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A NOVEL PURIFICATION PROCEDURE OF L-LYSINE 6-AMINOTRANSFERASE FROM *FLAVOBACTERIUM LUTESCENCE*

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Summary

A new method for the purification of L-lysine 6-aminotransferase (L-lysine: 2-oxoglutarate 6-aminotransferase, EC 2.6.1.36) was devised, in which affinity chromatography with L-lysylacetamidododecyl-Sepharose 6B, the most effective affinity adsorbent, was substituted for the heat treatment. The yield of the enzyme with the present procedure was approx. twice as high as that with the previous procedure (Soda, K. and Misono, H. (1968) Biochemistry 7, 4110—4119). The enzyme purified by this method was activated 2-fold by heat treatment (65 °C for 5 min). The enzyme has absorption maxima at 340 and 415 nm, derived from the bound pyridoxal 5′-phosphate, which are identical with those of the enzyme obtained with the procedure including heat treatment. These results rule out the possibility that the formation of the 340-nm pyridoxal 5′-phosphate of the enzyme is an artifact of heat treatment.

Introduction

L-Lysine 6-aminotransferase (L-lysine:2-oxoglutarate 6-aminotransferase, EC 2.6.1.36) catalyzes the transamination of terminal amino group of L-lysine to 2-oxoglutarate to produce Δ^1-piperideine-6-carboxylate (a cyclized form of 2-aminoadipate 5-semialdehyde) and L-glutamate [1]. The enzyme, which has been purified to homogeneity from *Flavobacterium lutescence* (*Achromobacter liquidum*) has a molecular weight of approx. 116 000 and contains 2 mols of pyridoxal 5′-phosphate [2]. 1 mol (λ_{max}; 415 nm) binds to the enzyme protein

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Abbreviation: pyridoxal-*P*, pyridoxal 5′-phosphate.

through an aldimine linkage with a 6-amino group of the lysine residue of the protein and is associated directly with catalytic action. This bound pyridoxal-*P* is released from the enzyme by incubation with L-lysine and then with 1 M KH_2PO_4 to yield a semi-apoenzyme, which is catalytically inactive and still contains 1 mol of pyridoxal-*P* (λ_{max}; 340 nm). To establish the role of the 340-nm pyridoxal-*P* it is necessary to rule out the possibility that this anomalous pyridoxal-*P* might be formed as an artifact during the enzyme purification, especially in a heat treatment at 55°C. The absorption maximum (360 nm (alkaline region) or 430 nm (acidic region)) of aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) is shifted to the shorter wavelength (340 nm) when the enzyme is subjected to prolonged aging or brief exposure to concentrated urea [3].

We describe here the mode of specific interaction between L-lysine 6-aminotransferase and various affinity adsorbents, and the purification of the enzyme by a novel procedure including affinity chromatography with L-lysylacetamidododecyl-Sepharose 6B substituted for heat treatment. Some properties of the enzyme purified are also described.

Materials and Methods

Materials. L-Lysine, L-glutamate, D-lysine and L-ornithine were obtained from Ajinomoto, Tokyo; pyridoxal-*P* from Kyowa Hakko Kogyo, Tokyo; α-aminoalkane and α,ω-diaminoalkanes from Nakarai Chemicals, Kyoto, and sodium 2,4,6-trinitrobenzenesulfonate from Wako Chemicals, Osaka. *o*-Aminobenzaldehyde was prepared from *o*-nitrobenzaldehyde [4]. The other chemicals were analytical grade reagents.

Derivatization of Sepharose 6B. Sepharose 6B (Pharmacia) was activated in a 5 or 10% (w/v) solution of CNBr (pH 10.5—11.0) at 4°C according to the method of Cuatrecasas and Anfinsen [5]. To the activated Sepharose 6B suspended in 25 ml of water was added 50 ml of 10% solution of α,ω-diaminoalkane (pH 9.5) in 50% dioxane. The reaction was allowed to proceed with gentle swirling at 4°C for 24 h. The ω-aminoalkyl-Sepharose 6B was filtered off and washed vigorously with 30% acetic acid and then with water. Alkylagaroses were prepared by the method described above except that 10% alkylamine solution (pH 9.5) in 50% dioxane was used.

Amino acid-coated Sepharose 6B was prepared from ω-aminoalkyl-Sepharose 6B through the bromoacetyl-Sepharose 6B [5]. To the bromoacetyl-Sepharose was added 2.2 M D- or L-lysine (pH 7.3), 2.0 M L-ornithine (pH 7.0) or 2.0 M L-glutamate (pH 8.6). The pH of the reaction mixture containing the basic amino acid was kept at approx. neutral in order to allow the α-amino group to react with the matrix arm. The reaction was carried out with gentle swirling at room temperature for 24 h. The adsorbent prepared was washed vigorously with 1.0 M NaCl and then with water. The Sepharose 6B derivatization was followed by the sodium trinitrobenzene sulfonate test [5].

Enzyme samples. F. lutescence (IFO 3084) was grown and disrupted as described previously [2], and the resulting homogenate was brought to 30% saturation with $(NH_4)_2SO_4$ and the precipitate removed by centrifugation. $(NH_4)_2SO_4$ was added to the supernatant solution to 70% saturation. The

precipitate was dialyzed against 0.01 M potassium phosphate buffer (pH 7.2), and was used for testing the retardation potency of the affinity matrix. Crystalline L-lysine 6-aminotransferase was prepared by the method described previously [2]. The enzyme was assayed as described previously [2]. 1 unit of the enzyme was defined as the amount of enzyme that catalyzes the formation of 1.0 μmol of Δ^1-piperideine-6-carboxylate.

Protein determination. Protein concentration was determined by the method of Lowry et al. [6] with bovine serum albumin as a standard. The concentration of crystalline enzyme was determined spectrophotometrically with a value of $A_{280}^{1\%} = 7.3$ [2].

Results

Effect of carbon-chain length on the strength of retardation

We prepared affinity adsorbents containing the amino donor of the enzyme as a ligand, L-lysylacetamidoalkyl-Sepharose 6B. The affinity of the matrixes for the enzyme depended markedly on the carbon-chain length of their alkyl groups (Table I). The dodecyl derivative showed the highest affinity. To examine the effect of CNBr concentration during the activation of Sepharose 6B on the strength of the retardation, the affinity matrixes activated with 10% CNBr were prepared. Their affinity for the enzyme are shown in Table II. The derivatives activated with 10% CNBr showed higher affinity than those activated with 5% CNBr. The most significant difference was observed with the dodecyl derivative; the enzyme applied to the column containing the matrix activated with 10% CNBr was not eluted with the buffer supplemented with 0.5 M NaCl but was with the buffer containing more than 1.0 M NaCl. The enzyme emerged in the buffer supplemented with 0.3—0.5 M NaCl when the matrix activated with 5% CNBr was used.

The specific activities of the eluted enzyme solutions were also determined. The preparation eluted with the buffer containing 1.0 M NaCl from the column

TABLE I

EFFECT OF CARBON-CHAIN LENGTH OF THE ALKYL GROUP OF L-LYSYLACETAMIDOALKYL-SEPHAROSE 6B ON THE AFFINITY FOR THE ENZYME

Sepharose 6B was activated with 5% CNBr and the derivative prepared. The crude enzyme solution (1.0 ml) containing approx. 50 mg protein was applied to the column packed with the matrix (0.9 × 8.0 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2) and stepwise elution was performed with approx. 50 ml of the buffer supplemented with 0, 0.1, 0.3, 0.5 and 1.0 M NaCl. Ethyl-, butyl-, hexyl-, octyl-, decyl- and dodecyl- represent the L-lysylacetamidoethyl-, L-lysylacetamidobutyl-, L-lysylacetamidohexyl-, L-lysylacetamidooctyl-, L-lysylacetamidodecyl- and L-lysylacetamidododecyl-Sepharose 6B, respectively. The total activity of the enzyme appllied was regarded as 100%.

NaCl concentration (M)	Activity in the eluted solution (%)					
	Ethyl-	Butyl-	Hexyl-	Octyl-	Decyl-	Dodecyl-
0	83	27	0	0	0	0
0.1	17	58	69	70	20	10
0.3	0	25	31	30	65	70
0.5	0	0	0	0	15	20
1.0	0	0	0	0	0	0

TABLE II

EFFECT OF CARBON-CHAIN LENGTH OF THE ALKYL GROUP OF L-LYSYLACETAMIDOALKYL-SEPHAROSE 6B ON THE AFFINITY FOR THE ENZYME

Sepharose 6B was activated with 10% CNBr and the derivative prepared. The chromatography was carried out as described in Table I. Octyl-, decyl and dodecyl- represent L-lysylacetamidooctyl-, L-lysylacetamidodecyl- and L-lysylacetamidododecyl-Sepharose 6B, respectively. The total activity of the enzyme applied was regarded as 100%.

NaCl concentration (M)	Activity in the eluted buffer (%)		
	Octyl-	Decyl-	Dodecyl-
0	0	0	0
0.1	32	0	0
0.3	55	73	0
0.5	42	27	0
1.0	0	0	81
2.0	0	0	15

packed with L-lysylacetamidododecyl-Sepharose 6B (activated with 10% CNBr) showed the highest specific activity. The recovery of the enzyme activity was more than 80%, as shown in Table II.

Interaction between various types of matrix and the enzyme

To elucidate the mode of interaction between the enzyme and the affinity matrix we examined the affinity of various matrixes for the enzyme (Table III). D-Lysyl and L-ornithyl derivatives showed high affinity. The L-glutamyl derivative, however, had no affinity. This shows that a free amino group is necessary in the matrix to bind the enzyme.

Dodecyl-Sepharose 6B was used to estimate the contribution of hydrophobicity of the matrix to the affinity. The enzyme was eluted with the buffer supplemented with 0.1—0.3 M NaCl. This suggests the occurrence of interaction between the enzyme and hydrophobic region of the matrix. Effectiveness

TABLE III

AFFINITY OF D-LYSYL, L-ORNITYL AND L-GLUTAMYLACETAMIDODODECYL-SEPHAROSE 6B AND DODECYL- AND ω-AMINODODECYL-SEPHAROSE 6B FOR THE ENZYME

Sepharose 6B was activated with 10% CNBr and the derivative prepared. The experimental conditions were described in Table I. D-Lysyl-, L-ornithyl-, L-glutamyl-, dodecyl- and ω-aminododecyl- represent D-lysyl, L-ornithyl, and L-glutamylacetamidododecyl-Sepharose 6B and dodecyl- and ω-aminododecyl-Sepharose 6B, respectively.

NaCl concentration (M)	Activity in the eluted solution (%)				
	D-Lysyl-	L-Ornithyl-	L-Glutamyl-	Dodecyl-	ω-Aminododecyl-
0	0	0	100	0	0
0.1	0	0	0	84	0
0.3	16	9	0	16	0
0.7	64	69	0	0	0
1.5	20	22	0	0	40

of the free amino group at the terminus of the matrix was shown by the fact that ω-aminododecyl-Sepharose 6B had very high affinity.

Activity of the matrix-bound enzyme

The results described above suggest that L-lysylacetamidododecyl-Sepharose 6B binds to an acidic site of the enzyme, which probably exists in an internal region of the enzyme protein. The activity of the matrix-bound enzyme was determined to see whether the acidic site exists in an active site of the enzyme (Table IV). The enzyme preparation in which 415-nm pyridoxal-*P* was half-resolved was bound to the L-lysylacetamidododecyl-Sepharose 6B and the activity was determined. The activity was reduced to one half of the activity of the original enzyme by binding. The matrix-bound enzyme was activated approx. 2-fold by the addition of pyridoxal-*P*. The enzyme was not dissociated from the matrix by the repeated enzyme reactions. The activity of the matrix-bound enzyme was not changed after being incubated five times with the reaction mixture followed by separation by centrifugation. These results show that the apoenzyme as well as the holoenzyme bind to the matrix, and that the matrix is not bound to the active site of the enzyme.

Purification of the enzyme

The enzyme was purified to homogeneity without heat treatment using L-lysylacetamidododecyl-Sepharose 6B as follows. The preparation of the crude extract and ammonium sulfate fractionation (30—70% saturation) were described in Materials and Methods. The enzyme obtained was dissolved in 0.01 M potassium phosphate buffer (pH 7.2) and dialyzed against the same buffer. The enzyme was applied to an L-lysylacetamidododecyl-Sepharose 6B column (1.5 × 20 cm) equilibrated with the buffer supplemented with 0.1 M NaCl. The elution pattern is shown in Fig. 1. The enzyme solution dialyzed against 0.01 M potassium phosphate buffer (pH 7.2)/0.01 mM pyridoxal-*P* was applied to a DEAE-cellulose column (1.2 × 20 cm) buffered with the dialysis buffer. The enzyme was eluted with the buffer containing 0.2 M NaCl. A summary of the purification is presented in Table V. The specific activity of

TABLE IV

ACTIVITY OF THE MATRIX-BOUND ENZYME

For the determination of the activity of the matrix-bound enzyme, the enzyme in which the 415-nm pyridoxal-*P* was half-resolved was adsorbed on L-lysylacetamidododecyl-Sepharose 6B (1.0 ml) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2). To the enzyme-binding matrix was added the reaction mixture containing 130 μmol of potassium phosphate buffer (pH 8.0)/10 μmol of L-lysine/10 μmol of 2-oxoglutarate/4.0 μmol of *o*-aminobenzaldehyde in a final volume of 2.0 ml. In the assay in the presence of pyridoxal-*P*, 40 nmol of pyridoxal-*P* was further added. After incubation at 37°C for 5 min with shaking, the absorbance at 465 nm was measured. The activity of free enzyme was assayed with the same reaction mixture except that Sepharose 6B was substituted for the matrix.

Assay mixture	Specific activity	
	Free enzyme	Matrix-bound enzyme
—Pyridoxal-*P*	489	220
+Pyridoxal-*P*	1000	450

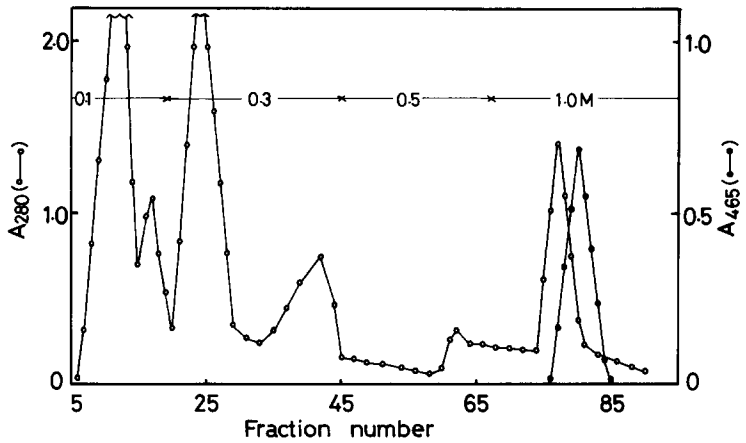

Fig. 1. L-Lysylacetamidododecyl-Sepharose 6B column chromatography. The enzyme solution containing about 500 mg of protein was applied to an L-lysylacetamidododecyl-Sepharose 6B column (1.5 × 20 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2) supplemented with 0.1 M NaCl. The column was eluted stepwise with the buffer containing 0.3, 0.5 and 1.0 M NaCl. The elution volume was 4 ml per tube. The absorbance at 280 nm (○———○) and 465 (●———●) was measured to determine the protein concentration and the enzyme activity, respectively.

the purified enzyme was half that of the enzyme obtained by the standard procedure involving heat treatment. The enzyme was activated twice by the heat treatment as described later. An overall yield of the enzyme using this purification procedure was nearly twice as high as that using the previous procedure [2].

Some properties of the enzyme

Spectrophotometric and fluorometric properties of the enzyme purified by this method were closely similar to those of the enzyme purified by the previous procedure; the enzyme exhibited absorption maxima at 280 (ϵ; 85, 260), 340 (ϵ; 10, 230) and 415 nm (ϵ; 9, 380) at pH 5.5—9.5, and showed the emission maxima at 380 and 495 nm when excited at 340 and 415 nm, respectively.

The profile of the heat activation of the enzyme is shown in Fig. 2. Although a separate addition of pyridoxal-*P*, 2-mercaptoethanol and 2-oxoglutarate enhanced the activation, the coexistence of the three compounds caused the highest activation. Total activity of the enzyme was increased to the maximum

TABLE V

PURIFICATION OF L-LYSINE 6-AMINOTRANSFERASE

Step	Total protein (mg)	Specific activity	Total units	Yield (%)
Cell-free extract	2292	4.8	11 000	100
$(NH_4)_2SO_4$ fractionation (30—70%)	1073	8.2	8 800	80
Affinity chromatography *	26	279	7 250	66
DEAE-cellulose chromatography	10	500	5 000	45

* L-Lysylacetamidododecyl-Sepharose 6B (activated with 10% CNBr) was used as the affinity matrix.

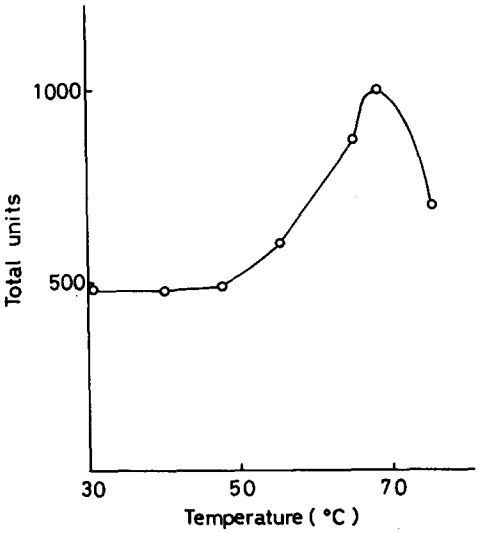

Fig. 2. Heat activation of the enzyme purified. 1 ml of the enzyme solution (0.01 M potassium phosphate buffer (pH 7.2)/0.01 mM pyridoxal-*P*/5.0 mM 2-oxoglutarate/0.01% 2-mercaptoethanol) containing approx. 1.0 mg of the protein was heated at various temperatures for 5 min and then immediately cooled in an ice bath.

by heat treatment at 65°C, but was decreased at 75°C. The enzyme solution after heat activation at 65°C was centrifuged and the specific activity of the supernatant solution was determined to be 1200 unit/mg. Thus, approx. 15% of the enzyme was denatured and precipitated by the heat treatment, but the specific activity of the remaining enzyme was increased approx. 2-fold. The enzyme was labile in the absence of pyridoxal-*P* or in the presence of L-lysine, which is effective for the coenzyme dissociation [2]; approx. 90% of the enzyme activity was lost irreversibly through the heat treatment (75°C for 5 min) in these conditions.

Discussion

L-Lysine 6-aminotransferase probably has an acidic site to which the terminal amino group of the affinity matrix is bound by an ionic bond. The site seems to exist in an internal region of the enzyme protein, but not in the active site. The length of alkyl side-chains significantly affected the retardation of the enzyme. However, in contrast to phosphorylase *b* [7], which also contains pyridoxal-*P*, the aminotransferase was adsorbed only slightly and not at all on dodecyl-Sepharose 6B and L-glutamylacetamidododecyl-Sepharose 6B, respectively. Thus, it is not likely that the hydrophobic interaction contributes significantly to the binding of the enzyme with an immobilized ligand.

The enzyme was purified to homogeneity without heat activation. The yield of the enzyme using this purification procedure was twice as high as that using the previous procedure [2]. L-Lysylacetamidododecyl-Sepharose 6B column chromatography was most effective; the enzyme was purified approx. 30-fold by the chromatography. For the purification of aspartate aminotransferase from wheat germ, 3-carboxypropionylaminododecyl-Sepharose 4B was used as

an affinity adsorbent [8]. However, the efficiency of this affinity matrix was not high: the enzyme was purified 8-fold with a 40% yield from the crude extract of wheat germ. Aspartate aminotransferase was eluted with 0.2% sodium cholate, showing the presence of strong hydrophobic interaction between the enzyme and the matrix. The affinity chromatography is highly effective in the purification of L-lysine 6-aminotransferase because both the long alkyl chain and free amino group in the ligand participate in binding of the enzyme to the matrix. The carboxyl group of the matrix probably accelerates release of the enzyme from the matrix because, only 40% of the enzyme was eluted with above 1.0 M NaCl from an ω-aminododecyl-Sepharose 6B column.

The enzyme purified by the present method has half the specific activity of the enzyme purified by the previous procedure, but the specific activity increased to the same level using heat treatment. Pyridoxal-*P*, 2-mercaptoethanol and 2-oxoglutarate stimulates the activation. The enzyme activity was lost using heat treatment in the absence of pyridoxal-*P* or in the presence of an amino donor. Thus, binding of pyridoxal-*P* to the enzyme probably makes its conformation more stable.

The absorption maxima at 340 and 415 nm arising from the bound pyridoxal-*P* was not affected by the heat treatment. Thus, the 340-nm pyridoxal-*P* is not an artifact of heat activation. Although the presence of similar anomalous pyridoxal-*P* has been reported for D-amino acid aminotransferase [9] and taurine aminotransferase [10], the role of the pyridoxal-*P* in the catalytic action has not been elucidated. We are now investigating this problem and also the mechanism of the heat activation of the enzyme.

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