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Short communication

Electroenzymatic reactions with oxygen on laccase-modified electrodes in anhydrous (pure) organic solvent

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Abstract

The electroenzymatic reactions of *Trametes hirsuta* laccase in the pure organic solvent dimethyl sulfoxide (DMSO) have been investigated within the framework for potential use as a catalytic reaction scheme for oxygen reduction. The bioelectrochemical characteristics of laccase were investigated in two different ways: (i) by studying the electroreduction of oxygen in anhydrous DMSO *via* a direct electron transfer mechanism without proton donors and (ii) by doing the same experiments in the presence of laccase substrates, which display in pure organic solvents both the properties of electron donors as well as the properties of weak acids. The results obtained with laccase in anhydrous DMSO were compared with those obtained previously in aqueous buffer. It was shown that in the absence of proton donors under oxygenated conditions, formation of superoxide anion radicals is prevented at bare glassy carbon and graphite electrodes with adsorbed laccase. The influence of the time for drying the laccase solution at the electrode surface on the electroreduction of oxygen was studied. Investigating the electroenzymatic oxidation reaction of catechol and hydroquinone in DMSO reveals the formation of various intermediates of the substrates with different electrochemical activity under oxygenated conditions. The influence of the content of aqueous buffer in the organic solvent on the electrochemical behaviour of hydroquinone/1,4-benzoquinone couple was also studied.

Keywords: Laccase; Electroenzymatic reaction; Anhydrous organic solvent; Dimethyl sulfoxide

1. Introduction

The interest in the combination enzymology and electrochemistry in anhydrous organic solvents, has increased during the last 15–20 years [1–9]. Enzymatic reactions in organic solvents have certain advantages compared with those in traditional aqueous based media. These include an increased solubility of organic substrates, reversion of hydrolytic reactions, and the possibility to influence the reaction mechanism [10]. Some factors influence the enzymatic reactions in non-aqueous solutions, such as the nature of the organic solvents and amount of water in the system [11–14]. The buffer composition can also be of principal importance for free radical enzymatic reactions. In the total absence of water the enzymes lose their catalytic activity since the hydration layer is essential for maintenance of the biocatalytic properties of the enzymes. The amount of water per enzyme molecule depends on the nature of the enzyme [10,15]. On the other hand electrochemical (non-enzymatic) reactions in aprotic media differ from their equivalent reactions in aqueous solutions. For example, in an aqueous medium the electrochemical reduction of oxygen at different electrodes materials results in the formation of hydrogen peroxide or water. In this case fast chemical reactions proceed the first electron transfer step. As a result only one stable intermediate, *viz.* hydrogen peroxide (or peroxide anion in basic solution) is formed in the reaction. If this reaction occurs in aprotic solvents superoxide anion radicals ($O_2^{\bullet -}$) will be the product [4,5,16]. The addition of protons (or proton donors) into the system leads to the formation of various intermediates and allows studying the stage of protonation in the reaction mechanism [4].

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Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), a blue multicopper oxidase, contains four copper ions and catalyses a four-electron reduction of dioxygen to water with concomitant oxidation of various organic and inorganic compounds [17].

It has been shown previously that carboneous electrodes with immobilised laccase catalyse the electroreduction of oxygen in aqueous buffer solutions directly to water without the intermediate stage of hydrogen peroxide formation [18–22]. In this case the electrode replaces the electron donors in the enzymatic reaction and four protons from the buffer solution take part in the direct oxygen electroreduction to water catalysed by laccase. In this context the following question is of great interest: in what process does the electroenzymatic reaction of oxygen reduction transform in an anhydrous solvent in the absence of protons?

At the present time, there is only very limited information concerning the electroenzymatic reaction in anhydrous organic solvents (in this context an anhydrous organic solvent means that it contains below 0.02% water), whereas in numerous papers the operation of electroenzymatic systems based on different enzymes, such as laccase, tyrosinase, and peroxidase, has been described in organic/aqueous buffer media, containing 2–20% of water [7,8,23–28]. It has been shown that an elaborated composite enzyme electrode consisting of laccase-Nafion retained its bioelectrocatalytic activity to a higher concentration of the organic component compared with that of the activity of the enzyme in aqueous solution, and it could be used to construct a bioelectrochemical sensor for determination of water-insoluble compounds [28].

The principal goals of the present investigation were the following:

(1) To accomplish oxygen electroreduction at graphite and glassy carbon electrodes without laccase, in an anhydrous organic solvent (DMSO), and in the absence of any organic proton donor.
(2) To study oxygen electroreduction at glassy carbon and graphite electrodes modified with adsorbed laccase in aprotic DMSO solutions through direct electron transfer between the electrode and the enzyme and without any proton donor, i.e., in the absence of laccase substrates. The results were compared with those obtained for electrodes modified with thermodenaturated laccase.
(3) To elucidate the mechanism of oxygen reduction on bare glassy carbon electrodes in anhydrous DMSO in the presence of a phenolic compound, i.e., a laccase substrate, e.g., hydroquinone, which is a weak acid in pure organic solvents.
(4) To investigate the electrochemical reactions with oxygen on laccase-modified electrodes in the presence of laccase substrates (hydroquinone and catechol).

2. Experimental

2.1. Materials

Fungal laccase (EC 1.10.3.2) from *Trametes* (*Coriolus*, *Polyporus*) *hirsuta* (*hirsutus*) was purified as described previously [29] and it had a specific activity of 100 U/mg measured in 0.1 M Na-citrate-phosphate buffer, pH 5.0 using 10 mM catechol as substrate. The physico-chemical, biochemical, and kinetic properties of the enzyme have previously been studied in detail [29,30].

Tetrabutylammonium perchlorate (TBAP) was purchased from Sigma (St. Louis, MO, USA) and was dried before use. Catechol and hydroquinone from Merck (Darmstadt, Germany) were used after distillation. Dried dimethyl sulfoxide (DMSO) from Merck contained a maximum of 0.02% water. All chemicals used were of analytical grade. The buffers were prepared using water (18 MΩ) purified with a Milli-Q system (Millipore, Milford, CT, USA). Laccase was denatured at 100 °C for 20 min in deionised water. The deionisation of laccase was performed by dialysis against deionised water (Milli-Q system).

2.2. Instrumentation

Rods of glassy carbon (outer diameter of 3 mm, type v.10, Le Carbone, Lorraine, France) and rods of spectroscopic graphite (outer diameter of 3.05 mm, type RW0, Ringsdorff–Werke GmbH, Bonn, Germany) were press fitted into Teflon holders and were then used as working electrodes. The end of the glassy carbon electrode was first polished with 0.25 μm alumina Stuers AP-paste (Copenhagen, Denmark), then sonicated and thoroughly washed with ethanol and deionised water. The end of the spectroscopic graphite rod was first polished on wet fine emery paper (Tufback Durite, P1200) and then with wet filter paper followed by washing with ethanol and deionised water. Before use in the electrochemical experiments laccase was dialysed against deionised water to remove buffer components that could influence the electrochemical reactions. Adsorption of the enzyme was accomplished by placing 6 μl aliquots of a 10 mg/ml stock solution of laccase on the surface of the working electrode followed by drying in the air at ambient temperature. The working solution of DMSO contained 0.1 M TBAP as supporting electrolyte.

Cyclic voltammetry was performed in a three-electrode electrochemical glass cell. The reference and counter electrode were a saturated calomel Radiometer K-901 electrode (SCE) for organic media (Copenhagen, Denmark) and a platinum net, respectively. The experiments were carried out using a home-made potentiostat connected to an X–Y recorder Omnigraph 2000 (Houston, TX, USA). All experiments were performed at room temperature. Anaerobic conditions were obtained by saturating the bulk solution with nitrogen (AGA Gas AB, Sundbyberg, Sweden) for at least 20 min before use. The solubility of O_2 at 1 atm pressure and 25 °C in DMSO was taken to 2.1 mM [4]. All the potentials in the graphs and in the text are consistently referred to SCE.

3. Results and discussion

It is well known fact that various bare carbonaceous electrodes are sufficiently inactive for direct electroreduction of oxygen at neutral pH. However, below −0.2 V *vs.* SCE, oxygen

electroreduction at bare carbonaceous electrodes results in the formation of hydrogen peroxide and water [19]. Adsorption of a fungal laccase on the surface of carbon electrodes significantly facilitates the electroreduction of molecular oxygen. The electrode modified with *T. hirsuta* laccase efficiently catalyses the oxygen electroreduction in the potential range between +0.7 and −0.2 V *vs.* SCE [19,21,22]. It was previously shown using a rotating ring-disk electrode that laccase catalyses the oxygen electroreduction on a carbon electrode directly to water without forming any intermediate hydrogen peroxide [18]. At the same time the electroenzymatic redox reactions with oxygen on laccase-modified electrode in anhydrous organic solvents are depended on various factors such as the nature of the electrode material and organic solvents, the water content supporting the hydrated layer of the enzyme, presence of proton donors or metal ions in the reaction medium, and the nature of the buffer components. Using cyclic voltammetry the possible effect of each of these parameters was investigated and determined.

3.1. Oxygen electroreduction on bare glassy carbon and graphite electrodes in DMSO

Cyclic voltammograms of the electroreduction of oxygen on bare glassy carbon and bare spectroscopic graphite electrodes in DMSO were recorded. The peak potentials for the cathodic formation and anodic oxidation of $O_2^{\bullet-}$ were −0.83 V and −0.65 V for glassy carbon and −0.80 V and −0.68 V for spectroscopic graphite, respectively. The separation between the cathodic and anodic peaks for both electrode materials was practical identically and found to be −0.18 V. These data are very close to those previously reported for oxygen electroreduction in DMSO on glassy carbon [4,5]. It should be taken into account that some difference between our and the literature data could be explained by the use of different reference electrodes. The amount of water up to 0.1% w/w did not affect the electrochemistry of oxygen electroreduction in anhydrous DMSO.

3.2. Oxygen electroreduction at laccase-modified glassy carbon electrode in anhydrous DMSO in the absence of proton donors

In an aprotic solution such as dry DMSO there are only two components of the electroenzymatic reaction of oxygen reduction present compared with when carried out in an aqueous buffer, *viz*. the electron donor (electrode) and oxygen (electron acceptor) and a lack of protons necessary to form water (or hydrogen peroxide). Therefore, the shape of the cyclic voltammograms in DMSO registered for the laccase-modified electrodes dramatically changed compared with the ones obtained without laccase on bare glassy carbon electrodes. Fig. 1a illustrates the oxygen electroreduction at successive potential scans on the laccase-modified glassy carbon electrode in pure DMSO. In the presence of laccase the cathodic peak potential of oxygen electroreduction was shifted about 100 mV in the negative potential range as compared with the corresponding bare electrode. It seems as

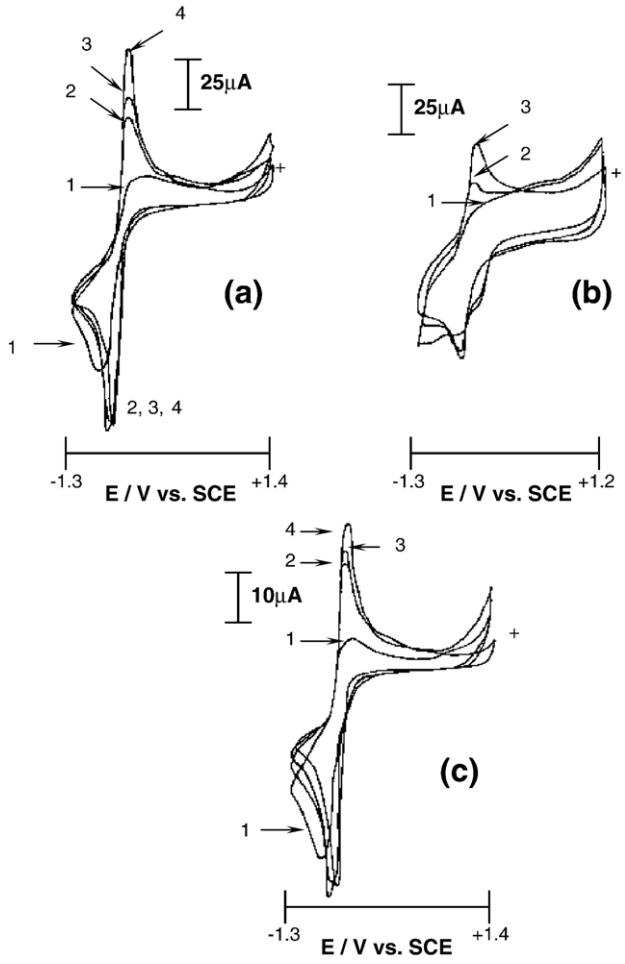

Fig. 1. The effect of native *T. hirsuta* laccase (a, b) compared with thermo-denatured laccase (c) adsorbed on glassy carbon (a, c) and graphite (b) electrodes on oxygen electroreduction in anhydrous DMSO. (a_1-a_4), (b_1-b_3) and (c_1-c_4) successive potential scans with oxygen on electrodes modified with *T. hirsuta* laccase. a_1; b_1; c_1 — first potential scans. Conditions: all solutions contained 0.1 M TABS as supporting electrolyte; sweep rate, 100 mV/s; 5 μl of the preparation of laccase dialysed against deionised water and containing 10 mg protein per ml were placed on the top of the glassy carbon electrodes and then dried for 60 min at room temperature; (+) — indicates the start potential.

though the reduction of oxygen at the electrode is a totally irreversible process (first scan) but after a few scans the electrode reaction is partially restored to reversibility in the $O_2/O_2^{\bullet-}$ reaction. Increasing the amount of *T. hirsuta* laccase deposited on the electrode surface in the range from 0.01 to 0.06 mg enhanced the suppressing effected in the reaction forming $O_2^{\bullet-}$. Similar results were received on laccase-modified graphite electrodes (Fig. 1b). These results strongly correlate with data obtained by pulse radiolysis in homogeneous phase with laccase in the reaction with $O_2^{\bullet-}$ obtained in aqueous buffer [31]. This method permits to observe in real time the change in concentration of $O_2^{\bullet-}$ generated after radiolysis of the solution. Laccase decreased the concentration of $O_2^{\bullet-}$ in the system and did not exert any kinetic influence on the decomposition of the $O_2^{\bullet-}$. To study the influence of the water content of the enzyme layer experiments were carried out drying the enzyme on the electrode surface for various

times (from 20 to 120 min) at room temperature. After 25 min of drying the responses of the laccase-modified electrodes were practically identical and laccase immobilised on the electrode suppressed the formation of $O_2^{\bullet-}$ at the electrode in anhydrous DMSO. The shape of the cyclic voltammograms with thermodenatured laccase is very close to the ones obtained with native enzyme (Fig. 1c). The main problem of the bioelectrochemical phenomenon of oxygen electroreduction with laccase in anhydrous organic solvent is the following: what is the electrochemical reaction observed at the surface of the laccase-modified electrodes in an oxygenated environment? It was reported previously that laccase forms transient adducts with $O_2^{\bullet-}$ [31]. In this connection comparing the results obtained in the present investigation with literature data it is proposed that the oxygen $O_2^{\bullet-}$ reacts with a limited number of sites on the polypeptide chain, e.g., the Thr, Cys, Tyr, Phe, His amino acid residues of laccase. Therefore, it is possible to observe a decreasing effect of the formation of $O_2^{\bullet-}$ on the surface of laccase-modified electrode. However, with time the cyclic voltammograms of the laccase-modified electrodes start to look like those registered for naked electrodes indicating that the possible sites of the enzyme that $O_2^{\bullet-}$ can react with are consumed.

3.3. Oxygen electroreduction in DMSO in the presence of weak acids (hydroquinone and catechol) without the enzyme

Initially, the electrochemical behaviour of hydroquinone was studied on a bare glassy carbon electrode in a nitrogen atmosphere in anhydrous DMSO. A typical cyclic voltammogram of hydroquinone is shown in Fig. 2a. The asymmetric and irreversible anodic (maximum peak potential of 0.88 V *vs.* SCE) and cathodic (maximum peak potential of −0.03 V *vs.* SCE) peak couple is displayed in this figure. It should also be noticed that a cyclic voltammogram of 1,4-benzoquinone on the glassy carbon electrode in anhydrous DMSO shows two cathodic and two anodic peaks (not shown in Fig. 2). Thus, the mechanism of the electrooxidation reaction of hydroquinone differs from the mechanism of electroreduction of 1,4-benzoquinone in pure DMSO. After addition of small amounts of aqueous 0.05 M Na-acetate buffer, the shape of the cyclic voltammograms of hydroquinone in DMSO saturated with nitrogen dramatically changed (*cf.* Fig. 2a and b). In this case the cathodic and anodic peaks were split as a result of protonation of the intermediates in the electrochemical reactions of hydroquinone. The same results were obtained with another week acid, catechol. The cathodic and anodic peak potentials and the ratio between the peak currents very strongly depended on the concentration of aqueous buffer. On the one hand, increasing the concentration of the aqueous buffer the shape of the voltammograms approaches the one registered in pure aqueous buffer (no splitting effect). On the other hand, no visible splitting effect (only a shift in peak potential) was observed for concentrations of aqueous buffer less then 0.5 w/w %. Similar effects were found using two other aqueous buffers, namely Na-phosphate and Na-citrate. Thus, in the present investigation the preparation of *T. hirsuta* laccase was dialysed against deionised water before used to modify the electrodes.

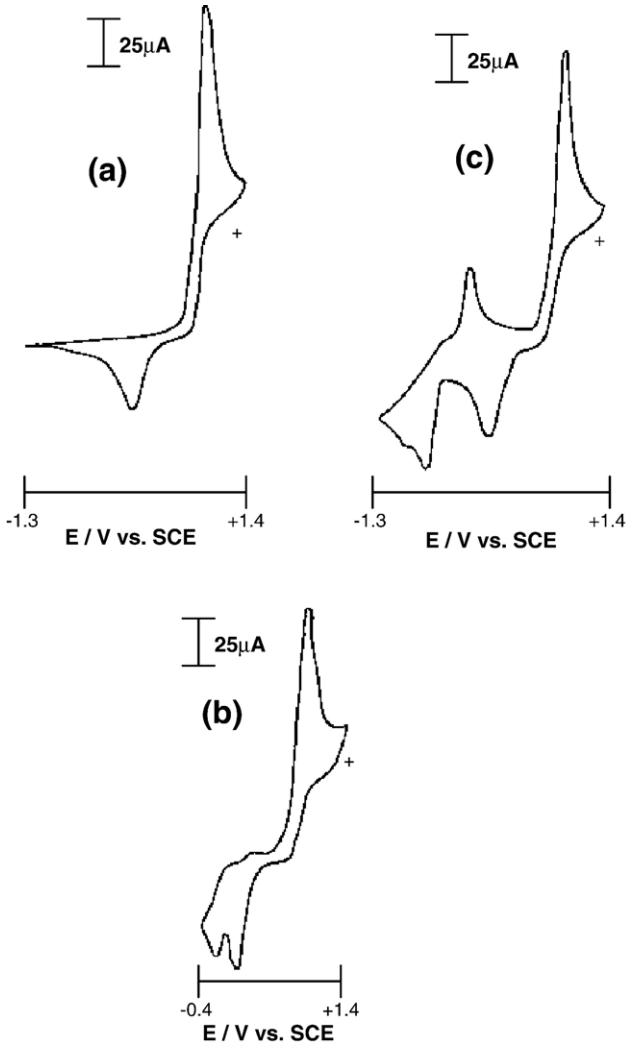

Fig. 2. Cyclic voltammograms for: (a) 11 mM hydroquinone on a glassy carbon electrode in anhydrous DMSO deaerated with nitrogen, (b) in the presence of 10 mM hydroquinone in DMSO containing 10 v/v % of 0.05 M Na-acetate water buffer at pH 5.0 in nitrogen atmosphere, (c) in oxygen saturated (1 atm) pure DMSO in the presence of 11 mM hydroquinone. Conditions: 0.1 M TBAP was used as supporting electrolyte; sweep rate 100 mV/s; (+) indicates the start potential.

Fig. 2c displays the oxygen electroreduction at a bare glassy carbon electrode in DMSO in the presence of 11 mM hydroquinone acting as a weak acid. At the same time the electrochemistry of hydroquinone under anaerobic conditions is presented in Fig. 2a. The addition of hydroquinone caused the appearance of a new irreversible redox process on the cyclic voltammogram with an anodic peak at a potential of about −0.33 V and an increase in the cathodic peak height at −0.83 V (Fig. 2c). The redox couple at −0.03 V and +0.88 V corresponds to the electrochemical redox transformation of hydroquinone. Thus, $O_2^{\bullet-}$ formed on the bare glassy carbon electrode can react with hydroquinone and lead to a change of the shape of the cyclic voltammograms of hydroquinone in contrast to when registered at anaerobic conditions.

3.4. Electrochemical reactions of oxygen with T. hirsuta laccase deposited on the electrode in the presence of laccase substrates, such as hydroquinone and catechol

As mentioned above, hydroquinone and catechol can serve both as laccase substrates (electron donors in the enzyme reaction) as well as being weak acids in aprotic organic solvents. Using cyclic voltammetry it is possible to detect intermediates formed in the enzymatic reaction in anhydrous DMSO. Fig. 3 displays the change of the appearance of the cyclic voltammograms registered after successive potential scans corresponding to the products of the enzymatic reaction of the oxidation of hydroquinone catalysed by laccase in pure DMSO under oxygenated conditions. Comparing the voltammograms recorded in the presence of laccase on the electrode in oxygen saturated DMSO with hydroquinone and with those without enzyme (see Fig. 2c) shows one more redox couple in the presence of the enzyme. The appearance of a pair of new cathodic (at a potential of about −0.38 V) and anodic (at a potential of about +0.53 V) peaks can be explained by the electrochemical transformation of intermediates in the enzymatic reaction. After a few potential scans these peaks disappeared and the shape of the voltammogram became identical to the one recorded on bare glassy carbon electrode without the enzyme (see Fig. 2c). It seems as though it occurs as a result of the loss of the hydrated layer of the laccase and its following inactivation (denaturation) in pure DMSO during repeated potential scans.

The following conclusions can be drawn from the results presented in this work. (i) Laccase adsorbed on a carbonaceous electrode surface modifies the mechanism of oxygen electroreduction in an anhydrous organic solvent without proton donors compared with oxygen electroreduction at a bare electrode, namely it prevents formation of $O_2^{\bullet-}$. (ii) Some electroactive intermediates formed in the enzymatic reaction of hydroquinone and catechol (laccase substrates) are oxidised by oxygen in pure DMSO. In both cases laccase loses its activity after a few potential scans due to the dehydration of the protein globule and the cyclic voltammograms become the same as when recorded on a bare electrode. (iii) The investigation of the electrochemical transformation of the hydroquinone/1,4-benzoquinone redox couple in DMSO with a low content of aqueous buffer shows the formation of intermediates. The extent of the formation of these intermediates was shown to depend on successive additions of an aqueous buffer, causing protonation steps having different activity in the electrochemical reaction. In the presence of aqueous buffer added to DMSO the cyclic voltammograms of hydroquinone and its oxidised form were distorted. Increasing the amount of water in DMSO (without buffer components) lead to a shift of the redox couple of the diphenols in the negative potential range without modification of the shape of the cyclic voltammograms. These factors are of great importance for the development of amperometric biosensors operating in organic-buffer media and when investigating the catalytic mechanism of some oxidoreductases in organic solvents. It should be taken into account that the catalytic parameters of the enzymatic reaction carried out via free radicals can be dependent on the percentage of aqueous inorganic buffer media not only due to various hydration steps of the enzymes but also due to the formation of several types of protonised intermediates of the enzymatic reaction, having different enzymatic and electrochemical activities in these solutions.

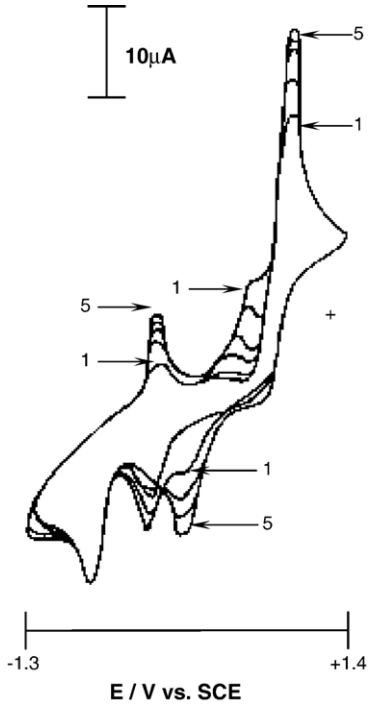

Fig. 3. Evolution of cyclic voltammograms during successive potential scans obtained on the laccase-modified glassy carbon electrode in oxygen saturated DMSO solution in the presence of 11 mM hydroquinone. (1) indicates the first potential scan. Conditions: supporting electrolyte, 0.1 M TBAP; sweep scan, 100 mV/s; (+) indicates the start potential.

The data obtained in the present investigation are important to understand what processes take place in a biological system in the presence of oxygen, $O_2^{\bullet-}$ and different organic compounds including proteins.

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