Stabilizing effects of sucrose–polymer formulations on the activities of freeze-dried enzyme mixtures of alkaline phosphatase, nucleoside phosphorylase and xanthine oxidase

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ABSTRACT

To stabilize two freeze-dried enzyme mixtures, consisting of alkaline phosphatase, nucleoside phosphorylase and xanthine oxidase, the effects of sucrose–polymer (bovine serum albumin, gelatin, dextran, polyethylene glycol and polyvinylpyrrolidone) formulations on the remaining activity of the enzyme mixtures were investigated. The enzyme mixtures were freeze-dried with the additives, and then stored at 25, 40 and 55 °C. The glass transition temperatures (T_g) of the freeze-dried samples were assessed in order to determine their physical stability. The T_g values of sucrose–polymer formulations, with the exception of sucrose–polyethylene glycol, were higher than that of sucrose alone. Comparison of the remaining activities of freeze-dried samples showed that sucrose–bovine serum albumin and/or –gelatin prevented activity loss more effectively than did sucrose. Sucrose–polyethylene glycol showed protective ability equivalent to that of sucrose. On the other hand, sucrose–dextran and/or –polyvinylpyrrolidone diminished the stabilizing effect of sucrose. During storage, sucrose–gelatin prevented gradual activity loss to a much greater degree than did sucrose alone.

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1. Introduction

Maintenance of enzyme activity is important for its application in biotechnology and the food industry. Freeze-drying is a commonly used technique for stabilizing enzymes. However, some labile enzymes are sensitive to both freezing and subsequent dehydration stresses encountered during freeze-drying (Anchordoquy, Izutsu, Randolph, & Carpenter, 2001; Carpenter, Prestrelski, & Arakawa, 1993; Kawai & Suzuki, 2007; Prestrelski, Arakawa, & Carpenter, 1993; Wang, 2000), resulting in a loss of activity.

Alkaline phosphatase (ALP), nucleoside phosphorylase (NP) and xanthine oxidase (XOD) have been widely used in biotechnology. For instance, two enzyme mixtures, NP–XOD and ALP–NP–XOD, are necessary in testing paper used to evaluate the freshness of fish, based on the change in the relative contents of nucleotides (e.g., inosine 5′monophosphate [IMP]) and nucleosides (e.g., inosine and hypoxanthine) in fish meat (Kaminashi, Nakaniwa, Kunimoto, & Miki, 2000; Srirangsan, Kawai, Hamada-Sato, Watanabe, & Suzuki, 2010).

$$IMP \xrightarrow{ALP} inosine \xrightarrow{NP} hypoxanthine \xrightarrow{XOD} xanthine \xrightarrow{XOD} uric\ acid$$
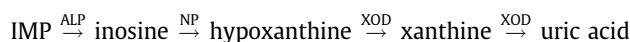
ALP is used to convert IMP to inosine. Likewise, inosine is converted to hypoxanthine by NP, and subsequently, XOD oxidizes hypoxanthine to xanthine and uric acid (Kalckar, 1947; Srirangsan et al., 2010). ALP, NP and XOD are, however, thermally unstable and become almost completely inactive during freeze-drying and subsequent storage. Thus, it is of practical importance to stabilize these enzymes.

Many efforts have been devoted to improving the stability of freeze-dried enzymes using additives (Allison, Chang, Randolph, & Carpenter, 1999; Anchordoquy & Carpenter, 1996; Anchordoquy et al., 2001; Izutsu, Yoshioka, & Kojima, 1995; Izutsu, Yoshioka, & Takeda, 1991; Kawai & Suzuki, 2007; Prestrelski et al., 1993; Schebor, Buera, & Chirife, 1996; Schebor, Burin, Buera, Aguilera, & Chirife, 1997; Srirangsan et al., 2010). Among the additives, sugar and polymer are usually effective (Allison et al., 1999; Anchordoquy et al., 2001; Chang, Beauvais, Dong, & Carpenter, 1996; Kreilgaard, Frokjaer, Flink, Randolph, & Carpenter, 1998; Prestrelski et al., 1993; Sampedro, Guerra, Pardo, & Uribe, 1998; Schebor et al., 1997; Wang, 2000). For example, Anchordoquy et al. (2001) investigated the activity of reconstituted freeze-dried lactate dehydro-

genase (LDH) formulations containing various kinds of stabilizing agents (namely, glucose, sucrose, trehalose, dextran and polyethylene glycol [PEG]) and demonstrated that the presence of these sugars or polymers greatly prevented LDH dissociation and preserved enzyme activity during freeze-drying. The use of 0.05% bovine serum albumin (BSA) resulted in approximately 80% activity in reconstituted freeze-dried LDH (Anchordoquy & Carpenter, 1996). Furthermore, the combined use of sugar and polymer can synergistically stabilize some types of enzymes. For example, mixtures of sugar (trehalose, lactose or mannitol) and 1% PEG maintained the activity of freeze-dried enzymes (LDH and phosphofructokinase) at a higher level than did their individual formulations (Prestrelski et al., 1993). The addition of 2% dextran to a sucrose-containing actin formulation significantly increased protein stability during freeze-drying (Allison et al., 1998).

In our previous study (Srirangsan et al., 2010), the effects of sugar, polymer and their mixtures on the remaining activity of freeze-dried XOD were investigated. The results indicated that sucrose and BSA were relatively good stabilizers. Furthermore, a combination of sucrose and BSA synergistically improved the remaining XOD activity. Freeze-dried XOD, however, is used with ALP and NP for the production of freshness test paper, and thus, not only XOD, but also ALP and NP, require stabilization. In addition, there are limited data on the stabilizing effects of sugar–polymer mixtures on enzymes. Therefore, we investigated the effects of various sucrose–polymer mixtures on the stability of freeze-dried NP–XOD and ALP–NP–XOD.

2. Materials and methods

2.1. Preparation of freeze-dried samples

NP (bacterial E.C. 2.4.2.1), BSA (fraction V) and dextran (MW = 10.4 kDa) were purchased from Sigma–Aldrich Co. (USA). ALP (from calf intestine E.C. 3.1.3.1), XOD (from butter milk E.C. 1.1.3.22), IMP disodium salt, inosine, sucrose, gelatin, PEG (MW = 0.6 kDa), polyvinylpyrrolidone k30 (PVP, MW = 40 kDa), and other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Japan).

ALP, NP and XOD were separately dialyzed against 20 mM potassium phosphate buffer (pH 7.6) at 4 °C for 48 h to remove stabilizing agents. Two enzyme mixtures, NP–XOD and ALP–NP–XOD, were prepared, for which the activity of NP:XOD and ALP:NP:XOD were determined to be in the proportion of 0.3:0.6 Uml^{-1} and 45:0.3:0.6 Uml^{-1}, respectively. The NP–XOD mixture was prepared with additives in the following formulations: 200 mM sucrose (SUC), 200 mM sucrose + 1% BSA (SUC–BSA), 200 mM sucrose + 0.5% gelatin (SUC–GE), 200 mM sucrose + 1% dextran (SUC–DE), 200 mM sucrose + 1% PEG (SUC–PEG), and 200 mM sucrose + 1% PVP (SUC–PVP). The ALP–NP–XOD mixture was prepared with SUC and SUC–GE. The concentrations of the polymers were as percentages (w/v). The gelatin was employed at a lower concentration than those of the other polymers, to prevent its gelation during freeze-drying. As the control, non-additive samples of both enzyme mixtures were also prepared. Aliquots (1 ml) of each solution were placed in 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 in 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 phosphorus pentoxide in a vacuum desiccator for 7 days at room temperature. The samples were

then hermetically sealed in a dry nitrogen-purged glove box. The enzyme activity of a part of the dried samples was assayed in order to evaluate the activity loss during freeze-drying. In addition, the moisture content and thermal properties were investigated, as described later. The other samples were stored at 25, 40 and 55 °C for up to 110 days, and then the remaining activities of the enzymes were determined for loss during storage.

2.2. Moisture content analysis

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

2.3. Assay of enzyme activity

The activities of NP–XOD and ALP–NP–XOD were separately assayed by the enzymatic conversion of the substrates inosine and IMP, respectively. The freeze-dried samples were rehydrated with distilled water to render the previous concentration, and the sample solution (75 μl) was added to 225 μl of 1.33 mM substrate (inosine for the NP–XOD samples and IMP for the ALP–NP–XOD samples) in 20 mM potassium phosphate buffer (pH 7.6). In this study, the enzymatic activities of NP–XOD and ALP–NP–XOD were evaluated as an apparent total activity for the production of uric acid from inosine and IMP, respectively. The time course for absorbance at 292 nm, which is the maximal absorption peak of uric acid, was measured at 20 °C, using a UV–VIS spectrophotometer (V-630BIO, Jasco, Tokyo, Japan). The activity was evaluated from the initial reaction rate. The remaining activity was expressed as a percentage of the activity relative to that determined before freeze-drying.

2.4. Differential scanning calorimetry

The physical properties of the freeze-dried samples were assessed by differential scanning calorimetry (DSC-50: Shimadzu, Co., Japan). The DSC was calibrated using pure samples of indium and distilled water. Alumina powder was used as a reference material. The sample (approximately 15 mg) was weighed into an aluminium DSC pan and hermetically sealed in a dry nitrogen-purged glove box. All measurements were performed from 0 to 180 °C at a scan rate of 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.

2.5. Statistical analysis

The data were interpreted by Bonferroni multiple comparison tests, using Kaleida Graph (Version 3.6, Synergy software). Statistical significance was expressed at the $P < 0.05$ level.

3. Results and discussion

3.1. Thermal properties of freeze-dried samples

Table 1 lists the abbreviations for each sample and the T_g, T_c, and moisture contents of the freeze-dried samples. The moisture content of the SUC samples was slightly higher than those of the other samples. All samples containing additive(s) showed glass transition. Anhydrous glassy sucrose is known to have T_g = 68 °C (Kawai, Hagiwara, Takai, & Suzuki, 2005). SUC and SUC–PEG showed lower T_g than did the anhydrous glassy sucrose. This

Table 1
Sample abbreviations, T_g, T_c, and moisture contents (%) of freeze-dried samples.

Enzyme	Formulation	Abbreviation	T_g (°C)[a]	T_c (°C)[b]	% Moisture content[b]
NP–XOD	200 mM sucrose	SUC	54 ± 2	105 ± 5	2.64 ± 0.76
	200 mM sucrose + 1%BSA	SUC–BSA	67 ± 4	137 ± 4	0.95 ± 0.38
	200 mM sucrose + 0.5%gelatin	SUC–GE	75 ± 4	139 ± 5	0.34 ± 0.05
	200 mM sucrose + 1%dextran	SUC–DE	75 ± 6	145 ± 3	0.27 ± 0.09
	200 mM sucrose + 1%PEG	SUC–PEG	46 ± 3	100 ± 2	0.49 ± 0.31
	200 mM sucrose + 1%PVP	SUC–PVP	75 ± 2	nd	0.22 ± 0.20
ALP–NP–XOD	200 mM sucrose	SUC	53 ± 3	103 ± 4	2.82 ± 0.13
	200 mM sucrose + 0.5%gelatin	SUC–GE	74 ± 2	134 ± 6	0.73 ± 0.20

nd = not determined.
[a] The values are expressed as means ± SD ($n = 2$).
[b] The values are expressed as means ± SD ($n = 3$).

may be because the respective moisture and PEG (MW = 0.6 kDa) play the role of plasticizer (Imamura et al., 2002; Roos, 1995). On the other hand, SUC–BSA, SUC–GE, SUC–DE and SUC–PVP had higher T_g than had SUC. This is because a high-molecular weight polymer has a higher T_g and greater resistance to a decrease in T_g, induced by the plasticizing effect of water, than had sucrose. Crystallization of sucrose was observed in most of the formulations, excluding SUC–PVP, in which no crystallization occurred. The T_c values of SUC–BSA, SUC–GE and SUC–DE were higher than those of the SUC samples. In contrast, SUC–PEG showed a slightly lower T_c than that of SUC. This means that the physical stability of amorphous sucrose is improved by the addition of BSA, gelatin, dextran and PVP, and diminished by the addition of PEG. Comparing NP–XOD and ALP–NP–XOD mixtures, it was noted that the presence of ALP had little or no effect on the T_g and T_c of the samples.

3.2. Activity of freeze-dried NP–XOD remaining immediately after preparation

The remaining activity of NP–XOD samples, immediately after preparation, is shown in Fig. 1. The non-additive sample showed a drastic decrease in enzyme activity, to approximately 34%. SUC, on the other hand, showed approximately 64% activity. SUC–BSA and SUC–GE stabilized NP–XOD more effectively and maintained approximately 86% and 84% activity, respectively. SUC–PEG showed about 61% activity, which was comparable to SUC. The other formulations, SUC–DE and SUC–PVP, had lower remaining activities than had SUC; the activity decreased to 48% and 42%, respectively.

The stabilizing mechanisms of additives on freeze-dried enzymes are mainly explained by "preferential exclusion", "water replacement" and "glass transition". Preferential exclusion involves the preferential exclusion of additives from the enzyme surface, and conformation of the enzymes is thermodynamically stabilized in the liquid and/or freezing state (Arakawa, Prestrelski, Kenney, & Carpenter, 2001; Arakawa & Timasheff, 1982; Carpenter & Crowe, 1988; Nema & Avis, 1992; Wang, 2000). Water replacement involves hydrogen bonding of the enzyme with additives instead of the removed water molecules, and native-like conformation of the enzyme is maintained in the dried state (Allison et al., 1999; Arakawa et al., 2001; Kawai & Suzuki, 2007; Kreilgaard et al., 1998; Prestrelski et al., 1993; Schebor et al., 1997; Wang, 2000). Glass transition leads to the prevention of physical and chemical enzyme degradation, due to the slowing down of conformational changes in freeze-concentrated and/or freeze-dried glassy matrices (Anchordoquy et al., 2001; Franks, 1993; Kawai & Suzuki, 2007; Prestrelski, Pikal, & Arakawa, 1995; Schebor et al., 1996, 1997). It is commonly known that both sugars and polymers effectively show preferential exclusion effects (Anchordoquy et al., 2001; Arakawa & Timasheff, 1982; Arakawa et al., 2001; Carpenter and Crowe, 1988; Heller, Carpenter, & Randolph, 1996; Wang, 2000). On the other hand, polymers show superior glass transition effects compared to sugars, while sugars show superior water replacement effects compared to polymers (Allison et al., 1998; Carpenter, Pikal, Chang, & Randolph, 1997;
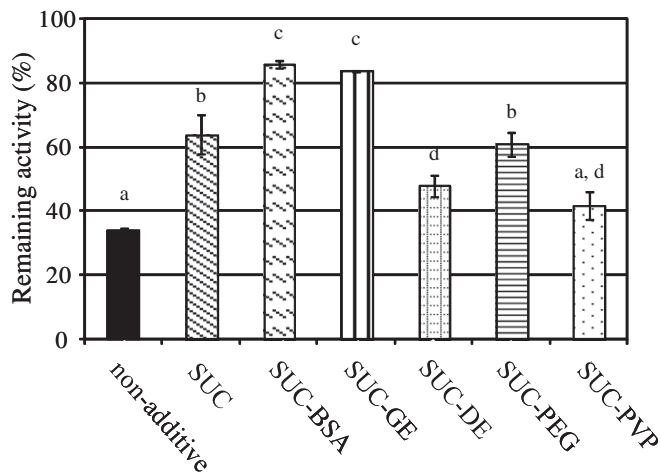

Fig. 1. Comparison of the NP–XOD activity remaining after freeze-drying. The values are expressed as means ± SD ($n = 3$). Different letters above bars indicate statistical differences ($P < 0.05$).
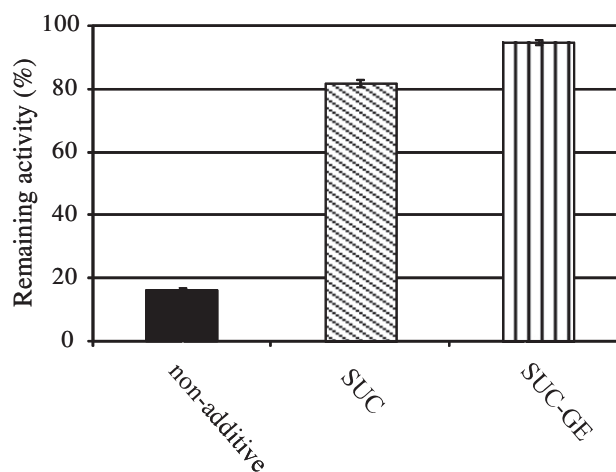

Fig. 2. Comparison of the ALP–NP–XOD activity remaining after freeze-drying. The values are expressed as means ± SD ($n = 3$). The data were significantly different ($P < 0.05$).
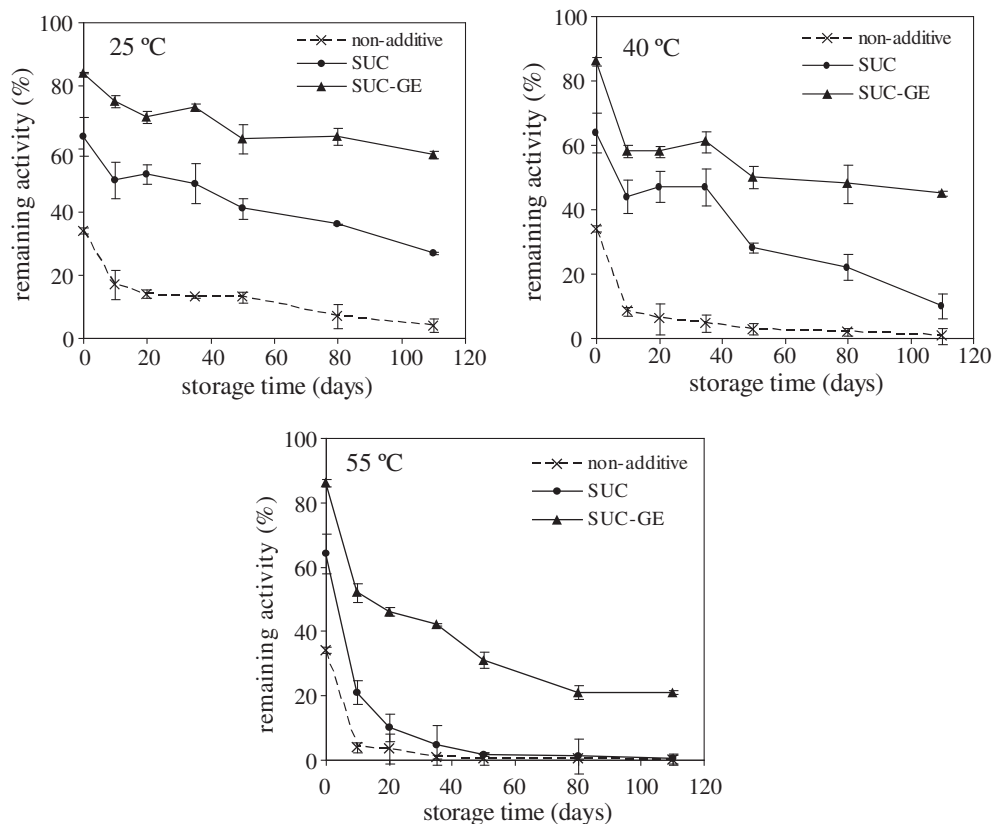
Fig. 3. Freeze-dried NP–XOD activity (%) remaining during storage at 25, 40 and 55 °C. The values are expressed as means ± SD (n = 3).
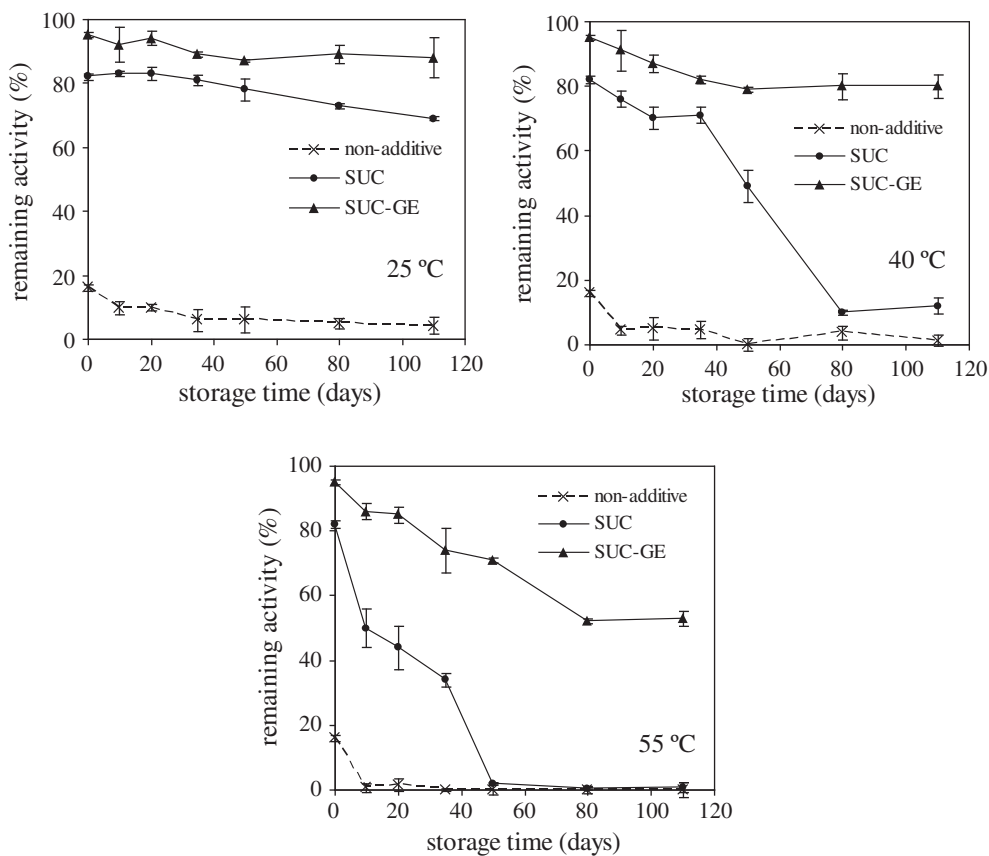

Fig. 4. Freeze-dried ALP–NP–XOD activity (%) remaining during storage at 25, 40 and 55 °C. The values are expressed as means ± SD (n = 3).

Prestrelski et al., 1995; Taylor & Zografi, 1998). A sugar–polymer mixture, therefore, will compensate for its individual weaknesses and be able to synergistically stabilize some types of enzyme (e.g., LDH and PFK, as reported by Prestrelski et al., 1993). The stabilizing effect of sucrose–polymer mixtures on NP–XOD, however, depend strongly on the type of polymer, and only BSA and gelatin could improve the stability of freeze-dried NP–XOD. These results are discussed as follows: both sucrose and polymers are excluded from NP–XOD in liquid and freezing states (Arakawa et al., 2001; Carpenter & Crowe, 1988; Nema & Avis, 1992). The excluded dextran, PEG, or PVP and sucrose are freeze-concentrated together, and the mixtures turn into a glassy state during freezing and/or subsequent dehydration. Then, the molecular mobility of sucrose is highly limited in the glassy matrix strengthened by polymer, and the water replacement effect of sucrose to NP–XOD is restricted. The fact that T_g and T_c of SUC–DE and SUC–PVP were higher than those of SUC suggests that the water replacement effect of sucrose to NP–XOD was highly restricted by dextran and/or PVP. Thus, SUC–DE and SUC–PVP showed the large activity loss of NP–XOD. The reason why SUC–PEG maintained higher NP–XOD activity than did SUC–DE and SUC–PVP is that SUC–PEG had a lower T_g than had SUC–DE and SUC–PVP; sucrose in SUC–PEG is more flexible and better at water replacement than in SUC–DE and SUC–PVP, as suggested by (Imamura, Ogawa, Sakiyama, & Nakanishi, 2003). BSA and gelatin, on the other hand, are proteins themselves, and sucrose is excluded, not only from NP–XOD but also from BSA or gelatin (Arakawa et al., 2001; Nema & Avis, 1992). As a result, sucrose effectively provides the water replacement effect to NP–XOD while BSA or gelatin strengthens the glassy matrix.

3.3. Activity of freeze-dried ALP–NP–XOD remaining immediately after preparation

A typical effective additive, SUC–GE, was further investigated with respect to freeze-dried stabilization of ALP–NP–XOD. For comparison, non-additive and SUC were also employed. The remaining activity of the samples, immediately after preparation, is shown in Fig. 2. Although the enzyme activity for the non-additive sample was lost (down to 16%), SUC and SUC–GE stabilized the enzyme mixture and resulted in 82% and 95% remaining activities, respectively. There were statistical differences ($P < 0.05$) among them. The results indicate that the amount of ALP–NP–XOD remaining activity was in the order of non-additive < SUC < SUC–GE, similar to that of NP–XOD. Comparing ALP–NP–XOD with NP–XOD, the non-additive sample showed less remaining activity for ALP–NP–XOD than for NP–XOD. This is because ALP was more sensitive to stresses encountered during freeze-drying than were NP and XOD; the loss of ALP activity causes a decrease in the production of inosine (substrate for NP), and thus the apparent total activity of ALP–NP–XOD decreased more than did that of NP–XOD. In contrast, the ALP–NP–XOD in SUC and SUC–GE showed more remaining activity than did the NP–XOD. This indicates that, not only was ALP stabilized by sucrose and/or sucrose–gelatin mixture, but also the stabilized ALP protected NP–XOD. ALP is a protein itself, and thus improves the water replacement effect of sucrose to NP and XOD (similarly to BSA or gelatin).

3.4. Storage stability of freeze-dried NP–XOD and ALP–NP–XOD

Time courses of the remaining activity of NP–XOD samples held at 25, 40 and 55 °C are shown in Fig. 3. Although non-additive samples became almost completely inactive, SUC and SUC–GE maintained activity to a certain extent, depending on the temperature and time. Probably the storage stability of freeze-dried enzyme is primarily affected by the T_g, because, the higher the T_g, the lower is the molecular mobility at a storage temperature (T). In other
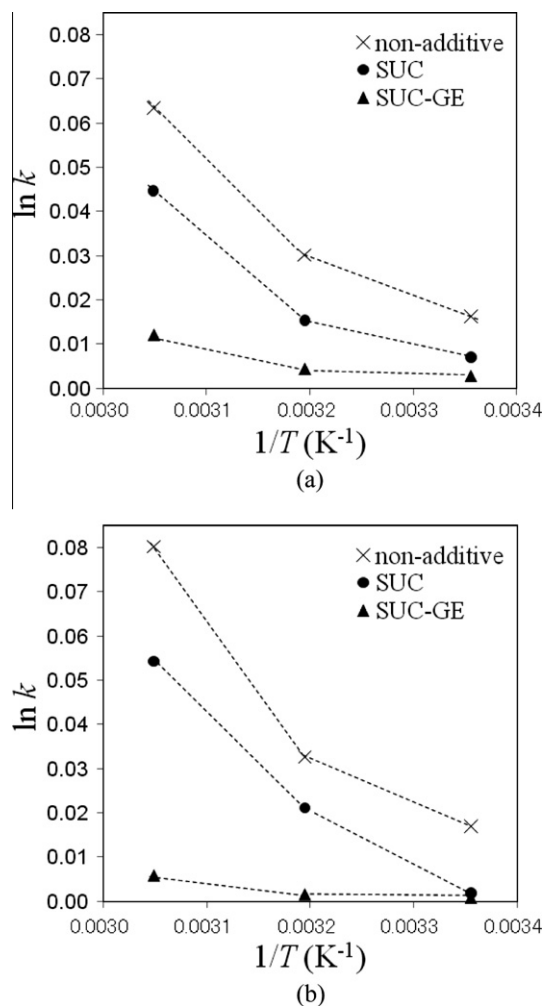

Fig. 5. Arrhenius plots of the degradation rate constant (k) of (a) NP–XOD and (b) ALP–NP–XOD.

words, the higher the difference between T_g and T (T_g–T), the greater is the expected storage stability. During storage at 55 °C, SUC had a T_g just lower than T (T_g–$T \approx -1$ °C). The sample was in a rubbery state and structural collapse (evidenced as a dramatic shrinkage of the matrix) had apparently occurred. On the other hand, SUC–GE had a slightly higher T_g than had T (T_g–$T \approx 20$ °C). Since molecular mobility of SUC was much higher than that of SUC–GE, the enzyme activity of SUC decreased more rapidly than did that of SUC–GE. During storage at 40 °C, the T_g values of SUC and SUC–GE were slightly (T_g–$T \approx 14$ °C) and considerably (T_g–$T \approx 35$ °C) higher than those of T, respectively. Thus, either SUC or SUC–GE showed a lower rate of activity degradation than that occurring at 55 °C. During storage at 25 °C, SUC and SUC–GE had T_g values that were higher (T_g–$T \approx 29$ °C) and much higher (T_g–$T \approx 50$ °C) than those of T, respectively. SUC and SUC–GE greatly maintained the enzyme activity compared to samples stored at 40 and 55 °C.

Time courses of the remaining activity of ALP–NP–XOD samples held at 25, 40 and 55 °C are shown in Fig. 4. The results showed trends similar to those of NP–XOD; SUC–GE preserved the enzyme activity more effectively than did SUC. The lower the storage temperature of samples, the higher is the enzyme activity. Although the T_g values of NP–XOD and ALP–NP–XOD were almost identical in the same formulation, the remaining activity of ALP–NP–XOD was considerably higher. This may be influenced by the higher activity remaining after freeze-drying, since more protection was obtained by the assistance of the ALP, as described previously.

The time courses of the remaining activities of NP–XOD and ALP–NP–XOD samples could be represented by first order kinetics, and a first order degradation rate constant (k) was reasonably evaluated ($R^2 = 0.83 \sim 0.95$). Furthermore, dependence of k on the storage temperature was described by an Arrhenius plot, as shown in Fig. 5. From the results, it was noted that non-additive and SUC samples showed a large deviation from Arrhenius law. Such a non-Arrhenius behaviour suggests that conformational changes of the systems (e.g., structural collapse) occurred with an increase in the storage temperature, resulting in the acceleration of degradation (Sun, Davidson, & Chan, 1998). In fact, the non-additive sample stored at 40 and 55 °C, and SUC sample stored at 55 °C showed structural collapse. On the other hand, SUC–GE samples showed a slight deviation from the Arrhenius law. Since SUC–GE samples had a much higher T_g than the storage temperature, there will have been little conformational change, even at high temperature.

4. Conclusions

This study demonstrated that a combination of sucrose and protein (BSA and gelatin) had excellent stabilizing effects in ALP, NP and XOD mixtures. Using a non-protein polymer (dextran, PEG, and PVP) with sucrose, on the other hand, did not improve the stability of enzyme mixtures during freeze-drying. The addition of gelatin to the sucrose formulation increased the T_g, providing beneficial effects on the storage stability of freeze-dried enzymes. The results of this study will be of practical use in the production of test paper for application in fish freshness determination.

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