Purification and properties of α-amino-ε-caprolactam racemase from *Achromobacter obae*

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We have purified a unique enzyme, α-amino-ε-caprolactam racemase 945-fold from an extract of *Achromobacter obae* by Octyl–Sepharose CL-4B and Thiopropyl–Sepharose 6B and some other chromatographies. The purified enzyme was found homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analytical ultracentrifugation. The enzyme has a monomeric structure with $M_r \sim 50000$ and a sedimentation coefficient ($s_{20,w}$) of 4.28 S. The enzyme contains pyridoxal 5′-phosphate as a coenzyme. The pH optimum for the enzyme activity is ~9.0. D- and L-α-amino-ε-caprolactams are the only substrates. The K_m values for the D- and L-isomers are, 8 and 6 mM, respectively.

Amino-caprolactam racemase *Non-carboxylic compound racemase* *Pyridoxal 5′-phosphate enzyme*
Purification (of a racemase) *Properties (of a racemase)* *Bacterial enzyme*

1. INTRODUCTION

α-Amino-ε-caprolactam is a chiral heterocyclic compound synthesized in chemical industry and is used as the substrate for the enzymatic production of L-lysine; L-α-amino-ε-caprolactam is hydrolyzed to form L-lysine by L-α-amino-ε-caprolactamase (EC class 3.5.2) of a yeast, and the unreacted D-isomer is racemized with bacterial α-amino-ε-caprolactam racemase (EC class 5.1.1) [1]. Thus, L-lysine is produced with almost 100% yield. We have purified the L-α-amino-ε-caprolactamase from *Cryptococcus laurentii* to homogeneity and characterized it [2].

Various amino acid racemases occur in bacteria and actinomycetes, and a few have been purified and characterized [3–6]. Lactate [7] and mandelate [8] racemases also are found in anaerobic and aerobic bacteria, respectively, and have been studied enzymologically. α-Amino-ε-caprolactam racemase is different from other racemases in acting on a non-carboxylic compound, i.e., an intramolecular cyclic amide with α-amino group and also on an industrial chemical that has not been found in nature.

We describe here the purification of this unique racemase from *Achromobacter obae* to homogeneity and its physico-chemical and enzymological properties.

2. MATERIALS AND METHODS

D-, L- and D,L-α-amino-ε-caprolactams and *Achromobacter obae* FERM-P 776 [9] were supplied by Dr Kyosuke Yotsumoto of Toray Industries: pyridoxal 5′-phosphate was obtained from Kyowa Hakko (Tokyo), DEAE-Toyopearl from Toyo Soda Manufacturers and DEAE–Sephadex A-50, Sephadex G-200, Octyl–Sepharose CL-4B and Thiopropyl–Sepharose 6B were from Pharmacia (Uppsala).

L-α-amino-ε-caprolactamase was purified from *Cryptococcus laurentii* as in [2]. L-Lysine-α-oxidase purified from *Trichoderma viridie* as in [10]

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Ultracentrifuge was carried out in a Spinco Model E Ultracentrifuge. SDS–Polyacrylamide gel electrophoresis was performed as in [11].

The activity of α-amino-ε-caprolactam racemase was routinely followed by measurement of the polarimetric rotation change of D- or L-α-amino-ε-caprolactam at 546 nm, 436 nm or 365 nm at 37°C with a Perkin-Elmer (model 241) recording polarimeter. The standard reaction mixture consisted of 200 μmol D- or L-α-amino-ε-caprolactam, 40 nmol pyridoxal 5′-phosphate, and 200 μmol potassium phosphate buffer (pH 8.0) and enzyme in 2.0 ml final vol. Enzyme was replaced by water in a blank. The activity was also followed with L-α-amino-ε-caprolactamase; L-α-amino-ε-caprolactam produced from the D-isomer by enzymatic racemization was determined after its conversion into L-lysine by L-α-amino-ε-caprolactamase [2]. L-Lysine produced was determined with L-lysine-α-oxidase [12]. One unit of enzyme was defined as the amount of enzyme that catalyzes the racemization of 1 μmol D- or L-α-amino-ε-caprolactam/min. The specific activity was expressed as units/mg protein. Protein was determined as in [13] with bovine serum albumin as a standard; with most column fractions, protein elution patterns were estimated by A_{280}.

3. RESULTS AND DISCUSSION

3.1. *Purification of the enzyme*

Achromobacter obae FERM-P 776 was grown as in [14] in a medium composed of 1.4% L-aspartate, 0.32% DL-α-amino-ε-caprolactam, 0.4% glucose, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% $MnCl_2 \cdot 4H_2O$, 0.003% $FeSO_4 \cdot 7H_2O$, 0.002% $CaCl_2 \cdot 2H_2O$, 0.05% yeast extract and 0.003% antifoam-AF-emulsion (pH 7.0). The cultures were grown at 28°C for 24 h with aeration. The harvested cells were washed twice with 0.85% NaCl solution.

We have established the stabilization conditions for α-amino-ε-caprolactam racemase activity [14], which enabled us to purify the enzyme. Unless otherwise stated, all operations were done at 0–5°C and the buffers used contained 0.25 M sucrose, 2×10^{-5} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol.

Step 1

The washed cells (~1 kg wet wt) were suspended in 850 ml of 10 mM potassium phosphate buffer (pH 7.2) and each 500 ml portion was disrupted by sonication for 30 min. DNase and RNase were added to the suspension followed by incubation at 25°C for 1 h with occasional stirring. The supernatant solution obtained by centrifugation was dialyzed against 2 changes of 40 l potassium phosphate buffer (pH 6.8). The precipitate formed during dialysis was discarded.

Step 2

The enzyme solution was passed through a DEAE–Toyopearl column (7.8 × 30 cm) equilibrated with the dialysis buffer. The active fractions were combined.

Step 3

The enzyme solution was diluted to 4 mg protein/ml in 0.15 M potassium phosphate buffer (pH 6.8) containing 0.5% 2-mercaptoethanol. The solution was heated at 55°C for 20 min followed by centrifugation. The supernatant solution was concentrated with an Amicon PM-10 membrane, and dialyzed against 100 vol. 10 mM potassium phosphate buffer (pH 7.2) containing 0.08 M KCl. The insoluble materials formed were removed by centrifugation.

Step 4

The enzyme solution was placed onto a DEAE–Sephadex A-50 column (2.6 × 23 cm) equilibrated with the dialysis buffer, and the column was washed with the same buffer containing 0.12 M KCl. The enzyme was eluted with the buffer supplemented with 0.14 M KCl. The active fractions were combined and concentrated by ultrafiltration.

Step 5

The enzyme solution was chromatographed with a Sephadex G-200 column (2.65 × 100 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.2). The active fractions were pooled and concentrated by ultrafiltration.

Step 6

The enzyme solution (2 mg protein/ml) in 0.4 M potassium phosphate buffer containing 2×10^{-4} M pyridoxal 5′-phosphate, 1.5% 2-mercaptoethanol

and 0.4 M sucrose was heated at 62°C for 15 min followed by centrifugation. The supernatant solution was concentrated by ultrafiltration and dialyzed against 10 mM potassium phosphate buffer (pH 7.2) containing 10^{-4} M pyridoxal 5′-phosphate, 0.10% 2-mercaptoethanol and 0.03 M sucrose.

Step 7

The enzyme solution was applied to an Octyl–Sepharose CL-4B column (0.9 × 4.0 cm) equilibrated with the above buffer containing 4.0 M KCl, and the column was washed with the same buffer. The enzyme was eluted by a linear gradient between the equilibration buffer and the buffer in which 4.0 M KCl was replaced by 2.0 M sucrose. The active fractions were concentrated by ultrafiltration followed by dialysis against 10 mM potassium phosphate buffer (pH 7.85) containing 0.05 M KCl.

Step 8

The enzyme solution was applied to a DEAE–Sephadex A-50 column (0.55 × 3.5 cm) equilibrated with the dialysis buffer, and the column was washed with the same buffer. The enzyme was eluted by a linear gradient between the buffers containing 0.05 M KCl and 0.14 M KCl. The active fractions were combined, concentrated and dialized against 10 mM potassium phosphate buffer (pH 7.4) containing 0.2 M KCl.

Step 9

The enzyme solution was placed onto a Thiopropyl–Sepharose 6B column (1.5 × 7 cm) equilibrated with the dialysis buffer, and the column was washed with the same buffer and the buffer containing 10 mM L-cysteine successively. The enzyme was eluted with the buffer supplemented with 40 mM dithiothreitol. The active fractions were combined and concentrated by ultrafiltration. A summary of the purification procedure is given in table 1.

3.2. *Properties of the enzyme*

The purified enzyme was shown to be homogeneous by the criterion of ultracentrifugation and SDS–polyacrylamide gel electrophoresis (fig. 1). The sedimentation coefficient ($s_{20,w}$) was calculated to be 4.28 S (20°C; 10 mM potassium phosphate buffer (pH 7.2) containing 2×10^{-5} M pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol; protein 1.2 mg/ml). The M_r of the enzyme was determined to be ~51000 by the Sephadex G-200 method [15] with beef muscle lactate dehydrogenase (M_r 130000), pig kidney D-amino acid oxidase (114700), bovine serum albumin (67000), horseradish peroxidase (44000), bovine pancreas α-chymotrypsinogen A (23650), and horse heart cytochrome *c* (13400) as standard proteins. The M_r of the polypeptide chain was calculated to be 49000 by sodium lauryl sulfate–polyacrylamide gel electrophoresis [16] with heamoglobin (M_r 15500),

Table 1

Purification of α-amino-ε-caprolactam racemase

Step	Total protein (mg)	Total units	Spec. act.	Yield (%)
1. Crude extract	24000	9100	0.4	100
2. DEAE-Toyopearl	6800	6800	1.0	75
3. 1st Heat treatment	2300	7900	3.4	87
4. 1st DEAE–Sephadex A-50	210	6000	29.0	66
5. Sephadex G-200	83	4800	58.0	53
6. 2nd Heat treatment	56	4700	84.0	52
7. Octyl–Sepharose CL-4B	14	3500	250.0	39
8. 2nd DEAE–Sephadex A-50	4.0	1200	300.0	13
9. Thiopropyl–Sepharose 6B	1.8	680	380.0	7.5

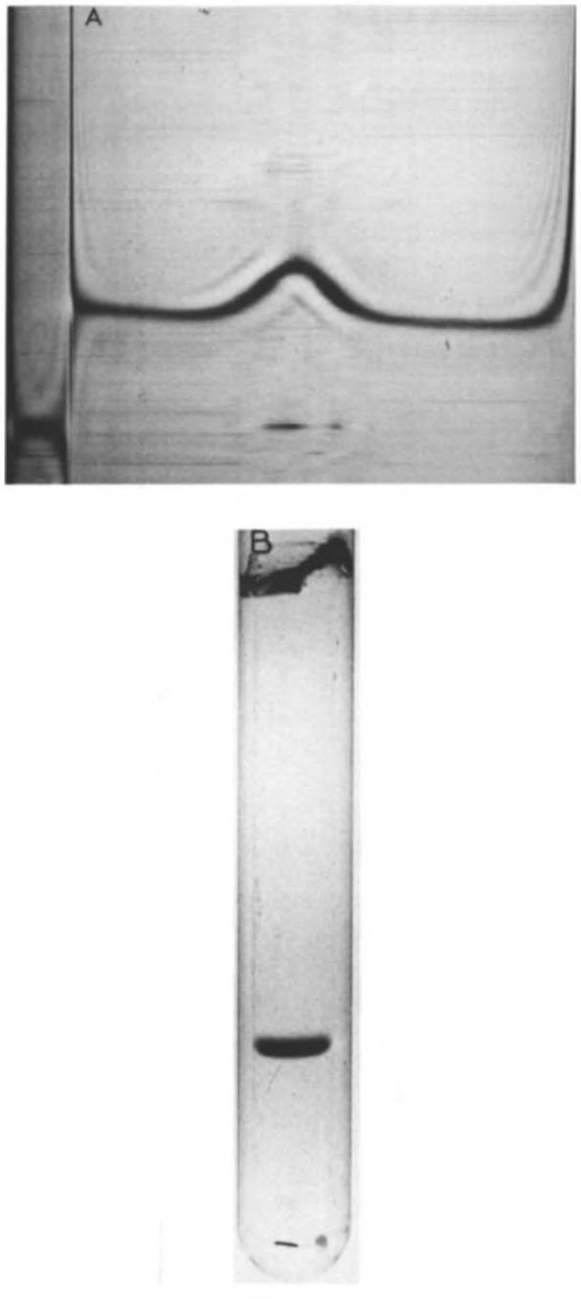

Fig. 1. Sedimentation pattern (A) and SDS–polyacrylamide gel electrophoresis (B) of α-amino-ϵ-caprolactam racemase. (A) Sedimentation pattern was obtained at 1.2 mg protein/ml in 10 mM potassium phosphate buffer (pH 7.2). The picture was taken at 23 min after achieving top speed (42040 rev./min). (B) 1% SDS-treated enzyme (12 μg protein) was subjected to electrophoresis under the conditions in [11]. The direction of migration is from cathode (top) to anode.

trypsin (23300), aldehyde dehydrogenase (41000) and serum albumin (67000) as marker proteins. These results indicate that the enzyme is composed of a single polypeptide chain.

The purified enzyme can be stored at −20°C for at least 6 months in the presence of 0.25 M sucrose with little loss of activity at >3 mg protein/ml, whereas it is inactivated significantly by freezing, in particular in a dilute solution (<1 mg/ml). The enzyme shows absorption maxima at 280 nm and 412 nm, which is characteristic of pyridoxal 5′-phosphate enzymes. The absorbance ratio at 280 nm and 412 nm is ~5 : 1. When the enzyme was incubated with 5 mM hydroxylamine in 10 mM potassium phosphate buffer for 12 h at 4°C, the enzyme was bleached and lost its activity (<5% of the original activity). However, the enzyme activity was restored fully by addition of 2×10^{-5} M pyridoxal 5′-phosphate and, the 412 nm band reappeared. The enzyme shows maximum activity at pH ~9.0. D- and L-α-amino-ϵ-caprolactams were racemized exclusively; lysine, ornithine, alanine and other α-amino-α-carboxy-amino acids were inert. The K_m values for D- and L-α-amino-ϵ-caprolactams were calculated to be 8 and 6 mM respectively.

REFERENCES

[1] Fukumura, T. (1977) Agric. Biol. Chem. 41, 1327–1330.
[2] Fukumura, T., Talbot, G., Misono, H., Teramura, Y., Kato, K. and Soda, K. (1978) FEBS Lett. 89, 298–300.
[3] Soda, K. and Osumi, T. (1969) Biochem. Biophys. Res. Commun. 35, 362–368.
[4] Yorufuji, T., Ogata, K. and Soda, K. (1971) J. Biol. Chem. 246, 5085–5092.
[5] Adams, E. and Norton, I.L. (1964) J. Biol. Chem. 239, 1525–1535.
[6] Svensson, M. and Gatenbeck, S. (1981) Arch. Microbiol. 129, 213–215.
[7] Cantwell, A. and Dennis, D. (1974) Biochemistry 13, 287–291.
[8] Fee, J.A., Hegeman, G.D. and Kenyon, G.L. (1974) Biochemistry 13, 2528–2532.
[9] Fukumura, T. (1977) Agric. Biol. Chem. 41, 1321–1325.
[10] Kusakabe, H., Kodama, K., Kuninaka, A., Yoshino, H., Misono, H. and Soda, K. (1980) J. Biol. Chem. 255, 976–981.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.

[12] Kusakabe, H., Kodama, K., Kuninaka, A., Yoshino, H. and Soda, K. (1979) Agric. Biol. Chem. 43, 1749–1752.
[13] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Ahmed, S.A., Esaki, N. and Soda, K. (1982) Bull. Inst. Chem. Res. Kyoto Univ. in press
[15] Andrews, P. (1951) Biochem. J. 91, 222–233.
[16] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.