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INACTIVATION OF ENZYMES UNDER HIGH PRESSURE

1. Inactivation of Salivary α-Amylase under High Pressure

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It has been found that α-amylase of human saliva is inactivated in aqueous solution by an application of high pressure above 5,000 kg/cm².

The inactivation by high pressure follows first order kinetics, with respect to the enzyme concentration. The rate of inactivation is strongly accelerated by an increase in pressure corresponding $-32\,cm^3$/mole of the activation volume at 30°. The activation enthalpy and the activation entropy are 8.2 kcal./mole and -44 e.u. respectively, at 7500 kg/cm² and pH 6.9. The rate of inactivation by pressure is also roughly proportional to hydrogen ion concentration in the solution, at a pH between 4 and 10. These features of the inactivation by pressure of salivary α-amylase, resemble closely those of bacterial α-amylase.

Introduction

The purpose of our investigations has been to clarify the behaviors of enzymes under high pressure and to elucidate the mechanism of the enzyme inactivations by high pressure.

Since the discovery of the enzyme inactivations under high pressure by Basset and Macheboeuf[1] in 1932, there have been only a few investigations concerning the effects of pressure, in enzyme chemistry, comparing with those concerning the effects of temperature, acids or alkalis. Although, the inactivations of pepsin, trypsin and chymotrypsin, by high pressure were studied in detail, by Curl and Jansen[2], and by Mathew, Dow and Anderson[3], kinetic investigations were scarcely done.

In our previous paper[4], inactivation of bacterial α-amylase under high pressure up to 10,000 kg/cm² has been examined, and the results have been discussed on the basis of the transition state theory.

In the present communication, the inactivation of α-amylase of human saliva under high pressure will be discussed, on which no work has been previously reported.

Experimentals

Enzyme Saliva from an adult man was used as the mother solution of salivary α-amylase.

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1) J. Basset and M. Macheboeuf, *Compt. rend.*, **196**, 1431 (1932)
2) L. Curl and E. Jansen, *J. Biol. Chem.*, **184**, 45 (1959); *ibid.*, **185**, 713 (1950)
3) J. E. Mathew, R. B. Dow and A. K. Anderson, *ibid*, **135**, 697 (1940)
4) K. Suzuki and K. Kitamura, *J. Biochem.*, **54**, 214 (1964)

without any purifications, which was stored in icebox, after collected, and used in the experiments within a week. Its enzyme activity and susceptibility to inactivation were not changed within a week or two. The test solutions to be compressed were prepared by diluting the saliva about 200 times with water or with buffer solutions; in the experiments at pH 6.9 the saliva was diluted with M/15 phosphate buffer, and at the other experiments, it was diluted with distilled water and the pH of the solution was adjusted with hydrochloric acid or with sodium hydroxide.

High pressure apparatus and procedures The high pressure apparatus and the procedures were essentially the same with those in the experiments of bacterial α-amylase[4]. The high pressure vessel was a thick-walled cylinder, made of hardened steel, with 16 mm. inner diameter and 130 mm. length. A sample solution packed in a sack made of polyvinylchloride, was charged in the high pressure vessel, and compressed hydrostatically, and the catalytic powers to hydrolyze starch were measured on the solutions before and after compression. The activity of α-amylase in a solution was measured by the method of Noelting and Bernfeld[5].

Results

Effect of pressure Fig. 1 shows the effect of pressure on the inactivation of salivary α-amylase at 30°C and pH 6.9. Pressure was applied on the 200 times diluted saliva for 5 minutes, and the enzyme activity after the sapplication of pressure was measured. As are shown in the figure, the enzyme activity is not changed, in the conditions, under a pressure below 4000 kg/cm². The inactivation is observed only at a pressure above 5000 kg/cm², and the retention activity after compression decreases with increasing magnitude of pressure, so that the activity is destroyed almost completely at 9000 kg/cm², within only 5 minutes.

The experiments were repeated, at the same conditions, using the samples of saliva which were different in the date of sampling and in the duration time in the ice box, and always obtained the reproducible same results, within experimental errors.

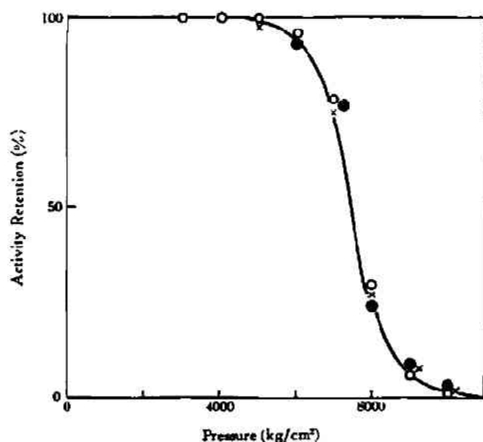

Fig. 1 Loss in activity of the pressure treated enzyme solutions

The solutions were pressed for 5 minutes at pH 6.9, 30°C

○ : A saliva soltion was used within 5 hrs. after collected.

● : The same stock ○ was compressed the next day.

× : Another saliva sample collected in the other day was used within the day.

5) G. Noelting and P. Bernfeld, *Helv. Chim. Acta*, 31, 286 (1948)

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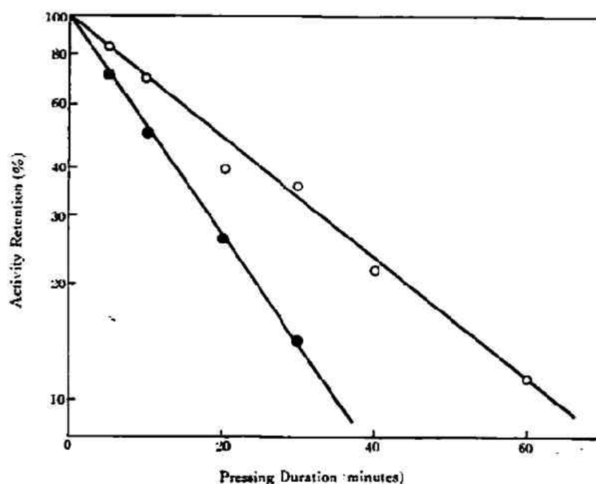

Fig. 2 Time course of the inactivations at 30°C pH 6.9 under pressures
○ : 7000 kg/cm² and ● : 7500 kg/cm²

Table 1 Restoration of enzyme activity after releasing pressure
Pressures were applied for 5 minutes at 30°C, pH 6.9. After releasing pressures, enzyme solutions were stored at 0C°, till activity measurements.

Pressure (kg/cm²)	Time after pressing (hrs.)	Enzyme activity (relative value)
7500	0	0.359
	7	0.359
	24	0.421
	48	0.381
8000	0	0.126
	19	0.135
	43	0.180
9000	0	0.043
	18	0.055

Effect of compressing duration In Fig. 2, the retention activity of the enzyme is plotted against time of compression, in semilogarithmic scale. A linear relationship satisfied at each pressure, shows that the inactivation follows apparently first order kinetics, with respect to enzyme concentration.

Recovery of the enzyme activity after releasing pressure Although the inactivation of salivary α-amylase follows irreversible first order kinetics under high pressure, the enzyme activity is restored partly, after releasing pressure, as are shown in Table 1.

Influence of temperature The influences of temperature on the inactivation were examined at 7000 kg/cm² and at 8000 kg/cm², both at pH 7.9. As are shown in Fig. 3, the inactivation is accelerated by an increase in temperature between 10° and 40°C.

Influence of pH The influences of pH of the enzyme solution on the pressure inactivation were

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examined, at 30°C and at pressures 7000 kg/cm² and 8000 kg/cm², by measuring the retention amylase activities of the saliva solutions of different pH, equally compressed for 5 minutes. As are shown in Fig. 4, the inactivation is accelerated by an lowering of pH between 4 and 10.

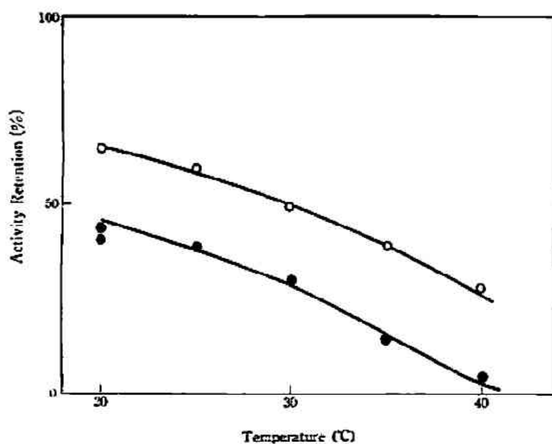

Fig. 3 Influences of temperature on the inactivations by pressures

○ : 7500 kg/cm²
● : 8000 kg/cm²
at pH 6.9, for 5 minutes compression

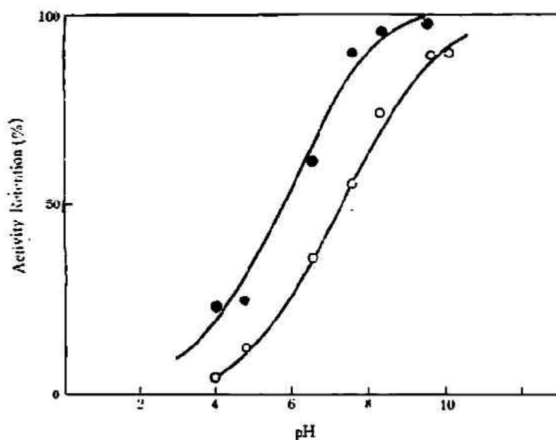

Fig. 4 Influences of pH on the inactivations by pressing for 5 minutes

● : 7000 kg/cm²
○ : 8000 kg/cm²
at 30°C

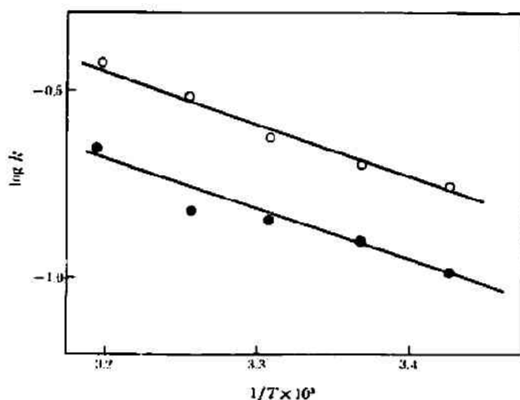

Fig. 5 The Arrhenius' plots for the inactivations by pressures

● : 7500 kg/cm²
○ : 8000 kg/cm²
at pH 6.9

Considerations

The apparent activation energies of the pressure inactivation E. at 7500 kg/cm² and 8000 kg/cm², are calculated from the slopes of Arrhenius plots in Fig. 5, to be 8.6 kcal/mole in both cases. The first order rate constants in the figure are obtained from the data in Fig. 3.

By applying the transition state theory, the activition free energy $\Delta G^{\neq}$, the activation enthalpy $\Delta H^{\neq}$ and the activation entropy $\Delta S^{\neq}$ are calculated, as are shown in Table 2, by the formulae;

Table 2 Kinetics of inactivation of salivary α-amylase by pressure (pH 6.9)

Temperature, °C	Pressure, kg/cm²	$\Delta G^{\neq}$(kcal/mole)	$\Delta H^{\neq}$(kcal/mole)	$\Delta S^{\neq}$(e. u.)	$\Delta V^{\neq}$(cm³/mole)
20	7500	21	8.2	−44	
30	6000	23	8.2	−49	−32
	7500	22	8.2	−44	−32
	9000	21	8.2	−41	−32
40	7500	22	8.2	−44	

$$\Delta G^{\neq} = -RT\ln(\boldsymbol{k}T/kh)\,. \quad (1)$$

$$\Delta H^{\neq} = E - RT\,. \quad (2)$$

$$\Delta S^{\neq} = (\Delta H^{\neq} - \Delta G^{\neq})/T\,, \quad (3)$$

where $\boldsymbol{k}$ is Boltzmann constant, h the Planck constant, R the gas constant, k the first order rate constant and T the absolute temperature.

The activation volume $\Delta V^{\neq}$ of the process, at 30°C and pH 6.9, is obtained from the data in Fig. 1, by the equation;

$$(\partial \ln k/\partial p)_T = -\Delta V^{\neq}/RT\,. \quad (4)$$

where p is pressure. As is shown in Fig. 6, the logarithm of the rate constant against pressure comes to a straight line, and the value of $\Delta V^{\neq}$ is calculated to be −35 cm³/mole.

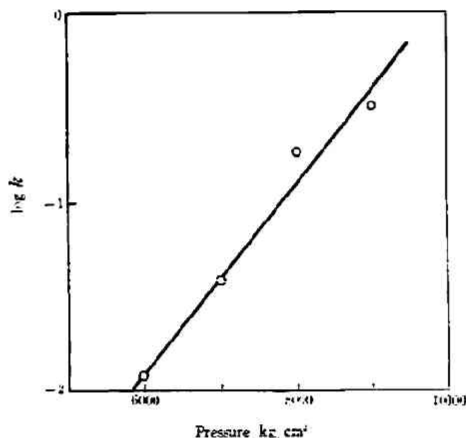

Fig. 6 Relationship between first order constant of the inactivation and magnitude of pressure applied, at 30°C, pH 6.9

Experiments were the same as in Fig. 1

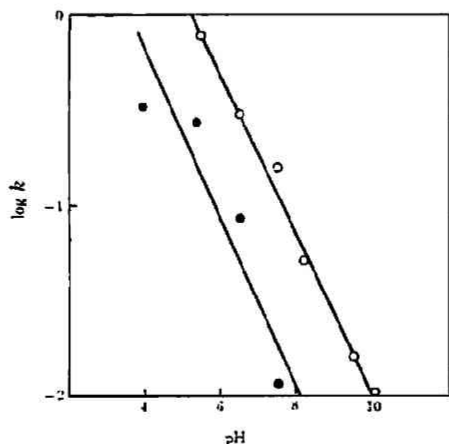

Fig. 7 Influence of pH on the rate of pressure inactivation

○ : at 7500 kg/cm²
● : at 8000 kg/cm²
Experiments were the same as in Fig. 4.

As are shown in Fig. 7, the plot of the rate constant in logarithmic scale against pH comes on a straight line with a slope about -1; namely, the apparent first order rate constant of the inactivation is approximately proportional to the hydronium ion activity of the solution.

These kinetic features of the inactivation of salivary α-amylase by high pressure are closely similar to those of bacterial α-amylase[4] and except for $\Delta H^{\ddagger}$ to the behaviors of denaturations of ovalbumin[6] and hemoglobin[7] by high pressure, in their negative activation volumes and negative activation entropies. It may be assumed, therefore, that the inactivation of this enzyme by high pressure also results by the denaturation of the enzyme protein, with the mechanism common in these proteins and enzymes.

Acknowledgement

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6) K. Suzuki, *This Journal*, **28**, 24 (1958)
7) K. Suzuki and K. Kitamura, *ibid.*, **29**, 81 (1960); *ibid.*, **29**, 86 (1960)