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Purification and Characteristics of Cytidine Deaminase from *Bacillus subtilis* ED 213

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The cytidine deaminase was purified by about 87 fold with a 10% yield from cell-free extract of *Bacillus subtilis* ED 213. The purified cytidine deaminase was confirmed to be pure, and showed a single band and a single peak on the polyacrylamide gel electrophoresis and HPLC, respectively. The molecular weight of the enzyme was determined to be about 56,000 by gel filtration and consisted of four identical subunits having molecular weights of about 14,600 by SDS-polyacrylamide gel electrophoresis. The isoelectric point of the enzyme was pH 4.27 and the absorption spectrum of the enzyme has a maximum at 274 nm and a minimum around 250 nm. The enzyme was relatively stable between pH 6.0 and pH 8.0, however, it was unstable at 50°C for 10 minutes resulting in 50% of the normal activity. The cytidine deaminase catalyzed the deamination of deoxycytidine, 5-methylcytidine, 5-fluorodeoxycytidine, 5-bromocytidine besides cytidine.

KEY WORDS □ **cytidine deaminase, *Bacillus subtilis* ED 213**

Cytidine deaminase (cytidine/deoxycytidine aminohydrolase, EC 3.5.4.5) catalyzes the irreversible hydrolytic deamination of various cytosine nucleosides to the corresponding uracil nucleosides (20) and the enzyme is widely distributed in microorganisms (12, 22). But the only two microorganisms which have been established to be devoid of the activity are *Pseudomonas acidovorans* and *Neisseria meningitidis* (18).

The synthesis of the enzyme is highly inducible in *E. coli* and *S. typhimurium* (10). The inducer is cytidine which acts by binding the repressor protein encoded by the unlinked *cyt*R gene synthesis (25). However, cytidine deaminase is not inducible in *B. subtilis* (18).

The *cdd* genes were cloned and characterized from *B. subtilis* (23), from *S. typhimurium* (15), from *B. stearothermophilus* (5) and from *E. coli* (14). In animal cells, the enzyme has been partially purified in preparations, from the liver (8), in normal and leukemic granulocytes (4), and in lymphoblasts (21). However the human placental cytidine deaminase has been purified homogeneously and the human placental enzyme was determined to be 52-KDa oligomeric protein composed of four apparently idenitical subunits (3).

In microorganisms, the enzyme has been partially purified from *E. coli* (2, 6, 11), from *S. typhimurium* (13, 15, 19) and from baker's yeast (12). Recently, the *E. coli* cytidine deaminase was purified homogeneously and the molecular weight of the *E. coli* enzyme was determined to be 56,000 by gel filtration, and it consisted of an oligomeric structure of two identical subunits of 33,000 by SDS-polyacrylamide gel electrophoresis (26), but it has not yet been purified from *B. subtilis*.

The purpose of this work was to purify the homogeneous cytidine deaminase and to characterize the *B. subtilis* enzyme.

MATERIALS AND METHODS

Strains

The transformant was *B. subtilis* ED 213 (*lys*, *cdd*$^{-1}$, *pyr*$^{-2}$) which harbored pSO 100 carrying the *cdd* gene originated from the wild type *B. subtilis* 168 (23).

Media

Luria broth (LB) (17) containing 0.5% glycerol and 10 μg/ml kanamycin was used as a complex media for bacterial growth. Minimal medium for *B. subtilis* was Spizizen minimal medium (SMM) (24), which contains 5 g of glycerol, 2 g of $(NH_4)_2SO_4$, 14 g of K_2HPO_4, 6 g of KH_2PO_4, 1 g of sodium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.27 mg of $MnSO_4 \cdot 4H_2O$, 0.2% casamino acids, lysine (50 μg/ml), cytidine (40 μg/ml) and kanamycin (10 μg/ml) per 1 *l* of distilled water.

Culture conditions

The bacterial cells were cultivated in 4 *l* of LB medium containing 0.5% glycerol and 10 μg/m*l* kanamycin adjusted to pH 7.0 in a 5 *l* jar-fermentor at 30°C for 24 hrs and Silicone KM-70 was added as an antiformer.

The cells were harvested by centrifugation at 5,000×*g* for 10 min and were washed twice with 0.9% saline solution. The growth was determinated by measuring the optical density of the culture at 660 nm.

Preparation of cell-free extract

The washed cells were suspended in 0.05 M potassium phosphate buffer (KPB, pH 7.0) containing 5 mM 2-mercaptoethanol, and disintegrated by sonicator (Lab-line Co.) below 10°C and centrifuged to remove cell debris at 9,000×*g* for 20 min. The resultant solution was referred to as the cell-free extract.

Cytidine deaminase activity

Cytidine deaminase activity was determinated by the procedure of Hammer-Jespersen *et al.* (9). One unit is defined as the amount of enzyme which will deaminate one nano mole of cytidine per min at 37°C.

Protein assay

Protein determination was performed by the method of Lowry *et al.* (16) using bovine serum albumin as a standard.

Electrophoresis

Polyacrylamide gel electrophoresis was performed by a modification of Davis's method (7). 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 10B (Merck), and electrophoretically destained and stored in 7% acetic acid. Sodium dodecyl sulfate (SDS)-electrophoresis was performed according to Weber and Osborn's method (29) in 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), and electrophoretically destained and stored in 7% acetic acid.

High performance liquid chromatography (HPLC)

Another method of protein purity was performed with HPLC (Jasco) on a C_8 reverse column with a linear gradient of CH_3CN containing 0.1% trifluoroacetic acid from 10 to 100 % at flow rate of 0.5 ml/min. Protein in the elute was monitored at 280 nm with a UV detector (Uvidec-100-V, Japan Spectroscopic Co. Ltd.).

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 (2.7×81 cm) previously equilibrated with 0.05 M KPB (pH 7.0). The standard proteins used were myoglobin (MW 17,200), papain (MW 23,000), egg albumin (MW 45,000), bovine serum albumin (MW 68,000) and alcohol dehydrogenase (MW 150,000). SDS-polyacrylamide gel electrophoresis in the presence of 0.1% SDS was also employed for the determination of the molecular weight of subunits of the enzyme according to the Weber and Osborn's method (29). The standard proteins used were from a kit of standard proteins of low molecular weight; phosphorylase (MW 94,000), bovine serum albumin (MW 68,000), ovalbumin (MW 45,000), carbonic anhydrase (MW 31,000), trypsin inhibitor (MW 21,500) and α-lactoalbumin (MW 14,400).

Isoelectric point

Isoelectric points were determined at 4°C using a Rotofore cell (Bio-Rad Co.) in a pH 3.5~10.0 ampholyte (Bio-Rad Co.). 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 total volume of 50 m*l* containing the purified enzyme, 2% ampholyte (pH 3.5~10.0) and 5 mM 2-mercaptoethanol, and adjusted at a constant current of 12 W.

After loading the sample for 12 hrs at 4°C, 20 fractions were collected from the focusing chamber, and the pH and the enzyme activity of fractions were immediately tested.

Chemicals

A standard kit of low molecular weight for electrophoresis was purchased from Pharmacia K.K. DEAE-cellulose, Sephadex G-100, DEAE-Sephadex A-50 and Phenyl-Sepharose CL-4B were obtained from Sigma Co.

RESULTS AND DISCUSSION

Purification of the cytidine deaminase

The stepwise purification of the enzyme is summarized in table 1. Unless otherwise indicated, all the purification steps were carried out at 4°C, and all buffers used contained 5 mM 2-mercaptoethanol.

Step 1. Ammonium sulfate fraction: To the cell-free extract, solid ammonium sulfate was added to give 40% saturation and the pH was adjusted 7.0. After standing for 4 hrs, the resulting precipitate was removed by centrifugation at 9,000×*g* for 10 min and discarded. And then solid ammonium sulfate was added to the supernatant to give 75% saturation and allowed to stand for 4 hrs. The resulting precipitate was collected in the same manner and dissolved in the minimal volume of 0.05 M KPB (pH 7.0), and dialyzed overnight against 50 volumes of the same buffer.

Step 2. DEAE-cellulose column chromatography: The dialyzed enzyme was applied to a DEAE-cellulose column (1.8×60 cm) equilibrated with the same buffer (pH 7.0). The column was washed with the same buffer, which removed much of the inactive protein. The enzyme was eluted in linear gradient with the same buffer containing from zero

Table 1. *Purification of the cytidine deaminase from B. subtilis ED 213.*

Purification steps	Total activity (mg)	Total activity ($\times 10^4$ units)	Specific activity ($\times 10^4$ units/mg)	Fold	Yield (%)
Cell free extract	800	222.144	0.278	1.0	100
$(NH_4)_2SO_4$	352	166.038	0.472	1.6	74
DEAE-cellulose	120	133.286	1.111	4.0	60
Sephadex G-100	50	115.482	2.310	8.0	51
DEAE-Sephadex A-50	10	63.795	6.380	23.0	28
Phenyl-Sepharose CL-4B	0.95	22.966	23.175	87.0	10

to 1 M sodium chloride at flow rate of 15 m*l* per hour and 3.8 m*l* fractions were collected. The active fractions were combined and concentrated by the addition of solid ammonium sulfate to give 75% saturation. The precipitate obtained by centrifugation at 9,000×*g* for 10 min was dissolved in 0.05 M KPB (pH 7.0).

Step 3. Sephadex G-100 column chromatography: The concentrated enzyme was applied to a Sephadex G-100 column (2.7×81 cm) equilibrated with the same buffer (pH 7.0). The buffer was allowed to flow at a rate of 14 m*l* per hour and 3.8 m*l* fractions were collected. The active fractions were combined and concentrated by the addition of solid ammonium sulfate to give 75% saturation. The precipitate obtained by centrifugation at 9,000 ×*g* for 10 min was dissolved in 0.05 M KPB (pH 7.0) and was dialyzed overnight against 3 changes of 20 volumes of the same buffer.

Step 4. DEAE-Sephadex A-50 column chromatography: The concentrated enzyme was applied to DEAE-Sephadex A-50 column (1.8×60 cm) equilibrated with the same buffer (pH 7.0). The enzyme was eluted in linear gradient with the same buffer containing from zero to 1 M sodium chloride at flow rate of 10 m*l* per hour and 3.8 m*l* fractions were collected. The active fractions were combined and concentrated by the addition of solid ammonium sulfate to give 75% saturation. The precipitate obtained by centrifugation at 9,000×*g* for 10 min was dissolved in the same buffer (pH 7.0) and was dialyzed overnight against 3 changes of 20 volumes of the same buffer.

Step 5. Phenyl-Sepharose CL-4B column chromatography: Phenyl-Sepharose CL-4B was packed in a column (2.2×18 cm) and equilibrated with the same buffer (pH 7.0). The dialyzed enzyme was placed on the column and then allowed in reverse linear gradient with the same buffer containing from 1.7 to zero M ammonium sulfate at a flow rate of 12 m*l* per hour and 3.8 m*l* fractions were collected. The elution pattern is shown in Fig. 1. The cytidine deaminase from cell-free extract was purified by about 87-fold with about a 10% yield as shown in Table 1.

Homogeneity of the purified enzyme

As shown in Fig. 2, the purified cytidine

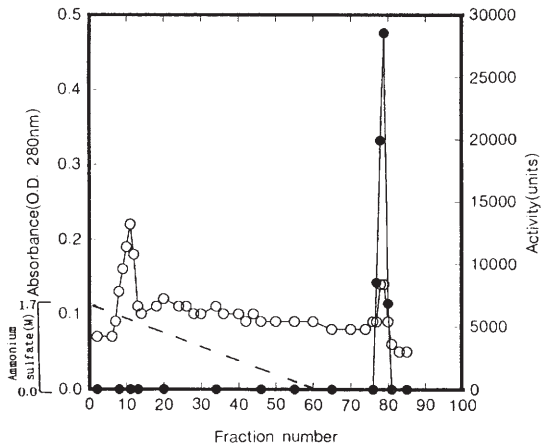

Fig. 1. *Elution profile of the cytidine deaminase on Phenyl-Sepharose CL-4B column chromatography.*
The active fractions from DEAE-Sephadex A-50 column were applied on Phenyl-Sepharose CL-4B column. The enzyme was eluted with linear gradient from 1.7 M to 0 M ammonium sulfate in 50 mM KPB (pH 7.0). ○, protein; ●, the cytidine deaminase activity.

deaminase showed a single band on acrylamide gel electrophoresis in absence of sodium dodecyl sulfate (SDS). Moreover, the enzyme appeared a single peak in the HPLC using C_8 reverse column as shown in Fig. 3. Those results indicated that the purified cytidine deaminase was homogeneous.

UV absorption spectrum

The ultraviolet absorption spectrum of the enzyme is as shown in Fig. 4. A typical UV spectrum for protein and absorption maximum and minimum were found at 274 nm and 250 nm, respectively. These results were consistent with the absorption spectrum of purified cytidine deaminase from *E. coli* B (26) and from chicken liver (27).

Substrate specificity

With various nucleotide-related substances, the

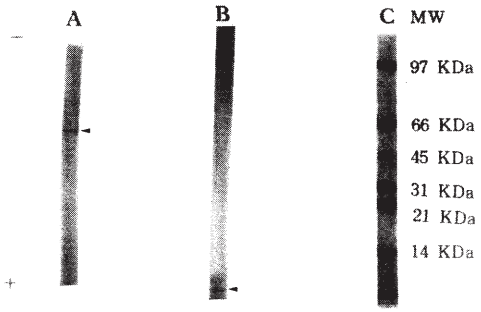

Fig. 2. *Polyacrylamide gel electrophoresis of the purified cytidine deaminase in the absence (lanes A) or presence (lanes B and C) of sodium dodecyl sulfate.*
Lanes A and B were purified cytidine deaminase and lane C was the standard proteins. The standard proteins used and their molecular weights are listed from top to bottom; myosine (97,400), bovin serum albumin (66,2000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400).

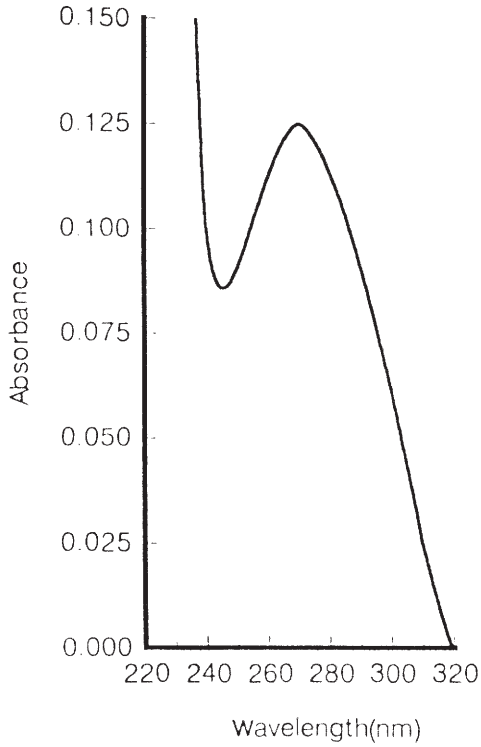

Fig. 4. *Ultraviolet absorption spectrum of the purified cytidine deaminase.*

deamination activity of the enzymes was examined. The relative reaction rates of deamination are presented in Table 2. The cytidine deaminase catalyzed the deamination of deoxycytidine, 5-methylcytidine, 5-bromocytidine and 5-fluorodeoxycytidine besides cytidine. However, the enzyme revealed no deamination activity with cytosine-β-D-arabinofuranoside (Ara-C), 5-iodocytidine, aza-deoxycytidine and 2-fluoro-5-iodo-β-D-arabinofuranosyl cytosine (FIAC). The cytidine deaminase

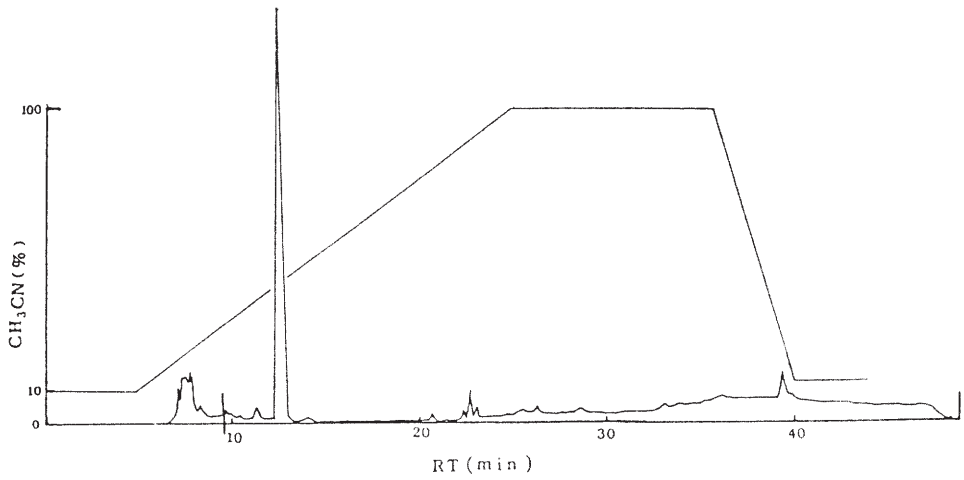

Fig. 3. *Identification of the purified cytidine deaminase by HPLC.*
Gradient profile of CH_3CN concentration was indicated by a line. Protein in the eluate was monitored at UV 280 nm. RT, retention time.

Table 2. *Substrate specificity of the cytidine deaminase.*

Cytidine analogue (1 mM)	Relative activity (%)
Cytidine	100
Deoxycytidine	110
5-Methylcytidine	100
5-Fluorodeoxycytidine	78
5-Bromocytidine	67
Ara-C	0
5-Iodocytidine	0
FIAC	0
Azadeoxycytidine	0

The cytidine deaminase activity was assayed under standard reaction conditions and the results were expressed as relative activity to that of cytidine. Ara-C, Cytosine-β-D-arabinofuranoside; FIAC, 2-fluoro-5-iodo-β-D-arabinofuranosyl cytosine.

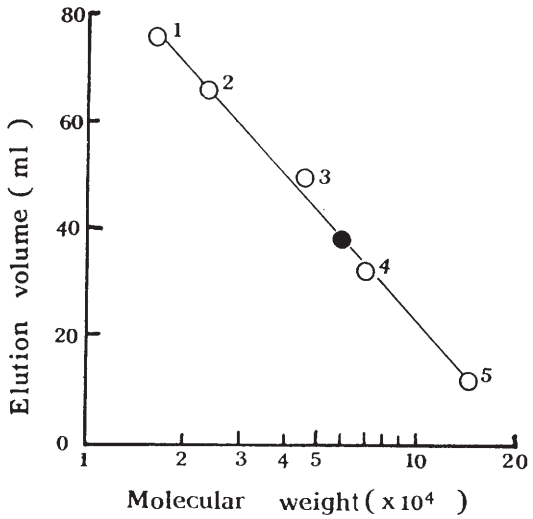

Fig. 5. *Determination of molecular weight of the cytidine deaminase by Sephadex G-100 gel filtration.*
The standard proteins used and their molecular weights were: 1, myoglobin (17,200); 2, papain (23,000); 3, egg albumin (45,000); 4, bovin serum albumin (68,000); 5, alcohol dehydrogenase (150,000); ○, standard proteins; ●, the cytidine deaminase.

from *E. coli* B was deaminated deoxycytidine and methylcytidine besides cytidine (26). The substrate specificity of the enzyme from *B. subtilis* ED 213 was more variable than that of the enzyme from *E. coli* B.

Molecular weight

The molecular weight of the cytidine deaminase was estimated to be about 56,000 by gel filtration,

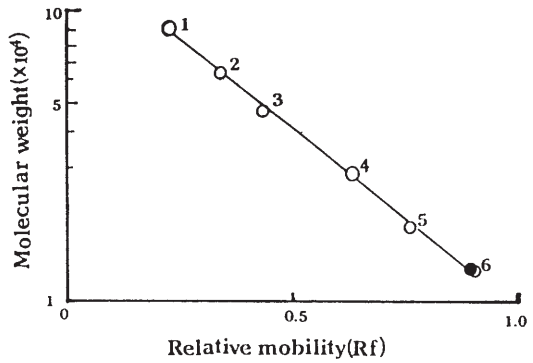

Fig. 6. *Determination of molecular weight of the cytidine deaminase by SDS-polyacrylamide gel electrophoresis.*
The standard proteins used and their molecular weights were: 1, myosine (97,400); 2, bovin serum albumin (66,200); 3, ovalbumin (45,000); 4, carbonic anhydrase (31,000); 5, soybean trypsin inhibitor (21,500); 6, lysozyme (14,400). ○, standard proteins; ●, the cytidine deaminase.

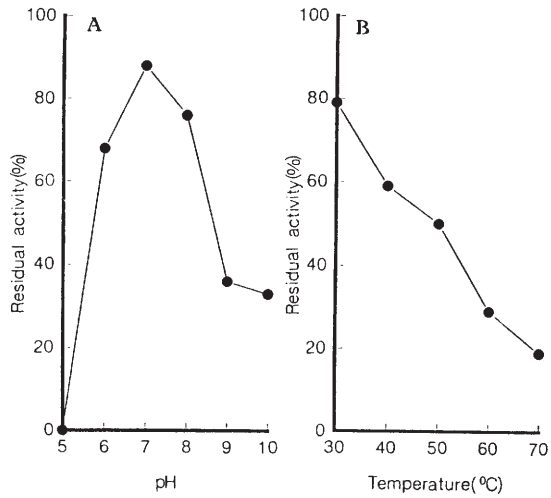

Fig. 7. *pH (A) and temperature (B) stability of the cytidine deaminase.*
A. Enzyme solutions were kept in 50 mM buffers of pH from 5.0 to 10.0 at 4°C for 24 hrs. The residual activity was assayed under standard conditions.
B. Enzyme solutions were kept in 50 mM buffers (pH 7.0), and were incubated at the indicated temperatures ranging from 30°C to 70°C for 10 min. After cooling, the residual activities were assayed under standard reaction conditions.

as shown in Fig. 5. The molecular weight of the enzyme was determined to be about 14,600 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, as shown in Fig. 6. From the above results, the cytidine deaminase from *B. subtilis* ED 213 should be composed of tetrameric identical subunits. This data corresponded well with that of Song and Neuhard (23) who calculated the molecular mass of 14,837 Da. from a nucleotide sequence of the open reading frame of *B. subtilis* cytidine deaminase gene. From the Stokes radius and the sedimentation constant of the enzyme, its molecular mass was estimated to be 58 kDa. This is very similar to the reported values for the purified enzyme from *E. coli* (26). Despite the similarities in molecular mass of the *B. subtilis* and *E. coli* holoenzyme, they differ in their subunit composition.

Isoelectric point

The activity and the pH profile of an elute from the isoelectric focusing column shows that one catalytically active component with an isoelectric pH value was present 4.27 (data not shown). A similar isoelectric point was reported for the purified enzyme from *E. coli* B, chicken liver, human granulocytes and human placenta (3, 26, 27, 28).

Stability of the enzyme

The purified enzyme was reasonably stable in pH range from 6.0 to 8.0 and the enzyme was relatively labile to thermal treatment, as shown by 50% inactivation through incubation at 50°C for 10 min (Fig. 7).

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초 록: *Bacillus subtilis* ED 2130이 생성하는 Cytidine Deaminase의 정제 및 특성

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Cytidine deaminase는 황산암모늄 염석(40～75%), DEAE-cellulose, Sephadex G-100, DEAE-Sephadex A-50, Phenyl-Sepharose CL-4B column chromatography로부터 10% 수율로, 약 87배 정제되었다. 정제 효소는 전기 영동과 HPLC 분석에 의하여 균일한 효소 단백질로 정제되었음을 확인하였다. 본 효소의 분자량은 gel 여과에 의하여 약 56,000으로 추정되었으며 SDS-polyacrylamide 전기 영동에 의한 분자량은 약 14,600으로 측정되어, 본 효소는 동일 분자량인 4개의 subunit로 구성된 tetramer로 추정되었다. 본 효소의 등전점은 pH 4.27이었으며, pH 6.0～8.0에서 비교적 안정하였으며, 50°C에서 10분간 열처리함으로써 효소 활성이 50% 실활되어 열 안정성이 비교적 낮은 효소였다. 본 효소는 cytidine 뿐 아니라 deoxycytidine, 5-methylcytidine, 5-fluorodeoxycytidine, 5-bromocytidine을 탈아미노화하였다.