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Enzymatic Properties of Cytidine Deaminase from *Aspergillus fumigatus* IFO 5840

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Abstract

Cytidine deaminase (EC 3.5.4.5) from *Aspergillus fumigatus* IFO 5840, which was the first cytidine deaminase to be found in a mold, was fractionated with ammonium sulfate (35~60%). When the enzyme solution in 0.25M of Tris-HCl buffer (pH 7.2) was preincubated at 37° C for 25min, the enzyme activity was reached to maximum state. The optimum pH and temperature for the enzyme activity were found to be 6.8 to 7.2 and near 37° C, respectively. The enzyme was stable in a pH 7.2 to 9.0, and was generally stable at 40° C, but after treating at 60° C for 20min at the optimal pH, 77% of the enzyme activity was inactivated, and disappeared completely by treating at 70° C for 25min. Activation energy (Ea) of fungal cytidine deaminase was calculated as 14.190 Kcal/mol by the Arrhenius plot, and temperate coeffient (Q_{10}) of the enzyme was calculated as 2.163.

Key words : cytidine deaminase, *Aspergillus fumigatus* IFO 5840

INTRODUCTION

In one of the salvage pathways[1,2] the cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) catalyzes the hydrolytic deamination of cytidine to uridine and ammonia.

The enzyme which was initially discovered in yeast[3], was identified from *Lactobacillus pentosus*[4] and has since been isolated and purified from *E. coli*[5,6,17,18], yeast[3,7], *Bacillus substilis*[8~10], corn[11], mouse's kidney[12], sheep's liver[13], human liver[14~16]. It has been difficult to purify because of a small presence of the enzyme in the cell[17].

Lowly-evolved motile flagellated bacteria, such as *Pseudomonas* and *Xanthomonas*, possess a nucleoside hydrolase-cytosine deaminase system, while highly-evolved bacteria, such as *Micrococcus*, *Staphylococcus* and *Streptomyces*, possess a cytidine deaminase-nucleoside phosphorylase system and halfway-evolved bacteria are of a mixed type in respect with the cytidine degradation[19].

Cytidine deaminase is an important enzyme in the catabolism of pyrimidine nucleotide, in the salvage synthesis of pyrimidine nucleotide, and in the classification of bacteria based on the kind of cytidine-degrading enzyme system. However, cytidine deaminase has never been found in molds, which are more highly evolved than bacteria or yeasts.

This paper deals with some properties of cytidine deaminase from the mold *Aspergillus fumigatus* IFO 5840.

MATERIALS AND METHODS

Microorganism and culture conditions

A strain, *Aspergillus fumigatus* IFO 5840[20~23] was used in this experiment. One loop of the stock culture was transfered into a 500ml shaking flask con-

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taining 100ml of medium composed 2% glucose, 1% yeast extract, 0.1% peptone and 0.1% (by vol.) antifoam (pH 5.6). The seed culture was carried out in a shaking incubator (reciprocal incubator ; 120 strockes/min) at 30° C for 3 days and 10ml of the seed culture were inoculated in shaking flasks, containing 100ml of the same medium, and cultivated under aerobic conditions for 22 to 24hrs.

Preparation of crude enzyme and ammonium sulfate fractionation

The cells were harvested with filter paper under reduced pressure and washed twice with 0.85% NaCl solution. The cell suspension was mixed 50% (v/v) of cells which were suspended in 0.02M Tris-HCl buffer (pH 6.5) containing 1mM 2-mercaptoethanol and 50% (v/v) of glass beads (φ 0.3~0.5 mm). It was disintegrated at below 10° C for 10 min by an Ace homogenizer and centrifuged at 12,000 x g for 20 min to remove cell debris. The resulting clarified solution was referred to as the crude enzyme. To the cell extract, ammonium sulfate was added to give 35% saturation, and the pH was adjusted to 6.5. The mixture was left at 4° C for 3 hrs, and the precipitate was removed by centrifugation at 12,000 x g for 10 min. To the supernatant, ammonium sulfate was added to give 60% saturation, and the mixture was kept at 4° C for 5 hrs. The precipitate was collected in the same way and dissolved in a small volume of the same buffer. The enzyme solution was dialysed at 4° C for 10hrs against three change of 20 volumes of buffer. The dialysed enzyme solution was centrifuged under the same conditions to remove insoluble material formed.

Enzyme and protein assay

Cytidine deaminase activity was measured by Tomchick's method[12]. The reaction mixture containing 1.3ml of 0.25M Tris-HCl buffer (pH 7.2), 1.4ml of distilled water and 0.2ml of dialyzed enzyme solution were preincubated at 37° C for 25min ; 0.1ml of 5mM cytidine were added to the reaction mixture and incubated at 37° C for 20min ; 0.2ml of 23% perchloric acid were added to stop the reaction ; the reaction enzyme solution was centrifuged at 12,000 x g for 15 min and was measured adsorption decreasing at 290nm (Fig. 1). One unit of activity was defined as the amount of enzyme that deaminates 1 μmol of cytidine per min. The specific activity was defined as the unit of activity per mg of protein.

Paper chromatography

Mixtures containing 0.4ml of 0.25M Tris-HCl buffer (pH 7.2), 0.3ml of distilled water and 0.2ml of dialyzed enzyme were preincubated at 37° C for 25 min ; 0.1ml of 5mM cytidine was added to the reaction mixture, and incubated at 37° C for 1 hr. The reaction mixtures were heated at 100° C for 3min to stop the reaction and were centrifuged at 12,000 x g for 15 min to remove denaturated protein. 200 μl of enzyme reaction solution were spotted on Toyo filterpaper No.50 and developed at room temperature for 18hrs. Developing solvent used for ispropanol : H_2O : HCl (65 : 18.4 : 16.6 v/v%). Detection of product used for UV lamp and UV absorbed material was detected.

Activation energy (Ea) and temperature coefficient (Q_{10})

Activation energy (Ea) of cytidine deaminase was

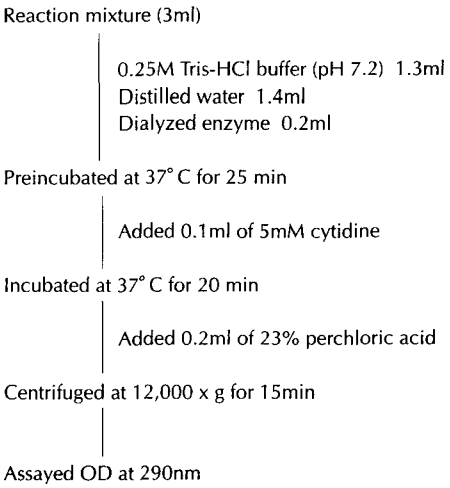

Fig. 1. Assay of cytidine deaminase activity.

calculated at 27° C to 37° C from Arrhenius equation[24]. Temperature coefficient (Q_{10})[25] was calculated at 27° C to 37° C from $Vmax_2/Vmax_1$.

Protein assay

Protein in the enzyme solutions was measured by the method of Lowry[26], with bovine serum albumin as the standard.

Chemicals

Cytidine and uridine used were obtained from Sigma Chemical Co. (U.S.A.). The other chemicals were purchased from Wako Pure Chemical Industries (Japan).

RESULTS AND DISCUSSION

Ammonium sulfate fractionation

In order to examine effects of pH and temperature, on cytidine deaminase from *Aspergillus fumigatus*. Ammonium sulfate was added to the crude enzyme solution to give 35~60% saturation and was fractionated. As shown in Table 1, the specific activity was increased to about 2.4 times and the yield was 38%.

Paper chromatography

Conversion of cytidine to uridine was examined by paper chromatography. As shown in Fig.2, the values of Rf were calculated 0.68, 0.769, and 0. 769, respectively. Therefore, presence of cytidine deaminase in *Aspergillus fumigatus* was detected.

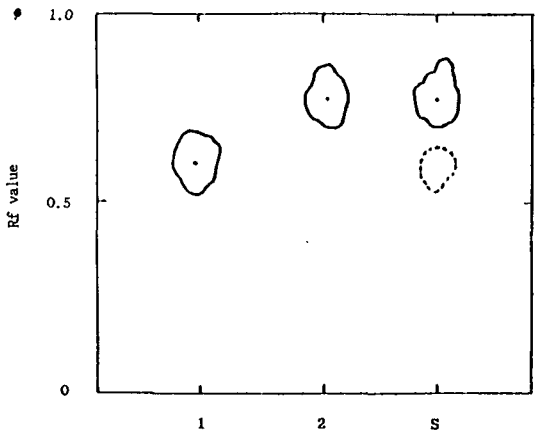

Fig. 2. Paper chromatogram for cytidine and uridine.
Developed in isopropanol : H_2O : HCl (65 : 18.4 : 16.4 v/v%), standard ; (1) cytidine, Rf = 0.608 ; (2) uridine, Rf = 0.769 Sample ; S, Rf = 0.769.

Preincubation time

Effect of preincubation time on cytidine deaminase activity was examined. As shown in Fig. 3, the enzyme activity was reached to maximum state when preincubated at 37° C for 25min.

The pH stability

In order to examine the pH stability on cytidine, deaminase enzyme solution was stored in 0.25M buffers of pH's ranging from 5.9 to 9.0 at 4° C for 24hrs. The residual activities were assayed under the standard conditions. Buffers used were potassium phosphate buffer (pH 5.9 to 7.2) and Tris-HCl buffer (pH 7.2 to 9.0). As shown in Fig. 4, the enzyme was stable between pH 7.2 and 7.4, but stability decreased sharply outside this range.

Temperature stability

In order to examine the thermostability of the cy-

Table 1. Partial purification of intracellular cytidine deaminase

Step	Enzyme vol. (ml)	Enzyme unit (1ml/hr)	Protein (mg/ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Fold
Crude enzyme	365	0.795	3,625	290.2	1,323.1	0.219	100	1
Ammonium sulfate (35~60%)	32.6	3.18	6,000	103.7	195.6	0.530	38	2.4

tidine deaminase, enzyme solution in Tris-HCl buffer (pH 7.2) was heated at the indicated temperature ranging from 40° C to 70° C for 5, 25, 40 min, respectively. The remaining activities of quickly cooled enzyme were measured under the standard assay condition. As shown in Fig. 5, the enzyme was stable below 40° C for 5 min. Thermal inactivation of cytidine deaminase stability at various temperatures was tested using the standard enzyme assay for the indicated time from 10 to 40 min. As shown in Fig. 6, the enzyme was stable at 40° C for the 10 min, but 45% of the enzyme activity was inactivated by heating at 50° C for 15 min, 77% was inactivated by heating at 60° C for 20min and was completly inactivated at 70° C for 25 min.

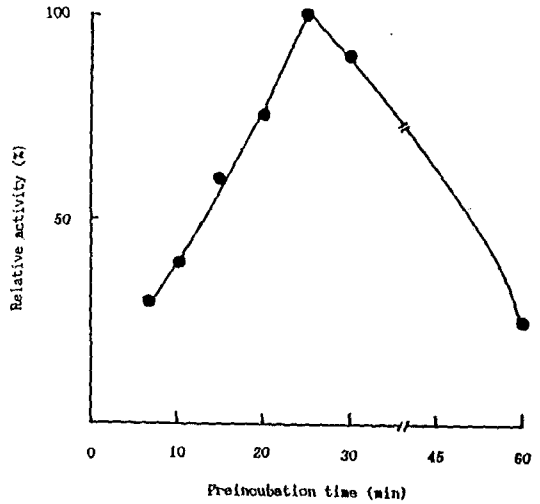

Fig. 3. Effect of preincubation on cytidine deaminase activity.
The enzyme solution in 0.25M Tris-HCl buffer (pH 7.2) was preincubated at 37° C for the indicated time and the enzyme activities were assayed by the standard assay conditions (Fig. 1).

Effect of pH

In order to examine the effect of pH on the enzyme activity, enzyme reaction solution was adjusted to various pH values from 4.4 to 9.0 and then measured under the standard assay conditions. As shown in Fig. 7, the enzyme activity was high from pH 6.8 to pH 7.2. The buffers (0.2M) used were acetic acid buffer (pH 4.4~5.6), phosphate buffer (pH 5.9~7.4), and Tris-HCl buffer solution (pH 7.2

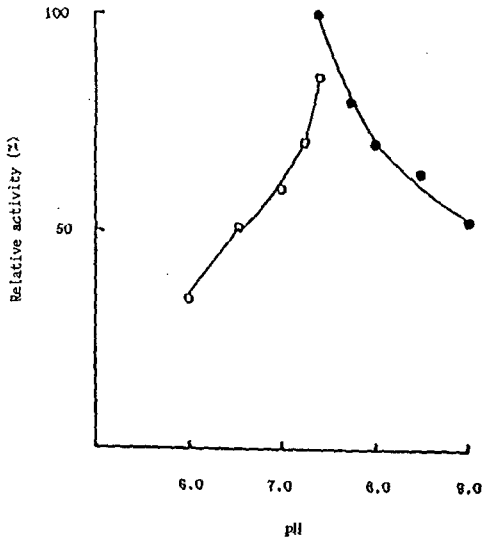

Fig. 4. The pH stability of cytidine deaminase.
Enzyme solution was storaged in 0.25M buffers of pH's ranged from 5.9 to 9.0 at 4° C for 24 hours. The residual activities were assayed under the standard conditions (Fig. 1). Buffers used were potassium phosphate buffer (pH 5.9 to 7.2), Tris-HCl buffer (pH 7.2 to 9.0).

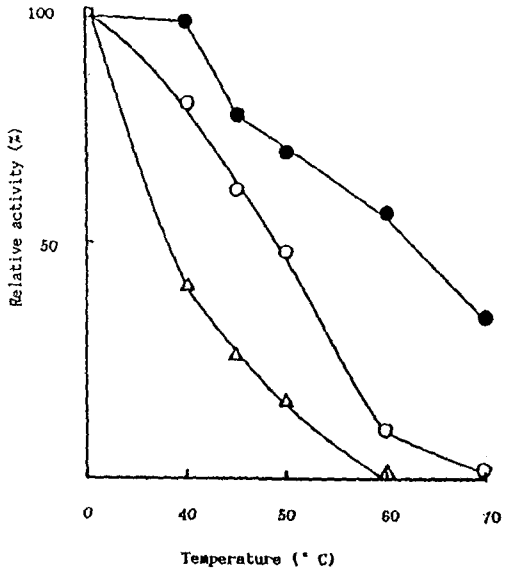

Fig. 5. Effect of temperature on cytidine deaminase stability.
The enzyme solution in 0.25M Tris-HCl buffer (pH 7.2) was incubated at the indicated temperatures ranges from 40 to 70° C for the indicated time (●-● ; 5min, ○-○ ; 25min, △-△ ; 40min). After being cooled, the reaction mixtures were added a cytidine and assayed for residual activities.

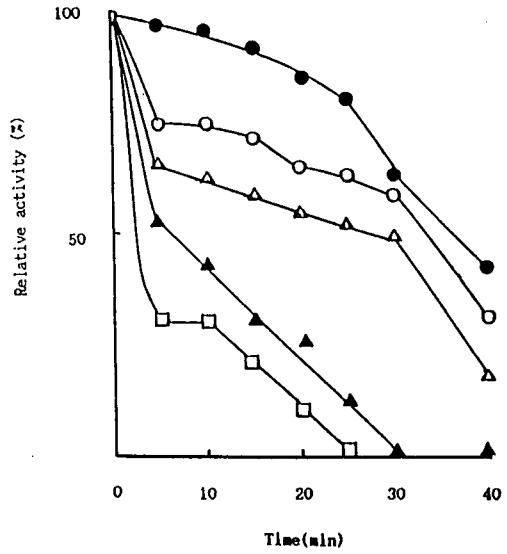

Fig. 6. Thermal inactivation of cytidine deaminase stability at various temperatures was tested using the standard enzyme assay as described in methods.
Symbols : 40° C (●), 45° C (○), 50° C (△), 60° C (▲), 70° C (□).

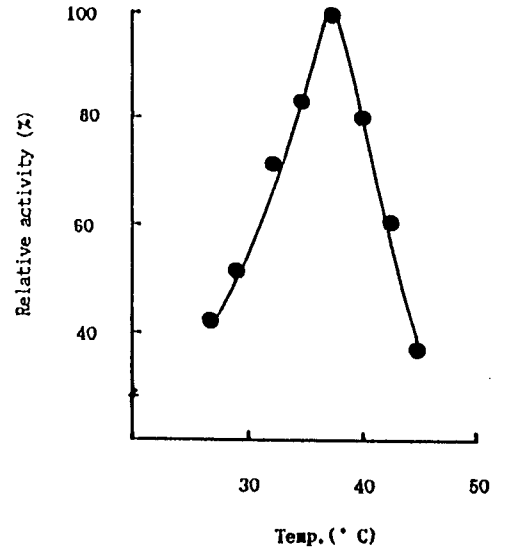

Fig. 8. Effect of temperature of cytidine deaminase activity.
Temperature dependence of the enzyme activity was tested at various temperatures using the standard enzyme assay as descrided in the methods.

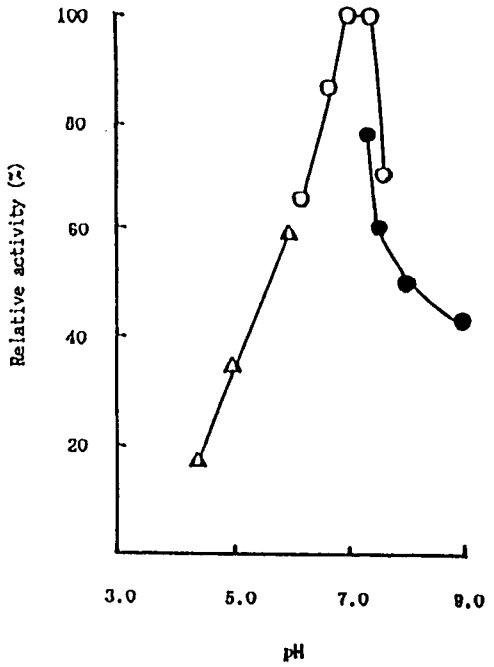

Fig. 7. Effect of pH on the cytidine deaminase activity.
The optimal pH for the enzyme activity was determined by the standard assay method (Fig. 1) using various pH buffers (0.2M) of acetate (pH 4.4 to 5.6), potassium phosphate (pH 5.9 to 7.4), Tris hydrochloride (pH 7.2 to 9.0) (symbols : acetate buffer (△), potassium phosphate buffer (○), Tris hydrochloride buffer (●).

~9.0). This result was similar to report optimum pH of Tomchick's[9] Kidney of mouse was determined from pH 6.0 to 7.0, Hosono's[6] *E. coli* from pH 6.0 to 7.5, and Ipata's[7] baker's yeast of near pH 7.0 ; but enzyme activity of strain used was demonstrated in weak alkalic region in comparison with optimum pH that Wisdom's[10] liver of sheep (pH5.0), Song's[6] *Bacillus* subtilis (pH 6.0).

Effect of temperature

As shown in Fig. 8, the cytidine deaminase activity was measured at various temperatures from 25° C to 45° C for a 20min reaction. The maximum cytidine deaminase activity was observed at 37° C.

Activation energy (Ea) and temperature coefficient (Q_{10})

The activation energy for the cytidine deaminase can be determined by measuring the reaction rate constant at a temperature range from 27° C to 37° C and by plotting logK versus 1/T. It was 14.190Kcal /mole determined by Arrehenius plot, as shown in Fig. 9. Temperature coefficient (Q_{10}) was determined to be 2.163 at a temperature between 27° C and 37° C. This value agreed with the fact that reaction velocity to an increase of temperature 10° C was double[27].

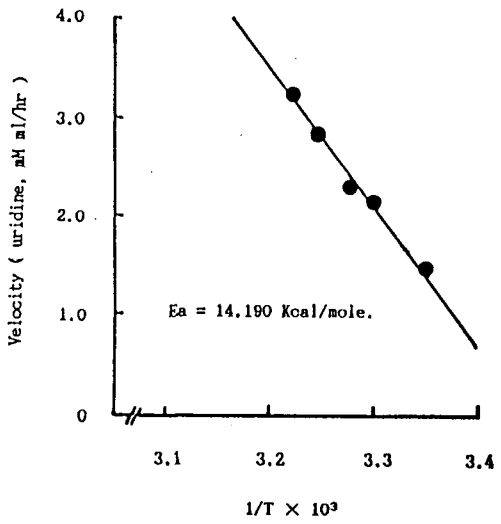

Fig. 9. Arrhenius plot of the effect of temperature on cytidine deaminase.
The date include initial velocity determinations at temperatures. Velocity was expressed in mmol of uridine formed per 1.0ml of the enzyme solution for 60min.

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Aspergillus fumigatus IFO 5840이 생산하는 Cytidine Deaminase의 효소학적 성질

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요 약

황산암모늄 분획(35~60%) 효소액을 사용하여 *Aspergillus fumigatus* IFO 5840의 cytidine deaminase에 대한 효소학적 성질을 조사하였다. 효소반응의 전처리시간은 25분이었으며 본 효소의 최적pH와 최적온도는 각각 6.8~7.2와 37°C부근이었다. pH에 대한 안정성은 pH7.2에서 9.0의 범위에서 안정하였으며 온도의 안정성은 40°C에서 10분 열처리시 대체로 안정하였으나 60°C에서 20분간 처리시는 77%의 효소실활을 나타내었고 70°C에서 25분간 처리하였을때 완전히 실활되었다. 본 효소의 활성화 에너지 값(Ea)은 14.190Kcal/mol이었고 온도계수 (Q_{10})는 2.163이었다.