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Application of cyclic voltammetry for determination of the mitochondrial redox activity during subcellular fractionation of yeast cultivated as biocatalysts

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In this study, cyclic voltammetry is proposed as an alternative method for determination of mitochondrial localization during subcellular fractionation of *Candida melibiosica* 2491 yeast cultivated under polarization. The obtained electrochemical activity of the fractions is compared to the determined cytochrome *c* activity in the presence and absence of rotenone.

Keywords: yeast-based biofuel cell; mitochondrial electrochemical activity; cytochrome *c* oxidase.

INTRODUCTION

Mitochondria play a crucial role in eukaryotic cellular bioenergetics. Most eukaryotic cells rely on mitochondrial oxidative phosphorylation as the major pathway for synthesis of adenosine triphosphate (ATP) – the main energy source for the cellular metabolism. Recent advances in the proteomic analyses provide important information about the mitochondrial function, as well as the species and tissue specificity of mitochondrial protein composition [1-3]. However, the precise mechanisms, by which mitochondrial respiration and ATP synthesis are controlled in the cells, are still not completely understood [4]. Besides elucidation of these mechanisms, in recent years there has been an increase in research of mitochondria, concerning their involvement in cell aging or apoptosis [5, 6]. A key technique used is the subcellular fractionation, by which the cellular proteins are separated into defined enriched subcellular fractions. In most cases, the protocols for purification of mitochondrial fractions are based on disruption (homogenization) of the intact cells or tissue samples followed by consecutive differential and density gradient centrifugation of the cell homogenate [7, 8]. The purity of the obtained mitochondrial fractions is usually demonstrated by enzymatic marker detection assay, most often by a cytochrome *c* oxidase (COX) assay [9, 10]. Although the COX activity is wide-accepted as a primary marker for localization of intact mitochondria in isolated subcellular fractions, some complications connected with the isolation procedure [11-13], rearrangement of light and heavy mitochondria in different subcellular fractions [14] and fast electron transport from the ferricytochrome *c* to the oxygen may compromise the data acquisition and interpretation.

In this paper, we propose an alternative approach for determination of mitochondria localization in subcellular fractions, based on the measurement of their electrochemical activity in the presence and absence of specific mitochondrial inhibitor. The presented results, obtained by means of cyclic voltammetry (CV) carried out with subcellular fractions isolated from *Candida melibiosica* 2491 yeast, cultivated under polarization conditions in biofuel cell in the presence and absence of rotenone, are compared with data collected by colorimetric COX activity assay.

EXPERIMENTAL

Cultivation of Candida melibiosica 2491 in biofuel cell

C. melibiosica 2491 yeast cells were cultivated in the anodic compartment of double-chamber biofuel cell. The yeast was suspended in 50 ml of a YP_{fru} sterile medium previously determined as optimal [15]. The quantity of yeast inoculum was unified by adjusting its optical density OD_{600} to 0.670. 0.1M $K_3[Fe(CN)_6)]$ in PBS, pH 7, served as a catholyte. The biofuel cell compartments were

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connected with a salt bridge to avoid any possible mixing of the anolyte and the catholyte. Pieces of carbon felt (SPC-7011, 30 g/m^2, Weibgerber GmbH & Co. KG) with a rectangular shape (4×4 cm) were used as both anodes and cathodes. The cultivation was carried out in a thermostat at 28°C on an orbital shaker at 100 rpm in the presence and absence of 30 µM rotenone (Sigma-Aldrich: R8875). The yeast suspension was cultivated in a batch-regime under constant polarization by switching on 1 kΩ load resistor.

Subcellular fractionation

Two ml of anolyte from yeast suspension cultivated in biofuel cells in the presence and absence of rotenone were collected and centrifuged at 5100 *g* for 5 min. After washing the cells with PBS buffer (pH 6.8), the obtained yeast pellets were re-suspended in homogenizing buffer, consisting of 0.4 M sucrose, 50 mM Tris-HCl (pH 7.5), 3 mM EDTA and 1 mM PMSF, in proportion 1:4, and disintegrated by sonication (5-times, 10 s, in an ice-bath). The homogenates were centrifuged at 1000 *g* for 10 min. After nuclei removal, the supernatants were centrifuged at 3000 *g* for 5 min. The obtained supernatants in this step were further centrifuged at 10000 *g* for 20 min. The obtained pellets (P_{10000}) were mixed with homogenization buffer and introduced on a freshly prepared sucrose density gradient, composed of overlayered 45%, 35% and 25% sucrose solutions. The fractionation was carried out by centrifugation at 13000 *g* for 30 min. The sucrose density fractions were collected and investigated for COX activity and protein content. In parallel, the fractions were analyzed by means of cyclic voltammetry.

Specific cytochrome c oxidase activity

The COX activity was determined by means of colorimetric assay, based on observation of the decrease in absorbance at 550 nm of ferrocytochrome *c* caused by its oxidation to ferricytochrome *c* by cytochrome *c* oxidase. The substrate stock solution was freshly prepared from cytochrome *c* (Sigma 30398) in concentration 2.7 mg/ml and reduced prior to use by addition of 12 µl of 10 mg/ml sodium hydrosulfite. The reaction mixture consisted of 0.25 % Triton X-100/PBS (pH 6.8), 0.25 M sucrose, reduced cytochrome *c* (90:9:10) and 10 µl sample. The absorption decay was recorded spectrophotometrically at 550 nm during the first 10 sec of the reaction. The COX activity, defined as 1.0 µmol oxidized cytochrome *c* per min at 25 ºC and pH 6.8, was estimated by the following equation:

$$\text{Units/ml} = (\Delta A_{550}/\text{min}) \times V_{react} / V_{samp} \times 18.5$$

where $\Delta A_{550}/\text{min} = A_{550}/\text{min (sample)} - A_{550}/\text{min (blank)}$; V_{react} is the reaction volume in ml; V_{samp} is the volume of sample in ml; 18.5 $mM^{-1}\,cm^{-1}$ is the difference in millimolar absorptivity between reduced and oxidized cytochrome *c* [16].

The specific cytochrome *c* oxidase activity is presented as enzyme activity per milligram protein (Units/mg). The protein content was determined by using a modified Bradford method (Merck 1.10306.0500). The intracellular protein concentration was estimated in mg/ml by using BSA- calibration curve.

Electrochemical analyses

The electrochemical behavior of the yeast fractions was investigated by means of cyclic voltammetry (CV). The CV was carried out in a three electrode arrangement, using Pt-wires as working and counter electrodes and Ag/AgCl as a reference electrode. PalmSens handheld potentiostat/galvanostat was used for the analyses. The potential was swept with a scan rate 10 mV/s.

The experiments were performed in duplicate.

RESULTS AND DISCUSSION

Yeast cells were harvested from the suspension used as an anolyte in the biofuel cell and fractionated by means of differential centrifugation for gathering the cellular organelles. The organelle enriched fractions were further fractionated by sucrose density centrifugation, i.e. rearranged due to the specific organelle density [17]. For determination of the mitochondrial location during this procedure, the COX activity of all obtained fractions was analyzed (Fig. 1, solid columns). The purification factor was expressed for the individual step - the specific COX activity of the P_{10000} fraction was admitted as unit of measurement. The highest specific activity was observed in 35%-sucrose gradient fraction. The purification factor of this fraction amounted to 0.9 and denoted as mitochondrial fraction. The results show that the most yeast mitochondria are situated in the fraction with density 1.1513 g/cm^3. The light mitochondria were located in the 25% fraction, however the purification factor was twice lower (0.4). The negligible values of the determined COX activity in the 15%- and 45%- fractions lead to the supposition that these fractions do not contain mitochondria.

In the presence of rotenone, the COX activity of P_{10000} was lower with two orders of magnitude. As an inhibitor of electron transport chain (ETC) complex I, the addition of rotenone to the yeast suspension leads to dysfunction of the ETCs. Having in mind that rotenone blocks the electron passage from Fe-S centers of complex I to ubiquinone (CoQ), the lower electron passage between the ETC complexes leads to COX activity deactivation (Fig. 1, sparse columns).

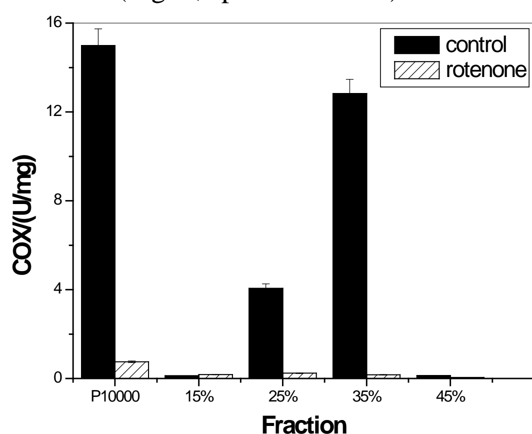

Fig. 1. Specific COX activity of the sucrose density gradient fractions in the presence and absence of rotenone.

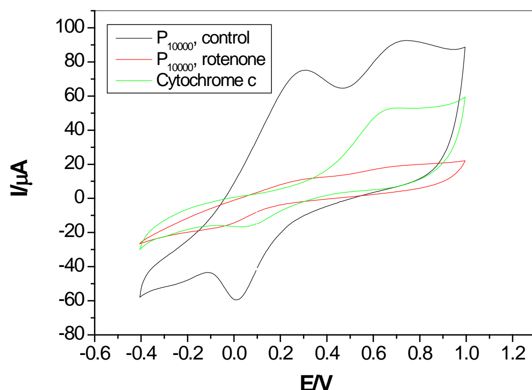

Fig. 2. Cyclic voltammograms of the organelle enriched fractions (P_{10000}) obtained in the presence and absence of rotenone compared to cytochrome *c* as a standard.

In parallel, the electrochemical activity of the organelle enriched fractions (P_{10000}) in the presence and absence of rotenone was analysed by means of cyclic voltammetry (Fig. 2). Two anodic peaks at potentials 0.3 V and 0.7 V and one cathodic peak at 0.03V were clearly observed on the voltammograms in the case without inhibition. We considered that the anodic peak at 0.7 V and the cathodic peak at 0.03 V represent the oxidation and reduction of cytochrome *c*. Fig. 2 clearly shows that the rotenone addition significantly decreased the redox activity of the fraction in comparison with that of the same fraction without inhibitor.

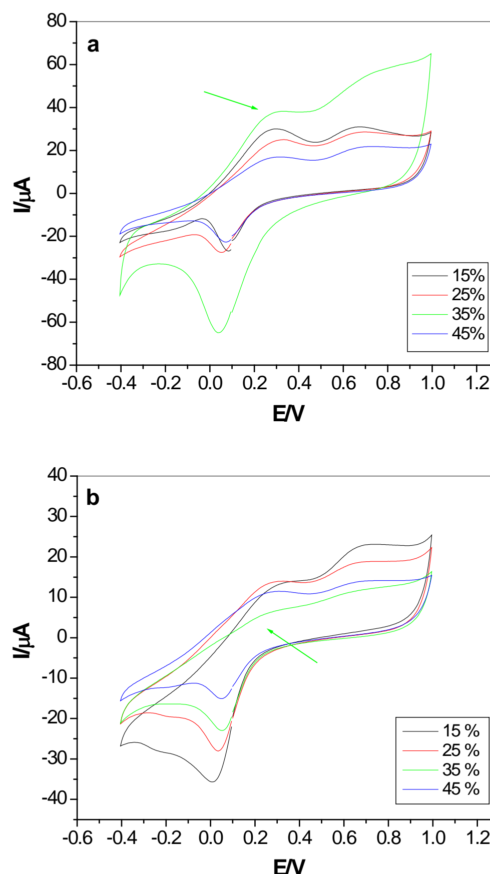

Fig.3. Cyclic voltammograms of the sucrose density gradient fractions obtained from yeast cultivated in biofuel cells: a) without inhibition; b) in the presence of rotenone.

After establishing the differences in the peaks on the CV in the presence and absence of inhibitor, cyclic voltammetry was carried out with the sucrose density fractions (Fig. 3). Identical to P_{10000} anodic peaks appeared on the voltammograms of the fractions in the case without inhibition (Fig. 3a). The observed electrochemical activity demonstrated the presence of cytochrome *c* type molecules. It was considered that the peaks appeared at relatively low concentrations of the protein (5×10^{-9} mol/1) as described by Brabec et al. [18], corresponding to a catalytic reaction, in which some of haemins of ferricytochrome *c* were reduced to haems. These haemins could be regenerated by chemical oxidation of the haem residues with oxygen [18]. The decreased current height of the peaks (I_p) of the 15 %, 25 %, and 45 % fractions showed significantly lower concentrations of redox components. At the same time, the obtained peaks` currents of the mitochondrial fraction were much higher than those of the rest fractions. The highest electrochemical activity of the mitochondrial fraction overlaps the determined highest COX activity.

The electrochemical activity of the sucrose density gradient fractions obtained from yeast cultivated in the presence of rotenone was also explored (Fig.3b). The current height of the observed peaks of the 15 %, 25 %, and 45 % fractions was 50 % lower in comparison with the same fractions without inhibitor. The 35 % (mitochondrial) fraction, however did not express the anodic peak at a potential of 0.3 V. As far as rotenone is a specific mitochondrial NADH:ubiquinone oxidoreductase inhibitor, the result reveals that this peak represents the activity of the oxidoreductase reaction [19]. The lack of anodic peak on the CV of the mitochondrial fraction coincided with the significantly decreased specific COX activity determined in the presence of rotenone.

CONCLUSION

The results obtained in this study offer a new fast approach for determination of the mitochondrial localization during fractionation. The conduction of cyclic voltammetry in the presence and absence of rotenone definitely shows the rearrangement of these organelles due to their maximal (without) and at the same time minimal (with inhibitor) redox activity. The cyclic voltammetry could be applied as an alternative method to the conventional enzymatic method for determination of cytochrome *c* oxidase activity.

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ПРИЛОЖЕНИЕ НА ЦИКЛИЧНАТА ВОЛТАМПЕРОМЕТРИЯ ЗА ОПРЕДЕЛЯНЕ НА МИТОХОНДРИАЛНА РЕДОКС АКТИВНОСТ ПО ВРЕМЕ НА ВЪТРЕКЛЕТЪЧНО ФРАКЦИОНИРАНЕ НА ДРОЖДИ КУЛТИВИРАНИ КАТО БИОКАТАЛИЗАТОРИ

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(Резюме)

В настоящата разработка е предложен алтернативен метод за определяне на митохондриалната локализация във фракции, получени чрез вътреклетъчно фракциониране на дрожди *Candida melibiosica* 2491, култивирани при поляризация. Електрохимичната активност на фракциите е сравнена с цитохром *c* оксидазната им активност в присъствие и отсъствие на ротенон.