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Tyrosinase Immobilization on Cu^{2+} Chelated Poly(ethylene glycol dimethacrylate-N-Vinyl Imidazole) Beads

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Abstract

Poly(ethylene glycol dimethacrylate-n-vinyl imidazole) [poly(EGDMA-VIM)] hydrogel beads (average diameter 150–200 μm) was prepared by copolymerizing of ethylene glycol dimethacrylate (EGDMA) with n-vinyl imidazole (VIM). Poly(EGDMA-VIM) beads had a specific surface area of 59.8 m^2/g. Poly-(EGDMA-VIM) beads were characterized by swelling studies, elemental and SEM. Cu^{2+} ions were chelated on the poly(EGDMA-VIM) beads, then these beads were used in the reversible adsorption of mushroom tyrosinase. The maximum tyrosinase adsorption capacity of the poly(EGDMA-VIM)–Cu^{2+} beads was observed as 14.04 mg/g at pH 6.5. Storage stability was found to increase with immobilization. It was observed that enzyme could be repeatedly adsorbed and desorbed without significant loss in adsorption capacity or enzyme activity.

Key Words: Enzyme immobilization; Tyrosinase; Chelating beads; n-Vinyl imidazole; Affinity beads.

INTRODUCTION

Immobilization of enzymes is one of the important faces of biotechnology. Enzymes have an enormous potential as catalysts in chemical processes in a wide range of industries and medicine. They offer a distinct advantage due to their specificity, high catalytic efficiency at low temperatures and, being biodegradable, these present fewer disposable problems. The use of immobilized enzymes lowers production costs as these can readily be separated from reaction mixtures and hence can be used repeatedly and continuously. Several methods have been employed for enzyme immobilization which include adsorption onto insoluble materials, entrapment in polymeric gels, encapsulation in membranes, cross linking with bifunctional or multifunctional reagents and linking to an insoluble carrier [1]. Among these, adsorption to a solid support material is the most general, easiest to perform and oldest protocol of physical immobilization methods. The most important advantages of this method are that the stability of enzyme activity after immobilization and reuse of the enzyme and support material for different purposes because of reversibility of the method. Reversible enzyme immobilization is a very powerful tool that may be considered to solve this cost problem. Reversible immobilization could provide the possibility of using such enzymes in an immobilized form and, in this way, having the advantages of the use of immobilized enzymes, saving time and cost [2,3].

Tyrosinase (EC 1.14.18.1) is a copper containing protein, known to be the key enzyme in melanogenesis as well as the browning

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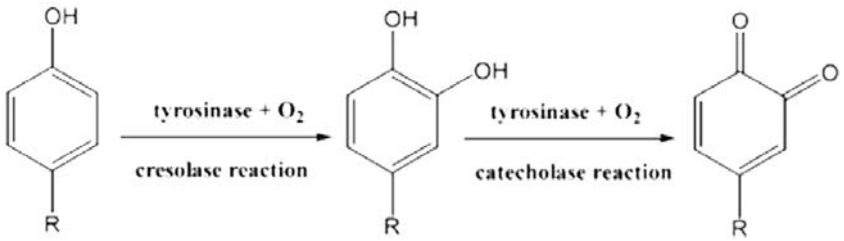


Figure 1. Mono-oxygenase and oxidase activities of tyrosinase also named as cresolase and catecholase reaction, respectively.

phenomenon in fruits [4]. The enzyme provides the major driving force towards melanin formation by o-hydroxylation of its phenolic substrate and successive oxidation of the produced o-dihydroxy compound to the corresponding o-quinone (Figure 1) in the presence of molecular oxygen [5]. The consecutive catalytic functions of tyrosinase are named cresolase and catecholase, respectively (6).

The enzyme has been proposed for synthesis of 3,4-dihydroxyphenylalanine (L-DOPA) [7], dephenolization of industrial wastewater [7-11], as a part of an enzyme electrode for the determination of phenol and its derivatives [12,13], bioremediation of contaminated soils [14,15], and fruit juice clarification [16].

In this study, tyrosinase was immobilized onto a metal affinity support via adsorption. For this purpose, poly(ethylene glycol dimethacrylate-n-vinyl imidazole) [poly(EGDMA-VIM)] hydrogel beads were prepared by copolymerizing of ethylene glycol dimethacrylate with n-vinyl imidazole. Poly(EGDMA-VIM)–Cu^{2+} chelate matrix was prepared by adding poly(EGDMA-VIM) beads to the aqueous solution of metal ion. Cu^{2+} ions coordinate to the vinyl imidazole chelating-ligand and the enzyme binds the polymer via the chelated metal ion. This approach for the preparation of enzyme carrier has several advantages over conventional immobilization methods. An expensive, time consuming and critical step in the preparation of immobilized metal-affinity carrier is coupling of a chelating ligand to the adsorption matrix. In this procedure, comonomer VIM acted as the metal-chelating ligand, and it is possible to load metal ions directly on the beads without further modification steps. In the present work, the protein adsorption capacity, coupling efficiency, enzymatic activity, reuse and storage stability of immobilized tyrosinase were analyzed.

EXPERIMENTAL

Materials

Tyrosinase [EC 1.14.18.1; polyphenol oxidase; monophenol monooxygenase, from mushroom, 2034 U/mg solid], 2 N Follin Reagent from Sigma was diluted to 1 N just before use. Ethylene glycol dimethacrylate (EGDMA) was obtained from Merck (Darmstadt, Germany), purified by passing through active alumina and stored at 4°C until use. n-Vinyl imidazole (VIM, Aldrich, Steinheim, Germany) was distilled under vacuum (74–76 °C, 10 mm Hg). 2,2' Azobisisobutyronitrile (AIBN) was obtained from Fluka A.G. (Buchs, Switzerland). Poly(vinyl alcohol) (PVAL; Mw: 100.000, 98% hydrolyzed) was supplied from Aldrich Chem. Co. (USA). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the chelation experiments was purified using a Barnstead (Dubuque, IA, USA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANO pure® organic/colloid removal and ion exchange packed-bed system.

Preparation of poly(EGDMA-VIM) beads

The poly(EGDMA-VIM) beads were selected as the carrier for the synthesis of metal-chelate affinity adsorbent for enzyme adsorption. The poly(EGDMA-VIM) beads were produced by suspension polymerization technique in an aqueous medium as described in our previous article [17]. EGDMA and VIM were polymerized in suspension by using AIBN and poly(vinyl alcohol) as the initiator and the stabilizer, respectively. Toluene was included in the polymerization recipe as the diluent (as a pore former). A typical preparation procedure was exemplified below. Continuous medium was prepared by dissolving poly(vinyl alcohol) (200 mg) in the purified water (50 ml). For the preparation of dispersion phase, EGDMA (6 ml; 30 mmol) and toluene (4ml) were stirred magnetically at 250 rpm for 15 min at room temperature. Then, VIM (3 ml; 30 mmol) and AIBN (100 mg) were dissolved inthe homogeneous organic phase. The organic phase was dispersed in the aqueous medium by stirring the mixture magnetically (400 rpm), in a ealed-pyrex polymerization reactor. The reactor content was heated to polymerization temperature (i.e., 70°C) within 4 h and the polymerization was conducted for 2 h with a 600 rpm stirring rate at 90°C. Final beads were extensively washed with ethanol and water to remove any unreacted monomer or diluent and then stored in distilled water at 4°C.

Incorporation of Cu^{2+} ions

Chelates of Cu^{2+} ions with poly(EGDMA-VIM) beads were prepared as follows: 1.0 g of the beads were mixed with 50 ml of aqueous solutions containing 50 ppm Cu^{2+} ions, at constant pH of 5.0 (adjusted with HCl and NaOH), which was the optimum pH for Cu^{2+} chelate formation at room temperature. A 1000 ppm atomic absorption standard solution (containing 10% HNO_3) was used as the source of Cu^{2+} ions. The flask was stirred magnetically at 100 rpm for 1 h (sufficient to reach equilibrium). The concentration of the Cu^{2+} ions in the resulting solution was determined with a graphite furnace atomic absorption spectrometer (Analyst 800/Perkin–Elmer, USA). The amount of adsorbed Cu^{2+} ions was calculated by using the concentrations of the Cu^{2+} ions in the initial solution and in the equilibrium. Cu^{2+} leakage from the poly(EGDMA-VIM) beads was investigated with media pH (3.0–8.0), and also in a medium containing 1.0 M NaCl. The bead suspensions were stirred 24 h at room temperature. Cu^{2+} ion concentration was then determined in the supernatants using an atomic absorption spectrometer. It should be also noted that metal-chelated beads were stored at 4°C in the 10 mM Tris–HCl buffer (pH 7.4).

Tyrosinase adsorption studies

Tyrosinase adsorption of the Cu^{2+}-chelated poly(EGDMA-VIM) beads was studied at various pH values, either in acetate buffer (50 mM pH 3.0–5.5) or in phosphate buffer (50 mM, pH 6.0–8.0). Initial tyrosinase concentration was 1 mg/ml. The adsorption experiments were conducted for 4 h at 25°C while stirring continuously. At the end of this period, the enzyme adsorbed beads was removed from the enzyme solution and was washed with the same buffer four times. It was stored at 4°C in fresh buffer until use. The amount of adsorbed tyrosinase was calculated as

$$Q=[(C_o - C)V] /m \quad (1)$$

Here, Q is the amount of tyrosinase adsorbed onto unit mass of beads (mg/g); C_o and C are the concentrations of tyrosinase in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/ml); V is the volume of the aqueous phase (ml); and m is the mass of the beads used (g). In order to determine the adsorption capacities of poly(EGDMA-VIM)–Cu^{2+} beads, the

concentration of tyrosinase in the medium was varied in the range 0.2-1.5 mg/ml.

Desorption of tyrosinase

In order to determine the reusability of the poly(EGDMA-VIM)–Cu^{2+} beads, the tyrosinase adsorption and desorption cycle was repeated 5 times. The tyrosinase desorption from the poly(EGDMA-ViM)–Cu^{2+} beads was carried out with 25 mM EDTA. The beads were washed several times with phosphate buffer (0.05 M, pH 6.5), and were then reused in enzyme immobilization.

Determination of immobilization efficiency

The Lowry method was used to determine the protein content in solution. The enzyme concentration was determined by comparing with the standard curve constructed using enzyme solutions with known concentrations. The amount of the enzyme coupled on poly(EGDMA-VIM)–Cu^{2+} was determined from the initial protein amount present in the enzyme coupling solution substracting the final total protein amounts present in the remaining coupling solution.

Activity assays of free and immobilized tyrosinase

Tyrosinase is a copper dependent enzyme that catalyses two different reaction 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 coloured polymeric pigments can be monitored spectrophotometrically, a measure of these coloured compounds produced in the presence of tyrosinase can be used as an indication of the monophenol levels present.

Free enzyme

The reaction was carried out in a quartz cuvette (3 ml) at 25°C and change in absorbance (A280 nm) were measured using a Shimadzu Model 2100 UV spectrophotometer. 2.9 ml of L-tyrosine solution (1 mM L-tyrosine in phosphate buffer (50 mM, 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 [18].

Immobilised enzyme

For the determination of immobilised tyrosinase activity, 0.05 g enzyme beads were introduced to L-tyrosine solution (1mM, 5ml). It was then incubated in a shaking waterbath and the reaction medium was purged with pure oxygen. At different time interval, the increase in absorbance was followed as 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 25°C in a reaction mixture containing L-tyrosine.

Storage stability

The activity of free and adsorbed tyrosinase in phosphate buffer (50 mM, pH 6.5) was measured in a batch operation mode at 4°C.

RESULTS AND DISCUSSION

Properties of polymer beads

In this study, we attempted to prepare a specific metal-chelate affinity adsorbent for tyrosinase adsorption. VIM was used as the metal-chelating affinity ligand to immobilize Cu^{2+} for specific binding of tyrosinase molecules. Details of preparation and

characterization of poly (EGDMA-VIM) beads were given in our previous paper [17]. The suspension polymerization procedure provided crosslinked poly(EGDMA-VIM) beads in the spherical form in the size range of 150–200 μm. The surface morphology and internal structure of polymer beads are investigated by the electron micrographs in Figure 2. As clearly seen here, the beads have a spherical form and very rough surface due to the pores which formed during the polymerization procedure. The roughness of the surface should be considered as a factor providing an increase in the surface area. In addition, these pores reduce mass transfer resistance and facilitate the diffusion of metal ions because of high internal surface area. This also provides higher metal chelation capacity. Specific surface area of the poly(EGDMA-VIM) beads was found to be 59.8 m^2/g. The poly(EGDMA-VIM) beads are crosslinked hydrogels. They do not dissolve in aqueous media, but do swell, depending on the degree of cross-linking and on the hydrophilicity of the matrix. The equilibrium swelling ratio of the chelating beads used in this study is 78%. The water molecules penetrate into the entanglement polymer chains more easily, resulting in an increase of polymer water uptake in aqueous solutions. It should be also noted that these beads are quite rigid, and strong enough due to highly cross-linked structure therefore they are suitable for column applications. The poly(EGDMA-VIM) beads were prepared by copolymerizing of EGDMA with VIM at a 1:1 molar ratio in the presence of the initiator AIBN according to the following reaction.

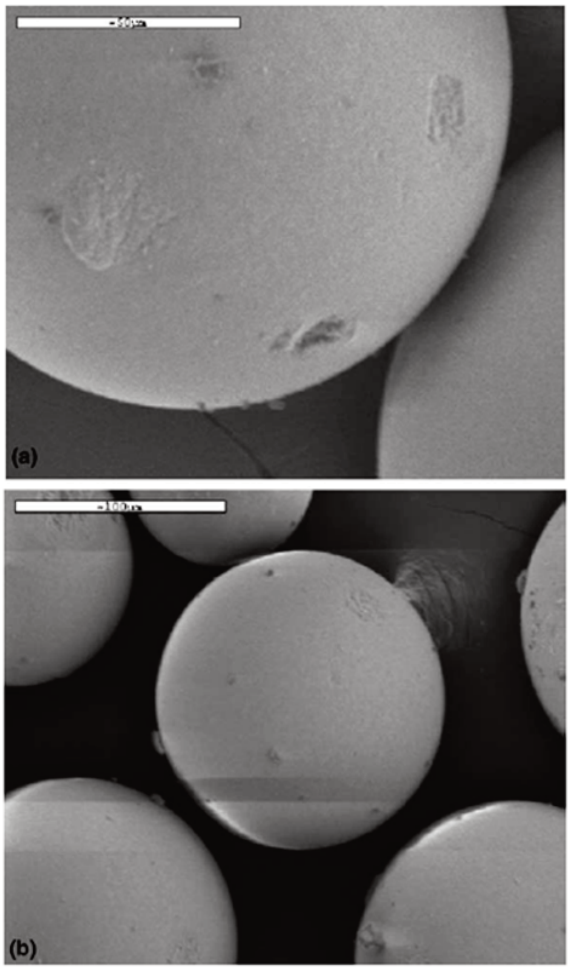


Figure 2. SEM micrographs of poly(EGDMA-VIM) beads.

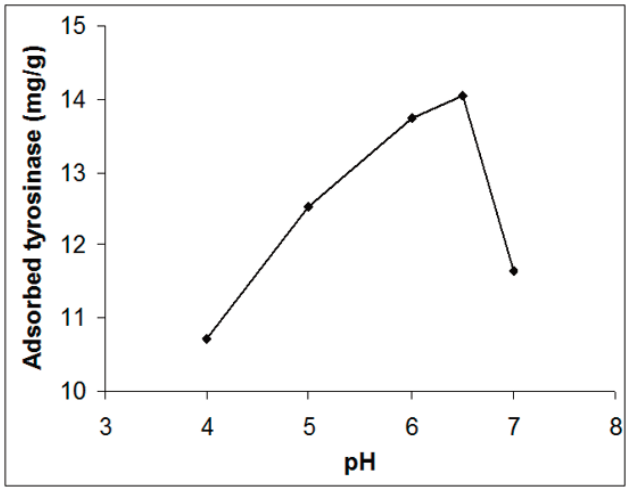


Figure 3. Effect of pH on tyrosinase adsorption on the poly(EGDMA-VIM)-Cu^{2+} beads. The immobilization of tyrosinase on beads at various pH was studied under following conditions; initial enzyme concentration 1.0 mg/mL, temperature 25°C.

Immobilization of tyrosinase on poly(EGDMA-VIM) beads

The effect of pH on the adsorption of tyrosinase onto poly(EGDMA-VIM)–Cu^{2+} beads was studied and presented in Figure 3. The maximum tyrosinase adsorption was obtained at pH 6.5 for poly (EGDMA-VIM)-Cu^{2+} beads (14.04 mg/g) when initial tyrosinase concentration was 1.0 mg/ml. The incorporation of Cu^{2+} ions on beads results in enzyme adsorption. This could be due to the

specificinteractions between tyrosinase molecules and Cu^{2+} ions, as tyrosinase is a copper dependent enzyme. The divalent metal ions such as Cu^{2+}, Zn^{2+} and Ni^{2+} 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 poly(EGDMA-VIM)-Cu^{2+} complexes therefore, yield substantially high enzyme adsorption. The decrease in the protein adsorption capacity in more acidic and more alkaline regions can be attributed to electrostatic repulsion effects between the opposite charged groups.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(EGDMA-VIM)-Cu^{2+} beads, but shifted to neutral pH values (6.5). This could result from preferential interaction between tyrosinase molecules and Cu^{2+} incorporated polymeric matrix at neutral pH. 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^{3+} would adsorb a distinct profile of proteins at acidic pH whereas immobilized Cu^{2+}at neutral pH [19]. Tyrosinase is classified as a copper dependent enzyme and contains two tetragonal Cu^{2+} atoms [20]. This phenomenon could also provide additional high binding affinity for tyrosinase to the poly(EGDMA-VIM)-Cu^{2+} beads. As presented in Figure 4 with increasing enzyme concentration in solution, the amount of per g of tyrosinase adsorbed by poly(EGDMA-VIM)-Cu^{2+} beads increases almost linearly at low concentrations, below 1 mg/ml, then reaches saturation and levels off.

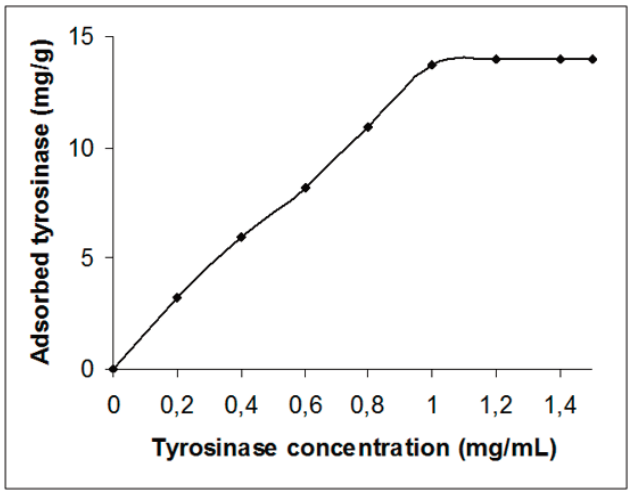


Figure 4. Effect of initial tyrosinase concentration on the enzyme loading on beads: the experimental conditions are: pH=6.5 , initial concentration of enzyme varied between 0.2-1.5 mg/ml, temperature, 25°C.

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

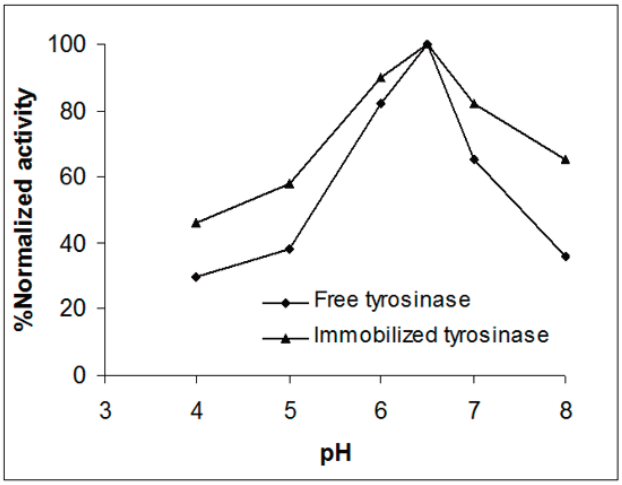


Figure 5. pH profiles of the free and immobilized tyrosinase preparations.

Figure 5. The pH value for optimum activity for free and adsorbed tyrosinase was found to be at 6.5. this results also show that the applied immobilization method stabilized the enzyme activity in a wider pH range.

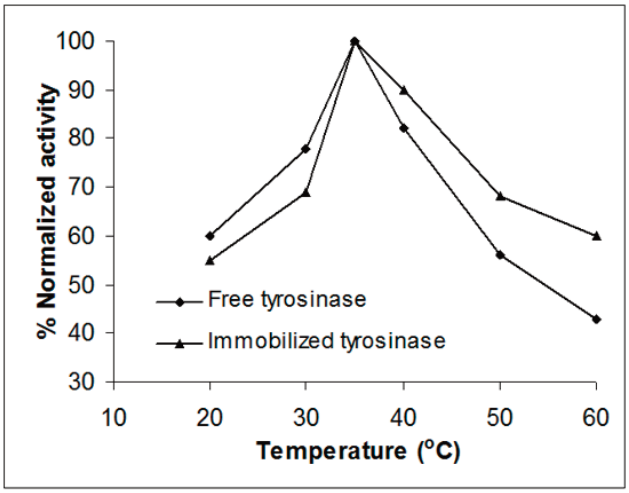


Figure 6. Temperature profiles of the free and immobilized tyrosinase preparations.

The activities obtained in a temperature range of 20–60°C were expressed as percentage of the maximum activity (Figure 6). As seen from the figure, the activity of the free and immobilized tyrosinase was strongly dependent on temperature, and the optimum temperature was observed at 35°C for free and adsorbed tyrosinase. Applied immobilization method was not change the optimum temperature and also stabilized the enzyme activity especially above 40°C.

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 and the activity measurements were carried out for a period of 50 days (Figure 7). No enzyme release was observed. The free enzyme lost its all activity with in 30 days. Immobilized preparation of poly(EGDMA-VIM)-Cu^{2+}-tyrosinase lost 29% of its activity during the same period. This decrease in enzyme activity was explained as a time dependent natural loss in enzyme activity and this was prevented to a significant degree upon immobilization.

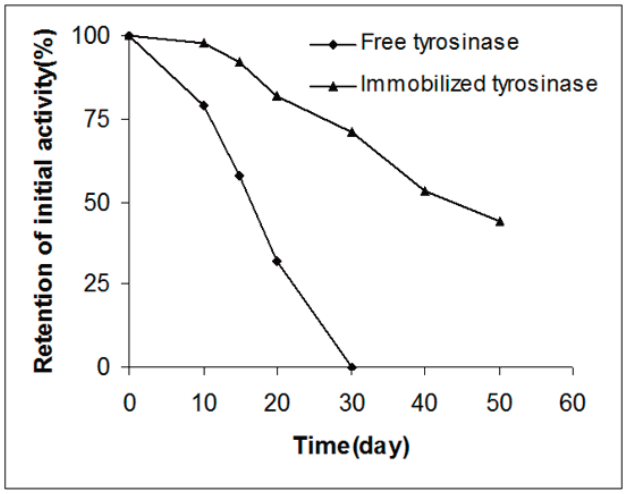


Figure 7. Stoge ability of free and immobilized tyrosinase.

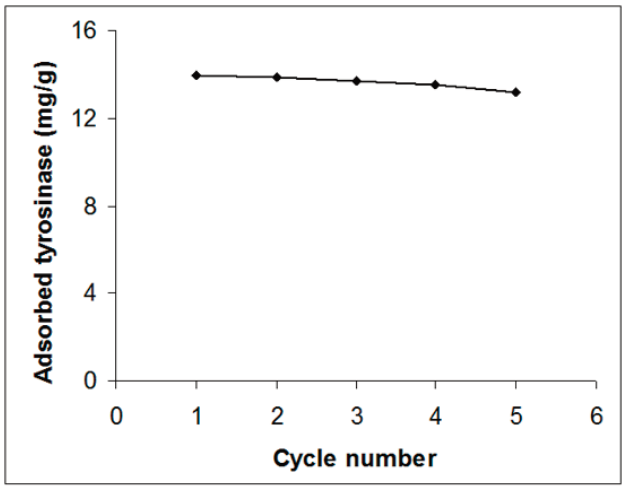


Figure 8. Repeated of adsorbed tyrosinase: tyrosinase concentration, 1 mg/mL; pH 6,5; time, 2 h; 25°C.

Repeated use

The most important advantage of immobilization is repeated use of enzymes. Desorptions of tyrosinase from Cu^{2+}-chelated poly(EGDMA-VIM) beads were carried out in a batch system. Poly(EGDMA-VIM)–Cu^{2+}-tyrosinase preparation was placed within the desorption medium containing 25 mM EDTA at room temperature for 2 h. It was then repeatedly used in adsorption of tyrosinase. The tyrosinase adsorption capacity was not change during the five successive

adsorption– desorption cycles (lost about %5 of its original capacity at the and of the five usage) (Figure 8). It should be also noted that the enzyme activities of preparations did not significantly change during these adsorption– desorption cycles. These results showed that Cu^{2+}-chelated poly(EGDMA-VIM) beads can be repeatedly used in enzyme immobilization.

CONCLUSION

The time consumption and high cost of metal chelating procedure has inspired a search for suitable low-cost adsorbents. The main advantage of IMAC consists in its simplicity, universality, stability and cheapness of the chelating supports [21-23]. In addition, the IMAC supports ensure the milder elution conditions of proteins, keep their biological activity. In this study, vinyl imidazole (VIM) containing affinity adsorbent for the separation of tyrosinase was prepared. This new approach for the preparation of metal-chelating adsorbent has many advantages over conventional techniques. An expensive and critical step in the preparation process of metal-chelating adsorbent is coupling of a chelating ligand to the adsorption matrix. In this procedure, comonomer VIM acted as the metal-chelating ligand, and there is no need to activate the matrix for the chelating-ligand immobilization. Another major issue is that of slow release of this covalently bonded chelators off the matrix. Metal-chelating ligand release is a general problem encountered in any immobilized metal-chelate affinity adsorption technique which caused a decrease in adsorption capacity. It is well known that metal-chelating ligand leakage from the adsorbent causes contaminations that will interfere with analysis of the purified protein. Metal-chelating ligand immobilization step was also eliminated in this approach. Metal-chelating ligand and/or comonomer VIM was polymerized with EGDMA and there is no leakage of the ligand. This purification method will come over the drawback of multi-step purification methods.

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