Lysine Decarboxylase from *Hafnia alvei*: Purification, Molecular Data and Preparation of Polyclonal Antibodies

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The purification and molecular properties of lysine decarboxylase from *Hafnia alvei* and the preparation of polyvalent antibodies specific for this enzyme are described. The enzyme was purified within two HPLC steps on a TSK G 4000 SW and a MonoQ column to homogeneity. The subunit of the enzyme has a molecular weight of approximately 80,000 d. Under "native" conditions it seems to form aggregates up to ten subunits. Lysine decarboxylase from *H. alvei* contains one mol pyridoxal phosphate per mol subunit. Antibodies against the lysine decarboxylase were purified by affinity chromatography.

Introduction

Production of secondary metabolites by plant cell cultures is often limited by the low activity of secondary pathway enzymes [1]. This is holds also true for the formation of quinolizidine alkaloids in cell cultures of *Lupinus polyphyllus* [2, 3]. One reason for the low synthesis of quinolozidine alkaloids may be seen in the low activity of lysine decarboxylase (LDC) [2, 3]. We intend to determine whether LDC-activity of *Lupinus* cells might be greatly increased by the introduction of a LDC-gene from a bacterium into *Lupinus* plants [4]. For cloning a corresponding gene we first had to purify lysine decarboxylase from *Hafnia alvei*, as this gram negative enterobacterium contains high amounts of LDC [5, 6].

In 1969 Soda and Moriguchi described a seven-step purification protocol of LDC from *Hafnia alvei* which nevertheless did not yield homogeneous LDC [7]. With the availability of FPLC or HPLC columns for protein purification the purification of LDC can rapidly be performed today. Using the newer methods we established a very efficient and fast procedure for yielding mg-amounts of homogeneous LDC. Since the final aim of our project is to transform plant cells with the LDC gene from *H. alvei*, the next step was the preparation of LDC specific antibodies needed as probes for detecting the LDC-gene of *H. alvei* in a gene bank of *E. coli*. A rapid purification based on affinity chromatography removed undesired antibodies of the serum.

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Materials and Methods

Purification of LDC from H. alvei

H. alvei strain (IFO 3731) was grown in 500 ml TY-medium (10 g bacto tryptone, 5 g yeast extract, 5 g NaCl). Culture conditions were 37 °C and 150 rpm on a New Brunswick shaker. After 10–14 h cells were harvested by centrifugation in a GS3-Sorvall rotor for 20 min at 7,500 rpm and 4 °C. The pellets were washed once with 330 ml 0.05 M Tris/acetate pH 6.0 (buffer A) and resuspended in 30 ml of the same buffer. The cells were lysed by sonication (10 boosts for 30 s). To avoid warming up, cells were cooled on ice. Cells and cell debris were removed by centrifugation in a SS34-Sorvall rotor at 10,000 rpm for 15 min at 4 °C. The proteins of the supernatant were precipitated at 55% ammonium sulfate saturation at 4 °C for 15 min and centrifugated at 14,000 rpm for 15 min at 4 °C. Pellets were resuspended in the minimum volume of buffer A being sufficient for the resolution of the proteins. This crude extract could be stored at −20 °C for months without significant loss of enzyme activity.

Gel filtration on a TSK G 4000 SW HPLC-column

The column was loaded with up to 1 ml of the crude extract. Proteins were eluted at a flow rate of 0.2 ml/min or 0.5 ml/min respectively with 0.05 M Tris/acetate pH 6.0 + 0.03% NaN_3. Elution of pro-

teins was monitored with an Uvicord monitor at 280 nm.

MonoQ ion exchange-chromatography

The LDC-activity containing fractions eluted by the HPLC-gel filtration column were directly loaded on the MonoQ-column (Pharmacia) at a flow rate of 0.1 ml/min. The column was equilibrated with 0.02 M piperazine-acetate pH 5.5 (buffer B). The column was washed with 7 ml of buffer B. Proteins were eluted in a linear gradient from 0–0.7 M NaCl in buffer B. The volume of the gradient was 40 ml. The column was washed with 1 M NaCl in buffer B.

Protein assay

The quantitative protein assay was performed as described by Bradford [8].

SDS-Polyacrylamide gel electrophoresis (SDS PAGE): SDS-PAGE was performed as described previously [9].

Enzyme activity

LDC-activity was measured as described previously [10] with minor modifications. The enzyme reaction was performed in a volume of 300 µl for 30 min at 37 °C and 50 mM L-lysine. The reaction was stopped with 300 µl 1 M K_2CO_3. Then 300 µl of 3% trinitrobenzosulfonic acid (TNBS) were added. After 20 min of incubation at 37 °C the N,N'bistrinitrophenylcadaverine was extracted with 0.6 ml toluene. Absorbance was measured at 340 nm against toluene.

Western blotting [11, 12]

Two sheets of chromatography paper (S&S) soaked with SDS-PAGE running buffer containing 20% methanol were placed on a refined steel plate (3.5 × 11.5 cm). Then the SDS gel, nitrocellulose soaked with destilled water and eight layers of chromatography paper soaked with SDS-PAGE running buffer were formed to a sandwich. The sandwich was closed by a second refined steel plate and was stabilized by two insulated tube clamps. Electrotransfer was performed at 40 mA for 2 h.

Preparation of anti-LDC antibodies

A rabbit was immunized with a three step protocol. The initial immunization was carried out with 400 µg homogeneous LDC, prepared as described in this paper, mixed with an equal volume of "Freund's adjuvant complete" (Difco). Two weeks later the rabbit was boosted with 500 µg homogeneous LDC mixed with an equal volume of "Freund's adjuvant incomplete". After additional two weeks the first serum was obtained. More serum was obtained in 14 day intervals. Two months after the first immunization, approximately 200 µg of LDC-immunoprecipitate were injected to obtain a high and consistent anti-LDC antibody titer [20]. The precipitate was prepared by double diffusion in an agarose gel.

Purification of anti-LDC antibodies by affinity chromatography

5 mg of purified LDC were immobilized on "Affi-Gel 10" (BioRad) as described by the supplier. The gel was stacked in a column with the size of 0.8 × 6 cm (= 3 ml). Up to 1 ml serum from an immunized rabbit was incubated over night on this column. The column was washed with PBS extensively until all unbound protein was removed from the column. Anti-LDC antibodies were eluted with 5 ml 4.5 M $MgCl_2$. Antibodies were washed *via* PD 10 columns (Pharmacia) equilibrated with PBS. The antibodies were precipitated with 40% ammonium sulfate. After centrifugation at 10,000 rpm and 4 °C the pellet was resuspended in 500 µl PBS containing 0.02% NaN_3.

Nitrocellulose immunoassay: After western blotting the nitrocellulose filter was washed with distilled water. The nitrocellulose filter was transferred to a 1:1000 dilution of affinity-purified anti-LDC antibodies in 0.02 M Tris/HCl pH 9.5 containing 2.5% BSA. After incubation at 4 °C over night the solution was decanted and the filter was washed twice with 15 ml 0.02 M Tris/HCl pH 9.5, 2.5% BSA. Then goat anti rabbit-peroxidase conjugate (GAR/Po (Sigma)) diluted 1:500 in Tris/HCl pH 9.5 + 10% horse serum (Boehringer) + 2.5% BSA was incubated for 1 h at room temperature on the nitrocellulose filter. The filter was washed with 20–50 ml 0.5 M acetate buffer pH 5.0. Color development was performed as described by Horejsci and Hilgert [13].

ELISA for LDC and crude extracts from H. alvei and E. coli

Saturating solution: PBS, 10% horse serum, 2.5% BSA. Staining solution: 10 mM Tris/HCl, 0.04%

5-aminosalicylic acid, pH 7.0. Hundred µl of antigen (1 mg/ml or 10 mg/ml respectively) were dried in the wells of a microtiter plate. The wells were filled with saturating solution and were kept over night at 4 °C. The saturating solution was then removed. Serum or specific antibodies were diluted in 1:2 steps up to 1:65536 and incubated for 2 h at ambient temperature. The wells were excessively washed with PBS. Goat anti rabbit peroxidase conjugate (GAR/Po Sigma) was diluted 1:500 and incubated for 2 h. After excessive washing with PBS the wells were filled with 100 µl of staining solution. The absorbance at 450 nm was measured with a "Microelisa Auto Reader" (MR 580 Dynatech) after 10–20 min.

Results and Discussion

Purification of the LDC from Hafnia alvei

For obtaining specific polyvalent antibodies homogeneous antigen is required. As first step of the enzyme purification we chose gel-filtration on a TSK G 4000 SW – HPLC column. As the volume applicable per run was limited to 1 ml [14], the crude extract was concentrated by ammonium sulfate precipitation. From 1 liter of culture 103 mg precipitated protein were obtained and a final protein concentration of 9.9 mg/ml was achieved. The total enzyme activity decreased during this step by 34%. Thus per run 9.9 mg protein were purified with the first step. As

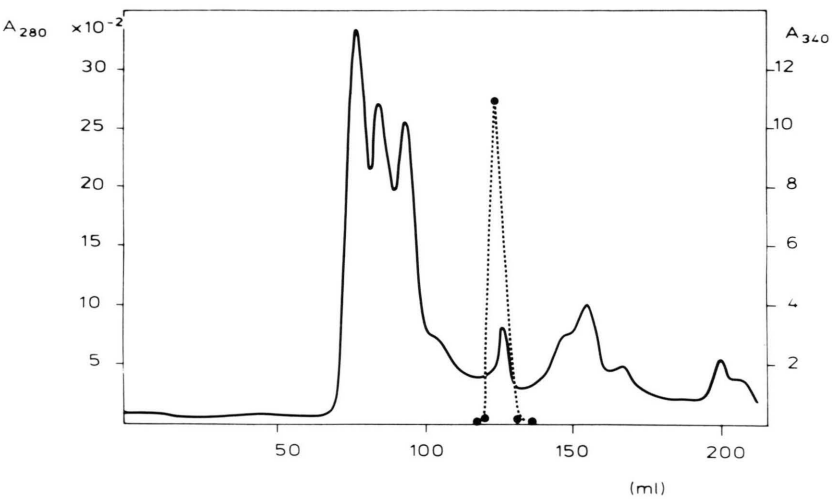

Fig. 1a. Elution profile of crude protein extracts from *H. alvei* on a TSK G 4000 SW column. LDC-activity is designated by the dotted line. Running conditions were as described in Materials and Methods.

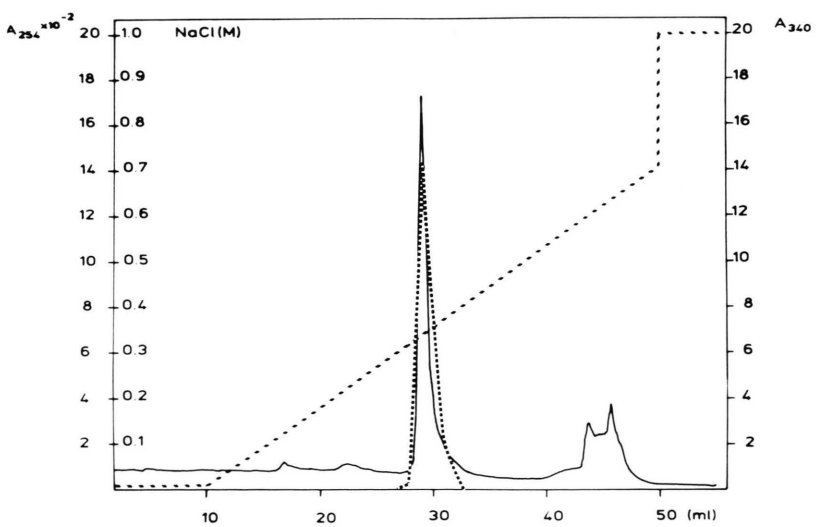

Fig. 1b. Elution profile of LDC, prepurified by TSK G 4000 SW HPLC, on a MonoQ ion exchange column. The continuous line indicates the protein. LDC-activity is designated by the dotted line. The dashed line indicates the NaCl-gradient. Running conditions see Materials and Methods.

shown in Fig. 1a, gel filtration on the TSK G 4000 SW generated already a highly purified LDC. The elution volume suggested a native molecular weight of approximately 545,000 d. The column was calibrated with thyroglobulin, ferritin, BSA and aldolase. Further purification by MonoQ-ion exchange chromatography yielded already homogeneous LDC as suggested by the shape of the peak (Fig. 1b) and as later proven by electrophoresis. Purification data are listed in Table I. It can be calculated that one liter culture yielded about 6.5 mg of homogeneous LDC. The LDC content in crude extracts of *H. alvei* is about 4% of total soluble protein.

As shown in Fig. 2, SDS PAGE revealed two protein bands. Native PAGE revealed one band at a molecular weight of 422 kD and a smear at approximately 50 kD (Fig. 3). Sabo *et al.* [15, 16] reported only one subunit for the LDC from *E. coli*, which is closely related to *H. alvei* [5, 6]. Therefore the question arose whether LDC from *H. alvei* consisted of two different subunits or was partially digested by proteases during preparation without affecting its enzymatic properties. This question was answered by two different approaches. As reported by Fecker *et al.* [4] the LDC-gene from *H. alvei* has in the meantime been cloned. The plasmid of the LDC$^+$ clone, designated pLD462 [4], had only one open reading frame coding for LDC. When protein extracts of *E. coli* HB101, transformed with pLD462, were analyzed by a nitrocellulose immunoassay, also two LDC-positive bands were detected and thus the same pattern as found in *H. alvei* crude extract (Fig. 4). As the gene codes for only one amino acid chain the second band must be caused by degradation. In the second approach the minicell-producing *E. coli* strain LF930 [17] was transformed with pLD462. As described by Fecker *et al.* [4] *de novo* protein synthesis in minicells revealed only one additional band

Table I. Protocol of the purification of lysine decarboxylase of *H. alvei* by HPLC-chromatography.

	Protein [mg]	LDC activity [μkat]	Specific activity [μkat/mg]	Purification factor
pre TSK	9.9	1.34	0.135	
post TSK	0.82	1.14	1.395	12.0
post MonoQ	0.63	1.14	1.81	15.7

"Pre TSK": Ammonium sulfate precipitated crude extract from *Hafnia alvei* prepared for TSK G 4000 SW purification.
"Post TSK": Pooled LDC-active fractions after TSK chromatography concentrated to the volume of "pre TSK" (1 ml).
"Post MonoQ": Homogeneous LDC after ion exchange chromatography with MonoQ HR 5/5. The LDC-activity was detected in one fraction with a volume of one ml.

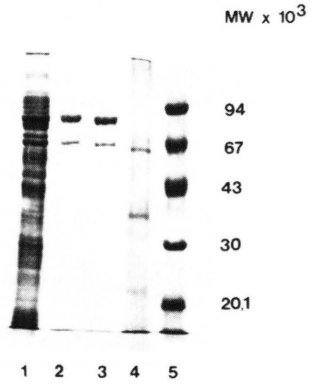

Fig. 2. SDS-PAGE of different LDC-preparations. Lane 1: protein pattern of the crude extract from *H. alvei*. Lane 2: pattern of LDC purified by HPLC with TSK G 4000 SW column. Lane 3: purified LDC after ion exchange chromatography with MonoQ column. Lanes 4–5: molecular weight marker proteins. Positions of the marker proteins are indicated on the right-hand margin. The LDC is represented by two bands which were caused by proteolytic degradation.

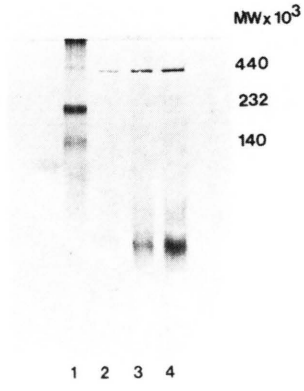

Fig. 3. Native PAGE of purified LDC. Lane 1: marker proteins; lane 2–4: purified LDC with 4 μg, 8 μg and 15 μg, respectively. The protein had an approximate molecular weight of 420,000 d. The diffuse protein spots at lower molecular weights seem to be dissociation products of LDC. The probability of contaminating proteins can be excluded, because the proteins should be visible on SDS-PAGE (Fig. 2).

Fig. 4. Nitrocellulose immunoassay of affinity-purified anti-LDC antibodies, separated by SDS-PAGE. Bands which contained peroxidase were stained as described by Horejsci and Hilgert [13]. Lane 1: crude protein extract from *E. coli* LD462 [4]; lane 2: crude protein extract from *H. alvei*. The arrow indicates non-denatured LDC. Cross reactivity against *E. coli* proteins is strongly reduced due to affinity purified antibodies. Nevertheless some faint bands, which were not related to LDC, were seen in the original.

related to LDC. Obviously LDC was not degraded in this experiment. It was therefore clear that the LDC exists of only one subunit.

Interestingly the enzymatic activity was not affected by this degradation. Variations in the relation of the two bands in SDS PAGE (Fig. 2) did not reflect any differences in specific activity.

Structural characteristics of the LDC

The molecular weight (MW) of native LDC was determined by analytical ultracentrifugation [18], gel filtration and native PAGE. As shown in Table II, the MW of LDC differed, depending on the analytical method used. The data suggested that the subunit of LDC formed aggregates with 5, 7, 10 or 15 molecules depending on the conditions of the environment. Probably aggregation is a function of protein concentration.

The pyridoxyl phosphate (PLP) concentration of the LDC was determined fluorometrically by the method of Adams [19]. A PLP/LDC (subunit = 78,000 d) ratio of 0.98–0.99 was measured. This indicated that every subunit contained one molecule of PLP. Sabo *et al.* [10] described the same PLP/LDC ratio for the LDC from *E. coli*.

Kinetic data of the LDC

For LDC from *H. alvei* or *E. coli* several K_m-values have been published [6, 7, 10, 15, 16, 21]. Since these data are determined with impure preparations we tried to reproduce these data with homogeneous LDC from *H. alvei*. We found a K_m-value of 1.70 mM for lysine as determined by "Michaelis Menten plot" (data not shown) which compared well with the published data calculating values of 1.2–2.1 mM. The inhibition coefficient (K_{is}) was 5.0 mM L-lysine. Since K_m and K_{is} are so close together, K_m cannot be calculated by using the Lineweaver-Burk plot. The kinetic data of the purified LDC of *H. alvei* are summarized in Table III.

Properties of the anti-LDC antibodies

Since the serum was needed to find an LDC^+-clone in an *E. coli*-gene bank it had to be tested for cross reaction with proteins from *E. coli*. Indeed the serum contained antibodies directed against *E. coli* proteins (data not shown). The antibodies specific

Table II. Comparison of molecular weights of LDC when using different analytical techniques.

Method	Molecular weight	Integer multiple of	Number of subunits
native PAGE	422,000	84,000	5
gel filtration	545,000	77,000	7
anal. ultracentrifugation			
at 6,800 rpm	760,959	76,000	10
at 3,600 rpm	795,679	79,500	10
at 2,600 rpm	1173,850	78,200	15

Table III. Some kinetic data for the purified LDC from *H. alvei*.

K_m (mM L-lysine)	1.70
Cooperativity	R_s = 7/30 n_H = 6.1
K_{is} (mM L-lysine)	5.0
Turnover number	10,500 /min

K_{is} = inhibition coefficient for L-lysine.

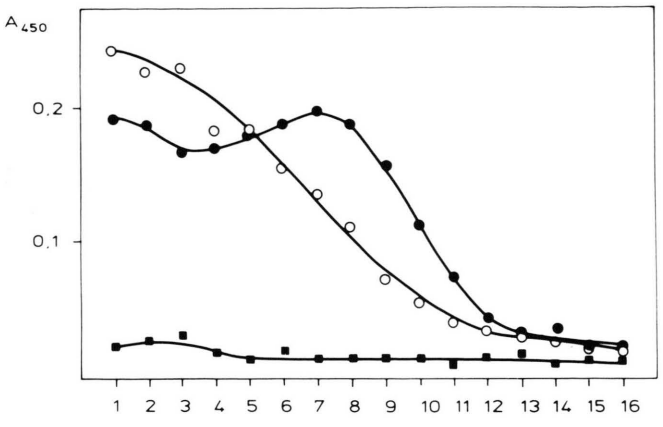

Fig. 5. ELISA control of the purification of the anti-LDC antibodies. Anti-LDC serum was purified by affinity chromatography with an LDC-loaded column. Two antibody-containing preparations were yielded: a) anti-LDC antibodies which had bound to the column b) anti-*E. coli*-protein antibodies which had not bound to the column. The antibody preparations were diluted in one to two steps. ●, anti-LDC antibodies tested against LDC. ■, anti-LDC antibodies tested against crude protein from *E. coli*. ⊙, anti-*E. coli*-protein serum tested against crude protein from *E. coli*. This figure shows, that no cross reactivity against *E. coli* proteins can be detected by this assay.

for the LDC were easily isolated from the crude serum by affinity-chromatography. As demonstrated in Fig. 5 there were nearly no cross reacting antibodies left in the affinity-purified antibody preparation. Additionally these figures demonstrate that the cross reactivity is not caused by proteins homologous to LDC from *H. alvei*. The usefulness of this purified antibody preparation for cloning the LDC-gene of *H. alvei* has been demonstrated [4].

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