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Effect of yeast pretreatment on the characteristics of yeast-modified electrodes as mediated amperometric biosensors for lactic acid

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ABSTRACT

Carbon paste electrode modified with baker' and wine yeast *Saccharomyces cerevisiae* (a source of flavocytochrome b_2) were investigated as amperometric biosensors for L-lactic acid. Before immobilization on the electrode surface, yeast cells were pretreated with various electrolytes, alcohols and weak organic acids. Electrode responses to L-lactic acid were tested in the presence of various mediators (potassium ferricyanide, phenazine methosulfate, 2,6-dichlorophenolindophenol sodium salt hydrate, 1,2-naphthoquinone-4-sulfonic acid sodium salt). The highest (144±7 nA per 0.2 mM L-lactic acid) and the most stable responses were obtained after yeast pretreatment with 30% ethanol using potassium ferricyanide as a mediator. Different electrode sensitivities with mediator phenazine methosulphate probably reflected diverse changes in yeast membrane (and/or cell wall).

1. Introduction

The importance of determination of lactic acid in clinical analysis, sports medicine and dairy industry has stimulated the development of amperometric biosensors based on enzymes active for lactic acid such as lactate oxidase [1–4], NAD+-dependent lactate dehydrogenase [5–8] and flavocytochrome b_2 [9–12]. All these enzymes catalyze the oxidation of L-lactic acid to pyruvic acid. The advantages of flavocytochrome b_2 are that this enzyme is specific for L-lactic acid; it does not require an additional cofactor and is non-specific for mediators. The scheme of mediated electrocatalytic oxidation of lactic acid can be represented as follows:

$$\text{L–}lactic\ acid + flavocytochrome\ b_{2(ox)} \rightarrow pyruvic\ acid + flavocytochrome\ b_{2(red)} \quad (1)$$

$$\text{flavorcytochrome } b_{2(red)} + \text{mediator}_{(ox)} \rightarrow \text{flavocytochrome } b_{2(ox)} + \text{mediator}_{(red)} \quad (2)$$

$$\text{mediator}_{(red)} \rightarrow \text{mediator}_{(ox)} + e^-, \quad (3)$$

where Eqs. (1) and (2) are chemical reactions of flavocytochrome b_2 with lactic acid and mediator, respectively, Eq. (3) is the electrochemical oxidation of the reduced form of mediator. This reaction is used for amperometric detection of lactic acid. The limitation of flavocytochrome b_2 (isolated mostly from yeast *Saccharomyces cerevisiae*, *Hansenula anomala*, *Hansenula polymorpha*) as a biosensing constituent is its poor stability. The possible ways to solve the stability problem are to seek the yeast strains producing stable forms of flavocytochrome b_2 [13,14] or to modify electrodes with the whole yeast cells, thus keeping the enzyme in its natural environment. Flavocytochrome b_2 is known to be located in the inter-membrane space of yeast mitochondria [15]. The catalytic activity of intracellular enzymes in intact yeast cells may be low due to the impermeability of membrane to the substrate and/or mediator [16]. Relatively thick cell wall (composed mostly from polysaccharides, chitin and proteins) is responsible for the cell's resistance to mechanical stress and presents no real barrier to the diffusion of small molecules and ions [17]. Research of redox processes occurring in the intact yeast cells [18–21] revealed that amperometric measurements using only negatively charged ions (such as ferricyanide) were not possible probably due to electrostatic interaction of mediator with phosphate moieties of membrane phospholipids. The double mediator system containing both lipophilic (such as menadione) and hydrophilic (ferricyanide) was needed to monitor the redox activity inside the yeast cell.

Another way to achieve significantly higher electrode responses compared to those when intact yeast cells were immobilized onto electrode surface is the permeabilization of yeast cells. Common permeabilization methods include cell treatment with solvents, detergents, salts, cell freezing and thawing or electropermeabilization [22,23].

Numerous publications of the effects of various electrolytes, alcohols and weak organic acids on “yeast life” (including possible perturbation of yeast cell membrane integrity) has motivated this investigation of the performance of yeast-modified electrodes

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Table 1
Current responses of baker's (BY) and wine (WY) yeast-modified electrodes to 0.2 mM L-lactic acid in phosphate buffer at pH 7.3 containing 0.5 mM $K_3[Fe(CN)_6]$ and 0.5 mM EDTA (operating potential 0.3 V, *vs.* Ag/AgCl, 3 N NaCl))

Solution used for yeast pretreatment	Current response (BY), nA	Current response (WY), nA
0.1 M KH_2PO_4+0.1 M KCl (pH 7.3)	17±4	12±6
0.1 M KH_2PO_4 (pH 7.3)	11±10	11±4
0.1 M KCl	17±4	10±6
0.1 M NaH_2PO_4 (pH 7.3)	28±8	12±8
0.1 M NaH_2PO_4+0.1 M NaCl (pH 7.3)	42±9	12±6
0.1 M LiCl	71±10	45±5
0.1 M LiCl+0.1 M NaH_2PO_4 (pH 7.3)	95±7	47±8
0.01 M LiCl+0.1 M KH_2PO_4+0.1 M KCl (pH 7.3)	11±6	11±6
0.025 M LiCl+0.1 M KH_2PO_4+0.1 M KCl (pH 7.3)	58±10	not tested
0.05 M LiCl+0.1 M KH_2PO_4+0.1 M KCl (pH 7.3)	73±9	not tested
0.1 M LiCl+0.1 M NaH_2PO_4+0.1 M NaCl (pH 7.3)	93±7	40±8
0.2 M LiCl+0.1 M NaH_2PO_4+0.1 M NaCl (pH 7.3)	99±10	45±4
0.1 M LiCl+0.1 M KH_2PO_4+0.1 M KCl (pH 4.6)	72±14	45±8
Water	11±4	12±4

containing yeast cells pre-treated with these substances prior to immobilization on the electrode surface. Electrode responses to L-lactic acid in the presence of mediators (potassium ferricyanide, phenazine methosulfate, 2,6-dichlorophenolindophenol sodium salt hydrate, 1,2-naphthoquinone-4-sulfonic acid sodium salt) probably reflected the changes in cell membrane and/or cell wall induced by the cell pre-treatment.

2. Experimental

Baker's yeast (BY) *S. cerevisiae* (SEMA, Panevezys, Lithuania) was obtained from local market (shelf life not less than 2 weeks as specified by the producer). Wine yeast (WY) wine strain type K2 killer Rom K-100 HM/HM wt [kill-K2] from the collection of Institute of Botany (Vilnius, Lithuania) was grown on the YEPD medium (1% yeast extract, 2% peptone, 2% glucose and 2.5% agar) until stationary phase. The plates were kept for 3 days at 30 °C. Afterwards the samples of yeasts were stored in the fridge.

Potassium ferricyanide, *N*-methylphenazonium methyl sulphate (or phenazine methosulphate, PMS), 2,6-dichlorophenolindophenol sodium salt hydrate (DCPIP) were obtained from Fluka, 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS) was from Merck. Phosphate buffer was prepared from 0.1 M KH_2PO_4 and contained additionally 0.1 M KCl (both from Fluka). The values of pH were adjusted with KOH.

Plain carbon paste was prepared by mixing 100 mg of graphite powder (Merck) with 50 μL of paraffin oil (Fluka). Bulk yeast-modified paste was prepared by mixing 40 mg of yeast with 60 mg graphite and 50 μL of paraffin oil. The pastes were packed into an electrode body consisting of a plastic tube (diameter 2.9 mm) and a copper wire serving as an electrode contact. The layers of the pre-treated yeast cells on the surfaces of a plain carbon paste electrodes were formed by dipping the electrode into the suspensions of yeast prepared from 40 mg yeast in 0.5 mL of solutions of various salts (KH_2PO_4, KCl, NaH_2PO_4, NaCl, LiCl (all from Fluka) in various combinations, or in solutions of methanol, ethanol or isopropyl alcohol (all from Reakhim, Russia) of various concentrations in phosphate buffer at pH 7.3 or in solutions of acetic acid, sodium and lithium acetate or benzoic acid (all from Reakhim, Russia) at various concentrations. The electrodes were allowed to dry at room temperature for 25–30 min. and then covered with a dialysis membrane (Aldrich-Sigma) pre-soaked in water. To test the effect of time of yeast pretreatment on electrode responses, electrode modifications with pre-treated cells were performed after 1 h and further after every second hour or as indicated (in the cases of methanol or ethanol). All experiments were repeated at least for 3 times. Yeast suspensions were stored at room temperature.

Electrochemical experiments were carried out with a BAS-Epsilon Bioanalytical system (West Lafayette, USA) and a three-electrode cell arranged with a magnetic stirrer. Modified carbon paste electrode served as a working electrode. Platinum wire and Ag/AgCl, 3 N NaCl were, respectively, counter- and reference electrodes. Amperometric measurements were carried out in a stirred solution at an operating potential 0.3 V (vs. Ag/AgCl, 3 N NaCl) in phosphate buffers at pH 7.3 containing 0.5 mM of mediator. In the case of potassium ferricyanide, the solution contained additionally 0.5 mM EDTA (Reakhim, Russia). For some experiments, solutions were deoxygenated by purging argon for 20 min. All measurements were performed at room temperature.

To test the viability of pre-treated cells, 10 mg of the yeasts were suspended with stirring in 0.2 mL of sterile water. Primary culture of the yeast was also suspended for comparison. Both suspensions were seeded on the YEPD medium (1% yeast extract, 2% peptone, 2% glucose and 2.5% agar). The plates were kept for 3 days at 30 °C. The cloning was carried out until separate colonies of the yeast were obtained. The resemblance of the cells was controlled by microscopy.

3. Results and discussion

3.1. The effect of various electrolytes

Previous our investigation on possibility to use baker's yeast as a cheap source of the enzyme flavocytochrome b_2 for the development of amperometic biosensor for lactic acid was focused on the properties of intact yeast-modified carbon paste electrode in combination with various mediators both in the solution or adsorbed on graphite [24,25].

The idea to pre-treat the yeast cells with various electrolytes before immobilization on the electrode surface was inspired by the effect of lithium salts on yeast transformation efficiency and yeast membrane permeability [26–29]. Therefore, yeast suspensions for electrode modification were prepared in solutions of LiCl and other salts (such as KH_2PO_4, NaH_2PO_4, KCl, and NaCl) commonly employed in bioelectrochemical analysis. Electrode response to 0.2 mM of L-lactic acid in phosphate buffer at pH 7.3 containing 0.5 mM potassium ferricyanide and 0.5 mM EDTA was taken as a measure of the effect of yeast pretreatment since it was determined (as described below) that the highest and the most stable electrode responses were obtained with potassium ferricyanide as a mediator. The results are summarized in Table 1. Current responses of electrodes with yeast (both BY and

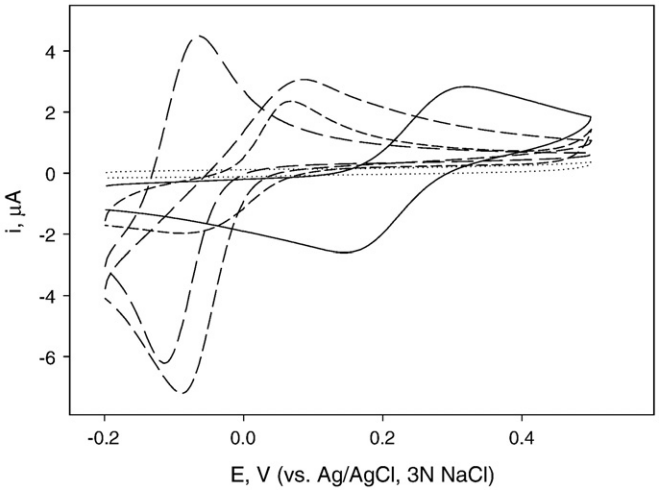

Fig. 1. Cyclic voltammograms of electrode modified with LiCl-pretreated BY in phosphate buffer pH 7.3 (dotted line) and in phosphate buffer pH 7.3 containing 1 mM $K_3[Fe(CN)_6]$ (solid line), 1 mM phenazine methosulphate (long-dashed line), 1 mM 2,6-dichlorophenolindophenol sodium salt hydrate (medium-dashed line), 1 mM 1,2-naphthoquinone-4-sulfonic acid sodium salt (short-dashed line). Potential scan rate 50 mV/s.