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Glutaraldehyde activated eggshell membrane for immobilization of tyrosinase from *Amorphophallus companulatus*: Application in construction of electrochemical biosensor for dopamine

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ABSTRACT

Tyrosinase from a plant source *Amorphophallus companulatus* was immobilized on eggshell membrane using glutaraldehyde. Among the three different approaches used for immobilization, activation of eggshell membrane by glutaraldehyde followed by enzyme adsorption on activated support could stabilize the enzyme tyrosinase and was found to be effective. K_m and V_{max} values for dopamine hydrochloride calculated from Lineweaver-Burk plot were 0.67 mM and 0.08 mM min^{-1}, respectively. Studies on effect of pH showed retention of more than 90% activity over a pH range 5.0–6.5. Membrane bound enzyme exhibited consistent activity in the temperature range 20–45 °C. Shelf life of immobilized tyrosinase system was found to be more than 6 months when stored in phosphate buffer at 4 °C. An electrochemical biosensor for dopamine was developed by mounting the tyrosinase immobilized eggshell membrane on the surface of glassy carbon electrode. Dopamine concentrations were determined by the direct reduction of biocatalytically liberated quinone species at −0.19 V versus Ag/AgCl (3 M KCl). Linearity was observed within the range of 50–250 μM with a detection limit of 25 μM.

1. Introduction

Designing new protocol that improves enzyme stability during immobilization is an exciting area of research for the application in construction of enzyme-based biosensors. Though there are different approaches to modulate enzyme characteristics including genetic engineering, chemical modification, inclusion of additives, immobilization of enzyme has been continued to be the most widely used method for stabilizing the enzyme [1–3]. Both, the choice of support material and immobilization method, can influence the enzyme activity and its kinetic properties. The conventional methods of enzyme immobilization include adsorption, cross-linking with glutaraldehyde, covalent attachment and entrapment/encapsulation within polymeric gels or carbon paste.

Tyrosinase (or polyphenol oxidase) is a binuclear copper containing metalloprotein (EC 1.14.18.1) that catalyzes, in presence of molecular oxygen, two different reactions: hydroxylation of monophenols into diphenols and oxidation of diphenols to *o*-quinones [4]. It is a broad substrate spectrum enzyme that finds application in several areas such as

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environment, foods, pharmaceuticals and clinical diagnostics [5–9]. Analytical role of tyrosinase in terms of biosensor was first reported by Macholan and Schanel, who measured O_2 consumption in determination of phenolic substrate [10]. Since then, tyrosinase based biosensors have gained major share of attention in monitoring phenols. Besides the different sources of tyrosinase, a wide variety of matrices including carbon paste [11], nafion membrane [12], hydrogel [13], graphite [14], conducting polymers [15,16], biopolymers [17] and nanoparticles [18] have been used in construction of sensor probes.

Biopolymers, in general, have unique properties such as hydrophilicity, biocompatibility, biodegradability, non-toxicity and susceptibility to chemical modification. Recently, we reported a novel biopolymer matrix, agarose-guar gum for immobilization of invertase [19]. Agarose and guar gum are naturally occurring biopolymers having high permeability towards water. The same support matrix was used for tyrosinase immobilization and subsequent development of electrochemical biosensors [20,21]. In the present investigation, we are reporting eggshell membrane as carrier matrix for tyrosinase immobilization. Martin Choi's group has reported eggshell membrane based biosensors wherein enzymes catalase, glucose oxidase, myrosinase, uricase, amino acid oxidase were immobilized using glutaraldehyde [22–26].

Here, we report immobilization of tyrosinase from *Amorphophallus companulatus* on eggshell membrane and its characterization for kinetic parameters and for optimum pH, temperature and storage stability. Immobilized tyrosinase eggshell membrane was then used as biocomponent for the development of electrochemical biosensor for dopamine.

2. Experimental

2.1. Materials

Tyrosinase derived from a plant source *A. companulatus* [27] was used. Raw membrane-bound eggshells were collected from a campus canteen and stored in water. Twenty five percent glutaraldehyde solution was purchased from Sisco Research Laboratory, India. The buffer solutions were sodium phosphate solutions with different pH and concentrations. All solutions were prepared with water from Millipore Milli-Q system.

2.2. Immobilization of tyrosinase on eggshell membrane

An eggshell membrane was carefully peeled from a broken fresh eggshell after the albumen and yolk had been removed. It was cleaned with distilled water. The membrane was cut into small pieces of 1 cm^2 area. Three different approaches were followed for tyrosinase immobilization:

(I) Adsorption onto the eggshell membrane
The eggshell membrane was immersed in eppendorf tube containing 500 μL of undiluted enzyme and incubated at 30 °C for 30 min. The membrane was then soaked on tissue paper to remove excess of unbound enzyme. This was followed by washing with phosphate buffer (pH 6.0). Finally, the tyrosinase adsorbed eggshell membrane was stored in phosphate buffer (pH 6.0) at 4 °C until further use.

(II) Adsorption followed by glutaraldehyde cross-linking
The eggshell membrane was incubated with 500 μL of undiluted enzyme in eppendorf tube for 30 min at 30 °C. It was washed with pH 6.0 phosphate buffer and then subsequently immersed in 500 μL of 25% glutaraldehyde for 5 min. Excess unbound enzyme was soaked on tissue paper, followed by washing with phosphate buffer and storing it in phosphate buffer (pH 6.0) at 4 °C until further use.

(III) Glutaraldehyde activation of eggshell membrane followed by adsorption
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 °C until further use.

2.3. Determination of enzyme activity

Eggshell membrane (1 cm × 1 cm) was placed in eppendorf tube containing 0.8 mL of phosphate buffer (pH 6.0, 0.1 M) and incubated at 30 °C for 30 min. 0.2 mL of dopamine hydrochloride solution (10 mM stock) was then added. The product dopachrome was measured at 475 nm after 8 min of dopamine addition. One unit of activity was defined as increase in absorbance by 0.001 per min at 475 nm.

2.4. Determination of kinetic parameters

Varying concentrations of dopamine hydrochloride were assayed to determine the maximum velocity of reaction (V_{max}) and Michaelis-Menten constant (K_m). The apparent K_m value was calculated from Lineweaver-Burk plot.

2.5. Catalytic characterization of immobilized tyrosinase

Optimum pH and optimum temperature were studied to check the effect of immobilization on catalytic activity. For optimum pH determination, acetate and phosphate buffers were used in the pH range 4.0–7.0. Temperature was varied in the range 20–60 °C for determining optimum temperature.

2.6. Determination of storage stability

The shelf life of immobilized membrane was evaluated over 7 months' period by storing the membrane at two temperatures, at 4 and 25 °C.

2.7. Assembly of dopamine biosensor 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 measurements were performed in a cell with a working volume of 1 mL. Autolab 30 potentiostat employing three-electrode system was used for the electrochemical experiments. Ag/AgCl (3 M KCl) and a platinum wire were used as a reference and an auxiliary electrode, respectively.

Determination of dopamine was carried out electrochemically by measuring the intensity of current, which corresponds to the electrochemical reduction of the enzymatically generated quinone. This was done by immersing the working electrode in a 0.1 M phosphate buffer at pH 6.0 and applying a polarization voltage of +100 mV to the platinum electrode against an Ag/AgCl (3 M KCl). When the background current had stabilized, an appropriate amount of dopamine was introduced in an electrochemical cell. All the measurements were carried out at room temperature. A sensitive cathodic reduction peak was used for quantitative determination. A good linear relationship was observed between cathodic peak currents and dopamine concentration. Low concentrations of dopamine were analyzed by differential pulse voltammetry (DPV) at modified GCE.

3. Results and discussion

Biomaterials have emerged as ideal platform for enzyme immobilization as they provide biocompatible microenvironment around the enzyme. Eggshell membrane is one such support matrix with highly cross-linked protein structure and excellent permeability to substrates and products. Hence, in the present work, tyrosinase immobilization on eggshell membrane was investigated. To our knowledge, this is the first report on tyrosinase immobilization employing eggshell membrane as the solid support.

3.1. Tyrosinase immobilization

Three different approaches namely physical adsorption, adsorption followed by glutaraldehyde crosslinking and glutaraldehyde activation followed by adsorption were studied to immobilize enzyme tyrosinase on eggshell membrane. Out of them, membrane prepared by physical adsorption showed the maximum activity followed by glutaraldehyde activated membrane, however, the membrane prepared by adsorption followed by glutaraldehyde treatment showed negligible activity.

There are two protocols for immobilization of proteins using glutaraldehyde: use of previously activated support and treatment with glutaraldehyde of previously adsorbed proteins on support. When immobilization is carried out on preactivated supports, the primary amino groups of enzyme would react with aldehyde groups that have been introduced by modification of amino groups of the support. On the other hand, when enzyme is firstly adsorbed on support and then treated with glutaraldehyde, all the primary amino groups of enzyme and support may be activated and this may lead to enzyme-support multipoint attachment. We tried both the strategies to stabilize enzyme tyrosinase on eggshell membrane. In addition, we also employed simple adsorption of enzyme onto the membrane. We observed that the preactivated eggshell membrane could stabilize enzyme tyrosinase. This is in agreement with previous results showing stabilization of proteins on preactivated supports [28–31].

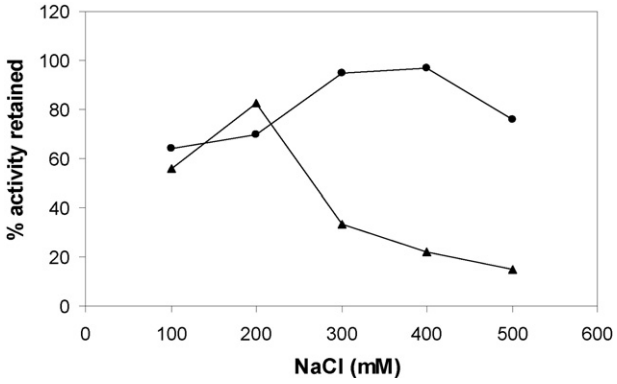

Fig. 1 – Desorption studies using NaCl on eggshell membrane prepared by physical adsorption (Δ) and glutaraldehyde activation followed by adsorption (●).

3.2. Salt tolerance

Though physical adsorption of enzyme is the oldest and the simplest immobilization method, loss of adsorbed enzyme is possible if changes in pH, ionic strength or temperature occurs. Therefore, membrane obtained with physical adsorption and the one preactivated with glutaraldehyde were subjected to NaCl treatment. The results are shown in Fig. 1. Glutaraldehyde activated membrane showed good stability compared to adsorbed membrane in the given NaCl concentration. Therefore, glutaraldehyde activated membrane was used for further studies.

3.3. Kinetic studies of tyrosinase immobilized on eggshell membrane

Effect of varying dopamine concentration on the velocity of reaction was studied using tyrosinase immobilized on eggshell membrane. Apparent K_m and V_{max} values were calculated from Lineweaver-Burk plot (Fig. 2). K_m was found to be 0.67 mM and V_{max} was 0.08 mM min^{-1}. Increase in K_m (compared to soluble enzyme for which K_m is 0.20 mM) indicate that the affinity of enzyme for substrate has decreased to some extent after immobilization, which may be due to glutaraldehyde treatment.

3.4. Effect of pH

The pH effect was investigated over the range 4.0–7.0. Fig. 3 shows the normalized activity of immobilized enzyme against pH when subjected to 2 mM dopamine hydrochloride. The

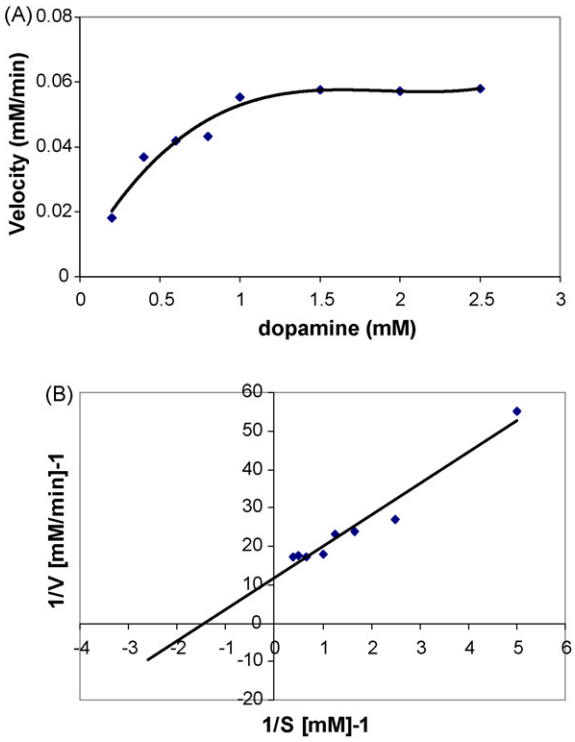

Fig. 2 – (A) Effect of varying dopamine concentration on the velocity of reaction and (B) corresponding Lineweaver-Burk plot for tyrosinase immobilized on preactivated eggshell membrane.

results showed that the activity could be maintained above 90% over a pH range 5.0–6.5.

3.5. *Determination of optimum temperature*

Optimum temperature was determined by carrying out enzyme substrate reaction at a given temperature after the incubation. Relatively constant and highest activity was observed in the range 20–40 °C, after that the gradual fall in activity was there (Fig. 4).

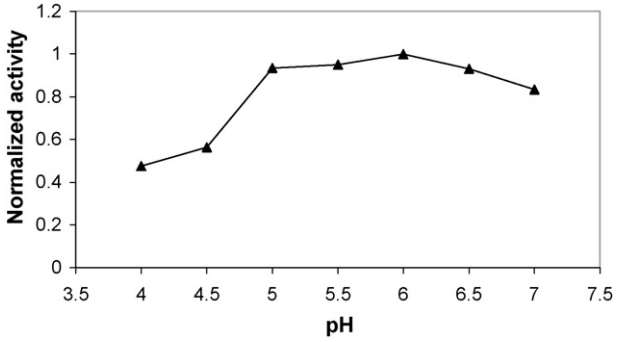

Fig. 3 – Effect of pH on immobilized tyrosinase eggshell membrane.

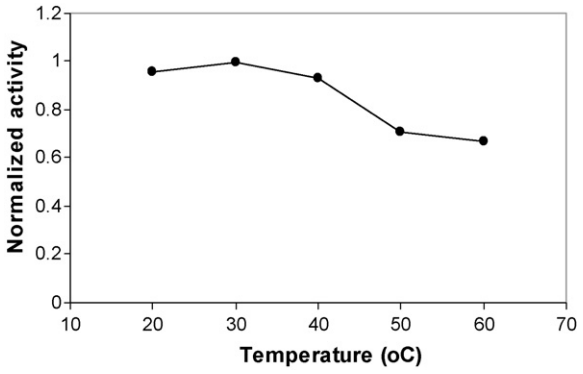

Fig. 4 – Effect of temperature on immobilized tyrosinase eggshell membrane.

3.6. *Shelf life of immobilized tyrosinase*

The storage stability was tested for 7 months' period at 4 and 25 °C. 83% and 50% of enzyme activity was found to be retained after 7 months when stored at 4 (Fig. 5) and 25 °C, respectively. Storage stability of this immobilized tyrosinase system was compared with those based on other carrier matrices, as shown in Table 1. It can be seen that the proposed immobilized system showed better long-term stability than that reported by other groups [32–36].

3.7. *Electrocatalytic dopamine biosensor based on tyrosinase immobilized eggshell membrane*

The dopamine biosensor was developed by mounting the tyrosinase immobilized eggshell membrane on the surface of glassy carbon electrode. The resulting dopamine biosensor was characterized electrochemically. Cyclic voltammograms of tyrosinase electrode in sodium phosphate buffer (0.1 M, pH 6.0) without dopamine and with increasing concentrations of dopamine are shown in Fig. 6. It was observed that the reduction peak increased after dopamine was added to phosphate buffer on enzyme immobilized electrode. Such an increase in reduction peak is due to the reduction of quinone species liberated from the enzymatic reaction catalyzed by tyrosinase on enzyme electrode. Reduction currents were measured at −0.19 V. Differential pulse voltammogramms of tyrosinase

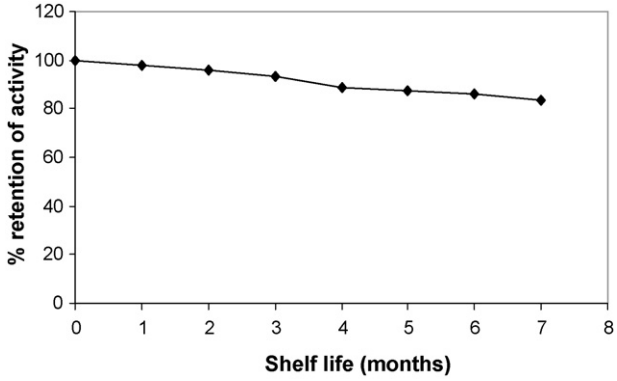

Fig. 5 – Shelf life of tyrosinase immobilized on eggshell membrane.

Table 1 – Comparison of shelf life of proposed method with others

Parameter	Ref. [32]	Ref. [33]	Ref. [34]	Ref. [35]	Ref. [36]	Proposed method
Shelf life	Retained 80% after 3 months	Retained 84% after 70 days	Useful lifetime 18 days	Retained 70% after 1 month	Retained 74% after 2 weeks	Retained 83% after 7 months
Support matrix	Electropolymerized PTS-doped polypyrrole film	Hybrid titania	Gold nanoparticles	Magnetic $MgFe_2O_4$ nanoparticles	Silicate/nafion composite film	Eggshell membrane
Immobilization method	Entrapment	Entrapment in sol–gel	Glutaraldehyde crosslinking	Glutaraldehyde crosslinking	Entrapment in sol-gel	Glutaraldehyde activation of support followed by adsorption

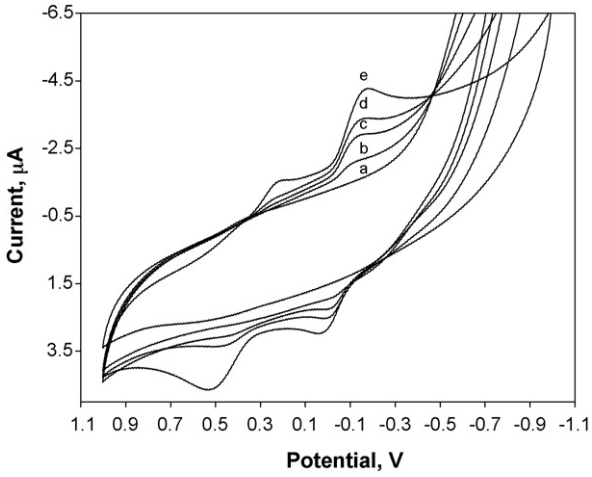

Fig. 6 – Cyclic voltammogramms obtained at immobilized tyrosinase eggshell membrane electrode for solutions of increasing dopamine concentration from 0 μM (a), 50 μM (b), 100 μM (c), 150 μM (d) and 250 μM (e). Scan rate: 100 mV s^{-1}. Supporting electrolyte: phosphate buffer (0.1 M, pH 6.0).

immobilized eggshell membrane-covered electrode in the presence of dopamine are shown in Fig. 7.

3.8. *Response characteristics of electrocatalytic dopamine biosensor*

A linear response for dopamine obtained at tyrosinase immobilized eggshell membrane electrode is shown in Fig. 8. Dopamine sensor gave a linear plot for the range 50–250 μM ($r^2 = 0.9719$). Sensitivity corresponding to the linear range was 10.6 μA mM^{-1}. A lower detection limit of 25 μM was obtained which was in good agreement with the results reported by other tyrosinase biosensors [37,38]. It can be attributed to the

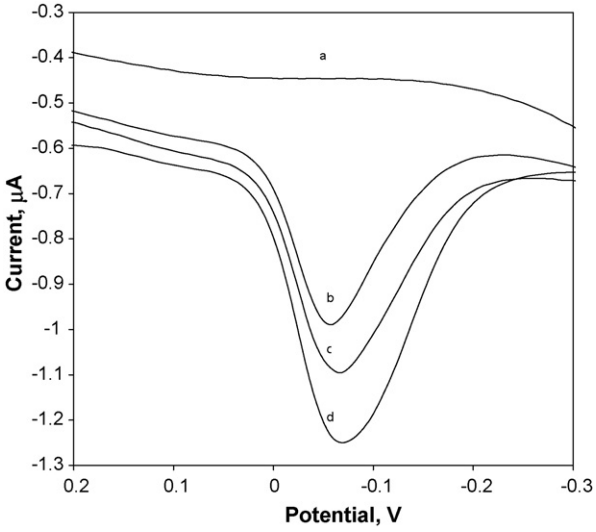

Fig. 7 – Differential pulse voltammogramms obtained at immobilized tyrosinase eggshell membrane electrode for solutions of increasing dopamine concentration from 0 μM (a), 100 μM (b), 150 μM (c) and 200 μM. (d).

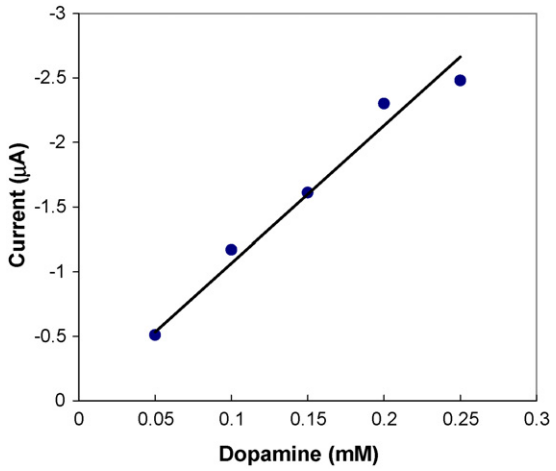

Fig. 8 – Linear response for dopamine obtained at immobilized tyrosinase eggshell membrane electrode. Potential: −0.19 V vs. Ag/AgCl (3 M KCl), scan rate: 100 mV s^{-1}. Supporting electrolyte: phosphate buffer (0.1 M, pH 6.0).

biocompatible microenvironment for the enzyme provided by the eggshell membrane.

4. Conclusions

Immobilization on preactivated eggshell membrane followed by enzyme adsorption was found to be a simple and a fast procedure to prepare stable immobilized tyrosinase. The reaction between primary amino groups of enzyme with glutaraldehyde activated support works well than the reaction between two glutaraldehyde molecules bound to primary amino group. Immobilized tyrosinase eggshell membrane was mounted on the surface of glassy carbon electrode to fabricate enzyme electrode. The work demonstrates a simple, easy and economical method to fabricate tyrosinase based electrochemical biosensor for dopamine.

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