Protein Ternary Phase Diagrams. 2. Effect of Ethanol, Ammonium Sulfate, and Temperature on the Phase Behavior of (*S*)-Ovalbumin

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The phase behavior of (*S*)-ovalbumin in aqueous ethanol and $(NH_4)_2SO_4$ solutions (pH 7.0) was investigated for temperatures ranging from 20 to 90 °C. The various morphologies observed were presented on ternary phase diagrams. At 20 °C, three morphologies were observed in aqueous ethanol: one-phase liquid, one-phase solid, and two-phase liquid and aggregate. Differential scanning calorimetry indicated that the (*S*)-ovalbumin denaturation temperature in buffer solution of 89.45 °C significantly decreased with ethanol addition and increased in the presence of $(NH_4)_2SO_4$. As a result, a pastelike morphology was observed at moderate protein (3−30 wt %) and ethanol (20−40 wt %) concentrations when (*S*)-ovalbumin solutions were heated above 50 °C. In contrast, $(NH_4)_2SO_4$ had a stabilizing effect on (*S*)-ovalbumin ternary structure and thus had little effect on (*S*)-ovalbumin phase behavior below the denaturation transition temperature. However, this salt encouraged solid phase formation once the denaturation transition temperature was reached.

Keywords: *Protein; ovalbumin; ethanol; salt; phase diagram*

INTRODUCTION

Under certain solution conditions, protein native structure can undergo transformations such as dissociation, denaturation, aggregation, polymerization, and coagulation (Lencki et al., 1992). Salt or solvent addition can strongly influence the temperature at which these transitions occur and the morphology of the final structures produced. As a result, solutes are often added to regulate these transformations during the processing of proteins or protein-containing food materials. To effectively control and optimize these food-processing unit operations, a broad understanding of how added solutes influence protein morphology is essential.

The effect of system parameters, particularly temperature, on structural transformations has been studied for many proteins. However, the precise system conditions under which each morphology exists has yet to be extensively outlined. A previous publication examined the effect of a solvent (ethanol) and salt [$(NH_4)_2SO_4$] on the solution behavior of gelatin, a non-heat-coagulable protein (Elysée-Collen and Lencki, 1996). This work will focus on a heat-coagulable globular protein: ovalbumin. At elevated temperatures, ovalbumin irreversibly gels. The opposite effect is observed with gelatin, which gels upon cooling but will liquify if reheated. Heat-induced ovalbumin gels become more elastic upon cooling but will not return to the liquid state (van Kleef, 1986).

The thermal denaturation and gelation behavior of egg white, and more specifically ovalbumin, has been extensively examined (Seideman et al., 1963; Holme, 1963; Nakamura et al., 1978; Egelandsdal, 1980; Johnson and Zabik, 1981). Ovalbumin is often chosen as a model protein for heat-induced gelling experiments because it is a well-characterized food protein which is known to aggregate easily (Holme, 1963; Egelandsdal, 1980). When stored for extended periods of time, the native *R*-form of ovalbumin converts to (*S*)-ovalbumin, which is more heat-stable (Egelandsdal, 1980). Commercial ovalbumin preparations typically contain a large percentage of the *S*-variant.

Hatta et al. (1986) investigated the conditions leading to different types of (*R*)-ovalbumin gels (turbid or translucent). They suggested that the molecular interaction caused by heating is mainly hydrophobic and that hydrogen bonding is less important than in gelatin gels. Therefore, disulfide bridges are not essential for forming the gel or coagulum; however, the formation of disulfide bonds is probably involved in the first step of coagulation.

The presence of salts or solvents is known to have a strong effect on the denaturation and coagulation behavior of ovalbumin (Mirsky and Pauling, 1936). Hegg et al. (1979) investigated the effects of salts and pH on the thermal denaturation and aggregation of (*R*)- and (*S*)-ovalbumin. Shimada and Matsushita (1981) prepared (*S*)-ovalbumin gels in the presence of salts and denaturants and found that the addition of ammonium sulfate increased the turbidity of heat-induced gels of (*S*)-ovalbumin while the addition of NaCl increased the hardness of the gels.

The studies outlined above are similar to most work examining protein solution behavior: They focus on the effect of only a few specific system variables or a particular range of component concentrations. For example, many studies have examined the effect of pH on gel formation and structure only at low salt concentrations (up to physiological ionic strength, typically 0.15 M). The ovalbumin concentrations used in most studies have also been lower than 15 wt %; however, van Kleef (1986) studied the gelation behavior of (*S*)-ovalbumin solutions using concentrations of up to 35 wt %. Because most studies have been limited in their scope, it is difficult to obtain an overall view of how the various ovalbumin morphologies interrelate. In a previous publication (Elysée-Collen and Lencki, 1996), we have demonstrated the utility of using ternary diagrams for illustrating the interrelationships between gelatin morphologies as a function of temperature, protein, and $(NH_4)_2SO_4$ or ethanol concentrations. This study will

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Table 1. Ovalbumin Phase Diagram Morphology Descriptions

morphology	description
A	transparent liquid, clear and colorless at low ovalbumin concentrations, increasingly yellow at higher concentrations (above 15%)
B	liquid containing protein aggregates that do not redissolve upon dilution
C	solid with no free liquid present, ranges from a dry powder to a soft hydrated gel, small quantities of solid salt sometimes present [in systems containing $(NH_4)_2SO_4$]
D	thick paste that is spreadable but possesses a microstructure that does not break apart with manipulation or redissolve upon dilution
E	liquid containing protein precipitate that redissolves upon dilution
F	liquid containing protein precipitate and salt crystals that redissolve upon dilution

extend this work by examining the effect of these system variables on the phase behavior of (*S*)-ovalbumin.

MATERIALS AND METHODS

Materials. Grade V ovalbumin (A-5503, lot no. 14H7035) was purchased from Sigma (St. Louis, MO) and used without further purification. Differential scanning calorimetry analysis indicated that this preparation principally contained (*S*)-ovalbumin. Added solutes consisted of reagent grade ammonium sulfate (Fisher Scientific, Mississauga, ON) and analytical grade ethanol (Commercial Alcohols, Toronto, ON). All solutions were made up with 50 mM sodium phosphate buffer (Fisher Scientific, Mississauga, ON) adjusted to pH 7.0; 1.0 mM DL-dithiothreitol (Sigma, St. Louis, MO) was used to determine the presence of cystine bonding.

Methods. *Sample Preparation.* All samples were made up by weight in duplicate. Solutions were vortexed for 5−15 s to ensure complete dissolution of the solvent or salt in the buffer before protein addition. Since the morphology obtained was sometimes dependent on the sequence of addition, the components were always added in the following order: (1) buffer, (2) $(NH_4)_2SO_4$ or ethanol, (3) protein.

Ovalbumin Phase Diagrams. Over 100 samples were prepared in 1.8 mL polypropylene tubes. Concentration increments of 10 wt % were initially used to identify the general morphological regions. However, when a phase change was observed from one solution to the next, solutions in this concentration range were then prepared, first in 5 wt % and then in 2 wt % increments to delineate the precise phase boundary. Therefore, the variability of the data was less than 2 wt % of the drawn phase boundaries. The samples were microcentrifuged for 5 min after addition of protein to effect protein solubilization and phase separation. The morphology of each sample at room temperature (20 °C) was determined by visual and tactile observations by two observers. All of the samples were made in duplicate to verify the morphologies observed. The samples were then heated in a water bath set at 30 °C for 10 min, and the morphology was recorded. This process was repeated at 40, 50, 60, 70, and 80 °C. A heating block was used to attain a temperature of 90 °C. Some cooling occurred when the samples were taken out of the water bath to determine the existing morphology. Morphology identification could have been affected if the temperature significantly decreased during this period because an increase in gel rigidity can occur upon cooling (Arntfield et al., 1990; van Kleef, 1986). However, since this cooling set is reversible (Beveridge et al., 1984), this effect was eliminated by reheating the solution in the water bath before reexamination.

At the end of the analysis, samples of the solid fractions of morphologies B, D, E, and F were diluted with buffer to determine whether solid formation was reversible. Grapher (version 1.26) by Golden Software (Golden, CO) was used to generate the ternary diagrams.

Differential Scanning Calorimetry (DSC). The denaturation temperatures of selected solute−water−ovalbumin solutions [15% ovalbumin in 15% ethanol; 25% and 35% ovalbumin in 13% ethanol; 15%, 25%, and 35% ovalbumin in 10% and 5% ethanol solution; 15%, 25%, and 35% ovalbumin in buffer; and 25% ovalbumin in 10 and 20% $(NH_4)_2SO_4$] were determined to ascertain whether denaturation played a key role in the formation of certain solution morphologies. All experiments were performed in duplicate. DSC thermograms were recorded on a Dupont Model 1090 thermal analyzer (Wilmington, DE) programmed for a rate of temperature increase of 5 °C min^{-1}. The instrument was calibrated with indium and gallium; 6−10 μL samples of protein solution (15%, 25%, or 35 wt % ovalbumin) were pressure-sealed in DuPont hermetic pans using air (i.e., an empty pan) as reference material. Endothermic heat flows (endotherms) were then obtained. The thermograms were analyzed using the supplier software (DuPont interactive DSC analysis, version 3.0). The denaturation temperature was taken as the downward peak of endothermic heat flow with temperature. The denaturation onset temperatures (where denaturation begins to occur) were also obtained from the data. The observed duplicate denaturation temperatures were within ±0.5 °C.

RESULTS

After examining several hundred solute−water−ovalbumin samples, a total of six morphologies could be distinguished visually (Table 1). Morphologies A and C were observed with both the ethanol−water−ovalbumin (Figure 1) and $(NH_4)_2SO_4$−water−ovalbumin (Figure 2) systems at all temperatures examined. However, morphologies B and D were specific to ethanol systems above 50 °C ,and morphologies E and F were only observed with $(NH_4)_2SO_4$.

Only three morphological regions were present in the ethanol−water−ovalbumin system below 40 °C (Figure 1a). At ethanol concentrations below 20% and ovalbumin concentrations below 56% (solubility limit), the samples were a transparent liquid (morphology A), increasing in yellow color above 15% protein. The high protein (>40%) concentration samples were very viscous but still transparent. Addition of ethanol up to 10% appeared to increase the solubility of ovalbumin (Figure 1a). However, at protein concentrations below 40%, the addition of 15−20% ethanol caused protein aggregation (morphology B). This aggregation was not reversible upon dilution, even when 1.0 mM DL-dithiothreitol was added to the buffer solution. Above 40% ovalbumin, approximately 15% ethanol was required for solidification (morphology C). The samples at protein concentration above 60% and low ethanol concentration (<10%) had a dry, pasty consistency. At ethanol concentrations above 15%, the opaque solid had a gellike appearance but was found to crumble when squeezed between the fingers. A gel should have a resilient and elastic nature (Almdal et al., 1993); therefore, this terminology could not be justified for morphology C.

At 50 °C, solid morphology C formed at protein concentrations above 20−25%, decreasing the size of the morphology B region (Figure 1b). These samples took on the consistency of a dry powder. The size of the liquid region (morphology A) was also reduced as moderate protein concentration (30%) samples in approximately 10% ethanol became a crumbly opaque solid. A new morphology was also observed at 50 °C at protein concentrations below 25% and ethanol concentrations between 10% and 30% (morphology D): a thick paste that was opaque but not as white as the heat-induced gels observed at the denaturation temperature of ovalbumin in buffer solution. This paste was distinguishable from the solid or aggregated samples because it was not dry like a powder but had a texture similar

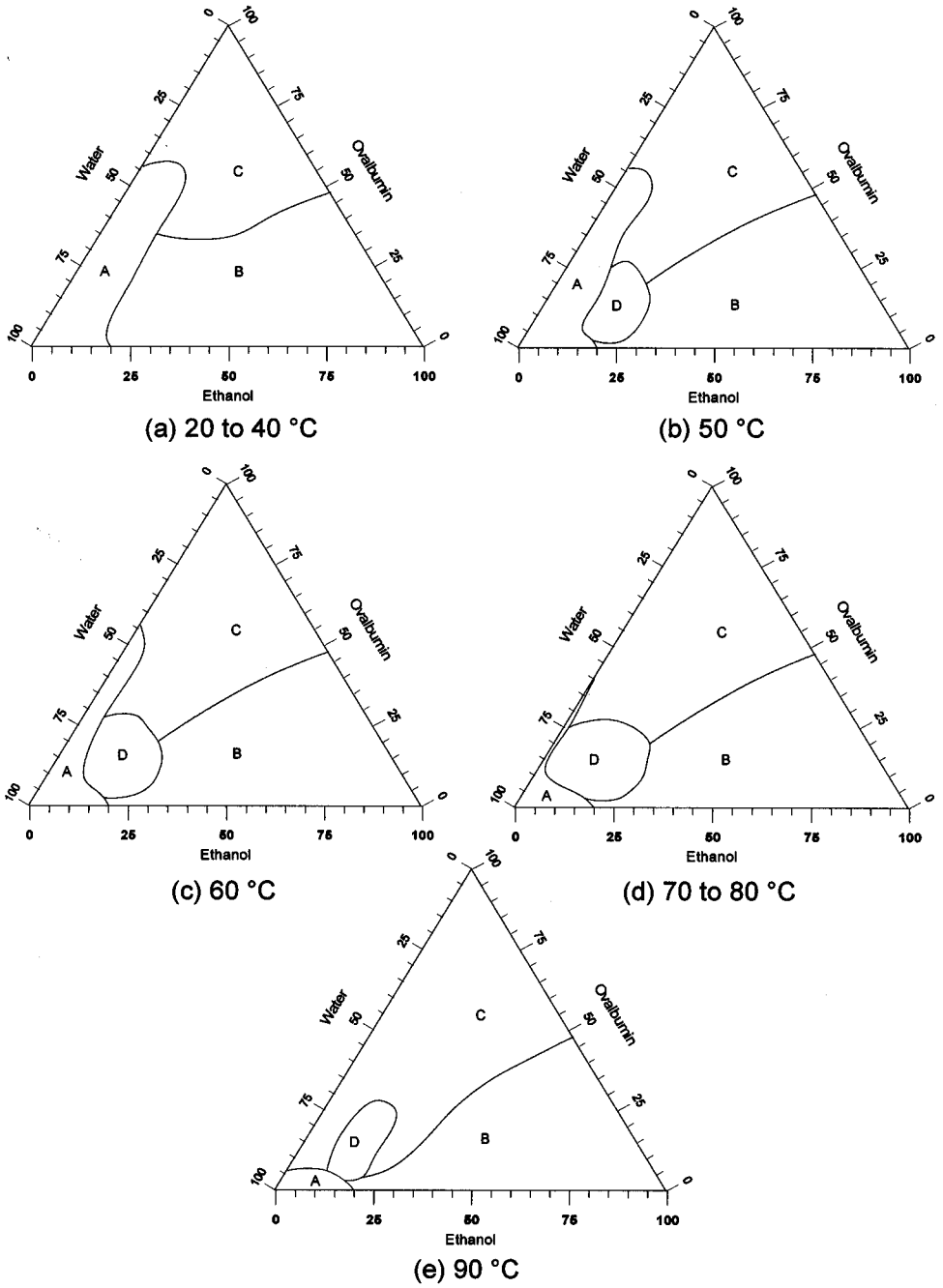
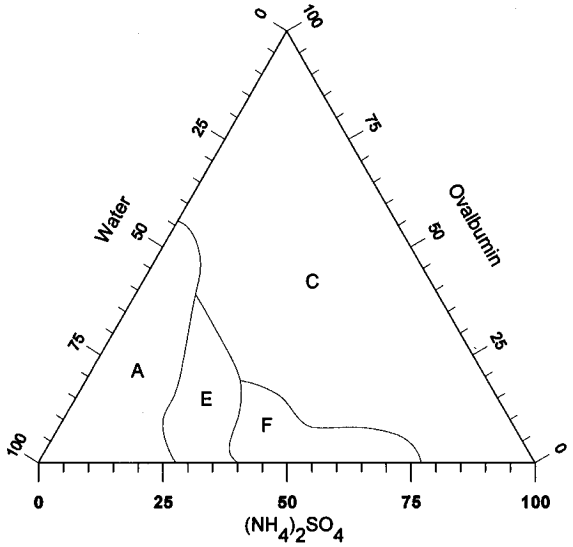


Figure 1. Ethanol−water−(*S*)-ovalbumin ternary system (see Table 1 for morphology descriptions).

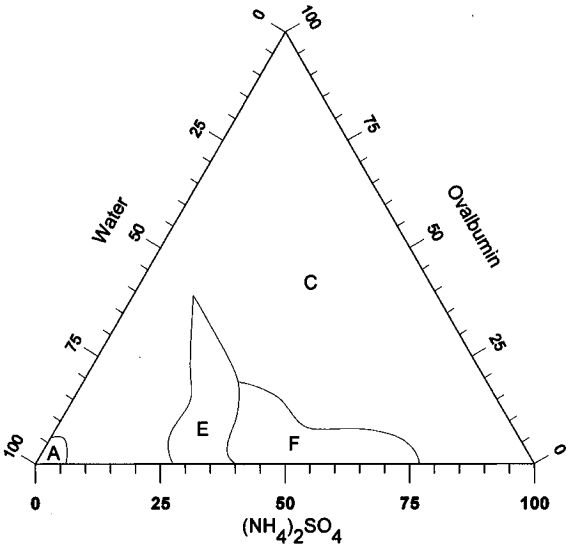
to very thick yogurt; it was spreadable and possessed a microstructure that would not break apart with manipulation. A sample of morphology D also did not redissolve upon dilution.

Some of the liquid samples (morphology A) became opaque and more viscous, turning into a paste (morphology D) after heating to 60 °C (Figure 1c). At 70 °C (Figure 1d), morphology D expanded to almost consume the whole liquid region. No noticeable changes in morphology were observed when the samples were further heated to 80 °C, and therefore the ternary diagram was the same as presented for 70 °C (Figure 1d). However, at 90 °C (Figure 1e), region C expanded because some of the paste samples hardened and therefore could then be classified as a solid. Samples with protein concentrations below the critical gelling concentration of ovalbumin (5.5%) (Arntfield et al., 1990) and below 20% ethanol remained liquid. In this liquid region, the low-ethanol (0−5%) samples remained transparent, while the rest of the samples in the morphology A region became more opaque.

Four different morphologies were observed for the $(NH_4)_2SO_4$-water-ovalbumin ternary system at room temperature (Figure 2a). A large part of the ternary diagram was solid (morphology C). These solid samples had a yellow/white powdery consistency and did not display a gellike structure. At protein concentrations below 56% and salt concentrations below 26%, a liquid solution was formed (morphology A). Addition of salt caused the solubility of ovalbumin to decrease below 56% until the protein formed a white powdery precipitate in the region between 15% and 40% salt and at protein concentrations below 40% (morphology E). This two-phase region (liquid−solid) was classified differently from morphology B observed with ethanol because the solid formed by salt addition could be resolubilized upon dilution of the solution. As the $(NH_4)_2SO_4$ concentration was increased above 40%, a solid salt layer was evident



(a) 20 to 80 °C



(b) 90 °C

Figure 2. $(NH_4)_2SO_4$−water−(*S*)-ovalbumin ternary system (see Table 1 for morphology descriptions).

(morphology F) with progressively less of a separate water layer until the mixture again became totally solid above 76% salt. The addition of ovalbumin also caused the solubility limit of ammonium sulfate to decrease from 43% in buffer solution to 31% at 19% ovalbumin.

The phase behavior of ovalbumin with $(NH_4)_2SO_4$ was not a strong function of temperature. The room temperature samples displayed essentially the same morphology even when they were heated up to 80 °C. However, at 90 °C, many of the liquid samples solidified into a hard, white, gellike solid, expanding the morphology C region (Figure 2b). The samples with a protein concentration below the critical gelling concentration of 5.5% for ovalbumin and at low salt concentrations (<6%) did not gel and remained a transparent liquid. The samples from 6% to 27% salt were an opaque white gel, but after tactile observation, it was apparent that this solid morphology had a very weak structure; liquid was easily exuded when the solid was disturbed. The samples in regions E and F did not change when heated to 90 °C.

DSC analysis (Figure 3) was performed on several solute−water−protein samples to determine whether

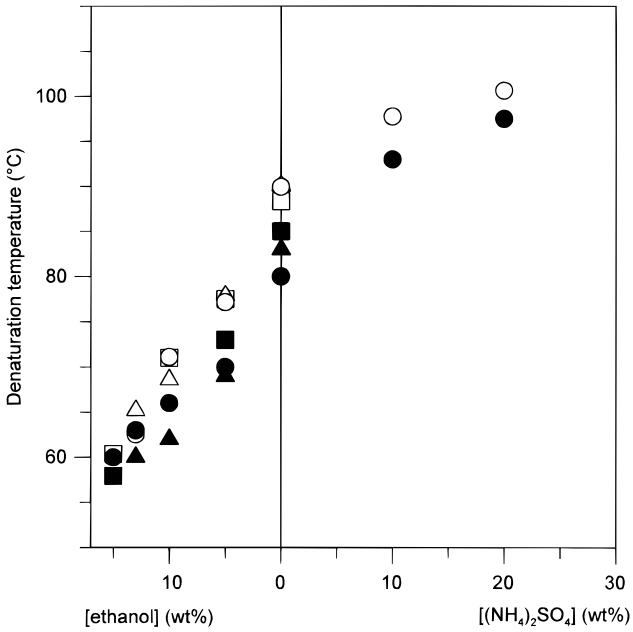


Figure 3. Denaturation temperature (onset and peak temperatures) as a function of solvent concentration. (*S*)-Ovalbumin concentration: □, 15%; ○, 25%; △, 35%. Peak temperatures: open symbols. Onset temperatures: filled symbols.

(*S*)-ovalbumin was present in the native or denatured form in the various solid morphologies present in Figures 1 and 2. In pH 7.0 buffer solution, the denaturation temperatures (T_d) were determined to be 88.35, 89.95, and 90.05 °C at 15%, 25%, and 35% protein, respectively. These results are similar to those of Donovan and Mapes (1976), who obtained a T_d value of 92.5 °C for (*S*)-ovalbumin at pH 8.8, while Hegg et al. (1979) found that (*S*)-ovalbumin denatured at 85 °C in pH 7 solution. Differences between our results and those found in the literature are most likely because our heating rate of 5 °C min^{-1} was slower than the 10 °C min^{-1} used in the above-mentioned studies. The denaturation temperature was not a strong function of protein concentration in the range studied. This agrees with the results of Arntfield et al. (1990) who found that in the range of 5−15%, protein concentration had no significant effect on the T_d values of (*S*)-ovalbumin. The results in Figure 3 also indicate that the presence of ethanol-destabilized (*S*)-ovalbumin. For example, the addition of 15% ethanol reduced the T_d by almost 30 °C. In contrast, $(NH_4)_2SO_4$ had a moderate stabilizing effect, with 20% salt increasing T_d by approximately 8 °C.

DISCUSSION

(*S*)-Ovalbumin was readily soluble in buffer solution (morphology A), with a solubility limit of 56%. The addition of up to 10% ethanol increased the solubility of (*S*)-ovalbumin by several percent, indicating that this protein is moderately hydrophobic (Shimada and Matsushita, 1981). The fact that moderate amounts of $(NH_4)_2SO_4$ did not salt-in but only salted-out this protein further indicates that (*S*)-ovalbumin has a relatively hydrophobic nature (Melander and Horvath, 1977).

At temperatures below 40 °C and ovalbumin concentrations less than 35%, when the ethanol concentration was increased to 17%, aggregation (morphology B) began to occur (Figure 1a). Ovalbumin is known to aggregate easily (Holme, 1963). At 17% ethanol and 40 °C, (*S*)-ovalbumin had not yet undergone heat denaturation (Figure 3) which is in agreement with the obser-

vation of Hegg et al. (1979) that ovalbumin aggregation principally occurs below the denaturation temperature. The presence of 1 mM DL-dithiothreitol could not break up these aggregates, indicating that cystine formation was not the principal mechanism of aggregate stabilization. This result was also found by van Kleef (1986).

When the temperature was increased to 50 °C, the stronger hydrophobic interactions that are present at higher temperatures would most likely accentuate ethanol−protein interactions, producing a more swollen native (*S*)-ovalbumin (Scopes, 1987). Water-binding ability is also enhanced by increasing temperature (Woodward, 1990). Therefore, at this higher temperature, protein−solute interactions would increase relative to interprotein interactions, leading to the formation of more solid morphology C at the expense of liquid morphology A and aggregate morphology B (Figure 1b). Paste morphology D also appeared at this temperature. This is perhaps not surprising since lower interprotein and higher protein−solute interactions would also transform aggregated morphology B to the more swollen, pasty structure of morphology D.

Protein−solute interactions would further increase as the temperature was raised to 60 °C, leading to the expansion of regions C and D (Figure 1c). DSC results with 15%, 25%, and 35% (*S*)-ovalbumin in ethanol solution (Figure 3) indicated that at 60 °C, denaturation of (*S*)-ovalbumin would occur at about 15% ethanol, which is approximately at the center of the region where paste morphology D is observed. However, the (*S*)-ovalbumin T_d was determined at the peak of the DSC endotherm, a point where the rate of denaturation was at its maximum. If paste morphology D formation only required a small amount of denatured (*S*)-ovalbumin, then the boundary of region D would more likely correspond to the temperature where denaturation began (i.e., the temperature at which the denaturation endotherm separated from the base line on a DSC thermogram). Figure 3 indicates that at 60 °C, denaturation onset began at an ethanol concentration of approximately 12%. At 60 °C and protein concentrations between 15% and 35%, the boundary between morphologies A and D occurred at 11% ethanol (Figure 1c). It would therefore appear that the coagulation of denatured (*S*)-ovalbumin played a role in the formation of this morphology. Denaturation would also expose hydrophobic groups that are buried in the core of the native structure, making the protein more hydrophobic (Scopes, 1987). This could explain why this pasty morphology was highly swollen at ethanol concentrations ranging from 20% to 30%. Beyond 30% ethanol, increased interprotein and intraprotein interactions, possibly resulting from the effect of increasing ethanol concentration on the dielectric constant, would contract the paste structure eventually leading to formation of separate dense solid and liquid phases (morphology B).

Beveridge et al. (1984) found that whey protein concentrate formed a distinct, visually strong solid upon heating. However, upon tactile examination, the gelled material lacked the springy, rubber elastic character of gelled albumen, consisting instead of drier, crumbly, dense material having some elastic character but overall was more pastelike. This description is similar to the paste morphology D found in the ovalbumin-ethanol samples in this study. Their inspection by electron microscopy revealed that the whey protein concentrate gels appeared much the same as the ovalbumin gels (Beveridge et al., 1984); therefore, it is most likely that this pastiness is a macroscopic phenomenon.

As the solutions were heated through 70 and 80 °C, the paste region (morphology D) further increased in size (Figure 1d). The region where solid morphology C was observed also expanded, once again most likely because of increased protein−solute interactions at higher temperatures. Finally, at 90 °C, morphology C took over a large portion of the phase diagram (Figure 1e). This would be expected because the denaturation temperature of (*S*)-ovalbumin in buffer solution was determined to be between 88 and 90 °C. The elevated temperature and high degree of denaturation would accentuate hydrophobic interprotein interactions, leading to the production of a solid structure. Only between 10% and 20% ethanol, when protein−solvent interactions appeared to be at their peak, did a paste morphology occur.

The addition of high concentrations of $(NH_4)_2SO_4$ to solutions of (*S*)-ovalbumin would accentuate hydrophobic interactions due to salting-out phenomena (Scopes, 1987). When the $(NH_4)_2SO_4$ concentration reached approximately 25%, interprotein hydrophobic interactions appeared to increase to the point where a solid precipitate (morphology E) was formed. The solubility limit of $(NH_4)_2SO_4$ in water was 43%, but this value decreased to 31% at a (*S*)-ovalbumin concentration of 19%, probably a direct consequence of the protein and salt competing for water of hydration (Bull and Breese, 1970).

Salting-out effects did not appear to be very temperature sensitive, since the $(NH_4)_2SO_4$−water−(*S*)-ovalbumin phase diagram remained essentially unchanged as the temperature increased from 20 to 80 °C (Figure 2a). However, at 90 °C and protein levels above the critical solidification concentration (i.e., 5.5%), liquid morphology A transformed to solid morphology C (Figure 2b). In the morphology C area between regions A and E, at moderate protein concentrations (8−30%), the opaque solid formed was very fragile. The presence of $(NH_4)_2SO_4$ stabilizes (*S*)-ovalbumin (Figure 3) so progressively less protein was in the denatured state as the salt concentration increased (Scopes, 1987). Consequently, less coagulation occurred, resulting in a weaker solid. Furthermore, because at higher salt concentrations (e.g., 20%) the protein molecules would interact more strongly but the level of protein−water interaction would be reduced, considerable water exuded from the solid upon tactile examination. At lower salt concentrations (<10%), when the protein molecules were not so strongly interacting, there would most likely be more protein−water interaction (solvent binding); thus, with the heat-induced solid of morphology C at salt concentrations less than 10%, tactile examination did not lead to a loss of solution (van Kleef, 1986).

CONCLUSIONS

The addition of ethanol or $(NH_4)_2SO_4$ principally affected the phase behavior of (*S*)-ovalbumin by altering the denaturation transition temperature and interprotein and protein−solute interactions of this globular, heat-coagulable protein. Increasing ethanol concentrations decreased the temperature of denaturation onset, leading to the formation of a paste morphology, whereas $(NH_4)_2SO_4$ had a stabilizing effect on ovalbumin, encouraging solid phase formation at elevated temperatures. This behavior was very different from that observed with non-heat-coagulable gelatin (Elysée-Collen and Lencki, 1996). With this protein, ethanol or $(NH_4)_2SO_4$ addition influenced the formation of coacervate and gel morphologies. In addition, increasing temperature caused a gradual change in gelatin phase

behavior, but with (*S*)-ovalbumin, temperature effects were more abrupt, particularly with the $(NH_4)_2SO_4$ ternary system. It would therefore appear that ternary phase diagrams are not only useful for characterizing the effect of various added solutes on the phase behavior of a particular protein but can also be used to compare the solubility and stability behavior of different types of proteins.

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