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Conducting Polymer Based Enzyme Electrodes Fabricated by Invertase and Polyphenol Oxidase

Elif DOĞAN [1], Ayşe Elif BÜYÜKBAYRAM [2,*]

[1] *Department of Chemistry, Karabük University, Karabük, 78050, Turkey,* ***ORCID:*** *0000-0002-3681-1345*
[2] *Department of Chemistry, Karabük University, Karabük, 78050, Turkey,* ***ORCID:*** *0000-0002-9085-6042*

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Abstract

Novel carbon fiber enzyme electrodes were constructed and evaluated. Fabrication of electrodes was performed via electrochemical deposition of a conducting matrice composed of polythiophene and polypyrrole (PTh–PPy) onto carbon fiber substrates. The enzyme was entrapped into the matrix during electropolymerization. Resultant biosensors represented higher kinetic parameters, V_{max} and K_m, in comparison to PPy matrice, which are 2.471 ± 0.150 µmol min^{1-} $electrode^{1-}$ and 30.60 ± 5.30 mM for invertase, 0.056 ± 0.012 µmol min^{1-} $electrode^{1-}$ and 842.00 ± 37.50 mM for polyphenol oxidase respectively. Optimum pH and temperature of the immobilized enzyme within PTh–PPy composite indicates that this matrice provides a more protective environment. The detection limit (LOD) of polyphenols was obtained as 0.037 mg mL^{1-}. Polyphenol oxidase enzyme electrodes were proved to be used for the determination of polyphenolic substances in real samples and the results were confirmed by the Folin-Ciocalteau method.

1. Introduction

Carbon fiber (CF) microelectrodes are considered useful substrates since they are inexpensive, possess high biocompatibility, and can be employed with a variety of surface modifications [1,2]. As an inorganic material, it possesses inertness against humidity, weak base, and acid at room temperature. In biosensor applications, its porous and high-surface-area microstructure allows CF to be loaded by large quantities of biomaterials, which makes the sensitivity of the resulting sensor enhances. Since their dimension is in micro and nano levels, they serve as a convenient tool for micro sample measurements (single droplet or single cell) [3,4]. They have been utilized in the construction of enzymatic biosensors, which involve studies on particularly glucose oxidase and polyphenol oxidase (tyrosinase) [1,5,6]. Here, polymer-modified CF substrates were selected to be used for the fabrication of enzymatic biosensors.

The use of immobilized enzymes has advantages over the use of free enzymes. Since the use of free enzymes makes the industrial processes expensive, there is a demand for more economical ways on the enzymatic applications in the industrial scale. Concerning this problem, the immobilization of the enzyme into a suitable supporting material provides the possibility of multiple uses of the enzyme and consequently reduces the cost of the enzymatic processes. Enzymes are sensitive biomolecules that can be denatured easily by external conditions. Immobilization in a matrix usually offers a protective environment for enzymes and it becomes more resistive to changes in the conditions. Besides, the reaction between enzyme and substrate can be easily stopped by removing the enzyme immobilized matrix from the reaction medium. The reaction medium is not contaminated with the enzyme and a clean product is obtained [7].

Conducting polymers are frequently used in biosensors and many studies have been devoted to the immobilization of enzymes to these matrices [8-10].

* Corresponding Author: aeboyukbayram@karabuk.edu.tr

Here, a composite material that consisted of polypyrrole and polythiophene (PTh-PPy) was used for enzyme immobilization because of the remarkable stability of these two polymers. PPy gains most of the interest because of its unique properties such as low oxidation potential, solubility in water rendering the polymerization possible in aqueous solutions, and long-lasting stability in room conditions [11]. In this study, entrapment of enzyme into a composite film is based on the codeposition during polymerization of PPy on previously PTh coated CF substrate in an aqueous enzyme solution containing supporting electrolyte. Among the immobilization methods, electrochemical polymerization offers a simple and rapid, one-step fabrication of enzyme electrodes [12].

One of the enzymes studied in this work is invertase (INV), an enzyme that is used as a model enzyme in immobilization studies, to have an idea about the performance of electrodes. The other enzyme, polyphenol oxidase (PPO) catalyzes hydroxylation and oxidation of monophenols and diphenols to corresponding *o*-quinones. Its substrate is polyphenolic substances; a large family of natural compounds which play a major role in the growth and reproduction of fruits and vegetables and supply protection against pathogens [13]. Phenolic compounds have a wide range of beneficial properties to health with their antioxidant activity [14,15]. Due to the large demand for antioxidants nowadays in city life, the phenolic content of foods acquires considerable importance. On the other hand, polyphenolics in natural waters are organic pollutants that have a detrimental influence on humans and animals. Phenolic derivatives are widely used chemicals in the manufacturing of industrial products. They are released to environmental waters through the wastewater of the production plants and have a hazardous effect on aquatic life. Phenolics are considered one of the priority pollutants by the American Environmental Protection Agency. Since they are toxic and persistent in the environment, their determination in natural waters is of great importance.

The study aimed to develop an alternative method to the reported biosensors for the quantification of polyphenolic compounds. CF substrates were used to construct micro-level electrodes for micro samples. The enzyme was immobilized to have a multiple-use tool to reduce the cost of the enzymatic applications. Immobilization was performed by using a conducting polymer composite to provide a protective microenvironment for the enzyme. After the fabrication, enzymatic microelectrodes were characterized by the investigation of the optimized conditions, and the kinetic parameters [16].

2. Materials and Methods

2.1. Materials

INV (E.C.3.2.1.26) Type V and Tyrosinase from mushroom (E.C.1.14.18.1) were purchased from Sigma. Pyrrole and sodium dodecyl sulfate (SDS) were supplied from Merck. Nelson's reagents constituents were of analytical grade, ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_2 \cdot 4H_2O$) and sodium arsenate di-basic-7-hydrate ($Na_2HAsO_4 \cdot 7H_2O$) were provided from Aldrich. 3-Methyl-2-benzothiazolinone hydrazone hydrochloride hydrate ($C_8H_9N_3S.HCl.H_2O$, MBTH) was bought from Aldrich and catechol from Sigma-Aldrich. GAMRY Instruments Interface 1000 Potentiostat/Galvanostat/ZRA and Wenking POS-88 potentiostats, Shimadzu UV-1201-V model spectrophotometer, and Carl ZEISS ULTRA PLUS GEMINI FESEM scanning electron microscope (SEM) were used for characterization.

2.2. Preparation of Enzyme Electrodes

CF was attached to a steel wire with Teflon tape. It was inserted into a tapered micropipette tip, fixed in place by an epoxy resin. A glass tube was placed around the wire. Prepared CF electrode was used as working electrode in a typical three-electrode cell in which electropolymerization was performed, containing also 0.5 cm^2 Pt foil as counter electrode and an Ag wire as a reference electrode. PTh was synthesized in acetonitrile containing 0.05 M tetrabutylammonium tetrafluoroborate and 6.5 µL/mL thiophene. Immobilization of enzyme was achieved via electropolymerization of PPy on previously PTh coated CF electrodes in a 10 mL buffer solution. Acetate buffer (pH 5.0) including 0.6 mg/mL SDS (as supporting electrolyte), 5.0 µL/mL pyrrole, 0.6 mg/mL INV and citrate buffer (pH 7.0) containing 1.0 mg/mL SDS, 5.0 µL/mL pyrrole and 0.4 mg/mL PPO (2687 U/mg solid) were used for INV and PPO immobilization respectively. It was carried out by cyclic voltammetry at a potential interval of 0.0 V – 2.4 V for PTh and -1.2 V – 0.6 V for PPy at room temperature. During electrochemical deposition, the enzyme was entrapped within the matrix. After construction, enzyme electrodes were kept at 4 °C in a buffer solution.

2.3. Enzyme Activity Assays and Kinetic Characterization

Immobilized INV activities were determined using the Somogyi-Nelson method [17] in which different

concentrations of sucrose were prepared, after immersing enzyme electrodes at certain times, Nelson's and arsenomolibdate reagents were added to form a complex compound with the enzymatic reaction product. Activities of immobilized PPO were obtained by Besthorn's Hydrazone method [18]. Different concentrations of catechol were prepared, after adding MBTH reagent, the enzyme electrode was immersed at certain times. Quinone products react with MBTH to form a complex compound. Enzyme activities were calculated by reaction rates of enzymatic reaction. Kinetic parameters of enzyme electrodes; maximum enzyme activity, V_{max} and Michaelis-Menten constant, K_m were obtained at optimum pH and 25 °C by using the Michaelis-Menten method and Lineweaver-Burk graphics [19].

2.4. Optimizations and Stability Studies

To find out optimum pH and temperature, electrode activities were measured by changing pH between 2 and 12 for INV, between 4 and 9 for PPO, and changing incubation temperature between 10 °C and 80 °C. The stability of enzyme electrodes was investigated by sequential 40 and 20 activity measurements for INV and PPO respectively. Measurements were performed at a substrate concentration of 5 K_m.

2.5. Determination of Polyphenols in Tea Extracts

Tea extracts were prepared as follows: 5.0 g tea leaves or 5.0 g tea bag was put in a beaker, 200 mL boiling water was poured and the mixture was placed into a 70 °C water bath for 10, 20, 30, and 40 min. infusion time. A sample of 5 mL extract was drawn at each infusion time, diluted by 1:3, and the enzyme activity procedure was applied. The calibration curve was drawn by applying the same procedure to catechol standards. The concentration of total polyphenolics in tea extracts was expressed as mg/mL catechol equivalent. Folin Ciocalteau method was used as a control method [20]. The method was adapted to catechol and the calibration curve was prepared by catechol standards having concentrations between 0.1 and 2.0 mg/mL. First, 40 µL of the standard solution was drawn, 3.16 mL water, and 200 µL Folin-Ciocalteau reagent were added. Then, the solution was mixed and 600 µL 20% Na_2CO_3 solution was added. After mixing and waiting for 2 hrs, absorbances were measured at 765 nm to form the calibration curve. The same procedure was applied to 40 µL tea extract samples and the total amount of polyphenolics was represented as mg/mL catechol equivalent.

3. Results and Discussion

CF electrodes SEM photos are shown in Figure 1. The diameters of bare and composite polymer-coated CFs are 8.22 µm and 55.35 µm respectively. The upper two photos belong to bare CF and the middle two belong to coated CF which has a typical cauliflower morphology. The lower micrograph shows the enzyme immobilized biosensor.

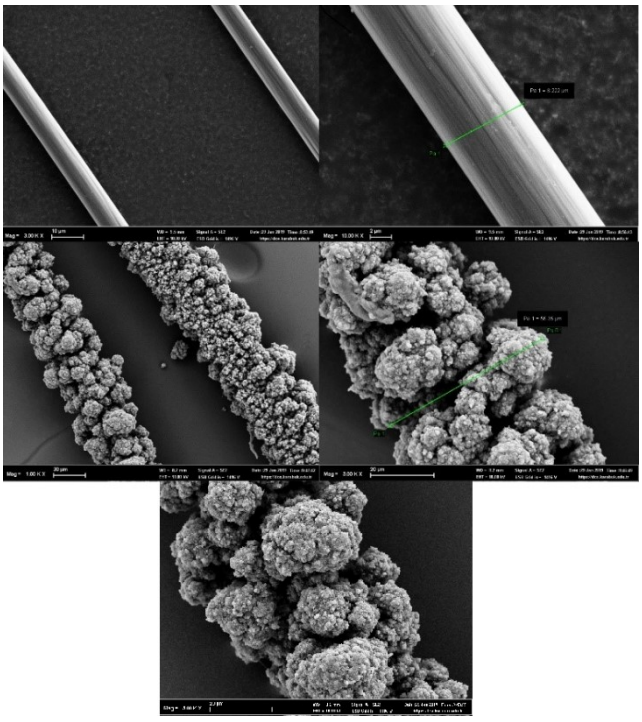

Figure 1. SEM micrographs of bare CF, coated CF, and the electrode with the immobilized enzyme.

Kinetic characterization of INV and PPO electrodes prepared by composite material (CF/PTh-PPy/INV and CF/PTh-PPy/PPO) was performed by Lineweaver-Burk graphics presented in Figures 2 and 3 and related kinetic parameters are given in Table 1. CF/PPy/INV and CF/PPy/PPO are the electrodes where the enzyme is immobilized to the PPy matrix [21] and their data was added to Table 1 for comparison.

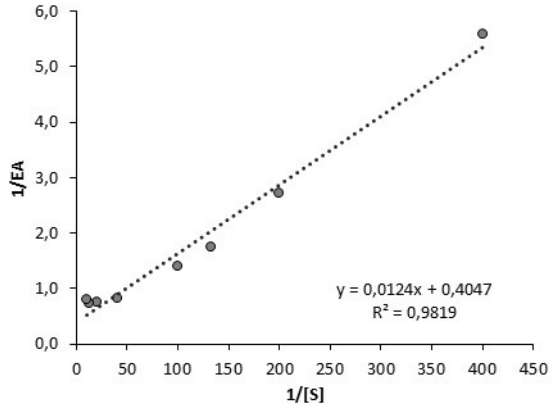

Figure 2. Lineweaver-Burk plot of CF/PTh-PPy/INV Electrode.

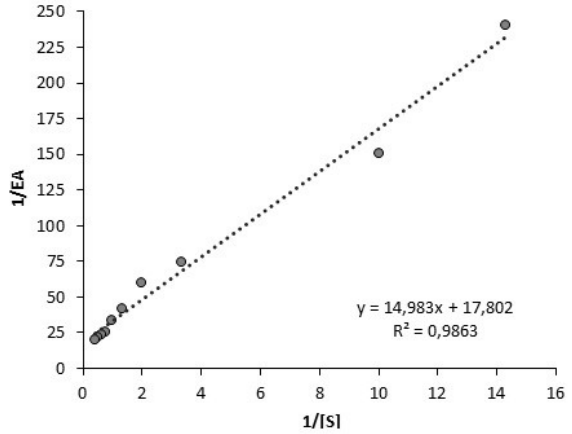

Figure 3. Lineweaver-Burk plot of CF/PTh-PPy/PPO electrode.

As shown in Table 1, the maximum activity of the immobilized enzyme, V_{max} is much lower than the V_{max} of the free enzyme as expected. This is a general trend for immobilized enzymes arising from a substrate diffusion barrier created by immobilizing matrix. In the comparison of the PPy matrix which was studied in our previous work and PTh-PPy composite material, it is observed that V_{max} of immobilized enzyme in composite material is three times higher than V_{max} of immobilized enzyme in PPy matrix that is related with the polymers specific surface area and porosity. When Michaelis-Menten constants are compared to each other, it is seen that K_m of immobilized enzyme is higher than K_m of free enzyme. K_m shows affinity and lower K_m means higher affinity between enzyme and its substrate. The free enzymes can meet with the substrate molecules easily when they both exist freely in the solution. An increase in K_m for immobilized enzyme means that the enzyme and its substrate have difficulty at becoming together and the extent of increase is directly related to the microenvironment where the enzymatic reaction takes place. Immobilized enzyme generally exhibits larger K_m values compared to the free enzyme, which points that there is tardiness in the diffusion of the substrate into the reaction space when the enzyme is immobilized into a matrix. On the other hand, in comparison PPy and PTh-PPy composite matrices, it is noticed that the K_m value of immobilized enzyme into PTh-PPy is higher than that of PPy matrix [21-24].

Table 1. Kinetic parameters, optimum pH and the temperature of free and immobilized INV and PPO.

	V_{max}	K_m (mM)	Opt. pH	Opt. Temp.
Free INV[a]	82,30 µmol/min.mL	24,30	4.6	50 °C
CF/PPy/INV electrode[b]	0,779 ± 0,120 µmol/min.electrode	27,20 ± 6,70	6.0	40 °C
CF/PTh–PPy/INV electrode	2,471 ± 0,150 µmol/min.electrode	30,60 ± 5,30	5.0	60 °C
Free PPO[c]	0,073 µmol/min.mL	4,00	5.0	40 °C
CF/PPy/PPO electrode[d]	0,017 ± 0,004 µmol/min.electrode	176,00 ± 26,60	7.0	60 °C
CF/PTh–PPy/PPO electrode	0,056 ± 0,090 µmol/min.electrode	842,00 ± 32,03	8.0	-

[a][22], [b][20], [c][21,23], [d][20]

The effect of pH on the activity of immobilized INV into PTh-PPy composite matrice is illustrated in Figure 4. In a previous study, the pH at which maximum activity was observed for the free enzyme was found to be 4.6 [23]. The immobilized enzyme within PTh-PPy composite matrix revealed a maximum activity at pH 5.0, a very close value to the free enzyme. pH 5.0 was used throughout the optimization and characterization studies of the CF/PTh-PPy/INV electrode.

The temperature influence on enzyme activity of immobilized INV within the PTh-PPy composite matrix was investigated and the maximum activity was observed at 60 °C. Enzyme activity rises slightly up, reaches a maximum, then declines sharply. Enzyme loses its activity completely at 70 °C as a result of temperature-induced denaturation of protein structure. The temperature of maximum activity of free INV was given as 50 °C by Alkan et al. [23] and that of PPy immobilized INV was found as 40 °C in our previous study [21]. Optimum temperature higher than free and PPy immobilized INV indicates a more protective structure of PTh-PPy composite material than PPy matrix concerning temperature stability.

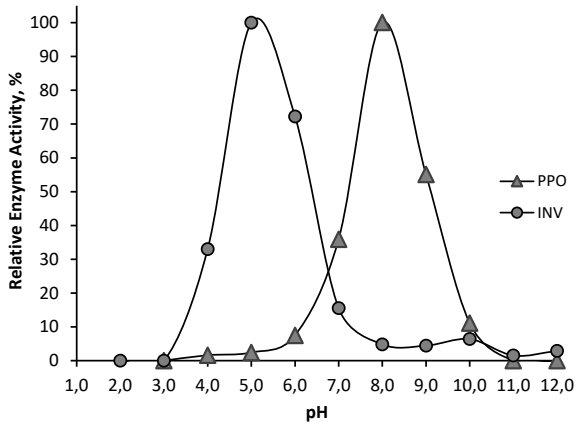

Figure 4. pH effect on CF/PTh-PPy/INV and CF/PTh-PPy/PPO electrodes activity.

According to the study performed by Kiralp et al., free PPO revealed a maximum activity at pH 5.0 [24]. The pH influence on enzyme activity of PPO immobilized within the PTh-PPy composite matrix is represented in Figure 4 and maximum activity is exhibited at a pH of 8.0. In our previous study, the maximum activity of immobilized PPO within the PPy matrix was given as 7.0 [21]. Results indicate a significant enhancement in enzyme stability towards the basic pHs upon immobilization. The reason for that, as explained by Erginer et al., could be the effect of charge distribution on the enzyme in the microenvironments of the composite matrix [25]. Throughout the studies, pH 7.0 was used for CF/PTh-PPy/PPO electrode.

Stability studies of enzyme electrodes show that immobilized INV is quite stable until the 40th measurement as shown in Figure 5a. On the other hand, immobilized PPO shows a rather alternating behavior as compared to INV that activity is relatively stable between 80 % and 100 % as seen in Figure 5b.

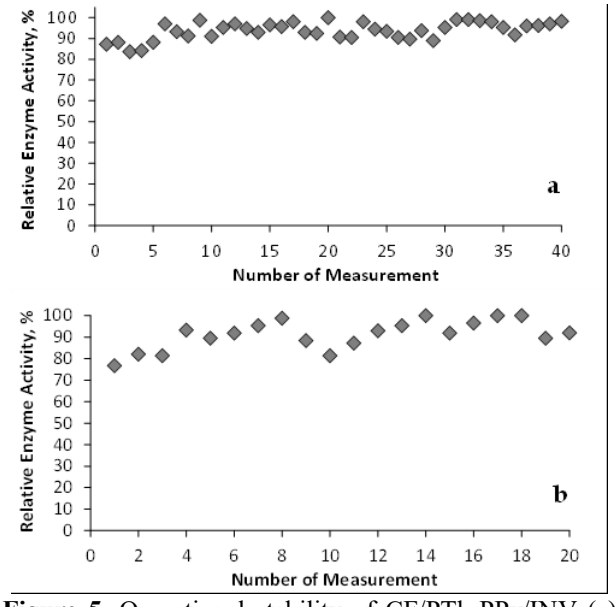

Figure 5. Operational stability of CF/PTh-PPy/INV (a) and CF/PTh-PPy/PPO (b) electrodes.

CF/PTh-PPy/PPO electrode was applied to the analysis of polyphenolic substances in tea extracts by using a calibration curve (y=0.3265x+0.0202, R^2=0.9930) prepared with catechol standards in concentration between 0.007 and 1.600 mg/mL. LOD was found as 0.037 mg/mL. The results with changing brewing times are given in Table 2.

The amount of polyphenolic substance infused into the water increases between 10 and 20 minutes. However, it stays constant at 30 min and 40 min infusion times. Therefore, 20 min is sufficient infusion time to obtain the maximum amount of phenolics in the extraction solvent, which are 0.641 mg/mL and 1.450 mg/mL for tea leaves and tea bags respectively.

The results were confirmed by the Folin Ciocalteau method of analysis. A calibration curve was drawn by different catechol standards (y=1.0998x+0.0704, R^2=0.9944). The results obtained in different brewing times are shown in Table 2.

Table 2. Total polyphenolics determined by CF/PTh-PPy/PPO electrode and the Folin Ciocalteau method as mg/mL catechol equivalent.

	Samples Brewing Time (min)				
	10	**20**	**30**	**40**	
Total Polyphenols (mg/mL), Tea Leaves	0.053	0.641	0.614	0.669	Analysis by Enzyme Electrode
	0.515	0.702	0.627	0.740	Analysis by Folin Ciocalteau Method
Total Polyphenols (mg/mL), Tea Bag	1.156	1.450	1.468	1.533	Analysis by Enzyme Electrode
	0.562	1.148	1.055	1.180	Analysis by Folin Ciocalteau Method

The optimum extraction time is 20 min similar to previous results obtained by CF/PTh-PPy/PPO electrode. There is no increase in the number of polyphenolics with increasing brewing time after 20 min. 0.702 mg/mL and 1.148 mg/mL are the maximum amounts of phenolic substances extracted from tea leaves and tea bags with 20 min infusion time.

4. Conclusion

Construction of novel CF enzyme electrodes modified with conducting PTh-PPy composite was achieved via electrochemical polymerization. Produced biosensors were characterized in terms of kinetic parameters. Composite-based CF enzyme electrodes revealed higher V_{max} and K_m values compared to PPy based CF enzyme electrodes due to the morphological differences. An increase in the maximum enzyme activity

means more enzymes immobilized in that matrix, which indicates that composite material has a more porous structure than the PPy matrix and holds more enzymes. Higher K_m means that cavities on the PTh-PPy surface are deeper than that on the PPy surface, which makes it less easy to meet enzyme and substrate on the PTh-PPy matrix. This situation is observed for both INV and PPO enzymes. Regarding the optimization studies, composite-based INV electrodes exhibited an optimum pH almost the same as a free enzyme which means that PTh-PPy matrix does probably not affect the charge distribution significantly on immobilized INV enzyme. CF/PTh-PPy/PPO electrodes optimum pH value is much higher than optimum pH of free enzyme. During oxidative electropolymerization, negatively charged dopant ions are received into the polymer structure. In micro spaces within the matrix, these dopant ions make the protons accumulate around the enzymes and protect them against hydroxide ions. This situation may cause a difference between the pH around the immobilized enzyme and the pH of the bulk. The increase in optimum temperature upon immobilization into PTh-PPy implies the protective environment supplied by PTh-PPy composite matrix against higher temperatures. With these advantages, CF/PTh-PPy/PPO electrode offers a cheaper and more practical alternative to conventional methods. As a conclusion, it is proposed to be used for the determination of phenolic substances, particularly in food samples and environmental waters.

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Declaration of Ethical Standards

The authors of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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