CRYSTALLINE L-LEUCINE DEHYDROGENASE

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Summary. The preparation of crystalline L-leucine dehydrogenase from the extract of *Bacillus sphaericus* is described. The enzyme is homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis. The molecular weight is 280,000. The enzyme catalyzes the oxidative deamination of L-leucine, L-valine, L-isoleucine, L-norleucine and L-norvaline, while none of D-leucine, D-valine, L-alanine and L-glutamate is deaminated. NAD only serves as the hydrogen acceptor.

L-Leucine dehydrogenase (L-leucine:NAD oxidoreductase (deaminating) EC class 1.4.1) catalyzes the oxidative deamination of L-leucine and certain other branched-chain L-amino acids and the reductive amination of their α-keto analogs. The enzyme was found in the vegetative cells and spores of various *Bacillus* species, and partially purified from *Bacillus cereus* (1) and *Bacillus subtilis* (2). The enzymological characterization of the partially purified enzyme was also reported.

This communication describes the purification, crystallization and some of the properties of L-leucine dehydrogenase from *Bacillus sphaericus*.

Purification and Crystallization.

Bacillus sphaelicus IFO 3525 was grown in the medium composed of 1.5% peptone, 0.1% glycerin, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.5% NaCl, 0.01% yeast extract, 0.01% $MgSO_4 \cdot 7H_2O$ and 0.01% meat extract (pH 7.2). The cultures were grown at 30° for 20 hr. The harvested cells were washed twice with 0.85%

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sodium hydrochloride solution. All subsequent operations were performed at 0-5°.

Step I. The washed cells (about 1 kg wet weight) were suspended in 1 liter of 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol, and subjected in 250-ml portions to sonication in a 19-kc Kaijo Denki oscillator for 20 min. The intact cells and cell debris were removed by centrifugation.

Step II. To the cell-free extract (2030 ml) was added 1.0 ml of 2% protamine sulfate solution (pH 7.2) per 100 mg of the protein with stirring. After 10 min, the bulky precipitate formed was removed by centrifugation.

Step III. The supernatant solution (3465 ml) was brought to 30% saturation with ammonium sulfate, and the resultant precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 70% saturation. The precipitate collected by centrifugation was dissolved in 1 liter of 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol. The enzyme solution was dialyzed overnight against 100 volumes of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

Step IV. The enzyme solution (1200 ml) was placed on a DEAE-cellulose column (6 x 50 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the same buffer, and then with the buffer containing 0.23 M sodium chloride, the enzyme was eluted with the buffer supplemented with 0.3 M sodium chloride. The active fractions were pooled, concentrated by addition of ammonium sulfate (80% saturation), and dialyzed against 100 volumes of 0.01 M potasium phosphate buffer (pH 7.0) containing 0.01% 2-mercaptoethanol.

Step V. The dialyzed enzyme solution was applied to a hydroxylapatite column (3 x 30 cm) equilibrated with 0.001 M potassium phosphate buffer (pH 7.0) containing 0.01% 2-mercaptoethanol. The enzyme was eluted with the same buffer. The active fractions were collected and concentrated by ammonium

sulfate precipitation (80% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol.

Step VI. Ammonium sulfate was added slowly to the enzyme solution until a faint turbidity was obtained. The pH of solution was kept constant at 7.2-7.4 with 10% ammonium hydroxide solution. On standing overnight, crystal formation occurred. The crystals took the form of the rhombic dodecahedrons (Figure 1). The crystalline enzyme was collected by centrifugation and dissolved in a small volume of the 0.01 M potassium phosphate buffer (pH 7.4).

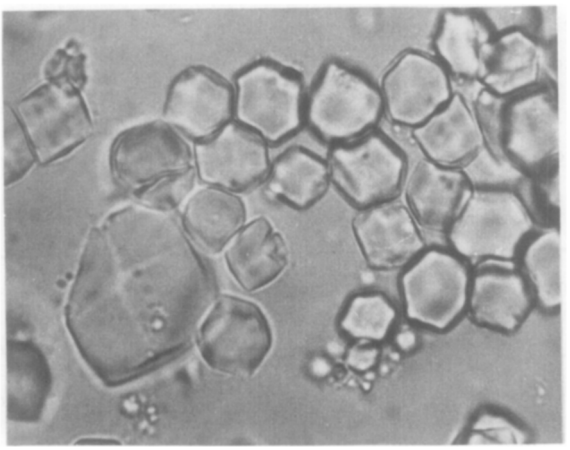

Figure 1. Crystals of L-leucine dehydrogenase.

containing 0.01% 2-mercaptoethanol. The enzyme was recrystallized as described above. A protocol of the purification procedure is presented in Table I.

Properties.

The crystalline enzyme is homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis (Figure 2). The sedimentation coefficient of the enzyme, calculated for water at 20° and zero protein concentration, is 9.0 S. The molecular weight was determined by the sedimentation equilibrium method of Van Holde and Boldwin (4). Assuming a partial specific volume of 0.74, a molecular weight of 280,000 $\pm$ 10,000 was obtained. The enzyme exhibits absorption maximum only at 280 mμ ($E^{1\%}_{1\,cm}$: 6.44). The enzyme catalyzes the

TABLE I. PURIFICATION OF L-LEUCINE DEHYDROGENASE

Step	Fraction	Total Protein	Total Units	Specific Activity	Yield
		(mg)		(Units/mg)	(%)
I.	Crude extract	85,400	205,000	2.4	100
II.	Protamine treatment	67,200	168,000	2.5	86
III.	Ammonium sulfate fractionation	36,500	153,000	4.2	74.6
IV.	DEAE-cellulose chromatography	1,020	91,500	89.4	44.6
V.	Hydroxylapatite chromatography	471	64,700	137.4	31.6
VI.	Crystallization	226	41,000	154.0	20.0

The enzyme was assayed by measuring the rate of the increase in absorbance at 340 mμ due to the reduction of NAD to NADH. The reaction mixture contained 10 μmoles of L-leucine, 1.25 μmole of NAD, 100 μmoles of glycine-KCl-KOH buffer (pH 11.3) and enzyme in a final volume of 0.8 ml. Incubation was carried out at 25° in a cuvette of 1-cm light path. The change in absorbance at 340 mμ was recorded with a Shimadzu spectrophotometer MPS-50L. One unit of the enzyme is defined as the amount of enzyme that causes the increase of 0.001 in absorbance at 340 mμ. Specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.* (3), or estimated from the absorbance at 280 mμ.

oxidative deamination of L-leucine, L-valine, L-isoleucine, L-norleucine and L-norvaline in this order, while D-leucine, D-valine, L-alanine and L-glutamate are inert. NAD is required specifically for the enzyme reaction and NADP cannot be substituted for it. The Km values of L-leucine and NAD are 1.2×10^{-3} M and 4.0×10^{-4} M, respectively.

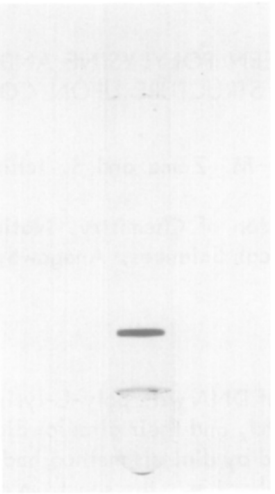

Figure 2. Disc gel electrophoresis of the enzyme. A sample of the crystalline enzyme preparation (20 μg) was electrophoresed under the conditions of Davis (5).

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