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Excipient crystallinity and its protein-structure-stabilizing effect during freeze-drying

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Abstract

The relationship between mannitol crystallization during freeze-drying and its effects on stabilizing protein structures was studied using lysozyme, bovine serum albumin, ovalbumin, β-lactoglobulin and lactate dehydrogenase as model proteins. FT-IR analysis of the protein secondary structure indicated perturbation of both α-helix and β-sheet regions in freeze-drying without cosolutes, whereas the proteins retained most of their native structure in co-lyophilization with sucrose. Mannitol protected the protein structure to different degrees depending on the crystallinity. The combination of mannitol with potassium phosphate buffer reduced the mannitol crystallinity and the structural changes occurring during freeze-drying, whereas mannitol by itself showed little stabilizing effect. Heat-treatment of the frozen solutions at -10°C resulted in a higher mannitol crystallinity and a smaller stabilizing effect in freeze-drying. The secondary structure perturbation was mostly reversible in rehydrated solutions. The varied structure-stabilizing effects of mannitol paralleled its effects on maintaining lower concentrations of enzyme activity during freeze-drying. These results confirm the contribution of molecular interactions between amorphous excipients and proteins (e.g. hydrogen bonding) to structure stabilization during freeze-drying.

Introduction

Freeze-drying is a popular method for stabilizing proteins that are physically or chemically unstable in aqueous solutions. Despite improvements in long-term stability, various proteins lose their biological activity during the freeze-drying process because of irreversible structural change or resulting aggregation in rehydrated solutions (Manning et al 1989; Carpenter et al 1997; Franks 1998; Pikal 1999). Proteins face stresses such as low temperature, cosolute concentrations, pH changes and dehydration in the freeze-drying process. Many protein formulations contain various excipients, including saccharides, polymers, salts and buffer components, to protect proteins during freeze-drying and subsequent storage. Elucidating the stresses and stabilizing mechanisms is important to the rational design of freeze-dried formulations.

Freezing an aqueous solution concentrates solutes into a supercooled phase among ice crystals. Various excipients possess different physical properties in frozen solutions and freeze-dried solids (e.g. crystallinity, glass transition temperature) (Franks 1998; Craig et al 1999; Pikal 1999). Although many solutes remain amorphous in the freeze concentrate, some (e.g. mannitol, glycine, poly(ethylene glycol)) tend to crystallize in single-solute frozen solutions (Gatlin & DeLuca 1980). The varied physical properties of excipients and correspondingmolecular interactions with proteins affect the protein-stabilizing effects against inactivation during freezing and freeze-drying.

Mannitol is a popular excipient in the freeze-drying of low-molecular-weight chemical pharmaceuticals, as the crystallization results in a good cake structure, but it provides less protection for proteins during freeze-drying compared with other amorphous sugars and sugar alcohols (Hellman et al 1983; Pikal et al 1991; Carpenter et al 1993; Prestrelski et al 1993; Costantino et al 1998). Studies employing different cosolute compositions or freeze-drying methods have revealed that only amorphous mannitol protects proteins against the stresses (Izutsu et al 1991, 1993; Jiang & Nail 1998).

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Crystallization of other solutes (e.g. inositol, PEG) also reduces the protein-stabilizing effects during freeze-drying and subsequent storage (Carpenter et al 1993; Izutsu et al 1994). Crystallization is considered to deprive excipients of the molecular interactions (e.g. hydrogen bonding) with proteins that are necessary to maintain the native protein structure against dehydration stresses (Carpenter & Crowe 1989; Tanaka et al 1991; Crowe et al 1998). It is important, however, to clarify the effects of mannitol crystallization on the protein structure, as the stabilizing effects have been studied only through activity changes in the lower concentrations of freeze-labile model enzymes (e.g. lactate dehydrogenase). In this study, the relationship between the mannitol crystallinity and its protein-structure-stabilizing effect during freeze-drying was examined. Changing the cosolute composition and the freeze-drying procedure varied the crystallinity of mannitol. The stabilizing effects of mannitol were studied through changes in the protein secondary structure as obtained by FT-IR analysis and changes in lactate dehydrogenase (LDH) activity.

Materials and Methods

Materials

Bovine serum albumin (BSA; fatty-acid free), chicken egg albumin (ovalbumin), lactate dehydrogenase (LDH; rabbit muscle) and β-lactoglobulin (bovine milk) were purchased from Sigma Chemical Co. (St Louis, MO). Chicken egg lysozyme was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Mannitol and all other chemicals were of analytical grade and obtained from Wako Pure Chemical Co. (Osaka, Japan). The proteins were dialysed against distilled water before the experiment.

Freeze-drying

Freeze-drying of the solutions was performed using a freeze-drier (Tozai Tsusho, Freezevac-1CFS, Tokyo, Japan). Aqueous solutions (200 μL) containing combinations of proteins (2.5 mg mL^{-1} for LDH and 10 mg mL^{-1} for other proteins), mannitol, and potassium phosphate buffer (pH 7.0) in flat-bottom polypropylene tubes (approx. 5 mm in depth) were frozen by immersion into liquid nitrogen. The protein solutions were dried under vacuum (6 Pa) immediately after the tubes were transferred to the shelf of the freeze-drier. The shelf temperature of the freeze-drier was maintained at -40°C for 12 h for the first drying and at 20°C for 24 h for a secondary drying step, heating at 0.5°C min^{-1} between the steps. Some frozen solutions were heat-treated at -10°C for 1 h before the first drying step at -40°C to achieve the mannitol crystallization in the frozen solutions.

Thermal analysis and powder X-ray diffraction

Thermal analysis of frozen solutions was performed by a differential scanning calorimeter (DSC Q10, TA Instrument, DE). Indium (156.6°C) and cyclohexane (-87.1°C) were used for temperature calibration. Aqueous solutions (10 μL) in hermetic aluminium cells (approx. 4 mm in diameter) were cooled at approximately 20°C min^{-1} and scanned from -100°C at 5°C min^{-1} under N_2 purging. The crystallinity of mannitol in freeze-dried solids was studied using a powder X-ray diffractometer (Rigaku type RAD-2C system, Tokyo) with Ni-filtered Cu-K radiation (30 kV, 10 mA) at a scanning rate of 2° min^{-1}. The diffraction data were processed using RADText software programmed by Dr Kobayashi (Numazu College of Technology).

FT-IR analysis

The secondary structures of proteins were analysed with an FT-IR system (MB104 spectrophotometer with Prota software, ABB Bomen, Quebec, Canada). Spectra of freeze-dried solids were obtained from pressed disks containing freeze-dried solids (approximately 1 mg protein) and dried potassium bromide (approximately 250 mg). Spectra of aqueous protein solutions (10 mg mL^{-1} in 50 mM potassium phosphate buffer) were recorded at 4 cm^{-1} resolution using infrared cells with CaF_2 windows and 6-μm film spacers (256 scans). LDH spectra were obtained at 2.5 mg mL^{-1}. Reference spectra were recorded with the corresponding buffer and excipient solutions. Second-derivative spectra were calculated as described by Susi & Byler (1983) using GRAMS/32 software (Galactic Ind. Co., Salem, NH) and smoothed with a 7-point smoothing function. The second-derivative amide I spectrum was area-normalized as described by Kendrick et al (1996).

LDH assay

Freeze-dried solids were rehydrated and diluted to 2.5 μg mL^{-1}. LDH activity was measured by monitoring the decrease in NADH absorption at 340 nm at 37°C (Seguro et al 1990). Each assay mixture contained 0.35 mM sodium pyruvate and 0.07 mM reduced nicotinamide-adenine dinucleotide (NADH) in 50 mM sodium phosphate buffer (pH 7.4). The results are expressed in terms of relative enzyme activity (%) to the initial solutions without excipient.

Results

Figure 1 shows thermograms of frozen solutions containing mannitol, BSA and potassium phosphate buffer (0–100 mM). Frozen 50 mg mL^{-1} mannitol solutions showed a small shift and a following exothermic peak at approximately -24°C. The exothermic peak has been attributed to the mannitol crystallization, although there is some debate on the interpretation due to the complex crystallization behaviour of frozen mannitol solutions (Gatlin & DeLuca 1980; Martini et al 1997; Kim et al 1998; Cannon & Trappler 2000). Addition of BSA (10 mg mL^{-1}) altered the peak shape to some extent. The phosphate buffer raised the peak temperature, and made the exothermic peak

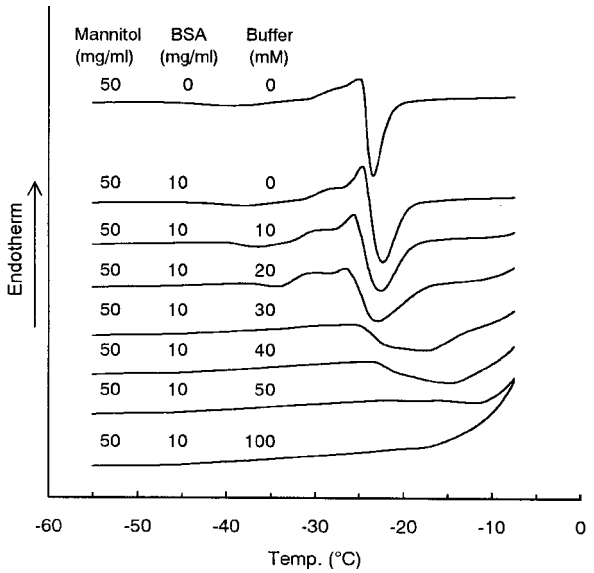

Figure 1 Thermograms of frozen solutions containing mannitol, BSA and different concentrations of potassium phosphate buffer (0–100 mM). Samples (10 μL) of solutions were scanned from -100°C at 5°C min^{-1}.

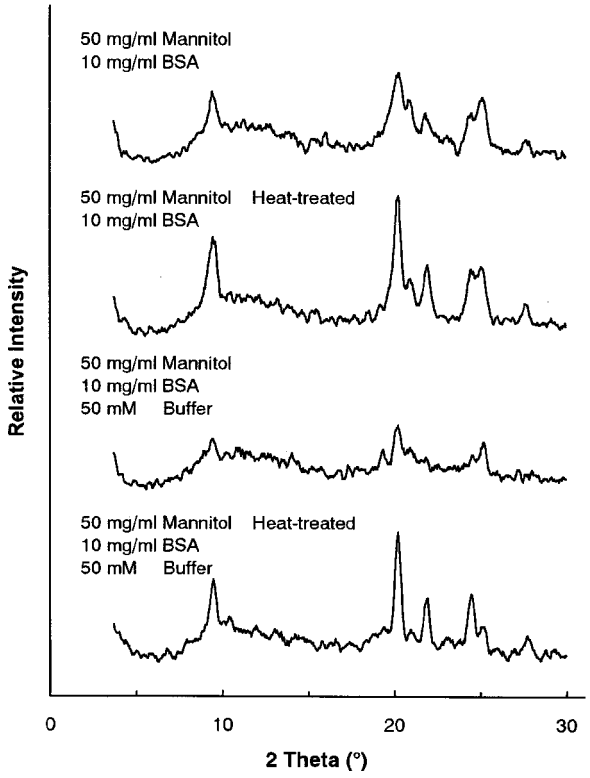

Figure 2 Powder X-ray diffraction patterns of freeze-dried solids containing mannitol, BSA and potassium phosphate buffer. The protein solutions were dried under vacuum immediately after freezing or after heat-treatment at -10°C.

disappear at 50–100 mM buffer. The thermograms indicate that phosphate buffer changes the physical property of the frozen mannitol solutions.

Figure 2 shows powder X-ray diffraction patterns of freeze-dried solids obtained from solutions containing mannitol, BSA and potassium phosphate buffer (0, 50 mM), with or without heat-treatment of the frozen solutions. The patterns show different mannitol crystallinity and similar polymorph composition (mainly δ-form) in each dried solid depending on the cosolute and freeze-drying procedure. The moderate diffraction peaks of the freeze-dried solid containing mannitol and BSA indicate a partial crystallization of mannitol. Heat-treatment of the frozen solution resulted in higher mannitol crystallinity in the freeze-dried solids. Co-lyophilization with both BSA and the buffer reduced mannitol crystallinity, whereas heat-treatment of the frozen solution resulted in extensive mannitol crystallization. The low mannitol crystallinity in freeze-drying without the heat-treatment suggested that the frozen solution was dried below the crystallization temperature of the system. Sucrose remained amorphous in the freeze-dried solids (data not shown).

Figures 3–5 show area-normalized second-derivative amide I infrared spectra of various proteins in initial aqueous solutions and freeze-dried solids. The protein spectra obtained in the aqueous solutions were consistent with literature data (Prestrelski et al 1993; Dong et al 1995; Carpenter et al 1997). The large peaks at 1656 cm^{-1} and 1628 (1638) cm^{-1} denote α-helix and β-sheet structures, respectively (Susi & Byler 1983; Dong & Caughey 1994). BSA and lysozyme consist primarily of an α-helix conformation, whereas a β-sheet is dominant in β-lactoglobulin. Ovalbumin and LDH contain both of the secondary structures. The excipients employed in this study did not largely alter the protein spectra obtained in aqueous solutions (data not shown). Freeze-drying of these proteins without cosolutes broadened the major bands of the second derivative spectra. The reduced intensity of both the large α-helix (BSA, lysozyme) and β-sheet (β-lactoglobulin) bands in the freeze-dried solids indicates perturbation of the protein secondary structure.

The excipients showed different effects in protecting the secondary structure of these proteins. Co-lyophilization of the proteins with the potassium phosphate buffer had only a minor effect on the perturbed protein structure (data not shown). The addition of sucrose provided freeze-dried protein spectra closer to the initial solution structures, indicating that sucrose maintains the protein secondary structure during freeze-drying (Prestrelski et al 1993; Dong et al 1995; Prestrelski et al 1995). The combination of sucrose and the buffer appeared to have a similar effect on the freeze-dried protein structures as sucrose alone (data not shown). The effect of mannitol on the freeze-dried protein structure depended on the cosolute composition and the freeze-drying procedure. Mannitol showed weak effects on maintenance of the protein structure during freeze-drying, whereas the combination of mannitol and the phosphate buffer retained the protein secondary structure to a larger extent. The mannitol and buffer combination produced freeze-dried protein spectra between

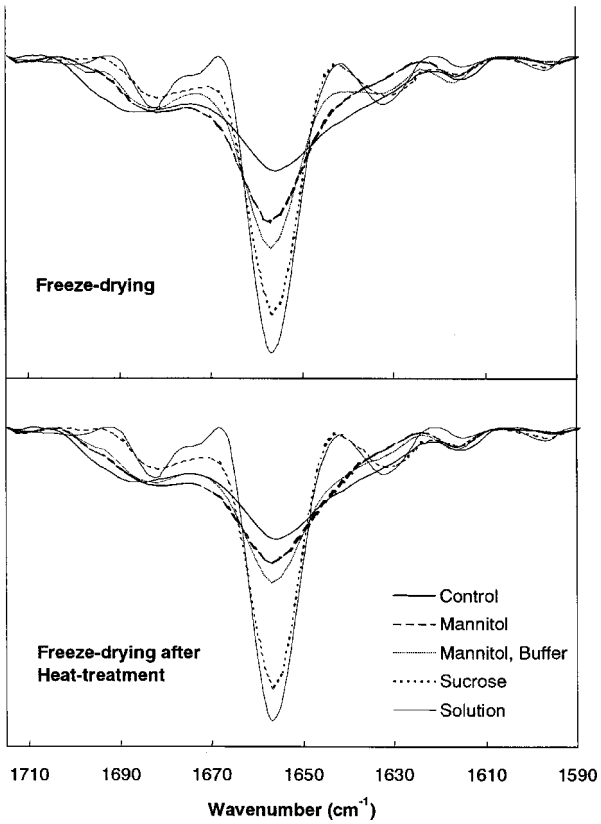

Figure 3 Area-normalized second-derivative infrared spectra of BSA in aqueous solution (10 mg mL^{-1}) and in freeze-dried solids without (control) or with cosolutes (50 mg mL^{-1} mannitol, 50 mg mL^{-1} sucrose, 50 mM potassium phosphate buffer). The proteins were dried under vacuum immediately after freezing or after heat-treatment at −10°C.

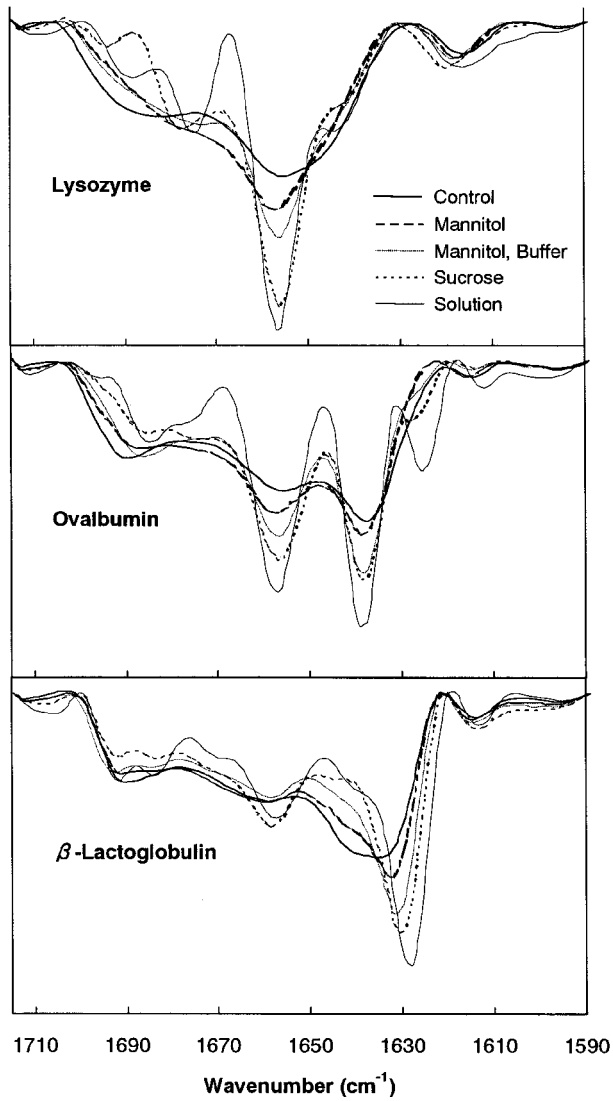

Figure 4 Area-normalized second-derivative infrared spectra of proteins (lysozyme, ovalbumin, β-lactoglobulin) in aqueous solution (10 mg mL^{-1}) and in freeze-dried solids without (control) or with cosolutes (50 mg mL^{-1} mannitol, 50 mg mL^{-1} sucrose, 50 mM potassium phosphate buffer). The proteins were dried under vacuum immediately after freezing.

those of protein co-lyophilized with mannitol and with sucrose. Heat-treatment of the frozen solutions did not significantly alter the spectra of proteins freeze-dried alone or with sucrose, but reduced the stabilizing effect of mannitol (Figures 3 and 5). The proteins co-lyophilized with mannitol, or the combination of mannitol and phosphate buffer, largely lost their original structures in the heat-treated freeze-dried solids. These results emphasize the importance of amorphous mannitol in maintaining protein secondary structure during freeze-drying. These proteins returned to their initial secondary structures in rehydrated solutions (data not shown), indicating the high reversibility of the perturbed protein structure.

Figure 6 shows the effects of cosolutes (mannitol, sucrose, potassium phosphate buffer) on the remaining activity of freeze-dried LDH (50 μg mL^{-1}). The protein retained approximately 40–50% of the initial activity in the absence of cosolutes. Co-lyophilization with phosphate buffer or heat-treatment of the frozen solution was found to have little effect on the activity of freeze-dried LDH. The enzymes lost most of their activity in co-lyophilization with mannitol, whereas the combination of mannitol and potassium phosphate buffer maintained the enzyme activity in freeze-drying. Heat-treatment of the frozen solution significantly reduced LDH activity only in freeze-drying with the combination of mannitol and phosphate buffer, suggesting that increased mannitol crystallinity reduces its stabilizing effects. The reduced LDH activity in freeze-drying with mannitol implied that the mannitol crystallization provides further stress for the enzyme structure. LDH maintained most (approximately 95%) of the original activity in freeze-drying without cosolutes at the higher concentration (2.5 mg mL^{-1}) employed for the FT-IR analysis (data not shown), supporting the high reversibility of the perturbed protein structure. The structure of LDH freeze-dried from the lower concentration initial solution was not available in the FT-IR analysis.

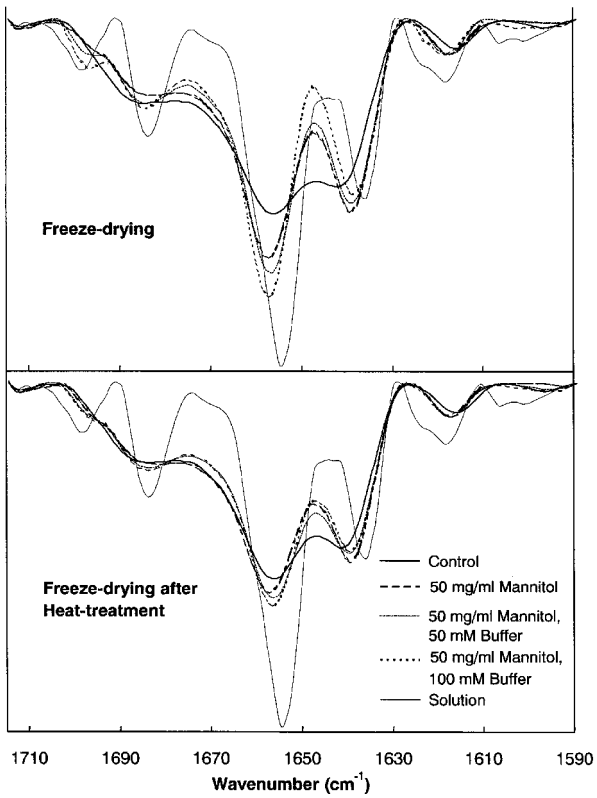

Figure 5 Area-normalized second-derivative infrared spectra of lactate dehydrogenase in aqueous solution (2.5 mg mL^{-1}) and in freeze-dried solids without (control) or with cosolutes (mannitol (50 mg mL^{-1}), sucrose (50 mg mL^{-1}), potassium phosphate buffer (50, 100 mM)). The proteins were dried under vacuum immediately after freezing or after heat-treatment at $-10°C$.

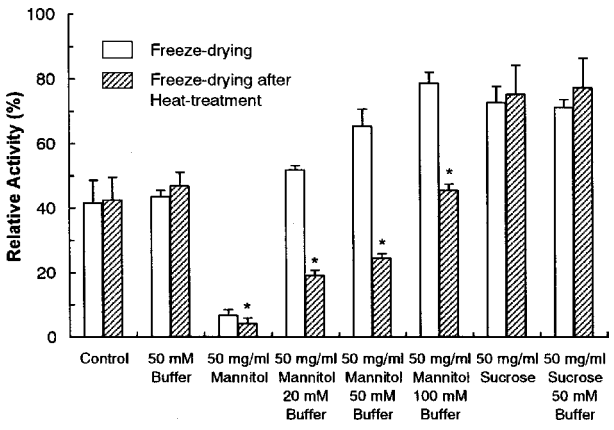

Figure 6 Effect of cosolutes on the activity (%) of freeze-dried lactate dehydrogenase (50 μg mL^{-1}). Error bars represent s.d. (n = 4). $^{*}P < 0.05$, comparing the same dose groups.

Discussion

Mannitol is one of the most popular excipients employed in the freeze-drying of low-molecular-weight pharmaceuticals, as its crystallization results in a good cake structure (Pikal 1999). Complex crystallization behaviour of mannitol in frozen solutions leads to various polymorphs (α, β and δ) and crystallinity in freeze-dried solids, depending on the formulation and processing conditions (Kim et al 1998; Yu et al 1999; Cannon & Trappler 2000). Co-lyophilization with other solutes often reduces the crystallinity of mannitol in freeze-dried solids (Ito 1971; Gatlin & DeLuca 1980; Izutsu et al 1993; Martini et al 1997; Kim et al 1998). The X-ray diffraction patterns and thermograms indicated that co-lyophilization with phosphate buffer reduced the crystallinity of mannitol by changing the thermal property of frozen solutions. Most of the mannitol and phosphate buffer should be freeze-concentrated into an amorphous mixture phase, which may also contain the proteins. The interaction between mannitol and the phosphate salts should reduce the molecular mobility and same-molecular interactions required for mannitol nucleation or crystal growth. Heat-treatment of the frozen solution should induce the mannitol crystallization by increasing the molecular mobility.

Loss of the protein-stabilizing effects of crystallized excipients has been reported in the freeze-drying of various proteins at low concentrations, since the large changes in the biological activity make the stabilizing effects more apparent. Mannitol often produces smaller protective effects on maintenance of the biological activity of asparaginase (Hellman et al 1983), β-galactosidase (Izutsu et al 1993), human growth hormone (Costantino et al 1998) and other proteins (Carpenter et al 1993; Prestrelski et al 1993) during freeze-drying than those of other polyols. This has been attributed to its crystallization. Contrarily, co-lyophilization of mannitol with some cosolutes results in a lower mannitol crystallinity and apparent stabilizing effects against aggregation of freeze-dried proteins (Pikal et al 1991; Cleland et al 2001). Varying mannitol crystallinity in lyophilization with cosolutes suggests enzyme stabilization by amorphous mannitol (Izutsu et al 1993; Jiang & Nail 1998).

Crystallization should deprive mannitol of its direct molecular interaction with proteins (e.g. hydrogen bonding). Amorphous polyols protect proteins by substituting surrounding water molecules that are necessary for structural stabilization (Carpenter & Crowe 1989; Tanaka et al 1991; Crowe et al 1998; Pikal 1999). Insufficient molecular interaction between protein and excipients by steric hindrance or phase separation in amorphous freeze-concentrate also leads to a limited stabilizing effect (Carpenter et al 1997; Randolph 1997; Pikal 1999). Besides the loss of stabilizing effects, mannitol crystallization can affect protein stability by changing the remaining solute composition around proteins or by inducing protein denaturation at the protein/mannitol crystal interfaces.

The results of this study have clarified the fact that crystallinity determines the structure-stabilizing effects of an excipient during freeze-drying under pharmaceutically relevant conditions. The second-derivative FT-IR spectra indicated that sucrose and lower-crystallinity mannitol protect proteins from structural perturbation during freeze-drying, whereas crystallized mannitol has minimal effects.

The structure-stabilizing effects of mannitol paralleled the stabilization of lower-concentration LDH during freeze-drying. The marked reversibility of perturbed protein structure in rehydrated solutions suggests that most of the protein molecules maintain their structural core in the freeze-dried solid, the collapse of which will result in misfolding and aggregation in rehydrated solutions (Dong et al 1995; Prestrelski et al 1995).

The FT-IR analysis provides protein structure in freeze-dried solids at initial protein concentrations often employed for pharmaceutical formulations (Dong et al 1995; Prestrelski et al 1995; Carpenter et al 1997; Costantino et al 1998). Maintaining the native protein structure in freeze-dried formulations is desirable, despite the marked reversibility of the perturbed structure, since it should not only reduce the chances for misfolding in the rehydrated solutions but also improve long-term storage stability of freeze-dried proteins (Prestrelski et al 1995; Chang et al 1996). The protein misfolding or aggregation can have significant effects on the quality of pharmaceutical formulations, since even small amounts of protein aggregates induce possible immunogenic reactions in the human body. Proteins encounter various stresses (e.g. low temperature, dehydration, increasing cosolute concentrations, pH changes) during the freeze-drying process (Carpenter et al 1997; Franks 1998; Pikal 1999). Some stresses (e.g. ice–protein or air–protein interfaces) have a larger effect in a lower initial protein concentration. Information on the protein structure in the freeze-dried solids is useful in designing an appropriate formulation with various excipients (e.g. polyols, surfactants, polymers) that protect proteins through different mechanisms (Manning et al 1989; Anchordoquy & Carpenter 1996; Carpenter et al 1997; Franks 1998; Pikal 1999). The results indicate that amorphous mannitol protects proteins during freeze-drying from the stresses in both of the protein concentrations.

Conclusion

This study showed that the crystallinity of mannitol, a popular excipient, determines its protein-structure-stabilizing effects during freeze-drying. Mannitol was found to crystallize differently during freeze-drying, depending on the cosolute composition and freeze-drying methods. Sucrose and lower-crystallinity mannitol (e.g. a combination of mannitol and phosphate buffer) significantly reduced the structural perturbation of the five proteins examined during freeze-drying. In contrast, highly crystallized mannitol (produced by heat-treatment of frozen solution above the crystallization temperature) resulted in minimal stabilization of the protein secondary structure. The effects of mannitol on the protein secondary structure paralleled the stabilizing effects against inactivation of lower-concentration enzyme (LDH) during freeze-drying. Crystallized mannitol most likely loses its molecular interaction with proteins, which is the key to maintaining protein structure. These results emphasize the importance of proteins and excipients existing in a single amorphous phase for stabilization against stresses arising during freeze-drying.

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