An Amperometric Xanthine Oxidase Enzyme Electrode Based on Hydrogen Peroxide Electroreduction

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Z. Naturforsch. **57c,** 883–889 (2002); received April 22/May 29, 2002

Xanthine Oxidase, Enzyme Electrode, Hydrogen Peroxide Electroreduction

A xanthine oxidase enzyme electrode (*xanthine oxidase immobilized on electrochemically modified graphite and conveniently coated with gelatine electrode working surface*) for quantitative analysis of xanthine is proposed. The detection of thus developed electrochemical system is based on the electroreduction of hydrogen peroxide generated in enzyme layer and offered L-ascorbic and uric acid reducing interference effect on the substrate determination. At a working potential –50 mV (vs. Ag/AgCl) the detection limit of 4.5 μM and the linearity of the amperometric signal up to substrate concentration of about 40 μM were found. At that working potential, the electrode is practically inert towards L-ascorbic- and uric acid present. The response time did not exceed 2 min.

Introduction

As products of nucleic acids degradation that could also undergo xanthine oxidase- catalyzed oxidation with molecular oxygen leading to the formation of H_2O_2 and uric acid, xanthine and hypoxanthine attracted much attention as indicators for estimating meat or fish freshness. Since both reaction products are electrochemically active, a variety of amperometric biosensors for xanthine/hypoxanthine analysis, based on electrooxidation of the uric acid (Lorenzo *et al.*, 1991; Kilinc *et al.*, 1998) or hydrogen peroxide formed (Horozova *et al.*, 2000; Niu and Lee, 2000; Mao *et al.*, 2001) have been proposed. It is the high potential applied on the working electrode, which makes these biosensors responsive to interfering substances. To avoid this disadvantage either elimination of electrochemically-active interferents by pre-treatment of the sample solutions or lowering of the applied potential were used. As reviewed by Gorton (1995) and described by Zhao (Zhao and Luong, 1994) use of artificial electron acceptors – mediators, permit a reaction control at potentials lower than these required for hydrogen peroxide or uric acid electrooxidation. For development of a xanthine biosensor with improved selectivity the unique ability of xanthine oxidase was used to generate a superoxide radical (Doblhoff-Dier and Rechnitz, 1989) and the detection was performed before its spontaneous dismutation occurred at a working potential of 50 mV. A further approach proposed is the detection of oxygen consumption (Hu and Liu, 1997; Okuma and Watanabe, 2002) but the electrochemical process in these cases takes place at high cathodic working potentials.

In contrast, enzyme electrodes based on H_2O_2 electroreduction offer the advantage of performing this transformation at applied potentials near 0 V, where a large variety of substances and typical components of biological fluids (*such as* L-*ascorbic acid, uric acid, glutathione, etc.*) exhibit no electrochemical activity. Unfortunately, general scope and feasibility of this approach remain obscure because of its still limited applications for only glucose monitoring (Jonsson-Pettersson, 1991; Gamburzev *et al.*, 1997; Celej and Rivas, 1998; Cosnier *et al.*, 1999; Cosnier *et al.*, 2000).

As a part of our investigations focusing on amperometric enzyme electrodes design (Horozova *et al.*, 2000), we now describe development and characterization of a biosensor with a XOD-immobilized on modified graphite electrode as related to a hydrogen peroxide electroreduction-based detection, for quantitative determination of xanthine.

0939–5075/2002/0900–0883 $ 06.00

Experimental

Materials

Xanthine oxidase (XOD) (E. C. 1.1.3.2) – from *buttermilk* (Fluka-Bio Chemika), with activity of 0.39 U × mg^{-1} (1 U corresponds to the amount of enzyme which oxidizes 1 μmol xanthine per min at pH 7.8 and 30° C). Xanthine ($C_5H_4N_4O_2$); hydrogen peroxide (H_2O_2) and chemicals for buffer solutions preparation: $Na_2HPO_4 \times 12H_2O$, citric acid, KOH, H_3PO_4, were purchased from Fluka and used as received. Gelatine-analytical grade (Chimtek, Dimitrovgrad, Bulgaria) was employed as a 5% suspension in phosphate-citrate buffer (pH = 8.4) for electrode coating formation. All solutions were prepared with bidistilled water.

Inert pads of graphite "GMZ"™ with geometric surface S = 1.6–1.8 cm^2 (0.7 × 0.7 × 0.3 cm) were used. The structural characteristics of graphite are as follows: specific surface 0.8 $cm^2 \times g^{-1}$, density 1.56–1.70 g × cm^{-3} and porosity 20–25%. Prof. Bogdanovskiy, the State University of Moscow, Russia, kindly provided the graphite pads.

Preparation of the electrodes

The enzyme electrode was prepared on the basis of a modified graphite electrode which catalyses hydrogen peroxide electroreduction. The graphite lamellae were modified with microquantities of (Pt + Pd). The catalytically active components were deposited in a potentiostatic regime ($E_r^{deposit}$ = + 0.05 V vs. reversible hydrogen electrode) by a brief electrolysis ($t_{deposit}$ = 10 s) from the following electrolyte: 2% $H_2PtCl_6 \times 6H_2O$ + 2% $PdCl_2$ + 0.1 M HCl in the ratio (Pt + Pd) (10:90%) (Horozova *et al.*, 1997).

Enzyme immobilization

Three different procedures for XOD immobilization on modified graphite electrode were investigated:

I. a 50 μl drop of XOD solution (with the enzyme concentration of 10^{-5} M) was deposited onto electrode surface and was allowed to dry at room temperature. Then the working surface was covered with two layers of gelatine;
II. two layers of xanthine oxidase suspended in gelatin (5 mg XOD in 1 ml 5%-gelatine solution at 37° C) were applied onto electrode surface and dried under argon; and
III. XOD adsorbed on electrochemically activated modified graphite electrode and then covered with two layers of XOD suspension in gelatine (5 mg XOD in 1 ml 5%-gelatine solution at 37° C).

The electrochemical pretreatment of the modified graphite electrode was a cathode-anode cyclization (30 min) within the potential range of –0.60 to +0.35 V (vs. Ag/AgCl). Just before immobilization, the graphite electrode was polarized for 2 min at E = 1.5 V. The adsorption of XOD was carried out under static conditions by immersing the electrochemically pretreated electrode in the enzyme solution with a 10^{-5} M concentration, in phosphate buffer (pH = 8.4) for 24 h at 4 °C. After adsorption the electrode was dried in air, at room temperature, for ~ 45 min. Then the working surface was coated with 2 layers of 5% gelatine solution containing XOD. After applying each layer, the electrode surface was dried under argon.

After the measurements the enzyme electrodes were carefully washed with bidistilled water, dried in air at room temperature for ~ 30 min and then stored in refrigerator at 4° C until next measurements. In case of necessity the immobilized enzyme can be removed from the electrode surface by treating of the electrode for ~ 20 min in hot bidistilled water (50–60° C) regenerating the uncovered modified graphite electrodes. Thus the processed electrode material can be stored more than one year in bidistilled water (at room temperature) and used repeatedly.

Apparatus and measurements

All electrochemical measurements were performed in a three-electrode water-jacketed glass cell with separated anode and cathode compartments (working volume 12 ml). An Ag/AgCl electrode was used as a reference electrode, and platinum wire – as a counter electrode. The electrochemical setup also involved a bipotentiostat, type BiPAD (TACUSSEL, Villeurbanne, France); a generator, type EG-20 (Elpan, Lubawa, Poland); a digital voltmeter, type 1AB105 (ZPU, Pravets, Bulgaria). The solutions were stirred by bubbling with argon during the measurements. The biosensor was characterized by the polarization curves' method in potentiostatic regime (background electrolyte: phosphate pH = 8.4).

The experimental points were obtained by consecutive addition of 10^{-3} M xanthine solution to the buffer in the cell with simultaneous registration of the current. The time to reach a steady-state value of the current did not exceed 2 min. For maintaining constant temperature a thermostat UH (VEB MLW Prüfgeräte-Werk, Medingen, Germany) was used.

Results and Discussion

Study of modified graphite electrode at hydrogen peroxide and xanthine present

The polarization curve of modified graphite electrode in hydrogen peroxide solution (Fig. 1, closed circles) indicates that within the potential range from −50 to 150 mV the cathodic current reached a limited value. The amperometric response is due to the H_2O_2 electroreduction at pH = 8.4 according to the equation: $H_2O_2 + 2e\text{-} \xrightarrow{\text{Electrode}} 2\ OH^-$.

The electrode response was found to depend linearly on hydrogen peroxide concentration up to approx. 300 μM at working potentials −50, 0 and 50 mV, while at $E = 100$ and $E = 150$ mV the linear portion was up to about 200 and about 170 μM, respectively (not shown).

To exclude electrochemical non-enzymatic transformation of the analyte of interest-xanthine, the current-potential dependencies of modified graphite electrode recorded in the background electrolyte (Fig. 1; triangles) and in the xanthine solution (Fig. 1; open circles) were investigated. Within the potential range from −100 to 200 mV the polarization curves in both cases were practically identical which proves that no electrochemical process is observed on modified graphite in xanthine solution.

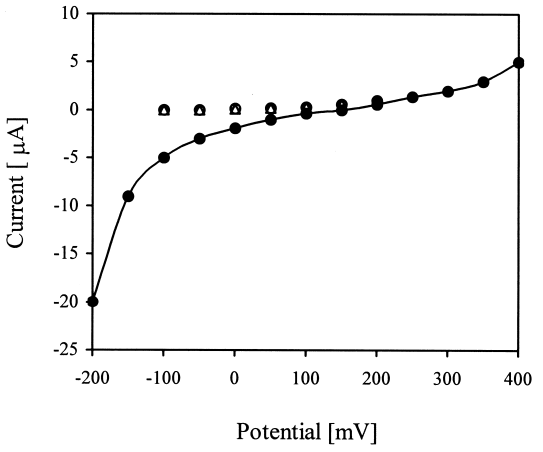

Fig. 1. Polarization curves of graphite electrode modified with microquantities of Pt + Pd (10 + 90%) in: 0.1 mM hydrogen peroxide (closed circles); background electrolyte-phosphate buffer, pH = 8.4 (triangles); and 1×10^{-5} M xanthine solution (open circles); temperature 20° C.

Enzyme electrode based on modified graphite. Optimization of enzyme immobilization

Generally, the enzyme loading affects the sensitivity of enzyme electrode. Increasing amounts of immobilized XOD (using the immobilization procedures I, II and III described in Experimental section) cause improved electrode sensitivity and an expanded linear range of the electrode response as a function of substrate concentration was detected. The dependency of the enzyme electrode response on xanthine concentration at $E = -50$ mV showed that higher sensitivity (determined as the slope of the linear portion, dI/dC) was registered using the third procedure for XOD immobilization:

- Sensitivity of 0.044 μA × μM^{-1} and linearity up to ~ 20 μM were obtained using the immobilization procedure I;
- Sensitivity of 0.119 μA × μM^{-1} and linearity up to ~ 30 μM were obtained with enzyme electrode prepared according to the immobilization procedure II;
- Sensitivity of 0.210 μA × μM^{-1} and linearity up to ~ 40 μM were obtained immobilizing XOD on the electrode using procedure III.

The last procedure was optimized by adsorbing the enzyme for 1, 2, 24 and 48 h. Preliminary experiments showed no substantial increase of the electrode sensitivity and linear portion of the electrode response as a function of adsorption time. Low stability of the immobilized biocatalyst was detected for the electrodes obtained by a one- and 2-h adsorption of XOD. In this case the enzyme electrode was inactivated after 5 working h. Increasing the time for enzyme adsorption from 24 to 48 h did effect neither electrode sensitivity and the linear portion of the response, nor the stability of immobilized xanthine oxidase. All further results reported were realized with an enzyme electrode obtained by procedure III of XOD immobilization, carrying out the enzyme adsorption for 24 h.

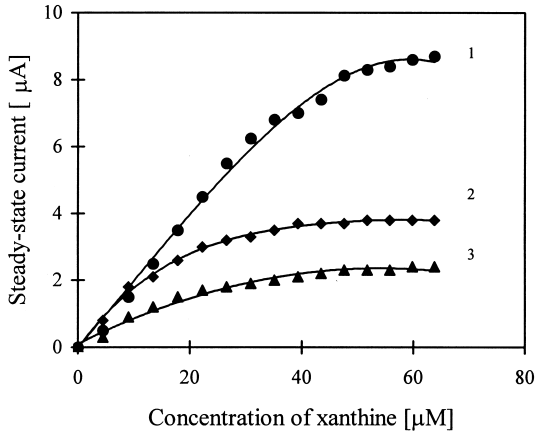

Fig. 2. Dependence of the enzyme electrode response on xanthine concentration; working potential: 1) −50 mV; 2) 0 mV; 3) 50 mV; temperature 23° C.

Operational characteristics of the enzyme electrode. Temperature effects

To determine the working potential of the enzyme electrode the dependency of the steady-state current on substrate concentration was studied within the potential range from $E = -50$ mV to 50 mV (Fig. 2). The highest electrode sensitivity as well as the widest linear portion of the response as a function of xanthine concentration was registered at $E = -50$ mV (curve 1). A significant decrease of the electrode sensitivity and a smaller linear portion of relationship than those detected at potential −50 mV (curve 1) were observed at higher working potentials ($E = 0$ mV, curve 2; $E = 50$ mV, curve 3). The values of the apparent Michaelis constants (calculated from the electrochemical Eadie-Hofstee plot) at different applied potentials are given in Table I. Both, the apparent Michaelis constant (representing approx. the linear part of the electrode response) and the saturated current (measured at high substrate concentrations) decreased as a function of the potential applied. The electrode sensitivities registered with the XOD-electrode in substrate solutions were practically identical with those detected in H_2O_2 solutions on bare modified graphite, covered with gelatin under the same experimental conditions (temperature and working potential). This finding indicates that most probably the amperometric response is due to hydrogen peroxide electroreduction.

As optimal working potential $E = -50$ mV was selected because of highest sensitivity and widest linearity of the electrode response depending on substrate concentration. The detection limit of 4.5 μM at the optimal working potential was determined at a signal to noise ratio 3:1.

The calibration graph of the electrode for xanthine (background current subtracted versus concentration) at working potential $E = -50$ mV, and the corresponding Eadie-Hofstee plot are given at Fig. 3. The electrode response increased linearly as a function of substrate concentration up to about 40 μM, and reached a constant value (saturation) at xanthine concentrations exceeding 60 μM. The values of the steady-state current from Fig. 3-a are presented in Fig. 3-b (the electrochemical Eadie-Hofstee plot) as a function of electrode sensitivity (determined as the ratio of the steady-state current divided by the substrate concentration which it is measured). The electrode sensitivity remained practically constant at xanthine concentrations up to ~ 30 μM. Probably this vertical region implies a diffusion control over the electrochemical process. The sloping region indicates that within the concen-

Table I. Operational and regression parameters of xanthine oxidase electrode, determined in xanthine solution at different working potentials; background electrolyte: phosphate buffer, pH = 8.4; temperature: 23° C.

Potential [mV]	Regression equation*	K_M^{app} [μM]	Saturated current [μA]	Sensitivity** [μA × μM^{-1}]	r^2
−50	$Y = -29.73X + 13.43$	30 ± 3.0	9	0.210	0.98_7
0	$Y = -22.174X + 5.77$	22 ± 1.9	3.6	0.106	0.98_4
50	$Y = -21.524X + 3.25$	20 ± 1.8	2.4	0.058	0.97_1

* The regression analysis of experimental results was performed for the sloped region of the Eadie-Hofstee plot, coordinates: $X = (I_S - I_0)/C_{xanth}$ and $Y = (I_S - I_0)$.

** The sensitivity was determined as the slope of the linear part of the electrode response as a function of xanthine concentration.

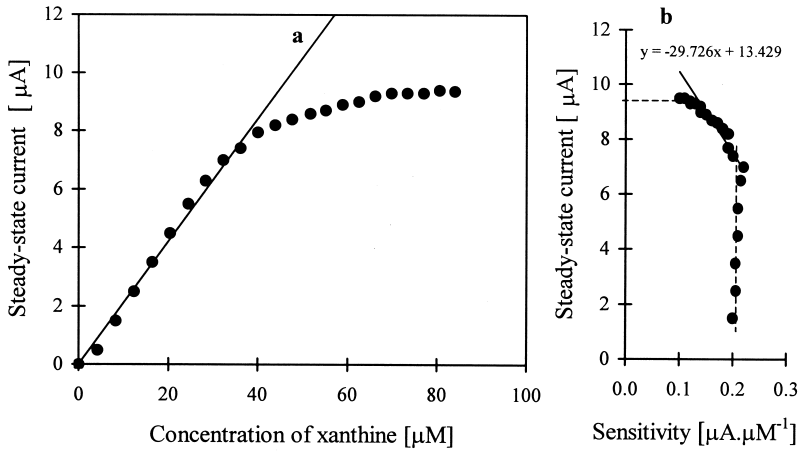

Fig. 3. Steady-state current (background subtracted) of the biosensor (at −50 mV vs. Ag/AgCl): (a) as a function of xanthine concentration; (b) as a function of the electrode sensitivity (background subtracted steady-state current divided by xanthine concentration at which it is measured); working temperature: 23° C.

tration range from 30 to approx. 60 μM the reaction is controlled by enzyme kinetics. The apparent Michaelis-Menten constant K_M^{app} = 30 ± 3 μM was calculated by regression analysis from experimental data for the sloping region. The horizontal region at Fig. 3-b observed at high xanthine concentrations is probably due to substrate saturation of the immobilized enzyme (saturated current). An analogous electrode behavior was detected at potentials E = 0 mV and E = 50 mV: diffusion-, activation-limited and substrate-saturated regions were observed on the electrochemical Eadie-Hofstee plot.

The temperature effect on the electrode response was studied at the working potential E = −50 mV within the temperature range from 15 to 30° C. The dependence of the steady-state current on substrate concentration remained linear within the temperature range investigated, and the electrode sensitivity (determined as the slope of the linear portion, dI/dC) increased as a function of temperature (Table II). The value of the apparent Michaelis constant determined at 30° C (K_M^{app} = 14 ± 1.5 μM) was approximately two times lower than that obtained at 25 °C. This result may be explained with decreased solubility of oxygen in the buffer solution at the higher working temperature. On the basis of the results discussed as working temperature interval we preferred 20 to 25° C at pH = 8.4.

Table II. XOD-enzyme electrode sensitivity registered in xanthine solution at different temperatures; working potential E = −50 mV (Ag/AgCl); background electrolyte: phosphate buffer, pH = 8.4.

Temperature [°C]	15	20	25	30
Electrode sensitivity [μA × μM^{-1}]	0.18	0.21	0.30	0.45

Electrode response in presence of interfering substances

The influence of interfering substances such as L-ascorbic acid and uric acid normally presented in biological samples on electrode performance was studied at a working potential E = −50 mV. The values of steady-state current (background subtracted) obtained in L-ascorbic acid and uric acid solutions were compared with these obtained in substrate solutions at the same experimental conditions (Fig 4). No current increase is observed with increasing L-ascorbic acid concentration (Fig. 4a). Increasing the concentration of uric acid (Fig 4b) under the same working conditions insignificant anodic currents were detected at E = −50 mV. At potentials higher than 0 mV both in L-ascorbic and uric acid solutions anodic currents were registered that increased as a function of its concentration.

Stability of the enzyme electrode

The lifetime of an enzyme electrode is an important characteristic concerning biosensor development. The stability of xanthine oxidase enzyme electrode was monitored during the period of approximately 500 h. The results obtained indicated that the electrode response decreased to

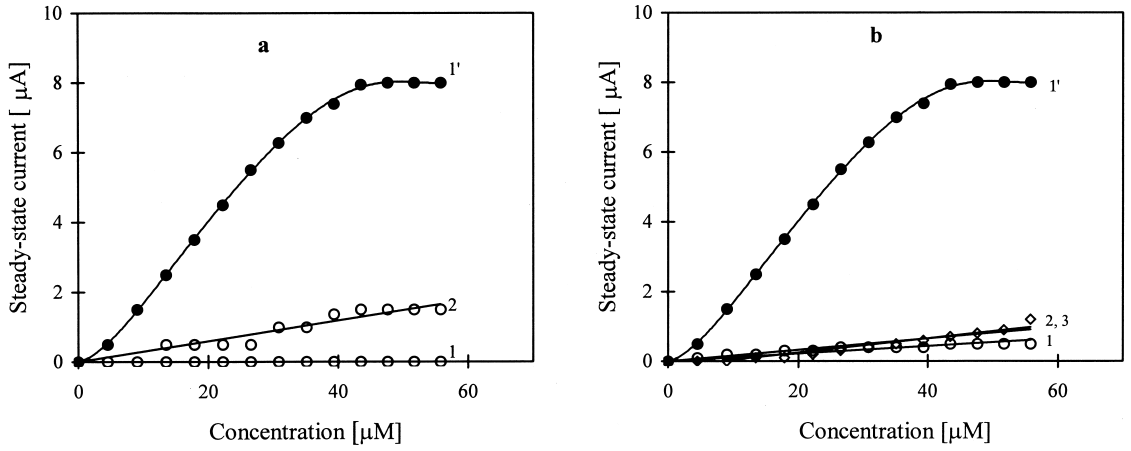

Fig. 4. Dependence of the steady-state current (background subtracted) of the enzyme electrode on concentrations of:
a) xanthine (1′) and L-ascorbic acid (1 and 2); working potentials: −50 mV (1, 1′); 0 mV (2);
b) xanthine (1′) and uric acid (1–3); working potentials: −50 mV (1, 1′); 0 mV (2); 50 mV (3). Background electrolyte: phosphate buffer (pH = 8.4); temperature 23° C.

about 35% of its initially registered value after 30 hours and remained practically unchanged during the next 470 h. Some authors noted that even immobilized xanthine oxidase rapidly looses its catalytic activity and recommend its stabilization with EDTA (Zhao and Luong, 1994). Stabilization of the XOD solutions before enzyme immobilization on the electrode surface probably will improve the long-term stability of the enzyme electrodes.

Acknowledgment

Financial support of this research from the University of Plovdiv Research Fund is appreciated.

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