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Molecular and kinetic characterization of lactate dehydrogenase enzyme in the heart and breast muscle of Japanese quail (*Coturnix japonica*)

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Abstract: In the present investigation, attempts were made to characterize lactate dehydrogenase (LDH) isozyme in quail heart and breast muscle by using electrophoresis, kinetic, and molecular techniques. Polyacrylamide gel electrophoresis of the heart and breast muscle revealed a single isozyme. Kinetic studies were carried out for further functional characterization of LDH isozyme in the heart and breast muscle. Substrate inhibition, enzyme kinetics, and inhibitor (urea) studies indicated that the isozymes present in these tissues differed in their functional properties. LDH isozyme present in the heart and breast muscle exhibited properties of LDH-A and LDH-B isozyme, respectively. Furthermore, RT-PCR amplification of Ldh-a and Ldh-b mRNA in both tissues confirmed that these isozymes were formed by the association of both H and M subunits and may be of H3M1 and M3H1 in the heart and breast muscle, respectively.

Key words: Lactate dehydrogenase, heart, breast muscle, enzyme kinetics, polymerase chain reaction

Introduction

Our previous investigation on lactate dehydrogenase (LDH, EC 1.1.1.27) in quail brain revealed the presence of 4 different isozymes. Among these isozymes, LDH-4 (75.6%) was predominant in quail brain (1), which was unlike chicken brain (2). This study indicated a difference in the distribution of tissue-specific LDH isozymes and a difference in energy metabolism in quail because the distribution of LDH isozymes was correlated with local oxygen tensions, pyruvate inhibition, and lactate accumulation (3). LDH isozymes (LDH-A and LDH-B) occur in all vertebrates; one of them is found principally in the skeletal (LDH-A) and the other in the heart (LDH-B) muscle. In mammals, the 5 isozymes of tetrameric LDH are combinations of M (muscle, LDH-A) and H (heart, LDH-B) subunit proteins, which are encoded independently by the *ldha* and *ldhb* genes, respectively (4). According to the compositional ratio of the 2 subunits in a tetramer and its 5 isozymes in vivo, the total LDH activity exhibits distinct physical and catalytic properties; the M4 and M3H1 isozymes preferentially reduce pyruvate to lactate in anaerobic tissues such as muscle, and the H4 and H3M1 isozymes oxidize lactate to pyruvate in aerobic tissues like the heart (5).

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These 2 isozymes are separate entities as judged by physical, enzymatic, and immunochemical criteria (6). In aerobic glycolysis, LDH acts as a terminative enzyme and promotes the breakdown of glucose to lactate, and therefore it is essential for ATP production. LDH catalyzes the interconversion of pyruvate and lactate, which are end-products in glycolysis, with NADH and NAD+, respectively, as redox cofactors. It is also involved in glucogenesis, in aerobic tissues such as the heart, where lactate is used as a fuel. It appears from our previous investigation that there is a need to study the LDH isozyme present in quail heart and skeletal muscle to explore the difference in tissue-specific distribution of isozymes because these 2 tissues contain 2 different isozymes with distinct physiological functions.

There are many reports about the distribution of LDH isozymes in chickens (2,7). Withycombe (7) reported the presence of only a single isozyme in adult chicken heart (LDH-B) and breast muscle (LDH-A), whereas Heinova et al. (2) described 5 different isozymes in heart and only a single isozyme in breast muscle. To the best of our knowledge, there is no literature available on quail heart and breast muscle specific LDH isozymes. Previously it was reported that the distribution of isozymes is tissue specific rather than species specific and is related to the physiological activity and microenvironment of the tissue (8). In contrast, equine and cheetah studies showed that the distribution and activity of LDH isozymes are species specific (9,10). These observations necessitate further studies on quail LDH isozymes present in heart and breast muscle. Therefore, the aim of this study was to characterize LDH isozymes present in heart and breast muscle in Japanese quail.

Material and methods

Chemicals

Nitro blue tetrazolium (NBT) and phenazine methosulphate (PMS), lactate, NAD, and NADH were purchased from Sigma Chemicals Co. Ltd., USA. All other chemicals were purchased from local manufacturers.

Experimental birds

Clinically healthy adult male Japanese quail (8 weeks old) weighing 180 g approximately were procured from the quail farm of the Central Avian Research Institute Izatnagar, India. They were maintained under a 14-h light cycle with standard quail ration and water ad libitum. The experimental birds were maintained as per the guidelines of the animal ethical committee of the institute.

Collection of samples

Four male Japanese quail were sacrificed and a small piece (1 cm long) of heart and a small piece (1 cm long) of breast muscle were collected aseptically and washed in chilled PBS. Special care was taken to remove blood from the samples. Each collected sample was divided into 2 parts: the first part was used immediately for RNA isolation while the second part was stored at –20 °C until required for tissue extract preparation.

Preparation of tissue extracts

Tissues were homogenized (10% w/v) in 0.02 M ice cold Tris–HCl buffer (pH 7.4) by using a Polytron homogenizer (PT 1600E) at 4 °C. Tissue homogenates were centrifuged at 40,000 × *g* for 20 min at 4 °C in a P40ST rotor using Himack CP80B-Hitachi ultracentrifuge. The supernatants were collected and stored at –20 °C until required for native PAGE and other enzymatic studies.

Kinetic studies

The effects of different substrate concentrations (lactate/pyruvate) on LDH enzyme activity were measured in both forward and reverse reactions. Lactate (0.46–166.0 mM) was used as a substrate to measure the forward reaction while pyruvate (0.05–5.0 mM) was used for reverse reaction. Km and Vmax values of the enzyme present in breast and heart muscle were calculated for lactate and pyruvate. The Km and Vmax values were calculated by using the Michaelis-Menten equation in GraphPad Prism 5 software (GraphPad Prism Software Inc., USA).

Forward reaction (oxidation of lactate to pyruvate)

The forward reaction was measured by using NAD as a co-enzyme and sodium lactate as a substrate in glycine-NaOH buffer (pH 10.0). The final concentrations of the reactants in the reaction

mixture were 50 mM glycine-NaOH buffer, 92 mM sodium lactate, 0.74 mM NAD, and 25 µL of suitably diluted sample. The final volume of the reaction mixture was 1 mL. The enzyme activity was measured in a spectrophotometer (UV5704SS, ECIL, India). An increase in absorbance at 340 nm for 3 min at 30-s intervals was measured at 25 °C.

Reverse reaction (reduction of pyruvate to lactate)

NADH as a co-enzyme and sodium pyruvate as a substrate in Tris-HCl buffer (pH 7.4) were used for assaying the reverse reaction. The final concentrations of the reactants in the reaction mixture were 20 mM Tris-HCl buffer, 1 mM sodium lactate, 0.33 mM NADH, and 25 µL of suitably diluted sample. The final volume of the reaction mixture was 1 mL. The enzyme activity was measured by a spectrophotometer (UV5704SS, ECIL; India) where a decrease in absorbance at 340 nm for 3 min at 30-s intervals was recorded at 25 °C.

The endogenous LDH enzyme activity in both forward and reverse reactions was calculated according to Fritz et al. (11).

Effect of inhibitor

Effect of urea was examined according to the method described by Withycombe (7). Urea to give a final concentration of 2 M was incorporated into the reaction mixture, which was allowed to stand for 30 min at 25 °C before determining the activity at 340 nm in a spectrophotometer (UV5704SS ECIL; India). The enzyme activity was measured in forward (50 mM glycine-NaOH buffer, 92 mM sodium lactate, and 0.74 mM NAD) and reverse (20 mM Tris-HCl buffer, 1 mM sodium pyruvate, and 0.33 mM NADH) reactions.

Native polyacrylamide gel electrophoresis (PAGE)

LDH isozymes were analyzed on non-denaturing 8% separating gel according to the method described by Trigun et al. (12). After electrophoresis, the gels were subjected to LDH enzyme specific stain. Staining solution consisted of 0.125 M Tris–HCl (pH 7.4), 0.5 mM $MgCl_2$, 0.1 mM Na-lactate, 1 mg/mL NAD, 0.01 M NaCl, 0.25 mg/mL nitro blue tetrazolium (NBT), and 0.025 mg/mL phenazine methosulfate (PMS). The isozyme bands in the gel were characterized by comparing their migration patterns.

Protein estimation

Total protein in the heart and breast muscle was assayed according to Smith et al. (13) by using bicinchoninic acid (BCA) in the presence of 1% sodium dodecyl sulfate.

RNA isolation and reverse transcription

Total RNA from individual samples was extracted by RNAgents Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Approximately 25-mg tissue samples were used for RNA isolation. The concentrations and purities of RNA preparations were determined spectrophotometrically at A260 and A280. The possible traces of genomic DNA were removed by treating 5 µg of each RNA sample with 5 U of RNase-free DNase at 37 °C for 1 h. The DNase was subsequently inactivated by incubation at 65 °C for 10 min. Each DNase treated total RNA sample (1 µg) was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. The resultant cDNA was stored frozen at –20 °C until used. Negative controls had all components except reverse transcriptase.

Expression of Ldh-a and Ldh-b mRNA

The amplification of Ldh-a and -b mRNA was carried out by reported gene specific primers for chicken (14) shown in the Table. PCR reactions were performed in a thermal cycler (iCycler Bio-Rad, Hercules, CA, USA) with equal amounts of cDNA samples in separate tubes. The amplification was carried out in

Table. Primer used for semi-quantitative RT-PCR.

Gene Name	Sense primer	Antisense primer	Amplicon size
LDH-A	5'CCATGTCTCTCAAGGATCATCTC3'	5'GCACCAGCAGTGACAATGAC3'	293
LDH-B	5'TTCCCAGCAACAAGATCACCGT3'	5' AACACCTGCCACATTAACTCCG3'	511

25 µL volume containing 10 pmol of each primer, 0.1 mM dNTPs mix, 1 unit of Taq DNA polymerase, and 2 µL of cDNA in 1X Taq polymerase buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$). PCR cycling conditions included an initial period of denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C (for LDH-A and LDH-B) for 1 min, and extension 72 °C for 1 min, and then a final extension at 72 °C for 10 min. Amplicons were analyzed on ethidium bromide stained with 2.0% agarose gel (gel documentation system, Bio-Rad, USA).

Results

Isozyme separation by electrophoresis

The zymogram of quail heart and breast muscle revealed only a single band when subjected to LDH enzyme specific staining. A small difference was observed in the electrophoretic mobility of these 2 isozymes. LDH isozyme present in the heart migrated more towards the anode as compared to the muscle counterpart (Figure 1).

Effect of substrate concentration on LDH enzyme activity

The LDH enzyme activity of quail heart and breast muscle was assayed in different lactate (0.46–166 mM) and pyruvate (0.05–5 mM) concentrations, respectively (Figures 2 and 3). The optimum concentrations of lactate for heart and breast muscle were 37 and 129 mM, respectively. The optimum concentrations of pyruvate for heart and breast muscle were 0.2 and 1.5 mM, respectively. The curves were obtained by plotting initial reaction velocity, which was expressed as percentage of maximal activity against substrate concentration. The pattern

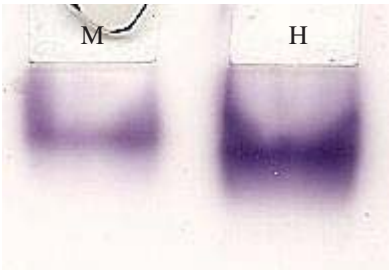

Figure 1. Zymogram of LDH isozyme present in Japanese quail breast muscle and heart (H-heart, M-breast muscle).

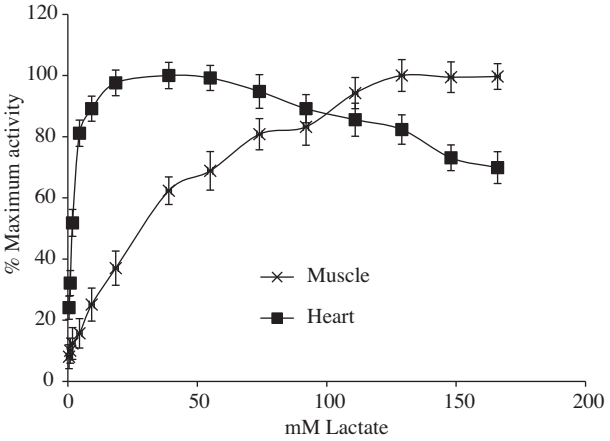

Figure 2. Effect of lactate concentration on LDH enzyme activity in breast and heart muscle homogenates. Initial reaction velocity, expressed as percentage of maximal activity, is plotted against lactate concentration. Final concentration of NAD was 0.74 mM, that of glycine-NaOH buffer, pH 10.0, 50 mM, and lactate concentrations range from 0.46 to 166 mM. Values are mean of enzyme activity of triplicate determination of 4 birds.

of LDH enzyme activity (forward reaction) in heart and muscle homogenates was different when lactate was used as a substrate (Figure 2). Enzyme activity

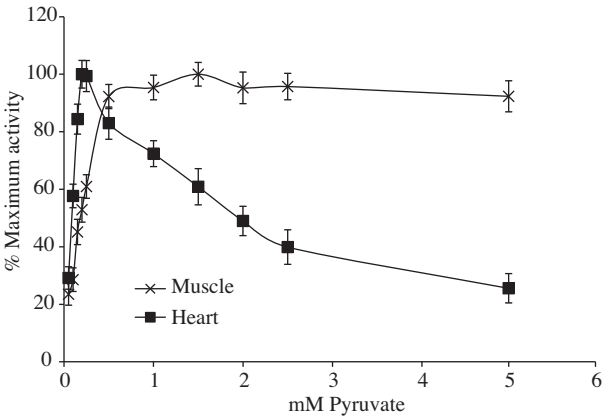

Figure 3. Effect of pyruvate concentration on LDH enzyme activity in breast and heart muscle homogenates. Initial reaction velocity, expressed as percentage of maximal activity, is plotted against pyruvate concentration. Final concentration of NADH was 0.33 mM, that of Tris-HCl buffer, pH 7.4, 20 mM, and pyruvate concentrations range from 0.05 to 5 mM. Values are mean of enzyme activity of triplicate determination of 4 birds.

of LDH present in the heart was inhibited in higher lactate concentrations. In contrast, no inhibition was observed in breast muscle LDH enzyme activity in higher lactate concentrations. A similar trend was noted in the reverse reaction where pyruvate was used as a substrate (Figure 3).

Km values for pyruvate and lactate of heart LDH isozyme were 0.100 ± 0.04 and 7.83 ± 0.52, respectively, whereas for breast muscle Km values were 0.350 ± 0.035 and 23 ± 1.21, respectively.

Urea, a known inhibitor of M-type LDH isozyme, in the presence of pyruvate inhibited enzyme activity in the breast muscle by 50.1% and 93.3% in forward and reverse reactions, respectively. However, only 19.3% and 4.4% inhibition were observed in LDH activity in the heart in forward and reverse reactions, respectively.

Expression of Ldh-a and Ldh-b mRNA

To characterize the expression of Ldh-a (M subunit protein) and Ldh-b (H subunit protein) in heart and breast muscle we used gene-specific primers. The amplifications of both Ldh-a and Ldh-b were detected in the heart and breast muscle (Figure 4). Electrophoresis of amplified products revealed no visual differences in band intensity in Ldh-a and Ldh-b mRNA in the heart and breast muscle. These results confirmed that LDH isozyme present in breast muscle and the heart was composed of H and M subunit proteins.

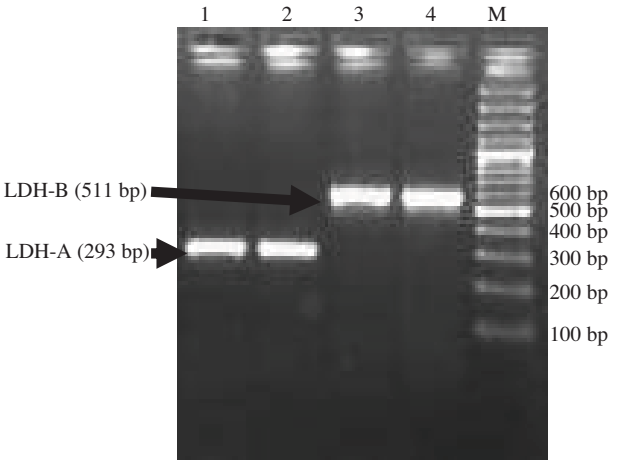

Figure 4. Expression of Ldh-a and ldh-b mRNA in breast muscle and heart tissues (Lane 1 = LDH-A in muscle, lane 2 = LDH-A in heart, lane 3 = LDH-B in muscle, lane 4 = LDH-B in heart and lane M = 100 base pair DNA ladder). Equal amounts of amplified product (8 µL) were loaded in lanes 1-4.

Discussion

In the present investigation, electrophoresis of LDH isozymes revealed a single band with a small difference in electrophoretic mobility in heart and breast muscle (Figure 1). Further, the kinetic studies revealed that these 2 isozymes differed in their biochemical activities. Our results match those of a previous study by Withycombe (7), who reported that chicken heart and breast muscle isozymes have nearly identical electrophoretic mobility with different biochemical properties.

Substrate inhibition has been widely used to differentiate catalytic properties of LDH isozymes (15). The difference in substrate inhibition has been suggested as a basis for a distinct physiological role of the 2 main (H and M) type of LDH (6,8). We plotted curves of reaction velocity against substrate concentration for heart and breast muscle extracts for both substrates (lactate and pyruvate). We observed that LDH present in the heart reached its maximum activity at lower substrate concentration and was slightly inhibited by increasing lactate concentrations in the forward reaction as compared to LDH isozyme present in muscle (Figure 2). These results match those for LDH isozymes present in the heart and muscle of rabbits and chickens (15,16). Further, the activity of LDH isozyme present in the heart was inhibited in the presence of higher pyruvate concentrations (Figure 3), whereas no inhibition was observed in muscle LDH isozyme, which was again similar to the H- and M-type LDH present in the heart and muscle of rabbits and chickens (15,16).

The maximum activity of LDH present in the heart was recorded at 39 mM lactate concentration in the forward reaction, which was close to the H-type LDH isozyme present in rabbit heart (25–50 mM), chicken heart (around 40 mM), and quail seminal plasma (37 mM) (15–17). The maximum activity of LDH present in muscle was recorded at 129 mM lactate concentration in the forward reaction, which was again close to the M-type LDH isozyme present in rabbit muscle (150–200 mM) (17). Similarly,

the maximum enzyme activity of LDH present in the reverse reaction was observed at 0.21 mM of pyruvate concentration in the heart, which was close to the maximum activity of H-type LDH enzyme (0.25 mM pyruvate) of human (18) and quail seminal plasma (0.20 mM pyruvate) (17). Further, the K_m value of LDH enzyme present in quail heart for pyruvate (0.1 mM) was close to that of the H-type LDH isozyme (0.120 mM) of rabbits (15), chickens (0.095 mM), and humans (0.080 mM) (7). The Km for lactate (7.83 mM) was little different from that of rabbit (5.5 mM), which may be due to the difference in coenzyme (NAD) concentration in the assay (15). The Km values of LDH enzyme present in muscle for pyruvate and lactate were also close to those of M-type LDH present in rabbit muscle (15).

Inhibitors also have been used to reveal differences in the properties of LDH isozymes. Urea, a potential M-type LDH inhibitor, does not inhibit H-type when pyruvate is used as a substrate (7). We observed that enzyme activity of LDH present in quail heart was slightly inhibited (4.4%) as compared to enzyme present in muscle (93.3%) by urea in the presence of pyruvate as substrate. Similar results were observed for H-type LDH isozyme of chickens (5%) (7) and rabbits (4%) (15). These results are further proof of the presence of 2 distinct LDH isozymes in the heart and breast muscle. The H-type isozyme may function as a lactate dehydrogenase in the heart, whereas the M-type isozyme may be geared to operate as a pyruvate reductase in breast muscle. Therefore, these results indicate an index of different physiological conditions in quail heart and breast muscle. H-type LDH isozyme would favor an aerobic type of metabolism, since an increase in pyruvate or lactate prevents further accumulation of lactate and would force the oxidation of pyruvate in the Krebs cycle. On the other hand, M-type isozyme would function at high pyruvate and lactate concentrations, allowing the re-oxidation of NADH and the supply of energy under anaerobiosis (8).

Furthermore, RT-PCR studies confirmed expression of Ldh-a (M subunit protein) and Ldh-b (H subunit protein) mRNA in the heart as well as in breast muscle. This indicated that LDH isozyme present in the heart and breast muscle is a heterotetramer formed by the association of both H and M subunit proteins, which is uncommon. In chickens, the heart contains a majority (94.6%) of the LDH in the form of H whereas M-type isozyme was observed in skeletal muscle (2). These 2 isozymes are virtually homotetramers formed by the association of only H/M subunit protein. According to the compositional ratio of the 2 subunits in a tetramer, the total LDH activity exhibits distinct physical and catalytic properties; the M4 and M3H1 isozymes preferentially reduce pyruvate to lactate in anaerobic tissues such as muscle, and the H4 and H3M1 isozymes oxidize lactate to pyruvate in aerobic tissues like the heart (5). Therefore, collectively based on the kinetic and RT-PCR results, we assume that the LDH isozyme present in quail heart and breast muscle is of heterotetramers of H3M1 and M3H1, respectively, with their unique biological functions.

References

1. Singh, R.P., Sastry, K.V.H., Pandey, N.K., Shit, N., Ahuja, A., Radha, D., Mohan, J., Singh, K.B., Moudgal, R.P. Characterization of Lactate dehydrogenase isozymes in Japanese quail brain (*Coturnix coturnix japonica*). J. Cereb. Blood Flow Metab., 2009; 29: S381–S393.
2. Heinova, D., Rosival, I., Avidar, Y., Bogin, E.: Lactate dehydrogenase isoenzyme distribution and patterns in chicken organs. Res. Vet. Sci., 1999; 67: 309–312.
3. Stambaugh, R., Post, D.: Substrate and product inhibition of rabbit muscle lactate dehydrogenases-heart (H4) and muscle (M4) isozymes. J. Biol. Chem., 1966; 241: 1462–1467.
4. Markert, C.L., Shaklee, J.B., Whitt, G.S.: Evolution of a gene. Science, 1975; 189: 102–114.
5. Everse, J., Kaplan, N.O.: Lactate dehydrogenase: structure and function. Adv. Enzymol., 1973; 37: 61–131.
6. Kaplan, N.O., Ciotti, M.M., Hamolsky, M., Bieber, R.E.: Significance of substrate inhibition of dehydrogenases. Ann. N.Y. Acad. Sci., 1968; 15: 400–412.
7. Withycombe, W.A.: Lactate dehydrogenase isozyme in chicken tissues. Nature, 1967; 213: 513–514.
8. Cahn, R.D., Kaplan, N.O., Levine, L., Zwilling, E.: Nature and development of lactic dehydrogenases. Science, 1962; 136: 962–969.

9. Karlström, K., Essén-Gustavsson, B., Lindholm, A.: Fibre type distribution, capillarization and enzymatic profile of locomotor and nonlocomotor muscles of horses and steers. Acta Anat. (Basel)., 1994; 151: 97–106.

10. Williams, T.M., Dobson, G.P., Mathieu-Costello, O., Morsbach, D., Worley, M.B., Phillips, J.A.: Skeletal muscle histology and biochemistry of an elite sprinter, the African cheetah. Comp. Biochem. Physiol., 1997; 167: 527–535.

11. Fritz, P.J., Morrison, W.J., White, E.L., Vesell, E.S.: Comparative study of methods for quantitation of lactate dehydrogenase isozymes. Anal. Biochem., 1970; 36: 443–453.

12. Trigun, S.K., Singh, A.P., Asthana, R.K., Pandey, S.M., Pandey, P., Singh, S.K., Singh, S.P.: Assessment of bioactivity of a Fischerella species colonizing Azadirachta Indica (neem) bark. Appl. Ecol. Environ. Res., 2006; 4: 119–128.

13. Smith, P.K., Krohn, R.I., Hormanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goek, N.M.: Measurement of protein using bicinconinic acid. Anal. Biochem., 1985; 150: 76–85.

14. Imagawa, T., Yamamoto, E., Sawada, M., Okamoto, M., Uehara, M.: Expression of lactate dehydrogenase-A and B messenger ribonucleic acid in chick glycogen body. Poult. Sci., 2006; 85: 1232–1238.

15. McIndoe, W.M., Mitchell, G.G.: Lactate dehydrogenase isozyme in the spermatozoa of the domestic fowl, Gallus domesticus and turkey, Meleagris gallopavo. Comp. Biochem. Physiol. 1978; 61: 433–437.

16. Battellino, L.J., Jaim, F.R., Blanko, A.: Kinetic properties of rabbit testicular lactate dehydrogenase isozyme. J. Biol. Chem. 1968; 243: 5185–5192.

17. Singh, R.P., Sastry, K.V.H., Pandey, N.K., Shit, N., Agrawal, R., Singh, K.B., Saxena, V.K., Mohan, J., Moudgal, R.P.: Characterization of lactate dehydrogenase enzyme in seminal plasma of Japanese quail (*Coturnix coturnix japonica*). Theriogenology, 2011; 75: 555–562.

18. Clausen, J., Oliver, B.: Lactate dehydrogenase isoenzymes of human semen. Biochem. J., 1965; 97: 513–517.