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Characterization of Cytosine Deaminase with Substrate Specificity to 5-Fluorocytosine

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5-Fluorocytosine에 기질특이성을 가지는 Cytosine Deaminase의 특성

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ABSTRACT: A cytosine deaminase from the cell-free extract of an isolate was examined after ethyl alcohol fractionation. The enzyme catalyzed the conversion of 5-fluorocytosine to 5-fluorouracil by the possession of specificity to the substrate. The optimum temperature and storage time on the stability of the enzyme were at below 50°C and near 2 days in tris-HCl buffer. The maximum activity was also presented at 9.0 in pH and 45°C in temperature. The pHs and temperatures for the enzyme activity ranged from 8.5-9.5 and from 40-50°C, respectively. The presence of Ag^+, Hg^{2+}, or Zn^{2+} in the reaction mixture resulted in the marked inhibition in the activity, but 1 mM of Fe^{3+}, K^+, or Na^+ increased the enzyme activity. The enzyme preparation was not affected by inhibitors used except N-ethylmaleimide of 1 and 10 mM, and considerably activated by 1 mM of pyrophosphate and 10 mM of phosphate.
KEY WORDS □ Cytosine Deaminase, Deamination of 5-Fluorocytosine.

After Hahn and Lentzel(1923), or Hahn and Schafer(1925) found firstly an endoenzyme, cytosine deaminase(cytosine aminohydrolase, EC 3.5.4.1) from yeast and *Escherichia coli,* the distribution of the enzyme in organisms, or in microorganisms and identification of the particular enzyme were investigated. Some suggestions were given with regard to the enzymes that extracts of animal tissues were unstable to degrade cytosine itself, or dietary cytosine, and that although cytosine deaminase was found in *Escherichia coli* as a cell-free extract, the properties didn't seem to be inherent in all strains.

The observations on the microorganism-originated cytosine deaminase were conducted after these findings.

From *Corynebacterium* and *Mycobacterium,* Hayaishi and Kornberg(1952) found that degradation of cytosine was done by deamination such as in soil bacteria described by Wang and Lampen in 1952.

More detailed cytosine deaminase have been since known by Kream and Chargaff(1952). They reported the partially purified preparations of cytosine deaminase in yeast and that cytosine was degraded with the production of uracil and ammonia and that, of many other pyrimidines, only 5-methylcytosine was converted to thymine and isocytosine acted as an inhibitor.

Besides the above investigations, Lara(1952a and 1952b) studied the induction of the enzyme of pyrimidine degradation from uracil and thymine substrate from *Nocardia corallina.* When cytosine or 5-methylcytosine were tested as substrate, it

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was postulated that cytosine deaminase was involved in a salvage pathway for pyrimidine nucleotide biosynthesis in yeast(Ipata *et al.*, 1971). After these researches, the enzyme that catalyzed the only cytosine from *Serratia marcescens* was reported, and other enzyme that catalyzed the deamination of both cytosine and 5-methylcytosine was also found(Sakai *et al.*, 1975a and 1975b).

The cytosine deaminases from these two bacteria were activated by phosphate and pyrophosphate but differed in the effects of some nucleosides and nucleotides on the enzyme activity(Yu *et al.*, 1976a and 1976b).

In other view points, deamination of 5-fluorocytosine, or its derivative to 5-fluorouracil or its derivative is now studied because of their antineoplastic agent. Nishiyama *et al.* (1985) reported the antineoplastic effects of 5-fluorocytosine in combination with cytosine deaminase. They observed the conversion of 5-fluorocytosine to 5-fluorouracil by the cell-free extract of *Escherichia coli* and the significant reduction of the brain tumor growth and cytotoxic changes without allergic reactions to the host.

However, because the above studies were completely limited to an endocellular enzyme originated from organism, it became specially important and necessary to find extracellular cytosine deaminase catalyzing the deamination of 5-fluorocytosine to 5-fluorouracil. In this regard, Yeeh (1985) reported the enzyme properties including the effects of enzyme inhibitors and Yeeh *et al.* (1985) did again the extracellular cytosine deaminase produced by *Arthrobacter* species. Besides there was a report on the properties of enzyme from *Corynebacterium*(Yeeh, 1986).

From the above backgrounds, the author continuously tried to obtain the more stable enzyme capable of catalyzing the deamination of 5-fluorocytosine by the possession of specificity to 5-fluorocytosine and found the endogenous enzyme originated from *Bacillus* species isolated from soil samples, and examined the properties of the particular enzyme.

Accordingly the author now reports here the experimental results.

MATERIALS AND METHODS

Chemicals

Cytosine and 5-fluorocytosine used were obtained from Sigma Chemical Company. All other chemicals employed through this work were guaranteed reagents, or products of the certified reagent grade such as extra pure reagent.

Microorganism and conditions of culture

Bacillus species isolated from soil was used throughout this work. This isolate was stored in Medium B at 4 °C as shown in Table 1 and used continuously in the experiments. For the constant production of cytosine deaminase, the enzyme levels were examined under some cultural conditions. As the cultural condition, precultivation was performed. First, one loopful of cells was inoculated in 10 m*l* of test tubes with Medium A (Table 1) without agar and the test tubes were cultivated at 30 °C for 24 hrs on the reciprocal shaker (110 rev. × 6 cm stroke). Second, 3 m*l* of the precultures were inoculated in 500 m*l* of shaking flasks containing 90 m*l* of medium B with 0.2% of NaCl and cultivated under aerobic conditions. The pH was adjusted to 8.0 with NaOH or HCl.

Preparation of crude enzyme solution

The grown cells of cultivated medium were centrifuged at 10,000 × g for 15 min. at 4 °C and the cells were harvested and washed twice with 0.85% of NaCl solution. The cells were suspended in 0.2 M tris-HCl buffer(pH 8.0). Then the cell suspension was subjected to sonication. Cells and debris were removed by centrifugation at 15,000

Table 1. *Composition of media for cultivation(pH 8.0)*

Medium for isolation(Medium A)	
Dextrose	0.1 %
Peptone	0.1 %
K_2HPO_4	0.05 %
Agar	1.7 %
Medium for stock culture(Medium B)	
Dextrose	1.0 %
Meat extract	0.1 %
Peptone	0.1 %
K_2HPO_4	0.05 %

× g for 30 min. The resulting supernatant was referred to as the cell-free extract and used as the crude enzyme preparation.

Substrate specificity

Cytosine, 5-methylcytosine, 5-fluorocytosine and other compounds as substrate were reacted with the enzyme solution and compared with one another. To make certain the presence, or absence of the substrate specificity, the reaction mixtures containing the fractionated enzyme solution with ammonium sulfate were examined by paper chromatography and spectrophotometry (Yeeh *et al.*, 1985).

Measurement of protein

The protein concentration was measured colorimetrically by the method of Lowry protein determination(Lowry *et al.*, 1951). An egg albumin was used as the standard protein.

Enzyme assay

The enzyme activity of cytosine deaminase to cytosine was measured by a spectrophotometric assay based on the differential absorption of cytosine and uracil(Kalcker, 1947). The activity of cytosine deaminase to 5-fluorocytosine was determined by the differential absorption spectra in the substrate and product at particular wave length (Yeeh *et al.*, 1985). One unit of the enzyme activity was expressed as enzyme quantity catalyzing the conversion of 1 μ mole cytosine or 5-fluorocytosine to uracil, or 5-fluorouracil under the standard assay system. The specific activity was expressed as μ moles of product formed per minute per mg of protein. The enzyme reaction was performed from 1 m*l* of reaction mixture containing 3 μ mole of substrate, 100 μ mole of tris-HCl buffer(pH 8.0) and appropriate volume of enzyme preparation.

Ammonium sulfate fractionation

The procedures of partial purification were carried out at 5 °C unless otherwise noted. Stirring gently the crude enzyme preparation with a magnetic stirrer, solid ammonium sulfate was slowly added to reach 0.2 saturation keeping pH 8.0 with 0.1 M NaOH. After standing for about 12 hours, the precipitate formed was removed by centrifugation at 15,000 × g for 30 min. and discarded.

The ammonium sulfate concentration then increased to 0.7 saturation by the addition of solid ammonium sulfate. After being kept overnight, the resulting precipitate formed was collected by centrifugation at 15,000 × g for 30 min. The collected pellet was dissolved in a minimal amount of 0.2 M of tris-HCl buffer (pH 8.0). The solution was dialyzed for 48 hours against the same buffer at 4 °C and the buffer solution for dialysis was changed three times. After dialysis, the solution was recentrifuged to remove the solid materials and the supernatant was used for the next experiment.

Ethyl alcohol fractionation

The cold 99% ethyl alcohol was slowly poured in the dialyzed enzyme solution with stirring to a final concentration of 45%(v/v) at less than 0 °C. After standing at −5 °C for 1 hour. The resultant precipitate was removed by centrifugation at 22,000 × g for 30 min. and discarded. Next, 1.5 parts by volume of cold 99% ethyl alcohol was added to 1 part by volume of the supernatant.

The resultant precipitate was collected by centrifugation in the same manner and dissolved in a minimal volume of 0.2 M tris-HCl buffer(pH 8.0) and the solution was dialyzed overnight against 3 changes of the same buffer.

RESULTS AND DISCUSSION

Ammonium sulfate and ethyl alcohol fractionation

For the preparation of cytosine deaminase, solid ammonium sulfate was added to 20-70% saturation to the crude enzyme solution and fractionated. As the results compared the fractionated enzyme with cell-free extract, the specific activity was increased to about 2.5 times and the yield was 77.1% as shown in Table 2. The specific activity of enzyme preparation after ethyl alcohol fractionation was increased to about 87 times of the culture filtrate.

Substrate specificity

The hydrolytic activity to various related substrates was tested. The enzyme solution fractionated with ethyl alcohol showed the hydrolytic

Table 2. *Partial purification of cytosine deaminase*

Step	Total protein (mg)	Total activity (units)	Specific activity	Yield
Culture filtrate	47400	62200	1.21	100
Ammonium sulfate	12600	48000	3.08	77
Ethyl alcohol	3250	32700	10.6	52

activity capable of catalyzing the deamination of cytosine, 5-methylcytosine, or 5-fluorocytosine into uracil, thymine, or 5-fluorouracil. Therefore these three compounds were likely to have the ability to serve as substrates for this fractionated enzyme as indicated in Table 3. But the hydrolytic activity was not observed in other compounds employed such as cytidine, adenine, guanine, or guanosine.

Stability of enzyme.

The partial purified preparation of enzyme solution was relatively stable in 0.2 M of tris-HCl buffer(pH 8.0) below 50 °C, but the stability on various temperatures was progressively decreased from above 60 °C and the residual activity at 70 °C was decreased to about 10% of the original one. These results that 20% of decrease in the original activity at 60 °C occurred, agreed relatively with those of other report(Yeeh *et al.*, 1985). The hydrolytic activity of this enzyme preparation was not completely vanished at 70 °C(Fig. 1).

The other properties were reported that

Table 3. *Substrate specificity of cytosine deaminase*

Substrate(3 mM)	Relative activity(%)
Cytosine	100
5-Methylcytosine	54
5-Fluorocytosine	80
Cytidine	0
Thymidine	0
Adenine	0
Adenosine	0
Guanine	0
Guanosine	0
5′-AMP	0
5′-CMP	0
5′-GMP	0

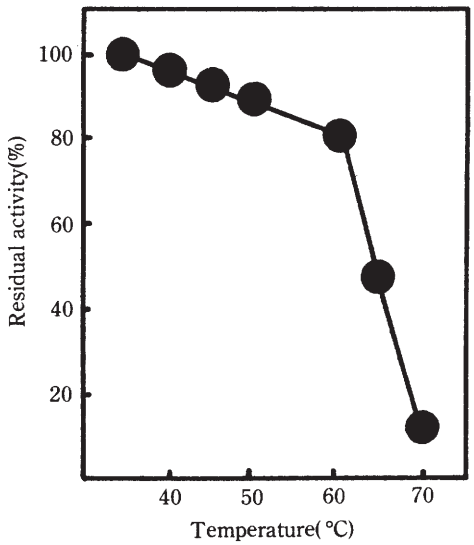

Fig. 1. *Effects of temperatures on the stability of cytosine deaminase (Enzyme solution in 0.2 M of tris-HCl buffer, pH 8.0, was incubated at various temperatures for 10 min. After being cooled, the mixtures were added to the substrate and assayed for residual activities)*

cytosine deaminase from *Serratia marcescens* (Sakai *et al.*, 1985a) maintained 75% of the original activity when treated at 70 °C for 10 min. and that cytosine deaminase from *Pseudomonas aureofaciens*(Sakai *et al.*, 1985b) did about 30% of the starting activity when treated at 60 °C for 5 min.

Therefore this enzyme is likely to stable to temperatures below 60 °C.

The effects of storage times on the stability of the enzyme were presented in Fig. 2. The enzyme preparation was stored in both 0.2 M of tris-HCl and 0.2 M of potassium phosphate buffer at 4 °C, respectively and the residual activity assayed was expressed as %.

This enzyme showed the better effects on the stability in tris-HCl buffer than in potassium phosphate buffer when being kept for 12 days. In tris-HCl buffer solution, the enzyme stability was nearly kept for 2 days, but decreased to about 15% of the starting activity after storage for 6 days. The maintenance of this enzyme in this buffer for above 6 days resulted in the rapid reduction in residual activity. These tendencies agreed relatively with other results exhibited by Yeeh *et al.*

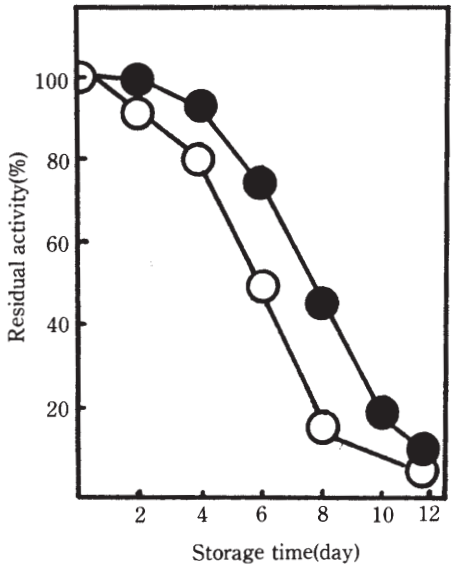

Fig. 2. *Effects of storage times on the stability of cytosine deaminase(The enzyme solution was stored at 4 C and then the residual activity was assayed).*
●, 0.2 M tris-HCl buffer(pH 8.0)
○, 0.2 M potassium phosphate buffer(pH 8.0)

(1985). Furthermore, the storage of the enzyme in 0.2 M of potassium phosphate buffer for above 6 days also resulted in the rapid reduction in the residual activity similar with that in tris-HCl buffer.

Effects of pHs and temperatures on the activity of the enzyme

The effects of changes in pHs on the activity of the enzyme were presented in Fig. 3. The cytosine deaminase activity was measured under the standard assay conditions except that pH values were varied by employing various buffer solutions. Various buffers of 0.2 M were used and tested for the effects of pHs on the activity of cytosine deaminase.

It was known that cytosine deaminase isolated from yeast(Kream *et al.*, 1952) showed the maximum activity at pH 6.0 to 9.0, and that other enzyme from *Pseudomonas aureofaciens*(Sakai *et al.*, 1975b) did the highest activity at pH 9.5 to 10.0. Furthermore, the maximum activity in cytosine deaminase from *Arthrobacter* species(Yeeh *et al.*, 1985) was presented at pH 8.0 to 8.5. But the enzyme showed the maximum activity at pH 9.0 and

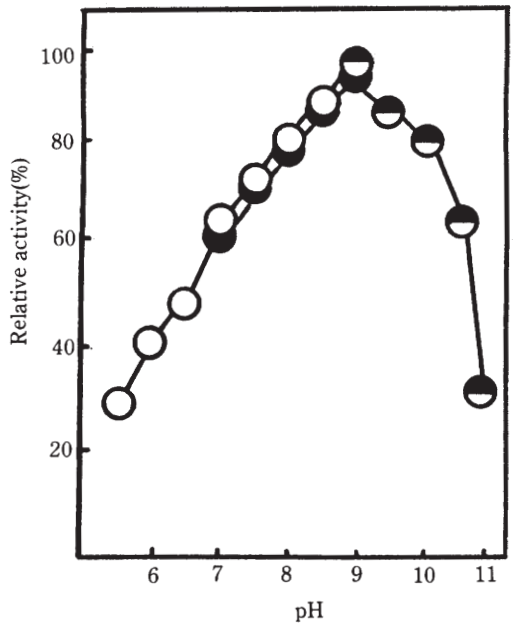

Fig. 3. *Effects of pHs on the activity of cytosine deaminase(0.2 M of each buffer was used).*
●, Tris-HCl buffer
○, Sodium phosphate buffer
◓, Glycine-sodium hydroxide-sodium chloride buffer

the range of pHs for optimum activity was 8.5 to 9.5.

The effects of temperatures on the activity of the enzyme were tested in 0.2 M of tris-HCl buffer, pH 8.0 and the results were presented in Fig. 4. Under the standard assay conditions except that the reaction temperatures were varied, the residual activities were compared. The maximum temperature for the activity was about 45 °C and the optimum temperatures ranged from 40-50 °C. These results were similar with those that the optimum temperature for the enzyme activity ranged from 40-50 °C in the enzyme of *Pseudomonas aureofaciens* and was around 45 °C (Sakai *et al.*, 1975a and 1975b). The enzyme activity was progressively reduced to 55 °C in lower rates according to the increase in temperature, but at below 35 °C and at above 55 °C, the enzyme activity was rapidly reduced and the relative activity of this enzyme was about 30% at around 70 °C.

From the above patterns, the enzyme was considered thermostable.

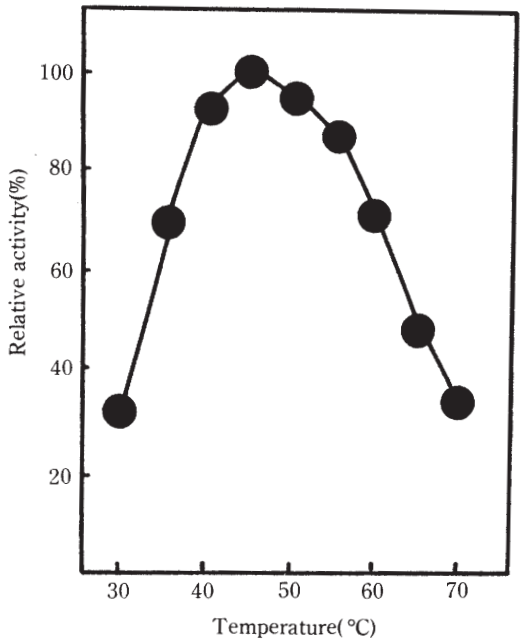

Fig. 4. *Effects of temperatures on the activity of cytosine deaminase(0.2 M of tris-HCl buffer, pH 8.0 was used).*

Table 4. *Effects of metal compounds on the activity of cytosine deaminase*

Metal compound	Relative activity(%)	
	1 mM	0.1 mM
None	100	100
$AgNO_3$	0	0
$BaCl_2 \cdot 2H_2O$	85	70
$CaCl_2 \cdot 2H_2O$	90	90
$CoCl_2 \cdot 6H_2O$	15	45
$Cr(NO_3)_2 \cdot 3H_2O$	60	58
$Cu(NO_3)_2 \cdot 3H_2O$	30	38
$FeCl_3 \cdot 6H_2O$	104	100
$HgCl_2$	0	0
KCl	106	104
$MgCl_2 \cdot 6H_2O$	85	80
$MnCl_2 \cdot 4H_2O$	90	80
NaCl	114	110
$NiCl_2$	40	24
$Pb(NO_3)_2$	15	20
$SnCl_2 \cdot 2H_2O$	24	38
$ZnCl_2$	0	0

Effects of metal compounds on the activity of cytosine deaminase

In order to examine the effects of metal compounds on the enzyme activity, the reactions were carried out at 1 mM, or 0.1 mM of various metal compound as exhibited in Table 4. The enzyme reaction was inhibited by some metal ions such as Ag^+, Co^{2+}, Cu^{2+}, Pb^{2+} Sn^{2+} and Zn^{2+}, and especially strongly inhibited by Ag^+, Hg^{2+} and Zn^{2+}. But the presence of 1 mM of $FeCl_3 \cdot 6H_2O$, KCl and NaCl in the reaction mixture increased the enzyme activity. This result corresponded generally with the reports from *Arthrobacter* species(Yeeh *et al.*, 1985) and *Pseudomonas* species(Sakai *et al.*, 1975b), but didn't with the other report that an enzyme from *Serratia* species was strongly inhibited by the presence of Mn^{2+}(Sakai *et al.*, 1975a). Therefore, from the properties of enzyme based on effects of metal compounds, the enzyme seemed to be slightly different from the other cytosine deaminases reported so far.

Effects of enzyme inhibitors and divalent anions on the activity of the enzyme

The reaction mixture contained various inhibitors and divalent anions as indicated in Table 5. Metal chelating reagents such as EDTA and o-phenanthroline didn't present heavy inhibitory effects on the enzyme activity even at the concentration of 10 mM, but N-ethylmaleimide showed strong inhibitory effect at both concentration of 10 mM and 1 mM. At the concentration of 10 mM, EDTA and monoiodoacetate as enzyme inhibitors accelerated the enzyme activity, but remarkable effects were not shown in reaction mixtures containing 10 mM of α, α'-dipyridyl, NaCN and TCA. The enzyme from *Arthrobacter* species revealed the strong inhibition to p-CMB, TCA, N-ethylmaleimide, EDTA, α, α'-dipyridyl and NaCN. The enzyme from *Arthrobacter* species (Yeeh *et al.*, 1985) was evaluated as that being highly influenced by the typical enzyme inhibitors. On the other hand, the enzymes from *Serratia* and *Pseudomonas* species were completely inhibited by p-CMB, but not inhibited by other enzyme in-

Table 5. *Effects of inhibitors and divalent anions on cytosine deaminase activity*

Inhibitor or divalent anion	Relative activity(%)	
	10 mM	1 mM
None	100	100
p-CMB[a]	85	90
α,α'-Dipyridyl	95	100
EDTA[b]	104	80
N-Ethylmaleimide	20	24
I_2	85	80
2-Mercaptoethanol	90	80
NaCN	100	100
NaF	58	60
o-Phenanthroline	85	100
TCA[c]	95	100
Monoiodoacetate	104	100
Borate	104	100
Carbonate	104	104
Nitrate	80	100
Sulfate	85	90
Sulfite	100	100
Phosphate	120	110
Pyrophosphate	85	160

[a]p-Chloromercuric benzoate
[b]Ethylenediaminetetraacetic acid
[c]Trichloroacetic acid

hibitors used in Table 5. Moreover the characteristic differences in enzyme property were observed. This enzyme preparation was not affected by most of enzyme inhibitors with the exception of N-ethylmaleimide of 10 mM and 1 mM. This properties to the typical enzyme inhibitors tested relatively resembled the other reports obtained from *Serratia marcescens* and *Pseudomonas aureofaciens*(Yu *et al.*, 1976a and 1976b). Moreover, phosphate and pyrophosphate activated the enzyme. The enzyme was 60% activated by 1 mM of pyrophosphate and 20% by 10 mM of phosphate. This result was alike in the effects examined in *Serratia* and *Pseudomonas* species. That is, Yu *et al.* (1976a and 1976b) reported that enzyme from *Serratia marcescens* was 70% activated by 1 mM of pyrophosphate and 50% by 10 mM of phosphate, and that enzyme from *Pseudomonas aureofaciens* was 30% activated by 1 mM of pyrophosphate and 30% by 10 mM of phosphate. They concluded that the enzyme activity didn't increase in the presence of phosphate beyond 50 mM and optimum concentration for activation were found to be 20 mM for pyrophosphate and 50 mM for phosphate.

The comprehensive interpretations of results from the experiments and comparison with the other reports indicated that this enzyme was considered more or less different from the other endocellular, or extracellular cytosine deaminase discussed so far in its enzymatic properties.

적 요

토양 분리균주의 세포추출물로 부터 얻은 cytosine deaminase 함유액을 황산암모늄 및 ethyl alcohol 분획후 그 효소성질을 검토하였다. 이 효소는 5-fluorocytosine에 기질 특이성을 가짐으로서 5-fluorocytosine으로의 전환을 촉매하였다. 본 효소의 안정성에 대한 온도 및 보존시간을 tris-HCl 완충액에서 검토한 결과 최적온도는 50℃ 이하에서, 보존시간은 2일정도가 가장 양호하였고 효소의 최대활성은 pH 9.0 및 45℃ 부근에서 나타났다. 또 효소활성에 대한 pH 및 온도의 범위를 검토한 결과 pH 8.5-9.5 및 40-50℃의 범위였다.

한편 이 효소는 Ag^+, Hg^{2+} 혹은 Zn^{2+} 이온을 첨가하면 그 활성이 강력하게 저해되었으나 1 mM의 Fe^{3+}, K^+ 혹은 Na^+은 효소활성을 촉진시키는 것으로 나타났다. 또한 본 효소는 사용한 대부분의 효소저해제에 대체로 영향을 받지 않는 것으로 보였으나 1 mM 및 10 mM의 N-ethylmaleimide에서는 저해되었으며, 특히 1 mM pyrophosphate 및 10 mM의 phosphate에 의해 그 효소활성이 현저히 촉진되었다.

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