

ARGINASE-BASED AMPEROMETRIC BIOSENSOR FOR MANGANESE IONS ANALYSIS

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Abstract

The development of simple cost-effective sensitive enzymatic methods for analysis of toxic metallic ions is an actual problem. Promising tools for elaboration of such methods are Mn^{2+} -dependent enzymes. A novel manganese(II)-sensitive amperometric bi-enzyme biosensor based on of recombinant human arginase I (arginase) isolated from the gene-engineered strain of methylotrophic yeast *Hansenula polymorpha* and commercial urease is described. The biosensing layer with urease and apo-enzyme of arginase was placed onto a polyaniline-Nafion composite platinum electrode. The developed sensor revealed a high sensitivity to Mn^{2+} -ions – 9200 ± 20 A/(M m²) with the apparent Michaelis-Menten constant derived from Mn^{2+} -ions calibration curve of 11.5 ± 1.0 μ M. A linear concentration range was observed from 1 μ M to 6,5 μ M $MnCl_2$, a limit of detection being of 0.15 μ M and a response time – 2.5 min. The proposed biosensor may be useful to monitor manganese compounds in laboratories of medicine, food industry and environmental control service.

Keywords: Amperometric biosensor, manganese(II) ion, polyaniline, arginase I, urease.

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1. Introduction

Manganese is considered as one of the most abundant element in the biosphere. It is widely distributed in soil, sediment, water, and live organisms [1, 2]. Although manganese is essential for humans and other species of the animal kingdom as well as for plants, it exhibits the high level of toxicity at higher concentrations. In human chronic manganese excess affects the central nervous system [3–8], with the symptoms resembling those of Parkinson's disease [9] and autism [10, 11]. It can also affect the ecosystem negatively, accumulating in the food chain. Thus to monitor manga-

nese compounds is actual problem for medicine, food industry and environmental control service. A majority of known chemical and physico-chemical methods of Mn^{2+} determination have a number of disadvantages such as a low sensitivity, high costs and complexity of the equipment [12–18]. So the development of simple cost-effective sensitive methods of quantitative analysis of metallic ions, including enzymatic ones, is the important task of analytical chemistry. Promising tools for elaboration of such methods are Mn^{2+} -dependent enzymes.

Arginase I (EC 3.5.3.1; L-arginine amidinohydrolase) is a manganese containing enzyme of the urea cycle. It catalyses the final cytosolic reaction of urea formation in the mammalian liver – the conversion of arginine to ornithine and urea. Arginase I (further – arginase) has recently been considered not only as a prospective pharmaceutical in enzymotherapy of some kinds of cancers auxotrophic for L-arginine (Arg) but also as an analytical instrument for Arg assay [19–23].

In our paper we demonstrate the possibility to use apoenzyme of Mn^{2+} -dependent arginase as a Mn^{2+} -recognizing bioelement of the corresponding amperometric biosensor. For this aim, the ammonium-selective PANi-Nafion film has been used in conjunction with urease-containing layer.

2. Material and methods

2. 1 Reagents

Urease (EC 3.5.1.5, type IX from Jack Beans, 26100 U·g⁻¹), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), sodium ethylenediaminetetraacetate (EDTA), $MnCl_2$, NaCl, aniline (99 %), 5 % Nafion® perfluorinated resin solution, chloroform and Butvar solution B-98 were purchased from Sigma-Aldrich. L-arginine was obtained from Merck (Darmstadt, Germany), cathodic polymer GY 83-0270 0005 was obtained from BASF Farben und Lacke, Germany. All buffers and standard solutions were prepared using the water purified by the Milli-Q system (Millipore).

2. 2 Apparatus

A three-electrode system was used for the electropolymerization of aniline and electrochemical studies. A piece of Pt wire and an Ag/AgCl/3M KCl electrode were used as the counter and reference electrodes, respectively. A platinum rod electrode (ALS Co., Tokyo, Japan) was applied as a working electrode (3.0 mm diameter). Amperometric measurements were carried out with a potentiostat CHI 1200A (IJ Cambria Scientific, Burry Port, UK) and performed in batch mode under continuous stirring in a standard 40 ml cell at room temperature.

Atomic Force Microscope (AFM-microanalyser Solver P47-PRO, NT-MDT, Netherlands) was used for morphological analyses of the PANi film on the surface of a Pt electrode.

2. 3 Arginase I

Human liver arginase I (further – arginase) was isolated from the recombinant yeast strain NCYC 495 – *pGAP1-HsARG1 (leu2car1 Sc:LEU2) Hansenula polymorpha* over-producing the enzyme, by column chromatography on the own-synthesized affinity sorbent as described in detail earlier [21, 23]. As a result, a 200-fold highly purified preparation of enzyme was obtained with a 11 % yield [23] and specific activity about 600 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein.

Apoenzyme of arginase (apo-arginase) devoid of manganese (II) ions was prepared by dialysis of the purified holoenzyme with protein concentration 0.1 mg/mL for 48 h against several changes of 50 mM Hepes-buffer, pH 8.0 (HB), containing 50 mM NaCl and 1 mM EDTA under stirring. EDTA was removed from the resulting apoenzyme solution by three dialysis steps against water, then – against HB.

2. 4 Mn^{2+} -sensitive bioelectrode construction and amperometric measurements

To construct a bi-enzymatic layer sensitive to Mn^{2+} urease and apo-arginase were dropped on the surface of the PANi-Nafion/Pt electrode and covered with a commercial cathodic polymer GY 83-0270 0005. The optimal enzymes' proportions were chosen in experiment. The calibration was performed by the stepwise addition of a standard analyte solution.

3. Results and discussion

3.1 Influence of metal ions on arginase activity

Native human liver arginase consists of 3 identical subunits with manganese (II) ions in active centre. It is worth mentioning that during the procedure of arginase' obtaining from yeast cells we did not add Mn^{2+} ions in buffers: neither under cells disruption nor during chromatographic fractionation of cell-free extract. Thus the obtained enzyme preparation may be named "semi-holoarginase". The purified recombinant holoarginase was shown to have optimal pH 9.0–9.5 and optimal temperature for its activity – 37–42 °C [23]. **Fig. 1** demonstrates the results of screening of metal ions as effectors of enzymatic activity. "Semi-holoarginase" was incubated in Hepes buffer, pH 7.5 (HB) in the presence of 1 mM or 10 mM metal ions during 10 min at 37 °C then enzymic activity was determined in standard conditions. As control (100 % of activity), enzyme with HB instead metal ions was used.

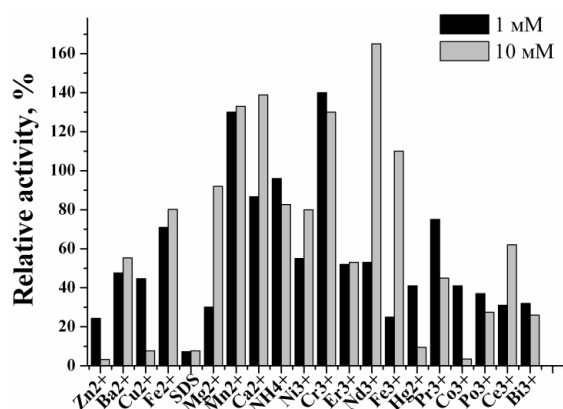


Fig. 1. The influence of metal ions on arginase activity

3.2 Apo-arginase reconstruction to holoenzyme

To appreciate the ability of enzyme for metal-binding reactions in biosensor analysis, apoenzyme of arginase (apo-arginase), obtained as described in 2.3, was used in experiments with Mn^{2+} . Characteristics of the process of arginase-holoenzyme reconstruction in the presence of Mn^{2+} -ions under different conditions of procedure are demonstrated in **Fig. 2**. It was shown that this process is strongly dependent on pH and temperature.

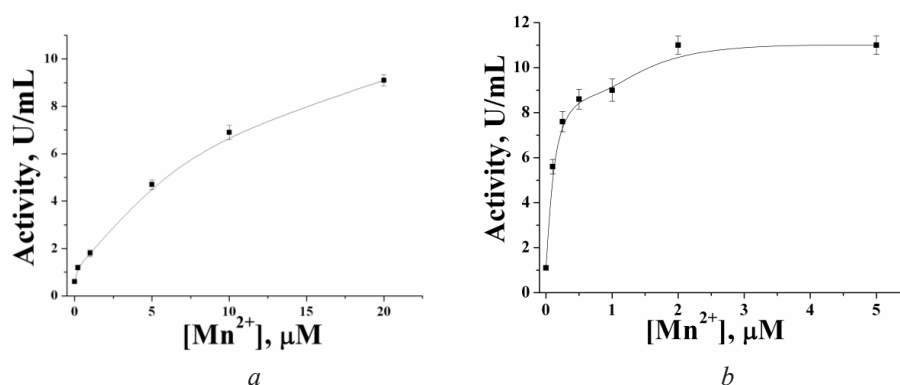


Fig. 2. The dependence of enzymatic activity on the concentration of Mn^{2+} and conditions of reconstruction: *a* – pH 7.5, 30 min, 37 °C; *b* – pH 9.5, 10 min, 56 °C

The results presented in **Fig. 2, b** demonstrate, that Mn^{2+} -ions begin to activate apo-arginase at low concentration (about 0.2 mkM) at optimal conditions for arginase activity: pH 9.5 and temperature 56 °C [23]. The highest enzymatic activity was shown to achieve at 2 μM Mn^{2+} in optimal conditions (**Fig. 2, b**) or at 20 μM Mn^{2+} at lower pH and temperature (**Fig. 2, a**). It is worth mentioning that holoenzyme could be reactivated in optimal conditions with a yield of 60 % compared to

an expected value. Thus the described experiments have proved the ability of apo-arginase to be a prospective manganese-sensitive enzyme in biosensor analysis.

3. 3 Construction and characterization of a manganese-sensitive biosensor

3. 3. 1 The main principle of bioelectrode construction

The apoenzyme of arginase (apo-arginase) was used as a Mn^{2+} -recognizing bioelement for construction of amperometric biosensor. For this aim the ammonium selective PANi-Nafion film has been used in conjunction with urease-containing layer. Binding of Mn^{2+} with the immobilized apo-arginase induces reconstruction of the holoenzyme (holo-arginase) followed by generation of ammonium ions from Arg in arginase-urease catalysed reactions. The resulting NH_4^+ -ions diffuse further to the PANi-Nafion film and trigger the reduction of PANi on the Pt electrode. The last product is monitored at the working potential of -200 mV. The schematic diagram of the bioselective membrane of amperometric biosensor is shown in **Fig. 3**.

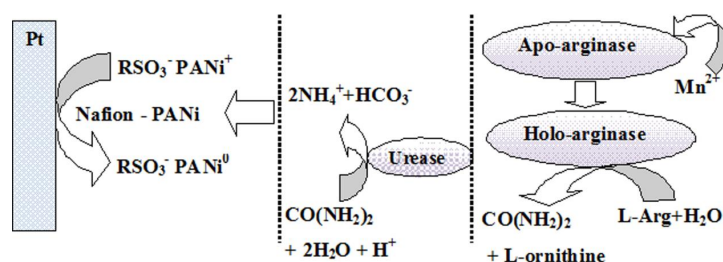


Fig. 3. Principal scheme of Mn^{2+} assay by the bi-enzyme PANi-Nafion/Pt electrode. PANi^+ and PANi^0 – oxidized and reduced forms of PANi, respectively; RSO_3^- – a skeleton of Nafion

3. 3. 2 The PANi-Nafion-modified Pt electrode

To construct the polyaniline (PANi)-based ammonium sensitive electrode the 3.0 mm Pt electrode was covered with Nafion as described in detail in our previous paper [22]. The PANi film was formed on the surface of Nafion/Pt rod electrode by 11 cycles of electrodeposition, the cation exchanged sulfonate groups of immobilized Nafion serve as a compensator of a negative charge generated during the anodic polymerization of PANi.

Electrochemical and morphological characteristics of the PANi film, formed by the potentiodynamic mode in the aniline-containing solution, as well as mechanisms of electron transfer in the proposed system, were described earlier [22]. Morphological properties presented in **Fig. 4** were shown to be typical for the PANi films.

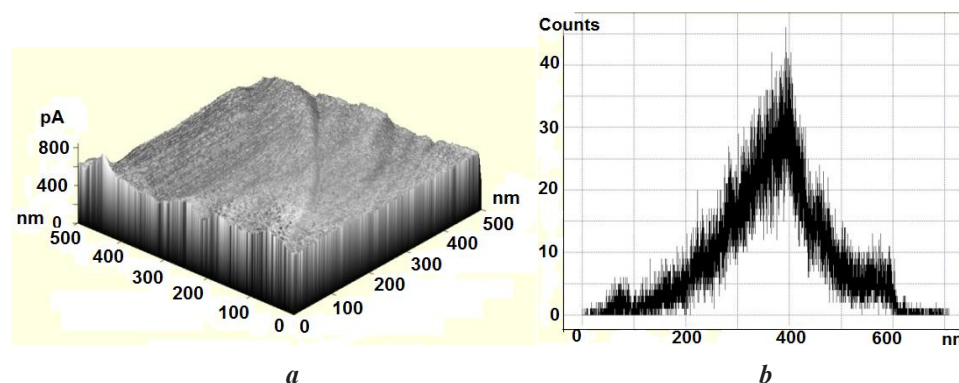


Fig. 4. Structure and thickness of the PANi film on the Pt electrode characterized with AFM. *a* – micrograph, resonance frequency – 160 kHz, scan rate of 1 Hz/s and resolution of 256×256 pixels; *b* – the Gaussian distribution by size

The surface of the Pt-rod electrode after 11 cycles of aniline electrodeposition became of a dark-green colour due to the PANi film formation. The morphology of the PANi film (**Fig. 4, a**)

was almost smooth and the structure of the surface was homogeneous. The Gaussian distribution by size from the AFM data (Fig. 4, b) proved the average thickness of the layer to be the 367 nm.

3. 3. 3 Mn^{2+} – sensitive bioelectrode

To construct a bi-enzymatic layer sensitive to Mn^{2+} urease and apo-arginase were dropped on the surface of the PANi-Nafion/Pt electrode and covered with a commercial cathodic polymer GY 83-0270 0005. The optimal enzymes' proportions were chosen empirically. The calibration was performed by the stepwise addition of a standard analyte solution. The corresponding chronoamperogram and calibration curve for Mn^{2+} biosensor are shown in Fig. 5.

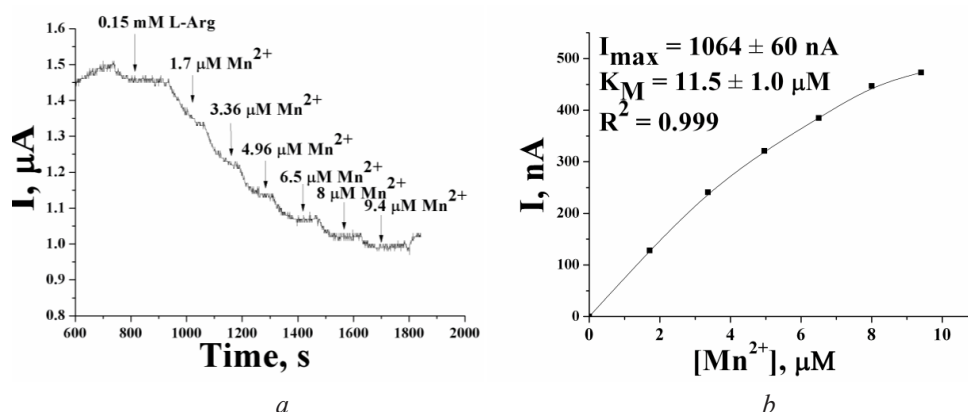


Fig. 5. Amperometric response on Mn^{2+} of the developed bi-enzyme PANi-Nafion/Pt electrode: chronoamperogram (a) and calibration curve (b).
Conditions: 200 mV vs Ag/AgCl electrode, pH 10.5 at 22 °C

An amperometric response on $MnCl_2$ of the developed bi-enzyme electrode was tested in the range of L-Arg concentration from 1 to 10 μM (Fig. 5, a). The dynamic range was linear between 1 μM to 6.5 μM $MnCl_2$. The K_M^{app} derived from the calibration curve was shown to be 11.5 ± 1.0 μM for Mn^{2+} . The maximal detected signal was found as 1064 ± 60 nA (Fig. 5, b). The sensitivity was calculated as 9200 ± 20 A/(M \cdot m 2), and LOD is of 0.15 μM . The full reconstruction time of arginase holoenzyme immobilized on the top of working electrode was about 2.5 min that highly correlates with the results of enzyme reconstruction in solution (2 min). The prolonged time of reconstruction of immobilized arginase can be explained by difficulties for diffusion of Mn^{2+} -ions through polymer film. So the obtained results clearly prove a possibility of using the apoenzyme of human liver arginase I for construction of Mn^{2+} -sensitive biosensor and analysis of Mn^{2+} in real samples.

4. Conclusions

A novel manganese(II)-sensitive amperometric bi-enzyme biosensor based on of recombinant human arginase I (arginase) isolated from the recombinant yeast *Hansenula polymorpha* and commercial urease was proposed. The biosensing layer (urease and apo-arginase) was placed onto a polyaniline-Nafion composite platinum electrode. The developed sensor revealed a high sensitivity to Mn^{2+} -ions and a low detection limit.

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STORAGE OF ACTIVITY OF THE HUMAN CHORIONIC GONADOTROPIN HORMONE IN SOLUTION AT ADDITION OF ORGANIC COMPOUNDS TO THE PHARMACOLOGICAL COMPOSITION

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