Conformational Changes in ι- and κ-Carrageenans Induced by Complex Formation with Bovine β-Casein

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The formation of electrostatic complexes between β-casein and ι- and κ-carrageenans is well-known. However, the molecular mechanism of the complexation has yet to be determined, particularly with respect to the conformational changes of the interacting macromolecules. High-sensitivity differential scanning calorimetry was used to study β-casein/carrageenan mixtures at different pH values (3.0 to 7.5), ionic strengths (0.03 and 0.15 M), and various molar protein/polysaccharide ratios (3−400). The effects of these variables on the temperature, enthalpy, and width of the helix−coil transition of ι- and κ-carrageenans were investigated. Neither pH nor the protein/polysaccharide ratio influenced the transition temperature of either carrageenan in the complexes. However, the transition enthalpy of both carrageenans in complexes with β-casein decreased to zero with both decreasing pH and increasing protein/polysaccharide ratio. This may reflect an unwinding of the polysaccharide double helix induced by β-casein, a conformational change which is fully reversible in conditions of sufficiently high ionic strength. The interaction of β-casein with ι- and κ-carrageenans was approximated in terms of the model of binding of large ligands to macromolecules, that provides the binding constants for these biopolymers.

Introduction

Protein−polyelectrolyte (DNA) complexes play important roles in living structures.[1−3] The increasing interest in these complexes results from their probable biological functions and their potential application in nanotechnologies,[4−6] drug and gene delivery,[7−10] encapsulation,[11,12] and the food industry.[13−15] Studies on protein−anionic polysaccharide complexes are summarized in several reviews.[3,14−16]

Electrostatic interactions play a key role in the formation of protein−anionic polysaccharide complexes which can therefore be controlled by pH, ionic strength, and the protein/polyelectrolyte ratio.[14,17,18] Soluble inter-biopolymer complexes exist mainly in dilute polymer solutions at pH values near or above the isoelectric point of the protein. A decrease in pH to below that of the isoelectric point of the protein and an increase in the bulk biopolymer concentration favor the formation of insoluble inter-biopolymer complexes. Information about conformational changes in protein−polyelectrolyte complexes in dilute solutions is limited. Formation of complexes with unstructured anionic polysaccharides decreases the conformational stability of some globular proteins.[19,20] Complex formation[3,21−24] and phase separation[3,16,25−28] in mixtures of helix-forming polysaccharides and proteins, as well as rheology[16,21,25,27,29] and gelation[25,30] of these mixtures, have been intensively studied. Nevertheless, conformational changes in ordered polysaccharides induced by complex formation with proteins have not been investigated despite their potential use as models of the nonspecific interactions between double stranded DNA, proteins, and amphiphilic polymers. The aim of this work therefore is to investigate conformational aspects of the polyelectrolyte complexes formed by β-casein with ι- and κ-carrageenans.

The carrageenans belong to a family of water-soluble, regular, linear polysaccharides originating from red marine algae. There are several types of carrageenans of differing primary structure.[31−33] κ-Carrageenan is composed of alternating α-(1−3)-D-galactose-4-sulfate and β-(1−4)-3,6-anhydro-D-galactose. Carrageenan chains of ι- and λ-types are composed of the same disaccharide units that have two and three sulfate groups, respectively, in 1,4 linked galactose units. κ- and ι-Carrageenans are gel-forming polysaccharides and are used extensively in food and pharmaceutical products as gelling, thickening, and stabilizing components.[32,33] In aqueous solutions, in the presence of cations and depending on the temperature, κ- and ι-carrageenans can have two conformations, a random coil conformation at elevated temperatures or a helical conformation at low temperatures. Thus far, however, the mechanism of the helix−coil conformational transition of the carrageenans has not been resolved.[31] In earlier work both single-helix[34−37] and intertwined double-helix[38−43] conformations were reported for ι- and κ-carrageenans. Later, the intramolecular double strand structure of ι-carrageenan appeared more probable.[44−49] For κ-carrageenan, the situation is more complicated, particularly because of the highly pronounced association of its helices.[31,50] In dilute solutions an increase in molecular weight of κ-carrageenan occurs due to the coil−helix transition.[46,49,51,52] However, other works[53−55] did not indicate changes in molecular weight of the polysaccharide related to its conformation. Thermodynamic approaches did not provide much progress in the problem of

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the helix−coil transitions in κ-carrageenan.[56] It is thought that the double helix−coil type of transition in this polysaccharide is more probable.[31,57,58] For elucidation of the mechanism of the helix−coil transition in κ-carrageenan in the intramolecular level it would be advisable to use high-sensitivity differential scanning calorimetry that was successfully applied to other helix forming polysaccharides.[45,59]

Bovine β-casein is a small protein (M = 24 kDa) constituting of about 35% of the casein fraction in cow's milk.[60] It is a polypeptide chain noted for its lack of tertiary structure,[61,62] which contains phosphoserine residues providing the Ca^{2+}-promoted precipitation of this protein.[63] At low temperatures, in dilute aqueous solutions of low ionic strength, β-casein exists as a monomer.[64−66] When the temperature increases, the monomers associate and form micelles.[64,65,67,68] The association of β-casein is sensitive to pH, ionic strength, and protein concentration,[65,69−71] but the mechanism of association[66,72,73] and the protein micelle structure[67,68,74,75] are unclear.

This work investigates conformational changes of ι- and κ-carrageenans that can be caused by the electrostatic interaction with β-casein in dilute solutions. The effects of pH and polymer ratio on the helix−coil transition of the polysaccharides were determined. The binding of the protein to the polysaccharides is described in terms of the model of binding of large ligands to macromolecules.

Experimental Section

Materials. Bovine β-casein (Eureal, France) was used without additional purification. κ-Carrageenan (Sigma) was dissolved in distilled water (5 g·L^{-1}) and mixed with 20% aqueous KCl solution to a final KCl concentration of 4%. The resulting gel was separated by centrifugation, washed several times with a 4% KCl solution, dialyzed against distilled water, and freeze-dried to obtain a potassium salt of κ-carrageenan.

ι-Carrageenan (Sigma) was dissolved in distilled water (10 g·L^{-1}), transferred to a column (250 × 25 mm) containing a cation exchanger KU-2 in H^+-form, and eluted with water. The acid eluate was immediately neutralized with solid KOH, dialyzed against distilled water, and freeze-dried to obtain a potassium salt of ι-carrageenan. The chemical composition of the polysaccharide samples was analyzed using ^{13}C and ^{1}H NMR spectroscopy. The sample of ι-carrageenan was essentially homogeneous while the κ-carrageenan sample contained about 6% ι-carrageenan. Molecular weights of the carrageenans were determined using the size-exclusion chromatography-multiangle laser light scattering (SEC-MALLS) as outlined by Tuinier et al.[76] The following molecular weights were obtained: M_n = 91 kDa for ι-carrageenan, and M_n = 294 kDa for κ-carrageenan.

Preparation of the Protein/Polysaccharide Complexes. A stock solution of β-casein was prepared in water and the pH adjusted to 9.0. The solution was filtered through a membrane (0.45 μm) and the protein concentration determined spectrophotometrically assuming $E_{280\ nm}$ = 4.6 (for 1%).[77]

Carrageenans were dissolved in 0.03 or 0.15 M KCl (for κ- and ι-carrageenan, respectively) and the solutions were heated to 90 °C and held at this temperature for 10 min. The polysaccharide concentration of the stock solution was ascertained by the dry residue method.

Protein/polysaccharide mixtures were prepared by the gradual addition of the protein stock solution to the annealed polysaccharide solution under continuous stirring. The mixture was titrated with HCl solution to a desirable pH, incubated for 30 min at room temperature, and then subjected to calorimetric measurements. The composition of the mixture was characterized by the protein/polysaccharide weight ratio, *r*, which was controlled by addition of the protein while the polysaccharide concentration was constant (0.5 and 1.0 mg·mL^{-1} for κ- and ι-carrageenan, respectively). Complexes of ι-carrageenan with β-casein were studied in 0.15 M KCl, whereas complexes of κ-carrageenan with β-casein were studied in 0.03 M KCl and in 0.03 M KCl containing 0.12 M NaCl. The KCl concentrations were chosen to provide the comparable stabilities of double helix conformations of ι- and κ-carrageenans.

High-Sensitivity Differential Scanning Calorimetry. HS-DSC measurements were carried out with differential adiabatic scanning calorimeters DASM-4 and DASM-4A ("Biopribor", Russia) within the temperature range 10−100 °C under a pressure in excess of 2 atm and a heating rate of 1 K·min^{-1}. Two consecutive scans were performed for most of the protein/polysaccharide mixtures. The remarkable difference between the first and second scans reflected the effects of thermal history of the biopolymer mixture. Where three consecutive scans were performed, the second and third scans were completely reproducible. The thermograms of the second scan were therefore used to determine the transition parameters using "Nairta" software from the Institute of Biochemical Physics, Moscow. The apparent heat capacity was normalized per gram of carrageenan in the mixture, since the contribution of the association of β-casein to the overall heat effect was negligible under given conditions. The excess heat capacity function was obtained by subtraction of a transition baseline from the apparent heat capacity function. The transition baseline was constructed by spline interpolation of the linear segments of the apparent heat capacity function before and after the transition. The transition parameters, i.e., the transition temperature, T_t, and enthalpy, $\Delta_t h$, were determined as the maximum temperature and area of the excess heat capacity peak, respectively. The transition width, $\Delta_t T$, was determined as the ratio of the transition enthalpy and the maximal value of the excess heat capacity.

Light Scattering. Measurements of light scattering, I_{90}, of the protein/polysaccharide mixed solutions were carried out at the 90° angle and at a wavelength of 436 nm using a spectrophotometer Specol (Karl Zeiss Jena, Germany) coupled with a pH-meter and titrator Radiometer (Type TTT1, Copenhagen). The mixtures were prepared at pH 9 in 0.03 M KCl (κ-carrageenan) and 0.15 M KCl (ι-carrageenan), placed into a 15 mL spectrophotometer cell, and stirred continuously. Aliquots of 0.025 M HCl were added automatically to decrease the pH of the solutions from 9.0 to 2.5 in 0.1 pH units. Computer acquisition of I_{90} values was performed as a function of pH using an analogue interface.

NMR Spectroscopy. The ^{13}C NMR spectra were recorded with a Bruker DRX 500 spectrometer at 125.76 MHz.[78] A solution of 0.5% carrageenan in H_2O at 80 °C was prepared. This solution was sonicated for 1 h in melting ice (Heat Systems XL 2020 sonicator, 1/2″ tip, power 475 W, frequency 20 kHz) and lyophilized. For the NMR analysis, the solution was dissolved in D_2O to concentrations of 5−10%, the pH was adjusted to pH 7−8 with solid Na_2HPO_4, and the solution was added to a 5 mm NMR tube.

^{1}H NMR spectra were recorded on a Bruker DRX600 spectrometer operating at 500.13 MHz at 65 °C. Typically 64 scans were taken with intervals of 20 s (the T_1 values for the resonances of κ- and ι-carrageenan were shorter than 1.5 s). Sample preparation for ^{1}H NMR experiments involved dissolving the carrageenan sample in D_2O up to a concentration of 0.5% at 80 °C and sonication for 3 (1 h) periods in a sonicator bath (Branson 2510).

SEC-MALLS Analysis. Size exclusion chromatography was performed using TSK-gel 6000PW and TSK-gel 3000PW columns (Phenomenex) in series with a TSK quard column (Phenomenex) with simultaneous detection from a ERC-7510 RI-detector (Erma Optical Works Ltd.) and a Dawn DSP-F multiangle laser light scattering detector (Wyatt Technology Corp.). A Shimadzu LC-10AT programmable HPLC pump with a Jour Research X-Act in-line degassing unit was used at a constant flow rate of 1 mL·min^{-1}. Elution was performed using a 0.1 M solution of $LiNO_3$. Data analysis was carried out using ASTRA for Windows software (Wyatt Technology Corp.). Samples were prepared by adding MilliQ water (5 mL) to carrageenan (10 mg). After storage overnight at 4 °C, samples were heated to 80 °C for 30

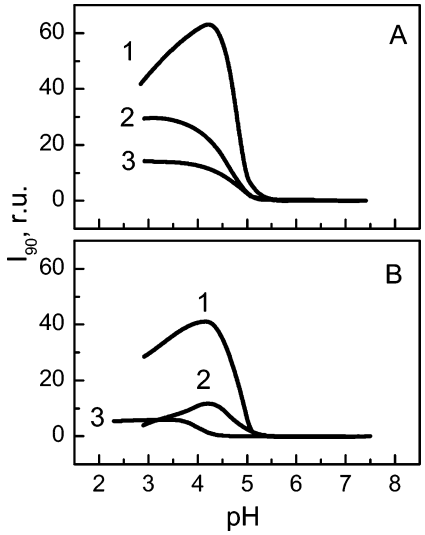

Figure 1. Nephelometric titration curves for β-casein (1) and its mixtures with ι-carrageenan (A) and κ-carrageenan (B). The protein/polysaccharide weight ratio $r = 2.5$ (2) and 0.5 (3). Solvent, 0.15 M KCl (A) and 0.03 M KCl + 0.12 M NaCl (B).

min. Prior to analysis samples were diluted with a concentrated $LiNO_3$ solution to the concentration of the eluent.

Results

Nephelometric Titration of Mixtures of β-Casein with ι- and κ-Carrageenans. β-Casein is soluble in aqueous media at basic and neutral pH values and precipitates sharply around pH 5.0, i.e., in the vicinity of its isoelectric point (pI 4.98). Figure 1 shows the β-casein solubility profile obtained by measurement of the light scattering intensity in the pH range 2.5 to 9.0. The maximum I_{90} corresponding to aggregation (precipitation) of the protein is observed at about pH 4.2 (Figure 1 A, B, curve 1). The titration curves for mixtures of β-casein with ι- (Figure 1A) and κ- (Figure 1B) carrageenans show that the light scattering intensity decreases with increasing polysaccharide content. This is particularly pronounced for the mixtures of β-casein with κ-carrageenan. For example, the mixture at $r = 0.5$ demonstrates a shift of the light scattering maximum to acid pH values (Figure 1B). This means that addition of carrageenans can extend the pH range of solubility of β-casein to a weak acid region.

HS-DSC Data for Mixtures β-Casein/ι-Carrageenan. Figure 2 displays apparent heat capacity curves for the mixture β-casein/ι-carrageenan ($r = 2.5$) in 0.15 M KCl at different pH values. At this salt concentration and in the absence of β-casein, the heat capacity curve of ι-carrageenan contains a single asymmetric peak at about 52 °C irrespective of the pH value (Figure 2A,F). This peak has been assigned to the melting of the carrageenan double helix.[45] The thermograms of the mixture β-casein/ι-carrageenan change markedly with pH (Figure 2A−F). At pH 5.8, the melting transition of ι-carrageenan is not affected by the addition of β-casein (Figure 2A) and the broad low-temperature peak in the thermogram of the mixture is related to heat-induced association of β-casein.[66] A decrease in pH reduces the height of the transition peak of ι-carrageenan without notable change in its position (Figure 2B−E). At pH 4.0 the peak of ι-carrageenan practically disappears (Figure 2F). Curves at pH 5.4 and pH 5.2 refer to soluble complexes β-casein/ι-carrageenan while insoluble complexes exist at pH < 5.0. A gradual change in the melting transition of ι-carrageenan was observed for both soluble and insoluble complexes.

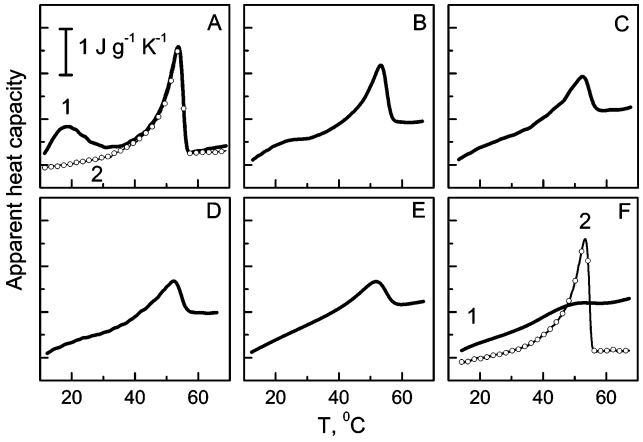

Figure 2. Heat capacity curves of ι-carrageenan (circles) and of its mixture with β-casein (solid lines) at different pH values: 5.8 (A); 5.4 (B); 5.2 (C); 5.0 (D); 4.5 (E); 4.0 (F). Solvent, 0.15 M KCl; the protein/polysaccharide weight ratio $r = 2.5$; the concentration of ι-carrageenan 1.0 mg·mL^{-1}.

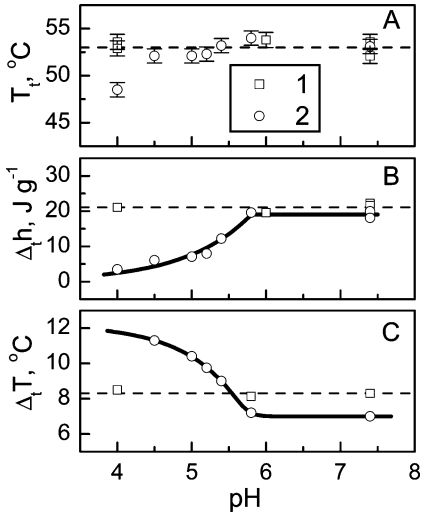

Figure 3. The helix−coil transition temperature (A), enthalpy (B), and width (C) of ι-carrageenan in the reference solution (1) and in the mixtures with β-casein (2) at different pH values. The dashed lines indicate the average values of the transition parameters for ι-carrageenan alone. Solvent, 0.15 M KCl; the protein/polysaccharide weight ratio $r = 2.5$; the concentration of ι-carrageenan 1.0 mg·mL^{-1}.

Thus, the formation of a complex with β-casein affects the ordered structure of ι-carrageenan. Formation of a complex also completely suppresses the micellization of β-casein (Figure 2B−F) thus shifting the monomer−micelle equilibrium toward the monomeric form of the protein.

Figure 3 shows three main helix−coil transition parameters of ι-carrageenan in the mixtures with β-casein at different pH values. These are the transition temperature, T_t; the transition enthalpy, $\Delta_t h$; and the transition width, $\Delta_t T$. The transition temperature of ι-carrageenan in the complexes with β-casein does not change significantly upon changing pH and is close to that of ι-carrageenan alone (Figure 3A). The transition enthalpy of ι-carrageenan decreases on decreasing pH and disappears at pH below 4.0 (Figure 3B). The transition width of ι-carrageenan in the mixtures with β-casein increases with decreasing pH (Figure 3C), which reflects changes in the cooperativity of the transition. Transition temperature, transition width, and enthalpy of the polysaccharide in reference solution do not change with pH.

Heat capacity curves of mixed β-casein/ι-carrageenan solutions when the protein/polysaccharide ratio r is increased, are

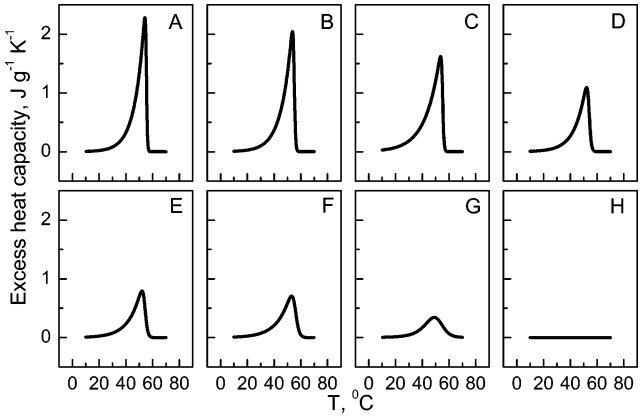

Figure 4. Excess heat capacity curves of ι-carrageenan in the reference solution (A) and in the mixtures with β-casein (B−H) at different values of the protein/polysaccharide weight ratio $r = 0.5$ (B); 1.0 (C); 2.0 (D); 2.5 (E); 3.5 (F); 5.0 (G); 10 (H) and pH 5.0. Solvent, 0.15 M KCl; the concentration of ι-carrageenan 1.0 mg·mL^{-1}.

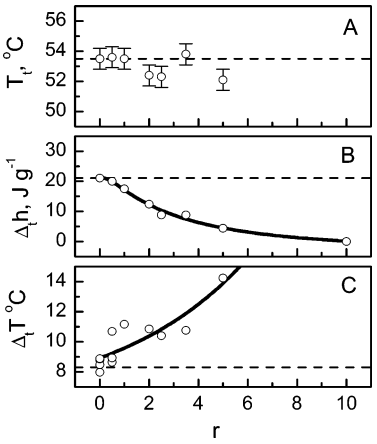

Figure 5. The helix−coil transition temperature (A), enthalpy (B), and width (C) of ι-carrageenan in the mixtures with β-casein as a function of the protein/polysaccharide weight ratio *r*. The dashed lines indicate the average values of the transition parameters for ι-carrageenan in the reference solution. Solvent, 0.15 M KCl; pH 5.0; the concentration of ι-carrageenan 1.0 mg·mL^{-1}.

shown in Figure 4. The overall effect at pH 5 (Figure 4) is similar to that observed on decreasing pH (Figure 2): the transition heat capacity peak of ι-carrageenan decreases in height with an increase in protein content in the mixture while the position of the peak does not change.

Figure 5 shows the effect of the protein/polysaccharide ratio on the transition parameters of ι-carrageenan for the mixed β-casein/ι-carrageenan solutions. The transition temperature of ι-carrageenan is not significantly dependent on the protein content (Figure 5A). The transition enthalpy decreases to zero on increasing the protein content (Figure 5B) while the transition width increases with an increase in the protein/polysaccharide ratio (Figure 5C).

Thus, both a decrease in pH and an increase in the protein/polysaccharide ratio similarly affect the helix−coil transition of ι-carrageenan in mixtures with β-casein. Both these factors significantly reduce the cooperativity of the helix−coil transition.

Figure 6 shows that in the β-casein/ι-carrageenan mixture with $r = 2.5$, at pH 4.0 and 0.15 M KCl, the ι-carrageenan helix is practically removed (curve 1). Addition of 0.45 M NaCl to the mixed solution results in a cooperative transition with a large enthalpy (curve 2). Both position and profile of the curve are close to those of ι-carrageenan in 0.15 M KCl + 0.45 M NaCl

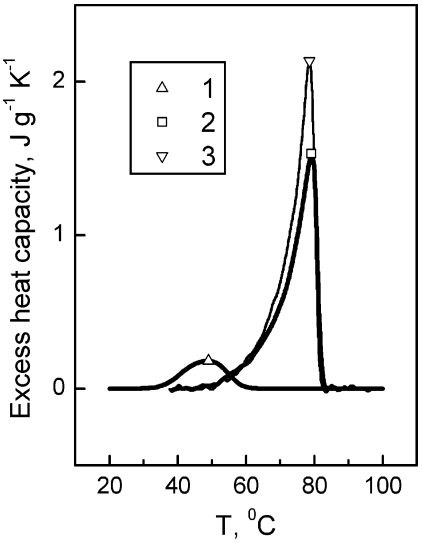

Figure 6. Excess heat capacity curves of ι-carrageenan in the mixture with β-casein ($r = 2.5$) in 0.15 M KCl (1), in the same mixture after addition of 0.45 M NaCl (2) and of ι-carrageenan alone in 0.15 M KCl + 0.45 M NaCl (3) at pH 4.0.

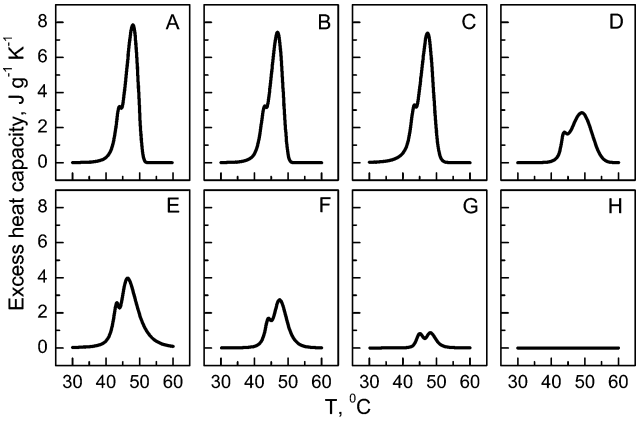

Figure 7. Excess heat capacity curves of κ-carrageenan in the reference solution (A) and in the mixtures with β-casein (B−H) at different pH values: 7.3 (A, B); 6.5 (C); 5.8 (D); 5.5 (E); 5.2 (F); 5.0 (G); 4.0 (H). Solvent, 0.03 M KCl; the protein/polysaccharide weight ratio $r = 2.5$; the concentration of κ-carrageenan 0.5 mg·mL^{-1}.

(curve 3). The enthalpy is recovered by about 80%. Consequently, screening of electrostatic interactions by the high salt concentration results in dissociation of the protein/polysaccharide complex and a recovery of the polysaccharide helical structure.

HS-DSC Data for Mixtures β-Casein/κ-Carrageenan. To compare the influence of β-casein on helical structure of κ- and ι-carrageenan, the effects of the protein/polysaccharide weight ratio and pH of mixed β-casein/κ-carrageenan solutions have been studied at two ionic strength values. The ionic strength of 0.03 M KCl provided approximately the same stability of double helix conformation of κ-carrageenan as that of ι-carrageenan in 0.15 M KCl. Additionally, the ionic strength 0.03 M KCl + 0.12 M NaCl used for κ-carrageenan seems to provide comparable screening of electrostatic interactions of κ- and ι-carrageenans with β-casein.

Figure 7 shows the excess heat capacity curves for β-casein/κ-carrageenan solutions over a range of pH values, at $r = 2.5$ and at KCl concentration of 0.03 M. At this ionic strength the heat capacity curve of κ-carrageenan has a main peak at about 50 °C and a small shoulder at 43 °C (Figure 7A). The minor transition may correspond to a cooperative dissociation of helical

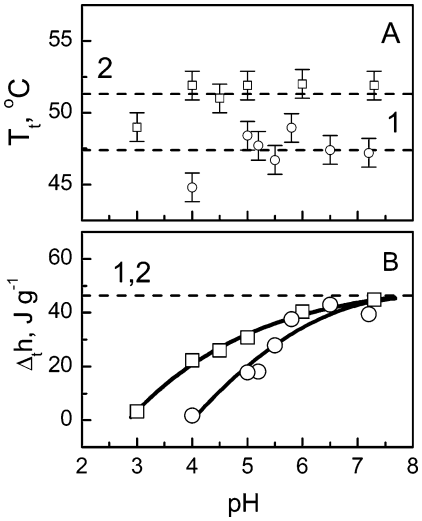

Figure 8. The order−disorder transition temperature (A) and enthalpy (B) of κ-carrageenan in the mixtures with β-casein at different pH values. The dashed lines indicate the average values of the transition parameters of κ-carrageenan in the absence of the protein. Solvent: 0.03 M KCl (circles, 1); 0.03 M KCl + 0.12 M NaCl (squares, 2). The protein/polysaccharide weight ratio r = 2.5; the concentration of κ-carrageenan 0.5 mg·mL^{-1}.

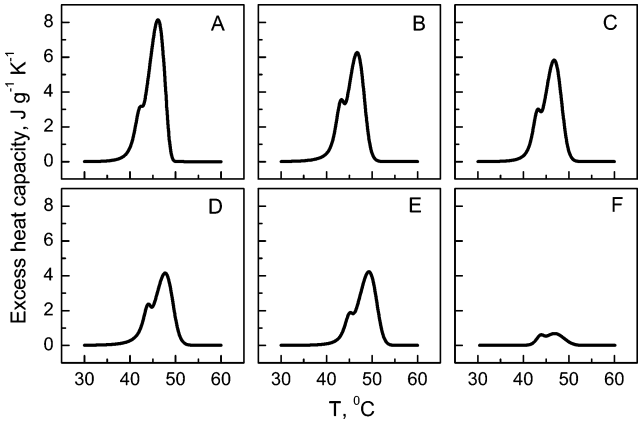

Figure 9. Heat capacity curves of κ-carrageenan in the reference solution (A) and in the mixtures with β-casein (B−F) at different protein/polysaccharide weight ratios, r = 0.6 (B); 1.2 (C); 1.4 (D); 2.5 (E); 5.0 (F) at pH 5.0. Solvent, 0.03 M KCl; the concentration of κ-carrageenan 0.5 mg·mL^{-1}.

aggregates, which have been observed in KCl solutions of κ-carrageenan.[51,52] The main peak seems to reflect the melting of κ-carrageenan helices. At pH 7.3 and 6.5 the order−disorder transition of κ-carrageenan is not affected by the added β-casein (Figure 7B,C), but a further decrease in pH specifically changes the thermograms of the β-casein/κ-carrageenan mixture. At pH values of 5.8, 5.5, and 5.2, the height of the κ-carrageenan transition peak decreases without apparent change in its position (Figure 7D−F). At pH 5.0 the main κ-carrageenan transition peak becomes similar in area to the low-temperature peak (Figure 7G). At pH 4.0 the β-casein/κ-carrageenan mixture does not reveal any cooperative transitions, perhaps due to the complete loss of the polysaccharide helical structure (Figure 7H). Similar effects of the protein on the heat capacity curves of κ-carrageenan were observed in 0.03 M KCl + 0.12 M NaCl (data not shown).

Figure 8 represents two calorimetric parameters of the κ-carrageenan order−disorder transition; the transition temperature, T_t, and enthalpy, $\Delta_t h$. The transition width was analyzed qualitatively, because of the bimodal profile of the κ-carrageenan

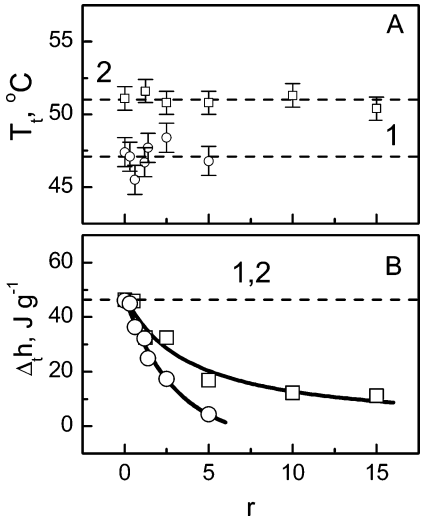

Figure 10. The order−disorder transition temperature (A) and enthalpy (B) of κ-carrageenan in the mixtures with β-casein as a function of the protein/polysaccharide weight ratio r at pH 5.0. The dashed lines indicate the average values of the transition parameters in the absence of the protein. Solvent, 0.03 M KCl (circles, 1) and 0.03 M KCl + 0.12 M NaCl (squares, 2); the concentration of κ-carrageenan 0.5 mg·mL^{-1}.

transition. The transition temperature of κ-carrageenan at an ionic strength of 0.15 M exceeds that at 0.03 M by 4 °C, while the transition enthalpy is the same at both ionic strengths. As in the case with ι-carrageenan, both transition parameters of κ-carrageenan in the reference solution do not depend on pH. The effects of pH on the transition temperature and enthalpy of κ-carrageenan in mixtures with β-casein at ionic strength of 0.03 and 0.15 M are also shown in Figure 8. The transition temperature of κ-carrageenan in the mixtures is close to that of κ-carrageenan alone and does not depend on pH (Figure 8A). This is typical of both low and high ionic strengths. The transition enthalpy of κ-carrageenan in the mixtures with β-casein decreases with a decrease in pH (Figure 8B), disappearing at pH about 4.0 and 3.0 at the low and high ionic strength, respectively. It is noteworthy that an increase in ionic strength from 0.03 to 0.15 M affects both transition parameters of κ-carrageenan in the mixtures with β-casein, similarly. This could mean that β-casein/κ-carrageenan complexes are rather stable to changes in ionic strength.

The heat capacity curves of β-casein/κ-carrageenan mixtures with increasing protein/polysaccharide ratio r, at pH 5.0 and ionic strengths of 0.03 and 0.15 M are shown in Figure 9. The overall effect is similar to that observed on decreasing pH (Figure 7). The height of the transition heat capacity peak of κ-carrageenan decreases with an increase in the protein content (Figure 9B−F), but the position of the peak does not change.

In Figure 10 the transition parameters of κ-carrageenan in mixtures with β-casein are shown as a function of the protein/polysaccharide ratio. The transition temperature of κ-carrageenan is not dependent on the protein content (Figure 10A), but the transition enthalpy decreases to zero with increasing protein content (Figure 10B). The decrease in enthalpy is less pronounced at an ionic strength of 0.15 M than at 0.03 M due to higher electrostatic screening of the protein−polysaccharide interaction.

Thus, conformational changes in ι- and κ-carrageenans induced by electrostatic complex formation with β-casein have a common feature: a reduction of the carrageenan helicity

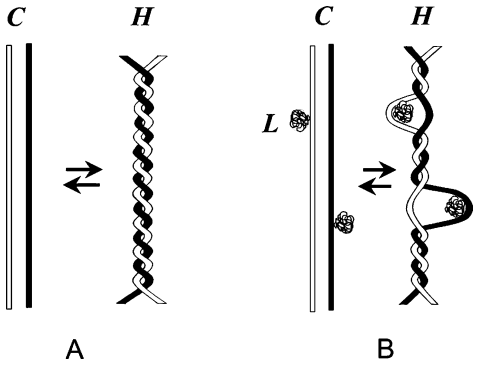

Figure 11. A simplified scheme of the coil−helix transition in carrageenans: A, in reference solution; B, in complexes with β-casein. *C* and *H* represent the random coil and double helix conformations of the polysaccharide, respectively; *L* is the monomeric form of β-casein.

without changing the helix stability. Factors responsible for this effect are pH and increasing content of the protein in the complexes.

Discussion

The main result from the complex formation of ι- and κ-carrageenans with β-casein is a decrease in the polysaccharide ordering degree. At ionic strengths of 0.15 M KCl for ι-carrageenan, and 0.03 M KCl and 0.03 M KCl/0.12 M NaCl for κ-carrageenan, both polysaccharides seem to exist at low temperatures in the form of double helices or associated single helices (helix dimers). Schemes of the carrageenan order−disorder transitions in the absence and presence of β-casein are shown in Figure 11.

The coil−helix conformational transition in the β-casein/carrageenan complexes can be represented by the following stoichiometric equation:

$$2[C]_{N-n(r)}L_{\nu(r)} \rightleftarrows \{[H]_{N-n(r)}L_{\nu(r)}\}_2 \quad (1)$$

where *C* and *H* are the coil and helical state of the disaccharide repeating unit, respectively; *N* is the total number of the repeating units in polysaccharide; $n(r)$ is the number of the repeating units excluded from the helix formation because of attachment of $\nu(r)$ molecules of the protein ligand *L*, *r* is the protein/polysaccharide weight ratio. If one protein molecule blocks *l* repeating units, then $n(r) = l\nu(r)$. It is reasonable to assume that the transition enthalpy is proportional to the number of the repeating units participating in the transition:

$$\Delta_t h(r) \propto N - n(r) \quad (2)$$

In this case the relative change of the transition enthalpy due to the complex formation can be expressed as follows:

$$\frac{\Delta_t h(r)}{\Delta_t h(0)} = \frac{N - n(r)}{N} = 1 - \frac{n(r)}{N} = 1 - \frac{l\nu(r)}{N} \quad (3)$$

The value $\phi(r) = 1 - l\nu(r)/N$ characterizes the fraction of sites (i.e., the polysaccharide repeating units) occupied by the bound protein molecules. It can be calculated in terms of the model of binding of large ligands by macromolecules.[79] According to this model the binding isotherm is written in the form:

$$\phi\left[1 - \left(1 - \frac{1}{l}\right)\phi\right] - Kl(L_T(r) - \nu_{max}\phi M_T)(1 - \phi)^l = 0 \quad (4)$$

Table 1. Parameters of Binding of β-Casein to ι- and κ-Carrageenans

polysaccharide	salt concentration	N[a]	l[b]	$l/2\langle R^2\rangle^{0.5}$ [c]	K,[d] M^{-1}
ι-carrageenan	0.15 M KCl	168	9.4	1.1	5.8×10^4
κ-carrageenan	0.03 M KCl + 0.12 M NaCl	693	9.5	1.1	2.2×10^4
κ-carrageenan	0.03 M KCl	693	15.2	1.7	1.7×10^7

[a] *N* is the number of disaccharide repeating units per polysaccharide chain calculated from the number-average molecular weight of the polysaccharide (91 and 294 kDa for ι- and κ-carrageenan, respectively, determined by SEC-MALLS) and the molecular weight of the K^+-form of the corresponding disaccharide (543 and 424 for ι- and κ-carrageenan, respectively). [b] *l* is the apparent size of the bound protein expressed as the number of the disaccharide repeating units. [c] $\langle R^2\rangle^{0.5} = 4.6$ nm is the gyration radius of the protein.[64] [d] *K* is the intrinsic binding constant.

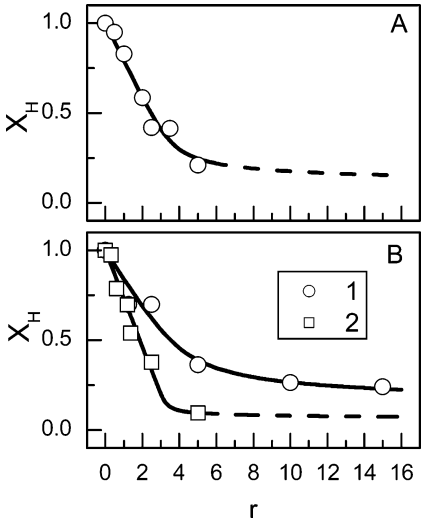

Figure 12. Helicity, $X_H = \Delta_t h(r)/\Delta_t h(0)$, of ι-carrageenan (A) and κ-carrageenan (B) in complexes with β-casein as a function of the protein/polysaccharide weight ratio *r*. Solvent: 0.15 M KCl (A); 0.03 M KCl + 0.12 M NaCl (B, 1) and 0.03 M KCl (B, 2). Lines represent the best fits of eqs 3 and 4 to the experimental data (see comments in the text).

where *K* is the intrinsic binding constant; $L_T(r)$ is the total molar concentration of the ligand that is a trivial function of the protein/polysaccharide weight ratio *r* at a constant weight polysaccharide concentration; $\nu_{max} = N/l$; M_T is the total molar concentration of the polysaccharide.

Equations 3 and 4 were used for approximation of the experimental dependences of the transition enthalpy on the protein/polysaccharide weight ratio upon variation of an apparent size of the protein ligand *l* and of the intrinsic binding constant *K*. The best fit values of these parameters are given in Table 1, and the corresponding calculated curves are shown in Figure 12.

At ionic strength 0.15 the values of the parameter *l* for ι- and κ-carrageenans coincide well (9.4 and 9.5 repeating units, respectively). Assuming the length of the repeating unit to be equal to the distance between the neighbor charges in κ-carrageenan, that is 1.03 nm,[80] an apparent length of the bound β-casein at ionic strength 0.15 will be of about 9.7−9.8 nm. This is in a good agreement with the diameter of this protein in a random coil conformation $2\langle R^2\rangle^{0.5} = 9.2$ nm.[64] However, for κ-carrageenan at ionic strength of 0.03, $l = 15.4$, i.e. the apparent length of the bound β-casein exceeds its coil size by a factor of 1.7. This is probably a consequence of the coil deformation (extension) upon binding. Such an extension is greatly possible at a very low ionic strength, when electrostatic interaction

between protein and polysaccharide is most pronounced. Upon binding, the protein adopts a more asymmetric conformation that could provide a more tight contact between charged groups of the protein and polysaccharide.

Under conditions of similar electrostatic screening (at ionic strength of 0.15 M) the binding constants for ι- and κ-carrageenan are equal to 5.8×10^4 and 2.2×10^4 M^{-1}, respectively. These values fall into the range of binding constants reported for nonspecific binding of proteins to the double helix of DNA.[81,82] The higher value of binding constant for ι-carrageenan is in accordance with the higher charge density of this polysaccharide as compared to κ-carrageenan. This fact is not surprising and obviously reflects an electrostatic nature of the β-casein/carrageenan binding. Decreasing the ionic strength from 0.15 to 0.03 M increases the binding constant for κ-carrageenan by about of 3 orders of magnitude (Table 1). Such a high sensitivity to salt concentration makes the protein−carrageenan interaction similar to the protein−DNA interactions. A sharp dependence of constants of nonspecific protein binding by DNA is known to be a typical feature of DNA,[83,84] suggesting that the main driving force of these interactions involves release of counterions accompanying the protein−polyelectrolyte complex formation.

Functional properties of carrageenans, in particular rheological properties of the gels, are determined by relative contents of helical regions and kinks in the polysaccharide molecule. Prevalence of any of these structural motifs adversely affects the rheological properties of the gels, but incorporation of optimal quantities allows desirable rheological properties to be obtained.[85,86] Partly helical complexes of carrageenans with proteins are therefore of great interest for various applications in food technology, pharmacology, and biotechnology.

Conclusions

β-Casein forms stable electrostatic complexes with κ- and ι-carrageenans which are soluble in a considerably wider pH range than β-casein alone. β-Casein reduces helicity of the carrageenans because of preferential interaction with unordered parts of the polysaccharide chains. Dissociation of the complexes and recovery of the helical structure of carrageenans may be attained by increasing the ionic strength. The effect of β-casein on the ordering of carrageenans can be important to obtain optimal functional properties, such as rheology, gel formation, and encapsulation, of these polysaccharides.

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References and Notes

(1) *Glycosciences. Status and Perspectives*; Gabius, H.-J.; Gabius, S., Eds.; Chapman and Hall: London, 1997.
(2) Toida, T.; Chaidedgumjorn, A.; Linhardt, R. J. *Trends Glycosci. Glycotechnol.* **2003**, *15*, 29.
(3) Tolstoguzov, V. B. *Crit. Rev. Biotech.* **2002**, *22*, 89.
(4) Yang, C. C.; Chen, C. C.; Chang, H. M. *J. Food Sci.* **1998**, *63*, 962.
(5) Stamatis, H.; Xenakis, A. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 399.
(6) Roy, I.; Gupta, M. N. *J. Chromatogr. A* **2003**, *998*, 103.
(7) Picker, K. M. *Drug Dev. Ind. Pharm.* **1999**, *25*, 339.
(8) Azzam, T.; Raskin, A.; Makovitzki, A.; Brem, H.; Vierling, P.; Lineal, M.; Domb, A. J. *Macromolecules* **2002**, *35*, 9947.
(9) Patil, R. T.; Speaker, T. J. *J. Pharm. Sci.* **2000**, *89*, 9.
(10) Miguel, M. G.; Pais, A. A. C. C.; Dias, R. S.; Leal, C.; Rosa, M.; Lindman, B. *Colloids Surf. A* **2003**, *228*, 43.
(11) Navratil, M.; Sturdik, E. *Chem. Listy* **2000**, *94*, 380.
(12) Van de Velde, F.; Lourenco, N. D.; Pinheiro, H. M.; Bakker, M. *Adv. Synth. Catal.* **2002**, *344*, 815.
(13) Glicksman, M. *Gum Technology in the Food Industry*; Academic Press: New York, 1969.
(14) Schmitt, C.; Sanchez, C.; Desorby-Banon, S.; Hardy, J. *Crit. Rev. Food Sci. Nutr.* **1998**, *38*, 689.
(15) De Kruif, C. G.; Tuinier, R. *Food Hydrocolloids* **2001**, *15*, 555.
(16) Doublier, J. L.; Garnier, C.; Renard, D.; Sanchez, C. *Curr. Opin. Colloid Interface Sci.* **2000**, *5*, 202.
(17) Weinbreck, F. *Whey protein/polysaccharide coacervates: structure and dynamics*; Utrecht University: Utrecht, The Netherlands, 2004.
(18) Tolstoguzov, V. B. In *Food Proteins and Their Applications*; Damodaran, S.; Paraf, A., Eds.; Marcel Dekker, Inc.: New York, 1997; pp 171−198.
(19) Burova, T. V.; Grinberg, N. V.; Grinberg, V. I.; Leontiev, A. L.; Tolstoguzov, V. B. *Carbohydr. Polym.* **1992**, *18*, 101.
(20) Burova, T. V.; Varfolomeeva, E. P.; Grinberg, V. Y.; Haertle, T.; Tolstoguzov, V. B. *Macromol. Biosci.* **2002**, *2*, 286.
(21) Dickinson, E. *Trends Food Sci. Technol.* **1998**, *9*, 347.
(22) Galazka, V. B.; Smith, D.; Ledward, D. A.; Dickinson, E. *Food Chem.* **1999**, *64*, 303.
(23) Galazka, V. B.; Smith, D.; Ledward, D. A.; Dickinson, E. *Food Hydrocolloids* **1999**, *13*, 81.
(24) Tadera, K.; Minami, Y.; Chohchi, M. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1840.
(25) Baeza, R. I.; Carp, D. J.; Perez, O. E.; Pilosof, A. M. R. *Lebensm.-Wiss. Technol.* **2002**, *35*, 741.
(26) Bourriot, S.; Garnier, C.; Doublier, J. L. *Carbohydr. Polym.* **1999**, *40*, 145.
(27) Garnier, C.; Michon, C.; Durand, S.; Cuvelier, G.; Doublier, J. L.; Launay, B. *Colloids Surf., B* **2003**, *31*, 177.
(28) Thaiudom, S.; Goff, H. D. *Int. Dairy J.* **2003**, *13*, 763.
(29) Haug, I.; Williams, M. A. K.; Lundin, L.; Smidsrod, O.; Draget, K. I. *Food Hydrocolloids* **2003**, *17*, 439.
(30) Langendorff, V.; Cuvelier, G.; Launay, B.; Parker, A. *Food Hydrocolloids* **1997**, *11*, 35.
(31) Piculell, L. In *Food Polysaccharides and Their Applications*; Stephan, A. M., Ed.; Marcel Dekker: New York, 1995; pp 205−244.
(32) Imeson, A. P. In *Handbook of Hydrocolloids*; Philips, G.; Williams, P. A., Eds.; Woodhead Publishing: Cambridge, 2000; pp 87−102.
(33) Van de Velde, F.; de Ruiter, G. A. In *Polysaccharides II: Polysaccharides from Eukaryotes*; Steinbuchel, A.; De Baets, S.; Van Damme, E. J., Eds.; Wiley-VCH: Weinheim, Germany **2002**, 245−274.
(34) Smidsrod, O.; Andresen, I.-L.; Grasdalen, H.; Larsen, B.; Painter, T. *Carbohydr. Res.* **1980**, *80*, C11.
(35) Rochas, C.; Rinaudo, M. *Carbohydr. Res.* **1982**, *105*, 227.
(36) Paoletti, S.; Smidsrod, O.; Grasdalen, H. *Biopolymers* **1984**, *23*, 1771.
(37) Smidsrod, O.; Grasdalen, H. *Hydrobiologia* **1984**, *116/117*, 178.
(38) Anderson, N. S.; Campbell, J. W.; Harding, M. M.; Rees, D. A.; Samuel, J. W. B. *J. Mol. Biol.* **1969**, *45*, 85.
(39) Rees, D. A.; Steele, I. W.; Williamson, F. B. *J. Polym. Soc. C* **1969**, *28*, 261.
(40) Rees, D. A.; Scott, W. E.; Williamson, F. B. *Nature* **1970**, *227*, 390.
(41) Rees, D. A.; Williamson, F. B.; Frangou, S. A.; Morris, E. R. *Eur. J. Biochem.* **1982**, *122*, 71.
(42) Jones, R. A.; Staples, E. J.; Penman, A. *J. Chem. Soc., Perkin Trans. 1* **1973**, *2*, 1608.
(43) Norton, I. T.; Goodall, D. M.; Morris, E. R.; Rees, D. A. *J. Chem. Soc. Faraday Trans.* **1983**, *79*, 2475.
(44) Abeysekera, R. M.; Bergstrom, E. T.; Goodall, D. M.; Norton, I. T.; Robards, A. W. *Carbohydr. Res.* **1993**, *248*, 225.
(45) Grinberg, V. Y.; Grinberg, N. V.; Usov, A. I.; Shusharina, N. P.; Khokhlov, A. R.; de Kruif, K. G. *Biomacromolecules* **2001**, *2*, 864.
(46) Hjerde, T.; Smidsrod, O.; Christensen, B. E. *Biopolymers* **1999**, *49*, 71.
(47) Lee, I.; Atkins, E. D. T.; Miles, M. J. *Ultramicroscopy* **1992**, *42−44*, 1107.
(48) Stokke, B. T.; Elgsaeter, A.; Kitamura, S. *Int. J. Biol. Macromol.* **1993**, *15*, 63.
(49) Viebke, C.; Borgstrom, J.; Piculell, L. *Carbohydr. Polym.* **1995**, *27*, 145.
(50) Wittgren, B.; Borgstrom, J.; Wahlund, K.-G. *Biopolymers* **1998**, *45*, 85.
(51) Mangione, M. R.; Giacomazza, D.; Bulone, D.; Martorana, V.; San Biagio, P. L. *Biophys. Chem.* **2003**, *104*, 95.

(52) Ueda, K.; Itoh, M.; Matsuzaki, Y.; Ochiai, H.; Imamura, A. *Macromolecules* **1998**, *31*, 675.
(53) Bongaerts, K.; Reynaers, H.; Zanetti, F.; Paoletti, S. *Macromolecules* **1999**, *32*, 675.
(54) Slootmaekers, D.; De Jonghe, C.; Reynaers, H.; Varkevisser, F. A.; Bloys van Treslong, C. J. *Int. J. Biol. Macromol.* **1988**, *10*, 160.
(55) Vanneste, K.; Slootmaekers, D.; Reynaers, H. *Food Hydrocolloids* **1996**, *10*, 99.
(56) Viebke, C.; Borgstrom, J.; Carlsson, I.; Piculell, L.; Williams, P. *Macromolecules* **1998**, *31*, 1833.
(57) Ciszkowska, M.; Kotlyar, I. *Anal. Chem.* **1999**, *71*, 5013.
(58) Ciszkowska, M.; Guillaume, M. D.; Kotlyar, I. *Chem. Anal. (Warsaw)* **2000**, *45*, 167.
(59) Burova, T. V.; Golubeva, I. A.; Grinberg, N. V.; Mashkevich, A. Y.; Grinberg, V. Y.; Usov, A. I.; Navarini, L.; Cesaro, A. *Biopolymers* **1996**, *39*, 517.
(60) Payens, T. A. J.; Vreeman, H. J. In *Solution behavior of surfactants*; Mittel, K. L.; Fendler, E. J., Eds.; Plenum Press: New York, 1982; pp 543−571.
(61) Ribadeau-Dumas, B.; Bringnon, G.; Grosclaude, F.; Mercier, J. C. *Eur. J. Biochem.* **1972**, *25*, 505.
(62) Berry, G. P.; Creamer, L. K. *Biochemistry* **1975**, *14*, 3542.
(63) Guo, C.; Campbell, B. E.; Chen, K.; Lenhoff, A. M.; Velev, O. D. *Colloids Surf. B* **2003**, *29*, 297.
(64) Andrews, A. Z.; Atkinson, D.; Evans, M. T. A.; Finer, E. G.; Green, J. P.; Phillips, M. C.; Robertson, R. N. *Biopolymers* **1979**, *18*, 1105.
(65) Arima, S.; Niki, R.; Takase, K. *J. Dairy Res.* **1979**, *46*, 281.
(66) Mikheeva, L. M.; Grinberg, N. V.; Grinberg, V. Y.; Khokhlov, A. R.; De Kruif, C. G. *Langmuir* **2003**, *19*, 2913.
(67) Kajiwara, K.; Niki, R.; Urakawa, H.; Hiragi, Y.; Donkai, N.; Nagura, M. *Biochim. Biophys. Acta* **1988**, *955*, 128.
(68) Leclerc, E.; Calmettes, P. *Phys. Rev. Lett.* **1997**, *78*, 150.
(69) Payens, T. A. J.; Brinkhuis, J. A.; Markwijk, B. W. *Biochim. Biophys. Acta* **1969**, *175*, 434.
(70) Schmidt, D. G.; Payens, T. A. J. *J. Colloid Interface Sci.* **1972**, *39*, 655.
(71) Pearce, K. N. *Eur. J. Biochem.* **1975**, *58*, 23.
(72) Tai, M.; Kegeles, G. *Biophys. Chem.* **1984**, *20*, 81.
(73) Farrell, H. M.; Wickham, E. D.; Unruh, J. J.; Qi, P. X.; Hoagland, P. D. *Food Hydrocolloids* **2001**, *15*, 341.
(74) Leclerc, E.; Calmettes, P. *Physica. B* **1997**, *241*, 1141.
(75) Leclerc, E.; Calmettes, P. *Physica B* **1997**, *234*, 207.
(76) Tuinier, R.; Zoon, P.; Olieman, C.; Cohen Stuart, M. A.; Fleer, G. J.; de Kruif, K. G. *Biopolymers* **1999**, *49*, 1.
(77) Thompson, M. P.; Pepper, I. J. *J. Dairy Res.* **1964**, *47*, 633.
(78) Van de Velde, F.; Knutsen, S. H.; Usov, A. I.; Rollema, H. S.; Cerezo, A. S. *Trends Food Sci. Technol.* **2002**, *13*, 73.
(79) McGee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469.
(80) Vreeman, H. J.; Snoeren, T. H.; Payens, T. A. J. *Biopolymers* **1980**, *19*, 1357.
(81) Lohman, T. M.; Mascotti, D. P. *Methods Enzymol.* **1992**, *212*, 400.
(82) Record, M. T., Jr.; Spolar, R. S. In *Nonspecific DNA−Protein Interactions*; Revsin, A., Ed.; CRC Press: Boca Raton, FL, 1990; pp 33−69.
(83) Lohman, T. M. *CRC Crit. Rev. Biochem.* **1986**, *19*, 191.
(84) Record, M. T., Jr.; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, *11*, 103.
(85) Van de Velde, F.; Rollema, H. S.; Grinberg, N. V.; Burova, T. V.; Grinberg, V. Y.; Tromp, R. H. *Biopolymers* **2002**, *65*, 299.
(86) Van de Velde, F.; Antipova, A. S.; Rollema, H. S.; Burova, T. V.; Grinberg, N. V.; Pereira, L.; Gilsenan, P. M.; Tromp, R. H.; Rudolph, B.; Grinberg, V. Ya. *Carbohydr. Res.* **2005**, *340*, 1113.

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