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Studies on Taka-Amylase A under High Pressure

II. Recovery of Enzymic Activity of Pressure Inactivated Taka-Amylase A and its Enhancement by Retreatment at Moderate Pressure[1]

KINJIRO MIYAGAWA,

From the Laboratory of Chemistory, Osaka Joshigakuen College, Tennoji, Osaka, Japan

KIYOTOSHI SANNOE AND KEIZO SUZUKI

From the Department of Chemistry, Faculty of Science and Enginering, Ritumeikan University, Kyoto, Japan

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The recovery of enzymic activity in pressure-inactivated Taka-amylase A and its enhancement by a moderate pressure treatment were examined. The enzyme concentration, pH, temperature, and ionic strength influence the velocity of the recovery of activity to a great extent. The recovery was found to be favored by a high concentration of enzyme, neutral pH, a temperature of about 30°C, and a low ionic strengh. Treatment at a moderate pressure of 600-4000 kg per square centimeter of the high pressure-inactivated Taka-Amylase A enhanced the recovery. The recovery of the partially inactivated Taka-amylase A (compression at 7500 or 6000 kg per square centimeter for 10 minutes) was greater than that of the completely inactivated Taka-Amylase A (compression at 9500 kg per square centimeter for 10 minutes). Below 1200 kg per square centimeter the extent of the recovery increased as the pressure increased, but above 1200 kg per square centimeter the extent of the recovery gradually decreased. Taka-amylase A inactivated by prolonged compression at 9500 kg per square centimeter at 30° did not recover enzymic activity on standing at atmospheric pressure, but the activity

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of this inactivated enzyme could be partly recovered by treatment at a moderate pressure. Some kinetic parameters of the recovering reaction were calculated.

In our previous paper (1) it was reported that taka-amylase A (TAA) (Aspergillus oryzae α-amylase) [EC. 3. 2. 1. 1.] is inactivated by compression up to 10,000 kg per square centimeter, and the pressure inactivation is dependent on pH, temperature, and ionic strength as well as on the magnitude of the pressure. The process of inactivation, and activation of the rate process were calculated. The recovery of activity in pressure-inactivated enzyme on release of the pressure was investigated.

Recently, Isemura and his co-workers reported that some denatured proteins, including TAA, can revert to the native state when the denaturant is removed (2), but the recovery of pressure-inactivated enzyme has not been observed except with TAA. It seemed to be worthwhile to study recovery of activity of pressure-inactivated TAA in detail, since recovery of activity is not only an unusual phenomenon but also provides information on the mechanism of the pressure inactivation of TAA itself. Moreover, it is well known that the molar volume of a protein increases when the protein is denatured and that its recovery from denaturation causes the volume to decrease. In fact, Tongur *et al.* (3) found that recovery of heat-denatured protein is accelerated by a moderate pressure treatment. It seemed possible, therefore, that the recovery of pressure-inactivated TAA might be enhanced by a moderate pressure treatment.

The recovery of pressure-inactivated TAA after the pressure was released, and the effect on the reactivation of recompression under a moderate pressure were investigated in the present work.

EXPERIMENTAL

MATERIALS

Thrice-crystallized TAA was prepared from Taka-diastase Sankyo according to the method of Akabori *et al.* (4). Rivanol treatment was repeated twice to minimize the contamination of acid Taka-protease (5). Aqueous TAA solution was dialyzed thoroughly against 0.1 *M* potassium chloride and stored as a stock solution (TAA concentration about 5% in a refrigerator. The concentration of TAA was estimated spectrophotometrically, assuming the extinction of TAA in water to be $E_{1m}^{1\%} = 22.1$ at 2785 Å. In each experiment, the stock solution was usually diluted with distilled water to give a concentration of $2 \times 10^{-3}\%$.

PROCEDURES

The high pressure apparatus and the procedures for inactivation were the same as previously reported (6). The Apparatus (Fig. 1), which is suited for

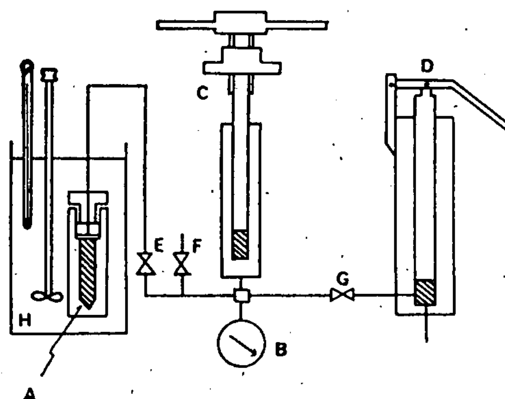

Fig. 1. Schematic layout of moderate pressure apparatus. (A) Reaction vessel; (B) Bourdon-type pressure gauge; (C) injection pump; (D) hand pump; (E, F, G) high-pressure valves; (H) thermostat.

relatively low pressures (up to 2000 kg per square centimeter), was used in the study of the effect of recompression at moderate pressure. An aqueous solution of TAA was sealed into a polyvinylchloride sack and set in the high pressure chamber. After a compression under defined conditions (usually for 10 minutes at 9500 kg per square centimeter at 30°), the sack was taken out. The inactivated samples were diluted threefold with a suitable buffer (0.05 *M* tris buffer above pH 7.0 or 0.05 *M* acetete buffer below pH 7.0). Therefore the enzyme concentration of the inactivated samples was $6.7 \times 10^{-3}\%$, except in experiments of the effet of initial concentration of TAA where the buffer concentration was over 0.033 *M*. One portion of the inactivated samples was

kept under atmospheric pressure, and the other was placed in the pressure chamber and recompressed at a pressure ranging from 600 to 4000 kg per square centimeter. In the case of activity measurements, the sample was diluted tenfold with distilled water to give a concentration of 6.7×10^{-4} %. Amylase activity was measured with soluble starch as the substrate at pH 5.3 and 30° by determination of the dextrinizing power with dinitrosalicylic acid acording to the method of Noelting and Bernfeld (7) : 1 ml of enzyme solution was mixed with 1 ml of 1 % soluble starch solution at pH 5.3 preheated in the thermostat at 30. After incubating for exactly 3 minutes, the enzyme reaction was stopped with 1 ml of 1 % dinitrosalicylic acid reagent. The mixture was boil d for 5 minutes and then cooled. After the solution was diluted tenfold with distilled water, the optical density was measured at 530 mμ. Under these conditions the increase in optical density can be taken directly as a measure of enzyme activity because the relation enzyme activity and optical density is linear.

RESULTS

HIGH-PRESSURE INACTIVATION OF TAA

The pressure dependence of the loss of enzymic activity is shown in Fig. 2. Unbuffered aqueous solutions of TAA (2×10^{-3}

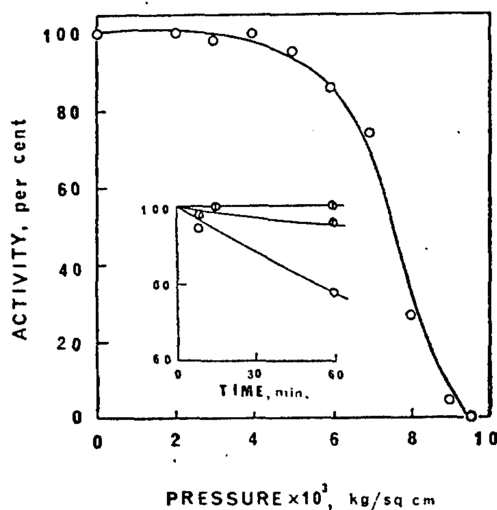

Fig. 2. Inactivation curves of TAA by pressure. Unbuffered aqueous solutioas of TAA (2×10^{-3}%) were compressed for 30 minutes at various pressures at 30° (—○—). The time course of the inactivation under 2000 kg per square centimeter (—◐—), 3000 kg per square centimeter (—◑—) and 4000 kg per square centimeter (—●—) at 30° was also examined.

%) were compressed for 10 minutes at various pressures at 30°, and time courses of the inactivation under 2000, 3000, and 4000 kg per square centimeter at 30° were also examined. The inactivation becomes noticeable in 60 minutes at about 3000 kg per square centimeter, is rapid above 6000 kg per square centimeter, and is complete in 10 minutes at about 9500 kg per square centimeter. The completely inactivated TAA used in this study was obtained by exposure to 9500 kg square centimeter for 10 minutes at 30° except where otherwise indicated; this material would not reactivate on standing under atmosphere pressure at 30°.

Effect of Initial Concentration of Enzyme on the Reactivation

One % of unbuffered aqueous solutions of TAA were completely inactivated by pressure, as described above. After the pressure was released, samples were diluted to various concentrations with tris buffer (pH 7.5; final concentration of buffer, 0.03 *M*). A portion of each sample was allowed to stand at atmospheric pressure at 30° and the other was recompressed at 800 kg per square centimeter at 30°. After 60 minutes the enzymatic activity was measured. The results are shown in Fig. 3. The extent of the reactivation is dependent on the initial concentration of TAA both at atmospheric pressure and at 800 kg per square centimeter: the higher the concentration the more extensive the reactivation.

Effect of Pressure Magnitude of Recompression

The pressure-inactivated TAA solution was recompressed under pressures up to 4000 kg per square centimeter at 30° for 20

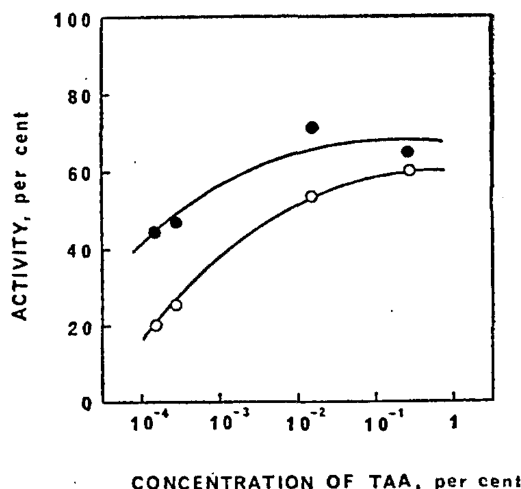

Fig. 3. The effect of the initial concentration of TAA on the recovery. One % unbuffered aqueous TAA solution was compressed for 10 minutes at 9500 kg per square centimeters at 30° and then diluted to each concentration indicated with tris buffer of pH 7.5 (final concentration 0.03 *M*). The inactivated TAA solutions were recompressed at 800 kg per square centimeter (—●—) or kept under atmospheric pressure (—○—) for 60 minutes, at 30°.

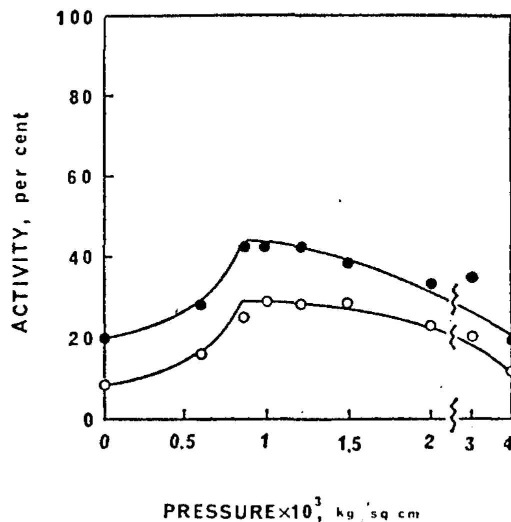

Fig. 4. The effect of pressure magnitude on the reactivation. Unbuffered aqueous solutions of TAA (2×10^{-3} %) were compressed for 10 minutes at 9500 kg per square centimeter at 30° and then diluted threefold with 0.05 *M* tris buff of pH 7.5. The inadtivated TAA solution was redompressed for 20 minute (—○—) and 60 minutes (—●—) under various pressures up to 4000 kg per square centimeter at 30°.

or 60 minutes. The results are shown in Fig. 4. The extent of the reactivation is enhanced by pressures up to 800—1000 kg per square centimeter. However, beyond 1200 kg per square centimeter or so the extent of reactivation seems gradually to lessen.

EFFECT OF pH

Pressure-inactivated, unbuffered aqueous solutions of TAA were adjusted to various pH values with tris buffer (above pH 7.0) or acetate buffer (below pH 7.0). A portion of the sample was recompressed at 800 kg pur square centimeter at 30° and the other was kept standing at 30° under atmospheric pressue for 60 minutes. The results are shown in Fig. 5. The reactivation is dependent on pH; the optimal pH for reactivation is in the

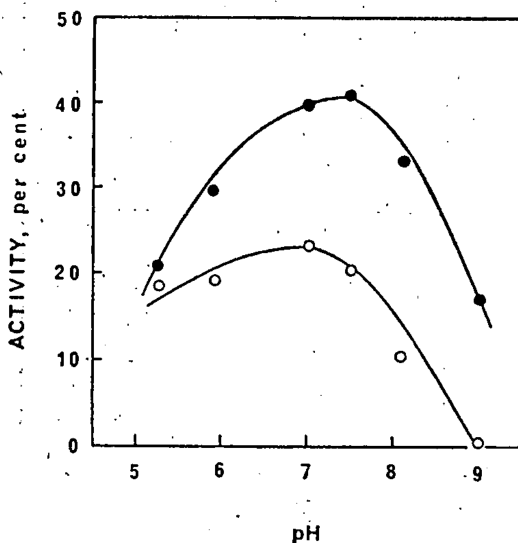

Fig. 5. The effedt of pH on the reactivation. Unbuffered aqueous solutions of TAA ($2\times10^{-3}\%$) were compressed for 10 minutes at 9500 kg per square centimeter at 30° and then diluted therefold with 0.05 *M* tris buffer (above pH 7.0) or 0.05 *M* acetate buffer (below pH 7.0). The inactivated TAA solution was recompressed at 800 kg per square centimeter (—●—) or kept under atmospheric pressure (—○—) for 60 minutes at 30°.

neutral range in both cases.

EFFECT OF TEMPERATURE AND AMOUNT OF INACTIVATION

Portions of completely inactivated TAA and partially inactivated TAA (7500 and 6000 kg per square centimeter for 10 minutes at 30°) were kept standing under atmospheric pressure, and other portions of these same samples were recompressed at 800 kg per square centimeter at various temperatures. The results are shown in Fig. 6. The extent of reactivation increases

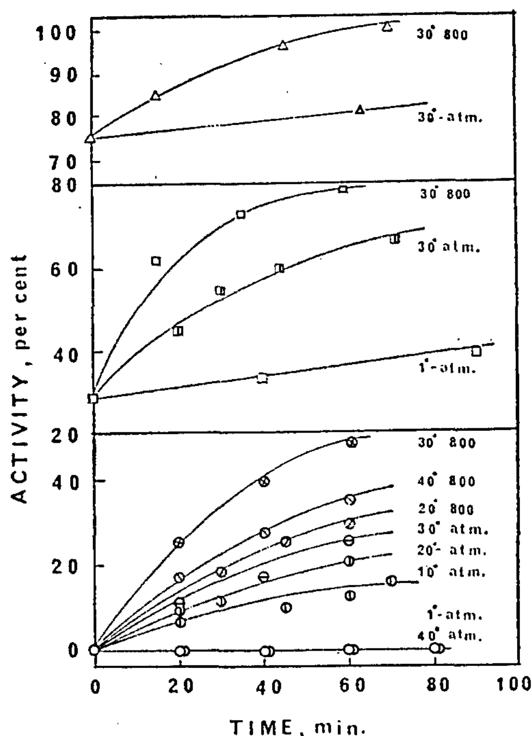

Fig. 6. Time course of the reactivation of completely and partially inactivated TAA. Unbuffered aqueous solutions of TAA (2×10^{-3} %) were compressed for 10 minutes at 30°C. at a pressure of 9500 (circles), 7500 (squares), and 6000 kg per square centimeter (triangles) and then diluted threefold with 0.05 *M* tris buffer of pH 7.5. The inactivated TAA solutions were recompressed at 800 kg per square centimeter or kept under atmospheric pressure at each temperature indicated up to 40°.

as the temperature increases up to 30°, but beyond 30° it is decreased. Recompression at 800 kg per square centimeter enhanced the reactivation over the whole temperature range studied. Although reactivation is not observed under atmospheric pressure at 40°, moderate recompression is still effective at this temperature. The activity regained is larger when inactivation is week (as seen in Fig. 6); activities after 60 minutes under recompression at 800 kg per square centimeter at 30° are 100, 70, and 45% when the original compression was at 6000, 7500, and 9500 kg per square centimeter, respectively.

SOME KINETIC PARAMETERS

The velocity constants k' were calculated from the slopes of the first order plots shown in Fig. 7. Values of k' and other parameters of the kinetics of recovery are listed in Tables I and II.

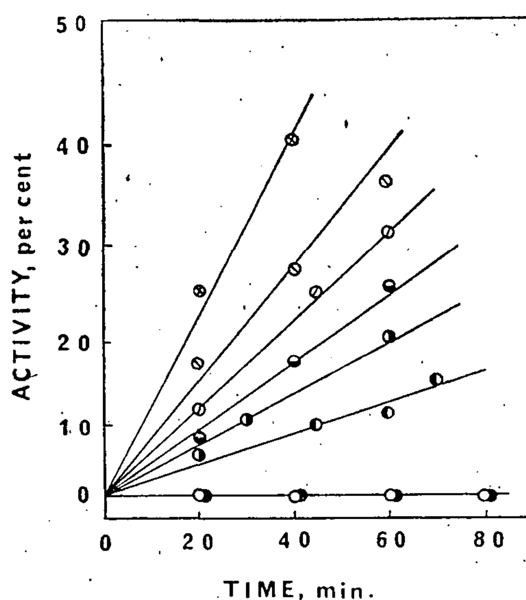

Fig. 7. First order plots of the reactivation. All symbols are the same as those in Fig. 6.

TABLE I

KINETICS OF THE RECOVERY OF PRESSURE-INACTIVATED TAA AT pH 7.5[a]

Pressure (kg/sq cm)	Temp. (°C)	k' (sec^{-1})	E (kcal/mole)	ΔF^* (kcal/mole)	ΔH^* (kcal/mole)	ΔS^* (cal/mole deg)
0	10	3.6×10^{-5}		22		−56
			6.7		6.7	
0	20	5.1×10^{-5}		23		−57
			4.3		4.3	
0	30	6.5×10^{-5}		24		−65
600	30	9.8×10^{-5}				
800	20	7.8×10^{-5}		23		−44
			10.6		10.0	
800	30	1.4×10^{-4}		23		−43
			−6.4		−7.0	
800	40	1.0×10^{-4}		24		−100
1200	30	1.5×10^{-4}				
1500	30	1.4×10^{-4}				

[a]Concentration of enzyme, 0.67×10^{-3} %

TABLE II

MOLAR VOLUME CHANGE OF ACTIVATION, ΔV^*, IN THE RECOVERY PROCESS AT pH 7.5 AT 30°C[a]

Pressure (kg/sq cm)	ΔV^* (cc/mole)
0~600	−6.0
600~800	−18.1
800~1200	1.1
1200~1500	1.6

[a]Concentration of enzyme, $2/3 \times 10^{-3}$ %.

EFFECT OF IONIC STRENGTH

To unbuffered aqueous solutions of completely inactivated

TAA, sodium chloride solution was added to give concentration of 1, 10^{-1}, 10^{-2}, and 10^{-3} mole per liter. The final enzyme concentration was always kept $2/3 \times 10^{-3}$ %. The solution was kept at 30° for 20 minutes or 60 minutes under recompression of 800 kg per square centimeter or under atmospheric pressure, and enzymic activity was then measured in each solution. The results are shown in Fig. 8. Under atmospheric pressure, reacti-

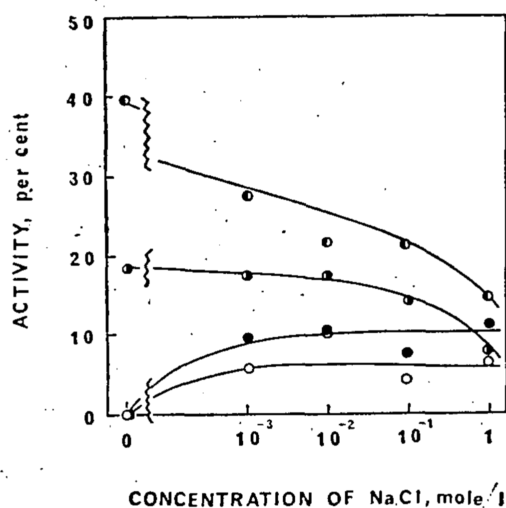

Fig. 8. The effect of ionic strength on the reactivation. To unbuffered aqueous solutions of TAA (2×10^{-3} %) compressed for 10 minutes at 9500 kg per square centimeter at 30°, sodium chloride was added to give each ionic strength indicated. The completely inactivaued TAA ($2/3 \times 10^{-3}$ %) was kept under atmospheric pressure at 30° for 20 minutes (—○—) and 60 minutes (—●—) or recompressed at 800 kg per square centimeter at 30° for 20 minutes (—◐—) and 60 minutes (—◑—).

vation takes place to a small extent in the presence of a small concentration of salt, though it is almost independent of the inoic strength above 10^{-3} mole per liter. On the other hand, a moderate recompression markedly increases the recovery only at the lower ionic strengths.

EFFECT OF COMPRESSION TIME FOR INACTIVATION

Unbuffered aqueous solutions of TAA were compressed under 9500 kg per square centimeter at 30° for two different times, 10 and 60 minutes. After the pressure was released, the pH was adjusted to 7.5 with tris buffer. The sample solutions thus prepared were kept at 30° under atmospheric pressure or under recompression at 800 kg per square centimeter. Enzymic activity was then measured at several times. The results are shown in Fig. 9. (Unbuffered TAA inactivated by 9500 kg per square

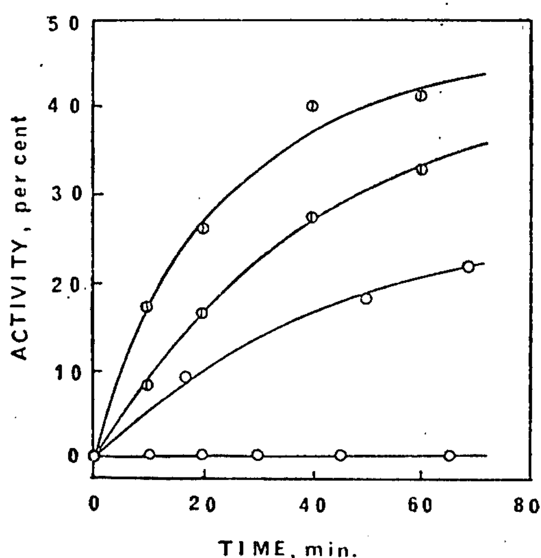

Fig. 9. The effect of compression time for inactivation on reactivation. Unbuffered aqueous TAA solutions (2×10^{-3} %) were compressed for 10 or 60 minutes at 9500 kg per square centimeter at 30° and then diluted threefold with 0.05 *M* tris buffer of pH 7.5. The inactivated TAA solutions were used for reactivation. Plots show the time course of the reactivation under atmospheric pressure at 30° of the TAA inactivated by compression for 10 minutes (—○—) and 60 minutes (—○—), and under recompression at 800 kg per square centimeter at 30° for 10 minutes (—⦶—) and 60 minuues (—◐—).

centimeter for 10 minutes at 30° would not reactivate on standing under atmospheric pressure; however, presence of buffer (pH 7.5) was responsible for reactivation.) The rate of the reactivation decreased with increasing compression time for inactivation. After compression for 60 minutes at 9500 kg per square centimete, the activity of TAA no longer recovered under atmospheric pressure; however, moderate recompression of the same inactivated TAA caused a distinct reactivation.

DISCUSSION

On the basis of the loss and recovery of enzyme activity on high-pressure treatment up to 10,000 kg per square centimeter, we can classify enzymes into four groups: (I) completely inactivated and irreversible; (II) completely inactivated and reversible (at least partially); (III) incompletely inactivated and irreversible; and (IV) incompletely inactivated and reversible. Several enzymes such as salivary amylase (8), barley β-amylase (9), and bacterial α-amylase (10) belong to type I, whereas trypsn (11), chymotrypsin (12), and, bacterial Al-proteinase (13) belong to type III. No enzyme which belongs to type IV is definitely known at present. Trypsin, chymotrypsin, and bacterial Al-proteinase may, however, belong to this type; it is difficult to be sure because of autodigestion.

According to our experimental results TAA is of type II under certain conditions. On campression for a short period (less than 60 minutes) at 9500 kg per square centimeter, the activity of TAA falls to zero, but when the pressure is released, the activity is recovered to a consideable extent. The extent of recovery of activity is dependent on the initial concentration of enzyme, pH, and temperature, but almost independent of ionic strength above a certain value, at least under atmospheric pressure.

We have found that a re-treatment under moderate pressure of pressure-inactivated TAA enhanced the recovery. As described in this paper, it is generally accepted that the molar volune of proteins increases on denaturation and decreases on renaturation. Below 800 kg per square centimeter in the recompression ΔV^* may be accounted for in view of the over-all volume change of activation in the recovery process. A moderate recompression may assist the folding of the protein which has been unfolded by very high pressure. Within this pressure range ΔV^* is negative and its absolute magnitude increases with increasing pressure; but above about 1200 kg per quare centimeter other conformational changes which are unfavorable to recovery may occur on the unfolded TAA, though a pressure up to 2000 kg per square centimeter (cf. Fig. 2) does not cause inactivation. If this is so, ΔV^* for the process of reactivation becomes positive, and enhancement of the reactivation may be decreased with increasing pressure. Above 3000 kg per square centimeter the pressure inactivation can occur by a prolonged compression, as shown in Fig. 2; a decrease in the extent of the reactivation is then to be expected.

As shown in Fig. 6, the extent of recouery is larger when inactivation is weak. This result is as expected because in the weak inactivation the conformational change of the protein molecule itself is presumed to be small, so that the recovery of enzyme activity as well as conformation is readily occurred. Prolonged compression at very high pressure, for example, 60 minutes at 9500 kg per square centimeter, as shown in Fig. 9, makes the inactivation at atmospheric pressure irreversible, though recompression at a moderate pressure causes reactivation. In the case of compression for 10 minutes at the same pressure, the activity is recovered gradually despite the complete initial loss of activity. This result may be explained if the conforma-

tion of the TAA molecule may be changed so drastically by a prolonged compression that the recovery to the native form is impossible even when the pressure is released. These results suggest that the recovery of enzymic activity may be dependent upon the re-establishment of the molecular conformation of the enzyme molecule as a whole.

As shown in Fig. 6, the reactivation proceeds well up to 30°, above which temperature the extent of the reactivation decreases. In our preliminary experiments, prolonged standing of TAA in buffered solution (pH 7.5 with 0.03 *M* tris buffer) at 40° indicates a slow heat inactivation. The reactivation of pressure-inactivated TAA may be prevented by this heat inactivation at a temperature above 30°. The most favorable range of pH for the reactivation is that of neutral pH. Takagi *el al.* (14, 15) suggested that carboxylate-phenolic hydrogen bonds play a part in stabilizing the native structure of TAA, that these bonds cannot be formed below pH 5.5, and that folding becomes impossible above pH 9.5. In the present investigation, results obtained on the pH effect are the same as the results on the recovery of acid-denatured and urea-denatured TAA (16, 17). As shown in Fig. 8 the reactivation of pressure-inactivated TAA under atmospheric pressure is influenced by salt concentrations below 10^{-3} mole per liter at least. But by recompression at 800 kg per square centimeter, the reactivation is noticeably retarded by increasing the salt concentration. Seemingly, the existence of a considerable concentration of salt prevents the unfolded enzyme molecule from being refolded to the enzymatically active canformation by recompression at a moderate pressure.

Unlike the results obtained in heat inactivation (18), it has been observed during pressure inactivation that the higher the initial concentration of TAA more extensive is the reaction. This difference may offer some clues to indicate the difference

in the inactivation mechanisms by heat.

A fuller interpretation of the relationship between enzymatic activity and molecular conformation will be postponed until the completion of work in which some physicochemical properties are being investigated, as well as activity.

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REFERENCES

1. MIYAGAWA, K.,; AND SUZUKI, K., *Arch. Biochem. Biophys.* **105**, 297(1964)
2. ISEMURA, T., *J. Japanese Biochem. Soc*' **37**, 1 (1962).
3. TONGUR, V. S., AND TONGUR, A. M., *Chem. Abstr.* **46**, 2102 (1952).
3a. TONGUR, V. S., *Chem. Abstr.* **47**, 643 (1953).
3b. TONGUR, V. S., AND KAZMINA, N. A., *Chem. Abstr,* **44**, 10012 (1950); **49**, 8679 (1955).
4. AKABORI, T., AND HAGIHARA, B. *J. Biochem.* **41**, 577 (1954).
5. AMANO, T., ISOJIMA, S., AND FUJINO, H., *Med. J. Osaka Univ.* **5**, 333 (1954).
6. SUZUKI, K., *Rev. Phys. Chem. Japan* **28**, 24 (1958).
7. NOELTING, G., AND BERNFELD, P., *Helw. Chim. Acta.* **31**, 286 (1948).
8. SUZUKI, K., KITAMURA, K., UTSUNOMIYA, H., AND IKEMOTO, H., *Memorirs Res. Inst. Sci. Eng. Ritumeikan Univ.* **6**, 21 (1961).
9. JONO, K., SUZUKI, C., K., AND KITAMURA, K., *Symp. High Pressure* **2**, 48 (1960).
10. SUZUKI, K., AND KITAMURA, K., *J. Biochem.* **54**, 214 (1963).
11. MIYAGAWA, K., AND SUZUKI, K., *Rev. Phys. Chem. Japan* **32**, 43 (1962).
12. MIYAGAWA, K., AND SUZUKI, K., *Rev. Phys. Chem. Japan* **22**, 51 (1962).
13. SUZUKI, AND KITAMURA, K., Unprublished Observation.
14. TAKAGI, T., AND ISEMURA, T., *J. Biochem.* **48**, 781 (1960).
15. TAKAGI, T., AND ISEMURA, T., *J. Biochem.* **49**, 43 (1961).
16. TAKAGI, T., AND ISEMURA. T., *J. Biochem.* **52**, 16 (1962).
17. TAKAGI, T., ISEMURA, T., *J. Biochem.* **52**, 314 (1962).
18. NAKAYAMA, S., AND KONN, Y., *J. Biochem.* **44**, 25(1957).