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# Stabilization of β-galactosidase by amphiphilic additives during freeze-drying

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## Summary

The effects of amphiphilic additives on inactivation of β-galactosidase during freeze-drying were studied in comparison with those of sugars and amino acids. The activity loss was greater when the enzyme was freeze-dried from solutions of lower enzyme concentration without additives. Well-known cryoprotectants such as sugars and amino acids provided concentration-dependent preservation of enzyme activity. Hydroxypropyl-β-cyclodextrin (HP-β-CD), 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD), 3-[ (3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and sucrose fatty acid monoester stabilized the enzyme at much lower concentrations than-sugars and amino acids. Polyethylene glycol 400 (PEG 400), polyoxyethylene 9 lauryl ether (polydocanol) and sodium dodecyl sulfate (SDS), however, were ineffective or rather induced inactivation. High-performance size-exclusion chromatography indicated the formation of soluble aggregates during freeze-drying without additives, which was inhibited by the sugar ester. SDS-PAGE indicated that the aggregates were formed by noncovalent bonding.

## Introduction

Breakthroughs in biotechnology are providing an increasing number of new protein-based parenteral drugs. Proteins are relatively unstable and follow many degradation pathways (Manning et al., 1989). Some proteins are formulated as freeze-dried products to avoid their inactivation during storage, since they readily lose their activity in solution (Geigert, 1989). In spite of their increased shelf-life after freeze-drying, some proteins are inactivated during the process. The apparent loss of activity is often accompanied by the formation of aggregates and precipitates (Pikal et al., 1991). Protein molecules are subject to many types of physico-chemical stress during freeze-drying, and this may cause protein denaturation (Yasui and Hashimoto, 1966; Nai-Teng Yu et al., 1972), leading to aggregation and precipitation.

The extent of inactivation during freeze-drying is affected by various extrinsic factors (Hanafusa, 1977) as well as the inherent characteristics of the proteins. These factors include pH, buffer components (Seguro et al., 1990) and the concentrations of protein and other ingredients. The freez-

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ing speed and drying temperature also affect the remaining activity (Hanafusa, 1977).

'Cryoprotectants' such as sugars (Yasui and Hashimoto, 1966; Carpenter et al., 1987), polyols (Hellman, 1983), and amino acids are often added to protein solutions to avoid inactivation during freeze-drying. Most of them are also known to protect proteins from heat denaturation. The stabilizing effect has been explained by the preferential exclusion hypothesis (Arakawa and Timasheff, 1981) and the amorphous-keeping hypothesis (Eckhardt et al., 1991; Pikal et al., 1991).

Surface-active agents are also used to stabilize proteins in solution, protecting the proteins from denaturation at liquid-air surfaces (Levine et al., 1991). Although some protein products contain surface-active agents (Hershenson et al., 1989), the mechanism of their effectiveness against inactivation during freeze-drying is still obscure.

In this study we investigated the inactivation of a model protein, β-galactosidase from *Aspergillus oryzae* (Tanaka et al., 1975), during freeze-drying, focussing on the effects of amphiphilic additives including surfactants. Our previous study had shown that the enzyme was partially inactivated during freeze-drying without protectants (Izutsu et al., 1991). In the present study, some amphiphilic additives were found to preserve the activity of the enzyme at much lower concentrations than sugars and amino acids.

## Materials and Methods

*Materials*

β-Galactosidase from *A. oryzae* was purchased from Toyobo, Co. (Osaka, Japan). Hydroxypropyl-β-cyclodextrin (HP-β-CD, approximate degree of substitution (DS): 6.3) was from Aldrich Chemical Co. (Milwaukee, WI). Polyoxyethylene 9 lauryl ether (polydocanol), polyethylene glycol 3350 (PEG 3350) and blue dextran (average molecular weight: 2000000) were from Sigma Chemical Co. (St. Louis, MO). Sucrose fatty acid monoester (DK ester SS®) was obtained from Dai-ichi Kogyo Seiyaku, Co. (Kyoto, Japan). The sugar ester contained more than 95% sucrose fatty acid monoester, and the fatty acid portion was palmitate and stearate (as stated on the product data sheet). 2,6-Di-*O*-methyl-β-cyclodextrin (DM-β-CD), PEG 400, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium dodecyl sulfate (SDS) and all other chemicals were from Wako Pure Chemical Ind., Ltd (Osaka).

*Freeze-drying of β-galactosidase*

β-Galactosidase was dissolved in 50 mM sodium phosphate buffer (pH 7.4) at a concentration of approx. 10 mg/ml and dialyzed at 4°C in seamless cellulose tubing (Union Carbide, Co.) in the buffer for 20 h. The protein solution was filtered through a 0.45 μm Millipore filter (SJHV 013 NS), and the concentration of the protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the protein standard. An aliquot of the enzyme solution was transferred to a polypropylene tube (flat bottom, bottom size 3.46 $cm^2$), and the buffer with or without additives was added to provide 1 ml of 20 μg/ml solution (unless otherwise stated).

The solutions were frozen by immersing the tube in liquid nitrogen for more than 1 min, and transferred to a Freezevac-1CFS lyophilizer (Tozai Tsusho, Co.). The samples were dried at −40°C for 1 h, −35°C for 12 h and 35 C for 4 h (shelf temperature). All samples were freeze-dried without apparent collapse, except those to which 100 mM proline was added.

*Freeze-thawing of β-galactosidase*

Solutions of β-galactosidase with or without additives were prepared as described above, and then frozen in liquid nitrogen. The samples were stored in a freezer (−20°C) for 20 h and thawed at room temperature.

*Reconstitution and assay of β-galactosidase activity*

The freeze-dried products were reconstituted with 1 ml of distilled water (unless otherwise stated) and diluted to 2 μg/ml with the buffer used for freeze-drying. The concentration of protein was calculated from that prior to freeze-drying. In some experiments for studying the effect of additives on dissolution after freeze-drying, the

enzyme was freeze-dried without additives and reconstituted with a solution of additives. The activity of β-galactosidase was determined at 30°C using 2-nitrophenyl-β-D-galactopyranoside as the substrate as described previously (Izutsu et al., 1990). The activity was measured within 30 min after reconstitution. The remaining relative activities were expressed as percentages of those before the freeze-drying.

*High-performance size-exclusion chromatography (HPSEC)*

HPSEC separation of proteins was carried out at 30°C on a Tosoh G3000SW-XL column (300 × 7.5 mm i.d.). The chromatographic system used consisted of a Hitachi 655 pump, a Tosoh RE-8000 column oven, a Hitachi L-4000 UV detector, and a Shimadzu C-R3A integrator. Phosphate buffer (0.2 M, pH 6.2) was used as the mobile phase at a flow rate of 1 ml/min. The protein was detected at 280 nm. β-Galactosidase was freeze-dried at a concentration of 0.1 mg/ml as described above. Freeze-dried samples were reconstituted with the same buffer as that used for the mobile phase to obtain a 0.1 mg/ml solution. The solution was then filtered (pore size, 0.45 μm) and injected through a 20 μl loop.

*Electrophoresis*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Phast System (Pharmacia LKB, Uppsala, Sweden) using 4–15% gradient acrylamide gels. The freeze-dried enzymes (freeze-dried at 0.1 mg/ml) and other proteins were solubilized in a protein solvent system (10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, pH 8.0) to obtain a 0.2 mg/ml solution for molecular standards and a 0.4 mg/ml solution of β-galactosidase, respectively. Protein solutions were heated at 100°C for 5 min with 5% 2-mercaptoethanol. To examine the existence of intermolecular disulfide bonding, some samples were prepared without reduction by 2-mercaptoethanol. After cooling, bromophenol blue was added to the solution (0.01%). Protein samples were then subjected to electrophoresis, and stained with Coomassie dye. The molecular weight standards were: rabbit muscle myosin (220000), *E. coli* β-galactosidase (subunit) (116000) and bovine serum albumin (BSA) (66200).

## Results

*Effect of enzyme concentration on residual activity after freeze-thawing and freeze-drying*

The effect of protein concentration on the residual activity after freeze-thawing and freeze-drying is shown in Fig. 1. β-Galactosidase freeze-dried at a concentration of 1 mg/ml maintained about 70% of its activity. The residual activity after freeze-drying decreased with decreasing concentrations of the protein. Although activity loss was observed during freeze-thawing at low concentration, it was smaller than that during freeze-drying.

*Effects of additives against inactivation during freeze-drying*

Fig. 2 shows the effects of additives against the inactivation of β-galactosidase freeze-dried at an enzyme concentration of 20 μg/ml. In the absence of additives, the activity of β-galactosidase was reduced to about 30% of its original value. In order to examine the effect of additives on the assay of the enzyme, activity was measured in the presence of these additives. The changes in ap-

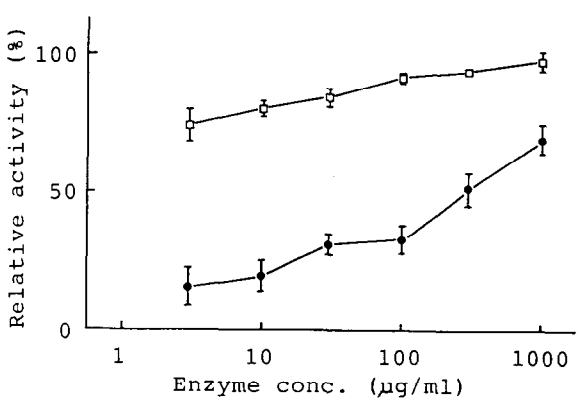


Fig. 1. Effects of enzyme concentration on residual activity after freeze-thawing (□) and freeze-drying (●). Relative activities are presented as percentages of the activity before freeze-drying, and values are given with their standard deviations ($n = 3$).

parent activity were less than 10% of the control up to the concentration used for freeze-drying.

Fig. 2A shows the effects of sugars (trehalose, glucose) and amino acids (L-proline, L-sodium glutamate). These sugars and amino acids are often used as cryoprotectants to prevent protein inactivation during freeze-drying. In this study, they provided dose-dependent activity preservation. Trehalose, glucose and sodium glutamate were effective at a concentration of more than 10 mM. Proline prevented inactivation at a lower concentration.

Fig. 2B shows the effects of cyclodextrin derivatives. Addition of HP-$\beta$-CD or DM-$\beta$-CD preserved the enzyme activity at 0.1 mM. On the other hand, $\alpha$-CD, $\beta$-CD and $\gamma$-CD had little effect on remaining activity. HP-$\beta$-CD is reported to protect recombinant tumor necrosis factor (rTNF; Hora et al., 1992) and interleukin-2 (Brewster et al., 1991) from inactivation during freeze-drying.

Fig. 2C and D shows the effects of some surfactants and PEGs. Sucrose fatty acid monoester and CHAPS stabilized the enzyme at 0.1–1 mM. Although PEG 3350 served as a weak protectant, PEG 400 produced obvious inactivation. Other surfactants such as SDS and polidocanol had little effect, or rather inactivated the enzyme. SDS and CHAPS are anionic and zwitterionic surfactants, respectively.

*Effect of additives on reconstitution of the freeze-dried enzyme*

Table 1 lists the effects of some additives upon reconstitution of the freeze-dried $\beta$-galactosidase. As shown above, the enzyme freeze-dried at 20 $\mu$g/ml and reconstituted with water possesed about 30% of its original activity. Reconstitution

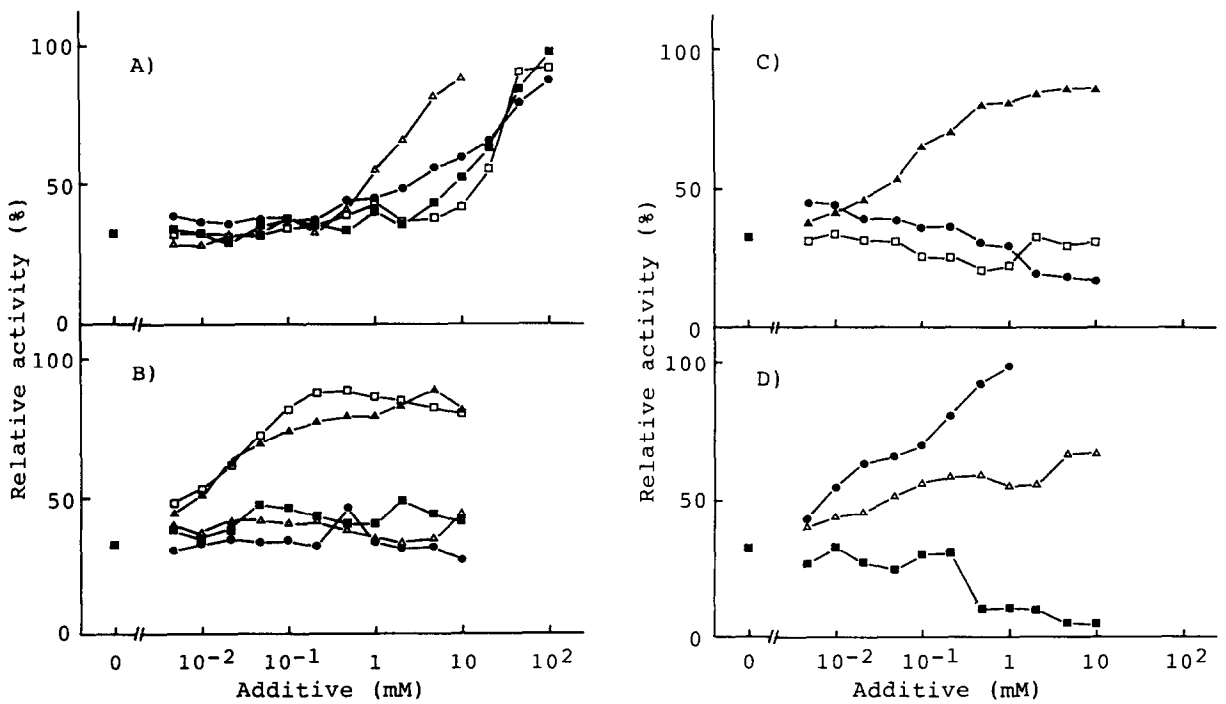


Fig. 2. The residual activity of $\beta$-galactosidase freeze-dried with additives. $\beta$-Galactosidase was freeze-dried with (A): (●) sodium glutamate, (■) glucose, (□) trehalose, (△) proline; (B): (□) DM-$\beta$-CD, (▲) HP-$\beta$-CD, (△) $\alpha$-CD, (●) $\beta$-CD, (■) $\gamma$-CD; (C): (●) polidocanol, (□) SDS, (▲) CHAPS; (D) (●) sucrose fatty acid monoester, (■) PEG 400, (△) PEG 3350. Relative activities are presented as percentages of the activity before freeze-drying, and each value is a mean of three determinations.

TABLE 1

*Remaining activity of freeze-dried β-galactosidase reconstituted with water or surfactant solution*

| | Concentration (mM) | Remaining activity (%) |
|---|---|---|
| Control | 0 | 28.7±6.5 |
| DK ester SS | 1 | 32.0±3.7 |
| CHAPS | 1 | 41.4±9.9 |
| | 10 | 29.6±8.0 |
| DM-β-CD | 1 | 42.8±7.1 |
| | 10 | 43.9±8.6 |
| HP-β-CD | 1 | 40.7±8.1 |
| | 10 | 43.5±7.2 |

Values are given with their standard deviations ($n = 3$).

of the freeze-dried enzyme with DM-β-CD, HP-β-CD, CHAPS and the sugar ester solutions increased the residual activity up to about 45%, whereas their effects were much smaller than when added prior to freeze-drying.

*HPSEC of freeze-dried β-galactosidase*

The enzyme solution and the reconstituted solution of freeze-dried enzyme were studied by HPSEC to elucidate the mechanism of enzyme inactivation. A typical chromatogram of HPSEC is shown in Fig. 3. Before freeze-drying, β-galactosidase eluted at around 7.8 min. The height of the original peak decreased and another peak which corresponded to a larger particle size appeared for the sample freeze-dried without additives. The retention time of the second peak was shorter than that of *E. coli* β-galactosidase (mol. wt. 465 000 (Fowler and Zabin, 1977); 6.4 min) and blue dextran (mol. wt. 2 000 000; 6.1 min). The peak height of the original enzyme was increased by addition of the sugar ester before freeze-drying. The increase in peak height was almost proportional to the residual activity.

*Electrophoresis*

Fig. 4 shows the gel pattern of SDS-PAGE. A dense band observed for β-galactosidase solution before freeze-drying was consistent with the molecular weight (105 000) reported in the literature (Tanaka et al., 1975). Two other weak bands were observed in the lower-molecular-weight region, which may have been due to impurities or degradation products of the protein. The reconstituted solution after freeze-drying showed a band identical to that before freeze-drying. No

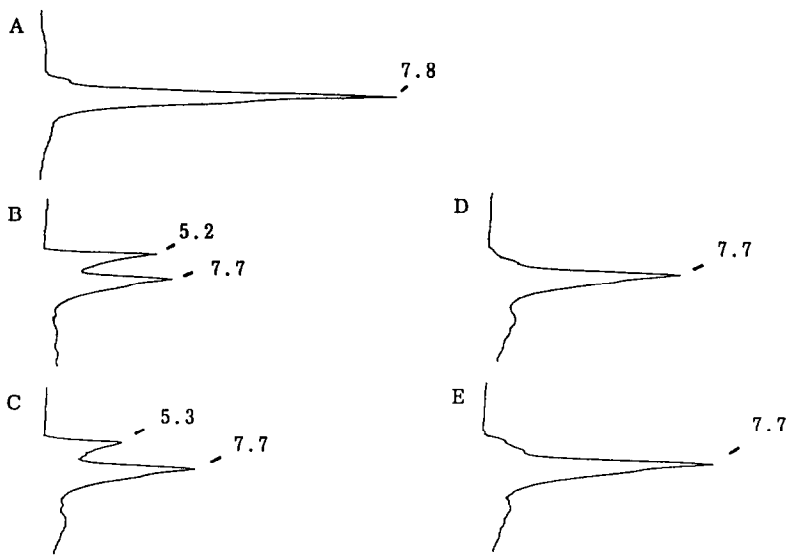


Fig. 3. High-performance size-exclusion chromatograms of β-galactosidase solution. (A) before freeze-drying, (B) freeze-dried without additives, (C–E) freeze-dried with 0.01, 0.1 and 1 mM sugar ester, respectively. The numbers beside the peaks represent the retention times (min).

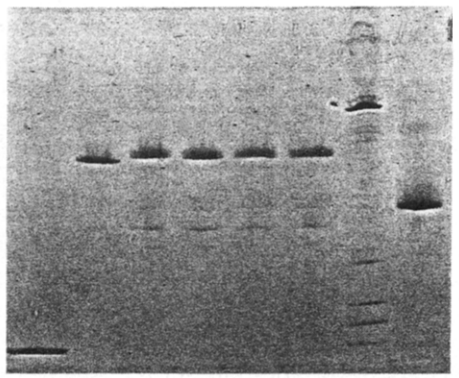


Fig. 4. SDS-PAGE of freeze-dried β-galactosidase. Lanes present (a) rabbit muscle LDH, (b) *E. coli* β-galactosidase, (g) rabbit muscle myosin and (h) bovine serum albumin as molecular weight standards. Lanes (c–f) represent: (c, d) original solution, (e, f) reconstituted solution of freeze-dried enzyme of *A. oryzae* β-galactosidase (d, f) Heat-treated without 2-mercaptoethanol.

other bands were observed in the higher-molecular-weight regions or insoluble material regions. Reduction with 2-mercaptoethanol did not affect the results.

## Discussion

β-Galactosidase lost its activity during freeze-drying, especially in diluted solutions. The activity loss during freeze-drying increased with decreasing enzyme concentration. The dependence of the inactivation on protein concentration is consistent with that reported for the inactivation of lactate dehydrogenase during freeze-thawing (Seguro et al., 1990) and rTNF during freeze-drying (Hora et al., 1992). The residual activity after freeze-thawing was greater than that after freeze-drying. This indicates that the inactivation occurred mainly during the drying process.

The results of HPSEC and electrophoresis give an insight into the mechanism of inactivation. As shown in HPSEC, some soluble aggregates were observed in samples freeze-dried without additives. The decrease in the activity was proportional to the decrease in peak height of the intact enzyme. These observations indicate that formation of aggregates is the main cause of inactivation during freeze-drying. Formation of aggregates accompanied by a decrease in activity has been reported for freeze-dried alcohol dehydrogenase (Ross et al., 1979). Chemical reactions such as the formation of intermolecular bonds (Manning, 1989) and physical changes such as denaturation (Yasui et al., 1966; Eckhardt et al., 1991) are known to cause aggregation. It is difficult to determine the nature of the aggregates only from the HPSEC chromatogram, but their short retention time suggests that they are not dimers of intact molecules. They appear more complex and larger in size.

Characterization of the freeze-dried enzyme by SDS-PAGE should help to determine the nature of the aggregates. If the aggregates have any intermolecular covalent bonding, SDS-PAGE should yield bands corresponding to dimers and oligomers. However, no significant change was observed in the SDS-PAGE pattern of the freeze-dried β-galactosidase. Dimers and tetramers were not detected in the samples heat-treated with or without 2-mercaptoethanol. These results suggest that the aggregates shown in HPSEC did not have intermolecular covalent bonds such as disulfide bridges. The aggregates may be due to complexing of denatured protein molecules by hydrophobic interaction. It has been reported that denaturation occurs during freeze-drying of ribonuclease A (Nai-Teng Yu et al., 1972) and myosin (Yasui et al., 1966). Denaturation, the appearance of a hydrophobic region on the surface of the protein, leads to the formation of aggregates by hydrophobic bonding. Sodium phosphate buffer, which is known to acidify frozen solution (Seguro et al., 1990), was used in the study. Although the enzyme is relatively stable in acidic region (Tanaka et al., 1975), the acidification should be one of the reasons for denaturation during freeze-drying.

Native PAGE showed that the intensity of the intact enzyme band was decreased by freeze-drying (data not shown), and that another band appeared at the application position instead. This suggests that the aggregates are too large to run on the gel. The difference in the pattern between SDS-PAGE and native PAGE may result from

the difference in sample preparation for electrophoresis. For SDS-PAGE, the proteins were heat-treated in SDS solution, which may cleave the aggregates into separate molecules.

Sugars and amino acids, which are known to be cryoprotectants, dose-dependently protected β-galactosidase from inactivation. These cryoprotectants thermodynamically stabilize the native conformation of proteins (Arakawa et al., 1991). The proteins remain amorphous in frozen solution, and maintain their protein-rich glassy state, resulting in protection during freeze-drying (Pikal et al., 1991; Hora et al., 1992).

The concentration of HP-β-CD, DM-β-CD, CHAPS and the sugar ester necessary for the protection of enzyme activity was much lower than that of other additives. In Fig. 2 the protective effect of the additives is plotted against molecular concentrations. These additives had a stronger stabilizing effect than the sugars studied, even when the effect per unit weight was taken into account. This difference in effective concentration suggests that their stabilization mechanism is somewhat different. The possibility of specific interaction of these reagents with the enzyme may-be excluded, since the structures of these additives are obviously different from one another.

HP-β-CD and DM-β-CD reduce surface tention (Imai et al., 1982; Yoshida et al., 1988). CHAPS and the sugar ester are often used as biological detergent. Some surfactants are known to inhibit protein absorption to the container. The stabilization effect of additives studied appears not to be due to inhibition of protein adsorption to the container, since no significant loss of activity was observed during storage of the enzyme solution without additives at 4°C for two weeks (data not shown).

Some surfactants have been used to dissolve hydrophobic proteins. For example, Laureth-12 has been used in a formulation of recombinant human interferon-β, Betaseron™ (Hershenson et al., 1989). Their effect was attributed to dissolution of proteins after freeze-drying. However, the results of this study indicate that the effect of additives studied cannot be explained simply by their dissolution effect, since their addition after freeze-drying was less effective. The findings that soluble aggragates were observed in the samples freeze-dried without additives and that some other surfactants were ineffective also exclude a possible dissolution effect. Therefore, these additives should protect the enzyme from denaturation during freeze-drying.

There are some possible mechanisms of protection of the enzyme activity. Additives with a stabilizing effect may maintain a protein-rich amorphous portion in frozen solution and inhibit denaturation by protecting the enzyme from unfavorable enviromental conditions such as ice-crystallization, acidification and removal of inevitable water. Although retention of amorphous nature should be important for the stabilization, it cannot explain the difference in effective concentration. The X-ray powder diffraction study showed that all of the freeze-dried cakes containing 1 and 10 mM of cyclodextrins were amorphous (data not shown). HP-β-CD and DM-β-CD stabilized the enzyme at very low concentration, but natural cyclodextrins had little effect. In addition to the property of retention of amorphousness, there may be some reason for the protection of the enzyme.

Another explanation is the difference in molecular interaction between additives and the enzyme. Cyclodextrins form an inclusion complex with hydrophobic residual amino acids in BSA (Matsuyama et al., 1988). HP-β-CD and DMβ-CD are surface active, and have different inclusion behavior compared to natural cyclodextrins. These cyclodextrin derivatives may bind move strongly to the enzyme than natural cyclodextrins. Although the manner in which binded additives protect the enzyme during freeze-drying is not clear, those molecular interactions may play an important role in stabilization. CHAPS and the sugar ester may also bind to the enzyme and protect from denaturation.

It is not clear why SDS, polidocanol and PEG 400 were ineffective. It is possible that these additives induced denaturation of the enzyme. The binding of some surfactants such as SDS is known to denature proteins (Tanford, 1980), while some other surfactants such as CHAPS bind to proteins without denaturation (Hjelmeland et al.,

1983). PEG 400 might work as co-solvent, and alter the physico-chemical conditions during freeze-drying.

In conclusion, HP-β-CD, DM-β-CD, CHAPS and sucrose fatty acid monoester were found to protect proteins against inactivation during freeze-drying at markedly low concentrations. These amphiphilic additives seem to bind to proteins and inhibit denaturation during freeze-drying. The mechanism of the stabilization effect, however, is still obscure and requires further clarification.

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