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Studies on Taka-Amylase A Under High Pressure

I. Some Kinetic Aspects of Pressure Inactivation of Taka-Amylase A[1]

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Pressure inactivation of Taka-amylase A was examined at a pressure range of 5000–10,000 kg./sq. cm., and at temperatures of 10–50°C. The inactivation of Taka-amylase A at pH 5.5 started at about 6000 kg./sq. cm. and ended at about 9000 kg./sq. cm. for 5 minutes pressing at 30°C. Inactivation was slightly influenced by the initial concentration of enzyme above concentration of about 0.006 mg N/ml., but in the lower concentration the enzyme was more resisant to pressure. The process of the inactivation shows first-order kinetics, and the thermodynamic properties of the rate process were calculated. The activation free energy, activation enthalpy, activation entropy, and activation volume were 21∼24 kcal./mole, 5∼11 kcal./mole, −32 ∼ −49 e.u., and −38∼ −52 cc./mole, respectively. These values are of the same order as those of enzymes previously reported by us. Activity of the pressure inactivated Taka-amylase A was recovered when pressure was released. And the extent of the reactivation was influenced by pH and ionic strength.

INTRODUCTION

A series of studies has been made on the inactivation and

denaturation of globular proteins and enzymes by high pressure. We have already reported on the kinetics of pressure inactivation of the enzymes trypsin (1), chymotrypsin (2), salivary α-amylase (3), and bacterial α-amylase (4). These enzymes were inactivated by compression under several thousand kilograms per square centimeter, and the process of inactivation followed first-order kinetics. The values of thermodynamic quantities were of the same order in those enzymes, except for a few cases,[2] and the approximate values of activation free energy, activation enthalpy, activation entropy, and activation volume were 20 $\sim$ 26 kcal./mole, 4 $\sim$ 8 kcal./mole, $-40 \sim -70$ e.u., and $-19 \sim -40$ cc./mole, respectively.

It is well known that several enzymes which are denatured by acid and urea are reversible under appropriate conditions. The reactivation, however, was not found on the above pressure-inactivated enzymes, at least under the conditions studied. It is generally accepted that the biological activity of an enzyme arises from an active site located in some part of the molecule, and also depends on the conformation of the molecule. Since protein denaturation is a phenomenon reflecting the change of the conformation of the molecule, it seems worthwhile to investigate the relation between the loss of the biological activity and the change of the conformation of molecule responsible for the denaturation. Proteolytic enzymes such as trypsin and chymotrypsin are not adequate for this study because of their autodigestion, i.e., the denatured enzyme protein is digested by the remaining active enzyme. Bacterial α-amylase is not adequate either, because, when pressure treated and at concentrations above 0.01%, it was turbid over the wide range of pH examined. Taka-amylase A seems to be a favorable case since it could be easily obtained in a large quantity in a crystalline state. Much valuable information on the enzymatic activity and structure

of Taka-amylase A has been published by Isemura and his co-workers, who studied the denaturation and recovery of Taka-amylaseA with several denaturants (5).

In this paper we shall first present the results of the pressure inactivation of Taka-amylase A from the standpoint of chemical kinetics in comparison with the previous results of the enzymes already experimented on by us. We shall also report the reversibility of the pressure-treated Taka-amylase A after the pressure is released.

[1]Presented at the 35th Annual Meeting of the Japanese Biochemical Society, Tokyo, October, 1962.

[2]The values of 21 kcal./mole in activation enthalpy and —2 e.u. in activation entropy in bacterial α-amylase are considerably larger than others (4).

EXPERIMENTAL

MATERIALS

Crystalline Taka-amylase A (TAA) (Asp. Oryzae α-amylase) [EC. 3.2.1.1.] was kindly supplied by Dr. Toda of the Institute for Proteins, Osaka University, who prepared it from Taka-Diastase Sankyo by the method of Akabori *et al.* (6). The enzyme was dissolved in 0.05 *M* acetate buffer at pH 5.5 to give about a 5% solution; this was stored in a refrigerator as a stock solution. For experiments, the stock solution was diluted with the same buffer to an appropriate concentration of the enzyme. In the experiments to examine the pH effect, the above stock solution was dialyzed against distilled water; the pH was then adjusted with 0.1 *N* HCl and 0.1 *N* NaOH. The concentration of TAA was estimated by the amount of nitrogen per mililiter of enzyme solution, which was determined by the micro-Kjeldhal method.

Procedure

Amylase activity was measured at pH 5.5 at 30°C. by determining its dextrinizing power with dinitro-salicylic acid according to the method of Noelting and Bernfeld (7); 1 ml. of enzyme solution and 1 ml. of 1% soluble starch solution at pH 5.5 preheated in the thermostat at 30°C. were mixed. After incubation for exactly 3 minutes, the enzyme reaction was stopped with 2 ml. of 1% dinitro- salicylic acid reagent. The mixture was boiled for 5 minutes and then cooled. After the colored solution was diluted tenfold with distilled water, the optical density of the solution was measured at 530 mμ. Under these conditions the optical density may be directly taken as the measure of enzyme activity, because all enzyme concentrations are within the range where the optical density is proportional to the enzyme activity.

The high-pressure apparatus and procedures were the same as in the previous report (8). A test solution of TAA was sealed into a polyvinylchloride sack and placed in the high-pressure chamber. After a definite compression, the sack was taken out, and the amylase activity was measured as soon as possible since the activity of pressure-treated TAA was recovered when the pressure was released, as described later.

RESULTS

Effect of Initial Concentration of Enzyme, Pressure Magnitude, Temperature, and pH

To examine the effect of initial concentration of enzyme on the pressure inactivation, the sample solution at pH 5.5 of diffe-

rent concentrations (0.0013–0.025 mg. N/ml.) were compressed for 5 minutes at a pressure of 8000 kg./sq.cm. at 30°C. The results are shown in Fig. 1, which illustrates that at the higher con-

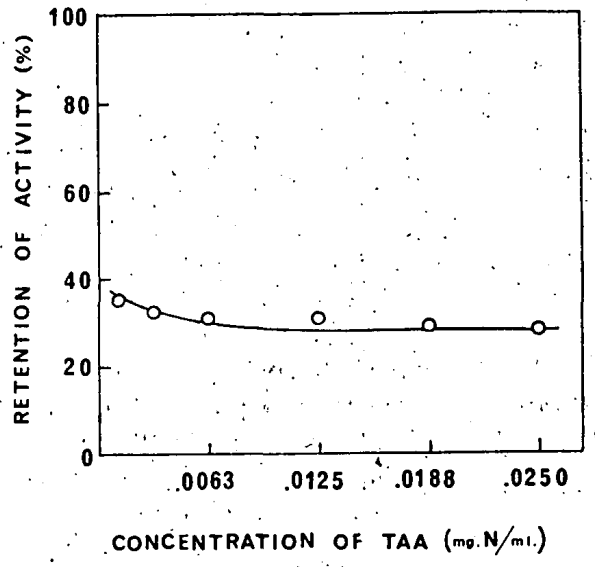

FIG. 1. The effect of initial concentration of TAA on pressure inactivation. Six samples of different concentrations of TAA in 0.05 *M* acetate buffer at pH 5.5 were compressed for 5 minutes at 8000 kg./sq. cm. at 30°C.

centration (above about 0.006 mg. N/ml.) the effect of initial concentration is almost absent; a slight effect is observed at the lower concentration, i.e., the enzyme at the lower concentration is more resistant to the pressure inactivation.

The effect of pressure magnitude on the pressure inactivation of the enzyme was examined in the range of pressure up to 10000 kg./sq.cm. The enzyme solutios of 0.0063 mg. N/ml. at pH 5.5 were compressed for 5 minutes at each pressure indicated, and at each temperature of 10–50°C. Figure 2 shows the results of this experiment. The inactivation, which lasted 5 minutes, started at about 6000 kg./sq.cm. and ended at about 9000 kg./sq.cm. at 30°C. The figures also show that the temperature coefficient of pressure inactivation is positive.

The pH dependence of the pressure inactivation was studied

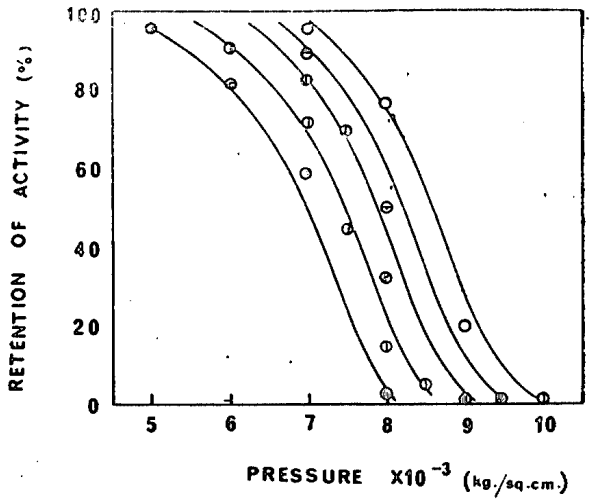

FIG. 2. The effect of pressure magnitude and temperature on the pressure inactivation. TAA solutions (0.0063 mg. N/ml.) in 0.05 *M* acetate buffer at pH 5.5 were compressed for 5 minutes at 5000-10,000 kg./sq. cm. at temperatures of 50°C. (—◉—); 40°C. (—◑—); 30°C. (—◐—); 20°C. (—⊖—); and 10°C. (—○—).

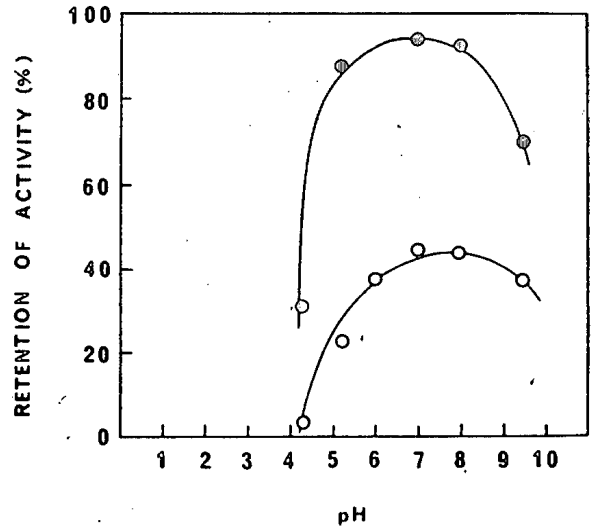

FIG. 3. The effect of pH on the pressure in activation and the reversibility of pressure inactivated TAA at various pH. TAA solution (0.0063 mg. N/ml.) at each pH, adjusted with 0.1 *N* HCl and 0.1 *N* NaOH, was compressed for 5 minutes at 8000 kg./sq. cm. at 30°C. After the pressure was released, in the first case the enzymatic activity was immediately measured (—○—), and in the second case after standing for 80 minutes at each pH at 30°C. (—◉—).

at various pH values at a pressure of 8000 kg./sq.cm. at 30°C. The results are shown in Fig. 3. In the neutral pH range, the enzyme was more stable against Pressure than in the acid or alkaline ranges.

Time Course of Inactivation by Pressure

TAA in acetate buffer at pH 5.5 was compressed at different pressures and tempratures, and residualy activities were plotted against time in a semilogarithmic scale. As illustrated in Fig. 4, straight lines were obtained under these experimental conditions, showing that the pressure inactivation of TAA follows first-order kinetics.

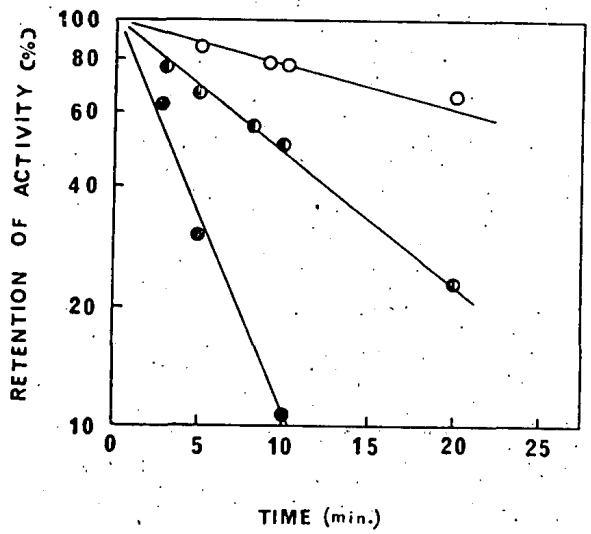

Fig. 4. Time course of the pressure inactivation. TAA solutions (0.0063 mg. N/ml.) in 0.05 *M* acetate buffer at pH 5.5 were compressed at pressures of 7000 kg./sq. cm. (—○—), 7500 kg./sq. cm. (—◐—), and 8000 kg./sq. cm. (—●—), at 30°C.

Recovery of Activity of ressure Inactivated Enzyme

In order to ascertain whether the inactivation of TAA by pressure treatment is reversible, the following experiment was

carried out: The enzyme solutions at various pH were compressed for 5 minutes at 8000 kg./sq.cm. at 30°C., and the pressure was then released. The pressure-treated samples were kept at 30°C. under atmospheric pressure, and after 80 minutes had elapsed, the enzymatic activties were measured. As shown in Fig. 3, the activity of pressure-inactivated TAA was recovered. Reversibility was appreciable, and the optimum pH range for recovery of activation was 6–8. Figure 5 shows that the extent

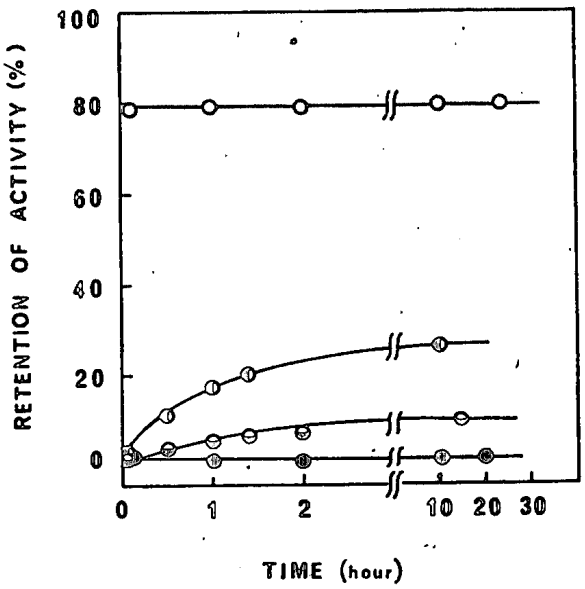

FIG. 5. The effect of ionicstrength on the reactivation of pressure-inactivated TAA. TAA solutions (0.0063 mg mg. N/ml.) in 0.05 *M* acetate buffer at pH 5.2 with ionic strengths of 0.1 *M* (—◐—), 0.01 *M* (—⊖—), and 0.001 *M* (—◐— by the addition of sodium chloride were compressedat 9500 kg./sq. cm., and a TAA solution of ionic strength of 0.1 *M* (—○—) was compressed at 7000 kg./sq.

of reactivation was influenced on the ionic strength of sodium chloride in buffer solution at pH 5.5 The lower the ionic strengths, the greater the reactivation. In ionic strength of 0.1, the recovery of enzymatic activity was almost not observable.

DISCUSSION

As shown in Fig. 4, the inactivation of TAA follows first-or-

der kinetics. Since a semilogarithmic plot of the rate constant against the pressure gives a straight line, as shown in Fig. 6, the actvation volume ΔV^* is calculated from this slope according to the equation:

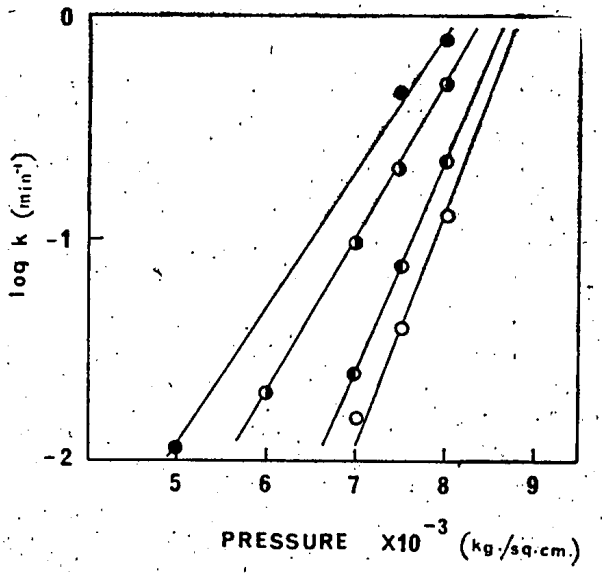

FIG. 6. Relation between logarithm of rate constant k and pressure at temperatures of 20°C. (—○—), 30°C. (—◐—), 40°C. (—◑—), and 50°C. (—●—). TAA solutions (0.0063 mg. N/ml.) in 0.05 M acetate buffer at pH 5.5 were used.

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^*}{RT},$$

where k is the rate constant, p the pressure, R the gas constant, and T the absolute temperature. The calculated values are listed

TABLE I

MOLAR VOLUME CHANGE OF ACTIVATION, ΔV^* (pH 5.5)

	Temperature (°C.)			
	20	30	40	50
ΔV^* (cc./mole)	−52	−50	−42	−38

in Table I. As shown in this table, the values of ΔV^* are negative over the whole range of temperature examined, but absolute values decrease as the temperature increases.

The studies on the dependence of the rate constant on tempe-

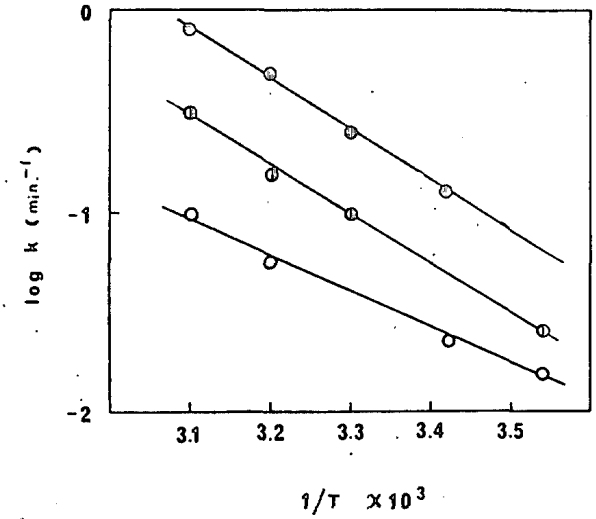

FIG. 7. Relation between logarithm of rate constant k and recipr oalɔof absolute temperature T at pressures of 7000 kg./sp. cm. (— ‸—), 7500 kg./sq. cm. (—◑—). and 8000 kg./sq. cm. (—◉—). TAA solutions (0.0063 mg. N/ml.) in 0.05 M acetate buffer at pH 5 were used.

rature give the values of apparent activation energy, E. As shown in Fig. 7, which gives the relation between the logarithm of rate constant and the reciprocal of the absolute temperature; straight lines were obtained. The apparent activation energies are calculated from the slopes and are listed in Table II.

TABLE II

APPARENT ACTIVATION ENERGY, E (pH 5.5)

	Pressure (kg.sq./cm.)		
	7000	7500	8000
E (kcal./mole)	8.3	10.6	12.0

TABLE III

KINETICS OF INACTIVATION OF TAA BY PRESSURE

(pH 5.5)

P (kg./sq. cm.)	Temp. (°C.)	k (sec^{-1})	ΔF^* (kcal./mole)	ΔH^* (kcal./mole)	ΔS^* (cal./deg. mole)
5000	50	1.7×10^{-4}	24		
6000	40	3.2×10^{-4}	23		
	50	5.8×10^{-4}	24		
7000	10	2.6×10^{-4}	22	8	−49
	20	3.8×10^{-4}	22	5	−45
	30	4.0×10^{-4}	23	8	−49
	40	9.6×10^{-4}	23	8	−48
	50	1.7×10^{-3}	23	8	−48
7500	10	6.2×10^{-4}	21	10	−39
	20	6.7×10^{-4}	22	10	−38
	30	1.1×10^{-3}	22	10	−39
	40	3.5×10^{-3}	22	10	−38
8000	10	6.7×10^{-4}	21	11	−32
	20	2.2×10^{-3}	21	11	−32
	30	3.8×10^{-3}	21	11	−32
	40	8.9×10^{-3}	21	11	−32

From the equations of the theory of absolute reaction rates, the thermodynamic properties of the activated state, i.e., the free energy ΔF^*, the enthalpy ΔH^*, and the entropy ΔS^* of activation, are calculated by the following equations:

$$\Delta F^* = RT \ ln\frac{KT}{hk}$$

$$\Delta H^* = E - RT$$

$$\Delta S^* = \frac{\Delta H^* - \Delta F^*}{T}$$

where k is the rate constant, K is the Boltzman constant, h is the Planck constant, T is the absolute temperature, E is the

apparent activation energy, and R is the gas constant. The results obtained are summarized in Table III.

The values of thermodynamic quantities found in these Tables are of nearly the same order as those found for trypsin (1), chymotrypsin (2), salivary α-amylase (3), and bacterial α-amylase (4). Moreover, it must be emphasized as common features of these reactions that ΔF^* has a comparatively small positive value, and ΔS^* and ΔV^* are both negative (the values for bacterial α-amylase are considerably larger than the others). However, the effect of pH on the pressure inactivation is very different in different enzymes, i.e., trypsin and chymotrypsin are more resistant at the acidic pH, while salivary α-amylase and bacterial α-amylase are more resistant at alkaline range. TAA is much more stable in the neutral pH range.

It is interesting to note that the pressure inactivation of TAA is reversed when the pressure is released, as shown in Fig. 3. This phenomenon was not observed in our previously examined enzymes under our experimental conditions. To explain these different results in reversibilty between enzymes, it will be necessary to investigate the relation between the loss of enzymatic activity and the change of the molecular conformation. Such studies are now in progress.

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