Enthalpic relaxation in frozen aqueous trehalose solutions

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Abstract

Our object was to investigate the effect of annealing on the glass transition temperatures and enthalpic recovery of frozen aqueous solutions of trehalose. Trehalose solutions were subjected to differential scanning calorimetry wherein they were first cooled from room temperature to $-60\,^{\circ}$C, and heated to the annealing temperature, which ranged between -34 and $-48\,^{\circ}$C. Following isothermal annealing for the desired time period, the glass transition temperatures and the enthalpic recovery were determined in the final heating scan. Frozen unannealed trehalose solutions were characterized by two glass transition events. At a heating rate of $2\,^{\circ}$C/min, the observed Tg'_1 and Tg'_2 were ~-45 and $-31\,^{\circ}$C, respectively. Annealing resulted in an increase in the lower transition temperature, Tg'_1, while the higher transition temperature, Tg'_2, was unaffected. Enthalpic recovery due to annealing was associated only with Tg'_2. Annealing at $-36\,^{\circ}$C resulted in the highest value of Tg'_1 and the maximum enthalpic recovery. Irrespective of the heating rates, the magnitude of enthalpic recovery and Tg'_1 showed a similar trend (first an increase, followed by a decrease) as a function of annealing temperature. This suggests that annealing led to crystallization of ice and subsequently the system became maximally freeze-concentrated. Annealing, at temperatures higher than $-36\,^{\circ}$C, led to a reduction in enthalpic recovery associated with Tg'_2 and a lowering of Tg'_1. These observations are consistent with the hypothesis that the higher transition temperature coincides with the onset of ice melting. We have attempted to bridge two conflicting schools of thought regarding the origin of multiple glass transitions in frozen aqueous sugar solutions. The two glass transitions are attributed to the formation of two "populations" in the freeze-concentrated phase during "non-equilibrium" or rapid cooling—one, that is maximally freeze-concentrated and the other that contains a higher amount of water. The higher transition temperature also overlaps with the onset of ice melting.
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1. Introduction

Amorphous substances are characterized by higher enthalpy, entropy and free volume compared to their crystalline counterparts [1,2]. Below the glass transition temperature, Tg, glassy materials are in non-

equilibrium, while, the supercooled liquid state above the Tg is considered to be in pseudo-equilibrium. When stored at temperatures below but close to Tg, glasses usually undergo relaxation, that is, they lose excess enthalpy and tend to approach a state of equilibrium [3–6]. On heating the "relaxed" glass, the lost enthalpy is recovered at the glass transition. In a differential scanning calorimeter, this recovery is manifested as an endotherm associated with the Tg. Enthalpic relaxation below Tg and subsequent recovery

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at Tg are also indicative of the existence of molecular mobility below the glass transition temperature [7]. Since the time scale of molecular motions have been shown to be directly related to stability and shelf-life of amorphous pharmaceuticals, it is important to understand the enthalpic relaxation processes [8].

The glass transition temperature is often considered the single most important determinant of processing and storage conditions [2,9,10]. It is well documented that significant molecular mobility exists below the Tg [7,11,12]. In frozen aqueous solutions, the glass transition temperature of the maximally freeze-concentrated amorphous phase is referred to as the Tg′ [13]. The Tg′ governs the choice of the critical processing variables. In order to prevent collapse of these systems, primary drying is usually carried out at temperatures <Tg′. Similarly, during secondary drying, it is advisable to keep the temperature below the Tg of the system to prevent the risk of crystallization and/or collapse [14]. Thus, in order to design an optimal freeze-drying cycle, it is worthwhile for a pharmaceutical formulator to evaluate the Tg′ of frozen aqueous systems.

When a dilute aqueous solution is cooled to sub-zero temperatures, it undergoes supercooling and ice crystallization occurs well below its equilibrium freezing temperature [15]. As the temperature is lowered further, ice crystallization continues. However, when a particular concentration (which is solute dependent) is reached, the unfrozen liquid undergoes glass transition and the viscosity increases by many orders of magnitude. When the system is maximally freeze-concentrated, further ice crystallization below the Tg′ is inhibited under practical time scales [16,17].

Literature in the area of frozen aqueous solutions abounds with examples of two apparent glass transitions. The physical significance of these two transitions is unclear. There are various schools of thought that provide different explanations. Luyet and Rasmussen regarded the lower temperature transition to be the true glass transition and the higher temperature transition to be due to an interfacial interaction between ice crystals and the amorphous phase [18]. They used the terms "ante-" and "incipient" melting to describe the higher temperature transition. Using electron spin resonance, LeMeste and Simatos, concluded that the ante-melting phenomenon was due to the onset of ice melting [19]. Levine and Slade suggested that the higher temperature transition was the

glass transition of the maximally freeze-concentrated solute, and that the lower temperature transition was due to an amorphous phase that was not maximally freeze-concentrated [13]. This is supported by the recent work with frozen aqueous solutions of sucrose by Chang et al. [20]. Shalaev and Franks have related the higher temperature transition to a softening phenomenon, which is due to an observable deformation and change in mechanical characteristics [21]. Ablett et al. and Izzard et al. also provided evidence in support of the suggestion that the lower temperature transition is the glass transition while the higher temperature transition was due to the onset of ice melting [16,22].

While considerable attempts have been made to explain the origin of multiple glass transitions in frozen aqueous solutions, there are only a few reports in the literature that investigate the phenomena of enthalpic relaxation in these systems (for example, [23–25]). The enthalpic recovery at Tg′ was found to be dependent on the heating and cooling rates of the sample—slower heating and cooling rates led to an increase in enthalpic recovery at Tg′ [23]. A systematic study of enthalpic relaxation and recovery will improve our understanding of the physical chemistry and thermal behavior of frozen aqueous solutions. Because of the presence of water, the behavior of these systems can be assumed to be different from that of their dry counterparts (for example, freeze-dried materials).

The objectives of this work were: (i) to study the factors influencing the enthalpic recovery in frozen aqueous trehalose solutions; (ii) to compare the behavior of trehalose in frozen aqueous solutions with that of freeze-dried trehalose; and (iii) to utilize the data obtained to understand the nature of double glass transitions.

Trehalose, a non-reducing disaccharide that is retained amorphous in frozen aqueous solutions, was chosen as the model compound. There has been considerable attention on trehalose in the recent past owing to its ability to serve as a lyoprotectant.

2. Experimental

2.1. Materials and methods

D(+)-Trehalose dihydrate ($C_{12}H_{22}O_{11} \cdot 2H_2O$, Sigma, St. Louis, MO) was used as received.

2.1.1. Differential scanning calorimetry

A differential scanning calorimeter (model 2920, TA Instrument, New Castle, DE), equipped with a refrigerated cooling system, was used in the standard mode. Nitrogen was used as the purge gas, at a flow rate of ~60 ml/min. The DSC was calibrated, at 2 and 5 °C/min heating rates, using indium, mercury and distilled water as standards.

2.1.2. Thermal analysis of trehalose solutions

Trehalose solution (45% (w/w)) was prepared in distilled water, filtered (0.45 μm) and used immediately. About 15–20 mg of the solution was weighed in an aluminum pan, which was then hermetically sealed. The solution was cooled from room temperature to −60 °C at 15 °C/min, held for 15 min, heated to the annealing temperature at 5 °C/min, and annealed for a predetermined time. It was then cooled back to −60 °C and reheated. The results presented were obtained from these final heating scans. The onset of the glass transition temperatures have been reported. While the general procedure is described here, the specific experimental details are provided in Section 3.

2.1.3. Preparation and analysis of lyophilized trehalose

Freeze-dried trehalose was obtained by lyophilization in a bench-top freeze-drier (Unitop 400L, Virtis). About 25 ml of aqueous trehalose solution (10% (w/v)) was placed in petri dishes, cooled to −45 °C and subjected to vacuum (100 mTorr/13.33 Pa) for 48 h in a tray freeze-drier (Model UNITOP 400L, Virtis). Over the next 24 h, the temperature was gradually increased to 50 °C. The secondary drying was carried out at 50 °C for 24 h, after which the temperature was raised to 60 °C and the drying continued for 24 h more. The samples were removed from the freeze-drier and stored in a desiccator at room temperature over anhydrous calcium sulfate (RH ~ 0%) until use. The residual water content was <0.2% (w/w).

About 4–5 mg sample was packed in an aluminum pan, crimped non-hermetically, and heated under dry nitrogen purge. In order to facilitate the removal of evolved water, several pinholes were made in the lid. The sample was dried in the DSC pan for 2 h at 100 °C and then the thermal history was removed by heating to 142 °C at 5 °C/min. It was cooled to 100 °C at 20 °C/min and annealed for the desired time. The

sample was cooled to room temperature at 20 °C/min and finally heated at 5 °C/min. The results presented were obtained from these final heating scans.

3. Results and discussion

3.1. Factors affecting the glass transition temperature in frozen aqueous solutions

Aqueous trehalose solutions were cooled from room temperature at different rates. Trehalose does not crystallize when its aqueous solutions are cooled and is reported to form an amorphous freeze-concentrate with a single Tg' value ranging between −35 and −22 °C [26]. However, as observed in frozen aqueous solutions of other disaccharides [13], we detected two transitions in the DSC heating profiles of frozen aqueous solutions of trehalose (Fig. 1). For the sake of simplicity, the transitions have been referred to as Tg'_1 (lower transition temperature) and Tg'_2 (higher transition temperature). An exotherm was usually observed between the two transitions and has been attributed to the crystallization of ice. Such an event, in frozen aqueous solutions of other sugars, has been referred to as "devitrification" [16,17].

Fig. 1 reveals the effect of cooling rate on the Tg'_1 and Tg'_2 of frozen aqueous solutions of trehalose. Higher cooling rates resulted in lower observed Tg'_1
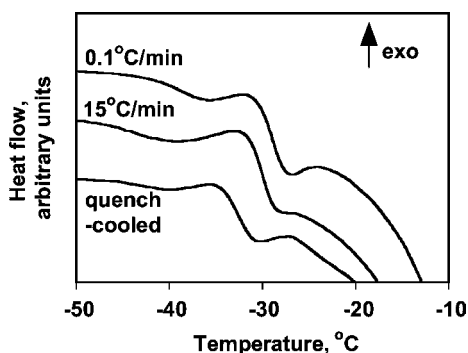

Fig. 1. Effect of cooling rate on the glass transition temperatures of frozen aqueous solutions of trehalose (45% (w/w)). The solutions were cooled from room temperature to −60 °C at 0.1 or 15 °C/min. One sample was quench-cooled by immersing in liquid nitrogen and the heating profiles are shown. All the solutions were heated at 2 °C/min to room temperature.

and Tg_2' values. For quench-cooled trehalose solutions, the Tg_1' and Tg_2' were ∼−46 and −34 °C, respectively, while for the solution that was cooled at 0.1 °C/min, the values were ∼−41 and −31 °C, respectively. The behavior of this frozen system is consistent with kinetically hindered ice formation in the rapidly cooled samples. Under moderate cooling rates, the Tg_1' and Tg_2' values were found to be independent of the initial trehalose concentration, strongly suggesting that in these systems, the unfrozen water fraction in the freeze-concentrate was constant. In an effort to determine the effect of solute content on the transition temperatures, a range of trehalose concentrations, from 5 to 45% (w/w), was studied. As expected, the two transitions became more discernable as the trehalose concentration became higher (data not shown). In order to maximize the signals, in all our subsequent studies of frozen systems, the trehalose concentration was 45% (w/w).

The glass transition is known to be highly dependent on the experimental conditions. Apart from the cooling rates used, as expected, the observed glass transition temperature(s) were also found to be dependent on the heating rates used in the DSC experiments. Since the Tg is reported to be the temperature where the experimental time scale equals the molecular relaxation time, an increase in the heating rate shifts the Tg to a higher temperature [27].

3.2. Factors affecting the enthalpic recovery in frozen aqueous solutions

The thermal history profoundly affects the observed enthalpic recovery of amorphous pharmaceuticals [28,29]. During isothermal annealing below Tg, the loss of enthalpy is dependent on the difference between Tg and the annealing temperature Ta, i.e. (Tg − Ta), as well as the annealing time. Relaxation is not significant at temperatures far below the glass transition temperature [7,30]. Thus, there exists an "aging range" where due to the existence of significant molecular mobility, enthalpic relaxation occurs. In pharmaceuticals, enthalpic relaxation was found to be negligible at ∼50 °C below the Tg [1]. In the case of frozen solutions, the aging range has not been determined.

Fig. 1 reveals the effect of cooling rate on enthalpic recovery. As the cooling rate decreased, the

material was allowed more time below the glass transition, including the "aging range". It is evident that the enthalpic relaxation increased as the cooling rate decreased. This is consistent with earlier observations of Her and Nail in frozen aqueous solutions of non-crystallizing solutes [23]. Our next object was to determine the temperature range where aging would occur, i.e. the "aging range" of freeze-concentrated amorphous trehalose. In addition, we were interested in determining the effect of annealing on the glass transition temperatures and the relationship, if any, between enthalpic relaxation and glass transition temperatures. Frozen aqueous trehalose solutions were annealed isothermally at temperatures ranging from −34 to −48 °C. The frozen solutions were held at the desired temperature for 2 h, and the enthalpic recovery associated with the glass transition was measured.

Fig. 2 contains the DSC profiles of frozen aqueous trehalose solutions annealed at various temperatures. The Tg_1' and Tg_2' of the unannealed samples were found to be ∼−45 and −31 °C, respectively. When the system was annealed at −34 °C, we observed enthalpic recovery associated *only* with Tg_2'. As the annealing temperature was decreased from −34 to −36 °C, the enthalpic recovery at Tg_2' increased. On lowering the annealing temperature further, there was a decrease in the enthalpic recovery. When aged at temperatures ≤−42 °C, we did not observe any recovery associated with either Tg_2' or Tg_1'. However, as is evident from the figure, there was a change in appearance of Tg_1' on annealing at these temperatures. The above results show that enthalpic recovery associated with Tg_2' was observed only when the samples were aged between −34 and −42 °C. Therefore, this represents the temperature range where, under the given set of conditions, significant relaxation associated with Tg_2' seemed to occur. In this experiment, we did not observe any enthalpic recovery associated with the lower temperature transition (Tg_1').

Fig. 3 is a plot of Tg_1', Tg_2' and the enthalpic recovery at Tg_2' as a function of annealing temperature. These solutions were heated at 2 °C/min. Since the observed enthalpic recovery is only due to the freeze-concentrate, it was necessary to normalize the obtained enthalpy value to the weight fraction of the freeze-concentrate in the system. The amount of unfrozen water associated with trehalose in the freeze-concentrate has been shown to be ∼20% (w/w)
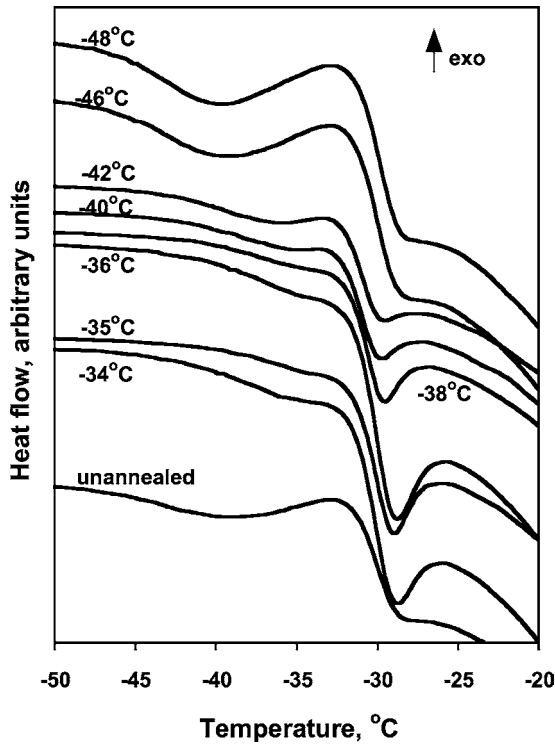
Fig. 2. Enthalpic relaxation in frozen aqueous solutions of trehalose (45% (w/w)) as a function of annealing temperature. Aqueous solutions were cooled to $-60\,^{\circ}$C at $15\,^{\circ}$C/min, heated to the annealing temperature at $2\,^{\circ}$C/min, held for 2 h, cooled back to $-60\,^{\circ}$C at $15\,^{\circ}$C/min and reheated at $2\,^{\circ}$C/min. The annealing temperature (shown along each DSC curve) varied from -34 to $-48\,^{\circ}$C. The DSC heating profile of the unannealed sample is also presented for comparison.

Fig. 3. Effect of annealing temperature on the Tg_1', Tg_2' and enthalpic recovery at Tg_2' of frozen aqueous solutions of trehalose (45% (w/w)). The solutions were cooled at $15\,^{\circ}$C/min to $-60\,^{\circ}$C, held there for 20 min, heated to the annealing temperature at $2\,^{\circ}$C/min, held for 2 h, cooled back and heated at $2\,^{\circ}$C/min.

of the solute [31–33]. This value was used in our calculation. It is evident from the figure that maximum enthalpic recovery associated with Tg_2' was observed for systems annealed at $-36\,^{\circ}$C. Though the endotherm due to relaxation occurred at the second transition only, this transition temperature (Tg_2') remained essentially unchanged after annealing. Aging at temperatures higher than $-48\,^{\circ}$C (Tg_1' of the unannealed sample) led to progressive changes in the DSC profiles (Fig. 2). Enthalpic recovery as well as Tg_1' increased up to an annealing temperature of $-36\,^{\circ}$C. At annealing temperatures $\geq -40\,^{\circ}$C, the devitrification exotherm between Tg_1' and Tg_2' disappeared which may be attributed to ice crystallization during annealing, resulting in a reduction in the unfrozen water
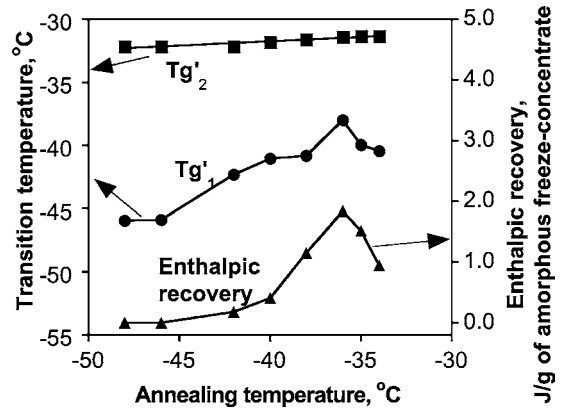
content of the system. If an aqueous solution does not form a maximally freeze-concentrated amorphous phase on cooling, annealing below the Tg_1' leads to ice crystallization, resulting in a decrease in the unfrozen water associated with the freeze-concentrate and an increase in Tg_1' [16,17,34]. Interestingly, the changes in Tg_1' as a function of annealing temperature followed the same trend as the enthalpic recovery observed at Tg_2' (Fig. 3).

The effect of a faster heating rate ($5\,^{\circ}$C/min), on the enthalpic recovery, was next investigated. Fig. 4 shows the effect of annealing temperature on Tg_1', Tg_2' and enthalpic recovery of solutions heated at $5\,^{\circ}$C/min. As observed at the slower heating rate of $2\,^{\circ}$C/min (Figs. 2 and 3), the enthalpic recovery was associated with the second transition (Tg_2') only. Again, maximum relaxation was observed in the sample annealed at $-36\,^{\circ}$C. At this heating rate, no recovery was detected at Tg_2' for systems annealed at $\leq -40\,^{\circ}$C (Fig. 4). Thus, at a heating rate of $5\,^{\circ}$C/min, the "aging range" appears to be between -38 and $-35\,^{\circ}$C. The enthalpic recovery at a heating rate of $5\,^{\circ}$C/min was always less than that at $2\,^{\circ}$C/min (compare Figs. 3 and 4). This is in agreement with the results of Her and Nail [23]. One possible explanation is the time spent by the sample in the aging range. At a slower heating rate, the system was allowed more time to relax and the enthalpic relaxation was higher. Another possible explanation is
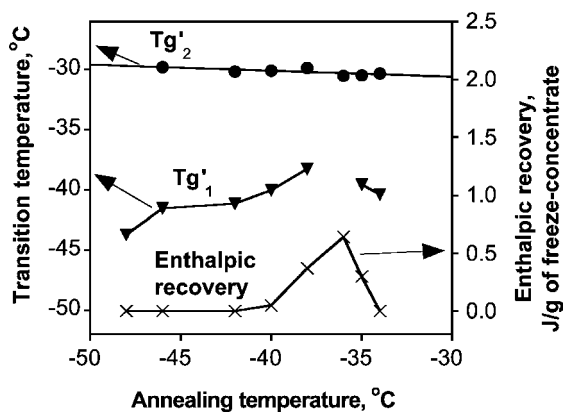
Fig. 4. Effect of annealing temperature on the Tg_1', Tg_2' and enthalpic recovery at Tg_2' of frozen aqueous solutions of trehalose (45% (w/w)) at a heating rate of 5 °C/min. The solutions were cooled at 15 °C/min to −60 °C, held there for 20 min, heated to the annealing temperature at 5 °C/min, held for 2 h, cooled back and heated at 5 °C/min.

Fig. 5. Isothermal annealing in frozen aqueous solutions. Plot of Tg_1' and Tg_2' and enthalpic recovery associated with Tg_2' after annealing the system at −36 °C. The annealed systems were cooled to −60 °C and heated at 5 °C/min (top panel) or at 2 °C/min (bottom panel).

the effect of heating rate on the observed DSC profile. At a faster rate, a more pronounced overlap of the two events, enthalpic recovery and onset of ice melting is expected. This issue is also discussed later. Interestingly, in the sample annealed at −36 °C, Tg_1' was not detected and Tg_2' appeared to be the only transition.

In order to study the effect of annealing time on the enthalpic recovery and glass transition values, frozen aqueous solutions were annealed at −36 °C for various time periods. The recovery was determined at two heating rates of 2 and 5 °C/min. The enthalpy regained at 2 °C/min was consistently higher than that at 5 °C/min (Fig. 5). At the faster heating rate, it was not possible to detect the lower transition temperature (Tg_1') for systems annealed for ≥2 h at −36 °C. While Tg_2' was unaffected on annealing, Tg_1' increased and merged with Tg_2' (Fig. 5). At the slower heating rate, the lower temperature transition, Tg_1' in these systems, was detected but with difficulty.

In previous work with frozen aqueous sucrose solutions, the higher transition temperature was attributed to the onset of ice melting [16]. The lower transition temperature depended on the annealing temperature. The authors explained that maximal freeze-concentration was not obtained at moderately high cooling rates. Sub-Tg' annealing led to ice crystallization, and on increasing the annealing tem-
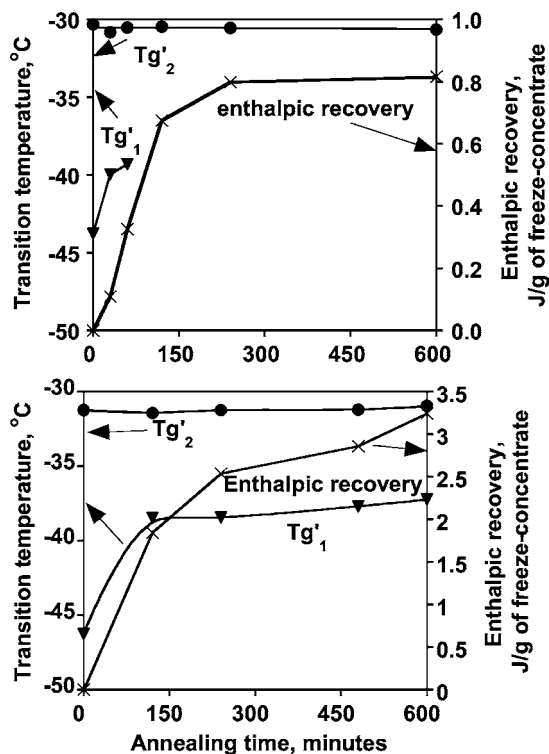
perature, more ice crystallized leading to an increase in the observed value of Tg'. When the annealing temperature was raised above the Tg', the ice started to melt resulting in an increase in the unfrozen water and a decrease in the observed Tg'. Following this rationale, the temperature of annealing that resulted in the highest glass transition temperature was considered the glass transition temperature of the maximally freeze-concentrated amorphous phase.

Our results too suggest that the second transition (denoted by Tg_2') is also the onset of ice melting. Frozen aqueous solutions of trehalose revealed a maximum enthalpic relaxation associated with Tg_2' when systems were annealed at −36 °C. After annealing at this temperature, the value of Tg_1' also reached a maximum. On increasing the annealing temperature to −35° and then to −34 °C, both the

enthalpic recovery and Tg' decreased (Fig. 3). This was an indication of the increase in the amount of unfrozen water in the system and is due to the onset of ice melting at temperatures higher than $-36\,°C$. This is the temperature where maximum relaxation of frozen aqueous solutions of trehalose was observed. At the faster heating rate ($5\,°C/min$), only one transition (Tg'_2, the higher temperature transition) could be detected in systems annealed at this temperature (Fig. 4). The observation that the Tg'_1 and enthalpic recovery follow similar trends as a function of annealing temperature, suggests that annealing led to crystallization of ice and subsequently the system became maximally freeze-concentrated. Applying Ablett's theory to our results, the actual Tg' of maximally freeze-concentrated amorphous trehalose is close to $-36\,°C$ [16]. The observed Tg' of the annealed system was $\sim -38\,°C$ (Fig. 3). The discrepancy in the results can be explained. An annealing time of 2 h at $-36\,°C$ was not enough to cause maximal freeze-concentration of trehalose. This is evident from the observation that annealing at $-36\,°C$ for 8 h caused an increase in the Tg'_1 of the system to $\sim -37\,°C$ (Fig. 5, bottom profile). It is also possible that the Tg'_1 of the system might have been higher on annealing for a longer period of time at $-36\,°C$.

Another theory, first proposed by Levine and Slade and more recently by Chang et al., hypothesized that the lower temperature transition is the glass transition of a phase containing higher amount of unfrozen water than a maximally freeze-concentrated phase and the higher temperature glass transition is that of the maximally freeze-concentrated amorphous solute [13,20]. In other words, there appears to be the formation of two "populations" of materials during "non-equilibrium" or rapid cooling—one, that is maximally freeze-concentrated and the other that contains a higher amount of water. Our results are in agreement in the following respects. (i) Chang et al. observed enthalpic recovery associated with the higher glass transition of frozen aqueous solutions of sucrose. Based on this observation, they believe that the Tg'_2 is also a glass transition. We also observed enthalpic relaxation associated with the higher temperature transition of trehalose (Tg'_2). (ii) The magnitude of enthalpic recovery depended on the annealing temperature. (iii) By crystallizing ice to different extents during annealing, Tg'_1 changed significantly. Crystal-

lization of ice would lower the amount of unfrozen water and raise the glass transition temperature.

There was one remaining question: Is there enthalpic relaxation associated with the lower transition temperature? Earlier, we had observed that on annealing at temperatures below the lower transition temperature, changes in the Tg'_1 were observed. Ice crystallization and enthalpic relaxation can both lead to an increase in the Tg'_1. In order to differentiate between the effects of ice crystallization and enthalpic relaxation, the system was consecutively annealed, first below Tg'_2 and then below Tg'_1.

As the first step, when the annealing was carried out only at $-48\,°C$, the DSC profile was substantially similar to that of the unannealed sample and there was no enthalpic recovery associated with Tg'_1 (compare curves 'a' and 'b' in Fig. 6). The frozen solutions were then consecutively annealed, first at $-36\,°C$ and then at $-48\,°C$. In Fig. 6, curves labeled 'c' and 'd' are the DSC heating curves of the samples that were annealed at $-48\,°C$ for 4 and 8 h, respectively, following annealing at $-36\,°C$ for 2 h. They were significantly
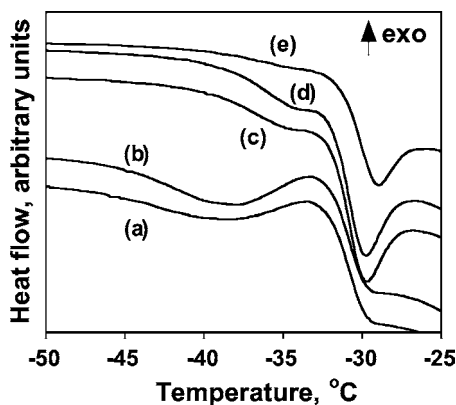

Fig. 6. The effect of consecutive annealing cycles on the thermal behavior of frozen aqueous trehalose solutions (45% (w/w)). All the solutions were cooled from RT to $-60\,°C$ at $15\,°C/min$. (a) DSC heating profile of the unannealed sample. Parts (b) and (e) are DSC heating profiles of frozen aqueous solutions of trehalose which were annealed at $-48\,°C$ for 4 h and at $-36\,°C$ for 2 h, respectively. (c) Frozen aqueous solutions were heated to $-36\,°C$, held there for 2 h, cooled back to $-48\,°C$, held for 4 h, cooled to $-60\,°C$ at $15\,°C/min$ and heated at $2\,°C/min$. (d) The experimental conditions were similar to that in (c) except that, the annealing was carried out at $-36\,°C$ for 2 h, then at $-48\,°C$ for 8 h.

different from that of the sample annealed only at $-36\,^\circ C$ (Fig. 6, curve 'e'). In contrast to the sample annealed only at $-36\,^\circ C$ (curve 'e'), the two glass transition events, at -39 and $-32\,^\circ C$, were clearly evident (curves 'c' and 'd'). Thus the extra annealing step at $-48\,^\circ C$ seems to have resulted in the reappearance of Tg_1' but at a higher temperature of $-39\,^\circ C$. This higher glass transition temperature indicates that there is a decrease in mobility due to enthalpic relaxation and/or a decrease in the amount of unfrozen water associated with this amorphous phase. However, based on the data presented in Fig. 2 (annealing at $-48\,^\circ C$; top curve) we do not expect any further ice crystallization at $-48\,^\circ C$. Thus the reappearance of the glass transition event can be attributed to relaxation.

Let us consider, in detail, the effect of the two annealing steps. After the first annealing at $-36\,^\circ C$, Tg_1' was much less discernable. This strongly suggests that the ice crystallization has resulted in a decrease in the "population" of the amorphous phase containing unfrozen water in excess of the maximally freeze-concentrated phase. When the system is then annealed at $-48\,^\circ C$, there is relaxation of this remaining amorphous phase containing higher amount of unfrozen water. While relaxation is usually reflected as an endotherm accompanying the glass transition, in this case, the amorphous phase constitutes a very small weight fraction of the total sample. As a result, we believe that the glass transition and the enthalpic recovery together are manifested only as a baseline shift, much as one would see in a glass transition event. In these systems (curves 'c' and 'd') this glass transition event was observed at a higher temperature $(-39\,^\circ C)$ than in the unannealed sample. This is consistent with the idea that the annealed system contains less unfrozen water than the unannealed system and is characterized by longer relaxation times.

This set of experiments, combined with the effects of annealing temperature (shown in Fig. 2) on the thermal behavior of frozen aqueous solutions around the lower glass transition temperature shows the possibility of enthalpic relaxation associated with Tg_1'. These results support the hypothesis that indeed the lower temperature transition is also a glass transition. From a freeze-drying perspective, Tg_2' is probably a more relevant temperature, since collapse is observed a few degrees above Tg_2'. The phenomenon of collapse may be observed while drying a system with reduced vis-

cosity (i.e. above the glass transition). The possibility of post-Tg_2' ice melting cannot be ruled out, especially in systems where the weight fraction of the amorphous solids in the freeze-concentrate is high. This needs to be kept in mind while designing freeze-drying cycles, especially, while deciding the annealing temperatures and/or primary drying temperatures based on thermal analysis.

3.3. Comparison with freeze-dried systems

We wanted to compare the annealing behavior of freeze-concentrated trehalose with that of freeze-dried trehalose. Not surprisingly, the two systems responded very differently to isothermal annealing. The effect of annealing time on the Tg and enthalpic recovery of freeze-dried trehalose ($Tg \sim 117\,^\circ C$) are presented in Fig. 7. Comparison of these results with those of freeze-concentrated trehalose (Figs. 3 and 4), strongly suggest that the extent of relaxation is much higher in the former. The freeze-concentrated systems contain unfrozen water and its influence, if any, on the enthalpic recovery is unknown. Another important factor is likely to be the difference in the annealing temperatures ($100\,^\circ C$ for the freeze-dried trehalose versus $\sim -40\,^\circ C$ for freeze-concentrated trehalose). This is because the glass transition temperatures of freeze-dried and freeze-concentrated trehalose are $\sim 150\,^\circ C$ apart. Thus it is not surprising that there is a pronounced difference in the mobility, and therefore, extent of relaxation, in the two systems.

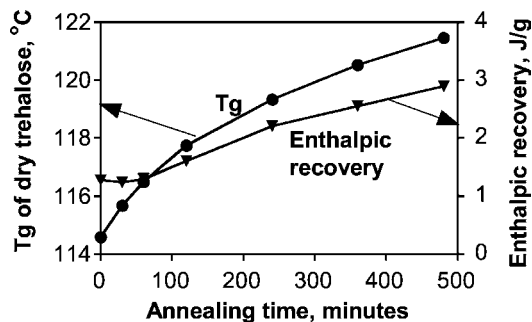Interestingly, the effect of heating rate on the enthalpic recovery in freeze-dried trehalose and

Fig. 7. Tg and enthalpic recovery after annealing freeze-dried trehalose at $100\,^\circ C$ ($\sim 17\,^\circ C$ below the Tg) for 0–8 h and reheating the annealed system at $5\,^\circ C/min$.

freeze-concentrated trehalose were also different. As mentioned earlier, at the slower heating rate, trehalose in the freeze-concentrated state regained higher enthalpy at Tg'_2 (Figs. 3 and 4). On the other hand, in freeze-dried trehalose, there was less observed enthalpic recovery at the slower heating rate (data not presented). This difference could be attributed to the presence of a significant amount of ice and the onset of ice melting in the frozen sample. In frozen solutions, Tg'_2 is believed to be the onset of ice melting [16]. This becomes obvious in concentrated solutions, wherein the onset of ice melting was more readily apparent. At rapid heating rates, there was a pronounced overlap of the enthalpic recovery and onset of ice melting. This may lead to a decrease in the observed enthalpic recovery.

3.4. Challenges and limitation in the studies of enthalpic relaxation in frozen solutions

The enthalpic relaxation of amorphous substances is difficult to study because of the following reasons. (i) Fast cooling rates do not lead to the formation of maximally freeze-concentrated solutes. As mentioned previously, since the observed enthalpic relaxation is only due to the freeze-concentrate, it was necessary to normalize the obtained enthalpy value to the weight fraction of the freeze-concentrate in the system. The amount of unfrozen water associated with trehalose in the freeze-concentrate has been shown to be ∼20% (w/w) of the solute. This value was used for normalizing the enthalpic relaxation in the freeze-concentrated systems. The determination of the unfrozen water content after annealing is not trivial and was outside the scope of this work. Moreover, the observed trends will not be significantly affected by small changes in the amount of unfrozen water. (ii) Ice crystallization during annealing below Tg', leads to potential problems in interpretation of the data. Thus, the observed changes in the thermal behavior may not be attributable to aging alone. For example, an increase in Tg'_1 in this system due to annealing is probably due to a combined effect of ice crystallization and increase in the molecular relaxation times with aging. (iii) The presence of a significant amount of ice that does not contribute to the enthalpic relaxation processes poses another challenge to sensitivity, especially in dilute solutions.

4. Conclusions

We have investigated the effects of some factors on the glass transition temperatures and enthalpic recovery in frozen aqueous systems of trehalose. We have also attempted to bridge two conflicting schools of thought regarding the origin of multiple glass transitions in frozen aqueous solutions of sugars. The conclusions are summarized below:

1. Frozen aqueous solutions of trehalose were characterized by two glass transition temperatures (Tg'_1 and Tg'_2).
2. Annealing below the transition temperatures, led to enthalpic recovery associated only with the higher temperature transition (Tg'_2).
3. Irrespective of the heating rates, the magnitude of enthalpic recovery and Tg'_1 showed a similar trend (first an increase, followed by a decrease) as a function of annealing temperature. This suggests that annealing led to crystallization of ice and subsequently the system became maximally freeze-concentrated. Annealing, at temperatures higher than −36 °C, led to ice melting resulting in a reduction in enthalpic recovery and a lowering of Tg'_1. This observation supports the hypothesis that the higher transition temperature coincides with ice melting.
4. In samples annealed at −36 °C and heated at 2 °C/min, it was difficult to detect Tg'_1. Interestingly, when a frozen aqueous solution of trehalose was consecutively annealed, first at −36 °C and then at −48 °C, it reappeared confirming that the thermal event at −45 °C is a glass transition (Tg'_1).
5. The two glass transitions are attributed to the formation of two "populations" in the freeze-concentrated phase during "non-equilibrium" or rapid cooling—one, that is maximally freeze-concentrated and the other that contains a higher amount of water. The higher transition temperature also overlaps with the onset of ice melting.

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