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Effects of sodium tetraborate and boric acid on nonisothermal mannitol crystallization in frozen solutions and freeze-dried solids

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Abstract

The purpose of the present study was to elucidate the effects of sodium tetraborate (borax) and boric acid on the crystallization of mannitol in frozen aqueous solutions and freeze-dried solids. Thermal analysis of frozen solutions showed that sodium tetraborate inhibits mannitol crystallization at sodium tetraborate/mannitol molar concentration ratios of approximately 0.05, which is much lower than the other co-solutes studied (boric acid, sucrose, sodium phosphate buffer). Inhibition of the mannitol crystallization in frozen solutions resulted in highly amorphous mannitol in the freeze-dried solids. Mannitol remained in an amorphous state in some of the combination freeze-dried solids, even at elevated temperatures. Changes in the thermal transition temperatures (glass transition temperature of maximally freeze-concentrated solute (T_g') and glass transition temperature of freeze-dried solid (T_g)) suggested reduced mannitol molecular mobility with increases in the sodium tetraborate ratio. Fourier-transform infrared spectroscopy (FT-IR) analysis of the bovine serum albumin secondary structure showed apparent protein structure-stabilizing effects of the amorphous mannitol and sodium tetraborate combination during the freeze-drying process. The mannitol and sodium tetraborate combination also protected lactate dehydrogenase (LDH) from inactivation during freeze-drying. We conclude that the complex formation and the accompanying reduction in molecular mobility make sodium tetraborate an effective mannitol crystallization inhibitor in frozen solutions and freeze-dried solids.

Keywords: Freeze-drying; Crystallization; DSC; Amorphous; Complexation

1. Introduction

Mannitol is a popular excipient commonly used in freeze-dried formulations. The intrinsic tendency of mannitol to crystallize in frozen solutions and during freeze-drying processes makes it a good bulking agent that grants efficient processing and pharmaceutically preferable, physically stable freeze-dried cakes (Nail et al., 2002). Mannitol crystallization profiles, including those of its various polymorphs, have been studied extensively (Martini et al., 1997; Kim et al., 1998; Yu et al., 1999; Burger et al., 2000; Kett et al., 2003). Despite its propensity for crystallization, mannitol shows different crystallinity depending on the formulation components and procedures. Amorphous-state mannitol possesses some physical and functional properties different from those of crystallized solids (Pikal et al., 1991; Izutsu et al., 1994; Hancock and Zografi, 1997; Martini et al., 1997; Crowe et al., 1998; Johnson et al., 2002; Nail et al., 2002). For example, amorphous-state mannitol protects protein conformations during freeze-drying through hetero-solute

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molecular interaction with proteins (e.g. hydrogen bonding). Chemical stability in extreme pH may provide the amorphous mannitol some advantages among various polyols and saccharides (Telang et al., 2003). It also serves as a good model to control the component crystallinity by formulation and process design.

Several approaches have been taken to produce amorphous-state mannitol-containing solids. Obtaining amorphous pure mannitol by rapid cooling of a hot-melt solution or freeze-drying aqueous mannitol solution are possible, but not practical, approaches because the amorphous mannitol is physically unstable, and mannitol molecules will transform to a more stable crystalline form even under ambient conditions (Telang et al., 2003; Yoshinari et al., 2003). Another and more practical approach is the freeze-drying or rapid cooling of mannitol and other amorphous component combinations (Pyne et al., 2002; Telang et al., 2003). Molecular-level mixing with other components may limit spatial rearrangement of the mannitol molecules required for crystallization. Various solutes that remain amorphous in frozen solutions and freeze-dried solids inhibit mannitol crystallization at high-concentration ratios (e.g., sucrose > 70%, w/w) (Kim et al., 1998). Some salts (e.g. NaCl) also inhibit mannitol crystallization from frozen solutions, although the underlying mechanism of the effects has not been well elucidated (Telang et al., 2003).

Altering molecular mobility in the amorphous phase by complex formation and/or molecular interaction changes is another approach to prevent mannitol crystallization. For example, the addition of a small amount of boric acid to hot-melt mannitol solution retards mannitol crystallization from the amorphous solid (Yoshinari et al., 2003). Sodium tetraborate (borax) is another candidate to effectively inhibit mannitol crystallization in frozen solutions and freeze-dried solids by the same mechanism. It dissociates into boric acid ($B(OH)_3$) and (tetrahydroxy)borate ion ($B(OH)_4^-$) in low-concentration solutions. The borate ion interacts with various polyols to form a complex in aqueous solutions (Boeseken, 1949; Deuel and Neukom, 1949; Conner and Bulgrin, 1967; Van Duin et al., 1985). The complexation reduces component molecular mobility in the amorphous freeze-concentrated saccharide phase (e.g. sucrose, trehalose) and its freeze-dried solids, as observed in higher saccharide frozen solution T_g' and freeze-dried solid T_g (Miller et al., 1998, 1999; Izutsu et al., 2003).

The purpose of the present study was to elucidate the effects of boric acid and sodium tetraborate on the crystallization of mannitol in frozen solutions and freeze-dried solids through thermal analysis. The crystallization-inhibiting effects of other solutes (e.g. sucrose, NaCl, sodium phosphate buffer) were also examined for comparison. While concerns regarding long-term safety limits the application of boric acid and sodium tetraborate in pharmaceutical formulations, the combinations serves as good model for controlling component crystallinity during freeze-drying (Hubbard, 1998).

2. Materials and methods

Sodium tetraborate anhydrate and decahydrate were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Bovine serum albumin (BSA) and rabbit muscle lactate dehydrogenase (LDH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). D-Mannitol and other chemicals were the product of Waco Pure Chemical (Osaka, Japan). BSA and LDH were dialyzed against 20 mM sodium phosphate buffer (pH 7.0) overnight before the sample preparation.

Freeze-drying of the aqueous solutions was performed by using a freeze-drier (Freezevac 1C, Tozai Tsusho). Aqueous mannitol and co-solute combination solution (200 μl) in a flat-bottomed glass vial was frozen by placing the vial in liquid nitrogen, after which freeze-drying was carried out. The freeze-drying was performed without shelf-temperature control for 12 h, and then maintaining the shelf at 35 °C for 8 h. The freeze-dried solids had a good cake structure except as mentioned. Freeze-dried samples for the protein secondary structure analysis were prepared from aqueous solutions containing 10 mg/ml BSA, 1 mM sodium phosphate buffer, and various concentrations of additives. Fourier-transform infrared spectroscopy (FT-IR) measurement of the protein secondary structure was performed by using a FT-IR system (Bomen Prota), as described previously (Izutsu and Kojima, 2002).

Thermal analysis of the frozen solutions and freeze-dried solid were performed using a differential scanning calorimeter (DSC-Q10, TA Instruments) with a

refrigerated cooling system. Aliquots (10 μl) of frozen solution in a hermetic aluminum cell were cooled from room temperature to −70 °C at 20 °C/min and scanned at 5 °C/min under nitrogen purge. The effect of co-solutes on the mannitol (50 mg/ml) crystallization in frozen solutions was expressed as the ratio (%) of the area under the baseline of the corresponding thermogram relative to that without co-solutes. Transition temperatures (T_g') of the frozen solutions were obtained from peaks in the derivative thermogram (Izutsu et al., 2003). Thermal analysis of the freeze-dried solids was performed in scanning sample in a hermetic aluminum cell from −20 to 200 °C at 10 °C/min.

Samples for LDH activity study were freeze-dried from solutions containing 0.01 mg/ml LDH, 10 mM sodium phosphate buffer (pH 7.0), 50 mg/ml polyol (mannitol, sucrose), and 0–50 mM sodium tetraborate. Freeze-dried LDH cakes were re-hydrated by adding 1 ml sodium phosphate buffer (50 mM, pH 7.4). The LDH was assayed spectroscopically within 30 min of reconstitution. Each 1 ml of 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 enzyme reaction was started by addition of 50 μl LDH solution, and the decrease in absorbance at 340 nm was monitored. The results were expressed as percentages of the remaining activity.

3. Results

Fig. 1 shows the effects of sodium tetraborate on the thermal profiles of frozen mannitol (50 mg/ml, ca. 277 mM) solutions. A single-solute frozen mannitol solution showed a large exothermic peak of mannitol crystallization at around −23 °C. A T_g'-like shift (−30 to −27 °C) and an endothermic peak (−25 °C) were also observed (Telang et al., 2003; Kett et al., 2003). No mannitol crystallization peak was observed in the cooling process. The addition of sodium tetraborate raised the crystallization peak temperature and concomitantly reduced the peak size. The exothermic peak disappeared in frozen solutions containing 15 mM sodium tetraborate, indicating that sodium tetraborate inhibited mannitol crystallization at the sodium tetraborate/mannitol molar concentration ratios of approximately 0.05. The mannitol crystallization peak also disappeared at identical concentration ratios in thermal analysis of solutions with other mannitol concentrations (25, 100 mg/ml, data not shown). Combinations of mannitol (50 mg/ml) and higher concentrations of sodium tetraborate (20–80 mM) showed a T_g' transition at approximately −36 to −32 °C. The single transition suggests freeze-concentration of the solutes into an amorphous phase surrounding ice crystals (Izutsu et al., 1998).

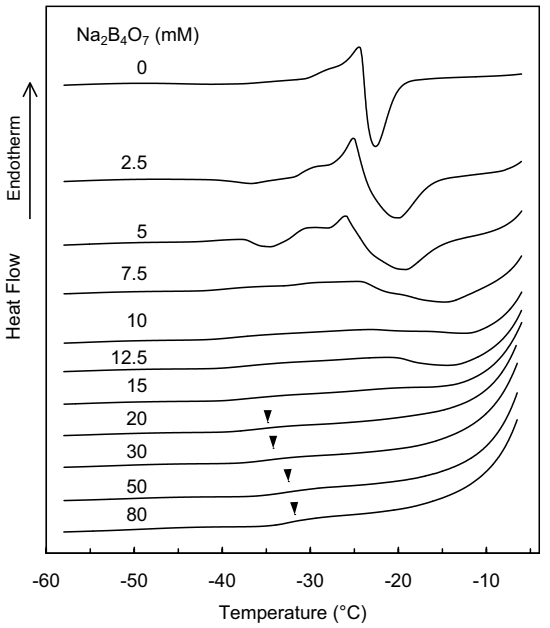

Fig. 1. DSC scans of frozen aqueous solutions containing mannitol (50 mg/ml) and different concentrations of sodium tetraborate. Glass transitions of maximally freeze-concentrated solutes (T_g' values) are marked with inverted triangles (▼).

Fig. 2 shows the effects of sodium tetraborate and boric acid on the T_g' values of frozen amorphous mannitol (50 mg/ml) solutions. Increasing the sodium tetraborate concentrations raised the T_g' to as high as approximately −22 °C (300 mM $Na_2B_4O_7$). Combination with mannitol also raises the sodium tetraborate solubility in the aqueous solutions (data not shown). Extrapolation of the transition temperatures suggested that T_g' of a single-solute frozen mannitol solution is below −35 °C, which is lower than the observed thermal shift (Fig. 1). It suggests that "real" mannitol T_g' is one of the smaller thermal events below

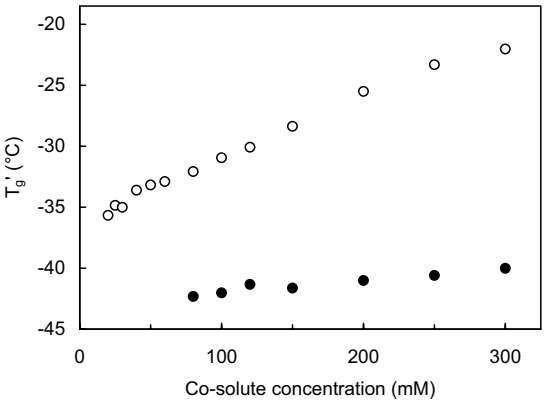

Fig. 2. Effects of sodium tetraborate (○) and boric acid (●) on T_g' (glass transition temperatures of maximally freeze-concentrated solutes) of frozen mannitol (50 mg/ml) solutions ($n = 2$).

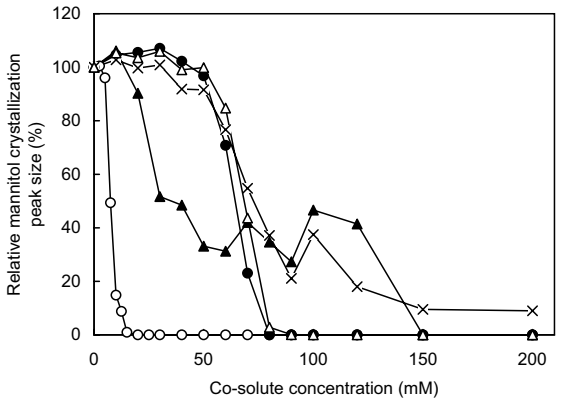

Fig. 3. Effects of co-solutes on the mannitol (50 mg/ml) crystallization exotherm in frozen solutions. Relative areas of the crystallization peaks under the baseline of each thermogram are expressed as the ratio (%) to that without co-solutes ($n = 2$, (○) $Na_2B_4O_7$, (●) H_3BO_3, (△) sucrose, (▲) sodium phosphate buffer, (×) NaCl).

the T_g'-like shift temperature. Frozen sodium tetraborate solutions have a T_g' transition at approximately −26 °C (data not shown) (Izutsu et al., 2003). The mannitol and high-concentration sodium tetraborate combinations showed T_g' transitions at temperatures above each of the single-solute solution T_g' values, indicating a larger effective solute molecular size by complexation rather than by simple mixing of the solutes in the freeze-concentrate (Levine and Slade, 1988; Izutsu et al., 2003). The high T_g' also suggests a high collapse temperature of the amorphous system in freeze-drying (Nail et al., 2002).

Fig. 3 shows the effects of various co-solutes on the mannitol (50 mg/ml) crystallization exotherm in frozen solutions. The different co-solute concentrations for the mannitol crystallization peak changes indicates their varied effects in inhibiting mannitol crystallization. The mannitol crystallization exothermic peak disappeared at 15 mM sodium tetraborate (ca. 3.0 mg/ml), 80 mM boric acid (ca. 4.9 mg/ml), and 80 mM sucrose (ca. 27 mg/ml). Sodium chloride and sodium phosphate buffer reduced the mannitol crystallization peak in a more complicated manner, probably due to overlapping of the salt crystallization and melting peaks (Murase and Franks, 1989; Nail et al., 2002). While the mannitol crystallization peak disappeared at 150 and 200 mM sodium phosphate buffer, a small endotherm at the mannitol crystallization temperature was observed up to 200 mM NaCl. The mannitol and NaCl combination showed T_g' values at temperatures much lower than those of other frozen solutions (e.g. 50 mg/ml mannitol, 200 mM NaCl; −52.8 °C). The relative exotherm peak size does not directly represent mannitol crystallinity because of the overlapping of several thermal events (e.g. glass transition, ice crystallization), whereas it is a useful indicator to compare the effects of co-solutes. Sodium tetraborate exerts a larger effect than other co-solutes in inhibiting mannitol crystallization in the frozen solutions, even when comparing the weight concentration ratios. Sodium tetraborate was also more effective than boric acid comparing in boron concentrations.

Fig. 4 shows thermograms of freeze-dried mannitol and sodium tetraborate combinations at elevated temperatures. The freeze-dried solids showed three different mannitol crystallization profiles, namely, mannitol already crystallized during the freeze-drying process, crystallization in exposure to elevated temperature, and maintenance of an amorphous state. Both original (reagent) mannitol crystal and its freeze-dried solids showed a β-polymorph crystal melting endotherm at approximately 166 °C (Ando et al., 1985; Burger et al., 2000). Similar melting enthalpies of the original and freeze-dried mannitol indicate that mannitol in the freeze-dried solid primarily crystallize during the freeze-drying process.

Co-lyophilization with sodium tetraborate (5–10 mM) reduced the mannitol crystal melting peak, indicating decreased mannitol crystallinity in the

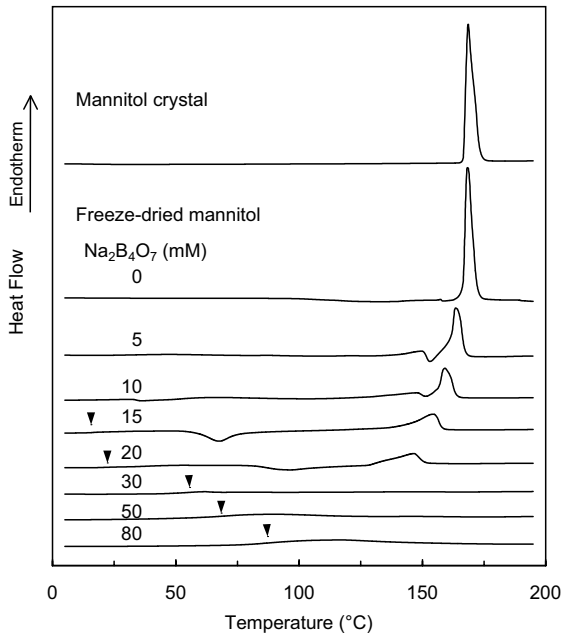

Fig. 4. Thermal profiles of freeze-dried mannitol (50 mg/ml) and various concentration sodium tetraborate combinations. Freeze-dried solid (0.8–1.4 mg) in an aluminum cell was scanned at 10 °C/min. Glass transition temperatures are marked with inverted triangles (▼).

freeze-dried solids. The broad and lower temperature endotherm (140–160 °C) just below the β-polymorph crystal melting temperature suggests melting of the δ-polymorph and/or temperature-depressed melting of other mannitol polymorphs in the presence of residual water (Yu et al., 1998; Telang et al., 2003). Mannitol crystallization during freeze-drying often leads to different anhydrous polymorphs, hydrates, and their mixtures as a result of co-solute composition and processing conditions (Kim et al., 1998; Yu et al., 1999; Burger et al., 2000; Torrado and Torrado, 2002).

Freeze-dried solids from solutions containing 50 mg/ml mannitol and 15 or 20 mM sodium tetraborate exhibited a glass transition-like thermogram shift at 10–30 °C, an exothermic peak at 60–80 °C, and a following endotherm at 140–160 °C. Some of these freeze-dried solids shrunk during freeze-drying, probably due to the secondary drying process at temperatures above the glass transition. The two peaks are attributed to be nonisothermal mannitol crystallization and crystal melting (Yu et al., 1999; Yoshinari et al., 2003). The small mannitol crystallization exotherm and the similar sized melting peak suggest that mannitol remains highly amorphous during freeze-drying. The glass transitions were observed near that of reported pure amorphous mannitol T_g (13 °C) (Kim et al., 1998; Yoshinari et al., 2003). Higher molecular mobility above the glass transition temperature may allow some spatial rearrangement of amorphous mannitol molecules required for crystallization. The mannitol crystallization temperature rose with increases in the sodium tetraborate concentrations. No mannitol crystallization and melting peaks were observed in thermal analysis of freeze-dried mannitol and higher concentration (30–80 mM) sodium tetraborate combinations, indicating practically stable amorphous freeze-dried cakes. These freeze-dried solids showed a broad glass transition at 50–90 °C, with the midpoint temperature rising with increases in the sodium tetraborate concentrations. The rising T_g is consistent with the sodium tetraborate effects on the freeze-dried saccharide solids (Miller et al., 1998, 1999).

Fig. 5 shows glass transition temperatures of freeze-dried mannitol and sodium tetraborate combinations as a function of composition (total: 25 mg/ml). The combinations showed large shift of T_g only in mannitol-rich concentration ratios (e.g. 0.1 weight fraction $Na_2B_4O_7$). Other freeze-dried solids, including that of single-solute sodium tetraborate, showed T_g midpoints between 70 and 95 °C. The T_g profile

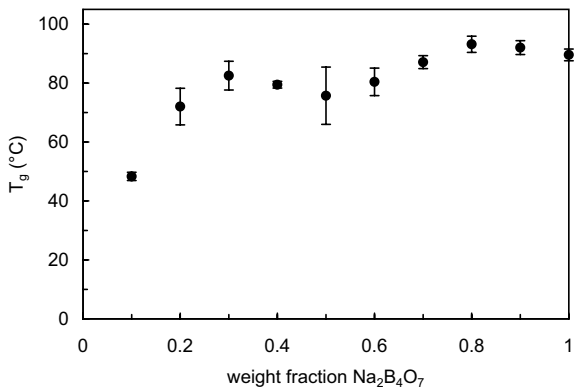

Fig. 5. Glass transition temperatures of co-lyophilized mannitol and sodium tetraborate combinations (25 mg/ml total) as a function of composition. Freeze-dried solids (0.5–1.0 mg/ml) were scanned from −20 °C at 10 °C/min. Symbols represent measured midpoint values ±S.D. ($n = 3$).

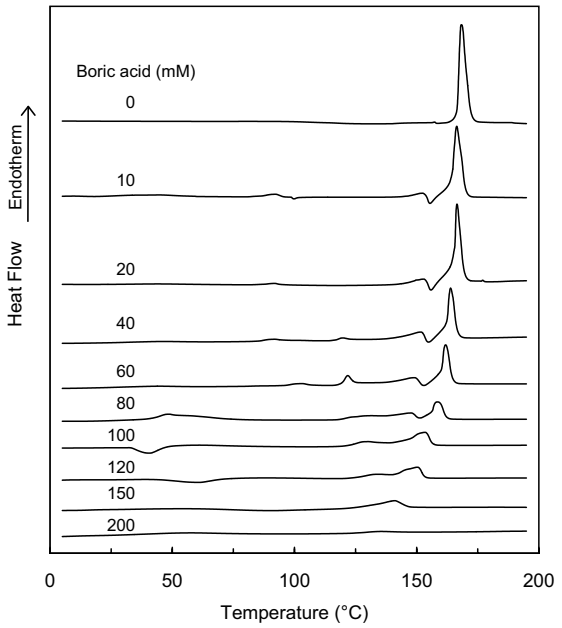

Fig. 6. Thermal profiles of freeze-dried mannitol (50 mg/ml) and various concentrations of boric acid combinations. Freeze-dried solid (0.8–1.4 mg) or mannitol crystal (1.03 mg) in an aluminum cell was scanned at 10 °C/min.

also suggested component complexation, rather than simple mixing, in the freeze-dried solids.

Fig. 6 shows thermograms of freeze-dried mannitol and boric acid combinations. Boric acid inhibited mannitol crystallization in the freeze-dried solids at much higher concentrations than sodium tetraborate. Combination with 150–200 mM boric acid kept the mannitol from crystallization during the freeze-drying process and at elevated temperatures. A small exotherm observed at 120 °C in some freeze-dried solids suggested a mannitol polymorph transition (Kim et al., 1998). Sucrose and sodium phosphate buffer (150–200 mM) also inhibited mannitol crystallization during the freeze-drying process and at elevated temperatures (data not shown) (Johnson et al., 2002). In contrast to other co-solutes, co-lyophilization with 60–200 mM sodium chloride resulted in highly crystallized δ-polymorph mannitol in the freeze-dried solids, presenting a sharp crystal melting peak at 155 °C (Telang et al., 2003). Combinations with 150 or 200 mM NaCl resulted in an apparent collapse of the freeze-dried solids.

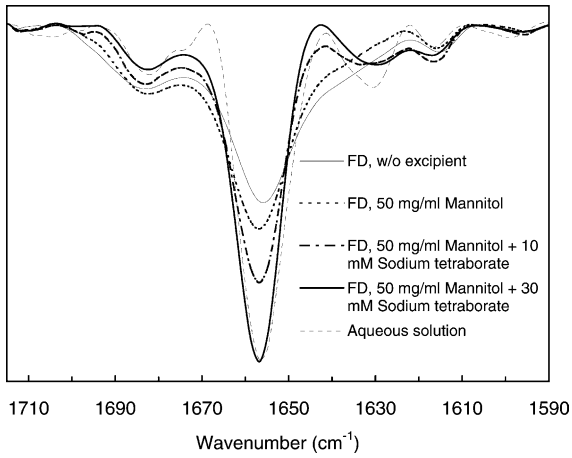

Fig. 7. Area-normalized second-derivative FT-IR spectra of BSA in an aqueous solution (10 mg/ml BSA, ca. 1 mM sodium phosphate buffer) and freeze-dried solids.

Fig. 7 shows area-normalized second-derivative amide I FT-IR spectra of aqueous and freeze-dried bovine serum albumin. Native BSA in initial aqueous solutions showed a large α-helix band at 1656 cm^{-1}. Freeze-drying of BSA without added solutes resulted in a broad α-helix band, indicating a perturbed protein secondary structure (Dong et al., 1995). Co-lyophilization with mannitol alone showed only a small protective effect on the BSA conformation, and highly crystallized mannitol in thermal analysis (Izutsu et al., 2003). The protein maintained most of its original conformation in freeze-drying with a 50 mg/ml mannitol and 30 mM sodium tetraborate combination. Co-lyophilization with 50 mg/ml sucrose or a combination of 50 mg/ml mannitol and 30–50 mM sodium tetraborate exhibited amide I spectra identical to that of BSA in an aqueous solution (data not shown). These freeze-dried solids remained in an amorphous state in the thermal analysis up to 180 °C. Sodium tetraborate alone showed a small structure-stabilizing effect in the freeze-drying of BSA (data not shown) (Izutsu et al., 1994). The FT-IR results indicated an apparent protein structure-stabilizing effect of the amorphous mannitol and sodium tetraborate combinations during the freeze-drying process. The mannitol–borate complex may have sufficient structurally accessible hydroxyl groups to interact with the protein surface as a water substitute (Izutsu et al., 2004).

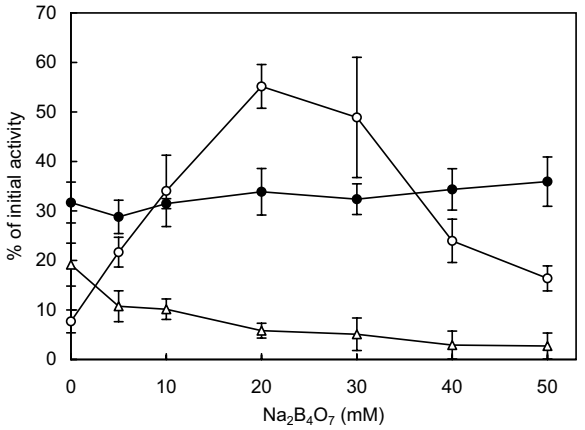

Fig. 8. Effects of excipients on the relative activity of freeze-dried LDH. Aliquots (10 μl) of LDH (0.01 mg/ml) solutions containing 10 mM sodium phosphate buffer (pH 7.0) and various co-solutes were freeze-dried. Each symbol and error bar represent the mean ± S.D. ($n = 5$, (△) w/o polyol, (○) 50 mg/ml mannitol, (●) 50 mg/ml sucrose).

Fig. 8 shows effects of co-solutes on the activity of freeze-dried LDH. Freeze-drying of the protein solution without protecting co-solutes reduced the enzyme activity to approximately 20% of the initial solutions. Sodium tetraborate alone (5–50 mM) lowered the activity of co-lyophilized LDH probably due to an alkaline pH shift of the solution. An aqueous solution containing 0.01 mg/ml LDH, 10 mM sodium phosphate buffer, and 50 mM sodium tetraborate had a pH of 9.0 (data not shown). Co-lyophilization with mannitol alone reduced the enzyme activity, whereas the combination of mannitol and 20 mM sodium tetraborate retained 50–60% of the initial enzyme activity. The amorphous combination may prevent the freeze-drying-induced LDH subunit dissociation and irreversible conformation change (Anchordoquy et al., 2001). Co-lyophilization with sucrose and 0–50 mM sodium tetraborate resulted in 30–40% of the initial LDH activity in the reconstituted solutions.

4. Discussion

The present results indicate that sodium tetraborate inhibits mannitol crystallization in frozen solutions, during freeze-drying, and in exposure of the freeze-dried solids to elevated temperatures at much lower concentrations than other co-solutes. We hypothesize that complex formation and reduced mannitol molecular mobility should allow the significant effect of sodium tetraborate to inhibit mannitol crystallization.

Many amorphous co-solutes (e.g. saccharides, polymers) inhibit mannitol crystallization in frozen solutions and during freeze-drying processes (Kim et al., 1998). They prevent formation of a supersaturated mannitol solution and/or the dried mannitol phase, a condition necessary for mannitol crystallization, through a molecular level mixing. Retaining amorphous-state mannitol by the molecular-level mixing with amorphous co-solutes (e.g. sucrose) usually requires a large amount of co-solute (Kim et al., 1998). Sodium tetraborate remained amorphous in frozen aqueous solutions with a T_g' transition at approximately −26°C (Izutsu et al., 2003). This intrinsic tendency is suitable for retaining mannitol in the amorphous state, whereas it does not explain the large effects of sodium tetraborate at low-concentration ratios.

Sodium tetraborate forms monodiol and didiol complexes with various chemicals containing hydroxyl groups (e.g. polyols, saccharides), including mannitol, in aqueous solutions (Boeseken, 1949; Deuel and Neukom, 1949; Conner and Bulgrin, 1967; Van Duin et al., 1985). The strong attractive force between borate ion and polyols for complexation can affect hydrogen-bonding network between polyol molecules. Various saccharide and sodium tetraborate combinations show physically stable amorphous mixture in frozen solutions and freeze-dried solids by the complexation and altered molecular interactions (Miller et al., 1998, 1999; Izutsu et al., 2003). The frozen solutions indicate complexation of a borate ion and two mono- or disaccharide molecules (Izutsu et al., 2003). The mannitol and sodium tetraborate combinations showed amorphous phases with high frozen solution T_g' and freeze-dried solid T_g in spite of the intrinsic tendency of mannitol toward crystallization. In addition to the difficulties of mannitol–borate complex to participate in the mannitol crystallization, the altered molecular interaction and reduced molecular mobility should prevent spatial arrangement required for mannitol crystallization. These mechanisms would explain the large effect of sodium tetraborate in inhibiting mannitol crystallization at lower concentration ratios.

The different extent of complexation and molecular mobility change should vary the mannitol crystallization profiles in frozen solutions and freeze-dried solids depending on the mannitol and sodium tetraborate concentration ratios. Sodium tetraborate may prevent crystallization of only surrounding mannitol molecules in frozen solutions at lower concentration ratios, thus resulting in partially crystallized mannitol in the freeze-dried solids. Maintaining the freeze-dried amorphous-state mannitol at elevated temperatures would require a further complexation and reduction of the molecular mobility than inhibiting crystallization during freeze-drying processes. Boric acid showed a smaller effect in inhibiting mannitol crystallization compared to sodium tetraborate, probably because the pH values of the combination solutions were not high enough for ionization and complex formation (Boeseken, 1949).

Sodium tetraborate exerted mannitol crystallization-inhibiting effects at elevated temperatures at much lower concentration ratios than other co-solutes. The amorphous mannitol and sodium tetraborate combination performed as a potent lyoprotectant. Although some concerns regarding possible long-term adverse effects limit usage of borate in pharmaceutical formulations (Hubbard, 1998), freeze-drying of the solute complexes should provide alternative ways of obtaining practically stable amorphous solids of not only mannitol but also various chemicals containing hydroxyl groups. Elucidating detailed mechanism of the crystallization inhibition and possible application of the complex system would require further studies.

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