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Reversible immobilization of glucoamylase by metal affinity adsorption on magnetic chelator particles

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Abstract

Magnetic Cu^{2+}-chelated particles, prepared by cerium initiated graft polymerization of tentacle-type polymer chains with iminodiacetic acid (IDA) as chelating ligand, were employed for glucoamylase immobilization. The particles had an obvious high adsorption capacity of glucoamylase with a great activity recovery of 84.0% after immobilization. The immobilized glucoamylase exhibited improved stability in reaction conditions over a wide pH region (pH 3.5–6.0) and a broad temperature range (45–75 °C). The value of the Michaelis constant (K_m) of the immobilized glucoamylase (1.77 mg/ml) was higher than that of the free one (1.07 mg/ml), whereas the maximum velocity (V_{max}) was lower for the adsorbed glucoamylase. Storage stability and temperature endurance of the immobilized glucoamylase were found to increase greatly, and the immobilized glucoamylase retained 75.7% of its initial activity after 30 successive batch reactions. The magnetic Cu^{2+}-chelated particles also exhibited excellent reusability, indicating the advantage of the magnetic metal-chelated particles in biocatalytic applications.

Keywords: Glucoamylase; Immobilization; Magnetic particles; Metal chelating

1. Introduction

Immobilized enzymes have been widely used in food, fine chemical and pharmaceutical industries because they provide many advantages over free enzymes including repeated or continuous reuse, easy separation of the product from reaction media, easy recovery of the enzyme and improvement in enzyme stability [1–4]. A wide variety of methods have been employed in the immobilization of enzymes, such as adsorption, entrapment, cross-link and covalent attachment [5–8]. Among these immobilization techniques, adsorption is the most general, easiest to perform and oldest protocol of physical immobilization methods [9]. Simplicity and reversibility are the most important advantages of this method. But a strong absorption between the enzyme and support should be achieved in the reversible immobilization methodology in order to prevent enzyme desorption from immobilization supports. Noncovalent immobilization technique such as metal-chelated adsorption of the enzyme on a metal-chelated adsorbent can be a good option because it saves time and labor for simple operation and the supports can be reused after desorption of the inactivated enzyme, in this way, reduce the final price and generate fewer residues [4,9,10]. The reversible metal-chelated immobilization has been succeeded in a few enzymes including catalase [9], α-amylase [11] and invertase [12].

Magnetic particles have many important applications in the fields of cell labeling and separation [13], magnetic resonance imaging (MRI) contrast agent [14], enzyme immobilization [15], protein separations [16], targeted drug delivery [17], and so forth. Magnetic particles promise to solve many of the problems associated with chromatographic separations in packed-bed and fluidized-bed systems [18]. Magnetic particles can be well separated and controlled with the help of magnetic fields. The use of magnetic particles reduces capital and operating costs combined with fluidized or fixed beds. The specific magnetic particles can

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be produced by immobilization of an affinity ligand on the surface of prefabricated magnetic beads and the use of the resulting conjugates for various applications [19].

Glucoamylase (GA), also known as amyloglucosidase or γ-amylase (EC 3.2.1.3), is a biocatalyst capable of hydrolyzing α-1,4 glycosidic linkages in raw or soluble starches and related oligosaccharides with the inversion of the anomeric configuration to produce β-glucose [20]. Glucoamylase is an industrially important enzyme and is used for large-scale saccharification of malto-oligosaccharides into glucose and various syrups required in the food, beverages and fuel ethanol industry [3]. Recent research of glucoamylase immobilization was mainly focused on entrapment of the cross-linked enzyme and covalent binding on different matrices in order to achieve industrial application [6,21]. However, glucoamylase immobilized by entrapment is easy to be washed out from the immobilization matrix [6]. Though the covalent methods may prevent enzyme leakage to the reaction medium, the adsorption amount was low and the immobilization matrix could not be reused [21].

In order to resolve all of the above questions, metal affinity magnetic particles have been proposed as a suitable method for both reversible and strong protein adsorption [9]. In this way, protein immobilized on this flexible coating matrix could produce minimal distortion of protein because the flexible polymer arm adapts itself to the protein structure during such an intense metal affinity adsorption. In addition, high specific surface area of these particles for its small size may induce good adsorption capacity. In this study, glucoamylase was immobilized onto metal affinity magnetic particles via adsorption. Nonporous micron-sized magnetic poly(vinyl acetate–divinylbenzene-*g*-glycidyl methacrylate–iminodiacetic acid) (PVA-DVB-*g*-GMA-IDA) particles were prepared by a modified suspension polymerization method and cerium initiated graft polymerization with chelating group. Magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} chelated particles were obtained by adding PVA-DVB-*g*-GMA-IDA beads to the aqueous solution of Cu^{2+} ion. Then glucoamylase was adsorbed onto the metal-chelating particles from aqueous enzyme solution. The optimization of immobilization conditions were carried out, and the enzymatic properties, reusability and storage stability of the immobilized glucoamylase were also investigated.

2. Materials and methods

2.1. Materials

Glycidyl methacrylate (GMA) and iminodiacetic acid (IDA) were purchased from Aldrich and Acros Organics, respectively (USA). All other materials were of analytical grade and were obtained from Beijing Chemical Reagents Company (Beijing, China). Vinyl acetate (VAc) was distilled under vacuum. Divinylbenzene (DVB) was used as a cross-linking agent. Benzoyl peroxide (BPO) was used as an initiator. Poly(vinyl alcohol) (PVA-1788, degree of polymerization 1700, degree of hydrolysis 88%) was used as a stabilizer. Glucoamylase (exo-l,4-α-D-glucosidase, EC 3.2.1.3 from *Aspergillus niger*, 50,000 U/ml, 100 mg protein/ml) was purchased from Wuxi Syder Bio-Products Limited Corporation (Wuxi, China).

2.2. Preparation of immobilization particles

Magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles were synthesized according to the method reported by our institute before [19]. Oleic acid-coated magnetite nanoparticles were synthesized by a coprecipitation method [22]. The magnetic PVAc-DVB particles were prepared by a modified suspension polymerization method and were transformed into magnetic PVA-DVB particles by ester exchange reaction. GMA-IDA monomer was first prepared by reaction of GMA with IDA [23] and then GMA-IDA grafted magnetic PVA-DVB particles by Cerium initiated graft polymerization. At last, the magnetic PVA-DVB-*g*-GMA-IDA particles were charged with copper ions, and then the fabricated magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles were washed several times with water and 20 mM sodium phosphate buffer (PBS, pH 8.0) to remove the excess unbound Cu^{2+}. The particle size and surface morphology of the magnetic polymer particles were observed by scanning electron microscopy (SEM, JEOL, JSM-6700F, Tokyo, Japan).

2.3. Immobilization of glucoamylase

Glucoamylase adsorption on the Cu^{2+}-chelated magnetic PVA-DVB-*g*-GMA-IDA particles was tested at various pH values, either in sodium acetate buffer (0.1 M, pH 3.0–5.5) or in phosphate buffer (0.1 M, pH 6.0–7.0). 10 mg of magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles was added to 20 ml of glucoamylase solution (0.2 mg/ml) prepared with the corresponding buffer. The resulting suspensions were subsequently incubated at 25 °C with shaking at 100 rpm for a given time in order to reach adsorption equilibrium. The glucoamylase-adsorbed particles were separated from the enzyme solution magnetically and washed with the same buffer three times. The elution solutions containing residual glucoamylase were collected. The activities of immobilized glucoamylase were evaluated by the assay of the activity recovery, relative activity and residual activity, respectively. The resulting immobilized glucoamylase were stored at 4 °C in fresh buffer until use. The amount of protein in the enzyme solution and in the washing solution was determined by the Bradford method [24], and the amount of protein (Q) bound on the particles was calculated from the formula:

$$Q = \frac{(C_i - C_f)V}{m} \quad (1)$$

where Q is the amount of glucoamylase adsorbed on a unit mass of the magnetic particles (mg/g); C_i and C_f are the concentrations of glucoamylase in the initial and final reaction medium, respectively (mg/ml); V is the volume of the reaction medium (ml); and m is the mass of particles used (g).

To determine the adsorption capacities of the Cu^{2+}-chelated magnetic PVA-DVB-*g*-GMA-IDA particles, the concentration of glucoamylase in the medium was varied in the range of 0.1–1.0 mg/ml.

2.4. Assay of glucoamylase activity

Activities of free and immobilized glucoamylase were assayed by the addition of 0.5 ml of diluted free enzyme or immobilized glucoamylase in 0.5 ml the acetate buffer solution (0.1 M, pH 4.5), using 0.5 ml soluble starch solution which contains 1.0% (w/v) soluble starch gelatinized in water (15 min, 100 °C, continuous mixing) as the substrate. The reaction was stopped by adding 5 ml of NaOH solution (0.1 M) after exactly 15 min of incubation at 60 °C, and then glucose content in the reaction medium was determined using DNS method [25]. All activity measurement experiments were carried out for three times and the relative standard deviation is less than 2.0%. One unit of glucoamylase activity is defined as the amount of enzyme that produces 1.0 μmol of glucose from soluble starch per minute under the assay conditions.

The activity recovery of the immobilized enzyme is calculated from the formula:

$$R(\%) = \left(\frac{A_i}{A_f}\right) \times 100\% \quad (2)$$

where R is the activity recovery of the immobilized enzyme (%), A_i the activity of the immobilized enzyme (U) and A_f is the activity of the same amount of free enzyme in solution as that immobilized on particles (U).

2.5. Effect of pH and temperature on free and immobilized glucoamylase activity

The optimum pH and reaction temperature of free and immobilized glucoamylase were determined as the relative activity after incubation for 15 min (as described above) under the variety of pH (0.1 M sodium acetate buffer for pH 2.0–5.5, 0.1 M phosphate buffer for pH 6.0–8.0) and temperature (from 30 to 80 °C).

2.6. Determination of kinetic parameters and properties of immobilized glucoamylase

Kinetic parameters (K_m and V_{max}) of the free glucoamylase and the immobilized glucoamylase were determined by measuring initial rates of the reaction with soluble starch [0.1–2.0% (w/v)] in acetate buffer (0.1 M, pH 4.5) at 60 °C. For this purpose, equivalent free and immobilized glucoamylase were added to soluble starch solution of different concentrations between 1.0 and 20 mg/ml and initial activities were determined as described above.

Thermal stability studies of the free and immobilized glucoamylase were carried out by measuring the residual activity of the enzyme exposed to three different temperatures (60–70 °C) in acetate buffer (0.1 M, pH 4.5), and the enzymatic activities of the free and immobilized glucoamylase were determined by the method described in Section 2.4. The activity of the immobilized glucoamylase was determined with the same method as Section 2.4 after repetitive use. For storage stability, the activities of free and immobilized glucoamylase in sodium acetate buffer (0.1 M, pH 4.5) stored at 4 °C were measured in batch operating mode under the experimental conditions given in Section 2.4.

2.7. Desorption and reusability of magnetic particles

In order to determine the reusability of magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles, glucoamylase adsorption and desorption cycle was repeated 10 times. Glucoamylase desorption from the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles were carried out with 50 mM EDTA. The particles were washed several times with phosphate buffer (50 mM, pH 7.0), and then were reused in enzyme immobilization.

3. Results and discussion

3.1. Magnetic PVA-DVB-g-GMA-IDA-Cu^{2+} particles

The specific magnetic metal-chelated affinity particles suitable for glucoamylase immobilization were prepared, and the particle has a spherical form and a micron size (several microns) which offers an appropriate surface area to bind affinity ligands and can sustain in aqueous suspension thus permitting reaction kinetics of a "quasihomogeneous" solution [19]. The strategy of noncovalent immobilization of glucoamylase on the metal-chelated particles is presented in Fig. 1. According to the Lewis acid–Lewis base concepts of Pearson [26], copper ion, as soft or borderline Lewis acids, exhibits preference for non-bonding lone pair electrons from nitrogen atoms in aromatic and aliphatic amino containing ligands. Thus, a strong binding can be established between amino acid side chain group of glucoamylase (i.e. especially imidazole groups of the histidine residues) and copper ions [27]. The particles are proposed suitable for glucoamylase immobilization and are favorable for column applications.

3.2. Glucoamylase immobilization parameters

The effect of pH on the immobilization of glucoamylase onto magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles was studied in the pH range 3.0–7.0, and the result is presented in Fig. 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 [28]. The isoelectric pH of glucoamylase used in this study is 4.5. As shown in Fig. 2, the maximum adsorption of glucoamylase was observed at pH 5.0, which had slightly shifted toward more neutral pH values. This phenomenon was due to preferential interactions between glucoamylase molecules and Cu^{2+} incorporated polymeric matrix

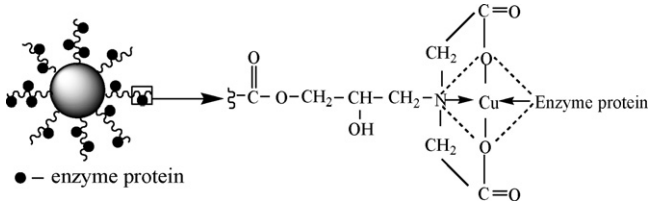

Fig. 1. Schematic diagram of glucoamylase immobilization on the metal-chelated particles.

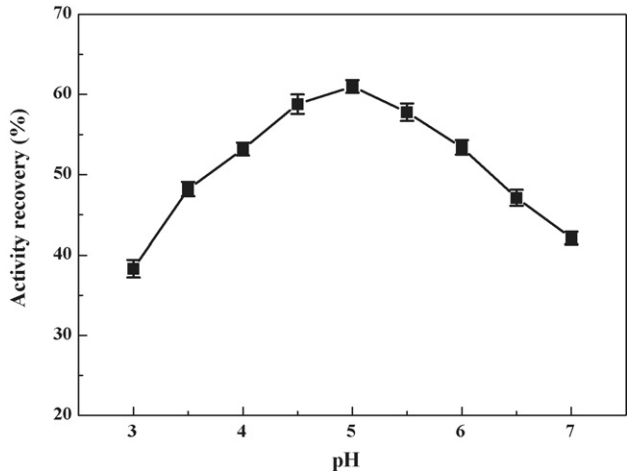

Fig. 2. Effect of pH on glucoamylase adsorption onto the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles. All immobilizations were carried out at 25 °C and 0.2 mg/ml of glucoamylase concentration for 3 h.

at neutral pH. The decrease in the enzyme adsorption capacity in more acidic and more alkaline regions was related to electrostatic repulsion effects between the oppositely charged groups [11].

Fig. 3 indicates the changes of the activity recovery and amount of adsorption with the reaction time. The amount of glucoamylase adsorption on the magnetic particles increased with prolonged reaction time and reached equilibrium after 3 h. The highest activity recovery was obtained under reaction allowed to proceeding for 3 h and the activity recovery declined slightly when reaction time prolonged further because of the increasing thermal deactivation.

Effect of the amount of enzyme added on activity recovery of the immobilization glucoamylase is shown in Fig. 4. The maximal activity recovery of the immobilized glucoamylase reached 84.0% when the enzyme-adding concentration was 0.5 mg/ml where the adsorbed glucoamylase reached 89 mg/g-magnetic particles. The activity recovery decreased gradually when the glucoamylase concentration was more than 0.5 mg/ml. It is due

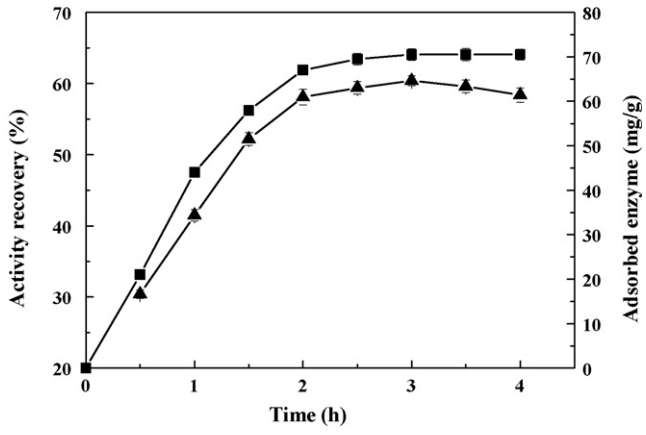

Fig. 3. Changes of the activity recovery and amount of adsorption with the reaction time. All reactions were carried out at pH 5.0 (0.1 M sodium acetate buffer), 25 °C and 0.2 mg/ml of glucoamylase concentration. (▲) Activity recovery; (■) adsorbed enzyme.

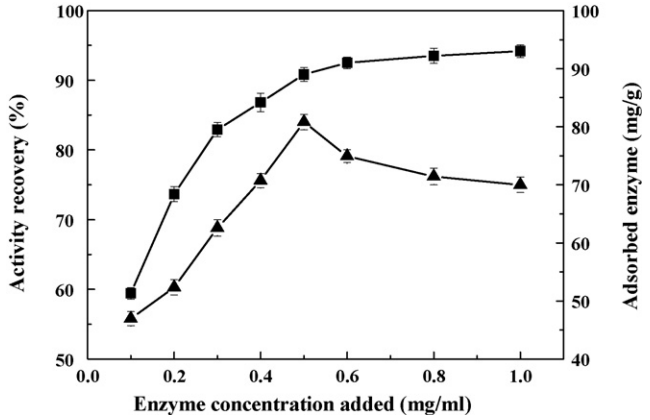

Fig. 4. Effect of the amount of enzyme added on glucoamylase immobilization onto the magnetic supports. All immobilizations were carried out at pH 5.0 (0.1 M sodium acetate buffer) and 25 °C for 3 h. (▲) Activity recovery; (■) adsorbed enzyme.

to more amount of enzyme immobilized to form an intermolecular space inhibition of the immobilized enzyme, which will restrain the dispersion of the substrate and product [4].

The adsorption isotherm of glucoamylase was illustrated for the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles in Fig. 5. The metal-chelated adsorption isotherm was well fitted to the Langmuir model which is expressed by Eq. (3).

$$q = \frac{q_m c}{K_d + c} \tag{3}$$

where c and q are the aqueous phase protein concentration and the adsorbed protein density in equilibrium, respectively, q_m the maximum adsorption capacity, and K_d is the dissociation constant. The parameters q_m and K_d were predicted to be 101.8 mg/g-magnetic particles and 0.075 mg/ml with the correlation coefficient R^2 being 0.9987.

The magnetic particles reported here have a good adsorption capacity for glucoamylase, which is higher than those of magnetic microbeads reported before in the literature: 26 mg/g [29],

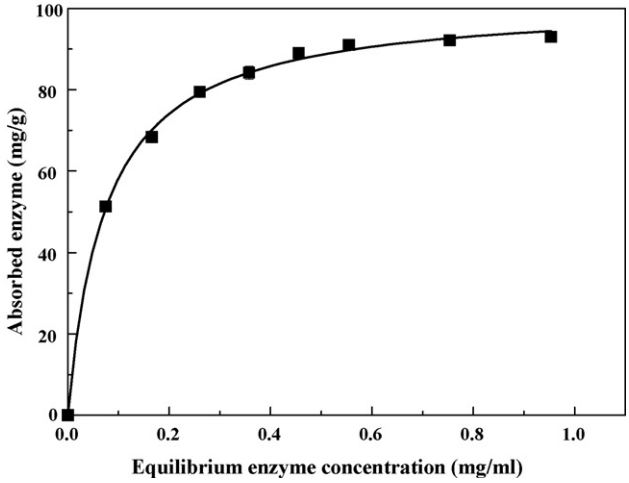

Fig. 5. Adsorption isotherm of glucoamylase on the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} supports (solid line represents Langmuir model). All immobilizations were carried out at pH 5.0 (0.1 M sodium acetate buffer) and 25 °C for 3 h.

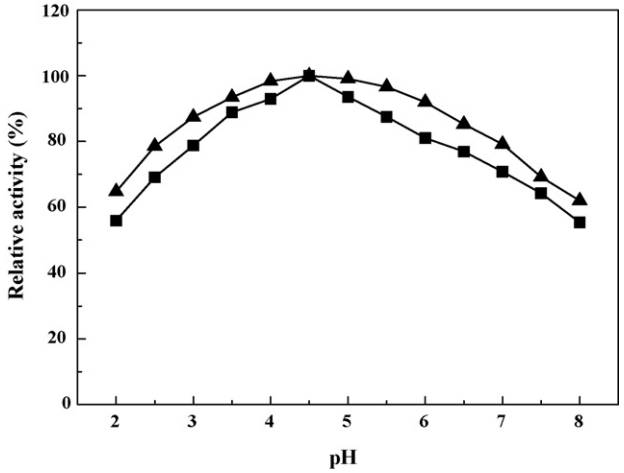

Fig. 6. Effect of pH on the activity of free and immobilized glucoamylase. (■) Free enzyme; (▲) immobilized enzyme.

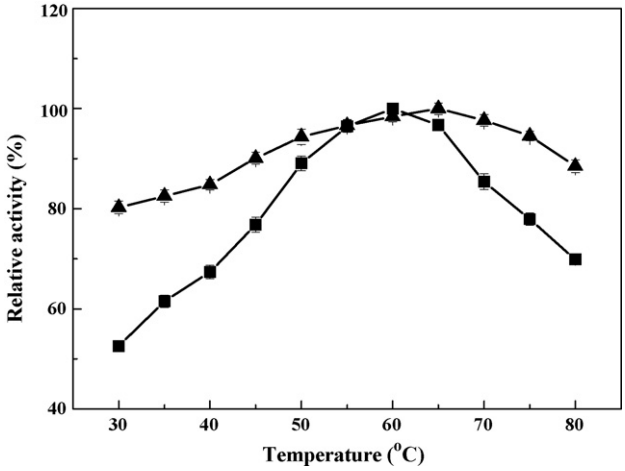

Fig. 7. Effect of temperature on the activity of free and immobilized glucoamylase. (■) Free enzyme; (▲) immobilized enzyme.

17 mg/g [30] and 8.5 mg/g [15]. Glucoamylase immobilizations on non-magnetic particles were studied by other researchers and the adsorption capacity of these particles varied in a wide range, e.g. 10 mg/g on montmorillonite [3], 3.97 mg/g on surface-modified carriers [31] and 35 mg/g on Sepabeads coated with polyethyleneimine [4]. Compared with their particles, the magnetic particles prepared in this study had a nonporous structure, which had the advantage of higher resistance to fouling and better mass transfer [19]. The high capacity of the magnetic particles was also attributed to the tentacle-type polymer chains grafted on the magnetic particle. Each of the chains possessed several affinity ligands, and the steric hindrance of enzyme protein to the ligands was reduced. In comparison with glucoamylase immobilization studies reported by other researchers [21,32–34], the activity recovery of the glucoamylase immobilized onto the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles reached a high level due to the nonporous structure and the flexible grafted tentacle chains reaching out of the solution on the magnetic polymer particles, which is beneficial for the substrate combining to the enzyme and the product diffusing to the reaction medium.

3.3. Conditions affecting on immobilized enzyme activity

The effects of pH on the hydrolysis activity of free and immobilized glucoamylase for soluble starch were determined in the pH range between 2.0 and 8.0, and the results are illustrated in Fig. 6. The maximum relative activity of the free glucoamylase was observed at pH 4.5, whereas the optimal pH value to get which of that was at 4.0–5.0 pH region for the immobilized glucoamylase. The immobilized glucoamylase showed excellent adaptability in a wider pH region comparing to the free enzyme, which was due to the stabilization of glucoamylase molecules resulting from multi-point ionic complex formation with the grafted GMA-IDA monomers [35].

As shown in Fig. 7, the optimal temperature of free glucoamylase was at 60 °C and the immobilized enzyme was shifted to 65 °C. This shift toward higher temperatures with immobilized glucoamylase was probably due to multipoint chelated interactions which consequently led to an increase in the activation energy for reorganization of the enzyme to an optimum conformation for binding to its substrate [9,12]. Compared with the free glucoamylase, the immobilized glucoamylase also exhibited a higher endurance for reaction temperature, of which the relative activity is more than 90% within 45–75 °C. The reason for the increased stability of immobilized glucoamylase was due to the restricted conformational mobility of the molecules following immobilization [36].

It is well known that enzymes in solution are not stable and their activities would also decrease gradually during use. Thermal stability was carried out with the free and immobilized glucoamylase in acetate buffer at various temperatures, and the results are shown in Fig. 8. The immobilization remarkably enhanced heat and denaturation resistance of glucoamylase. After incubation at 60–70 °C for 180 min, the immobilized glucoamylase remained higher activity in comparison with the free one. The improvement of the thermal stability for glucoamy-

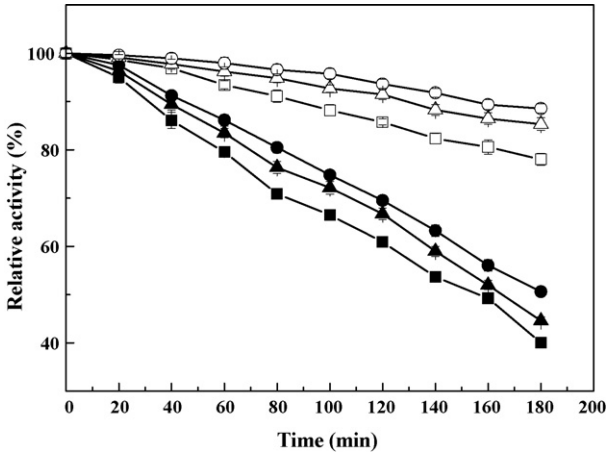

Fig. 8. Thermal stability of free and immobilized glucoamylase. (●) Free enzyme, 60 °C; (▲) free enzyme, 65 °C; (■) free enzyme, 70 °C; (◯) immobilized enzyme, 60 °C; (△) immobilized enzyme, 65 °C; (□) immobilized enzyme, 70 °C.

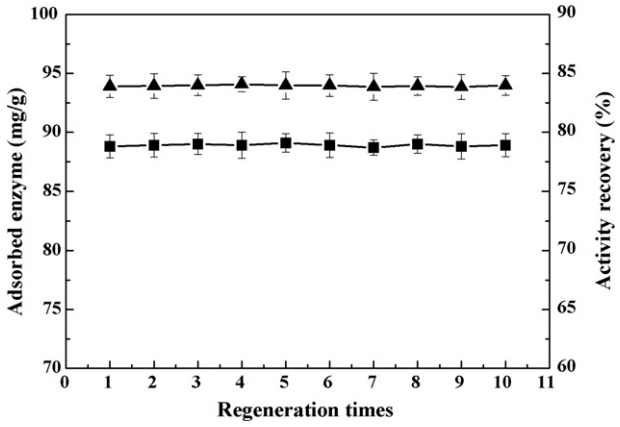

Fig. 9. Reuse of the magnetic PVA-DVB-*g*-GMA-DA-Cu^{2+} particles. Glucoamylase immobilization was carried out at 0.5 mg/ml of glucoamylase in 0.1 M acetate buffer (pH 5.0) at 25 °C. (▲) Activity recovery; (■) adsorbed enzyme.

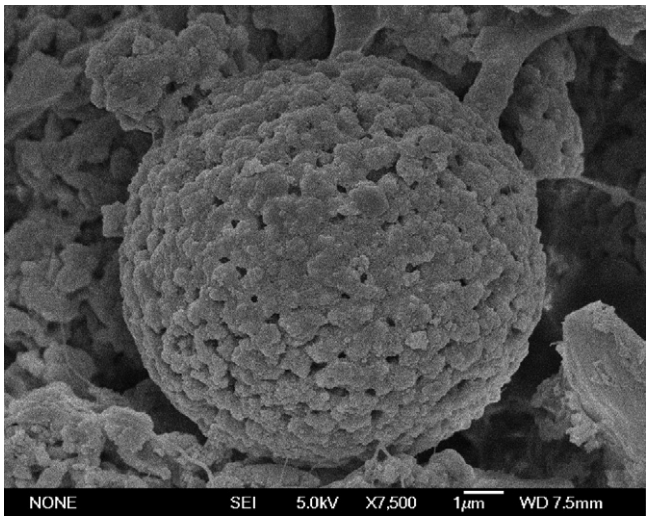

Fig. 10. SEM micrograph of the magnetic enzyme-immobilized particles after the tenth reuse.

lase was probably due to a reduction in molecular mobility by multi-point binding between the enzyme and the metal-chelated particles [37].

Storage stability is one of the important advantages for immobilized enzymes over the free enzymes because free enzymes can lose their activities fairly quickly. 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 42 days. No enzyme release from the magnetic particles was observed during this storage period. The free glucoamylase lost its whole activity within 6 weeks. However, the immobilized glucoamylase on the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles lost only 5% of its activity during the same period (data not shown). This result indicated that the stability of the immobilized glucoamylase was greatly improved 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 [8].

Compared with free enzyme, immobilized enzyme can be easily separated from product solution and reused [38]. It was observed that the immobilized glucoamylase was still maintained 75.7% of their original activities after the 30th reuse (data not shown). This result indicated that the immobilized glucoamylase onto the magnetic particles had a good stability.

An important advantage of the magnetic enzyme-immobilized particles is their reusability. Desorption of glucoamylase from the magnetic Cu^{2+}-chelated PVA-DVB-*g*-GMA-IDA particles was carried out in a batch system. The immobilized glucoamylase preparation was placed in a desorption medium containing 50 mM EDTA at room temperature for 2 h. It was then repeatedly used for adsorption of glucoamylase. No remarkable change was observed on the adsorption capacity and activity recovery of glucoamylase during 10 successive adsorption–desorption cycles (Fig. 9). The magnetic enzyme-immobilized matrix kept its spherical shape after the tenth reuse, and the structure of the magnetic particles was not damaged or collapsed (Fig. 10). These results showed that the magnetic PVA-DVB-*g*-GMA-IDA-Cu^{2+} particles can be repeatedly used in enzyme immobilization, without detectable losses in their initial adsorption capacity and recovered activity.

3.4. Kinetic parameters

Kinetic constants, the Michaelis constant (K_m) and the maximal initial rate of the reaction (V_{max}) for the free and the immobilized glucoamylase were determined by using soluble starch as a substrate, and the results are shown in Table 1. The K_m value of the immobilized glucoamylase was 1.5-fold higher than that of the free one. The V_{max} value of the free glucoamylase (2.91 μmol/(mg protein min)) was found to be higher than that of the immobilized glucoamylase (2.45 μmol/(mg protein min)). The change in the affinity of the glucoamylase to its starch is probably caused by structural changes in the enzyme introduced by

Table 1
Kinetic constants of free and immobilized glucoamylase

Form of enzyme	K_m (mg/ml)	V_{max} (μmol/(mg protein min))	EC[a] (mg/g-support)	V_{max}/K_m[b]	R (%)[c]
Free	1.07 ± 0.01	2.91 ± 0.03	–	2.73	–
Immobilized	1.77 ± 0.02	2.45 ± 0.02	89.0 ± 1.2	1.39	84.0 ± 1.1

[a] Enzyme content of per gram particles.
[b] Catalytic efficiency was defined as the ratio of V_{max}/K_m.
[c] R (%) is the activity recovery of the immobilized enzyme.

the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme [9,21,39].

4. Conclusion

The magnetic PVA-DVB-*g*-GMA-IDA particles were prepared from magnetic PVA-DVB particles and GMA-IDA monomer via Cerium initiated graft polymerization. The metal-chelated magnetic particles were used for the reversible immobilization of glucoamylase via metal-affinity adsorption. These particles not only can be operated magnetically but also exhibit a high adsorption capacity of glucoamylase. The immobilized glucoamylase had a good recovered activity and reached the "optimal compromise" at 0.5 mg/ml of glucoamylase concentration. Much of the activity of immobilized glucoamylase retained over wider ranges of temperature and pH than that of the free enzyme. A stable reuse capacity achieved with the immobilized glucoamylase indicated that the immobilized glucoamylase was suitable to be used in a continuous system for the production of glucose. The storage stability of the immobilized glucoamylase was also greatly improved at 4 °C. After inactivation of enzyme upon use, the adsorbed enzyme can be desorbed with EDTA, and the regenerated magnetic particles can be reused for the reversible immobilization of the same enzyme. Together with these results, the reusable magnetic beads can provide economic advantages for large-scale biotechnological applications.

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