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Improvement in the remaining activity of freeze-dried xanthine oxidase with the addition of a disaccharide–polymer mixture

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ABSTRACT

In order to improve the remaining activity of a practically important freeze-dried enzyme, xanthine oxidase (XOD), the effects of disaccharide (sucrose and trehalose), polymer (bovine serum albumin: BSA and dextran) and a mixture of them on the loss of XOD activity during freeze-drying and subsequent storage were investigated. All samples were amorphous solids and their glass transition temperatures (T_g) were evaluated by using differential scanning calorimetry. Although dextran showed no stabilizing effect on the freeze-dried XOD, the others protected XOD from the activity loss during freeze-drying to a certain extent. It was found that the mixture of disaccharide (sucrose or trehalose) and BSA improved the XOD activity synergistically. The XOD activity of the samples decreased gradually during storage at a temperature range of between 25 and 60 °C. Samples stored at temperatures below the T_g showed a lower loss of XOD activity than those stored at just the T_g.

1. Introduction

Evaluation of fish freshness is a very important quality control measure for raw fish (e.g., sashimi) and marine products (e.g., surimi). Fish freshness can be evaluated chemically from the amount of nucleotides and nucleosides produced by the adenosine-5′-triphosphate (ATP) degradative pathway and expressed as a *K*-value index (Hanna, 1992; Kaminashi, Nakaniwa, Kunimoto, & Miki, 2000; Saito, Arai, & Matsuyoshi, 1959)

$$K\text{-value}\ (\%) = \frac{[\mathrm{HxR}] + [\mathrm{Hx}]}{[\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}] + [\mathrm{IMP}] + [\mathrm{HxR}] + [\mathrm{Hx}]} \times 100$$

where ADP, AMP, IMP, HxR, and Hx are adenosine-5′-diphosphate, adenosine-5′-monophosphate, inosine-5′-monophosphate, inosine, and hypoxanthine, respectively. The contents of these nucleotides and nucleosides have been conventionally measured using high-performance liquid chromatography and column chromatography (Kaminashi et al., 2000; Valle, Malle, & Bouquelet, 1998). However, these methods require complicated and time-consuming procedures. Alternatively, the *K*-value has been practically simplified to K^*-value by excluding ATP and ADP due to their very low contents, and the K^*-value can be readily measured using freshness testing paper (FTP). The FTP, which contains several freeze-dried enzymes including xanthine oxidase (XOD), enables us to evaluate the relative content of nucleotides and nucleosides in fish muscle. The freeze-dried enzymes, however, are unstable and lose their activities during freeze-drying and subsequent storage. Therefore, it is necessary to maintain the activity of the enzymes.

The freeze-drying process generates a variety of stresses, such as low temperature stress, formation of ice crystals and dehydration stress, which can destabilize proteins by several degrees (Kawai & Suzuki, 2007; Prestrelski, Pikal, & Arakawa, 1995; Wang, 2000). To diminish the destabilization of freeze-dried proteins, many types of stabilizers have been used (Arakawa, Prestrelski, Kenney, & Carpenter, 2001; Carpenter, Pikal, Chang, & Randolph, 1997; Wang, 2000). As for the stabilizing mechanisms at work on freeze-dried proteins during the freezing process, the initial step of freeze-drying, these are referred to as "preferential exclusion" and "freeze-concentrated glass transition". The former involves preferential interaction of protein with water rather than stabilizers, which are preferentially excluded from the protein's hydration shell; unfolding of the protein is prevented and its native conformation is stabilized (Arakawa et al., 2001; Wang, 2000). The latter, "freeze-concentrated glass transition", involves the frozen protein being embedded in a high-viscous amorphous (i.e., glassy) matrix formed by a freeze-concentrated stabilizer and, consequently, the rate of protein degradation is decreased due to the restrictive molecular mobility (Anchordoquy, Izutsu, Randolph, & Carpenter,

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2001; Franks, 1993; Imamura, Iwai, Ogawa, Sakiyama, & Nakanishi, 2001; Suzuki, Imamura, Yamamoto, Satoh, & Okazaki, 1997; Wang, 2000).

In the dehydration step of freeze-drying and subsequent storage, the stabilizing mechanisms of stabilizers on the dried protein are described by "water substitution" and "glass transition". The water substitution hypothesis involves the native-like structure of protein being maintained by the formation of hydrogen bonds between dried protein and stabilizers in place of the removal of water molecules (Arakawa et al., 2001; Imamura et al., 2001; Kreilgaard, Frokjaer, Flink, Randolph, & Carpenter, 1998; Schebor, Burin, Buera, Aguilera, & Chirife, 1997; Suzuki et al., 1997; Wang, 2000). The interrelated stabilization mechanism, "glass transition", is intrinsically similar to the "freeze-concentrated glass transition" mechanism, involving embedding of the protein molecules in a glassy matrix. Therefore, the physical and chemical degradations of protein will be prevented due to the slowing down of the conformational change. The glass transition temperature (T_g) of stabilizers is one of the most significant parameters, because the glassy matrix changes to a liquid-like rubber state at temperatures above the T_g.

Disaccharides and/or polymer are known to be effective stabilizers (Allison, Chang, Randolph, & Carpenter, 1999; Anchordoquy et al., 2001; Chang, Beauvais, Dong, & Carpenter, 1996; Kreilgaard, Frokjaer, Flink, Randolph, & Carpenter, 1998; Prestrelski, Arakawa, & Carpenter, 1993; Sampedro, Guerra, Pardo, & Uribe, 1998; Wang, 2000). For example, the recovery of glucose-6-phosphate dehydrogenase activity increased from 40% to approximately 90% by adding 5.5% sugar mixture (glucose:sucrose = 1:10, w/w) as reported by Sun and Davidson (1998). Dextran (Mw: 40 kDa) at 10% level significantly protected freeze-dried elastase, and the activity remained near 82% during storage for 2 weeks at 40 °C with a relative humidity of 79% (Chang, Randall, & Lee, 1993). Some types of enzymes lose their activity during freeze-drying and subsequent storage even in the presence of disaccharide or polymer. One of the possible reasons for this is that disaccharides and polymers have different strengths and weaknesses in the stabilization of freeze-dried enzymes, and those disaccharides are inferior in preferential exclusion and glass transition but superior in water substitution than polymers (Allison et al., 2000; Prestrelski, Pikal, & Arakawa, 1995). Therefore, a mixture of disaccharides and polymers is sometimes useful for the improvement in the stability of freeze-dried enzymes (Carpenter, Prestrelski, & Arakawa, 1993).

Up until now there have been few studies on the stabilization of freeze-dried enzymes used for FTP. Therefore, this study employed XOD as a typical enzyme used for FTP, and aimed to elucidate the effects of disaccharide, polymer and their mixtures on the stability of XOD during freeze-drying and storage in the dried amorphous solid.

2. Materials and methods

2.1. Preparation of freeze-dried XOD samples

Reagent grade trehalose (anhydrous) was provided by Hayashibara, Co., Ltd., Japan. Bovine serum albumin (BSA) fraction V and dextran (Mw: 10.4 kDa) were obtained from Sigma–Aldrich, Co., USA. Analytical grade sucrose, xanthine (sodium salt), and XOD from buttermilk and other reagents were purchased from Wako Pure Chem. Ind., Ltd., Japan.

XOD was dialysed against 20 mM potassium phosphate buffer (pH 7.6) at 4 °C for 48 h in order to remove stabilizing agents. The XOD activity of the dialysed solution was evaluated as an initial activity, details of which are given later. The following samples were prepared: 200 mM sucrose, 200 mM trehalose, 1% and 5% BSA, 1% and 5% dextran, 200 mM sucrose + 1% BSA, 200 mM sucrose + 5% BSA, 200 mM trehalose + 1% BSA, 200 mM trehalose + 5% BSA, 200 mM sucrose + 1% dextran, 200 mM sucrose + 5% dextran, 200 mM trehalose + 1% dextran, 200 mM trehalose + 5% dextran. As the control, a non-additive sample was also prepared. Aliquots of 1 ml of each solution were placed into 2 ml-polypropylene tubes and frozen instantaneously with liquid nitrogen for at least 1 min. The frozen solids were transferred to a pre-cooled freeze-drier. Freeze-drying was performed with a gradual increase of the temperature by 5 °C from −40 to 5 °C followed by the gradual increase of 10 °C from 5 to 25 °C. At each step, the temperature was held for 3 h. The chamber pressure was maintained at 3.0×10^{-2} Torr throughout the drying process. After freeze-drying, the residual water in all samples was further removed over P_2O_5 in a vacuum desiccator for 7 days at room temperature. The XOD activity of a part of the freeze-dried samples was assayed in order to evaluate the activity loss during freeze-drying. In addition, the moisture content and thermal properties were investigated, details of which are given later. The other samples were hermetically sealed in a dry nitrogen-purged glove box and stored at 25 °C for a period of up to 110 days and at 40, 50, and 60 °C for up to 53 days, and then the XOD activity was assayed in order to determine the activity loss during storage.

2.2. Moisture content analysis

A Metrohm Karl Fisher coulometer (737 KF, Herisau, Switzerland) was used to measure the moisture content of the freeze-dried samples. The samples were prepared in a dry nitrogen-purged glove box.

2.3. Differential scanning calorimetry

The thermal properties of the freeze-dried samples were examined by a differential scanning calorimetry (DSC-50: Shimadzu, Co., Japan). Indium and distilled water were used to calibrate the temperature and heat capacity for the DSC measurements. Alumina powder was used as a reference material. The sample (approximately 15 mg) was weighed on an aluminium DSC pan in a dry nitrogen-purged glove box and sealed hermetically. All measurements were performed from 0 to 180 °C at a scan rate 5 °C/min. The values of the glass transition temperature (T_g) and crystallization temperature (T_c) were determined from the onset temperatures of endothermic shift and exothermic peak, respectively.

2.4. Assay of XOD activity

XOD activity was assayed by the enzymatic conversion of substrate xanthine to uric acid. The freeze-dried samples were rehydrated with distilled water to render a previous concentration, and the solution (10 μl) was added into 300 μl of 0.12 mM xanthine (sodium salt) in a 20 mM sodium phosphate buffer (pH 7.6). The time course for absorbance of 292 nm of the mixture was measured at 25 °C by using a UV–VIS spectrophotometer (V-630BIO: Jasco, Tokyo, Japan), and XOD activity was evaluated from the initial reaction rate. The remaining XOD activity was expressed as a percentage of the activity prior to freeze-drying.

3. Results and discussion

3.1. Thermal properties of freeze-dried samples

Table 1 shows the abbreviation of each sample and the results of glass transition (T_g), crystallization temperatures (T_c), and moisture content of the freeze-dried samples. The moisture contents of most of the samples were less than 1%. The T_g values of SUC and TRE were lower than those of anhydrous ones; T_g values of the

Table 1
Sample abbreviations, T_g, T_c and the moisture content of XOD in various formulations.

Formulation	Abbreviation	T_g (°C)[a]	T_c (°C)[a]	% Moisture content[b]
200 mM sucrose	SUC	60.8 ± 3.0	132.4 ± 1.6	0.96 ± 0.13
200 mM sucrose + 1% BSA	SUC + 1% BSA	65.1 ± 1.2	141.6 ± 0.7	0.85 ± 0.42
200 mM sucrose + 5% BSA	SUC + 5% BSA	76.0 ± 1.8	nd	0.43 ± 0.12
200 mM sucrose + 1% dextran	SUC + 1% DE	71.4 ± 1.1	nd	0.61 ± 0.31
200 mM sucrose + 5% dextran	SUC + 5% DE	91.2 ± 3.6	nd	0.52 ± 0.33
200 mM trehalose	TRE	87.7 ± 3.6	nd	0.66 ± 0.50
200 mM trehalose + 1% BSA	TRE + 1% BSA	94.7 ± 6.5	nd	0.50 ± 0.09
200 mM trehalose + 5% BSA	TRE + 5% BSA	97.7 ± 6.5	nd	0.41 ± 0.37
200 mM trehalose + 1% dextran	TRE + 1% DE	94.9 ± 4.7	nd	0.55 ± 0.39
200 mM trehalose + 5% dextran	TRE + 5% DE	113.1 ± 8.3	nd	0.46 ± 0.48
1% BSA	1% BSA	nd	nd	0.52 ± 0.31
5% BSA	5% BSA	158.1 ± 4.6	nd	0.45 ± 0.41
1% dextran	1% DE	nd	145.1 ± 3.5	0.63 ± 0.29
5% dextran	5% DE	134.0 ± 0.4	nd	0.59 ± 0.18

nd = not determined.
[a] The values are mean ± SD (n = 2).
[b] The values are mean ± SD (n = 3).

anhydrous sucrose and trehalose are reported to be 68 °C and 113 °C, respectively (Kawai, Hagiwara, Takai, & Suzuki, 2005). This is because the moisture plays the role of plasticizer (Imamura et al., 2002; Roos, 1995). Although SUC showed crystallization at a temperature above T_g, TRE maintained an amorphous state in the range of the measured temperature. This indicates that trehalose is more resistant to crystallization than sucrose. On the other hand, 1% BSA and 1% DE showed non-apparent glass transition. This is because the polymer exhibits a too small change in heat capacity due to glass transition in the wide temperature range. One percent DE showed an exothermic peak at 145 °C. This peak is attributed to the crystallization of the buffer, because dextran, an amorphous polymer, does not crystallize easily. Glass transitions of 5% BSA and 5% DE could be detected, and their T_g was much higher than those of SUC and TRE because of greater molecular interaction induced by its entanglement. The T_g values of disaccharide–polymer formulations were considerably higher than those of the individual disaccharide formulations, especially when the ratio of polymer to disaccharide is increased. In addition, SUC + 1% BSA showed higher T_c than SUC, and SUC + 5% BSA, SUC + 1% DE, and SUC + 5% DE showed no crystallization. This means that the physical stability of amorphous sucrose is improved by the addition of polymer as reported in a previous study (Imamura, Suzuki, Kirii, Tatsumichi, & Okazaki, 1998).

3.2. XOD activity of freeze-dried formulations after the preparation

The remaining activity of XOD in various formulations was observed immediately after the preparation as shown in Fig. 1. A non-additive sample and DE showed drastic decreases in XOD activity to approximately 20–26%. Using disaccharides and BSA individually, on the other hand, maintained XOD activity of 40 to 66%. Disaccharide–BSA mixtures improved the remaining XOD activity synergistically; SUC + BSA and TRE + BSA maintained XOD activity of approximately 90% and 83%, respectively. In contrast, disaccharide–DE mixtures did not have synergistic stabilizing effects, and maintained XOD activity of 35–50%. The polymer concentration had minimal effect on the remaining XOD activity.

As mentioned above, disaccharide and polymer have different strengths and weaknesses in the stabilization of freeze-dried enzymes. It is expected that disaccharide and polymer play a role as excellent stabilizers during drying and freezing, respectively. This interpretation is strongly supported by previous studies (Imamura, Ogawa, Sakiyama, & Nakanishi, 2003; Nema & Avis, 1992). Since disaccharide and polymer play different roles as stabilizers, the disaccharide–polymer mixture can compensate for each weakness. For example, polyethylene glycol (PEG) could prevent the activity losses of phosphofructokinase and lactate dehydrogenase during freezing, but not during freeze-drying. Although disaccharide (trehalose and lactose) could not also prevent their activity losses during freeze-drying, disaccharide–PEG mixtures could maintain high activities. From these results, it is suggested that PEG and disaccharide protect the enzymes during freezing and drying, respectively (Carpenter et al., 1993). This also reasonably explains why the results obtained in this study show that disaccharide–BSA mixtures greatly prevented the activity loss of XOD.

Although it is demonstrated that dextran is a good stabilizer for some frozen and freeze-dried enzymes (Anchordoquy et al., 2001; Chang et al., 1993; Nema & Avis, 1992), there was no stabilizing effect on the freeze-dried XOD. In addition, a synergistic effect induced by the disaccharide–polymer mixture was not observed in the case of disaccharide–DE. From these results, it is suggested that dextran is an unsuitable polymer as stabilizer for freeze-dried XOD. Moreover, it is known that freezing polymer solutions may cause phase separation due to polymers' altered solubilities at low temperatures (Wang, 2000). It seems that dextran phase-separates from proteins, which consequently reduces its efficiency to protect proteins (Allison et al., 1999, 2000; Heller, Carpenter, & Randolph, 1996).

In comparison, between sucrose and trehalose, it was found that sucrose protected XOD from activity loss better than trehalose. This result was supported by a previous study published by Allison et al. (1999). The sucrose formed hydrogen bonds with lysozyme to a larger extent than did trehalose as determined by infrared spectra. It may be stated that structural differences between sucrose and trehalose may influence the extent and intimacy of hydrogen bond formation to the XOD, consequently causing the difference in stabilizing the dried enzyme.

3.3. XOD activity of freeze-dried formulations during long-term storage

Long-term storage stability of XOD in SUC, TRE, SUC + 1% BSA, TRE + 1% BSA, and non-additive samples at various temperatures were further investigated. Fig. 2 shows the duration of the remaining XOD activity of the samples stored at 25, 40, 50, and 60 °C. Although non-additive samples lost nearly all of their remaining XOD activity during storage, samples containing stabilizers maintained XOD activity to a certain extent. The XOD activity decreased gradually with an increase in storage time. The rate of decrease in XOD activity was almost comparable regardless of the type of

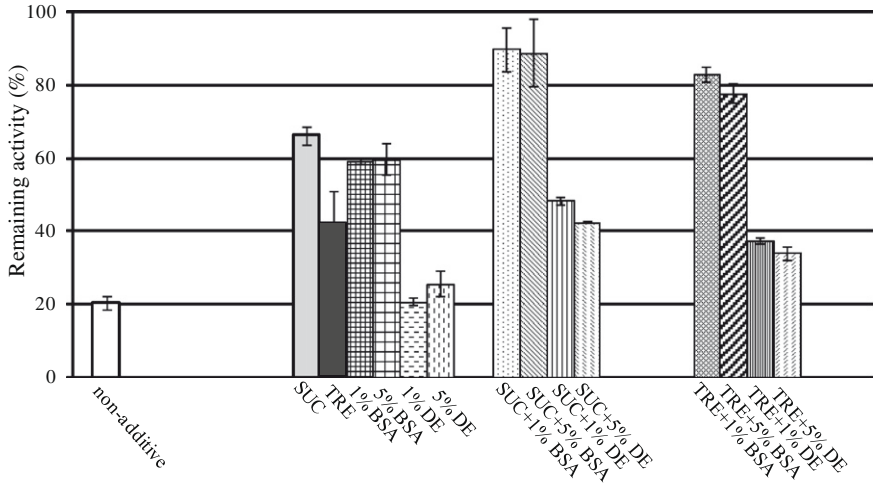

Fig. 1. Comparison of the remaining activity of XOD (%) in various freeze-dried formulations observed immediately after their preparation. The values are mean ± SD (n = 3).

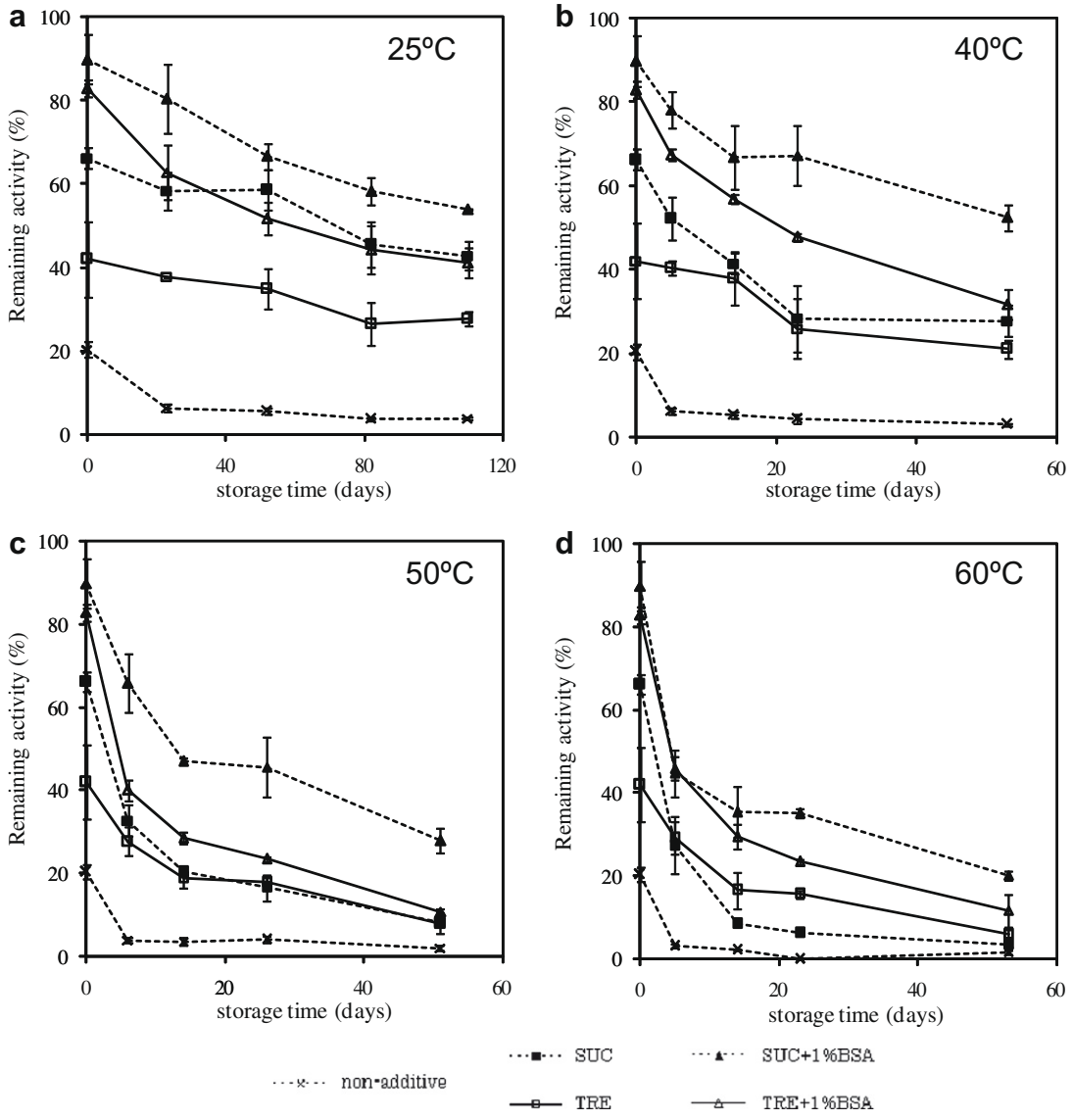

Fig. 2. Remaining activity of XOD with various stabilizers during storage at: (a) 25 °C, (b) 40 °C, (c) 50 °C, (d) 60 °C. The values are mean ± SD (n = 3).

stabilizer. In addition, SUC showed a more rapid loss of XOD activity than the others during storage at 60 °C. The T_g of SUC was the lowest amongst the samples, and the storage temperature was due to its T_g (60.8 °C). Since SUC has greater molecular mobility

than the other samples, the loss of XOD activity may have been accelerated.

The activity of XOD decreased gradually even at storage temperatures below T_g. Similar results were also reported in previous studies (Allison et al., 2000; Kawai & Suzuki, 2007). These results may be explained by the degradation of enzymes during storage being impacted by the damage gained previously upon the freeze-drying process as argued by Chang et al. (1996). This study tried to improve the remaining activity of freeze-dried XOD with the addition of stabilizers, and thus the effects of the operational condition of freeze-drying on the enzyme stability were not investigated. There may be more optimal conditions for freeze-drying. In addition, the molecular mobility of freeze-dried solids may also affect the gradual decrease of the XOD activity. Glassy materials show molecular dynamics in the time scale of hour to day at temperatures near T_g (Duddu, Zhang, & Dal Monte, 1997; Hancock, Shamblin, & Zografi, 1995; Kawai et al., 2005). The change in the molecular structure happens too slowly, but may not be negligible during long-term storage. Further study is necessary to solve this problem.

4. Conclusions

XOD, which is one of the practically important enzymes in food industry, loses almost all of its activity during freeze-drying. This study demonstrated that sucrose, trehalose and BSA protected XOD from the activity loss during freeze-drying. Furthermore, it was found that disaccharide–BSA mixture improved the XOD activity synergistically and that sucrose–BSA mixture was the most effective amongst the examined stabilizers. During subsequent storage, it was confirmed that the samples stored at temperatures below T_g showed a lower loss of XOD activity than those stored just at T_g. The XOD activity, however, gradually decreased with the increase in storage time even at temperatures below T_g. In order to store the freeze-dried XOD at ambient temperature for longer period of time, further improvement in the stabilization of XOD is required.

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