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Thermostable amperometric lactate biosensor with *Clostridium thermocellum* L-LDH for the measurement of blood lactate

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ABSTRACT

The gene for *Clostridium thermocellum* L-lactate dehydrogenase enzyme was cloned into pGEX-4T-2 purification vector to supply a source for a thermostable enzyme in order to produce a stable lactate biosensor working at relatively high temperatures. The purified thermostable enzyme (*t*-LDH) was then immobilized on a gold electrode via polymerization of polygluteraldehyde and pyrrol resulting in a conductive co-polymer. *t*-LDH working electrode (*t*-LDHE) was used for determination of lactate in CHES buffer. Amperometric response of the produced electrodes was measured as a function of lactate concentration, at a fixed bias voltage of 200 mV in a three-electrode system. The linear range and sensitivity of the biosensor was investigated at various temperatures in the range of 25–60 °C. The sensitivity *t*-LDHE increased with increasing the temperature and reached its highest value at 60 °C. The calculated value was nearly 70 times higher as compared to the sensitivity value of the same electrode tested at 25 °C. The sensing parameters of *t*-LDHE were compared with the electrodes produced by commercially available rabbit muscle LDH (*m*-LDH). The sensitivity of *t*-LDHE was nearly 8 times higher than that of *m*-LDHE. *t*-LDHE was found to retain its activity for a week incubation at refrigerator (+5 °C), while *m*-LDHE lost its activity in this period. *t*-LDHE was also tested in the presence of human blood serum. The results showed that the current increased with increasing concentrations of lactate in the human blood serum and the biosensor is more sensitive to serum lactate as well as the commercial lactate dissolved in serum as compared to the commercial lactate dissolved in CHES buffer.

1. Introduction

Blood lactate levels are indicative of various pathological states, including shock, respiratory insufficiencies, and heart and liver diseases. Normal range of lactate in blood is 0.5–2.5 mM. Any change or increase of blood lactate is an indication of surviving capability [1]. Lactate sensors are mainly applied in critical cases, during surgical operation and intensive therapy [2,3]. In addition to this, reliable measurements of lactate are becoming very important for the various biotechnological applications of the dairy or wine industries.

While blood lactate is a useful diagnostic indicator, there are some hindrances in its use since the required time for a lactate determination is relatively long. Even under favourable conditions a lactate measurement typically takes 30 min or longer, which is too long for many clinical decisions, particularly with critically ill patients [4]. Because of that, the demand for simple, inexpensive and selective L-lactate sensors continues.

Naturally thermostable enzymes or proteins isolated from thermophilic microorganisms have intrinsically stable structural features. In general, most enzymatic reactions also benefit from increased diffusion and thermodynamics of catalysis at higher operating temperatures. Because of those factors thermostable enzymes can be considered as ideal biosensors agent. Microbial thermostable enzymes are cloned and purified for many biotechnological applications and biosensor development [5–7]. Microbial enzymes are usually considered to be more useful than that of plants or animals because of the great variety of catalytic activities, the high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. They are also more stable than their corresponding plant and animal enzymes, and their production is more convenient and safer [8,9].

L-lactate dehydrogenase is an ubiquitous enzyme, usually a tetramer of 35 kDa subunits which catalyzes the interconversion of lactate and pyruvate using NAD^+ as a coenzyme [10]. The reaction catalized by lactate dehydrogenase is shown below.

$$\text{Lactate} + NAD^+ \overset{\text{LDH}}{\longleftrightarrow} \text{pyruvate} + NADH$$

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Allosteric L-lactate dehydrogenases (LDH; EC 1.1.1.27) are regulated by fructose 1,6-diphosphate (FDP). The biosensors with lactate dehydrogenase enzymes base on three main steps for the transferring of electron during the reaction under a defined electrical potential. As shown above, NAD^+ is reduced in the reaction between LDH and lactate through which lactate is converted to pyruvate, and NADH is produced. The conductive polymeric matrix, which connects the enzyme to the surface of the electrode, accept an electron from NADH. Then the polymeric matrix transfers its electron back to the electrode.

There are several studies on lactate biosensors with various polymeric materials [7,11,12]. Few reports exist on immobilization of thermostable lactate dehydrogenase enzymes to produce biosensors. In this study, a thermostable L-lactate dehydrogenase (LDH) cloned from a thermophilic bacterium *Clostridium thermocellum* was used as a bioagent for an amperometric lactate biosensor. The stability of the working electrode prepared with C. *thermocellum* LDH was investigated and compared with that of rabbit muscle LDH immobilized via polymerization of polygluteraldehyde-polypyrrole onto a gold electrode. The effect of relatively high temperatures on sensitivity of *thermostable* LDH electrode (*t*-LDHE) was studied. The *t*-LDHE was also tested for lactate measurements in the human blood serum sample at 50 °C.

2. Experimental

2.1. Cloning of C. thermocellum ldh (t-LDH) into pGEX-4T2 vector

2.1.1. Bacterial strains, plasmids and chemicals

Escherichia coli FMJ39 (*thr-1*, *leuB6*(Am), *fhuA21*, *lacY1*, *glnV44*(AS), *pflB1*, *rfbD1*, *dld-1*, r*psL175* (strR), *thi-1) and E.coli* DH5α (F′ϕ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F)U169 *sup*E44λ− *thi*-1 *gyr*A *rec*A1 *rel*A1 *end*A1 *hsd*R17) were requested from *E. coli* Genetic Stock Center and American Type Culture Collection, respectively. The cells were grown in Luria Bertani (LB) medium [13] and stored on LB agar plates suplemented with 100 ug/ml ampicillin. pMLSK carrying *t*-LDH gene [14] was used as template DNA in PCR reactions for amplification of *t*-LDH gene. pGEM-T vector (Promega) and pGEX-4T-2 (Amersham Life Sciences) were used as PCR cloning vector and purification vector, respectively. NAD^+, lactate and rabbit muscle LDH representing mesosphilic enzyme were obtained from Fluka. All other chemicals were obtained from Sigma.

2.1.2. Cloning of C. thermocellum ldh (t-LDH) gene for purification

Plasmid DNA of bacterial cells was isolated by using Qiagen Miniprep plasmid DNA isolation kits, and the procedure in the instruction manual was followed. *t*-LDH gene in pMLSK was amplified using forward (5′-GGTCGACTATGAACAATAACAAAG-3′, underlined bases show SalI restriction site), and reverse primer (5′-GGCGGCCGCTCATATATCTAGTGTTTTTAT-3′, underlined bases show NotI restriction site) by PCR. PCR reaction mixtures contained 14.5 μL d H_2O, 2 μL 10×PCR Mg^{+2} buffer, 50 pmol of each primers, 1.0 μL of 1 mM dNTPs, 0.5 μg genomic DNA and 5 units of Taq polymerase. Reaction for the amplification of L-lactate dehydrogenase gene fragments was carried out as 45 cycles: 1 min at 94 °C, 1 min at 72 °C and 1 min at 50 °C. First cycle of the PCR was performed as 4 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, and last cycle was performed as 1 min at 94 °C, 1 min at 52 °C and 4 min at 72 °C.

The fragment was extracted from gel by using Qiagen gel extraction kit and ligated into pGEM-T which was then amplified in *E. coli* DH5∝. Amplified plasmid was digested with SalI and NotI. In order to create pGEX*t*-LDH vector, released *t*-LDH was ligated into pGEX-4T-2 purification vector, which was previously cut with same enzymes. The enzyme, *t*-LDH is expressed linked to GST (glutation S-transferase) found in pGEX-4T-2. *E.coli* FMJ39 cells were transformed with the constructed plasmid. Competent cells of *E. coli* DH5α and mutant *E. coli* FMJ39 were prepared using the procedure developed by Inoue et al. [15]. Recombinants were selected on ampicillin containing LB agar medium.

Sequencing reactions were carried out by the chain termination method with dye-labelled dideoxy terminators from Thermo Sequenase II Dye terminator Cycle Sequencing Kits (Amersham) according to the manufacturer's protocol and analysed on the ABI Prism 377 Sequencer (PerkinElmer-ABI Prism, US). The DNA sequences and amino acid sequences deduced from open reading frames (ORFs) were compared with sequences in the GenBank database using BLAST [16].

2.1.3. Purification of t-LDH Enzyme

Recombinant *E. coli* FMJ39 cells were grown in LB containing 100 mg ampicillin/L for 10 h and IPTG was added into the culture to a final concentration of 0.3 M. After 24 h incubation crude extracts was prepared and *t*-LDH was purified from *E. coli* cells by using Sepharose 4B beads as described by the manufacturer (Amersham Life Sciences). Denaturing SDS-PAGE [17] was performed, and protein concentration was determined by the method described by Bradford [18]. Proteins were visualized with Commasie blue staining of the gels. Enzyme assays were performed as described by Contag et al. [19]. The reactions were monitored spectrophotometrically at 340 nm to determine the rate of NAD reduction to NADH. The enzymes *t*-LDH and *m*-LDH were assayed for lactate oxidation to pyruvate at pH 8.5. The assay mixture for lactate oxidation contained 10 mM dithiothreitol, 50 mM sodium 2 [N-cyclohexylamino] ethane sulfonate (CHES) buffer (pH 8.5), 10 mM NAD, 1 mM fructose 1,6-diphosphate (FDP) and 50 μg of purified enzyme. The substrate, 50 mM sodium lactate, was added last to start the reaction.

2.2. Biosensor preparation

2.2.1. Apparatus

Electrochemical experiments were performed by using a CHI Model 800B electrochemical analyzer. A gold working electrode (1 cm^2), a platinum wire counter electrode, an Ag/AgCl (3 M NaCl) reference electrode, and a conventional three-electrode electrochemical cell were obtained from CH instruments.

2.2.2. Preparation of polypyrrole (PPY)-polygluteraldehyde (PGA) film coated electrode and Immobilization of lactate dehydrogenase

PGA solution was prepared by adding 2 mL of 0.1 M NaOH and 2 mL of 25% glutaraldehyde to 10 mL of distilled water [20,21]. The mixture was stirred at 600 rpm for 30 min (final pH of the solution must be 9–10). The resulted PGA solution containing 0.1 M pyrrole and 0.6 mg\mL sodium dodecyl sulphate (SDS) was used for co-polimerization of pyrrol and PGA in a three-electrode system. Polymerization on a gold plate (1 cm^2) was performed by using a potansiostat (CHI Instruments) at a potential of 1.0 V for 5 min. A platinum and Ag/AgCl electrode were used as the counter and reference electrode, respectively. The gold plate coated with PGA-PPY polymeric film was immersed into 25% gluteraldehyde solution for 3 h to increase aldehyde groups linked to the film surface. *t*-LDH enzyme of 500 μL 5.7 mg/ml was spread evenly onto glutaraldehyde activated PGA-PPY polymeric film and kept for 24 h at 4 ◦C for coupling. The two types of working electrodes which consist of the enzymes *t*-LDH and mesophilic rabbit muscle LDH (*m*-LDH)), were prepared by using same immobilization method in order to compare the results. In this study, the produced working electrodes are represented as *t*-LDH electrode (*t*-LDHE) and *m*-LDH electrode (*m*-LDHE).

2.3. Electrochemical measurements

Electrochemical batch measurements for the detection of lactate were performed by using two different types of working electrodes which were produced with *t*-LDH and *m*-LDH at 0.2 V in the same system mentioned above section. 50 mM CHES buffer (pH 7.5), containing 10 mM NAD^+ and 1 mM FDP as enzyme cofactor and activator, respectively was used as working buffer. If otherwise mentioned, measurements were carried out at room temperature. The working vessel was tightly covered with a cap in order to prevent evaporation. *I*–*t* curves of chronoamperometric measurements were done by increasing concentrations of lactate. The current values is expressed as a function of lactate concentration in the reaction medium not in the sample.

The characteristics of the calibration plots obtained for lactate biosensing, as well as the corresponding limits of detection calculated according to the $3s_b/m$ criteria in Korkut et al. [20] where *m* is the slope of the linear range of the respective calibration plot, and s_b is estimated as the standard deviation of the signals from the plot of stability.

3. Lactate measurements in the presence of human blood serum

The change in current values by using *t*-LDHE was measured at 50 °C in 10 ml of Chess Buffer at bellowed conditions:

1. Each addition was conducted with a 100 μL of lactate at a concentration of 200 mM
2. Each addition was conducted with a 100 μL of human blood serum at an unknown concentration of lactate (normally blood serum is expected to contain 1–3 mM lactate).
3. Each addition was conducted with a 100 μL of human blood serum containing 200 mM lactate additional to its original lactate content, which was called as spiked serum. Then the results were plotted as volume of addition versus current value.

4. Results

4.1. Cloning and Purification of t-LDH

t-LDH gene found in pMLSK vector was amplified by PCR and ligated into purification vector, pGEX-4T-2 as described in experimental section. DNA sequencing of the gene in pGEX-4T-2 verified that *t*-LDH followed Gluthathione S transferase gene present in pGEX-4T-2 vector and no frameshift mutation occurred during cloning. Purified *t*-LDH (2.7 mg) was obtained from 808 mg crude extract. Specific activity of the purified enzyme was found to be 0.6 U mg^{-1}, which was about 8 fold of the specific activity in the crude extract of the recombinant organism. Activity yield of purified enzyme was calculated to be 2.7%. Specific activities (the number of activity units per unit mass of protein) were reported as milimoles of NADH formed per minute per milligram of protein.

GST*t*-LDH of 635 kDa was digested with trombine in order to prove that *t*-LDH gene was cloned into pGEX-4T-2 vector successfully. After digestion with trombin enzyme, 28 kDa GST and 35.5 kDa *t*-LDH proteins were obtained as shown in Fig. 1A. The activity of GST *t*-LDH was found to be higher than that of *t*-LDH separated from GST. GST *t*-LDH was used as bioagent for the preparation of thermostable working electrodes for the enhanced measurement of lactate at high temperature-usage, and it was referred in this article in a shortened form of *t*-LDH.

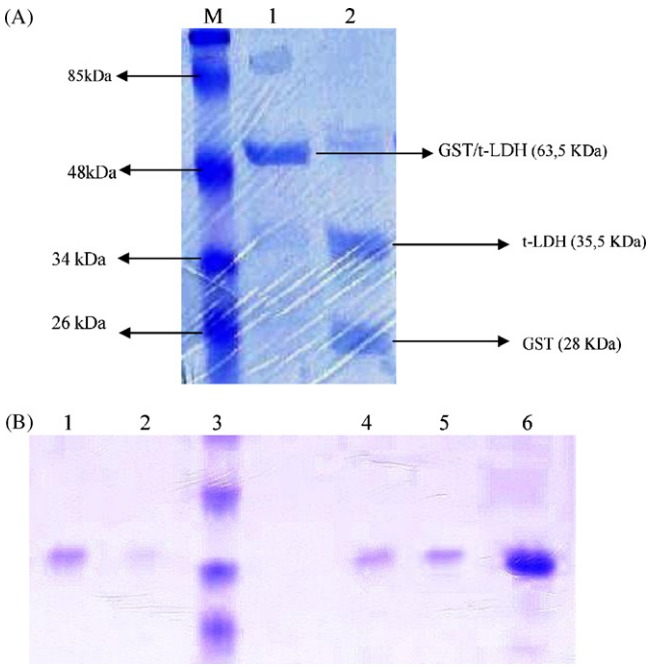

Fig. 1. (A) SDS PAGE Analysis after trombin digestion of GST*t*-LDH protein. GST*t*-LDH (1), GST and *t*-LDH enzymes after trombine digestion (2), Protein molecular weight marker (M). (B) The amount of the *m*-LDH left in the medium after immobilization at various numbers of segments: (1): enzyme residue for 30 segment-polypyrrole coating; (2): enzyme residue for 20 segment-polypyrrole coating; (3): LDH marker in the medium (no immobilization); (4): enzyme residue for 10 segment-polypyrrole coating; (5): enzyme residue for 5 segment-polypyrrole coating; 6: 2 mg/ml *m*-LDH (no immobilization).

4.2. Immobilization of the enzymes, t-LDH and m-LDH

Immobilization yield was optimized by changing the number of segment through the electropolymerization of polymeric film on the gold plate. Segment number characterizes the thickness of the coated film. The gold plate electrodes were coated with PGA-PPY co-polymer at various numbers of segments ranging 5–30 in a three-electrode cell system. Then, the immobilization procedure given under section of *Biosensor Preparation* was applied to each electrode coated with polymeric film at various numbers of segments. The optimum thickness of the film for enzyme coupling was found to be formed at 20 segments. It means that the residue of the enzyme in the medium was at the lowest level when the segment number reached to 20 through film coating. Immobilization yield of the enzyme for this application is calculated to be 61% from the initial activity of the enzyme in the medium before immobilization and the residual activity of the enzyme in the medium after the immobilization. The gel photograph shows the amount of the enzyme remained in the solution after the enzyme immobilization (Fig. 1B). After enzyme immobilization, specific activity of the residual enzyme in the medium and the current response of each electrode at various film thicknesses were also measured. The lowest enzyme activity (0.250 mM NADH/min/mg *m*-LDH) was obtained with the residual enzyme after immobilization. The current response was the highest (2.125×10^{-6} mA) for the electrode coated with 20 segments of polymeric film.

4.3. The comparison of t-LDHE and m-LDHE

Fig. 2 illustrates the CV of the polymerised PGA-PPY film at the scan rate of 100 mVs^{-1}. The conducted polymeric film was covered properly on the surface of the gold electrode which provides well surface characteristics as an immobilization matrix for the

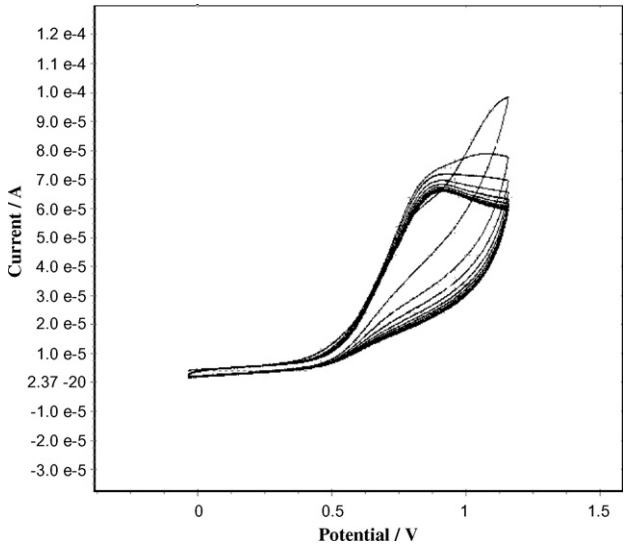

Fig. 2. CV of PGA-PPY film covered onto the surface of the gold electrode as a conductive matrix for enzyme immobilization.

enzyme. Polymerized PGA is used here as a cross linking agent. The electropolimerization reaction between PGA and PPY results in formation of a conductive co-polymer. Thus, enzyme was bounded chemically via the conductive polymeric film consisting aldehyde groups onto the gold electrode.

4.3.1. Effect of potential

The monitoring of the enzymatic reaction is carried out by the oxidation of lactate to pyruvate and reduction of NAD^+ to NADH. The oxidation current is proportional to concentration of lactate in the buffer. Since the responses of the amperometric biosensors are potential dependent, a wide potential range between −0.1 to +0.4 V was applied to the *t*-LDHE and *m*-LDHE. A clear peak at 0.2 V was obtained for both of the electrodes prepared with two different enzymes (data not shown). Further experiments were performed at that potential. No response was obtained at the potential of 0.2 V for the following two cases: 1. Enzyme electrode was immersed in 50 mM CHES buffer (10 mM NAD^+, 1 mM fructose 1,6-diphosphate (FDP)) which did not contain any lactate, 2. A gold electrode covered with PGA-PPY composite film (without immobilized enzyme) was immersed in 50 mM CHES buffer containing 10 mM NAD^+, 1 mM fructose 1,6-diphosphate (FDP) and 10 mM lactate (data not shown).

4.3.2. Preparation of the calibration curves for the t-LDHE and m-LDHE

Fig. 3 shows the dependence of the biosensors on lactate concentration. In the study of Ozkan et al. [14] *C. thermocellum* LDH was shown to be a reversible allosteric enzyme activated by FDP. Especially lactate oxidation to pyruvate was affected by the presence of FDP in the assay medium. A sigmoidal curve was obtained with increasing concentration of lactate between the concentrations of 0–160 mM at 1 mM FDP, and Hill coefficient was found to be 2.2. Sigmoidal shape of the curve appeared between the concentrations of 40–160 mM lactate. In this study, *t*-LDHE reflects slightly the allosteric character of *C. thermocellum* LDH since the curve was not exactly linear.

It was observed that the sensitivity of *t*-LDHE to lactate is higher (0.3 nA/μM) than that of *m*-LDHE (0.04 nA/μM) at room temperature. The calculated limit of detection (LOD) of *t*-LDHE and *m*-LDHE is 0.155 mM and 1.16 mM, respectively. *t*-LDHE showed nearly 7 times lower value than *m*-LDHE for the parameter of LOD regarding

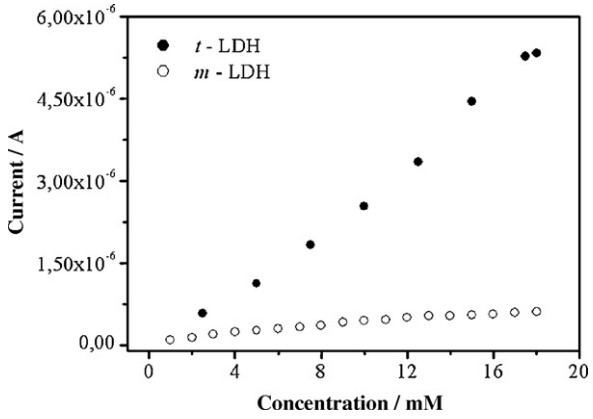

Fig. 3. Dependence of the biosensors using *t*-LDHE and *m*-LDHE on lactate concentration (0.2 V applied).

to its higher sensitivity. Linear range of *t*-LDHE and *m*-LDHE were found as 1–17.5 mM and 1–13 mM, respectively.

4.3.3. The effect of temperature on the biosensor response

For the *t*-LDHE, temperature was also found to be an important parameter influencing the sensitivity. For the detection of lactate at different temperatures the electrode was tested at 25 °C, 37 °C, 50 °C and 60 °C. The highest sensitivity was obtained at 60 °C (Fig. 4). In our previous study, optimum pH and temperature of partially purified *C. thermocellum* LDH were found to be 75 °C and 50 °C, respectively [22]. Influence of pH at a range of 5–9 on the response of *t*-LDHE was investigated to optimize the reaction conditions. Both types of working electrodes (*t*-LDHE and *m*-LDHE) showed maximum activity at pH 7.2 (data not shown).

Characteristics of the calibration plots of the biosensor using *t*-LDHE at various temperatures were analysed for the parameters of sensitivity, linear range and correlation coefficient (*r*). The highest sensitivity was obtained as 2 nA/μM with a high value of *r*, 0.99 (linear range: 2–14 mM) at 60 °C whereas the sensitivity was the lowest (0.03 nA/μM) at 25 °C. The widest linear range of 2–22 mM was found at 50 °C among the tested temperatures whereas it was between 2 mM and 10 mM at 25 °C. Increasing the temperature drastically increased the sensitivity of the developed lactate biosensor. At 25 °C, sensitivity was almost 70 times lower than the one obtained at 60 °C. Since the enzymes are generally very fragile at relatively high temperatures, construction of thermostable electrodes can directly increase the values of sensitivity and the effectiveness of the lactate measurement. Moreover, measuring lactate concentration at high temperatures can eliminate the false positive results which can be caused by the mesophilic enzymes or proteins found

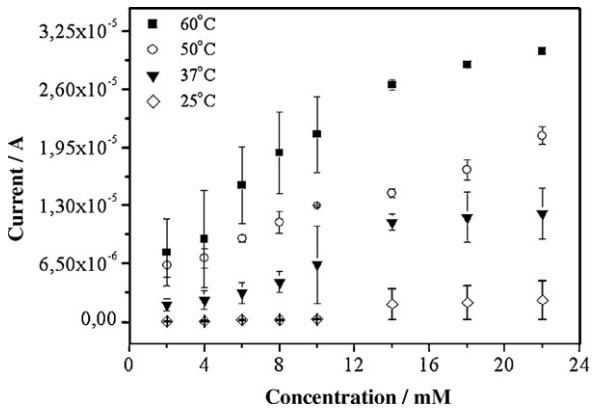

Fig. 4. The dependence of the response of the *t*-LDHE on temperature.

in human blood serum. However, not only enzyme but also prepared working electrode should be stable at high temperatures; otherwise, it cannot be used as a biosensor.

It was reported in the literature that above 50 °C NADH can be oxidized due to some non-biological factors [23,24]. In our previous studies, it was shown that *C. thermocellum* LDH has an optimum temperature of 50 °C for its activity. It lost 70% of its activity when incubated at 75 °C for 5 min and showed no activity at 80 °C [22]. In this study, we did not experience any disturbance in the current values for the *t*-LDHE at 50 or 60 °C. As expected from the nature of the thermostable enzyme used as bioagent, sensitivity of the biosensor increased with increasing the temperature, and it reached its highest value at 60 °C. The current values obtained at 60 °C showed a similar trend to those obtained at lower temperatures.

Amount of immobilized enzyme, pH and temperature are among the factors affecting biosensor sensitivity. Chaubey et al. [11] reported that increasing the concentration of immobilized LDH enzyme caused significant increase in the response current. They reported that the electrode prepared by immobilizing mesophilic LDH (*m*-LDH) on polyvinil polymer could detect 0.5–6 mM lactate. In our study, we cloned, purified and immobilized thermostable Lactate dehydrogenase enzyme as bioagent and showed that chance of working at high temperatures increases the biosensor sensitivity. Previously Halliwell et al. [7] prepared lactate biosensor using thermostable LDH from *B. stearothermophilus* immobilized on polyanyline-polyvinyl sulphonate film. They measured the current as 6 μA at 0.4 M lactate concentration and 9 μA when the lactate concentration in working buffer is 0.55 M. We obtained 5 μA at the concentration of 0.018 M lactate with *C. thermocellum* LDH immobilized on PGA-PPY film. It should be noted that the sources and the amount of the immobilized enzymes used in these two studies are different. Therefore the reaction capabilities of the enzymes are not expected to be the same. However, PGA-PPY composite film might supply better surface conditions for enzyme immobilization, since stable cross links were formed between enzyme and composite polymeric film via the aldehyde groups. PPY has some advantages over the other conducting polymers such as fast polymerization, easy membrane formation, high conductivity and chemical stability [11]. In addition to this, PGA might contribute to the mechanical strength of PPY film. Therefore, enzyme could easily be entrapped on the porous structure of PGA-PPY composite film.

4.4. Lactate measurement in the presence of human blood serum

In this study, lactate was also measured in the presence of human blood serum for testing the performance of *t*-LDHE in real samples. Fig. 5 shows three curves for the experiments carried out at three different conditions, namely as control lactate, serum and spiked serum. After addition of defined level of lactate to the samples of human blood serum in order to supply spiked serum sample, current response increased and provided detection of low lactate level found in human blood serum.

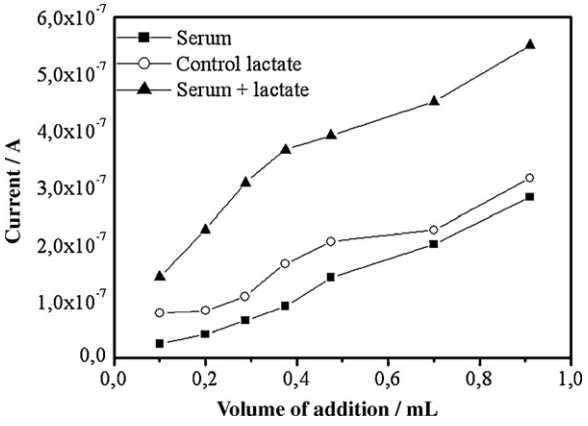

Fig. 5. Measurements of lactate in serum sample with the biosensor using *t*-LDHE.

The main aim of biosensor studies on lactate measurement is of course to develop a working electrode which could directly measure lactate concentration in blood samples. In literature there are several studies showing the performance of developed lactate biosensors in serum samples [23,12]. Suman et al. [12] reported that 8-hydroxyquiniline, urea, ammonium molibdate and uric acid caused a decrease in the response of biosensor prepared with lactate oxidase. Choi [25] tested the effects of 22 possible interferants (at a concentration of 0.1 mM) that can be found in plasma or dairy products on lactate biosensor with immobilized LDH enzyme and reported that only L-ascorbic acid and L-cysteine hydrochloride caused a slight signal drift. On the other hand, there are not enough reports on effect of serum itself on LDH activity. In this study, current obtained at 50 °C increased with increasing the amount of original human blood serum sample (with no additional lactate) in working buffer. Highest signal was obtained in linear range when the volume of serum sample was about 1/20 of the volume of working buffer. The results show that the biosensor is more sensitive to serum lactate as well as the commercial lactate dissolved in serum as compared to the commercial lactate dissolved in CHES buffer. This result can be caused by some impurities which might be added into the content of the commercial lactate during processing. Spiking the serum lactate with known amount of commercial lactate facilitated the detection of lactate concentration under the detection limit of the biosensor.

4.4.1. Stability of the biosensor

In order to see the stability of working electrodes, they were incubated at 5 °C for about 25 days. *t*-LDHE did not loose any activity

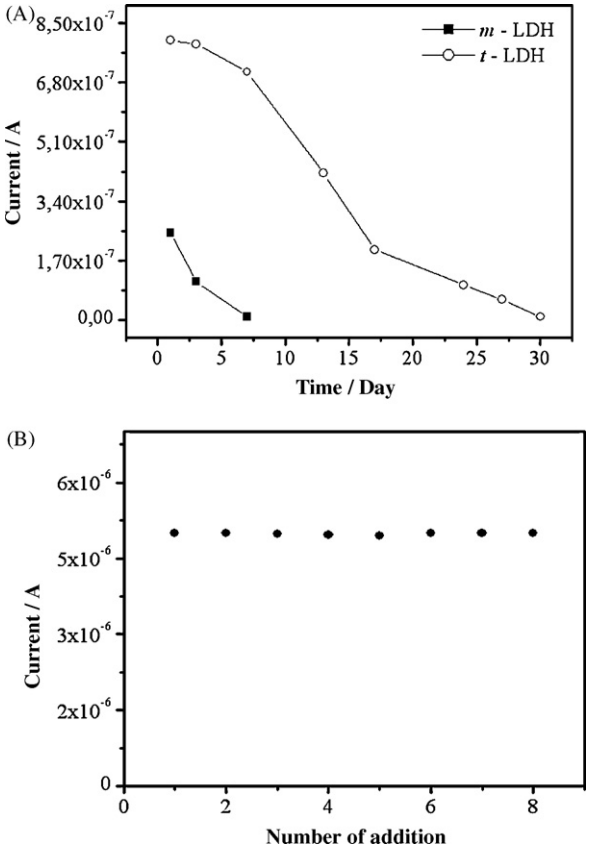

Fig. 6. (A) Stability of working electrodes *t*-LDHE and *m*-LDHE (B) Stability of thermostable lactate biosensor using the *t*-LDHE for sequential measuring at lactate concentration of 10 mM.

for a week period whereas almost no current was obtained with *m*-LDHE at the end of 7 days of incubation (Fig. 6A). Fig. 6B shows the stability of the *t*-LDHE for 8 sequential measurements at lactate concentration of 10 mM. As can be seen from the figures, using *t*-LDH enhanced the stability of the working electrode as compared to *m*-LDH. A good standard deviation at a level of 15.5 nA was obtained for the electrode at the end of the 8 sequential measurements.

5. Conclusion

The gene for *C. thermocellum* L-lactate dehydrogenase enzyme was cloned into pGEX-4T-2 purification vector to supply a continuous source of a thermostable enzyme. The highest sensitivity was obtained as 2 nA/μM with a high value of *r* (0.99) between the concentrations of 2–14 mM lactate at 60 °C. The widest linear range of 2–22 mM was found at 50 °C. It was observed that the sensitivity of *t*-LDHE to lactate is higher (0.3 nA/μM) than that of *m*-LDHE (0.04 nA/μM) at room temperature. At 25 °C, sensitivity was almost 70 times lower than the one obtained at 60 °C.

Since the enzymes are generally very fragile at relatively high temperatures, construction of thermostable electrodes with the thermostable enzymes can directly increase the shelf life of the electrodes and the values of sensitivity at high temperatures. Any interference caused by serum proteins or enzymes can also be eliminated by measuring concentration of blood lactate at high temperatures. The experiments performed in the presence of blood serum pointed out the potential use of the developed biosensor for blood lactate measurements. *t*-LDHE was found to be more sensitive to serum lactate as compared to commercially available lactate. After addition of defined level of lactate to the samples of human blood serum, current response of *t*-LDHE increased. This technique facilitated measuring of low level of lactate found in human blood serum.

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