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Research paper

Formulation of proteins in vacuum-dried glasses. I: Improved vacuum-drying of sugars using crystallising amino acids

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Abstract

Aqueous solutions of maltose or sucrose containing phenylalanine, tryptophan or arginine were vacuum-dried in 2 ml glass vials (fill-volume = 1 ml) at 20°C over 24 h at pressures down to 0.1 Pa. The dried products were examined using DSC, X-ray scattering and SEM. The inactivation of rhG-CSF and LDH on storage at 4, 30, 40 and 50°C was determined. Pure maltose or sucrose remained in the rubbery state at room temperature, since their water contents could not be reduced below $\approx 6\%$ w/w under the conditions used. The inclusion of phenylalanine reduced the residual water to $\approx 1\%$ giving glass transitions of maltose of > 80°C. X-ray diffraction and SEM showed that the phenylalanine crystallises during vacuum-drying as a network structure, which becomes coated with a thin film of the amorphous sugar. The small diffusional pathlength within the sugar film produces rapid drying at 20°C and T_g climbs to > 80°C in 12 h. rhG-CSF was stable in these glasses at 40°C for at least 1 year. LDH was less stable, but still much better than the raw enzyme. © 1997 Elsevier Science B.V.

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1. Introduction

Freeze-drying is a standard procedure for stabilising peptides and proteins during storage. Sufficient water must be removed from an aqueous solution of protein and lyoprotectant (typically a disaccharide) to give an amorphous product having a glass transition well above room temperature [1]. The stabilisation of the protein in such a dry glass is thought to be a result of water substitution [2] combined with a degree of molecular immobilisation [3]. The freeze-drying of peptides and proteins has, however, some serious drawbacks [4]. First, the initial freezing subjects them to various stresses, for example, freeze-concentration of the protein within the interstitial solution and pH-shifts because of altered solubility of buffer salts [3] that may lead to their unfolding and inactivation. Secondly, the exact control of the freeze-drying conditions is vital, as the product temperature most not exceed either the collapse temperature during primary drying or the glass transition temperature at any particular residual water content during secondary drying [5]. Thirdly, freeze-drying is an expensive process, since the capital outlay for a steam-sterilisable production-scale freeze-dryer is large, and the maintenance and running costs are high. For this reason the attention of some researchers has been attracted to evaporative drying as an alternative to freeze-drying for stabilising proteins. The aim is the same, ie to achieve a glassy product having a high glass temperature [4]. Recognition of this condition for

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protein stabilisation was not always evident in early work on evaporative drying, and special properties were attributed to trehalose [6]. Enzymes and antibodies were air-dried at 37°C in μl-quantities with trehalose, with a reported full retention of activity compared with > 90% loss of activity when dried alone [7]. Similarly impressive results were obtained with the air-drying of 50 μl aliquots of an aqueous solution of phosphofructokinase at 30–40°C containing up to 500 mM of either trehalose, maltose or sucrose [8]. These sugars ameliorated to different degrees the otherwise total inactivation of the enzyme by air-drying. Although the stabilising action of trehalose was thus proven not to be unique, it still gave the best results, with some 70% of enzyme activity being retained during 2 h air-drying.

Franks' lucid exposition of the physical changes occuring during the drying of a protein/sugar formulation provided the basis for understanding these remarkable results. He recognised that the extent of protein inactivation depends on the time taken for the amorphous adjuvent to reach its glass transition, T_g, as water is removed from the system [4]. This time can be long during the initial freezing step of freeze-drying. In contrast, the air-drying of small volumes of a solution of protein plus sugar can reach the glass transition after much shorter times, resulting in high maintained levels of protein activity. Aliquots (25 μl) of an aqueous solution of lactate dehydrogenase with a sucrose/epichlorohydrin-copolymer could, for example, be vacuum-dried at 37°C to give a glassy product showing 98% residual enzyme activity after 6 months' storage [9]. During evaporative-drying the formulation transverses the rubbery state, where non-Arrhenius kinetics govern degradation rate [10], rapidly to yield glasses of high glass temperature. As recognised by Franks [4,11], the time taken to reach the glassy state during evaporative drying depends not only on temperature and pressure, but also on the nature and concentration of the glass-forming adjuvent, its gradient of $T_g(w)$, and the total surface area available for evaporation. The latter factor limits the utility of evaporative-drying to small volumes (up to $\approx$ 1 ml is claimed [11]), where the surface/volume ratio is large enough to give acceptable drying times to the glassy state at ambient temperatures.

During our investigations of the freeze- and air-drying of protein drugs we have discovered a phenomenon that greatly reduces the drying time to the glassy state during vacuum-drying without having to raise the temperature. The addition of phenylalanine to aqueous sugar solutions produces vacuum-dried glasses of low residual water content and very high glass temperatures. The characterisation of the dried products using thermal analysis, x-ray diffraction and scanning electron microscopy shows how partial crystallisation of the amino acid is responsible for this improved vacuum-drying, which is now possible at 20°C and is not restricted to small volumes. The storage stability of recombinant human granulocyte-colony stimulating factor and lactate dehydrogenase within the vacuum-dried amino acid/sugar glass was good, if not quite as dramatic as that reported elsewhere [4].

2. Materials and methods

2.1. Materials

Water was double distilled from an all-glass apparatus. Maltose monohydrate (Merck, D-Darmstadt), sucrose (Südzucker, D-München) and various L-amino acids (all D-Clintec Salvia) each of pa grade, were used as received. Recombinant human granulocyte-colony stimulating factor (rhG-CSF) was obtained from Boehringer Mannheim (D-Mannheim). It has a molecular weight of 18.6 kD, and was received as a phosphate-buffered aqueous solution of 4.4 mg/ml. Lactate dehydrogenase (LDH) of molecular weight 34 kD from porcine muscle was also obtained from Boehringer Mannheim, stabilised as a suspension in 3 M NH_4SO_4. Before use this was dialysed at 4°C against 0.1 M NH_4PO_4 buffer of pH 7.0 to remove the NH_4SO_4 (cellulose membrane, MW 12 000–14 000). The LDH content was then determined by the LDH-activity test described later.

2.2. Vacuum- and freeze-drying proceedures

Solutions of sugar and amino acids were prepared by stirring with subsequent filtration through a 0.22-μm membrane. The samples were vacuum-dried in a freeze dryer (Fa. Schäfer and Hof, D-Lohra) with 0.6 m^2 shelf area. The shelf temperature was kept at a constant 20°C while the chamber pressure was reduced stepwise to 0.1 Pa. By way of comparison, some samples were also freeze-dried at a shelf temperature of −25°C during the freezing step, increasing stepwise to room temperature during primary drying. In all cases 1 ml of solution was dried in a 2-ml vial. At the end of any vacuum-drying or freeze-drying cycle the chamber was flooded with dry nitrogen and the stoppers pressed into place.

2.3. Physico-chemical characterisation of dried samples

The water content of each dried sample was determined by Karl Fischer titration (Mettler, CH-Greifensee). Their thermal behaviour was determined using a Perkin Elmer DSC 7 differential scanning calorimeter. Samples of 10–15 mg were sealed in Al pans and repeatedly cooled and heated between −50°C

Table 1
Residual water contents and glass transition temperatures (T_g) of maltose, either vacuum-dried or freeze-dried under various conditions

Process	Container	Fill volume (ml)	Drying time (h)	Shelf temperature (°C)	Water content of dried sample (% w/w)	T_g (°C)
Vacuum-dried	Vials	1	48	20	9.7 ± 0.14	3.1 ± 2.0
Vacuum-dried	Vials	1	72	20	9.5 ± 0.28	5.0 ± 1.1
Vacuum-dried	Vials	1	48	20–40	8.1 ± 0.15	13.4 ± 0.8
Vacuum-dried	Vials	1	48	20–50	6.4 ± 0.18	20.3 ± 0.7
Freeze-dried	Vials	1	–	–	1.02 ± 0.11	83.8 ± 1.1
Vacuum-dried	Vials	0.5	48	20–50	5.3 ± 0.18	29.9 ± 0.7
Vacuum-dried	Glass plate		48	20	5.7 ± 0.09	33.7 ± 0.8
Vacuum-dried	Glass plate	–	48	20–50	2.0 ± 0.10	76.8 ± 1.2

The vials were of 2 ml capacity and contained an aqueous maltose solution (50 mg/ml). The glass plates were coated with a film of the same maltose solution by pouring ($n = 4$).

and 120°C at 10°C/min. T_g was calculated as the midpoint of the respective transition. A measure of sample crystallinity was obtained using wide angle X-ray diffraction (WAXS, PW 1730, Phillips, D-Kassel; Cu-K_α radiation of $\lambda = 0.15418$ nm; 40 kV). Dried samples of 300 mg were examined at 25°C ± 1°C. The ultrastructure of the dried samples was examined using scanning electron microscopy (SEM) on a Zeiss DSM 902 microscope. Each sample was fixed to an Al sample holder and gold sputtered.

2.4. Storage stability of rhG-CSF and LDH

Solutions of sugar and amino acid were prepared as described above, also containing either 0.35 mg rhG-CSF or 165 units LDH per 1 ml fill-volume in a 2-ml vial. The samples were either vacuum- or freeze-dried, and the dried products stored at 4, 30, 40 50 and 60°C. Samples were examined at various times up to 1 year's storage.

The stability of the rhG-CSF in the dried products was assessed by determining of the amounts of monomer and aggregated dimer using size exclusion chromatography (SEC-HPLC). Each dry sample was first reconstituted in water and 20 μl injected into a Shimadzu HPLC with refrigerated autosampler (Waters TM 717). A TSK-Gel G 2000 SW (7.5 × 300 mm) column was used (Toso Haas) with detection at 214 nm (Shimadzu LC-GA). The mobile phase was 0.1 M Na/K-phosphate buffer of pH 6.2, with a flow rate of 0.6 ml/min at room temperature. Under these conditions the retention time of the monomer was 14.5 min against an rhG-CSF external standard. With LDH a direct determination of enzymatic activity was possible. Potassium phosphate buffer (2.5 ml 0.1 M, pH 7.0), pyruvate (0.1 ml 0.02 M) and NADH (0.05 ml 0.011 M) were mixed at 25°C in a plastic cuvette. To this 0.05 ml of the test LDH-solution was added, and, after mixing, the extinction was measured at $\lambda = 365$ nm over a period of 5 mins (Perkin-Elmer 552 UV/VIS photometer). The enzymatic activity [U/ml] was then calculated from the rate of change in extinction pro min, $\Delta E/\Delta t$, using: U/ml = ($\Delta E/\Delta t$•Volume of solution)/(Extinction coefficient · Pathlength · Volume of LDH-solution).

3. Results and discussion

3.1. Vacuum-drying of pure maltose

Vacuum-drying at 20°C of 1 ml of an aqueous maltose solution (50 mg/ml) in 2 ml vials did not produce satisfactory results. Even after 48 h drying time the maltose still contains > 9% water, resulting in a T_g of only 3°C (Table 1). Increasing the drying time to 72 h barely improves this result. An increase in the shelf temperature up to 50°C produces a better product, but still leaves > 6% water giving a hardly adequate T_g (Table 1). These vacuum-dried maltoses remain, therefore, in the rubbery state at room temperature, whereas adequate stabilisation of proteins requires rapid transition to a glass during drying. A glass is readily achieved by freeze-drying the maltose solution, yielding a T_g of > 80°C (Table 1), in agreement with literature values [12]. Only by reducing the fill-volume of the vials (Table 1) could a better vacuum-dried product be obtained, now having a T_g of almost 30°C. Previous success with the air-drying of protein/sugar solutions has been achieved with small liquid volumes, for example a 'few' μl [7], 50 μl [8] or 25 μl–1000 μl [9]. The smaller the liquid layer thickness, the shorter will be the diffusional pathlength of water to the rubber/air interface where evaporation occurs. The rate of water loss will, therefore, be inversely proportional to layer thickness in a vial [13], and reduced fill-volume will reduce the time taken to reach the glassy state under given conditions. We confirmed this by drying a thin layer (≈ 1 mm) of the maltose solution poured on to a glass plate, a technique essentially the same as drying small volumes

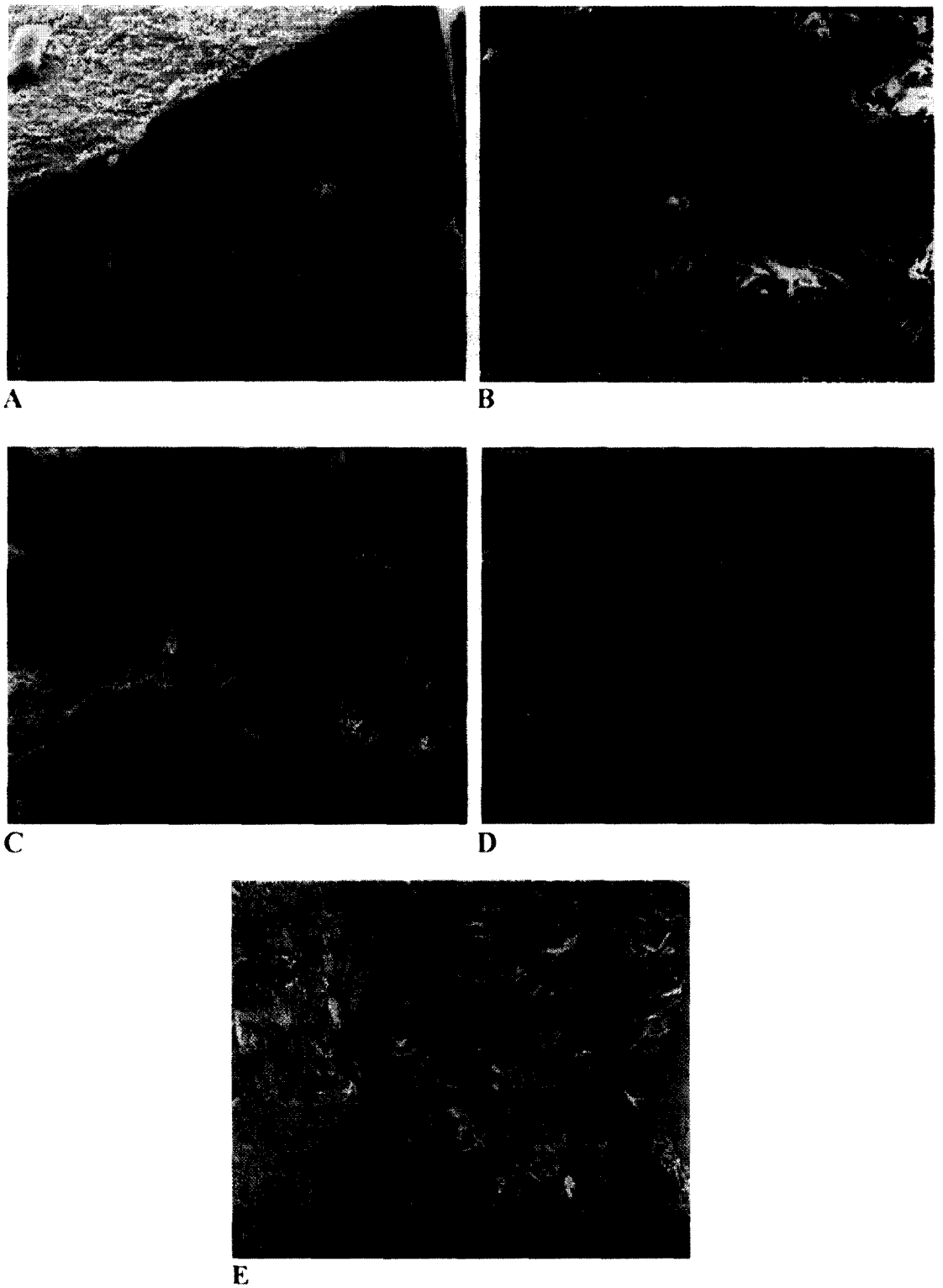


Fig. 1. Scanning electron micrographs of various vacuum-dried and freeze-dried products. (a) Vacuum-dried pure maltose, (b) freeze-dried pure maltose, (c) vacuum-dried phenylalanine/maltose (2:5), (d) vacuum-dried pure phenylalanine and (e) vacuum-dried tryptophan/sucrose (2.5:5). Initial solution concentrations were 50 mg/ml sugar plus amino acid, or 50 mg/ml amino acid. Fill volumes were 1 ml in 2 ml vials. The drying conditions are given in Materials and Methods. Note scale bars and magnification (×).

[7–9]. The resulting residual water content of > 6% for a drying temperature of 20°C (Table 1) yields a maltose glass at room temperature with a higher T_g. Especially an increase in drying temperature up to 50°C produces a substantially higher T_g, this being an already known air-drying process [9]. The product still did not, how-

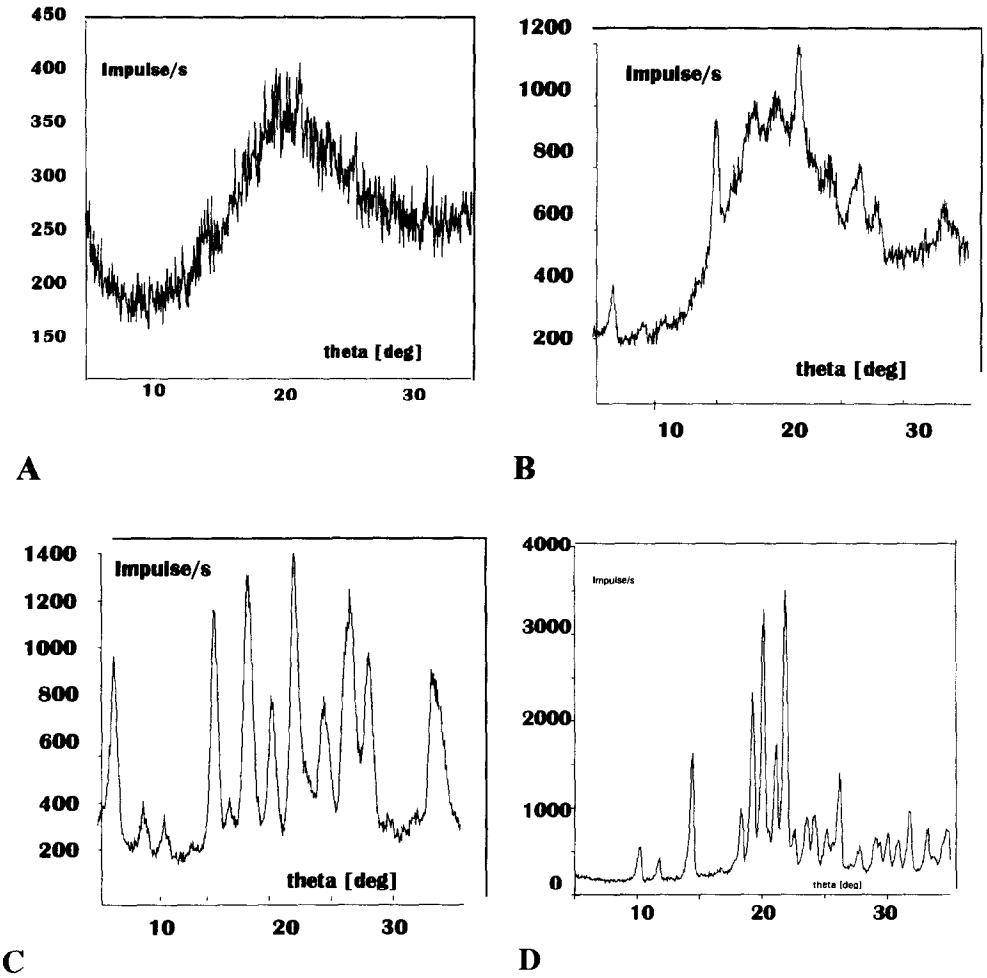


Fig. 2. Wide angle X-ray diffraction patterns of various products. (a) Pure vacuum-dried maltose, fully amorphous, (b) vacuum-dried phenylalanine/maltose (1:5), partially crystalline, (c) Pure phenylalanine, fully crystalline and (d) pure maltose monohydrate, fully crystalline. Theta is the diffraction angle during measurement.

ever, reach the level of dryness achieved with freeze-drying the maltose solution (Table 1).

This generally unsatisfactory behaviour arises because a non-porous monolith is formed by the supersaturated maltose solution during vacuum-drying, as shown by the SEM in Fig. 1a. The corresponding X-ray diffraction pattern (Fig. 2a) shows the characteristic halo typical of a fully amorphous structure. Under the vacuum-drying conditions used (viz, 0.1 Pa, 20°C) sufficient water is removed after 48 h drying only to form the rubbery state. The critical point is that the drying rate of the rubbery monolith (water loss/h) within the vial will be low because of its large layer thickness (≈ 150 μm in Fig. 1a) and its small surface/volume ratio. The time required to reach the glass transition [4] under these conditions thus exceeds the 48 h used, and the product's T_g remains below room temperature. This fundamental problem with vacuum-drying of large volumes of sugar solutions can be clearly appreciated by comparing the SEMs of the vacuum-dried (Fig. 1a) and freeze-dried maltose (Fig. 1b). The latter has a highly porous structure, as a result of sublimation during primary drying of the phase-separated ice [5]. The filaments appear to be some 10 μm thick, and will clearly have a much larger surface/volume ratio than the vacuum-dried monolith. Similar ultrastructures have been seen with freeze-dried sucrose [14], where filament thickness was only some 2 μm, but this may depend on freeze-drying conditions. Its X-ray diffraction pattern (not shown) is identical to that in Fig. 2a for the vacuum-dried maltose, but it has a T_g in Table 1 of > 80°C. The small filament thickness and large surface/volume ratio of this amorphous maltose phase allows the reduction of its residual water content during secondary drying to $\approx 1\%$.

3.2. Vacuum-drying of phenylalanine/maltose

The addition of phenylalanine (Phe) to the maltose solution produces a dramatic improvement in vacuum-drying under the same conditions (viz, 0.1 Pa, 20°C). Fig. 3a shows how an increase in the amount of Phe

added to 50 mg/ml of maltose reduces linearly the residual water content of the vacuum-dried product to below 1% w/w. At a weight ratio Phe/maltose of 1:5 the T_g lies well above 80°C. Further increase in the weight ratio of Phe up to 2:5 does not reduce the residual water content further, and T_g stays in the range 80–90°C. These values at $\approx 1\%$ water correspond with the T_g of 94°C cited for fully-dried, amorphous maltose [4]. The Phe/maltose mixtures $\geq (0.75{:}1)$ are thus in the glassy state at room temperature. Recall that these vacuum-drying conditions gave only a rubber with pure maltose (cf. Table 1).

The X-ray diffraction pattern for the Phe/maltose (1:5) mixture in Fig. 2b clearly shows numerous crystalline peaks superimposed on the amorphous halo. Fig. 2b gives a degree of crystallinity of $\approx 10\%$ (calculated from relative peak areas), this value lying towards the bottom end of the resolution range for wide-angle X-ray diffraction [15]. This crystallinity originates with the Phe, as can be appreciated from a comparison with X-ray diffraction patterns of fully-crystalline Phe and maltose. Thus, the positions of the crystalline peaks in Fig. 2b correspond exactly with those of fully-crystalline Phe (Fig. 2c), for example the characteristic double peak of Phe between 25°C and 28°C. Also, none of the peaks of fully-crystalline maltose monohydrate in Fig. 2d can be identified in Fig. 2b. The SEM of the Phe/maltose mixture shows the remarkable picture seen in Fig. 1c. Whereas the pure maltose was monolithic (Fig. 1a), the presence of Phe leads to a heterogeneous, porous ultra-structure. A scaffolding-like structure of anatomising, needle-shaped crystals is visible, with amorphous material forming a pellicle and filling partially the spaces within the scaffolding. The crystals have the same appearance as those of fully-crystalline Phe shown in Fig. 1d. We conclude that the mechanism of drying of the Phe/maltose solution differs from that of a sugar solution [4]. The evaporative loss of water increases the concentrations of the maltose and Phe from their initial values of 50 mg/ml and ≥ 7.5 mg/ml (cf. Fig. 3a). The aqueous solubility of Phe (≈ 29 mg/ml at 20°C) is reduced by the presence of equivalent concentrations of disaccharides [16]. The increasing maltose concentration will, therefore, continually depress the solubility of the Phe in the remaining solution, until at some point the Phe begins to crystallise. The continued loss of water and formation of a super-saturated maltose rubber [11] depresses the solubility further, causing the Phe crystals to grow and form the structure seen in Fig. 1c. The maltose rubber coats the Phe crystal scaffolding forming a pellicle. The vacuum-dried Phe/maltose is, therefore, porous, and its structure resembles that of freeze-dried (Fig. 1b) rather than vacuum-dried pure maltose (Fig. 1a). Judging from Fig. 1c, the pellicle of maltose that coats the Phe is only ≤ 2 μm thick, and the diffusional pathlength for water will be short, yielding a higher drying rate than found with the pure monolithic maltose. Additionally, the surface area for evaporation is larger and under given conditions the glass transition of the maltose will be reached more rapidly. The result is a Phe crystal scaffold covered with a pellicle of dry ($w_g < 1\%$) maltose glass with a $T_g > 80$°C.

If this explanation is correct, then Phe increases the drying rate (water loss/h) of the maltose rubber. The rate of change in T_g during vacuum-drying (1 ml fill-volume in 2 ml vials, 0.1 Pa, 20°C) is shown in Fig. 3b, as determined by examining samples at regular time intervals. The T_g of the pure maltose rises only to ≈ -10°C after 20 h drying time, reflecting the high residual water content of 12% of these samples. Judging from the shape of the $T_g(t)$-curve, the glass transition cannot be reached under these drying conditions. With Phe/maltose (1:5), however, the T_g of the maltose in-

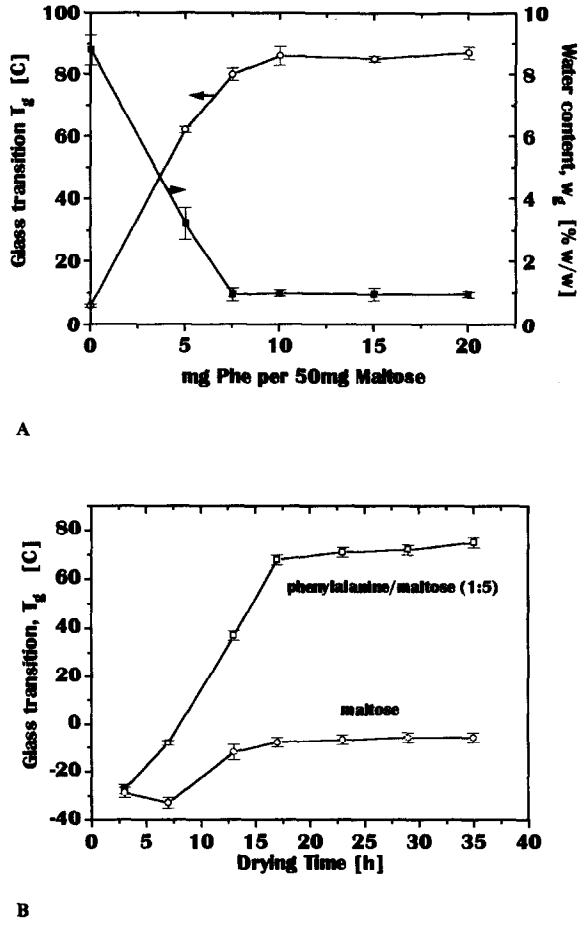


Fig. 3. (a) Residual water content, w_g, and glass transition temperature, T_g, of vacuum-dried phenylalanine/maltose mixtures in dependence of their phenylalanine content. Each initial solution contained 50 mg/ml maltose plus increasing amounts of phenylalanine. Fill volumes were are 1 ml in 2 ml vials ($n = 4$). (b) Change in glass transition temperature, T_g, during vacuum-drying of pure maltose (50 mg/ml) and phenylalanine/maltose (1:5). Fill volumes were 1 ml in 2 ml vials ($n = 4$).

Table 2
Residual water contents and glass transition temperatures (T_g) of various vacuum-dried and freeze-dried systems

Process	Components	Weight ratio	Water content of dried sample (% w/w)	T_g (°C)
Vacuum-dried	Sucrose	–	10	−6.5
Vacuum-dried	Phenylalanine/Sucrose	2:5	1.1 ± 0.17	63 ± 0.8
Vacuum-dried	Tryptophan/Sucrose	0.8:5	7.2 ± 0.5	14.5 ± 1.5
		1.25:5	5.5 ± 0.1	22.5 ± 1.0
		2.5:5	3.1 ± 0.4	37 ± 2.0
Vacuum-dried	Arginine/Maltose	1:5	8.5 ± 0.3	8.0 ± 2.0
		2:5	8.7 ± 0.2	7.0 ± 2.0
Freeze-dried	Phenylalanine/Maltose	1:5	0.85 ± 0.11	93 ± 0.7
		2:5	0.79 ± 0.05	92 ± 0.7

Fill-volume = 1 ml in 2 ml vial ($n = 4$). Vacuum-drying conditions were 0.1 Pa, 20°C for 24 h.

creases to over 70°C after 15 h. This illustrates dramatically the importance of the time taken to reach the glass state for air-drying [4], and how it is reduced greatly under the conditions used by inclusion of Phe. This remarkable improvement in vacuum-drying does not necessitate a higher drying temperature [8,9] and, more importantly, it apparently allows large volumes (≥ 1 ml) to be effectively dried.

3.3. Does this phenomenon occur with other sugars or amino acids?

As seen in Table 2, pure sucrose (1 ml fill-volume in a 2-ml vial) could also not be vacuum-dried satisfactorily after 24 h at 0.1 Pa and 20°C, and formed a rubbery state at room temperature. The addition of Phe to the sucrose (2:5) effects the same improvement in drying as seen with maltose, and T_g rises to > 60°C at a residual water content of $\approx 1\%$ w/w. This quite high T_g compares with 57–69°C for fully-dried sucrose [17]. The use of Phe to improve vacuum-drying under the conditions used here is, therefore, not restricted to maltose. Indeed, one expects that the physical nature of the crystallisation within the sugar rubber will make its action unspecific; the second component just crystallises as a scaffolding for the amorphous component. We examined briefly the plausibility of this supposition using some other amino acids. L-tryptophan (Trp) reduced, for example, the residual water content of sucrose to $\approx 3\%$ at a weight ratio Trp/sucrose of (2.5:5), giving a T_g of 37°C (Table 2). The mechanism of improved vacuum-drying here is to be the same as that with Phe/maltose, since the Trp/sucrose mixtures also have partially crystalline X-ray diffractograms (not shown) and a porous internal structure under SEM (Fig. 1e). We surmise that the flake-shape of the Trp crystals leads to slower vacuum-drying and higher residual water contents than with Phe, owing to their smaller surface area as a scaffolding.

No improvement in vacuum-drying is seen with non-crystallising amino acids, since these clearly do not form a scaffolding within the rubber phase. Mixtures of arginine/maltose, for example, show residual water contents similar to that obtained with pure maltose ($\approx 9\%$) and their T_g remain well below room temperature (Table 2). These products are fully amorphous and give X-ray diffraction patterns and SEMs identical to that for pure vacuum-dried maltose (cf. Fig. 1a).

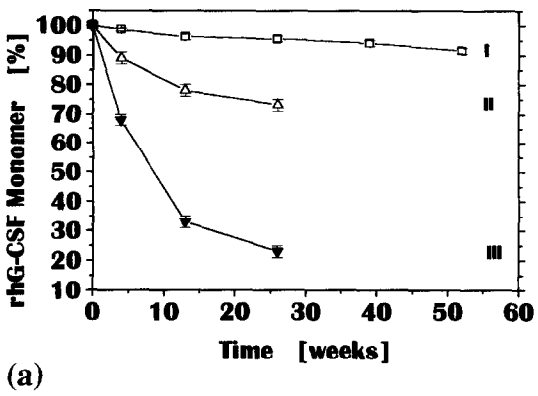
The addition of Phe produces only a minimal reduction in water content for freeze-drying of maltose (Table 2). The existence of Phe crystals was proven by X-ray diffraction (not shown). A highly-porous structure is, however, already formed during freezing and primary drying (cf. Fig. 1b), independent of the presence of Phe.

3.4. Stability of rhG-CSF and LDH

The extent of rhG-CSF degradation during vacuum-drying and subsequent storage was determined from the reduction in area of the native protein peak on the SEC-chromatogram. This was always accompanied by the appearance of small peaks immediately prior to or after the native peak. Thus the proportion of monomer of non-protected rhG-CSF decreases after vacuum-drying (20°C, 0.1 Pa, 24 h, 1 ml fill-volume in 2 ml vial) on storage at 40°C, leaving only 75% intact protein after 26 weeks (Fig. 4a). Aggregates and degradation products were clearly evident using SDS-Page with silver-stain colouring. Previous studies have already demonstrated large protein destabilisation during air-drying (using phosphofructokinase [8]) or on storage at 35°C after air-drying (using LDH [11]) without sugar protectants. rhG-CSF shows only the latter instability, and survives the vacuum-drying process initially intact. It was also vacuum-dried under the usual conditions in a maltose/Phe/arginine (5:1:1) formulation with residual water content of $\approx 1\%$ w/w and a T_g of > 80°C. This formulation shows identical behaviour to the Phe/maltose (1:5) system, the arginine dissolving in the maltose glass. After 1 year's storage at 40°C (Fig. 4a) more than 93% of rhG-CSF monomer could still be found in the

reconstituted aqueous solution, which was completely free of turbidity. In contrast to this stabilising action of the glass, the stability of rhG-CSF in a vacuum-dried maltose rubber is poor (Fig. 4a), the residual protein falling to below 30% after just 26 weeks' storage at 40°C. Non-arrhenius kinetics may govern reaction rate in the rubbery state [4,10], making long-term storage of the protein highly damaging.

The X-ray diffraction pattern of the vacuum-dried maltose/Phe/arginine glass remained unchanged during the 52 weeks' storage at 40°C, and we conclude that no substantial change (say $\pm$ 5% [15]) in the mixed amorphous/crystalline structure occurred. The water content increased with time, however, from 1% w/w up to some 1.2% w/w for the sample stored at 4°C, but up to 2.4% for those stored at the higher temperatures. As the vials were sealed in a N_2-atmosphere immediately after drying, this can only be a result either of sorption of water released from the rubber stoppers, or the stoppers not giving a hermetical seal [18]. Whatever the cause, the increased water contents effected reductions in T_g, from > 80°C to ≈ 70°C in the worst case for the samples



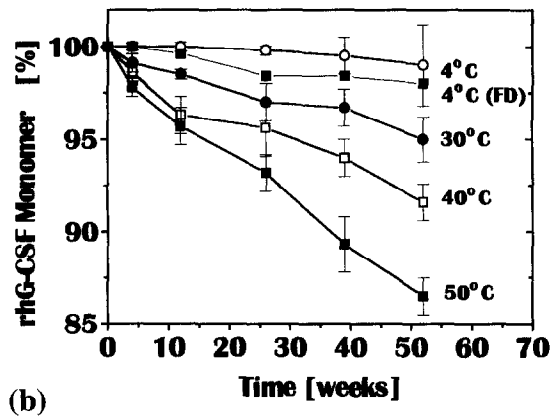


Fig. 4. Storage stability of rhG-CSF. (a) Selected formulations stored at 40°C. I = vacuum-dried maltose/Phe/arginine (5:1:1), II = pure rhG-CSF and III = vacuum-dried pure maltose (T_g = 9.4°C). (b) Vacuum-dried maltose/Phe/arginine (5:1:1) stored at 4, 30, 40 and 50°C. The same formulation freeze-dried and stored at 4°C is also shown. In all cases $n = 4$. Note different y-axis scales in (a) and (b).

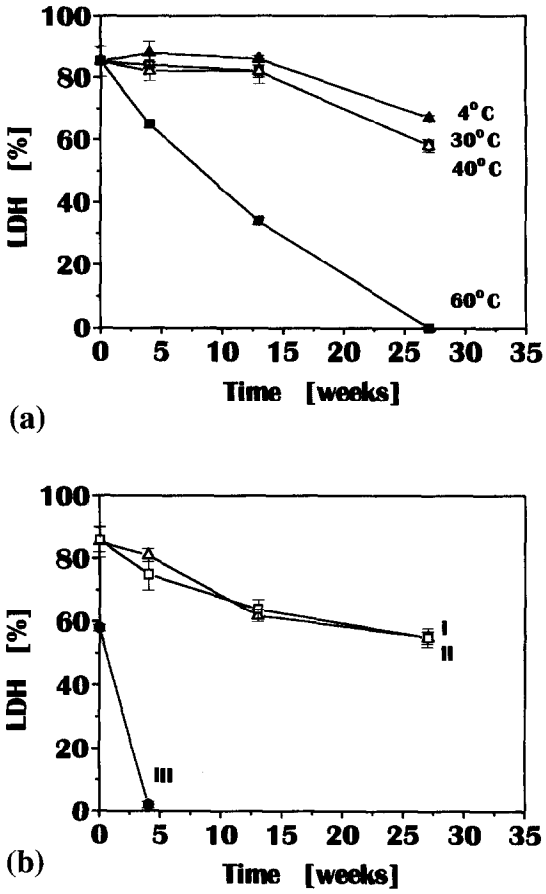


Fig. 5. Storage stability of LDH. (a) Vacuum-dried maltose/Phe/arginine (5:1:1) stored at 4, 30, 40 and 60°C. (b) Selected formulations stored at 40°C. I = freeze-dried maltose/Phe/arginine (5:1:1), II = vacuum-dried maltose/Phe/arginine (5:1:1) and III = pure LDH. In all cases $n = 4$.

stored at ≥ 30°C. Despite T_g remaining much higher than storage temperature there is a marked temperature dependence of the rhG-CSF storage stability in the glass (Fig. 4b). The degradation rate increases with higher storage temperature from 4 - 50°C, still well below T_g. Above T_g (i.e., in the rubbery state) chemical degradation has been found to correlate with Williams-Landel-Ferry-type kinetics [19], depending on $(T - T_g)$ rather than T. Below T_g, it may, however, be more appropriate to scale reaction rates with Vogel-Tammann-Fulcher-type kinetics [15], which specify that molecular mobility does not cease immediately below T_g in the glassy state [10]. The storage stability of the rhG-CSF at 4°C in the vacuum-dried formulation is slightly better than that in the same formulation after freeze-drying (Fig. 4b). A similar phenomenon has been found with LDH [11] and attributed to the longer time to reach the glass transition during freeze-drying [4].

LDH has been air-dried in small volumes of a sucrose/epichlorohydrin copolymer solution and found to retain 98% residual activity after 26 weeks' storage at ambient temperature [9], although the scatter in the

results is large. The stability of LDH in our vacuum-dried maltose/Phe/arginine formulation is not so good. Fig. 5a shows $\approx 85\%$ active LDH immediately after vacuum-drying ($t = 0$) at 20°C for 24 h, compared with 100% in the cited study [9]. In the range 4–40°C (Fig. 5a) temperature has little effect on LDH stability, and the maltose/Phe/arginine formulation ($T_g = 81$°C) is much more stable than pure LDH (Fig. 5b) which is completely inactivated after 4 weeks at 40°C. There is, however, a dramatic reduction in stability at 60°C (Fig. 5a). The inferior stability of LDH in the maltose/Phe/arginine glass compared with that in an air-dried sucrose-copolymer [9] occurs despite the former's much higher T_g. This may be a result of the longer time taken to reach the glassy state (10 h in Fig. 3b) than that with the small volumes in the previous study [9]

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