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Enzymatic Properties of Cytidine Deaminase Encoded by *cdd* Gene in *Bacillus subtilis*

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*Bacillus subtilis*의 *cdd* 유전자에 의해 코드되는 Cytidine Deaminase의 효소학적 성질

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The cloned *B. subilis cdd* gene encoding cytidine/2'deoxycytidine deaminase (EC 3.5.4.5) was expressed in the *cdd* deficient *B. subtilis* mutant ED40. The gene was isolted from the *cdd* complementing plasmid pSO21, and inserted into the EcoR1/Pvu1 sites of pGB215-110 ΔB, which is a temperature sensitivie *E. coli-B.subtilis* shuttle vector. In the transformed *B.subtilis* ED40 harboring the resulting plasmid pSO100, *cdd* was expressed at several hundred fold elevated levels, and the cytidine deaminase activity in *E. coli* containing pSO100 was twice the level in *B. subtilis*/pSO100. The Km value for cytidine of the partially purified enzyme is 1.88×10^{-4}M at pH7.0 and the $V_{max} = 11.1$ μmol/min/mg of protein. The enzyme was completely inhibited by 0.1M mercaptoethanol and $HgCl_2$. The inhibition by *p*-chrolomercurybenzoic acid showed a Ki = 5 uM. These results suggest that sulfhydryl reagents block an active site thiol group, and/or disturb the formation of the tetrameic holoenzyme.

Cytidine deaminase(deoxycytidine/cytidine aminohydrolase, EC 3.5.4.5) encoded by the *cdd* microorganisms catalyzes the conversion of cytosine nucleosides to the corresponding uracil nucleosides (1). The enzyme is widely distributed in microorganisms (2,3) with the exception of *Pseudomonas acidovorans* and *Neisseria meningitidis* (4), in yeast (5), and in animals (6,7,8,9). The synthesis of the enzyme is highly inducible in *Escherichia coli*. The Inducer is cytidine which act by binding the repressor protein encoded by the unlinked *cytR* gene synthesis (10). Cytidine deaminase is not inducible in *Bacillus subtilis* (4).

The *cdd* gene of *B. subtilis* was cloned from a λD69 library of *B. subtilis* genes into *E. coli* cells on pBR322 and the nucleotide sequence of the gene including its promoter region was previously determined (11). The open reading frame of *cdd* consists of 408 base pairs encoding a 136 amino acids polypeptide with a calculated molecular mass of 14,837 Da. From the Stokes radius and the sedimentation constant of the enzyme its molecular mass was estimated to 58 kDa. This is very similar to the reported values for the homologous enzymes from *E. coli*. (54 kDa(12) or 57 kDa(13)). Despite the similarities in molecular mass of the *B. subtilis* and *E. coli* holoenzymes they differ in their subunit composition. The *B. subtilis* enzyme consists of four identical subunits of molecular mass 14,837, whereas the purified *E. coli* enzyme was reported to

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be composed of two identical subunits of molecular mass 35 kDa(12) or 33 kDa(13). Because of the unknown sequence of the *E. coli* enzyme, a comparison of amino acid sequences of the monomers in the two organisms is not yet feasible.

This reason was conducted to study the *B. subtilis* cytidine deaminase by cloning and expression of the *cdd* gene complementing shuttle plasmid, pSO100, in *Bacillus* itself. The enzymatic properties of the amplified gene product were also characterized.

Materials and Methods

Strains

The *Bacillus subtilis* strains employed were all derived from strain 168. They are listed together with the *Escherichia coli* K-12 strains in Table 1. A *pyr*⁻ *cdd*⁻ double mutant of *E. coli* can utilize cytidine as sole pyrimidine source, but it cannot utilize deoxycytidine(4).

Media and growth conditions

Luria broth(14) was used as a complex media for bacterial growth. Minimal medium for *E. coli* was AB medium(15). For *B. subtilis* MG1 and MG2 media were employed for transformations and for transductions(16). Spizizen minimal medium(17) supplemented with 5 μM $MnSO_4$, 0.2% L-glutamate and 0.4% glucose was employed for *B. subtilis* minimal media were supplemented with appropriate requirements, antibiotics and glucose (0.2%) or glycerol(0.2%) as a carbon source. For the selection of *cdd* positive cells, uracil(20 μg/ml), cytidine or deoxycytidine (40 μg/ml) and antibiotics (ampicillin 50 μg/ml, tetracyclin 10 ug/ml, and kanamycin 10 μg/ml) were added to the medium. When required, 0.2% vitamin free casamino acids were added to the minimal medium. Most of the reagents were purchased from either KOSCO Biotech., Sigma Co. or Boehringer Mannheim.

DNA techniques

The restriction endonuclease digestions and T_4 DNA ligase reactions were performed according to the manufacturers specifications. For the isolation of plasmid in μg quantities from *E. coli* transformants, single colonies were transferred into 5 ml of L-broth supplemented with antibiotics and cultured for overnight at 37℃. Plasmids were prepared by a modified alkaline/SDS lysis procedure(18). Transformation in *B. subtilis* was performed essentially as described by Boylan *et al.*(19) with minor alterations(16). *E. coli* was made competent for transformation as described by Maniatis(20). Restriction endonuclease, RNase, proteinase K, and T_4 DNA ligase etc. were purchased from Jechul Chemicals, Takara Shuzo Co, and Boehringer Manheim.

Cytidine deaminase assay

Crude cell extracts prepared from sonic disrup-

Table 1. Lists of bacteria and plasmid DNA used.

Bacterial strains	Genotype	Source
Escherichia coli		
SO 003	*met*B1, *rel*A1, *spo*T, *rps*L	Lab collection[a)]
JF 611	*pyr*E60, *cdd*, *thi*-1, *arg*E3, *his*-4, *pro*A2, *thr*-1, *leu*-6, *mdl*-1, *xyl*-5, *ara*-14, *gal*K2, *lac*Y1, *rps*L, *sup*E44	J. Friesen
SO3838	JF 611/pSO21 Ap^r	Lab collection
SO3856	JF 611/pSO100 Km^r	This work
Bacillus subtilis		
ED40	*pyr*-2, *cdd*-1, *lys*	Rima and Takahashi
ED213	*pyr*-2, *cdd*-2, *lys*/pSO100, Km^r	This work

a) Strain collection of Lab. of Microbiology, Dept. of Biology, Teachers College, Kyungpook University, Taegu.

tion and/or cultured medium were used as an enzyme source. Cytidine deaminase activities were determined by the procedure of Hammer-Jespersen *et al.*(20). One unit is defined as the amount of enzyme which will deaminate one nano mole of cytidine per min at 37°C. Protein determination was performed by the method of Lowry *et al.*(21) using bovine serum albumin as a standard. If there were not mentioned about the enzyme source in each section, partially purified enzyme from the cultured medium of concentrated culture was used.

Results

Expression *cdd* in *B. subilis*

The 1.2 kbp EcoR1/Pvul restriction fragment from the pSO21 containing the *B. stutilis cdd* gene was inserted into the corresponding sites of the shuttle vector,pGB215-110 ΔB(22), and the resulting hybrid plasmid pSO100(Fig. 1) was transformed into both *E. coli* JF611 and *B. subtilis* ED40. In both cases pSO100 complemented the *cdd* mutation of the host strains by enabling them to grow with deoxycytidine as the sole pyrimidine source. As

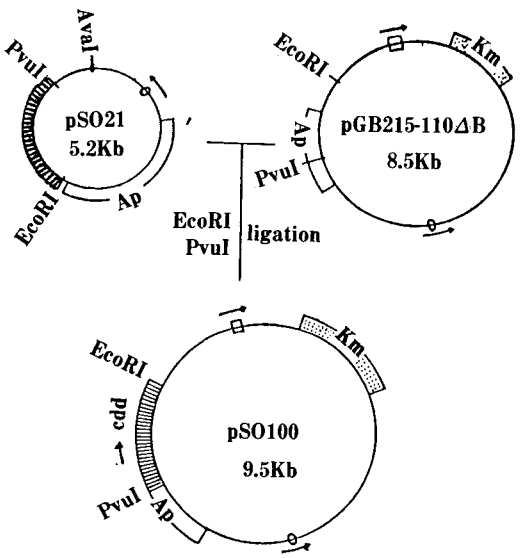

Fig. 1. Schematic diagram showing the construction of the shuttle plasmid pSO100.
The open segment(□) and the dotted segment(▨) indicate the genes for resistance to ampicillin(Ap) and kanamycin(Km), respectively. The hatched box(▨) indicates the *cdd* gene segment. Arrows indicate the direction of transcription of *cdd*(▨), and the replication origins for *B. subtilis*(⊖) and *E. coli*(⊖).

shown in Table 2 the levels of cytidine deaminase, in both *E. coli* and *B. subtilis* carrying the *cdd* gene on the multicopy plasmid pSO100, are very high compared to the basal levels observed in *E. coli* carrying the *cdd* gene in single copy on the chromosome.

Time courses of *cdd* expression in *B. subilis* ED213

Cytidine deaminase activity was almost undetectable in the wild type cells of *B. subtilis,* however, activity was present at high levels in the sonic extracts of *B. subtilis,* however, activity was present at high levels in sonic extracts of cells harboring pSO100(Table 2). The transformant strain ED213 produced cytidine deaminase from the mid of the logarithmic growth phase (Table 3). At this stage no

Table 2. Expression of *B. subtilis* cytidine deaminase in *B. subtilis* and *E. coli* harboring pSO100.[a]

Strain/Plasmid	Relevant genotype[b]	Cytidine deaminase Specific activity[c]
SO 003	cdd^+_{Ec}(wild type)	26
JF 611/pSO100	cdd/pcdd^+_{Bs}	7400
ED 40/pSO100	cdd/pcdd^+_{Bs}	3700

a) *E. coli* were grown at 37 °C in AB medium containing 0.2% glycerol, 0.2% casamino acids, 20 μg uracil per ml, and for pOS 100 carring strains also. 10 μg/ml of kanamycin. *B. subtilis* was grown in SMM medium supplemented with 0.2% casamino acids, and 20 μg/ml of cytidine, and 10 μg/ml of kanamycin.
b) cdd_{Ec} indicates the wild type *E. coli cdd* gene and cdd_{Bs} the wild type *B. subtilis cdd* gene.
c) Values the activity for hydrolyzing nanomoles cytidine deaminated per min per mg protein.

Table 3. Time course of cytidine deaminase production in *E. coli* SO3856 and *B. subtilis* ED213 harboring pSO100.[a]

Strains	*cdd*	Specific	activity[b]	
	Cultured Hours			
	9	12	24	48
E. coli SO3856	7655	7382	8054	7592
B. subtilis ED213	2797	3712	3770	3741

a) Culture media for both strains are the same as described in legend to Table 2. *cdd* units exhibit hydrolyzing activity for nanomoles of cytidine/min per mg protein.
b) cytidine deaminated permin per mg protein.

activity is found in the cultured medium. However, if cells in early stationary phase were concentrated 10-fold in fresh medium and cultured in the same conditions, a large amount of cytidine deaminase was found in the cultured medium. Whether this is the result of active secretion of enzyme through the cytoplasmic of intact cells membrane, or it is due to autolysis of cells in the concentrated cultures, has not yet been clarified. The enzyme was partially purified from the culture medium of the concentrated culture by ammonium sulfate precipitation and Sephadex column chromatography (Fig. 2).

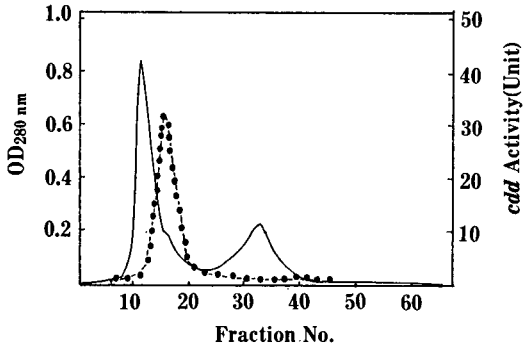

Fig. 2. Sephadex G-200 column chromatograpy pattern of cytidine deaminase.

After pricipitation of the concentrated cultured medium with 40-80% ammonium sulfate, the precipitate was dialysised and then applied to a Sephadex G-200 column (1.5 × 80 cm) equilibrated with 20 mM Tris buffer pH(8.0).Fractions of 2m*l* were collected with a flow rate of 22 m*l*/hr. (–) indicate protein in OD_{280} and *cdd* activity (● – ●).

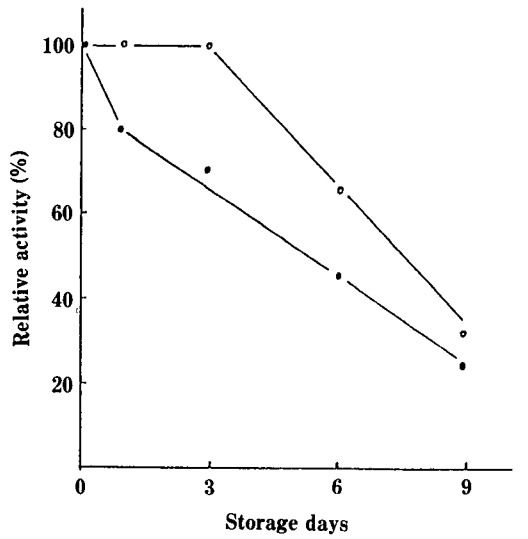

Fig. 3. Degradation of cytidine deaminase during storage at 4℃.

Sonic extracts of logarithmic cells of *B. subtilis*(○ – ○) and of *E. coli* (● – ●) were stored at 4C. At the indicated times, aliquiots were withdrawn, diluted 50-fold with 0.02M Tris-HCl buffer, pH 7.2 and the cytidine deaminase activities were measured.

Purification of cytidine deaminase

The cytidine deaminase was purified from the cultured medium of *B. subtilis* ED213 by conventional procedures. After ammonium sulfate precipitation, the *cdd* fraction was applied to a Sephadex G-200 column. The elution pattern is shown in Fig. 2. From the mobility of the enzyme in Sephadex G-200 the molecular mass was calculated to be 54 kDa(11). For further purification, the active pool of cytidine deaminase was transferred to a DEAE-sephadex column after concentration with ultrafilter O-1T (ULVAC Co. Japan); however, no activi-

Table 4. Inhibition of cytidine deaminase activity by various compounds.

Reagent	mM	*cdd* Specific activity[a]	Relative Activity (%)
None	—	3995	100
Mercaptoethanol	0.1	0	0
	1.0	0	0
DTT	0.1	4000	100
	1.0	3915	98
	10.0	3995	100
pCMB	0.05	319	8
	0.1	0	0
PMSF	0.2	3835	96
EDTA	1.0	4074	107
L-Cystein	0.2	958	24
Triton X-100	0.2	439	11
Urea	0.1	3755	94
	0.4	2676	67
	1.2	1757	44

a) A cytidine deaminase activity was assayed by using sonic extracts after preincubation with each compound for 10 min, at 37℃.Specific activity as in Table 3.

ty could be recovered from the eluate. It is not known yet whether the tetramer dissociating during this ion-exchange chromatographic step (Fig. 2).

pH optimum and heat stability

The pH dependence of cytidine deaminase in various buffers shows a pH optimum around 6.8 with 50% maximal activity at pH values of 5.0-8.0. When sonic extracts were stored at 4°C for 9 days, appreciable loss of activity was observed as shown in Fig. 3. Treating sonicates at 50°C inactivates cytidine deaminase rapidly.

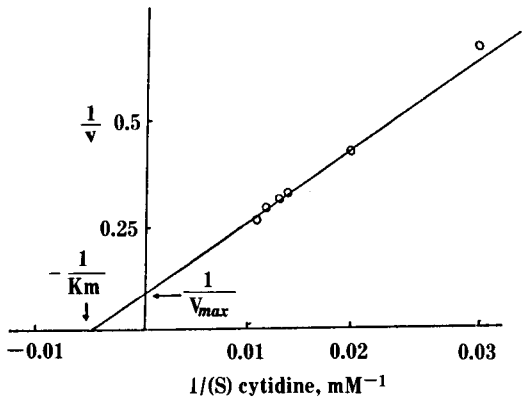

Fig. 4. Lineweaver-Burk plot of cytidine deaminase activity as a function of the concentration of cytidine.
The enzyme activity(v) is given as μ moles cytidine deaminated per min per mg protein.

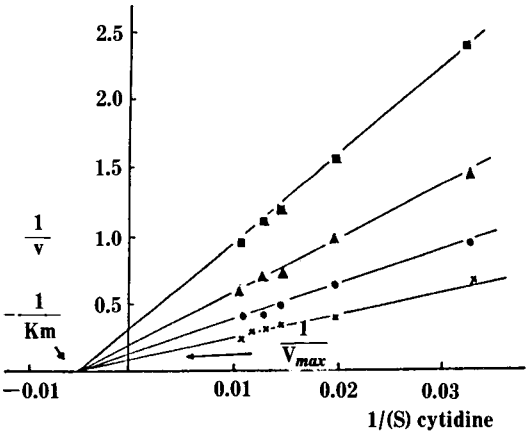

Fig. 5. Lineweaver-Burk plot of cytidine deaminase activity at different concentrations of *p*-chloromercuribenzoic acid(pCMB).
Enzyme activity(v) is given as hydrolyzing μmoles of cytidine deaminated per min per mg protein in the absence(x) and presence of 0.01(●), 0.02(▲), and 0.03(■) mM pCMB.

Kinetics of cytdine deaminase.

Double reciprocal plots of the initial rate of cytidine deamination versus substrate concentration were linear, yielding $Km^{-1} = 1.88 \times 10^{-4}$M at pH 7.0 and $V_{max} = 11.1\mu$mol/min/mg protein (Fig. 4).

Effectors of cytidine deaminase

In contrast to mammalian and yeast cytidine deaminase (5,23), *Bacillus* cytidine deaminase was completely inhibited by 0.1 mM mercaptoethanol. In contrast dithiothreitol (DTT) is without any effect. The sulfhydryl reagent *p*-chloromercurybenzoate(pCMB) also inhibited the enzyme completely. Phenylmethansulfonylfluoride(PMSF) frequently used as an inhibitor of serine proteases did not affect the enzyme activity. L-Cystein, Triton X-100 and high concentrations of urea inhibited the enzyme (Table 4). Calcium and magnesium ions did not inhibit, in fact it seems as if magnesium ions gave some stimulation(Table 5). Mercury and copper ions inhibit the enzyme action completely at concentration lower than 1 mM. The kinetics of pCMB inhibition of cytidine deaminase is shown in Fig. 5 as a lineweaver-Burk plot of enzyme activity versus cytidine concentration in the presence of different concentrations of pCMB. A non-competitive inhibition was found, in the standard assay with

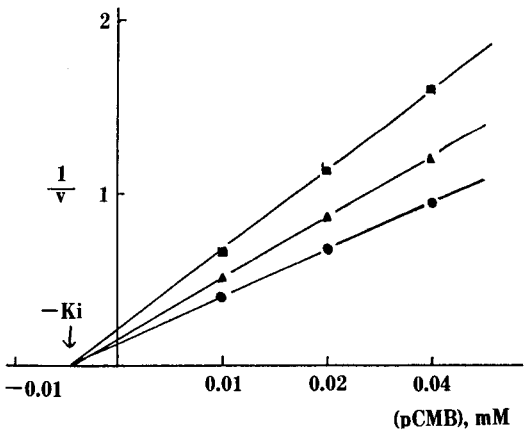

Fig. 6. Dixon plot for the determination of the inhibition constant(Ki) for *p*-chloromercuribenzoic acid on the cytidine deaminase activity.
Cytidine Concentrations: 50(■), 60(▲) and 70(●) μM, were used.

Table 5. Effect of metal ions on cytidine deaminase activity

Metals	Concentration (mM)	Specific Activity[a]	Relative Activity (%)
None	—	2610	100
$CuSO_4$	1.0	0	0
$CaCl_2$	1.0	3264	125
	10	2728	104
$FeCl_3$	1.0	2612	100
	10	2620	100
$ZnSO_4$	1.0	1660	64
	10	1300	49
$MgCl_2$	1.0	3999	152
	10	5160	197
$MnSO_4$	1.0	2670	102
	10	2858	109
$HgCl_2$	0.1	0	0

a) As Table 4.

low concentration of cytidine (Fig. 5), and the Ki value was determined to be 5 μM from a Dixon plot (Fig. 6).

Discussion

The *B. subtilis cdd* gene encoding cytidine/2'-deoxycytidine deaminase(EC 3.5.4.5) was expressed from a plasmid in the *cdd* deficient strain *B. subtilis* ED40. The gene was isolated from the *cdd* complementing plasmid pSO21 and inserted into the EcoR1/Pvu1 sites of pGB215-110ΔB, which is a temperature-sensitive *E. coli-B. subtilis* shuttle vector. The resulting plasmid, pSO100, was transformed into ED40 which can not utilize cytidine as a pyrimidne source(4). Accordingly, the transformed cells were selected on plates with Spizizen medium supplemented with cytidine and antibiotics. One such clone was retained as ED213.

The *cdd* gene was originally cloned from a XD69 library of *B. subtilis* genes by lysogenic complementation of an *E. coli cdd* mutation. Subsequently the *cdd* gene was transferred from λD69 $cdd_{B.\ subtilis}$ particles into the EcoR1 sites of pBR322 yielding pSO1.

Following subcloning and deletion of pSO1, the *cdd* complementing plasmid pSO21 was obtained as dscribed previously(26).

Late stationary, concentrated cultures of strain ED213, was shown to contain cytidine deaminase activity in the culture medium, wheras all the activity was intracellullar logarithmic cells.

The *B. subtilis* cytidine deaminase has a molecular mass of 58 kDa as determined by sucrose density gradient centrifugation and Sephadex G-200 column chromatography(11). From the nucleotide sequence of the *cdd* gene and from minicell experiment, the molecular weight of the cytidine deaminase subunit was shown to be 14 kDa. This means that the native enzyme may be a tetramer of identical subunits. This may be the reason why it was so difficult to purify in a homogeneous form. The monomer has 6 cysteine residues and is composed of 136 amino acids with a calculated molecular mass of 136 amino acids with a calculated molecular mass of 14837Da. The enzyme is completely inhibited by low concentrations of mercaptoethanol and pCMB. Accordingly, the cysteine residues in the structure may be very important for the molecular conformation of the tetramer and/or the enzyme contains an active site thiol. Whereas the cytidine deaminase from *E. coli* is similar to the *B. subtilis* enzyme in its inhibition pattern by heavy metals like mercury and copper ions and the dissociation patterns by urea, it differs significantly in its response to mercaptoethanol and pCMB(24).

The cytidine deaminase from *B. subtilis,* still grossly impure as described here, exhitits a Km value for cytidine similar to that of the *E. coli* enzyme(25). It is similarly inhibited by mercury and cupper ions. These results suggest that the sulfhydryl reagent block the active site thiol group, which contained six molecules of cysteine residues in the monomer structure, and/or disturbs the formation of the tetramer as a native enzyme.

Cytidine deaminase serves two different functions in metabolism. One is to scavange the pyrimidine moiety for nucleotide synthesis, and the other is catabolic leading to the formations of compounds which may serve as carbon and nitrogen sources. One might therefore expect that metabolites of cytidine or cytidine itself would influence

the expression of the *cdd* gene in *B. subtilis*. Although we have shown that the synthesis of the enzyme is not induced by cytidine(unpublished results), as it is in *E. coli* (10), it remains to be shown whether alterations in pyrimidine or nitrogen metablism may influence the rate of synthesis of the enzyme in *B. subtilis*.

요 약

고초균(*Bacillus subtilis*)의 cytidine/2'-deoxycytidine deaminase(EC 3.5.4.5)를 코드하는 *cdd* 유전자를 *cdd* 결손변이주 *B. subtilis* ED40에서 발현시켰다. 이 *cdd* 유전자는 *Bacillus*의 λD69 유전자은행으로부터 처음 클로닝된 것으로서 *B. subtilis-Escherichia coli*의 *shuttle vector* pGB 215-110ΔB의 EcoRI/PvuI 부위에 삽입시켰다. 형질전환된 ED40는 야생주에 비해 3700 unit의 강한 *cdd* 활성을 나타내었으며 이 클론된 백터 pSO100을 *E. coli*에서 발현시키면 *B. subtilis* 비해 2배의 강한 활성을 나타내었다. 겔 여과로 부분정제한 본 효소의 Km치는 1.88×10^{-4}M이었으며 Vmax=11.1 μmol/min/mg 단백이었다. 이 효소는 0.1M mercaptoethanol과 수은에 의해 완전저해되었으며 *p*-chloromercurybenzoic acid에 대해 Ki=5μM로 나타났다.

본 효소의 활성상실은 monomer에 함유된 6개의 cysteine 잔기의 일부가 활성단으로 작용하는 과정이 저해되었거나 tetramer로서의 회합과정이 저해되었기 때문인 것으로 추측되었다.

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