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Research Paper

Influence of Processing Conditions on the Physical State of Mannitol—Implications in Freeze-Drying

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Purpose. To study the effect of processing conditions on the physical state of mannitol during various stages of the lyophilization cycle of a protein formulation.
Materials and Methods. Mannitol and trehalose were used as the bulking agent and lyoprotectant, respectively. The physical state of mannitol during various stages of freeze-drying cycle, in the absence and presence of a model protein, was characterized using low temperature X-ray powder diffractometry (XRD) and differential scanning calorimetry (DSC).
Results. Mannitol did not crystallize even when the solution for lyophilization was cooled to −45°C at a slow cooling rate of 1°C/min. Annealing facilitated mannitol crystallization, and in the absence of the protein, a mixture of δ-mannitol and mannitol hemihydrate was obtained at both low (−18°C) and high (−8°C) annealing temperatures. However, in the presence of protein, the high annealing temperature promoted δ-mannitol crystallization and inhibited formation of mannitol hemihydrate, while the low annealing temperature facilitated the formation of mannitol hemihydrate. Interestingly, the hemihydrate in the frozen solution was retained in the final lyophile, even when the primary and secondary drying temperatures were as high as −5 and 65°C, respectively.
Conclusions. The presence of protein as well as the processing conditions (annealing temperature and time, primary and secondary drying temperatures) influenced the physical form of mannitol in the final lyophile. The protein promoted formation of δ-mannitol while inhibiting the formation of mannitol hemihydrate. Since the physical form of mannitol was greatly influenced by the presence of protein, it will be prudent to conduct the preliminary lyophilization cycle development studies in the presence of the protein. If mannitol hemihydrate is formed during annealing, its dehydration may require high secondary drying temperature.

KEY WORDS: annealing; lyophilization; mannitol; processing; protein; trehalose.

INTRODUCTION

Mannitol is a very popular bulking agent in freeze-dried formulations, since it readily forms a crystalline cake and its high eutectic melting temperature with ice (−1.5°C) enables primary drying at relatively high temperatures. Crystalline mannitol provides a robust matrix during freeze-drying, which endures "micro-collapse" of amorphous content without compromising the quality of the final lyophiles (1). Therefore, primary drying can be conducted at relatively high temperatures. A recent study showed that a sucrose–mannitol formulation was successfully primary-dried at −10°C without visual collapse (2). The physical form of mannitol in the final lyophile will depend on formulation variables and processing conditions.

Mannitol can exist in numerous physical forms including three anhydrous polymorphs (α-,β-, and δ-), mannitol hemihydrate and amorphous mannitol. Among the anhydrous polymorphs, β-mannitol is the stable form under ambient conditions. Studies have shown that the physical form of mannitol in the final lyophile can be influenced by the initial mannitol concentration in the solution for lyophilization (3,4), active pharmaceutical ingredient (API) concentration (5), cooling rate (4,6), and the buffer concentration (4). Low mannitol concentration (1.5 and 3% w/w) in the solution for lyophilization favored the β-form, while higher concentration (7.5% w/w) led to α-form (3). Trappler *et al.* reported that slow cooling rate caused a mixture of δ- (predominant) and α-forms (minor) (7). On the contrary, rapid cooling generated a mixture of α- (predominant) and δ-forms (minor). However, an extra annealing step (−20°C) could eliminate both α- and δ-forms, leading to the stable β-form. Polysorbate 80 was shown to not only enhance the crystallinity of mannitol, but also influence the polymorphic forms of mannitol (8). Mannitol hemihydrate was first observed by Yu *et al.* and was believed

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to be a product formed only at low temperatures (9). Cavatur *et al.* showed that slow cooling (1°C/min) caused the formation of mannitol hemihydrate while fast cooling (20°C/min) retained mannitol amorphous (6). It was also found that mannitol hemihydrate could survive at 27°C but dehydrated at ≥50°C. Johnson *et al.* reported the existence of mannitol hemihydrate in several protein formulations (2). Their results suggested that mannitol hemihydrate could transform to anhydrous mannitol *during* secondary drying at temperatures ≥40°C.

Mannitol can be retained in the amorphous state if the other formulation components inhibit mannitol crystallization. Previous reports showed that lyoprotectants such as sucrose and trehalose could inhibit mannitol crystallization depending on their concentrations (4). Bulking agents like glycine and sodium chloride can also play a role in inhibiting mannitol crystallization (10,11). Phosphate buffer showed inhibitory effect on mannitol crystallization when its concentration was 100 mM or higher (6). In addition, a recent study in our laboratory reported that a monoclonal antibody inhibited mannitol crystallization and the inhibitory effect was concentration-dependent (12). However, if mannitol crystallizes in an undesired physical state, for example as mannitol hemihydrate, it could still have dire consequences.

First, mannitol hemihydrate contains water, stoichiometrically incorporated in the crystal lattice, which may not be completely removed unless secondary drying is conducted at ≥40°C (2,9). On the other hand, many proteins cannot withstand such harsh secondary drying conditions (13,14). The secondary drying will therefore have to be conducted at lower temperatures. Second, during product storage, mannitol hemihydrate may transform to anhydrous mannitol with release of lattice water. The water will then be available for interaction with the other formulation ingredients including the API. This may lead to loss of protein activity. Finally, batch-to-batch variations may result from variable mannitol hemihydrate content in the lyophile.

Since the existence of mannitol hemihydrate poses a threat to the long-term stability of the final product, it would be of great interest to understand the mechanism of formation of mannitol hemihydrate. Hydrate formation during lyophilization is not unique to mannitol. Pyne *et al.* reported that disodium hydrogen phosphate crystallized as a dodecahydrate during freezing and then transformed into an amorphous phase during primary drying (15). Several studies revealed that processing conditions and formulation variables directly affected the physical state of solutes (16,17).

The impetus for this work came out of preliminary studies of a lyophilized formulation of a therapeutic protein containing mannitol and trehalose. The model protein used for the studies described in this work belongs to a novel class of molecules generated through the fusion of human serum albumin and a therapeutic molecule (18–20). Characterization of the final lyophiles revealed that some sample vials contained mannitol hemihydrate while others did not. Interestingly, while the composition was identical in all the formulations, the processing conditions were not. This observation suggested the processing conditions could influence the physical state of mannitol in the final lyophiles. However, based on the characterization of the final lyophiles, it was not possible to identify the critical processing parameters influencing the physical state of mannitol. Low temperature XRD has been proven to be a powerful tool to identify the phases that crystallize during cooling and annealing of frozen solutions. By attaching a vacuum pump and a low temperature stage to the XRD sample chamber, the entire freeze-drying cycle can be carried out, and the phase changes during the process can be monitored. The purpose of this study is to monitor the physical state of mannitol *during* the various stages of freeze-drying and to determine the influence of processing conditions on the physical form of mannitol. The processing conditions investigated were: (1) cooling rate, (2) annealing condition, (3) primary drying, and (4) secondary drying temperatures.

EXPERIMENTAL

Materials

Unless otherwise stated, the solution for lyophilization contained 1.7 mg/ml protein, 200 mM mannitol, 60 mM trehalose, 0.01% (w/v) polysorbate 80, and 10 mM phosphate buffer (pH=7.2).

Methods

Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (MDSC, Model 2920, TA Instruments, New Castle, DE) with a refrigerated cooling accessory was used. The DSC cell was calibrated using mercury and tin. About 10–15 mg of the sample solution was weighed in an aluminum pan and then hermetically sealed. The solutions were cooled from room temperature to −70°C at 20°C/min, held at −70°C for 30 min to ensure attainment of temperature equilibrium, and 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 either −28 or −18°C for periods of 15 to 60 min. Specific details are provided in the "Results and Discussion" section.

In case of lyophiles, the powder was filled into the aluminum pan in a glove box under nitrogen purge, sealed non-hermetically, and heated from −30 to 200°C, at 10°C/min, under a stream of nitrogen.

Thermogravimetric Analysis (TGA)

About 4 mg of lyophile was heated in an open pan from 25 to 250°C, under nitrogen purge, at 10°C/min, in a thermogravimetric analyzer (Q50, TA instruments, New Castle, DE).

Ambient Temperature X-ray Powder Diffractometry (XRD)

The powder was filled into an aluminum holder and exposed to CuKα radiation (45 kV×40 mA) in a wide-angle X-ray powder diffractometer (Model D5005, Siemens). The instrument was operated in the step scan mode, in increments of 0.04°2θ. The angular range was 5 to 40°2θ, and counts were accumulated for 1 s at each step. The data collection program used was JADE 7.0.

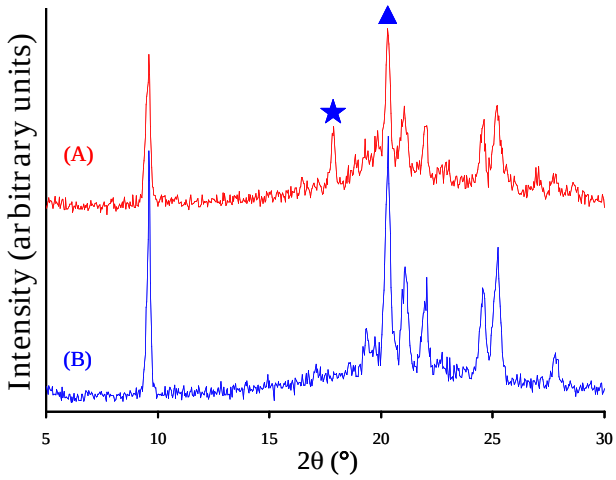

Fig. 1. XRD patterns of lyophiles obtained from solution containing 2.5 mg/mL protein, 200 mM mannitol and 60 mM trehalose in 10 mM phosphate buffer (pH=7.2). The solutions were processed under different conditions. (A) Annealed at −18°C for 1 h, primary dried at −5°C for 14 h and secondary dried at 35°C for 10 h (lyophile A). (B) Annealed at −18°C for 5 h, primary dried at 5°C for 12 h and secondary dried at 45°C for 10 h (lyophile B). One characteristic peak of mannitol hemihydrate (★) and δ-mannitol (▲) are pointed out.

Variable Temperature X-ray Powder Diffractometry (XRD)

An X-ray powder diffractometer (Model XDS 2000, Scintag) with a variable temperature stage (Micristar®, Model 828D, R.G. Hansen & Associates, Santa Barbara, CA; working temperature range −190 to 300°C) was used. An accurately weighed aliquot of sample solution (~100 mg) was filled into a copper holder and cooled from room temperature to −45°C at 1°C/min. The frozen solutions were then subjected to a controlled temperature program. Specific details are provided in the "Results and Discussion" section. XRD patterns were obtained by exposing the sample to CuKα (45 kV×40 mA) radiation. The angular range was 5 to 30°2θ, the step size was 0.04°2θ, and counts were accumulated for 1 s at each step. The samples were maintained under isothermal conditions at the selected temperatures. After annealing, the frozen solutions were subjected to primary drying *in situ* in the sample chamber of the XRD at a pressure of ~100 mTorr. Primary drying was carried out until the sublimation of crystalline ice was complete. The sample was then heated to the selected secondary drying temperature where the drying was continued for the desired time period. Again, the specific details are provided in the "Results and Discussion" section.

Freeze-Drying

The solution for lyophilizaion was filled in 3 ml glass vials (1 ml of solution per vial), cooled from room temperature to −45°C at 1°C/min, in a freeze-dryer (Virtis). It was then heated to −18°C, at 1°C/min, and annealed. The drying, at a pressure of 100 mTorr, was carried out under two conditions. In the first case, primary drying was carried out at −5°C for 14 h followed by secondary drying at 35°C for 10 h (referred to as lyophile A). In the second case, primary drying was conducted at 5°C for 12 h, followed by secondary dried at 45°C for 10 h (lyophile B). At the end of the secondary drying, the vials were sealed under vacuum. While the general experimental details are provided here, some of the specific details are provided in the "Results and Discussion" section, and also in the figure legends.

RESULTS AND DISCUSSION

Solid State Characterization of Final Lyophiles

As mentioned in the "Introduction" section, the lyophilization conditions influenced the physical form of mannitol in the final lyophiles. While the presence of δ-mannitol (characteristic peak at 9.6°2θ) was evident in both the lyophiles, the mannitol hemihydrate content in lyophile A (characteristic peak at 17.9°2θ) was much higher than in lyophile B (Fig. 1). This was also evident from DSC, wherein a pronounced endotherm at ~80°C, attributable to dehydration of the hemihydrate, was observed only in sample A (Fig. 2). We believe that dehydration of mannitol hemihydrate leads to lattice collapse and the formation of an amorphous anhydrate, which then rapidly crystallized (exotherm). We have not seen any evidence of trehalose crystallization in any of our experiments. While the buffer crystallization cannot be ruled out, the concentration is so low that its detection is unlikely. The weight loss in the TGA occurred in two stages, with the first step predominantly attributable to the loss of

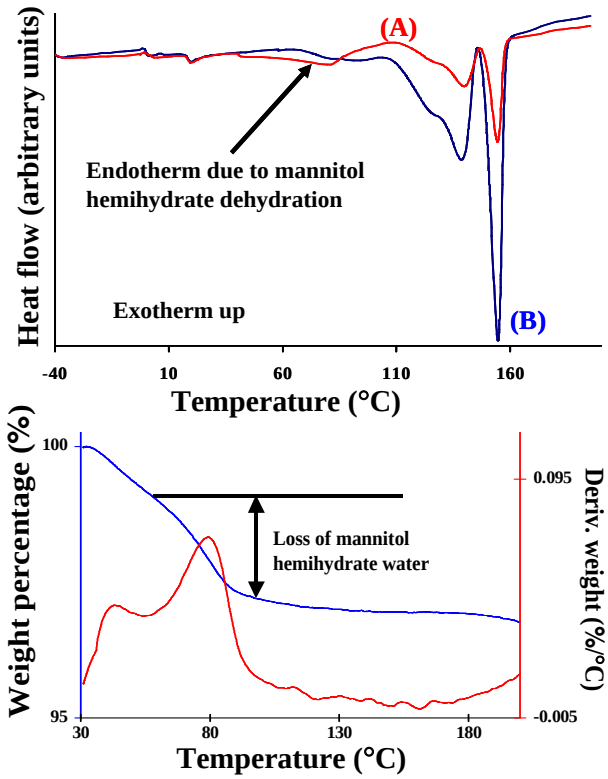

Fig. 2. (*Top*) DSC heating curves of lyophiles A and B. (*Bottom*) TGA and DTGA curves of lyophile A. For the DSC analyses, the samples were cooled from room temperature to −40°C, and then heated to 200°C, both at 10°C/min. For the TGA analysis, the sample was heated from 35 to 200°C at 10°C/min.

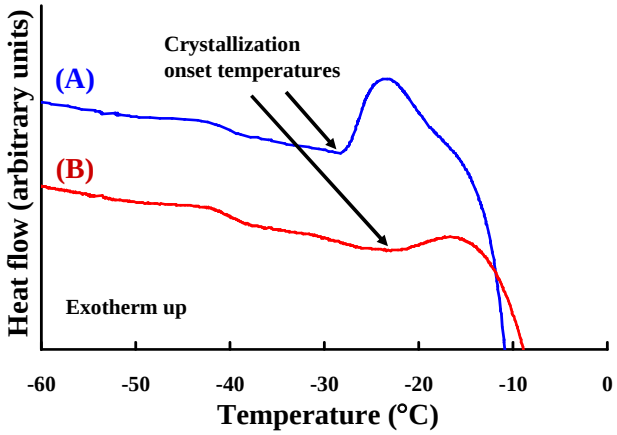

Fig. 3. DSC heating curves of frozen solution (A) containing 1.7 mg/ml protein and (B) placebo. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min, and heated to room temperature at 5°C/min. The mannitol, trehalose and phosphate buffer concentrations were 200, 60 and 10 mM, respectively. The crystallization onset temperature of mannitol is pointed out.

sorbed water and the second (over the temperature range of ~70 to 85°C) predominantly to dehydration (Fig. 2). The above observation clearly indicated that processing conditions are critical to the physical state of mannitol in the final lyophiles. In order to identify the critical processing parameter(s), each stage of the freeze-drying cycle was monitored starting with the frozen system.

Characterization of Frozen Systems

The solution to be lyophilized was either quench-cooled with liquid nitrogen or cooled to −70°C, at controlled rates ranging from 1 to 10°C/min. When the XRD patterns of the frozen solutions were obtained, only the characteristic peaks of ice were observed (not shown). Crystalline mannitol was not detected in the frozen solution. Irrespective of the cooling rate, when the frozen solution was heated at 5°C/min to 25°C, the following thermal events were observed: (a) glass transitions with onset at ~−41°C and ~−32°C; (b) an exotherm with onset at ~−26°C, ascribed to mannitol crystallization; and (c) overlapping eutectic and ice melting endotherms (Fig. 3). The observed Tg′ values were in reasonable agreement with the reported values of −41 and −33°C in systems with similar but not identical compositions (21). A placebo solution, subjected to the same treatment, exhibited similar Tg′ values (Fig. 3). Thus the Tg′ values seemed to be unaffected by the presence of the protein which is not surprising since the protein concentration in the solution before lyophilization was low (1.7 mg/ml). However, the crystallization onset temperature was shifted to a lower temperature (−22.6 to −25.7°C) in the presence of protein suggesting that the protein facilitated mannitol crystallization.

Effect of Annealing

Annealing, an isothermal step to promote solute crystallization, is commonly conducted at temperatures above Tg′. Two temperatures, −28 and −18°C, were chosen for annealing. When annealed at −28°C, crystallization enthalpy gradually decreased as the annealing time increased from 15 to 45 min (Fig. 4; upper panel). The crystallization peak completely disappeared when the annealing time reached 60 min. On the other hand, crystallization was very rapid when annealing was conducted at −18°C, and was complete in 15 min (Fig. 4; lower panel). Irrespective of the annealing temperature, there was a shift in Tg′ to a higher temperature of ~−32°C. Because mannitol has a strong tendency to crystallize, there was a progressive increase in the crystalline mannitol content with annealing and a consequent decrease in the uncrystallized (amorphous) mannitol content in the freeze-concentrate. Therefore, the composition of amorphous phase in the frozen system is increasingly "trehalose-rich" and then Tg′ is close to that of trehalose (−32°C, 21). XRD was then used to identify the phases crystallizing from

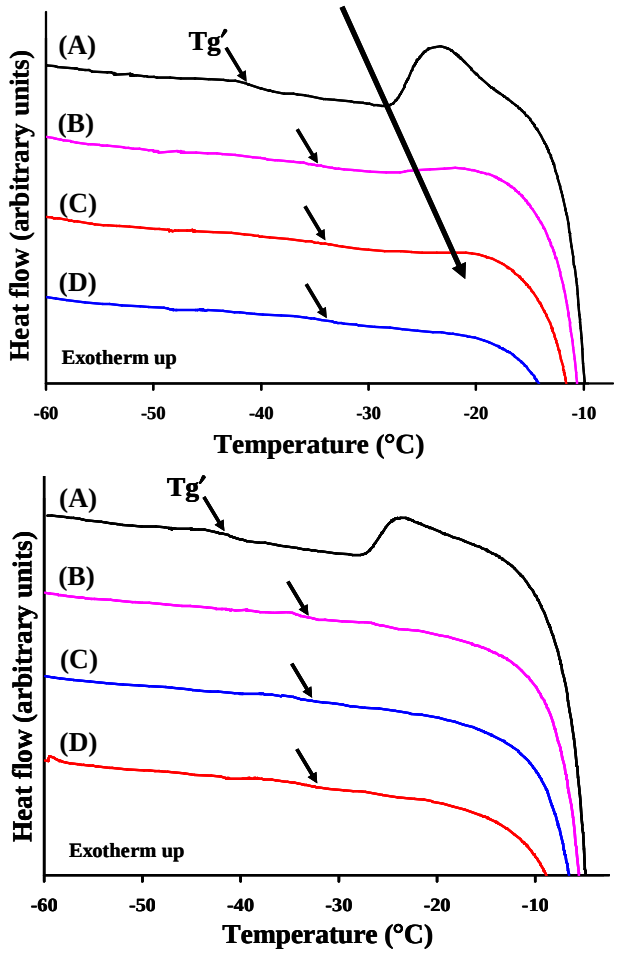

Fig. 4. The effect of annealing temperature on mannitol crystallization. DSC heating curves of frozen solution containing 1.7 mg/ml protein. The mannitol, trehalose and phosphate buffer concentrations were 200, 60 and 10 mM, respectively. The solutions were initially cooled from room temperature to −70°C at 20°C/min, held for 30 min, heated either to −28°C (*top panel*) or to −18°C (*bottom panel*) at 5°C/min and annealed for (A) 0, (B) 15, (C) 30 and (D) 60 min. The solutions were then cooled to −65°C at 20°C/min, held for 15 min, heated to room temperature at 5°C/min. The second heating scans are shown here. The *line* shows the trend in the crystallization exotherm of mannitol as a function of annealing time. The Tg′ is also pointed out.

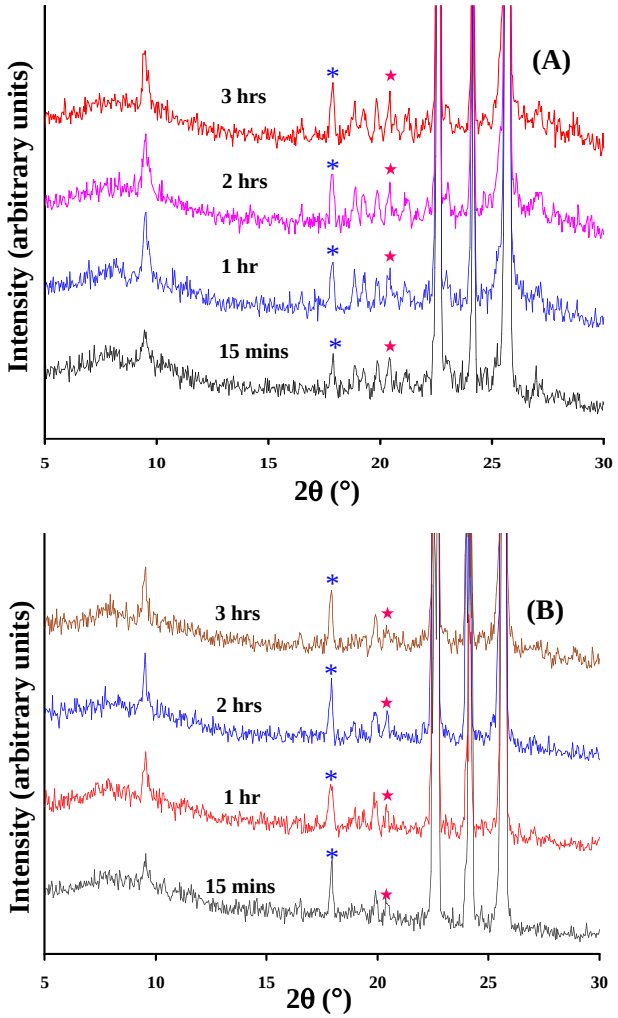

Fig. 5. XRD patterns of frozen aqueous solutions (A) containing 1.7 mg/ml protein and (B) placebo. The mannitol, trehalose and phosphate buffer concentrations were 200, 60 and 10 mM, respectively. The solutions were initially cooled from room temperature to −45°C at 1°C/min, heated to −18° at 1°C/min, and annealed for 3 h. One characteristic peak of mannitol hemihydrate (*asterisk*) and δ-mannitol (★) are pointed out.

solution. The solutions were cooled to −45°C (at 1°C/min), held for 15 min, and heated at 1°C/min to the desired annealing temperature, and held for 3 h, and XRD patterns were obtained at regular intervals. There was no solute crystallization in the unannealed frozen solutions. After annealing for 15 min at −18°C, solute crystallization, both as mannitol hemihydrate and as δ-mannitol was evident (Fig. 5A and B). As annealing time increased, the intensity of the characteristic peaks of both mannitol hemihydrate and δ-mannitol increased. In order to obtain a semi-quantitative trend of the mannitol phases during annealing, the integrated intensity ratio (intensity of 20.4°2θ peak of δ-mannitol/ intensity of 17.9°2θ peak of mannitol hemihydrate) was plotted as a function of annealing time (Fig. 6). The presence of protein seemed to selectively inhibit the formation of mannitol hemihydrate. In order to determine if this inhibitory effect was temperature dependent, annealing was also conducted at a higher temperature of −8°C.

As before, after annealing for 15 min at −8°C mannitol hemihydrate and δ-mannitol crystallized, both in the presence and absence of protein (Fig. 7A and B). The inhibitory effect of protein on mannitol hemihydrate was evident at longer annealing times. There was a dramatic increase in the δ-mannitol peak intensity (20.4°2θ) at the expense of mannitol hemihydrate (Fig. 6, curve 1; Fig. 7A). After 3 h of annealing, the characteristic peak of mannitol hemihydrate had almost completely disappeared (Fig. 7A). In the placebo, both mannitol phases were still present after 3 h of annealing (Fig. 7B). Our DSC experiments showed that the crystallization onset temperature was lowered in the presence of protein, suggesting that the protein facilitated mannitol crystallization (Fig. 3). This was also confirmed by XRD, wherein the intensity of mannitol peaks (both δ- and hemihydrate) was observed to be much higher in the presence of protein (Fig. 5A and B). XRD revealed the additional information that the protein facilitated formation of δ-mannitol possibly at the expense of mannitol hemihydrate.

Effect of Primary Drying

By attaching a vacuum pump to the low-temperature stage, the entire freeze-drying cycle was simulated in the sample chamber of the X-ray diffractometer. When the solution annealed at −8°C was primary dried at −5°C, which is slightly below the eutectic temperature, there was a pronounced increase in the intensity of δ-mannitol peaks (Fig. 8B). The solution annealed at −8°C for 3 h contained very little mannitol hemihydrate (Fig. 7A), and the primary

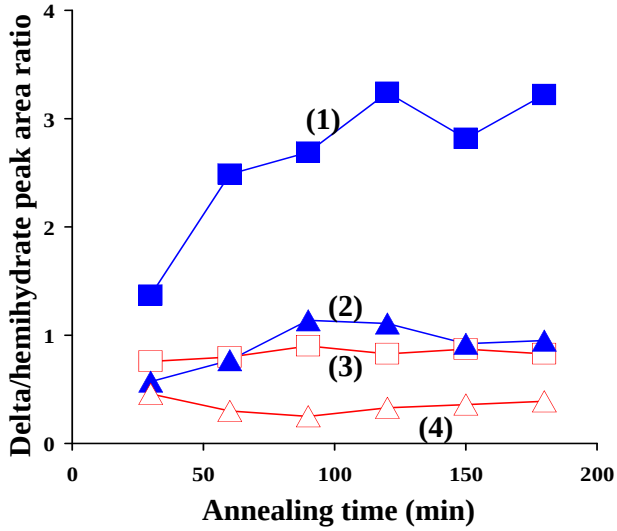

Fig. 6. The effect of the protein on mannitol phases crystallizing from solution as a function of annealing time. (1) Protein solution annealed at −8°C, (2) placebo solution annealed at −8°C, (3) protein solution annealed at −18°C, and (4) placebo solution annealed at −18°C. The solutions were initially cooled at 1°C/min from room temperature to −45°C, and then heated to selected annealing temperatures at 1°C/min. The protein concentration was 1.7 mg/ml and the mannitol, trehalose and phosphate buffer concentrations were 200, 60 and 10 mM, respectively. *(intensity of 20.4°2θ peak of δ-mannitol/intensity of 17.9°2θ peak of mannitol hemihydrate).

drying also seemed to favor the formation of δ-mannitol. Thus, even if mannitol hemihydrate was formed during primary drying, it may be unstable at the high primary drying temperature, and may be transformed to δ-mannitol. In order to test this possibility, primary drying was conducted at a much lower temperature of −20°C. The pronounced peak of mannitol hemihydrate suggests that the hemihydrate phase can be retained at low primary drying temperatures (Fig. 8C).

Annealing at −18°C had resulted in a mixture of the anhydrous and hemihydrate phases (Fig. 5A). While both the phases were retained on primary drying at −5°C, the hemihydrate content was higher in the final lyophile (Fig. 8A). Thus the presence of significant concentration of the hemihydrate in the frozen solution enabled the retention of the hemihydrate in the final lyophile, even when the primary drying temperature was high. These results suggest that if the hemihydrate is not desired in the final lyophile, it will be best to eliminate it *before* initiating the primary drying.

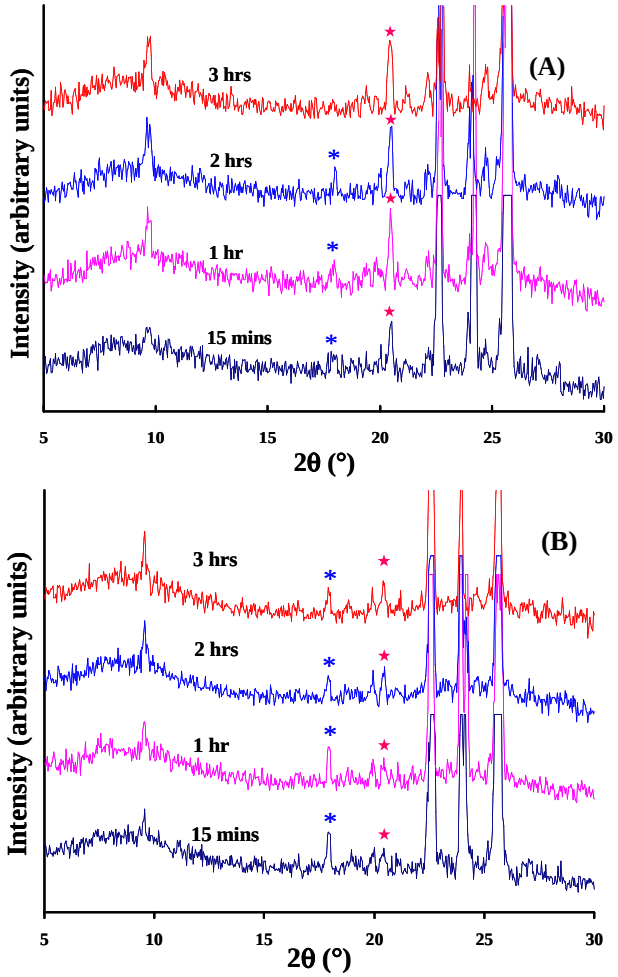

Fig. 7. XRD patterns of frozen aqueous solutions (A) containing 1.7 mg/ml protein and (B) placebo. The mannitol, trehalose and phosphate buffer concentrations were 200, 60 and 10 mM, respectively. The solutions were initially cooled from room temperature to −45°C at 1°C/min. The temperature was raised to −8° at 1°C/min, annealed for 3 h. One characteristic peak of mannitol hemihydrate (*asterisk*) and δ-mannitol (★) are pointed out.

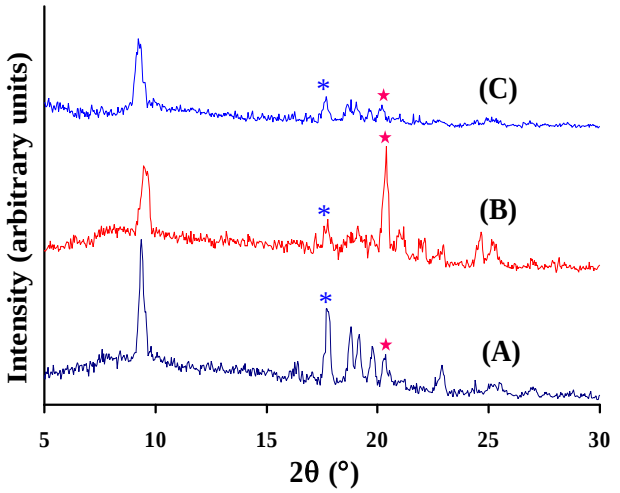

Fig. 8. XRD patterns of protein lyophiles primary dried for 1 h at (A) −5°C after annealing at −18°C, (B) −5°C after annealing at −8°C, and (C) at −20°C after annealing at −8°C. The protein, mannitol and trehalose concentrations were 1.7 mg/ml, 200 mM and 60 mM, respectively. The solutions were initially cooled from room temperature to −45°C at 1°C/min. The temperature was raised to the desired annealing temperatures at 1°C/min, annealed for 3 h. Primary drying was conducted at a chamber pressure ~100 mTorr. The characteristic peaks of mannitol hemihydrate (*asterisk*) and δ-mannitol (★) are marked.

Effect of Secondary Drying

Once mannitol hemihydrate was formed, it was very difficult to eliminate it from the final lyophile unless secondary drying was conducted at a very high temperature. Figure 9 contains the XRD patterns of lyophile secondary

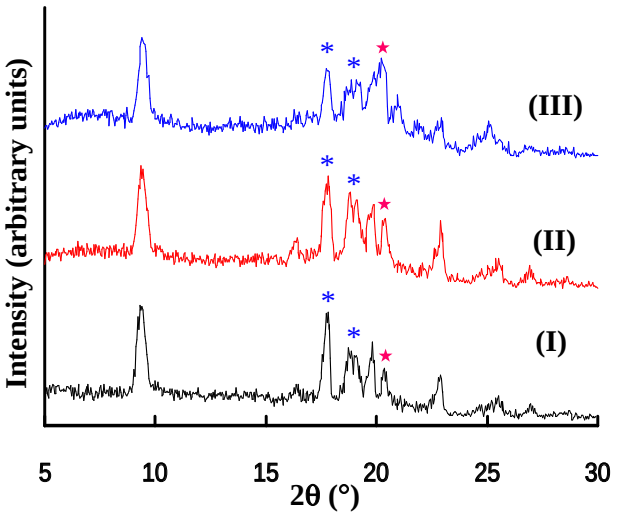

Fig. 9. XRD patterns of protein lyophiles after different secondary drying for 30 mins at (I) 25°C, (II) 45°C, and (III) 65°C. The protein, mannitol and trehalose concentrations were 1.7 mg/mL, 200 mM and 60 mM, respectively. The solutions were initially cooled from room temperature to −45°C at 1°C/min. The temperature was raised to −18° at 1°C/min, annealed for 3 h. The annealed solution was heated to −5°C and subjected to primary drying for 1 h at a chamber pressure ~100 mTorr. The characteristic peaks of mannitol hemihydrate (*asterisk*) and δ-mannitol (★) are marked.

dried at progressively higher temperatures up to 65°C. While secondary drying at 25 and 45°C did not cause dehydration, drying at 65°C for 30 min caused partial dehydration and possibly conversion to δ-mannitol. The resistance to dehydration in the presence of protein was earlier reported by Johnson *et al.* (2), while preparing a freeze-dried formulation of daniplestim (a recombinant protein) containing sucrose and mannitol. A shelf temperature of 40°C and a drying time of 13 h were the mildest secondary drying conditions for elimination of mannitol hemihydrate.

SIGNIFICANCE

The reported observations have several implications. Traditionally, solid-state and physical characterization studies have been restricted to the final freeze-dried product. This does not provide information about physical changes (glass transition, crystallization, and solid-state transition) during the various stages of the freeze-drying process. We have investigated the crystallization behavior of mannitol in the presence and absence of the protein during the various stages of the freeze-drying process. Our experimental results indicated that the physical state of mannitol was influenced by the processing conditions as well as the formulation components including the API. It will therefore be prudent to conduct the lyophilization cycle development studies in the presence of the API since results from the placebo studies can be misleading. Moreover, it is valuable to monitor the phase transitions during the various stages of lyophilization cycle. This will enable us to identify the critical process parameters that influence the physical form of the formulation components. The shipping and storage conditions of the final product could facilitate phase transitions in mannitol, especially the dehydration of the hemihydrate. The product stability could then be affected by the released water. In addition, such transitions might lead to differences in the stability profile between lots even when the production of the API and the freeze-drying process are tightly controlled.

CONCLUSIONS

We have investigated the effect of processing conditions on the phase behavior of mannitol in a model protein formulation during freeze-drying. Mannitol did not crystallize during cooling even at a slow cooling rate of 1°C/min. Annealing facilitated mannitol crystallization. In the absence of the protein, elevating annealing temperature did not change the outcome of physical form of mannitol. In the presence of protein, high annealing temperature promoted delta mannitol crystallization and inhibited formation of mannitol hemihydrate, while low annealing temperatures facilitated the formation of mannitol hemihydrate. Mannitol hemihydrate can survive even at a relatively high secondary drying temperature (45°C), but there was evidence of dehydration at 65°C. Therefore, the physical form of mannitol in the final lyophile appeared to be a complex function of annealing conditions, primary drying and secondary drying temperatures.

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