Protein Ternary Phase Diagrams. 1. Effect of Ethanol, Ammonium Sulfate, and Temperature on the Phase Behavior of Type B Gelatin

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The effect of ethanol or $(NH_4)_2SO_4$ addition on aqueous gelatin solution (pH 7.0) phase behavior was examined in the temperature range from 10 to 70 °C for protein and solute concentrations of 0−100 wt %. Ternary phase diagrams were used to effectively illustrate the relationship between the seven protein morphologies observed. Gelatin only exists as a random coil structure above 40 °C. As a result, in ethanol and salt systems below 30 °C, one-phase or two-phase gel and liquid morphologies dominated. In contrast, above 50 °C, one-phase sol or two-phase coacervate morphologies occupied significant portions of the two phase diagram systems. Between 30 and 50 °C, a wide range of morphologies was observed in both systems, as the gelatin gradually transformed to a more random structure. Differences observed between the various gelatin phase diagrams were a result of altered protein−solute, intraprotein, and interprotein interactions caused by changing temperature, ethanol, and $(NH_4)_2SO_4$ concentrations.

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

INTRODUCTION

Controlling how a protein interacts with itself, other proteins, and its surroundings is an important element of many food production and analysis procedures. One method of manipulating these interactions is by the addition of solutes. For example, salts can be added to protein-containing food preparations to modify attributes such as texture and stability. Several protein separation or purification processes (e.g., precipitation, crystallization) also require the addition of salts or solvents (Scopes, 1987). For example, ethanol precipitation is used to purify bovine serum albumin (Cohn et al., 1947). The control of solute concentration is also important in protein analysis techniques such as hydrophobic or ion exchange chromatography (Kennedy, 1990; Rossomando, 1990).

Care must be taken, however, when using solutes during processing or analysis because these compounds can strongly influence protein native structure stability. Protein destabilization involves processes such as dissociation, denaturation, aggregation, coagulation, and polymerization (Lencki et al., 1992), and at high protein concentrations, these reactions can lead to the formation of gel structures (Zeigler and Foegeding, 1990). The effect of salt concentration on protein stability is very ion specific, with stabilizing or destabilizing effects typically following the Hofmeister series (von Hippel and Wong, 1964). Solvents such as ethanol tend to destabilize proteins at elevated temperatures (Herskovits and Jaillet, 1969). The addition of solutes to hydrophobic proteins such as gelatin can also lead to the formation of two-phase liquid systems (Bungenberg de Jong, 1949). The creation of these coacervate structures is a key step in the production of many encapsulated gelatin products (Wood, 1977).

Temperature strongly influences a wide variety of protein solution phenomena (Schellman, 1987). However, most of the work on temperature effects has focused on either native-to-denatured transitions of globular proteins (Adams, 1991) or gel formation and structure (Zeigler and Foegeding, 1990). Globular proteins such as ovalbumin are heat coagulable, meaning they will denature at elevated temperatures and then irreversibly coagulate to form a gel. In contrast, nonheat coagulable proteins (e.g., gelatin) create a gel structure at reduced temperatures. Unlike gels produced using ovalbumin, gelatin gel formation is reversible and can be disrupted upon reheating.

Even though the formation mechanisms and structures of many of the above-mentioned protein morphologies have been addressed in detail, the precise temperature and solute concentration conditions under which each of these morphologies exists have yet to be extensively delineated. Therefore, our first goal was to experimentally determine the effect of temperature and added solutes on the morphological transition boundaries for a heat coagulable and nonheat coagulable protein. Our second goal was to effectively represent these transition boundaries. Ternary phase diagrams are widely used to depict the effect of a third component on the phase behavior of a particular compound in aqueous solution (Walas, 1985). While most of this work has focused on inorganic systems, phase diagrams have also been constructed for biological compounds. For example, Larsson and Puang-Ngern (1979) constructed a ternary diagram for two wheat lipids and water to understand the molecular organization of biological membranes. Carlson et al. (1976) used a ternary system to illustrate the structures formed when lipid aggregates in flour react with water. Ternary diagrams effectively delineate polymer incompatibility (Hsu and Prausnitz, 1974) and have been used to characterize two-phase liquid regions in concentrated aqueous two-protein (Polyakov et al., 1985) or protein−polysaccharide (Muchin et al., 1978) systems. The effect of temperature on biological molecules can also be illustrated using phase diagrams [e.g., critical point behavior of aqueous γ_{11}-crystalline solutions (Thomson et al., 1987); phase behavior of chlorophyll *a* (Eigenberg et al., 1982)]. Unfortunately, the protein phase behavior studies involving added salts or solvents published in

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the literature only focused on particular regions of the ternary diagram. For example, the effect of salts and solvents on protein phase behavior has been previously determined for gelatin (Holleman et al., 1933; Dervichian, 1954). However, these studies were limited to the regions of the diagram where coacervation phenomena dominate. Other gelatin phase change studies examined only dilute gelatin solution (0−2% gelatin) (Hayashi and Oh, 1983). Thus, fragments of gelatin solution behavior information can be found in the literature. Unfortunately, a global picture of the effect of temperature, salts, and solvents on gelatin phase behavior has not been developed.

$(NH_4)_2SO_4$ and ethanol are solutes typically used to purify or alter protein functionality (Scopes, 1987). Therefore, we wish to generate ternary diagrams that characterize the effect of these solutes on the phase behavior of various proteins as a function of temperature over the entire range of protein, water, and solute concentrations. This work will focus on gelatin, a hydrophobic, nonheat coagulable protein commonly used in the food industry. A subsequent publication (Elysée-Collen and Lencki, 1996) will examine the effect of these solutes as well as temperature on a heat coagulable protein (ovalbumin).

MATERIALS AND METHODS

Materials. Type B gelatin (1.25% moisture and 0.45% ash) from bovine skin (G-9382, lot no. 53H0271, approximately 225 bloom) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Added solutes consisted of reagent grade ammonium sulfate (Fisher Scientific, Mississauga, ON) and analytical grade ethanol (Commercial Alcohols, Toronto, ON). All solutions were prepared with 50 mM sodium phosphate buffer (Fisher Scientific, Mississauga, ON) adjusted to pH 7.0.

Methods. *Visual Analysis.* Gelatin solutions were prepared at room temperature by mixing sodium phosphate buffer with either ammonium sulfate or ethanol, vortexing for 5−15 s to ensure complete dissolution, and then adding the appropriate weight of gelatin. Solute concentration increments of 5% were initially used to identify the general morphological regions. When a phase change was seen from one solution to the next, however, solutions in 2% increments were prepared to delineate the precise phase boundary. Solutions within 2% of phase boundary lines were duplicated, and therefore the variability of the data is less than 2% of the drawn phase boundaries; 30 mL disposable glass vials containing the gelatin mixtures were placed in a water bath set at 70 °C. The physical state of each sample was periodically determined by visual and tactile observations by two observers. Phase behavior was recorded once the samples had attained a constant consistency, a process that took approximately 15 min. The water bath temperature was then decreased to 60 °C and the phase behavior recorded when the solutions once again reached equilibrium. This cooling and reequilibrating process was repeated at 50, 40, 35, 30, 25, 20, and 10 °C. According to the definition presented by Almdal et al. (1993), a gel must appear elastic and resilient to the human touch and, on a time scale of seconds, should exhibit no flow under its own weight. Therefore, the gelation point (within 5%) was taken as the temperature where the solution ceased to flow when the vial was inverted for 5 s. This method was similar to the standard method described by Wainewright (1977) where the gelatin sample was visually examined at regular time intervals for signs of gelation. The formation of the various morphologies due to cooling was reversible upon reheating of the solution in all cases. Consequently, some solutions were repeatedly heated and cooled to precisely delineate phase transition boundaries.

The software package Grapher (version 1.26) by Golden Software (Golden, CO) was used to generate the ternary diagrams. Ternary phase diagrams are constructed such that the concentrations in weight percent of the three components add up to 100% at every point. Therefore, the very top of the diagrams in this study represents 100% protein, the left corners represent 100% water, and the right corners represent 100% solute (either ethanol or salt in this study). The protein concentration increases along lines parallel to the bottom axis, where the bottom axis represents 0% protein. Similarly, water concentration increases along lines parallel to the right axis, where the right axis represents 0% water, and ethanol concentration increases along lines parallel to the left axis. The concentrations are indicated by the values on the respective labeled axes.

Table 1. Gelatin Phase Diagram Morphology Descriptions

morphology	description
I	transparent liquid, clear and colorless at low gelatin concentrations, increasingly yellow at higher concentrations (above 10%)
II	opaque liquid containing small aggregates
III	coacervate region, upper phase a clear liquid, lower phase very viscous and either transparent yellow (ethanol) or opaque white (ammonium sulfate)
IV	transparent or opaque lower gel phase with an upper liquid sol phase
V	homogeneous, transparent gel phase with no upper liquid sol phase
VI	powdery aggregates with no free liquid, small quantities of solid salt sometimes present in samples containing ammonium sulfate
VII	transparent or opaque lower gel phase with an upper liquid sol phase, lower phase also contains solid salt (only occurs with ammonium sulfate)

Rheological Analysis. Gel formation was also measured by oscillatory rheometry. Temperature sweeps were performed in triplicate on selected samples using a Carri-Med CSL 100 rheometer (Carri-Med Ltd., Surrey, England) to verify gel points determined visually (i.e., samples which changed from morphology I to morphology V). A 1.8 mL, 70 °C sample was placed between two 4 cm parallel plates separated by a 500 μm gap. The gap was set at the middle of the temperature range (45 °C) to minimize the error resulting from plate expansion and contraction. This setup method produced a gap that varied with temperature by less than 5% (Beveridge et al., 1984). A solvent trap sealed with corn oil was used to prevent sample drying. A stress value (Pa) was chosen for each sample from the midpoint of the linear viscoelastic region (LVER) determined in preliminary testing at 1 Hz, such that the sample displacement was within the limits of detection of the instrument ($1.0-3.6 \times 10^{-4}$) and the strain was within 0.15−1.0%. The LVER for each sample was determined at the visually determined gelling temperature. Because the gelation point is dependent on the heating rate (Stading and Hermansson, 1990), the temperature was decreased from 70 to 10 °C over a 2 h period to reproduce the conditions used for the visual determination of the gel point. The rheological characteristics storage (G') and loss modulus (G'') were monitored during cooling at a frequency of 1 Hz. In addition, the solidification point of the gelatin coacervates was verified. Only the coacervate (viscous liquid) portion was analyzed in order to obtain a homogeneous sample and prevent slippage of the plates during the experiment.

RESULTS

Inspection of several hundred solute−water−gelatin combinations indicated that a total of seven morphologies could be visually distinguished (Table 1 provides precise descriptions). Only zones with morphologies I−VI were observed with ethanol systems (Figure 1); $(NH_4)_2SO_4$ systems displayed all seven morphologies (Figure 2).

The precision of the visually determined zone transition lines was verified using rheological analysis. Fig-

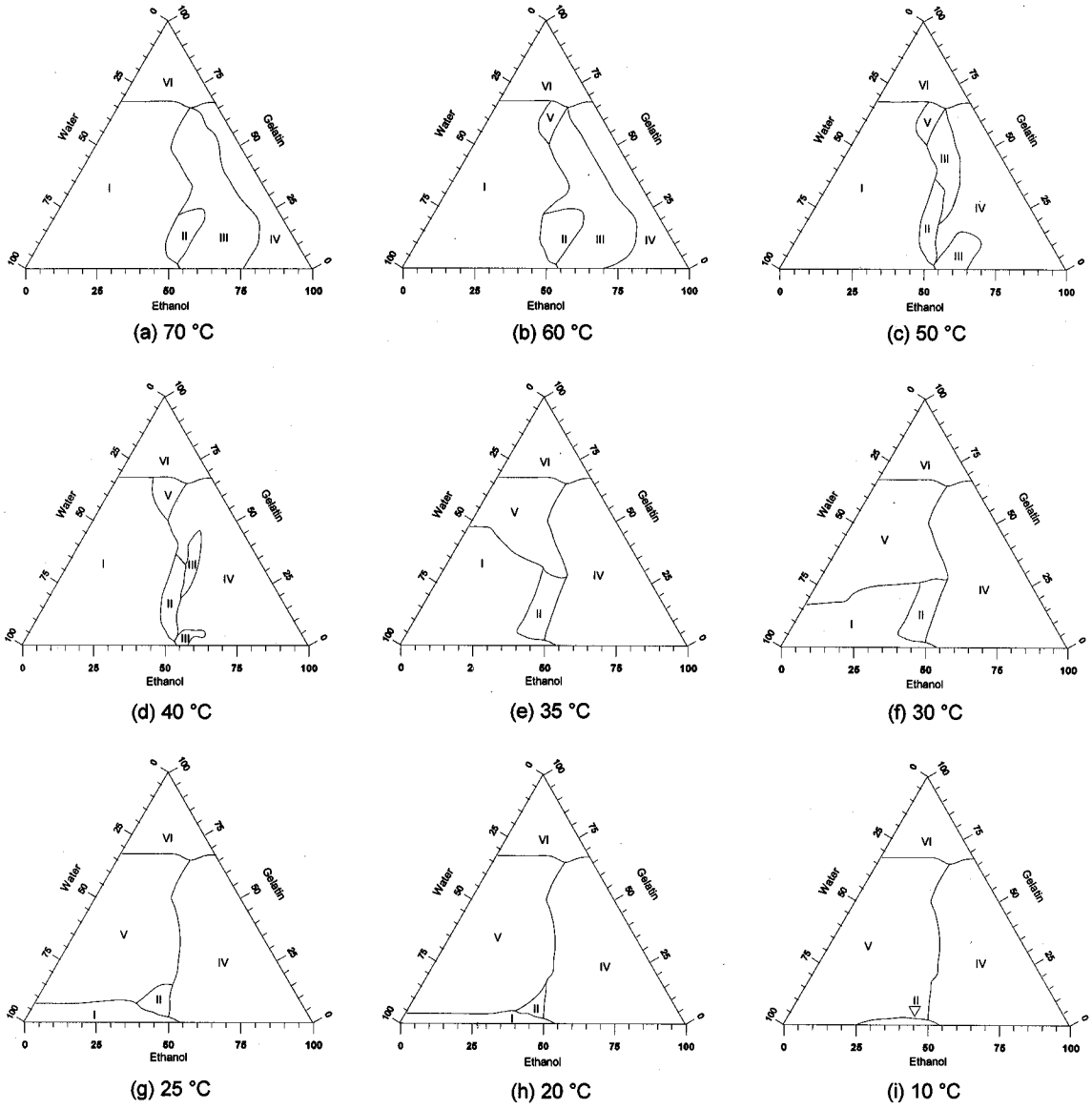

Figure 1. Ternary phase diagrams of the system ethanol−water−gelatin (see Table 1 for gelatin phase diagram morphology descriptions).

ure 3 illustrates a typical Carri−Med temperature sweep for a solution that was visually observed to form a gel at 20 °C. Several methodologies have been suggested for determining the gel point using temperature sweeps; these are reviewed by Stading and Hermansson (1990). For example, the gel temperature can be calculated by extrapolating the steeply rising portion of the G' versus temperature curve (Clark, 1991; Steventon et al., 1991). The value where the extrapolated line intercepts the temperature axis is then taken as the gel temperature. The gel point can also be defined as the temperature where G' first becomes detectable (Richardson and Ross-Murphy, 1981; Stading and Hermansson, 1990) or first starts to increase from a well-defined plateau (Fernandes et al., 1991; Moritaka et al., 1991).

Table 2 lists the gel temperatures determined using the three methodologies for several representative solutions. As expected, the gel temperatures determined by extrapolation were in most cases lower than those determined by the plateau−break method. The good agreement between the plateau−break and visual methods indicates that gel point determination by visual inspection can accurately detect changes in phase behavior.

At 70 °C, a large section of the ethanol−water−gelatin phase diagram (Figure 1a) consisted of a transparent liquid sol (morphology I). However, when the ethanol concentration approached 50%, at gelatin concentrations ranging from 5% to 25%, the solution became opaque (morphology II). When gelatin or ethanol concentrations were further increased above 20% gelatin or 55% ethanol, a visually discernable separate transparent yellow coacervate phase (morphology III) formed. As the water concentration decreased below approximately 15% (at 20% gelatin and above), the lower layer solidified (morphology IV). This lower solid phase took on the form of a dense opaque gel. The gelatin solutions made with only ethanol were different from the solutions containing water. The mixtures formed a liquid and an insoluble powdery aggregate phase. However, as soon as any amount of water was introduced, the powdery aggregate became an opaque gel. The ratio of upper liquid to lower gel volumes in morphology IV decreased as the amount of gelatin increased, until, at gelatin concentrations greater than 65%, no upper liquid phase was observed for all ethanol−water combinations. In addition, the protein aggregates found in this region no longer formed one solid mass but took on a powdery consistency (morphology VI).

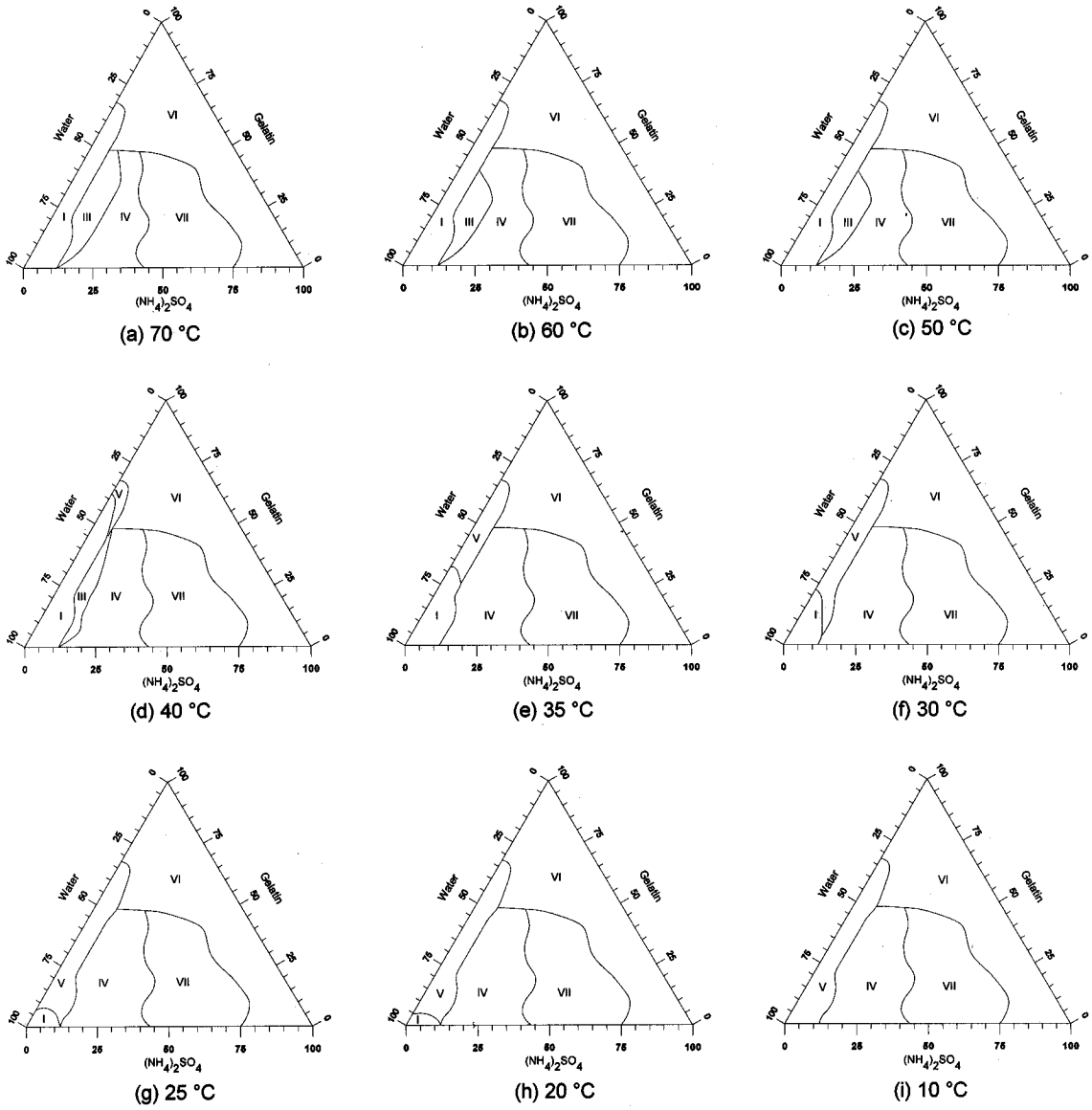

Figure 2. Ternary phase diagrams of the system $(NH_4)_2SO_4$−water−gelatin (see Table 1 for gelatin phase diagram morphology descriptions).

When the ethanol−water−gelatin system was cooled to 60 °C, the boundary between morphologies III and IV remained relatively stationary whereas zone II increased in size (Figure 1b). The boundaries of morphology VI also did not change noticeably as temperature decreased. Morphology V (a homogeneous transparent yellow gel with no upper liquid phase) was first observed at this temperature, occurring at between 20% and 27% ethanol and 50% and 66% gelatin. Between 50 and 40 °C, morphology IV grew substantially to occupy much of the right section of the phase diagram (Figure 1c,d). The boundary area between zones I and IV also became quite complex. The characteristics of this transition area are illustrated in three dimensions in Figure 4 (the transition zone is best visualized if temperature, which is plotted on the *z*-axis, decreases with increasing values of *z*). The coacervation phase splits into two regions at 50 °C, with a coacervate forming at either low gelatin (0−15%) and moderately high ethanol (52−65%) or high gelatin (20−66%) and moderate ethanol (25−45%) concentrations. From 45% to 52% ethanol and lower gelatin concentrations (5−20%), the lower phase solidified. Below 40 °C, the coacervate morphology disappeared and the single-phase homogeneous gel structure grew substantially (Figure 1e). This growth continued until, at 20 °C (Figure 1h), only a small zone I band existed at low gelatin concentrations (less than 5%). Cooling further to 10 °C (Figure 1i) resulted in the complete disappearance of morphology I.

The addition of $(NH_4)_2SO_4$ to gelatin−water solutions greatly extended the range in which gelatin forms an opaque powdery solid (morphology VI). As was observed with the ethanol phase diagrams, the boundaries of this region were not noticeably affected by temperature (Figure 2). At 70 °C, the transparent liquid sol region (morphology I) occupied a narrow band between 0% and 10% $(NH_4)_2SO_4$ (Figure 2a). As the salt concentration increased above 10%, a coacervate with a white opaque lower phase (zone III) was observed. Salt concentration above the 12−20% range caused a liquid upper and solid lower phase (zone IV) to form. Morphology VII was finally produced when the salt concentration reached the 30−40% range. This morphology was characterized by the appearance of salt crystals in the solid gel phase, so it was only found on the $(NH_4)_2SO_4$ diagrams. The boundary locations of zone VII were also not a strong function of temperature.

Decreasing temperature caused the size of the coacervate region to decrease (Figure 2b−d). This coacer-

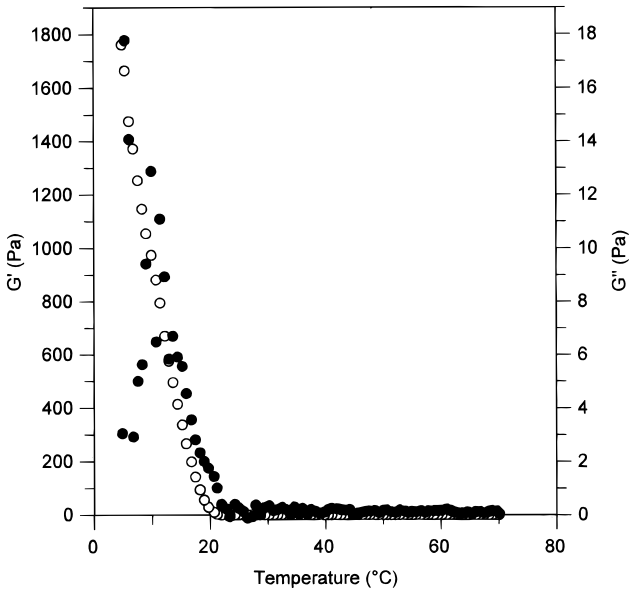

Figure 3. Gelation curve for a mixture of 5% gelatin, 5% ethanol, and 90% water: ○, storage modulus G'; ●, loss modulus G''.

Table 2. Comparison of Gelatin Solution Gel Points Determined by Various Methods

concentrations (wt %)			gel temperature (°C)		
gelatin	ethanol	salt	extrapolation to *x*-axis	plateau break	visual inspection
20	10	0	25.2 ± 3.2	30.6 ± 0.4	30
10	20	0	23.3 ± 1.5	23.3 ± 1.5	25
5	5	0	18.8 ± 0.5	20.5 ± 0.7	20
30	0	5	34.6 ± 2.0	34.9 ± 1.3	35
10	0	10	26.5 ± 2.0	29.9 ± 4.5	30
10	0	5	26.8 ± 1.0	29.0 ± 2.5	25
25[a]	0	15	38.6 ± 4.4	38.3 ± 4.6	40

[a] Coacervate sample.

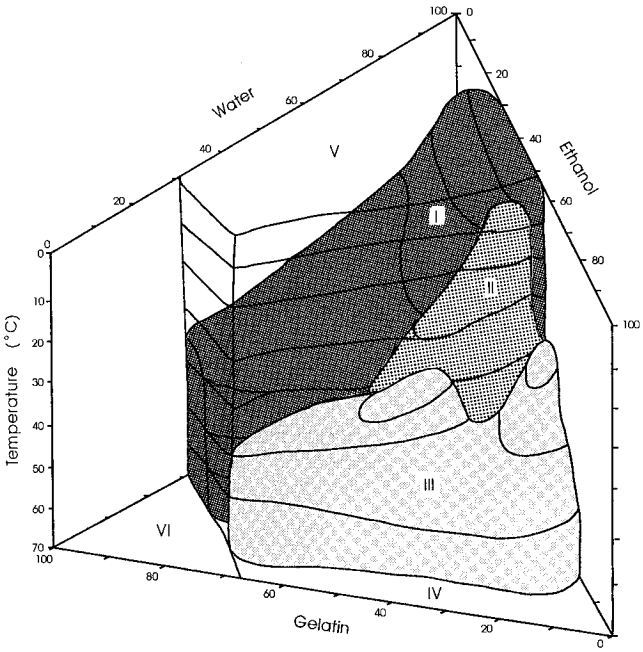

Figure 4. Phase behavior of the system ethanol−water−gelatin as a function of temperature (see Table 1 for gelatin phase diagram morphology descriptions).

vate region was no longer present at temperatures below 35 °C (Figure 2e), which was similar to what was observed with ethanol (Figure 1). Once again, this

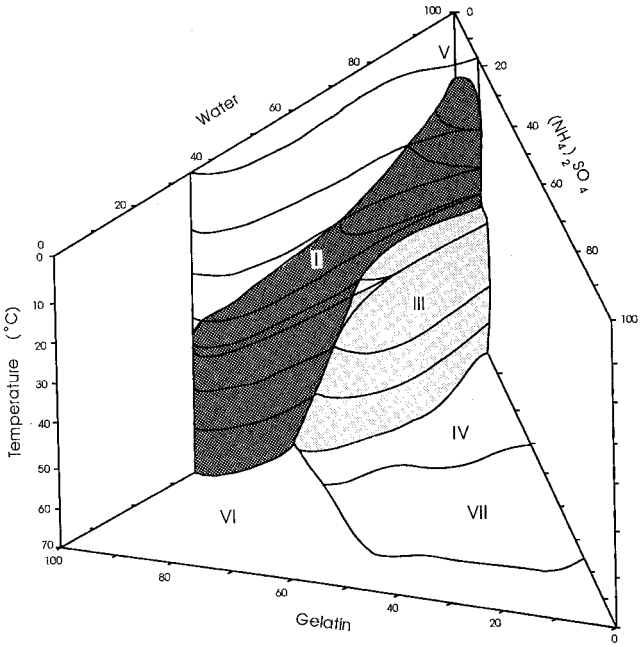

Figure 5. Phase behavior of the system $(NH_4)_2SO_4$−water−gelatin as a function of temperature (see Table 1 for gelatin phase diagram morphology descriptions).

transformation phenomenon is best observed on a three dimensional diagram (Figure 5). Upon cooling, the lower portion of the coacervate solidified, leaving a liquid and a gel-like structure (morphology IV). At 40 °C, morphology I began to solidify, forming a homogeneous transparent yellow gel (morphology V). Decreasing the temperature below 40 °C caused zone V to slowly consume zone I, to the point where, at 10 °C, no morphology I was present.

DISCUSSION

The results from the ternary diagrams presented in this study can be analyzed in terms of the extent to which a protein interacted with itself, other proteins, the solvent (water), and solute [ethanol or $(NH_4)_2SO_4$] because the morphology present under a particular set of solution conditions is determined by these factors (van Holde, 1977). Our results indicated that a water concentration of at least 33% was needed to break apart the proteins, hydrate the gelatin, and form a solution. Below this concentration, only a hydrated powder was present (morphology VI). These results were comparable to the published solubility limit of 65% for gelatin (Hayashi and Oh, 1983; Slade and Levine, 1987). The addition of ethanol did not appear to significantly affect the amount of total solvent required to hydrate the protein because the solubility limit for gelatin at all temperatures remained relatively constant at a combined ethanol−water concentration of 33%. This would indicate that the compact amorphous gelatin powder (morphology VI) showed little preference for ethanol or water and, therefore, was relatively hydrophobic.

In contrast, the addition of $(NH_4)_2SO_4$ reduced the solubility of gelatin. NaCl, another water structure former, is known to destabilize gelatin structure (Naftalin and Symons, 1974; Slade and Levine, 1987). In this study, at salt concentrations above 5%, almost 50% water was required to form a liquid phase. This was most likely a direct consequence of both the protein and salt competing for water of hydration. Similarily, increasing protein concentrations led to a decrease in

salt solubility. For example, the solubility limit of $(NH_4)_2SO_4$ in water was 43%, but this value decreased to 20% at a gelatin concentration of 45%. The fact that this salt demonstrated such a strong salting-out effect once again indicates that the gelatin powder appeared to have a hydrophobic character (Melander and Horvath, 1977).

At temperatures above 40 °C and high water (greater than 50%) concentrations, gelatin formed a transparent sol (morphology I). However, as the concentration of ethanol or $(NH_4)_2SO_4$ was further increased, coacervation occurred, producing a protein-rich lower and solute-rich upper phase (morphology III). With coacervate systems, ethanol and $(NH_4)_2SO_4$ selectively partition away from gelatin, concentrating in the upper phase (Dervichian, 1954). It is well known that at temperatures above 40 °C, gelatin takes on a random coil structure (Hayashi and Oh, 1983). Because ethanol appears to have a lower affinity for the protein than water, the open gelatin configuration is probably more hydrophilic than the compact powder structure observed in morphology VI. Gelatin protein−protein interactions are predominantly a result of hydrogen bonding (Johnston-Banks, 1990). Thus, the solubilization of gelatin would expose more functional groups that are capable of hydrogen bonding to the solvent, creating a more hydrophilic molecule.

An intermediate phase (morphology II) was also observed between the liquid sol (morphology I) and coacervate (morphology III) or liquid/solid two-phase (morphology IV) zones on the ethanol phase diagrams at all temperatures examined. Gelatin is not a homogeneous preparation but a mixture of proteins with varying molecular weights and charges (Johns and Court, 1977). Therefore, at low protein concentrations (<20%), the high molecular weight, highly charged gelatin molecules will drop out of solution first. In fact, ethanol has been used to fractionate gelatin preparations (Stainsby, 1977). Because at low total protein concentrations a very small amount of protein was present in the second phase, the creation of a distinct coacervate or gel structure was probably not possible, so small aggregates were then formed. However, when the total amount of gelatin in solution was increased to about 20%, it seems that enough protein was then present in the solid phase to produce a discernable coacervate or gel structure.

The liquid sol phase (morphology I) began to transform into a transparent gel (morphology V) at 60 °C and high gelatin (55−66%) and ethanol (75−90%) concentrations. However, with $(NH_4)_2SO_4$, this transition did not begin until the solution was cooled to 40 °C. It is well documented in the literature that in aqueous solution, below 40 °C, gelatin molecules begin to interact with each other, creating protein−protein linkages via microcrystalline junction zones (Slade and Levine, 1987) stabilized by hydrogen bonds (Croome, 1953). Hydrogen-bonding solvents like ethanol encourage gel formation (Finch and Jobling, 1977); therefore, gelatin formed a gel (morphology V) at a higher temperature in ethanol solution than in $(NH_4)_2SO_4$ solution. Ethanol will also compete for hydrogen-bonding sites; this is most likely why a transparent fine gel was formed. Since $(NH_4)_2SO_4$ is a water structure former, it would accentuate both intraprotein and interprotein interactions (Ledward, 1985), leading to a more coarse, opaque gel. The critical gel concentration for gelatin is 0.5% (Hayashi and Oh, 1983; Slade and Levine, 1987). The lowest protein concentration used in this study was 2%; therefore all of the samples were gelled at the lowest temperature examined.

The concentration ranges of salt, water, and gelatin in this study yielding a coacervate (morphology III) at 50 °C were approximately the same as those found in a previous study using sodium sulfate (Na_2SO_4) at 50 °C (Bungenberg de Jong, 1949). Dervichian and Van den Berg (1948) studied gelatin below the isoelectric point at pH 4, from 0% to 12.5% gelatin and 4−14% $(NH_4)_2SO_4$. At 37 °C, they found a coacervate in the narrow range from 7% to 10% salt. The coacervate found at pH 7 in this study occurred from 12% to 16% $(NH_4)_2SO_4$ in the same protein concentration range.

The salt-induced coacervate had a distinctly different appearance than the one induced by ethanol. The ethanol coacervate was a very transparent yellow liquid, while the salt coacervate was white and turbid. This difference is most likely due to the different solution environments present in ethanol and salt solutions. In ethanol solutions, a clear structure would be obtained because protein−solvent and interprotein interactions are balanced (Hatta et al., 1986), while a turbid structure is most likely produced with $(NH_4)_2SO_4$ because intraprotein and interprotein interactions are stronger, leading to a higher degree of aggregation (Ziegler and Foegeding, 1990). The turbidity of the salt-induced coacervate may also be due to multiplication of junction points through salt bridges (Tar and Wolfram, 1979).

CONCLUSIONS

Ternary phase diagrams are a useful method for characterizing protein morphology behavior in salts and solvents. Because of the flexibility of its random coil structure above 40 °C, gelatin can demonstrate a wide variety of phase behaviors and morphologies. The heterogeneity of gelatin also leads to complex morphological behavior. Nevertheless, protein ternary phase diagrams can clearly illustrate under which conditions the various solution behaviors are observed and the relationships between the various morphologies. Therefore, ternary phase diagrams are useful for comparing the effect of different solvents or salts on protein phase behavior. This knowledge will be useful for developing improved protein separation and stabilization protocols in the food industry.

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