge density parameter ξ of the polyion is lower than unity. If we use $\xi = 0.64$, which was obtained for κ-carrageenan in our previous paper,[33] the ionic strength of a 0.1% κ-carrageenan solution in 10 mM KCl can be calculated to be 11.2 mM. In a similar manner, the ionic strength of the 0.025% solution can be calculated to be 10.3 mM. This indicates that the ionic strength lowers only 8% even if the solution is diluted 4 times from the 0.1% solution.

These results indicate that the dilution during the column separation may not affect our conclusion. However, it is not to say that a direct measurement of the optical rotation at the time of detection by the LALLS and RI detectors is desirable. Although it is known that the optical rotation lacks the sensitivity needed to perform precise measurement at the low concentrations used in SEC, we will try to improve our LALLS system to measure all the above values simultaneously with desirable sensitivity. Further studies will be needed in order to obtain the concrete conclusion for this problem.

Our present experiment was performed with the same time scale as the ORD measurements in order to avoid the time effect for the coil-to-helix transition process. However, it is also interesting to investigate the kinetic effects of the coil-to-helix or aggregation processes. In our next work, we plan to investigate this time effect.

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