Freeze-Drying of Proteins From a Sucrose-Glycine Excipient System: Effect of Formulation Composition on the Initial Recovery of Protein Activity

Wei Liu,[1,3] D.Q. Wang,[2] and Steven L. Nail[1,4]

[1]Department of Industrial and Physical Pharmacy, Purdue University, 1336 Robert Heine Building, West Lafayette, IN
[2]Pharmaceutical Development, Bayer Corporation, 800 Dwight Ave, Berkeley, CA
[3]GlaxoSmithKline, 709 Swedeland Rd, King of Prussia, PA
[4]Current Address: Lilly Corporate Center, Indianapolis, IN

ABSTRACT

The purpose of this study was to investigate the effect of sucrose-glycine excipient systems on the stability of selected model proteins during lyophilization. Recovery of protein activity after freeze-drying was examined for the model proteins lactate dehydrogenase and glucose 6-phosphate dehydrogenase in a sucrose-glycine-based excipient system in which the formulation composition was systematically varied. In a sucrose-only excipient system, activity recovery of both model proteins is about 80% and is independent of sucrose concentration over a range from 1 to 40 mg/mL. When both sucrose and glycine are used and the ratio of the 2 excipients is varied, however, activity recovery decreases in a pattern that is consistent with the inhibition of activity recovery by glycine crystals, despite the presence of an adequate amount of sucrose to afford protection. Annealing of sucrose-glycine formulations causes a small but significant decrease in activity recovery relative to unannealed controls, whereas no annealing effect is observed with sucrose-only formulations. Addition of 0.01% polysorbate 80 to the formulation resulted in complete recovery of activity, irrespective of the sucrose-glycine ratio or annealing. Addition of the same concentration of polysorbate 80 to the reconstitution medium caused an increase in activity recovery for each formulation, but the overall pattern remained unchanged. The data are consistent with an interfacial model for lyophilization-associated loss of protein activity involving denaturation at a solid/freeze-concentrate interface.

KEYWORDS: interfacial denaturation, protein formulation, X-ray powder diffraction, crystallization, annealing, lyophilization

INTRODUCTION

Nearly all freeze-dried pharmaceutical proteins are formulated in an excipient system because the excipient system is required for stabilizing the protein against the stresses of freezing and freeze-drying or because a bulking agent is needed to provide a suitable matrix into which a small quantity of protein is dispersed. The process of selecting a suitable formulation, which, in addition to the protein, may consist of a buffer, a bulking agent, one or more stabilizers, a surfactant, and perhaps sodium chloride or another inorganic salt for tonicity adjustment, has traditionally been developed through trial and error. In the last few years, however, there have been significant advances in understanding the physical chemistry of freeze-drying protein systems, and broad formulation guidelines are emerging as a result of this research.[1] Rational design of a freeze-dried protein formulation should consider including an amorphous protective agent, a crystalline bulking agent to improve the freeze-drying characteristics and the physical stability of the cake, and a surfactant to provide further stabilization and inhibition of protein loss by adsorption to surfaces. The addition of salts to the formulation should be justified, and is generally based on solubility[2] or stability considerations. In particular, combinations of sucrose (an amorphous protectant) and glycine (a crystallizing excipient) have been shown to provide a relatively robust excipient system.[3,4]

Although these formulation guidelines are useful, there are many unanswered questions concerning the role of formulation composition in both the short-term (immediately after freeze-drying) and long-term (during storage) stability of freeze-dried proteins. The purpose of this study is to examine the role of the composition of a sucrose-glycine-based excipient system in the short-term stability of 2 model proteins, lactate dehydrogenase and glucose-6-phosphate dehydrogenase.

MATERIALS AND METHODS

Materials

Lactate dehydrogenase (LDH) (type II, from rabbit muscle) crystalline suspension in ammonium sulfate solution and glucose-6-phosphate dehydrogenase (G-6-PDH) (both from Sigma Chemical Co, St. Louis, MO) were dialyzed (approximately 10 000 MW cutoff) at 4°C for 24 hours against a sodium phosphate solution (0.05 mol/L, pH 7.4). The protein concentration was determined using a kit

Corresponding Author: Steven L. Nail, Lilly Corporate Center, Indianapolis, IN 46285; Tel: (317) 655-2829; Fax: (317) 277-0833; E-mail: nailsl@lilly.com

purchased from Sigma Chemical Co. based on the Lowry method. Bovine serum albumin, included in the kit, was used as a standard.

Formulations used in this study usually contained 25 μg/mL of protein, except for a preliminary study in which the protein concentration was systematically varied. Excipients included 30 mmol/L NaCl and combinations of sucrose and glycine with and without polysorbate 80. Sodium chloride, sodium hydroxide, sodium phosphate monobasic and dibasic, sucrose, and glycine (all analytical grade) were purchased from Mallinckrodt Baker Chemical, Inc (Paris, KY). Polysorbate 80 was used as received from Sigma Chemical Co. The concentration of phosphate in the final formulation was less than 1 mmol/L.

Freeze-Drying

One milliliter of formulation solution containing dialyzed LDH or G-6-PDH was filled into 5-mL serum vials. A preliminary study was conducted using LDH in which samples were analyzed after freezing and thawing to distinguish between freezing-induced and freeze-drying-induced loss of activity. Freeze-drying experiments were conducted using a laboratory freeze-dryer (Dura Stop-Dura Dry; FTS Systems, Inc, Stone Ridge, NY). Pressure was monitored and controlled using a capacitance manometer. Type T thermocouples (30-gauge; FTS Systems, Stone Ridge, NY) were placed in 3 vials with the thermocouple tip touching the bottom center of the vial. Samples were loaded onto the shelves at room temperature. The shelf temperature was ramped to −42°C and held for 6 hours. Annealing, where used, was conducted by ramping the shelf temperature to −20°C at a rate of 0.5°C/min and holding it for 6 hours after the initial freeze. When all thermocouple readings were below −40°C, primary drying was initiated by first evacuating to 0.07 mm Hg followed by increasing the shelf temperature to −35°C at 0.5°C/min. Primary drying was conducted at −35°C for 24 hours. The shelf temperature was then increased to 25°C at 0.5°C/min and held at 25°C for 8 hours. Chamber pressure was controlled at 0.07 mm Hg throughout the drying cycle.

Enzyme Activity Assay

LDH activity was measured using a kit purchased from Sigma Chemical Co. The assay is based on the interconversion of lactate and pyruvate. During the reduction of pyruvate, an equimolar amount of NADH is oxidized to NAD. The oxidation of NADH results in a decrease in absorbance at 340 nm. The rate of decrease of absorbance at 340 nm is directly proportional to LDH activity in the sample, where one unit of LDH activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole/L of NAD per minute under the conditions of the assay.

The activity of G-6-PDH, also measured using a kit purchased from Sigma Chemical, is based on NADP reduction to NADPH by G-6-PDH in the presence of G-6-P, which results in an increase in absorbance at 340 nm. The rate of increase of absorbance at 340 nm is directly proportional to the activity of G-6-PDH in the sample. One unit of G-6-PDH activity is defined as the amount of enzyme that converts 1 μmol/L NADP per minute under conditions of the assay.

After lyophilization, 3 freeze-dried samples of each formulation were reconstituted with 1 mL of distilled, reverse osmosis water, and the enzyme activity was measured for each sample. Samples were taken from the center of the array of vials. The activity recovery of the freeze-dried samples was expressed as a percentage of the activity before freeze-drying and is the mean of the activity recovery of 3 samples plus or minus the standard deviation.

Residual Moisture Measurement

The moisture content of the freeze-dried products was determined by Karl Fisher titration (model 150; Fisher Scientific, Pittsburgh, PA). The freeze-dried material was quickly transferred to the titration vessel containing anhydrous reagents. The end-point persistence time and the extraction time were 15 seconds and less than 3 minutes, respectively.

X-ray Powder Diffraction

X-ray powder diffraction patterns of freeze-dried powders were determined using a Siemens D-500 Kristalloflex diffractometer (Madison, WI). CuKα radiation (λ = 0.15406 nm) was used with a current of 20 mA and a voltage of 40 kV. A Kevex Psi Peltier cooled silicon (SiLi) detector was used. Slits I, II, III, and IV were at 1°, 1°, 1°, and 0.15°, respectively. Alignment of the equipment was conducted before each measurement using a silicon reflection peak at 28.466° (2 θ). The powders were gently broken up and mounted on a low background aluminum holder. Samples were scanned from 2° to 40° (2 θ) at a rate of 6°/min, 0.05° step/sec.

RESULTS

Freeze-drying of both model proteins in the excipient systems examined here resulted in pharmaceutically acceptable solid cakes with residual moisture levels of less than 1.5%. The purpose of measuring residual moisture was to verify that differences in stability between different formulations did not arise from differences in the residual moisture level. There was no significant difference in residual moisture levels between different formulations.
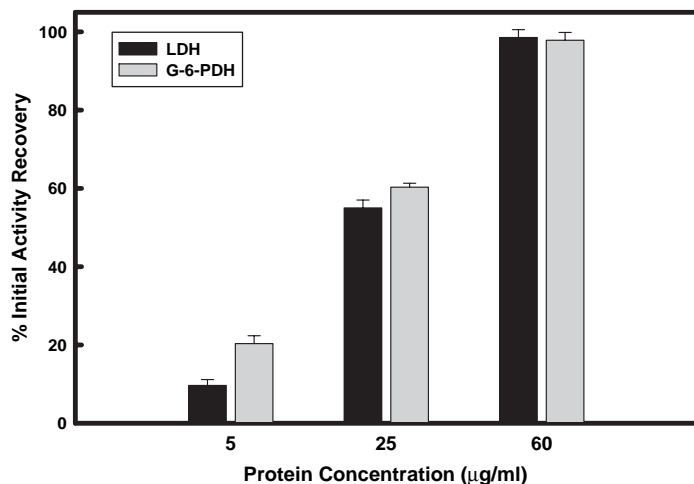
Figure 1. Activity recovery of freeze-dried LDH and G-6-PDH as a function of protein concentration in an excipient system consisting of 10 mg/mL sucrose, 21.7 mg/mL glycine, and 30 mmol/L NaCl.

Freeze-drying of both LDH and G-6-PDH at a concentration of 25 μg/mL in the absence of any stabilizing solute results in a complete loss of enzymatic activity (data not shown). Figure 1 shows the effect of protein concentration on activity recovery in an excipient system consisting of 10 mg/mL of sucrose (0.029 mol/L), 21.7 mg/mL of glycine (0.29 mol/L), and 30 mmol/L NaCl. Clearly, recovery of activity is very concentration-dependent. This seems to be a common observation in the freezing and freeze-drying of proteins, although we are not aware of any detailed research studying this aspect of freezing and freeze-drying of proteins. Chang et al[5] reported that increasing the concentration of interleukin-1 receptor antagonist (from 1 to 100 mg/mL) diminished the level of aggregates from 50% to 0.01%. This effect has also been reported for β-galactosidase,[6] catalase,[6] LDH,[6] and bovine IgG.[7]

The influence of formulation composition on initial recovery of activity for both LDH and G-6-PDH is shown in Figure 2. The general pattern is the same for both proteins. Not surprisingly, glycine alone has a minimal protective effect because glycine tends to crystallize during freezing. The low level of activity observed in this system is probably attributable to a small fraction of glycine that remains amorphous during freeze-drying. Sucrose at a concentration of 10 mg/mL (0.029 mol/L) consistently results in a recovery of about 80% of the activity before freeze-drying. Addition of sodium chloride causes a small but significant decrease in activity recovery relative to sucrose alone. The effect of sodium chloride on recovery of activity was not investigated further in this study. Possible mechanisms include the effect of sodium chloride on the degree of super-cooling (with subsequent effect on the freezing rate), Hofmeister series effects, and specific ion effects.[7,8]

An unexpected result illustrated in Figure 2 is that the addition of 0.29 mol/L glycine to the sucrose-sodium chloride system caused a small but significant additional decrease in activity recovery. Subsequent experiments were focused on a better understanding of this effect.

The influence of systematic variation of the ratio of sucrose to glycine in a formulation containing 30 mmol/L sodium chloride on activity recovery of LDH and G-6-PDH is shown in Figure 3. The ratio (wt/wt) of the 2 solutes is shown on the *x*-axis. The total concentration of both solutes is 20 mg/mL. At glycine to sucrose ratios of up to about 30:70, the recovery of activity is roughly independent of composition and not significantly different from that of sucrose alone. As the relative amount of glycine increases, however, the initial recovery of enzyme activity decreases continuously. Annealing the system at −20°C results in a further decrease in activity recovery of 5% to about 10% for all sucrose-glycine combinations (Figure 3). There is a measurable annealing effect even in the range in which no effect of weight ratio of excipients is observed when annealing is not done.

The effect of increasing the relative concentration of glycine on activity recovery is largely a freeze-drying effect rather than an effect observed following freeze-thaw. In Figure 4, the recovery of activity of LDH after freeze-thawing is shown as a function of the sucrose-glycine ratio. There is a small but significant decrease in activity recovery as the relative amount of glycine increases above 50%; however, all recoveries are greater than 80%. The magnitude of the freeze-thaw effect is small compared with the magnitude of the effect observed after freeze-drying.

One possible explanation for the results summarized in Figure 3 is that, by keeping the total concentration of solute constant, there is not a high enough molar ratio of sucrose
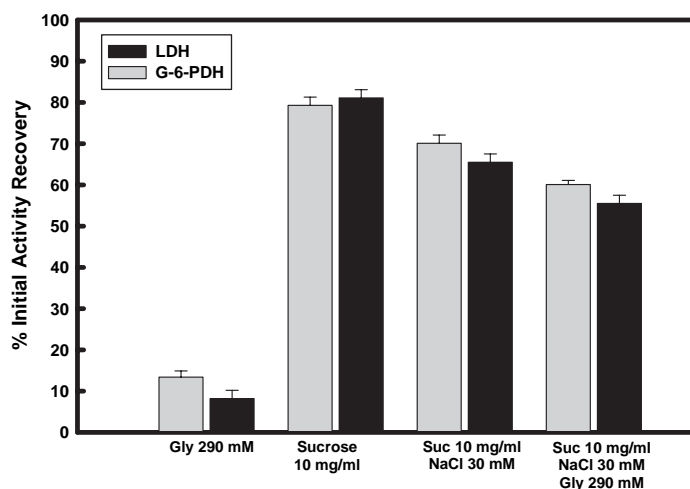

Figure 2. Activity recovery of LDH and G-6-PDH as the formulation is systematically varied. Gly, glycine; Suc, sucrose.
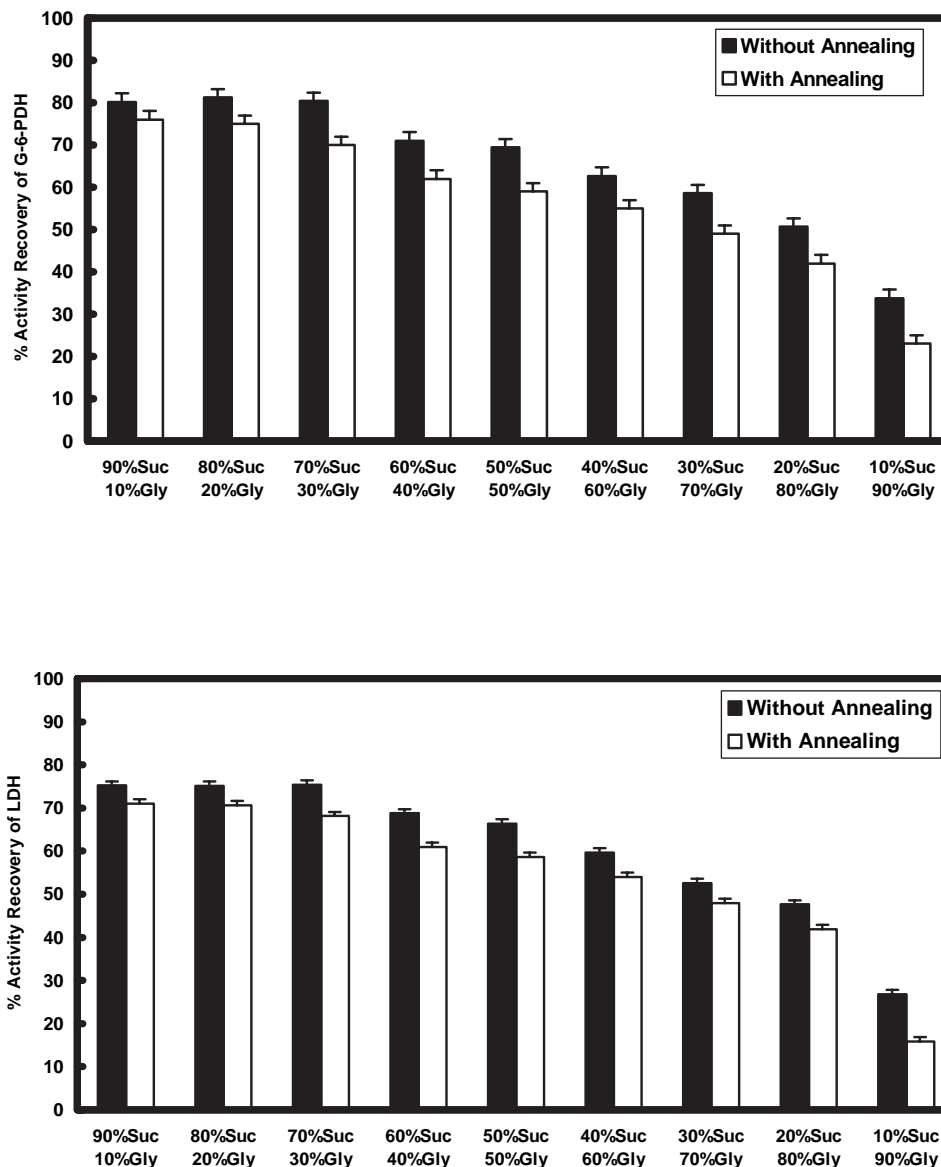
Figure 3. Activity recovery of G-6-PDH (top panel) and LDH (bottom panel) as a function of the sucrose (Suc)-glycine (Gly) ratio for both unannealed and annealed material.

to protein to provide maximum protective effect. As shown in Figure 5, however, the activity recovery for both proteins is independent of sucrose concentration over a range of 1 to 40 mg/mL sucrose. The reason that recovery of activity does not exceed about 80% for either protein in the presence of sucrose is not known at present. The loss of protein by adsorption to surfaces was examined by measuring absorbance at 280 nm; no significant adsorptive loss was observed (data not shown). The main conclusion supported by Figure 5, however, is that depletion of sucrose does not explain the results shown in Figure 3. Even at a 10:90 weight ratio of sucrose to glycine, there is 2 mg/mL sucrose present, which is in the concentration range covered by the data in Figure 5. Furthermore, there is no significant effect of annealing of sucrose-only formulations. This is in sharp contrast to the annealing effect observed in sucrose-glycine formulations.

The X-ray diffraction patterns for the formulations represented in Figure 3 are shown in Figure 6. The X-ray diffraction patterns of freeze-dried solids containing 10:90, 20:80, and 30:70 weight ratios of glycine to sucrose are amorphous as measured by X-ray diffraction. As the relative concentration of glycine increases, however, increasing levels of crystallinity are observed in the freeze-dried solid. The peak positions are consistent with the β polymorph of glycine. These results are consistent with studies of water-glycine-sucrose systems at temperatures below 0°C using differential thermal analysis and X-ray diffraction methods reported by Shalaev et al.[9,10] These investigators reported that only β-glycine crystal formation was detected in a sucrose-glycine system. Crystallization of glycine is hindered for compositions with an elevated content of sucrose, and annealing at about −25°C promotes the crystallization of remaining amorphous glycine.
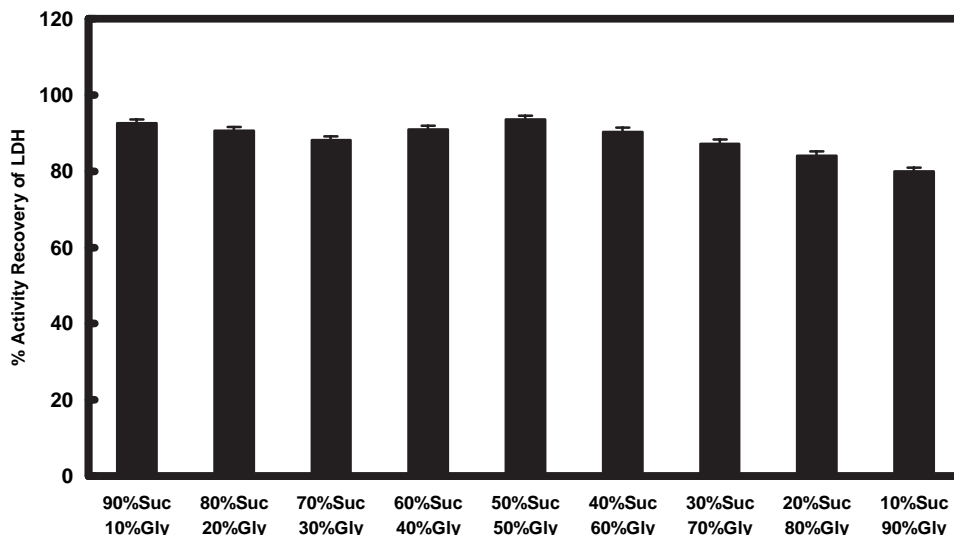
Figure 4. Activity recovery of LDH as a function of the sucrose (Suc)-glycine (Gly) ratio following freeze-thawing.

Figure 7 shows the effect of adding a surfactant, polysorbate 80, to the formulation at a concentration of 0.1 mg/mL (0.01% wt/vol). When a surfactant is used, quantitative recovery of activity is observed, irrespective of the ratio of sucrose to glycine and annealing. There was no observable effect of the surfactant on the pattern of crystallization of glycine (data not shown). To distinguish between the protective effect of polysorbate 80 in the formulation before freeze-drying versus the surfactant in the water used to reconstitute the lyophilized solids, a separate experiment was conducted with LDH, which was similar to the experiment summarized in Figure 3 except that 0.1 mg/mL polysorbate 80 was added to the water used to reconstitute the freeze-dried solids. The results are summarized in Figure 8. Note that there is a significant increase in recovery of activity for all combinations of sucrose and glycine; recovery is nearly quantitative at glycine to sucrose ratios less than about 30:70. Nevertheless, the same general pattern as that shown in Figure 3 is observed; that is, the higher the ratio of sucrose to glycine, the lower the recovery of activity. This supports the idea that the surfactant works best when included in the formulation.

DISCUSSION

There is a small but significant body of literature supporting the idea that loss of protein integrity during freezing and freeze-drying is, at least in part, an interfacial phenomenon involving partial denaturation of protein at the ice/freeze-concentrate interface. Strambini and Gabellieri[11] studied phosphorescence lifetimes of tryptophan residues in several proteins and demonstrated that freezing of
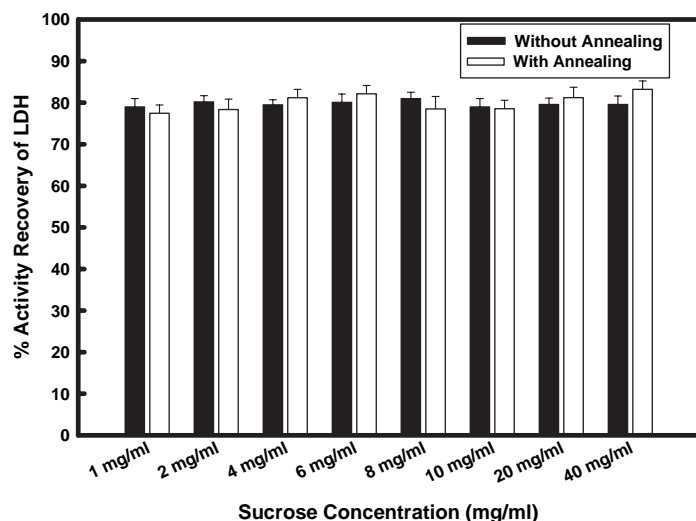

Figure 5. Activity recovery of LDH as a function of the sucrose concentration for both unannealed and annealed systems.
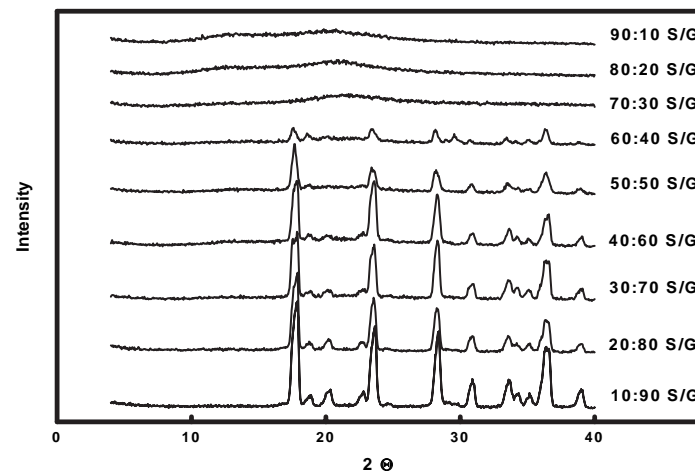

Figure 6. X-ray powder diffractograms of formulations containing a range of weight ratios of sucrose to glycine (S/G).
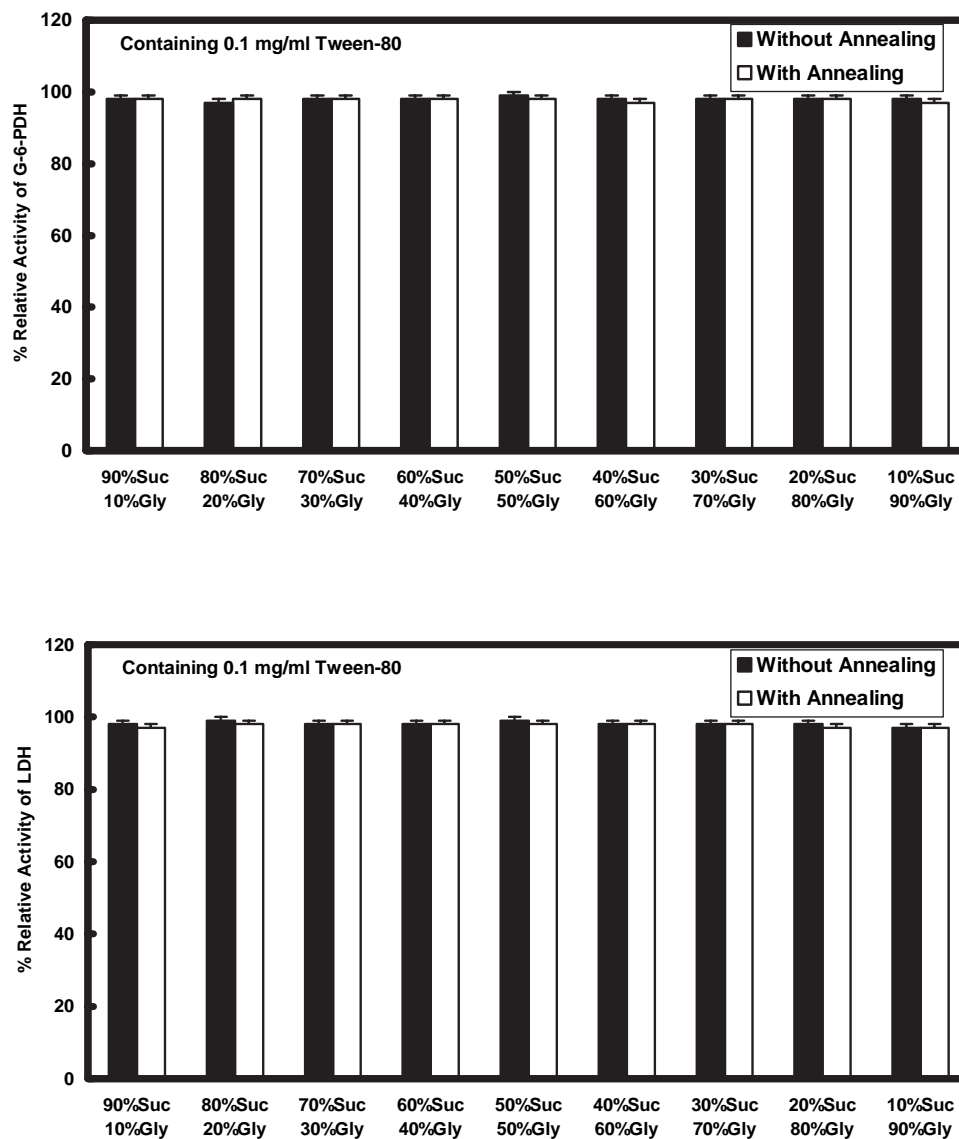
Figure 7. Activity recovery of G-6-PDH (top panel) and LDH (bottom panel) for varying sucrose (Suc)-glycine (Gly) ratios with 0.1 mg/mL Tween 80 for both unannealed and annealed material.

aqueous solutions of these proteins is accompanied by loosening of the native fold and considerable loss of secondary and tertiary structure. The phenomenon is largely reversible after thawing, although in some cases a fraction of the protein recovers neither the original phosphorescence properties nor the original activity. Cryoprotectants, such as sucrose and glycerol, were found to reduce or eliminate the perturbation caused by freezing. These investigators did not study freeze-drying effects.

If protein activity loss is strongly affected by interfacial adsorption at the ice/freeze-concentrate interface, then the extent of activity loss should be a function of the freezing rate in which rapid freezing generates smaller ice crystals and a higher interfacial area during freezing. Eckhardt et al[12] studied the effect of freezing rate (from 0.5°C/min to about 50°C/min) on the formation of soluble and insoluble aggregates of human growth hormone using combina-

tions of phosphate buffer and mannitol as an excipient system in a pH range of 7.4 to 7.8. The effect of freezing rate on formation of insoluble aggregates (measured turbidimetrically) was dramatic, with considerably more aggregation at higher freezing rates. The effect of freeze-drying on aggregation was not studied.

Chang et al[13] reported that there is a strong correlation between the tendency of a protein to denature at surfaces and its tendency to denature during freezing, and their data support the conclusion that the ice/freeze-concentrate interface can play a significant role in protein denaturation during freezing. Slow freezing (3°C/h) resulted in markedly less turbidity after freezing than dipping vials in liquid nitrogen (quench freezing), presumably because the relative area of the ice-liquid interface is smaller after slow freezing. Furthermore, addition of 0.01% polysorbate 80 resulted in essentially complete protection of all the
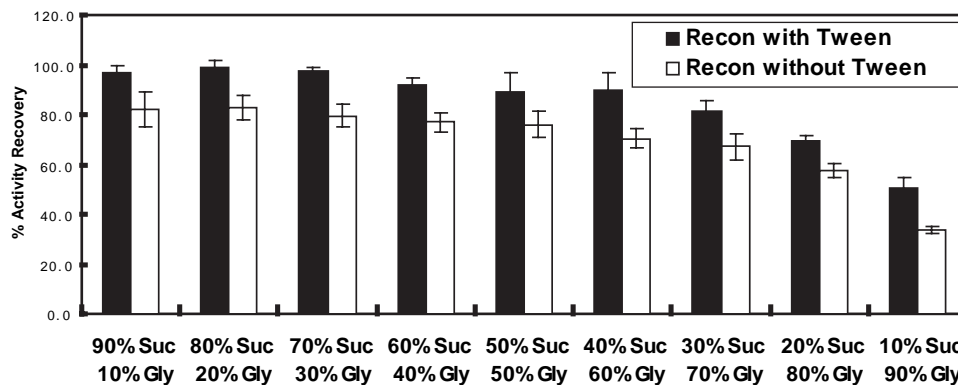
Figure 8. Activity recovery of LDH as a function of the sucrose (Suc)-glycine (Gly) ratio following reconstitution with and without 0.1 mg/mL Tween 80 in the reconstitution medium.

model proteins after freeze-thawing, presumably because surfactants can compete with proteins for the available ice surface.

Jiang and Nail[6] measured recovery of enzymatic activity of lactate dehydrogenase and β-galactosidase as a function of freezing rate and found that the rapid freezing of solutions of these proteins by placing vials in liquid nitrogen consistently resulted in decreased recovery of activity relative to slower freezing methods. Sarciaux et al[7] reported lower levels of insoluble aggregate in bovine IgG after freeze-drying following slow cooling relative to quench cooling in liquid nitrogen. Annealing of quench-cooled samples resulted in a significant reduction in the level of insoluble aggregates after freeze-drying, along with a corresponding decrease in the specific surface area of the freeze-dried solids.

Millqvist-Fureby and co-workers[14] used electron spectroscopy for chemical analysis to examine freeze-drying of proteins with various carbohydrates by measuring the surface composition of the amorphous solid. The proteins were found to be enriched at the solid surface relative to the bulk concentration of protein in the powders, and the mechanism was believed to be preferential adsorption of the proteins to the ice/freeze-concentrate interface.

These data are also consistent with the idea that the loss of protein integrity after freeze-drying is an interfacial phenomenon in which there are 2 interfaces that must be considered: the ice/freeze-concentrate interface and the glycine/freeze-concentrate interface. The observed concentration effect is, of course, consistent with loss of protein activity as an interfacial phenomenon because, at higher protein concentrations, a smaller percentage of protein would be expected to reside at either the ice/freeze-concentrate interface or at the glycine/freeze-concentrate interface. Recovery of activity of the model proteins in sucrose-glycine combinations is essentially the same as that in sucrose alone when the glycine remains amorphous. As the relative concentration of glycine increases and glycine crystallizes

from the frozen matrix, activity recovery decreases. Annealing, which promotes crystallization of glycine, is accompanied by decreased recovery of enzyme activity relative to that of the same system that was not annealed. Annealing has no such effect when glycine is not present. Annealing would be expected to decrease the ice/freeze-concentrate interfacial area of a sucrose-only formulation, with a subsequent increase in recovery of protein activity. It may be that the decreased interfacial area was not large enough to be measured by the methods used in this study. Finally, addition of a surfactant eliminates the effects of glycine crystallization, presumably by competing for occupancy of the freeze-concentrate/solid interface, thus inhibiting protein denaturation. Near quantitative recovery of activity when a surfactant is included in the sucrose formulation without glycine (compared with about 80% recovery in the sucrose-only formulation) is consistent with partial denaturation of protein at the ice/freeze-concentrate interface.

Practical Considerations

The observation of decreased activity of the model proteins at certain glycine/sucrose combinations does not mean that it is a poor formulation practice to incorporate glycine into a formulation, particularly because the adverse effect of glycine is eliminated by the use of a surfactant. The benefits of including a crystallizing solute in formulations include improved freeze-drying characteristics by preventing macroscopic collapse during freeze-drying as well as improved physical stability of the freeze-dried solid.

The results of this study add to the body of literature on the role of interfacial phenomena in freeze-drying of proteins and extends this knowledge to include crystal surfaces other than ice. Much more research is needed to accomplish the long-term goal of a molecular level understanding of the physical chemistry of protein freeze-drying; in particular, a better understanding is needed of the nature of

the solid surfaces present and the driving force for interaction of proteins with these surfaces.

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