PURIFICATION AND CRYSTALLIZATION OF BACTERIAL ω-AMINO ACID-PYRUVATE AMINOTRANSFERASE

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1. Introduction

Although ω-amino acids occur widely in a variety of animal tissues, plants and microorganisms in the free or conjugated forms [1], little attention has been given to the enzymic transamination of ω-amino acids. Taurine-α-ketoglutarate aminotransferase was found in *Achromobacter superficialis* [2], purified to homogeneity, and crystallized to elucidate the physicochemical and enzymologic properties [3]. In addition to taurine, several other ω-amino acids can serve as an amino donor, but α-ketoglutarate is the exclusive amino acceptor in this amino-transferase reaction.

Recently we demonstrated the occurrence of a new ω-amino acid aminotransferase, taurine-pyruvate aminotransferase, in the cell-free extract of *Pseudomonas* sp. F-126 [4].

In the present communication the purification, crystallization, and some properties of the enzyme are described.

2. Materials and methods

Pyridoxal 5′-phosphate was obtained from Kyowa Hakko Kogyo, Tokyo; β-alanine from Nakarai Chemicals, Kyoto; sodium pyruvate from Ajinomoto, Tokyo; DEAE–cellulose from Midori Juji Company, Osaka, and Sephadex G-150 from Pharmacia, Uppsala. Hydroxyapatite was prepared by the procedure of Tiselius et al. [5]. *o*-Aminobenzaldehyde was synthesized by reduction of *o*-nitrobenzaldehyde according to the method of Smith and Opie [6].

2.1. *Enzyme assay*

The standard assay system consisted of 20 μmol of β-alanine or other amino acid, 20 μmol of sodium pyruvate, 1 μmol of pyridoxal 5′-phosphate, 100 μmol of potassium phosphate buffer (pH 8.0) and enzyme in a final volume of 1.0 ml. In a blank β-alanine was replaced by water. After incubation was performed at 30°C for 30 min, the reaction was terminated by addition of 0.1 ml of 25% trichloroacetic acid followed by centrifugation. The enzyme was assayed by determining alanine (method A) or malonic semialdehyde (method B) as follows. Method A. Alanine in the supernatant was determined with ninhydrin after separation by circular paper chromatography [7] or with an amino acid analyzer (Nihon Denshi JLC-6AH). Method B. To 0.5 ml of the supernatant were added 0.1 ml of 0.8 M KOH, 1.0 ml of 1 M glycine–KCl-KOH (pH 8.0), and 1.0 ml of 0.025 M *o*-aminobenzaldehyde (in 10% ethanol) [8]. The mixture was incubated at 37°C for 30 min to develop yellow color, and then an absorbance was measured at 440 nm with a Toshiba-Beckman Specta 20 spectrophotometer.

One unit of enzyme was defined as the amount of enzyme which catalyzes the formation of 1 μmol of alanine or the change in A_{440} of 1.0. Specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry et al. [9].

Pseudomonas sp. F-126 was grown, harvested and washed as described previously [4]. β-Alanine was the best inducer for formation of the enzyme.

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3. Results and discussion

3.1. *Purification and crystallization*

All subsequent operations were performed at about 5°C. Step 1. Sonic extraction. The washed cells were suspended in 0.02 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol, and subjected to sonication for 20 min in a 19 kHz oscillator followed by centrifugation. Step 2. Polyethyleneimine treatment. To the cell-free extract was added 0.1 ml of 10% polyethyleneimine solution (pH 7.4) per 100 mg of the protein with stirring. After 10 min, the bulky precipitate was removed by centrifugation. The supernatant solution was brought to 70% saturation with ammonium sulfate. The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) and dialyzed overnight against 100 vol. of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation. Step 3. First DEAE–cellulose column chromatography. The enzyme solution was placed on a DEAE–cellulose column (11 × 100 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). After the column was washed thoroughly with the same buffer, the enzyme was eluted with the buffer supplemented with 0.05 M NaCl. The active fractions were pooled and concentrated by addition of ammonium sulfate (70% saturation). The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) and dialyzed against 100 vol. of the same buffer. Step 4. Second DEAE–cellulose column chromatography. The enzyme solution was subjected to DEAE–cellulose column chromatography (a column: 6 × 70 cm) in the same manner as mentioned above. The active fractions were concentrated by ammonium sulfate (70% saturation) and dissolved in 0.01 M potassium phosphate buffer (pH 7.4) followed by dialysis against 100 vol. of the same buffer. Step 5. Hydroxyapatite column chromatography. The enzyme solution was applied to a hydroxyapatite column (4 × 45 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). The column was washed with 0.1 M potassium phosphate buffer (pH 7.4), and the enzyme was eluted with 0.3 M potassium phosphate buffer (pH 7.4). The active fractions were concentrated by ammonium sulfate (70% saturation) and dissolved in a small volume of 0.02 M potassium phosphate buffer (pH 7.4). Step 6. Sephadex G-150 column chromatography. The enzyme was applied to a Sephadex G-150 column (2.5 × 100 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 7.4), and eluted with the same buffer. The active fractions were collected. Ammonium sulfate was added gradually

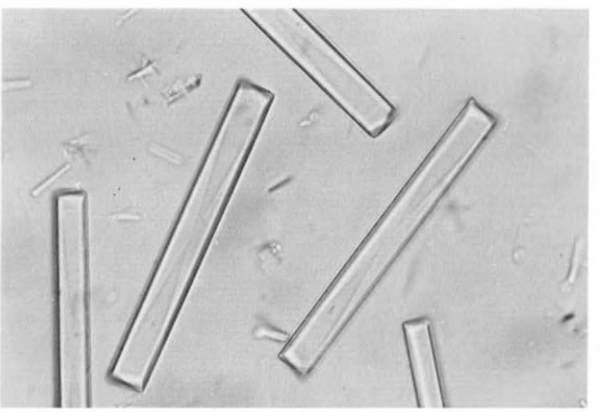

Fig.1. Crystals of ω-amino acid-pyruvate aminotransferase.

Table 1
Purification of ω-amino acid aminotransferase

Step	T. protein (mg)	T. activity[a] (units)	S. activity[a] (units/mg)	Yield (%)
Sonicate	140 000	9800	0.07	100
Polyethyleneimine	102 025	7243	0.07	78
First DEAE–cellulose	6205	5026	0.81	53
Second DEAE–cellulose	2760	4305	1.56	46
Hydroxyapatite	992	3204	3.23	33
Sephadex G-150	736	2590	3.52	28

[a] The enzyme activity was determined by method A

to the enzyme solution until a faint turbidity was obtained. The pH of solution was kept constant at about 7.4 with 1 N NaOH solution. On standing overnight crystal formation occurred. The crystals took the form of rectangular plate (fig.1). A protocol of the purification is presented in table 1.

3.2. *Properties*

The purified enzyme was shown to be homogeneous by the criteria of disc-gel electrophoresis (fig.2) and ultracentrifugation (fig.3). The molecular weight of the enzyme was determined to be approximately 178 000 by the gel filtration method of Andrews [10]. The enzyme exhibits absorption maxima at 280 and 345 nm and a shoulder at 390 nm (fig.4) with molecular absorption coefficients of 174 400, 23 890 and 13 952, respectively (fig.4). The enzyme activity was inhibited by phenylhydrazine, hydroxylamine, D-cycloserine and aminoxyacetate.

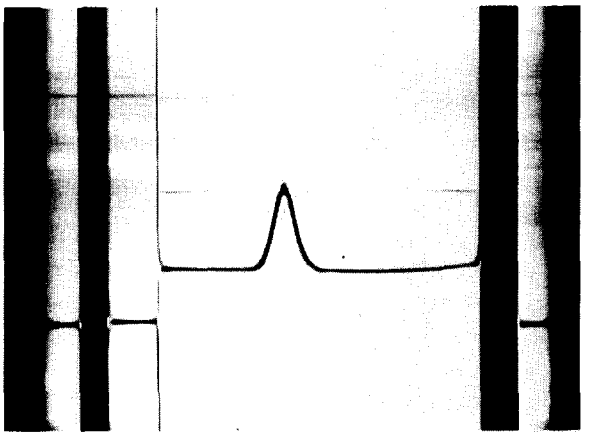

Fig.3. Sedimentation pattern of ω-amino acid aminotransferase. Sedimentation pattern was obtained at 5.2 mg/ml of protein concentration in 0.02 M potassium phosphate buffer (pH 7.4). Picture was taken at 50 min after achieving top speed (59 780 rev/min).

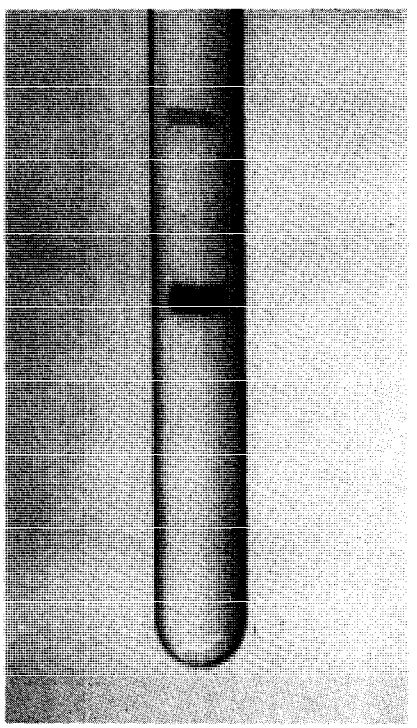

Fig.2. Disc-gel electrophoresis of ω-amino acid aminotransferase. The crystalline enzyme preparation (60 μg) was electrophoresed under the conditions of Davis [11].

The aminotransferase was resolved when incubated with the above mentioned inhibitors (e.g. 0.005 M phenylhydrazine, at 37°C for 30 min) followed by Sephadex G-25 gel filtration with 0.02 M potassium phosphate buffer (pH 7.4). The apoenzyme does not have a soulder at 390 nm, but does still a 345 nm

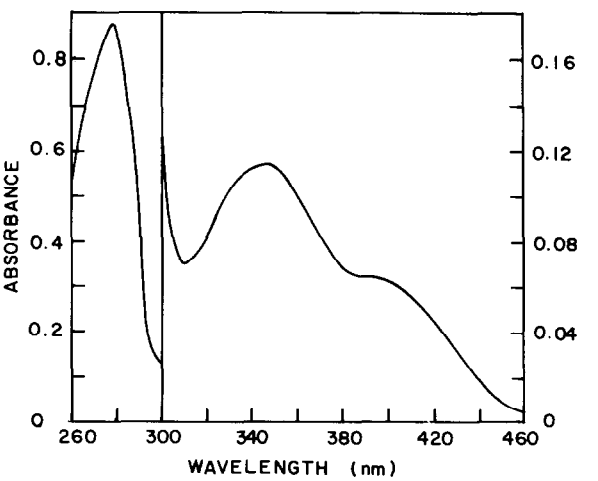

Fig.4. Absorption spectrum of ω-amino acid aminotransferase. Absorption spectrum of the enzyme was measured in 0.05 M potassium phosphate buffer (pH 7.4).

peak. Reconstitution of the enzyme with pyridoxal 5′-phosphate was carried out with difficulty, though the more detailed investigation is now in progress.

The enzyme has a maximum reactivity in the pH range of 8.0–8.5 for β-alanine-pyruvate transamination.

The aminotransferase catalyzes the transfer of amino groups of various ω-amino acids to pyruvate. The relative activity is 100 for β-alanine, 3.6 for taurine, 79.8 for 3-aminopropanesulfonate, 39.7 for γ-amino-*n*-butyric acid, 35.2 for 7-aminoheptanoic acid, 96.4 for DL-β-amino-*n*-butyric acid, 54.2 for β-aminoisobutyric acid and 0.36 for glycine. Aminomethane sulfonate, *p*-aminobenzoic acid, D- and L-lysine, L-ornithine, DL-α-amino-*n*-butyric acid, L-phenylalanine, L-glutamic acid, and L-carnosine are not amino donors. α-Ketoglutarate is inert as amino acceptor.

Although the enzyme was designated previously taurine-pyruvate aminotransferase [4], 'ω-amino acid-pyruvate aminotransferase' is employed here because this terminology is more relevant from the viewpoint of the substrate specificity.

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