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The effects of additives on the stability of freeze-dried β-galactosidase stored at elevated temperature

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Summary

The effects of sugars, polyols and amino acids on the stability of freeze-dried β-galactosidase were investigated. β-Galactosidase from *Aspergillus oryzae* was freeze-dried with these compounds as additives then stored at 70 °C over silica gel. The additives studied demonstrated three different types of effect. Some prevented inactivation of the enzyme, some accelerated inactivation and others had no effect. X-ray diffraction showed that additives that prevented inactivation were amorphous in freeze-dried cakes, whereas crystal-forming additives did not exert such effect. Crystallinity of additives would thus appear to be an important factor which determines the stabilizing effect. Possible mechanisms of β-galactosidase inactivation are also discussed.

Introduction

Due to recent bio-technological advances, protein preparations for pharmaceutical purposes are being used increasingly. Many of these preparations are in the form of a 'freeze-dried cake' and directed to be dissolved before use. Some proteins are unstable and lose their activity during freeze-drying and storage (Manning et al., 1989), so it is important that proteins are stabilized in order to develop effective preparations. Proteins can be stabilized by two methods. One is by mutagenesis or chemical modification of the protein (reviewed by Alber, 1989) and the other is to change the environment around it. Sugars and albumin are added to these preparations as 'stabilizers', but the mechanism of their protective effect has yet to be elucidated (Townsend and DeLuca, 1988).

In this report the stabilization of proteins by carbohydrates and amino acids as additives is described. β-Galactosidase was selected as a model protein for evaluation of the effect of these additives on protein stability during freeze-drying and storage at high temperature. The correlation between enzyme inactivation and the crystallinity of additives, and possible inactivation mechanisms are discussed.

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Materials and Methods

Materials

Lyophilized powders of β-galactosidase from *Aspergillus oryzae* and *Escherichia coli* were purchased from Toyobo Co., Ltd (Osaka) and was used without further purification. Bovine serum albumin (BSA) fraction V and phosphofructokinase (from rabbit muscle, type III) were obtained from Sigma (St. Louis, U.S.A.), amino acid arylamidase (from hog kidney) and glucose-6-phosphate dehydrogenase (from yeast) were from Boehringer Mannheim Yamanouchi Co. (Tokyo), L-arginine monohydrochloride and L-histidine monohydrochloride monohydrate were from Nippon Rikagaku Yakuhin Co. (Tokyo), and silica gel for desiccation was from Kokusan Chemical Co. (Tokyo). All other chemicals were of reagent grade and obtained from Wako Pure Chemical Industries Ltd (Osaka). The polypropylene tubes used for freeze-drying were purchased from the Iuchi Seieido Co. (Osaka).

Methods

Freeze-drying, storage and reconstitution of β-galactosidase

β-Galactosidase and additives were dissolved separately in 50 mM phosphate buffer (pH 7.4), the ionic strength of which was adjusted to 0.15 by the addition of NaCl. β-Galactosidase concentration was measured by the method of Lowry et al. (1951) using BSA as protein standard.

An aliquot of the protein solution was added to the additive or additive-free solution to give a final β-galactosidase concentration of 1 mg/ml, a final concentration of BSA of 10 mg/ml and 100 mg/ml for the other additives. The pH of each solution was measured.

1 ml of the test solution was transferred to a polypropylene tube (flat bottomed, bottom area 3.46 cm^2), frozen in liquid nitrogen for more than 1 min and then freeze-dried overnight at 1 Pa, using a Freezevac C-1 type lyophilizer (Tozai Tsusho Co., Ltd, Tokyo). At the beginning of freeze-drying, shelf temperature was controlled to under $-30\,^{\circ}C$, and freeze-drying was continued without further temperature control. The freeze-dried samples were dried further in vacuo over P_2O_5 for 24 h at room temperature, then stored at $70\,^{\circ}C$ over dried silica gel around polypropylene tubes. At appropriate intervals samples were removed for testing, reconstituted with 1 ml of distilled water and diluted with 50 mM phosphate buffer (pH 7.4). The enzyme concentration in this solution was calculated from the amount present prior to freeze-drying.

Assay for β-galactosidase activity

β-Galactosidase was diluted to 2 μg/ml and its activity was measured at $30\,^{\circ}C$ as described previously (Izutsu et al. ,1990). The activity remaining after freeze-drying and storage was expressed as a percentage of the activity determined before freeze-drying.

Absorbance of protein solutions

The absorbance of 0.2 mg/ml concentrations of protein solutions was measured in 1 cm light path quartz cuvettes using a Shimadzu UV-260 spectrophotometer (Kyoto).

X-ray powder diffraction

X-ray powder diffraction measurements were carried out on a Rigaku RAD-2C system (Tokyo) with Ni-filtered Cu-K radiation (30 kV, 10 mA) at a scanning rate of $2\,^{\circ}$/min.

High-performance size-exclusion chromatography (HPSEC)

HPSEC separation of proteins was carried out at $30\,^{\circ}C$ on a Tosoh G3000SW-XL column (300 × 7.5 mm i.d., Tokyo). The chromatographic system used for analyses consisted of a Tosoh CCPD pump, a Tosoh RE-8000 column oven and a Hitachi L-4000 variable-wavelength UV detector (Tokyo). A Shimadzu C-R3A integrator was used for quantitative analysis. Phosphate buffer (0.2 M, pH 6.2) was used as the mobile phase at a flow rate of 1 ml/min. Chromatographic peaks were measured at 280 nm (absorbance of aromatic amino acids). Freeze-dried additive-free β-galactosidase was dissolved to 0.2 mg/ml and injected through a pre-column filter (pore size: 0.45 μm). Solutions of BSA, glucose-6-phosphate dehydro-

genase, amino-acid arylamidase, phosphofructokinase, and β-galactosidase from *E. coli* (0.2 mg/ml) were injected as molecular standards.

SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition was performed on PhastGel® homogeneous 12.5 with PhastSystem® (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) at 250 V for 0.8 h at 15°C. β-Galactosidase samples were prepared and stained according to the method of Townsend and DeLuca (1988).

Differential scanning calorimetry (DSC)

Calorimetric measurements were performed using a Shimadzu heat-flux type differential scanning calorimeter (DSC-41M) at a scan rate of 5°C/min. Freeze-dried additive-free β-galactosidase was dissolved with distilled water to produce a protein concentration of 10 mg/ml. Aliquots (25 μl) of solutions were packed in pre-autoclaved aluminum cells. Calorimetric data were analyzed by microcomputer software prepared by Shimadzu.

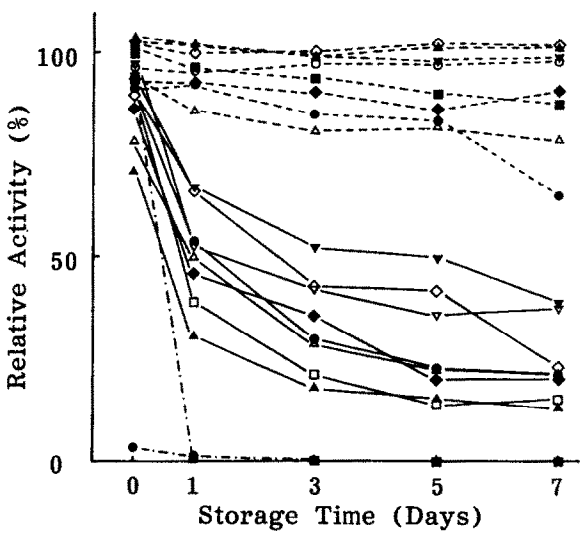

Fig. 1. Activity of β-galactosidase freeze-dried with additives during storage at 70°C over silica gel. β-Galactosidase was freeze-dried with (◇) L-arginine monohydrochloride, (▲) sucrose, (▼) lactose monohydrate, (○) trehalose dihydrate, (◆) maltose monohydrate, (■) raffinose pentahydrate, (△) D-cellobiose, (●) L-histidine monohydrochloride monohydrate for (------); (▼) *myo*-inositol, (▽) L-serine, (●) without additives, (◇) BSA, (△) glycine, (◆) L-threonine, (□) L-proline, (▲) mannitol for (———); and (▲) D-fructose, (△) D-glucose, (◇) D-xylose, (●) L-arginine for (–·–·–). Each point is a mean of two experiments and represents percentage of activity before freeze-drying.

Results

Activity of β-galactosidase

A. oryzae β-galactosidase is a monomeric protein of 105 kDa (Tanaka et al., 1975). When freeze-dried without additives, it retained about 85% of its initial activity (Fig. 1 and Table 1). During storage at 70°C, over silica gel, the activity of β-galactosidase freeze-dried without additives gradually declined to about 50 and 20% of its initial activity after 1 and 7 days, respectively.

β-Galactosidase freeze-dried with disaccharides or trisaccharide, such as trehalose, sucrose, maltose, lactose, cellobiose and raffinose, did not lose activity during freeze-drying and storage, whereas with monosaccharides, such as glucose, fructose and xylose, the enzyme maintained its activity during freeze-drying, but lost it immediately upon storage at 70°C. It is known that at high temperatures reducing sugars undergo the Maillard reaction with proteins, resulting in a brown coloration (Kato et al., 1986). The increase

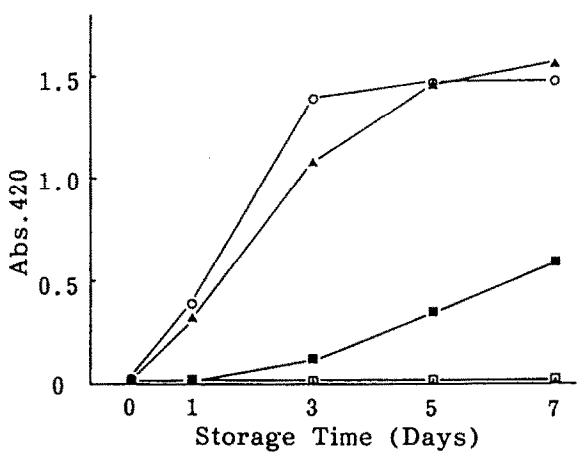

Fig. 2. Brown color development of β-galactosidase freeze-dried with (○) D-xylose, (▲) D-fructose, (■) D-glucose, (□) without additives during storage at 70°C. Each point is a mean of two experiments.

TABLE 1

Relative activity (%) of β-galactosidase freeze-dried with additives during storage at 70°C over silica gel

Additive	Storage time (days)				
	0	1	3	5	7
Trehalose	96.2	95.1	97.3	96.5	97.7
Sucrose	104.1	102.1	98.5	101.1	101.7
Maltose	99	96.6	93.8	89.1	88
Lactose	101.4	101.6	98.1	97.3	97.8
D-Cellobiose	94.3	85.7	80.3	82.3	79
Raffinose	93.9	92.1	90.3	85.9	90.8
D-Fructose	102.2	0.4	0	0.1	0.3
D-Glucose	103.3	0.8	0	0	0.1
D-Xylose	90.3	0	0.3	0.5	0.9
Mannitol	71.37	31.41	17.7	15.4	13.5
myo-Inositol	97.9	52.8	41.6	35.8	37.4
L-Arginine · HCl	102.3	99.6	100.7	104	103.9
L-Histidine · HCl	91.6	92.4	84.22	83.8	64.62
Glycine	77.4	49.9	28.3	23	21.6
L-Proline	93.5	38.6	20.8	13.8	15.1
L-Threonine	86.8	45.8	35.6	19.5	19.7
L-Serine	94.2	67.6	52.4	49.4	38.3
L-Arginine	2.7	1.3	0	0.1	0
BSA	89.2	66.2	43.8	42.8	24
No additive	86	53.4	29.1	22.5	21.8

Relative activities are represented as percentage of activity before freeze-drying and each value is a mean of two experiments.

in absorbance at 420 nm observed for freeze-dried cake with glucose, fructose or xylose (Fig. 2) indicates that inactivation of β-galactosidase, in the presence of these reducing monosaccharides, was caused mainly by the Maillard reaction. The absorbance of cakes with reducing disaccharides, such as maltose, lactose and cellobiose, did not increase during storage. Samples freeze-dried with arginine hydrochloride or histidine hydrochloride showed little loss of activity.

When freeze-dried with *myo*-inositol, serine, glycine, proline, threonine, mannitol or BSA, the enzyme lost activity at a rate similar to or slightly slower than the samples without additives.

β-Galactosidase lost activity during freeze-drying with arginine. This may have been due to the increased pH of the solution containing arginine (Table 2), causing some physical and/or chemical degradation.

X-ray powder diffraction

The X-ray diffraction scans of freeze-dried cakes are shown in Fig. 3, and the data for the degree of crystallinity are summarized in Table 2. Freeze-dried cakes of β-galactosidase with sugars such as trehalose and sucrose were amorphous. Samples with arginine, arginine hydrochloride or histidine hydrochloride were also amorphous. These samples remained amorphous during storage. In contrast, the scans of samples freeze-dried with serine, glycine, proline or mannitol showed peaks identical to these of the crystalline forms of these additives, although their smaller peak heights suggest that some additives were not fully crystallized in the freeze-dried cake.

X-ray scans of freeze-dried samples containing *myo*-inositol showed that it was amorphous initially but crystallized during storage. Samples with threonine also crystallized during storage.

TABLE 2

Effect of crystallinity on β-galactosidase inactivation

Additive	pH	Effect on inactivation	Crystallinity		
			a	b	c
Trehalose	7.3	prevent	−	−	+ +
Sucrose	7.3	prevent	−	−	+ +
Maltose	7.4	prevent	−	−	+ +
Lactose	7.4	prevent	−	−	+ +
D-Cellobiose	7.4	prevent	−	−	+ +
Raffinose	7.4	prevent	−	−	+ +
D-Fructose	7.3	accelerate	−	−	+ +
D-Glucose	7.3	accelerate	−	−	+ +
D-Xylose	7.3	accelerate	−	−	+ +
Mannitol	7.3	no effect	+	+	+ +
myo-Inositol	7.3	no effect	−	+ +	+ +
L-Arginine · HCl	6.7	prevent	−	−	+ +
L-Histidine · HCl	5.0	prevent	−	−	+ +
Glycine	7.0	no effect	+	+	+ +
L-Proline	7.3	no effect	+	+	+ +
L-Threonine	6.9	no effect	−	+	+ +
L-Serine	6.9	no effect	+	+	+ +
L-Arginine	10.8	* 1	−	−	+ +
BSA	7.4	no effect	n.d.	n.d.	n.d.

* 1, inactivation during freeze-drying; −, amorphous; +, partially crystallized; + +, crystallized.
(1) pH of solution before freeze-drying, (2) effect on inactivation during storage and (3) crystallinity of β-galactosidase freeze-dried with additives. Crystallinity columns a–c correspond to panels a–c in Fig. 3.

HPSEC and SDS-PAGE

We tried to ascertain possible mechanisms of inactivation of β-galactosidase stored at 70°C. Fig. 4 shows the high-performance size-exclusion chromatogram obtained for the stored sample. A peak corresponding to intact β-galactosidase was observed at about 7.2 min. Peaks corresponding to greater molecular size grew with storage time. The peak height of the original β-galactosidase peak decreased in parallel with the decline in enzyme activity (Fig. 5). The retention times of proteins used as molecular weight standards are listed in Table 3. The apparent sizes of degradation products were larger than that of native β-galactosidase from *E. coli*, which was reported as 540 kDa (Craven et al., 1965).

Results of SDS-PAGE indicate that β-galactosidase that had been freeze-dried and stored at 70 °C for 1 and 7 days had only one band which corresponds to enzyme the monomer, as did non-freeze-dried β-galactosidase (data not shown). The band faded a little and no other bands appeared in samples stored at 7 days.

TABLE 3

Retention time of proteins

Protein	Molecular weight	Retention time (min)
Serum albumin (bovine)	67000	7.8
Glucose-6-phosphate dehydrogenase (yeast)	104000 [a]	7.0
β-Galactosidase (*A. oryzae*)	105000	7.2
Amino acid arylamidase (hog)	280000 [b]	6.4
Phosphofructokinase (rabbit)	360000 [c]	6.3
β-Galactosidase (*E. coli*)	540000 [d]	6.0

[a] Engel et al. (1969).
[b] Kim et al. (1976).
[c] Aaronson et al. (1972).
[d] Carven et al. (1965).

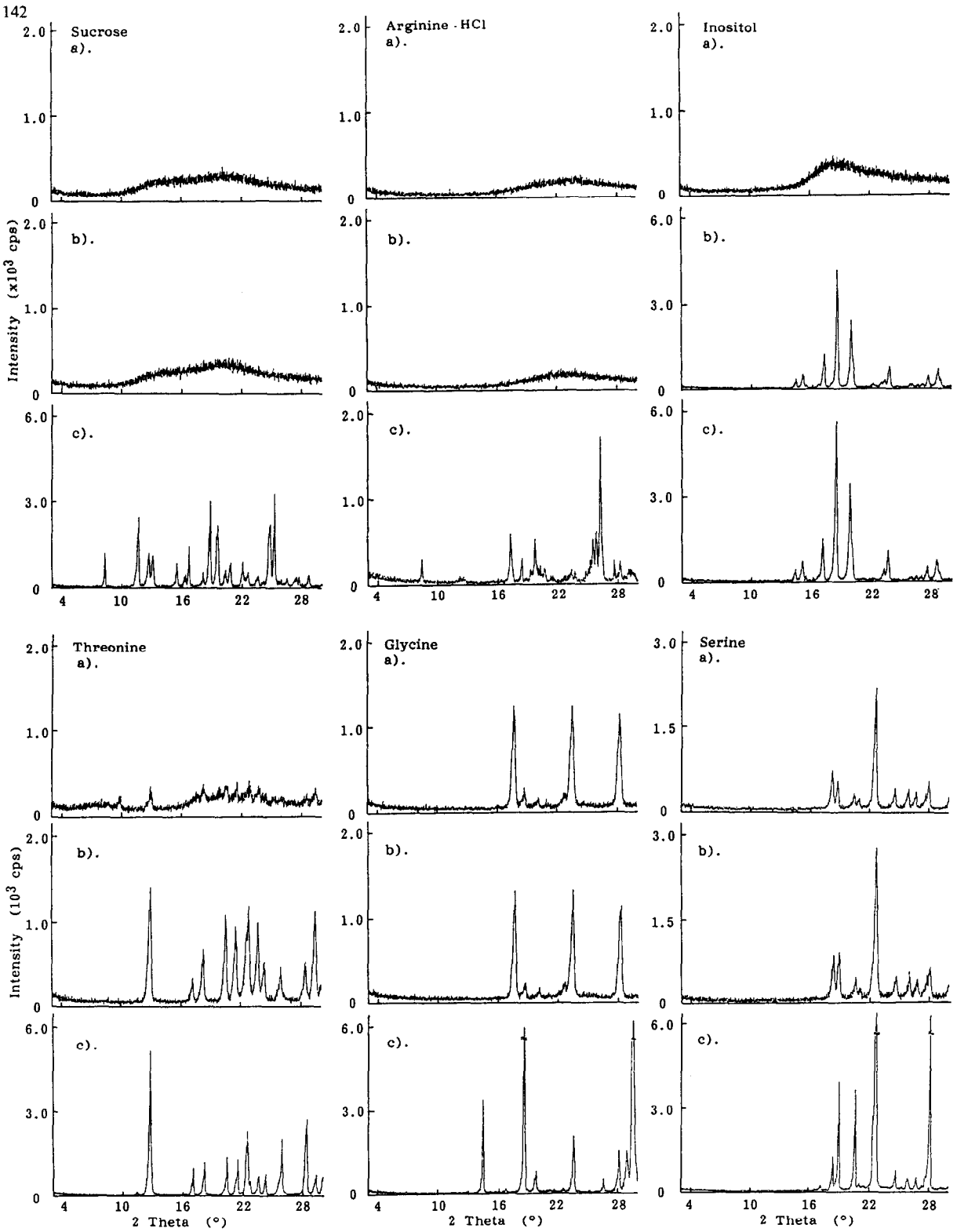

Fig. 3. Powder X-ray diffraction pattern of (a) freeze-dried cake of β-galactosidase with sucrose, L-arginine monohydrochloride, *myo*-inositol, L-threonine, glycine or L-serine. (b) Freeze-dried cake stored for 7 days at 70 °C. (c) Crystalline additives that were not freeze-dried.

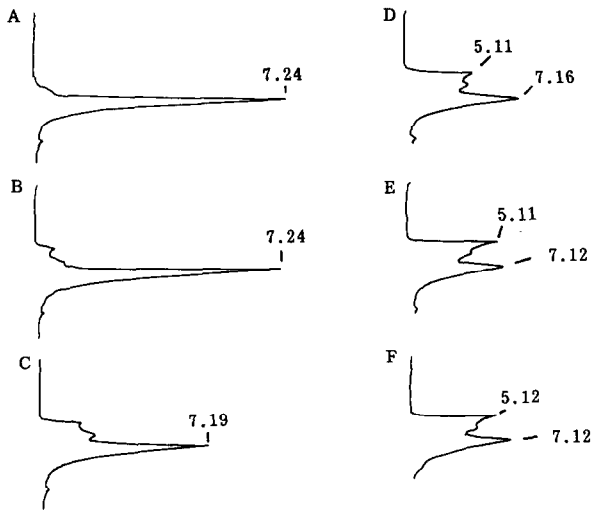

Fig. 4. Changes in high-performance size-exclusion chromatograms. (a) β-Galactosidase in phosphate buffer, (b) solution of freeze-dried cake without additives, chromatograms (c–f) represent solution of freeze-dried cakes without additives stored at 70 °C for 1, 3, 5 and 7 days, respectively. Numbers beside peaks represent retention time (min).

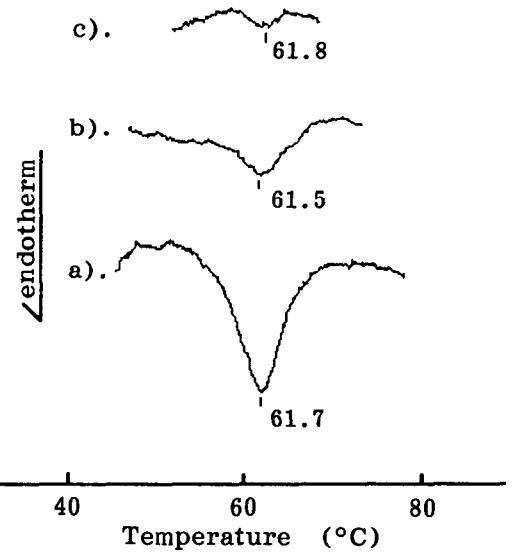

Fig. 6. DSC curves of β-galactosidase. (a) Solution of freeze-dried cake, (b) solution of freeze-dried cake stored at 70 °C for 3 days, (c) solution of freeze-dried cake stored at 70 °C for 7 days.

DSC

DSC curves for thermal denaturation of β-galactosidase are shown in Fig. 6. Peak temperatures of denaturation curves were 61–62 °C and remained constant for samples stored at high temperatures. The observed denaturation enthalpy, which represents the amount of folded proteins in the sample, decreased as storage time increased. The result suggests that unfolding of protein (denaturation) occurred during storage. Denaturation peaks at other temperatures were not observed. The change in the observed denaturation enthalpy was small in samples freeze-dried with sucrose (data not shown).

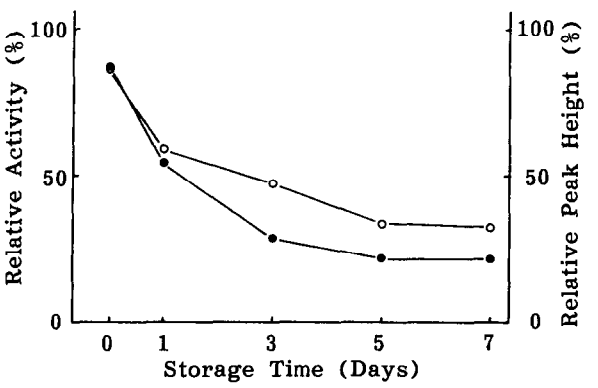

Fig. 5. Activity (●) and peak height (○) of the native enzyme. β-Galactosidase was freeze-dried without additives and stored at 70 °C. Each point is a mean of two experiments and expressed as a percentage of data before freeze-drying.

Discussion

Inactivation of enzymes may occur in solution, in frozen solution, during freeze-drying and during storage after freeze-drying. It has been reported that many solutes, including sugars, polyols and amino acids, stabilize proteins in solution and increase denaturation temperatures (Gerlsma, 1968; Arakawa and Timasheff, 1983). These compounds also protect proteins from cold denaturation and are described as 'cryoprotectants'. Phosphofructokinase (PFK) has been shown to be protected against denaturation during freeze-thawing by these compounds (Carpenter et al., 1986, 1988a), but was protected only by sugars during freeze-drying (Carpenter et al., 1987). It has been

emphasized that freezing and dehydration are different stress vectors (Crowe et al., 1990).

Townsend and DeLuca (1988) reported that sucrose, Ficoll® and polyvinylpyrrolidone (PVP) protected ribonuclease A in freeze-dried cake from inactivation during storage at 45 °C. Such 'lyoprotectants' protect proteins not only from freezing stress but also from the stresses of drying and storage at high temperature.

We investigated the effects of additives on inactivation of freeze-dried β-galactosidase stored at elevated temperature. It was found that, of the compounds studied, disaccharides and trisaccharide prevented inactivation of β-galactosidase during storage. In contrast, some amino acids and polyols did not exert a stabilizing effect. These results are consistent with those reported for the protective effects of these additives against freeze-drying denaturation of PFK. Histidine hydrochloride and arginine hydrochloride were also found to prevent inactivation of β-galactosidase.

With respect to the protective mechanism of additives against protein denaturation in solution, sugars and amino acids are considered to increase preferential interaction of proteins with water (Arakawa and Timasheff, 1982, 1983). In this study, only some sugars, arginine hydrochloride and histidine hydrochloride demonstrated a protective effect in freeze-dried cakes. In this type of formulation, the difference in protective effect cannot be explained by the protective mechanism, which has been proposed for these additives in solution.

The protective effect in freeze-dried cakes seems to correlate with the crystallinity of the additives. Additives which demonstrated a protective effect maintained their amorphous state during freeze-drying and storage, whereas freeze-dried additives with a crystalline structure had no or less marked protective effects.

It is known that water molecules surrounding proteins play an important role in protein stability (Sanches et al., 1986; Hageman, 1988). Two explanations for the protective effects of additives have been proposed (Carpenter and Crowe, 1988b). One is that additives retain water molecules indispensible to the protein and resist excess drying. The other is that additives interact directly with proteins and replace water by hydrogen bonding to the protein's polar groups.

How then does the crystallinity of additives relate to their stabilizing effect? First, the degree of crystallinity may determine the apparent water content in the cake. Amorphous cakes of many compounds are known to contain a larger amount of water than crystalline cakes (Ahmed and Lelievre, 1978). The difference in water content may result in a difference in the rate of protein inactivation caused either by dehydration or by some chemical reactions. Beside the amount of water around protein, the conformation of water is an important factor for protein stability (Franks and Eagland, 1975). Crystallization of additives may affect not only the amount of water around protein but also the ordering of water molecules.

Second, crystallization of additives may change the interaction between proteins and additives. Some direct interaction between sugars and proteins in freeze-dried cake has been detected by FT-IR PAS (Carpenter and Crowe, 1989). This interaction appears to be favorable for protein stabilization. However, crystallization of additives may cause rearrangement of molecules resulting in a loss of the favorable interaction and a significant change in the stabilizing effect. Crystallization of additives during freeze-drying (Franks, 1982) must be prevented in order to produce stable freeze-dried preparation.

Solid-state decomposition of proteins is known to involve several mechanisms (Hageman, 1988). Chromatograms of HPSEC obtained in this study suggest that the activity loss of stored β-galactosidase was accompanied by an increase in apparent protein size. Formation of aggregated species (Watson and Kenny, 1988) and denaturation (Corbett, 1984) of proteins provide the peaks of larger apparent size in HPSEC chromatograms. The total concentration of protein was almost constant and no hydrolysis products were formed. The apparent size increase may therefore be due to covalent bond formation between protein molecules, aggregation of molecules without covalent bonding and/or protein denaturation to the unfolded state. Townsend and DeLuca (1988) reported, based on their SDS-PAGE results, that the specific activity loss of freeze-dried ribonuclease A

was ascribed to the formation of covalently bonded aggregates of the enzyme, and that molecular oxygen was involved in the process (Townsend et al., 1990). In the present study with β-galactosidase freeze-dried and stored at 70 °C, the band of the monomeric enzyme faded a little, but we could not find other bands that correspond to covalently bonded enzyme.

Although no change in the total concentration of soluble protein was observed by HPSEC, the observed denaturation enthalpy was found to decrease during storage. This may be ascribed to loss of folded protein (Izutsu, et al., 1990) caused by the unfolding of protein (denaturation) during storage.

Conclusion

Freeze-dried β-galactosidase lost activity during high-temperature storage and some sugars protected against it. Arginine hydrochloride and histidine hydrochloride also had a stabilizing effect. Additives with a protective effect maintained their amorphous state during freeze-drying and storage, whereas crystal-forming additives demonstrated less marked protective effects. Therefore, crystal formation of additive may be an important factor affecting loss of enzyme activity. Data from HPSEC chromatograms, SDS-PAGE and DSC curves suggested that inactivation mechanism may involve bond formation between enzymes and/or denaturation of enzymes.

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