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The effect of charge density and conformation on the polyelectrolyte complex formation between carrageenan and chitosan

Andreas Hugerth, Ninus Caram-Lelham[1] and Lars-Olof Sundelöf*

Physical Pharmaceutical Chemistry, Faculty of Pharmacy, Uppsala University, Uppsala Biomedical Center, Box 574, S-751 23 Uppsala, Sweden

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The formation of polyelectrolyte complexes (PECs) between chitosan varying in degree of deacetylation (DD) and κ-, ι- and λ-carrageenan, respectively, was investigated, in moderately concentrated solutions, with emphasis on the effect of charge density and conformation. Results obtained indicate that macroscopically electroneutral PECs were formed when chitosan interacted with carrageenan molecules in coil or non-aggregated helical conformation. When the κ-carrageenan existed in an aggregated helical conformation the interaction with chitosan produced PECs with a charge ratio of chitosan to carrageenan below unity. Furthermore, the PEC formation process proved to be significantly affected by the presence of low molecular weight polyelectrolyte components. © 1997 Elsevier Science Ltd

INTRODUCTION

Polyelectrolyte complexes (PECs) (Bungenberg de Jong & Kruyt, 1930), formed by mixing polysaccharides of opposite charge, have recently attracted considerable attention because of their potential for use in drug delivery systems as well as in various biotechnological applications (Yoshioka *et al.*, 1990; Takaharu *et al.*, 1993; Tomida *et al.*, 1994; Ikeda *et al.*, 1995). The multitude of reactions in biology that involve polyelectrolyte–polyelectrolyte interaction increases the interest in investigating this area. The characteristic properties of the polyelectrolytes, e.g. charge density, chain conformation and the response of PECs to variation in pH, ionic strength and temperature, offer a wide set of variables with a potential to be employed in pharmaceutical formulations. Interaction between a completely dissociated polyanion and a polycation, both of comparatively high charge densities, may be considered primarily to be of an electrostatic nature since such interactions are strong as well as of relatively long-range order. The possible influence of, for example, hydrophobic interactions and Van der Waals forces on the formation of PECs must also be considered, especially when the charge–charge interaction is screened by a supporting electrolyte of high ionic strength. Not only is the intermolecular interaction governed by the nature and structure of the polyelectrolytes involved but it can indeed be significantly affected by the prevailing solute–solvent and solvent–solvent conditions.

In the preparation of a new drug delivery system it is naturally an advantage if substances that already are permitted for use in the pharmaceutical or food industries can be utilized. Carrageenans and chitosans are two polysaccharide families belonging to this category that are commercially available, have diverse features and are available at a reasonable cost. The carrageenans constitute a class of linear, sulfated polysaccharides extracted from various species of the Rhodophyta (marine red algae). Their backbone is based on a repeating galactan sequence with alternating 1→3-α-D and 1→4-β-D linkages. One normally differentiates between three main types of carrageenans, κ-, ι- and λ-carrageenan, differing essentially with respect to sulfate content (charge density) and amount of anhydro-galactose. Carrageenans are extensively used in the food industry because of their gelling and viscosity enhancing

*Author to whom correspondence should be addressed.
[1]Center for Bioengineering, University of Washington, Box 352255, Seattle, WA 98195, USA.

properties. The gelling capacity of the lower sulfated κ- and ι-carrageenans supposedly emanates from the ability to adopt helix conformation. However, although the conformational ordering seems to be a prerequisite it alone does not necessarily cause gel formation. Chitosan is a linear polysaccharide composed of β-(1→4)-linked 2-amino-2-deoxy-D-glucopyranose (GlcN) and 2-acetamido-deoxy-D-glucopyranose (GlcNAc), and it can be derived by alkaline N-deacetylation of chitin (poly-GlcNAc), which is a structural polysaccharide in the exoskeleton of many arthropods and marine invertebrates. Chitosan has been the subject of intense study during the last decade since it is one of the few abundantly available and naturally derived biocompatible cationic polysaccharides. Furthermore chitosan has proved to be effective as an absorption enhancer, depending on factors such as degree of deacetylation and molecular weight (Schipper *et al.*, 1996).

Previous reports on the chitosan–carrageenan system have not to any greater extent dealt with the effect of variation of charge density on the formation of PECs and were carried out at rather high polymer concentrations (Mireles *et al.*, 1991). In addition, conformational aspects have, to our knowledge, not been elucidated at all. The objective of this study is to examine the intermolecular complex formation between chitosan and carrageenan with respect to charge density and, to some extent, its conformation dependence, in a moderately concentrated regime, at a pH where both types of polymers are completely dissociated.

MATERIALS AND METHODS

Materials

Commercial samples of ι- and λ-carrageenan (designations C-4014, lot no. 27F-0373 and C-3889, lot no. 21H0322) were obtained from Sigma Chemicals Co. κ-carrageenan (Genugel type X-8944 batch no. 44807920) was a gift from Copenhagen Pectin A/S. Partly deacetylated chitosans (22743, lot no. 317641/1 195; 22742; 22741, lot no. 340315/1 595) were purchased from Fluka BioChimika. Chitosan labeled Seacure 244 (batch no. 010-370-02R) was kindly donated by Pronova Biopolymer AS. All other chemicals used were of analytical grade.

METHODS

Charge density

The charge densities of the carrageenan samples were determined by a method described in a previous paper (Caram-Lelham & Sundelöf, 1994a) and the degree of deacetylation of the chitosan samples was determined by first-derivative ultraviolet spectrophotometry at 202 nm in 0.02 M HAc (Muzzarelli & Rocchetti, 1985) on a HP 8452A diode array spectrophotometer.

Helix–coil transition

Optical rotation was measured at 546 nm on a Perkin Elmer 241 polarimeter, using a 10 cm jacketed cell and a circulating water bath for accurate temperature control. Care was taken to allow readings to become steady, i.e. to allow the system to reach conformational equilibrium, before the final reading was made. The helix–coil transition was monitored through cooling runs. The fraction of helix conformation (β) was calculated from

$$\beta = (\alpha - \alpha_{coil})/(\alpha_{helix} - \alpha_{coil})$$

where α equals the measured optical rotation, α_{coil} was determined from the plateau value at high temperatures and α_{helix} was estimated from the highest observed value of α.

PEC experiments

Chitosan stock solutions were prepared by adding a 0.3 M HAc solution, varying with respect to amount and type of added salt, to a weighed amount of chitosan. The solution was stirred magnetically for 20 h at ambient temperature in order to ensure as complete dissolution of the chitosan as possible. The resulting solution was filtered through a 0.22 μm (Millipor Millex GP) filter to rid the stock solution of insoluble material. This provided solutions of chitosan in the completely dissociated form since the pKa of chitosan is ≈6.5 (Rinaudo & Domard, 1989). A series of solutions was prepared from the stock solution by diluting to the desired concentration. Chitosan concentrations used in all experiments were chosen such that self-aggregation was prevented (Amiji, 1995). The carrageenan solutions were placed in a 50°C water bath and magnetically stirred for 30 min, then allowed to cool down to ambient temperature and filtered as above before the experiments were carried out.

The complex formation between carrageenan and chitosan was studied by mixing equal volumes of chitosan solution, varying in concentration, and carrageenan solution, with a final fixed concentration of 0.200 mg/g, in centrifuge tubes. Immediately upon mixing the solutions were vigorously shaken by hand and placed on a shaking table for a minimum period of 150 min. The resulting solutions were thereafter centrifuged for 120 min at 41 000 **g**. The supernatants obtained were analyzed quantitatively with respect to carrageenan by a modified anthrone method (Caram-

Lelham & Sundelöf, 1994b) and with respect to chitosan by means of a ninhydrin reagent (Curotto & Aros, 1993).

RESULTS AND DISCUSSION

Results from experiments on the interaction between chitosan with a DD of 73% and κ-, ι- and λ-carrageenan, respectively, in a solvent containing 0.3 M HAc and 0.03 M NaCl are presented in Fig. 1. The polysaccharide concentration in the supernatant after mixing and centrifugation is plotted against the total amount of chitosan added. The results indicate that all chitosan added binds to the carrageenan until a point is reached where the entire amount of carrageenan present has reacted. Beyond this point further addition of chitosan results in an increasing amount of unreacted chitosan in the supernatant. In these preliminary experiments, and when necessary, the concentration of chitosan was recorded in order to confirm and complement the results obtained in the determination of the carrageenan content by the anthrone method, which in previous investigations had proved to be a precise and reliable method. The scattering of data in the case of chitosan was at least partly caused by the uncertainty in the colorimetric method employed. Hence only the carrageenan concentration was monitored in most instances. In order to calculate the ratio of interacting charges in the PECs formed, the linear charge densities, defined as the average amount of charge per disaccharide, were determined (Table 1). The water content of the polysaccharides (determined by colorimetric Karl Fisher titration and gravimetric measurements) was

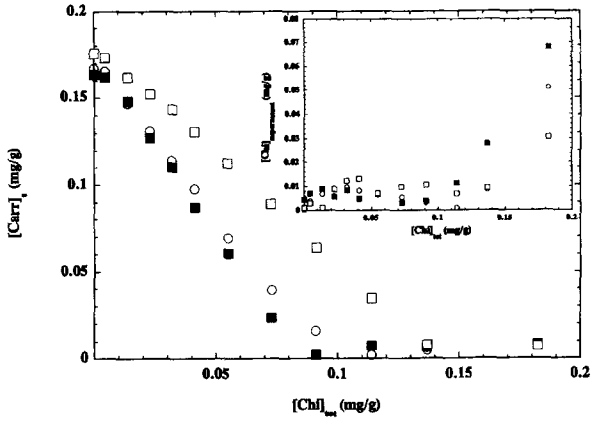

Fig. 1. Complex formation between chitosan (DD 73%) with κ- (■), ι- (○) and λ- (□) carrageenan in 0.3 M HAc with 0.03 M NaCl. In this and the following figures the total concentration of chitosan $[Chi]_{tot}$ (mg/g) is plotted as a function of the carrageenan concentration in the supernatant $[Carr]_s$ (mg/g). The insert shows the concurrently recorded concentration of chitosan in the supernatant $[Chi]_s$ (mg/g).

Table 1. Average amount of charges per disaccharide

Sample	Charge density (no. of charges per disaccharide)
κ-carrageenan	0.96
ι-carrageenan	1.49
λ-carrageenan	2.09
Fluka, chitosan high molecular weight	1.46 (DD 73%)
Fluka, chitosan low molecular weight	1.69 (DD 84%)
Pronova, Seacure 244	1.86 (DD 93%)

taken into account when calculating the charge ratios. The PEC structure can be characterized by its three-dimensional arrangement of charges. This is difficult to determine more precisely and this determination lies outside the scope of this paper. However, due to the sensitivity of the spatial segment distribution to the linear charge density of any flexible polymer in solution, even the spatial distribution of charge interaction in PEC formation can be expected to be related to the linear charge density. Consecutive phase separation experiments between chitosans varying in DD (73%, 84% and 93%) and κ- (a), ι- (b) and λ-carrageenan (c), respectively, are displayed in Fig. 2. All complexes formed (Figs 1 and 2) were rather compact water-insoluble hydrous precipitates. The ratio of charges in all the PECs obtained (Figs 1 and 2) proved to be close to unity (0.96±0.05) irrespective of the order of mixing. These findings are in accordance with previously published results concerning the mixing of oppositely charged pendent-type polyelectrolytes, i.e. where the charges are not carried directly on the polymer backbone, which are totally dissociated at the prevailing pH (Tsuchida & Abe, 1982). The equimolar pairing of oppositely charged groups deduced from Figs 1 and 2 indicates the formation of electroneutral complexes, at least on the macro scale, which do not seem to be dependent on the position of the charges. The distance between charges on both types of polyelectrolytes used varies with charge density. Furthermore, κ- and ι-carrageenan can exist either in coil or helix conformation which, naturally, will affect the intercharge distances (Andersson *et al.*, 1969; Paoletti *et al.*, 1984; Kvam & Grasdalen, 1989). Deviations from a one-to-one charge ratio would therefore not have been inconceivable, especially if the PECs formed had been of a ladder-type structure. Denuziere *et al.* (1996) investigated a similar system and they found the most likely structure to be a scrambled mixture of coils. The data presented here favor the scrambled coil model of the complexes formed although this is only implicated.

A variable not considered in the present investigation but which might affect the PEC structure is the precise distribution of structurally different monomeric

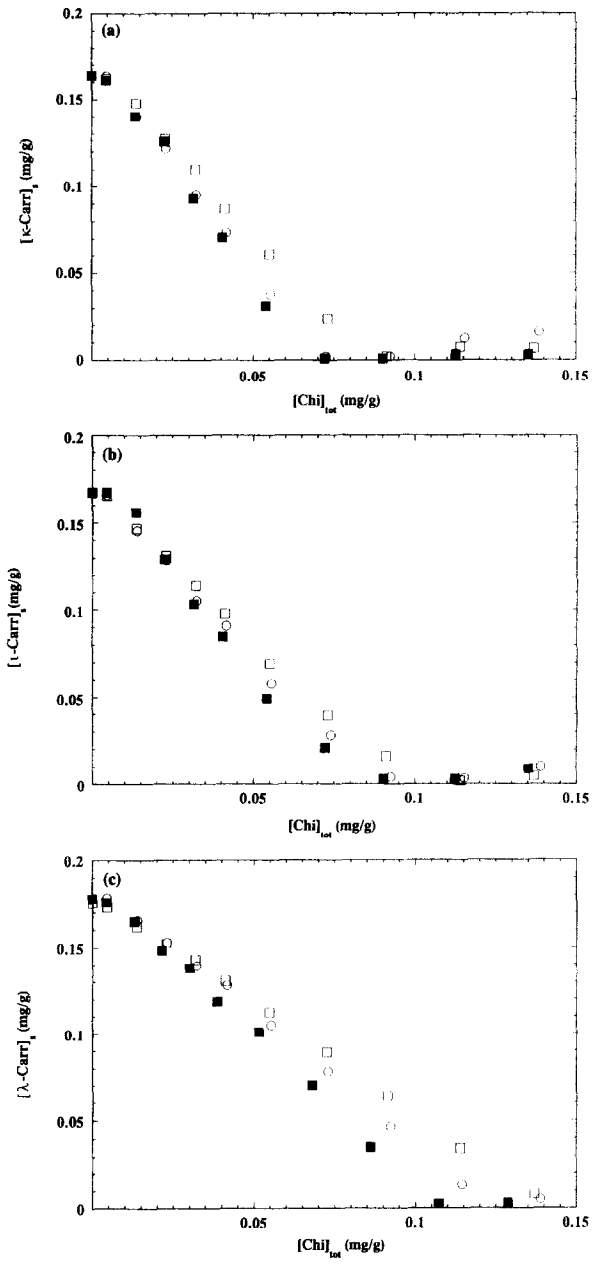

Fig. 2. Complex formation between (a) κ-carrageenan, (b) ι-carrageenan and (c) λ-carrageenan with chitosan DD 73% (□), 84% (○) and 93% (■) in 0.3 M HAc with 0.03 M NaCl.

moieties (especially if PECs of the ladder-type were to be formed). In the case of chitosan, for example, the N-acetyl- and D-glucosamine units can be either randomly distributed or arranged in blocks, depending on the origin of the sample (Aiba, 1990). In addition, κ-, ι- and λ-carrageenans, as well as the chitosans, are natural products with inherent structural heterogeneity and with a batch-to-batch variation with respect to charge density. If PECs with a known charge ratio are to be produced then the charge density of every batch must be determined.

The close relationship between charge density, other primary structural characteristics and polymer–solvent interaction on one hand and conformational properties on the other is particularly interesting when considering the carrageenans. It is well known that κ- and ι-carrageenans can undergo a thermal as well as a salt-induced helix–coil transition (McKinnon *et al.*, 1969; Rees *et al.*, 1969). This order–disorder transition of κ-carrageenan has been demonstrated to be dependent on the specific type of cation (Morris *et al.*, 1980b; Rochas & Rinaudo, 1980) as well as anion (Grasdalen & Smidsröd, 1981; Kvam & Grasdalen, 1989) used. K^+ ions are well known for their capacity to induce helix conformation and to promote helix–helix aggregation, thereby facilitating gel formation. Iodide ions, on the other hand, have been noted for their ability to stabilize κ-carrageenan in an intramolecular, ordered conformation and at the same time prevent a helix–helix aggregation process (Grasdalen & Smidsröd, 1981). Recent findings in the case of ι-carrageenan suggest, in contrast to κ-carrageenan, that ι-carrageenan does not exhibit any pronounced cation or anion dependence in the helix–coil transition which thus depends mainly on ionic strength (Austen *et al.*, 1985; Piculell *et al.*, 1987). An investigation of the effect on the formation of PEC of the conformational state of the carrageenans, as well as of the differences between ι- and κ-carrageenan with respect to ion specificity and aggregation behavior, was therefore undertaken in a variety of solvents.

Prior to the polyelectrolyte–polyelectrolyte interaction studies, the helical content at various temperatures was monitored by optical rotation (OR) measurements to ascertain the conformational state of carrageenan at ambient temperature. The fraction of κ- (0.1% w/w) and ι-carrageenan (0.4% w/w) having helical conformation (β) in the presence of KCl and NaI, at various concentrations, is given in Fig. 3 as a function of temperature. Optical rotation

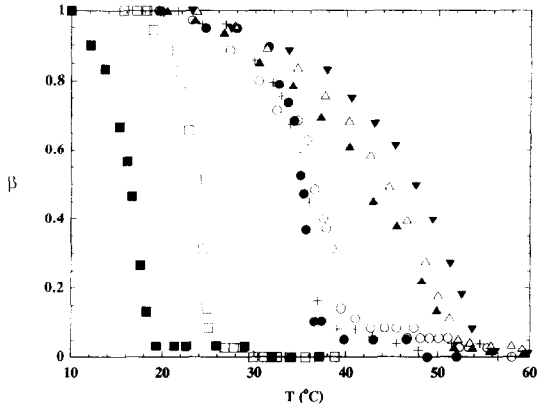

Fig. 3. Thermally induced helix–coil transition of κ-carrageenan in 0.30 M HAc + 5 mM KCl (■), 10 mM KCl (□), 30 mM KCl (+), 50 mM KCl (○) and 100 mM NaI (●), and of ι- carrageenan in 0.30 M HAc + 30 mM KCl (▲), 50 mM KCl (△) and 100 mM KCl (▼).

measurements conducted to monitor the order–disorder transitions were carried out at concentrations of about one order of magnitude higher than used in the phase separation experiments since working at the same concentrations would have resulted in barely detectable transitions. Although the coil–helix transition is displaced towards higher temperatures as the carrageenan concentration is increased (Rochas & Rinaudo, 1980; Nilsson *et al.*, 1989), the displacement is only a few degrees and will therefore not influence the following discussion. The onset of helix formation (Fig. 3) shifts towards higher temperatures both for ι- and κ-carrageenan as the ionic strength is increased for one and the same electrolyte. The transition of κ-carrageenan in 0.03 M KCl shows only minor differences compared to the 0.05 M KCl case. A probable reason for this observation is degradation in the case of 0.05 M KCl since in this instance the κ-carrageenan sample was heated on a 80°C water bath instead of the 50°C one otherwise used. Association–dissociation curves recorded for ι-carrageenan in NaCl (not shown in Fig. 3) differed negligibly compared to the KCl case, which is in agreement with the less pronounced ion specificity as described above. Furthermore ι-carrageenan proved to exist in an all-helical conformation at ambient temperature even in the solvent 0.3 M HAc without other electrolytes added. The NaCl concentrations used in the PEC experiments did not induce helix formation in the κ-carrageenan. It should be noted that the onset of helix formation of κ-carrageenan in 0.03 M KCl occurs at about the same temperature as in the case of 0.10 M NaI.

Figure 4b illustrate the effect of κ-carrageenan conformation on the intermacromolecular complex formation. It is evident from Fig. 4a that the formation of PECs is significantly affected by the fraction of κ-carrageenan present in potassium-induced helical conformation. In 0.03 and 0.05 M KCl, where κ-carrageenan is in an all-helical conformation at ambient temperature, interaction with non-equimolar amounts of chitosan produced gel-like PECs with a charge ratio less than unity with respect to chitosan. A probable scenario is that the chitosan molecules act as bridging elements between helix–helix aggregated κ-carrageenan, thus inducing gel formation. However, when an increasing amount of chitosan was added all of it was incorporated into the PEC until a stoichiometric charge ratio of unity was obtained. The interaction in 0.005 M KCl (Fig. 4a), where all κ-carrageenan existed in coil conformation, was practically identical to complexes formed in NaCl (Fig. 4b). In a subsequent experiment in 0.010 M KCl, a composition which facilitated the presence of κ-carrageenan in both coil and helix conformation, resulted in a pattern of polyelectrolyte–polyelectrolyte interaction intermediate compared to the all-helix and all-coil case. Results obtained for the interaction

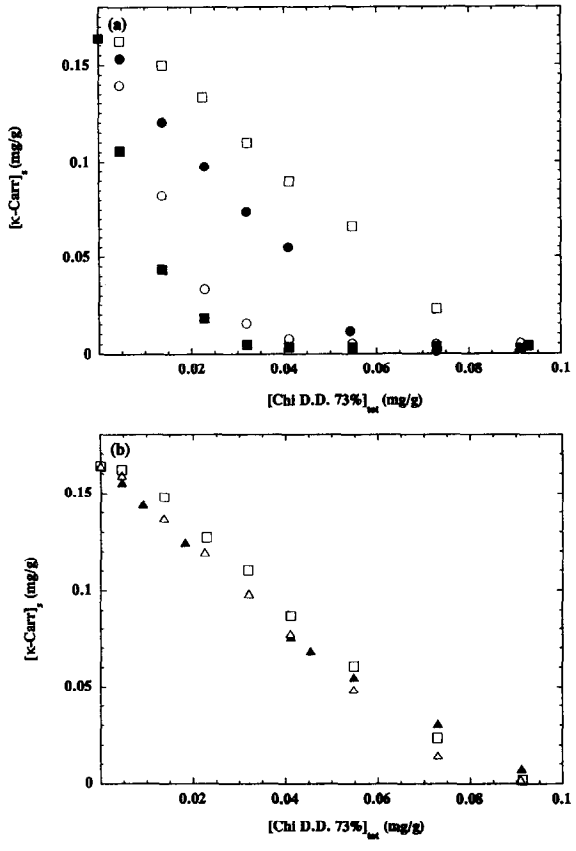

Fig. 4. (a) Effect of κ-carrageenan conformation on the intermacromolecular complex formation. κ-carrageenan in 0.3 M HAc in the presence of 5 mM KCl (□), 10 mM KCl (•), 30 mM KCl (■) and 50 mM KCl (○). (b) Effect of κ-carrageenan conformation on the intermacromolecular complex formation. κ-carrageenan in 0.3 M HAc in the presence of 30 mM NaCl (□), 100 mM NaCl (△) and 100 mM NaI (▲).

between κ-carrageenan in the (iodide-induced) non-aggregating helical state with chitosan were indistinguishable from those where κ-carrageenan was present in coil conformation at the same ionic strength (Fig. 4b). Thus the decisive conformational factor of PEC formation seems to be whether or not κ-carrageenan is in a helix–helix aggregated state.

Complex formation between ι-carrageenan and chitosan in solvents varying with respect to ionic strength and type of cation is shown in Fig. 5. Consistent with the data presented above, it appears as if the kind of cation present (if monovalent) is of secondary importance. When the ionic strength increased, less chitosan was required to bind all the available carrageenan. This effect, although limited, is evident from both Fig. 4b and Fig. 5. Thus it applies to the κ-carrageenan as well as to the ι-carrageenan case. Both carrageenan and chitosan owe their solubility in water-based solvents to their charged groups since, except for these groups, the polymer backbones are quite hydrophobic. An increase in ionic

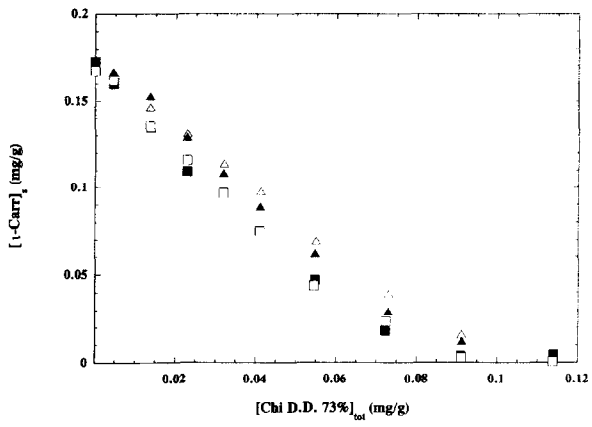

Fig. 5. The dependence of amount and type of salt added on the interaction between ι-carrageenen and chitosan (DD 73%). Concentration of salt added: 30 mM NaCl (△), 30 mM KCl (▲), 100 mM NaCl (□) and 100 mM KCl (■).

strength, therefore, which will screen the charges of the macroions, should be accompanied by a decrease in solubility. Thus a possible explanation of the results could be that the induced increase in hydrophobic character of the carrageenan molecules is larger than that of chitosan. In addition, a decrease in the dielectric constant of the solvent, caused by the acetic acid and the inorganic electrolytes present (Robinson & Stokes, 1965), has been stated to increase the tendency for phase separation (Michaeli *et al.*, 1957). As previously indicated by the helix–coil transition experiments, ι-carrageenan is always present in an all-helical conformation. This is probably a combined effect of the acetic acid, which decreases the dielectric constant of the solvent, and the carrageenan sample containing excess salt that had not been removed. The helical conformation of ι-carrageenan has been stated to be thermodynamically more stable (Morris *et al.*, 1980a; Norton *et al.*, 1983; Austen *et al.*, 1985) than its κ-carrageenan equivalent and therefore not as susceptible to the formation of helix–helix aggregates. When compared to the κ-carrageenan case the absence of any induced gel formation of the ι-carrageenan in Fig. 5 is notable. These results strengthen the hypothesis that the decisive factor is not whether the carrageenan molecules are in the helical or coil state but if aggregated helical elements are present in the solution or not.

An additional conformational parameter that might affect the polymer–polymer interaction is the stiffness of the polyelectrolytes involved. Concerning chitosan, Anthonsen *et al.* have presented data indicating that the stiffness of chitosan increases with N-acetyl content (Anthonsen *et al.*, 1993). Although the stiffness of the chitosans investigated in this study was not evaluated per se, they varied in N-acetyl content and therefore also in stiffness. However, the effect, if any, on the end result of the PEC-forming process was negligible compared to effects caused by the electrostatic interactions and the effect of κ-carrageenan conformation.

The molecular weight of the interacting polyelectrolytes could also be a factor of importance in the formation of intermacromolecular complexes (Tsuchida & Abe, 1982). However, the variation in molecular weight of the chitosans used (≈60 000–350 000, estimated by viscosimetry in accordance with a procedure described by Rinaudo *et al.* (1993)) did not affect the PEC process in our case. Concerning the carrageenans it must be taken into consideration that under the prevailing experimental conditions (pH≈2.6) the κ-, ι- and to some extent even λ-carrageenans are susceptible to hydrolytic degradation. In the case of λ-carrageenan this is mainly due to degradation of κ-carrageenan-like moieties present as impurities, either incorporated in the λ-carrageenan chain or existing as separate entities. These results have been deduced from the kinetics of degradation of κ- and λ-carrageenan samples since κ-carrageenan is degraded more rapidly than λ-carrageenan (unpublished data from work in progress in our laboratory). An experiment in which chitosan interacted with a degraded sample of κ-carrageenan resulted in the formation of a non-centrifugable fraction of carrageenan detected in the supernatant coexisting with a precipitated one. Considering the strong columbic forces involved it does not seems unlikely that soluble complexes were been formed. On fractionation by a Superdex™30 preparation grade column (Caram-Lelham *et al.*, 1995) the κ-carrageenan sample turned out to contain about 30% of oligosaccharides of the size of seven disaccharides or less. This fraction of oligosaccharides corresponded well with the amount of soluble complexes formed in the PEC experiment with degraded κ-carrageenan. Considering the results presented above it seems plausible that the PEC formation process is significantly dependent on the presence of low molecular weight components either in the form of polyelectrolyte degradation products or a low molecular fraction initially present in the sample. In preparing a polyelectrolyte complex gel from chitosan and carrageenan, caution must therefore be exercised with regard to the conditions of reaction such as pH, temperature and time to avoid an extensive formation of low molecular weight degradation products of carrageenan, the more so since the molecular weight of carrageenan also affects its toxicity.

CONCLUSION

The results presented indicate that interaction between κ-, ι- and λ-carrageenan in either coil or non-aggregating helical conformation and chitosans varying in DD normally results in the formation of PECs with

stoichiometric charge ratios of unity. If, however, the κ-carrageenan sample exists in the helix–helix aggregated state then the interaction with chitosan produces PECs with a charge ratio below unity with respect to chitosan, thereby providing a means of producing complexes with a surplus of negative charge. A moderate increase in ionic strength, which does not induce coil–helix transition, generates complexes with a charge ratio slightly less than unity, possibly due to hydrophobic effects. The investigation emphasizes the need to use well-characterized material if the PEC process is to be controlled, e.g. degradation of carrageenan resulting in the formation of low molecular weight material provides an obstacle in the regulation of the PEC process. These findings could thus be of use when selecting appropriate conditions for production of gels formed from κ-carrageenan and chitosan and may also provide a means of regulating the extent of interaction between the polyelectrolytes.

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