Solid-State Rehydration-Induced Recovery of Bilirubin Oxidase Activity in Lyophilized Formulations Reduced during Freeze-Drying

Yuji Nakai,[1] Sumie Yoshioka,* Yukio Aso, and Shigeo Kojima

National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan.

Dehydration during freeze-drying often alters the native conformation of proteins, leading to a loss of activity. Solid-state rehydration of lyophilized protein formulations was studied to examine the recovery from structural damage caused by freeze-drying. Bilirubin oxidase was used as a model protein. The enzymatic activity of bilirubin oxidase decreased markedly when freeze-dried with polyvinylalcohol (PVA), but this loss in activity was partially recovered by subsequent solid-state rehydration of the lyophilized formulations. This recovery of activity upon solid-state rehydration was not observed in lyophilized formulations containing dextran that exhibited a similar loss of activity during freeze-drying. Thus, PVA appears to have a greater ability to act as a water substitute than dextran, resulting in less structural damage during dehydration. This in turn allows a recovery in activity upon solid-state rehydration.

Key words bilirubin oxidase; freeze-drying; rehydration; activity recovery

Lyophilization is a well known method of preparing stable protein formulations. However, dehydration during lyophilization often alters the native conformation of proteins, leading to a loss of activity. Fourier-transform infrared spectroscopic studies have indicated that secondary structure rearrangement of various proteins occurs during dehydration.[2—6] These involve both reversible and irreversible rearrangements. Some proteins such as α-lactoalbumin unfold during dehydration but regain their native conformation upon rehydration, whereas other proteins such as lactate dehydrogenase unfold during dehydration and aggregate upon rehydration.[7] Rehydration upon reconstitution of lyophilized proteins in aqueous solutions has been studied to examine its effect on protein structure and/or activity. It is of great interest to discover if solid-state rehydration can lead to recovery of the protein structure damaged by freeze-drying to the same extent as achieved by solution-state rehydration.

The present paper describes the solid-state rehydration-induced recovery of bilirubin oxidase (BO) activity in lyophilized formulations reduced by dehydration. The effect of polyvinylalcohol (PVA) and dextran on the recovery of activity was compared with that of trehalose. A marked recovery of activity was observed for lyophilized formulations that contained PVA, but not for those containing dextran. The stability of the lyophilized formulations during storage at elevated temperatures after solid-state rehydration was also examined in order to better understand the protein damage caused by dehydration.

Experimental

Materials Lyophilized BO (*Myrothecium verrucaria*) powder containing 25% sucrose and 25% ammonium sulfate was kindly provided by Amano Pharmaceutical Co. (Nagoya). The lyophilized BO powder was dissolved in water to make a solution of approximately 200 mg/ml, and dialyzed using cellulose tubing (Viskase Sales Co.) against water at 4 °C to remove sucrose and ammonium sulfate. The solution was immersed in liquid nitrogen for 15 min and dried in a vacuum below 5 Pa for 20 h in a lyophilizer (Freezevac C-1, Tozai Tsusho Co., Tokyo). The shelf temperature was between −35 and −30 °C for the first 1 h, and ambient for the subsequent 19 h. The lyophilized BO was stored below −20 °C.

Bovine serum albumin (BSA) (fraction V) and dextran (average molecular weight of 39000) were purchased from Sigma Chemical Co.,

Inc. (St. Louis, MO). PVA (average molecular weight of 31000—50000) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI), respectively. All other chemicals were of reagent grade and purchased from Wako Pure Chemical Industries Ltd. (Osaka).

Preparation of Lyophilized BO Formulations Lyophilized BO was dissolved in a 0.2 mg/ml Triton X-100 solution (0.4 mg/ml), and an excipient (trehalose, dextran or PVA) was added (40 mg/ml). Three hundred microliters of the solution was frozen in a polypropylene sample tube (10 mm diameter) by immersion in liquid nitrogen for 10 min, and then dried in a vacuum below 5 Pa for 23.5 h. The shelf temperature was between −35 and −30 °C for the first 1 h, 20 °C for the subsequent 19 h, and 30 °C for the last 3.5 h.

The lyophilized samples were stored at 15 °C for 24 h in a desiccator with 12%, 23.4% or 60.2% relative humidity (RH). Saturated solutions of LiCl, potassium acetate and NaBr, respectively, were used. The water content was determined by the Karl Fisher method (684 KF Coulometer, Switzerland) and shown in Table 1. No physical collapse was observed for the lyophilized samples with various water contents except for the samples containing trehalose stored at 60.2% RH.

Stability Study of Lyophilized BO Formulations The lyophilized BO formulations with various water contents were stored at 70 °C. BO activity remaining after 5 h-storage was determined spectrophotometrically as described below.

Determination of BO Activity in Lyophilized Formultions The lyophilized BO formulation in a polypropylene sample tube was reconstituted with 1.5 ml pH 7.0 buffer (50 mM phosphate buffer containing 0.05 mM EDTA), and diluted with pH 7.0 buffer containing 0.2 mg/ml Triton X-100 to make a 3.2 mg/ml BO solution. Two hundred microliters pH 7.0 buffer containing 30 mg/ml BSA and 0.15 mg/ml bilirubin[8] was added to 3.0 ml pH 7.0 buffer containing 10 mg/ml sodium cholate, and preincubated at 37 °C for 10 min. One hundred microliters of the BO solution was added, and the decrease in absorbance at 460 nm was monitored.[9] The remaining activity was expressed as a percentage of that before freeze-drying.

BO activity was determined within 30 min of reconstitution in order to exclude the effect of activity recovery in aqueous solution. Storage of the reconstituted solution at 5 °C for 1 d caused a small degree of activity recovery.

Table 1. Water Content of Lyophilized Formulations (mg/g solid)

RH (%)	PVA	Trehalose	Dextran
—	7.82 ± 3.58[a]	11.4 ± 5.15	13.3 ± 4.84
12	21.99 ± 3.99	30.5 ± 4.82	53.6 ± 3.49
23.4	38.46 ± 4.68	52.9 ± 3.37	84.5 ± 2.58
60.2	100.50 ± 4.5	ND	162.7 ± 4.7

a) Average ± S.D. ND: not determined.

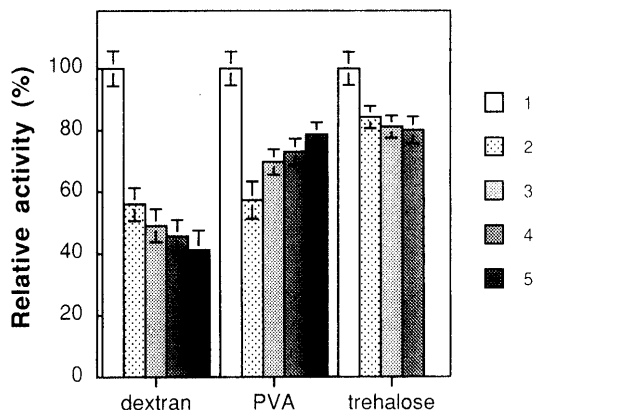* To whom correspondence should be addressed.

Fig. 1. Enzyme Activity after Freeze-Drying and Subsequent Rehydration Compared with That of a BO Solution Prior to Freeze-Drying

(1) BO solution prior to freeze-drying; (2) after freeze-drying; rehydrated by storing at 15 °C and 12% (3), 23.4% (4) and 60.2% RH (5) for 24 h.
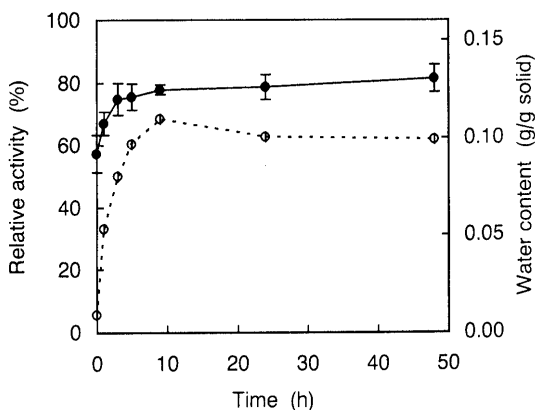

Fig. 2. Time-Course of Water Adsorption (○) and Activity Recovery (●) of Lyophilized BO Formulations that Contained PVA Stored at 15 °C and 60.2% RH

Fig. 3. Enzyme Activity Loss of Lyophilized BO Formulations That Contained Trehalose (△), Dextran (□), or PVA (●) Stored at 70 °C

Before rehydration (a), rehydrated at 12% (b), 23.4% (c) and 60.2% RH (d).

Results

Figure 1 shows the BO activity after freeze-drying and subsequent rehydration compared with that of the BO solution prior to freeze-drying. The smallest loss of activity during freeze-drying was observed in the BO formulations with trehalose. Freeze-drying with dextran and PVA resulted in greater damage to BO. Solid-state rehydration of the lyophilized BO formulations by storing under various humidity conditions caused further loss of activity in the formulations that contained dextran and trehalose. Formulations stored under higher humidity exhibited a greater loss of activity. In contrast, BO formulations freeze-dried with PVA showed recovery of activity upon solid-state rehydration, and it was found that a larger degree of water adsorption brought about a greater recovery of activity.

Figure 2 shows the time-course for water adsorption and activity recovery when the lyophilized BO formulations that contained PVA were stored at 60.2% RH. Water content increased with time and reached an equilibrium at around 10 h. The BO activity of the formulation increased with increasing water content. The loss of activity caused by freeze-drying recovered partially following water adsorption to a similar level to that of the BO formulations freeze-dried with trehalose (Fig. 1).
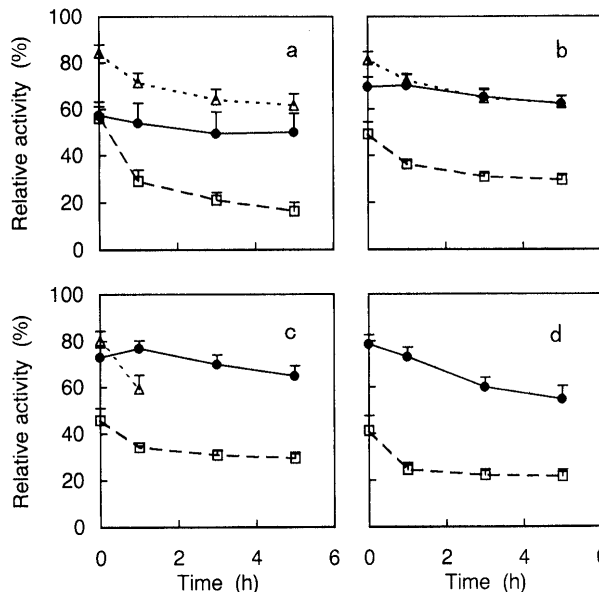
The BO activity shown in Figs. 1 and 2 was determined using BO formulations freeze-dried from a buffer-free BO solution containing trehalose, dextran or PVA. The lyophilized BO formulations that contained PVA prepared using pH 7.0 phosphate buffer instead of water exhibited similar patterns of activity loss during freeze-drying and activity recovery upon subsequent solid-state rehydration. The absence of a buffer did not appear to significantly affect the loss or recovery of activity.

Figure 3 shows the stability of the lyophilized BO formulations that contained PVA, dextran and trehalose with various water contents when stored at 70 °C. The formulations with trehalose, which exhibited the least loss of activity during freeze-drying, showed a significant loss of activity during the initial stage of storage at 70 °C. Partial dissolution occurred in the formulations with a higher water content when stored at 70 °C. The data obtained from the partially dissolved samples were not included in Fig. 3.

The lyophilized BO formulations that contained dextran, which exhibited a marked loss of activity during freeze-drying and a further loss of activity upon solid-state rehydration, showed a significant loss of activity during the initial stage. In contrast, the formulations that contained PVA, which exhibited a similar degree of activity loss to that of dextran formulations during freeze-drying, showed better stability. Activity did not decrease markedly during the initial stage for either the lyophilized formulations or formulations that demonstrated a recovery of activity due to solid-state rehydration, although the rate of activity loss appeared to increase gradually with increasing water content.

Discussion

The loss of BO activity brought about by freeze-drying with PVA was partially recovered by rehydration in the solid-state to a level similar to that observed for freeze-

drying with trehalose, which is a well known stabilizer that is effective against protein denaturation during dehydration (Fig. 1). This finding suggests that reversible alteration of the BO structure was involved in the activity loss of the lyophilized BO formulations that contained PVA. In contrast, no recovery of activity due to solid-state rehydration was observed for the BO formulations that contained dextran, suggesting that freeze-drying with dextran causes a more dramatic protein structure alteration, even if the BO activity remaining after freeze-drying is similar to that of the formulations that contains PVA.

The stability data in Fig. 3 support the notion that there is less structural protein damage in the lyophilized BO formulations that contained PVA. The stability of the PVA formulations without rehydration was greater than that of the dextran formulation (Fig. 3a). The BO with less structural damage in the PVA formulations may be less susceptible to denaturation than the severely damaged BO in the dextran formulations. The BO activity of the lyophilized PVA formulations, which showed recovery as a result of solid-state rehydration, was also stable, although an increase in water content tended to reduce the stability, indicating that upon solid-state rehydration the BO with less structural damage can revert to the native structure that is less susceptible to denaturation at elevated temperatures.

Why is the dehydration-induced alteration of the BO structure reduced when freeze-dried with PVA compared with dextran? A glassy state (less molecular mobility) and water replacement are known to be important factors determining protein stability during dehydration.[7] A previous NMR study showed that lyophilized protein formulations that contained PVA exhibited a Lorentzian relaxation process due to microscopically liquidized protons at lower temperatures than those containing dextran, indicating that the molecular mobility of PVA formulations was higher than that of dextran formulations.[10] This suggests that molecular mobility in the final stage of dehydration is also higher in the PVA formulations than in dextran formulations. Despite this higher molecular mobility, the structural alteration that occurs during dehydration was less for the PVA formulations. This suggests that the higher molecular mobility of the PVA formulations led to a greater ability to act as a water substitute, resulting in less damage during dehydration. The destabilizing effect of increased molecular mobility

appeared not to be serious at the sample temperatures during dehydration. The reduced stabilizing effect of dextran during dehydration may also be explained by reduction in the ability to act as a water substitute because of greater steric hindrance and/or phase separation.[11]

A destabilizing effect of the increased molecular mobility of the PVA formulations was observed at elevated temperatures. The storage stability of the PVA formulations decreased with increasing water content (Figs. 3c and 3d), indicating that the molecular mobility, which increased due to the plasticizing effect of water, resulted in protein destabilization.[12]

Conclusion

The enzymatic activity of bilirubin oxidase decreased markedly when freeze-dried with PVA, but the activity loss was partially recovered by subsequent solid-state rehydration of the lyophilized formulations. Activity recovery upon solid-state rehydration was not observed in the lyophilized formulations that contained dextran, which exhibited a similar degree of activity loss during freeze-drying. PVA appears to have a greater ability to act as a water substitute than dextran, which resulted in less structural damage during dehydration and, ultimately, led to a recovery of activity upon solid-state rehydration.

References and Notes
1) Present address: The Institute of Physical and Chemical Research (RIKEN), 3-1-1, Koyadai, Tsukuba, Ibaraki 305, Japan.
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