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Formation of Hydrogel Particles by Thermal Treatment of β-Lactoglobulin−Chitosan Complexes

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Molecular complexes based on proteins and ionic polysaccharides have considerable potential for encapsulation of functional food components, but their widespread utilization is limited because their structure is highly sensitive to pH and ionic strength. We have investigated the possibility of creating stable hydrogel particles by thermal treatment of protein (β-lactoglobulin) and cationic polysaccharide (chitosan) mixtures. Mixed solutions of β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) were prepared at various pH's (3−8) and were heated (80 °C for 20 min). Prior to heating, the biopolymer mixtures formed molecular complexes at pH values where there was an electrostatic attraction between the protein and the polysaccharide: soluble complexes at pH 4.5; complex coacervates at pH 5.0 and 5.5; precipitates at pH > 5.5. After heating, relatively small ($d \approx 140$ nm) and cationic ($\zeta > +20$ mV) hydrogel particles were formed at pH 4.5, but much larger aggregates were formed at pH 5.0 and higher ($d > 1000$ nm). The thermally treated hydrogel particles formed at pH 4.5 maintained their initial particle size when the pH was subsequently adjusted within the range pH 3−5, but they aggregated when the pH was adjusted to >pH 5 because of a reduction in the magnitude of their electrical charge. This study suggests that hydrogel particles can be formed by heating mixed protein−polysaccharide systems under controlled conditions. These hydrogel particles may be useful for encapsulation of functional food components.

KEYWORDS: chitosan; β-lactoglobulin

INTRODUCTION

When two oppositely charged polyelectrolytes are mixed together, they may form either a one-phase or a two-phase system depending on the nature of the polyelectrolytes involved, the solution composition, and the prevailing environmental conditions (*1*). If the attractive electrostatic interactions are relatively weak, then a one-phase system is formed, where the two polyelectrolytes exist either as individual molecules or as soluble complexes. On the other hand, if the attractive electrostatic interactions are relatively strong, a two-phase system is formed, with one phase being rich in both polyelectrolytes and the other phase being depleted in both polyelectrolytes. The polyelectrolyte-rich phase may be either a complex coacervate or a precipitate depending on the strength of the attraction and the charge characteristics of the polyelectrolytes involved (*1*). Complex coacervation has been widely used industrially as a means of encapsulating functional components, including oils, flavors, drugs, cosmetics, pesticides, live cells, and vaccines (*1*−*4*).

A variety of food-grade proteins and ionic polysaccharides are capable of forming electrostatic molecular complexes under appropriate solution conditions (*2*, *5*, *6*). Typically, complex coacervates are formed under conditions where there is a moderately strong electrostatic attraction between the protein and polysaccharide molecules. Consequently, the formation and properties of coacervates are strongly influenced by solution pH and ionic strength as well as by the total polyelectrolyte concentration and the protein/polyelectrolyte ratio (*1*, *2*, *7*−*10*). There has been a considerable amount of research on the formation and properties of complex coacervates produced by mixing proteins with anionic polysaccharides (*2*). For example, there have been studies of complex coacervation between β-lactoglobulin (β-Lg) and acacia gum (*11*−*13*), between β-Lg and pectin (*14*−*16*), between β-Lg and sodium alginate (*17*), between whey proteins and acacia gum (*18*−*22*), and between whey proteins and carrageenan (*23*). Coacervates have also been formed between proteins and cationic polysaccharides. For example, there have been studies of complex coacervation between BSA and chitosan (*24*), between ovalbumin and chitosan (*25*−*27*), and between β-Lg and chitosan (*28*).

Complex coacervation provides food scientists with a relatively simple method of creating novel hydrogel particles that can be utilized for encapsulation purposes. A major advantage

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of these systems is that the hydrogel particles can be assembled entirely from food-grade ingredients (proteins and polysaccharides) using simple processing operations (e.g., pH adjustment and mixing). Nevertheless, there are several factors that currently limit the more widespread application of complex coacervation in the food industry. First, the coacervate phase is only stable over a relatively narrow range of pH values, and a protein−polysaccharide coacervate will tend to either disintegrate (when the pH is adjusted so that the molecules have strong similar charges) or form precipitates (when the pH is adjusted so that the molecules have strong opposite charges). Second, the coacervate phase is held together by relatively weak electrostatic interactions that may be disrupted when the ionic strength is increased, which may limit their application in some foods. Third, the particles in a coacervate suspension will tend to coalesce over time, leading to a gradual increase in the mean particle size and eventually to macroscopic phase separation. Fourth, the current methods used to form more stable hydrogel particles by cross-linking the coacervate phase, such as gluteraldehyde treatment, are only approved for a limited range of food applications, for example, gelatin and gum arabic. There is, therefore, a need to find alternative methods of forming stable hydrogel particles that are more suitable for utilization within foods.

Recently, it has been shown that nanometer-sized hydrogel particles can be formed by thermal treatment of ovalbumin−chitosan solutions under pH conditions where the protein and polysaccharide molecules have opposite charges (*27*). The hydrogel particles formed were shown to have good stability over a wide range of pH conditions (i.e., they did not dissociate), which can be attributed to cross-linking of the thermally denatured globular proteins. It was therefore proposed that these hydrogel particles would be good candidates for development of delivery systems for cosmetic and pharmaceutical applications. The thermal treatment of molecular complexes of proteins and polysaccharides may also be useful for the fabrication of novel hydrogel particles for application as delivery systems in the food industry. In this study, we examined the impact of pH (pH 3−8) and thermal treatment (80 °C for 20 min) on the properties of aqueous mixed biopolymer solutions consisting of a globular protein (β-Lg) and a cationic polysaccharide (chitosan), with the aim of identifying conditions where stable hydrogel particles could be formed. The hydrogel particles formed using this process may be a useful alternative to the traditional coacervation process.

The globular protein β-Lg was used in this study because its molecular characteristics and functional properties are well established. It is a relatively small globular protein (monomer = 18 400 Da) with an isoelectric point around pH 5. In addition, it is known to form molecular aggregates upon heating above its thermal denaturation temperature because of exposure of nonpolar and disulfide groups (*29*−*33*). Chitosan is a cationic polysaccharide whose charge characteristics are determined by amino side groups ($pK_a \approx 6.3$) (*34*). At relatively low pH (<6.3), chitosan is positively charged and tends to be soluble in dilute aqueous solutions, but at higher pH, it tends to lose its charge and may precipitate from solution because of deprotonation of the amino groups (*35*). Recent studies have shown that hydrogel particles can be formed using β-Lg and chitosan using a different approach than the one used in the present study (*36*, *37*). In these previous studies, the β-Lg was first thermally denatured by heating a protein solution, then this solution was mixed with a chitosan solution to form molecular complexes, and then tripolyphosphate (TPP) was used to cross-link the chitosan and form hydrogel particles.

MATERIALS AND METHODS

Materials. Powdered β-Lg was obtained from Davisco Foods International (lot# JE 001-1-922, Le Sueur, MN). As stated by the manufacturer, the protein content was 97.4 wt % (dry basis) of which 95.0 wt % was β-Lg. Soluble chitosan powder was obtained from Cargill Acidulants R & D (sample # B-75, Eddyville, IA). According to the manufacturer, the molecular weight of the chitosan was approximately 62 900 Da and the degree of deacetylation was high, >90%. Distilled and deionized water from a water purification system (Nanopure Infinity, Barnstead International, IA) was used for preparation of all solutions. Analytical grade acetic acid, sodium acetate, hydrochloric acid, and sodium hydroxide were purchased from the Sigma Chemical Co. (St. Louis, MO).

Solution Preparation. A β-Lg solution was prepared by dispersing 0.5 wt % powdered β-Lg in 10 mM acetate buffer (pH 3.0 ± 0.1) and by stirring for 3 h to ensure complete dissolution. A chitosan solution was prepared by dispersing 0.1 wt % powdered chitosan in 10 mM acetate buffer (pH 3.0 ± 0.1) and by stirring overnight to ensure complete dissolution. A β-Lg and chitosan solution was prepared by dispersing 0.5 wt % β-Lg and 0.1 wt % chitosan in 10 mM acetate buffer (pH 3.0 ± 0.1) and by stirring for 3 h to ensure complete dissolution. These protein and polysaccharide concentrations were selected because a previous study in our laboratory showed that this amount of chitosan is required to saturate 0.5 wt % β-Lg (*28*).

ζ-Potential Measurements. Samples were placed in a disposable cuvette that acted as the measurement chamber of the particle electrophoresis instrument (Zetasizer Nanoseries ZS, Malvern Instruments, Worcestershire, United Kingdom). The ζ-potential was determined by measuring the direction and velocity that the molecules or complexes moved in the applied electric field. The Smoluchowsky mathematical model was used by the software to convert the electrophoretic mobility measurement into ζ-potential values. It was assumed that the viscosity and dielectric constant of the aqueous solution surrounding the particles was the same as that of pure water, which is a reasonable assumption for low biopolymer concentrations. All measurements were made on at least two freshly prepared undiluted samples.

Particle Size Measurements. The mean particle size of the biopolymer systems was determined using a commercial dynamic light scattering instrument (Zetasizer NanoZS, Malvern Instruments, Worcestershire, United Kingdom). This instrument infers the size of the particles from measurements of their translational diffusion coefficients. All measurements were made on at least two freshly prepared undiluted samples. Dynamic light scattering measurements on samples containing highly aggregated materials are often irreproducible because the large particles diffuse slowly but scatter light strongly and because large aggregates may be sensitive to handling and sampling.

Turbidity Measurements. The turbidity of solutions contained in a 10 mm path length quartz cuvette was measured using a UV−vis spectrometer (Ultrospec 3000pro, Amersham-Pharmacia, Uppsala, Sweden) at 600 nm. For temperature-scanning measurements, sample solutions were poured into the cuvettes and then were covered with a thin layer of mineral oil and a plastic lid to retard evaporation during the experiments. The cuvette was then placed in the sample holder of the spectrometer and was allowed to equilibrate to 30 °C for 5 min. The turbidity of the solutions was then recorded as they were heated from 30 to 90 °C at 1.5 °C min^{-1}. The turbidity was defined from the following equation: $\tau = -1/d \times \log(P/P_0)$, where d is the path length of the cell (1 cm), and P and P_0 are the powers of the beams that pass through the sample and reference cells, respectively. Measurements were carried out on two or three sample solutions prepared at different times from the same materials (replicates).

RESULTS AND DISCUSSION

Properties of β-Lactoglobulin and Chitosan in Isolation. Initially, we characterized the electrical charge and aggregation

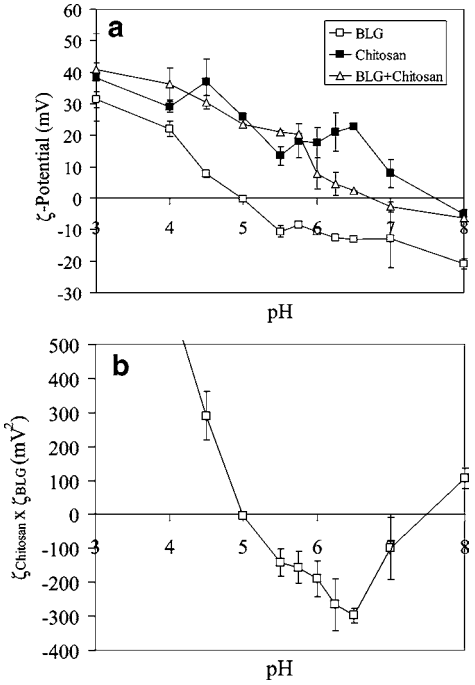

Figure 1. The pH dependence of the electrical characteristics (ζ-potential) of aqueous solutions containing β-lactoglobulin (0.5 wt %), chitosan (0.1 wt %), or their mixture (10 mM citrate buffer): (**a**) ζ-potential versus pH; (**b**) the product of the ζ-potentials of the pure β-lactoglobulin and chitosan systems versus pH.

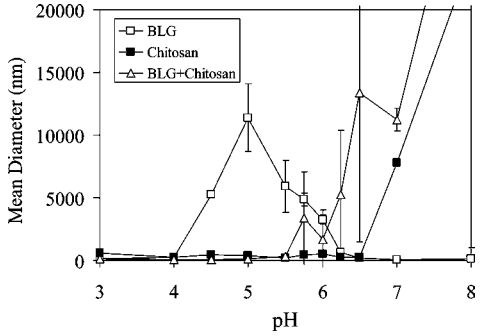

Figure 2. The pH dependence of the mean particle diameter (*z*-average) determined by dynamic light scattering of aqueous solutions containing β-lactoglobulin (0.5 wt %), chitosan (0.1 wt %), or their mixture (10 mM citrate buffer).

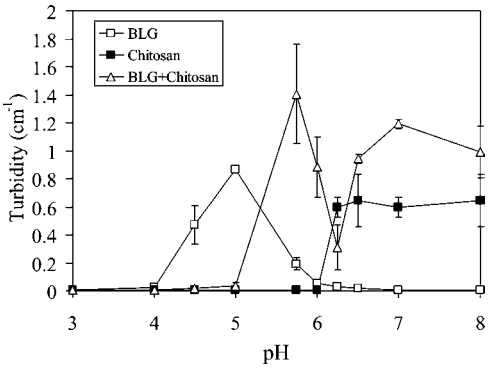

Figure 3. The pH dependence of the turbidity (at 600 nm) of aqueous solutions containing β-lactoglobulin (0.5 wt %), chitosan (0.1 wt %), or their mixture (10 mM citrate buffer).

behavior of the polyelectrolytes used to form the molecular complexes: β-Lg (0.5 wt %) and chitosan (0.1 wt %). The pH dependence of the electrical charge (ζ-potential) of the two biopolymers is shown in **Figure 1a**. The ζ-potential of the β-Lg changed from positive to negative as the pH was increased from 3 to 8, with the point of net zero charge being around pH 5, which is because β-Lg has an isoelectric point (pI) around pH 5. The ζ-potential of the chitosan was positive from pH 3 to 7 but was slightly negative at pH 8, as has been reported by other workers (*27*). The progressive loss of positive charge on chitosan with increasing pH is due to deprotonation of amino groups ($pK_a \approx 6.3$). The measurements of the electrical properties of the β-Lg and chitosan in aqueous solution indicate that the signs of their charges are opposite between pH 5 and 7, and hence, one would expect them to form complexes in this range. The pH dependence of the product of the ζ-potentials of the β-Lg and chitosan molecules indicates that maximum binding should occur around pH 6.5 (**Figure 1b**).

The pH dependence of the mean particle diameter (*z*-average) and turbidity of the two biopolymers in aqueous solution is shown in **Figures 2** and **3**, respectively. The mean diameter of the particles in the β-Lg solution was relatively small at pH 3−4 and pH 6−8, which can be attributed to the fact that it predominantly exists as either monomers or dimers under these conditions (*38*). The turbidity of the β-Lg solutions is also relatively low across these pH ranges because of the relatively weak light scattering caused by the small size of the particles in the system. On the other hand, there is a large increase in the mean particle diameter and solution turbidity around pH 5 (**Figures 2** and **3**), which can be attributed to the self-association of some of the β-Lg molecules when their electrical charge is relatively small (*38*). Previous studies show that β-Lg forms octamers around its isoelectric point (*39*). The mean diameter of the particles in the chitosan solution was relatively small from pH 3 to 6 but then increased appreciably at higher pH. This effect can be attributed to the fact that the chitosan molecules are highly charged at the lower pH values, and so there is a strong electrostatic repulsion between them. At pH 7 and 8, the magnitude of the electrical charge on the chitosan molecules was relatively small (**Figure 1**), so that there was little electrostatic repulsion between them, which would account for the formation of insoluble aggregates that strongly scattered light in this pH range (**Figures 2** and **3**).

Properties of β-Lactoglobulin−Chitosan Mixtures. The electrical charge and aggregation of mixed biopolymer solutions containing β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) were measured (**Figures 1−3**). At pH 3, the ζ-potential of the mixed system was highly positive, which can be attributed to the fact that both β-Lg and chitosan are positively charged at this pH. The pure β-Lg (0.5 wt %) solution gave an appreciably higher intensity signal in the ζ-potential instrument (~350 counts per second) than the pure chitosan (0.1 wt %) solution (~150 counts per second). Consequently, one would expect the signal from the protein to dominate the overall signal in a mixed biopolymer system. Nevertheless, the particle electrophoresis instrument only provides an overall measure of the ζ-potential of the system, which may have contributions from both the protein and chitosan. When the pH of the solution was increased from 4 to 8, the ζ-potential of the mixed system was more positive (or less negative) than that of the pure β-Lg solution, indicating that a molecular complex had been produced. Presumably, this complex was formed between negatively charged groups on the protein (e.g., $-COO^-$) and positively charged groups on the chitosan (e.g., $-NH_4^+$). At pH 3, most of the carboxyl groups on the protein would be protonated ($-COO^- + H^+ \leftrightarrow -COOH$, $pK_a \sim 4$), and therefore little binding occurred. However, when the pH was increased, there

would be an increasingly large number of negatively charged groups on the protein surface which could act as binding sites for the positively charged groups on chitosan. This would explain why complexation was observed at pH values below the isoelectric point of the protein (where both the protein and chitosan had a net positive charge).

The pH dependence of the mean particle diameter and turbidity of the mixed biopolymer solutions was appreciably different from that of the pure β-Lg and chitosan solutions (**Figures 2** and **3**). At pH 3, the mean particle diameter and turbidity of the mixed biopolymer solutions was relatively low indicating that no insoluble aggregates were formed. At pH 4.5−5.0, the mean particle diameter and the turbidity of the mixed biopolymer solution were considerably less than that of the pure β-Lg solution, which indicates the formation of a molecular complex between the protein and the chitosan that increased the protein's solubility in this pH range. The magnitude of the ζ-potential on the molecular complexes was considerably higher than that on the protein alone (**Figure 1a** and **1b**), which would prevent the protein molecules from aggregating. We attribute the origin of this interaction to an electrostatic attraction between negatively charged groups on the protein surface and positively charged groups on the chitosan mentioned above. At pH > 5.75, the turbidity of the mixed solutions increased appreciably, often being higher than the turbidity of either of the individual biopolymer solutions, indicating the formation of large insoluble complexes between the biopolymers.

Impact of Heating on β-Lactoglobulin and Chitosan Solutions. We examined the impact of thermal treatment on the aggregation behavior of β-lactoglobulin and chitosan mixtures in aqueous solution because previous studies have shown that this approach can be used to form novel hydrogel particles (*27*). In this series of experiments, each mixed biopolymer solution was prepared, heated, and analyzed at a particular pH rather than being prepared and heated at one pH and then being analyzed at a different pH (see next section). The impact of pH and heating (80 °C for 20 min) on the mean particle diameter and turbidity of the mixed β-lactoglobulin and chitosan solutions was measured (**Figure 4**). At pH 3 and 4, the mean particle diameter and turbidity were relatively low for the β-Lg−chitosan mixtures both before and after heating, which suggested that large aggregates were not formed because of the thermal treatment. On the other hand, at pH 5−6, heating caused an appreciable increase in the particle diameter and turbidity of the β-Lg−chitosan mixtures, indicating that appreciable aggregation was induced by thermal treatment. The relatively low turbidity of the unheated solutions over this pH range can be attributed to the fact that mixed molecular complexes were formed that inhibited protein aggregation near its isoelectric point (*1*). These molecular complexes would have been either soluble complexes or coacervates depending on the solution pH. The increase in mean particle diameter and turbidity because of heating may have occurred for several reasons. First, the molecular complexes may have aggregated with each other forming bigger particles that scattered light more effectively. Second, the molecular complexes may have dissociated at higher temperatures, releasing the protein molecules and thereby initiating protein aggregation. Nevertheless, it is not possible to determine which of these mechanisms is responsible for the observed effects from our experimental data. At pH 7 and higher, large aggregates were formed in the unheated and heated mixed biopolymer systems, which can be attributed to the formation of precipitates involving β-Lg and chitosan.

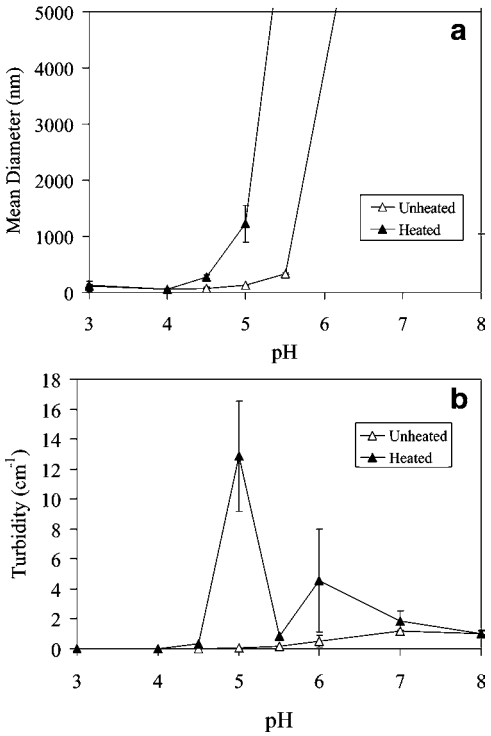

Figure 4. (**a**) The pH dependence of the mean particle diameter (*z*-average) determined by dynamic light scattering for aqueous solutions containing β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) mixtures, which were either unheated or heated (80 °C, 20 min). These solutions were prepared, heated, and analyzed at the pH specified on the *x*-axis. (**b**) The pH dependence of the turbidity (at 600 nm) for aqueous solutions containing β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) mixtures, which were either unheated or heated (80 °C, 20 min). These solutions were prepared, heated, and analyzed at the pH specified on the *x*-axis.

Turbidity versus temperature measurements were carried out to provide further insights into the origin of the aggregation observed in the mixed biopolymer systems after heating (**Figure 5**). In these experiments, we selected pH 4.5, 5.0, and 5.5, since the protein and polysaccharide were believed to form molecular complexes (soluble complexes or coacervates) in this pH range. We also measured the turbidity profiles of pure β-Lg and chitosan solutions to compare with the mixed biopolymer systems. Pure chitosan solutions (0.1 wt %) gave no turbidity across the entire temperature range (30−90 °C) at all three pH values studied (data not shown).

pH 4.5. At pH 4.5 and 30 °C, the pure β-Lg solution had a relatively high turbidity (~0.5 cm^{-1}), which can be attributed to self-association of the globular protein molecules into molecular complexes near their isoelectric point (*38*). Upon heating, the pure β-Lg solution showed a slight decrease in turbidity from 50 to 60 °C, followed by a steady increase from 60 to 80 °C, followed by a sharp increase at higher temperatures (**Figure 5a**). The slight decrease in turbidity from 50 to 60 °C suggests that there may have been some partial dissociation of the molecular complexes induced by mild heating. The progressive increase in turbidity from 60 to 80 °C suggests that there was a gradual increase in aggregation, which may have been due to partial unfolding of the protein molecules increasing their surface hydrophobicity and thereby promoting intermolecular association (*29*, *40*). The steep increase in turbidity above 80 °C can be attributed to extensive protein aggregation following irreversible unfolding (thermal denaturation) of the globular protein, since this leads to a strong increase in the

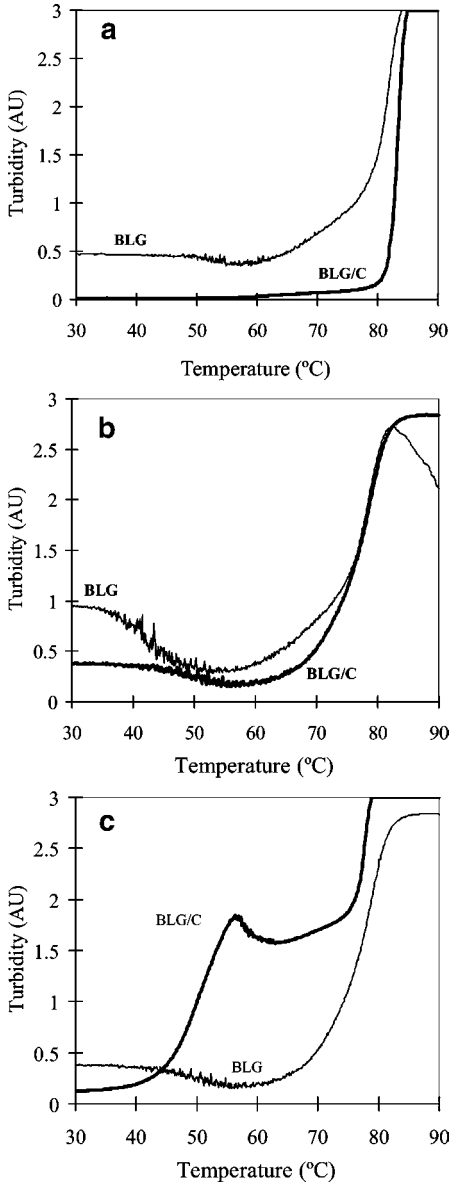

Figure 5. The temperature dependence of the turbidity (at 600 nm) of aqueous solutions containing β-lactoglobulin (0.5 wt %) and β-lactoglobulin (0.5 wt %) + chitosan (0.1 wt %). Measurements were carried out for solutions at different pH so as to have different kinds of molecular complexes: (**a**) pH 4.5; (**b**) pH 5.0; (**c**) pH 5.5.

hydrophobic attraction between the molecules (*29*, *40*). Previous studies have shown that the thermal denaturation temperature (T_m) of β-Lg at pH 5 is around 80 °C (*41*), which is close to the temperature where a rapid increase in turbidity was observed.

At pH 4.5 and 30 °C, the β-Lg−chitosan solution had a relatively low turbidity (∼0.0 cm^{-1}), which can be attributed to the formation of soluble complexes (*1*). Upon heating, the β-Lg−chitosan solution remained clear up to around 55 °C suggesting that the soluble complexes remained intact during this fairly mild heat treatment. From 55 to 80 °C, the turbidity of the mixed biopolymer system increased gradually, which suggested that there were some changes in either the size or concentration of the biopolymer particles in the system that increased light scattering. Previous studies have shown that β-Lg undergoes a reversible conformational change in this temperature range that increases the surface hydrophobicity of the protein. Consequently, it is possible that the protein molecules are beginning to associate with one another through hydrophobic attraction, leading to an increase in the size of the molecular aggregates in the system (*40*). Alternatively, the protein molecules may be released from the molecular complexes at these higher temperatures, which would allow them to self-associate since the pH is quite close to the protein's isoelectric point. When the mixed biopolymer solutions were heated to temperatures >80 °C, there was a steep rise in the turbidity, which may be attributed to irreversible thermal denaturation of the globular protein leading to a large increase in protein aggregation. Again, it is unclear whether the β-Lg molecules remain within the molecular complexes or are released prior to aggregation.

pH 5.0. At pH 5.0 and 30 °C, the pure β-Lg solution had a high turbidity (∼0.9 cm^{-1}), which can be attributed to extensive aggregation of β-Lg molecules at their isoelectric point. Upon heating, the pure β-Lg solution showed a significant decrease in turbidity from 40 to 55 °C, followed by a steady increase from 55 to 70 °C, followed by a sharp increase at higher temperatures (**Figure 5b**). These results suggest that there may have been some initial dissociation of the protein aggregates upon heating up to 55 °C, followed by progressive aggregation upon heating to higher temperatures. As mentioned earlier, the initial reduction in turbidity upon heating may have been due to some dissociation of molecular aggregates because of the increased thermal energy of the system, which would have increased the thermodynamic tendency for aggregates to breakdown associated with the increased entropy of mixing of a nonaggregated system. At higher temperatures, the protein molecules unfolded and exposed nonpolar groups to the surrounding aqueous phase, which led to an increase in the hydrophobic attraction between the molecules and larger aggregates. The decrease in turbidity observed above 80 °C can be attributed to sedimentation of the large aggregates formed in the measurement cell (which was observed visually when the samples were removed).

At pH 5.0 and 30 °C, the β-Lg−chitosan solution had a lower turbidity (∼0.4 cm^{-1}) than the pure β-Lg solution (∼0.9 cm^{-1}), which can be attributed to the formation of molecular complexes that increased the protein solubility. Nevertheless, the turbidity was higher than for the mixed biopolymer system at pH 4.5 (∼0.1 cm^{-1}), which suggests that the molecular complexes were appreciably bigger. On the basis of previous studies (*1*), we suggest that soluble complexes were formed at pH 4.5, but complex coacervates were formed at pH 5.0. Upon heating, the β-Lg−chitosan solution showed a slight decrease in turbidity from 40 to 55 °C, and then its turbidity increased gradually from 55 to 70 °C, and then it increased steeply at higher temperatures (**Figure 5b**). The measurements made at pH 5.0 therefore followed a similar trend to those made at pH 4.5 (**Figure 5a**). The large increase in turbidity occurred at a similar temperature in the pure protein and mixed biopolymer system, suggesting that protein aggregation was the cause of the observed change. As mentioned earlier, the increase in turbidity because of heating may have occurred because of several reasons, for example, aggregation of soluble complexes or coacervates or dissociation of complexes or coacervates followed by aggregation of the proteins released.

pH 5.5. At pH 5.5 and 30 °C, the pure β-Lg solution had a relatively high turbidity (∼0.4 cm^{-1}), which can be attributed to some aggregation of the β-Lg molecules close to their isoelectric point. Upon heating, the pure β-Lg solution showed a similar trend to the pure protein solutions at pH 4.5 and 5.0, that is, there was a significant decrease in turbidity from

40 to 55 °C, followed by a steady increase from 55 to 70 °C, followed by a sharp increase at higher temperatures (**Figure 5c**).

At pH 5.5 and 30 °C, the β-Lg−chitosan solution had a lower turbidity (~0.13 cm^{-1}) than the pure β-Lg solution (~0.4 cm^{-1}), which can be attributed to formation of molecular complexes. Again on the basis of the results of previous studies (*1*), we propose that these molecular complexes were coacervates formed because of electrostatic attraction between the protein and polysaccharide molecules. The β-Lg−chitosan solution at pH 5.5 showed a qualitatively different turbidity−temperature profile (**Figure 5c**) than the mixed biopolymer solutions at pH 4.5 or 5.0 (**Figure 5a** and **5b**). Upon heating, there was a steep increase in turbidity from 40 to 55 °C, followed by a region where the turbidity remained fairly constant from 55 to 70 °C, followed by another steep increase in turbidity at higher temperatures (**Figure 5c**). Presumably, the steep increase in turbidity above 70 °C can be attributed to thermal denaturation of the globular protein, but the origin of the steep increase that begins at 40 °C is currently unknown. At this pH, the β-Lg has a high negative charge and the chitosan has a high positive charge (**Figure 1**), and so one would expect a relatively strong electrostatic attraction between them. Further studies are needed to elucidate the molecular and physicochemical origin of this interesting effect.

Thermoreversibility of Aggregate Formation. The changes in turbidity of the pure β-Lg and mixed β-Lg/chitosan samples were irreversible: the turbidity remained high and did not return to its original value when the samples were cooled back to room temperature (data not shown). In some samples, there was a slight decrease in turbidity after heating, but this was due to sedimentation of the large aggregates out of the path of the light beam in the UV−vis spectrometer. Indeed, sediments were clearly visible in the bottom of the measurement cells in these samples when they were removed from the spectrometer. The irreversible change in turbidity after thermal treatment indicates that the molecular complexes formed during heating were different from those present in the unheated samples. We called the new particles formed by thermal treatment of the mixed biopolymer systems "heat-induced molecular complexes" (HI−MC).

pH Stability of Heat-Induced Molecular Complexes. The molecular complexes formed by heating β-Lg and chitosan mixtures together may be useful for creating novel hydrogel particles for utilization in the food and other industries, for example, for encapsulation of active ingredients, for controlling protein digestibility, or as a fat mimetic (*36*). For practical applications, it is important to establish the range of pH conditions where the hydrogel particles remain stable. We therefore characterized the pH stability of the molecular complexes formed by heat treatment. In this study, we compared unheated and heated systems at pH 4.5 (assumed to be soluble complexes at ambient temperature prior to heating) since this system formed relatively small (<1000 nm) hydrogel particles after heating. The heat treatment involved holding a solution of the molecular complexes at 80 °C for 20 min to form the complexes and then cooling to room temperature prior to analysis. This holding temperature is slightly below the temperature where a rapid increase in solution turbidity occurred in the β-Lg and chitosan mixtures (pH 4.5) during thermal scanning (**Figure 5a**). Consequently, it may be possible to control the size or concentration of the aggregates formed by using different holding temperatures and times.

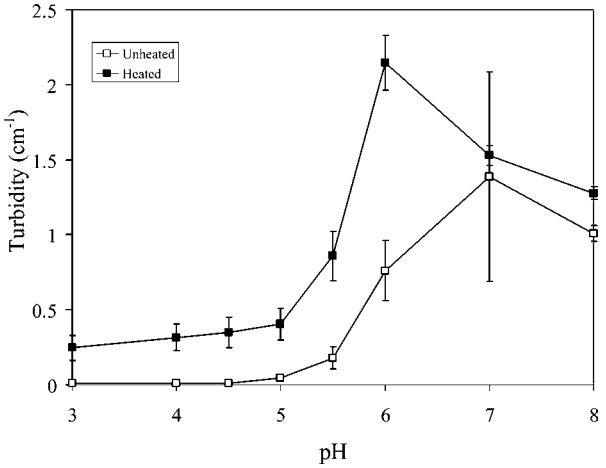

Figure 6. The pH dependence of the turbidity (at 600 nm) determined of aqueous solutions containing β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) mixtures, which were either unheated or heated. These solutions were prepared and heated (if necessary) at pH 4.5 and then were adjusted to and analyzed at the pH specified on the *x*-axis.

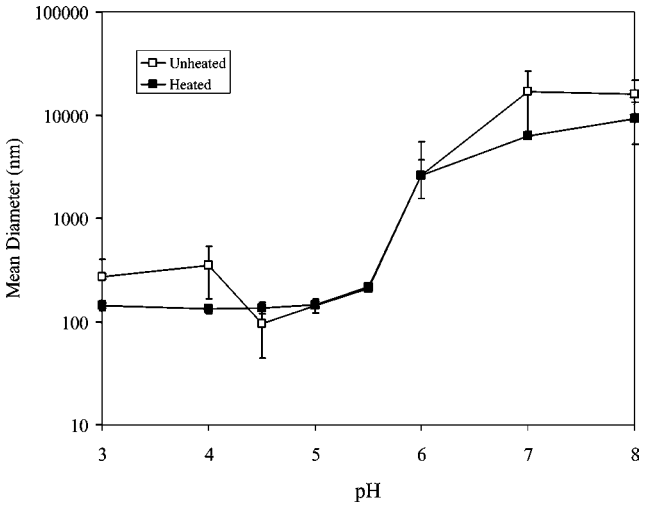

Figure 7. The pH dependence of the mean particle diameter (*z*-average) determined by dynamic light scattering for aqueous solutions containing β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) mixtures, which were either unheated or heated (80 °C, 20 min). These solutions were prepared and heated (if necessary) at pH 4.5 and then were adjusted to and analyzed at the pH specified on the *x*-axis.

The pH dependence of the turbidity and mean particle diameter (*z*-average) of unheated and heated molecular complexes formed at pH 4.5 is compared in **Figures 6** and **7**. For the unheated β-Lg−chitosan system, the turbidity and particle size were relatively low between pH 3.0 and 5.0 indicating that there was either no interaction or soluble complex formation. At pH 5.5, there were slight increases in turbidity and particle size, which can be attributed to complex coacervate formation. At higher pH, there were large increases in turbidity and particle size, which can be attributed to the formation of insoluble precipitates between the oppositely charged protein and polysaccharide. The turbidity of the heat-treated β-Lg−chitosan system was ~0.25 cm^{-1} at the initial pH of 4.5 (compared to 0.01 cm^{-1} for the unheated system), which indicated the formation of heat-induced molecular complexes. The hydrogel particles formed after heating were initially around 140 nm in diameter at pH 4.5 (**Figure 7**). The turbidity (~0.25−0.4 cm^{-1}) and the mean particle diameter (135−145 nm) remained relatively constant

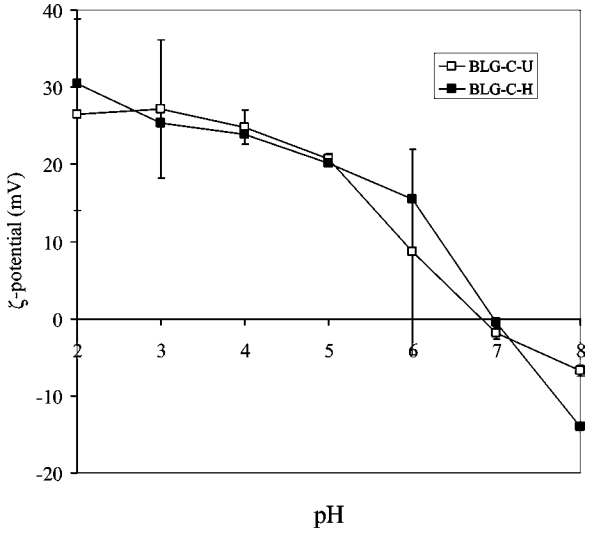

Figure 8. The pH dependence of the ζ-potential of aqueous solutions containing β-lactoglobulin (0.5 wt %) and chitosan (0.1 wt %) mixtures, which were either unheated or heated (80 °C, 20 min). These solutions were prepared and heated (if necessary) at pH 4.5 and then were adjusted to and analyzed at the pH specified on the *x*-axis.

when the pH was adjusted from pH 4.5 to values in the pH range 3.0−5.0 indicating that the heat-induced molecular complexes formed during heating remained intact. Nevertheless, when the pH was raised to pH 5.5 and above, there was an appreciable increase in turbidity and mean particle diameter (**Figures 6** and **7**), which suggested that the heat-induced molecular complexes aggregated. This aggregation can be attributed to a reduction in the magnitude of the ζ-potential on the β-Lg−chitosan complexes at higher pH values (**Figure 8**), which would reduce the electrostatic repulsion between the hydrogel particles.

Conclusions. This study has shown that sub-micrometer sized hydrogel particles can be formed by heating molecular complexes of a globular protein (β-lactoglobulin) and a cationic biopolymer (chitosan) under certain pH conditions (pH 4.5). The β-lactoglobulin and chitosan formed soluble complexes at pH 4.5 prior to heating because of electrostatic attraction between cationic groups on the polysaccharide and anionic patches on the protein surface. Upon heating, it is proposed that the β-lactoglobulin molecules partially unfolded and aggregated with one another, leading to the formation of hydrogel particles consisting of a network of aggregated protein molecules with chitosan molecules trapped inside. The precise nature of the molecular and physicochemical mechanisms that lead to the formation of these hydrogel particles is currently unknown and requires further study. The hydrogel particles formed may have several potential applications in the food industry, for example, as delivery systems, to control protein digestibility, or as fat mimetics. The formation of these particles requires a heat-processing step, which may limit their application for heat labile functional food components.

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