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Preparation of biosensors by immobilization of polyphenol oxidase in conducting copolymers and their use in determination of phenolic compounds in red wine

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Abstract

Electrochemically produced graft copolymers of thiophene capped polytetrahydofuran (TPTHF1 and TPTHF2) and pyrrole were achieved by constant potential electrolysis using sodium dodecylsulfate (SDS) as the supporting electrolyte. Characterizations were based on Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). Electrical conductivities were measured by the four-probe technique.

Novel biosensors for phenolic compounds were constructed by immobilizing polyphenol oxidase (PPO) into conducting copolymers prepared by electropolymerization of pyrrole with thiophene capped polytetrahydrofuran. Kinetic parameters, maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) and optimum conditions regarding temperature and pH were determined for the immobilized enzyme. Operational stability and shelf-life of the enzyme electrodes were investigated. Enzyme electrodes of polyphenol oxidase were used to determine the amount of phenolic compounds in two brands of Turkish red wines and found very useful owing to their high kinetic parameters and wide pH working range.

Keywords: Conducting polymer; Copolymer; Biosensor; Polyphenol oxidase; Immobilization; Electrochemistry; Determination of phenolic compounds; Red wine

1. Introduction

Conducting polymers have widespread applications, however, they are poor in mechanical and physical properties in practical use. To improve the processibility of conducting polymers, various methods such as preparation of conducting polymer composites, blends and copolymers can be used [1–5]. Copolymers of pyrrole capped polytetrahydrofuran and pyrrole, in the presence of various supporting electrolytes, were synthesized and characterized [6]. Thiophene capped polytetrahydrofuran was synthesized in previous studies. Living polytetrahydrofuran initiated by methyl triflate was terminated with sodium thiophenemethonate to yield a polymer with thiophene group at one end (TPTHF1) [7]. For the synthesis of polymer with thiophene groups at both ends, living polytetrahydrofuran was initiated by triflic anhydride and terminated with sodium thiophenemethonate (TPTHF2) [8]. In this work graft copolymers of thiophene capped polytetrahydrofuran and pyrrole; TPTHF1-co-Py and TPTHF2-co-Py were prepared by constant potential electrolysis using SDS as the supporting electrolyte. Characterization of resultant products was performed by FTIR and SEM. Electrical conductivities were measured by four-probe technique.

Immobilized enzyme has many operational advantages over free enzyme such as reusability, enhanced stability, continuous operational mode, rapid termination of reaction, easy separation of biocatalyst from product and reduced cost of operation. Through the many methods for immobilization of enzymes on solid support, physical entrapment by electrochemical polymerization has an easier way of fabrication and may provide relatively small perturbation of the enzyme native structure and function [9].

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Tyrosinase is a copper containing oxidoreductase type enzyme with EC number of 1.14.18.1. It is probably present in all plants and catalyzes two different oxygen-dependent reactions that occur consequently: the *o*-hydroxylation of monophenolics to yield *o*-diphenolics and the oxidation of *o*-diphenolic compounds to the corresponding *o*-quinones, called respectively as cresolase and catecholase activity [10]. Tyrosinase has widespread applications in industry such as analysis of phenolic pollutants found in surface waters and in the effluents of industrial discharges [11], cleaning of polluted waters by the filtration of tyrosinase precipitated phenolics [12], construction of sensors to determine the phenolic amount in waste water [13,14], to detect catechols in the urine [15] and to obtain the concentration of total phenolics in red wine [16–20]. In this study, fabricated enzyme electrodes were applied for determination of phenolics in red wine.

2. Experimental

2.1. Materials

Tyrosinase and SDS were purchased from Sigma. Citrate buffer was prepared from tri-sodium citrate-2 hydrate and citric acid. Pyrrole was purchased from Aldrich and distilled before use. Catechol, 3-methyl-2-benzothiozolinone hydrazone (MBTH), acetone and sulfuric acid were obtained from Sigma and used in spectrophotometric PPO activity assay.

2.2. Synthesis and characterization of graft copolymers

Grafting of pyrrole into the insulating polymers was performed in a conventional three-electrode cell equipped with platinum working, platinum counter (1.5 cm^2 each) and Ag^0/Ag^+ reference electrodes. Working electrode was coated by insulating polymer solution prepared as 0.5% (w/v) TPTHF1 or TPTHF2 in dichloromethane. Copolymers of TPTHF1-co-Py and TPTHF2-co-Py were produced by the reaction of pyrrole and thiophene end of TPTHF1 or TPTHF2 as shown on Fig. 1. Synthesis was carried out in a SDS-water medium (1 mg/ml) in the presence of pyrrole (0.01 M) at constant potential of 1.0 V for 20 min. Resulted copolymer films were washed in dichloromethane several times and dried at room temperature.

FTIR spectra of the products were recorded on a Nicolet 510 FTIR spectroscopy. Surface morphologies were obtained by a JSM-6400 scanning electron microscopy. Conductivity measurements were performed by the four-probe technique for both solution and electrode sides of the copolymer films.

2.3. Preparation of enzyme electrodes

The matrices described above were used for immobilization of tyrosinase. It was achieved by electropolymerization of pyrrole on previously TPTHF1 or TPTHF2 coated platinum working electrodes. Constant potential electropolymerization was performed by Wenking POS-73 model potentiostat at room temperature in the presence of tyrosinase. The oxidation potential of pyrrole (+1.0 V) was applied for 20 min and immobilization was carried out in optimized medium [16] which is citrate buffer (pH 6.5) solution containing 1 mg/ml SDS as the supporting electrolyte, 0.01 M pyrrole and 0.3 mg/ml enzyme. Tyrosinase was entrapped within the conducting copolymer produced. After immobilization, enzyme electrodes were washed several times with buffer to remove the supporting electrolyte and unbound enzyme. Electrodes were kept at 4 °C in citrate buffer solution when not in use.

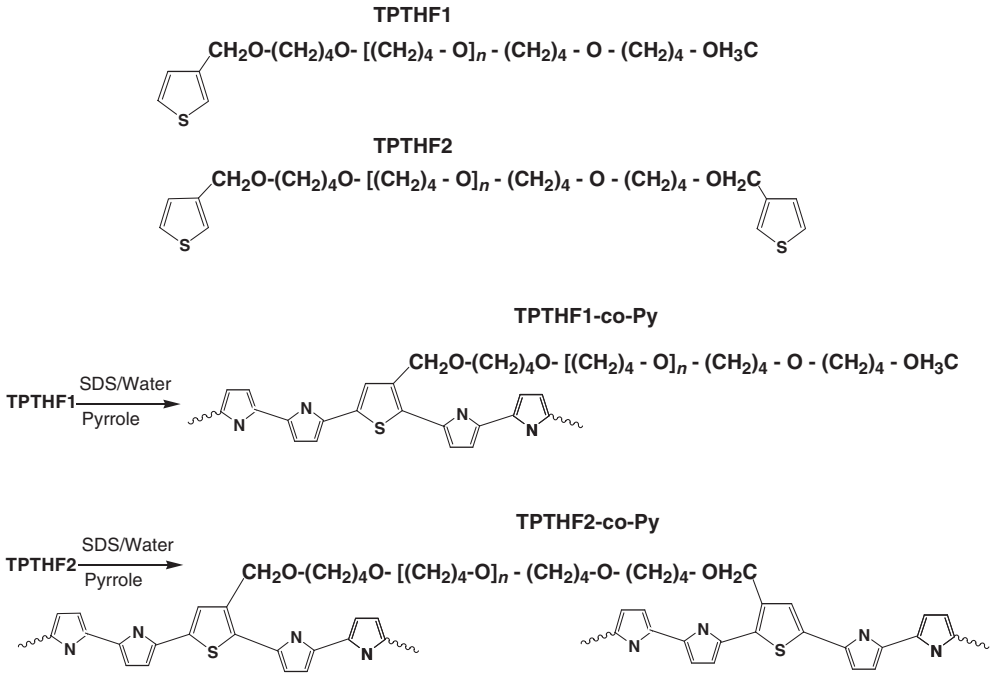

Fig. 1. Electrochemical synthesis route for copolymerization.

2.4. PPO activity determination

Besthorn's hydrazone method was used as enzyme assay to determine the activities of immobilized tyrosinase [21]. The method includes the spectrophotometric measurement of red complex produced by the interaction (Fig. 2) between MBTH and the quinones liberated from catecholase activity [22].

Different concentrations of catechol solutions were prepared in citrate buffer (pH 6.5). 3.0 ml aliquots in test tubes were placed in water bath at 25 °C. 1 ml MBTH solution was added to the test tubes and enzyme electrode was immersed into the solutions for specific reaction times of 5, 10 and 15 min. After removing the electrode, 1.0 ml sulfuric acid (5% v/v) was added to stop the enzymatic reaction. 3 ml acetone was added to dissolve the red color complex produced by reaction of MBTH with quinone. Total volume of 6 ml solutions was obtained. After mixing, their absorbances were measured at 495 nm by using a Shimadzu UV-1601 spectrophotometer. All activity determination results are average of three determinations with a relative standard deviation in the range of 3–8%.

The same procedure was utilized for the measurements of polyphenols in red wines except catechol solutions were replaced by red wines.

2.5. Determination of optimum temperature and pH

Optimum temperature was determined by changing the incubation temperature from 10 °C to 80 °C by 10 °C interval at constant pH of 6.5. Optimum pH was determined by changing pH from 2 to 11 at 25 °C. Substrate concentration was kept constant at 5 km for both optimizations. The rest of the procedure was same as in the activity assay.

2.6. Determination of operational stability and shelf life

Operational stability of enzyme electrode was determined by measuring its activity for 40 repetitive uses on the same day in the optimum conditions. Storage stability was investigated for a 50-day period of time by preserving the electrodes in buffer solution at 4 °C when not in use.

2.7. Determination of phenolics in red wine

TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes were used for determination of phenolic compounds in two brands of Turkish red wine; Brands K and D. Wine samples were diluted by buffer to set the sample to pH 6.5. Assay procedure explained in Section 2.4 was applied in the same way.

3. Results and discussion

3.1. Characterization of copolymers

FTIR spectra of insulating polymers TPTHF1 and TPTHF2 exhibit characteristic C–O–C asymmetric stretching peak at 1111 cm^{-1}. Aliphatic CH_2 vibrations show three peaks between 2750 cm^{-1} and 3000 cm^{-1}. Aromatic C=C stretching peak of thiophene moieties is at 780 cm^{-1}. Copolymers, TPTHF1-co-Py and TPTHF2-co-Py exhibited the characteristic band of Py namely N-H peak at about 900 cm^{-1} and characteristic peaks of insulating polymers except the ether peak. Instead, it was observed a broad band in the range of 1000–1200 cm^{-1} which might belong to both SDS dopant anion and C–O–C group or to merely dopant anion. In order to distinguish the existence of C–O–C peak, copolymer which was produced by electrochemical oxidation, was reduced back electrochemically. As the dopant ion was removed from the copolymer by reduction, ether peak

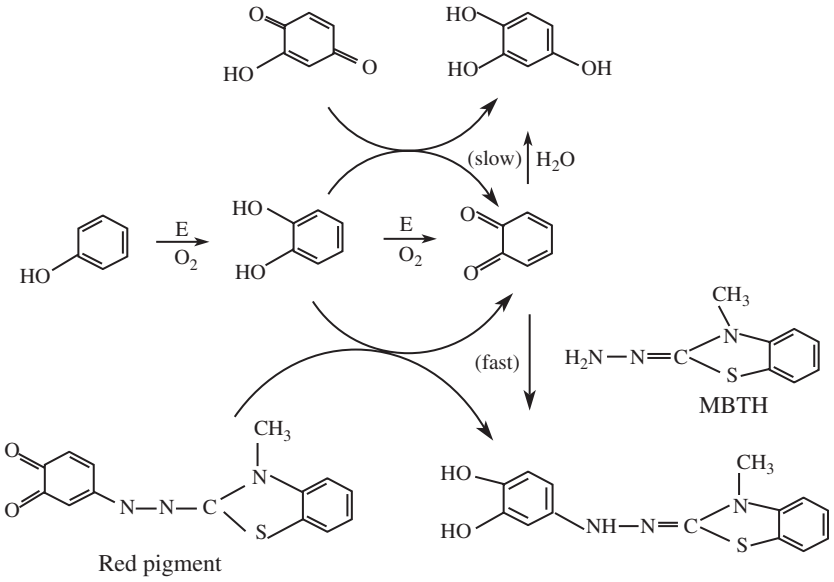

Fig. 2. Reaction mechanism of Besthorn's hydrazone method.

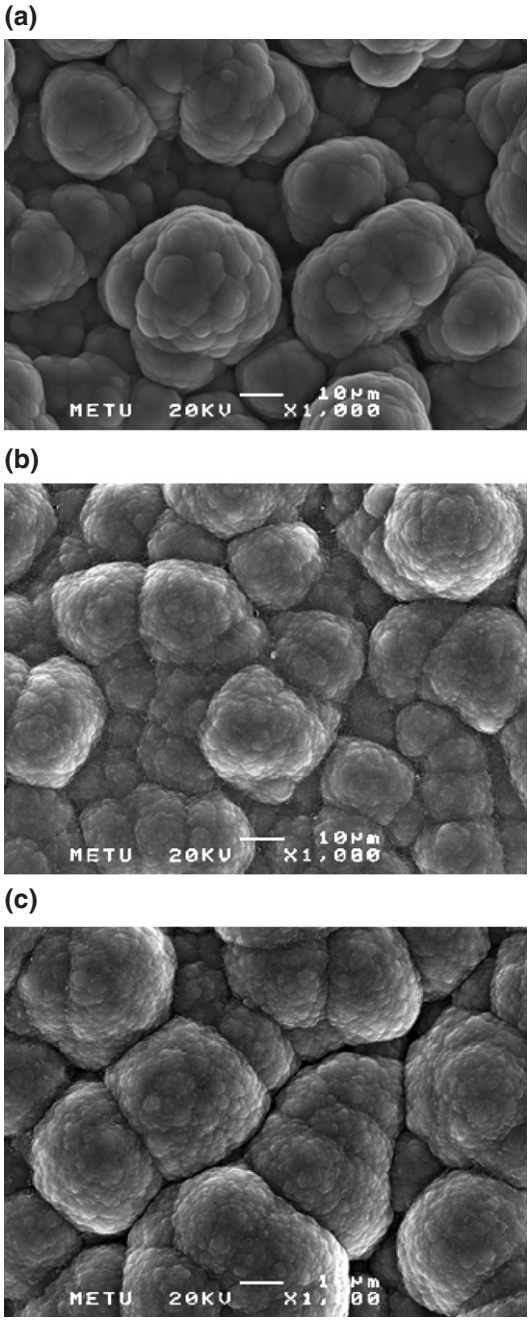

Fig. 3. SEM micrographs of (a) solution side of washed PPy film, (b) solution side of washed TPTHF1-co-Py film, (c) solution side of washed TPTHF2-co-Py film.

appeared with respect to the reduction time. This manner proved the existence of the copolymers and was observed for both of them.

Table 1
Conductivities of the films

Matrices (in SDS)	Conductivity (S/cm)
PPy	18.1
TPTHF1-co-Py	1.2
TPTHF2-co-Py	1.0

Table 2
Kinetic parameters of free and immobilized tyrosinases

	V_{max}	K_m (mM)
Free PPO	0.073 (µmol/min ml)	4
PPy	0.031 (µmol/min electrode)	96
TPTHF1-co-Py	0.021 (µmol/min electrode)	25
TPTHF2-co-Py	0.022 (µmol/min electrode)	25

In SEM photographs, typical cauliflower-like structure was observed for the solution side of SDS doped PPy film (Fig. 3 (a)). However, morphology of the solution sides of washed

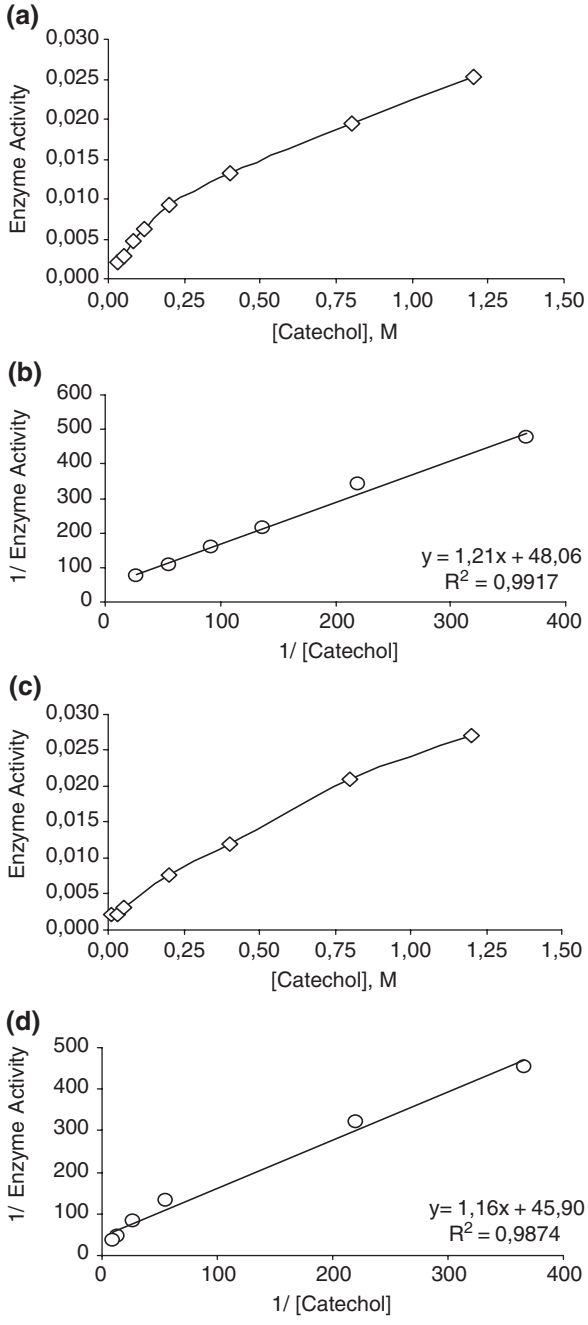

Fig. 4. Activity versus substrate concentration curves of immobilized PPO in TPTHF1-co-Py (a), in TPTHF2-co-Py (c), Lineweaver–Burk plot of immobilized PPO in TPTHF1-co-Py (b), in TPTHF2-co-Py (d).

TPTHF1-co-Py and TPTHF2-co-Py copolymers (Fig. 3(b) and (c)) exhibited a little different surface structure than PPy. They show more compact structure but no significant difference was observed between the two copolymers films.

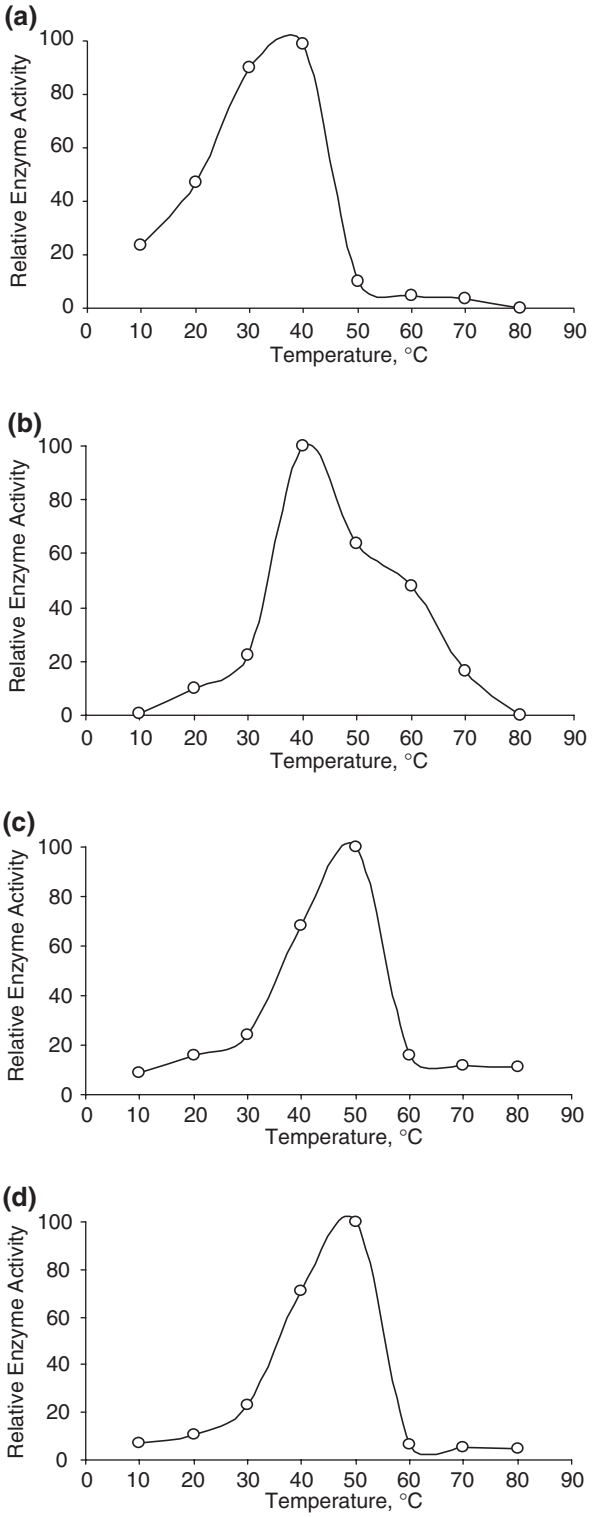

Fig. 5. Effect of temperature on PPO enzyme activity of free (a) and immobilized enzyme in PPy (b), TPTHF1-co-Py (c) and TPTHF2-co-Py (d) enzyme electrodes.

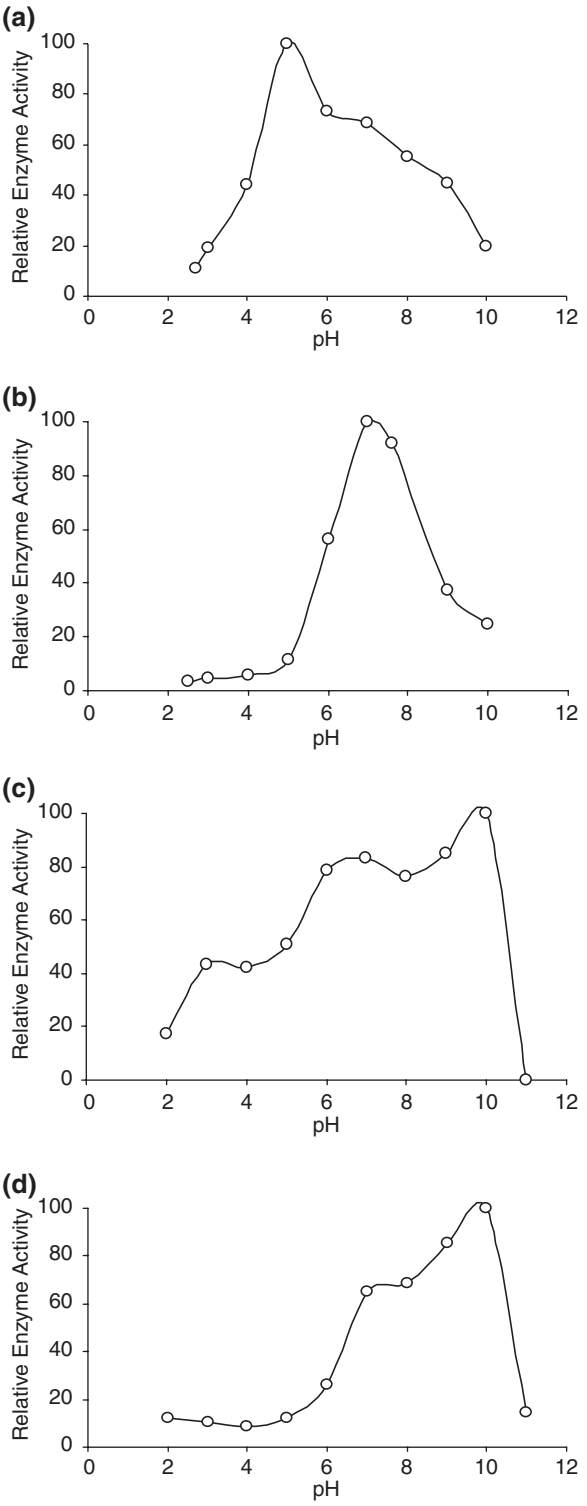

Fig. 6. Effect of pH on PPO enzyme activity of free (a) and immobilized enzyme in PPy (b), TPTHF1-co-Py (c) and TPTHF2-co-Py (d) enzyme electrodes.

Conductivities of TPTHF1-co-Py and TPTHF2-co-Py were shown on Table 1. No significant difference was observed between the two copolymers. The conductivities of both electrode and solution sides were also in the same order of magnitude, which reveal the homogeneity of the films. Difference between conductivities of PPy and copolymer

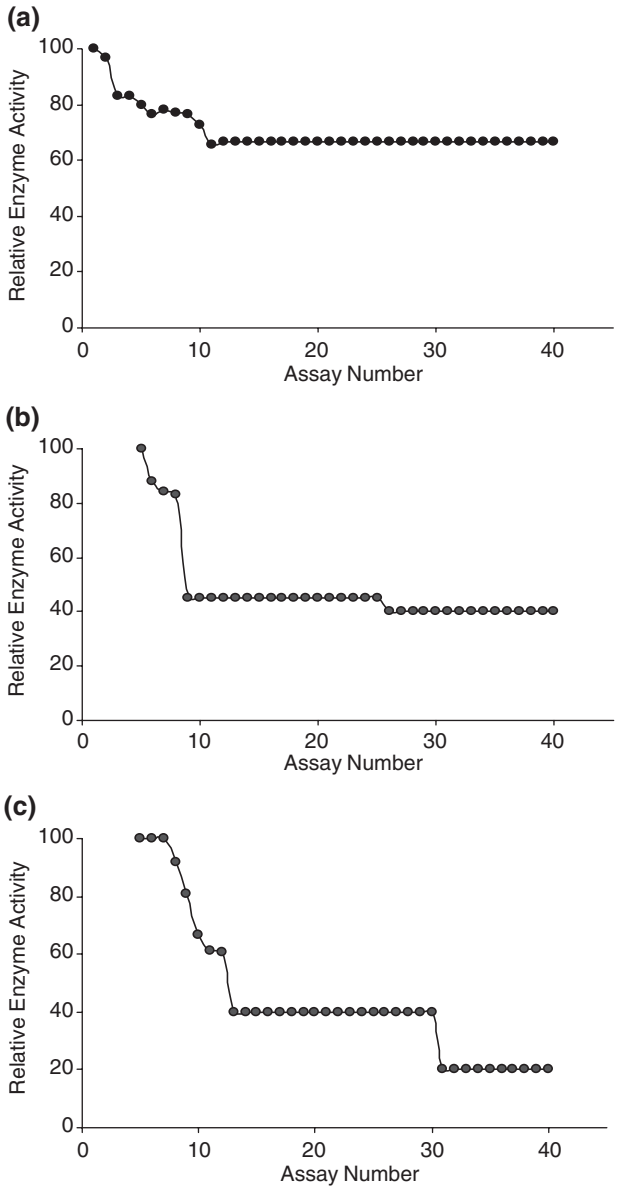

Fig. 7. Effect of repetitive use on PPO enzyme activity immobilized in PPy (a), TPTHF1-co-Py (b) and TPTHF2-co-Py (c) enzyme electrodes.

films shows that copolymerization of pristine polymer and pyrrole was achieved and an intermediate value of conductivity which is lower than conductivity of pyrrole.

3.2. Kinetic parameters of immobilized enzyme

Kinetic parameters; V_{max} and K_m (Table 2) for free and immobilized PPO were obtained from TPTHF1-co-Py and TPTHF2-co-Py enzyme electrode activity curves (Fig. 4(a), (b) respectively) and corresponding Lineweaver–Burk plots (Fig. 4 (c), (d)).

It was observed that the rate of reaction decreases due to immobilization of enzyme. The diffusion of substrate from the bulk solution to the micro-environment of an immobilized enzyme can limit the rate of the enzyme reaction. Thus, enzymes entrapped in PPy and copolymers have comparable reaction rates with each other but lower than that of the free enzyme. K_m values of immobilized enzyme in both PPy and copolymer matrices are higher than that of free enzyme, thus showing a lower affinity between the substrate and immobilized enzyme compare to the one between substrate and free enzyme. On the other hand, copolymers have much better affinity constants than PPy. Enzyme entrapped in copolymer has much better affinity towards its substrate means that enzyme can meet its substrate in a more convenient medium most probably because of more porous structure of copolymers.

3.3. Effect of temperature on enzyme activity

Both free and immobilized polyphenol oxidase (in PPy) showed an optimal temperature of 40 °C. Free PPO loses its activity completely at 50 °C but PPO immobilized in PPy has 60% of original activity at this temperature. However, temperature of maximum activity shifts to 50 °C for immobilized PPO in TPTHF1-co-Py and TPTHF2-co-Py

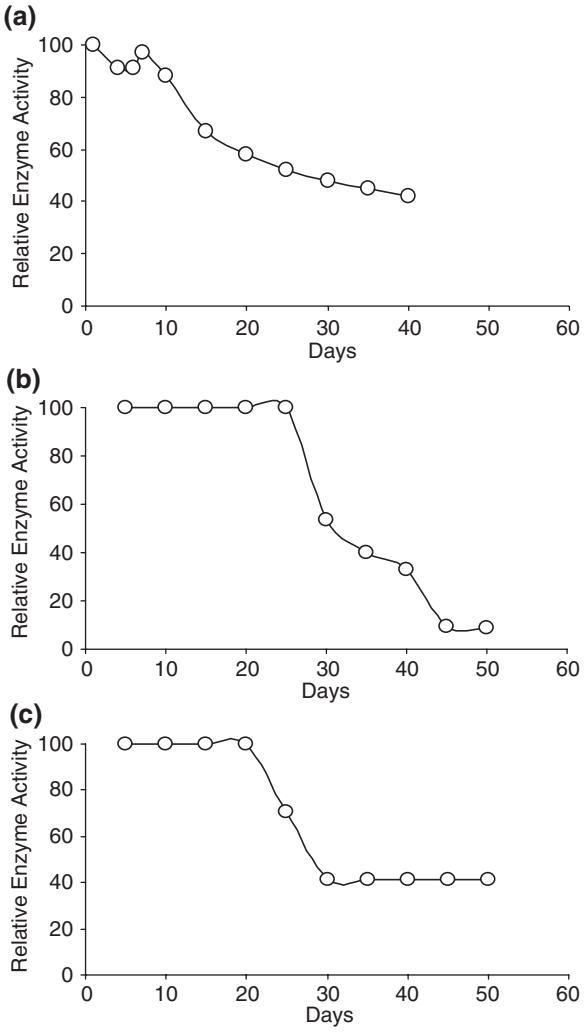

Fig. 8. Shelf-life of PPO immobilized in PPy (a), TPTHF1-co-Py (b) and TPTHF2-co-Py (c) enzyme electrodes.

electrodes (Fig. 5). Copolymer matrices protect the enzyme against high temperatures.

3.4. Effect of pH on enzyme activity

Free PPO had a maximum activity at pH of 5. Immobilized PPO in PPy revealed an optimal pH of 7 and thus, pH stability is increased upon immobilization in PPy. The optimum pH values for both TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes were shifted towards the alkaline side compared to that of the free enzyme (Fig. 6). This reveals a high degree of stability for both electrodes against higher pH values as observed in a previous study about PPO immobilization [16]. This might be explained by the partitioning of protons. The negative groups of the matrices are concentrated around the enzymes and attract the protons in the solution. This tendency makes the pH around enzyme lower than that of the bulk. A higher tendency of matrices to concentrate protons within makes the pH stability of enzyme higher in the matrices [16]. The activity of PPO immobilized in PPy and copolymers was almost insensitive to the pH change, at pH levels lower than 5. Immobilized enzyme molecules show no considerable activity in acidic range because of ionization in acidic pH values [16].

3.5. Operational and storage stability of enzyme electrodes

Operational stability is an important consideration for immobilized enzymes. To determine this parameter, activities of the enzyme electrodes were studied for 40 successive measurements. Fig. 7 shows the operational stability of PPy and copolymer electrodes respectively. The rate of loss of activity of the immobilized enzyme is high for the first 10 uses retaining 60% of the initial activity, since most of the loosely bound enzyme was leached during early stages of the well-entrapped enzyme in the films. The PPO immobilized in copolymer matrices retained about 45% of its initial activity after 10 uses.

Since enzymes can easily lose their catalytic activity and get denatured, stability of electrodes as a function of time over a period of 40 days was also investigated. Before performing the enzyme assays, the electrodes were washed with buffer solution to remove any loosely bound enzyme that might have diffused to the surface of the film in time. Fig. 8 shows the effect of time on the activity of the immobilized PPO. The rate of loss of activity of the PPy immobilized enzyme was high for the first days, retaining 60% of the initial activity after 10 days. On the other hand, PPO immobilized in copolymer matrices retained 100% activity during 20 days. Since copolymers have more compact structure as seen in SEM micrographs. We believe diffusion of enzyme towards the substrate solution is inhibited during use. However, they have a limited time of 20 days for use, their totally reserved activity of 100% for this period makes TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes very useful.

3.6. Determination of phenolic compounds in red wines

Total phenolic compounds in Turkish wines were reported as 2000–3000 mg/l in literature [23–25]. For the same purpose, Folin Ciocalteau Method was used in this work and total phenolic amount was obtained as 4040 mg/l and 2360 mg/l for the two Turkish red wines; Brands K and D respectively. Free PPO, PPy [16], TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes were used to determine total phenolic compounds in the same brands (Table 3). Free PPO enzyme gives misleading results for phenolic determination when compared to immobilized enzyme electrodes due to the inhibitors present in the wine. Benzoates found naturally in wines act as inhibitors for free PPO [26,27]. However, PPO protected via matrix entrapment was not affected by the inhibitors and the results are found to be comparable to the literature values of phenolic compounds in Turkish red wines. Brand K contains twice the amount of phenolics compared to that of Brand D. This result was confirmed by all enzyme electrodes. PPy enzyme electrode gave a result of 4000 mg/l and 2200 mg/l phenolics in Brands K and D respectively. However, TPTHF1-co-Py and TPTHF2-co-Py enzyme electrodes gave lower values for the same brands since enzyme electrodes have different sensitivity ranges according to their Michaelis–Menten constants (K_m).

Table 3
Phenolic compounds in Turkish red wines

	Brand K	Brand D
Free PPO [16]	0.004 M –OH 220 mg/l	0.005 M –OH 270 mg/l
PPy/PPO [16]	0.072 M –OH 4000 mg/l	0.04 M –OH 2200 mg/l
TPTHF1-co-Py/PPO	0.048 M –OH 2670 mg/l	0.027 M –OH 1460 mg/l
TPTHF2-co-Py/PPO	0.040 M –OH 2200 mg/l	0.022 M –OH 1200 mg/l

Since tyrosinase act on –OH groups on phenolic compounds, total amount of -OH groups in red wines was obtained through activity determination of enzyme electrodes. Results are reported in Gallic Acid Equivalent (GAE) as mg/l [28].

4. Conclusion

The synthesis of graft copolymers of TPTHF1 and TPTHF2 and pyrrole were achieved via constant potential electropolymerization. Thermally stable and electrically conducting polymer films were obtained. Immobilization of tyrosinase enzyme was carried out successfully in conductive matrices. In terms of the immobilized enzyme activity good results were obtained with the conducting copolymer matrices, means that enzyme electrodes exhibited a similar V_{max} but much better K_m values than PPy. Although they have limited storage capacities, these enzyme electrodes are very useful as their high kinetic parameters point. In addition to that, they exhibit a wide pH working range through the alkaline side after pH 6. Since they eliminate the sample preparation and separation procedures necessary for other determination methods, their application for analysis of phenolic compounds in red wine as an alternative method to the conventional methods is reasonably possible.

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