Effect of Sodium Tetraborate (Borax) on the Thermal Properties of Frozen Aqueous Sugar and Polyol Solutions

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The effect of sodium tetraborate ($Na_2B_4O_7$, borax) on the thermal property of frozen aqueous sugar and polyol solutions was studied through thermal analysis. Addition of borax raised the thermal transition temperature (glass transition temperature of maximally freeze-concentrated solutes; T_g') of frozen sucrose solutions depending on the borax/sucrose concentration ratios. Changes in the T_g' of frozen mono- and disaccharide solutions suggested various forms of complexes, including those of a borate ion and two saccharide molecules. Borax exerted the maximum effect to raise the oligosaccharide and dextran T_g's at borax/saccharide molar ratios of approximately 1—2 (maltose and maltooligosaccharides), 2 (dextran 1060), 5 (dextran 4900), and 10 (dextran 10200). Further addition of borax lowered T_g's of the saccharide solutions. Borax also raised T_g and T_g' temperatures of frozen aqueous glycerol solutions. The decreased solute mobility in frozen solutions by the borate-polyol complexes suggested higher collapse temperature in the freeze-drying process and improved stability of biological systems in frozen solutions.

Key words glass transition; borate; freeze-drying; frozen solution; thermal analysis; formulation

Recent advances in biotechnology have focused attention on the stable storage of the biological polymers (*e.g.*, proteins) and biological systems (*e.g.*, cells) for medical use. Freezing and freeze-drying are popular methods to archive their long-term stability.[1—4] Various co-solutes such as sugars (*e.g.*, sucrose, trehalose) and other polyols protect proteins and cells through different mechanisms against stresses in aqueous solutions, frozen solutions, and freeze-dried solids.[5,6] Freezing an aqueous solution concentrates the solutes into a supercooled solution surrounded by ice crystals.[7,8] The sugars and polyols protect the proteins and cell membranes against dehydration-induced structural perturbation by substituting water molecules through direct molecular interactions (*e.g.*, hydrogen bonding). Decreased molecular mobility in the polyol-based glass-state amorphous phase reduces chemical changes of the components.[3,7] Controlling the physical properties of the freeze-concentrates and subsequently freeze-dried solids should provide the key to production of stable protein and other biopolymer formulations.[1,2,7]

Thermal analysis of frozen aqueous carbohydrate solutions often shows several transitions of the amorphous supercooled phase including the "real" glass transition (T_g) and the glass transition of maximally freeze-concentrated solutes (T_g') at temperatures depending on the solute compositions.[9,10] The terming and implications of these thermal transitions are still under some debate, whereas it has been established that the changes in the solute and surrounding water mobility at the transition temperatures have significant impact on the physical and chemical stability of the components.[11] The T_g' transition, which involves ice melting around the solute molecule, is often the most apparent, and is the most important in the development of freeze-dried formulations because the increased solute mobility above the T_g' typically induces cake collapse during the freeze-drying process.[1,2] The "real" glass transition of the frozen solution (T_g) occurs without ice melting, and is often less apparent in thermal analysis.

It is well known that borate forms chemical complexes with polyhydroxy compounds.[12—14] Addition of sodium tetraborate decahydrate (borax), which hydrolyzes to boric acid and sodium borate ($Na_2B_4O_7+7H_2O \rightarrow 2B(OH)_3+2B(OH)_4Na$), to aqueous sugar and/or other polyol solutions results in various complexes between the borate ion and the polyols. Increase in the "effective" molecular size and changes in the molecular interactions by the complex formation (*e.g.*, borate-trehalose) leads to reduced component mobility in the amorphous freeze-dried solids and hydrated mixtures.[15—17] Co-lyophilization with the borate–sugar combinations improves the protein stability at higher temperatures.[15]

The large dependence of the frozen saccharide solution T_g's on their molecular weights has suggested alternation of the T_g' through the borate–polyol complex formation.[18,19] The purpose of this study was to investigate how complexation with borate affects the physical properties of frozen sugar and polyol solutions. The effect of borax was studied through thermal analysis of various solute combinations and concentration ratios. Possible applications of the complex formation in the freeze-drying of aqueous biopolymer solutions and frozen storage of biological systems were discussed.

Experimental

Bovine serum albumin (BSA, essentially fatty acid free), dextran (average MW 10200), trehalose, and maltose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dextrans (average molecular weights 1090 and 4900) and sodium tetraborate decahydrate were from Serva Electrophoresis GmbH (Heidelberg, Germany) and Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), respectively. Maltotriose, maltotetraose, maltopentaose, and maltoheptaose were purchased from Hayashibara Biochemical Laboratories Co. (Okayama, Japan). Other chemicals were of analytical grade, and were obtained from Wako Pure Chemical Co. (Osaka, Japan). BSA was dialyzed against a potassium phosphate buffer solution (20 mM, pH 7.0) before the thermal analysis.

Thermal analysis of the frozen solutions was carried out using a differential scanning calorimeter (DSC Q10, TA Instruments). Aliquots (10 μl) of the solutions in aluminum cells were cooled at approximately -20 °C/min and scanned from -100 °C at a heating rate of 5 °C/min under dry nitrogen purging. Frozen solutions containing glycerol were scanned from -120 °C. Derivative thermograms were obtained by using Thermal Solutions software (TA Instruments). The thermal transition temperatures of frozen solutions were obtained from the peak in the corresponding derivative thermograms. In experiments designed to study the effect of heat-treatment (annealing), the first scanning of the frozen solutions was paused and maintained at

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−15 °C for 30 min. The samples were re-scanned from −100 °C at 5 °C/min.

Results and Discussions

Figure 1 shows derivative thermograms of frozen solutions containing sucrose (20 mg/ml, approx. 58 mM) and various concentrations of borax. The frozen sucrose solution had a thermal transition (T_g') peak at −33.7 °C.[9,18,19] The lower-temperature "real" glass transition (T_g) was not apparent in the thermogram. Thermal analysis of frozen borax solutions (25—125 mM) showed T_g' peaks at approximately −26 °C, suggesting transition of amorphous-state hydrolyzed borax components in the freeze-concentrate. Addition of borax raised the T_g' peak temperature of the frozen sucrose solution (−19.1 °C, in 20 mg/mlsucrose and 70 mM borax), and enlarged the T_g' peak size in the derivative thermograms. The T_g' temperatures of the sucrose and borax combinations above each of the component T_g's indicate the freeze-concentration of the solute complexes, rather than that of the simple solute mixture. Re-scanning of a frozen solution (20 mg/ml sucrose, 50 mM borax) after heat-treatment above the T_g' (annealing, −15 °C for 30 min) showed a T_g' peak at a temperature similar to that of the initial scanning. This suggested that the complex is practically stable in the frozen solution.

Figure 2 shows the effect of borax on the T_g' peak temperatures of various frozen sucrose and trehalose solutions. The addition of borax raised the sucrose solution T_g' in a similar manner at the three initial sucrose concentrations (10, 20, 50 mg/ml) depending on the borax/sucrose concentration ratios. Limited solubility of borax in the initial solutions prevented the thermal analysis at some high borax/sucrose ratio solutions (50 mg/ml sucrose, 175 or 250 mM borax). The T_g' peak temperature reached a plateau at a borax/sucrose molar ratio of approximately 1 : 1, suggesting completion of the complex formation at around that ratio. As can be observed in the Fig. 1, the T_g' transition peak size got larger at the particular temperature by further addition of borax. Borax also raised the T_g' peak temperature of frozen trehalose (20 mg/ml) solution. The effect of the boric acid on the frozen sucrose solution T_g' was also studied in order to elucidate the contribution of hydrolyzed borax components (boric acid and sodium borate).[15] The minimal effects of up to 400 mM boric acid, which has a single-solute T_g' at around −11 °C, on the sucrose (20 mg/ml) T_g' indicated that the borate ion plays a dominant role in the complex formation and the resulting T_g' rise (data not shown). Several other salts (*e.g.*, NaCl) lowered the sucrose T_g' by increasing the quantity of the ice-concentrated matrixes (data not shown).[20]

Figure 3 shows the effect of borax on the T_g's of frozen monosaccharide to oligosaccharide solutions (20 mg/ml). The frozen saccharide solutions showed varied single-solute T_g' peaks at −45.5 °C (glucose), −30.9 °C (maltose), −24.5 °C (maltotriose), −20.0 °C (maltotetraose), −17.6 °C (maltopentaose), and −13.9 °C (maltoheptaose).[19] Addition of borax raised the T_g's of frozen glucose and maltose solutions to as high as −26.5 °C (140 mM borax) and −18.9 °C (70 mM borax), respectively. The highest T_g' temperatures of these combination solutions were close to the single-solute maltose and maltotetraose solution T_g's, suggesting that a complex of the two saccharide molecules being concentrated in the frozen solutions. The T_g' peak temperatures of frozen maltooligosaccharide solutions also rose in association with increasing borax up to certain concentrations, beyond which the addition of borax lowered the T_g' peak temperatures. Plotting the T_g' change data against the solute molar concentration ratios showed that the maximum effect of borax was achieved at the borax/oligosaccharide molar ratios between 1 and 2 in the frozen maltose to maltoheptaose solutions (data not shown). Increasing ratio of lower T_g' "excess" borax (single-solute T_g': approximately −26 °C) and the borate complex with a single polyol molecule may lower the T_g's at the higher borax concentration ratios.[21,22]

Freeze-dried pharmaceutical protein formulations often

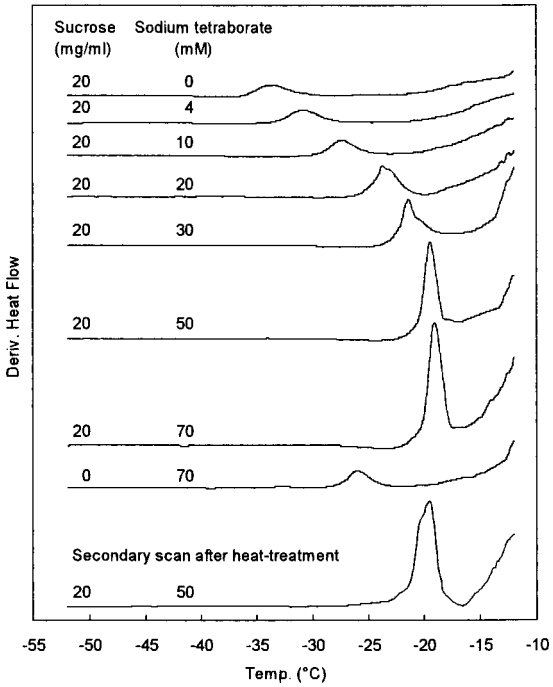

Fig. 1. Derivative Thermograms of Frozen Aqueous Solutions Containing Sucrose, Sodium Tetraborate, and Combinations Thereof

Aliquots (10 μl) of frozen solutions in an aluminum cell were scanned from −100 °C at a scanning rate of 5 °C/min. Secondary scan of a frozen solution was performed after the heat-treatment at −15 °C for 30 min.

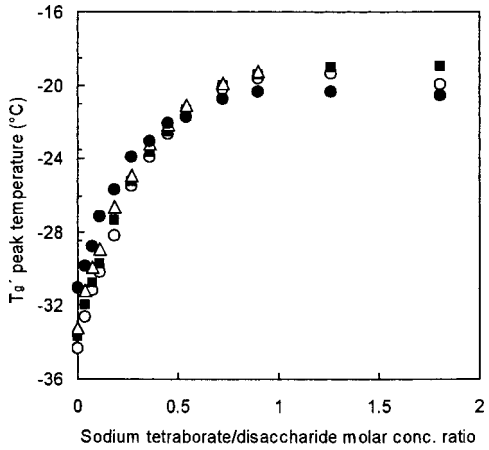

Fig. 2. Effect of Sodium Tetraborate on the Tallest T_g' Peak Temperatures in the Derivative Thermograms of Frozen Aqueous Disaccharide Solutions

○: 10 mg/ml sucrose, ■: 20 mg/ml sucrose, △: 50 mg/ml sucrose, ●: 20 mg/ml trehalose.

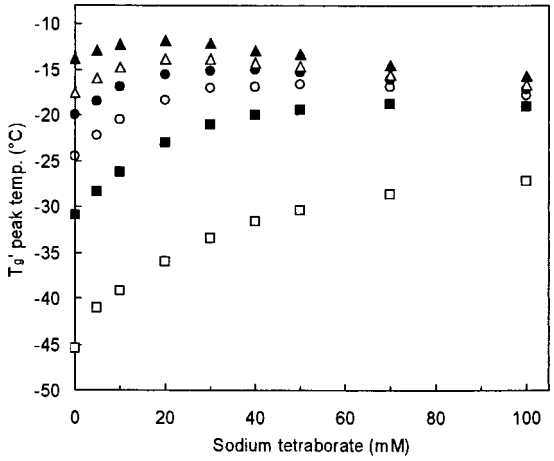

Fig. 3. Effect of Sodium Tetraborate on the T_g' Peak Temperatures of Frozen Aqueous Monosaccharide to Oligosaccharide Solutions (20 mg/ml)

□: glucose, ■: maltose, ○: maltotriose, ●: maltotetraose, △: maltopentaose, ▲: maltoheptaose.

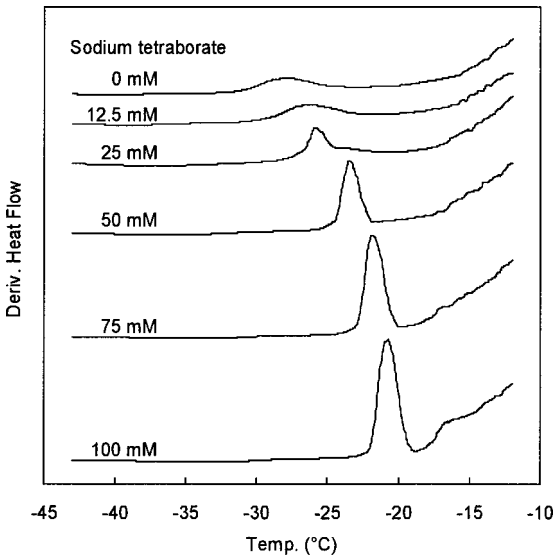

Fig. 4. Derivative Thermograms of Frozen Aqueous Solutions Containing 50 mg/ml Sucrose, 20 mg/ml BSA, 20 mM Potassium Phosphate Buffer, and Various Concentrations of Sodium Tetraborate

contain various excipients such as sugars, polymers, buffer salts, and surfactants.[1—4] The effect of borax on the thermal properties of a multi-component frozen solution was studied (Fig. 4). Frozen solutions containing 20 mg/ml BSA, 50 mg/ml sucrose, and 20 mM potassium phosphate buffer showed a single T_g' peak at a temperature (−28.0 °C) between the sucrose T_g' and putative BSA T_g' (approximately −10 °C), indicating a freeze-concentration of the solutes into a supercooled mixture phase.[18] Addition of borax raised the T_g' peak temperature gradually to approximately −21 °C, and enlarged the transition magnitude. The borate–sugar complex raised the T_g' of the multi-component frozen solution that mimics a protein formulation.[1,3]

Various polymers and non-saccharide polyols are potent cryoprotectants and/or lyoprotectants, alone or in combination with other solutes. The different stabilizing mechanisms and physical properties of these solutes provide a wide range of choices for the appropriate design of biopolymer formulations and cryopreservation media. How the borax affects the physical properties of the frozen aqueous polysaccharide and low-molecular-weight polyol solutions was studied. Figure 5 shows the effect of borax on the T_g' of various molecular weight dextran (20 mg/ml) solutions, plotted against the borax/dextran molar concentration ratios. The frozen single-solute dextran solutions showed T_g' peaks at −22.6 °C (dextran 1090), −16.7 °C (dextran 4900), and −13.9 °C (dextran 10200). As was observed in the oligosaccharide and borax combinations, the lower concentration of borax raised the T_g' peak temperatures of the frozen dextran solutions, whereas the T_g's dropped at higher borax concentrations. The largest effect of borax were observed at borax/dextran molar ratios of approximately 2 (dextran 1060, 40 mM borax), 5 (dextran 4900, 20 mM borax), and 10 (dextran 10200, 20 mM borax). The balance of the changing complex size through the inter-chain (raising T_g') or intra-chain (lowering T_g') crosslinks, and concentration of free borax components (lowering T_g') should determine the transition temperature. Dextran by itself is not a potent stabilizer in freeze-drying of many proteins because of difficulties to form appropriate molecular interaction due to the steric hindrance,[5,23] whereas it protects some oligomer proteins against the freezing stresses by raising the integrity of the quaternary structures.[24] Combinations of dextran and low-molecular-weight saccharides archive protein stabilization by the structure-stabilizing mechanisms in the glass-state amorphous solids.[23,25]

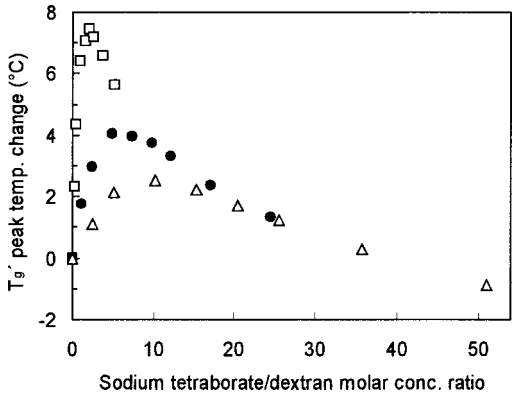

Fig. 5. Effect of Sodium Tetraborate on the T_g' Temperatures of Frozen Dextran Solutions (20 mg/ml)

□: dextran 1090, ●: dextran 4900, △: dextran 10200.

Figure 6 shows the effect of borax on the thermal property of frozen aqueous glycerol solutions. Several low-molecular-weight cell-permeating polyols (*e.g.*, glycerol, ethylene glycol, propylene glycol) and dimethyl sulfoxide (DMSO) are potent cryoprotectants for storage of frozen cells, tissues and microorganisms.[26] The derivative thermogram of the frozen glycerol solution showed T_g and T_g' peaks at −99.8 °C and −70.5 °C, respectively.[19,27] Addition of borax shifted both of the transitions to higher temperatures. The higher temperature T_g' peak became more apparent at higher borax concentrations.

The effects of borax to shift the thermal transition temperatures (T_g and T_g') of frozen aqueous sugar and polyol solutions were consistent with the reported borax-induced T_g change of unfrozen hydrated sugar (*e.g.*, trehalose) matrixes.[15,16] The borax/sugar concentration ratios should deter-

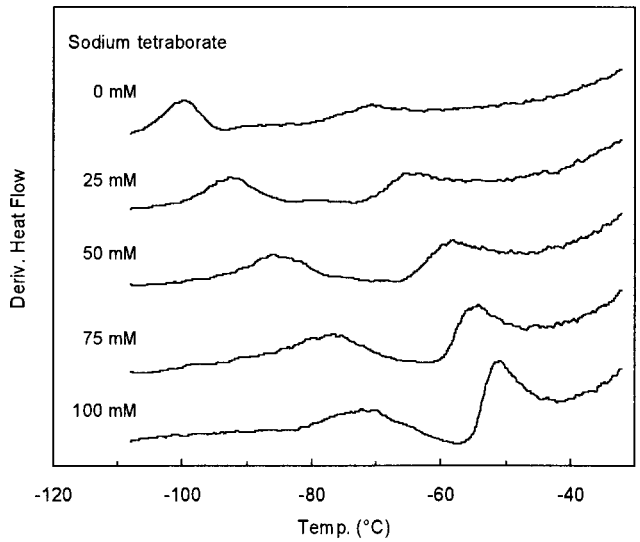

Fig. 6. Derivative Thermograms of Frozen Aqueous Solutions Containing 50 mg/ml Glycerol and Various Concentrations of Sodium Tetraborate

mine the transition temperatures of the equally freeze-concentrated matrixes irrespective of their total concentrations in the initial solutions, whereas the amount of water also greatly affects T_g of the unfrozen borax-sugar mixtures. The complex formation mainly in the initial solutions should raise the frozen solution T_g' and the subsequently freeze-dried solid T_g by increasing the effective molecular size and the concomitant reduction of the component mobility. The frozen solutions should contain a mixture of the different complex forms since the tetrahydroxyborate ion ($B(OH)_4^-$) has universal ease of complexation through interaction with various hydroxyl groups in a polyol molecule and those with two polyol molecules.[21,22] The effect of borax on the T_g' of frozen low-molecular-weight saccharide (*e.g.*, glucose and maltose) solutions suggested formation of a complex of a borate ion and two saccharide molecules. A greater number of hydroxyl groups and larger molecular size should allow formation of the intra-chain borate–polymer complexes and the multi-molecular complexes through the inter-chain crosslinks. The reproducible T_g' transition after the heat-treatment indicated stability of the complex in the frozen solution. The effect of borax should depend on the solution pH, in that it affects the borate ion concentration active for the complex formation.[15] Most of the experiments in this study were performed without controlling the pH of the solutions.

The borate–polyol complex formation should have practical importance in freeze-drying and frozen storage of biological polymers and biological systems. Protecting the native protein conformation from various stresses and obtaining good structure cake are particularly important in freeze-drying of protein solutions.[1—3] Increase in the component molecular mobility above the T_g' often induces cake collapse during the freeze-drying process. The higher collapse temperature and consequently acceptable product temperature for the primary drying process, afforded by the complex formation, should significantly reduce the time and energy requirements for freeze-drying. The complex formation also improves storage stability of co-lyophilized proteins by raising the glass transition temperature (T_g) of the freeze-dried solids.[15] Borax has been used as an antiseptic agent, and as a pH adjuster in some medical products.[28] The borax–polyol combination will not be appropriate for frequently administrated pharmaceutical injections because of the safety concerns,[29,30] whereas it provides further choice to stabilize proteins in other applications.

Higher transition temperatures (T_g' and T_g) of frozen borax and polyol combination solutions should improve the stability of biopolymers and biological systems at a particular temperature and/or allow storage at higher temperatures. Raising the acceptable storage temperature should be desirable especially in the cryopreservation media containing low-molecular-weight cell-permeating polyols (*e.g.*, glycerol) that often requires special apparatus (*e.g.*, a liquid nitrogen storage vessel) to maintain the low storage temperatures. Application of the complex formation requires further study regarding the direct and indirect effects of borax on biopolymers and biological systems.[29,30]

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