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Studies on Taka-Amylase A under High Pressure

IV. Influence of Inital Concentration of Enzyme upon Pressure Inactivation and Reactivation

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The influence of initial concentration of enzyme (1-10^{-4}%) at pH 6.5, 7.5, and 9.0 upon pressure-inactivation and reactivation was studied. Inactivation and reactivation appeared to follow first-order kinetics; however, the more concentrated the enzyme solution, the greater the rate of inactivation and reactivation. In the equation, $k=\alpha Ci^{\beta}$, for the fist-order rate constants of both processes, k, and initial concentration of TAA, C_i, where α and β are constants at a definite pH, temperature, and pressure, the values of β were 0.14, 0.08, and 0 for inactivation and 0.06, 0.06, and 0 for reactivation at pH 6.5, 7.5. and 9.0, respectively. The rate constants are independent of concentration at pH 9.0, while an increasing dependence is found at lower pH values. In sedimentation studies, pressure-denatured TAA did not associate at pH 9.0, while association was observed at pH 7.5 and 6.5, with the degree of association increasing with decrease of pH. The influence of concentration on pressure-inactivation and reactivation may be attributed to association of the enzyme.

This paper reports a continuation of investigations on the high pressure inactivation and reactivation of Taka-amylase A (Asp. Oryzae α-amylase, TAA) (1—3), In an earlier paper (1), it was shown that the rate of pressure-inactivation increases with concentration at pH 5.5 in spite of the fact that the reaction odeys first-order kinetics. This phenomenon has also

been found in the pressure-inactivation of trypsin (4) and chymotrypsin (5). By contrast, in the heat-inactivation of sweet potato β-amylase and Taka-amylase A (6), the rates decrease with increase of enzyme concentration.

Earlier sedimentation experiments (3) showed that pressure-modified TAA is associated at pH 7.5, a pH far from the isoelectric point, 3.8, though at pH 9.0 no association occurs.

Thus it may be expected that at lower pH values, association may be one of the causes of inactivaion. In our earlier experiments measurements of concentration dependence were in a lower and narrower range, and the pH was only 5.5.

Experiments reported in this paper show that intermolecular-interaction in fact may be one of the causes for inactivation.

MATERIALS AND METHODS

TAA was prepared from "TAKA-diastase Sankyo" according to the method of Akabori *et al.* (7), and thrice-crystallized with acetone. Rivanol treatment was repeated twice to eliminate the contamination of acid Taka-protease (8). Aqueous TAA solution was dialyzed against 0.1 *M* potassium chloride and stored as about 5 per cent solution in a refrigerator. The concentrion of TAA was estimated spectrophotometrically, assuming the extinction coefficient in water to be $E^{1\%}_{1cm} = 22.1$ at 278.5mμ. The stock solution was dialyzed against distilled water and then diluted to a given concentration with 0.05 *M* acetate buffer (below pH 6.5) or 0.05*M* tris buffer (above pH 7), The high pressure apparatus and procedures for inactivation were the same as previously reporterd. After compression at several thousand kilograms at 30°, the pressure was released and the following measurements were carried out.

Amylase activity was measured at pH 5.5 (0.1 *M* acetate buffer) at 30° with

soluble starch as substrate according to the method of Noelting and Bernfeld (9).

Turbidity was measured by transmission at 500 mμ in a Hitachi EPU2A- photoelectric spectrometer.

Sedimentation experiments were conducted at 23° at 54, 700 rpm in a Hitachi model UCA-1 ultracentrifuge. Ionic strength of the solvent was kept at 0. 15 in the sedimentation experiments.

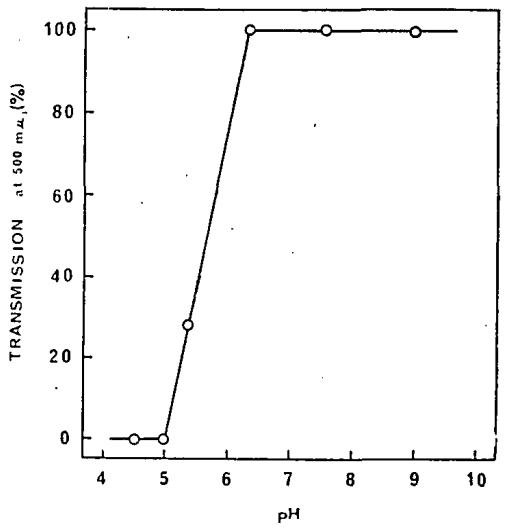

FIG. 1. Turbidty of TAA induced by pressure at various pH values after release of pressure. TAA solutions (1%) at various pH values (below pH 6. 5, 0. 05 *M* acetate buffer and above pH 7, 0. 05 *M* tris buffer) compressed at 9500 kg/sq. cm. for 10 miantes. After release of pressure the absorbance at 500 mμ was measured.

RESULTS

Turbidity and association of TAA at various pH due to pressure treatment. Figure 1 shows the turbidity of TAA solutions at various pH values due to pressure treatment. One per cent TAA in buffer was compressed at 9500 kg per square centimeter for 10 minutes at 30° After the pressure was released, the tur-

bidity was measured. Below about pH 6.0, the turbidity rapidly increases and at pH 4.5 TAA forms a gel. Above pH 6.0 no appreciable turbidity was observed.

In earlier sedimentation experiments (3), association of TAA induced by high pressure was found at pH 7.5, though turbidity was apparently not observed. In the present work, a sedimentation experiment was carried out on pressure-denatured TAA (1%) which had been compressed at 9500 kg per square centimeter for 120 minutes at pH 6.6. As shown in Fig. 2 [photographs are shown with earlier results (3)], pressure-denat-

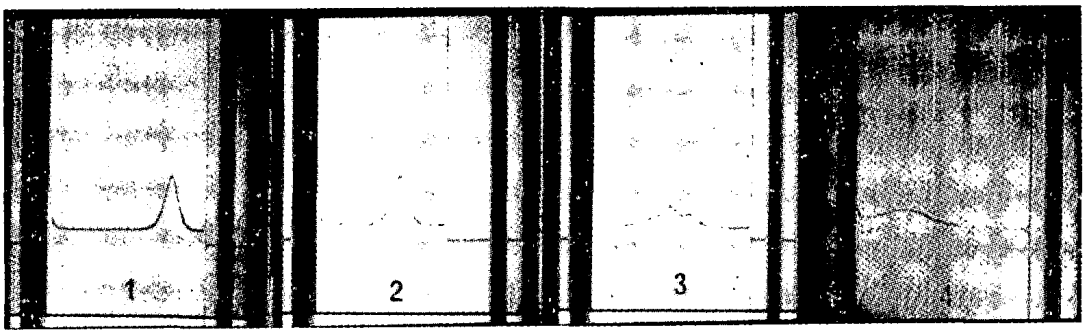

Fig. 2. Sedimentation diagrams of native TAA at pH 9.0 (1), pressure-denatured TAA (9500 kg/sp. cm for 120 minntes) at pH 9.0 (2), pH 7.5 (3), and pH 6.5 (4). Concentration of TAA was 1%. Photographs of (1), (2), and (3) were taken at 60 minutes (55,400 rpm) and (4) at 30 minutes (54,700 rpm) after reaching full speed.

ured TAA at pH 9.0 was monodisperse, while at pH 7.5 and 6.5 it was polydisperse. The sedimentation constants are given in Table I. The high values for the sedimentation constants and the very broad sedimentation pattern at pH 6.5 suggest that pressure-denatured TAA at pH 6.5 is highly associ-

TABLE I

SEDIMENTATION CONSTANTS OF NATIVE AND PRESSURE-DENATURED TAA WHICH WAS COMPRESSED AT 9500 KG/SQ. CM FOR 120 MINUTES AT 30°

	pH	S
Native TAA	9.0	3.9
	9.0	3.5
Pressure-denatured TAA	7.5	(f)7.6
		(s)6.0
	6.5	23,6

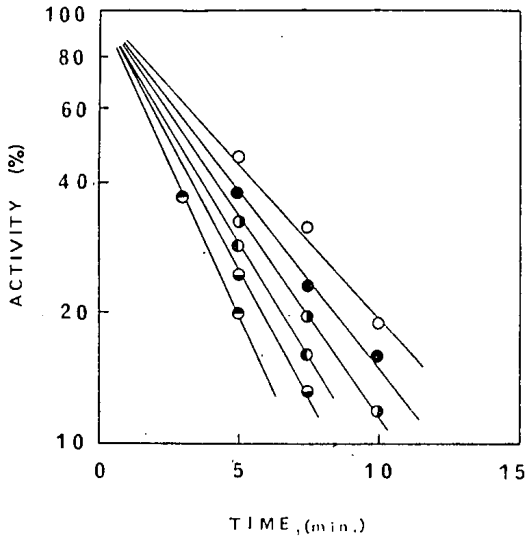

FIG. 3. Time courses of pressure-inactivation at various concentrations of TAA. TAA at pH 9.0, 10^{-4}% (●), pH 7.5, 10^{-4}% (○), 10^{-3}% (◑), 10^{-2}% (◑), 10^{-1}% (◑). and 10^{0}% (◒) compressed at 8000 kg/sq. cm at 30°.

ated; however, at pH 9.0 no association was observed.

Effect of initial concentration on pressure-inactivaation. The time course of inactivation was examined at various initial concentrations of TAA at pH 6.5, 7.5, and 9.0. TAA solutions were compressed at 8000 kg per square centimeter at 30°.

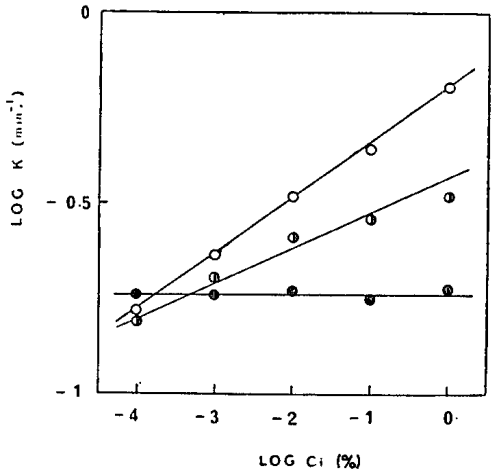

FIG .4. Plots of log k vs. log Ci. TAA solutions of various concentrations at pH 6.5 (○), 7.5 (◑), and 9.0 (●) were compressed at 8000 kg/sp. cm at 30°. Rate constant is k, initial concetration Ci.

Some of the results are shown in Fig. 3. The figure shows that a linear relation is satisfied, and the process is consistent with first-order kinetics. Logarithms of rate constants, k, versus logarithms of initial concentration of TAA, Ci, are plotted as shown in Fig. 4. At pH 6.5, and 7.5, the rates of pressure-inactivation are dependent on the initial concentration of TAA; however, no dependence is found at pH 9.0.

It is convenient to show the relationship between the rate constant, k, and the initial concentration of enzyme, Ci in the equation

$$k = Ci\beta,$$

where α and β are constants under the inactivation condition studied. From Fig. 4, the slopes at pH 9.0, 7.5, and 6.5 were found to be 0, 0.08, and 0.14, respecttvely. Moreover, the rates of inactiva iontseem to converge at lower initial con-

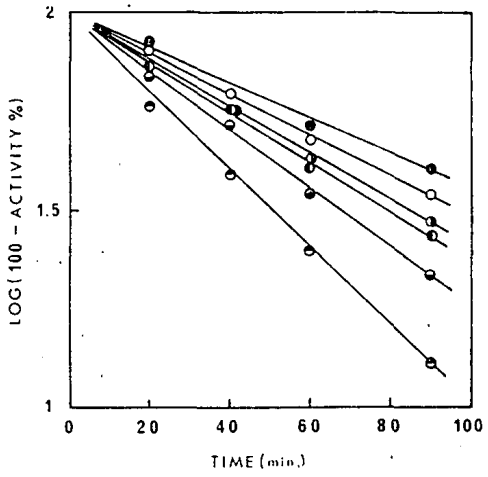

FIG. 5. Time courses of reactivation at various concentrations of TAA. TAA at pH 9.0, 10^{-4}% (●), pH 7.5, 10^{-4}% (○), 10^{-3}% (◑), 10^{-2}% (◑), 10^{-1}% (◒), and 10^{0}% (◓) compressed at 9500 kg/sq. cm for 10 ninutes at 30°.

centrations, about 10^{-4}%.

Effect of initial concentration on recovery of activity. Dependence of the recovery of activity on the initial concentration was examined. TAA solutions at various initial concentrations at

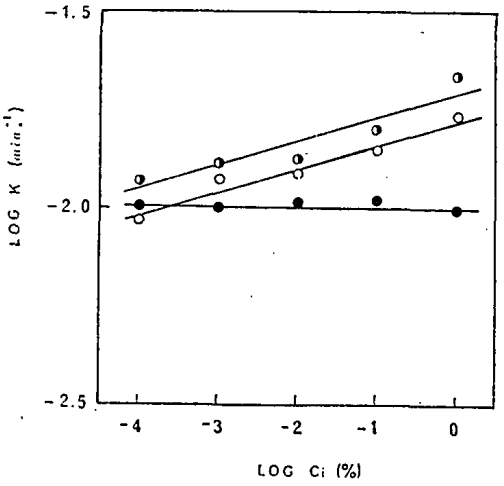

FIG. 6. Plots of log k vs. log Ci for reactivation. TAA at pH 6.5 (○), 7.5 (◑). nd 9.0 (●) compressed at 9500 kg/sq. cm for 10 minutes at 30°, Rate constant is k, initial concentration Ci,

pH 6.5, 7.5, and 9.0 were compressed at 9500 kg per square centimetre for 10 minutes at 30°, and, after release of pressure, each solution was kept at 30°. Figure 5 shows some kinetics of the reactivation process. Linear relations are satisfied at various initial concentrations of TAA. Logarithms of the initial concentration Ci, are plotted in Fig. 6. The rates of reactivation were dependent on the initial concentration at pH 6.5 and 7.5, but not at pH 9.0. The relationship between the rate constant, k, and the initial concentration of enzyme Ci, was formulated as before and the values of β were found to be 0.06, 0.06, and 0, at pH 6.5, 7.5 and 9.0, respectively.

DISCUSSION

Since processes of protein denaturation involve the transformation of a single reactant species, the protein itself, it is to be expected that the reaction would be unimolecular. In fact, a number of experimental results have shown that denaturation is first order. However, there are many cases in which denaturation is dependent on the initial concentration of protein, though the process itself is first order. For example, heat inactivation of pancreatic amylase (10), sweet potato β-amylase and Taka-amylase A (6) exhibit first order kinetics but an order less than unity with respect to concentration. These enzymes are more stable with increasing concentration. By contrast, in pressure-inactivation, the rate increases with initial concentration of enzyme. As shown in Fig. 4, concentration dependence was observed at pH 6.5 and 7.5, but not at pH 9.0.

Moreover, from the results of sedimentation experiments (Fig. 2 and Table I), it was found that TAA was associated by pressure at pH 6.5, and 7.5, but not at pH 9.0 and the degree of association was higher at pH 6.5 than 7.5. It seems probable that the dependence of pressure-inactivation on the initial concentration of enzyme may be attributed to association. Active sites of TAA molecules may be masked in the aggregated state. Intermolecular interaction would be facilitated at higher concentration. At lower initial concentration (about 10^{-4}%), the rates of inactivation at pH 6.5, 7.5, and 9.0 seem to converge. Presumably, at high dilution, TAA does not associate, even at lower pH.

As shown in Fig 6, at pH 6.5 and 7.5 the extents of recovery were dependent on the initial concentration of TAA. It may be reasonable to assume that the extent of alteration at higher concentrations might be smaller than that at lower concentrations. At higher concentration, though inactivation is rapid, intramolecular alteration might be prevented by intermoleculear associtionn. If this is so, it is conceivable that renaturation of TAA which was denatured at higher concentration should occur easily after releasing the pressure.

As already described, at pH 6.5, the degree of association is higher than at pH 7.5, and the extent of reactivation should be greater than at pH 7.5. However, reactivation should also be controlled by the concentration of hydrogen ion (2), so that at pH 6.5 no greater reactivation might occur than at pH 7.5.

A mechanism may be postulated to explain the main features

of the pressure-denaturation and renaturation of TAA. The essential feature of the proposed mechanism is that pressure-denaturation is a cooperative phenomenon, involving the participation of n molecules of TAA which undergo denaturation. Moreover, denaturation is reversible. The process may be represented as,

$$nP \xrightarrow{(1)} Pn \xrightarrow{(2)} Pnd \quad \text{(under high pressure)}$$

$$nP \xleftarrow{(1)'} Pn \xleftarrow{(2)'} Pnd \quad \text{(after releasing the pressure)},$$

where P is the native TAA, Pn the clustered form, and Pnd the clustered and denatured TAA.

Under high pressure, n molecules of native TAA cluster to Pn at a time, so that the concentration of Pn becomes one-nth that of native enzyme. Pn reverts to native enzyme at once when the pressure is released; therefore, the measured activity corresponds to n times the concentration of Pn. The denaturation process is step (2), so that the over-all kinetics will be first order.

It is not unreasonable to suppose that n is a function of pH and the initial concentration of TAA at constant temperature and pressure; n would become larger with decreasing pH and increasing concentration of TAA. The rate of denaturation (step 2) therefore would become larger with increase of n. After releasing the pressure, the renaturation process is step $(2)'$ and the rate of step $(2)'$ is controlled by the state of Pnd; intramolecular alteration would be smaller with increase of n

as already postulated and the rate of renaturation would become larger with increase of n.

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