Oligosaccharide-based Surfactant/Citric Acid Buffer System Stabilizes Lactate Dehydrogenase during Freeze-drying and Storage without the Addition of Natural Sugar

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Abstract: Experiments were conducted to assess the maintenance effects of oligosaccharide-based surfactants on the enzymatic activity of a model protein, lactate dehydrogenase (LDH), during freeze-drying and room temperature storage using the citric acid buffer system. Oligosaccharide-based surfactants, which exhibit a high glass transition temperature ($T_g$), promoted the eminent retention of enzymatic activity during these protocols, whereas monosaccharide-based surfactants with a low $T_g$ displayed poor performance at high concentration, albeit much better than that of Tween 80 at middle concentration. The increase in the alkyl chain length did not exert positive effects as observed for the maintenance effect during freeze-thawing, but an amphiphilic nature and a glass forming ability were crucial for the effective stabilization at a low excipient concentration during freeze-drying. Even a low oligosaccharide-based surfactant content (0.1 mg mL$^{-1}$) could maintain LDH activity during freeze-drying, but a high surfactant content (1.0 mg mL$^{-1}$) was required to prevent buffer precipitation and retain high LDH activity on storage. Regarding storage, glass formation restricted molecular mobility in the lyophilized matrix, and LDH activity was effectively retained. The present results describe a strategy based on the glass-forming ability of surfactant-type excipients that affords a natural sugar-free formulation or an alternative use for polysorbate-type surfactants.

Key words: Freeze-drying, sugar ester, glass transition, citric acid buffer, lactate dehydrogenase

1 INTRODUCTION

 Freeze-drying ([lyophilization]) is a successful technology for manufacturing protein therapeutics, as exemplified by numerous products in the market¹, ². However, labile proteins often lose their enzymatic activities in those protocols, and lyoprotectants are needed for the formulation to attenuate this inactivation³-⁴. Sugar-based surfactants are known to exhibit less toxicity and biodegradability⁵-⁷. Recently, several sugar fatty acid esters derived from natural sugars have become available, and their applications in biochemical fields have been explored⁸-¹⁰. Concerning their application for protein preservation during freeze-drying, sugar-based surfactants have been reported to effectively prevent the inactivation of various water soluble proteins during both freeze-thawing and freeze-drying processes, even in the absence of natural sugars¹¹-¹⁴. However, to date the effect of sugar-based surfactants on a long-term storage of lyophilized protein without the addition of natural sugars has not been assessed.

Recently, one of the properties distinguishing sugar-based surfactants from other non-ionic surfactant like polysorbate-type surfactants has been proposed to be the former’s excellent glass-forming ability¹⁵, ¹⁶. A variety of alkylated sugars exhibit inhibitory effects on the formation of eutectics consisting of electrolytes and ice via glass formation under a freeze-concentrated state of the aqueous electrolyte solution¹⁵, ¹⁷, as similarly observed for an...
aqueous natural sugar solution\textsuperscript{18, 19}. In addition, a typical sugar-based surfactant is less likely to crystallize itself under arid conditions\textsuperscript{20}. These properties are naturally received as important functions for protein stabilization during both freeze-drying and storage. We expected that rationally designed formulations consisting of sugar-based surfactants effectively stabilize labile proteins in these protocols via the formation of glassy matrices.

In the present study, we studied the maintenance effects of sugar-based surfactants on the enzyme activity of lactate dehydrogenase (LDH) using low-concentration (1 or 10 mM) sodium citric acid buffer (pH 6.5) during freeze-drying and room temperature storage; pH-adjusting buffer salt such as sodium citric acid buffer is reported to exhibit a low crystallizing tendency\textsuperscript{21}, and form glassy matrices with a high $T_g$ themselves in solid forms\textsuperscript{4, 22}. We selected the corresponding acyl derivatives because we expected them to possess potent glass-forming ability in consideration of the high $T_g$ of trehalose ($T_g$: 111.8°C)\textsuperscript{23} and raffinose ($T_g$: 103.2°C)\textsuperscript{24} among non-reduced oligosaccharides. Mono-saccharide-based surfactants, namely alkyl β-D-glucosides, were used for comparison as the representative sugar-based surfactants that display low $T_g$s among sugar-based surfactants\textsuperscript{25}.

2 EXPERIMENTAL PROCEDURES

2.1 Materials

LDH from rabbit muscle (type II, suspension in 3.2 M ammonium sulfate solution, pH 6.0), β-nicotinamide adenine dinucleotide (premium grade), reduced disodium salt, and sodium pyruvate (Reagent Plus\textsuperscript{TM}) were purchased from Sigma-Aldrich Co. (Tokyo, Japan). Trehalose dihydrate and pure water were purchased from Wako Pure Chemicals Ltd. (Tokyo, Japan). Tween 80 (polyoxyethylene sorbitan monooleate) was purchased from Tokyo Kasei Industry Co. Ltd. (Tokyo, Japan). n-Alkyl β-D-glucosides (alkyl chain length, n = 4, 6, or 8) and 6-O-alkanoyl trehaloses (n = 8, 10, 12, or 14) were obtained as described in our previous reports\textsuperscript{25, 26}. 6-O-Lauroyl raffinose (n = 12) was obtained by reacting raffinose with vinyl laurate in a mixture of tert-butyl alcohol and pyridine using Thermomyces lanuginosus lipase (Lipzyme TL IM) according to the published procedures\textsuperscript{27}. All surfactants were purified by silica gel column chromatography using a mixed solvent (methanol:acetone:chloroform:water = 4:4:9:1). The surfactants used in this study contained more than 99.5% mono-substitution, as confirmed by $^1$H-nuclear magnetic resonance spectroscopy (300 MHz; Varian Inc.), thin-layer chromatography (methanol:acetone:chloroform:water = 4:4:9:1), and high-performance liquid chromatography (acetoniitrile: water = 85:15 or 80:20, Inertsil NH$_2$ column: GL Sciences Inc.). To determine $T_g$s of anhydrate sugar-based surfactants, a DSC-8500 (Perkin Elmer Inc.) was used. The corresponding non-crystalline samples in an open aluminum container were heated to 120°C under nitrogen gas flow (20 mL min$^{-1}$) for 40 min to remove any adsorbed water moisture. Traces of water greatly decrease $T_g$s of sugar-based surfactants\textsuperscript{28}. The samples were later cooled to −30°C and then reheated to 120°C at the scanning rate of 20°C min$^{-1}$. The measurement was conducted twice to confirm the reproducibility for each sample.

2.2 Preparation of LDH solution

LDH suspension in 3.2 M ammonium sulfate solution was dialyzed against 1 or 10 mM sodium citric acid buffer (pH 6.5) to completely remove ammonium sulfate. In addition, any remaining insoluble precipitates were eliminated by filtration using a 0.45-μm filter. The suspension was diluted to approximately 9.0 μg mL$^{-1}$ against a similar buffer solution. At this concentration, LDH activity was completely maintained for as long as 40 min at 25°C in the buffer solution (see Fig. S1 in Supplementary Information). The LDH concentration was spectroscopically determined by ultraviolet absorption (280 nm) using an extinction coefficient of E$280 = 1.96 \times 10^5$ M$^{-1}$ cm$^{-1}$\textsuperscript{29}. Sugar-based surfactants were added to the LDH solution to adjust the concentration from 0.001 mg mL$^{-1}$ to 1.0 mg mL$^{-1}$.

2.3 Freeze-drying treatment

An approximately 9.0 μg mL$^{-1}$ LDH solution (0.5 mL) was added to a polypropylene microtube vial. Subsequently, the vial was dipped into a liquid N$_2$ bath for 3 min. Then, the solution was lyophilized overnight in a stored container of the lyophilizer (FD-1000, Tokyo Rikakikai Co., Ltd.), and lyophilized powder was obtained. The powders were reconstituted by adding the same amount of water evaporated, and the solution was assessed within 15 min.

2.4 Room temperature storage test under different humidities

For the storage test, several selected lyophilized powders were stored for 20 days in a desiccator containing diphosphorus pentoxide [relative humidity (RH) = nearly 0%] or saturated magnesium chloride (RH = approximately 33%) at 25°C. The powders were reconstituted by adding the same amount of water evaporated, and the solution was assessed within 15 min.

2.5 Assay of relative LDH activity

Activity assays were conducted at 25°C as follows. The LDH solution with and without freeze-drying treatment was added to a 10-fold volume reaction mixture consisting of 0.2 mM NADH and 2.0 mM sodium pyruvate buffer solution in a 2-mL cylindrical quartz cuvette. As manual mixing often induces foam formation, which leads to non-negligible human-dependent experimental error, to eliminate this
obstacle, we moderately stirred for 5 s using a magnetic stirring bar (2-mm diameter) and a magnetic stirrer set at 1400 rpm (F-201N, TGK Co., Ltd.), permitting mixing without any bubble generation (Fig. S2). Within an additional 5 s, the cuvette was transferred to the cell holder of a UV-vis spectrometer (V-550, JACSO Corp.), and the reaction behavior was then monitored by the absorbance decrease of NADH at 340 nm. The remaining LDH activity was calculated by the initial reaction rate from 10 s after the LDH solution was added to the reactant. The relative LDH activity percentage (%) after freeze-drying was calculated as a percentage of that of the non-lyophilized sample. The value was presented as the mean based on three determinations.

2.6 Thermal analysis of lyophilized samples

The thermal properties of several selected lyophilized powders were investigated using differential scanning calorimetry (DSC) (DSC-60, Shimadzu Co., Ltd.). The sample was hermetically sealed in an aluminum pan, and it was heated to 150°C at a scanning rate of 5°C min⁻¹, after which it was cooled to −40°C.

3 RESULTS and DISCUSSION

3.1 Determination of Tg of oligosaccharide-based surfactants in anhydrate state

Firstly, we determined Tg of the prepared oligosaccharide-based surfactants by DSC. As shown in Fig. 1, the glass transition was clearly observed for every oligosaccharide-based surfactant studied in this work as changes in the heat flow appeared to be a step in both the cooling and heating DSC curves. Tg was determined as the temperature corresponding to the middle point of the height of the heat flow change, and the results are summarized in Table 1.

Of note, although Tg values were lower than those of the corresponding natural sugars as observed for alkyl β-D-glucosides, the oligosaccharide-based surfactants studied in this work exhibited high Tg values. A raffinose derivative, 6-O-

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Tg/°C</th>
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<tbody>
<tr>
<td>6-O-octanoyl trehalose</td>
<td>8</td>
<td>84.7</td>
</tr>
<tr>
<td>6-O-decanoyl trehalose</td>
<td>10</td>
<td>81.5</td>
</tr>
<tr>
<td>6-O-lauroyl trehalose</td>
<td>12</td>
<td>81.0</td>
</tr>
<tr>
<td>6-O-myristoyl trehalose</td>
<td>14</td>
<td>79.5</td>
</tr>
<tr>
<td>6-O-lauroyl raffinose</td>
<td>12</td>
<td>86.5</td>
</tr>
</tbody>
</table>

Fig. 1 Differential scanning calorimetry thermograms of (a) 6-O-octanoyl trehalose, (b) 6-O-decanoyl trehalose, (c) 6-O-lauroyl trehalose, (d) 6-O-myristoyl trehalose, and (e) 6-O-lauroyl raffinose.
lauroyl raffinose ($n = 12$), exhibited the highest temperature of approximately 86.5°C, and 6-O-octanoyl trehalose ($n = 8$) displayed a slightly higher $T_g$ (approximately 84.7°C) than the other trehalose derivatives ($n = 10, 12, 14$). Thus, excellent glass-forming properties of oligosaccharide-based surfactants such as trehalose and raffinose fatty acid esters were identified in terms of $T_g$ compared with those of other sugar-based surfactants such as n-alkyl β-D-glucoside ($T_g = 11.4–16.3°C$) and sucrose derivatives ($T_g$ less than 50°C under the definition of this study).

### 3.2 Maintenance effect of sugar-based surfactants on LDH activity during freeze-drying

LDH was inactivated by freeze-drying treatment by approximately 50% based on the initial activity in the absence of stabilizing excipients. The well-known lyoprotectant trehalose started to exhibit protective effects at higher concentrations exceeding 32 mg mL$^{-1}$ and displayed a maximum maintenance effect of approximately 80% at 60 mg mL$^{-1}$, indicating that a large amount of sugar is required for the effective retention of enzymatic activity (Fig. 2b). Tween 80, which is the representative nonionic surfactant used in many marketed formulations, did not function as a lyoprotectant, and it destabilized LDH (Fig. 2b). These results in the citric acid buffer system accorded with those of a previous study in which another buffer system was used. Then, we evaluated the maintenance effects of sugar-based surfactants on LDH activity during freeze-drying (Fig. 3).

Regarding a series of n-alkyl β-D-glucosides ($n = 4, 6, 8$), the relative LDH activity increased with an increase in the concentration of the surfactant up to 0.05–0.1 mg mL$^{-1}$. Hexyl glucoside displayed the greatest effect, maintaining enzymatic activity at approximately 80%, whereas the other glucosides maintained enzymatic activity at a maximum of approximately 70% and 60%, respectively. However, the maintenance effect weakened as the concentration was further increased (Fig. 3a). The marked reduction in LDH activity at high concentrations of alkyl β-D-glucoside, as shown in Fig. 3a, indicated that the destabilizing effect was similar to that of Tween 80. Concerning a series of 6-O-alkanoyl trehalose derivatives ($n = 8, 10, 12, 14$), the remaining LDH activity increased up to approximately 90% as the concentration of the derivatives was increased to approximately 0.3–0.5 mg mL$^{-1}$. Subsequently, the relative activity became constant or gradually increased to approximately 100% at higher concentrations (Fig. 3b). As shown in Figs. 3a and 3b, the maintenance effects of sugar-based surfactants during freeze-drying was not positively correlated with the increase in alkyl chain length ($n$), which is different from that during freeze-thawing. A stronger retention effect was observed during freeze-thawing by increasing the alkyl chain length of sugar-based surfactants, whereas a weak performance of 6-O-myristoyl trehalose ($n = 14$) was observed during freeze-thawing as well as freeze-drying in Fig. 3b, which was assumed to be attributable to its low solubility at subzero temperatures. This finding indicated that the maintenance effect during freeze-drying is not defined by surfactant properties such as adsorption, the effect of which is logarithmically influenced by variation in the alkyl chain length. In addition, it should be noted that less relative activity was retained in the presence of a low surfactant content of 0.001–0.005 mg mL$^{-1}$ than the 50% activity retained in the control in Figs. 3a and 3b. This suggested that the maintenance effects of sugar-based surfactants possess concentration dependence in the citric acid buffer system. Meanwhile, the trisaccharide-based surfactant 6-O-lauroyl raffinose exhibited a better performance than 6-O-lauroyl trehalose at every concentration studied (Fig. 3c). Almost complete retention of LDH activity exceeding 90% was attained at a surfactant concentration of approximate-

**Fig. 2** Relative lactate dehydrogenase activities remaining after lyophilization of the 9.0 μg mL$^{-1}$ LDH solution in the presence of (a) trehalose and (b) Tween 80 in 10 mM citric acid buffer.

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ly 0.05 mg mL\(^{-1}\), and the retention of enzymatic activity continued at higher concentrations. From this result, it was found that the maintenance effects of sugar-based surfactants depend on the sugar rather than the alkyl chain length, whereas some amphiphilic characteristic is crucial for an effective maintenance effect. The latter fact was readily confirmed by a comparison of the maintenance effects of trehalose (Fig. 2a) and trehalose derivatives (Fig. 3b). By summarizing the results of Table 1 and Fig. 3, it was believed that oligosaccharide-based surfactants promote glass formation during freeze-drying, resulting in their excellent retention of LDH activity, whereas owing to the lack of sufficient glass-forming ability, alkyl \(\beta\)-D-glucosides exhibited weak effects at high concentrations among the sugar-based surfactants studied here. On the other hand, the effects of sugar-based surfactants including alkyl \(\beta\)-D-glucosides were much superior to those of Tween 80 around 0.1 mg mL\(^{-1}\), and it may be resulted from the preferential exclusion of them or providing a hydrogen network as water substitutes by them. The preferential exclusion have been proposed for various excipients as the effective protection mechanism during freeze-drying.

3.3 Maintenance effect of sugar-based surfactants during room temperature storage

A 20-day storage test was conducted using different humidity levels for several lyophilized formulations consisting of oligosaccharide-based surfactants with a 12-alkyl chain length (Fig. 4). When the lyophilized powder was stored under P\(_2\)O\(_5\) (RH = approximately 0%) in the absence of oligosaccharide-based surfactants, the control formulation retained 68% of its enzymatic activity. Compared with the standard value, a small improvement was recognized for the formulations prepared with 0.1 mg mL\(^{-1}\) 6-O-lauroyl raffinose or 6-O-lauroyl trehalose, which retained 76% and 74%, respectively, of the enzymatic activities. However, approximately 100% and 90% of the enzymatic activity were retained for lyophilized powders containing 6-O-lauroyl raffinose and 6-O-lauroyl trehalose, respectively, after freeze-drying (Fig. 4). These results indicate that the requirements for the retention of activity differ between freeze-drying and room temperature storage. On the con-
trary, 94% and 89% of the enzymatic activities were maintained after room temperature storage for 20 days for the formulations containing 1.0 mg mL$^{-1}$ 6-O-lauroyl raffinose and 6-O-lauroyl trehalose, respectively, indicating that a sufficient amount of oligosaccharide-based surfactant (1.0 mg mL$^{-1}$) enabled the maintenance of LDH activity during room temperature storage. Meanwhile, storage under a humidifying condition of 33% RH accelerated the loss of activity. The water moisture plasticized the lyophilized matrix and increased the mobility of the components, thus accelerating the chemical reaction or degradation\textsuperscript{35,36}.

3.4 Glass transition behavior of lyophilized matrices

Subsequently, we studied the thermal behavior of the lyophilized powders, which consisted of a surfactant content of 0.1 or 1.0 mg mL$^{-1}$, because the assay results were recognized to depend on the surfactant content in the storage test (Fig. 4). For this experiment, the sample was stored under 0% RH in a desiccator for 24 h after lyophilization, and the thermal behavior was then analyzed by DSC. In the thermograms for the formulations consisting of 0.1 mg mL$^{-1}$ surfactant content [thermograms (a) & (c) in Fig. 5], an apparent endothermic peak was observed at approximately 130°C. This peak can be categorized as the melting or dehydration of crystalline formed in the citric acid buffer because lyophilized formulations containing citric acid buffer alone (control) displayed a similar endothermic peak in the heating process (please see Fig. S3). Thus, the appearance of the peak for the formulations containing 0.1 mg mL$^{-1}$ surfactant content must indicate precipitation during the 24-h storage. On the contrary, in the thermograms (b) and (d) in Fig. 5 obtained for the formulations containing 1.0 mg mL$^{-1}$ surfactant content, this endothermic peak was not observed, indicating the prevention of crystallization under these conditions. The difference in the crystallization behavior must be associated with mobility in the lyophilized matrix. It is well known that crystallization frequently occurs at temperatures exceeding $T_g$, whereas it is repressed at lower temperatures in the amorphous matrix\textsuperscript{37} with some exceptions such as mannitol, which crystallizes even below $T_g$\textsuperscript{38}. Of note, a step-like phenomenon was observed at approximately 60°C as denoted by an arrow in the thermogram of (d), indicating the presence of $T_g$ greatly exceeding room temperature. On the contrary, the step-like phenomenon indicated by arrows in the thermograms (a) and (c) occurred at approximately room temperature, indicating a lower $T_g$. Such low $T_g$s correlated with the poor performance in terms of the retention of LDH activity. The molecular mobility in the formulation containing a low surfactant content (0.1 mg mL$^{-1}$) must allow the components to readily crystallize and protein activity to be quickly inactivated. Instead, a sufficient amount of oligosaccharide-based surfactant (1.0 mg mL$^{-1}$) contributed to the formation of glassy matrix with a high $T_g$ and prevented the mobility of the components as well as the chemical reaction that induces the loss of protein activity at room temperature. To permit a high $T_g$, contributions of both the sugar-based surfactant and sodium citric acid buffer are considered because of their high glass-forming properties. However, the weight ratios of the sugar-based surfactant against the citric acid buffer were approximately 2 and 20 for surfactant contents of 0.1 and 1.0 mg mL$^{-1}$, respectively, in the presence of 1.0 mM sodium citric acid buffer; therefore, it can be considered that oligosaccharide-based surfactants must greatly contribute to enhancing $T_g$s of those lyophilized formulations. On the other hand, the step-like behavior was also recognized for the lyophilized powder consisted of alkyl β-D-glucoside (1.0 mg mL$^{-1}$) (Fig. S4), despite that the LDH activity was not maintained during the freeze-drying process (Fig. 3). It was assumed that owing to the low glass forming ability of alkyl β-D-glucosides ($T_g$, 11.4–16.3°C)\textsuperscript{39}, the amount of amorphous water remaining after ice freezing decreased, and an acidification in the non-frozen matrix or the formation of strong interaction between sugar-moiety and buffer components occurred. Hence, the alkyl β-D-glucosides might be hydrolyzed under freeze-concentrated state, and formed hemiacetal structure, which induces the Maillard reaction that loss the LDH activity even in the lyophilized condition\textsuperscript{40}. Alternatively, the formation of specific interaction between alkyl β-D-glucoside and citric acid buffer can be assumed, as observed for the lyophilized system consisted of natural sugar and phosphate buffer\textsuperscript{41}. 

![Fig. 5 Heating thermograms of lyophilized samples after room temperature storage under 0% relative humidity for 24 h. Each sample was prepared via the addition of (a) 0.1 mg mL$^{-1}$ 6-O-lauroyl raffinose, (b) 1.0 mg mL$^{-1}$ 6-O-lauroyl trehalose, (c) 0.1 mg mL$^{-1}$ 6-O-lauroyl raffinose, or (d) 1.0 mg mL$^{-1}$ 6-O-lauroyl raffinose to 1.0 mM citric acid buffer.](image-url)
4 CONCLUSION

This work was performed to gain insight into the use of oligosaccharide-based surfactants such as trehalose or raffinose fatty acid esters for the protein therapeutic formulation based on freeze-drying. The addition of 1.0 mg mL$^{-1}$ oligosaccharide-based surfactants, which exhibit high $T_g$ ($>80^\circ C$) promoted the excellent retention of LDH activity during freeze-drying and 20-day storage at room temperature. The appropriately formulated matrix formed the glassy state after lyophilization, and the part of maintenance effect of oligosaccharide-based surfactants during room temperature storage was explained by the glassy state. The results demonstrated provide a formulating strategy directed toward the manufacturing of natural sugar-free formulations or the alternative use of oligosaccharide-based surfactants to polysorbate-type surfactants in lyophilized formulations.

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Supporting Information

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15) Ogawa, S.; Osanai, S. Glass transition behavior of Oligosaccharide-based Surfactant Stabilizes Lactate Dehydrogenase during Freeze-drying and Storage

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