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Purification and Properties of Intracellular Cytosine Deaminase from *Chromobacterium violaceum* YK 391

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Abstract Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) stoichiometrically catalyzes the hydrolytic deamination of cytosine and 5-fluorocytosine to uracil and 5-fluorouracil, respectively. The intracellular cytosine deaminase from *Chromobacterium violaceum* YK 391 was purified to apparent homogeneity with 272.9-fold purification with an overall yield of 13.8%. The enzyme consisted of dimeric polypeptides of 63 kDa, and the total molecular mass was calculated to be approximately 126 kDa. Besides cytosine, the enzyme deaminated 5-fluorocytosine, cytidine, 6-azacytosine, and 5-methylcytosine, but not 5-azacytosine. Optimum pH and temperature for the enzyme reaction were 7.5 and 30°C, respectively. The enzyme was stable at pH 6.0 to 8.0, and at 30°C for a week. About 70% of the enzyme activity was retained at 60°C for 5 min. The apparent K_m values for cytosine, 5-fluorocytosine, and 5-methylcytosine were calculated to be 0.38 mM, 0.87 mM, and 2.32 mM, respectively. The enzyme activity was strongly inhibited by 1 mM Hg^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+}, and by *o*-phenanthroline, α,α'-dipyridyl, ρ-chloromercuribenzoate, *N*-bromosuccinimide, and chloramine-T. In addition, the enzyme activity was strongly inhibited by 1 mM 2-thiouracil, and weakly inhibited by 2-thiocytosine, or 5-azacytosine. Finally, intracellular and extracellular cytosine deaminases from *Chromobacterium violaceum* YK 391 were found to have a different optimum temperature, apparent K_m value, and molecular mass.

Key words: Cytosine deaminase, *Chromobacterium violaceum* YK 391, 5-fluorocytosine

Nucleobases such as uracil, thymine, adenine, and guanine are converted into nucleotides *via* Salvage synthesis, or are directly degraded by biooxidation into barbituric acid or uric acid.

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Unlike other nucleobases such as uracil, thymine, adenine, and guanine, cytosine is not only degraded directly, but also does not act as a substrate for Salvage synthesis. Cytosine is hydrolyzed to uracil by cytosine deaminase, and therefore, is involved in the recycling of pyrimidine bases to the nucleotide pool or is completely degraded. Because of these reasons, cytosine deaminase seems to be a unique enzyme not only in the catabolism of pyrimidine nucleotides, but also in the Salvage synthesis of pyrimidine nucleotides. Cytosine aminohydrolase (cytosine deaminase; EC 3.5.4.1) stoichiometrically catalyzes the hydrolytic deamination of cytosine, 5-fluorocytosine, and 5-methylcytosine to uracil, 5-fluorouracil, and thymine, respectively. 5-Fluorocytosine (5-FC) has no antitumoral or bacteriostatic activity and little clinical toxicity [4]. On the other hand, 5-fluorouracil (5-FU) is toxic, and has antitumoral activity [20] and a strong broad-range antimicrobial spectrum [6]. 5-FC, after its conversion to 5-FU by cytosine deaminase, has antineoplastic activity and acts as a selective fungicide [5, 24]. This antifungal activity of 5-FC has been attributed to the participation of the cytosine deaminase in the fungi themselves in which the enzyme deaminates 5-FC to 5-FU. Cytosine deaminase activity has not been found in mammalian and plant cells, therefore 5-FC is not metabolized [14]. However, a small amount of 5-FU has been detected in the blood, and it has been proposed that deamination of 5-FC might be catalyzed by cytosine deaminase of intestinal microflora [15, 16].

Cytosine deaminase was first identified in 1923 [7], and has been studied since in yeast [8, 9, 17], some bacteria [21, 22, 26], and mold [30]. Subsequently, cytosine deaminases from *Serratia marcescens* [21] and *Pseudomonas aureofaciens* [22] were first purified to homogeneity in 1975, and then from *Escherichia coli* [10], *Salmonella typhimurium* [26], and *Aspergillus fumigatus* [30]. Furthermore, extracellular cytosine deaminase was

purified from *Chromobacterium violaceum* YK 391 [28]. *C. violaceum* YK 391 produced not only extracellular enzyme, but also intracellular cytosine deaminase [13]. In order to distinguish the extracellular and intracellular enzymes of *C. violaceum* YK 391, some properties of the extracellular enzyme have previously been studied [28]. The present study dealt with the enzymatic properties of intracellular cytosine deaminase purified from *C. violaceum* YK 391. Our ultimate goals of the present research were i) to analyze the genes of the extracellular and intracellular enzymes, and ii) to increase the production of an enzyme, between these two enzymes, with excellent enzymatic properties.

MATERIALS AND METHODS

Materials

A standard kit of low molecular weight markers for electrophoresis was purchased from Bio-Rad Co., U.S.A. DEAE-Cephacel, Ultrogel A6, and Sephadex G-100 were purchased from Sigma Co., U.S.A., and peptone, meat extract, and yeast extract from Difco (Lab. Inc., U.S.A.).

Microorganism and Growth Conditions

C. violaceum YK 391 [13] was grown at 28°C for 3 days in a 5-l mini jar fermentor (SY-500, Korean Fermentor Co., LTD., Korea) containing 4 l of the medium (pH 7.0), which consisted of 0.75% soluble starch, 1.5% peptone, 0.1% meat extract, 0.1% yeast extract, 0.05% K_2HPO_4, 0.01% NaCl, 0.01% $MgCl_2 \cdot 7H_2O$, and 0.1% (v/v) silicone KM-70 (Shin-etsu Chemical Industry Co., Tokyo) as an antifoamer under aeration. The cells were harvested at the early stationary phase of growth by centrifugation at 13,000 ×*g* for 20 min, washed twice with saline, and stored at −20°C until use.

Preparation of Cell-Free Extract

Washed cells were suspended in 0.2 M Tris-HCl buffer (pH 7.5), and the suspension was subjected to 120 kHz ultrasonic oscillator (Sonics and Materials Inc. U.S.A.) at below 5°C. Cells and debris were removed by centrifugation at 15,000 ×*g* for 30 min. The resultant was referred to as cell-free extract and used as the crude enzyme preparation.

Enzyme Assay

Cytosine deaminase activity was assayed spectrophotometrically, as described elsewhere [13, 31]. A mixture of the enzyme and 1 mM cytosine in 1.0 ml of 0.2 M Tris-HCl buffer (pH 7.5) was incubated at 37°C for 30 min. The reaction was then terminated by the addition of 4 ml of 0.1 N HCl. If a precipitate formed, it was removed by centrifugation. One unit of cytosine deaminase activity was defined as the amount of enzyme decomposing 1 μmol of cytosine per hour, and the specific activity was defined as the number of units of enzyme activity per mg of protein.

Protein Assay

Protein was determined by the method of Bradford [2], using a protein assay kit (Bio-Rad Co., U.S.A.), with bovine serum albumin as a standard protein, or by measuring absorbance at 280 nm.

Molecular Weight

The molecular mass of the enzyme was estimated by gel filtration, as described by Andrews [1]. Gel filtration was performed in a Sephadex G-100 column (1.8×81 cm), equilibrated previously with 0.2 M Tris-HCl buffer (pH 7.5). Carbonic anhydrase (MW 29 kDa), bovine serum albumin (MW 66 kDa), alcohol dehydrogenase (MW 150 kDa), and β-amylase (MW 200 kDa) were used as standard proteins (Sigma Co. U.S.A.). The void volume (V_o) was determined by the elution of blue dextran. Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was also employed for determination of the molecular mass of the subunits of the enzyme, as described by Weber and Osborn [25]. A kit (Pharmacia Biotech Co., U.S.A.) of standard proteins of low molecular weight was used: myosin (MW 212 kDa), α_2-macroglobulin (MW 170 kDa), β-galactosidase (MW 116 kDa), transferrin (MW 76 kDa), and glutamate dehydrogenase (MW 53 kDa).

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed, using the modified method of Davis [3]. Stacking and running gels were polymerized in a test tube (0.5×10 cm). After running with a constant current of 8 mA per gel, the gel was stained with 1% Amido black 10 B (E. Merck, Darmstadt, Germany), electrophoretically destained, and then stored in 7% acetic acid. Sodium dodecyl sulfate (SDS)-electrophoresis was performed at 5 mA per gel, using the method of Weber and Osborn [25] on a 10% gel with normal amount of cross-linker. After running, the gel was stained with Coomassie brilliant blue R-250 (Sigma Co., U.S.A.), electrophoretically destained, and then stored in 7% acetic acid.

Purification of Cytosine Deaminase

The cytosine deaminase from *C. violaceum* YK 391 was comparatively thermostable, but all operations were done at below 4°C.

Step 1: Ammonium Sulfate Fractionation. Powdered ammonium sulfate at 20% saturation was added in the crude enzyme suspension, and the pH was adjusted to 7.5. After 1 h, the precipitate formed was removed by centrifugation at 15,000 ×*g* for 30 min. The ammonium sulfate concentration was then increased to 40% saturation

by the addition of powdered ammonium sulfate. After standing overnight, the resulting precipitate was collected by centrifugation at 15,000 ×*g* for 30 min and resuspended in 0.2 M Tris-HCl buffer (pH 7.5). The dissolved enzyme suspension was dialyzed overnight at 4°C. Insoluble materials formed were removed by centrifugation as under the above conditions.

Step 2: DEAE-Sephacel Column Chromatography. The dialyzed enzyme suspension was applied to a DEAE-Sephacel column (2.2×30 cm) equilibrated with 0.2 M Tris-HCl buffer (pH 7.5). The column was washed thoroughly with the same buffer, containing 0.1 M NaCl, to remove inactive protein. The enzyme was subsequently eluted with the same buffer, containing a gradient formed by 0.1 M to 0.3 M NaCl at a flow rate of 15 ml per h. The active fractions were combined and concentrated by pervaporation at 4°C.

Step 3: Ultrogel A_6 Column Chromatography. The above concentrated enzyme preparation was loaded onto a Ultrogel A_6 column (1.8×81 cm) equilibrated with 0.2 M Tris-HCl buffer (pH 7.5), and the column was eluted with the same buffer at a flow rate of 15 ml per h. The active fractions were combined and concentrated by Amicon ultrafiltration.

Step 4: Sephadex G-100 Column Chromatography. The concentrated enzyme suspension was finally purified, using a Sephadex G-100 column (1.8×81 cm) equilibrated with 0.2 M Tris-HCl buffer (pH 7.5), and the column was eluted with the same buffer at a flow rate of 10 ml per h and 3 ml-fractions were collected.

RESULTS AND DISCUSSION

Purification of Cytosine Deaminase

As shown in Table 1, the cytosine deaminase of *C. violaceum* YK 391 was purified 272.9 folds from 4,000 ml of culture broth with an overall yield of 13.8%. The elution profile of the final step in Ultrogel A_6 gel filtration demonstrated a single symmetrical protein peak, and the enzyme activity was entirely associated with the peak (Fig. 1). The homogeneity of the purified cytosine deaminase was investigated by disc- and SDS-PAGE. As shown in Fig. 2, the final preparation showed a single band on a polyacrylamide gel both in the presence and absence of SDS, indicating that the cytosine deaminase from *C. violaceum* YK 391 was purified to homogeneity. UV spectrum of the purified enzyme preparation showed maximum absorption at 275 nm and a minimum at around 255 nm (data not shown).

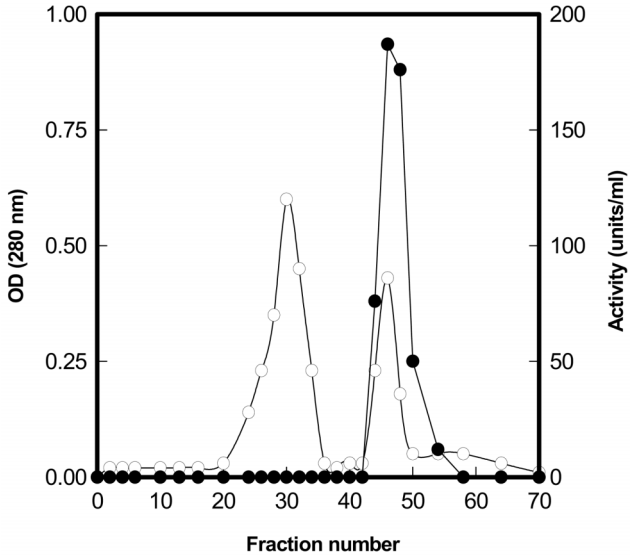

Fig. 1. Elution profile of the cytosine deaminase on Sephadex G-100 column chromatography.
The column was stabilized with 0.2 M Tris-HCl buffer (pH 7.5). The elution was performed with the same buffer at a flow rate of 10 ml/h, and 3.0 ml fractions were collected. Symbols: ○, absorbance at 280 nm; ●, cytosine deaminase activity.

Molecular Mass and Subunit Composition

Molecular mass of the purified enzyme was found to be about 126 kDa, estimated by gel filtration on a Sephadex G-100 (Fig. 3A). On the other hand, the molecular mass of the enzyme was about 63 kDa, when measured by PAGE in the presence of SDS (Fig. 3B). These results indicate that the native cytosine deaminase of *C. violaceum* YK 391 is composed of identical dimeric subunits. The molecular mass of the enzymes of *C. violaceum* [28], *E. coli* [10], *S. typhimurium* [26], *S. marcescens* [21], and *P. aureofaciens* [22] have been reported to be 156 kDa, 200 kDa, 230 kDa, 580 kDa, and 630 kDa, respectively. The molecular mass of *A. fumigatus* [30] and Baker's yeast enzymes are 34 kDa [9] and 41 kDa [12], respectively. The molecular weight of prokaryotic bacterial cytosine

Table 1. Summary on purification of the intracellular cytosine deaminase from *Chromobacterium violaceum* YK 391.

Procedure	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification factor (fold)	Recovery (%)
Cell-free extract	1,340	1,227	0.92	1.0	100
$(NH_4)_2SO_4$	37.0	1,161	31.4	34.3	94.3
DEAE-sephacel	13.4	544.3	40.6	44.3	44.4
Ultrogel A6	1.92	220.1	114.6	125.1	17.9
Sephadex G-100	0.68	170.0	250.0	272.9	13.8

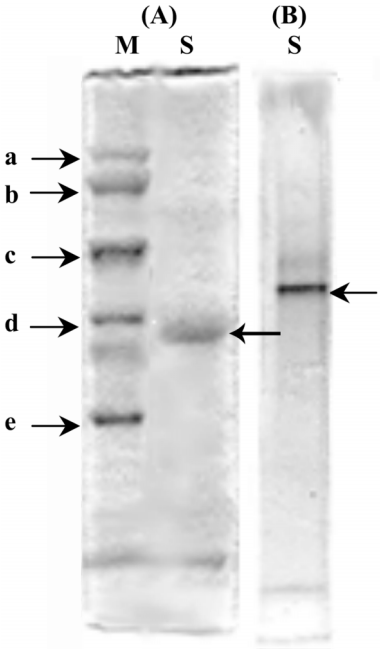

Fig. 2. Polyacrylamide gel electrophoresis of the purified cytosine deaminase.
(A) SDS-polyacrylamide gel. Lane 1, marker protein: a, myosin (M.W. 212 kDa); b, α_2-macroglobulin (M.W. 170 kDa); c, β-galactosidase (M.W. 116 kDa); d, transferrin (M.W. 76 kDa); e, glutamic dehydrogenase (M.W. 53 kDa); Lane 2, purified cytosine deaminase. The purified enzyme and standard proteins were treated with SDS at 100°C for 2 min, and then electrophoresis was carried out at 20 mA for 2 h with 10% SDS. (B) Disc-polyacrylamide gel in the absence of SDS.

deaminase is generally high, whereas those of eukaryotic enzymes are low. The molecular mass of intracellular cytosine deaminase from *C. violaceum* YK 391 was also similar to those of prokaryotic bacterial enzyme. The molecular mass of the presently described intracellular enzyme (126 kDa) is smaller than that of extracellular cytosine deaminase (156 kDa) [28].

Effects of pH and Temperature on Enzyme Stability and Activity

The purified cytosine deaminases are stable in the pH range of 6.5 to 8.0. In the present study, even after incubating the enzyme for 10 min at 60°C (pH 7.5), 70% of the enzyme activity remained (data not shown). In order to examine the effect of pH on the enzyme activity, the enzyme reaction was carried out in the pH range of 4.0 to 10.0. As shown in Fig. 4, the enzyme showed maximum activity at pH 7.5. The optimum temperature for the enzyme activity was found at 30°C, and the enzyme activity was decreased to half at 63°C for 10 min.

The activation energies (E_a) of cytosine deaminases from Baker's yeast [17], *A. fumigatus* [30, 32], Baker's yeast [9], and *S. typhimurium* [24] were reported to be 19.5, 13.24, 7.74, and 4.45 kcal/mol, respectively. Activation energy of the present cytosine deaminase, determined from an Arrhenius plot [23], was at 16.5 kcal/mol between 25°C and 30°C (data not shown), and this E_a value is lower compared with those of other sources.

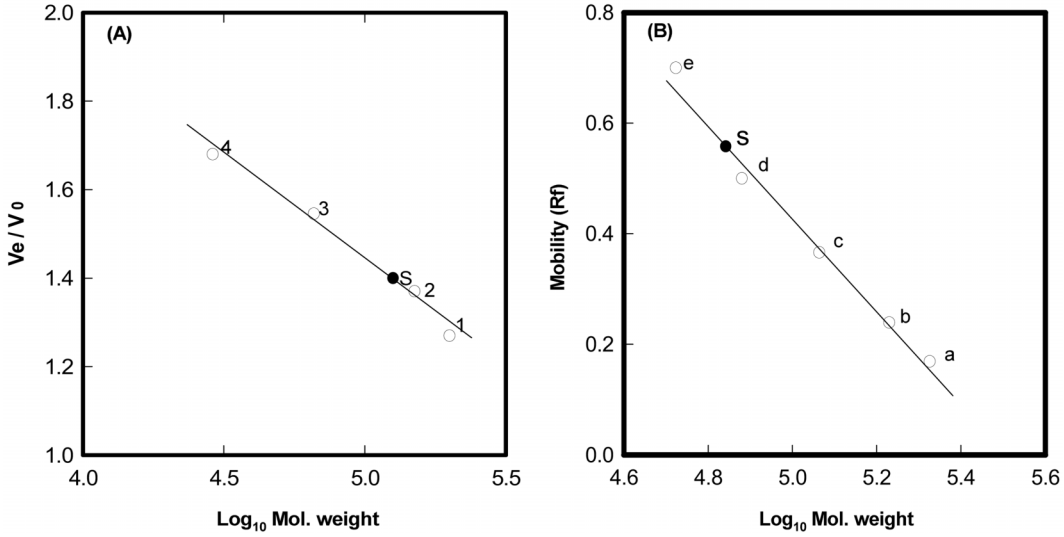

Fig. 3. Determination of molecular mass of the cytosine deaminase by gel filtration (A) and by SDS-PAGE (B).
(A) A column of Sephadex G-100 equilibrated with 0.2 M Tris-HCl (pH 7.5) was eluted at a flow rate of 5 ml per h. The fractions of 3 ml were collected. Each standard protein was applied as a solution of 1 mg in 1 ml of buffer. V_e is the elution volume. The void volume (V_0) was determined by elution of blue dextran. Molecular standard molecular marker: 1, carbonic anhydrase (M.W. 29 kDa); 2, bovine serum albumin (M.W. 66 kDa); 3, alcohol dehydrogenase (M.W. 150 kDa); 4, β-amylase (M.W. 200 kDa). (B) Electrophoresis was performed in 7.5% PAGE containing SDS at pH 8.8 with a current of 30 mA per plate. About 20 μg of protein were applied. Standard molecular marker: a, myosin (M.W. 212 kDa); b, α_2-macroglobulin (M.W. 170 kDa); c, β-galactosidase (M.W. 116 kDa); d, transferrin (M.W. 76 kDa); e, glutamic dehydrogenase (M.W. 53 kDa); S, cytosine deaminase. Symbols: ○, standard proteins; ●, cytosine deaminase (S).

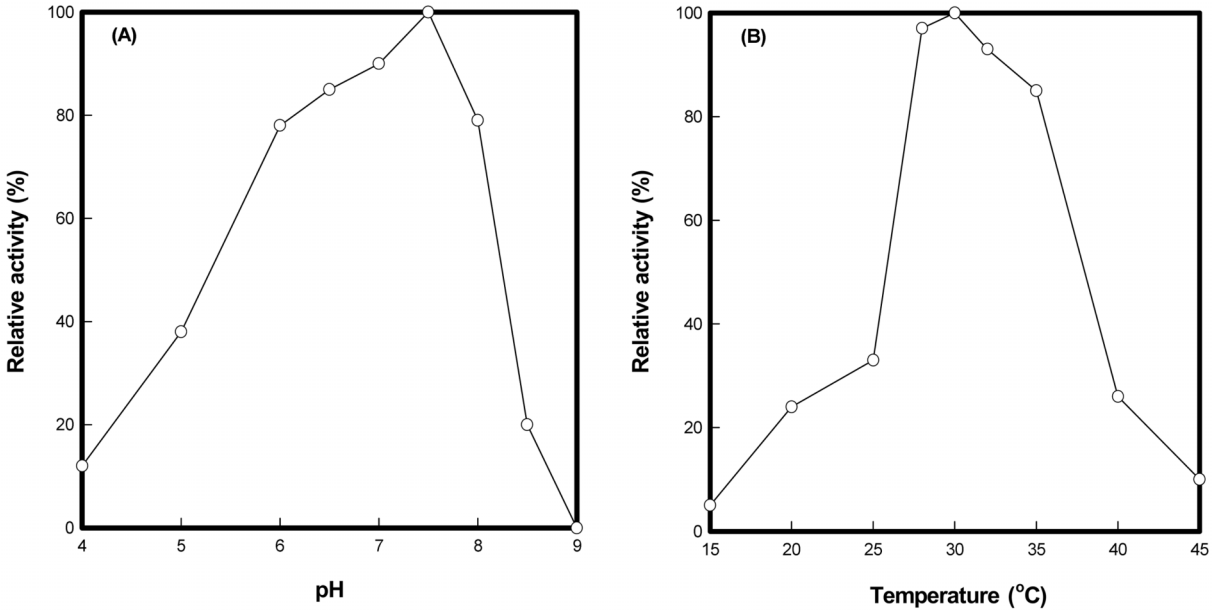

Fig. 4. Effect of pH (A) and temperature (B) on the cytosine deaminase activity.
(A) The enzyme activity was assayed under standard reaction conditions, using acetate buffer (pH 4.0 to 5.0), citrate-phosphate buffer (pH 5.5 to 7.0), Tris-HCl buffer (pH 7.0 to 8.0), and glycine-NaOH buffer (pH 8.0 to 10.0). (B) The enzyme activity was assayed under standard reaction conditions, and the reaction temperature was varied from 15°C to 45°C.

Substrate Specificity

The enzymes from *S. typhimurium* [26], *B. polymyxa* YL 38-3 [29], and *S. marcescens* [21] did not catalyze deamination of 5-methylcytosine, whereas the enzymes from *A. fumigatus* [30], Baker's yeast [12], *E. coli* [10], and *P. aureofaciens* [22] catalyzed 5-methylcytosine. In the present study, pyrimidine compounds were tested as substrates for the cytosine deaminase from *C. violaceum* YK 391.

As shown in Table 2, the purified enzyme catalyzed deamination of 5-fluorocytosine, cytidine, 5-methylcytosine, and 6-azacytosine in addition to cytosine, but did not catalyze 5-azacytosine. Based on 100% activity for cytosine, the activities for 5-fluorocytosine, cytidine, 5-methylcytosine, and 6-azacytosine were 69, 21, 7, and 10%, respectively. The enzymes from *S. marcescens* [21], *P. aureofaciens* [22], *A. fumigatus* [30], *S. typhimurium* [26], *E. coli* [10], and extracellular enzymes from *Arthrobacter* sp. JH-13 [27], and *B. polymyxa* YL 38-3 [29] did not catalyze cytidine. Baker's yeast enzyme was inhibited 50% by

Table 2. Substrate specificity of the cytosine deaminase.

Substrate (5 mM)	Relative activity (%)
Cytosine	100
5-Fluorocytosine	69
5-Methylcytosine	7
6-Azacytosine	10
5-Azacytosine	0
Cytidine	21

The cytosine deaminase activity was assayed under standard reaction conditions in the presence of substrates at indicated concentrations, and the results are expressed as relative activity to that of cytosine.

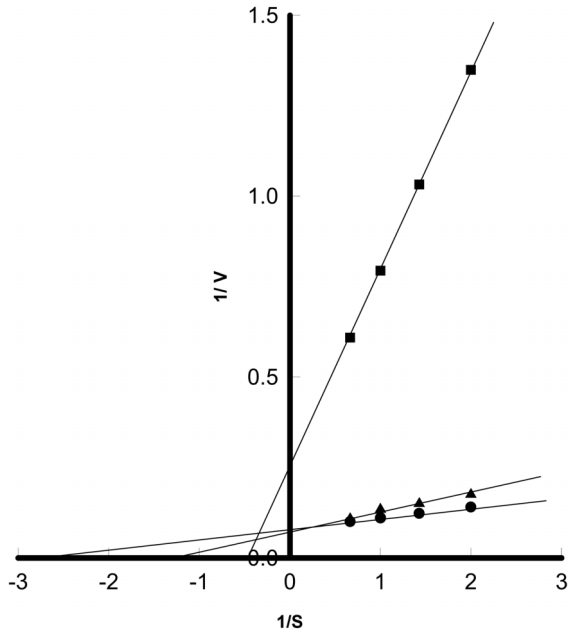

Fig. 5. Determination of K_m value for the cytosine deaminase by Lineweaver-Burk plot.
The plot is based on the rearrangement of the Michaelis-Menten equation into a linear form. Reaction mixtures containing purified cytosine deaminase and various concentrations of substrate were incubated at 37°C for determination of cytosine deaminase. Symbols: ●, cytosine; ▲, 5-fluorocytosine; ■, 5-methylcytosine.

0.517 mM cytidine [9]. The intracellular cytosine deaminase from *C. violaceum* YK 391 catalyzed cytidine, suggesting that this enzyme is unique.

Michaelis Constants

The effect of concentration of substrates on the enzyme activity was measured for cytosine, 5-fluorocytosine, and 5-methylcytosine. From Lineweaver-Burk double reciprocal plots [18], the apparent Michaelis constants (K_m) were calculated to be 0.38 mM, 0.87 mM, and 2.32 mM for cytosine, 5-fluorocytosine, and 5-methylcytosine, respectively (Fig. 5). In addition, the kinetics showed classical Michaelis-Menten type. The K_m values of the enzymes from *S. typhimurium* [26]. *A. fumigatus* [30], Baker's yeast [9], *S. marcescens* [21], and *P. aureofaciens* [22] were 0.74 mM, 2.0 mM, 2.5 mM, 3.4 mM, and 4.5 mM cytosine, respectively. Therefore, the results indicate that the rate constant of cytosine for the cytosine deaminase from *C. violaceum* YK 391 was higher than those from other sources. The apparent K_m value of intracellular enzyme from *C. violaceum* YK 391 was 2.32 mM for 5-methylcytosine, which is extremely smaller than that of the extracellular enzyme from *C. violaceum* YK 391 (67.2 mM) [28].

Effects of Metal Ions and Chemical Reagents on Enzyme Activity

The effects of metal ions and chemical reagents on the enzyme activity were examined.

As shown in Table 3, the purified enzyme did not require the presence of metal ions, and a number of metal ions inhibited the activity of purified cytosine deaminase. The purified enzyme activity was strongly inhibited by heavy metals such as Cd^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, and Fe^{3+} at 1 mM concentration.

Furthermore, the enzyme activity was completely inhibited by ρ-chloromercuribenzoate (ρ-CMB), *N*-bromosuccinimide, and chloramine-T, and strongly inhibited by 1 mM concentration of α,α'-dipyridyl, phenylmethylsulfonyl fluoride (PMSF), *o*-phenanthroline (Table 4). From Lineweaver-Burk double reciprocal plots [18], the apparent inhibitor constants (K_i) of chloramine-T, ρ-CMB, and *N*-bromosuccinimide were calculated to be 5.5×10^{-2} mM, 2.1×10^{-3} mM, and 1.8×10^{-4} mM, respectively (data not shown). Sodium cyanide, trichloroacetate, monoiodoacetate, and sodium azide showed no effect on the purified enzyme activity.

The cytosine deaminase from *P. aureofaciens* [31] and Baker's yeast [9, 11, 12] were also completely inhibited by 1 mM ρ-CMB. Inhibition of an enzyme activity by mercury compounds, ρ-CMB, suggests that the sulfhydryl group is involved in the active site of an enzyme [19]. These results indicated the presence of a sulfhydryl group in the catalytic sites of this cytosine deaminase.

Effects of Cytosine Analogues on Enzyme Activity

Table 5 demonstrates the effect of various cytosine analogues on the enzyme activity. The enzyme activity was completely inhibited by 2-thiouracil, and strongly

Table 3. Effect of metal ions on the cytosine deaminase activity.

Metal ion (1 mM)	Relative activity (%)
$CdCl_2$	0
$HgCl_2$	0
$ZnCl_2$	3
$CuCl_2$	5
$PbCl_2$	15
$FeCl_3$	24
$CaCl_2$	84
$FeCl_2$	90
$MnCl_2$	92
$MgCl_2$	97
None	100

The enzyme activity was assayed under standard reaction conditions in the presence of metal ions at indicated concentrations.

Table 4. Effect of inhibitors on the cytosine deaminase activity.

Inhibitors (1 mM)	Relative activity (%)
Trichloroacetate	102
Monoiodoacetate	102
Sodium cyanide	100
Sodium azide	100
EDTA	98
Sodium fluoride	89
o-phenanthroline	23
α,α'-Dipyridyl	22
PMSF	20
ρ-CMB	0
N-bromosuccinimide	0
Chloramine-T	0
None	100

The enzyme activity was assayed under standard reaction conditions in the presence of inhibitors at indicated concentrations. EDTA, ethylenediamine tetra acetic acid; PMSF, phenylmethylsulfonyl fluoride; ρ-CMB, ρ-chloromercuribenzoate.

Table 5. Effect of cytosine analogues on the cytosine deaminase activity.

Cytosine analogue (1 mM)	Relative activity (%)
5-Azacytosine	105
Deoxycytosine	105
Isocytosine	105
6-Azacytosine	50
2-Thiocytosine	40
2-Thiouracil	0
None	100

The cytosine deaminase activity was assayed under standard reaction conditions in the presence of cytosine analogues at indicated concentrations, and the results are expressed as relative activity to that of none.

by 50% and 60% by 6-azacytosine and 2-thiocytosine, respectively. The enzyme activity was not influenced by the presence of 5-azacytosine, deoxycytosine, and isocytosine. However, isocytosine is not deaminated by the yeast cytosine deaminase, but rather acts as an inhibitor [14].

Differences between Extracellular and Intracellular Enzymes

In our earlier paper [28], we reported some properties of extracellular cytosine deaminase from *C. violaceum* YK 391. The molecular mass and optimum temperature of the extracellular enzyme were 156 kDa and 40 to 45°C, however, those of the intracellular enzyme were 126 kDa and 30°C, respectively. The apparent K_m values of the extracellular enzyme were 1.55 mM, 5.52 mM, 67.2 mM for cytosine, 5-fluorocytosine, and 5-methylcytosine, respectively. Therefore, the results indicate that the rate constants of cytosine, 5-fluorocytosine, and 5-methylcytosine for the intracellular enzyme were lower compared with those of the extracellular enzyme. Furthermore, the extracellular enzyme was completely inhibited by a metal-chelating agent, such as α,α'-dipyridyl at 1 mM, and very weakly (89%) inhibited by a specific tryptophan modification reagent, such as *N*-bromosuccinimide at 1 mM. In contrast to the extracellular enzyme, the intracellular enzyme was completely inhibited by 1 mM *N*-bromosuccinimide, and was strongly (22%) inhibited by 1 mM α,α'-dipyridyl. These results suggest that a tryptophan residue might be involved in the active site of the intracellular enzyme, but the extracellular enzyme does not appear to involve a tryptophan residue. In addition to enzymatic properties of two enzymes, other differences were also observed between these two enzymes (Table 6). Based on these results, the intracellular enzyme appears to be quite different from the extracellular enzyme. The continued detailed studies will be presented in subsequent papers.

Table 6. Differences between extracellular and intracellular cytosine deaminases from *Chromobacterium violaceum* YK 391.

Properties	Extracellular [28]	Intracellular
Molecular mass (enzyme)	156 kDa	126 kDa
Molecular mass (subunit)	78 kDa	63 kDa
Optimum temperature	40 to 45°C	30°C
K_m values		
Cytosine	1.55 mM	0.38 mM
5-Fluorocytosine	5.52 mM	0.87 mM
5-Methylcytosine	67.20 mM	2.32 mM
Relative activity (%) by inhibitors		
N-bromosuccinimide	89%	0%
α,α'-Dipyridyl	0%	22%
PMSF	70%	20%

PMSF; phenylmethylsulfonyl fluoride.

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