Stabilization of Lactate Dehydrogenase Following Freeze-Thawing and Vacuum-Drying in the Presence of Trehalose and Borate

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Purpose. The purpose of this work was to investigate the effects of trehalose and trehalose/sodium tetraborate mixtures on the recovery of lactate dehydrogenase (LDH) activity following freeze-thawing and centrifugal vacuum-drying/rehydration. The storage stability of LDH under conditions of either high relative humidity or high temperature was also studied.

Methods. LDH was prepared in buffered aqueous formulations containing trehalose alone and trehalose/"borate" mixtures. Enzymatic activity was measured immediately following freeze-thawing and vacuum-drying/rehydration processes, and also after vacuum-dried formulations were stored in either high humidity or high temperature environments. Also, glass transition temperatures (T_g) were measured for both freeze-dried and vacuum-dried formulations.

Results. The T_g values of freeze-dried trehalose/borate mixtures are considerably higher than that of trehalose alone. Freezing and vacuum-drying LDH in the presence of 300 mM trehalose resulted in the recovery of 80% and 65% of the original activity, respectively. For vacuum-dried mixtures, boron concentrations below 1.2 mole boron/mole trehalose had no effect on recovered LDH. After several weeks storage in either humid (100% relative humidity) or warm (45°C) environments, vacuum-dried formulations that included trehalose and borate showed greater enzymatic activities than those prepared with trehalose alone. We attribute this stability to the formation of a chemical complex between trehalose and borate.

Conclusions. The high T_g values of trehalose/borate mixtures offer several advantages over the use of trehalose alone. Most notable is the storage stability under conditions of high temperature and high relative humidity. In these cases, formulations that contain trehalose and borate are superior to those containing trehalose alone. These results have practical implications for long-term storage of biological materials.

KEY WORDS: protein stabilization; cryopreservation; lyophilization; trehalose; borate.

INTRODUCTION

Preservation of labile biological products - such as proteins, vaccines, blood, or other cells—has important applications in the fields of biology, biochemistry, pharmacy, medicine, and food science. In this work, we have formulated and tested some agents that not only protect proteins against the stresses induced by freeze-thawing and centrifugal vacuum-drying ("vacuum-drying") but also provide effective long-term storage media. We have chosen the enzyme lactate dehydrogenase (LDH) as our test system. Previous work has shown that LDH is particularly sensitive to the stresses induced during freezing and drying processes (1–4).

Much effort has been spent toward elucidating the mechanisms of protein stabilization in solution, during freeze-thawing, and during freeze-drying. Recent work asserts that the stresses that arise during freezing and drying are fundamentally different, thereby necessitating separate mechanisms to explain protein stabilization during each of these processes (5).

Unfortunately, many agents that preserve proteins in solution and during freeze-thawing fail to preserve them in the dried state. Simple saccharides are some of the few additives known to stabilize proteins in the dried state. Of these agents, trehalose, a non-reducing disaccharide of glucose, has received considerable attention (13–15). Clegg and other workers have noticed that many organisms that have the ability to undergo complete desiccation also accumulate large amounts of trehalose (16,17). These examples provided by nature have led us and other workers to focus on this disaccharide as a protective agent for biological structures.

One possible mechanism of preservation in the dried state was first suggested in 1971 by Crowe and has come to be known as the "water replacement hypothesis" (18). To account for the ability of certain organisms to withstand desiccation, Crowe proposed that certain physiological solutes "replace" the lost water around polar residues of macromolecules. Carpenter and Crowe suggested that this may be the mechanism by which certain solutes maintain dried proteins in their native conformations (19). According to those authors, some of the hydroxyl groups on the glucose rings of trehalose form hydrogen bonds with the polar residues of the proteins.

In the past, some workers have argued that the stabilization mechanism is due to the formation of an amorphous, or glassy matrix (20–22). The glassy "phase" severely restricts the translational and relaxational motions of the protein. Indeed, Green and Angell noted a correlation between the glass transition temperature (T_g) of several mono- and disaccharides and their ability to protect liposomes (22); of the simple sugars examined by those authors, trehalose was found to have the highest T_g.

We note, however, that additional correlations can be made between protective capacity and other physical quantities. An observed sequence of protective capacity of simple sugars (glucose < sucrose < maltose < trehalose) not only follows the order of T_g but also the order of the infinite dilution heat of solution of the amorphous saccharide (unpublished).

It is also important to note that there are examples of good glass formers that are poor protective agents. Previous workers have found that dextran, a good glass former, does not provide nearly as much cryoprotection as disaccharides (23). It is our opinion (and that of others (24,25)) that the ability of a chemical system to *both* form an amorphous solid and to favorably interact with proteins are the necessary requirements of an effective protective system. This view has led us to propose the protectant mixtures presented in this work.

Most of the studies reported so far have involved four components: water, a buffer medium, a protective agent and a biological entity. Matters are further complicated by addition

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of other components to the system. It has been shown, for example, that combinations of trehalose and divalent cations of certain transition metals are superior to trehalose alone (26). The possibility of a synergism involving additional components has shifted our focus toward more complex mixtures. From a practical point of view, this becomes necessary since the buffer or electrolytes found in most biological systems inherently make them complex mixtures. In light of the aforementioned results, the minimal criteria for design of an effective protectant mixture include low toxicity, good glass forming ability, and favorable interactions with proteins. Based on the results of several preliminary trials with various compounds, we believe that certain mixtures of trehalose and borate ions satisfy these criteria. Borate ions dramatically increase the T_g of trehalose. The T_g of pure, dry trehalose is approximately 115°C, while it increases to 150°C for a freeze-dried trehalose/sodium tetraborate mixture with a boron to trehalose mole ratio of 0.33 (see Figure 2). Using ^{11}B NMR, we have verified that the borate ions form crosslinks with the hydroxyl groups of the trehalose molecule, thereby forming trehalose:borate complexes. This crosslinking raises the solution viscosity and, at sufficiently low temperatures, promotes the formation of a glass. This effect, we speculate, inhibits the motions of the protein that lead to loss of activity during freezing and drying. Furthermore, we have confirmed that the degree of trehalose:borate complexation can be controlled by adjusting the pH of the aqueous medium (unpublished). This borate chemistry has been previously reported, but not in connection with this application (27–30). Interestingly, some of that work has involved uses in the petroleum industry, where borate/polysaccharide gels are used as high viscosity fluids during hydraulic fracturing of oil and natural gas wells.

In this work, we report results of several experiments in which LDH was frozen/thawed and vacuum-dried/rehydrated. These results illustrate the recovery of enzymatic activity as a function of protective agent concentration. We also introduce a novel protective agent, a mixture consisting of trehalose and sodium tetraborate, and characterize its effect on the recovery of LDH after freeze-thawing and vacuum-drying/rehydration. Finally, we show results of experiments in which various LDH/protective agent formulations were vacuum-dried and stored in controlled-humidity and high-temperature environments over extended periods of time.

MATERIALS AND METHODS

Lactate dehydrogenase (LDH, EC 1.1.1.27, Type II, rabbit muscle) was purchased from Sigma Chemical Co. (St. Louis, MO). The LDH assay reagents were β-nicotinamide adenine dinucleotide (NADH) and pyruvic acid; LDH and all reagents were prepared in 50 mM "Tris" buffer (Tris[hydroxymethyl]aminomethane and Tris hydrochloride, pH 7.5 at 25°C). D-(+)-trehalose dihydrate was obtained from Pfanstiehl Laboratories, (Waukegan, IL). Sodium tetraborate decahydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI). Purified water (10^{18} MΩ · cm) was used in all experimental work.

Preparation and Assay of Lactate Dehydrogenase

LDH was dialyzed overnight at 4°C in 50 mM Tris-HCl buffer (pH 7.5). Total protein content was assayed using the modified Biuret method of Ohnishi and Barr (31). The LDH assay was based on the characteristic absorption of NADH at 340 nm. Measurements were performed at 25°C with a Shimadzu UV-1601 spectrophotometer. The 1.5-ml reaction mixture contained 50.0 mM Tris buffer (pH 7.5), 0.150 mM NADH, and 1.20 mM pyruvic acid.

A stock solution of LDH was prepared with a protein concentration of 16 μg/mL. As a control, 50 μl of this stock solution was mixed with 50 μl of Tris buffer (no protective agents). To prepare samples for freezing or vacuum-drying, 50 μl of the stock enzyme solution was gently mixed (at low speed on a vortexer) with 50 μl of a solution containing twice the desired solute concentration.

To avoid adsorption of the enzyme to glass, all work was done using polyethylene containers. Samples with enzyme and protective agent were put in polyethylene microcentrifuge vials. Freezing was done by immersion in liquid nitrogen for at least one minute. All centrifugal vacuum-drying ("vacuum-drying") was performed in a Savant Speed Vac Concentrator for more than 36 hours at 13 Pa. Evaporative cooling during vacuum-drying did not result in freezing. The dried samples were stored in a desiccator until rehydration, which was performed within two hours after completion of vacuum-drying.

Frozen samples were slowly thawed at room temperature (approximately 23°C) and immediately assayed. Aqueous NADH (1.3 ml, 0.173 mM) was added and the solution was poured into a methacrylate UV-cuvet and warmed to 25°C in the spectrophotometer. Vacuum-dried samples were rehydrated by adding aqueous NADH (1.4 ml, 0.161 mM). For both frozen and dried samples, the reaction was initiated by adding pyruvic acid (0.1 ml) to the cuvet and the resulting decrease in absorbance was monitored. The "method of initial rates" was used to obtain the activity via the rate of change in the sample absorbance at 340 nm. The relative activity was calculated by dividing the initial rate of a sample by that of the control sample.

To study the effect of storage in a humidified environment on enzymatic activity, several vacuum-dried LDH samples were stored in a desiccator at 25°C. An environment of 100% relative humidity (RH) was maintained by placing a fibrous cloth saturated with water at the bottom of the desiccator. In a separate desiccator, a saturated $MgCl_2$ solution was used to maintain a relative humidity of approximately 32% at 25°C. Relative humidity was monitored with a humidity probe (±0.1% RH, Model HM34C, Vaisala, Finland). Residual water content of vacuum-dried samples was measured by Karl Fischer titration.

To study the effect of high temperature storage on enzymatic activity, several vacuum-dried LDH samples were sealed and stored in a vacuum oven maintained at 45°C (±1°C).

Freeze-dried mixtures were prepared in an FTS Systems tray dryer coupled with a Dura-Dry MP condenser module. Aqueous 10 wt% mixtures were cooled to −45°C, and then held under vacuum (10^{-2} mbar) for 48 hours. The shelf temperature was then increased to 0°C over 4 days. Secondary drying was performed in a vacuum oven for three 24 hour segments at 25°C, 35°C, and 50°C. All subsequent handling was done in a dry nitrogen atmosphere. The water content of the freeze-dried samples was determined to be less than 0.3 wt%, as measured by Karl Fischer titration.

Glass transition temperatures of the vacuum-dried and freeze-dried mixtures were measured during heating at 10°C/minute in a Netzsch DSC-200 differential scanning calorimeter.

Details of sample preparation and instrument calibration are as previously described (13).

RESULTS AND DISCUSSION

All enzyme activity data in this section represent an average of at least five measurements; reported uncertainties are the standard deviation of these measurements. In the cases where error bars are not shown, the size of the symbol indicates the uncertainty. In reporting results for the vacuum-drying experiments we use the phrase "trehalose concentration" to denote the *initial* trehalose concentration. There have been many studies of the effects of freezing and freeze-drying of LDH. Comparison of our results with literature data is semi-quantitative due to differences in laboratory techniques and purities of reagents and LDH. The buffer type, concentration and pH can profoundly influence results. Furthermore, freezing and thawing rates not only depend on the technique used but also on the quantity of sample and container geometry. For this work, we have chosen to use a Tris-HCl buffer since previous studies have shown that, during freezing, precipitation of phosphate buffers (particularly sodium phosphate) can cause extreme pH changes (32,33).

Preservation of biological samples typically involves the removal of water through such processes as freeze-drying, spray-drying, vacuum-drying, or simple evaporative drying at ambient temperature. Of these techniques, we have chosen vacuum-drying because of its fast processing time, low energy requirements, and simplicity, all of which make it attractive for large-scale commercial processes.

It has previously been shown that, at sufficient concentrations, some enzymes exhibit a self-protection mechanism during freezing and freeze-drying (2,34,35). We found that at LDH concentrations greater than 10 μg/ml, a substantial fraction of the original activity was recovered following freezing and vacuum-drying. For this reason, we used a protein concentration of 8 μg/ml in all experiments. At this concentration, LDH is particularly susceptible to inactivation in the absence of stabilizing agents.

Trehalose

Figure 1 shows the relative LDH activity after freeze-thawing and vacuum-drying/rehydration in the presence of trehalose. For the freeze-thaw experiments, the recovered activity increases with increasing trehalose concentration. The activity begins to level off at about 80% recovery at a 300 mM trehalose concentration. In Figure 1, the vacuum-drying/rehydration data exhibit an interesting peak in recovered activity (76%) at an initial trehalose concentration of approximately 100 mM. The addition of more trehalose diminished the recovered activity. We attribute this to partial crystallization of trehalose. Presumably, crystalline trehalose would not be able to interact with the enzyme in a protective capacity. Using differential scanning calorimetry, we found that partial trehalose crystallization occurred in mixtures with an initial trehalose concentration greater than 200 mM (not shown). Such samples require longer drying times due to slow diffusion of water through the sample. We suggest that, from a kinetic point of view, a long drying time increases the probability and degree of trehalose crystallization.

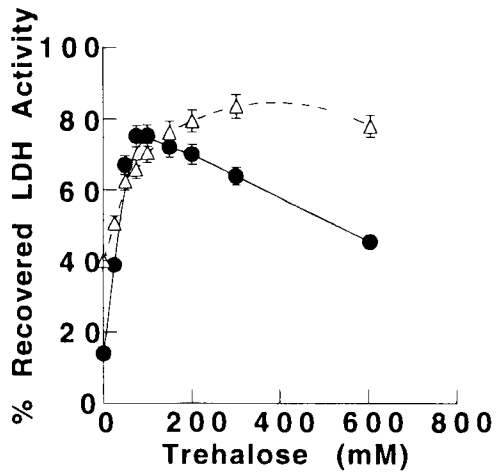

Fig. 1. Recovery of LDH activity following freeze-thawing (Δ) and vacuum-drying (●) as a function of trehalose concentration. Prepared in Tris buffer.

Trehalose/Borate

The "simple" aqueous trehalose mixtures studied in Figure 1 not only contained trehalose, but also LDH and Tris buffer. Addition of a salt, sodium tetraborate, further complicates matters. For this reason, we have chosen to begin by investigating the glass-forming properties of a simpler system. Figure 2 shows the T_g values of freeze-dried and vacuum-dried trehalose/sodium tetraborate mixtures as a function of R, the mole ratio of boron to trehalose. Although the borate ion, $B(OH)_4^-$, is the active species in solution, we have expressed concentrations in terms of boron since aqueous sodium tetraborate ($Na_2B_4O_7$) is not completely dissociated to borate ions. Figure 2 not only includes T_g data for trehalose/borate mixtures prepared in water,

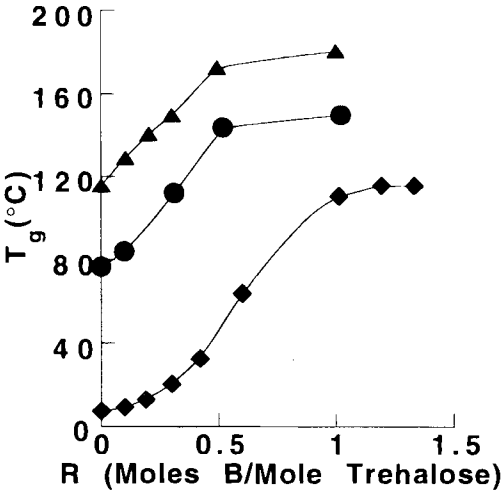

Fig. 2. The glass transition temperature of freeze-dried and vacuum-dried mixtures of trehalose and sodium tetraborate as a function of the mole ratio of boron to trehalose, R. Freeze-dried mixtures were prepared from solutions that included either unbuffered water (▲) or LDH and Tris buffer (●). Vacuum-dried mixtures (◆) contained LDH and Tris buffer. Initial trehalose concentration of the vacuum-dried samples was 100 mM.

but also for freeze-dried and vacuum-dried formulations that contain Tris and LDH.

In the freeze-dried trehalose/borate mixtures prepared in water, T_g increases from 115°C for trehalose to 170°C for a mixture with R = 0.5 mole B/mole trehalose. This increase is remarkable, given the modest amounts of sodium tetraborate present. In contrast, the freeze-dried formulations that included Tris and LDH had somewhat lower T_g values. For all values of R, these mixtures had T_g values about 40°C below those prepared with water. Since the concentration of borate ions depends on pH, we expect that the buffer concentration and pH strongly influence the degree of interaction between borate ions and trehalose. We speculate that the borate interacts with the hydroxyl groups of the Tris[hydroxymethyl]aminomethane molecule. Furthermore, we deduce that Tris interacts with trehalose even in the absence of borate, as evident from the reduction of T_g of the formulations for which R = 0.

Comparison of the vacuum-dried and freeze-dried trehalose/borate/Tris/LDH mixtures at the same boron:trehalose ratio shows considerably different T_g values. In the vacuum-dried trehalose/borate/Tris/LDH mixtures, the presence of residual water (from 8 to 10 wt%) considerably reduced the T_g values; T_g varied from 7°C for the trehalose formulation (R = 0) to 115°C for the mixture with R = 1.2. We also note that the T_g curves for all three types of formulations appear to reach a plateau. We suggest that this is a consequence of partial miscibility of trehalose and borate.

Figure 3 shows the dependence of T_g on the water content of a trehalose/sodium tetraborate/Tris/LDH mixture with R = 0.3. These data demonstrate the strong effect of residual moisture on the T_g of the formulation. The data for intermediate water contents in Figure 3 were obtained by periodically stirring a sample during vacuum-drying. This resulted in a drier product than is typically encountered during vacuum-drying. The water content of the datum at approximately T_g = 19°C (Figure 3) is representative of the samples processed by vacuum-drying. This mixture was left undisturbed in the vacuum-dryer for more than 36 hours. The datum at 0.2% water is for a freeze-dried trehalose/sodium tetraborate/Tris/LDH mixture.

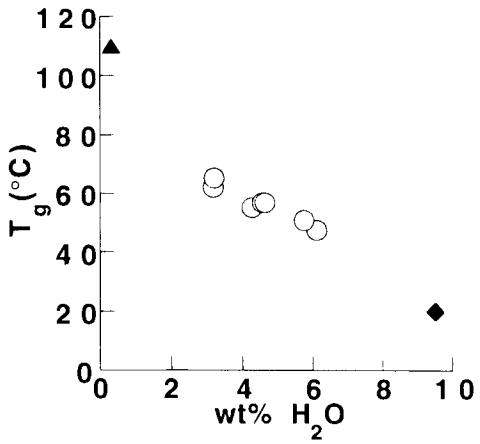

Fig. 3. The variation of the glass transition temperature of a trehalose/sodium tetraborate/Tris/LDH mixture (R = 0.30 mol boron/mol trehalose) with water content. Results shown for a rigorously dried freeze-dried sample, (▲), vacuum-dried samples with intermittent stirring, (○), and an undisturbed vacuum-dried sample, (◆). Initial trehalose concentration was 100 mM.

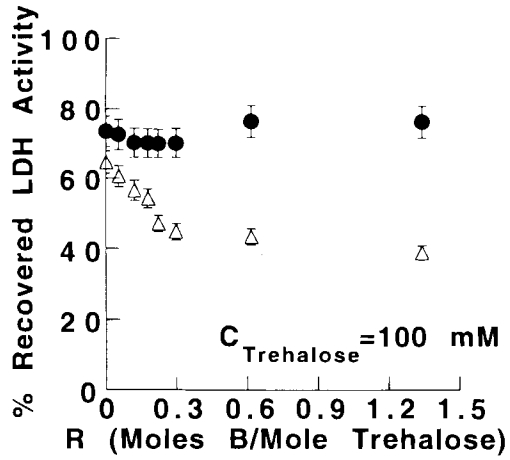

Fig. 4. Recovery of LDH activity following freeze-thawing (Δ) and vacuum-drying (●) as a function of the mole ratio of boron to trehalose. Trehalose concentration is 100 mM.

Figure 4 shows the relative LDH activity after freeze-thawing as a function R. The trehalose concentration is 100 mM in all cases. Without "borate", 62% of the activity was recovered following freeze-thawing. The presence of borate has a negative effect on recovery of LDH after freeze-thawing. Figure 4 also shows the relative LDH activity recovered after vacuum-drying/rehydration as a function of R; for these mixtures, about 75% of the activity is recovered. The apparent increase in the recovery of LDH over the range of concentrations studied is statistically insignificant. As compared to freezing, the greater overall recovery of activity indicates that, for the formulations studied, vacuum-drying is less destructive to the enzyme.

Figure 5 shows the relative LDH activity after freeze-thawing as a function of R. The trehalose concentration is 300 mM in all cases. The freeze-thaw results indicate that borate has a minor beneficial effect for $R < 0.3$; a loss of LDH activity, however, is observed at higher borate concentrations. For the vacuum-dried/rehydrated formulations, 62% of the original activity is recovered for mixtures prepared without borate. As

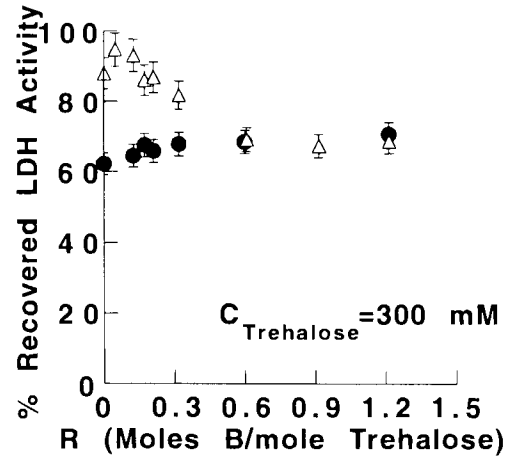

Fig. 5. Recovery of LDH activity following freeze-thawing (Δ) and vacuum-drying (●) as a function of the mole ratio of boron to trehalose. Trehalose concentration is 300 mM.

with the results for 100 mM trehalose (Figure 4), the slight increase of the recovery of LDH over the range of concentrations studied is statistically insignificant.

Together, the results of Figures 4 and 5 suggest that an optimum concentration of trehalose and borate exists for maximal recovery after freeze-thawing. The origins of this optimum, however, are unclear as several factors are at play in such a process, including viscosity enhancement, precipitation of solutes, and pH changes.

Time Denaturation Studies

The ultimate practical test of a protective system is whether it maintains enzymatic activity during storage under conditions of high temperature and/or humidity. Figure 6 shows the effect of storage time in a humidified environment (25°C) on the recovered activity of LDH. We used an environment of 100% relative humidity to accelerate the "aging" of vacuum-dried samples. These results demonstrate that the recovered activity of LDH prepared with a mixture of trehalose and sodium tetraborate is superior to that of trehalose alone. After more than seven weeks in this environment, the formulations containing trehalose and borate lost less than 25% of their original activity, while those containing only trehalose lost more than 75% of their original activity. It is interesting to note that, due to the high relative humidity used in this study, all samples absorbed a considerable amount of water after several days. We observed that all samples eventually became "rubbery" solids, which indicates that the T_g of the formulation was slightly below the storage temperature.

We suggest that the resistance to degradation of the mixtures prepared with borate is kinetic in origin. The glassy or highly viscous network of the trehalose/borate systems retards the motions that lead to protein denaturation. The substantially higher glass transition temperatures of trehalose/borate mixtures over trehalose alone play a critical role. For a given amount of absorbed water, the mixtures containing borate maintain an environment of much greater viscosity and higher T_g than those prepared with trehalose alone.

Part of the explanation for the stabilization property of trehalose/borate formulations could also be attributed to borate's affinity for water. The thermodynamically stable form of sodium tetraborate is the decahydrate. Thus, water absorbed by the sample during storage may preferentially bind to the borate. This would prevent both the glassy matrix and the protein from gaining the molecular mobility which leads to denaturation. This explanation is similar to that offered by Aldous et al. for the protective properties of the sugars trehalose and raffinose, both of which also exist as stoichiometric hydrates (21). In the cases where the formulations became "rubbery" solids, we observed that the trehalose/borate formulations were much less soft than those containing trehalose alone (unpublished).

Figure 7 shows the effect of storage time in an environment of lower relative humidity (RH = 32%, 25°C) on the recovered activity of LDH. These results show that vacuum-drying LDH in the presence of a boron/trehalose mixture (R = 1.2) results in a formulation with superior storage characteristics over that prepared with trehalose alone.

Perhaps a more pharmaceutically relevant storage situation is one in which a product is stored at low relative humidity,

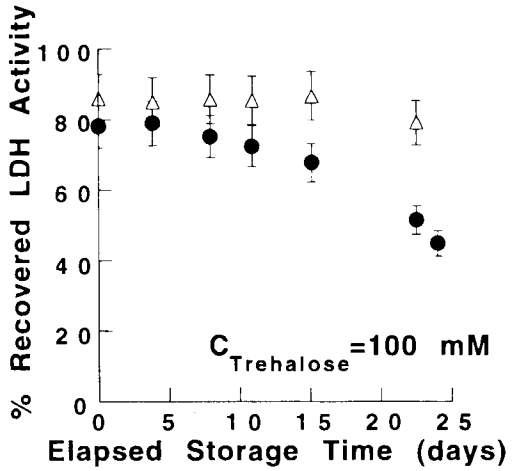

Fig. 7. Recovery of LDH activity over time for vacuum-dried trehalose/sodium tetraborate/Tris/LDH samples stored in a controlled humidity environment (RH = 32%, 25°C): R = 0 (●), R = 1.2 (Δ). Initial trehalose concentration is 100 mM.

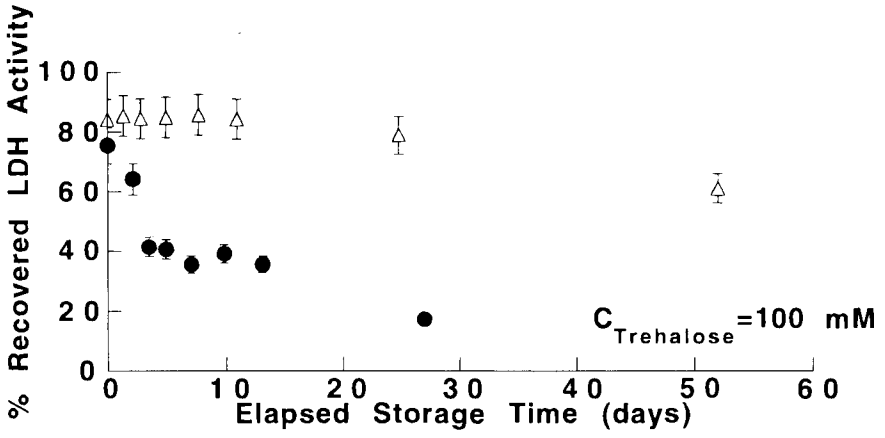

Fig. 6. Recovery of LDH activity over time for vacuum-dried trehalose/sodium tetraborate/Tris/LDH samples stored in a controlled humidity environment (RH = 100%, 25°C): R = 0 (●), R = 1.2 (Δ). Initial trehalose concentration is 100 mM.

but at an elevated temperature. We have chosen 45°C as an upper bound on temperature that may be experienced during shipping, for example. Figure 8 shows the effect of storage in an arid environment (RH < 1%) at 45°C on the recovered activity of LDH. (From Figure 2, the T_g values of the vacuum-dried mixtures of R = 0.0, 0.3, and 1.2 moles boron/mole trehalose are 7.0, 19.0, and 115°C, respectively).

These results demonstrate that vacuum-drying LDH in the presence of trehalose/borate mixtures (R = 0.3, R = 1.2) results in a formulation with superior storage characteristics over that prepared with trehalose alone. The mixture prepared with just trehalose lost nearly all its activity within two days. Those prepared with trehalose and borate retained a significant fraction of their activity even after more than 40 days of storage. We also note that vacuum-dried samples appear spatially heterogeneous. That is, because of more efficient mass transfer during vacuum-drying, the material at the air interface is drier than the material at the bottom of the container. Thus, proteins or subunits of a protein may experience different local environments depending on their position in the container. Sample heterogeneity may be responsible for some of the initial loss of activity of samples stored at a temperature below T_g. For relatively large boron:trehalose ratios, we already noted that phase separation may have occurred between the trehalose and the sodium tetraborate (see Figure 2). This phenomenon may have also resulted in a loss of stabilizing interactions between the LDH and the trehalose:borate matrix.

CONCLUSIONS

The enzyme lactate dehydrogenase is easily denatured by freezing and drying processes. Its labile nature, along with its simple assay technique, make it an ideal candidate for testing protective agents. We have verified previously reported results by showing that, following a freeze/thaw cycle, recovered activity increases with trehalose concentration.

We have also investigated a new class of additives that combine synergistically with trehalose. In an aqueous mixture containing trehalose and sodium tetraborate, the borate ion interacts with the hydroxyl groups of trehalose to form a chemical complex and, possibly, a network. These interactions persist in the dry state (<0.3 wt% H_2O), as evident from the elevated T_g values of freeze-dried trehalose/borate mixtures. We emphasize that the Tris buffer profoundly affects the T_g of the vacuum-dried and freeze-dried formulations.

We have shown that the use of borate results in a decreased recovery of LDH following freeze-thawing and no significant change in LDH recovery after vacuum-drying. While the differences are modest, the results have practical significance when considering the relative costs of trehalose and borate.

We suggest that the differences between the freeze-thawing and vacuum-drying results (see either Fig. 4 or Fig. 5) are due to the lower capacity of the trehalose/borate mixtures to protect LDH during freezing processes. These results are similar to those of Carpenter et al., in which they found that trehalose concentrations (up 100 mM) failed to protect LDH during *freeze-drying* (25). However, addition of small amounts of polyethylene glycol (PEG) provided protection during freeze-drying, as long as trehalose was present. They concluded that PEG and trehalose provided protection to the protein during the freezing and drying steps, respectively. We propose that a similar scheme which combines a *cryo*protectant (such as PEG) with a trehalose/borate mixture could provide a more effective formulation for freeze-drying.

The most remarkable results involve the storage stability of formulations containing both trehalose and borate. Those mixtures, in comparison with formulations containing trehalose alone, showed substantially greater LDH activities after more than seven weeks in an environment of 100% relative humidity. Similar results were found for mixtures stored at a lower relative humidity. We have also shown that trehalose/borate mixtures protect proteins against stresses in a high temperature environment. Enzymes prepared in trehalose/borate mixtures, vacuum-dried, and then stored in an arid environment at 45°C always showed significantly greater activities over those prepared in trehalose alone.

We suspect that similar results will be found for other combinations of saccharides (and other polyhydroxy compounds) and other boron compounds (e.g., potassium, calcium, magnesium and lithium borate salts, boric acid). We are currently performing tests of these systems on other enzymes. We also plan to undertake conductivity and X-ray studies to help elucidate the physical state of the trehalose and borate in aqueous solution and in the dried products, respectively. Finally,

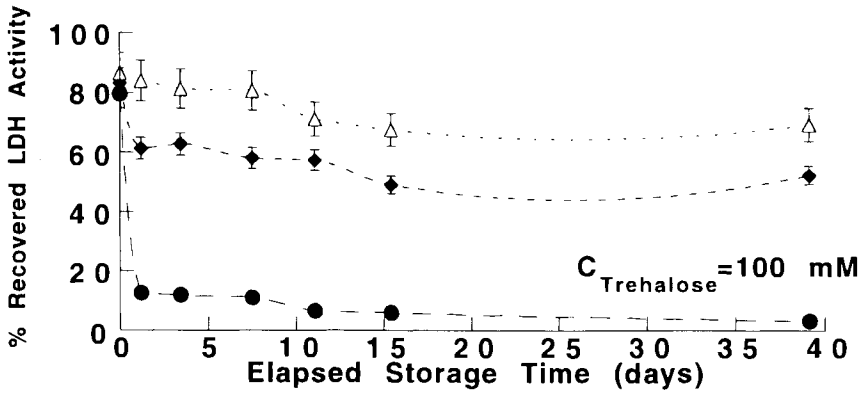

Fig. 8. Recovery of LDH activity over time for vacuum-dried trehalose/sodium tetraborate/Tris/LDH samples stored at 45°C: R = 0 (●), R = 0.3 (♦), R = 1.2 (Δ). Initial trehalose concentration is 100 mM.

we are currently evaluating the protective properties of the trehalose/borate system for freeze-drying of enzymes.

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