Physical Stability and Protein Stability of Freeze-Dried Cakes During Storage at Elevated Temperatures

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The relationship between physical stability of freeze-dried cakes and protein stability during storage was studied using β-galactosidase as a model protein and inositol as an excipient. Amorphous samples freeze-dried from solutions containing the enzyme and various concentrations of inositol in sodium phosphate buffer (50 mM, pH 7.4) were stored for 7 days over P_2O_5 at 40 to 70°C. Structural collapse and inositol crystallization were observed in some of the samples, depending on the formulation and storage temperature. The physical stability of freeze-dried samples was also studied by differential scanning calorimeter (DSC). Inositol showed a protein-stabilizing effect when its amorphous form was retained during storage, regardless of structural collapse. However, crystallization of inositol during storage removed its stabilizing effect. Addition of water-soluble polymers such as dextran, Ficoll and carboxymethyl cellulose sodium salt (CMC-Na) preserved activity of the enzyme by preventing inositol crystallization.

KEY WORDS: storage; crystallization; protein stability; freeze-drying; excipients.

INTRODUCTION

The inherent instability of many proteins including recombinant proteins necessitates intensive formulation studies for pharmaceutical purposes (1,2). Freeze-dried formulations containing excipients such as sugars, polyols and amino acids are often selected for protein pharmaceuticals (3,4). These excipients protect the proteins against inactivation during freezing (4), freeze-drying (4,5) and storage (3,6). Some excipients often crystallize during freeze-drying, depending on their concentration and the freeze-drying method employed (7,8). Ideally, excipients should remain amorphous during freeze-drying in order to exert their stabilizing effect, since upon crystallization excipients lose their essential molecular interactions with proteins (4,5,9).

In spite of their protein-stabilizing effect, amorphous cakes have problems associated with their physical stability during storage (10,11). Above the glass transition temperature (T_g), their physical state changes from a glassy solid to a highly viscous liquid (rubber). Storage of freeze-dried cakes at high temperature and/or high humidity often results in collapse of the cake structure, accompanied by crystallization of the excipients. It remains unclear how these physical changes affect protein stability in freeze-dried cakes.

Previously, we reported the stabilizing effect of various excipients during storage of freeze-dried enzyme at elevated temperature, using β-galactosidase from *Aspergillus oryzae* as a model protein (12). Among the excipients used, only those that maintained an amorphous state were capable of preserving the enzyme activity.

In this study, the relationship between physical stability of amorphous freeze-dried cakes and inactivation of β-galactosidase was studied. We used inositol as an excipient because, 1) the physical stability of freeze-dried cakes containing inositol can be altered easily by changing the formulation, and 2) the crystallization temperatures of the resulting freeze-dried cakes are relatively low in comparison with those made of sucrose or trehalose, allowing experimentation at elevated temperatures, as used in accelerated stability testing.

MATERIALS AND METHODS

Materials

β-Galactosidase from *Aspergillus oryzae* was purchased from Toyobo Co. (Osaka, Japan). Ficoll (nonionic synthetic polymer of sucrose, Type 400, average MW: 400,000) and dextran (D-9260, average MW: 11,000) were from Sigma Co. (St. Louis, USA). Inositol, carboxymethyl cellulose sodium salt (CMC-Na), 2-nitrophenyl-β-D-galactopyranoside (ONPG) and all other chemicals were of reagent grade and purchased from Wako Pure Chemical Co. (Osaka).

Freeze-drying and Storage of β-galactosidase

β-Galactosidase solution was prepared as described previously (8). Solutions (1 ml) in polyethylene tubes (flat bottom, 2.1 cm in diameter) containing the enzyme (20 μg/ml), phosphate buffer (50 mM, pH 7.4) and designated concentrations of inositol were frozen by immersion in liquid nitrogen for more than 1 min, and transferred to a pre-cooled shelf in a freeze-drier (Freezevac-1FCS, Tozai Tsushio, Tokyo, Japan). The samples were then freeze-dried, maintaining the shelf temperature at −40°C for 1 h, −35°C for 20 h and 35°C for 4 h. The shelf temperature was altered at 1°C/min. The samples were stored over P_2O_5 for 7 days at 40, 50, 60 or 70°C. In some experiments to study the effect of polymers, dextran, Ficoll or CMC-Na was added (1 mg/ml) to the solutions, freeze-dried and stored as described above.

Enzyme Activity and Crystallinity of Inositol

The samples were reconstituted by addition of 1 ml water, and diluted to obtain a 2 μg/ml solution of the enzyme with 50 mM phosphate buffer. The enzyme concentration was calculated from that before freeze-drying. The remaining activities were assayed at 30°C using ONPG as a substrate (13).

An X-ray diffraction study was carried out using a Rigaku RAD-2C system with Ni-filtered Cu-Kα radiation (30 kV, 10 mA, Tokyo) at a scanning rate of 2°/min. The samples were scanned from 3 to 30° (2θ). Samples showing peaks higher than twice the intensity of the corresponding amorphous samples were judged to be crystalline.

Thermal analysis of the freeze-dried products was done with a Shimadzu DSC-41 (Kyoto, Japan), using samples (1.3–1.7 mg) sealed in aluminum cells. After the furnace had cooled to 10°C, the samples were scanned at 1°C/min.

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RESULTS AND DISCUSSION

Effect of Storage Temperature on Excipient Crystallinity and Enzyme Activity

β-Galactosidase freeze-dried from solutions containing various concentrations of inositol in 50 mM phosphate buffer (pH 7.4) was stored for 7 days over P_2O_5 at elevated temperatures. Figure 1 shows the residual enzyme activities and inositol crystallinities of samples before and after storage. Table I shows the changes in appearance of samples during storage. All the freeze-dried samples prior to storage had a cylindrical structure, and the inositol in them was amorphous. The activity of the enzyme freeze-dried without inositol was about 15% that of the original solution (data not shown), whereas about 70–100% of the activity was retained when samples were freeze-dried from solutions containing 50 to 500 mM inositol.

Storage at elevated temperatures brought about a change in the physical appearance and crystallization of the inositol in the samples. The cakes freeze-dried from lower initial concentrations (50 to 160 mM) of inositol solution maintained their cylindrical structure (Table I), and no crystals of inositol were observed after storage between 40 and 70°C (Fig. 1B, 1C). Structural collapse and inositol crystallization were observed in cakes freeze-dried from higher concentrations (above 250 mM) of inositol solution after storage at 60 and 70°C.

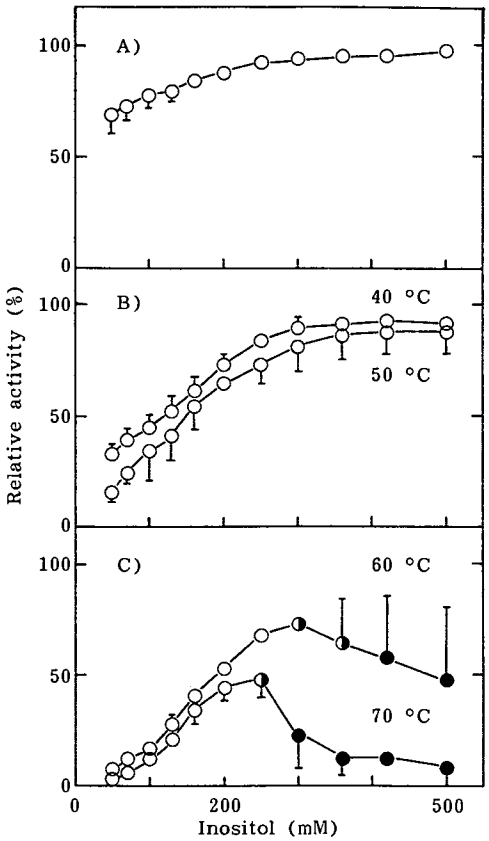

Fig. 1. Residual activity of β-galactosidase and crystallinity of inositol in freeze-dried cakes stored at elevated temperatures. β-Galactosidase (20 μg/ml) was freeze-dried from 50 mM phosphate buffer (pH 7.4) containing various concentrations of inositol. Figures A to C show the remaining activity immediately after freeze-drying (A), after 7 days of storage at 40, 50°C (B), and 60, 70°C (C), respectively. The values are mean ± SD (n = 3). The open and closed symbols represent amorphous and crystallized samples analyzed by X-ray diffraction, respectively. Half-filled symbols denote that crystallization was observed in some of the samples.

The remaining enzyme activity of the samples freeze-dried from lower concentrations (50–200 mM) of inositol solution decreased with decreasing inositol concentration, and it appeared that the amount of inositol in these samples was insufficient for stabilization. Samples freeze-dried from initial inositol concentrations above 300 mM retained more than 75% of their activity after 7 days of storage at 40 or 50°C, whereas they lost their activity rapidly at 60 and 70°C. The remaining activity after 7 days of storage at 60 or 70°C was maximal in samples freeze-dried from solutions containing around 250 mM inositol.

The results shown in Table I and Fig. 1 suggest that enzyme inactivation is relate to cake collapse and inositol crystallization. Among these forms of physical degradation, crystallization should be related more closely to inactivation than structural collapse. Although cakes freeze-dried from more than 200 mM inositol solution collapsed during storage at 70°C, no significant decrease in remaining enzyme activity was observed except in crystallized samples. In other words, amorphous inositol still retains its stabilizing effect despite the structural collapse of the freeze-dried cake. Molecular interactions such as hydrogen bonding between excipients and protein are reported to be important for stabilization during freeze-drying and storage of freeze-dried products (4,9). Crystallization may remove inositol molecules from the vicinity of the enzyme, reducing the molecular interaction between inositol and the enzyme, and thus leading to destabilization (5).

DSC Scans of Freeze-dried Cakes Containing Inositol

The thermal behavior of freeze-dried cakes was analyzed by DSC to determine the glass transition and crystallization temperatures (10,14). Figure 2 shows DSC scans of samples freeze-dried from various concentrations of inositol solution. Exothermic peaks due to inositol crystallization appeared around 60°C to 110°C, depending on the inositol concentration before freeze-drying. The crystallization temperature decreased with the increase in inositol concentration before freeze-drying, consistent with the tendency for crystallization of the samples stored at elevated temperatures.

Discontinuities in heat capacity, which can be ascribed to glass transition of the systems, were observed over a relatively wide range below the crystallization temperature. The end-points of the glass transition temperatures revealed in the DSC scans decreased with the increase in inositol concentration before freeze-drying, which may have accounted for the differences in appearance among samples stored at elevated temperatures (10).

Inhibition of Inositol Crystallization by High Concentration of Phosphate Buffer

It has been reported that raising the glass transition temperature by a change in formulation is a practical way to

Table I. Changes in appearance of freeze-dried cakes stores at elevated temperatures.

Inositol (mM)	50 mM buffer							200 mM buffer
	40 °C	50 °C	60 °C	70 °C	dextran 70 °C	Ficoll 70 °C	CMC-Na 70 °C	70°C
70	–	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–
130	–	–	–	–	–	–	–	–
160	–	–	–	–	–	–	–	–
200	–	–	–	+	+	–	+	–
250	–	–	+	+ +	+ +	+	+ +	–
300	–	–	+ +	+ +	+ +	+ +	+ +	–
360	–	–	+ +	+ +	+ +	+ +	+ +	–
420	–	–	+ +	+ +	+ +	+ +	+ +	+
500	–	–	+ +	+ +	+ +	+ +	+ +	+ +

Freeze-dried cakes from the described solutions were stored over P_2O_5 at elevated temperatures for 7 days. The structure of the samples was unchanged (−) or collapsed (+ or + +) during storage.

improve the physical stability of freeze-dried cakes (15). Various ingredients such as buffer salts, sugars and polymers are known to inhibit crystallization of freeze-dried carbohydrates (8,10). We employed a high concentration of phosphate buffer to prevent inositol crystallization during high-temperature storage, and studied whether preserved amorphous inositol retained its stabilizing effect.

The physical stability of freeze-dried cakes was improved by freeze-drying from higher concentrations of buffer (200 mM phosphate buffer, pH 7.4) compared to those from 50 mM buffer (Table I, Fig. 3). The number of samples which retained their cylindrical structure increased, and all the samples remained amorphous during storage at 70°C. DSC scans showed that the glass transition and crystallization temperatures of samples freeze-dried from 500 mM inositol solution were increased when 200 mM buffer was employed (D in Fig. 4) compared with freeze-drying from 50 mM buffer (A in Fig. 2).

Though freeze-drying from 200 mM buffer improved the physical stability, it was not effective for enzyme stabilization. The relative activities of samples preserved after storage at 70°C were below 40%. The remaining activity of the enzyme freeze-dried from 200 mM buffer was smaller than that from 50 mM buffer (Fig. 3). These results show that freeze-drying from a higher concentration of buffer may not be a practical approach for enzyme stabilization.

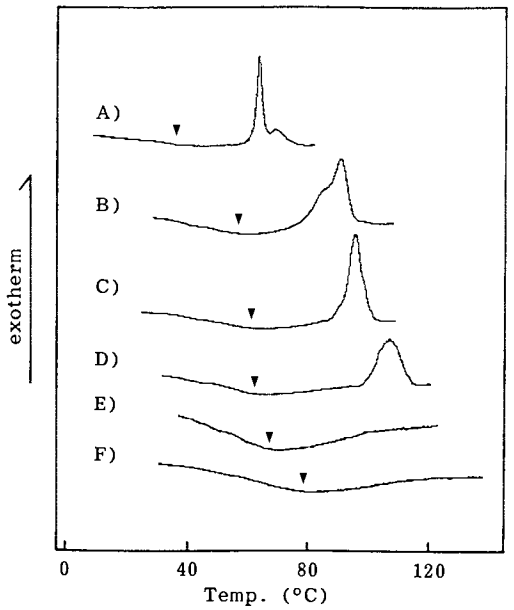

Fig. 2. Effect of inositol concentration on the thermal behavior of freeze-dried cakes. Samples (1.3–1.7 mg) freeze-dried from 500 (A), 360 (B), 250 (C), 160 (D), 100 (E) and 50 (F) mM inositol solutions containing β-galactosidase (20 μl) and phosphate buffer (50 mM, pH 7.4) were sealed in aluminum cells, and scanned at 1°C/min. The end-points of glass transition are indicated by ▼.

Inhibition of Inositol Crystallization by Polymers

Polymers such as dextran and gelatin are reported to inhibit the crystallization of carbohydrates in freeze-dried cakes at high temperature (10,16). The relationship between the physical stability of freeze-dried cakes and enzyme stability during storage was studied using dextran, Ficoll and CMC-Na as inhibitors of inositol crystallization. β-Galac-

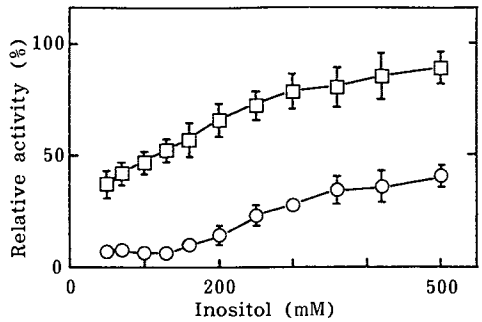

Fig. 3. Effect of 200 mM buffer on the enzyme stability during storage. β-Galactosidase (20 μg/ml) was freeze-dried from 200 mM phosphate buffer (pH 7.4) containing various concentrations of inositol. Residual activity was assayed immediately after freeze-drying (□) or after 7 days of storage at 70°C (○). The open symbols represent amorphous samples analyzed by X-ray diffraction. The values are mean ± SD. (n = 3)

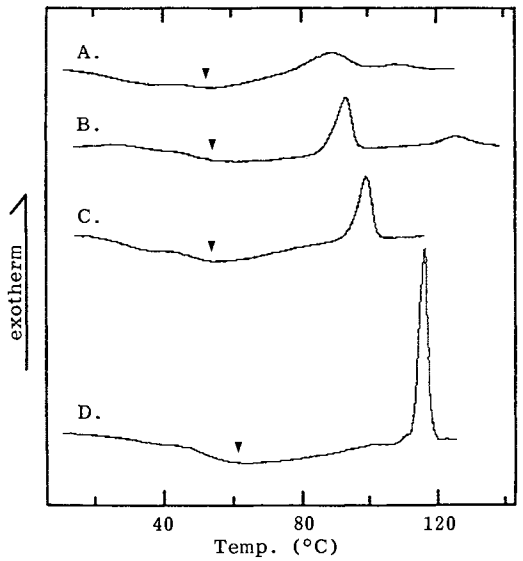

Fig. 4. Effect of polymers and 200 mM buffer on thermal behavior of freeze-dried cakes. Freeze-dried samples (1.3–1.7 mg) were sealed in an aluminum cell and scanned at 1°C/min. Scans A to C are for cakes freeze-dried from solutions containing β-galactosidase (20 μg/ml), 500 mM inositol and polymer (1 mg/ml) in 50 mM phosphate buffer. Polymers in the samples were dextran (A), Ficoll (B) and CMC-Na (C). Scan D is for a cake freeze-dried from solution containing 500 mM inositol and β-galactosidase (20 μg/ml) in 200 mM phosphate buffer (pH 7.4). The end-points of glass transition are indicated by ▼.

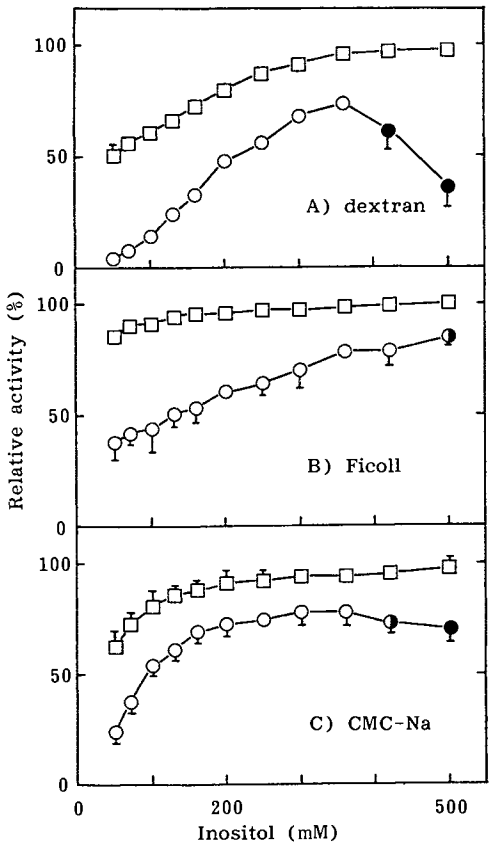

Fig. 5. Effect of storage on the stability of β-galactosidase cakes containing polymers. β-Galactosidase (20 μg/ml) was freeze-dried from 50 mM phosphate buffer (pH 7.4) containing polymers (1 mg/ml) and various concentrations of inositol. Polymers in the samples were dextran (A), Ficoll (B) and CMC-Na (C). Residual activity was assayed immediately after freeze-drying (□) or after 7 days of storage at 70°C (○, ●). The values are mean ± SD (n = 3). The open and closed symbols represent amorphous and crystallized samples analyzed by X-ray diffraction, respectively.

tosidase was freeze-dried from solutions containing polymers (1 mg/ml) and various concentrations of inositol in 50 mM buffer. Figure 5 (A to C) shows the effects of polymers on the inositol crystallinity and remaining enzyme activity immediately after freeze-drying and after 7 days of storage at 70°C.

Dextran, Ficoll and CMC-Na inhibited inositol crystallization during high-temperature storage. The number of samples that retained their amorphous state during storage at 70°C was increased by the addition of these polymers (Fig. 5) compared to polymer-free samples (Fig. 1C). Among the polymers studied, Ficoll showed the greatest ability to prevent inositol crystallization at a concentration of 1 mg/ml (Ficoll > CMC-Na > dextran). Even for the samples where inositol crystal was observed, crystallization peaks in the X-ray diffraction patterns were smaller than those for polymer-free cakes. This indicated that polymers inhibited the complete crystallization of inositol, and that a proportion of inositol remained amorphous (data not shown).

The effects of polymers on the thermal behavior of cakes freeze-dried from 500 mM inositol solution are shown in scans A to C in Fig. 4. The peak temperatures of the crystallization exotherms for the freeze-dried cakes containing polymers were higher than those of polymer-free samples (Fig. 2, scan A). The increased crystallization temperature may be related to the suppressed crystallization during storage at 70°C.

Addition of these polymers had little effect on the enzyme inactivation during freeze-drying (Fig. 5). However, the enzyme activity remaining after 7 days of storage at 70°C was increased considerably by addition of dextran, Ficoll or CMC-Na. In spite of these differences, there was a similarity between inositol crystallinity and remaining enzyme activity. In the presence of polymers, amorphous inositol showed a concentration-dependent stabilization effect during storage. These polymers may prevent inositol crystallization, and stabilize the enzyme indirectly by maintaining the stabilizing effect of amorphous inositol.

In addition to their indirect stabilizing effect, polymers such as Ficoll and CMC-Na may protect the enzyme by interacting with it. Freeze-dried samples from lower concentrations of inositol retained their amorphous form during storage at 70°C, regardless of polymer addition. The addition of dextran, which is reported to have little molecular interaction with proteins in freeze-dried cakes (9), had little effect on the inactivation of these samples during storage, whereas the addition of Ficoll or CMC-Na markedly increased the remaining enzyme activity. It has been reported that Ficoll alone protects freeze-dried ribonuclease A from inactivation during storage (6). These results suggest that, as has been reported for other excipients, molecular interactions with proteins contribute to the stabilization effect of Ficoll and CMC-Na.

The present results also show that crystallization is a more important factor for the stabilizing effect of inositol than structural collapse. Although polymers inhibited the crystallization of inositol during storage, they had little effect on the number of collapsed samples (Table I). The decrease in the stabilization effects of inositol was observed only in crystallized samples.

In conclusion, enzyme inactivation as well as physical degradation such as structural collapse and inositol crystallization were observed during storage of freeze-dried cakes at elevated temperatures. Although amorphous inositol showed a stabilizing effect, crystallization during storage deprived inositol of this effect. Dextran, Ficoll and CMC-Na preserved the enzyme activity through interaction with the enzyme and inhibition of inositol crystallization.

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