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Purification and Properties of Leucine Dehydrogenase of *Bacillus natto* KMD 1126[1]

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Leucine dehydrogenase of *Bacillus natto* KMD 1126 was purified by ammonium sulfate fractionation, diethylaminoethyl cellulose chromatography, Sephadex G 200 gel filtration, and preparative electrophoresis. The homogeneity of purified enzyme was demonstrated by disc gel electrophoresis. Optimum pH for oxidative deamination was 10.7, whereas it was 9.5 for reductive amination. The molecular weight of the enzyme was 360000 daltons as determined by gel filtration on Sephadex G 200. Nicotinamide adenine dinucleotide (NAD^+) in the oxidative deamination and reduced NAD in the reductive amination assay could not be replaced by nicotinamide adenine dinucleotide phosphate ($NADP^+$) or reduced NADP. L-Leucine, L-isoleucine, L-valine, and L-alanine were accepted as substrates but not the following amino acids: L-glutamic acid, L-aspartic acid, L-glutamine, L-asparagine, L-serine, L-threonine, L-cysteine, L-lysine, and D-amino acids. *p*-Chloromercuribenzoate (PCMB) inactivated the enzyme and it was reversed by cysteine. This enzyme had not antitumor activity on Ehrlich ascites carcinoma bearing mice.

Keywords——*Bacillus natto*; leucine dehydrogenase; antitumor activity; purification; properties of enzyme; substrate specificity

L-Leucine dehydrogenase (L-leucine: nicotinamide adenine dinucleotide (NAD^+) oxidoreductase (deaminating) EC 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,[3,4] and purified from *Bacillus cereus*,[5] *Bacillus subtilis*,[6] and *Bacillus sphaericus*.[7]

Ohshima *et al.*[8] reported that crystalline leucine dehydrogenase of *Bacillus sphaericus* has an antitumor activity to Ehrlich ascites carcinoma *in vivo*.

In a previous paper,[9] the authors reported that a strain of *Bacillus natto* (tentatively called KMD 1126), which was newly isolated from "Natto" (fermented beans), has a contact antitumor effect on solid type Ehrlich carcinoma cells. As the continuation of this work, it was now of interest to examine whether the leucine dehydrogenase of *Bacillus natto* has an antitumor activity on Ehrlich ascites carcinoma, or not. Then, the enzyme activity in the vegetative cells of *Bacillus natto* KMD 1126 was measured according to the method of Sanwal, *et al.*[5] and it was shown that there is high leucine dehydrogenase activity in the microorganisms. The present paper deals with the purification and some properties of the enzyme and effect on Ehrlich ascites carcinoma *in vivo*.

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Materials and Methods

Chemicals——Amino acids were purchased from Tanabe Seiyaku Company. NAD^+ and reduced NAD (NADH) were obtained from Sigma Chemical Company. Sephadex G-25 and Sephadex G-200 were products of Pharmacia Fine Chemicals, and diethylaminoethyl (DEAE) cellulose was product of Brown Company. All of other chemicals were of reagent grade and used without further purification.

Bacterial Strain and Culture Condition——*Bacillus natto* KMD 1126 is the stock strain kept in this laboratory on nutrient agar slants. The culture medium consisted of K_2HPO_4, 10 g, NaH_2PO_4, 0.5 g, $MgSO_4 \cdot 7H_2O$, 0.2 g, sodium citrate, 1 g, yeast extract, 1 g, glucose, 5 g, L-isoleucine, 3 g, in 1000 ml of distilled water. Culture was carried out by the following manner. To 3 liters shaking flasks, 800 ml of the culture medium (pH 8.0) were added, sterilized in an autoclave under 0.5 kg/cm^2 pressure for 20 min, and inoculated with 50 ml of seed culture of the bacteria, which was prepared by shaking culture of the organisms at 37° for 15 hr in the same medium. The culture was carried out at 37° with reciprocal shaking (130 c/min, strock 7 cm) for 7 hr.

Assay of Leucine Dehydrogenase Activity——The enzyme activity was assayed by either the increase in absorbance in the oxidative deamination assay or the decrease in the reductive amination assay at 340 nm (Hitach Model 101 Spectrophotometer). Quartz cuvettes of 1 cm light path were used throughout. The reaction mixture in the oxidative deamination assay consisted of 0.1 ml of 0.2M L-leucine, 0.05 ml of 7.6×10^{-3} M NAD^+ and 2.8 ml of 0.1M glycine–NaOH buffer, pH 10.7 (preheated at 25°). The reaction was started by adding 0.05 ml of a properly diluted enzyme solution, and extinction measurements were made every 30 sec for 2 min. The reaction mixture in the reductive amination assay consisted of 0.1 ml of 0.2M α-ketoisocaproate, 0.1 ml of 1.2M $(NH_4)_2SO_4$, 0.05 ml of 4.6×10^{-3}M NADH, and 2.7 ml of 0.1M Tris–HCl buffer, pH 9.5. At zero time, 0.05 ml of a properly diluted enzyme preparation was added, and the decrease in absorbancy at 340 nm was measured at 30 sec intervals for 2 min. In calculating the rate of the reaction, the first two 30 sec figures were used. One unit of the enzyme is defined as the increase of 0.001 in absorbancy per min in the oxidative deamination assay at 25°. Specific activity is the number of enzyme units per mg of protein.

Estimation of Protein——Protein was assayed by Lowry's method,[10] with crystalline bovine serum albumin as standard.

Purification of Leucine Dehydrogenase——Unless otherwise stated all steps in the purification were carried out at 4° in 0.01M sodium phosphate buffer (pH 7.5) containing 0.01% mercaptoethanol. *Bacillus natto* KMD 1126 was cultured in above mentioned medium for 7 hr and cells in late exponential phase were harvested by centrifugation at 9600 ***g*** for 15 min. Cells from 8 liters of culture were washed with 400 ml of 0.01M phosphate buffer (pH 7.5) containing 0.8% NaCl and 0.01% mercaptoethanol and centrifuged. The washed cells were suspended in 200 ml of the buffer and raptured with sonic oscillation (Tomy Model UR 2000 P). Two portions of the suspension (100 ml each) were subjected to three 2 min sonic treatments with 5 min intervals under ice cooling. The suspension of the ruptured cells was centrifuged at 20000 ***g*** for 15 min and the supernatant fraction (cell free extract) was collected. Solid $(NH_4)_2SO_4$ was added to the cell free extract with constant stirring until the concentration reached 25% of saturation and pH was adjusted to 7.5 with NH_4OH. After 1 hr, the precipitated protein was collected by centrifugation at 20000 ***g*** for 15 min and the supernatant fraction, which contained essentially all of the leucine dehydrogenase activity, was collected. Solid $(NH_4)_2SO_4$ was added to the supernatant fraction until the concentration reached 60% saturation. After 1 hr, the precipitated protein was collected by centrifugation at 20000 ***g*** for 15 min, and dissolved in small amount of the buffer. After removal of insoluble impurities by centrifugation, the clear supernatant was applied to a column of Sephadex G 25 (4×90 cm), which had been equilibrated with the buffer. The same buffer was used to elute the enzyme. The active fraction (6 PS) was collected, and applied to a column of DEAE cellulose (3×40 cm), which had been equilibrated with the buffer. The column was washed with the buffer containing 0.2M NaCl until no more protein emerged and the enzyme was eluted with an increasing linear gradient of NaCl (0.2 to 0.5M) contained in the buffer. The active fractions (DEAE) were collected, concentrated to about 15 ml by ultrafiltration, applied to a column of Sephadex G 200 (2.5×90 cm) equilibrated with the buffer, and eluted by upward flow at 15 ml per hr. The active fractions (G 200) were pooled, concentrated to 10 ml by ultrafiltration and submitted to preparative electrophoresis.

Preparative Electrophoresis——Preparative electrophoresis was carried out in Slab electrophoresis apparatus ($15 \times 15 \times 0.2$ cm) from Toyo Co. SPG-1400. The gel system was comprised of a spacer gel (4 ml of 2.5% polyacrylamide) and separating gel (50 ml of 7.5% polyacrylamide). All procedures were carried out in a cold room at 4°. The concentrated enzyme sample in 10% glycerol (2 ml) was loaded on top of the spacer gel and 0.05M Tris-glycine buffer, pH 8.3, added to the upper and lower reservoirs. Electrophoresis was performed at a constant current of 25 mA with a potential difference of about 100 V. After electrophoresis, the separating gel was quickly removed and sliced into 3 mm sections. A sample of each sections were stained leucine dehydrogenase by the method of Moore and Villee.[11] The gel slice containing leucine

10) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) R.O. Moore and C.A. Villee, *Science*, **142**, 389 (1963).

dehydrogenase were crushed and extracted with the buffer containing 0.2M NaCl. Gel debris was removed by centrifugation at 20000 *g* for 10 min. The supernatant was concentrated by ultrafiltration, applied to a column of Sephadex G 200 (2.5×90 cm), and eluted by upward flow at 15 ml per hr. The active fractions (2 G 200) were concentrated by ultrafiltration and pooled.

Disc Electrophoresis——For the purpose of checking each purification step of the enzyme, disc electrophoresis was carried out in 7.5% polyacrylamide gel with 0.05M Tris-glycine buffer (pH 8.3) by the method of Davis.[12] Gels were stained with amidoblack dye and destained by gently shaking in frequent changes of 7% acetic acid over 48 hr. The enzymatic stain for leucine dehydrogenase was carried out by the method of Moore and Villee.[11] The gels were stained by submerging it in a solution containing 0.2 ml of 0.2M L-leucine, 0.1 ml of 7.6 mM NAD^+, 1 ml of nitroblue tetrazolium (1.6 mg/ml), 1 ml of phenazine methosulfate (0.4 mg/ml), and 2 ml of 0.1M glycine–NaOH buffer, pH 10.7. When the purple band denoting enzyme activity became visible, the gels were washed with water and stored in 7% acetic acid.

Determination of Molecular Weight——The molecular weight of the enzyme was estimated by gel filtration on a calibrated column of Sephadex G 200 according to the method of Andrews.[13] Gel filtration was performed with a column of Sephadex G 200 (2.5×90 cm) previously equilibrated with the buffer. The sample, in 2 ml, was applied onto the column and 5 ml fractions were collected. A calibration curve was obtained by applying several marker proteins on the same column.

Antitumor Activity——Ehrlich ascites carcinoma cells were inoculated by intraperitoneal injection of 0.2 ml aliquots containing 5×10^6 cells in 20 to 22 *g* DD female mice. The enzyme was injected intraperitoneally once daily for 10 days consecutively, starting 24 hr after tumor inoculation. Antitumor activity was evaluated by the increase in life span.

Results and Discussion

Effect of Culture Medium and Culture Time on the Production of Leucine Dehydrogenase

It has been a common experience that the quantity of enzyme present in the bacteria depends strongly on the composition of the culture medium. Then, the authors investigated the effect of medium composition on the formation of leucine dehydrogenase in *Bacillus natto* KMD 1126. As shown in Fig. 1, it was observed that isoleucine is the best nitrogen source for the enzyme formation. This observation agrees with the results of Hermier, *et al.*[6] Therefore, the organisms were cultured in glucose isoleucine medium at 37° with shaking and appropriate aliquots of the culture fluid were taken out at different culture times and centrifuged at 20000 *g* for 15 min. The cells were suspended in the buffer and ruptured by sonic oscillation and leucine dehydrogenase activity of the resulting crude extracts was measured. It was shown from these experimental results, that the production of the enzyme is maximum at 6 to 8 hr of culture.

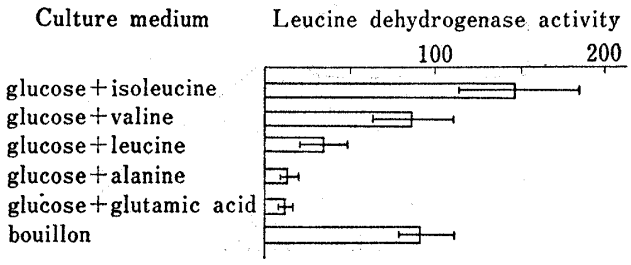

Fig. 1. Effect of the Composition of Culture Medium on the Production of Leucine Dehydrogenase

Activities are shown by leucine dehydrogenase activities in the bacteria which were obtained from 1 ml of culture medium.

Purification of Leucine Dehydrogenase

Purification of the enzyme involved ammonium sulfate fractionation followed by DEAE cellulose chromatography, Sephadex G 200 gel filtration, preparative electrophoresis, and finally Sephadex G 200 gel filtration, as described in Materials and Methods. Leucine dehydrogenase and alanine dehydrogenase were well separated by preparative electrophoresis. A summary of a typical purification is presented in Table I. The final preparation moved as a single band on polyacrylamide gel disc electrophoresis at pH 8.3. The enzyme was

12) B.J. Davis, *Ann. New York Acad. Sci.*, **121**, 404 (1964).
13) P. Andrews, *Biochem. J.*, **96**, 595 (1965).

Table I. Purification of Leucine Dehydrogenase of *Bacillus natto* KMD 1126

Enzyme fraction	Total protein mg	Total activity u	Specific activity u/mg	Recovery %	Purification
Cell free extract	4624	860000	186	100	1
$(NH_4)_2SO_4$ 6PS	2385	770000	323	89.5	1.7
DEAE	162	660000	4070	76.7	21.8
G 200	30	432000	14400	50.3	77.5
Electrophoresis		231000		26.9	
2 G 200	5.7	192000	33700	22.3	181

purified up to about 180 fold from the original crude extract with a recovery of 22% by these procedures. The specific activity of the purified enzyme was 33700 u/mg and comparable to leucine dehydrogenase of *Bacillus subtilis* (56000 u/mg), reported by Zink, *et al.*,[3] but higher than the enzyme of *Bacillus sphaericus* (154 u/mg) reported by Soda, *et al.*[7] Molecular weight of the native enzyme by calibrated Sephadex G 200 gel filtration was estimated to be about 360000 daltons (Fig. 2). This value differs from that of the leucine dehydrogenase of *Bacillus sphaericus* (280000)[7] determined by sedimentation equilibrium.

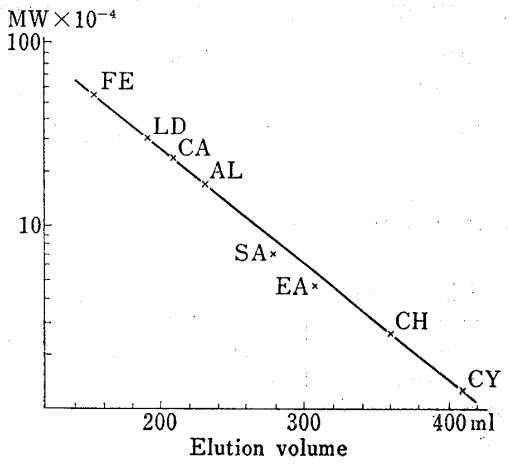

Fig. 2. Molecular Weight Determination of Native Leucine Dehydrogenase by Gel Filtration

Sample of leucine dehydrogenase (LD), ferritin (FE), catalase (CA), aldolase (AL), serum albumin (SA), egg albumin (EA), chymotrypsinogen (CH), and cytochrome C (CY) were run on a Sephadex G 200 column as described in the text.

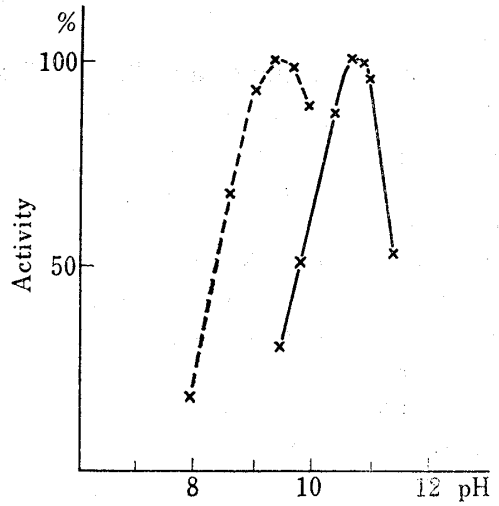

Fig. 3. Effect of pH on the Reductive Amination (------) and Oxidative Deamination (———) Raections Catalyzed by Purified Leucine Dehydrogenase

Assays were done at 25° in glycine-NaOH buffer or Tris-HCl buffer at indicated pH. Results are plotted as percentage of maximum activity.

Optimal pH

The effect of pH was examined for both the amination and deamination reactions. As shown in Fig. 3, the pH optimum of the oxidative deamination of leucine was about 10.7. The pH optimum of the reductive amination of α-ketoisocaproate occurred at about 9.5. This agrees with data to other leucine dehydrogenases from *Bacillus subtilis*,[6] *Bacillus cereus*,[5] and *Bacillus sphaericus*.[7] The enzyme was inactive above pH 12.0.

Coenzyme Specificity

NAD^+ in the oxidative deamination and NADH in the reductive amination assay couldn't be replaced by $NADP^+$ or NADPH respectively. In this regard the enzyme is similar to leucine dehydrogenases of *Bacillus subtilis*[6] and *Bacillus sphaericus*.[7]

Substrate Specificity

Deamination of several amino acids were maesured in 0.1 M glycine-NaOH buffer (pH 10.7) and NAD^+. The results were presented in Table II. L-Isoleucine, L-valine, and L-alanine were deaminated by leucine dehydrogenase at slower rates than that found for L-leucine. No

Table II. Substrate Specificity of Leucine Dehydrogenase of *Bacillus natto* KMD 1126

Substrate	Relative activity	Substrate	Relative activity
L-Leucine	100	D-Leucine	0
L-Isoleucine	87	D-Isoleucine	0
L-Valine	56	D-Valine	0
L-Alanine	0.3	L-Serine	0
L-Glutamic acid	0	L-Threonine	0
L-Aspartic acid	0	L-Cysteine	0
L-Glutamine	0	L-Lysine	0
L-Asparagine	0	L-Ornithine	0

deamination was observed for other amino acids, even when the enzyme concentration was increased 20 folds. D-Leucine, D-isoleucine, and D-valine were not a substrate for the leucine dehydrogenase but caused inhibition of the oxidative deamination of L-leucine.

Effects of Metal Ions, Chelating Agent, and Sulfhydryl Reagent on the Enzyme Activity

Oxidative deamination of leucine was measured in the presence of metal ions, chelating agent or sulfhydryl reagent. As shown in Table III, Mg^{++}, Ca^{++}, or Cu^{++} did not inhibit or activate the enzyme activity at 10^{-3} M. Also the enzyme was insensitive to metal chelating agent such as EDTA. However, the enzyme was completely inhibited by *p*-chloromercuribenzoate (PCMB) and Hg^{++} at 10^{-3} M. The PCMB inhibition was reversed by L-cysteine. These results might mean that SH groups are necessary for the activity.

Table III. Effect of Metal Ions, Chelating Agent, and Sulfhydryl Reagent

Add (10^{-3}M)	Relative activity
None	100
$MgSO_4$	93
$CaCl_2$	102
$CuSO_4$	93
$HgCl_2$	0
EDTA	102
PCMB	0

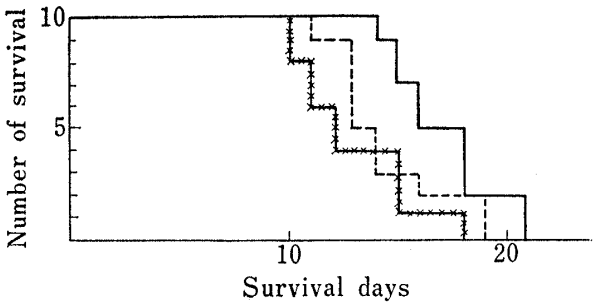

Fig. 4. Assay of Antitumor Activity of Leucine Dehydrogenase of *Bacillus natto* KMD 1126

Survial number of mice after inoculation of Ehrlich ascites carcinoma cells (control ———) and treatment with purified leucine dehydrogenase (1.4 mg/kg/day ·····, 2.8 mg/kg/day —×—×) were shown.

Michaelis Constants

The effect of different concentrations of substrates and the coenzyme on the velocity of oxidative deamination was studied and the *K*m values were calculated graphically.[14] In the pH 10.7 assay, the apparent *K*m values for L-leucine was 6.3 mM and for NAD^+ was 0.14 mM. These values are in close agreement with those reported for the enzyme from *Bacillus cereus* (6.2 mM and 0.16 mM)[5] but do not agree closely with those reported for *Bacillus sphaericus* (1.2 mM

14) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

and 0.4 mM).[7] It is assumed that this enzyme differ from leucine dehydrogenase of *Bacillus sphaericus* from these results and its specific activity.

Antitumor Activity

Ehrlich ascites carcinoma cells were inoculated by intraperitoneal injection in DD mice. The purified enzyme solution (2.8 mg/kg/day or 1.4 mg/kg/day) was injected intraperitoneally once daily for 10 days consecutively, starting 24 hr after tumor inoculation. As shown in Fig. 4, all animals in the control groups died of tumor growth between 14 and 21 days after inoculation. In the groups treated with leucine dehydrogenase, animals died of tumor growth between 10 and 20 days after inoculation. In contrast to above groups, the group which was not inoculated with carcinoma cells but treated with the enzyme, all animals were alive 40 days after leucine dehydrogenase treatment. That is, leucine dehydrogenase of *Bacillus natto* KMD 1126 had not antitumor activity. This result does not agree with the result reported for leucine dehydrogenase of *Bacillus sphaericus*.[8] However, leucine dehydrogenase of *Bacillus natto* have different specific activity and Michaelis constants to the enzyme of *Bacillus sphaericus* as mentioned above. That is, it is assumed that these two enzymes differ on antitumor activity.