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Production and Stabilization of α-Amino-ε-caprolactam Racemase from *Achromobacter obae*

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Achromobacter obae FERM-P 776 grown in both organic and inorganic nitrogen media produced α-amino-ε-caprolactam racemase, and DL-α-amino-ε-caprolactam stimulated the enzyme production. The enzyme was labile, but was stabilized considerably by addition of 40% ethylene glycol, 0.25 M sucrose and a mixture of glycerol, dithiothreitol, superoxide dismutase and a pepsin inhibitor S-PI. Approximately 90% of the enzyme activity was retained in the presence of sucrose even after storage at −20°C for four months.

KEY WORDS: Amino-caprolactam racemase/ Bacterial enzyme/ Enzyme production/ Enzyme stabilization/

INTRODUCTION

α-Amino-ε-caprolactam racemase (EC class 5.1.1) was demonstrated by Fukumura[1] in some bacteria. *Achromobacter obae* that produces the enzyme most abundantly is utilized industrially to produce L-lysine from DL-α-amino-ε-caprolactam with a high yield in a coupled system with L-α-amino-ε-caprolactamase (EC class 3.5.2) of *Cryptococcus laurentii*.[2] This enzyme has been purified to homogeneity and characterized.[3] However, Fukumura[4] has made an attempt to purify the racemase with little success due to its lability.

In order to purify the enzyme and study its enzymological and physicochemical properties, it is essential to find out the optimum conditions for the enzyme production and stabilization. We here describe the studies of the growth medium for *Achromobacter obae* to produce the enzyme effectively, and its stabilization.

MATERIALS AND METHODS

Chemicals

DL-α-Amino-ε-caprolactam, L- and D-α-amino-ε-caprolactam hydrochlorides were obtained from Toray Industries Inc., pyridoxal 5′-phosphate from Kyowa Hakko and superoxide dismutase was from Sigma. A pepsin inhibitor S-PI, produced by a *Streptomyces sp.*[5,6] was a kind gift from Drs. Murao and Arai of Osaka Prefecture University. L-α-Amino-ε-caprolactamase was purified by a procedure previously described.[3] L-Lysine-α-oxidase purified as described previously[7] was kindly given by Dr. H. Kusakabe of Yamasa Shoyu, Choshi. The other

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chemicals were of analytical grade.

Microorganism and cultural conditions

Achromobacter obae FERM-P 776[1] was obtained from Toray Industries Inc., and used throughout.

Unless otherwise stated, cells were grown aerobically at 28°C with shaking in a medium containing glucose 5.0 g, KH_2PO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $MnCl_2 \cdot 4H_2O$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 0.03 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, yeast extract 0.5 g, antifoam-AF-emulsion 0.03 g and indicated amounts of various nitrogen sources in 1 liter of tap water. The pH was adjusted to about 7.0 with NaOH or HCl. The harvested cells were washed with 0.85% NaCl and then with 10 mM potassium phosphate buffer (pH 7.3) containing 20 μM pyridoxal 5'-phosphate and 0.01% 2-mercapto-ethanol.

Preparation of cell-free extracts

We prepared cell-free extracts either by homogenizing the cells in a morter with sea sand and powdered alumina, and extracting the soluble protein with 10 mM potassium phosphate buffer (pH 7.3) containing 20 μM pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol or by disruption of the cells by sonication (Kaijo Denki Oscillator, 19 KHz) in the same buffer for 5 min. The supernatant solution obtained was dialyzed against 500 volumes of the same buffer, and used as a crude α-amino-ε-caprolactam racemase.

Enzyme assay

The reaction mixture (2.0 ml, pH 8.0) contained 320 μmol of D- or L-α-amino-ε-caprolactam, 40 nmol of pyridoxal 5'-phosphate, 320 μmol of potassium phosphate buffer and enzyme. The reaction was started by addition of enzyme. The racemization was usually followed at 37°C by measurement of an initial optical rotation change at 546 nm with a Perkin-Elmer polarimeter (model 241). The reaction mixture containing D-α-amino-ε-caprolactam as a substrate was also incubated at 37°C for 5 ~40 min, and then the L-isomer formed was determined by a coupled reaction[7] with L-α-amino-ε-caprolactamase[3] and L-lysine-α-oxidase.

Definition of enzyme unit

One unit of α-amino-ε-caprolactam racemase was defined as the amount of enzyme that catalyzes the racemization of one μmole of D- or L-α-amino-ε-caprolactam per min. The specific activity was units per mg of protein. Protein was determined by the method of Lowry *et al.*[8] with bovine serum albumin (Sigma) as a standard.

RESULTS AND DISCUSSION

Cells were grown in various media containing glucose or glycerol a as carbon source and DL-α-amino-ε-caprolactam or peptone as a nitrogen source (Table I). The highest total and specific activities were obtained in the medium supplemented with 1.2% DL-α-amino-ε-caprolactam and 0.5% glucose (medium H).

The cells were grown in the basal medium containing 2.18 g of nitrogen of organic

Table I. Effect of medium composition on α-amino-ε-caprolactam racemase production

Nutrients (g/l)	Medium							
	A	B	C	D	E	F	G	H
Glucose	—	—	—	—	0.5	—	5.0	5.0
Glycerol	2.0	—	2.0	—	—	—	—	—
Peptone	2.0	2.0	—	—	—	—	—	—
DL-ACL	12.0	12.0	12.0	12.0	12.0	12.0	—	12.0
K_2HPO_4	1.0	1.0	1.0	1.0	—	—	—	—
KH_2PO_4	1.0	1.0	1.0	1.0	2.0	2.0	2.0	2.0
$MnCl_2 \cdot 4H_2O$	—	—	—	—	0.2	0.2	0.2	0.2
$FeSO_4 \cdot 7H_2O$	—	—	—	—	0.03	0.03	0.03	0.03
$CaCl_2 \cdot H_2O$	—	—	—	—	0.02	0.02	0.02	0.02
Yeast extract	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antifoam-AF-emulsion	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
	Results							
Cell yield (g/l)	2.8	1.3	2.8	0.5	2.3	1.6	2.0	4.7
Activity (U/g cell)	5.0	3.0	2.7	4.0	8.3	1.4	n.d.	11.6
Specific activity	0.17	0.06	0.1	0.1	0.3	0.1	0.4	0.6

Cells were grown in 250 ml of each medium. n.d.=not determined. ACL=α-amino-ε-capvolactam

Table II. Effect of nitrogen source on α-amino-ε-caprolactam racemase production.

Nitrogen (2.18 g/l) source	Cell yield (wet) (g/l)	Racemase activity (Units/g wet cell)	Specific activity
$NaNO_3$	3.1	2.7	0.18
$(NH_4)_2SO_4$	7.5	1.1	0.11
Urea	3.7	0.6	0.17
α-Amino-ε-caprolactam	2.9	2.6	0.44
α-Pyrrolidone	2.9	0.9	0.09
L-Aspartic acid	6.6	2.2	0.21
L-Glutamic acid	3.8	1.2	0.25
L-Lysine	3.7	1.2	0.20

Cells were grown in 250 ml of each medium as described in the MATERIALS AND METHODS. Inoculum (6 ml each) was prepared in a medium without nitrogen added.

and inorganic nitrogen sources per liter. The cells grew well on all the media containing any nitrogen source tested, and all the extracts of the cells grown showed α-amino-ε-caprolactam racemase activity more or less (Table II). Although ammonium sulfate supported the highest cell growth, the specific activity of the cells grown in the ammonium sulphate medium was very low. When L-aspartic acid was used as a nitrogen source, the highest total racemase activity was obtained. Though DL-α-amino-ε-caprolactam added to the medium did not affect the total activity, the specific activity of the cells grown in this medium was about twice higher than that of the cells grown in the basal medium supplemented with L-aspartic acid. Thus, the cells can produce α-amino-ε-caprolactam racemase irrespective of addition of

DL-α-amino-ε-caprolactam to the medium, but the presence of DL-α-amino-ε-caprolactam stimulates the production of enzyme.

We have varied the ratio of glucose and DL-α-amino-ε-caprolactam (as a sole nitrogen source) added to the medium and examined the influence on the enzyme production (Table III). The highest total and specific activities of the enzyme were obtained when a medium containing 1.0 g per liter of glucose and 8.0 g of DL-α-amino-ε-caprolactam was used. The addition of DL-α-amino-ε-caprolactam to the basal medium is required for the maximum production of the racemase.

Table III. Effect of ratio of concentrations of glucose and DL-α-amino-ε-caprolactam in the growth medium on α-amino-ε-caprolactam racemase production.

Concentration (g/l) of		Cell yield (wet) (g/l)	Racemase activity (Units/g wet cell)	Specific activity
Glucose	DL-ACL			
5.0	0.0	2.6	1.5	0.21
4.0	2.0	5.6	9.7	0.74
3.0	4.0	4.7	11.3	0.88
2.0	6.0	4.1	11.0	0.78
1.0	8.0	4.5	12.5	0.93
0.0	10.0	1.9	9.0	0.20

Cells were grown in 250 ml of each medium. ACL=α-amino-ε-caprolactam

α-Amino-ε-caprolactam racemase is unstable as reported previously[4] and this instability prevents one from purifying the enzyme effectively. Therefore, we have attempted to minimize a loss of the enzyme activity during its storage and purification.

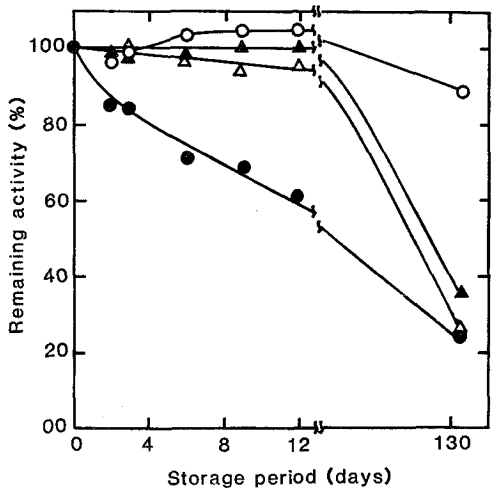

Fig. 1. Effect of various compounds on α-amino-ε-caprolactam racemase stability. Various compounds were added to crude α-amino-ε-caprolactam racemase preparations (protein 15 mg/ml) and stored at −20°C. Activities of the enzyme preparations (0.2 ml) were measured as described in MATERIALS AND METHODS. —●—, None; —△—, ethylene glycol; —▲—, a mixture of superoxide dismutase, S-PI, dithiothreitol and glycerol; and —○—, sucrose.

Various cations, *i.e.*, NH_4^+, Zn^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+} (2～10 mM), and pyridoxal 5′-phosphate (20～200 μM) were added to the crude preparation in 10 mM potassium phosphate buffer (pH 7.3), and the enzyme was stored at various temperatures (25°C, 4°C, −20°C and −195°C) for 3, 7, 15 and 32 days. None of them protected the enzyme from a decrease in the activity. Superoxide dismutase (0.2 mg/ml), S-PI (0.2 mg/ml) or glycerol (0.4～0.8 g/ml) was also ineffective, when added to the crude enzyme in 10 mM potassium phosphate buffer (pH 7.3) containing 20 μM pyridoxal 5′-phosphate and 0.01% 2-mercaptoethanol, and the mixture was stored at −20°C. However, when the enzyme solution was supplemented with a mixture of superoxide dismutase (0.2 mg/ml), S-PI (0.2 mg/ml), dithiothreitol (2.0 mM) and glycerol (0.5 g/ml) and stored at −20°C, its activity was retained fully after 10 days (Fig. 1). Ethylene glycol (40%) also protected the enzyme from inactivation. However the enzyme lost its activity gradually on storage for a long period (*i.e.*, more than 4 months) even in the presence of the effective stabilizers shown above. Sucrose (0.25 M) was found to stabilize the enzyme most effectively; about 90% of the enzyme activity was retained after storage at −20°C for four months. Sucrose and other polyhydroxy alcohols are known to stabilize labile enzymes owing to an increase in solvent cohesive force,[9] hydration of the protein molecule,[10] or by induction of a conformational change in a protein molecule.[11] α-Amino-ε-caprolactam racemase of *Achromobacter obae* probably is stabilized by sucrose through a similar mechanism, although further investigations are needed to clarify the detailed mechanism.

The purification of α-amino-ε-caprolactam racemase is currently in progress to elucidate it enzymologically.

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