EFFECT OF PROCESSING AND COSOLUTES

ON

MANNITOL PHASE BEHAVIOR IN FROZEN SOLUTIONS

A THESIS

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DEDICATION

For all the people who love and support me in my life

ABSTRACT

Mannitol is widely used as a bulking agent in lyophilized protein formulations. It can crystallize in different forms, including three anhydrous forms (α-, β- and δ) and a hemihydrate ($C_6H_{14}O_6 \bullet 0.5H_2O$, MHH). The effect of cosolutes (sugars and proteins), their concentration and processing (annealing) on mannitol phase behavior was investigated using differential scanning calorimetry (DSC) and X-ray diffractometry (XRD). Based on the DSC results, sugars exhibited a stronger inhibitory effect than proteins on mannitol crystallization. The freeze concentrate was heterogeneous when mannitol crystallization was not completely inhibited, evident from the multiple glass transitions in the heating curves. On annealing the frozen solutions at -25 °C, MHH crystallization was extensively observed while the δ-form was favored at a higher temperature (-10 °C). The higher annealing temperature also enabled rapid mannitol crystallization. XRD results show that mannitol predominantly crystallized as MHH in the presence of sugars. Interestingly, proteins selectively facilitated the crystallization of δ-mannitol. The crystallization inhibitory effect of proteins could be overcome with annealing while, at a high sugar concentration, a substantial fraction of mannitol was retained amorphous even after annealing.

Keywords: freeze-drying, mannitol, crystallization, sucrose, trehalose, protein, glass transition

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CHAPTER 1

INTRODUCTION

Freeze-drying (lyophilization)

Protein therapeutics have gained significant attention in recent years. A versatile class of proteins (monoclonal antibodies, protein hormones, enzymes, etc.) have been observed to be highly potent and effective in the treatment of different types of diseases with fewer side effects than is typically observed with small molecule drugs[1]. However, the complex structure (secondary, tertiary, and quaternary structure) of protein molecules and their thermolabile nature poses challenges with respect to their formulation into dosage forms[2]. Protein destabilization can lead to not only a loss in activity but also potential immunogenicity[1]. Retention of protein stability across various stages (e.g. manufacturing, shipping, storage, and use by the patient[3,4]), therefore, has become one of the major challenges in the development of protein-based products.

When formulated as solutions, some proteins are not adequately stable. As a result, the formulation may not have an acceptably long shelf life. Freeze-drying, also known as lyophilization, is a commonly used low-temperature drying technique to prepare stable protein formulations. Lyophilization consists of three stages – (i) freezing to facilitate water (and solute) crystallization, (ii) primary drying to enable ice sublimation, and (iii) secondary drying to remove absorbed water (Figure 1.1). However, the stresses imposed on proteins during freezing and drying can lead to their instability. For example, during

freezing, proteins may undergo cold denaturation, causing unfolding[5]. The adsorption of proteins at the ice/water interface can be another reason for protein denaturation[6]. The formation of ice is accompanied by an increase in the concentration of the non-crystallizing solutes, including the protein, in solution. This is referred to as the "freeze-concentrate". One consequence of freeze-concentration is an increase in ionic strength as well as potential pH shifts due to selective crystallization of buffer components[7]. Both of these can be detrimental to protein stability. The drying process can also cause protein destabilization because of the stresses association with the removal of water. This instability can be attributed to the loss of hydrogen bonding between the proteins and water[8]. In addition to the consideration of stresses experienced by the protein during lyophilization, the manufacturability of protein formulations also warrants attention. For instance, from a cost perspective, reducing the primary drying time is one of the goals since it is the longest stage of freeze drying[9]. These challenges require appropriate formulation and processing design of lyophilization.

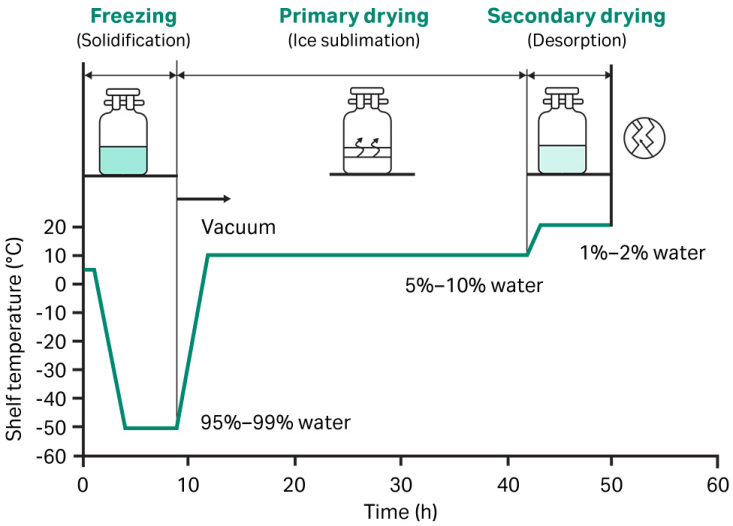

Figure 1.1 The process of lyophilization contains three stages

Figure reference:

https://www.cytivalifesciences.com/en/us/solutions/genomics/knowledge-center/advantages-of-lyophilization

After lyophilization, several product quality attributes should be evaluated, including residual water content, reconstitution time, and cake appearance. The residual moisture level in the freeze-dried cake can affect protein stability. The sorbed water may (i) change the conformation stability of proteins by increasing their mobility or (ii) act as a reactant or medium for enhancing the mobility of other reactants and facilitate processes such as the Maillard reaction[10]. Besides, from the end-user perspective, the cake must have a short reconstitution time. Partially crystalline cakes appear to have a shorter reconstitution time than fully amorphous cakes. For instance, the partially crystalline mannitol improved cake wettability and promoted fluid penetration as well as cake disintegration[11]. Ideally, the acceptable cake is supposed to be “elegant”. However, cake deformations may not

necessarily suggest product stability issues. One of the common consequences of deformation is the formation of collapsed cakes (Figure 1.2). During primary drying, collapse occurs when the product temperature exceeds the glass transition temperature of the freeze-concentrate (Tg'). There is data indicating that cake collapse is just a cosmetic imperfection[12]. However, collapsed cakes may result in high residual moisture, and thus protein stability can be affected. Therefore, avoidance of collapse is another important consideration during the design of formulation and processing parameters[13].

Figure 1.2 Collapsed cake: total collapse (left) and partial collapse (center). The vial on the right shows no evidence of collapse.

Figure reference: Sajal et al. Journal of Pharmaceutical Sciences, 2017.

Excipients

Excipients, each with an intended functionality, are included in freeze-dried protein products (Figure 1.3). The addition of excipients is supposed to be protein-specific and the goal is to keep the formulation simple.

Stabilizers

Non-reducing sugars, such as sucrose and trehalose, act as lyoprotectants and stabilize proteins during freezing drying and subsequent storage. Maillard-reaction with amines limits the use of reducing sugars (such as lactose and maltose). The proposed stabilization mechanisms of sugars include: i) preferential exclusion, ii) formation of a glassy matrix, and iii) the water replacement theory[14].

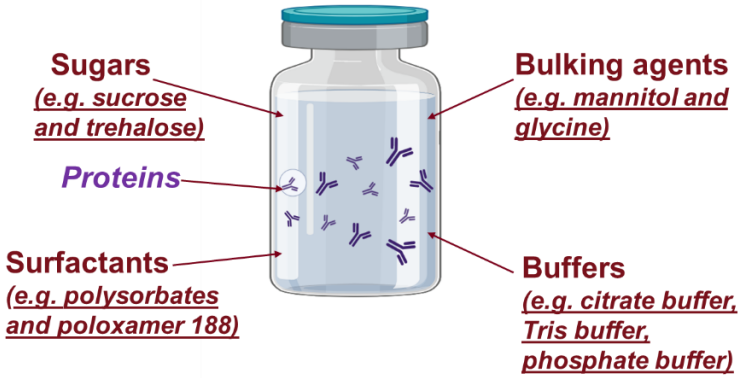

Figure 1.3 Some commonly used excipients in protein formulations.

A specific stabilization mechanism may be invoked at each stage of lyophilization. At the beginning of freezing, the exclusion of sugars increases the free energy of denaturation. Thus, the protein is preferentially hydrated, stabilizing it in its native state. From a kinetic perspective, the formulation viscosity (viscosity of the freeze-concentrate) increases with ice crystallization. Immobilization of dispersed protein in the rigid glassy matrix formed by sugars slows the rate of protein unfolding and/or degradation. This kinetic mechanism can be applied to both frozen and dried states. Besides, when there is insufficient water around the protein, sugars can interact with proteins via hydrogen bonds, serving as water substitutes.

Amino acids are another class of stabilizers. When combined with sugars, they typically serve as a secondary stabilizer since their stabilization effect is weaker when compared to sugars[15]. However, their stabilization mechanism is not well understood. One of the most commonly used amino acids is arginine. Based on solid-state nuclear magnetic resonance (NMR) spectroscopy, its stabilization effect can be attributed to hydrogen bonding and ion-dipole interaction with protein[16]. The systems containing arginine HCl, trehalose, and mannitol exhibited improved protein stability, evident from the lower degree of aggregation, when compared to formulation with trehalose and mannitol[17].

The addition of other excipients such as buffers, surfactants, and antioxidants may also be warranted based on the need for protein stabilization. Buffers maintain the pH during processing and in the solution after reconstitution, while surfactants are widely used to

prevent ice surface-induced denaturation in frozen solutions. When oxidation is the main degradation pathway of proteins, adding antioxidants is supposed to mitigate such impact.

Bulking agent

In the case of low-concentration protein formulations, bulking agents are necessary to prevent blowout and ensure an elegant product. Sugar can also be added to the formulation as an amorphous bulking agent. However, an amorphous cake is not ideal due to the long primary drying time dictated by the glass transition temperature of the freeze concentrate (T_g'). Primary drying at a temperature above T_g' results in viscous flow, which has the potential to cause cake collapse[9]. A crystalline bulking agent is therefore preferred. For example, mannitol serves as a bulking agent due to its high crystallization propensity, thereby providing mechanical strength and thus, an elegant cake appearance. In a frozen solution containing amorphous solutes as well as a crystalline bulking agent (for example, mannitol), cake collapse can be avoided by conducting primary drying at temperatures above the T_g' but below the eutectic melting temperature. Therefore, the high eutectic melting temperature of mannitol-ice binary mixture (at -2.2 ºC) enables efficient primary drying compared to the formulations with only sugars[18]. Glycine is another popular crystalline bulking agent, since it also readily crystallizes from solution. One of the limitations of glycine is the pH dependent speciation of glycine[19]. It may crystallize as glycine, glycine HCl, diglycine HCl, or sodium glycinate[20].

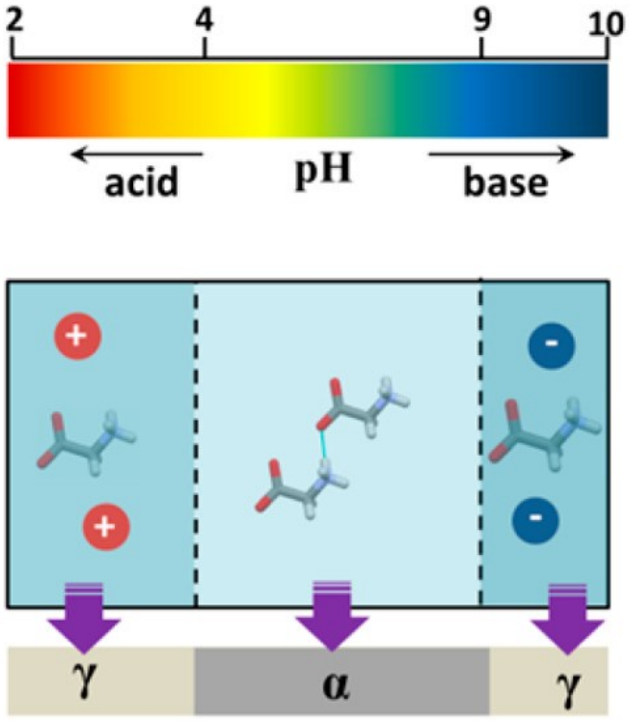

Figure 1.4 pH-dependent speciation of glycine

Figure reference: Tang et al, Crystal Growth & Design, 2017

Excipient-excipient interactions

The functionality of excipients in lyophilized formulations is associated with their physical forms. Lyoprotectants (e.g. sucrose and trehalose) need to be retained in an amorphous state during lyophilization so as to stabilize the protein. In contrast, bulking agents, such as mannitol, need to have high crystallinity to confer an elegant cake appearance. As we discussed before, protein-excipient interactions (e.g. hydrogen boding, preferential hydration, and electrostatic interactions) are critical for improving protein stability. However, the physical states of an excipient can also be influenced by proteins. The

inhibition of mannitol crystallization by proteins was observed in a concentration dependent manner[21].

In addition, excipients can also interact with each other, affecting their phase behavior and thus their functionality in formulations. For example, mannitol and trehalose, in a concentration dependent manner, influenced the crystallization behavior of each other in frozen solutions[22]. At a 1:1 weight ratio, mannitol induced trehalose crystallization, while mannitol crystallization was inhibited when the mannitol to trehalose ratio was 1:3. If trehalose crystallizes, its stabilization effect can be compromised and can lead to potential protein instability upon storage. From another perspective, there is potential for mannitol to be a stabilizer if it can be retained in amorphous state[23].

Besides, lyoprotectants and bulking agents can also interact with buffer salts. In phosphate buffered saline, the pH shift due to the crystallization of disodium hydrogen phosphate was reduced with the addition of mannitol or trehalose[24]. Similar results also demonstrated that mannitol or glycine could prevent the pH shift of succinic acid during cooling if they could remain in the amorphous state[25]. In addition, in the presence of sugars, the crystallization of poloxamer 188 (P188), a surfactant, can be inhibited in frozen solutions, retaining it in the amorphous state. However, drying promoted its crystallization, and thereby P188 can act as a stabilizer in frozen solutions and a bulking agent in freeze-dried products[26].

Mannitol

Mannitol is a diuretic and can also be used to reduce intracranial pressure. However, it is predominantly used as an excipient in multiple types of pharmaceutical formulations. In liquid formulations, the role of mannitol is osmolarity modification, while in tablet formulations, it is widely used in orally disintegrating tablets[27]. As we mentioned before, in lyophilized products, it functions as a bulking agent. The high crystallinity of mannitol, therefore, is necessary to obtain non-collapsed cake. Therefore, the annealing step, an isothermal treatment at a temperature above T_g' is included in the freezing step to promote mannitol crystallization and thus provide support for the cake (Figure 1.5)[28].

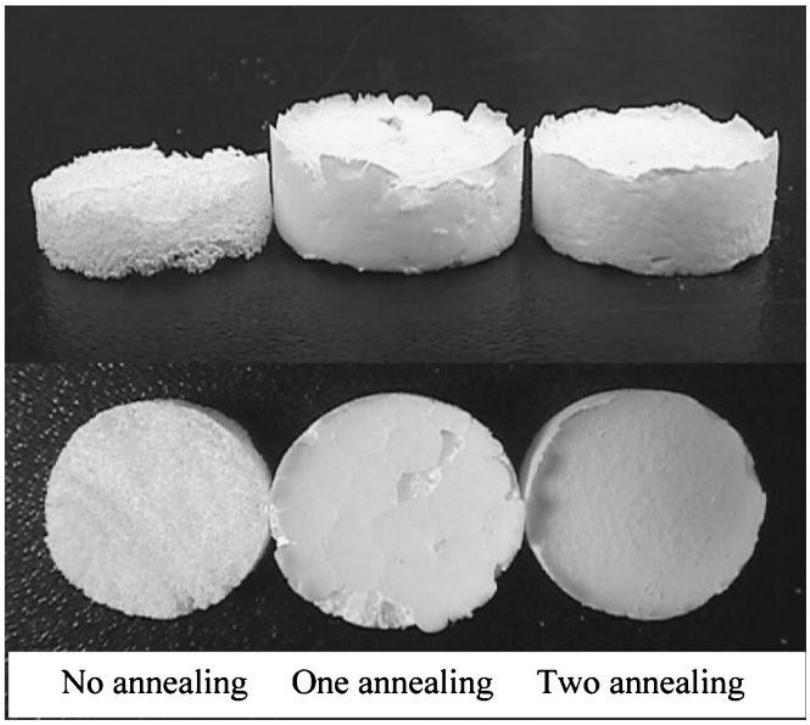

Figure 1.5 The effect of annealing on visual appearance of freeze-dried cakes.

Figure reference: Lu et al, Pharmaceutical Development and Technology, 2004

Several crystalline forms of mannitol may be detected during freeze drying. It can exist in three anhydrous forms (α-, β-, and δ-), and also as mannitol hemihydrate (MHH) in frozen solutions. MHH is kinetically stable, and may therefore be retained in final lyophilized products. However, since MHH is not thermodynamically stable under ambient conditions, the water incorporated into the lattice may be released upon the storage of final products[28]. Accordingly, the released water can lead to (i) protein destabilization and (ii) sucrose crystallization. Sucrose crystallization can cause a loss in its lyoprotectant function and lead to protein instability[29].

Due to the potential consequence of metastable MHH on protein stability, efforts have been directed towards the investigation of mannitol phase behavior under subambient conditions. The formation of MHH during cooling is attributed to Ostwald's rule of stages, where MHH has lower solubility than anhydrous forms at < -15 °C[30]. The phase diagram for the mannitol-water system has been established recently (Figure 1.6). The solid phases of interest are: hexagonal ice (lh), β-mannitol (β-m), δ-mannitol (δ-m), and MHH (HH). However, this phase diagram is of limited practical use since the crystallization of mannitol is dominated by kinetics. The control of physical forms of mannitol can be challenging[31]. Overall, the phase behavior of mannitol in mannitol-water systems depends on the initial concentration of mannitol, cooling rate, and annealing conditions[28].

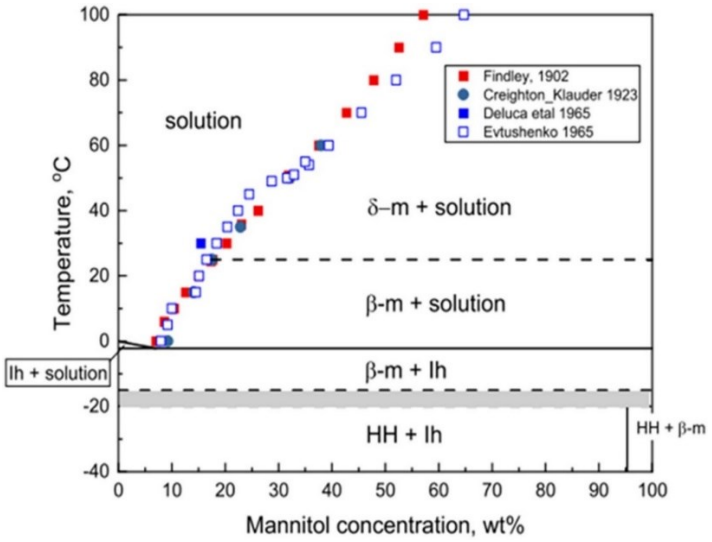

Figure 1.6 Temperature-composition phase diagram of mannitol-water system.

Figure reference: Miguel R, et al, The Journal of Physical Chemistry Letters, 2021

HYPOTHESIS AND SPECIFIC AIMS

Non-crystallizing solutes, such as sugars and macromolecules, can cause incomplete crystallization of mannitol[21,22,32]. When mannitol is intended to function as a bulking agent, it is desired in a highly crystalline state. If mannitol is retained in a partially crystalline state in the final product, it can crystallize during storage. The release of the unfrozen water can result in protein instability. On the other hand, vial breakage can also be attributed to mannitol crystallization during freeze-drying[33]. Hence, it is imperative to facilitate mannitol crystallization by annealing. *It will be ideal to cause complete mannitol crystallization during the freezing step.* However, a recent study indicated that annealing might be insufficient to ensure complete mannitol crystallization - a second crystallization of mannitol was shown at temperatures > -10 ºC during freeze-thawing[31].

Moreover, the physical form of mannitol crystallizing from solutions is influenced by the addition of non-crystallizing solutes. In the lyophilized product, MHH was not observed in a protein (an antibody) formulation with a high mannitol:sucrose (5:1) weight ratio. While MHH was detected in the final products with lower mannitol:sucrose ratios, an increase in protein concentration inhibited the formation of MHH[34]. In addition, in mannitol-sucrose-NaCl-buffer systems, with an increase in the Fc-fusion protein concentration, δ-mannitol crystallization was promoted[35]. Similar results were observed in the mannitol-NaCl system in the presence of human serum albumin[36]. A design of experiment (DoE) approach further revealed the importance of interactions between relative mannitol ratio, the protein concentration (lysozyme and bovine serum albumin), and annealing conditions on MHH

content in the lyophiles[37]. Hence, the effect of cosolutes, especially proteins, on the design of formulations and lyophilization processing parameters needs more attention.

Our previous studies showed that in the presence of proteins, on annealing, crystallization of δ-mannitol was promoted while inhibiting the formation of MHH in mannitol-sucrose-buffer systems. Therefore, in the current study, we hypothesize that the effect of proteins on mannitol phase behavior is distinct from that of sugars (sucrose and trehalose).

We explored the phase behavior of mannitol in the presence of sugars (sucrose and trehalose) and proteins (immunoglobulin G, bovine serum albumin, and lysozyme) under different annealing conditions (-25 or -10 °C). Our goal was to modulate the mannitol phase behavior by the addition of cosolutes and modulating the annealing conditions. The study of the individual effect of cosolutes can further improve our mechanistic understanding of mannitol phase behavior and facilitate the rational design of formulation and process parameters.

We had two main objectives:

1. To understand the impact of cosolutes (sugar and protein) and annealing temperature on the physical form of mannitol crystallizing in frozen solutions.
2. To investigate the impact of cosolutes (sugar and protein) as well as the annealing temperature on the mannitol crystallization kinetics.

CHAPTER 2

INTRODUCTION

Freeze-drying, also known as lyophilization, is a commonly used low temperature drying technique for the stabilization of protein pharmaceuticals. It consists of three stages – (i) freezing to facilitate water (and solute) crystallization, (ii) primary drying to enable ice sublimation and (iii) secondary drying to remove sorbed water. Several excipients are used in a lyophilized formulation, each with a unique function. Sugars, such as sucrose and trehalose, act as lyoprotectants to stabilize proteins during both freezing and drying[14]. Mannitol, due to its high crystallization propensity, is added to provide mechanical strength for elegant product appearance. This is specifically important for low-concentration protein formulations[28]. As a bulking agent, the addition of mannitol enables efficient primary drying due to the high eutectic melting temperature of mannitol-ice binary mixture (-2.2 °C)[18]. In high-concentration protein formulation, mannitol could accelerate cake reconstitution by promoting disintegration[11].

Mannitol has three anhydrous forms (α-, β- and δ), with their thermodynamic stability rank ordered as: β > α > δ under ambient condition[38]. In frozen solutions, mannitol can also crystallize as a hemihydrate ($C_6H_{14}O_6 \bullet 0.5H_2O$, abbreviated as MHH). The formation of MHH poses challenges to lyophilized protein products. This is because MHH can dehydrate during product storage, and the released water can induce protein denaturation[39]. MHH dehydration, by promoting sucrose crystallization, could also undermine product stability[29]. In addition, during lyophilization, the elimination of MHH cannot be achieved unless the secondary drying temperature is ⩾ 40 °C[40]. Such a high temperature is usually

detrimental to proteins. In order to avoid the formation of MHH, mannitol crystallization should be carefully controlled in frozen solutions. However, MHH is observed in frozen solutions, especially at ⩽ -20 °C[30].

The presence of noncrsytallizing solutes (sugar and/or protein) can inhibit mannitol crystallization. However, if mannitol crystallization is not complete in frozen solutions, the mannitol-sugar-water freeze concentrate usually exhibits multiple glass transitions, reflecting its heterogeneity. This heterogeneity can be one reason for the protein instability[41]. Moreover, during product storage, crystallization of mannitol and the release of the water associated with amorphous mannitol can lead to protein destabilization[28].

Therefore, mannitol is desired as a highly crystalline anhydrate in the final lyophile. It is necessary to understand the effect of cosolutes and processing to modulate mannitol's crystallization behavior. Cosolutes impact the physical form of mannitol in the frozen solution. Mannitol in combination with a sugar (sucrose or trehalose) has been used in freeze-dried protein formulations wherein the sugar can inhibit mannitol crystallization. Proteins facilitated the crystallization of anhydrous mannitol upon annealing at -8 °C [42].

However, these studies characterized crystallization behavior of mannitol in complex formulations. The phase behavior of mannitol could be affected by multiple solutes. Systematically understanding the impact of each non-crystallizing solute on mannitol crystallization has not been attempted.

We aim to systematically study the individual effect of several cosolutes as well as processing conditions on mannitol phase behavior in frozen solutions. Additionally, we

plan to investigate the impact of cosolutes (sugar and protein) and annealing temperature on mannitol crystallization kinetics.

MATERIALS AND METHODS

Materials

D-Mannitol ($C_6H_{14}O_6$), sucrose ($C_{12}H_{22}O_{11}$), trehalose dihydrate ($C_{12}H_{22}O_{11} \bullet 2H_2O$), lyophilized lysozyme (Mw = 14 kDa) from chicken egg white, bovine serum albumin (BSA, Mw = 66.5 kDa), and immunoglobulin G from human serum (IgG, Mw = 150 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). The sample solutions were prepared by dissolving mannitol (6% w/v) and one of the above cosolutes (2% or 6% w/v) in Milli-Q® water. Thus, the mannitol to cosolute weight ratios were 3:1 or 1:1.

Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (Q2000, TA Instruments, New Castle, DE) equipped with a cooling accessory was used. The instrument was calibrated with indium. Each aqueous sample (ca. 20 mg) was sealed hermetically in an aluminum pan cooled to −50 °C at 1 °C/min, after holding isothermally for 15 min, and heated to +20 °C at 5 °C/min. The experiment was conducted under nitrogen flow at 50 mL/min. When there was an annealing step, the frozen solution after the isotherm hold at -50 °C for 15 min, was heated to -10 or to -25 °C and held for the desired time period (either 0.5 or 2 h). The annealed solution was cooled back to -50 °C and finally heated to +20 C at 5 °C/min. The glass transition temperature (T_g) is reported at the transition midpoint. The crystallization temperature (T_c) was the onset of the transition.

X-ray Diffractometry (XRD)

An X-ray diffractometer (SmartLab, Rigaku Americas, Texas) with a low temperature chamber (TTK 450, Anton Paar, Virginia) was used. Cu Kα radiation (45 kV × 140 mA) was used with a scanning rate of 5° 2θ/min and a step size of 0.1° 2θ. The angular range was 5° to 30° 2θ. The aqueous solutions (50 μL) were placed onto a copper sample holder. The samples were cooled from room temperature to −50 °C at 1 °C/min and then heated to either -25 or to -10 °C at 5 °C/min. After isothermally annealing for 2 hours, the samples were heated to room temperature. During freeze as well as thawing, XRD patterns were obtained at predetermined temperatures. Upon annealing, the samples were scanned every 10 min. Selected characteristic peaks (MHH at 17.9° 2θ; δ-mannitol at 20.4° 2θ) were used and the area under the integrated peak intensities was determined using MDI Jade 7.8.2.

RESULTS AND DISCUSSION

Phase Behavior of Mannitol Aqueous Solution during Freeze-Thawing

Figure 2.1a is the DSC cooling curve of aqueous mannitol (6% w/v) solution. The exotherms at ~ -11 °C °C and at ~ -31 °C are attributed to ice and mannitol crystallization, respectively. Confirmatory evidence was obtained from XRD (discussed later). The DSC results are summarized in **Table 2.1**. When the frozen solution was heated (**Figure 2.1, panel b**), glass transitions were observed at -29 and -26 °C, followed by an exotherm at -24 °C. Cavatur et al. reported two glass transitions in frozen mannitol solutions, and our results are in broad agreement with previous studies[43,44]. When the solution was heated above the higher glass transition temperature (-26 °C), there was rapid solute crystallization. It is instructive to recognize that the unfrozen water associated with the solute will crystallize along with the solute. Based on the above observations, mannitol crystallization was incomplete at the current cooling rate (1 °C/min).

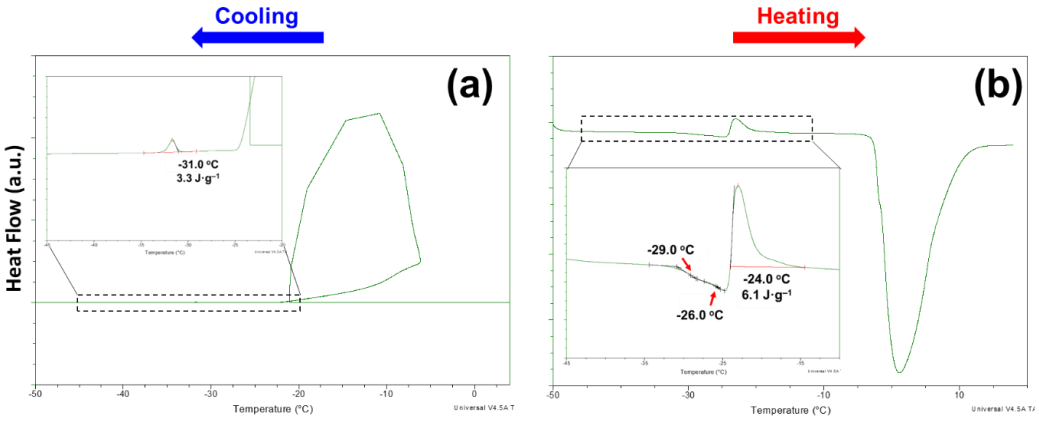

Figure 2.1 (a) DSC cooling curve of aqueous mannitol (6.0% w/v) solution from RT to -50 °C. (b) DSC heating curve of frozen mannitol solution from -50 to 20 °C. The glass transition events are highlighted with red arrows. The crystallization temperature and enthalpy (ΔH) are provided. The cooling and heating rates were 1 and 5 °C/min, respectively.

Effect of Cosolute on Mannitol Crystallization

The cosolutes used were two widely used lyoprotectants, sucrose and trehalose, and three model proteins, lysozyme, bovine serum albumin (BSA) and immunoglobulin G (IgG). The effect of each cosolute on the mannitol crystallization behavior was investigated. For the sake of simplicity, they are each abbreviated with a single capital letter: for example, "S" for sucrose, "L" for lysozyme, etc. The mannitol-cosolute ratio is also provided in the sample name. For instance, mannitol-sucrose solution at a weight ratio of 3:1 is labeled as "M:S_3:1". We will first look at the thermal events during cooling and then the behavior of this frozen solution when it was heated.

Cooling

The DSC cooling curves of mannitol-cosolute solutions are shown in **Figure 2.2**. At 2% w/v cosolute concentration (i.e., mannitol to sugar weight ratio of 3:1), sucrose (S) and trehalose (T) completely inhibited mannitol crystallization during cooling, revealed by the absence of solute crystallization exotherm (**Figure 2.2, panel a**). On the other hand, the impact of proteins on mannitol crystallization was much less pronounced, if any. While the mannitol crystallization temperature (~ -32 °C) was unchanged, there appeared to be a small decrease in the crystallization enthalpy (2.2 ~ 2.5 J/g) when compared with that of the solution containing mannitol alone (**Table 2.1, Samples 1, 6, 8 and 10**). However, mannitol crystallization was completely inhibited as the protein concentration was increased to 6% (i.e., mannitol to sugar weight ratio of 1:1) (**Table 2.1, Samples 7, 9 and 11**). The results suggest that sucrose and trehalose exhibited a stronger inhibitory effect on mannitol crystallization than the proteins.

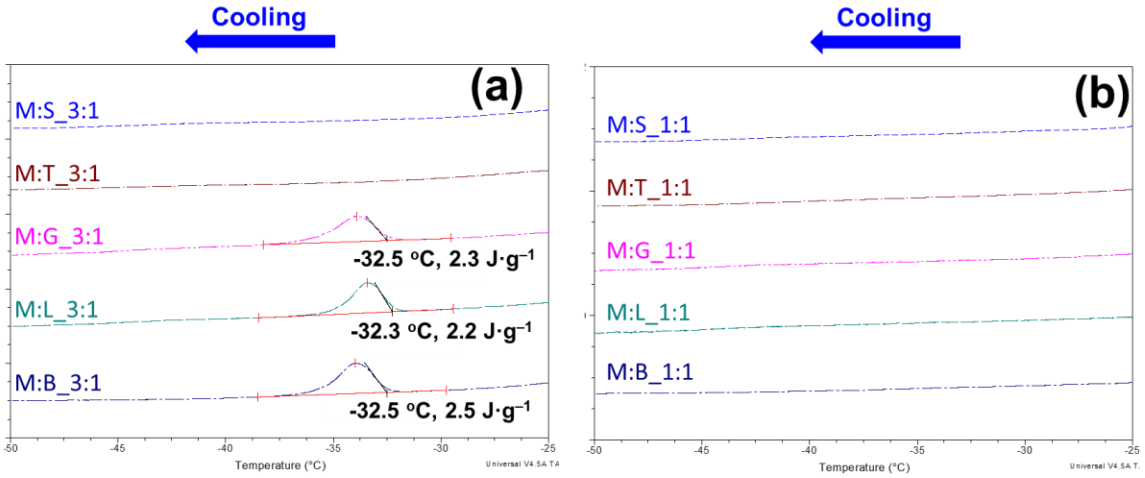

Figure 2.2 DSC cooling curves of mannitol-cosolute solutions from RT to -50 °C. The results of solutions containing mannitol/cosolute at ratios of 3:1 and 1:1 are shown in panel (a) and (b), respectively. The mannitol crystallization temperature and enthalpy (ΔH) are provided. The samples are named with the following abbreviations: mannitol-"M"; sucrose-"S"; trehalose-"T"; lysozyme-"L"; bovine serum albumin-"B"; Immunoglobulin G-"G".

Table 2.1 Mannitol crystallization upon freezing followed by thawing. The solutions were cooled from RT to -50 °C, held for 15 min, and then heated to 20 °C.

No.	Mannitol (% w/v)	Cosolutes (% w/v)	Cooling	Heating
			T_{cry} (°C) (ΔH: J·g^{-1})	T_{cry} (°C) (ΔH: J·g^{-1})
1	6	0	-31.0 (3.3)	-24.0 (6.1)
		Sucrose		
2	6	2	ND*	-23.9 (11.4)
3	6	6	ND	ND
		Trehalose		
4	6	2	ND	-25.2 (10.9)
5	6	6	ND	ND
		IgG		
6	6	2	-32.5 (2.3)	-22.7 (7.4)
7	6	6	ND	-19.2 (8.1)
		Lysozyme		
8	6	2	-32.3 (2.2)	-23.3 (5.3)
9	6	6	ND	-19.6 (11.5)
		BSA		
10	6	2	-32.5 (2.5)	-20.8 (9.7)
11	6	6	ND	-21.3 (9.5)

The cooling and heating rates were 1 and 5 °C/min respectively. The crystallization enthalpy (ΔH) is in parenthesis beside the relevant temperature. *ND – Not detected. T_{cry} – temperature of the solute crystallization exotherm

Heating

Figure 2.3 shows the DSC heating curves of mannitol-cosolute solutions. We had earlier observed that a frozen solution of amorphous mannitol was characterized by two glass transition events (at -29 and -26 °C) (**Figure 2.1, panel b**), indicating the formation of a heterogeneous freeze concentrate. With the addition of a cosolute, up to three glass transition temperatures can be observed. For example, in the mannitol-sucrose solution with a solute weight ratio of 3:1 (referred to as "MS_3:1", **Figure 2.3, panel a**), glass transitions were observed at -42, -33, and -29 °C. Similar results were reported with the

mannitol-sucrose solution at a weight ratio of 4:1 by Hawe et al[44]. In the presence of trehalose, two glass transitions were detected earlier[45]. The lack of the glass transition temperature of freeze concentrate, close to the onset of mannitol crystallization, may be due to the overlap with the subsequent crystallization[44]. These glass transitions can be ascribed to the formation of freeze concentrates with different sugar-mannitol-water compositions. Based on previous studies, the transition at -42 °C was assigned to a "water-rich" freeze concentrate[46]. Determining the solute compositions in such ternary frozen systems is challenging and has not yet been accomplished. Interestingly, with the addition of 6% sugar (**Figure 2.3, panel b**), a homogenous freeze concentrate was observed, with a glass transition temperature at ~ -40 °C. In the presence of protein (2% or 6%), the freeze concentrated solutions were heterogeneous, evident from the two or three glass transition temperatures in the DSC heating curves (**Figure 2.3, panels c and d**).

At low sugar content (2%, w/v), mannitol crystallized at ~ -24 °C during heating (**Figure 2.3, panel a; Table 2.1, Samples 2 and 4**). When the sugar concentration was increased to 6% (mannitol/sugar = 1:1), mannitol crystallization was completely suppressed (**Figure 2.3, panel b; Table 2.1, Samples 3 and 5**). Based on the crystallization temperature and the enthalpy, the magnitude of the inhibitory effect of sucrose and trehalose on solute crystallization was similar (**Figure 2.3, panel a; Table 2.1, Samples 2 and 4**). Proteins were less effective in suppressing mannitol crystallization. Even at a high protein concentration (6%), pronounced solute crystallization was observed at ~ -20 °C (**Figure 2.3 panel d; Table 2.1, Samples 7, 9 and 11**). This again suggests that proteins had a weaker inhibitory effect than sugars on mannitol crystallization. The three model proteins

used in the current work did not reveal any pronounced differences in their inhibitory effect on mannitol crystallization, evident from the similar solute crystallization temperatures (~ -20 °C) and enthalpy (8.1–11.5 J/g) in the DSC heating curves (**Figure 2.3, panel d; Table 2.1, Samples 7, 9 and 11**).

Mannitol can crystallize during cooling as well as heating. We can assume that the total enthalpy of crystallization is a measure of the crystallization inhibition potential of cosolute. For example, the solution containing 6% lysozyme showed similar crystallization exotherm (11.5 J/g) to either sucrose or trehalose at 2%. This weaker inhibitory effect of protein on mannitol crystallization can be linked to the amorphous-amorphous phase separation in a freeze concentrate. With ice crystallization during freezing, the concentration of the solutes rises and can cause phase separation. It is common when the systems are in the presence of macromolecules[47]. Besides, based on solid-state NMR, polymers have a high tendency to phase separate[48]. Our group previously also found that alditols have a stronger inhibitory effect on mannitol crystallization than polymers[49]. Proteins, much like polymers, have the ability to foster phase separation from small molecules[47]. In other words, polymers (proteins and synthetic polymers) appear to have a higher phase separation propensity than small molecules (sugars and sugar alcohols) from mannitol. The high phase separation tendency of proteins results in their weaker inhibitory effect on mannitol crystallization.

Based on the DSC results, sugars exhibited a stronger inhibitory effect than proteins on mannitol crystallization during both freezing and thawing. A large fraction of the

compositions were heterogeneous, evident from the multiple glass transitions (**Figure 2.3**). However, at a high sugar concentration (6%), mannitol did not crystallize during heating and a homogenous freeze concentrate was obtained (**Figure 2.3**; **panel b**). Building upon the insights provided by DSC, the next steps were to: (i) characterize the physical form(s) of mannitol crystallizing from solution using XRD, (ii) anneal the frozen solutions for 2 hours, either at -25 or -10 °C and determine the impact of annealing on the physical form of mannitol crystallizing from solution, and (iii) monitor the mannitol crystallization kinetics.

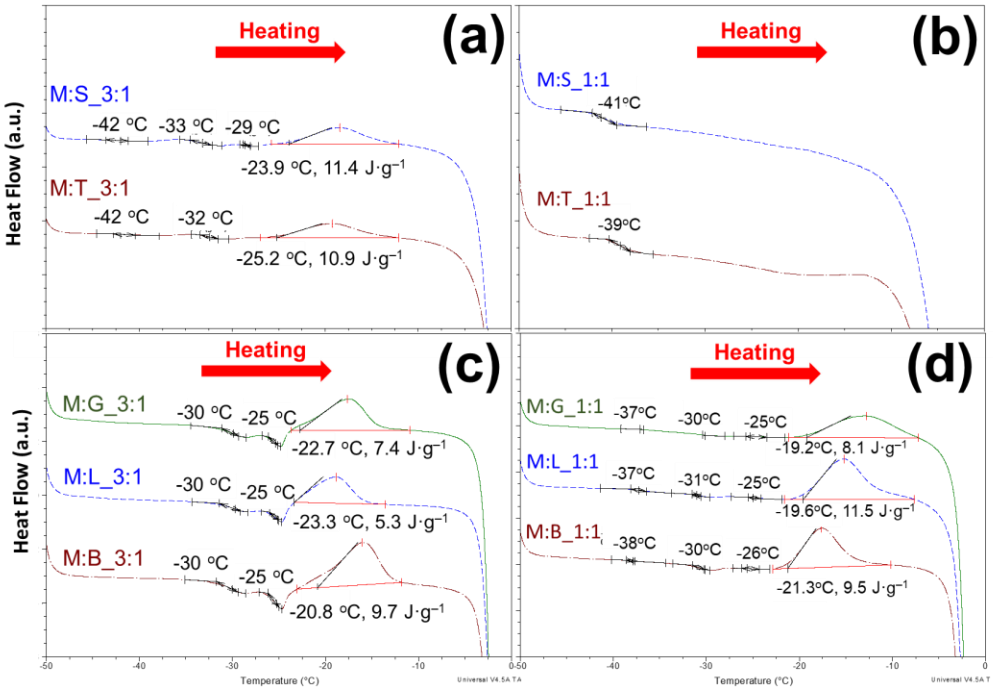

Figure 2.3 DSC heating curves of mannitol-cosolute solutions. The solutions were heated from -50 to 20 °C at 1 °C/min. The results of solutions containing mannitol and sugar at the ratios of 3:1 and 1:1 are shown in panels (a) and (b), respectively. Panels (c) and (d) present the results of mannitol and protein at the ratios of 3:1 and 1:1, respectively. Mannitol crystallization temperature and enthalpy (ΔH), as well as the glass transition temperatures are provided. The samples are named with the following abbreviations: mannitol-"M"; sucrose-"S"; trehalose-"T"; lysozyme-"L"; bovine serum albumin-"B"; Immunoglobulin G-"G".

Physical Forms of Mannitol in Frozen Solutions

The XRD patterns of mannitol solutions upon freeze-thawing are shown in **Figure 2.4**, **S1,** and **S2**. After cooling to -50 °C, the frozen solutions were heated to -25 or -10 °C and annealed for 2 h. This annealing step was conducted to study the impact of annealing temperature on the physical form of mannitol crystallizing from solution.

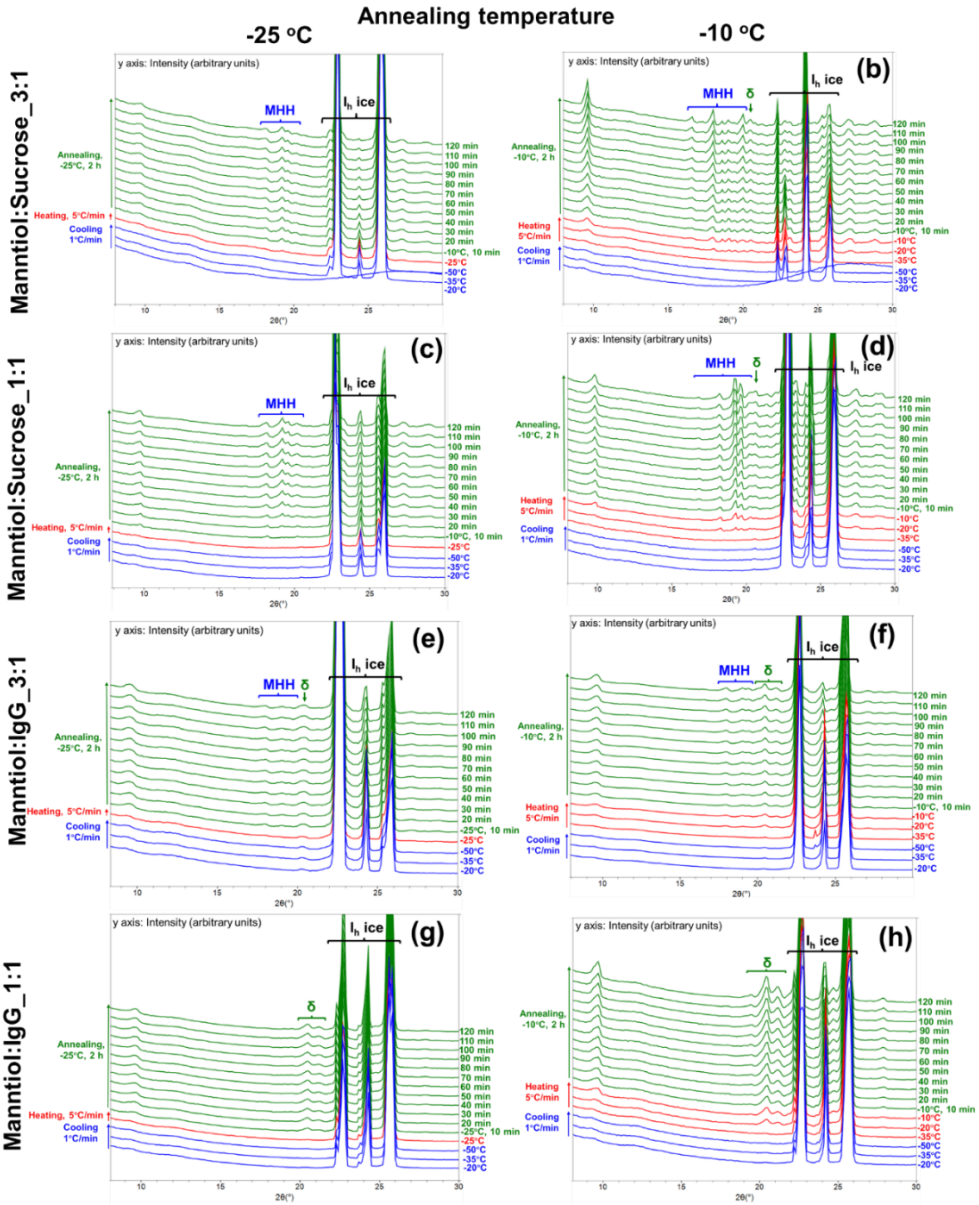

Figure 2.4 XRD patterns of mannitol solutions containing either sucrose (panels a – d) or IgG (panels e – h) [mannitol + sucrose] and [mannitol + IgG]. The solutions were cooled from room temperature to −50 °C at 1 °C/min, held for 15 min, and then heated to -25 or -10 °C at 5 °C/min. After isothermally annealing for 2 hours, the samples were heated to room temperature. For the sake of clarity, only selected XRD patterns are provided, with an emphasis on the phase behavior during annealing. The compositions are provided in the left margin.

Upon cooling, ice crystallized in the hexagonal form (I_h) (RT to -50 °C, 1 atm), characterized by the diffraction peaks at ~ 22°, 24° and 26° 2θ (*d*-spacings of 3.9, 3.7 and 3.4 Å). However, "splitting" of ice diffraction peak was frequently observed (**Figure 2.4, panels a, b, g and h; Figure S1, panels b, c, e and f; Figure S2, panels c and h**). This is possibly due to the pronounced disorder in ice crystal lattice, resulting from local stress at ice/container and ice/ice interfaces[50]. Poor ice crystallization may also become evident from the absence of one of the characteristic peaks of the hexagonal ice[51]. For example, upon cooling mannitol-BSA (1:1) solution, the diffraction peak of ice at ~ 24° 2θ (*d*-spacing, 3.7 Å) was not detected **(Figure S2, panel g)**. However, the analysis of mannitol phase behavior would not be affected by the above observations, as the major characteristic peaks of mannitol (at < 22° 2θ) did not overlap with those of ice.

In the absence of cosolutes, mannitol crystallized as MHH upon cooling and did not transform to other physical forms during heating and annealing (**Figure S1, panels a and b**). Sugars (2% w/v) inhibited mannitol crystallization during cooling (**Figure 2.4, panels a and b; Figure S1, panels c and d**), which is in excellent agreement with the DSC results (**Figure 2.2, panel a**). As expected, there was no evidence of crystallization of either sucrose or trehalose upon cooling. During heating and annealing, MHH crystallization was detected (**Figure 2.4, panels a and b; Figure S1, panels c and d; red and green patterns**). In addition to MHH, δ-mannitol crystallized upon annealing at -10 °C, but not at -25 °C (**Figure 2.4, panels a and b; Figure S1, c and d**). Thus, the crystallization of δ-mannitol was favored at a higher annealing temperature. The results were in excellent agreement

with earlier observation, wherein crystallization of anhydrous mannitol was favored when annealed at -10 °C[30]. The effect of annealing temperature on mannitol crystallization was not evident at the higher sugar concentration (6% w/v). For example, with the addition of 6% trehalose, the diffraction peak of δ-mannitol was not detected, possibly due to the substantial inhibitory effect of sugar on solute crystallization (**Figure S1, panel f**).

At a low protein content (2%), crystallization of both MHH and δ-mannitol were observed, regardless of the annealing temperature (**Figure 2.4, panels e and f; Figure S2, panels a, b, e and f**). At 6% content of either IgG or BSA, irrespective of the annealing temperature, mannitol predominantly crystallized in the δ-form (**Figure 2.4, panels g and h; Figure S2, panels g and h**). The presence of protein and a high annealing temperature (-10 °C) appeared to have a synergistic effect in promoting the crystallization of δ-mannitol. For example, with the addition of 6% lysozyme, both MHH and δ-mannitol crystallized upon annealing at -25 °C **(Figure S2, panel c)**. An increase in annealing temperature to -10 °C selectively facilitated crystallization of the δ-form (**Figure S2, panel d**).

As pointed out earlier, mannitol crystallizes as MHH at temperatures ≤ −20 °C, while anhydrous mannitol is formed at temperatures ≥ −10 °C. The effect of sucrose and trehalose on mannitol phase behavior was similar. The addition of sugar, irrespective of its concentration (2 or 6%), did not have an impact on the mannitol physical form when annealed at -25 °C – only the crystallization of MHH was observed. However, when the annealing temperature was increased to -10 °C, there was crystallization of both MHH and the δ- form.

At a low sugar concentration (2%), more than one glass transition was observed, reflecting heterogeneity in the freeze-concentrate (**Figure 2.3**). However, when the sugar concentration was increased (6%), only one glass transition was revealed, but at a low temperature (~ -40 °C). This suggests that the system was not "maximally" frozen. One indirect evidence was the absence of mannitol crystallization when these frozen compositions were heated (**Figure 2.3, panel b**). When annealed, the microenvironment in the freeze concentrate may have an adequate amount of unfrozen water to facilitate MHH crystallization.

The three proteins had a similar effect on the mannitol phase behavior. At low protein concentration (2%), irrespective of the annealing temperature, a mixture of MHH and δ-mannitol was observed. Thus, the freeze concentrate microenvironment was conducive to anhydrous mannitol crystallization (as a mixture with MHH) even at a low annealing temperature of -25 °C. When the protein concentration was increased to 6%, and the solution was annealed at -25 °C, only the δ- form was observed in the presence of IgG and BSA. Thus, at the higher protein concentration, there was an increased propensity for anhydrous mannitol crystallization. When the annealing temperature was increased to -10 °C, as expected, only the δ- form crystallized. The frozen mannitol-protein systems, particularly at high protein concentration, appeared to be quite heterogeneous with multiple glass transition temperatures (**Figure 2.3, panel d**). When the solution was heated above the highest glass transition temperature (~ -25 °C), there was pronounced mannitol crystallization. Thus, in the presence of protein, it appears that substantial mannitol crystallization can be accomplished, either by annealing or by heating above the highest

glass transition temperature. Moreover, because of the small difference in T_g' values obtained from the three proteins, it is reasonable to presume similar mannitol crystallization behavior irrespective of the freeze concentrate compositions. As anhydrous mannitol forms were obtained, we speculate that in the presence of proteins, anhydrous mannitol crystallization is favored by the "drier" microenvironment. The water content of the maximally freeze concentrated solution could be indirect evidence. Compared to sugars, the unfrozen water content of monoclonal IgG_1 antibody freeze-concentrate is less than that of sucrose or trehalose[52].

Our next interest was to understand the kinetics of mannitol crystallization during the different stages of processing – cooling, heating, and annealing.

Crystallization Kinetics of Mannitol in Frozen Solutions

Differential Scanning Calorimetry

The effect of annealing on mannitol crystallization was first studied by DSC. After annealing the frozen solutions, they were cooled back to -50 °C, and the second heating curves are shown in **Figure 2.5 and S3**. In the absence of cosolutes, mannitol substantially crystallized after 0.5 h annealing (either -25 or -10 °C), revealed by the disappearance of glass transitions and solute crystallization in the second DSC heating curve (**Figure S4**). With the addition of sugar (2%), irrespective of the annealing temperature, mannitol crystallization was facilitated by 0.5 h of annealing. In the second DSC heating curves, only a single glass transition was observed over the temperature range of -35 to -30 °C (**Figure 2.5, panel a and b**). The temperatures are close to the T_g' of the sugar freeze concentrate (-31 and -34 ºC for trehalose[53] and sucrose[54], respectively), indicating

substantial mannitol crystallization upon annealing. Increasing the annealing time to 2 h did not further increase the glass transition temperature (**Figure 2.5, panel a and b**). The results indicate the rapid crystallization of mannitol upon annealing in solutions containing 2% sugar. The effect of annealing on mannitol crystallization was not as pronounced at a higher sugar concentration (6%), wherein 0.5 h of annealing at -25 °C did not bring about a substantial increase in the glass transition temperature (~ -40 °C) compared to that of the unannealed solution (**Figure 2.5, panel c**). However, the inhibitory effect of sugars on mannitol crystallization could be overcome by annealing at a higher temperature (-10 °C) or over a longer time period (2 h). In mannitol-protein solutions, no discernible thermal events were observed in the second DSC heating curves (**Figure S3**). Therefore, we could not draw any conclusions about mannitol crystallization based on the DSC results. The systems were further evaluated by XRD.

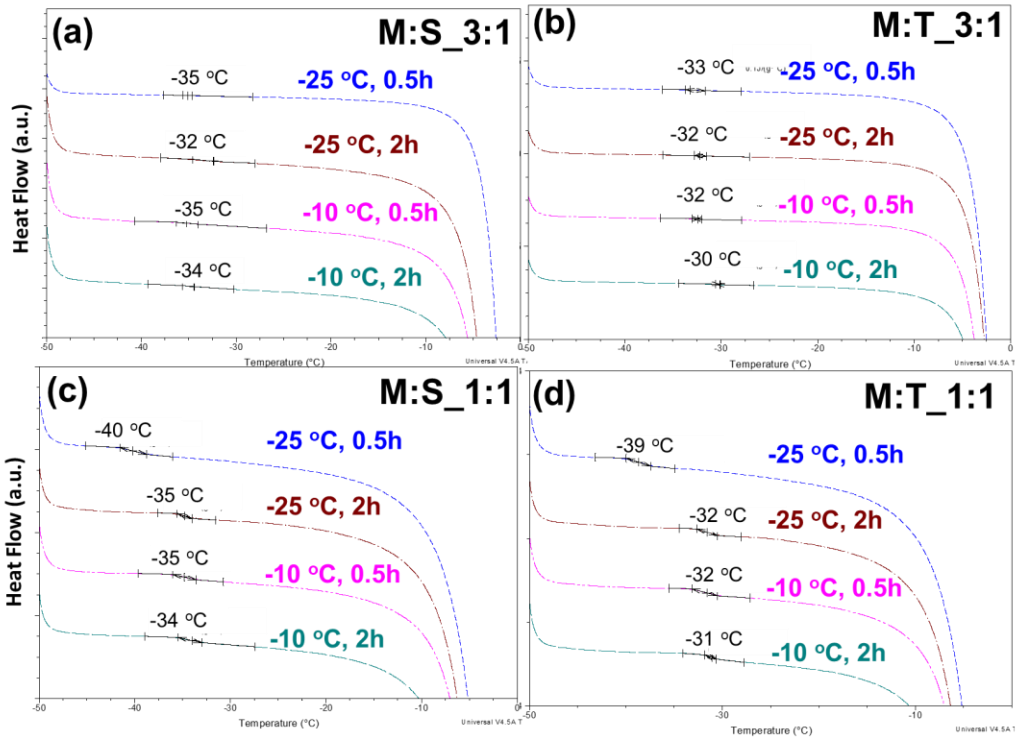

Figure 2.5 DSC heating curves of mannitol-cosolute solutions after annealing. The solutions were first cooled to -50 °C, held for 15 min, and then heated to the annealing temperature. After annealing, the solutions were cooled back to -50 °C followed by heating to 20 °C. The second DSC heating curves are provided. The DSC of solutions containing mannitol and sucrose at the ratios of 3:1 and 1:1 are shown in panel (a) and (c), respectively. Panel (b) and (d) present the results of mannitol and trehalose at the ratios of 3:1 and 1:1, respectively. The abbreviations used are: mannitol- "M"; sucrose-"S"; trehalose-"T".

X-ray Diffractometry

In order to quantify mannitol crystallization in the presence of cosolutes in the frozen solutions, the integrated intensity of the characteristic peak of MHH at 17.9° 2θ and of δ-mannitol at 20.4° 2θ were plotted as a function of time (**Figure 2.6, Figure 2.7 and Figure** 2.8).

We had earlier observed the formation of MHH during freezing of the solutions containing no cosolute. Annealing at -25 °C did not cause additional mannitol crystallization (**Figure**

2.6, panel a). However, when the annealing temperature was higher (-10 °C), mannitol progressively crystallized (**Figure 2.6, panel b**).

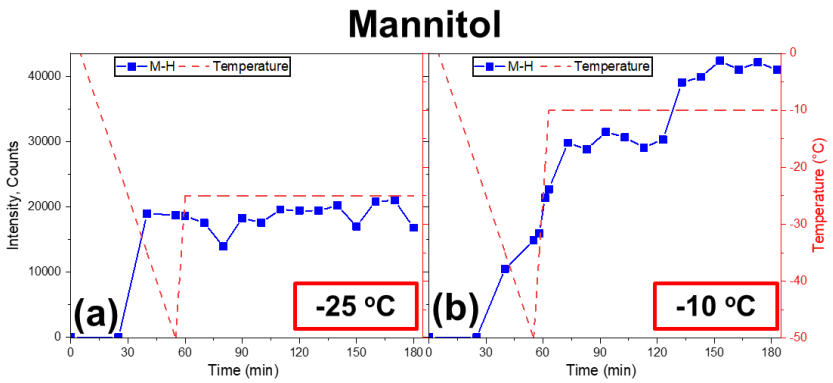

Figure 2.6 The intensity of 17.9° 2θ peak of MHH (blue symbols; left y-axis) during cooling, heating, and annealing of solutions containing mannitol alone (6%). The right y-axis (red line) is the processing temperature. Panel (a) and (b) reveal the results of samples annealed at -25 and at -10 °C, respectively.

Consistent with the DSC results (**Table 2.1**), the presence of sugar (2%; sucrose or trehalose) inhibited mannitol crystallization upon cooling (**Figure 2.7**). In the presence of sucrose, the solute rapidly crystallized during the initial stage of annealing at -25 °C, but slowed down after ~30 min. Mannitol appeared to continue crystallizing after 2 h when the annealing temperature was increased to -10 °C. In addition, crystallization of both MHH and δ-mannitol were observed. Based on the peak intensities, we can conclude that a larger fraction of the mannitol crystallized at the higher annealing temperature. The effect of trehalose (2%; **Figure 2.7, panels e and f**) was substantially similar to that of sucrose. With both the sugars, δ-mannitol crystallization increased at the high annealing temperature (-10 °C).

However, when the sugar concentration was increased to 6%, annealing was not as effective in promoting mannitol crystallization (**Figure 2.7, panels c, d, g and h**). The crystallization rate as well as the amount of crystalline mannitol (based on crystalline mannitol peak intensities) were substantially decreased. The higher sugar concentration also seems to favor crystallization of MHH – δ-mannitol was observed only in the presence of sucrose and when annealed at -10 °C.

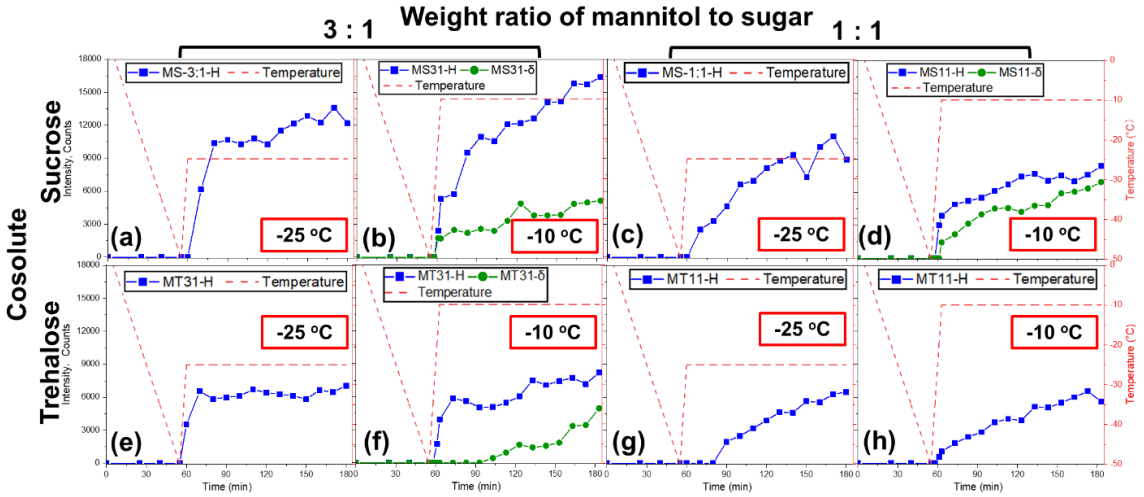

Figure 2.7 The intensity of 17.9° 2θ peak of MHH (blue symbols; left y-axis) and 20.4° 2θ peak of δ-mannitol (green symbols; left y-axis) during cooling, heating, and annealing of solutions containing mannitol (6%) and sugar (2% or 6%). The right y-axis (red line) is the processing temperature. Panels (a) to (d) and (e) to (h) reveals the results of mannitol solutions with the addition of sucrose and trehalose, respectively.

As we mentioned in the previous section (see "*Effect of Cosolute on Mannitol Crystallization*"), proteins were less effective than sugars in inhibiting mannitol crystallization in frozen solutions. Unlike solutions containing sugars, δ-mannitol was extensively detected in the presence of proteins (**Figure 2.8**). At a low protein concentration (2%), both MHH and δ-mannitol crystallized in the frozen solutions (**Figure**

2.8, panels a, b, e, f, i and j). Annealing at -10 °C selectively facilitated the rapid crystallization of the δ-form. On the other hand, MHH crystallization was not evidently affected by the annealing temperature (**Figure 2.8, panels a, b, e, f, i, and j, blue symbol**). Even at a high protein concentration (6%), there was substantial mannitol crystallization upon annealing at -10 °C (**Figure 2.8, panels c, d, g, h, k and l, green symbol**). While MHH was occasionally detected upon annealing at -25 °C (**Figure 2.8, panel g**), an increase in annealing temperature to -10 °C facilitated crystallization of the δ-form (**Figure 2.8, panel d, h, and l**). Overall, the three model proteins exhibited an essentially similar effect on the physical form of crystallizing mannitol as well as the crystallization kinetics in frozen solutions.

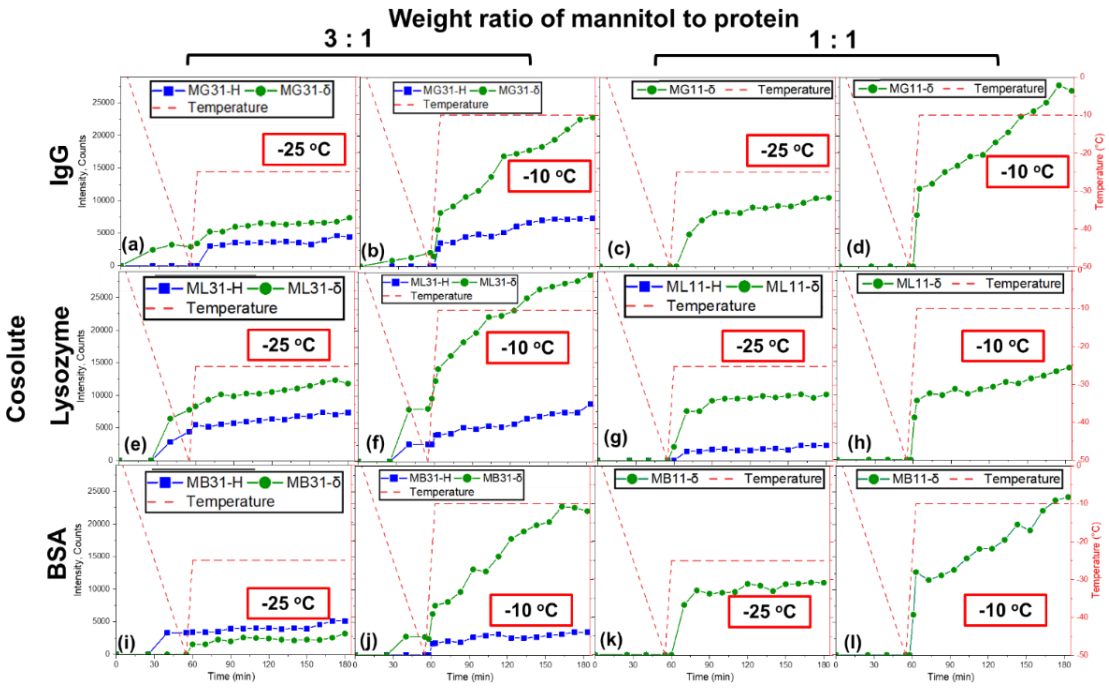

Figure 2.8 The intensity of 17.9° 2θ peak of MHH (blue symbols; left y-axis) and 20.4° 2θ peak of δ-mannitol (green symbols; left y-axis) during cooling, heating, and annealing of solutions containing mannitol (6%) and a protein (2% or 6%). The right y-axis (red line) is the processing temperature. Panels (a) to (d), (e) to (h), and (i) to (l) reveal the results of mannitol solutions with the addition of IgG, lysozyme, and BSA, respectively.

SIGNIFICANCE

Mannitol is widely used as a bulking agent in lyophilized protein formulations. In the final lyophiles, it is desired in a highly crystalline state. However, both sugars and proteins are known to inhibit mannitol crystallization in frozen solutions. Our study shows that all the three proteins exhibited a weak inhibitory effect. Substantial mannitol crystallization occurred when the frozen solutions were heated (even in the absence of annealing). However, if a substantial amount of sugar (e.g. at 6%) is added, highly crystalline mannitol was obtained only by annealing at -10 °C.

The formation of MHH and its subsequent dehydration during storage can induce product destabilization. Our results reveal that at a high protein concentration (e.g., mannitol to protein ratio of 1:1), crystallization of MHH can be prevented by annealing at a high temperature (-10 °C). On the other hand, sucrose and trehalose, while substantially inhibiting mannitol crystallization, barely affected MHH crystallization. The selectivity of anhydrous mannitol crystallization in the presence of protein was occasionally observed in previous systems. However, in this study, we provide direct evidence that proteins can modulate the mannitol crystallization behavior. Thus, when mannitol is added as a bulking agent, a protein formulation should be carefully designed based on the (i) noncrystallizing solute content, including the relative amounts of protein or sugar to the bulking agent, and (ii) annealing conditions, to facilitate mannitol crystallization in an anhydrous form.

CONCLUSION

As a bulking agent, mannitol has a high crystallization propensity in frozen solutions. Compared to proteins, sugars exhibited a stronger inhibitory effect on mannitol crystallization. In the absence of a cosolute, mannitol crystallized as MHH. Proteins selectively facilitated δ-mannitol crystallization, while sugars did not have such an effect. A high annealing temperature (-10 °C), in addition to accelerating mannitol crystallization, also selectively enabled crystallization of the δ-form. Overall, cosolute and annealing temperature played a synergistic effect on mannitol crystallization in frozen solutions.

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APPENDIX

Figure S1 to S4 included in this section

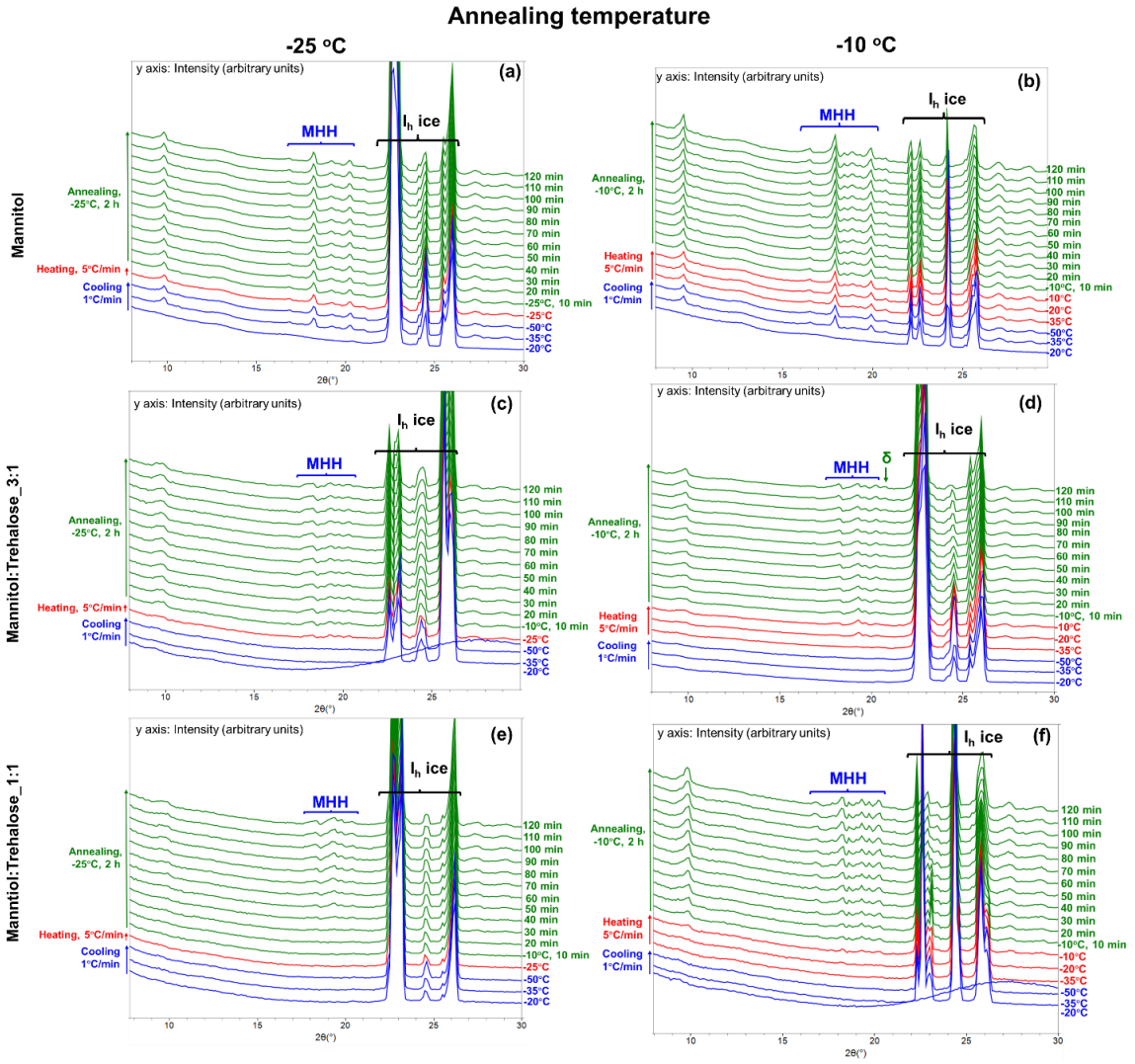

Figure S1. XRD patterns of solutions containing mannitol alone and [mannitol + trehalose] upon annealing at -25 or -10 °C. Panels (a) and (b) show the results of solutions with mannitol alone during cooling, heating, and annealing for 2 h at -25 or -10 °C, respectively. The results of [mannitol + trehalose] solution (weight ratio = 3:1) are shown in panel (c) to (d). Panels (e) and (f) reveal the results of [mannitol + trehalose] solution (weight ratio 1:1). The solutions were cooled from room temperature to −50 °C at 1 °C/min, held for 15 min, and heated to -25 °C or -10 °C at 5 °C/min and isothermal annealed for 2 h.

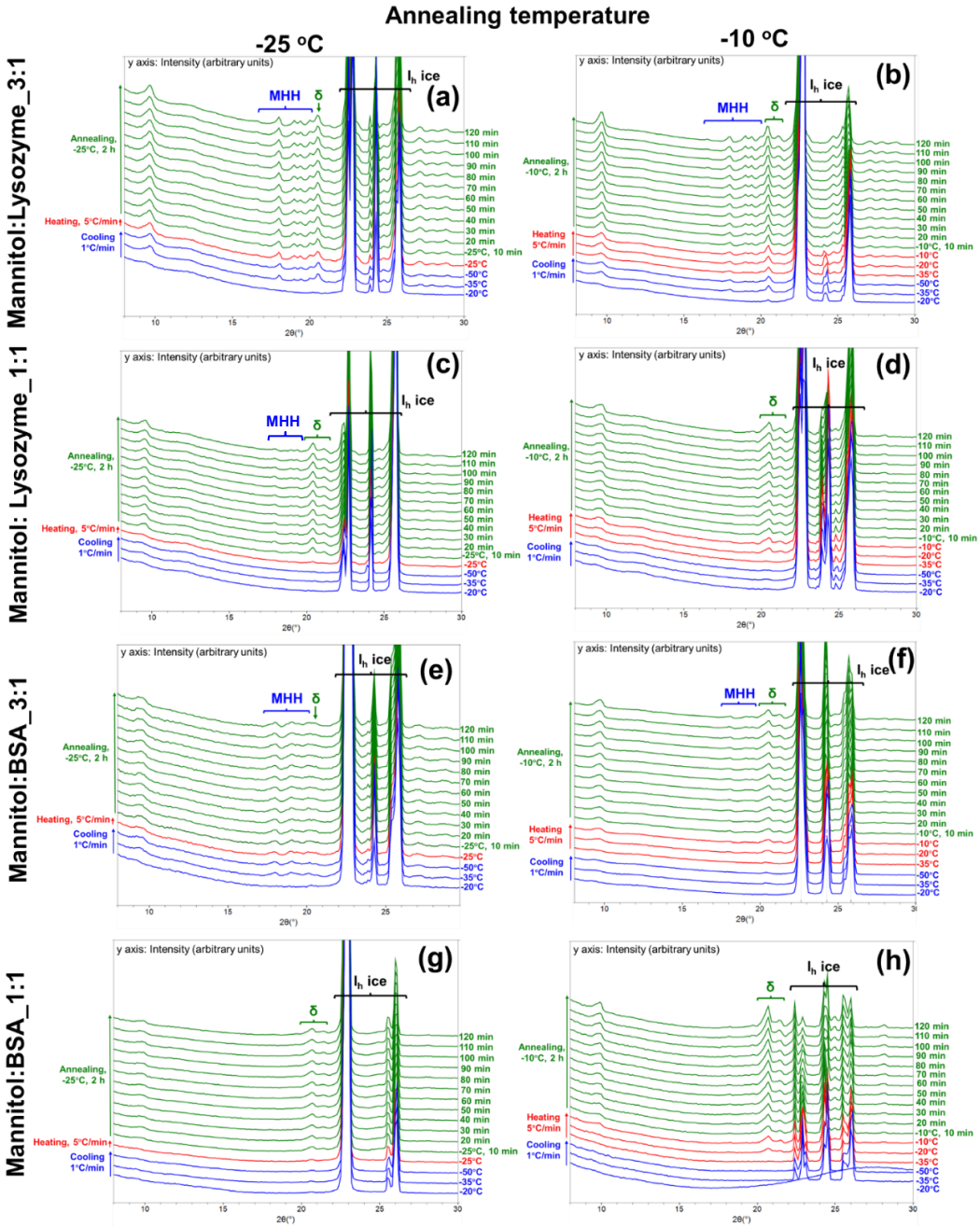

Figure S2. XRD patterns of mannitol-protein (lysozyme and BSA) solution upon annealing at -25 or -10 °C. Panels (a) and (b) show the results of solutions with the addition of lysozyme (3:1, w/w) during cooling, heating, and annealing for 2 h at -25 or -10°C, respectively. Panel (c) and (d) exhibit the results of solution containing mannitol and lysozyme (1:1, w/w). Panels (e) and (f) present the results of [mannitol + BSA] solutions at a ratio of 3:1. The results of [mannitol + BSA] solutions at a ratio of 1:1 were demonstrated in panels (g) and (h). The solutions were frozen from room temperature to −50 °C at 1 °C/min, held for 15 min, heated to -25 °C or -10 °C at 5 °C/min and isothermal annealed for 2 h.

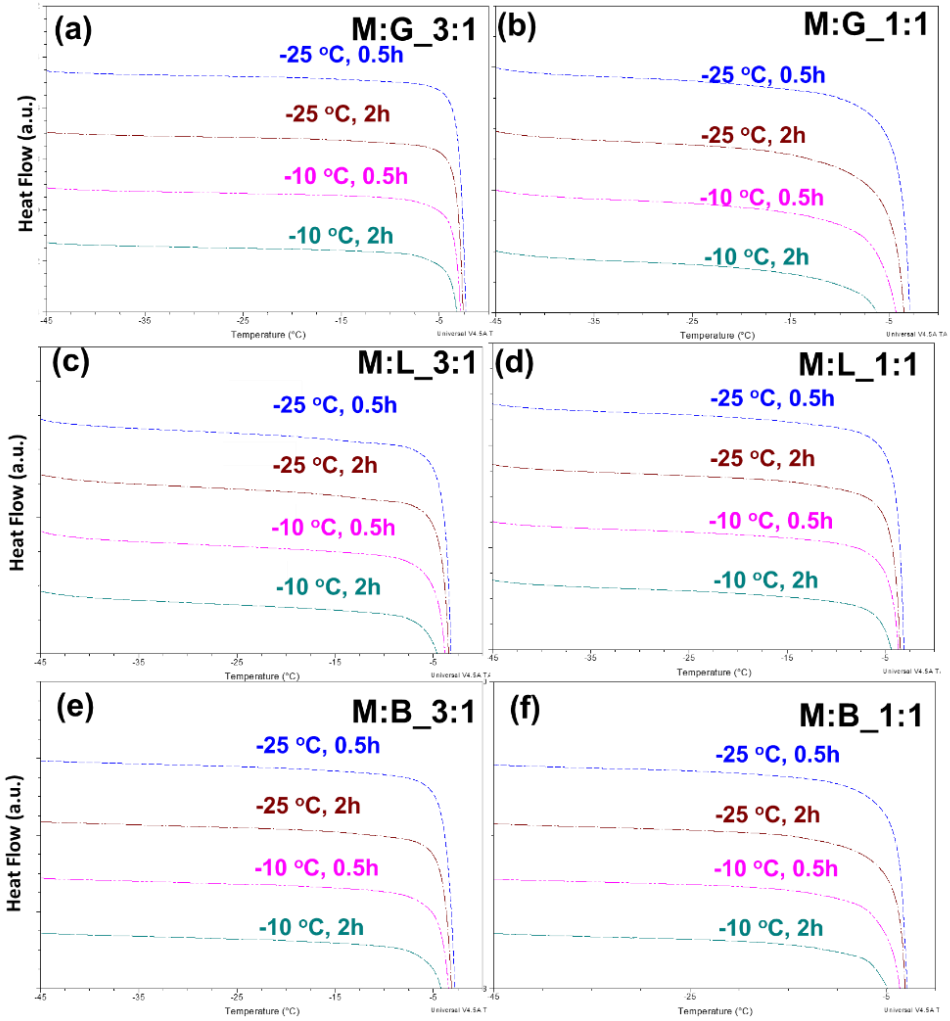

Figure S3. DSC heating curves of mannitol-protein solutions after annealing. The solutions were first cooled to -50 °C, held for 15 min, and then heated to the annealing temperature. After annealing, the solutions were cooled back to -50 °C followed by heating to 20 °C. The second DSC heating curves are provided. The DSC of solutions containing mannitol and IgG at the ratios of 3:1 and 1:1 are shown in panel (a) and panel (b), respectively. Panel (c) and (d) present the results of mannitol and lysozyme at the ratios of 3:1 and 1:1, respectively. Panels (e) and (f) present the results of mannitol and BSA at the ratios of 3:1 and 1:1, respectively. The abbreviations used are: mannitol- "M"; Immunoglobulin G-"G"; Lysozyme-"L"; bovine serum albumin-"B".

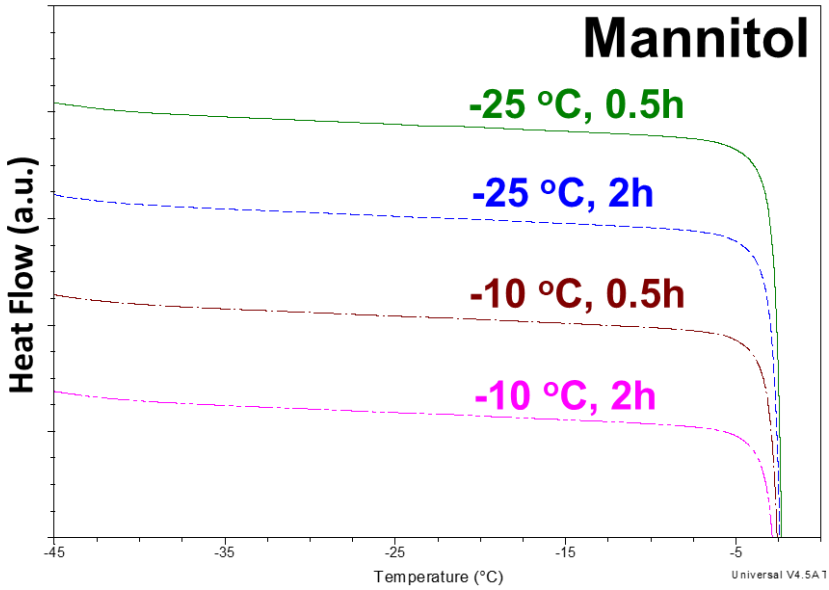

Figure S4. DSC heating curves of solution containing mannitol alone after annealing. The solutions were first cooled to -50 °C, held for 15 min, and then heated to the annealing temperature. After annealing, the solutions were cooled back to -50 °C followed by heating to 20 °C. The second DSC heating curves are provided.