PURIFICATION AND CRYSTALLIZATION OF ADENOSINE DEAMINASE IN *PSEUDOMONAS IODINUM* IFO 3558

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1. Introduction

The purification and properties of adenosine deaminase (adenosine aminohydrolase: EC 3.5.4.4), have been described from calf intestinal mucosa [1], takadiastase and various animal sources [2–9]. However, there are few studies of microbial intracellular adenosine deaminase. Recently, the attempt was made to purify the enzymes from the cytoplasm of *Halobacterium cutirubrum* and from the cytoplasm and the membrane of *Micrococcus sodonensis*, but these enzymes were unstable and could be purified only partially [10,11].

We describe here a purification and crystallization method for adenosine deaminase from *Pseudomonas iodinum* IFO 3558. We found that although adenosine deaminase in *Ps. iodinum* cells was quite unstable, it could be stabilized by ethyl alcohol. The enzyme was purified by ammonium sulfate and ethyl alcohol precipitation and column chromatography on DEAE-cellulose, Sephadex G-200, DEAE–Sephadex A-50, Sephacryl S-200 superfine and hydroxyapatite, in the presence of 15% ethyl alcohol. By these procedures, adenosine deaminase was purified 1800–2000-fold with a recovery of 25–30% activity originally present in the cell-free extract.

2. Materials and methods

2.1. *Materials*

All chemicals not otherwise noted were the products of Wako Pure Chemical Industry, Osaka and of certified reagent grade. Adenosine and related compounds were obtained from Sigma, St Louis.

2.2. *Enzyme assay*

Adenosine deaminase activity was assayed spectrophotometrically based on the differential absorption of adenosine and inosine at 265 nm. Enzyme activity was assayed at 37°C in a mixture containing 5 μmol adenosine, a suitable amount of the enzyme, 2 mmol ethyl alcohol, and 50 μmol Tris–HCl buffer (pH 7.0), in total vol. 1.0 ml. The reaction was initiated by the addition of the enzyme solution, and terminated by heating for 3 min in a boiling water bath. Inosine was determined by measuring A_{265} in 0.05 M Tris–HCl buffer (pH 7.0). One unit of activity represents the activity to form 1 μmol inosine in 1 h and specific activity represents the activity possess by 1 mg protein Protein concentration was measured by the method in [12], with bovine serum albumin as the standard.

2.3. *Gel electrophoretic analysis*

Acrylamide gel electrophoresis was performed by the method in [13].

2.4. *The microorganism and the conditions of culture*

Pseudomonas iodinum IFO 3558 was grown in a medium composed of 2% peptone, 1.0% starch, 0.2% NaCl, 0.1% KH_2PO and 0.1% K_2HPO (pH 7.0). The submerged cultures were grown at 30°C for 30 h in a 500 ml shaking flask containing 100 ml medium. The cells were washed twice with 0.85% NaCl solution and then with 0.05 M Tris–HCl buffer (pH 7.05).

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3. Results and discussion

3.1. *Purification procedure*

Table 1 summarizes the procedures used. All operations were performed at 4°C, unless otherwise noted, and the buffer contained 15% ethyl alcohol, except for steps 1 and 2.

3.1.1. Step 1

The washed cells were suspended in 0.05 M Tris–HCl buffer (pH 7.0), and subjected to the action of a sonic oscillator (20 kHz, Kaijyo Denki, Osaka). Debris was removed by centrifugation at 10 000 × *g* for 20 min and discarded. The resultant supernatant, referred to as the cell-free extract, was used as the crude enzyme preparation.

3.1.2. Step 2

To the cell-free extract, solid ammonium sulfate was added to give 0.35 saturation and the pH was adjusted to 7.0. After standing 2 h, the precipitate was removed by centrifugation at 10 000 × *g* for 20 min and discarded. The ammonium sulfate concentration was then increased to 0.65 saturation by the addition of solid ammonium sulfate. After 2 h, the precipitate was collected by centrifugation at 10 000 × *g* for 20 min, dissolved in 0.05 M Tris–HCl buffer (pH 7.0) and dialyzed overnight against about 20 vol. 0.05 M Tris–HCl buffer (pH 7.0) containing 10% ethyl alcohol.

3.1.3. Step 3

The dialyzed enzyme solution was next fractionated with ethyl alcohol and the precipitate which formed at concentrations of between 50–80% ethyl alcohol was collected, as follows:

To the dialyzed enzyme solution obtained in step 2 ethyl alcohol was added slowly, keeping temperatur below 0°C, to final concentration 50%. After standing at −20°C for 15 min, the precipitate was removed by centrifugation at 10 000 × *g* for 20 min and discarded. To the supernatant, ethyl alcohol was then slowly added to make final concentration 80%. The precipitate was collected and dissolved in 0.05 M Tris–HCl buffer (pH 7.35).

3.1.4. Step 4

The enzyme solution was applied to two columns (5 × 50 cm) of packed DEAE-cellulose equilibrated with 0.05 M Tris–HCl buffer (pH 7.35). The columns were then washed thoroughly with the same buffer, and then with buffer supplemented with 0.35 M NaCl. The enzyme was eluted with buffer supplemented with 0.5 M NaCl. Chromatography was carried out at flow-rate 2 ml/min and 15 ml fractions were collected.

3.1.5. Step 5

The active fractions were pooled and dialyzed against 0.05 M Tris–HCl buffer (pH 7.05) and applied to a column (2.8 × 45 cm) of packed

Table 1
Purification scheme for adenosine deaminase

Step	Total prot. (mg)	Total act. (units)	Spec. act. (units/mg protein)	Relative purification	Yield (%)
1. Cell free extract	84 056	724 186	8.6	1	100
2. Ammonium sulfate	32 000	694 847	21.7	2.5	96
3. Ethanol fract.	3700	566 145	153	17.8	78
4. 1st DEAE-Cellulose	518	456 826	882	102.6	63
5. 2nd DEAE-Cellulose	249	393 000	1579	183.6	54
6. Sephadex G-200	168	336 000	2000	232.6	46
7. DEAE-Sephadex A-50	46.2	286 154	6194	720.2	40
8. 1st Sephacryl S-200	24.5	240 000	9796	1139.0	33
9. 2nd Sephacryl S-200	14.5	236 153	16 286	1893.7	32
9. Hydroxyapatite	12.6	197 308	15 659	1820.8	27
10. Crystallization	9.5	148 390	15 620	1816.3	20

DEAE-cellulose equilibrated with 0.05 M Tris–HCl buffer (pH 7.05). The column was washed with Tris–HCl buffer (pH 7.05) containing 0.2 M NaCl, then chromatographed. The linear gradient was made up of two 4.5 column volumes, one of 0.2 M NaCl and the other of 0.7 M NaCl in Tris–HCl buffer (pH 7.05) with flow-rate 1 ml/min. Fractions, 15 ml, were collected, and those containing the enzyme were combined and concentrated to 10 ml by a collodion bag (Sartorius, Göttingen).

3.1.6. Step 6

The condensed enzyme solution was applied to two Sephadex G-200 columns (2.5 × 92 cm) which were equilibrated with 0.05 M Tris–HCl buffer (pH 7.05). The column was eluted with the same buffer at a flow-rate of 5 ml/h and fractions of 5 ml were collected. The fractions containing enzyme activity were pooled and dialyzed against 0.05 M Tris–HCl buffer (pH 7.5).

3.1.7. Step 7

The dialyzed enzyme solution was applied to a DEAE–Sephadex A-50 column (2.1 × 14 cm) which was equilibrated with 0.05 M Tris–HCl buffer (pH 7.5). After the column was washed with the same buffer, and then with the buffer containing 0.24 M NaCl and 0.28 M NaCl, the enzyme was mainly eluted with 0.32 M NaCl. The fractions containing enzyme activity were pooled and concentrated to 2 ml in a collodion bag.

3.1.8. Step 8

The concentrated enzyme preparation was applied to a Sephacryl S-200 superfine column (1.7 × 98 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.05). Chromatography was performed at flow rate 3 ml/h. Active fractions of 3 ml were collected and rechromatographed on the same column in the same manner.

3.1.9. Step 9

Active fractions were pooled and dialyzed against 0.04 M potassium phosphate buffer (pH 7.0). The dialyzed enzyme solution was applied to a hydroxyapatite column equilibrated with 0.04 M potassium phosphate buffer. All of the enzyme activity passed through the column and was pooled.

3.1.10. Step 10

Active fractions were pooled and concentrated to 3 ml in a collodion bag. Ammonium sulfate was added gradually to the enzyme solution until a faint turbidity was obtained. The pH of the solution was kept constant at around 7.0. On standing several days, rectangular plate crystals (fig.1) were formed.

3.2. *Properties of the enzyme*

Adenosine deaminase of *Ps. iodinum* was very unstable. However, as shown in fig.2, ethyl alcohol was found to protect the enzyme almost completely from inactivation. Ionic strength was also found to be a factor in protection from inactivation: low ionic strength caused inactivation of the enzyme even in the presence of 15% ethyl alcohol, although even high ionic strength could not substitute for ethyl alcohol.

Adenosine deaminase from *M. sodonensis* is unstable, particularly at low-protein concentrations (0.1–10 μg/ml). It requires Mg^{2+} or Mn^{2+} ions for stabilization [11]. However, stabilizing effects of neither Mg^{2+} nor Mn^{2+} were observed in the case of adenosine deaminase from *Ps. iodinum.* The purified enzyme is homogeneous according to the criteria of ultracentrifugation and SDS disc-gel electrophoresis (fig.3,4). Its sedimentation coefficient ($s_{20,w}$) is 8.0. The mol. wt was determined by the method in [14], using Sephadex G-200 gel and Sephacryl S-200 superfine gel filtration, with α-chymotrypsinogen A (25 000), ovalbumin (43 000), hemoglobin (68 000), alcohol dehydrogenase (140 000), bovine serum albumin (dimer, 136 000) and catalase (240 000) as

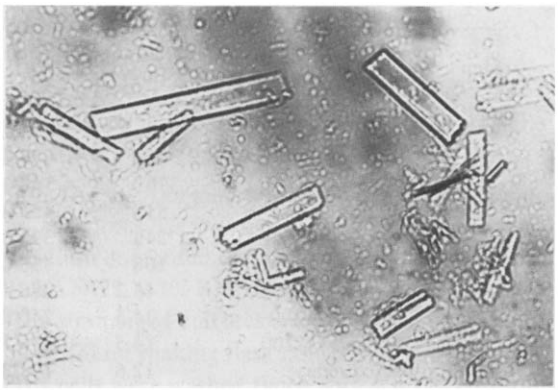

Fig.1. Crystals of adenosine deaminase.

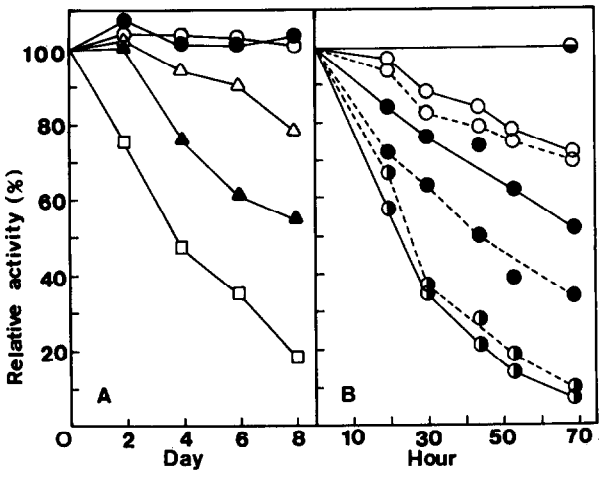

Fig.2. Effects of ethyl alcohol and ionic strength on the stability of adenosine deaminase. (A) The purified enzyme was stored at 4°C in 0.05 M Tris–HCl buffer, pH 7.0, containing different concentrations of ethyl alcohol: (○) 20%; (●) 15%; (△) 10%; (▲) 5%; (□) 0. (B) The purified enzyme was stored at 8°C in different concentrations of potassium phosphate: (- - ◒ - -) 0.05 M; (- - ○ - -) 0.02 M; (- - ● - -) 0.01 M; (- - ◑ - -) 0.001 M, or Tris–HCl buffer, pH 7.0; (—◒—) 0.05 M; (—○—) 0.02 M; (—●—) 0.01 M; (—◑—) 0.001 M, containing 15% ethyl alcohol. After storage for indicated period, activities were measured.

standard proteins. Different mol. wt values were obtained in the presence and absence of ethyl alcohol, 240 000 in the case of the former and 160 000 in the case of the latter. The enzyme easily dissociates into subunits during the course of polyacrylamide gel electrophoresis (fig.4). This phenomenon suggests that the stabilizing effect of ethyl alcohol may be in preventing the dissociation of the enzyme molecule. However, a detailed study was not performed and the role of ethyl alcohol is still unclear.

The mol. wt of adenosine deaminase from *M. sodonensis* has been reported to be 130 000, and the value is similar in size to that of the *Asp. oryzae* enzyme. In general, adenosine deaminase if obtained from animal origins is much smaller than that from microbial origins [11]. The enzyme from this source, *Ps. iodinum*, is the largest adenosine deaminase to be found so far.

The enzyme exhibits an A_{283} max with a shoulder around 290 nm and a minimum at 251 nm (fig.5). Th enzyme stoichiometrically converted adenosine to inosine. In addition to adenosine, 2′ and 3′-deoxy-adenosine, 2′,3′-isopropyridene adenosine and 2-amino-6-chloropurine riboside serve as effective

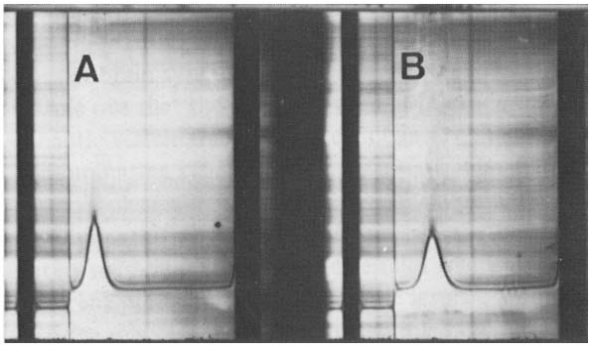

Fig.3. Sedimentation patterns of adenosine deaminase. Sedimentation patterns were obtained at 6 mg/ml protein concentration in 0.05 M potassium phosphate buffer, pH 7.0, containing 15% ethyl alcohol. Pictures were taken at 60 (A) and 80 min (B) after achieving top speed (52 000 rev/min).

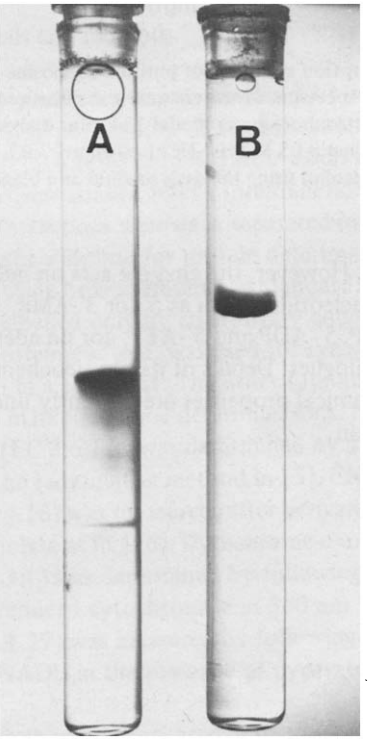

Fig.4. Acrylamide gel electrophoresis in the absence (A) or presence (B) of sodium dodecyl sulfate.

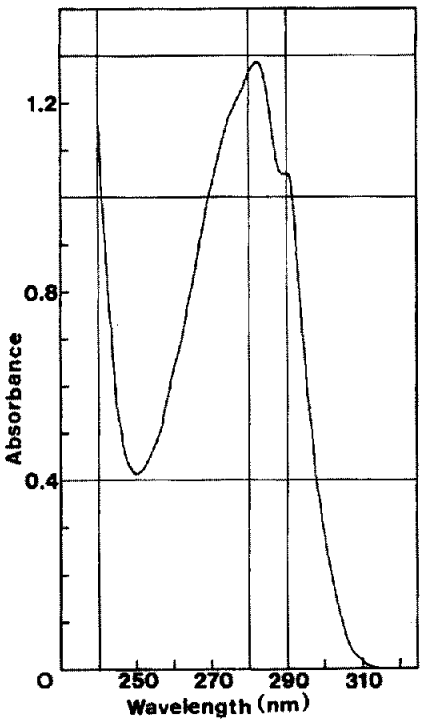

Fig.5. Absorption spectrum of purified adenosine deaminase. Absorption spectrum of the enzyme was measured with a Hitachi spectrophotometer model 124 after dialysis of the enzyme against 0.05 M Tris–HCl buffer, pH 7.05, containing 15% ethyl alcohol using the dialysis fluid as a blank.

substrates. However, the enzyme acts on neither adenine nucleotides, such as 5′- or 3′-AMP, 3′,5′-cAMP, 5′-ADP and 5′-ATP, nor on adenine and its analogues. Details of its physicochemical and biochemical properties are currently under investigation.

References

[1] Brady, T. G. and O'Connel, W. (1962) Biochim. Biophys. Acta 62, 216–229.
[2] Minato, S., Tagawa, T. and Nakanishi, K. (1965) J. Biochem. (Tokyo) 58, 519–525.
[3] Rockwell, M. and Maguire, M. H. (1966) Mol. Pharmacol. 2, 574–584.
[4] Coddington, A. (1965) Biochim. Biophys. Acta 99, 442–451.
[5] Hoagland, V. D., jr and Fisher, J. R. (1967) J. Biol. Chem. 242, 4341–4351.
[6] Piggott, C. O. and Brady, T. G. (1971) Biochem. J. 125, 112p.
[7] Akedo, H., Nishihara, H., Shinkai, K., Komatsu, K. and Ishikawa, S. (1972) Biochim. Biophys. Acta 276, 257–271.
[8] Bishop, S. H., Barnes, L. B. and Kirkpatrick, D. S. (1972) Comp. Biochem. Physiol. 43B, 949–963.
[9] Osborne, W. R. A. and Spencer, N. (1973) Biochem. J. 133, 117–123.
[10] Bauer, R. J. and Carlberg, D. M. (1973) Can. J. Biochem. 51, 621–626.
[11] Pickard, M. A. (1975) Can. J. Biochem. 53, 344–353.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Sakai, T., Yū, T., Tabe, H. and Omata, S. (1975) Arg. Biol. Chem. 39, 1623–1629.
[14] Andrews, P. (1965) Biochem. J. 96, 595.