Encapsulation of Milk β-Lactoglobulin by Chitosan Nanoparticles

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Effect of Chitosan Degradation on Its Interaction with β-Lactoglobulin

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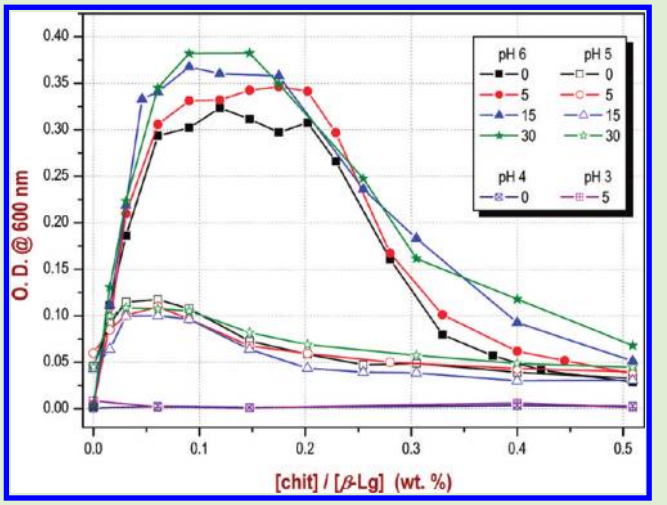
ABSTRACT: Complexes between chitosan and β-lactoglobulin (β-Lg) were investigated, and their formation was found to depend on pH and ionic strength. The electrostatic attraction between the cationic polysaccharide and the negatively charged protein above its isoelectric point has been identified as the main driving force in the molecular recognition process. At low protein concentration, soluble complexes were shown to be formed, and their structural features were characterized by circular dichroism (CD) and steady-state fluorescence. Both the overall secondary structure of the protein and the local environment probed by its tryptophan residues are not affected by the presence of chitosan in the complex. Furthermore, the formation of the complex does not lead to a net stabilization of the native state of the protein over its denatured state due to formation of a similarly stable complex between the polyelectrolyte and the denatured state of the protein. The formation of coacervates between β-Lg and chitosan was also characterized as a function of average molecular weight of chitosan (subjected to ultrasonication for different periods of time: 0, 5, 15, and 30 min) by means of both turbidimetric and calorimetric techniques. The combination of turbidimetric as well as isothermal calorimetric titrations have allowed the deconvolution of two processes usually coupled in the formation of protein−polyelectrolyte coacervates: the formation of complex coacervates as the protein sites become saturated by polyelectrolyte molecules and the redissolution of the coacervates as the polyelectrolyte-to-protein ratio increases.



INTRODUCTION

The interactions between proteins and polysaccharides have been widely studied in the last several years because of their relevance in many biological systems and their applications in the development of products for the pharmaceutical and food industries.[1] The molecular recognition process arises mainly from the establishment of favorable intermolecular electrostatic, hydrogen bonding, and hydrophobic interactions. The intensity of such interactions has been shown to be highly dependent on factors such as the degree of ionization of the polymer, the concentration ratio between polymer and protein, the bulk solution composition, and so on.

Bovine β-lactoglobulin (β-Lg) is the major protein in cow whey mixtures and is frequently used by the dairy industry as an ingredient in many food products. β-Lg is a globular protein composed of 162 amino acid residues with a molecular weight of 18 363 D[2] and an isoelectric point around 4.9.[3] The structure of the protein has been well characterized[4,5] and shown to be composed of mainly β-sheets, some β-turns, and a single α-helix. Under physiological conditions, β-Lg is predominantly dimeric, although the monomeric form is favored below pH 3 and low ionic strength without a decrease in the conformational stability of the protein.[6,7]

Chitin is a linear polysaccharide polymer of *N*-acetylglucosamine being the second most abundant polymer in nature after cellulose.[8] Chitosan is produced by partial deacetylation of chitin and therefore is composed of randomly distributed β-(1→4)-linked acetylated and deacetylated units (namely *N*-acetylglucosamine and D-glucosamine).[9] The amino group introduced in each deacetylated monomeric unit has a pK_a value of ∼6.5,[10] which results in the polymer acting as a cationic polyelectrolyte at acidic pH. The increase in the charge of the polymer at low pH leads to the expansion of the macromolecule due to the electrostatic repulsion among its charged amine groups.[11,12]

Chitosan is commonly used in food processing, water purification for agricultural uses, immobilization of enzymes and other proteins, control of cholesterol levels, some antimicrobial applications, and so on.[13−18] Recently, several studies have addressed the effect of chitosan molecular weight on its physical

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properties and on its efficiency in the tasks associated to some of the applications cited above.[17,19] Among the different methods commonly used for the degradation of long polymeric macromolecules, ultrasonication emerges as a simple and economical approach. The handling of several experimental parameters (temperature, ultrasound frequency and intensity, polymer concentration, etc.) has shown to allow us to control the extent of polymer degradation yielding hydrolyzed macromolecules within a certain range of molecular weight.[20,21] The effects of these ultrasonic conditions on the degradation of chitosan via chain scission through the β-(1→4)-linkage have been already reported by several studies. Strategies consisting of keeping constant some of the ultrasonication conditions have shown that the molecular weight distribution of chitosan is dependent on the ultrasonication time.[12,19,21,22]

The formation of soluble complexes and coacervates between protein and chitosan has been addressed in previous reports.[3,26−30] For example, the interaction of chitosan with porcine myofibrillar proteins and β-lactoglobulin has been attributed to the establishment of favorable electrostatic interactions between cationic chitosan and anionic proteins above their isoelectric point.[26,29] It has long been recognized that inmobilization of enzymes in water-soluble nonstoichiometric polyelectrolyte complexes may lead to the stabilization of enzymes against thermal denaturation.[45]

We have studied the formation of soluble complexes between β-Lg and chitosan by means of circular dichroism (CD) and steady-state fluorescence at low protein concentration (below 0.1 $mg \cdot mL^{-1}$). It has been found that the binding of the polysaccharide to β-Lg does not involve important changes in the secondary structure of the protein or the local environment of its tryptophan residues. Furthermore, the thermal stability of the protein remains unaffected by the formation of the complex with chitosan, which suggests that the polyelectrolyte is recognized by both the native and thermally denatured states of the protein with virtually the same affinity. As the concentration of protein is increased, the phase separation between β-Lg−chitosan complexes and the bulk solution is observed. We have studied the formation of these coacervates monitoring the increase in solution turbidity under a variety of solvent conditions (pH, ionic strength, etc.) to gain insight into the nature of the interactions responsible for the molecular recognition process. These studies were complemented using isothermal titration calorimetry (ITC) to monitor the amount of heat released when β-Lg was continuously titrated with chitosan until complete saturation of the protein. We characterized the role played by electrostatics in the recognition of chitosan by β-Lg by analyzing the effect of the charge of the protein (varying the pH below and above the isoelectric point of the protein) and the ionic strength of the solution on the energetics of complex formation.

MATERIALS AND METHODS

Materials. β-Lactoglobulin (β-Lg), was obtained as a commercial sample (PSDI 2400) from Arla Foods Ingredients, Ambh (Denmark) and used as the protein source without further purification. According to the producer, the protein content of the supplied material was 92% (obtained by determining the total nitrogen content using the Kjeldahl digestion method), and the β-Lg content (variants A and B) was >95%, as obtained by reverse-phase HPLC chromatography. The β-Lg molecular weight (M_w) is equivalent to 18 363 D. Chitosan samples with degree of deacetylation 90 were obtained from Primex (Siglufjordur, Iceland). The approximate molecular mass provided by the supplier was in the range 250 000−300 000 D. The actual average molecular masses (M_v) of the polysaccharide was obtained from viscosity measurements in our previous work[27] and estimated to be 303 000 D. All chemicals (sodium acetate, glacial acetic, sodium chloride, urea, etc.) were analytical grade and used without further purification. Purified water produced by a Milli-Q filtration system was used for the preparation of all solutions.

Solution Preparation. All acetate buffer solutions (3.0 < pH < 6.0) were prepared at a concentration of 0.10 $mol \cdot dm^{-3}$ in water, and the pH of the resulting solution was adjusted, when necessary, by the addition of aliquots either 0.1 $mol \cdot dm^{-3}$ acetic acid or 0.1 $mol \cdot dm^{-3}$ sodium acetate. Chitosan solutions were prepared by dissolving weighted amounts of the solid sample in 0.10 $mol \cdot dm^{-3}$ acetate buffer adjusted to the desired pH. The solutions were gently stirred for at least 2 h to ensure complete dissolution and stored at 4 °C. Before the experiments were performed, chitosan solutions were incubated at room temperature for more than 60 min and centrifuged at relative centrifugal force of 6300*g* during 30 min to ensure the elimination of the insoluble matter that could eventually exist in the chitosan solutions using an Eppendorf MiniSpin centrifuge.

Ultrasonic Degradation of Chitosan. Chitosan samples with M_v 303 000 D[27] were submitted to ultrasonic degradation for defined periods of time using an ultrasonic probe (BRANSON, model 250) equipped with a 1/8" microtip. In a typical experiment, 50 mL of a 0.7% (w/w) chitosan solution in 0.10 $mol \cdot dm^{-3}$ acetate buffer at the desired pH was sonicated on ice for a specific period of time (0, 5, 15, and 30 min) using a constant duty cycle and output ultrasound power of 200 W.

METHODS

Circular Dichroism. CD spectra of β-Lg (in the presence and absence of chitosan) were collected on a Jasco J810 spectropolarimeter (Tokyo, Japan) fitted with a thermostatted cell holder and interfaced with a Peltier unit. The instrument was periodically calibrated with (+) 10-camphorsulphonic acid. All CD spectra were collected at 25 °C (unless it is stated otherwise) using a 0.1-cm-path-length quartz cell (Hellma). Protein concentration was 0.1 $mg \cdot mL^{-1}$ to avoid the formation of coacervates, and the protein-to-polyelectrolyte ratio was 1:1 on a w/w basis. The concentration of buffer was also lowered to 0.01 $mol \cdot dm^{-3}$ because of its strong absorbance in the far UV region. Isothermal wavelength CD spectra were acquired at a scan speed of 50 nm/min with a response time of 2 s and averaged over six scans at 25 °C. All spectra were corrected by subtracting the proper baseline. The molar ellipiticity, [Θ], and the helical content were calculated, as described elsewhere.[31] In addition to changes in secondary structure and aromatic side chains, far-UV CD is also sensitive to distortions of helices such as bends or tilting of the amide planes relative to the helix axis.[31−33] Modern methods of CD analysis, such as CDNN[34] or DICHROWEB,[35,36] were used to get estimations of the secondary structure contents of the free protein and the protein−chitosan complex.

Fluorescence. Fluorescence spectra were collected on a Cary Eclipse spectrofluorometer (Mulgrave, Australia) interfaced with a Peltier temperature-controlling system. Sample concentrations (both β-Lg and chitosan) were identical to the ones used in CD to allow for direct comparison of the results. All fluorescence spectra were collected at 25 °C (unless it is stated otherwise) using 1 cm path length quartz cells (Hellma).

(a). Steady State Fluorescence Measurements. β-Lg emission spectra (in the absence and presence of chitosan) were recorded at 25 °C between 300 and 450 nm after excitation of the sample at 280 nm using excitation and emission slits equal to 5 nm. The signal was acquired for 1 s, and the wavelength increment was 1 nm.

(b). Thermal Denaturation. Thermal denaturations of β-Lg in the absence and presence of chitosan (at different urea concentrations) were performed at a constant heating rate of 60 °C/h between 20 and 100 °C by monitoring the emission of the protein at 335 and 350 nm after excitation at 280 nm as a function of temperature. As in the steady-state

experiments, the excitation and emission slits were set to 5 nm, and the signal was averaged.

Isothermal Titration Calorimetry (ITC). The energetics of the interaction between chitosan and β-Lg were measured using an isothermal titration calorimeter (VP-ITC, MicroCal, Northampton, MA).[37] The protein (or chitosan) solution was loaded into the calorimetric cell (1.4189 mL), equilibrated at 25 °C, and titrated adding 40−50 successive 5 μL injections of chitosan (or protein) while continuously stirring the solution at 310 rpm. After each chitosan (or protein) addition, a certain amount of heat evolved as a consequence of concomitant formation of the protein−polyelectrolyte complex, and the temperature of the measuring cell either increased (for an exothermic reaction) or decreased (for an endothermic reaction) as compared with the temperature of the reference cell. The amount of electrical power ($\mu cal \cdot s^{-1}$) sent to the reference cell (for an exothermic reaction) or to the measuring cell (for an endothermic reaction) to maintain a match to the temperatures of both cells was monitored as a function of time. Typically, the injections were made at a constant flow rate of 0.5 $\mu L \cdot s^{-1}$ (usually 5 μL in 10 s) to minimize both mechanical heat and temperature gradients within the calorimetric cell upon ligand injection. The time between injections was adjusted to 210 s because the kinetics of β-Lg−chitosan complex formation was shown to be fast enough to ensure the completion of the reaction within this time interval. The amount of power required to maintain the reaction cell at constant temperature after each injection was monitored as a function of time. The integration of each calorimetric peak yields the apparent enthalpy change for coacervates formation using the software package Origin 7.0 from MicroCal. All experiments were performed at 25 ± 0.1 °C and were repeated at least three times.

Turbidity Measurements. The turbidity (optical dispersion, OD) of chitosan/β-Lg solutions was measured using a Shimadzu UV-1603 UV−visible spectrophotometer at a wavelength of 600 nm using quartz cells (HELMA) with a light path of 10 mm. Turbidity values were recorded only when the signal became stable and were corrected using the buffer as a blank. Each reported value is the average of three consecutive readings. Three different experimental approaches were used to monitor the formation of chitosan/β-Lg coacervates by the increase in the turbidity of the solution: (i) to study the effect of the chitosan concentration on the extent of coacervate formation with β-Lg, several solutions were prepared in 2 mL of disposable centrifuge tubes containing a constant concentration of protein (either 0.05% or 0.025% (w/w)) and increasing concentrations of chitosan delivered from a common stock solution (0.07% (w/w)). The solutions were prepared in the pH range of 3−6 (measured using a Crison pHmeter, model GLP 21) and were allowed to stand at room temperature for at least 30 min to ensure that the reaction was completed before its turbidity was recorded. (ii) To account for the effect that the charge of the protein has on the formation of chitosan/β-Lg complexes, coacervate solutions containing both β-Lg at a concentration of 0.025% (w/w) and chitosan at a concentration at which the maximum of turbidity was detected (0.00368% (w/w)) were equilibrated at pH 6.0 and titrated with acetic acid (at a concentration of 1 $mol \cdot dm^{-3}$) until the solution reached pH 3. Both the pH and the turbidity of the solution were measured after each addition of acetic acid. (iii) Finally, to get insight into the nature of the interactions involved in the formation of the chitosan/β-Lg complexes, coacervate solutions were titrated with a concentrated NaCl solution (4 $mol \cdot dm^{-3}$) at both pH 6 (with protein and polyelectrolyte concentrations of 0.025 and 0.00368 $g \cdot dL^{-1}$, respectively) and at pH 5 (being the concentrations used for β-Lg and chitosan 0.025 and 0.00152 $g \cdot dL^{-1}$, respectively).

RESULTS AND DISCUSSION

Effect of Chitosan on the Structure and Thermal Stability of β-LG. Figure 1A shows the far-UV CD spectra of β-Lg in the absence and presence of chitosan at pH 6. The CD spectrum of β-Lg is strongly dependent on pH[5] because e between pH 2 and 13 the protein undergoes a number of pH-induced structural transitions. The CD spectrum of the free protein is consistent with its known secondary structure (Table 1) with a large percentage of β-sheet, β-turns, and random coil and a low content of α-helix and compares well with the CD spectra reported in the literature under similar conditions.[5,44] More importantly, though, the negative dichroic signal of the free protein seems to be slightly enhanced with respect to one of its complexes with chitosan (either as the soluble complex or the resulting supernatant of the protein/chitosan filtered solution), the deconvolution of those spectra (Table 1) yields virtually the

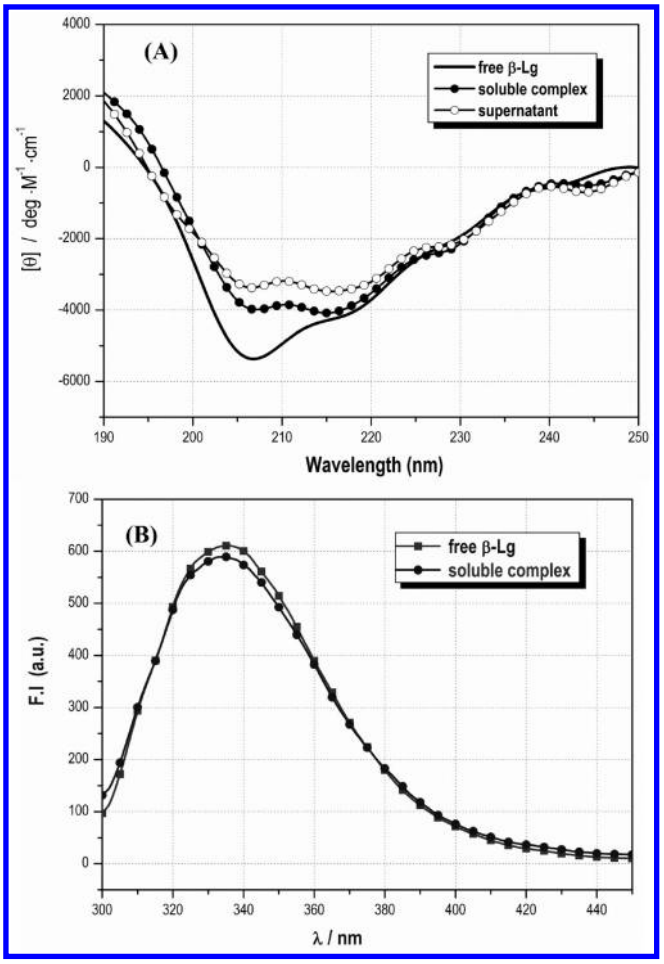

Figure 1. (A) Far-UV CD spectra of β-Lg solution (solid line), chitosan/β-LG soluble complex (●), and chitosan/β-LG supernatant (○) in 0.01 $mol \cdot dm^{-3}$ acetate buffer, pH 6. (B) Fluorescence emission spectrum of β-Lg solution (blue) and chitosan/β-LG soluble complex (red). The concentrations of both protein and polymer solutions are 0.1 $mg \cdot mL^{-1}$ in 0.1 $mol \cdot dm^{-3}$ acetate buffer.

Table 1. Percentage of the Different Elements of the Secondary Structure of β-Lg Free in Solution and Complexes with Chitosan

sample	% (α-helix)	% (β-sheet)	% (β- turn)	% (random coil)
β-Lg (no chitosan)	18.4	38.2	20.4	41.4
β-Lg (complex)	19.3	36.1	20.2	40.3
β-Lg (supernatant)	18.3	38.2	20.4	41.7

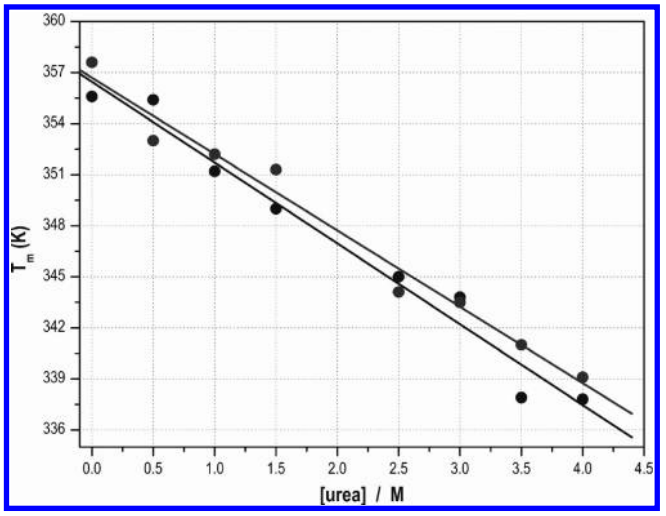

Figure 2. Dependence of the thermal stability of β-Lg (0.1 mg·mL^{-1}) on the concentration of denaturant (urea) in absence and presence (0.1 mg·mL^{-1}) of chitosan at pH 6.0 obtained from temperature scans monitoring the fluorescence of the protein as a function of temperature. The melting temperature decreases linearly with the concentration of urea. The slope of this decrease, $(\partial T_m/\partial[D])_p$, is virtually the same, within experimental error, for the free protein, -4.75 ± 0.32 deg·M^{-1} (blue), and the complex between β-Lg and chitosan, -4.49 ± 0.27 deg·M^{-1} (red).

same percentage of secondary elements (α-helix, β-sheet, β-turns, and random coil), which suggests that the binding of chitosan to the protein has a negligible effect on its overall structure. Increasing the protein concentration leads to the characteristic phase separation when coacervates between proteins and oppositely charged polyelectrolytes are formed.[1] Therefore, the slightly enhanced ellipticity of the free protein with respect to that observed for the complex can be simply ascribed to the coexistence in solution of both a fraction of soluble protein−polyelectrolyte complex (a major component at this concentration) and a fraction of a protein involved in a coacervate complex that is opaque to the electromagnetic radiation. This interpretation is consistent with the results obtained for the emission spectra of the free and chitosan-complexed protein presented in Figure 1B.

Figure 1B shows the emission spectra of β-Lg (0.1 mg·mL^{-1}) at pH 6.0 in the absence and presence of chitosan (0.1 mg·mL^{-1}). Both spectra are characterized by a single emission band centered at around 335 nm, which is characteristic of a well-folded protein in which the tryptophan residues (that dominate the fluorescence signal under the excitation conditions used) are mainly buried within the interior of the protein. Both the intensity of the emission band and the position of its maximum are very sensitive to changes in the local environment of the tryptophan residues. The exposure of the tryptophan residues that is induced upon protein denaturation (either by heat or the presence of high concentrations of denaturants) leads to a red shift of the fluorescence spectra of the protein concomitant with an increase in the emission intensity. The similarity of the fluorescence spectra of the protein presented in Figure 1B is indicative that the local environment of the tryptophan residues of the protein is very similar in both the free protein and the complex. Again, as was shown in the CD spectra (Figure 1A), the slightly smaller intensity of the fluorescence spectrum of the protein forming a complex with chitosan as compared with the one shown by the protein free in solution may reflect the existence of a small fraction of protein complexed with chitosan in the coacervate phase.

Therefore, we can conclude from the results presented in Figure 1 and Table 1 that the binding of chitosan to β-Lg involves neither a significant change in the overall secondary structure of the protein nor a change in the local environment probed by the tryptophan residues of the protein.

Because the formation of the β-Lg−chitosan complex does not seem to involve significant structural changes in the native protein conformation, we turned our attention to the effect that the formation of the (soluble) complex could have on the thermal stability of the protein. Figure 2 shows the decrease in the melting temperature of the protein (T_m) with the concentration of the neutral denaturant urea obtained from the temperature dependence of the fluorescence intensity of the protein. To destabilize the native state of the protein (therefore decreasing its apparent T_m), we chose urea as denaturant because of its neutral nature to minimize the influence of the presence of molar amounts of the denaturant on the protein−chitosan free energy of binding (mainly due to the electrostatic attraction between the two molecules, see below). At any given urea concentration, the temperature of melting of the protein, T_m, is virtually the same, within experimental error, for the free protein and the chitosan-complexed one. Furthermore, the melting temperature for both free β-Lg and the complexed one decreases linearly with the concentration of denaturant (urea), yielding very similar slopes in the linear correlation of T_m versus [urea]; $(\partial T_m/\partial[D])_p$ equals -4.75 ± 0.32 deg·M^{-1} for the free protein and -4.49 ± 0.27 deg·M^{-1} for the protein complexed with chitosan.

According to the results obtained in the thermal denaturation experiments, the formation of the β-Lg−chitosan complex does not lead to any significant stabilization (or destabilization) of the native state of the protein with respect to its denatured one because no change in the T_m of the protein is observed at any given concentration of denaturant. Because under the experimental conditions probed (low ionic strength and pH 6.0 above the isoelectric point of the protein) the native state of the protein forms a stable complex with chitosan (that at concentrations >0.1 mg·mL^{-1} leads to the formation of coacervates), we must conclude that the thermally denatured state of β-Lg forms an almost equally stable complex with chitosan. In other words, the formation of the complex between β-Lg and chitosan leads to no net stabilization of the native state of the protein over its denatured state because of the fact that on the one hand no significant change in the conformational state of the native state of the protein is induced upon complex formation and on the other hand the overall free energy change, ΔG, upon chitosan binding to the native state of the protein is mainly balanced by a similar ΔG of the polyelectrolyte to the denatured state of the protein.

The consequences of these results are far from being elucidated; clearly, further work needs to be done in this respect. The formation of complexes between polyelectrolytes and partially or fully unfolded proteins opens new perspectives in the use of these ligands under conditions in which the interaction between a given protein and a polyelectrolyte leads to the formation of a stable and soluble complex. The change in the surface density charge of the protein as a consequence of its binding to a polyelectrolyte would have an important and positive impact in the mitigation of problems such as the irreversibility of thermal-denaturation processes coupled to the aggregation of partially or

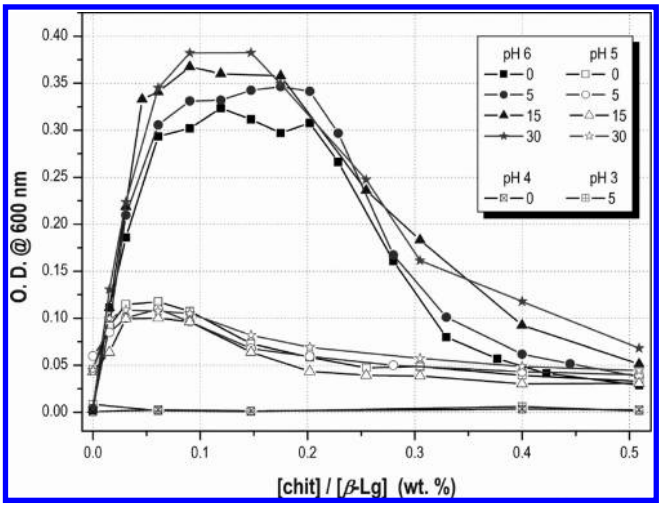

Figure 3. Turbidity of the samples containing different chitosan to β-Lg weight % ratio. The concentration of β-Lg was 0.025 wt %, and the concentration of chitosan was varied from 0 to 0.0125 wt %. The turbidity (optical dispersion at 600 nm) was measured for chitosan samples previously submitted to different sonication times (0, ■; 5, ●; 15, ▲; and 30 min, ★, respectively). The filled symbols represent the turbidity of the samples at pH 6.0 and the open symbols represent the turbidity of the samples at pH 5.0. The turbidity of two additional pH 4 samples at pH 4.0 (square with ×) and pH 3.0 (square with +) are also presented (chitosan not submitted to sonication).

fully unfolded proteins, the control of protein solubility (especially at pH values close to its isoelectric point), the solubilization of inclusion bodies frequently obtained in the large-scale purification of proteins after their overexpression using molecular biology techniques, and so on.

We monitored the formation of complex coacervates between β-Lg and chitosan by measuring the turbidity of the solution induced by the dispersion of the incident light (600 nm) by the coacervate particles formed. To address the effect of chitosan molecular weight on its capability of forming coacervates with β-Lg polysaccharide, samples were degraded by ultrasonication for different periods of time (0, 5, 15, and 15 min.). The reduction of the molecular weight of chitosan by high-intensity ultrasound has been the subject of quite detailed studies[23−25] leading to the description of the mechanism of the sonolysis process and the characterization of both the process parameters and the physicochemical properties of the fragments. The mechanism of chitosan degradation is shown to be adequately described by a random scission model.[25] The degree of deacetylation of the fragments remained has been shown to remain unchanged compared with the initial polysaccharide sample[24] (and therefore their charge density).

Figure 3 shows turbidity of solution containing a constant concentration of β-Lg (0.025 wt %) and increasing concentration of chitosan (degraded by sonolysis for different periods of time) at different pH values in the range 3−6. The formation of coacervates is shown to be maximal at pH 6, and the effect largely decreased upon reduction of the pH of the solution. On average, the turbidity for the same chitosan-to-β-Lg wt % ratio is reduced by a factor of 3 when the pH of the solution is decreased from 6 to 5, becoming barely detectable when the pH of the solution is below this value. At pH 6, the addition of chitosan to the buffered solution containing β-Lg (0.025 wt %) induces a rapid increase in the turbidity of the solution reaching a maximum when the wt % ratio is around 0.10 to 0.15 and decreasing monotonically when the concentration of chitosan increases (at constant protein concentration). The increase in the turbidity of the solution can be ascribed to the formation of coacervates containing protein−chitosan complexes,[26,27,30] whereas its decrease after reaching a maximum can be interpreted by the redissolution of the protein−polyelectrolyte complexes as the number of chitosan molecules per protein molecule decreases for concentrations of the polysaccharide higher that the ones needed for saturation of the protein.

The overall trend observed at pH 5 is similar to the one described above, although the coacervates formation seems to be greatly reduced. At pH 5, the turbidity reaches a maximum (with an intensity three-fold lower than at pH 6) at a wt % ratio of ∼0.05 (where saturation of the protein by chitosan is completed) and decreases for larger concentrations of chitosan, which is consistent with the redissolution of the complex coacervates as the molar ratio of chitosan to β-Lg increases above its characteristic value for complete saturation.

At pH 3 and 4, the formation of complex coacervates is barely detectable, as shown for the negligible turbidity of the solution containing chitosan and β-Lg at different wt % ratios. Similar results were obtained for β-Lg samples containing 0.05 wt % (data not shown).

The pH dependence of the complex coacervates (for any given extent of degradation of chitosan) clearly indicates that electrostatic attraction between the positively charged chitosan and the negatively charged protein plays a key role in the molecular recognition process. Whereas the (partial) deacetylation of the N-acetylglucosamine monomeric units of chitin yields a polymer that would act as a cationic polyelectrolyte below physiologic pH, the negative charge borne by the protein at pH 6 (above its isoelectric point, pI, ∼4.9) rapidly diminishes as the pH decreases becoming positive below 4.9. Therefore, the strength of the electrostatic attraction between the cationic polyelectrolyte and β-Lg within the pH interval studied would be maximal at pH 6. At pH values below the isoelectric point of the protein, the electrostatic attraction between the positive charge of chitosan and the negative patches in the protein surface would be opposed by the repulsion of the cationic polyelectrolyte and the protein bearing an overall positive charge that increases as the pH of the solution is lowered.

When chitosan is submitted to controlled degradation via ultrasonication for defined periods of time, the polydispersity of the samples increases because of the unspecific hydrolysis of the β-(1→4) glucosidic bonds favoring the population of lower molecular weight chitosan polymers.[38−40] According to the results presented in Figure 3, there is a clear correlation between the degree of degradation of chitosan and its tendency to form complex coacervates with β-Lg. At pH 5 (around the isoelectric point of the protein), the interaction becomes detectable by turbidimetric methods (Figure 3), even at concentrations of protein as low as 0.025% (w/w), although the small effect detected precludes the identification of any dependence of the turbidity with the average molecular weight of chitosan fragments. At pH 6, the turbidity of samples containing identical concentration of both chitosan and protein increases with the degree of degradation of chitosan. This can be more clearly seen in Figure 4, where the turbidity of the maximum in Figure 3 (corresponding to the saturation point of β-Lg with chitosan) is plotted against the time of ultrasonication to which the chitosan sample was submitted. The slope of the apparent linear correlation scales with the concentration

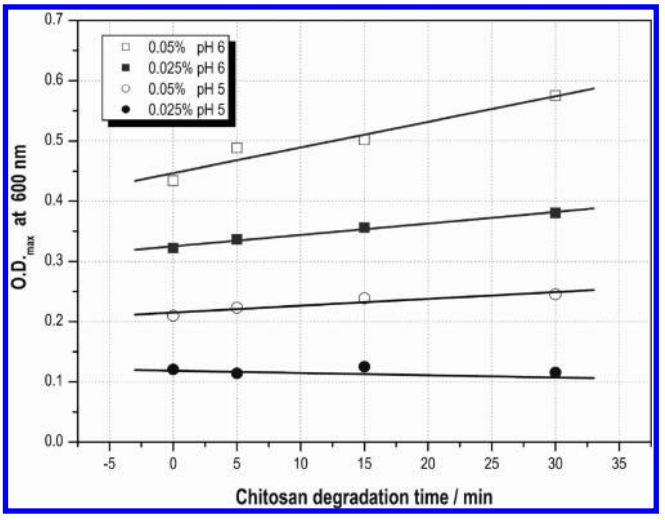

Figure 4. Maximun turbidity for the titration of β-Lg with chitosan versus the time of degradation of the polysaccharide. From top to bottom, the protein samples contained 0.05 wt % at pH 6 (□), 0.025 wt % at pH 6 (■), 0.05 wt % at pH 5 (○), and 0.025 wt % at pH 5 (●).

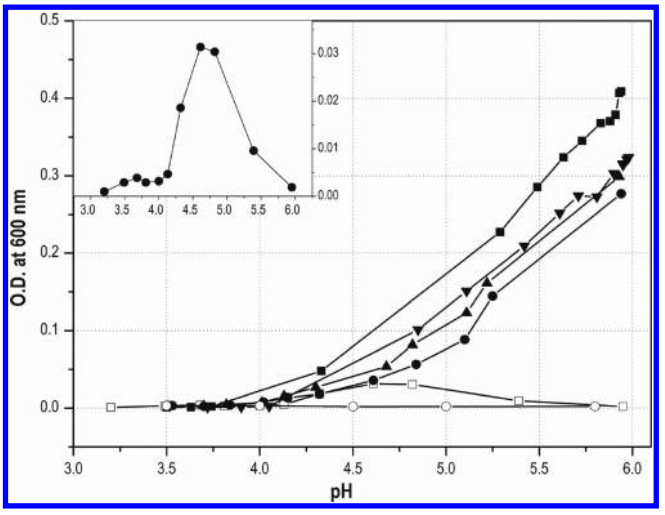

Figure 5. pH dependence of the turbidity of solutions containing β-Lg at a concentration of 0.025 wt % and chitosan (with different degrees of degradation) at a wt % ratio of 0.17 (maximum in Figure 3). The samples contained no chitosan (□), intact chitosan (no β-LG) (○), and β-Lg with chitosan submitted to ultrasonic degradation for different periods of time: 0 (●), 5 (▲), 15 (▼), and 30 min (■). Inset: pH dependence of the turbidity of β-Lg (no chitosan). Note that the scale has been enlarged by a factor of 15. As expected, the turbidity of β-Lg solution reflects the decrease in the solubility of the protein as the pH of the solution approaches its isoelectric point.

of β-Lg and is shown to be highly dependent on protein concentration and the pH of the solution, being four times higher at pH 6 than at pH 5.

To get a deeper insight into how the degradation of chitosan influences its interaction with β-Lg and concomitant formation of complex coacervates, the pH dependence of the turbidity of solutions containing a constant concentration of β-Lg (0.025 wt %) and chitosan at the a concentration of 0.0043 wt % is determined (corresponding to a wt % ratio of 0.17 at which the maximum in turbidity for the titration of β-Lg with chitosan is obtained, see Figure 3). The results are presented in Figure 5.

As could be expected from the results presented above, the formation of complex coacervates between β-Lg and chitosan is almost negligible at low pH (below the isoelectric point of the protein) and increases monotonically as the pH is increased above this value. The inset in Figure 5 shows the turbidity of the solution containing β-Lg at a concentration of 0.025 wt % (without chitosan) as a function of pH. The turbidity of the protein solution (in the absence of chitosan) clearly shows how the solubility of the protein decreases rapidly as the pH of the solution approaches its isoelectric point (~4.9 for the conditions used here, 100 mM acetate buffer). The concentration of chitosan in the experiments presented in Figure 5 (0.00425 wt %) is low enough to ensure that the polysaccharide remains soluble within the pH interval studied here. (See Figure 5.)

The results obtained for the β-Lg/chitosan samples showed a similar general trend in all cases. The tendency to form complex coacervates is highly reduced at pHs below the isoelectric point of the protein (~4.9 for the experimental conditions used here) because the electrostatic attraction of the cationic polyelectrolyte (chitosan) by the negatively charged protein residues is largely overcome by the repulsion of the more numerous positively charged residues. As the pH of the solution increases, the balance between these two forces becomes more favorable to the formation of the complex due to the increase in the number of negative charges in the surface of the protein. The average size of the cationic polysaccharide is shown to have a non-negligible effect on the tendency of chitosan to form complex coacervates with β-Lg. For any given pH, the turbidity of the solution scales with the time of degradation of the chitosan sample (i.e., the average molecular weight of the polysaccharide). The turbidity of a sample is a function not only of the number of dispersing particulates but also their average size. Therefore, the results presented in Figure 5 might be understood as a balance between these two factors. On one hand, the total number of particles in solution for samples having a fixed wt % of both chitosan and β-Lg would increase as the average molecular weight of chitosan decreases (increasing in the ultrasonic degradation time). On the other hand, the average size of these complex protein−polyelectrolyte coacervates would decrease with the molecular weight of chitosan because the charge density of the initial chitosan polymer and the fragments obtained by sonolysis is basically the same. (The fragmentation does not affect the degree of acetylation of the polysaccharide[23,25].) The sum of both contributions to the observed turbidity of the solution leads to a slight increase in this observable. Noticeably, the formation of complex coacervates below the isoelectric point of the protein is favored for chitosan samples with a larger degree of degradation. The reduction in the average molecular weight of the polysaccharide with the time of sonication may propose a plausible explanation for this behavior: chitosan molecules with lower molecular weight might be favored in the balance between its electrostatic attraction by the negatively charged residues of the β-Lg and the repulsion by the overall positively charged protein below its isoelectric point.

One of the major conclusions deduced from the results presented above, is the key role played by the electrostatic attraction in the molecular recognition process between β-Lg and chitosan at pHs above the isoelectric point of the former. To gain insight into the nature of the interaction, we studied the effect of the ionic strength of the solution on the formation of complex coacervates between the protein and the polysaccharide at pH 6 and a constant wt % ratio of 0.14. The results are presented in

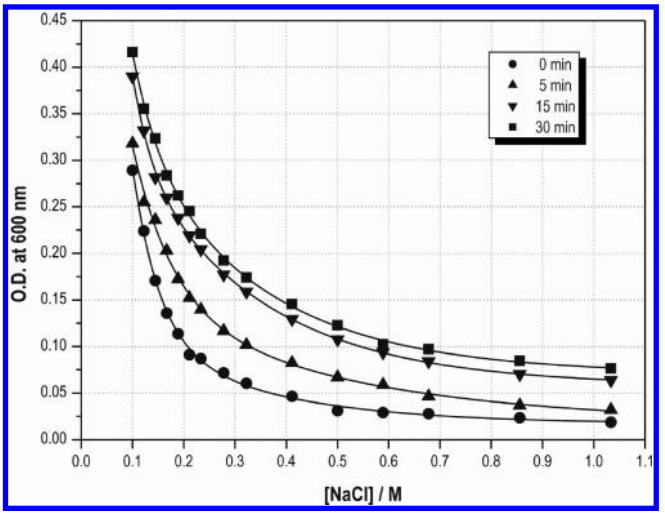

Figure 6. Ionic strength dependence of the complex coacervates formation between β-Lg and chitosan samples subjected to ultrasonic degradation for different periods of time: 0 (●), 5 (▲), 15 (▼), and 30 min (■). The turbidity of the each solution was recorded as its optical density at 600 nm and 25 °C.

Figure 6. As could be expected for a molecular recognition process dominated by the electrostatic attraction between a cationic polyelectrolyte (chitosan) and a protein molecule bearing and overall negative charge above its isoelectric point, the intensity of the interaction is highly affected by the increase in the ionic strength of the solution. The presence of increasing concentrations of inert ions in the solution has the effect of screening the electrostatic attraction between the interacting charged polyelectrolyte species responsible for the formation of complex coacervates.[41−43] Similar results were obtained for the same samples at pH 5 with the only difference in the lower turbidity interval monitored.

To study further the nature of the interaction between β-Lg and chitosan, we carried out calorimetric titrations characterizing the energetics of complex formation. Figure 7 shows a series of experiments necessary for the calorimetric characterization of the reaction at pH 6 and 25 °C. Panel A shows the calorimetric output for the titration of β-Lg (0.25 wt %, in the calorimetric cell) with intact chitosan (0.7 wt %, in the syringe) together with the dilution experiments (chitosan injected into buffer and buffer injected into protein) necessary for correction. Panel B shows heat evolved as a consequence of the formation of the chitosan−protein complex (corrected for the dilution effects, as in our previous work[24]) plotted against the wt % ratio, [chitosan]/[β-Lg].

The formation of the β-Lg−chitosan complex is characterized by an exothermic enthalpy change, as seen by the release of heat upon polysaccharide binding to the protein and subsequent coacervate formation. The corrected heat effect for complex formation decreases (in absolute value) as the protein becomes saturated with the polysaccharide, reaching a negligible value for a wt % ratio of 0.25.

It is important to note that whereas the turbidity of the solution is an indirect measure of the coacervate formation, the heat evolved throughout the titration is a direct measure of all of the events involved in the molecular recognition process, namely, the formation of soluble protein−polysaccharide complexes and the subsequent formation of coacervates and their eventual redissolution as the concentration of chitosan increases above the one needed for complete saturation of the protein. The comparison between the turbidimetric titration of β-Lg with chitosan (Figure 3) and the calorimetric one (Figure 7) allows us to understand better the nature of the processes taking place throughout the titration reaction. For the turbidimetric titration (monitoring the amount and size of dispersing particles in solution as a function of chitosan concentration), the turbidity of the solution increased sharply with the concentration of chitosan until a maximum was reached at a wt % ratio close to 0.17. The increase in the concentration of polysaccharide above this point led to a decrease in the turbidity, which was ascribed to the redissolution of the coacervates when the protein-to-polyelectrolyte stoichiometry of the initially formed complexes decreased and the solubility of the complex increased as a consequence of the larger number of free charges now exposed to the solvent. The picture given by Figure 7 (calorimetric titration) seems to be at odds with the one described above: the heat evolved by the formation of the protein−polysaccharide coacervates decreases (in absolute value) as the protein becomes saturated by chitosan, and the process seems to be completed at a wt % ratio of 0.25 with the redissolution process being detected. To reconcile both interpretations, one needs to take into account the fact that during the redissolution process the increase in the concentration of chitosan does not change the number of protein−polyelectrolyte contacts because the establishment of new interactions between β-Lg and the chitosan molecules added to the solution implies the disruption of similar interactions between protein molecules and chitosan molecules previously present in the solution. As a consequence, the enthalpy change due to the redissolution can be anticipated to be close to zero because of the similar energetics of the newly formed interactions and the ones previously disrupted. Therefore, the calorimetric titration monitors mainly the formation of the protein−polyelectrolyte complexes without the contribution of the redissolution process.

The wt % ratio observed for complete saturation of the protein in the calorimetric titration (0.25) is higher than the one corresponding to the maximum in the turbidimetric titration (0.17). This difference can be understood if we consider that the redissolution of the initially formed β-Lg−chitosan complexes (in which the number of protein molecules per chitosan molecule is high) takes place before the protein becomes completely saturated with the polysaccharide. In other words, as soon as the first complexes are formed, the addition of chitosan to the solution leads to the competition between complex formation (increasing the turbidity of the solution) and their redissolution to give complexes with a lower protein to polyelectrolyte stoichiometry that would be characterized by an enhanced solubility due to the exposure to the solvent of a larger number of charged moieties (reducing the turbidity of the solution).

We have also investigated how the average molecular weight of the chitosan sample affects its ability to form complex coacervates by means of the ITC in a similar way as the one presented above for intact chitosan. The calorimetric results obtained here are in line with the turbidimetric titrations discussed above. For all chitosan samples (regardless its degree of degradation), the complex formation was barely undetectable for pH values below 5, in agreement with the idea that electrostatic repulsion between the cationic polysaccharide and positively charged protein below its isoelectric point dominates the interaction precluding the formation of a stable complex.

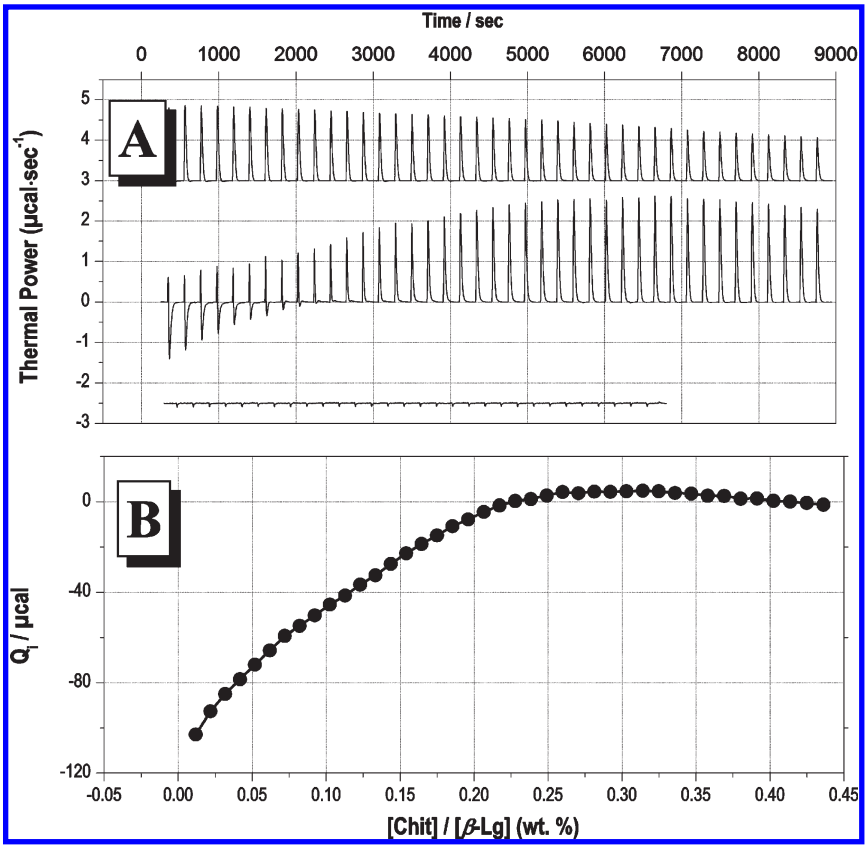

Figure 7. Typical microcalorimetric titration experiments performed for the characterization of the complex formation of chitosan and β-Lg complexes at pH 6 and 25 °C. (A) Calorimetric output (thermal power vs time) for the titration of β-Lg (0.25 wt % in the calorimetric cell) with intact chitosan (0.7 wt % in the syringe) in acetate buffer at pH 6 (center) together with the signals obtained for the dilution of chitosan (0.7 wt % injected into the calorimetric cell containing buffer, top) and the dilution of the protein (buffer injected into the calorimetric cell containing β-Lg at a concentration of 0.25 wt %) (bottom). (B) Heat for the formation of the complex obtained by integration of the calorimetric outputs in panel A plotted against the wt % ratio ([Chitosan]/[β-Lg]. The heats obtained for each injection in the titration experiment (chitosan injected into the calorimetric cell containing protein, center panel A, was corrected by the heats of dilution of both chitosan and protein (top and bottom isotherms in panel A)).

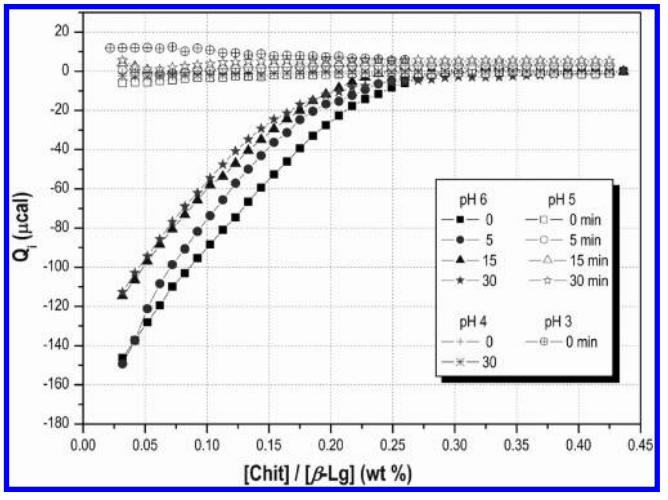

Figure 8. Interaction enthalpies for the titration of β-Lg at a concentration of 0.25 wt % with chitosan solutions (all at a concentration of 0.7 wt %) in 0.1 M acetate buffer at pH 6.

Similarly to the results presented in Figure 7, at pH 6, the formation of protein−chitosan complexes is characterized by an exothermic enthalpy that decreases (in absolute value) monotonically as the protein becomes saturated with chitosan for all chitosan samples (regardless of the degree of degradation). The enthalpy change upon complex formation was exothermic for all chitosan samples tested, although it was less exothermic for those samples with smaller chitosan molecular weight (Figure 8). Nonetheless, no major difference was observed in the wt % ration ([chitosan]/[β-Lg]) needed for complete saturation of the protein.

CONCLUSIONS

The formation of soluble complexes between chitosan and β-lactoglobulin does not lead to a significant change in the structure of the native state of the protein. Both the overall secondary structure of the protein (monitored by the far-UV spectra CD spectra of the protein) and the local environment probed by its tryptophan residues (shown by the protein emission spectra) are not affected by the presence of chitosan in the complex.

Moreover, according to the results obtained in the thermal denaturation experiments, the formation of the β-Lg−chitosan complex does not lead to any significant stabilization (or destabilization) of the native state of the protein with respect to its denatured one because no change in the T_m of the protein is observed at any given concentration of urea denaturant. Because chitosan binds with fairly high affinity to the native state of the protein, these results clearly suggest that chitosan is also able to bind to the heat-denaturated state of the protein because the

overall free energy changes upon binding of chitosan to either conformational state (native and heat-denaturated) are almost identical.

The formation of insoluble complexes between chitosan and β-Lg is shown to be highly dependent on pH, ionic strength, protein/polysaccharide concentration ratio, and molecular weight of chitosan. In addition, because sonolysis of chitosan does not affect its degree of deacetylation (because the charge density for intact chitosan and its fragments essentially the same), the average molecular weight of the polysaccharide was shown to be an important factor in the efficiency of the polyelectrolyte to form insoluble coacervates with β-lactoglobulin at constant pH and ionic strength as well as to form soluble complexes at high polyelectrolyte to protein ratios (redissolution).

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REFERENCES

(1) Turgeon, S. L.; Schmitt, C.; Sanchez, C. *Curr. Opin. Colloid Interface Sci.* **2007**, *12*, 166–178.
(2) Onwulata, C. I.; Huth, P. J. *Whey Processing, Functionality and Health Benefits*; Wiley-Blackwell: Hoboken, NJ, USA, 2008.
(3) Harnsilawat, T.; Pongsawatmanit, R.; McClements, D. J. *Food Hydrocolloids* **2006**, *20*, 577–585.
(4) Sawyer, L.; Kontopidis, G.; Wu, S.-Y. *Int. J. Food Sci. Technol.* **1999**, *34*, 409–418.
(5) Townend, R.; Kumosinski, T. F.; Timasheff, S. N. *J. Biol. Chem.* **1967**, *242*, 4538–4545.
(6) Uhrinova, S.; Smith, M. H.; Jameson, G. B.; Uhrin, D.; Sawyer, L.; Barlow, P. N. *Biochemistry* **2000**, *39*, 3565–3574.
(7) Qin, B. Y.; Bewley, M. C.; Creamer, L. K.; Baker, H. M.; Baker, E. N.; Jameson, G. B. *Biochemistry* **1998**, *37*, 14014–14023.
(8) Dupuis, G.; LeHoux, J. G. *Carbohydr. Polym.* **2007**, *68*, 287–294.
(9) Aranaz, I.; Mengíbar, M.; Harris, R.; Paños, I.; Miralles, B.; Acosta, N.; Galed, G.; Heras, A. *Curr. Chem. Biol.* **2009**, *3*, 203–230.
(10) Muzzarelli, R. A. A.; Muzzarelli, C. *Adv. Polym. Sci.* **2005**, *186*, 151–209.
(11) Signini, R.; Desbrières, J.; Campana Filho, S. P. *Carbohydr. Polym.* **2000**, *43*, 351–357.
(12) Czechowska-Biskup., R.; Rokita, B.; Lotfy, S.; Ulanski, P.; Rosiak, J. M. *Carbohydr. Polym.* **2005**, *60*, 175–184.
(13) Shahidi, F.; Arachchi, J. K. V.; Jeon, Y.-J. *Trends Food Sci. Technol.* **1999**, *10*, 37–51.
(14) Campiña, J. M.; Souza, H. K. S.; Borges, J.; Martins, A.; Goncalves, M. P.; Silva, F. *Electrochim. Acta* **2010**, *55*, 8779–8790.
(15) Liu, H.; Bao, J.; Du, Y.; Zhou, X.; Kennedy, J. F. *Carbohydr. Polym.* **2006**, *64*, 553–559.
(16) Rabea, E. I.; Badawy, M. E. T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W. *Biomacromolecules* **2003**, *4*, 1457–1465.
(17) Gallaher, C. M.; Munion, J.; Hesslink, R., Jr; Wise, J.; Gallear, D. D. *J. Nutr.* **2000**, *130*, 2753–2759.
(18) Gallaher, D. D.; Gallaher, C. M.; Gregory, J. M.; Carr, T. P.; Hollingshead, C. H.; Hesslink, R., Jr; Wise, J. *J. Am. Coll. Nutr.* **2002**, *21*, 428–433.
(19) Rong, H. C.; Hwa, H.-D. *Carbohydr. Polym.* **1996**, *29*, 353–358.
(20) Striegel, A. M. *J. Biochem. Biophys. Methods* **2003**, *56*, 117–139.
(21) Cravotto, G.; Tagliapietra, S.; Robaldo, B.; Trotta, M. *Ultrason. Sonochem.* **2005**, *12*, 95–98.
(22) Min, L. T.; Rong, H. C. *J. Appl. Polym. Sci.* **2003**, *90*, 3526–3531.
(23) Kassai, M. R.; Arul, J.; Charlet, G. *Ultrason. Sonochem.* **2008**, *15*, 1001–1008.
(24) Li, J.; Cai, J.; Fan, L. *J. Appl. Polym. Sci.* **2008**, *109*, 2417–2425.
(25) Wu, T.; Zivanovic, S.; Hayes, D. G.; Weiss, J. *J. Agric. Food Chem.* **2008**, *56*, 5112–5119.
(26) Guzey, D.; McClements, D. J. *Food Hydrocolloids* **2006**, *20*, 124–131.
(27) Souza, H. K. S.; Bai, G.; Gonçalves, M. P.; Bastos, M. *Thermochim. Acta* **2009**, *495*, 108–114.
(28) Zhang, H.; Oh, M.; Allen, C.; Kumacheva, E. *Biomacromolecules* **2004**, *5*, 2461–2468.
(29) Chen, Y. C.; Wang, C. H.; Lai, L. S.; Lin, K. W. J. *Food Sci.* **2003**, *68*, 826–831.
(30) Bastos, D. S.; Barreto, B. N.; Souza, H. K. S.; Bastos, M.; Rocha-Leão, M. H. M.; Andrade, C. T.; Gonçalves, M. P. *Food Hydrocolloids* **2010**, *24*, 709–718.
(31) Kelly, S. M.; Price, N. C. *Curr. Protein Pept. Sci.* **2000**, *1*, 349–384.
(32) Woody, R. W.; Kenneth, S. *Methods Enzymol.* **1995**, *246*, 34–71.
(33) Sreerama, N.; Woody, R. W.; Ludwig, B.; Michael, L. J. *Methods Enzymol.* **2004**, *383*, 318–351.
(34) Böhm, G.; Muhr, R.; Jaenicke, R. *Protein Eng.* **1992**, *5*, 191–195.
(35) Whitmore, L.; Wallace, B. A. *Nucleic Acids Res.* **2004**, *32*, W668–673.
(36) Whitmore, L.; Wallace, B. A. *Biopolymers* **2008**, *89*, 392–400.
(37) Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131–137.
(38) Jin, L.; Jun, C.; Lihong, F. *J. Appl. Polym. Sci.* **2008**, *109*, 2417–2425.
(39) Liu, H.; Du, Y.-M; Kennedy, J. F. *Carbohydr. Polym.* **2007**, *68*, 598–600.
(40) Czechowska-Biskup, R.; Rokita, B.; Lotfy, S.; Ulanski, P.; Rosiak, J. M. *Carbohydr. Polym.* **2005**, *60*, 175–184.
(41) Boeris, V.; Romanini, D.; Farruggia, B.; Picó, G. *Int. J. Biol. Macromol.* **2009**, *45*, 103–108.
(42) Hongliang, J.; Yijuan, W.; Qian, H.; Yan, L.; Chaonan, X.; Kangjie, Z.; Weiliam, C. *Macromol. Biosci.* **2005**, *5*, 1226–1233.
(43) Vasconcelos, C. L.; Bezerril, P. M.; Santos, D. E. S; Dantas, T. N. C.; Pereira, M. R.; Fonseca, J. L. C. *Biomacromolecules* **2006**, *7*, 1245–1252.
(44) Taulier, N; Chalikian, T. V. *J. Mol. Biol.* **2001**, *314*, 873–889.
(45) Margolin, A. L.; Sherstyuk, S. F.; Izumrudov, V. A.; Zezin, A. B.; Kabanov, V. A. *Eur. J. Biochem.* **1985**, *143*, 625–632.