Purification and Some Properties of Inducible Lysine Decarboxylase from *Vibrio parahaemolyticus*

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Inducible lysine decarboxylase from *Vibrio parahaemolyticus* AQ 3627 was purified to apparent homogeneity and characterized. The enzyme displayed a molecular weight of 531000 by gel filtration and 79000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme required pyridoxal phosphate as a cofactor, and the pH optimum was 5.5. The K_m value for L-lysine was 3.2 mM, and the enzyme was inhibited by 6-aminocaproic acid and α -fluoromethyl analogs of cadaverine. δ -Hydroxylysine and S-aminoethyl-L-cysteine was active as substrates to a lesser extent than L-lysine. The amino-terminal amino acid sequence was determined to be Met–Asn–Ile–Phe–Ala–Ile–Leu. These properties were compared with those of other bacterial lysine decarboxylases.

Keywords lysine decarboxylase; *Vibrio parahaemolyticus*; purification; property; amino-terminal sequence; enzyme induction

Lysine decarboxylase (LDC), which catalyzes the formation of cadaverine and CO_2 from L-lysine, is produced by certain bacteria in response to growth in an acid environment,¹⁾ and it has been suggested that bacteria use this reaction either to increase intracellular pH²⁾ or to control the intracellular concentration of CO_2 , which would otherwise be exceedingly low under acidic conditions.³⁾ In addition to the acidic conditions, the presence of its substrate is known to favor LDC induction.²⁾ As a consequence of these observations, Moeller⁴⁾ and others⁵⁾ have developed qualitative, colorimetric tests for the presence of inducible LDC, which are being used routinely for the differentiation of genera and species within the bacterial families. Despite the widespread utilization of the inducible LDC in *Vibrio* species for this purpose,⁶⁾ it has not been enzymologically well-characterized.

In this paper, we describe the purification and characterization of the inducible LDC from *V. parahaemolyticus*, and compare some properties of this enzyme with those of the corresponding enzymes from *Escherichia coli* and *Hafnia alvei* (formerly designated *Bacterium cada-veris*).

Materials and Methods

Chemicals Cadaverine analogs as inhibitors of LDC⁷⁾ were kindly supplied by Merrell Dow Institute (Strasbourg Cedex, France); standard proteins for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration and pyridoxal phosphate (PLP) were from Sigma; hydroxyapatite and lysine analogs were from Wako; L-[U- ^{14}C]lysine (6.36 GBq/mmol) was from ICN. All other chemicals were of analytical or reagent grade.

Bacterial Strain and Growth Conditions The strain used was *V. parahaemolyticus* AQ3627, which was supplied by the Research Institute for Microbial Disease, Osaka University (Osaka). It was precultured at 37 °C for 12 h in medium A [0.45% yeast extract (Difco); 0.1% Bactocastone (Difco); basal inorganic salts⁸⁾; 2% NaCl; final pH 7.5]. Several 2 l Erlenmeyer flasks, each containing 1 l of medium A supplemented with L-lysine and glucose at final concentration of 50 mM and 0.5%, respectively, were inoculated with 20 ml of the preculture and then incubated at 37 °C with a rotary shaker. The concentrations of L-lysine and glucose were as recommended by Moeller.⁴⁾ After 6 h growth, the cells were harvested by centrifugation for 10 min at 5300 $\times g$ at 4 °C and washed once with ice-cold 2% NaCl containing 10 mM MgCl_2 . Cell pellets were stored at –70 °C before enzyme purification. No change in enzyme activity was noted for at least one month.

Enzyme Assay The standard assay mixture (total volume 1 ml) contained 100 mM sodium acetate buffer (pH 5.5), 10 mM L-lysine, 0.1 mM PLP and enzyme protein (0.1–100 μg). After incubation at 37 °C for 10 or 20 min, the reaction was stopped by the addition of 0.4 ml of 20% HClO_4 . Cadaverine formed was determined by a gas liquid chromatography (GLC) method⁹⁾ using 1,7-diaminoheptane as an internal standard.

Under the conditions used, the reaction was proportional to the amount of enzyme added. The decarboxylation of δ -hydroxylysine (DL and DL allo), S-aminoethyl-L-cysteine, N^α - and N^ϵ -acetyl-L-lysine was followed manometrically by measuring the CO_2 evolution (Warburg apparatus). Enzyme assays involving α -fluoromethylcadaverines were carried out by a modification of the method of Boeker and Fischer¹⁰⁾ in which radioactive CO_2 released from L-[^{14}C]lysine (10 mM, ca. 1.1×10^4 dpm/ μmol) was measured, since these compounds interfered with the GLC analysis of cadaverine. One unit of activity was defined as the amounts of enzyme that catalyzed the formation of 1 mmol cadaverine/h under standard conditions. Protein was determined by the method of Lowry with bovine serum albumin as the standard.

Purification of LDC All operations were done at 4 °C or in an ice bath, and all dialyses were done against 100 volumes of the specified buffers with two changes for 12 h. The final procedures adopted for the purification of LDC are shown in Table II. The frozen cells (43 g) were thawed and suspended in 200 ml of 10 mM Tris–HCl buffer (pH 7.5) containing 0.04 mM PLP, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 0.1% Tween 80 (buffer A), and sonicated with an ultrasonic homogenizer. Cell debris was removed by centrifugation at 40000 $\times g$ for 30 min. A small portion of the supernatant was dialyzed against buffer A to assay the activity (crude extract). The supernatant was fractionated with solid ammonium sulfate, and the protein precipitated between 50–60% saturation was collected by centrifugation, dissolved in buffer A and dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-Sephacrose CL-6B column (2.6 $\times$ 20 cm) equilibrated with 10 mM Tris–HCl (pH 7.5) containing 1 mM DTT (buffer B). After washing with 200 ml of the same buffer, the column was eluted with a linear gradient of NaCl, 0–1 M in 500 ml of the same buffer. Fractions (8 ml) were collected in tubes containing 80 μl of 4 mM PLP. The active fractions were pooled and dialyzed against buffer B containing 0.04 mM PLP. This preparation was rechromatographed in the same way except that a linear gradient of NaCl, 0–0.75 M, was applied. The pooled active fraction was dialyzed against 20 mM potassium phosphate buffer (pH 7.5) containing 0.04 mM PLP and 1 mM DTT and then applied to a hydroxyapatite column (2.1 $\times$ 10 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.5). After washing the column with 3 column volumes of the same buffer, the enzyme was eluted with a 200 ml gradient of 20–200 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT. Fractions (5 ml) were collected in tubes containing 50 μl of 4 mM PLP. The pooled active fraction, which had been concentrated to 3 ml using a Centricon 30 (Amicon), was applied to a Sephacryl S-300 column (2.1 $\times$ 85 cm) equilibrated with buffer B containing 0.04 mM PLP, 0.4 M NaCl and 0.02% sodium azide. The peak fraction (2 ml) was dialyzed for 4 h against buffer B containing 0.04 mM PLP and 0.02% sodium azide and then stored at 4 °C.

Electrophoretic Analysis and Gel Filtration SDS-PAGE was done by an 8.5% polyacrylamide slab gel according to the method of Laemmli.¹¹⁾ Before loading, the enzyme sample was heated for 3 min with 0.1% SDS and 10% glycerol in a boiling-water bath. The gel was stained for protein with Coomassie brilliant blue R-250. The relative mobility was determined for the molecular weight markers as well as for the enzyme. Analytical gel filtration for the molecular weight determination of the native enzyme was done using a Sephacryl S-300 (2.1 $\times$ 90 cm) calibrated with the standard proteins, as described by Andrews.¹²⁾ The column was equilibrated and eluted with buffer B containing 0.04 mM PLP, 0.4 M NaCl and 0.02%

sodium azide.

Amino-Terminal Amino Acid Sequencing This was determined with an Applied Biosystems model 477A protein sequencer.

Results and Discussion

In order to define a good enzyme source for purification, LDC activity was determined in cells grown under various medium conditions (Table I). At an initial pH of 7.5, glucose relatively strongly induced the enzyme with a concomitant drop of the medium pH, whereas L-lysine did not induce it at all. However, at an initial pH of 6.0, L-lysine alone produced and appreciable induction, but to a lesser extent than glucose. Simultaneous addition of L-lysine and glucose resulted in a maximal induction with a similar specific activity, regardless of the initial pH of the medium. These results suggest that the low pH of the medium, which would perturb intracellular pH homeostasis, or lowering the intracellular and extracellular pH by the acid products of glucose fermentation may contribute to LDC induction. When the bacterium was grown for 6 h at an initial pH of 7.5, the total (intra- and extracellular) cadaverine content increased from 0.47 in the absence of L-lysine and glucose to 88.1 $\mu\text{mol/mg}$ cell protein in the presence of both. In the latter case, more than 95% of cadaverine produced was detected in the medium. Recently, Ruzgiene *et al.*¹³⁾ reported the factors affecting LDC induction in *Vibrio* species.

The purification of LDC from *V. parahaemolyticus* AQ 3627 is summarized in Table II. The purified enzyme had a specific activity of 19.5 units/mg of protein, and showed 279-fold purification over the crude extract, with about 30% recovery. The purified enzyme gave a single protein band on SDS-PAGE (Fig. 1).

Stability of the enzyme was enhanced by the presence of

PLP in the solution. Freezing and thawing almost completely inactivated the enzyme. The purified enzyme could be stored at 1 °C in the presence of 1 mM DTT, 40 μmol PLP and 0.02% sodium azide for at least 2 weeks without any appreciable loss of activity.

The molecular weight of the native enzyme was determined to be 531000 by gel filtration (Fig. 2), and the band on SDS-PAGE was estimated to have a molecular

Fig. 1. SDS-PAGE of the Purified LDC

A, purified enzyme (6 μg); B, standard proteins (from the top: myosin; β -galactosidase, phosphorylase b; bovine serum albumin; egg albumin; carbonic anhydrase).

Fig. 2. Estimation of the Molecular Weight of the Purified Enzyme by Gel Filtration

The standard proteins and their molecular weights were: A, thyroglobulin (669000); B, apoferritin (443000); C, β -amylase (200000); D, alcohol dehydrogenase (150000). $\circ$: the purified enzyme.

Fig. 3. Effects of pH (A) and Temperature (B) on the Purified LDC Activity

The standard assay mixture containing 0.3 μg of the purified enzyme was incubated at various pHs and temperatures as indicated. The buffers used were: 100 mM sodium acetate buffer (pH 4.0–5.6); 100 mM sodium phosphate buffer (pH 5.8–8.0).

TABLE I. Effects of Glucose, L-Lysine and pH on LDC Induction in *V. parahaemolyticus*

Addition to the medium	LDC activity ($\mu\text{mol/mg}$ protein/h) ^{a)}	
	Initial pH of the medium 7.5	6.0
None	0.49 (7.43)	2.32 (6.35)
L-Lysine	0.52 (7.41)	15.0 (7.05)
Glucose	13.8 (6.63)	31.2 (5.27)
L-Lysine + glucose	71.4 (7.18)	70.1 (6.91)

a) LDC activity was determined with cells grown for 6 h in the absence or presence of L-lysine (50 mM) and/or glucose (0.5%). Values shown in parentheses represent the pHs of the media after 6 h growth.

TABLE II. Purification of Inducible LDC from *V. parahaemolyticus*

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Crude extract	2087	146	0.070	100	1
Ammonium sulfate (50–60%) precipitation	834	171	0.205	117	2.9
1st DEAE-Sephacrose CL-6B	334	137	0.410	94	5.9
2nd DEAE-Sephacrose CL-6B	16.0	119	7.44	82	106
Hydroxyapatite	5.5	88	16.0	60	229
Sephacryl S-300	2.3	45	19.5	31	279

A typical purification from 43 g of wet weight cells is shown.

weight of 79000. The molecular weight of this band was unaltered when the protein was treated with 2-mercaptoethanol before electrophoresis. Thus, the active form may be composed of seven subunits of identical size which are not linked by disulphide bonds. Similarly, a heptameric structure has been suggested for the *H. alvei* LDC when calibrated by gel filtration.^{15a)}

The effects of pH and temperature on enzyme activity were investigated. The enzyme was most active at pH 5.5, as shown in Fig. 3A, but, unlike that from *E. coli*,^{2b)} showed no bell-shaped curve. The maximum activity, which was about 1.6-fold higher than that observed at 37 °C, was seen at 45–50 °C (Fig. 3B). Furthermore, when the enzyme was incubated at various temperatures for 10 min at pH 5.5, and then the remaining activity was measured under the standard assay conditions, 65% of its initial activity was retained after heating even at 80 °C. A similar heat stability was reported for the *E. coli* LDC.^{2b)}

D-Lysine, L-ornithine and lysine analogs at 10 mM were tested as substrates under the standard assay conditions. S-Aminoethyl-L-cysteine and δ -hydroxylysine were decarboxylated at rates of 23% and 17% that observed for L-lysine, whereas D-lysine, L-ornithine, *N*²- and *N*^ε- acetyl-L-lysine were not. Lineweaver-Burk plots gave a K_m of 3.2 mM for L-lysine and a V_{max} of 20.5 mmol cadaverine/mg protein/h.

Overnight dialysis of the purified enzyme against buffer B (devoid of PLP) resulted in about 60% irreversible loss of activity. When PLP was omitted from the assay mixture, the activity of such a dialyzed preparation decreased to 30% of the control activity in the presence of 0.1 mM PLP. These results indicate that PLP is indispensable for both stability and full activity of the enzyme.

The effect of various inhibitors on enzyme activity was examined by adding them to the incubation mixture (Table III). At a final concentration of 5 mM, 6-aminocaproic acid and carboxymethoxylamine, a PLP-dependent enzyme inhibitor, produced a more than 80% inhibition of the *V. parahaemolyticus* LDC. It is of interest that the *V. parahaemolyticus* LDC, as well as the *E. coli* LDC,^{2b)} is inhibited by 6-aminocaproic acid which lacks an α -amino group expected to be important in the interaction with the enzyme. On the other hand, at a final concentration of 1 mM, α -monofluoromethylcadaverine (MFMC) inhibited the enzyme to a much lesser extent than the other α -fluoromethylcadaverines. However, when preincubated with MFMC at the same concentration in the absence of the substrate, the enzyme was inhibited by about 80%. The same treatment of the enzyme with α -difluoromethylcadaverine (DFMC) or α -trifluoromethylcadaverine (TFMC) brought about no appreciable inhibition. Moreover, TFMC appeared to be more potent than DFMC in inhibiting the enzyme. These results are in agreement with the finding⁷⁾ that MFMC acts on the *E. coli* LDC as an enzyme-activated irreversible inhibitor and two other α -fluoromethylcadaverines as competitive inhibitors.

Addition of EDTA, DTT, Mg^{2+} or Ca^{2+} (all 1 mM) to the assay mixture did not affect enzyme activity. DTT was required for stabilizing the enzyme during purification and storage.

The amino-terminal sequence of the enzyme was determined to be Met-Asn-Ile-Phe-Ala-Ile-Leu.

TABLE III. Effect of Inhibitors on Inducible LDC from *V. parahaemolyticus*

Inhibitor	Concentration (mM)	Relative activity (%) ^{a)}
None (Control)	—	100
6-Aminocaproic acid	1	42
	5	23
Carboxymethoxylamine	1	37
	5	9
α -Monofluoromethylcadaverine	1	92
	1	16 ^{b)}
α -Difluoromethylcadaverine	1	77
	1	77 ^{b)}
	2	59
α -Trifluoromethylcadaverine	1	53
	1	47 ^{b)}
	2	12

Each assay was carried out with 0.3 μ g of the purified enzyme. a) The values are means of triplicate measurements, which are expressed as percentages of the control activity. b) The standard assay mixture without L-lysine as the substrate was preincubated with individual inhibitors for 20 min at 37 °C, and then the activity was assayed after addition of the substrate to the reaction mixture.

TABLE IV. Comparison of Some Properties and Amino-Terminal Sequences of LDCs from Different Bacteria

LDC source	K_m (mM)	pH optimum	Mol.wt. (native) ($\times 10^{-3}$)	Subunit mol.wt. ($\times 10^{-3}$)	Amino-terminal sequence
<i>V. parahaemolyticus</i>	3.2	5.5	531	79	Met-Asn-Ile-Phe-Ala-Ile-Leu-
<i>E. coli</i>	1.5	5.7	780	80	Met-Asn-Val-Ile-Ala-Ile-Leu-
<i>H. alvei</i>	1.7	5.8	542	77	Met-Asn-Ile-Ile-Ala-Ile-Leu-

Table IV shows some properties and the amino-terminal sequences of the inducible LDCs from *E. coli*^{2b,14)} and *H. alvei*¹⁵⁾ in Enterobacteriaceae, together with those of the *V. parahaemolyticus* LDC. There are several similarities among the enzymes from these bacteria, including cofactor requirement, optical pH, subunit molecular weight and amino-terminal sequence. However, the molecular structure of the enzyme in *V. parahaemolyticus* is somewhat different from that in *E. coli*, but rather similar to that in *H. alvei*. The molecular weights of the native enzymes of *V. parahaemolyticus* and *H. alvei*, as judged by gel filtration, are 531000 and 545000, respectively, and hence they may consist of seven identical subunits, whereas that of *E. coli* is characterized by a decameric structure with a molecular weight of 780000, consisting of ten identical subunits with a molecular weight similar to *V. parahaemolyticus*. The K_m value of the *V. parahaemolyticus* LDC was higher than those of the *E. coli* and *H. alvei* LDCs. Moreover, the amino-terminal sequence of the *V. parahaemolyticus* LDC differs from that of *H. alvei* LDC only in the fourth position, but differs from that of *E. coli* in the third and fourth positions. These substitutions in amino acids, however, may be caused by only one base pair exchange, and the amino-terminal regions appear to be well conserved among these species to remain rich in hydrophobic amino acids.

References

- 1) E. F. Gale, *Biochem. J.*, **34**, 392 (1940); D. R. Morris and R. H.

- Fillingame, *Annu. Rev. Biochem.*, **43**, 303 (1974).
- 2) a) E. F. Gale, *Adv. Enzymol.*, **6**, 1 (1946); b) D. L. Sabo, E. A. Boeker, B. Byers and E. H. Fischer, *Biochemistry*, **3**, 662 (1974).
 - 3) B. M. Guirard and E. E. Snell, *Compr. Biochem.*, **14**, 138 (1964).
 - 4) V. Moeller, *Acta Pathol. Microbiol. Scand.*, **36**, 158 (1955).
 - 5) S. Falkow, *Am. J. Clin. Pathol.*, **29**, 598 (1958); H. L. Smith, Jr. and P. Bhat-Fernandes, *Appl. Microbiol.*, **26**, 620 (1973).
 - 6) J. J. Farmer III, F. W. Hickman-Brenner and M. T. Kelly, "Manual of Clinical Microbiology," ed. by A. Balows, W. J. Hausler, Jr. and H. J. Shadomy, American Society of Microbiology, Washington, D. C., 1985, p. 282.
 - 7) P. Bey, C. Danzin and M. Jung, "Inhibition of Polyamine Metabolism," ed. by P. P. McCann, A. E. Pegg and A. Sjoerdsma, Academic Press, Inc., San Diego, 1987, p. 1.
 - 8) S. Yamamoto, S. Shinoda, M. Kawaguchi, K. Wakamatsu and M. Makita, *Can. J. Microbiol.*, **29**, 724 (1983).
 - 9) S. Yamamoto, Y. Suemoto, Y. Seito, H. Nakao and S. Shinoda, *FEMS Microbiol. Lett.*, **35**, 289 (1986).
 - 10) E. A. Boeker and E. H. Fischer, *Methods Enzymol.*, **94**, 180 (1983).
 - 11) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
 - 12) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
 - 13) A. Ruzgiene, A. Ragaricius and S. Bereckiene, *Prikl. Biokhim. Mikrobiol.*, **26**, 81 (1990) [*Chem. Abstr.*, **112**, 155032s (1990)].
 - 14) D. L. Sabo and E. H. Fischer, *Biochemistry*, **13**, 670 (1974).
 - 15) a) H. Beier, L. F. Fecker and J. Berlin, *Z. Naturforsch.*, **42c**, 1307 (1987); b) L. F. Fecker, H. Beier and J. Berlin, *Mol. Gen. Genet.*, **203**, 177 (1986); c) K. Soda and M. Moriguchi, *Biochem. Biophys. Res. Commun.*, **34**, 34 (1969).