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Macromolecular complexes of the main storage protein of *Vicia faba* seeds with sulfated polysaccharide

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ABSTRACT

Specific interaction between legumin from *Vicia faba* seeds and kappa carrageenan at neutral pH (7.3) and low ionic strength (0.01) and the mechanism that leads to increased conformation stability and a decreased aggregation of the protein by addition of the polysaccharide were investigated using high sensitivity differential scanning calorimetry (DSC), circular dichroism (CD), fluorescence (FLU), the size exclusion chromatography equipped with an online multi-angle light scattering detector (SEC-MALS), turbidimetry and a methylene blue spectrophotometric methods (MBS). Kappa carrageenan (kCG) molecules are able to form with legumin at room temperature a water soluble interpolymeric complex. Complex formation leads to a small perturbation and loosening of the secondary structure of the protein. On the contrary, conformation stability and the stability of the protein molecules with respect to thermo aggregation are noticeably increased. It also reduces the extent of thermal modification of secondary and tertiary structure of legumin due to growth of the net charge of a protein and weakening interaction between similar protein molecules. The joint analysis of SEC-MALS and MBS shown the process is proceeded with a high the degree of conversion (>0.8) and the complexes formed are equimolar.

1. Introduction

The interactions of proteins with polyelectrolytes are important in natural biological systems as well as in biotechnological applications. In such biological systems specific interactions between macromolecules are quite important and the various functions realized in these systems are closely related to higher-order structures and assemblies of macromolecules controlled by intra- and intermacromolecular interactions (Cantor & Schimmel, 1980).

Weak intermacromolecular interactions are responsible for dramatical changes in thermodynamic compatibility of biopolymers (Antonov & Friedrich, 2007; Antonov, Lefebvre, & Doublier, 1999; Antonov, Dmitrochenko, & Leontiev, 2006; Antonov & Soshinsky, 2000). Technologically, protein–polyelectrolyte interactions can be utilized for isolation of proteins (Dubin, Gao, & Mattison, 1994; Hidalgo & Hansen, 1971; Serov, Antonov, & Tolstoguzov, 1985) and enzymes (Kiknadze & Antonov, 1998). Complex formation of proteins and polysaccharides affects the structure and physical properties of biopolymers mixtures and plays an important role in protein processing in food products (Tolstoguzov, 1998). Current research activity in this area is a reflection of these considerations.

Usually Coulomb complexes are formed between the oppositely charged macroions although in some cases water soluble complexes were detected at pH slightly above isoelectrical point (IEP; see for example Antonov & Friedrich, 2007; Antonov & Soshinsky, 2000, 2006; Kayitmazer, Seyrek, Dubin, & Staggemeier, 2003; Mattison, Dubin, & Brittain, 1998). This particularly concerns mixtures containing sulfated polysaccharide the charge density of which is higher than those of carboxyl containing polyelectrolytes.

A beneficial consequence of complexation of sulfated polysaccharide with caseins at pH values above IEP is the protection afforded against loss of solubility as a result of protein aggregation during heating or following high-pressure treatment (Dickinson, 1998; Galazka, Ledward, Sumner, & Dickinson, 1997). The mechanism of this protection has been unclear till now. Snoeren, Payens, Jevnink, and Both (1975) assumed that there is a nonstatistical distribution of positively charged amino acid residues along the polypeptide chain of kappa casein molecules and, as a consequence, the existence of a dipole interacting by its positive pole with sulfur polysaccharide is responsible for complex formation in such systems. Interactions between proteins and sulfur polysaccharides, especially kappa carrageenans (kCG) turned out to be of significant interest last time due to the establishment of the capacity these polysaccharides to decrease aggregation of the proteins (BSA, β-lactoglobulin) having a statistical distribution of the charged amino acid residues (Chung et al., 2007; Zhang,

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Foegeding, & Hardin, 2004). Meanwhile we need to establish the fact that extensive studies of intermacromolecular interactions in such systems have been performed using mainly gelatin and some milk proteins.

This work studies on possible specific interactions between a legumin (or 11 S family), the main storage protein, and kappa carrageenan (kCG). Legumin, which has a molecular weight of 300–400 kDa, is composed of six nearly identical subunits with molecular weights of 50–60 kDa, and as received notable interests because of their molecular properties, diffusion, and applied importance. On the other hand, kCG is one of the most important polysaccharides with a wide range of food applications, including water jellies and dairy desserts as a gelling agent, processed meat products as a water holding agent, sauces, dressings, and beverages as a stabilizing and a thickening agent, etc. Studies on the interaction of carrageenans with proteins may contribute to spread its application area in the industries (Tolstoguzov, 1998).

The following methods were used: differential scanning calorimetry (DSC), circular dichroism (CD), fluorescence (FLU), the size exclusion chromatography equipped with an online multi-angle light scattering detector (SEC-MALS), turbidimetry and a methylene blue spectrophotometric method (MBS).

In this study, we have investigated the complexation between legumin and kCG at neutral condition, being considerably higher than the IEP of legumin ($\approx$4.7). Although some researchers suppose that protein–polysaccharide complexes can be formed only in the vicinity of the isoelectric point of the protein, soluble protein–polysaccharide complexes at pH 6.5–8.0 have been registered and studied by several methods for legumin–sodium salt carboxymethylcellulose (Antonov et al., 2006), gelatin–kappa carrageen (Antonov & Gonçalves, 1999) and gelatin–iota carrageen systems (Michon, Konate, Cuvelier, & Launay, 2002; Michon,Vigouroux, Boulenger, Cuvelier, & Launay, 2000).

2. Experimental section

2.1. Materials

Commercial Russian variety "Agate" of white common bean (*Vicia faba* L.) was purchased from a local market (Orel, Russia). Legumin from faba beans flour was isolated by combined salt fractionation and isoelectric precipitation as described before (Schwenke, Dudek, Seifert, Mothes, & Staatz, 1994). The homogeneity of 11 S globulin was confirmed by sedimentation analysis. The IEP of the protein is about 4.7. Kappa carrageenan is a commercial product supplied by Cargill, France, Lot 321973-HZ06-143. The polysaccharide was purified according to the method described before (Eremenko, Theunissen, Mortensen, & Reynaers, 2001; Slootmaekers et al., 1991). Such methodology has diminished the content of gel forming potassium ion more then 130 time (Ca^{2+} 0.01%, K^{+} 0.1%, Mg^{2+} 0.02%, Na^{+} 3.9%).

To prepare legumin stock solutions, the biopolymer was gradually added to the monobasic potassium phosphate–NaOH buffer (pH 7.3, $I = 0.01$) and stirred at 298 K for 1 h. The solutions were centrifuged at 20,000*g* for 1 h at 298 K to remove insoluble particles. Subsequently, the concentration of the biopolymer was determined by measuring the dry weight residue. In some cases, the final protein concentration was determined also by spectrophotometric measurements. All measurements were performed after equilibrating the biopolymer solutions and their mixtures for 15 h. kCG solutions were prepared by dispersing the gum in monobasic potassium phosphate–NaOH (pH 7.3, $I = 0.01$) and heating at 358 K for 20 min. Subsequent manipulations were the same as those described above for the preparation of the legumin solutions.

2.2. Methylene blue spectrophotometric method (MBS)

The MBS used to study interactions between legumin and kCG at the molecular level was previously developed by Michon et al. (2002) and Snoeren (1976). The attractive electrostatic interaction between the chains of a sulfated polysaccharide and methylene blue molecules modifies the visible spectrum (Michon, Cuvelier, Launay, & Parker, 1997; Schoenberg & Moore, 1964; Snoeren, 1976). A methylene blue solution shows an optical density maximum at about 663 nm corresponding to the absorption of the free methylene blue molecules. When a small amount of carrageenan is added to the methylene blue solution, the peak at 663 nm decreases and a plateau appears at 554 nm which can be attributed to the absorption of the methylene blue interacting with the sulfated groups of carrageenans chains. If legumin, which interacts associatively with carrageenan, is introduced in a methylene blue/carrageenan solution, a competition between the planar cationic and the legumin molecules for the sulfated groups of carrageenan takes place. Some methylene blue molecules are removed from the sulfated group sites and then released in the solution, the peak at 663 nm increases and the shoulder at 554 nm decreases.

The procedure was adapted from Michon et al. (2002). A methylene blue (MB) solution in phosphate buffer (pH 7.3, $I = 0.01$) was prepared by stirring at room temperature for 1 h. Its concentration ($\sim$0.001 wt%) was adjusted such as its absorption at 663 nm ($A_{663\ nm}$) and 20 °C had a value of about 1.3 in order that, for all experiments in which kCG is added in the MB solution, the $A_{663\ nm}$ value remains lower than 1.0, the absorption range where a linear response is obtained with the spectrophotometer.

Methylene blue/kCG (MB/kCG) solutions were prepared by adding the different amount of the stock solution of carrageenan in the MB solution and by stirring at 70 °C for 10 min and then at 80 °C for 20 min. Series of MB/kCG solutions were prepared in the range of carrageenan concentrations from 7.8×10^{-4} wt% to 2.4×10^{-2} wt%. On the other hand, MB/kCG/legumin solutions were prepared by adding at 56 °C and stirring for 10 min a small volume of a 5.0 wt% legumin solution in phosphate buffer (pH 7.3, $I = 0.01$).

Optical density sweeps as a function of the wavelength in the range 450–750 nm were performed at 25 °C in 1 cm glass cells using a Jasco U-500 UV/VIS Spectrophotometer.

2.3. Circular dichroism measurements

CD measurements of legumin solution alone and in the presence of kCG were performed using a Jasco J-720-S instrument. Far-UV CD spectra (from 185 nm to 250 nm) were measured in 0.5 mm cells, whereas near-UV CD spectra (from 250 nm to 320 nm) were determined in 1 cm cells in the temperature range from 25 °C to 85 °C and the temperature gradient of $\sim$60 °C h^{-1}. After achievement of the necessary temperature, the solution was kept in a cell at this temperature 15 min prior to measurement to stabilize the temperature. Similar manipulation was performed to study reversibility of CD spectra of biopolymer solutions and the mixtures after heating to 85 °C and subsequent cooling to 25 °C.

The solutions were scanned at 20 nm min^{-1} using 2 s as the time constant with a sensitivity of 20 mdeg and a step resolution of 0.1. The average of four scans was recorded and the experimental error involved in measurements was $<$1.5%. Data were corrected for the effect of buffer and polysaccharide, and results were expressed as mean residue ellipticity [θ], in units of degrees cm^2 dmol^{-1}. Mean residue ellipticities [θ] were obtained using the mean residue weight (MRW) calculated on the basis of the amino acid composition of legumin. The MRW was taken 113.6 for native legumin (Schwenke, Knopfe, Seifert, Gornitz, & Zirwer, 2000).