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Electrochemical Biosensor Based on Horseradish Peroxidase for the Determination of Oxidizable Drugs

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Abstract : An amperometric biosensor based on a carbon paste electrode modified with horseradish peroxidase (HRP) is defined as a device for the quantitative and qualitative detection of oxidizable drugs. This paper demonstrates the advantages of using HRP as the biocatalyst in the in vitro detection of oxidizable drugs in the presence of hydrogen peroxide. Amperometric analysis and batch experiments were carried out in acetate buffer (pH 4.8) in the presence of hydrogen peroxide. Amperometry was performed by applying a potential of -0.1 V (vs. Ag/AgCl), in the presence of hydrogen peroxide and adding a drug which has an oxidizable group (OxG; e.g. -OH, -NH, -SH). The results show that the HRP-modified electrode responds rapidly to micromolar changes in the OxG addition. The current signals obtained are proportional to the OxG concentration. Experimental variables, such as pH, operating potential, change in the percentage of HRP, and reproducibility are discussed. The stability of the HRP-modified electrode is also demonstrated.

Key Words: Biosensor, Amperometry, Horseradish peroxidase, Oxidizable drugs

Introduction

The development of the biosensor is an interdisciplinary area for which sharp limits cannot be drawn easily. The concept of the biosensor has evolved as a self-contained analytical device that responds selectively and reversibly to the concentration or activity of chemical species in biological samples. A biosensor can be defined simply as a device that intimately associates a biological sensing element and a transducer (1).

Amperometry is a classical method in electrochemistry. The application of a potential between a reference and an indicator electrode enables a current to be measured when an electroactive analyte is oxidized or reduced, depending on the voltage at the indicator electrode. The current is related to the rate of the electrochemical reaction that occurs (2).

Amperometric biosensors based on the incorporation of biological entities within carbon paste are gaining considerable attention. Coupling plant tissue and pure enzymes as sensitive agents to biosensors enables the detection of a wide range of chemicals from the agricultural, pharmaceutical and fermentation industries (3-9). Such a versatile strategy allows the coimmobilization of the enzyme, its mediator or cofactor, and another enzyme or stabilizer, as needed for a reagentless biosensing device. Short response times result from the absence of supporting membranes and the close proximity of the biocatalytic and graphite sites. The bulk of the carbon paste serves as the source of the biocatalytic activity, and fresh biosurfaces can easily be obtained by renewing the surface (10).

Molecular recognition by enzymes coupled with electrochemical transduction have proved to be a useful approach in the design of chemical sensors for a wide variety of analytes (11). Peroxidases are used in catalyzing the oxygenation of a variety of organic and inorganic substrates by hydrogen peroxide or related compounds (12). With the most commonly used peroxidase, horseradish peroxidase (HRP), virtually any reducing agent may work in this respect, e.g., ferrocyanide, phenolic compounds, o- and p-phenylendiamines, iodide, ascorbic acid, etc. The oxidized reaction product can in turn be electrochemically reduced at an applied potential substantially lower than that of the direct oxidation of hydrogen peroxide (13).

The use of as many as three consecutive enzymatic reactions to generate the amperometric response has also been described. In these types of biosensor, the relative

conversion efficiency of each enzymatic step must be considered (14). We also constructed bienzyme sensors because glucose oxidase undergoes electron transfer with a variety of drugs with OxG. With glucose oxidase and HRP coimmobilized in a carbon paste, OxG can be detected via both a monoenzyme equation (Equation I) and a bienzyme equation (Equation II).

Equation I

$$OxG + H_2O_2 \xrightarrow{HRP} OxG^+ + 2H_2O$$

$$OxG^+ + e^- \xrightarrow{-0.1V} OxG$$

Equation II

$$Glucose + O_2 \xrightarrow{GOX} Gluconolactone + H_2O_2$$

$$H_2O_2 + 2OxG \xrightarrow{HRP} 2H_2O + 2OxG^+$$

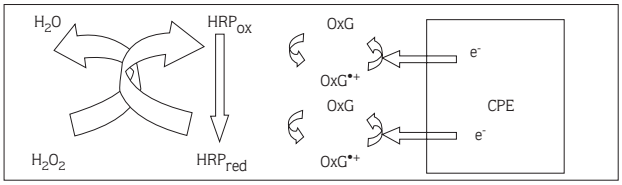
$$OxG^+ + e^- \xrightarrow{-0.1V} OxG$$

With a single sensor, therefore, it is possible to switch between the two detection schemes simply by adjusting the substrate with respect to the enzyme in the carbon paste matrix. At -0.1 V, the bienzyme scheme (Equation II) is referred to. Thus, the HRP/GOX sensor is a convenient model system for the comparison of mono- and bienzyme sensors (14). We demonstrated the utility of a HRP/GOX sensor with successive batch additions of acetaminophen.

In bioelectrochemistry, peroxidase studies have been numerous, and since the late eighties considerable interest has been paid to the development and characterization of HRP-modified electrodes (15-17). Such electrodes allow both direct and mediated electron transfer reactions for substrates, inhibitors and activators of the enzyme. The interest in HRP is derived from the possibility of direct electron transfer between the enzyme and the electrode matrix, allowing the construction of "reagentless" electrochemical biosensors (18).

In the presence of H_2O_2, immobilized peroxidase (HRP Fe^{3+}) is oxidized to compound 1 (oxyferryl iron and porphyrin cation radical). The latter is directly electroreduced (via an intermediate redox state; compound 2) to its native resting state (Fe^{3+}) at the electrode surface (16). The oxidation products formed during the peroxidase reaction depend on the nature of the substrate.

In this study, the bioelectrochemistry of peroxidases was directed for a more detailed presentation of the electrochemical reactions of peroxidase, providing the basis for an amperometric HRP-modified biosensor. The mechanism of amperometric response at the HRP-CPE, in the presence of hydrogen peroxide in solution with electroreduction of HRP accelerated by the oxidizable group (OxG), is shown below:



In parallel to a previous study on the analysis of some phenothiazines by using a HRP-modified carbon paste electrode (HRP-CPE) (18), the analysis of drugs with oxidizable groups (OxG) in the presence of H_2O_2; acetominophen, epinephrine, pyrogallol, pyrocatechol, resorcinol (shown in Figure 1), and also the determination of H_2O_2 using the same procedure was realized. The biosensor response to the drugs above was examined. The experimental parameters of pH, operating potential, reproducibility, change in the percentage of HRP and stability were investigated.

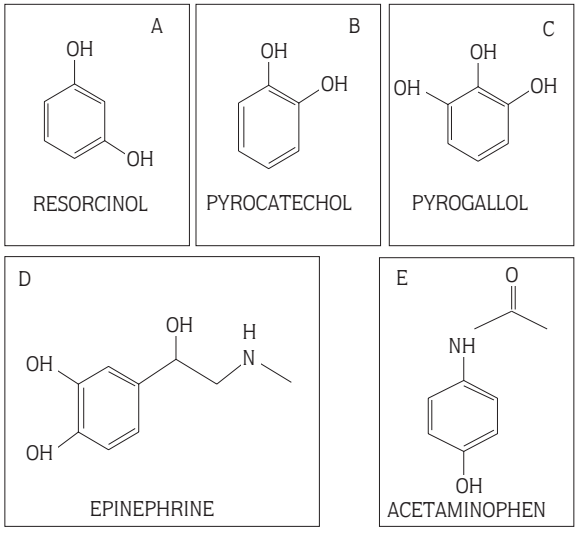


Figure 1. Molecular structure formulas of (A) resorcinol, (B) pyrocatechol, (C) pyrogallol, (D) epinephrine, (E) acetaminophen.

Material and Methods

Reagent

Horseradish peroxidase (EC 1.11.1.7, 51 U/mg) and glucose oxidase (EC 1.1.3.4, 8.4 g solid) were purchased from Sigma. Acetaminophen was obtained from Atabay Chemical Co. TURKEY. Glucose, pyrocatechol, pyrogallol, epinephrine and resorcinol, hydrogen peroxide solution (35 %) were purchased from Merck. The other chemicals were of analytical reagent grade. Double-distilled and deionized water was used in all solutions. 0.5 M acetate buffer solution (pH 4.8) was used as the supporting electrolyte. The stock solutions of acetaminophen, pyrocatechol, pyrogallol, epinephrine and resorcinol were prepared daily, $1x10^{-3}$ M with distilled water, and kept in darkness.

Apparatus

All experiments were performed in a Bioanalytical Systems (BAS) Model VC-2 electrochemical cell. For amperometric measurements, the three electrode system was used; CPE, Ag/AgCI reference electrode, and platinum wire as the auxiliary electrode were used in connection with a Methrom 626 Polarecord Analyzer. A magnetic stirrer and a stirring bar provided convective transport.

Electrode Preparation

Carbon paste was prepared in the usual way by hand-mixing graphite powder (Fisher) and mineral oil (Acheson 38) in a 70:30 weight ratio. The HRP-modified electrode was prepared by thorough mixing of HRP and unmodified carbon paste. The HRP-GOX modified electrode was prepared by thorough mixing of HRP, GOX and unmodified carbon paste. The resulting pastes were packed into the well of the working electrode to a depth of 1 mm. The surfaces were polished on a weighing paper to give a smooth finish before use. The body of the working electrode was a glass tube (3 mm. i.d.) tightly packed with the carbon paste. The electrical contact was provided by a copper wire. The carbon paste was stored at 4 °C when not in use.

Procedure

All measurements were performed at room temperature (25.0±0.5)°C. The solution was stirred with a magnetic bar at approximately 200 rpm. The amperometric measurements were carried out by applying the desired potential and allowing the transient current to decay to a steady-state value before the injection of the drug. At the same time, the amperometric monitoring was carried out.

Results

Analysis of Hydrogen Peroxide:

From an analytical point of view, one interesting feature of a HRP immobilized electrode is the low operating potential (close to 0 V. vs. Ag/AgCl) which offers low background currents and minimizes the risk of surface fouling and interference by electroactive species (18).

In order to select an adequate operating potential, a hydrodynamic voltammogram, as shown in Figure 2, was performed with 5 % (w/w) HRP-CPE by the injection of $1x10^{-2}$ M hydrogen peroxide.

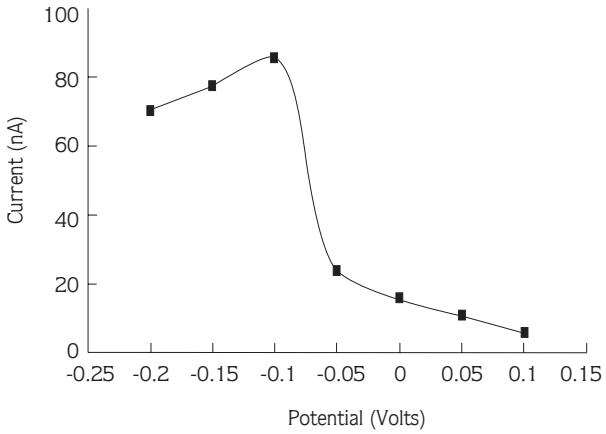


Figure 2. Hydrodynamic voltammogram for 100 µM H_2O_2 at the HRP-modified CPE. Batch experiment, stirring the solution at 200 rpm in 0.5 M acetate buffer (pH 4.8).

The dependence of the 100 µM hydrogen peroxide response on the operating potential in the range from -0.2 V to +0.1 V was also investigated. The current rapidly increased and reached a peak at -0.1 V then sharply decreased between the potentials -0.1 V and +0.1 V. The optimum operating potential selected for further experiments was 0.1 V (vs. Ag/AgCl). This potential corresponds to the electroreduction of the oxidized forms of HRP (HRP_{ox}) (11-13).

Effect of pH:

The optimum operational pH for the HRP-CPE was determined in 0.5 M acetate buffer in the pH range 3.0-9.0 using 100 µM solution of hydrogen peroxide. Amperometric experiments were carried out in the batch setup. The optimum pH range for hydrogen peroxide bioelectroreduction was observed at 4.8, as shown in Figure 3. Therefore, a pH of 4.8 was chosen for further

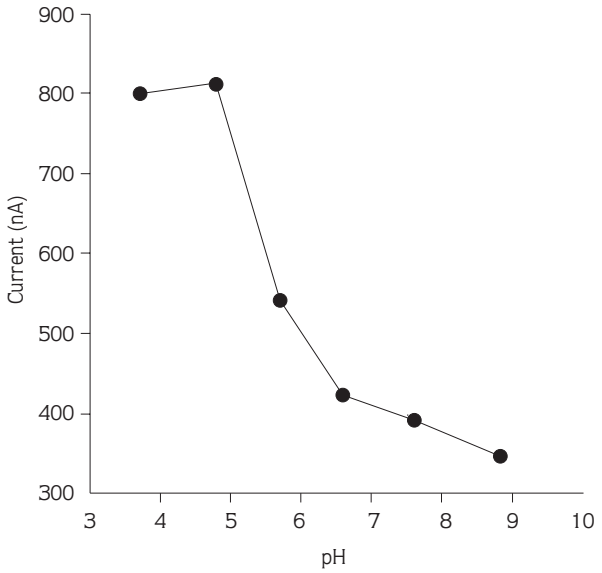


Figure 3. Dependence of the 100 µM H_2O_2 response on the solution pH. Batch experiment, stirring the solution at 200 rpm and -0.1 V operating potential in 0.5 M acetate buffer.

experiments. A similar result was reported by Petit et al. (18) with a HRP-modified CPE.

Effect of change in the percentage of HRP:

The response of the HRP-modified electrode is strongly affected by its composition. The effects of HRP contents in carbon paste were evaluated from calibration graphs for hydrogen peroxide (Figure 4). The optimum percentage of HRP added to the carbon paste was determined in 0.5 M acetate buffer (pH 4.8) by varying the quantity of enzyme between 2.5 % (w/w) and 10.0 % (w/w). Amperometric experiments were carried out in the batch setup. Carbon pastes containing 2.5, 5.0 and 10.0 % (w/w) HRP yielded steady-state currents of 230, 350 and 845 nA, respectively, for 100 µM hydrogen peroxide. As expected from the increased biocatalytic activity of the electrode, the response increased on increasing the amount of enzyme in the paste; however, this occurred nonlinearly. The increased analytical signal was accompanied by a larger amperometric background current. In parallel to Petit et al. (18), the 5 % HRP electrode yielded the most favorable amperometric response characteristics (minimum noise, S/N=3) and was thus employed in all subsequent work.

Repeatability of Intrasurfaces and Intersurfaces:

The validation of the HRP-modified carbon paste electrode was obtained by the relative standard deviation (RSD) of 6.71 % (n=6) in the same surface (between each measurement, the HRP-modified carbon paste

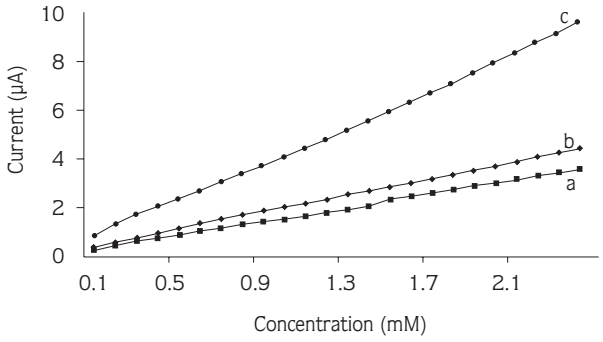


Figure 4. Effect of HRP content in the carbon paste on the hydrogen peroxide response. HRP loadings 2.5 (a), 5.0 (b), 10.0 (c) % (w/w) for carbon paste with 100 µM H_2O_2 increments. Other conditions as in Figure 2.

electrode was rinsed with deionized water) and 12.19 % (n=6) from surface to surface, for 100 µM hydrogen peroxide.

The HRP-modified carbon paste electrode shows amperometric response to successive increments of OxG at an applied potential of -0.1V. The plain electrode gives no response to the addition of OxG. In contrast, if the drug consists of oxidizable groups, the HRP-modified electrode responds rapidly to the change in the substrate concentration and approaches a steady-state response. This procedure provides us with the qualitative detection of OxG.

Figure 5 (A) shows the dependence of the steady state of the HRP-modified carbon paste electrode on the concentrations of five different oxidizable drugs: acetaminophen, pyrocatechol, pyrogallol, epinephrine,

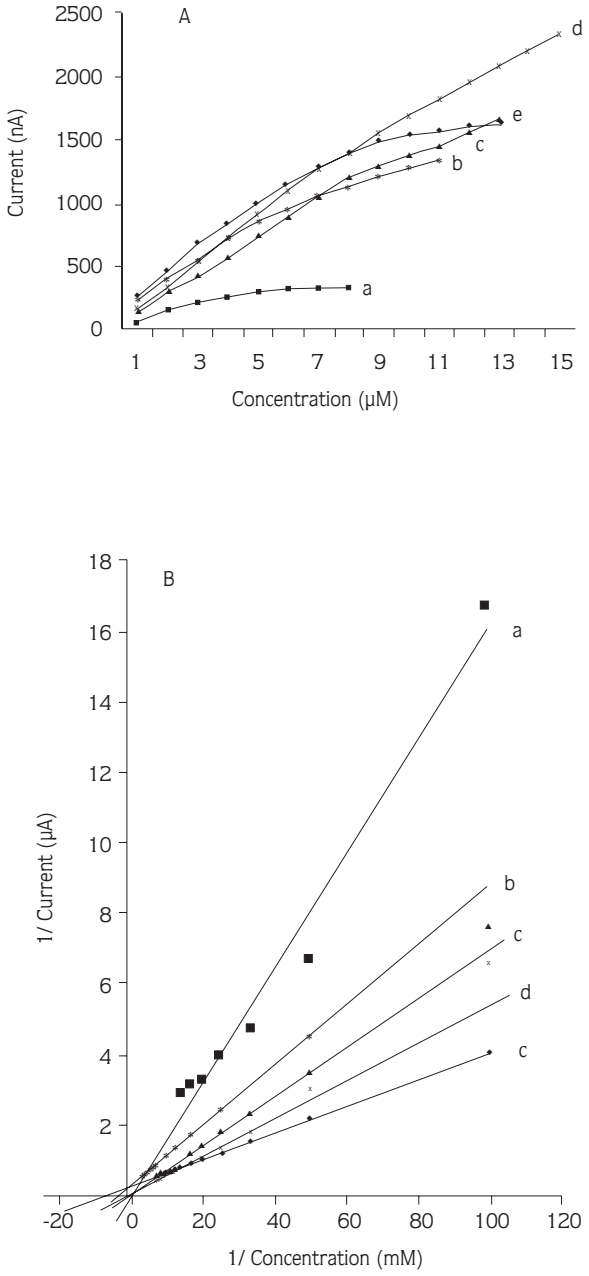


Figure 5. Dependence at the steady state current on the concentration of (a) resorcinol, (b) epinephrine (c) pyrogallol, (d) acetaminophen and (e) pyrocatechol (A) i vs. C; (B) $1/i_{ss}$ vs. 1/C. Other conditions as in Figure 2.

and resorcinol. The increase in response was linear up to 9 μM for acetaminophen, 8 μM for epinephrine and 6 μM for resorcinol, but for pyrocatechol and pyrogallol, it was nonlinear over the 1-13 μM range examined. The detection limits were found to be 1.25 μM, 1.23 μM, 1.15 μM, 1 μM, 0.36 μM for resorcinol, epinephrine, pyrogallol, pyrocatecthol and acetaminophen. Also shown in Figure 5(B) are the corresponding reciprocal plots of $1/I_{ss}$ vs. 1/C. These plots exhibit excellent linearity over the range examined. The slopes of these Lineweaver-Burke type plots allowed calculation of the apparent Michaelis constants; 0.13 mM acetaminophen, 0.24 mM epinephrine, 0.6 mM pyrocatechol, 1.2 mM pyrogallol and 2.4 mM resorcinol. According to these apparent Michaelis constants, the following trend in sensitivity was observed: acetaminophen > epinephrine > pyrocatechol > pyrogallol > resorcinol.

In parallel to Ruzgas et al. (16), it was found in our study that reducing substrates such as uric acid and ascorbic acid in concentrations higher than 10 μM were demonstrated to be interfering for HRP-modified CPE. The amperometric response of the plain and 2.5 % (w/w) HRP and 2.5 % (w/w) GOX containing CPEs to successive increments of 10 μM acetaminophen was demonstrated in the presence of 10 mM glucose at an applied potential -0.1 V. A plain electrode gave no response to the addition of acetaminophen. In contrast, the HRP/GOX-modified CPE responded to the change in the substrate concentration.

The stability of the HRP-modified CPE was observed over a one-month period. A fast decay with up to 30 % depression within 12 days, and a slow decay occurred with the storage of the electrode, dry at 4ºC in between two measurements on the same surface for 100 μM hydrogen peroxide. The loss of response could be because of the degradation of the enzyme, or fouling of the graphite sites by electropolymerized and adsorbed product might account for this failure.

Discussion

The HRP-modified CPE represents an interesting tool for studying the peroxidation of drugs with oxidizable groups. In the present study, the use of HRP-modified CPE in the detection of oxidizable drugs was demonstrated. The high sensitivity and reproducibility, together with the simplicity and low cost, make the HRP-modified electrode very attractive. We envisage that an analytical method such as the one reported here will have widespread applications as it not only provides a direct

assay for hydrogen peroxide, but could also be a component of other drug formulation assay systems in which hydrogen peroxide is a product of one or more further chemical reactions.

Emergency drug assays demand an analytical technique that is simple, rapid and specific for the parent drug. Technological improvements may be foreseen for exploiting the biosensor in therapeutic drug monitoring and diagnosis.

Further modification of the electrode configuration would significantly influence its performance. We are now working toward the development of the biosensor for the determination of oxidizable groups in various drug formulations.

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