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Research paper

Effect of process conditions on recovery of protein activity after freezing and freeze-drying

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Abstract

The objective of this research was to gain a better understanding of the degree to which recovery of activity of model proteins after freeze-drying can be maximized by manipulation of freeze-dry process conditions in the absence of protective solutes. Catalase, β-galactosidase and lactate dehydrogenase (LDH) were used as model proteins. All of the three proteins exhibited a concentration-dependent loss of activity after freezing, with significantly higher recovery at higher concentration. The freezing method and the type of buffer were also important, with sodium phosphate buffer and freezing by immersion of vials in liquid nitrogen associated with the lowest recovery of activity. Differential scanning calorimetry was predictive of the onset of collapse during freeze-drying only for β-galactosidase. For the other proteins, either no T_g' transition was observed, or the apparent glass transition did not correlate with the microscopically-observed collapse temperature. The time course of activity loss for β-galactosidase and LDH was compared during freeze-drying under conditions which produced collapse of the dried matrix and conditions which produced retention of microstructure in the dried solid. Recovery of activity decreased continuously during primary drying, with no sharp drop in recovery of activity associated with the onset of collapse. The most important drying process variable affecting recovery of activity was residual moisture level, with a dramatic drop in activity recovery associated with residual moisture levels less than about 10%. © 1998 Elsevier Science B.V.

Keywords: Catalase; β-Galactosidase; Lactate dehydrogenase; Thermal analysis; Collapse; Residual moisture

1. Introduction

Freeze-drying is often a critical unit operation in the production of protein pharmaceuticals. Much of the research emphasis in freeze-drying of proteins has dealt with the use of lyoprotectants, or solutes which improve the recovery of protein activity after freeze-drying, and this has been the subject of comprehensive reviews by Carpenter, Prestrelski, Arakawa, and coworkers [1,2]. The broad objective of the work reported here is to use model proteins to better understand the influence of processing conditions on recovery of protein activity after freezing and freeze-drying in the absence of protective solutes. Specific objectives were (1) to determine the relationship between protein concentration and recovery of activity both after freezing and after freeze-drying, (2) to measure the degree to which recovery of activity after freeze-drying can be maximized by manipulating primary and secondary drying conditions, and (3) to determine whether the onset of structural collapse during freeze-drying is necessarily associated with activity loss. This type of information is particularly relevant to freeze-drying of proteins during downstream processing of the bulk active material.

Three model proteins were chosen for this work: β-galactosidase, catalase, and lactate dehydrogenase (LDH). The criteria for choosing these proteins included partial loss of activity after freeze-drying and the availability of rapid and precise spectrophotometric methods to measure biological activity. β-Galactosidase from *Aspergillus oryzae* is a monomer with apparent molecular weight of 105 000, and has been reported to lose most of its activity after freeze-

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drying in the absence of protective solutes [3]. The reported mechanism of inactivation of β-galactosidase resulting from freeze-drying is partial unfolding followed by the formation of soluble aggregates [4]. Various excipients, such as sugars, polyols, amino acids, and amphiphilic additives have been shown to protect against activity loss during freezing, freeze-drying and storage [3,5].

Catalase contains four identical subunits, each monomer consisting of 505 amino acid residues. Hanafusa reported that the recovery of activity of catalase after freezing was 40%, while after freeze-drying the recovery was only 13% [6]. In studies reported by Pikal, pure catalase retains 80% of its initial activity after freeze-thawing, but only 30% is retained after freeze-drying [7]. Tanford's studies demonstrated that the mechanism of activity loss during freeze-drying is partial dissociation into monomers and dimers [8]. Glucose, maltose, and maltotriose showed a similar protective effect during freeze-drying, while saccharides with longer glucoside chains showed a diminishing protective effect with increasing molecular weight [7].

Lactate dehydrogenase, which is also composed of four identical subunits, tends to lose its activity after either freezing or freeze-drying, and has been used as a model protein for distinguishing between the protective effects of solutes against freezing and drying-induced denaturation [9]. Izutsu and co-workers reported concentration-dependent loss of activity of LDH after freezing which was less than 10% of the original activity at protein concentrations less than 0.01 mg/ml [10]. The same investigators reported recovery of activity after freeze-drying of LDH which was significantly higher than the recovery after freeze-thawing.

2. Experimental

2.1. Materials

Catalase from bovine liver and β-galactosidase from *Aspergillus oryzae* were used as received from Sigma Chemical Co. (St.Louis, MO). Lactate dehydrogenase (Type II, from rabbit muscle) crystalline suspension in ammonium sulfate solution (Sigma) was dialyzed at 4°C for 24 h against sodium phosphate solution (0.05 M, pH 7.4). The final concentration was determined using a kit supplied by Sigma based on the Lowry method. Bovine serum albumin was used as a standard.

O-Nitrophenyl β-D-galactopyranoside (ONPG), the total protein determination kit, and the LDH assay kit were from Sigma. Sodium phosphate and hydrogen peroxide (analytical grade) were from J.T. Baker (Philipsburg, PA).

2.2. Enzyme activity assays

Catalase was assayed at 25°C using a procedure provided by Sigma, based on the first-order decomposition of peroxide by catalase, which was monitored by the decrease in absorbance at 240 nm. Catalase was dissolved in 0.05 M potassium phosphate buffer (pH 7.0) to a final concentration of about 50 units per ml. The substrate solution was 4.5×10^{-3}% hydrogen peroxide in the same phosphate buffer.

The activity of β-galactosidase was determined by hydrolysis of β-nitrophenyl-β-D-galactopyranoside (ONPG) using the method described by Izutsu et al. [11].

LDH activity was measured using a kit supplied by Sigma. The assay is based on the interconversion of lactate and pyruvate. During reduction of pyruvate, an equimolar amount of NADH is oxidized to NAD. The oxidation of NADH results in a decrease in absorbency at 340 nm. The rate of decrease of absorbency at 340 nm is directly proportional to LDH activity in the sample.

2.3. Thermal analysis and freeze-dry microscopy

The DSC thermograms of frozen protein solutions were measured using a Perkin-Elmer Series 7 DSC (Norwalk, CT). Catalase was dissolved in phosphate buffer at 50 mg/ml, pH 7.0. β-Galactosidase and LDH were dissolved in phosphate buffer (pH 7.4) at 50 mg/ml and 56 mg/ml, respectively. Twenty μl of sample solution was sealed in an aluminum pan. The sample was cooled to −70°C at 20°C/min, then heated to 20°C at 10°C/min. The thermogram was recorded during the heating cycle.

Freeze-dry microscopy was used to observe the collapse temperature of protein solutions using a procedure described by Nail et al. [12]. Composition of the samples was the same as that used for thermal analysis.

2.4. Freeze-thaw studies

Catalase was dissolved in 0.05 M potassium phosphate solution (pH 7.0) at different concentrations, ranging from 0.1 mg/ml to 4.0 mg/ml. β-Galactosidase and LDH were dissolved in 0.05 M sodium phosphate buffer, pH 7.4, at a concentration range of 2 to 100 μg/ml. The minimum concentration of each protein was determined by the minimum concentration requirement of the activity assay, and the high end of the range was based on a 40- to 50-fold increase over the minimum concentration. The enzyme solutions were frozen at −40°C for 20 h, then thawed at room temperature. The activity was assayed before and after freezing, and relative activity was expressed as a percentage of the activity prior to freezing.

Additional studies were carried out to determine the influence of buffer on freeze-thaw stability. β-galactosidase and LDH were dissolved at 5, 25, and 50 μg/ml in phosphate buffer (0.05 M, pH 7.4), citrate buffer (0.05 M, pH 7.4) and Tris buffer (0.05 M, pH 7.4). All these solutions were frozen at −40°C for 20 h. The pH of the frozen solution was monitored (Mettler-Toledo Process Analytical, Wilmington, MA) using a technique described by Gomez and Rodriquez-Hornedo [13].

To compare different freezing methods, β-galactosidase and LDH solutions (5, 25 and 50 μg/ml) in phosphate buffer (0.05 M, pH 7.4) were frozen using the following methods: (1) placing directly in a freezer at −40°C, (2) immersion in liquid nitrogen followed by transfer to a −40°C freezer, and (3) placing vials on the shelf of a freeze-dryer and freezing to −40°C at 0.5°C/min. After 20 h, all the frozen solutions were thawed at room temperature.

2.5. Freeze-drying

Solutions were filled into 10-ml serum vials (2 ml fill volume) and transferred to either an Edwards Lyoflex® 08 (Tonawanda, NY), or an FTS Dura-Stop® (Stone Ridge, NY) freeze-dryer. Type T thermocouples (32 gauge) were placed in the bottom center of three vials for each batch. The cycle consisted of freezing at −45°C (the approximate lower product temperature limit for pharmaceutical freeze-drying) for 6 h, then drying under varied conditions for both primary and secondary drying. The time course of activity loss for β-galactosidase and LDH was examined by pre-weighing several vials and removing them using a sample extractor throughout the drying cycle. Distilled water was then added back to each vial to restore the original weight prior to the activity assay. For vials removed during secondary drying, residual moisture was measured by Karl Fisher titration using coulometric end point detection (Model 150, Denver Instruments, Arvada, CO). Three vials were removed after freezing as controls. Activity of the freeze-dried samples was then expressed as a percentage of the activity of the freeze-thawed control.

2.6. Scanning electron microscopy observation of freeze-dried products

The freeze-dried products were coated with gold for 15 min (Hummer® 6.2 sputtering system, Anatech, Alexandria, VA) before observation by scanning electron microscopy (Jeol Model JSM-35CF, Tokyo, Japan). The accelerating voltage was 25 kV.

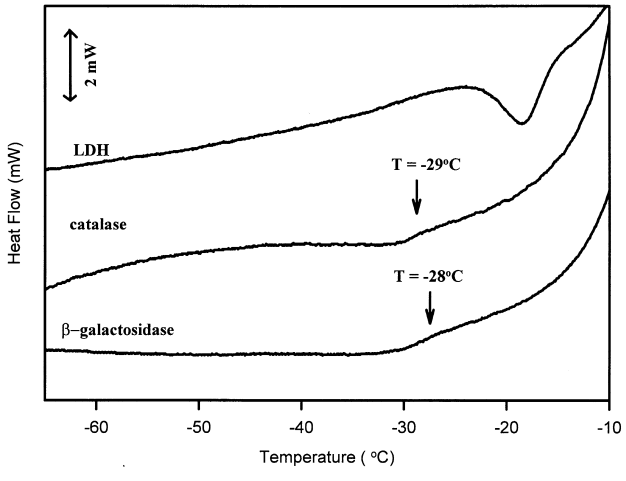

Fig. 1. DSC thermograms of β-galactosidase (50 mg/ml), catalase (50 mg/ml), and LDH (56 mg/ml) in phosphate buffer (0.05 M, pH 7.4).

3. Results and discussion

3.1. Glass transition temperature (T_g') measurement of protein solutions

Differential scanning calorimetry often provides useful information concerning the physical state of solutes in frozen solutions as well as an estimate of the maximum allowable product temperature during primary drying. For protein formulations, which generally contain an amorphous component, the maximum allowable product temperature during primary drying is the collapse temperature, which is closely related to the glass transition temperature of the maximally freeze concentrated solution, or T_g'. DSC thermograms of β-galactosidase, catalase and LDH are shown in Fig. 1. The thermogram of catalase contains a weak transition – which appears to be a glass transition – with a midpoint temperature of approximately −29°C. The thermogram of β-galactosidase contains a transition of about the same magnitude at approximately the same temperature, −28°C. The thermogram of LDH, on the other hand, reveals no detectable glass transition. The exotherm centered at about −18°C is believed to arise from crystallization of phosphate buffer during the thermal analysis experiment. Failure to detect a glass transition in frozen solutions containing amorphous solutes is not unusual based upon our experience. This may be caused by a change in heat capacity at in the glass transition region which is too small to be detected by the instrumentation, or because the glass transition region is broad enough to be 'lost' in baseline drift. Heat capacity changes at the glass transition are tabulated below for catalase and β-galactosidase.

Collapse temperature (T_c) as observed using freeze-dry microscopy of the same protein solutions are compared with apparent T_g' data in Table 1.

The only model protein for which good agreement between apparent T_g' and observed collapse temperature is β-galactosidase. Given that the reported collapse temperature is based on direct observation of the material during freeze-drying, this must be considered the most meaningful of the two sets of data. In comparing the thermograms of catalase and β-galactosidase, both contain an apparent glass transition with about the same change in heat capacity at approximately the same temperature, yet one is predictive of collapse in freeze-drying while the other is not. These results underscore the need for caution in interpreting thermograms containing weak, albeit reproducible, transitions, particularly when basing selection of processing conditions on these measurements. It is also clear that further work is needed using other model proteins to better evaluate the

Table 1

Comparison of T_g' and T_c of protein solutions

Protein	T_g' (°C)	T_c (°C)	ΔC_p (J/gm-°C)
Catalase	–29	–15	0.15
β-Galactosidase	–28	–29	0.11
LDH	None detected	–28	–

predictivity of low temperature thermal analysis as a formulation and process development tool.

3.2. Freeze-thawing of protein solutions

Fig. 2 shows the recovery of activity of catalase (a), β-galactosidase (b) and LDH (c) in phosphate buffer after freezing by transferring vials to a freezer at –40°C. β-Galactosidase and LDH were also examined with citrate and Tris buffers. Absorbance at 280 nm was used to distinguish between loss of protein activity and physical loss of protein due to adsorption to the container wall. No loss due to adsorption was observed. The same pattern of activity loss was observed for all three proteins. Significant activity loss was observed at low concentration, and recovery increased with increasing concentration up to a plateau, where recovery of activity became roughly independent of concentration. Within the plateau region, recovery of activity after freezing was approximately 90%. This reduced activity loss with increasing concentration is consistent with observations of other researchers [3,10], although the effect has not, to the best of our knowledge, been systematically investigated. Perhaps the effect of concentration is a particular example of the preferential exclusion thermodynamic argument advanced by Timasheff and coworkers [14]. This mechanism has been shown to apply to polymers such as dextran and polyethylene glycol, and to be predictive of the ability of solutes to protect proteins from freezing-induced damage. It seems reasonable that the concept of preferential exclusion would apply to other proteins as well.

A significant buffer effect is seen, particularly for LDH, where recovery is lowest for the phosphate buffer system. Recovery of LDH activity at a concentration of 50 μg/ml when frozen in 0.05 M sodium phosphate buffer at pH 7.4 is approximately 85%. This is seemingly in sharp contrast to data reported by Izutsu et al. [11], where the recovery was well below 20%. However, the data in Fig. 3 seem to explain this discrepancy, as well as to point out the need for attention to detail in defining freezing processes for protein solutions. Izutsu and coworkers froze LDH by immersion in liquid nitrogen, then transferring to a precooled freeze-dryer shelf at –40°C. Our study involved three different freezing methods, as described above. As seen in Fig. 3, freezing by immersion in liquid nitrogen consistently gave the lowest recovery. For LDH at a concentration of 5 μg/ml, recovery of activity was less than 10%, which is in good agreement with the data reported by Izutsu et al. The best recovery was obtained by transferring vials directly to a freezer at –40°C. Intermediate levels of activity recovery were observed when vials were transferred to the shelf of a freeze-dryer at room temperature and the shelf temperature was ramped downward slowly at 0.5°C/min. It would seem that there is no clear pattern relating activity recovery to freezing rate, since what would appear to be the intermediate freezing rate gave the highest recovery of activity. One hypothesis which needs to be tested is that the slowest freezing method actually resulted in a greater degree of supercooling and better thermal equilibration throughout the volume of liquid such that, when ice crystals nucleate, the freezing rate is actually faster than when placing vials in a freezer at –40°C. Placing thermocouples in vials of product would not be useful in comparing thermal history of freezing, since the thermocouple acts as a site for nucleation of ice crystals. Future work will use a thermocouple fixed to the outside of vials in order to better understand freezing rate effects for proteins.

The observation of lower recovery of protein activity

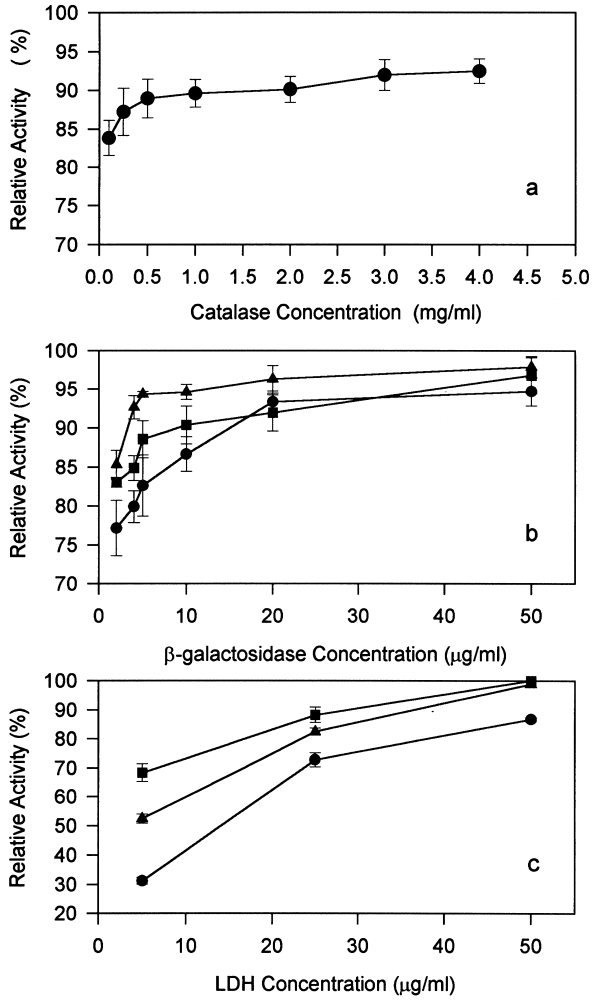

Fig. 2. Activity recovery vs. concentration after freeze-thaw in buffer solutions: (a) catalase, (b) β-galactosidase, and (c) LDH. (●, phosphate buffer; ■, citrate buffer; ▲, Tris buffer).

after fast freezing is consistent with at least two recent reports of ice-induced partial unfolding of proteins. Strambini and Gabellieri [15] used the phosphorescence lifetime of tryptophan residues to demonstrate that freezing of aqueous protein solutions is accompanied by a loosening of the native fold and loss of secondary and tertiary structure. This phenomenon is largely reversible, but a small fraction of proteins studied recovered neither the initial phosphorescence properties nor enzymatic activity. Slow cooling resulted in a smaller perturbation of phosphorescence lifetime, presumably because larger ice crystals are formed with a relatively low ice surface area. Chang et al. [16] studied freeze denaturation of several proteins, and reported increased levels of insoluble precipitate when solutions were frozen by dipping in liquid nitrogen as opposed to slower freezing. These investigators further reported a strong correlation between the tendency of a protein to freeze denature and its tendency to surface denature, and that addition of small amounts of surface active agents protected proteins from both freeze- and surface-induced denaturation.

The pH shifts associated with freezing observed in this investigation are consistent with values reported by other investigators, particularly when considering that the pH shift is affected by composition, concentration, and the thermal history of freezing. In our studies, the pH shifts of buffer systems after freezing to –20°C are from 7.4 to 3.9, 7.03, and 8.04 for sodium phosphate, citrate, and Tris, respectively. Van den Berg and Rose reported a pH shift in sodium phosphate buffer from 7.0 to 3.6 after freezing [17]; Orii and Morita reported that the pH of sodium phosphate buffer decreased by more than 3 pH units, while the pH of Tris buffer increased by one unit after freezing [18], and Larsen reported that Tris buffer undergoes a shift from 7.5 to 8.0 after freezing to –14°C, citrate buffer pH decreased from 6.5 to 6.2 after freezing, and that the pH of sodium phosphate decreased by more than 3 units [19].

Fig. 2b and c show the comparison of recovery of β-galactosidase and LDH in these three buffer solutions after freezing at –40°C. For all the concentrations tested, phosphate buffer resulted the lowest recovery. While this does not establish a causal relationship between pH shift and activity loss for these systems, it does point to the importance of seeking alternatives to sodium phosphate as a buffer in systems intended for freezing or freeze-drying.

3.3. Effect of freeze-drying on activity recovery

Fig. 4 shows the relative activity of catalase, β-galactosidase and LDH after freezing drying as a function of protein concentration. Constant conditions were used for all proteins; i.e. freezing for 6 h at –45°C, drying at –30°C for 24 h and at 25°C for 4 h. A chamber pressure of 70 mTorr was used throughout the cycle. The activity recovery is expressed as a percentage of the control group, which was removed after freezing, so that these data represent activity loss due to drying only. The data support the conclusion that recovery of activity after freeze-drying is, like freezing, very concentration dependent. While recovery is clearly dependent on the nature of the protein, recovery is consistently enhanced at higher concentrations. Again, this is an effect which needs further investigation. The preferential exclusion hypothesis cannot apply to explanation of effects of solutes on protection of proteins against damage by dehydration. However, many of the same solutes which afford protection from freezing are also effective lyoprotectants. The most credible explanation for this effect is water replacement, where the solute acts as a substitute for water in forming hydrogen bonds with the surface of a protein as water is removed, helping to maintain the native structure. Again, there is no reason to reject the idea that other protein molecules can act in the same way.

The time course of activity loss of β-galactosidase during freeze-drying under different primary drying conditions is shown in Fig. 5. Primary drying shelf temperatures were –30, 0, and 25°C in Fig. 5a,b,c, respectively. The product temperature at which collapse begins is also shown on each plot. In Fig. 5a, product temperature remained well below the collapse temperature throughout primary drying, whereas at higher shelf temperatures, product temperature exceeds the temperature at which collapse begins prior to

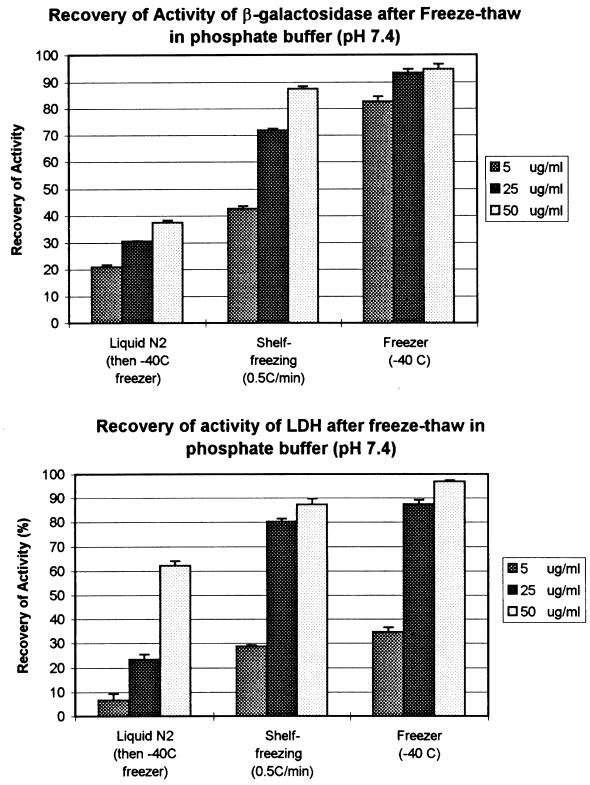

Fig. 3. Recovery of activity of β-galactosidase and LDH after freezing by different methods. Samples were frozen by: (1) placing directly in a freezer at –40°C, (2) immersion in liquid nitrogen followed by transfer to a –40°C freezer, and (3) freezing on the freeze-dryer shelf ramped from 25 to –40°C at 0.5°C/min.

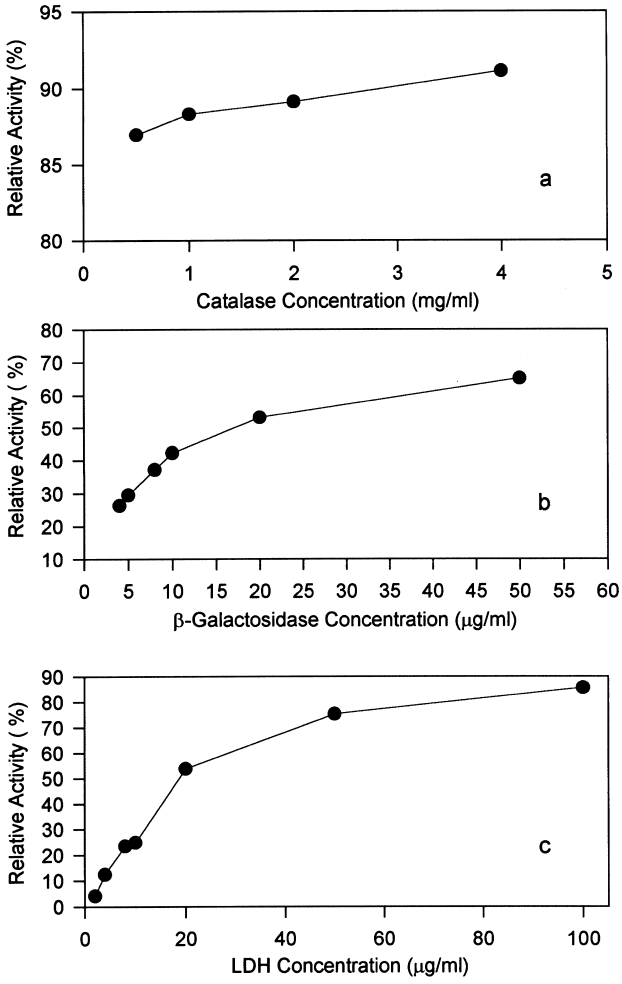

Fig. 4. Activity recovery after freeze-drying for (a) catalase, (b) β-galactosidase, and (c) LDH. Each data point represents the average of three measurements.

the end of primary drying. It is clear from Fig. 5 that loss of recoverable activity is slower at lower shelf temperatures. However, primary drying temperature does not seem to have a significant impact on recovery of activity after the drying process; i.e., the slower the rate of loss of activity in primary drying, the faster it appears to be during secondary drying. In addition, the onset of collapse does not seem to be associated with a sharp reduction in activity recovery. Thus, the commonly held view that collapse causes a sharp reduction in recovery of activity does not seem to be supported by these data.

Electron photomicrographs of freeze-dried solids for which primary drying took place both below the collapse temperature and at the early stages of collapse are shown in Fig. 6. The onset of collapse is characterized by development of holes in the plate-like amorphous structure, the number and size of which increase with an increasing degree of collapse. This is presumably caused by surface tension forces which become active as the viscosity of the glassy phase decreases, thereby opening holes at thin points in the solid material. This is consistent with data reported by Milton et al. [20] for freeze-dried lactose.

None of the freeze-dried solids reported in Fig. 5 underwent gross collapse of the solids during primary drying. In order to explore the relationship between collapse and loss of activity further, experiments were done with β-galactosidase, catalase, and LDH where the protein solutions were dried under conditions resulting in total collapse. This was done by carrying out primary drying at a shelf temperature of −10°C, but at pressures sufficiently high to produce total collapse. Secondary drying was carried out at 20°C for 4 h under full vacuum. For the control group, freeze-drying was carried out under primary drying conditions which resulted in retention of microstructure. Percent recovery results are summarized in Table 2.

For two of the proteins studied, there is only a modest decrease in activity recovery between drying conditions of

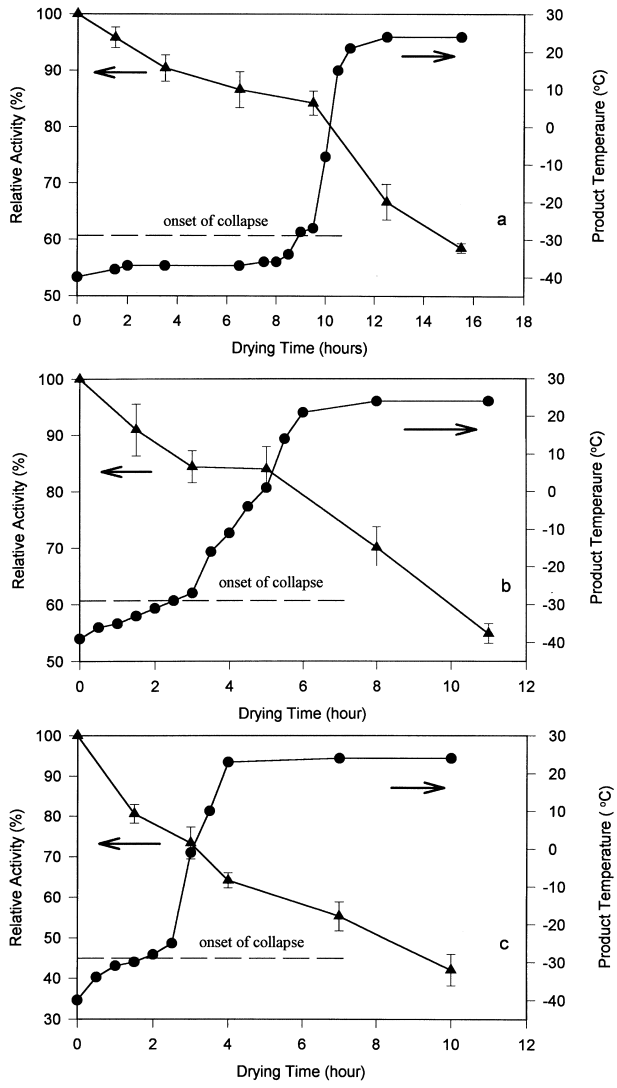

Fig. 5. Time course of activity loss of β-galactosidase during freeze-drying at primary drying shelf temperatures of (a) −30°C, (b) 0°C, and (c) 25°C. Each data point represents the average of three measurements.

Table 2

Recovery of protein activity after freeze drying (%)

Protein	Structure retention	Collapse
Catalase	92	88
β-Galactosidase	95	85
LDH	95	50

structure retention and total collapse. For LDH the recovery associated with collapse is significantly lower; however, this may be at least partially associated with the unique profile of activity loss of LDH during freeze-drying, which is discussed below.

Fig. 7 is a series of plots analogous to Fig. 5 showing the activity loss profile for LDH during freeze-drying. This loss profile is different from that of β-galactosidase in that there is an apparent sharp decrease in activity during primary drying, and activity recovery increases to a maximum value at the end of primary drying and early in secondary drying. Activity recovery then drops off sharply as secondary drying proceeds. The sharp initial drop in activity is associated with removal of samples in which ice is still

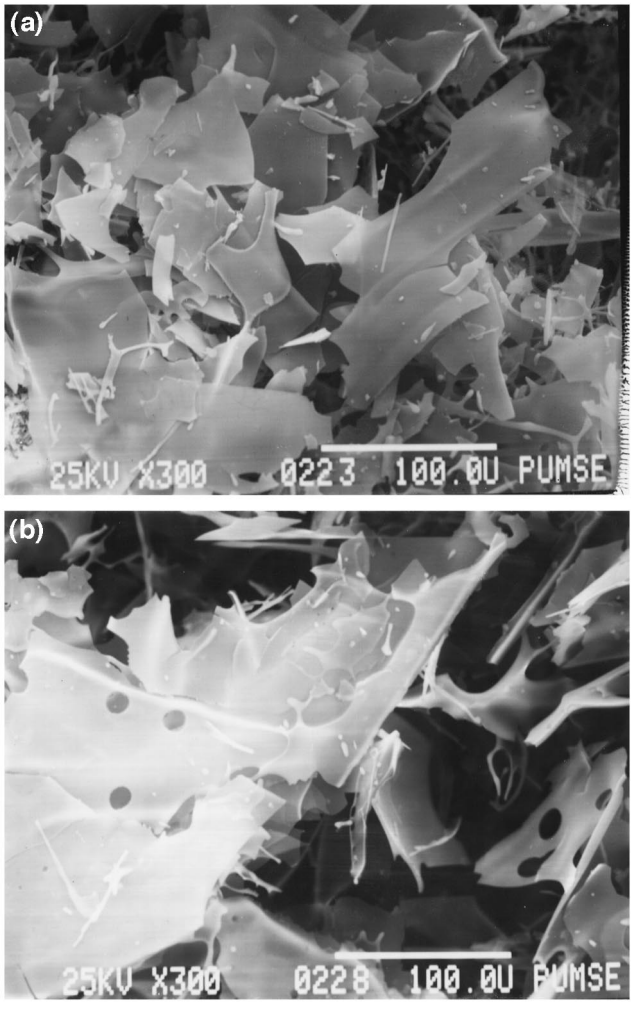

Fig. 6. Scanning electron photomicrographs of freeze-dried protein solid showing microstructure (a) when dried below the collapse temperature, and (b) at the onset of collapse.

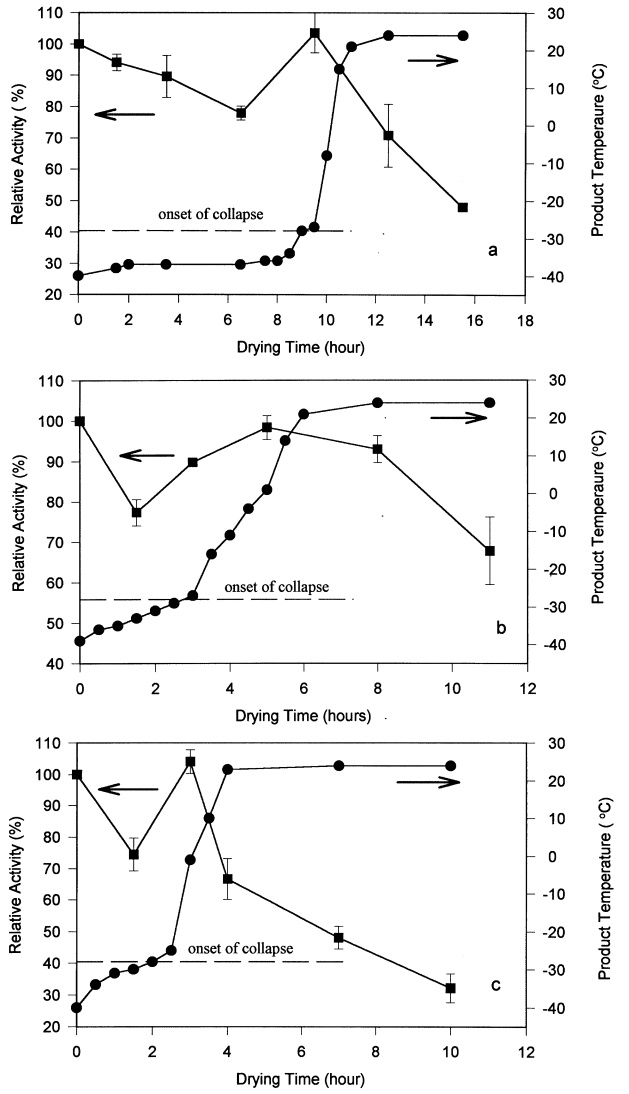

Fig. 7. Time course of activity loss of LDH during freeze-drying at primary drying shelf temperatures of (a) –30°C, (b) 0°C, and (c) 25°C. Each data point represents the average of three measurements.

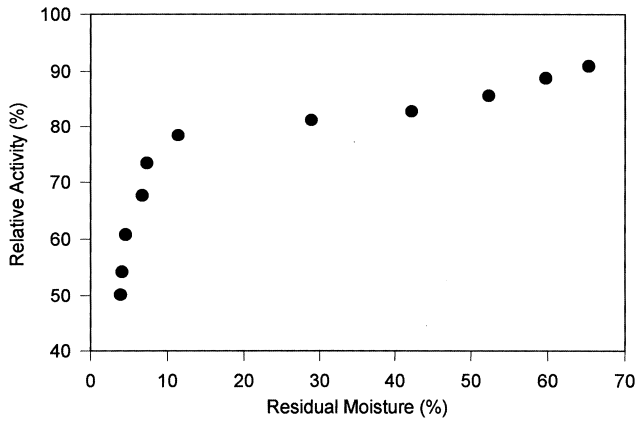

Fig. 8. Activity recovery of β-galactosidase versus residual moisture after freeze-drying.

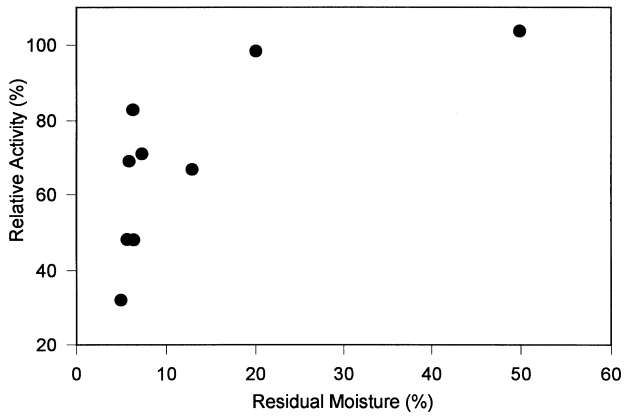

Fig. 9. Activity recovery of LDH versus residual moisture after freeze-drying.

present. Given that activity recovery during freeze-drying is expressed as a percentage of activity recovery following freeze-thawing of this system, it seems unlikely that the activity loss during primary drying is associated with freeze-thaw damage during primary drying. Further work is needed to better understand this effect. However, the data in the table above for LDH dried under conditions of collapse should be regarded with some caution, since partial melting during primary drying under the aggressive drying conditions used cannot be ruled out.

Despite the marked difference in the activity loss profiles for β-galactosidase and LDH, both point to secondary drying as a critical aspect of the process with respect to recovery of activity. Further experiments were carried out, again using a sample extractor, but carrying out the process at lower, and constant, shelf temperatures in order to focus on the relationship between residual moisture and recovery of activity. Results are shown in Fig. 8 for β-galactosidase, and show a high level of activity recovery above a residual moisture level of about 10%. However, activity recovery decreases dramatically below this moisture level. Comparable results were obtained for LDH (Fig. 9), although more scatter is apparent in the data. These data for LDH, along with the data in Fig. 7, may explain the results reported by Izutsu et al. [10], where a higher activity recovery was seen for freeze-dried LDH than for freeze-thawed LDH. The data also support the conclusion that careful attention must be given to avoiding overdrying in order to maximize recovery of activity from proteins when no lyoprotectant is present.

4. Conclusions

When freezing or freeze-drying proteins under conditions in which no protective solute is present, it was consistently observed that recovery of protein activity can be maximized by using the highest practical protein concentration. Alternatives to the sodium phosphate buffer system should be considered. Thermal history of freezing also appears to be important. In this study, the best recovery of activity was obtained by placing vials directly at −40°C in a freezer. Fast freezing by exposing product to liquid nitrogen should be approached with caution. Primary and secondary drying conditions seem to be important only through their effect on residual moisture after a given drying time. Maximizing recovery of activity depends upon halting the process before 'over-drying' takes place.

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