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Alanine Functionalized Magnetic Nanoparticles for Reversible Amyloglucosidase Immobilization

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Supporting Information

ABSTRACT: A hydrophobic group/comonomer *N*-methacryloyl-(L)-alanine (MAAL) containing 2-hydroxyethyl methacrylate (HEMA)-based magnetic hydrophobic nanoparticles was synthesized by using a microemulsion polymerization technique for immobilization of amyloglucosidase (AMG). The magnetic hydrophobic nanoparticles with an average diameter of 79 nm were characterized with Fourier transform infrared spectroscopy, transmission electron microscopy, zeta-size analysis, and electron spin resonance spectroscopy. Hereby, the effecting factors such as initial concentration of AMG, pH, and temperature on the immobilization were investigated. After determining the optimum immobilization conditions, the kinetic constants (K_M and V_{max}) and properties of free and immobilized AMG were investigated in batch studies. The maximum AMG adsorption capacity of the magnetic hydrophobic nanoparticles was 294.42 mg/g at pH 3.0. The optimum pH and temperature for free and immobilized AMG were found to be pH 5.0 and 60 °C. The K_M value for immobilized AMG was higher than that of the free enzyme, whereas the V_{max} value was lower for the immobilized AMG preparations.

1. INTRODUCTION

Amyloglucosidase or glucoamylase (EC 3.2.1.3) is an industrial enzyme which hydrolyzes α-(1 → 4) and α-(1 → 6) glucosidic bonds of starch from the nonreducing ends. It is used for the production of glucose from starch[1] and is exploited as a sweetener, substrate producing alcohol for microorganisms, as a foundation of energy in health drinks, and the production of high fructose corn syrup.[2] It is also used in huge amounts for the hydrolysis of malto-oligosaccharides into glucose, for different syrups needed in foods and soft drinks, and also in the fuel ethanol industry.[3] The use of a free enzyme causes various difficulties in applications such as stability, recovery from reaction system, reusability, separation from substrates, and products and process cost. A solution to overcome these drawbacks is to immobilize the enzyme on the solid support. Enzyme immobilization obtains a multifunctional physicochemical part that permits the reuse of enzymes, simplifies the reactor design, prevents product contamination, and develops stability or tolerability.[4] Immobilized enzymes rather than free enzymes have been extensively used in several industries such as food, fine chemical, and pharmaceutical industries owing to their many superior features such as reuse, ease of separation of the output from reaction mixture, ease of recovery of the enzyme, and development in enzyme stability. There are several important factors which effect immobilization processes, such as carrier features (particle diameter and pore size), enzyme stability, and immobilization parameters (pH, temperature, protein concentration).[5] An extensive diversity of approaches has been used in the immobilization of enzymes, such as chromatography, adsorption, entrapment, cross-linking, and covalent attachment.[6] These methods used of operation of enzyme immobilization are selected to provide the highest retention of enzyme activity, stability, and durability. From this point of view, hydrophobic interaction chromatography (HIC) is one of the most suitable methods to immobilize enzyme because the stability of the enzyme activity is retained after immobilization. Also the enzyme and support material are reusable due to the reversibility of the method. HIC is the basis of hydrophobic interactions between nonpolar and hydrophobic parts of target molecules and polymeric matrices that carry immobilized hydrophobic ligands.[7−10] Efficient adsorption of biomolecules on the hydrophobic supports in the aqueous solution is generally based on van der Waals interactions.[11] Proteins or enzymes that have hydrophobic residues are capable of interacting with and binding to the hydrophobic groups found on the solid support through the separation process.[12] The combination of an enzyme and magnetic nanoparticles has the advantages of ease of production and separation and being nontoxic and biocompatible.[13,14] Magnetic nanoparticles have already been known for several decades and got popular as immobilizing supports because of their magnetic responsibility, chemically modifiable surface, large enzyme loading capacity, large specific surface area, low mass transfer resistance, high binding capacity, and good reusability.[15,16] In recent years, they had many important applications such as cell labeling,[17] magnetic resonance imaging contrast agent,[18] enzyme immobilization,[19] protein separations,[20] targeted drug delivery,[21] filtration,[22] and so on.

This article shows novel magnetic hydrophobic nanoparticles as a support to reversibly immobilize AMG. For this aim, 2-hydroxyethyl methacrylate (HEMA)-based magnetic hydrophobic nanoparticles with a hydrophobic group/comonomer *N*-methacryloyl-(L)-alanine (MAAL) were synthesized by using a microemulsion polymerization technique in the presence of magnetite (Fe_3O_4) nanopowder. Because alanine has a small

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nonpolar side chain, we have selected it to introduce a slightly hydrophobic functionality into the polymeric backbone. The slight hydrophobic character allows us to immobilize the enzyme molecules onto the nanoparticle in a reversible manner. The magnetic hydrophobic nanoparticles were characterized with Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), zeta-size analysis, and electron spin resonance (ESR) spectroscopy. Hereby, the effecting factors such as initial concentration of AMG, pH, and temperature on the immobilization were investigated. After determining the optimum immobilization conditions, the kinetic constants (K_M and V_{max}) and properties of free and immobilized AMG were investigated in batch studies.

2. EXPERIMENTAL SECTION

2.1. Materials. Amyloglucosidase (EC 3.2.1.3) from *Rhizopus mold* (11600 U/g solid), magnetite nanopowder (Fe_3O_4, diameter, 5−50 nm), poly(vinyl alcohol) (PVA), sodium dodecyl sulfate (SDS), ammonium persulfate (APS), sodium bicarbonate ($NaHCO_3$), sodium bisulfite ($NaHSO_3$), 2-hydroxyethyl methacrylate (HEMA), and ethylene glycol dimethacrylate (EGDMA) were bought from Sigma Ltd. (St. Louis, USA). *N*-Methacryloyl-(L)-alanine (MAAL) monomer that was in advance synthesized and characterized was provided from Nanoreg Ltd. (Ankara, Turkey). The others were of analytical grade and bought from Merck AG (Darmstadt, Germany) and the water used in the experiments was purified using a Barnstead (Dubuque, IA) purifier.

2.2. Preparation of Magnetic Hydrophobic Nanoparticles. The polymerization process consisting of two aqueous phases was carried out in a microemulsion system. In short, the first aqueous phase consisted of PVA (0.187 g) as a stabilizer, SDS (28.3 mg) as a surfactant to form emulsion, and $NaHCO_3$ (23.4 mg) as an agent to adjust pH in 10 mL of water. The second aqueous phase consisted of PVA (0.1 g) and SDS (0.1 g) in 200 mL of water. The monomer phase, consisting of MAAL (260 mg) as a hydrophobic functional monomer, HEMA (1.0 mL) as a basic monomer in the main polymeric backbone, and EGDMA (2.1 mL) as a cross-linker, was mixed with the first phase. The mixture was then homogenized at 25000 rpm for approximately 10 min to obtain a microemulsion. This last mixture was quite slowly added to the second phase, and Fe_3O_4 nanoparticles (75 mg) were retained if magneticity was desired. The final mixture was transferred to a polymerization system. After the addition of $NaHSO_3$ (115 mg) and $(NH_4)_2S_2O_8$ (126 mg) as initiators, the polymerization system was carried out at 40 °C for 24 h while the reaction mixture was mechanically stirred at 500 rpm. The polymerized solution comprising magnetic hydrophobic nanoparticles was centrifuged at 14500 rpm for 1.5 h (Eppendorf Mini Spin Plus). The obtained magnetic hydrophobic nanoparticles were completely washed with ethanol and ultrapure water three times. The washed magnetic hydrophobic nanoparticles were dispersed in ultrapure water by using an ultrasound sonicator and kept at 4 °C until use.

2.3. Characterization of Magnetic Hydrophobic Nanoparticles. The presence of magnetite in the magnetic hydrophobic nanoparticles was investigated with ESR spectroscopy (Varian E-L X band spectrometer, USA) at 25 °C in a magnetic field range of 1000−5000 G. The ESR spectrum was shown as the first derivative of absorption curve by altering the magnetic field. The morphology of the magnetic hydrophobic nanoparticles was determined with TEM (TEM, FEI Company Tecnai). The FTIR spectrum was recorded for dried magnetic hydrophobic nanoparticles by a FTIR spectrophotometer (PerkinElmer, Spectrum One, USA). The range of 4000 cm^{-1}−600 cm^{-1} was selected for the wavenumber range.

Average particle size and size distribution of the magnetic hydrophobic nanoparticles were determined by NanoZetasizer (NanoS, Malvern Instruments, London, England). The results were a mean of three measurements.

2.4. AMG Immobilization Studies. AMG immobilization on magnetic hydrophobic nanoparticles was tested at several pH values (2.0−7.0) adjusted by using appropriate buffer couples, phosphate, and acetate. Initial AMG concentration was 1.0 mg/mL. The adsorption experiments were conducted at 25 °C for 2 h while stirring continuously. At the end of this period, the enzyme adsorbed magnetic hydrophobic nanoparticles were removed from the enzyme solution and washed with the same buffer three times. The amount of adsorbed AMG was calculated as

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

Here Q refers the quantity of adsorbed AMG on a unit mass of the magnetic hydrophobic nanoparticles (mg/g); C_i and C_f refer the concentrations of AMG in the initial solution and in the final solution, (mg/mL); V refers the volume of the aqueous phase (mL); and m refers the mass of the magnetic hydrophobic nanoparticles used (g). To calculate adsorption capacities of magnetic hydrophobic nanoparticles, the concentration of AMG in the medium was altered between 0.25 mg/mL to 3.0 mg/mL. To examine the thermal stability, AMG adsorption on the magnetic hydrophobic nanoparticles was tested at 4−60 °C in pH 3.0 at 1.0 mg/mL initial AMG concentration for 2 h. Reusability is the one feature mostly required for cost-friendly adsorbents. AMG molecules were eluted to show reusability of the magnetic hydrophobic nanoparticles. The magnetic hydrophobic nanoparticles were removed from the desorption medium, washed with pH 10.0 Tris-HCl in 1.5 h and were then used over and over in enzyme immobilization. The reusability of magnetic hydrophobic nanoparticles is given in the Supporting Information.

2.5. Activity Measurements of Free and Immobilized AMG. Activities of free and immobilized AMG were performed by the addition of 0.5 mL of diluted free and immobilized AMG in 0.5 mL of acetate solution (0.1 M, pH 5.0), using 1.0 mL of soluble starch solution which involved 1.0% (w/v) soluble starch gelatinized in water (15 min, 100 °C, continuous mixing) as the substrate. The reaction was ended by adding sodium hydroxide solution (0.1 M, 0.5 mL) after incubation at 60 °C for 15 min, and then the amount of glucose in the reaction mixture was defined by using dinitrosalicylic acid (DNS) method.[23] One unit of AMG activity is defined as the quantity of enzyme which produces 1.0 μmol of glucose from soluble starch per minute at pH 5.0 at 40 °C.[24] The activity assays were carried out over the pH range of 2.0−8.0, temperature range of 10−60 °C, and substrate concentration range of 1.0−5.0 mg/mL to determine the pH, temperature, and substrate concentration profiles for free and immobilized AMG. The relative activity of the immobilized AMG is calculated by using the accepted maximum activity as 100%.

2.6. Thermal Stability. The thermal stability of free and immobilized AMG was determined by a measurement of the residual enzymatic activity of the AMG in an acetate buffer (pH 5.0) for 210 min at two different temperatures (50 and 60 °C).

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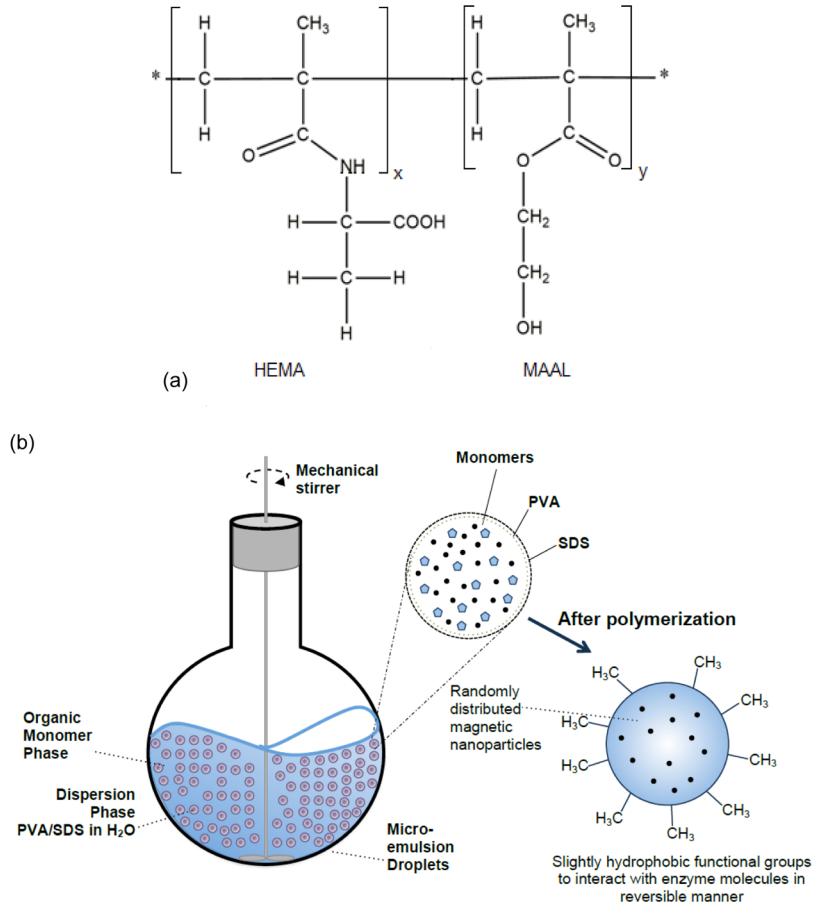

Figure 1. Molecular formula of the PHEMAAL magnetic nanoparticle (a). Schematic representation of nanoparticle production and enzyme immobilization steps (b).

After every 30 min time interval, a sample was removed and assayed for enzymatic activity.

2.7. Storage Stability. This experiment was conducted to determine the stabilities of free and immobilized AMG preparations that are stored in an acetate buffer (pH 5.0) for 60 days. The activity of each preparation was expressed as a percentage of its residual activity compared to the initial activity.

3. RESULTS AND DISCUSSION

3.1. Properties of Magnetic Hydrophobic Nanoparticles. The possible molecular formula of the magnetic hydrophobic nanoparticles is givin in Figure 1. To characterize chemical and physical properties of magnetic hydrophobic nanoparticles produced in this study, several methods were applied. FTIR, ESR spectra and zeta-size measurements are given in Figure 2. As seen in this figure, the most important adsorption band at 1635 cm^{-1} represents amide I (due to C═O stretching), respectively. The FTIR bands observed at 1455 and 2957 cm^{-1} were assigned to the aliphatic stretchings of C−H, respectively. Other bands for the spectrum were the −OH band at 3413 cm^{-1} and the carbonyl band by reason for ester and amide groups at 1720 cm^{-1}. The structure of the magnetite is demonstrated by peaks at around 751 cm^{-1} due to Fe−O groups.

The presence of magnetism in the hydrophobic nanoparticle structure was also verified by ESR. A peak of magnetite Fe_3O_4 fine particle was detected in the ESR spectrum (Figure 2B). In this spectra, the internal magnetic field change of hydrophobic nanoparticles was clearly seen with respect to the external magnetic field variation. As seen in this figure, the resonance magnetic field ($\mathbf{H}_r$) value for these nanoparticles was measured as 3270 G. Also, this curve was very symmetrical which indicates that the magnetic component could adsorb/release an external magnetic field easily. This feature is very helpful for designing and using the magnetic materials in biomedical and biochromatographic applications.[20] In addition, the response intensity to an external magnetic field is a very significant parameter when designing a magnetically stabilized reactor with immobilized enzyme systems for the magnetic filtration systems. The value of this magnetic field is a function of the flow velocity, particle size, and magnetic susceptibility of the solids to be displaced.[25] As seen from Figure 2C, the average particle size and size distribution of the magnetic hydrophobic nanoparticle were about 79 nm with a polydispersity index measured as 0.130. The final characterization was performed using TEM imaging and also indicated that the sizes of magnetic hydrophobic nanoparticles were approximately 79 nm. As seen in Figure 3, it can be clearly observed that the magnetic hydrophobic nanoparticles have a dark center region in contrast to a pale edge. The magnetic attraction, hydrogen bonding, and hydrophobic interaction might cause of the aggregation of some nanoparticles. Also, drying conditions may cause those aggregation problems.

3.2. Optimization of AMG Immobilization onto Magnetic Hydrophobic Nanoparticles. The batch experiments were performed in 2 mL of eppendorf placed in a

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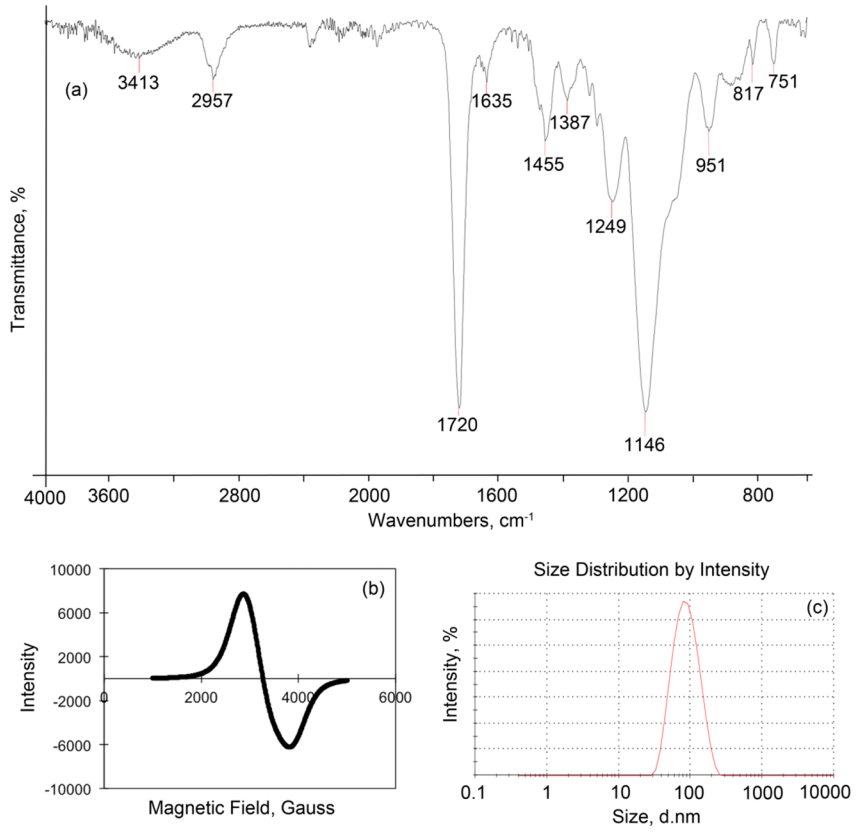

Figure 2. FTIR (a) and ESR (b) spectra and size distribution (c) of magnetic hydrophobic nanoparticles.

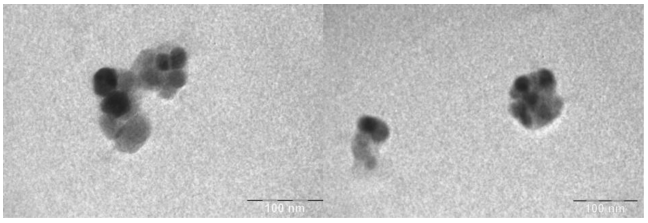

Figure 3. TEM images of magnetic hydrophobic nanoparticles.

rotator. 1.5 mL of AMG solution was interacted with 0.5 mL of nanoparticle suspension (concentration: 1.0 mg/mL) for each run while rotating them at 20 rpm. The AMG immobilization efficiency of magnetic hydrophobic nanoparticles at different pH's in the range of 2.0 to 7.0 is given in Figure 4. According to the results, the maximum amount of bound AMG is 294.42 mg/g at pH 3.0 that is accepted as optimal value and used for all immobilization processes. The results mainly depend on the hydrophobic interaction between comonomer (MAAL) and target enzyme (AMG). AMG has an isoelectrical point around 3.6 and functional ligand alanine has pK_a values as 2.35/9.69 (pI 6.02). At pH 3.0 although both groups have slightly positive charges, this result may depend on the conformation and charge distribution through the AMG molecules.

The effect of AMG concentration on immobilization efficiency was studied at different AMG concentrations in the range of 0.25−3.0 mg/mL at pH 3.0. It can be seen from Figure 5 that the adsorption capacity of magnetic hydrophobic nanoparticles increased with an increase in AMG concentrations from 0.25 to 3.0 mg/mL. For optimum conditions, 1.0

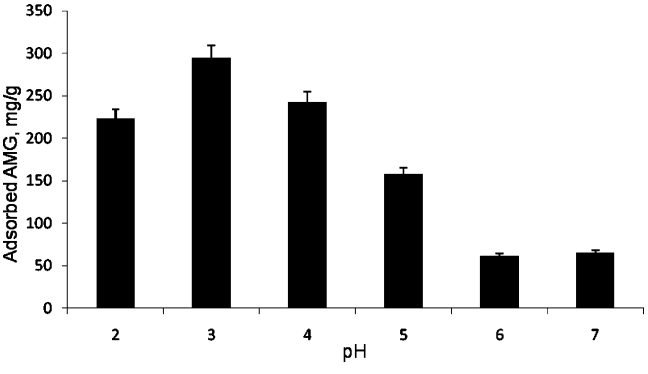

Figure 4. Effect of pH on AMG immobilization onto magnetic hydrophobic nanoparticles.

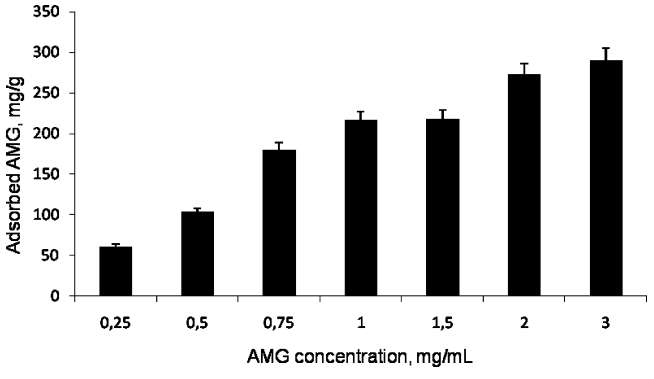

Figure 5. Effect of AMG concentration on AMG immobilization onto magnetic hydrophobic nanoparticles.

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mg/mL AMG concentration was chosen for further experiments. An increase in AMG concentration in the adsorption medium led to an increase in adsorption efficiency, but this leveled off at an AMG concentration of 2.0 mg/mL. The maximum AMG adsorption capacity of the magnetic hydrophobic nanoparticles was determined as 294.42 mg/g at pH 3.0. Although this increase could be due to the specific interactions between AMG and magnetic hydrophobic nanoparticles, the main reason for this behavior could be explained by concentration differences between solid and liquid phases. This difference caused a gradient force that drove the AMG through a solid matrix, and AMG molecules easily interacted with the hydrophobic group/comonomer (MAAL) and bound on the nanoparticle specifically.

The effect of temperature on immobilization efficiency was also studied at different temperatures in the range of 4−60 °C at pH 3.0. As seen from Figure 6, the adsorption capacity of

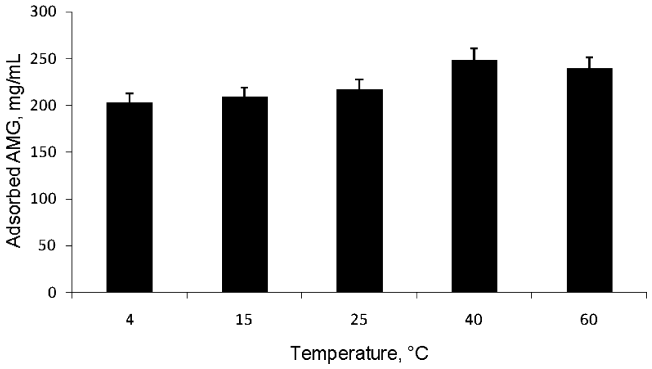

Figure 6. Effect of temperature on AMG immobilization onto magnetic hydrophobic nanoparticles.

magnetic hydrophobic nanoparticles increased until 40 °C. Then, a small decrease in the adsorption capacity was observed. This adsorption tendency may be explained by temperature dependency of hydrophobic interactions. With increasing temperature, the water molecules around hydrophobic surfaces (hydrophobic residues on biomolecules and hydrophobic comonomers) were more random. So, the interaction between hydrophobic counter sites were enhanced by the increasing temperature, which caused an increase in adsorption capacity. In addition, inner hydrophobic residues in the biomolecules were more accessible at higher temperature, which also promotes hydrophobic interactions and causes the increase in adsorption capacity.

3.3. Effect of pH, Temperature, and Substrate Concentration on Activity. An important parameter for successful immobilization is the binding capacity of the magnetic hydrophobic nanoparticles, so the immobilization method was optimized in terms of immobilization pH, temperature, and enzyme concentration. Herein, the effects of pH, temperature, and substrate concentration onto enzyme activity were also evaluated to discuss the feasibility of hydrophobic magnetic nanoparticles as an enzyme carrier. The activity of the immobilized enzyme with respect to pH variation depends on the characteristics of the nature of the enzyme, the carrier, and the method of immobilization. The effect of pH on the activity of free and immobilized AMG in glucose hydrolysis was determined in the pH range from 2.0 to 8.0. As seen in Figure 7 the maximum relative activity for free and immobilized AMG was observed at pH 5.0. The difference may depend on the method of immobilization as well as the structure and charge of the matrix and low electrostatic charge distribution on both enzyme support and surrounding substrate solution.

As seen in Figure 8, the maximum activities of free and immobilized AMG were observed at 60 °C, respectively. The

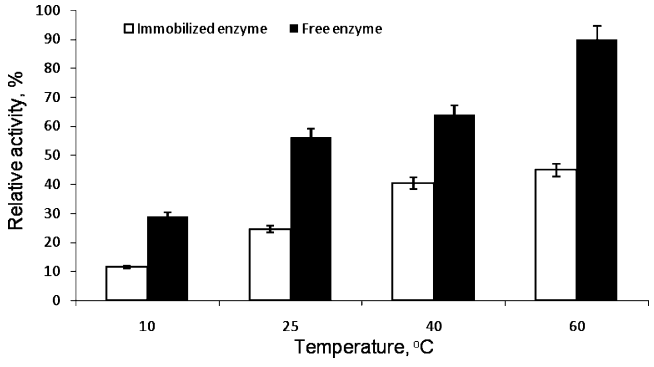

Figure 8. Effect of temperature on the relative activity of the immobilized and free AMG.

raise in optimum temperature gave rise to the altering physical and chemical features of the AMG. This result could be explained by creation of conformational limitations on the

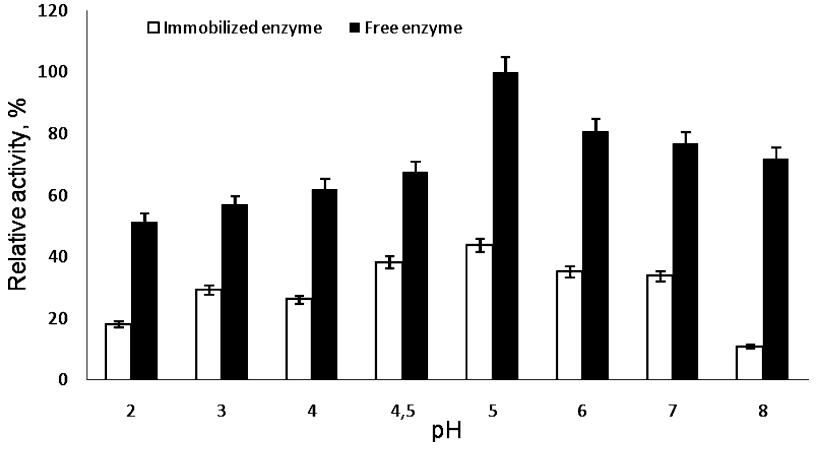

Figure 7. Effect of pH onto the relative activity of the free and immobilized AMG.

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AMG movements as a result of formation of noncovalent bonds between AMG and the support. As seen in Figure 9, the

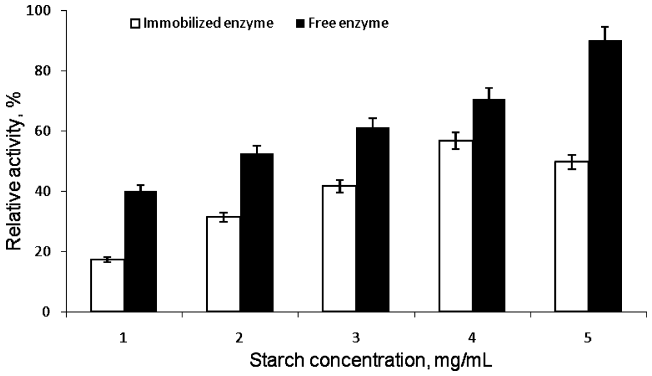

Figure 9. Effect of substrate concentration on the relative activity of the immobilized and free AMG.

increase in substrate concentration caused increase in enzyme activities for both enzyme systems as expected. The results confirmed that immobilization process did not inhibit enzyme activity although causing some decrease in activity of immobilized AMG. As a conclusion, the activity of AMG immobilized onto magnetic nanoparticles directly depended on these parameters including pH, temperature, and substrate concentration. By adjusting the external parameters, it is possible to obtain optimal enzyme activity as well. Significant enhancement in the enzyme activity with the immobilization process revealed that steric hindrances around the active site, diffusion of substrate molecules into the active site, and conformation changes causing denaturation of the enzyme molecules did not occur during the immobilization process, meanwhile stability, active structural conformation, and proper orientation onto magnetic nanoparticles were achieved. Also, because of the excellent feature of high specific surface area-to-volume ratio of the nanoparticle, the enzyme activity was obviously improved.

3.4. Thermal Stability. The thermal stability of free and immobilized AMG is shown in Figure 10. The graph shows that a lower rate of thermal inactivation was observed for immobilized AMG than for free AMG on the magnetic hydrophobic nanoparticles. At 60 °C, the free AMG retained 35% of its initial activity after 210 min of heat treatment, while

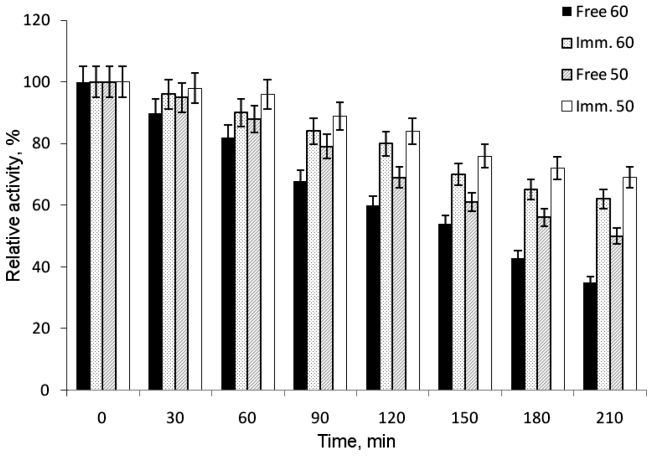

Figure 10. Thermal stability of free and immobilized AMG.

immobilized AMG showed important resistance to thermal inactivation. It retained 62.0% of its initial activity after the same period. At 50 °C, the free AMG retained 50% of its initial activity after 210 min of heat treatment, while immobilized AMG showed important resistance to thermal inactivation. It retained 69% of its initial activity after the same period. These results showed that the thermostability of immobilized AMG became a significant challenge at higher temperature. On the basis of these observations, it can be determined that magnetic hydrophobic nanoparticles form a hydrophobic group in a hydrophilic network which causes higher thermal stability compared to that of its free AMG.

3.5. Storage Stability. Free and immobilized AMG samples were stored in pH 5.0, and activity measurements were performed for a period of 60 days (Figure 11). Although

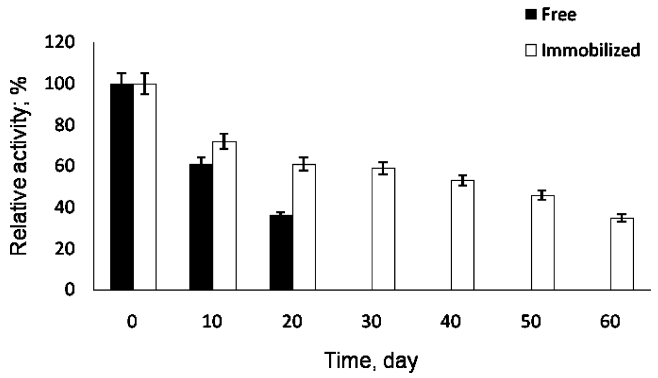

Figure 11. Storage stability of free and immobilized AMG.

free AMG lost all its initial activity within 30 days, immobilized AMG lost only 40% activity during the same period. Adsorption definitely appears to hold AMG in a stable position in comparison to the free AMG. By this way, immobilized AMG could preserve initial activity more than 40% during the storage period which means that the shelf-life of the enzyme has been extended and the cost would be decreased.

3.6. Kinetic Constants. Two kinetic constants, the Michaelis constant, K_M (mg/mL) and the maximum reaction rate, V_{max} (U/mg) for free and immobilized AMG were determined with Lineweaver–Burk plots. These constants exhibit the effective characteristics of the AMG and depend on both partition and diffusion effects.[26] Water-soluble starch was used as a model substrate. The K_M values for free and immobilized AMG were 1.56 (mg/mL) and 3.22 (mg/mL), respectively. This result shows that the affinity of AMG against its substrate was partially decreased by adsorption. The V_{max} value of free (11.1 U/mg) was found to be lower than that of immobilized AMG (11.2 U/mg). As seen in Table 1, K_M and V_{max} values were changed after the adsorption of AMG onto the magnetic hydrophobic nanoparticles, but the changes were quite low. The change in the affinity of the AMG to its substrate is certainly induced by several factors, such as protein conformational changes induced by the support, steric

Table 1. Properties of Free and Immobilized AMG onto the Magnetic Hydrophobic Nanoparticles

type of enzyme	K_M (mg/mL)	V_{max} (U/mg)	R^2
free	1.56	11.1	0.982
immobilized	3.22	11.2	0.932

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hindrance, structural changes in the AMG introduced by the immobilization procedure, lower accessibility of the large substrate to the active site of the immobilized AMG and diffusion limitation of the protein introduced by immobilization. But, the V_{max} values were almost the same for free and immobilized AMG. This result confirms that the diffusion limitation is low and not effective in our magnetic hydrophobic nanoparticles,[27−29] and the change in the affinity does not affect the enzymatic conversion performance of AMG, and these changes may occur far away from the active site.[30]

4. CONCLUSION

In recent years, there has been a huge interest in magnetic nanoparticles in different areas, especially in diagnostics and therapy, because of their original and significant magnetic features and their talent to interact with several biomolecules. The easy separation of an enzyme from a reaction mixture due to magnetic features may supply cost-effective benefits for various biotechnological applications. In this paper, we advanced a novel and simple process for the synthesis of magnetic hydrophobic nanoparticles. The process immobilizes the enzyme molecules onto the nanoparticle in a reversible manner, which causes important advantages with respect to some parameters such as reusability, reproductivity, ease of use, and cost. Also, this method was created to produce functional nanoparticles in narrow size distribution, which is an important parameter in the acquisition of reproducible adsorption dynamics in an enzyme immobilization process. Herein, a comparison of the obtained results showed that the activities of both the free and immobilized AMG as a function of pH were optimized at the same pH. The optimum temperature and starch concentration for free and immobilized AMG were also examined. Thermal stability results showed that immobilized AMG had important resistance at high temperatures and also lost only 40% of activity, while free AMG lost all its activity within the same time.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 and Figure S2 showing that the magnetic hydrophobic nanoparticles could be repeatedly used in AMG immobilization without significant losses in the initial adsorption capacities and AMG activities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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