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Effects of Salt Concentration on Formation and Dissociation of β-Lactoglobulin/Pectin Complexes

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The formation and dissociation of β-lactoglobulin/pectin complexes at various sodium chloride concentrations (C_{NaCl}) have been studied by turbidimetric titration. An increase of C_{NaCl} up to 0.1 M shifts the critical $pH_{\varphi 1}$, which designates the formation of β-lactoglobulin/pectin coacervates, to higher pH values, whereas further increase of C_{NaCl} from 0.1 to 0.8 M decreases $pH_{\varphi 1}$ values. These salt effects can be explained in terms of a salt-enhanced effect at lower salt concentrations or a salt-reduced effect at higher salt concentrations, respectively. On the other hand, the value of $pH_{\varphi 2}$, which corresponds to the dissociation of β-lactoglobulin/pectin coacervates, tends to have smaller pH values when C_{NaCl} increases from 0.1 to 0.3 M. No observable $pH_{\varphi 2}$ values are found at C_{NaCl} higher than 0.3 M. The disappearance of $pH_{\varphi 2}$ is mainly attributed to the strong self-aggregation capability of β-lactoglobulin at higher C_{NaCl}. The aggregation of β-lactoglobulin at high C_{NaCl} is reversible, as suggested by the atomic force microscopy results.

KEYWORDS: β-Lactoglobulin; pectin; complexes; self-aggregation; salt effect

INTRODUCTION

The technological applications involving complexes formed by polyelectrolytes and oppositely charged colloids (e.g., proteins and surfactants) have received considerable attention since the pioneering works of Tiebackx (*1*) and Bungenberg de Jong (*2*). The term "complexes" encompasses soluble complexes, complex coacervation, which is a liquid/liquid type of phase separation, and precipitation, which is a solid/liquid type of phase separation. The complexes formed by proteins and polyelectrolytes have already been used in protein separation *3a*, microencapsulation of food ingredients *3b*, enzymes *3c*, cells *3d*, and pharmaceuticals *3e–f*. More recently, polyelectrolyte multilayers are used as substrates for the immobilization of proteins, such as enzymes and antibodies, for the biosensor applications *4a–b*. Despite the widespread industrial applications and some fascinating biological implications, the complexation between polyelectrolytes and proteins is still an intriguing and important topic of investigation *4a–b*.

The general picture for the coacervation between protein and anionic polysaccharide is that primary soluble protein/polysaccharide complexes are initially formed at the first critical pH (pH_c, corresponding to the onset of soluble complex), and then soluble protein/polysaccharide complexes start to aggregate into insoluble protein/polysaccharide complexes at the second critical pH ($pH_{\varphi 1}$), which ultimately sediment to generate the dense coacervate phase (*6*, *7*). When the pH decreases to the third critical pH ($pH_{\varphi 2}$), protein/polysaccharide coacervates will dissociate into soluble protein/polysaccharide complexes again (*8*). Because the coacervates formed by proteins and oppositely charged polysaccharides are usually considered to be mainly electrostatic in nature, the addition of salt has been demonstrated to strongly influence the protein/polysaccharide coacervation (*9*).

In most of the previously published work (*10*), it was found that the added salt would weaken the formation of coacervates between proteins and anionic polysaccharides. In another word, the critical pH values (pH_c and $pH_{\varphi 1}$) decreased with increasing salt concentration, and the addition of excess salt could completely suppress the formation of protein/polysaccharide coacervates. These salt-reduced effects on the protein/polysaccharide coacervation are generally explained in terms of the reduction or complete screening of the electrostatic attraction between proteins and polysaccharides. However, different from the salt screening effect, some authors noted that the added salt could enhance the formation of protein/polysaccharide complexes at certain salt concentrations (*7*, *11*), as indicated by the increase of either pH_c or $pH_{\varphi 1}$. On the other hand, the unique protein properties, such as the self-aggregation for β-lactoglobulin, may compete with the protein/polysaccharide coacervation. This competition could add extra complication to the process of protein/polysaccharide coacervation driven by electrostatic interaction.

The associative phase separation of a β-lactoglobulin/pectin mixed solution has been studied by small-angle light scattering *12a* and isothermal titration calorimetry *12b*. The results suggested that the complexation between β-lactoglobulin and

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pectin was a two-step process, where enthalpic/entropic contributions were different. Very recently, the structure and rheological properties of β-lactoglobulin/pectin coacervates were investigated by Huang et al. using small-angle neutron scattering *13a* and rheology *13b*. Their results indicated that the high self-aggregation ability of β-lactoglobulin could promote the formation of protein domains in β-lactoglobulin/pectin coacervates. The composition and storage modulus of β-lactoglobulin/pectin coacervates were found to be significantly influenced by protein self-aggregation. In the present work, the effects of salt on the formation and dissociation of β-lactoglobulin/pectin complexes upon the addition of sodium chloride has been studied by turbidimetric titration, a typical way of monitoring complex formation *6a*. β-Lactoglobulin is a model globular protein with a well-known structure (*14*, *15*), whereas pectin is an anionic polysaccharide broadly used in food, cosmetics, and pharmaceutical applications (*16*, *17*). For comparison, the self-aggregation of β-lactoglobulin has also been studied by atomic force microscopy (AFM).

MATERIALS AND METHODS

Materials. β-Lactoglobulin powder (JE 003–3–922) was provided by Davisco Foods International, Inc. (Le Sueur, MN) and used without further purification. Pectin with 31% esterification was obtained from Danisco A/S, Denmark and was purified by dialysis against Milli-Q water for five days using Spectra/Por dialysis membrane with a molecular weight cutoff equal to 12,000, followed by freeze-drying. The weight-average molecular weight (M_w) of pectin determined by gel permeation chromatography was 7.0×10^5 (relative to protein standards), and the polydispersity (M_w/M_n) was 1.3. Sodium chloride (NaCl, purity >99%) and standard hydrochloric acid (HCl, 0.5 N) were purchased from Fisher Scientific (Pittsburgh, PA). Milli-Q water (18.3 Ω) was used in all experiments.

Turbidimetric Titrations. The pH-dependent turbidity measurements, reported as 100 − *T*%, were carried out at 420 nm using a Brinkmann PC910 colorimeter equipped with a 1 cm path length optical probe. The colorimeter was calibrated to read 100% transmittance with Milli-Q water. Pectin and β-lactoglobulin solutions prepared in 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.8 M NaCl were adjusted to pH 8.0 and were filtered using 0.45 μm Whatman filters prior to use. A 0.5 N HCl solution was used to adjust the pH of the mixed solutions containing 1 g/L β-lactoglobulin and 0.1 g/L pectin or the pure 1 g/L β-lactoglobulin solutions at various salt concentrations under gentle stirring. After each small droplet of HCl was added, the turbidity value was collected, and the pH was monitored with a Thomas Scientific pH meter (Model 8025) calibrated with two buffers of pH 4 and 7. All the measurements were conducted at 25 °C.

Immobilization of β-Lactoglobulin. AT-cut quartz crystal coated with gold (fundamental frequency of 5 MHz) was obtained from Q-Sense AB (Sweden). The linkage of β-lactoglobulin onto the gold-coated crystal was carried out using the procedure modified from a previous paper (*18*). Gold-coated quartz crystal was first cleaned in an UV/ozone chamber for 10 min, followed by immersion in a 1:1:5 mixture of ammonium hydroxide (NH_4OH, 25%), hydrogen peroxide (H_2O_2, 30%), and Milli-Q water for 5 min at 75 °C; finally, it was placed in an UV/ozone chamber for another 10 min. The gold-coated quartz crystal was then rinsed with Milli-Q water and dried with nitrogen gas (N_2); It was subsequently soaked in a 10 mM 11-mercaptoundecanoic acid solution (11-MUA) in absolute ethanol at 60 °C for at least 24 h. The excess amount of 11-MUA was rinsed off with absolute ethanol, and the surface was dried under N_2. Just before the immobilization of protein, the 11-MUA-coated surface was activated by a mixed solution containing 1:1 (v/v) of 100 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 100 mg/ml N-hydroxysuccinimide (NHS) in Milli-Q water for 1 h. The solution of 10 mg/ml β-lactoglobulin was used to incubate the activated quartz crystal surface at 4 °C for at least 24 h. The β-lactoglobulin chemically linked quartz crystal surface was finally rinsed out with Milli-Q water and dried under N_2. The success of immobilization was confirmed by grazing-angle Fourier transform infrared (FTIR) measurements (see Supporting Information).

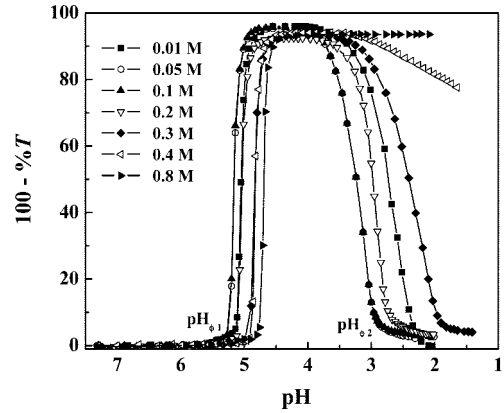

Figure 1. Turbidity (100 − *T*%) of the mixtures of β-lactoglobulin with pectin as a function of pH at different C_{NaCl} values.

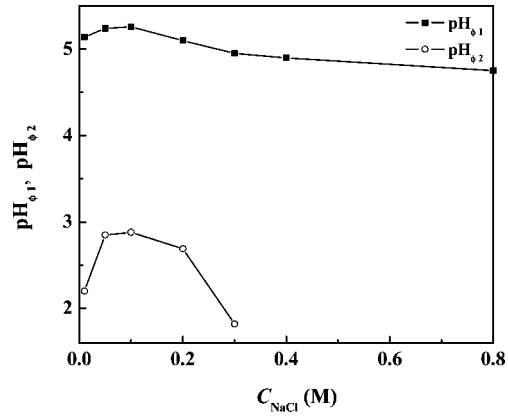

Figure 2. The variation of $pH_{\varphi 1}$ and $pH_{\varphi 2}$ as a function of sodium chloride concentration.

Tapping Mode Atomic Force Microscopy (TM-AFM). Images of β-lactoglobulin were collected in a liquid cell using a commercial Nanoscope IIIa Multi-Mode AFM (Veeco Instruments, CA), which was operated in tapping mode using silicon nitride cantilevers.

RESULTS AND DISCUSSION

Figure 1 shows turbidimetric titration curves of mixtures of β-lactoglobulin/pectin as a function of pH at 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.8 M sodium chloride concentrations (C_{NaCl}). The turbidities for all the turbidimetric titration curves are very small and remain constant until the pH reaches a critical value designated as $pH_{\varphi 1}$, beyond which the global phase transition occurs, and the turbidity abruptly increases and eventually reaches a plateau at all salt concentrations. Nevertheless, a further decrease of pH makes turbidity change differently at different salt concentration regions. At 0.01 M ≤ C_{NaCl} ≤ 0.3 M, the plateau is followed by a large decrease in turbidity with decreasing pH ending at another critical value $pH_{\varphi 2}$. At C_{NaCl} = 0.4 M, the decrease in turbidity after the plateau is much smaller, and $pH_{\varphi 2}$ is not observed. Moreover, at C_{NaCl} = 0.8 M, the plateau height remains constant with decreasing pH, and $pH_{\varphi 2}$ is also not observed.

It is generally accepted that the abrupt change of turbidity arises mainly from the change of either the mass or the size of aggregates in the solution (*19*, *20*). The abrupt increase of turbidity at $pH_{\varphi 1}$ is supposed to denote the point of global phase transition, where the aggregation of soluble protein/polysaccharide complexes results in the formation of insoluble protein/

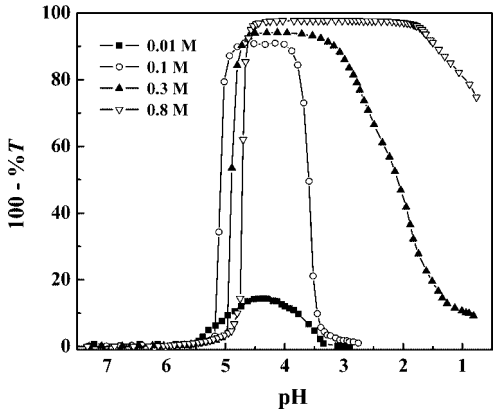

Figure 3. Turbidimetric titration curves of a 1 g/L β-lactoglobulin solution at various salt concentrations.

polysaccharide complexes. The point of soluble complex formation, initiated at pH_c (*21*), which is viewed as the point of incipient binding of pectin and β-lactoglobulin, can not be clearly determined from the nearly constant turbidity prior to $pH_{\varphi 1}$ in the current pectin and β-lactoglobulin mixtures, and is not the focus of this paper. Instead, we mainly focus on the effects of salt concentration on the coacervate formation (at $pH_{\varphi 1}$) and dissociation (at $pH_{\varphi 2}$) in the pectin and β-lactoglobulin mixtures.

Usually, $pH_{\varphi 1}$ values may be used as the starting point of protein/polysaccharide coacervation. The higher the $pH_{\varphi 1}$ value, where β-lactoglobulin carries less positive charge for interacting with negatively charged pectin, the stronger the tendency for β-lactoglobulin and pectin to form coacervates. The variation of $pH_{\varphi 1}$ and $pH_{\varphi 2}$ values as a function of C_{NaCl} is given in **Figure 2**. From **Figure 2**, it is found that an increase in C_{NaCl} up to 0.1 M causes the $pH_{\varphi 1}$ to shift to higher pH, whereas a further increase of C_{NaCl} to higher than 0.1 M shifts the $pH_{\varphi 1}$ to a lower value. Similarly, $pH_{\varphi 2}$ also shows a maximum at $C_{NaCl} = 0.1$ M. Our low-salt concentration results are consistent with other people's work (*10*). However, they did not investigate the effects of salt on β-lactoglobulin/pectin coacervation at high salt concentration ($C_{salt} > 0.1$ M). The added micro-ions could screen the charges of proteins and polysaccharides. As a result, the coacervation between β-lactoglobulin and pectin occurs at a lower $pH_{\varphi 1}$, where β-lactoglobulin carries more positive charges. However, the tendency of increasing $pH_{\varphi 1}$ values with C_{NaCl} at C_{NaCl} lower than 0.1 M suggests an interesting salt-enhanced effect that the small amount of salt could promote the β-lactoglobulin/pectin coacervation.

Although the added salt was reported to enhance the formation of protein/polysaccharide complexes at certain salt concentrations (*7, 11*), the reason is still unclear. The protein molecules are essentially amphoteric polyelectrolytes containing both positive and negative charges. Therefore, there simultaneously exists electrostatic attraction and electrostatic repulsion between the charges in protein molecules and polysaccharide chains. According to Dubin's model of electrostatic interaction of protein with polyeletrolyte (*11, 22*), the electrostatic attraction and electrostatic repulsion may be related to the average distance between the protein's positive sites and the polysaccharide's negative sites (R_+), the average distance between the protein's negative sites and the polysaccharide's negative sites (R_-), and the Debye length (R_d). The Debye length is defined as the distance over which significant charge separation can occur, outside of which charges are screened. If R_+, and R_- are independent of salt concentration, the presence of salt leads to Coulombic screening through influencing R_d (at room temperature, $R_d \approx 0.3/(C_{NaCl}^{1/2})$). At lower salt concentrations, during β-lactoglobulin/pectin coacervation, there may be $R_+ < R_d < R_-$, and the addition of salt is mainly to screen electrostatic

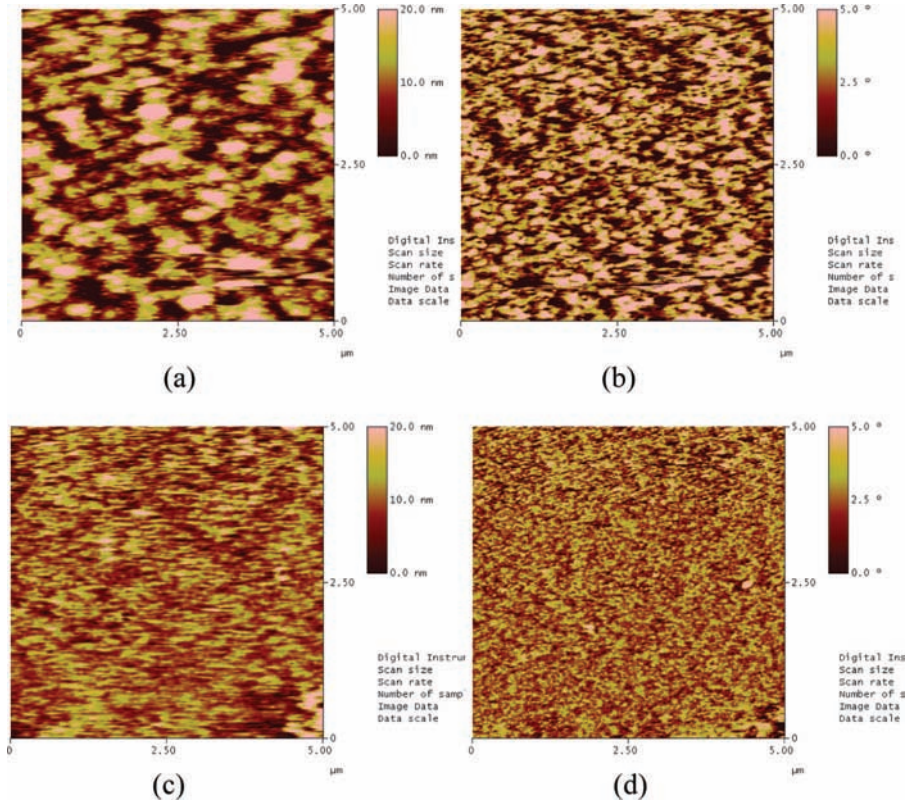

Figure 4. Tapping mode atomic force microscopy images of surface-grafted β-lactoglobulin in 0.8 M NaCl solution [(**a**) and (**b**)] and pure DI water [(**c**) and (**d**)] at pH 4.0. Panels (**a**) and (**c**) are height images, whereas panels (**b**) and (**d**) are phase images.

repulsion, instead of disturbing the electrostatic attraction between β-lactoglobulin and pectin. Therefore, the total interaction will be enhanced with increasing C_{NaCl}. This salt-enhanced effect thus causes β-lactoglobulin/pectin coacervation to occur at higher $pH_{\varphi 1}$ values when C_{NaCl} increases from 0.01 to 0.1 M. On the contrary, when C_{NaCl} is above 0.1 M, $R_d < R_+ < R_-$ could make both electrostatic attraction and repulsion be screened significantly because of the higher amount of salt, as shown in most of the previously published works (*10*). Therefore, an increase in C_{NaCl} from 0.1 to 0.8 M leads to decreased values of $pH_{\varphi 1}$.

It is worthy of noting in **Figure 2** that the $pH_{\varphi 2}$ values change to smaller values at C_{NaCl} values from 0.1 to 0.3 M. The $pH_{\varphi 2}$ cannot be determined from the turbidity curves for C_{NaCl} of 0.4 and 0.8 M. Generally, $pH_{\varphi 2}$ is used to denote the dissociation of protein/polysaccharide coacervates. The smaller $pH_{\varphi 2}$ values indicate that β-lactoglobulin/pectin coacervates are more difficult to dissociate at higher salt concentration, which seems to be in contrast to our above discussions about salt-reduced β-lactoglobulin/pectin coacervation at C_{NaCl} values higher than 0.1 M. Nevertheless, the changes of $pH_{\varphi 2}$ may be because of the unique character of self-aggregation for β-lactoglobulin. At normal physiological pH, β-lactoglobulin mainly exists in the dimer form (*14*, *15*). β-Lactoglobulin dimers have been reported to easily aggregate into oligomeric structure as pH decreases to lower than the isoelectric point (pI) of β-Lactoglobulin at around pH 5.2 (*23*, *24*), which is similar to the pH window for the formation of β-lactoglobulin/pectin coacervates. Therefore, the competition between self-aggregation of β-lactoglobulin and β-lactoglobulin/pectin coacervation must be considered.

Figure 3 indicates the formation of β-lactoglobulin aggregates from the dramatic increase of turbidity for pure 1 g/L β-lactoglobulin at 0.01, 0.1, 0.3, and 0.8 M NaCl solutions. The onset of protein aggregation from high pH occurs for 0.01 M NaCl and persists through 0.8 M NaCl, suggesting salt-reduced protein aggregation around pH 5.2 (*25*), and should be attributed to a decrease of the isoelectric point of β-lactoglobulin at higher salt concentrations (*26*). However, at acidic pH values lower than 4.5, the bigger turbidity values at higher salt concentrations indicate that the higher salt concentration tends to promote the self-aggregation of β-lactoglobulin (*27*, *28*). Furthermore, an increase of C_{NaCl} up to 0.8 M is observed to extend the pH range for β-lactoglobulin aggregates. This salt-enhanced protein aggregation at pH values lower than 4.5 may be because of the salt screening effect on β-lactoglobulin dimers (*25*). The similar shape of turbidity curves in **Figures 1** and **3** suggests that the observed turbidity values in the mixture of β-lactoglobulin and pectin contribute from both the formation of β-lactoglobulin/pectin complexes and from β-lactoglobulin aggregates, especially at higher salt concentration. The increasing salt concentration above 0.1 M NaCl has dual effects: (1) to screen the charges of β-lactoglobulin and pectin and then reduce β-lactoglobulin/pectin interactions, and (2) to promote the formation of larger β-lactoglobulin aggregates. These competing effects result in the formation of larger β-lactoglobulin domains within β-lactoglobulin/pectin coacervates at higher salt concentration, as indicated in our recent small-angle neutron scattering (*12*) and dynamic rheology (*13*) studies.

To better understand the nature of self-aggregation of β-lactoglobulin in high-salt solutions (i.e., are they reversible?), we carry out TM-AFM measurements in a liquid cell. β-lactoglobulin molecules are chemically immobilized on a gold-coated quartz surface to ensure the reproducible results. **Figure 4** shows the AFM height and phase images of surface-grafted β-lactoglobulin in both a 0.8 M NaCl solution (pH = 4.0) and water. It is found that β-lactoglobulin molecules form large aggregates in 0.8 M NaCl solution, and these large aggregates disappear when the 0.8 M NaCl solution is replaced with water. This aggregation−dissociation cycle monitored by AFM is found to be reproducible. The reversibility of the self-aggregation of β-lactoglobulin, depending upon the solvent used, is consistent with other people's work (*27*, *29*).

In summary, the addition of salt has been found to play an important role on the pH boundaries of β-lactoglobulin/pectin coacervation, which is accompanied with the self-aggregation of β-lactoglobulin. The change of $pH_{\varphi 1}$ values with salt concentrations suggests that small added salt lower than 0.1 M could enhance the formation of β-lactoglobulin/pectin coacervates, whereas salt concentrations higher than 0.1 M could reduce β-lactoglobulin/pectin coacervation. At salt concentrations higher than 0.1 M, the addition of salt can strongly enhance the self-aggregation of β-lactoglobulin at pH values smaller than 4.5, which could inversely reduce β-lactoglobulin/pectin coacervation. TM-AFM results suggest that the self-aggregation of surface-grafted β-lactoglobulin is solvent-dependent and that the high-salt solution promotes the formation of β-lactoglobulin aggregates.

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Supporting Information Available: FTIR spectra of 11-MUA and 11-MUA-β-Lg. This material is available free of charge via the Internet at http://pubs.acs.org.

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