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Studies on the Production of Enzymes by Thermophilic Actinomycetes

(PART II) Some Properties of α-Amylase from Thermophilic *Actinomycetes*

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高溫性 放線菌에 의한 酵素生産에 관한 硏究

(第2報) α-Amylase의 酵素學的 性質

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Abstract

During the course of studies on the production and utilization of thermostable α-amylase from a thermophilic actinomycete species isolated from soil, partial characterization of the α-amylase has been carried out. The optimum pH for the dextrinogenic activity of the enzyme was found to be 6.5 and the maximum reaction rate was achieved at a temperature range of 55° to 65°C. Calcium ion was recognized to have a slight effect in activating the enzyme, while heavy metal salts especially ferrous and cupric ions showed a remarkable inhibition effect. The enzyme was best protected from thermal denaturation at pH 8.0 with tris-HCl buffer; inactivation was rapid at higher or lower pH values. Furthermore, its thermal stability was greatly increased by calcium ion, particulary at the final concentration of 1×10^{-2} mole in the reaction mixture. The Km value for the α-amylase was calculated to be 2.17×10^{-4}g per ml and the energy of activation for the dextrinogenic reaction to be 12,000±580 cal per mole.

Introduction

It has been postulated that the ability of certain microorganisms to grow at high temperatures is due to the relative thermal stability of their cellular components.[1] Actually, extensive studies on enzymes, especially α-amylases from thermophilic bacteria have shown their unusual heat stability.[2~11]

Thermophilic actinomycetes, however, had escaped detailed attention in the literature, although some species were known to hydrolyze starch.[12] Only recently, Kuo and Hartman reported a work on the production of α-amylase by *Thermoactinomycetes vulgaris* and purification and partial

characterization of the enzyme.[13,14] Yamada et al. also studied on microbiological properties and amylase formation environments of *Thermoactinomycetes vulgaris* V-17 strain.[15] No other reports on amylase production by thermophilic actinomycetes have been found.

The authors, therefore, have isolated a thermophilic actinomycete from soil which can produce substantial quantities of thermostable α-amylase, and investigated on some microbiological properties of the selected strain. An optimal procedure for the formation of α-amylase by the strain has also been proposed as in the previous paper.[16] In the present work, we have studied some of the enzymatic properties of the α-amylase.

Materials and Methods

Organism: The organism used throughout this investigation was a thermophilic actinomycete isolated from soil as described previously.[16] Stock cultures were maintained on nutrient agar slants containing 2.0% of soluble starch and stored at 5°C.

Culture Medium: The culture medium used for the α-amylase production was composed of soluble starch 3.0%, peptone 1.0%, yeast extract 0.5%, NaCl 0.5%, $MgSO_4 \cdot 7H_2O$ 0.1%, K_2HPO_4 0.02 %, and $FeSO_4 \cdot 7H_2O$ 0.002%. The initial pH of the medium was adjusted to 7.0 with dilute NaOH solution.

Chemicals: All the chemicals used in this work were commercial products.

Preparation of Enzyme Solution: The selected thermophilic actinomycete was inoculated in 300 ml Erlenmeyer flask containing 30ml of culture medium and incubated at 50°C for about 16 hours with reciprocal shaking (120 strokes per minute). The cells were then removed with centrifuging at 3,500 rpm for 10 minutes. The resultant cell free fluid was used as a crude α-amylase solution in this investigation. The activity of the enzyme solution showed about 3,000 dextrinogenic units per ml of the enzyme solution.

Assay for α-Amylase Activity: α-Amylase activity was determined by a modification[17] based on blue value method. The incubation was carried out in a reaction mixture containing 1.0ml of diluted enzyme solution, 200μ moles of phosphate buffer (pH 6.5) and 1.0ml of soluble starch solution equivalent to 10μ moles of glucose in a total volume of 3.0ml. The reaction was done for 10 minutes at 37°C and stopped by the addition of 5.0ml of acidic iodine solution. The extinction of the resultant blue solution was measured at 720mμ and blanks lacking enzyme solution was run with each batch of assays. One dextrinogenic unit (DU) was defined as the amount of enzyme which was able to reduce 10% of blue value of iodine color in one minute, in acting on 1.0ml of soluble starch solution under the assay conditions.

Results and Discussions

Effect of pH on α-Amylase Activity: The activity of the enzyme preparations at different pH values was determined by the use of buffered starch substrates prepared with 0.1M acetate buffer, 0.1M phosphate buffer and 0.1M tris HCl

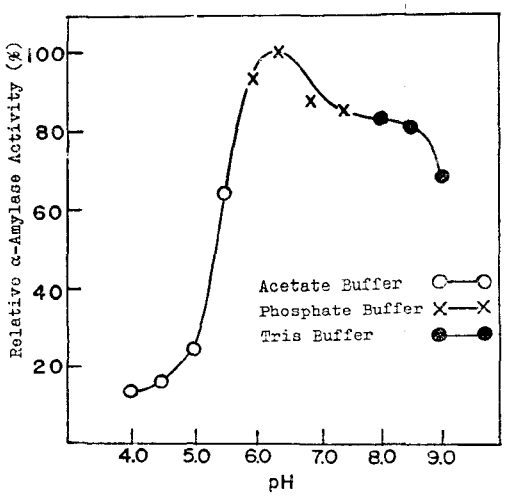

Fig. 1. The Effect of pH on α-Amylase Activity. The enzyme preparations (6.0 DU per ml) were added to buffered substrate solutions maintained at appropriate pH values with 100μ moles of various buffers. After incubation for 10 minutes at 37°C, the enzyme activity was assayed under the standard conditions.

buffer. The pH range covered was from 4.5 to 9.0. The incubation was carried out with the reaction mixture containing 6.0 DU of the enzyme under the standard conditions.

As shown in Fig. 1, maximum enzyme activity was within the pH range of 6.0 to 8.0, whereas the enzyme was inactive at lower pH values than 5.0. Maximum reaction rate was observed at pH 6.5 when phosphate buffer was used, thus a pH of 6.5 was selected for all the amylase assays.

Effect of Metal ions on Amylase Activity: To examine the effect of metal ions on the enzyme activity, various metal salts were added in the reaction mixtures to the final concentration of 2×10^{-2} mole. After incubation for 10minutes at 37°C, the α-amylase activity was measured under the optimal conditions.

As shown in Table 1, no significant activation can be realized with any of the metal ions tested but only a slight effect on the activation of the enzyme was seen in the presence of calcium salt. Heavy metal ions, especially ferrous and cupric ions rather inhibited the enzyme reaction remarkably. These findings are in agreement with those of others[4,18~22] that all α-amylase investigated contained at least 1g atom of firmly bound calcium per mole of enzyme and calcium, had an important function in α-amylases irrespective of their origins.

Stability of the Enzyme in Solution: The stability of the α-amylase in solution was tested by storing the crude enzyme solutions at various temperatures for a range of periods indicated. Then the residual activities were assayed under the optimal conditions.

Table 1. Effect of Various Metal ions on the Activity of α-Amylase. The incubation was done with a reaction mixture containing 270.5 DU of α-amylase, 60μ moles of various metal salts, 100μ moles of phosphate buffer (pH 6.5), and 1.0 ml of substrate solution. After reacting, the residual activity was assayed under the standard conditions.

Metal Ions	Activity (DU/ml)	Relative Activity
$ZnSO_4$	68.0	25.1%
$MgSO_4$	273.5	101.1
$FeSO_4$	10.0	3.7
NaCl	286.5	105.9
$CaCl_2$	366.3	135.4
KCl	270.5	100.0
$CuSO_4$	12.0	4.4
$Pb(CH_3COO)_2$	63.0	23.3
$MnSO_4$	134.5	49.7
None	270.5	100.0

As illustrated in Table 2, the enzyme lost about 10% of its activity by standing for 96 hours at 0°C and about 15% at 40°C. The crude enzyme preparation, therefore, was found to be relatively unstable in solution.

Effect of pH on the Stability of α-Amylase To investigate the effect of pH on the stability of the enzyme, the α-amylase solution was incubated at 70°C for a 10-minute period in 0.1M buffers at a range of pH values showed. After incubati ng, the remained activity of the enzyme solution was measured. The enzyme was found to be stable over the pH range from 7.0 to 8.5 as shown in Fig. 2. Maximum stability of the

Table 2. Stability of the Enzyme in Solution. The enzyme solutions containing 300 DU per ml were held at various temperatures for a range of period indicated. After 50 fold dilution of the incubated enzyme solutions, the residual activities were determined.

Activity / Hours	Activity (DU /ml)	Relative Activity (%)	Activity (DU /ml)	Relative Activity (%)	Activity (DU /ml)	Relative Activity (%)	Activity (DU /ml)	Relative Activity (%)	Activity (DU /ml)	Relative Activity (%)
Tem. (°C)	12		24		48		72		96	
0	366.0	100.0	358.2	97.9	347.1	94.8	338.5	92.5	328.5	89.8
10	360.4	98.5	355.6	97.2	342.5	93.6	337.0	92.1	320.4	87.5
30	358.1	97.8	349.8	95.6	338.0	92.3	329.8	90.1	320.0	87.4
40	357.5	97.7	349.0	95.4	337.8	92.3	316.5	86.5	310.7	84.9

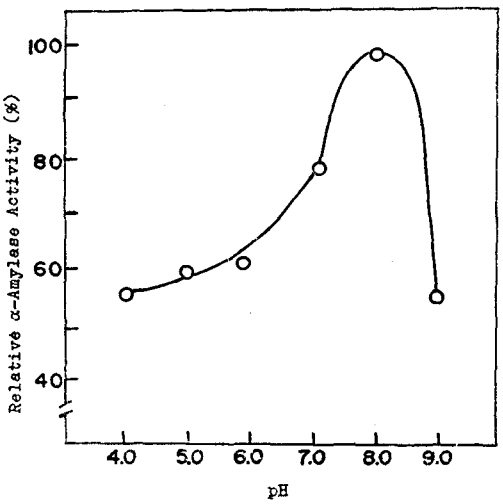

Fig. 2. The Effect of pH on the Stability of α-Amylase. α-Amylase solution (12 DU /ml) was incubated in 0.1M buffers at the appropriate pH values for 10 mins. at 70°C. The amount of α-amylase activity remained after incubation at each pH value was determined under standard assay conditions.

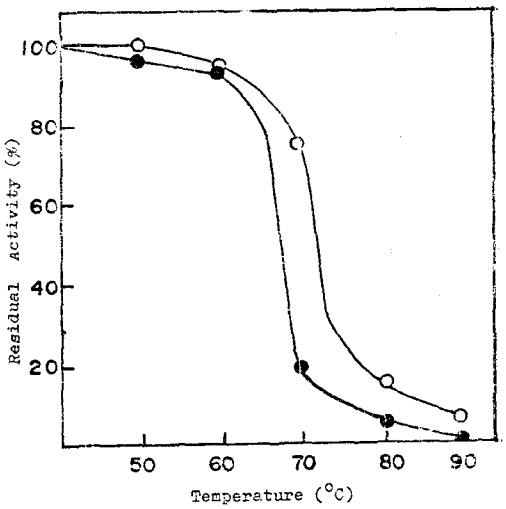

Fig. 3. Thermostability of α-Amylase. The enzyme solutions containing 12.0 DU of α-amylase per ml in 0.1M tris-HCl buffer (pH 8.0) and in distilled were incubated at the temperatures indicated for 10 minutes and cooled immediately. The residual activities were measured under the standard assay conditions. ●—●: Distilled water, ○—○: Tris buffer (pH 8.0).

enzyme showed at pH 8.0 with tris-HCl buffer. The α-amylase was rapidly inactivated at higher pH values than the optimum pH value for the enzyme stability.

Thermostability of the Enzyme: The thermal stability of the enzyme was studied in the presence of distilled water and in tris-HCl buffer (pH 8.0). The α-amylase solutions were placed in a water bath at the desired temperatures for 10 minutes. After treatment samples were removed and their remaining activities tested.

As illustrated in Fig. 3, the enzyme was proved to be more stable in tris-HCl buffer than in distilled water. The data also revealed that the enzyme lost about 23% of its activity by standing at 70°C for 10 mins. in the buffer but it was completely denaturated at higher temperatures.

Effect of Metal ions on the Thermal Stability of the Enzyme: The buffered enzyme solutions were incubated in the presence of various metal ions at 70°C for a range of period indicated. After treatment the remaining activities were determined.

As shown in Table 3, only calcium salts among the metal salts investigated were found to have an protective effect from the thermal inactivation of the enzyme. Especially calcium chloride showed the greatest effect in increasing the thermotolerance of the α-amylase.

Examination of the data in Table 4 reveals that the maximal stability of the enzyme was attained at the concentration of 1×10^{-2} mole of $CaCl_2$ in the reaction mixture. However, the heat resistance of the amylase fell off as the concentration of $CaCl_2$ was increased more than the optimum level.

These data confirmed the suggestion of others [1,2,4,10,21] that calcium has been shown to protect α-amylases from thermal denaturation and proteolytic degradation. On the other hand, heavy metal ions particularly cupric and ferrous salts were showed rather to accelerate thermal inactivation of the enzyme markedly.

Effect of Ca ion on Thermal Stability of the Amylase: As illustrated in Table 3 and 4, calcium chloride was realized to have the best

Table 3. Effect of Metal ions on the Thermal Stability of α-Amylase. The enzyme solution containing 93.0 DU of α-amylase per ml was treated in 0.1M tris-HCl buffer (pH 8.0) supplemented with 1×10^{-2} mole of metal ions at 70°C for 10 and 30 minutes. The residual activity was then measured.

Metal Ion	Activity (DU/ml)	Relative Activity (%)	Activity (DU/ml)	Relative Activity (%)
	(at 70°C for 10min)		(at 70°C for 30min)	
None	71.8	100.0	54.5	100.0
$ZnSO_4$	45.1	62.8	32.8	60.2
$MgSO_4$	23.2	32.3	15.9	29.2
$MnSO_4$	29.8	41.5	21.8	40.0
$FeSO_4$	20.4	28.4	14.1	25.9
NaCl	67.8	94.4	39.2	71.9
$CaCl_2$	231.8	322.9	169.5	311.0
$CaCO_3$	118.5	165.0	55.0	100.9
$Ca(CH_3COO)_2$	158.4	220.6	102.9	188.8
KCI	53.4	74.4	47.7	87.5
$CuSO_4$	12 1	16.9	11.1	20.4

Table 4. Effect of the Concentration of $CaCl_2$ on the Thermostability. The enzyme solutions (40.0 DU/ml) containing various concentration of $CaCl_2$ were incubated at 70°C for 10, 30 and 60 minutes. Then the residual activities were determined.

Activity / Temp. (°C) / Conc.	Activity (DU/ml)	Relative Activity (%)	Activity (DU/ml)	Relative Activity (%)	Activity (DU/ml)	Relative Activity (%)
	(at 70°C for 10min)		(at 70°C for 30 min)		(at 70°C for 60 min)	
None	30.6	100.0	22.8	100.0	16.8	100.0
1×10^{-3}M	86.2	281.7	60.7	266.2	32.8	195.2
1×10^{-2}M	98.0	320.3	80.2	351.8	52.2	310.7
2×10^{-2}M	84.0	274.5	73.0	320.2	51.6	307.1
4×10^{-2}M	82.8	270.6	70.6	309.6	49.8	296.4
6×10^{-2}M	82.0	268.0	68.0	298.2	44.0	261.9
8×10^{-2}M	76.8	250.9	38.2	167.5	35.2	209.5

effect in protecting the enzyme from thermal denaturation. Therefore, this work was to examine the effect of calcium on the stability of the amylase at various temperatures. The buffered enzyme solution containing 1×10^{-2} mole of $CaCl_2$ was incubated for 10 minutes at various temperatures and then the remaining activity was tested under the standard assay conditions.

Fig. 4 shows that the enzyme was greatly protected from thermal inactivation by the addition of calcium ion and it lost only 25% of its activity at 80°C and about 60% at 90°C for 10 minutes incubation.

Effect of Substrate Concentration on α-Amylase Activity: To investigate the effect of sub strate concentration on α-amylase activity and to determine Michaelis-Menten Constant of the enz yme reaction, α-amylase solution buffered at 6.5 was incubated with the indicated amount of starch at 37°C and then the activity was determined under the standard assay conditions.

Fig. 5 shows the effect of substrate concentration on α-amylase activity plotted according to Lineweaver and Burk.[23] The Km value was calculated to be 2.17×10^{-4}g per ml. The Km values recorded for α-amylases of animal, plant and

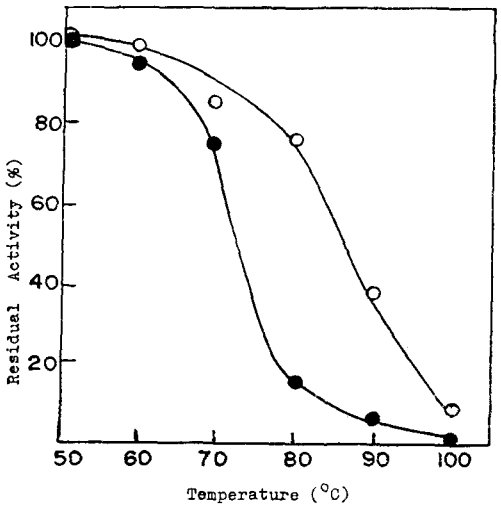

Fig 4. Effect of Ca ion on the Thermostability of α-Amylase. The enzyme solutions containing 12.0 DU of α-amylase per ml in 0.1M tris-HCl buffer supplemented with 1×10^{-2}M $CaCl_2$ and without Ca ion were reacted at the temperatures indicated for 10 minutes and then the residual activities were assayed. ●—●; Tris buffer (pH 8.0) ○—○; Ca ion (1×10^{-2}M)

microbial origin have ranged from 1.8×10^{-4} to 6.5×10^{-4} g per ml.[2,18,24,25]

Energy of Activation of Amylase: Activity measurements of α-amylase were carried out at pH 6.5 over a range of temperatures between 30°C and 60°C at 5°C intervals. The optimal temperature for α-amylase activity was found to extend over a range of 55°C to 60°C at pH 6.5.

A plot of the logarithm of the velocity as a function of the reciprocal of the absolute temperature reveals a straight line over the temperature range of 30° to 55°C and indicates that enzyme denaturation occurred at higher temperatures as shown in Fig. 6. By means of Arrhenius' equation, the energy of activation of the dextrinogenic reaction was calculated and found to be 12,000± 580 cal per mole. The temperature coefficient (Q_{10}) of the enzyme reaction was found to be 1.80 between 35° and 45°C, 2.06 between 45° and 55°C, and 1.04 between 55° and 65°C.

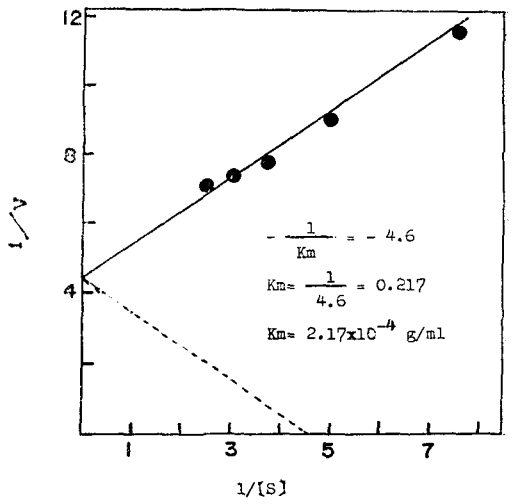

Fig. 5. Lineweaver-Burk Plot of Starch Hydrolysis by α-Amylase. The reactions were carried out at 37°C for 10 minutes in reaction mixtures containing 6.0 DU of α-amylase per ml, 100μ moles of potassium phosphate buffer, pH 6.5, and variable amounts of soluble starch indicated in a total volume of 3.0ml. Velocity (V) was expressed as the amount of enzyme which is able to reduce 10% of blue value of iodine color per min. and substrate concentration (S) as g per liter.

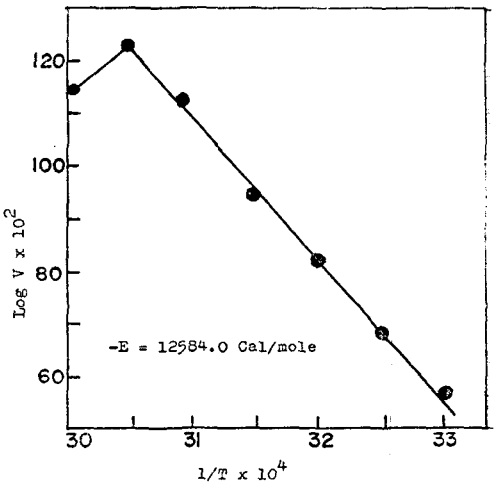

Fig. 6. Effect of Temperature on α-Amylase Activity. Dextrinogenic activity measurements were carried out at pH 6.5 over the temperature range of 30° to 60°C with the reaction mixture containing 6.0 DU of α-amylase per ml. The logarithm of the velocity of the dextrinogenic reaction is plotted as a function of the reciprocal of the absolute temperature.

要　　約

高溫性 放線菌으로부터 耐熱性 α-Amylase의 生産과 그 利用 可能性을 검토하기 위하여 土壤試料로부터 α-Amylase 生産能力이 극히 우수한 菌株를 分離하여 分離菌의 몇가지 菌學的 性質내지는 酵素生産을 위한 培養條件을 調査하여 前報에 發表하였으며 本報에서는 供試放線菌이 生産하는 α-Amylase의 酵素學的 一般性質을 검토하여 다음과 같은 結果를 얻었다.

1) 本酵素의 最適活性 pH는 6.5이었으며 最適活性溫度는 55°~65°C이었다.
2) 本酵素의 安定 pH 범위는 7.0~8.0에 위치 하였으며
3) 活性에 대한 금속 ion의 影響은 Ca^{++} 및 Na^{+} ion에 의하여 促進되었으나 중금속 ion인 Fe^{++} Cu^{++} ion등은 현저히 阻害함을 보였으며 熱安定性에 대한 금속 ion의 效果는 Ca^{++} ion에 의해 증가했다.
4) 本 酵素의 Km value는 2.17×10^{-4}g per ml이었으며
5) Activation energy는 12,000±580 cal per mole이었다.

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