

Effect of Mannitol Crystallinity on the Stabilization of Enzymes during Freeze-Drying

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The stabilizing effect of mannitol during the freeze-drying of proteins was studied using L-lactate dehydrogenase (LDH, rabbit muscle), β -galactosidase (*Escherichia coli*) and L-asparaginase (*Erwinia chrysanthemi*) as model proteins. Crystallization of mannitol was studied by powder X-ray diffraction and differential scanning calorimetry (DSC), in relation to the stabilizing effect. All the enzymes were protected concentration-dependently by amorphous mannitol, but the stabilizing effect was decreased with an increase in mannitol crystallinity. The heat-treatment of frozen solutions above crystallization temperature prior to drying enhanced mannitol crystallization and LDH inactivation. The importance of maintaining excipients in an amorphous state during freeze-drying, previously reported for *Aspergillus oryzae* β -galactosidase (K. Izutsu *et al.*, *Pharm. Res.*, 10, 1233 (1993)), was confirmed using three different enzymes.

Keywords freeze-drying; cryoprotectant; crystallization; mannitol; lactate dehydrogenase

Freeze-dried products are often preferred to guarantee sufficient shelf-lives of protein pharmaceuticals. Some excipients are added prior to freezing to act as stabilizers because proteins tend to lose their activity during freeze-drying.^{1,2)} Many sugars, polyols and amino acids protect proteins from inactivation during freeze-drying.³⁾ These excipients are preferentially excluded from the surface of proteins, and stabilize them thermodynamically against heat-denaturation and cold-denaturation.⁴⁾ Molecular interactions, such as hydrogen bonding, between proteins and excipients are reported to be necessary for stabilization during the drying process.^{3,5)}

Some drugs and excipients tend to crystallize during freeze-drying, and components in freeze-dried products exist in either a crystalline or amorphous state.⁶⁾ Crystallization is one of the major concerns in the freeze-drying of chemical pharmaceuticals, because it affects the physicochemical properties of formulations, including their solubility and stability.⁷⁾

Crystallization of excipients during freeze-drying is an important factor in the stability of protein pharmaceuticals, too.⁸⁾ Amorphous compounds such as sucrose, trehalose and hydroxypropyl- β -cyclodextrin (HP- β -CD) preserve the activity of proteins during freeze-drying, while mannitol, which tends to crystallize, had a smaller effect.^{8,9)} The crystallization of excipients also affects protein stability during storage.^{10–12)} In our previous paper we studied the stabilizing effects and crystallization of mannitol, inositol and glucose during freeze-drying, using *Aspergillus oryzae* β -galactosidase as a model enzyme.¹³⁾ It showed that the stabilizing effect of the excipients depended on their crystalline morphology.

To ascertain in general the importance of maintaining an amorphous state, we studied the relationship between the crystallization of mannitol and its stabilizing effect using three different enzymes: β -galactosidase from *Escherichia coli*, rabbit muscle L-lactate dehydrogenase (LDH), and L-asparaginase from *Erwinia chrysanthemi*. Mannitol is a widely used bulking agent for freeze-drying.¹⁾

Materials and Methods

Materials Rabbit muscle LDH (LDH-5, type V-S) and L-asparaginase (*Erwinia chrysanthemi*) were purchased from Sigma Chemical Co. β -Galactosidase from *E. coli* was purchased from Toyobo Co. Mannitol and all other chemicals used were of reagent grade.

Freeze-Drying and Freeze-Thawing Enzymes were dissolved in 50 mM of a sodium phosphate buffer (pH 7.4) to about 10 mg/ml. The enzyme solutions were dialyzed against the same buffer overnight. The concentration of dialyzed solutions was measured by the method of Lowry *et al.*¹⁴⁾ Aliquots of the enzyme solutions and excipient solutions were transferred to polypropylene tubes (flat bottom, 2.1 cm in diameter), to get designated concentration of enzymes (20 μ g/ml for LDH and β -galactosidase, 10 μ g/ml for L-asparaginase) and mannitol (0–500 mM). Solutions were frozen by immersion in liquid nitrogen and transferred to a pre-cooled shelf in a freeze-drier (Freezevac-1CFS, Tozai-Tsusho). The shelf temperature was maintained at -40°C for 1 h, -35°C for 12 h, and 35°C for 4 h. The temperature was altered at $1^{\circ}\text{C}/\text{min}$.

The effect of heat-treatment before freeze-drying was studied with LDH. LDH solutions were prepared and frozen as described above. Samples were transferred to the pre-cooled freeze-drier (-40°C), heat-treated at -20°C for 1 h, re-cooled to -40°C and freeze-dried. To study the effect of freeze-thawing with heat-treatment, samples were removed before drying and thawed at room temperature.

Enzyme Assay Freeze-dried cakes were reconstituted with distilled water (1 ml), and the enzyme solutions were diluted with 50 mM of a phosphate buffer (pH 7.4) to 2 μ g/ml. Activities of β -galactosidase were assayed at 25°C using 2-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate. An enzyme solution (50 μ l) was added to 1 ml of the substrate solution (5.7 mM ONPG in a sodium phosphate buffer (50 mM, pH 7.4)). The change in absorbance at 420 nm was monitored with a spectrophotometer (Shimadzu, UV-260). LDH activity was measured by monitoring the decrease in NADH absorption at 340 nm as described by Seguro *et al.*¹⁵⁾ L-Asparaginase was assayed by an enzymatic spectrophotometric technique utilizing L-glutamate oxaloacetate transaminase and L-malate dehydrogenase.¹⁶⁾ The remaining activity of each enzyme was expressed as the percentage of the activity before freeze-drying. The variation in apparent activity by the addition of mannitol was less than 10% of the original activity.

X-Ray Powder Diffraction The powder X-ray diffraction patterns of lyophilized enzymes were obtained using a Rigaku RAD-2C diffractometer system with Ni-filtered $\text{CuK}\alpha$ radiation (30 kV, 10 mA). The samples were scanned from 3 to 30° (2θ) at $2^{\circ}/\text{min}$. Diffraction patterns that have peak heights more than double of the baseline are judged to be crystalline cakes.

Differential Scanning Calorimetry (DSC) The thermal behavior of the frozen solutions and freeze-dried cakes was studied by DSC (DSC-41, Shimadzu). DSC studies were done using the equivalent weight of alumina as a control, and data were processed with software (Shimadzu). To study the thermal behavior of frozen solutions, aliquots (25 μ l) of

solutions in aluminum cells were scanned by heating from -60°C at $1^{\circ}\text{C}/\text{min}$. The samples in furnaces were cooled with liquid nitrogen in a cooling unit. To study the crystallization of freeze-dried samples, cakes (1.3–1.7 mg) in aluminum cells were scanned from 0°C at $1^{\circ}\text{C}/\text{min}$.

Results and Discussion

The Effect of Mannitol Crystallization during the Freeze-Drying of Enzymes Figure 1 shows the effect of mannitol on the remaining activity of LDH after freeze-drying. The enzyme lost most of its activity when freeze-dried from mannitol-free buffers (50 or 200 mM). The remaining activity was increased by the addition of mannitol. When the enzyme was freeze-dried from a 50 mM buffer, maximal recovery was observed around 100 mM mannitol. At more than 200 mM of mannitol, the remaining activity was decreased with the appearance of mannitol crystals observed in X-ray powder diffraction. Freeze-dried samples from the 200 mM buffer were amorphous up to 400 mM mannitol, and no decrease in stabilizing effect was observed up to 500 mM mannitol.

Residual activities of *E. coli* β -galactosidase after freeze-drying are shown in Fig. 2. The stabilizing effect of mannitol was reduced with the appearance of mannitol crystals. The relationship between the crystallinity and the remaining activity was in accordance with that of *A. oryzae* β -galactosidase.¹³⁾ β -Galactosidase from *E. coli*^{17,18)} (MW: 540000) is a tetramer having a structure and an optimal pH different from that of *A. oryzae*¹⁹⁾ (MW: 105000) studied previously. A similar relationship between mannitol crystallization and recovered activity was also observed for L-asparaginase (Fig. 3). These results show that, at least in freeze-drying from a sodium phosphate buffer, maintaining mannitol in an amorphous state is necessary for the stabilization of many enzymes.

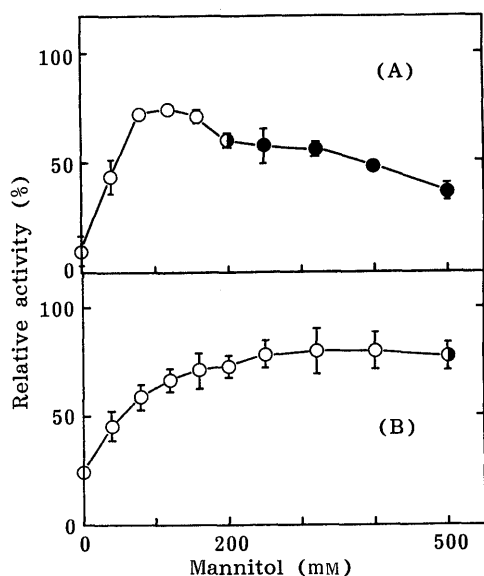


Fig. 1. The Effect of Mannitol on the Residual Activity of Freeze-Dried LDH

LDH (20 $\mu\text{g}/\text{ml}$) in 50 mM (A) or 200 mM (B) of a sodium phosphate buffer (pH 7.4) containing various concentrations of mannitol was freeze-dried. Each point is the mean of three experiments \pm standard deviation. The open and closed circles denote amorphous and crystallized samples, respectively. The half-filled circles show that some samples were crystallized.

Thermal Analysis of Frozen Solutions The thermal behavior of frozen solutions was studied by DSC (Fig. 4). The LDH solutions (20 $\mu\text{g}/\text{ml}$) in a phosphate buffer (50 mM, pH 7.4) containing various concentrations of mannitol were cooled to -60°C and scanned at $1^{\circ}\text{C}/\text{min}$. Gatlin and DeLuca reported⁶⁾ that mannitol in an aqueous frozen solution presents a glass transition at -65°C , and crystallization exotherm begins at -29°C . Mannitol crystallizes gradually above the glass transition temperature, whereas it crystallizes rapidly at the crystallization temperature.^{6,20)} The crystallization exotherm of mannitol (200 mM) in aqueous frozen solution was observed at around -25°C , which was altered by various phosphate buffer concentrations.¹³⁾ The thermal behavior of frozen mannitol solutions containing 50 mM phosphate buffer depended on mannitol concentrations (Fig. 4). Exotherm peaks were observed between -25 and -8°C , but a glass transition temperature could not be clearly observed. The crystallization exotherms of solutions with more than 200 mM mannitol moved to lower temperatures. This phenomena may be responsible for the crystallization of mannitol during freeze-drying.

Effect of Heat-Treatment of Frozen Solutions before Freeze-Drying

Figure 5 shows the effect of heat-treat-

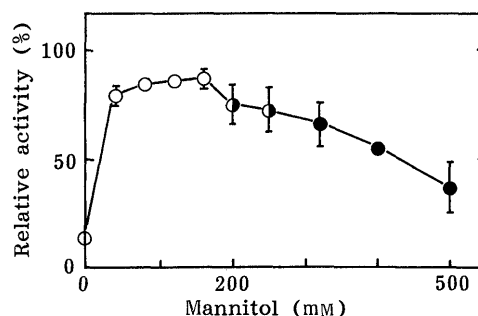


Fig. 2. Effect of Mannitol on the Residual Activity of Freeze-Dried *E. coli* β -Galactosidase

β -Galactosidase (20 $\mu\text{g}/\text{ml}$) from *E. coli* in a sodium phosphate buffer (50 mM, pH 7.4) containing various concentrations of mannitol was freeze-dried. Each point is the mean of three experiments \pm standard deviation. The open and closed circles denote amorphous and crystallized samples, respectively. Half-filled circles show that some sample were crystallized.

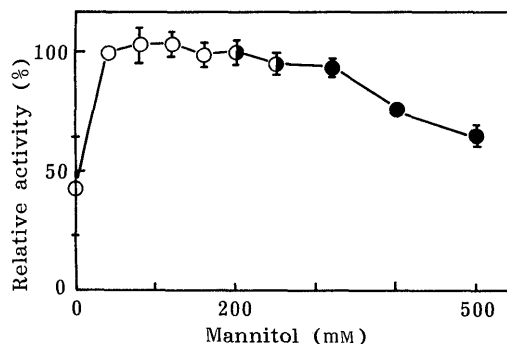


Fig. 3. Effect of Mannitol on the Residual Activity of Freeze-Dried L-Asparaginase

L-Asparaginase (10 $\mu\text{g}/\text{ml}$) in a sodium phosphate buffer (50 mM, pH 7.4) containing various concentrations of mannitol was freeze-dried. Each point is the mean of three experiments \pm standard deviation. The open and closed circles denote amorphous and crystallized samples, respectively. Half-filled circles show that some sample were crystallized.

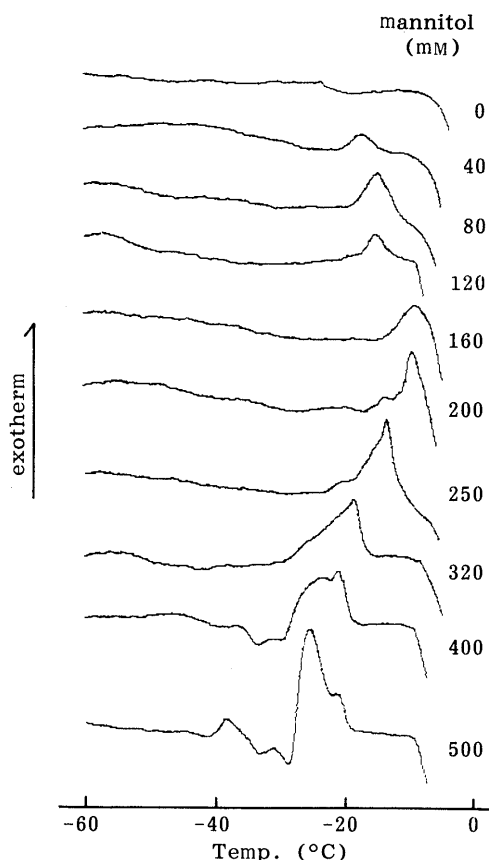


Fig. 4. DSC Scans of Frozen Mannitol Solutions

Mannitol solutions containing *E. coli* β -galactosidase (20 μ g/ml) in 50 mM phosphate buffer (pH 7.4) were cooled to -60°C , and scanned by heating at $1^\circ\text{C}/\text{min}$.

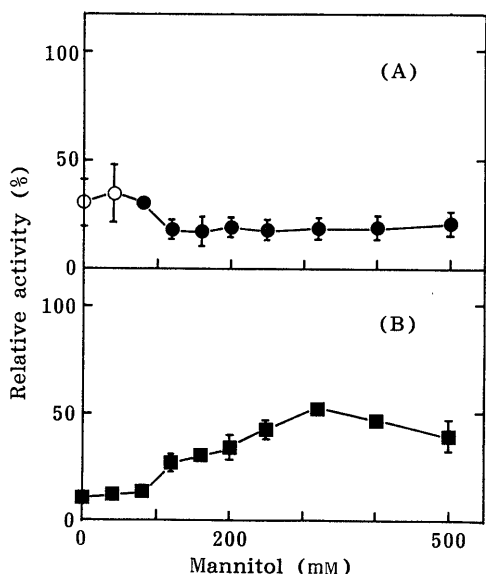


Fig. 5. Effect of Mannitol on the Residual Activity of LDH Freeze-Dried (A) or Freeze-Thawed (B) after Heat-Treatment

LDH (20 μ g/ml) in a sodium phosphate buffer (50 mM, pH 7.4) containing various concentrations of mannitol was frozen and heat-treated at -20°C for 1 h. Samples were re-cooled to -40°C , and freeze-dried (A) or thawed (B). Each point is the mean of three experiments \pm standard deviation. The open and closed circles denote amorphous and crystallized samples, respectively.

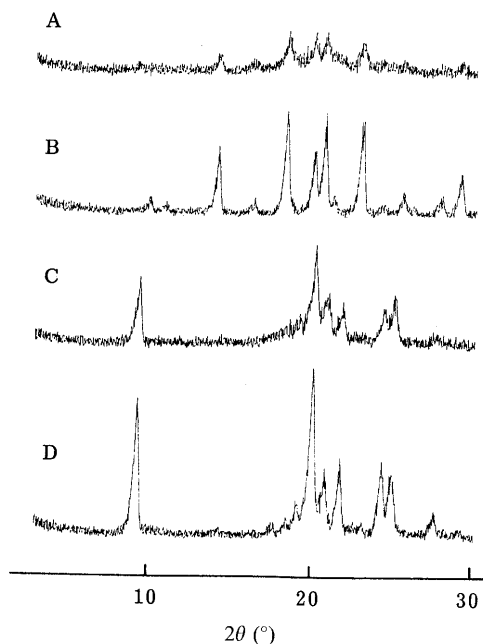


Fig. 6. Powder X-Ray Diffraction Patterns of LDH Containing Mannitol Freeze-Dried with or without Heat-Treatment

LDH (20 μ g/ml) in a sodium phosphate buffer (50 mM, pH 7.4) containing 250 mM (A, B) or 500 mM (C, D) of mannitol was freeze-dried with (B, D) or without (A, C) heat-treatment at -20°C for 1 h.

ment before freeze-drying. As shown in Fig. 4, the exotherms of mannitol crystallization in most samples began below -20°C . Frozen solutions were heat-treated at -20°C for 1 h before freeze-drying in order to crystallize mannitol in a frozen solution. The heat-treatment is also termed as annealing, and is often used to achieve crystalline freeze-dried products of pharmaceuticals, including antibiotics. The number of freeze-dried cakes with crystallized mannitol was increased by the heat-treatment (Figs. 1A and 5A). Mannitol in freeze-dried cakes from the lower concentrations of mannitol solutions (80 to 200 mM) were crystallized only in the heat-treated samples. The remaining activity of LDH after freeze-thawing with heat-treatment is shown in Fig. 5B. The activity of freeze-thawed LDH with high concentrations of mannitol was greater than that of the freeze-dried samples. This indicates that some activity was lost during the drying process.

Though mannitol freeze-dried from a solution of more than 250 mM crystallized regardless of the heat-treatment, the remaining activity of LDH was much smaller in heat-treated cakes (Figs. 1A, 5A). Besides inactivation during the heat-treatment, physical properties of the cakes may be changed by the heat-treatment. To investigate the physical properties of freeze-dried products, mannitol crystallinity of heat-treated or untreated cakes was studied by powder X-ray diffraction and DSC. Figure 6 shows the powder X-ray diffraction of freeze-dried cakes. As reported in the previous paper, the crystalline form of mannitol in freeze-dried cakes was different depending on the mannitol concentration before freeze-drying.¹³⁾ The mannitol crystals from a 250 mM solution were β -form, whereas those from a 500 mM solution were α -form. The reason for the formation of different crystalline forms is unclear.

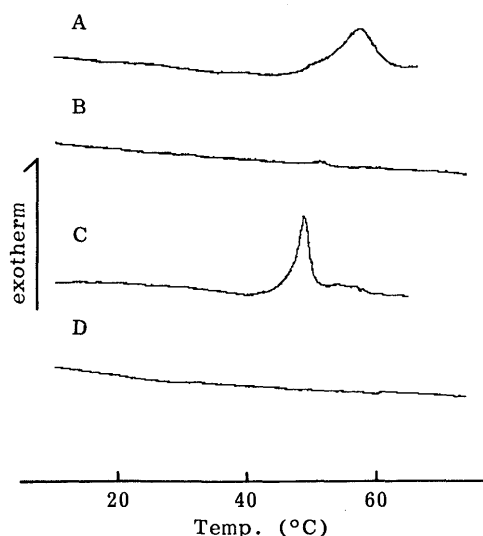


Fig. 7. DSC Scans of LDH Containing Mannitol Freeze-Dried with or without Heat-Treatment

LDH (20 $\mu\text{g}/\text{ml}$) in a sodium phosphate buffer (50 mM, pH 7.4) containing 250 mM (A, B) or 500 mM (C, D) of mannitol was freeze-dried with (B, D) or without (A, C) heat-treatment at -20°C for 1 h. Samples (1.3–1.7 mg) were scanned at $1^\circ\text{C}/\text{min}$.

The peak heights of mannitol crystals were increased in heat-treated cakes, which indicates that mannitol crystallinity was increased by the heat-treatment before freeze-drying.

Amorphous sugars prepared by freeze-drying display crystallization exotherm in thermal analysis.^{21,22} DSC scans of the cakes freeze-dried without the heat-treatment (Fig. 7) showed an exothermic peak corresponding to the crystallization of mannitol, which was not observed in the heat-treated samples. The results of X-ray diffraction and DSC show that freeze-dried samples without heat-treatment were a mixture of amorphous and crystallized mannitol, whereas mannitol in the heat-treated samples was almost completely crystallized. A difference in crystallinity may be one of the factors causing further inactivation.

Molecular interactions between excipients and proteins are important for stabilization during freeze-drying.^{3,5} Mannitol may lose its stabilizing effect as a result of crystallization, because it separates the phases and destroys the interaction inevitable for stabilization.⁸ In addition to a loss of stabilizing effect, the crystallization of excipients may also alter the environment of proteins in other ways. For example, crystallization may change the degree of freeze-concentration, which affects remaining activity. These results illustrate the importance of the

precise control of the freeze-drying procedure in order to freeze-dry proteins without inactivation.

Conclusion

Amorphous mannitol protected LDH, *Escherichia coli* β -galactosidase and L-asparaginase during freeze-drying, but this protective effect was decreased by mannitol crystallization. The number of crystallized samples and the degree of crystallinity were increased by the heat-treatment of frozen solutions. Fully crystallized mannitol showed no stabilizing effect on the freeze-drying of LDH. The obtained results show that the importance of maintaining amorphous states is not specific for *A. oryzae* β -galactosidase reported previously, but applies to proteins in general.

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