PURIFICATION AND PROPERTIES OF A NOVEL ENZYME, L-α-AMINO-ε-CAPROLACTAMASE FROM *CRYPTOCOCCUS LAURENTII*

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Received 23 March 1978

1. Introduction

A novel synthetic process of L-lysine from DL-α-amino-ε-caprolactam with almost 100% yield has been established [1]. This process is composed of two new enzymatic reactions, the selective hydrolysis of L-α-amino-ε-caprolactam to L-lysine, and the racemization of α-amino-ε-caprolactam, which proceed in the same vessel. The L-α-amino-ε-caprolactam hydrolyzing enzyme (L-α-amino-ε-caprolactamase (EC 3.5.2.)) has been found in the cells of *Cryptococcus laurentii* and other yeasts [2,3]. α-Amino-ε-caprolactam racemase has been found in the cells of *Achromobacter obae* and other bacteria [4]. Both enzymes were partially purified from *C. laurentii* and *A. obae*, respectively [5,6].

In this paper, we describe the purification of the new amidohydrolase from *C. laurentii* to homogeneity and some of its properties.

2. Materials and methods

L-, D- and DL-α-amino-ε-caprolactams were supplied by Toray Industries, Otsu, Shiga. DEAE-cellulose was obtained from Serva, Heidelberg and DEAE Sephadex and Sephadex G-200 from Pharmacia Fine Chemicals, Uppsala. Hydroxyapatite was prepared as in [7]. L-Lysine-α-ketoglutarate ε-aminotransferase was purified from *Flavobacterium lutescens* IFO 3085 (*Achromobacter liquidum*) to homogeneity as in [8].

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Ultracentrifugation was carried out in a Spinco Model E ultracentrifuge. Disc-gel electrophoresis was as in [9].

The enzyme was assayed as follows. The standard reaction mixture consisted of 250 μmol L-α-amino-ε-caprolactam·HCl (pH 8.5, adjusted with N NaOH), 1 μmol $MnCl_2$, 200 μmol Tris–HCl buffer (pH 8.5) and enzyme in final vol. 1.0 ml. Enzyme was replaced by water in a blank. Incubation was carried out at 37°C for 30 min, and the reaction was terminated by immersing the test tubes in boiling water for 5 min. After cooling, L-lysine formed was determined spectrophotometrically with L-lysine-α-ketoglutarate ε-aminotransferase as in [10]. One unit of enzyme was defined as the amount of enzyme which catalyzes the formation of 1 μmol L-lysine/min. Specific activity was expressed as units/mg protein. Protein was determined as in [11] using bovine serum albumin as a standard; with most column fractions, protein elution pattern was estimated by A_{280}.

3. Results and discussion

3.1. *Purification of the enzyme*

Cryptococcus laurentii (Toray 2001) was grown in a medium composed of 1.0% DL-α-amino-ε-caprolactam, 1.0% sodium L-glutamate, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $MnCl_2 \cdot 4H_2O$ and 0.05% yeast extract (pH 7.2). The cultures were grown at 28°C for 20 h under aeration. The harvested cells were washed twice with 0.85% NaCl solution. The yield of cells was approx. 5 g (wet wt)/l medium.

Elsevier/North-Holland Biomedical Press

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

3.1.1. Step 1

The washed cells (about 830 g, wet wt) were suspended in 4 liters 0.01 M potassium phosphate buffer (pH 7.4), and disrupted continuously with a Dyno-Mill (Willy A, Switzerland) at a 4 l/h flow rate followed by centrifugation. The supernatant solution was dialyzed overnight against 50 liters 0.01 M potassium phosphate buffer (pH 7.4). The precipitate formed during dialysis was discarded.

3.1.2. Step 2

The enzyme solution was applied to a DEAE-cellulose column (8.5 × 40 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the buffer and then with the buffer containing 0.15 M NaCl, the enzyme was eluted with the buffer supplemented with 0.20 M NaCl. The active fractions were combined and brought to 70% saturation with ammonium sulfate. The precipitate was dissolved in 1 mM potassium phosphate buffer (pH 7.4). The enzyme solution was dialyzed overnight against 100 vol. same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

3.1.3. Step 3

The enzyme solution was applied to a hydroxyapatite column (3.7 × 25 cm) equilibrated with 1 mM potassium phosphate buffer (pH 7.4). After the column was washed with 0.01 M potassium phosphate buffer (pH 7.4), the enzyme was eluted with 0.03 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 vol. 0.01 M potassium phosphate buffer (pH 7.4).

3.1.4. Step 4

The enzyme was applied to a Sephadex G-200 column (2.5 × 150 cm) equilibrated with 0.01 M potassium buffer (pH 7.4) and eluted with the same buffer. The active fractions were pooled and concentrated by addition of ammonium sulfate (60% saturation). The precipitate was dissolved in a small vol. 0.01 M Tris–HCl buffer (pH 8.5) containing 0.1 M NaCl and dialyzed overnight against the same buffer.

3.1.5. Step 5

The dialyzed enzyme solution was placed on a DEAE–Sephadex A-50 column (0.7 × 3.0 cm) equilibrated with the dialysis buffer, and the column was washed with 0.01 M Tris–HCl buffer (pH 8.5) containing 0.15 M NaCl. The enzyme was eluted with the buffer supplemented with 0.2 M NaCl. The active fractions were combined and concentrated by ultrafiltration. A summary of the purification is given in table 1.

3.2. *Properties of the enzyme*

The purified enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc-gel electrophoresis (fig.1). The sedimentation coefficient of the enzyme, calculated for water at 20°C and zero protein concentration, is 8.7 S. The molecular weight was determined to be 185 000 by

Table 1
Purification of L-α-amino-ε-caprolactamase

Step	Total protein (mg)	Total units	Spec. act.	Yield (%)
1. Crude extract	35 500	79 500	2.24	100
2. DEAE-cellulose chromatography	390	26 400	67.6	30.2
3. Hydroxyapatite chromatography	125	25 600	205	32.2
4. Sephadex G-200 chromatography	76	15 700	206	19.7
5. DEAE–Sephadex A-50 chromatography	42.9	10 500	244	13.2

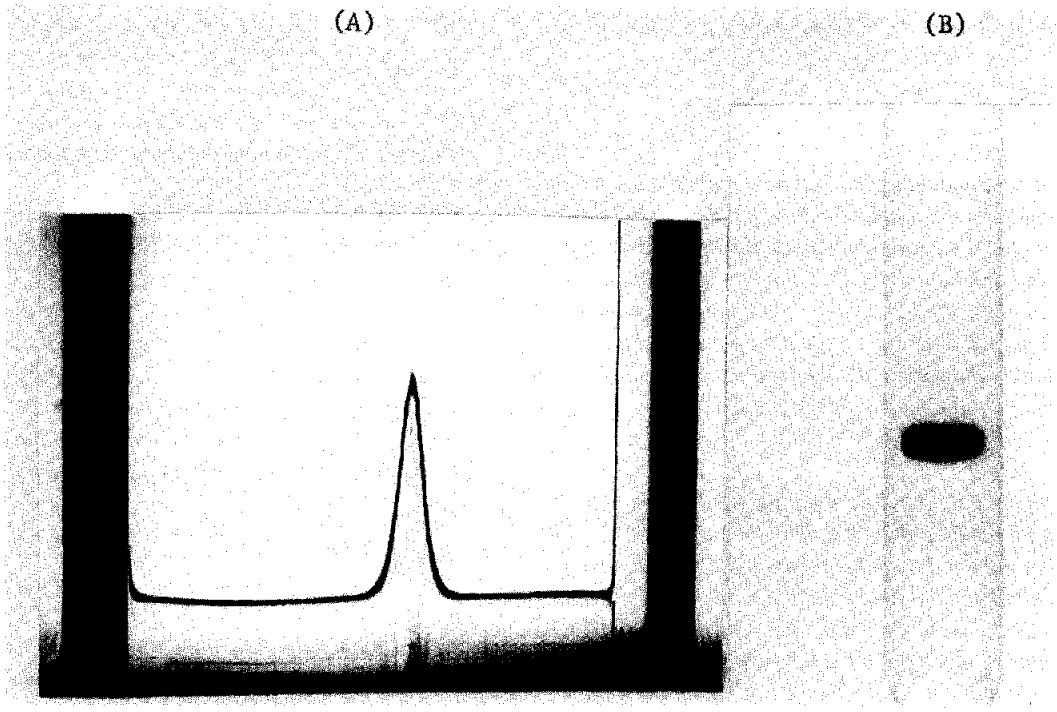

Fig.1. Sedimentation pattern (A) and disc-gel electrophoresis (B) of L-α-amino-ε-caprolactamase. (A) Sedimentation pattern was obtained at 0.68% protein concentration in 0.01 M potassium phosphate buffer (pH 7.2). Picture was taken at 56 min after achieving top speed (59 780 rev./min). (B) A sample of the enzyme preparation (50 μg) was electrophoresed under the conditions in [9].

the Sephadex G-200 gel filtration method [12], with bovine liver catalase (240 000), bacterial methioninase (180 000) [13], bovine heart lactate dehydrogenase (140 000), bovine serum albumin (monomer, 68 000) and ovalbumin (43 000) as standard proteins. The enzyme shows an absorption spectrum of a simple protein with an A_{420} max. ($A^{1\%}_{1\,cm}$ 8.80).

The enzyme was activated by addition of $MnCl_2$ and $MgCl_2$. The enzyme has a maximum reactivity at about pH 9.0. The K_m value for L-α-amino-ε-caprolactam was calculated to be 2.6 mM. D-α-amino-ε-caprolactam and ε-caprolactam were not substrates.

The reaction mechanism and subunit structure of the enzyme are currently under investigation.

Acknowledgements

The authors thank Dr T. Yamamoto, Dr E. Sato and Mr Y. Kawabata for their helpful discussion.

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