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Salting-in characteristics of globular proteins

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ABSTRACT

Protein solubility, and the formation of various solid phases, is of interest in both bioprocessing and the study of protein condensation diseases. Here we examine the the phase behavior of three proteins (chymosin B, β-lactoglobulin B, and pumpkin seed globulin) previously known to display salting-in behavior, and measure their solubility as a function of pH, ionic strength, and salt type. Although the phase behavior of the three proteins is quantitatively different, general trends emerge. Stable crystal nucleation does not occur within the salting-in region for the proteins examined, despite the crystal being observed as the most stable solid phase. Instead, two types of amorphous phases were found within the salting-in region; additionally, an analog to the instantaneous clouding curve was observed within the salting-in region for chymosin B. Also, protein solutions containing sulfate salts resulted in different crystal morphologies depending on whether Li_2SO_4 or $(NH_4)_2SO_4$ was used.

1. Introduction

The stability of protein solutions is important in many biological and industrial processes. Metastable protein solutions phase-separate over a range of time scales and can thereby impair purification or formulation steps during pharmaceutical development. A number of diseases have also been attributed to or characterized by protein phase separation and aggregation, including cold cataracts [1], hemoglobin sickle-cell disease [2], and Alzheimer's disease [3]. Consequently, there is a need to understand better the thermodynamics of protein aggregation and to be able to adjust solution conditions rationally to control it.

A key measure of the stability of protein solutions is the solubility, which by implication is that of the most stable condensed phase, namely protein crystals. Crystallization as a process can be an effective method of protein purification due to the exclusion of impurities from the equilibrium crystal phase [4]; however, adjusting protein solution conditions to attain metastable states that produce a crystalline phase is usually a trial-and-error procedure without obvious relationships to the protein structure. The most common method for adjusting the stability of homogeneous protein solutions (and by extension, protein–protein interactions) is by the addition of salts such as sodium chloride. The pioneering work of Cohn [5] and Green [6–9] described generalized trends for protein solubility as a function of ionic strength. At low ionic strength, they observed that protein solubility was increased by the addition of salt, in what is termed the salting-in region. The protein solubility increases up to a certain ionic strength, beyond which point it decreases as a function of ionic strength; this is the salting-out region. Although the behavior outlined in these early studies is often presented as being generally applicable, salting-in behavior is in fact a relatively unstudied phenomenon for globular proteins, and as such a mechanistic understanding remains unclear.

It is generally observed that salting in occurs near the isoelectric point (pI) [10] of the protein; proteins such as α-amylase from *Bacillus halmapalus* [11], serum globulin [12], edestin [13], and β-lactoglobulin [14,15] display salting-in behavior in NaCl solutions, albeit over differing ranges of ionic strength. Tanford suggested that protein salting-in is controlled by nonspecific electrostatic interactions resulting from the increased screening of attractive electrostatic interactions with increasing salt concentrations [16]. However, the observation of salting-in behavior at high ionic strengths, where electrostatics are completely screened, makes this explanation unlikely to be applicable in those cases; here hydration effects are typically invoked. More recent work has focused on how ions affect the water associated with the protein [17–19]. Ions that alter the protein–water interface such that the water becomes a "better" solvent would result in an increase in protein solubility.

In addition to the electrostatic effects suggested by the observation of salting-in phenomena near the isoelectric point, the limited charge on the protein in that region also affects the amount of water associated with the protein and the affinity of the solvent for the protein surface. Recent work has suggested that salting-in may be a result of direct binding of ions to increase the net charge; strongly-hydrated cations are thought to interact with the polar protein surface, particularly the amide group, and increase the solubility [20]. Further aspects of salting-in behavior are still unclear, such as the

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steep increase in solubility often observed with very small increases in ionic strength and the transition between salting-in and salting-out behavior.

The purpose of this work is to determine experimentally the salting-in region for three proteins and to map solubility trends as a function of pH, ionic strength, salt type, and precipitate morphology. Although the emphasis is on one of these proteins, chymosin B, the more limited data on the other two are important for exploring the generality of the observations. We also explore whether crystallization occurs within the salting-in region and hypothesize possible mechanisms for the lack of stable crystallites. The transition between salting-in and salting-out behavior with changing solution parameters such as pH and ionic strength is examined in order to provide insight into these different solubility trends. Finally, the influence of multivalent sulfate salts on protein solubility and precipitate structure is studied and compared to the behavior with monovalent NaCl to explore the role of charge-dense ions in determining salting-in and salting-out trends. Although the possible mechanisms involved are discussed here only qualitatively, the classification of the observations in terms of the equilibrium phase diagram can help in the development of a more thorough thermodynamic analysis of protein phase separation.

2. Materials and methods

2.1. Materials and solutions

Deionized water prepared using a Milli-Q Plus deionization system from Millipore (Billerica, MA) was used for protein dissolution, dialysis, and buffer preparation. All buffer and protein solutions were filtered to remove biological matter and other large impurities. VacuCap® 60 filter units (4632-Pall Corporation, East Hills, NY) were used for filtering large buffer volumes into two-liter roller bottles (431133-Corning Inc., Corning, NY). Protein solutions were filtered using Millex GV PVDF filters from Millipore (Bedford, MA). Protein concentrations were measured using UV/vis spectrometry with a Lambda 4B spectrophotometer (Perkin-Elmer, Norwalk, CT) with samples in quartz cuvettes (Starna Cells, Atascadero, CA). The absorbance was measured at 280 nm using extinction coefficients calculated to be 1.32 mL mg^{-1} cm^{-1} for chymosin B, 0.97 mL mg^{-1} cm^{-1} for β-lactoglobulin B, and 1.18 mL mg^{-1} cm^{-1} for pumpkin seed globulin [21].

The salts and buffers, ammonium sulfate (A-2939), bis-tris (B-7535), lithium sulfate (C-3881), and sodium citrate (S-4641), were purchased from Sigma. Sodium chloride (S-271), sodium hydroxide (S-318), sodium phosphate dibasic (S-374), potassium phosphate monobasic (P-285) and hydrochloric acid (A144-212) were purchased from Fisher Scientific. Tris (819620) was purchased from INC (Aurora, OH). These chemicals were used without further purification.

2.2. Purification of chymosin B

Chymosin B is the major isozyme of chymosin, and while it is capable of cleaving the A isozyme, it cannot be cleaved by itself or by other isozymes [22,23]. Bovine chymosin B is a 323 residue, 36 kDa protein with an isoelectric point of approximately 4.6. The protein, an aspartic protease found in calf stomachs with a β-hairpin structure at the active site, was obtained from a commercial rennet powder (Renco, New Zealand). Because of structural differences between chymosin B and other isozymes, as well as stability concerns, we developed a multi-step purification technique based on early purifications performed by Bunn et al. [24]. Crude rennet powder was dissolved in pH 6.0 solution buffered with 50 mM sodium phosphate. The protein was precipitated by saturation with NaCl, followed by centrifugation at 3220 rcf for 5 min to recover the precipitate, which was then redissolved in 3 times its volume of pH 6.0 solution buffered with 50 mM sodium phosphate. These steps were repeated twice more. The protein solution was precipitated at the isoelectric point of chymosin (pH 4.6) to remove any pepsin and recovered using centrifugation at 3220 rcf for 5 min. The precipitate was then dissolved in 3 times its volume of pH 6.0 solution buffered with 50 mM sodium phosphate. The isoelectric precipitation was then repeated.

Following the second isoeletric precipitation, the protein solution was precipitated at pH 5.0, 5 mM sodium acetate with 0.7 M NaCl. The precipitate, which appeared to be crystalline, was recovered using centrifugation, washed with high-salt buffer (5 mM sodium acetate, 2 M NaCl, pH 5.0) over a Whatman 0.22 μm PVDF filter to remove excess protein, then dissolved in a 50 mM sodium phosphate, pH 6.0 buffer solution to a concentration of ~2 mg/mL and purified chromatographically using an ÄKTA Purifier with fraction collector Frac-950 from GE Healthcare (Piscataway, NJ). A chromatographic separation was performed using the anion-exchange resin Q Sepharose FF (GE Healthcare) in an XK16 column (GE Healthcare) at pH 7 using 5 mM tris, pH 7 as the low-salt buffer and 2 M sodium chloride, 5 mM tris, pH 7 as the high-salt buffer. The chromatographic peak corresponding to chymosin B was recovered and the protein solution was again crystallized at pH 5.0, 0.7 M NaCl as a final purification step.

2.3. Purification of β-lactoglobulin B

β-lactoglobulin B (BLG-B) from bovine milk (L-0130) was purchased from Sigma (St. Louis, MO). It is a relatively small protein (162 residues, 18.3 kDa) with an isoelectric point of approximately 5.3 and β-barrel structure [25–27]. BLG-B normally exists as a dimer, except at low pH [28]. The protein solution was purified chromatographically on the equipment described earlier, following the same method as for chymosin. The fraction corresponding to BLG-B was recovered and precipitated using ammonium sulfate (A-2939, Fisher Scientific). The solution was centrifuged to recover the precipitate, which was redissolved in 3 times its volume of 5 mM bis-tris buffered solution, pH 6.0. The protein was precipitated using 3 M ammonium sulfate at pH 6.0. The precipitate, which appeared to be crystalline, was recovered using centrifugation, washed with high-salt buffer over a Whatman 0.22 μm PVDF filter to remove any excess protein and dissolved in a 50 mM sodium phosphate, pH 6.0 buffered solution.

2.4. Purification of pumpkin seed globulin (PSG)

The storage protein pumpkin seed globulin (PSG) was obtained from dried raw pumpkin seeds purchased from the Newark Food Cooperative (Newark, DE). PSG is a larger globular protein (MW 112 kDa, pI 6.3) that consists of two subunits, and was recovered and purified using a method modified from that of Blagrove and Lilley [29]. 100 g of ground seed meal was defatted by soaking in ligroin for 1 h; the solvent and all fat-soluble proteins were separated by centrifugation at 3220 rcf for 8 min and then discarded. The ground seeds were then soaked for 2 h in 1 L of deionized water to remove any proteins that were water soluble at very low salt concentrations. PSG was then extracted by soaking the seed meal in a 100 mM sodium phosphate, 1.7 M NaCl solution at pH 8.0 for 2 h. The protein was precipitated from the clarified extract by the gradual addition of 800 mL of water. The precipitate was dissolved in 100 mL standard buffer (1 M KCl, 0.1 M sodium phosphate buffer, pH 7), the solution was clarified by centrifugation and PSG was precipitated by the addition of 200 mL of water.

2.5. Solubility measurements

Solubility measurements in solutions that were not seeded with crystals were carried out by preparing series of samples at constant salt concentration with increasing protein concentration, and also at constant protein concentration with increasing salt concentration.

The protein stock solutions were dialyzed extensively against buffers at the desired pH and salt concentration; these buffer solutions also contained 5 mM NaN_3 to inhibit biological growth. Because of the sensitivity of these experiments to ionic strength, the dialyzed solutions were then diluted to three times their volume in buffer of the appropriate salt concentration and pH. These solutions were then reconcentrated to the necessary protein concentration using Amicon ultrafiltration centrifuge tubes. Final solution concentrations were measured using UV/vis spectrometry at 280 nm. Volumes of 0.5 mL were prepared by mixing appropriate amounts of concentrated stock solutions (high-salt buffer, low-salt buffer and concentrated protein solution) in 0.5 mL tubes (05-408-120) from Fisher Scientific. From a sample at a given solution condition, 7.5 μL were pipetted into each of the 6 wells in the first row of a 72-well microplate (HR3-087 from Hampton Research, Aliso Viejo, CA) so as to provide replicates for visual observation. These 6 sample wells were then covered with paraffin oil (HR3-421, Hampton Research). This procedure was repeated such that each of the 12 rows on the microplate contained different solutions with 6 replicates each.

The phase behavior of the samples in microplates was recorded periodically throughout the first 48 h; additional observations were spaced based upon these initial trends. Micrographs showing the phase behavior were obtained using a Leitz Laborlux S microscope and a Nikon Coolpix 8700 digital camera. Protein solubility was determined from the supernatant concentration of samples prepared in the 0.5 mL tubes and measured by UV absorbance at 280 nm after 72 h. The supernatant was quantitatively diluted in buffer solution so as to measure an absorbance signal within the linear region. The supernatant solubility was then sampled every 24 to 48 h to confirm that the system had reached equilibrium.

Fluorescence measurements were performed using an ISS fluorimeter (Champaign, IL), with 750 μL quartz cuvettes from Starna Cells used to hold samples. Precipitated protein samples redissolved to approximately 0.25 mg/mL were filtered immediately prior to measurement using a 0.22 μm filter (G75, Millipore, Bedford, MA). All protein samples were excited at 280 nm, with emission recorded between 290 and 400 nm.

Circular dichroism measurements were performed using a Jasco 810 spectrometer with a Peltier RT temperature controller on redissolved precipitate at a protein concentration of 0.25 mg/mL. Solutions were filtered using Millex GV PVDF filters from Millipore, then injected into a quartz cuvette (Starna Cells).

3. Results and discussion

3.1. Chymosin B in NaCl

The solubility data obtained for chymosin B (Fig. 1A) show both salting-in and salting-out behavior at all pH conditions studied. Salting-in is observed up to approximately 0.3 M NaCl, where the system then transitions to salting-out behavior. Results are shown only down to 0.1 M NaCl; solubilities could not be determined reliably at 0 M NaCl. We observe a slight decrease in solubility when the system pH is closer to the pI (ca. 4.6) [24], consistent with the frequent general observation that protein solubility increases as the pH is further from the pI due to increasing electrostatic repulsion that helps to stabilize the solution [30–35]. Although slightly lower average solubilities were measured at pH 4.0 (and not the pI), the differences are within the statistical uncertainty for these points. The classical picture of protein solubility predicts that the minimum would occur at the pI [16], although this generalization does not always apply [15].

The equilibrium solid phase for all data points in Fig. 1A is an orthorhombic crystal. Solutions within the salting-in region were seeded with crystals to determine the solubility with respect to the crystalline solid phase, as we were unable to nucleate crystals within the salting-in region. Seeding was also used to confirm that data points obtained within the salting-out region had reached their true equilibrium value.

Both salting-in and salting-out with respect to the crystalline solid phase were observed for chymosin in NaCl solutions at each pH. Protein systems exhibiting both salting-in and salting-out have been previously observed but primarily for systems containing salts with multivalent cations [36]. The solubility approaches zero at low ionic strength (<0.1 M) at all pH. Fig. 1B shows the extremely low solubility of chymosin below 0.1 M NaCl at pH 5.0, conditions where electrostatic repulsion would be expected to stabilize the protein solution. This suggests that there may be a strong attractive electrostatic component involved, consistent with Tanford's proposed mechanism [16].

The factors that make salting-in behavior possible appear to become less important when the solution pH is sufficiently far from the isoelectric point. At pH 3.0, only salting-out is observed (Fig. 1C), presumably because the acidic residues (Asp and Glu) are protonated. The resulting dominant positive charge would eliminate any attractive electrostatic configurations that may be responsible for salting-in.

When the solution pH is increased to pH 6.0, we observe analogous behavior to that at pH 3.0, with only salting-out observed for chymosin (Fig. 1D), in agreement with the equilibrium solubility curve determined by Bunn et al. [24]. Solubility values were not determined below 0.1 M NaCl, and although it is possible that salting-in occurs in this range, unseeded solutions below this salt concentration showed no precipitation or crystallization. Seeding proved difficult due to the amount of crystalline material needed to observe any phase behavior, making estimates of the salt concentration unreliable. The absence of salting-in at pH 6.0 again suggests attractive electrostatic interactions as a possible mechanism driving association at lower pH. Above the pK_a for one or more His residues, deprotonation may disrupt attractive electrostatic configurations that lead to low-salt aggregation at lower pH. The salting-out trend in the solubility data at pH 6.0 appears to follow the Cohn relationship ($\beta = 3.5 \pm 0.2$, $K = -1.4 \pm 0.6$), whereas the salting-out trend at pH 3.0 does not [37].

Fig. 1E shows the salting-out region for chymosin in NaCl solutions at pH 5.0, which contains two distinct boundaries characteristic of molecules with short-range attractive interactions [15]. One is the crystal solubility, reflecting the solid–fluid equilibrium, and the other is a clouding boundary defined as the protein concentration at which visible clouding was observed essentially instantaneously in well-mixed samples. Solution conditions between the solubility curve and the clouding boundary produced a crystalline solid phase from a single-phase solution. A sample micrograph of this crystalline solid is shown in Fig. S1 in the Supplementary Material.

The instantaneous clouding curve found at higher protein concentrations indicates a region of spontaneous demixing into a transparent supernatant and an opaque protein-dense phase that appears qualitatively as an amorphous solid. However, the amorphous dense phase can evolve over time into the crystalline solid phase. Protein crystals nucleate over varying time scales (as seen in Fig. S2), depending upon the solution conditions, and continue to grow at the apparent expense of the amorphous solid phase. Growth continues until the amorphous solid phase is consumed and only crystals are in equilibrium with the protein-lean phase.

These observations within the salting-out region are interesting because they indicate that, given sufficient time, the presence of a metastable dense phase does not inhibit the nucleation and growth of crystals. Dense-phase formation within the salting-in region, however, displays remarkably different behavior. Fig. 2 shows the location of the instantaneous clouding boundary within the salting-in region. Solutions formed within this boundary spontaneously demix, analogous to the instantaneous clouding boundary within the salting-out region except that no stable crystal nucleation is observed from the amorphous dense phase (Fig. S3). Inside the phase envelope, solutions along the salting-in

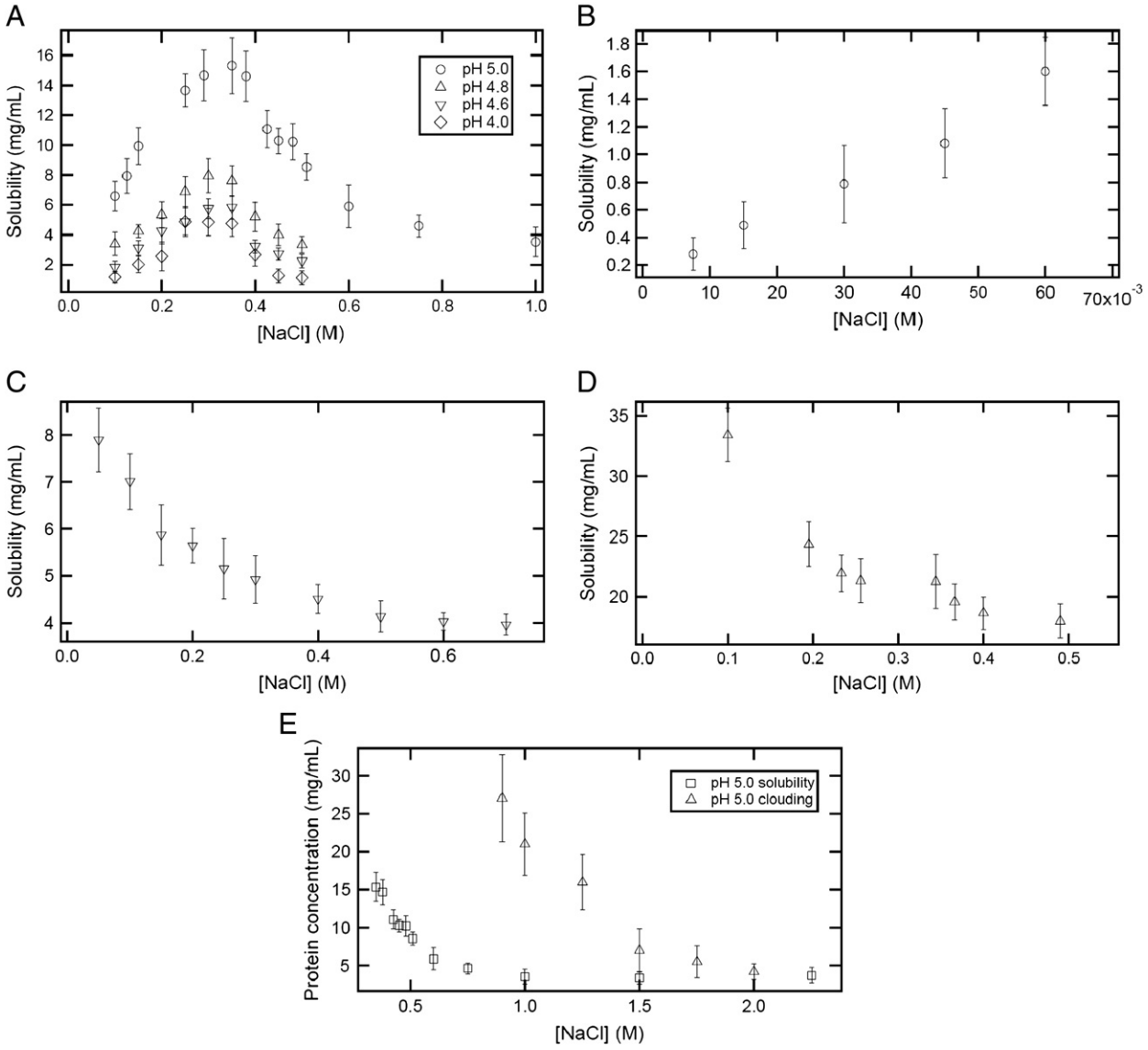

Fig. 1. The solubility of chymosin as a function of NaCl concentration. A) pH 4.0, 4.6, 4.8, and 5.0. The equilibrium solid phase for all data points is an orthorhombic crystal. B) pH 5.0 in solutions with a salt concentration <0.1 M NaCl. C) pH 3.0. D) pH 6.0. Note the absence of salting-in and the high solubility at low ionic strength (0.1 M NaCl). E) Solubility line and instantaneous clouding boundary at pH 5.0 in the salting-out region.

curve are stable over short time periods. The observed precipitate is also a non-crystalline dense phase that takes the form of a viscous liquid or gel droplet (Fig. S4), instead of a highly-disordered amorphous solid. In contrast to the behavior in the salting-out region, neither non-crystalline dense phase evolves over time to nucleate stable crystals. Aggregate formation appears to be irreversible upon dilution in the mother liquor; dissolution requires an increase in ionic strength.

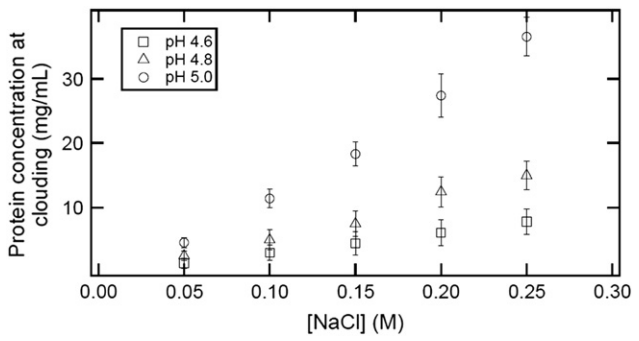

Fig. 2. Approximate location of the instantaneous clouding boundaries of chymosin in NaCl at pH 4.6, 4.8, and 5.0.

The location of the clouding boundary within the salting-in region varies as a function of both pH and ionic strength. As shown in Fig. 2, the effect of pH on the clouding boundary becomes difficult to distinguish at low salt concentrations. At 0.1 M NaCl, the clouding boundary occurs at low concentrations (1.3–4.5 mg/mL), such that these points are not readily distinguishable within error. As the conditions approach the salting-in to salting-out transition, the clouding boundaries show distinct separation as a function of pH, such that at pH 5.2 concentrated chymosin solutions can be prepared within the salting-in region that do not display the demixing or microphase separation associated with clouding.

The nature of the protein in these low-salt dense phases was investigated using fluorescence emission spectroscopy and circular dichroism (CD) on redissolved protein to observe possible changes in the protein structure as a function of salt concentration. For intrinsic tryptophan fluorescence excited at 290 nm, no discernable shift was observed in the emission maximum at 330 nm or in the spectral

center of mass. This suggests that chymosin does not undergo any measurable structural changes as a function of NaCl concentration that would expose internal tryptophan residues to the solvent. Far-UV circular dichroism spectroscopy was used to determine if the secondary structure of the protein undergoes any significant secondary structure changes as a function of NaCl concentration. Although some slight changes were observed in the mean residue ellipticity as a function of increasing NaCl concentration, no statistically significant changes were seen in the spectra to conclude that a conformational change had occurred. The presence of chloride ions also limited the wavelength range that could be reliably explored.

3.2. Chymosin in sulfate salt solutions

Empirical evidence in the literature for a wide variety of proteins [4,11,15] suggests that the protein solubility in solutions of sulfate salts should be lower than observed with chloride salts. By changing the accompanying cation, changes in phase behavior can provide insight into the role of the cation. Two sulfate salts with distinctly different cations were thus chosen to magnify any observable trends: the weakly-hydrated NH_4^+ and the strongly-hydrated Li^+ [38,39].

The solubility of chymosin at pH 5.0 (Fig. 3) does not show any major differences between the two salts. The overall apparent protein solubility is lower for the sulfate salts at pH 5.0 than for the NaCl solutions discussed previously; this is not surprising as sulfate salts have repeatedly been observed to precipitate proteins more efficiently [31–33,35]. Similar behavior is seen at pH 4.0. The monovalent NH_4^+ and Li^+ ions result in similar salting-out behavior, with the only statistically significant difference in the measured solubilities being at the highest salt concentrations and reflecting a reverse Hofmeister trend for the cations.

Salting-in behavior is observed at low ionic strength in the presence of both sulfate salts; again, non-crystalline aggregates are formed under these low-salt conditions in the absence of seeded crystals. Replacing NaCl with the charge-dense sulfate anion did not lead to true crystalline equilibrium at low ionic strength. The equilibrium solid phase also appears to be independent of cation type for the cations studied. Crystal formation is, however, seen along each salting-out curve. Also, apparently identical amorphous precipitates are observed within the salting-in region at the same ionic strength for both $(NH_4)_2SO_4$ and Li_2SO_4. Solutions at sufficiently high protein concentrations or at very low salt conditions display rapid demixing and result in the amorphous precipitate shown in Fig. S5. Solutions that are close to the equilibrium boundary or near the salting-in to salting-out transition form viscous liquid/gel droplets that display no qualitative difference and do not appear to depend on the cation in solution (Fig. S6). Further these droplets are similar to those formed within the salting-in region for chymosin in NaCl solutions, suggesting that the ions involved do not play a dominant role in determining the dense phase morphology in the salting-in region; this would be consistent with a simple electrostatic screening role for the salt. These dense phases are stable over long times (4–8 months) and do not evolve into the equilibrium crystalline solid. Although we cannot conclude whether the droplets are a kinetically trapped phase, crystal nucleation appears to be unfavorable at low ionic strength regardless of the ion type used to precipitate the protein.

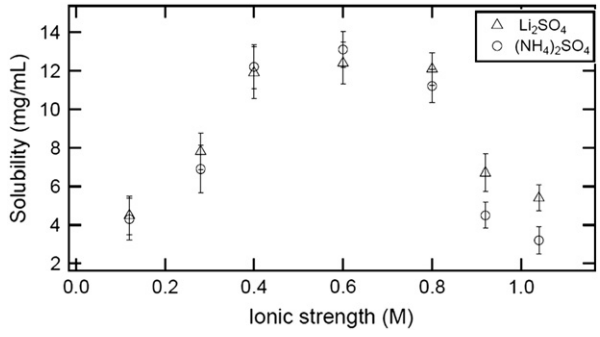

Fig. 3. The solubility of chymosin at pH 5.0 as a function of ionic strength in $(NH_4)_2SO_4$ and Li_2SO_4 solutions.

Although the type of ion used as a precipitant has little effect on the dense phase formed within the salting-in region, it does have an effect in more concentrated sulfate salt solutions. As the solutions become more concentrated with Li_2SO_4, chymosin solutions show an interesting and complex progression towards a stable equilibrium. The solutions first give rise to a transparent dense phase that appears to be continuous with an increased viscosity. This dense phase evolves over the course of weeks to form heterogeneous regions of increased size; these regions eventually separate to form crystals. An example of this metastable dense-phase evolution is shown in Fig. S7.

The growth of protein crystals from an apparent amorphous dense phase has been observed previously in this work for chymosin in NaCl solutions. However, the dense phase for chymosin/Li_2SO_4 solutions is qualitatively different from that observed in the salting-out region in NaCl solutions, namely an apparently continuous, transparent dense phase instead of an opaque aggregate. Evolution towards crystallization progresses through a number of complex steps compared to the relatively rapid nucleation that occurs in the presence of the metastable chymosin aggregates in NaCl.

Chymosin/$(NH_4)_2SO_4$ solutions within the salting-out region display distinctly different paths toward equilibrium from those of Li_2SO_4 solutions; this observation indicates that the cation plays a critical role in this process. The behavior of the $(NH_4)_2SO_4$ and Li_2SO_4 systems in the salting-out region diverges as the solution approaches the instantaneous clouding region. Chymosin solutions that show clouding in the presence of $(NH_4)_2SO_4$ do not show the metastable evolution that characterizes chymosin/Li_2SO_4 solutions. Solutions that are sufficiently far inside the instantaneous clouding boundary do not immediately sediment, but remain as opaque mixtures over long times. Less concentrated solutions precipitate over the course of hours, forming a dense phase that is a particulate aggregate rather than a continuous gel-like structure (Fig. S8). All these dense phases were shown to be reversible, suggesting that they are not a kinetically trapped solid but rather a metastable equilibrium phase. This behavior presents a parallel to the observed formation of chymosin crystals in NaCl solutions following precipitation of an amorphous dense phase.

Chymosin solutions with higher concentrations of $(NH_4)_2SO_4$ or Li_2SO_4 also show changes in the gross morphology of the crystals that evolve from these metastable dense phases. Orthorhombic cubic crystals, similar to those formed in NaCl solutions, are observed near the salting-in to salting-out transition. When the chymosin/SO_4 salt solution is sufficiently concentrated to form the previously shown dense phase, the crystals that nucleate in the presence of these phases have a variety of gross morphologies that appear to depend on the cation.

As shown in Fig. S9, chymosin crystals grown from the $(NH_4)_2SO_4$-precipitated dense phase are rectangular in gross morphology rather than the expected cubic form. Over long time periods (6 to 8 months), the entire dense phase is incorporated into crystals. Similarly, the chymosin dense phase in Li_2SO_4 solutions results in large crystals with apparently different morphologies. We have not determined if these crystals have different space groups or if the different gross morphologies simply reflect different growth rates for different faces.

3.3. Comparison to β-lactoglobulin and pumpkin seed globulin

β-lactoglobulin B phase behavior has been studied extensively [14,15,27,40,41], although the majority of this work was performed using ammonium sulfate as a precipitant. BLG-B solutions in NaCl show some similarities in their solubility trends to those of chymosin solutions at low ionic strength (Fig. 4). Strong salting-in behavior is observed at low ionic strength with respect to a seeded crystal, for all

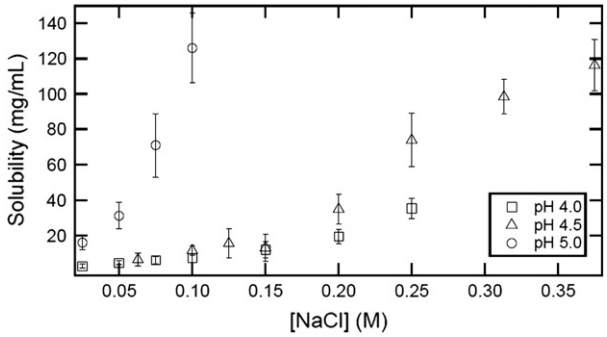

Fig. 4. Solubility of β-lactoglobulin B as a function of NaCl concentration at pH 4.0, 4.5, and 5.0.

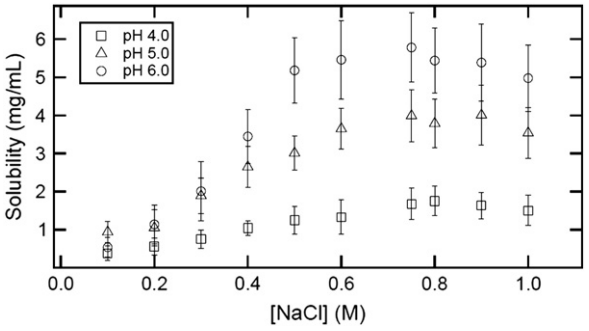

Fig. 5. Solubility of pumpkin seed globulin as a function of NaCl concentration at pH 4.0, 5.0, and 6.0.

pH conditions studied, although the overall solubility is much higher than that of chymosin. The increase in solubility is especially large between pH 4.5 and 5.0, presumably as a result of deprotonation of Asp and/or Glu residues.

As evidenced in Fig. 4, β-lactoglobulin B displays a strong increase in solubility within the salting-in region as a function of ionic strength. The high solubility at low ionic strength made it impractical to perform phase behavior experiments beyond 0.4 M NaCl due to the amount of material required. Within the salting-in region, no amorphous precipitates were observed at pH 5.0. However, amorphous precipitates are observed at certain ionic strengths at both pH 4.0 and 4.5, depending on the initial protein concentration. Crystal nucleation was not observed for any of the experimental conditions in the salting-in region.

Pumpkin seed globulin exhibits solubilities (Fig. 5) comparable to those of chymosin B in the pH range 4.0–6.0, but with some differences in overall behavior. The minimum solubility for the pH range examined is not near the isoelectric point (~6.3), but around pH 4.0. Strong salting-in behavior is observed, with a salting-in to salting-out transition that occurs in the range of 0.8 M NaCl, higher than that observed for chymosin. This transition is consistent with the condition of maximum solubility observed by Pericin et al. [42]. Seeded crystals exist as the stable solid phase across the phase diagram, consistent with behavior in the salting-in region for both chymosin and β-lactoglobulin B.

Pumpkin seed globulin precipitates within the salting-in region are non-crystalline in nature and, like the low-salt aggregates for chymosin, remain in this form in their mother liquor indefinitely. Crystal nucleation again appears to be retarded under these conditions. The dense phases are qualitatively different from those observed in the chymosin system, as shown in Fig. S10. Small, irregular beads are formed at near-neutral pH; decreasing the pH results in a transition to a defined network structure. The amorphous precipitates follow the previously established trend in that they do not redissolve on dilution at the conditions at which they were formed. However, these precipitates completely dissolve on addition of NaCl to the system and can later be crystallized if the salt concentration is sufficient to raise the solution into the salting-out regime.

The salting-in regions for the three proteins examined appear to be characterized by a lack of stable crystal nucleation. Malkin and McPherson observed the existence of aggregates in undersaturated PSG solutions [43], and these aggregates may be the origin for the non-crystalline solid phases observed at low ionic strength. The variation in location of the salting-in to salting-out transition remains unclear, and suggests that there are additional contributions to the repulsive potential beyond screening of long-range attraction.

4. Conclusions

The results for chymosin show that salting-in behavior is observed in NaCl solutions within the pH range of 4.0 to 5.0. Disruption of attractive electrostatic configurations by protonation of acidic residues (pH 3.0) or increased negative charge (pH 6.0) is a likely cause for this change in low-salt behavior. The concentration dependence of the amorphous precipitates within the salting-in region, in conjunction with the apparent irreversibility of formation of both amorphous aggregate morphologies in their mother liquor, indicates that there may be a kinetic aspect to this phenomenon that at present remains elusive.

The limited effect of ion type on both salting-in behavior and the corresponding dense phase morphology suggests that salting-in is primarily driven by attractive configurations that are sensitive to the electrostatic changes induced by pH effects. Ion effects, particularly those due to the multivalent sulfate anion, become pronounced at high salt concentrations (>1.0 M) in the salting-out region and manifest themselves in apparent changes to gross crystal morphology as well as observed time scales for crystallization.

Similar salting-in phenomena were observed for β-lactoglobulin B and pumpkin seed globulin. The salting-in region was not observed to nucleate stable crystals for either of these two proteins, analogous to the behavior observed for chymosin. The formation of liquid/gel-like droplet phases for both chymosin and pumpkin seed globulin near the solubility line suggests that a nucleation process is occurring, but one that does not appear to involve stable crystal contacts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2011.02.002.

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