ELSEVIER

International Journal of Pharmaceutics 166 (1998) 211–221

international journal of pharmaceutics

Water sorption behavior of lyophilized protein–sugar systems and implications for solid-state interactions

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Received 22 August 1997; received in revised form 13 January 1998; accepted 14 January 1998

Abstract

This study examines the water sorption behavior of proteins co-lyophilized with sugar/polyol excipients. Gravimetric sorption analysis (GSA) was used to measure water sorption of the lyophilized mixtures and these data allowed for calculation of the water monolayer (M_0). Lyophilized protein–mannitol mixtures behaved as predicted from the data for the pure components. Mannitol was shown to crystallize upon lyophilization. For protein co-lyophilized with sucrose or trehalose, which remain amorphous upon lyophilization, M_0 tended to be lower than that expected based on contributions of the pure protein and sugar. This negative deviation supports the view that amorphous sugars and pharmaceutical proteins interact in the solid state in such a way as to reduce the availability of water-binding sites. At high relative humidities (rh), sucrose and trehalose were susceptible to moisture-induced crystallization. When co-lyophilized protein was present, the GSA data revealed that this crystallization required a higher rh, or did not occur. For the temperature-induced (non-isothermal) sucrose crystallization, which was studied by differential scanning calorimetry, it was found that the temperature of crystallization tended to increase with an increasing amount of protein. The tendency to crystallize rose in the presence of elevated moisture, whether or not protein was present, likely due to the ability of water to plasticize the solid phase.

Keywords: Lyophilization; Gravimetric sorption analysis; Protein; Sugar; Water monolayer

1. Introduction

Sugars or polyols are commonly employed as excipients for lyophilized protein pharmaceuticals (Arakawa et al., 1993). Such agents can provide a 'bulking' effect to maintain the lyophilized cake's

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0378-5173/98/$19.00
PII S0378-5173(98)00050-7

structure, as well as enhance the shelf-life of therapeutic proteins. Various mechanisms have been invoked to describe how sugars improve the stability of lyophilized proteins. For example, Franks (1994) contends that sugars provide a 'glassy' matrix in which protein mobility and reactivity are reduced. Another view is that this matrix dilutes the protein in the solid state, reducing intermolecular contacts and thus inhibiting deleterious intermolecular pathways (i.e. aggregation) (Liu et al., 1991). Carpenter and Crowe (1989) propose that sugars directly interact with proteins via hydrogen-bonding in the solid state. In this manner, sugars aid in preserving protein structure, which is prone to dehydration-induced alteration (Prestrelski et al., 1993; Costantino et al., 1995a; Griebenow and Klibanov, 1995).

Besides the presence of excipients, another important factor influencing the stability of lyophilized pharmaceutical proteins is the water content. A useful concept in this regard is the water monolayer (M_0). Lyophilized proteins generally exhibit increased instability when hydrated above this level. Protein reactivity increases with additional water due to its ability to enhance conformational flexibility, mobilize reactants and participate in degradation pathways (Hageman, 1992; Costantino et al., 1994). Therefore, it is advisable to maintain a dried protein's water content so as not to exceed the monolayer water coverage. Drying to well below M_0 may also lead to protein instability (Hsu et al., 1991).

In addition to the protein's sorption behavior, the excipient's affinity for water will influence protein stability. If the hydration level of the excipient is above its monolayer level, the additional water drawn into the system is likely to be shared with the protein and thus affect stability. For instance, this scenario was put forward to explain the potency of various excipients in stabilizing recombinant human albumin against moisture-induced solid-state aggregation (Costantino et al., 1995b).

Thus, it is important to investigate the sorption behavior of lyophilized protein–sugar systems. To this end, we have employed gravimetric sorption analysis (GSA) to measure the water vapor sorption isotherm for various lyophilized protein–sugar systems. The scope of this study encompassed three model pharmaceutical proteins and three model excipients. From analyses of the sorption data for pure protein/excipient and co-lyophilized systems, insights were gained regarding their interaction in the solid state.

2. Materials and methods

2.1. Materials

Excipient-free recombinant human insulin-like growth factor I (rhIGF-I, $M_w = 7.4$ kDa), recombinant human growth hormone (rhGH, $M_w =$ 22.2 kDa) and recombinant humanized monoclonal antibody (rhuMAb, a glycosylated protein with $M_w \approx 150$ kDa) were produced at Genentech. All other chemicals were of reagent grade and purchased from commercial suppliers.

2.2. Lyophilization

All solutions were freeze dried in a Leybold (Germany) model GT20 unit using various lyophilization cycles (Costantino et al., 1997). In a typical procedure, 1 ml of aqueous solution contain protein and/or excipient at 5 mg ml^{-1} solids concentration was dispensed in 5-cc vials, frozen at -50°C for 4 h, and then dried at -25°C for 50 h (primary stage) and 20°C for 10 h (secondary stage), both at 150 μmHg pressure. All dried samples were stoppered under dry N_2 when the vacuum pressure was increased to 127 mmHg.

2.3. Water vapor sorption isotherm measurements and M_0 calculation

Water sorption behavior was examined using a gravimetric sorption analyzer (GSA; VTI Corporation, MB 300G). Samples (typically 10 mg) were weighed into glass sample holders and loaded rapidly (2–3 min) into the unit. The sample was then equilibrated (i.e. no change in sample weight of less than 5 μg over 5 min) with dry air, and then equilibrated sequentially at increasing relative humidity (rh) up to 85% rh. For rhIGF-I and rhGH-containing samples, a ramp with incre-

ments of 10% rh was used; for rhuMAb the step size was 5% rh. The monolayer water, M_0, was calculated from the water vapor sorption data using a modified form of the Brunauer–Emmett–Teller (BET) equation (Hsu et al., 1991). The BET equation and its various extensions have proven useful in describing the moisture-sorption of pharmaceutical solids, as reviewed elsewhere (Zografi, 1988; Hageman, 1992). It should be noted that solution and free-volume theory provide an alternative model for moisture sorption on polymeric solids (Vrentas and Vrentas, 1991; Hancock and Zografi, 1993), although the application to low-molecular-weight (e.g. sugar excipient) and heterogeneous polymeric (e.g. proteins) species remains relatively unexplored. Herein, we have chosen to employ the modified BET equation due to its straight-forward and proven application in describing pharmaceutical solids, in particular therapeutic protein and sugar systems (Hsu et al., 1991; Costantino et al., 1997).

2.4. Differential scanning calorimetry (DSC)

DSC was performed using a Seiko DSC 120 unit. Sample preparation was conducted in glove box. For typical runs, the presence of moisture was avoided by continuously flowing dry air (approximately 25 l min^{-1}) though the glove box. For runs where equilibration at a controlled humidity was desired, the glove box was filled with air of equilibrated rh, achieved by the presence of saturated salt solutions within the chamber (Greenspan, 1977): saturated LiBr, 6% rh; LiCl, 22%; and NaI, 39%. Samples (approximately 3 mg) were sealed (silver DSC pans) in the glove box and then loaded into the calorimeter.

2.5. Fourier-transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD)

Both FTIR spectroscopy and XRD were used to examine the physical state of the solid, i.e. amorphous or crystalline nature. FTIR spectra were measured using an ATI-Mattson Galaxy 5022 IR spectrophotometer at a resolution was 4 cm^{-1} over the scanning range of 4000–400 cm^{-1}. Samples were purged with dry air flowing at 25 l min^{-1}. Sample preparation was carried out in the dry glove box described above. Approximately 5 mg of solid sample was added to 30 mg of a fluorinated hydrocarbon (Spectra-Tech Flurolube), mixed using a mortar and pestle, and this paste was pressed between two BaF_2 windows. All spectra were ratioed against a Flurolube background. XRD was carried out as described previously (Costantino et al., 1997).

3. Results and discussion

Recently, we demonstrated the utility of GSA for determining the water sorption isotherm and water monolayer for lyophilized pharmaceutical proteins (Costantino et al., 1997). In this approach, lyophilized samples are placed on a microbalance contained in a controlled-humidity environment. The relative humidity is increased in a stepwise fashion, and at each level the sample weight (i.e. water sorption/desorption) is allowed to stabilize before increasing to the next humidity. Herein, we more fully explore the use of GSA to examine various lyophilized protein–sugar systems.

The first model system under consideration is that of the pharmaceutical protein rhGH lyophilized in the presence of mannitol. Data for the water vapor sorption of rhGH:mannitol lyophilized at various ratios is depicted in Fig. 1A. In the case of pure mannitol, essentially no water uptake was seen, consistent with the formation of anhydrous crystals upon lyophilization (Costantino et al., 1997). This was confirmed by FTIR spectroscopy (Fig. 2A), showing essentially no change in the region around 3600–2800 cm^{-1}, which includes O–H stretching vibrations, and yields a distinct pattern if the material is crystalline. X-ray diffraction (XRD) of the mannitol samples yielded the same conclusion (Costantino et al., 1997). For each rhGH:mannitol isotherm, the data at low relative humidity (up to 40% rh) was used in determining the water monolayer (M_0) using a modified form of the BET equation. A summary of these fits is presented in Fig. 1B. The experimentally determined water monolayer for the various rhGH:mannitol samples are con-

sistent with the prediction based on the values for the pure components and the ratio of protein-to-mannitol (dashed line in Fig. 1B).

To test the generality of this observation, we examined another pharmaceutical protein, namely rhIGF-I, co-lyophilized in the presence of mannitol. This system exhibited behavior similar to that of rhGH:mannitol. The sorption isotherm curves (Fig. 3A) followed the same trend, from little to no water sorption at low protein-to-mannitol weight ratios to the highest curve for that of pure rhIGF-I. In addition, the values for M_0 calculated from the experimental data (up to 40% rh) (Fig. 3B) are in-line with that predicted from the contributions of pure protein and mannitol.

In addition to mannitol, another model excipient for formulating pharmaceutical proteins is the sugar sucrose. We followed the same approach described above to determine the water monolayer for systems of sucrose co-lyophilized with rhGH, rhIGF-I, and another therapeutic protein, a recombinant humanized monoclonal antibody (rhuMAb). The plots of the calculated water monolayers for these systems are shown in Fig. 4A–C.

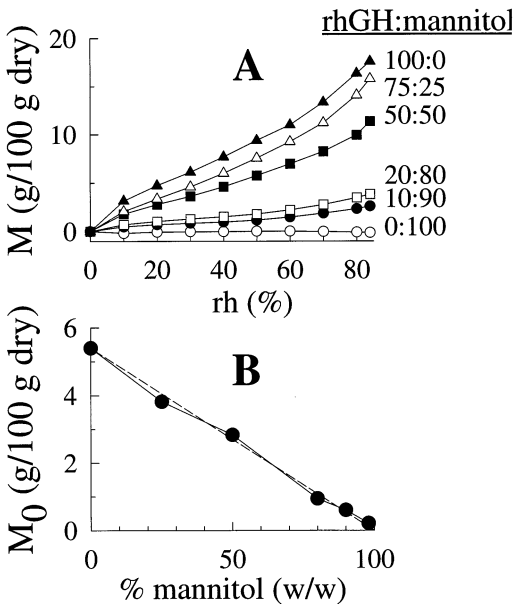

Fig. 1. Water sorption behavior of lyophilized rhGH:mannitol. A. The water vapor sorption isotherm for lyophilized powders consisting of (from top to bottom) 100:0, 75:25, 50:50, 20:80, 10:90 and 0:100 rhGH:mannitol (w:w, dry basis), as indicated. The data were obtained using the GSA method. B. The water monolayer (M_0) for lyophilized rhGH:mannitol. The values for M_0 were calculated from the GSA data using the modified BET plot.

The data reveal a different behavior for sucrose compared to mannitol. For example, as sucrose is added to rhGH (Fig. 4A), the M_0 calculated from the water sorption data decreases slightly, even though the experimentally determined water monolayer for pure sucrose is above that of the protein. According to the hypothesis that sugars (e.g. sucrose) can replace water on a dried protein, it is possible that there is a solid-state interaction between the protein and sugar which is masking water monolayer sites from the humidified atmosphere. M_0 begins to increase with increasing sucrose at approximately 50:50 (rhGH:sucrose, dry basis). At this weight ratio, there are approximately 65 mol sugar per mol protein, very similar to the amount of water required to satisfy the rhGH monolayer (5.3 g /100 g protein) or 66 mol mol^{-1} protein). This suggests that beyond this point, the proposed interaction has reached saturation, i.e. the added sucrose molecules exhibit their full water monolayer.

Similar behavior was observed for rhIGF-I and rhuMAb co-lyophilized with sucrose (Fig. 4B and C, respectively), namely M_0 was found to be generally lower than expected based on the values for the sugar and protein alone. The data support the view that sucrose and these proteins are interacting such that water monolayer sites in the system are being masked from the humidified atmosphere. In order for such an interaction to be possible, the sucrose molecules must exist in the amorphous phase in intimate contact with protein molecules. To ascertain this, we measured the FTIR spectra for lyophilized sucrose over the spectral range of 3600–2800 cm^{-1}. The relatively smooth spectra obtained for lyophilized sucrose indicates that it is amorphous compared to the crystalline form (compare curves a and b in Fig. 2B). It was seen that lyophilized sucrose was susceptible to crystallization at elevated rh (curve c in Fig. 2B). This observation has reported previously by others (teBooy et al., 1992; Saleki-Gerhardt and Zografi, 1994; Sarciaux and Hageman,

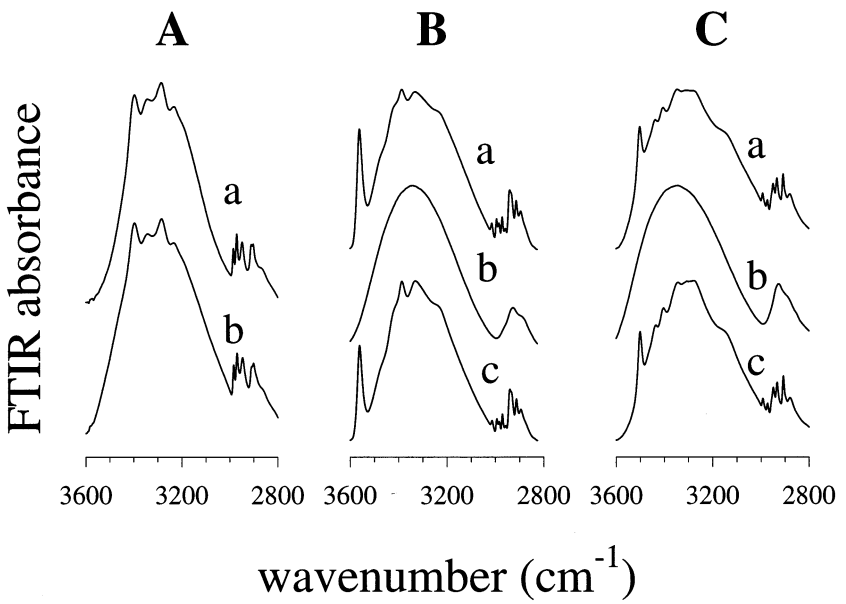

Fig. 2. FTIR spectra for various excipients (3600–2800 cm^{-1}). A. Mannitol, from top to bottom: (a) anhydrous crystals (as provided by the commercial supplier) and (b) the lyophilized powder. B. Sucrose, from top to bottom: (a) anhydrous crystals (as provided by the commercial supplier), (b) the lyophilized powder and (c) the lyophilized powder following high-humidity storage (1-week storage at room temperature and 84% rh). C. Trehalose, from top to bottom: (a) dihydrate crystals (as provided by the commercial supplier), (b) the lyophilized powder and (c) the lyophilized powder following high-humidity storage.

1997) and will be discussed later. Our observations regarding the physical state of lyophilized sucrose were further confirmed by XRD (data not shown).

In addition to mannitol and sucrose, yet another potential excipient for therapeutic proteins is the sugar trehalose, which may be particularly potent in stabilizing dried proteins (Crowe et al., 1996). Thus, we next investigated the moisture sorption behavior of lyophilized rhGH:trehalose. From the GSA data, M_0 was calculated for this system at various ratios of protein-to-trehalose (Fig. 4D). The trend observed was very similar to that for the various pharmaceutical proteins co-lyophilized with sucrose. As more sugar was added to the protein, the water monolayer tracks lower than predicted based on M_0 for pure rhGH and trehalose alone. At approximately an equal weight ratio of rhGH-to-trehalose, a different behavior can be seen, namely as the relative amount of sugar increases beyond this point, the data more closely fit the ideal case (dashed line in Fig. 4D). Note that lyophilized rhGH:sucrose exhibited a similar transition in the M_0 data (Fig. 4A). These findings suggest that trehalose, like its isomer sucrose, can interact with rhGH in the solid state such that some water monolayer-binding sites are not available. Furthermore, the point at which this effect reached saturation was similar for the two sugars.

As was the case for sucrose, trehalose also remains amorphous upon lyophilization as evidenced by XRD (Costantino et al., 1997) and FTIR (Fig. 2C) data. Furthermore, trehalose is susceptible to moisture-induced crystallization. For example, the FTIR spectra reveal that trehalose crystallized upon storage at 84% rh (Fig. 2C, curve c). This event can also be discerned in the GSA data. Consider the set of sorption data for the various lyophilized rhGH:trehalose systems Fig. 5. For clarity, the data are offset-plotted using a common scale for the ordinate. In the case of trehalose alone, the GSA data show a typical sorption up to about 50% rh, then a slight decrease in the level of moisture as the rh was raised from 50–60%, and no further increase at higher

rh. This suggests a solid-phase transition to another form which can retain less sorbed water than the disordered amorphous matrix. The level of water sorbed at this plateau, corresponding to approximately 11 g /100 g) trehalose, is very similar to the 10.5 g /100 g) expected for the dihydrate crystal.

The data reveal that the presence of co-lyophilized rhGH acts to inhibit trehalose crystallization. It is interesting to reflect that even though amorphous sugars are employed to protect proteins (against solid-state degradation), the protein may act to preserve the sugar in the amorphous form, thus 'protecting' it from crystallization. For example, the lyophilized sample comprised of 25:75 rhGH:trehalose (w:w) crystallized at 70% rh and 40:60 at 80% rh, compared to 60% rh for the sugar alone. For mixtures of at least equal ratio of protein-to-trehalose, no crystallization was seen, i.e. these samples exhibited

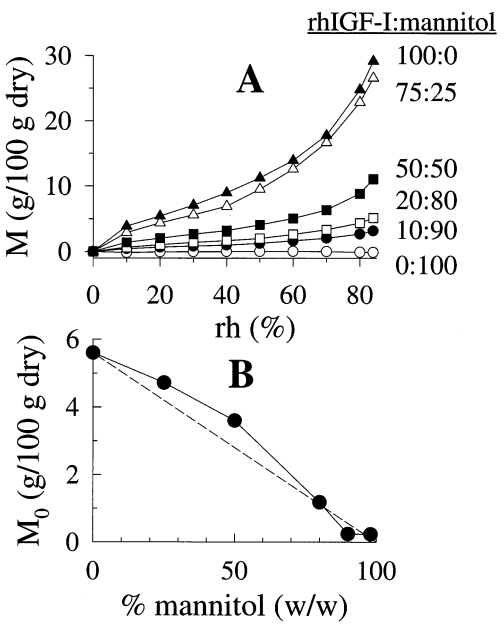

Fig. 3. Water sorption behavior of lyophilized rhIGF-I:mannitol. A. The water vapor sorption isotherm for lyophilized powders consisting of (from bottom to top) 0:100, 10:90, 20:80, 50:50, 75:25 and 100:0 rhIGF-I:mannitol (w:w, dry basis), as indicated. The data were obtained using the GSA method. B. The water monolayer (M_0) for lyophilized rhIGF-I:mannitol. The values for M_0 were calculated from the GSA data using the modified BET plot.

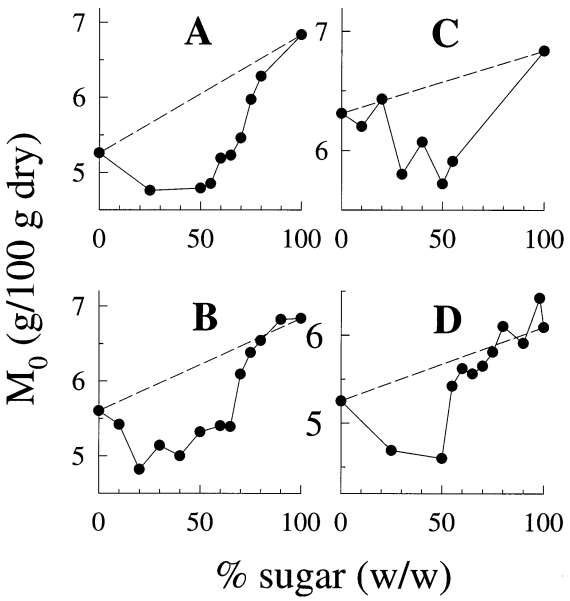

Fig. 4. Determination of M_0 for various lyophilized protein–sugar systems. A. rhGH:sucrose. B. rhIGF-I:sucrose. C. rhuMAb:sucrose. D. rhGH:trehalose. The values for M_0 were calculated from the GSA data using the modified BET plot.

continued moisture uptake throughout the GSA experiment. If an interaction exists between rhGH and trehalose in the solid state, this could provide some resistance to crystallization.

Our observations are consistent with the findings of French et al. (1995) regarding the crystallization behavior of spray-dried mixtures of trehalose and the pharmaceutical proteins recombinant human granulocyte colony stimulating factor (rhG-CSF) and recombinant consensus interferon (rConIFN). In that study, it was found that both rhG-CSF and rConIFN delayed the onset of spray-dried trehalose crystallization, as determined by a GSA approach. Since the amount of water absorbed at the transition increased with increasing amount of protein, it was proposed that either the sugar was experiencing reduced hydration (due to preferential binding of water by the protein) or that protein–trehalose interactions did not permit the crystallization. When protein was present in the spray-dried system above some 35–50%, no trehalose crystallization was observed.

In the present investigation, we found that lyophilized rhGH:sucrose exhibited behavior similar to that of the protein freeze-dried in trehalose. The water sorption isotherms for the lyophilized rhGH:sucrose systems are presented in Fig. 6. For pure sucrose, the moisture sorption increased with increasing rh up to about 50% at which point the sample then expelled water upon further rh increase in the process of forming anhydrous sucrose crystals. Addition of co-lyophilized rhGH retarded the moisture-induced sucrose crystallization. For example, 30:70 rhGH:sucrose crystallized when the rh was raised from 60–70% rh, and for ratios of 35:65 and above, no evidence of sugar crystallization was seen by GSA.

To examine whether the retardation of sucrose crystallization requires intimate contact between sugar and protein molecules, we prepared several mechanically mixed rhGH:sucrose systems and compared their GSA data to the co-lyophilized case. We choose three ratios to study, 10:90, 35:65 and 75:25 rhGH:sucrose (w:w). The GSA data for the mechanically mixed and co-lyophilized samples are depicted in Fig. 7. At 10:90, the amount of protein present is insufficient to prevent sucrose crystallization, even in the co-lyophilizate. The minimal relative amount of protein for complete inhibition of sucrose crystallization for the co-lyophilized case, 35:65 protein:sugar, was not effective in the mechanically mixed case. For instance, the GSA data clearly demonstrate a decrease in weight (i.e. moisture content) as the rh increased from 50 to 60%, indicative of sucrose crystallization. Even for the instance of 75:25, it was observed that mechanically mixing rhGH and sucrose was not sufficient to keep the sugar amorphous at elevated rh. These data are consistent with the view that solid-state interactions between protein and sugar molecules retard the sucrose crystallization, and that mechanical mixing is not sufficient to provide such contact.

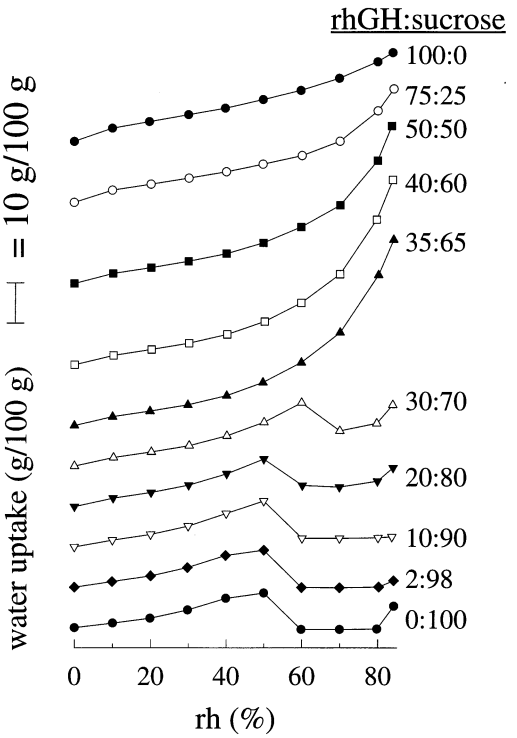

Fig. 6. GSA water uptake for lyophilized rhGH:sucrose. The ratio of protein-to-sugar was varied as indicated.

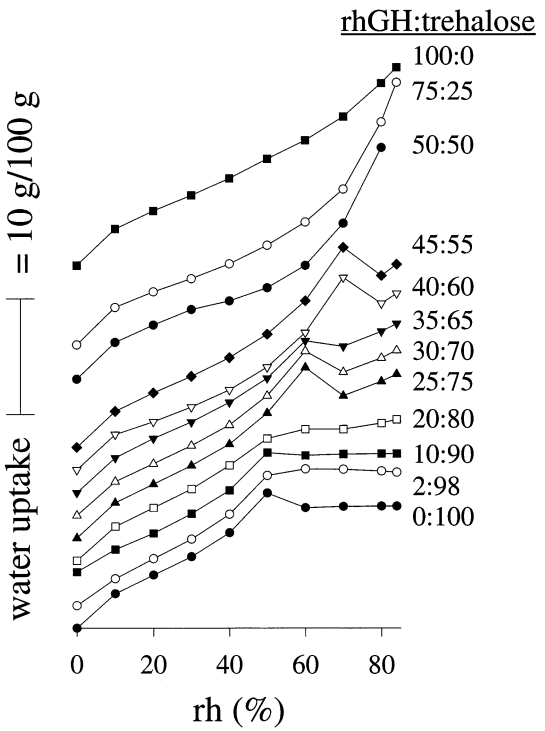

Fig. 5. GSA water uptake for lyophilized rhGH:trehalose. The ratio of protein-to-sugar was varied as indicated.

Recently, data has been published on the crystallization behavior of sucrose co-lyophilized with bovine somatotropin (rbSt) (Sarciaux and Hageman, 1997). It was found that rbSt increased the sugar's resistance towards crystallization. Kinetic gravimetric sorption studies (monitoring the sample weight at a constant rh) revealed that both the nucleation and growth phases of moisture-induced crystallization were significantly retarded by the presence of protein. For example, the addition of 10% (w:w, dry basis) rbSt in sucrose resulted in approximately 10-fold increase in induction time (i.e. nucleation phase) and 3-fold decrease in rate of water loss (i.e. growth phase) for the lyophilized powder incubated at 23°C and 45% rh (Sarciaux and Hageman, 1997).

To test the generality of these findings, we have used a similar approach. Lyophilized rhGH:sucrose powders were placed in the GSA apparatus at room temperature and 60% rh and

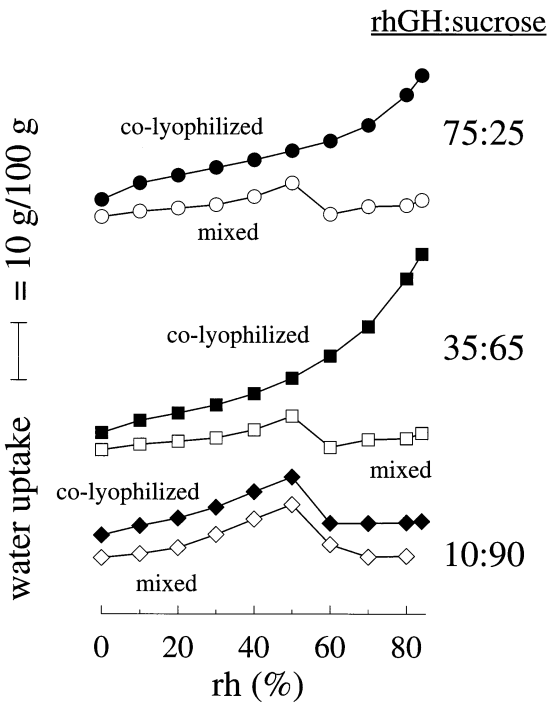

Fig. 7. GSA water uptake for co-lyophilized vs. mechanically mixed rhGH:sucrose. From top to bottom: (**X**) co-lyophilized and (**X**) mechanically mixed 75:25; (**X**) co-lyophilized and (**X**) mechanically mixed 35:65; and (**X**) co-lyophilized and (**X**) mechanically mixed 10:90 rhGH:sucrose, as indicated

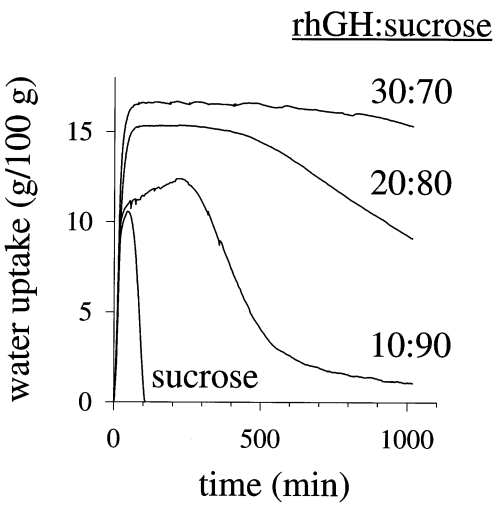

Fig. 8. Water loss of lyophilized rhGH:sucrose at 60% rh and 23°C due to crystallization of sucrose. Shown are data for (a) pure sucrose, (b) 10:90, (c) 20:80 and (d) 30:70 rhGH:sucrose.

moisture contents (water sorption/desorption or weight gain/loss) was monitored over time (Fig. 8). Compared to sucrose alone, 10:90 and 20:80 rhGH:sucrose have significantly longer induction times (period of relatively constant sample weight following initial sorption) and lower rates of weight loss, i.e. crystal growth (which can be approximated as the slope during the period where the sample weight is decreasing). At 30:70, no crystallization was seen over the time period of the kinetic experiment. This is in-line with the GSA isotherm, which shows that crystallization was delayed until the rh was raised from 60 to 70% for 30:70 rhGH:sucrose (Fig. 9).

From the sorption isotherm data, it is possible to compare rhGH, rhIGF-I and rhuMAb in terms of their ability to inhibit sucrose crystallization. For example, Fig. 9 depicts the data for all three proteins at the same protein-to-sugar ratio of 30:70. It can be seen that sucrose crystallization was delayed until the rh was raised from 50–60% for 30:70 rhIGF-I:sucrose whereas some 10% rh higher was required for the same ratio of rhGH in sucrose. The 30:70 rhuMAb:sucrose did not crystallize over the rh range studied (up to 85%), suggesting it had additional capability to inhibit the isothermal crystallization of sucrose compared to the other two proteins.

Another, more quantitative, approach to compare the proteins is to test their ability to inhibit non-isothermal crystallization. To this end, we employed differential scanning calorimetry (DSC) which has been demonstrated to be useful in studying sucrose crystallization (Saleki-Gerhardt et al., 1994). Lyophilized sucrose and protein–sugar systems were equilibrated at various rh (water activities) prior to DSC measurement, in order to study the effects of composition and moisture on the crystallization. Typical data are presented in Fig. 10 for lyophilized rhGH:sucrose (in this case, the samples were equilibrated at 6% rh). The thermogram for pure sucrose exhibits a glass transition at roughly 50°C, followed by crystallization (the sharp exothermic event occurring some 40°C higher) and finally the melting exotherm at approximately 180°C. With addition of rhGH from 10 to 30 wt%, the temperature of the glass transition (T_g) exhibited little change whereas the crystallization temperature (T_c) increased dramatically and the peak became somewhat less sharp. This indicates that the presence of protein inhibited the non-isothermal crystallization of sucrose. The melting behavior reveals no definite trend, except some increased complexity, probably due to some influence of the protein.

Table 1 summarizes the DSC data for the lyophilized protein:sugar systems. Samples of sucrose co-lyophilized with rhGH, rhIGF-I and

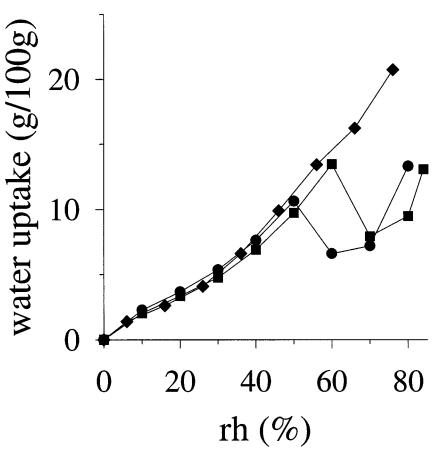

Fig. 9. Water sorption isotherms for (**X**) rhIGF-I, (**X**) rhGH and (**X**) rhuMAb, all lyophilized at 30:70 protein:sucrose.

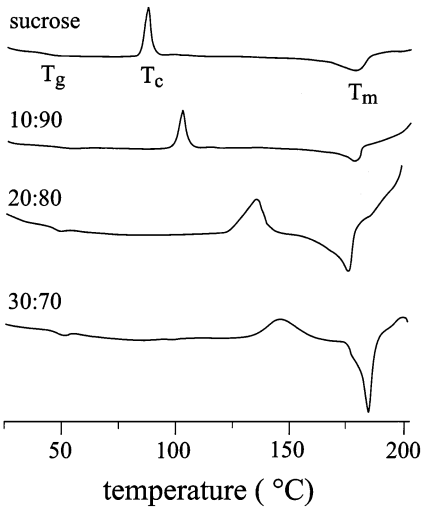

Fig. 10. DSC of lyophilized rhGH:sucrose (equilibrated at 6% rh). From top to bottom, thermograms for pure sucrose, 10:90, 20:80 and 30:70 rhGH:sucrose, as indicated. The temperature corresponding to the sucrose glass transition (T_g), crystallization (T_c) and melting (T_m) are denoted.

rhuMAb were equilibrated at 0 (continuously flowing dry air), 6, 22 and 39% rh. The data show that both T_g and T_c decrease with increasing rh. Moisture's role in lowering the T_g for sucrose has been reported previously in several reports (teBooy et al., 1992; Saleki-Gerhardt and Zografi, 1994; Sarciaux and Hageman, 1997). The melting temperature (T_m) displayed no clear trend and this endotherm occurred at approximately 180°C, as expected from the data of others (Saleki-Gerhardt and Zografi, 1994). Likewise, the measured enthalpy of crystallization, roughly some 60 J g^{-1} sucrose, was similar to the value previously reported in the literature (teBooy et al., 1992).

The effect of moisture on sucrose crystallization has been rationalized using the model of nucleation-controlled crystallization (Jolley, 1970). In this view, the crystallization occurs roughly halfway between the glass transition and the melt where the contributions of nuclei formation (which decreases with increasing temperature) and diffusion to the growing nuclei (which increases with increasing temperature) are balanced. Thus, when water is added, which has a much lower T_g than sucrose, i.e. −139°C (Sugisaki et al., 1968), both T_g and T_c decrease. This may also explain

Table 1
Summary of DSC data for lyophilized protein:sucrose[a]

Sample	0% rh[b]		6% rh		22% rh		39% rh	
	T_g	T_c	T_g	T_c	T_g	T_c	T_g	T_c
Sucrose	72 ± 1	117 ± 1	53 ± 5	86 ± 1	49 ± 1	82 ± 2	—[d]	63 ± 2
rhIGF-I:sucrose								
10:90	73 ± 3	125 ± 3	50 ± 1	101 ± 1	52 ± 6	85 ± 5	nd[c]	nd
20:80	76 ± 1	142 ± 3	51 ± 1	124 ± 1	45 ± 2	98 ± 5	—	73 ± 2
rhGH:sucrose								
10:90	74 ± 1	126 ± 1	51 ± 1	102 ± 1	—	84 ± 1	nd	nd
20:80	74 ± 3	133 ± 3	51 ± 1	132 ± 1	44 ± 2	101 ± 1	—	81 ± 2
30:70	77 ± 2	154 ± 2	52 ± 1	141 ± 3	48 ± 2	121 ± 5	nd	nd
rhuMAb:sucrose								
10:90	70 ± 2	124 ± 3	51 ± 1	105 ± 1	58 ± 2	90 ± 1	nd	nd
20:80	70 ± 2	146 ± 1	51 ± 1	141 ± 1	49 ± 2	122 ± 3	—	87 ± 2

[a] The heating rate was 3°C min^{-1}.
[b] The equilibration of samples at various rh is described in Section 2.
[c] nd, not determined.
[d] —, not detected.

how various agents which raise the T_g for sucrose also raise the T_c (Saleki-Gerhardt and Zografi, 1994). However, our data show that all three proteins studied had little effect on the T_g (and T_m), but increased the T_c (for a given set of data at constant water activity). In addition, the plasticizing effect of water on T_g and T_c for sucrose was similar regardless of the presence of protein.

The results clearly show that addition of pharmaceutical proteins inhibits the non-isothermal crystallization of sucrose, that is, increases the T_c. It has been reported that other proteins, such as gelatin (Jolley, 1970; Van Scoik and Carstensen, 1990) and rbSt (Sarciaux and Hageman, 1997), and also polymers such as poly(vinylpyrrolidone) and Ficoll (Shamblin et al., 1996) can also inhibit sucrose crystallization. Based on the limited number of proteins studied, no conclusions can be drawn regarding the physicochemical properties which govern the degree to which crystallization is inhibited. The molecular weight may play some role: the protein of largest molecular size, rhuMAb ($M_w \approx 150$ kDa), had the greatest effect, whereas the two smaller proteins, rhGH (22.2 kDa) and rhIGF-I (7.4 kDa), had roughly the same ability to increase the sucrose T_c. For example, the T_c for 20:80 rhuMAb:sucrose equilibrated at 22% rh was 122 ± 3°C compared to 98 ± 5 and 101 ± 1 for the case of rhIGF-I and rhGH under the same conditions (Table 1). Another factor which may be relevant is the content of sites on the protein which can interact with the sugar. Those sites which comprise the water monolayer may also be those involved in protein–sugar interactions (see discussion above). Additional studies are necessary to further examine the relation between a protein's nature, e.g. its water-sorbing capacity, and its ability to inhibit the transformation of co-lyophilized sugars from the amorphous to crystalline state.

It has been noted by Sarciaux and Hageman (1997) that many of the agents known to inhibit sucrose crystallization are hydrophilic molecules which form strong hydrogen bonds. This would tend to place restrictions on the diffusion, collisions and spatial orientation of the sucrose molecules. As a result, crystal nucleation and growth would be retarded. Since sugars have been shown to hydrogen-bond to proteins in the solid state (Carpenter and Crowe, 1989), it is reasonable to hypothesize that these interactions also play a role in the inhibition of sucrose crystallization. This same interaction can also account for the shielding of water monolayer-binding sites in lyophilized rhGH:sucrose as discussed above.

In summary, we have studied the moisture sorption behavior of sugars co-lyophilized with three different pharmaceutical proteins. The findings suggest that there is some interaction between amorphous sugars and pharmaceutical proteins in the solid state. Sorption isotherms show that monolayer water-binding sites were masked as a result of this interaction. In addition, the transformation of amorphous sugars to the crystalline state is inhibited by the presence of co-lyophilized protein, as revealed from both isothermal and non-isothermal experiments.

Acknowledgements

The authors thank Karen Kostick for assistance in sample preparation, Dr Tue Nguyen for supporting this work, and Bob Jones and Dr Gordon Brown of the Department of Geology at Stanford University for cooperation in XRD studies.

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