Protection of Protein Secondary Structure by Saccharides of Different Molecular Weights during Freeze-Drying

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The protective effects of saccharides with various molecular weights (glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltoheptaose, dextran 1060, dextran 4900, and dextran 10200) against lyophilization-induced structural perturbation of model proteins (BSA, ovalbumin) were studied. Fourier transform infrared (FT-IR) analysis of the proteins in initial solutions and freeze-dried solids indicated that maltose conferred the greatest protection against secondary structure change. The structure-stabilizing effect of maltooligosaccharides decreased in increasing the number of saccharide units. Larger molecules of dextran also showed a smaller structure-stabilizing effect. Increasing the effective saccharide molecular size by a borate–saccharide complexation reduced the protein structure-stabilizing effect of all of the saccharides except glucose. The results indicate that the larger saccharide molecules, and/or the complex formation with borate ion, reduce the free and accessible hydroxyl groups to interact with and stabilize the protein structure by a water-substitution mechanism.

Key words freeze-drying; protein formulation; stabilization; borate

Development of recombinant therapeutic proteins requires rational design of both the formulations and manufacturing processes.[1—5] Freeze-drying is a popular method to retain long-term stability of various proteins that are not sufficiently stable in aqueous solutions, although the stresses incurred during the freeze-drying process often induce partial unfolding and resulting protein aggregation in the rehydrated solutions.[6—8] Stable formulation design is required to avoid the biological activity loss and possible immunogenic effect of the structurally and/or chemically altered protein molecules.

Many polyols (*e.g.*, saccharides and sugar alcohols) protect proteins from inactivation during freeze-drying and subsequent storage. They protect native protein conformation against dehydration stresses by replacing essential water molecules through molecular interaction (*e.g.*, hydrogen bonding) with the protein molecules.[1—4,9] Some polyols also improve the physical and chemical stability of proteins during the freeze-drying process and subsequent storage by keeping the protein within a highly viscose glass-state amorphous solid with limited molecular mobility.[10,11]

The choice of an appropriate saccharide and/or saccharide combination is crucial to the formulation design.[1,2,12] Sucrose has been added to many freeze-drying formulations because of its potent structure-stabilizing effect and proven safety, whereas the low glass transition temperatures of the sucrose-based amorphous freeze-dried solids result in stability problems in storage at higher temperature or higher humidity.[13] Various saccharides posses different physical properties and protein-stabilizing effects.[1—3] Large saccharide molecules often provide freeze-dried solids with high glass transition temperatures (T_gs), which are suitable for protein storage stability. In contrast, they often show smaller effect to protect lower concentration enzymes (*e.g.*, 1 μg/ml catalase) from inactivation during freeze-drying.[14]

Protein molecules face different stresses during the freeze-drying process depending on the initial concentrations, whereas the relationship between the saccharide molecular weights and the protein-stabilizing effect in freeze-drying of pharmaceutically relevant, high concentration (*e.g.*, greater than 1 mg/ml) protein solutions has not been well elucidated. In addition to the low temperature and dehydration stresses, which are independent of the initial protein concentrations, lower concentration proteins are more likely to undergo physical stress on ice, air, and container surfaces.[3,4,15] In the present work, we used Fourier transform infrared (FT-IR) spectroscopy to study the effects of saccharides with different molecular weights on the secondary structure of proteins (BSA, ovalbumin) freeze-dried in high concentration. Addition of sodium tetraborate ($Na_2B_4O_7$, borax) to aqueous saccharide solutions raises the "effective" saccharide molecular size through complex formation between a tetrahydroxy borate ion and one or two saccharide molecules.[16—18] How the borate–saccharide complexation affects the protein structure-stabilizing effect was also studied.

Experimental

Glucose, maltose, dextran (average MW: 10200), bovine serum albumin (BSA, essential fatty acid free), and chicken egg albumin (ovalbumin) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Maltotriose, maltotetraose, maltopentaose, and maltoheptaose were obtained from Hayashibara Biochemical Lab. (Okayama, Japan). Sodium tetraborate decahydrate and dextrans of different molecular weights (average MW: 1060, 4900) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and Serva Electrophoresis GmbH (Heiderberg, Germany), respectively. Other chemicals were from Wako Fine Chemical Industries (Osaka, Japan). BSA and ovalbumin were dialyzed against the potassium buffer before the sample preparation.

Freeze-drying of the protein solutions was performed using a freeze-drier (Freezevac-1C, Tozai Tsusho). Aqueous protein solutions (200 μl) containing BSA (10 mg/ml), saccharide (0—50 mg/ml), potassium phosphate buffer (20 mM), and sodium tetraborate (0—50 mM) in flat bottom glass vials were frozen by immersion into liquid nitrogen. The vials on a pre-cooled iron container were dried under vacuum without shelf temperature control for 16 h and at 35 °C for 6 h. All the solutions were freeze-dried without apparent collapse.

Thermal analysis of frozen solutions and freeze-dried solids was carried out using a differential scanning calorimeter (DSC Q10; TA Instruments) and Universal Analysis software (TA Instruments). An aliquot (10 μl) of the solution in a hermetic aluminum cell was cooled at approximately 20 °C/min, and scanned from −80 °C at 5 °C/min under dry nitrogen pursing. The thermal transition temperature of the frozen solution was obtained from the peak in the derivative thermogram. A freeze-dried solid (approximately 1.3—1.9 mg) in a hermetic aluminum cell was scanned from −20 °C at 10 °C/min to obtain the glass transition temperature (T_g).

The secondary structures of proteins were analyzed with an FT-IR system

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(MB104 spectrophotometer with Prota software; ABB Bomen). Spectra of aqueous protein solutions (10 mg/ml in 20 mM potassium phosphate buffer) were recorded at 4 cm^{-1} resolution using infrared cells with a 6 μm spacer (512 scans, room temperature). Reference spectra were recorded with a corresponding buffer and excipient solutions. Spectra of the freeze-dried solids were obtained from pressed disks containing approximately 1 mg protein and 250 mg potassium bromide (256 scans). Second-derivative spectra were calculated as described by Byler and Susi[19] using GRAMS/32 software (Galactic Industries) and smoothed with a 7-point smoothing function. The second-derivative amide I spectrum was area-normalized as described by Kendrick *et al.*[20] The amide II absorption peak of the freeze-dried solids was obtained from the spectra.

Results

Figure 1 shows area-normalized second-derivative amide I FT-IR spectra of BSA in aqueous solutions and freeze-dried solids. The native BSA in a potassium phosphate buffer solution (solution control) showed a large α-helix band at approximately 1656 cm^{-1}. The saccharides did not alter the BSA conformation in the aqueous solutions. Freeze-drying of BSA in the potassium phosphate buffer resulted in a broad derivative spectrum, indicating perturbation of the protein secondary structure.[6,7] Among the maltooligosaccharides, maltose showed the greatest ability to maintain the sharp BSA α-helix band in freeze-drying. The finding that the α-helix band became broader as the number of saccharide units increased suggests that the structure-stabilizing effect decreased as a result of the freeze-drying stresses. Freeze-drying of BSA with glucose resulted in an α-helix band shape comparable to that of maltotriose. The structure-stabilizing effect of the saccharides reached a plateau at concentrations lower than those employed in the present study (50 mg/ml, data not shown).[21]

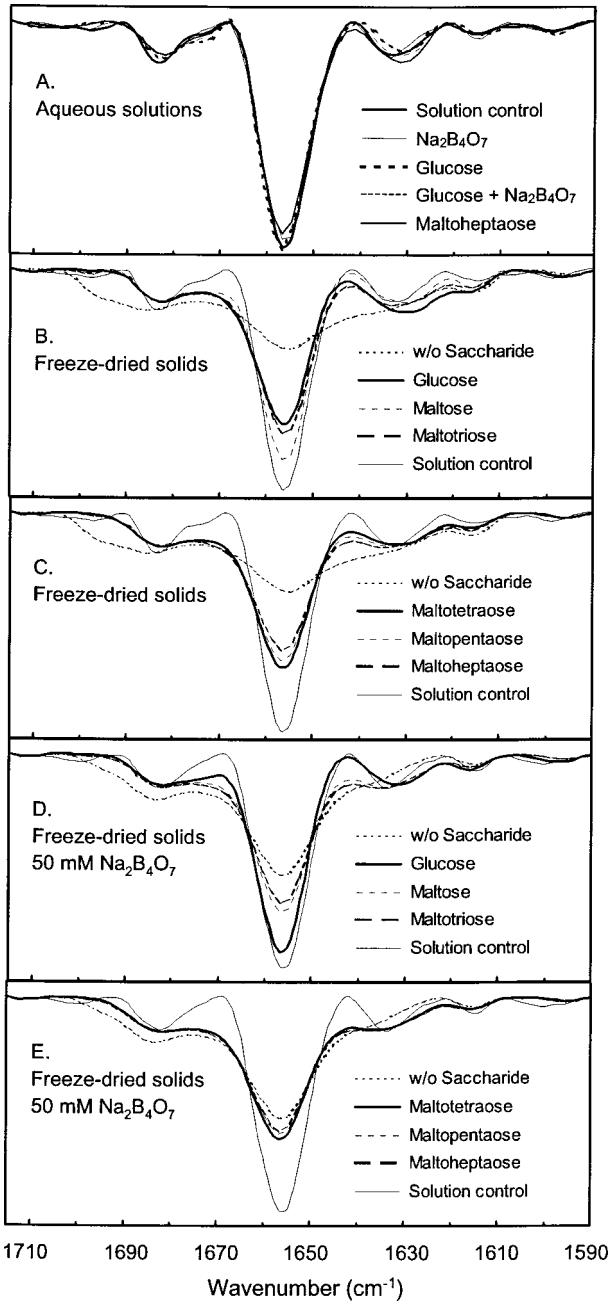

Fig. 1. Effect of Mono- to Oligosaccharides on the Area-Normalized Second-Derivative FT-IR Spectrum of Aqueous and Freeze-Dried BSA

Freeze-dried solids from aqueous solutions containing BSA (10 mg/ml), saccharides (50 mg/ml), potassium phosphate buffer (20 mM), and sodium tetraborate (0, 50 mM) were subjected to FT-IR analysis.

Addition of 50 mM sodium tetraborate did not alter the BSA structure in the aqueous solution, whereas it showed a slight structure-stabilizing effect during the freeze-drying process. Co-lyophilization with sodium tetraborate (50 mM) and most of the maltooligosaccharides resulted in a BSA α-helix band broader than by maltooligosaccharide alone, indicating that the complex formation reduced the structure-stabilizing effect. In contrast, the combination of glucose and sodium tetraborate resulted in a clear increase in structure stabilization compared to that by glucose alone.

Figure 2 shows the amide II absorption peak of freeze-dried BSA. Co-lyophilization with saccharides shifted the amide II peak to higher wavenumbers, suggesting hydrogen-bonding between BSA and the saccharides in the freeze-dried solids.[9] Glucose induced the largest shift in the absorption peak, followed by maltose and the other maltooligosaccharides. Co-lyophilization with sodium tetraborate and the saccharides reduced the peak shift.

Figure 3 shows the effect of sodium tetraborate on the thermal transition temperatures of frozen solutions containing BSA, various saccharides, and potassium phosphate buffer. The frozen BSA and saccharide combination solutions showed T_g' transitions at −38.1 °C (glucose), −26.5 °C (maltose), −22.2 °C (maltotriose), −19.6 °C (maltotetraose),

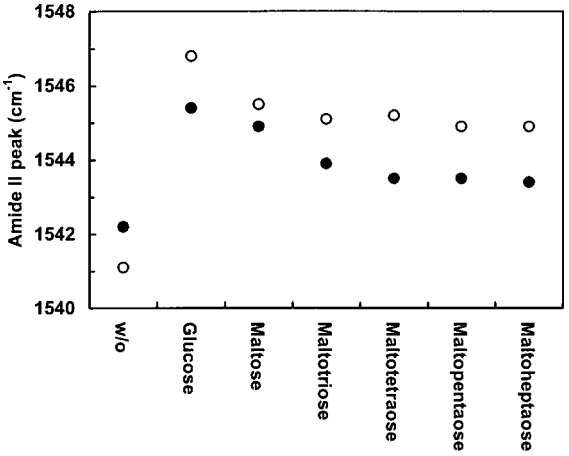

Fig. 2. Amide II Absorption Peaks of BSA (10 mg/ml) Freeze-Dried with Various Saccharides (50 mg/ml) and Potassium Phosphate Buffer (20 mM)

○: w/o $Na_2B_4O_7$; ●: 50 mM $Na_2B_4O_7$.

−17.7 °C (maltopentaose), and −14.9 °C (maltoheptaose) in the absence of sodium tetraborate.[22,23] The shape and temperature of the T_g' transition suggested freeze-concentration of the solutes into a single mixed phase.[24,25] A frozen solution containing 50 mM sodium tetraborate and 20 mM potassium phosphate buffer showed a transition peak at −32.5 °C (data not shown). Addition of sodium tetraborate raised the T_g' peak temperatures of the frozen BSA and saccharide combination solutions. Some combination frozen solutions presented transitions at temperatures above those of the single-solute solutions, suggesting a strong interaction between the components.[18]

Freeze-dried solids from solutions containing BSA (10 mg/ml) and saccharides (50 mg/ml) showed a broad glass transition in thermal analysis (Fig. 4). The glass transition temperature of a freeze-dried maltose and BSA combination was higher than that of the glucose and BSA. Other saccharide combinations also showed T_g midpoints above room temperature (data not shown). Co-lyophilization with sodium tetraborate raised the glass transition temperatures, indicating a reduced saccharide molecular mobility by the complexation.[16—18]

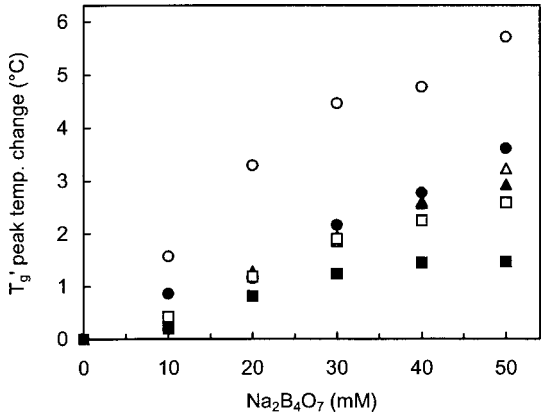

Fig. 3. Effect of Sodium Tetraborate on the Glass Transition Temperatures of the Maximally Freeze-Concentrated Phase (T_g') of Frozen Solutions Containing BSA (10 mg/ml), Saccharides (50 mg/ml), and Potassium Phosphate Buffer (20 mM)

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

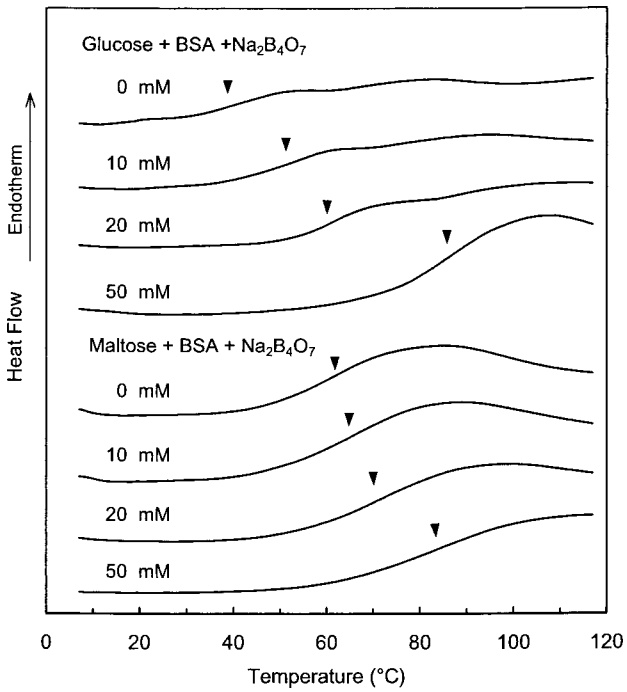

Fig. 4. Thermal Profiles of Freeze-Dried Solids from Solutions Containing BSA (10 mg/ml), Saccharides (50 mg/ml Glucose, Maltose), Potassium Phosphate Buffer (20 mM), and Sodium Tetraborate (0—50 mM)

Freeze-dried solids (1.3—1.9 mg) in hermetic aluminum cells were scanned from −20 °C at 10 °C/min.

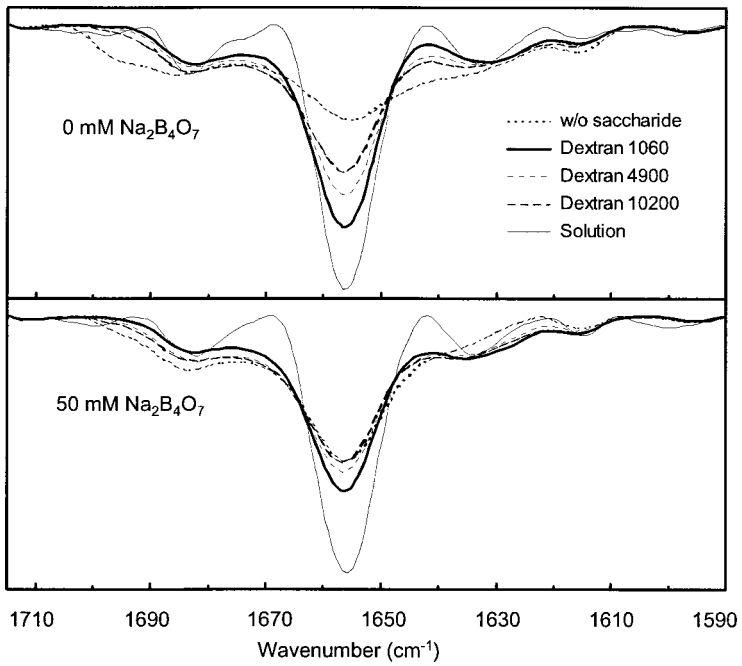

Fig. 5. Effect of Dextran and Sodium Tetraborate on the Area-Normalized Second-Derivative FT-IR Spectrum of Freeze-Dried BSA

Freeze-dried solids from aqueous solutions containing BSA (10 mg/ml), dextrans (50 mg/ml), potassium phosphate buffer (20 mM), and sodium tetraborate (0—50 mM) were subjected to FT-IR analysis.

Figure 5 shows the area-normalized second-derivative FT-IR spectrum of BSA freeze-dried with three molecular-weight dextrans. Dextran 1060 maintained the BSA α-helix band against the lyophilization stress to a degree similar to comparable molecular-weight maltooligosaccharides (*e.g.*, maltopentaose and maltoheptaose).[26] The effect decreased with increasing molecular weight of dextran. Consistent with other reports, the large dextran molecule (dextran 10200) was not very effective at stabilizing the protein structure during freeze-drying.[27,28] Sodium tetraborate reduced the structure-stabilizing effect of dextran 1060 and dextran 4900, whereas it did not alter the low stabilizing effect of dextran 10200.

Figure 6 shows the effects of various saccharides on the conformation of co-lyophilized ovalbumin. The native ovalbumin in aqueous solution presented a spectrum with two major bands at approximately 1656 cm^{-1} (α-helix) and 1638 cm^{-1} (β-sheet).[6,19] Freeze-drying of ovalbumin without saccharides resulted in a broader spectrum, indicating a perturbed secondary structure. Co-lyophilization with maltose gave a sharper α-helix and β-sheet bands that were closer to those of the native protein spectrum in the aqueous solution. The α-helix and β-sheet bands gradually became broader as the molecular weights of the co-lyophilizing oligosaccharides were increased. Freeze-drying of ovalbumin with sodium tetraborate resulted in sharper bands, suggesting a slight structure-stabilizing effect. Co-lyophilization with sodium tetraborate reduced, to some degree, the structure-stabilizing effect of most of the saccharides, whereas the salt increased the structure-stabilizing effect of glucose.

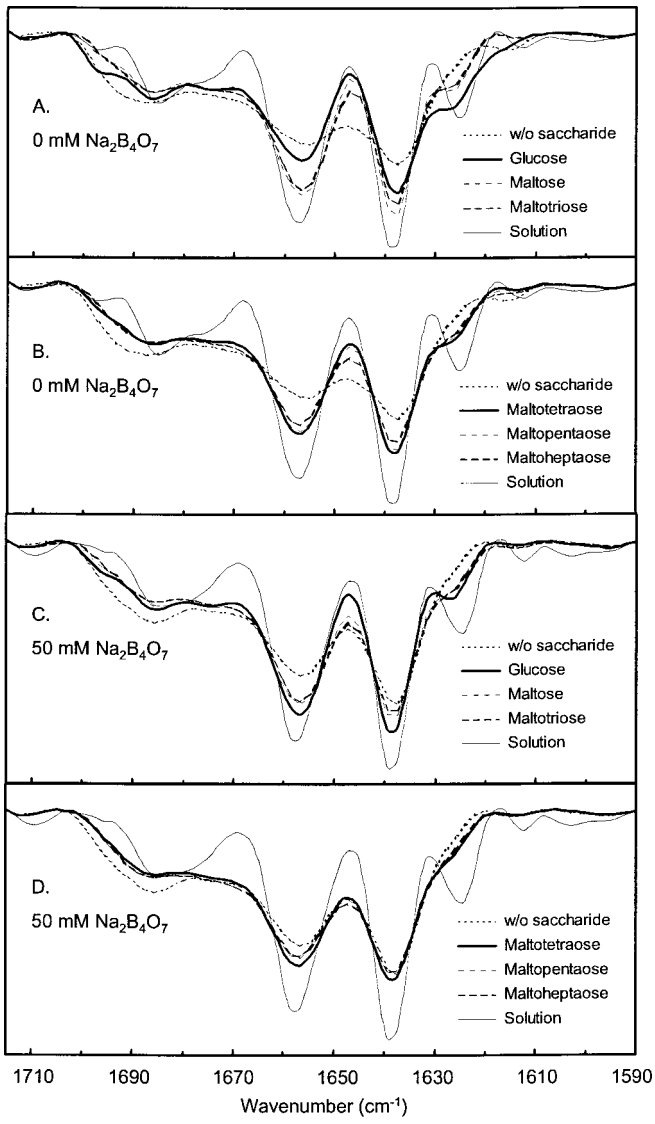

Fig. 6. Effect of Mono- to Oligosaccharides on the Area-Normalized Second-Derivative FT-IR Spectrum of Freeze-Dried Ovalbumin

Freeze-dried solids from aqueous solutions containing ovalbumin (10 mg/ml), saccharides (50 mg/ml), potassium phosphate buffer (20 mM), and sodium tetraborate (0—50 mM) were subjected to FT-IR analysis.

Discussions

FT-IR study of BSA and ovalbumin indicated secondary structure perturbation in the freeze-dried solids, which was consistent with other reports.[4,6,7] The method is often used in freeze-dried protein formulation studies since the resolution enhancement of amide I absorption (self-deconvolution and second derivative) allows comparison of protein conformation in diverse environments. Whereas the dehydration-induced conformation changes has been supported by other analytical methods (*e.g.*, solid circular dichroism),[29] there are some debates on the sample preparation and data interpretation (*e.g.*, band assignment) of the dried protein FT-IR analysis.[30] The partial conformation change can lead to protein aggregation during the rehydration processes, whereas most of the protein molecules return to native structure.[6,7] The structural alteration can also increase chemical degradation during storage.[8]

Among the maltooligosaccharides and dextrans, maltose showed the greatest ability to maintain BSA and ovalbumin conformation during freeze-drying. The structure-stabilizing effect decreased with increasing molecular weight of the saccharide. This result was consistent with a previous report that oligosaccharides protect lower-concentration catalase from inactivation during freeze-drying.[14] A large structure-stabilizing effect of disaccharides has also been demonstrated in freeze-drying of liposomes.[31]

Differing ability of saccharide molecules to interact with protein is expected to result in the different degrees of structural stabilization during freeze-drying. The saccharides protect native protein conformation against dehydration stresses by replacing essential water molecules through molecular interaction (*e.g.*, hydrogen bonding) with the protein molecules.[1—4,9] Larger saccharide molecules should have a lesser ability to form effective molecular interaction with proteins by the increasing steric hindrance and possible phase separation in the freeze-concentrates.[14,24,27] Dehydration is one of the most significant of the various stresses—pH change, co-solute concentration, low temperature, contact with surfaces, dehydration, *etc.*—that proteins face during the freeze-drying process.[1—5,15,32] Large molecular mobility in frozen solution and dried solid have been suggested to limit the structure-stabilizing effect of glucose, in spite of the considerable molecular interaction with the proteins.[33]

Co-lyophilization with sodium tetraborate reduced the protein structure-stabilizing effect of most of the saccharides studied. Complex formation between a tetrahydroxy borate ion and one or two saccharide molecules are expected to reduce the accessible saccharide hydroxyl groups for the structure-stabilizing molecular interaction, both by increasing the

steric hindrance and by occupying some of the hydroxyl groups by complexation. In contrast to other saccharides, the glucose and borate combination resulted in an increased structure-stabilizing effect during freeze-drying. Improved physical properties (*e.g.*, reduced molecular mobility) and sufficient structurally accessible free hydroxyl groups for the molecular interaction should result in the apparent structure-stabilizing effect of the borate-glucose complex. Many other borate-saccharide complexes are expected to achieve the kinetic protein stabilization at the expense of the structure-stabilizing effect.[16—18] Because of this drawback, borate-saccharide combinations will be useful in only a limited number of applications, such as for high temperature storage. Possible health risks of boron-containing compounds should be also taken into consideration in pharmaceutical formulation design.

The saccharides showed a decreased protein structure-stabilizing effect with increasing molecular weight, or by the borate-saccharide complexation, against the lyophilization-induced protein structural perturbation. The appropriate formulation composition should thus be determined by balancing various characteristics of the protein, application, and storage conditions.

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