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Molecular characterization of lysine 6-dehydrogenase from *Achromobacter denitrificans*

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An inducible lysine 6-dehydrogenase (Lys 6-DH), which catalyzes the oxidative deamination of the 6-amino group of L-lysine in the presence of NAD^+, was purified to homogeneity from *Achromobacter denitrificans*, yielding a homodimeric protein of 80 kDa. The enzyme was specific for the substrate L-lysine and NAD^+ served as a cofactor. The dimeric enzyme associated into a hexamer in the presence of 10 mM L-lysine. The K_m values for L-lysine and NAD^+ were 5.0 and 0.09 mM, respectively. The *lys 6-dh* gene was cloned and overexpressed in *E. coli*. The open reading frame was 1,107 nucleotides long and encoded a peptide containing 368 amino acids with 39,355 Da. The recombinant enzyme was purified to homogeneity and characterized. Enzyme activities and kinetic properties of the recombinant enzyme were almost the same as those of the endogenous enzyme obtained from *A. denitrificans*. Crystals of the enzyme were obtained using the hanging drop method. [BMB reports 2008; 41(11): 790-795]

INTRODUCTION

Amino acid dehydrogenases have been studied intensively because of their value in industrial applications (1). So far, two types of lysine dehydrogenases, lysine 2-dehydrogenase (Lys 2-DH, EC 1.4.1.15) and lysine 6-dehydrogenase (Lys 6-DH, EC 1.4.1.18), have been reported. It was proposed that human liver cells possessed Lys 2-DH activity (2). However, this enzyme has not been characterized because its occurrence remains elusive. In contrast, Lys 6-DH activity is present in many microorganisms (3-5). Misono and Nagasaki demonstrated that Lys 6-DH from *Agrobacterium tumefaciens* catalyzes the oxidative deamination of the 6-amino group of L-lysine in the presence of NAD^+ to yield delta-1-piperideine-6-carboxylate (P-6-C), along with the reaction intermediate L-2-aminoadipate 6-semialdehyde (3). Among microbial Lys 6-DH enzymes, the *A. tumefaciens* protein has been extensively studied (4,6, 7-10) and is presently used for L-lysine determination (11,12) and P-6-C preparation (13). Recently, *Geobacillus stearothermophilus* was characterized as a lysine dehydrogenase producer (14). However, it is not clear which amino group of L-lysine the enzyme cleaves because the reaction product from L-lysine was only examined via thin layer chromatography. By using this chromatographic technique, delta-1-piperideine-2- carboxylate (P-2-C), the product of the Lys 2-DH reaction, was not separated from P-6-C (15).

This paper documents the isolation of a new Lys 6-DH producer, *Achromobacter denitrificans*. The properties of the enzyme, its gene cloning, the effective production of Lys 6-DH, and its crystallization are presented.

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RESULTS AND DISCUSSIONS

Isolation of a bacterium having lysine dehydrogenase activity

A soil bacterium that possessed lysine dehydrogenase activity was isolated. It was identified as a gram negative, rod, aerobic, motile (with polar flagella), and non-sporing bacterium which could grow at 42°C. Reduction and denitrification of nitrate were positive. The 16S ribosomal RNA gene sequence showed a similarity of 98% to that of *Achromobacter denitrificans*. Therefore, this strain was designated to be *A. denitrificans*. Lysine dehydrogenase production was induced by the addition of L-lysine to the medium. The highest specific and total activities were obtained by cultivating the cells in a 1.0% peptone medium containing 1.0% L-lysine at 30°C for 20 h on a reciprocal shaker.

Purification of lysine dehydrogenase from *A. denitrificans*

The enzyme was purified approximately 100-fold to homogeneity with a 15% yield (Table 1). The purified enzyme migrated as a single band on SDS-PAGE (Fig. 1).

Molecular mass, subunit structure, and amino acid sequences

The molecular mass of the subunit was 40 kDa as determined

Table 1. Purification of lysine 6-dehydrogenase from *A. denitrificans* K-1

Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Lys 6-DH from *A. denitrificans* K-1				
Cell extract	8,810	425	0.05	100.0
60%$(NH_4)_2SO_4$	3,350	216	0.06	50.8
DEAE-cellulose	314	185	0.59	43.5
40%$(NH_4)_2SO_4$	49	196	4.00	46.1
Sephadex G-150	36	73	2.03	17.1
Mono Q	18	89	4.94	21.0
Phenyl Sepharose	13	63	4.85	14.8
Lys 6-DH from *E. coli* clone				
Cell extract	697	1,500	2.15	100.0
DEAE-Toyopearl	317	1,382	4.36	92.1
DEAE-Sephadex A50	182	816	4.48	54.4

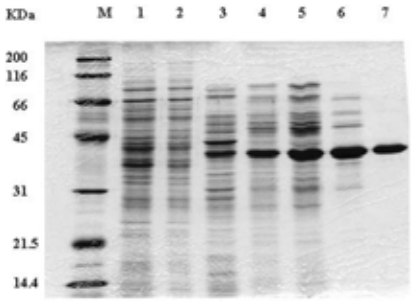

Fig. 1. SDS-PAGE of the lysine dehydrogenase from *A. denitrificans* K-1. Lane M, molecular marker proteins; lane 1, crude enzyme (80 μg); lane 2, 60% ammonium sulfate fraction (80 μg); lane 3, DEAE-cellulose pool fraction (50 μg); lane 4, 40% ammonium sulfate fraction (40 μg); lane 5, Sephadex G-150 pool fraction (40 μg); lane 6, Mono Q pool fraction (20 μg); and lane7, Phenyl Sepharose pool fraction (10 μg).

by SDS-PAGE. The molecular mass of the purified enzyme was 80 kDa by gel filtration on a TSK gel G3000SW column. These results indicated that the enzyme was a dimer, similar to the *A. tumefaciens* enzyme (78 kDa) (7). This was in contrast to the hexameric enzyme (260 kDa) isolated from *G. stearothermophilus* (14). However, the *A. denitrificans* enzyme eluted as hexamer (240 kDa) when a high concentration of L-lysine (10 mM) was supplemented to both the enzyme solution and the elution buffer. In contrast, NAD^+ (0.1 mM) was ineffective at promoting the quaternary structure of the enzyme. Our observations suggest that the dimeric enzyme itself exhibits catalytic activity and it assembled into a hexamer in the presence of L-lysine. Previous studies indicated that the *A. tumefaciens* enzyme associated as a tetramer (160 kDa) in the presence of L-lysine (7) and the *G. stearothermophilus* enzyme was a hexamer in the absence of L-lysine (14). Thus, the *A. denitrificans*

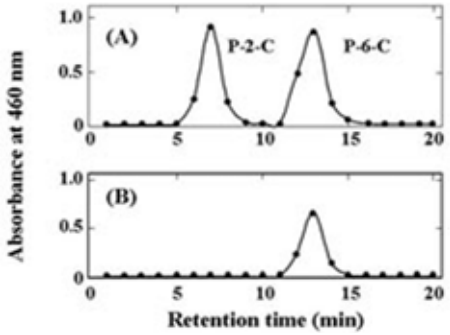

Fig. 2. Ion-exchange chromatogram of the reaction product formed from L-lysine. The reaction product was analyzed. (A) standard: P-2-C and P-6-C; (B) the reaction product.

enzyme is different from the *A. tumefaciens* enzyme and the *G. stearothermophilus* enzyme. The N-terminal amino acid sequence of the enzyme was determined to be MSRTQHAITVLG AGKIGFAIALLLQRTGDYA. The amino acid sequence of an internal peptide, which was isolated from the lysyl endopeptidase-digested protein, was KVPPMEGYETFTLDGVEYEAFNTSG GLGTLPQTLLG.

Identification of the lysine dehydrogenase reaction product

Since a cyclized form of the oxo or aldehyde analog of L-lysine, P-2-C or P-6-C, should be formed from L-lysine in the reaction (3, 16), the reaction products were analyzed by ion-exchange chromatography. The product from L-lysine separated at the same point as P-6-C (Fig. 2). This result indicates that this enzyme catalyzes the deamination of the 6-amino group of L-lysine.

Effects of pH and temperature on stability and activity of the enzyme

The enzyme was most stable in the pH range of 5.5 to 7.0 when assayed at 50°C for 10 min. When the enzyme was kept at 30°C in 10 mM KP buffer (pH 7.4), the full activity was maintained for 3 days and 70% of the activity remained after 7 days. Upon storage at 4°C, the full activity was maintained for 2 months. The enzyme was stable up to 50°C when heated for 10 min in 10 mM KP buffer (pH 7.4), whereas the enzyme was stable up to 60°C in the presence of 10 mM L-lysine. These results show that the presence of L-lysine significantly increased its thermal stability. The *A. denitrificans* enzyme was determined to be more stable than the *A. tumefaciens* enzyme. Moreover, the stability of the *A. denitrificans* enzyme incubated with L-lysine is near that of the *G. stearothermophilus* enzyme. The enzyme showed the maximum activity at approximately pH 9.7 and 50°C. The optimum pH of the *A. denitrificans* enzyme (pH 9.7) is similar to that of the enzyme from *A. tumefaciens* (pH 9.7) (7) and *G. stearothermophilus* (pH 10.0) (14).

Coenzyme and substrate specificities

The enzyme required NAD^+ as a coenzyme for the oxidative deamination of L-lysine, whereas no activity was observed with $NADP^+$. Thus, in its specificity for $NADP^+$, the *A. denitrificans* enzyme is obviously different from the *G. stearothermophilus* enzyme (14). Some analogs of NAD^+ could also serve as coenzymes. In the presence of deamino-NAD^+ (K_m, 0.11 mM), 3-acetylpyridine-NAD^+ (K_m, 1.32 mM), and nicotinamide guanine dinucleotide (K_m, 0.37 mM), the enzyme activities were 64, 55, and 70% of the activity with NAD^+, and their affinities for the enzyme were lower than that of NAD^+ (K_m, 0.09 mM).

The enzyme acts exclusively on L-lysine. None of the following amino acids and amines (20 mM) were substrates: D-lysine, delta-hydroxy-L-lysine, alpha-*N*-acetyl-L-lysine, epsilon-*N*-acetyl-L-lysine, 6-amino-*n*-caproate, L-ornithine, L-2, 4-diaminobutyrate, DL-homoserine, saccharopine, cadaverine, putrescine, L-phenylalanine, L-leucine, L-isoleucine, L-norleucine, L-norvaline, L-valine, L-glutamate, L-aspartate, L-alanine, L-serine, L-threonine, L-histidine, or L-arginine.

When the dimeric enzyme was used, the NAD^+ saturation curve was hyperbolic (K_m, 0.09 mM), but a sigmoidal saturation curve was obtained for L-lysine. The Hill coefficient was 2.2 and the K_m for L-lysine was 8.1 mM. These results suggest that L-lysine acts as an allosteric effector, similar to that of the *A. tumefaciens* enzyme (7). In additon to L-lysine, the L-lysine analogs L-norleucine and 6-amino-n-caproate, which were not substrates, induced the association of the *A. denitrificans* enzyme as found in the *A. tumefaciens* enzyme (9). The apparent K_m of the hexameric enzyme for L-lysine was 5.0 mM. The specific activity of the hexameric enzyme (10.4) was higher than that of the dimeric enzyme (4.8) in the presence of 20 mM L-lysine. The V_{max} of the hexameric enzyme (14 μmol/min/mg) was higher than that of the dimeric enzyme (8.2 μmol/min/mg). The allostericity found in the *A. tumefaciens* and *A. denitirificans* enzymes was not reported for the *G. stearothermophilus* enzyme (14).

Inhibitors

The enzyme was not affected by 1 mM EDTA, sodium azide, sodium arsenate, or sodium fluoride. The enzyme was completely inactivated by 0.01 mM *p*-chloromercuribenzoate, 0.01 mM $HgCl_2$, and 0.1 mM *N*-ethylmaleimide, when the enzyme was incubated with these reagents in 100 mM KP buffer (pH 7.4) at 30°C for 10 min. The non-substrate amino acids and amines (10 mM) described above were not inhibitors, except for L-ornithine, 6-amino-*n*-caproate, L-norleucine, and L-leucine. The enzyme was inhibited 10% by 10 mM L-ornithine and 6-amino-*n*-caproate, 20% by 10 mM L-norleucine, and 50% by 10 mM L-leucine. L-Leucine inhibited the enzyme noncompetitively against L-lysine (K_i, 3.2 mM).

Gene cloning and sequencing

The gene encoding the Lys 6-DH from *A. denitrificans* was cloned and sequenced (GenBank DQ165182). The gene encoded a protein consisting of 368 amino acid residues (Fig. 3). The deduced amino acid sequence of the first 20 amino acids at N-terminus was identical with that of the enzyme purified from *A. denitrificans*. The predicted molecular mass (39,355 Da) was in good agreement with the apparent subunit molecular mass (40 kDa) of the enzyme purified from *A. denitrificans*.

Amino acid sequence alignment

The predicted amino acid sequence of the enzyme from *A. denitrificans* K-1 was 52% and 26% similar to Lys 6-DH from *A. tumefaciens* (AB288379) (Unpublished data) and the lysine dehydrogenase from *G. stearothermophilus* (BAB39707) (14), respectively. A typical NAD^+-binding motif (βαβ-fold) was found in the N-terminal region (17). SH-modifying reagents inactivated the enzymes from *A. denitrificans*, *A. tumefaciens* (7), and *G. stearothermophilus* (14). However, cysteine residues were not conserved in these enzymes, suggesting that cysteine residues do not play a role in the catalysis.

Fig. 3. Alignment of the deduced amino acid sequences of lysine dehydrogenases from *A. denitrificans* (DQ165182) (Ad), Lys 6-DH from *A. tumefaciens* (AB288379) (At), and lysine dehydrogenase from *G. stearothermophilus* (BAB39707) (Gs). Gray shading indicates residues identical among the three sequences. The glycine residues in the NAD^+-binding site are indicated by asterisks.

Overproduction of recombinant Lys 6-DH and its properties

The overproduction of the *A. denitrificans* Lys 6-DH was performed using the *E. coli* BL21(DE3)/pET-ADK strain. The recombinant enzyme was abundantly produced in this *E. coli* clone and was found to be soluble. A crude extract of the recombinant cells (2.15 units per mg of protein) had 43-fold higher enzyme activity than that of wild type (0.05 units per mg of protein). The recombinant enzyme was purified to homogeneity with a 54% yield (Table 1). The molecular mass of the recombinant enzyme was 80 kDa in the absence of L-lysine, 240 kDa in the presence of L-lysine, and 40 kDa under denatured conditions. The enzymological and kinetic properties of the recombinant enzyme were almost the same as those of the endogenous enzyme purified from *A. denitrificans*.

Crystallization

Crystals of the enzyme were obtained using the hanging drop method (Fig. 4). The crystallization of the *A. denitrificans* enzyme will help in the establishment of the three-dimensional structure of Lys 6-DH.

Recently, much attention has been paid to the asymmetric synthesis of L-pipecolate (18, 19), a key component of the immunosuppressant rapamycin (20) and the antitumor agent sandramycin (21). L-Pipecolate can be derived from P-6-C (18). Therefore, the *E. coli* BL 21(DE3)/pET-ADK clone that can overproduce Lys 6-DH of *A. denitrificans* should be useful for the bioproduction of L-pipecolate from L-lysine. We are now studying the molecular structure of Lys 6-DH in more detail.

MATERIALS AND METHODS

Materials

A TSK gel G3000SW column was obtained from Tosoh (Japan). A YMC-Pack C4 column was obtained from YMC (Japan). The pET 17-b plasmid was supplied by Novagen (USA). Ex*Taq* DNA polymerase was purchased from Takara Shuzo (Japan). P-2-C and P-2-C were prepared from lysine using lysine α-oxidase from *Trichoderma viride* (16) and Lys 6-DH from *A. tumefaciens* (6) respectively. The *o*-Aminobenzaldehyde was synthesized from *o*-nitrobenzaldehyde (22).

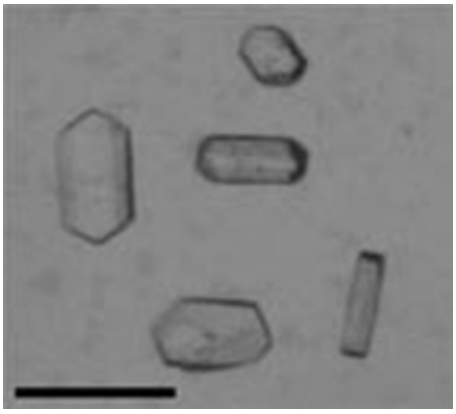

Fig. 4. Crystals of the enzyme grown using the hanging drop vapor diffusion method. The size of the bar is 0.5 mm. The size of a big crystal is 0.5 mm x 0.25 mm.

Bacteria and culture conditions

A. denitrificans K-1 was cultured in peptone medium pH 7.2 (1.5% peptone, 1.0% L-lysine, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.2% NaCl, 0.015% $MgSO_4 \cdot 7H_2O$, and 0.015% yeast extract) at 30°C for 20 h with shaking. The cells were harvested by centrifugation and washed twice with 0.85% NaCl solution.

Enzyme activity assays

The reaction mixture (1 ml) for determination of lysine dehydrogenase activity consisted of 0.2 M glycine-KOH buffer (pH 10.0), 20 mM L-lysine, 2 mM NAD^+, and the enzyme. The reaction was started by the addition of NAD^+ and the increase in the absorbance at 340 nm was monitored for 1 min using a Beckman DU-640 spectrophotometer. As a blank control, the reaction mixture without L-lysine was used. One unit of enzyme activity was defined as the amount that catalyzed the formation of 1 μmol of NADH per minute. Protein concentration was measured by the method of Lowry *et al.* (23).

Enzyme purification

All steps were performed at 4°C. A standard buffer (10 mM potassium phosphate (KP) buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 1 mM EDTA) was used in all steps unless otherwise stated. Washed cells of *A. denitrificans* K-1 (684 g) were suspended in 100 mM KP buffer (pH 7.4) containing 0.02% 2-mercaptoethanol, 2 mM EDTA, and 1 mM PMSF, then disrupted by sonication. After centrifugation, the supernatant was dialyzed against the standard buffer and used as the crude extract. The crude extract was subjected to ammonium sulfate fractionation. The 0-60% saturation fraction was dissolved in standard buffer and dialyzed against the same buffer. The supernatant was applied to a DEAE-cellulose column (5 x 50 cm). The column was then washed stepwise with the standard buffer containing 0, 0.05, and 0.1 M KCl, respectively. The enzyme was eluted with the buffer containing 0.15 M KCl. The active fractions were pooled and subjected to ammonium sulfate fractionation (40% saturation). The precipitate was dissolved in the standard buffer and applied to a Sephadex G-150 column (3 x 95 cm) that had been equilibrated with the standard buffer containing 10 mM L-lysine. The active fractions were pooled, dialyzed, and applied to a Mono Q column (0.5 x 5 cm) connected to an AKTA prime FPLC system (Amersham Bioscience). After the column was washed with standard buffer, the enzyme was eluted with a linear gradient of KCl (0 to 0.3 M) in the buffer. The active fractions were pooled, dialyzed, and concentrated by ultrafiltration with an Amicon PM-10 membrane. The enzyme solution, which was dialyzed against the standard

buffer containing ammonium sulfate (20% saturation), was subjected to the FPLC system equipped with a HiTrap Phenyl Sepharose column (0.5 x 5 cm) that had been equilibrated with standard buffer containing ammonium sulfate (20% saturation). After the column had been washed with the same buffer, the enzyme was eluted with a linear gradient of ammonium sulfate (20% to 0% saturation) in the buffer. The active fractions were pooled, dialyzed, and concentrated by ultrafiltration.

Molecular mass determination

The molecular mass was estimated by gel filtration on a TSK gel G3000SW column (0.75 x 60 cm) at a flow rate of 0.7 ml per min with 0.1 M KP buffer (pH 7.0) containing 0.2 M NaCl. The proteins glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome c (12.4 kDa) were used as molecular mass standards. The molecular mass of the enzyme subunit was estimated by SDS-PAGE (24) using the myosin (200 kDa), β-galactosidase (116 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) as marker proteins.

Kinetic measurement

The reactions were performed under the standard conditions as described above. Initial velocity experiments were carried out by varying the concentration of one substrate at different fixed concentrations of the other substrate. The Michaelis constant was calculated from the secondary plot of intercepts versus the reciprocal concentration of the substrate.

Isolation of peptides obtained by lysyl endopeptidase-digestion of the enzyme and amino acid sequence analysis

The purified enzyme (1 nmol) was dialyzed against water and lyophilized. The protein was dissolved in 20 µl of 8 M urea and incubated at 37°C for 1 h. Then 60 µl of 0.2 M Tris-HCl buffer (pH 9.0) and 5 pmol of lysyl endopeptidase were added and incubated at 37°C for 18 h. The digested peptides were separated on a Shimadzu HPLC system equipped with a YMC-Pack C4 column using a solvent system of 0.1% trifluoroacetic acid (A) and acetonitrile containing 0.1% trifluoroacetic acid (B). A 90-min linear gradient from 5 to 50% B was used to elute peptides at a flow rate of 1.0 ml per min. The absorbance at 210 nm of the effluent was continuously monitored. The isolated peptides were collected and lyophilized.

The N-terminal amino acid sequence of the enzyme and the isolated peptides were analyzed with an Applied Biosystems model 492-protein sequencer linked to a phenylthiohydantoin derivative analyzer.

Identification of the reaction product

The reaction mixture (1.0 ml) composed of 0.02 µM of L-lysine, 0.02 µM of NAD^+, 0.2 µM of sodium carbonate buffer (pH 9.5), and 800 µg of the purified enzyme was incubated at 30°C for 30 min. After the reaction was terminated by the addition of 0.2 ml of 50% trichloroacetic acid, the reaction mixture was centrifuged at 10,000 g for 10 min. The supernatant (0.5 ml) was applied to a standard column (0.4 x 15 cm) of a Hitachi 835 amino acid analyzer, equilibrated with the citrate buffer at pH 3.25 (7.74 g of sodium citrate dehydrate, 7.07 g of NaCl, 20 g of citric acid monohydrate, 20 ml of ethanol, 5 ml of thiodiglycol, and 4 ml of 25% Brij-35 per liter). The amino acids were eluted with the same buffer at a flow rate of 1.0 ml per min at 55°C. The eluate was collected in 1.0-ml fractions and mixed with 0.2 ml of 50 mM o-aminobenzaldehyde in 0.2 M KP buffer (pH 8.0). After incubation at 37°C for 1 h, absorbance was measured at 460 nm.

Genetic manipulation

Two mixed primers were designed from the N-terminal amino acid sequence and an internal peptide of the enzyme. The sense and antisense primers were 5'-ACTCA(A,G)CA(C,T)GCTATTAC(A,C,G,T)GT(A,C,G,T)CTT-3' and 5'-AATGT(C,T)TC(A,G)TATCC(C,T)TCCATTGG(A,C,G,T)GG(A,C,G,T)AC-3', respectively. PCR was performed with Ex*Taq* DNA polymerase using chromosomal DNA of *A. denitrificans* as a template. The nucleotide sequence of the amplified DNA fragment (600 bp) was determined by using the PRISM kit with an Applied Biosystems 373A DNA sequencer. Then, the inverse PCR was carried out. The chromosomal DNA was digested with each of various restriction enzymes, and then the digested fragments were incubated with T4 DNA ligase to promote self-circulation for serve as DNA templates. Two primers 5'-CGTGCATTACTTCGACCTGA-3' and 5'-ATGCACTGCGCTACAACCTT-3' were designed from the determined nucleotide sequence of the amplified DNA. PCR was conducted and the nucleotide sequence of the amplified 1.8 kb fragment containing a putative structural gene of the enzyme was determined.

To overproduce the enzyme, two single primers were designed from the nucleotide sequences at both the immediate up and downstream sequences, with the sense primer 5'-GGGAATTCCATATGTCCCGTACGCAACACGCCATCACCGTC-3') containing a *Nde*I site and the antisense primer 5'-CGCGGATCCTCAGGCGGCCAGCAGGGCGTCGAG-3' containing a *Bam*HI site. The amplified DNA fragment was digested with both *Nde*I and *Bam*HI and ligated into the *Nde*I-*Bam*HI site of pET17-b. The constructed plasmid, pET-ADK, was introduced into *E. coli* BL21 (DE3) by electrotransformation. The sequence of the insert in pET-ADK was verified in both directions.

Isolation of recombinant Lys 6-DH

An *E. coli* transformant harboring pET-ADK was inoculated into 100 ml of LB broth containing ampicillin (50 µg per ml). The cultivation was done at 37°C on a reciprocal shaker. At the log phase of cell culture, IPTG (final concentration of 0.2 mM) was added to the cell suspension to induce enzyme production and the culture was grown for 5 h.

Cells (6 g) were suspended in 100 mM KP buffer (pH 7.4) containing 0.02% 2-mercaptoethanol, 2 mM EDTA, and 1 mM PMSF,

then disrupted by sonication at 4°C. The cell debris was removed by centrifugation and the supernatant was dialyzed. This solution was used as the crude extract. The recombinant enzyme was purified using DEAE-Toyopearl and DEAE- Sephadex A50 columns. The purified enzyme was concentrated and stored at 4°C.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper is available in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number DQ165182.

Crystallization

Crystallization of the enzyme was performed using the hanging drop vapor diffusion method. Each droplet was prepared by mixing 5 μl of protein solution (10 mg per ml) in 0.01 M KP buffer (pH 7.4) with an equal volume of each precipitant solution of the Wizard I, II, and III (Emerald BioSystems, USA).

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