[Chem. Pharm. Bull. 25(8)2061—2066(1977)]

UDC 577.158.07 : 576.85.098

Purification and Properties of Alanine Dehydrogenase from *Bacillus natto* KMD 1126[1)]

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(Received December 23, 1976)

Alanine dehydrogenase [L-alanine: NAD^+ oxidoreductase (deaminating) EC.1.4.1.1.] from *Bacillus natto* KMD 1126 was purified 160-fold by ammonium sulfate fractionation, diethylaminoethyl-cellulose chromatography, Sephadex G-200 gel filtration, and adenosine-5′-phosphate Sepharose 4B affinity chromatography. The purified enzyme preparation showed a single band on polyacrylamide gel disc electrophoresis. Specific activity of the purified enzyme was 21 u/mg for oxidative deamination of L-alanine and lower than that of *Bacillus subtilis* (1350 u/mg). The molecular weight of the enzyme was 280000 daltons as determined by gel filtration on Sephadex G-200. Optimum pH for oxidative deamination of L-alanine was 10.4—10.7, whereas it was 8.2—8.4 for reductive amination of pyruvate. The enzyme was completely inhibited by Hg^{2+} and *p*-chloromercuribenzoate at 10^{-3} M. Nicotinamide adenine dinucleotide and its reduced form were essential as coenzymes and could not be replaced by nicotinamide adenine dinucleotide phosphate and its reduced form. L-Alanine was oxidatively deaminated by the enzyme and L-sereine was also deaminated at the rate of 0.5% of L-alanine, but D-alanine and other amino acids were not deaminated. That is, the enzyme has relatively high substrate specificity.

Keywords——alanine dehydrogenase; *Bacillus natto*; purification of enzyme; affinity chromatography; molecular weight; optimal pH; substrate specificity; Michaelis constant

L-Alanine dehydrogenase [L-alanine: NAD^+ oxidoreductase (deaminating) EC 1. 4. 1. 1.] catalyzes the oxidative deamination of L-alanine and reductive amination of pyruvate. As regard to alanine dehydrogenase from microorganisms, the enzymes from *Bacillus subtilis*,[3)] *Mycobacterium tuberculosis*,[4)] *Bacillus cereus*,[5,6)] *Bacillus licheniformis*,[7)] and *Desulfovibrio desulfuricans*[8)] were partially purified and characterized. Halvorson, *et al.* investigated L-alanine dehydrogenase from spores[5)] and vegetative cells[6)] of *Bacillus cereus* T, which is believed to function in the trigger mechanism for alanine induced germination. Yoshida, *et al*[9)] obtained crystalline alanine dehydrogenase from vegetative cells of *Bacillus subtilis* and its enzymatic properties were studied. The enzyme preparations from different sources exhibit considerable variations in their properties. When the authors engaged in the study of leucine dehydrogenase of *Bacillus natto* KMD 1126,[10)] it has been found that the cell free extract of this strain has high alanine dehydrogenase activity. Then, alanine dehydrogenase from *Bacillus natto* KMD 1126 was purified by diethylaminoethyl (DEAE) cellulose

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1) A part of this work was reported at 49th Annual Meeting of Biochemical Society of Japan, Sapporo, September, 1976.
2) Location: *3 Ho, Kanagawa-machi, Kanazawa, 920, Japan.*
3) J.M. Wiame and A. Pierard, *Nature*, **176**, 1073 (1955); A. Pierard and J.M. Wiame, *Biochim. Biophys. Acta*, 37, 490 (1960).
4) D.S. Goldman, *Biochem. Biophys. Acta*, **34**, 527 (1959).
5) R.J. O'Connor and H.O. Halvorson, *Arch. Biochem. Biophys.*, **91**, 290 (1960).
6) N.G. McCormick and H.O. Halvorson, *J. Bacteriol.*, **87**, 68 (1964).
7) S.M. McCowen and P.V. Phibbs, Jr., *J. Bacteriol.*, **118**, 590 (1974).
8) G.J. Germano and K.E. Anderson, *J. Bacteriol.*, **96**, 55 (1968).
9) A. Yoshida and E. Freese, *Biochim. Biophys. Acta*, **92**, 33 (1964); *idem*, *Biochim. Biophys. Acta*, **96**, 248 (1965).
10) K. Matsui, Y. Kameda, T. Atsusaka, M. Higaki, and K. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **25**, 761 (1977).

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chromatography, Sephadex G 200 gel filtration and affinity chromatography on adenosine 5′ phosphate (5′AMP) Sepharose 4B and the enzymatic properties were investigated. The present paper describes the purification and some properties of L-alanine dehydrogenase from vegetative cells of *Bacillus natto* KMD 1126.

Materials and Methods

Chemicals——Amino acids were purchased from Tanabe Seiyaku Company. Nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and their reduced forms (NADH, NADPH) were obtained from Sigma Chemical Company. Sephadex G-25, Sephadex G-200, and 5′AMP Sepharose 4B were products of Pharmacia Fine Chemicals, and 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, 14 g; $NaH_2PO_4 \cdot 2H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; sodium citrate, 1 g; yeast extract, 1 g; glucose, 5 g; and DL-alanine, 3 g in 1000 ml of distilled water. To 3 liter shaking flasks, 800 ml of the culture medium (pH 7.2) 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 cultivation was carried out at 37° with reciprocal shaking (120 cycle/min, strock 7 cm) for 7 hr.

Assay of Alanine 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 (HITACHI 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 0.2 M L-alanine, 0.05 ml of 7.6×10^{-3} M NAD^+, and 2.8 ml of 0.1 M glycine–NaOH buffer, pH 10.6 (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 0.2 M sodium pyruvate, 0.1 ml of 1 M NH_4Cl, 0.05 ml of 4×10^{-3} M NADH, and 2.7 ml of 0.1 M Tris-HCl buffer (pH 8.4). 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 alanine dehydrogenase activity is defined as the amount of enzyme which caused the reduction of 1 μmol of NAD^+/min or the oxidation of 1 μ mole of NADH/min under the above assay conditions. An estimated extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} was used for NADH.[11] Specific activity is defined as units per mg of protein.

Estimation of Protein——Protein was assayed by Lowry's method,[12] with crystalline bovine serum albumin as standard.

Purification of Alanine Dehydrogenase——Unless otherwise stated all steps in the purification were carried out at 4° in 0.05 M sodium phosphate buffer (pH 7.5) containing 0.01% mercaptoethanol. *B. natto* KMD 1126 was cultured in the above mentioned medium for 7 hr and cells in late exponential phase of growth were harvested by centrifugation at 9600 ***g*** for 15 min. Cells from 8 liters of culture were washed by centrifugation with 400 ml of 0.05 M phosphate buffer (pH 7.5) containing 0.8% NaCl and 0.01% mercaptoethanol. The washed cells were suspended in 200 ml of the buffer and ruptured with sonic oscillation (TOMY Model UR 2000P). 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 20% of saturation and pH was adjusted to 7.5 with NH_4OH. After 1 hr, the precipitated protein was removed by centrifugation at 20000 ***g*** for 15 min. 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 fractions were collected, and applied to a column of DEAE-cellulose (3 × 50 cm), which had been equilibrated with the buffer. The column was washed with the buffer containing 0.15 M NaCl until no more protein emerged and the enzyme was eluted with an increasing linear gradient of NaCl (0.15 to 0.5 M) contained in the buffer. The active fractions (DEAE-cellulose fraction) 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/hr. The active fractions (Sephadex G-200 fraction) were concentrated to 10 ml by ultrafiltration and applied to a column of 5′AMP Sepharose 4B (2 × 30 cm) which had been equilibrated with the buffer containing 0.2 M NaCl. When the elution was

11) B.L. Horecher and A. Kornberg, *J. Biol. Chem.*, **175**, 385 (1948).
12) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

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continued with same buffer, alanine dehydrogenase was eluted as an extremely broad peak after a peak of inactive proteins. The active fractions were collected and concentrated by ultrafiltration.

Disc Electrophoresis——For the purpose of checking purity in each purification steps of the enzyme, disc electrophoresis was carried out in 7.5% cross-linked polyacrylamide gel with 0.05M Tris-glycine buffer (pH 8.3) by the method of Davis.[13] The concentrated enzyme sample (in 10% glycerol) was loaded on top of the spacer gel and electrophoresis was performed at a constant current of 5 mA/tube. After electrophoresis, the gels were quickly removed, stained with amidoblack dye, and destained by gently shaking in frequent changes of 7% acetic acid over 48 hr. The enzymatic stain for alanine dehydrogenase was carried out by the method of Moore and Villee.[14] The gels were stained by submerging it in a solution containing 0.2 ml of 0.2M L-alanine, 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 10.6. 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.[15] 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.

Results

Purification of Alanine Dehydrogenase

Purification of alanine dehydrogenase involved ammonium sulfate fractionation followed by DEAE-cellulose chromatography, Sephadex G-200 gel filtration, and 5′AMP Sepharose 4B affinity chromatography, as described in Materials and Methods. Since 5′AMP Sepharose had relatively weak affinity for alanine dehydrogenase, tight adsorption of the enzyme did not occur. As shown in Fig. 1, after a peak of inactive proteins, alanine dehydrogenase was eluted as a broad peak. However, the enzyme preparation moved as a single band on polyacrylamide gel disc electrophoresis at pH 8.3 after this affinity chromatography. A summary of a typical purification is presented in Table I. The enzyme was purified about 160-fold from the original cell free extract with a recovery of 40% by these procedure. The specific activity of the purified enzyme preparation was 21.0 u/mg. Molecular weight of the intact alanine dehydrogenase by calibrated Sephadex G-200 gel filtration was estimated to be about 280000 daltons (Fig. 2).

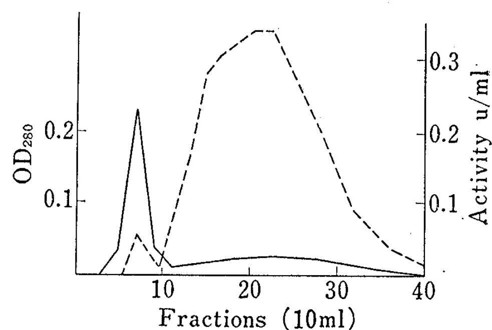

Fig. 1. Elution Pattern from 5′ AMP Sepharose 4B Column

Partially purified alanine dehydrogenase (purified through DEAE-cellulose and Sephadex G-200 column chromatography) was placed on a 5′ AMP Sepharose 4B column (2×30 cm) and eluted with 0.05 M phosphate buffer containing 0.01 % mercaptoethanol and 0.2 M NaCl.
(——), absorbancy at 280 nm; (----), alanine dehydrogenase activity.

TABLE I. Purification of Alanine Dehydrogenase from *Bacillus natto* KMD 1126

Purification step	Total protein mg	Total activity u	Specific activity u/mg	Recovery %	Purification
Cell free extract	5600	740	0.132	100	1
$(NH_4)_2SO_4$ fraction	1950	572	0.293	77.3	2.2
DEAE-cellulose	396	450	1.14	60.8	8.6
Sephadex G-200	62.0	417	6.73	56.4	51.0
5′ AMP sepharose	14.3	299.6	21.0	40.5	159.1

13) B.J. Davis, *Ann. New York Acad. Sci.*, **121**, 404 (1964).
14) R.O. Moore and C.A. Villee, *Science*, **142**, 389 (1963).
15) P. Andrews, *Biochem. J.*, **96**, 595 (1965).

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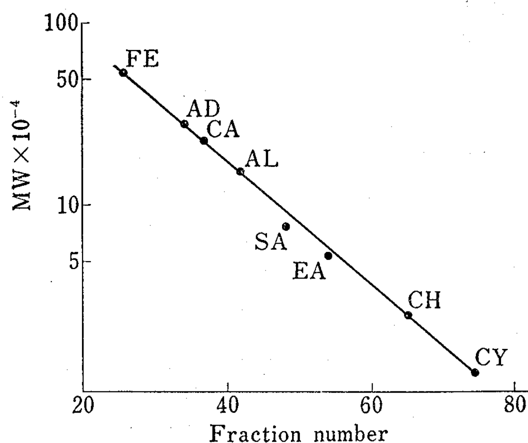

Fig. 2. Molecular Weight Determination of Native Alanine Dehydrogenase by Sephadex G-200 Gel Filtration

Sample of alanine dehydrogenase (AD), 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 and 5 ml fractions were collected as described in the text.

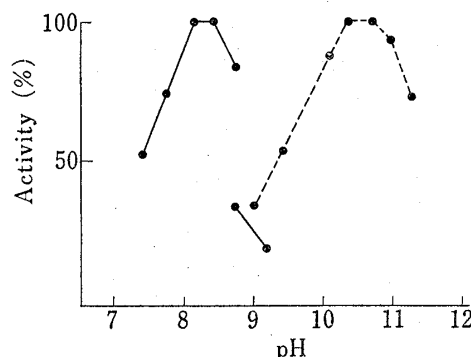

Fig. 3. Effect of pH on the Reductive Amination (——) and Oxidative Deamination (------) Reactions Catalyzed by Purified Alanine Dehydrogenase

Assays were done at 25° in Tris-HCl buffers or glycine-NaOH buffers 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 for the oxidative deamination of alanine was about 10.4—10.7. The pH optimum for the reductive amination of pyruvic acid was about 8.2—8.4. These values agree with data to the enzymes from *B. subtilis*[9] and *B. cereus*.[6] The present enzyme was inactive above pH 12.0.

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

Oxidative deamination of L-alanine was measured in the presence of metal ions, a chelating agent or a sulfhydryl reagent. As shown in Table II, Mg^{2+}, Ca^{2+}, Co^{2+}, or Ni^{2+} did not inhibit or activate the enzyme activity at 10^{-3} M, but Cu^{2+}, Hg^{2+} or Ag^{+} inhibited the enzyme activity at 10^{-3} M. The enzyme was insensitive to metal chelating agent such as ethylene diamine tetraacetate (EDTA). However, the enzyme was completely inhibited by *p*-chloromercuribenzoate (PCMB) at 10^{-3} M. The PCMB inhibition was reversed by L-cysteine. These results might mean that sulfhydryl groups may be necessary for the activity. These results agree with the enzymes from other bacteria.[3-9]

Table II. Effects of Metal Ions, a Chelating Agent, and a Sulfhydryl Reagent

Addition (10^{-3} M)	Relative activity	Addition (10^{-3} M)	Relative activity
None	100	Cu^{2+}	3
Mg^{2+}	104	Hg^{2+}	0
Ca^{2+}	96	Ag^{+}	0
Co^{2+}	100	EDTA	96
Ni^{2+}	92	PCMB	0
Mn^{2+}	65		
Zn^{2+}	50		

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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 alanine dehydrogenases from other bacteria.[3-9]

Substrate Specificity

Deamination of several amino acids were measured in 0.1 M glycine–NaOH buffer (pH 10.6) and 7.6 mM NAD^+. L-Alanine was oxidatively deaminated by the enzyme and L-serine was also deaminated at the rate of 0.5% of L-alanine. However, no deamination was observed for other amino acids such as β-alanine, L-leucine, L-isoleucine, L-valine, L-threonine, L-glutamic acid, L-glutamine, L-aspartic acid, L-asparagine, L-cysteine, L-methionine, L-lysine, L-arginine, and L-phenylalanine even when the enzyme concentration was increased 40 fold. D-Alanine was not a substrate for the alanine dehydrogenase, but caused inhibition of the oxidative deamination of L-alanine.

Michaelis Constants

The effect of different concentrations of substrates and coenzymes on the velocity of the enzyme reaction were studied and the apparent *K*m values were calculated by graphically.[16] In the pH 10.6 assay of oxidative deamination and pH 8.4 assay of reductive amination, the apparent *K*m values for L-alanine, NAD^+, pyruvate, NADH, and NH_4^+ were 5 mM, 0.15 mM, 1.1 mM, 0.04 mM, and 17 mM, respectively. These values resemble with those reported for the enzyme from *B. subtilis* vegetative cells (1.7 mM, 0.18 mM, 0.5 mM, 0.02 mM, and 38 mM).[9]

Discussion

Alanine dehydrogenase from *B. natto* KMD 1126 was purified easily in good yield by DEAE-cellulose chromatography, Sephadex G–200 gel filtration, and 5′AMP Sepharose 4B affinity chromatography. Especially, affinity chromatography was useful and the enzyme preparation showed a single band on polyacrylamide gel disc electrophoresis by affinity chromatography. 5′AMP Sepharose affinity chromatography was used for the purification of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase.[17] In that case, the enzymes adsorbed tightly on 5′AMP Sepharose and the buffer containing NAD^+ or NADH required for the elution of these enzymes. Since 5′AMP Sepharose had relatively weak affinity for alanine dehydrogenase, it was eluted as a broad peak after a peak of inactive proteins. Leucine dehydrogenase was not adsorbed on 5′AMP Sepharose 4B under these conditions, so it was revealed to be suitable for good separation of leucine dehydrogenase and alanine dehydrogenase by this chromatography. The specific activity of the present purified enzyme was 21.0 u/mg and this value was lower than the enzymes of *B. subtilis* (1350 u/mg)[9] or *B. cereus* (196 u/mg).[6] The purified enzyme preparation showed a single protein band, which corresponds to the enzymatically stained alanine dehydrogenase, on polyacrylamide gel disc electrophoresis. That is, the present enzyme differed from the enzyme of *B. subtilis* in the specific activity. Molecular weight of the intact alanine dehydrogenase was estimated to be about 280000 daltons by calibrated Sephadex G–200 gel filtration. This value was higher than that of the alanine dehydrogenase from *B. subtilis* (228000)[9] or *B. cereus* (248000)[6] determined by sedimentation equilibrium. However, this is a small difference which may be within the margin of experimental error, since we did not use the sedimentation equilibrium method. Concerning the active site of the present enzyme, it was suggested that cysteine residues are involved in the active site, since the addition of PCMB gave rise to complete

16) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
17) K. Mosbach, H. Guilford, R. Ohlsson, and M. Scott, *Biochem. J.*, **127**, 625 (1972).

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inactivation and this inactivation was restored by addition of L-cysteine. The fact that Hg^{2+} which are known to coordinate with sulfhydryl group also inhibited the enzyme activity, further suggested the participation of cystein residue in the active site. These results agree with the enzymes from other bacteria.[3-9] Optimum pH, active site, coenzyme specificity, and apparent *K*m of the L-alanine dehydrogenase in our strain were almost same with those reported by other investigators.[5-9] However, there were some differences in substrate specificity. That is, it had been reported that the enzymes from *B. subtilis*[9] or *B. cereus*[6] is able to oxidatively deaminate L-valine, L-isoleucine, and L-leucine other than L-alanine. The present enzyme could not deaminate these amino acids. The discrepancy between them may be due to difference in species or to insufficient separation of leucine dehydrogenase. As mentioned above, alanine dehydrogenase from *B. natto* has some differeces to the enzyme from *B. subtilis*. However, more investigation require for the demonstration of difference between these two enzymes.

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