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Author(s)	UCHIJIMA, Yutaka; YOSHIDA, Ken-Ichiro; SAITO, Hiroshi
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## A FURTHER STUDY OF HUMAN SEMINAL PLASMA LACTATE DEHYDROGENASE-C<sub>4</sub> (LDH-C<sub>4</sub>): KINETIC PROPERTIES OF LDH-C<sub>4</sub>

Yutaka UCHIJIMA, Ken-Ichiro YOSHIDA and Hiroshi SAITO

*From the Department of Urology, Saitama Medical Center, Saitama Medical School*

Lactate dehydrogenase-C<sub>4</sub> (LDH-C<sub>4</sub>) from human seminal plasma was purified by 40~60% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation, Oxamate-Sepharose 4B affinity column chromatography and AMP affinity column chromatography.

Enzyme kinetic studies of human seminal plasma LDH-C<sub>4</sub> were performed with purified LDH-C<sub>4</sub>. The human LDH-C<sub>4</sub> exhibits a much higher affinity for pyruvate and  $\alpha$ -ketovalerate than lactate. Apparent Km values for pyruvate,  $\alpha$ -ketovalerate and lactate were  $7.7 \times 10^{-5}$  M,  $1.3 \times 10^{-3}$  M and  $6.7 \times 10^{-2}$  M, respectively. The human seminal plasma LDH-C<sub>4</sub> was severely inhibited by increasing concentration of substrate, especially lactate.

These kinetic properties were compared with those of previous reports of human spermatozoa.

**Key words:** Human LDH-C<sub>4</sub>, Kinetic study, Seminal plasma

### INTRODUCTION

Lactate dehydrogenase (LDH) is an important enzyme in regulating cell metabolism by the interconversion of lactate to pyruvate under both aerobic and anaerobic conditions. The enzyme can be distinctively separated into five forms, LDH1 to LDH5, by electrophoresis<sup>1)</sup>. In addition to these isoenzymes, testis, seminal plasma and spermatozoa contain another isoenzyme named LDH-C<sub>4</sub>, commonly known as LDH-X<sup>2,3)</sup>. Because of the important role of LDH-C<sub>4</sub> in the metabolic pathways that provide energy for spermatozoa motility and survival, the enzyme in spermatozoa has been widely investigated in many species<sup>4-6)</sup>. On the other hand, there are only a few reports on LDH-C<sub>4</sub> of human seminal plasma. We have already reported the purification of the enzyme of human seminal plasma with affinity column chromatographies<sup>7)</sup>.

In this study, we examined the kinetic properties of LDH-C<sub>4</sub> using the purified enzyme.

### MATERIALS AND METHODS

Semen samples were obtained from the infertility clinic of Saitama Medical School. After separating seminal plasma

from sperm, seminal plasma was kept at  $-20^{\circ}\text{C}$  until use. AH-Sepharose 4B was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). The adenosine 5'-monophosphate-Sepharose 4B (AMP) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Pyruvic acid, DL-lactic acid, NAD, NADH,  $\alpha$ -ketovalerate and 1-ethyl-3-carbodiimide were purchased from Sigma Chemical Co.

Purification procedures of human seminal plasma LDH-C<sub>4</sub>

The details of the isolation and the purification procedures of human seminal plasma LDH-C<sub>4</sub> have been reported previously<sup>7)</sup>. In brief, after centrifugation of seminal plasma at 10,000 G for 60 minutes at  $4^{\circ}\text{C}$ , the enzyme was precipitated from the supernatant fraction between 40 and 60% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Then the precipitate was dialysed vs. 0.05 M phosphate buffer solution containing 0.5 M NaCl (PBS, pH 6.8) for over 24 hours. After mixing the dialysed sample with PBS containing  $0.5 \times 10^{-6}$  M NADH, the preparation was loaded on to a column of Oxamate-Sepharose 4B which had been prepared by coupling the potassium oxamate with AH-Sepharose 4B

according to Spielmann et al<sup>8)</sup>. The column was eluted with PBS containing  $1.6 \times 10^{-3}$  M NAD. The eluted sample was applied to further affinity column chromatography of AMP.

The elution was performed with PBS containing  $0.5 \times 10^{-6}$  M NADH. The eluted samples thus prepared contained purified LDH-C<sub>4</sub>. This preparation was used as the enzyme source for kinetic studies.

#### The assay of LDH activity

LDH activity was determined at 37°C by recording the optical density change at 340 nm on a spectrophotometer (UV-180, Joko Co.) according to the methods of Wroblewski and LaDuce<sup>9)</sup> and of Market and Ursprung<sup>10)</sup> for the direct and reverse reactions, respectively. The incubation medium for the direct reaction (pyruvate to lactate) contained the substrate, enzyme preparation,  $0.115 \times 10^{-3}$  M NADH and 0.1 M sodium phosphate buffer (pH 7.4), and for the reverse reaction (lactate to pyruvate),  $0.9 \times 10^{-3}$  M NAD<sup>+</sup> and 0.1 M Tris HCl buffer (pH 9.0) were used instead of NADH and sodium phosphate buffer. The final volume of incubation medium was adjusted to 3 ml. The activity of LDH was expressed in units. One unit of enzyme activity was defined as the amount of enzyme producing conversion of  $1 \times 10^{-6}$  M NADH per minute or  $1 \times 10^{-6}$  M NAD<sup>+</sup> per minute in the direct or reverse reaction, respectively.

#### The electrophoresis of LDH

The agar gel electrophoresis was performed in 0.8% agar barbiturate buffer solution with the technique proposed by Nagamine<sup>11)</sup>. After electrophoresis, gels were stained with nitrotetrazolium blue and scanned with a Densitron IM (Joko Co.).

The disc gel electrophoresis was performed in 7.5% polyacrylamide gel. After electrophoresis, LDH was stained with the technique proposed by Dietz and Lubrance<sup>12)</sup>.

#### Protein content

Protein concentration was determined

by the method of Lowry et al<sup>13)</sup>.

### RESULTS

The purity of LDH-C<sub>4</sub> following the precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the affinity column chromatography was examined by both agar gel electrophoresis and acrylamide disc gel electrophoresis.

As shown in Fig. 1a and 1b, a single band of LDH-C<sub>4</sub> is stained on both electrophoreses. The purification increased the specific activity of seminal plasma LDH-C<sub>4</sub>  $512 \pm 130$  fold (n=5). The specific activity of  $31.8 \pm 9.5$  U/mg protein was obtained in final.

Fig. 2a shows the saturation curve for LDH-C<sub>4</sub> with pyruvate in the presence of

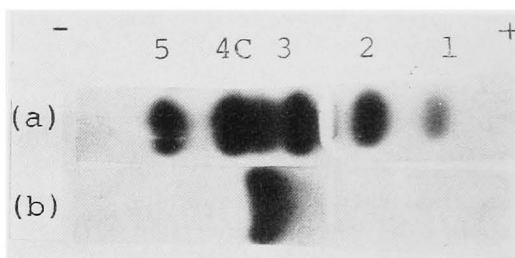


Fig. 1a. Demonstration of LDH isoenzyme patterns. (a) seminal plasma, (b) purified LDH-C<sub>4</sub> isoenzyme in agar gel electrophoresis.

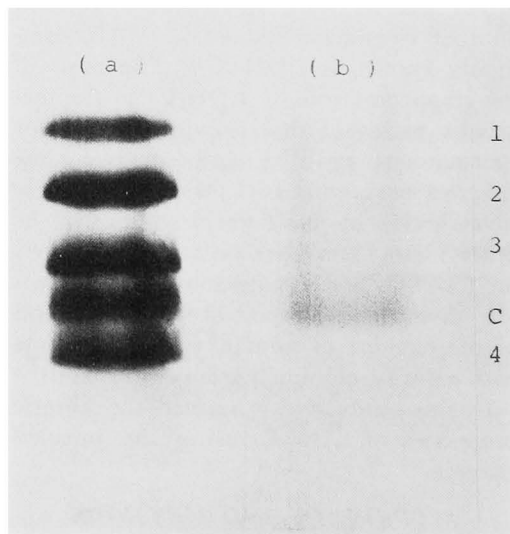


Fig. 1b. Demonstration of LDH isoenzyme patterns. (a) seminal plasma, (b) purified LDH-C<sub>4</sub> isoenzyme in polyacrylamide gel disc electrophoresis.

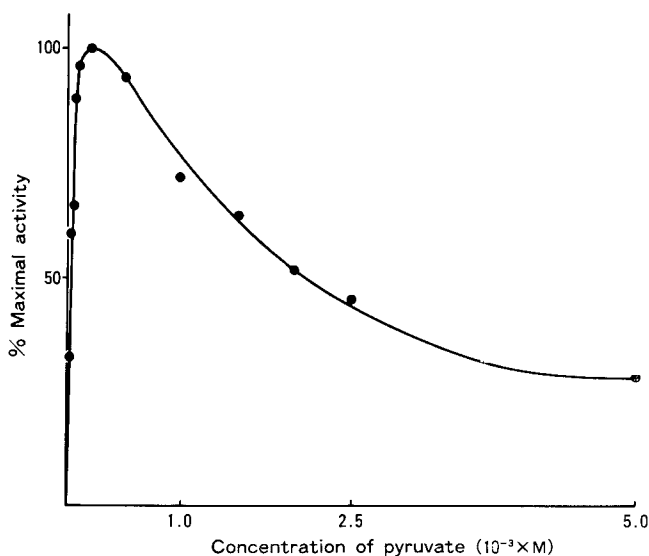


Fig. 2a. Effect of pyruvate concentration upon activity of human seminal plasma lactate dehydrogenase-C<sub>4</sub>. Initial reaction velocity, expressed as percentage of maximal activity, is plotted against pyruvate concentration ( $5 \times 10^{-5} \sim 5 \times 10^{-3} M$ ). For direct reaction, the final concentration of NADH is  $0.115 \times 10^{-3} M$ .

$0.115 \times 10^{-3} M$  NADH. Activity increased linearly with the substrate, up to  $0.2 \times 10^{-3} M$ . With higher substrate concentration, LDH-C<sub>4</sub> activity declined. The LDH-C<sub>4</sub> activity showed 95.5% of its maximal activity with  $0.4 \times 10^{-3} M$  of pyruvate (two-fold concentration of the optimum) and 82.0% of its maximal activities with  $0.8 \times 10^{-3} M$  of pyruvate concentration (four-fold concentration of the optimum). This observation demonstrates the inhibitory effect of excess substrate on LDH-C<sub>4</sub> activity. The optimal pyruvate concentration was  $0.2 \times 10^{-3} M$  and apparent  $K_m$  value for pyruvate was  $7.7 \times 10^{-5} M$  (Fig. 2b).

Fig. 3a shows the saturation curve for LDH-C<sub>4</sub> with  $\alpha$ -ketovalerate, an analogue of pyruvate, under the same conditions studied for pyruvate. As expected, the pattern of the saturation curve resembles that of pyruvate, while the LDH-C<sub>4</sub> activity showed 49% of its maximal activity with  $5 \times 10^{-3} M$  of  $\alpha$ -ketovalerate (two-fold concentration of the optimum) and 30.0% of its maximal activity with  $10 \times 10^{-3} M$  of  $\alpha$ -ketovalerate (four-fold concentra-

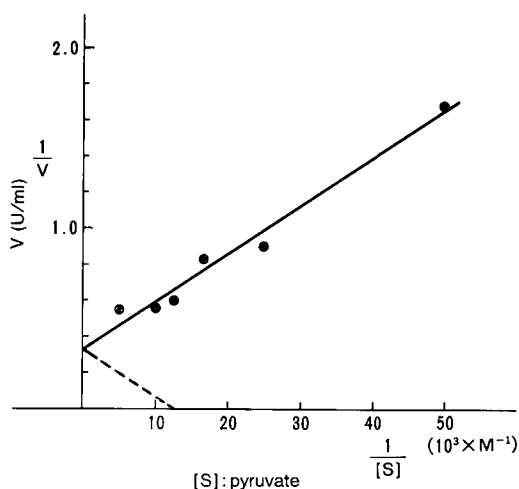


Fig. 2b. Apparent  $K_m$  for pyruvate is  $7.7 \times 10^{-5} M$ .

tion of the optimum). The optimal concentration was  $2.5 \times 10^{-3} M$  and apparent  $K_m$  value was  $1.3 \times 10^{-3} M$  (Fig. 3b).

Fig. 4a shows the saturation curve for the enzyme with lactate in the presence of excess  $NAD^+$ . Substrate inhibition of the enzyme was also observed. LDH-C<sub>4</sub> activity showed 59.2% of its maximal activ-

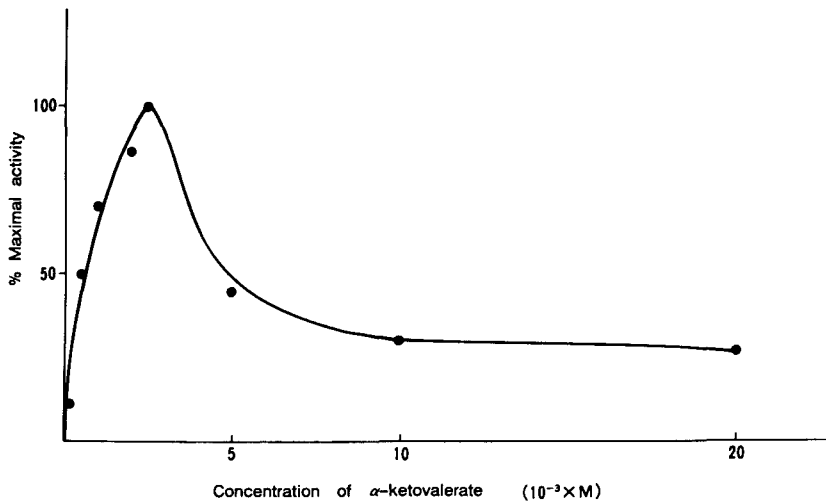


Fig. 3a. Effect of  $\alpha$ -ketovalerate concentration upon activity of human seminal plasma lactate dehydrogenase- $C_4$ . Initial reaction velocity, expressed as percentage of maximal activity, is plotted against  $\alpha$ -ketovalerate concentration ( $1 \times 10^{-4} \sim 5 \times 10^{-3} M$ ). For reaction, the final concentration of NADH is  $0.115 \times 10^{-3} M$ .

ity with 0.1M lactate (two-fold concentration of the optimum) and 26.5% of its maximal activity with 0.2 M lactate (four-fold concentration of the optimum). The substrate inhibition was greater in the reverse reaction than the direct reaction. The optimal lactate concentration was  $5 \times 10^{-2} M$  and apparent  $K_m$  value for lactate  $6.7 \times 10^{-3} M$  (Fig. 4b).

Table 1 shows the  $V_{max}$  and apparent  $K_m$  values for each substrate. The  $K_m$  value for lactate was one hundred times higher than the value for pyruvate and five times higher than that for  $\alpha$ -ketovalerate.

#### DISCUSSION

Although the LDH- $C_4$  isoenzyme has been detected in seminal plasma, testis and spermatozoa<sup>2,3</sup>, this enzyme is not detectable in semen from the patients with azoospermia and vasectomized men<sup>14,15</sup>. On the other hand LDH- $C_4$  has been reported as the predominant isoenzyme of LDH in spermatozoa, which represents 80~100% of the total LDH activity<sup>16</sup>. These observations suggest that LDH- $C_4$  originates mainly from the spermatozoa. However, recently, Virji<sup>17</sup> demonstrated that there is no leakage of LDH- $C_4$  from

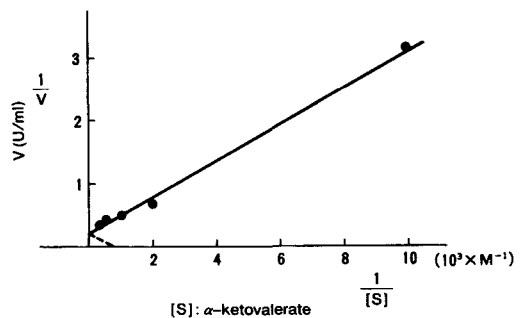


Fig. 3b. Apparent  $K_m$  for  $\alpha$ -ketovalerate is 1.  $3 \times 10^{-3} M$ .

the ejaculate sperm. These reports raise the question whether the LDH- $C_4$  in spermatozoa is identical to the enzyme in seminal plasma.

The enzyme properties of LDH- $C_4$  in spermatozoa have been widely studied in many species including men. Coronel et al.<sup>6</sup> reported the optimal concentration and  $K_m$  values for substrate, inhibition by substrate, and activity against analogue substrate for LDH- $C_4$  of human spermatozoa; the optimal concentration of substrate pyruvate and apparent  $K_m$  were  $0.5 \times 10^{-3} M$  and  $0.114 \times 10^{-3} M$ , respectively. These values are different from our present values. In addition, there are some differences between the previous reports

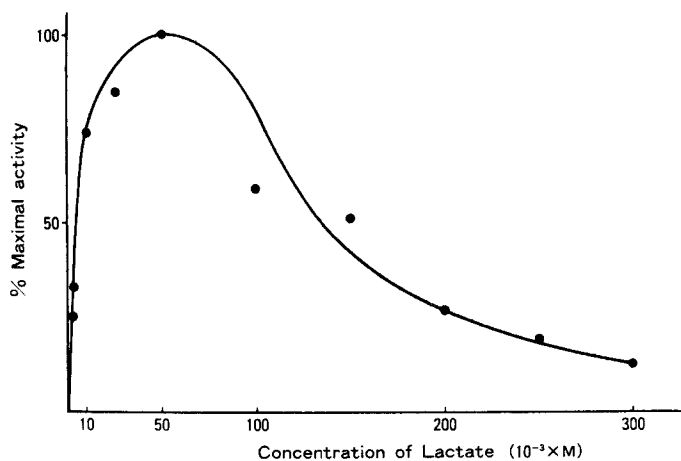


Fig. 4a. Effect of lactate concentration upon activity of human seminal plasma lactate dehydrogenase-C<sub>4</sub>. Initial reaction velocity, expressed as percentage of maximal activity, is plotted against pyruvate concentration ( $2 \times 10^{-3} \sim 300 \times 10^{-3} \text{M}$ ). For direct reaction, the final concentration of NAD is  $0.9 \times 10^{-3} \text{M}$ .

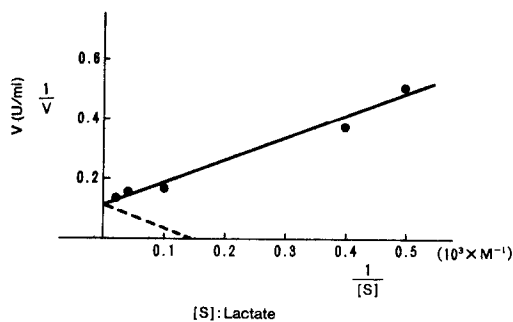


Fig. 4b. Apparent  $K_m$  for lactate is  $6.7 \times 10^{-3} \text{M}$ .

on spermatozoa<sup>18,19)</sup> and our present study. The phenomenon of substrate inhibition for LDH-C<sub>4</sub> of spermatozoa has been reported to be effective in the direct reaction than in the reverse reaction, while the opposite has been reported for LDH-C<sub>4</sub> of seminal plasma. Moreover, the saturation curve for  $\alpha$ -ketovalerate with LDH-C<sub>4</sub> of spermatozoa is different from that of seminal plasma. The discrepancy between the previous reports and the present study may be due to the difference in enzyme preparations. These observations suggest the structural difference of the LDH-C<sub>4</sub> in spermatozoa from that in seminal plasma. Recently, the sequences of amino acids have been studied<sup>20,21)</sup>. These studies will elucidate

Table 1.  $V_{max}$  and apparent  $K_m$  for each substrates

substrate	$V_{max}$ (U/ml*)	$K_m$ ( $10^{-3} \times \text{M}$ )
Pyruvate	3.0	0.077
$\alpha$ -ketovalerate	4.4	1.3
Lactate	8.9	6.7

\* 1 ml of enzyme preparation contains 20  $\mu\text{g}$  of protein.

the structural difference of LDH-C<sub>4</sub> between seminal plasma and spermatozoa.

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## 和文抄録

ヒト精漿中乳酸脱水素酵素—C<sub>4</sub> (LDH-C<sub>4</sub>) の研究：酵素反応速度の研究

埼玉医科大学総合医療センター泌尿器科 (主任：斉藤 博教授)

内島 豊, 吉田謙一郎, 斉藤 博

ヒト乳酸脱水素酵素 LDH-C<sub>4</sub> は硫酸塩析, Oxamate-Sepharose 4B および AMP のカラムクロマトグラフィーを使用して精製・分離された。この精製・分離された LDH-C<sub>4</sub> について反応速度が研究された。

ヒト LDH-C<sub>4</sub> は lactate より  $\alpha$ -ketovalerate と pyruvate の方に高い親和性を示した。Km 値は各々,

pyruvate,  $\alpha$ -ketovalerate および lactate について  $7.7 \times 10^{-5}$  M,  $1.3 \times 10^{-3}$  M,  $6.7 \times 10^{-3}$  M であった。

ヒト LDH-C<sub>4</sub> は基質濃度が増加するにつれて酵素活性は抑制され、特に lactate の場合強く抑制された。ヒト精漿中の LDH-C<sub>4</sub> と精子中の LDH-C<sub>4</sub> を比較検討し両者に違いのある可能性を検討した。

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