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Freeze-drying of proteins with glass-forming oligosaccharide-derived sugar alcohols

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ABSTRACT

Physical properties and protein-stabilizing effects of sugar alcohols in frozen aqueous solutions and freeze-dried solids were studied. Various frozen sugar alcohol solutions showed a glass transition of the maximally freeze-concentrated phase at temperatures (T_g's) that depended largely on the solute molecular weights. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) formed glass-state amorphous cake-structure freeze-dried solids. Microscopic observation of frozen maltitol and lactitol solutions under vacuum (FDM) indicated onset of physical collapse at temperatures (T_c) several degrees higher than their T_g's. Freeze-drying of pentitols (e.g., xylitol) and hexitols (e.g., sorbitol, mannitol) resulted in collapsed or crystallized solids. The glass-forming sugar alcohols prevented activity loss of a model protein (LDH: lactate dehydrogenase) during freeze-drying and subsequent storage at 50 °C. They also protected bovine serum albumin (BSA) from lyophilization-induced secondary structure perturbation. The glass-forming sugar alcohols showed lower susceptibility to Maillard reaction with co-lyophilized L-lysine compared to reducing and non-reducing disaccharides during storage at elevated temperature. Application of the oligosaccharide-derived sugar alcohols as alternative stabilizers in lyophilized protein formulations was discussed.

1. Introduction

Freeze-drying is a popular method to ensure long-term stability of therapeutic proteins that are not stable enough in the aqueous solutions during distribution and long-term storage (Manning et al., 1989; Nail et al., 2002). Removal of surrounding water molecules by lyophilization significantly reduces gradual chemical and physical degradation of proteins, whereas the dehydration often induces structural perturbation that leads to misfolding and/or aggregation in the re-hydrated solutions (Arakawa et al., 2001). Protecting the protein conformation in freeze-dried formulations through appropriate process control and ingredient optimization is essential to ensure the pharmacological effects, as well as to reduce the risk of product immunogenicity (Hermeling et al., 2004).

Some non-reducing saccharides (e.g., sucrose, trehalose) are popular stabilizers that protect proteins from the chemical and physical degradations in aqueous solutions, during freeze-drying, and in subsequent storage (Arakawa and Timasheff, 1982; Carpenter and Crowe, 1989; Franks, 1992; Wang, 2000). They protect protein conformation in the solids thermodynamically through direct interactions (e.g., hydrogen bonds) that substitute surrounding water molecules and reduce protein chemical degradation kinetically by embedding the protein in a glass-state lower molecular mobility environment. High molecular mobility of the glass-state disaccharide solids, however, induces slow but not negligible chemical degradation of embedded proteins over pharmaceutically relevant timescales. Unexpected exposure of the solids to humid or high-temperature environments often induces physical changes of the dried cakes (e.g., shrinkage) (Breen et al., 2001; Tian et al., 2007).

Application of other excipients that stabilize proteins by themselves and/or in combination with disaccharides would provide further choices to improve the formulation quality (Wang, 2000; Costantino, 2004). Some excipients (e.g., polymers, sodium phosphates) raise the glass transition temperature (T_g) of co-lyophilized disaccharide-based solids, which limited molecular mobility should confer robustness against undesirable storage conditions (Ohtake et al., 2004). Some amino acids and their salts (e.g., L-arginine citrate) form glass-state amorphous solids that protect proteins from inactivation during freeze-drying (Tian et al., 2007; Izutsu et al., 2009). In addition to the structural stabilization, varied physicochemical properties of amino acids would provide some unique effects (e.g., reducing the protein aggregation in aque-

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ous solutions) that are preferable for pharmaceutical formulations (Arakawa et al., 2007).

The purpose of this study was to systematically examine the physical properties and protein-stabilizing effects of oligosaccharide-derived sugar alcohols for their application in freeze-dried formulations. It has been established that various sugar alcohols (e.g., sorbitol, xylitol, lactitol) protect proteins from heat-induced denaturation in aqueous solutions through a thermodynamic mechanism (preferential exclusion) identical to that of saccharides and other polyols (e.g., glycerol) (Arakawa and Timasheff, 1982; Gekko, 1982). Some pentitols and hexitols (e.g., xylitol, sorbitol) protect biological macromolecules (e.g., proteins) and microorganisms from inactivation and/or viability loss during freeze-thawing and during freeze-drying (Tamoto et al., 1961; Carpenter and Crowe, 1988). Varied physical properties (i.e., crystallinity, molecular mobility) have been considered as key factors that determine effects of sugar alcohols to stabilize proteins in frozen solutions and freeze-dried solids (Griebenow and Klibanov, 1995; Carrasquillo et al., 2000; Liao et al., 2002). For example, high propensity to crystallize in the frozen solution (e.g., mannitol) or to collapse during primary drying (e.g., sorbitol, xylitol) makes them inappropriate for main stabilizer in freeze-drying. Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) should have greater opportunities to structurally and kinetically stabilize proteins during freeze-drying and subsequent storage. Maltitol and lactitol are popular excipients for oral (tablet) formulations, and are also widely used in food industries as glass-forming additives upon cooling of edible hot-melt compositions (Slade et al., 2006). Information on the physical properties (e.g., thermal transition temperatures) and protein-stabilizing effects (e.g., enzyme activity, protein secondary structure) should be relevant in the application of sugar alcohols to the freeze-dried formulations.

2. Materials and methods

2.1. Materials

All chemicals employed in this study were of analytical grades and were obtained from the following commercial sources: L-lactic dehydrogenase (LDH, rabbit muscle), bovine serum albumin (BSA, essentially fatty acid free), glucose, trehalose dihydrate, sorbitol, and sucrose (Sigma Chemical, St. Louis, MO); maltitol, maltotriitol and maltotetraitol (Hayashibara Biochemical Laboratories, Okayama, Japan); maltose, lactose, mannitol, xylitol, lactitol monohydrate, and other chemicals (Wako Pure Chemical, Osaka, Japan); methanol dehydrate (Kanto Kagaku, Tokyo, Japan). The protein solutions were dialyzed against 50 mM sodium phosphate buffer (pH 7.0), and then centrifuged (1500 g × 5 min) and filtered (0.45 μm PVDF filters, Millipore, Bedford, MA) to remove insoluble aggregates before the freeze-drying study.

2.2. Freeze-drying

A freeze-drier (FreeZone-6; Labconco, Kansas City, MO) was used for lyophilization. Aliquots (0.3 ml) of aqueous solutions in flat-bottom glass vials (13 mm diameter, SVF-3; Nichiden-Rika Glass, Kobe, Japan) were placed on the shelf of the lyophilizer. The shelf was cooled to −40 °C at 0.5 °C/min, and then maintained at this temperature for 2 h before the primary drying process. The frozen solutions were dried under a vacuum (4.0 Pa) while maintaining the shelf temperature at −40 °C for 15 h, −30 °C for 6 h, and 35 °C for 6 h. The shelf was heated at 0.2 °C/min between the thermal steps. The vials were closed with rubber stoppers under a vacuum.

2.3. Thermal analysis

Thermal analysis of frozen solutions and dried solids was performed by using a differential scanning calorimeter (Q-10; TA Instruments, New Castle, DE) and software (Universal Analysis 2000; TA Instruments). Aliquots of aqueous solutions (10 μl) in hermetic aluminum cells were cooled from room temperature to −70 °C at 10 °C/min, and then scanned by heating at 5 °C/min. Freeze-dried solids (1–2 mg) in hermetic aluminum cells were subjected to the thermal analysis from −20 °C at 5 °C/min under a nitrogen gas flow. Cooled-melt saccharide and sugar alcohol solids obtained by a brief period of heating (1 min at 160 °C for maltose monohydrate, xylitol, sorbitol, maltitol, and lactitol monohydrate; at 180 °C for glucose; at 200 °C for sucrose, mannitol, and maltotriitol; and at 220 °C for lactose and trehalose monohydrate) and subsequent rapid cooling (−50 °C) in hermetic aluminum cells were scanned at 5 °C/min to obtain the glass transition temperatures. The glass transition temperatures were determined as the maximum inflection point of the discontinuities in the heat flow curves.

2.4. Freeze-drying microscopy (FDM)

We observed the behavior of frozen aqueous excipient solutions under a vacuum using a freeze-drying microscope system (Lyostat2; Biopharma Technology, Winchester, UK) with an optical microscope (BX51; Olympus, Tokyo). Aqueous solutions (2 μl) sandwiched between cover slips (70 μm apart) were frozen at −40 °C and then maintained at that temperature for 5 min. Each sample was heated under a vacuum (12.9 Pa) at 5 °C/min to a temperature approximately 5 °C below its T_g' as obtained by thermal analysis, and then scanned at an angle speed of 1 °C/min after reaching T_g'. The collapse onset temperature (T_c) of the frozen solution was determined from the first appearance of translucent dots behind the ice sublimation interface ($n = 3$).

2.5. Powder X-ray diffraction (XRD) and residual water measurements

The powder X-ray diffraction patterns were measured at room temperature by using a Rint-Altima diffractometer (Rigaku, Tokyo, Japan) with Cu Kα radiation at 40 kV/40 mA. The samples were scanned in the area of $5^\circ < 2\theta < 35^\circ$ at an angle speed of 5°/min. The lyophilized solids were suspended in dehydrated methanol to obtain residual water by a volumetric Karl-Fischer titrator (AQV-6; Hiranuma Sangyo, Ibaraki, Japan). Residual water contents were shown as ratios (%) to the estimated solid weights in the vials.

2.6. Freeze-drying and activity measurement of LDH

Aqueous solutions (0.5 ml) containing LDH (0.05 mg/ml), excipients (100 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0) were lyophilized in the flat-bottom glass vials. Some freeze-dried solids plugged with rubber stoppers were stored at 50 °C for 7 days in a temperature chamber (Model SH-221, Espec, Osaka, Japan). Pyruvate and NADH were used as substrates to obtain LDH activity from the absorbance reduction at 340 nm (25 °C). Residual enzyme activity was shown as the ratio (%) to that of the solution before freezing ($n = 6$) (Izutsu et al., 1994).

2.7. Fourier-transform infrared (FT-IR) analysis of freeze-dried BSA

A Fourier-transform infrared spectrophotometer (MB-104; Bomen, Quebec, Canada) with a dry gas generator (Balston, Haverhill, MA) and software (PROTA; BioTools, Jupiter, FL and GRAMS/32; Galactic Ind., Salem, NH) was used to obtain mid-infrared spectra of

Table 1
Physical properties of saccharides and sugar alcohols in frozen solutions and freeze-dried solids.

	Excipient				Excipient + BSA + Buffer		
	Frozen solution	Freeze-dried solid		Cooled-melt Solid	Frozen solution	Freeze-dried solid	
	T_g' (°C)	T_g (°C)	Residual water (%, w/w)	T_g (°C)	T_g' (°C)	T_g (°C)	Residual\water (%, w/w)
w/o excipients					n.d.	n.d.	6.3 ± 0.4
Glucose	−42.7 ± 0.5	Collapsed	–	37.3 ± 0.8	−41.4 ± 1.6	41.5 ± 2.0	2.6 ± 0.4
Lactose	−29.1 ± 0.1	90.9 ± 6.6	1.2 ± 0.1	112.0 ± 1.9	−27.8 ± 1.8	105.3 ± 2.2	1.0 ± 0.4
Sucrose	−33.5 ± 0.1	62.0 ± 2.6	1.8 ± 0.9	46.4 ± 0.3	−32.0 ± 0.7	68.2 ± 0.8	1.9 ± 0.0
Maltose monohydrate	−31.1 ± 0.0	86.2 ± 1.1	0.9 ± 0.0	68.8 ± 1.5	−28.8 ± 1.6	95.6 ± 1.2	1.2 ± 0.4
Trehalose dihydrate	−30.6 ± 0.1	80<	1.0 ± 0.2	117.3 ± 0.3	−27.4 ± 0.5	90<	1.2 ± 0.4
Xylitol	−48.5 ± 0.5	Collapsed	–	−21.9 ± 0.2	−45.9 ± 1.2	Collapsed	–
Sorbitol	−45.0 ± 0.4	Collapsed	–	−1.9 ± 0.2	−39.9 ± 0.7	Collapsed	–
Mannitol	Crystallized	Crystallized	–	Crystallized	Crystallized	Partially Crystallized	–
Maltitol	−36.7 ± 0.2	40.6 ± 0.4	1.1 ± 1.1	47.3 ± 0.8	−35.7 ± 0.6	56.3 ± 1.0	1.3 ± 0.4
Lactitol monohydrate	−31.8 ± 0.1	54.9 ± 2.5	0.3 ± 0.5	48.4 ± 3.3	−29.2 ± 1.5	63.3 ± 1.9	1.2 ± 0.2
Maltotriitol	−29.5 ± 0.0	72.8 ± 2.8	0.3 ± 0.2	88.6 ± 0.8	−26.6 ± 0.7	85.3 ± 3.1	1.5 ± 0.2
Maltotetraitol	−24.9 ± 0.2	n.d.	1.2 ± 0.3	–	−24.8 ± 0.3	n.d.	0.9 ± 0.1

Average ± s.d. (n = 3).

BSA in the aqueous solution and freeze-dried solids (Prestrelski et al., 1993; Dong et al., 1995; Izutsu et al., 2004). Spectra of aqueous BSA solutions (10 mg/ml in 50 mM sodium phosphate buffer, pH 7.0) were recorded at 4 cm^{-1} resolution using infrared cells with CaF_2 windows and 6 μm film spacers (256 scans). Spectra of freeze-dried BSA solids were obtained from pressed disks containing the sample (approximately 1 mg BSA) and dried potassium bromide (approx. 250 mg). Area-normalized second-derivative amide I spectra (1600–1715 cm^{-1}, 7-point smoothing) were employed to elucidate the integrity of the protein secondary structure.

2.8. Non-enzymatic color development of freeze-dried solids

Lyophilized solids containing L-lysine (5 mg/ml) and a saccharide or a sugar alcohol (100 mg/ml, 0.3 ml) were stored at 80 °C for 4 days. Changes in the absorbance (280 nm) of the re-hydrated solutions (5-times diluted) were obtained by using a UV–visible spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan).

3. Results

3.1. Physical property of frozen solutions

Most of the frozen aqueous solutions containing a saccharide or a sugar alcohol (100 mg/ml) showed typical thermograms that indicated an amorphous freeze-concentrated phase surrounding ice crystals (Table 1). An increase in the solute molecular weight shifted the glass transition of the maximally freeze-concentrated phase (T_g') to higher temperatures, which trend was consistent with literature (Levine and Slade, 1988). Addition of BSA (10 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0) raised the T_g' of the frozen excipient solutions except for that of maltotetraitol. The frozen mannitol solution showed an exotherm peak that indicated eutectic crystallization at around −23 °C (Cavatur et al., 2002).

Freeze-drying of some disaccharides or oligosaccharide-derived sugar alcohol solutions (trehalose, sucrose, maltitol, lactitol, maltotriitol) resulted in cake-structure solids. Conversely, frozen solutions containing smaller solute molecules (e.g., glucose, sorbitol, xylitol, $T_g' < -40$ °C) collapsed during the process. Addition of BSA prevented glucose from physical collapse during the freeze-drying process. Freeze-drying microscopy indicated dynamic changes of frozen solutions under vacuum (Fig. 1). Heating of a frozen lactitol solution showed ice sublimation from the upper right corner of the image, leaving a structurally ordered dark dried region behind (−30 °C). Further heating induced transparent dots that indicated loss of the local structure (collapse onset temperature, T_c: −27.8 ± 0.3 °C), followed by larger structural damage. Other frozen solutions also showed T_cs (trehalose: −24.3 ± 0.7 °C; maltitol: −31.2 ± 2.1 °C) several degrees higher than their T_g's.

3.2. Characterization of freeze-dried solids

The physical properties of the freeze-dried solids were studied by thermal analysis, powder X-ray diffraction, and residual water measurement. Thermal analysis showed glass transition of some lyophilized oligosaccharide-derived sugar alcohol solids (maltitol, lactitol, maltotriitol) at above room temperature (Table 1) (Shirke et al., 2005). No apparent transition was observed in freeze-dried maltotetraitol solid. Some freeze-dried disaccharides (e.g., lactose, maltose) showed T_gs higher than those of the structurally relating sugar alcohols. Freeze-dried solids containing BSA, buffer components, and disaccharides or oligosaccharide-derived sugar alcohols (maltitol, lactitol, maltotriitol) showed halo powder X-ray diffraction (XRD) patterns typical for non-crystalline solids (Fig. 2). Some peaks in the XRD pattern, as well as the combination of an exotherm peak (51.7 °C) and an exotherm peak (164.1 °C) in the thermogram, indicated partially crystallized mannitol lyophilized with BSA and the buffer salts. The small peaks in the XRD patterns also suggested partial crystallization of glucose and sorbitol during the freeze-drying process and/or during sample preparation for the analysis. The residual water contents of the cake-structure dried solids were less than 2%. The protein lyophilized without the stabilizing excipients showed higher residual water contents.

3.3. Effects on protein stability

The effects of the oligosaccharide-derived sugar alcohols on the protein stability during the freeze-drying process and subsequent storage were studied through the enzyme activity (LDH) and secondary structure (BSA) measurements. The enzyme (0.05 mg/ml) freeze-dried from the sodium phosphate buffer solution (50 mM, pH 7.0) retained approximately 60% of its initial activity (Fig. 3). The disaccharides and oligosaccharide-derived sugar alcohols (100 mg/ml sucrose, trehalose, maltitol, lactitol, maltotriitol) protected LDH from the activity loss during freeze-drying. In contrast, sorbitol and mannitol did not show any apparent effect on the co-lyophilized enzyme activity. The enzyme lyophilized with sorbitol or in the absence of polyols lost most of its activity during storage at 50 °C for 7 days. The disaccharides and oligosaccharide-

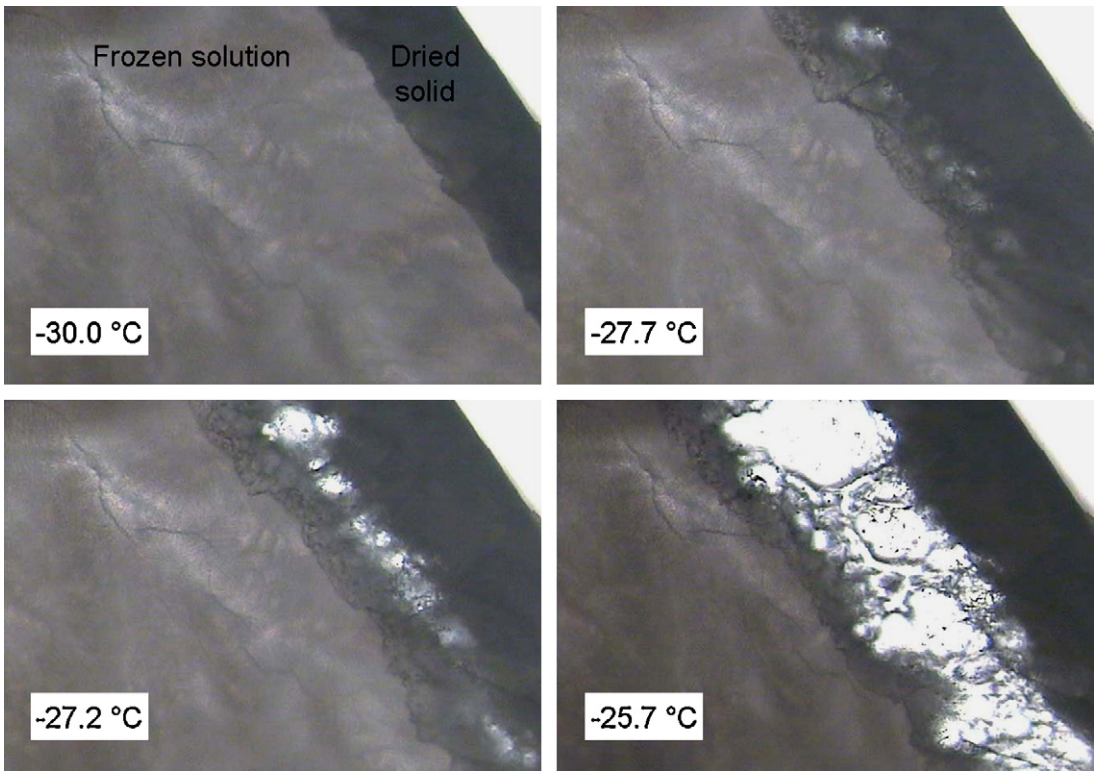

Fig. 1. Freeze-drying microscopy images of a frozen lactitol solution (100 mg/ml) obtained during a heating scan (1 °C/min). The frozen solution (2 μl) in a thin cell was dried under a vacuum (12.9 Pa) from the upper right corner of the figures.

derived sugar alcohols retained the enzyme activity during the high-temperature storage. The freeze-dried maltitol formulation shrunk during the storage near its glass transition temperature. The enzyme lyophilized with mannitol retained its activity to some extent in the largely crystallized solid during the high-temperature storage.

The effects of the saccharides and sugar alcohols on the secondary structure of freeze-dried BSA were studied (Fig. 4). The area-normalized second-derivative amide I spectra of BSA in the sodium phosphate buffer solution (50 mM, pH 7.0) showed a large band at 1656 cm^{-1} that denoted a predominant α-helix structure in the native conformation (Dong et al., 1995). Lyophilization of the protein from the buffer resulted in a reduction of the α-helix band intensity and broadened the overall spectra, indicating a perturbed secondary structure (Prestrelski et al., 1993). Maltitol and

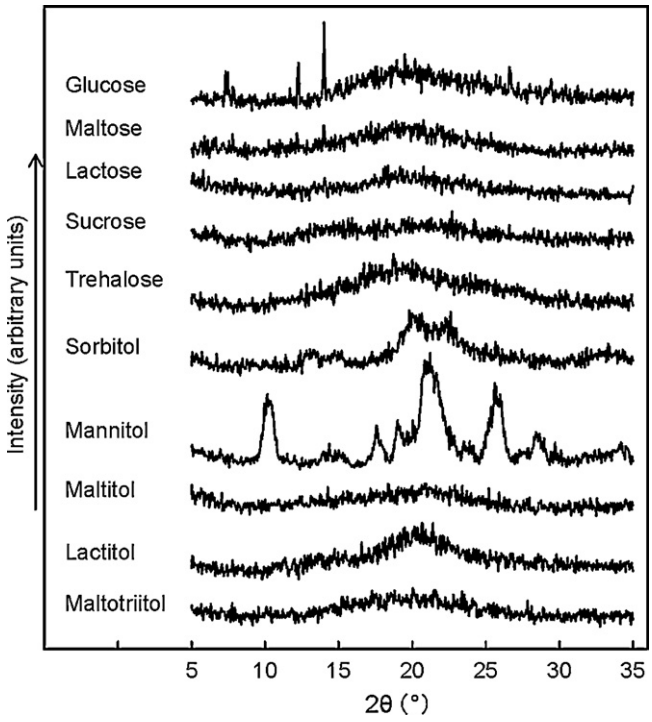

Fig. 2. Powder X-ray diffraction patterns of solids freeze-dried from solutions containing BSA (10 mg/ml), excipient (100 mg/ml) and sodium phosphate buffer (50 mM, pH 7.0).

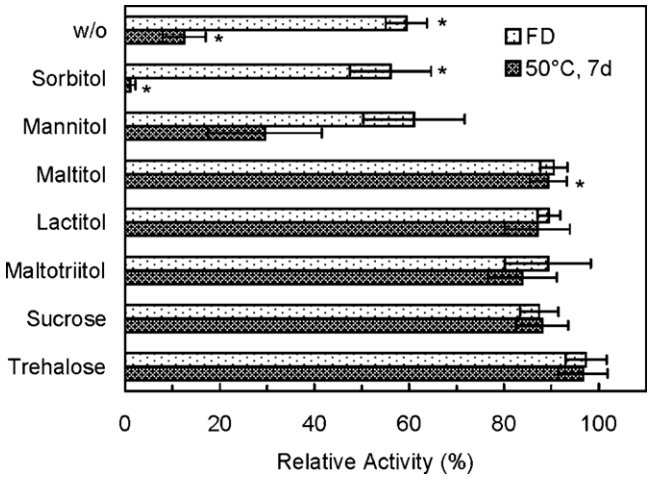

Fig. 3. Effects of excipients on the relative activity of rabbit muscle lactate dehydrogenase after freeze-drying and subsequent storage at 50 °C for 7 days ($n = 3$). Aqueous solutions containing LDH (0.05 mg/ml), excipient (100 mg/ml) and sodium buffer salt (50 mM, pH 7.0) were freeze-dried in glass vials. Asterisks indicate collapsed or shrunk solids.

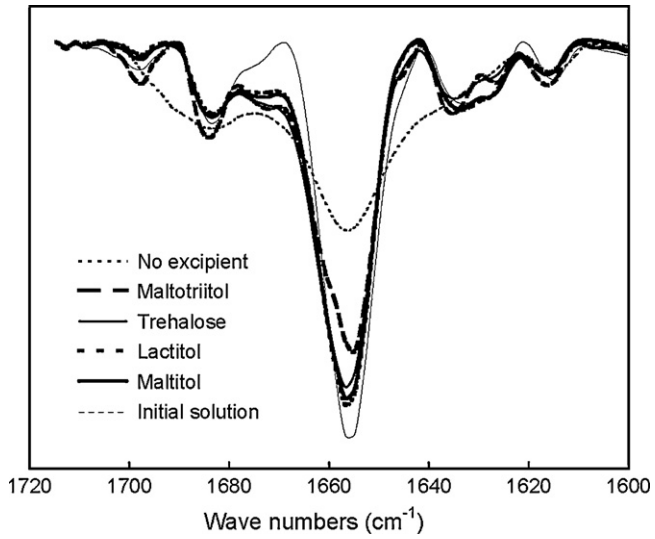

Fig. 4. Area-normalized second-derivative amide I spectra of BSA (10 mg/ml) in a sodium phosphate buffer solution (50 mM, pH 7.0) and in solids freeze-dried with or without co-solutes (100 mg/ml).

lactitol were as effective as trehalose at retaining the conformation of the co-lyophilized protein. The smaller α-helix band of the protein lyophilized with maltotriitol suggested insufficient structure stabilization.

3.4. Chemical stability in freeze-dried solids

The possible reactivity of the sugar alcohols with proteins (e.g., Maillard reaction) in the dried solids was studied by using model freeze-dried systems containing the excipients and L-lysine (Fig. 5) (Kawai et al., 2004). The co-lyophilized solids maintained the cake-structure (e.g., trehalose, lactose) or shrunk (other excipients) during the storage at an elevated temperature (80 °C for 4 days). The solids turned brown to varying degrees irrespective of the solid structure. The high-temperature storage of solids containing the reducing saccharides (glucose, maltose, lactose) and L-lysine induced apparent absorbance changes of the re-hydrated solutions (3 < Abs.280, data not shown). The oligosaccharide-derived sugar alcohols (maltitol, lactitol, maltotriitol) showed lower chemical reactivity with co-lyophilized L-lysine

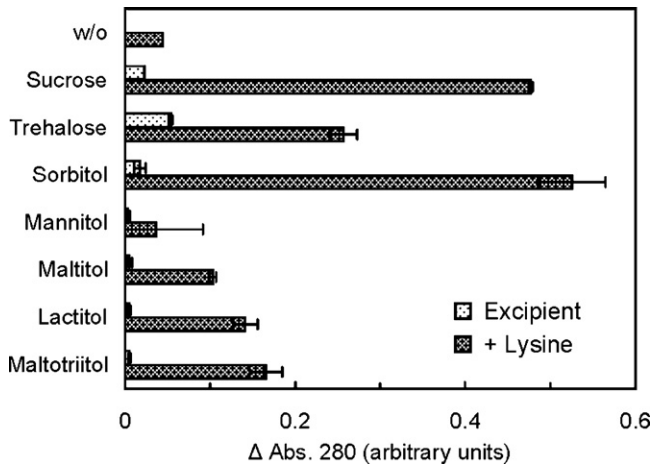

Fig. 5. Effect of storage (80 °C, 3 days) on non-enzymatic color development of solids freeze-dried from solutions containing L-lysine (5 mg/ml) and saccharides or sugar alcohols (100 mg/ml). Changes in the absorbance of re-hydrated solutions were obtained at 280 nm ($n = 3$).

compared to the non-reducing saccharides (sucrose, trehalose). Lower absorbance suggested limited reactivity of the partially crystallized mannitol in the dried solids.

4. Discussion

The results indicated the relevance of some oligosaccharide-derived sugar alcohols as principal stabilizers in the freeze-drying of proteins. An improved understanding the varied physical properties and protein-stabilizing mechanisms in the frozen solutions and freeze-dried solids, in comparison with those of disaccharides, will be indispensable for the rational application of the sugar alcohols.

The thermal transition and collapse onset temperatures (T_g', T_c) of frozen disaccharide-derived sugar alcohol solutions, comparable with those of structurally related saccharides, should allow freeze-drying by ordinary lyophilizers that are designed to cool their shelves down to −40 °C. Decreasing local viscosity of non-crystalline concentrated solute phases above the thermal transition (T_g') induces physical collapse from the drying interface (Pikal and Shah, 1990; Meister and Gieseler, 2009). The collapsed solids are not usually pharmaceutically acceptable because of their inelegant appearance and other changes in their physical properties (e.g., higher residual water, component crystallization) (Costantino et al., 1998). Controlling the shelf temperature to achieve a product slightly below T_g' or T_c (maximum allowable product temperatures) is usually recommended for efficient ice sublimation without collapse, since the ice sublimation speed increases significantly depending on the temperature (approx. 13% at 1 °C interval) (Pikal and Shah, 1990; Nail et al., 2002). Frozen saccharide solutions often show a T_c several degrees higher than the corresponding T_g', which difference depends on various factors, including the component composition and measurement methods (e.g., vacuum pressure, cell structure, type of microscope). Technical difficulties in distinguishing the changes at the collapse onset may partly explain the relatively large difference between the T_g' and T_c in the higher concentration (100 mg/ml) frozen excipient solutions.

The disaccharides and oligosaccharide-derived sugar alcohols formed cake-structure glass-state solids upon freeze-drying. Varied solid densities, degradation products, and residual water contents originating from the hydrated crystals may explain the different T_gs of some excipients prepared by freeze-drying and quench-cooling of the heat-melt. Addition of BSA and the buffer salts raised the transition temperature of the frozen solutions (T_g') and the dried solids (T_g) containing the oligosaccharide-derived sugar alcohols, suggesting their molecular-level mixing in the freeze-dried solid. Possible large molecular mobility during primary (low T_g' of frozen solutions) and/or secondary (low T_g of partially dried solids) drying processes should explain the partial crystallinity of glucose and sorbitol in the solids (Piedmonte et al., 2007).

The retention of the enzyme activity (LDH) and secondary structure (BSA) indicated that the oligosaccharide-derived sugar alcohols protected the proteins against stresses in each step of the freeze-drying process. LDH is a typical enzyme that irreversibly loses its activity by freeze-thawing and freeze-drying-induced subunit dissociation and conformation changes (Jaenicke, 1990; Anchordoquy et al., 2001; Bhatnagar et al., 2008). Various sugar alcohols (e.g., sorbitol, xylitol, maltitol) favor the native conformation of proteins over the unfolded states in the aqueous solutions in the same thermodynamic mechanism with those of saccharides (e.g., preferential exclusion) (Arakawa and Timasheff, 1982; Gekko, 1982; Gekko and Idota, 1989). In addition to the stabilization of aqueous proteins prior to freeze-drying and after re-hydration, some sugar alcohols (e.g., xylitol, sorbitol) is considered to protect proteins from low-temperature-induced conformational changes in frozen solutions through the thermodynamic mechanism (Carpenter and Crowe, 1988; Arakawa et al., 2001). Sta-

bilization of proteins and cell membranes makes sorbitol a popular additive for food cryopreservation (e.g., minced fish meat) (Suzuki, 1981).

Extent of conformation changes by dehydration during secondary drying usually determines the lyophilization-induced protein inactivation (Jiang and Nail, 1998). The oligosaccharide-derived sugar alcohols (e.g., lactitol, maltitol) should substitute water molecules surrounding proteins that are essential to maintain the conformation during the freeze-drying process, as has been reported in oligosaccharides (Carpenter and Crowe, 1989). Insufficient number of water-substituting hydrogen bonds due to steric hindrance may explain the smaller structure-stabilizing effect of maltotriitol compared to maltitol and lactitol. Similar reductions of the structure-stabilizing effects have been reported in some larger oligosaccharides (e.g., maltotriose, maltotetraose, maltopentaose) and polysaccharides (e.g., dextran) (Tanaka et al., 1991; Izutsu et al., 2004). Crystallization during the freeze-drying process and storage deprives some sugar alcohols (e.g., mannitol, sorbitol) of the water-substituting molecular interaction (Izutsu et al., 1993; Cavatur et al., 2002; Piedmonte et al., 2007). Some non-crystallizing pentitols and hexitols (e.g., sorbitol) can provide additional protein-stabilizing water-substituting interactions in the co-lyophilization with some glass-forming or crystallizing excipients (Chang et al., 2005). Crystallization of mannitol in the frozen mixture solutions allows fast lyophilization that results in cake-structure microporous solids and dispersing amorphous regions containing proteins and protein-stabilizing excipients (e.g., sucrose) (Johnson et al., 2002).

The glass-state freeze-dried oligosaccharide-derived sugar alcohol solids should also protect embedded proteins from the chemical and physical degradation during storage. The high T_g and sufficient water-substituting interactions should make lactitol a preferable protein stabilizer over maltitol and maltotriitol for long-term storage of lyophilized solids (Hancock et al., 1995). The lower T_g amorphous solids are prone to faster chemical degradation and physical changes by the larger molecular mobility during storage and occasional exposure to temperatures above their T_g. Our present results also indicate the superior robustness of freeze-dried trehalose against the high-temperature stresses over the other saccharides and sugar alcohols studied. Co-lyophilization with some high T_g excipients (e.g., polymers) or excipients that intensify molecular interactions between stabilizing excipients (e.g., sodium phosphate) should be a potent method to raise the T_g of the amorphous sugar alcohol solids (Miller et al., 1998; Ohtake et al., 2004). The low enzyme activity remaining in the stored mannitol formulation suggested protection of the protein by rubber-state amorphous mannitol moiety dispersed in the physically stable crystalline cake.

In addition to the water-substitution and glass-embedding mechanisms, the oligosaccharide-derived sugar alcohols should protect protein structure in several other ways. They should dilute the non-ice phase in frozen solutions, and thus prevent protein denaturation by various stresses, including excess concentration of unfavorable co-solutes (e.g., inorganic salt), pH change by buffer opponent crystallization, and contact with ice surfaces. The sugar alcohols should also prevent crystallization of co-lyophilized saccharides (e.g., sucrose) during storage (Bhugra et al., 2007). The higher exclusion volume of larger sugar alcohol molecules (e.g., maltotriitol) should help to retain the integrity of the quaternary structure of LDH against the low-temperature-induced subunit dissociation that leads to irreversible structural change (Jaenicke, 1990; Anchordoquy et al., 2001).

The suggested lower susceptibility for the Maillard reaction should be an advantage to applying the oligosaccharide-derived sugar alcohols for freeze-drying of chemically labile proteins. The Maillard reaction, which often appears as non-enzymatic browning, is one of the major pathways of protein chemical degradation that also leads to biological activity loss (Manning et al., 1989; Kawai et al., 2004). The lower hydrolysis rate compared to some oligosaccharides should explain the limited reactivity of the sugar alcohols (Desai et al., 2007). Sucrose tends to be degraded into reactive reducing monosaccharides (glucose, fructose), as well as highly reactive fructofuranosyl cations during storage (Perez Locas and Yaylayan, 2008).

The oligosaccharide-derived sugar alcohols should be potent options in the formulation design as principal stabilizers that alternate disaccharides and/or an additional excipient to optimize the physical properties of the disaccharide-based formulations. Excipients appropriate for a particular therapeutic protein should vary depending on their chemical and physical stability, as well as their intended use. Further information on the safety and long-term protein stability would facilitate application of the oligosaccharide-derived sugar alcohols for freeze-dried protein formulations.

5. Conclusion

Some oligosaccharide-derived sugar alcohols (e.g., maltitol, lactitol, maltotriitol) formed glass-state amorphous cake-structure solids that protect model proteins from secondary structure perturbation (BSA) and activity loss (LDH) during freeze-drying and subsequent storage. Thermal and FDM analysis indicated applicability of ordinary lyophilizer for their freeze-drying without physical collapse during the process. The dried sugar alcohol solids have lower glass transition temperatures than the structurally related oligosaccharides, whereas lower susceptibility to Maillard reaction during storage should be an apparent advantage for particular applications.

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