Development of Dry-preservation Technology for Biological Protein at Room Temperature
—Measurements of Glass Transition Temperature and Residual Activity of Lactate Dehydrogenase—

Kiyoshi TAKANO*†    Ryo SHIRAKASHI*

* Institute of Industrial Science, The University of Tokyo
(4-6-1, Komaba, Meguro-ku, Tokyo 153-8505)

Summary
The glass transition temperatures of the several kinds of materials which are considered to be effective as protective agents were measured before dry preservation experiment. The following four types of protective agents were selected to assess the dry-protective ability with Lactate Dehydrogenase (LDH): trehalose, trehalose with ε-Poly-L-Lysine (PLL) and trehalose with boron. The sample solutions of LDH with the various combinations of protective agents were rapidly vacuum-dried at the room temperature, followed by measuring their moisture content. After that, the dried samples were rehydration to measure the enzymatic activity by biochemical assay. The residual activity of LDH sample with trehalose was about 50% when its moisture content was 11 to 60 %. This dried-sample, however, suddenly lost its residual activity down to about 20 % when the moisture content decreased to 11 wt% or less. On the other hands, LDH samples with PLL alone and with trehalose + PLL kept their residual activities around 75 to 80 % in the wide range of moisture content. LDH samples with boron + trehalose kept their average residual activity from 69 to 74 %.

Keywords: Dry-preservation, Room temperature, Glass transition temperature, LDH, Residual activity

1. Introduction
Cryopreservation and lyophilization are widely used for the high quality and long-term preservation of biological materials, such as foods, pharmaceuticals and clinical analytes3). These technologies are composed of several processes of pre-freezing, freezing, storage, and of thawing. In contrast to these technologies, the dry-preservation at non-cryogenic temperature has the advantages of the energy free-storage and of the simple operation. Previous studies3) reported the hypothesis that trehalose suppresses the degradation of proteins by replacing with their hydrated water (water replacement effect), and by forming amorphous state around the proteins. Miller et al.3) reported that glass transition temperature increased about 60 °C by adding a small amount of boron to trehalose solution. They also indicated that when the boron + trehalose mixture was used as a protective agent, the activity of vacuum dried Lactate Dehydrogenase (LDH) at room temperature after rehydration was not decrease for a long time. However, they did not measure the glass transition temperature which changed the mixing ratio and concentration of boron + trehalose mixture, systematically.

In this study, we measured the glass transition temperature of the several kinds of materials which are considered to be effective as protective agents before the dry preservation experiment3). As a result, the following four types of protective agents were selected, and we performed an experiment to verify the protective effects of trehalose, ε-Poly-L-Lysine (PLL), PLL + trehalose mixture and borax + trehalose mixture on the dry-preservation of LDH at room temperature. We selected LDH as a biological protein, which is the very common biomarker of liver diseases including cancer in a blood test. Because this biomarker is so fragile to environmental change that it is often used as a benchmark biomarker-molecule to assess the storage-tolerance of clinical analytes. For these reason, this enzyme has been measured in many other studies. For the measurement of the protective effect, we compared the enzymatic activity of immediate-rehydrated LDH with the activity of the undried one by the enzymatic analysis. We also investigated the enzymatic activity of rehydrated samples for various moisture contents in the dried state. The data obtained in this study should helpful for screening the dry-protective agent and for judging the critical moisture content in the sample for keeping its enzymatic activity3).

2. Materials and Methods
2.1 Measurement of glass-transition temperature
The following sample protective agent were used in the glass-transition temperature measurements. Trehalose (dihydrate: Hayashibara, Japan), boron...
The glass transition temperatures of the samples with various water contents were defined by detecting the drastic change of their specific heat by differential scanning calorimetry (Shimadzu DSC-60A). The samples were, first, rapidly cooled (>100 °C/min) down to -90 °C to be vitrified, followed by the calorimetry in the condition of 20 °C/min warming. The glass transition temperature of the each sample concentration was measured for 3 to 6 times to obtain the averaged value.

2.2 Sample solutions for activity measurement

LDH solution used in the experiment was L-Lactate dehydrogenase from pig heart (Wako; 305-50951 : Act. 5000 U/mL, the suspension contains: Protein = 14.3 mg/mL, K-PO₄ = 0.1 mol/L, (NH₄)₂SO₄ = 3.0 mol/L, pH = 7.0). Four kinds of protective agents were used in the experiment. Each sample includes different protective agents. The ingredients of each sample in detail are shown in Table 1. The boron + trehalose mixture was prepared so that the molar ratio of boron in sodium tetraborate decahydrate to trehalose anhydride, R, was 1.2. Prior to the experiment, we performed a preliminary experiment of vacuum-drying and rehydration with the LDH solution that was diluted with trehalose aqueous solution to various dilution ratio. This result confirmed that the residual activity of LDH varies depending on the dilution ratio. Hence, in the sample used in this experiment, the LDH stock solution was always diluted 50 times (equivalent to 100 U/mL) with the solution including protective agent.

2.3 Vacuum-drying and measurement of moisture content in the samples

The LDH stock solution from the glass vial was diluted 50 times with the protective agent solution to obtain the sample solution followed by the vacuum-drying to prepare the sample of certain moisture content. The vacuum-dried samples were immediately rehydrated for measuring their enzymatic activities.

The vacuum-drying process was precisely explained in elsewhere⁶). In brief, the dried sample was prepared on a cleaned glass plate with 52 mm × 76 mm and 1 mm thickness. First, the unmasked center part (40 mm × 60 mm) of the glass surface was reformed by a plasma treatment for 5 minutes.

<table>
<thead>
<tr>
<th>Protective agent</th>
<th>Mixing ratio</th>
<th>Total wt% of protective agent</th>
<th>Dilution ratio of LDH</th>
<th>Protective agent/LDH protein (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td></td>
<td>31.8</td>
<td>50-fold (100 U/mL)</td>
<td>1000</td>
</tr>
<tr>
<td>Boron + trehalose</td>
<td>R=1.2</td>
<td>30.0</td>
<td></td>
<td>1086</td>
</tr>
<tr>
<td>PLL</td>
<td></td>
<td>25.0</td>
<td></td>
<td>828</td>
</tr>
<tr>
<td>PLL + trehalose</td>
<td>1:1</td>
<td>25.0</td>
<td></td>
<td>884</td>
</tr>
</tbody>
</table>

Fig. 1 Glass transition curves of boron + trehalose mixture.

Fig. 2 Glass transition curves of PLL and PLL + trehalose mixture.
using a plasma surface treatment apparatus (plasma processor: YHS-R, Sakigake, Japan) to improve its hydrophilicity. Then, 50 μL of the sample solution was uniformly spread exactly on the area of hydrophilic region, and was vacuum dried in the desiccator chamber at room temperature with $1.3 \times 10^{-1}$ Pa for various drying periods.

By using a precision electronic balance (Shimadzu AUW120D, resolution 10 μg), the moisture content of dried sample were calculated from the differences of the samples mass before and after the drying. During the mass measurement, the surface of the dried sample was sealed with the glass plate with rubber-flange to prevent the sample from absorbing the atmosphere moisture.

2.4 Activity measurement

Equation (1) shows the enzymatic reaction of LDH. The reaction rate of this equation is proportional to the increasing rate of NADH concentration that is defined by an enzymatic analysis. In this analysis, the absorbance at 340 nm which is specific to NADH is measured every second to obtain the kinetics of LDH enzymatic activity.

\[
\text{Lactic acid} + \text{NAD}^+ \xrightarrow{LDH} \text{Pyruvic acid} + \text{NADH} + H^+ \quad (1)
\]

In the absorbance measurement, first, ultra purified water was added to the dried sample on the glass plate to obtain the rehydrated sample that was 10 folds diluted (= 10 U/mL equivalent) from the sample before drying (= 100 U/mL equivalent). Next, a lactic acid dehydratase kit (KAINOS, Japan; Aqua auto Kainos LDH II) was added to the sample solution followed by measuring the absorbance of sample using an absorption microplate reader (CORONA Electric MTP-310 Lab type). Since the absorbance changed according to the Michaelis-Menten equation, we approximated its initial stage absorbance profile with linear approximation. Residual activity Ra was calculated from the change rate of absorbance for dried sample (Dry in Fig.3), negative control i.e. pure water (Water (NC)), and for fresh LDH solution without additives (Fresh) using equation (2).

\[
Ra = \frac{\text{slope of Dry} - \text{slope of NC}}{\text{slope of Fresh}} \times 100 \quad (2)
\]

3. Experimental Results

3.1 Glass transition temperature

Figure 1 shows the experimental results of the glass transition temperature vs. the concentration for trehalose solution and for boron + trehalose solution. The dehydrate limit illustrated in Fig. 1 is defined as the trehalose wt% of trehalose dihydrate crystal. The measured value was approximated by a curve by Gordon-Taylor equation as follows.

\[
T_g = \frac{C_1 \cdot T_{g1} + \kappa \cdot C_2 \cdot T_{g2}}{C_1 + \kappa \cdot C_2} \quad (3)
\]

$C_1$: Solute concentration (wt%)

$T_{g1}$: Glass transition temperature of solute (°C)

$C_2$: Concentration of solvent (water) (wt%)

$T_{g2}$: Glass transition temperature of water (-135 °C)

$\kappa$: Influence coefficient of water

Table 2 shows the glass transition temperature at 100 wt% trehalose measured by various researchers. Measured glass transition

![Fig. 3 Absorbance change in enzymatic reaction of LDH without addition of protective agent. (Dry: dried LDH sample, Fresh: fresh LDH solution without additives, Water (NC): water as negative control)](image)
temperature agreed with not only the value of 100 wt% but also the concentration tendency to the result of Cesa’ro et al.⁸) Therefore, we considered that the measurement of the glass transition temperature by DSC in present experiment could sufficiently reliable. The glass transition curves of boron + trehalose mixture increased almost parallel to the curve of trehalose single component as boron increased.

Figure 2 shows the experimental results of the glass transition temperature vs. the concentration for PLL single component and PLL + trehalose mixture. The inclination of the glass transition temperature to the concentration for PLL is less steep than that for trehalose. In addition, the value of $T_g$ for PLL was smaller than that for trehalose in the concentration range of 75 to 95 wt%. In the measured mixing conditions, the glass transition temperatures of the PLL + trehalose mixture are independent from the mixing ratio, and are about 15 °C higher than the PLL single component.

3.2 Residual activity without addition of protective agent

As a preliminary experiment, the activity of LDH without protective agent, that was vacuum dried for various periods (1 - 15 minutes), was measured. The retained water and the sample mass after drying was too little to measure even with the precision balance. Fig. 3 (1) and (2) are the typical absorbance from 0 to 200 seconds of the samples that were rehydrated immediately after vacuum dried for 1 (Fig.3 (1)) and 15 minutes (Fig.3 (2)). In both cases, the enzymatic activities of 6 dried samples (Dry) were averaged in a single experiment. The experiment was carried out 10 times for 1 min.-drying and 6 times for 15 min.-drying to calculate the population means. As the result, the residual activity of the drying time of 1 minute was 35.9 % ± 3.27 (S. E.), and the residual activity of the drying time of 15 minutes was 11.6 % ± 1.03 (S.E.). Because the moisture content decreases as the drying time increases, it was found that the residual activity remarkably decreases as the moisture content decreases.

3.3 Residual activity and moisture content with addition of protective agent

The samples with various moisture contents were prepared by changing the period of vacuum-drying of the sample solution. In Fig. 4 (1) - (3), the obtained moisture contents after the various period

<table>
<thead>
<tr>
<th>$T_g$ (°C)</th>
<th>Researchers</th>
</tr>
</thead>
<tbody>
<tr>
<td>104.2</td>
<td>Cesa’ro et al.⁸)</td>
</tr>
<tr>
<td>113.3</td>
<td>Oka et al.⁹)</td>
</tr>
<tr>
<td>115</td>
<td>Saleki-Gerhardt¹⁰)</td>
</tr>
<tr>
<td>105</td>
<td>Imamura¹¹)</td>
</tr>
<tr>
<td>108</td>
<td>Wolkers et al.¹²)</td>
</tr>
<tr>
<td>115</td>
<td>Zang et al.¹³)</td>
</tr>
<tr>
<td>104.4</td>
<td>Present work</td>
</tr>
</tbody>
</table>

Table 2 Comparison of $T_g$ in 100 wt% trehalose
of vacuum-drying are plotted for the solution of trehalose, PLL + trehalose (1:1), PLL and boron + trehalose (R=1.2), respectively. The shoulder like inflection point around 30 seconds in Fig. 4 (1) - (3) may be caused by the partial break of vitrified surface of the samples, which affects the local moisture transfer in the sample. Although all the sample solutions were vacuum-dried with the same pumping power and for the same periods, the moisture content of each sample depends on the protective agent in the sample solution. For example, when the samples are vacuum-dried for 1000 seconds, the moisture content is about 5 wt% in trehalose (Fig. 4 (1)), about 1.5 wt% in PLL + trehalose or PLL (Fig. 4 (2)), about 15 wt% in boron + trehalose (Fig. 4 (3)). These results show that the difference of moisture content could be an order of magnitude between the samples containing PLL and boron.

In Fig. 5 (1) - (4), the enzymatic activities of the vacuum-dried LDH samples with protective agents at their various moisture contents are plotted. For the each moisture content, the residual activities of 6 samples were measured to be averaged. The standard error of each single experiment that was calculated from 6 samples is comparable to the symbol size. This suggests that the measurement error of each experiment was quite small. Nevertheless, the enzymatic activities between the experiments are widely dispersed. This dispersion is calculated to be about ±25%. Fig. 5 (1) shows the experimental results of moisture content and residual activity in the case of trehalose + LDH. The result indicates that the averaged residual activity in the moisture content ranging from 11 to 60 wt% was about 50%. For this protective agent, the moisture content less than 4.5 wt% was not achieved even after the vacuum-drying for 1000 seconds. When the moisture content decreased less than 11 wt%, the residual activity sharply decreased to about 20%.

![Fig. 5 Residual enzymatic activity of LDH with protective agents at various moisture contents.](image-url)
Because the moisture content of 11 wt% of trehalose-water system is nearly the concentration of trehalose-dihydrate (see Fig. 1), this rapid degradation of enzymatic activity may be caused by the crystallization of trehalose in the sample. Fig. 5 (2), (3) show the residual activities of LDH with PLL + trehalose (1:1) and PLL respectively. As it can be seen from Fig. 4 (1) and (2), the addition of PLL as dry-protective agent increases the drying speed. The residual activities in Fig. 5 (2) and (3) were 75 to 80% in the moisture content ranging from 0 to 60 wt%. Fig. 5 (4) shows the residual activities of LDH with boron + trehalose. The sample with boron + trehalose could not be vacuum-dried to the moisture content less than 13 wt% in 1000 sec because of the high water retention ability of boron. The residual activity of these samples in the moisture content ranging from 13 to 60 wt% was 69 to 74%. This value is slightly lower than the result of Miller et al. (80 % or more) that was stored in a high humidity environment at 25 °C after vacuum-drying.

4. Summary

We performed the experiments to confirm the feasibility of the room temperature dry-storage technology, which has the advantages of energy conservation comparing with the cryopreservation and the lyophilization. It is considered that the material which vitrified at higher temperature is effective as a dry-protective agent for room temperature storage. Therefore, the relation between the concentration and the glass transition temperature of trehalose single component, boron + trehalose mixture, PLL single component and PLL + trehalose mixture were measured in detail. As a result, it was clarified that the conditions of mixing ratio and the concentration of materials which vitrify at higher temperature than trehalose single component. Thus, we used these materials as protective agents, and measured the rehydrated LDH activity after the vacuum-drying.

It was confirmed the higher LDH residual activity of 75 to 80% was obtained by adding PLL + trehalose (1:1) or PLL as protective agents rather than by adding only trehalose, which is considered to be effective as a cryo-protective agent.

Although PLL has potential high protective ability, PLL is cationic at pH~7. Thus, PLL may bind to anionic biomolecules in the clinical analytes and in the enzymes for chemical examination. This should prevent the accurate enzymatic examination of analytes. Boron + trehalose also indicated relatively high residual activity after the quick drying-rehydration, although the long-term protective effects of these molecules have not been confirmed yet. Furthermore, because borax is toxic to human, it is difficult to use for food preservation or pharmaceuticals.

The effects of moisture content to the residual activity, except the trehalose + LDH case, were not clear, because of largely scattered measured values. This scattering may be caused by the instability of drying process and by the temperature where the measurement took places. For practical application of room-temperature drying, the residual activity after rehydration should be stabilized. To this end, the drying process should be investigated in detail with moisture transfer and phase transition in the sample.

References

5) Takano, K. and Shirakashi, R., Proceedings of the 9th Asian Conference on Refrigeration and Air Conditioning, Sapporo, Japan (2018), A114