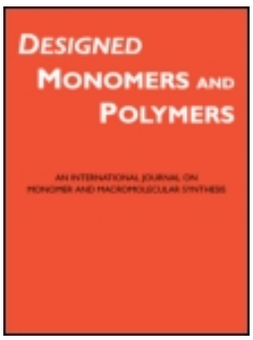

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Determination of phenolic compounds in wines with enzyme electrodes fabricated by immobilization of polyphenol oxidase in conducting copolymers

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Abstract—A copolymer of pyrrole with a new monomer (MBTA) containing an ester group derived from 3-thiophene acetic acid and (*S*)-(−)-2-methylbutanol was used as the matrix for immobilization of polyphenol oxidase. Enzyme electrodes were constructed by entrapment of enzyme in the conducting copolymer during the electrochemical polymerization of pyrrole. The performance of enzyme electrodes was optimized by examining the effects of pH and temperature on enzyme activity. The changes in the maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) upon immobilization were investigated in addition to shelf-life and operational stability. By using these enzyme electrodes the total amount of phenolic compounds in red wines of Turkey was also analyzed.

Keywords: Polyphenol oxidase; immobilization; electrochemistry; phenolic determination; wine.

1. INTRODUCTION

The study of phenolic compounds is of great interest because of their contribution to the properties of fruits and beverages, such as color, astringency, bitterness, flavor and browning [1, 2]. Wine is one of the products that is at the center of interest in order to optimize wine quality [3, 4].

Polyphenol oxidase (PPO) is a bifunctional enzyme responsible for the formation of the natural macromolecule pigment melanin in different species [5]. In its first reaction, monooxygenase activity, PPO hydoxylates a phenolic substrate at the *ortho*-position to the hydroxyl group. In the second reaction, oxidase activity, the *o*-dihydroxy compound is oxidized to the pertinent *o*-quinone derivative (Scheme 1).

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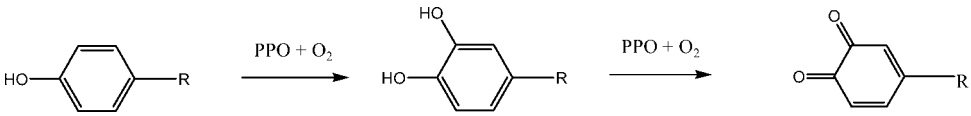


Scheme 1.

Immobilization of PPO has been proven to be an alternative method to typical methods based on chromatographic techniques for determination of amount of phenolic compounds.

In our previous study [6] immobilization of PPO was achieved in matrices of polypyrrole (PPy) and a copolymer of menthyl ester of 3-thiophene acetic acid (MM) with pyrrole. Results were compared with the MBTA matrice. Synthesis and characterization of both copolymers were studied earlier [7, 8].

2. EXPERIMENTAL

2.1. Materials

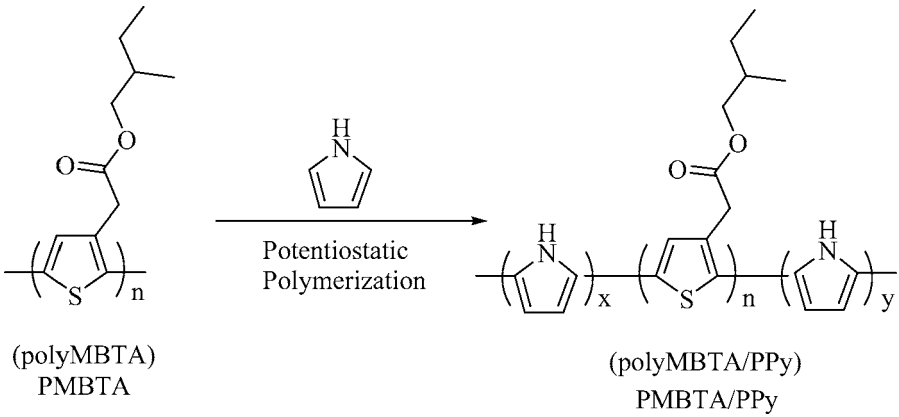
Tyrosinase (polyphenol oxidase, PPO, EC 1.14.18.1) was purchased from Sigma. Pyrrole was purchased from Aldrich and sodium dodecyl sulfate (SDS) from Sigma. Pyrrole was distilled before use. 3-methyl-2-benzothiozolinone (MBTH), acetone and sulfuric acid, used in spectrophotometric activity determination of PPO, were also obtained from Sigma. For preparation of citrate buffer, tri-sodium citrate-2 hydrate and citric acid were used as received.

Catechol was purchased from Sigma. All catechol solutions were prepared in citrate buffer.

2.2. Immobilization of PPO in PMBTA

The homo-polymerization of MBTA (Scheme 2) was achieved by constant current electrolysis in one compartment cell consisting of platinum working and counter electrodes. Experiments were carried out in dichloromethane (10 ml)/tetrabutylammonium tetrafluoroborate (TBAFB) (0.2 M) solvent-electrolyte system with 50 mg monomer at 0°C using 30 mA for 10 min.

Immobilization of PPO was achieved by electropolymerization of pyrrole on a previously PMBTA-coated platinum electrode. The solution consists of 0.3 mg/ml PPO, 0.6 mg/ml supporting electrolyte (sodium dodecyl sulfate), 0.01 M pyrrole and 10 ml citrate buffer (pH 6.5). Immobilization was performed in a typical three-electrode cell, consisting of the Pt working and counter electrodes and a Ag/Ag^+ reference electrode. Immobilization was carried out at a constant potential of +1.0 V for 1 min at room temperature. Enzyme electrodes were kept at 4°C in citrate buffer solution when not in use.



Scheme 2.

2.3. Determination of PPO activity

The activities of free and immobilized PPO were determined by using Besthorn's hydrazone method [9]. For determination of activity of immobilized PPO, different concentrations of catechol were prepared (3.0 ml) and put in a water bath at 25°C. 1 ml MBTH solution was added. The enzyme electrode was immersed in the solution and shaken for 5 min. 1 ml sulfuric acid and 1 ml acetone were added for a total volume of 6 ml. After mixing, absorbances were measured at 495 nm.

2.4. Determination of optimum temperature and pH

Optimum temperature and pH determinations were carried out by changing incubation temperature between 20 and 80°C and pH between 2 and 11, respectively. The rest of the procedure was the same as the determination of PPO activity.

2.5. Protein determination

Protein determination measurements were performed by Bradford's method [10]. The protein determination procedure is explained in detail elsewhere [6].

3. RESULTS AND DISCUSSION

3.1. Protein determination of enzyme electrodes

As it was mentioned in the previous study [6], the reaction rate for free enzyme was 11.2 μmol/min per mg protein. Protein determination results for PMBTA/PPO electrode showed that 0.0043 mg protein was entrapped in the matrix.

3.2. Kinetic studies

V_{max} and K_m are parameters that give maximum reaction rate and Michaelis–Menten constant, respectively. These parameters were obtained from Lineweaver–Burk plots [11].

V_{max} for PPy/PPO and MM/PPO was 0.11 and 0.10 μmol/min per electrode, respectively [6], V_{max} for the PMBTA/PPO enzyme electrode was found to be 0.048 μmol/min per electrode. When we compared this result with other two enzyme electrodes in the previous study we saw that V_{max} of immobilized enzyme in PMBTA/PPO electrode was half that of the other two electrodes. These results were also confirmed by the protein amount entrapped in the electrodes.

K_m values of enzyme electrodes studied in the previous work were very high when compared to that of free enzyme. K_m is a parameter that is directly related with morphology of the matrice. When we examine the scanning electron micrographs of the three electrodes in Fig. 1, one can see that both PPy/PPO and MM/PPO electrodes have very compact morphology that make it difficult for the substrate to diffuse into the matrix. However, PMBTA/PPO electrode gives rise to easy diffusion for substrate by making enzymes more available, resulting in a small K_m value, 18 mM.

3.3. Effect of temperature on enzyme electrode

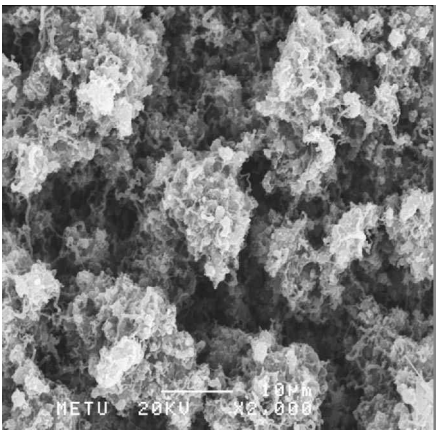
Figure 2 shows the temperature dependence of the activity of immobilized enzyme. At temperatures between 50°C and 70°C, the enzyme electrode exhibits high resistivity to temperature change. Almost no activity loss was observed between these temperatures.

3.4. Effect of pH on enzyme electrodes

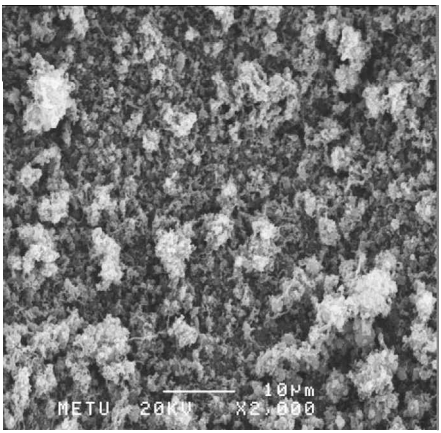
In the previous work [6], where PPy/PPO and MM/PPO electrodes were studied, the shift in the optimum pH values towards the alkaline side was explained as the partitioning of protons. The same behavior in pH dependence was observed for the PMBTA/PPO electrode, but this electrode exhibits greater stability towards high pH (Fig. 3). From pH 7 to 11 there is no change in the enzyme activity. This shows that this electrode can protect enzymes against high OH concentration.

3.5. Operational stability and shelf-life of enzyme electrodes

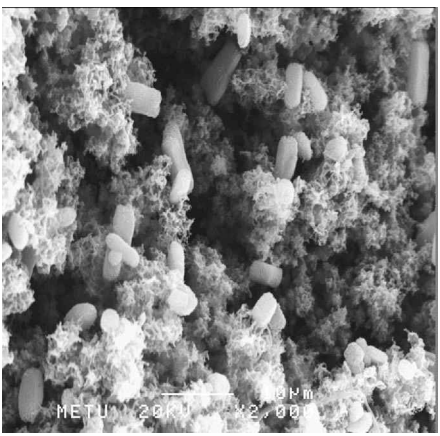
Enzymes can easily lose their catalytic activity and denatured, so careful storage and handling are essential. To determine the stability against repetitive use and shelf-life of PMBTA/PPO electrode the activity of electrode was checked. 40 measurements were done on the same day to test the operational stability. Gradual decrease was observed up to the 15th assay and then stayed constant at 60% activity (Fig. 4a). Upon examining the activity change with time (Fig. 4b), we see that there is a rapid decrease in the activity which slows down after the 20th day.



(a)



(b)



(c)

Figure 1. Scanning electron micrographs of (a) Ppy/PPO, (b) MM/PPO and (c) PMBTA/PPO enzyme electrodes.

3.6. Determination of total phenolic amount in red wines

The PMBTA/PPO electrode was used for analysis of phenolic amount in two Turkish red wines, Brand K and Brand D.

When we compare the phenolic amount of two brands, results reveal that Brand K contains twice the amount of phenols of that of Brand D (Table 1). This result agrees with the result of other two enzyme electrodes studied previously [6]. Results are reported in Gallic Acid Equivalent (GAE) [12].

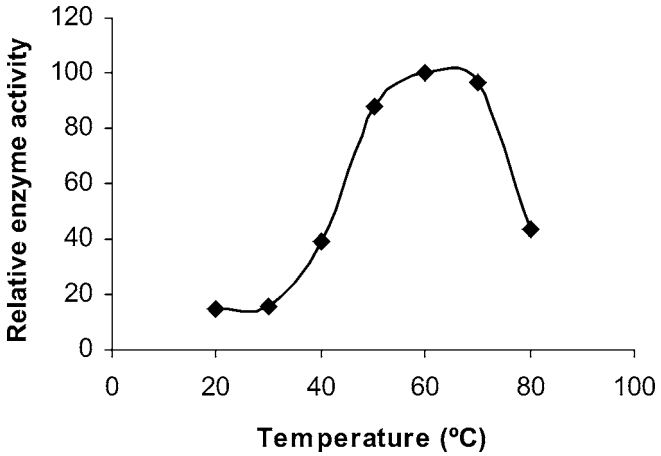


Figure 2. Temperature dependence of PMBTA/PPO electrode.

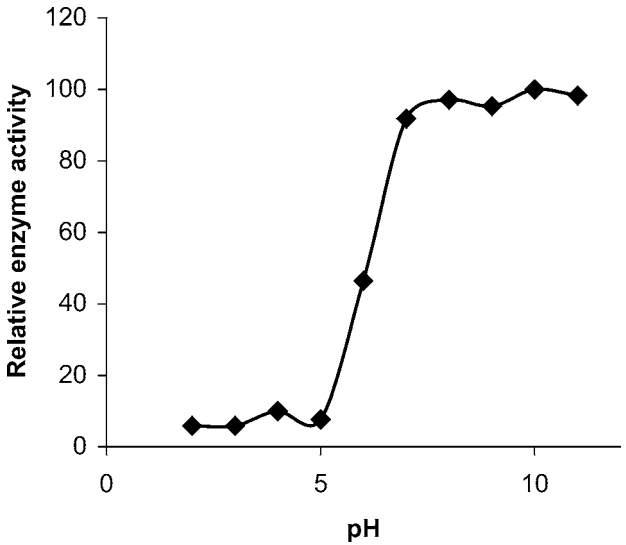
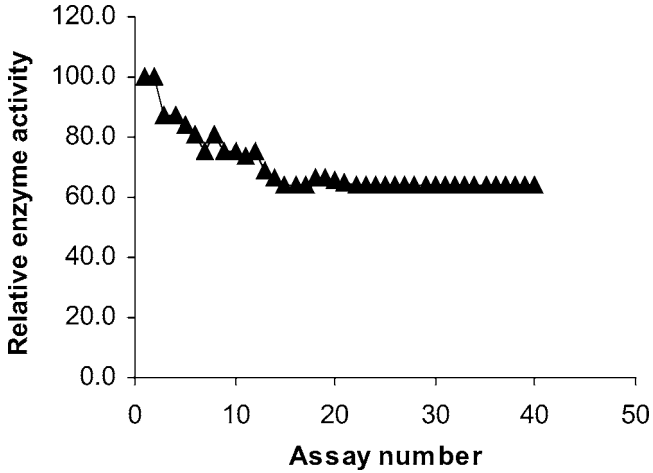


Figure 3. pH dependence of PMBTA/PPO electrode.

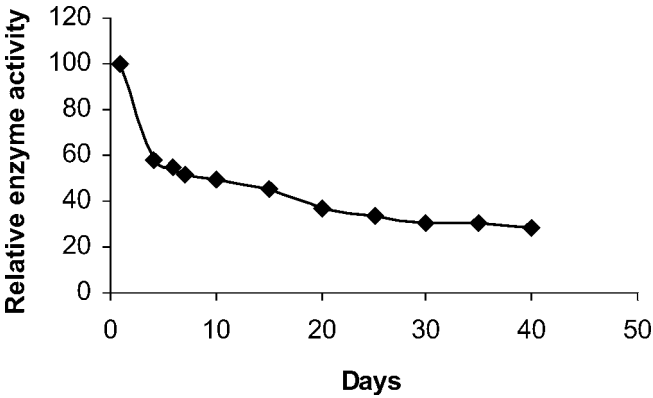
Table 1.
Total phenolics in two different red wines, determined by the enzyme electrode

	−OH (M)	PMBTA/PPO (mg/l)[a]
Brand K	0.075	4160
Brand D	0.035	1950

Values are the average of four determinations.
[a] GAE.



(a)



(b)

Figure 4. (a) Operational stability. (b) Shelf-life of the PMBTA/PPO electrode.

4. CONCLUSIONS

This study shows that PMBTA electrode can be successfully used for the immobilization of PPO. We can conclude that obtained results from the analysis based on kinetic studies, temperature and pH optimization studies and stability studies are very good. Like the electrodes studied in the previous work this electrode also can be used as an alternative method for the determination of total phenolic compounds.

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