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A new metal-chelated beads for reversible use in uricase adsorption

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Abstract

Poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) [poly(EGDMA-VIM)] beads (average diameter 150–200 μm) was prepared by copolymerizing ethylene glycol dimethacrylate (EGDMA) with *n*-vinyl imidazole (VIM). Average pore size of poly(EGDMA-VIM) beads was 550 nm. The copolymer beads composition was characterized by elemental analysis and found to contain five EGDMA monomer units each VIM monomer unit. Poly(EGDMA-VIM) beads had a specific surface area of 59.8 m^2/g. Poly-(EGDMA-VIM) beads were characterized by swelling studies and SEM. Cu^{2+} ions were chelated on the poly(EGDMA-VIM) beads, then these beads were used in the adsorption of uricase from Porcine Liver in batch system. The maximum uricase adsorption capacity of the poly(EGDMA-VIM)-Cu^{2+} beads was observed as 118.3 mg/g at pH 6.0. The K_m values for immobilized uricase (poly(EGDMA-VIM)-Cu^{2+}) (91.95×10^{-3} mM) was higher than that of free enzyme (7.5×10^{-3} mM). V_{max} was calculated as 0.012 μmol/min mg protein for the free enzyme. For the immobilized enzyme, V_{max} was calculated as 1.44 μmol/min mg protein. Free enzyme lose all of original activity in 35 days. On the other hand immobilized enzyme preserved 80% of original activity in same time. 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.

Keywords: Uricase; Enzyme immobilization; IMAC

1. Introduction

The advantages of the use of immobilized enzymes are many, and some of them have a special relevance in the area of food technology [1–3]. In this industrial area the control of the expenses must be very strict because of the low added value of products [4]. Different procedures have been developed for enzyme immobilization [5–11]. These include adsorption to insoluble materials, entrapment in polymeric matrix, encapsulation, crosslinking with a bifunctional reagent, or covalent linking to an insoluble carrier. 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 the stability of enzyme activity after immobilization and reuse of the enzyme and support material for different purposes because of reversibility of the method [12–14]. 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 [15,16]. The support matrix for binding of chelator ligand should consist of a molecular network that is permeable to proteins, therefore this method provide one step purification of proteins [17,18]. Among reversible methodologies, metal-chelate immobilization seems to be the simplest way to immobilized proteins [15]. However, scarce work is found referring to the reversible metal-chelate immobilization. Because of the easily polarised nature of their d-electron shells due to orbital valancies, 1st row transition metal ions such as Cu^{2+}, Zn^{2+} and Ni^{2+} function as soft or borderline Lewis acids according to the Lewis acid–Lewis base concepts of Pearson [19].

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) catalyzes the oxidative opening of the purine ring of urate to yield allantoin, carbon dioxide, and hydrogen peroxide. The equation of

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the enzymatic reaction is as follows;

$$\text{Uric acid} + O_2 + H_2O \rightarrow \text{Allantoin} + H_2O_2 + CO_2$$

Uricase is an enzyme participating in the final step of purine degradation. Uric acid represents the major catabolite of purine breakdown in humans and for this reason remains an important marker molecule for disorders associated with purine metabolism, most notably gout, hyperuric aemia and the Lesch–Nyhan syndrome [20]. Determining the urate concentration in blood and urine is very important for these reasons [21]. Uricase is useful for enzymatic determination of urate in clinical analysis by coupling with 4-aminoantipyrineperoxidase system [22]. As a copper protein, this enzyme is a tetramer composed of two types of different subunits with a final molecular weight in the range 145–150 kDa [23–26]. The subunit size, as calculated from the cDNA sequence, is 35 kDa [26,27]. Uricase can be also used as a protein drug to reduce the toxic urate accumulation [28–30].

In this study, uricase was immobilized onto a new support via adsorption. For this purpose, poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) [poly(EGDMA-VIM)] beads was prepared by copolymerizing ethylene glycol dimethacrylate with *n*-vinyl imidazole. Cu^{2+}-poly(EGDMA-VIM) 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 and enzymatic activity, reuse and storage stability of immobilized uricase were analyzed.

2. Experimental

2.1. Materials

Uricase (urate:oxygen oxidoreductase, EC 1.7.3.3) obtained from Sigma was used in this study. 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 mmHg). 2,2′-Azobisisobutyronitrile (AIBN) was obtained from Fluka A.G. (Buchs, Switzerland). Poly(vinyl alcohol) (PVAL; M_w: 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 metal 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 NANOpure® organic/colloid removal and ion exchange packed bed system.

2.2. 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 previous article of our research group [31]. EGDMA and VIM were copolymerized 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 given below. Continuous mediumwas 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 (4 ml) were stirred magnetically at 250 rpm for 15 min at room temperature. Then, VIM (3 ml; 30 mmol) and AIBN (100 mg) were dissolved in the homogeneous organic phase. The organic phase was dispersed in the aqueous medium by stirring the mixture magnetically (400 rpm), in a sealed 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.

2.3. Chelation 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+} ionswas 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) beadswas investigated with media pH (3.0–7.0), 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 spectrophotometer. It should be also noted that metal-chelated beads were stored at 4 °C in the 10 mM Tris–HCl buffer (pH 7.4).

2.4. Uricase adsorption studies

Uricase adsorption of the Cu^{2+}-chelated poly(EGDMA-VIM) beads was studied at various pH values, either in acetate

buffer (0.1 M, pH 4.0–5.5) or in phosphate buffer (0.1 M, pH 6.0–8.0). Initial uricase concentration was 1.0 mg/ml. The adsorption experiments were conducted for 3 h at 20 °C while stirring continuously. At the end of this period, the enzyme adsorbed beads were removed from the enzyme solution and they were washed with the same buffer three times. The beads were stored at 4 °C in fresh buffer until use. The amount of adsorbed uricase was calculated as:

$$Q = \frac{[(C_0 - C)V]}{m} \quad (1)$$

Here, Q is the amount of adsorbed uricase onto unit mass of the beads (mg/g); C_0 and C, the concentrations of uricase in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/ml); V, the volume of the aqueous phase (ml); and m is the mass of the beads used (g). In order to obtain adsorption capacities of poly(EGDMA-VIM)-Cu^{2+} beads, the concentration of uricase in the medium was varied in the range 0.05–1.0 mg/ml.

2.5. Desorption of uricase

In order to determine the reusability of poly(EGDMA-VIM)-Cu^{2+} beads, uricase adsorption and desorption cycle was repeated five times. Uricase 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 (0.1 M, pH 7.0), and were then reused in enzyme immobilization.

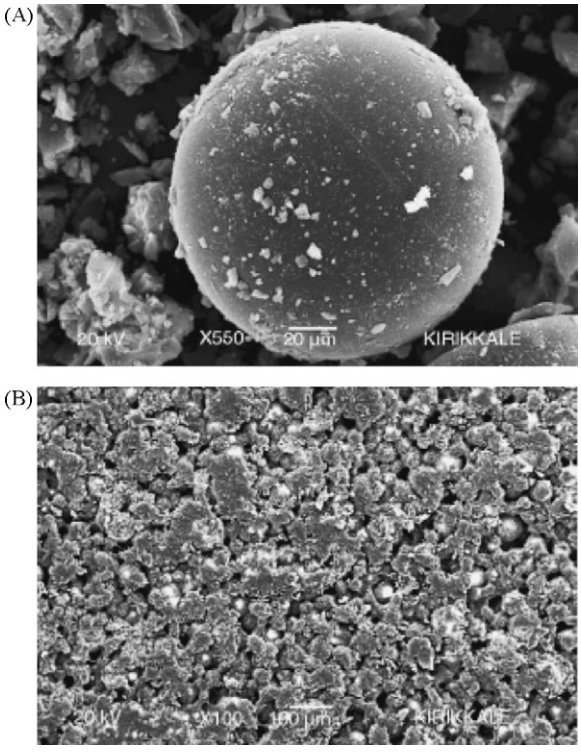

Fig. 1. SEM images showing the surface morphology (A) and internal structure (B) of the poly(EGDMA-VIM) beads.

2.6. Assay of uricase activity

Uricase was assayed by following the disappearance of urate, detected by a decrease in absorbance at 290 nm in the presence of enzyme. The assay mixture contained 0.5 ml enzyme solution (50 mM borate buffer containing 0.001% Triton X-100 and 1.0 mM EDTA (pH 8.0)) and 40 μM urate in a final volume of 3.0 ml. The unit of activity was defined as the amount of enzyme that catalyzed the transformation of 1 μmol substrate per min at 37 °C and pH 8.0. Reaction mixtures containing 5–50 μM substrate solution were used to determine the kinetic parameters.

2.7. Storage and thermal stability

The activity of free and immobilized uricase in acetate buffer (50 mM, pH 5.0) were measured in a batch-operation mode at 4 °C under the experimental conditions given above. Thermal stability studies of the free and the adsorbed uricase were carried out by measuring the residual activity of the enzyme exposed at 60 °C in acetate buffer (50 mM, pH 5.0). After every 10 min time interval, a sample was removed and assayed for enzymatic activity as described above.

3. Results and discussion

3.1. Properties of polymer beads

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 scanning electron micrographs which were given in Fig. 1. As clearly seen here, the beads have a spherical form and very rough surface due to the pores which formed during the polymerization. The roughness of the surface should be considered as a factor providing an increase in the surface area. According to mercury porosimetry data, the average pore size of the poly(EGDMA-VIM) beads was 550 nm. This pore diameter range is possibly available for diffusion of the uricase molecules. The general shape of uricase can be viewed as eliptic. The size of the uricase is 8.0 nm × 9.6 nm × 10.6 nm [32]. Based on this data, it was concluded that the poly(EGDMA-VIM) beads had effective pore structures for diffusion of uricase. Specific surface area of the poly(EGDMA-VIM) beads was found to be 59.8 m^2/g. The poly(EGDMA-VIM) beads are cross-linked beadss. 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 strong enough due to highly cross-linked structure therefore they are suitable for column applications.

3.2. Uricase adsorption

Because of the easily polarized nature of their delectron shells due to orbital valancies, 1st row transition metal ions such as Cu^{2+}, Zn^{2+} and Ni^{2+} function as soft or borderline Lewis acids according to the Lewis acid–Lewis base concepts of Pearson [19]. They thus exhibit preferance for non-bonding lone pair electrons from nitrogen atoms in aromatic and aliphatic amino-containing ligands. In the case of amino acid residues with in a polypeptide or protein, histidine, tryptophan and the α-amino group at the N-terminus are particularly favoured by these borderline metal ions [33]. Taking the advantage of this properties, Cu^{2+} ions was coordinated to the *n*-vinyl imidazole ligand and the enzyme was bound the polymer via Cu^{2+} ions. Studies aimed at detecting leakage of Cu^{2+} from the poly(EGDMA-VIM) beads revealed no leakage in any of the adsorption media, and implied that the washing procedure was satisfactory for the removal of the non-specific adsorbed Cu^{2+} ions from the beads. Firstly, the effect of pH on the adsorption of uricase onto poly(EGDMA-VIM)-Cu^{2+} beads was studied and presented in Fig. 2. 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. Proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points. The isoelectric pH of uricase is 6.3. In the present study, the maximum adsorption was observed at pH 6.0 as expected.

The adsorption isotherm of uricase is presented for Cu^{2+}-chelated poly(EGDMA-VIM) beads in Fig. 3. A point worth noting that, there was a negligible uricase adsorption onto the poly(EGDMA-VIM) which was about 1.4 mg/g. This may be due to weak binding of the enzyme to poly(EGDMA-VIM) beads through van der Waal's and/or hydrogen binding interactions. On the other hand, much higher adsorption capacity was observed when the Cu^{2+} chelated poly(EGDMA-VIM) beads was used. An increase in uricase concentration in the adsorption medium led to an increase in adsorption efficiency but this levelled off at uricase concentration of 1.0 mg/ml. Maximum uricase adsorption was obtained for poly(EGDMA-VIM)-Cu^{2+}

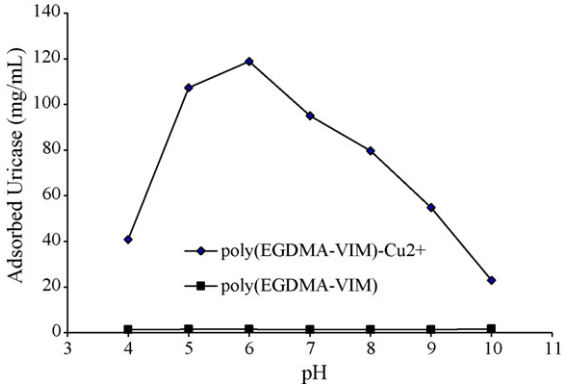

Fig. 2. Effect of pH on the adsorption efficiency of poly(EGDMA-VIM)-Cu^{2+} beads: Initial Cu^{2+} concentration: 1.0 mg/mL; Cu^{2+} loading: 452 μmol/g; pH: 6.0 and *T*: 20 °C.

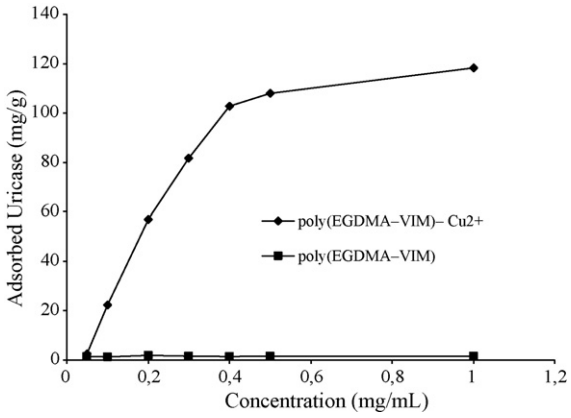

Fig. 3. Effect of uricase concentration on the adsorption efficiency of poly(EGDMA-VIM)-Cu^{2+} beads: Cu^{2+} loading: 452 μmol/g; pH: 6.0 and *T*: 20 °C.

beads (118.3 mg/g). This increase could be due to the specific interactions between uricase and chelated-Cu^{2+} ions. Porath suggested that the molecular interaction in metal-affinity adsorption may be classified as follows: (i) ionic bond formation due to electrostatic forces; (ii) coordinative bonds with electrons in overlapping orbitals; (iii) hydrophobic interaction [34].

Kinetic parameters, Michaelis constants K_m and V_{max} for free and immobilized uricase were determined using Lineweaver–Burk plot. Uric acid was used as the substrate. For the free enzyme, K_m was found to be 7.5×10^{-3} mM, whereas V_{max} was calculated as 0.012 μmol/min mg protein. Kinetic constants of the immobilized uricase were also determined in batch system. K_m values were found to be 91.95×10^{-3} mM for poly(EGDMA-VIM)-Cu^{2+}-uricase preparation. The V_{max} values of immobilized uricase for poly(EGDMA-VIM)-Cu^{2+}-uricase preparation was estimated from the data as 1.44 μmol/min mg protein of adsorbed protein onto poly(EGDMA-VIM) beads. As expected, the K_m and V_{max} values were significantly affected after adsorption onto the Cu^{2+} incorporated poly(EGDMA-VIM) beads. The change in the affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure or by lower accessibility of the substrate to the active site of the immobilized enzyme.

3.3. Effect of pH on the activity

The pH effect on the activity of the free and immobilized uricase preparations for hydrolysis of uric acid was studied. The effect of pH on the free and the immobilized preparations were investigated in the pH range between 3.0 and 6.0 in acetate and phosphate buffers and the results are presented in Fig. 4. The data show that poly-(EGDMA-VIM)-Cu^{2+}-uricase preparation has the same optimum as the free enzyme (pH 8.5). The immobilized preparations gave a significantly broad profile than that of the free enzyme. It was between 3.5 and 8.5. The pH profiles of the immobilized enzymes were much broader with respect to the free enzyme, probably due to the production of oxygen, forming bubbles and causing external diffusional limitations on the enzyme-polymer beads surface.

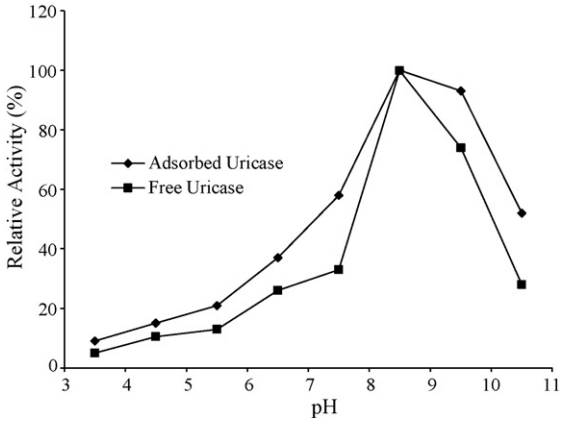

Fig. 4. pH profiles of free and adsorbed uricase: Cu^{2+} loading: 452 μmol/g; uricase concentration: 1.0 mg/mL; *T*: 20 °C.

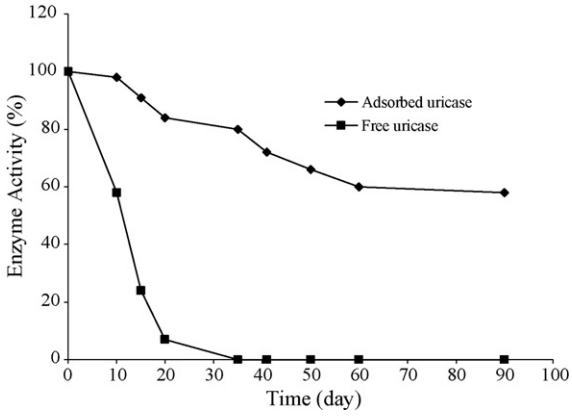

Fig. 6. Storage stability of free and adsorbed uricase: Cu^{2+} loading: 452 μmol/g; uricase concentration: 1.0 mg/mL; pH: 6.0, *T*: 20 °C.

3.4. Effect of temperature on the catalytic activity

The maximum activity for free and adsorbed enzyme preparations was observed at 35 and 45 °C, respectively (Fig. 5). The activities obtained in a temperature range of 4–60 °C were expressed as percentage of the maximum activity. For the free enzyme, the relative activity increased with increasing temperature in the range of 4–35 °C and exhibited a maximum at 35 °C. In this temperature range, the thermal deactivation was probably slow and had no appreciable effect on the rate of the catalysed reaction. Then, an increase was observed in the relative activity with the increasing temperature. The activity of free enzyme decreased at temperatures higher than 35 °C, probably due to thermal deactivation. However, the activity of adsorbed uricase continuously increased with increasing temperature in the range of 4–45 °C.

This shift towards higher temperatures with adsorbed uricase could be explained by multipoint chelate interactions, which consequently leads to an increase in the activation energy of the enzyme to reorganize an optimum conformation for binding to its substrate. Therefore, it is concluded that the adsorption caused a significant improvement in the thermal stability of uricase.

3.5. Storage and thermal stability

Storage stability is an important advantage of immobilized enzymes over the free enzymes, because free enzymes can lose their activities fairly quickly. In general, if an enzyme is in aqueous solution, it is not stable during storage, and the activity is gradually decreased. Free and adsorbed uricase preparations were stored in an acetate buffer (50 mM, pH 5.0) at 4 °C and the activity measurements were carried out for a period of 90 days. No enzyme release was observed. The free enzyme lost its all activity within 35 days. Adsorbed preparation of metal-chelated beads lost 20% of its activity during the same period (Fig. 6). 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 adsorption. The result readily indicates that the immobilized uricase exhibits an improved stability over that of the free enzyme. Of the immobilization methods, fixation of enzyme molecules on a surface often gives rise to the highest stabilization effect on enzyme activities because the active conformation of the immobilized enzyme is stabilized by multipoint bond formation between the substrate and the enzyme molecules [35] (Fig. 7).

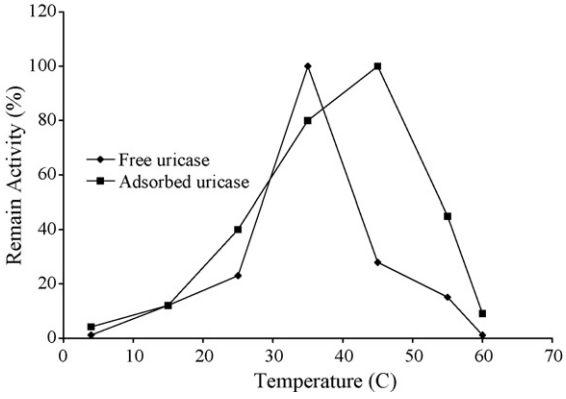

Fig. 5. Temperature profiles of the free and adsorbed uricase: Cu^{2+} loading: 452 μmol/g; uricase concentration: 1.0 mg/mL; pH: 6.0.

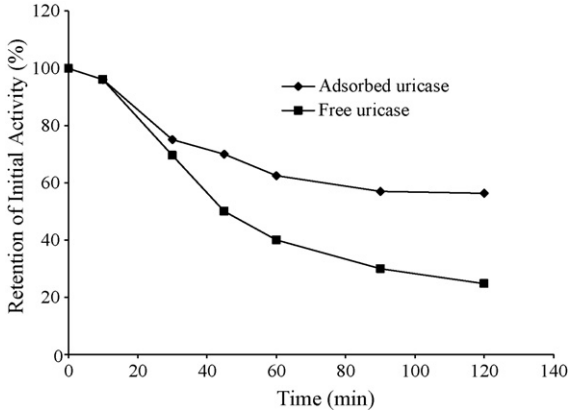

Fig. 7. Influence of temperature on the stability of the free and adsorbed uricase: Cu^{2+} loading: 452 μmol/g; uricase concentration: 1.0 mg/mL; pH: 6.0, *T*: 60 °C.

Thermal stability was carried out with the free and adsorbed enzymes, incubated in the absence of substrate at 60 °C. Fig. 5 shows the heat inactivation curves for the free and adsorbed enzymes. At 60 °C the adsorbed and the free uricase retained their activity about to a level 56 and 25% during a 90 min incubation period, respectively. The adsorbed form was inactivated at a much slower rate than the native form. These results showed that, the activity of the adsorbed preparation is more resistant than that of the soluble form against heat and denaturing agents.

3.6. Repeated use

The most important advantage of immobilization is repeated use of enzymes. Desorptions of uricase from Cu^{2+}-chelated poly(EGDMA-VIM) beads were carried out in a batch system. Poly(EGDMA-VIM)-Cu^{2+}-uricase 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 uricase. The uricase adsorption capacity was not changed during the 10 successive adsorption–desorption cycles. It should be noted that the enzyme activities of preparations did not significantly change during these adsorption–desorption cycles. Adsorbed uricase retains an activity of 82% after 10 batch successive reactions, demonstrating the usefulness of the enzyme-loaded beads in biocatalytic applications. These results showed that Cu^{2+}-chelated poly(EGDMA-VIM) beads can be repeteadly used in enzyme immobilization, without detectable losses in their initial adsorption capacities and activities.

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