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PURIFICATION OF LACTATE DEHYDROGENASE-C4 FROM SEMINAL PLASMA OF MALE INFERTILITY

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ABSTRACT

Male factor infertility contributes partially and solely to the problem of childlessness in around 50% of the cases. Unfortunately, 30 -- 50% of the etiologies of male infertility are unknown and therefore, no specific therapy can be instituted. Sperm count and sperm motility are prime parameters that determine the functional ability of spermatozoa.This study included determination of lactate dehydrogenase-C(LDH) activity in male infertility (n=23)and its relationship with the different parameters of the seminogram. Also deals with partial purification lactate dehydrogenasefrom semen plasma of male infertility patients by dialysis, and ion exchange DEAE-cellulose techniques. There was statistically significant correlation was found between LDH activity and Sperm Count , morphology % and Motility % (r=0.486), (r=0.263)and (r=0.444) in fertile group respectively, also that the correlation in infertile group was statistically significant between LDH activity and Sperm Count , morphology % and Motility % (r=0.431), (r=0.671)and (r=0.336) respectively.

Oneproteinous peak of lactate dehydrogenaseactivity is obtained with specific activity of 0.861u\mg protein and purification fold 6.10 compared to crude enzyme. The kinetic characteristics of partially purified lactate dehydrogenaseare studied. The maximum activity is obtained at 250µg of enzyme, Tris-Hcl buffersolution at pH 7.9, temperature 37^{o}C, incubation time 5 min., with 10mMK^{+}, Vmax

and Km values of partially purified lactate dehydrogenase1.176 unit/ml and 27.02 mM with the substrate sodium pyruvateare found respectively.

INTRODUCTION

Male infertility represents a very challenging area of clinical medicine. Many different types of medications have been tried and very few have had satisfactory results. There is a huge need to advance and develop andrologic diagnostic techniques, focusing on the metabolomics and proteomics of the sperm, seminal plasma, and testicular tissue. Clarification of the causes of idiopathic male infertility and the discovery of novel molecular targets will help guide future innovative development of new pharmacologic agents(1). Infertility is defined as the lack of ability to conceive within one year of unprotected intercourse with the same partner. It is estimated that nearly 8–12% of couples are infertile, and approximately 30–40% of infertility cases are caused by male factors(2). Several risk factors are involved in the pathogenesis of infertility, some of which include alterations in spermatogenesis due to testicular cancer, aplasia of the germinal cells, varicocele, defects in the transport of sperm, or environmental factors(3.5).As well as congenital anomalies, infectious diseases, bilateral spermaducts, pregnancy-related infections, alterations in the characteristics of semen such as a decrease in sperm motility and sperm count, the presence of antisperm antibodies (ASAs), and nutritional deficiency of trace elements such as selenium and zinc (Zn). (3).The lactic dehydrogenase C4 (LDH-C4), also named LD H -X, is an isoenzyme of lactic dehydrogenase (EC 1.1.1.27) specific in mature testicles and sperm , whose beginning is closely associated with the starting of active spermatogenesis, being specific for certain types of germinal cells of the seminal epithelium (4). Reports have been published in which LDH activity in the seminal fluid is shown to be the highest for all corporal fluids, LD H -C 4 making the major contribution to the total LDH activity in fertile individuals. The present study was carried out to demonstrate the practical usefulness of the quantitative analysis ofLDH-C4 activity in seminal plasma as a tracer of germinal activity and to prove with a simple and slight noninvasive test that it is possible to get an idea of in-depth physiological events that differentiate the spermatogenesis in fertile and infertile men(5)

MATERIAL AND METHODS

The study was conducted in Al-Mosul governorate, 23 infertile menpatients with different infertility potential. Those selected infertile men group include 23 patientswith age range (33-41) years with primary infertility and sperm count less than 4 million/milliliter (grade A according to World Health Organization (1999).The control group include 25 apparently healthy fertile volunteers with normal seminal parameters according to WHO (1999)(6).All semen samples were obtained by masturbation after 3 to 4 days of abstinence. The samples were collected at the laboratory and examined after liquefaction at 37°C. The initial analysis included assessment of the volume, viscosity, pH, number, motility, viability (expressed as stained spermatozoa percentage) and morphology of the spermatozoa. Seminal plasma was obtained by centrifugation for 15 minutes at 400 X g and then for 5 minutes at 6000 Xg.(5)

Measurement of Seminal Plasma lactate dehydrogenase–Cactivity:

LDH activity was measured by the method of Brown et al 1975.The assay mixture, directly made in a 3 mL cuvette, contains 1.8 ml of Tris-buffer; 1.0 ml of 1.8 mM sodium pyruvate; and 0.1 ml of 5 mM NADH. The reaction is initiated by the addition of 0.1 ml of diluted enzyme from the different purification steps and the decrease in A340 as a function of time is monitored(7).

Purification of LDH:

Purification of LDH from seminal plasmaof Infertile Men.

Step I: Dialysis

Ten milliliter of serum was dialyzed against 10mM Tris-Hcl buffer (pH 8.6).The solution was stirred overnight with a magnetic stirrer at 4oC. The buffer was changed every 6 hrs. during dialysis (8).

Step II: Ion Exchange Chromatography

Ten milliliter of dialyzed enzyme solution was applied on DEAE cellulose anion exchanger column (2.5x40) cm, followed by Tris-Hcl buffer (pH 8.6). Elution of the protein was carried out at a flow rate (50) ml/hour with a definite time (6) min, using Tris-Hcl buffer as eluent. The fractions were collected and the protein in each fraction was detected by following the absorbance at wavelength 280 nm. peak was combined separately from the plot of an absorbance versus elution volumes and LDH was determined in each fraction (9)

Lyophilization: The fractions which contain LDH activity were collected and concentrated by lyophilizer at -20'C.

Properties of the purified LDH:

Enzyme concentration: The partially purified LDH was added to the reaction mixture with different concentrations to study the best concentration of enzyme. The partially concentrated-elution volume used were 50,100,150,200,250,300and 350 µl.

Buffers type: The partially purified enzyme reaction mixture were incubated with the following buffers: Tris-HCl, Na-Na-phosphate, Na-K-phosphate، Citric acid, Sodium acetate.

pH range: The purified enzyme reaction mixture was incubated at different pH values 6.3,6.9,7.3, 7.9,8.5and 9.1using more effective buffer above on enzyme reaction.

Temperature: The partially purified LDH activity was measured at 10,20، 30،37,50,60,70and 80C`.

Incubation time: The partially purified enzyme is incubated at different times (3,4,5,6,7,8,9,10,11,12 and 13 min) to study the best time for reaction

Metal ions: All these metal ions Na+l, K+1, Mg+2 and Ca+2 were applied on the reaction mixture with a concentration of 10mM.

Substrate concentration: The substrate, Sodium pyruvatewas applied on the reaction mixture at different concentrations (10,,20,30,40 and 50mM).

Statistical analysis: All values are reported as mean ± SEM. Statistical significance was assessed using Student's t-test. P value less than 0.05 was accepted as the significance level. The correlation coefficient was analyzed statistically using spss-program ($p<0.001$).

RESULTS AND DISCUSSION

Levels of some biomarkers in fertileand infertile men . The concentration of seminal LDH was significantly decreased in infertile males 1792U/mg against 820U/mg in fertile males.

Table 1: Seminal fluid analysis and LDH parameters[Mean ± SD] of fertile and infertile groups.

Semen parameter	Fertile group N=25	Infertile group N=23
Age (years)	36±4.3	35±5.4
Volume (ml)	3.77±0.78	3.42±0.6
Sperm Count (Million/ml)	60± 8.9	3.3±0.7*
morphology %	43.6±3.5	17.9 ±3.7*
Motility %	60.6±6.29	31.9±7.03*
LDH (U/mg)	1792±68.55	820±183.0*

Data are reported as mean ± SD. *P<0.05 compared to control (Student t-test).* significant.

There is controversy about the role of LDH activity in sperm viability. Different studies have reported that: absent and diminished LDH activity in seminal plasma was associated with male infertility and there was a strong positive correlation between LDH-C4 activity and sperm density (10).Human seminal plasma contains several enzymatic systems that play an important role in the normal function of sperm. Many studies suggested that decreased levels of this enzyme in seminal plasma might be a potential cause of infertility but there were always contradictory between reports (11). In our research we tried to explore the seminal activity of LDH in seminal plasma of our collected samples , we noted that the activity of seminal LDH was lower in abnormal groups than normal groups. Noguera 1993 showed also that the seminal LDH activity from healthy subjects wasthree times greater than that from infertile males. Efficiently, the increased activity of LDH in seminal plasma of normospermic men suggested that higher activity of this enzyme catalyzes the ROS which might protect sperm against per-oxidative damage (5)and also plays role in sperm maturation from the early events up to the onset of fertilization (10). Reduction of LDH in seminal plasma may lead to reduce fertilizing capacity and defective sperm quality (12).

Significantly lower levels of lactate dehydrogenase have been observed in infertile males.Also a significant positive correlation was found between LDH activity and Sperm Count , morphology % and Motility % (r=0.486), (r=0.263)and (r=0.444) in Fertile group respectively , also that the correlation in Infertile group was a significant positive between LDH activity and Sperm Count , morphology % and Motility % (r=0.431), (r=0.671)and (r=0.336) respectively.

Purification LDH**:** A representative purification profile of the LDH active fractions is summarized in Table 3. male infertilityLDH was purified to about 6.10 fold compared to crude enzyme with 173% activity recovery. From the elution profile of proteins, which is shown in Fig.1, LDH active fractions were represented in the fractions between 24 to 35.

Table 3: LDH-C4 purification steps from seminal plasma of Infertile Men.

Purification steps	Volume (ml)	Totalprotein (mg)	Total activity (U)*	Specific activity (U/mg protein)	Yield %	Purification fold
Crude	10	93	13.2	0.141	100	1
Dialysis	9	81.9	13.9	0.169	105	1.19
Ion exchange	82	26.61	22.93	0.861	173	6.10

U: A unit is defined as that amount of enzyme which reduce I micrornole of LDH per min.

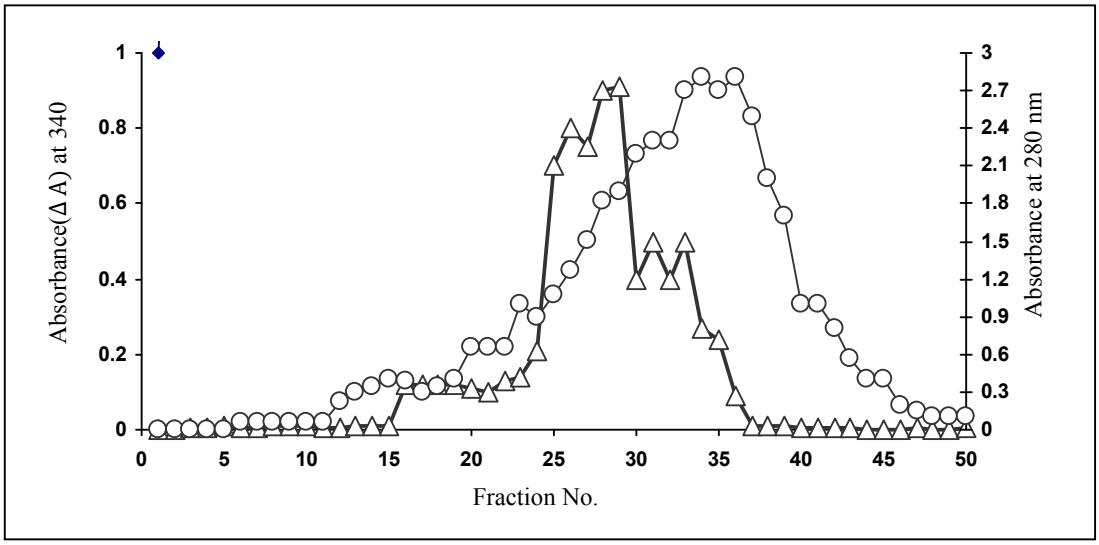
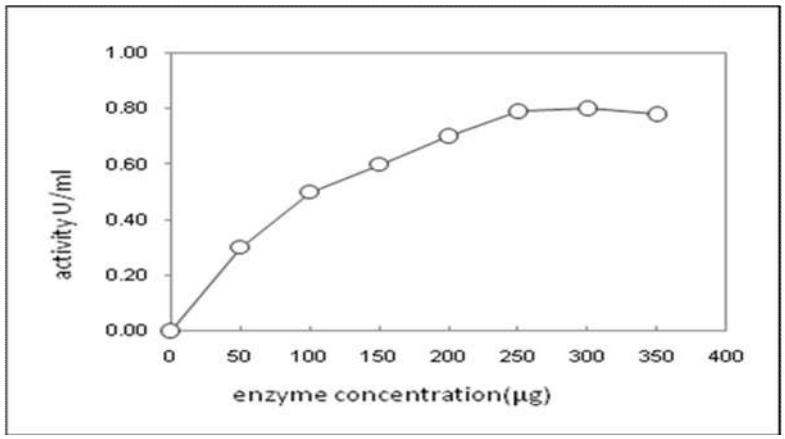


Fig. 1: Elution profile of LDH purification enzyme activity seminal plasma of Infertile Menby DEAE- cellulose chromatography column (40x2.5 cm). LDH activity at 270nmΔ , ProteinO

Effect of enzyme conc. on reaction velocity: Fig. (2) showed that LDH activity was linearly proportional to the amount of protein up to250µg .



(Fig.2) Effect of enzyme concentration on the activity of the partiallyLDH-C4

Effect of buffers and pH on the reaction velocity: LDH-C4 activity assayed with different buffers. It was found that LDH activity exhibited maximum activity Tris-Hcl buffer Fig. (3)

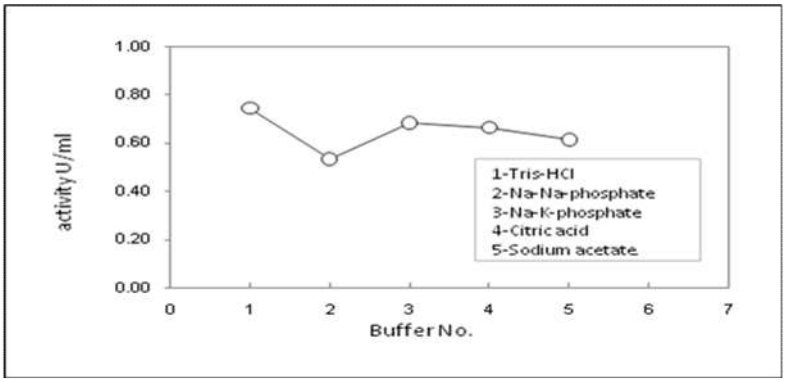


Fig.3: Effect of pH values on the activity of the partially purified LDH-C4

When assayed the LDH-C4 activity at different pH (6.3 to 9.1) of Tris-Hcl buffer, it exhibited optimum activity at pH:7.9Fig. (4)This value was different from those of human pH 7.5 (13)and liver pH 7.8 (14).The optirnum pH for different enzymes varies depending on the nature of catalytic

groups. The stability of the tertiary and I or quaternary structures of the enzyme may also be pH dependent and may affect the velocity of the enzyme reaction, especially at extreme alkaline or acidic pH values (11).

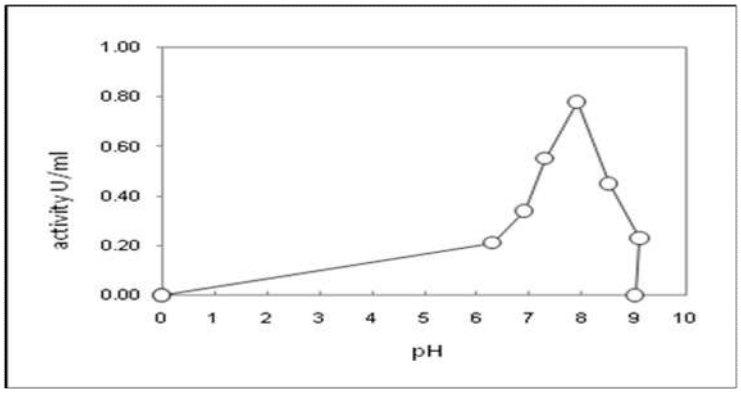


Fig. 4: Effect of buffer type on the activity of the partially purified LDH

Effect of temperature on the reaction velocity: The activity of LDH-C4 was determined at different temperature (10-70)^{o}C. It is clear from the results presented in Fig. (5) that LDH exhibited a maximum temperature at 37^{o}C .

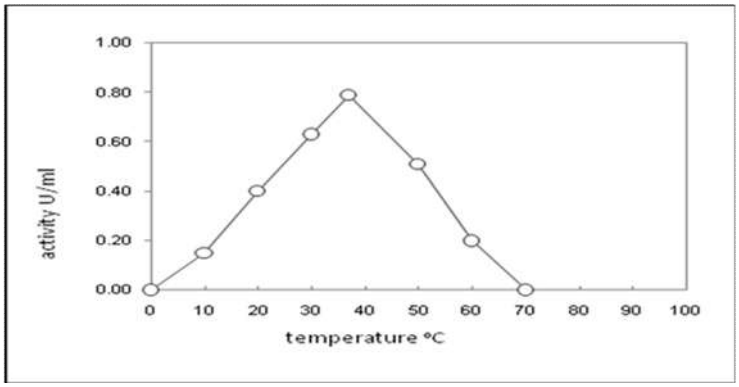


Fig. 5: Temperature effect on the activity of the partially purified LDH-C4

The optimum temperature for the enzyme activity from the heart of Karamanos 2014 was around 40'c (15). The enzyme reaction has optimum temperatures and then rapidly decrease with further temperature increase. The loss of activity at the higher temperatures is due to thermal conformational (denaturation) changes of the enzyme. Most enzymes are inactivated at temperatures above 55-60.(16).

Effect of enzyme incubation time on the reaction velocity: The activity of LDH-C4 was determined every 1 min. The maximum activity was at 5 min (Fig.6)

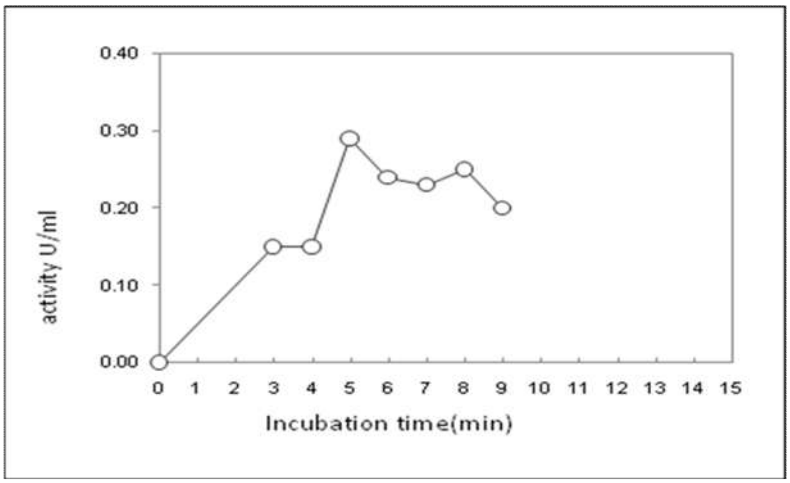


Fig.6 Effect of incubation time on the activity of the partially purifiedLDH-C4

Effect of ions on the activity of LDH **:**LDH activity was determined with different ions (**1-Na^{+1} ,2-K^{+1},3-Mg^{+2}, 4-Ca^{+2}**) conc. at l0mM. As shown in Fig (7)

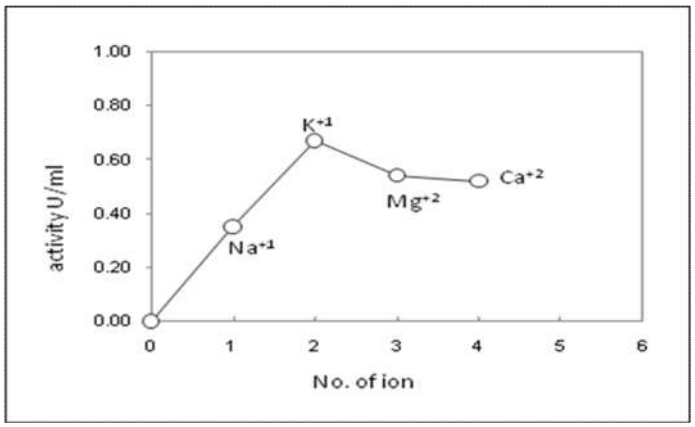


Fig.7: Effect of ions (10mM) on the activity of the partially purifiedLDH-C4

Effect of substrate concentration on enzymatic activity: The effect of different substrate concentration was tested by incubating different substrate concentration (10 to 50mM) with the same amount of the enzyme. The enzyme activity was plotted against substrate concentration. The

results in Fig.(8) demonstrated that 30 mM of substrate was the best of the optimal LDH activity.Our study illustrated the values of Vmax and Km were 1.176 U/ml and 27.02mM respectively .

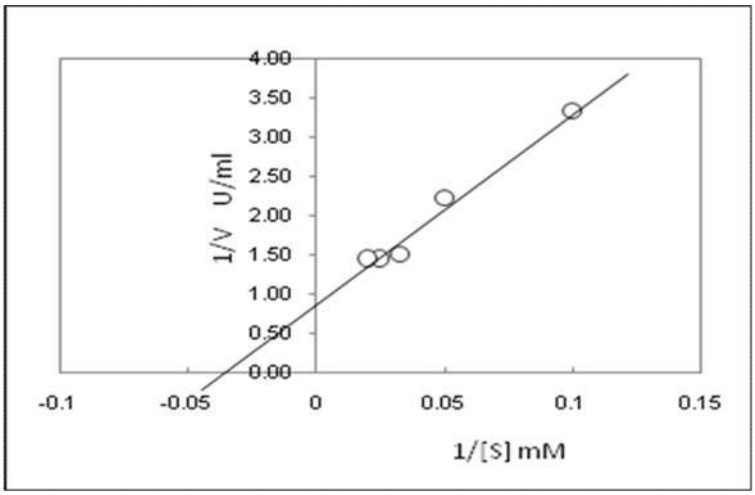


Fig. 8: Linewaver-Burk plot of LDH-C4

The Km value of pig heart LDH was 6mM by using pyruvate، as substrate (15),The velocity increases with the increase in substrate concentration up to a certain point and then becomes constant and reaches a maximum velocity. The high value of Km indicates that there is a low enzyme affinity toward substrate. The Km value is affected by substrate, pH and temperature (17)

CONCLUSION

In conclusion, the LDH activities in sperm and semen were significantly correlated with seminal qualityinfertile samples . A seminal LDH stimulation may be a useful tool for determining sperm fertilization potential. However, the results of this study could provide a database for further research into the effects of LDH on sperm. fertility is a complex process involving many factors. The real roles played by LDH in sperm quality merit further investigation.

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