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Reactivation of Pressure-Inactivated α-Amylase of Bacillus sudtilis under Moderate Pressure[1]

KINJIRO MIYAGAWA

Laboratory of Chemistry, Osaka Joshigahuen College, Tennoji, Osaka, Japan

KIYOTOSHI SANNOE AND KEIZO SUZUKI

Department of Chemistry, Faculty of Science and Engineering Ritumeikan University, Kyoto, Japan

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α-Amylase of *Bacillus subtilis* was remarkably stabilized against pressure inactivation by calcium ion (in most of experiments, an enzyme solution containing 10^{-2} *M* calcium ion was used). After releasing the pressure, partially or completely pressure-inactivated enzyme was not observed to regain activity under atmospheric pressure; however, the activity was recovered to a considerable extent by recompression at a moderate pressure. A prssure of about 3000 kg per square centimeter (above this pressure the extent of reactivation gradually decreased because of competing pressure inactivation), a pH of about 7.5, a temperature of about 30°, and high concentration of enzyme were optimal for the recovery. Reactivation was almost in dependent of ionic strength.

In our earlier work (1) dealing with high pressure inactivation of α-amylase of *Bacillus subtilis* [EC. 3.2.1.1. α-1, 4-glucan 4-glucano-hydrolase] (B. α-amylase), it was found that the enzyme (in the presence of 10^{-2} *M* calcium ion) was completely inactivated by pressures up to 10,000 kg per square centimeter, and recovery of enzymic activity was not observed after releasing the pressure. By contrast, pressure-inactivated Take-amylase A (TAA) spontaneously regained activity after pre-

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ssure release (2, 3.) Moreover, the reactivation of TAA was enhanced by recompression at moderate pressures up to 3000 kg per square centimeter (3). Similar effects of recompression have been reported Suzuki *et al.* (4, 5) and Tongur *et al.* (6-10) with pressure-denatured and heat-denatured proteins, respectively. However, the interpretation of these phenomena has not been fully clarified. Accordingly, in the present investigation the reactivation of pressureinactivated B. α-amylase by recompression under a moderate pressure was studied.

EXPERIMENTAL

Enzyme. Crystalline B. α-amylase was obtained from Nagase Industrial Co. Ltd., and twice recrystallized according to the method of Hagihara (11). The aqueous enzyme solution was dialyzed against 0.01 *M* calcium chloride solution and stored in a refrigerator as a stock solution. The concentration of enzyme was estimated spectrophotometrically, assuming the extinction of enzyme in water to be $E^{1\%}_{1cm} = 25.6$ at 297 mμ (12). For experiments, the stock solution was usually diluted with 0.01 *M* calcium cholride solution to give a enzyme concentration of 4×10^{-4} %.

Procedures. The high pressure equipment was the same as previously reported (3, 13). Unbuffered aqueous solutions of enzyme were sealed into a polyvinyl-chloride sack and set in the high pressure chamber. After a compression under defined conditions, the inactivated sample was diluted threefold with a suitable buffer (0.05*M* tris buffer above pH 7.0, or 0.05 *M* acetate buffer below pH 7.0). Therefore the enzyme concentration and buffer concentration used in the following experiments were $\frac{1}{3}\times10^{-4}$% and $\frac{5}{3}\times10^{-2}$ *M*, respectively, except for special cases. One portion of the inactivated sample was kept under atmospheric pressure, and the other was placed in the pressure chamber and recompressed at a moderate pressure. Amylase activity was measured with soluble starch as the substrate by the method of Noelting and Bernfeld (14) in which 3,5-dinitrosalicylic acid is used.

RESULTS

Preliminary experiments were performed to obtain a sutiable pressure-inactivated sample. Unbuffered aqueous solution of the enzyme containing calcium chloride in different concentrations were compressed at various pressures for 5 minutes at 30° (Fig. 1). The enzyme was remarkably stabilized against pressure inactivation by addition of calcium ion. Reproducibility of the results was poor when the content of calcium ion was small. Therefore, in subsequent experiments, an enzyme solut-

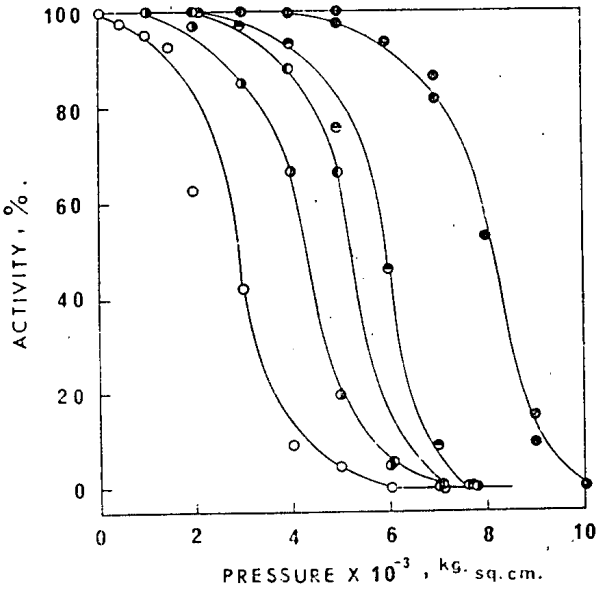

FIG 1. Effect of calcium ion on pressure inactivation. Unbuffered aqueous enzyme solutions (concentration of enzyme, 4×10^{-4}%), in which calcium chloride was added to give a concentration of 0 (no calcium ion was added; however, a small amount of calcium ion was present in the stock solution) (—○—), 10^{-5} (—◑—), 10^{-4} (—◐—), 10^{-3} (—◒—), and 10^{-2} (—●—) mole per liter were compressed at various pressures up to 10,000 kg per square centimeter for 5 minutes, at 30°

ion containing 10^{-2} *M* calcium ion was used, and to get a completely inactivated enzyme, the solution was compressed for 5 minutes at 10,000 kg per square centimeter and 30°.

Effect of pressure. A part of the completely inactivated enzyme solution (pH 7.5) was recompressed at various pressures up to 5000 kg per square centimeter, and the other part was kept under atmospheric pressure. After 1 and 3 hours, activity was measured (Fig. 2). No reversal occurred under atmospheric pressure; however, partial reversal was obtained by recompression. A pressure of about 3000 kg per square centimeter was optimal for the recovery of activity; above this pressure the extent of the reactivation decreased gradually.

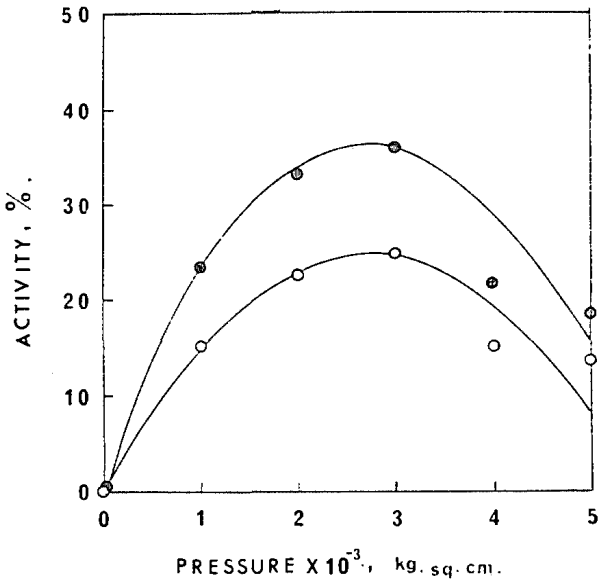

FIG. 2. Effect of pressure on reactivation. Completely inactivated enzyme solution (pH 7.5, concentration of enzyme: $\frac{1}{3} \times 10^{-4}$ %) was recompressed at various pressures up to 5000 kg per spuare centimeter for 1 hour (—○—) and 3 hours (—●—), at 30°

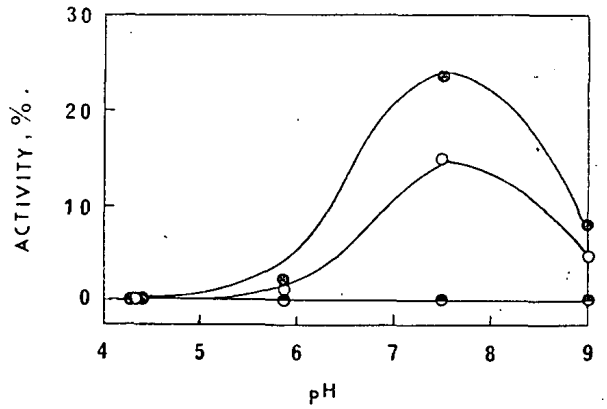

FIG. 3. Effect of pH on reactivation. After compression, the enzyme solution was adjusted to various pH values (final conc. of enzyme: $\frac{1}{3} \times 10^{-4}$ %). A portion of the sample was recompressed at 1000 kg per square centimeter for 1 hour (—○—) and 3 hours (—●—); the remainder was kept under atmospheric pressure for 3 hours (—◒—) at 30°.

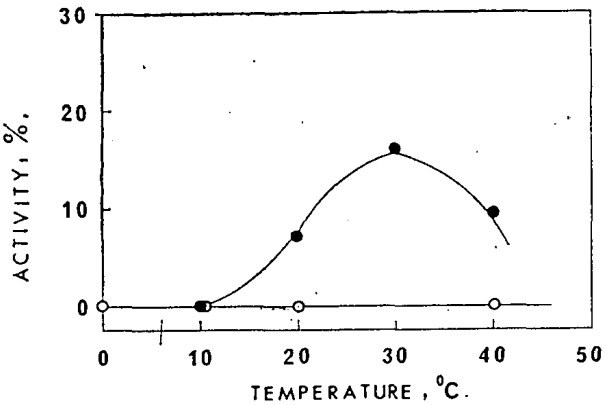

FIG. 4. Effect of temperature on reactivation. A portion of the completely inactivated enzyme (pH 7.5; conc. of enzyme: $\frac{1}{3} \times 10^{-4}$%) was recompressed at 1000 kg per square centimeter at various temperatures up to 40° for 1 hour (—●—); a control was kept under atmospheric pressure (—○—) for the same time.

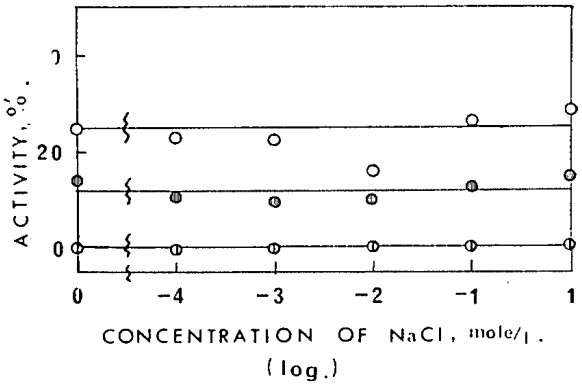

FIG. 5. Effect of addition of salt on reactivation. To the inactivated enzyme solution, sodium chloride was added to a final concentration of 0-1 *M* (pH7.5; final conc. of enzyme: $\frac{1}{3} \times 10^{-4}$ %). Portions of these samples were recompressed at 1000 kg per square centimeter for 1 hour (—●—) and 3 hours (—○—); controls were kept under atmospheric pressure (—◑—) for 3 hours at 30°.

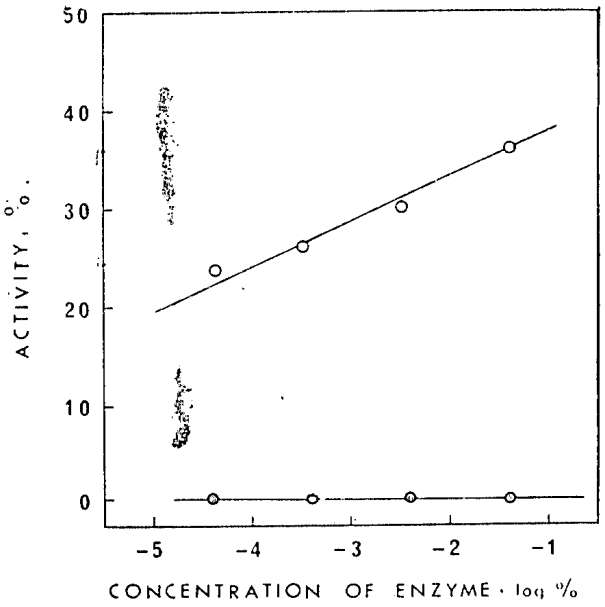

FIG. 6. Effect of concentration of enzyme on reactivation. Unbuffered enzyme solutions at four different concentration were compressed at 10,000 kg per square centimeter for 5 minutes at 30°,

then diluted threefold with tris buffer at pH 7.5. A portion of each sample was recompressed at 1000 kg per square centimeter for 3 hours (—○—); the remainder remained at atmospheric pressure for 3 hours (—●—) at 30°.

Effect of pH. After compression, the completely inactivated enzyme solution was adjusted to each pH value with a suitable buffer. A portion of the sample was recompressed at 1000 kg per square centimeter, and the remainder was kept under atmospheric pressure. The results are shown in Fig. 3. No reversal was obtained at any pH value at atmospheric pressure. Recompression reactivated the enzyme to a considerable extent, especially in the neutral pH renge.

Effet of temperature. A portion of the enzyme (pH 7.5) was recompressed at 1000 kg per square centimeter, and the remainder was kept under atmospheric pressure at various temperatures from 0° to 40°. As shown in Fig. 4, reactivation was optimal at about 30°.

Effect of addition of salt. After inactivation, sodium chloride was added to various concentrations (pH 7.5), and reactivation was studied as described above (Fig. 5). The extent of recovery was independent of the salt concentration.

Effect of initial concentration of enyume. Unbuffered enzyme solutions at four different concentrations were completely inactivated by pressure. Each solution was diluted threefold with tris buffer to adjust the pH to 7.5. The conditions for reactivation were the same as described above. Activity was

measured after 3 hours (Fig. 6). The extent of recovery on recompression was dependent on the initial concentration of enzyme: the higher the concentration, the more extensive the recovery.

Effect of extent of inactivation and compression time for inactivation. Unbuffered aqueous solutions of enzyme were compressed at 6500, 7500, and 10,000 kg per square centimeter for 5 minutes at 30° to obtain enzyme samples which differed in the extent of inactivation. The results of recompression are shown in Fig. 7. The absolute extents of recovery of partially inactivated enzyme (Fig. 7a and b) and completely inactivated enzyme (Fig. 7c) were not significantly different, but the percentage recovery increased as the degree of inactivation decreased. A partly inactivated enzyme (inactivation was 20%) compressed at 6500 kg per spuare centimeter recovered almost full activity by recompression at 1000 kg per square centimeter for 3 hours, whereas a completely inactivated enzyme (compressed at 10,000 kg per square centimeter for 5 minutes) recovered only 25% of full activity under the same condition. The extent of reactivation of a sample compressed at 10,000 kg per square centimeter for 60 minutes was less than that of a sample inactivated for 5 minutes at the same pressure (see Fig.7c).

Some kinetic parameters. The velocity constants k' were calculated from the initial rates of reactivation assuming a first-order reaction (the reactivation process followed first-order kinetics over the first 80 minutes at moderate pressure). Some of the other parameters of the kinetics of the reactiva-

tion were also calculated and are listed in Tables I and II.

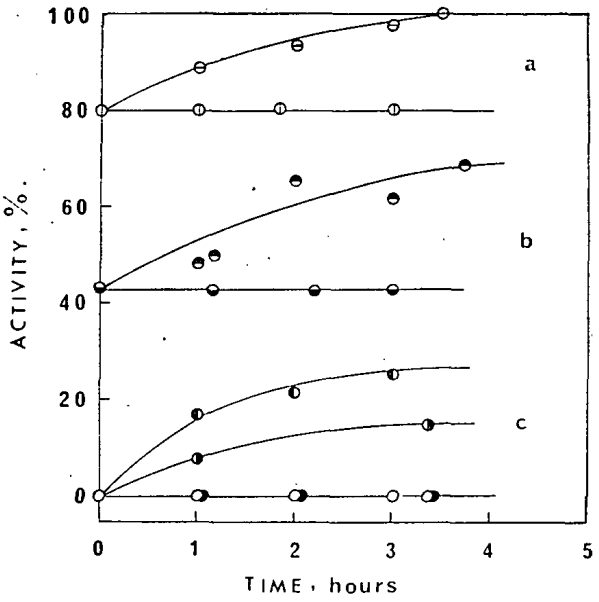

FIG. 7. Effect of extent of inactivation and of compression time for inactivation on reactivation. Conditions were:

Inactivation (unbuffered; enzyme conc., 4×10^{-4}%)		Reactivation (pH 7.5; enzyme conc., $4/3\times10^{-4}$%)
Pressure (kg/sq cm)	Duration (min)	Pressure (kg/sq cm)
(−◐−) 10,000	5	1000
(−◑−) 10,000	60	1000
(−○−) 10,000	5	atm.
(−●−) 10,000	60	atm.
(−◓−) 7500	5	1000
(−◒−) 7500	5	atm.
(−⊖−) 6500	5	1000
(−⊕−) 6500	5	atm.

TABLE I

KINETICS OF REACTIVATION OF PRESSURE-INACTIVATED B.α-AMYLASE AT PH 7.5

(CONCENTRATION OF ENZYME: $\frac{4}{3}\times10^{-4}$%)

Pressure (kg/sq cm)	Temp. (°C)	k' (sec^{-1})	E (kcal/mole)	$\triangle F^*$ (kcal/mole)	$\triangle H^*$ (kcal/mole)	$\triangle S^*$ (cal/mole deg)
1000	20	5.4×10^{-4}	6.4	23	5.8	−59
1000	30	7.5×10^{-4}	6.4	23	5.8	−57
1000	40	6.1×10^{-4}	−2.8	23	−3.4	−66
2000	30	8.7×10^{-4}		23		
3000	30	8.9×10^{-4}		23		
4000	30	7.9×10^{-4}		23		
5000	30	6.4×10^{-4}		23		

*Activated state

TABLE II

MOLAR VOLUME CHANGE OF ACTIVATION, $\triangle V^*$, IN THE REACTIVATION PROCESS AT PH 7.5 AT 30° (CONCENTRATION OF ENZYME: $4/3\times10^{-4}$ %)

Pressure (kg/sq cm.)	$\triangle V^*$ (cc/mole)
1000-2000	−3.7
2000-3000	−0.6
3000-4000	3.0
4000-5000	5.2

DISCUSSION

As shown in Fig. 1, calcium ion greatly stabilized the enzyme toward inactivation. Proteins and enzymes which have been reported to be denatured and inactivated by compression do so only under several thousand atmospheres. It was therefore surpring to find that, in the absence of calcium ion, the inactivation of B. α-amylase begins at about 500 kg per square

centimeter, an unusually low pressure. In the presence of about 10^{-2} *M* calcium ion, the more usual behavior is observed. Accordingly, it would be of interest to investigate the correlation between the loss of activity and conformational changes in the molecule. Such information is not readily accessible because the enzyme solution above a concentration of about $10^{-2}\%$ becomes turbid over a wide range of pH under compression. A pressure of 500 kg per square centimeter does not appear to cause conformational changes in the over-all molecule. It is therefore presumed that only the active site is affected at low pressure, and that it is strongly protected by calcium ion.

Activity was not recovered after compression even if the enzyme was only partially inactivated. However, by recompression at a moderately low pressure, recovery occurred to a considerable extent. The extent of recovery was dependent on pH, temperature, pressure, and the initial concentration of enzyme, but was almost independent of ionic strength. Higher concentrations of enzyme, a pH value of about 7.5, a temperature of about 30°, and a pressure of about 3000 kg per square centimeter were optimal for reactivation. These conditions were almost the same as those for TAA (3). However, a significant difference between TAA and B. α-amylase is that the former recovers activity spontaneously after compression even if enzymic activity was completely lost, while with B. α-amylase recovery is only observed after recompression at a moderate pressure. In this respect the behavior of B. α-amylase

resembles the redissolution of pressure-coagulated β-lactoglobulin (5). The difference between B. α-amylase and TAA may be attributed to the structure of the enzyme molecules. Taka-amylase A is considered to have a particularly compact structure with four disulfide linkages, while B. α-amylase contains neither sulfhydryl groups nor disulfide bonds in tde molecule.

As shown in Fig. 7c, the rate of recovery was lower when the compression time for inactivation increased, although both compressed samples were completely inactivated. A similar result was also found in TAA (3). It is presumed that in prolonged compression, alterations of the protein molecule continue to occur beyond a point from which recovery can proceed. The opposite action of pressure around 3000 kg per square centimeter is noteworthy. Similar results have been reported in TAA (3), γ-globulin (4), β-lactogloblin (5), and carbonyl hemoglobin (15). Moreover, Tongur *et al.* (6-10) have shown that heat-denatured proteins were renatured by compression at moderate pressure. Inactivation and reactivation may have equilibrium characteristics:

Native enzyme ⇄ Inactivated enzyme.

Under high pressure(above about 3000 kg per square centimeter) the equilibrium favors inactivation, but at moderate pressures (below about 3000 kg per square centimeter) it favors the left. However, the rate of reactivation might be very slow. Moreover, the process of reactivation accompanied a decrease in volume. Thus increasing pressures up to 3000 kg per square centimeter favor reactivation.

The kinetics of the reactivation of B. α-amylase are almost of the same order as with TAA (3), and substantially identical processes of inactivatlon and reactivation under pressure are presumed to exist in both enzymes. As already reported (1), pressure-inactivation of B. α-amylase involves a decrease in volume. Thus both inactivation and reactivation by compression are associated with a negative volume change. Interesting examples have been found in the effect of pressure on micelle formation in detergents solution (11, 12). The critical micelle concentration (c.m.c.) increases with increasing pressure up to 1000 atm.; however, beyond this pressure the c.m.c. decreases with increasing pressure. The behavior of proteins under pressure is undoubtedly more complex, and it will be necessary to study the interrelation between the bonds constituting the structure of proteins and water molecles under pressure through an investigation of appropriate systems. (This suggestion was made by Professor W. Kauzmann, Princeton University).

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