Evaluation of the Physical Stability of Freeze-Dried Sucrose-Containing Formulations by Differential Scanning Calorimetry

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Freeze-dried samples of sucrose with buffer salts, amino acids, or dextran have been analyzed with differential scanning calorimetry (DSC) to evaluate the use of DSC thermograms in predicting the physical storage stability. The glass transition temperature, T_g, of the amorphous cake, crystallization, and melting of sucrose are observed with DSC. T_g appeared to be an important characteristic of the physical stability of the amorphous freeze-dried cake. A storage temperature above T_g results in collapse or shrinkage of the cake, which for a sucrose-based formulation, may be accompanied by crystallization of the sucrose. The T_g of the amorphous sucrose is influenced by other components present in the cake. Dextran-40 raised T_g, while the addition of glycine to the formulation lowered T_g. The residual moisture content strongly influences T_g, since water acts as a plasticizer of the system; the higher the moisture content, the lower the T_g and the less physically stable the freeze-dried cake. Crystallization of amorphous sucrose is shown to be inhibited by high molecular weight components or ionic compounds. DSC analysis of freeze-dried cakes proved to be a powerful tool in formulation studies.

KEY WORDS: freeze-drying; differential scanning calorimetry; glass transition temperature; collapse; crystallization.

INTRODUCTION

Sugars are known to stabilize proteins to heat and are used to provide bulk to freeze-dried proteins (1). However, two main problems are encountered in the freeze-drying of sugar solutions. First, dependent upon the freeze-drying conditions, a loss in structure of the freeze-dried cake can be observed immediately after freeze-drying. This loss in structure is described as a collapse phenomenon and may be ascribed to a too rapid increase in the temperature of the product during the freeze-drying cycle (2). Second, to investigate the effect of a formulation on the stability of freeze-dried proteins, accelerated stability studies are often conducted at higher storage temperatures. However, most freeze-dried carbohydrate systems show, at elevated temperatures (40–60°C), a clear change in the physical appearance of the cake related to the viscous flow of the dried powder mixture. This change in appearance or shrinkage, which has been termed "stickiness" (3), is also a result of collapse phenomena. This observed collapse at higher temperatures makes it difficult to predict the stability behavior of a freeze-dried protein formulation at ambient temperatures. An additional problem with freeze-dried sucrose solutions is that, at temperatures causing a collapse of the cake, the collapse is rapidly accompanied by crystallization of the sucrose (4).

Recent literature (5–8) postulates a new approach to the collapse problems encountered in the freeze-drying of carbohydrate systems. The behavior of concentrated carbohydrate systems has been described in terms of nonequilibrium thermodynamics as applied in polymer technology (9).

As most freeze-dried carbohydrate systems, polymers are often noncrystalline amorphous systems. These amorphous systems are characterized by three composition-dependent or material-specific temperatures, i.e., T_g, T_h, and T_m. T_h is the homogeneous nucleation temperature. In the absence of impurities or catalytic surfaces, nuclei are formed at this temperature. The formation of nuclei initiates the crystallization of amorphous material. In the presence of impurities nucleation will start somewhere between T_h and T_m, i.e., the crystalline melting temperature. T_g is defined as the glass transition temperature. T_g does not describe the temperature at which a phase transition occurs, but the temperature above which an amorphous system is changed from the glassy solid state to a highly viscous liquid state. The T_g can be characterized thermodynamically by a second-order change in the free enthalpy, represented by a discontinuity in the heat capacity. This change in heat capacity can be measured with differential scanning calorimetry (DSC) (10).

The T_g theory as summarized above offers a good starting point for the evaluation of carbohydrate containing formulations to be freeze-dried. T_g seems to be a valuable parameter indicating the maximum temperature at which a solution should be frozen, the maximum temperature at which the product can be dried, and the maximum temperature at which the final product can be stored. The aim of this study is to evaluate the suitability of DSC in formulation studies, especially with regard to the physical stability of freeze-dried sucrose-containing formulations.

MATERIALS AND METHODS

Materials

Chemicals used (sucrose, amino acids, dextran-40, citric acid, and dibasic sodium phosphate salts) were all of analytical grade. Sucrose was purchased from Coöperatieve Suiker Unie, Breda, The Netherlands, sodium citrate was obtained from Hoffman-La Roche, Mydrecht, The Netherlands, the sodium phosphate salts from CN Schmidt-BV, Amsterdam, The Netherlands, Polysorbate 20 from Chemag AG, Laren, The Netherlands, and glycine and alanine from J. T. Baker, Deventer, The Netherlands.

Methods

Freeze-Dried Samples. The solutions described in this study were dispensed in 1-ml vials [0.5 ml per vial and varying from 5 to 7% (w/v) total concentration of compounds], semistoppered, and freeze-dried in a Leybold GT20. Freeze-drying proceeded following a freezing step of 2 hr to −50°C,

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a primary drying step at 30 mTorr with a shelf temperature of −35°C (for up to 20 hr), and finally, a secondary drying step at 30 mTorr while raising the temperature gradually to ambient temperatures.

Preparation of Samples for Thermal Analysis. Rubber-stoppered vials containing samples of freeze-dried material was opened in a nitrogen flushed chamber. Immediately after opening, a sample of the freeze-dried cake (3–20 mg) was transferred to an aluminum pan (50 μl). An aluminum cover (10 μl, without holes) was placed on top to avoid adsorption of moisture. Aluminum pan and cover were pre-weighed. The sample was hermetically sealed in a press, just outside the nitrogen flushed chamber and weighed, to determine the sample weight.

DSC Analysis. The differential scanning calorimeter used was a 7 series thermal analysis system for Perkin-Elmer. The sample was placed in the preequilibrated DSC oven (35°C). After an equilibration period of 5 min, the sample was heated to 200°C at a speed of 10°C/min. The heat flow was measured using the same type of sealed pan and cover with air as a reference. Some samples were cooled again to 35°C and subsequently reheated to 135°C, at the same speed. Samples were analyzed at least twice. For measurements in the −50 to 100°C region, the oven was cooled with liquid nitrogen.

Sucrose Analysis. Sucrose was analyzed by a reversed-phase HPLC method. Typical chromatographic conditions are depicted in Table I. The chromatographic system used for analysis consisted of a HP 1090L pump, a HP 1090 column oven, and a filter-photometric detector. A chromatography/laboratory automation system (Perkin-Elmer) was used for quantitative analysis.

Residual Moisture. The residual moisture content has been determined using a Karl Fischer titrimetric method.

X-Ray Powder Diffraction. X-ray powder diffraction measurements were carried out on a Philips system with Cu Kα radiation (40 kV, 40 mA).

RESULTS AND DISCUSSION

Crystalline Sucrose

Figure 1A shows a DSC heating power–time curve of crystalline sucrose. The only event detected is melting of sucrose at 190°C with a melting enthalpy of 132 J/g. These data are in accordance with literature data (T_m, 188°C; dH_m, 120 J/g) (11). According to this reference the melting of sucrose is immediately followed by decomposition of the sugar

Table I. HPLC Conditions for the Determination of the Sucrose Content

Column	Supelco, LC-NH_2, 5 μm, length = 25 cm, i.d. = 4.6 mm
Mobile phase	Acetonitril:purified water (75:25)
Flow rate (ml/min)	2
Column temperature	Ambient
Detector temperature	30°C
Detector	Refractometer
Injector volume	10 μl
Instrument	HP 1090 liquid chromatograph

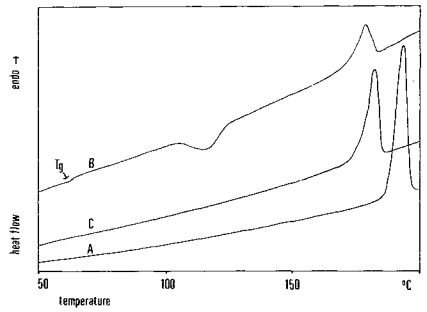

Fig. 1. DSC heating power–time curves of crystalline sucrose (A), freeze-dried sucrose (B), and freeze-dried sucrose stored for 1 month at 60°C (C).

starting at 195°C (reaction enthalpy, −770 J/g). Probably due to sample preparation in a nitrogen atmosphere and the high heating rate, decomposition of the sugar has not been detected for the crystalline samples upon heating to 200°C.

Frozen Solutions of Sucrose

Figures 2A and B show the heating power–time curves of a sucrose solution upon freezing and rewarming, respectively. Upon freezing, only crystallization of water can be observed, whereas upon rewarming a discontinuity in heat capacity at −34°C followed by a melting endotherm of water has been observed. The discontinuity at −34°C can be ascribed to the glass transition temperature of a water-containing sucrose system, which will be formed during freezing of the solution. This discontinuity is often described as the collapse temperature, i.e., the temperature below which the product temperature must be maintained during freeze-drying in order to avoid a collapse. Since no crystallization of sucrose can be observed during freezing, it is most likely that a freeze-dried sucrose system will be in an amorphous state.

Freeze-Dried Sucrose

In Fig. 1B, a DSC thermogram of freeze-dried amorphous sucrose is shown. A T_g at 59–65°C is observed. At 80–110°C, a broad exothermic peak has been detected, which can be attributed to the crystallization of sucrose. Different freeze-dried sucrose samples have been allowed to reabsorb moisture and have been analyzed. The observed T_g's and the temperatures at which crystallization starts are

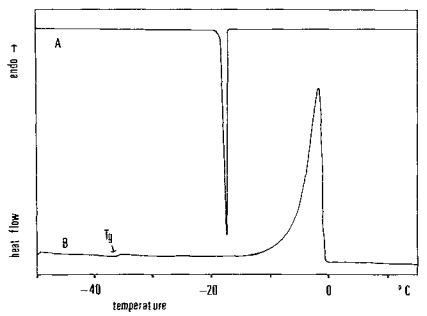

Fig. 2. DSC heating power–time curve of a frozen sucrose solution (200 mg/ml) upon freezing (A) and rewarming (B).

Table II. Dependence of T_g and T_{cr}[a] on Moisture Content of Amorphous Freeze-Dried Sucrose

Residual moisture (wt%)	T_g (°C)	T_{cr} (°C)	Enthalpy of crystallization (J/g)
8.5	16	80	−52.6
2	59 ± 2	101	−62
1	63 ± 1	105	−63
0.7	64 ± 1	107	−56

[a] Temperature at which crystallization of sucrose starts.

summarized in Table II. The T_g detected seems to be dependent on the residual moisture content of the various samples. As shown in Table II, the observed start of the crystallization also seems to depend upon the moisture content.

At 175–185°C the crystalline sucrose melts. The melting enthalpies measured (80–121 J/g) are of the same order of magnitude as measured for crystalline sucrose. Differences in melting enthalpies may be attributed to incomplete crystallization or decomposition reactions occurring (almost) simultaneously with melting of the sugar, evidenced by baseline noise on the right-hand side of the melting peak.

Samples of freeze-dried sucrose stored at 60°C have completely collapsed within 1 month without discoloration. DSC analysis of this collapsed cake has revealed (Fig. 1C) that during storage the sucrose has crystallized. No T_g or crystallization has been detected. Only a melting peak for crystalline sucrose has been found. X-ray diffraction measurements support DSC observations. Figure 3 shows typical X-ray diffraction patterns of some freeze-dried samples. A sucrose cake, just after freeze-drying, is completely amorphous. When this sample is stored at 60°C (stoppered vial) or at 20°C in a moist environment (RH 50–60%, unstoppered vial) it collapses and becomes crystalline.

When crystalline sucrose after melting is cooled down immediately to 25°C, no recrystallization of sucrose has been detected. Yet a discontinuity in heat flow is observed at 67°C. When the sample is reheated to 135°C, a discontinuity is again observed (see Fig. 4). The sudden change in heat flow indicates a T_g of molten amorphous sucrose.

In contrast to amorphous freeze-dried sucrose, molten sucrose is not able to crystallize. This may be explained by either the presence of heterogeneous nuclei in freeze-dried sucrose (12) or the higher water content in freeze-dried sucrose. A higher water content will facilitate the movement and collisions of sucrose molecules necessary to build up the critical nucleus.

Freeze-Dried Sucrose with High Molecular Weight Compounds

The DSC heating power–time curve of a freeze-dried sucrose solution, where a high molecular weight compound has been added, i.e., sucrose/dextran-40 solution (3:2, w:w) does not show a change in heat capacity within a limited temperature range but, rather, within a broader temperature range around 80°C (see Fig. 5). Although the T_g is more difficult to estimate, the T_g of the sample (residual moisture of 0.6%) is much higher than the T_g measured for freeze-

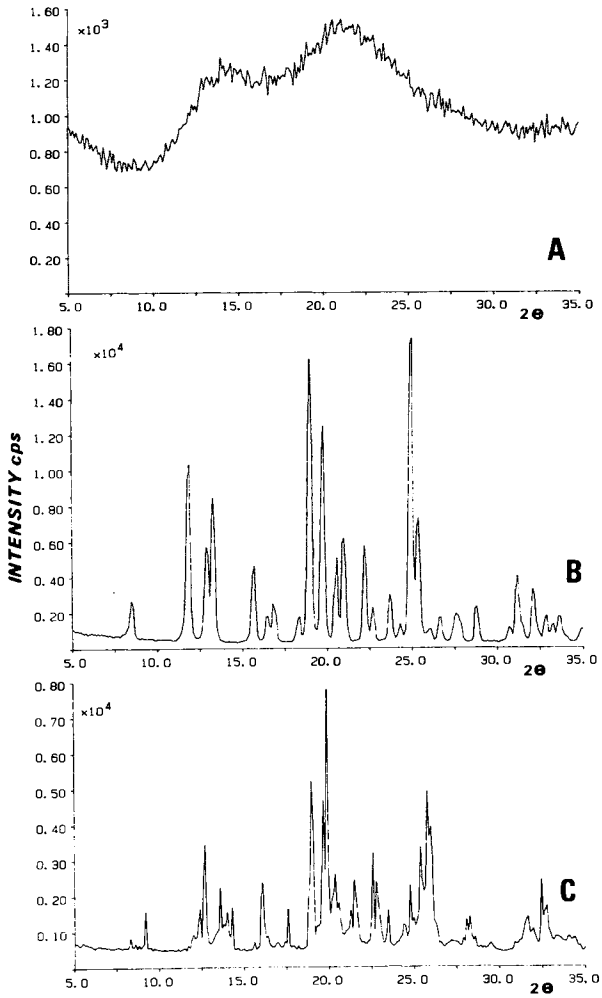

Fig. 3. X-ray diffraction patterns of freeze-dried sucrose (A), freeze-dried sucrose stored at 60°C (B), and freeze-dried sucrose stored at 20°C in a moist environment (RH 50–60%) (C).

dried sucrose without additives (65°C). Clearly the dextran polymer raises the T_g of the system.

Since T_g is defined as the temperature at which a system will have a viscosity of 10^{12} Pa · sec (5), and viscosity can be described as a function of molecular weight, high molecular weight compounds will increase T_g (13–15). Measurements of T_g of pure dextran-40 have revealed that the polymer, as was expected, has a high T_g of 94°C. The relatively high T_g of the sucrose/dextran mixture is in agreement with the good physical stability at 60°C found for these samples: freeze-dried cakes of sucrose/dextran appeared to be stable for at least 3 months at 60°C. That the T_g has not been measured as a distinct discontinuity is probably caused by the polymeric

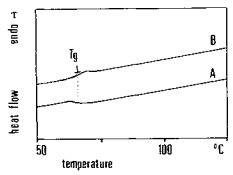

Fig. 4. DSC heating power–time curves of molten sucrose, when cooled (A) and when warmed (B).

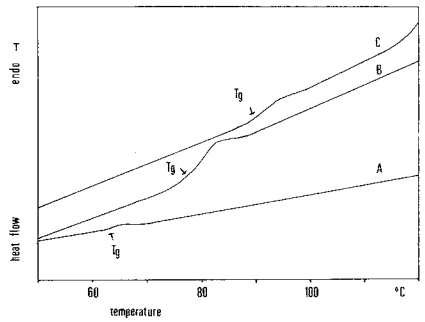

Fig. 5. DSC heating power–time curve of molten sucrose (A), a freeze-dried sucrose/dextran mixture (3:2, w:w), (B) and dextran (C).

character of the dextran molecules (MW distribution, instead of distinct MW). No crystallization of sucrose is observed after the addition of high molecular weight components to the mixture. Apparently, T_g is raised to such an extent that the viscosity of the system around the nucleation temperature is too high to enable crystal growth.

Freeze-Dried Sucrose with Buffer Salts

From the DSC heating power–time curve of a sample of freeze-dried sucrose with Na_2HPO_4 and citric acid (15:5:1, w:w:w), it appears that the freeze-dried material is amorphous, and although the residual moisture content is relatively high (1.9%), it has a T_g of 64–65°C. Crystallization and melting of the sucrose have not been detected. The buffer salts obviously are preventing the sucrose from crystallization. A minor broad peak is observed at approximately 170°C. This peak may be related to the loss of water at that temperature. The DSC heating power–time curve is not changed when citric acid is replaced by KH_2PO_4. The sucrose still remains amorphous and no crystallization is observed upon heating to 200°C. The added buffer salts prevent the sucrose from crystallization.

Samples based upon sucrose/phosphate buffer (pH 7) with a residual moisture content of 2% stored at 60°C have shown extensive browning. Although no crystallization of phosphates is by DSC, this nonenzymatic browning (16) can be caused by the crystallization of Na_2HPO_4 (17).

Crystallization of Na_2HPO_4 will be accompanied by a drop in pH, thus allowing acid hydrolysis of sucrose. To confirm this hypothesis sucrose solutions of pH 3 and 9, adjusted to pH with either diluted hydrochloric acid or sodium hydroxide, have been freeze-dried and analyzed for appearance and sucrose content (by a HPLC method). The results are summarized in Table III. Sucrose, as a freeze-dried cake, will be hydrolyzed under acid conditions, even though the meaning of pH in a freeze-dried product is questionable. These results support the hypothesis of the crystallization of Na_2HPO_4 during the freeze-drying process.

The DSC profiles of the sucrose samples at pH 3 and 9 are given in Fig. 6. Remarkably, at pH 3, crystallization is not observed, only a water evaporation peak can be detected. At pH 9, both crystallization and melting are present, however, the crystallization temperature is shifted toward much higher temperatures than the plain sucrose formulation. These results indicate that crystallization of amorphous sucrose is influenced by the presence of ions, which may exert its action by influencing the water activity.

Table III. Physical Characteristics of Freeze-Dried Sucrose Solution (50 mg/ml) at Low and High pH

pH	Appearance after 1-month storage at 60°C	Sucrose content (±SE) as percentage theoretical amount (1-month storage)	
		4°C	60°C
3	Complete loss of structure extensive browning	78.6 ± 0.2	0 ± 0
9	Shrinkage, no browning	94.7 ± 0.6	95.5 ± 0.5

Freeze-Dried Sucrose with Amino Acids

Cakes of sucrose and glycine (1:1, w:w) are amorphous. Although the residual moisture content of the sucrose/glycine cake has been found to be relatively low (0.7%), the measured T_g at 54°C is lower than for pure amorphous freeze-dried sucrose (59–65°C). Apparently, glycine will lower the T_g of the sucrose system. Further, the added component, glycine, also seems to prevent the crystallization of sucrose at higher temperatures. Decomposition reactions are observed at higher temperatures (>170°C).

No crystallization of sucrose was detectable with cakes composed of sucrose and alanine (1:1, w:w); however, a significant amount of sucrose is melting at 180°C. Whether the sucrose already has crystallized during the freeze-drying process (18) or during warming in the DSC oven (in which case the signal disappears in the baseline) remains unclear.

Freeze-Dried Sucrose with Alcohols

Alcohols are known to promote crystallization of solutes during freezing (19,20). Freeze-drying of sucrose solutions, with isopropyl alcohol (IPA), has resulted in a powder instead of a cohesive cake, which is physically stable at higher temperatures (±60°C), suggesting crystallization of the material. Two IPA:sucrose ratios have been used, 1:2.5 and 1:9 (w:w). The heating power time curves are shown in Fig. 7. In both cases, a T_g occurred at 64–65°C, indicating that still amorphous sucrose is present. In the case with the

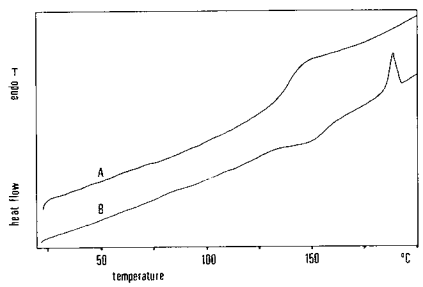

Fig. 6. DSC heating power–time curves of freeze-dried sucrose adjusted to pH 3 (A) or 9 (B).

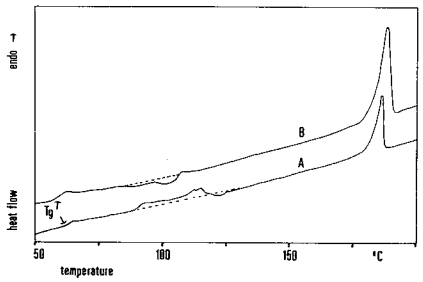

Fig. 7. DSC heating power–time curves of sucrose, freeze-dried in the presence of IPA. IPA:sucrose (w:w) = 1:2.5 (A) and 1.9 (B).

lower initial IPA:sucrose ratio (1:9), sucrose has crystallized at 90°C, followed by a melting of the sucrose at 188°C. The sample with the higher IPA:sucrose ratio has shown a different pattern in the 70–130°C region. A broad endothermic peak is followed by a small exothermic peak. The origin of this pattern is not fully understood but can be a combination of evaporation of IPA closely followed by crystallization of sucrose.

To study the efficacy of different alcohols to promote crystallization, a concentrated sucrose solution (180 mg/ml), which is difficult to freeze-dry, has been freeze-dried with the addition of ethanol, IPA, and tertiary-butyl alcohol (TBA). The appearance of the final cake becomes more cohesive with an increase in chain length of the alcohol. Higher ethanol concentrations (5% in the sucrose solution) have resulted in extensive foaming of the cake, indicating that the solution is boiling under reduced pressure during the sublimation phase of the freeze-drying cycle.

The effect of different concentrations of TBA has been studied. The addition of 5% TBA to a 180 mg/ml sucrose solution will result in a pharmaceutical acceptable freeze-dried cake. The cake made with 5% TBA is physically stable without collapse at higher temperatures. However, DSC analysis reveals a glass temperature with a crystallization peak at 109°C, indicating that still amorphous material is present. A peculiar phenomenon is observed if the sample is preheated to a preset temperature (below the observed crystallization temperature), immediately cooled back to 30°C and heated again to 200°C. It turns out that the crystallization peak largely disappears, depending upon the prewarming temperature. Some enthalpy data are presented in Table IV. Apparently, by prewarming the sample, the TBA is allowed to evaporate, thus facilitating complete crystallization.

Table IV. Dependence of Enthalpy of Crystallization and Temperature upon Prewarming Condition (180 mg/ml Sucrose, Plus 5% TBA)

Prewarming temperature (°C)	Enthalpy of crystallization (J/g)	T_{cr} (°C)
—	−58.4	109
60	−12.9	99
70	nm[a]	nm
80	nm	nm
93	−5.2	92

[a] Not measurable.

CONCLUSIONS

The results show that the glass transition temperature, T_g, is related to the physical stability of the dried cake: samples with an apparent T_g <65°C collapsed during storage at 60°C, while samples with a T_g of 80°C showed no collapse in the same period of time at 60°C.

T_g is composition dependent: buffer salts, amino acids, or polymeric species added to the sugar solution prior to freeze-drying influence the T_g of the cake. Glycine added to sucrose was found to lower the T_g, whereas addition of dextran resulted in an increase in T_g as compared to freeze-dried sucrose only. An increase in residual water content of the "dried" cake was accompanied by a lowering of T_g, and in this way water can be considered as a plasticizer of the carbohydrate system.

Besides influencing the T_g, the additives also seem to influence the extent and/or rate of the crystallization of amorphous sucrose, which may be related to the water content or water activity. High molecular weight compounds and ionic substances impede the crystallization of sucrose. More insight in the influence of additives and residual moisture on the T_g of a freeze-dried carbohydrate system opens possibilities for adjusting T_g, gaining better physical stability, and determining a critical moisture content for the aimed storage condition. Formulation studies can be guided by knowledge of T_g of a system. For proteins, a physically stable freeze-dried cake will itself not assure sufficient stability, but a physically unstable system will inevitably result in a rapid decay of protein activity, especially if crystal formation occurs during storage (21).

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