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LACTATE DEHYDROGENASE FROM GASTROCNEMIUS
MUSCLE OF TURTLE

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Five bands of lactate dehydrogenase (LDH) isoenzymes were seen by
polyacrylamide gel electrophoresis in gastrocnemius muscle of the turtle (Kachuga
smithi). The major band was of M₂H₂ type and was partially purified by gel filtration
and affinity chromatography. The specific activity of the enzyme was 2.6 units/mg
protein. The half-life of the enzyme at 4°C, was about 7 days. The optimum
temperature for enzyme activity was 30°C and the enzyme was irreversibly inactivated
at 40°C. The optimum pH for the forward reaction (pyruvate to lactate) was 5.5, while
for reverse reaction it was between 8.0 to 9.5. The apparent Kₘ values for pyruvate,
NADH, lactate and NAD⁺ were 0.20, 0.013, 25 and 0.333 mM, respectively. Oxalate
was found to be the inhibitor of LDH with Kᵢ of about 4.2 mM.

Lactate dehydrogenase (1-lactate:NAD⁺ oxidoreductase, LDH, EC
1.1.1.27), the terminal enzyme of anaerobic glycolysis, plays an important role
in the oxidation of reduced NADH [1]. In mammals, five tetrameric LDH
isoenzymes, LDH-1 (H₄=heart), -2(M₁H₃), -3(M₂H₂), -4(M₃H₁), and
-5(M₄=muscle) are found in various proportions among different somatic
tissues [2-5]. Although all the LDH isoenzymes catalyze the interconversion
of lactate to pyruvate, the LDH-M₄ (muscle) and LDH-H₄ (heart) isoenzymes
possess distinct physicochemical, enzymatic, and immunological properties
[2, 3].

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Different striated muscles were found to vary greatly in their composition of LDH isoenzymes. It was found that the type LDH-H4 isoenzyme was nearly absent in the pectoralis muscle but found in abundant quantities in anterior latissimus dorsi [6]. In soleus (slow) muscle, LDH pattern resembles the heart type [7]. During development, the diaphragm also shows predominantly LDH-H4 subunits [8]. The LDH present in the tissues have been correlated with their physiological functions [9, 10]. Muscles which contract tonically or rhythmically (slow) have an LDH composition like heart; muscles which contract more abruptly (fast) have LDH-M4 [6-8].

This study describes the type and properties of LDH in gastrocnemius muscle of the turtle.

MATERIALS AND METHODS

Materials. The turtles, *Kachuga smithi*, were obtained from the river Chenab, near Multan (Pakistan) and were kept in the pond of the Department. NAD\(^+\), NADH, and 5'-AMP-Sepharose-4B were obtained from Sigma Chemical Co. (U.S.A.). Ultrogel A\(_6\) was from LKB (France). Acrylamide, lithium lactate, Nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) were from Merck (F.R.G.). Bisacrylamide was from Fluka (Switzerland). All other chemicals were of reagent grade.

Extraction of LDH. Fresh gastrocnemius muscle of the turtle was dissected out after anaesthetizing the animals in chloroform. Four grams of muscle was homogenized in about three volumes of cold distilled water. The homogenized extract was filtered through two layers of cheese cloth and then centrifuged at 8000 \(\times g\) for one hour at 4°C. The supernatant was the source of enzyme.

Electrophoresis. To check the types of isoenzymes in the muscle extract, polyacrylamide gel electrophoresis was performed according to the method described by Cooper [11] using only separating gel of 6.8% polyacrylamide at 4°C. The electrophoretic pattern of LDH isoenzymes was compared with that of human plasma. After completion of the electrophoresis, the LDH isoenzymes were visualized with a staining solution containing 10 mM Tris/HCl buffer (pH 7.5), 10 mg NAD\(^+\), 180 mg Li-lactate, 6 mg Nitroblue tetrazolium and 0.05 mg phenazine methosulfate in a final volume of 20 ml. The gels were immersed in staining solution at 37°C in the dark for about 30 min. The stained gels were fixed in 5% acetic acid and photographed.

Purification of LDH. LDH type M\(_4\)H\(_2\) was partially purified from other isoenzymes by affinity chromatography and gel filtration.

Affinity Chromatography. This chromatography was performed using 5'-AMP-Sepharose 4B according to the method of Brodelius & Mosbach [12] with some modifications. Two millilitres of freshly prepared muscle homogenate was mixed with 2 ml 0.2 M phosphate buffer (pH 7.0) containing
2 mM 2-mercaptoethanol and 2 M NaCl. The whole sample (4 ml) was applied on top of the column equilibrated with 0.1 M phosphate buffer (pH 7.5) containing 1 mM 2-mercaptoethanol. After washing the column with about 50 ml of 0.1 M phosphate buffer (pH 7.5) the enzyme was eluted with a step-wise gradient of 0.1 mM - 0.3 mM NADH in water. All fractions were analyzed for LDH activity and those with high activity were pooled.

Gel Filtration. To remove NADH and to further purify the enzyme, 2 ml of pooled fractions with the highest activity was applied to a column of Ultrogel A₅ previously equilibrated with 0.1 M phosphate buffer (pH 7.5) containing 2 mM 2-mercaptoethanol. The enzyme was eluted with the same buffer and the active fractions were again pooled. The enzyme was stored at 4°C for further studies.

LDH Activity. Enzyme activity was usually measured in the forward reaction (NADH to NAD⁺) at 37°C in 1 ml of reaction mixture containing 50 mM Tris/HCl (pH 7.5), 10 μM NADH, 50 μM sodium pyruvate and the required amount of enzyme extract. The production of NAD⁺ was followed by a decrease in absorbance at 340 nm using a Shimadzu, UV-Vis recording spectrophotometer equipped with a thermostatically controlled cell housing. One unit of enzyme was defined as the amount of enzyme that produced one micromole of NAD⁺ per minute under the assay conditions (molar absorption coefficient for NADH being 6.22 × 10³ liter mol⁻¹ at 340 nm [13]). Specific activity of LDH was calculated by the method of Narang & Narang [14].

For the reverse reaction (NAD⁺ to NADH), the enzyme activity was determined by measuring the increase in absorbance at 340 nm. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 25 mM lithium lactate, 0.2 mM NAD⁺ and 20 μl of the enzyme per ml.

Determination of Kₘ for reactions (pyruvate, NADH, lactate and NAD⁺) was carried out at pH 7.5. The kinetic parameter, K_m, was determined from the Lineweaver-Burk plot.

For inhibition studies, a known amount of sodium oxalate was added to the reaction mixture. The activity of the enzyme was then measured with NAD⁺ plus lactate as a substrates.

Protein Determination. Protein concentration was determined by the method of Lowry et al. [15], with bovine serum albumin as the standard protein.

RESULTS AND DISCUSSION

The isoenzymes of LDH in the extract of gastrocnemius muscle of the turtle were examined by PAGE. Five isoenzymes were seen (Fig. 1). The electrophoretic mobilities of these isoenzymes were compared with those from human plasma under identical conditions. The main activity band in the turtle muscle
extract was \( M_2H_2 \) whose mobility was slightly less than that of \( M_1H_3 \) of human plasma. The mobilities of \( M_4 \) and \( M_3H_1 \) were higher while the mobilities of \( M_1H_3 \) and \( H_4 \) were much lower as compared with the corresponding isoenzymes in human plasma (Fig. 1). The number of LDH

![Image](image)

Fig. 1. LDH isoenzymes of gastrocnemius muscle of turtle. PAGE was carried out at 4°C according to the method of Cooper [11] with certain modifications (see Materials and Methods). Other conditions were as follows: dimensions: 0.5 x 7 cm; volume of the sample applied: 20 µl; voltage applied: 300 V; and duration: 1 h. Electrophoretic migration is shown from bottom (cathode) to top (anode). The lines along the photographs indicate the isoenzymes (1-5) of LDH. A: human plasma; B: crude muscle extract; C: pooled fractions of affinity chromatography; D: pooled fractions of gel filtration chromatography.
isoenzymes is in accordance with that for other animals while the type of major isoenzyme (M₂H₂) is different [1, 2, 4-6, 16, 17]. It is well known that skeletal muscles from most sources have M₄ as the major isoenzyme of LDH [2, 4, 6]. The purification procedure is summarized in Table 1. The specific activity of the purified enzyme was shown to be 2.6 units/mg protein.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Purification protocol of LDH from turtle gastrocnemius muscle</th>
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<tr>
<td></td>
<td>Activity (units/ml)</td>
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<tr>
<td>Crude Extract</td>
<td>3.215</td>
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<tr>
<td>AMP-Sepharose</td>
<td>1.547</td>
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<tr>
<td>Ultrogel A₆</td>
<td>0.416</td>
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</table>

Fig. 2. Effect of temperature on the activity of LDH. Enzyme activity was measured at desired temperature according to the procedure described in Materials and Methods. LDH activity is expressed in arbitrary units.
The purified enzyme was stable in the presence of 1 mM 2-mercaptoethanol at 4°C. The half-life at 4°C was about seven days but only three days at room temperature (25-30°C), without mercaptoethanol the enzyme was almost completely inactivated in a few hours. The optimum temperature was found to be 30°C (Fig. 2). This property is in accordance with previous findings [2, 17, 18]. When the effect of pH on LDH activity was studied, it was found that at physiological pH (pH 7.40), the activity of the enzyme was less than maximum (Fig. 3). The relative rates of the forward and backward reactions were shown to be dependent upon pH. It is suggested that in a situation of changing intracellular pH (due to accumulation, movement and utilization of lactate), the activity of M₅H₂ changes. Changes of intracellular pH during anaerobiosis have been demonstrated for certain animals [1, 20].

To check the thermal stability, the enzyme was incubated at 40, 45, 50 and 55°C for five minutes. The activity of the enzyme was then measured at 37°C. The enzyme showed inactivation above 40°C which was directly related to the temperature of incubation. The enzyme was almost totally inactivated at 55°C (Fig. 4). This heat-stability is quite different than in many other reports [2, 4]. It is known that H₄ is heat-stable and its activity is unaffected at 60°C for 30 min, whereas that of M₄ is largely destroyed [17, 21].

![Diagram](image_url)

**Fig. 3.** Effect of pH on the activity of LDH. 50 mM phosphate buffer of designated pH was added in the reaction mixture instead of 50 mM Tris/HCl buffer, pH 7.5. Activity was then measured by adding NADH, forward reaction •; or NAD⁺, reversed reaction ○; as described under Materials and Methods. Activity is expressed as described in Fig. 2
Fig. 4. Heat inactivation of LDH. The enzyme was heated for about 5 min at designated temperature and then assayed for enzymatic activity at 37°C. Activity of LDH is expressed as described in Fig. 2.

<table>
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<th>Substrate</th>
<th>$K_m$</th>
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<tr>
<td>Lactate</td>
<td>25 mM</td>
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<tr>
<td>NAD$^+$</td>
<td>333 µM</td>
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<tr>
<td>Pyruvate</td>
<td>200 µM</td>
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<tr>
<td>NADH</td>
<td>13 µM</td>
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</table>

It should be noted that the characteristics of enzymes are easily changed under various experimental conditions [16-18]. Many studies (including ours) on LDH have been performed under different conditions; it is therefore difficult to compare the data quantitatively [18]. The $K_m$ values for different substrates of LDH are shown in Table 2, which shows that the enzyme has more affinity
for pyruvate and NADH than for lactate and NAD\(^+\). It is known that the enzyme has higher affinity for pyruvate than for lactate as substrate, i.e., under physiological conditions, the reaction is favoured towards lactate production in muscle [16, 17]. Heteropolymers made up of both H and M subunits have intermediate values of \(K_m\) [1, 4, 17, 19]. Oxalate inhibition has been shown for LDH from many other sources [22-24]. \(H_4\), but not \(M_4\), is strongly inhibited by 0.2 mM oxalate [21]. In our case the \(K_i\) for oxalate inhibition is about 4.2 mM (Fig. 5).

![Graph showing inhibition of LDH activity as a function of oxalate concentrations. A desired amount of oxalate was added in the reaction mixture. Activity was then measured by adding NAD\(^+\) to the reaction mixture using lactate as substrate (see Materials and Methods). Activity is expressed as described in Fig. 2.](image)

In conclusion, we can say that most of the properties studied resemble those for the intermediate types of LDH, i.e., \(M_2H_2\) which is seen in electrophoretic experiments (Fig. 1).

The LDH in skeletal muscle is correlated with their physiological functions [9, 10]. Muscles which contract tonically or rhythmically (slow) have an LDH composition like heart, while muscles which contract more abruptly (fast) have \(M_4\) type LDH [6-8]. In some animals, all limb muscles are uniformly slow at birth, whereas in adult animals they are differentiated into fast and slow muscles [25]. In the cat's pale-fibered gastrocnemius muscle, there are three types of fibres most of them strongly and swiftly contracting, though a few are slow and some very small [26]. The turtle is an aquatic and slow moving
animal, its gastrocnemius muscles may be made up of intermediate type of fibres (i.e., fast and slow). Therefore, the gastrocnemius muscle of the turtle contains the intermediate type (M₃H₂) of LDH. However to confirm the types of fiber in gastrocnemius muscle of turtle, we require further research especially on its contractile and histological properties.

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REFERENCES