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Purification and Kinetic Properties of Skeletal Muscle Lactate Dehydrogenase from the Lizard *Agama stellio stellio*

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Abstract—Lactate dehydrogenase isoenzyme LDH-5 (M4) was purified to homogeneity from the skeletal muscle of lizard *Agama stellio stellio* as a poikilothermic animal, using colchicine-Sepharose chromatography and heat inactivation. The purified enzyme showed a single band after SDS-PAGE, corresponding to a molecular weight of 36 kD. The K_m values for pyruvate, NADH, lactate, and NAD^+ were 0.020, 0.040, 8.1, and 0.02 mM, respectively. Pyruvate showed maximum activity at about 180 μM, with a decline at higher concentrations. The enzyme was stable at 70°C for 30 min, but was rapidly inactivated at 90°C. The optimum pH for the forward reaction (pyruvate to lactate) was 7.5, and for the reverse reaction (lactate to pyruvate) was 9.2. Oxalate, glutamate, Cu^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+} were inhibitory in both forward and reverse reactions.

Key words: lactate dehydrogenase, skeletal muscle, lizard, *Agama stellio stellio*

Most poikilotherms exhibit biochemical and physiological adaptation which tend to maintain vital function at a relatively constant level against external temperature. These adaptation can be categorized with regard to the time span over which they function as instantaneous, acclimation, or evolutionary and may involve biogeographic, behavioral, physiological metabolic, or genetic characteristics of these species [1-4].

Acclimation changes in cellular metabolic rates may be in part explained by changes in the activities of the enzymes involved in the major pathway of energy metabolism, glycolysis and the Krebs cycle. Moreover, isoenzyme patterns of some enzymes such as lactate dehydrogenase were reported to be affected by various acclimations of poikilotherms [5-8].

Lactate dehydrogenase (L-lactate:NAD-oxidoreductase, EC 1.1.1.27; LDH) is a glycolytic enzyme that catalyses the conversion of pyruvate and lactate.

The structure of LDH was investigated by Cahn et al. [9], who showed the enzyme to be tetramer composed of two subunits, types M (or muscle) and H (or heart) which combined to form five isoenzymes, LDH-1 (H_4), LDH-2 (H_3M_1), LDH-3 (H_2M_2), LDH-4 (H_1M_3), and LDH-5 (M_4).

Abbreviations: EDTA) ethylenediamine tetraacetic acid; KPE) potassium phosphate (containing EDTA); LDH) lactate dehydrogenase.

LDH isoenzymes are not formed by random subunit combination, and differences in the proportions of isoenzymes in different organs suggest a physiological basis for their existence [10-12]. Thus LDH-4 and LDH-5, which contain mainly M subunits, permit rapid accumulation of lactate and are found in tissues such as skeletal muscle where anaerobic glycolysis predominates, whereas LDH-1 and LDH-2, containing mainly H subunits, are found in tissues such as heart, where pyruvate is oxidized via the tricarboxylic acid cycle [5, 13]. Very little information is available on purified LDH from reptiles, especially lizards, as poikilothermic animals which exhibit biochemical and physiological adaptations and tend to maintain vital functions at a relatively constant level against the environment conditions. In this study we describe for the first time the basic properties of this enzyme from a skeletal muscle of the lizard *Agama stellio stellio* and show that they differ from the properties of this enzyme in other species.

MATERIALS AND METHODS

The lizard *A. stellio stellio* of either sex having weight ranging from 38-71 g were obtained from the north area of Jordan. All chemicals used were of analytical grade and purchased from Sigma (USA) or Fluka (Germany).

Skeletal muscle was dissected from freshly killed specimens. All extraction and purification procedures of LDH

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were performed at 0-4°C unless indicated otherwise. Muscles were homogenized (1 : 5 w/v) in cold 0.05 M potassium phosphate buffer containing 1 mM EDTA (KPE buffer, pH 7.2). After centrifugation for 1 h at 20,000*g*, the supernatant was fractionated with ammonium sulfate (40-80% saturation) and the protein precipitates were dissolved in a minimum volume of the KPE buffer. The extract was dialyzed overnight at 4°C against the same buffer, centrifuged for 30 min at 10,000*g*, then applied to a colchicine-Sepharose column (2 × 1.5 cm) pre-equilibrated with 50 mM KPE containing 2.5 M NaCl. In order to remove glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.1), the column was washed with KBE buffer till A_{280} became zero. LDH was then eluted from the column using 1 mM NADH in KPE, and the fractions containing maximum LDH activity were pooled and dialyzed against KPE buffer to remove NADH. The purified LDH was heated in a water bath at 80°C for 30 min and then centrifuged for 10 min at 10,000*g*. The supernatant was used for LDH activity measurements.

To resolve the type of LDH isoenzyme in the muscle extract and fractions, SDS-PAGE (7.5%) was used [14]. LDH isoenzymes were visualized with a staining solution containing 10 mM Tris-HCl buffer (pH 7.5), 0.75 mM NAD^+, 90 mM lithium lactate, 0.37 mM nitroblue tetrazolium, and 8 µM phenazine methosulfate. The stained gels were fixed in 5% acetic acid.

Enzyme activity was measured in the forward reaction (pyruvate reduction in the presence of NADH with NAD^+ formation) at 25°C in 1 ml reaction mixture containing 50 mM potassium 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. The production of NAD^+ was followed by a decrease in absorbance at 340 nm. One unit (U) of enzyme is defined as the amount of enzyme that produced 1 µmole of NAD^+ per 1 min under the assay conditions. The molar absorption coefficient for NADH of $6.2 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ was used [7]. The specific activity of LDH was calculated by the method of Narang and Narang [15]. For the reverse reaction (lactate oxidation in the presence of NAD^+ with NADH formation), the enzyme activity was determined by measuring the increase in absorbance at 340 nm. The final concentration of the reactants in 1 ml reaction mixture was 50 mM Tris-HCl buffer (pH 9.5), 50 mM lithium lactate, 0.1 mM NAD^+ and suitable amount of enzyme. In the determination of K_m for the forward reaction, NADH concentration was varied in the range of 3-96 µM at a constant concentration of pyruvate (400 µM), or the pyruvate concentration was varied in the range of 3-96 µM at a constant concentration of NADH. For the reverse reaction, NAD^+ concentrations of 0.1-1.0 mM were used at a constant concentration of lactate (20 mM), or the lactate concentrations 1-10 mM were used at NAD^+ concentration of 2 mM. Protein concentration was determined by Bradford's method [16] with bovine serum albumin as a standard protein.

RESULTS AND DISCUSSION

When dialyzed extract of the skeletal muscle of *A. stellio stellio* was applied on a colchicine-Sepharose column, the entire LDH activity was retained by the column. Addition of 1 mM NADH in KBE buffer resulted in the elution of a sharp peak of active LDH.

The LDH could not be separated into its isoenzymes by the methods normally used for mammalian

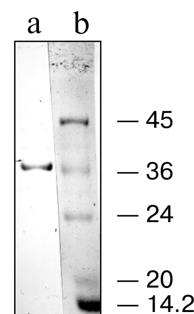

Fig. 1. SDS-PAGE electrophoresis of LDH purified from the skeletal muscle of the lizard *A. stellio stellio*: a) purified LDH; b) reference proteins: egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), trypsinogen (24 kD), trypsin inhibitor (20 kD), lactalbumin (14.2 kD).

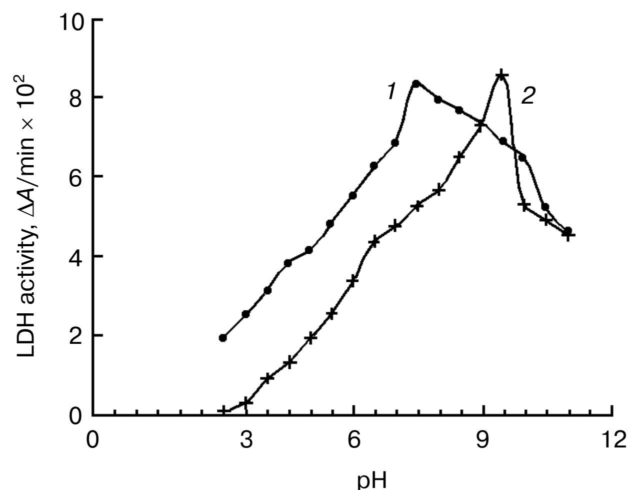

Fig. 2. Effect of varying pH on the activity of LDH from skeletal muscle of lizard *A. stellio stellio*. The reaction mixture consisted of the optimal concentration of pyruvate and NADH (forward reaction) (*1*) or the lactate and NAD^+ (reverse reaction) (*2*).

LDH [7, 17, 18]. The purity of this enzyme was checked by SDS-PAGE (7.5%) which showed a single band corresponding to the molecular weight of about 36 kD (Fig. 1). The details of the purification results are given in Table 1.

Table 2 summarized the results of experiments in which the concentration of pyruvate, lactate, NADH, and NAD^+ were varied. With pyruvate as a variable substrate, the enzyme showed maximum activity at approximately 180 μM of pyruvate, and it was then inhibited by higher concentrations of pyruvate.

As shown in Fig. 2, the optimum pH for the conversion of pyruvate to lactate was 7.5 and for the reverse reaction, lactate to pyruvate was 9.5.

Table 1. Purification of LDH from *Agama stellio stellio* skeletal muscles

Step	Protein, mg/ml	Activity, U/ml	Specific activity, U/mg	Yield, %
Crude extract	36.0	68.2	1.9	100
Ammonium sulfate fractioning (40-80%)	18.3	48	2.7	71.4
Colchicine-Sepharose chromatography	1.8	41.7	23.2	61.2
Heating (80°C)	0.4	21.2	53	28.2

Table 2. Kinetic constants of LDH from skeletal muscle of *Agama stellio stellio*

Substrate	K_m, mM	V_{max}, μmol/min per ml
Pyruvate	0.020	0.44
NADH	0.040	2.07
Lactate	8.1	0.41
NAD^+	0.2	0.33

Table 3. Effect of inhibitors on LDH activity

Inhibitor	Concentration, mM	Inhibition, %	
		forward reaction	reverse reaction
Control		0	0
Oxalate	0.5 1.0	75 75	75 75
Glutamate	0.5 1.0	33 36	60 60
Cu^{2+}	0.5	28	22
Co^{2+}	0.5 1.0	14 22	11 41
Mn^{2+}	0.5 1.0	14 37	12 18
Mg^{2+}	0.5 1.0	23 44	26 44

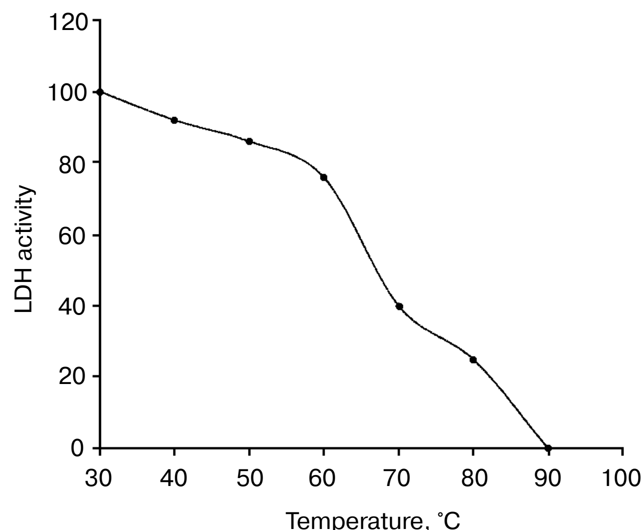

Fig. 3. Effects of temperature on the activity of lactate dehydrogenase in forward reaction.

This enzyme was stable at 70°C for 30 min and it was destroyed at 90°C (Fig. 3). The most striking property of this enzyme was its resistance to preheating [19]. The K_m values of LDH from *A. stellio stellio* skeletal muscle for studied substrates were found to be within the range reported for other animals [19, 20].

A. stellio stellio is a cold-blooded animal, and it goes into hibernation during winter. It has been shown that the K_m values of LDH depend on the temperature of the environment of cells or organisms [19]. Table 3 shows the effect of various inhibitors on the LDH activity. Oxalate was found to be a strong inhibitor both for forward and reverse directions, the apparent K_i was found to be about 0.44 mM for oxalate in accordance with reports of others [21]. Glutamate was a more effective inhibitor for the reverse reaction (about 60% inhibition). The metals used (Cu^{2+}, Co^{2+}, Mn^{2+}, and Mg^{2+}) showed inhibitory effects, both in forward and reverse directions. It has been shown [7] that glutamate inactivates LDH from the skeletal muscle of fish, probably it affects the M-type of enzyme as it is the main isoenzyme in skeletal muscles. Cu^{2+} and Co^{2+} have also been shown to be inhibitors of LDH from other sources [21, 22]. However, Co^{2+}, Mg^{2+}, and Mn^{2+} have been shown to be activators of LDH from *Nocardia asteroides* [23], the reason possibly being that LDH from *N. asteroides* was shown to be a membrane-bound enzyme, while in vertebrates this enzyme is found in cytosol [24].

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