Enzyme Electrodes for Determination of Total Phenolic Capacity of Red Wines

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Received 24 May 2004; accepted 5 January 2005
DOI 10.1002/app.22165
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A tyrosinase-modified electrode, based on physical entrapment of enzyme in copolymer matrix of decanedioic acid bis(4-pyrrol-1-yl-phenyl) ester (DAPE) with pyrrole was constructed and used to investigate the total amount of phenolics in red wines. Kinetic parameters, V_{max}, and K_m, were determined. Also, temperature, pH optimizations, operational stability and shelf life of the enzyme electrode were examined. Surface morphologies of the films were studied by scanning electron microscope. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 98: 521–524, 2005

Key words: electrochemistry; enzymes; copolymerization; conducting polymers

INTRODUCTION

Wines, particularly red wines, contain numerous biologically active compounds, the most important of which are polyphenols, whose nutritional importance is attributed to their antioxidant power. This property attracts great interest in evaluating the antioxidant capacity of wines in relation to their phenolic constituents.[1]

Polyphenol oxidase (tyrosinase) is an enzyme containing binuclear copper, which catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity) (Scheme 1).[2] Several bioelectrodes incorporating PPO were proposed for phenols.[3–11] Use of conducting copolymers for this purpose is another method for fabrication of enzyme electrodes. Advantages of immobilization of enzymes in a conducting polymer by electropolymerization are the easy one-step procedure, accurate control of the polymer thickness via the electrical charge passed during the film formation process, localization of the electrochemical reaction exclusively on the electrode surface allowing precise modification of microelectrodes and surfaces of complex geometry, and the possibility to build up multilayer structures.[12]

By using conducting copolymers, a biosensing approach with advantages of high specificity, high sensitivity, and rapid detection mechanism may provide an alternative method for determination of phenolic compounds.[13]

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Journal of Applied Polymer Science, Vol. 98, 521–524 (2005)

EXPERIMENTAL

Materials

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

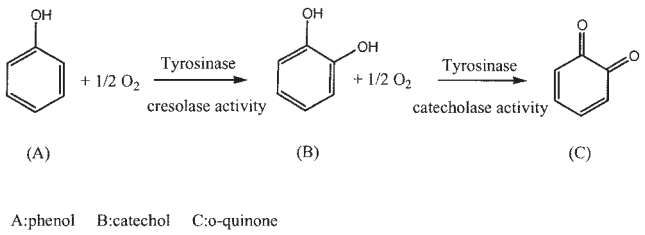
Catechol was purchased from Sigma. All catechol solutions were prepared in citrate buffer.

Preparation of enzyme electrodes

In the present study, decanedioic acid bis(4-pyrrole-1-yl-phenyl) ester (DAPE) was used to synthesize copolymers with pyrrole to form a matrix (Ppy/DAPE) for immobilization of PPO. Synthesis and characterization of the copolymer of DAPE with pyrrole were described (Scheme 2) in an earlier study.[14] Immobilization of PPO was achieved by electropolymerization of pyrrole with a previously DAPE-coated platinum (Pt) electrode.

Electropolymerization was performed in a typical three-electrode cell, consisting of the Pt foils (1.5 cm^2 each) as the working and counter electrodes and an Ag^0/Ag^+ reference electrode.

Enzyme electrodes were prepared in 10 mL citrate buffer (pH 6.5) containing 1 mg/mL SDS as the supporting electrolyte, 0.01*M* pyrrole, and 0.3 mg/mL PPO. Immobilization was carried out at a constant potential of +1.0 V (a potentioscan Wenking POS-73



Scheme 1

model potentiostat was used) for 20 min at room temperature. Enzyme electrodes were kept at 4°C in citrate buffer solution when not in use.

Determination of PPO activity

The activities of free and immobilized PPO were determined by using Besthorn's Hydrazone Method.[15] 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 of MBTH solution was added. Besthorn's Hydrazone Method includes spectrophotometric measurements. In this method, MBTH interacts with the quinones produced by the enzyme to yield red products instead of brown color pigments in the absence of the color reagent.[16] 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.

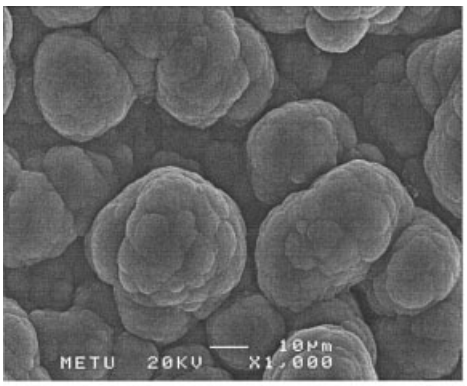
Determination of optimum temperature and pH

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

RESULTS AND DISCUSSION

Morphologies of the films

Scanning electron microscopy (SEM; JEOL JSM-6400) was used to examine the surface morphologies of the enzyme-entrapped polymer films. These were compared with films prepared in the absence of PPO. The films were washed before analysis to eliminate unbound enzymes. Whenever an enzyme is successfully entrapped in a matrix having polypyrrole chains, the cauliflower-like structure is noticeably damaged [Figure 1(a)]. As is observed in Figure 1(b and c), enzyme immobilization significantly changed the surface morphology.



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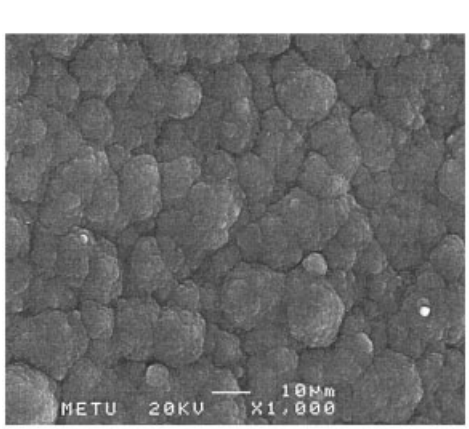

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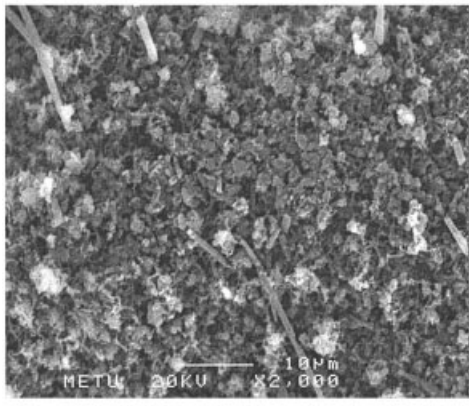
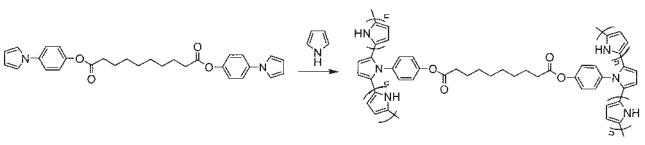


(c)

Figure 1 Scanning electron micrograph of (a) polypyrrole film representing cauliflower-like structure; (b) solution side of the film without the enzyme, (c) solution side of the film with the enzyme.



Scheme 2

TABLE I
Kinetic Data

	V_{max}	K_m (mM)
Free PPO	11.2 μmol/min · mg protein	4
Ppy/PPO	0.110 μmol/min · electrode	100
Ppy/DAPE/PPO	0.04 μmol/min · electrode	14

Kinetic parameters of immobilized enzyme

The maximum reaction rate, $V_{max,}$ and Michaelis–Menten constant, K_m, were obtained from Lineweaver–Burk plots.[17]

K_m is a parameter that is inversely proportional to the affinity of enzyme to substrate. Large K_m values indicate that substrate and enzyme do not prefer to stay together for a long time. For the Ppy/DAPE matrix, PPO has a K_m value of 14 mM while the free enzyme has a K_m value of 4 mM (Table I). As expected, the affinity of the enzyme to substrate decreased when it was entrapped in a polymer matrix. However, Ppy/DAPE electrode still provides a better environment to PPO compared to Ppy electrode, which has a K_m value of 100 mM. Ppy/DAPE enzyme electrode exhibits the best K_m among the matrices that were studied previously.[13]

V_{max} for Ppy/PPO was 0.11 μmol/min·electrode; that for Ppy/DAPE enzyme electrode was 0.040 μmol/min·electrode. When we compared this result with that of the Ppy matrix we saw that the V_{max} of immobilized enzyme in Ppy/DAPE electrode was one third that of the Ppy matrix. These results were also confirmed by the protein amount entrapped in the electrodes, which were 9.6×10^{-3} mg protein for Ppy and 3.6×10^{-3} mg protein for the matrix.

Influence of temperature on the enzyme electrode

The effect of temperature on the enzyme activity was investigated and is shown in Figure 2. Both free and immobilized PPO in the Ppy/PPO electrode showed a maximum activity at about 40°C, whereas the Ppy/DAPE electrode revealed maximum activity at 70°C and, after this temperature, up to 80°C it lost only 20% of its activity. These results imply that enzyme entrapped within this matrix showed high stability against temperature.

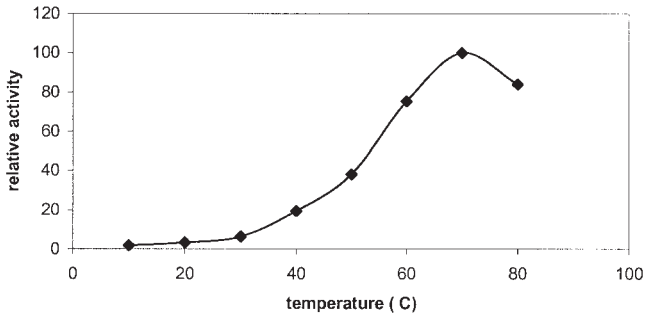


Figure 2 Temperature dependence of DAPE enzyme electrode.

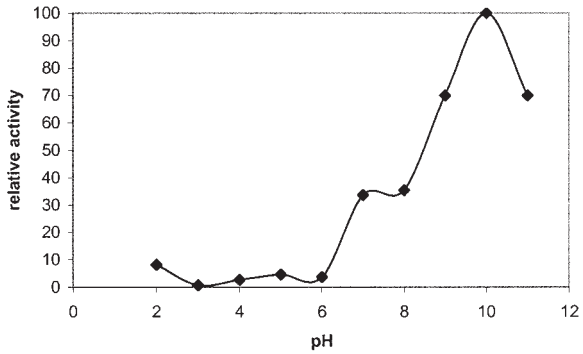


Figure 3 pH dependence of DAPE enzyme electrode.

Influence of pH on the enzyme electrode

In previous work,[18] the shift in the maximum pH values toward the alkaline side was explained with the partitioning of protons. The same behavior in pH dependence was observed for the Ppy/DAPE electrode, yet this electrode exhibits greater stability against high pH (Figure 3). Maximum enzyme activity was observed at pH 10. This shows that this electrode can protect enzymes against high hydroxide concentration.

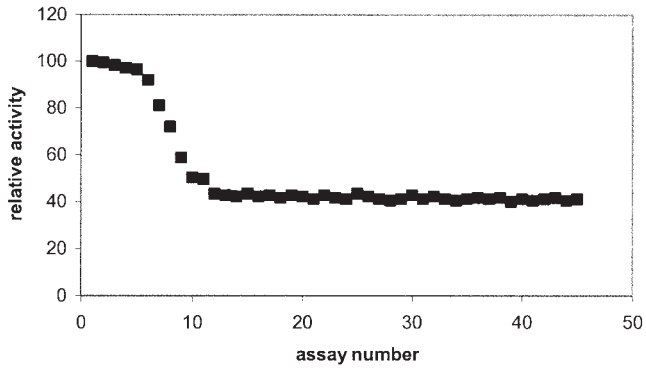
Operational stability and shelf life of the enzyme electrode

The stability of the enzyme electrode in terms of repetitive uses was studied by performing 45 successive measurements at 25°C in 1 day. The Ppy/DAPE electrode showed activity that gradually decreased up to the 10th use and then stayed constant at 45% activity [Figure 4(a)].

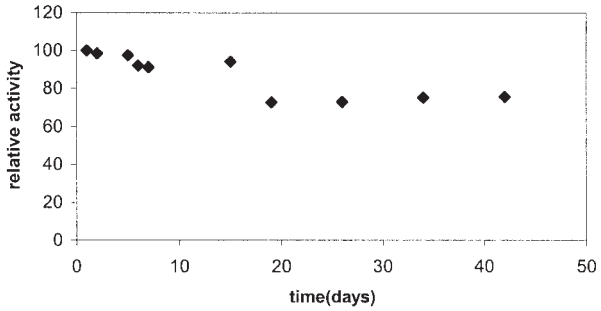
For the shelf-life determination of the enzyme electrode, the activity was checked every day for a week and then once in 5 days throughout 40 days. The enzyme maintained 70% of its initial activity after 18 days and stayed constant until the 40th day [Figure 4(b)].

Determination of total phenolic amount in red wines

Total phenolic compounds in Turkish wines were reported as 2000–3000 mg/L.[19–21] Polyphenol oxidase enzyme acts on –OH groups on phenolic compounds. Via activity determination of enzyme electrodes in red wines we obtain the total –OH groups.



(a)



(b)

Figure 4 (a) Operational stability and (b) shelf life of the DAPE enzyme electrode.

By using the Ppy/DAPE electrode, two Turkish red wines (brand K and brand D) were analyzed for their concentration of phenolic compounds.

Analyzing the red wines with the enzyme electrode, it was found that brand K contains 0.06*M* –OH group, which gives 3350 mg/L total phenolic compound according to gallic acid equivalent calculations.[19] The other brand contains 0.026*M* –OH group, which is equal to 1450 mg/L phenolics. Brand K contains twice the amount of phenolics compared with brand D.

CONCLUSIONS

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

This work was financially supported by DPT 2003 K120920–02 and TUBA grants.

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