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ARTICLES

# An Amperometric Biosensor Based on Laccase Immobilized in Polymer Matrices for Determining Phenolic Compounds<sup>1</sup>

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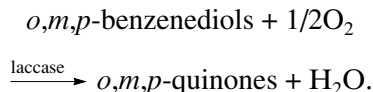
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**Abstract**—An amperometric enzyme electrode based on laccase for determining phenolic compounds is proposed. The following three types of polymer materials were used for enzyme immobilization on the surface of a glassy-carbon electrode: positively charged cetyl ethyl poly(ethyleneimine) (CEPEI) and negatively charged commercial Nafion and Eastman AQ 29D polymers. The advantages and disadvantages of each of the above polymers for enzyme immobilization are discussed. The detection limits of the model phenolic compounds hydroquinone and pyrocatechol in a buffer solution on laccase immobilization in a Nafion membrane were  $3.5 \times 10^{-8}$  and  $5.0 \times 10^{-8}$  M, respectively, at a signal-to-noise ratio of 3. Electrodes with laccase immobilized in Nafion and Eastman AQ 29D membranes exhibited the shortest response time. The operating stability and the stability in storage can be significantly improved by the additional incorporation of gelatin in the polymer matrices. Gelatin prevents enzyme inactivation as a result of enzyme modification by the free-radical oxidation products of phenolic compounds.

Phenolic compounds are widespread in nature, and they play a crucial role in living organisms. They are used in medicine and industries, including wood processing and pesticide production. The degradation products of nonionic surfactants, which are used both in household products and in textile, food, and varnish-and-paint industries, are another source of phenol derivatives. Because of this, phenols can occur in wastewater, enter into soil, and pollute the environment. Many of these compounds are highly toxic, and their determination in low concentrations is an important problem. Various methods for determining these compounds are well known [1, 2]. New procedures for determining phenols with biosensors have been described [3–15]. The sensitivity of analysis and the stability of an enzyme electrode can be improved using new matrices for enzyme immobilization. Enzyme immobilization on electrode surfaces due to physical adsorption, the covalent linking of laccase to the surfaces of carbon materials and gold with bifunctional reagents, incorporation in a gel of bovine serum albumin crosslinked with glutaraldehyde, and incorporation into a polyaniline matrix have been described [13, 16–18]. The electrodes developed exhibited various characteristics in terms of stability, sensitivity, and analysis time.

In this paper, we propose an enzyme electrode with laccase immobilized in the following three polymers supported on a glassy-carbon electrode for determining phenolic compounds: negatively charged polyelectrolytes (Nafion and Eastman AQ 29D) and a positively charged poly(ethyleneimine) polymer successively modified with cetyl bromide and ethyl bromide.

The oxidation of phenolic compounds with the participation of laccase can be represented by the following reaction scheme:

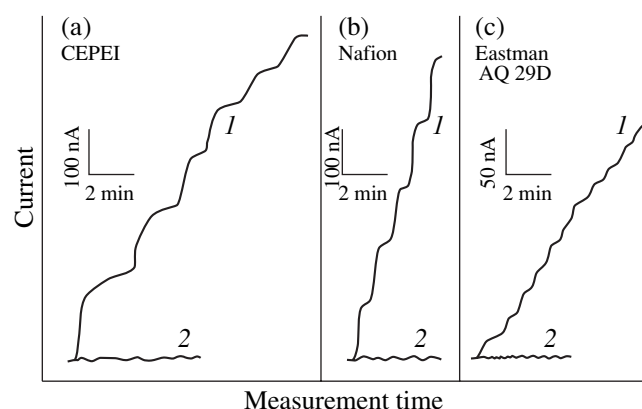


Model analytes (hydroquinone and pyrocatechol) were determined in a system with stirring by the electroreduction of an enzymatic reaction (quinines) at the electrode.

## EXPERIMENTAL

**Materials.** Laccase from the basidial fungus *Trametes hirsute* was purified to an electrophoretically homogeneous state in accordance with a well-known procedure [19]. It had a specific activity of 100 U/(mg protein) at pH 5.0. The enzyme activity unit (U) was taken to be a change in the absorbance of a 10 mM pyrocatechol solution in a 0.1 M sodium citrate buffer at 410 nm by unity in 1 min. The enzyme concentration was determined at a wavelength of 610 nm ( $\epsilon =$

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**Fig. 1.** (1) Current-time relationships obtained using the following modified glassy-carbon electrodes in a system with stirring while successively increasing the concentration of hydroquinone in the cell by 1  $\mu\text{M}$ : (a) laccase/CEPEI, (b) laccase/Nafion, and (c) laccase/Eastman AQ 29. (2) The relationships obtained for the specified electrodes in the absence of the enzyme. Experimental conditions: working electrode potential, 0.05 V; 0.1 M sodium citrate buffer solution, pH 5.0;  $T = 20^\circ\text{C}$ ; rate of a magnetic stirrer, 300 rpm.

4900  $\text{M}^{-1} \text{cm}^{-1}$ ). Pyrocatechol, citric acid monohydrate, and sodium acetate from Merck (Germany) and hydroquinone from Riedel-de Haen (Germany) were used. All solutions were prepared in deionized water prepared with Milli-R and Milli-Q systems (United States).

The polymer compounds Nafion and Eastman AQ 29D were obtained from Aldrich Chemie (United States) and Kodak Eastman Chemical (United States), respectively. Poly(ethyleneimine) modified with cetyl bromide and ethyl bromide (CEPEI) was synthesized in accordance with the procedure described in [20].

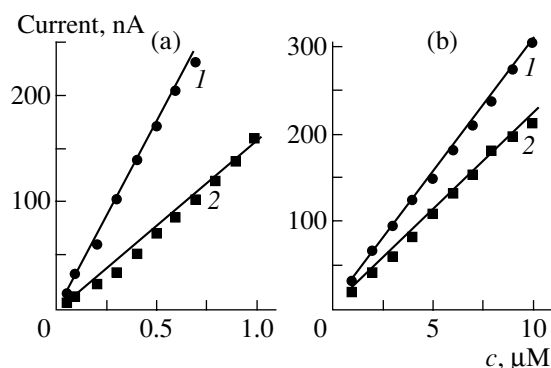
#### Instrumentation and experimental procedures.

A glassy-carbon rod ( $d$  3 mm) press-fit in Teflon (Le Carbon, France) was used as a working electrode. Before each particular experiment, the electrode was initially polished with alumina paste with a particle size of 0.25  $\mu\text{m}$  (Struers, Denmark), washed with deionized water, and dried. Enzyme immobilization was usually performed as follows: 4  $\mu\text{L}$  of laccase (40  $\mu\text{g}$ ) was applied to the end of a polished glassy-carbon electrode and dried for 15 min. Next, the electrode surface was coated with a 0.7 wt % Nafion solution, which was prepared by diluting a 20 wt % commercial preparation with water; an aqueous 0.7 wt % Eastman AQ 29D suspension; or a 0.7 wt % CEPEI solution in benzene-1-butanol (2 : 1). Before the use of the polymer Eastman AQ 29D, its suspension in water was heated to  $90^\circ\text{C}$  and then cooled to room temperature. The modified electrodes were dried at room temperature for 1 h, repeatedly washed with deionized water, and placed in an electrochemical cell. In some experiments, 5  $\mu\text{L}$  of a

solution containing 0.7 wt % Nafion or Eastman AQ 29D in water and 40  $\mu\text{g}$  of laccase were applied to the surface of the working electrode. The electrochemical experiments were performed in a three-electrode glass cell under steady-state conditions at room temperature. A potentiostat (Zata Electronics, Sweden) equipped with a Model BD111 recorder (Kipp and Zonen, the Netherlands) was used. A saturated calomel electrode (SCE) and platinum gauze with a geometric surface area of 0.7  $\text{cm}^2$  served as reference and auxiliary electrodes, respectively. The kinetic parameters of the homogeneous enzymatic reactions of hydroquinone and pyrocatechol oxidation and the stability of the enzyme in storage were evaluated using a Clark oxygen electrode and a potentiostat from BAS (United States).

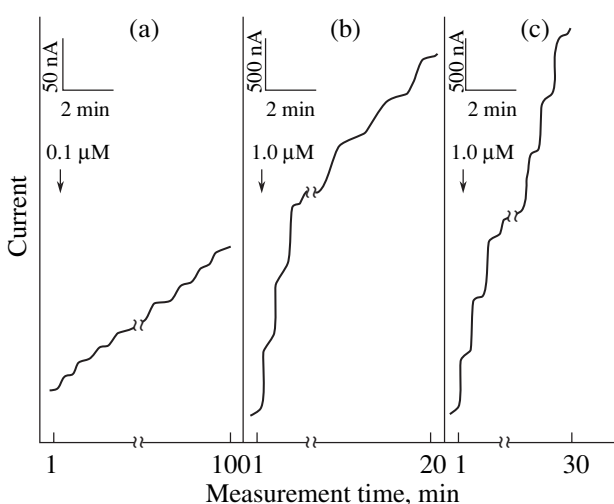
## RESULTS AND DISCUSSION

The following three types of polymers different in physicochemical parameters were used for immobilizing laccase on the surface of a glassy-carbon electrode and for comparing the characteristics of the resulting enzyme electrodes: CEPEI, Nafion, and Eastman AQ 29D. Poly(ethyleneimine) successively modified with cetyl bromide and ethyl bromide is an amphiphilic cationic polyelectrolyte capable of forming polymer micelles in organic solvents. Laccase incorporated into the inverse polymer micelles of CEPEI exhibited high catalytic activity in an organic system (benzene-1-butanol) [20]. The use of CEPEI for enzyme immobilization on the working surface of an electrode makes this biosensor attractive for the following reasons: First, the positively charged matrix of this polymer can partially remove the effect of side electrochemical processes related to positively charged components in the test solution. Second, the hydrophobic properties of the polymer due to the presence of cetyl residues make it possible to concentrate a hydrophobic analyte (for example, phenolic compounds) in the enzyme membrane and to improve the sensitivity of analysis. The concentration of phenolic compounds in a system with stirring was found by the reduction of enzymatic reaction products at the electrode. The working-electrode potentials are specified in the figure captions. Figure 1a shows a typical steady-state response of a laccase/CEPEI electrode (1) to a successive increase in the concentration of hydroquinone in the working solution by 1  $\mu\text{M}$  and the result of a control measurement under the same conditions in the absence of the enzyme from the electrode (2). The response time was sufficiently short, and the signal reached no less than 95% of a maximum value in 2 min. The deviation of current from linearity after several repeated injections of hydroquinone into the solution was likely due to the accumulation of a hydrophobic product (1,4-benzoquinone) of the enzymatic reaction in the hydrophobic polymer layer. It is our opinion that CEPEI as a matrix for laccase immo-



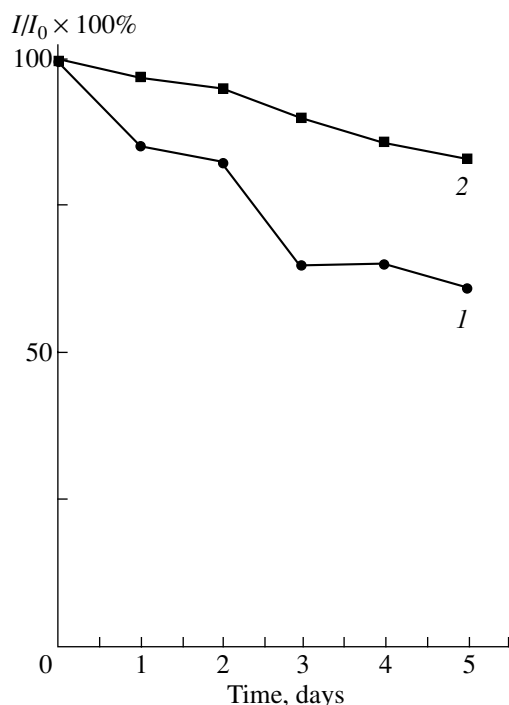
**Fig. 2.** Calibration graphs for modified electrodes: (a) laccase/Nafion and (b) laccase/Eastman AQ 29. Analytes: (1) hydroquinone and (2) pyrocatechol. The experimental conditions are specified in Fig. 1.

bilization in determining phenolic compounds is suitable for the development of disposable biosensors (for example, made using screen print or spray technologies) and biosensors functioning in aqueous organic media. Nafion and Eastman AQ 29D are polyanions, and they are occasionally used in biosensors for the immobilization of enzymes or as films that cover the layer of an enzyme immobilized on the electrode in order to prevent undesirable electrooxidation reactions of negatively charged components of the test solution (such as ascorbic acid) [21, 22], which impaired analytical results. Figures 1b and 1c show the steady-state responses of electrodes with laccase immobilized in Nafion and Eastman AQ 29D films, respectively, to a successive increase in the concentration of hydroquinone in solution by  $1 \mu\text{M}$ . The response times of laccase electrodes coated with these polymers were similar and approximately three times shorter than that of the laccase/CEPEI modified electrode. The current intensity at the laccase/Nafion electrode was several times higher than that at the laccase/Eastman electrode but lower than that at the laccase/CEPEI electrode at the same hydroquinone concentration in solution. The difference in the sensitivity of the enzyme electrodes to the test component can be explained by different mass transfers in these membranes. The immobilization of laccase in the bulk of both of the polymers followed by the application of  $5 \mu\text{L}$  of a polymer-enzyme mixture containing  $40 \mu\text{g}$  of laccase to the surface of a glassy-carbon electrode led to analogous results obtained upon coating an electrode with supported laccase with a polymer film. The enzyme electrode with a laccase/Nafion film can determine no less than  $0.1 \mu\text{M}$  laccase substrate. The detection limits of hydroquinone and pyrocatechol were  $3.5 \times 10^{-8}$  and  $5.0 \times 10^{-8}$  M, respectively, at a signal-to-noise ratio equal to 3. Figure 2 shows calibration graphs for (1) hydroquinone and (2) pyrocatechol obtained at (a) laccase/Nafion and (b) laccase/Eastman electrodes. In both cases, the slope



**Fig. 3.** Comparison of operation stabilities: (a) the laccase/Nafion modified electrode upon a successive increase in the concentration of pyrocatechol in the cell by  $0.1 \mu\text{M}$ , (b) the laccase/Nafion modified electrode upon a successive increase in the concentration of pyrocatechol in the cell by  $1 \mu\text{M}$ , and (c) the laccase/gelatin/Nafion modified electrode upon a successive increase in the concentration of pyrocatechol in the cell by  $1 \mu\text{M}$ . The experimental conditions are specified in Fig. 1.

of the calibration graph for hydroquinone was greater than that for pyrocatechol. This difference can be explained by a lower value of  $K_M$  for laccase from *Trametes hirsuta* with respect to hydroquinone than that with respect to pyrocatechol ( $0.17$  and  $0.22$  mM, respectively) and by a higher catalytic constant ( $98$  and  $87$   $\text{s}^{-1}$ , respectively). The calibration graph for hydroquinone at the laccase/Nafion modified electrode was linear over the range  $1.0 \times 10^{-7}$ – $3.0 \times 10^{-6}$  M. Other important performance characteristics of enzyme electrodes are their operating stability and stability in storage. The operating stability implies the number of continuous measurements that can be performed with an enzyme electrode without loss of its activity. This parameter mainly depends on the properties of the enzyme and on the immobilization procedure. Figure 3a demonstrates the operating stability of the laccase/Nafion electrode on the successive addition of  $0.1 \mu\text{M}$  pyrocatechol to the solution. It can be seen that the activity of the biosensor remained unchanged upon the successive addition of at least 100 portions of the test substrate to the cell. However, the activity of the enzyme electrode decreased after 10 successive additions of  $1 \mu\text{M}$  pyrocatechol to the electrochemical cell (Fig. 3b). It is well known [23] that the stability of laccase from the fungus *Polyporus versicolor* in a homogeneous reaction increased in the presence of gelatin. We used this property in order to improve the operating stability of a laccase/Nafion electrode. Figure 3c demonstrates an increase in the stability of the enzyme electrode based on a Nafion film in the presence of gelatin



**Fig. 4.** Stability in storage: (1) laccase/Nafion and (2) laccase/gelatin/Nafion modified electrodes. The experimental conditions are specified in Fig. 1.

in the enzyme membrane. The biosensors did not lose activity upon the successive addition of at least 30 pyrocatechol samples with a concentration of 1  $\mu\text{M}$  each to the working solution. A small decrease in the limiting current of reduction of the enzymatic oxidation product of pyrocatechol (1,2-benzoquinone) was observed, as compared with the electrode without gelatin; this decrease can be explained by a decrease in the mass-transfer rate of the substrate. It is likely that the increase in the operating stability of the enzyme electrode in the presence of gelatin was due to a decrease in laccase inactivation by enzymatic reaction products (quinines) or free radicals formed in the catalytic process, which modify laccase. In this case, gelatin is a trap for the products of enzymatic substrate oxidation. Figure 4 (curve 1) demonstrates the stability of the laccase/Nafion electrode in storage at room temperature. The enzyme electrode retained approximately 60% of its initial activity after storage for five days. The introduction of 200  $\mu\text{g}$  of gelatin into the enzyme membrane allowed us to retain the electrode activity at a level of 80% (Fig. 4, curve 2).

Thus, we found that the electrode fabricated using the Nafion polyelectrolyte was the best in terms of the entire set of parameters: low detection limits for the model phenolic compounds to be determined, a short response time, high operating stability, and high stability in storage. The two last-named parameters were sig-

nificantly improved using gelatin as a trap for the enzymatic oxidation products of phenolic compounds. The electrode with laccase immobilized in modified poly(ethyleneimine) determined low concentrations of phenolic compounds because of analyte preconcentration in a hydrophobic polymer matrix. However, the operating stability of this system was low, and the electrode response decreased after the successive addition of the samples of phenolic compounds to the reaction system. The procedure proposed for laccase immobilization in polymer matrices considerably simplified the procedure of the preparation of enzyme electrodes for determining phenolic compounds and provided high stability and sensitivity. The biosensor proposed detected phenolic compounds in aqueous solutions at a concentration lower than 1  $\mu\text{M}$ .

#### ACKNOWLEDGMENTS

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