KOR. JOUR. MICROBIOL February, 1993 p. 54~62 Vol. 31, No. 1

Purificaion and Characterization of Adenosine deaminase from *Aspergillus oryzae*

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Intracellular adenosine deaminase (ADA) from *Aspergillus oryzae* was purified using ammonium sulfate fractionation, a DEAE-Sephadex A-50 anion exchange chromatography, an ultrafiltration using a PM 10 membrane and two times of Sephadex G-100 gel filtration chromatography. The enzyme was purified 151 fold with a 9% recovery. Purified enzyme gave a single protein band with a molecular weight of 105,000 dalton. The enzyme was reasonably stable. The enzyme activity was kept even after 1 hr incubation at 55°C, but decreased significantly at 60°C. The pH optimum was found to be from 6.5 to 7.5. Among tested compounds, the substrate activity was found with adenosine, adenine arabinofuranoside, formycin A, 2′-deoxyadenosine, 3′-deoxyadenosine, 2′,3′-isopropylidene adenosine, 2,6-diaminopurine deoxyriboside, β-nicotinamide adenine dinucleotide (reduced form), 6-chloropurine riboside, 2′-adenine monophosphate (AMP), 3′-AMP and 5′-AMP. The values of Km of adenosine and 2′-deoxyadenosine were calculated to be 500 and 710 μM, respectively. ADA was sensitive to Zn^{2+}, Cu^{2+} and Fe^{3+}. *p*-chloromercuribenzoate and mersalyl acid inactivated the enzyme. The activity of enzyme was not changed when ADA was incubated with dithiothreitol, 2-mercaptoethanol, N-ethylmaleimide, iodoacetic acid and iodoacetamide.

KEY WORDS □ **Adenosine deaminase, *Aspergillus oryzae*, intracellular enzyme, adenosine, inosine**

Adenosine deaminase (ADA) catalyzes the irreversible deamination of adenosine to produce inosine and ammonia. The enzyme plays a key role in the normal catabolic pathway for adenine nucleotides (4). The generation of hypoxanthine, when coupled to nucleoside hydrolase or phosphorylase, can be utilized for the purine salvage pathway. ADA is widely distributed in animal tissues. Purified enzymes were obtained from various sources such as calf intestinal mucosa, chicken duodenum, bovine placenta, chicken-egg yolk, human erythrocyte, marine mussel and scallop muscles (7, 13, 16, 17, 20, 22). However, there are a few studies on ADA in microorganisms. In contrast to ubiquity of the enzyme in animal tissues, ADA was detected only in several microorganisms. *Bacillus cereus* contained ADA to participate in the utilization of exogenous purine compound (23). The enzyme levels were subjected to genetic control with adenine derivatives as inducers. For the degradation of AMP, ADA was considered to play a role in *Azotobacter vinelandii* (24). In *Escherichia coli*, ADA was induced by adenine, hypoxanthine and their nucleosides and went through purification procedures (15). The extreme halophilic *Halobacterium cutirubrum* contained ADA activity which acted on only adenosine, but not on other bases and nucleotides (2). In addition, the microbial enzymes were purified from *Pseudomonas iodinum* (19), *Klebsiella* sp. (10), *Micrococcus sodonensis* (18), *Saccharomyces cerevisiae* (14), extracellular source of *Streptomyces* sp. (8) and Takadiastase from *Aspergillus oryzae* (25). However, nothing has been done for ADA in intracellular source of *A. oryzae*. In the present paper, we described the purification procedures for ADA from intracellular *A. oryzae*. The stability of the enzyme, the effects of various compounds and substrate specificities were determined for the preliminary characterization of intracellular ADA from *A. oryzae*.

MATERIALS AND METHODS

Cultivation of *Aspergillus oryzae*

The organism used in the preparation of this enzyme was *Aspergillus oryzae* KCTC 2114. The mold, *Aspergillus oryzae* was maintained on MGY slant (malt extract 10g; yeast extract 4g; glucose 4g; agar 15g per 1 liter) at 4°C and cultured in MGY broth for 3~4 days (200 ml medium in a 1 L of Erlenmeyer flask). After inoculation with

1~2 loops of spores, a culture was incubated at 37°C in a reciprocating shaker.

Enzyme Purification

To prepare fungal extracts, two liters of cultured broth was filtered through four layers of gauze and the mycelium was washed twice with distilled water. The mycelium was suspended in 10 mM KPO_4 buffer, pH 7.2 and disrupted for 10 min with a pulse in an ice-chilled bead beater (Biospec Products, USA) with 0.5 mm diameter of glass beads.

The homogenate was centrifuged at 10,000×g for 40 min in a refrigerated Centrikon, T-324 using an A 8.24 rotor (Kontron Instruments).

Ammonium sulfate was added to the supernatant fluid to make an 35% saturated solution and the suspension was stirred in an ice bucket for 30 min and then centrifuged as described above. The pellet had a very small amount of activity and was discarded. The supernatant fluid was brought to 80% saturation with more ammonium sulfate and stood for two hours at 4°C. The precipitate was collected after centrifugation and dissolved in a minimum volume of 10 mM KPO_4, pH 7.2. The solution was dialyzed overnight against the 4 L of same buffer with two changes.

The dialyzed enzyme was centrifuged at 10,000 ×g for 10 min to remove some insoluble material and then chromatographed on a DEAE-Sephadex A-50 column (3×22 cm). The column was equilibrated with 10 mM KPO_4, pH 7.2. After the sample loading, the column was washed with 350 ml of the same buffer until the absorbance at 280 nm decreased below the value of 0.1. A linear gradient from 0 to 1.2 M NaCl in 10 mM KPO_4, pH 7.2 was applied and fractions of 7 ml each were collected. The fractions containing enzyme activity were pooled and concentrated by ultrafiltration with a membrane filter, PM 10 (Amicon).

The concentrated enzyme solution was transferred to a Sephadex G-100 column (3×43 cm) equilibrated with 20 mM Tris-Cl, pH 7.2. Fractions of 1.5 ml each were collected and the active fractions were pooled and concentrated. The fractions were rechromatographed on a Sephadex G-100 (3×43 cm) equilibrated with the same buffer.

Measurement of Protein Concentration

Protein concentrations were measured by the method of Lowry *et al.* (12) with bovine serum albumin as a standard or the method in which the absorbances at 280 and 260 nm were multiplied by factors of 1.55 and 0.76, respectively and the latter was subtracted to obtain the protein concentration in mg per ml.

Adenosine Deaminase Assay

All enzyme assays were carried out at 30°C by the modifications of method of Agarwal *et al.* (1). Adenosine deaminase activity was determined by the measuring the rate of decrease in absorbance at 265 nm resulting from the conversion of adenosine to inosine. A temperature controlled spectrophotometer (Kontron, UVKON 860) equipped with RS 232C-interface was used. The reaction mixture contained 0.1 mM adenosine, 50 mM Tris-Cl, pH 7.2 and the enzyme in a final volume of 1 ml. All reaction mixture except ADA were preincubated for 3 min. The reaction was initiated by the addition of the enzyme, and continued for about 2 min to get the linear region. One unit of ADA is the amount of enzyme catalyzes the deamination of 1 μmole of adenosine per minute ($-\Delta E = 8.6\ min^{-1}ml^{-1}$) under the conditions of assay. Specific activity was expressed as units of enzyme per milligram of protein.

Polyacrylamide Gel Electrophoresis

Non-denaturing discontinuous polyacrylamide gel (10%) electrophoresis was conducted by the method of Davis (5). Molecular weight determinations were carried out according to the method of Laemmli (9) on 10% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. The following proteins were used as standards; myosine, 200,000; β-galactosidase, 116,250; phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 45,000. The gels were stained for proteins with Coomassie brilliant blue G-250.

High Pressure Liquid Chromatography (HPLC)

Analytical reversed phase HPLC was used to show the deamination of adenosine to inosine by the purified enzymes. HPLC was run by the modification of method of Bohacek and Hosek (3). Separation of adenine, adenosine and inosine was monitored by reversed phase HPLC, using a Gilson 305 HPLC equipped with a Waters μ Bondapak C_{18} analytical column (3.9 mm×15 cm). The isocratic elution of 10 mM potassium phosphate, pH 5.5 with 10% methanol was performed. The flow rate was 1 ml/min. Commercially available adenosine, inosine and adenine were used as standards and had retention times of 10 min, 4 min and 6 min, respectively. The incubation mixture contained final concentrations of 100 mM KPO_4, pH 7.0, 100 μM adenosine and the adequate amounts of purified adenosine deaminase. At the indicated time points, an aliquot of 0.5 ml was added to 0.1 ml of ice cold 60% perchloric acid, vortexed and put on ice for 15 min. After centrifugation for 2 min in a microfuge, supernatant was neutralized with 5 N KOH. Following a second centrifugation to remove potassium perchlorate, the supernatant was stored at −28°C prior to analysis.

RESULTS

Enzyme Purification

The overall purification procedures were summarized in Table 1. Adenosine deaminase (ADA) was purified approximately 151 fold with a 9%

Table 1. *Purification of adenosine deaminase from Aspergillus oryzae*

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Culture Homogenate	56.7	3610	0.016	100	1.0
35-80% $(NH_4)_2SO_4$ fractionation	34.2	799	0.043	60	2.7
Dialysis	31.5	531	0.059	56	3.7
DEAE-Sephadex A-50	21.5	104	0.210	38	13.1
Ultrafiltration (PM 10)	17.6	66	0.270	31	16.9
Sephadex G-100 (I)	9.1	7.3	1.250	16	78.1
Sephadex G-100 (II)	5.1	2.1	2.430	9	151.2

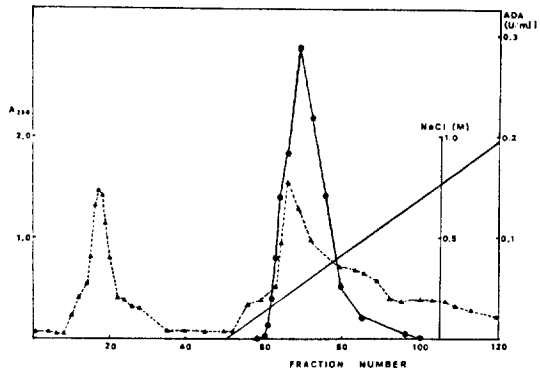

Fig. 1. *Elution profile of adenosine deaminase from anion exchange column, DEAE-Sephadex A-50.*
The dialyzed enzyme solution was washed and eluted as described in Materials and Methods.
A_{280} for protein concentration (---); Adenosine deaminase activity (—)

activity yield. The enzyme was bound on a DEAE-Sephadex A-50 column and no activity was found in the washing steps as shown in Fig. 1. The activity of ADA was found in the elution buffer containing 0.15 to 0.5 M NaCl with a single peak. However, the attempts to use a hydroxyapatite column and a CM-Sephadex C-50 column for the purification was unsuccessful, since ADA was failing to bind to both of absorbants. The active fractions after first Sephadex G-100 column chromatography (Fig. 2-A) were applied to a Sepharose 4B-200 column or second Sephadex G-100 column (Fig. 2-B). A Sepharose 4B-200 column did not improve the specific activity of the final product. The production of inosine from adenosine with the final purified enzyme was shown in the change of absorption spectrum from 220 to 300 nm (Fig. 3), by the analytical HPLC (Fig. 4) and in the coupled reaction with purine nucleoside phosphorylase and xanthine oxidase to produce uric acid. Purity of the enzyme was analyzed by nondenaturing PAGE (Fig. 5-A) and

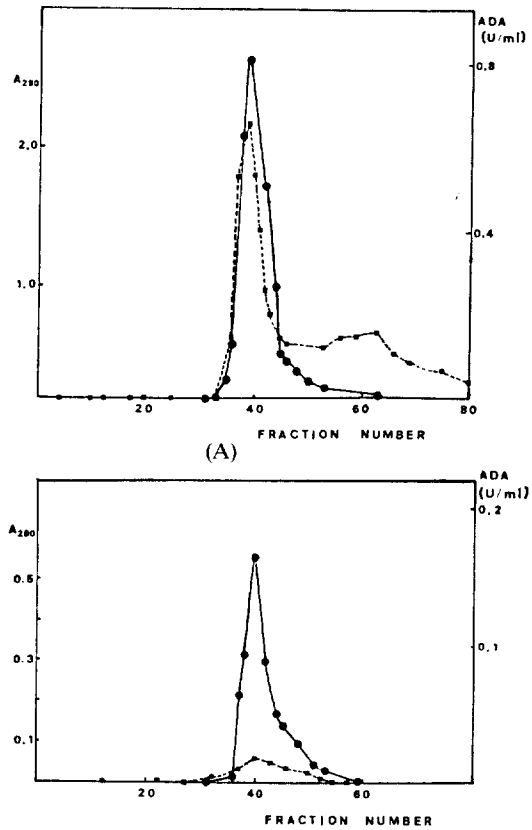

Fig. 2. *Elution profile of adenosine deaminase from gel filtration column, Sephadex G-100.*
(A) The pooled fractions from DEAE-Sephadex A-50 was concentrated by ultrafiltration using a PM-10 membrane. The concentrate was applied and the column was eluted as described in Materials and Methods.
(B) The portion of the fractions from the first gel filtration column was applied and the column was eluted as described in Materials and Methods.
A_{280} for protein concentration (---); Adenosine deaminase activity (—)

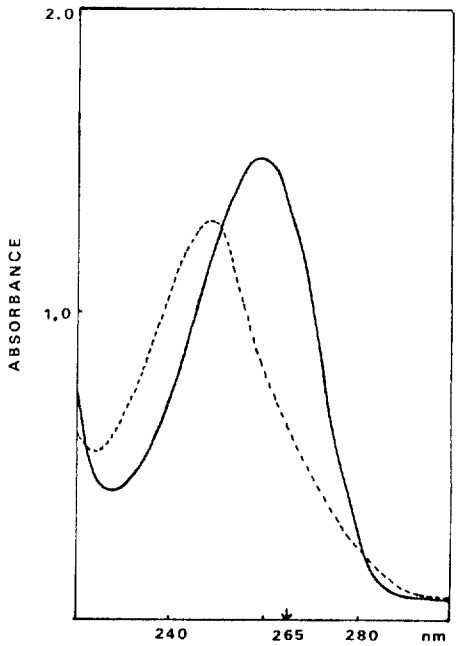

Fig. 3. *Absorption spectrum of the conversion of adenosine to inosine by adenosine deaminase (ADA).*
The reaction mixture contained 0.1 mM adenosine, 50 mM Tris-Cl, pH 7.2 and the appropriate amount of ADA in a final volume of 1 ml. Immediately after adding the enzyme solution, the absorption spectrum was taken from 220 to 300 nm as a 0 hr sample (—). The reaction mixture was incubated at 30°C and the spectrums at 1 hr and 4 hr (---) were overlapped.

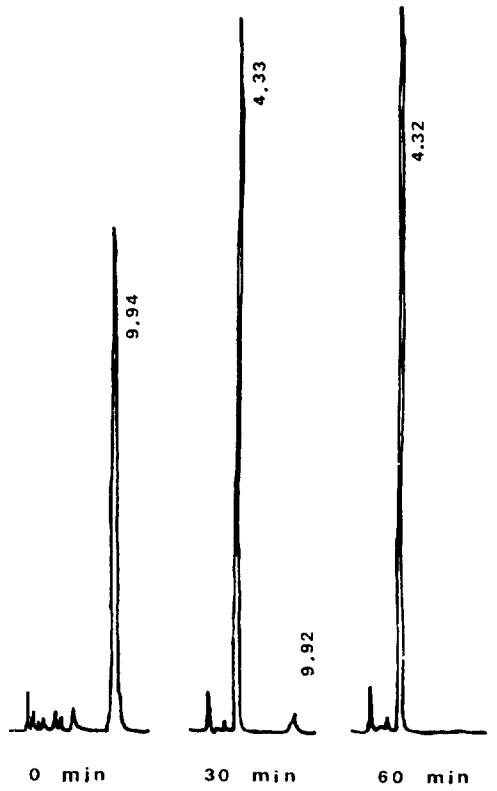

Fig. 4. *HPLC chromatogram of conversion of adenosine to inosine.*
The reaction mixture was prepared and analyzed as described in Materials and Methods. The peak of adenosine at 0 hr was converted to inosine completely at 1 hr. Commercially available adenosine and inosine were used as standards and had retention times of 10 min and 4 min, respectively.

SDS-PAGE (Fig. 5-B) and the active fraction after second Sephadex G-100 column chromatography gave a single protein band with more than 90% homogeneity. The molecular weight of the enzyme was determined to be 105,000 dalton on SDS-PAGE (Fig. 6).

Some Properties of the Enzyme

Stability: Purified ADA was reasonably stable. In the absence of any ligand, the enzyme was stable at 4°C for 7 days and at −20°C for several months.

Effect of pH: Enzyme activity was determined in the potassium phosphate buffers of pH 4.0 to 9.0. A typical bell shaped curve was obtained with optimal region of pH 6.5 to 7.5 (Fig. 7). After the enzyme was preincubated in 200 mM KPO_4 of pH 4.0 to 9.0 for 5 min and assayed under the standard conditions. No effect on activity was observed, indicating that ADA was not irreversibly inactivated at low or high pH.

Effect of Temperature: Effects of temperature on initial velocity were determined between 15 to 42.5°C. As temperature went up, ADA activity increased under the assayed conditions. As shown in Fig. 8, a plot of log (velocity) against 1/T gave a sharp change in slopes at 30°C. The values of activation energy for the reaction were calculated from the Arrhenius equation (21) and to be 6.7×10^3 cal/mol from 30 to 42.5°C and 13.4×10^3 cal/mol from 15 to 30°C (Fig. 8), indicating that the enzyme existed in two convertible forms. When ADA was incubated at different temperatures for 1 hr, the enzyme was stable up to 55°C, but 50% of the activity was lost at 60°C after 15 min as shown in Fig. 9.

Substrate Specificity: The substrate specificity of the enzyme was determined with a series of known or potential substrates. All substrates were used at 100 μM final concentration and the changes of absorption spectrum from 220 to 300

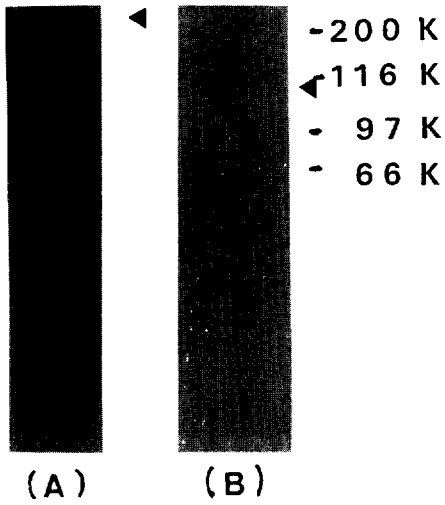

Fig. 5. (A) *Non-denaturing PAGE analysis of adenosine deaminase (ADA).* (B) *SDS-PAGE analysis of ADA.*
The active fraction after second Sephadex G-100 column chromatography was applied on 10% polyacrylamide gel. Gels were run as described in Materials and Methods. Molecular weight markers were run on a separate gel. The arrow indicates the position of ADA.

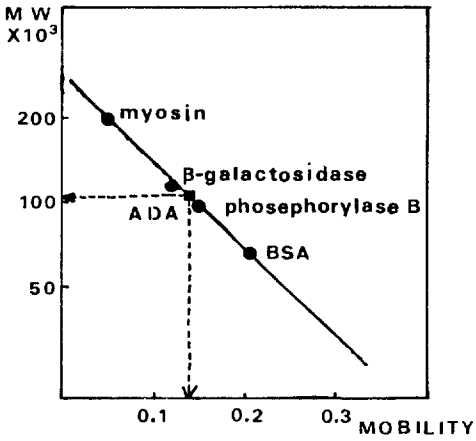

Fig. 6. *Estimation of molecular weight of adenosine deaminase by SDS-PAGE.*
The molecular standard markers consisted of myosine (200,000), β-galactosidase (116,250), phosphorylase B (97,400) and bovine serum albumin (66,200). The molecular weight of ADA was calculated to be 105,000 dalton.

nm by the deamination for each compound were measured. Among the tested compounds, those

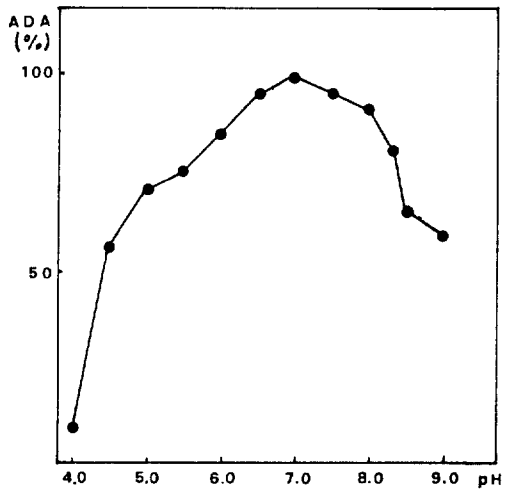

Fig. 7. *Effect of pH on adenosine deaminase activity.*
The reaction mixture contained 0.1 mM adenosine and an appropriate pH of 50 mM potassium phosphate buffer. After 3 min preincubation at 30°C, the enzyme was added into the reaction mixture.

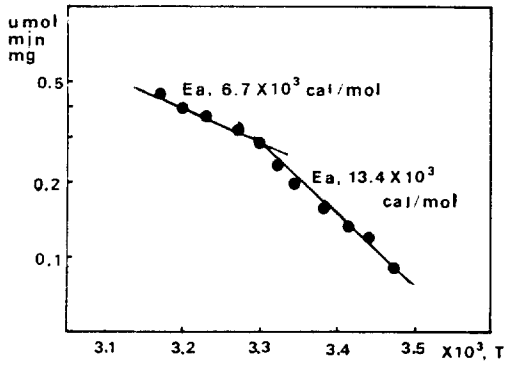

Fig. 8. *Effect of temperature on the initial velocity of adenosine deaminase.*
The reaction mixture as described in Materials and Methods was preincubated for 3 min. The reaction was started by adding the enzyme. Each of the mixture was incubated in the temperature controlled cell of the spectrophotometer. Activation energy, Ea was calculated to be 6.7×10^3 cal/mol and 13.4×10^3 cal/mol from 30~42.5°C and 15~30°C, respectively.

acted as substrates were adenosine, adenine arabinofuranoside, formycin A, 2'-adenosine monophosphate (AMP), 3'-AMP, 5'-AMP, β-nicotinamide adenine dinucleotide (reduced form), 2'-deoxyadenosine, 3'-deoxyadenosine, 2,6-diaminopurine deoxyriboside, 6-chloropurine riboside,

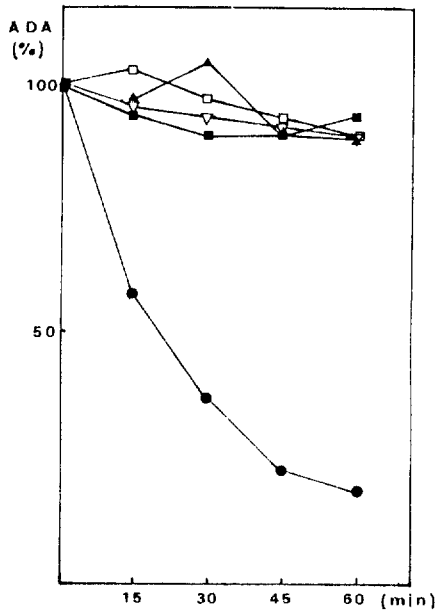

Fig. 9. *Effect of temperature on the stability of adenosine deaminase.*
Each of the appropriate amount of the enzyme solution was incubated at 37°, 42°, 50°, 55° and 60°C. The aliquot was taken at the indicatied time points and the activity of ADA was measured under the standard assay conditions.
37°C (□—□); 42°C (▽—▽); 50°C (▲—▲); 55°C (■—■); 60°C (●—●)

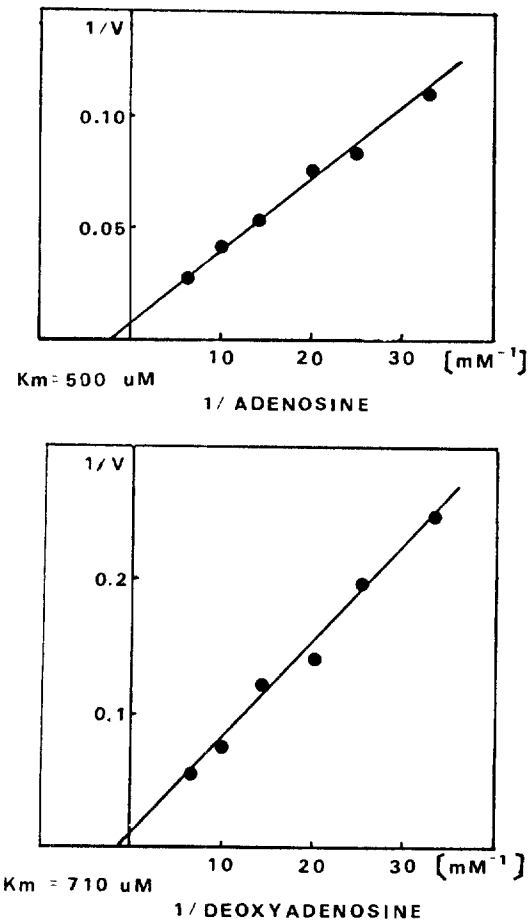

Fig. 10. *Lineweaver-Burk plot of deamination of adenosine by adenosine deaminase.*
The reaction was carried out as described in Materials and Methods. The reaction mixture contained 50 mM Tris-Cl, pH 7.2 and adenosine or 2′-deoxyadenosine ranging from 30 to 150 μM as a substrate. The values of Km were calculated to be 500 μM and 710 μM for adenosine and 2′-deoxyadenosine, respectively.

and 2′,3′-isopropylidene adenosine. The effect of various concentrations of adenosine and 2′-deoxyadenosine on the initial velocity of deamination was shown in Fig. 10. The values of Km for adenosine and 2′-deoxyadenosine were calculated to be 5.0×10^{-4} M and 7.1×10^{-4} M, respectively. Apart from the substrates shown, a number of compounds were inactive as substrates even after 5 hrs incubation at 30°C. These were adenine, cytosine, cytidine, guanosine, N^6-methyladenosine, N^6, N^6-dimethyladenosine, purine riboside and 6-methylpurine riboside.

Effects of Metal Ions and Various Chemicals: Effects of various compounds on the activity of the purified enzyme were investigated (Table 2). The enzyme was significantly inhibited by Zn^{2+}, Cu^{2+} and Fe^{3+} and Mn^{2+} and sodium dodecyl sulfate decreased the enzyme activity, but no stimulation of the activity was observed with any tested metal ions. Among the sulfhydryl group reactive reagents, *p*-chloromecuribenzoate and mersalyl acid were found to be potent inhibitors of ADA, but N-ethylmaleimide showed no effect on enzyme and $HgCl_2$ decreased the activity of enzyme slightly. Alkylating agents, iodoacetic acid and iodoacetamide did not show any effect on ADA. The thiol reagents, glutathione, cystein, dithiothreitol (DTT) and 2-mercaptoethanol did not change activity of the enzyme at all.

DISCUSSION

Adenosine deaminase (ADA) has been isolated from a variety of animals and microorganisms. Microbial intracellular ADA has been reported in *Escherichia coli* (15), *Pseudomonas iodinum* (19), *Klebsiella* sp. (10), *Bacillus cereus* (6), *Micrococcus sodonensis* (18) and *Saccharomyces cerevisiae* (14).

Table 2. *Effect of various chemicals on activity of adenosine deaminase from Aspergillus oryzae.*

Chemicals	Conc. (mM)	Relative Activity (%)
None	–	100
Dithiothreitol	1.0	91
2-Mercaptoethanol	1.0	95
Glutathione	1.0	93
L-Cysteine	1.0	95
EDTA	1.0	95
Sodium dodecyl sulfate	1.0	63
5,5′-Dithionitrobenzoate	1.0	–
	0.2	82
	0.1	92
Mersalyl acid	1.0	5
	0.1	41
p-Chloromercuribenzoate	1.0	5
	0.1	82
N-Ethylmaleimide	1.0	91
$HgCl_2$	1.0	84
$CaCl_2$	1.0	91
$MgCl_2$	1.0	91
$CoCl_2$	1.0	80
$MnCl_2$	1.0	68
$ZnCl_2$	1.0	0
$FeCl_3$	1.0	0
$CuSO_4$	1.0	0
$AlK(SO_4)_2$	1.0	86
NaCl	1.0	86
KCl	1.0	91
NH_4Cl	1.0	91

The studies of ADA in *Streptomyces* sp. (8) and *Aspergillus oryzae* (25) were from extracellular sources. However, nothing has been done for intracellular and extracellular ADA from same species. Microbial intracellular ADA was relatively unstable and stabilized by the presence of ethanol and ethylene glycol for *Pseudomonas* and *Escherichia* ADA, respectively. Maximal stabilization of ADA from *Bacillus cereus* occurred with monovalent cations such as NH_4^+ or K^+ (6). Extracellular ADA from *A. oryzae* did not need any cofactors or metal ions (25). Intracellular ADA from *A. oryzae* was relatively stable and did not change the activity with or without various monovalent cations. Incubation of the enzyme in the presence of the thiol reagents, DTT or 2-mercaptoethanol did not stimulate or inhibit the activity of ADA significantly. However, ADA from scallop muscle, *Patinopecten yessoensis* was rapidly inactivated when stored without DTT (20). Comparing with other enzymes, intracellular ADA from *A. oryzae* was quite stable and temperatures greater than 60°C were required for total inactivation. The values for the activation energy for the enzymatic hydrolysis of adenosine were 6.7 and 13.4×10^3 cal/mol at 30~42.5°C and 15~30°C, respectively. It suggested the transition of two interconvertible forms of the enzyme. Published values for the Arrehnius constant were in the ranges of $4\sim9\times10^3$ cal/mol, except for the chicken intestinal ADA (7) and the soluble form from *Micrococcus sodonensis* (18) which reported to be 17×10^3 and 13×10^3 cal/mol, respectively. No value for the extracellular enzyme from *A. oryzae* has been reported.

The molecular weight of intracellular ADA by SDS-PAGE was 105,000 dalton similar in size to the extracellular ADA, 110,000 by gel filtration (25), to the extracellular *Streptomyces* ADA, 90,000 by gel filtration (8) and to the intracellular and membrane bound enzyme from *Micrococcus sodonensis*, 130,000 by gel filtration (18). Certainly, the enzyme appeared larger than the calf intestinal ADA, 52,000 (25), the intracellular enzyme from *Klebsiella*, 26,000 (10), from *Bacillus cereus*, 53,700 (6), from *Azotobacter vinelandii*, 66,000 (24) and from *Escherichia coli*, 29,000 (15).

In addition to the affinity of substrate, substrate activities were quite different depending on the origin of the enzyme. Generally, bacterial enzymes were inactive to adenine and adenine nucleotides, but the extracellular enzyme from *A. oryzae* was active to adenine and various adenine nucleotides (25). However, intracellular ADA from *A. oryzae* did not use adenine as a substrate, but several adenine mononucleotides such as 2′-AMP, 3′-AMP and 5′-AMP acted as substrates. Extracellular ADA from *Streptomyces* was active to 3′-AMP and 5′-AMP, but not to 2′-AMP and adenine (8). Adenine acted as a substrate for human erythrocytic, calf intestinal, scallop muscle and marine mussel ADA. The values of Km of adenosine were in the range of 20~80 μM for ADA from animal tissues and several microorganisms. However, ADA from extracellular *Streptomyces*, extracellular *Aspergillus* and intracellular *Aspergillus* showed one order higher Km values for adenosine, 670 μM (8), 250 μM (25) and 500 μM, respectively. ADA from animal tissues generated inosine from 6-chloropurine riboside, but no microbial enzyme showed substrate activity with 6-chloropurine riboside except intra- and extracellular origin of *Aspergillus*.

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(Received November 12, 1992)
(Accepted December 2, 1992)

초 록: *Aspergillus oryzae*에서 Adenosine Deaminase의 정제와 특성
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*Aspergillus oryzae*의 세포내 효소인 adenosine deaminase(ADA)를 ammonium sulfate fractionation, DEAE-Sephadex A-50 음이온 교환 크로마토그래피, PM 10을 이용한 ultrafiltration과 2차례의 Sephadex G-100 겔여과 크로마토그래피를 거쳐 정제했다. 효소는 151배로 정제되었고 회수율은 약 9%였다. 정제된 효소는 전기영동법에 의해 1개의 띠로 나타났고 SDS를 첨가한 전기영동법에 의해 분자량이 105,000달톤으로 계산되었다. 효소는 대체적으로 안정하고 최적 pH는 6.5에서 7.5정도 되며 열처리시 55°C에서 1시간 배양에도 크게 활성이 저하되지 않았지만 60°C에서부터 활성이 급격히 저하되었다. 조사된 화합물중 adenosine, adenine arabinofuranoside, formycin A, 2′-deoxyadenosine, 3′-deoxyadenosine, 2′,3′-isopropylidene adenosine, 2,6-diaminopurine deoxyriboside, 6-chloropurine riboside, β-nicotinamide adenine dinucleotide의 환원형, 2′-adenine monophosphate (AMP), 3′-AMP와 5′-AMP가 기질로서 작용했고 그중 adenosine과 2′-deoxyadenosine은 Km 값이 각각 500 μM과 710 μM로 측정되었다. ADA는 Zn^{2+}, Cu^{2+}, Fe^{3+}에 의해 활성이 심각하게 저해되고 *p*-chloromercuribenzoate와 mersalyl acid도 효소 저해제로 작용했다. 그러나, dithiothreitol, 2-mercaptoethanol, N-ethylmaleimide, iodoacetic acid와 iodoacetamide는 별 영향을 미치지 않았다.