Increased Stabilizing Effects of Amphiphilic Excipients on Freeze-Drying of Lactate Dehydrogenase (LDH) by Dispersion into Sugar Matrices

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Purpose. The stabilizing effect of amphiphilic excipients and sugars against protein inactivation during freeze-drying was studied in relation to their physical states in freeze-dried cakes. ***Methods.*** Physical states of amphiphilic excipients were studied by powder X-ray diffractometry and differential scanning calorimetry (DSC). Stabilizing effects of excipients were studied using lactate dehydrogenase (LDH) as a model protein. ***Results.*** Although poly(ethylene glycols) (PEGs) 1000 to 20000 crystallized when freeze-dried alone, the addition of sugars decreased their crystallinity by dispersing PEG into sugar-dominant matrices. Sugars species, molecular weight of PEGs, and buffer concentration also affected the crystallinity of PEGs. Sugars also dispersed some of other amphiphilic excipients, which tended to crystallize or become "microscopically liquid" when freeze-dried without sugar. Only the amphiphilic excipients that remained amorphous solid state protected the enzyme during freeze-drying in the absence of sugars. However, combinations of sucrose and all the amphiphilic excipients studied increased the stabilizing effects markedly. The remaining activities were greater than the sum of their individual ones. ***Conclusions.*** Various amphiphilic excipients are good stabilizers for freeze-drying of proteins when dispersed into sugar-dominant matrices.

KEY WORDS: freeze-drying; amphiphilic excipients; solid dispersion; protein formulation; stabilization.

INTRODUCTION

The development of many recombinant proteins has brought about an increasing need for formulations that stabilize proteins adequately (1). Although freeze-dried formulations are often chosen because of their good stability during storage, the freeze-drying process inactivates some proteins. The stabilizing effects and responsible stabilization mechanisms of many excipients during freeze-drying have been studied actively these days (1-12).

Many polyols, including sugars, are good stabilizers that inhibit protein inactivation during freeze-thawing, freeze-drying, and storage (1-3,13). Sugars and other polyols stabilize proteins in solution thermodynamically by virtue of their preferential exclusion from the protein surfaces (2,13). They protect proteins during freeze-drying and storage through hydrogen bonding between protein and polyol (2). The polyols must be amorphous, at least in the immediate neighborhood of the proteins, so as to interact with and protect proteins during freeze-drying (3,4,14) and storage (5,14,15).

As well as the conventional cryoprotectants like polyols, some amphiphilic excipients such as poly(ethylene glycol) (PEG), poly(vinylpyrrolidone)(PVP), and nonionic surfactants also protect proteins against inactivation during storage in the frozen state and freeze-drying (6-8,16). The mechanism of stabilization by amphiphilic excipients is different from that of conventional cryoprotectants. In solution, amphiphilic excipients protect proteins from inactivation caused by dilution (17), adsorption to container surfaces (9,18), denaturation at the liquid-air interface (19), and thermal denaturation (20,21). In addition to these protective effects, some amphiphilic excipients assist refolding of denatured proteins (22,23). Binding and/or direct interaction of amphiphilic excipients to proteins contribute to some of these effects (6,7,17,22,23). Some protein formulations contain amphiphilic excipients as stabilizers and/or solubilizers (9,10). Of the many amphiphilic excipients that protect proteins against inactivation during freeze-thawing, only some do so during freeze-drying (7,8). It has been demonstrated that some amphiphilic excipients exhibit stabilizing effects at much lower concentrations than conventional cryoprotectants (6,7), but the mechanism responsible remains to be elucidated.

Carpenter *et al.* reported that the combination of PEG and trehalose had an positive effect against lactate dehydrogenase (LDH) inactivation during freeze-drying (8). Sugars have been reported to disperse antibiotics into freeze-dried cake matrices (24). In other words, sugars inhibit crystallization of other components. This study investigated the effect of sugars on the physical states, such as miscibility and crystallinity, of PEGs and other amphiphilic excipients in freeze-dried cakes. The relationships between the physical states of the excipients and their protein-stabilizing effects are discussed.

MATERIALS AND METHODS

Chemicals

Polyoxyethylene 9 lauryl ether (polidocanol) and L-lactate dehydrogenase (LDH, L-5132) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium cholate, 3-[(3-cholamidepropyl)-dimethylammonio]-1-propanesulfate (CHAPS) and sucrose monolaurate were from Dojin Chemical Co. (Kumamoto, Japan). Hydroxypropyl-β-cyclodextrin (HP-β-CD, molecular substitution = 0.9) was purchased from Aldrich Chemical Company (Milwaukee, WI). Poly(ethylene glycol) (PEG, average molecular weight: 400, 1000, 3000, 7500, and 20000), polyoxyethylene (10) octylphenyl ether (Triton X-100), polyoxyethylene (20) cetyl ether (Brij 58), sucrose, and all the other chemicals used were of reagent grade from Wako Pure Chemical Co. (Osaka, Japan).

Freeze-Drying of Samples

Solutions (1 ml) in polyethylene tubes (flat bottom, 2.1 cm in diameter) containing the required concentrations of solutes were frozen by immersion in liquid nitrogen for at

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least 1 min and transferred to a pre-cooled shelf of a freeze-dryer (Freezevac-1CFS, Tozai Tsusho, Tokyo). The samples were freeze-dried by maintaining the shelf temperature at -40 °C for 1 h, -35 °C for 12 h and 35 °C for 4 h. The shelf temperature was altered at the rate of 1 °C/min.

Powder X-Ray Diffraction and Thermal Analysis of Freeze-Dried Cakes

The powder X-ray diffraction study of freeze-dried samples was carried out using a Rigaku (Tokyo) Rad-2C system with Ni-filtered Cu-Kα radiation (30 kV, 10 mA). The samples were scanned from 3 to 30° (2θ) at the rate of 2° /min.

The freeze-dried samples were subjected to thermal analysis using a differential scanning calorimeter (Shimadzu DSC-41M, Kyoto). Freeze-dried samples (1.0-2.0 mg) containing PEGs with various molecular weights (400-20000) were sealed in aluminum cells. Samples containing PEG 3000, PEG 7500, and PEG 20000 were scanned from room temperature at 2 ° C/min. For samples containing PEG 400 and PEG 1000, the furnace was cooled to -50 and -20 °C respectively with liquid nitrogen, and the samples were scanned at 2 °C/min. Freeze-dried samples were stored at -20 °C until the thermal analysis was carried out.

The crystallinity of PEG in each freeze-dried cake was obtained from the heat absorption at the melting temperature and expressed as a ratio (%) relative to fully crystallized PEG. Beaumont *et al.* reported that the enthalpy of fusion of 100% crystallized PEG was 51.5 cal/g (215.5 J/g, 25). This value has been used as the standard by many workers to estimate PEG crystallinity (26). Indium was used as the calorimetric standard. The PEG crystallinity in each freeze-dried cake was calculated using the following equation:

$$\text{Crystallinity of PEG (\%)} = \frac{\text{Heat absorption of melting peak (J)}}{\text{Weight of PEG (g)} \times 215.5\ \text{(J/g)}} \times 100$$

Thermal analysis of freeze-dried cakes containing Triton X-100, Brij 58 and polidocanol was carried out from -50, -30, and -10 °C respectively at a scanning rate of 2 °C/min.

Effects of Excipients on the Freeze-Drying of LDH

The concentrations of LDH in dialyzed solutions were determined as described previously (7). Solutions (1 ml) containing LDH (2 μg/ml), 50 mM sodium phosphate buffer (pH 7.4), and the required concentrations of excipients were freeze-dried, as described above, then reconstituted immediately by adding water (1 ml). The remaining LDH activity was assayed at 30 °C and expressed as activity (%) relative to that before freezing.

RESULTS AND DISCUSSION

Effect of Sucrose Concentration on PEG Crystallization During Freeze-Drying

In frozen solutions, PEGs tend to crystallize, yielding crystalline freeze-dried cakes (8,27). The effect of sucrose on the crystallinity of freeze-dried PEG 3000 was studied by powder X-ray diffractometry and differential scanning calorimetry (DSC). Figure 1 shows the powder X-ray diffraction patterns of PEG 3000 (20 mg/ml) freeze-dried with 50 mM phosphate buffer and sucrose (0-500 mM). Although diffraction peaks due to PEG 3000 crystals were observed in the samples freeze-dried with 0 to 200 mM sucrose, these peaks disappeared with sucrose concentrations higher than 200 mM. No peaks due to crystalline sucrose were observed, indicating that sucrose was amorphous in all the cakes. Figure 2 shows the DSC scans of the freeze-dried samples. The endothermic peaks of PEG 3000 melting observed for samples freeze-dried with the lower concentrations of sucrose (up to 200 mM) disappeared when over 200 mM sucrose was used. These endothermic peak temperatures were identical to that of melting of crystallized PEG 3000 reported in the literature. The appearance of melting peaks showed that the molecules of PEG 3000 in these freeze-dried cakes had separated from the other components and crystalline at room temperature. Freeze-drying with high concentrations of sucrose inhibited PEG 3000 crystallization by dispersing it into the sucrose-dominant amorphous matrices of the freeze-dried cakes. Aqueous frozen solutions of PEG behave as binary eutectic mixtures, which may be responsible for the PEG crystals in freeze-dried cakes (28). Sucrose should inhibit the eutectic crystallization of PEG in the frozen solutions through inhibiting phase separation of components.

The PEG 3000 crystallinity in freeze-dried samples was calculated from the DSC data using the enthalpy of fusion of 100% crystallized PEG reported by Beaumont *et al.* (25). Figure 3 shows the crystallinity of PEG 3000 (1-50 mg/ml) freeze-dried with 50 mM phosphate buffer and 0 to 500 mM sucrose. The results indicate that the higher the PEG concentration, the more sucrose is required to disperse PEG in freeze-dried cakes.

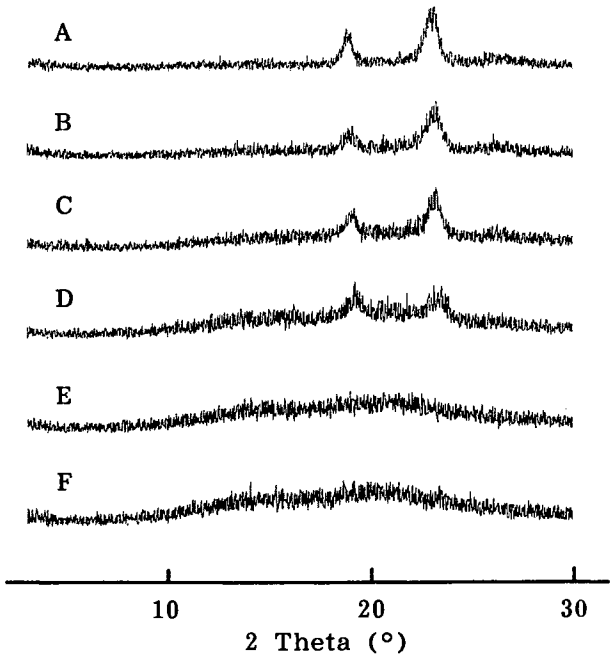

Fig. 1. Powder X-ray diffraction patterns of PEG 3000 freeze-dried with sucrose. Freeze-dried samples from solutions containing PEG 3000 (20 mg/ml), 50 mM sodium phosphate buffer, and 0 (A), 50 (B), 100 (C), 200 (D), 300 (E), and 500 (F) mM sucrose were studied by powder X-ray diffractometry.

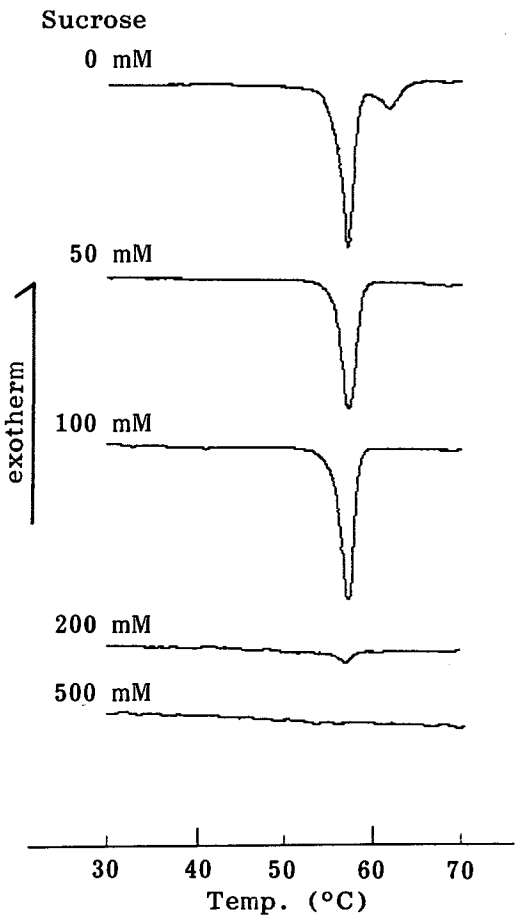

Fig. 2. Differential scanning calorimetry scans of PEG 3000 freeze-dried with sucrose. Freeze-dried samples (1.0-2.0 mg) from solutions containing PEG (20 mg/ml), 50 mM sodium phosphate buffer, and various concentrations of sucrose (0-500 mM) were freeze-dried and the crystallinity of PEG 3000 was studied by DSC. The samples were sealed in aluminum cells and scanned from room temperature at 2 °C/min.

Figure 4 shows the effect of sucrose on the crystallinities of PEGs with various molecular weights in freeze-dried cakes. The melting temperatures of the PEGs studied ranged from around 0 °C (PEG 400) to 60 °C (PEG 20000). The DSC scans of the freeze-dried cakes containing PEG 3000, PEG 7500, and PEG 20000 with the lower concentrations of sucrose showed melting peaks at around 55 to 60 °C, which indicated that the PEGs in these cakes were crystalline at room temperature. The crystallinities of PEG 3000 to PEG 20000 decreased in samples freeze-dried with higher concentrations of sucrose.

Samples from solutions containing PEG 400, phosphate buffer and 0 to 50 mM sucrose collapsed during freeze-drying; however, others retained their cylindrical structure during freeze-drying. The DSC scans of some cakes containing PEG 400 and PEG 1000 showed endothermic peaks (at around 0 and 25 °C, respectively), which corresponded to their melting (data not shown). The melting peaks observed were below room temperature. Therefore, at least a portion of both PEG 400 and PEG 1000 should have separated from the other components and should have become "microscopically liquid" at room temperature. The structure of these cakes were relatively unstable against storage, which may be due to an excess of "microscopically liquid" components.

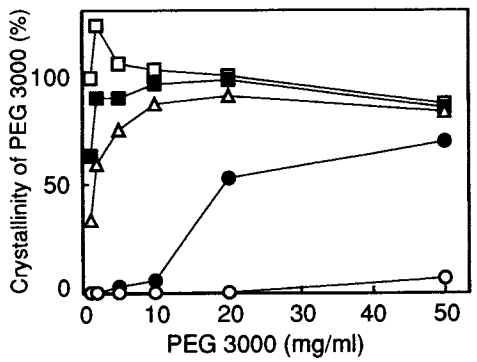

Fig. 3. Effect of sucrose on the crystallinity of freeze-dried PEG 3000. Freeze-dried cakes from solutions containing PEG 3000 (1-50 mg/ml), 50 mM sodium phosphate buffer (pH 7.4), and sucrose (□: 0 mM, ■: 50 mM, △: 100 mM, ●: 200 mM, ○: 500 mM) were studied by DSC. Samples (1.0-2.0 mg) were sealed in aluminum cells and scanned from room temperature at 2 °C/min. The PEG 3000 crystallinities were calculated from the melting endotherm areas as described in the methods. The values are the means of two experiments.

As was observed with high MW PEGs (>3000), the melting peaks of PEG 400 and PEG 1000 disappeared when the samples were freeze-dried with higher concentrations of sucrose (>200 mM), which means they were dispersed within the matrices. These results also revealed that higher concentrations of sucrose were required to disperse the higher molecular weight PEGs.

Effects of Various Sugars and Buffer Concentrations on PEG 3000 Crystallization During Freeze-Drying

Figure 5 shows the crystallinities of PEG 3000 (1-50 mg/ml) freeze-dried with various sugars in the presence of 50

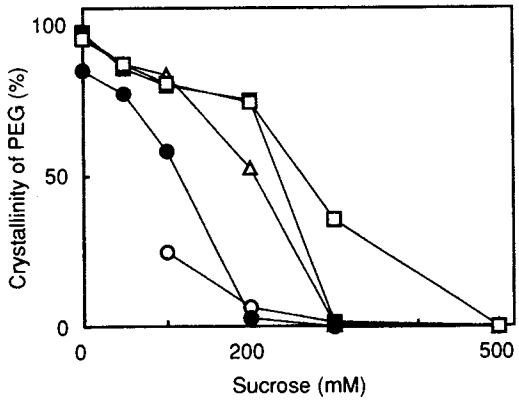

Fig. 4. Effect of sucrose on the crystallinities of freeze-dried PEGs with various molecular weights. Freeze-dried cakes (1.0-2.0 mg) from solutions containing various PEGs (○: PEG 400, ●: PEG 1000, △: PEG 3000, ■: PEG 7500, □: PEG 20000, 20 mg/ml), 50 mM sodium phosphate buffer (pH 7.4), and 200 mM sucrose were scanned from room temperature at 2 °C/min. Samples containing PEG 400 and PEG 1000 were scanned from -50 °C and -20 °C respectively. The PEG crystallinities were calculated as described in Fig. 3. The values are the means of two experiments.

mM phosphate buffer. Of the sugars studied, sucrose demonstrated the greatest ability to disperse PEG 3000, followed in descending order, by trehalose, maltose and lactose. All these sugars were amorphous in all the cakes. Less PEG 3000 was dispersed in the mannitol matrix, probably due to mannitol crystallization during freeze-drying (4,29).

Figure 5 also shows the crystallinities of PEG 3000 (1-50 mg/ml) freeze-dried with 200 mM sucrose in the absence of sodium phosphate buffer. The effect of sucrose on the crystallinities of PEG 3000 was altered by the buffer concentration. These results indicate that various factors affect the dispersing effect of sugars.

Thermal Analysis of Freeze-Dried Cakes Containing Amphiphilic Excipients

Figure 6 shows the DSC scans of freeze-dried cakes containing Triton X-100, Brij 58, and polidocanol. All the cakes remained cylindrical during freeze-drying, but some shrunk during storage at room temperature. In the samples freeze-dried with mannitol, endothermic melting peaks of amphiphilic excipients were observed at the same temperatures as those of the pure excipients. The melting temperatures of Triton X-100 and polidocanol were below 30 °C, so they would be expected to become "microscopically liquid" at room temperature. Moreover, no melting peaks were observed when these excipients were freeze-dried with sucrose. Therefore, the excipients have been dispersed within the sucrose-dominant matrices of the freeze-dried cakes. These results indicate that the physical states of amphiphilic excipients are altered by other components in freeze-dried cakes.

The results obtained in this study and the existence of hydrogen bonding between protein and sugar in freeze-dried cakes (2) suggest that proteins, as well as excipients, are dispersed into the sucrose-dominant matrices of freeze-dried cakes. The dispersion may provide molecular interaction between protein and amphiphilic excipients that are lost by excipients crystallization.

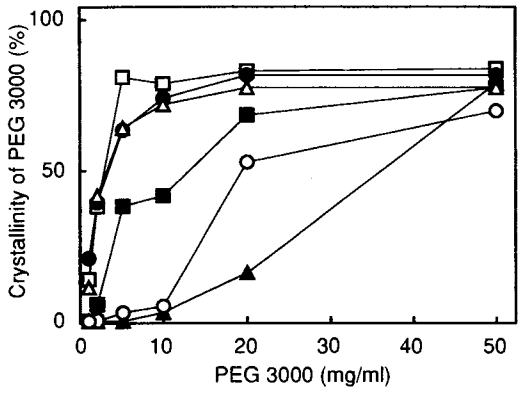

Fig. 5. Effects of various sugars and buffer concentration on the crystallinity of freeze-dried PEG 3000. Freeze-dried cakes (1.0-2.0 mg) from solutions containing PEG 3000 (1-50 mg/ml), 50 mM sodium phosphate buffer (pH 7.4), and various sugars (○: 200 mM sucrose, ■: 200 mM trehalose, ●: 200 mM maltose, □: 200 mM lactose, △: 400 mM mannitol) were studied by DSC. Some samples (▲) were freeze-dried from solutions containing PEG 3000 (1-50 mg/ml) and 200 mM sucrose. Samples were scanned from room temperature at 2 °C/min. The PEG crystallinities were calculated as described in Fig. 3. The values are the means of two experiments.

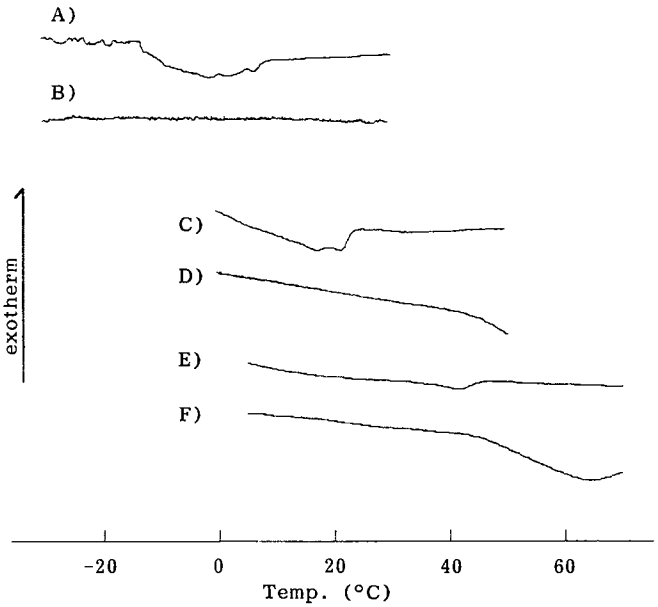

Fig. 6. Differential scanning calorimetry scans of freeze-dried cakes containing amphiphilic excipients. Freeze-dried cakes (2.0-5.0 mg/ml) were scanned at 2 °C/min. Each scan represents freeze-dried cakes from solutions containing A: Triton X-100 (10 mg/ml) and 400 mM mannitol, B: Triton X-100 (10 mg/ml) and 200 mM sucrose, C: polidocanol (5 mg/ml) and 400 mM mannitol, D: polidocanol (5 mg/ml) and 500 mM sucrose, E: Brij 58 (2 mg/ml), and 400 mM mannitol and F: Brij 58 (2 mg/ml) and 200 mM sucrose.

Effects of Excipients on the Activity of Freeze-Dried LDH

Figure 7 shows the lactate dehydrogenase (LDH) activities remaining after freeze-drying with amphiphilic excipients in the absence (A) and presence of 400 mM mannitol (B) and 200 mM sucrose (C). All the samples were cylindrical during freeze-drying. In the absence of sucrose or mannitol (A), HP-β-CD, CHAPS, sodium cholate, and sucrose monolaurate showed concentration-dependent protective effects against inactivation during freeze-drying. All these excipients (from 10 mg/ml solutions) were amorphous solids in the freeze-dried cakes. Both PEG 3000 and PEG 20000, which crystallize easily during freeze-drying, showed weak stabilizing effects. The addition of higher concentrations of Triton X-100, Brij 58, polidocanol, and PEG 400 inactivated LDH. These results are consistent with those obtained with β-galactosidase (6) and LDH (7) reported in a previous paper. In the absence of sugars, only the amphiphilic excipients that remained in an amorphous solid state protected LDH against inactivation during freeze-drying.

Similar results were obtained when 400 mM mannitol was added to the solutions before freeze-drying (B). The powder X-ray diffraction patterns of the freeze-dried cakes show that most of the mannitol in these freeze-dried cakes had crystallized (data not shown). The stabilizing effect of excipients that became "microscopically liquid" (PEG 400, Triton X-100, Brij 58, and polidocanol) decreased as their concentrations increased. Excess liquid components in freeze-dried cakes may enhance protein inactivation due to

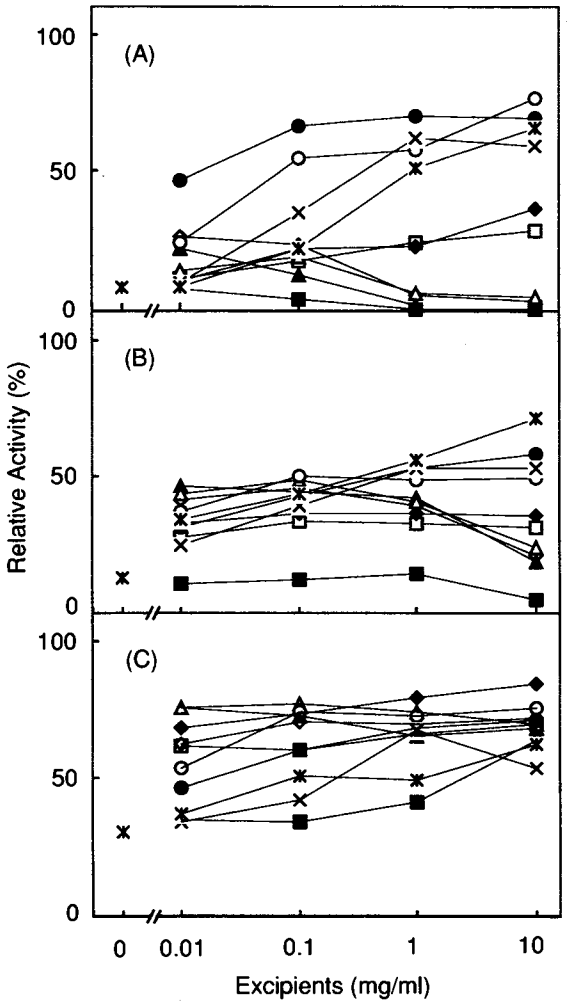

Fig. 7. Effects of excipients on the activity of freeze-dried LDH. Lactate dehydrogenase (2 μg/ml) in 50 mM sodium phosphate buffer (pH 7.4) was freeze-dried with various amphiphilic excipients in the absence (A) and presence of 400 mM mannitol (B) and 200 mM sucrose (C). Samples were reconstituted by adding water (1 ml) immediately after freeze-drying and assayed at 30 °C (■: PEG 400, □: PEG 3000, ◆: PEG 20000, ◇: Brij 58, ▲:polidocanol, △: Triton X-100, ●: HP-β-CD, ○: CHAPS, ×: sodium cholate, *: sucrose monolaurate). The values are the means of five experiments.

denaturation or enhancement of some chemical reactions (1,30).

In the presence of 200 mM sucrose (C), all the amphiphilic excipients used in this study showed apparent stabilizing effects. Sucrose protected the enzyme independently; less than 40% of the LDH activity was retained after freeze-drying with 50 to 500 mM sucrose (data not shown). However, the LDH activities remaining after freeze-drying with combinations of some amphiphilic excipients and sucrose were much greater than the sum of the activities obtained with the agents alone. The increased LDH activities remaining were particularly apparent with those excipients that crystallized or became "microscopically liquid" in freeze-dried cakes without sucrose.

The enhanced stabilizing effects of combinations of PEGs and sugars are consistent with the results reported by Carpenter *et al.* (8), and suggest that such combinations exert effects greater than the sums of the individual excipients. The DSC scans of such samples indicated that PEG 400, PEG 3000, and PEG 20000 freeze-dried with sucrose were dispersed in the matrices (data not shown). Sucrose may maintain the molecular interactions between LDH and PEGs requisite for stabilization through inhibiting phase separation of components during freeze-drying.

Broad melting peaks of Triton X-100, Brij 58, and polidocanol make it difficult to determine their physical states in freeze-dried cakes. Although endothermic melting peaks were observed with cakes from solutions containing 200 mM sucrose and the highest concentration (10 mg/ml) of these excipients, no peaks were observed in cakes from 1 mg/ml solutions (data not shown). These results suggest that at least portions of these excipients were dispersed in the sucrose-dominant matrices of freeze-dried cakes, especially when freeze-dried from lower concentration solutions. The knowledge of factors that determine the dispersion such as kind and concentration of excipients and freeze-drying method may contribute to design the stable protein formulations.

Hydroxypropyl-β-cyclodextrin (HP-β-CD) has been demonstrated to be one of the most effective stabilizers for freeze-drying proteins (9,11,12). The effect of HP-β-CD depends on its amphiphilic character and ability to maintain an amorphous state during freeze-drying (11,18). Combinations of amphiphilic excipients and sucrose may provide an environment around proteins similar to that produced by HP-β-CD. Such combinations have advantages over a single excipient. They provide a greater choice of amphiphilic excipients that depend less on their physical properties, and lower concentrations of such excipients are required for stabilization, which should help to develop more stable and safer protein formulations.

In conclusion, sugar disperse PEG and other amphiphilic excipients in the amorphous matrices of freeze-dried cakes by inhibiting the phase separation of components that causes crystallization or formation of "microscopic liquid" in the cakes. This dispersion may be one of the reasons why combinations of sucrose and amphiphilic excipients exhibit greater stabilizing effects during freeze-drying of LDH than the summated effects of the individual agents.

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