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Permeabilized cells of flavocytochrome b_2 over-producing recombinant yeast *Hansenula polymorpha* as biological recognition element in amperometric lactate biosensors

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Abstract

A L-lactate-selective microbial biosensor was developed using permeabilized cells of gene-engineered thermotolerant methylotrophic yeast *Hansenula polymorpha*, over-producing L-lactate:cytochrome *c*-oxidoreductase (EC 1.1.2.3, flavocytochrome b_2, FC b_2). The construction of FC b_2-producers by over-expression of the gene *CYB2 H. polymorpha* encoding FC b_2 is described. The *HpCYB2* gene under the control of the strong *H. polymorpha* alcohol oxidase promoter in the frame of a plasmid for multicopy integration was transformed to the recipient strain *H. polymorpha* C-105 (*gcr1 catX*) impaired in glucose repression and devoid of catalase activity.

The permeabilized cells were either immobilized on the graphite working electrode by physical entrapment of the cell suspension by means of a dialysis membrane or by integration of the cells in an electrochemically generated layer using a cathodic electrodeposition polymer. Phenazine methosulphate was used as a free-diffusing redox mediator. It was assumed that the mediator reacts with mitochondrial FC b_2 after entering the cells in the presence of L-lactate. The biosensor based on recombinant yeast cells exhibited a higher K_M^{app} value and hence expanded linear range toward L-lactate as compared to a similar sensor based on the initial cells of *H. polymorpha* C-105.

Keywords: Flavocytochrome b_2; Gene-engineering; *Hansenula polymorpha*; Permeabilized cells; Amperometric biosensor; L-Lactate

1. Introduction

Reliable determination of L-lactate is important in food technology, fermentation and wine industries, as well as in clinical chemistry and sport medicine. All these facts convincingly demonstrate a need for analytical devices for the accurate L-lactate measuring. Use of enzyme- and cell-based amperometric biosensors looks very promising due to the favourable coupling of the selectivity of the biological recognition element and the sensitivity of electrochemical transducer.

Biosensors for the detection of L-lactate are often based on either NAD^+-dependent lactate dehydrogenase (LDH) from mammal muscles (Arvinte et al., 2006; Hong et al., 2002), bacterial lactate oxidase (LOX) (Hirano et al., 2002; Iwuoha et al., 1999) or bi-enzyme systems combining peroxidase (HRP) and LOX (Herrero et al., 2004; Zaydan et al., 2004; Serra et al., 1999). However, due to the equilibrium constant of the LDH-catalysed reaction and the need to add free-diffusing NAD^+ as well as the problems arising from the in general high working potentials of LOX-based amperometric biosensors, there is still a need for the development of alternative sensor concepts for the determination of L-lactate. For decreasing the impact of interfering compounds, related sensors were, for example, covered with an additional permselective membrane (Madaras and Buck, 1996). Despite the more complex sensor preparation, this pathway is especially hampered for the development of L-lactate sensors due to the fact that negatively charged L-lactate is simultaneously prevented to reach the electrode surface. These limitations provoke the development of biosensors based on novel enzymes, including proteins isolated from recombinant microbial cells. Besides LDH and LOX, L-lactate: cytochrome *c*-oxidoreductase (EC 1.1.2.3; flavocytochrome b_2, FC b_2) is

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known for participating in the lactic acid metabolism in yeasts (Brooks, 2002). FC b_2 catalyses the electron transfer from L-lactate to cytochrome *c* in yeast mitochondria. The enzyme can be isolated from *Saccharomyces cerevisiae* and *Hansenula anomala* (Labeyrie et al., 1978; Haumont et al., 1987; Silvestrini et al., 1993) as a tetramer with four identical subunits, each consisting of FMN- and heme-binding domains. FC b_2 has been previously used as biological recognition element in bioanalytical devices in using artificial redox mediators for establishing the electron transfer between the immobilized enzyme and the electrode surface (Kulys and Svirmickas, 1980; Bartlett and Caruana, 1994; Staskeviciene et al., 1991). However, the broad application of FC b_2 was hampered by its instability and difficulties in purification of the enzyme (Labeyrie et al., 1978). Recently, we have described the use of FC b_2 isolated from the thermotolerant methylotrophic yeast *Hansenula polymorpha* as biological recognition element in amperometric biosensors (Smutok et al., 2005). Although the stability of FC b_2 isolated from *H. polymorpha* is significantly better than the one from baker's yeast, related biosensors still lack from sufficient stability.

In this paper, we describe a L-lactate-selective microbial amperometric biosensor based on permeabilized cells of the recombinant yeast *H. polymorpha* over-producing flavocytochrome b_2. The constructed strain is thermotolerant and able to produce a high quantity of FC b_2 (sixfold to eightfold when compared to the initial strain). The FC b_2 is located in the intermembrane space of mitochondria and hence not easily accessible for redox mediators. After permeabilizing the cells free-diffusing phenazine methosulphate can effectively diffuse into the cell to the site of FC b_2 and hence efficiently couple enzyme catalyzed L-lactate oxidation with the electrode. In addition, permeabilization of the cells facilitates L-lactate diffusion into the cells.

2. Materials and methods

2.1. Materials

L-Lactate:cytochrome *c*-oxidoreductase (flavocytochrome b_2) (EC 1.1.2.3) was isolated and purified from the constructed recombinant strain "tr1" of the methylotrophic yeast *H. polymorpha*. The enzyme was purified to the specific activity of 5 U mg^{-1} and stored as a suspension in 30%-saturated ammonium sulfate, pH 7.2 according to (Smutok et al., 2005). One unit of the enzyme activity is defined as that amount of the enzyme which oxidizes 1 μmol of L-lactate in 1 min under standard conditions of the assay (20 °C; 30 mM phosphate buffer, pH 7.5; 0.33 M L-lactate, 0.83 mM $K_3[Fe(CN)_6]$, 1 mM EDTA) (Appleby and Morton, 1959). The isolated enzyme was used to compare the catalytic properties of the recombinant cells with those of the purified enzyme.

L(+)-Lactic acid was obtained from Acros Organics (Geel, Belgium); L(+)-malic acid monosodium salt, α-ketoglutaric acid, uric acid and phenazine methosulphate, phenylmethylsulfonyl fluoride (PMSF) and cetyltrimethylammonium bromide were obtained from Sigma (Deisenhofen, Germany). Pyruvic acid sodium salt was from Fluka (Buchs, Switzerland). Citric acid monohydrate and D(+)-glucose monohydrate were purchased from J.T. Baker (Deventer, The Netherlands). Ethanol absolute was from Riedel-de Haën (Seelze, Germany) and EDTA from Serva (Heidelberg, Germany); $(NH_4)_2SO_4$, Na_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$ was obtained from Merck (Darmstadt, Germany). Dialysis membranes (cut off 10 kDa) were from Biomol (Hamburg, Germany). The cathodic electrodeposition polymer, EDP, (*CP9*) was synthesized following previously published procedures) (Ngounou et al., 2004; Smutok et al., 2006). In short, the cathodic EDP (*CP9*) was obtained by copolymerizing butylacrylate (BA) (2 mmol), methylmethacrylate (MMA) (2 mmol), 2-(dimethylamino)ethylmethacrylate (DMAEA) (4 mmol), 1-imidazol-1-yl-hex-5-en-3-oxy-2-ol (AGE-IM) (4 mmol) in 500 μL isopropanol–water (1:1) using di-tert-butylperoxide (DTBP) (40 μL) as radical starter. The radical copolymerisation was initiated by heating the mixture in a specifically designed reaction tube with reflux condenser at a temperature of 80–90 °C for 5 h. The viscous and sticky products were diluted with HPLC-grade water to decrease the viscosity of the reaction mixture. The obtained copolymer grade emulsion was vigorously stirred to form an aqueous polymer emulsion.

All chemicals were of analytical grade, and all solutions were prepared using HPLC-grade water. Solutions of L-lactic acid, ketoglutaric acid, citric acid and uric acid were prepared in 50 mM phosphate buffer, pH 7.8 followed by neutralization using concentrated NaOH.

2.2. Strains and media

H. polymorpha CBS4732 (Lahtchev et al., 2002) and C-105 (*gcr1 catX*) (Gonchar et al., 1998, 2002) were grown on YPD at 37 °C. The latter yeast strain defective in glucose repression and void of catalase activity was used as a recipient for transformation experiments. For the selection of yeast transformants on YPD, 0.5–1.5 mg L^{-1} of the antibiotic G418 was added. The *Escherichia coli* DH5α strain (Φ80d*lac*ZΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169) was used as a host for propagation of plasmids. The strain DH5α was grown at 37 °C in LB medium as described previously (Sambrook et al., 1989). Transformed *E. coli* cells were maintained on a medium containing 100 mg L^{-1} ampicillin or 200 mg L^{-1} erythromycin.

2.3. Plasmid construction and molecular techniques

The recombinant plasmid pHIPX2_CYB2 (Fig. 1A(a)) was constructed on the basis of the plasmid pHIPX2 which was kindly provided by Dr. M. Veenhuis (Faber et al., 1994). Primers Sm1 5′-CCC AAG CTT ATG TGG AGA ACC TCC TAT AG-3′ (with included *Hind*III site) and Sm2 5′-CCC GGT ACC GGA TCC CAA AAT AGA GCG CAA GAT TGC-3′ (with included *Kpn*I site) were used for obtaining the *H. polymorpha CYB2* ORF with terminator region (orf 230 hp_contig28, in the *H. polymorpha* genome database, Rhein Biotech GmbH) by PCR amplification of corresponding ~1.9 kb fragment from the genomic DNA of *H. polymorpha* CBS 4732.