Effect of Sucrose/Raffinose Mass Ratios on the Stability of Co-Lyophilized Protein During Storage Above the T_g

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Purpose. To examine the potential of raffinose as an excipient in stabilizing protein and to study the effect of sucrose/raffinose mass ratios on the stability of co-lyophilized protein and amorphous solids during storage at an elevated temperature.

Methods. Glucose-6-phosphate dehydrogenase (G6PDH) was co-lyophilized with sucrose and raffinose mixed at different mass ratios. The activity of dried G6PDH was monitored during storage at 44°C. Thermal properties of sucrose/raffinose matrices were determined by differential scanning calorimetry (DSC).

Results. Mass ratios of sucrose to raffinose did not affect the recovery of G6PDH activity after freeze-drying, but significantly affected the stability of freeze-dried G6PDH during storage. The sucrose-alone formulation offered the best enzyme stabilization during storage. With increasing fraction of raffinose, the G6PDH stability decreased, sugar crystallization inhibited, and crystal-melting temperature increased.

Conclusions. Despite the higher T_g of the formulations with higher fraction of raffinose, they provided less protection for G6PDH than did sucrose alone during storage. Our data do not support the prediction from recent thermophysical studies that raffinose should be superior to sucrose and trehalose as a potential excipient or stabilizer.

KEY WORDS: freeze-drying; glass transition; protein stabilization; raffinose; sucrose.

INTRODUCTION

Many proteins are unstable during freezing, drying, and subsequent storage. Protein denaturation and degradation could occur under diverse stresses encountered in solution and in the frozen or dried state (1). Protective effects of several sugars and other polyhydroxy compounds on proteins and other biomaterials during freezing, dehydration and subsequent dry storage have been well studied (2–4).

Recently, increased attention has been paid to raffinose as a potential excipient in stabilizing biomaterials (5–9). Raffinose and other oligosaccharides accumulate in many organisms that can survive extreme desiccation. The longevity of such organisms in the dry state was found to be associated with the content of these oligosaccharides and their ratio to sucrose (10,11). Raffinose has several interesting properties that make it a promising excipient. Firstly, raffinose appears to be more effective in hydrogen bonding with biomolecules than do sucrose and trehalose (12). The direct hydrogen bonding between sugars and biomolecules is required for the stabilization of proteins, membranes and cells during freezing, dehydration, and subsequent storage (1,2,13–15). Secondly, upon freezing, aqueous raffinose solution is able to form a freeze-concentrated amorphous solid (FCAS) with a higher glass transition temperature (T_g) and higher unfrozen water content than other disaccharide solutions (16,17). The collapse temperature (T_c) of the raffinose FCAS during freeze drying is much higher than those of lactose, maltose, sucrose, and trehalose (18). T_g and T_c are important parameters that are associated with physical and chemical stability of labile therapeutic substances during processing (1,19,20). At low moisture level (<15 %), freeze-dried amorphous raffinose has comparable or higher T_g than trehalose glass at the same moisture content (6,21,22). Third, raffinose forms the most "fragile" glass among those pharmaceutical excipients for which data are available. The "zero mobility" temperature (T_0) of anhydrous raffinose glass is much higher than those of sucrose and trehalose (8,23). Lastly, amorphous raffinose is probably the best-known "water sequester" among soluble carbohydrates. When raffinose crystallizes, it forms pentahydrate crystals. Each raffinose molecule is able to sequester up to five water molecules or ~18% of its anhydrous mass. The existence of lower raffinose hydrates was also reported (7). Water acts as a plasticizer in amorphous, freeze-dried products. Crystallization as hydrate sequesters water in the dry sugar matrix, increases the T_g of the remaining amorphous phase, and therefore may offer greater chemical and physical stability for dried pharmaceutical products during long-term storage (5,6,24). According to these thermophysical properties, raffinose was thought to be the preferred choice as an excipient or stabilizer (8).

The objective of the present study was to examine the potential of amorphous raffinose as an excipient in stabilizing protein during storage. The stability of co-lyophilized protein and solid sugar matrices was investigated at an elevated temperature for the sucrose/raffinose mixtures at different mass ratios.

MATERIALS AND METHODS

Enzyme samples were made using glucose, raffinose pentahydrate, sucrose, and glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49) (Sigma, ST Louis, MO, USA). G6PDH suspension (50 mM Tris-HCl buffer containing 1 mM $MgCl_2$ and 3.2 M $(NH_4)_2SO_4$, pH 7.5) was desalted using a 10-DG column (cut-off, 6 kDa) (Bio-Rad, Hercules, CA, USA) with 50 mM Tris-HCl buffer (pH 7.5). Desalted G6PDH was then mixed with stock solutions of glucose, sucrose and raffinose to the final concentrations: 670 units/L G6PDH (~8 mg protein), 10 g/l glucose, and 100 g/l sucrose plus raffinose at different mass ratios of 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. Aliquots (15 μl) of sugar/enzyme solution were pipetted into 1.5-ml Eppendorf tubes. Each sample contained 0.01 unit G6PDH (~0.12 μg protein), 0.15 mg glucose and 1.5 mg sucrose plus raffinose. Samples were frozon at −80°C before freeze drying in a lyophilizer, Freezemobile 25EL, (Virtis Co., Gardiner, NY, USA) under the chamber pressure of 100 to 200 mTorr for 15 hours. The temperature of heat source was about −15°C at first 2 hours, and then gradually increased to room temperature (~24°C).

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Dry mass of samples was measured after samples were dried at 105°C to constant weight. Residual moisture of freeze-dried samples, as determined gravimetrically, was ~0.05 to 0.06 g water per gram dry matter (g/g).

After freeze drying, samples were sealed immediately. Sealed sample tubes were then packed into jars where RH was maintained at ~20% with silica gel, and were stored at 44°C for up to 81 days. Samples were regularly taken during storage for the determination of residual enzyme activity. For each sample, 1-ml assay reagent (containing 0.38 mM NADP and 6.3 mM $MgCl_2$ and 3.3 mM glucose-6-phosphate in 50 mM Tris-HCl buffer, pH 7.5) was added to the Eppendorf tube. The absorbance at 339 nm was recorded with a Shimadzu UV-160A spectrophotometer, and enzyme activity was calculated according to the change in absorbance between 60 and 180 seconds after mixing. At least triplicate samples were used for each determination.

To study the effect of the mass ratios between sucrose and raffinose on the thermal stability of amorphous sugar matrices during storage, a separate set of glucose/sucrose/raffinose samples were prepared at the same time. Aliquots of 60 μl sugar solution were pipetted into 1.5-ml Eppendorf tubes, frozen at –80°C, and then freeze dried. Therefore, each sample contained 0.6 mg glucose and 6 mg sucrose plus raffinose at different mass ratios. The thermal stability of matrices was studied during storage at 44°C. Samples of 4 to 5 mg were loaded into 30-μl aluminium crucibles and the pans were crimped and measured by differential scanning calorimetry (DSC-131, Setaram, France). All samples were first cooled with liquid nitrogen to below –80°C, and then scanned at 10°C/min to 120°C. The mid-temperature of glass transition was taken as the T_g.

The small amount of glucose (1%) was added to all samples for the following reasons. Firstly, raffinose alone had a strong tendency to crystallize during freezing and freeze-drying at temperature above the T_g'. Even if a small percentage of sample materials crystallized during freezing and freeze drying, it would affect the comparison of the stabilization among various formulations. The incorporation of small amount of glucose could prevent the possibility of sugar crystallization during freezing and freeze-drying. Secondly, glucose would gradually separate from amorphous matrices during storage (25). The incorporation of glucose offered a convenient method to study the physical stability of samples during storage. The crystallization of glucose could be more easily detected by differential scanning calorimetry. Thirdly, glucose, a reducing sugar, was known to attack enzyme via the Maillard reactions, and hence its incorporation facilitated the study of enzyme preservation by sucrose and raffinose during storage.

RESULTS

Activities of Enzyme and the Physical State of Freeze-Dried Products

The recovery of enzyme activity after freeze drying was very high for all sucrose/raffinose mixtures, ranging from 94 to 98% (Table I). No significant difference was observed for the recovery of G6PDH activity after freeze-drying among six mass ratios between sucrose and raffinose. As such, the present study permitted a fair comparison for enzyme preservation during subsequent storage in sucrose/raffinose matrices with different mass ratios. However, for control samples (freeze-dried without excipient), the recovery of G6PDH activity after freeze-drying was extremely low, only 3.1 ± 1.2 % of the original activity (Table I). Therefore, no comparison in storage stability can be made between control samples and other six treatments.

The crystallization of raffinose was avoided during the freeze-drying process, and the dried product remained in completely amorphous phase (Fig. 1). Visual examination of the freeze-dried products did not show any sign of collapse. The T_g of freeze-dried products increased slightly from 21.0 to 24.3°C as the sucrose/raffinose mass ratio changed from 100:0 to 20:80 (Table I). However, the T_g of samples with a sucrose/raffinose mass ratio of 0:100 was much higher (36.5°C). DSC thermograms showed that the width of the glass to liquid transition was broader with the increasing fraction of raffinose (Fig. 1).

Stability of Freeze-Dried G6PDH During Storage Above the T_g

The loss of G6PDH activity in samples was monitored regularly during storage for all preparations (Fig. 2). Sucrose

Table I. Recovery of Glucose-6-Phosphate Dehydrogenase Activity and Corresponding Glass Transition Temperatures (T_g) of Freeze-Dried Samples After Co-Lyophilization with Sucrose and Raffinose Mixed at Different Mass Ratios and After Storage at 44°C. Data Are Mean ± SE of at Least Three Measurements. Numbers in Parentheses Denote the Number of Independent Measurements

Sucrose:Raffinose mass ratio	Activity (%) after freeze-drying	Glass transition temperature (°C)	
		Before storage	After 45-d storage
100:0	95.6 ± 3.1	21.0 ± 1.2 (5)	——[a] (3)
80:20	94.5 ± 4.9	20.8 ± 1.5 (5)	17.1 ± 1.6 (3)
60:40	98.2 ± 4.4	22.3 ± 2.7 (3)	18.6[b] (3)
40:60	93.7 ± 4.2	24.6 ± 2.4 (5)	21.9[b] (3)
20:80	98.2 ± 3.8	24.3 ± 0.9 (4)	——[a] (3)
0:100	97.6 ± 4.1	36.5 ± 2.8 (4)	38.8 ± 3.7 (2)
No excipient	3.1 ± 1.2		

[a] No glass transition was detected.
[b] Glass transition was detected in only one sample.

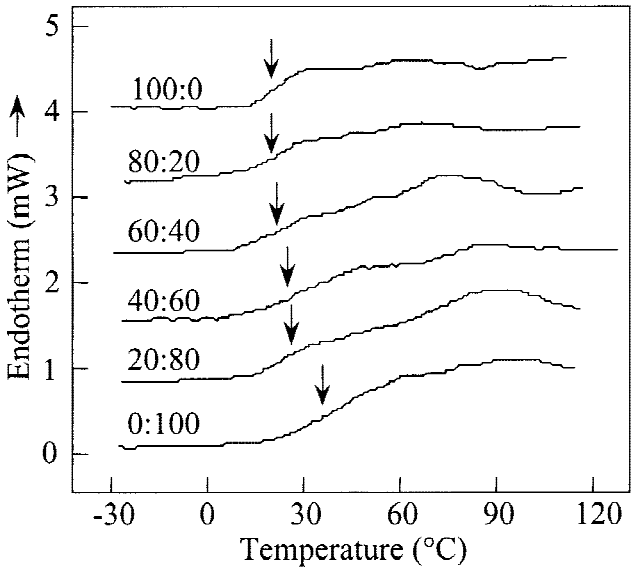

Fig. 1. DSC thermograms of freeze-dried samples with different mass ratios between sucrose and raffinose. Scan rate was 10°C/min. Thermograms are normalized to a mass of 5 mg. Glass transition is indicated by arrows.

alone (i.e., the mass ratio of 100:0) offered the best protection, with about 80% of initial G6PDH activity retained after 81 days of storage at 44°C. The stability of G6PDH decreased significantly as the mass ratio of sucrose to raffinose changed from 100:0 to 0:100. Samples with the sucrose/raffinose mass ratio of 0:100 retained only about 50% of initial G6PDH activity after 81days of storage. For all six preparations, the activity of G6PDH decreased much faster during the first 20 days of storage at 44°C (Fig. 2). The inactivation of G6PDH during the first 20 days could be described by the first-order kinetics. For subsequent 60 days of storage, the loss of G6PDH activity resembled the zero-order inactivation. Figure 3 shows the effect of the sucrose/raffinose mass ratios on the rate constant of G6PDH inactivation during storage. Rate constants calculated with the "square root of time kinetics" (26) were presented for comparison. The stability of G6PDH decreased with the increasing fraction of raffinose.

Physical Stability of Freeze-Dried Products During Storage

The physical state (or structure) of sugar matrices also changed during storage at 44°C. Before storage, the glass transition event was clearly detected in all samples, and no sharp endothermic melting peak was observed (Fig. 1). After 45 days of storage, the glass transition became undetectable in many samples, and in those samples where glass transition was found, the T_g decreased by 4 to 5°C, except for the formulation with the sucrose/raffinose mass ratio of 0:100. Endothermic (melting) peaks were observed from 70 to 100°C (Fig. 4A). The mass ratios between sucrose and raffinose affected the thermophysical properties of sugar matrices. With the increasing fraction of raffinose, the melting temperature shifted from 75 to 92°C, whereas the melting enthalpy decreased from 7.4 J/g to 4.5 J/g (Fig. 4B). The melting enthalpy was reported to be in the range of 175 to 191 J/g for glucose hydrate (27) and of 143 to 150 J/g for raffinose pentahydrate (6,7,28). If the melting peak was solely glucose hydrate,

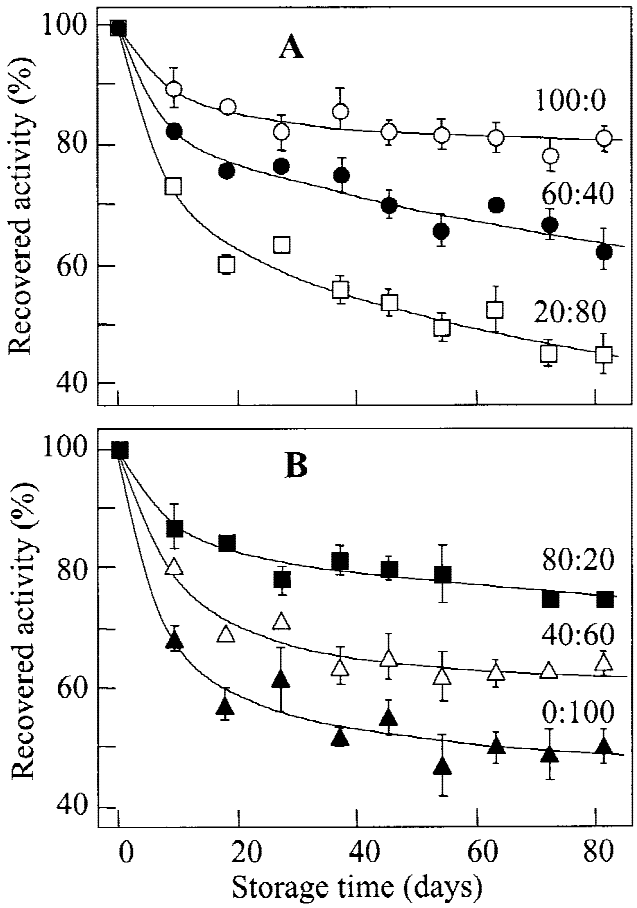

Fig. 2. Effect of mass ratio between sucrose and raffinose on the inactivation of glucose-6-phosphate dehydrogenase during storage at 44°C. Mass ratios are shown near individual curves. Data are mean ± SE of three measurements.

roughly 26 to 44% of glucose in samples crystallized. On the other hand, if the melting peak was solely raffinose pentahydrate, only 3 to 5% of raffinose in samples crystallized during 45 days of storage. Therefore, the majority of carbohydrate matrix still remained in amorphous phase. The failure to detect the glass transition by DSC in many samples might be attributed to the annealing effect (molecular and structural relaxation), which reduced the change in specific heat capacity ($_{\Delta}Cp$) during the glass transitions.

DISCUSSION

Raffinose previously was reported to be as effective as trehalose in stabilizing several enzymes from the inactivation during storage at high temperatures and was superior to lactose, maltose and sucrose (9,21,29). More recently, studies on the thermophysical properties of raffinose have predicted that raffinose should be the preferred choice as an excipient for the stabilization of biomaterials (8). However, our study on protein stabilization during storage suggested that raffinose may not be superior to sucrose and trehalose. Our data showed that, when the freeze-dried samples were stored at a temperature that was 10 to 20°C above the T_g of amorphous products, the formulation with the highest sucrose/raffinose ratio offered the best stabilization, and with the increasing fraction of raffinose, the loss of enzyme activity actually in-

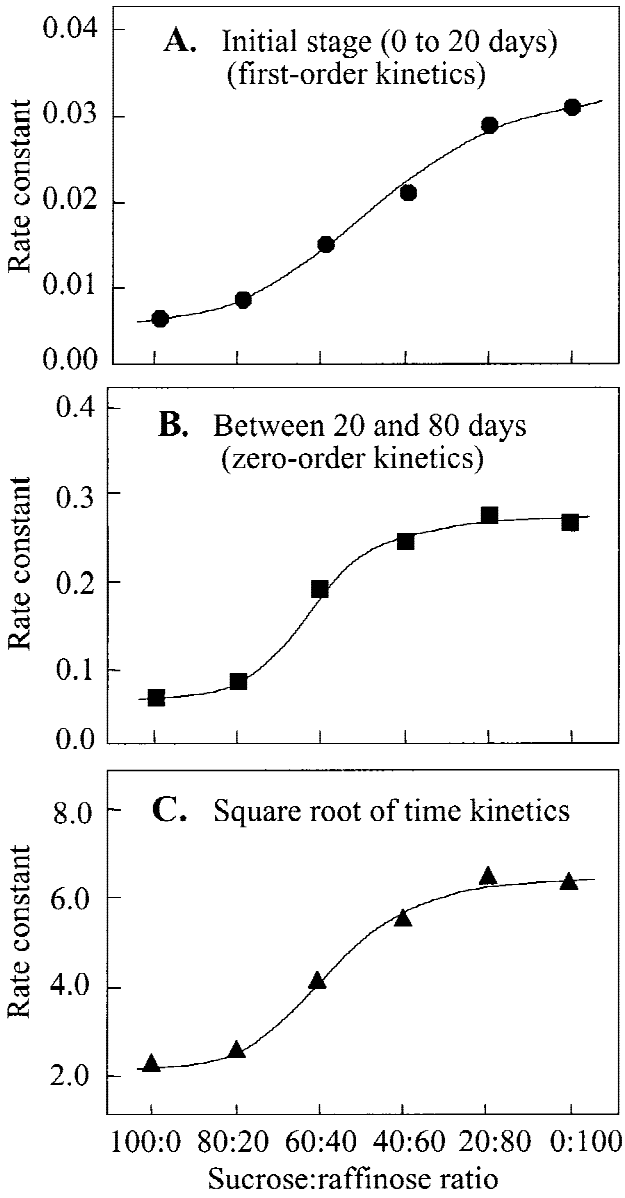

Fig. 3. Effect of mass ratio between sucrose and raffinose on the inactivation rate constant of glucose-6-phosphate dehydrogenase during storage at 44°C. (A) The first-order rate constant during the first 20 days. (B) The zero-order rate constants between 20 and 80 days. (C) The rate constants calculated with the "square root of time model."

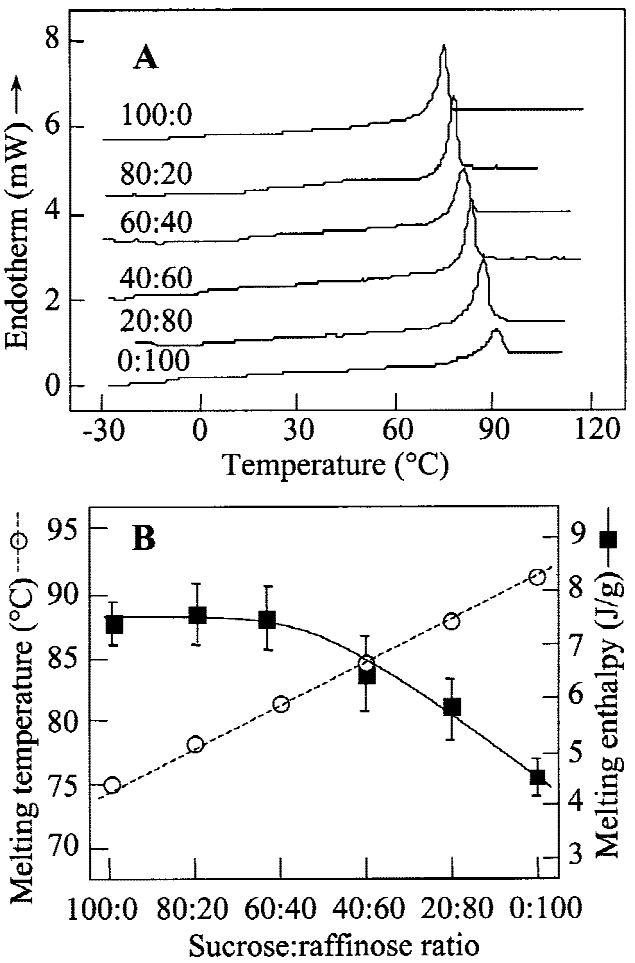

Fig. 4. (A) DSC thermograms of freeze-dried samples after 45-day storage at 44°C. Mass ratios between sucrose and raffinose are shown near individual thermograms. Scan rate, 10°C/minute. Thermograms are normalized to a mass of 5 mg. (B) Effect of mass ratios between sucrose and raffinose on melting temperature and melting enthalpy of samples after 45-day storage. Data of melting enthalpy are mean ± SE of three measurements.

creased during storage despite the slightly higher T_g of the formulations with higher fraction of raffinose. The discrepancy in results between the present study and previous studies may be attributed to different dehydration and storage conditions used. In the study of Rossi *et al.* (21), the solutions of restriction enzyme *Eco*RI were vacuum dried in the presence of substantial amount of glycerol, and the dried samples were stored at 45°C for 21 days. In another study (29), the initial water content of sucrose samples before storage was 1.5% higher than samples prepared with maltose, lactose, raffinose and trehalose, and therefore, the T_g of sucrose formulation was much lower than those of other sugar formulations. In addition, freeze-dried enzymes were stored at 65°C in the presence of silica gel as the desiccant (29). Under such conditions, the isothermal dehydration would likely increase the T_g of amorphous glass domain, thus increasing enzyme stability during subsequent storage period. The amorphization of raffinose pentahydrate was also documented under isothermal dehydration at temperature above 60°C (6,8). The retardation of protein inactivation with the increased T_g during storage was also reported (25). It should be pointed out that previously published studies used only one or two excipients, whereas in the present study an additional excipient glucose was used. All of those factors might contribute to different results between the present study and previous studies.

The complex behavior of protein degradation kinetics was observed previously, and was interpreted due to the presence of a degradation intermediate (26). The shift of protein stability kinetics during storage is interesting and deserves further investigation (Fig. 2). We tried first to calculate the rate constant of activity loss during storage, using the "square root of time kinetics" proposed by Pikal and Rigsbee (26). The "square root of time kinetics" described four of six degradation curves quite well, although the last two points (72 and 81 days) consistently deviated from the fitted curves

(higher than the expected values). A closer examination, however, indicated that simple first-order and zero-order kinetics could describe the experimental data even better. The mechanism for the transition from the first-order-kinetics to the zero-order-kinetics after 20 days of storage remains unclear, and we suspect that it might be related to the structural collapse of the carbohydrate matrices. To examine this possibility, we had carried out an additional storage experiment using sucrose alone. Freeze-dried sucrose/enzyme samples were stored at several temperatures. We observed that the transition from the initial faster degradation kinetics to subsequent slower kinetics was roughly correlated to the structural collapse (data not shown). As the carbohydrate matrices collapse, the specific surface area that was related to reactivity would be reduced, and therefore adventitious protein degradation processes were expected to slow down significantly.

The T_g of freeze-dried products was not the only factor determining protein stability. The slight increase in the T_g as the raffinose mass ratio increased (Table I) was not accompanied with greater protein stability during storage. In fact, protein stability during storage decreased as the raffinose mass ratio increased (Figs. 1 and 2). It has been suggested that the fragility of a glass is relevant to the protein stabilization (23). In general, a strong glass displays a broad transition, whereas a fragile glass gives rise to a narrow transition. In the present study, we observed that, as the raffinose ratio increased, the width of the glass to liquid transition became broader, suggesting the presence of a less fragile glass (Fig. 1). The observed decrease in the fragility as the raffinose ratio increased was well correlated to the reduced protein stability during storage (Fig. 3). It should be pointed out that two earlier studies reported that the glass of pure raffinose was the most "fragile" among those carbohydrates commonly used as pharmaceutical excipients (8,23). Our result may be due to the presence of glucose and residual moisture (~0.05 to 0.06 g/g dw) in all freeze-dried samples. Water-plasticized glasses are much less fragile, and the effect of plasticization by water may vary among different carbohydrates.

Phase separation of the amorphous sugar matrices would lead to fast protein inactivation during storage (25). However, DSC measurements did not see sufficient evidence of phase separation in the present study. If significant phase separation had occurred during storage, we should have detected two or more separate glass transitions. The glucose-rich domain would have a much lower T_g than that of the sucrose-rich domain or the raffinose domain. Other structural changes of the amorphous sugar matrices, such as the loss of the glassy state and sugar crystallization, were detected during storage at 44°C (Fig. 4). Sugar crystallization was reflected by the presence of additional sharp endothermic (melting) peaks in DSC thermograms of samples after storage, which were not detected before storage. No attempt was made in the present study to identify the specific carbohydrate that crystallized and the cause for the gradual increase in melting temperature from 75°C to 92°C as the raffinose mass ratio increased. However, data available in literature suggested that endothermic peaks between 75°C and 92°C were probably from the melting of hydrates of glucose and raffinose. The monohydrate of glucose melts at 83°C, whereas the pentahydrate, tetrahydrate and trihydrate of raffinose have their melting points at 80°C, 84.3°C, and 85.4°C, respectively (7,28,30). The decrease in melting enthalpy with increasing raffinose mass ratio indicated that sugar crystallization became more difficult as raffinose content increased. Therefore, the trend of declining protein stability with the increasing fraction of raffinose was unlikely related to the crystallization of glucose and/or raffinose. The crystallization of raffinose as hydrate might not be accompanied with greater protein stability during storage.

In conclusion, the present study has found the sucrose/raffinose mass ratio did not affect the recovery of protein activity after freeze drying, but the stability of freeze-dried protein during storage was affected. With increasing raffinose mass ratio, protein stability decreased. Sugar matrices underwent significant structural changes during storage. However, the loss of enzyme activity was not simply correlated with the T_g, phase separation, and crystallization of sugar matrices.

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