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Enzymatic Properties of Intracellular Adenosine Deaminase from *Nocardioides* sp. J-326TK

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Abstract The properties of purified intracellular adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) of *Nocardioides* sp. J-326TK isolated from soil have been studied. The enzyme deaminated adenosine and 2′-deoxyadenosine and the respective K_m values were 4.0×10^{-4} M and 5.0×10^{-4} M, but the enzyme was not active on 8-bromoadenosine, 6-methylaminopurine riboside, ATP, ADP, 2′-AMP, 3′-AMP, 5′-AMP, dAMP, cAMP, NAD, FAD, NADP and adenine. The enzyme activity was strongly inhibited by the addition of Hg^{2+} and Ag^{+}. Cu^{2+}, Co^{2+} and Mn^{2+} also inhibited the activity but much less extent. The effect of alkyl reagents, metal chelating reagents and certain other compounds on the enzyme activity were also examined. No reagent activated the enzyme. On the contrary, the enzyme reaction was slightly inhibited by *o*-phenanthroline and 6-benzyladenosine.

Key words: Adenosine deaminase, *Nocardioides* sp. J-326TK, enzymatic properties.

Introduction

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) has a wide distribution in nature and catalyzes the irreversible conversion of various adenine nuclesides to their respective inosine analogue [32]. Hereditary lack of adenosine deaminase activity is associated with severe impairment of cellular and humoral immunity (severe combined immunodificiency disease, SCID) [8,19,29,34]. A partial genetic deficiency of adenosine deaminase with lower activity in peripheral lymphocytes may be due to mutations in the enzyme. These mutations have been associated with an unstable adenosine deaminase [9] or an electrophoretically altered protein [5].

Homogeneous preparation of adenosine deaminase have been obtained from chicken duodena [10], mucosa of calf duodena [2], calf serum [4], calf spleen [27], and takadiastase [35]. In addition to studies on extensively purified ox heart [30], partially purified preparations from brain [25], bovine thyroid [6], Yoshida ascites cells [3], and cat lung [24] have been made. Adenosine deaminase have been identified and characterized from several microorganisms as well as a variety of biological sources. Adenosine deaminase from *Escherichia coli* [15], *Mycobacterium tuberculosis* [16], *Bacillus cereus* [7], *Halobacterium cutirubrum* [1], *Micrococcus sodonensis* [28], *Pseudomonas iodinum* [31], *Azotobacter vinelandii* [33], *Streptomyces* sp. J-845S [13], *Klebisella* sp. LF 1202 [17] and *Nocardioides* sp. J-326TK [11,12,14] have been studied. Recently, the use of adenosine deaminase in chemotheraphy for immunodeficiency diseases has motivated us to search for a stable form of this enzyme. Previously we reported the purification and partial characteristics of intracellular adenosine deaminase prepared from *Nocardioides* sp. J-326TK [12]. In the present study, the enzymatic properties of the purified intracellular adenosine deaminase from *Nocardioides* sp. J-326TK are reported.

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Materials and Methods

Chemicals

Nucleotide-related compounds were obtained from Sigma Chemicals (St. Louis). Other chemicals were purchased from Wako Pure Chemiclas Industries (Osaka). All compounds were certified reagent grade.

Organism and growth of cells

Nocardioides sp. J-326TK, isolated from soil [11,12], was used as the enzyme producer. The culture medium consisted of 0.5% glycerol, 0.5% yeast extract, 0.05% $MnCl_2$ and 0.001% $FeSO_4$ (pH 7.0). Three milliliters of seed culture incubated at 30℃ for 30 hours were then transferred into 100 ml of the medium in a 500-ml shaking flask and incubated at 30℃ for 30 hours on a vigorous shaker.

Preparation and assay of adenosine deaminase

Adenosine deaminase from *Nocardioides* sp. J-326TK was

purified as described in the previous paper [12]. A spectrophotometric assay based on the differential absorption of adenosine and inosine was employed. For the determination of adenosine deaminase activity, the 1 ml assay mixture containing 5 μmol of adenosine and 3-5 units of enzyme solution was used in 50 μmol of potassium phosphate buffer (pH 7.0). After incubation at 37℃ for 30 min, the reaction was stopped by heating for 4 min in a boiling-water bath. The inosine formed was assayed by measurement of the absorbance at 265 nm. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μmol of inosine per milliliter in 60 min under these conditions.

The enzyme kinetics and calculation of the activiation energy were determined by measuring the initial velocity. The standard assay (3 ml) was performed with 100 mM potassium phosphate buffer (pH 7.0) and 100 mM substrate at 37℃. The reaction was started by the addition of enzyme solution, and the enzyme activity was found from the initial velocity in change of absorbance at 265 nm.

Protein determination

Protein concentration was measured by the method of Lowry *et al.* [18] with bovine serum albumin as the standard protein.

Results and Discussion

Activation energy

The activation energy was calculated from the influence of temperature on adenosine deaminase activity (Fig. 1). The Arrhenius plots obtained were biphasic, with a change in slope between 40 and 45℃. From the slopes, the apparent activation energies were found to be 0.90 Kcal/mol between 20 and 30 ℃, and 0.411 Kcal/mol between 30 and 40℃. Our values were lower than those of the extracellular adenosine deaminase from *Nocardioides* sp. J-326TK, but higher than those from *Streptomyces* species. The activation energies of extracellular adenosine deaminase from *Nocardioides* sp. J-326TK were 4.1 Kcal/mol between 20 and 35 ℃, 1.20 Kcal/mol between 35 and 40℃ and 0.14 Kcal/mol between 40 and 50℃ [14]. Those from *Streptomyces* were 0.6 Kcal/mol between 20 and 50℃ and 0.34 Kcal/mol between 50 and 55℃ [13].

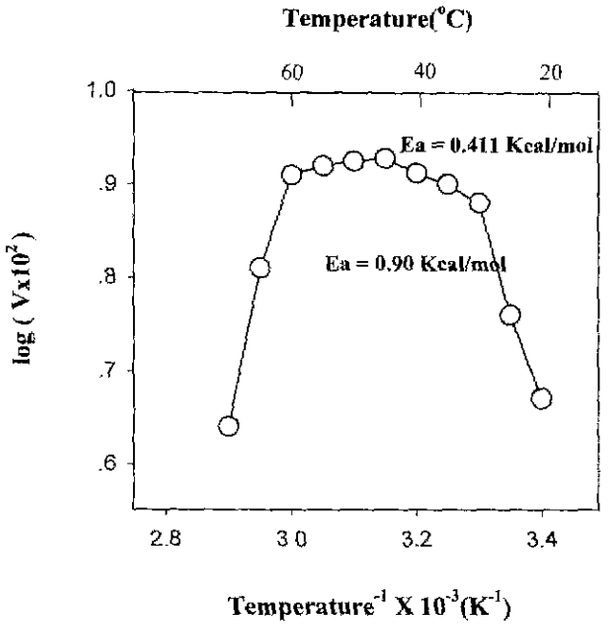

Fig. 1. Effect of temperature on the activity of intracellular adenosine deaminase from *Nocardioides* sp. J-326TK.

Substrate specificity

Various purine compounds were tested as substrates for enzyme. Compounds serving as substrates are listed in Table 1 with their K_m values and the relative activity for each substrate. Substrate specificities of microbial adenosine deaminases are different depending on the origin of the enzymes. It is well documented that bacterial intracellular adenosine deaminases have relatively narrow ranges of substrate specificity and that they are inactive toward adenine and adenine nucleotides [17,26,28,31]. In contrast, it is known that the extracellular enzymes from *A. oryzae* [20,21,23], *Streptomyces* sp. [13] and *Nocardioides* sp. J-326TK [14] have much broader ranges of substrate specificity than intracellular enzymes. In this respect it was apparent that our enzyme is a relatively high-specific.

The extracellular enzyme from *A. oryzae* is more active toward adenosine-2′-monophosphate (2′-AMP), adenosine-3′-monophosphate (3′-AMP), and cAMP than toward adenosine, and less active toward 2′-deoxyadenosine [23]. However, our adenosine deaminase was active toward adenosine, 2′-deoxyadenosine, 2′,3′-isopropylidene adenosine and 6-(γ,γ′-dimethylallylamino)purine riboside, as shown Table 1. Similar results were obtained with the extracellular adenosine deaminase from *Nocardioides* sp. J-326TK [14].

The effect of adenosine and 2′-deoxyadenosine concentration on adenosine deaminase activity was tested. The Michaelis constants of the enzyme for adenosine and 2′-deoxyadenosine were determinated from Lineweaver-Burk plots. As shown in Fig. 2, linear lines were obtained in

Table 1. Substrate specificity of intracellular adenosine deaminase from *Nocardioides* sp. J-326TK

Substrates	K_m (mM)	Relative activity (%)
Adenosine	0.40	100.0
2′-Deoxyadenosine	0.50	50.7
2′,3′-Isopropylidene adenosine	-	4.2
6-(γ,γ′-Dimethylallylamino)-purine riboside	-	3.3

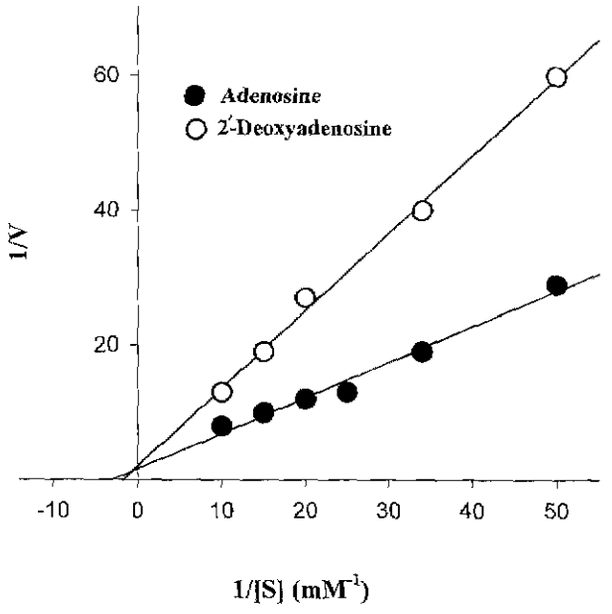

Fig. 2. Effect of substrate concentration on the intracellular adenosine deaminase from *Nocardioides* sp. J-326TK. Enzymatic activity was assayed by a standard assay method.

double reciprocal plots. The K_m values for adenosine and 2′-deoxyadenosine were calculated to be 4.0×10^{-4} M and 5.0×10^{-4} M, respectively, while those of the extracellular enzyme from *Nocardioides* sp. J-326TK were 2.2×10^{-2} M for adenosine and 2.0×10^{-4} M for 2′-deoxyadenosine [14].

Apart from the compounds listed there, a number of other compounds were tested as substrates. However, the enzyme did not affect 8-bromoadenosine, ATP, ADP, 2′-AMP, 3′-AMP, 5′-AMP, NAD, NADP, 6-benzyladenosine, FAD, dAMP, cAMP, adenine and 6-methylaminopurine riboside as substrates.

Effect of metal ions

The effects of various metal ions on adenosine deaminase activity were examined (Table 2). In order to assay the effect of metal ions on enzyme activity, each metal ion was added to the reaction mixture at final concentrations of 0.1 and 1.0 mM. The enzyme was activated with Fe^{3+}. However, Co^{2+}, Mn^{2+} and Cu^{2+} slightly inhibited the enzyme. In the presence of 0.1 mM Hg^{2+} and Ag^{+}, the enzyme was completely inactivated. Therefore, these metal ions had an accelerating effect on the inactivation of the enzyme from strain J-326TK just as observed in the extracellular adenosine deaminase from *Streptomyces* sp. [13] and *Nocardioides* sp. J-326TK [14]. Besides these metal ions, Ca^{2+}, K^{+}, Fe^{3+}, Cd^{2+}, Mg^{2+}, Ba^{2+} and Sn^{2+} had no effect on the enzyme activity.

Effect of various chemicals

The effects of alkyl reagents, metalchelating reagents, and certain other compounds on the enzyme activity were examined. No reagent activated the enzyme. The enzyme reaction was inhibited by *o*-phenanthroline (Table 3). Likewise, the extracellular enzymes of *Streptomyces* sp. [13] and *Nocardioides* sp. J-326TK [14] were inhibited by *o*-phenanthroline. The adenosine deaminase from *A. oryzae* [23] and *Pseudomonas iodinum* [31] is reported to be inhibited by *p*-chloromercuribenzoaete, whereas our enzyme was not affected by that. *o*-Phenanthroline forms a complex with ferrous and ferric ions and inhibits the reaction of enzymes containing these ions. That the enzyme reaction is promoted by $FeCl_3$ and inhibited with *o*-phenanthroline

Table 2. Effect of metal ions on the activity of intracellular adenosine deaminase from *Nocardioides* sp. J-326TK

Metal ions	Relative activity (%)		
	1.0 mM	0.1 mM	0.01 mM
$CaCl_2$	99	94	
NaCl	89	93	
KCl	91	92	
$FeCl_2$	108	39	
$FeCl_3$	109	98	
$CuCl_2$	47	77	
$CdCl_2$	91	91	
$ZnCl_2$	75	91	
$NiCl_2$	86	86	
$CoCl_2$	47	95	
$MgCl_2$	91	96	
$MnCl_2$	58	94	
$HgCl_2$	0	0	51
$BaCl_2$	102	85	
$SnCl_2$	95	96	
$MgSO_4$	97	87	
$AgNO_3$	0	0	12
$SrCl_2$	92	93	

Table 3. Effect of various chemicals on the activity of intracellular adenosine deaminase from *Nocardioides* sp. J-326TK

Reagents	Relative activity (%)	
	1.0 mM	0.1 mM
2-Mercaptoethanol	105	100
Ethylenediaminetetraacetate	96	96
o-Phenanthroline	77	85
α,α'-Dipyridyl	107	91
Trichloroacetate	100	98
p-Chloromercuribenzoate	-	101
NaF	86	91
NaN_3	96	91
Na_2HAsO_4	92	93
NaCN	87	93
Pantachlorophenol	87	85
N-Ethylmaleimide	87	88
L-Cystein	96	94
Dithiothreitol	91	93
Monoiodoacetate	102	108

suggests participation of the ferrous (or ferric) ion in the adenosine deaminase reaction.

Effect of nucleoside-related compounds on the enzyme activity

The effects of various nucloside-related compounds on the enzyme activity were examined by their addition to the reaction mixture to a final concentration of 0.1 mM. Adenine, caffein, 8-bromoadenosine, 2-amino-6-chloropurine riboside, adenosine-N'-oxide, 6-(γ-γ'-dimethylallyamino)purine riboside, guanosine, 6-benzyladenosine, 6-mercaptopurine riboside, 6-methylaminopurine riboside, 6-chloropurine, theobromine, theophylline, 8-azaadenine, 2',3'-isopropylidene adenosine and inosine were tested. The enzyme reaction was inhibited in the presence of 6-benzyladenosine by about 15%, but none of them besides 6-benzyladenosine inhibited the enzyme reaction (Table 4).

In the reaction of the extracellular adenosine deaminase from *A. oryzae*, the 6-amino group of the adenine molecule is involved in the bonds with the enzyme molecule, and the nitrogen atom at the 7-position is also important in the binding [22]. 8-Bromoadenosine, adenosine-N'-oxide and 8-azaadenine have the same structure as adenine at the 6 and 7 positions. The extracellular enzyme from *Streptomyces* sp. [13] is reported not to be affected by the presence of the nucleoside-related compounds tested here. Moreover, the adenosine deaminases from *A. oryzae, Ps. iodinum* and *Nocardioides* sp. J-326TK were inhibited by 8-bromoadenosine [14,23,31]. As compared with these, our enzyme was not inhibited by 8-bromoadenosine. Besides this, adenosine-N'-oxide, which inhibits the *A. oryzae* enzyme, did not affect our enzyme. These findings lead us to believe that the structure of the active site of our enzyme differs from that of the adenosine deaminases from the *A. oryzae*, *Ps. iodinum* and *Nocardioides* sp. J-326TK.

Table 4. Inhibition of adenosine deaminase activity by various nucleoside related compounds

Compounds (0.1mM)	Relative activity (%)
Adenine	92
Caffein	92
8-Bromoadenosine	100
2-Amino-6-chloropurine riboside	97
Adenosine-N'-oxide	97
6-(γ-γ'-Dimethylallyamino)purine riboside	97
Guanosine	98
6-Benzyladenosine	84
6-Mercaptopurine riboside	96
6-Methylaminopurine riboside	92
6-Chloropurine	98
Theobromine	94
Theophylline	96
8-Azaadenine	90
2',3'-Isopropylidene adenosine	90
Inosine	100

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