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Vinyl imidazole carrying metal-chelated beads for reversible use in yeast invertase adsorption

Bilgen Osman [a], Ali Kara [a], Lokman Uzun [b], Necati Beşirli [a], Adil Denizli [b,*]

[a] Department of Chemistry, Uludağ University, Bursa, Turkey
[b] Department of Chemistry, Hacettepe University, Ankara, Turkey

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Abstract

Poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) [poly(EGDMA–VIM)] hydrogel (average diameter 150–200 μm) was prepared copolymerizing 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 and scanning electron microscope (SEM). Cu^{2+} ions were chelated on the poly(EGDMA–VIM) beads (452 μmol Cu^{2+}/g), then the metal-chelated beads were used in the adsorption of yeast invertase in a batch system. The maximum invertase adsorption capacity of the poly(EGDMA–VIM)–Cu^{2+} beads was observed as 35.2 mg/g at pH 4.5. The adsorption isotherm of the poly(EGDMA–VIM)–Cu^{2+} beads can be well fitted to the Langmuir model. Adsorption kinetics data were tested using pseudo-first- and -second-order models. Kinetic studies showed that the adsorption followed a pseudo-second-order reaction. The value of the Michaelis constant K_m of invertase was significantly larger upon adsorption, indicating decreased affinity by the enzyme for its substrate, whereas V_{max} was smaller for the adsorbed invertase. The optimum temperature for the adsorbed preparation of poly(EGDMA–VIM)–Cu^{2+}-invertase at 50 °C, 10 °C higher than that of the free enzyme at 40 °C. Storage stability was found to increase with adsorption. Adsorbed invertase retains an activity of 82% after 10 batch successive reactions, demonstrating the usefulness of the enzyme-loaded beads in biocatalytic applications.
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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 [1–4]. In this industrial area the control of the expenses must be very strict because of the low added value of products [5]. Different methods have been developed for enzyme immobilization [6–14]. These include adsorption to insoluble materials, entrapment in polymeric matrix, encapsulation, cross-linking with a bifunctional reagent, or covalent linking to an insoluble support. Among these, adsorption to a solid support material is the most general, easiest to perform and oldest protocol of physical adsorption methods. The most important advantages of this method are the simplicity and reversibility. Reversible immobilization of enzymes is a very convenient immobilization protocol because it provides a very easy route for enzyme immobilization and permits the reuse of the adsorbent with the subsequent reduction of industrial waste [15–17]. Therefore, 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. Among reversible methodologies, metal–chelate immobilization seems to be the simplest way to immobilized proteins [18]. 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]. 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

* Corresponding author. Tel.: +90 312 297 7983; fax: +90 312 299 2163.
E-mail address: denizli@hacettepe.edu.tr (A. Denizli).

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a polypeptide or protein, histidine, tryptophan and the α-amino group at the *N*-terminus are particularly favoured by these borderline metal ions [20].

Invertase (β-D-fructofuranosidase fructohydrolase, EC 3.2.1.26) is a highly efficient enzyme that has been described as specific for converting sucrose to glucose and fructose. The hydrolized sugar mixture obtained by invertase has the advantage of being colourless in contrast to the coloured products obtained by acid hydrolysis [21,22]. Immobilization of invertase on corn grits, gelatine and various agarose polymers has already been achieved, while its immobilization onto hydrogel polymer has been limited. The latter have good chemical properties and mechanical stability and are not susceptible to microbial attack [23–25].

In this study, yeast invertase 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 copolymerizing ethylene glycol dimethacrylate with *n*-vinyl imidazole. Cu^{2+}-poly(EGDMA–VIM) chelate matrix was prepared adding poly(EGDMA–VIM) beads to the aqueous solution of Cu^{2+} 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 matrix 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 activation and ligand immobilization steps. In the present work, the protein adsorption capacity, coupling efficiency and enzymatic activity, reuse and storage stability of immobilized yeast invertase were analyzed.

2. Experimental

2.1. Materials

Invertase (β-fructofuranosidase fructohydrolase, E.C. 3.2.1.26, Grade VII from baker's yeast) obtained from Sigma was used in this study. 2N Folin reagent from Sigma was diluted to 1N 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; 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 our previous article [26]. 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 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 (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+} 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 (3.0–7.0), 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. Invertase adsorption studies

Invertase adsorption of the Cu^{2+}-chelated poly(EGDMA–VIM) beads was studied at various pH values, either in acetate buffer (0.1 M, pH 3.0–5.5) or in phosphate buffer (0.1 M, pH 6.0–8.0). Initial invertase 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 invertase was calculated as:

$$Q = [(C_0 - C)V]/m \quad (1)$$

Here, Q is the amount of adsorbed invertase onto unit mass of the beads (mg/g); C_0 and C, the concentrations of invertase 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 invertase in the medium was varied in the range 0.1–1.0 mg/ml.

2.5. Desorption of invertase

In order to determine the reusability of poly(EGDMA–VIM)–Cu^{2+} beads, invertase adsorption and desorption cycle was repeated 10 times. Invertase 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.

2.6. Activity assays of free and adsorbed invertase

The kinetic parameters K_m and V_{max} constants were determined by measuring initial reaction rates at optimum conditions (pH 5.0, 35 °C) for adsorbed invertase and free invertase with sucrose in acetate buffer. For this purpose, 0.6 mg free or adsorbed invertase was added to 50 ml sucrose solution of different concentrations between 30 and 300 mM and initial activities were determined as described below. The activities of both the free and the adsorbed invertase preparations were determined by measuring the amount of glucose liberated from the invertase-catalysed hydrolysis of sucrose per unit time. In the determination of the activity of the free enzyme, the reaction medium consisted of acetate buffer (2.5 ml, 50 mM, pH 5.0) and sucrose (0.1 ml, 300 mM). Following a pre-incubation period (5 min at 35 °C), the assay was started by the addition of the enzyme solution (0.1 ml, 10 mg/ml) and incubation was continued for 5 min. In order to terminate the enzymatic reaction, the reaction medium was then placed in a boiling water bath for 5 min.

The same assay medium was used to determine the activity of the adsorbed enzyme. The enzymatic reaction was started by the introduction of 0.5 g of metal-chelated beads into the assay medium (10 ml) and was carried out at 35 °C with shaking in a water bath. After 15 min, the reaction was terminated by removal of the beads from the reaction mixture.

Sucrose hydrolysis performances of the free and adsorbed enzyme preparations were determined by measuring the glucose content of the medium according to a method described previously [27]. Assay mixture contained GOD (25 mg), POD (6 mg) and *o*-dianisidine (13.2 mg) in phosphate buffer (100 ml, 0.1 M, pH 7.0). An aliquot (2.5 ml) of enzymatically hydrolyzed sample was mixed and then incubated in a water bath at 35 °C for 30 min. After addition of sulfuric acid solution (1.5 ml, 30%) absorbance was measured by a UV–vis spectrophotometer (Shimadzu, Model 1601, Tokyo, Japan) at 530 nm. The activity of the adsorbed invertase preparations was presented as a percentage of the activity of free enzyme of same quantity.

2.7. Storage and thermal stability

The activity of free and immobilized invertase 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 invertase were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (50–70 °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 cross-linked 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 invertase molecules. The general shape of invertase can be viewed as eliptic. The size of the invertase is 9.4 nm × 11.3 nm × 12.9 nm [28]. Based on this data, it was concluded that the poly(EGDMA–VIM) beads had effective pore structures for diffusion of invertase. 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 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 strong enough due to highly cross-linked structure therefore they are suitable for column applications.

3.2. Invertase adsorption

Taking the advantage of metal–chelate properties, Cu^{2+} ions was coordinated to the *n*-vinyl imidazole ligand and the enzyme

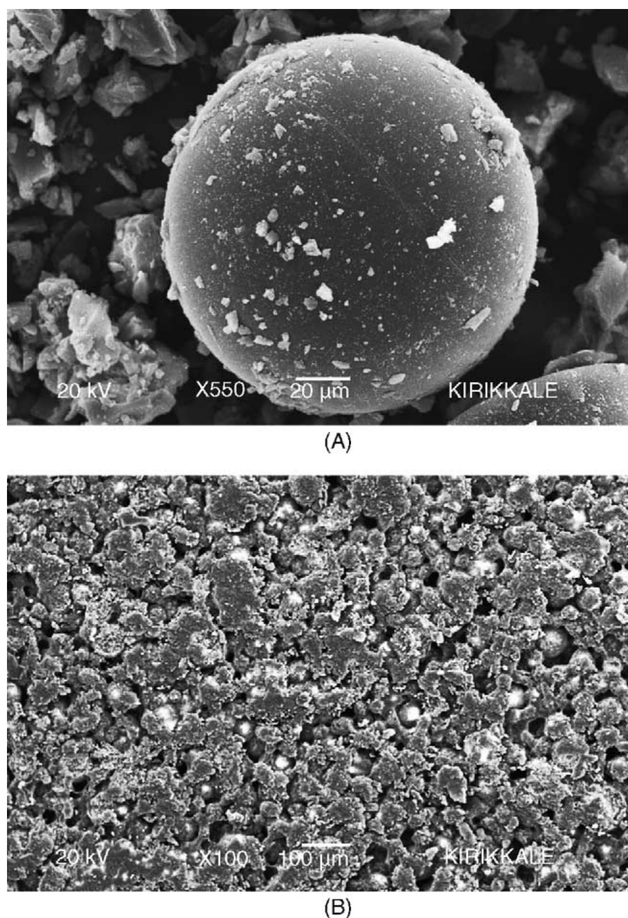

Fig. 1. SEM images showing the surface morphology (A) and internal structure (B) of the poly(EGDMA–VIM) beads.

was bound the polymer via Cu^{2+} ions. Maximum Cu^{2+} loading was found to be 452 μmol/g support. Studies aimed at detecting leakage of Cu^{2+} from the poly(EGDMA–VIM) beads revealed no leakage in any of the adsorption media even in long period of time (more than 4 weeks), and implied that the washing procedure was satisfactory for the removal of the non-specific adsorbed Cu^{2+} ions from the beads.

The adsorption isotherm of invertase is presented for Cu^{2+}-chelated poly(EGDMA–VIM) beads in Fig. 2. A point worth noting that, there was a negligible invertase 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 invertase concentration in the adsorption medium led to an increase in adsorption efficiency but this levelled off at invertase concentration of 0.75 mg/ml. Maximum invertase adsorption was obtained for poly(EGDMA–VIM)–Cu^{2+} beads (35.2 mg/g). This increase could be due to the specific interactions between invertase 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 [29].

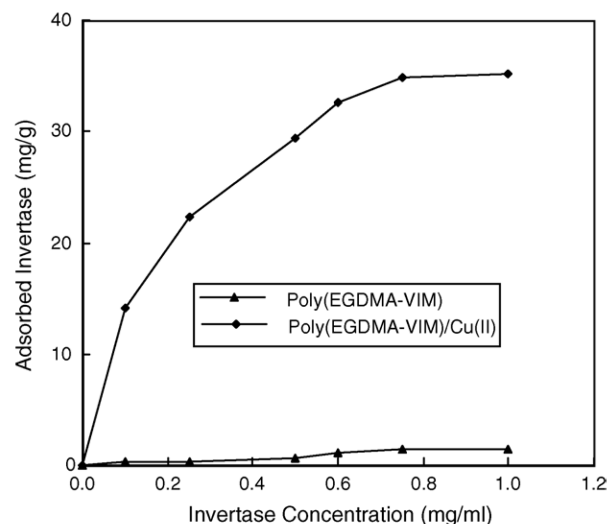

Fig. 2. Effect of invertase concentration on the adsorption efficiency of poly(EGDMA–VIM)–Cu^{2+} beads: Cu^{2+} loading: 452 μmol/g; pH: 4.5 and T: 20 °C.

An adsorption isotherm is used to characterize the interactions of each molecule with the adsorbents. This provides a relationship between the concentration of the molecules in the solution and the amount of ion adsorbed on the solid phase when the two phases are at equilibrium.

During the batch experiments, adsorption isotherms were used to evaluate adsorption properties. For the systems considered, the Langmuir model was found to be applicable in interpreting invertase adsorption by metal chelated beads. The Langmuir adsorption isotherm is expressed by Eq. (2). Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which can only hold one molecule. These sites are also assumed to be energetically equivalent, and distant to each other so that there are no interactions between molecules adsorbed to adjacent sites [30]. The corresponding transformations of the equilibrium data for invertase molecules gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

$$Q = Q_{\max} b C_e / (1 + b C_e) \quad (2)$$

where, Q is the concentration of bound invertase in the adsorbent (mg/g), C_e, the equilibrium invertase concentration in solution (mg/l), b, the Langmuir constant (g/ml) and $Q_{\max}$ is the adsorption capacity (mg/g). This equation can be linearized:

$$1/Q = [1/(Q_{\max} b)][1/C_e] + [1/Q_{\max}] \quad (3)$$

The plot of $1/C_e$ versus $1/Q$ was employed to generate the intercept of $1/Q_{\max}$ and the slope of $1/Q_{\max} b$.

The maximum adsorption capacity ($Q_{\max}$) data for the adsorption of invertase was obtained from the experimental data. The correlation coefficient (R^2) was 0.9947. The Langmuir adsorption model can be applied in this affinity adsorbent

system. It should be also noted that the maximum adsorption capacity (Q_{max}) and the Langmuir constant were found to be 39.1 mg/g and 10.7 g/ml, respectively.

The prediction of the rate-limiting step is an important factor to be considered in the adsorption models [31]. For a solid–liquid adsorption process, the solute transfer is usually characterized by external mass transfer (boundary layer diffusion), or intraparticle diffusion, or both. The kinetic models (pseudo-first- and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution.

The adsorption first-order rate constant was found to be 0.056 min for initial invertase concentration of 1.0 mg/ml. The regression coefficient value was found to be 0.93. The theoretical q_e value (50.6 mg/g) calculated from pseudo-first-order model was not very close to the experimental value (35.2 mg/g). The calculated q_e value (45.0) for pseudo-second-order model agree well with the experimental value, and a regression coefficient of above 0.98 shows that the pseudo-second-order model can be applied for the entire adsorption process and confirms the chemi-sorption of invertase onto poly(EGDMA–VIM)–Cu^{2+} beads.

3.3. Kinetic constants

Kinetic parameters, the Michaelis constant (K_m) and the maximal initial rate of the reaction (V_{max}) for the free and the adsorbed invertase preparations were determined using sucrose as a substrate. V_{max} defines the highest possible rate when all the enzyme is saturated with substrate, therefore this parameter reflects the intrinsic characteristics of the adsorbed enzyme, but may be affected by diffusion constrains. K_m is defined as the substrate concentration that gives a reaction rate of 1/2 V_{max}. This parameter reflects the effective characteristics of the enzyme and depends upon both partition and diffusion effects [32].

K_m values were found to be 24 mM for the free invertase and 456 mM for the adsorbed invertase. There was approximately a 19-fold increase in K_m value for the adsorbed enzyme. This increase in the K_m value was either due to the conformational changes of the resulting in a lower possibility of forming a substrate–enzyme complex, or to the lower accessibility of the substrate to the active sites of the adsorbed enzyme caused by the increased diffusion limitation. The V_{max} value of the free invertase (0.21 mg enzyme/ml min) was found to be higher than that of the adsorbed invertase (0.07 mg enzyme/ml min^{-1}). V_{max} values of enzymes demonstrate a decrease upon adsorption [23].

3.4. Effect of temperature on the catalytic activity

The maximum activity for free and adsorbed enzyme preparations was observed at 40 and 50 °C, respectively (Fig. 3). The activities obtained in a temperature range of 20–60 °C were expressed as percentage of the maximum activity. For the free

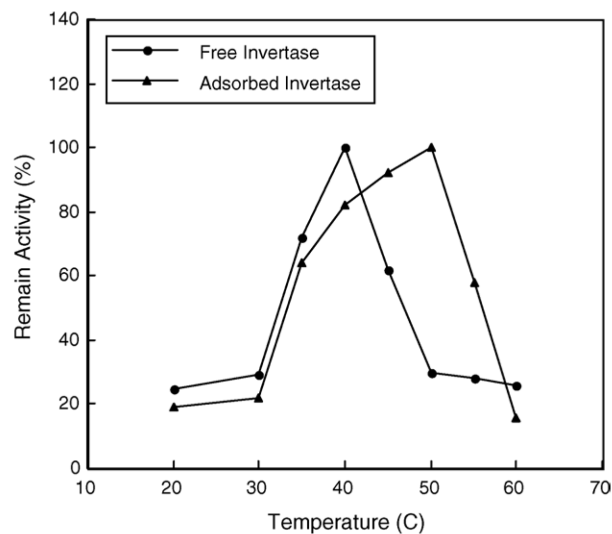

Fig. 3. Temperature profiles of the free and adsorbed invertase: Cu^{2+} loading: 452 μmol/g; invertase concentration: 1.0 mg/ml; pH: 4.5 and T: 20 °C.

enzyme, the relative activity increased with increasing temperature in the range of 20–40 °C and exhibited a maximum at 40 °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 40 °C, probably due to thermal deactivation. However, the activity of adsorbed invertase continuously increased with increasing temperature in the range of 20–50 °C. This shift towards higher temperatures with adsorbed invertase 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 invertase.

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 invertase 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 30 days. Adsorbed preparation of metal-chelated beads lost 20% of its activity during the same period (Fig. 4). 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 invertase 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

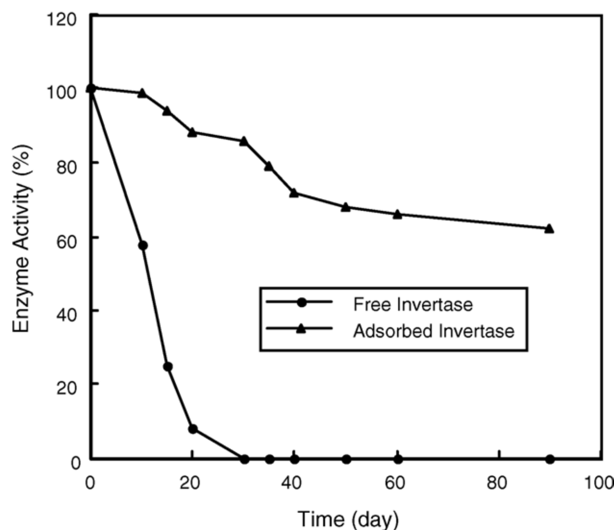

Fig. 4. Storage stability of adsorbed invertase; Cu^{2+} loading: 452 μmol/g; invertase concentration: 1.0 mg/ml; pH: 4.5 and T: 20 °C.

activities because the active conformation of the immobilized enzyme is stabilized by multipoint bond formation between the substrate and the enzyme molecules [33].

Thermal stability was carried out with the free and adsorbed enzymes, incubated in the absence of substrate at various temperatures. Fig. 5 shows the heat inactivation curves between 50 and 70 °C for the free and adsorbed enzymes, respectively. The adsorbed invertase preserved its activity at 50 °C and free enzyme retained its initial activity about 92% during a 90 min incubation period. At 60 °C the adsorbed and the free invertase retained their activity about to a level 78 and 45%, 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.

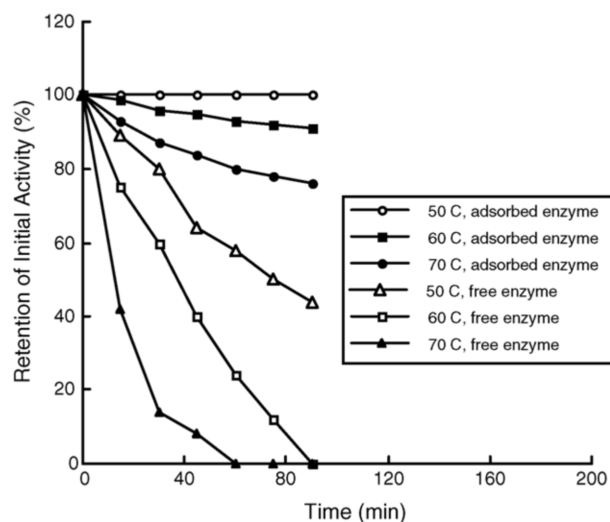

Fig. 5. Influence of temperature on the stability of the free and adsorbed invertase; Cu^{2+} loading: 452 μmol/g; invertase concentration: 1.0 mg/ml; pH: 4.5.

3.6. Repeated use

The most important advantage of immobilization is repeated use of enzymes. Desorptions of invertase from Cu^{2+}-chelated poly(EGDMA–VIM) beads were carried out in a batch system. Poly(EGDMA–VIM)–Cu^{2+}-invertase 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 invertase. The invertase 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 invertase 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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