Reversibility of Heat-Inactivation of *Bacillus subtilis* α -Amylase

By Takehiko YAMAMOTO, Akimasa NISHIDA and Juichiro FUKUMOTO

Faculty of Science, Osaka City University

Received April 2, 1966

Inactivation of *Bacillus subtilis* α -amylase by heat was found to be reversible under a certain condition, and the factors affecting there were investigated, distinguishing into two groups: those influencing on the inactivation process by heat and those on the reactivation at the subsequent incubation after heating. Generally, the amylase heated in borate buffer solution was best in the reactivation degree. For reactivation of the heat-inactivated enzyme there was found an optimum in temperature, pH and concentration of enzyme, respectively. The reactivation was temporarily prevented by urea, but irreversibly inhibited by either calcium salts or calcium binding agents. In the reversible heat-inactivation of the enzyme was also found a reversible change in the absorption spectra as well as in the behavior of the enzyme toward proteinase.

Many experimental results have indicated that denatured enzyme is renatured under a certain condition. The study of reversible denaturation of enzyme protein is of importance to approach the study on the relationship between the conformational structure of enzyme protein and enzyme activity.

As for reversible denaturation of enzyme by heat, there have been several papers such as on Taka-amylase,¹⁾ Trypsin,²⁾ *Proteus vulgaris* nucleotide pyrophosphatase,³⁾ etc. Our previous paper⁴⁾ has reported that *Bac. subtilis* α -amylase inactivated by heat restores its activity under a certain condition. Recently, it was found that the reactivation of heat-inactivated amylase was reversibly inhibited by urea.

It is well-known that *Bac. subtilis* α -amylase contains neither cysteine nor cystine and that the enzyme possesses one gram atom of calcium as an indispensable constituent for enzyme activity. The enzyme thus loses its

activity when made free from the calcium and the inactivated amylase is reactivated upon addition of calcium ions.^{5~7)} This fact clearly indicates that the active structure of the enzyme is achieved by chelating in a certain way with calcium. Thus, there may be the possibility that the metal in the enzyme protein also plays an important role for reactivation of the heat-inactivated amylase.

The present paper deals with the condition of reversible heat-inactivation of *Bac. subtilis* α -amylase and some properties of the amylase reversibly inactivated by heat.

MATERIALS AND METHODS

Amylase. The crystalline *Bac. subtilis* α -amylase used in the present work was obtained according to the method reported by one of the present authors.⁸⁾ In order to remove contaminated protease, the crystalline amylase was dissolved in a dilute sodium hydroxide solution and dialyzed against a slightly alkaline solution (pH 8.4) for four days in the cold. The dialyzed amylase was then heated at 70°C for 30 min. and, after cooling by allowing to stand at

1) E. Ohlsons and T. Sweitchin, *Bull. Soc. Chem. Biol.*, **11**, 333 (1929); **15**, 470 (1933).

2) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **17**, 393 (1934).

3) M. N. Swartz, N. O. Kaplan and M. F. Lanborg, *J. Biol. Chem.*, **232**, 1051 (1958).

4) T. Yamamoto, A. Nishida and J. Fukumoto, *This Journal*, **28**, 656 (1964).

5) E. H. Fischer et al., *The 4th International Congress of Biochemistry*, Symposium 8~3, 1958.

6) T. Yamamoto, *This Journal*, **23**, 68 (1959).

7) T. Yamamoto, *ibid.*, **24**, 16 (1960).

8) T. Yamamoto, *ibid.*, **19**, 121 (1955).

room temperature for several hours, was dialyzed against deionized water for two days in the cold. The dialyzed amylase was centrifuged if any precipitate occurred and calcium acetate solution was added in a minimum amount sufficient to crystallize out the enzyme. The crystals thus obtained were again dissolved in a slightly alkaline solution and dialyzed against deionized water for several days to remove excessive calcium. The dialyzed enzyme was then freeze-dried; the preparation was very soluble in deionized water (ca. 30% by weight) and one per cent solution of the enzyme gave a value of 25.0 at 280 m μ per cm. The activity of the enzyme preparation was 480 units per mg when determined by the following assay method.

Enzyme activity assay. To 4 ml of a 0.5% soluble starch solution buffered with M/50 acetic acid-ammonium acetate at pH 5.9 was added one ml of enzyme and incubated at 40°C. After 3 min., the reducing sugar formed was determined by the Shaffer-Somogyi's method.⁹⁾ One unit of enzyme activity was defined as the enzyme quantity that produced one μ mole of reducing sugar as glucose per min. under the condition.

Difference spectra. Difference spectra were measured in matched quartz cells of one cm path on the to Chotampa QU-2 model spectrophotometer. In the spectroscopy, solutions containing about 0.5 mg of α -amylase per ml in M/400 borate buffer at pH 8.6 were used and the absorbancy of heated amylase solution was measured against unheated amylase as the control. Because of considerably rapid change in absorbancy of amylase during heating, the absorption was followed as a function of time measuring at given wavelengths, and the difference spectra were obtained from the results. The change in absorbancy with change in temperature was also determined by quipping a special thermostat for cuvette, the temperature of which was raised at a constant rate by the aid of the Haake circulating thermostat.

Fractional determination of native and denatured amylase. The well-known fact, that, under a certain condition, native enzyme is quite resistant to proteolytic enzyme while denatured enzyme is extremely sensitive, was applied here. At varied times of incubation of amylase after heating, 0.5 ml of *Bac. subtilis*' alkaline protease (Nagarse, 0.12 mg/ml of M/10 phosphate, pH 7.0) was mixed with 3 ml of amylase

and, after a given time at 30°C, 3.5 ml of TCA mixture (0.33 N trichloroacetic acid, 0.66 N acetic acid, 0.99 N sodium acetate) was added. The filtrate of the mixture obtained after 30 min. was subjected to colorimetric determination according to the modified Folin-Ciocalteu's method.¹⁰⁾

EXPERIMENTAL RESULTS

I. Inactivation and Reactivation of Amylase

There were found various factors which affected on reversible inactivation of *Bac. subtilis*' α -amylase by heat. They were clearly distinguished into two groups, one those affecting at heating and the other those affecting after heating.

A. Factors affecting at heating

1) **pH, sort of buffer solutions or salts.** The freeze-dried amylase was dissolved in M/60 buffer solution of borate, tris (trihydroxymethyl-aminomethane), phosphate or acetate of various pH values and, after heating at 80°C for 10 min. and subsequently cooling at 24°C, the enzyme activity was assayed at varied times of incubation at 24°C. The borate buffer used here was prepared without any addition of NaCl. As shown in Fig. 1, the best recovery of enzyme activity was found with amylase heated in M/60 borate buffer solution. On the other hand, the amylase in acetic acid-sodium acetate solution lost its activity by heating without showing any activity recovery. In solutions containing Ca^{++} , Na^+ and Cl^- which are known to increase the thermal stability of amylase, the loss of amylase activity was small, but no increase in enzyme activity was found during subsequent incubation after heating. However, the amylase dissolved in acetate buffer of concentrations less than M/120 showed an appreciable recovery of amylase activity (Fig. 2). The amylase solution slightly alkalized with NaOH (pH 8.4) also showed reactivation. However, the amylase which was dissolved only in deionized water (pH 5.2), M/400 Ca-acetate or solutions of various inorganic

9) P. A. Shaffer and M. Somogyi, *J. Biol. Chem.*, **100**, 695 (1933).

10) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

FIG. 1. Effect of pH and Various Buffer Solutions on Reversible Heat-Inactivation of Amylase.

■, immediately after heating; ▨, after 30 min; □, after 150 min.
 a, M/60 borate buffer; b, M/60 phosphate buffer; c, M/60 tris buffer;
 d, M/100 Ca-acetate + M/20 NaCl; e, M/90 acetic-Na-acetate.

FIG. 2. Effect of Concentration of Buffer.

The amylase dissolved in the buffer solutions indicated was heated at 85°C for 10 min. and then incubated at 30°C.

a, acetic-sodium acetate (pH 5.95~6.05);
 b, borate (pH 8.2~8.4).
 1, M/15; 2, M/30; 3, M/60; 4, M/120; 5, M/240; 6, M/480.

FIG. 3. Temperature of Heating and Reversibility of Amylase.

The amylase dissolved in a borate buffer of M/240, pH 8.6, was heated for 10 min. at the temperatures indicated and subsequently incubated at 30°C.

salts of concentrations more than M/400 resulted in complete loss of enzyme activity and no reactivation was found.

2) **Temperature.** The recovery of amylase activity after heating was found to differ

according to the temperature to which the enzyme was exposed. As shown in Fig. 3, the activity recovery was best with the enzyme heated at 80°C and the higher the temperature,

the less recovery of enzyme activity was obtained. However, it was worthy to note that even the amylase heated at 100°C for 10 min. clearly showed restoration of activity up to a 15% of the original.

FIG. 4. Restoration of Activity after Heating at Various Temperatures for 10 min.

The solid lines and the broken lines indicate the amylase activity determined right after heating and after incubation of the heated amylase for 150 min. at 25°, respectively.

- , M/400 acetate buffer (pH 6.0);
- △—, M/400 borate buffer (pH 8.6);
- , M/400 Ca-acetate (pH 6.0).

In Fig. 4 are shown the activities of amylase immediately after heating at various temperatures for 10 min. and those after leaving the heated amylases at 25°C for 150 min. The results indicate that, in solutions of borate buffer of M/400 (pH 8.6) or of acetic-sodium acetate buffer of M/400 (pH 6.0), the best reactivation of enzyme occurs on heating at temperatures between 80° and 85°C. Fig. 4 again indicates that the thermal stability of amylase is clearly increased by the presence of calcium acetate, but no reactivation is found with the enzyme heated with calcium ions.

3) **Period of heating.** The reversibility of heat-inactivation of amylase was also found to be affected by the period of heating, even at a given temperature. As shown in Fig. 5, the longer the period of heating, the less re-

FIG. 5. Effect of Heating Period on the Reversibility.

The amylase dissolved in M/400 borate buffer, pH 8.4, was heated at 85°C for the period indicated and then incubated at 30°C.

A, 10 min.; B, 20 min.; C, 30 min.

activation was obtained.

4) **Concentration of enzyme.** The concentration of enzyme is an important factor affecting on the reversibility degree of the enzyme after heating. Fig. 6 illustrates the relationship between the concentration of enzyme at heating and the degree of reactivation on the subsequent incubation, indicating that there is an optimum concentration for amylase to be reversibly inactivated by heat. The best

FIG. 6. Concentration of Amylase and the Degree of Reactivation.

Solutions of M/200 borate buffer, pH 8.4, containing various amounts of amylase were heated at 85°C for 10 min. and subsequently incubated at 30° for the period indicated. A, activity immediately after heating; B, activity after 30 min. on the subsequent incubation; C, activity after 150 min. on the subsequent incubation.

reversibility was found with a solution of around 0.5mg amylase per ml and, at concentrations more than 8mg per ml, no reversibility was observed.

5) **Effect of addition of urea or alcohol.** The effect of addition of urea or alcohol at heating on the reversible inactivation of amylase was investigated. A mixture solution of amylase in $M/200$ borate buffer, pH 8.6, and urea or alcohol (3M or 12.5% in the final conc., respectively) was heated at 85°C for 10 min. and the enzyme activity was assayed at varied times during the subsequent incubation at 30°C. However, the presence of urea or alcohol at heating resulted in the complete inactivation of enzyme and no reactivation was observed on the subsequent incubation.

B. Factors affecting on the reactivation of the heat-inactivated amylase

The reactivation of heat-inactivated amylase was found to be affected by several factors. Unless otherwise indicated, the amylase used in the experiment was the one that was inactivated by heating at 85°C for 10 min. in $M/400$ borate buffer at pH 8.6.

1) **Temperature on incubating the heat-inactivated amylase.** The heat-inactivated amylase was incubated at various temperatures and the recovery of enzyme activity was periodically investigated. In Fig. 7 are shown the results, indicating that there is an optimum

FIG. 7. Effect of Temperature on Reactivation of the Heat-Inactivated Amylase.

The numbers indicate the temperature °C, at which the enzyme was incubated after heating.

in the temperature for reactivation and the best reactivation occurs at 30°C.

2) **pH of incubation for reactivation.** To the amylase heated in a borate buffer solution ($M/400$, pH 8.6) was added acetate buffer in about $M/60$ immediately after heating and the pH was adjusted to 5.8. The amylase heated in an acetate buffer solution ($M/400$, pH 5.8) was, on the other hand, added with $M/60$ borate buffer in the final concentration to adjust the pH to 8.6 and the change of enzyme activity was investigated. As shown in Fig. 8, the inactivated amylase the pH of which was altered from acid to alkaline side caused reactivation (curve 4); while the enzyme treated in the reverse showed no reactivation (curve 2). This result indicates that the presence of $M/60$ acetate interferes the process of reactivation of the amylase reversibly inactivated by heat.

FIG. 8. Effect of pH on Reactivation of the Heat-Inactivated Amylase.

Curve	pH	
	at heating	at subsequent incubation
1	8.6	8.6
2	8.6	5.8
3	5.8	5.8
4	5.8	8.6

3) **Effect of urea and ethanol.** Urea or ethanol was added to the amylase immediately after heating at 85°C for 10 min. and the mixture was allowed to stand at 30°C, but there was observed no reactivation of amylase. In this experiment, a solution of 6M urea or 25% ethanol previously cooled at 0°C was added in a equal volume to the amylase right

TABLE I. REACTIVATION OF AMYLASE INACTIVATED BY HEAT AND SUBSEQUENTLY MIXED WITH UREA (3 M)

Treatment	Activity		Treatment	Activity
Before heating	1000		After dilution and incubation at 25°C for	
Right after heating	3	+ Urea (3 M) $\rightarrow$	0 hr.	15
After incubating the heated enzyme at 25°C, 2 hr.	620		2 hr.	490
			After dialysis for 48 hr.	420

after heating. However, dialysis of the mixture against deionized water caused an appreciable recovery of enzyme activity, as is seen in Table I. Dilution of the mixture with water to decrease the concentration of the additions resulted in a similar effect of reactivation. By diluting with water, a similar result but with less degree of reactivation was observed with the enzyme mixed with ethanol after heating.

In Table II is presented the effect of various concentrations of urea on the heat-inactivated amylase, showing that the addition of urea in 3 M in the final concentration to the heated amylase completely inhibits reactivation of the enzyme, while, in the case of the addition of 1 M urea, the heated enzyme is spontaneously reactivated to a similar degree to that found with the amylase without any addition of urea. The activity of native amylase was not affected by 3 M urea on incubation over 20 hours at 25°C. It is followed that these facts make it possible to

preserve the heated amylase in a reversibly inactivated state, since the reactivation of amylase inactivated by heat is reversibly prevented by the presence of 2 to 3 M urea and the preventive effect of urea is removed by only diluting with water decreasing the concentration of urea in less than 1 M. However, the degree of reversibility of the amylase inactivated by heat and subsequently mixed with 3 M urea was less than that of amylase

FIG. 9. Effect of Incubation Period on Reactivation of the Amylase Inactivated by Heating and Subsequently Mixed with 3 M Urea.

—●—, without urea;
---○---, with urea; the curves show that the amylase is reactivated in 1 to 2 hrs after diluting the amylase-urea mixture.

FIG. 10. Effect of pH on Reactivation of the Amylase Inactivated by Heat and Mixed with Urea.

TABLE II. EFFECT OF VARIED CONCENTRATIONS OF UREA ON REACTIVATION OF THE HEAT-INACTIVATED AMYLASE

Conc. of urea, M	Activity		
	Immediately after heating	After the subsequent incubation* for	
		3 hr.	20 hr.
0	5	600	600
1	—	595 (600)	600 (600)
2	—	310 (590)	310 (600)
3	—	5 (470)	5 (230)
4	—	5 (410)	5 (95)

* Incubated at 25°C. The number in parenthesis indicates the enzyme activity determined after diluting the mixture to 1 M of urea.

without urea, and the longer the period of incubation with 3 M urea, the less reactivation of amylase was observed (Fig. 9). The reactivation of amylase inactivated by heat and mixed with urea was also influenced by pH. To the heat-inactivated amylase being incubated with 3 M urea at 30°C for 150 min. was added a twice-volume portion of veronal buffer (M/60) of various pH values and the activity recovery was investigated. As shown in Fig. 10, the reactivation occurred at pH values between 7 and 8.8.

4) **Effect of the addition or removal of calcium.** The effect of calcium ions on amylase at heating was already found to result in irreversible inactivation of enzyme. Also, it has been found that the amylase reversibly inactivated by removal of calcium with EDTA is sensitive to heat.⁷⁾ In this section, the effect of addition or removal of calcium on the amylase inactivated by heat was investigated. To the amylase (1 mg/ml) immediately after heating at 85°C for 10 min. in a dilute sodium hydroxide solution (pH 8.6) was added a cold solution of calcium acetate or ethylenediaminetetraacetic acid ammonium salt (EDTA) with or without urea. After certain period of incubation at 25°C, the mixture was diluted with water and again incubated for further 3 hours. The enzyme activity was then assayed on a soluble starch solution with or without Ca^{++} . As given in Table III, only

the heat-inactivated amylase which was incubated without calcium salt or EDTA showed reactivation. In comparison, a similar experiment was carried out with native amylase and the enzyme mixed with EDTA in the presence of urea was irreversibly inactivated with sharp contrast to the amylase incubated with only EDTA; the latter enzyme showed quite the same activity as the original when assayed on a starch solution containing calcium ions.

II. Some Properties of the Amylase Reversibly Inactivated by Heat

The properties of the heat-inactivated amylase may be seen in part from the investigation on the factors affecting on reactivation of the inactivated amylase which have been described above. In this section were investigated some other properties of the heat-inactivated amylase.

1) **Spectroscopical properties of the amylase.** On heating amylase at 85°C for 10 min., a blue shift was clearly observed in the absorption spectra of the enzyme. Fig. 11 illustrates the difference spectra which were measured at given wavelengths as a function of incubation period at 30°C after heating, showing that the difference in the spectra appears most remarkably at regions of 292~293 and 283~285 $\text{m}\mu$. The result also indicates that the value of difference in the absorption spectra gradually decreases approximately in-

TABLE III. EFFECT OF ADDITION OF CALCIUM IONS OR EDTA ON THE HEAT-INACTIVATED AMYLASE

Amylase	Treatment (diluent, pH, incubation period at 25°C)	Activity recovered, % on starch	
		without Ca^{+++}	with Ca^{+++}
Heat-inactivated	H_2O , pH 7.4, 3 hr.	38	60
"	Ca-acetate (M/200), pH 7.6, 4 hr.	0	0
"	EDTA (M/300), pH 7.6, 4 hr.	0	0
"	EDTA (M/300) + urea (3 M), pH 7.6, 4 hr.	0	0
Native	H_2O , pH 6.0, 3 hr.	75	100
"	EDTA (M/300), pH 6.0, 24 hr.	0	100
"	EDTA (M/300) + urea (3 M), pH 6.0, 6 hr.	0	0

* The soluble starch used for activity assay was dissolved in M/500 $\text{EDTA} \cdot (\text{NH}_4)_2$ and buffered with M/50 acetic acid-ammonium acetate at pH 5.9.

** To the soluble starch used as the standard was added M/500 Ca-acetate.

FIG. 11. Difference Absorption Spectra of *Bac. subtilis*' α -Amylase Reversibly Inactivated by Heat.

A solution of amylase (0.47 mg amylase/ml of M/400 borate buffer pH 8.6, $E_{280m\mu}^{1.18}$) was heated at 85°C for 10 min. and, immediately after cooling at 25°C, its absorbancy was determined against the unheated amylase solution at a given wavelength as a function of time.

FIG. 12. Changes with Temperature of Absorption of *Bac. subtilis*' α -Amylase at Various Wave Lengths.

Amylase; 0.424 mg per ml of M/400 borate buffer, pH 8.6. The temperature was raised at a rate of approximately 5°C in every 2 min., up to 65°C, but at temperatures higher than this, the rate was gradually decreased and 5 min. was taken between 80°C and 85°C.

versely with reactivation of amylase.

The change in absorption spectra was also investigated as a function of temperature. As shown in Fig. 12, the decrease in the absorption already began at 50° to 60°C and, the higher the temperature, the more decrease was observed. In the experiment, the absorption spectra were measured against water as the control, raising the temperature of the thermostat for cuvette by the aid of Haake's water-circulating thermostat. Since the rate of rising in temperature could not be uniformly regulated and a longer time was required to get 85°C, the result obtained there seems not to be comparable with those in Fig. 11, but the reversibility of change in the absorption spectra is observed.

2) **Susceptibility of the heat-inactivated amylase to proteolytic enzyme.** The amylase reversibly inactivated by heat was extremely sensitive to proteolytic enzyme. A solution of amylase (0.5 mg/ml of M/240 borate buffer, pH 8.6) was heated at 85°C for 10 min. and then incubated at 30°C. At varied times of incubation, a 3 ml aliquote was taken into 0.5 ml of *Bac. subtilis*' alkaline protease (Nagarse, 0.06 mg) and the mixture was again incubated at 30°C, assaying the amylase activity. As shown in Fig. 13, the amylase

FIG. 13. Effect of Protease on the Heat-Inactivated Amylase.

The heated amylase on the subsequent incubation was mixed with protease at the time indicated by arrows.

exposed to the protease right after heat-treatment showed no activity even on a long incubation. On the other hand, the amylase restoring activity on the subsequent incubation was stopped to recover more activity by the addition of protease. A test of the fractional determination of native and denatured amylase revealed that the amylase activity remaining in the incubation mixture with protease was just comparable to the amount of indigestive amylase protein.

DISCUSSION

The present works demonstrated the heat-inactivated *Bac. subtilis* α -amylase to be reactivated to a great extent under a certain condition. From the experiment on the absorption spectra of the enzyme and the behavior toward proteinase, it is evident that the reversibility of inactivation of the enzyme by heat and reactivation on the subsequent incubation are achieved, accompanying a certain marked change in conformation of the enzyme protein.

It is of interest that the reversibility of heat-inactivation of the enzyme occurs easily at alkaline sides. The result shown in Fig. 8 indicated the reactivation of the inactivated amylase to be prevented by the presence of a certain concentration of buffer at acid sides. A similar phenomenon has been known with Taka-amylase¹¹⁾ and several other amylases which will be reported elsewhere.

It is also interesting that the addition of calcium ions either before and after heating significantly inhibits the reversibility of the enzyme, in spite of a marked stabilization effect for the native amylase under various conditions. Generally, the reversible inactivation of the enzyme was not obtained where some precipitates occurred on heating the enzyme solution, and the addition of calcium salts, even to the amylase right after heating, always resulted in formation of precipitate under the condition described above. The added calcium may affect the enzyme protein so as for the conformation not to transform reversibly. However, the calcium originally

FIG. 14. A Diagram Showing the Process of Reversible Inactivation of *Bac. subtilis* α -Amylase.

11) A. Tanaka, This Journal, **24**, 152 (1960).

contained in the enzyme preparation seems to play an important role in the reversibility, because the amylase freed from calcium is extremely sensitive to heat and, on the other hand, the addition of EDTA to the heat-inactivated amylase leads to irreversible inactivation of the enzyme.

The fact that the addition of urea to the heat-inactivated amylase inhibits reactivation of the enzyme and removal of the urea makes to cause reactivation, seems important in the investigation of the reversible inactivation of enzyme. Early in 1936, Anson had found reversibility of heat-inactivation of trypsin.²⁾ His findings might have been favored by use of hemoglobin as the substrate which contained

urea in order to denature the substrate. By applying this effect of urea it was found¹²⁾ that the heat-inactivated amylase showed no reactivity with the antisera to native *Bac. subtilis*' α -amylase and, as the inactivated amylase was reactivated, the reactivity with the antisera was also recovered, the details of which experiments will be published elsewhere.

From the facts in the present work together with those ever reported by several workers, the nature of *Bac. subtilis*' α -amylase in special reference to the stability may be summarized as shown in Fig. 14.

12) T. Maruyama, M. Niwa, T. Yamamoto, A. Nishida and J. Fukumoto, *J. Biochem., Tokyo*, **60**, 286 (1969).