J. Microbiol. Biotechnol. (1999), **9**(2), 173–178

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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

YU, TAE-SHICK* AND TAE-HYUN KIM[1]

Department of Microbiology, College of Natural Science, Keimyung University, Taegu 704-701, Korea
[1]Department of Herbs and Food Science, Traditional Food Institute, Kyongbuk College of Science, Kyungpook 718- 850, Korea

Received: December 30, 1998

Abstract The extracellular cytosine deaminase (EC 3.5.4.1) from *Chromobacterium violaceum* YK 391 was purified 264.7-fold with an overall yield of 14.3%. The enzyme was for the first time homogeneous by the criteria of polyacrylamide gel electrophoresis performed in the absence and in the presence of sodium dodecyl sulfate. The molecular weight of the purified enzyme was estimated to be about 156 kDa. The enzyme consisted of two identical subunits of approximate molecular weight 78 kDa. The isoelectric point of the enzyme was pH 5.55. The enzyme had a pH optimum of 7.5 and a temperature optimum of around 40 to 45°C. Besides cytosine, the enzyme deaminated 5-fluorocytosine, cytidine, 5-methylcytosine, and 6-azacytosine, but not 5-azacytosine. The extracellular cytosine deaminase is believed to be unique because it was active not only on cytosine but also on cytidine. The apparent K_m values for cytosine, 5-fluorocytosine, cytidine, and 5-methylcytosine were determined to be 1.55 mM, 5.52 mM, 10.4 mM, and 67.2 mM, respectively. The enzyme activity was strongly inhibited by heavy metal ions such as Fe^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} at 1 mM, and completely by α,α'-dipyridyl, and ρ-chloromercuribenzoate at 1 mM, and weakly inhibited by 1 mM *o*-phenanthroline. The enzyme activity was not affected by various nucleosides and nucleotides.

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

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

Cytosine, unlike other nucleobases, is not only degraded directly, but also does not act as a substrate for Salvage synthesis. Cytosine is certainly hydrolyzed to uracil by cytosine deaminase, and therefore is involved in the recycling of pyrimidine bases to the nucleotide pool or is entirely degraded. For 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. 5-Fluorocytosine (5-FC) has no antitumoural or bacteriostatic activity, and little clinical toxicity [3]. Furthermore, while 5-fluorouracil (5-FU) has antitumoural activity and a strong broad-range antimicrobial spectrum, it is toxic [5]. 5-FC after its conversion into 5-FU by cytosine deaminase has antineoplastic activity and acts as a selective fungicide [4, 18]. 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.

However, in mammalian and plant cells, cytosine deaminase activity has not been found in any organ of the body [11]. On the other hand, a small amount of 5-FU has been detected in the blood, probably arising from intestinal microflora. The idea, then, was to devise a way to use 5-FC as a depot form of 5-FU in the treatment of cancer by infecting or implanting cytosine deaminase capsules near the tumor of a cancer patient [15]. Such therapy would be advantageous, especially in the brain.

Cytosine deaminase was first identified in 1923 [6], and it has since been studied in yeast [7], some bacteria [16, 17, 20], and mould [24]. Cytosine deaminases from *Serratia marcescens* [16] and *Pseudomonas aureofaciens* [17] were first purified to homogeneity in 1975 by polyacrylamide gel electrophoresis and ultracentrifugal analysis. These enzymes were also purified to homogeneity from *Escherichia coli* [8], *Salmonella typhimurium* [20], and *Aspergillus fumigatus* [24].

The above cytosine deaminases were intracellular enzymes. Extracellular cytosine deaminase was recently observed in *Arthrobacter* sp. JH-13 [21] and in *Bacillus polymyxa* YL 38-3 [23], but these extracellular enzymes were not purified. This paper deals with the purification

*Corresponding author
Phone: 82-53-580-5252; Fax: 82-53-580-5164;
E-mail: tsyu@kmucc.keimyung.ac.kr

and enzymatic properties of extracellular cytosine deaminase isolated from *Chromobacterium violaceum* YK 391.

MATERIALS AND METHODS

Materials

A standard kit of low molecular weight for electrophoresis was purchased from Bio-Rad Co., U.S.A. DEAE-cellulose, Ultrogel A6, and Sephadex G-100 were obtained from Sigma Co. Pepton, meat extract, and yeast extract were purchased from Difco. The Tris-HCl buffer used was 0.02 M Tris-HCl (pH 7.5) containing 1 mM 2-mercaptoethanol.

Microorganism and Growth Conditions

C. violaceum YK 391 [25] isolated from soil in Taegu city was grown at 30°C for 3 days in a 3-l fermentor (SY-500, Korean Fermentor Co., LTD., Korea) containing 2.5 l of the medium (pH 7.5) consisting of 1% soluble starch, 1% 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 antiformer under aeration [26]. The cells were removed by centrifugation at 13,000×g for 20 min. The resulting supernatant was used as the crude enzyme source.

Enzyme Assay

Cytosine deaminase activity was assayed spectro-photometrically based on the absorbance of cytosine and uracil in 0.1 N HCl at 290 nm as previously described [23, 25, 26]. A mixture of the enzyme and 1 mM of cytosine in 1.0 ml of 0.02 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 h. The specific activity was defined as the number of units of enzyme activity per mg of protein.

Purification of Cytosine Deaminase

The extracellular cytosine deaminase from *C. violaceum* YK 391 was thermo-unstable, and so all operations were done at temperatures below 10°C.

Step 1: Evaporation and ammonium sulfate fractionation. The crude enzyme solution was evaporated with a rotary evaporator at 30°C. The evaporated crude enzyme solution was dialyzed overnight at 4°C against 50 volumes of 0.02 M Tris-HCl buffer (pH 7.5). To the dialyzed enzyme solution was added solid ammonium sulfate to give 25% saturation, and the pH was adjusted to 7.5. After 1 h, the precipitate formed was removed by centrifugation at 13,000×g for 10 mins. Additional ammonium sulfate was then added to the resultant supernatant to give 45% saturation. After 5 h, the resultant precipitate was collected, dissolved in a small volume of buffer, and dialyzed at 4°C overnight. Insoluble materials formed were removed by centrifugation in the above conditions.

Step 2: Sephadex G-100 column chromatography. The dialyzed enzyme solution was applied to a Sephadex G-100 column (2.7×81 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 7.5). The active fractions were combined and concentrated by amicon ultrafiltration.

Step 3: DEAE-cellulose column chromatography. The concentrate was applied to a DEAE-cellulose column (2.2×30 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 7.5). The column was washed thoroughly with buffer. After removing much of the inactive protein, elution was carried out with the same buffer containing 0.5 M NaCl. The active fractions were pooled and concentrated by pervaporation at 4°C.

Step 4: Ultrogel A_6 column chromatography. The extracellular cytosine deaminase from *C. violaceum* YK 391 was finally purified from the concentrate of the active fractions from step 3 by pervaporation at 4°C The concentrated enzyme solution was introduced into a Ultrogel A_6 column (1.8×81 cm) equilibrated with the same buffer.

Isoelectric Point (pI)

The isoelectric point was determined at 4°C using a Rotofore cell (Bio-rad Co. U.S.A.) in a pH range of 3.5 to 10.0 ampholyte (Bio-rad Co. U.S.A.). The anodic solution was 0.1 M H_3PO_4 and the cathodic solution was 0.1 M NaOH. The focusing chamber was loaded with a total volume of 50 ml containing the purified enzyme, 2% ampholyte (pH 3.5 to 10.0), and 5 mM 2-mercaptoethanol. The current was adjusted to a constant 12 W. After loading the sample for 12 h at 4°C, 20 fractions were collected from the focusing chamber, and the pH and enzyme activity of the fractions were immediately tested.

Molecular Weight

The molecular weight of the enzyme was estimated by gel filtration according to Andrews [1]. Gel filtration was performed in a Sephadex G-100 column (1.8×81 cm) previously equilibrated with 0.02 M Tris-HCl buffer (pH 7.5). The standard proteins used were cytochrome C (MW 12.4 kDa), carbonic anhydrase (MW 29 kDa), bovine serum albumin (MW 66 kDa), alcohol dehydrogenase (MW 150 kDa), and β-amylase (MW 200 kDa). 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 the determination of the molecular weight of the subunits of the enzyme according to Weber and Osborn [19]. The standard proteins used came from a kit of standard proteins of low molecular weight: myosin (MW 205 kDa), β-galactosidase (MW 116 kDa), phosphorylase b (MW 97.4 kDa), bovine albumin

(MW 66 kDa), egg albumin (MW 45 kDa), and carbonic anhydrase (MW 29 kDa).

Protein Assay

Protein determination was performed by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

Electrophoresis

Polyacryamide gel electophoresis was performed by a modified version of Davis' method [2]. 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 stored in 7% acetic acid. SDS-PAGE was performed according to Weber and Osborn [19] on a 10% gel with the normal amount of cross-linker at 5 mA per gel. After running, the gel was stained with Coomassie Brilliant blue R-250 (Sigma Co., U.S.A.), electrophoretically destained, and stored in 7% acetic acid.

Paper Chromatography of Reaction Mixtures

For the assay of products produced with the enzyme, paper chromatography was performed according to Sakai *et al.* [16]. The 50 µl of the incubated reaction mixture was developed on Toyo Roshi No. 50 filter paper with a solvent (*n*-butanol-acetic acid-water, 4:1:1 by vol.) at room temperature for 18 h. The paper was dried and the spots were detected under a UV lamp (SL 2537, Black Light Eastern Co., U.S.A.). The spots of product on the chromatography paper were cut and immersed in 5 ml of 0.1 N HCl for 3 h at room temperature. The papers were removed by centrifugation and $OD_{260\,nm}$ was used to ascertain the concentration of products.

Results and Discussion

Purification of Extracellular Cytosine Deaminase

The extracellular cytosine deaminase from *C. violaceum* YK 391 was purified 264.7 fold from 3,000 ml of culture broth with an overall yield of 14.3% the first time, as shown in Table 1. The elution patterns of the final step with Ultrogel A_6 gel filtration yielded a single symmetrical protein peak and the enzyme activity was entirely associated with the peak. The homogeneity of the purified preparation was investigated by polyacrylamide gel electrophoresis. As shown in Fig. 1, the final preparation showed a single band on a polyacrylamide gel both in the presence and in the absence of sodium dodecyl sulfate. These results indicate that the purified extracellular cytosine deaminase from *C. violaceum* YK 391 was homogeneous. The ultraviolet absorption spectrum of the purified extracellular cytosine deaminase has a maximum at 273 nm and minimum around 257 nm (data not shown).

Table 1. Purification of extracellular cytosine deaminase from *C. violaceum* YK 391.

Purification steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield (%)
Cell free extract	2462.4	1680	0.68	1.0	100
$(NH_4)_2SO_4$	76.25	1600	21.0	30.9	95.2
Sephadex G-100	17.07	873.81	51.19	75.3	52.0
DEAE-cellulose	4.93	601.95	122.1	179.6	35.8
Ultrogel A_6	1.33	239.4	180.0	264.7	14.3

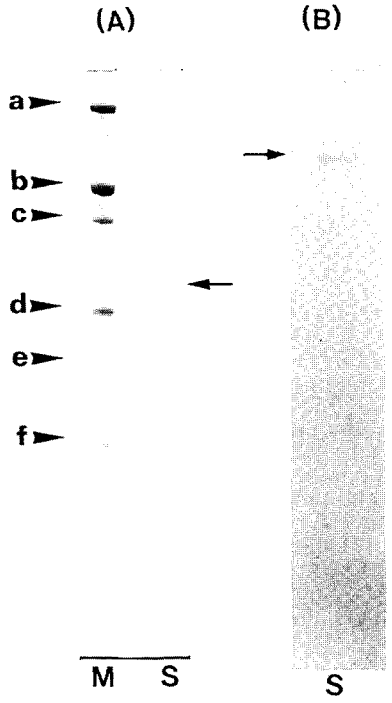

Fig. 1. Polyacrylamide gel electrophoresis in the presence (A) or absence (B) of sodium dodecyl sulfate.
M: standard protein; a, myosin (205 kDa); b, β,-galactosidase (116 kDa); c, phosphorylase b (97.4 kDa); d, bovine albumin (66 kDa); e, egg albumin (45 kDa); f, carbonic anhydrase (29 kDa). S: purified extracellular cytosine deaminase.

Molecular Weight and Isoelectric Point

The molecular weight of the extracellular cytosine deaminase was estimated to be about 156 kDa by gel filtration, as shown in Fig. 2. The molecular weight of the enzyme was determined to be about 78 kDa by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, as shown in Fig. 1. Based on the above results, the extracellular cytosine deaminase from *C. violaceum* YK 391 is probably composed of dimeric identical subunits. The molecular weights of bacterial cytosine deaminases are generally high, but those of eukaryotic enzymes are low. The molecular weights of *E. coli* [8], *S. typhimurium* [20], *S. marcescens* [16], and *P. aureofaciens* [17] enzymes are

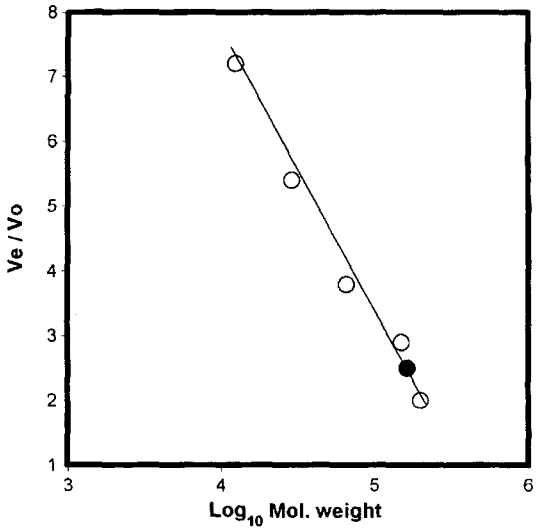

Fig. 2. Determination of molecular weight of the cytosine deaminase by Sephadex G-100 gel filtration.
A column (1.8×81 cm) of Sephadex G-100 was equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. 1, cytochrome C (12.4 kDa); 2, carbonic anhydrase (29 kDa); 3, bovin serum albumin (66 kDa); 4, alcohol dehydrogenase (150 kDa); 5, β-amylase (200 kDa). ○, standard proteins; ●, the cytosine deaminase. The void volume (V_o) was determined by the elution of blue dextran.

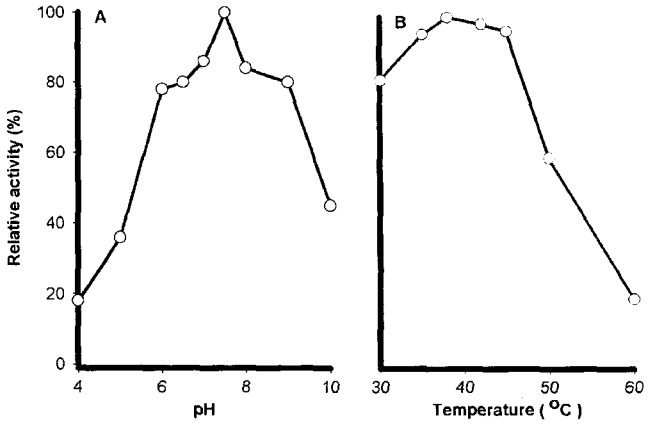

Fig. 3. Effect of pH (A) and temperature (B) on the cytosine deaminase activity.
A. The enzyme activity was assayed under standard reaction conditions using citric acid-sodium phosphate buffer (pH 4.0 to 7.0), Tris-HCl buffer (pH 7.0 to 8.0), and glycine-NaOH buffer (pH 8.0 to 10.0). B. The enzyme activity was assayed under standard reaction conditions and the reaction temperature was varied from 25°C to 60°C.

200, 230, 580, and 630 kDa, respectively, and the molecular weights of *A. fumigatus* [24] and baker's yeast enzymes [9] are 32 and 34 kDa, respectively. The molecular weight of extracellular cytosine deaminase from *C. violaceum* YK 391 was also similar to those of bacterial enzymes. The activity and pH profile of an elute from the isoelectric focusing column show that one catalytically active component with an isoelectric point value was present at pH 5.55 (data not shown). The isoelectric point of the enzyme is similar to that of the *E. coli* enzyme (pH 5.8) [8] and of the baker's yeast enzyme (pH 4.7) [9], respectively.

Effects of pH and Temperature on Enzyme Stability and Activity

The crude and purified extracellular cytosine deaminases were stable in the pH range of 6.0 to 8.0. Even after incubating the enzyme for 10 min at 50°C (pH 7.0), 50% of the enzyme activity was retained. In order to examine the effect of pH on the enzyme activity, enzyme reactions were carried out in the pH range of 4.0 to 10.0.

As shown in Fig. 3, the enzyme showed maximum activity at pH 7.5. Moreover, the optimum temperature for the extracellular enzyme was found to be between 40 to 45°C, and enzyme activity was decreased to half at 50°C for 30 min.

The activation energies (E_a) of intracellular cytosine deaminases from baker's yeast [12], *A. fumigatus* [22], baker's yeast [7], and *S. typhimurium* [20] were 19.5, 13.24, 7.74, and 4.45 kcal/mol, respectively. The activation energy of extracellular cytosine deaminase determined from an Arrhenius plot was 3.1 kcal/mol between 25°C and 37°C (data not shown) and this E_a value was lower compared with those of other sources.

Substrate Specificity

Some pyrimidine compounds were tested as substrates of the extracellular cytosine deaminase. The enzymes from *S. typhimurium* [20], *B. polymyxa* [23], and *S. marcescens* [16] did not catalyze the deamination of 5-methylcytosine. The enzymes from *A. fumigatus* [24], baker's yeast [9], *E. coli* [8], and *P. aureofaciens* [17], however, catalyzed 5-methylcytosine.

As shown in Table 2, the purified enzyme catalyzed the deamination of 5-fluorocytosine, cytidine, 5-methylcytosine, and 6-azacytosine besides cytosine, but did not catalyze 5-azacytosine. The relative values of the activities for 5-fluorocytosine, cytidine, 5-methylcytosine, and 6-azacytosine were 57, 47, 16, and 11%, respectively, compared with 100 for cytosine. The intracellular enzymes from *S. marcescens* [16], *P. aureofinosa* [17], *A. fumigatus* [24], *S. typhimurium* [20], *E. coli* [8], and extracellular enzymes from *Arthrobacter* sp. JH-13 and *B. polymyxa* YL 38-3 [23] did not catalyze cytidine. Furthermore, baker's yeast enzyme was inhibited 50% by 0.517 mM cytidine [7]. Based on these results, the extracellular cytosine deaminase from *C. violaceum* YK 391 is believed to be unique because it deaminated not only cytosine and cytosine analogues but also cytidine.

Michaelis Constants

The effect of the concentration of substrates on the enzyme activity was examined for cytosine, 5-fluorocytosine, and

Table 2. Substrate specificity of the cytosine deaminase from *C. violaceum*.

Substrate (5 mM)	Relative activity (%)
Cytosine	100
5-Fluorocytosine	57
Cytidine	47
5-Methylcytosine	16
6-Azacytosine	11
Adenosine	0
AMP	0
5-Azacytosine	0
CMP	0

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

5-methylcytosine. From Lineweaver-Burk double reciprocal plots [10], the apparent Michaelis constants (K_m) were calculated to be 1.55, 5.52, 10.4, and 67.2 mM for cytosine, 5-fluorocytosine, cytidine, and 5-methylcytosine, respectively (Fig. 4). Also, the kinetics were of the classical Michaelis-Menten type. The *Km* values of *S. typhimurium* [20], *A. fumigatus* [24], baker's yeast [7], *S. marcescens* [16], and *P. aureofaciens* [17] were 0.74, 2.0, 2.5, 3.4, and 4.5 mM cytosine, respectively. From the results, the rate constant of cytosine against the extracellular cytosine deaminase from *C. violaceum* YK 391 was higher compared with those of other sources.

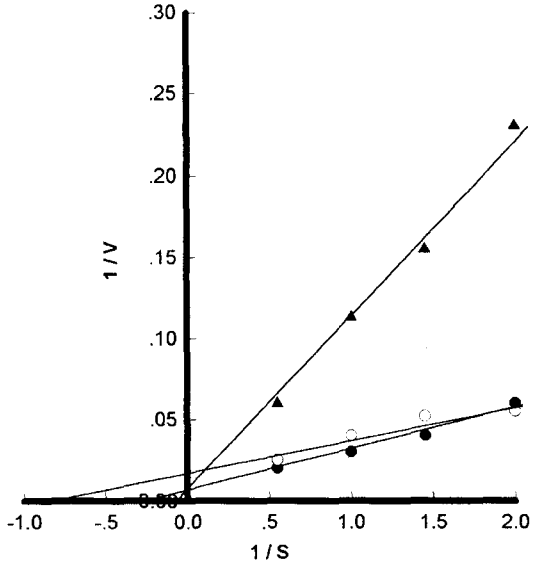

Fig. 4. Determination of *Km* value for the cytosine deaminase by Lineweaver-Burk plot.

The plot is based on the rearrangement of the Michaelis-Menten equation into a linear form. Reaction mixture of purified extracellular cytosine deaminase and various concentrations of substrate were incubated at 37°C for determination of the conversion of the cytosine deaminase. ○, 5-methylcytosine; ●, cytosine; △, 5-fluorocytosine.

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, this extracellular enzyme did not require the presence of metal ions and was strongly inhibited by some heavy metals such as Fe^{2+}, Pb^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} at concentrations of 1 mM.

Furthermore, the enzyme was strongly inhibited by ρ-chloromercuribenzoate (ρ-CMB) and α,α'-dipyridyl, and weakly inhibited by *o*-phenanthroline at concentrations of 1 mM, as shown in Table 4. Sodium cyanide, trichloroacetate, and monoiodoacetate had no effect on the enzyme activity.

The extracellular cytosine deaminase from *Arthrobacter* sp. [21] and *B. polymyxa* [23] were also completely inhibited by 1 mM ρ-CMB. Inhibition of enzyme activity by mercury compounds, ρ-CMB, suggests that a sulfhydryl group is involved in the active site of an enzyme [14]. From these results, we suggest that a sulfhydryl group exists in the catalytic sites of these extracellular enzymes. Baker's yeast enzyme [7] was inhibited by GMP, CMP, CDP, and guanosine, and *A. fumigatus* [24] by ATP and UTP, but *S. marcescens* [16] was strongly activated by IDP, ITP, GTP, and ATP, respectively. However, the extracellular enzyme activity was not affected by ATP,

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

Metal ion (1 mM)	Relative activity (%)
$CaCl_2$	105
$MgCl_2$	86
$MnCl_2$	75
$CuCl_2$	8
$HgCl_2$	6
$ZnCl_2$	5
$CdCl_2$	5
$PbCl_2$	2
$FeCl_2$	0
None	100

The enzyme activity was assayed in the presence of metal ions at indicated concentrations.

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

Inhibitors (1 mM)	Relative activity (%)
Sodium cyanide	108
Sodium azaide	106
EDTA	103
Trichloroacetate	102
Sodium fluoride	100
Monoiodoacetate	99
o-Phenanthroline	47
α,α'-Dipyridyl	0
ρ-Chloromercuribenzoate	0
None	100

The enzyme activity was assayed in the presence of inhibitors at indicated concentrations.

GMP, IMP, adenosine, guanosine, adenine, UDP, CMP, UMP, and thymine at concentrations of 1 mM (data not shown).

REFERENCES

1. Andrews, P. 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96:** 595-606.
2. Davis, B. J. 1964. Disc electrophoresis. 2. Method and application to human serum proteins. *Ann. New York Acad. Sci.* **121:** 404-427.
3. Duschinsky, R., E. Pleven, and C. Heiderberger. 1957. The synthesis of 5-fluoropyrimidines. *J. Am. Chem. Soc.* **79:** 4559-4560.
4. Giege, R. and J. H. Weil. 1970. Elude des tRNA de levureagent incorpore du 5-fluorouracile provenant de la desamination in vivo de la 5-fluorocytosine. *Bull. Soc. Chim. Biol.* **52:** 135-144.
5. Grunberg, E., E. Titsworth, and M. Bennett. 1964. Chemotherapeutic activity of 5-fluorocytosine, pp. 566-568. *In Antimicrobial Agents and Chemotherapy,* Ann Arbor, U.S.A.
6. Hahn, A. and W. Lentzel. 1923. Uber das verhalten von pyrimidinderivaten in den organismen. I. Einfluss von hefe auf pyrimidinderivate. *Z. Biol.* **79:** 179-190.
7. Ipata, P. L., F. Marmochii, G. Magni, R. Felio, and G. Polidoro. 1971. Baker's yeast cytosine deaminase. Some enzymic properties and allosteric inhibition by nucleosides and nucleotides. *Biochemistry* **10:** 4270-4276.
8. Katsuragi, T., T. Sakai, K. Matsumoto, and K. Tonomura. 1986. Cytosine deaminase from *Escherichia coli*-production, purification, and some characteristics. *Agric. Biol. Chem.* **50:** 1721-1730.
9. Katsuragi, T., T. Sonoda, K. Matsumoto, T. Sakai, and K. Tonomura. 1989. Purification and some properties of cytosine deaminase from bakers' yeast. *Agric. Biol. Chem.* **53:** 1313-1319.
10. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56:** 658-666.
11. Koechlin, B. A., F. Rubio, S. Palmer, T. Gabriel, and R. Duschinsky. 1966. The metabolism of 5-fluorocytosine-2^{14}C and of cytosine-^{14}C in the rat and the disposition of 5-fluorocytosine-2^{14}C in man. *Biochem. Pharmacol.* **15:** 435-446.
12. Kream, J. and E. Chargaff. 1952. On the cytosine deaminase of yeast. *J. Am. Chem. Soc.* **74:** 5157-5160.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. L. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265-275.
14. Nakanishi, Y., F. Isohaschi, S. Ebisuno, and Y. Sakamoto. 1989. Sulfhydryl groups of an extramitochondrial acetyl-CoA hydrolase from rat liver. *Biochim. Biophys. Acta* **996:** 209-213.
15. Nishiyama, T., Y. Kawamura, K. Kawamoto, H. Matsumura, N. Yamamoto, T. Ito, A. Ohyama, T. Katsuragi, and T. Sakai. 1982. Antineoplastic effect of 5-fluorocytosine and cytosine deaminase on brain tumors. *Neurol. Med. Chirs.* **22:** 344-352.
16. Sakai, T., T. S. Yu, H. Tabe, and S. Omata. 1975. Purification of cytosine deaminase from *Serratia marcescens. Agric. Biol. Chem.* **39:** 1623-1629.
17. Sakai, T., T. S. Yu, K. Taniguchi, and S. Omata. 1975. Purification of cytosine deaminase from *Pseudomonas aureofaciens. Agric. Biol. Chem.* **39:** 2015-2020.
18. Wallace, P. M., J. F. MacMaster, V. F. Smith, D. E. Kerr, P. D. Senter, and W. L. Cosand. 1994. Intratumoral generation of 5-fluorourcil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. *Cancer Res.* **54:** 2719-2723.
19. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406-4412.
20. West, T., M. S. Shanley, and G. A. O'Donovan. 1982. Purification and some properties of cytosnie deaminase from *Salmonella typhimurium. Biochim. Biophys. Acta* **719:** 251-258.
21. Yeeh, Y., J. H. Park, and H. K. Jun. 1985. Properties of extracellular cytosine deaminase from *Arthrobacter* sp. JH-13. *Kor. J. Microbiol.* **23:** 177-183.
22. Yu, T. S., J. K. Kim, and T. Sakai. 1986. Cytosine deaminase of fungus. *Kor. J. Appl. Microbiol. Bioeng.* **14:** 169-174.
23. Yu, T. S., T. H. Kim, J. M. Park, H. I. Song, and K. T. Chung. 1988. Optimum culture conditions for production of extracellular cytosine deaminase by *Bacillus polymyxa* YL 38-3. *Kor. J. Microbiol.* **26:** 368-374.
24. Yu, T. S., J. K. Kim, T. Katsuragi, T. Sakai, and K. Tonomura. 1991. Purification and properties of cytosine deaminase from *Aspergillus fumigatus. J. Ferment. Bioeng.* **72:** 266-269.
25. Yu, T. S. and T. H. Kim. 1997. Isolation and identification of bacterium producing extracellular cytosine deaminase. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 9-14.
26. Yu, T. S. and T. H. Kim. 1997. Optimization of extracellular cytosine deaminase production from *Chromobacterium violaceum* YK 391. *Kor. J. Biotechnol. Bioeng.* **12:** 89-95.