THERMAL STABILIZATION OF FREEZE-DRIED ENZYMES BY SUGARS

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Key Words: Freeze-Drying, Enzyme, Sugar, Thermal Stabilization, Amorphous

The thermal stabilization effect of sugars on freeze-dried proteins is studied, particularly the relation between stabilizing effect and the degree of sugar crystallinity. Three kinds of enzymes, alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH), were used as model proteins. Aqueous solutions of enzymes with sugars were freeze-dried and stored in dry air at 65°C. The stabilities of freeze-dried enzymes improve remarkably by addition of trehalose or raffinose. By measurement of X-ray diffractometry, the sugars are found to form fully amorphous matrix in freeze-dried samples. Furthermore, sucrose stabilizes enzymes to a great extent when it is amorphous in samples, though it shows little stabilizing effect when it is crystalline. These results indicate that the stabilizing effect of sugars closely relates to the amorphous matrix formed by the sugars.

Introduction

Due to advances in bioscience and biotechnology, numerous proteins that may be excellent pharmaceutical raw materials have been found. Though some of them have been applied to pharmaceutical purposes, many of them cannot be used because of their instability (Manning *et al.*, 1989). Thus it is very important to maintain the bioactivity of labile proteins during drug manufacturing processes and storage.

It is known that sugars are able to protect proteins from the stresses of drying and thermal inactivation during storage. Hence, one useful method to keep protein activity is to freeze-dry protein solutions containing sugars. As for the mechanism of the stabilizing effect of sugars, many studies have been done and two hypotheses have been suggested. One is the water replacement hypothesis and another is glassy state theory. Water replacement hypothesis argues that sugar molecules hydrogen-bond to the dried protein instead of the water molecules that play an important role in maintaining the tertiary structure of the protein, and so "water substitution" may protect the protein from the stresses of drying and storage (Carpenter and Crowe, 1988, 1989). On the other hand, glassy state theory suggests that the stabilizing effect by sugars may be caused by the amorphous structure formed by sugars (Franks *et al.*, 1991; Levine and Slade, 1992).

Although the two hypotheses are different in describing the stabilizing mechanism, we think that these two hypotheses are complementary for a description of the phenomenon of stabilization effect of sugars. Hydrogen-bonds between sugars and proteins should be essential for the stabilization effect of sugars. Hence it is quite natural that water substitution occurs in freeze-dried samples. On the other hand, such water substitution will occur more readily when the degree of crystallinity of sugars is low, rather than high.

According to the consideration described above, there would be a tendency for freeze-dried proteins to be stabilized with the amorphous matrix formed by sugars. Hence we are interested in the relation between the degree of crystallinity of sugars and the stabilizing effect. A similar theory, that the amorphous matrix of sugars stabilizes freeze-dried proteins, is also argued by the glassy state theory. However, little is known about the relation between crystallinity of sugars and the stabilizing effect of them. For example, to what degree does the stability of freeze-dried samples coincide with the order of the degree of crystallinity of sugars? Moreover, it is not known whether the stabilizing effect of a certain sugar changes by changing the degree of crystallinity of the sugar in the freeze-dried sample. Our present study is a preliminary one to discuss such problems.

We study the high-temperature inactivation of proteins freeze-dried with various sugars and the degree of crystallinity of sugars in the freeze-dried samples. Alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH) were used as model proteins. Enzymes were freeze-dried with sugars and stored in dry air at 65°C.

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The thermal stabilities of enzymes were evaluated with the change in the residual activities during storage. The degree of crystallinity of sugars in the freeze-dried samples was determined with X-ray powder diffractometry. The relationship between residual activities of enzymes during storage and the degree of crystallinity of sugars is examined. Furthermore, by using sucrose we investigate difference of stabilizing effect for samples of same sugar but different degree of crystallinity.

1. Experimental

1.1 Materials

ADH was purchased from Sigma (St. Louis, U.S.A.). LDH and MDH were from Toyobo Co. (Osaka). These enzymes were in the form of lyophilized powders and were used without further purification. All sugars used in this study, that is, glucose, sucrose, trehalose, maltose, lactose, raffinose, and maltotriose, were obtained from Nacalai Tesque (Kyoto). Silica gel for desiccation, and sodium pyruvate and ethanol for assay were from Wako Pure Chemical Industries Ltd. (Osaka). Glutathione was from Sigma and all other reagents were from Nacalai Tesque.

1.2 Freeze-drying and storage of enzymes

For each enzyme and each sugar, an aqueous solution containing 0.40 mg/ml enzyme and 0.055 mmol/ml sugar was prepared. Each 0.5 ml of prepared solution was transferred to a glass test tube (inside diameter 6 mm, thickness 1 mm) and frozen in liquid nitrogen. The frozen samples were soaked in an ethanol bath, whose temperature was kept at -10°C , and freeze-dried for 24 h at about 1 Pa. We also prepared a sample containing only 0.40 mg/ml enzyme without sugars in the same manner.

Sucrose samples obtained by the method described above were highly crystalline, that is, sucrose in the samples was almost fully crystalline. In order to study the different stabilizing effects of sucrose for samples with different crystallinity, we need sucrose samples which have a low degree of crystallinity. Hence we prepared such samples by the following method: 0.250 ml enzyme-sucrose solution (containing 0.40 mg/ml enzyme and 0.055 mmol/ml sucrose) was frozen in liquid nitrogen and soaked in a -30°C ethanol bath, and then freeze-dried for 48 h at 1 Pa. Then the sample was freeze-dried soaking in a -10°C ethanol bath for 24 h.

After the procedure of freeze-drying, moisture content was 5.4 ± 0.3 wt% for all samples with sugars except samples with amorphous sucrose, and 6.9 wt% for samples with amorphous sucrose. Moisture contents of samples without sugars were 6.3 wt% for ADH, 8.5 wt% for LDH, and 6.9 wt% for MDH, respectively. These samples were stored in a des-

iccator at 65°C . Silica gel was used for desiccation of air in the desiccator. We examined the changes of moisture contents of the samples containing ADH over the storage period. Moisture contents decrease to less than 0.5 wt% in a few days. Moisture contents of the LDH or MDH samples should also decrease over extended storage periods, though we did not examine this. After appropriate storage periods, enzyme activities were measured by the methods described in the next section.

1.3 Assays of enzyme activity

ADH was assayed at 25°C by the following method: ADH preparation was made by rehydrating freeze-dried ADH samples with distilled water to give an ADH concentration of 0.40 mg/ml. The 20 μl of ADH preparation was added to the 3.0 ml reaction mixture containing 0.1 M sodium pyrophosphate buffer (pH 9.0), 76.5 mM semicarbazide, 0.58 M ethanol, 2.1 mM NAD^{+} and 1.0 mM glutathione. After the addition of enzyme preparation, the increase in absorbance at 340 nm was measured immediately by a Shimadzu UV2200 spectrometer. From the results, initial reaction rate was determined, and ADH activity was estimated.

LDH activity was also measured at 25°C . 3.0 ml of the reaction mixture contained 67 mM potassium phosphate buffer (pH 7.4), 0.49 mM pyruvate and 0.1 mM NADH. The reaction was initiated by the addition of 10 μl of LDH preparation prediluted to 1.33 $\mu\text{g}/\text{ml}$ and monitored by measuring the decrease in absorbance at 340 nm.

MDH was assayed at 37°C . 3.0 ml of the reaction mixture contained 97 mM potassium phosphate buffer (pH 7.5), 0.49 mM oxaloacetate and 0.2 mM NADH. The reaction was initiated by the addition of 10 μl of MDH preparation prediluted to 1.33 $\mu\text{g}/\text{ml}$ and monitored by measuring the decrease in absorbance at 340 nm.

1.4 X-ray diffraction

X-ray diffraction measurements were carried out on a Rigaku Ultima⁺ system with graphite monochromator and Cu-K α radiation. The degree of crystallinity was determined by the Ruland method (Ruland, 1961).

2. Results and Discussion

2.1 Activities of enzymes freeze-dried with sugars

In this study, all the samples of freeze-dried enzymes did not lose their activities during freeze-drying. The activities of freeze-dried enzymes during storage are shown in Fig. 1. The values of the ordinates indicate residual activities of the enzymes, that is, relative values to the initial activity before freezing.

From Fig. 1 we can see that activities of freeze-dried enzymes strongly depend on the kind of sugars

Fig. 1 Residual activity of (a) ADH, (b) LDH, and (c) MDH freeze-dried with (+) glucose, (□) sucrose, (○) trehalose, (●) maltose, (■) lactose, (△) raffinose, (▲) maltotriose, and (×) without sugars

added. Trehalose and raffinose were superior in maintaining enzyme activity. Addition of maltose and maltotriose were effective in stabilizing ADH and LDH. Addition of lactose was only effective for LDH. Addition of sucrose was effective for ADH, but inactivated MDH. Glucose inactivated three enzymes strongly. Figure 1 also shows that significant inactivation of enzymes occurred for samples containing glucose, lactose, and maltose in the early stages of storage. Actually, brown coloration was found for these samples. It is known that proteins with reducing sugars undergo the Maillard reaction, which causes brown coloration at high temperature (Kato *et al.*, 1986). Thus, it seems that not only the inactivation caused by deformation of tertiary structure, but also inactivation via the Maillard reaction occurs for samples containing these sugars.

Table 1 Degree of crystallinity of freeze-dried ADH with sugars

Sugar	Degree of crystallinity (%)	
	After freeze-drying	During storage
Glucose	49	—
Sucrose	92	100 (15 day)
Trehalose	0	0 (15 day)
Maltose	0	0 (7 day)
Lactose	51	62 (7 day)
Raffinose	0	0 (15 day)
Maltotriose	0	0 (7 day)

Originally, it was thought to be difficult to select additives for stabilizing dried proteins. Only a few kinds of solute, such as sugars and amino acids, are effective for preserving proteins from inactivation caused by drying and storage at high temperature (Carpenter *et al.*, 1987; Carpenter and Crowe, 1988; Crowe *et al.*, 1990), as well as minimizing protein denaturation in aqueous systems and during freeze-thawing (Gekko and Morikawa, 1981; Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a, b, 1983, 1985). Moreover, according to the present results, only certain sugars can be used for stabilizing proteins. This confirms that selection of a proper sugar is both difficult and important.

2.2 Results of the measurement of X-ray diffraction

The X-ray diffraction patterns of freeze-dried samples containing ADH are shown in Fig. 2. The values of degree of crystallinity determined from the X-ray diffraction patterns are summarized in Table 1. The crystallinities of all freeze-dried samples did not change during storage.

According to these results, and taking account of the results of the above section, it is found that there is a tendency that enzyme activity is preserved more samples of low crystallinity than samples of high crystallinity. Addition of trehalose and raffinose is remarkably effective to preserve the enzyme activity. As described above, the freeze-dried samples for these sugars are fully amorphous, judging from the results of measurement of X-ray powder diffractometry. Samples of maltose and maltotriose are also fully amorphous, and these sugars show stabilizing effects. In contrast, sucrose, lactose and glucose are inferior in stabilizing freeze-dried enzymes, and are found to be completely or partially crystallized in the freeze-dried samples.

2.3 Thermal stability of enzymes with amorphous sucrose

The results described above indicate that there should be a close relationship between the stabilizing

Fig. 2 X-ray powder diffraction patterns of freeze-dried samples containing ADH immediately after freeze-drying

effect of sugars and the amorphous matrix of sugars. However, we can interpret the results in two ways: One way is that the amorphous matrix formed by sugars should stabilize dried proteins. Another way is that

sugars with little crystal-forming nature may stabilize dried proteins effectively, whether the sugars form amorphous matrices or crystallized structures. In order to clarify which interpretation is true, we

Fig. 3 Residual activity of (○ and ●) ADH, (△ and ▲) LDH, and (□ and ■) MDH in the samples of (open key) amorphous sucrose and (closed key) crystallized sucrose

prepared freeze-dried enzymes with amorphous sucrose and evaluated the thermal stability of enzymes during high-temperature storage.

In Fig. 3, the activities during storage of enzymes with amorphous sucrose are shown. Compared to the samples of enzymes with crystal sucrose, the samples of enzymes with amorphous sucrose show remarkably high stability. This result confirms that the stabilization of dried proteins is caused by the amorphous matrix formed by sugars.

As we described in the introduction, the mechanism of stabilizing dried proteins by sugars is as follows. Proteins are surrounded by sugar molecules in freeze-dried samples. At the interface between the protein and sugar molecules, the shape of interface formed by the sugar molecules is a more suitable form in amorphous samples than in high crystallinity samples. Thus protein activity should be preserved more in amorphous matrices of sugars.

Conclusion

We investigate the relation between stabilizing effect of sugars on freeze-dried enzymes and the degree of crystallinity of sugars in freeze-dried samples. It is found that sugars of low crystallinity preserve enzyme activities more than sugars of high crystallinity. Furthermore, sucrose is found to stabilize enzymes effectively when sucrose forms on amorphous matrix,

though it shows little stabilizing effect when it is crystalline. These results indicate that the effect of stabilizing dried protein is caused by the amorphous matrix formed by sugars.

Acknowledgment

The authors acknowledge Dr. S. Fukuda, Mr. M. Ikeda, Mr. H. Yamauchi, Mr. T. Tatefuji, and other members of Hayashibara Biochemical Laboratories, Inc., for their useful discussions and encouragement. This work was supported by a Grant-in-Aid for Scientific Research (A), No. 06403017 and No. 07305034 from the Ministry of Education, Science, Sports and Culture of Japan, and also by a Nestlé Research Grant.

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