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RESEARCH ARTICLE

Formulation of Proteins in Vacuum-Dried Glasses. II. Process and Storage Stability in Sugar-Free Amino Acid Systems

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ABSTRACT

The purpose of this research was to investigate the freeze- and vacuum-drying behavior of L-amino acids of current/potential use as adjuvants for formulating proteins. The analytical methods used were wide-angle x-ray diffraction, differential scanning calorimetry, and scanning electron microscopy. Protein analysis was performed either as an activity assay (lactate dehydrogenase [LDH]) or by size-exclusion chromatography (granulocyte colony-stimulating factor [rhG-CSF]). After samples were freeze-dried, only the four basic amino acids (arginine, lysine, histidine, and citrulline) formed amorphous solids, which, however, were partially crystalline. The remaining amino acids all formed fully crystalline solids. After samples were vacuum-dried, (20°C, 0.1 mbar, 1 ml fill volume in 2-ml vials) fully crystalline solids were formed by all of the amino acids. For arginine, the addition of either HCl, H_3PO_4, or H_2SO_4 sufficient to form the respective salt produced amorphous solids after vacuum-drying, but they had high residual water contents and low glass transition temperatures (T_g). Addition of phenylalanine to arginine base inhibited crystallization of the latter at low concentrations during vacuum-drying procedure, leading to formation of a pure rubbery solid. At higher concentrations the phenylalanine crystallized, producing dry products with glass transition temperatures of >60°C. The process and storage stability of LDH and rhG-CSF in the vacuum-dried phenylalanine/arginine glasses was greatly improved at temperatures up to 40°C compared with the unprotected proteins. Uptake of moisture during storage was, however, a complicating factor, reducing T_g, promoting crystallization, and leading to decreased protein stability. The PO_4 salt of arginine produced especially high glass transition temperatures after it was vacuum-dried. These sugar-free amino acid formulations thus are potential stabilizes for proteins.

KEY WORDS**:** *Amino acid; Freeze-drying; Phosphoric acid; Protein stabilization; Vacuum-drying.*

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INTRODUCTION

A number of amino acids are frequently cited as being suitable bulking agents for freeze-dried formulations. There exists some evidence that certain amino acids also act as cryoprotectants. These protect sensitive active agents, especially peptides and proteins, against various destabilizing effects occurring in aqueous solution during the initial freezing phase, e.g., freeze concentration and pH shifts (1). Carbohydrates, amino acids, glycerol, and polyethylene glycols (2) are all thought to act by their so-called preferential exclusion from the protein/water interface (3). This maintains native protein structure, provided the cryoprotectants are present in sufficient quantities (4). There is no evidence that amino acids alone (i.e., not combined with carbohydrates) can also act as lyoprotectants, which protect proteins against physical and chemical instabilities in the dried state after they are freeze-dried. Indeed, it is carbohydrates, especially disaccharides such as sucrose, maltose, and trehalose, which are the most effective lyoprotectants (5).

It was found previously that vacuum-drying could be improved by the judicious combination of disaccharides with the crystallizing amino acid phenylalanine (Phe), to produce partially amorphous phases having residual moisture of $<1.5\%$ (6). Proteins could be rendered storage stable at room temperature in these vacuum-dried sugar/amino acid glasses. The question then arose whether pure amino acids also form amorphous glasses of sufficiently low residual moisture and high glass transition temperature when vacuum- or freeze-drying procedures are used. If so, do the amino acids act as lyoprotectants and can proteins be stabilized in such sugar-free systems? The literature indicates that glycine, for example, crystallizes during freeze-drying (7), its behavior being dependent on pH and salt form. The literature did not, however, yield a comparative study of a wider spectrum of amino acids regarding their behavior during freeze-drying. In this paper we identify those amino acids which form amorphous glasses during either freeze- or vacuum-drying procedures. The importance of salt forms for the glassy amino acids is a feature of this work, as is the use of mixed amino acid systems to reduce residual water content. The lyoprotective ability of the vacuum-dried amino acid glasses was investigated using lactate dehydrogenase (LDH) and recombinant human granulocyte colony-stimulating factor (rHG-CSF).

MATERIALS AND METHODS

All of the L-amino acids used were of analysis grade and obtained from either Merck (Darmstadt, Germany), Sigma (Munich, Germany), Clintec Salvia (Frankfurt, Germany), or Degussa (Frankfurt, Germany). Hydrochloric and phosphoric acids were obtained from Merck. Water was double-distilled from an all-glass apparatus. LDH and rhG-CSF were used as described previously (6).

Preparation and Physicochemical Characterization

All solutions of the amino acids investigated were first filtered through a 0.22-μm membrane filter and filled into 2 ml vials. These were then either freeze- or vacuum-dried using a freeze-dryer with 0.6 m^2 shelf area (Schäfer & Hof, Lohra, Germany), as described previously (6). Vacuum-drying was conducted for 24 hr at 20°C and down to 0.1 mbar. Each 2 ml vial contained 1 ml of solution, the rubber stoppers being pressed into place at the end of the drying cycle after the chamber was flooded with dry air. The freeze-drying conditions were as follows: shelf temperature −25°C, primary drying at 0.1 mbar, secondary drying at shelf temperature rising to 10°C and at 0.01 mbar.

Karl Fischer (Mettler, Greifensee, Switzerland) titration was used to determine the residual water content of each dried sample. Glass transition and crystallization temperatures were determined by differential scanning calorimetry (DSC 7, Perkin-Elmer, Munich, Germany). Samples (10–15 mg) in sealed Al pans were repeatedly cooled and heated at 10°C/min in the range −50–150°C. The crystallinity of the dried samples was examined using wide-angle x-ray diffraction (PW 1720, 40 kW, Cu-Kα, $\lambda = 0.15418$ nm; Phillips, Kassel, Germany). Powdered samples (300 mg) were examined at 25 ± 1°C. The dried samples were also examined using scanning electron microscopy (SEM, DSM 902, Zeiss, Germany) after gold sputtering was performed in an Al sample holder.

Stability of LDH and rhG-CSF in Dried Amino Acids

Solutions of the amino acids of interest were prepared containing either 165 units LDH/ml or 0.35 mg rhG-CSF per 1 ml of solution. After vacuum-drying was performed, these samples were stored at various temperatures and examined periodically during up to 9 months 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 was injected into an HPLC system (Shimadzu, Munich, Germany) with a re-

frigerated autosampler (TM 717, Waters, Frankfurt, Germany). A TSK-Gel G 2000 SW (7.5 × 300 mm) column was used (Toso Haas) with detection at 214 nm (LC-GA, Shimadzu). 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 reduced nicotinamide adenine dinucleotide (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 the solution was mixed, the extinction was measured at $\lambda = 365$ nm over a period of 5 min (552 UV/VIS photometer, Perkin-Elmer). The enzymatic activity (U/ml) was then calculated from the rate of change in extinction pro min, $\Delta E/\Delta t$, using

$$\begin{aligned} \text{U/ml} = {} & (\Delta E/\Delta t \cdot \text{volume of solution})/ \\ & (\text{extinction coefficient} \cdot \text{pathlength} \\ & \cdot \text{volume of LDH solution}) \end{aligned} \quad (1)$$

RESULTS AND DISCUSSION

Freeze-Drying of Amino Acids

Only the four basic amino acids (lysine, arginine [Arg], histidine, and citrulline) showed conspicuous glass transitions after they were freeze-dried. For the example of L-arginine, we found 1.2% w/w residual water and a glass transition at $T_g = 42$°C, followed by an exothermic crystallization peak at $T_c = 61$°C. This behavior is typically seen with amorphous phases of, for example, carbohydrates (8). All of the other amino acids examined (Table 1) showed no detectable T_g or T_c and were apparently fully crystalline after being freeze-dried under the conditions used here. This finding underscores the frequently cited use of amino acids as crystalline bulking agents (2) [for example, glycine (6)]. Wide-angle x-ray diffraction reveals that the four basic amino acids were not, however, fully amorphous directly after being freeze-dried. The x-ray diffractogram of the freeze-dried L-arginine [Fig. 1(a)], for example, shows several crystalline reflections superimposed on the characteristic amorphous halo. The positions of these peaks are identical to those of anhydrous crystalline L-arginine (not shown). The degree of crystallinity in Fig. 1(a) is approximately 10%, which lies within the range quantifiable using standard methods of x-ray diffraction (8). Heating to above a sample's T_c produced spontaneous conversion to the fully crystalline form of the respective basic amino acid, as could be confirmed from its x-ray diffractogram. Thus, under the standard freeze-drying conditions used here, the four basic amino acids form amorphous solids, but with some crystalline content.

The addition of mineral acids to the L-arginine base solution before freeze-drying was performed greatly altered the properties of the dried product. The amount of acid added was chosen to give the correct ratio of Arg base to acid required to form the corresponding salt. An equimolar solution of L-arginine and HCl is thus assumed to form Arg Cl salt, and resulted in a more than doubled residual water content, and T_g was accordingly sharply reduced from 42 to 18°C (Table 2). The use of a third molar part of H_3PO_4 in the L-arginine solution (Arg PO_4) also resulted in a higher residual water content than with the base, but there was a dramatic increase in T_g from 42 to 93°C (Table 2). In addition, the dried product of Arg PO_4 was fully amorphous [Fig. 1(b)], as there were no signs of the crystalline reflections seen with Arg base [Fig. 1(a)]. The large increase in T_g is, therefore, independent of the change in residual water content, indicating clearly that Arg base and Arg PO_4 have quite different abilities as glass-formers. Presumably, the higher water solubility of a salt compared with the base reduces nucleation tendencies during freezing. Indeed, salt forms of glycine were also found to crystallize more slowly than the base (7).

Vacuum-Drying of Amino Acids

In contrast to the primary drying stage of freeze-drying, water loss during vacuum-drying is by evaporation. The properties of the dried product (state and residual water content) are determined essentially by the drying pressure, temperature, and time (9), as well as the volume to be dried in a specific container (6). Carbohydrates were found to form amorphous structures after being vacuum-dried under the same conditions used here, although they remained as rubbers, owing to high residual water content (6). The amino acids that crystallized here during freeze-drying also crystallized during vacuum-drying. The residual water contents for the two processes (Table 1) do not differ within the experimental error expected using Karl Fischer titration of these small amounts of water (2% w/w residual water ≈ 1 mg water). The vacuum-drying conditions fortuitously were good enough to give the same results as the freeze-drying conditions. In each case the x-ray diffractogram was identical to that of the fully crystalline amino acid, as with freeze-drying, and no T_g or T_c was discernable on the DSC scan (result not shown). Differences between freeze- and vacuum-drying only emerge with the four basic amino acids, which

Table 1

Results of Freeze-Drying or Vacuum-Drying Aqueous Amino Acid Solutions

	After Freeze-Drying		After Vacuum-Drying	
Amino Acid	Residual Water Content (% w/w)	DSC Behavior	Residual Water Content (% w/w)	DSC Behavior
Glycine	1.1 ± 0.1	Crystalline	0.8 ± 0.2	Crystalline
L-Alanine	0.9 ± 0.1	Crystalline	0.8 ± 0.2	Crystalline
L-Valine	0.7 ± 0.2	Crystalline	0.6 ± 0.2	Crystalline
L-Leucine	1.3 ± 0.2	Crystalline	1.6 ± 0.3	Crystalline
L-Isoleucine	0.8 ± 0.3	Crystalline	1.1 ± 0.3	Crystalline
L-Serine	0.6 ± 0.3	Crystalline	0.4 ±0.1	Crystalline
L-Threonine	1.1 ± 0.2	Crystalline	0.5 ± 0.2	Crystalline
L-Cysteine	7.8 ± 0.1	Crystalline	2.6 ± 0.8	Crystalline
L-Lysine	0.9 ± 0.1	T_g = 68 ± 2.1	0.8 ± 0.2	Crystalline
L-Arginine	1.3 ± 0.2	T_g = 42 ± 2	0.5 ± 0.1	Crystalline
L-Histidine	2.8 ± 0.1	T_g = 37 ± 5.6	0.8 ± 0.2	Crystalline
L-Citrulline	1.8 ± 0.1	T_g = 64 ± 0.7	4.9 ± 0.1	Crystalline
L-Proline	0.7 ± 0.1	Crystalline	1.9 ± 0.4	Crystalline
L-Phenylalanine	1.9 ± 0.1	Crystalline	1.5 ± 0.1	Crystalline
L-Methionine	0.9 ± 0.2	Crystalline	1.6 ± 0.4	Crystalline
γ-Aminobutyric acid	0.8 ± 0.2	Crystalline	3.0 ± 2.6	Crystalline

In all cases a 0.24 M solution was used, with the exception of L-histidine, L-isoleucine, L-leucine, and L-methionine, which were prepared as 0.12 M solutions because of their low solubility. During freeze-drying at a shelf temperature of −25°C the L-arginine and L-lysine collapsed during primary drying, and were experiments repeated at −60°C.

formed freeze-dried amorphous solids, but fully crystalline products after they were vacuum-dried (Table 1). The residual water contents, therefore, tend to be lower than those observed after the material is freeze-dried. Only L-citrulline showed a higher residual water content in the crystalline state. The lower rate of water removal from the amino acid solution during vacuum-drying allowed sufficient time for complete nucleation and crystal growth.

The addition of either HCl, H_3PO_4, or H_2SO_4 to the basic amino acid solutions to form the respective salts causes crystallization to be completely suppressed during vacuum-drying. The results, given in Table 2 for the example of L-arginine, show fully amorphous solids that are rubbers at room temperature, owing to their high residual water contents. The x-ray diffractograms (not shown) are identical to that of the fully amorphous freeze-dried Arg PO_4 shown in Fig. 1(b). As seen with freeze-drying, salt-formation reduces the tendency to nucleation; in this case, in the supersaturating amino acid solution during evaporative water loss. This effect is, however, salt specific; neither nitrate nor acetate counter ions inhibited crystallization during vacuum-drying and the dried products were fully crystalline with high residual water contents (Table 2).

Vacuum-Drying of Phe/Arg Base Mixtures

The addition of HCl, H_2SO_4, or H_3PO_4 inhibits crystallization of Arg during vacuum-drying. The dried products were, however, all still in the rubbery state at room temperature, and as such, would certainly be unsuitable for stabilizing peptides or proteins (2). The reason for the rubbery products is as follows. Under the vacuum-drying conditions used here (0.1 mbar, 20°C, 1 ml fill volume in 2 ml vial), the time required for the Arg/acid solution to reach the glass transition exceeds the 24-hr drying time. Of those factors that determine the time to the glass transition during vacuum-drying [temperature, pressure, nature and concentration of glass former, gradient of T_g (w), and surface area of evaporation (10)], it is the last-named that is responsible for this poor vacuum-drying behavior. As already found with carbohydrates, the small surface-to-volume ratio of the solution contained in the vial prevents sufficient water being lost after 24 hr at 20°C and 0.1 mbar to achieve the glassy state (6).

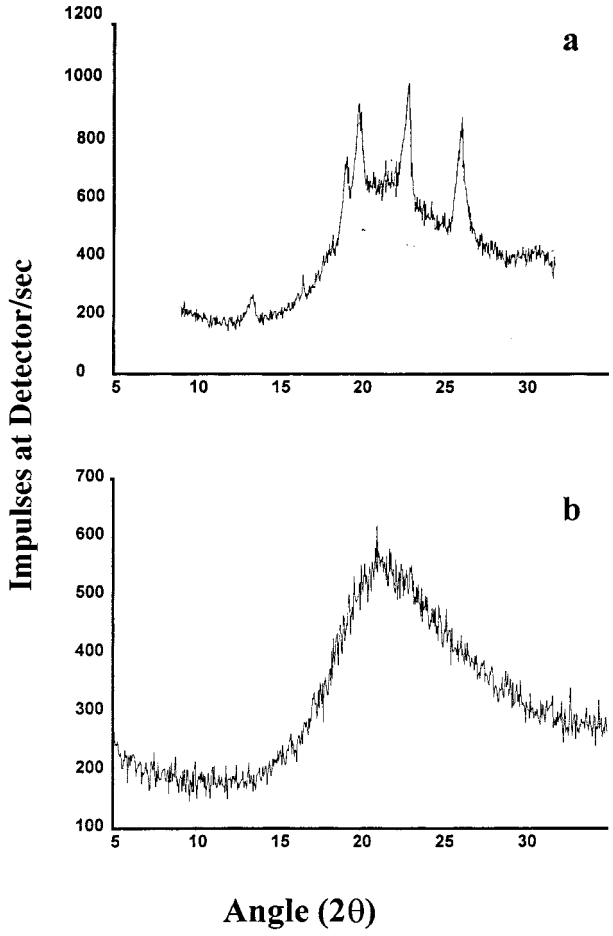

Figure 1. Wide-angle x-ray diffractograms of freeze-dried amino acids taken at 25°C. (a) L-Arg; (b) L-Arg (0.24 M) + H_3PO_4 (0.12 M).

A combination of the Arg base with Phe, both of which form fully crystalline products when vacuum-dried separately without acid (see Table 1), can, however, greatly reduce residual water content. Figure 2 shows the residual water content (w_g) and T_g of the dried Phe/Arg mixtures as a function of increasing molar proportion of Phe in the Arg. Recall that vacuum-dried Arg base is fully crystalline with a residual water content of <1% (Table 1). The addition of 1 mole-part Phe to 7 mole-parts Arg yields a clearly discernable glass transition at approximately 2°C (Fig. 2), indicating a change away from fully crystalline toward amorphous character. This is confirmed by the x-ray diffractogram for this mixture in Fig. 3(a), where crystalline peaks, identifiable from their angular positions as Arg and not Phe, lie superimposed on an amorphous halo. This small proportion of added Phe has, therefore, partially suppressed the crystallization of the Arg during vacuum-drying. Consequently, w_g jumps from <1% for pure fully crystalline Arg to approximately 12% for this partially amorphous product (Fig. 2), which as explained above cannot then be effectively vacuum-dried under the conditions used. Further increase in the mole fraction of Phe in the mixture produces, however, remarkable changes in w_g and T_g. Figure 2 shows how w_g then decreases, at first accompanied by only a marginal increase in T_g. Thus, 1 mole-part Phe to 5 mole-parts Arg is still a rubbery solid at room temperature (T_g = 2°C), but is fully amorphous [Fig. 3(b)]. The Phe completely suppresses crystallization of the Arg at and above this mole fraction. With 1 mole-part Phe to 2 mole-parts Arg, however, new crystallization peaks emerge from the amorphous halo [Fig. 3(c)], identifiable as being those of crystalline Phe. This incipient crystallization of Phe within the amorphous Arg leads to a change in behavior of the vacuum-dried Phe/Arg product. Thus, further in-

Table 2

Influence of Counter Ions on the Freeze-Drying or Vacuum-Drying Behavior of L-Arginine

	After Freeze-Drying		After Vacuum-Drying	
Arginine/Counter Ion	Residual Water Content (% w/w)	T_g (°C)	Residual Water Content (% w/w)	T_g (°C)
L-Arg base (0.24 M)	1.3 ± 0.2	42 ± 2	0.5 ± 0.1	Crystalline
L-Arg (0.24 M) + HCl (0.24 M)	3.5 ± 0.18	18 ± 0.2	6.5 ± 0.1	3.5 ± 0.3
L-Arg (0.24 M) + H_3PO_4 (0.12 M)	2.2 ± 0.07	93 ± 1	3.3 ± 0.2	5.2 ± 0.6
L-Arg (0.24 M) + H_2SO_4 (0.12 M)			3.2 ± 0.3	6.7 ± 0.3
L-Arg (0.24 M) + HNO_3 (0.24 M)			2.7 ± 0.1	Crystalline
L-Arg (0.24 M) + Ch_3COOH (0.24 M)			11.1 ± 0.8	Crystalline

Drying conditions were as in Table 1, n = 3.

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