Study of Xanthine Oxidase Immobilized Electrode Based on Modified Graphite

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Xanthine oxidase (E. C. 1.2.3.2) was immobilized by adsorption on electrochemically modified graphite plate to obtain an enzyme electrode. The current of the enzyme electrode in substrate (xanthine) solutions was found to be a result of the electrooxidation of H_2O_2 generated in the enzyme layer. The linearity of the amperometric signal was up to a substrate concentration of 65 μM at 0.6 V (vs. Ag/AgCl). The response time was 2 minutes. The enzyme electrode preserves 80% of its initial activity after a three-week storage in air at room temperature.

Introduction

Xanthine oxidase (XOD) is referred to the group of metal-containing flavoproteins (Ganelin and Lvov, 1994; Rubin and Ladiguina, 1974). Two molecules of flavin adenine dinucleotide (FAD) and two atoms of molybdenum are bound forming its prostethic group. Furthermore, eight atoms of nonheme iron are also bound to the molecule of xanthine oxidase. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid in presence of molecular oxygen.

$$Xanthine + H_2O + O_2 \xrightarrow{XOD} Urate + H_2O_2$$

The enzyme also catalyses the oxidation of other purines, pteridines and aldehydes. By oxidation of these substrates xanthine oxidase can transfer electrons and hydrogen not only to O_2 but also to other acceptors.

In biocatalytic and electrochemical systems the enzyme is generally used in immobilized state. The biocatalytic process of oxidation of the substrates of this enzyme was carried out on a glassy carbon electrode modified with redox polymers and *p*-tetracyanoquinodimethane (TCNQ) (Kulys and Razumas, 1983; Cenas *et al.*, 1984). An enzyme-substrate system with xanthine oxidase based on conducting organic salts was described (Turner *et al.*, 1987; Albery and Knovoles, 1987). In this system the membrane electrode is sensitive to the increase of xanthine concentration.

In electrochemical systems xanthine oxidase is used in amperometric biosensors for determination of various substrates. A membraneless amperometric biosensor for hypoxanthine, based on immobilized xanthine oxidase, conducting organic salts and silicon oil was described (Korell and Spichiger, 1994). An amperometric biosensor for hypoxanthine, xanthine and phosphates based on deflavo xanthine oxidase and 1,1′-dimethylferricinium redox mediator was reported (Zhao and Luong, 1994). Xanthine oxidase and peroxidase, both immobilized on glassy carbon electrodes (Kulys *et al.*, 1983) were used to determine hypoxanthine and uric acid.

Both xanthine and hypoxanthine are important indicator compounds for determination of food freshness. They can be monitored through enzymatic oxidation which produces H_2O_2 and uric acid. For that purpose xanthine oxidase was immobilized on spectroscopically pure graphite (Lorenzo *et al.*, 1991), on graphite soot (Martin and Rechnitz, 1990) and on carbon paste (Doblhoff-Dier and Rechnitz, 1989). The enzymatic reaction in these studies was followed by electrochemical oxidation of the uric acid formed. The reaction can also be followed by use of mediators allowing a control at +0.3 V (Gorton, 1995). A sensor for determination of allopurinol, an inhibitor of xanthine oxidase, was described (Martin and Rechnitz, 1990).

XOD is also used in co-immobilization with other enzymes. XOD and peroxidase, together

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with a mediator – ferrocene – were co-immobilized in an electrode matrix of teflonized graphite (Cayela *et al.*, 1998). The bienzyme amperometric sensor was used for determination of hypoxanthine in fish samples. Bienzyme electrode of XOD and nucleoside phosphorylase co-immobilized on glassy carbon coated with polymer film of Nafion™, was also used for determination of hypoxanthine and inosine in the presence of phosphates (Hu and Liu, 1997). The same authors (Hu and Liu, 1997) reported an amperometric biosensor for hypoxanthine detection, developed on the basis of a chemically modified glassy carbon electrode, coated with a film of Nafion™. It determines the oxygen consumption in the enzyme reaction catalyzed by the xanthine oxidase immobilized on the electrode.

The objective of the present work is to prepare and characterize an enzyme electrode, based on electrochemically modified graphite and to examine the possibility for quantitative determination of xanthine.

Materials and Methods

Xanthine oxidase (XOD) (E. C. 1.2.3.2) – from milk (Fluka Bio-Chemika), with activity of 0.39 $U\times mg^{-1}$ and M_r = 275 000; xanthine ($C_5H_4N_4O_2$); hydrogen peroxide (H_2O_2); for preparing buffer solutions: $Na_2HPO_4\times 12H_2O$, KOH, H_3PO_4, purchased from Fluka-Chemika; and gelatin (Chimtec – Bulgaria). All chemicals were used without further purification. All solutions were prepared with double distilled water.

Inert pads of graphite "GMZ"™ with a geometric surface of 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 $g\times cm^3$ and porosity 20–25%. The graphite plates were kindly provided by Prof. G. Bogdanovskiy, The State University of Moscow, Russia.

The enzyme electrode was prepared on the basis of a modified graphite electrode which catalyses the electrooxidation of hydrogen peroxide. The graphite pads were modified with microquantities of (Pt + Pd). The catalytically active components were deposited by a potentiostatic regime ($E_r^{deposit}$ = +0.05 V vs. reversible hydrogen electrode) of a brief electrolysis ($t_{deposit}$ = 10 s) using the following electrolyte: 2% $PtCl_6.6H_2O$ + 2% $PdCl_2$ + 0.1 M HCl in the ratio (Pt+Pd) (10:90%) (Horozova *et al.*, 1997). XOD is adsorbed on the electrochemically activated, modified graphite electrode. The electrochemical pretreatment of the modified graphite electrode was a cathode-anode cyclization (30 min) in 0.1 M phosphate buffer (pH=8.4); potential range of −0.58 – +0.35 V (vs. Ag/AgCl). Just before immobilization, the graphite electrode was polarized for 2 min at E = 1.5 V in the same buffer solution. The adsorption of XOD was carried out by immersing the graphite electrode in the solution of the enzyme with a 10^{-5} M concentration, in 0.1 M phosphate buffer (pH = 8.4) for 60 min. After the adsorption the electrode was dried in air at room temperature for 45 min. The working surface of the enzyme electrode was coated with three layers of gelatin (30 $mg\times ml^{-1}$) to prevent desorption of the enzyme. After application of each layer, the electrode surface was dried with argon.

All electrochemical measurements were performed in a three-electrode cell with separated anode and cathode compartments. 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 (Priborostroitelen zavod, Pravets, Bulgaria). The solutions were

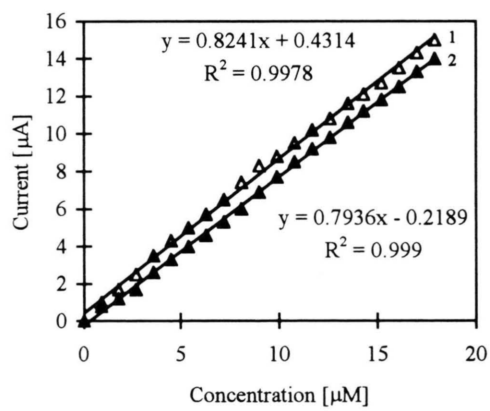


Fig. 1. Dependence of the steady-state current on the concentration of H_2O_2 (curve 1) and the concentration of xanthine (curve 2) at E = 0.6 V (vs. Ag/AgCl). Electrodes: 1) Modified graphite without adsorbed enzyme, covered with gelatin; 2) Enzyme electrode covered with gelatin.

purged with argon during the measurements. The electrode was characterized by the polarization curves method in a potentiostatic regime (0.1 M phosphate buffer, pH = 8.4).

For maintaining constant temperature a thermostat UH (VEB MLW Prüfgeräte Werk, Medingen, Freital, Germany) was used. Xanthine determination was performed spectrophotometrically at λ = 275 nm.

Results and Discussion

Fig. 1 shows the dependence of the background subtracted steady current of the enzyme electrode on the concentration of xanthine (curve 2) at a potential of +0.6 V and at room temperature. Comparing the oxidation rate of H_2O_2 on the modified graphite electrode without adsorbed enzyme (curve 1) and the current values of the enzyme electrode for the substrate (curve 2), can be concluded that the amperometric signal of the electrode is caused by the electrooxidation of H_2O_2 formed in the enzyme-catalytic oxidation of xanthine. Previous electrochemical examination of XOD immobilized on carbon materials showed that in the presence of xanthine the current is due to the electrooxidation of H_2O_2 generated in the enzymatic layer. In addition, the rate of the electrooxidation at 0.6 V with 18 μM xanthine on naked modified graphite was 3.5 μA, while under the same conditions the electrooxidation rate of H_2O_2 was 16 μA.

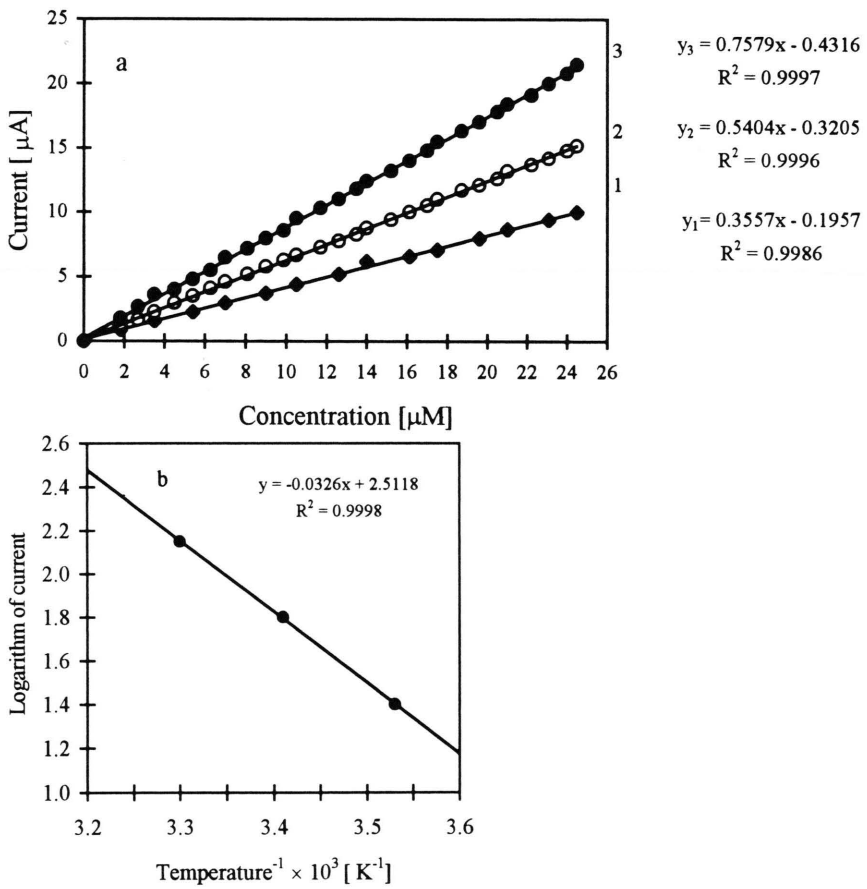


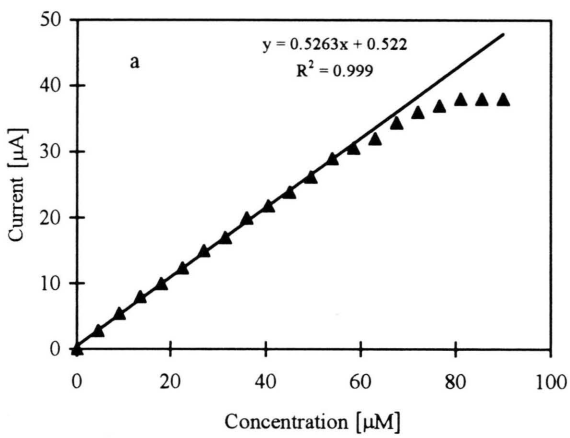
Fig. 2. (a) Dependence of the steady-state current of enzyme electrode on the concentration of the substrate at various temperatures, K: 1) 283; 2) 293; 3) 303.
(b) Relationship logarithm of the steady-state current – temperature^{-1} ($\ln I_S$-1/T) for the same electrode at a potential E = 0.6 V. Concentration of xanthine – 10 μM.

To determine the limiting step, the effect of temperature on the rate of the multistep enzymatic-electrochemical process was studied at 0.6 V. The electrode response remained linear for the three temperatures (T = 283, 293 and 303 K) (Fig. 2a), and the sensitivity ($\partial I/\partial c$) of the electrode increased with temperature increase. The relationship between the current of the enzyme electrode and the temperature is presented in Arrhenius coordinates in Fig. 2b. The graphically determined value for the activation energy $E_a = 26 \pm 1$ kJ×mol^{-1} is very close to E_a of the electrooxidation of H_2O_2 (E_a = 23 kJ×mol^{-1}) on modified graphite electrode with no enzyme adsorbed at 0.6 V (Horozova *et al.*, 1997). This fact indicates that the adsorption immobilization of XOD on electrode surface does not change substantially the mechanism of the electrochemical process and probably the electrooxidation of H_2O_2 is the limiting factor.

Figure 3a presents the steady-state current of the above-described enzyme electrode as a function of the xanthine concentration at a constant potential +0.6 V versus Ag/AgCl. The steady state current of the electrode increased linearly with the increase of the substrate concentration up to a concentration of 65 µM, and reached a constant value at xanthine concentration above 80 µM. The experimental points were obtained by consecutive addition of portions of 10^{-3} M xanthine solution to the 0.1 M phosphate 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.

The values of the steady-state current from Fig. 3a are presented in Fig. 3b as a function of the ratio between the steady-state current and the xanthine concentration at which it is measured (Eadie-Hofstee plot). This ratio expresses the sensitivity of the enzyme electrode. It is seen that the sensitivity of the enzyme electrode remained practically constant up to 45 µM xanthine. This shows that a diffusion control of the process in the enzyme electrode dominates in this range of substrate concentrations. The sloping region indicates that in the concentration range from 45 to 80 µM the reaction is controlled by enzyme kinetics and the value calculated of the apparent Michaelis-Menten constant for this region is K_M^{app} = 3.12×10^{-4} M. The horizontal region of the Eadie-Hofstee plot observed at high xanthine concentrations is probably connected with a substrate saturation of enzyme in the electrode.

The linear course of the concentration dependence of steady-state current also remained in the potential range E = 0.55 to E = 0.70 V (Fig. 4).



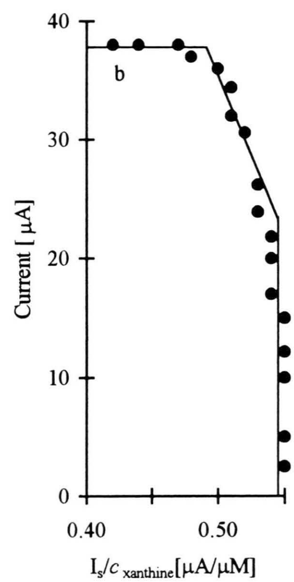


Fig. 3. Steady-state current of the enzyme electrode (at +0.6 V vs. Ag/AgCl): (a) as a function of xanthine concentration; (b) as a function of the sensitivity of the electrode; working temperature: 299 K.

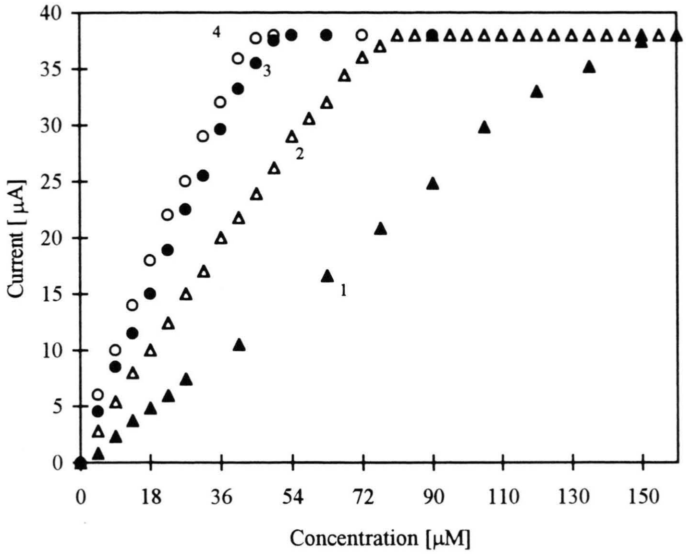


Fig. 4. Dependence of the steady-state current on the concentration of the substrate at various potentials: curve 1- at 0.55 V; curve 2- at 0.60 V; curve 3- at 0.65 V; curve 4 -at 0.70 V.

With the increase in the potential applied to the working electrode there was an increase in the slope of the linear range of the relationship $I_S = f(c)$. On the other hand, a higher potential applied led to a shorter linear range of the concentration dependence of the current (Table I). This finding can be explained with the polarization curves of electrooxidation of H_2O_2 on modified graphite (Fig. 5). From the figure it is seen that within the potential range from 0.45 to 0.70 V (vs. Ag/AgCl) the electrooxidation rate is in the limiting current region.

At potentials $E \leq 0.50$ V, the increase in the concentration of the substrate – xanthine cause an insignificant current change. At potentials $E \geq 0.70$ V the background current is strongly increased which is probably due to some parallel electrolysis processes taking place in the background electrolyte, or to activation of some oxygen-containing groups on the electrode surface.

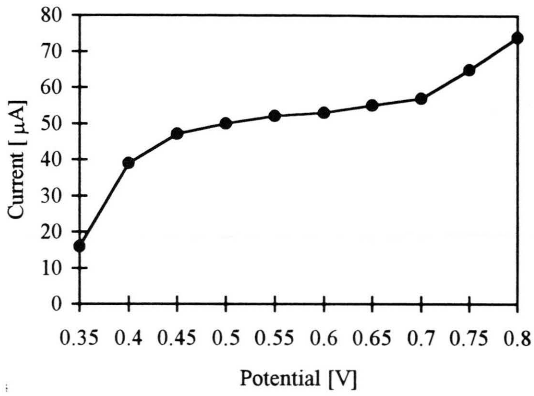


Fig. 5. Polarization curves of the electrooxidation of 10^{-4} M H_2O_2 on bare modified graphite electrode (without gelatin cover), 0.1 M phosphate buffer, pH = 7.0.

The life time of an enzyme electrode and its operational and storage stability are of great importance for practical application. As it is seen from

Table I. Slope ∂I/∂c, and range of the linear concentration dependence of the steady-state current at various potentials at the working electrode.

E [V]	Slope = ∂I/∂c [μA×μM^{-1}]	Linear range $I_S = f(c)$ [μM]
0.55	0.26	up to 120
0.60	0.59	up to 65
0.65	0.86	up to 40
0.70	0.98	less than 35

Table II. Results of parallel determination of xanthine in model solutions by UV-absorbance and with the enzyme electrode.

$c_{xanthine}$ [μM], determined by UV-spectrophotometry	$c_{xanthine}$ [μM], determined with enzyme electrode
11.0	12.3
21.5	24.6
32.5	34.4
43.1	46.7

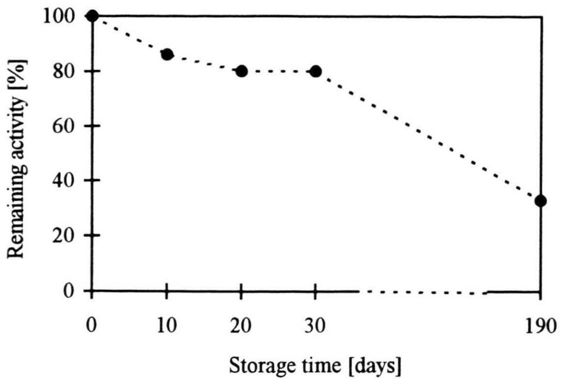


Fig. 6. Dependence of the remaining activity of enzyme electrode on storage time at constant concentration of the substrate (9 μM).

Fig. 6, the prepared enzyme electrode preserves 80% of its initial activity after three weeks and approximately 33% after 190 days storage in air at room temperature.

The concentrations of xanthine, determined with the enzyme electrode in model solutions were compared to the concentrations of xanthine determined by UV-spectrophotometry in the same solutions (Table II). The results obtained with the enzyme electrode are higher with 6–15% than those determined spectrophotometrically. This probably is due to the electrooxidation of the uric acid generated in the enzymatic reaction. (The rate of the electrooxidation of 18 μM uric acid on naked modified graphite at 0.6 V is I_s = 3.5 μA, while the electrooxidation rate of H_2O_2 under the same conditions is I_s = 16 μA.)

Acknowledgements

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