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RESEARCH PAPER

Assessment of Degree of Disorder (Amorphicity) of Lyophilized Formulations of Growth Hormone Using Isothermal Microcalorimetry

Mitra Mosharraf, Ph.D. *

Product and Process Development, Pfizer Global Manufacturing, Stockholm, Sweden

ABSTRACT

When determining the degree of disorder of a lyophilized cake of a protein, it is important to use an appropriate analytical technique. Differential scanning calorimetry (DSC) and X-ray powder diffraction (XRPD) are the most commonly used thermoanalytical techniques for characterizing freeze-dried protein formulations. Unfortunately, these methods are unable to detect solid-state disorder at levels $<10\%$. Also, interpretation of DSC results for freeze-dried protein formulations can be difficult, as a result of the more complex thermal events occurring with this technique. For example, proteins can inhibit the thermally induced recrystallization of the lyophilized cake, resulting in potential misinterpretation of DSC degree of disorder results. The aim of this investigation was to study the use of isothermal microcalorimetry (IMC) in the assessment of degree of solid-state disorder (amorphicity) of lyophilized formulations of proteins. For this purpose, two formulations of growth hormone were prepared by lyophilization. These formulations consisted of the same amounts of protein, mannitol, glycine, and phosphate buffer, but differed in the freeze-drying procedure. After lyophilization, the recrystallization of the samples was studied using IMC at 25°C under different relative humidities (58–75%). The effect of available surface area was studied by determining the heat of recrystallization (Q) of the samples before and after disintegration of the cakes. The results showed that, in contrast to DSC, IMC allowed detection of the recrystallization event in the formulations. Although both formulations were completely disordered and indistinguishable according to XRPD method, IMC revealed that formulation B had a different solid-sate structure than formulation A. This difference was the result of

*Correspondence: Mitra Mosharraf, Ph.D., Product and Process Development, Pfizer Global Manufacturing, Lindhagensgatan 133, SE-112 87, Stockholm, Sweden; Fax: +46-8-695-41-47; E-mail: mitra.mosharraf@pfizer.com.

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differences in the freeze-drying parameters, demonstrating the importance of choosing appropriate analytical methodology.

Key Words: Isothermal microcalorimetry; Freeze-drying; Solid-state structure; Recrystallization; Lyophilization; Degree of disorder.

INTRODUCTION

In order to increase the stability of proteins used in pharmaceutical preparations, the protein is usually lyophilized with a lyoprotectant such as a carbohydrate to produce an amorphous or partially amorphous matrix. Since the mobility and reactivity of the protein in such a glassy solid-state is reduced,[1] protein-protein interactions and aggregation may also be reduced,[2] resulting in increased protein stability. Protein stability in lyophilized cakes may also be enhanced by molecular interaction via hydrogen bonding between the carbohydrate and the protein in the solid state.[3]

Sugars such as sucrose and trehalose are potentially useful excipients for lyophilization techniques because they tend to produce amorphous cakes. Although mannitol has a strong crystallizing tendency, it is also known to exist in a fully or partially amorphous state in certain drug formulations.[4] The main advantage of using mannitol in lyophilized products is its chemical stability: in contrast to many disaccharides, it does not undergo hydrolysis. The relatively high Tg′ (i.e., the glass transition temperature of the maximally freeze-concentrated amorphous phase[5]) of mannitol is also favorable, as the formulations can be lyophilized at higher temperatures than those used for sucrose, for example. Another advantage of using mannitol is that it can allow rapid lyophilization.

Various processing conditions such as the cooling rate[6] and the primary drying temperature[7] also affect the solid state of the lyophilized cake. For example, when manufacturing amorphous cakes, it is critical to conduct primary drying below the Tg′ in order to avoid collapse of the system. It is important to use appropriate analytical techniques for determining the degree of disorder of the lyophilized cake and testing its storage stability. Differential scanning calorimetry (DSC) and X-ray powder diffraction (XRPD) are the thermo-analytical techniques most commonly used to characterize freeze-dried protein formulations. Unfortunately, these methods are limited in their ability to detect solid-state disorder at levels less than 10%.[8] Also, interpretation of DSC results for freeze-dried protein formulations can be difficult, because of the more complex thermal events occurring in the process. It has also been reported[9] that, in some cases, proteins inhibit the thermally induced recrystallization of the cake, resulting in increased or completely absent recrystallization temperatures (Tc) in the DSC scan, thus making it difficult to determine the degree of disorder. An alternative method, isothermal microcalorimetry (IMC), has also been used to study the solid-state structure and stability of lyophilized protein formulations. In this method, the solid-state structure of the sample is determined by exposing the powder to a specific relative humidity (RH)[10,11] using either ampoules or an RH perfusion unit. Isothermal microcalorimetry is a much more sensitive method than DSC and XRPD.[12]

Several factors can be named that can affect the moisture-induced recrystallization of the samples. The relative humidity, the amount of surface area available, and the solid-state structure of the sample are some of these parameters. It is known that the greater the relative humidity and available surface area, the faster the recrystallization rate. In 1994, Sebhatu, Angberg, and Ahlneck[11] measured the heat of recrystallization (Q) of 100% amorphous lactose by IMC and showed that Q was constant at 57–100% RH at constant temperature. Buckton and Darcy[13] showed that at higher temperatures there is a difference in the Q values at different humidities. This was explained to be due to a lower amount of water required to lower Tg below T at higher temperatures and the greater rates of evaporation and diffusion of the water vapor at higher temperature. According to these authors, if the supply of water vapor is slow, the net exotherm in the microcalorimeter is the same at each RH studied at 25°C. In cases where the supply of water vapor is rapid, the amorphous lactose will equilibrate to a different water load at each RH, prior to crystallization.[13]

The aim of this investigation was to study the use of IMC in the assessment of the degree of solid-state disorder of lyophilized formulations of a model protein, recombinant human growth hormone.

MATERIALS

Recombinant human growth hormone (rhGH) with a molecular weight of 22 KDa was used as the model protein. RhGH was produced at Pfizer (Strängnes, Sweden) from bacterial fermentation of a strain of *Escherichia coli*. The protein contained 191 amino acid residues similar to the natural pituitary-derived GH. Crystalline mannitol was purchased from Pfizer (Stockholm, Sweden) and glycine was obtained from Merck

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(Damstadt, Germany). The buffer salts monobasic and dibasic sodium phosphate were obtained from VWR International, Stockholm, Sweden. Sodium bromide (Sigma-Aldrich, Steinheim, Germany), cupric chloride (Sigma, Germany), and sodium chloride (Merck, Germany) were used to obtain 58%, 68%, and 75% RH environments, respectively.

METHODS

Freeze-Drying

A stock solution containing growth hormone, glycine, and mannitol was prepared in 5 mM phosphate buffer (pH 7.0). (The weight ratio of mannitol:rhGH in this solution was 0.7:1.) This solution was passed through a 0.22 μm Millipore sterile filter into special glass cartridges to a volume of 0.775 mL/cartridge, and freeze-dried using an Edward freeze drier (Edward 28, Edward, Kniese & Co., Marburg, Germany). Thermocouples were positioned in representative cartridges for monitoring the temperature during the process.

The freeze-drying cycles used for the samples were either A, in which the solutions were cooled to $-40^{\circ}C$ for 3 h, followed by primary drying at $-20^{\circ}C$ for 58 h, or B, in which freezing occurred at $-40^{\circ}C$ for 1.45 h followed by primary drying at $-35^{\circ}C$ for 120 h. The secondary drying process was the same in both cases.

Samples of each formulation were disintegrated and tested along with whole intact freeze-dried (lyophilized) cakes. In order to mimic the effect of variations in the milling procedure, five cakes from each batch were pooled and milled by hand for a few seconds using a mortar and pestle.

Scanning Electron Microscopy (SEM)

The cakes were scanned before and after disintegration using a Stereoscan 420 scanning microscope under an acceleration voltage of 20 kV and 300 X magnification. In order to scan the internal structure and porosity of the whole cakes, each cake was sliced into three pieces; the middle slice was then studied. In order to study the effect of disintegration and milling on the surface morphology, the lyophilized powder from disintegrated cakes was also examined.

Determination of Total Surface Area by Gas Adsorption

About 100 mg of sample was weighed in a Brunauer, Emmett, and Teller (BET) tube and degassed under vacuum overnight. The total surface area of sample A (before and after disintegration) was determined using Krypton gas adsorption (ASAP 2010, Micrometrics, Georgia, USA) according to the BET method. Since there was no access to sample B during the BET measurements, this sample was not analyzed by this method.

Determination of Residual Moisture

The residual moisture in each formulation was determined using a 684 Karl Fischer coulometer (Metrohm, Switzerland). A solution of methanol and formamide (70:30) was used as solvent. Three samples were tested for formulation A and one for formulation B.

Characterization of the Solid-State Structure

Isothermal Microcalorimetry

The IMC experiments were performed in a 2277 Thermal Activity Monitor (TAM) (Thermometric AB, Järfälla, Sweden) using the ampoule method, according to Sebhatu, Angberg, and Ahlneck, 1994.[11]

One whole cake (approximately 30 mg) was removed from the cartridge, inserted carefully into a microcalorimeter vial, and accurately weighed. At least three cakes were analyzed for each formulation.

A sample of about 30 mg of the disintegrated cake (powder) was also placed into a vial and weighed carefully and tested in the microcalorimeter. A 30 mg sample of raw crystalline mannitol was also tested. The experiments were performed at RH values of 58–75%. In order to obtain the various RH values, specific saturated salt solutions[14] (see Materials section) were placed into vials that were then sealed. An empty, freshly sealed vial was used as reference. The sample and reference vials were equilibrated in the TAM before starting the experiment. All experiments were performed at 25°C. The heat flow signal (dQ/dt in μw) was monitored as a function of time. In this study, the heat of recrystallization (Q, measured in J) was calculated by integrating the total area under the heat flow curve,[13] because of overlapping absorption and recrystallization peaks. The values were normalized for eventual weight differences.

The relative degree of disorder (amorphicity) of the samples was calculated as the ratio of the heat of recrystallization of B to that of A.

X-Ray Powder Diffraction

The solid-state structure of the lyophilized samples, before and after exposure to 75% RH in the

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microcalorimeter and also the solid-state structure of raw material of mannitol were investigated using XRPD. Diffraction patterns of the materials were obtained using a Siemens D5000 diffractometer (Siemens, Germany), with Cu Kα radiation at 45 kV and 40 mA. The samples were scanned in steps from 10° to 45° (2θ).

The starting material was scanned over the range of 2° to 80° 2θ (step size 0.02°) using a SCINTAG X'TRA powder diffractometer (Scintag, Ecublens, Switzerland), with Cu Kα radiation (45 kV and 40 mA).

The resulting patterns for raw material of mannitol and lyophilized cakes after exposure to 75% RH were then compared to theoretical diffraction patterns (calculated from single crystal data) for mannitol polymorphs[15] in order to determine the polymorphic form of mannitol in the samples.

Differential Scanning Calorimetry

These experiments were performed on a DSC 220 (Seiko Instruments, Seiko, Japan). The freeze-dried cakes were gently disintegrated. A sample of approximately 2.5–3.5 mg was accurately weighed in an aluminium pan and sealed. An empty pan was used as reference. The sample and the reference were exposed to a linear heating ramp in the temperature range 5–220°C and a heating rate of 5°C/min under nitrogen atmosphere. For each formulation, duplicated samples were analyzed. Exothermic signals were given positive values.

RESULTS AND DISCUSSION

Visual Inspection

The cakes did not show any sign of collapse or shrinkage after lyophilization.

Scanning Electron Microscopy

It is difficult to draw definitive conclusions from the SEM pictures (see Fig. 1). Both formulations contained a large number of pores, finely dispersed throughout the cakes. No significant differences in the surface morphology of the disintegrated samples were observed after milling the cakes.

BET

The BET results are listed in Table 1. The results show that after disintegration the total surface

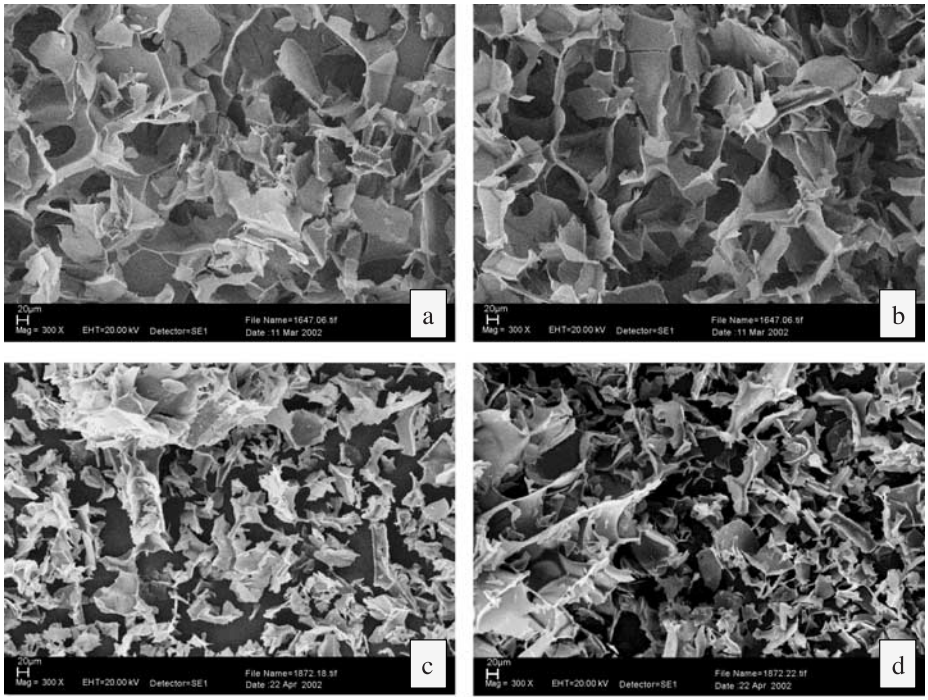

Figure 1. SEM pictures of the middle slice of lyophilized whole cakes of A (a) and B (b) for comparison with the corresponding powders (c) and (d).

Table 1. Primary characterization.

Sample	Residual moisture[a] (%)	Total surface area[b] (m^2/g)	
		Cake	Powder
Lyophile A	0.7	0.47	0.89
Lyophile B	0.4	–	–

[a]Determined by Karl Fischer.
[b]Determined by gas adsorption (BET).

area of sample A is increased from 0.47 m^2/g to 0.89 m^2/g.

Residual Moisture

In both formulations (A and B), the amount of residual moisture in the cakes was very low (< 1%; Table 1).

X-Ray Powder Diffraction

The raw material of D-mannitol had sharp peaks indicative of a very crystalline structure (Fig. 2). It is well known that D-mannitol has three polymorphs; α, β, and δ.[15] The XRPD pattern of the starting material of mannitol used in this study was in accordance with the theoretical diffraction pattern of β mannitol. The XRPD patterns of both lyophilized samples A and B (Fig. 2) had broad diffuse spectra typical of an amorphous material. According to the XRPD patterns, there were no detectable differences in the amorphous solid-state structures of the two formulations. After IMC measurements and moisture uptake, the samples had sharp peaks indicative of crystalline regions in the sample. These observations suggest a phase transformation from amorphous to crystalline mannitol. Comparing the collected patterns to the theoretical diffraction patterns for mannitol polymorphs, it was shown that mannitol in these samples had crystallized to the δ form due to the exposure to moisture at 75% RH.

Differential Scanning Calorimetry

Representative DSC curves for the samples are shown in Fig. 3. The raw crystalline mannitol showed a typical sharp melting endotherm at 166.5°C. No clear melting endotherm was seen for samples A and B (Fig. 3A). This is typical of amorphous materials and suggests that samples A and B were both amorphous. However, no recrystallization exotherm was observed for the test samples. It seems, thus, that the rhGH protein may have prevented the thermally induced recrystallization of the cake. This observation corroborates other reported results[9,16] and suggests that DSC is not a suitable method for determination of amorphicity of formulations containing proteins.

Magnification of DSC scans for lyophilized samples A and B (Fig. 3B) showed two weak endothermic peaks. This suggests that at least a small portion of the cakes was crystalline. The largest endotherm, the melting endotherm of mannitol, occurred at 159°C for both A and B. A much smaller endotherm was also observed before this peak, at 144.2°C in A and 147.5°C in B. Two melting endotherms for mannitol lyophilized with protein have also been reported by Souillac et al. (2002).[16]

The values obtained for enthalpy of fusion (ΔH) for these endotherms are compared in Table 2. The heat of fusion of the first endotherm (ΔH_1) in A is twice that in B. The differences shown in the table suggest that the solid-state structures of the samples may be slightly different. However, because there is no recrystallization exotherm, it is almost impossible to determine the exact degree of disorder of the formulations by DSC.

These endotherms suggest the presence of a direct interaction between protein and mannitol. On the other hand, it has also been reported[17,18] that pure crystalline mannitol has three polymorphs, which melt at 158.0, 166.0, and 166.5°C.[19] The smaller initial peak demonstrated in this study could represent the melting point of one of the mannitol polymorphs or may be the result of a mixed mannitol/protein partially crystalline form.

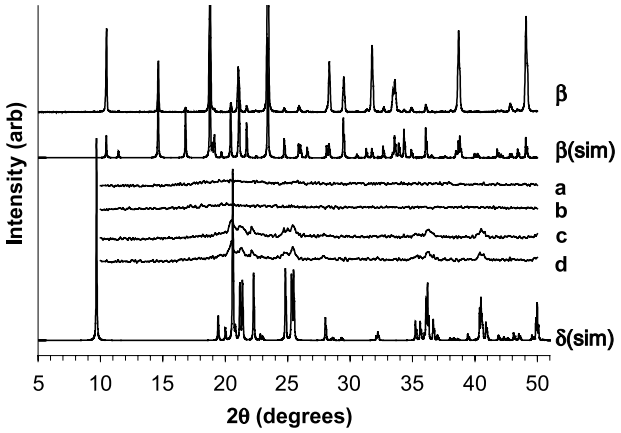

Figure 2. X-ray diffraction patterns of lyophilized samples A and B, before (a and b) and after (c and d) exposure to 75% relative humidity, compared to XRPD pattern of raw material of mannitol (β) and simulated patterns of β (sim) and δ (sim) mannitol.

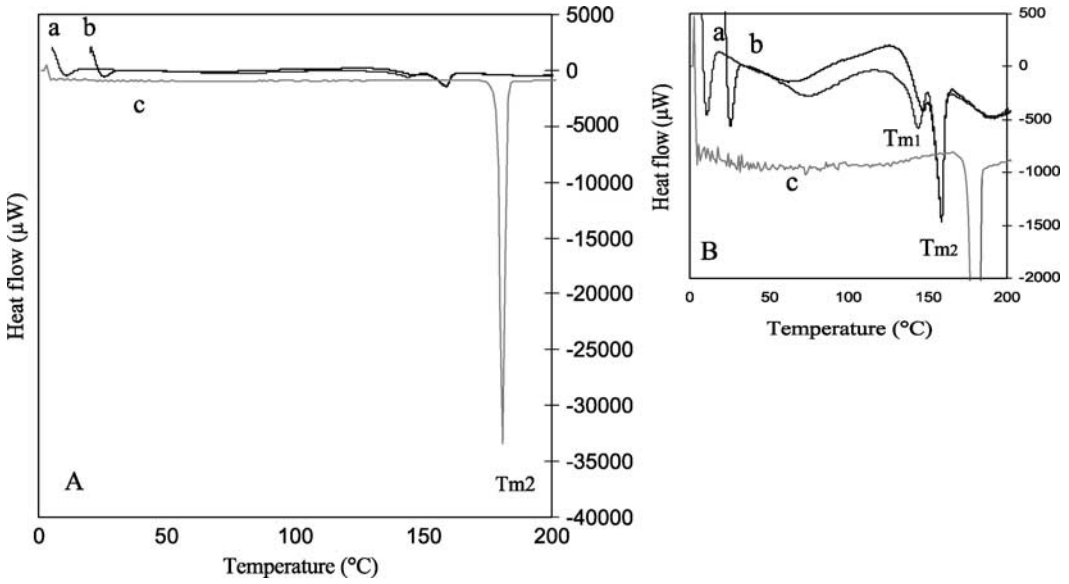

Figure 3. A) Representative DSC curves for the samples; lyophilized samples A (a) and B (b) and raw crystalline mannitol (c). B) Magnification of DSC scans of Fig. 3A. (*View this art in color at www.dekker.com.*)

The Effect of Relative Humidity and Surface Area on the Heat of Crystallization Obtained by IMC

Typical microcalorimetry responses for the cakes and the corresponding powders are presented in Fig. 4. These curves show that lyophilized protein formulations A and B reacted with moisture, resulting in a positive heat flow. This flow of heat is the result of a recrystallization event, due to moisture uptake.[11,20] The starting material of D-mannitol did not show any heat flow at 75% RH, confirming that this sample was crystalline prior to lyophilization. These results were also in good agreement with the DSC and XRPD results. The heat evolved or absorbed (Q) by IMC was calculated by integrating the total heat flow curve. The values obtained for heat of crystallization were compared at different relative humidities before and after disintegration of the cake to a fine powder; the results are listed in Table 3 and plotted in Fig. 5.

Table 2. DSC data.

	Melting point (°C)		Heat of fusion (J/g)	
Sample	T_{m1}	T_{m2}	ΔH_{m1}	ΔH_{m2}
Mannitol	–	166.5	–	284.0
Lyophile A	144.2	158.9	14.2	29.1
Lyophile B	147.5	159.0	7.1	26.4

In order to see if differences between the Q values for each sample at the different RH were significant, the results in Table 3 were evaluated using both analysis of variance (ANOVA) and Mann-Whitney tests. The differences between the Q-values obtained at 58% and 75% RH for cakes A and B were statistically significant, when post hoc tests in ANOVA were used, i.e., the p values were < 0.015 for cake A and <0.016 for cake B. Using the nonparametric Mann-Whitney test, only the difference between Q values obtained at 58% and 75% RH for cake A remained statistically significant ($p < 0.029$). The difference for cake B was marginally significant ($p < 0.071$).

It was thus shown (Table 3, Fig. 5) that for cake A, the heat of crystallization decreased as a function of RH. The highest mean Q value was obtained at 58% RH (22.1±3.6 mJ/mg). This value decreased to 15.1 and 11.7 mJ/mg at 68% and 75% RH, respectively.

For cake B, the heat of crystallization was slightly increased as RH was increased. These values were 26.7 (±6.4) at 58%, 32.9 (±0.7) at 68% RH, and 35.0 (±3.0) mJ/mg at 75% RH. The highest Q value obtained for the formulation (i.e., 35 mJ/mg obtained for cake B, at 75% RH) is very close to the heat of crystallization obtained for other totally amorphous carbohydrates such as spray-dried lactose (31 mJ/mg, as shown by

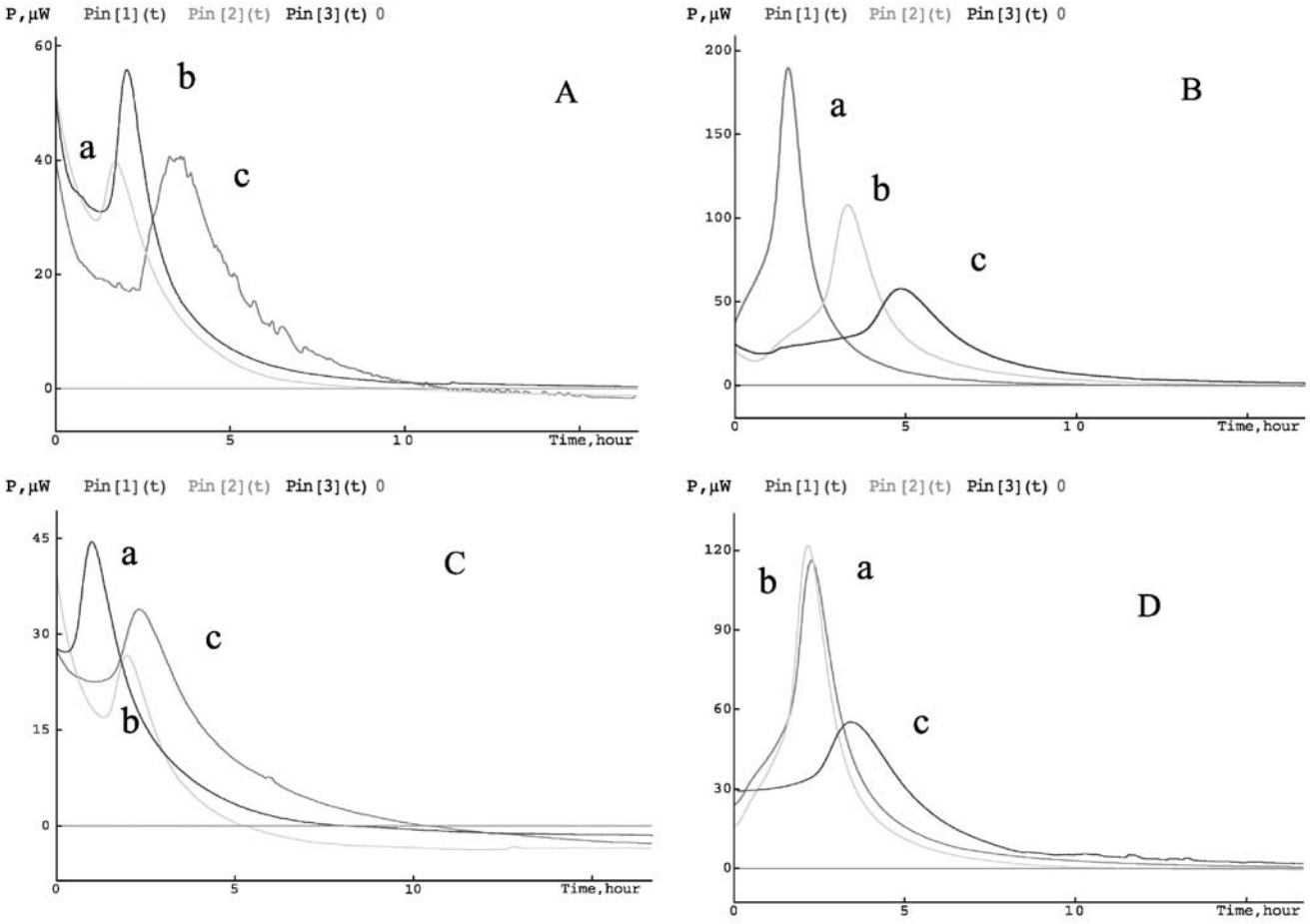

Figure 4. Heat flow curves obtained by IMC, for lyophilized cakes A (A) and B (B) and the corresponding powders (C) and (D) at 75% (a), 68% (b), and 58% (c) relative humidity. (*View this art in color at www.dekker.com.*)

Sebhatu, Angberg, and Ahlneck, 1994[11]) and lyophilized and quenched sucrose (33 and 49 mJ/mg, respectively, unpublished data), suggesting that sample B is very amorphous.

When the cakes were gently milled to a fine powder prior to microcalorimetry studies, the mean Q values differed less and were independent of RH. It was also interesting to note that in all these cases the amount of

Table 3. IMC data.

	Heat of crystallization Q (mJ/mg)		
Sample	58% RH Mean, (*CI*[a]), n[b]	68% RH Mean, (*CI*[a]), n[b]	75% RH Mean, (*CI*[a]), n[b]
Mannitol	–	–	0
Lyophile A			
Cake	22.1 (±3.6), 4	15.1 (±4.5), 3	11.7 (±5.9), 4
Powder	12.9 (±3.6), 4	11.1 (±4.6), 3	14.3 (±2.9), 3
Lyophile B			
Cake	26.7 (±6.4), 3	32.9 (±0.7), 3	35.0 (±3.0), 5
Powder	33.9 (±6.5), 4	28.5 (±2.3), 3	32.4 (±3.8), 3

[a]*CI* denotes the 95% confidence interval limits.
[b]n Denotes the number of experiments.

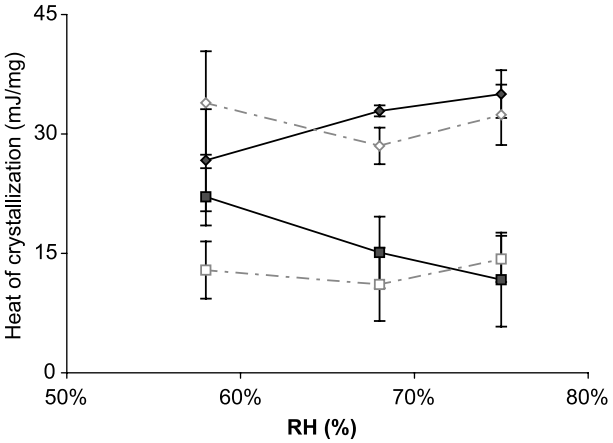

Figure 5. Heat of crystallization of lyophilized samples A (■) and B (◆) as a function of relative humidity (RH), before (closed symbols) and after (open symbols) disintegration of the cakes. Bars = 95% confidence intervals.

heat of crystallization (Q) obtained by IMC was at the same level of that obtained for the corresponding cakes at higher RH (75% RH) (Table 3 and Fig. 5).

In Fig. 6, the crystallization peak times are plotted against RH. As would be expected, the higher the relative humidity, the shorter was the crystallization peak time (especially for the intact cakes). For sample A, after gentle milling of the cakes to a fine powder, the peak times were similar (3.3, 2.9, and 2.5 at 58%, 68%, and 75% RH, respectively). Also, for sample B similar peak times were obtained for the powders after exposure to 68%, and 75% RH (i.e., 3.5 and 3.1 h, respectively). All samples (cakes and corresponding powders) had similar peak times at 75% RH (Table 4). According to Table 1, gentle milling of the cakes increased the total surface area of sample A by almost two-fold. Thus, a faster reaction with moisture was expected for the powder. This was the case when cake A was studied at 58% RH, before and after gentle milling to a fine powder (i.e., the peak time was reduced from 4.9 to 3.3 h, see Table 4). There was no clear relationship between the crystallization peak time and Q for sample B, when it was studied in powder form.

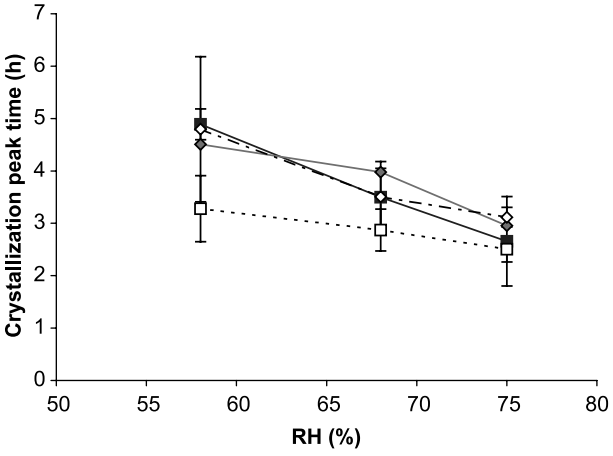

Figure 6. Peak time for crystallization of lyophilized cakes A (A) and B (B) and the corresponding powders (C) and (D) as a function of relative humidity (RH). Bars = standard deviation. (*View this art in color at www.dekker.com.*)

A probable explanation for the results in Fig. 5, is to assume that the degree of disorder (amorphicity) is not the same throughout the cakes, i.e., there are areas in the lyophilized cake that are more disordered (amorphous) and other areas that are less disordered.

For the intact cakes exposed to 58% RH, the reaction between moisture and the cake seems to be limited to a peripheral layer (surface) of the cake, due to a lower amount of moisture uptake and a slower crystallization at 58% RH compared to that at 75% RH. This suggests that the amount of water absorbed by the cake at 58% RH is not enough to cause a total recrystallization of the cake. Therefore, it would appear that at 58% RH, the Q value likely reflects the solid surface structure of the cake.

Table 4. The crystallization peak times obtained from IMC curves.

Sample	RH (%)	Sample weight[a] (mg)	Mean peak time[a] (h)
Lyophile A			
Cake			
	58	31.7 (0.2)	4.9 (0.3)
	68	31.5 (0.3)	3.5 (0.5)
	75	31.9 (0.2)	2.7 (0.9)
Powder			
	58	31.1 (0.2)	3.3 (0.6)
	68	30.9 (0.4)	2.9 (0.4)
	75	31.2 (0.8)	2.5 (0.2)
Lyophile B			
Cake			
	58	30.8 (0.1)	4.5 (1.4)
	68	30.7 (0.4)	4.0 (0.7)
	75	31.0 (0.1)	3.0 (0.2)
Powder			
	58	31.1 (0.7)	5.3 (0.5)
	68	30.3 (0.5)	3.5 (0.3)
	75	31.7 (0.3)	3.1 (–)[b]

[a]The values in parentheses are standard deviations. In all cases n is the same as listed in Table 3.
[b]In this case n = 2.

The results also suggest that in cake B, the peripheral parts (external surface layer) are less disordered than the bulk. In contrast, with cake A, the peripheral parts of the cake are more disordered than the interior of the cake. This conclusion is based on the variations in the heat of recrystallization obtained at 58% RH compared to that obtained at 75% RH for the cakes and their corresponding powders.

In other words, for cake A, at 58% RH, the degree of disorder seems to be overestimated, since only the more amorphous peripheral parts are being recrystallized, preventing the interior parts of the cake to react with moisture.

In sample A, when the amount of moisture reacting with the sample is increased, either by an increase in RH (ex. to 75%) or an increase in the exposed surface area (by disintegrating the cake prior to IMC measurements), the Q value is decreased. This shows that in these cases the whole material is being recrystallized and Q is reflecting the bulk properties of the cake, i.e., both amorphous and crystalline surfaces are exposed to moisture, affecting the final heat of crystallization and reactivity with moisture.

For sample B, since the cake is more amorphous in the interior parts than on the external surface, after gentle milling of the cake to a fine powder or at higher RH, the amount of amorphous surfaces exposed to moisture is increased and thus the reactivity is increased and Q is increased.

In a simplified model, the net area under the microcalorimetry curve (Q_{Total}) can be assumed to be the mean value of the heat obtained from all the surfaces that are in equilibrium with moisture.

$$Q_{Total} = \Sigma Q/n \text{ or } (Q_{surface} + Q_{core})/2$$

If the recrystallization process is not completed before the equilibrium between the moisture and the whole solid mass is reached, then $Q_{total}=Q_{surface}$. If the surface is more disordered than the core, then Q_{Total} is overestimated (cake A at 58% RH). If the surface is less disordered than the core, Q_{Total} is underestimated (cake B at 58% RH).

If the intact cake is exposed to a higher RH or if the exposed surface area of the sample is increased (i.e., by gentle milling of the cake prior to IMC measurements), a complete recrystallization of the sample occurs and the obtained Q would reflect the bulk properties of the sample.

These studies conclude that several factors may affect the obtained heat of crystallization as measured by IMC: 1) the reactivity of the surface in contact with moisture, 2) the amount of exposed surface area, 3) the relative humidity, 4) the amount of absorbed moisture required in order to establish an equilibrium between the solid and the vapor, 5) the recrystallization rate, and 6) the initial solid-state structure of the material.

Assessment of Degree of Disorder by Isothermal Microcalorimetry

Because of the high tendency for mannitol to crystallize, it was not possible to obtain an amorphous mannitol reference by melt-quenching in liquid nitrogen. Thus, in calculation of degree of disorder, a relative degree of disorder was calculated in each case from the ratio of Q_B/Q_A. The results (Table 5) suggested that sample B is almost twice as disordered as sample A, in all cases studied, except when the cakes are compared at 58% RH.

As the distribution of Q_B/Q_A is not known, simulations have been performed to estimate the error in the relative degrees of disorder listed in Table 5. A standard statistical package (SPSS) (V11.0, SPSS Inc. Chicago, IL) was utilized for this statistical analysis. Using the estimated means and standard deviations presented for the 12 variables in Table 3, the SPSS random number generator produced 4000 normally distributed numbers for each of the 12 variables under the assumption that the variables were normally distributed. In this way, it was possible to achieve 4000 simulated ratios for each of the ratios in Table 5. The 2.5 and 97.5 percentiles then are presented as the 95% upper and lower limits for the ratios in Table 6.

The Effect of Formulation on the Solid-State Structure

Mannitol commonly crystallizes during freeze-drying in a vial.[21] Nonetheless, despite this strong

Table 5. Estimation of the relative degree of disorder of B:A.

	$Q_{(B)}/Q_{(A)}$	
RH (%)	Powder	Cake
58	2.6	1.2
68	2.6	2.2
75	2.3	2.9

Table 6. Point and interval estimates of relative degree of disorder calculated from simulations (n = 4000).

	Powder			Cake		
	Mean ratio	Percentiles[b]		Mean ratio	Percentiles[b]	
RH (%)	$Q_{(B)}/Q_{(A)}$[a]	2.5	97.5	$Q_{(B)}/Q_{(A)}$[a]	2.5	97.5
58	2.68	1.92	3.78	1.21	0.88	1.60
68	2.71	1.72	4.66	2.23	1.69	3.05
75	2.29	1.81	2.96	3.24	1.97	6.02

[a]The mean ratios found through simulations do not necessarily correspond to the values found in Table 5.
[b]The 2.5 and 97.5 percentiles are presented as the 95% upper and lower limits for the ratios.

tendency to crystallize, mannitol can also exist in a fully or partially amorphous state in certain drug formulations.[4] In contrast, when growth hormone is lyophilized without any excipient, it remains in an amorphous state.[21] The effect on the solid-state structure of a mixture of mannitol and a strong glass, such as a protein, has also been studied, with varying results. Mixing amorphous components at the molecular level results in an amorphous phase with properties (such as Tg) that are related to the properties of the individual components.[22] According to Pikal, Dellerman, and Roy (1991),[23] when mannitol, growth hormone, and glycine were combined in a weight ratio of 5:1:1, mannitol crystallizes, but glycine remains amorphous, presumably molecularly dispersed in the amorphous protein phase.[21] In contrast, Kim, Akers, and Nail (1998)[17] reported that, in freeze-dried formulations containing lysozyme plus less than 30% mannitol, no mannitol crystallization took place (as shown by X-ray diffraction). Costantino et al. (1998)[24] demonstrated that at mannitol:rhGH molar ratios of less than 131:1, the obtained lyophilized cake is amorphous. The results of the present study substantiate the results obtained by Costantino et al. (1998).[24] In the formulations studied here, the weight ratio and molar ratio of mannitol:rhGH in the solution were 0.7:1 and 110:1, respectively, and XRPD and IMC demonstrated a resultant amorphous solid. It seems, thus, that the degree of disorder in lyophilized formulations containing mannitol may depend on the amount of mannitol in the formulation, i.e., the weight ratios of protein, mannitol, and other excipients.

However, it is not only the molar ratio of mannitol:rhGH that will affect the solid-state structure of the cake, but the freeze-drying process also has an effect. Despite identical formulation ingredients, sample B has a higher reactivity than sample A, i.e., sample B is more disordered (amorphous).

The Effect of the Freeze-Drying Process on the Solid-State Structure

Since both A and B have the same ingredients, the differences in the solid-state structure should depend on the different freeze-drying cycles used. The differences in the product temperature during the lyophilization are shown in Table 7. The product temperatures during subcooling were −9°C and −12°C for A and B, respectively. The final temperatures during the freezing step were −28°C and −35°C. The product temperatures during the primary drying period were −19°C and −32°C for A and B, respectively. According to the freeze-drying microscopy results (not shown), the collapse temperature of the formulation is about −10°C. Thus, in both cases, the final temperature during the freezing step and the maximum temperature during primary drying were below −10°C, indicating that the lyophilization process was performed successfully. A difference in the lyophilization process of A and B was in the cooling rate. The freezing step was twice as long in A. Thus, the ice crystals had a chance to grow larger in A and could also develop more slowly (faster cooling rates lead to the formation of smaller ice crystals, which have a higher surface-area-to-volume ratio than larger crystals[25]). Theoretically, since the

Table 7. Product temperatures during lyophilization.

	Freezing step		Primary drying	Sec drying
Sample	Sub cooling (C)	Final temp (C)	Max temp (C)	Final temp (C)
Lyophile A	−9	−28	−19	24
Lyophile B	−12	−35	−32	25

solidification stage occurred more slowly in A than in B, a more crystalline (ordered) solid-state structure would be expected for A.

CONCLUSIONS

Although growth hormone prevented thermally induced recrystallization of the lyophilized cake samples, preventing determination of the degree of solid-state disorder using DSC, exothermic recrystallization peaks were obtained by IMC, indicating that growth hormone did not prevent moisture-induced recrystallization of the lyophilized cake. Thus, in contrast to DSC, IMC was capable of demonstrating a recrystallization event, indicating a disordered/partially disordered solid-state structure for both rhGH/mannitol/glycine samples. This was verified by XRPD, although it was not possible to detect any difference in the degree of solid-state disorder of the samples using this method. Isothermal microcalorimetry, however, showed that sample B was twice as disordered as sample A. This difference in the solid-state structure was caused by differences in the freeze-drying parameters, the only differences between the formulations.

It was also shown that the use of IMC for studying the solid-state structure of lyophilized cakes containing proteins does not require milling (disintegration) of the cake prior to analysis, if the study is performed at RH >58%. In fact, if the aim of the study is to determine recrystallization behavior of the cake on storage, it is perhaps better to conduct the measurements on the cakes without disintegration.

Exposed surface area and RH were not only of importance for the crystallization kinetics, but also affected the heat of crystallization obtained by IMC.

In the cases where the whole intact cakes were studied, the heat of crystallization varied by RH and crystallization time. For cake A, heat of crystallization was decreased as RH was increased and for cake B, it was slightly increased. When the cakes were gently milled to a fine powder prior to IMC studies, the heat of crystallization was independent of RH and crystallization time.

Isothermal microcalorimetry thus appears to be an alternative method for studying the solid-state structure of and batch-to-batch variations (caused by up-scaling or changes in the formulation or process parameters) in lyophilized protein formulations. However, assessment of degree of disorder of lyophilized protein formulations by IMC should be done by a proper choice of RH and sample form.

It was concluded that several factors may affect the obtained heat of recrystallization by IMC: 1) the reactivity of the surface in contact with the moisture, 2) the amount of exposed surface area, 3) the relative humidity, 4) the amount of absorbed moisture required in order to establish an equilibrium between the solid and the vapor, 5) the recrystallization rate, and 6) the initial solid-state structure of the material.

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