PURIFICATION AND CRYSTALLIZATION OF D-AMINO ACID AMINOTRANSFERASE OF *BACILLUS SPHAERICUS*

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1. Introduction

Enzymatic transamination of D-amino acids was demonstrated in bacilli [1–4], *Rhodospirillum rubrum* [5], and mammalian liver [6]. D-Alanine aminotransferase was purified to near homogeneity from the extract of *Bacillus subtilis* to elucidate the enzymological properties [7–9]. We found the occurrence of high activity of D-amino acid transaminase in the cell-free extract of *Bacillus sphaericus* IFO 3525 [10]. The present communication describes the purification and crystallization of the enzyme from *B. sphaericus*, and some of its properties.

2. Materials and methods

Pyridoxal 5-phosphate (PLP) was obtained from Kyowa Hakko, Tokyo, Japan, and DEAE-cellulose and Sephadex G-150 were products of Midori Juji Company, Osaka, Japan, and Pharmacia, Uppsala, Sweden, respectively. Hydroxyapatite was prepared by the method of Tiselius et al. [11].

Ultracentrifugation was carried out in a Spinco Model E ultracentrifuge, and the molecular weight of the enzyme was determined by the sedimentation equilibrium method of Van Holde and Baldwin [12]. Disc gel electrophoresis was performed by the procedure of Davis [13].

The enzyme was assayed as follows. The standard reaction system consisted of 25 μmoles of D-amino acids, 25 μmoles of α-keto acids, 1 μmole of PLP, 80 μmoles of potassium phosphate buffer (pH 8.0) and enzyme in a final volume of 1.0 ml. Enzyme was replaced by water in a blank. After the mixture was incubated at 37°C for 20 min, the amount of pyruvate or amino acids formed was determined with salicylaldehyde [8] or ninhydrin [14], respectively. One unit of enzyme was defined as the amount of enzyme which catalyzes the formation of 1 μmole of pyruvate or amino acids per min. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry et al. [16].

3. Results and discussion

3.1. *Purification and crystallization*

B. sphaericus IFO 3525 was grown in a medium composed of 1.5% peptone, 0.1% glycerol, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.5% NaCl, 0.01% yeast extract and 0.01% meat extract (pH 7.2). The cultures were grown at 30°C for 20 hr under aeration. The harvested cells were washed twice with 0.85% NaCl solution.

All subsequent operations were performed at 0–5°C. The buffers used contained 10 μM PLP and 0.01% 2-mercaptoethanol.

Step 1. Sonic extraction

The washed cells were suspended in 0.01 M potassium phosphate buffer (pH 7.4), and subjected to sonication

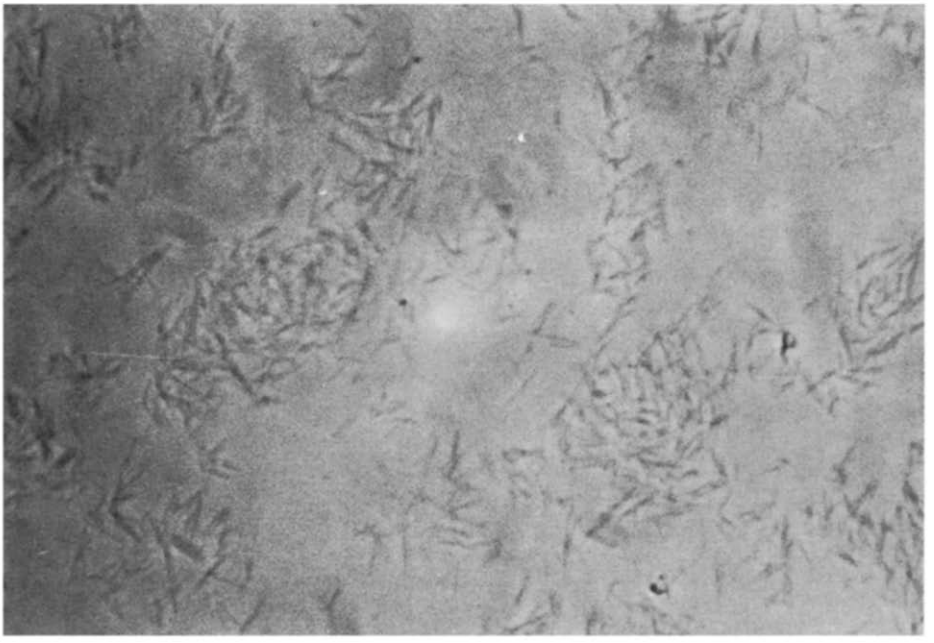

Fig. 1. Crystals of D-amino acid aminotransferase.

in a 19-kc oscillator for 20 min. The intact cells and cell debris were removed by centrifugation.

Step 2. Protamine sulfate treatment

To the cell-free extract was added 1.0 ml of 2% protamine sulfate solution (pH 7.4) per 100 mg of the protein with stirring. After 10 min, the bulky precipitate was removed by centrifugation.

Step 3. Ammonium sulfate fractionation

The supernatant solution was brought to 30% saturation with ammonium sulfate. After the precipitate was removed, ammonium sulfate was added to the supernatant to 70% saturation. The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.4). The enzyme solution was dialyzed overnight against 100 vol of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

Step 4. DEAE-cellulose column chromatography

The enzyme solution was placed on a DEAE-cellulose column (6 × 50 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer, and then with the buffer containing 0.1 M NaCl, the enzyme was eluted with the buffer supplemented with 0.15 M NaCl. The active fractions were pooled, concentrated by addition of ammonium sulfate (70% saturation), and dialyzed against 100 vol of 0.01 M potassium phosphate buffer (pH 7.4).

Step 5. Hydroxyapatite column chromatography

The dialyzed enzyme solution was applied to a hydroxyapatite column (3 × 30 cm) equilibrated with 1 mM potassium phosphate buffer (pH 7.4). The enzyme was eluted with 0.01 M potassium phosphate buffer (pH 7.4). The active fractions were collected and concentrated by ammonium sulfate (70% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.4).

Step 6. Sephadex G-150 column chromatography

The enzyme was applied to a Sephadex G-150 column (1.5 × 55 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.4), and eluted with the same buffer. The active fractions were pooled and concentrated by addition of ammonium sulfate (70%

Table 1
Purification of D-amino acid aminotransferase

Purification Step	Total Protein	Total Units	Specific Activity	Yield
	(mg)			(%)
1. Crude extract	38 410	4610	0.12	100
2. Protamine treatment	20 000	4400	0.22	95.6
3. Ammonium sulfate fractionation	14 160	4320	0.31	93.5
4. DEAE-cellulose	1 480	3100	2.10	62.0
5. Hydroxyapatite	54	1580	29.30	34.3
6. Sephadex G-150	7	808	115.40	17.5
7. Crystallization	5	580	116.00	12.6

saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.4).

Step 7. Crystallization

Ammonium sulfate was added gradually to the enzyme solution until the solution became slightly turbid. The pH of the solution was kept constant at 7.2–7.4 with 10% NH_4OH solution. On standing overnight crystals formed. The crystals took the form of fine needles (fig. 1). Approximately 970-fold purification was achieved with an over-all yield of 12.6%. A protocol of the purification procedure is presented in table 1.

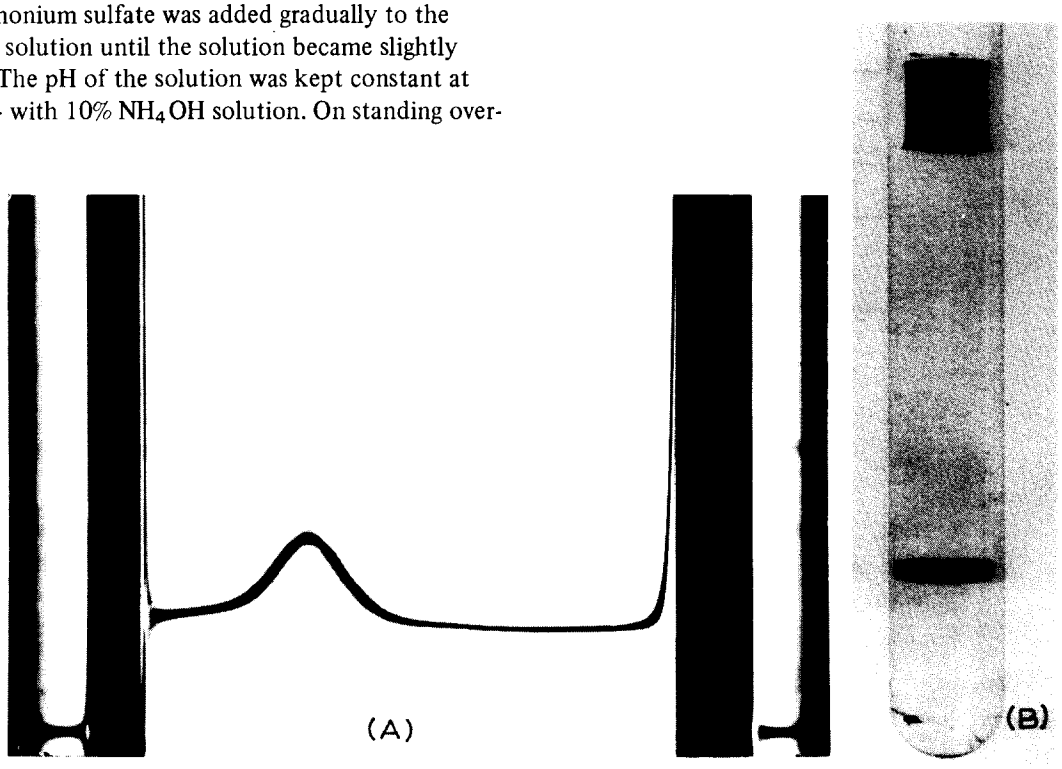

Fig. 2. Sedimentation pattern (A) and disc gel electrophoresis (B) of D-amino acid aminotransferase. Sedimentation pattern was obtained at 8 mg/ml of protein concentration in 0.01 M potassium phosphate buffer (pH 7.4). The picture was taken at 72 min after achieving top speed (50 740 rpm).

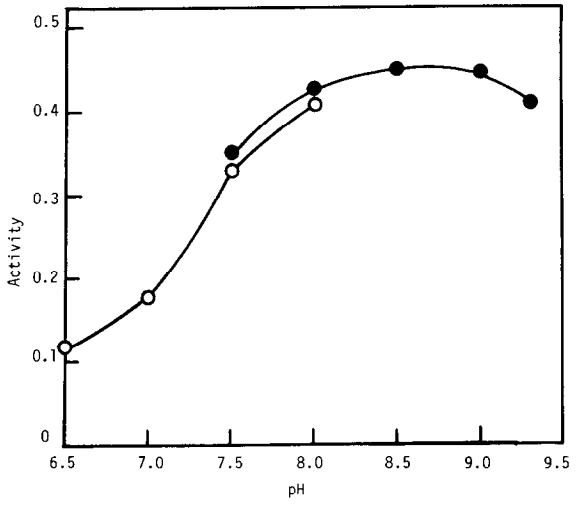

Fig. 3. Effect of pH on D-alanine-α-ketoglutarate transamination. D-Amino acid aminotransferase was assayed by measuring the amount of pyruvate formed per min (enzyme; 2.5 μg). (○——○) 0.1 M potassium phosphate buffer (●——●) 0.1 M Tris–HCl buffer.

3.2. *Properties of enzyme*

The crystalline 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 zero protein concentration, is 4.30 S. Assuming a partial specific volume of 0.74, a mol. wt. of 58 000 ± 2000 was obtained. The enzyme exhibits absorption maxima at 280, 330 and 415 nm (the molecular absorption

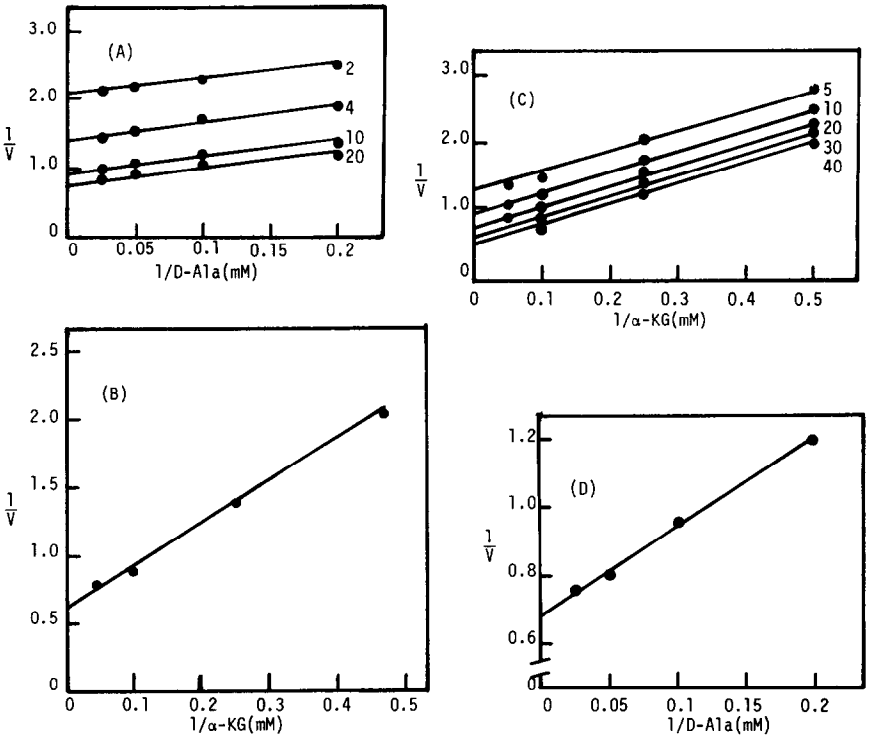

Fig. 4. Effect of concentrations of D-alanine and α-ketoglutarate on the activity. The reaction mixture contained 0.1 μmole of PLP, 60 μmoles of potassium phosphate buffer (pH 8.0), 1 μg of enzyme and variable amounts of the substrates as indicated in the figure. The enzyme activity was assayed by measuring the amount of pyruvate formed for 20 min. (A) Double-reciprocal plots of initial velocity against D-alanine 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 D-alanine (mM). (D) Secondary plot from the intercept 1/V of (C).

coefficients are 65 000 at 280 nm, 14 700 at 330 nm and 5650 at 415 nm) and the identical spectra were obtained when recorded in a series of buffers in the pH range from 5.0–9.0. The PLP content of the enzyme was determined by the phenylhydrazine method [15] to be 2 moles per mole of enzyme. The enzyme was partially resolved to apoenzyme, when incubated with 0.1 M D-alanine in the presence of high concentration of phosphate (a final concentration; 1.0 M) at pH 5.0 according to the method of Scardi et al. [17]. The enzyme thus treated still had peaks at 280 and 330 nm, but absorbance at 415 nm was diminished.

This aminotransferase catalyzes the transfer of amino groups of various D-amino acids to α-ketoglutarate, pyruvate or α-ketobutyrate. When α-ketoglutarate was used as an amino acceptor, the relative activity was 100 for D-alanine, 83 for D-α-aminobutyrate, 46 for D-theanine (γ-glutamylethylamide), 44 for D-methionine, 31 for D-asparagine, 30 for D-aspartate, and 25 for D-glutamine. In the reaction systems with pyruvate, the following activities were obtained; 157 for D-α-aminobutyrate, 142 for D-glutamate, 90 for D-methionine, 70 for D-glutamine, 41 for D-theanine, 40 for D-asparagine and 34 for D-aspartate. In the reactions with α-ketobutyrate, the relative activity was 161 for D-alanine, 126 for D-glutamate, 74 for D-methionine, 48 for D-glutamine, 34 for D-aspartate, 31 for D-theanine and 26 for D-asparagine. However, L-amino acids such as L-alanine, L-glutamate, L-asparagine and L-glutamine, and β-alanine were inert as an amino donor.

The enzyme has a maximum reactivity in the pH range of 8.3–9.0 for D-alanine-α-ketoglutarate transaminaton (fig. 3). The K_m values were calculated to be 4.2 mM for D-alanine and 3.4 mM for α-ketoglutarate (fig. 4), when examined according to the method of Velick and Vavra [18].

Acknowledgements

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