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Purification and characterization of lactate dehydrogenase from *Varanus* liver

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Abbreviations: LDH, lactate dehydrogenase; AGE, Agarose gel electrophoresis; KPE, potassium phosphate buffer containing EDTA

Abstract

Lactate dehydrogenase was purified 21-fold from liver of Varanus bengalensis using colchicine-sepharose column chromatography. The crude enzyme showed two isoenzymes (LDH-5 and LDH-4) by agarose gel electrophoresis (AGE). The purified enzyme showed a single band after SDS-PAGE corresponding to molecular mass of 35 kDa. The molecular mass of native enzyme was about 140 kDa. The optimum pH for the forward reaction was 7.5 while that for the reverse reaction was pH 9.5. The K_m values for pyruvate, NADH, lactate and NAD^+ were 0.17 ± 0.037, 0.02 ± 0.004, 12.4 ± 3.05 and 0.38 ± 0.032 mM, respectively. Pre-heating of enzyme showed that its t_{50} was 40-50˚C. Oxalate and n-hexanediol were inhibitors for both forward and reverse reactions. Among divalent ions, Cu^{++} was shown to be more effective inhibitor for the forward reaction.

Keywords: LDH isoenzyme, liver, properties, purification, *Varanus bengalensis*

Introduction

Lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27; LDH) plays an important role in the regulation of anaerobic glycolysis through reoxidation of NADH (Everse and Kaplan, 1973; Javed *et al*., 1995). The structure of vertebrate LDH has been investigated by a number of investigators (Cahn *et al*., 1962; Everse and Kaplan, 1973; Li *et al*., 1989)) who have shown that it is a tetramer composed of two subunits, A (M or muscle or type-5) and B (H or heart or type-1). The combinations of various forms of subunits produce five isoenzymes (Maekawa, 1988; Javed *et al*. 1995). These isoenzymes differ in various physicochemical, immunological and physiological properties (Everse and Kaplan, 1973; Maekawa, 1988; Javed and Waqar, 1993).

LDH isoenzymes are not formed by random subunits combinations, and differences in the proportions of subunit for isoenzymes in different tissues (organs) suggest a physiological basis for their existence. LDH-4 and LDH-5 which contain mainly M subunits permit rapid accumulation of lactate, and are found in skeletal muscle where anaerobic glycolysis predominates, whereas LDH-1 and LDH-2 containing mainly H subunits are found in heart, where pyruvate is oxidized via the Krebs cycle (Basaglia, 1989). Very little information is available on LDH from reptiles. In our previous studies (Javed *et al*., 1995), we have shown that LDH from a reptile, *Uromastix hardwickii*, has four isoenzymes in the liver which differ in physicochemical and electrophoretic properties from livers of other animals. Similarly, the characteristics of LDH isoenzyme purified from testes of *U. hardwickii* are also different from other animals (Javed *et al*., 1994; Javed and Waqar, 1996). In this paper, we have described the properties of purified LDH from liver of another reptile, *Varanus bengalensis*.

Materials and Methods

Materials

Fresh livers were obtained from *Varanus bengalensis* and frozen at -20˚C until use. No distinctions were made between male and female animals. NADH, NAD^+, agarose, lithium lactate, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), agarose type-1 low EEO, MW SDS-200 kit and sodium pyruvate were from Sigma Chemical Co., U.S.A. 1,6-Hexanediol (extra pure) was from Aldrich Chemical Co. Inc., U.S.A. Colchicine-CH-Sepharose was a gift from Dr. Tomoji Kocha (Department of Hygienic Chemistry, Showa College of Pharmaceutical Sciences, Tokyo, Japan). All other chemicals were of analytical grade.

Purification of LDH

All purification procedures of LDH were performed at 0-4˚C, otherwise indicated. Frozen liver was thawed and the LDH was purified to homogeneity by ammonium sulphate fractionation and colchicine-sepharose column

chromatography according to the methods previously described (Javed *et al*. 1995).

Gel electrophoresis

To resolve the types of LDH isoenzymes in liver extract and chromatographic fractions, we used horizontal agarose gel electrophoresis. One percent ultrathin (0.4 mm) horizontal agarose gel (7.5×10cm) was prepared on gel bond and electrophoresis and LDH staining were carried out as described elsewhere (Javed *et al*., 1995). To check the purity of the LDH, SDS-PAGE was done according to the method described by Gorg *et al*. (1985) using a gradient gel from 7.5% to 25%.

Molecular mass determination

The molecular mass of the LDH was estimated using a 1.5×73 cm column packed with approximately 125 ml of Sepharose CL-6B in phosphate buffer containing EGTA (KPE) buffer. All standard proteins and dextran blue were passed through column separately.

Enzyme and protein assays

Enzyme activity was measured in the forward reaction (NADH to NAD^+) at 25˚C in a reaction mixture containing 50 mM phosphate buffer (pH 7.5), 0.18 mM NADH, 0.6 mM sodium pyruvate and a suitable amount of enzyme to obtain a measurable decrease in absorbance at 340 nm using Beckman DU70 UV-Vis spectrophotometer. One unit of enzyme was defined as the amount of enzyme that produced one micromol of NAD^+ per min under the assay conditions. A molar absorption coefficient for NADH of 6.22×10^3 $M^{-1}cm^{-1}$ at 340 nm was used for calculations (Voorter *et al*., 1993). For the reverse reaction (NAD^+ to NADH), the enzyme activity was determined by measuring an increase in absorbance at 340 nm. The final concentration of the reactants in a standard reaction mixture was 50 mM Tris-HCl buffer (pH 9.5), 50 mM lithium lactate and 0.1 mM NAD^+. It contained 20 μl of suitably diluted enzyme. The volume of reaction mixtures in both cases was 1 ml. K_m and V_{max} (K_{cat}) values were calculated from initial velocity measurements, using Enzfitter (R. J. Leather-barrow), a non-linear regression program that integrates the data into the Michaelis-Menton equation, with simple weighting. Proteins were determined by the method of Lowry *et al*. (1951) with bovine serum albumin as standard.

For inhibition studies, a known concentration of the test modulator (after adjusting pH 7.5 for forward reaction and pH 9.5 for reverse reaction) was added to the reaction mixture. The activity of the enzyme was then measured by the forward or reverse reactions. The results are expressed as remaining activity (in %) compared to control (100%). The effect of these chemicals were also observed on the oxidation/reduction of NADH/NAD^+ in the absence of enzyme. The nature of oxalate inhibition was determined using Lineweaver-Burk plots.

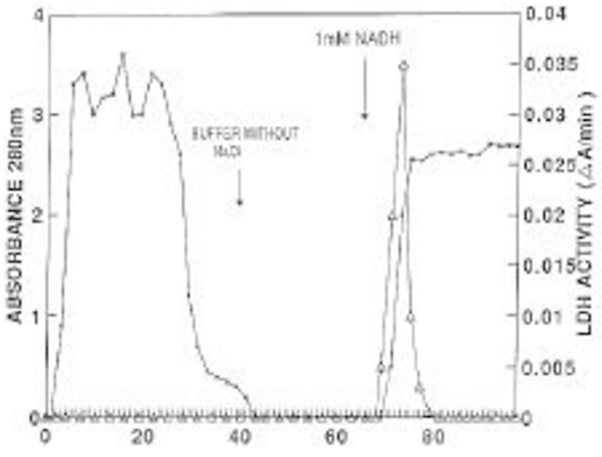

Figure1. Colchicine-Sepharose column chromatography of LDH from *Varanus* liver. Sample was loaded onto a column (1.5 × 2 cm) of immobilized colchicine-Sepharose equilibrated with 2.5 M NaCl-KPE buffer. The column was washed with the same buffer. To wash out glyceraldehyde 3-phosphate dehydrogenase, KPE buffer without NaCl was passed through the column. LDH was then eluted with 1 mM NADH in KPE buffer. The flow rate was about 40 ml/h and 1 ml fractions were collected. The protein concentration (□) was measured as A_{280}. The enzyme activity (▲) was determined by measuring the change in absorbance at 340 nm min-1 at 25˚C.

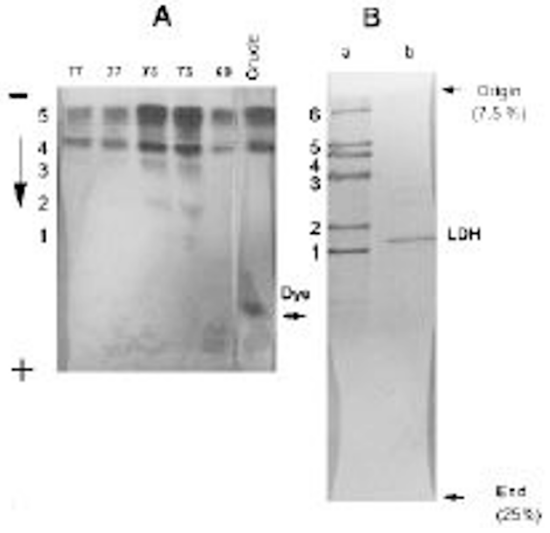

Figure 2. A. Agarose gel electrophoresis (AGE) of LDH from *Varanus* liver. Lanes 69, 73 and 77 are tube number after elution of LDH from colchicine-Sepharose column by NADH. Crude sample is liver extract. B. SDS-PAGE of purified LDH. a, reference proteins are; 1, carbonic anhydrase (29 kDa); 2, egg albumin (45 kDa); 3, BSA (66 kDa); 4, phosphorylase B (97.4 kDa); 5, β-galactosidase (116 kDa); 6, myosin (205 kDa); b, purified LDH.

Results

Colchicine-Sepharose chromatography

When dialyzed liver extract was applied on a colchicine-sepharose column, the entire LDH activity was retained by the column. Addition of 1 mM NADH in KPE buffer eluted LDH in a sharp peak (Figure 1). The agarose gel electrophoresis (AGE) of LDH is shown in Figure 2. Two bands of LDH (LDH4 and 5) were identified in 40-80% ammonium sulphate fraction, while five LDH isoenzymes were resolved in peak fraction (Number 73) after colchicine-Sepharose column chromatography. The major isoenzyme in all cases was LDH-5 (about 70%). The purity of LDH was checked by SDS-PAGE which showed a single band corresponding to the molecular mass of about 35 kDa (Figure 2B, lane b). The molecular mass of native LDH was calculated to be about 140 kDa (Table 1) by Sepharose column chromatography.

Table 1. Elution volumes from Sepharose CL-6B column

Sample	Mol.Wt. (KDa)	Elution volume (ml)
Blue dextran	2000	42.5
Thyroglobulin	669	64.0
LDH	140[a]	79.0
BSA	67	85.5
Cytochrome c	12	96.5

[a] calculated

Table 2. Purification of LDH from *Varanus* liver

Step	Protein (mg/ml)	Activity (U/ml)	Specific Activity (U/mg)
Crude Extract	12.2	83.0	6.8
40-80% $(NH_4)_2SO_4$ Fraction	2.77	18.5	6.7
Colchicine-Sepharose Chromatography	0.055	8.0	145.45

Table 3. Kinetic parameters of LDH. The assay of enzyme was performed as described in the Materials and Methods at 25˚C. The amount of enzyme was adjusted so as to give a change in absorption from 0.05 to 0.06 min^{-1}.

Variable substrate	K_m (mM)	V_{max} (μmol min^{-1} ml^{-1})
Pyruvate	0.17 ± 0.037	0.56 ± 0.05
NADH	0.02 ± 0.004	0.33 ± 0.02
Lactate	12.4 ± 3.05	0.27 ± 0.036
NAD^+	0.38 ± 0.032	0.165 ± 0.005

The results from a representative purification according to the procedure described in the "Materials and Methods" section are shown in Table 2. The specific activity of purified LDH was 145.45 U/mg protein. The purified enzyme was stable at 4˚C for at least 2 years while it was unstable at room temperature.

Kinetic studies

The pH profiles of LDH for the forward and reverse directions are shown in Figure 3. In the forward direction the optimum pH was 7.0. For the reverse reaction, the enzyme showed an optimum pH at 9.5. The activity was still very high (about 55%) even at pH 11.0.

Table 3 summarizes the results of experiments in which kinetic parameters of LDH for pyruvate, lactate, NADH and NAD^+. Before analyzing the effects of the concentrations of various substrates, LDH was dialyzed against KPE for overnight to remove NADH. With pyruvate as the variable substrate, the apparent K_m was about 170 ± 37 μM while the V_{max} was 0.56 ± 0.05 μmol/min. Similarly, the K_m and V_{max} for NADH were 20 ± 4 μM and 0.33 ± 0.02 μmol/min, respectively. The K_m values for NAD^+ and lactate were 0.38 ± 0.032 μM and 12.4 ± 3.05 mM, while V_{max} values were 0.165 ± 0.005 μmol/min and 0.27 ± 0.036 μmol/min, respectively.

Heat stability studies

Purified LDH was incubated at a concentration of about 0.4 U/ml in water bath at given temperature for 5 min as shown in Figure 4. The enzyme was then cooled in ice and the residual activity was measured. Figure 4 shows the heat-inactivation profile of LDH for the forward and the reverse reactions. The t_{50} values (the temperature

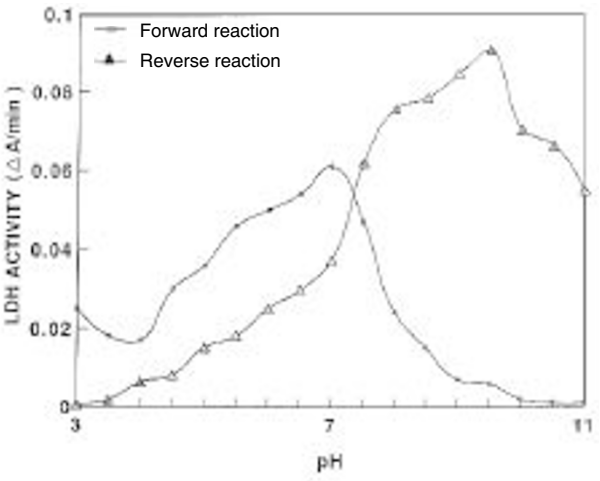

Figure 3. Effect of varying pH on activity of LDH from *Varanus* liver. The composition of reaction mixture was same as explained in the 'Materials and Methods'.

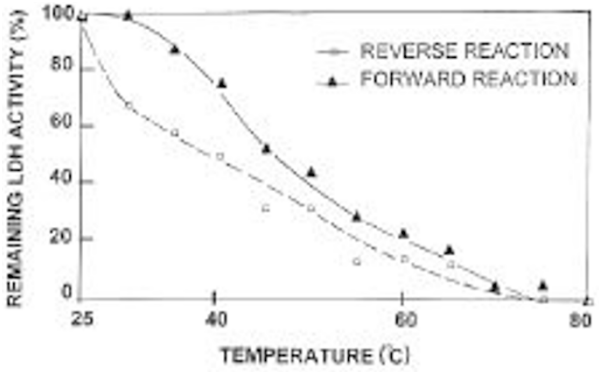

Figure 4. Effect of pre-heating upon activity of LDH. The purified LDH was incubated for 5 min at various temperatures. An aliquot was then taken and kept in ice. After completing the experiment, the activity of LDH was measured both for forward and reverse reactions as explained in the 'Materials and Methods'.

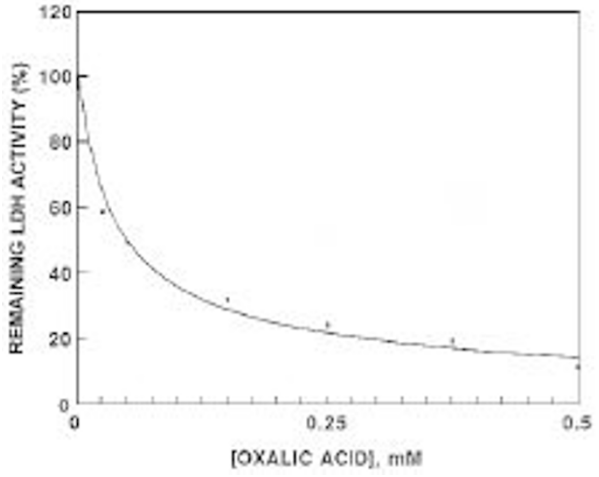

Figure 5. Effect of oxalate on inhibition of LDH. An indicated amount of oxalate was added in the reaction mixture, and then the activity of LDH was determined as described for the forward reaction in the 'Materials and Methods'.

at which 50% of the activity remains after an incubation of 5 min) for forward-reaction is about 50˚C and for reverse-reaction, it is 40˚C.

Effect of Inhibitors

The effect of various modulators on the LDH activity is shown in Table 4. Oxalate was found to be a strong inhibitor, both for the forward and reverse directions particularly at 1 mM concentrations. The apparent IC_{50} (concentration where 50% inhibition was observed) was found to be about 0.05 mM (Figure 5). Glutamate was a more effective inhibitor in the reverse reaction (100% inhibition) as compared to the forward reaction (22.3% inhibition) at 20 mM concentration. Cu^{++} and Co^{++} were

Table 4. Effect of inhibitors on LDH activity

Inhibitors	Concentration (mM)	Remaining activity[a](%)	
		Forward reaction	Reverse reaction
Control	0.0	100.0	100.0
Oxalate	0.5	19.0	98.0
	1.0	7.5	35.0
Glutamate	10.0	89.0	5.0
	20.0	77.7	0.0
Cu^{2+}	0.5	59.0	80.0
	1.0	23.8	70.0
Co^{2+}	0.5	95.0	100.0
	1.0	0.0	100.0
Mn^{2+}	0.5	100.0	100.0
	1.0	84.0	100.0
Mg^{2+}	0.5	99.9	100.0
	1.0	88.8	90.0
n-Hexanediol	400.0	81.5	75.0
	800.0	68.5	55.0

[a] A known amount of modulator was added in the reaction mixture and then the activity of LDH was determined as explained in the 'Materials and Methods'. Values are mean for three determinations for all except for *n*-hexanediol which were repeated for four times.

more effective inhibitors for forward reactions as compared to the reverse reactions. Mn^{2+} and Mg^{2+} had almost no effect on LDH activity on either sides of reaction. *n*-Hexanediol has also shown inhibition for forward and reverse reactions with IC_{50} of about 1.5 M and 0.75 M, respectively (Figure 6).

Discussion

Lactate dehydrogenase from various mammalian species have been shown to be made up of five isoenzymes. We have observed that LDH from skeletal muscles of a reptile, *Uromastix hardwickii* consisted of six isoenzymes and they can not be separated by usual methods of electrophoresis (Javed *et al*., 1992). The purified LDH isoenzymes from liver and testes of *Uromastix* were quite different from other species (Javed *et al*., 1994; Javed *et al*., 1995). Considering the results of *Uromastix* LDH, we have decided to study the properties of LDH purified from another reptile, *Varanus*. It has been well established that the liver of rat, and man contains two LDH isoenzymes (LDH-5 and LDH-4) with 98% as LDH-5, i.e., M-type. The rabbit liver showed three isoenzymes (LDH-5, LDH-4 and LDH-3) with LDH-5 as predominant isoenzymes (Maly and Toranelli, 1993). The cattle liver showed three isoenzymes (LDH-4, LDH-3 and LDH-2) while pig liver showed four isoenzymes (LDH-4, LDH-3,

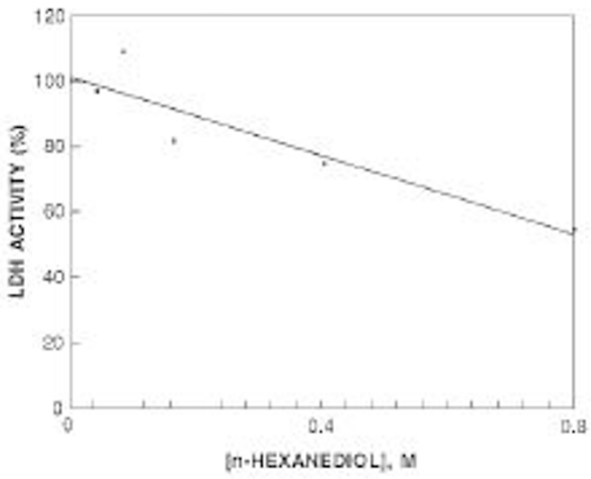

Figure 6. Effect of n-hexanediol on the inhibition of LDH. A known amount of n-hexanediol was added in the reaction mixture, and the LDH activity was determined for forward and reverse reactions as explained in the 'Materials and Methods'.

LDH-2 and LDH-1) with LDH-3 and LDH-2 as predominant isoenzymes (Maly and Toranelli, 1993). *Varanus* liver showed two isoenzymes (LDH-5 and LDH-4) same as in human liver. The K_m values for pyruvate, NADH and NAD^+ were quite different in compared to *Uromastix* liver LDH which are about 15 μM, 40 μM and 10 μM, respectively (Javed *et al.* 1995). The reason is probably the type of LDH isoenzyme. We purified LDH-1 from *uromastix* liver previously (Javed *et al*., 1995). It has been known that the kinetic properties of LDH-1 and LDH-5 are quite different (Everse and Kaplan, 1973; Maekawa, 1988). However, the K_m for lactate in *Varanus* LDH was almost similar to LDH from *Uromastix* (Javed *et al*., 1995). One of the main functions of liver is to convert lactate into pyruvate for energy or gluconeogenesis (Stryer, 1995). The pH and temperature effects on *Varanus* LDH were almost similar to LDH-5 from other sources (Hayashi *et al*. 1985; Javed, *et al*. 1995). The molecular weight of native LDH (140 kDa) from *Varanus* liver was same as previously reported. (Everse and Kaplan, 1973; Maekawa, 1988) and that of subunits was found to be about 35 kDa. Thus the *Varanus* liver LDH is also made up of four subunits.

The inhibition of this LDH by oxalate is in accordance with previous reports (Everse and Kaplan, 1973). In most of the cases, oxalate has been shown to be either competitive or non-competitive types of inhibitor (Saxena *et al.,* 1986; Javed and Waqar, 1993). Glutamate has been shown to protect the inactivation of LDH from the skeletal muscles of Carp (Nakajima *et al.,* 1993). In *Varanus* liver LDH it was strong inhibitor for the reverse-direction only. We had also observed less inhibition by glutamate in case of LDH from *Uromastix* liver for the forward-reaction (Javed *et al*., 1995). Cu^{2+} and Co^{2+} inhibition was more evident for forward reaction as compared to reverse-reaction while Mn^{2+} and Mg^{2+} were least effective which were shown to inhibitors of LDH from some other sources (Yoshido, 1965; Saxena, 1986; Javed *et al*., 1995). However, CO^{2+}, Mg^{2+} and Mn^{2+} have been shown to be activators of LDH from *Norcadia asteroides* (Ike *et al*., 1992). The reason may be that LDH from *N. asteroides* was shown to be a membrane-bound enzyme, while we have purified the LDH from the cytosol of liver. *n*-Hexanediol has been shown to be a non-competitive inhibitor for human LDH-5 with pyruvate as substrate (Tanishima *et al*., 1985).

From electrophoretic and other biochemical properties, it seems that the LDH of *Varanus* liver resembles mostly human liver LDH and quite different from the liver LDH of *Uromastix*, another reptile (Javed *et al*., 1995).

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