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Stabilization of Nucleoside phosphorylase-Xanthine oxidase Mixture in Dried Glassy Matrices

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To stabilize freeze-dried mixture of nucleoside phosphorylase (NP) and xanthine oxidase (XOD), effects of sucrose and sucrose-bovine serum albumin (BSA) mixture on the remaining activity of the enzymes were investigated. Furthermore, glass transition temperatures (T_g) of the dried samples were examined. The T_g of sucrose sample was lower than that of sucrose-BSA one. This indicates that physical stability of the dried samples was improved by the addition of BSA. The activity of NP-XOD mixture was greatly protected by sucrose. Furthermore, the combination of sucrose and BSA improved the enzymes stability.

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INTRODUCTION

Due to recent bio-technological advances, the need for stabile enzyme preparations is growing. Many of these preparations are in the form of a freeze-dried solid. However, some enzymes are sensitive to both freezing and subsequent dehydration stresses encountered during freeze-drying [1, 2], which causes them to lose most of their activity during the process.

Nucleoside phosphorylase (NP) and xanthine oxidase (XOD) have been widely used in biotechnology. For example, mixtures of them have been used in a test paper to evaluate freshness of fish based on the change of the relative content of nucleosides, e.g., inosine, hypoxanthine and xanthine, in fish meat[3, 4]. Inosine is oxidized to hypoxanthine by NP; likewise XOD is used to convert hypoxanthine to xanthine and consequently to uric acid [5]. The NP and XOD, however, are unstable thermally and almost completely lose their activities during freeze-drying, so it is practically important that the enzymes are stabilized.

Many efforts have been devoted to improve the stabilization of freeze-dried enzymes by using additives[3, 6-9]. Stabilizing mechanisms of additives on freeze-dried enzymes during freezing, the first step of freeze-drying, are described by "solute exclusion" [2, 7, 10] and "freeze-concentrated glass transition" [7, 8, 11]. The former involves that additives are preferentially excluded from the enzyme's hydration shell, thus the enzyme unfolding is prevented. The latter involves that additives form a high-viscous glassy matrix by

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freeze-concentration, and thus, molecular rearrangement of the enzyme and the rate of degradation are immobilized. On the other hand, stabilizing mechanisms during dehydration step of freeze-drying are described by "water replacement" [2,6,7,10] and "glass transition" [7,8,11]. The "water replacement" hypothesis involves the formation of hydrogen bonds between the dried enzyme and additives in place of the removal water molecules, and thus the dried enzyme maintains its native-like conformation. The "glass transition" mechanism is intrinsically similar to "freeze-concentrated glass transition" mechanism, involving embedding of the enzyme molecules in a glassy matrix. Therefore, the physical and chemical degradations of the enzyme will be prevented due to the slowing down of the conformational change.

In our previous study, effects of disaccharides, polymer, and their mixture on the remaining activity of freeze-dried XOD were investigated. From the results, it was found that sucrose and bovine serum albumin (BSA) were relative good stabilizers. Furthermore, sucrose-BSA mixture improved the remaining XOD activity synergistically. Freeze-dried XOD, however, is used with NP for the production of freshness test paper, and thus not only XOD, but also NP is required to be stabilized. As the next subject of our study, effects of sucrose and sucrose-BSA on the remaining activity of freeze-dried NP and XOD mixture were investigated.

MATERIALS AND METHODS

Preparation of freeze-dried samples

Nucleoside phosphorylase (NP, E.C. 2.4.2.1) and xanthine oxidase (XOD, E.C. 1.1.3.22) were separately dialysed against 20 mM potassium phosphate buffer (pH 7.6) at 4 °C for 48 h in order to remove stabilizing agents. Mixture of both dialyzed enzyme solutions was prepared by which the activity of NP and XOD was determined to be in the proportion 0.3 : 0.6 Uml^{-1}. Two types of freeze-dried formulations were prepared as 200 mM sucrose and 200 mM sucrose + 1% BSA. As the control, a non-additive sample was also done. Aliquots of 1 ml of each solution were placed into 2 ml-polypropylene tubes and frozen instantaneously with liquid nitrogen. The frozen solids were transferred to a pre-cooled freeze-drier. Freeze-drying was performed with warming the temperature from -40 to 25 °C at 3.0×10^{-2} Torr over a 2-day period. 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.

Moisture content analysis

A Metrohm Karl Fisher coulometer (737 KF, Herisau, Switzerland) was used to measure the moisture content of the dried samples.

Differential scanning calorimetry

Thermal properties of the freeze-dried samples were examined by a differential scanning calorimetry (DSC-50: Shimadzu, Co., Japan). Alumina powder was used as a reference material. The sample (approximately 15 mg) was weighed on an aluminum DSC pan and sealed hermetically. All measurements were performed from 0 to 180 °C at a scan rate of 5 °C/min.

Assay of the enzymes activity

The freeze-dried samples were rehydrated with distilled water to give a previous concentration, and the sample solution (75 μl) was added into 225 μl of 1.33 mM inosine in a 20 mM potassium phosphate buffer (pH 7.6). In this study, the enzymatic activity of NP-XOD mixture was evaluated as "total enzymatic activity" for catalyzing reduction of inosine and the produced hypoxanthine to uric acid. The absorbance of 292 nm, which is the maximal absorption peak of uric acid, was measured at 20 °C by using a UV-VIS spectrophotometer (V-630BIO: Jasco, Tokyo,

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Japan). The activity was evaluated from the initial reaction rate. The remaining activity was expressed as a percentage of the activity prior to freeze-drying.

RESULTS AND DISCUSSION

The moisture content of the individual sucrose formulation was approximately 2.3% (w/w), which was considerably greater than that of the sucrose-BSA (0.9%, w/w). Both of the samples showed glass transition at a temperature above 25 ℃. The obtained glass transition temperature (T_g) values for the sucrose and sucrose-BSA samples were 58 and 67 ℃ (Fig. 1), respectively. The T_g of the sucrose-BSA sample was significantly higher than that of the sucrose alone sample, this will be because BSA has higher T_g and greater resistance to a decrease of T_g induced by plasticizing effect of water than sucrose. Moreover, crystallization of sucrose in the presence of BSA occurred at a temperature higher than that of the sucrose alone sample. This means that the physical stability of amorphous sucrose is improved by the addition of BSA as reported in a previous study [12].

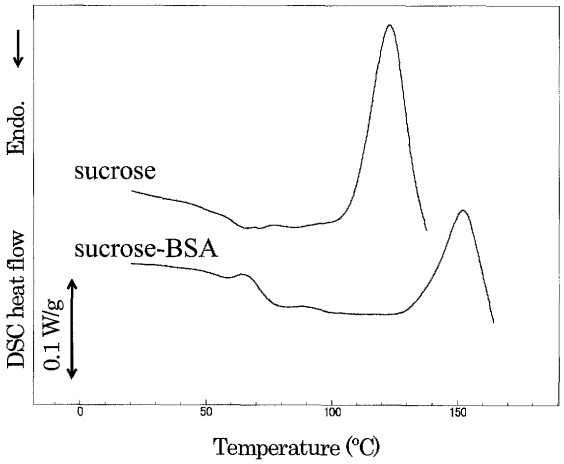

Fig. 1. DSC thermograms for the freeze-dried samples

The remaining activity of NP-XOD mixture is shown in Fig. 2. A non-additive sample showed a drastic decrease in the activity to approximately 34%. Using the given additives greatly preserved the activity. Although sucrose was effective in protecting the enzymes from the activity loss, a combination of sucrose and BSA improved the stability of the enzymes more synergistically and effectively. The sucrose-BSA maintained the enzymes activity of approximately 86%, whereas the activity was remained about 64% in the individual sucrose formulation.

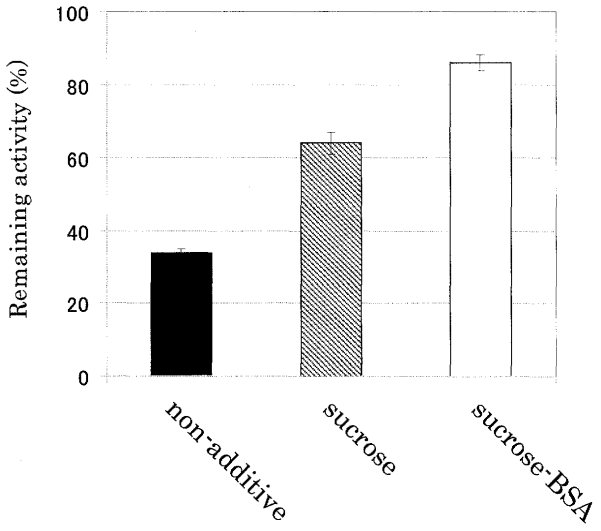

Fig. 2. Comparison of the remaining activity of the enzymes. The values are mean±SD (n=3).

It is thought that sucrose and BSA have different main roles as stabilizers during freeze-drying. BSA is an excellent cryoprotectant, which is preferentially excluded from the enzyme's hydration shell and the native conformation of the enzymes is stabilized during freezing process. Furthermore, it has a good glass-forming ability, which the rate of the enzyme degradation is decreased due to the restrictive molecular mobility. On the other hand, sucrose plays a main role in hydrogen bonding to the enzyme instead of the removal water during drying. Thus, the combined using of sucrose and BSA may compensate for each weakness and stabilize the enzyme synergistically. This result provides the possibility of producing freshness test paper.

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CONCLUSIONS

This study demonstrated that sucrose greatly protected NP and XOD from activity loss during freeze-drying. Furthermore, a combined using of sucrose and BSA preserved the enzymes more effectively. Taking results in previous studies into account, it is thought that sucrose and BSA stabilize the enzymes synergistically. This result provides the possibility of producing freshness test paper.

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