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Purification and Characterization of Lactate Dehydrogenase from the Heart Ventricles of River Buffalo (*Bubalus bubalis*)

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Abstract.- The present study describes purification and characterization of lactate dehydrogenase (LDH) from the heart ventricles of river buffalo (*Bubalus bubalis*). Heart specific isozyme of LDH has been purified to apparent homogeneity on SDS-PAGE using ion-exchange column chromatography, selective precipitation in the presence of ammonium sulfate and hydrophobic-interaction chromatography. The enzyme was purified up to 48 fold with 16% recovery. The maximum activity of purified enzyme was observed at pH 7.0 and it has shown reasonable stability at a broad range of temperature with maximum activity at 30°C. The K_m value with pyruvate is 41 µM, it has only 18% activity with lactate as compared to its activity with pyruvate at pH 7.0. The molecular weight of a subunit of enzyme is 36416.5±2 Da as determined by MALDI-TOF analysis.

Key words: LDH, heart muscles, river buffalo.

INTRODUCTION

Lactate dehydrogenase (LDH; EC 1.1.1.27) is found in a wide range of living organisms. It catalyzes the interconversion of pyruvate and lactate. The enzyme has been purified and characterized from animal, plant and bacterial sources using various types of chromatographic methods (Eventoff *et al*., 1974; Pettit *et al*., 1981; Marchat *et al*., 1996; Mulcahy and O'Carra, 1997)**.** The enzyme exists as a tetramer with a subunit molecular weight of 35 to 36 kDa (Huston *et al*., 1972; Sommer *et al*., 1985; Al-Jassabi, 2002). LDH exists as five isoenzymic forms that are composed of two types of subunits, H (heart) and M (muscle), the subunits are encoded by different genes. There is a significant difference in the kinetic properties of isoenzymes from a variety of animal sources like their thermal stability and sensitivity to inhibitors (Lippert and Javadpour, 1981; Hagberg and Siegbahn, 1983). Clinically, LDH is an important enzyme because of its applications as a biomarker for the diagnosis of some diseased conditions (Kato *et al*., 2006; Chen *et al*., 2007; Torres *et al*., 2009).

Present study deals with the purification and characterization of LDH from the heart ventricles of river buffalo (*Bubalus bubalis*), which is a domestic animal not only important in live stock, dairy and agriculture but is also the predominant slaughter house animal in Pakistan. The present work was aimed at purifying and characterizing the LDH from this previously unexplored species.

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MATERIALS AND METHODS

Chemicals

Fresh *Bubalus bubalis* heart tissue used as raw material in the present study was obtained from the main slaughterhouse at Lahore Pakistan, ammonium sulfate was purchased from ARŌS Organics, Diethyaminoethyl-Sephadex, Laboratory grade sodium pyruvate, Nicotinamide Adenine dinucleotide (NADH) and chromatography related reagents were purchased from Sigma-Aldrich and Calzyme Laboratories. Inc. California. USA.

Purification of enzyme

Fresh heart ventricle tissue (1350g) was homogenized in 2700 mL of 10 mM phosphate buffer pH 7.5 (buffer-A). The sample was centrifuged at 9000x *g* and 4°C for 20 min. The residue was discarded and the supernatant was processed as crude extract. The enzyme was batch adsorbed to diethylaminoethyl-Sephadex and eluted with a linear gradient from 0 to 0.5 M NaCl prepared in buffer-A. The fractions were analyzed for purity on 12.5% SDS-PAGE and those

containing considerably pure enzyme were pooled together. The impurities observed on the SDS-PAGE were removed by repeated ammonium sulphate precipitations at 35% and 80% saturation followed by hydrophobic interaction chromatography on FPLC using Resource PHE column.

Measurement of enzyme activity

Spectrophotometer (SHIMADZU BioSpec-1601) was adjusted at 340 nm and 35°C. The experimental and control cells were added with 220µM NADH, 200µM sodium pyruvate in a reaction mixture of 3.0 mL prepared in phosphate buffer pH 7.0. The reagents were mixed and incubated for 2 min to record the change in absorbance (if any). The enzyme solution (a dilution that can cause 0.03 to 0.1 change in absorbance at 340 nm per min) was mixed with the reaction components and change in absorbance at 340 nm was monitored for 5 min. Activity was calculated by using Beer-Lambart law, using the value of extinction coefficient for NADH as 6220 M^{-1} cm^{-1}.

Study of kinetics properties

The effect of pH variation on enzyme activity was determined by using different pH buffer solutions (pH 5.0 to 10.5) in making the reaction mixture. Temperature stability of enzyme and its activity at different temperatures was determined by incubation of enzyme sample at different temperatures for 5 min and adjusting the temperature of reaction mixture at different temperatures respectively. The K_m value for pyruvate was calculated in 50 mM phosphate buffer pH 7.0. The substrate concentration was increased from 8 µM to 200 µM in the presence of 220 µM of NADH in the reaction mixture. The enzyme activity was also measured for reversible reaction using 400 µM NAD^+ and 200 mM sodium lactate at pH 7.0.

MALDI-TOF analysis

The purified enzyme sample containing 1.5 mg of protein per mL was subjected to MALDI-TOF analysis for the determination of molecular weight of a subunit of enzyme. The enzyme sample (1.5 µL) containing 2 µg of enzyme sample was mixed with 20 µL of matrix-B (5 mg sinapinic acid dissolved in 1 mL of 30% acetonitrile containing 0.1% trifluroacetic acid). From this mixture, 5 µL of sample was spotted on stainless steel mass spectrometric plate and allowed to dry for 20-30 min. The mass spectrum of purified enzyme was recorded. The mass spectrophotometer used in this study was of Bruker Autoflex MALDI-TOF (Bruker Daltonics Inc. MA 01821 USA-Billerica). The procedure used in this study was provided by the company.

RESULTS

Lactate dehydrogenase is purified from the heart ventricles of river buffalo by ion-exchange chromatography followed by the removal of some unwanted proteins by selective ammonium sulfate precipitations. After repeated ammonium sulfate fractionation, the sample was loaded on to the hydrophobic column (Resource PHE), and it eluted as a single peak (Fig.1). The crude extract prepared from the heart tissue was brought to 48 fold purity with a 16% yield as given in Table I. The purified enzyme has a specific activity of 410 international units/ mg of pure enzyme and it appeared as a single protein band on SDS-PAGE with a molecular weight of 35 kDa (Fig.2). MALDI-TOF analysis gave us a more accurate molecular weight and also confirmed the purity of the enzyme indicating a single peak (Fig.3). When the purified enzyme was studied for kinetic parameters, it displayed an optimum pH and temperature of 7.0 and 30˚C

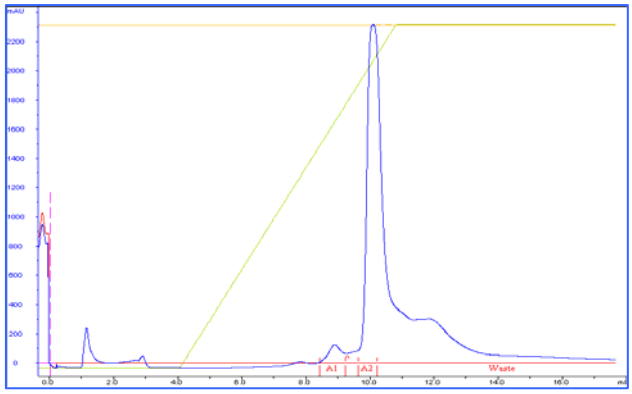

Fig. 1. Hydrophobic-interaction chromatograph of LDH using Reource PHE on FPLC (AKTA Purifier).

Table I.- Purification steps of LDH with specific activity, percentage yield and fold purification at every stage. One enzyme unit is the amount of enzyme that can convert one micromole of pyruvate to lactate in one minute at 35ºC in 50 mM sodium phosphate buffer pH 7.5.

Purification stages	Volume (mL)	Activity (U/mL)	A280* /mL)	Specific activity (U/mg)	Total units	% age yield	Fold purification
Crude extract	4050	68.8	8.1	8.4	278924	100	1.0
Anion-exchange column	490	486.7	8.0	60.5	238508	85.5	7.2
80% A/S ppt	320	542.3	5.4	100.0	173555	62.2	11.9
CM column	400	393.4	3.2	122.9	157360	56.4	14.6
DEAE column	400	219.5	1.3	165	87812	31.5	19.6
80% A/S ppt	100	489.4	2.8	173	48947	17.5	20.5
Hydrophobic column	65	698.3	1.7	410	45395	16.3	48.7

*These values were assined at by multiplying the O.D. of diluted enzymes with their dilution factors.

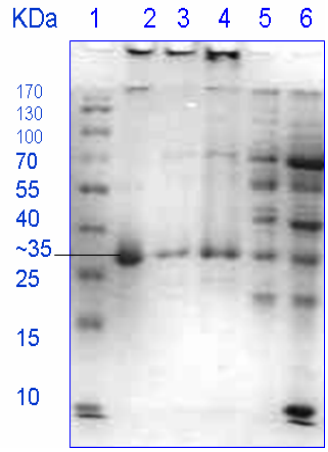

Fig. 2. SDS-PAG photograph: Lane. 1-Protein marker. Lane- 2, 3, 4, purified LDH. Lane-5, and 6-After 1st chromatography and crude extract respectively.

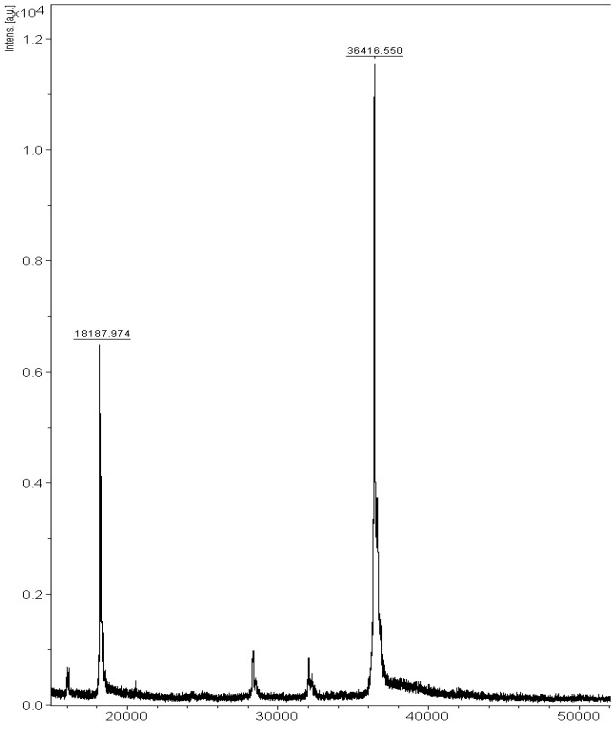

Fig. 3. MALDI-TOF analysis of the purified sample showing a clear peak indicating a molecular mass of 36416.5±2 Da

respectively (Figs. 4 and 5). The results have shown that the purified enzyme remains active in a broad range of pH and temperature. The K_m value for sodium pyruvate in a phosphate buffer of pH 7.0 was 41µM (Fig.6). When sodium pyruvate was used as a substrate with NADH coenzyme, the enzyme activity was 5 times greater than the activity measured with sodium lactate and NAD^+ under the conditions given in the assay procedure.

DISCUSSION

LDH, an enzyme of clinical importance has been purified and characterized from a variety of living organisms. The present study was aimed at characterizing this enzyme from *Bubalus bubalis*, a species unexplored for its proteins and related DNA sequences. The procedure used for the purification of LDH, described in this report is economical and simple as it does not require the complex methods such as biomemetic dye affinity

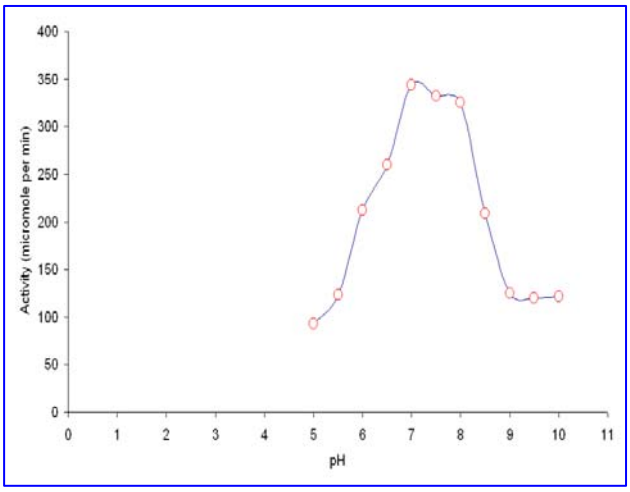

Fig. 4. The effect of pH on the activity of purified enzyme. The reaction mixture was prepared in the buffer solutions adjusted at pH 5 to 10.5.

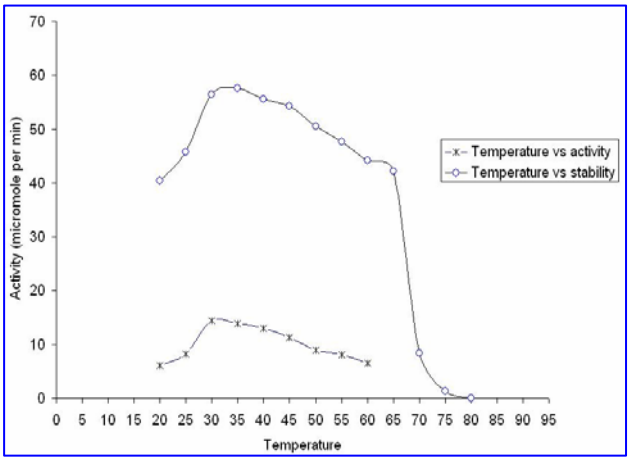

Fig. 5. The effect of temperature on the activity and stability of purified LDH. For the determination of temperature stability, the enzyme sample was incubated at each given temperature for 5 min the activity was measured under the conditions described in assay method. For the determination of optimum temperature for enzyme activity, the temperature of the reaction mixture was adjusted for 5 min before the addition of enzyme and activity was measured.

chromatography, general-ligand affinity chromatography or displacement chromatography on a Tris Acryl DEAE (Pridgar *et al.,* 1984., Ghose and BoMattiasson, 1993; Labrou and Clonis, 1995).

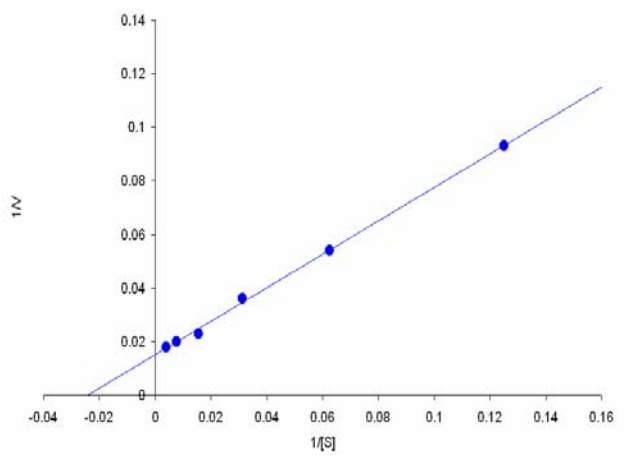

Fig. 6. The K_m value for pyruvate was determined by increasing the concentration of sodium pyruvate in a linear way under the constant pH, temperature and unlimited concentration of NADH. The inverse of enzyme activity calculated was plotted against the inverse of pyruvate concentrations to obtain the Lineweaver-Burk plot.

The purified enzyme is compared for its physiochemical properties with that from other species. The molecular weight of a subunit of the enzyme is about 36 kDa (Schwert *et al*., 1967; Allison *et al*., 1969; Sommer *et al*., 1985). In the present study, the molecular weight has been determined by SDS-PAGE and MALDI-TOF analysis, the molecular weight of a subunit of LDH is 36416±2 Da as determined by MALDI-TOF, the molecular weight determined in the present study is more close to the actual molecular weight of the enzyme (Fig. 3). The optimum pH for the purified enzyme is 7.0 while pyruvate is used as a substrate with NADH (Fig. 4), which correlates with the pH studies with human enzyme (Gay *et al.*, 1968) and differ with the studies on ovine and lizard lactate dehydrogenase (Doughty, 1998; Al-Jassabi, 2002). At pH 7.0, K_m value for pyruvate is 41 µM (Fig. 6). The results are comparable with the reports in literature (Boland and Gutfreund, 1975; Marchat *et al*., 1996). Only 18% enzyme activity was recorded at this pH while lactate was used as a substrate. The present study not only provides the first report describing the basic characteristics of heart lactate dehydrogenase from water buffalo (*Bubalus bubalis*).

REFERENCES

Al-Jassabi, S. 2002. *Biochem.* (Moscow). **67:** 786-789.

Allison, W.S., Admiraal, J. and Kaplan, N.O., 1969. *J. biol. Chem.* **244:** 4743-4749.

Boland, M.J. and Gutfreund, H., 1975. *Biochem. J.***151:** 715-727.

Chen, Y., Zhang, H., Xu, A.J., Liu, J.F., Li, N., Wu, S.S., Huang, L.Y., He, D.C. and Xiao, X.Y. 2007. *Zhonghua Jie He He Hu Xi Za Zhi*. **8:** 577-581.

Gay, R.S., MaComb, R.B. and Bowers, G.N., Jr., 1968. *Clin. Chem*. **14:** 740-747.

Doughty, M.J., 1998. *Exp. Eye Res*. **66:** 231-239.

Eventoff, W., Olsen, K.W. and Hacker, M.L., 1974. *Biochim. Biophys. Acta – Enzymol*. **341:** 327-331

Ghose, S. and Bo Mattiasson, 1993. *Biotech. Tech*. **7:** 615-620.

Hagberg, H. and Siegbahn, A., 1983. *Scand. J. Haematol*., **31:** 49-56.

Huston, J.S., Fish, W.W., Mann, K.G. and Tanford, G., 1972. *Biochemistry*, **11:** 1609-1612.

Kato, G.J., McGowan, V., Machado, R.F., Little, J.A., Taylor, J. VI, Morris, C.R., Nichols, J.S., Wang, Poljakovic, X.M., Morris, S.M. Jr, and Gladwin, M.T. 2006. *Blood*, **107:** 2279-2285.

Labrou, N.E. and Clonis, Y.D., 1995. *J. Chromatograph. A.***7:** 35-44.

Lippert, M.C. and Javadpour, N., 1981. *Cancer,* **48:** 2274-2278.

Marchat, P.M. Loiseau and Petek, F., 1996. *Parasitol. Res*., **82:** 672-680.

Mulcahy, P. and O'Carra, P., 1997. *Phytochemistry,* **45:** 889-896.

Pettit, S.M., Nealon, D.A. and Henderson, A.R. 1981. *Clin. Chem.,* **27:** 88-93.

Pridgar, E.M., Moses, G.C. and Henderson, A.R., 1984. *Clin Chem.,* **30:** 1353-1357.

Schwert, G.W., Miller, B.R. and Peanasky, R.J,. 1967. *J. biol. Chem*. **242:** 3245-3252.

Sommer, P., Klein, J.P., Scholler, M. and Frank, R.M., 1985. *Infec. .Immun.* **47:** 489-495.

Torres, S.H., DeOca, M.M., Loeb, E., Zabner-Oziel, P., Wallis, V. and Hernández, N., 2009. *Arch Bronconeumol*., **45:** 75-80.

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