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Incorporation of EDTA for the Elimination of Metal Inhibitory Effects in an Amperometric Biosensor Based on Mushroom Tissue Polyphenol Oxidase

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Enzyme based biosensors are highly selective devices which rely on the specific binding of the target analyte (the substrate) to the active-site regions of the enzyme. The enzymatic reaction between mushroom tissue polyphenol oxidase and its substrate phenol is coupled with the use of potassium ferrocyanide ($K_4Fe(CN)_6$) as the mediator in ammonia buffer solution (pH 8.80). The response of devices is often affected by the presence of inhibitors, which combine with the free enzyme in a manner that prevents substrate binding.

The objective of this study is to describe an efficient and yet simple strategy to eliminate the interference of metals in amperometric biosensing. The methods relies on in situ removal of the interfering metal by a surface-bound complexing agent, disodium ethylenediaminetetraacetate (EDTA). EDTA is incorporated into the electrode by mixing it within the enzyme-containing carbon paste matrix. Mixed enzyme/carbon paste electrodes are receiving considerable attention for the preparation of fast responding biosensors.

Introduction

Biosensors are an interdisciplinary area for which sharp limits cannot be drawn easily. The concept of biosensors 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 simply defined, in a first approach, as a device that intimately associates a biological sensing element and a transducer[1].

Amperometry is a well-described method in electrochemistry. The application of a potential between a reference and a working electrode allows one to measure a current when electroactive analyte is oxidized or reduced, depending on the voltage at the working electrode. The current is related to the rate of the electrochemical reaction that occurs[2].

Amperometric sensors based on tissues and pure enzymes continue to be a topic of interest due to their high potential for sensitivity and selectivity[3−9]. Coupling tissues and pure enzymes as sensitive agents for biosensors to enable detection of chemicals of agricultural and pharmaceutical interest has been the subject of a considerable research effort. Tissue-based amperometric biosensors use tissues as enzyme sources while having the advantage of low cost, and higher biocatalytic and graphite sites. The bulk of the carbon paste

serves as a source of the biocatalytic activity, and fresh biosurfaces can easily be obtained by renewing the surface[8−9].

One drawback of tissue-based carbon paste electrodes (CPEs) is the low sensitivity. This can be addressed with the use of mediators[8]. The practical sensing utility of these biosensors has been greatly facilitated by the introduction of mixed tissue/carbon paste amperometric devices, which couple high sensitivity with fast response and recovery times.

The response of the amperometric biosensors is often affected by the presence of inhibitors which combine with the free enzyme in a manner that prevents substrate binding. In particular, inorganic ions such as silver, mercury or lead possess strong affinity for many enzymes and thus greatly influence the rate of many biocatalytic reactions[10]. Indeed, such inhibitory effects have been exploited for indirect assays of toxic metals [11]. However, for measurements of the substrate, it is desirable to investigate means for circumventing the effect of such cations upon the performance of enzyme electrodes.

The objective of the present study is to describe an efficient and yet simple strategy to eliminate the interference of metals in amperometric biosensing. The method relies on the in situ removal of the interfering metal by a surface-bound complexing agent, disodium ethylenediaminetetraacetate (EDTA)[12]. EDTA is incorporated into the surface by mixing it within the enzyme-containing carbon paste matrix. Ligand-containing carbon paste electrodes have also been developed in connection with preconcentration/voltammetric measurements of target metal analytes[13−15].

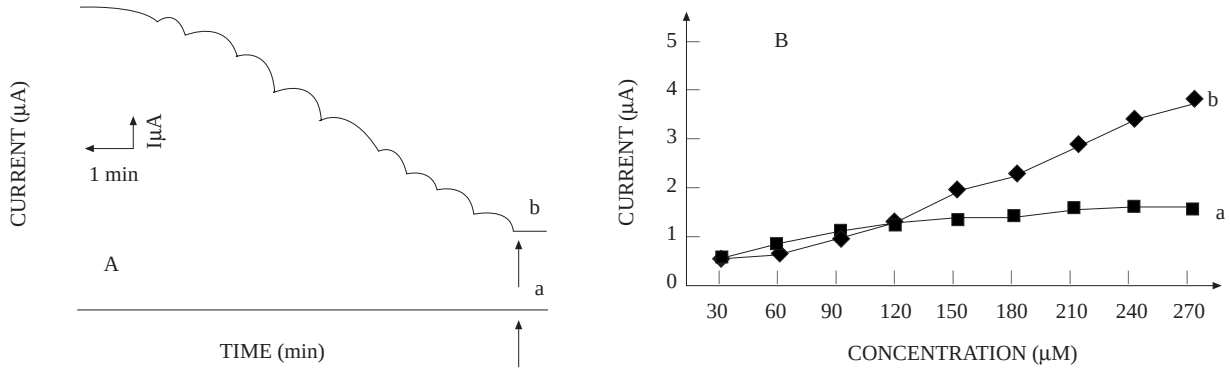
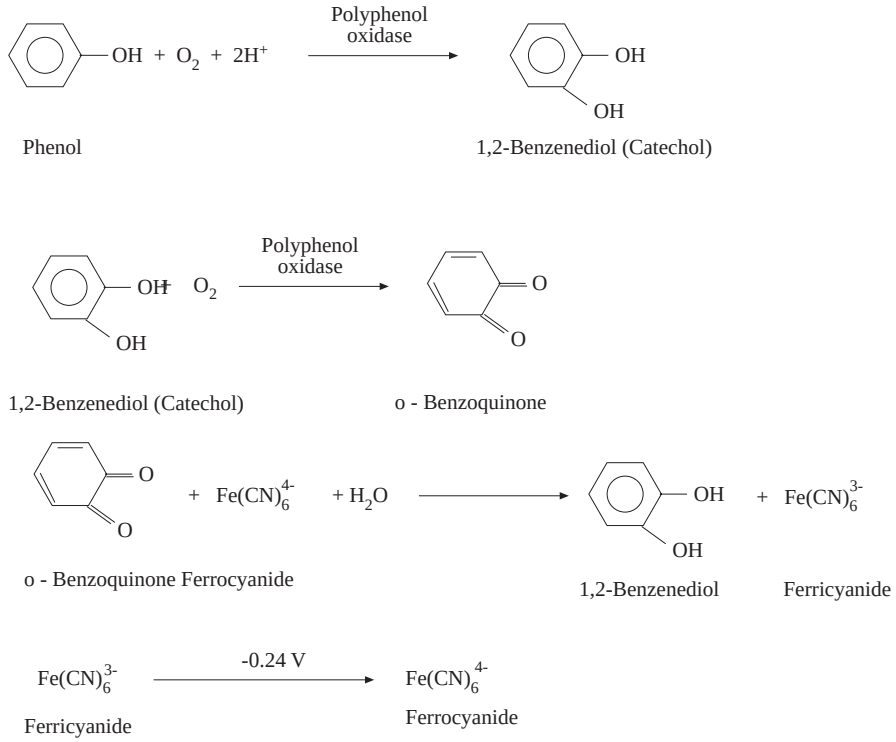


Figure 1. **(A)** Current-time recording obtained at **(a)** the plain and **(b)** 5% (w/w) mushroom tissue containing CPE with increasing concentrations of phenol in 3 x 10^{-5} M steps. Batch experiment, stirring the solution at 400 rpm and -0.24 V operating potential in 0.10 M ammonia buffer; NH_3/NH_4Cl (pH 8.80) containing 30 μM $K_4Fe(CN)_6$. **(B)** The resulting calibration plots at **(a)** the plain CPE and **(b)** the 5% (w/w) mushroom tissue containing CPE with increasing the concentration of phenol in 3 x 10^{-5} M steps. Other conditions are as in Figure 1A.

Because of the toxicological significance of phenolic compounds, a reliable method is desired for their determination in complex environmental and food matrices. Amperometric biosensors, based on the mushroom (Agaricus campestris) polyphenol oxidase (EC 1.14.18.1), have proved to be very useful for this task[3−6]. Polyphenol oxidase is a copper-containing protein which is widely distributed in plants, animals and humans. Polyphenol oxidase catalyzes the hydroxylation of tyrosine in the liver and in melanin-forming cells to 3,4-dihydroxyphenylalanine (dopa). It also catalyzes several reactions in the biosynthesis of melanin pigments and is deficient in patients with type 1 oculocutaneous albinism (OCA 1). Polyphenol oxidase is thought to bind two copper ions, one at each of two conserved sequence motifs, termed CuA and CuB, but

to date this has been directly proved only for the nuerospora and mushroom enzyme[16].

The electrochemical redox reactions taking place with phenolic compounds can be summarized in the scheme below :



A plain electrode gives no response to the addition of phenol. In contrast, the mushroom tissue modified electrode responds rapidly to the change in the substrate concentration, approaching a steady state response within one minute as shown in Figure 1A. K_4 $Fe(CN)_6$ was used as the electron mediator because it is electrochemically reversible[4−6] and water soluble and needs a low applied potential. As observed in the scheme above, K_4 $Fe(CN)_6$ reduces the oxidized analyte and permits the analyte cycling. Then K_3 $Fe(CN)_6$ is reduced back to K_4 $Fe(CN)_6$ under the operating potential of -0.24 V.

A flow probe integrating the sample manipulations and the electrochemical detection process in a Lab-on-Cable form was reported by Wang et al. The enzymatic amperometric assays of phenolic compounds and hydrodynamic voltammetric detection of hydrazine and peroxide species was demonstrated with the elimination of errors (e.g., pH or natural-convection effects) common to remote sensors[17].

Wang et al. also described a new approach for the amperometric determination of hydrazine compounds. The inhibitory action that the hydrazine compounds exerted on the activity of polyphenol oxidase provided the basis for the procedure. The biocatalytic activity acquired from the inhibitor-enzyme interaction was observed from the decreased current response of polyphenol oxidase to the phenol[18].

In the following sections, we will illustrate that the coimmobilization of enzymes and complexing agents within the carbon paste environment represents a convenient approach for circumventing metal inhibition problems characterizing amperometric biosensors. Such development should facilitate the application of biosensors in complex samples containing high metal concentrations.

Experimental

Apparatus

Experiments were performed in a 10 mL cell. For amperometric measurements, the three electrode system was used, which was composed of the working electrode, Ag/AgCl reference electrode, and platinum wire as auxiliary electrode in connection with a Methrom 626 Polarecord Analyzer. A Velp Scientifica magnetic stirrer and a stirring bar provided convective transport. A glassy carbon electrode (GCE) was obtained from Metrohm.

Electrode preparation

The modified carbon paste was prepared in two steps. The first involved a thorough mixing of the desired amounts of graphite powder and mineral oil (Acheson 38, Fisher) (30/70% (w/w) carbon/oil). Then 150 mg of the initial paste was mixed with 10 mg of the mushroom tissue and 40 mg of EDTA disodium salt. A portion of the resulting paste was packed into the end of a 3-mm-i. d. glass tube. Electrical contact was established via a copper wire. The surface was smoothed on a weighing paper. Ordinary mushroom tissue modified CPE was prepared as explained above without the incorporation of EDTA. Ordinary CPE was prepared in a similar fashion without the mushroom tissue in the carbon paste matrix.

The coating of the working electrodes with Nafion was performed by using 0.5 % Nafion solution, which was prepared by diluting 50 μL of the commercial Nafion (Dupont) solution in 400 μL of ethanol and 50 μL of deionized water. Then 10 μL of 0.5% Nafion solution was placed on the surface of the working electrode and the solvent was allowed to evaporate at room temperature for 10 min.

GCE pretreatment

Before each measurement, the GCE was first polished on a polishing pad with 1 μm alumina and rinsed with water; then it was smoothed with 0.15 μm alumina and rinsed with water. Residual polishing material was removed from the surface by sonication in a water bath for 1 min after each polishing process.

Reagents

Solutions were prepared from reagent grade chemicals with deionized water. Phenol was obtained from Sigma. Potassium ferrocyanide was obtained from Aldrich. The disodium salt of EDTA was obtained from Fisher. The nitrate salts of the metals were used to prepare metal stock solutions. Mushrooms (Agaricus campestris) were obtained daily from local grocers

Procedure

All measurements were performed at room temperature (25.0°C±0.5). Amperometric biosensing proceeded under batch conditions with 400 rpm stirring. The desired working potential was then applied and transient currents were allowed to decay to steady state values before the injections of the substrates. At the same time, amperometric measurement was completed.

An amperometric calibration curve of phenol at a pretreated GCE was realized by applying - 0.24 V operating potential in the 0.1 M ammonia buffer: NH_3/NH_4Cl (pH 8.80) solution containing 20% (w/w)

EDTA and 30 μM $K_4Fe(CN)_6$ in the presence and absence of 1 x 10^{-4} M magnesium ions with increasing concentrations of phenol in 3 x 10^{-5} M steps.

Results and Discussion

The ability to circumvent the inhibition interference of metal cations at biocatalytic electrodes is illustrated in the following sections using mushroom polyphenol oxidase as a model enzyme and in the presence of EDTA as the complexing agent. The broad coordination action of EDTA makes it an ideal choice for the task of removal of most cationic interferences. The CPE configuration is particularly useful for this task, as it allows controlled loading of polyphenol oxidase and EDTA. The amount of incorporated EDTA and the immediate proximity of the enzyme and ligand sites greatly facilitate the rapid and effective removal of metal cations approaching the surface. The biosensor operates in the amperometric mode for monitoring the phenol substrate through cathodic detection of the enzymatically liberated ferricyanide species (the oxidized form of the mediator) and electrochemical regeneration of the ferrocyanide. Various metal cations, e.g., Ca^{2+}, Mg^{2+} and Zn^{2+}, known to inhibit this biocatalytic process[19], have been tested. The high stability of the EDTA complexes of these cations ($\log K_f$ of 10.7, 8.7 and 16.5, respectively) greatly minimizes the competitive formation of the metal (inhibitor)-enzyme complex and hence circumvents the metal inhibiton process.

The validation of the Nafion coated enzyme-EDTA containing CPE was obtained by the relative standard deviation (RSD) of 10.8% (n=5) in the same surface (between each measurement, the working electrode was rinsed with deionized water) and 12.5% (n=5) from surface to surface, for 3 x 10^{-5} M phenol. The validation of the ordinary CPE was obtained by the RSD of 9.6% (n=5) in the same surface (between each measurement, the working electrode was rinsed with deionized water) and 11.3% (n=5) from surface to surface, for 3 x 10^{-5} M phenol. The signal-to-noise (S/N=3) characteristics indicate a detection of 0.90 μM phenol and the resulting calibration plot is also shown in Figure 1B.

The efficiency of the metal removals was also strongly affected by the paste composition. It was found that the effect of the interferents such as uric acid and ascorbic acid was eliminated by the coating of Nafion onto the CPE surface. The amperometric response of the ordinary and Nafion coated enzyme-EDTA containing CPEs to successive increments of 10 μM ascorbic acid and 10 μM uric acid was demonstrated at an applied potential of -0.24 V. The Nafion coated enzyme-EDTA containing CPE gave no response to the addition of uric acid and ascorbic acid. In contrast, the ordinary electrode with no Nafion coating responded to the changes in the interferent concentration. The coating of Nafion on the CPE surface had no effect on the voltammetric signal of phenol in comparison with the voltammetric signal of phenol obtained with the ordinary CPE. Nafion-coated enzyme-EDTA containing CPEs were employed for all further experiments.

An amperometric calibration curve of phenol under the same experimental condition at a non-enzymatic GCE was realized in the presence and in the absence of magnesium ions by applying - 0.24 V (not shown). Since magnesium showed the lowest stability of EDTA-metal complex ($\log K_f$), the possible interaction between $K_4Fe(CN)_6$ and the studied metal would affect the voltammetric signal of phenol most. The slope of the amperometric curve in the presence of magnesium ions was found to be 2.15 and the slope of the amperometric curve in the absence of magnesium ions was found to be 2.08 under the same experimental conditions. It was observed that there was no interaction between the mediator and the studied metal, because the slopes of the curves were nearly identical.

The possible interaction of the mediator with magnesium was also investigated at the enzymatic electrode in the absence of the mediator. The detection of o-quinone was realized and the amperometric signals showed inhibition suppression because of the EDTA incorporated within the CPE matrix (not shown). The amperometric signals were considerably lower than the ones obtained in the presence of the mediator in the solution, because the mediator catalyzes the electron transfer to the surface of the working electrode as shown in the scheme. The other reason for using $K_4Fe(CN)_6$ is the small amount of enzyme provided by the mushroom tissue in the carbon paste matrix. The same experiments using commercially available pure enzymes may obviate the use of a water soluble mediator.

The effect of the EDTA content upon the suppression of the metals on the biosensing signal was also studied. The enzyme inhibition by the metals rapidly decreased upon increasing the percentage of EDTA within the carbon paste. Negligible inhibition effects were observed for pastes containing more than twenty percent (w/w) of the complexing agent. Such profiles were expected from the increased binding capacity of the electrode. 20% (w/w) of EDTA within the carbon paste was used for further experiments. It was also observed that the incorporation of EDTA in the carbon paste matrix was more advantageous than just having EDTA in the solution (not shown). The amperometric signals obtained with the EDTA-modified CPE were higher than the ones obtained with the EDTA in the solution. These results showed that the inhibiton by metals on the polyphenol oxidase activity was still observed with EDTA in the solution. Since the electrochemical reactions took place at the surface of the working electrode, the EDTA incorporated within the carbon paste matrix provided better inhibition suppression.

Figure 3 illustrates the prevention of interferences by metal ions through the incorporation of EDTA within the carbon paste matrix. It displays the current response of polyphenol oxidase modified biosensors, with (a) and without (b) the incorporated ligand, to 3 x 10^{-5} M addition of phenol(s), followed by five repetitive additions of 1 x 10^{-4} M of calcium (A), magnesium (B) and zinc (C) inhibitors (in). Both electrodes offered a similar response to the phenol substrate, indicating that the presence of EDTA was not influencing the sensitivity and speed of the response. The steady-state signal was achieved within 20 s. However, at the plain (ligand-free) electrode, the response decreased rapidly upon adding the metal ions. In contrast, the phenol signal at the ligand-containing CPE was not affected by the additions of the inhibitors. Apparently, the added cations were rapidly being collected by the surface-bound EDTA (Figure 2).

The severity of the metal inhibition problem in the absence of EDTA precluded quantitation of the phenol substrate. In contrast, the ligand-containing biosensor offered convenient measurement of these micromolar concentrations. A fast and sensitive response and a low noise level were observed in this study.

Calibration plots from the data of Figure 2 are shown in Figure 3. Several points were noted from Figure 3. First, the curves at the EDTA-containing electrode in the presence of the metals were nearly identical with the range of 0.03-0.09 μM phenol. The second point observed in Figure 3 is that in the absence of metals the plain enzyme electrode yielded a response larger (by 15%) than the ligand-containing surface. Such behavior was attributed to the higher content (% w/w) of the graphite sensing sites at the plain enzyme electrode. The third point that can be observed in Figure 3, poor sensitivity was observed in the presence of the metals at the plain enzyme electrode, in comparison to the high sensitivity inherent to the EDTA -modified biosensor. It was also demonstrated that as the stability of EDTA-metal complexes ($\log K_f$) increased, the voltammetric signal of the phenol in the presence of the metal increased. The efficiency of the collection of calcium (A), magnesium (B) and zinc (C) ions by the surface-bound ligand is illustrated in Figure 3. In addition, these data indicated that interactions of the metals with the ammonia buffer or with

the ferrocyanide had a negligible effect upon the behavior reported in this paper.

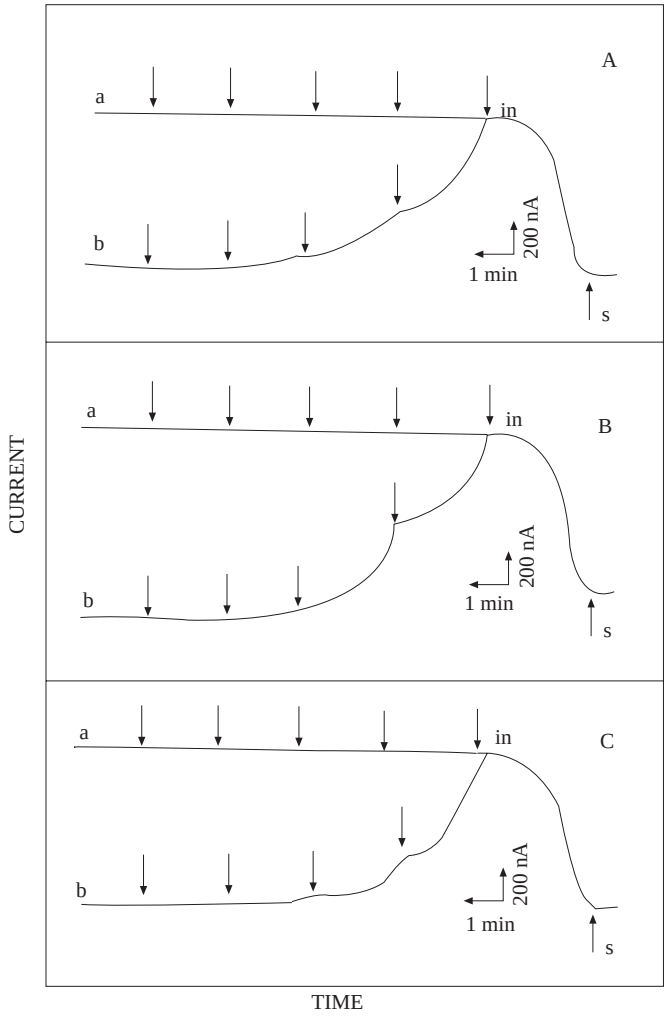


Figure 2. Current-time recordings at 5% (w/w) mushroom tissue/CPE containing **(a)** 20% (w/w) EDTA and **(b)** 0 % (w/w) EDTA upon increasing the calcium **(A)**, magnesium **(B)** and zinc **(C)** ion concentration in 1 x 10 $^{-4}$ M steps. **S** represents the initial responds to the substrate (3 x 10^{-5}M phenol), while represents first inhibitor (metal ion) addition. Other conditions are as in Figure 1A.

The use of a mushroom tissue modified CPE illustrates the fundamentals of electrochemical and biosensor concepts. It reinforces the underlying principles of ligand-containing electrodes which are employed to circumvent enzyme inhibition processes. EDTA provided high stability to the substrate determination in the presence of interfering metals. In this experiment, mushroom tissue polyphenol oxidase was chosen as a model system because the homogeneous enzyme kinetics are well characterized and is reasonably stable. The mixed carbon paste biosensor configuration permits simultaneous incorporation of additional modifiers, as needed, to eliminate other interferences. Other ligands (e. g., dithizone) may offer similar advantages when inhibition by certain metals (e.g., mercury or silver) is concerned. The experiment can easily be modified for other important enzyme electrodes and either the electrochemical or analytical information or both can be emphasized.

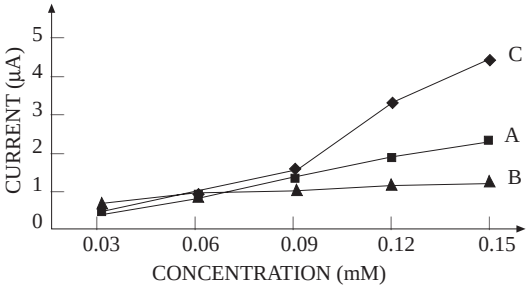


Figure 3. Calibration plots for phenol at 5% (w/w) mushroom tissue/CPE containing 20% (w/w) EDTA in presents of 1 x 10^{-4} M **(A)** calcium, **(B)** magnesium and **(C)** zinc ions. Other conditions are as in Figure 1A.

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