Denaturation of Enzyme Protein by Freeze-Thawing and Freeze-Drying

II. Freeze-thawing and freeze-drying of catalase*

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Abstract

The effects of freeze-thawing and freeze-drying on catalase, one of globular proteins, were examined for the purpose of investigating relations of the conformation of protein to its stability against freeze-thawing and freeze-drying. Also the water content of the freeze-dried egg albumin, a representative protein, was measured with or without some additives, for the clarification of the mechanism of their protective ability.

Freeze-thawing reduced the enzymatic activity of catalase to the extent dependent on the final freezing temperature but did not change the conformation, suggesting that the structure of a globular protein is more stable than that of a rod-like protein against freeze-thawing.

Freeze-drying unfolded the conformation and dissociated into 3.8 S or 5.8 S subunits of a single component independently of freezing conditions of catalase showing that the unfolding and the dissociation was due only to drying. Additives with the protective ability seemed to decrease the residual water content of freeze-dried albumin. It might be supposed that additives with protective ability were substituted reversibly for hydration water and inhibited the disturbance of hydration shell of the protein molecule.

I. Introduction

On the basis of several experimental facts, it was suggested in the

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previous paper[1] that water might play an important role in the denaturation of proteins such as myosin by freeze-thawing and freeze-drying. It was also pointed out there that for clarifying the role of water it was important to find relations of the shape of protein to its stability against freeze-thawing and freeze-drying, and some preliminary research for the relations was tried.

In the present paper the change in some physico-chemical properties such as viscosity, optical rotatory dispersion (ORD), circular dichroism (CD), difference spectra, sedimentation constant and enzymatic activity of freeze-thawed or freeze-dried catalase, one of globular proteins, were examined so that the denaturation mechanism of a globular protein might be discussed.

OGAWA[2] and SHIKAMA[3] respectively reported that catalase activity was decreased by freeze-thawing. It was, however, not clear in their papers whether the conformation of catalase changed or not. TANFORD *et al*[4] and DOUNCE *et al*[5] reported that freeze-drying dissociated catalase into subunits and decreased its enzymatic activity, but they did not explain the mechanism of dissociation in detail in relation with freezing or drying conditions. It was not also clarified whether the conformation of protein moiety of dissociated subunits by freeze-drying changed or not.

Some additives were expected to protect catalase from the dissociation by freeze-drying. To find such protective additives and to elucidate their protective mechanism might be useful to understand the role of water in the denaturation of protein by freeze-drying. The effects of some additives on the dissociation of catalase by freeze-drying were examined, and the water content of freeze-dried egg albumin, as a representative protein, was measured with or without additives. On the basis of the measurements, the protective mechanism were discussed in relation with the role of water in protein structure.

II. Materials and Methods

Materials

Catalase was prepared from beef liver according to KITAGAWA and SHIRAKAWA's method[6] with some modifications. Recrystallization procedure was repeated three times. Crystallized catalas ($\times 3$ crystallized specimen. Lot No. 18B–8084) purchased from Sigma Co. was also used after recrystallization. The final products were prepared as aqueous solution adjusted by NaOH to pH 7.3.

Crude crystal of egg albumin purchased from Kishida Co. was used as a solution in 0.075 M phosphate buffer (pH 7.0).

Before experiments, all the specimens were centrifuged for 10 min at

12000 rpm in order to remove insoluble fractions.

Methods

The procedures of freeze-thawing and freeze-drying were the same as described in the previous paper except that beside the ordinary 6-hour drying procedures with a rotary pump, a procedure with additional 6-hour drying with an oil diffusion pump was used (The former procedure will be conventionally denoted by DI and the latter by DII).

The concentration of catalase was determined photometrically with the value[4], $E_{278\,m\mu}=4.0\times10^5$ and $E_{405\,m\mu}=3.1\times10^5$. The value[7], $E^{1\%}_{405\,m\mu}$, was also used.

The catalase activity was measured photometrically in 0.075 M phosphate buffer solution (pH 7.0) at 20°C by a modified BEERS and SIZER's method[7], where the specimen used was of catalase concentration 1×10^{-9} M and was of substrate H_2O_2 concentration 1×10^{-4} M.

The viscosity of the specimen was measured at 26°C in an Ubbelohde type viscosimeter in the range of protein concentration between 0.43 and 0.14%.

The measurements of CD and ORD were carried out with a Jasco ORD/UV-5 recording spectropolarimeter equipped with a CD attachment at room temperature. The cells were 10 and 1 mm in length. The CD and ORD data were expressed in terms of circular ellipticity, $[\theta]$, and the mean residue rotation, [m'], taking 129 for mean residue weiget, M_0[8]. Helix content of catalase was determined following formula[8]:

$$\text{Helix } \% = ([m']_{233}+2000)\times100/13000$$

The difference spectrum and other photometrical properties were measured with either a Shimadzu Model 5V-50A recording spectrophotometer or a Hitachi-Perkin Elmer Model 139 spectrophotometer using 10 mm quartz cells. In few cases, the Jasco ORD/UV-5 was also used.

Ultracentrifugal analysis was made with a Hitachi Model UCA-1 ultracentrifugal apparatus using a standard cell.

The water content was measured with a Karl Fisher's apparatus[9] made by Kyoto Electric Co. with Mitsubishi Karl Fisher's reagent of factor 0.05 mg water eq/ml.

III. Results

Freeze-thawing of catalase

Two milliliters of 0.5% catalase in aqueous solution in a glass container

were frozen to a given temperature slowly by stepwise transfer of the container to the baths of appropriate temperature and thawed rapidly. Neither precipitation nor turbidity was observed after thawing.

Ultracentrifugal analysis of freeze-thawed catalase showed no changes in sedimentation constant and sedimentation pattern, as shown in Figs. 1

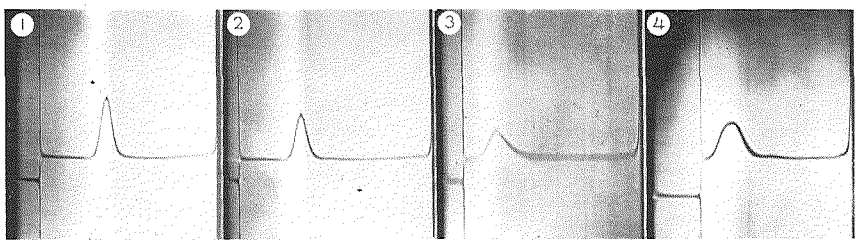

Fig. 1. Sedimentation diagrams of frozen-thawed and freeze-dried catalase
1 Native 32 min after full speed reached. $S_{20,w}=11.5$
2 Freeze-thawing. Freezing temp. −60°C. 32 min. $S_{20,w}=12.4$
3 Freeze-drying. Freezing temp. −80°C. 30 min. $S_{20,w}=3.8$
4 Freeze-drying. Freezing temp. −80°C. 32 min. $S_{20,w}=5.8$
Protein concentration, 0.42%; Solvent, 0.075 M phosphate buffer (pH 7.0); 55430 r.p.m.; 20°C

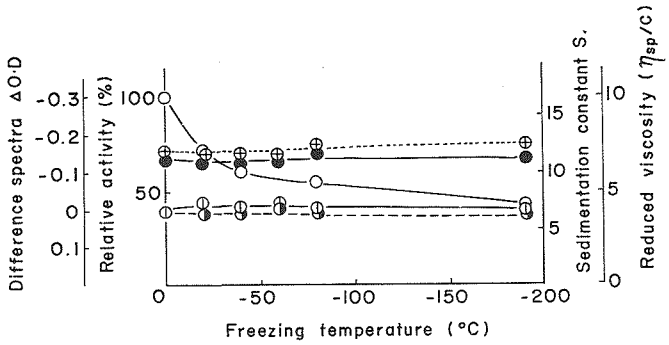

Fig. 2. Changes in enzymatic activity and physico-chemical properties of frozen-thawed catalase
○, enzymatic activity; ⊕, reduced viscosity $\eta_{sp/c}$;
●, sedimentation constant $S_{20,w}$; ⓘ, $\Delta O \cdot D_{405\,m\mu}$;
◑, $\Delta O \cdot D_{278\,m\mu}$

and 2. This indicated that freeze-thawing caused no dissociation or association of catalase molecule. As shown in Fig. 2, the catalase activity decreased with the lowering of the freezing temperature, as found in the case of myosin, but about 40% of the activity was retained even in the freezing at −196°C. The helix parameters obtained by CD and ORD spectra in far ultraviolet region were shown in Table 1. It was observed only very slight

Table I. Helix parameters of freeze-thawed catalase[a]

Freezing Temperature °C	$[m']_{233}$	$[\theta]_{222}$	Helix Content[b] %
Control	−8600	−10000	51
− 20	−8600	−10000	51
− 80	−8500	− 9600	50
−196	−8600	−10000	51

a, Solvent, 0.075 M phosphate buffer (pH 7.0)
b, Calcurated from the value of $[m']_{233}$

change in the difference spectra and CD spectra in near ultraviolet region and in Soret band. Very slight changes were observed of the reduced viscosity, helix parameter and other measured physico-chemical properties shown in Fig. 2. These results suggested that freeze-thawing of catalase solution caused only very slight change in the conformation of the molecule, although the enzymatic activity was decreased.

It was obtained the same result either in aqueous solution or in 0.075 M phosphate buffer solution of catalase.

Freeze-drying of catalase

Catalase of 0.5% in aqueous solution was freeze-dried according to the procedure DI or DII, and was resolved in 0.075 M phosphate buffer solution (pH 7.0) to make the final solution of catalase concentration 0.5%. The freeze-dried catalase could be easily resolved in the buffer solution without any turbidity or precipitation.

Figure 1 illustrated sedimentation diagram of freeze-dried catalase. As seen in this figure, freeze-dried catalase showed a single peak of 3.8 S or 5.8 S component, instead of 11.5 S of native catalase. From the result, it was considered that freeze-drying dissociated catalase molecule into 3.8 S or 5.8 S subunits of a single component. In a few case, dissociation into two component of 4.3 S and 6.2 S subunits was observed. The extent of the dissociation was independent of the conditions of freezing or drying at least in the examined range and also of the catalase concentration in the investigated range between 0.1 and 2%.

The catalase activity was decreased extremely even in the specimen DI and further in the specimen DII, as illustrated in Fig. 3. Its dependence on the freezing temperature was similar to that in the case of freeze-thawing.

In the difference spectra of freeze-dried catalase, it was observed slight

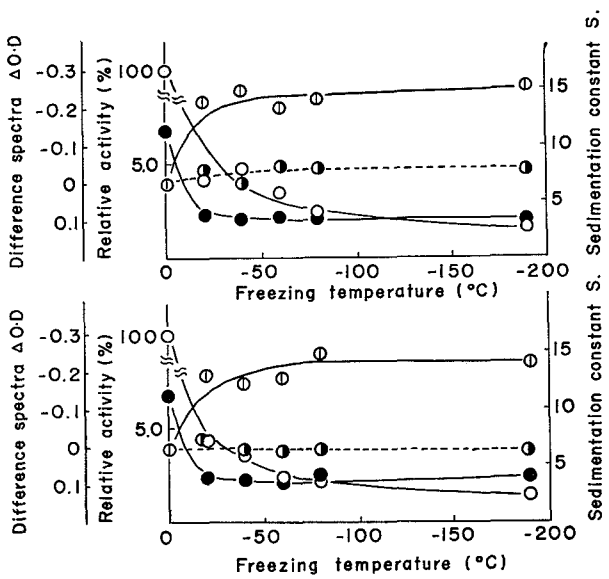

Fig. 3. Changes in enzymatic activity and physico-chemical properties of freeze-dried catalase

○, enzymatic activity; ●, sedimentation constant $S_{20,w}$; ⓘ, $\Delta O \cdot D_{405\,m\mu}$; ◑, $\Delta O \cdot D_{278\,m\mu}$

upper, Freeze-dried with oil ratary pump; protein concentration, 1% in water

lower, Freeze-dried with oil diffusion pump; protein concentration, 0.5%, in water

blue shift with slight decrease of the adsorption intensity in a near ultraviolet region and remarkable reduction of the absorption intensity in Soret band as shown in Fig. 4. The CD spectra in the both regions of freeze-dried specimen were shown in Fig. 5. The negative CD band at 390 mμ for natve catalase, which corresponded to the Soret band, decreased remarkably. In the near ultraviolet region a positive CD band at 290 mμ for native catalase, which may be ascribed to amino acid group in the protein moiety, shiftted slightly to shorter wave length toward 280–285 mμ with slight decrease in the intensity. The extent of the shift and the intensity of CD and absorption spectra in both regions altered depending slightly on the freezing temperature. The reduction of the intensity of CD and absorption spectra in Soret band suggests some changes of the environment around haem group in the catalase molecule as a result of the dissociation into subunits. The blue shift of CD spectra in the near ultraviolet region with the reduction of the intensity may be corresponded to the blue shift of UV

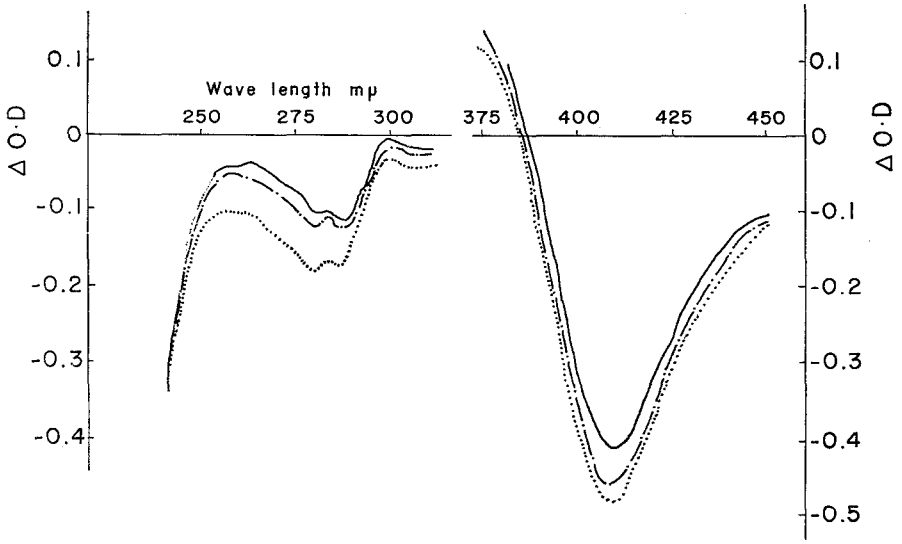

Fig. 4. Difference spectrum of freeze-dried catalase I Effect of freezing temperature
Freezing temperature: ——, −20°C; —·—, −80°C; ···, −196°C
Protein concentration, 0.17%: Solvent, 7.5×10^{-2} M phospate buffer (pH 7.0)
Reference, non-treated catalase

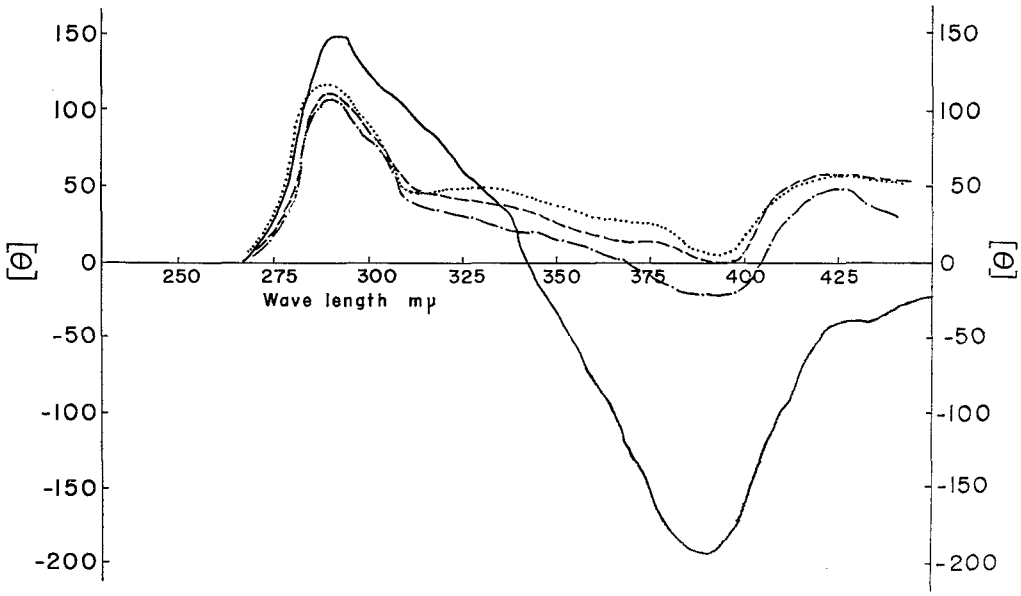

Fig. 5. CD spectrum of freeze-dried catalase. Effect of freezing temperature
——, non-treated catalase; —·—, freezing at −20°C; ---, −80°C; ···, −196°C; Protein concentration, 0.17%; Solvent, 7.5×10^{-2} M phosphate buffer (pH 7.0)

absorption band with slight decrease of the intensity. This result suggests some alteration of the environment of non-polar groups in the protein moiety due to the dissociation.

As shown in Table II, the helix parameters, which were obtained by CD and ORD spectra in far ultraviolet region, decreased by freeze-drying

Table II. Helix parameters of freeze-dried catalase[a]

Freezing Temperature °C	$[m']_{233}$	$[\theta]_{222}$	Helix Content[b] %
Control	−8700	−10900	51
− 20	−7100	− 8900	39
− 80	−6900	− 8800	38
−196	−6700	− 8300	35

a, Solvent, 0.075 M phosphate buffer (pH 7.0)
b, Calcurated from the valus of $[m']_{233}$

depending slightly on the freezing temperature. It was estimated that about 30% of the helix in the molecule unfolded with freezing temperature at −196°C.

The phosphate buffer solution (pH 7.0) was used as an additive, the extent of denaturation decreased with the increase in the buffer concentration, whereby denaturation is defined by the reduction of catalase activity

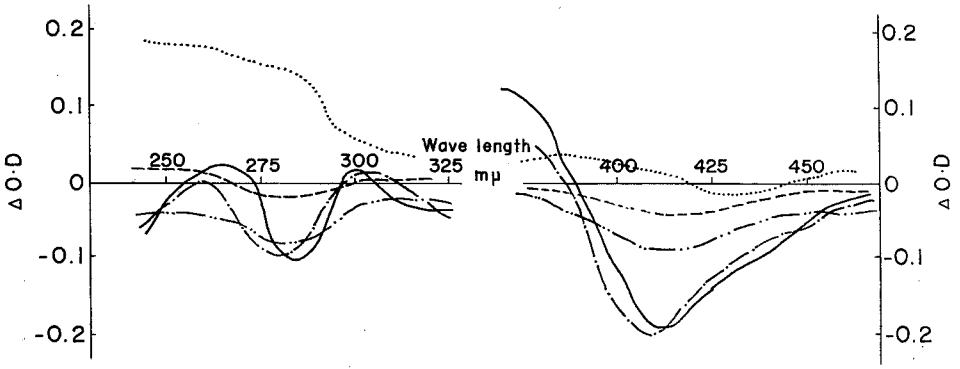

Fig. 6. Difference spectrum of freeze-dried catalase II Effect of the concentration of phosphate buffer solution
Concentration of phosphate buffer solution (pH 7.0): ——, O; —·—, 7.5×10^{-3} M; —··—, 1.5×10^{-2} M; ---, 3.7×10^{-2} M; ···, 5.6×10^{-2} M. Protein concentration, 0.14%; Solvent, 7.5×10^{-2} M phosphate buffer (pH 7.0)
Reference, non-treated catalase

by the values of $-\Delta O \cdot D_{278\,m\mu}$ and $-\Delta O \cdot D_{405\,m\mu}$ and the extent of the dissociation. As shown in Fig. 7 (upper), ultracentrifugal analysis showed two peaks of 11.6 S and 6 S component at low concentration of phosphate buffer, proving partial dissociation of 11.6 S native catalase into 6 S subunits. With the increase in the buffer concentration, 6 S component decreased and 11 S component increased. The values of $-\Delta O \cdot D_{278\,m\mu}$ and $-\Delta O \cdot D_{405\,m\mu}$ in the difference spectra of the same specimen decreased with increasing in the buffer concentration, as shown in Fig. 6. The residual enzymatic activity was also increased with increasing of the buffer concentration, as shown in Table III. As shown in these results, the change in difference spectra and

Table III. Effect of the concentration of phosphate buffer (pH 7.0) on the dissociation of catalase by freeze-drying

Sample	Phosphate buffer concentration (M)	$S_{20,w}$	$\Delta O \cdot D_{280m\mu}$ [a]	$\Delta O \cdot D_{410m\mu}$ [a]	Relative catalase activity (%)
Control	5.6×10^{-2}	11.6	—	—	100
1.	0	5.9	− 0.10	− 0.20	12
2.	7.5×10^{-3}	11.8 5.1	− 0.20	− 0.20	52
3.	1.5×10^{-2}	11.5 6.0	− 0.08	− 0.09	62
4.	3.7×10^{-2}	12.5 8.1	− 0.02	− 0.04	74
5.	5.6×10^{-2}	12.0	+ 0.15	+ 0.01	81

Protein concentration, 0.5%, freezing temperature, −80°C (except control)
a. Protein concentration reduced to 0.12%

the dissociation vanished and 80% of the activity was retained at the concentration of 0.056 M.

In the previous paper, the protective ability of sugars and amino acids against the denaturation of myosin by freeze-drying was reported. The effects on catalase were examined. The effects of sucrose of various concentration in the presence of 0.01 M phosphate buffer were shown in Fig. 7 (lower). A small peak of 6 S component and a peak of 11 S native component were observed for the sucrose concentration 0.01 M.

When the sucrose concentration was increased, 6 S component decreased and 11 S component increased. Thus, the extent of dissociation, as well as the change in catalase activity and in difference spectra, decreased with the increase in sucrose concentration, vanishing at the sucrose concentration 0.03 M.

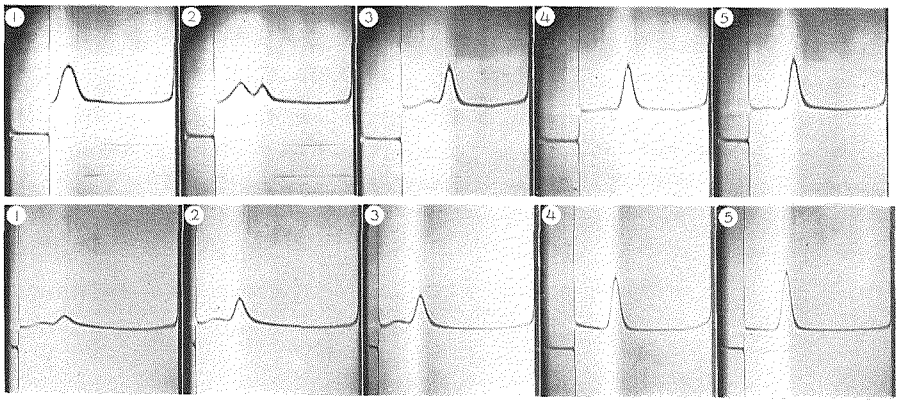

Fig. 7. Sedimentation diagrams of freeze-dried catalase I
Upper: Effect of the concentration of phosphate buffer (pH 7.0). 1) None; 2) 7.5×10^{-3} M; 3) 1.5×10^{-2} M; 4) 3.7×10^{-2} M; 5) 5.6×10^{-2} M
Lower: Effect of the concentration of sucrose with 1×10^{-2} M phosphate buffer (pH 7.0). 1) None; 2) 5×10^{-3} M; 3) 1×10^{-2} M; 4) 3×10^{-2} M; 5) Native catalase. Protein concentration, 0.42%; Solvent, 7.5×10^{-2} M phosphate buffer (pH 7.0); 55430 rpm; 24 min after reaching full speed at 20°C

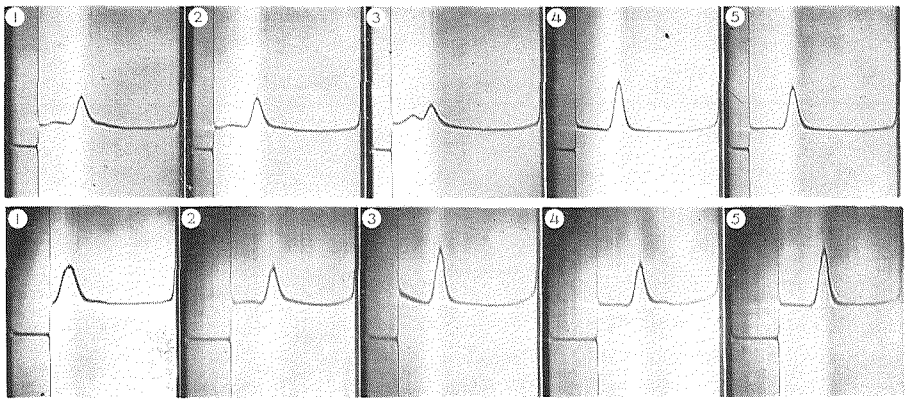

Fig. 8. Sedimentation diagrams of freeze-dried catalase II
Upper: Effect of 3×10^{-2} M additives with 1×10^{-2} M phosphate buffer (pH 7.0). 1) None; 2) NaCl; 3) Na pyrophosphate; 4) Sucrose; 5) K-glutamate
Lower: Effect of 3×10^{-2} M additives in aqueous solution 1) None; 2) KCl; 3) Inositol; 4) Glycine; 5) Native. Protein concentration, 0.42%, Solvent, 7.5×10^{-2} M phosphate buffer (pH 7.0); 55430 rpm; 24 min after reaching full speed at 20°C

The effect of several additives with 0.01 M phosphate buffer (pH 7.0) were shown in Fig. 8 (upper) and Table IV. Freeze-drying dissociated catalase partly into a 6 S subunit in this buffer concentration without an additive. When sugar or amino acid was added, the sedimentation constant the difference spectrum and catalase activity were the same as those of the

Table IV. Effect of some additives with 0.01 M phosphate buffer (pH 7.0) on the dissociation of catalase by freeze-drying

No.	Additives [a] (0.03 M)	$S_{20,w}$	$\Delta O \cdot D_{280m\mu}$ [b]	$\Delta O \cdot D_{410m\mu}$ [b]	Catalase Relative activity (%)
Cont.	None	11.9	0	0	100
1.	None	13.0 6.2	− 0.12	− 0.18	57
2.	NaCl	11.5 6.4	0	− 0.13	56
3.	Na-pyrophosphate	11.3 6.5	0.06	− 0.26	56
4.	Sucrose	11.5	0	− 0.05	96
5.	Glucose	11.7	0.03	− 0.04	95
6.	Lactose	12.3	0.03	− 0.06	95
7.	Glycine	12.8	0	− 0.09	87
8.	Na-glutamate	12.1	0.04	− 0.07	95
9.	Egg albumin [c]	13.1 (3.8) [d]	—	− 0.17	66

Protein concentration, 0.5%, freezing temperature, −80°C (except control)
a, 0.01 M phosphate buffer solution (pH 7.0)
b, Protein concentration reduced to 0.12%
c, 0.05%
d, Mixture of catalase and albumin

nontreated, though the ultracentrifugal analysis showed a slight peak of a 6 S subunit in few cases. In contrary to sugars or amino acids, NaCl and KCl showed no protective ability and pyrophosphate remarkably increased the extent of dissociation and decreased enzymatic activity. Egg albumin which was widely used as a protectant for freeze-drying of microorganism did not show any protective effects against dissociation and the loss of activity.

The effect of 0.03 M additives in an aqueous solution without buffer on the freeze-drying of catalase was examined. By the addition of sugars and amino acids, 4 S and 6 S components vanished and only 12 S native component was observed, as shown in Fig. 8 (lower). The values of $-\Delta O \cdot D_{278m\mu}$ and $-\Delta O \cdot D_{405m\mu}$ decreased and the enzymatic activity retained near the same

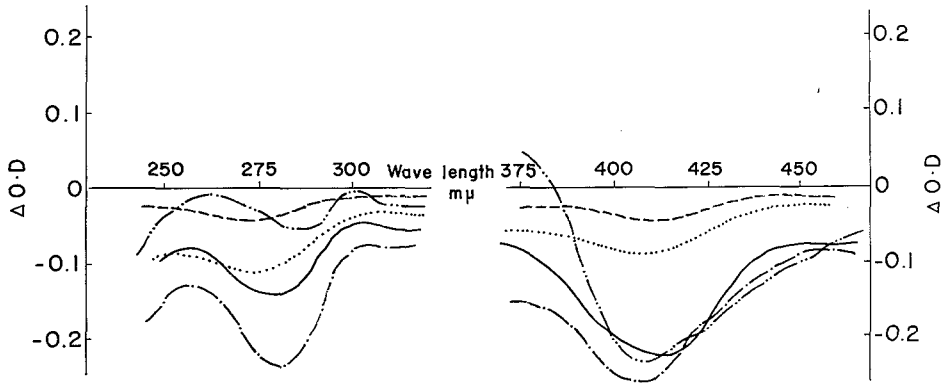

Fig. 9. Difference spectrum of freeze-dried catalase III Effect of some additives (0.03 M). ——, None; —·—, NaCl; —··—, Na-pyrophosphate; ---, Glucose; ···, Na-glutamate. Protein concentration, 0.14%; Solvent, 7.5×10^{-2} M phosphate buffer (pH 7.0); Reference, non-treated catalase

Table V. Effect of certain additives on the dissociation of catalase by freeze-drying

Sample	Additives[a] (3×10^{-2} M)	$S_{20,w}$	$\Delta O\cdot D_{280m\mu}$[b]	$\Delta O\cdot D_{410m\mu}$[b]	Relative catalase activity (%)
Control	None	11.8	—	—	100
1.	None	6.2	− 0.14	− 0.23	13
2.	None	12.2 8.0	− 0.14	− 0.23	34
3.	KCl	11.4 5.4	− 0.20	− 0.20	38
4.	Na-pyrophosphate	5.7	− 0.10	− 0.23	10
5.	Sucrose	12.0	− 0.04	− 0.05	85
6.	Inositol	12.3	− 0.11	− 0.18	71
7.	Glycine	12.4	− 0.05	− 0.11	91
8.	K-glutamate	11.9	− 0.10	− 0.07	90

Protein concentration, 0.5%; freezing temperature, −80°C (except control)
a, All in aqueous solution
b, Protein concentration reduced to 0.12%

value of the control, as shown in Fig. 9 and Table V. From these results, it is considered that, even without buffer solution, 0.03 M sugars and amino acids protected catalase from dissociation and the loss of activity by freeze-drying.

Effect of additives on the water content of freeze-dried protein

Sugars and amino acids might be considered to protect protein from the denaturation by retaining some water necessary to sustain the conformation during freeze-drying because of their hydrophilic ability. For confirming this assumption, the water content of freeze-dried protein was measured with

Table VI. The effect of sucrose concentration on the water content of freeze-dried egg alubumin[a]

	Sucrose concentration (M)[b]	Water content (H_2O mg/mg protein)	
		DI	DII
Cont.	None	0.042	0.036
1	0.001	0.038	0.031
2	0.005	0.039	0.03
3	0.010	0.038	0.031
4	0.05	0.036	0.027
5	0.1	0.029	0.027
6	0.25	0.050	0.030

a, Protein concentration, 1%
b, Aqueous solution
Pre-freezing temperature, −196°C
DI, freeze-dried with oil rotary pump
DII, Freeze-dried with oil diffusion pump

Table VII. The effect of some additives on the water content of freeze-dried egg albumin[a]

	Additives[b] (0.03 M)	Water content (H_2O mg/mg protein)	
		DI	DII
Cont.	None	0.039	0.025
1	Sucrose	0.037	0.025
2	Glucose	0.035	0.027
3	Glycine	0.036	0.025
4	K-glutamate	0.034	0.029
5	NaCl	0.039	0.030
6	Na-pyrophosphate	0.038	0.030

a, Protein concentration, 1%
b, Aqueous solution
Pre-freezing temperature, −196°C
DI, Freeze-dried with oil rotary pump
DII, Freeze-dried with oil diffusion pump

or without additives.

Egg albumin of 1% was used as a representative protein. The water content was measured by Karl FISHER's method immediately after freeze-drying.

The relation of the water content to the concentration of added sugar was shown in Table VI, where the values in the column denoted by DI and DII were the water contents of the specimens treated with the procedure DI and DII, respectively. The residual water content was rather larger in the specimen without sucrose, except for the highest sucrose concentration. The procedure DII dehydrated only a little more water than the procedure DI did.

The effects of several additives of 0.03 M concentration on the water content were shown in Table VII. Additives with the protective ability seemed to decrease slightly the residual water content.

IV. Discussion

Concerning the freeze-thawing of catalase, OGAWA[2] reported the simple dependency of the reduction on catalase activity on the freezing temperature, while SHIKAMA[3] reported that there was a critical temperature region of the complete loss of activity just like in case of myosin. In the present experiment, catalase activity decreased smoothly with the lowering of the freezing temperature under the constant rate of cooling. This does not necessarily contradict SHIKAMA's result becaues of the difference between his cooling rate and the cooling rate of the present experiment, as discussed in the previous paper.

As for the relation of the stability of protein to its shape, the present experiment showed that freeze-thawing did not cause any large conformational changes in a catalase molecule, one of globular proteins. Hence, the reduction of the activity of catalase by freeze-thawing might be considered to be due to some local disturbance near active sites. Contrary to the results on myosin, it was shown that the enzymatic activity of catalase, one of the function of the protein molecule, was affected remarkably by freeze-thawing without the change in the protein conformation. Concerning this result, it was noted that the rate of polymerization of freeze-thawed G-actin decreased depending on the freezing temperature and that freeze-thawing of G-actin did not cause any large conformational change[1]. In the difference spectra of freeze-thawed catalase, very slight disturbances were observed in a ultraviolet region and Soret band region. They might be related to some local disturbance.

In the previous paper a hypothesis was proposed that the destruction of hydrophobic bonds of protein by partial or complete dehydration and the breakdown of intramolecular hydrogen bonds by disturbance of hydration shell around protein molecule might be the main cause of the denaturation of protein by freeze-thawing or freeze-drying. The conformational stability of catalase against freeze-thawing might be explained on the hypothesis as follows: In a globular protein, hydrophobic bonds are formed in the interior of the molecule as compared with a rod-like protein, and, moreover, the surface area of protein contacting water is naturally considered smaller in the globular protein than in the rod-like protein. Hence, the hydrophobic bonds or hydration shell of globular protein might be less disturbed by partial dehydration such as freezing than those of rod-like protein was.

Dissociation of catalase by freeze-drying into 4.2 S and 7.6 S subunit was reported by TANFORD[4] and that into a 5.8 S subunit by DOUNCE[5] In the present experiment, freeze-drying dissociated catalase into either 3.8 S or 5.8 S subunits independent of the conditions of freeze-drying. The 3.8 S subunit is considered as a monomer while the 5.8 S a dimer. The latter may be identical to the component II reported by DOUNCE *et al.* The reason for the occurrence of two modes of dissociation, 3.8 or 5.8 S, even under the same freeze-drying conditions, has not yet been explained.

Concerning about the measurement of CD, ORD and absorption spectra of freeze-dried catalase, the evidences that the reduction of helix parameters, the reduction of the intensity of CD and absorption band in Soret band, and the shift with the decrease of the intensity of CD and absorption band in a ultraviolet region, are similar to the result of the denaturation with urea[4]. But, the extent of decrease of helix content and other changes are not so remarkable as compared with that result. All of the changes depended slightly on the freezing temperature.

The changes of CD and absorption spectra in Soret band seem to be due to dissociation, which makes the haem group to be exposed to a solvent environment. The alteration of CD and absorption in a near ultraviolet region may be the alteration of the environment around non-polar proups in the protein moiety, probably tyrosine residues[7]. These results show that freeze-drying causes not only the dissociation of catalase into subunits but also the unfolding and conformational change of protein moiety of dissociated subunits. Concerning these results, the electron microscopic observation[10] showed that the freeze-dried catalase had irregular amorphous shapes instead of the regular shape of the native catalase. The dissociation into subunits and the destruction of subunits structure might cause the complete loss of

enzymatic activity of freeze-dried catalase.

The dissociation of freeze-dried catalase into subunits suggests that water plays a role to bind subunits, *i.e.* the quarternary structure of catalase. Perutz and his coworker[11] recently showed that the binding sites between subunits of hemoglobin were composed of two non-polar and two polar interactions, using X-ray analysis of 2.8 Å resolution. Since catalase is haem protein composed of four subunits similarly to hemoglobin, the binding sites of catalase may be similar to those of hemoglobin. Should it be so assumed, the hydrophobic bonds between subunits might be destroyed reversibly during freezing, because the polar interaction sustained the quarternary structure. Since freeze-drying dehydrates protein more completely than freeze-thawing does, the destruction of hydrophobic bonds and the disturbance of intramolecular hydrogen bonds will be impelled extremely. In other words, freeze-drying will bring about extreme unfolding or dissociation of protein, because hydrophobic bonds can not exist without environmental water. Moreover, the hydration shell will be destroyed compretely. In such situation, the reversibility of hydrophobic bonds will be lost. As an example, it was reported that, like catalase haemocyanin was denatured by freeze-drying but not by freeze-thawing[12].

Inorganic salts such as NaCl or KCl did not show any protective effects on myosin and catalase from denaturation by freeze-drying; sodium pyrophosphate even accelerated denaturation. It was reported that inorganic salts excluded the hydration water of polar groups in amino acid residues[13]. This might affect polar interactions in the protein molecule. The accelerating effect of pyrophosphate may be due to the strong charge effect of a pyrophosphorous group in a non-aqueous state. But, it can hardly be explained as yet that phosphate buffer, one of inorganic salts and phosphate compounds, had the protective effect.

Sugars and amino acids have been widely used as protective additives for microorganisms in the process of freeze-thawing and freezd-drying. The protective effect of sucrose on myosin from the reduction of ATPase activity by freeze-thawing was reported by SHIKAMA[3], and that by freeze-drying by YASUI[12]. In the present experiment, not only sucrose but glucose, lactose, inositol, as well as such amino acids as glycine and glutamate inhibited the denaturation of protein.

It has been usually assumed that sugars and amino acids retain some water during freezing or drying because of their hydrophilic properties. The assumption can hardly explain the evidence that the water content of freeze-dried protein changed little with such an additive or that it rather

decreased with an additive. YASUI considered that the protective effect of sucrose might be related to its dielectric property of sucrose. But it is difficult to explain the result that amino acids, weak dipolar electrolytes, also showed the protective effect.

As an alternative hypothesis, it is proposed here that these effective additives may be substituted reversibly for hydration water binded to in the protein molecule because of their large hydrogen bonding ability, so as to protect binding sites from exposing them to the environment during freeze-drying.

There are many proteins, especially globular ones, which are stable against not only freeze-thawing but also freeze-drying. Such proteins seem to be rich in intramolecular disulphide bonds, which play the role of skeleton to sustain the protein conformation during freezing or drying. It is hoped that further study will be made on the relations of the specificity of the shape or conformation of protein and the stability against freeze-thawing and freeze-drying.

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