

Research Paper

Stabilizing Effect of Four Types of Disaccharide on the Enzymatic Activity of Freeze-dried Lactate Dehydrogenase: Step by Step Evaluation from Freezing to Storage

Kiyoshi Kawai^{1,3} and Toru Suzuki²

Received May 10, 2006; accepted October 31, 2006; published online May 8, 2007

Purpose. In order to understand the stabilizing effects of disaccharides on freeze-dried proteins, the enzymatic activity of lactate dehydrogenase (LDH) formulations containing four types of disaccharide (trehalose, sucrose, maltose, and lactose) at two relative humidity (RH) levels (about 0 and 32.8%) was investigated after three processes: freeze-thawing, freeze-drying, and storage at three temperatures (20, 40, and 60°C) above and/or below the glass transition temperature (T_g).

Materials and Methods. The enzymatic activity was determined from the absorbance at 340 nm, and T_g of the samples was investigated by differential scanning calorimetry.

Results. At each RH condition, T_g values of sucrose formulations were lower than those of other formulations. Although effects of the disaccharides on the process stability of LDH were comparable, storage stability was dependent on the type of disaccharide. All the formulations were destabilized significantly during storage at temperature above T_g . During storage at temperature below T_g , the LDH activity decreased with increases in the storage temperature and moisture. Maltose and lactose formulations showed significant destabilization with the change of color to browning.

Conclusions. Taking the storage stability of freeze-dried proteins under the various conditions (temperature and RH) into consideration, trehalose is better suited as the stabilizer than other disaccharides.

KEY WORDS: cryostabilization; disaccharide; freeze-drying; glass transition; lyostabilization; storage stability.

INTRODUCTION

Freeze-drying is a commonly used technique for stabilizing labile biological and pharmaceutical proteins. However, some proteins are sensitive to chemical and physical stresses induced by freeze-drying, which causes them to lose most of their functional activity during the process. Extensive efforts have been made to improve the stabilization of freeze-dried proteins by using stabilizers (1–3).

Since freezing is the first step of freeze-drying, cryostabilizing effect of stabilizers should be considered first. The most widely accepted cryostabilizing mechanisms are “solute exclusion” (2–5) and “freeze-concentrated glass transition” (1,2,6–8) mechanism. The “solute exclusion” mechanism involves solute exclusion of protein with water

than stabilizers, which are preferentially excluded from its hydration shell, and thus, unfolding of the protein is prevented and its native conformation is stabilized. The “freeze-concentrated glass transition” mechanism involves embedding of the frozen protein in a glassy matrix formed by freeze-concentrated stabilizers leading to a highly viscous state, and thus, molecular rearrangement of the protein and the rate of chemical and physical degradations are immobilized. In contrast, mechanisms of lyostabilization and subsequent storage stabilization have often been explained by the “water substitute” (2,3,5) and “glass transition” (2,6–8) mechanisms. The “water substitute” mechanism involves intermolecular hydrogen bonds of exposed polar groups of the freeze-dried protein with amorphous stabilizers as water substitutions, and thus the freeze-dried protein maintains its native-like conformation. The maintenance of native-like conformation decreases damage induced by freeze-drying process, and thus the storage stability is also improved more or less. The “glass transition” mechanism is intrinsically similar to “freeze-concentrated glass transition” mechanism; the glassy matrix formed by the freeze concentration maintains its state even at an elevated temperature due to the decrease in the number of water molecules that play the role of a plasticizer. In the “glass transition” mechanism, the glass transition temperature (T_g) of stabilizers is one of the most

¹ Tokyo University of Technology Institution, School of Bionics, 1404-1 Katakura, Hachioji, Tokyo, 192-0982, Japan.

² Department of Food Science and Technology, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo, 108-8477, Japan.

³ To whom correspondence should be addressed. (e-mail: kawai@bs.teu.ac.jp)

ABBREVIATIONS: DSC, differential scanning calorimetry; LDH, lactate dehydrogenase; PFK, phosphofructokinase; RH, relative humidity (%); T_g , glass transition temperature (°C).

significant parameters, because the glassy formulations change to liquid-like rubber state at the T_g .

The stabilizing effect of disaccharide on frozen and freeze-dried proteins is often greater than that of other agents such as polyol, monosaccharide, carbohydrate polymer, synthetic polymer, amino acid, and salt (2,4). Carpenter and Crowe (4) investigated the activity recovery of frozen and/or freeze-dried phosphofructokinase (PFK) formulations containing various kinds of stabilizing agents and demonstrated that while each of these agents maintained a high activity of the frozen-thawed PFK formulations, only disaccharide showed a significant stabilizing effect on the freeze-dried formulations. Similarly, Izutsu *et al.* (9) investigated the effects of various stabilizing agents on the long term stability of freeze-dried β -galactosidase at an elevated temperature. They found that while most agents were able to stabilize the protein during the freeze-drying process, only disaccharide was able to improve its long term stability. The significant stabilizing effect of disaccharide on frozen and freeze-dried proteins has been explained by the above-mentioned mechanisms: disaccharide exhibits effective solute exclusion (3–5) and the ability to form intermolecular hydrogen bonds (2,4,5) as well as having a relatively high T_g (2,6–8). Furthermore, disaccharide is known to show a significant lyostabilizing effect on phospholipids by similar mechanisms (10,11). Therefore, to stabilize freeze-dried biomaterials, the four typical types of disaccharide: trehalose, sucrose, maltose, and lactose, have generated technical as well as fundamental interest.

The literature indicates large variations in the process and storage stabilizing effects of the four types of disaccharide on frozen and freeze-dried proteins (12–32). This is because the cryo- and lyo-stabilizing effects of disaccharide depend on various experimental parameters. Furthermore, it has been suggested that damage to proteins during freeze-drying process affected their subsequent storage stability (18). Therefore, it is necessary to evaluate not only process stability but also storage stability of freeze-dried proteins. Previous studies have extensively investigated the effects of the composition of freeze-dried formulations [for example, their disaccharide (13–15,18,20,23,25,27,29,31) and moisture contents (23,26,28,32), and combination with other agents (12,15,16,18,21,22,25,29,30)] on the stabilization of proteins immediately after freezing or freeze-drying. The storage stability of the freeze-dried protein-disaccharide formulations has also been investigated under limited storage conditions (9,18,20,21,30–32). However, to the best of our knowledge, few, if any studies have been conducted on the stabilizing effects of disaccharides on the freeze-dried proteins from freezing as a first step of the freeze-drying process to storage under the various conditions.

In this study, a stepwise evaluation of the stability of freeze-dried protein-disaccharide formulations was performed. Lactate dehydrogenase (LDH) was employed as a model protein, and the activity of freeze-dried LDH containing four types of disaccharide (trehalose, sucrose, maltose, and lactose) and two relative humidity (RH) levels (about 0 and 32.8%) were investigated at three stages: after freeze-thawing, after freeze-drying, and after storage, at 20, 40, and 60°C over a period of up to 90 days. The stabilization effect of the four disaccharides was compared at each stage.

MATERIALS AND METHODS

Preparation of Freeze-dried Formulations and the Experimental Procedure

Analytical grade sucrose, maltose monohydrate, lactose monohydrate, and LDH from rabbit muscle were purchased from Wako Pure Chem. Ind. Ltd., Japan. Reagent grade D-trehalose dihydrate was provided by Hayashibara Co. Ltd., Japan. Other reagents were purchased from Sigma-Aldrich Co., USA.

The experimental procedure is illustrated in Fig. 1. LDH suspension was dialyzed against 50 mM sodium phosphate buffer (pH=7.5) at 5°C for 24 h. The LDH concentration was determined from the absorbance at 280 nm by a UV-spectrophotometer (U-3200: Hitachi, Co. Ltd., Japan) using an extinction coefficient of $0.648 \text{ l cm}^{-1} \text{ g}^{-1}$. A solution containing 100 μg LDH and 100 mg disaccharide (trehalose, sucrose, maltose, or lactose) per 1 ml was prepared, and 1 ml aliquot of this solution was pipetted into 2 ml-polyethylene tubes (denoted as initial formulation in Fig. 1). The initial formulation was frozen by immersion into liquid nitrogen for at least one min. A part of the frozen formulations was thawed in a water bath at 25°C, before assaying LDH activity in order to examine the loss of LDH activity after freezing. The other frozen formulations were transferred to a precooled freeze-drier, and then the freeze-drying was carried out with a chamber pressure of 3.0×10^{-2} torr by increasing the shelf temperature from -40 to -25°C at the rate of 5°C per 6 h, and from -25 to 25°C at the rate of 5°C per 3 h. For moisture control, the freeze-dried formulations were either kept in a vacuumed desiccator containing diphosphorus pentoxide (RH = about 0%) or in a desiccator containing saturated magnesium chloride (RH=32.8%) at 25°C for 7 days. The polyethylene tubes containing freeze-dried sample were screw-capped in a bag with flowing nitrogen gas as soon as possible. The two formulations are denoted in Fig. 1 as fully dried formulation and slightly wet formulation, respectively. At this stage,

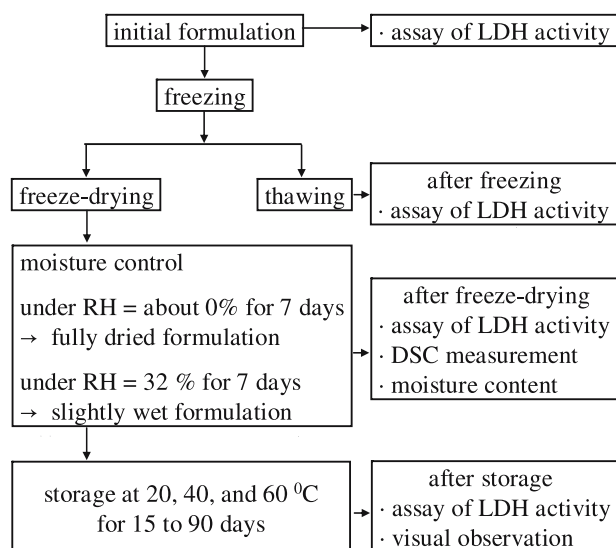


Fig. 1. Experimental procedure used in this study.

moisture content of the freeze-dried formulations was checked gravimetrically by vacuum drying by increasing the temperature from 25 to 60°C over a 3-day period. Additionally, T_g of the freeze-dried formulations was examined by differential scanning calorimetry (DSC), details of which are given later. A part of these formulations was rehydrated with distilled water, and LDH activity was assayed in order to examine the loss of LDH activity immediately after the preparation of the freeze-dried formulations. The other formulations were stored at 20, 40, and 60°C for a period of up to 90 days. Chemical and physical changes in the formulations were observed visually. The stored formulations were rehydrated with distilled water and LDH activity was assayed in order to examine the loss of LDH activity associated with during storage.

Assay of LDH Activity

The LDH concentration of formulations was adjusted to 10–100 µg/ml and 10 µl of this solution was then added to a quartz cell containing 3 ml reaction solution (50 mM sodium phosphate buffer (pH=7.5), 0.49 mM pyruvate and 0.1 mM NADH) kept at 25°C. The quartz was set into UV-spectrophotometer (U-3200: Hitachi, Co. Ltd., Japan), and decrease in absorbance at 340 nm was immediately measured for 3 min with stirring by using a magnetic stirrer. The “initial rate method” was used to obtain the LDH activity via the rate of change in the absorbance. The relative LDH activity was expressed as a percentage of the LDH activity of freeze-thawed, freeze-dried, or stored formulations to that of the initial formulations. The variation of relative LDH activity based on this measurement was in the range of ±4.5%.

DSC Measurement

Thermal properties of freeze-dried formulations were examined by a DSC (DSC-50: Shimadzu Co. Ltd., Japan). The instrument was purged with nitrogen gas and calibrated for temperature and enthalpy with indium and distilled water. The α -alumina powder was used as a reference and liquid nitrogen was used as a coolant of the instrument. The sample (approximately 10 mg) was weighed in an aluminum DSC pan in a bag with flowing nitrogen gas and hermetically sealed. Fully dried formulations were heat-scanned at 5°C/min from ambient temperature to 120°C. Slightly wet

formulations were pre-cooled from ambient temperature to –20°C, and then heat-scanned at 5°C/min to 70°C. DSC thermograms were analyzed by TA-60 software interfaced with the DSC.

RESULTS AND DISCUSSION

Glass Transition Behavior of Freeze-dried Formulations

Moisture contents of the fully dried formulations were below 0.5% (w/w). DSC thermograms of these formulations are shown in Fig. 2. All formulations indicated an endothermic shift due to the glass transition, and the T_g was determined from the onset point of this shift. The obtained T_g values were listed in Table I, along with values for their pure disaccharide systems reported in previous studies (7,33). In comparison with the T_g values, it was noted that the T_g values of the freeze-dried formulations showed various tendencies compared with the pure disaccharide systems; the trehalose and lactose formulations were lower T_g , and the sucrose and maltose formulations were higher T_g than those of the pure systems. The variation of T_g values has been often reported in previous studies. For example, Urbani *et al.* pointed out that literature indicated large variations between 52 and 70°C in the T_g value of pure sucrose systems (34). This is because the T_g value of amorphous materials is affected by various experimental factors (*e.g.*, thermal history). Furthermore, the T_g value of multi-component systems shows more complicated variation. It is known well that only a little residual moisture decreases significantly the T_g value of freeze-dried sugars (6,8). In addition, Ohtake *et al.* (11,35) reported that freeze-dried disaccharide-phosphate mixture showed a drastic change of the T_g value (54–101°C for sucrose system and 96–119°C for trehalose system) depending on the pH and amount of counter ion in the initial solution. Taking these observations into consideration, it is noted that the T_g value of the freeze-dried formulations cannot be determined strictly. However, as an obvious trend of the T_g values, it was confirmed that the T_g value of the trehalose, maltose and lactose formulations was higher than that of the sucrose formulation as well as the pure disaccharide systems.

Moisture contents of the slightly wet formulations were in the range between 5.5 and 6.5% (w/w). DSC thermograms of these formulations are shown in Fig. 3. Sucrose formulation exhibited an endothermic shift due to the glass transition

Table I. T_g and Moisture Content of Freeze-dried LDH Formulations

Formulation	T_g of Formulation in this Study (°C)	T_g of Pure System in Reference (°C)	Moisture Content (% w/w)
Fully dried (RH=about 0%)			
Trehalose	99.7	100(8), 113 (34)	<0.5
Sucrose	80.3	62 (8),68 (34)	<0.5
Maltose	95.0	87 (8), 90 (34)	<0.5
Lactose	93.0	101 (8)	<0.5
Slightly wet (RH=32.8%)			
Trehalose	40.3	Unknown	6.5
Sucrose	22.8	Unknown	5.6
Maltose	40.1	Unknown	5.5
Lactose	39.9	Unknown	5.8

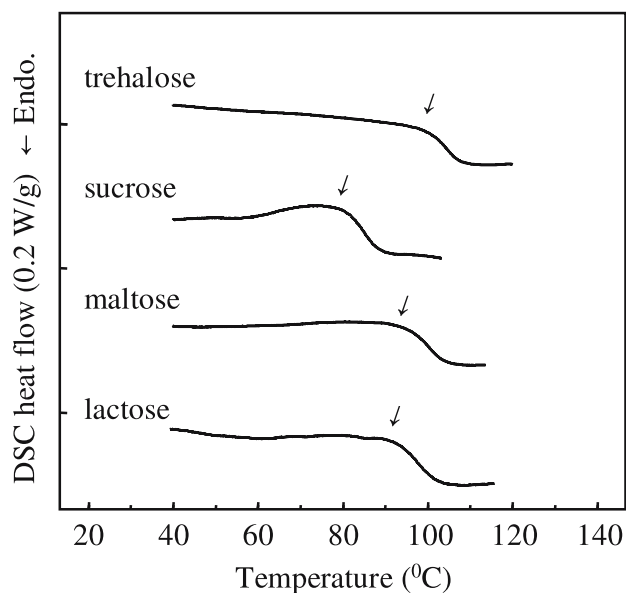


Fig. 2. DSC thermograms for fully dried formulations. Arrows indicate T_g .

similar to those shown in Fig. 2. In contrast, trehalose, maltose, and lactose formulations exhibited an endothermic peak in the first scan. These samples were re-scanned for confirmation, and then an endothermic shift due to the glass transition appeared instead of the endothermic peak. It should be noted that the endothermic peak in the first scan is due to not the melting of crystallized disaccharide and/or buffer, but the recovery of relaxed enthalpy. The enthalpy relaxation is a characteristic thermal event observed in glassy materials stored at temperature below T_g , and the degree of relaxed enthalpy increases with increases in the storage temperature and time (33,36). The enthalpy relaxation of the trehalose, maltose, and lactose formulations will have occurred during the preparation, because their T_g values were slightly higher than the prepared temperature (25°C). On the other hand, the sucrose formulation turned into rubber state during the preparation, and thus enthalpy relaxation was not observed in the DSC thermogram. The T_g values determined from the results in the first scan are listed in Table I. It was noted that the T_g value of the slightly wet trehalose, maltose and lactose formulations was higher than that of the sucrose formulation as well as the fully dried formulations.

LDH Activity of Frozen and Freeze-dried Formulations

The relative LDH activity of freeze-thawed formulations is shown in Fig. 4. The relative LDH activity of the no-additive formulation decreased to approximately 80% of the initial activity. This result was comparable to or higher than those found in literature; the relative LDH activities of freeze-thawed no-additive formulations were reported to be approximately 40% for 8 $\mu\text{g/ml}$ LDH in 50 mM tris-HCl buffer (24), 30% for 25 $\mu\text{g/ml}$ LDH in 10 mM phosphate buffer (15), 20–90% for 25–500 $\mu\text{g/ml}$ LDH in 20 mM tris-HCl buffer (37), and 30–85% for 5–50 $\mu\text{g/ml}$ LDH in 50 mM phosphate buffer (38). The differences in the relative LDH activity will be caused mainly by differences in the LDH

concentration. It is known that the enzymatic activity of freeze-thawed proteins increases with an increase in the protein concentration (i.e., self-protection of protein). Actually, our result (80% LDH activity for 100 $\mu\text{g/ml}$ LDH in 50 mM phosphate) was in good agreement with the data for a similar formulation (85% LDH activity for 50 $\mu\text{g/ml}$ LDH in 50 mM phosphate buffer) (38). Although effects of buffer, freezing rate, and freezing time on the cryostabilizing of LDH were also suggested as other possible contributions (37,38), it was beyond the scope of this paper to discuss these effects. On the other hand, the formulations containing disaccharide fully-almost completely maintained their relative LDH activity. It was suggested that these formulations showed the tendency to maintain just a little higher relative LDH activity than the no-additive formulation. However, taking experimental error into consideration, the cryostabilizing effect of the disaccharides on the LDH activity was unclear, because the no-additive formulation showed comparatively high LDH activity. From these results, it was found that not only the disaccharide-additive formulations but also the no-additive one maintained sufficiently high LDH activity at this stage.

Relative LDH activities of fully dried and slightly wet formulations are shown in Fig. 4. In all the formulations, destabilization of LDH by freeze-drying was much greater than that by freezing. The relative LDH activity of the no-additive formulations decreased to approximately 15% of initial activity. The result was comparable to or lower than those found in literature; the relative LDH activities of freeze-dried no-additive formulations were reported to be approximately 20% for 25 $\mu\text{g/ml}$ LDH in 10 mM phosphate buffer (15), 46% for 2 mg/ml LDH in 20 mM phosphate buffer (16), 11%

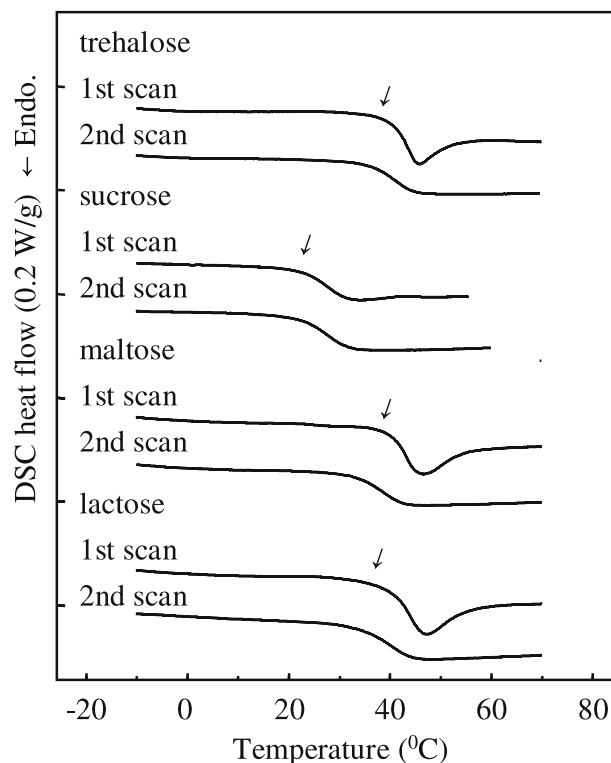


Fig. 3. DSC thermograms for slightly wet formulations. Arrows indicate T_g .

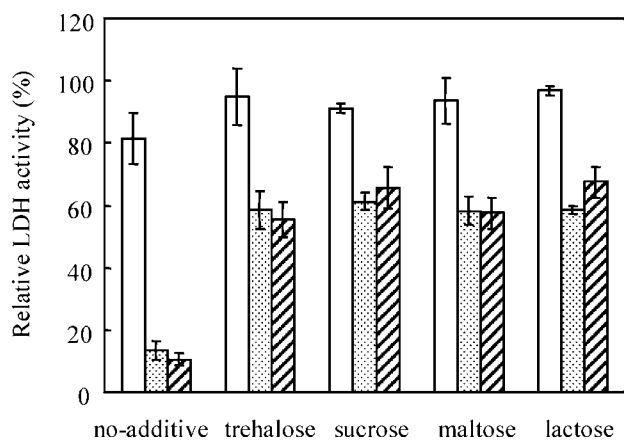


Fig. 4. The relative activity of freeze-thawed and freeze-dried LDH formulations. *Open*, *dotted*, and *slash* bars indicate the result of frozen formulation, fully dried formulation and slightly wet formulation, respectively.

for 1 mg/ml LDH in 10 mM tris-HCl buffer (17), 20–60% for 25–500 μ g/ml LDH 20 mM tris-HCl buffer (37), and 5–90% for 25–100 μ g/ml LDH in 50 mM phosphate buffer (38). The differences in the results between this study and previous ones will have been caused by differences in initial LDH concentration, damage during freezing process, drying temperature, and residual moisture content (37,38). On the other hand, the relative LDH activity of disaccharide-additive formulations decreased to approximately 60% of initial activity. It was noted that the lyostabilizing effect of the disaccharides was significant, but independent of the type of disaccharide and the levels of moisture content. Among the disaccharides, trehalose has attracted significant attention as a stabilizer because organisms that live under harsh conditions utilize trehalose for self-preservation of membranes (39). The stabilizing effects of trehalose have been attributed to a number of its physical properties: high T_g (40), ability to form intermolecular hydrogen bonds (41), and low molecular mobility below T_g (33). However, it should be noted that after freeze-drying, the stabilizing effect of trehalose on LDH formulations was not unique compared to other disaccharides. On the other hand, it has been reported that the moisture of freeze-dried proteins can affect its stability both indirectly (plasticizer or reaction medium) and directly (reactant or product) (2). However, it was observed that the effect of moisture on the relative LDH activity was not significant at this stage.

LDH Activity of Freeze-dried Formulations Stored under Various Conditions

Time courses of the relative LDH activity of fully dried and slightly wet formulations stored at 20, 40, and 60°C are shown in Figs. 5, 6, and 7, respectively. Although all the no-additive formulations lost the relative LDH activity within 15 or 30 days, the disaccharide-additive formulations maintained the relative LDH activity more or less. The stabilizing effect of the disaccharides on the LDH activity was strongly dependent on the types of disaccharide and the levels of moisture content. On the other hand, visual observation results of the disaccharide-additive formulations are listed in Table II. These results were discussed as follows.

During storage at 60°C (Fig. 7), all slightly wet formulations were in rubber state ($T > T_g$). From visual observation, while structural collapse occurred in all the formulations, the color of the formulation changed to brown also occurred in the maltose and lactose formulations. The relative LDH activity of all the slightly wet formulations was almost completely lost within 15 days. In contrast, all fully dried formulations were in glassy state ($T < T_g$). During the storage period of up to 90 days, the trehalose and sucrose formulations showed no visual change, and their relative LDH activities decreased to approximately 35 and 45% of initial activity. On the other hand, the maltose and lactose formulations showed the color of the formulation changed to brown, and their relative LDH activities decreased to approximately 22 and 15% of initial activity. These results indicate that the storage stability of freeze-dried LDH formulations in glassy state was much greater than that in rubber state at the storage temperature of 60°C. The destabilization in the fully dried maltose and lactose formulations was greater than that in the fully dried trehalose and sucrose formulations, which could be related to the color of the formulation changed to brown observed visually in the maltose and lactose formulations. The color of the formulation changed to brown may be indicative of the progress of a non-enzymatic browning reaction. A non-enzymatic browning reaction, which is a chemical reaction between carbonyl compounds (for example, reducing sugar such as maltose and lactose) and amino compounds, results in the formation of browning pigments. It is known that the progress of a non-enzymatic browning reaction causes destabilization of a protein (9,19), and that this reaction can progress even at a temperature much lower than T_g within an hour to a day (42,43). In the fully dried

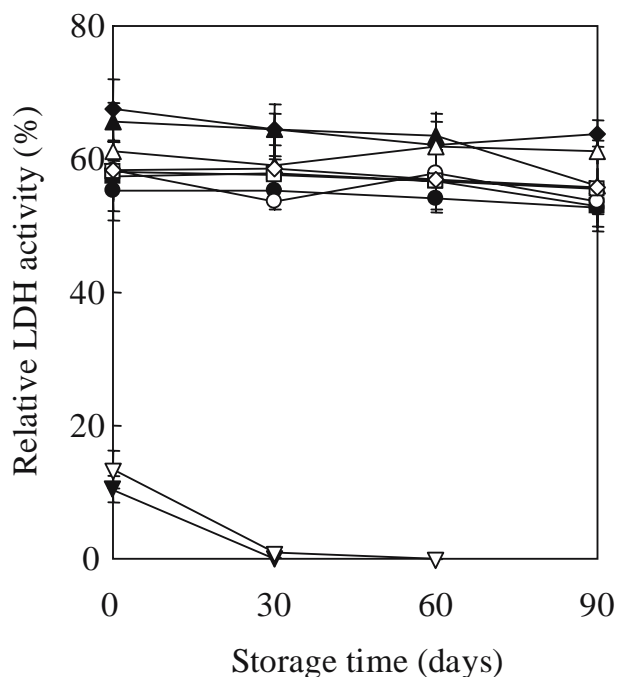


Fig. 5. Time courses of the relative activity of LDH formulations stored at 20°C: *lower triangle*, no-additive; *circle*, trehalose; *upper triangle*, sucrose; *square*, maltose; *diamond*, lactose. *Open* and *closed* symbols indicate a fully dried formulation and a slightly wet formulation, respectively.

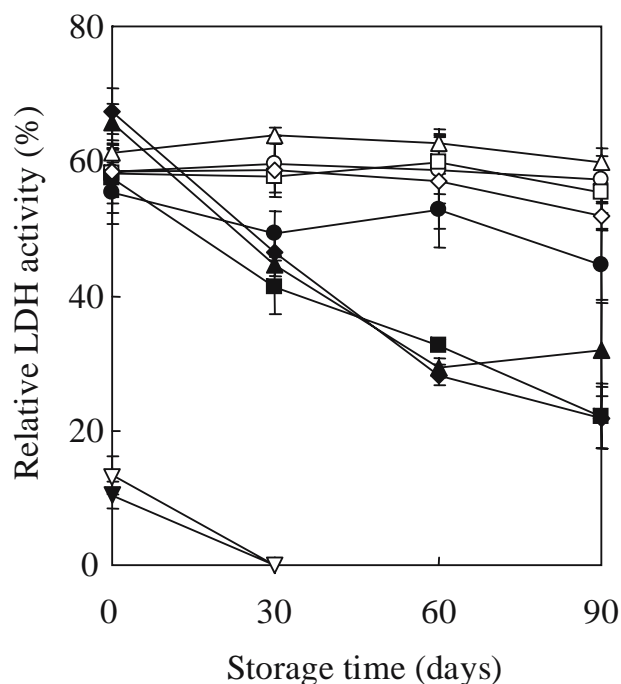


Fig. 6. Time courses of the relative activity of LDH formulations stored at 40°C: lower triangle, no-additive; circle, trehalose; upper triangle, sucrose; square, maltose; diamond, lactose. Open and closed symbols indicate a fully dried formulation and slightly wet one, respectively.

trehalose and sucrose formulations which exhibited no browning change, there was a gradual loss of the relative LDH activity at the storage temperature of 60°C. The effect of trehalose and sucrose on the storage stability of LDH was almost comparable. It should be noted that destabilization was not completely prevented at 60°C, even if the formulations were in glassy state.

During storage at 40°C (Fig. 6), all fully dried formulations were in glassy state ($T < T_g$). These formulations showed no change upon visual observation and only a slight if any loss in the relative LDH activity, though the formulations stored at 60°C showed various destabilizations as mentioned above. This is because a decrease in storage temperature diminished the destabilization of LDH; the effect of the decrease in storage temperature will imply decreases in the molecular mobility of glassy matrix and the rate of various degradations. The slightly wet trehalose, maltose and lactose formulations, the T_g of which were close to the storage

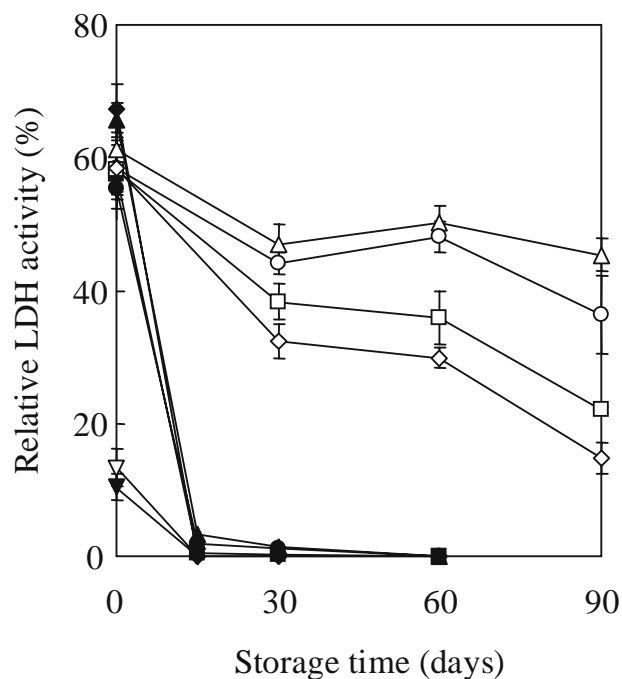


Fig. 7. Time courses of the relative activity of LDH formulations stored at 60°C: lower triangle, no-additive; circle, trehalose; upper triangle, sucrose; square, maltose; diamond, lactose. Open and closed symbols indicate a fully dried formulation and slightly wet one, respectively.

temperature ($T \approx T_g$), existed in either in glassy or in rubber state, while the slightly wet sucrose formulation was in rubber state ($T > T_g$). It was observed visually that no changes occurred in trehalose formulation, structural collapse occurred in the sucrose formulation, and the color of the formulation changed to brown occurred in the maltose and lactose formulations. The relative LDH activity of the trehalose formulation was markedly greater than that of the sucrose, maltose, and lactose formulations. It is assumed that the destabilization of the maltose and lactose formulations was related to the progress of the non-enzymatic reaction. In contrast, the destabilization of the sucrose formulation probably occurred because it was stored at temperature above T_g . Since viscosity of a rubber matrix is considerably lower than that of a glassy matrix at a reference temperature, various destabilization (e.g., thermal degradation, aggregation, and crystallization of the rubber matrix) probably

Table II. Visual Observations of LDH Formations Stored at Various Temperatures

Formulation	Visual Observation after Storage		
	At 60°C	At 40°C	At 20°C
Fully dried (RH=about 0%)			
Trehalose	—	—	—
Sucrose	—	—	—
Maltose	Browning	—	—
Lactose	Browning	—	—
Slightly wet (RH=32.8%)			
Trehalose	Collapse	—	—
Sucrose	Collapse	Collapse	—
Maltose	Collapse & browning	Browning	—
Lactose	Collapse & browning	Browning	—

occurred in the sucrose formulation. On the other hand, the relative LDH activity of the slightly wet trehalose formulation was a little lower than fully dried one at the storage temperature of 40°C. This result suggests that an increase in the moisture content caused the destabilization of LDH during the storage. It is known that the moisture of freeze-dried proteins can affect its stability both indirectly (plasticizer or reaction medium) and directly (reactant or product) (2). It should be noted that destabilizing effects induced by moisture were not completely prevented even at temperature below T_g .

During storage at 20°C (Fig. 5), all fully dried and slightly wet formulations were in glassy state, showing no change on visual observation and only a slight if any loss of relative LDH activity over the duration of the storage period. As mentioned above, the slightly wet formulations showed various destabilizations during storage at 40°C. The destabilizations induced by the storage at 40°C will have diminished enough at 20°C. At such the low-temperature, the stabilizing effect of the disaccharides was comparable.

CONCLUSIONS

In conclusion, the stabilizing effect of the four types of disaccharide (trehalose, sucrose, maltose, and lactose) on freeze-dried LDH can be summarized as follows. Although the effects of the disaccharides on the process stability of LDH were comparable, the storage stability of LDH was strongly dependent on the storage conditions (temperature and humidity) and the physical and chemical properties of the disaccharides (T_g and chemical reactivity). In terms of chemical reactivity, trehalose and sucrose were superior to maltose and lactose which play the role of reactants in non-enzymatic browning reactions. In terms of T_g , trehalose, maltose and lactose were superior to sucrose, which has a lower T_g than the other disaccharides. Taking the storage stability of freeze-dried proteins under the various conditions (temperature and RH) into consideration, trehalose is better suited as the stabilizer than other disaccharides.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support from a Grant-in-Aid for JSPS Fellows provided by The Ministry of Education, Culture, Sports, Science and Technology. The authors would also like to thank Drs. Rikuo Takai and Tomoaki Hagiwara of the Tokyo University of Marine Science and Technology for their valuable discussions, Drs. Munehiko Tanaka and Shoichiro Ishizaki of the Tokyo University of Marine Science and Technology for the use of UV-spectrometer, and Dr. Hiroto Chaen of Hayashibara Biochemical Lab. Inc., Japan, for providing the trehalose dihydrate reagent.

REFERENCES

1. J. F. Carpenter, M. J. Pikal, B. S. Chang, and T. W. Randolph. Rational design of stable lyophilized protein formulations: some practical advice. *Pharm. Res.* **14**:969–975 (1997).
2. W. Wang. Lyophilization and development of solid protein pharmaceuticals. *Int. J. Pharm.* **203**:1–60 (2000).
3. T. Arakawa, S. J. Prestrelsky, W. C. Kenney, and J. F. Carpenter. Factors affecting short-term and long-term stabilities of proteins. *Adv. Drug Deliv. Rev.* **46**:307–326 (2001).
4. J. H. Crowe, J. F. Carpenter, L. M. Crowe, and T. J. Anchordoguy. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology* **27**:219–231 (1990).
5. T. Arakawa, Y. Kita, and J. F. Carpenter. Protein-solvent interactions in pharmaceutical formulations. *Pharm. Res.* **8**:285–291 (1991).
6. F. Franks. Freeze drying: from empiricism to predictability. *Cryo-Lett.* **11**:93–110 (1990).
7. H. Levine, and L. Slade. Principles of “cryostabilization” technology from structure/property relationships of carbohydrate/water system—a review. *Cryo-Lett.* **9**:21–63 (1988).
8. Y. H. Roos. Phase Transitions in Foods. Academic, San Diego, CA, 1995.
9. K. Izutsu, S. Yoshioka, and Y. Takeda. The effects of additives on the stability of freeze-dried β -galactosidase stored at elevated temperature. *Int. J. Pharm.* **71**:137–146 (1991).
10. J. H. Crowe, S. B. Leslie, and L. M. Crowe. Is vitrification sufficient to preserve liposomes during freeze-drying? *Cryobiology* **31**:355–366 (1994).
11. S. Ohtake, C. Schebor, S. P. Palecek, and J. J. Pablode. Effect of sugar-phosphate mixtures on the stability of DPPC membranes in dehydrated systems. *Cryobiology* **48**:81–89 (2004).
12. J. F. Carpenter, L. M. Crowe, and J. H. Crowe. Stabilization of phosphofructokinase with sugars during freeze-drying: characterization of enhanced protection in the presence of divalent cations. *Biochim. Biophys. Acta* **923**:109–115 (1987).
13. J. F. Carpenter and J. H. Crowe. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochem.* **28**:3916–3922 (1989).
14. K. Tanaka, T. Takeda, and K. Miyajima. Cryoprotective effect of saccharides on denaturation of catalase by freeze-drying. *Chem. Pharm. Bull.* **39**:1091–1094 (1991).
15. J. F. Carpenter, S. J. Prestrelski, and T. Arakawa. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization. 1. Enzyme activity and calorimetric studies. *Arch. Biochem. Biophys.* **303**:456–464 (1993).
16. S. J. Prestrelski, T. Arakawa, and J. F. Carpenter. Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization II. Structural studies using infrared spectroscopy. *Arch. Biochem. Biophys.* **303**:465–473 (1993).
17. S. J. Prestrelski, N. Tedeschi, T. Arakawa, and J. F. Carpenter. Dehydration-induced conformational transition in proteins and their inhibition by stabilizers. *Biophys. J.* **65**:661–671 (1993).
18. B. S. Chang, R. M. Beauvais, A. Dong, and J. F. Carpenter. Physical factors affecting the storage stability of freeze-dried interleukin-1 receptor antagonist: glass transition and protein conformation. *Arch. Biochem. Biophys.* **331**:249–258 (1996).
19. C. Schebor, L. Burin, M. P. Buera, J. M. Aguilera, and J. Chirife. Glassy state and thermal inactivation of invertase and lactase in dried amorphous matrices. *Biotechnol. Prog.* **13**:857–863 (1997).
20. J. G. Sampedro, G. Guerra, J.-P. Pardo, and S. Uribe. Trehalose-mediated protection of the plasma membrane H^+ -ATPase from *Kluyveromyces lactis* during freeze-drying and rehydration. *Cryobiology* **37**:131–138 (1998).
21. L. Kreilgaard, S. Frokjaer, J. M. Flink, T. W. Randolph, and J. F. Carpenter. Effects of Additives on the stability of recombinant human factor XIII during freeze-drying and storage in the dried solid. *Arch. Biochem. Biophys.* **360**:121–134 (1998).
22. B. Lueckel, B. Helk, D. Bodmer, and H. Leuenberger. Effects of formulation and process variables on the aggregation of freeze-dried interleukin-6 (IL-6) after lyophilization and on storage. *Pharm. Dev. Technol.* **3**:337–346 (1998).
23. T. Suzuki, K. Imamura, H. Fujimoto, and M. Okazaki. Relation between thermal stabilizing effect of sucrose on LDH and sucrose-LDH hydrogen bond. *J. Chem. Eng. Jpn.* **31**:565–570 (1998).

24. D. P. Miller, R. E. Anderson, and J. J. de Pablo. Stabilization of lactate dehydrogenase following freeze-thawing and vacuum-drying in the presence of trehalose and borate. *Pharm. Res.* **15**:1215–1221 (1998).
25. S. D. Allison, B. Chang, T. W. Randolph, and J. F. Carpenter. Hydrogen bonding between sugar and protein is responsible for inhibition of dehydration-induced protein unfolding. *Arch. Biochem. Biophys.* **365**:289–298 (1999).
26. E. D. Breen, J. G. Curley, E. D. Overcashier, C. C. Hsu, and S. J. Shire. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. *Pharm. Res.* **18**:1345–1353 (2001).
27. T. J. Anchordoquy, K. Izutsu, T. W. Randolph, and J. F. Carpenter. Maintenance of quaternary structure in the frozen state stabilizes lactate dehydrogenase during freeze-drying. *Arch. Biochem. Biophys.* **390**:35–41 (2001).
28. K. Imamura, T. Ogawa, T. Sakiyama, and K. Nakanishi. Effects of types of sugar on the stabilization of protein in the dried state. *J. Pharm. Sci.* **92**:266–274 (2003).
29. Y. Liao, M. B. Brown, and G. P. Martin. Investigation of the stabilization of freeze-dried lysozyme and the physical properties of the formulations. *Eur. J. Pharm. Biopharm.* **58**:15–24 (2004).
30. S. Passot, F. Fonseca, M. Alarcon-Lorca, D. Rolland, and M. Marin. Physical characterization of formulations for the development of two stable freeze-dried proteins during both dried and liquid storage. *Eur. J. Pharm. Biopharm.* **60**:335–348 (2005).
31. L. Chang, D. Shepherd, J. Sun, D. Ouellette, K. L. Grant, X. Tang, and M. J. Pikal. Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix?. *J. Pharm. Sci.* **94**:1427–1444 (2005).
32. K. Kawai, T. Hagiwara, R. Takai, and T. Suzuki. The role of residual water for the stability of protein freeze-dried with trehalose. In M. P. Buera, J. Welti-Chanes, P. J. Lillford, and H. R. Corti (eds.), *Water Properties of Food, Pharmaceutical, and Biological Materials*, CRC Press, Boca Raton, 2006, pp. 543–550.
33. K. Kawai, T. Hagiwara, R. Takai, and T. Suzuki. Comparative investigation by two analytical approaches of enthalpy relaxation for glassy glucose, sucrose, maltose and trehalose. *Pharm. Res.* **22**:490–495 (2005).
34. R. Urbani, F. Sussich, S. Prejac, and A. Cesàro. Enthalpy relaxation and glass transition behavior of sucrose by static and dynamic DSC. *Thermochim. Acta* **304/305**:359–367 (1997).
35. S. Ohtake, C. Schebor, S. P. Palecek, and J. J. de Pablo. Effect of pH, counter ion, and phosphate concentration on the glass transition temperature of freeze-dried sugar-phosphate mixtures. *Pharm. Res.* **21**:1615–1621 (2004).
36. R. Surana, A. Pyne, and R. Suryanarayanan. Effect of aging on the physical properties of amorphous trehalose. *Pharm. Res.* **21**:867–874 (2004).
37. T. J. Anchordoquy and J. F. Carpenter. Polymers protect lactate dehydrogenase during freeze-drying by inhibiting dissociation in the frozen state. *Arch. Biochem. Biophys.* **332**:231–238 (1996).
38. S. Jiang and S. L. Nail. Effect of process conditions on recovery of protein activity after freezing and freeze-drying. *Eur. J. Pharm. Biopharm.* **45**:249–257 (1998).
39. K. C. Fox. Putting proteins under glass. *Science* **267**:1922–1923 (1995).
40. J. L. Green, and C. A. Angell. Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly. *J. Phys. Chem.* **93**:2880–2882 (1989).
41. A. Patist, and H. Zoerb. Preservation mechanisms of trehalose in food and biosystems. *Colloids Surf., B Biointerfaces* **40**:107–113 (2005).
42. K. Kawai, T. Hagiwara, R. Takai, and T. Suzuki. Maillard reaction rate in various glassy matrices. *Biosci. Biotechnol. Biochem.* **68**:2285–2288 (2004).
43. K. Kawai, T. Hagiwara, R. Takai, and T. Suzuki. The rate of non-enzymatic browning reaction in model freeze-dried food system under the glassy state. *Innov. Food Sci. Emerg. Technol.* **6**:346–350 (2005).