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Determination of lactate dehydrogenase isoenzymes in single rat glioma cells by capillary electrophoresis with electrochemical detection

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Abstract

A method for determination of lactate dehydrogenase (LDH) isoenzymes in single rat glioma cells (C6) was developed. In this method, a whole cell was electrokinetically injected into the front end of the separation capillary. After that, the cell was lysed by ultrasonication and the isoenzymes in the cell were pre-separated at 20 kV for 5 min and then incubated for 2 min with the enzyme substrates nicotinamide adenine dinucleotide (NAD^+) and lactate in the capillary electrophoresis running buffer. The electroactive product NADH generated by the isoenzymes through on-capillary enzyme-catalyzed reaction was detected at the outlet of capillary by using the end-capillary amperometric detection with a constant potential mode at a carbon fiber bundle microdisk electrode. Since the amplification of signal via the enzyme reaction, the concentration of nicotinamide adenine dinucleotide (NADH) is much higher than that of LDH. The external standardization was used to quantify isoenzymes in individual cells. Three LDH isoenzymes in single rat glioma cells (C6) were determined and quantified.

Keywords: Single-cell analysis; Electrochemical detection; Lactate dehydrogenase; Isoenzyme

1. Introduction

Lactate dehydrogenase (LDH) is a very important enzyme with its isoenzymes in life science. It has been found to be very valuable in diagnosing different kinds of diseases, such as liver disease, myocardial infarction, etc. [1]. LDH might show different activity in tumor cells compared to that in normal cells, and some isoenzymes even show different patterns [2]. The activity of LDH isoenzymes in single cells can provide useful chemical and biological information and help to elucidate whether the cell is in a normal state. Electrophoresis fluorometric method [3] and differential amperometric measurement [4] have been used to detect total LDH. Capillary electrophoresis (CE) has many inherent features of its operation suitable for analysis of single cells such as extremely small sample size, high separation speed and efficiency, biocompatible environments [5,6]. Laser-induced fluorescence (LIF) detection and electrochemical (EC) detection are two most useful detection techniques in single-cell analysis with CE. Yeung and co-worker have separated and determined LDH isoenzymes in single cells by using CE with LIF detection [7,8]. In the previous paper, we described a method to detect total LDH activity in human hemolysate by CE–EC detection based on the enzyme-catalyzed reaction [9]. In the present work, we develope a capillary electrophoretic assay with EC detection for determination of activity of LDH isoenzymes in individual rat glioma cells (C6). In this method, a single cell, following by a cell lysis solution, is injected into the front end of the separation capillary by electromigration. The cell is lysed by applying a high voltage. The LDH isoenzymes in the cell are preseparated by applying a separation voltage and then allow to react with its enzyme-catalyzed substrate, nicotinamide adenine dinucleotide (NAD^+) and lactate. LDH has three isoenzymes in rat glioma cells (C6) [10], thus, three zones of the reaction product nicotinamide adenine dinucleotide (NADH) corresponding to the three LDH isoenzymes are formed and are monitored by EC detection. Since the LDH activity in the single rat glioma cells (C6) is very high and two of three isoenzymes cannot be completely separated, the quantification of three LDH isoenzymes in single cells is discussed.

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2. Experimental

2.1. Reagents and solutions

L-LDH (EC 1.1.1.27, 135000 molecular weight) solution (8.6 mg/ml, 1145 U/mg) was purchased from Sigma (St. Louis, MO, USA). The dilute solutions were obtained by a serial dilution with the CE running buffer before use. Tris (>99.8%), NAD^+ (>97%) and NADH (>92%) were purchased from Amresco (Solon, OH, USA). DL-Lithium lactate (chemical pure grade) was obtained from Shanghai Reagents Co. (Shanghai, China). 0.50 mol/1 Tris, 0.5 mol/1 NAD^+ and 0.01 mol/1 NADH stock solutions were prepared with water, respectively. The CE running buffer consisted of 5.0×10^{-3} mol/1 NAD^+ (NAD^+ was added in the CE running buffer before use), 5.0×10^{-2} mol/1 lactate and 5.0×10^{-2} mol/1 Tris–HCl (pH 9.3). Other reagents were of analytical grade.

All solutions were prepared with double-distilled water and stored at 4 °C. Several steps were taken to minimize contamination from the outside environment. All buffers and vessels were disinfected under 1.4 kg/cm^2 (to obtain high temperature) for 20 min in an electrothermal-pressure vessel before use, in order to prevent the growth of microorganisms and denature any enzyme contaminants. Latex gloves were worn during sample preparation to minimize transfer of enzyme from skin. All solutions were prepared in disposable plastic wares with use of disposable micro-pipettips. In order to prevent the contamination from the possible repeat sampling, the commercial original enzyme solution was divided into several small packs in the disinfected plastic vessels before use.

2.2. CE–EC detection system

Details of the CE separation system used in this work were similar to our previous description [9]. Briefly, a high-voltage power supply (Model 9323-HVPS, Beijing Institute of New Technique, Beijing, China) was used to apply a voltage of 20 kV across a fused silica capillary with 10 μm i.d. (Yongnian Optical Conductive Fiber Plant, Yongnian, China) to separate the LDH isoenzymes. An electrochemical analyzer (Model CHI802, CH Instruments, Austin, TX, USA) with a three-electrode system consisting of a carbon fiber microdisk bundle electrode as the working electrode, a coiled Pt wire as the auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode was used to perform the electrochemical detection. The electrochemical detection cell with the three electrodes was housed in a Faraday cage in order to minimize the interference from noise of external sources. The electrochemical detection cell and the working electrode were described in references [11,12], respectively.

2.3. Preparation of cell and its extract

Rat glioma cells (C6) with a diameter of about 20 μm were provided by School of Life Science, Shandong University. In order to remove the culture medium, glioma cells suspended in approximately 3 ml of the culture medium were centrifuged for 10 min at 1000 rpm and the supernatant was discarded. After that, about 1 ml of the CE running buffer was added. After the cell mixture was disrupted by pipetting, it was centrifuged for 10 min at 1000 rpm. The supernatant was discarded again. Following two washing steps the cells were suspended in ca. 500 μl of the CE running buffer. This was the cell suspension for single-cell analysis and it was stored in ice water. In order to obtain the extract of glioma cell, after the cell number in the cell suspension was counted using a hemocytometer (Shanghai Medical Optical Instrument Plant, Shanghai, China), the cell suspension was diluted five times with the CE running buffer and sonicated for ca. 10 min to cytolysis. Such the extract of glioma cell was obtained.

2.4. CE–EC detection of activity of standard LDH solution and LDH isoenzymes in the cell extract

Before each run, the capillary was flushed with 0.2 mol/1 NaOH, water and the CE running buffer for 5 min, respectively, by means of a syringe. The carbon fiber microdisk bundle electrode cemented onto a microscope slide was aligned with the outlet of capillary in the horizontal direction by adjusting a laboratory-made three-dimensional micro-manipulator under a stereo microscope. With the aid of a small normal mirror, the vertical alignment was carried out using the micro-manipulator. Then a voltage of 20 kV was applied across the capillary and a detection potential of 1.00 V versus SCE was applied at the working electrode. After the electroosmotic flow reached a constant value, the electrokinetic injection of the standard LDH solution or the cell extract was carried out at 5.0 kV for 10 s. After the solution was injected into the capillary, the capillary was carefully moved from the solutions into a reservoir containing the CE running buffer that consisted of 5.0×10^{-2} mol/l Tris–HCl, 5.0×10^{-2} mol/1 lactate and 5.0×10^{-3} mol/l NAD^+ (pH 9.3). The reservoir was placed in an ultrasonicator and LDH solution or the cell extract was ultrasonicated for 10 s, to have the same conditions as in single-cell analysis. Then, LDH solution was incubated for 2 min with lactate and NAD^+ in the CE running buffer. For the cell extract, the separation voltage of 20 kV was applied for 5 min to pre-separate LDH isoenzymes. Then the high voltage was turned off and the LDH isoenzymes were incubated for 2 min with lactate and NAD^+ in the CE running buffer. Finally, the separation voltage of 20 kV and the detection potential of 1.00 V were applied across the capillary and at the working electrode, respectively, and the electropherogram was recorded.

2.5. Single-cell analysis

In order to inject single cells into the capillary, the cell suspension must be transferred directly into the injection end of the separation capillary. A droplet of the cell suspension of 10 μl was, therefore, placed on a clean microscope slide. After the microscope slide was placed on an inverted biological microscope with a magnification of 400×, the injection end of the capillary filled with the CE running buffer was gently immersed in the droplet under the guidance of a three-

dimensional micro-manipulator. In order to see the opening of the injection end, a ca. 5 mm section of the polyimide coating at the injection end of the capillary was removed by burning before use. A platinum wire was placed in the cell suspension to serve as the electrophoresis anode. As soon as one cell was drifting towards the injection end under the field of vision of the inverted biological microscope, an injection voltage of 2.0 kV was applied to draw the whole cell into the capillary tip by electroosmotic flow. The cell was adsorbed on the wall of the front end of the capillary. Then the capillary was gently moved from the cell suspension into a backer of 10 ml with the CE running buffer. The backer with the capillary was put in an ultrasonicator. The cell adsorbed on the capillary was lysed by ultrasonication for 10 s. After that, the capillary was manipulated up, out of the backer, and immersed into the anodic reservoir of the CE system. Then the preseparation voltage of 20 kV was applied for 5 min to separate LDH isoenzymes. The high voltage was turned off and the LDH isoenzymes were incubated for 2 min with lactate and NAD^+ in the CE running buffer. The high voltage of 20 kV was turned on and the detection potential of 1.00 V was applied again. At the same time, the electropherogram was recorded.

2.6. Data analysis

For two electrophoretic peaks not separated well, their areas were measured according to the following method described in reference [13]. When the point of intersection of the two peaks is lower than their half-height of peak like curve 1 in Fig. 1, q is the product of the peak current, i_p, and the width at the peak half-height, $W_{1/2}$. When the point of intersection of the two peaks is higher than the half-height of peak of the small peak like curve 2 in Fig. 1, q is calculated according to the product of their own peak area and the correction coefficient, f. In this case, the two peaks are separated by the vertical line from the point of intersection of the two peaks to the base line shown in Fig. 1, curve 2. f depends on the distance between the two peaks, $W_{1/2}$ of any peak and the peak area ratio of both peaks and can be obtained from the list in reference [13].

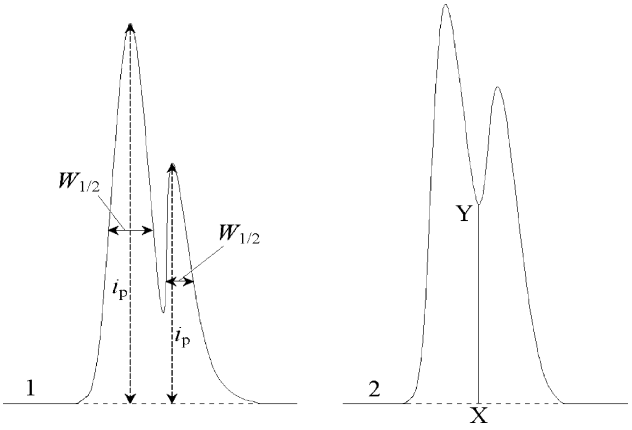

Fig. 1. Schematic representation of measurement of electrophoretic peak area. The intersection of the two peaks: (1) lower and (2) higher than their half-height of peak.

3. Results and discussion

3.1. Optimization of detection of LDH

It was found that the separation voltage of 20.0 kV and the detection potential of 1.00 V were suitable for detection of NADH. Therefore, these values were used in the subsequent experiments. Since pH 9 is suitable to the enzyme catalytic reaction [14], the electrophoretic behavior of LDH in six solutions with pH values around pH 9 was investigated. The migration time, t_m, the peak current, i_p and the peak area, q, on the electropherograms, and the number of theoretical plates, N, at different pH are listed in Table 1. Both q and i_p had the maximum value at pH 9.3. Therefore, pH 9.3 was selected in our experiments. Table 2 lists q on the electropherograms at different concentrations of lactate, C_l, or NAD^+, C_{NAD}. When the concentrations of lactate and NAD^+ were 5.0×10^{-2} and 5.0×10^{-3} mol/l, respectively, the maximum q was obtained. Therefore, these concentrations were used for determination of LDH. Under these conditions, the electropherograms of LDH after ultrasonication for 10 s and incubation for 2 min with NAD^+ (curve 1) and without NAD^+ (curve 2) are shown in Fig. 2. For comparison the electropherogram of NADH is also shown in Fig. 2 (curve 3), in which the peak eluting at 4.2 min was corresponding to impurity in the reagent NADH.

The peak A in curve 1 and 2 was confirmed to result from the difference between the CE running buffer and the standard LDH solution that was prepared in $(NH_4)_2SO_4$ solution (see the product label). Peak B came from impurity in the reagent LDH. It was noted that there was a plateau on the electropherogram after ca. 8 min. The enzyme-catalyzed reaction caused by the LDH zone migrating in the capillary should be responsible for the plateau [15]. The peak on the curve 1 produced during the incubation, eluting at 13 min, could be identified as the peak of LDH on the basis of the migration time by comparing the NADH peak shown in curve 3. q was used to quantify. Since the standard LDH solution was stored at 4 °C and was diluted before use, its activity did not change during experiments. It was also demonstrated by reproducible experiments for measurement of q indicated below. The concentration and mass linear range of LDH were 0.982–97.5 U/ml and 1.24×10^{-7}–1.23×10^{-5} U, which was calculated using the injection volume of 126 pl, with a correlation coefficient of 0.999. The concentration limit of

Table 1
The values of t_m, i_p, q and N of the electropherograms of 0.448 U/ml LDH in 5.0×10^{-2} mol/l Tris–HCl at different pH

pH	t_m (min)	i_p (pA)	q (pC)	N (10^4)
8.9	7.25	33.5	412	1.5
9.1	7.15	35.5	423	1.7
9.3	7.10	42.3	442	2.1
9.5	7.10	33.2	339	2.2
9.7	7.17	30.8	320	2.5
10.0	7.22	25.3	279	2.7

5.0×10^{-2} mol/l lactate; 5.0×10^{-3} mol/l NAD^+; reaction, 10 min at 18 °C; capillary, 35 cm × 10 μm i.d.; separation voltage, 20 kV; injection, 5.0 kV for 10 s; detection potential, 1.00 V (vs. SCE).

Table 2
Peak area, q, on the electropherograms of LDH at different concentrations of lactate, C_l, or NAD^+, C_{NAD}

C_l[a] (10^{-2} mol/l)	1.0	2.0	3.0	4.0	5.0	6.0	8.0
q (pC)	31	144	188	298	442	407	324
C_{NAD}[b] (10^{-3} mol/l)	1.0	2.0	3.0	4.0	5.0	6.0	8.0
q (pC)	73	136	232	306	442	432	423

[a] $C_{NAD} = 5.0 \times 10^{-3}$ mol/l.
[b] $C_l = 5.0 \times 10^{-2}$ mol/l, 5.0×10^{-2} mol/l Tris–HCl (pH 9.3), Other conditions as in Table 1.

detection (LOD) calculated from the electrophoretic peak area obtained for the concentration at the low end of their linear range was 0.3 U/ml, when the signal to noise ratio was 3. Its mass LOD was 40 nU for the incubation time of 2 min. If the incubation time was prolonged, the mass LOD can be reduced. The response for a series of six injections of 19.7 U/ml LDH resulted in relative standard deviations of 1.1% for t_m and 2.4% for q.

3.2. Detection of activity of LDH isoenzymes in glioma cell extract

To determine whether electroactive compounds such as cysteine, histidine, tryptophan, tyrosine, dopa, dopamine, serotonin, epinephrine and norepinephrine, which can be directly oxidized at the working electrode, interfered with the determination of LDH, their electrophoretic behavior was investigated. It was found that no peak of cysteine for the concentration of 5×10^{-5} mol/l was detected and the peaks for other compounds eluted before 5 min. They could be well separated with NADH at 13 min, implying that they did not interfere with the determination of LDH. A typical electropherogram of the extract from glioma cell after ultrasonication for 10 s and incubation for 2 min is shown in Fig. 3, curve 2. In this case, the LDH isoenzymes were not separated. The shape of the electropherogram with a plateau and then a peak, and the migration time of the peak were the same as those of standard LDH shown in Fig. 3, curve 1. The peak, eluting at 13 min, should be the peak of all LDH isoenzymes not separated. Fig. 3, curve 3 shows the electropherogram of the extract, in which LDH isoenzymes were separated at 20.0 kV for 5 min and then incubated for 2 min. Three peaks could be observed. Since only LDH can

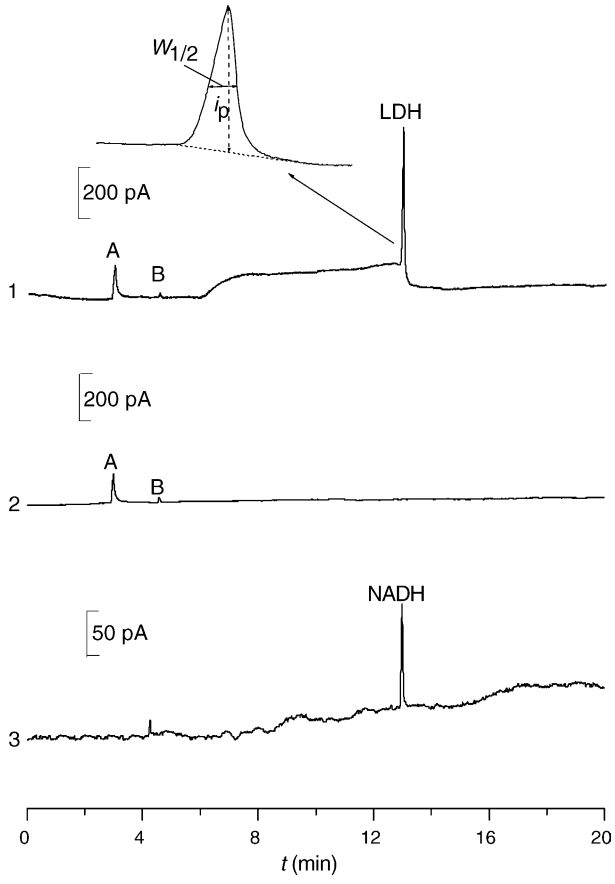

Fig. 2. Electropherograms of 19.7 U/ml LDH (1) with and (2) without 5.0×10^{-3} mol/l NAD^+, and (3) 1.00×10^{-4} mol/l NADH. 5.0×10^{-2} mol/l Tris–HCl (pH 9.3); ultrasonication for 10 s; incubation for 2 min; other conditions as in Table 1.

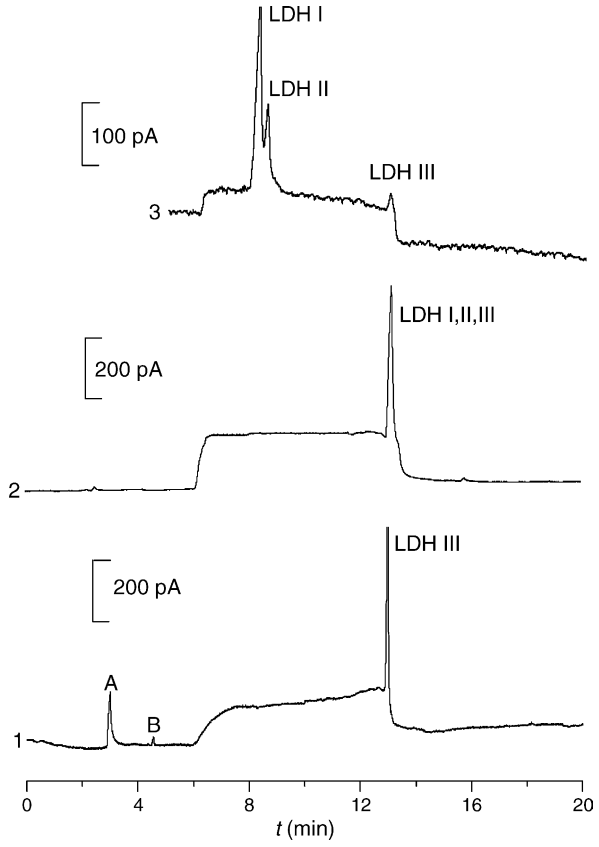

Fig. 3. Electropherograms of: (1) 19.7 U/ml LDH and the cell extract (2) without and (3) with preseparation at 20 kV for 5 min before incubation. Other conditions as in Fig. 2.

catalyze NAD^+ to NADH and NADH can be detected, LDH isoenzymes should be responsible for the three peaks. Moreover, the area sum of the three peaks was equal to that of the LDH peak detected in the extract shown in Fig. 3, curve 2, which was not separated before incubation. This means that three isoenzymes in the glioma cell extract were found, which was in agreement with the conclusion reported in reference [10]. Here the three LDH isoenzymes were defined as LDH I, II and III according to their elution sequence on the electropherogram. When the same conditions as curve 3 were used to record the electropherogram of the standard LDH solution, only one peak was recorded (Fig. 3, curve 1). The fact indicated that LDH in the reagent was LDH III according to its migration time.

The activity concentrations of the LDH isoenzymes were quantified using the calibration curve of the peak area, q. Due to the peaks LDH I and II could not be well separated, their areas were measured according to the method described in Section 2.6. The peak area of LDH III was measured based on the product of i_p and $W_{1/2}$. The mean activity concentrations determined for LDH I, II and III were 9.6 ± 0.09, 6.6 ± 0.12 and 3.3 ± 0.09 U/ml (mean ± standard deviation), respectively ($n = 3$). The ratios of the activity concentration for LDH I, II and III in the cell extract were 3:2:1. The total mean activity concentration of LDH in the extract was 19.5 U/ml. To prove the reliability of the method, a certain amount of standard LDH was added to the cell extract and then the extract was determined. From the detected activity concentration in the extract with and without the standard LDH, the recovery calculated was between 95 and 104%. Since the cell concentration in the cell extract was 2.9×10^6 cell/mL, the mean activities of LDH isoenzymes I, II and III, and the total mean activities of LDH in a single glioma cell could be calculated to be 3.3, 2.3, 1.1 and 6.7×10^{-6} U, respectively.

3.3. Analysis of activity of LDH isoenzymes in single glioma cells

In order to prevent some chemical reagents such as NaOH, SDS and some organic solvents that can lyse cells may denature enzyme, the cell injected into the capillary was lysed by ultrasonication. Fig. 4 shows the electropherograms of single glioma cells at different preseparation time. It could be noted that when the preseparation time of 5 min was used, the peaks corresponding to LDH isoenzymes I, II and III could be separated. The reproducible peak areas, together with the large linear dynamic range obtained from the measurements of LDH standard solution made it suitable to use external standardization for the quantification of the LDH isoenzymes in individual glioma cells. The activities of the LDH isoenzymes determined consecutively in four single cells are summarized in Table 3. It was observed that the activity of LDH I was the highest, which was identical to that found in the cell extract. The activities of LDH isoensymes determined in the four cells are 2.4–11, 0.60–7.9 and $0.81–3.8 \times 10^{-6}$ U, which were reasonable as compared with the values in the cell extract (3.3, 2.3 and 1.1×10^{-6} U for LDH I, II and III, respectively).

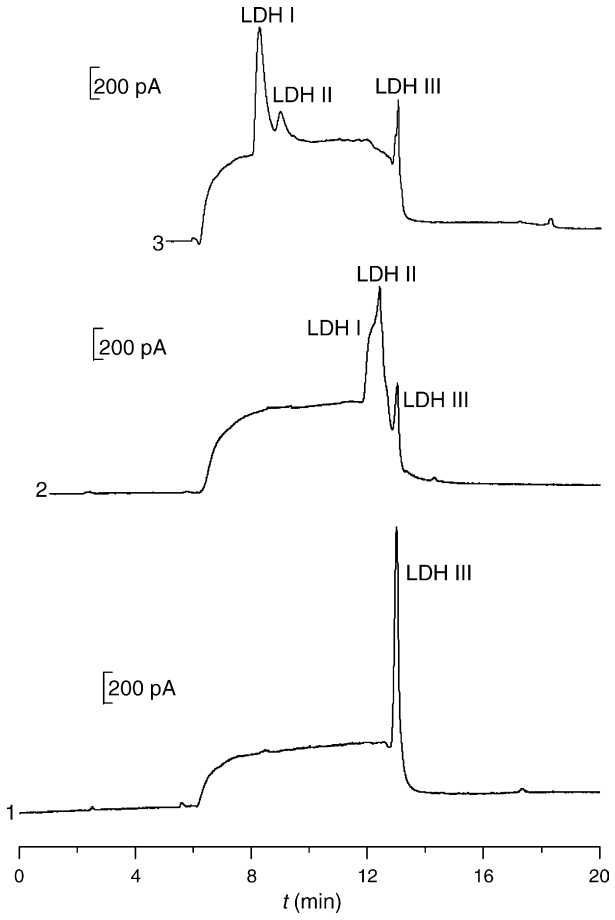

Fig. 4. Electropherograms of LDH isoenzymes in individual glioma cells at different preseparation times of: (1) 0; (2) 1 and (3) 5 min before incubation. Conditions as in Fig. 2.

Table 3
Activities of LDH isoenzymes I, II and III in four single rat glioma cells (10^{-6} U)

Cell	LDH I	LDH II	LDH III	Total LDH
1	4.1	1.4	3.3	8.8
2	2.4	0.60	0.81	3.8
3	3.1	2.0	2.7	7.8
4	11	7.9	3.8	22

4. Conclusion

Capillary electrophoresis with electrochemical detection at a carbon fiber microdisk bundle electrode coupled with on-capillary enzyme-catalyzed reaction is capable of analysis of LDH isoenzymes in single glioma cells. Ultrasonication lysis can prevent the possibility of denaturing enzymes. When the three LDH isoenzymes in single cells are preseparated at 20 kV for 5 min and incubated for 2 min with the enzyme substrates NAD^+ and lactate in the CE running buffer, they can be determined via measuring electroactive product (NADH) zones generated by the enzyme-catalyzed reaction.

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