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Reactive Brown 10-attached Polyamide Hollow Fiber for Reversible Amyloglucosidase Immobilization

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Abstract

Amyloglucosidase (AMG) [α-1, 4-D-glucan glucohydrolase (E.C.3.2.1.3)] is an exo-enzyme, which is used in the hydrolysis of starch to glucose in industry. In this study, AMG was immobilized onto Reactive Brown 10-attached polyamide hollow fibers. Dye-attached hollow fibers were characterized by scanning electron microscopy. The amount of attached dye was determined by elementel analysis considering the sulphur stoichiometry. It was found as 18.4 μmol/g hollow fiber. Affecting factors, pH and initial AMG concentration, on the immobilization was investigated. Maximum AMG immobilization was achieved at pH 4.0, and adsorption capacity of hollow fibers increased by increasing initial AMG concentration and reached the plateau at 1.5 mg/mL. The effects of temperature and pH on the enzyme activity were also investigated. Immobilized enzyme has a wider pH dependence around pH 4.0-5.0, but free enzyme has optimal pH at its p*I* value, pH 4.5. Optimal temperature for immobilized enzyme was determined as 65°C, which is higher than free enzyme, 60°C. The immobilization process increased the thermal stability of AMG. In 180 min, although free enzyme lost the activity about 60% at 60°C, immobilized enzyme lost only 20% at same temperature. Significant amount of the immobilized AMG was desorbed using 1.0 M NaCl at pH 3.0. In order to determine the effects of adsorption and desorption conditions on possible conformational changes of AMG, fluorescence spect-rophotometry was employed. It was concluded that polyamide dye-affinity hollow fibers can be applied for AMG immobilization without causing any significant conformational changes. K_M and V_{max} values of immobilized and free enzymes were found to be 1.08 and 1.31 mg/mL and 4.53 and 1.94 μmol/mg.min, respectively. The K_M value of the immobilized AMG was 1.2-fold higher than that of the free one. The V_{max} value of the free AMG was found to be higher than that of the immobilized AMG. These results showed that Reactive Brown 10-attached hollow fiber can be used for reversible immobilization of AMG in biotechnological applications.

Key Words: Hollow fibers; Reactive Brown 10; amyloglucosidase; polyamide; reversible immobilization

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 and oldest protocol of physical immobilization methods [9]. Simplicity and reversibility are the most important advantages of this method. But a strong adsorption 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 dye-ligand affinity adsorption of the enzyme on a reactive dye-attached 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].

Amyloglucosidase (AMG, EC 3.2.1.3), also known as glucoamylase, acid maltase, lysosomal α-glucosidase, exo-1,4-α-glucosidase, is one of the most economically important industrial enzymes. AMG hydrolases α-(1→4) and α-(1→6) glucosidic bonds of starch from the non-reducing ends successively to produce glucose and is used for the production of glucose from starch. At present, glucose is mainly produced by enzymatic hydrolysis of starch using α-amylase and AMG [11]. Thus, AMG 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]. Another advantage of the AMG process, pure product is obtained in the final stages of the process.

In industrial conventional enzymatic reactions, a mixture of the substrate and the soluble enzyme is incubated. After completion of each batch of reaction, the amylases are inactivated. Naturally, the process would be more economical if the enzyme could be reused, for example, by immobilization [11]. The immobilization of enzymes is a widely used approach for obtaining reusable forms of biocatalysts that provide easy separation from products

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and convenient handling [12-14]. The most important factors influencing immobilization processes are as follows: (i) carrier properties (material, particle diameter, pore size, available anchor groups and their amount); (ii) enzyme stability, available anchor groups on a protein surface; (iii) immobilization conditions (pH, ionic strength, protein concentration, carrier activators) [15].

Immobilization of amyloglucosidase have been studied using various supports, such as activated carbon [16], polymers [17-20], and inorganic materials [21,22]. Disadvantage of enzyme immobilization contain the activity decrease and diffusion limitation of substrate, intermediate and product [19]. The surface reaction or the diffusion of substrate and product in the pore may be the rate-controlling step in the immobilized enzyme system [23]. If the pore size higher or the particle size smaller, the effect of pore diffusion of the substrate and intermediates would be expected to decrease, and this would lead to results closer to those obtained with free enzyme [16,24].

Commercially available triazinyl-based reactive dyes were screened for their binding affinity to the enzymes. Desorption of the adsorbed enzymes from the dye ligands by enzyme substrates or salts were allowed to immobilize the enzyme, reversibly [25]. In recent years, porous membranes were used for support material [26-29]. Microporous membranes have the advantages of large surface area, short diffusion path and low pressure drop. As a result of the convective flow of solution through the pores, the mass transfer resistance is reduced and the binding kinetics dominates the adsorption. This results in a rapid processing, which improves adsorption, washing, elution, regeneration steps and decreases the probability of inactivation of biomolecules. Membranes which used for protein separation or enzyme immobilization must fulfill the requirements of high hydrophilicity, low non-specific adsorption, large pore size, chemical and mechanical stability as well as having enough functional groups.

In this study, a dye affinity support, Reactive Brown 10-attached polyamide hollow-fibers have been prepared. This support was used for reversible immobilization of AMG. AMG was immobilized onto the dye attached hollow fiber from aqueous enzyme solution. The optimization of immobilization conditions were carried out, and the enzymatic properties, reusability and storage stability of the immobilized AMG were also investigated.

Materials and Methods

Materials

Microporous polyamide hollow fibers (PA386C) were a gift from Akzo (Wuppertal, Germany). Reactive Brown 10 (Procion Brown MX-5BR) and amyloglucosidase ((AMG), exo-1,4-α-D-glucosidase, EC 3.2.1.3 from *Aspergillus niger* 94.5 U mg^{-1}) were obtained from Sigma (St. Louis, MO, USA) and used as received. All other chemicals were of analytical reagent grade and supplied from Merck (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA, USA) RO-Pure 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. The resulting purified water (deionized water) has a specific conductivity of 18 MΩ/cm.

Reactive Brown 10-attached hollow-fibers

Polyamide hollow-fibers were cut into small segments (1 cm in length, normal pore size: 0.20 μm; maximum pore size: 0.43 μm). Reactive Brown 10 was dissolved in 10 ml distilled water (initial dye concentration 0.6 mg/ml). Dye solution was transferred to polyamide hollow fibers (total length: 50 cm) in 90 ml distilled water, and then 4.0 g of NaOH added. The medium consisting of the fiber segments and dye was magnetically stirred (at 400 rpm) in a sealed reactor at 80°C for 4 h. After dye attachment, in order to remove the non-specifically attached dyes, a cleaning procedure was applied, which was as follows: the hollow fibers were first washed with deionized water. The hollow fibers were then dispersed in methanol, and the dispersion was sonicated for 2 h in an ultrasonic bath (200 W, Bransonic 200, USA). At the last stage, hollow fibers were washed again deionized water. The modified hollow fibers were then stored at 4°C with 0.02% sodium azide to prohibit microbial contamination.

The release of the Reactive Brown 10 was investigated at different pH values in the range of 4.0–8.0. It should be noted that these media were the same which were used in the enzyme immobilization experiments given below. Reactive Brown 10 release was also determined in the medium at pH 3.0 solution containing 1.0 M NaCl which was the medium used for the AMG elution experiments.

Characterization of hollow-fibers

Elemental analysis

The amount of Reactive Brown 10 attached on the hollow fiber was evaluated by using on elemental analysis instrument (Leco, CHNS-932, USA) by considering the sulphur stoichiometry.

SEM studies

Microscopic observations of the hollow fibers were performed by using a scanning electron microscope (Jeol, JEM 1200 EX, Tokyo, Japan). Hollow fibers were dried at room temperature and coated with a thin layer of gold (about 100 Å) in vacuum and photographed in the electron microscope.

Immobilization of amyloglucosidase

Amyloglucosidase adsorption on the dye-attached hollow fibers 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). Dye-attached hollow fibers were added to 20 ml of AMG solution (1.0 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 AMG-immobilized hollow fibers were separated from the enzyme solution via decantation and washed with the same buffer three times. The elution solutions containing residual AMG were collected. The activities of immobilized AMG were evaluated by the assay of the relative activity and residual activity, respectively. The resulting immobilized AMG 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 [30], and the amount of protein (Q) bound on the particles was calculated from the formula:

$$Q= [(C_i-C_f).V]/m \quad (1)$$

where Q is the amount of AMG bound on a unit mass of the dye-attached hollow fibers (mg/g); C_i and C_f are the concentrations of AMG 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 dye-attached hollow fibers used (g).

To determine the adsorption capacities of the dye-attached hollow fibers, the concentration of AMG in the medium was varied in the range of 0.5–2.5 mg/ml.

Assay of amyloglucosidase activity

Activities of free and immobilized AMG were assayed by the addition of 0.5 ml of diluted free enzyme or immobilized AMG in 0.5 ml the acetate buffer solution (0.1 M, pH 4.5), using 1.0 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 by using dinitrosalicylic acid (DNS) method [31]. All activity measurements were carried out for three times and the relative standard deviation is less than 2.0%. One unit of AMG 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 relative activity of the immobilized enzyme is calculated from the formula:

$$R (\%) = (A_{im}/A_{free}) \times 100 \quad (2)$$

where R is the relative activity of the immobilized enzyme (%), A_{im} is the activity of the immobilized enzyme (U) and A_{free} is the activity of the same amount of free enzyme in solution as that immobilized on holow fibers (U).

Effect of pH and temperature on free and immobilized amyloglucosidase activity

The optimum pH and reaction temperature of free and immobilized AMG 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 3.0–5.5, 0.1 M phosphate buffer for pH 6.0–7.0) and temperature (from 30 to 70 °C).

Determination of kinetic parameters and properties of immobilized amyloglucosidase

Kinetic parameters (K_M and V_{max}) of the free and immobilized AMG were determined by measuring initial rates of the reaction with soluble starch [0.05–3.0% (w/v)] in acetate buffer (0.1 M, pH 4.5) at 60°C. For this purpose, equivalent free and immobilized AMG 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 and storage stability of amyloglucosidase

Thermal stability studies of the free and immobilized AMG were carried out by measuring the residual activity of the enzyme at 65°C in acetate buffer (0.1 M, pH 4.5), and the enzymatic activities of the free and immobilized AMG were determined by the method described above. The activity of the immobilized AMG was determined with the same method as above after repetitive use. For storage stability, the activities of free and immobilized AMG 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 above.

Desorption and reusability of dye-attached hollow fibers

In order to determine the reusability of dye-attached hollow fibers, AMG adsorption and desorption cycle was repeated 5 times. AMG desorption from the dye-attached hollow fibers were carried out with 1.0 M NaCl solution, pH 3.0. The fibers were washed several times with acetate buffer (50 mM, pH 4.0), and then were reused in enzyme immobilization.

Results and Discussion

Characteristics of polyamide hollow-fiber membrane

Reactive Brown 10 is a dichlorotriazine dye (Figure 1), and it has a sulphonate, carboxylic, secondary amino group and a pendant hydroxyl group. In addition of these groups, it also contains a chelated Cr^{2+} ion through the carboxyl and hydroxyl groups. The schematic representation of dye-attachment reaction is shown in Scheme 1. After the dye attachment step, orginally chelated Cr^{2+} ion was desorbed with ethylenediamine tetraacetic acid solution (EDTA, 25 mM, pH 5.0) to avoid non-specific interactions and other effects of the ion.

SEM micrographs given in Figure 2 show the surface structure and the cross-section of the polyamide hollow fibers. The hollow fiber has large pores; the micropore radius is around 4.0 µm. The hollow fiber has rough and heterogeneous surface. This large pores reduce diffusional resistance and facilitate mass transfer because of high internal surface area. In contrast to the packed bed columns, the membrane chromatography brings the protein molecules into the proximity of the attached ligand molecules into the large pores by convection, thus reducing the resistance to mass transfer. It should be noted

that no changes of polyamide hollow fibers cross-section and surface morphology were observed after dye modification.

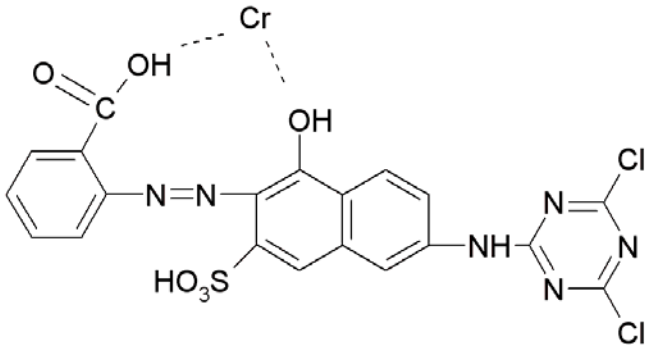
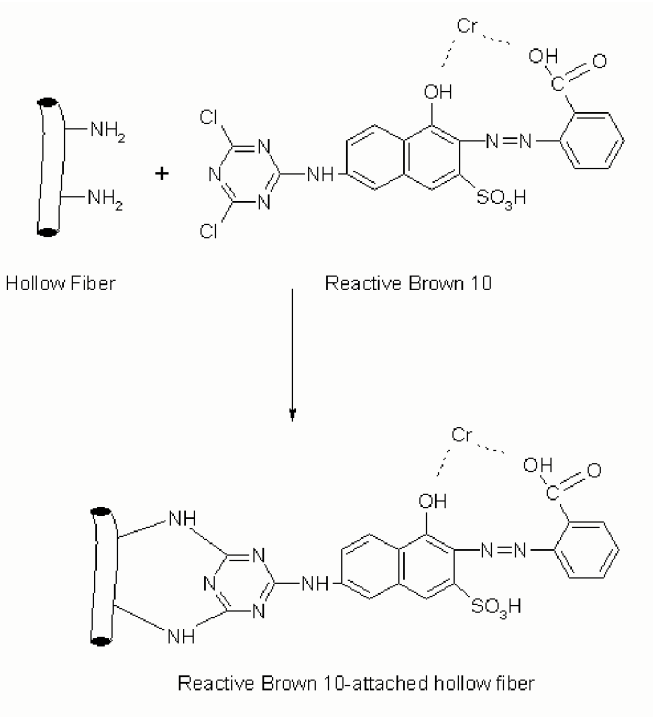


Figure 1. Chemical structure of Reactive Brown 10.



Scheme 1. Binding reaction of Reactive Brown 10 onto the hollow fibers.

Reactive Brown 10-attached hollow fibers were subjected to elemental analysis. The amount of Reactive Brown 10 attached to the hollow fibers was found as 18.4 μmol/g. Note that the Reactive Brown 10-attached hollow-fibers were extensively washed until to ensure that there is no dye leakage from any of the dye-attached hollow fibers and in any media used at adsorption and/or desorption steps.

Glucoamylase immobilization parameters

Effects of initial concentration

Figure 3 shows the effects of initial AMG concentration on enzyme immobilization. It was observed that the amount of immobilized AMG was increased with the initial AMG concentration. Maximum adsorption capacities of the hollow fibers for AMG was found to be 108.9 mg/g, respectively, and the adsorbed amount of AMG per unit mass of hollow fibers increased and reached plateau value at about 1.5 mg/ml.

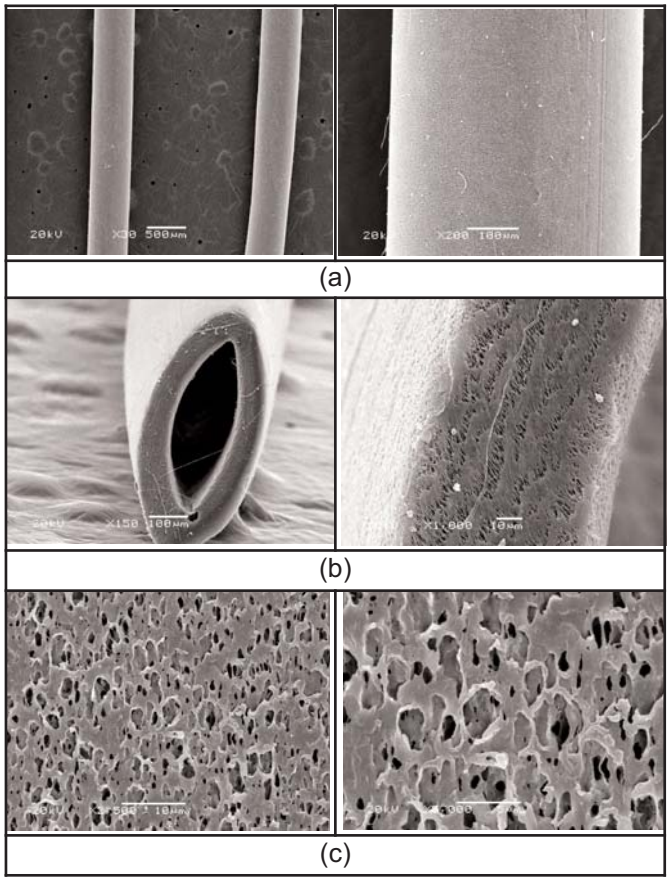


Figure 2. The representative SEM micrographs of polyamide hollow fibers: (a) outer surface; (b) cross-section; (c) inner surface.

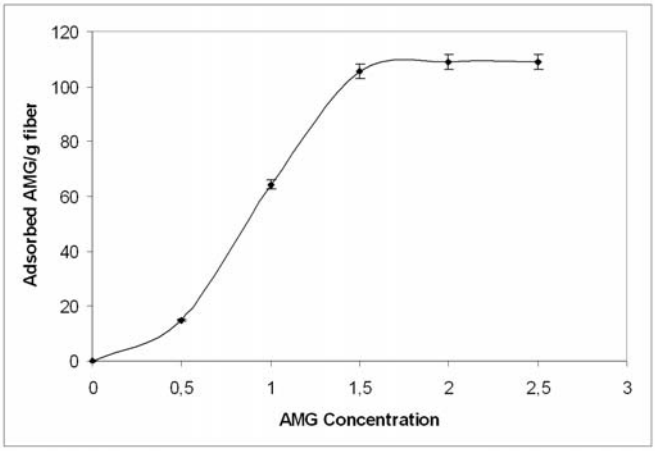


Figure 3. Effects of initial AMG concentration on immobilization onto dye-attached polyamide hollow fibers: pH 4.0; Reactive Brown 10 loading: 18.4 μmol/g; T: 25°C.

Effects of pH

The effects of pH on adsorption are presented in Figure 4. Proteins have no net charge at their isoelectric points, and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric points [25,32]. The isoelectric pH of AMG used in this study is 4.5. As seen in Figure 4, the maximum adsorption of AMG was observed at pH 4.0. At this pH value, AMG was positively charged but dye-attached hollow fibers were negatively charged due to their -SO_3H and –CO_2H acid groups. So we can speculate that dominant force contributing to dye and protein interactions is electrostatic rather than other forces. The conformational changes of protein molecules due to specific interactions at this pH may also contribute the specific interaction.

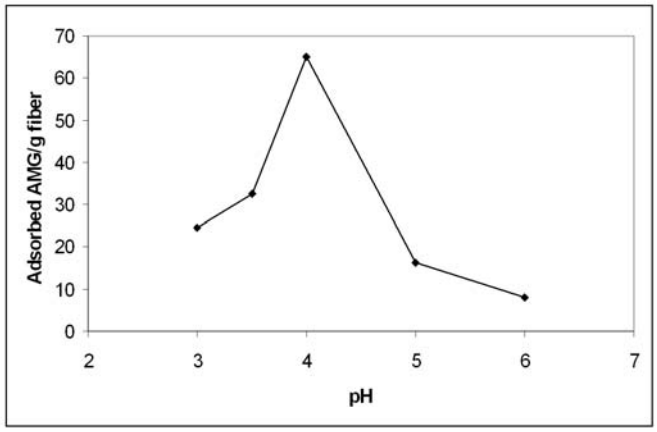


Figure 4. Effects of medium pH on AMG immobilization onto dye-attached polyamide hollow fibers. Reactive Brown 10 loading: 18.4 μmol/g; initial AMG concentration: 1 mg/ml; T: 25°C.

pH effect on immobilized enzyme activity

The effects of pH on the hydrolysis activity of free and immobilized AMG for soluble starch were determined in the pH range between 3.0 and 7.0, and the results are shown in Figure 5. The maximum relative activity of the free AMG was observed at pI (pH 4.5), but that for immobilized AMG was observed as wider around pH 4.0-5.0 range. It shows the immobilization process increased the stability of AMG and decreased the pH sensitivity [33].

Temperature effect on enzyme activity

Figure 6 shows that activity dependence of free and immobilized AMG. As seen in figure, maximum relative activity of immobilized enzyme was shifted to higher temperature. Immobilization can effect on the structure of enzyme and led to an increase in the activation energy for reorganization of the enzyme to an optimum conformation for binding to its substrate [9].

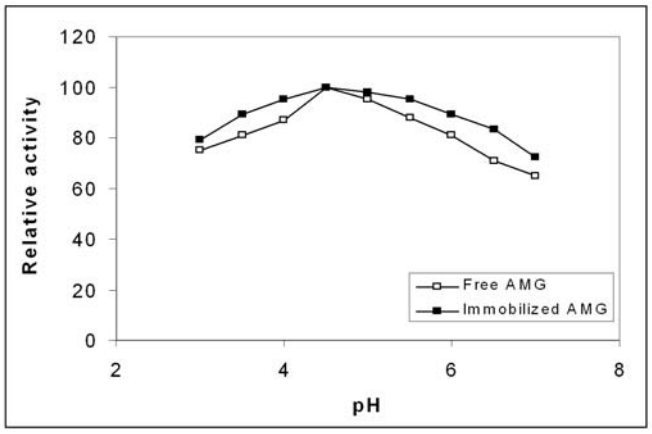


Figure 5. Effects of pH on the activity of free and immobilized AMG. (□) Free enzyme; (■) immobilized enzyme.

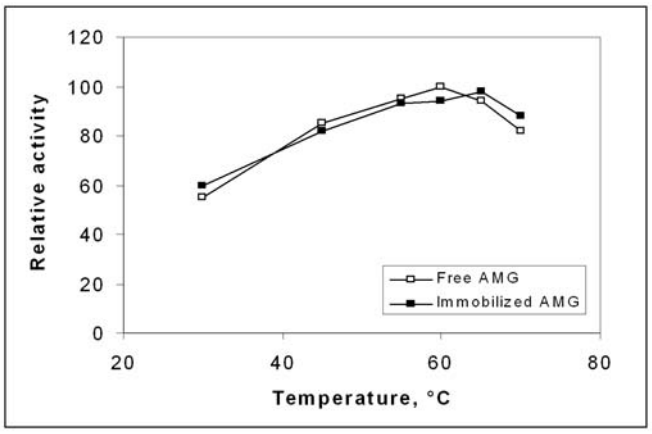


Figure 6. Effects of temperature on the activity of free and immobilized AMG. (□) Free enzyme; (■) immobilized enzyme.

Thermal Stability of Free and Immobilized Enzyme

Enzymes in aqueous solutions are not stable as known. Thermal stability was carried out with the free and immobilized AMG in acetate buffer at 60°C. Figure 7 shows thermal stability of free and immobilized enzyme. The immobilization process increased the heat and denaturation resistance of AMG. After incubation at 60°C for 180 min, the immobilized AMG remained higher activity in comparison with the free one. These results suggest that the thermostability of immobilized enzyme increased considerably as a result of immobilization via adsorption onto the dye-attached hollow fiber [25].

Reuse and stability studies

The desorption experiments were performed by using 1 M NaCl at pH 3.0. The results showed that about 89% of the adsorbed AMG are successfully desorbed. Adsorption-desorption cycles were repeated 5 times by using same hollow fibers. No significant decrease in adsorption capacity was observed. This is an important feature indicating the possibility of reversible immobilization onto hollow fiber. Figure 8 shows the reuseability of the Reactive Brown 10-attached hollow fibers.

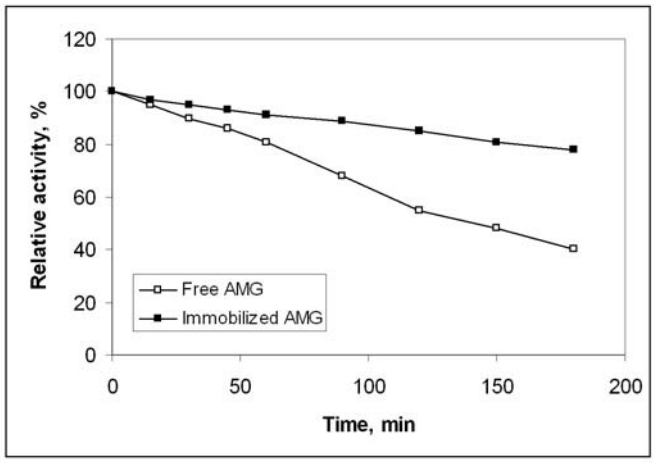


Figure 7. Thermal stability of free and immobilized AMG. (□) Free enzyme; (■) immobilized enzyme. T: 60°C.

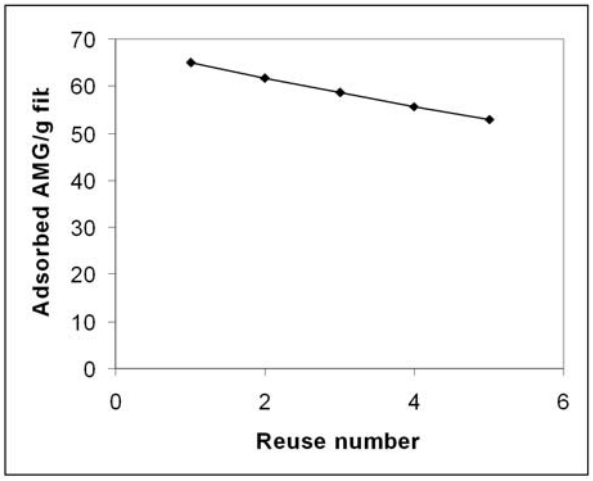


Figure 8. Reuseability of dye-attached hollow fibers. Reactive Brown 10 loading: 18.4 μmol/g; initial AMG concentration: 1 mg/ml; pH: 4.0; T: 25°C.

In order to evaluate the effects of adsorption and desorption conditions on protein structure, fluorescence spectrophotometry was employed. The fluorescence spectra of protein samples obtained from the desorption step were recorded. The fluorescence spectra of native AMG was also taken. The fluorescence spectra of the samples withdrawn from the desorption step were very close to those of native one and no shift of maximum wavelength was detected in the spectrum (Figure 9). It may be concluded that Reactive Brown 10-attached polyamide hollow fibers can be applied for reversible AMG immobilization without causing any conformational changes and denaturation.

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 AMG 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 Reactive Brown 10-attached hollow fibers was observed during this storage period. The free AMG lost its whole activity within 6 weeks. However, the immobilized AMG lost only 8% of its activity during the same period. This result indicated that immobilization process improved the enzyme stability and prevented the active conformation of AMG [33].

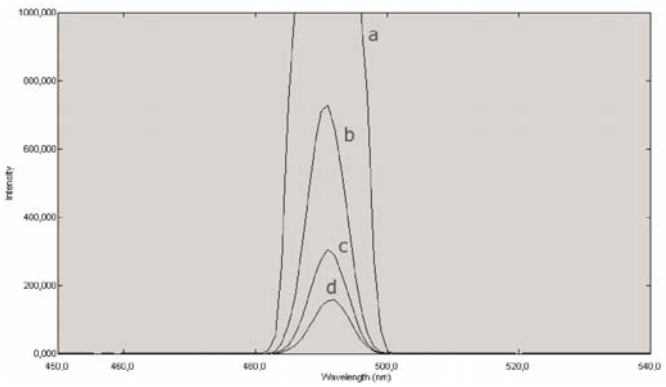


Figure 9. Spectrofluorimeter spectra of (a) denaturated; (b) native; (c) adsorbed; (d) desorbed AMG. Extinction wavelength: 488 nm; emission wavelength: 520 nm.

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 AMG were determined by using soluble starch as a substrate, and the results are shown in Table 1. The K_M value of the immobilized AMG was 1.2-fold higher than that of the free one. The V_{max} value of the free AMG (4.55 μmol mg^{-1} min^{-1}) was found to be higher than that of the immobilized AMG (1.94 μmol mg^{-1} min^{-1}). The change in the affinity of the AMG may come from the structural change that occurred in immobilization. Other reason for this situation may be that AMG should interact with Reactive Brown 10-attached hollow fibers where is closed the active side for starch conversion.

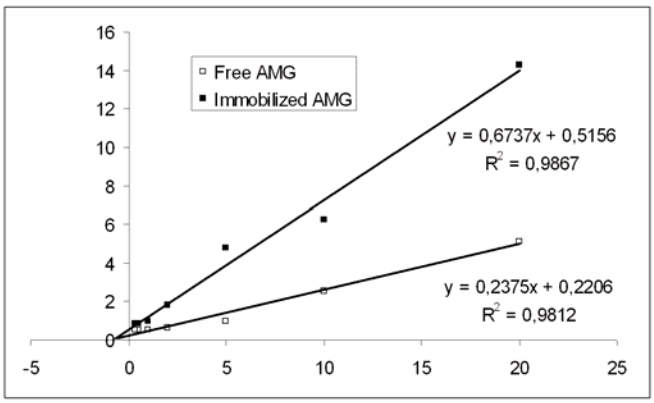


Figure 10. Lineweaver-Burk curves for free and immobilized AMG.

Conclusion

An important limiting factor of enzyme immobilization is the diffusion limitation of the substrate and product. Hollow fiber membranes with their large pores are alternative sorbents for these limitations. The hollow fiber has rough and heterogeneous surface. This large pores reduce diffusional resistance and facilitate mass transfer because of high internal surface area. Triazinyl-based reactive dyes are very popular pseudo-specific ligands for

Table 1. Kinetic parameters for free and immobilized AMG.

Enzyme Type	K_M, mg/mL	V_{max}, μmol mg^{-1} min^{-1}	R^2
Free	1.077	4.533	0.9812
Immobilized	1.307	1.939	0.9867

protein separation and enzyme immobilization due to their binding affinity to the biomolecules. Reactive Brown 10-attached hollow fibers were used for the reversible immobilization of AMG. With the increase in initial AMG concentration, AMG immobilization increased, also. Optimum pH for AMG immobilization was observed at pH 4.0. Much of the activity of immobilized AMG retained over wider ranges of temperature and pH than that of the free enzyme. Desorption of immobilized enzyme was achieved by 1.0 M NaCl solution without any denaturation. Immobilization of enzyme onto dye-attached hollow fibers increased the thermal and storage stability. Reuseability of the dye-attached hollow fibers provides economical advantages for decreasing the cost. Together with these results, we can say that Reactive Brown 10-attached hollow fiber can be used for reversible immobilization of AMG in biotechnological applications.

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