PURIFICATION AND CRYSTALLIZATION OF L-ORNITHINE:α-KETOGLUTARATE δ-AMINOTRANSFERASE FROM *BACILLUS SPHAERICUS*

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1. Introduction

L-Ornithine δ-aminotransferase (L-ornithine:2-oxoacid aminotransferase (EC 2.6.1.13)) catalyzes the δ-transamination of L-ornithine with α-ketoglutarate to produce glutamic-γ-semialdehyde, which is spontaneously converted to Δ^1-pyrroline-5-carboxylate, and L-glutamate and plays an important role in ornithine and proline metabolism. This enzyme has been purified to homogeneity from rat liver and kidney, and its enzymological properties and physiological functions extensively studied [1–8]. Although microbial ornithine δ-aminotransferases have been demonstrated [9–11], little effort has been devoted to their purification and characterization. Here we describe the purification and crystallization of the enzyme from *Bacillus sphaericus* and some of its properties.

2. Materials and methods

Pyridoxal 5′-phosphate was obtained from Kyowa Hakko, Tokyo, DEAE-cellulose from Serva, Heiderberg, Sephadex G-150 from Pharmacia, Uppsala and polyethylenimine (mol. wt 60 000–80 000) from Nakarai Chemicals, Kyoto. Hydroxyapatite was prepared by the method in [12].

Ultracentrifugation was in a Spinco Model E ultracentrifuge, and the molecular weight of the enzyme determined by the sedimentation equilibrium method [13]. Disc gel electrophoresis was as in [14] except that 10% gel concentration was used.

The enzyme was assayed spectrophotometrically by a modification of the method for L-lysine-α-ketoglutarate ε-aminotransferase [15]. The standard reaction system consisted of 20 μmol L-ornithine, 20 μmol sodium α-ketoglutarate, 0.1 μmol pyridoxal 5′-phosphate, 50 μmol Tricine (*N*-tris (hydroxymethyl)methylglycine) buffer (pH 8.5) and enzyme in 1.0 ml final vol. Enzyme was replaced by water in a blank. After the mixture was incubated at 37°C for 20 min, the amount of Δ^1-pyrroline-5-carboxylate or L-glutamate formed was determined with *o*-aminobenzaldehyde or ninhydrin [16], respectively. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of one micromole of Δ^1-pyrroline-5-carboxylate or L-glutamate per minute. Specific activity was expressed as units per milligram of protein. Protein was determined by the Lowry method [17].

3. Results and discussion

3.1. *Purification of the enzyme*

Bacillus sphaericus IFO 3525 was grown in a medium composed of 0.4% L-arginine·HCl, 0.5% peptone, 0.2% glycerol, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl and 0.05% yeast extract (pH 7.2). The cultures were grown at 28°C for 15 h under aeration. The harvested cells were washed twice with 0.85% NaCl solution. All subsequent operations were performed at 0–5°C. The buffer used contained 10 μM pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol.

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Step 1. Enzyme extraction: The washed cells (~1 kg wet wt) were suspended in 4 liters 10 mM potassium phosphate buffer (pH 7.4), and disrupted continuously with Dyno-Mill (Willy A, Switzerland) at a flow rate 4 liters/h, followed by centrifugation. The supernatant solution was dialyzed against 50 liters of 10 mM potassium phosphate buffer (pH 7.4). The precipitate formed during dialysis was discarded.

Step 2. Polyethylenimine treatment: To the enzyme solution 0.1 ml 10% polyethylenimine solution (pH 7.4)/100 mg protein was added with stirring. After 30 min, the precipitate was removed by centrifugation.

Step 3. Ammonium sulfate fractionation: The enzyme solution was brought to 60% saturation with ammonium sulfate. After the precipitate was removed by centrifugation, ammonium sulfate was added to the supernatant solution to 80% saturation. The precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.4). The enzyme solution was dialyzed against 100 vol. of the same buffer. The insoluble materials formed during the dialysis was removed by centrifugation.

Step 4. DEAE-cellulose column chromatography: The enzyme solution was placed on a DEAE-cellulose column (7.0 X 27 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer and then with the buffer containing 0.15 M NaCl, the enzyme was eluted with the buffer supplemented with 0.20 M NaCl. The active fractions were combined and brought to 80% saturation with ammonium sulfate. The precipitate was dissolved in 1 mM potassium phosphate buffer (pH 7.4). The enzyme solution was dialyzed against 100 vol. of the same buffer.

Step 5. Hydroxyapatite column chromatography: The enzyme solution was applied to a hydroxyapatite column (4.2 X 22 cm) equilibrated with 1 mM potassium phosphate buffer (pH 7.4). After the column was washed with 20 mM potassium phosphate buffer (pH 7.4), the enzyme was eluted with 40 mM potassium phosphate buffer (pH 7.4). The active fractions were collected and concentrated by ammonium sulfate (80% saturation). The precipitate was dissolved in a small volume of 10 mM Tris–HCl buffer (pH 8.5) containing 0.10 M NaCl and dialyzed against the same buffer.

Step 6. DEAE–Sephadex A-50 column chromatography: The enzyme solution was chromatographed on a DEAE–Sephadex A-50 column (1.2 X 9.0 cm) equilibrated with the dialysis buffer. After application of the enzyme and washing of the column with 10 mM Tris–HCl buffer (pH 8.5) containing 0.10 M NaCl, the enzyme was eluted with the buffer supplemented with 0.12 M NaCl. The active fractions were concentrated by ultrafiltration then by addition of ammonium sulfate (80% saturation). The precipitate was dissolved in a small volume of 20 mM potassium phosphate buffer (pH 7.4).

Step 7. Sephadex G-150 column chromatography: The enzyme was applied to a Sephadex G-150 column (1.5 X 104 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4) and eluted with the same buffer. The active fractions were concentrated by addition of ammonium sulfate (80% saturation) and dissolved in a small volume of 20 mM potassium phosphate buffer (pH 7.4).

Table 1
Purification of L-ornithine:α-ketoglutarate δ-aminotransferase

Step	Total protein (mg)	Total units	Specific activity	Yield (%)
1. Crude extract	50 600	31 900	0.63	100
2. Polyethylenimine	30 000	28 400	0.947	88.9
3. Ammonium sulfate (60–80%)	8720	27 500	3.15	86.2
4. DEAE-cellulose	3050	24 200	7.93	75.9
5. Hydroxyapatite	420	13 900	33.1	43.6
6. DEAE–Sephadex A-50	116	5850	50.4	18.3
7. Sephadex G-150	50	2680	53.6	8.4

Approximately 85-fold purification was achieved with an overall yield of ~8%. A protocol of the purification is presented in table 1.

Crystallization: Ammonium sulfate was added gradually to the enzyme solution until the solution became slightly turbid. The solution was kept constant at pH 7.2–7.4 with 10% NH_4OH solution. On standing for a few days crystals formed. The crystals took the form of needles (fig.1).

3.2. *Properties of the enzyme*

The purified enzyme was shown to be homogeneous by the criteria of disc-gel electrophoresis and ultracentrifugation (fig.2). The sedimentation coefficient of the enzyme, calculated for water at 20°C and zeroprotein concentration, is 5.3 S. Assuming $\bar{v} = 0.74$, a mol. wt 85 000 was obtained by the sedimentation equilibrium method [13]. This value is < 50% that of the rat liver enzyme (mol. wt 180 000) [2]. The enzyme shows A_{max} at 278, 343 and 425 nm with an absorbance ratio of 100:14.3:4.3. No appreciable spectral shifts occurred on varying the pH from 5.0–9.0.

The aminotransferase catalyzes the transfer of δ-amino group of L-ornithine to α-ketoglutarate. In addition to L-ornithine, β-lysine slightly reacted with α-ketoglutarate, but none of D-ornithine, L-lysine, D-lysine, L-arginine, L-citrulline, glycine, L-alanine, L-aspartate, L-valine, L-leucine, L-α-aminobutyrate, γ-aminobutyrate, β-alanine, taurine, butylamine, cadaverine and putrescine were the substrates. When L-ornithine was used as an amino donor, pyruvate,

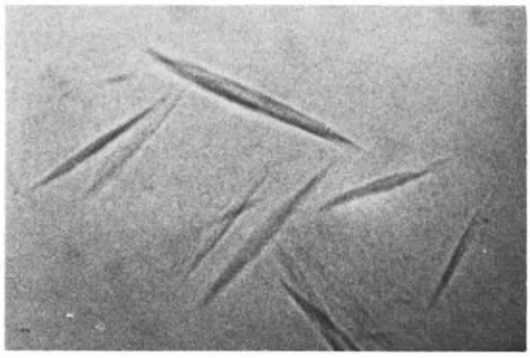

Fig.1. Crystals of L-ornithine:α-ketoglutarate δ-aminotransferase.

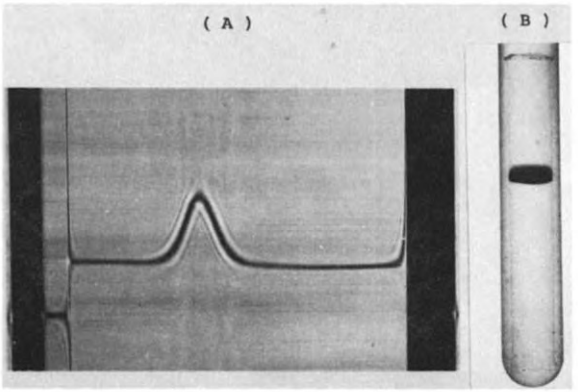

Fig.2. Sedimentation pattern (A) and disc gel electrophoresis (B) of L-ornithine:α-ketoglutarate δ-aminotransferase. (A) Sedimentation pattern was obtained at 5.4 mg protein/ml in 10 mM potassium phosphate buffer (pH 7.4). The picture was taken at 78 min after achieving top speed (59 780 rev./min). (B) A sample of the enzyme preparation (50 μg) was electrophoresed under the conditions in [14] except that 10% gel was used.

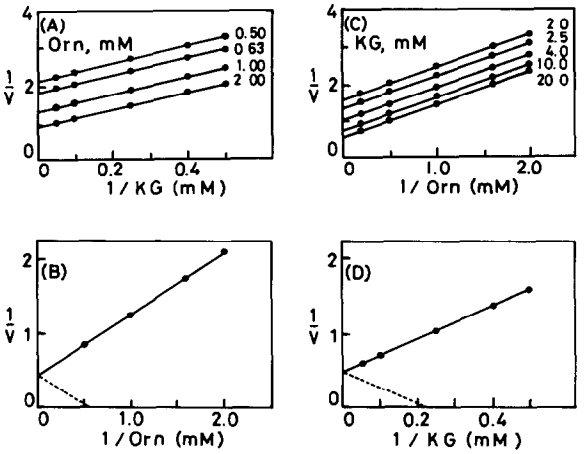

Fig.3. Determination of the Michaelis constants for L-ornithine and α-ketoglutarate. The reaction mixture contained 0.1 μmol pyridoxal 5'-phosphate, 50 μmol Tricine buffer (pH 8.5) and variable amounts of the substrates as indicated in the figure. (A) Double-reciprocal plots of initial velocity against L-ornithine concentration at a series of fixed concentrations of α-ketoglutarate (mM). (B) Secondary plot from the intercepts $1/V$ of (A). (C) Double-reciprocal plots of V against α-ketoglutarate concentration at a series of fixed concentrations of L-ornithine (mM). (D) Secondary plot from the intercepts $1/V$ of (C). *Abbreviations:* Orn, L-ornithine; KG, α-ketoglutarate

glyoxylate and oxaloacetate were very poor substrates. α-Ketobutyrate, α-ketovalerate, α-ketoisovalerate, α-ketocaproate, α-ketoisocaproate and β-phenylpyruvate were not the amino acceptors.

The enzyme has the maximum reactivity at about pH 8.5 for the L-ornithine : α-ketoglutarate δ-transamination. The K_m values were calculated to be 1.8 mM for L-ornithine and 4.6 mM for α-ketoglutarate according to [16] (fig.3).

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