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Electrooxidation and bioelectrooxidation of L- and D-ascorbic acids

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Electrooxidation and bioelectrooxidation of L- and D-iso ascorbic acids over gold nanoparticles-modified glassy carbon electrode was examined at pH 5.6 (optimal for enzyme activity). The glassy carbon electrode was modified through electrodeposition of gold nanoparticles under potentiodynamic conditions. The enzyme ascorbate oxidase was then chemisorbed onto their surface after electrochemical pre-treatment in 0.5 M H_2SO_4. Electrochemical oxidation of the enzyme substrates L- and D-isoascorbic acid was investigated by cyclic voltammetry under both aerobic and anaerobic conditions and by constant potential amperometry at the same pH. The experimental results showed that there is no difference in the electrooxidation rates of both enantiomers when electrooxidised on bare gold nanoparticles-modified glassy carbon electrode; whilst the enzyme electrode showed ca. 30% higher electrocatalytic activity to D-isoascorbic acid as deduced from cyclic voltammograms and ca. 12% higher sensitivity towards D-isoascorbic acid than to L-ascorbic acid as estimated by constant potential amperometry.

Keywords: biosensor, L- and D-ascorbic acid, ascorbate oxidase, gold nanoparticles-modified glassy carbon electrode

INTRODUCTION

Ascorbate oxidase (AOx) belongs to the group of blue multicopper oxidases, and catalyses the oxidation of L-ascorbic acid to dehydroascorbic acid in the presence of molecular oxygen with its concomitant reduction to water. The enzyme has two active sites consisting of three spectroscopically distinct copper centres which comprise one type 1 (T1), one type 2 (T2), and two type 3 (T3) copper ions [1].

As many oxidoreductases, the enzyme has been utilized in a wide variety of first generation biosensors for ascorbic acid, employing enzyme immobilization on various matrices: collagen membrane[2]; nylon net [3]; glassy carbon [4]; carbon nanotubes [5]; screen-printed carbon electrode [6]. In all these studies the measurable biosensor response was either the current from ascorbate oxidation or the detection of oxygen consumption.

Similarly to other multicopper oxidases, ascorbate oxidase has been reported to be electrochemically active [7-12] under both aerated and de-aerated conditions [11]. However, the observed electrochemical activity was associated only with the enzyme capability to catalyse the electrochemical oxygen reduction.

On this background, there are two literature precedents communicating not only the electrochemical activity of the immobilized AOx, but also its capability to act as a catalyst of the electrochemical oxidation of L-ascorbate [13, 14]. In both studies the enzyme was immobilized through chemisorption on electrochemically deposited gold structures onto carbonaceous electrodes.

In this paper, the electrooxidation and bioelectrooxidation of L- and D-ascorbic acid over Au modified glassy carbon electrode with and without chemisorbed AOx were compared in order to get a deeper insight into the mechanism of bioelectrocatalytic oxidation process.

EXPERIMENTAL

The materials used were ascorbate oxidase (AOx) (E.C. 1.10.3.3) from *Cucurbita sp.* (Sigma - Aldrich); L-ascorbic acid and D-isoascorbic (erythorbic) acid (Acros, Belgium); K_2HPO_4 and KH_2PO_4 (Sigma - Aldrich); $HAuCl_4.H_2O$ (Acros), all of analytical grade and used without further pre-treatment.

The working surface of the cleaned and polished glassy carbon electrodes was modified through electrodeposition under potentiodynamic conditions at a scan rate of 0.100 V s^{-1} from electrolyte containing 0.4 mM $HAuCl_4$ dissolved in 0.1M HCl.

Before the enzyme immobilization the gold-modified electrode was cleaned electrochemically in 0.5 M H_2SO_4 by cyclic voltammetry (CV) over the potential range from 0 to 1.7 V (*vs.* Ag/AgCl, 3 M KCl) for at least 10 cycles. The chemisorption of AOx was carried out under static conditions by immersing the modified electrodes in solutions containing 5 mg mL^{-1} of AOx dissolved in 0.1 M potassium phosphate buffer, pH =5.6, for 24 hours.

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All electrochemical experiments were performed in a conventional three-electrode cell with a working volume of 25 mL. The working modified glassy carbon (with or without enzyme), the Ag/AgCl reference electrode and a platinum wire (auxiliary electrode), were connected to a computer-controlled electrochemical workstation PalmSens (Palm Instruments BV, The Nederland). Cyclic voltammograms (CVs) were registered at a scan rate of 5 mV s^{-1}. When necessary, the solutions were purged with either Ar gas or air. All the potentials are reported *vs*. Ag/AgCl (3 M KCl) reference electrode.

The amperometric detection of ascorbic acid was performed by successive addition of aliquots of 0.1 M solution of L- and D-ascorbic acid with the simultaneous registration of the current at a constant potential of 200 mV (*vs*. Ag/AgCl). The solution was stirred at 350 rpm during the measurements with a magnetic stirrer.

Buffer solutions (0.1 M) were made of potassium phosphates (K_2HPO_4 and KH_2PO_4**)** dissolved in double distilled water, pH = 5.6, adjusted with a pH meter pH 211 (Hanna Instruments, USA).

RESULTS AND DISCUSSION

The enzyme electrode produced on the basis of the gold-modified glassy carbon electrode through chemisorption of AOx was screened for electrochemical activity by cyclic voltammetry at a scan rate of 5 mV s^{-1} performed under both aerobic and anaerobic conditions (Fig. 1) at the optimal for the enzyme functioning pH = 5.6. On the CVs recorded under deaerated conditions (dashed line) an anodic peak at 245 ± 5 mV and a cathodic hump spanning over 100 mV to 50 mV were observed. In presence of oxygen a pronounced catalytic wave (solid line) appears on the CV, which lacks under deoxygenated conditions. Based on these observations one can account this wave for the catalytic oxygen reduction associated with the redox transformation of the (T2/T3) copper cluster of the immobilized AOx. The current of the anodic peak at 245 ± 5mV remains unchanged by the presence and absence of oxygen and therefore it could be assigned to the redox transformation of the T1 copper site. The appearances of the anodic peak and cathodic catalytic wave present an indication that the enzyme is not denatured during the immobilization, since it is both electrochemically and enzymatically active.

The addition of either L-ascorbate (red) or D-isoascorbate (blue), results in well-defined oxidative peaks (Fig. 2) with identical peak potentials that slightly exceed the potential of the T1 site redox transformation. Surprisingly, ca. 30% higher oxidative current was noted upon addition of D-isoascorbic acid as compared to the oxidative peak current for L-ascorbic acid with the same concentration. This finding implies preference of the immobilized AOx for the D-iso enantiomer over the L- one.

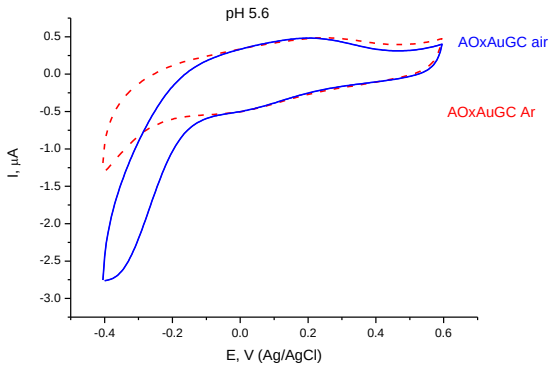

Fig. 1. CVs of the enzyme electrode in 0.1 M potassium phosphate buffer pH=5.6 under deoxygenated with Ar buffer (dashed line) and under aerobic conditions (solid line); v = 5 mVs^{-1}; reference electrode Ag/AgCl, 3M KCl.

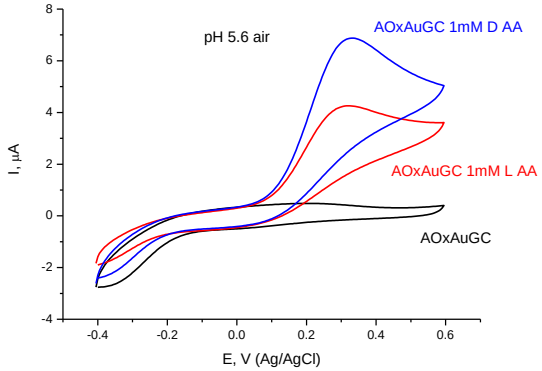

Fig. 2. CV of the enzyme electrode in 0.1 M potassium phosphate buffer pH 5.6 - background (black); in the presence of 1 mM D-isoascorbic acid (blue) and 1 mM L-ascorbic acid (red); v = 5 mV s^{-1}; reference electrode Ag/AgCl, 3M KCl.

Comparative studies on the oxidation of L-ascorbic or D-isoascorbic acid on a gold – modified GC electrode without enzyme showed (Fig. 3) that the peak currents are identical, i.e. there are no differences in the rates of the electrochemical oxidation of both enantiomers. The potentials of the oxidative peaks for the two enantiomers were found virtually the same (±5%), however negatively shifted with ca. 80 mV as compared to the peak potential for the oxidation of these compounds on the enzyme electrode (Fig. 4). Bearing in mind that the potential of the T1 copper site of the

immobilized AOx falls in the middle between the oxidative potentials of the L-, D-enantiomers onto enzyme-less electrode and on the enzyme electrode, we can only hypothesize that the phenomenon observed in Fig. 2. (different oxidative currents generated on the enzyme electrode in the presence of L- and D-enantiomers) results not from the oxidation of the substrates themselves, but is rather due to the oxidation of the T1 site of the immobilized enzyme. Therefore, the T1 copper site gains one electron (and gets reduced) every time it reacts with a substrate molecule (biocatalytic substrate oxidation), then the reduced T1 site sends one electron to the electrode surface (electrochemical process) and becomes oxidized. In this case both processes – the biocatalytic and the electrochemical one are conjugated: a phenomenon which is denoted as bioelectrocatalysis, because the immobilized enzyme acts as an electrocatalyst. The manifested differences in the peak maxima for the two enzyme substrates (Fig. 2), suggest different rates of the biocatalytic interaction, which precedes the electron transfer process. Provided that the biocatalytic stage is much slower than the charge transfer stage, this may eventually result into a positive shift of the oxidative peak potential in the presence of the enzyme substrate as compared to the redox transformation of the active site in its absence, as it was observed in the present study.

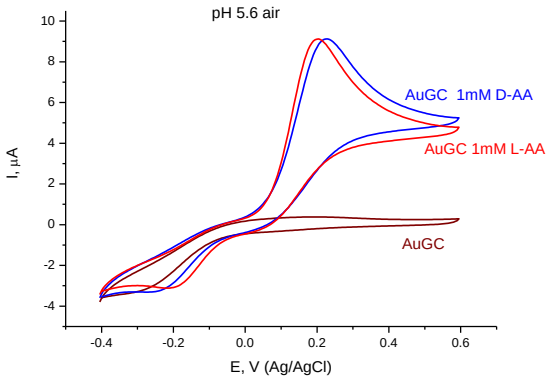

Fig. 3. CV of the modified glassy carbon in 0.1 M potassium phosphate buffer pH= 5.6 (black); in the presence of 1 mM D- isoascorbic acid (blue) and 1 mM L- ascorbic acid (red); v = 5 mV s^{-1}; reference electrode Ag/AgCl, 3M KCl.

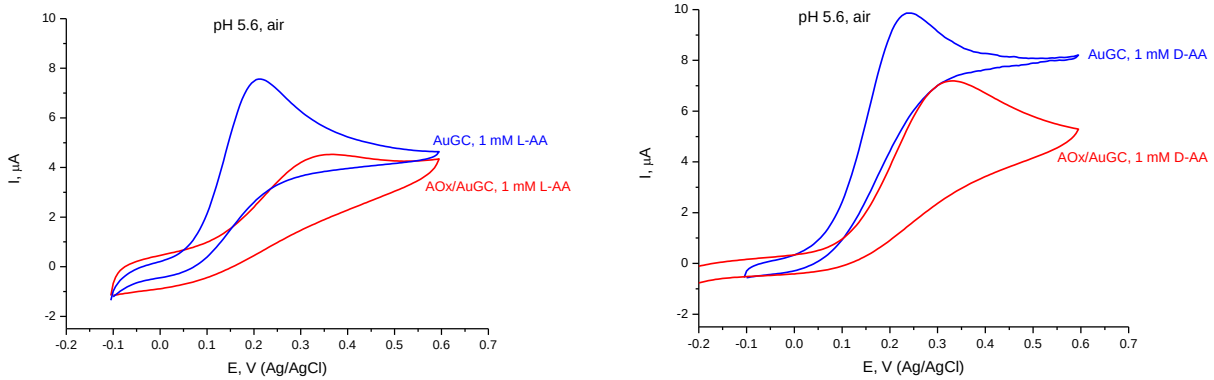

Fig. 4. CVs of the modified glassy carbon electrode without (blue) and with immobilized AOx(red) in 0.1 M potassium phosphate buffer pH = 5.6; in the presence of 1 mM L- ascorbic acid (on the left) and 1 mM D- isoascorbic acid (on the right); v = 5 mV s^{-1}; reference electrode Ag/AgCl, 3M KCl.

The enzyme electrode was further tested under the same conditions as in previous studies as an amperometric biosensor prototype for quantification of L- or D-enantiomer at an applied potential of 0.2 V where the noise level was the lowest (Fig. 5).

The results show that the oxidative current increased stepwise upon introducing in the buffer aliquots of the 0.1 M L- and D-ascorbic acid. Values of the slope (biosensor sensitivity calculated from the regression analysis of the experimental points, were respectively 1380.0 ± 16.7 µA mM^{-1} for substrate L-ascorbic acid and 1547.5 ±26.1 µA mM^{-1} for substrate D-isoascorbic acid.

These values of the biosensor sensitivity indicate that the immobilized enzyme possesses high affinity towards both substrates, but under the given experimental conditions it exhibits certain "preferences" for D-isoascorbic acid.

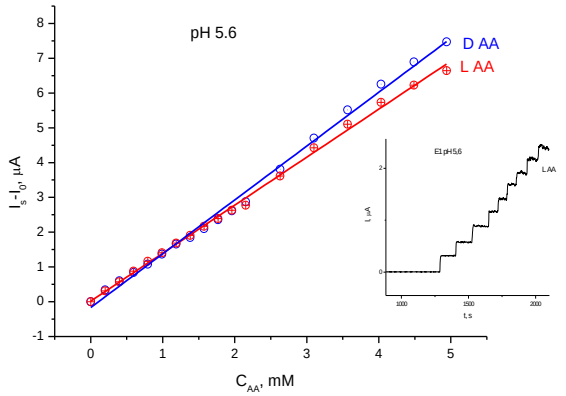

Fig. 5. Background-corrected steady-state response of the enzyme electrode as a function of ascorbic acid concentration at an operating potential of 200 mV (*vs.* Ag/AgCl, 3M KCl); stirring rate: 350 rpm; supporting electrolyte: 0.1 M potassium phosphate buffer, pH= 5.6; Inset: part of the authentic record of the current change upon addition of L-ascorbic or D- iso-ascorbic acid aliquots.

CONCLUSIONS

The results presented in this study showed that: there is no difference in the oxidation rates of both enantiomers of ascorbic acid when electrooxidized on enzyme-less gold nanoparticles modified glassy carbon electrode; however the immobilized enzyme acts as bioelectrocatalyst for electrochemical oxidation of L- and D-enantiomers of ascorbic acid, and is capable of discriminating between them.

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