Freeze-Concentration Separates Proteins and Polymer Excipients into Different Amorphous Phases

Ken-ichi Izutsu[1,2] and Shigeo Kojima[1]

Received March 16, 2000; accepted June 19, 2000

Purpose. To study the miscibility of proteins and polymer excipients in frozen solutions and freeze-dried solids as protein formulation models.

Methods. Thermal profiles of frozen solutions and freeze-dried solids containing various proteins (lysozyme, ovalbumin, BSA), nonionic polymers (Ficoll, polyvinylpyrrolidone [PVP]), and salts were analyzed by differential scanning calorimetry (DSC). The polymer miscibility was determined from the glass transition temperature of maximally freeze-concentrated solute (T_g') and the glass transition temperature of freeze-dried solid (T_g).

Results. Frozen Ficoll or PVP 40k solutions showed T_g' at –22°C, while protein solutions did not show an apparent T_g'. All the protein and nonionic polymer combinations (5% w/w, each) were miscible in frozen solutions and presented single T_g's that rose with increases in the protein ratio. Various salts concentration-dependently lowered the single T_g's of the proteins and Ficoll combinations maintaining the mixed amorphous phase. In contrast, some salts induced the separation of the proteins and PVP combinations into protein-rich and PVP-rich phases among ice crystals. The T_g's of these polymer combinations were jump-shifted to PVP's intrinsic T_g' at certain salt concentrations. Freeze-dried solids showed varied polymer miscibilities identical to those in frozen solutions.

Conclusions. Freeze-concentration separates some combinations of proteins and nonionic polymers into different amorphous phases in a frozen solution. Controlling the polymer miscibility is important in designing protein formulations.

KEY WORDS: protein formulation; freeze-drying; phase separation; excipient.

INTRODUCTION

The increasing number and diversity of biotechnology-derived proteins for medical use requires rational formulation design to assure better quality and prompt development. Although freeze-drying is often a preferred method for improving the shelf life of proteins unstable in aqueous solutions, many proteins lose their activity in freezing and drying. In order to achieve sufficient stability during processing and storage, various excipients (e.g., polyols, surfactants, salts, and polymers) are added to protein formulations (1). These excipients protect proteins against freezing and drying stresses in different mechanisms. For example, various polyols thermodynamically stabilize proteins in both liquid and frozen solutions by being preferentially excluded from the protein vicinity (2,3). In addition, they protect proteins from drying stress by substituting themselves for water molecules essential to maintaining the protein structure (4–6).

Molecular interactions between ingredients in amorphous multicomponent freeze-dried solids have received a great deal of attention as a major factor in determining the quality of various formulations (7,8). These interactions are particularly important in freeze-dried protein formulations since the stabilization mechanisms, especially the water-substitution to reduce the degree of drying stress, are based on direct interactions between proteins and co-solutes. It should be possible to control various factors affecting the molecular interactions in formulation processes (7,9,10).

Recent studies have shown that solutes in a freeze-concentrated non-ice phase are miscible or immiscible depending on their mutual solubility (9–15). Because freezing concentrates solutes, some combinations of solutes with steric hindrance or repulsive interactions separate into different phases among ice crystals, just as some concentrated polymer solutions separate to form aqueous two-layer systems (10,16,17). While certain concentrations of polymers are required to form an aqueous two-layer system, the freeze-induced phase separation can occur even with diluted initial solutions. Single-phase solutions containing combinations of two nonionic polymers (e.g., PVP and dextran), polyelectrolytes and nonionic polymers (e.g., DEAE–dextran and dextran), and nonionic polymers and buffer salts (e.g., PVP and phosphate buffer) have been observed to separate into two amorphous phases in a frozen solution (9–15). Salts and low-molecular weight co-solutes in the initial solutions have a large effect on the polymer miscibility in frozen solutions (1,14,15).

The freeze–concentration-induced phase separation between proteins and polymer excipients is an interesting area of study. While many polymers often prevent protein inactivation in freeze-thawing and freeze-drying, some polymers (e.g., poly(ethylene glycol) [PEG]) crystallize in frozen solutions and lose the stabilizing effect (9,12,18,19). The polymer miscibility in amorphous freeze-concentrate has not been well elucidated, despite its possible importance, due to the lack of an appropriate analytical method. Although many proteins are miscible with various polymers at low concentrations because of their small, spherical, polyelectrolyte nature, some of them separate to form aqueous two-layer systems or to precipitate proteins at high concentrations (20–22). The immiscibility in aqueous solutions suggests a possible phase separation in the freeze-concentrate. The freeze-induced phase separation can directly or indirectly affect the protein stability in freeze-drying by changing the component interaction and/or by acting as a possible site for surface denaturation (12,16,17). Elucidating and manipulating the component miscibility and its effects should be of significant importance in protein formulation design.

We examined the miscibility of proteins and nonionic polymers that remain amorphous in frozen aqueous solutions through thermal analysis. An amorphous freeze-concentrated

[1] National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya, Tokyo, 158-8501, Japan.

[2] To whom correspondence should be addressed. (e-mail: izutsu@nihs.go.jp)

ABBREVATIONS: BSA, bovine serum albumin; DEAE-dextran, diethylaminoethyl-dextran; DSC, differential scanning calorimetry; LDH, lactate dehydrogenase; PEG, poly(ethylene glycol); PVP, polyvinylpyrrolidone; T_g', glass transition temperature of maximally freeze-concentrated solute; T_s, softening temperature of freeze-concentrated phase; T_g, glass transition temperature of freeze-dried solid.

0724-8741/00/1000-1316$18.00/0

phase shows a noncontinuity in the thermogram at a temperature referred to as the "glass transition temperature of maximally freeze-concentrated solute (T_g')" or the "softening temperature (T_s)" (23). Since the intrinsic T_g' of many polymers is independent of the initial concentrations, the temperature, shape, and number of the transitions are good indicators of the solute miscibility in multisolute frozen solutions (14,15). Although the proteins do not show an apparent T_g', the transition shape and temperature of the polymer combinations provide important information regarding the polymer miscibility in frozen solutions.

MATERIALS AND METHODS

Bovine serum albumin (essential fatty acid-free), chicken egg albumin (ovalbumin, grade V), PVP (average molecular weight [MW]: 40,000), and Ficoll (average MW: 400,000) were obtained from Sigma Chemical (St. Louis, Missouri). Chicken egg lysozyme (6× crystallized) was purchased from Seikagaku Kogyo (Tokyo, Japan). Salts and other chemicals were obtained from Wako Pure Chemical (Osaka, Japan). The proteins and polymers were dialyzed against distilled water before the experiments.

The thermal analysis of frozen solutions and freeze-dried solids was carried out using a differential scanning calorimeter (DSC 2920, TA Instrument, New Castle, DE). An aliquot of sample solution (approximately 10 μl) in an aluminum cell was cooled at approximately 20°C/min and scanned from –100°C at 5°C/min. The protein and polymer excipient concentrations employed were 5% (w/w) unless otherwise stated. T_g' of the frozen solution was determined from the peaks in the derivative thermograms. The effects of co-solutes were studied with inorganic salts (0–200 mM), buffers (0–100 mM), and sucrose (0–5 %, w/w). The pH of sodium phosphate buffer (pH 7.0) and of Tris–HCl buffer (pH 7.4) was adjusted before mixing with the polymers.

A freeze-drier was used to freeze-dry the polymer solutions (Freezevac 1CF3, Tozai Tsuso, Tokyo). Aliquots of the polymer solutions (180 μl) in polypropylene tubes were frozen by immersion into liquid nitrogen and then freeze-dried at –35°C (24h) and at 35°C (12h). The shelf temperature was raised at 0.5°C/min. Approximately 2 mg of freeze-dried sample in an aluminum cell was initially heated from room temperature to 185°C at 10°C/min for thermal analysis. After being cooled to room temperature at approximately 50°C/min, the sample was scanned again to 220°C at 10°C/min under nitrogen purging. The data obtained during the second scans were used to determine the glass transition temperature (T_g) of the freeze-dried cakes (7,24).

RESULTS

Miscibility of Proteins and Nonionic Polymers in Frozen Solutions

The miscibility of various combinations of proteins (ovalbumin, lysozyme, BSA) and polymer excipients (PVP 40k, Ficoll) in frozen solutions were studied by thermal analysis. Figures 1 and 2 show the derivative thermograms of aqueous frozen solutions containing PVP 40k and ovalbumin (Fig. 1), or various combinations of proteins and nonionic polymers (Fig. 2).

Single-solute nonionic polymer solutions showed glass transition temperatures of maximally freeze-concentrated solute (T_g') peaks in derivative thermograms (Fig. 1). Frozen 10% (w/w) PVP 40k and Ficoll solutions had T_g' at –22.1°C (11) and –21.9°C, respectively. In contrast, the frozen protein solution showed no apparent T_g' in the thermograms. The derivative thermogram tilted to the endothermic side from approximately –20°C and continuously increased. The gradual increase suggested a broad transition within the temperature range. The protein T_g', which was reported to be approximately –10°C, is often obscure due to the inherent heterogeneity and the large subsequent ice-melting endotherm in thermal analysis (25,26). Although single-solute frozen solutions often show a smaller "real" glass transition (T_g) below T_g' (1,25,26), T_g was observed only in the 10% lysozyme solution (–64°C).

Combinations of ovalbumin and PVP 40k showed single T_g' peaks in the derivative thermograms in which the temperature rose with increases in the protein ratio until the transition became unclear. A frozen solution containing 5% PVP 40k and ovalbumin had T_g' at –17.5°C. Single T_g's that shift between the component's individual T_g's indicate their high miscibility in the freeze-concentrate (14).

The miscibility of other combinations of proteins and nonionic polymers have also been studied at various concentration ratios. Some of the derivative thermograms are shown in Fig. 2. Most of the protein and PVP or Ficoll combinations were miscible in frozen solutions, presenting T_g' shifts similar to those of ovalbumin and PVP combination (data not shown). The only exception was the solution containing 2% BSA and 8% PVP 40k. While all the polymer combination solutions employed were miscible at room temperature, the solution (2% BSA, 8% PVP 40k) turned cloudy when cooled to 4°C. The phase separation in the initial solution resulted in a T_g' (–22.0°C) identical to that of PVP 40k. While the polymer combination (BSA and PVP 40k) was miscible in solutions at other concentration ratios, the limited mutual solubility between the polymers appeared as an insufficient homogeneity of freeze-concentrated phases. For example, a 5% BSA and 5% PVP 40k combination showed a broader T_g'

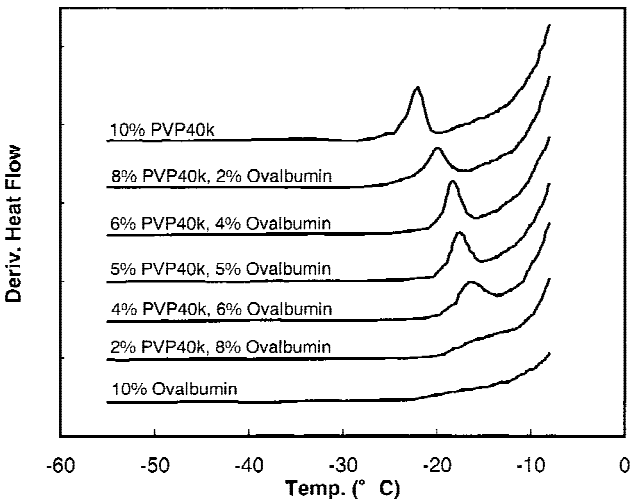

Fig. 1. Derivative thermograms of frozen solutions containing PVP 40k and ovalbumin. Aliquots (10 μl) of frozen polymer solutions were scanned from –100°C at 5°C/min.

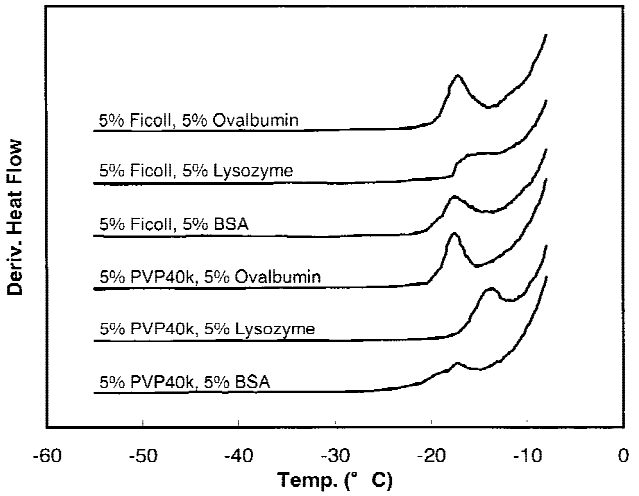

Fig. 2. Derivative thermograms of frozen solutions containing proteins and nonionic polymers. Frozen solutions containing combinations of polymers (5% each) were scanned from –100°C at 5°C/min.

peak in the derivative thermogram than an ovalbumin and PVP combination (Fig. 2). Combinations of lysozyme and nonionic polymers showed a higher T_g' (5% lysozyme and PVP 40k) or "shoulder" (5% lysozyme and Ficoll) in the derivative thermograms. These results suggest that lysozyme makes a larger contribution to the mixture T_g' than ovalbumin or BSA. The high miscibility of proteins and nonionic polymers in frozen solutions is consistent with their tendency to form complexes in media with low ionic strength and at pH values different from the protein's isoelectric point (20,21). The interaction between these proteins, typical polyampholytes and polyelectrolytes, and nonionic polymers should be favorable because of the electrostatic effect (27).

Effect of Co-Solutes on the Protein and Nonionic Polymer Miscibility in Frozen Solutions

The effects of various salts on the T_g's of single polymer (PVP 40k, Ficoll) solutions are shown in Fig. 3. The salts altered the T_g' differently depending on their lyotropy. The addition of NaCl, NaSCN, or Tris-HCl buffer almost linearly lowered the T_g' of the nonionic polymers. The salting-in and "intermediate" salts let the T_g' of the mixed phase down since they have intrinsic T_g' lower than the polymers (11,26). While the polymer T_g's were decreased in response to the low concentrations of Na_2SO_4, the salt had little effect on the T_g' at higher concentrations. The appearance of an exothermic peak at –3°C indicates that Na_2SO_4 is separated from the polymer vicinity and crystallizes in frozen solutions (26)(data not shown). Sodium phosphate buffer (pH 7.4) presented a more complex effect. For example, two T_g' peaks in a derivative thermogram (–26.4 and –22.4°C, at 50 mM buffer) suggest two amorphous phases in a frozen solution (11).

Figure 4 shows derivative thermograms of frozen aqueous solutions containing 5% PVP 40k, 5% ovalbumin, and various concentrations of NaCl. After the addition of 2 mM NaCl, which slightly lowered the polymer combination T_g', the transition peak became unclear at 5 mM NaCl. The T_g' peak then reappeared at approximately –22°C in the presence of 10 mM or 20 mM NaCl. The relationship between the salt concentrations and the polymer combination T_g's (Fig. 5)

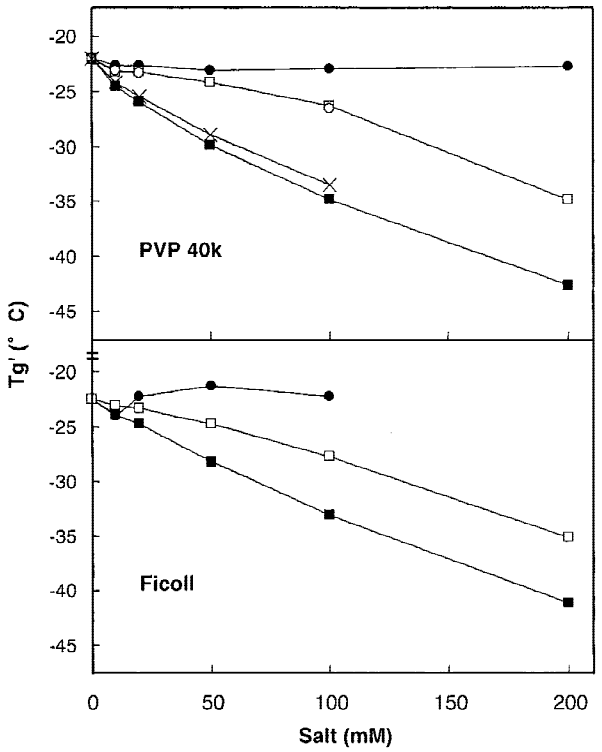

Fig. 3. Effect of salts on the T_g' of PVP 40k and Ficoll. Frozen solutions containing 5% nonionic polymers and various concentrations of salts were scanned from –100°C at 5°C/min. The T_g's were obtained from the peak temperatures of derivative thermograms. The signals in each figure indicate, ■: NaCl, □: NaSCN, ●: Na_2SO_4, ○: sodium phosphate buffer, X: Tris-HCl buffer.

clearly shows a significant T_g' change at 10–20 mM NaCl. The T_g' change of the polymer combination was even larger than that in the PVP 40k alone (Fig. 3). The further addition of NaCl (50–200 mM) gradually lowered the T_g'.

These results strongly suggest that NaCl separates the amorphous ovalbumin and PVP 40k combination into oval-

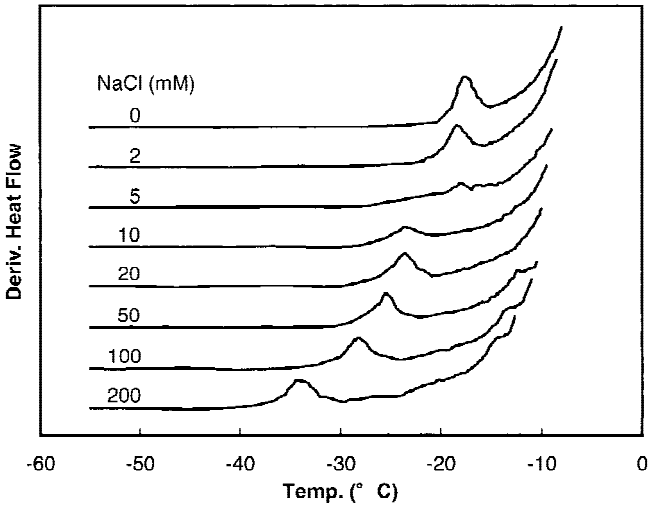

Fig. 4. Derivative thermograms of frozen solutions containing 5% ovalbumin, PVP 40k, and various concentrations of NaCl. Aliquots (10 μl) of frozen polymer solutions were scanned from –100°C at 5°C/min.

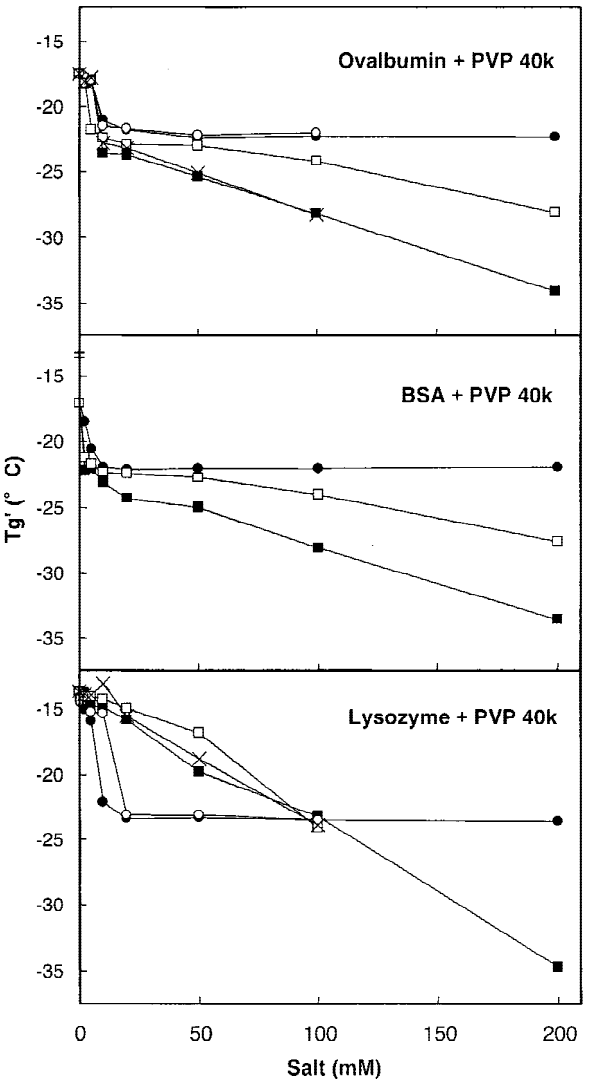

Fig. 5. Effect of salts on the T_g' of protein and PVP 40k (5% each) combinations. The signals in each figure indicate, ■: NaCl, □: NaSCN, ●: Na_2SO_4, ○: sodium phosphate buffer, X: Tris-HCl buffer.

bumin-rich (without apparent T_g') and PVP-rich (T_g' at –22 °C) phases in a frozen solution. The salt-induced sudden T_g' change resembles that of the T_g' splitting previously observed in DEAE–dextran and dextran combinations. The single T_g' of the polyelectrolyte and nonionic polymer combination was split into two transitions at certain salt (e.g., NaCl) concentrations due to the freeze-induced phase separation. It is plausible that separation into ovalbumin-rich and PVP-rich phases in a frozen solution appears as a sudden T_g' change rather than as T_g' splitting, since only the mixed and PVP-rich phases show T_g' in the thermograms. The polymer combinations in a single-phase initial solution may separate due to cooling and/or the freeze-concentration. Some polymer combinations can separate in the cooling process since the polymer interactions depend on temperature (17). Freezing significantly concentrates solutes and causes the phase separation of some polymer combinations (10,12–15). The solutions containing ovalbumin, PVP, and NaCl remained transparent in storage at 4°C, which suggests the contribution of the freeze-concentration in the phase separation. The slower T_g' drop above the phase-separating NaCl concentration indicates that some of the NaCl is distributed to the PVP-dominant amorphous phase in the frozen solutions.

The effects of various co-solutes on the T_g' of protein and nonionic polymer combinations are shown in Figs. 5 and 6. All the salts and buffers employed (NaCl, NaSCN, Na_2SO_4, Tris-HCl buffer, and sodium phosphate buffer) showed similar effects on the T_g' of ovalbumin and PVP 40k combinations at low concentrations (Fig. 5). They significantly lowered the T_g' of the polymer combination at 5–20 mM, indicating the salt-induced phase separation in the frozen solutions. While the further addition of NaCl or NaSCN lowered the T_g', the T_g' stayed at –22°C at high Na_2SO_4 concentrations. The effect of high salt concentrations should vary due to their different miscibilities with the PVP-dominant phase. In contrast to the salting-in or "intermediate" salts (NaSCN, NaCl), the salting-out salt (Na_2SO_4) should be immiscible with the phase-separated PVP (Fig. 3). The T_g' of the BSA and PVP 40k combination was also significantly shifted in response to the low concentrations of salts. The T_g' data for the polymer combination with 200 mM NaSCN wasn't incorporated into the figure since the salt denatured and precipitated the protein at room temperature. The concentration of salts required for the T_g' "jump" was lower in the polymer combination compared to that required with ovalbumin and PVP 40k. All the salts employed (NaCl, NaSCN, Na_2SO_4) significantly lowered the T_g' at 2–10 mM. The effects of other co-solutes on the ovalbumin and PVP 40k miscibility were also studied. Sucrose concentration-dependently lowered the single T_g' of the polymer combination, which is indicative of its high level of miscibility in the freeze-concentrate (data not shown).

In order to clarify whether the phase separation of poly-

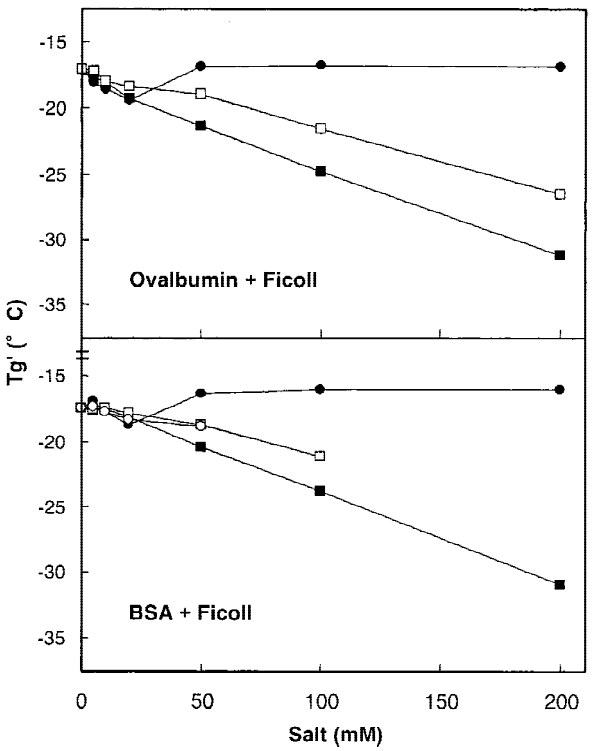

Fig. 6. Effect of salts on the T_g' of protein and Ficoll (5% each) combinations. The signals in each figure indicate, ■: NaCl, □: NaSCN, ●: Na_2SO_4, ○: sodium phosphate buffer.

mers occurs before or after freezing, the miscibility of the polymer combinations was also studied at lower ovalbumin and PVP 40k concentrations. A frozen 1% polymer combination showed a T_g' at −17.2°C, which was slightly (0.3°C) higher than the 5% combination. The sudden T_g' change of the frozen solution was observed in 1–4 mM salts (NaCl, NaSCN, Na_2SO_4), with the salt-to-polymer concentration ratio being similar to that in the 5% polymer combinations (data not shown). The significance of the solute concentration ratio in determining the polymer miscibility strongly suggests the contribution of the freeze-concentration to the phase separation observed in the frozen solutions.

The salts were observed to have one of two primary effects on the T_g' of lysozyme and PVP 40k combinations depending on their position in the lyotropic series. The addition of NaCl, NaSCN, or Tris-HCl almost linearly lowered the T_g' of the system, indicating that the miscible freeze-concentrated phase contained the polymers and the salts. On the other hand, the rapid T_g' change at low concentrations of Na_2SO_4 (10 mM) or sodium phosphate buffer (20 mM) was indicative of the phase separation of the polymers. The different effects between sodium phosphate buffer and Tris-HCl buffer suggest that the choice of buffer salts is an important factor in determining the solute miscibility in some protein formulations.

Figure 6 shows the effects of salts on the T_g' of frozen solutions containing 5% Ficoll and proteins (ovalbumin, BSA). Ficoll was more miscible with the proteins than PVP 40k. The addition of NaCl or NaSCN linearly lowered the T_g' in both of the polymer combinations, indicating a mixed amorphous phase in the frozen solutions. A high concentration (200 mM) of NaSCN denatured lysozyme and precipitated as a white solid before freezing. The T_g's of Ficoll and the protein combinations stayed at −16 to −17°C at Na_2SO_4 concentrations above 50 mM, suggesting that the polymers remain miscible while excluding the salting-out salt from their vicinity.

The miscibility of proteins and nonionic polymers varied depending on the polymer combinations and salts in the solution. The added salts may induce a phase separation of proteins and nonionic polymers by concealing the electrostatic effects between polymers and by modifying their hydration states (20,21). The electrostatic effect contributes largely to the delicate balance of interactions that determines protein and nonionic polymer miscibility (20,21). Salts should suppress the effects favorable to protein–nonionic polymer interactions and enhancing the self-association of proteins. It makes various combinations of proteins and nonionic polymers separate to form an aqueous two-layer system at above certain salt concentrations. The phase separation of ovalbumin and PVP in a frozen solution indicates that the interaction is thermodynamically unfavorable without the electrostatic effect. The salting-in and salting-out salt should increase and reduce the polymer miscibility, respectively, by changing the polymer hydration states (14,15,17). The altered hydration state may explain why the freezing separates the combination of lysozyme and PVP 40k only in the presence of Na_2SO_4 or sodium phosphate buffer. In the present study, Ficoll showed better miscibility with proteins than with PVP 40k. The presence of protein, Ficoll, and salts in the mixed freeze-concentrated amorphous phase suggests their favorable interactions even in the altered hydration state or without the electrostatic effect. PVP is often used to precipitate proteins by phase separation (20–22,28).

Miscibility of PVP and Proteins in Freeze-Dried Solids

The miscibility of proteins and PVP 40k in freeze-dried solids was studied to elucidate the drying effects on polymer miscibility. Figure 7 shows the thermograms of ovalbumin, PVP 40k, and their combinations freeze-dried with or without NaCl. The physical properties of a single-component amorphous freeze-dried solid change between the glass and rubber states at the glass transition temperature (T_g) (1). The triangles in the figure indicate the T_gs obtained from the peaks in derivative thermograms. The number and temperature of T_g are good indicators of the component miscibility in freeze-dried solids, just as with T_g' in frozen solutions (29).

The thermogram of freeze-dried PVP 40k showed a broad T_g at approximately 171°C (24,29) and a subsequent thermogram inclination change above that temperature. Freeze-dried ovalbumin presented a smooth thermogram between 150 and 200°C, with a broad glass transition at 200–210°C. The absence of T_g for PVP in a freeze-dried polymer combination (ovalbumin and PVP 40k) suggests the good miscibility of the two components. On the other hand, discontinuity in the thermogram (170°C) and a following inclination change appeared in freeze-dried solids containing the polymers and 10–50 mM NaCl. The transitions were small but reproducible in repeated experiments. The transition at PVP 40k's intrinsic T_g indicates a fluctuation of local composition in these freeze-dried solids due to a phase separation between

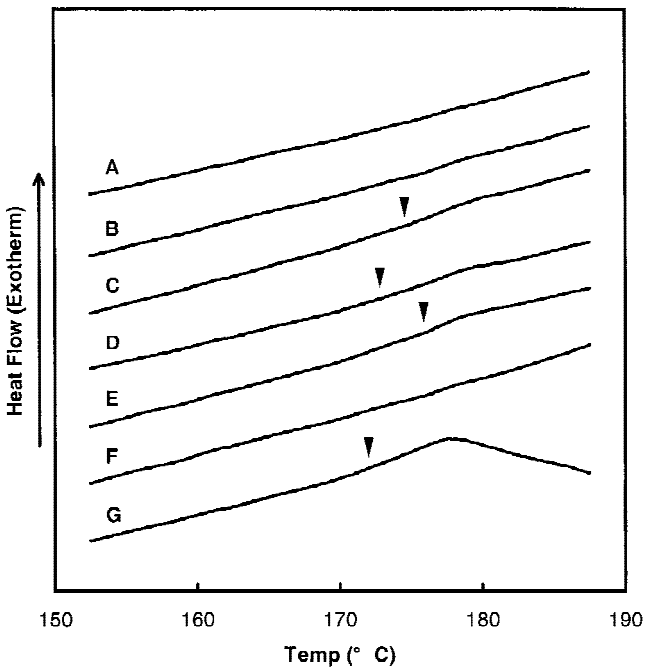

Fig. 7. Effect of sodium chloride on the glass transition of freeze-dried ovalbumin and PVP 40k. Approximately 2 mg of freeze-dried sample was scanned at 10°C/min after initial scanning up to 185°C. The thermograms A to E represent freeze-dried solids from solutions containing 5% ovalbumin, 5% PVP 40k, and (A) 0 mM, (B) 5 mM, (C) 10 mM, (D) 20 mM, and (E) 50 mM NaCl. Thermograms F and G were from (F) 5% ovalbumin and (G) 5% PVP 40k. The glass transition temperatures (T_g) obtained from the derivative thermograms are marked with triangles (▼).

PVP 40k and ovalbumin. The polymer miscibility observable in Figs. 4 and 7 indicates that the salt-induced phase separation of ovalbumin and PVP 40k in frozen solutions results in phase-separated heterogeneous freeze-dried solids. The miscibility of PVP 40k and other proteins (lysozyme, BSA) in the freeze-dried solids also followed those in frozen solutions (data not shown).

DISCUSSION

This study has shown how some combinations of proteins and nonionic polymers separate into different amorphous phases in frozen solutions. As has been observed in other polymer combinations (14,15), the miscibility between proteins and nonionic polymers in frozen solutions should be determined by molecular interactions that depend on their structure, size, and surface properties. The unfavorable polymer interactions and freeze-concentration separate proteins and nonionic polymers in frozen solutions. Salts should alter the molecular interactions between polymers by covering the electrostatic effect and/or changing their hydration state (17,20,21). Concentrations of not only polymers but also of the salts needed for the freeze-induced phase separation are much lower than those for aqueous two-layer formation, as freezing equally concentrates all solutes. The miscibility of proteins and nonionic polymers in amorphous freeze-concentrate is practically important in freeze-drying protein formulations, as it determines various physical and chemical properties of the multicomponent pharmaceutical dosage forms (8). The freeze-induced phase separation may also change the composition of the low-molecular weight molecules surrounding the proteins since they often distribute differently into separated phases (17).

Many of the "model" solutes employed in the present study are popular or potential excipients for protein pharmaceuticals. The freeze-induced phase separation between protein and polymer excipients should directly or indirectly alter the protein stability in frozen solution and during freeze-drying. The phase-separating polymer excipients may stabilize or destabilize proteins in various ways. Two opposing hypotheses have been formed regarding their effects in frozen solutions. Phase-separating polymer excipients may protect the multimeric proteins (e.g., lactate dehydrogenase [LDH]) by stabilizing the subunit interactions (19). The strong repulsive interaction between the protein and polymer co-solutes, which cause phase-separation in frozen solution, shift the equilibrium between the subunit association (i.e., tetramers, dimers, and monomers) towards the more stable fully polymerized form (18). In contrast, the freeze-induced phase separation can inactivate proteins at the interface between phases and/or eliminate the stabilizing effects of excipients in the freeze-drying process. Proteins exposed to the interface can face additional stress to their structure in frozen solutions (10,12,13). Separation between polymers and a possible uneven distribution of low-molecular weight co-solutes should prevent the molecular interaction required to stabilize proteins against the drying stress (3,4,6,17,30).

The freeze-induced phase separation may also occur in various systems containing combinations of proteins or a protein and other biological macromolecules. Some protein/protein combinations are immiscible at high concentrations, depending on the solution conditions (e.g., pH, ionic strength, temperature) (20). Molecular interactions between biologically active and inert proteins in some freeze-dried formulations can vary in terms of their possible phase separation in frozen solutions. In addition, the freeze-induced phase separation between polymers in biological systems can be one of the reasons for various kinds of damage in freezing cells and organisms.

In conclusion, freeze-concentration separates some combinations of proteins and nonionic polymers into different amorphous phases in frozen solutions. The miscibility of proteins and nonionic polymers in frozen solutions varies depending on their combination and the composition of the co-solutes. The inherent importance of polymer miscibility in protein stability and formulation quality requires a more systematic study of related phenomena and rational formulation design.

REFERENCES

1. F. Franks. Freeze-drying of bioproducts: Putting principles into practice. *Eur. J. Pharm. Biopharm.* **45**:221–229 (1998).
2. S. N. Timasheff. Solvent stabilization of protein structure. *Methods. Mol. Biol.* **40**:253–269 (1995).
3. J. F. Carpenter, S. J. Prestrelski, T. J. Anchordoguy, and T. Arakawa. Interactions of stabilizers with proteins during freezing and drying. *ACS Symp. Ser.* **567**:134–147 (1994).
4. J. F. Carpenter and J. H. Crowe. An infrared spectroscopic study of interactions of carbohydrates with dried proteins. *Biochemistry* **28**:3916–3922 (1989).
5. K. Tanaka, T. Takeda, and K. Miyajima. Cryoprotection effect of saccharides on denaturation of catalase by freeze-drying. *Chem. Pham. Bull.* **39**:1091–1094 (1991).
6. J. M. Sarciaux, S. Mansour, M. J. Hageman, and S. L. Nail. Effects of buffer composition and processing conditions on aggregation of bovine IgG during freeze-drying. *J. Pharm. Sci.* **88**: 1354–1361 (1999).
7. L. S. Taylor and G. Zografi. Sugar-polymer hydrogen bond interactions in lyophilized amorphous mixtures. *J. Pharm. Sci.* **87**: 1615–1621 (1998).
8. B. C. Hancock and G. Zografi. Characteristics and significance of the amorphous state in pharmaceutical systems. *J. Pharm. Sci.* **86**: 1–12 (1997).
9. K. Izutsu, S. Yoshioka, S. Kojima, T. W. Randolph, and J. F. Carpenter. Effects of sugars and polymers on crystallization of poly(ethylene glycol) in frozen solutions: Phase separation between incompatible polymers. *Pharm. Res.* **13**:1393–1400 (1996).
10. T. W. Randolph. Phase separation of excipients during lyophilization: Effects on protein stability. *J. Pharm. Sci.* **86**:1198–1203 (1997).
11. L. M. Her, M. Deras, and S. L. Nail. Electrolyte-induced changes in glass transition temperatures of freeze-concentrated solutes. *Pharm. Res.* **12**:768–772 (1995).
12. M. C. Heller, J. F. Carpenter, and T. W. Randolph. Effects of phase separating systems on lyophilized hemoglobin. *J. Pharm. Sci.* **85**:1358–1362 (1996).
13. M. C. Heller, J. F. Carpenter, and T. W. Randolph. Protein formulation and lyophilization cycle design: Prevention of damage due to freeze-concentration induced phase separation. *Biotechnol. Bioeng.* **63**:166–174 (1999).
14. K. Izutsu, M. C. Heller, T. W. Randolph, and J. F. Carpenter. Effect of salts and sugars on phase separation of polyvinylpyrrolidone-dextran solutions induced by freeze-concentration. *J. Chem. Soc., Faraday Trans.* **94**:411–417 (1998).
15. K. Izutsu and S. Kojima. Phase separation of polyelectrolytes and non-ionic polymers in frozen solutions. *Phys. Chem. Chem. Phys.***2**:123–127 (2000).
16. P.-A. Albertsson. *Partition of cell particles and macromolecules,* Wiley, New York, 1986.
17. B. Y. Zaslavsky. *Aqueous two-phase partitioning,* Marcel Dekker, New York, 1995.
18. T. J. Anchordoquy and J. F. Carpenter. Polymers protect lactate

dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state. *Arch. Biochem. Biophys.* **332**:231–238 (1996).
19. M. W. Townsend and P. P. DeLuca. Use of lyoprotectants in the freeze-drying of a model protein, ribonuclease A. *J. Parenter. Sci. Technol.* **42**:190–199 (1988).
20. V. B. Tolstoguzov. Concentration and purification of proteins by means of two-phase systems: Membraneless osmosis process. *Food Hydrocolloids* **2**:195–207 (1988).
21. V. Tolstoguzov. Compositions and phase diagrams for aqueous systems based on proteins and polysaccharides. *Int. Rev. Cytol.* **192**:3–31 (2000).
22. A. Polson, G. M. Potgieter, J. F. Largier, G. E. F. Mears, and F. J. Joubert. The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochim. Biophys. Acta* **82**: 463–475 (1964).
23. E. Y. Shalaev and F. Franks. Structural glass transition and thermophysical processes in amorphous carbohydrates and their supersaturated solutions. *J. Chem. Soc., Faraday Trans.* **91**:1511–1517 (1995).
24. D. T. Turner and A. Schwartz. The glass transition temperature of poly(N-vinylpyrrolidone) by differential scanning calorimetry. *Polymer* **26**:757–762 (1985).
25. A. P. MacKenzie. The physico-chemical basis for the freeze-drying process. *Dev. Biol. Stand.* **36**:51–57 (1977).
26. B. S. Chang and C. Randall. Use of thermal analysis to optimize protein lyophilization. *Cryobiology* **29**:632–656 (1992).
27. C. Vinches, A. Parker, and W. F. Reed. Phase behavior of aqueous gelatin/oligosaccharide mixtures. *Biopolymer* **41**:607–622 (1997).
28. W. R. Gombotz, S. C. Pankey, D. Phan, R. Drager, K. Donaldson, K. P. Antonsen, A. S. Hoffman, and H.V. Raff. The stabilization of a human IgM monoclonal antibody with poly(vinylpyrrolidone). *Pharm. Res.* **11**:624–632 (1994).
29. S. L. Shamblin, L. S. Taylor, and G. Zografi. Mixing behavior of colyophilized binary systems. *J. Pharm. Sci.* **87**:694–701 (1998).
30. L. Kreilgaard, S. Frokjaer, J. M. Flink, T. W. Randolph, and J. F. Carpenter. Effects of additives on the stability of Humicola lanuginosa lipase during freeze-drying and storage in the dried solid. *J. Pharm. Sci.* **88**:281–290 (1999).