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# Epinephrine Biosensor Using Tyrosinase Immobilized Eggshell Membrane

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**Abstract:** Electrochemical biosensor based on immobilized tyrosinase was developed for the determination of epinephrine. The enzyme was extracted from a plant source *Amorphophallus companulatus* and immobilized on eggshell membrane. Tyrosinase catalyses oxidation of epinephrine to epinephrinequinone which is electrochemically reduced at -0.17 V versus Ag/AgCl (3 M KCl). The resulting current was used for epinephrine quantification. The sensor showed linearity in the range 3 x $10^{-5}$ to 3 x $10^{-4}$ M with a detection limit of 1 x $10^{-5}$ M. It has reusability up to 15 cycles and a shelf life of more than 6 months when stored at 4° C. *Copyright © 2009 IFSA.*

**Keywords:** Tyrosinase, Epinephrine, Biopolymers, Electrochemical biosensor

## 1. Introduction

Epinephrine (EN) is one of the important members of catecholamine family that acts as neurotransmitter and hormone. A number of methods have been developed for the quantification of epinephrine such as spectrophotometry, fluorimetry, liquid chromatography, chemiluminescence and piezoelectric detection [1-5]. Since epinephrine is an electroactive molecule, many electroanalytical methods based on chemically modified electrodes have also been reported [6-9]. Electrochemical biosensors based on immobilized tyrosinase represent potential alternatives to these techniques as they offer advantages such as low cost and fabrication simplicity [10, 11].

Tyrosinase (or Polyphenol oxidase) is a binuclear copper containing metalloprotein (EC 1.14.18.1) that catalyzes the oxidation of phenolic compounds via hydroxylation with molecular oxygen to catechols and subsequent dehydrogenation to *o*-quinones [12]. Quinones are electroactive species that can be reduced at low potentials [13].

Use of biocompatible matrix for enzyme immobilization is crucial for maintaining the functionality of biomolecule while providing accessibility towards the target analyte. We have reported different biopolymer matrices for immobilization of various enzymes [14-18]. Recently we have shown that eggshell membrane is a suitable bioplatform for tyrosinase immobilization [19]. In the present work, we report development of epinephrine biosensor using simple, easy and economical method of tyrosinase immobilization on eggshell membrane.

## 2. Materials and Methods

### 2.1. Materials

Tyrosinase derived from a plant source *Amorphophallus companulatus* [20] was used. Raw membrane-bound eggshells were collected from a campus canteen and stored in water. Epinephrine was purchased from Sigma chemical (USA). Twenty five percent glutaraldehyde was purchased from Sisco Research Laboratory, India. Sodium phosphate buffer solutions with different pH and concentrations were prepared with water from Millipore Milli-Q system.

### 2.2. Immobilization of Tyrosinase on Eggshell Membrane

Tyrosinase was immobilized on eggshell membrane as described earlier [19]. Briefly, an eggshell membrane was carefully peeled from a broken fresh eggshell after the albumin and yolk had been removed. It was cleaned with distilled water. The membrane was cut into small pieces of 1 $cm^2$ area. An aliquot of 500 μL of 25 % glutaraldehyde was taken in eppendorf tube. The eggshell membrane piece was then dipped into glutaraldehyde solution. After 5 min. incubation, glutaraldehyde activated membrane was then transferred to an eppendorf tube containing 500 μL of undiluted tyrosinase extract for 20 min. The membrane was then immersed and washed with phosphate buffer (pH 6.0). Finally, the tyrosinase-immobilized eggshell membrane was stored in phosphate buffer (pH 6.0) at $4^o$ C until further use.

### 2.3. Fabrication of Enzyme Electrode and Electrochemical Measurement

The tyrosinase-immobilized eggshell membrane was positioned on the surface of a glassy carbon electrode (GCE) and kept in a steady position by an O-ring. Electrochemical experiments were done in a cell employing three-electrode system with Ag/AgCl (3 M KCl) and a platinum wire as a reference and an auxiliary electrode, respectively. Cyclic voltammetric measurements were performed by scanning the potential between + 1.0 V to – 1.0 V at a scan rate of 100 mV/s. Cathodic peak currents of epinephrine were used for analytical measurements. Low concentrations of epinephrine were analyzed by differential pulse voltammetry (DPV) at modified GCE.

## 3. Results and Discussion

### 3.1 Tyrosinase Catalyzed L-dopa Oxidation

L-dopa in contact with enzyme tyrosinase is oxidized to dopaquinone. Spectrophotometric investigation shows a very interesting behaviour of tyrosinase catalyzed reaction (Fig. 1). L-dopa is oxidized by tyrosinase to form leucodopachrome, which shows high absorptivity in the UV region ($\lambda$ = 310 nm). This product forms dopachrome, a scarlet red colored compound, with maximum absorption at $\lambda$ = 475 nm. There are at least three wavelengths where dopa can be monitored: 280, 310 and 475 nm. Measurements at 280 nm can be easily affected by the presence of other aromatic species (example: benzoic acid). At 310 nm, the molar absorptivity is much higher and in this wavelength the interference of aromatic compounds will be smaller. The last absorption peak (maximum at $\lambda$ = 475 nm was attributed to the formation of dopachrome. This species is dependent of the formation of leucodopachrome and the signal measured was smaller. Our preference in the spectrophotometric study was for recording absorbance at 475 nm. Felix et al reported similar observations for epinephrine [21].

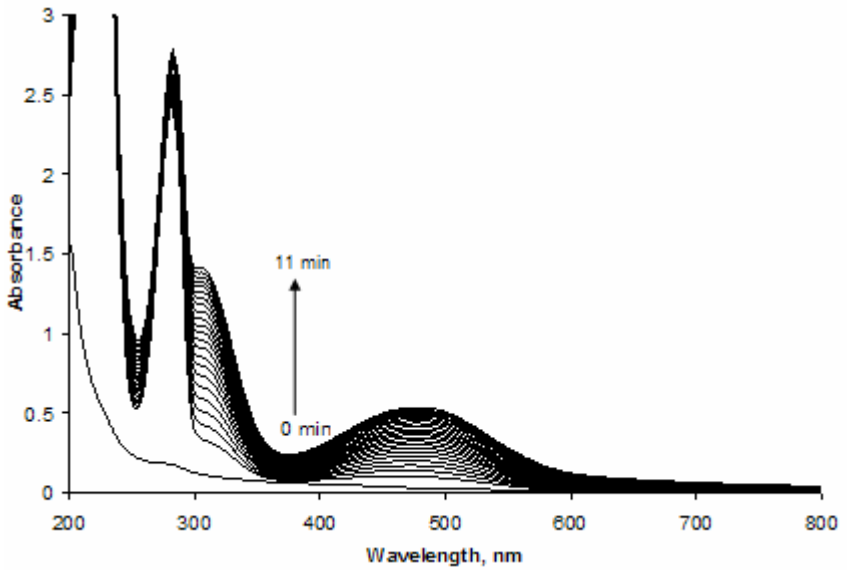
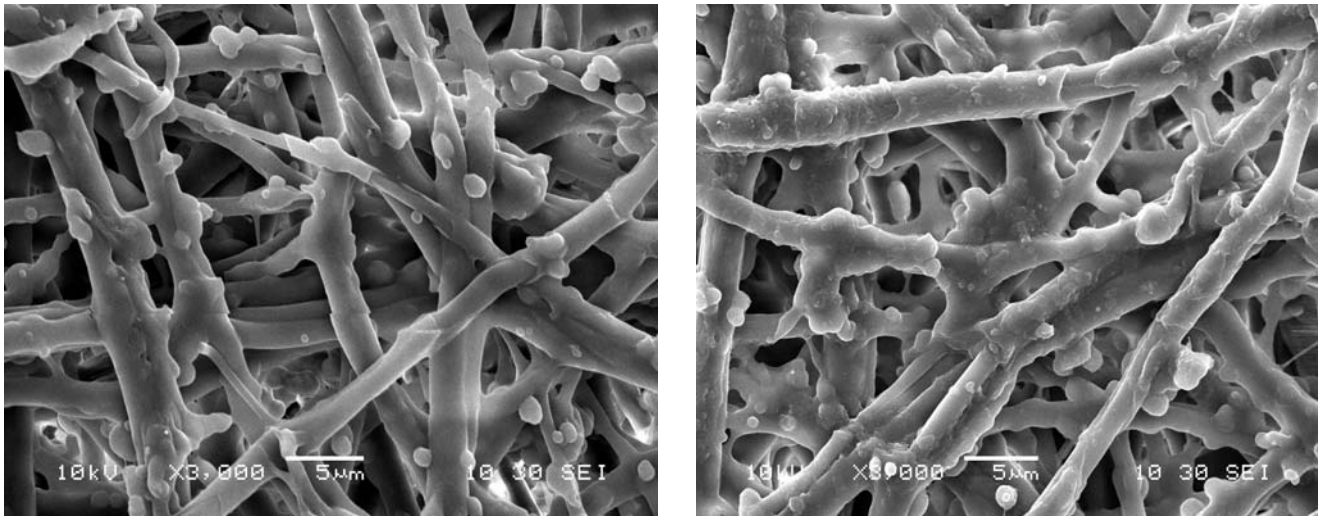



**Fig. 1.** Absorption spectra of 6.7 x $10^{-4}$ M L-dopa solution in phosphate buffer (0.1 M, pH 6.0) reacting with enzyme tyrosinase recorded for 11 min with 30 sec interval.

### 3.2. The microstructure of the Eggshell Membrane

Fig. 2 shows the scanning electron micrographs of the eggshell membranes without (Fig. 2(a)) and with (Fig. 2(b)) the immobilized tyrosinase.

The eggshell membrane consisting of highly cross-linked protein fibers (ca. diameter 1–5 μm) and cavities make it gas and water-permeable. Some specks or clusters of enzymes can be clearly seen on the protein fibers (Fig. 2(b)) whereas Fig. 2 (a) does not show the attachment of enzymes on the protein fibers. The immobilized enzyme can only be observed as some condensed spots on the fibers as SEM images cannot visualize the individual size of tyrosinase. The results are in agreement with earlier report on microstructure of eggshell membrane before and after uricase immobilization [22]. It is clear that tyrosinase has been successfully immobilized on the eggshell membrane as it responds well to its substrate dopamine hydrochloride and this will be discussed in the following section.




(a) (b)

**Fig. 2.** The scanning electron micrographs of the eggshell membranes without (Fig. 2(a)) and with (Fig. 2(b)) the immobilized tyrosinase.

### 3.2. Kinetic Characterization of Immobilized Tyrosinase Eggshell Membrane

The influence of pH was investigated over the range 4.0-7.0. The optimum pH was found to be 6.0. The effect of temperature on enzyme activity was also investigated, and the optimum temperature was found to be 30 °C. The maximum reaction rate ($V_{max}$) and the Michaelis –Menten constant ($K$m) for tyrosinase were calculated for epinephrine using Lineweaver–Burk plot (Fig. 1). Km was found to be 1.67 mM and Vmax was 0.142 mM/min. All these parameters were determined spectrophotometrically.

### 3.3. Electrochemical Response Characteristics of Epinephrine Biosensor

The cyclic voltammogramms for varying epinephrine concentrations at unmodified eggshell membrane-covered glassy carbon electrode are shown in Fig. 2. As can be seen, a well defined redox wave of EN was observed. The oxidation peak appeared at + 0.65 V and the reduction peak was seen at – 0.45 V. This confirms the suitability of eggshell membrane as effective support matrix for electron transfer between the electrode surface and the electroactive species in the solution.

The cyclic voltammogramms of tyrosinase immobilized eggshell membrane-covered electrode in sodium phosphate buffer (pH 6.0, 0.1 M) without EN and with varying EN concentrations are shown in Fig. 3. Reduction peak at -0.25 V is due to the reduction of quinone species liberated from the reaction catalysed by tyrosinase on enzyme electrode. Oxidation peak of epinephrine appeared at + 0.45 V, which is 200 mV less negative than that at unmodified eggshell membrane-covered electrode; reduction peak appeared at – 0.25 V at the electrode is 200 mV less positive than that at eggshell membrane-covered electrode. The results implied that the tyrosinase immobilized eggshell membrane efficiently catalyze the oxidation of epinephrine. The sharp oxidation and reduction peaks associated with an enhancement in peak current reflect a fast electron transfer at tyrosinase immobilized eggshell membrane-covered electrode.

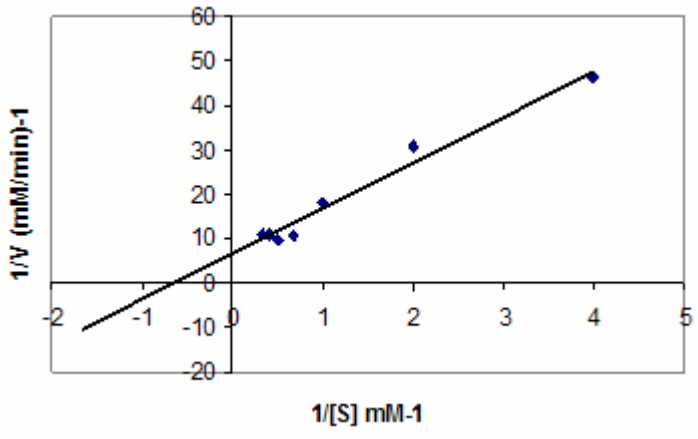



**Fig. 3.** Lineweaver-Burk plot obtained for epinephrine with immobilized tyrosinase eggshell membrane.

DPV peak current increased with increasing epinephrine concentration (Fig. 4). Reduction current measured at -0.17 V was indicative of enzymatic activity. The calibration curve for epinephrine is shown in Fig. 5. The linear range of 3 x $10^{-5}$ to 3 x $10^{-4}$ M (r = 0.9734) with a detection limit of 1 x $10^{-5}$ M was observed (Fig. 5 inset). The sensor performance is comparable to earlier report on epinephrine biosensor [11]. From the Lineweaver–Burk plot (based on the data of Fig. 5), an apparent *K*m value of 0.2 mM was obtained for epinephrine.

The sensor showed reusability up to 15 cycles and a long-term storage stability of 6 months when stored at 4$^{o}$ C.

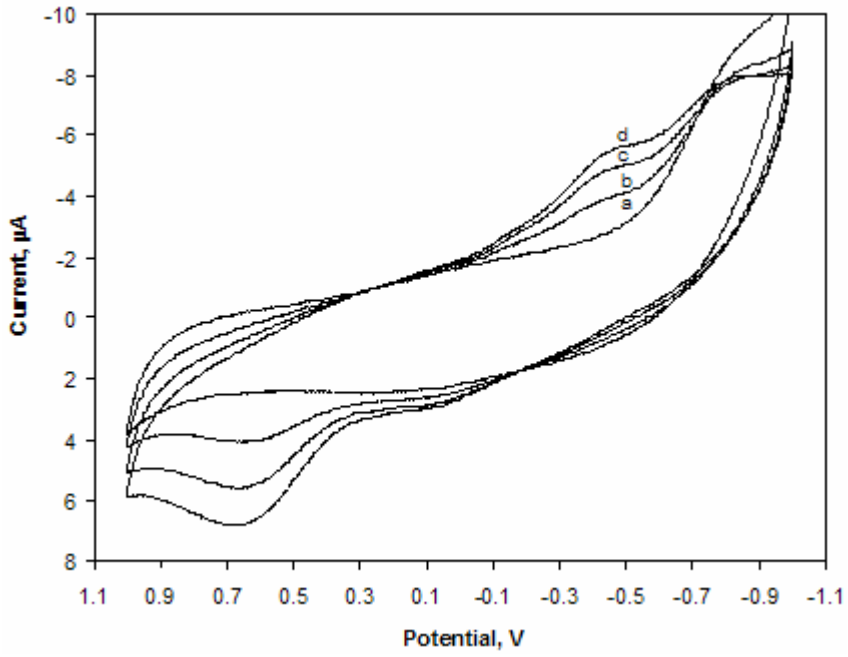



**Fig. 4.** Cyclic voltammogramms obtained at unmodified eggshell membrane-covered glassy carbon electrode for solutions of increasing epinephrine concentration from 0 µM (a), 100 µM (b), 200 µM (c) and 300 µM (d). Scan rate: 100 mV/s. Supporting electrolyte: Phosphate buffer (0.1 M, pH 6.0).

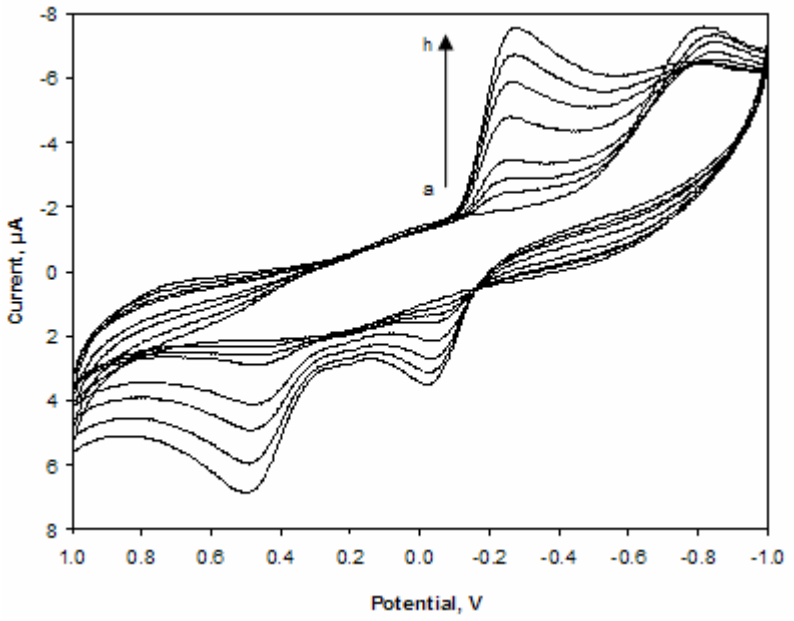



**Fig. 5.** Cyclic voltammogramms obtained at immobilized tyrosinase eggshell membrane electrode for solutions of increasing epinephrine concentrations (a – h): 0, 30, 50, 100, 200, 300, 400 and 500 µM. Scan rate: 100 mV/s. Supporting electrolyte: Phosphate buffer (0.1 M, pH 6.0).

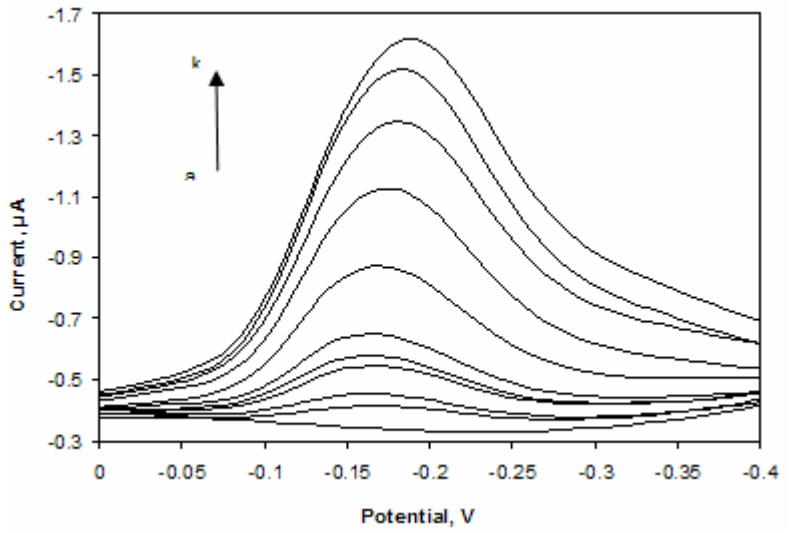



**Fig. 6.** Differential pulse voltammogramms obtained at immobilized tyrosinase eggshell membrane electrode. EN concentrations (a-k): 0, 10, 20, 30, 40, 50, 100, 200, 300, 400 and 500 µM. Potential: -0.17 V, Scan rate: 0.020 V/s, amplitude: 0.025 V, Electrolyte: Phosphate buffer (0.1 M, pH 6.0).

### 3.4. Differential Electrochemical Detection of Dopamine and Epinephrine

Dopamine (DA) and epinephrine (EN) are two important catecholamines which are structurally similar; therefore, difficult to differentiate electrochemically. Electrochemical behavior of dopamine at tyrosinase immobilized eggshell membrane was studied earlier [19]. The cathodic peak was observed at -0.08 V for dopamine whereas for epinephrine it is -0.17 V. This peak separation ($\Delta E_a$) of about -0.09 V indicates that the tyrosinase immobilized eggshell membrane can separate the cathodic peak of DA and EN.

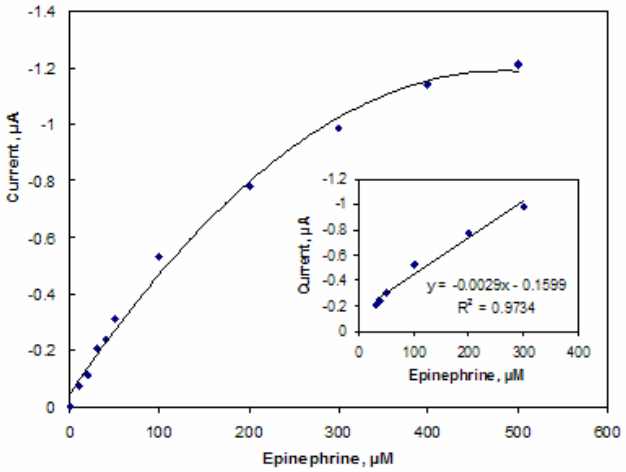



**Fig. 7.** Calibration curve for epinephrine obtained with immobilized tyrosinase eggshell membrane electrode. Inset showing linear response. Potential: -0.17 V versus Ag/AgCl (3 M KCl), Supporting electrolyte: Phosphate buffer (0.1 M, pH 6.0).

## 4. Conclusions

A favorable use of tyrosinase immobilized eggshell membrane as support matrix for the development of epinephrine biosensor is described. The advantages of this sensor are its low cost, fabrication simplicity and short time for enzyme electrode assembly. The analytical characteristics of this sensor, including linear range, lower detection limit and kinetic constants are described. The biosensor exhibited good performance in terms of reusability and storage stability.

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- Sensor instrumentation;
- Virtual instruments;
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