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Research Paper

Influence of the Active Pharmaceutical Ingredient Concentration on the Physical State of Mannitol—Implications in Freeze-Drying

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Purpose. The aim of this study was to investigate the effect of the concentration of the active pharmaceutical ingredient on the physical state of mannitol in frozen aqueous systems.
Methods. A human monoclonal antibody was used as the model protein. Mannitol and sucrose were used as the bulking agent and the lyoprotectant, respectively. The thermal behavior of frozen mannitol–sucrose solutions *during* and after annealing, in the absence and presence of the protein, were characterized by low-temperature powder X-ray diffractometry and differential scanning calorimetry. The influence of the protein on the crystallization behavior of mannitol was also evaluated.
Results. The excipient concentration had a pronounced effect on the glass transition temperature of maximally freeze-concentrated amorphous phase (T_g'). At fixed excipient compositions, the protein had no effect on the T_g' if the protein concentration was $\leq$20 mg/ml. However, at higher protein concentrations, there was a marked increase in T_g' as a function of protein concentration. The inhibitory effect of the protein on mannitol crystallization was concentration dependent and was directly evident from X-ray diffractometry experiments. Annealing facilitated both mannitol nucleation and crystal growth even in the presence of the protein.
Conclusions. The ratio of mannitol to sucrose and the protein concentration have an impact on the T_g' and may therefore influence the primary drying temperature. The protein inhibits both the nucleation and growth of mannitol crystals and this effect seems to be concentration dependent. The presence of the protein and the protein concentration dictate the processing conditions, i.e., annealing time, annealing temperature, and primary drying temperature.

KEY WORDS: annealing; lyophilization; mannitol; protein; sucrose.

INTRODUCTION

The lyophilization process has attracted a great deal of attention recently because of the increasing pharmaceutical importance of proteins and other biotechnology products (1,2). The ultimate goal of lyophilization is to retain the activity of the therapeutic agent [active pharmaceutical ingredient (API)] while obtaining a pharmaceutically elegant end product. This goal usually is achieved by adding excipients, such as lyoprotectants and bulking agents. Often it is necessary to use a combination of excipients. Typical examples contain a crystalline material (bulking agent) and a noncrystallizing agent (lyoprotectant) (3,4). The rationale behind such a combination is that the crystalline material offers a robust matrix so that the primary drying can be conducted at high temperatures while the noncrystallizing agent serves as a lyoprotectant (3,4).

Mannitol–sucrose combination is a popular choice because mannitol readily forms a crystalline cake and its high eutectic melting temperature with ice (−1.5°C) enables primary drying at relatively high temperatures, whereas sucrose is a widely used lyoprotectant. A recent study showed that a sucrose–mannitol formulation was successfully primary dried at −10°C without visual collapse (3). This excipient combination also provided the most effective protection against aggregation of interleukin-6 (IL-6) (5). However, for such an approach to be successful, mannitol should be retained in a substantially crystalline state and there should be a sufficient amount of amorphous sucrose for maximal lyoprotection.

In formulations containing mannitol and sucrose, retention of mannitol in a substantially crystalline state is essential and incomplete mannitol crystallization can have serious consequences. The amorphous mannitol in the lyophile may crystallize during manufacture, transport, or storage. This can result in the release of the sorbed water associated with the amorphous phase. The water may then be available for interaction with the other formulation ingredients including the API. This may not only bring about instability of the API, but may also result in product collapse. Second, batch-to-batch variations can be brought about by incomplete crystallization during storage (6).

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Complete crystallization of mannitol may be very difficult because mannitol crystallization can be influenced by many factors. Kim *et al.* showed that the physical form of mannitol in the final lyophile was influenced by the formulation variables, the processing conditions (freezing rates), as well as the presence of a noncrystallizing cosolute (7). Sodium chloride, a partially crystallizing "doubly unstable" glass, could inhibit mannitol crystallization even at low concentrations (8,9). In lyophilized aspirin formulations, mannitol crystallization was affected by the ratio of acetylsalicylic acid to mannitol (10).

Mannitol crystallization is further complicated when protein is included in the formulation, especially when the concentration of the protein is relatively high. Proteins are structurally complex and possess numerous functional groups that can react nonspecifically with the formulation components, and thereby influence the crystallization behavior of mannitol. In other words, the presence of protein may inhibit mannitol crystallization *during* lyophilization. As a result, mannitol crystallization may be incomplete in the final lyophile. At high protein concentrations, as is the case with formulations of human monoclonal antibody, this effect may become quite pronounced (11). The inhibitory effect on mannitol crystallization therefore poses a potentially serious challenge while designing lyophilization cycles of protein formulations. Interestingly, little attention has been paid to the effect of proteins on the physical form of mannitol, whereas the influence of mannitol crystallization on the stability and activity of proteins has been widely studied (12,13).

To study the effect of proteins on the physical state of mannitol, a human monoclonal antibody was used as the model protein. In the prelyophilization solution, the protein concentration ranged between 10 and 50 mg/ml, which is high compared to many other protein formulations (14,15). Our objectives are to investigate (i) the effect of the model protein on the behavior of the mannitol–sucrose frozen solutions under subambient conditions, (ii) the effect of protein on the crystallization behavior of mannitol, and (iii) the effect of annealing on mannitol crystallization. The thermal behavior of frozen mannitol–sucrose solutions during and after annealing, in the absence and presence of the protein, were characterized using low-temperature powder X-ray diffractometry (XRD) and differential scanning calorimetry (DSC).

MATERIALS AND METHODS

Materials

D-Mannitol ($C_6H_{14}O_6$, Sigma, St. Louis, MO, USA), sucrose ($C_{12}H_{22}O_{11}$, Aldrich, Milwaukee, WI, USA), and citric acid ($C_6H_8O_7$, Sigma) were used as received. The purified monoclonal antibody was provided by Human Genome Sciences (Rockville, MD, USA).

The studies were divided into three groups, with a progressive increase in the number of components. The aqueous solutions prepared contained (i) different weight ratios of mannitol to sucrose, (ii) the same weight ratio of mannitol to sucrose in the presence of citrate buffer, and (iii) mannitol, sucrose, citrate buffer, and the protein. The sucrose and mannitol concentrations were in the range of 2–5% (w/w). The mannitol to sucrose weight ratios (R) were 0.45, 1.10, 1.50, 1.95, 2.50, and 3.00. The detailed solution compositions are given in Table I. The citrate buffer concentration was 10mM and the solution pH was 6.5. When the human monoclonal antibody was added (hereafter referred to as "protein"), its concentration ranged between 10 and 50 mg/ml. All solutions were subjected to membrane filtration (0.45 μm) except the protein solution.

Methods

Differential Scanning Calorimetry

A differential scanning calorimeter (MDSC, Model 2920, TA Instruments, New Castle, DE, USA) with a refrigerated cooling accessory was used. The DSC cell was calibrated using mercury and distilled water. About 13 mg of the sample solution was weighed in an aluminum pan, sealed hermetically, cooled from room temperature to −70°C at 20°C/min, and maintained at −70°C for 30 min to ensure attainment of temperature equilibrium. The frozen solutions were then heated at 5°C/min to room temperature. Only the DSC heating curves were recorded. When there was an annealing step, the frozen solutions were annealed at a selected temperature ranging from −25 to −45°C for periods of 15 to 480 min. Specific details are provided in the "Results and discussion" section.

X-ray Powder Diffractometry

An X-ray powder diffractometer (Model XDS 2000, Scintag, Cupertino, CA, USA) with a variable temperature stage (Micristar, Model 828D, R.G. Hansen & Associates, Santa Barbara, CA, USA; working temperature range −190 to 300°C) was used. An accurately weighed aliquot of sample solution (~100 mg) was filled into a copper sample holder, which was placed on the stage and cooled from room temperature to −70° at 10°C/min. The samples were then normally held for 30 min and heated to the annealing temperature at 5°C/min.

RESULTS AND DISCUSSION

Characterization of Frozen Aqueous Mannitol–Sucrose Solutions

Initial studies focused on the thermal events *during* the cooling and heating of aqueous solutions containing different

Table I. Composition of Aqueous Solutions Containing Mannitol and Sucrose

Mannitol to sucrose ratio (R)	Mannitol (% w/w)	Sucrose (% w/w)
0.45	2.26	5.00
1.10	3.54	3.21
1.50	4.04	2.70
1.95	4.16	2.13
2.50	5.00	2.50
3.00	5.00	1.67

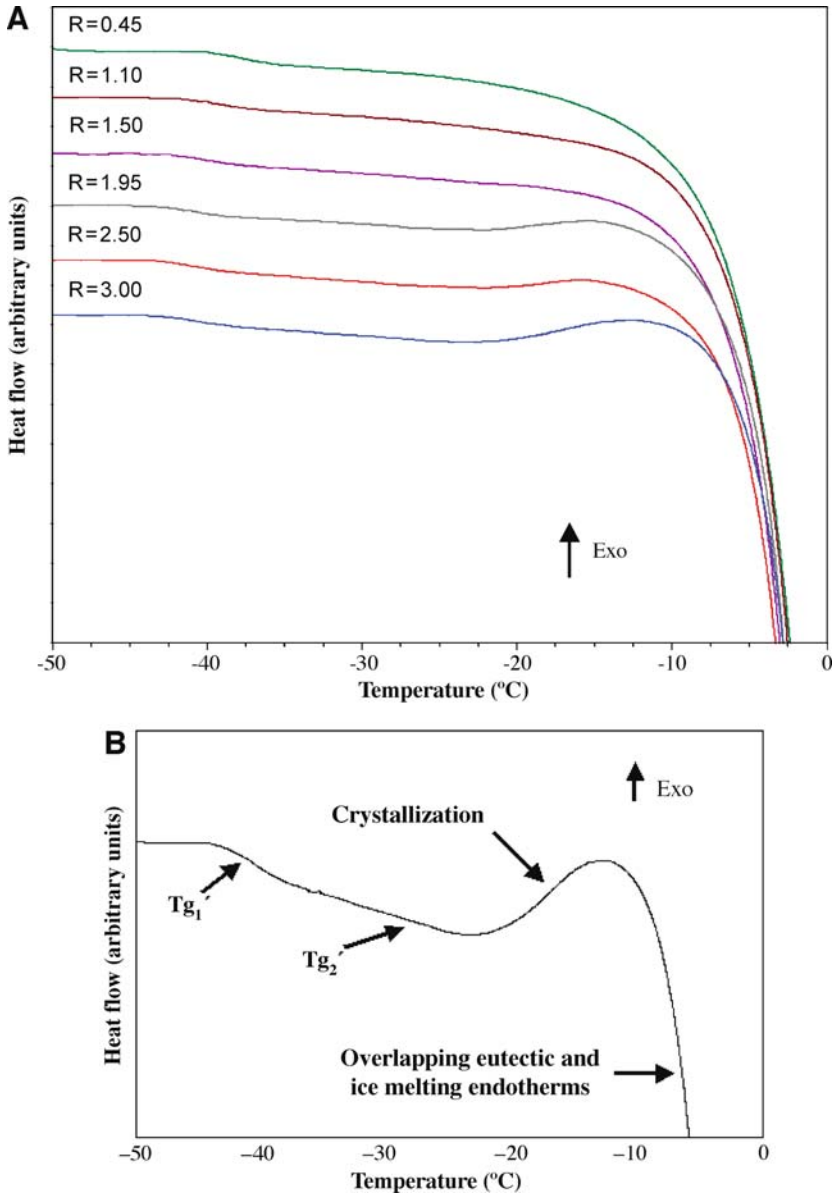

Fig. 1. (A) Overlaid DSC heating profiles of frozen aqueous mannitol–sucrose solutions. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min, and heated to room temperature at 5°C/min. (B) Expanded view of DSC heating profile of frozen aqueous mannitol–sucrose solution (R = 3.00) showing the various thermal events.

ratios of mannitol to sucrose. It has been reported and confirmed in this work that mannitol crystallization is inhibited at cooling rates ≥20°C/min (16). On the other hand, when the frozen aqueous solution was heated to room temperature at 5°C/min, several thermal events were observed (Fig. 1): (i) glass transition with onset between −40 and −44°C (T'_{g1}; Fig. 1B), (ii) a possible second glass transition with onset at approximately −27°C (T'_{g2}; Fig. 1B), (iii) depending on the mannitol to sucrose weight ratio (R), an exotherm attributable to solute crystallization at $R \geq 1.95$ (Fig. 1A), and (iv) an endotherm due to eutectic melting of mannitol and ice (Fig. 1A, B). In a recent study, glass transition temperatures of −41 and −31°C were reported for an aqueous mannitol (4% w/w)–sucrose (1% w/w) system containing 10 mM Tris–HCl buffer (17). The origin of multiple glass transitions is not fully understood and is a subject of debate (18). However, many reports have attributed the lower temperature transition to be the true glass transition (19,20). Therefore, this transition (hereafter referred to as T'_g) was the focus of our studies.

The glass transition temperature of maximally freeze-concentrated amorphous phase (T'_g) is one of the important thermophysical parameters in the lyophilization cycle design because it may be close to the collapse temperature (21). Therefore, our studies initially focused on the effect of the mannitol to sucrose weight ratio (R) on the T'_g. As shown in Fig. 2, as R increased from 0.45 to 1.50, the T'_g decreased from approximately −40 to −43°C. At $R \geq 1.5$, the T'_g

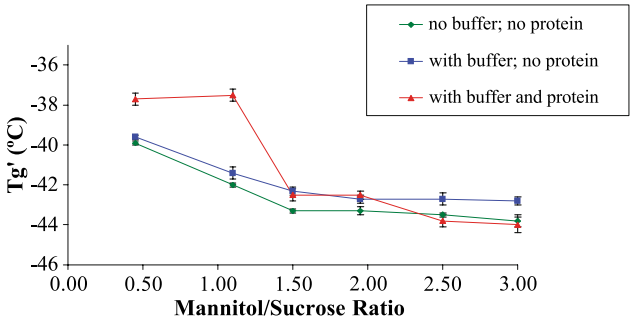

Fig. 2. The effect of mannitol to sucrose weight ratios on the T_g'. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min and heated to room temperature at 5°C/min. The protein concentration was 20 mg/ml. Each point is the mean of three determinations. Error bars represent standard deviations (n = 3).

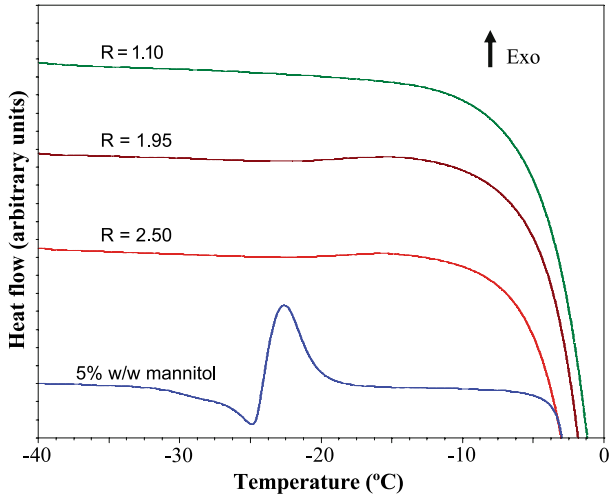

Fig. 3. DSC heating profiles of frozen aqueous mannitol–sucrose and 5% mannitol-only solutions. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min, and heated to room temperature at 5°C/min.

reached a plateau (approximately −43.5°C). Interestingly, the T_g' of the mixture is lower than the T_g' of the individual components, i.e., mannitol and sucrose. The glass transition temperature of the maximally freeze-concentrated mannitol was reported to be in the range of −30 to −32°C (16,22), whereas the reported T_g' of sucrose was −35°C (23,24). It was speculated that the unfrozen water content increased as R increased (25). This seems to be valid between R values of 0.45 to 1.5. However, further increase in R did not seem to increase the unfrozen water content.

The effect of buffer on the T_g' was also examined by replacing water with 10 mM aqueous citrate buffer (pH = 6.5). A similar trend was observed in the presence of citrate buffer. However, it raised the T_g', typically by ~1°C. The T_g' value at R = 1.95 (approximately −42°C) was in reasonable agreement with previously reported value of approximately −41°C (R = 2) (22).

The effect of protein at a concentration of 20 mg/ml was next investigated. As shown in Fig. 2, the T_g' stayed almost unchanged when R increased from 0.45 to 1.10, followed by a sharp drop at R = 1.50. The T_g' continued to decrease until the R reached 2.50. T_g' normally forms the basis for selection of the primary drying temperature. However, in the mannitol–sucrose formulations, although the T_g' is low (approximately −42°C), the primary drying can still be conducted at a relatively high temperature (approximately −10°C). It is postulated that crystalline mannitol supports the weight of the lyophile and prevents macroscopic collapse. The primary drying temperature thus seems to depend on the fraction of crystalline phase in the formulation, as has been demonstrated in recent several examples (3,26).

The inhibitory effect of sucrose on mannitol crystallization is also a subject of study. It is well known that sucrose, a noncrystallizing solute, prevents mannitol crystallization (22). The extent of inhibition depends on the concentration ratio of mannitol to sucrose. As shown in Fig. 3, at R = 1.10, no crystallization exotherm was observed in the DSC profiles. A very small exotherm appeared just before the eutectic melting endotherm, at $R \geq 1.95$. A control experiment was conducted, using 5% mannitol without sucrose. A much sharper exotherm was seen, with an onset at approximately −25.0°C. Mannitol crystallized only at $R > 1.95$, a result in good agreement with previous studies (3).

Effect of Protein Concentration on T_g' and Mannitol Crystallization

From Fig. 2, it is evident that the protein, at a concentration of 20 mg/ml, influences the T_g' at $R < 1.50$. The effect of protein concentration on the T_g' was determined at R values of 0.45, 1.5, and 3.0. As shown in Fig. 4, at R = 0.45, T_g' increased 4.4°C, from −39.1 to −34.7°C as the protein concentration increased from 10 to 50 mg/ml. At higher R values (1.5 and 3.0), at low protein concentrations (10 and 20 mg/ml), there seemed to be no effect on the T_g'. However, at higher protein concentrations, there was a marked increase in T_g' as a function of protein concentration. At R values of 1.5 and 3.0, if the protein concentration is high (>20 mg/ml), the T_g' is sensitive to protein concentration. This issue can be very important in the design of lyophilization cycles if formulations with different strengths of protein (API) are contemplated.

The inhibitory effect of protein on mannitol crystallization was also investigated. A solution with a mannitol to sucrose weight ratio of 3.00 was chosen because mannitol crystallization was evident at this composition. As shown in

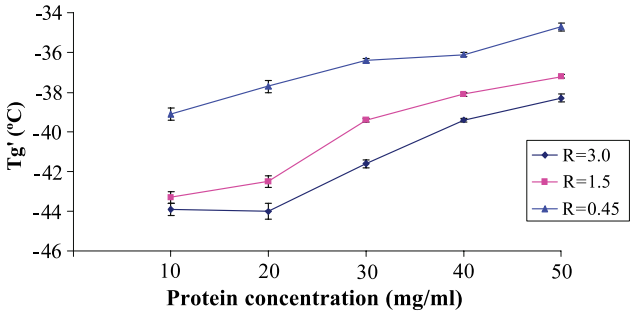

Fig. 4. The effect of protein concentrations on the T_g'. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min and heated to room temperature at 5°C/min. The mannitol to sucrose weight ratios (R) were 0.45, 1.5, and 3.0. Each point is the mean of three determinations. Error bars represent standard deviations (n = 3).

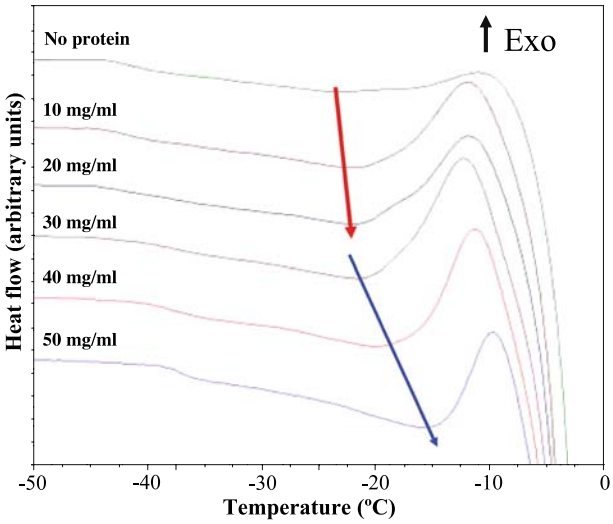

Fig. 5. The inhibitory effect of protein on mannitol crystallization. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min, and heated to room temperature at 5°C/min. The mannitol to sucrose weight ratio (R) was 3.00. The protein concentration is shown above each DSC curve. The lines show the trend in the crystallization onset temperature as a function of the protein concentration.

Fig. 5, when the protein concentration increased from 0 to 20 mg/ml, the crystallization onset temperature of mannitol shifted slightly to higher temperatures. As the protein concentration increased from 30 to 50 mg/ml, the onset temperature increased from −21 to −15°C. This shift is a clear indication of the inhibitory effect of the protein on mannitol crystallization.

Effect of Annealing

Nucleation is a prerequisite for crystallization (27). Previous studies of mannitol–trehalose systems in our laboratory revealed that sub-T_g' annealing facilitated ice crystallization and mannitol nucleation (28). Our first objective here is to investigate the effect of sub-T_g' annealing in the absence and the presence of the protein. The solution with a mannitol to sucrose weight ratio of 3.00 was studied in detail because it had the most pronounced crystallization event in the DSC profile. The annealing temperature (T_a) was −45°C, one degree below the T_g'. Because the mannitol–sucrose solution and the mannitol–sucrose–protein solution have almost the same T_g', the difference between the annealing temperature and the glass transition temperature (T_a–T_g') is negligible. In Fig. 6, the enthalpy of crystallization is plotted as a function of annealing time in solutions annealed at −45°C. In unannealed solutions, the crystallization onset was delayed so that the crystallization exotherm overlapped with the huge eutectic melting endotherm, thus making the accurate measurement of crystallization enthalpy difficult. In annealed samples, annealing led to nucleation, resulting in crystallization at lower temperatures. This separated the crystallization exotherm from the eutectic melting endotherm, thereby enabling the accurate measurement of enthalpy. Incidentally, the effect of annealing was much less pronounced at lower temperatures of −47 and −49°C.

Figure 6 shows that the crystallization enthalpy increased with annealing time. In the absence of the protein, the enthalpy increased from 13.1 to 19.1 J/g when the

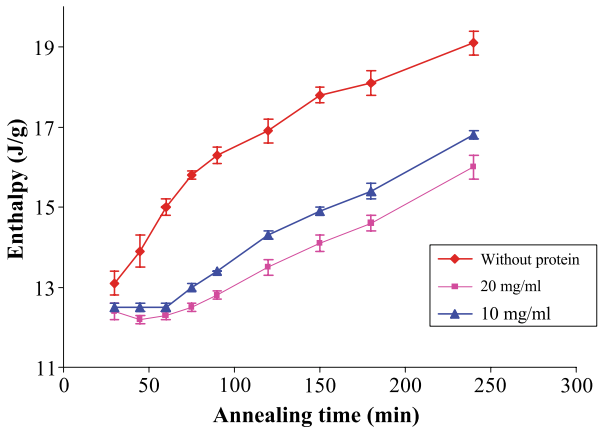

Fig. 6. The effect of protein concentration on the enthalpy of crystallization as a function of annealing time. The solutions were initially cooled from room temperature to −70°C at 20°C/min. They were annealed at −45°C and then heated to room temperature at 5°C/min. The mannitol to sucrose weight ratio (R) was 3.00. Each point is the mean of three determinations. Error bars represent standard deviations (n = 3).

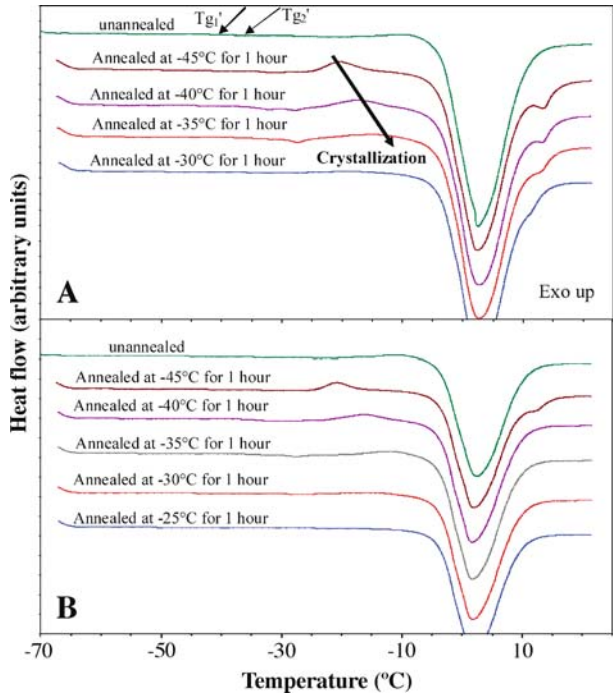

Fig. 7. The effect of annealing temperature on the crystallization behavior of mannitol in frozen aqueous (A) mannitol–sucrose and (B) mannitol–sucrose–protein solutions. The solutions were cooled from room temperature to −70°C at 20°C/min. It was held at −70°C for 30 min and heated to the annealing temperature at 5°C/min, annealed for 60 min, and cooled back to −70°C. The solutions were reheated to room temperature at 5°C/min. The second heating scans are shown here. The mannitol to sucrose weight ratio (R) was 3.00 and the protein concentration was 20 mg/ml. The line shows the trend in the crystallization exotherm as a function of annealing temperature.

annealing time was increased from 30 to 240 min. With addition of the protein (10 mg/ml), the enthalpy increased from 12.5 to 16.8 J/g in the same time period. As the protein concentration increased to 20 mg/ml, the enthalpy increased from 12.4 to 16.0 J/g. The increase in the enthalpy of crystallization with annealing time was more pronounced in the absence of the protein. It can be inferred that the protein exhibits a concentration-dependent inhibition of mannitol crystallization. In the absence of the protein, mannitol crystallization was initiated almost immediately. On the other hand, there was a lag time of 30 and 60 min at protein concentrations of 10 and 20 mg/ml, respectively.

Effect of Annealing on Mannitol Crystallization

As discussed above, sub-T'_g annealing facilitated nucleation of mannitol. What will be the effect of the protein when the annealing temperature is higher than the T'_g? To answer the question, we decided to examine in detail the physical stability of the amorphous freeze concentrate under more aggressive annealing conditions. The samples were annealed at temperatures ranging from −45 to −25°C. As shown in Fig. 7A, in the absence of protein, as the annealing temperature increased from −45 to −30°C, the enthalpy of mannitol crystallization during the second heating decreased. This indicated that during the isothermal annealing, the extent of mannitol crystallization increased as a function of the annealing temperature. Annealing at −30°C caused complete mannitol crystallization. Consequently, there was no exotherm attributable to mannitol crystallization during the second heating (Fig. 7A). In the presence of the protein, annealing at −30°C did not cause complete crystallization of mannitol. As a result, crystallization was evident during the second heating (Fig. 7B). However, annealing at a higher temperature of −25°C caused complete crystallization of mannitol. Figure 8 is another good example of the inhibitory effect of the protein on mannitol crystallization. In the absence of protein, when annealed at −35°C, mannitol

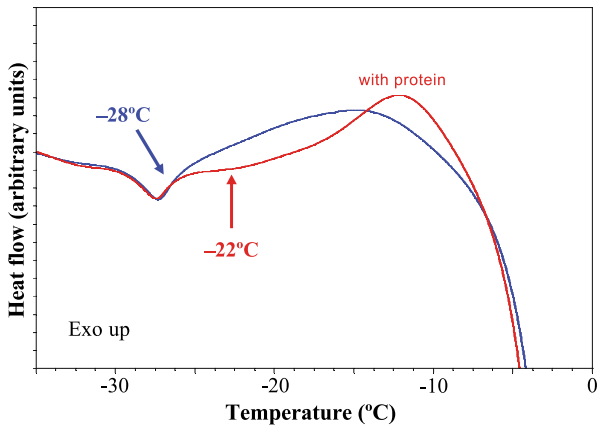

Fig. 8. DSC heating profiles of frozen aqueous mannitol–sucrose solutions in the absence and presence of the protein. The solutions were cooled from room temperature to −70°C at 20°C/min. They were annealed at −35°C for an hour, cooled back to −65°C, and then heated to room temperature at 5°C/min. The final heating curves are shown. The mannitol to sucrose weight ratio (R) was 3.00. The protein concentration was 20 mg/ml. The crystallization temperatures are marked with arrows.

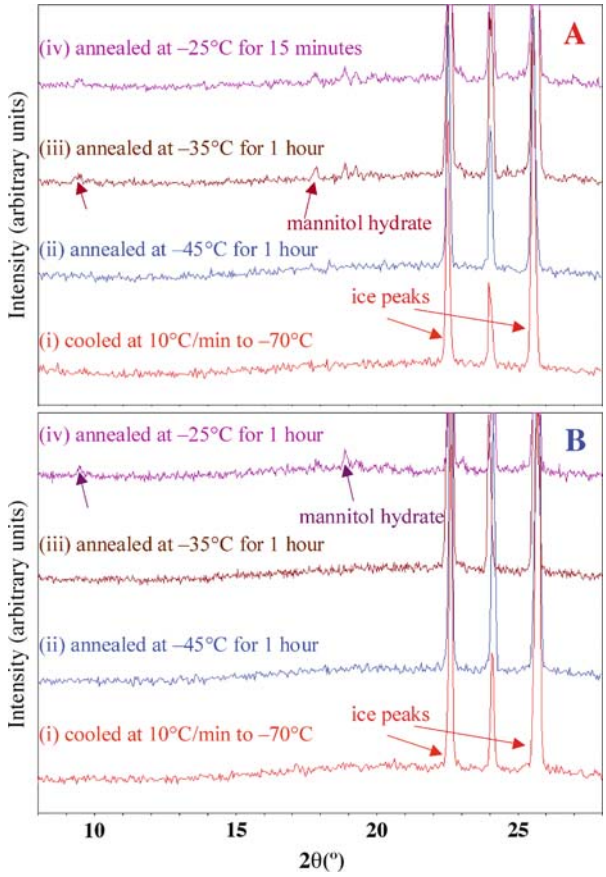

Fig. 9. XRD patterns of frozen aqueous mannitol–sucrose solutions (A) in the absence and (B) presence of the protein. The mannitol to sucrose weight ratio (R) was 3.00. The protein concentration was 20 mg/ml. (i) The solutions were cooled from room temperature to −70°C and XRD pattern was obtained. (ii) The temperature was raised to −45°C and the sample was annealed for 1 h. (iii) To remove the thermal history, the sample was heated to room temperature, cooled back to −70°C, then raised to −35°C and annealed for 1 h. (iv) After again heating to room temperature and cooling back to −70°C, the temperature was raised to −25°C and the sample was annealed for 15 min (in the absence of protein) and 1 h (in the presence of protein). All heating and cooling rates were 5 and 10°C/min, respectively.

crystallization peak was observed immediately after the enthalpy recovery (about −28°C). On the contrary, in the presence of protein, the mannitol crystallization peak did not emerge until the temperature reached about −22°C. This comparison demonstrated that the protein prevents mannitol crystallization even after annealing at −35°C (9°C above the T'_g) for 60 min.

Low-temperature XRD provided direct evidence of the inhibitory effect of the protein on mannitol crystallization. Figure 9 shows the XRD patterns of frozen mannitol–sucrose (R = 3.00) solutions in the presence and absence of the protein. No solute crystallization was detected after cooling to −70°C. In the absence of protein (Fig. 9A), after annealing for 60 min at −45°C, mannitol crystallization was not observed. The mannitol hydrate peak (9.6° and 17.9° 2θ) was observed after annealing at −35°C for an hour. However, the protein was effective in inhibiting mannitol crystallization at this temperature. There was no evidence of

mannitol crystallization after annealing for 60 min. Characteristic peaks of mannitol hydrate (9.6° and 17.9° 2θ) emerged only after annealing at −25°C for 60 min (29).

SIGNIFICANCE

The results of this study demonstrate that the API, human monoclonal antibody, dramatically inhibited mannitol crystallization even under fairly aggressive annealing conditions. The inhibitory effect of the API was observed at a moderate concentration (20 mg/ml). In addition, this effect was concentration dependent and would be even more pronounced if the protein concentration were increased (>20 mg/ml). These results pose some serious challenges to the design of freeze-drying cycles. In such systems, it will be prudent to fully understand the influence of the API concentration on the finished product characteristics, and by extension its performance. These issues become particularly relevant when the API concentration is sufficiently high so as to influence the physical state of one or more excipients.

This study is one of the first to demonstrate the inhibitory effect of the API on mannitol crystallization and points out some approaches to solve the problem. Aggressive annealing conditions facilitated mannitol crystallization. However, this may not always be feasible. On the contrary, the inhibitory effect of the protein can be exploited in the formulation design. If the protein facilitates retention of a fraction of mannitol in the amorphous state, then the mannitol would act both as a lyoprotectant and as a bulking agent.

CONCLUSIONS

The ratio of mannitol to sucrose and the protein concentration have an impact on the T_g' and may therefore influence the primary drying temperature. The protein inhibits both the nucleation and crystallization of mannitol and this effect seems to be concentration dependent. As a result, the protein concentration may also dictate the processing conditions (annealing time, annealing temperature, and primary drying temperature).

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