Studies on Thermophilic α-Amylase from Bacillus Stearothermophilus

[I] Some Physico-chemical Properties of Thermophilic α-Amylase

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Kyoko Ogasahara

Division of Physical Chemistry,

Institute for Protein Research,

Osaka University

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[I] Some Physico-chemical Properties of Thermophilic α-Amylase *

Temperature is one of the most important environmental variable, affecting the growth, activity, and evolution of organisms. Ordinary organisms can grow only over a relatively narrow range of temperature (around 25 °C). On the other hand, there are living organisms, thermophiles, which preferably survive at high temperature where proteins are generally denatured. Thermophilic bacteria were first described by Miquel in 1888. Scince then, these bacteria or other thermophiles, e.g. blue green algae, have been objects of scientific interest. How do these organisms manage to live at temperature where ordinary heat-sensitive cellular constituents are destroyed ? The most widely distributed and studied thermophilic forms have been microorganisms.

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* Abbreviation used ; thermophilic α-amylase, α-amylase from B.stearothermophilus Donk, strain BS-1

The studies on them in early time were limited to observations of spectacular growths in hot springs. After that, as the remarkable progress of biochemistry, the knowledge on thermophiles have been accumulated. There have been two general types of explanation for this remarkable faculty offered by Gaughran (1) and Allen (2). The first explanation is that the essential cell components of thermophiles are relatively more heat-stable than those of mesophiles. This thermal stability may be an inherent property of these molecules and molecular complexes or may be brought about through the presence of protective factors or the absence of labilizing materials in the external or internal environment of the cell. The other is that the survival of thermophilic organisms at higher temperature might be due to their ability to replace the heat-damaged intracellular material at such a rate that overall metabolism is not affected.

Campbell and his co-workers have crystallized a heat-stable α-amylase from B.stearothermophilus, strain 503-4 (3) and studied on some general properties of this enzyme(4,5). Their investigations provided the first clue to elucidate the thermostability of the enzyme on the molecular basis. It has been concluded that the α-amylase of B. stearothermophilus in the native state exists as a semi-random- or random-coiled

form which is characterized a large negative optical rotation (4) and an unusually high proline content(5). A suggestion has been also made that the thermostability of the protein molecule may be due to the characteristic less ordered strucuture of the protein itself.

The α-amylase obtained by Campbell et al was, however, very different in some physico-chemical properties from the α-amylase in the present investigation. In the present study, to clarify the cause of thermostability of α-amylase from B.stearothermophilus Donk, strain BS-1 in the relation to the physico-chemical properti es, these properties were studied comparing with those of α-amylase from the same genus Bacillus, B.subtilis.

EXPERIMENTAL

Bacterial Strain ------ The thermophilic bacteria isolated from soil in Japan was kindly supplied by Dr. Endo, Daiwa Kasei Co. Ltd.. Identification of this bacteria was carried out by Drs. Iizuka and Furuta, Institute of Applied Microbiology, the University of Tokyo. The physiological properties of this bacteria were found to be similar to those of four standard strains, B.stearothermophilus, strain 231-3 IAM 11001, 231-4 IAM 11002, 231-5 IAM 11003, and 231-6 IAM 11004. However, some cultural morphological properties

of this bacteria were somewhat different from those of the four standard strains. For example, the formation of spore of this bacteria was slower and worse than the standard strains and the irregular forms of spore were observed during the cultivation of this bacteria. The detailed study on this problems has not yet been accomplished. According to the classification method by Smith (6), this bacteria were identified to be B.stearothermophilus Donk, strain BS-1 by considering that the bacteria were able to grow at 65 °C by Drs. Iizuka and Furuta.

Materials ------ Preparation and crystallization of α-amylase from B.stearothermophilus Donk, strain BS-1 cultured at 55 °C was carried out according to the method of Hagihara (7) and found to be homogeneous as judged by the measurements of the cellulose acetate electrophoresis, sedimentation and Sephadex gel-filtration.

Crystalline B.subtilis α-amylase was supplied by Daiwa Kasei Co. Ltd..

β-Amylase (Merk) from potato was purchased.

Methods ------ Concentrations of thermophilic and B.subtilis α-amylases were estimated from absorption at 280 mμ assuming $E_{1\%}^{1\,cm}$ = 28.7 and 25.6 determined experimentally, respectively.

Type of thermophilic α-amylase was determined by the measurement of optical mutarotation of the enzymatic products and saccharifying power (S.P.) of the enzyme by Fuwa's method (8) in comparing with those of β-amylase from potato.

α-Amylase activity was determined by the measurement of dextrinizing power (D.P.) using amylose as a substrate according to Fuwa's method (8) with a slight modification.

Molecular weight of thermophilic α-amylase was determined by the method of sedimentation equilibrium described by Yphantis (9). The experiment was made using a Spinco model E analytical ultracentrifuge and carried out at a rotor speed of 12,590 rpm and 25 °C with standard schlieren optics.

The sedimentation constant was determined on a Hitachi model UCA-1 type ultracentrifuge equipped with schlieren optics. The experiment was carried out at a rotor speed of 54,200 rpm and 25 °C.

Optical rotatory dispersion (ORD) measurements in the 365-578 mμ region were made on an O.C.Rudolph and Sons model 200S photoelectric spectropolarimeter. Concentrations of thermophilic and B.subtilis α-amylases for the measurement of optical rotatory dispersion were between 0.4 and 0.8 per cent. The despersion data were analyzed by the Moffitt-Yang's eqration (10),

$$[m']_\lambda = \frac{3}{n^2+2}\frac{M_0}{100}[\alpha]_\lambda = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \quad (1)$$

where $[m']_\lambda$ is the mean residue rotation at any wavelength corrected for the refractive index (n) of the solvents. The parameters a_0 and b_0 in equation (1) were obtained from the intercepts and slopes of $[m']_\lambda(\lambda^2-\lambda_0^2)/\lambda_0^2$ versus λ_0^2 $(\lambda^2-\lambda_0^2)$ plots, with λ_0 value taken as 212 mμ. The mean residue weight of thermophilic and B.subtilis α-amylases were assumed to be 115.

The measurements of ORD in the 210-250 mμ region and the circular dichroism (CD) were made using a Jasco spectropolarimeter model ORD/UV-5 with a CD attachment. Molecular ellipticity, [θ], was obtained by the equation,

$$[\theta] = 3{,}300\ (\varepsilon_l - \varepsilon_r) \quad (2)$$

where $(\varepsilon_l - \varepsilon_r)$ is the difference between the molar extinction coefficients for left and right circularly polarized light. In the calculation $(\varepsilon_l - \varepsilon_r)$, the mean residue weight was used instead of molecular weight.

An apparent content of right-handed α-helix was calculated as follows,

$$= \frac{b_0}{-630} \times 100 \quad (11)$$

$$= \frac{|[m']_{233}| - 1,700}{14,000} \times 100 \qquad (12)$$

$$= \frac{-[\theta]_{222}}{40,000} \times 100 \qquad (13, 14)$$

Viscosity of thermophilic α-amylase was measured with an Ostwald type viscometer having the flow time of 300 seconds with 10.0 ml water. The temperature was controlled in a water bath at 25.0 ± 0.01 °C.

Assay of metals contained in thermophilic α-amylase was carried out with a Perkin-Elmer model 303 atomic absorption spectrophotometer after dialysing the enzyme against deionizing water for 4 and 7 days.

Amino acid composition of the enzyme was determined with a Beckman/Spinco model MS amino acid analyzer after hydrolysis at 110 °C for 20 hours. Cystein was determined by a colorimetric method using the reagent DTNB (15). The content of tryptophan residues were determined by the spectrophotometric method (16).

RESULTS

Determination of Type of Thermophilic Amylase ------ To determine the type of thermophilic amylase (i.e. α or β), the optical mutarotation of enzymatic products was measured. The enzymatic products of thermophilic amylase mutarotated downward and were estimated to have an α-configuration. As shown in Fig. 2, the mode of hydrolysis of amylose by thermophilic amylase was compared with that by β-amylase. The additional thermophilic amylase added to each reaction mixture at point a in Fig. 2 did not increase the saccharifying power. While the β-amylase added at point b increased the saccharifying power in the medium of thermophilic amylase. These results indicate that the thermophilic amylase is of an α-type of dextrinization.

Fig. 2

Effect of pH on Enzymatic Activity ------ Enzymatic activity of thermophilic α-amylase was tested at different pH's . The pH profile of the activity of thermophilic α-amylase

are shown in Fig. 3. The pH range of the maximal enzyme activity was found to be 4.5 to 6.5 and 5.0 to 6.0 at 37 and 60 °C, respectively. The optimun pH range at 60 °C was somewhat narrower than that at 37 °C.

Fig. 3

The optimun pH of B.subtilis α-amylase have been reported to be 5.6 to 6.0.

Effect of Temperature on Enzymatic Activity ------

The enzymatic activity of thermophilic and B.subtilis α-amylases at various temperatures was tested at pH 5.5 for 10 minutes. The temperature profiles of the activity of thermophilic and B.subtilis α-amylases are shown in Fig. 4. Temperature for the maximal activity of thermophilic α-amylase extended over the range from 65 to 73 °C. In the case of B.subtilis α-amylase, however, it was in the range from 43 to 58 °C. Optimum temperature range of maximal activity for thermophilic α-amylase was 15-20 °C higher than that for B.subtilis α-amylase . It was shown that even at 96 °C the same order of activity as that at 37 °C still remained within the 10 minutes incubation.

Fig. 4

Effect of pH on Stability ------ To study the effect of pH on the stability of thermophilic α-amylase, after the enzyme was incubated in 0.1 M buffer at various pH's at 37 °C for 30 minutes and 20 hours, the enzymatic activity was tested. The results are shown in Fig. 5. After incubation for 30 minutes, the enzyme was stable in the pH range between 6 and 11. After the incubation for 20 houres, the stable pH region became narrower than that in the former in acid side.

Fig. 5

The pH range where B.subtilis α-amylase is stable have been found to be from 5 to 12 (7).

Thermal Stability ------ To study the thermal stability of thermophilic α-amylase in comparison with that of B.subtilis α-amylase, both enzymes were incubated in a buffer solution of pH 6.0 at 90 °C and the remaining activity was measured after incubation for various intervals. The results are shown in Fig. 6. B.subtilis α-amylase was completly inactivated after 6 minutes, while thermophilic α-amylase lost only 17 % of original activity. After 30 minutes, thermophilic α-amylase still remained 20 % of original activity. It was shown that thermophilic α-amylase was more resistant to heat than B.subtilis α-amylase.

Fig. 6

Molecular Weight ------ The sedimentation patterns of thermophilic α-amylase are shown in Fig. 7. Single peak in the figure indicated that the enzyme behaved as a homogereous component in the sedimentation runs. The value of the sedimentation constant, $S_{20\ w}$, was calculated to be 4.2 S.

Fig. 7

The experiment of sedimentation equilibrium were carried out at three different enzyme concentration in 0.1 M acetate buffer solution of pH 6.0 at 25 °C. Fig. 8 shows the relation between distance from the center of rotation and log c at respective concentration. A linear relationship was shown in all cases. This result shows that molecular

Fig. 8

weight of the enzyme is independent on the concentration. Molecular weight of thermophilic α-amylase was calculated to be 48,000 by assuming a patial specific volume to be 0.72 ml per g (17). These values are very close to 49,000 (18) and 4.4 S (19) for B.subtilis α-amylase. Molecular weight

of 15,600 for α-amylase from B.stearothermophilus, strain 503-4 obtained by Campbell et al (4) is very smaller than that obtained here.

Intrinsic Viscosity ------ Viscometry of thermophilic α-amylase was carried out with 10.0 ml of the enzyme solution at various concentrations in 0.003 M acetate buffer of pH 8.0 at ionic strenght of 0.1. Fig. 9 shows the reduced viscosity, η_{sp}/C, as a function of concentration of the enzyme. The intrinsic viscosity, $[\eta]$, was estimated to be 0.032 (dl/g) by extrapolating the reduced viscosity to zero concentration. This value is very colse to 0.03 (dl/g) for B.subtilis α-amylase (20) and also to the value observed for

Fig. 9

the many other golbular proteins.of similar size.

Analysis of the Metal Bound to the Enzyme ------ Analysis of various metals contained in thermophilic α-amylase was carried out after dialyzing the enzyme against deionizing

water for 4 and 7 days at 4 °C. Mg, Zn, Fe, Ni, Sr, and Pb were not detected but one gram atom of calcium per mole was detected to be contained in the enzyme molecule.

Optical Rotatory Dispersion and Circular Dichroism ------ The ORD curves in the 210-250 mμ region for thermophilic and B.subtilis α-amylases were measured at pH 8.0 at 25 °C. As shown in Fig. 10 (a), the ORD curve for thermophilic α-amylase showed a negative Cotton effect with a trough at 233 mμ , cross over point at 223 mμ , and shoulder at 115 mμ.

Fig. 10

The ORD curve shows that thermophilic α-amylase contains a right-handed α-helical structure in the molecule (12). The mean residue rotation at 233 mμ , $[m']_{233}$, was found to be -5,090 °. The apparent content of the right-handed α-helix in thermophilic α-amylase was esticated to be about 24 per c cent. B.subtilis α-amylase showed also the ORD curve which is characteristic of proteins having a right-handed α-helical structure (Fig.10 b). The value of $[m']_{233}$ of B.subtilis

α-amylase was found to be -5,240 ° and the content of α-helix was estimated to be about 25 per cent.

The CD spectra in the 200-250 mμ region for thermophilic and B.subtilis α-amylases were measured at pH 8.0 at 25 °C. As shown in Fig. 11, the CD spectrum for thermophilic α-amylase showed two troughs at 210 and 225 mμ. These position of the troughs are characteristic of proteins having an α-helical structure (13, 14) as well as in the ORD curve (Fig. 10 a). The molecular ellipticity at 225 mμ, $[\theta]_{225}$,

Fig. 11

for thermophilic α-amylase was -8,070 deg. cm^2 per dmol. The content of α-helix in the enzyme was estimated to be about 20 per cent. The CD spectrum of B.subtilis α-amylase showed also the characteristic pattern of α-helical structure. The value of $[\theta]_{223}$was -9,500 deg. cm^2 per dmol and the content of α-helix in this enzyme was estimated to be about 24 per cent.

The Moffitt-Yang's parameters, a_o and b_o, of thermophilic and B.subtilis α-amylases were determined from the

ORD measurement in the 365-578 mμ region at pH 8.0 at 30 °C. The values of a_o and b_o for thermophiilic α-amylase were -225 ° and -115 °, respectively. Those for B.subtilis α-amylase were -130 ° and -150 °, respectively. The values of $[\alpha]_D$ of thermophilic and B.subtilis α-amylases were -42° and -32 °, respectively. It should be noted that the value of $[\alpha]_D$ of thermophilic α-amylase obtained by the authors is very different from that of α- amylase obtained by Campbell et al (4) which has a larger levorotation of -98.5 ° at D line.

Optical properties of thermophilic α-amylase are summarized in Table I in comparison with those of B.subtilis α-amylase and Campbell et al's α-amylase. The results suggest

Table I

that about 20 per cent of poly peptide chain assumes α-helical structure in thermophilic α-amylase and the content is very similar to that in B.subtilis α-amylase molecule.

Fig. 12 shows the CD spectra in the 250-320 mμ region of thermophilic and B.subtilis α-amylases at pH 8.0 at 25 °C.

It should be noted that the spectra of two enzymes are very differnt from each other.

Fig. 12

Amino Acid Composition ------ To compare the amino acid compositions of three kinds of α-amylase , i.e. α-amylases from B.stearothermophilus Donk, strain BS-1, B.stearothermophilus, strain 503-4 (5), and B.subtilis (18), the data are expressed in number of residues per 1,000 residues of the enzymes . Table II gives the amino acid compositions according to a tentative classification of the side chains of amino acid (18). In contrast with B.subtilis α-amylase lacking

Table II

the cysteine residue, it was found that thermophilic α-amylase contained one mole cysteine per mole by the denaturation by acidic (0.1 N HCl) 8 M urea. Cysteine was not detected in

the native thermophilic α-amylase molecule. Therfore, it seems that a cystein residue in thermophilic α-amylase is buried in the enzyme molecule.

In the class of amino acid with hydrophilic side chain, it can be seen that thermophilic α-amylase is lower in glutamic acid content relative to B.subtilis α-amylase. In the hydrophobic class, the contents of proline, leicine, and tyrosin in thermophilic α-amylase is somewhat higher than those in B.subtilis α-amylase. It can be said that the content of amino acids with ionizable side chain in thermophilic α-amylase is slightly lower but the content of amino acid with hydrophobic side chain is higher than that in B.subtilis α-amylase. An unusual higher content of prolin, 16 per cent, is characteristic of α-amylase obtained by Campbell and Manning (5). In thermophilic α-amylase obtained here, however, such anomaly could not be found, although the proline content is somewhat higher than that in B.subtilis α-amylase.

DISCUSSION

As shown in Figs. 4 and 6, thermophilic α-amylase maintained its catalitic function at higher temperature

and had higher thermal stability than B.subtilis α-amylase. These results are in agreement with the findings by others on α-amylases that the enzymes from thermophiles are more heat-resistant than those from mesophiiic sources (21-25). Some physico-chemical quantities with calcium content of thermophilic α-amylase were summarized in Table III in comparison with those of B.subtilis α-amylase and β-amylase obtained by Campbell et al. It was shown that the molecular weight, sedimentation constant and intrinsic viscosity of thermophilic α-amylase were similar to those of B.subtilis α-amylase but remarkably different from those of β-amylase obtained by Campbell et al. The value of intrinsic viscosity

Table IIII

of thermophilic α-amylase, 0.032 (dl/g), is that ordinarily observed for many globular proteins. The content of α-helix in thermophilic α-amylase was found to be about 20 per cent which was similar to that in B.subtilis α-amylase . It is apparent that thermophilic α-amylase is a compact globular

protein having α-helical structure as well as B.subtilis α-amylase . Thermophilic protease by Ohta et al (26) have been also reported to assume a compact globular structure with α-helical structure. The thermostability of enzymes from thermophiles are concluded not always to relate with the random structure of enzymes.

The CD spectra in the 250-320 mμ region for thermophilic and B.subtilis α-amylases were very differnet. This fact suggests that the local conformation in both enzyme molecules are very differnt from each other.

Another difference of thermophilic α-amylase from B.subtilis α-amylase was in the amino acid composition: The former had a slightly higher content of hydrophobic amino acid residues than the latter. A cysteine residue per mole was found in the former but not in the latter. It is noted that a slight abundance of amino acid with hydrophobic side chain and content of cysteine in thermophilic α-amylase may play a role on thermal stability.

SUMMARY

Some physico-chemical and biochemical properties of a crystalline α-amylase from B.stearothermophilus Donk, strain BS-1 (thermophilic α-amylase) were investigated. Thermophilic α-amylase was more resistant to higher temperature than B.subtilis α-amylase. Optimum temperature range for the activity of thermophilic α-amylase was found to be from 65-73 °C within 10 minutes incubation and it was almost 17 °C higher than that of B.subtilis α-amylase. Molecular weight and intrinsic viscosity of thermophilic α-amylase were 48,000 and 0.032 (dl/g), respectively, Thermophilic α-amylase showed a negative Cotton effect at 233 mμ in the optical rotatoty dispersion and two negative troughs at 210 and 225 mμ in the circular dichroic spectrum suggesting that the molecule contained a right-handed α-helical structure. The apparent content of α-helix in thermophilic α-amylase was estimated to be about 20 per cent. Molecular weight, intrinsic viscosity and content of α-helix of thermophilic α-amylase were different from those of α-amylase obtained by Campbell et al and very close to those of B.subtilis α-amylase. It may be concluded that thermophilic α-amylase has such a globular structure as those of many other α-amylase except α-amylase obtained by Campbell et al. The

difference of thermophilic α-amylase from B.subtilis α-amylase was found in the circular dichroic spectra in the 250-320 mμ region and in the amino acid composition.

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Fig. I

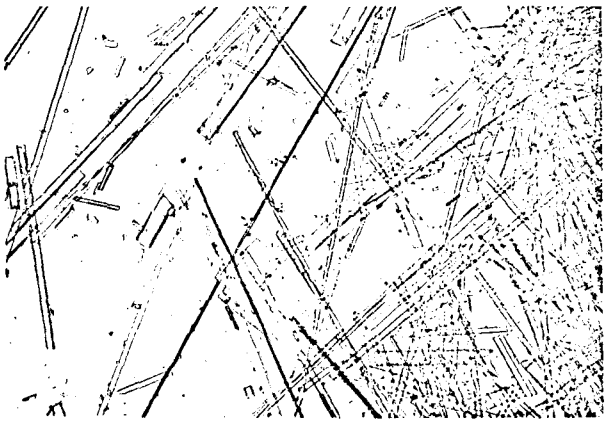


Fig. 2

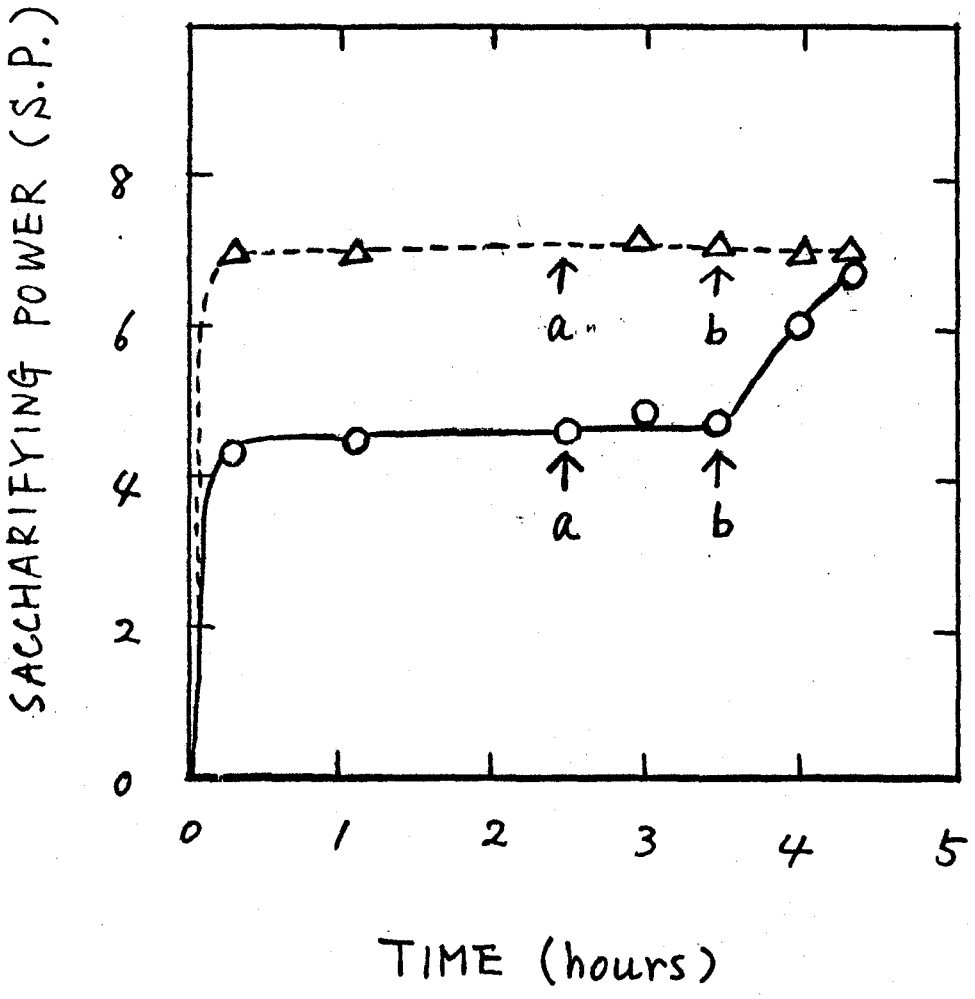
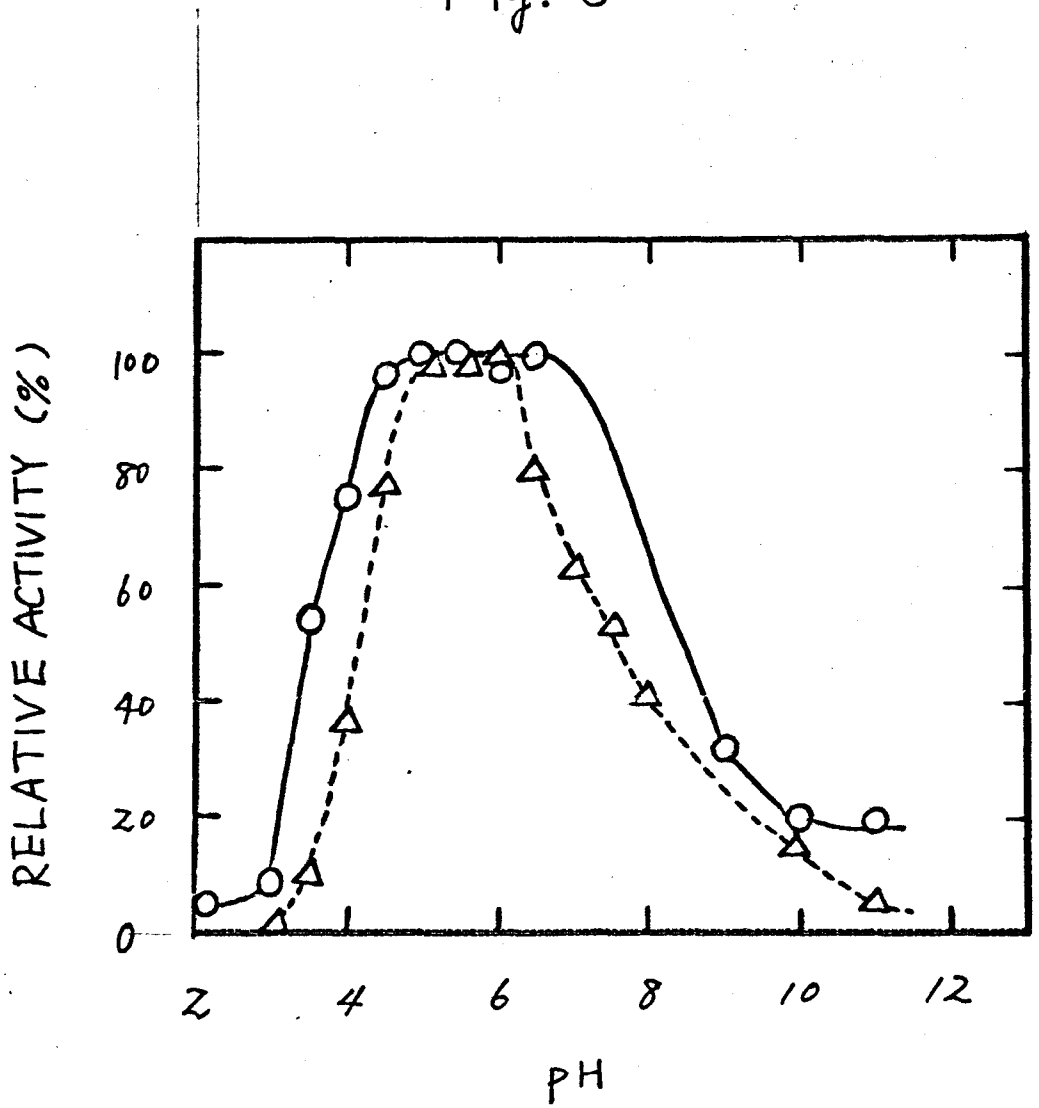


Fig. 3



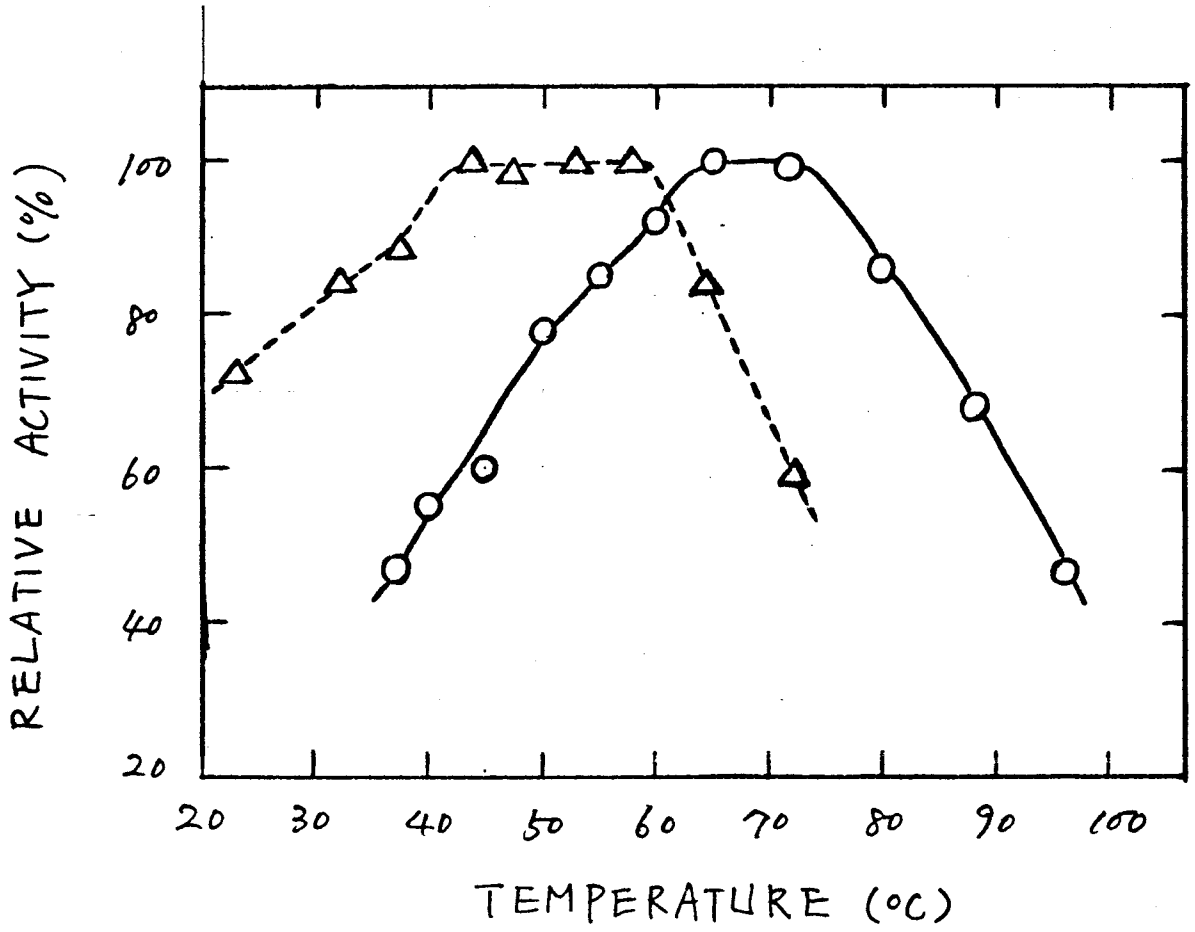

RELATIVE ACTIVITY (%)
100
80
60
40
20
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)

Fig.4

Fig. 5

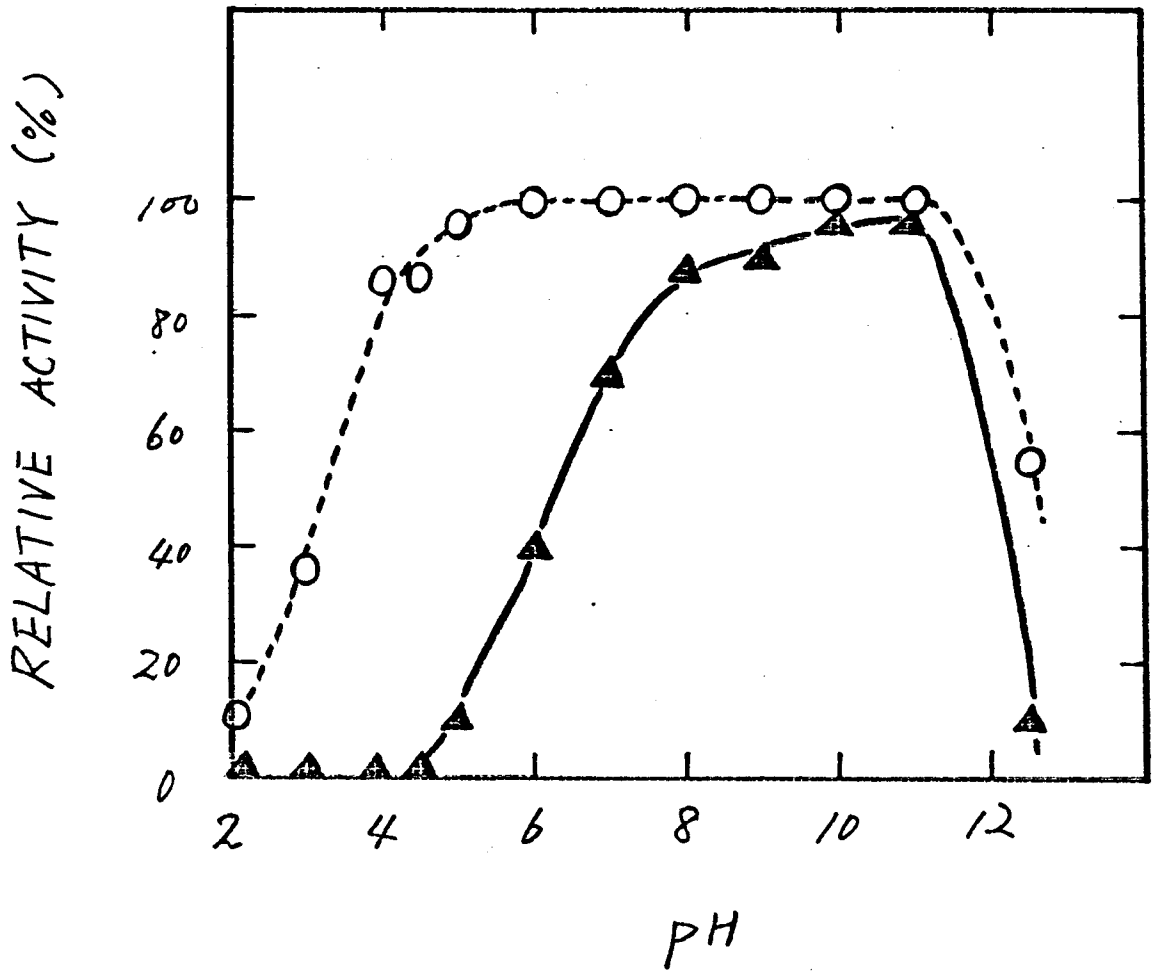
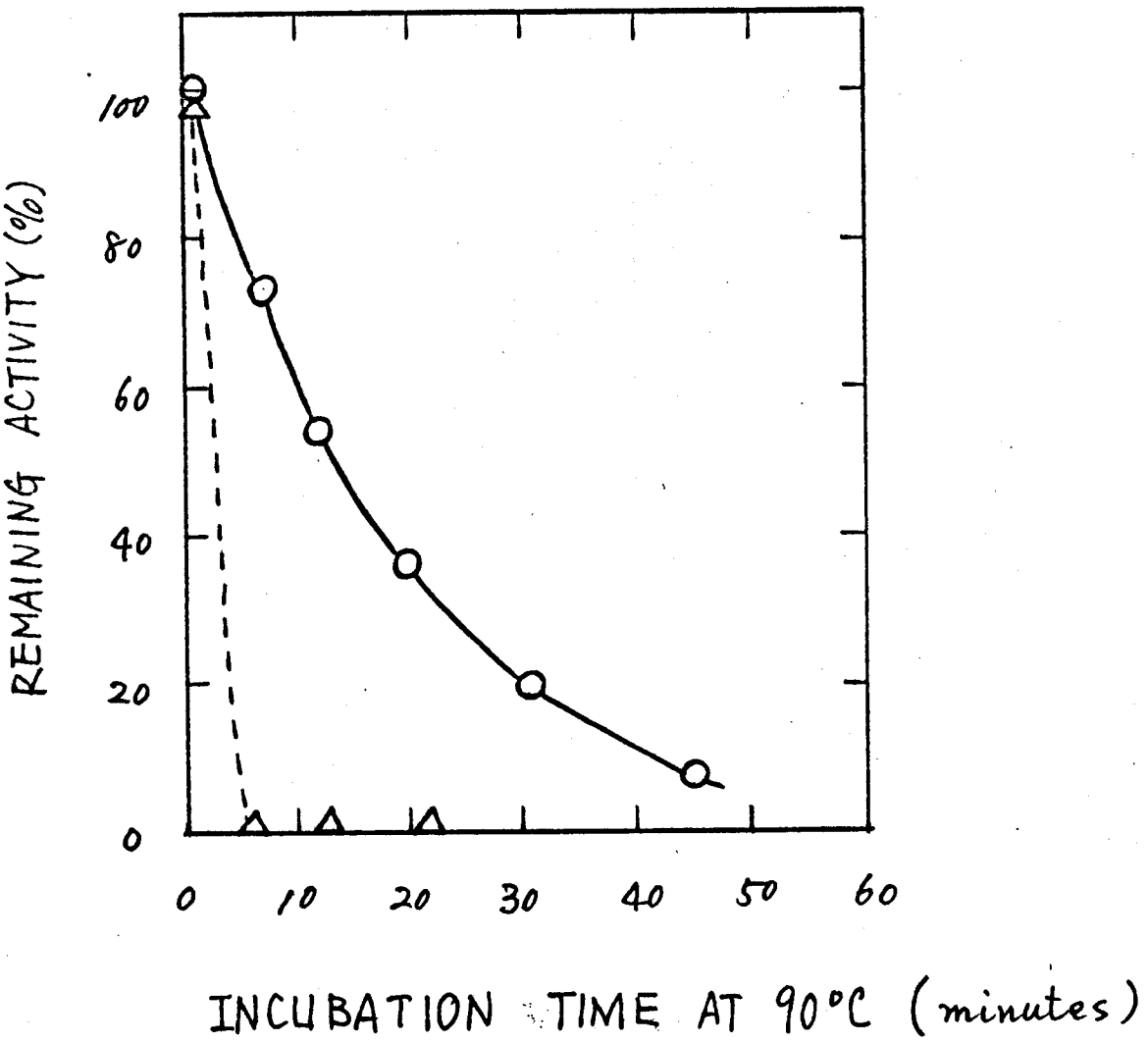


Fig. 6


REMAINING ACTIVITY (%)
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80
60
40
20
0
0
10
20
30
40
50
60
INCUBATION TIME AT 90°C (minutes)

Fig. 7

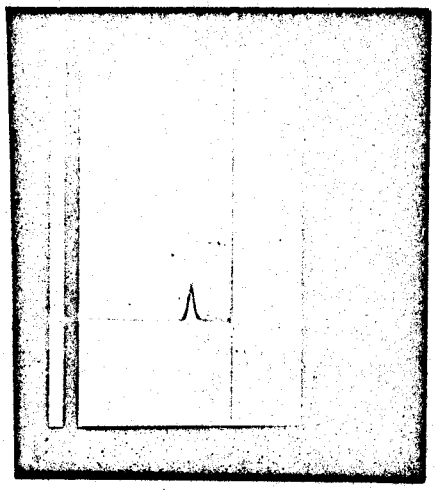


Fig. 8

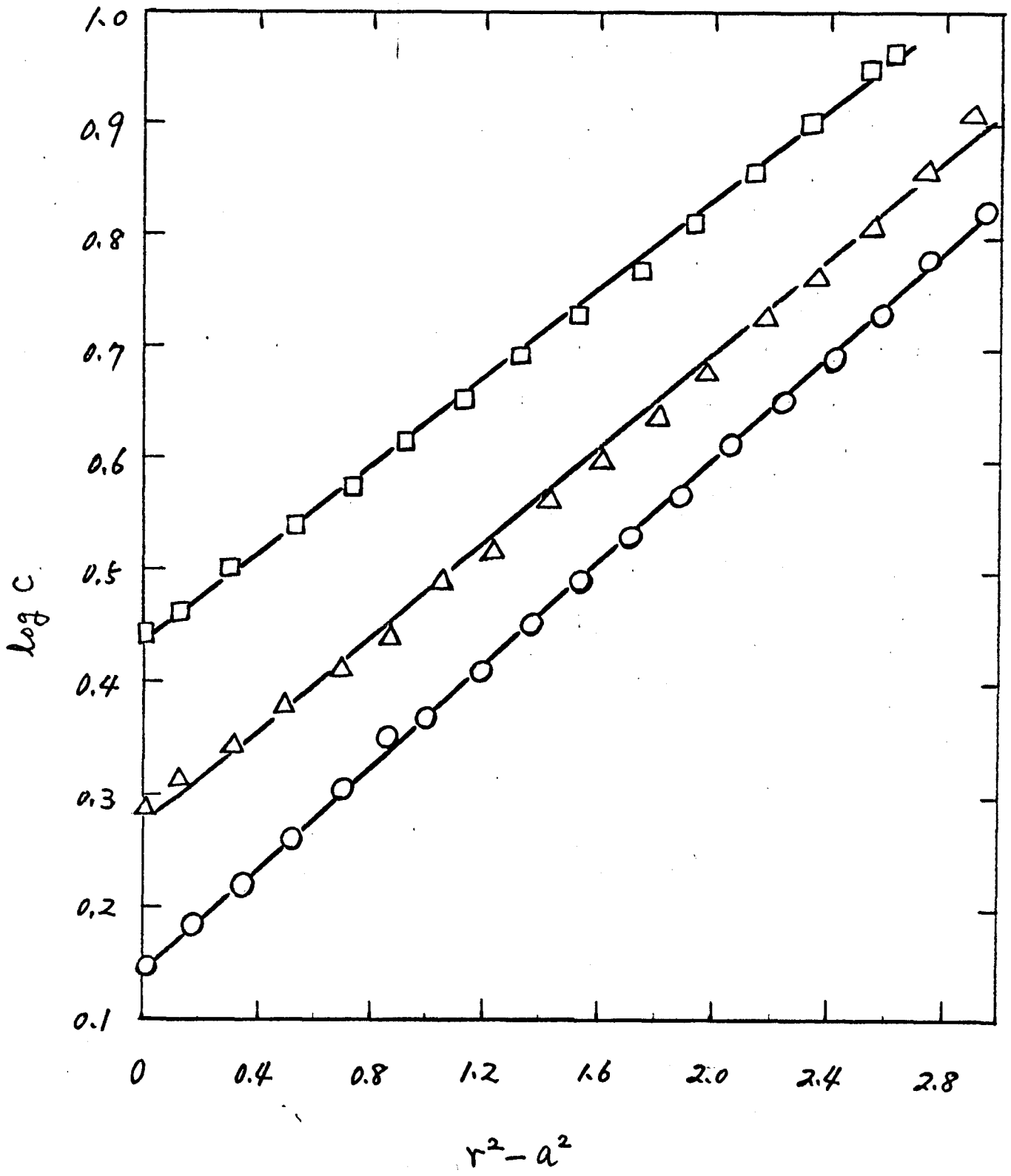
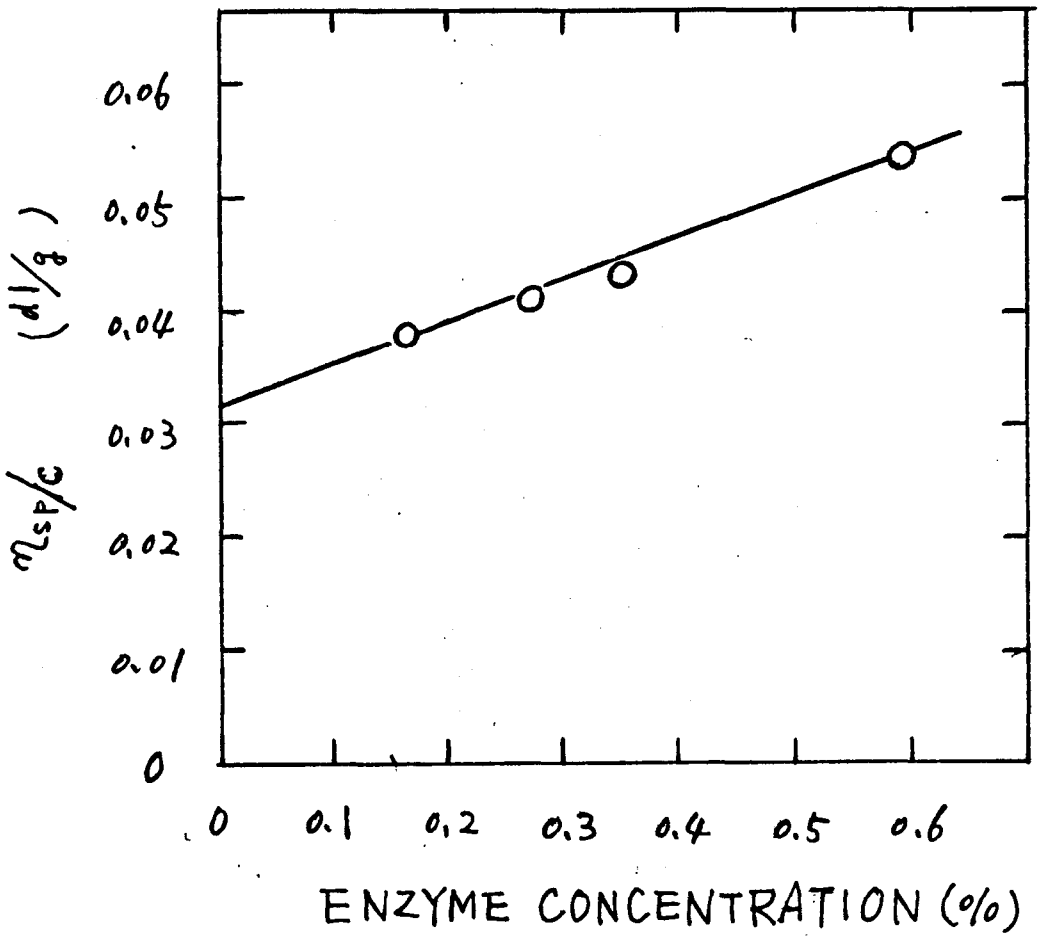


Fig. 9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0.04
0.03
0.02
0.01
0
$\eta_{sp/c}$ (dl/g)
0
0.1
0.2
0.3
0.4
0.5
0.6
ENZYME CONCENTRATION (%)

Fig. 10

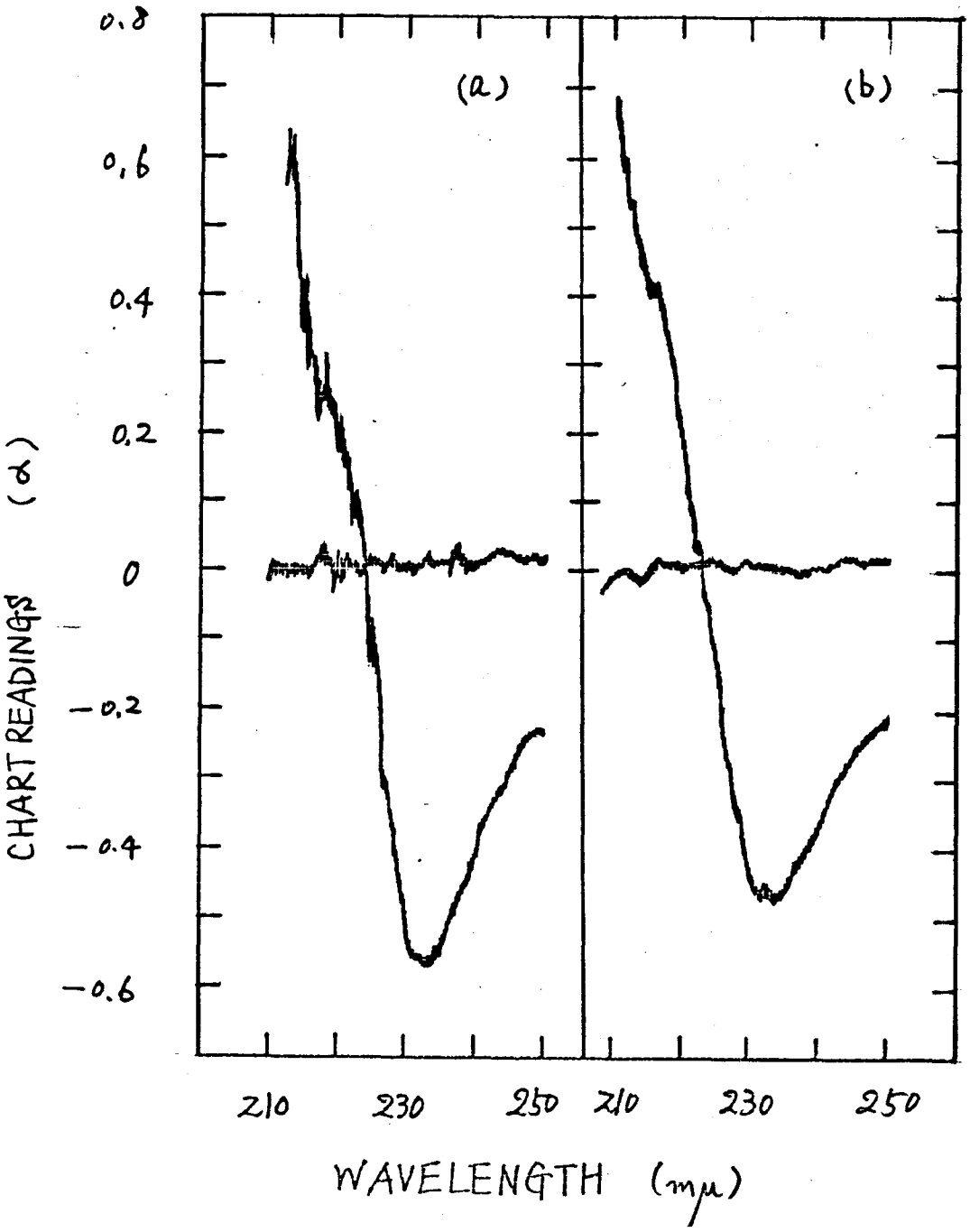
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
CHART READINGS (α)
(a)
(b)
210
230
250
210
230
250
WAVELENGTH (mμ)

Fig. 11

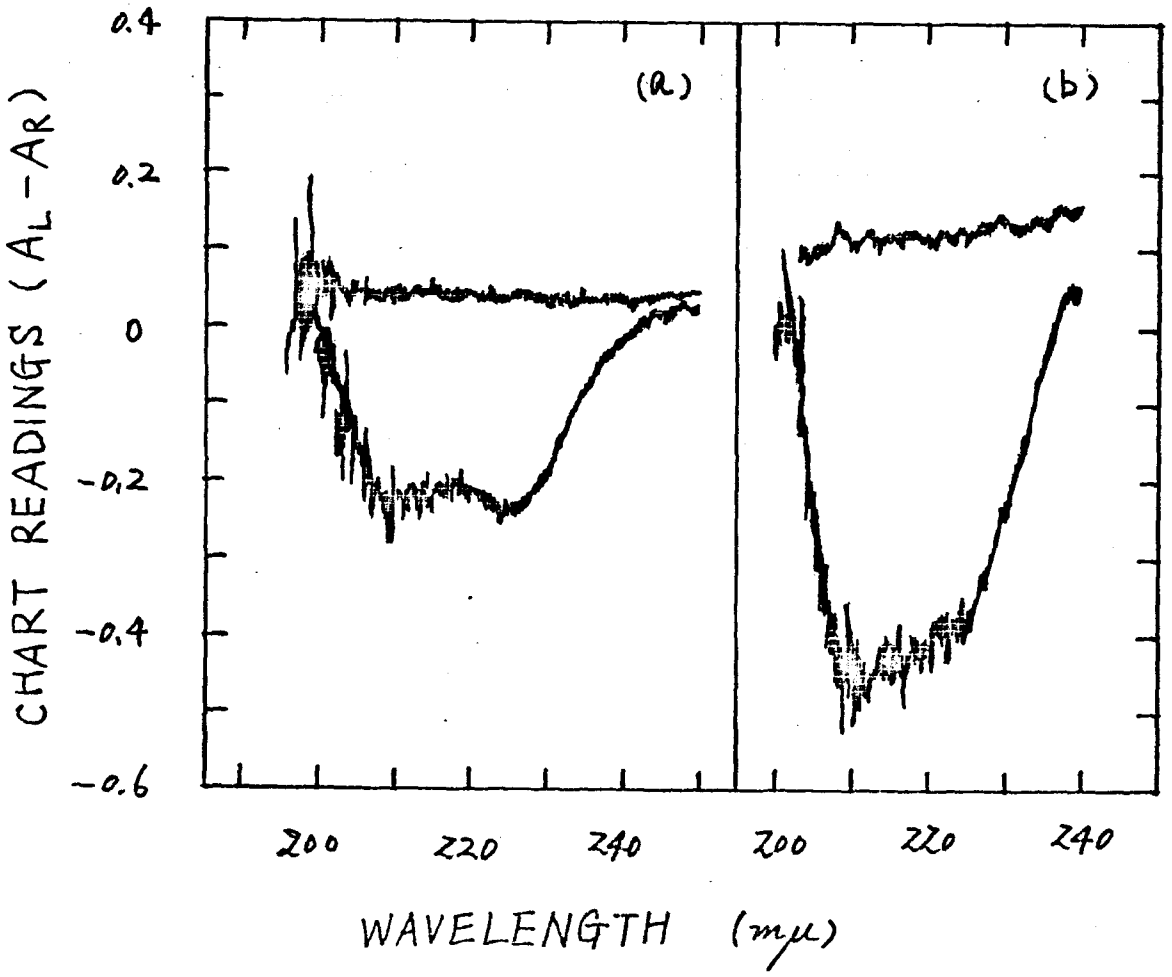
0.4
0.2
0
-0.2
-0.4
-0.6
CHART READINGS ($A_L - A_R$)
(a)
(b)
200
220
240
200
220
240
WAVELENGTH (mμ)

Fig. 12

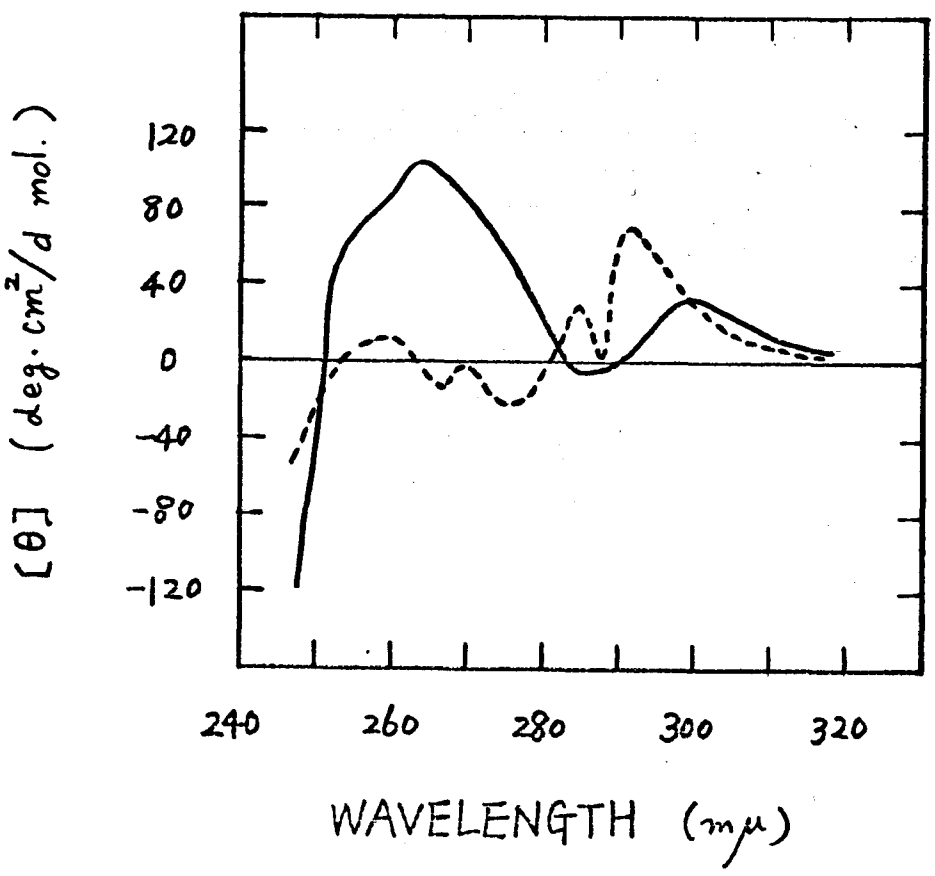
[θ] (deg·cm²/d mol.)
120
80
40
0
-40
-80
-120
240
260
280
300
320
WAVELENGTH (mμ)

Table I

Optical rotatory parameters of three kinds of α-amylase

	$-[\alpha]_D$ (deg.)	$-a_\circ$ (deg.)	$-b_\circ$ (deg.)	$-[m']_{233}$ (deg.)	$-[\theta]_{223-5}$ (deg. cm^2/dmol)
a Thermo.	42	225	115 (18 %)	5,090 (24 %)	8.070 (20 %)
b B.sub.	32	130	150 (25 %)	5,240 (25 %)	9,500 (24 %)
c Thermo.	98.5 (4)				

a, α-amylase from B.stearothermophilus Donk, strain BS-1

b, α-amylase from B.subtilis

c, α-amylase from B.stearothermophilus, strain 503-4

( ), α-helical content

Table II

Classification of side chains in three kinds of α-amylases

	Amino Acid	No.of Residues per mole of Thermophilic Amylase	No. of Residues/1,000 Residues		
			a Thermo.	b B.sub.	c Thermo.
Hydrophilic	Arg	15	36	42	21
	Lys	19	45	62	42
	His	10	24	30	28
	Asp	55	131	131	77
	Glu	32	76	106	155
			(31 %)	(37 %)	(32 %)
	Ser	20	48	59	42
	Thr	36	86	57	56
			(13 %)	(12 %)	(10 %)
	Gly	41	98	96	63
	Ala	28	67	71	56
			(17 %)	(17 %)	(12 %)
Hydrophobic	Val	24	57	62	77
	Leu	30	72	57	63
	Ileu	16	38	42	49
	Pro	18	43	34	155
	Met	8	19	12	21
	1/2Cys	1	2	0	28
	Phe	20	48	44	42
	Tyr	29	69	59	21
	Try	17	41	37	0
			(39 %)	(34 %)	(46 %)

a, α-amylase from B.stearothermophilus Donk, strain BS-1

b, α-amylase from B.subtilis (18)

c, α-amylase from B.stearothermophilus, strain 503-4 (5)

Table III

Some physico-chemical quantities of three kinds of α-amylase

	Mw.	$[\eta]$ (dl/g)	Calcium content (g atom/ mole)	$S_{20\,w}$
a Thermo.	48,000	0.032	1	4.16
b B.sub.	49,000 (18)	0.030 (20)	1-3 (20)	4.4-5.2 (19)
c Thermo.	.5,600 (4)	0.10-0.14 (4)	2 (3)	0.762 (4)

a, α-amylase from B.stearothermophilus Donk, strain BS-1

b, α-amylase from B.subtilis

c, α-amylase from B.stearothermophilus, strain 503-4

Fig. 1. Crystalline α-amylase from B.stearothermophilus Donk, strain BS-1.

Fig. 2. Comparison of the mode of hydrolysis of amylose by thermophilic amylase and β-amylase.
0.3 ml of thermophilic amylase(-o-)and β-amylase (--Δ--) at concentration of 10^{-2} % , respectively, ~~was~~ were added in 10 ml of 1 % of amylose solution at pH 5.5 and 37 °C. The arrows at a and b points show that additional 0.3 ml of thermophilic amylase and β-amylase, respectively, were added in each medium at the indicated time. 0.2 ml of aliquots of reaction mixtures were removed at various intervals and the saccharifying power (S.P.) were determined.

Fig. 3 Effect of pH on thermophilic α-amylase activity. The enzymatic activity was measured at 37 °C and 60 °C at 10 minutes after incubation with amylose at respective pH's. Concentrations of the enzyme during assay were 3.83 $X10^{-6}$ % at 37 °C and 1.53 x 10^{-6} % at 60 °C, respectively. (-o-)·, at 37 °C ; (--△--) , at 60 °C

Fig. 4. Effect of temperature on the activity of thermophilic and B.subtilis α-amylases.
The enzymatic activity was measured at pH 5.5 at 10 minutes at each temperature. Concentrations of thermophilic and B.subtilis α-amylases during assay were 1.53×10^{-6} % and 3.09×10^{-6}%, respectively.
(— o —), thermophilic α-amylase ;
(--Δ--), B.subtilis α-amylase

Fig. 5. Effect of pH on the stability of thermophilic α-amylase.

Thermophilic α-amylase was incubated in 0.05 M buffer of various pH's at 26 °C. After the enzyme was incubated for 30 minutes and 20 hours, enzymatic activity was tested at pH 5.5 at 37 °C for 10 minutes. Concentration of the enzyme during incubation at 26 °C was 1.40×10^{-6} %.

(--O--), for 30 minutes ;

(-▲-), 20 houres

Fig. 6. Thermal stability of thermophilic and B. subtilis α-amylases at 90 °C as a function of time.

The enzymes were incubated in 0.017 M acetate buffer of pH 6.0 at 90 °C. At various times the enzymatic activity was tested at pH 5.5 at 37 °C for 3 minutes. Concentrations of thermophilic and B. subtilis α-amylases during incubation were 1.19×10^{-4} % and 0.461×10^{-4} %, respectively. (—o—) , thermophilic α-amylase ;(··Δ··), B. subtilis α-amylase

Fig. 7. Sedimentation patterns of thermophilic α-amylase at 54,200 rpm at 25.7 °C, at 30 minutes
The sample solution of the enzyme was 0.228 % in 0.1 M acetate buffer of pH 6.0.

Fig. 8 Plot of logarithms of C vs $(r^2 - a^2)$ in sedimentation eqilibrum of thermophilic α-amylase.

C, concentration of solute ;

$(r^2 - a^2)$, the distance from the center of rotation.

(-○-), 0.0915 % of the enzyme ;

(-△-), 0.128 % ;

(-□-), 0.183 %

Fig. 9. Plots of the reduced viscosity of thermophilic α-amylase as a function of the enzyme concentration. Measurements of viscosity were carried out in 0.025 M Tris-Cl buffer of pH 8.0, ionic strenght of 0.1 at 25 °C.

Fig. 10. The ORD curves of thermophilic and B.subtilis α-amylases in the far ultraviolet region.
Concentrations of thermophilic and B.subtilis α-amylases were 0.0162 and 0.0130 %, respectively, in 0.05 M Tris-Cl buffer (pH 8.0). The light path of the cell used was 5 mm.
(a), thermophilic α-amylase ;
(b), B.subtilis α-amylase

Fig. 11. The CD spectra of thermophilic and B.subtilis α-amylases in the far ultraviolet region.

Concentrations of thermophilic and B.subtilis α-amylases were 0.0261 and 0.0420 %, respectively, in 0.05 M Tris-Cl buffer of PH 8.0. The light path of the cell used was 1 mm.

(a), thermophilic α-amylase ;

(b), B.subtilis α-amylase

Fig. 12. The CD spectra of thermophilic and B.subtilis α-amylases in the 250-320 mμ region.
Concentrations of thermophilic and B.subtilis α-amylases were 0.0697 and 0.102 %, respectively, in 0.05 M Tris-Cl buffer of pH 8.0. The light path of the cell used was 10 mm.

(——) , thermophilic α-amylase ;

(-----) , B.subtilis α-amylase

Studies on Thermophilic α-Amylase from Bacillus Stearothermophilus

[II] Thermal Stability of Thermophilic α-Amylase

In a previous paper (1), some physico-chemical properties of a crystalline α-amylase from B.stearothermophilus Donk,. strain BS-1 (thermophilic α-amylase)* were investigated and also compared with those of B.subtilis α-amylase. It was found that thermophilic α-amylase was so thermostable as to catalyze the hydrolysis of amylose even at 96 °C. Neverthless, it was found that thermophilic α-amylase contained a right-handed α-helical structure in contrast with α-amylase from B.stearothermophilus, strain 503-4 by Campbell et al (2) which exists as a (semi-)randomly coiled structure. It should be noted that thermophilic α-amylase had the molecular weight,

---

* Abbreviation used ; thermophilic α-amylase, α-amylase from B.stearothermophilius Donk, strain BS-1

sedimentation constant, and intrinsic viscosity which are very close to those of B.subtilis α-amylase. It was concluded that thermophilic α-amylase had a globular structure like many other α-amylase except α-amylase obtained by Campbell et al (2,3,4).

In the present investigation, to know the factor which stabilizes thermophilic α-amylase molecule, the effect of pH, urea and metal chelating agent on the thermal feature of the enzyme were studied by the measurements of enzymatic activity, optical rotatory dispersion, and circular dichroism with those of B.subtilis α-amylase.

EXPERIMENTAL

Materials ------ The crystalline α-amylase from B.stearothermophilus Donk, strain BS-1 cultured at 55 °C was prepared by Hagihara's method (5) with slight modifications and finally lyophilzed. Crystalline B.subtilis α-amylase was supplied by Daiwa Kasei Co. Ltd.. Urea was once recrystallized from 70 per cent aqueous ethanol.

Methods ------ The concentrations of thermophilic and B.subtilis α-amylases were estimated from absorption at 280 mμ assuming $E^{1\,cm}_{1\,\%} = 28.7$ and 25.6, respectively, determined

experimentally.

The α-amylase activity was determined by the measurement of dextrinizing power (D.P.) using amylose as a substrate according to Fuwa's method (6) with slight modifications. Incubation was carried out at 37 °C at pH 4.5 for 30 minutes.

Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements were made using a Jasco spectropolarimeter model ORD/UV-5 with a CD ataachment. Specific rotation was expressed in terms of the reduced mean residue rotaion, [m'], corrected the effect of the refractive index (n) of the solvent. The mean residue weight of thermophilic and B.subtilis α-amylases were assumed to be 115. Molecular ellipticity, [θ], was calculated using the equation,

$$[\theta] = 3.300\ (\varepsilon_l - \varepsilon_r)$$

where $(\varepsilon_l - \varepsilon_r)$ is the difference between the molar extinction coeffeicents left and right circularly polarized lights. In the calculation $(\varepsilon_l - \varepsilon_r)$, the mean residue weight was used instead of the molecular weight.

Right-handed α-helical content was esticated as follows,

α-helix per cent

$$= \frac{[m']_{233} - 1,700}{14,000} \times 100 \qquad (7)$$

$$= \frac{-[\theta]_{222}}{40,000} \times 100 \qquad (8,9)$$

RESULTS AND DISCUSSION

Enzymatic Activity ------ It was found previously (1) that thermophilic α-amylase contained one gram atom of calcium per mole of the enzyme. It has been reported that α-amylases isolated from various sources contain a few gram atoms of firmly bound calcium (10), and that these calcium atoms play an important role in the catalytic activity and stabilization of the enzyme molecule (11,12). Therfore, the effect of calcium ion on the stability of thermophilic α-amylase was studied. After the enzyme was incubated in a buffer solution of pH 7.9 containing EDTA, and EDTA and additional $CaCl_2$, at 37 and 60 °C, respectively, the enzymatic activity was measured at various intervals. The results are shown in Fig. 1. When the enzyme

Fig. 1

was incubated in a buffer solution without EDTA, thermophilic α-amylase remained 100 % of original activity after incubation for 180 minutes at 37 °C, while the enzyme was slowly inactivated during the incubation at 60 °C. This fact may indicates that the isolated thermophilic α-amylase itself is more stable thermodynamically at temperature lower than optimum growing temperature, 55-65 °C, for this bacteria.

In the buffer solution containing 0.005 M EDTA, the enzyme was more rapidly inactivated than in the buffer without EDTA and lost completely its activity after keeping the enzyme at 60 °C for 15 minutes. In the buffer containing 0.005 M EDTA with additional 0.025 M $CaCl_2$, on the other hand, the enzyme maintained its activity in full or slightly higher than the original. It was found that thermophilic α-amylase was stabilized by the presence of calcium ion as obserbed in other α-amylases from various sources (10,11,12).

Thermal stability of thermophilic α-amylase in various conditions was investigated in comparison with that of B.subtilis α-amylase. The enzymes were incubated at various temperatures for 15 and 30 minutes and the enzymatic activity remained was measured. The activity remained was pressented as a value in percentage refered to that of native enzyme at the same concentration.

Fig. 2 shows the thermal profiles of thermophilic and B.subtilis α-amylases at pH 7.9. Thermophilic α-amylase retained 100 % of its original activity up to 60 °C for 30 minutes, while B.subtilis α-amylase lost about 60 % of its original activity. From the curves for the 30 minutes incubation,

Fig. 2

transition temperature,Tm, defined as that retaining 50 % of the original activity was found to be 74 °C for thermophilic α-amylase while 58 °C for B.subtilis α-amylase.

Fig. 3 shows the thermal profiles of thermophilic and B.subtilis α-amylases at pH 4.6. The Tm points for thermophilic and B.subtilis α-amylases were 58.5 and 30.5 °C, respectively.

Fig. 3

Thermal stability of both α-amylases at pH 4.6 was lower tnan that at pH 7.9 (Fig. 2). This may be due to the weaker

binding of calcium to the enzyme at pH 4.6 (12).

Fig. 4 shows the thermal profiles of both α-amylases in 8 M urea at pH 8.5. After incubation for 30 minutes at 30 °C, B.subtilis α-amylase lost about 40 % of the original activity, whereas thermophilic α-amylase retained 90 % of the original activity. At 60 °C where B.subtilis α-amylase was completly inactivated during 30 minutes incubation, thermophilic α-amylase still retained 75 % of its activity. It seems that the presence of 8 M urea does not so affect the thermal stability of thermophilic α-amylase much as it does the stability of B.subtilis α-amylase. It has been also reported that the presence of urea does no cause a complete inactivation of α-amylase (2) and protease (13) from other thermophilic bacteria at room temperature. The values of Tm after incubation for 30 minutes were 67.5 and 35 °C for thermophilic and B.subtilis α-amylases, respectively.

Figl 4

Fig. 5 shows the thermal profiles of both α-amylases in 10^{-2} M EDTA solution of pH 6.5. The values of Tm for thermophilic and B.subtilis α-amylases were 41 and 32 °C, respectively. In this case, difference in the Tm points between both enzymes was smaller than those shown in Figs. 2.3. and 4. It seemed that the themal profiles of B.subtilis α-amylase was little affected by the conditions, i.e. pH 4.6, 8 M urea, and 10^{-2} M EDTA. In the case of thermophilic α-amylase, it was revealed

Fig. 5

that 10^{-2} M EDTA was remarkably effective than 8 M urea.

CD Spectra in Various Conditions ------ It was found previously (1) that thermophilic α-amylase was a compact globular protein having α-helical structure from the measurements of physico-chemical properties. In the present studies, the effect of urea, EDTA, and pH on the circular dichroic property of thermophilic α-amylase were investigated. The results are shown in Fig. 6. In the presence of 8 M urea at room temperature at pH 8.0, the CD specturm of thermophilic

α-amylase was almost the same as that of the native enzyme.

Fig. 6

Therfore, it seems that 8 M urea does not cause a destruction of the α-helical conformation at room temperature. It has been reported that the optical rotation at D-line of α-amylase from B.stearothermophilus, strain 503-4 is not significantly affected by 8 M urea or 4 M guanidine (2). Ohta has also reported that a protease from thermophilic bacteria is very stable against 8 M urea (13).

In the presence of 10^{-2} M EDTA at pH 8.0, the CD spectrum of thermophilic α-amylase at room temperature also showed the similar pattern as that of the native one. This may indicate that the removal of calcium does not cause a conformational alternation such as to detectable in the CD measurement at room temperature. Imanishi has reported that the ORD parameters and difference spectra for both "calcium-full" and "calcium-depleted" B.subtilis α-amylases are essentially identical with each other below 15 °C (12).

Effect of Temperature on the Conformation of Thermophilic α-Amylase ------ The effect of temperature on the ORD curve of thermophilic α-amylase was studied. Fig. 7 shows the ORD curves at pH 8.0 at three different temperatures. The ORD curve and the valus of a negative trough at 233 mμ did not change on geating up to 50 °C. On heating at 60 °C, the negative value of $[m']_{233}$ decreased from -5,090 ° to -3,610 ° although the ORD curve still showed a negative trough

Fig. 7

at 233 mμ. At 72 °C, the negative value of $[m']_{233}$ further decreased to -2,460 °. Scince the enzyme solution at pH 8.0 became turbid at elevated temperature above 72 °C, the ORD measurement of the enzyme could not be done.

Fig. 8 shows the ORD curves at three different temperatures in the presence of 8 M urea at pH 8.0. At 51 °C, the trough at 233 mμ became shallower to the value of -3,270 ° in the $[m']_{233}$. At 61 °C, the ORD curve of thermophilic α-amylase became simple and no longer showed a negative trough at 233 mμ. The value of $[m']_{233}$ in this case was found to be -2,700°.

Although this negative value was larger than that of randomly-coiled synthetic polypeptide chain, -1,700 °, most α-helical sturcture in thermophilic α-amylase might be disrupted at 61 °C in the presence of 8 M urea. From the results shown in

Fig. 8

Figs. 7 and 8, it was apparent that the structural destruction of thermophilic α-amylase by heating is promoted in the presence of 8 M urea than in the absence of urea as observed in thermophilic protease (13).

SUMMARY

The effects of pH, urea and EDTA on the thermal stability of thermophilic α-amylase were studied by the measurements of enzymatic activity and optical activity in the far ultraviolet region at various temperatures. Thermophilic α-amylase became unstable by removing calcium and protected against an inactivation by the presence of saturation level of calcium. Thermal stability of the enzyme was affected by 10^{-2} M EDTA than 8 M urea. This was more remarkable in thermophilic α-amylase than in B.subtilis α-amylase. These results suggested that calcium ion plays an important role in thermal stability of thermophilic α-amylase molecule. In the presence of 8 M urea, thermophilic α-amylase scarcely showed an alteration on the CD spectrum at room temperature, although the presence of 8 M urea promoted the denaturation of thermophilic α-amylase at elevated temperature.

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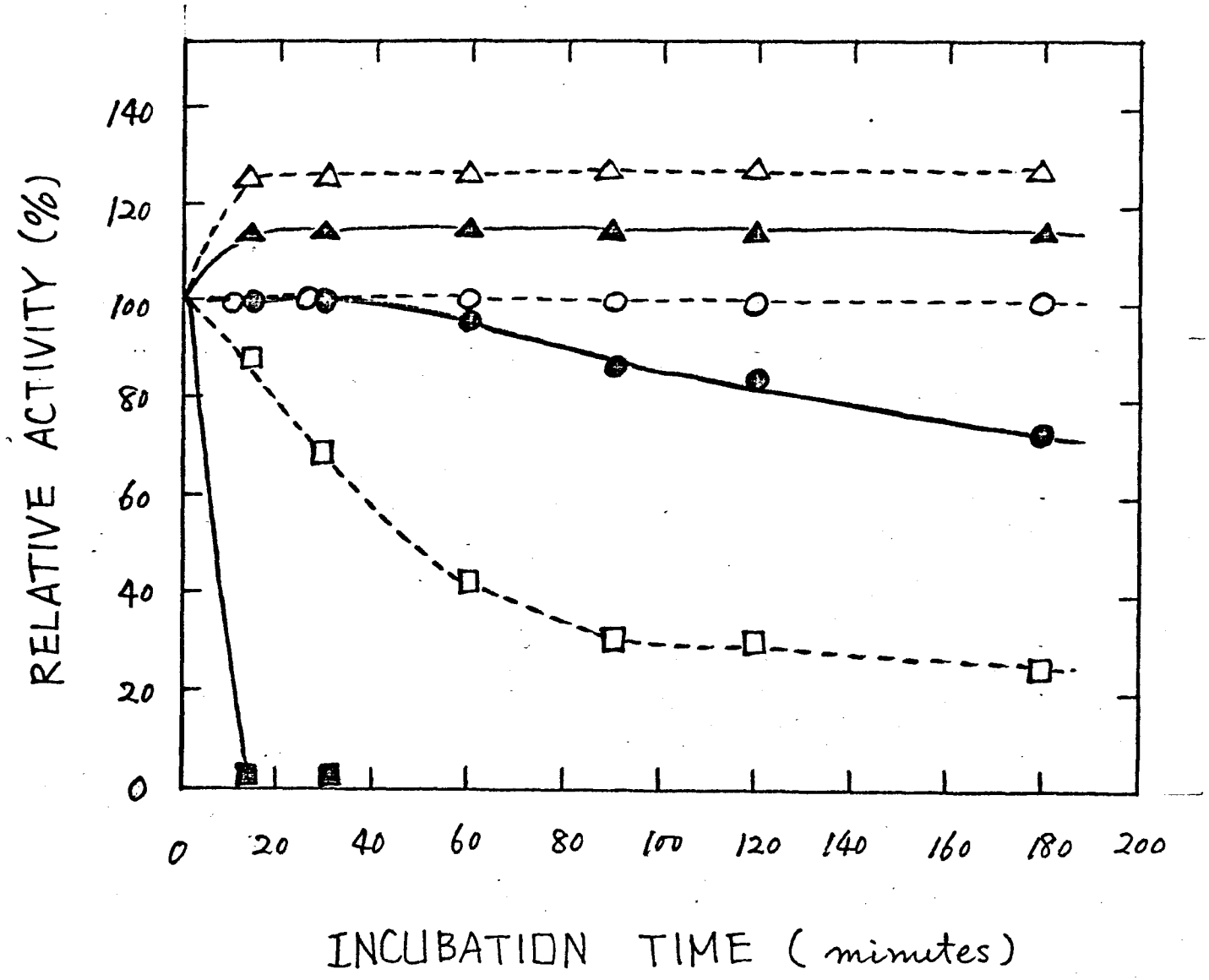
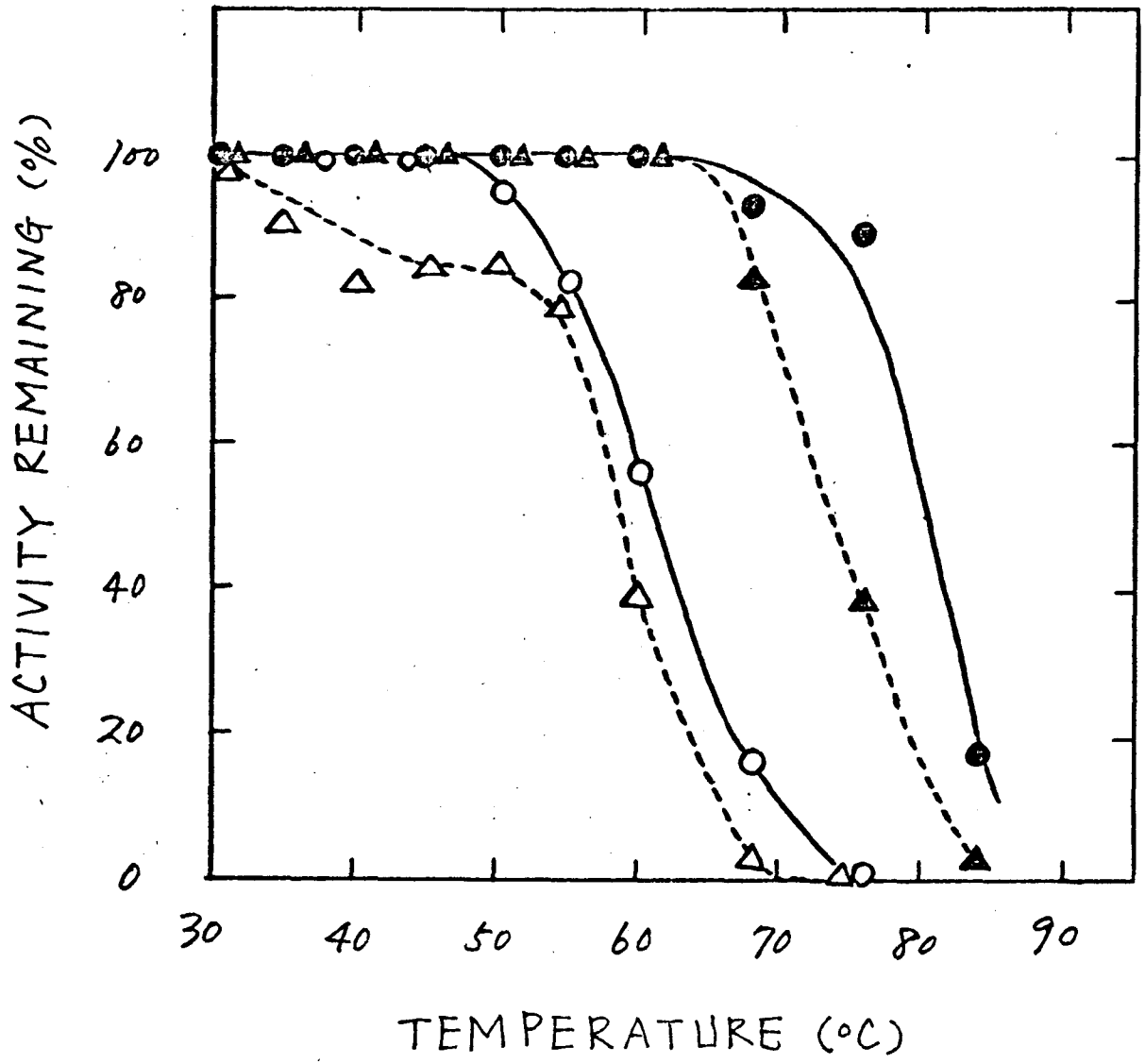

RELATIVE ACTIVITY (%)
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
INCUBATION TIME (minutes)

Fig. 1

Fig. 2


ACTIVITY REMAINING (%)
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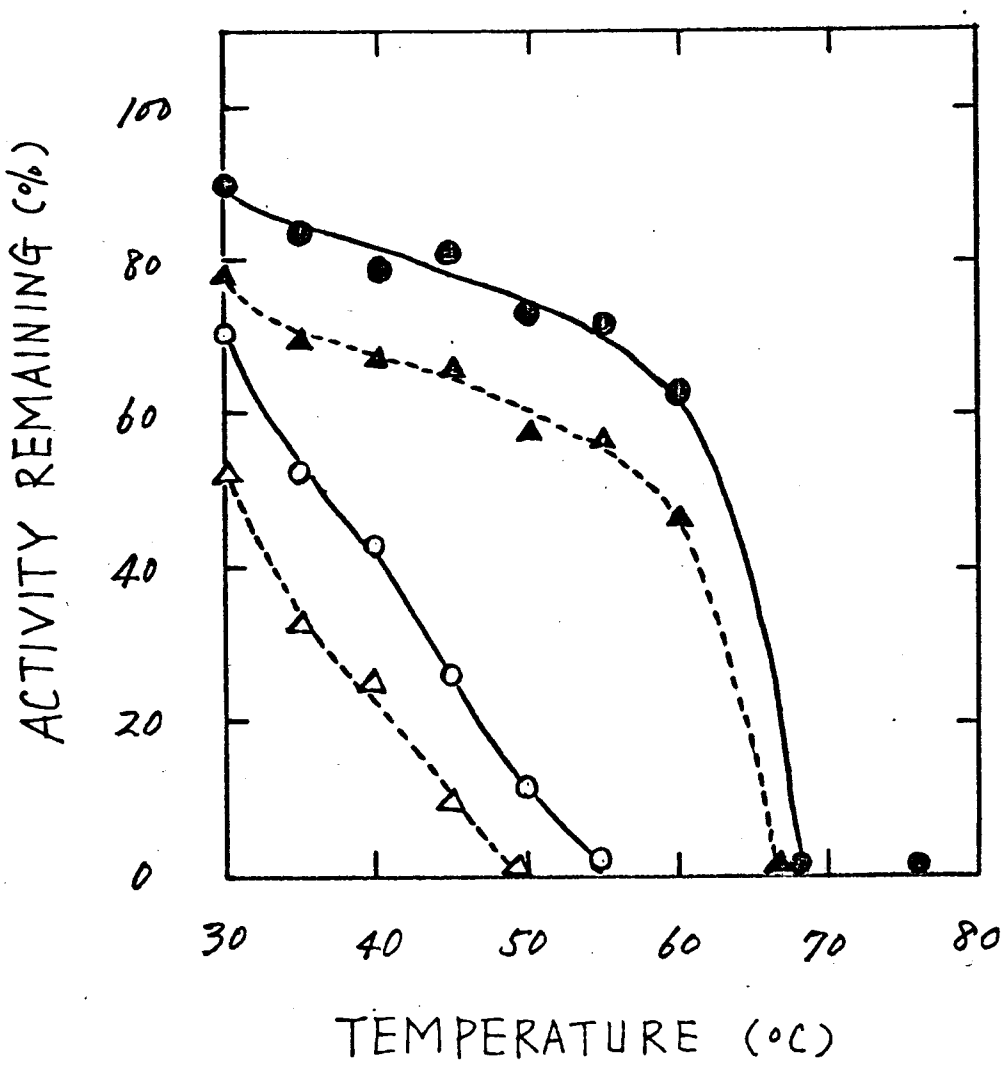
80
60
40
20
0
30
40
50
60
70
80
90
TEMPERATURE (°C)


ACTIVITY REMAINING (%)
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60
40
20
0
30
40
50
60
70
80
TEMPERATURE (°C)

Fig. 3

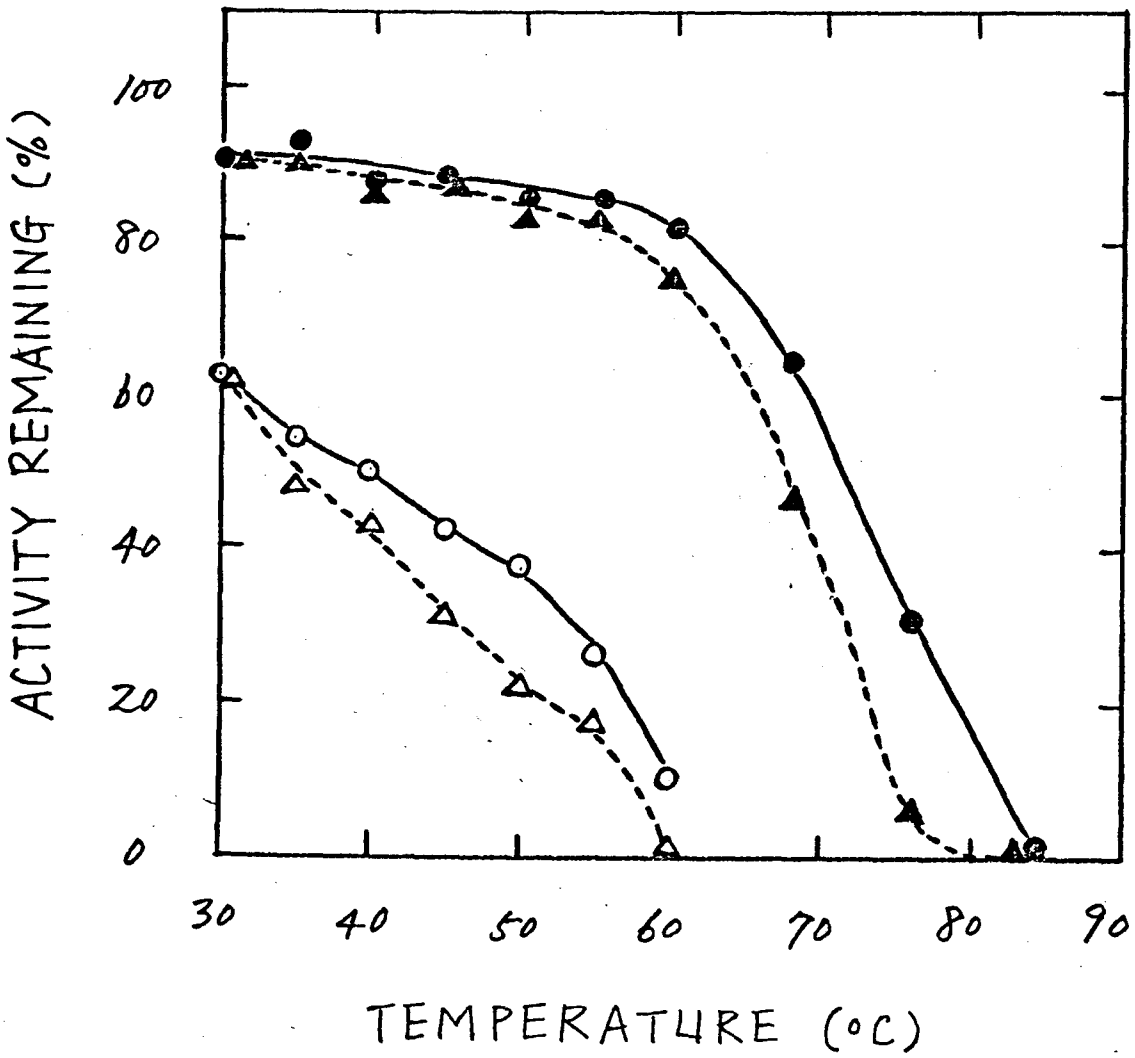
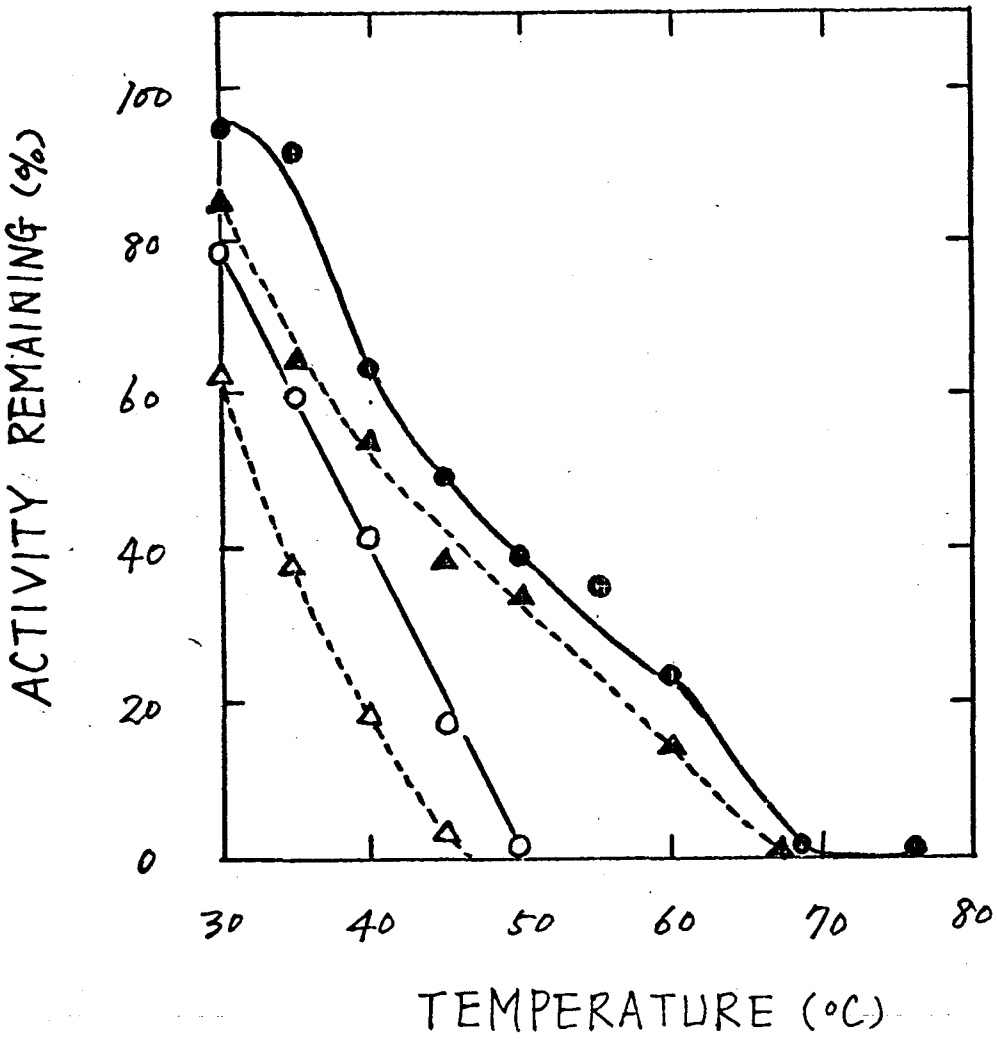

ACTIVITY REMAINING (%)
100
80
60
40
20
0
30
40
50
60
70
80
90
TEMPERATURE (°C)

Fig. 4


ACTIVITY REMAINING (%)
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80
60
40
20
0
30
40
50
60
70
80
TEMPERATURE (°C)

Fig. 5

Fig 6

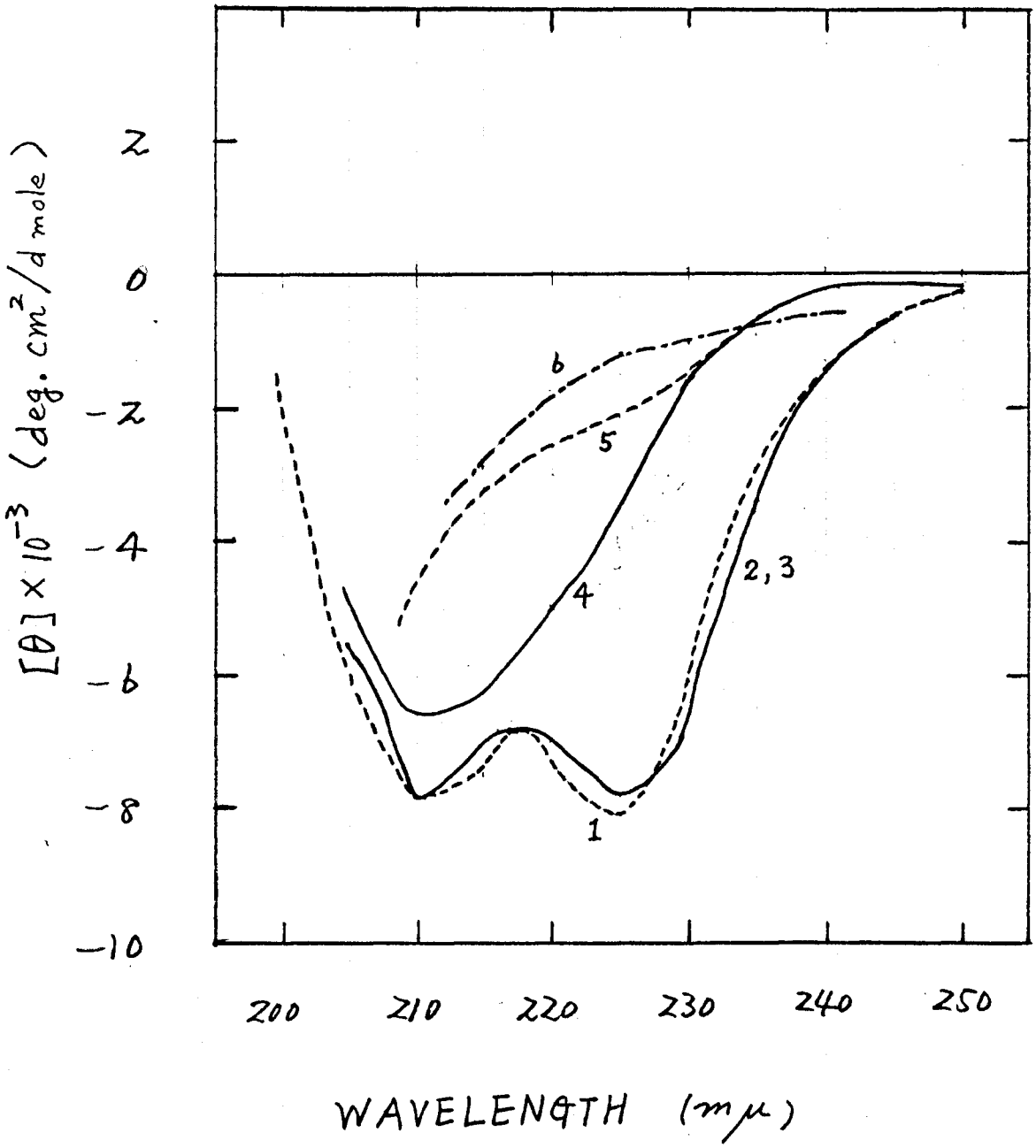
2
0
-2
-4
-6
-8
-10
[θ] × 10^-3 (deg. cm^2/d mole)
200
210
220
230
240
250
WAVELENGTH (mμ)
6
5
4
2,3
1

Fig. 7

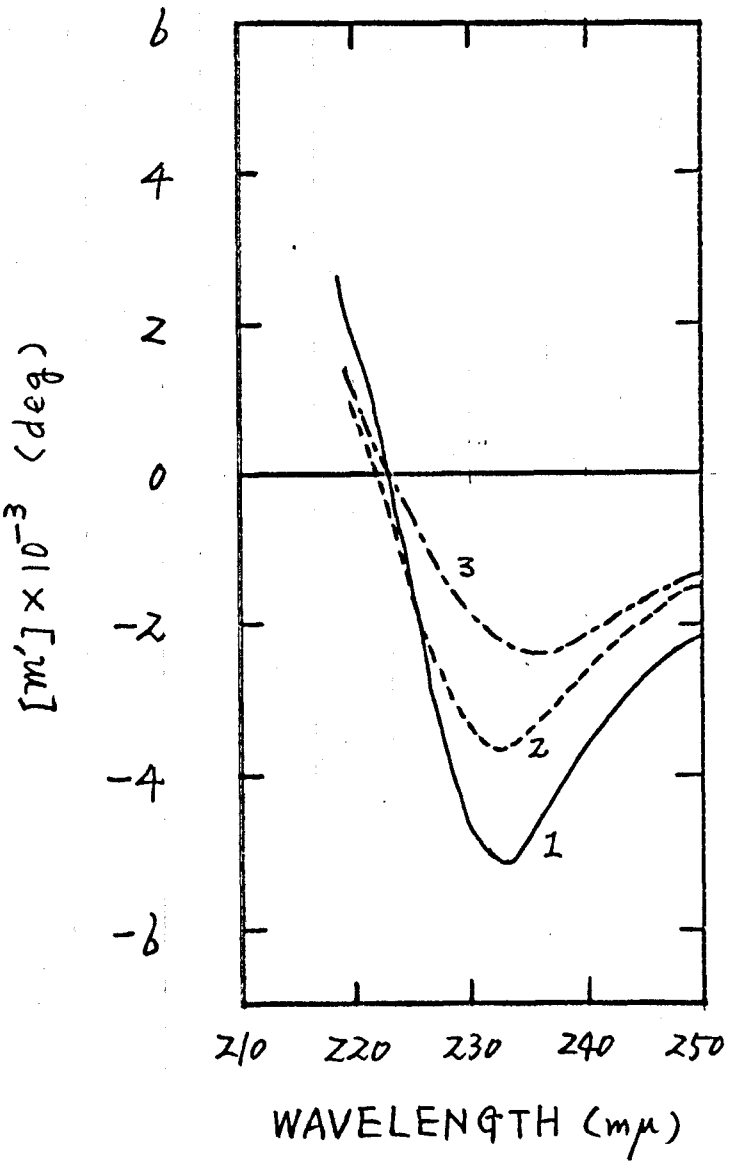

Fig. 8

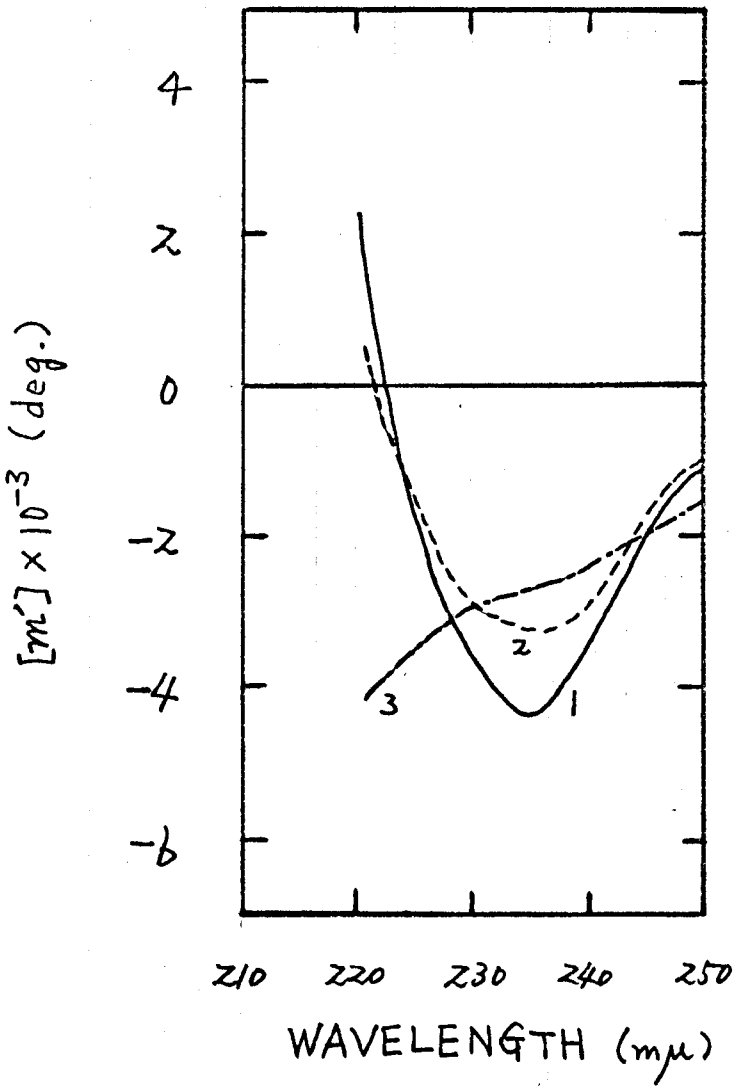

Fig. 1. Effect of calcium and EDTA on the thermal stability of thermophilic α-amylase.

Thermophilic α-amylase was incubated in 0.1 M Tris-Cl buffer solution of pH 7.9 containing 0.005 M EDTA, and 0.005 M EDTA and additional 0.025 M $CaCl_2$. 0.2 ml of aliquots of incubation mediums were removed at various intervals and the enzymatic activity was measured. Concentration of thermophilic α-amylase during incubation was 1.29×10^{-4} %.

Closed symbols represent the activity of the enzyme incubated at 60 °C and open symbols that incubated at 37 °C.

(○, ●), buffersolution alon ;

(□, ■), buffer solution with 0.005 M EDTA

(△, ▲), buffer solution with 0.005 M EDTA and additional 0.025 M $CaCl_2$

Fig. 2. Thermal stabilities of thermophilic and B.subtilis α-amylases at pH 8.0.

Both enzymes were incubated in 0.025 M Tris-Cl buffer at various temperatures. After incubation for 15 and 30 minutes, 0.2 ml of aliquot of the solution was taken and the enzymatic activity was measured. Concentrations of thermophilic and B.subtilis α-amylases during incubation were 0.732×10^{-4} and 1.41×10^{-4} %, respectively.

Closed symbol, thermophilic α-amylase ;

Open symbol, B.subtilis α-amylase.

Circles (o , ●) represent the data for the incubation for 15 minutes. Triangles (△ , ▲) for the incubation for 30 minutes.

Fig. 3. Thermal stabilities of thermophilic and B.subtilis α-amylases at pH 4.6.

Both enzymes were incubated in 0.025 M acetate buffer at various temperatures. Concentrations of both α-amylases during incubation and assay were the same as those of Fig. 2.

Closed symbol, thermophilic α-amylase ;

Open symbol, B.subtilis α-amylase.

Circles (○, ●) represent the data for the incubation for 15 minutes, Triangles (△, ▲) for the incubation for 30 minutes.

Fig. 4. Effect of urea on thermal stabilities of thermophilic and B.subtilis α-amylases.

Both α-amylases were incubated in the presence of 8 M urea at pH 8.5. Other conditions and symbols were the same as those of Fig. 2 .

Fig. 5. Effect of EDTA on thermal stabilities of thermophilic and B.subtilis α-amylases.

Both α-amylases were incubated in a buffer solution of pH 6.5 with 10^{-2} M EDTA. Other conditions and symbols used were the same as those of Fig.2.

Fig. 6. The CD spectra of thermophilc α-amylase at room temperature.

Curve 1, in 0.025 M Tris-Cl buffer of pH 8.0;

Curve 2, in 8 M urea at pH 8.0;

curve 3, in 10^{-2} M EDTA at pH 8.0;

curve 4, in 0.1 N HCl;

curve 5, in 0.1 N NaOH;

curve 6, acidic (0.1 N HCl) 8 M urea

Concentration of thermophilic α-amylase in each case, 0.0261 %.

Light path of the cell , 1 mm.

Fig. 7. Effect of temperature on the ORD curve of thermophilic α-amylase at pH 8.0.

Curve 1, at 27-50 °C; curve 2, at 60 °C; curve 3 , at 72 °C.

Concentration of thermophilic α-amylase in each case, 0.00662 %.

Light path of the cell , 10 mm.

Fig. 8. Effect of temperature on the ORD curve of thermophilic α-amylase in the presence of 8 M urea at pH 8.0. Curve 1, at 27 °C; curve 2, at 51 °C; curve 3, at 61 °C. Concentration of thermophilic α-amylase in each case, 0.00662 %. Light path of the cell, 10 mm.

Studies on Thermophilic α-Amylase from Bacillus Stearothermophilus

III Effect of Temperature on the Renaturation of Denatured Thermophilic** α-Amylase

In privious papers (1,2), some physico-chemical properties and thermal stability of a crystalline α-amylase from B.stearothermophilus Donk, strain BS-1 ~~was~~ were investigated and also compared with those of B.subtilis α-amylase. It was found that thermophilic α-amylase could catalyze the hydrolysis of amylose at higher temperature and is more heat-stable than B.subtilis α-amylase. However, some physico-chemical characteristics such as molecular weight, intrinsic viscosity and α-helical content of thermophilic and B. subtilis α-amylases were found to be similar to each other.

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**Abbreviation used: thermophilic α-amylase, α-amylase from B.stearothermophilus Donk, strain BS-1

It is now generally believed that the specific configuration which is assumed by a polypeptide chain in the formation of a native protein is determined principally by its amino acid sequence (3). In view of this hypothesis, the process of renaturation of denatured enzyme may show the final step of enzyme biosynthesis. The study on the renaturation of the denatured enzyme may provide a clue to mechanism of formation of spatial structure of nascent polypeptide chain in the cell. Because each organism has an inherent optimum growing temperature, the process of the renaturation of the denatured enzyme from various sourees may be affected by temperature. The study on the effect of temperature on the renaturation may also provide a clue to thermostability of the enzyme from themophiles.

In the present paper, from the point of view mentioned above, the effect of temperature on the renaturation of the denatured thermophilic α-amylase was investigated and also compared with that of B.subtilis α-amylase.

Experimental

Materials ——— The crystalline α-amylase from B.stearothermophilius Donk, Strain BS-1, grown at 55°C, was prepared by Hagihara's (4) method with slight modifications and finally

lyophilized. Crystalline B.subtilis α-amylase was supplied by Daiwa Kasei Co. Ltd. Bovine serum albumin (BSA) (Fraction V, The Armour Lab.) was purchased. Urea once recrystallized from 70 per cent aqueous ethanol.

Methods ——— Concentrations of thermophilic and B. subtilis α-amylases were estimated from absorption at 280 mμ assuming $E^{1\ cm}_{1\ \%}$ = 28.7 and 25.6, respectively.

α-Amylase activity of the enzyme was determined by the measurement of dextrinizing power using amylose as a substrate according to the method of Fuwa (5). Incubation was carried out at pH 4.5 and 37°C for 3 minutes. In this assay condition, reactivation of the denatured α-amylase during the assay was found to be negligible. The optical density at 700 mμ used for determination of the activity was unaffected by the presence of BSA under the experimental conditions.

For denaturation of the enzyme, crystalline α-amylases were dissolved in acidic (0.1 N HCl) 8 M urea solution at room temperature and kept for 5 hours. By this procedure the enzyme was completely unfolded and lost the enzymatic activity (2). For renaturation of the enzyme, the solution of denatured enzyme was diluted 50 fold with 0.0025 M buffer containing 10^{-4} M $CaCl_2$. The degree of recovery of the denatured enzyme was represented by a ratio of the activity of the enzyme to that

of the native enzyme of the same concentration with the former.

Results

Effect of pH on the Renaturation ——— Denatured thermophilic and B.subtilis α-amylases were diluted with a buffer containing 10^{-4} M $CaCl_2$ at various pH's at 37°C. The effect of pH on the reactivation of both enzymes are shown in Fig. 1. The reactivation of the denatured thermophilic α-amylase within the first one minutes after dilution was most remarkable at pH 7.0. Recovery of the activity at three different intervals increased with increasing of pH except that the recovery curve after one minute decreased with pH above 7. Thus, the pH profile of reactivation of the denatured thermophilic α-amylase showed the time dependence.

On the other hand, the pH of the maximum recovery of the denatured B.subtilis α-amylase after 5 minutes was 6.8.

Fig. 1

Effect of Temperature on the Renaturation ——— The renaturation experiments of denatured thermophilic and B.subtilis α-amylases by dilution with buffer containing 10^{-4} M $CaCl_2$ were carried out at various temperatures. Fig. 2 shows the time course of the reactivation of the denatured thermophilic

Fig. 2

α-amylase at pH 5.5. At 44°C, most of the reactivation was achieved within the first 2 minutes. The rate of reactivation was lowered with the decrease of temperature.

Fig. 3 shows temperature dependence of the recovery of the denatured thermophilic α-amylase at various reactivation periods at pH 5.5. Time dependence of the curve was observed below 45°C. The temperature where the maximum recovery was

Fig. 3

observed, was lowered with the reactivation time after dilution. The optimum temperature was 40-49°C at first one minute and shifted to 35-44°C during the following 9 minutes. On the other hand, at temperature above 50°C, the recovery after 2 minutes was the same value for one minute.

Fig. 4 shows the temperature dependence of the recovery of the denatured B.subtilis α-amylase at pH 5.4. Time dependence of the curve was also observed and the optimum temperature for reactivation shifted from 30°C for the curve at first one minute to 26°C for that at 10 minutes. The optimum temperature for reactivation was apparently lower than that for thermophilic α-amylase. It should be noted that in the range of temperature such as 40-50°C which is most suitable for reactivation of thermophilic α-amylase, B.subtilis α-amylase was no longer reactivated.

Fig. 4

The temperature dependence of the recovery of the denatured thermophilic α-amylase at pH 7.9 is shown in Fig. 5. The tempera-

ture profile of the recovery at pH 7.9 was similar to that at pH 5.5 (Fig.3). Maximum recovery at one and 10 minutes in the course of reactivation was observed at 45-50° and 34-45°C, respectively.

Fig. 5

Effect of BSA on the Renaturation ——— Optimum growing temperature of B.stearothermophilus Donk, strain BS-1 is in the range from 55 to 65°C. As shown in Figs. 3 and 5, however, the temperature suitable for the reactivation of denatured thermophilic α-amylase was found to be around 45°C and it was about 10°C lower than the optimum growing temperature. This difference might be due to the difference in condition or mechamism of folding of the polypeptide chain in vitro and in vivo. One of the difference of the conditions in vivo from that in vitro is the co-existence of other various kinds of proteins in the cell. It might be probable that these substances affect the folding of nascent polypeptide chain of the enzyme. Yutani et al. (6) have reported that the reactivation of denatured B.subtilis α-amylase was accelerated remarkably by the presence

of BSA. To see the effect of the BSA on the renaturation of denatured thermophilic α-amylase, the renaturation experiments of denatured thermophilic α-amylase by dilution with buffer containing 10^{-4} M $CaCl_2$ in the presence of BSA of 0.1 % were carried ou

Fig. 6 shows the time cource of the reactivation of the denatured thermophilic α-amylase in the presence of 0.1 %

Fig. 6

BSA at pH 7.9 comparing that in the absence of BSA. In both cases, i.e. in the presence and the absence of BSA, the recovery of the enzyme at 45°C reached the constant value within 2 minutes after dilution. But the final value reached in the presence of BSA was found to be 1.5 fold of the value in the absence of BSA. It was apparently suggested that BSA promoted the reactivation of the denatured thermophilic α-amylase. Similar effect of BSA was also observed for B.subtilis α-amylase. Fig. 7 shows

Fig. 7

temperature dependence of the recovery in the presence and in the absence of BSA. The optimum temperature for the recovery at one minute after dilution was scarcely dependent on the presence of BSA. In the absence of BSA and in the presence of 0.1 % BSA, the maximum recovery at first one minute was observed at 45-50°C and 40-50°C, respectively. It should be noted that 90 % of the activity were recovered one minute after dilution at 50°C in the presence of 0.1 % BSA and that 20 % of the activity were recovered after one minute even at 70°C.

Fig. 8 shows the effect of BSA on the temperature dependence of the reactivation of denatured thermophilic α-amylase at pH 5.5. Although the recovery at pH 5.5 was lower than that at pH 7.9, temperature profile similar with that at pH 7.9 was also observed at pH 5.5

fig. 8

Effect of BSA on the thermal Stability of B.subtilis α-Amylase ——— As described above, the reactivation of the denatured thermophilic α-amylase was promoted by the presence of 0.1 % of BSA and 90 % of the activity were recovered after one minute even at 50°C where the reactivation of denatured B.subtilis α-amylase did not occur. As shown in Fig. 9, 0.1 % BSA considerably retarded the loss of enzymatic activity of B.subtilis α-amylase at 60°C. In the absence of BSA, B.subtilis α-amylase lost 92 % of the activity at 60°C for 150 minutes, while the activity was lost 72 % in the presence of 0.1 % BSA. It seems that BSA has an effect protecting the enzyme from de - naturation at elevated temperature.

Fig. 9

DISCUSSION

The denatured thermophilic α-amylase was rapidly

reactivated at around 45 °C where the denatured B.subtilis α-amylase was not reactivated (Figs. 3 and 4). Thermophilic α-amylase may have an inherent property, so that the tertiary structure of the enzyme can rapidly form at higher temperature than that of B.subtilis α-amylase. According to the hypothesis by Anfinsen (3), it is thought that this inherent property of thermophilic α-amylase is contained in the primary structure of the enzyme, i.e. in the gene of this organism, and that the tertiary structure of thermophilic α-amylase may be constituted by the secondary and tertiary bonds (7) which can rapidly form at higher temperature.

As shown in Fig. 1, the pH profile of renaturation of denatured thermophilic α-amylase showed time dependence. The optimum pH of renaturation at first one minute after dilution is in good agreement with the optimum growing pH, 6.8, of this bacteria. From the result of temperature effect of renaturation shown in Fig. 3, the maximum recovery was observed at around 45 °C in earlier period of reactivation, while the temperature of maximum recovery was gradually lowered with reactivation time, i.e. time dependence of recovery was shown below 40 °C. In Fig. 10, time dependence of the reacti-

vation was shown as a difference of extent of recovery at one and 10 minutes, after dilution of the denatured thermophilic and B.subtilis α-amylases at various temperatures. The extent of recovery for 9 minutes between one and 10 minutes after dilution was plotted against temperature. The maximum recovery was found at 28 and 23°C for thermophilic and B.subtilis α-amylases, respectively. In the case of thermophilic α-amylase, temperature where the most remarkable reactivation was observed duting 9 minutes lowered remarkably, i.e. 17°C, than that at first one minutes. It has been reported that isolated thermophilic α-amylase itself is more stable at lower temperature and in the pH region (8-11) higher than physiological conditions

Fig. 10

of the organism (55-65°C, pH 6.8). We assumed the following equilibrium between native (N) and denatured (D) enzymes in the renaturation process

$$D \rightleftharpoons N \qquad (1)$$

From the results of time dependence of the reactivation, it may be said that in the cell of thermophilic bacteria the nascent polypeptide chain of the enzyme folds very rapidly to the native conformation at higher temperature than B.subtilis α-amylase and at physiological pH, but that the renaturation of denatured enzyme shifts the D $\rightleftharpoons$ N equilibrum of the enzyme to N state in the stable condition of the enzyme. According to a hyposethesis which proteins are stabilized by both thermodynamic and kinetic factors by Kauzmann (7), thermophilic α-amylase has a kinetic stability at the temperature and pH of physiological conditions, while the enzyme has its thermodynamic stability at lower temperature than 50 °C and in the pH region between 8 and 11. Allen (8) suggested that thermophilic bacteria are able to survive at high temperature because of their ability to replace the heat-damaged components at a rapid rate. Such a mechanism would requir a high "turnover" of the components and the material most liable to thermal damage would be the proteins. Bubela and Holdsworth (9) have reported that the turnover of proteins and nucleic acids in B.stearothermophilus is greater than in E.coli and suggested that B.stearothermophilus has a mechanism for providing a high rate of protein synthesis to enable replacement of cell constituents when organism is

growing at 63 °C. The very rapid turnover of protins and nucleic acids may be due to the kinetic stability of these molecules.

As shown in Fig. 7, in the presence of 0.1 % BSA at 55 °C and pH 7.9, 80 % of reactivation was achieved within the first one minute, while in the absence of BSA the reactivation was achieved only a half extent, In the both case, however, further reactivation was searcely observed at 55 °C. The presence of considerable amount of BSA retarded the denaturation of B.subtilis α-amylaseas shown in Fig. 9. This result seems to suggest the probable existence of a week interaction of BSA with the enzyme. This interaction may protect the enzyme from thermal denaturation and it may be suggest that the presence of BSA shifts effectively the D $\rightleftharpoons$ N equilibrium to N state in the reactivation process. Militzer and Burns (10) have reported that the pyruvic acid oxidation system of thermophiles is easily inactivated at the growing temperature of the organism (60-65 °C), but that the system in the intact cell withstood heating for an hour at 65 °C. It has also reported that pyruvate, magnesium ion, and oxygen increase the thermostability of the cell-free system. These facts strongly suggest that

enzymes from thermophiles are stabilized by the interaction with co-existed many materials in the cell.

Acknowledgements

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Summary

In order to know the mechanism of thermostability of α-amylase from B.stearothermophilis Donk, strain BS-1, the effect of temperature on the renaturation of acidic 8 M urea-denatured thermophilic α-amylase was investigated and compared with that of B.subtilis α-amylase. The rate of reactivation of denatured thermophilic α-amylase was most rapid at 40-50°C where denatured B.subtilis α-amylase was not reactivated. The reactivation of denatured thermophilic α-amylase was promoted by the presence of bovine serum albumin at concentration of 0.1 %. At 55°C which is optimum growth temperature of the organism, the ~~degree~~ recovery of denatured thermophilic α-amylase within the first one minutes was 35 % in the absence of BSA at pH 7.9, while it increased to 83 % in the presence of 0.1 % BSA. The presence of BSA retarded the heat denaturation of B.subtilis α-amylase. The results obtained here suggest that thermophilic α-amylase is strongly stabilized by the interaction with co-existed many materials in the cell.

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Fig. 1

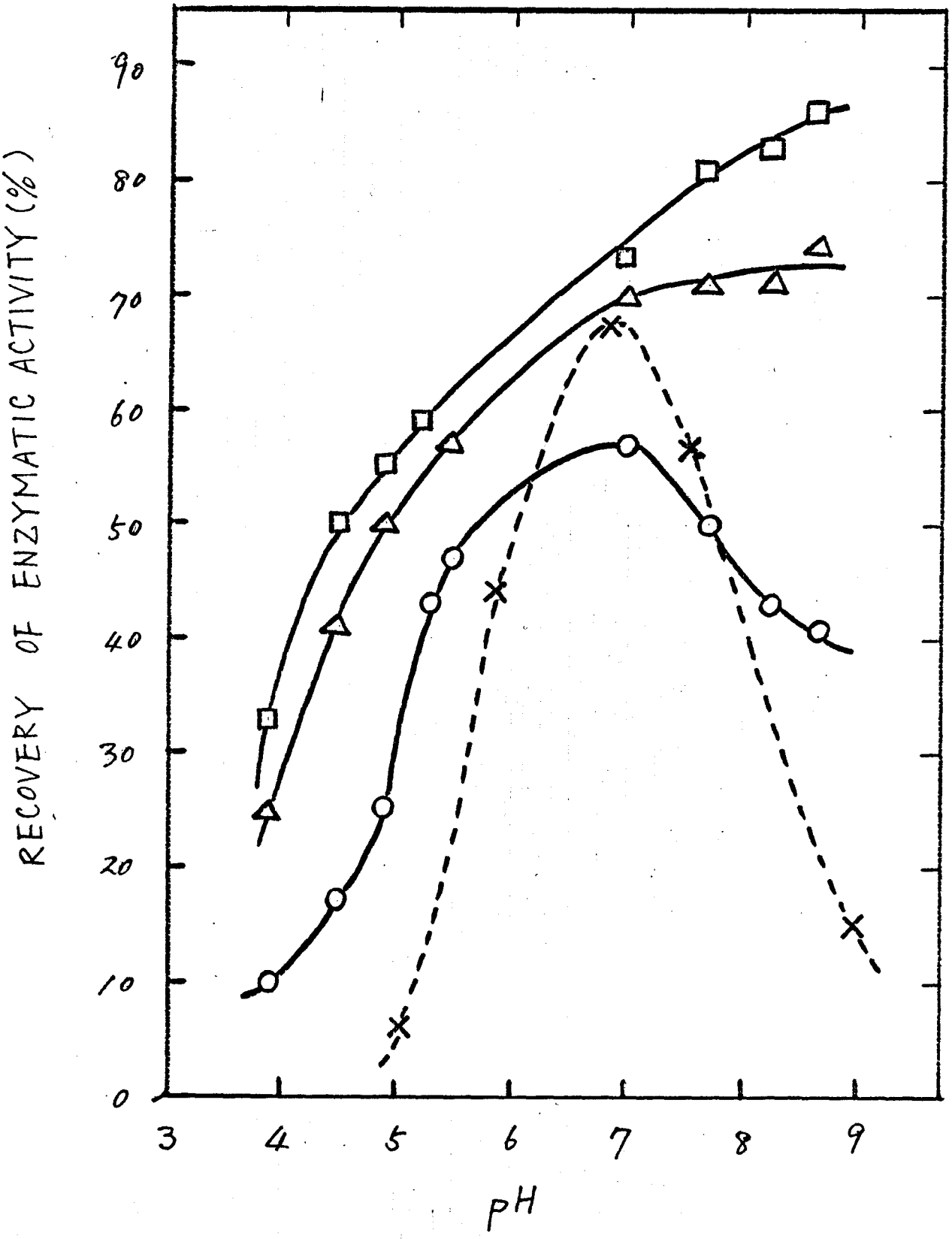

RECOVERY OF ENZYMATIC ACTIVITY (%)
90
80
70
60
50
40
30
20
10
0
3
4
5
6
7
8
9
pH

Fig. 2

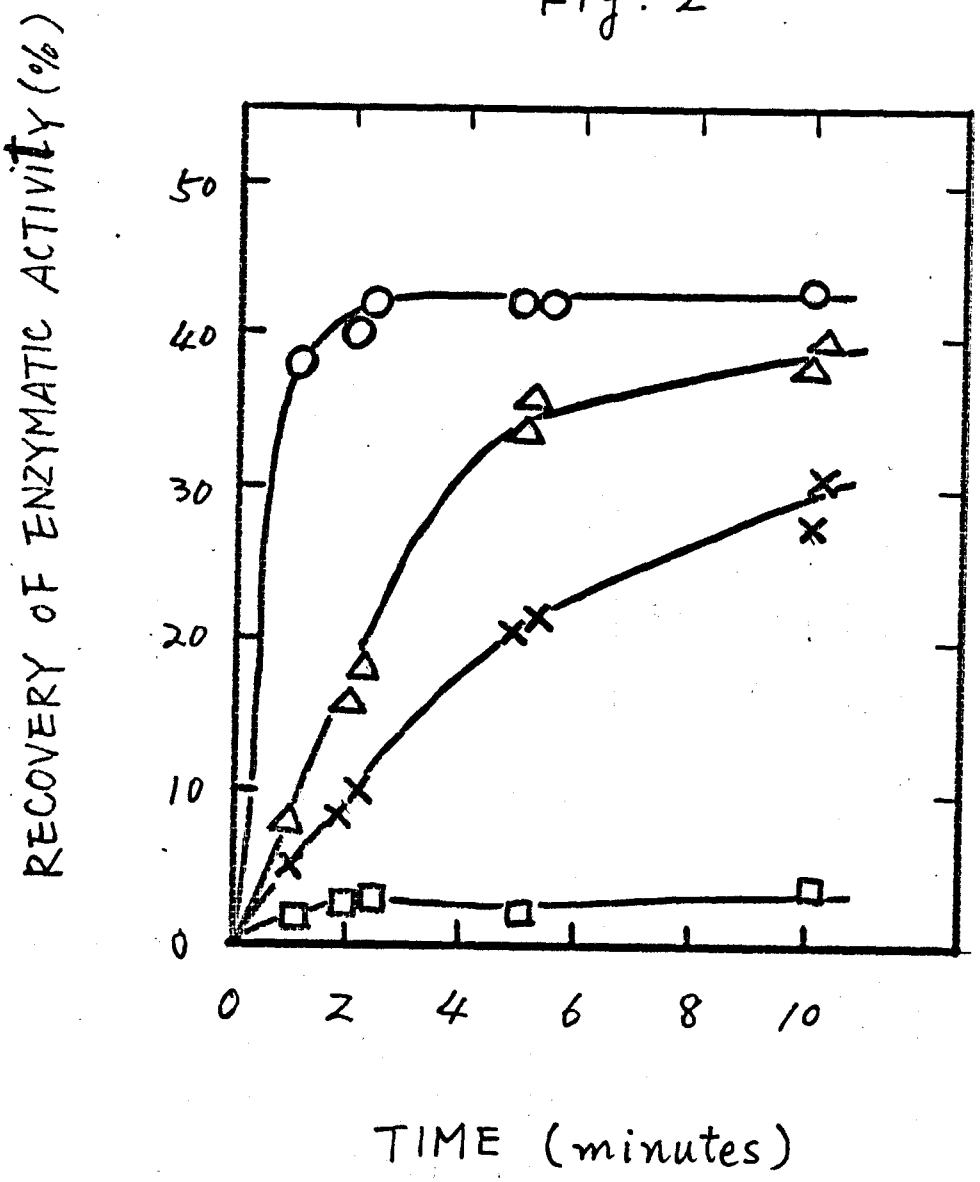

RECOVERY OF ENZYMATIC ACTIVITY (%)
50
40
30
20
10
0
0
2
4
6
8
10
TIME (minutes)

Fig. 3

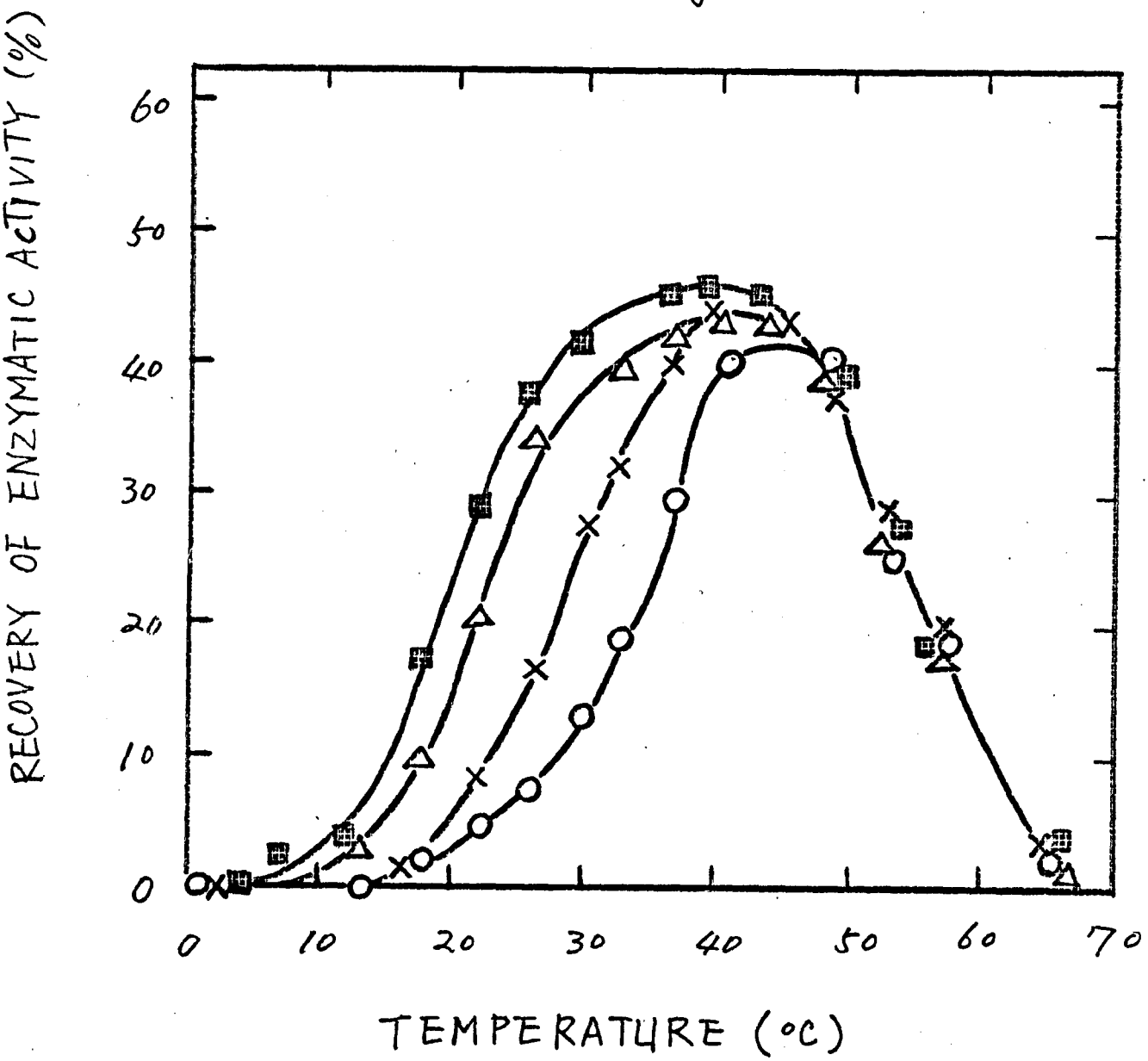
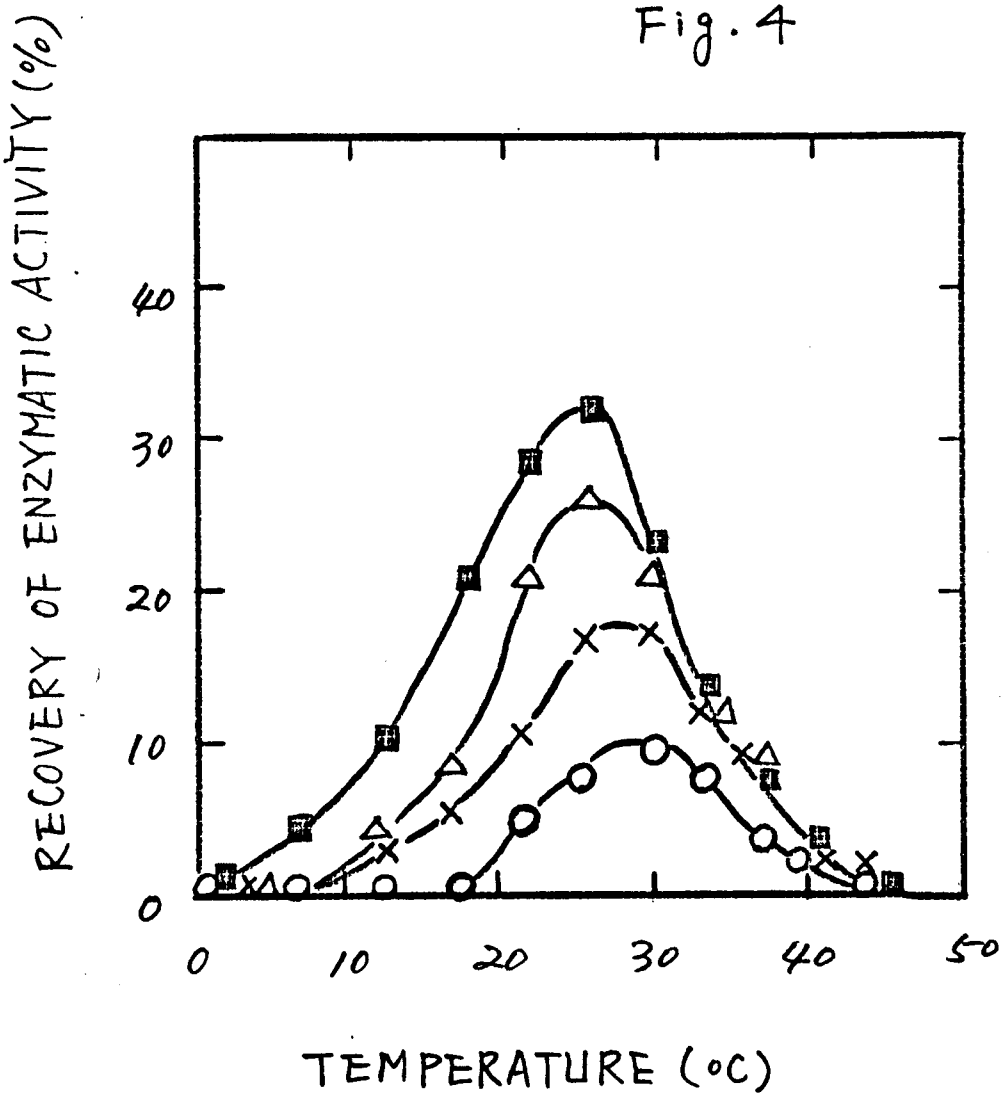

RECOVERY OF ENZYMATIC ACTIVITY (%)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
TEMPERATURE (°C)

Fig. 4


RECOVERY OF ENZYMATIC ACTIVITY (%)
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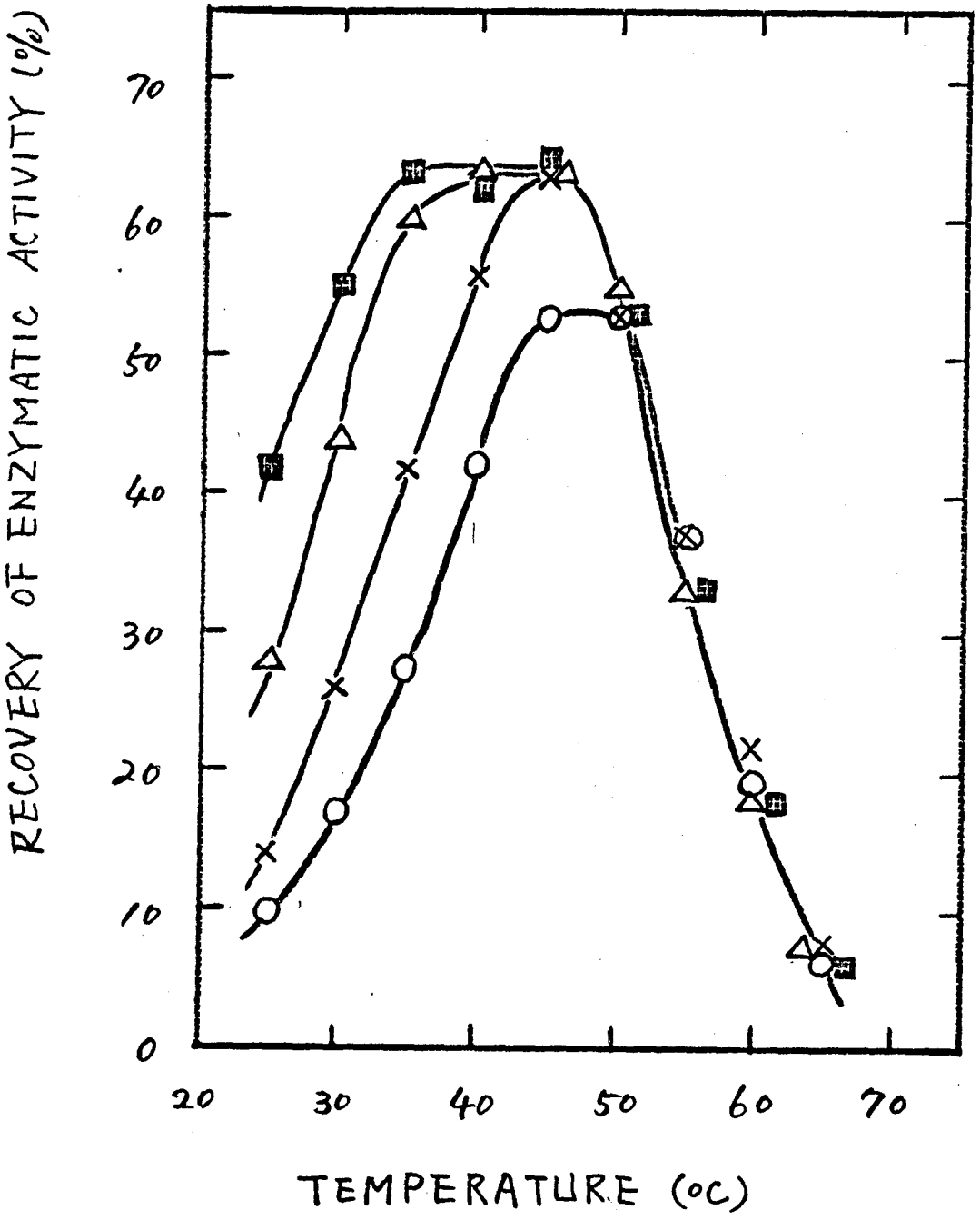
10
20
30
40
0
10
20
30
40
50
TEMPERATURE (°C)

Fig. 5


RECOVERY OF ENZYMATIC ACTIVITY (%)
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20
30
40
50
60
70
20
30
40
50
60
70
TEMPERATURE (oC)

Fig. 6

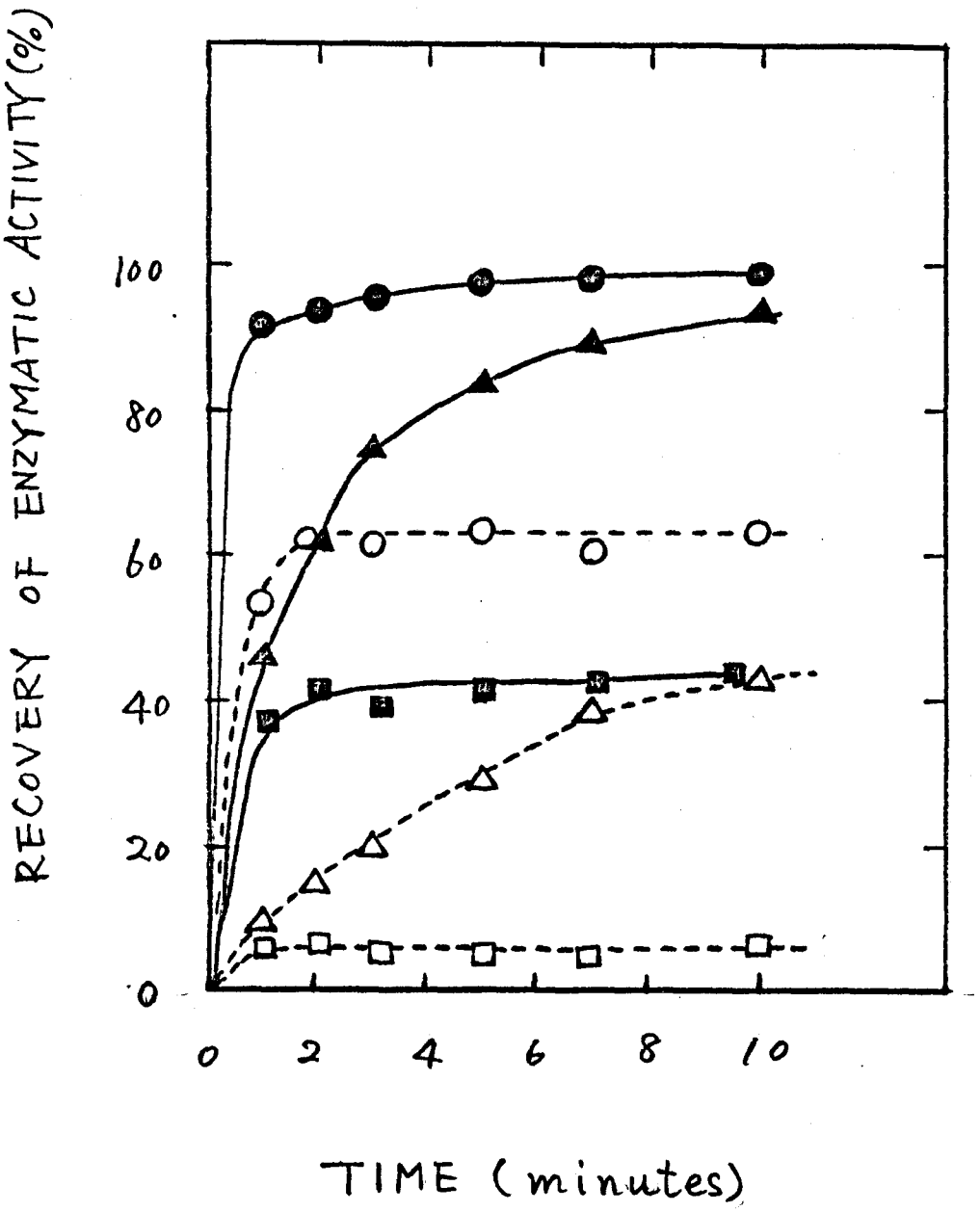

RECOVERY OF ENZYMATIC ACTIVITY (%)
100
80
60
40
20
0
0
2
4
6
8
10
TIME (minutes)

Fig. 7

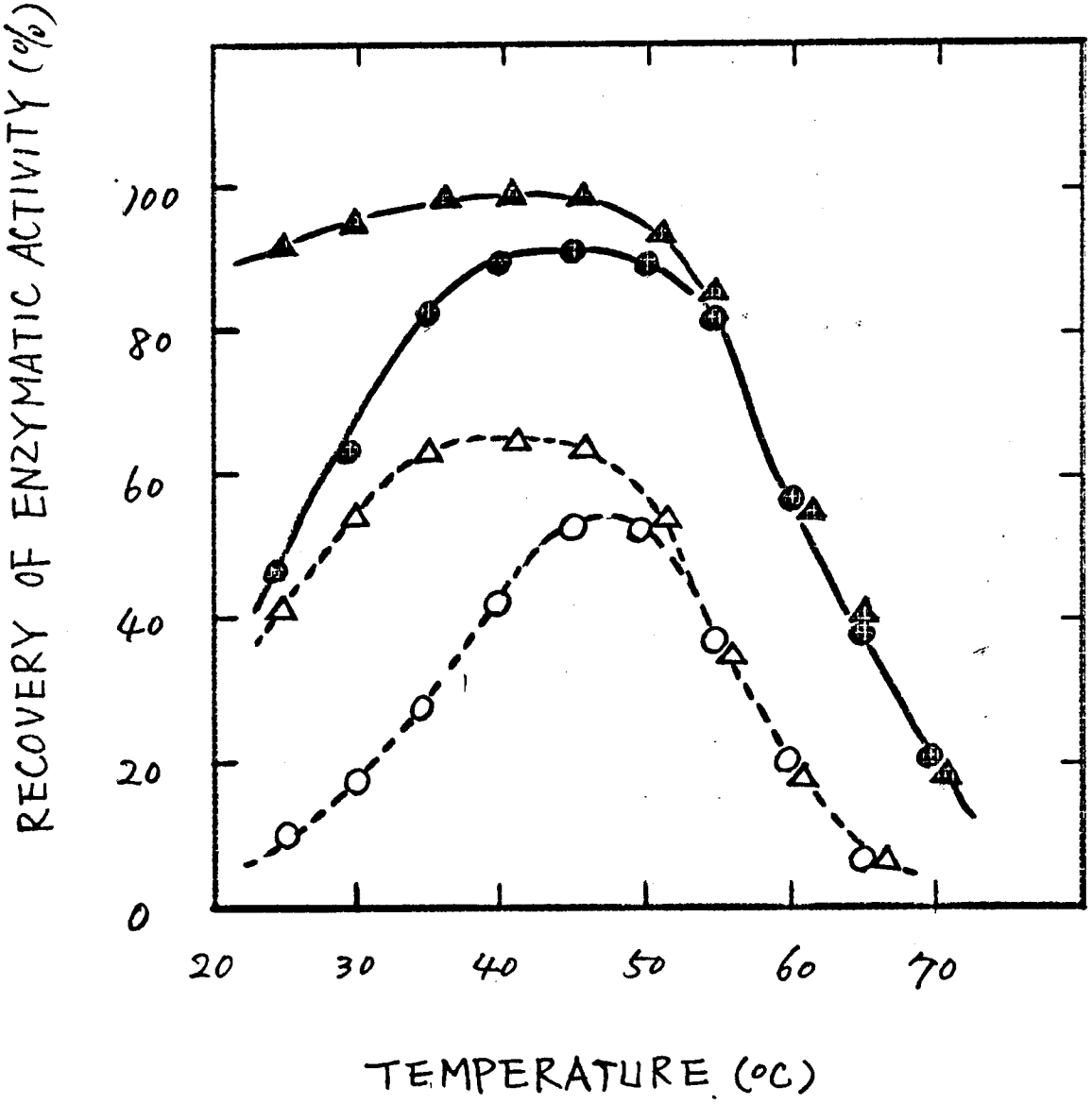
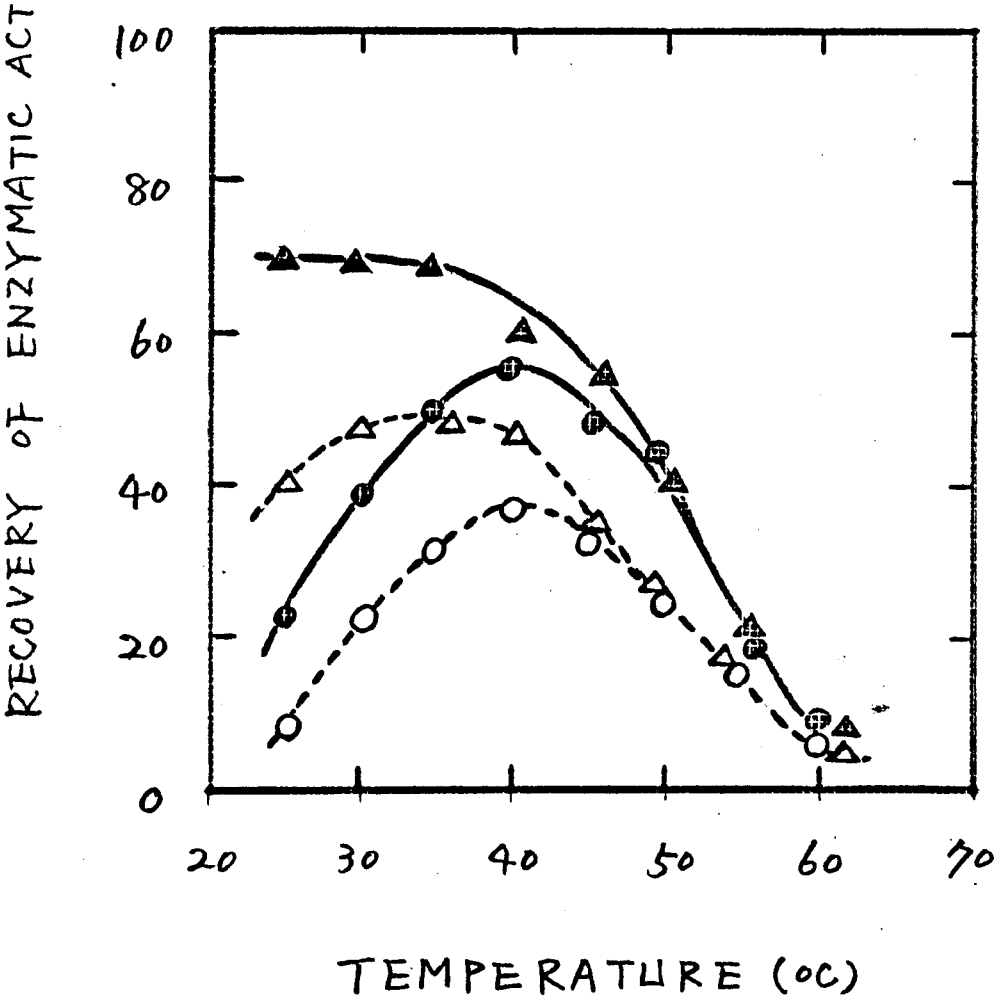

RECOVERY OF ENZYMATIC ACTIVITY (%)
100
80
60
40
20
0
20
30
40
50
60
70
TEMPERATURE (°C)

Fig. 8


RECOVERY OF ENZYMATIC ACTIVITY (%)
100
80
60
40
20
0
20
30
40
50
60
70
TEMPERATURE (°C)

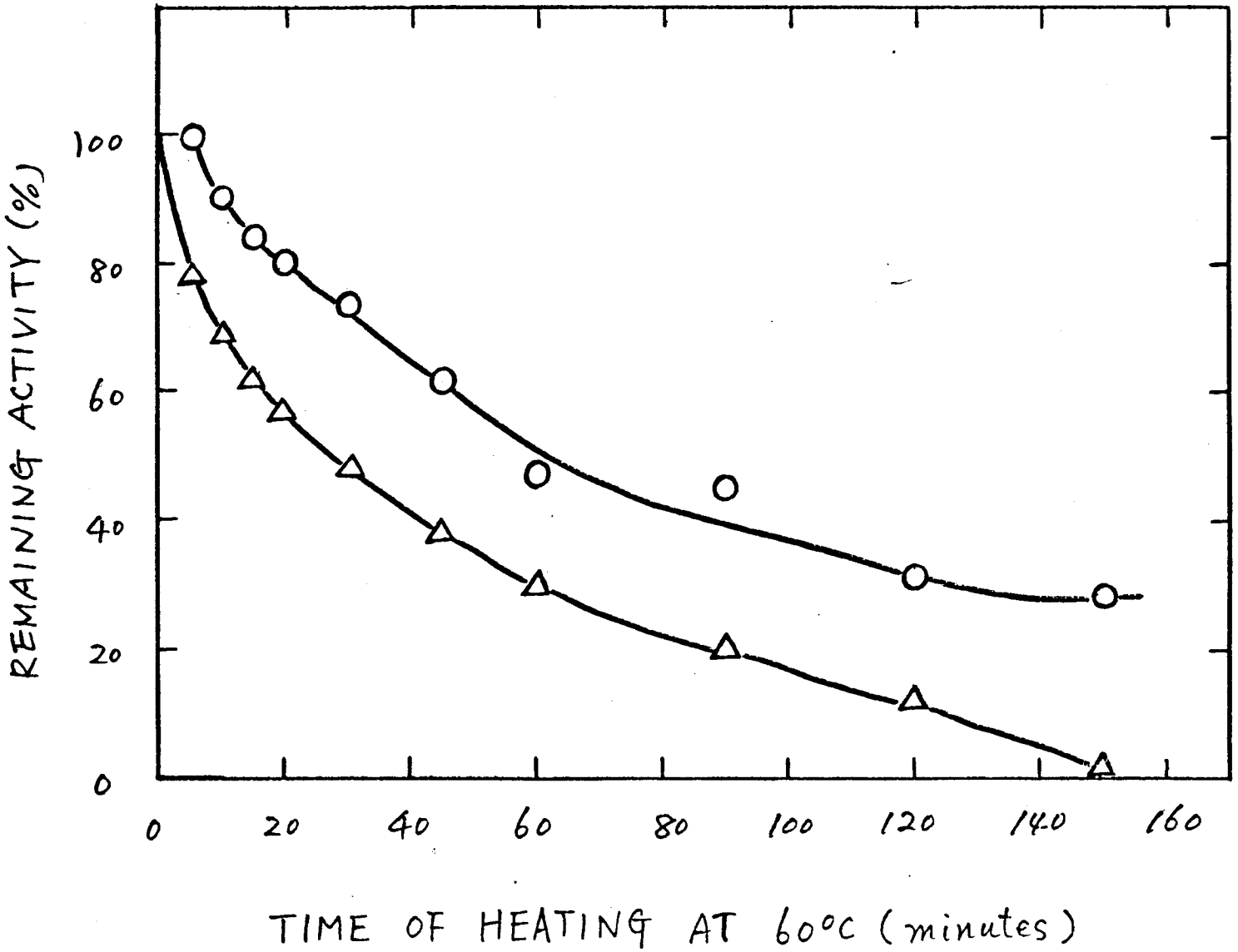

REMAINING ACTIVITY (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
TIME OF HEATING AT 60°C (minutes)

Fig. 9

Fig. 10

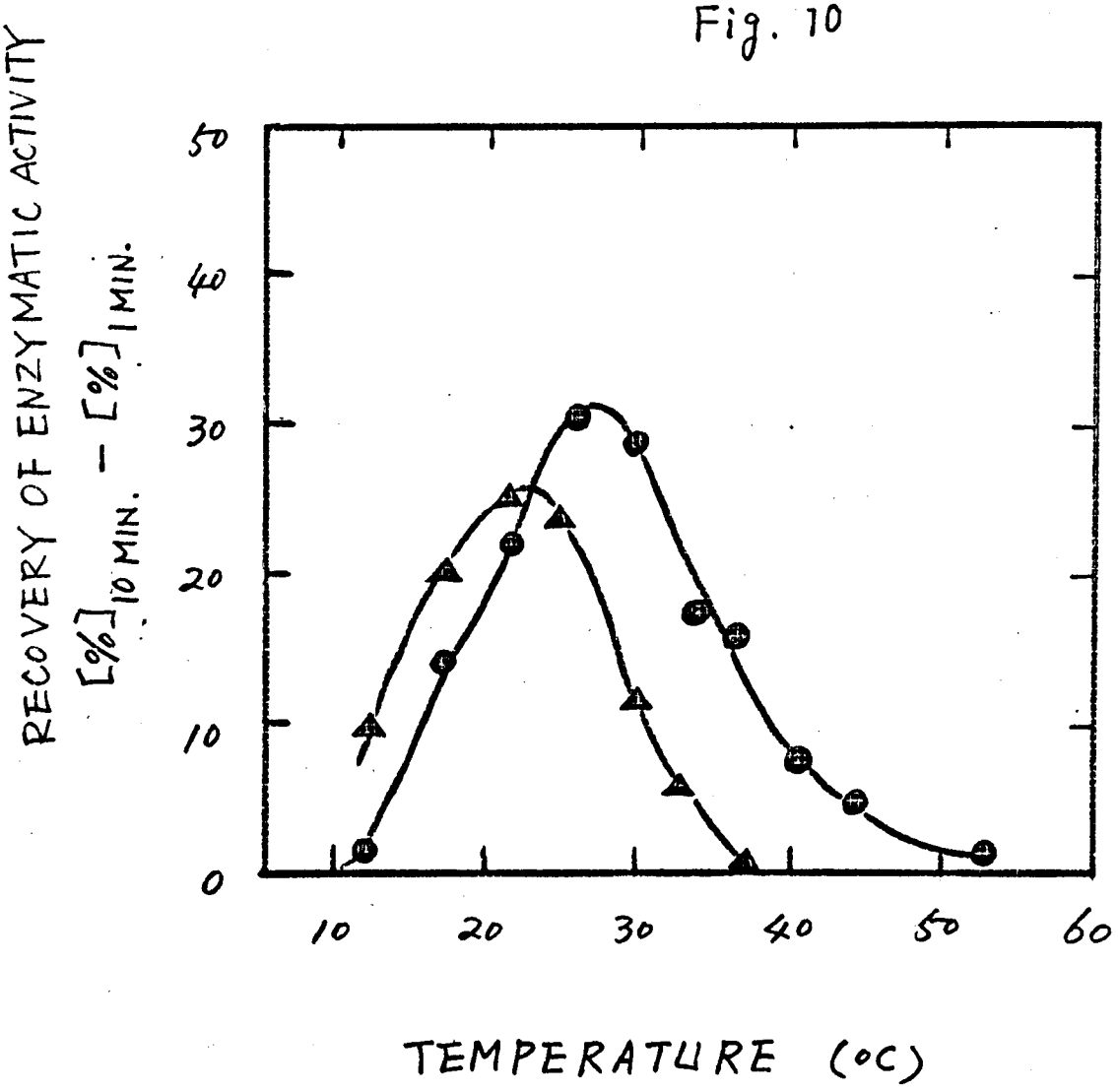

RECOVERY OF ENZYMATIC ACTIVITY
[%]10 MIN. − [%]1 MIN.
50
40
30
20
10
0
10
20
30
40
50
60
TEMPERATURE (°C)

Fig. 1. Effect of pH on the reactivation of denatured thermophilic and B.subtilis α-amylases.
Reactivation temperature, 37 °C. Concentrations of thermophilic and B.subtilis α-amylases during the reactivation, 0.802×10^{-4} %,
(-o-), at one minute after dilution with buffer for thermophilic α-amylase; (-Δ-), 3.5 minutes; (-□-), 9 minutes. (··x··), 5 minutes for B.subtilis α-amylase

g. 2. Time course of the reactivation of denatured ermophilic α-amylase at various temperatures at pH 5.5. ncentration of thermophilic α-amylase during the activation, 0.732×10^{-4} %.

X), 21.7 °C; (Δ), 25.6 °C; (O), 44.0 °C; □), 65.0 °C

Fig. 3. Effect of temperature on the reactivation of denatured thermophilic α-amylase at various periods in the reactivation at pH 5.5.
Concentration of thermophilic α-amylase during the reactivation, 0.732×10^{-4} %.
(O), one minute; (×), 2 minutes; (△), 5 minutes; (■), 10 minutes

ig. 4. Effect of temperature on the reactivation
f denatured B.subtilis α-amylase at various periods
n the cource of reactivation at pH 5.4.
oncentration of B.subtilis α-amylase during the react-
vation, 1.76×10^{-4} %.
○), one minute; (×), 2 minutes; (△), 5 minutes;
■), 10 minutes

Fig. 5. Effect of temperature on the reactivation of denatured thermophilic α-amylase at various periods in the cource of reactivation at pH 7.9.
Concentration of thermophilic α-amylase during the reactivation, 0.822×10^{-4} %.
(○), one minute ; (×), 2 minutes ;
(△), 5 minutes;
(■), 10 minutes

Fig. 6. Time course of the reactivation of denatured thermophilic α-amylase at pH 7.9 in the absence of BSA and in the presence of 0.1 % BSA.

Concentration of thermophilic α-amylase during the reactivation, 0.822×10^{-4} %.

Closed symbols, in the presence of 0.1 % BSA.

Open symbols, in the absence of BSA.

(△ , ▲), 25 °C ; (○, ●), 45 °C ;

(□, ■), 65 °C

Fig. 7. Effect of temperature on the reactivation of denatured thermophilic α-amylase in the absence of BSA and in the presence of 0.1 % BSA at various periods in the reactivation at pH 7.9.

Concentration of thermophilic α-amylase during the reactivation, 0.822×10^{-4} %.

Closed symbols, in the presence of 0.01 % BSA.

Open symbols, in the absence of BSA.

(○, ●), one minutes ;

(△, ▲), 10 minutes

Fig. 8. Effect of temperature on the reactivation of denatured thermophilic α-amylase in the absence of BSA and in the presence of 0.1 % BSA at various periods in the reactivation at pH 5.5.

Concentration of thermophilic α-amylase during the reactivation, 0.822×10^{-4} %.

Closed symbols, in the presence of 0.1 % BSA.

Open symbols, in the absence of BSA.

(○ , ●), one minutes ;

(△ , ▲), 10 minutes

Fig. 9. Effect of BSA on the thermal stability of B.subtilis α-amylase.

B.subtilis α-amylase at concentration of 1.27×10^{-4} % was incubated at 60 °C in the medium of 10^{-4} M $CaCl_2$, pH 7.9, containing 0.1 % BSA or not containing BSA. The enzymatic activity was measured after incubation for various time intervals.

(△), in the absence of BSA ;

(○), in the presence of 0.1 % BSA

Fig. 10. Temperature dependence of the recovery of enzymatic activity of denatured thermophilic and B.subtilis α-amylases during 9 minutes between one and 10 minutes after dilution with buffer of pH 5.5. Concentrations of thermophilic and B.subtilis α-amylases during the reactivation were the same as those of Figs. 3 and 4, respectively.

(●), thermophilic α-amylase ;

(▲), B.subtilis α-amylase