Vinyl Triazole Carrying Metal-Chelated Beads for the Reversible Immobilization of Glucoamylase

Senay Kök, Bilgen Osman, Ali Kara, Necati Beşirli

Department of Chemistry, Uludag University, Bursa, Turkey

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ABSTRACT: Poly(ethylene glycol dimethacrylate–1-vinyl-1,2,4-triazole) [poly(EGDMA–VTAZ)] beads with an average diameter of 100–200 μm were obtained by the copolymerization of ethylene glycol dimethacrylate (EGDMA) with 1-vinyl-1,2,4-triazole (VTAZ). The copolymer hydrogel bead composition was determined by elemental analysis and was found to contain 5 EGDMA monomer units for each VTAZ monomer unit. The poly-(EGDMA–VTAZ) beads were characterized by swelling studies and scanning electron microscopy (SEM). The specific surface area of the poly(EGDMA–VTAZ) beads was found 65.8 m^2/g. Cu^{2+} ions were chelated on the poly-(EGDMA–VTAZ) beads. The Cu^{2+} loading was 82.6 μmol/g of support. Cu^{2+}-chelated poly(EGDMA–VTAZ) beads with a swelling ratio of 84% were used in the immobilization of *Aspergillus niger* glucoamylase in a batch system. The maximum glucoamylase adsorption capacity of the poly(EGDMA–VTAZ)–Cu^{2+} beads was 104 mg/g at pH 6.5. The adsorption isotherm of the poly(EGDMA–VTAZ)–Cu^{2+} beads fitted well with the Langmuir model. Adsorption kinetics data were tested with pseudo-first- and second-order models. The kinetic studies showed that the adsorption followed a pseudo-second-order reaction model. The Michaelis constant value for the immobilized glucoamylase (1.15 mg/mL) was higher than that for free glucoamylase (1.00 mg/mL). The maximum initial rate of the reaction values were 42.9 U/mg for the free enzyme and 33.3 U/mg for the immobilized enzyme. The optimum temperature for the immobilized preparation of poly-(EGDMA–VTAZ)–Cu^{2+}–glucoamylase was 65°C; this was 5°C higher than that of the free enzyme at 60°C. The glucoamylase adsorption capacity and adsorbed enzyme activity slightly decreased after 10 batch successive reactions; this demonstrated the usefulness of the enzyme-loaded beads in biocatalytic applications. The storage stability was found to increase with immobilization. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 2563–2570, 2011

Key words: adsorption; enzymes; metal–polymer complexes

INTRODUCTION

Immobilized enzymes have been used in food technology, biotechnology, biomedicine, and also analytical chemistry. They provide various advantages over free enzymes, including easy separation of the reactants and products from reaction media, easy recovery of the enzyme, and repeated or continuous reuse. Enzymes can be immobilized to a large number of different carriers by covalent binding, entrapment, adsorption, and ionic binding. The methods and support used for enzyme immobilization are chosen to ensure the highest retention of enzyme activity, stability, and durability. Among the immobilization methods, adsorption may have a higher commercial potential than other methods because it is simpler and less expensive and retains a high catalytic activity.[1–4] The most important advantages of this method are the stability of the enzyme activity after immobilization and the reusability of the enzyme and support material for different purposes because of the reversibility of the method.[5–7] Reversible immobilization could allow the use of enzymes in an immobilized form and, in this way, could have advantage of the use of immobilized enzyme, which would save time and money.[8,9]

Immobilized metal-affinity chromatography (IMAC) has become a widespread analytical and preparative separation method for proteins, peptides, nucleic acids, hormones, and enzymes.[4,10–12] IMAC is based on the affinity of the surface functional groups of protein for immobilized metal ions, such as Zn^{2+}, Ni^{2+}, Cu^{2+}, and Fe^{3+}, which complex with suitable metal chelating carriers. The technique of metal-chelate immobilization permits the reuse of the carrier after its regeneration without the removal of the support from the column. This procedure has great significance for the continuous enzyme catalysis involved in food processing because it decreases carrier consumption and disposal problems.[13]

Glucoamylase (amyloglucosidase, EC 3.2.1.3) is a biocatalyst capable of hydrolyzing α-1,4-glycosidic

Correspondence to: A. Kara (akara@uludag.edu.tr).
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linkages in raw (sparsely soluble) or soluble starches and related oligosaccharides with the inversion of the anomeric configuration to produce β-glucose. Glucoamylase is an industrially important enzyme and is used in dextrose production, the baking industry, the brewing of low-calorie beer, and whole grain hydrolysis for the alcohol industry. This enzyme is used mainly in soluble form, and the industry has been reluctant to move to a process with immobilized enzyme because of the difficulty of obtaining the high-conversion yields required and because they wish to avoid redesigning already well-established processes.[14] To solve the former problem and achieve industrial application, recent research in this area has mainly focused on the improvement of efficient immobilization procedures. The entrapment of the crosslinked enzyme and covalent binding on different matrices have been studied.[15,16] However, glucoamylase immobilized by entrapment is easily washed out from the immobilization matrix.[15] Although covalent methods may have prevented enzyme leakage to the reaction medium, the adsorption amount was low, and the immobilization matrix could not be reused.[16]

In this study, *Aspergillus niger* glucoamylase was immobilized onto a metal-affinity support via adsorption. For this purpose, poly(ethylene glycol dimethacrylate–1-vinyl-1,2,4-triazole) [poly(EGDMA–VTAZ)] hydrogel beads were prepared by the copolymerization of ethylene glycol dimethacrylate (EGDMA) with 1-vinyl-1,2,4-triazole (VTAZ). A Cu^{2+}–poly(EGDMA–VTAZ) chelate matrix was prepared by the addition of the poly(EGDMA–VTAZ) beads to an aqueous solution of Cu^{2+} ions. The Cu^{2+} ions coordinated to the VTAZ chelating ligand, and the enzyme bound the polymer via the chelated metal ion. This approach for the preparation of the enzyme matrix had several advantages over conventional immobilization methods. An expensive, time-consuming, and critical step in the preparation of immobilized metal-affinity carriers is coupling of a chelating ligand to the adsorption matrix. In this procedure, the comonomer VTAZ acted as the metal-chelating ligand, and it was possible to load metal ions directly on the beads without further activation and ligand immobilization steps. In this study, we carried out the optimization of the immobilization conditions, and the enzymatic properties, reusability, and storage and thermal stability of the immobilized glucoamylase were also investigated.

EXPERIMENTAL

Materials

Glucoamylase (exo-l,4-∝-d-glucosidase, EC 3.2.1.3 from *A. niger*, 67.4 U/mg of solid) was purchased from Fluka (Buchs, Switzerland). Potato starch (soluble) from Sigma (Steinheim, Germany) was used as a substrate. EGDMA was obtained from Merck (Darmstadt, Germany), purified by passage through active alumina, and stored at 4°C until use. VTAZ (Aldrich, Steinheim, Germany) was distilled *in vacuo* (74–76°C,10 mmHg). 2,2′-Azobisisobutyronitrile (AIBN) was obtained from Fluka A. G. (Buchs, Switzerland). Poly(vinyl alcohol) (PVAL; weight-average molecular weight = 100,000, 98% hydrolyzed) was supplied from Aldrich Chemical Co. (Milwavkee, WI) All other chemicals were reagent grade and were purchased from Merck. All water used in the experiments was purified with a Barnstead ROpure LP reverse osmosis unit (Dubuque, IA) with a high-flow cellulose acetate membrane (Barnstead D2731); this was followed by pure organic/colloid removal with a Barnstead D3804 NANO and ion-exchange packed-bed system.

Preparation of the poly(EGDMA–VTAZ) beads

The poly(EGDMA–VTAZ) beads were selected as the carrier for the synthesis of the metal-chelate affinity adsorbent for the enzyme adsorption. The poly(EGDMA–VTAZ) beads were produced in an aqueous solution, as described in our previous article.[17] EGDMA and VTAZ were polymerized in suspension with AIBN and PVAL as the initiator and stabilizer, respectively. Toluene was included in the polymerization recipe as the diluent (a pore former). A typical preparation procedure was as follows. A continuous medium was prepared by the dissolution of PVAL (200 mg) in purified water (50 mL). For the preparation of the dispersion phase, EGDMA (6 mL; 32 mmol) and toluene (4 mL) were stirred for 15 min at room temperature. Then, VTAZ (3 mL; 35 mmol) and AIBN (100 mg) were dissolved in the homogeneous organic phase. We dispersed the organic phase in the aqueous medium by stirring the mixture magnetically (400 rpm) in a sealed cylindrical Pyrex polymerization reactor. The reactor content was heated to the polymerization temperature (i.e., 70°C) within 4 h, and the polymerization was conducted for 2 h at a 600-rpm stirring rate at 80°C. The final beads were washed extensively with ethanol and water to remove any unreacted monomer and diluent and then stored in distilled water at 4°C.

Characterization of the poly(EGDMA–VTAZ) beads

Elemental analysis

To evaluate the degree of VTAZ incorporation, the poly(EGDMA–VTAZ) beads were subjected to elemental analysis with a Leco elemental analyzer (model CHNS-932, St. Joseph, MI).

Surface area and pore size measurements

The polymeric beads were dried in a vacuum oven at 50°C for 24 h. Pore diameters greater than 20 Å were determined by a Carlo Erba model 200 mercury porosimeter (Milano, Italy) up to 2000 kg/cm^2. The specific surface area of the polymeric beads was determined in a Brunauer–Emmet–Teller isotherm of nitrogen with an ASAP2000 instrument (Micromeritics, Atlanta, GA). The average size and size distribution of the beads were determined by screen analysis performed with Tyler standard sieves (Retsch Gmbh, Haan, Germany).

Swelling test

The water-uptake ratio of the poly(EGDMA–VTAZ) beads was determined in distilled water. The experiment was performed as follows. Initially, dry beads were carefully weighed before they were placed in a 50-mL vial containing distilled water. The vial was put into an isothermal water bath at a fixed temperature (25 ± 0.5°C) for 2 h. The bead sample was taken from the water, wiped with filter paper, and weighed. The weight ratios of the dry and wet samples were recorded.

Scanning electron microscopy studies

The surface morphology and internal structure of the poly(EGDMA–VTAZ) beads were observed via a scanning electron microscope (JEOL, JEM 1200EX, Tokyo, Japan). The poly(EGDMA–VTAZ) beads were dried at room temperature and coated with a thin layer of gold (ca. 100 Å) *in vacuo* and photographed in the electron microscope at 1000× magnification. We determined the particle size by measuring at least 100 beads on photographs taken by a scanning electron microscope.

Chelation of the Cu^{2+} ions

Chelates of the Cu^{2+} ions with poly(EGDMA–VTAZ) 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 a 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 (this was sufficient for the reaction 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, PerkinElmer, Vernon Hills, IL). The amount of adsorbed Cu^{2+} ions was calculated with the concentrations of the Cu^{2+} ions in the initial solution and at equilibrium. Cu^{2+} ion leakage from the poly(EGDMA–VTAZ) beads was investigated in the media at different pH's (3.0–7.0) and also in a medium containing 1.0*M* NaCl. The bead suspensions were stirred 24 h at room temperature. The Cu^{2+} ion concentration was then determined in the supernatants with an atomic absorption spectrophotometer. The metal-chelated beads were stored at 4°C in 10 m*M* Tris-HCl buffer (pH 7.4).

Glucoamylase adsorption studies at different pH's

Glucoamylase adsorption on the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads was tested 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). The Cu^{2+}-chelated poly(EGDMA–VTAZ) beads (1.0 g) were mixed with 30 mL of buffer solution containing 1 mg/mL glucoamylase at different pH values. The adsorption experiments were conducted at 25°C for 4 h with continuous stirring. 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 Lowry method was used to determine the protein content in solution, and the amount of adsorbed glucoamylase was calculated as follows:

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

where Q is the amount of adsorbed glucoamylase on a unit mass of the beads (mg/g); C_0 and C are the concentrations of glucoamylase in the initial solution and in the aqueous phase after treatment for a 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).

To obtain the adsorption capacities of the poly(EGDMA–VTAZ)–Cu^{2+} beads, the concentration of glucoamylase in the medium was varied in the range 0.5–2.5 mg/mL.

Desorption of glucoamylase from the metal-chelated beads

To determine the reusability of the poly(EGDMA–VTAZ)–Cu^{2+} beads, a glucoamylase adsorption–desorption cycle was repeated 10 times with the same Cu^{2+}-chelated beads. The glucoamylase desorption from the poly(EGDMA–VTAZ)–Cu^{2+} beads was carried out with a 50 m*M* ethylene diamine tetraacetic acid (EDTA) solution with magnetic stirring at 100 rpm at room temperature for 3 h. The beads were removed from the desorption medium, washed

several times with phosphate buffer (0.1*M*, pH 7.0), and then reused in enzyme immobilization.

Activity assays of the free and adsorbed glucoamylase

The activities of the free and immobilized glucoamylase were assayed by the addition of 0.5 mL of diluted free glucoamylase or immobilized glucoamylase in 0.5 mL of an acetate buffer solution (0.1*M*, pH 4.5) with 0.5 mL of a potato starch solution that contained 15 mg/mL starch gelatinized in water (15 min, 100°C, continuous mixing) as the substrate. The reaction was stopped by the addition of an NaOH solution (0.1*M*, 5 mL) after incubation at 60°C for 15 min, and then, the glucose content in the reaction medium was determined with a DNS method.[18] All activity measurement experiments were carried out three times. One unit of glucoamylase activity was defined as the amount of enzyme that produced 1.0 μmol of glucose from soluble starch per minute under the assay conditions.

The activity assays were carried out over the pH range 3.0–8.0 and the temperature range 25–80°C to determine the pH and temperature profiles for the free and immobilized enzymes.

The kinetic parameters [Michaelis constant (K_m) and maximal initial rate of the reaction (V_{max})] of the free and immobilized glucoamylase samples were determined by measurement of the initial rates of the reaction with soluble starch (1.0–20 mg/mL) in acetate buffer (0.1*M*, pH 4.5) at 60°C.

Storage and thermal stability

The thermal stabilities of the free and immobilized glucoamylase were studied by measurement of the residual activity of the enzyme exposed to three different temperatures (60, 65, and 70°C) in acetate buffer (0.1*M*, pH 4.5) for 160 min. After every 20-min time interval, a sample was removed and assayed for enzymatic activity.

The activities of the free and immobilized glucoamylase preparations after storage in acetate buffer (0.1*M*, pH 4.5) at 4°C were measured in a batch operating mode under the experimental conditions given in the previous section.

RESULTS AND DISCUSSION

Properties of the copolymer beads

The suspension polymerization procedure provided spherical poly(EGDMA–VTAZ) beads in the size range 100–200 μm. The surface morphology and bulk structure of the polymer beads were investigated with the scanning electron photograph given in Figure 1. The polymeric beads had a rough surface. The roughness of the surface probably caused an increase in the surface area. According to mercury porosimetry data, the average pore size of the beads was 740 nm. The major form of glucoamylase contained three distinct regions, a catalytic domain, a starch-binding domain, and a linker. The total length of the linker was 10 nm, with the globular catalytic and binding domains having approximate diameters of 6 and 2 nm, respectively.[19] The first two steps involved substrate binding, and the third was a rate-determining catalytic step in the enzymatic reaction. On the basis of these data, we concluded that the poly(EGDMA–VTAZ) beads had effective pore structures for the diffusion of glucoamylase and that the enzymatic reaction occurred efficiently. The specific surface area of the poly(EGDMA–VTAZ) beads was found to be 65.8 m^2/g. The equilibrium swelling ratio of the poly(EGDMA–VTAZ) beads was 84%. The water molecules penetrated the entanglement polymer chains more easily; this resulted in an increase in the polymer water uptake in aqueous solutions. Also, these beads were strong enough because of their highly crosslinked structure; therefore, they would be suitable for column applications.

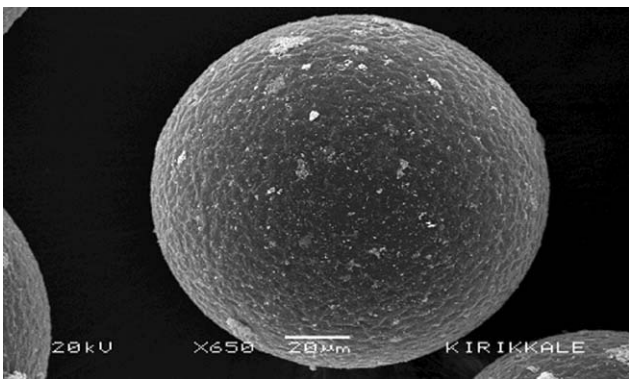

Figure 1 Scanning electron microscopy photograph of the poly(EGDMA–VTAZ) beads.

To evaluate the degree of VTAZ incorporation into the polymeric structure, elemental analysis of the synthesized poly(EGDMA–VTAZ) beads was performed. The molar ratio of EGDMA to VTAZ in the poly(EGDMA–VTAZ) beads was calculated from nitrogen stoichiometry to be 5 : 1. This result suggests that most of the polymer was made of repeat units of EGDMA at a ratio of 5 : 1 with VTAZ units. This result also shows that the copolymerization reaction was complete, as VTAZ monomers that did not form a part of the polymer would have been removed during the extensive washing. The incorporation of VTAZ was found with nitrogen stoichiometry to be 234 μmol/g of polymer. The maximum Cu^{2+} loading was found to be 82.6 μmol/g of polymer for the

TABLE I
Metal and VTAZ Determination in the Poly(EGDMA–VTAZ)–Cu^{+2} Beads

μmol of Cu^{+2}/g of polymer	82.6
External surface area (m^2/g of polymer)	65.8
μmol of Cu^{+2}/m^2	1.25
μmol of VTAZ/g of polymer	234
μmol of Cu^{+2}/μmol of VTAZ	0.352

poly(EGDMA–VTAZ) beads used in the glucoamylase immobilization (Table I).

Glucoamylase adsorption

Metal-chelate immobilization is based on the interaction between certain superficial protein residues (histidines, cysteines, and to a lesser extent, tryoptophans) and transition-metal cations (Ni^2, Co^{2+}, Zn^{2+}, Cu^{2+}) to form chelates. Taking advantage of these properties, we coordinated the Cu^{2+} ions to the VTAZ ligand, and the enzyme was bound the polymer via the Cu^{2+} ions. First, the effect of pH on the immobilization of glucoamylase onto the poly(EGDMA–VTAZ)–Cu^{2+} beads was studied in the pH range 3.0–7.0; this is presented in Figure 2. Proteins have no net charge at their isoelectric points, and therefore, the maximum adsorption from aqueous solutions is usually observed at their isoelectric points.[20] The isoelectric pH of the glucoamylase used in this study was 4.5. As shown in Figure 2, the maximum adsorption of glucoamylase was observed at pH 6.5; this was a shift toward more neutral pH values. This phenomenon was due to preferential interactions between glucoamylase molecules and the Cu^{2+}-incorporated polymeric matrix at neutral pH.

The adsorption isotherm of glucoamylase is presented for the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads in Figure 3. There was a negligible glucoamylase adsorption onto the poly(EGDMA–VTAZ) of

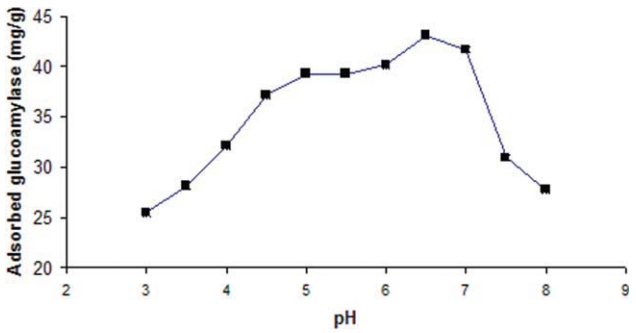

Figure 2 Effect of pH on glucoamylase adsorption onto the poly(EGDMA–VTAZ)–Cu^{2+} beads: glucoamylase concentration = 1 mg/mL, time = 4 h, and temperature = 25°C.

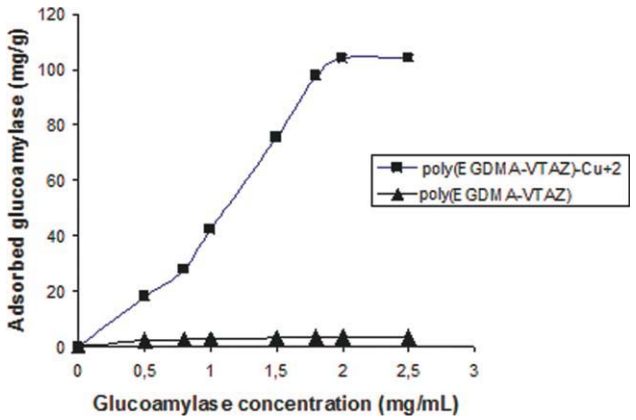

Figure 3 Effect of the glucoamylase concentration on the adsorption efficiency of the poly(EGDMA–VTAZ) and poly(EGDMA–VTAZ)–Cu^{2+} beads: pH = 6.5; time = 4 h, and temperature = 25°C.

about 5.4 mg/g. This may have been due to weak binding of the enzyme to the poly(EGDMA–VTAZ) beads through van der Waals and/or hydrogen-bonding interactions. On the other hand, a much higher adsorption capacity was observed when the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads were used. An increase in the glucoamylase concentration in the adsorption medium led to an increase in the adsorption efficiency, but this leveled off at a glucoamylase concentration of 2 mg/mL. The maximum glucoamylase adsorption capacity of the poly(EGDMA–VTAZ)–Cu^{2+} beads was determined as 104 mg/g at pH 6.5. This increase could have been due to the specific interactions between the glucoamylase and chelated Cu^{2+} ions. When the glucoamylase concentration was 2 mg/mL, the activity recoveries of the poly(EGDMA–VTAZ) and poly(EGDMA–VTAZ)–Cu^{2+} immobilized glucoamylase were determined to be 8.7 and 72%, respectively. This result confirms that glucoamylase adsorption on the poly(EGDMA–VTAZ) beads was not specific.

An adsorption isotherm was used to characterize the interactions of each molecule with the adsorbents. During the batch experiments, adsorption isotherms were used to evaluate the adsorption properties. For the systems considered, the Langmuir model was found to be applicable for interpreting the glucoamylase adsorption by the metal-chelated beads. The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which can hold only 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.[21] The Langmuir isotherm is expressed as

$$C_e/q_e = 1/q_m K + C_e/q_m \quad (2)$$

TABLE II
Properties of the Free and Adsorbed Glucoamylases onto the Cu^{2+}-Chelated Poly(EGDMA–VTAZ) Beads

Form of enzyme	K_m (mg/mL)	V_{max} (U/mg)	Recovered activity (%)
Free	1.00	42.9	100
Immobilized	1.15	33.3	77

where q_e is the amount of glucoamylase adsorbed on the adsorbent at equilubrium, q_m is the maximum adsorption capacity or maximum amount of glucoamylase per unit weight of poly(EGDMA–VTAZ)–Cu^{2+} (mg/g) to form a complete monolayer coverage on the surface bound at a high equilibrium glucoamylase concentration (C_e); K is the Langmuir constant, which is related to the affinity of the binding sites (L/mg); and q_m represents the particle-limiting adsorption capacity when the surface is fully covered with glucoamylase and assists in the comparison of adsorption performance. The plot of C_e/q_e versus C_e was used to generate the intercept of $1/q_m$ and the slope $1/q_mK$. q_m data for the adsorption of glucoamylase was obtained from the experimental data. The correlation coefficient was 0.9939. The Langmuir adsorption model could be applied in this affinity adsorbent system. Also, q_m and K were found to be 108.69 mg/g and 8.36 L/mg, respectively. The prediction of the rate-limiting step is an important factor to be considered in adsorption models.[22] For a solid–liquid adsorption process, the solute transfer is usually characterized by external mass transfer (boundary layer diffusion), intraparticle diffusion, or both. The kinetic models (pseudo-first- and second-order equations) could be used in this case with the assumption that the measured concentrations were equal to the 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 0.0084/min for an initial glucoamylase concentration of 1.0 mg/mL. The regression coefficient value was found to be 0.9834. The theoretical q_e value (77.05 mg/g), calculated from the pseudo-first-order model, was not very close to the experimental value (104 mg/g). The calculated q_e value (117.7 mg/g) for pseudo-second-order model agreed well with the experimental value, and a regression coefficient of 0.9974 showed that the pseudo-second-order model could be applied to the entire adsorption process and confirmed the chemisorption of glucoamylase onto the poly(EGDMA–VTAZ)–Cu^{2+} beads.

Kinetic parameters

The kinetic parameters (K_m and V_{max}) for the free and immobilized glucoamylase preparations were determined with soluble starch as a substrate (Table II). For the free enzyme, K_m was found to be 1 mg/mL, whereas V_{max} was calculated as 42.9 U/mg of enzyme. The kinetic constants of the immobilized glucoamylase were also determined in the batch system. The K_m value was found to be 1.15 mg/mL. The V_{max} value of the enzyme adsorbed onto the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads was estimated from the data to be 33.3 U/mg of enzyme. In general, when an enzyme is adsorbed, the kinetic parameters, K_m and V_{max}, undergo changes with respect to the corresponding parameters of the free form. These changes are caused by several factors, such as protein conformational changes induced by the support, steric hindrance, and diffusional effects.[23] As expected, the K_m and V_{max} values were affected after the adsorption of glucoamylase onto the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads, but the changes were quite small, and the adsorption procedure enabled a stabilized enzyme in a reversible way with low cost in terms of activity.

Effect of pH on the catalytic activity

The pH dependence of an immobilized enzyme activity is characteristic of the nature of the enzymes, the immobilization method, and the carrier. Figure 4 shows the effect of pH on the relative activity of the immobilized enzyme and its free counterpart. Upon immobilization, the optimal pH value for the immobilized enzyme shifted up from 4.0 to 4.5. The immobilized glucoamylase gave a broader profile than that of the free enzyme, probably because of the production of oxygen, which formed bubbles and caused external diffusional limitations on the enzyme–polymer bead surface.[24–26]

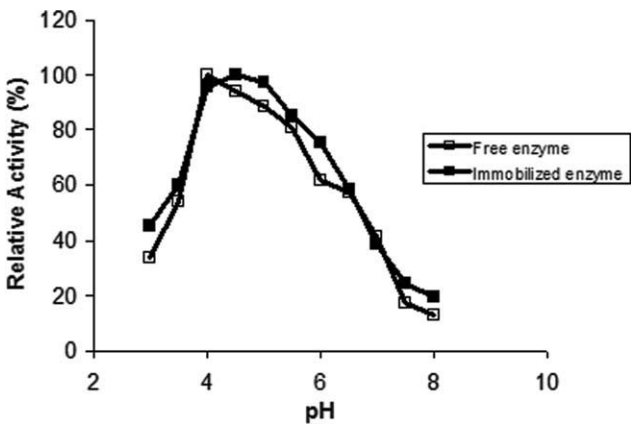

Figure 4 Effect of pH on the activity of the free and immobilized glucoamylase.

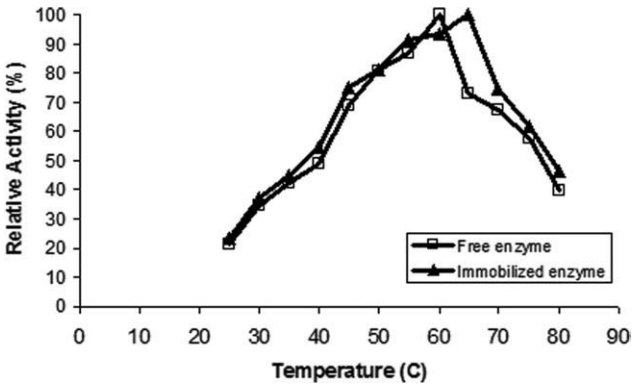

Figure 5 Effect of the temperature on the activity of the free and immobilized glucoamylase.

Effect of the temperature on the catalytic activity

The maximum activities for the free and adsorbed enzyme preparations were observed at 60 and 65°C, respectively (Fig. 5). The activities obtained in the temperature range 25–80°C were expressed as a percentage of the maximum activity. For the free enzyme, the relative activity increased with increasing temperature in the range 25–60°C and exhibited a maximum at 60°C. In this temperature range, the thermal deactivation was probably slow and had no effect on the rate of the catalyzed reaction. The activity of the free enzyme decreased at temperatures higher than 60°C. However, the activity of immobilized glucoamylase increased continuously with increasing temperature in the range 25–65°C. This shift toward higher temperatures with immobilized glucoamylase could be explained by multipoint chelate interactions, which consequently led to an increase in the activation energy for the reorganization of the enzyme to an optimum conformation for binding to its substrate.[27,28] Therefore, we concluded that the adsorption caused an improvement in the thermal stability of glucoamylase.

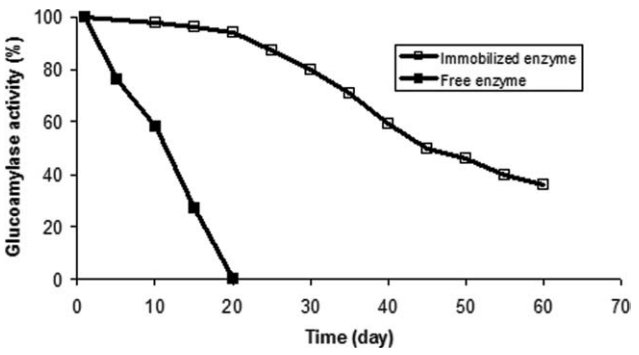

Figure 6 Storage stability of the free and immobilized glucoamylase.

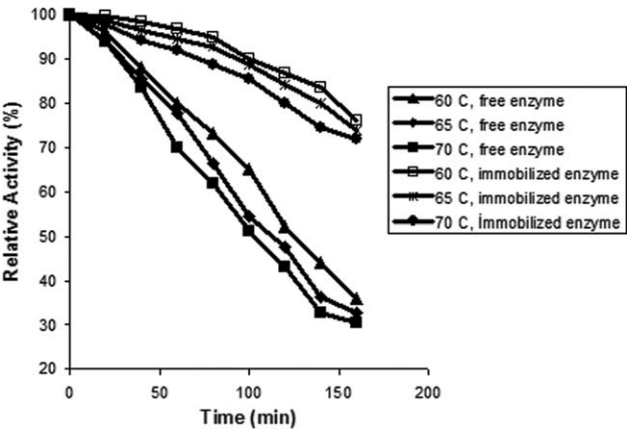

Figure 7 Thermal stability of the free and immobilized glucoamylase.

Storage and thermal stability

Storage stability is one important advantage for immobilized enzymes over free enzymes because free enzymes can lose their activities fairly quickly. The free and immobilized glucoamylase preparations were stored in an acetate buffer (0.1*M*, pH 4.5) at 4°C, and their activities were measured for a period of 60 days. No enzyme release from the beads was observed during this storage period. The free glucoamylase lost its whole activity within 20 days (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 results indicate that the immobilized glucoamylase exhibited improved stability over the free enzyme. Among immobilization methods, the 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.[29]

A thermal stability experiment were carried out with the free and immobilized enzymes; they were incubated in the absence of substrate at various temperatures. Figure 7 shows the heat-inactivation curves for the free and adsorbed enzymes. During a 100-min incubation period, the adsorbed glucoamylase preserved its activity at 95% at 60°C, and the free enzyme retained its initial activity at about 70%. At 70°C, the adsorbed and free glucoamylases retained their activities at levels of about 90 and 50%, respectively. The adsorbed form was inactivated at a much slower rate than the native form. These results show that the activity of the adsorbed preparation was more resistant than that of the soluble form against heat and denaturing agents.

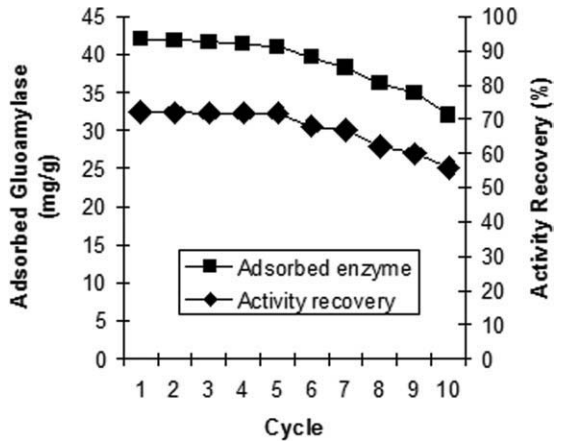

Figure 8 Repeated use of the poly(EGDMA–VTAZ)–Cu^{2+} beads for the glucoamylase adsorption: glucoamylase concentration = 1 mg/mL, pH = 6.5, time = 4 h, temperature = 25°C, and repeated use of poly(EGDMA–VTAZ)–Cu^{2+} immobilized glucoamylase.

Repeated use

The most important advantage of immobilization is repeated use of the enzyme. Desorptions of glucoamylase from the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads were carried out in a batch system. The poly(EGDMA–VTAZ)–Cu^{2+}–glucoamylase preparation was placed within a desorption medium containing 50 m*M* EDTA at room temperature for 3 h. It was then repeatedly used in the adsorption of glucoamylase. The glucoamylase adsorption capacity decreased slightly during the 10 successive adsorption–desorption cycles (Fig. 8). The enzyme activities of the preparations also slightly decreased during these adsorption–desorption cycles. These results show that the Cu^{2+}-chelated poly(EGDMA–VTAZ) beads could be repeatedly used in enzyme immobilization without excessive losses in their initial adsorption capacities and activities.

CONCLUSIONS

The main advantages of IMAC exist in its simplicity, universality, stability, and low cost of the chelating supports. The metal-chelated poly(EGDMA–VTAZ) beads presented herein were prepared by the copolymerization of EGDMA with VTAZ and used for glucoamylase adsorption. An expensive and critical step in the preparation process of a metal-chelating adsorbent is the coupling of a chelating ligand to the adsorption matrix. In this procedure, the comonomer VTAZ acted as the metal-chelating ligand, and there was no need to activate the matrix for the chelating-ligand immobilization. The metal-chelated poly(EGDMA–VTAZ) beads exhibited quite a high glucoamylase adsorption capacity. The immobilized glucoamylase preparation retained much of its activity over wider ranges of pH than the free form. The optimum temperature for the immobilized preparation was 65°C; this was 5°C higher than that of free enzyme at 60°C. The storage stability was found to increase with immobilization. After inactivation of the enzyme upon use, the adsorbed enzyme could be desorbed with EDTA under mild conditions. The regenerated beads could be reused for the immobilization of the same enzyme without excessive losses in their initial adsorption capacities and enzyme activities. The reusability of the support may provide economic advantages for large-scale biotechnological applications.

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