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Amperometric Biosensor for D-Lactate Assay

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Summary

A carbon paste electrode biosensor for D-lactic acid analysis in biological samples was developed. D-lactate cytochrome c oxidoreductase from baker's yeast (EC 1.1.2.4) was used as biorecognition element of the electrode in combination with phenazine methosulphate (PMS)–Reinecke's salt precipitate as mediator. The enzyme was bound on paste due to Enzacryl using glutardialdehyde. The electrode response to D-lactate addition was linear in the concentration range of 0.015–100 mM. Limit of detection was equal to 56 μM when signal *vs*. noise criterium (S/N=3) is considered. Specificity for only D-lactate was proven. Relative standard deviation of 6.6 % for 12 mM D-lactate was found. The biosensor exerts long-term stability and also good stability during repeated measuring cycles. The electrode has a good potential for use in practical samples.

Key words: D-lactate, D-LCR, carbon paste electrode, amperometric biosensor

Introduction

Lactic acid forms two natural enantiomers. The L-lactate is well known human metabolite, while D-lactate is principally produced by microorganisms such as *Lactobacillus acidophilus*, *Lactobacillus fermenti*, *Lactobacillus casei* and *Escherichia coli* as racemate with L-isomer (*1*,*2*) and in form of pure D-isomer by some algae and plants (*3*). D-lactate is metabolized only in small scale in humans. Large amount of D-lactate in human organism can cause acidosis so the World Health Organization (WHO) recommends a daily intake of this isomer in man of <100 mg/kg body mass (*4*).

There is a demand for the determination of D-lactate isomer in food technology such as in production of beer or wine, dairy fermentation *etc*. as an indicator of product quality. Thus, the amount of D-lactate in beer varies from 55 to 80 mg/L (0.60–0.90 mM) (*5*), increased content indicates a bacterial contamination. Such contamination can cause undesirable changes in taste, health hazard and result in economic losses.

The other field of interest is clinical diagnosis. D-lactate level can become elevated in a model of intestinal ischemia (*6*) or short bowel syndrome (*7*). Diagnoses of these diseases are usually based on nonspecific clinical symptoms. D-lactate estimation in body fluids could serve as efficient diagnostic tool for such diseases that are difficult to diagnose, as well as in the cases of acidosis of various origins.

There are not a lot of methods which can distinguish between the lactate enantiomers. It is possible to use chromatographic separation (*8*) to determine both forms but a pretreatment of samples is necessary to distinguish between D- and L-lactate. Two types of enzymatic methods were used for determination of D-lactate solely: photometric (*5*) or amperometric (*9*,*10*) sensors, and they both included D-lactate dehydrogenase (D-LDH, EC 1.1.1.28) which catalyzes NADH reduction. Some works using chiral chromatography or capillary electrophoresis have appeared recently (*11*). Modern chromatographic and electrophoretic methods are promising good possibilities but probably due to long time of analysis are rare in practical labs.

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This paper describes an amperometric biosensor for determination of D-lactate which is based on D-lactate cytochrome c oxidoreductase (D-LCR, EC 1.1.2.4) with cheap alternative substrate: phenazine methosulphate (PMS), precipitated by Reinecke´s salt as mediator, applying different voltage. The PMS could displace cytochrome c in oxidoreduction reaction allowing electron transfer between D-LCR and electrode.

Materials and Methods

Reagents

D-lactate, methylglyoxal (40 % in water), Triton X-100 and graphite powder were obtained from Sigma-Aldrich (St. Louis, MO, USA). Baker´s yeast (*Saccharomyces cerevisiae*) was obtained from Noli (Olomouc, The Czech Republic). Glutardialdehyde, Reinecke's salt, dichlorophenolindophenol (DCPIP) and PMS were obtained from Serva (Heidelberg, Germany).

Protein and enzyme activity determination

The total protein content was determined by UV assay (280 nm). Enzyme activity was measured by photometer at ambient temperature using DCPIP as electron acceptor with PMS as a mediator. Reaction medium contained 0.1 M phosphate buffer, pH=7.0, 33 μM DCPIP, 100 μM PMS, and 20 mM D-lactate. Reaction was started by the addition of enzyme and the decrease of absorbance at 600 nm was measured. The activity was calculated using the value of 21 000 for molar absorption coefficient of DCPIP.

Enzyme preparation

D-LCR induction

A mass of 40 g of commercial baker's yeast paste was suspended in 2 L of neutralized medium containing $(NH_4)_3PO_4$ 1.0 g, KH_2PO_4 0.7 g, NaCl 10 g, 200 μL of each 0.1 M $ZnSO_4$ and 0.1 M ferric citrate, and 2 mL of 40 % methylglyoxal. The suspension was aerated for 4 h, cells were harvested by centrifugation for 5 min at 3000×*g*, pellet was washed twice with water and suspended in 160 mL of 10 mM phosphate buffer, pH=7.2.

Purification of D-LCR

Suspended cells in aliquots of 40 mL were disrupted by X-press (AB Biox, Stockholm, Sweden) and the homogenate was centrifuged for 20 min at 3000×*g*. Supernatant was then centrifuged for 60 min at 12 000×*g*. The sediment was homogenized in 2 mL of 10 mM phosphate buffer, pH=7.0, with 1 % Triton X-100 and extracted for 45 min at 0 °C with occasional stirring. The D-LCR was purified by affinity chromatography (using fast protein liquid chromatography (FPLC) arrangement LCC 500, Pharmacia, Uppsala, Sweden). The extract was passed through a column (10/100) of AH-Sepharose 4B covalently modified with 4-hydroxy-α-cyanocinnamic acid equilibrated with 10 mM phosphate buffer, pH=7.0, containing 0.01 % Triton X-100. After washing with 5 mL of buffer, gradient of NaCl (0–2.0 M) was applied. Active fractions eluted at about 1.2 M NaCl were collected and stored in aliquots at –20 °C.

Construction of electrochemical biosensor

Carbon paste was prepared by friction of 2.0 g of graphite powder, 0.9 mL of paraffin oil, 100 mg of Enzacryl AH and 300 mg of PMS-Reinecke´s salt precipitated to homogeneity. A volume of 200 mL (the highest amount allowing to prepare a consistent paste) of purified enzyme (0.4 nkat) was then added and the material was mixed again. The D-LCR containing carbon paste was exposed to glutardialdehyde vapour by incubating with a piece of filter paper soaked with glutardialdehyde overnight in cold. The electrode body (own construction) consisted of the Teflon tube (3 mm bore) with platinum wire as contact and a holder with pressing screw. The Teflon tube was filled with active carbon paste, which was consequently stamped down with the screw. The surface of salient carbon paste was smoothed by wax paper when electrode surface was mechanically damaged.

Electrochemical measurements

All measurements were performed at 25 °C in a stirred cuvette containing 4 mL of 50 mM sodium phosphate buffer, pH=7.0, with 60 mM NaCl. A three-electrode set-up was chosen for measurement mode. The calomel electrode served as reference in all experiments and the platinum wire was installed as auxiliary electrode. The amperometric unit model ADCL-2 was used and the output signal was recorded by the TZ 5000 line recorder (both from Laboratory Instruments, Prague, The Czech Republic).

The initial background signal was allowed to stabilize (2 to 5 min). The analyzed samples were added to the cuvette and the response left to stabilize for 2 min. When the highest concentrations were applied, resulting in signal drift, the response could be measured up to 10 min.

Results and Discussion

Optimalization of measuring conditions

Working, or applied, potential is a parameter of principal importance determining the signal magnitude and stability. The electrode response may be strongly influenced by this parameter in the presence of PMS used as mediator. Intensity of the signal was measured using working potential varying from –60 to 60 mV. The results presented in Fig. 1 show strong dependence of

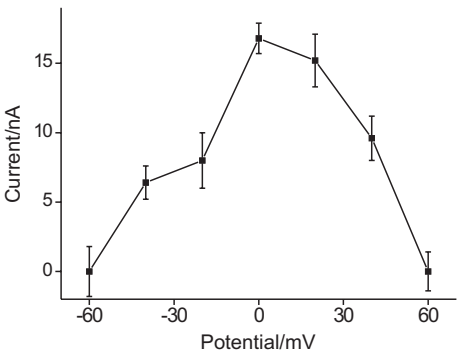

Fig. 1. Dependence of electrode response on working potential. D-lactate 10 mM was added to the reaction medium

the current on the potential with maximum at the value of 0.0 V. We expected strong increase of the current when plus potential is adjusted; nevertheless, the decrease of outputting signal is probably caused by mediator polarization. It follows from these results that D-LCR is efficient enough in transferring electrons from D-lactate to the next mediators. Any applied potential interferes with that transfer leading to the decrease of the current. So, all experiments were carried out with zero working potential.

The response of enzyme electrode is influenced by pH. The dependence of the signal on the pH value of the measured sample is presented in Fig. 2. The maximum was found at pH=7.0 with a sharp decrease to the lower values; the decrease between 7.0 and 8.0 was slight. Similar values of pH optima were found in BRENDA database for free D-LCR from *S. cerevisiae.* Thus, pH=7.0 was used in all experiments.

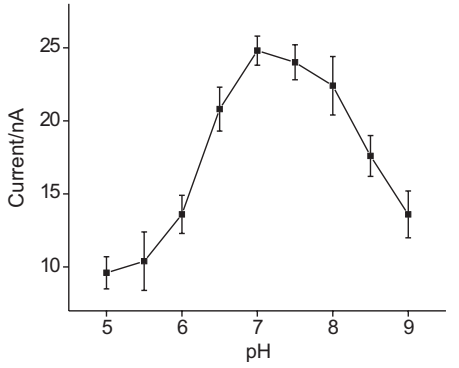

Fig. 2. The effect of pH on the electrode response. Reaction medium set to appropriate pH, working potential 0 V. Responses after addition of 10 mM D-lactate (pH was set to the same value as buffer) were recorded

Another parameter influencing the electrode response is ionic strength, which modifies the sample conductivity and consequently the current. The influence of ionic strength on the signal magnitude was followed by measuring the current in the presence of increasing NaCl concentration. NaCl was chosen because it represents an indifferent electrolyte with good solubility and corresponding ions are predominant in natural samples. Results are presented in Fig. 3. The electrode response after D-lactate addition was the highest in the absence of NaCl. Increasing ionic strength caused sharp decrease of the signal to about 2/3 at 40–50 mM NaCl; this value was not further influenced by increasing ionic strength. The molarity of 60 mM NaCl seemed to be optimal to eliminate the influence of ionic strength of the measured sample on the electrode response. The sensitivity of the electrode is lower in this case, but almost uniform conditions of measurement and suppression of ohmic polarization make the estimation of analyte reliable.

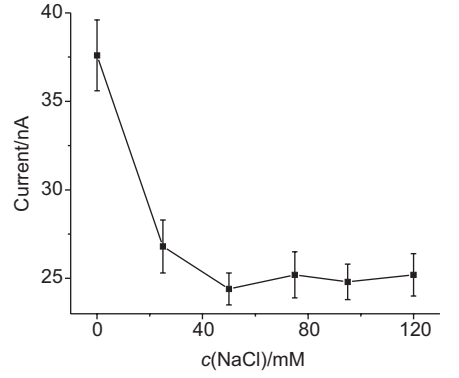

Fig. 3. Effect of ionic strength (expressed as NaCl concentration) on the electrode response. Sodium chloride was added into reaction medium in the amount creating indicated concentration. Working potential 0 V, 10 mM D-lactate

Estimation of D-lactate

The electrode response upon the addition of D-lactate was measured in the concentration range of 0–200 mM. Linear part of the dependence of signal on the D-lactate concentration is presented in Fig. 4. This dependence was used as calibration curve for D-lactate analysis. Decreasing of sensitivity occurred at the upper part of the linear range resulting in the maximum obtained signal of (248 ±14) nA for 200 mM D-lactate. Linear regression coefficient was 0.998 and relative standard deviation (RSD) was 6.6 % for 15 mM D-lactate and intraday measurement. The lowest limit of detection (LOD; S/N=3) was estimated as 56 mM D-lactate. The response was specific; L-lactate, succinate, citrate, and tartrate in equimolar concentrations gave no signal nor influenced the D-lactate response. The most important negative control is the one obtained by allowing the detection of L-lactate solution. As it is obvious from Fig. 4, distinguishing the D-lactate from L-lactate is statistically relevant in full range of calibration plot beginning above the LOD of D-lactate.

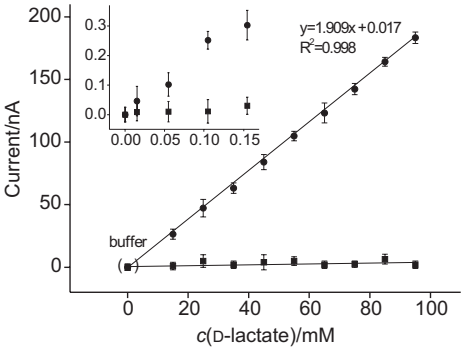

Fig. 4. Calibration curve for detection of D-lactate using the carbon paste electrode with immobilized D-LCR. Reaction medium contained 60 mM NaCl and 10 mM phosphate buffer pH=7.0. The experimental points represent mean response for the given concentration of D-lactate (•) and error bars indicate estimated standard deviations (*N*=3). Negative control was created by potential natural interferent L-lactate (■)

The enzyme electrode was stable for a long time in cold. It retained 80 % of activity after storage at 4 °C for 4 weeks. Also, operation stability allowed 100 consecutive measurements with measuring sensitivity decrease lower than 10 % for 10 mM D-lactate sample (the first *vs.* the last measuring cycle).

The sensor was practically examined on the real sample: randomly elected commercial Czech light beer

with alcohol content of 4.4 %. The amount of D-lactate was found as 0.83 mM, which responds to expectancies of 0.60–0.90 mM (*5*).

Conclusions

A novel tool for rapid and simple assay of D-lactate has been developed. The carbon paste electrode-based biosensor with immobilized D-LCR seems to be promising for measuring practical samples. Used mediator is cheaper than habitual NAD used in sensors with D-LDH. The zero working potential seems to be optimal for analysis, representing significant advantage due to simplification of the whole device. The sensor exerts lower limit of detection under supposed concentration of D-lactate in real samples.

Other parameters like RSD, linear range and detection limit are similar to other biosensors based on D-LDH, but low material demands, simplicity of D-LCR biosensor and short total time of analysis of about 2 min are the advantages of our method. All these facts are pointing at the sensor possibilities in commercial applications.

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