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Influence of pH on the inactivation kinetics of isoamylase[1]

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Abstract. The theraml stability of isoamylase from *Pseudomonas amyloderamosa* JD210 was quite sensitive to pH changes. The stability of the enzyme at 50°C and various pH values followed the order: pH 4 > pH 5 > pH 6 > pH 3 > pH 7. The thermal inactivation followed first order kinetics. The inactivation rate constants at pH 4 and temperature 50°C, 53°C, 55°C and 57°C were 0.0043, 0.0184, 0.0561 and 0.1602 min^{-1}, respectively. Those at pH 5 and temperature 46.5°C, 48°C, 50°C and 53°C were 0.0045, 0.0069, 00156 and 0.0715 min^{-1}, respectively. Those at pH 7 and temperature 33°C, 35°C and 38.5°C were 0.0048, 0.0110 and 0.02802 min^{-1}, respectively. The Arrhenius plots for the inactivation rate constants of isoamylase at pH 4 and pH 5 intersected at isokinetic temperature 61°C, while the two lines for pH 4 and pH 7 were parrallel. Activation energies obtained for pH 4, pH 5 and pH 7 were 98.7, 89.3 and 98.1 kcal/mol, respectively. We proposed that aspartyl residue in deprotonated form (-COO-) and histidyl residue in protonated form were important for maintaining isoamylase at stable conformation.

Key words: Isoamylase; *Pseudomonas amyloderamosa*; pH; stability.

Introduction

Isoamylase (EC.3.2.1.68) is a debranching enzyme which is very useful for the industrial production of maltose or glucose from starch and for elucidating the structures of glucans when is used in combination with other glycosidic enzymes (Harada, 1984). Recently, it has been used for the synthesis of branched cyclomalto-oligosaccharides (Abe *et al.*, 1986; Yoshimura *et al.*, 1987; Abe *et al.*, 1988). Isoamylase from *Pseudomonas amyloderamosa* can attack amylopectin and glycogen directly by catalyzing exolytic hydrolysis of (α-1,6)-glucosidic linkages from nonreducing ends. The enzyme stability is very important for its application. In the present work, we studied the effect of pH on isoamylase inactivation kinectics which would provide some insight for the choice of pH in industrial process.

Materials and Methods

Materials

Isoamylase (from *Pseudomonas amyloderamosa* JD210), which had a specific activity of 33,200 units/mg, was obtained from Dr. W. H. Hsu, Food Industry Research Development Institute, Hsinchu. Potato amylopectin was obtained from Sigma Co. All other chemicals were of reagent grade.

Enzyme Assay of Isoamylase

The enzyme was assayed according to Yokobayashi *et al.* (1979) with a slight modification. The standard reaction mixture (0.2 ml) containing 0.8% potato amylopectin and enzyme in 0.1 M acetate

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buffer (pH 5.0) was incubated at 40°C for 10 min and then stopped by adding 0.2 ml of 0.02 N I_2-KI solution (prepared by mixing 0.125 g of I_2 and 2.5 g of KI in 50 ml of H_2O). The mixture was diluted with 3.6 ml of distilled water and the increase in optical density at 610 nm was measured 20 min later. For blank, the enzyme was inactivated by heating at 100°C for 10 min. One unit of this enzyme was defined as △OD 610nm/h = 0.1 under the reaction condition.

Thermal Inactivation of Isoamylase

Isoamylase in a mixed buffer of sodium acetate and potassium phosphate (5 mM each), which was preadjusted to various pH values, was immersed in a thermostated water bath for a certain time, and then removed, cooled by immersion in an ice bath. Each residual enzyme activity remaining in this solution was assayed in triplicate and compared to an identically prepared but unheated control sample. First order rate constants for inactivation (k) were obtained from the slopes of least square fits of ln (fractional activity) remaining versus time, ln Et/Eo = -kt, where Eo and Et represent the initial enzyme activity and residual enzyme activity at time t, respectively.

Calculation of Theramodynamic Parameters for isoamylase Inactivation

Thermodynamic parameters were calculated by applying Eyring's transition state theory (Eyring, 1935).

$$k = \frac{k_b T}{h} e^{-\triangle F^*/RT} \quad (1)$$

$$\triangle F^* = \triangle H^* - T\triangle S^* \quad (2)$$

$$Ea = \triangle H^* + RT \quad (3)$$

Where $\triangle F^*$, $\triangle H^*$, $\triangle S^*$ and Ea represent the free energy, enthalpy, entropy and energy of activation respectively for enzyme denaturation; k_b is Boltzmann' constant (1.3087×10^{-23} J·°K^{-1}), h is Plank's constant (6.6262×10^{-34} J·°K mol^{-1}), T is the absolute temperature (°K). Since inactivation rate constant k can be obtained from the aforementioned experiment, $\triangle F^*$ was readily calculated from equation (1). Energy of activation (Ea) can be obtained from the slope of Arrhenius plot ln k vs. 1/T according to equation $\ln k = -\frac{Ea}{R}\frac{1}{T}$ + constant, therefore $\triangle H^*$ can be calculated from equation (3). Consequently, $\triangle S^*$ was calculated from equation (2).

Results and Disscusion

As shown in Fig. 1, pH greatly affected the stability of isoamylase at various temperatures. It was clear that stability of the enzyme at various pH values followed the order: pH 4 > pH 5 > pH 6 > pH 3 > pH 7. In solutions with pH above 7 or below 3, the enzyme was very unstable. The enzyme in pH 2 or pH 8 solution completely lost its activity after incubation at 35°C for 8 min. This is consistent with the results of isoamylase from *Pseudomonas* strain SB-15 which showed maximal stability at pH 3.5 to 5.5 when incubated at 40°C (Yokobayashi *et al.*, 1979). The pH for maximal stabilization is close to the optimal pH for isoamylase activity (pH 3.5) and pI (4.4).

Thermal inactivations as a function of time at pH 4, pH 5 and pH 7 are presented in Fig. 2, Fig. 3 and Fig. 4, respectively. Under the experimental conditions, inactivation follows first-order kinetics. These suggest that the inactivation of isoamylase is a simple unimolecular process in which active enzyme was directly transformed into inactive enzyme form. Neither intermediate enzyme form nor heterogeneous

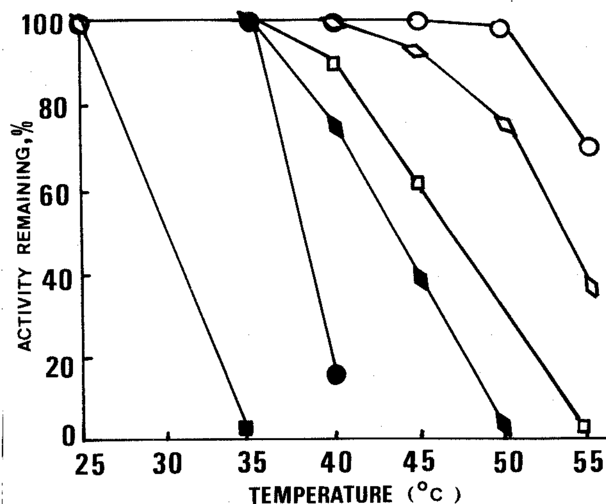

Fig. 1. Residual activity of isoamylase after incubation at various temperatures for 8 minutes. The isoamylase was dissolved in a mixed buffer of sodium acetate and potassium phosphate (5 mM each) which was preadjusted to various pH values. (■, pH 2 or 8; ◆, pH 3; ○, pH 4; ◇, pH 5; □, pH 6; ●, pH 7)

enzyme was involved. The inactivation rate constants of the enzyme in pH 4 buffer at 50°C, 53°C, 55°C and 57°C were 0.0043, 0.0184, 0.0561 and 0.1602 min^{-1}, respectively. Those in pH 5 buffer at 46.5°C, 48°C, 50°C and 53°C were 0.0045, 0.0069, 0.0156 and 0.0715 min^{-1}, respectively. Those in pH 7 buffer at 33°C, 35°C and 38.5°C were 0.0048, 0.0110 and 0.0802 min^{-1}, respectively.

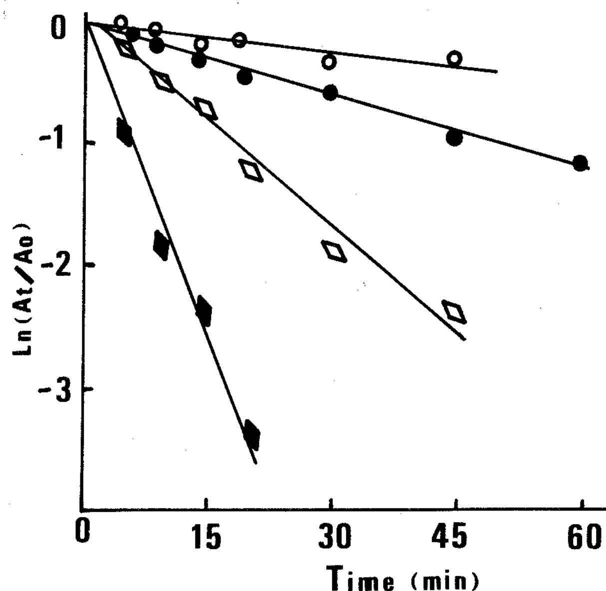

Fig. 2. Thermal inactivation of isoamylase at pH 4 and temperature 50°C (○), 53°C (●), 55°C (◇), 57°C (◆).

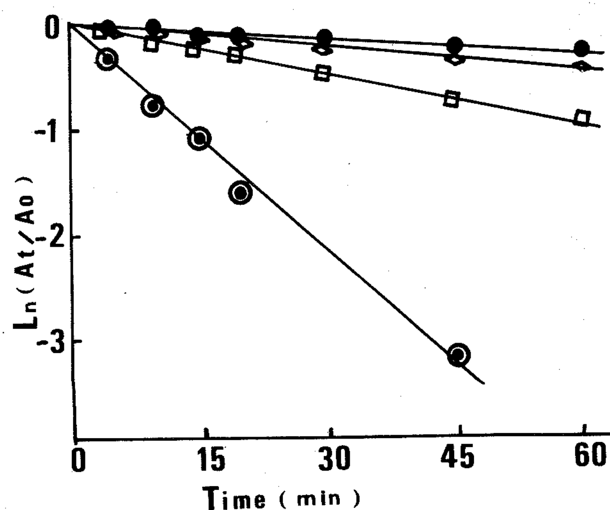

Fig. 3. Thermal inactivation of isoamylase at pH 5 and temperature 46.5°C (●), 48°C (◇), 50°C (□), 53°C (⊙).

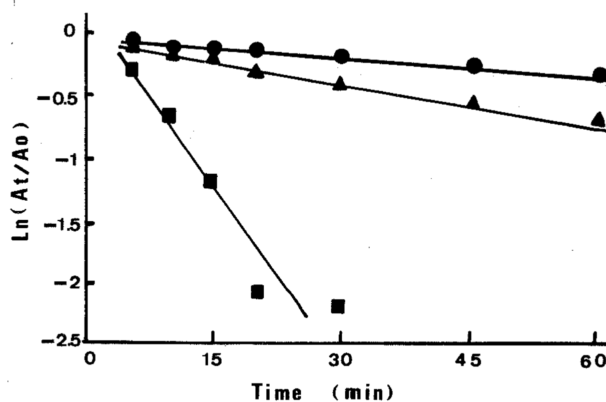

Fig. 4. Thermal inactivation of isoamylase at pH 7 and temperature 33°C (●), 35°C (▲), 38.5°C (■).

Arrhenius plots for thermal inactivation at pH 4, pH 5 and pH 7 are shown in Fig. 5. Activation energies obtained from these data are : Ea (pH 4), 98.7 kcal/mol; Ea (pH 5), 89.3 kcal/mol and Ea (pH 7), 98.1 kcal/mol. The thermodynamic parameters for isoamylase denaturation at 50°C based on Eyring's transition state theory are presented in Table 1. As shown in Table 1, the enzyme at pH 7 is much less stable (4784 times) than the enzyme at pH 4 solely due to the higher positive activation entropy (△S*) which suggests the enzyme conformation at pH 7 is very unstable. it is quite possible that the histidyl residue with a pKa of about 6 was very important for the confor-

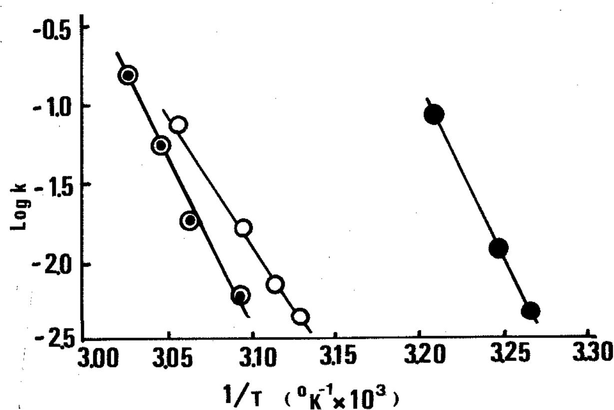

Fig. 5. Arrhenius plots for thermal inactivation rate constants (k) of isoamylase at pH 4 (⊙), pH 5 (○) and pH 7 (●).

Table 1. *Thermodynamic parameters for the denaturation of isoamylase at 50°C and various pHs.*

pH	k (s^{-1})	$\triangle F^*$ (kcal)	Ea (kcal)	$\triangle H^*$ (kcal)	$\triangle S^*$ (cal/°K)
4	7.17×10^{-5}	25.1	98.6	98.0	226
5	2.60×10^{-4}	24.3	89.3	88.7	199
7	0.343	19.6	98.1	97.5	241

mational stability of isoamylase. Although the enzyme at pH 5 had smaller activation entropy change ($\triangle S^*$) than the enzyme at pH 4, the smaller activation enthalpy change ($\triangle H^*$) overweighed $\triangle S^*$ effect and made the enzyme at pH 5 less stable (3.6 times) at 50°C. Since the Arrhenius plots for the inactivation rate constants of isoamylase at pH 4 and pH 5 intersect at higher temperature (61°C), the enzyme at pH 4 is more stable than that at pH 5 only below this isokinetic temperature. Thus, pH 4 exhibited a "low temperature stabilization" effect compared with pH 5 (Kutuzova *et al*., 1984).

From the aforementioned results, we propose that aspartyl residue in dissociated form (-COO-) and histidyl residue in protonated form (imidazolium ion) are essential for the stability of isoamylase. Since the pKa of β-carboxyl group of aspartyl residue and the imidazolium group of histidyl residue are about 3.5 and 6.0 respectively, the sharp decrease of isoamylase stability at pH < 3 or > 6 (as shown in Fig. 1) is consistent with our hypothesis. The higher entropy of activation ($\triangle S^*$) at pH 7 than that at pH 4 (Table 1) further suggests that the deprotonation of imidazolium ion lead to loosened enzyme conformation and instability. The gradual decrease of enzyme stability from pH 4 to 6 could be due to weakened salt bridge between -COO- and positively charged imidazolium ion or due to the dissociation of glutamyl (γ-carboxyl) group (its pKa is about 4.5). Further experiment by chemical modification of isoamylase or site specific mutation on its gene is required to prove our hypothesis.

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Literature Cited

Abe, J.I., N. Mizowaki, and S. Hizukuri. 1986. Synthesis of branched cyclomalto-oligosaccharides using *Pseudomonas* isoamylase. Carbohydrate Research **154:** 81-92.

Abe, J.I. and S. Hizukuri. 1988. Enzymic synthesis of doubly branched cyclomaltoheptaoses through the reverse action of *Pseudomonas* isoamylase. Carbohydrate Research **176:** 87-95.

Eyring, H. 1935. Activated complex theory for bimolecular reactions. J. Chem. Phys. **3:** 107-119.

Harada, T. 1984. Isoamylase and its industrial significance in the production of sugars from starch. Biotechnol. Genet. Eng. Rev. **1:** 39-63.

Kutuzova, G.D., N.N. Ugarova, and I.V. Berezin. 1984. General characteristics of the changes in the thermal stability of proteins and enzymes after the chemical modification of their functional groups. Russian Chem. Rev. **53:** 1852-1890.

Yokobayashi, K., A. Misaki, and T. Harada. 1979. Purification and properties of *Pseudomonas* isoamylase. Biochim. Biophys. Acta **212:** 458-469.

Yoshinura, Y., S. Kitahata, and S. Okada. 1987. FormationO of 6-O-maltosylcyclomalto-oligosccharides by transfer action of three debranching enzymes. Carbohydrate Research **168:** 285-294.

酸鹼度對異澱粉酶熱變性動力學之影響

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假單胞菌異澱粉酶之熱穩定性對 pH 值之變化非常敏感。在 50°C 下之酵素穩定性爲：pH4＞pH5＞pH6＞pH3＞pH7。此酵素之熱變性爲一級反應。在pH 4 下，50°C，53°C，55 °C與 57 °C之熱變性常數各爲 0.0043，0.0184，0.0561 與 0.1602min^{-1}。在 pH 5 下，46.5°C，48°C，50 °C與 53 °C者各爲 0.0045，00069，0.0156與 0.0715min^{-1}。在 pH 7 下，33 °C，35 °C與 38.5 °C者各爲0.0048，0.0110 與 0.0802min^{-1}。

Arrhenius 式作圖顯示 pH 4 與 pH 5 直線交於同溫度 61 °C，而 pH 4 與 pH 7 直線則爲平行。在 pH 4，pH 5 與 pH 7 之熱變性活化能各爲 98.7，89.3 與 98.1 kcal/mol，我們推論天門冬胺酸基在陰離子狀態而組織胺酸基存在陽離子狀態爲保持異澱粉酶於穩定構形所必需。