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Journal of Molecular Catalysis B: Enzymatic 27 (2004) 255–265

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Reversible immobilization of tyrosinase onto polyethyleneimine-grafted and Cu(II) chelated poly(HEMA-co-GMA) reactive membranes

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Received 3 March 2003; received in revised form 11 December 2003; accepted 11 December 2003

Abstract

The present study describes the preparation of poly(HEMA-co-GMA) reactive membranes that were grafted with polyethylenimine (PEI) following UV photo-polymerization. The immobilization of tyrosinase was carried out via multi-point ionic interactions based on $-NH_2$ groups of PEI and Cu(II) ions. Tyrosinase is a copper-dependent enzyme, which should show a binding affinity for the chelated Cu(II) ions on the membrane surfaces. The tyrosinase immobilization was positively correlated with the input enzyme amount in the immobilization medium. The maximum tyrosinase immobilization capacities of the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes were 19.3 and 24.6 mg/m^2, respectively. The enzyme activity when assessed at various pH and temperatures gave broader range for immobilized preparations when compared to free enzyme. The poly(HEMA-co-GMA)–PEI–Cu(II) tyrosinase membranes retained 82% of their initial activity at the end of 120 h of continuous reaction. Moreover, upon storage for 3 months the activity of the immobilized membranes retained 46% of their initial levels. After deactivation of the enzyme, the poly(HEMA-co-GMA)–PEI membrane was easily regenerated, re-chelated with the Cu(II) ions and reloaded with the enzyme for repeated use. The mild immobilization conditions, easy and rapid membrane preparation, one-step enzyme adsorption at substantially higher levels and membrane reusability are the beneficial properties of such systems and offers promising potential in several biochemical processes.
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Keywords: Immobilization; Adsorption; Polyethylenimine; Tyrosinase; Hydrophilic membranes

1. Introduction

In recent years, hydrogel membranes are widely used for immobilization of enzymes, especially in the construction of enzyme reactors and enzyme electrodes [1–4]. Various methods exist for the immobilization of enzymes and these may be divided into two main categories: physical methods based on molecular interactions between the enzyme and support, and chemical methods based on the formation of covalent bonds. The methods and supports employed for enzyme immobilization are chosen to ensure the highest retention of enzyme activity and its stability and durability.

Among the immobilization techniques, adsorption may have a higher commercial potential than other methods because it is simpler, less expensive, and it retains high catalytic activity. A number of methods for reversible immobilization of enzymes have been reported [5–8]. The method also allows for the reusability of expensive support after inactivation of immobilized enzyme. However, the adsorption is not generally very strong. Some of the adsorbed proteins will desorb during washing and operation. Thus, reversible enzyme immobilization via adsorption requires a strong hydrophobic or ionic interaction between the enzyme and support. The polyethylenimine coated Sepabead supports were used for the reversible immobilization of invertase and β-galactosidase [9,10]. Desorption of enzymes from the polyethylenimine coated support was found to require the use of denaturing conditions (under low pH and high ionic strength), but this desorption would be necessary after inactivation of the enzyme upon use [9,10]. On the other hand, immobilized metal affinity chromatography is based on the affinity of the surface functional groups of protein for immobilized metal ions. Divalent metal ions such as Cu(II) and Zn(II) are used as ligands after immobilization on the

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doi:10.1016/j.molcatb.2003.12.006

chromatographic supports [11–13]. It has been reported that Cu(II) ions bind very strongly to ethyleneimine molecules [14]. This complex should be able to bind tyrosinase, which is a copper dependent enzyme. Thus, this system provides specific binding sites for the reversible immobilization of tyrosinase.

The enzyme tyrosinase (E.C.1.14.18.1; monophenol monoxygenase) is a copper-dependent enzyme and widely distributed throughout the phylogenetic scale from bacteria to mammals [15]. Tyrosinase catalyzes two different oxygen-dependent reactions via separate copper-dependent active sites: the o-hydroxylation of monophenols to yield o-diphenols (cresolase activity) and the subsequent oxidation of o-diphenols to o-quinones (catecholase activity) [16–18]. The enzyme has been proposed for synthesis of 3,4-dihydroxyphenylalanine (L-DOPA) [19], dephenolization of industrial wastewater [20–23], as a part of an enzyme electrode for the determination of phenol and its derivatives [24,25], bioremediation of contaminated soils [26,27], and fruit juice clarification [28], thus it is an industrially important biomolecule.

The production of L-DOPA from L-tyrosine is possible by using immobilized tyrosine [19,29], L-DOPA is currently produced by chemical methods [30]. One other application of the immobilized tyrosinase is phenol removal from wastewater. Atlow et al. demonstrated that tyrosinase immobilized membranes can transform phenols and its derivatives from wastewaters to o-quinones and low molecular weight polymers [18,31–35].

In this study, acrylic hydrogel copolymers were synthesized in the membrane form from the monomers 2-hydroxyethylmethacrylate (HEMA) and glycidyl methacrylate (GMA). The polyethylenimine (PEI) polymer was covalently attached onto membrane surfaces. Cu(II) ions were then chelated with the amino groups of the PEA molecules. The amino groups of the PEI Cu(II) ions on the surface of the membrane were used for the reversible immobilization of tyrosinase. Our results demonstrate that tyrosinase can be efficiently and reversibly adsorbed on this novel support, its activity at the end continuous reaction was retained over 80%. The stability and activity at adverse reaction conditions were significantly superior to the native free enzyme. This study shows that mild reaction environments used in adsorbing enzymes offer a potential approach for continuous reactor applications.

2. Experimental

2.1. Materials

Tyrosinase [EC 1.14.18.1; Polyphenol oxidase; Monophenol monooxygenase, from Mushroom, 2250 U/mg solid], polyethylenimine (PEI, Mw: 25.000) and L-tyrosine were obtained from the Sigma–Aldrich (St. Louis, USA) and used as received. HEMA, glycidyl methacrylate (methacrylic acid 2,3 epoxypropyl isopropyl ether; GMA), and α-α'-azo-isobisbutyronitrile (AIBN) were obtained from Fluka Chemie, AG (Buchs, Switzerland). The monomers (i.e. HEMA and GMA) were distilled under reduced pressure before use. All the other analytical grade chemicals were purchased from Merck AG and were used as received. (Darmstadt, Germany).

2.2. Preparation of poly(hydroxyethyl methacrylate-co-glycidyl methacrylate) membranes

The poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate), poly(HEMA-co-GMA) hydrogels were prepared by photo-polymerization. The polymerization was carried out in a round glass mold (diameter: 9.0 cm) at 25 °C under a nitrogen atmosphere for 1 h. The membrane preparation mixture (5 ml) contained HEMA (1.6 ml or 1.32×10^{-2} mol), GMA (0.4 ml or 3.31×10^{-2} mol), isopropyl alcohol (1.0 ml), AIBN (10 mg) and phosphate buffer (2 ml, 50 mM, pH 7.0). The mixture was equilibrated at 25 °C for 15 min in a waterbath. The mixture was then poured into the mold and exposed to long-wave ultraviolet radiation for 20 min. After polymerization, the poly(HEMA-co-GMA) membranes were washed several times with distilled water and cut into circular pieces (diameter: 1.0 cm) with a perforator. The thickness of the wet membranes was about $450 \pm 50\ \mu$m.

2.3. Grafting of poly(HEMA-co-GMA) membrane with PEI

The poly(HEMA-co-GMA) membrane discs (10 g, diameter 1.0 cm) were swelled in phosphate buffer (50 mM, pH 8.0) for 2 h, and then transferred to fresh medium containing PEI (1.0%). Grafting of PEI onto the poly(HEMA-co-GMA) membrane discs was carried out at 65 °C for 5 h, with continuous agitation. The poly(HEMA-co-GMA)–PEI membrane discs were then washed first with 1.0 M NaCl and then with phosphate buffer (0.1 M, pH 7.0).

2.4. Incorporation of Cu(II) ions on the poly(HEMA-co-GMA)–PEI membrane

Cu(II) ion was chelated with the amino groups of the poly(HEMA-co-GMA)–PEI membrane. A 100 ppm solution of Cu(II) ions was prepared from nitrate salts in distilled water at pH 4.1. The poly(HEMA-co-GMA)-PEI membranes were suspended in the Cu(II) ions solution at 25 °C for 1.0 h followed by several washings with phosphate buffer (50 mM, pH 7.0) and were then stored at 4 °C until use.

The concentration of the Cu(II) ions in the resulting solution was determined with a flame atomic absorption spectrophotometer [(AAS), Shimadzu AA 6800, Japan]. The Cu(II) ions desorption experiments were performed in a buffer solution containing 25 mM EDTA at pH 4.9. The Cu(II) ions chelated poly(HEMA-co-GMA)–PEI

membranes were placed in a medium for 60 min. The final Cu(II) ion concentration in the medium was determined by AAS.

2.5. Immobilization of tyrosinase onto poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes

The initial immobilization concentration of tyrosinase was adjusted to 1.0 mg/ml. The immobilization experiments were conducted at 22 °C for 3 h with continuous shaking. Efficiency of immobilization was determined by measuring the initial and final absorbance of tyrosinase in the immobilization medium. A calibration curve was prepared using tyrosinase as a standard (0.05–3 mg/ml). The concentrations of tyrosinase were measured at 280 nm by UV/Vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601) obtained from the standard curve.

2.5.1. Effect of pH on the immobilization efficiency

Immobilization of tyrosinase (as described above) on the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes via adsorption were studied at various pH's, in either acetate (7.5 ml, 50 mM, pH 4.0–5.5) or in phosphate buffer (7.5 ml, 50 mM, pH 6.0–8.0). The initial concentration of tyrosinase was kept at 1.0 mg/ml in each corresponding buffer solution.

2.5.2. Effect of initial tyrosinase concentration on the immobilization efficiency

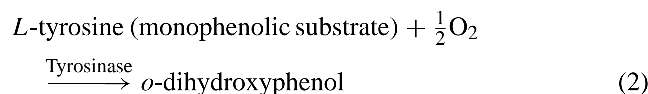
The tyrosinase concentration in the immobilization medium ranged from 0.2 to 1.0 mg/ml. The poly(HEMA-co-GMA)–PEI and/or the poly(HEMA-co-GMA)–PEI–Cu(II) membrane discs were added. The pH of the immobilization medium was 7.0 for the poly(HEMA-co-GMA)–PEI and this was 6.0 for the poly(HEMA-co-GMA)–PEI–Cu(II) membrane. After the immobilization was done as described above the membranes were separated from the enzyme solution and the amount of immobilized tyrosinase was obtained by using the following equation:

$$Q = \frac{[(C_o - C)V]}{S} \quad (1)$$

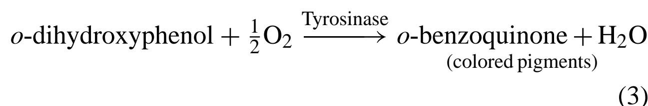
where Q is the amount of tyrosinase immobilized onto membrane (mg/m^2); C_o and C are the concentrations of the tyrosinase in the initial and final solutions, before and after immobilization, respectively (mg/ml); V is the volume of the aqueous solution (ml); S is the surface area of the membrane (m^2).

2.6. Activity assays of free and adsorbed tyrosinase

The tyrosinase reaction is:



$$L\text{-tyrosine (monophenolic substrate)} + \tfrac{1}{2}O_2 \xrightarrow{\text{Tyrosinase}} o\text{-dihydroxyphenol} \quad (2)$$



$$o\text{-dihydroxyphenol} + \tfrac{1}{2}O_2 \xrightarrow{\text{Tyrosinase}} \underset{\text{(colored pigments)}}{o\text{-benzoquinone}} + H_2O \quad (3)$$

Tyrosinase is a copper dependent enzyme which catalyzes two different reactions using molecular oxygen; the hydroxylation of monophenols to o-diphenols (monophenolase activity) and the oxidation of the o-diphenols to o-quinones (diphenolase activity). These quinones are reactive and can undergo subsequent conversions to form other intermediates such as hydroxylated biphenyls. These colored polymeric pigments can be monitored spectrophotometrically and can be used as an indication of the monophenol levels present.

2.6.1. Free enzyme

The reaction was carried out in a quartz cuvette (3 ml) at 25 °C and change in absorbance ($A_{280\,nm}$) was measured spectrophotometrically. A 2.9 ml of L-tyrosine solution (1.0 mM L-tyrosine in phosphate buffer (0.05 M, pH 6.5)) was saturated with pure oxygen at 25 °C for 2 min. The reaction was started by adding 0.1 ml of enzyme solution (1 mg tyrosinase/ml) and the increase in absorbance at 280 nm was measured for 10 min.

2.6.2. Immobilized enzyme

For the determination of immobilized tyrosinase activity, five pieces of enzyme-membrane discs were introduced to L-tyrosine solution (1.0 mM, 5 ml), were then incubated in a shaking waterbath and the reaction medium was purged with pure oxygen. The increase in absorbance at different time intervals was followed as described above.

One unit of enzyme activity is defined as an increase in absorbance at 280 nm, of 0.001/min at pH 6.5 and at 35 °C in a reaction mixture containing L-tyrosine.

2.7. Dependence of enzyme activity on pH and temperature

The effect of pH on the activity of the free and the immobilized enzymes was determined using the pH range 4.0–8.0 and at 35 °C. The effect of temperature on enzyme productivity was studied in the range 20–50 °C with a L-tyrosine concentration of 5.0 mM in 50 mM phosphate buffer pH 6.5. The results for pH and temperature were presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

The concentration of the L-tyrosine solution was 5.0 mM and was prepared in 50 mM acetate buffer in the pH range 4.0–5.5, and 50 mM phosphate buffer in the pH range 6.0–8.0. The ionic strength values are calculated for acetate and phosphate buffers as 0.05 and 0.15, respectively. The ionic strength of the acetate buffer (0.05) was adjusted to the same value of phosphate buffer (0.15) using a neutral salt KCl. The experimental ionic strength value in each buffer solution was about 0.15.

2.8. Enzyme reactor and operation

The glass reactor (length 12 cm, diameter 1.2 cm, total volume 13.5 ml) was made from Pyrex®. The enzyme-membranes (7.0 g) were equilibrated in phosphate buffer (50 mM, pH 6.5) at 4 °C for 1 h and were loaded into the reactor yielding a void volume of about 6.0 ml. To determine operational stability of immobilized tyrosinase, the reactor was run at 35 °C for 120 h. L-tyrosine solution (5.0 mM, in the phosphate buffer (50 mM, pH 6.5) was fed into the reactor at a rate of 20 ml/h with a peristaltic pump (Cole Parmer, Model 7521-00, USA) through the lower inlet part. The product formation was assayed hourly as described above.

2.9. Thermal and storage stability measurements of free and immobilized enzymes

Thermal stabilities of the free and immobilized tyrosinase were determined by measuring the residual activity of the enzyme exposed to two different temperatures (50 and 60 °C) in phosphate buffer (50 mM, pH 6.5) for 120 min. The residual activity was defined as the fraction of the total activity recovered after the immobilization of tyrosinase on the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes compared with the same quantity of free enzyme. Five membrane discs were removed from incubation solutions with a subsequent time interval of 15 min and assayed for enzymatic activity. The results were converted to relative activities (percentage of the maximum activity obtained in that series). To examine the storage stabilities of the enzyme immobilized on membranes, similar experiments were performed on 3 month-old membranes that were incubated at 4 °C.

2.10. Reusability of poly(HEMA-co-GMA) membranes

In the reusability experiments, adsorption and desorption cycles of tyrosinase were repeated five times on the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes. The enzyme desorption was performed in a KSCN solution (1.0 M, pH 4.0, 5.0 ml) while mixing at 25 °C for 3 h. The same membrane discs were then reused in the enzyme immobilization. The final tyrosinase concentration within the desorption medium was determined as described above and the desorption ratios of the enzyme were calculated by using the following expression:

$$\text{Desorption ratio} = \frac{[(\text{Amount of tyrosinase released}) \times 100]}{[\text{Amount of tyrosinase adsorbed on the membrane}]} \qquad (4)$$

2.11. Characterization of poly(HEMA-co-GMA) membrane

Basic characterization of the poly(HEMA-co-GMA)–PEI membranes was carried out as described briefly below and reported in Table 1.

Table 1
Properties of poly(HEMA-co-GMA) membranes

Epoxy group content (mmol/m^2)	9.02×10^{-1}
Free amino group content (mmol/m^2)	1.03×10^{-3}
Cu(II) ions content (mmol/m^2)	4.9×10^{-1}
Water content (%)	47
Water content after PEI-grafting (%)	51
Contact angle to water (°)	61.4
Contact angle to water after PEI-grafting (°)	58.7
Specific surface area (m^2/g)	1.43
Thickness (μm)	450 ± 50

2.11.1. Determination of the content of the epoxy group

The available epoxy group content of the poly(HEMA-co-GMA) membrane was determined by pyridine–HCl method as described previously [36].

2.11.2. Elemental analysis

The amount of covalently bound PEI on the membrane was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA), by considering the nitrogen stoichiometry.

2.11.3. Determination of the content of the free amino group

The free amino group content of the poly(HEMA-co-GMA)–PEI membrane was determined by potentiometric titration. The poly(HEMA-co-GMA)–PEI membrane discs (about 1.0 g) were transferred into hydrochloride acid solution (0.1 M, 20 ml) and were then incubated in a shaking water-bath at 35 °C for 6 h. After this reaction period, the final HCl concentration in the solution was determined by a potentiometric titration with 0.05 M NaOH solution.

2.11.4. FT-IR spectra

FT-IR spectra of the poly(HEMA-co-GMA) and poly(HEMA-co-GMA)–PEI membranes were obtained by using a FT-IR spectrophotometer (FT-IR 8000 Series, Shimadzu, Japan). The dry membrane (about 0.1 g) was mixed with KBr (0.1 g) and pressed into a tablet form. The FT-IR spectrum was then recorded.

2.11.5. Water contents of membranes

The water contents of the poly(HEMA-co-GMA) and poly(HEMA-co-GMA)–PEI membranes were determined at room temperature in phosphate buffer (0.1 M, pH 7.0) by using a gravimetric method. The water contents were defined as the weight ratio of water contained within swollen to dry membrane. The water content of the membranes was calculated by using the following expression:

$$\text{Swelling ratio}\,\% = \left\{\frac{(W_s - W_d)}{W_d}\right\} \times 100 \qquad (5)$$

where W_d and W_s are the dry and swollen weights of membranes, respectively.

2.11.6. Scanning electron microscopy

The dried poly(HEMA-co-GMA) membranes were coated with gold under reduced pressure and their scanning electron micrographs were obtained using a JEOL (Model, JSM 5600, Japan) scanning electron microscope.

2.11.7. Surface area measurement

The surface areas of the poly(HEMA-co-GMA) membrane samples were measured with a surface area apparatus (BET method).

2.11.8. Contact angle measurement

Contact angle is a quantitative measure of the wetting of a solid by a liquid and it is defined geometrically as the angle formed by liquid at the three-phase boundary where a liquid, gas and solid intersect. The primary focus of the contact angle studies is the assessment of the wetting characteristics of solid/liquid interactions. These studies are commonly used as the most direct measure of wetting. Contact angles to water of the dry poly(HEMA-co-GMA) and poly(HEMA-co-GMA)–PEI membrane samples were measured by sessile drop method at ambient temperature by using a digital optical contact angle meter CAM 200 (KSV Instruments, Helsinki, Finland).

3. Results and discussion

3.1. Properties of poly(HEMA-co-GMA)

In the present study, the epoxide groups carrying poly(HEMA-co-GMA) membrane was prepared from HEMA and GMA via photo-polymerization in the presence of an initiator (AIBN). The amount of available epoxy groups was determined by titration of pyridine–HCl solution with 0.1 M NaOH solution. It was found to be 9.02×10^{-1} mol/m^2 (or 1.29×10^{-2} mol/g) membrane. The epoxide groups are very convenient for the covalent immobilization of macromolecules. The O–C and N–C bonds formed by the epoxide groups are extremely stable, so that the epoxide-containing support matrices could be used for the immobilization of various macromolecules and proteins. Furthermore, epoxy activated supports are able to form covalent linkages with different side chain groups (amino, thiol, phenolic ones) on the macromolecule structure under suitable experimental conditions (e.g. pH 7.0) [37].

The PEI was grafted on the poly(HEMA-co-GMA) membrane surface via reaction between epoxy groups of the membrane and amino groups of the polyethylenimine. Elemental analyzes of the PEI-grafted membranes were performed, and the amounts of grafted PEI were found to be 1.03×10^{-3} mmol/m^2 (or 1.48×10^{-3} mmol/g) membrane from the nitrogen stoichiometry and potentiometric titration.

FTIR spectra of plain and PEI-grafted poly(HEMA-co-GMA) membranes are presented in Fig. 1. The broad band in

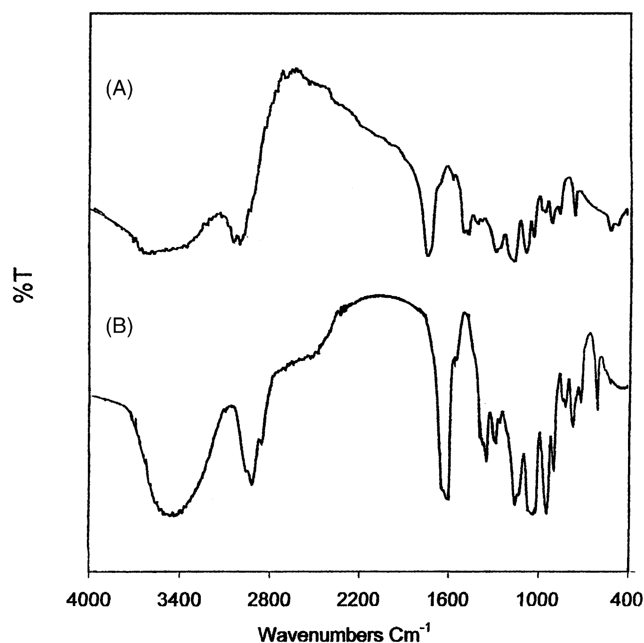


Fig. 1. FT-IR Spectra (A) poly(HEMA-co-GMA) membrane; (B) poly(HEMA-co-GMA)–PEI membrane.

the 3300–3500 cm^{-1} ranges indicates –OH stretching vibrations in the structure of the modified membranes. Among the characteristic vibrations of GMA are the methylene vibration at ~2930 cm^{-1} and the methyl vibration at 2960 cm^{-1}. The vibration at 1740 cm^{-1} represents the ester configuration of both HEMA and GMA. The epoxide group gives the band at 910 cm^{-1}. The FT-IR spectra of the PEI-grafted membranes have some absorption bands different from those of the plain membranes. The most important absorption bands at 1550 cm^{-1} representing N–H bending is due to the PEI bonded to the poly(HEMA-co-GMA) membrane.

Suitable matrices include hydrogels that are highly compatible for immobilization of enzymes due to their hydrophilic nature and high water content to provide the enzymes with a microenvironment similar to that in vivo. The functional poly(HEMA-co-GMA) membranes presented here are rather hydrophilic, i.e. they are hydrogels. The simple incorporation of water weakens the secondary bonds within the hydrogels. This enlarges the distance between the polymer chains and causes the uptake of water. The water content of the poly(HEMA-co-GMA) membrane was determined by the weight difference between the water-swollen membrane and the dry membrane, and it was found to be 47%. The water content of the grafted membrane increased about 4% with respect to unmodified membrane.

Scanning electron microscopy (SEM) micrographs presented in Fig. 2A and B show the surface structure of the poly(HEMA-co-GMA) and poly(HEMA-co-GMA)–PEI membranes, respectively. The SEM micrographs show that membranes have a porous surface structure. The surface morphology of the poly(HEMA-co-GMA)–PEI was slightly different to that of the untreated poly(HEMA-co-GMA)

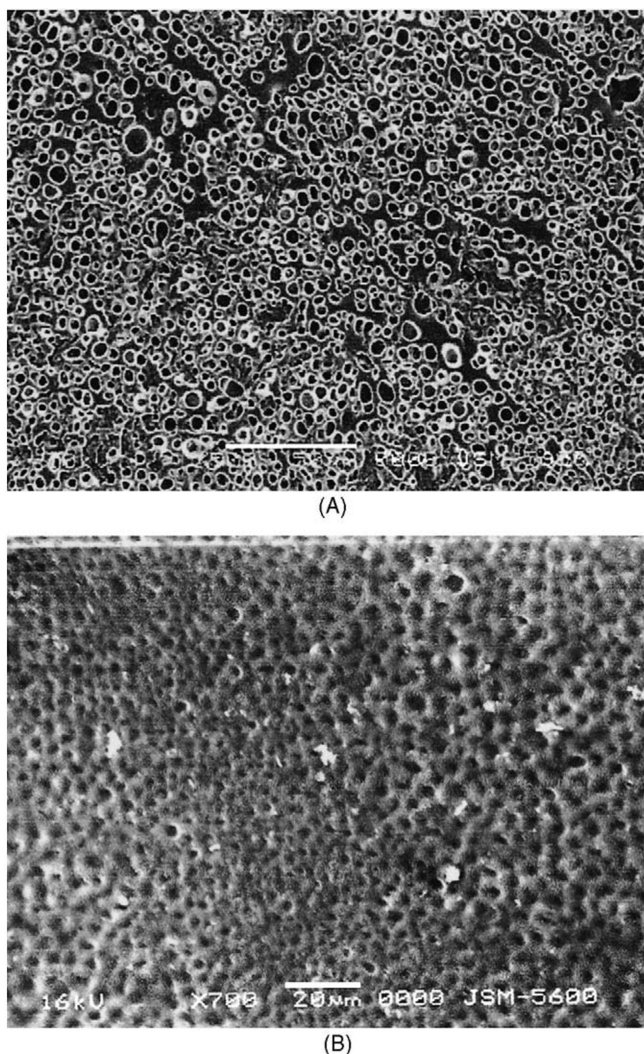


Fig. 2. SEM micrographs of membranes (A) poly(HEMA-co-GMA) (B) poly(HEMA-co-GMA)–PEI.

membranes; the smoothness on the membrane surface was observed as seen in Fig. 2B after PEI-grafting.

The surface area of the poly(HEMA-co-GMA) membranes was measured by BET method and was found to be 1.43 m^2/g membrane. The surface properties of the poly(HEMA-co-GMA) membranes would favor higher immobilization capacity for PEI and the enzyme due to the increase in the surface area.

The membranes were also characterized by contact angle measurements. The protein adsorption is dependent on the chemical nature of matrix. Considering that low contact angle represents hydrophilic surfaces and high contact angle represents hydrophobic surfaces, the poly(HEMA-co-GMA) membranes can be defined as relatively hydrophilic. We had expected changes in surface properties of the membrane after grafting with PEI. The values of contact angle to water were 61.4 ± 1.4 for the poly(HEMA-co-GMA) and 58.7 ± 1.7 for the poly(HEMA-co-GMA)–PEI membranes. PEI grafting of the membrane resulted in a significant difference on the value of contact angle to water. The data revealed that PEI grafting increased the hydrophilicity of the membrane with respect to the polymer structure surface. The properties of the poly(HEMA-co-GMA) membranes are summarized in Table 1.

3.2. Immobilization of tyrosinase onto membranes

The immobilization scheme of tyrosinase on the membrane grafted with PEI is presented in Fig. 3. To maximize the enzyme immobilization onto membranes, optimum pH and initial enzyme concentration were determined (Fig. 4). The maximum tyrosinase immobilization was obtained at pH 6.0 for poly(HEMA-co-GMA)–PEI (19.3 mg/m^2 membranes) when initial tyrosinase concentration was 1.0 mg/ml and at pH 7.0 for poly(HEMA-co-GMA)–PEI–Cu(II) (26.6 mg/m^2 membranes) when 1.0 mg/ml tyrosinase was included in the immobilization medium (Fig. 4).

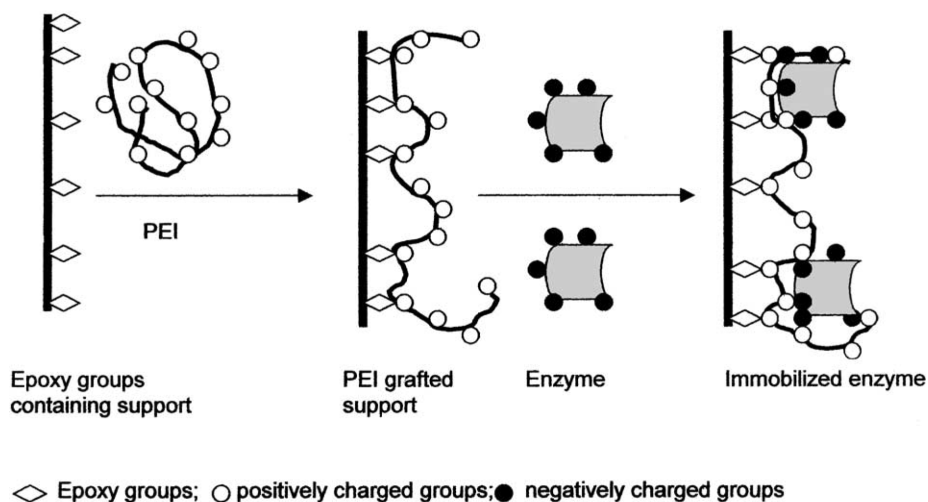


Fig. 3. Immobilization scheme of tyrosinase on the PEI-grafted poly(HEMA-co-GMA) membrane.

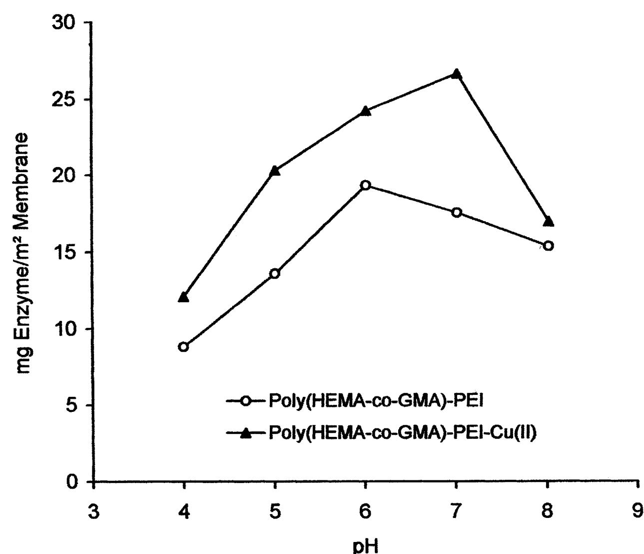


Fig. 4. Effect of pH on tyrosinase adsorption on the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes: the immobilization of tyrosinase on the membranes at various pH values was studied under the fallowing conditions; initial concentration of enzyme 1.0 mg/ml, Temperature 22 °C.

The amount of Cu(II) ions chelated on the surface of PEI-grafted membrane was 0.49 mmol/m^2. The incorporation of Cu(II) ions onto membrane significantly increased enzyme adsorption capacity of the membrane (from 19.3 to 26.6 mg/m^2). This could be due to the specific interactions between tyrosinase molecules and Cu(II) ions, as tyrosinase is a copper dependent enzyme. The divalent metal ions such as Cu(II), Zn(II) and Ni(II) are considered as soft Lewis acids and interact with soft Lewis bases such as nitrogen and sulfur that is found in histidine and cysteine. The surface functional groups of tyrosinase (nitrogen and sulfur groups) could easily chelate with the PEI–Cu(II) complexes on the membrane surface [11], therefore, yield substantially high enzyme adsorption. It should be noted that the optimal immobilization pH and the amount of immobilized enzyme on the poly(HEMA-co-GMA)–PEI–Cu(II) membrane were very different from the poly(HEMA-co-GMA)–PEI membrane. From this observation, the immobilization of enzyme on the poly(HEMA-co-GMA)–PEI–Cu(II) membrane should be a combination of metal co-ordination and ion-exchange interactions.

It has been shown that enzymes have no net charge at their isoelectric points (pI), and therefore the maximum adsorption from aqueous solution is usually observed at their pI. The pI of tyrosinase is 4.5. In the present study, the maximum adsorption was not observed at this pH for the poly(HEMA-co-GMA)–PEI membrane, but shifted to less acidic pH values. This could result from preferential interaction between tyrosinase and grafted PEI molecules at pH 6.0. These interactions may result from ion-exchange effects, caused by the amino groups on the grafted PEI and the amino acid side-chains of the enzyme molecules. On the other hand, the maximum tyrosinase adsorption was achieved at pH 7.0 for PEI grafted and Cu(II) ions chelated membrane. Based on immobilized metals affinity chromatography concept, the hard and borderlines metal ions would yield different adsorption selectivity when applying to the same sample. For example, immobilized Fe(III) would adsorb a distinct profile of proteins at acidic pH whereas immobilized Cu(II) at neutral pH [38]. Tyrosinase is classified as a copper dependent enzyme and contains two tetragonal Cu(II) atoms [17]. This phenomenon could also provide additional high binding affinity for tyrosinase to the poly(HEMA-co-GMA)–PEI–Cu(II) membrane. In this way, on the basis of metal recognition, an additional strong binding could be established between chelated Cu(II) ions and the enzyme molecule on the membrane surface.

As presented in Fig. 5 with increasing enzyme concentration in solution, the amount of per unit area of tyrosinase adsorbed by poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes increases almost linearly at low concentrations, below 0.8 mg/ml, then reaches saturation and levels off. The adsorption data revealed a difference in the binding of tyrosinase to the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes (Fig. 5). Tyrosinase binding becomes constant when the enzyme concentration is greater than 0.8 mg/ml. This could be explained by saturation of interacting groups of the grafted PEI molecules with the adsorbed tyrosinase molecules, achieving maximum immobilization capacity.

As presented in Table 2, the poly(HEMA-co-GMA)–PEI–Cu(II) membranes have a higher enzyme loading (24.2 mg enzyme/m^2 membrane) and a higher enzyme activity (42.2×10^3 U/m^2) then the poly(HEMA-co-GMA)–PEI membranes

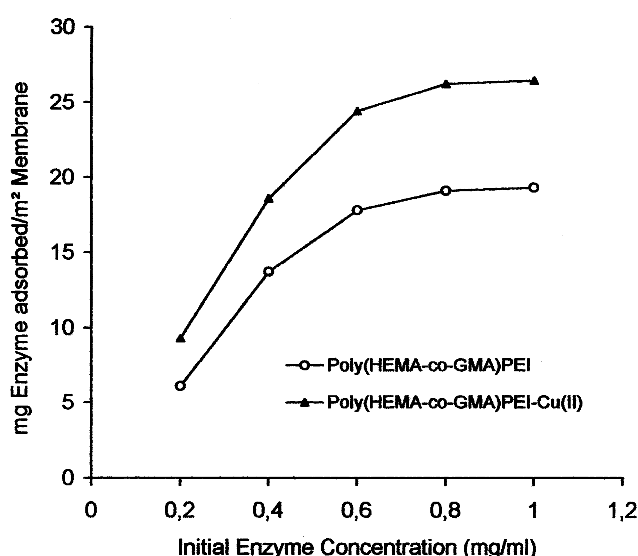


Fig. 5. Effect of tyrosinase initial concentration adsorption on the enzyme loading on membranes: the experimental conditions are: pH values was 6.0 for the poly(HEMA-co-GMA)–PEI and pH 7.0 for the poly(HEMA-co-GMA)–PEI–Cu(II) membranes, initial concentration of enzyme varied between 0.2 and 1.0 mg/ml, Temperature 22 °C.

Table 2
Properties of the adsorbed tyrosinase on poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes

Form of enzyme	Activity (10^3 U/mg enzyme)	Recovered activity (%)	Enzyme loading (mg/m^2)	Activity (10^3 U/m^2)
Free tyrosinase	2250	100	–	–
Membrane–PEI–enzyme	1440	64	19.3	27.8
Membrane–PEI–Cu(II)–enzyme	1755	78	24.2	42.7

(19.3 mg/m^2) and (27.8×10^3 U/m^2), respectively. The incorporation of Cu(II) ions on the poly(HEMA-co-GMA)–PEI membrane significantly increased the tyrosinase immobilization capacity of the membrane (by about 20%). It is clear that this increase is due to the ternary complex formation between PEI, Cu(II) and enzyme molecules (i.e. Cu(II) ions promotes the adsorption of tyrosinase).Several reports described new supports for tyrosinase immobilization. In one study, tyrosinase was covalently immobilized on the carboxymethylcellulose hydrogel beads, and the amount of the immobilized enzyme to 1.0 g of beads was 603 μg and immobilized enzyme retained about 44% of its initial activity [4]. Munjal and Sawhney [29] used Cu–alginate, polyacrylamide and gelatine gels for entrapment of tyrosinase, and the recovered enzyme activities for Cu–alginate, polyacrylamide and gelatine gels were 88, 67 and 57%, respectively . Chitosan-coated polysulphone capillary membranes were used for the immobilization of tyrosinase and the specific activity of the immobilized enzyme was 86 U/mg [20]. Wada et al. [22] reported that the retained activity of covalently immobilized tyrosinase on the weakly acidic cationic exchange resin was 16.3%. Naidja et al. [14] studied the immobilization of tyrosinase on the hydroxyaluminum–montmorillonite complexes, and the retained activity was about 62%. Sarkar et al. [39] reported that about 75% activity was retained when tyrosinase was immobilized on the various types of clay and soil. Chitosan beads and flakes were used for the immobilization of tyrosinase, and the immobilization capacity of the beads was about 14 times greater than that of the flakes [40]. In the present work, the poly(HEMA-co-GMA)–PEI–Cu(II) membranes resulted in a higher enzyme loading (24.6 mg/m^2 membrane) and a higher recovered activity (78%) than the poly(HEMA-co-GMA)–PEI membranes (19.3 mg/m^2) and (64%), respectively. All the immobilization studies published in the literature have been performed under different conditions. Therefore, it is almost impossible to compare the immobilization results.

3.3. Effect of pH and temperature on the catalytic activity

The change in optimum pH depends on the charge of the enzyme and/or of the matrix. This change is useful in understanding the structure–function relationship of the enzyme and to compare the activity of free and immobilized enzyme as a function of pH. The effect of pH on the activity of the free and immobilized tyrosinase preparations for L-tyrosine oxidation was examined in the pH range 4.0–8.0 at 25 °C and the results are presented in Fig. 6. The pH value for optimum activity for the free tyrosinase was found to be at 6.5, which was similar to that reported previously [4]. In this work, the shift toward a neutral pH for the adsorbed tyrosinase can be explained by the presence of positively charged amino groups on the PEI molecules at this pH value. Furthermore, the pH profiles of the immobilized tyrosinase preparations display significantly improved stability on both sides of the optimum pH values, in comparison to that of the free enzyme. This result implies that the applied immobilization methods stabilized the enzyme activity in a wider pH range. These results could probably be attributed to the stabilization of tyrosinase molecules resulting from multi-point ionic complex formation with the grafted PEI molecules.

The activities obtained in a temperature range of 20–60 °C were expressed as percentage of the maximum activity (Fig. 7). As seen from the figure, the activity of the free tyrosinase was strongly dependent on temperature, and the optimum temperature was observed at about 25 °C. The optimum reaction temperature for immobilized tyrosinase preparations was at 35 °C, and the temperature profiles of the immobilized enzymes were broader than that of the free enzyme. This shift towards higher temperatures with adsorbed enzyme could be explained by multi-point ionic interaction, which consequently leads to an increase in the activation energy of the enzyme to reorganize an optimum

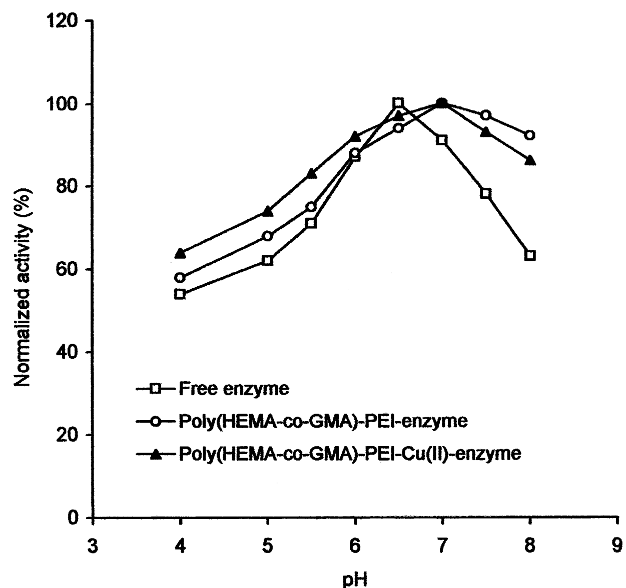


Fig. 6. pH profiles of the free and immobilized tyrosinase preparations.

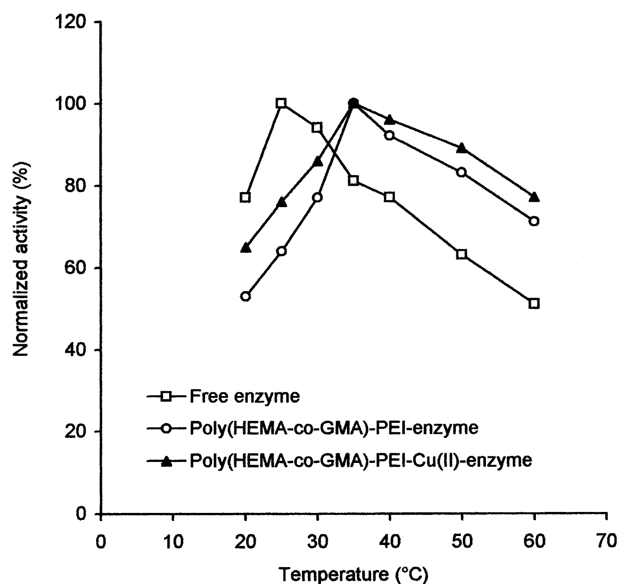


Fig. 7. Temperature profiles of the free and immobilized tyrosinase preparations.

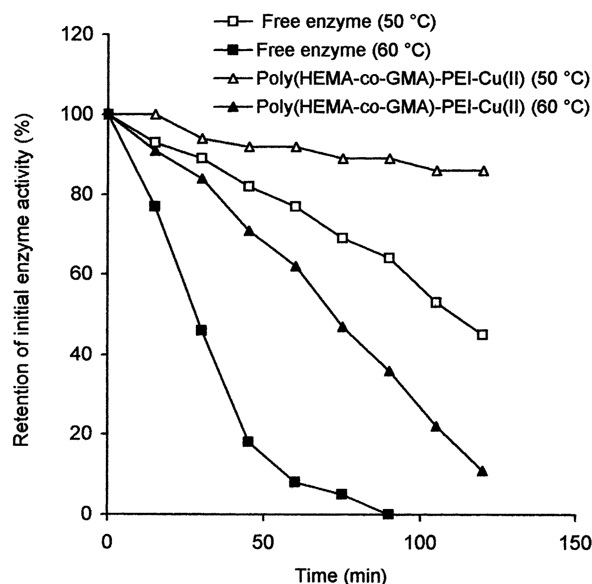


Fig. 8. Effect of temperature on the stability of the free and immobilized tyrosinase preparations.

conformation for binding to its substrate. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivating force due to restricted conformational mobility of the molecules following immobilization [41].

3.4. Enzyme reactor productivity

It is important for economical use of an enzyme, as a means for the mass production of the desired product, that the enzyme reaction is continuous. One of the problems in continuous enzyme reactions is the operational stability of the enzyme immobilized on the support. The operational stability of the adsorbed tyrosinase onto poly(HEMA-co-GMA)–PEI–Cu(II) membrane was monitored in a continuous system at 35 °C for 120 h. During the initial 70 h continuous operation the immobilized tyrosinase preserved all of its initial activity. After this period, a small decrease in enzyme activity was observed with time. After 120 h, the immobilized enzyme lost about 18% of its initial activity.

3.5. Thermal stability

The inability to enhance the thermal stability of a native enzyme is one of the most important limitations for their application in continuous reactors. Therefore, experiments were designed to establish the stability of free and the poly(HEMA-co-GMA)–PEI–Cu(II) adsorbed tyrosinase preparations. When incubated in the absence of substrate at various temperatures retained activity of the two enzyme preparations were plotted. As seen in Fig. 8, following 120 min of incubation at 50°C, the free and immobilized tyrosinase preparation retained their activity up to a level of 45 and 86%, respectively. At 60 °C, the immobilized enzymes were inactivated at a much slower rate than that of the free form.

3.6. Storage stability

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. The free and the immobilized tyrosinase preparations were stored in phosphate buffer solution (50 mM, pH 6.5) at 4 °C for a predetermined period. Under the same storage conditions, the activity of the immobilized tyrosinase decreased at a slower rate than that of the free tyrosinase. The free enzyme lost all its activity within a month. The immobilized tyrosinase preserved about 46% of its initial activity during a three months storage period. Thus, the immobilized tyrosinase exhibits a higher stability than that of the free enzyme. The higher stability of the immobilized tyrosinase could be attributed to the prevention of autodigestion and thermal denaturation as a result of multi-point attachment of the tyrosinase on the PEI-grafted membranes. Several researchers reported that hydrogel carriers such as carboxymethylcellulose, Sepharose, and poly(hydroxyethylmethacrylate) provide a protective microenvironment for enzymes and yield higher stabilities [4,41,42–45]. On the basis of these observations, PEI grafted and/or Cu(II) chelated hydrophilic membrane support should provide a stabilization effect, minimizing possible distortion effects which might be imposed from aqueous medium on the active site of the immobilized enzyme. The generated multi-point ionic interactions between enzyme and modified membrane should also convey a higher conformational stability to the immobilized enzyme. Thus, the PEI grafted and/or Cu(II) incorporated hydrophilic membrane supports and the immobilization

methods provide higher shelf life for the immobilized forms compared to that of its free form.

3.7. Repeated loading of poly(HEMA-co-GMA) membrane with the enzyme

Desorptions of adsorbed tyrosinase from poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membrane were carried out in a batch system. The immobilized-enzyme preparations were placed within the desorption medium containing 1.0 M KSCN at pH 4.0 at room temperature for 3 h as described above and were then repeatedly used in adsorption/desorption cycle of tyrosinase. The tyrosinase adsorption capacities were not changed during five successive adsorption–desorption cycles of the enzyme membrane preparations. The adsorption capacities of the membrane preparations did not significantly change during these adsorption–desorption cycles (lost about 5% of its original capacity at the end of five usages). These results showed that novel PEI grafted and/or Cu(II) ions incorporated membranes can be repeatedly used in enzyme immobilization without detectable losses in their initial adsorption capacities.

More than 95% (up to 97%) of the adsorbed tyrosinase was removed when KSCN was used as a desorption agent. The desorption result showed that KSCN is a suitable desorption agent for the PEI grafted and Cu(II) ions chelated membranes, and allows repeated use of the poly(HEMA-co-GMA)–PEI and poly(HEMA-co-GMA)–PEI–Cu(II) membranes developed in this study.

4. Conclusion

The poly(HEMA-co-GMA) membrane was prepared from HEMA and GMA monomer via UV-initiated photopolymerization. The PEI polymer was grafted on the membrane discs with a membrane phase concentration of 1.03×10^{-3} mmol/m^2 as an ionic polymer ligand, and some of the PEI-grafted membrane discs was chelated Cu(II) ions. The PEI grafted and/or Cu(II) ions chelated membranes were used for the reversible immobilization of tyrosinase via ionic binding and/or metal ions interactions. The desired amount of enzyme can be loaded on the membranes by changing the initial concentration of enzyme in the immobilization medium. The immobilized tyrosinase preparations retained much of their activities in wider ranges of temperature and pH than those of the free enzyme. A high operational stability obtained with the immobilized tyrosinase indicates that the immobilized tyrosinase could successfully be used in a continuous system for the production of L-DOPA from L-tyrosine in the presence of ascorbic acid. The storage stabilities of the immobilized tyrosinase preparations were also increased at 4 °C compared to the free enzyme. After inactivation of enzyme upon use, the adsorbed enzyme and the chelated Cu(II) ions can be desorbed from the membrane. The regenerated membranes can be reused for several cycles for the reversible immobilization of the same or different enzyme. The PEI grafted and/or Cu(II) ions chelated poly(HEMA-co-GMA) membranes revealed significantly enhanced adsorptive properties and will be useful for the immobilization of several enzymes via ionic interaction. In addition, the reusability of the membrane supports may provide economic advantages for large-scale biotechnological applications, and could be also used as affinity membrane supports for the separation of a target protein from biological fluids.

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