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Properties of an Extracellular 5-Fluorocytosine Deaminase

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세포외 5-Fluorocytosine Deaminase의 특성

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Abstract — Some properties of an extracellular cytosine deaminase excreted from an isolate from soil samples were examined after 20~80% ammonium sulfate fractionation. The enzyme catalyzed the conversion of cytosine and 5-fluorocytosine into uracil and 5-fluorouracil by substrate specificity, respectively. The optimum temperature and storage time on the stability of the enzyme preparation were below 50℃ keeping above 90% of the residual activity and near 4 days keeping above 80% of the residual one in Tris-HCl buffer. The maximum activity was also obtained at 8.0 in pH and 37℃ in temperature. The pHs and temperatures for enzyme activity ranged from 8.0~8.5 and from 37~45℃, respectively. The presence of Ag^{+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Sn^{2+}, or Pb^{2+} in the reaction mixture resulted in the marked inhibition in enzyme activity, but 1 mM of K^{+}, Fe^{3+}, Mg^{2+}, or Na^{+} slightly increased the activity. The enzyme preparation was heavily affected by most of inhibitors tested such as 1 mM of EDTA, NaCN and pentachlorophenol, and completely inactivated by *p*-CMB and TCA of 1 mM, or 10 mM.

Hahn and Lentzel found firstly an endoenzyme, cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) from the cells of yeast and bacteria (1). After these findings, more detailed properties of this enzyme have been known by reports of other researchers (2-13). Although cytosine deaminase was found in microorganisms as a cell-free extract, it was suggested that the enzyme seemed not to be inherent in all strain.

In other points of view, deamination of 5-fluorocytosine (5-FC), or its derivatives is now studied because of antineoplastic agent and the mode of action with 5-fluorouracil (5-FC) was previously described (14). Recently, Nishiyama *et al.* (15) reported antineoplastic effects of 5-FC in combination with 5-fluorocytosine deaminase capsules in rats. They observed the deamination of 5-FC by this particular enzyme extracted from cultivated *Escherchia coli*, and the significant reduction of tumor growth and cytotoxic changes without allergic reactions to the host. From the above backgrounds, the authors found an exogenous 5-fluorocytosine deaminase excreted by an isolate assumed as *Corynebacterium* and the experimented results are now reported here.

Key words: Cytosine deaminase, 5-fluorocytosine
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Materials and Methods

Chemical

Cytosine used was product of Kohjin Co. and 5-FC was that of Sigma. The other chemicals were guaranteed reagents, or products of the certified reagent grade such as extra pure reagent.

Cultural condition

Corynebacterium Y-3, an isolate, was used throughout this work. As cultural conditions, precultivation was performed. Firstly, one loopful of cell mass was inoculated in 10 m*l* of test tube with medium A (Table 1) without agar and the test tubes were cultivated at 30℃ for 24 hours on the reciprocal shaker (110 rev. ×6 cm stroke). Secondly, 3 m*l* of the cultures were inoculated in 500 m*l* of shaking flasks containing 90 m*l* of medium B (Table 1) and cultivated at 30℃ for about 60 hours.

Preparation of crude enzyme solution

The cultured fluid was centrifuged at 10,000×g for 10 min. at 4℃ and the resulting supernatant was used as crude enzyme solution. The crude enzyme solution was subsequently fractionated with ammonium sulfate.

Substrate specificity

Cytosine, 5-FC, cytidine and other compounds as substrate were reacted with the enzyme. To make certain the presence, or absence of the substrate specificity, the reaction mixture containing the fractionated enzyme preparation with ammonium sulfate was examined by paper chromatography and the detection of product was exerted by ultraviolet lamp (short wave).

Measurement of protein

The protein concentration was measured colorimetrically by the method of Lowry protein determination with folin-phenol solution (16). As the standard protein, an egg albumin was used.

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

Medium for isolation (Medium A)	
Dextrose	0.10%
Peptone	0.10%
K_2HPO_4	0.05%
Agar	1.50%
Medium for stock culture (Medium B)	
Dextrose	1.00%
Malt extract	0.50%
Meat extract	0.10%
K_2HPO_4	0.05%

Ammonium sulfate fraction

Stirring gently the crude enzyme solution with a magnetic stirrer, solid ammonium sulfate was slowly added to reach 0.2 saturation keeping pH 8.0. After standing for about 12 hours, the precipitate formed was removed by centrifugation at 12,000×g for 20 min. The collected pellet was dissolved in a minimal amount of 0.2 M potassium phosphate buffer, pH 8.0 or tris-HCl buffer. The solution was dialyzed for 48 hours against the same buffers at 4℃ and the buffer solution for dialysis was changed three times. After dialysis, the solution was centrifuged again to remove the solid materials and the centrifuged solution was used for the next experiment.

Measurement of enzyme activity

The activity of 5-fluorocytosine deaminase was determined by the differential absoption spectra in the substrate and the product at particular wave length. One unit of the enzyme activity was expressed as enzyme quantity catalyzing the conversion of 1 μmole 5-FC to 5-FU under the standard assay system. The specific activity was expressed as μmoles of product formed per minute per mg of protein. The enzyme reaction was performed from 1 m*l* of reaction mixture containing 3 μmoles of substrate, 100 μmoles of Tris-HCI buffer (pH 8.0) and appropriate volume of enzyme preparation.

Results and Discussion

Ammonium sulfate fractionation

For the preparation of an extracellular cytosine deaminase, solid ammonium sulfate was added to 20~80% saturation to the crude enzyme solution and fractionated. As compared the fractionated enzyme solution with culture filtrate, the specific

Table 2. Partial purification of an extracellular cytosine deaminase

Step	Total protein (mg)	Total activity (units)	Specific activity	Yield
Culture filtrate	9.7	141	14.5	100
Ammonium sulfate (20~80%)	2.7	59	21.8	41

Table 3. Substrate specificity of an extracellular cytosine deaminase

Substrate (3 mM)	Relative activity (%)
Cytosine	100
5-Fluorocytosine	56
Cytidine	0
Thymidine	0
Adenine	0
Adenosine	0
Guanine	0
Guanosine	0
5′-AMP	0
5′-CMP	0
5′-GMP	0

activity was increased to about 1.5 folds and the yield was 41.8% as shown in Table 2.

Substrate specificity

The substrate specificities of the enzyme solution fractionated with ammonium sulfate were examined to various nucleic acid derivatives, including 5-FC. The enzyme solution was likely to have ability to serve as catalyser to cytosine and 5-FC. But the hydrolytic activity was not observed in other compounds employed such as cytidine, thymidine, adenine, adenosine, guanine, guanosine, 5′-CMP, or 5′-GMP (Table 3).

Stability of enzyme

The effects of temperature on the stability of the enzyme were provided in Fig. 1. The results were obtained from the following procedures. The enzyme solution in 0.2 M of tris-HCl buffer, pH 8.0, was incubated at the indicated temperatures for 10 min. After being cooled, the activity was determined. As the results of this determination, the enzyme seemed to be relatively stable below 50℃, but the stability was progressively decreased from above 55℃ and completely vanished around 70℃. These results agreed with those of other report (9) from *Arthrobacter* sp. JH-13. In the case of *Arthrobacter* strain, about 20% of the original activity was vanished at about 60℃, however, about 45% of the original activity was vanished from this enzyme at about 60℃. Therefore this enzyme seemed

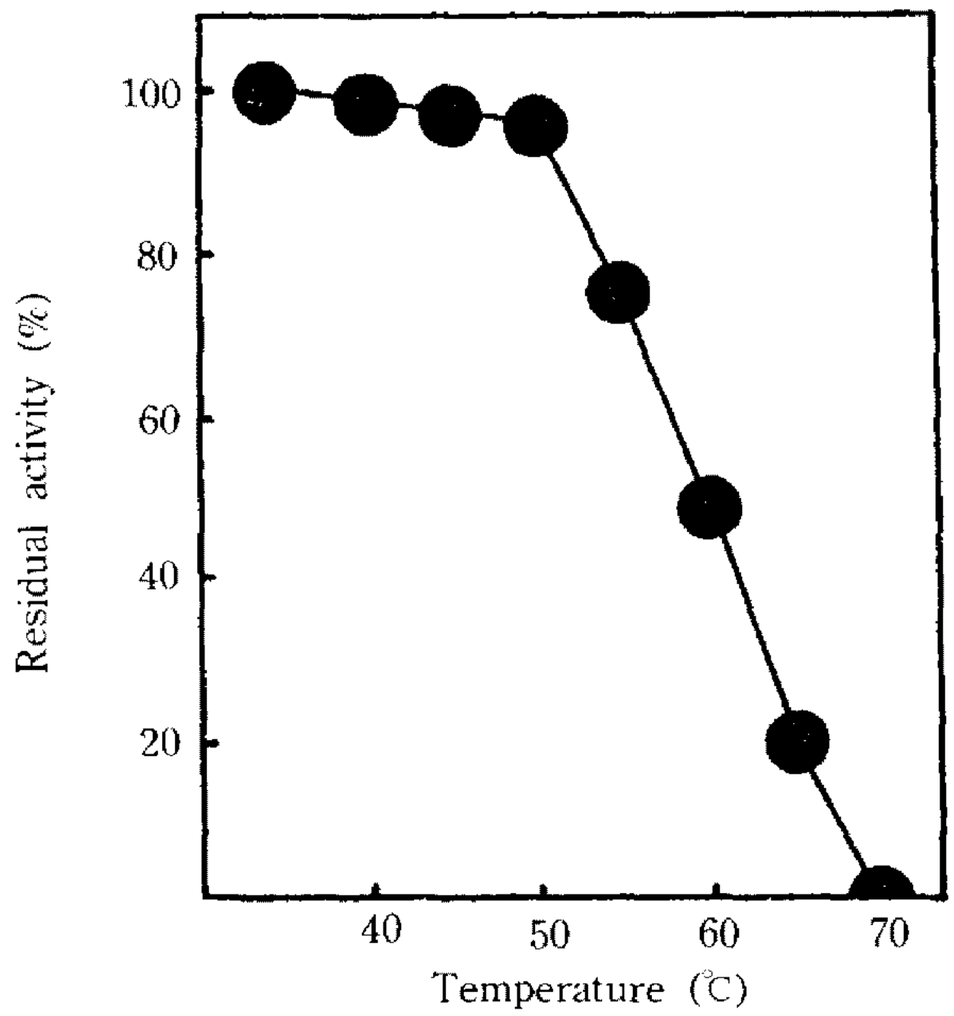

Fig. 1. Effects of temperature on the stability of an extracellular cytosine deaminase.

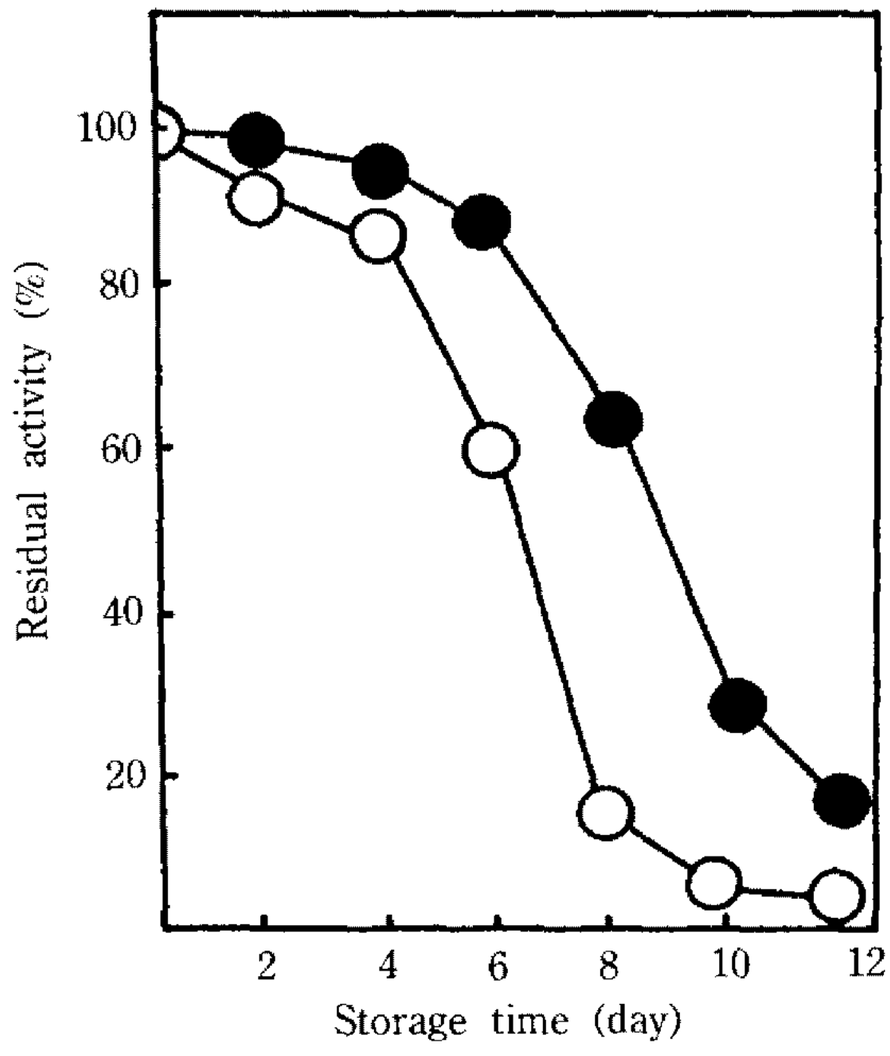

Fig. 2. Effects of storage time on the stability of an extracellular cytosine deaminase.
●, 0.2 M tris-HCl buffer (pH 8.0)
○, 0.2 M potassium phosphate buffer (pH 8.0)

to be generally stable to about 50℃.

The effects of storage time on the stability of this enzyme were exhibited in Fig. 2. The enzyme was stored in both 0.2 M of tris-HCl and 0.2 M of potassium phosphate buffer at 4℃, respectively and the residual activity was measured under the standard assay conditions. From Fig. 2, this enzyme

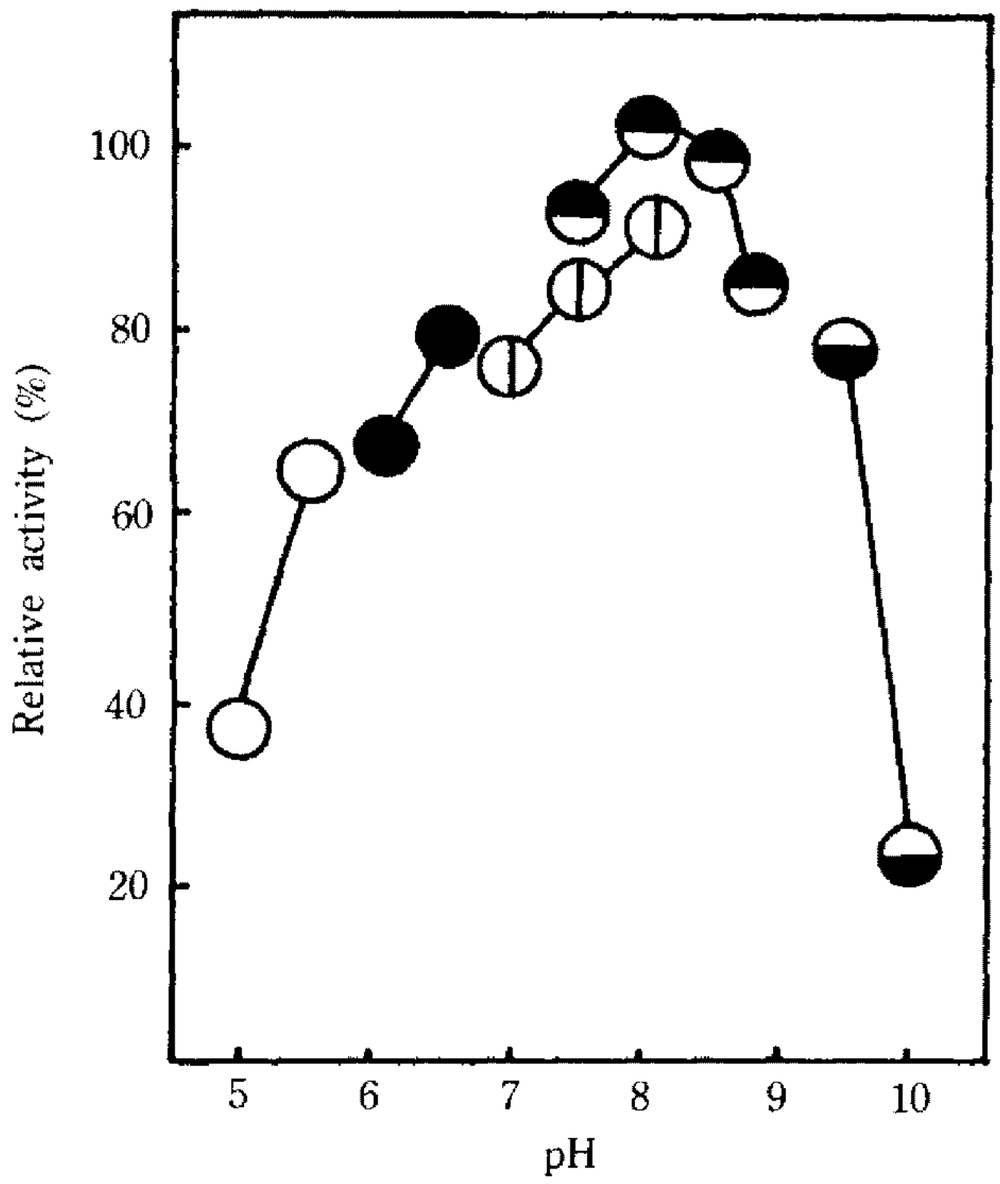

Fig. 3. Effects of the pH on the activity of an extracellular cytosine deaminase. 0.2 M of each buffer was used.

○, Sodium acetate buffer
●, Sodium phosphate buffer
◌, Potassium phosphate buffer
◓, Tris-HCl buffer
◒, Sodium carbonate buffer

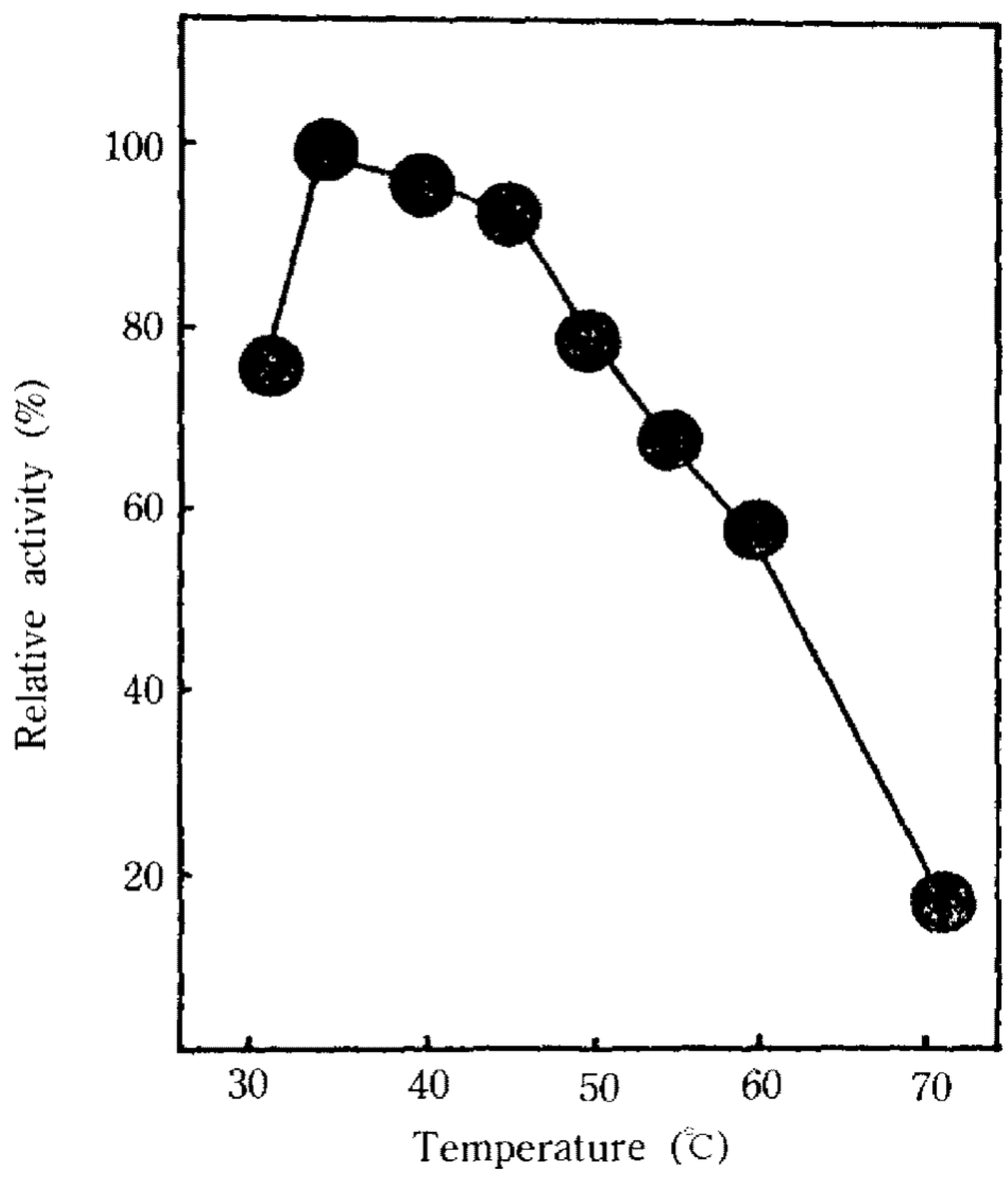

Fig. 4. Effects of temperature on the activity of an extracellular cytosine deaminase. 0.2 M of tris-HCl buffer (pH 8.0) was used.

showed general stability in tris-HCl buffer when being stored for 6 days and the residual activity was appeared to be above 85%. The storage of this enzyme for above 6 days in 0.2 M of tris-HCl buffer resulted in the rapid reduction of residual activity. When being stored for 4 days in potassium phosphate buffer, the residual activity was about 85%. The storage of this enzyme in this buffer for above 4 days resulted in the rapid reduction of residual activity not similar with that in tris-HCl buffer. Accordingly, when being compared the storage of this enzyme in tris-HCl buffer with that in potassium phosphate buffer, tris-HCl buffer seemed to be slightly more effective than potassium phosphate buffer in the stability of this enzyme. These tendencies did not agree relatively with other results exhibited by Yeeh and Park (12).

Effects of pH and temperature on the stability of the enzyme

Fig. 3 presented the results of the effects of changes in pHs on the activity of the enzyme. The enzyme activity was determined by the standard assay method except that pH values were varied. 0.2 M of various buffers were tested. It was reported that the pH showing the maximum activity of the endocellular cytosine deaminase was 6.0 to 9.0 from yeast (2) and 10.0 and 9.5 from *Pseudomonas aureofaciens* (6). Furthermore, the pH range of maximum activity in cytosine deaminase from *Arthrobacter* species (9) was 8.0 to 8.5. Being compared with the other endocellular enzymes, this extracellular enzyme showed different results. The range of pHs for maximum activity was from 7.5 to 8.5.

The effects of temperature on the activity of an extracellular cytosine deaminase were exhibited in Fig. 4. The buffer used in the test was 0.2 M of tris-HCl, pH 8.0 and the enzyme activity was determined under the standard assay conditions except that the reaction temperature was varied. The optimum temperature for the enzyme activity was from 40 to 45℃ in *Pseudomonas aureofaciens* (6) and around 45℃ in *Serratia marcescens* (5).

Table 4. Effects of metal ions on the activity of an extracellular cytosine deaminase

Inhibitor	Relative activity (%)	
	1 mM	0.1 mM
$AgNO_3$	0	0
$BaCl_2 \cdot 2H_2O$	84	62
$CaCl_2 \cdot 2H_2O$	90	74
$CoCl_2 \cdot 6H_2O$	33	62
$Cr(NO_3)_2 \cdot 3H_2O$	18	39
$Cu(NO_3)_2 \cdot 3H_2O$	3	25
$FeCl_3 \cdot 6H_2O$	101	91
$HgCl_2$	0	0
KCl	110	105
$MgCl_2 \cdot 6H_2O$	105	100
$MnCl_2 \cdot 4H_2O$	98	75
NaCl	103	96
$NiCl_2$	48	21
$Pb(NO_3)_2$	15	17
$SnCl_2 \cdot 2H_2O$	0	7
$ZnCl_2$	0	0
None	100	100

Table 5. Effects of enzyme inhibitors on the activity of an extracellular cytosine deaminase

Inhibitor	Relative activity (%)	
	10 mM	1 mM
p-CMB[a]	0	0
2,2′-Dipyridyl	20	34
EDTA[b]	10	15
N-Ethylmaleimide	3	2
I_2	20	31
2-Mercaptoethanol	50	91
NaCN	25	60
NaF	43	50
NaN_3	40	83
o-Phenanthroline	11	26
Pentachlorophenol	25	50
TCA[c]	0	0
None	100	100

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

Effects of metal ions on the activity of an extracellular cytosine deaminase

The effects of metal compounds examined on the activity of the enzyme were shown as in Table 4. The reaction mixtures contained various metal ions as indicated in the table. As shown in the table, the presence of 1 mM of K^+, Fe^{3+}, Mg^{2+} and Na^- in the reaction mixture increased more or less the enzyme activity. And some ions such as Ba^{2+}, Cr^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+} affected the enzyme activity by the dilution of the metal ions. Furthermore, the presence of Ag^+, Hg^{2+}, Pb^{2+}, and Zn^{2+} in the reaction mixtures showed the complete, or nearly complete inactivation of the enzyme activity. This result corresponded generally report from *Arthrobacter* (9), but didn't with the other reports from *Serratia marcescens* (5) and from *Pseudomonas aureofaciens* (6). It was reported that the enzyme activity from *Serratia marcescens* was inhibited by the presence of 0.1 mM of Mg^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Cd^{2+}, and also suggested that the enzyme from *Pseudomonas aureofaciens* was strongly inactivated by the presence of 1.0 mM of Cu^{2-}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+}.

Effects of enzyme inhibitors on the activity of an extracellular cytosine deaminase

This enzyme was completely inhibited in the presence of 1.0, or 10 mM of *p*-chloromercuric benzoate, N-ethylmaleimide and trichloroacetic acid, and also strongly inhibited in the presence of 10 mM of I_2, 2-mercaptoethanol, NaCN, NaF, 2,2′-dipyridyl and ethylenediaminetetraacetic acid. And in the cases of 2,2′-dipyridyl, NaN_3, o-phenanthroline and pentachlorophenol, the enzyme activity was increased as the dilution of the concentrations of these inhibitors was made. These properties of this enzyme to inhibitors tested were generally similar with the other results (9). In addition, it was suggested that the endocellular enzyme obtained from *Pseudomonas aureofaciens* (6) was not inhibited by other inhibitors with the exception of *p*-chloromercuric benzoate and that from *Serratia marcescens* (5) was not inhibited by some enzyme inhibitors tested in this experiment. Accordingly this enzyme was considered more or less different from the other endocellular, or extracellular enzyme reported so far in its enzymic properties.

요 약

토양 분리균주로부터 얻은 배양액을 20~80% 황산암모늄으로 분획한 후 세포외 cytosine deaminase의 성질을 검토하였다. 이 효소는 cytosine과 5-fluorocytosine(5-FC)에 기질 특이성을 가짐으로서 각각 uracil과 5-fluorouracil(5-FU)로의 전환을 촉매하였다. 효소안정성에 대한 온도와 보존시간을 tris-HCl 완충용액에서 검토한 결과 최적온도는 50℃ 부근이하에서 90% 이상의 잔존활성을, 보존시간은 4일정도에서 80% 이상의 잔존활성을 유지하였다. 최대활성은 pH 8.0과 37℃의 온도에서 나타났다. 활성에 적당한 pH는 8.0~8.5였고 온도는 37~45℃의 범위였다. 한편 이 효소는 Ag^{+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Sn^{2+}와 Pb^{2+} 등의 금속이온이 존재하면 그 활성이 강력하게 저해 되었으며, K^{+}, Fe^{3+}, Mg^{2+}, 또는 Na^{+} 등은 효소활성을 다소 증가시키는 것으로 보였다. 또한 이 효소는 1 mM의 EDTA, NaCN, 또는 pentachlorophenol 등과 같은 검증된 대부분의 효소저해제에 의해 그 활성이 상당한 영향을 받았으며, 특히, 1 mM과 10 mM의 *p*-CMB와 TCA 등에는 완전한 불활성화를 보였다.

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