CRYSTALLIZATION AND PROPERTIES OF ASPARTATE AMINOTRANSFERASE FROM *ESCHERICHIA COLI* B

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1. Introduction

Asparate aminotransferase (EC 2.6.1.1) has been extensively studied as a representative of the pyridoxal 5'-phosphate (pyridoxal-P)-dependent enzymes. The enzyme catalyzes the reversible transfer of the amino group between L-aspartic acid and α-ketoglutaric acid, and plays an important role in nitrogen metabolism. Animal and plant tissues contain both mitochondrial and cytosolic isoenzymes [1]. The primary structures of both isoenzymes from pig heart muscle have been elucidated [2,3]. Preliminary crystallographic data have been reported for the large and single crystals of cytosolic [4,5] and mitochondrial isoenzymes [6]. Mammalian aspartate aminotransferases are immunochemically distinct from the bacterial enzymes [7]. Structural studies of the bacterial enzymes have not been done. The enzyme has been purified to homogeneity from *Pseudomonas striata* and crystallized [8]. However, its low content in cells and low yield in purification prompted a search for other bacterial sources to produce it more abundantly. The aspartate aminotransferases have been highly purified from *Escherichia coli* K-12 [9], its mutant [10] and Crooks strain [11], but none of them have been obtained in a crystalline form.

We describe here high yield purification, crystallization and some properties of aspartate aminotransferase from *Escherichia coli* B as a first approach to the comparative studies on the enzyme.

2. Materials and methods

Escherichia coli B was grown in a medium composed of 0.5% succinate, 0.5% L-aspartate, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl and 0.01% $MgSO_4 \cdot 7\,H_2O$. The pH was adjusted to 7.5 with NaOH. After cultivation at 28°C for ~18 h under aeration, the cells were harvested and washed with a 0.85% NaCl solution. Malate dehydrogenase was purchased from Boehringer-Mannheim; polyethylene glycol (av. mol. wt 4000) and NADH from Sigma; and amino acids from Kyowa Hakko Kogyo, Tokyo.

The enzyme was assayed by a modification of the coupled malate dehydrogenase method [12] as follows: The reaction mixture contained 100 μmol Tris–HCl buffer (pH 8.5), 50 μmol L-aspartate, 5 μmol α-ketoglutarate, 100 nmol NADH, 15 nmol pyridoxal-P and 25 μg malate dehydrogenase in 1.0 ml final vol. The decrease in A_{340} was measured at 25°C with a Union Giken SM-401 recording spectrophotometer. For the determination of amino donor specificity, the reaction system consisted of 200 μmol Tris–HCl buffer (pH 8.5), 300 μmol amino acids except L-tyrosine (30 μmol), L-tryptophan (200 μmol) and L-aspartate (100 μmol), 10 μmol α-ketoglutarate and 30 nmol pyridoxal-P in 2.0 ml final vol. L-Glutamate produced was determined with a JEOL JLC-6AH automatic amino acid analyzer.

One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1.0 μmol L-glutamate or NAD/min. The specific activity was expressed as units/mg protein. Protein was determined by the Lowry method [13] using serum albumin as a standard.

3. Results and discussion

3.1. *Purification and crystallization*

The washed cells were suspended in 0.02 M potassium phosphate buffer (pH 7.0) containing 2×10^{-5} M pyridoxal-P and 0.01% 2-mercaptoethanol and subjected to sonication followed by centrifugation. The cell-free extracts were fractionated with ammonium sulfate (30–75% saturation). The resulting precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.0) containing 10^{-5} M pyridoxal-P and dialyzed against the same buffer. The dialyzed enzyme was applied to a DEAE-cellulose column equilibrated with the dialysis buffer. The enzyme fraction eluted with the buffer supplemented with 0.15 M NaCl was heated at 58–63°C for 5 min and the resulting precipitate discarded. The supernatant was concentrated by addition of ammonium sulfate (75% saturation). The precipitate was dissolved in a minimum volume of distilled water and applied to a Sephacryl S-200 column buffered with 0.01 M potassium phosphate buffer (pH 7.0) containing 10^{-5} M pyridoxal-P. The active fractions were concentrated and dialyzed against 0.02 M sodium acetate buffer (pH 4.8) containing 10^{-5} M pyridoxal-P. After centrifugation the enzyme was applied to a DEAE-cellulose column equilibrated with the dialysis buffer. The elution was carried out with a linear gradient (0–0.3 M NaCl) and the enzyme was eluted with the buffer containing ~0.15 M NaCl. The active fractions were collected and applied to a hydroxyapatite column equilibrated with 0.001 M potassium phosphate buffer (pH 6.0). The enzyme was eluted with ~0.01 M potassium phosphate buffer, when the elution was performed with a linear gradient (0.001–0.1 M). Thus, the enzyme was purified about 200-fold. A summary of the purification is presented in table 1.

The enzyme preparation with spec. act. 200 was crystallized from a polyethylene glycol solution by the vapour diffusion technique [14]. Protein solution, 20 μl (10 mg/ml 0.01 M potassium phosphate buffer (pH 7.0)) was placed in a well of a hollow slide glass. To the droplet were added 10 μl 10^{-2} M pyridoxal-P and 10 μl of a 9% (w/v) polyethylene glycol solution. The slide glass was placed on a small plastic sandwich box which was enclosed in a large sandwich box, and the box was sealed and maintained at 4°C for ~7–10 days. The large crystals measuring up to about $1.5 \times 0.4 \times 0.2$ mm^3 were formed as shown in fig.1.

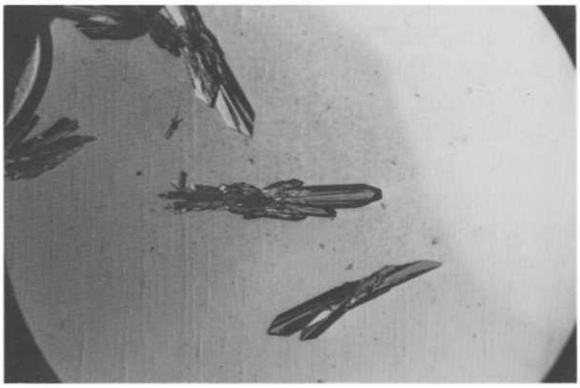

Fig.1. Crystals of aspartate aminotransferase from *Escherichia coli* B.

Table 1
Purification of aspartate aminotransferase from *Escherichia coli* B

Fraction	Total protein (mg)	Total unit	Spec. act.	Yield (%)
Crude extract	26 000	23 000	0.88	100
DEAE-cellulose chromatography (pH 7.0)	4700	17 300	3.68	75
Heat treatment	1500	17 000	11.33	74
Sephacryl S-200 chromatography	590	12 420	21.05	54
DEAE-cellulose chromatography (pH 4.8)	137	9200	67.15	40
Hydroxyapatite chromatography	23	4600	200.00	20

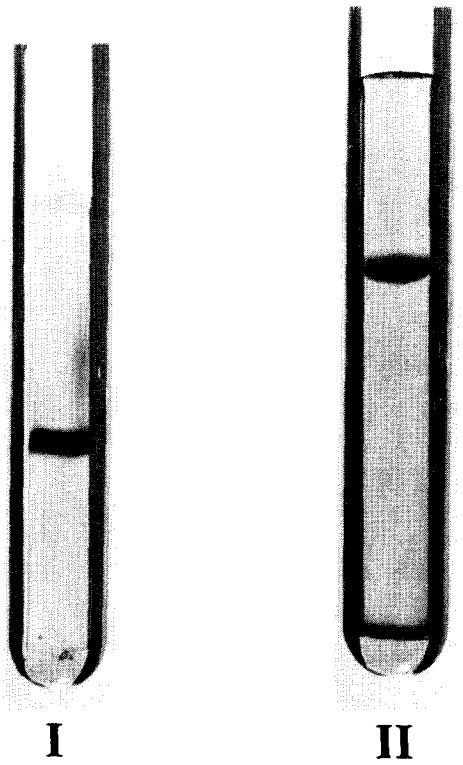

Fig.2. Disc gel electrophetic patterns of the enzyme. The samples were electrophoresed under the conditions in [18] gel (I), and in [19] gel (II). In the estimation of the subunit molecular weight were used bovine serum albumin (M_r 68 000), catalase (6000), ovalbumin (43 000), aldolase (40 000) and α-chymotrypsinogen (25 000) as standards.

Many attempts were made to crystallize the enzyme with ammonium sulfate without success.

3.2. *Properties*

The purified enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc-gel electrophoresis (fig.2(I)). The molecular weight was estimated to be 80 000 by the gel filtration with Sephadex G-150 [15] and 84 000 by the meniscus depletion sedimentation equilibrium method [16] assuming a partial specific volume of 0.74. SDS polyacrylamide disc gel electrophoresis (fig.2(II)) gave a single band with mol. wt 43 000 ± 1000 suggesting that the enzyme was composed of two subunits identical in molecular weight. The enzyme showed absorption maxima at 280 nm (ε 74 800) and 360 nm (ε 13 500) at pH 8.5, and 280 nm and 440 nm (ε 12 900) at pH 5.5. The enzyme had a maximum activity in the pH range of 7.5–8.5. The K_m values determined by spectrophotometric determination of oxalacetate by the method in [17] were 0.01 mM for oxalacetate, 15 mM for L-glutamate, 0.24 mM for α-ketoglutarate and 1.3 mM for L-aspartate. The same values for L-aspartate and α-ketoglutarate were obtained by the assay method with malate dehydrogenase. These properties are similar to those reported for the mammalian isoenzymes [1].

The enzyme from *E. coli* B catalyzed the transamination of aromatic amino acids as well as L-glutamate and L-aspartate with α-ketoglutarate. The relative activity was 100 for L-glutamate, 30 for L-aspartate, 11 for L-tryptophan, 6.2 for L-phenylalanine, 1.5 for L-tyrosine and 1.0 for L-methionine. Similar high reactivity toward aromatic amino acids has been reported with other bacterial aspartate aminotransferases [8–11]. The mammalian enzymes show higher substrate specificity [1].

Table 2
Amino acid composition of aspartate aminotransferase from *Escherichia coli* B

Amino acid	Residues[a]/subunit
Tryptophan[b]	4.4
Lysine	16.7
Histidine	5.7
Arginine	21.8
Aspartate	42.4
Threonine	22.3
Serine	18.1
Glutamate	47.6
Proline	14.6
Glycine	29.6
Alanine	45.5
Valine	25.4
Methionine	7.9
Isoleucine	17.3
Leucine	35.9
Tyrosine	11.3
Phenylalanine	19.9
Cysteine[c]	6.3

[a] The average or extrapolated (threonine and serine) values from 24, 48 and 72 h hydrolysis in 5.7 N HCl [20]. The molecular weight of The subunit was assumed to be mol. wt 42 000

[b] Value determined spectrophotometrically [21]

[c] Determined as cysteic acid [22]

The amino acid composition of the enzyme is shown in table 2. The enzyme is similar to *E. coli* Crooks enzyme [11] in the amino acid composition, although tryptophan residue was not determined in the latter enzyme. On the other hand, the amino acid composition resembles none of those reported with pig heart isoenzymes.

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