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PRESSURE INACTIVATION OF ENZYME*

Some Kinetic Aspects of Pressure Inactivation of Trypsin

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The inactivation process of trypsin by high pressure of 5000–10000 kg/cm² has been examined kinetically at temperature of 15–45°C.

The results are as follows: the inactivation of trypsin increases with pressure increase, but above the critical pressure (about 8000 kg/cm²) no more inactivation occurs. The process of inactivation is of the first order kinetics, and thermodynamic quantities of inactivation process are similar to the protein denaturation of ovalbumin and hemoglobin by pressure except the sign of enthalpy of activation, i. e. $\Delta F^* > 0$, $\Delta H^* > 0$, $\Delta S^* < 0$, $\Delta V^* < 0$.

Introduction

In our previous papers[1~3], the kinetics of the denaturation of alubumin and hemoglobin under high pressure was studied mainly by examining solubility of the proteins and it was observed that the rate process of the denaturation of the proteins is of the first order with respect to protein concentration and the values of ΔS^* and ΔV^* are negative.

As for enzymes, early investigators, Basset and Macheboeuf[4] investigated the pressure inactivation of yeast invertase, fungal and tree laccase, and Mathews, Dow and Anderson[5] of pepsin and rennin, and Curl and Jansen[6] of trypsin, chymotrypsin, pepsin and chymotrypsinogen. Kinetic treatment, however, has not been performed, and the mechanism of pressure inactivation still remains unclarified.

Though it has been already reported that trypsin is not perfectly inactivated under pressure range 6000–9000 bars, it seems to be interesting to investigate whether we can treat this reaction kinetically and to see whether its kinetic behavior is of the same nature as those observed for the pressure denaturation of protein.

This paper is concerned with confirming the results of Curl and Jansen[6] and trying the kinetic treatment of the inactivation of trypsin under high pressure.

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1) K. Suzuki, *Memoires Res. Inst. Sci. and Eng., Ritumeikan Univ.*, **2**, 19 (1957); *ibid.*, **3**, 1 (1958)
2) K. Suzuki, *This Journal*, **28**, 24 (1958); *ibid.*, **29**, 91 (1959)
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5) J. E. Mathews, R. B. Dow and A. K. Anderson, *J. Biol. Chem.*, **135**, 697 (1940)
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Experimentals

Material and procedures A weighed sample of commercial crystalline trypsin (Washington Co., U. S. A.) was dissolved in 1/15 *M* phosphate buffer of pH 6.5 and 7.6 of distilled water, in the latter case pH being adjusted with 0.1 *N* NaOH or 0.1 *N* HCl. The concentration of enzyme was determined by micro Kjeldahal method.

The high pressure apparatus was the same as previously described[1]. A sample solution was enclosed in a polyvinylchloride sack and set in the high pressure chamber, which filled up with water, and hydrostatically compressed up to 10000 kg/cm². Water from the thermostat was circulated through the water jecket surrounding the high pressure chamber to maintain desired temperature.

Method of activity measurement Trypsin activity was measured by determining its casein digestability; 1 ml of enzyme solution (0.009 mg *N*/ml) with 5 ml of 1% casein solution (pH 7.6) was incubated for 10 minutes at 30°C and the enzyme reaction was stopped by adding 0.44 *M* trichloroacetic acid and the precipitate was filtered off. Two ml of the filtrate was incubated with Folin reagent at 30°C, and after 30 minutes, the optical density was measured at 750 mμ by using Hitachi spectrophotometer Model EPU 2A. Under these conditions the optical density was found to be proportional to the enzyme concentration, and so the value of the optical density might be directly taken as the measure of enzyme activity.

Results

Irreversibility of inactivation In order to ascertain whether the inactivation by pressure is reversible or not, the following experiment was carried out. The enzyme solution at pH 6.5 and 7.6 was compressed to 7000 kg/cm² for 5 minutes at 25°C and the pressure was released. The samples were kept at 20°C and the enzyme activity was measured at some time intervals. As seen in Fig. 1 the inactivation of trypsin is irreversible.

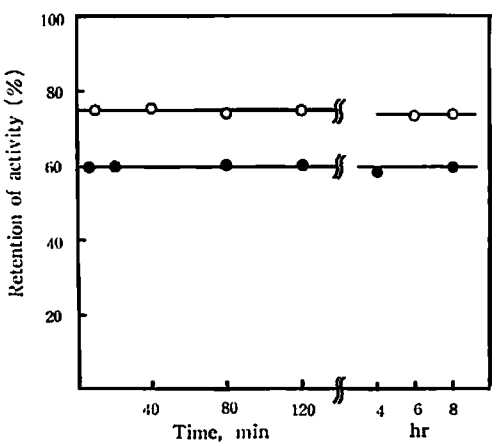

Fig. 1 Irreversibility of inactivation
Pressure: 7000 kg/cm²
Temperature: 25°C
Duration of time: 5 minutes
pH: 6.5 (-○-), 7.6 (-●-)

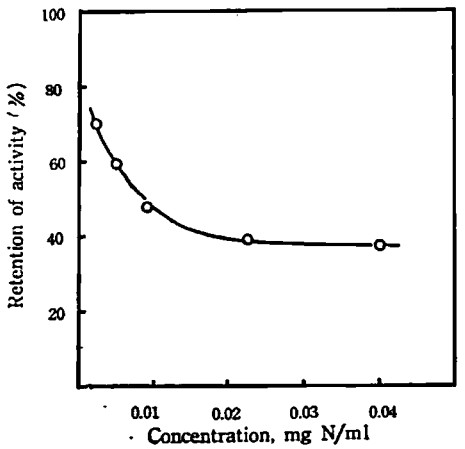

Fig. 2 Effect of concentration
Magnitude of pressure: 7500 kg/cm²
Duration of time: 5 minutes
Temperature: 25°C pH: 7.6

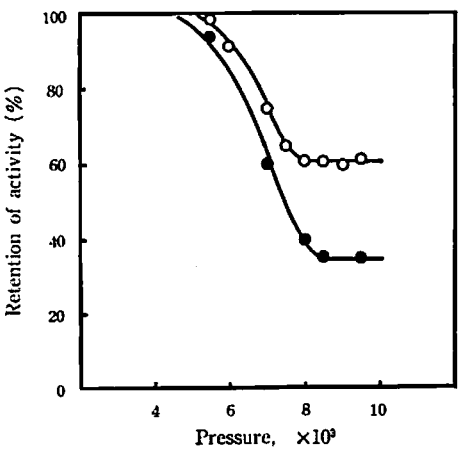

Fig. 3 Effect of magnitude of pressure
Temperature: 25°C
Duration of time: 5 minutes
pH: 6.5 (- ○ -), 7.6 (- ● -)

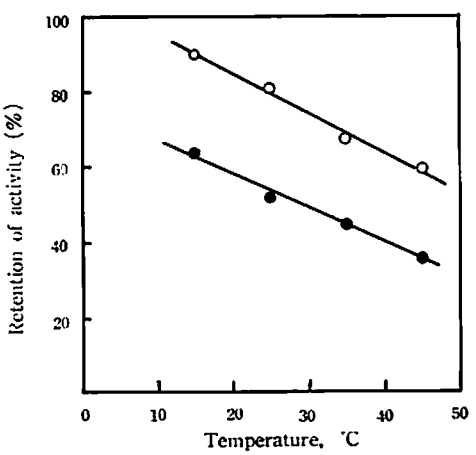

Fig. 4 Effect of temperature on the pressure inactivation of enzyme
Magnitude of pressure: 6500 kg/cm² (- ○ -)
7500 kg/cm² (- ● -)
Duration of time: 5 minutes pH: 7.6

Effect of concentration Five samples of different concentration of trypsin (pH 7.6) were compressed for 5 minutus at 7500 kg/cm² and 25°C. The results given in Fig. 2 show

that the lower the concentration of trypsin the larger the activity is.

Effect of magnitude of pressure Fig. 3 shows the effect of the magnitude of pressure under 5 minutes compression at pH 6.5 and 7.6. The pressure inactivation started at about 5000 kg/cm², and reached a constant activity value at about 8000 kg/cm² (let us call it a critical pressure), without undergoing complete inactivation. This value depends on pH.

Effect of temperature Fig. 4 shows the effect of temperature on the pressure inactivation of enzyme at 6500 kg/cm² and 7500 kg/cm². All compression was made at pH 7.6 for 5 minutes. In the temperature range studied from 15°C to 45°C, it was found that the rate of inactivation becomes larger at higher temperature.

Effect of pH Fig. 5 shows the effect of pH on the pressure inactivation of trypsin in the pH range 3.2 to 10.2. Pressures of 7500 kg/cm² and 9000 kg/cm² were applied for 5 minutes at 25°C. The inactivation of trypsin by pressure was much pronounced in alkaline side than in acid side.

At pH about 3, no inactivation was observed but eighty percent of activity were lost at pH 10.

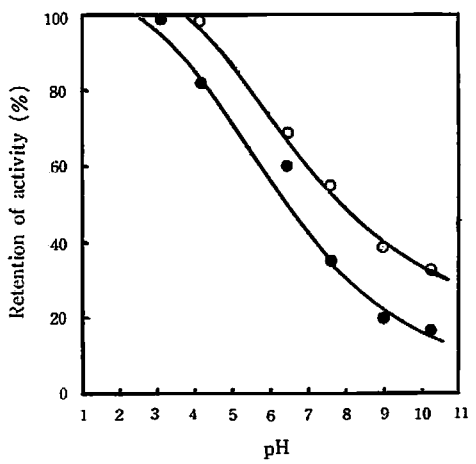

Fig. 5 Effect of pH on the pressure inactivation
pH was adjusted by 0.1 *N* HCl and 0.1 *N* NaOH
Magnitude of pressure: 9000 kg/cm² (- ○ -)
7500 kg/cm² (- ● -)
Duration of time: 5 minutes
Temperature: 25°C

Effect of repeated compression Fig. 6 shows the effect of repeated compression on trypsin at 25°C at pH 6.5. The pressure was raised initially to 6500 kg/cm² or 8500 kg/cm² for 5 minutes, then released to zero, then pressure raised again to 6500 kg/cm² or 8500 kg/cm² for 5 minutes. This procedure was repeated four times.

First, at 6500 kg/cm² which below the critical pressure (see Fig. 3), the repeated compression yields the same degree of inactivation as that resulted from a single compression, if the total duration of compression is the same (20 minutes in this case) for both cases. On the other hand, at 8500 kg/cm² which is just above the critical pressure, repeated compression caused much more inactivation than that caused by a single compression of totally the same duration.

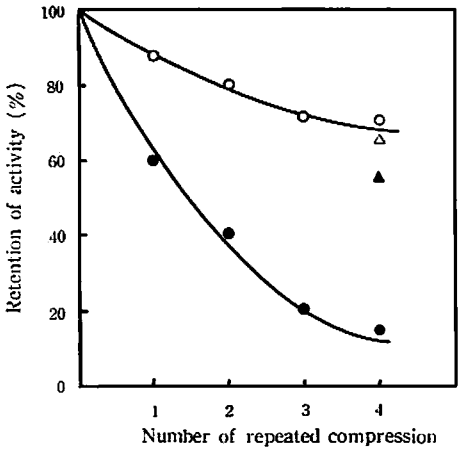

Fig. 6 Effect of repeated compression
(-○-) 6500 kg/cm² for 5 minutes
(-△-) 6500 kg/cm² for 20 minutes
(-●-) 8500 kg/cm² for 5 minutes
(-▲-) 8500 kg/cm² for 20 minutes
pH: 6.5
Temperature: 25°C

Discussion

The inactivation of trypsin by high pressure was very different from the examined protein denaturation by pressure in having a critical pressure; activities above 8000 kg/cm² take a constant value without undergoing complete inactivation. (cf. Fig. 3)

We shall express the optical density which is proportional to the activity above the critical pressure as *Ce*, which is the final value of inactivation by pressure at a given condition. Fig. 7 shows semilogarithmic plot of time course of inactivation, where C_0 is the optical density which is proportional to the activity at zero time, C is that at time t. This kinetics shows that the time course of inactivation follows a first order.*

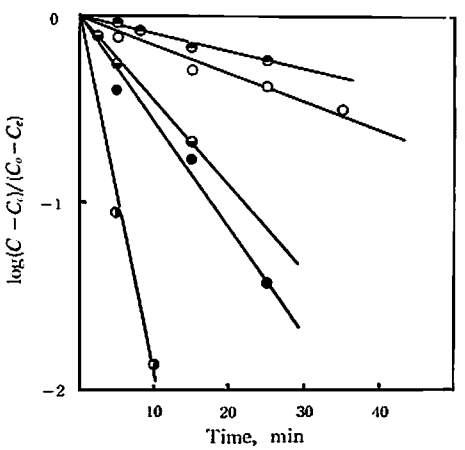

Fig. 7 Time course of inactivation
(-◓-) 6500 kg/cm² at 15°C
(-◒-) 7500 kg/cm² at 15°C
(-○-) 6500 kg/cm² at 25°C
(-●-) 7000 kg/cm² at 25°C
(-◐-) 7500 kg/cm² at 25°C
pH: 7.6
Co: initial activity
C: activity at time *t*
Ce: final value of *C*

* Strictly speaking, the kinetics does not follow a first order, because the inactivation of trypsin by pressure depends on the initial concentration of the enzyme (cf. Fig. 2). Such confliction is often found in the other denaturation of proteins and inactivation of enzymes (such as by urea or heat).

The rate constant k is calculated from the following equation,

$$k=1/t\ \ln(Co-Ce/C-Ce).$$

The influence of pressure p on the rate is related by the equation

$$\partial \ln k/\partial p=-\Delta V^{*}/RT,$$

where R is the gas constant, T the absolute temperature and ΔV^{*} the molar volume change of activation. The value of ΔV^{*} can be calculated from the slope of log k versus p plot, as shown in Fig. 8, which are given in Table 1.

We can calculate the apparent activation energy E from the temperature dependence of the

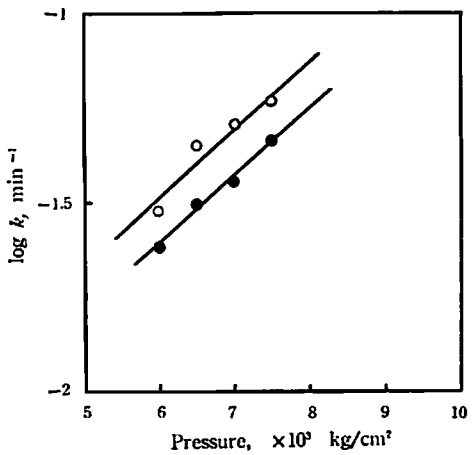

Fig. 8 Relation between logarithm of rate constant k (min.$^{-1}$) and pressure
(-●-) 15°C
(-○-) 25°C
pH: 7.6

Table 1 Molar volume change of activation, ΔV^{*}cc/mole (pH 7.6)

Pressure kg/cm²	Temperature	
	15°C	25°C
6500 7000 7500	−18.6	−20.8

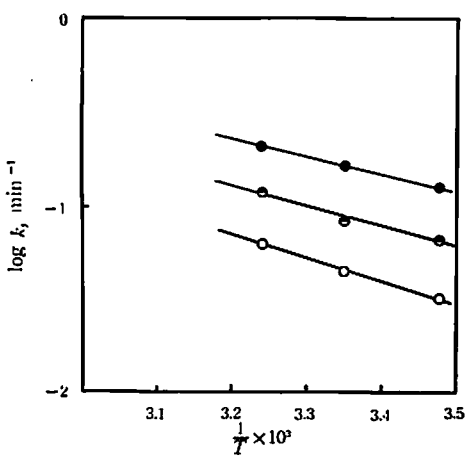

Fig. 9 Relation between logarithm of rate constant k (min^{-1}) and reciprocal of absolute temperature
(-○-) 6500 kg/cm²
(-◓-) 7000 kg/cm²
(-●-) 7500 kg/cm²
pH: 7.6

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rate constant k. Fig. 9 shows the relations between the reciprocal of absolute temperature and the logarithm of the rate constant k. Straight lines are obtained, and from the slopes the apparent activation energies are calculated and listed in Table 2.

Table 2 Apparent activation energy, E kcal/mole (pH 7.6)

Pressure kg/cm²	6500	7000	7500
E, kcal/mole	6.0	4.9	4.5

From the equation of absolute reaction rates, the thermodynamic quantities of activation, i. e. the free energy of activation, ΔF^*, the enthalpy of activation, ΔH^*, and the entropy of activation, ΔS^* concerned with the inactivation reaction, are calculated,

$$\Delta F^* = RT \cdot \ln KT/hk, \quad \Delta H^* = E - RT \text{ and } \Delta S^* = (\Delta H^* - \Delta F^*)/T,$$

where K is the Boltzman constant, h the plank constant, E is the apparent activation energy stated above. The results obtained are summarized in Table 3.

Table 3 Kinetics of inactivation of trypsin by pressure (pH 7.6)

Temperature °C	Pressure kg/cm²	k sec^{-1}	ΔF^* kcal/mole	ΔH^* kcal/mole	ΔS^* cal/mole. deg.
15	6500	2.2×10^{-3}	22	5.4	−58
	7000	6.0×10^{-3}	20	4.0	−57
	7500	1.1×10^{-1}	20	3.9	−56
25	6500	3.3×10^{-3}	22	5.4	−55
	7000	1.3×10^{-2}	21	4.0	−57
	7500	1.4×10^{-2}	21	3.9	−56
35	6500	5.5×10^{-3}	22	5.4	−55
	7000	2.2×10^{-2}	22	4.0	−58
	7500	9.0×10^{-2}	21	3.9	−57

These features except for ΔH^* are nearly the same as those found for ov albumin and hemoglobin, that is, ΔF^* are comparatively small positive value and ΔS^*, ΔV^*, are negative[2]. On the other hand, ΔH^* of trypsin is positive, but that of those proteins has negative value.

For pressure inactivation of trypsin, the following mechanism is presented according to the suggestion of Curl and Jansen[6];

$$iE \longleftarrow aE \rightleftarrows rE,$$

where aE refers to the active enzyme, rE to reversible inactive enzyme, and iE to irreversible inactive enzyme. That is, a definite portion of the enzyme, of which amount depends on pH is converted to the reversible inactive form at the moment when the pressure is applied, while the remainder to the irreversible inactive enzyme. And the reversible inactive enzyme would be expected to revert to the active form by releasing pressure.

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