# The Enzymatic Characteristics of the Cytidine Deaminase in *Salmonella typhimurium*

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**Abstract - The cytidine deaminase was partialy purified with sephadex G-200 and the characteristics of the enzyme were clarified. The molecular mass of the plasmid-encoded protein was identified as about 30 kDa in a minicell system. The native enzyme was estimated to have the molecular mass of 60 kDa by gel filtration. This indicates that the native enzyme may exist as a dimer composed of two identical subunits. The enzyme was reasonably stable in the pH range of 6 to 9, and was labile under high temperature above 50°C. Mercaptoethanol, pCMB, mercury and copper were found to inhibit the enzyme activity. The cytidine analogues of bromo- and iodo-(deoxy)-cytidine were also found to inhibit the activity, while fluorodeoxycytidine and azacytidine were found to activate it. Deoxycytidine, cytidine, ara-C and Methyldeoxycytidine have excellent substrates for the enzyme.**

**Key words : cytidine deaminase, *Salmonella typhimurium*, inhibitor, molecular weight**

## INTRODUCTION

Prokaryotic as well as eukaryotic cells have been shown to command numerous enzymatic reactions, through which they can scavenge nucleic acid precursors from the surroundings and metabolize them according to their immediate need. The physiological function of the cytidine deaminase (CDA : EC 3.5.4.5) is to scavange exogeneous and endogeneous cytidine and deoxycytidine for pyrimidine nucleotide synthesis (Hammer-Jesperson 1983).

The enzyme has since been identified in a variety of biological source. The characteristic of the enzyme was studied in mouse kidney (Tomchick *et al.* 1968) *E. coli* (Hosono and Kuno 1973), *Neurospora crassa* (Shaffer *et al.* 1975), *Mycoplasma* (Mitchell and Finch 1977), *Bacillus subtilis* (Song *et al.* 1989), *Arabidopsis thaliana* (Vincenzetti *et al.* 1999), Human (Demontis *et al.* 1998) and *Bacillus caldolyticus* (Woo *et al.* 2001). But the physiological and biochemical characteristics of *Salmonella* cytidine deaminase was not known yet, except the whole chromosome of *Salmonella typhimurium* LT2 was determined on 2001 (McClelland *et al.* 2001). The putative sequence of the *cdd* gene was identified with homology search with other known *cdd* gene. For elucidate whether the putative *cdd* sequence is correct or not, the cloned *cdd* gene was expressioned in *E. coli* and the characteristics of the enzyme was studied in this paper.

## MATERIALS AND METHODS

### 1. Strain and media

*E. coli* JF611/pSAM21 which harboring the plasmid containing the *cdd* gene was used as the experiment. *E.*

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*coli* and *S. typhimurium* strains were grown in a L broth consisting of 1% tryptone, 0.5% yeast extract and 0.5% NaCl. For enzyme assay *E. coli* and *S. typhimurium* strains were cultured in AB minimal medium (Chang *et al.* 1989).

### 2. Plasmid coded protein detection

Minicells, prepared from cultures of *E. coli* BD1854 (*min* A, *min* B, *thi, his, rps* L, *lac, man, mal, xyl, ton* A) containing the desired plasmids, were labled with ($^{35}$S)-Methionine, and then extracted by heating at 90°C for 10 min in buffer containing SDS and 2-mercaptoethanol (Jensen *et al.* 1984). Proteins were analyzed by electrophoresis through 12.5% polyacrylamide/SDS gels. The position of the bands was visualized by autoradiography after drying of the gels.

### 3. Partial purification of the enzyme

The sonic extract was treated with solid ammonium sulfate. The concentration was increased to 75% saturation. After increasing the percentage of saturation from 40 to 75; the mixture was centrifuged, and the resulting pellet was dispersed in 0.02 M Tris-Cl buffer (pH 7.5). This suspension was dialyzed in the 0.02 M potassium phosphate buffer (pH 7.5) overnight. After dialysis, the enzyme solution was applied to a column of Sephadex G-200 (1.5 × 68 cm) equilibrated with 0.02 M Tris-EDTA buffer (pH 7.5). It was eluted with the same buffer in 5 ml fractions at a flow rate of approximately 7.5 ml/hr. The protein was measured by spectrophotometer at 280 nm and the activity of cytidine deaminase was estimated with standard condition.

### 4. Enzyme activity assay

Assay mixtures present in a volume of 360 μl : 250 μl of Tris-Mg (2.35 ml of 100 mM Tris-Cl at pH 7.0 and 50 μl of 0.2 M of $MgCl_2$), 10 μl of 0.05 M cytidine and 100 μl of appropriate diluted enzyme. At 4, 8, and 12 min, 100 μl samples were pipetted into 0.9 ml of 0.5 N perchloric acid. The samples were centrifuged and the absorbance of the supernatant was measured at 290 nm. Protein determination was performed using bovine serum albumin as a standard (Lowry *et al.* 1951).

## RESULTS AND DISCUSSION

### 1. Partial purification of the enzyme

*E. coli* JF611/pSAM21 cells, which were grown overnight in AB minimal medium supplemented with the appropriate requirements and antibiotics, were suspended in 20 ml of 100 mM Tris-EDTA buffer (pH 7.5) and disrupted with a sonicator for 5 min. The sonic lysate was centrifugated for 20 min at 10,000 rpm and the cell debris was discarded. By stepwise precipitation with ammonium sulfate, most of the enzyme was recoverd from the 40 to 75 fraction. After dialysis of the dissolved precipitation, the crude solution was introduced to a column of Sephadex G-200 (1.5 × 68 cm) equilibrated with 0.02 M Tris-EDTA buffer (pH 7.5). The elution profile is shown in Fig. 1. A wide peak containing cytidine deaminase was recovered.

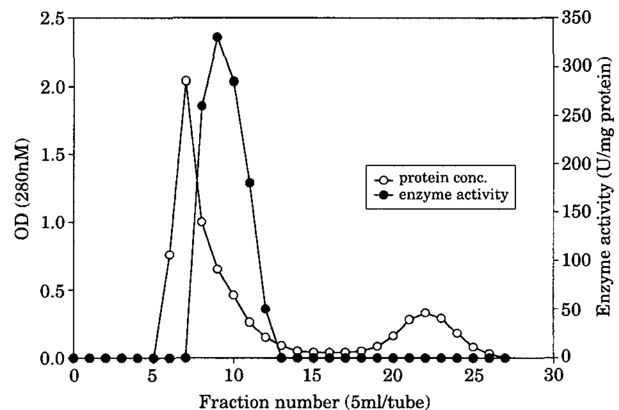


**Fig. 1.** Elution profile of the enzyme on Sephadex G-200.

### 2. Molecular mass determination

In order to determining the molecular mass of the *cdd* gene product, *cdd* complementing plasmids pSAM21 and pUC18 as a control were transformed into the minicell of *E. coli* BD1854. The $^{35}$S-methionine labelled proteins synthesized in the minicells were analysed by autoradiography after SDS polyacrylamide gel electrophoresis. As a result, one major polypeptide with a molecular mass of about 30 kDa appeared specifially in cells harboring the plasmid pSAM21 containing the *cdd*

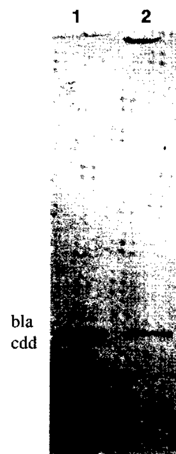


**Fig. 2.** Indentification of the polypeptide encoded by the *cdd* gene. $^{35}$S-methionine labeled extracts of mini-cells harboring the *cdd* gene cloned plasmid were electrophoresis in 12.5% SDS PAGE and then autoradiograph. lane1, pSAM21; lane2, pUC18.

gene. This band is not found in cells containing pUC18 (Fig. 2). Based on this, the polylpeptide is assumed to be the *Salmonellar* cytidine deaminase subunit encoded by the *cdd* gene. However the molecular weight of the cytidine deaminase in the native state was estimated at 60 kDa by gel filtration (Data not shown). This means that two identical subunits with molecular mass of 30 kDa exist as a dimer in the native state. Based on deduced amino acid sequences obtained from the data banks, the size of the CDA subunits from different organisms is either approximately 30 kDa as for *Escherichia coli* (Yang *et al.* 1992) and *Arabidopsis thaliana* (Vincenzetti *et al.* 1999) or half of that, i.e., approximately 15 kDa as for *Mycoplasma pneumoniae* (Himmelreich *et al.* 1996), *Bacillus subtilis* (Song *et al.* 1989), and human (Caccianmani *et al.* 1991).

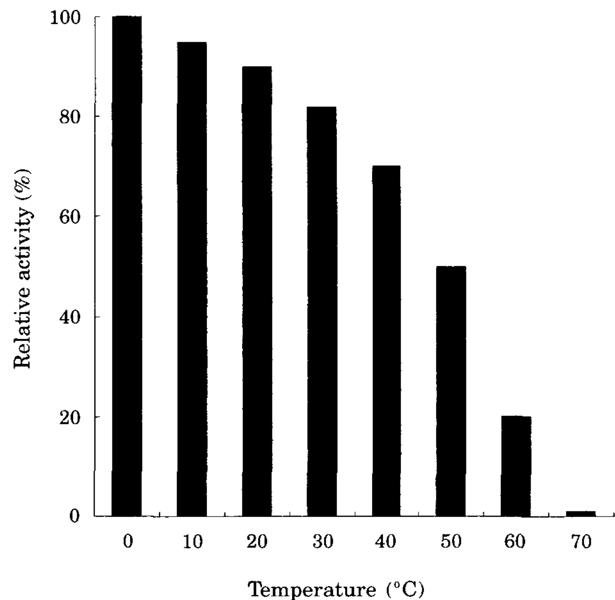


**Fig. 3.** Effect of temperature on stability of the cytidine deaminase.

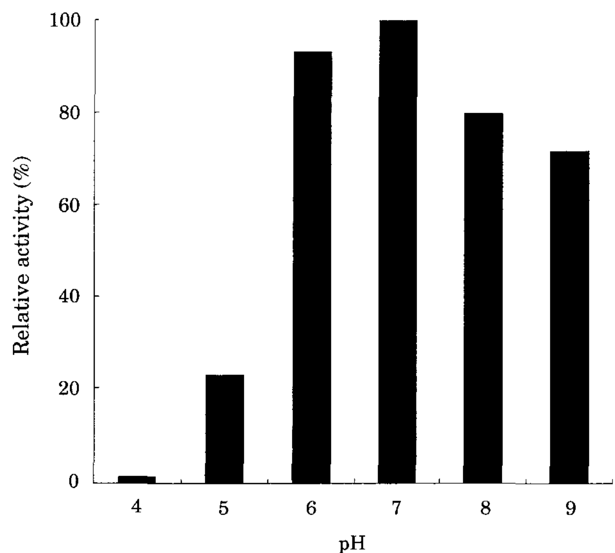


**Fig. 4.** Effect of pH on stability of the cytidine deaminase.

### 3. Temperature and pH stability

Fig. 3 shows the enzyme activity variation at various temperatures ranging from 30°C to 80°C. After preincubating the enzyme at a given temperature for 30 min, the remaining activity was assayed. The enzyme was labile to heat, complete inactivaion was observed at 70 °C. The heat stability of the enzyme also corresponded to previous results about the spleen (Rothman *et al.* 1978) and kidney (Tomchick *et al.* 1968) in mouse. The pH stability after preincubating the enzyme at given pH values at 37°C for 3 hrs was observed. The cytidine deaminase was stable at pH levels between 6 to 9, and the enzyme was inactivated sharply at the acidic pH (Fig. 4). The pH optimal for the cytidine deaminase was defined as around 7.0 and broad optimal pH range between 6 to 9 was revealed. This optimal pH range was similar to previous reports of yeast (Ipata *et al.* 1970),

mouse kidney (Tomchick *et al.* 1968), and *B. subtilis* (Song *et al.* 1988). These wide spectrums of pH ranges suggested that uridine and ammonia can be formed from cytidine even at the pH 9.2.

## 4. Effect of metal ions and reagents

The effect of various metal ions and reagents on the cytidine deamenase activity was determined by adding 0.1 mM to 1.0 mM of metal ions to the standard assay mixture (Fig. 5). The enzyme was slightly activated by the addition of $Mg^{++}$. $Hg^{++}$ inactivated the enzyme completely by addition of 1.0 mM concentration. $Cu^{++}$ and $Fe^{++}$ were shown to have a negative effect on the enzyme activity. The inhibition spectrum by heavy metal ions is considered one of widespread character in the *Salmonella* enzyme. The enzyme was completely inhibited by the addition of 1.0 mM pCMB to the reaction mixture. This result indicates some possibility for the presence of an active thiol group in the cytidine deaminase and/or indicates participation of the sulfhydryl group for multimer formation. Triton X-100 acted as a moderate inhibitor, while L-cysteine and DTT were not effected on the enzyme activity (Fig. 6). The inhibition pattern by mercaptoethanol on the enzyme was very similar with that of *B. subtilis* (Song *et al.* 1988). The enzyme is highly sensitive to mercaptoethanol, while it is not to dithiothreitol and EDTA. These results are also one of the similar characters adopted by various cytidine deaminase, suggesting that the enzyme molecule consists of a multimer formation.

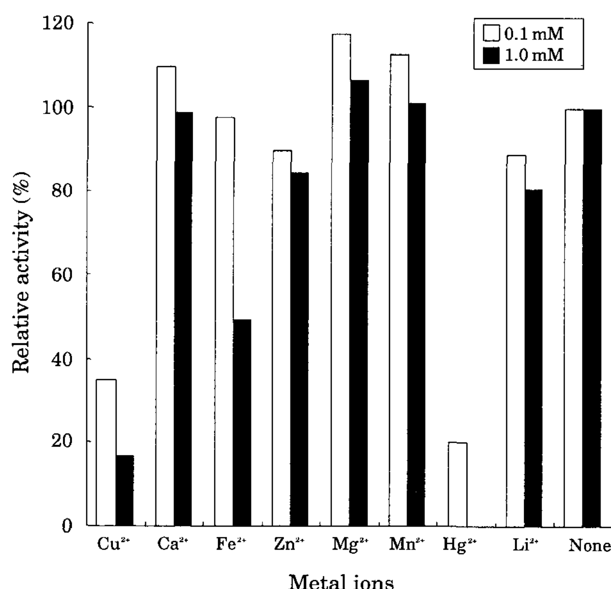


**Fig. 5.** Effect of metal ions on the cytidine deaminase activity.

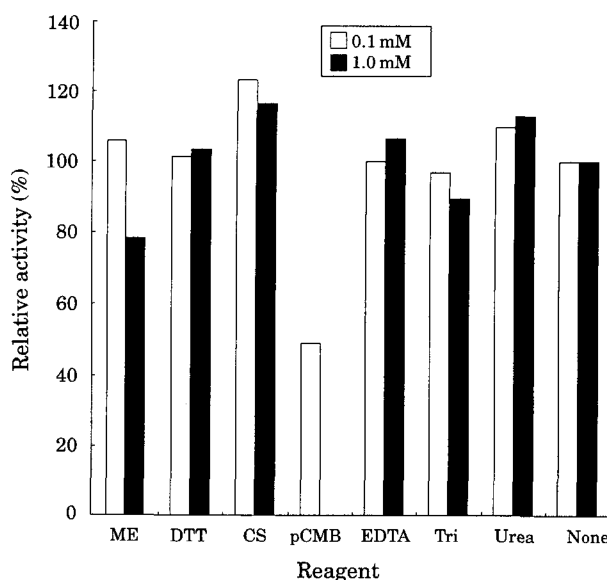


**Fig. 6.** Effect of various reagents on the cytidine deaminase activity. ME, mercaptoethanol; CS, Cysteine; Tri, Triton X-100.

## 5. Effect of cytosine analogues

As shown in Fig. 7, the compounds chlorodeoxy-, thio-, methyldeoxy-cytidine and Ara-C were not recognized as significant inhibitors at 1.0 mM concentrations. Fluoro- and Aza-cytidine somewhat activated the enzyme activity, while iodo- and bromo-(deoxy)cytidine inhibited it. The methylcytidine and 1-β-D-ribofuranosyl-4-sulfonyl-2-pyrimidine reacted as significant inhibitors to the enzyme activity in *E. col* (Wentwort and Wolfenden 1978). Cytidine and deoxycytidine acted as substrates for the *Salmonella* cytidine deamenase as shown in Fig. 8. Among the cytidine analogues, thio-, bromo(deoxy)-, methyl-, iododeoxy-, and fluorodeoxycytidine were metabolized moderately by the *Salmonella* cytidine deaminase while iodo-, chlorodeoxycytidine and cytosine were not deaminated. The cytosine analogues that reacted efficiently to *Salmonella* also had an affinity to the enzymes of *E. coli* and leukemic mouse spleen (Evans *et al.* 1975, Trimble and Maley 1971).

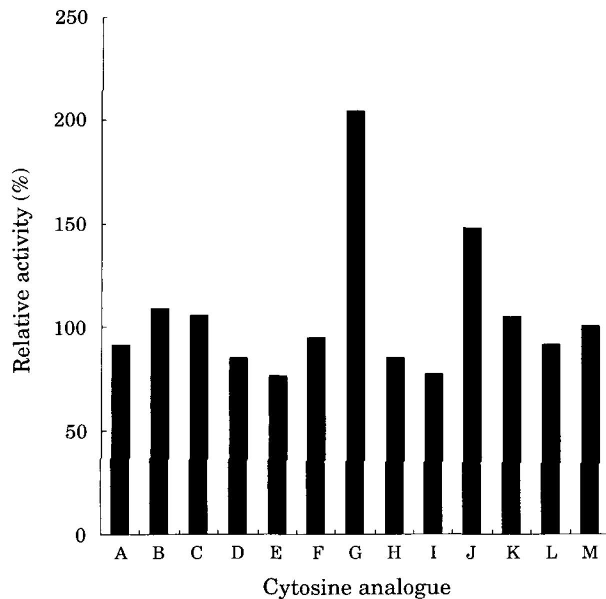


**Fig. 7.** Effect of cytosine analogues on the cytidine deaminase activity. A, Chlorodeoxycytidine; B, Thiocytidine; C, Ara-C; D, Bromodeoxycytidine; E, Bromocytidine; F, Azadeoxycytidine; G, Azacytidine; H, Iododeoxycytidine; I, Iodocytidine; J, Fluorodeoxycytidine; K, Methyldeoxycytidine; L, Methylcytidine; M, None.

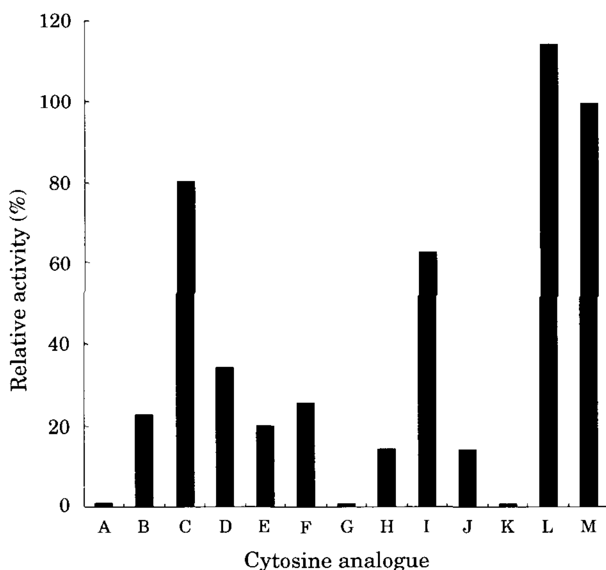


**Fig. 8.** Effect of cytosine analogues on cytidine deaminase activity as a substrate. A, Chlorodeoxycytidine; B, Thiocytidine; C, Ara-C; D, Bromodeoxycytidine; E, Bromocytidine; F, Iododeoxycytidine; G, Iodocytidine; H, Fluorodeoxycytidine; I, Methyldeoxycytidine; J, Methylcytidine; K, Cytosine; L, Deoxycytidine; M, Cytidine.

However, in the velocity of deamination rate, iodinated derivatives are faster than brominated ones in deamination by the enzyme of human liver cell, mouse kidney and leukemic mouse spleen (Wentwort and Welfenden 1975, Tomchick *et al.* 1968, Rothman *et al.* 1978) and these derivatives also revealed a different affinity in comparison with cytidine.

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