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Macromolecular complexes of lysozyme with kappa carrageenan

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Abstract: We present a structural study of the complexation and binding of lysozyme (Lys) with kappa carrageenan (kCG) by means of turbidity measurements, phase analysis, dynamic and electrophoretic light scattering, differential scanning microcalorimetry (DSMC), confocal laser scanning (CLSM) microscopy, fluorescence and circular dichroism measurements. Complexation is governed by both electrostatic interactions and secondary forces, and exhibits a maximum at the kCG to Lys ratio for which mutual compensation of charges occurs. The effect of the ionic strength (I) on complexation has a nonmonotonic character displaying a maximum in complex formation at $I \approx 0.03$. The specific pH value at which complex formation is completely suppressed (pH_{Set}), is only slightly dependent on the I value. Turbidity measurements indicate complexation of Lys with kCG at a pH as high as 11.5 ($I=0.01$). Molecules of Lys are placed mainly on the periphery of the complex particles and the localisation of kCG has an irregular character without formation of a single center of binding. Complexation in dilute solutions leads to a spectacular increase in the helix content, whereas in semidilute solutions complexation causes a decrease of the temperature of denaturation, suggesting that kCG has a higher affinity for the unfolded state than for the native state of Lys.

Keywords: *lysozyme, kappa carrageenan, complexation, structure, thermal denaturation*

1. Introduction

Many physical properties of food preparations such as their clarity, stability and gel-forming ability mainly depend on the interactions between proteins and polysaccharides in solution. Interactions between these biopolymers are also an essential element in food texture (Glicksman, 1983; de Ruiter & Rudolph, 1997). Protein-polysaccharide interactions often lead to formation of water soluble complexes, water insoluble complexes or coacervation as described by Bungenberg de Jong (1949). During the coacervation process, a homogeneous aqueous solution undergoes liquid-liquid phase separation giving rise to a dense protein-rich phase. This phenomenon is of interest from a basic physicochemical point of view as well as from the perspective of the development of a large variety of possible applications in the food industry (Doublier, Garnier, Renard, & Sanchez, 2000; Tolstoguzov, 1998; Dickinson, 1998; De Kruif, 2001), in encapsulation (Kabanov, 1994) and in purification of proteins by selective precipitation or coacervation with polyelectrolytes (Strege, Dubin, West, & Flinta, 1990; Tolstoguzov, 1998). Therefore, the complexation of these biopolymers is at the center of intense scientific interest. Numerous studies have focused on the investigation of protein-polyelectrolyte complexation, including different polysaccharide structures under diverse conditions, and there is a number of reviews concerning this subject (see for example Doublier et al., 2000; Kayitmazer et al., 2013; Kabanov, 1994; De Kruif, Weinbreck, & de Vries, 2004; Cooper, Dubin, Kayitmazer, & Turksen, 2005).

Many proteins are known to form complexes with sulfated polysaccharides, for example, ovalbumin (Galazka, Smith, Ledward, & Dickinson, 1999), casein (Garnier et al., 2003), and acidic fibroblast growth factor (Boyle & Moore, 1959). The ability of sulfated polysaccharides to selectively precipitate low density lipoproteins from serum has been widely utilized for the isolation and estimation of the lipoproteins (Cornvell & Kruger, 1961; Oncley, Walton, & Cornwell, 1957) and for the determination of lipid distribution in low and high density lipoproteins (Kritchevsky, Tepper, Alaupovic, & Furman, 1963). However, the above mentioned proteins all have a specific sulfated polysaccharide binding site. This prompted us to investigate the potential of using lysozyme (Lys), which does not have a well-defined binding site, but which does form complexes upon addition of sulfated polysaccharides (Antonov, Zhuravleva, Cardinaels, & Moldenaers, 2015; Xu et al., 2014).

Lys is a 14.3 kDa protein with a pI of ~10.5. The protein molecule is a compact complex with a more or less ellipsoidal shape with dimensions of 4.5 x 3.0 x 3.0 nm. Analysis of the protein surface reveals a close to homogeneous distribution of positive charges over the surface (Van de Weert, Andersen & Frokjaer, 2004), suggesting that the interaction with a sulfated polysaccharide will not occur at a very specific site on the lys molecule. Although complex formation of lys with some unstructured anionic polysaccharides has been studied (Van de Weert et al., 2004; Antonov

et al., 2015; Antonov et al., 2017), interaction and complexation of this protein with ordered polysaccharides as well as the conformation and structural changes of these biopolymers induced by complex formation have not been systematically investigated despite their potential use in food technology. Therefore the aim of this work is to consider complex formation of Lys with kappa carrageenan (kCG) and more in particular the structural and conformational aspects of the polyelectrolyte complexes formed by Lys with kCG. These complexes have potential for encapsulation as has been shown for example with curcumin (Xu et al. 2014).

kCG is a hydrophilic sulfated polygalactan extracted from red seaweeds (Knutsen, Myslabodski, Larsen, & Usov, 1994; Piculell, 2006) with 15% ester-sulfate content and an average molecular mass well above 100 kDa. It consists of an alternating linear chain of (1→3)-β-D-galactose-4SO₃⁻-(1→4)-3,6-anhydro-α-D-galactose. kCG is soluble in hot water (> 75 °C) and even low concentrations (0.1 to 0.5%) of this polysaccharide yield high viscosity solutions (Larsen, & Usov, 1994). kCG can adopt different conformations in solution, e.g. random coil and double helix, depending on the temperature and concentration (Piculell, 2006). kCG can also form gels in the presence of counterions (Larsen, & Usov, 1994). The sulfate groups are located on the periphery of the double helix, and are interacting with other ions. In the disordered state κ-carrageenan exists as a random coil, expanded as a result of the effect of the excluded volume and electrostatic repulsions between chain segments (Vreeman et al., 1980) with a high water absorption capacity (Harding et al., 1996).

This work characterizes the interaction and complexation processes in aqueous kCG/Lys mixtures. The complexation at different compositions, pH and ionic strength values was mapped out by means of turbidity measurements. Moreover, the size and shape of the complexes, distribution of the biopolymers inside the complex particles, the stability of the secondary and tertiary structures of Lys within the complex as well as the stability of the protein in the complex against heat-induced denaturation and aggregation are investigated by a combination of static, dynamic and electrophoretic light scattering, confocal imaging, differential scanning microcalorimetry, circular dichroism, fluorescence measurements and phase analysis. The binding of the protein to the polysaccharide is characterized on the basis of the modified Stern–Volmer equation. A potassium phosphate buffer with a low ionic strength (*I*) = 0.01 was chosen to provide a stable double helix conformation of kCG (Burova et al., 2007).

2. Materials and methods

2.1. Materials

Lys from chicken egg white (dialyzed, lyophilized powder) was purchased from Sigma-Aldrich and used without further purification. The sample of kCG (94% dry weight, 17.03% ash, 5.28%

Na⁺; 0.33% K⁺, 0.006% Ca⁺⁺, 0.009% Mg⁺⁺, 0.2179 degree of sulphation, Cl⁻ not detected; dn/dc = 0.115 cm³/g; M_w = 567 kD; M_n = 356 kD (in 0.1 M LiNO₃, 60°C) was supplied by Sanofi Bio-Industrie (France). Milli-Q ultrapure water was used throughout the experiments.

Lys solutions were prepared by dispersing Lys in a mono/bisphosphate (KH₂PO₄+ K₂HPO₄) buffer with *I*=0.01 and stirring at room temperature for 1h. The final solutions were subjected to centrifugation at 50.000g for 1h to remove insoluble aggregates. Finally, the concentration of the biopolymer was determined by measuring the dry weight residue. For the stock solution, the final protein concentration was also determined by spectrophotometric measurements.

kCG stock solutions were prepared by dispersing the gum in a mono/bisphosphate (KH₂PO₄+K₂HPO₄) buffer with *I*=0.01, followed by strong stirring for 40 min at room temperature and subsequently 20 min stirring at 85°C. After cooling the kCG solution to room temperature the pH of the solutions was adjusted by addition of 0.1 M solutions of KOH. Subsequent manipulations were the same as those described above for the preparation of the Lys solutions. Many experiments were performed in a dilute mono/bisphosphate (KH₂PO₄+K₂HPO₄, pH 7.0) buffer with ionic strength *I*=0.01. In the experiments with various ionic strength values this parameter was adjusted with NaCl or KCl (in the case of higher *I* values) in the same buffer. The final concentrations of the solutions were determined from the dry weight residue on the basis of the material balance taking into account the amount of added buffer and salt. To prepare mixed solutions of Lys and kCG with the required concentrations, weighed amounts of the Lys stock solution were added to a kCG solution and stirred for 1h at 23°C.

2.2. Methods

2.2.1. Turbidity measurements

Turbidity values of aqueous Lys solutions and complex kCG/Lys mixtures as functions of the kCG/Lys weight ratio (*q*), pH, and *I* were measured at 500 nm using a Unico SQ2800 UV/VIS spectrometer. The error of the turbidity measurements is typically about 2%–3%, in the charge ratio range from 0.2 to 0.8 the errors are markedly larger (6-8%).

With increasing kCG/lys ratio *q*, the complexation behavior undergoes three transitions characterized by *q*_{Onset}, *q*_φ, and *q*_{Max} (Carlsson, Lines, Malmsten, 2001). These correspond to respectively the transition from the absence of complexation to formation of water soluble complexes, from water soluble complexes to water insoluble complexes and their phase separation, and maximal complexation. To obtain accurate values for these transition points, additional characterizations were performed. The *q*_{Onset} value was determined as the minimum *q* value at which the size of the complexes, as determined by dynamic light scattering exceeds that of pure kCG by 10%. The *q*_φ value was determined as the minimum *q* value at which the turbidity increases

with time, which was quantified as an increase of >2% during 15 min under quiescent conditions for samples that had been stirred for 30 min before the test.

2.2.2. *Electrophoretic Mobility*

ζ -potential measurements of kCG, Lys and kCG/Lys complexes at different kCG/Lys weight ratios (q) were performed at 23°C with a 90 Plus particle size analyzer (Brookhaven instruments Inc.) using a rectangular quartz capillary cell. For each sample the ζ -potential was determined at least ten times and the average value is reported.

2.2.3. *Phase analysis*

Phase analysis of kCG/Lys mixtures was performed at a total biopolymer concentration of 0.3 wt% in pure water at various q values. Substitution of the phosphate buffer by water was necessary to exclude errors in composition due to possible binding of kCG with ions of the buffer, that can be unequally distributed over the different phases. The yield of the macromolecular components in the complex phase was determined by measuring the masses of the complex phase and the supernatant, and the total concentrations of biopolymer in these phases after phase separation of the complex system by centrifugation at 10000g for 20 min at 23°C. The total concentrations of biopolymer in the complex phase and the supernatant were determined by measuring the dry weight residue. The concentration of Lys in the supernatant was determined by measuring the absorption value at 280 nm at high ionic strength (0.5 M NaCl). From a calibration curve, the extinction coefficient of free lysozyme under these conditions was determined to be 2.693 ml mg⁻¹ cm⁻¹. The concentration of Lys in the complex phase was calculated from the total amount of Lys introduced in the kCG/Lys mixture and the amount of Lys found in the supernatant. The concentrations of kCG in the supernatant and complex phase were established by subtraction of the concentration of Lys in these phases from the total concentration of biopolymers in these phases. The experimental errors were approximately 8-10%.

2.2.4. *Light Scattering*

Determination of the intensity size distribution functions of kCG and Lys solutions as well as kCG/Lys mixtures was performed by means of dynamic light scattering (DLS) with an ALV/CGS-3 compact goniometer system (ALV GmbH, Germany). The system is equipped with an ALV-5000/EPP multi tau digital correlator, a HeNe laser operating at a wavelength of 632.8 nm, and an avalanche photodiode detector. Buffer and samples of the binary buffer/kCG and buffer/Lys solutions were filtered through 0.22 μ m DISMIC-25cs (cellulose acetate) filters (Millipore) to remove dust particles. Subsequently, the samples were centrifuged for 30s at 2000g to remove air bubbles and placed in the cuvette housing, which was kept at 23°C in a toluene bath. The detected

scattering light intensity was processed by digital ALV-5000 Correlator software. To process the DLS data the cumulant method was used. For each sample the measurement was repeated three times and the average values are reported. In addition to DLS, also static light scattering (SLS) was performed. Based on the SLS data, the asymmetry coefficient (Z) of the complex associates at low q values (under conditions of relatively limited aggregation of the complex particles) was estimated by the Debye method. The scattering intensity I was determined at two angles (45° and 135°) symmetric to 90° and subsequently the ratio $I(45^\circ)/I(135^\circ)$ was extrapolated to zero concentration (Storey, Lee, Papa, Rosen, & Simon, 1976) to determine Z .

2.2.5. *Fluorescent imaging*

Fluorescent imaging was performed using a multi-beam confocal microscope (VisiTech, UK), equipped with an oil-immersion objective (20x, 0.85 NA, Olympus, Japan) using 532 nm and 642 nm as excitation wavelengths. Full-frame (512x512 pixel resolution) images were acquired at 2Hz. Before imaging, Lys and kCG were fluorescently labeled by storing a Lys solution containing Rhodamine B dye at 5°C during 3 days, whereas a kCG solution containing Atto 647N dye (ATTO Tec. Germany) was kept under the same conditions. This labeling allowed to spectrally separate the signal from Lys (green) and kCG (red). Image analysis was performed using ImageJ v1.43r software.

2.2.6. *CD measurements*

CD measurements of a Lys solution and kCG/Lys mixtures were performed using a Chiroscan Applied Photophysics instrument equipped with a temperature control unit. A quartz cuvette with a light path length of 0.1 cm was filled with the sample solution. Circular dichroism spectra were recorded in the range of 195 to 250 nm with an interval of 0.2 nm. The solutions were scanned at a rate of 50 nm/min using a 2s time constant and a sensitivity of 20 mdeg and step resolution of 0.1. The mean content of helix, beta sheet and random structures was calculated with the SOMCD method which is an update of the k2D algorithm (Unneberg, Merelo, Chacón & Morán, 2001). Four scans were averaged to obtain one spectrum. The experimental error was 1.82%.

2.2.7. *Fluorescence spectroscopy*

Fluorescence emission spectra in the range of 300 to 450 nm were recorded on a RF 5301 PC Spectrofluorimeter (Shimadzu, Japan) at 23°C with the excitation wavelength set to 280 nm, slit widths of 3 nm for both excitation and emission, and an integration time of 0.5s. The fluorescence intensity was corrected for absorption of exciting light and re-absorption of the emitted light to decrease the inner filter effect using the relationship (Weiping, Wenao, Jianrong, Xiaohua and Zhide, 2011):

$$F_{cor} = F_{obs} \times e^{\frac{A_{ex} + A_{em}}{2.303}} \quad (1)$$

where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities respectively, and A_{ex} and A_{em} are the absorptions of the systems at the excitation and the emission wavelength, respectively. The reported intensity values are the corrected fluorescence intensities. The experimental error was 1.9%.

2.2.8. High-Sensitivity DSMC

Thermal denaturation of Lys in aqueous solutions in the absence and in the presence of kCG was monitored with a highly sensitive differential scanning microcalorimeter (DSMC) (DASM-4 M, Puschino, Russia). Thermograms were obtained between 10°C and 90°C, at a scan rate of 2 °C/min. For all measurements, the pH was 7.0. Degassing during the calorimetric experiments was prevented by application of an additional constant pressure of 172.25 kPa over the liquids in the cells. The data were sampled and processed using the service program WSCAL Version 2.0 based on the principles described by Filimonov, Potekhin, Matveev, & Privalov (1982). Effective (van't Hoff) enthalpy of denaturation was calculated according to (Velicelebi & Sturtevant, 1979):

$$\Delta H_{vH} = 4RT_m^2 \Delta C_m / Q_m \quad (2)$$

where T_m is the thermal midpoint, ΔC_m the peak height (in JK⁻¹), and Q_m the area under the peak (in J).

3. Results and discussion

3.1. Characterization of complexation at pH 7.0, ionic strength 0.01 and 23°C

3.1.1. Weight ratio range for complexation, zeta potential and phase analysis

The effect of the kCG/Lys weight ratio on complex formation was mapped out by means of turbidity measurements. Thereto, 0.04 wt% solutions of kCG and Lys in phosphate buffer at pH 7.0 and with ionic strength $I = 0.01$ were combined into complex mixtures with a total concentration of Lys and kCG (C_{Tot}^M) of 0.04 wt%. At pH 7.0, the net positive ζ -potential of Lys was determined to be +6.4 mV, which is close to the literature value (Kuehner, Engmann, Fergg, Wernick, Blanch, & Prausnitz, 1999 ; Sophianopoulos & Vanholde, 1964) whereas the ζ -potential of kCG under these conditions was found to be -28.6 mV. Based on this, electrostatically driven complexation is expected to readily occur under the present conditions.

Figure 1a shows the turbidity values at 500 nm (τ_{500}) as a function of the kCG/Lys weight ratio (q), which varies between 0.000348 and 2.85. The turbidity value at 500 nm (τ_{500}) for a 0.04 wt% solution of Lys or kCG was 0.002. The significant increase of τ_{500} upon mixing of kCG and Lys solutions, shown in Figure 1, clearly demonstrates complex formation. The extent and details of

the complex formation are dependent on the kCG/Lys weight ratio (q), with a maximum in τ_{500} occurring at $q=0.304$. The mixture behavior clearly depends on the kCG/Lys weight ratio (q), and consequently, on the charge ratio. The “molar” ratio Lys/kCG in the complex phase can be roughly determined from the molecular weights of Lys (Rezwan, Meier & Gauckler, 2005) and kCG, which are 14.3 kDa and 567 kDa respectively, which results in a molar ratio of 12:1. The Lys molecules are clearly much smaller than the kCG molecules and hence multiple Lys molecules can associate with one kCG molecule, like ligands on a nucleus, which is similar to other interacting weak polyelectrolytes (Sato, Maeda & Nakajima, 1979).

FIGURE 1

In Figure 1, two transitions in the state of the mixed system can be pinpointed. The first one characterizes the transition between absence of complexation (region I) and soluble complex formation (region II) and is denoted “ q_{Onset} ” (Carlsson, Lines, & Malmsten, 2001) whereas the second one, denoted “ q_{ϕ} ” (Carlsson et al., 2001), indicates the transition between soluble complex formation (region II) and phase separation of an insoluble complex (region III). The values of the transition points are approximately the same as in the complexation process of Lys with dextran sulfate ($q=0.001$ and 0.006 respectively), which has been studied before (Antonov et al., 2015).

The ζ -potential of the complex systems, determined as a function of the kCG/Lys weight ratio (q), is shown in Figure 2a. The positive charge of Lys is neutralized at $q = 0.27$ - 0.29 , and the surface charge of the formed complexes turns into negative values at higher q . Since complete neutralization of charges takes place at a q ratio close to that of maximal complexation according to turbidity measurements (Fig. 1), it can be concluded that this situation corresponds to a mutual compensation of negatively charged groups of kCG and positively charged groups of Lys.

Next, the complexation process between Lys and kCG was characterized by means of phase analysis. Figure 2b presents the yield of Lys (Y_{lys}) in the biopolymer rich complex phase (curve 1) and supernatant (curve 2) as a function of q as well as the kCG/Lys weight ratio in the biopolymer rich complex phase ($q^* \frac{kCG}{Lys}$) as a function of q (curve 3). It can be seen that the yield of lysozyme

Y_{lys} in the complex phase reaches its maximum (97.8%) at a weight ratio of 0.3, which approximately corresponds to the composition resulting in maximum complex formation, as determined from turbidity measurements, and charge neutralization, as determined from zeta potential measurements. Phase analysis measurements reveal two domains of q corresponding at

first approximation to constant $q^* \frac{kCG}{Lys}$ (domain I'), and increasing $q^* \frac{kCG}{Lys}$ and Y_{lys} in the

supernatant (domain II'). Since the composition of the kCG/Lys complexes in the first domain only

weakly depends on the composition of the initial mixture, we can suppose an “all or none” type complex formation mechanism, which is well-known for oppositely charged polyelectrolyte systems (Michaels, Mir & Schneider, 1965). This implies that the reactivity of a kCG chain partially covered by Lys may be considered higher than that of a free chain. Therefore, completely neutralized polyelectrolyte complexes and completely free polyelectrolyte coexist in the complex mixture. In the second domain (Fig. 2b), the content of kCG in the complex phase steeply increases with q while the yield of lysozyme in the complex phase decreases. Hence, the number of protein molecules interacting with each kCG chain decreases, leading to limited charge neutralization, which hinders the aggregation of the complexes, as illustrated by the reduced turbidity τ_{500} . Therefore, the formed complexes consist of decreasing total numbers of kCG chains and protein molecules (Y_{lys} in complex phase decreases), and the relative content of kCG in the complex precipitate increases with q . This behaviour is similar to that exhibited by kCG/Gelatin, dextran sulfate/Lys and casein/Lys systems that have been studied before (Antonov and Gonçalves, 1999; Antonov et al., 2015; Antonov et al., 2017).

FIGURE 2

3.1.2. The sizes of the complex particles

DLS measurements were performed on the complex mixtures to determine the size of the complex particles. Figure 3a shows the intensity size distribution functions for a Lys solution (0.1 wt %) and a kCG solution (0.1 wt %) and Figure 3b shows the same for a complex kCG/Lys mixture at a q ratio equal to 0.05, also with a Lys concentration of 0.1 wt%. The scattering correlation functions indicated that the data is good enough to calculate the mean size of the particles. As can be seen in Figure 3, Lys is present in solution mainly as a monomer, but also some aggregates are present (volume contribution of 0.2 %). Such aggregates are extremely difficult to avoid (Sophianopoulos and Vanholde, 1964; Bruzzesi, Chiancone and Antonini, 1965). The average radius of the Lys monomer was 1.9 nm, which is in accordance with known data (Parmar & Muschol, 2009; Valstar, Brown & Almgren, 1999). The kCG sample is clearly polydisperse (Fig. 3a). The intensity size distribution function shows a main peak corresponding to an average size of 62 nm, and a smaller peak corresponding to an average size of 842 nm, which probably originates from kCG aggregates. Analysis of the volume size distribution function for the kCG solution shows that the volume fraction of these aggregates does not exceed 10% (data not presented).

Since the main peak of the Lys monomer with an average radius of 1.9 nm is far enough from the main kCG peak (Fig. 3a), possible changes of each peak after complexation can be

discerned. DLS measurements of the kCG/Lys systems were strongly limited by the concentration of kCG in the system. On one hand, the sensitivity of the method was too low at low kCG concentrations, and on the other hand, formation of too large ($>10\ \mu\text{m}$) complex particles, even at q values as low as 0.06, hampered data analysis at high kCG concentrations. Therefore all DLS measurements of the complex system were performed at $q=0.05$. As can be seen in Fig. 3b, in the presence of even a small amount of kCG in the Lys solution ($q=0.05$), a new dominant wide peak appears in the size range of 360-4000 nm corresponding to an average radius of 860 nm. The asymmetry coefficient (Z) of the complex associates was estimated by the Debye method (Antonov, Moldenaers, 2009) based on determination of the scattering intensity (R) at two angles symmetrical to 90° namely 45° and 135° and subsequent extrapolation of the ratio $R_{45^\circ}/R_{135^\circ}$ to zero concentration. The results obtained are presented in Figure 3c. The complex associates are very asymmetric with Z values equal to 0.148. Microscopy images (data not presented) obtained at the same Lys concentration in the system clearly demonstrate formation of thin and long fiberlike complex particles. These particles increase in size and quantity at higher q values, reaching a maximal size at $q=0.3$. Further increase of q leads to a significant decrease in size and quantity of the complex particles (data not presented).

FIGURE 3

3.1.3. Structure of the complexes

The distribution of kCG and Lys within the kCG/Lys complexes was monitored at various q values by using specific fluorescent labelling of both proteins, as illustrated for three representative q values in Fig. 4. Figure 4a at $q=0.05$ corresponds to an excess of Lys, Fig. 4b at $q=0.3$ corresponds to the maximal yield of the complex according to phase analysis and turbidity measurements (Figs. 1 and 2b), and finally, Fig. 4c at $q=1.0$ corresponds to an equal quantity of both biopolymers in the complex system. It can be seen that in all cases the Lys molecules are placed mainly on the periphery of the complex particles (green signal comes mainly from Lys) and the distribution of kCG within the complex particles has an irregular character without formation of a single center of binding (red signal comes mainly from kCG). An important feature which can be observed from Fig. 4 is that only at $q=0.3$, which is the composition corresponding to zero zeta potential, a substantial part of the complex particles consists of mixed kCG/Lys regions, as demonstrated by the yellow color. At the other q values, the complex particles are dominated by patches of Lys covering a large kCG cluster, as can be concluded from the presence of a high intensity signal of both Lys and kCG. The relative contribution of the region within the complex particles which is rich in kCG increases from $\sim 30\%$ up to $\sim 80\%$ when q increases from 0.05 to 1.0 (based on information from multiple images).

FIGURE 4

3.1.4. Protein structure within the complexes and the binding constant

A last aspect of the complex formation that has been studied is the conformation of the Lys protein within the complexes. This is a relevant characteristic for many applications involving protein-polyelectrolyte complexes since it governs the enzymatic activity. Several complementary spectroscopic techniques have been used to monitor the structure of Lys within the formed complexes, and representative results at pH 7.0 and $I=0.01$ are presented. Figure 5a presents the results of CD spectroscopy on dilute solutions. A negative circular dichroism band was observed in the wavelength region below 250 nm. The CD spectrum of Lys(0.005 wt%) exhibits two negative peaks in the far-UV region namely at 208 and at 222 nm, which are characteristic of an α -helical structure in the protein. It is generally accepted that these peaks originate from the n/π^* transition of the peptide bond of the α -helix (Woody, 1995). The ellipticity at 220 nm is a standard measure of the helical content of a protein. Based on the analysis carried out using the SOMCD method, which is an update of the k2D algorithm, the secondary structure of Lys in the Lys solution and complex kCG/Lys systems is estimated. At 20°C lys contains 28.1% alpha helical structure, 9.0% beta sheets and 62.9% random structure. In literature, the secondary structure of Lys is reported to contain about 28-30% helices and 19 % beta sheets (Greenfield, 2007). The CD spectrum of a kCG solution (0.005 wt%) in the range of 200-250 nm is very close to the base line. At a 10 times higher concentration of kCG (0.05 wt%) the CD spectrum exhibits one positive peak at 208 nm (data not presented). The negative band of the kCG/ Lys mixtures at 220 nm increases in intensity with increasing values of q , and the absolute value of the peak is maximal at q equal to 0.36. At this kCG/Lys weight ratio in the complex system, the structure of Lys becomes very regular. It contains 49.3 % alpha helical structure, 25.7% beta sheets and 25% random structure. Further increase of q up to 0.75 does not lead to a significant change in the negative band at 220 nm.

FIGURE 5

Further investigation of the protein structure within the complexes was performed with fluorescence spectroscopy. Fluorescence emission spectra in the range of 280-450 nm upon excitation at 270 nm are shown in Fig. 5b for dilute kCG/Lys mixtures at different values of q . The wavelength of maximum emission (λ_{max}) for Lys is about 340 nm. This fluorescence peak exclusively arises from the six tryptophan residues of Lys, whose absorbency at the wavelength of excitation and quantum yield of emission are considerably larger than the respective values for

tyrosine and phenylalanine (Kuramitsu, Kurihara, Ikeda, & Hamaguchi, 1978; Lehrer & Fasman, 1966). Under the same conditions, no fluorescence of kCG was observed. When different amounts of kCG are titrated into a fixed concentration of Lys, the fluorescence intensity of Lys decreases without appreciable change in the position of the fluorescence peak (Fig. 5b). Such decrease in fluorescence intensity is called quenching. The fluorescence quenching data was not successfully analyzed using the well-known Stern-Volmer equation (Stern & Volmer, 1919) for dynamic (collisional) quenching:

$$F_0/F = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, $[Q]$ is the concentration of the quencher, and K_{SV} is the Stern–Volmer dynamic quenching constant. This suggests the presence of static quenching.

A UV study was performed to obtain the absorption spectra of Lys with the addition of different concentrations of kCG. The absorbance of Lys was found to increase with the presence of kCG (data not shown). This indicates that there is an interaction between Lys and kCG, which involves the formation of a ground state complex of the type kCG/Lys, since dynamic quenching only affects the excited state while it has no effect on the absorption spectrum (Wang, Min, Chen, Wu, & Hu, 2011). Therefore, the quenching mechanism is confirmed to be static quenching (complex formation quenching), which is in accordance with the fluorescence quenching results. Hence, the fluorescence quenching data were analyzed using a modified Stern–Volmer equation for static quenching, which allows to determine the fraction of inaccessible fluorophores (Timaseff, 1972):

$$\frac{F_0 - F}{F_0 - F} = \frac{1}{QfK} + \frac{1}{f} \quad (4)$$

where f is the fraction of the initial fluorescence that is accessible to the quencher and K is the Stern–Volmer binding constant. For Lys, $f=1$ (Lakowicz, 1999) suggesting that all the tryptophan residues are accessible to the quencher. The binding constant determined by this method is equal to $3.21 \cdot 10^6 \text{ M}^{-1}$. This value falls in the range of binding constants reported for binding of proteins to acidic linear polysaccharides (Bram, Sperber, Stuart, Schols, Voragen, & Norde, 2009).

3.2. Ionic strength and pH stability of complexes

Subsequently, the effect of the ionic strength (I) on the complexation process in the kCG/Lys systems was investigated. Since potassium ions have a significant affinity to kCG (Marcelo, Saiz, Tarazona, 2005), it was first investigated whether the presence of KCl affects

the complexation i.e. the turbidity τ_{500} and more in particular the q ratio corresponding to the turbidity maximum. Figure 6a shows the values of τ_{500} as a function of q at various ionic strength values (I). All initial solutions of Lys and kCG were prepared using the same phosphate buffer with addition of the necessary amount of KCl to obtain the desired ionic strength. Figure 6a shows that the turbidity values do not vary monotonically with ionic strength. At low ionic strength values ($I=0.01$), the turbidity values τ_{500} of the complex system increase with ionic strength whereas at $I>0.01$, the turbidity values decrease with increasing ionic strength. Moreover, increasing the ionic strength from 0.01 to 0.5 leads to a 3.1 times increase in the q_{Max} value ($q_{\text{Max}}=0.3$ at $I=0.01$ and $q_{\text{Max}}=0.93$ at $I=0.50$). However, further increasing the ionic strength does not appreciably affect the turbidity of the complex system. Finally, it can be noted that the turbidity values of the complex system always remain at least slightly above that of the binary buffer/kCG and buffer/Lys systems, even at $I=0.50$. To summarize the effects of the ionic strength on the complex formation, q_{onset} and q_{max} for complex formation are plotted as a function of ionic strength I in Figures 6b and 6c. For both cases, the data are well approximated by a linear fit. It is interesting to note that an increase of the ionic strength leads to dramatic changes in the stoichiometry of the complex (the maximum of the turbidity values shifts to higher kCG concentrations). Such changes in the stoichiometry of the complex can result from a decrease in the charge of kCG at high ionic strength due to the interaction of the carboxyl groups of kCG with the cationic groups of potassium salts (Takemasa, & Nishinari, 2004).

FIGURE 6

The fact that the turbidity values of the complex system in Figure 6a always remain above that of the binary solutions suggests that non-electrostatic forces such as hydrophobic forces or hydrogen bonds play a significant role in the complexation of kCG with Lys. To examine this hypothesis, the turbidity values τ_{500} of the kCG/Lys systems were determined as a function of I with and without 6M urea. A large ionic strength only suppresses electrostatic interactions between biopolymers, whereas urea suppresses both hydrophobic and hydrogen interactions. This allows us to analyze the contributions of different intermacromolecular bonds in complexation. In order to exclude a possible competition of potassium and Lys cations for the interaction with kCG, sodium chloride was used to increase the ionic strength of the kCG/Lys system. Figure 7 demonstrates that the turbidity values do not vary monotonically with ionic strength, both for $q=0.1$ and 0.3, which corresponds to the results of Figure 7a. The maximum in complex formation is situated at an ionic strength I around 0.025. The turbidity values of the complex system without 6 M urea are always higher than that of the binary buffer/kCG

($\tau_{500}=0.007$) and buffer/Lys systems ($\tau_{500}=0.004$), even at high ionic strength. It can be seen in Figure 7 that in the presence of 6 M urea and $I=0.5$ the difference between the turbidity values of the complex system and that of the binary buffer/kCG and buffer/Lys systems disappears. This confirms that secondary forces such as hydrogen or hydrophobic forces also take part in the formation of water insoluble complexes of kCG with Lys. Such behavior of the kCG/Lys system is different from that established for the sodium dextran/Lys system studied before (Antonov et al, 2015). Formation of water insoluble complexes in the last system is completely suppressed at high ionic strength (at $I= 0.5$).

FIGURE 7

It is well known that ionic strength and the presence of counterions strongly modulate the affinity between macromolecules (Manning,1969). Seyrek and co-workers (2003) have shown that binding of polyanions to a positive domain results in a combination of short-range attractive interactions coupled with longer range repulsive interactions due to the presence of charged patches on the protein. Since lysozyme is known to exhibit a multipolar charge distribution (Van de Weert, et al., 2004), both interactions are expected in the kCG/Lys mixtures. Hence, at low ionic strength, when the Debye length $\kappa^{-1} \approx 0.3/\sqrt{I}$ (nm) is large, the effect of salt is to screen the repulsions and thus to strengthen association. At higher ionic strength values, corresponding to a small Debye length, addition of salt weakens the attractions thereby suppressing complexation. The maximum in complexation occurs around $I = 0.03$ (Fig. 7), which corresponds to a Debye length of 1.7 nm, a value comparable to the size of the lysozyme molecule. The latter confirms the hypothesis that the maximum originates from a transition of the salt effect from screening repulsions to weakening attractions. Similar non-monotonic behaviour was found for several other protein-polyelectrolyte systems, as listed by Antonov et al. (2015).

Turbidity results as a function of pH for kCG/Lys systems at various ionic strength values are presented in Figure 8a. All initial solutions of Lys and kCG were prepared at the desired pH and ionic strength before mixing them together. Figure 8b presents the dependence of the pH_{Set} values of the system on the ionic strength, where pH_{Set} is defined as the pH at which the water insoluble kCG/Lys complexes disappear. It can be seen from Figure 8a that Lys can associate with kCG even at pH 11.8 at which the net charge of Lys is negative (Sato, Mattison, Dubin, Kamachi, & Morishima, 1998). This is caused by the presence of positively charged residues (11 arginyl residues whose pK_a value is 12.5), even at this pH. Park et al. (1992) reported that Lys interacted with Sodium poly[[2-(acrylamido)propyl]methyl] sulfate even above its pI when the net charge of

Lys was -2.6 ± 1 mV in 0.1M KCl. The data obtained show that the pH_{Set} values decrease from 11.8 to 10.6 when the ionic strength increases from 0.01 to 0.1. This behavior of the kCG/Lys system is different from that of the dextran sulphate/Lys system studied before (Antonov et al., 2015). The pH_{Set} values of the latter system are much more sensitive to ionic strength as demonstrated by a decrease in the pH_{Set} values from 11.5 to 7.8 when the ionic strength increases by a factor 7.5. The results presented in Figure 8 confirm the above conclusion that the complexation between Lys and kCG is not only governed by electrostatic interactions.

FIGURE 8

3.3. Thermal stability of the protein within the complexes

Differential scanning microcalorimetry (DSMC) was employed to study the thermal stability of the protein within the complexes. The DSMC traces for 0.125 wt% solutions of kCG or Lys as well as complex kCG/Lys systems are presented in Figure 9. Three characteristic compositions of the complex system were considered namely $q=0.1$, corresponding to an excess of Lys, $q=0.3$, corresponding to maximal yield of the complex according to the phase analysis and turbidity results (Figs. 1 and 2b), and finally $q=1.0$, corresponding to an equal quantity of both biopolymers in the complex system. The heat capacity curve of kappa-carrageenan contains a single asymmetric peak at about 45.8°C and overall transition enthalpy of 42.3 J/g (Fig. 9c). This peak has been assigned to the melting of the carrageenan double helix (Grinberg, Grinberg, Usov, Shusharina, Khokhlov, de Kruif, 2001). The DSMC thermogram of Lys could be fitted using one single transition with a melting temperature (T_m) of approximately 75.2°C (Fig. 9c) and overall unfolding enthalpy of 36.3 J/g or 521 kJ/mole. The results obtained are similar to those presented by Van de Weert et al. (2004). The ratio of the calorimetric to the van't Hoff enthalpy was 1.27, suggesting that, at first approximation, Lys molecules denaturate as a single cooperative unit (Antonov et al., 2015).

FIGURE 9

The thermograms of the complex kCG/Lys mixtures at $q=0.1$ and $q=0.3$ can be fitted using peaks with a melting temperature $T_m = 74.8^{\circ}\text{C}$ and 71.4°C respectively and unfolding enthalpies of 26.5 J/g and 23.6 J/g respectively, which are appreciably less than those for pure Lys. The disappearance of the calorimetric peak in consecutive heating scans of the kCG/Lys complex systems indicates that heat denaturation in this case is an irreversible process. Therefore, a direct comparison of the thermodynamic stability between the systems is not possible. The irreversible character of the thermal unfolding may be attributed to hydrophobic interactions of the unfolded protein molecules with the polyelectrolyte chains. The data

obtained allow to conclude that kCG in the complex kCG/Lys system at $q=0.1$ and 0.3 appreciably affects the thermodynamic parameters of the protein. In addition, Lys also affects the thermal transition of kCG. The thermogram of the complex kCG/Lys mixture at $q=1.0$ (high content of kCG) was best fitted using two partially merged peaks. The high temperature peak ($T_m=67.8^\circ\text{C}$) can be assigned as melting of Lys in the complex kCG/Lys system because the conformation transition of pure kCG in a solution with a concentration equal to that of the kCG in the kCG/Lys system at $q=1.0$ ends at 58°C (Fig. 9 c). It is thus clear that kCG decreases the stability of Lys against heat-induced denaturation. According to Waldron and Murphy (2003), a decrease in melting temperature upon ligand binding indicates a higher affinity of the ligand for the unfolded state than for the native state. The data obtained are in agreement with previous findings for the complexation of Lys with anionic polyelectrolytes such as heparin (van de Weert et al, 2004), polyvinyl sulfate (van de Weert, et al, 2004; Sedlak, Fedunova, Vesela, Sedlakova and Antalík, 2009), polyacrylic and methacrylic acid (Ivinova, Izumrudov, Muronetz, Galaev and Mattiasson, 2003). The shape of the low temperature peak ($T_m=59.4^\circ\text{C}$) in Fig. 9c is very similar to that of the pure kCG solution ($T_m=45.8^\circ\text{C}$), including the presence of a small shoulder on the left flank. Therefore, the low temperature peak of the kCG/Lys system at $q=1.0$ may be attributed to the thermal transition of kCG, free and partially bonded with Lys. The higher T_m values of the bonded kCG as compared to that of free kCG reflect the increased conformation stability of the kCG helical structure within the complex with Lys.

3.4. The effect of helix-coil transition of kCG on its complexation with lysozyme

In order to investigate the effect of the helix-coil transition of kCG on its complexation with Lys, solutions of kCG and Lys were initially separately preheated at 58°C (according to the data in Fig. 9) in order to induce a transition from helix to coil conformation of the kCG. Then the solutions were mixed in different concentrations at 58°C and the τ_{500} values of these mixed solutions were determined at 58°C . The results are presented in Figure 10 as a function of the kCG/Lys weight ratio (q). The data obtained at 23°C are added for comparison. It can be seen that the conformation transition of kCG does not appreciably affect the position of q_{Max} but the turbidity values of the kCG/Lys mixtures containing kCG in the coil state are much higher than that of the mixtures comprising kCG in the helix state. Up to the author's knowledge, the mechanism of the helix-coil conformational transition of carrageenans has not been resolved (Piculell, 1995). In dilute solutions an increase in molecular weight of kCG occurs due to the coil-helix transition (Ueda, Itoh, Matsuzaki, Ochiai, Imamura, 1998). This is caused by the fact that the conformational transition from coil to helix conformation leads to a dimerized state (Ueda et al, 1998). However, other works (Bongaerts, Reynaers, Zanetti, Paoletti, 1999; Vanneste, Sloodmaekers,

Reynaers,1996) did not indicate changes in molecular weight of the polysaccharide related to its conformation. Thermodynamic approaches did not provide much progress in the understanding of the helix-coil transitions in kCG (Viebke, Borgstrom, Carlsson, Piculell, Williams,1998). It is thought that a helix-coil type transition is most probable in this polysaccharide (Piculell, 1995; Ciszowska, Kotlyar, 1999). Our data do not confirm an increase in the molecular weight of kCG during helix-coil transition because the q_{Max} value after helix-coil transition did not change (Fig. 10). We assume that the considerable increase in the turbidity of the kCG/Lys mixtures after the helix-coil transition of kCG is due to the increased effect of the hydrophobic forces on the aggregation of kCG/Lys complexes.

FIGURE 10

3. Conclusion

The present study has provided important insights in the interactions between a sulfated polysaccharide with one sulphate group per disaccharide, a linear chain and various (helix–coil) conformations in solution (kCG), and a protein (Lys) that does not have a specific binding site for sulphated polysaccharides. The experimental results show that at pH 7.0 and $I = 0.01$ these biopolymers form water insoluble complexes with a stoichiometric kCG/Lys composition equal to 1:12 (mole/mole). Both electrostatic interactions as well as secondary forces contribute to complex formation. The complexation process, the solution behavior, structure and composition of the formed complexes depend on the kCG/Lys weight ratio of the complex mixtures as well as the ionic strength of the solution. Complex formation can be considered as an association in which a few Lys molecules (ligands) successively join one molecule of kCG (nucleus). When $10^{-3} < q < 5 \cdot 10^{-3}$, i.e. when the average zeta potential of the complex particles is positive, complexation leads mainly to the formation of water-soluble complexes. The process results in a sharp increase in the mean helix content of Lys without significant changes in its tertiary structure. The q range corresponding to formation of water soluble kCG/Lys complexes is about ten times lower as compared to that obtained for a dextran sulfate/Lys system comprising a polysaccharide with a similar molecular weight (500 kDa) but having a branched chain and about two sulfate groups per glucosyl residue of the dextran molecule (Antonov et al., 2015). At $q > 0.005$ formation of large (>1000 nm in radius) asymmetric water insoluble complex particles takes place. When $q \geq 1.0$, the formed complexes consist of a decreasing total number of kCG chains and protein molecules, and contain a relatively higher content of kCG. The effect of I on complexation has a nonmonotonic character displaying a maximum in complex formation at ionic strength I around 0.03. This results from a suppression of long-range repulsions at low salt content coupled with a weakening of attractions at large salt content. Complexation of Lys with kCG takes place even at pH 11.5, i.e at a pH above the

isoelectrical point of lys. This can be explained by the local interaction of the kCG molecules with the positively charged arginyl residues of Lys, which have a pKa value of 12.5. The specific pH values at which soluble complex formation is inhibited (pH_{Set}) only slightly depend on the ionic strength (I). CLSM clearly shows that the Lys molecules are placed mainly on the periphery of the complex particles and the distribution of kCG has an irregular character without formation of a single center of binding. Complexation in dilute solutions leads to a spectacular increase in the helix structure and stabilization of Lys within the complexes against thermal aggregation, whereas in semidiluted solutions the temperature of denaturation decreases, suggesting that kCG has a higher affinity for the unfolded state than for the native state of Lys. This was also confirmed by the lowering of the melting temperature of kCG. This observation is contrary to the increase in thermal stability of proteins with a specific binding site for sulphated polysaccharides (Paulikova, Molnarova and Podhradsky, 1998; Lookene, Chevreuil, Ostergaard and Olivecrona, 1996; Volkin, Tsai, Dabora, Gress, Burke, Linhardt and Middaugh, 1993). Besides partial denaturation of the protein, structural rearrangements upon kCG binding could be a factor in the modified thermal behaviour. The results obtained can be used in protein processing to generate fibre-like materials and phase separation technology. Lys molecules can be reversibly taken up by kCG at low ionic strength, and released by raising the amount of added salt and urea. This allows using systems based on a sulphated polysaccharide and Lys in biotechnology as a carrier of enzymes. Finally, the use of microscopic and spectroscopic methods at well-defined points of the phase diagram could be a means of studying the microstructure of the mixed food systems, the respective location of each macromolecule in the complexes, and the molecular structure of food macromolecules.

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Captions to figures

Figure 1. Turbidity values at 500 nm (τ_{500}) as a function of the kCG/Lys weight ratio (q) for kCG/Lys (a, b) mixtures at pH 7.0, $I=0.01$, and 23°C. The total concentrations of Lys and kCG in the ternary mixtures (C^M_{Tot}) and the concentrations of Lys and kCG in the binary solutions (C^0_{lys} , C^0_{kCG}) were kept constant at 0.04 wt%. In the inset (b) q_{Onset} indicates the transition between no complex formation and formation of soluble complexes whereas q_ϕ indicates the transition between formation of soluble and insoluble complexes.

Figure 2 (a) Zeta potential (ζ) as a function of the kCG/Lys weight ratio (q) for kCG/Lys mixtures. (b) The yield of Lys in the biopolymer rich complex phase (curve 1) and supernatant (curve 2) as well as the kCG/Lys weight ratio in the complex phase as a function of kCG/Lys weight ratio q (curve 3). $C^M_{tot}=0.3$ wt%, pH 7.0, $I=0.01$, and 23°C.

Figure 3. Intensity size distribution (a) for Lys (0.1 wt%) and kCG (0.1 wt%) and (b) for a kCG/Lys mixture at $q=0.05$ and $C^M_{lys}=0.1$ wt%. (c) Ratio of the scattering intensities (R) at angles 45° and 135° for kCG solutions as a function of kCG concentration and for kCG/Lys mixtures as a function of Lys concentration. pH 7.0, $I=0.01$ and 23°C.

Figure 4. Confocal microscopy images of kCG/Lys mixtures at different q values. (a) $q=0.05$, (b) $q=0.3$ and (c) $q=1.0$ for $C_{\text{tot}}^{\text{M}}=0.04\text{wt\%}$, pH 7.0, $I=0.01$ and 23°C . Lys is labeled green and kCG is labeled red. Full length of images is $208\text{ }\mu\text{m}$.

Figure 5. (a) Circular dichroism spectra for a Lys solution and complex kCG/Lys mixtures at various q values. (b) Fluorescence emission spectra for a Lys solution and complex kCG/Lys mixtures at various q values, $\lambda_{\text{exc}}=280\text{ nm}$. $C_{\text{lys}}^{\text{M}}=0.005\text{ wt\%}$, pH 7.0, $I=0.01$ and 23°C .

Figure 6. (a) Turbidity values τ_{500} of kCG/Lys mixtures as a function of kCG/Lys weight ratio q for various ionic strength (I) values. The inset (b) provides q_{Onset} values as a function of I , and (c) q_{Max} as a function of I for kCG/Lys mixtures. $C_{\text{kCG}}^{\text{O}}=C_{\text{lys}}^{\text{O}}=C_{\text{tot}}^{\text{M}}=0.04\text{ wt\%}$, pH 7.0 and 23°C . Ionic strength was varied with KCl.

Figure 7. Turbidity values τ_{500} of kCG/Lys mixtures (after subtraction of the τ_{500} values of kCG solutions with the same concentration) as a function of I at (a) $q=0.1$ and (b) $q=0.3$. Curve 1 is in the absence of urea, curve 2 is in the presence of 6M urea. $C_{\text{kCG}}^{\text{O}}=C_{\text{lys}}^{\text{O}}=C_{\text{tot}}^{\text{M}}=0.04\text{ wt\%}$, pH 7.0, and 23°C . Ionic strength was varied with NaCl.

Figure 8. (a) Turbidity values τ_{500} as a function of pH for kCG/Lys mixtures at various ionic strength values I . The inset (b) shows pH_{Set} values as a function of I . $C_{\text{tot}}^{\text{M}}=0.02\text{ wt\%}$, $q=0.3$ and 23°C . Ionic strength was varied with NaCl.

Figure 9. The DSC traces for the first heating scans of Lys, kCG and complex kCG/Lys mixtures at different kCG/Lys weight ratio values q : (a) 0.1, (b) 0.3 and (c) 1.0. The concentration of Lys in the Lys solution is $0.255\text{ wt\%}$ and that in the kCG/Lys mixtures is $0.25\text{ wt\%}$ at $q=0.1$, $0.417\text{ wt\%}$ at $q=0.3$ and $0.125\text{ wt\%}$ at $q=1$. The concentration of kCG in the kCG solution is $0.125\text{ wt\%}$ and that in the kCG/Lys mixtures is $0.026\text{ wt\%}$ at $q=0.1$ and $0.125\text{ wt\%}$ at $q=0.3$ and 1.0 . pH 7.0 and $I=0.01$.

Figure 10. Turbidity values at 500 nm (τ_{500}) as a function of the kCG/Lys weight ratio q for kCG/Lys mixtures at $C_{\text{kCG}}^{\text{O}}=C_{\text{lys}}^{\text{O}}=C_{\text{tot}}^{\text{M}}=0.02\text{ wt\%}$, pH 7.0, $I=0.01$ and 58°C or 23°C .

Highlights (for review)

1. Dependence of complex formation on ionic strength is not monotonic
2. Secondary forces take part in complex formation
3. Complexation leads to an increase in the helix structure content of lysozyme
4. Complexation leads to a decrease in the thermal stability of lysozyme
5. Complexation depends on the state of carrageenan (coil versus helix)

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