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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 nonmonotonous 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 localization 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.

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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 (Dickinson, 1998; Doublier, Garnier, Renard, & Sanchez, 2000; Tolstoguzov, 1998; de Kruif & Tuinier, 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, Seeman, Minsky, Dubin, & Xu, 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 (Galazkaa, 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

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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 × 3.0 × 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 (Antonov et al., 2015, 2017; Van de Weert et al., 2004), 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-4SO_3–(1 → 4)-3,6,anhydro-α-D-galactose. kCG is soluble in hot water (>75 °C) and even low concentrations (0.1–0.5%) of this polysaccharide yield high viscosity solutions (Knutsen et al, 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 (Knutsen et al., 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 (Vreenan, Snoeren, & Payens, 1980) with a high water absorption capacity (Harding, Day, Dhami, & Lowe, 1997).

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, allocation 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 cm^3/g; M_w = 567 kD; M_n = 356 kD (in 0.1 M $LiNO_3$, 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 (KH_2PO_4+ K_2HPO_4) buffer with I = 0.01 and stirring at room temperature for 1 h. The final solutions were subjected to centrifugation at 50.000g for 1 h 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 (KH_2PO_4+K_2HPO_4) 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 (KH_2PO_4+K_2HPO_4, 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 1 h 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, Linse, & 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 with 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

ς-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 ς-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

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