Novel Magnetic Nanoparticles for the Hydrolysis of Starch with *Bacillus licheniformis* α-Amylase

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ABSTRACT: Novel magnetic nanoparticles with an average size of 350–400 nm with *N*-methacryloyl-(L)-phenylalanine (MAPA) as a hydrophobic monomer were prepared by the surfactant-free emulsion polymerization of 2-hydroxyethyl methacrylate, MAPA, and magnetite in an aqueous dispersion medium. MAPA was synthesized from methacryloyl chloride and L-phenylalanine methyl ester. The specific surface area of the nonporous magnetic nanoparticles was found to be 580 m^2/g. Magnetic poly[2-hydroxyethyl methacrylate–*N*-methacryloyl-(L)-phenylalanine] nanoparticles were characterized by Fourier transform infrared spectroscopy, electron spin resonance, atomic force microscopy, and transmission electron microscopy. Elemental analysis of MAPA for nitrogen was estimated as 4.3×10^{-3} mmol/g of nanoparticles. Then, magnetic nano-poly[2-hydroxyethyl methacrylate–*N*-methacryloyl-(L)-phenylalanine] nanoparticles were used in the adsorption of *Bacillus licheniformis* α-amylase in a batch system. With an optimized adsorption protocol, a very high loading of 705 mg of enzyme/g nanoparticles was obtained. The adsorption phenomena appeared to follow a typical Langmuir isotherm. The inverse of enzyme affinity for free amylase (181.82 mg/mL) was higher than that for immobilized enzyme (81.97 mg/mL). Storage stability was found to increase with adsorption. It was observed that the enzyme could be repeatedly adsorbed and desorbed without a significant loss in the adsorption amount or enzyme activity. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 2574–2581, 2012

Key words: adsorption; enzymes; nanoparticle

INTRODUCTION

In recent years, magnetic nanoparticles have received great attention for such biotechnological applications as sorting or separating cells, proteins, and DNA;[1–3] medical diagnosis;[4] and controlled drug delivery.[5] Magnetic nanoparticles have several advantages as support materials: (1) they have good mass-transfer properties, (2) they allow easy recovery and reuse of immobilized enzymes, and (3) they have a large specific surface area, which can be used for the immobilization of enzymes.[6] Using the enzyme-immobilized magnetic nanoparticles makes the recovery of enzyme more effective and easy, as there is no need to apply high-speed centrifugation,[7] but a moderate magnetic field causes almost complete and easy removal of enzyme from the reaction system. Therefore, enzyme immobilized onto the surface of magnetic nanoparticles is preferred to native enzyme for the purpose of using industrial applications nowadays.[8–11]

Compared with chemical methods for the immobilization of enzymes onto carriers, physical methods, especially adsorption, may have a higher commercial potential because it they are simpler and less expensive and can retain a high catalytic activity.[12] Additionally, noncovalent adsorption techniques, such as hydrophobic adsorption of the enzyme on a hydrophobic adsorbent, may be a good option because adsorption is very simple, produces very little work, and takes little time and the supports may be reused after desorption of the enzyme and, by this way, reduce the final price and generate fewer residues. However, scarce works were found that refer to reversible hydrophobic adsorption.[13–15]

Among starch hydrolyzing enzymes that are produced on an industrial scale, α-amylases (EC 3.2.1.1) are of considerable commercial interest. α-Amylases randomly hydrolyze α-1,4-glycosidic linkages in starch or its hydrolysis products. The sale of amylolytic enzymes accounted for almost US$25 million worldwide.[16] α-Amylases are an important industrial bulk enzyme for the food processing industry. They find wide applications in the conversion of starch into dextrins, in the baking industry for dough modification, and in laundry aids.[17] Thermostable α-amylases have had many commercial applications for several decades. The enzymes are used in the textile and paper industries, starch liquefaction, food, adhesives, and sugar production.[18] *Bacillus subtilis*, *Bacillus amyloliquefacines*, *Bacillus licheniformis*, and

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Aspergillus oryzae are very important α-amylase sources because their enzymes are highly thermostable.[19]

Highly thermostable *B. licheniformis* α-amylase is widely used in biotechnology for the initial steps of starch degradation at temperatures up to 110°C. The primary substrate for *B. licheniformis* α-amylase is starch, but similar compounds, such as glycogen or smaller oligosaccharides, are readily converted as well. Furthermore, *B. licheniformis* α-amylase, like other α-amylases, shows pronounced transglycosylation activity and is, therefore, also used in synthetic chemistry for the enzymatic synthesis of oligosaccharides.[20]

Immobilized α-amylase has been widely studied with different solid support materials and immobilization methods to optimize its catalytic features. For example, poly(hydroxyethyl methacrylate)-based porous membranes,[19] glass beads,[21] poly(ethylene glycol dimethacrylate-n-vinyl imidazole)–Cu^{2+} hydrogel,[22] Amberlite MB 150 and chitosan beads,[16] superporous CELBEADS,[23] cyclic carbonate bearing hybrid materials,[24] and a recently developed magnetic nanostructure matrix[8] have been used for α-amylase immobilization as a support materials.

In this study, novel magnetic nano-poly[2-hydroxyethyl methacrylate–*N*-methacryloyl-(L)-phenylalanine] [mag-nano-poly(HEMA–MAPA)] nanoparticles were prepared and used for efficient adsorption of *B. licheniformis* α-amylase for the first time. In the first part, *N*-methacryloyl-(L)-phenylalanine (MAPA) was synthesized by the reaction of methacryloyl chloride and L-phenylalanine methyl ester. Then, mag-nano-poly(HEMA–MAPA) nanoparticles were produced by the surfactant-free emulsion polymerization of 2-hydroxyethyl methacrylate (HEMA) and MAPA in the presence of magnetite (Fe_3O_4) nanopowder. The mag-nano-poly(HEMA–MAPA) nanoparticles were characterized with Fourier transform infrared (FTIR), transmission electron microscopy (TEM), atomic force microscopy (AFM), elemental analysis, and electron spin resonance spectrophotometry. After that, α-amylase adsorption properties of the mag-nano-poly(HEMA–MAPA) nanoparticles from aqueous solutions were investigated at different experimental conditions.

EXPERIMENTAL

Materials

α-Amylase (*B. licheniformis*, EC 3.2.1.1), starch, 3,5-dinitrosalicylic acid, phenylalanine methyl ester, methacryloyl chloride, and Fe_3O_4 nanopowder (average diameter = 20–50 nm) were supplied by Sigma Chemical Co. (St. Louis, MO). HEMA and ethylene glycol dimethacrylate (EGDMA) were obtained from Fluka AG (St. Gallen, Switzerland). Commercial HEMA contains residual methacrylic acid and crosslinkers due to fabrication processes. The polymerization inhibitor 4-methoxyphenol also needs to be removed. HEMA and EGDMA were purified and distilled under reduce pressure (0.01 mbar, 70°C). The monomers were stored at 4°C until use. Poly(vinyl alcohol) (molecular weight = 100,000, 98% hydrolyzed) was obtained from Aldrich. All other chemicals were guaranteed analytical-grade, commercially available reagents and were used without further purification. The water used throughout this work was produced with a Barnstead ROpure LP reverse osmosis unit (Dubuque, IA) with a high-flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead 3804 NANOPURE organic/colloid removal and ion-exchange packed-bed system.

Synthesis of MAPA

Phenylalanine (5.0 g) and sodium nitrite ($NaNO_2$, 0.2 g) was dissolved in a potassium carbonate solution (30 mL, 5% w/v). This solution was cooled down to 0°C, 4 mL of methacryloyl chloride (density = 1.07 g/mL) was poured slowly into it under a nitrogen atmosphere, and then, the resulting solution was stirred magnetically at room temperature for 2 h at 100 rpm. At the end of this chemical reaction period, the pH of this solution was adjusted to pH 7.0, and then, the product was extracted with ethyl acetate. The aqueous phase was evaporated in a rotary evaporator. The residue (MAPA) was crystallized from ether and cyclohexane.[13]

Preparation of the mag-nano-poly(HEMA–MAPA) nanoparticles

Magnetic poly[2-hydroxyethyl methacrylate–*N*-methacryloyl-(L)-phenylalanine] [mag-poly(HEMA–MAPA)] nanoparticles were produced by surfactant-free emulsion polymerization. For the synthesis of mag-nano-poly(HEMA–MAPA), the following experimental procedure was applied: 0.5 g of poly(vinyl alcohol) was dissolved in 45 mL of deionized water and added to a glass sealed polymerization reactor. Then, 0.6 mL of HEMA, 0.3 mL of EGDMA, and 80 μL of MAPA comonomer were added to this solution, and the solution was slowly shaken for 30 s. Fe_3O_4 particles (0.5 g) and potassium peroxodisulfate (0.0198 g in 45 mL) were added to the reactor, and the process was conducted at 70°C for 7 h. After completion of the polymerization, the reactor contents were cooled to room temperature. Nanoparticles were cleaned by washing with ethanol and water several times to remove the unreacted monomers. For this purpose, the nanoparticles were precipitated and collected with the help of a centrifuge

at 18,000*g* for 1 h and resuspended in ethanol and water several times. After that, the mag-poly (HEMA–MAPA) nanoparticles were further washed with deionized water. Poly(2-hydroxyethyl methacrylate) nanoparticles were produced by same formulation without MAPA comonomer.

Characterization of the mag-nano-poly(HEMA–MAPA) nanoparticles

The characteristics functional groups of mag-nano-poly(HEMA–MAPA) nanoparticles were analyzed with an FTIR spectrophotometer (Varian FTS 7000, Palo Alto, CA). The samples were prepared by mixing with approximately 100 mg of dry, powdered KBr (IR grade, Merck, Dramstadt, Germany) and pressed into a pellet form.

The surface morphology of the magnetic beads was examined by TEM and AFM. For TEM, the dispersion of nanoparticles was dropped onto a formvar-coated copper grid and dried at room temperature. The dried nanoparticles were then imaged with TEM (FEI Co., Tecnai, G2 Spirit, Biotwin, Oregon, USA, 20–120 kV). For AFM, the magnetic nanoparticles were precipitated and covered on a glass plate (1.0×1.0 cm^2). Dried magnetic nanoparticles was mounted in an atomic force microscope (MMSPM Nanoscope IV, Digital Instruments) and scanned with tapping mode.

An amount of MAPA incorporated into the mag-nano-poly(HEMA–MAPA) nanoparticles was evaluated with an elemental analysis instrument (CHNS-932, Leco, St. Jaseph, MI) by nitrogen stoichiometry.

The surface area of the mag-nano-poly(HEMA–MAPA) nanoparticles was calculated with the following expression:

$$N = 6 \times 10^{10} S/\pi \rho_s d^3 \qquad (1)$$

where N is the number of nanoparticles per milliliter, S is the percentage of solids, ρ_s is the density of the bulk polymer (g/mL), and d is the diameter (nm). The weight of nanoparticles in milliliters of suspension was determined from a mass–volume graph of the nanoparticles. From all these data, the specific surface area of the mag-nano-poly(HEMA–MAPA) nanoparticles was calculated by multiplication of N and the surface area of one nanoparticle.

The presence of Fe_3O_4 particles in the synthesized polymer was investigated with an electron spin resonance spectrophotometer (EL 9, Varian).

Adsorption of α-amylase onto the magnetic nanoparticles

Adsorption of α-amylase onto the mag-nano-poly (HEMA–MAPA) nanoparticles was performed in a batch experimental setup. The adsorption experiments were carried out for 2 h at 25°C at a stirring rate of 120 rpm. The effects of the pH of the medium, α-amylase concentration, temperature, and salt type on the adsorption capacity were studied. The adsorption was followed by monitoring of the decrease in α-amylase concentration by ultraviolet absorbance at 280 nm. α-Amylase adsorption on the mag-nano-poly(HEMA–MAPA) was investigated at various pHs. The pH of the adsorption medium was changed between 3.0 and 9.0 with different buffer systems (0.1*M* acetate for pH 3.0–5.0, 0.1*M* phosphate for 6.0–8.0, and 0.1*M* carbonate for 9.0). To investigate the effects of the initial concentration of α-amylase on adsorption, the α-amylase concentration was changed between 0.05 and 2.0 mg/mL. To observe the effects of the temperature on the adsorption, adsorption studies were performed between 4 and 95°C. Sodium chloride (NaCl), ammonium sulfate [$(NH_4)_2SO_4$], and sodium sulfate (Na_2SO_4) were used to investigate the effect of salt type on the adsorption capacity.

Activity of free and adsorbed α-amylase

Activities of the free and immobilized α-amylase were determined in the presence of 1% (w/v) soluble starch as the substrate in 20 m*M* phosphate buffer (pH 6.9) at 25°C according to the Bernfeld method.[25] Briefly, 0.5 mL of enzyme sample was incubated for 5 min at 25°C with 0.5 mL of the substrate solution, and the enzymatic reaction was interrupted by the addition of 1 mL of 3,5-dinitrosalicylic acid reagent. The tube containing this mixture was heated for 5 min in boiling water and then cooled in running tap water. After the addition of 10 mL of deionized water, the absorbance of the digested products was measured spectrophotometrically at 540 nm (model 1601, Shimadzu, Tokyo, Japan). A blank was prepared in the same manner without free α-amylase. A calibration curve established with maltose (0.1–1 mg in 1 mL of deionized water) was used to convert the absorbance readings into the concentration of maltose.

One unit of activity was defined as the amount of α-amylase that liberated 1 μmol of maltose from starch per minute at pH 6.9 and 25°C. The specific activity was expressed as activity units per milligram of protein. The protein concentration was determined according to the Bradford method.

These activity assays were performed over the pH range 3.0–9.0 and the temperature range 4.0–95.0°C to determine the pH and temperature profiles for the free and immobilized α-amylase. The effect of the substrate concentration was investigated in the 0.05–10.0 mg/mL starch solutions.

Thermal, operational, and storage stability of the immobilized α-amylase

The thermal stability of free and adsorbed α-amylase was investigated by measurement of the residual enzymatic activity at different temperatures (55.0–95.0°C) in acetate buffer (0.1*M*, pH 5.0) for 7 h. After a time interval, the sample was removed and assayed for activity.

To test the operational stability of the immobilized α-amylase, the magnetic nanoparticles were used 10 times, and the enzyme activities were measured after each use. α-Amylase-immobilized magnetic nanoparticles were removed and washed with acetate buffer (0.1*M*, pH 5.0) after each reaction run. The enzyme activities of the freshly prepared magnetic nanoparticles measured in the first run were defined as 100%.

For the storage stability, free and adsorbed α-amylase was stored in a 0.1*M* acetate buffer (pH 5.0) at 4°C for 90 days. After time interval, free and adsorbed α-amylase was assayed for activity.

Desorption and repeated use

To determine the reusability of the mag-nano-poly (HEMA–MAPA) nanoparticles, the α-amylase adsorption and desorption cycle was repeated 10 times. α-Amylase desorption from mag-nano-poly (HEMA–MAPA) nanoparticles was performed with 50% ethylene glycol. The final α-amylase concentration in the desorption medium was determined by the absorbance at 280 nm.

RESULTS AND DISCUSSION

Characterization of the mag-nano-poly(HEMA–MAPA) nanoparticles

In recent years, nanostructured materials have been used as supports for enzyme adsorption because the high surface-area-to-volume ratios of the nanoparticles can effectively improve the enzyme loading and the catalytic efficiency of the adsorbed enzyme. However, the recovery of enzyme adsorbed onto nanoparticles is often limited. To overcome this limitation, magnetic nanoparticles may be used. Fe_3O_4-loaded enzymes are easy to recover by a magnetic field, which may optimize the operational cost and enhance the product's purity.[26]

In this study, mag-nano-poly(HEMA–MAPA) nanoparticles containing a hydrophobic amino acid (i.e., phenylalanine) were prepared via the copolymerization of HEMA monomer with MAPA ligand. Details of the characterization of mag-nano-poly (HEMA–MAPA) nanoparticles were given in our previous article.[27] The FTIR spectra of both nano-poly(2-hydroxyethyl methacrylate) [nano-poly (HEMA)] and mag-nano-poly(HEMA–MAPA) had characteristic stretching vibration bands of hydrogen-bonded alcohol (O—H) around 3440 cm^{-1}. The sharp peak around 748 cm^{-1} was due to aromatic groups in the MAPA comonomer. Additionally, the characteristic stretching vibration band of the carbonyl group at 1700 cm^{-1} in mag-nano-poly (HEMA–MAPA) was excessively sharp because of extra carbonyl groups. The size and morphology of the resultant magnetic nanoparticles were observed by TEM and AFM. The average particle size of mag-nano-poly(HEMA–MAPA) ranged from 350 to 400 nm. Such small-sized magnetic nanoparticles are desirable, as they provide a large surface area available for the adsorption of the enzyme.[8] Additionally, the mag-nano-poly(HEMA–MAPA) nanoparticles were highly uniform in size, spherical in form, and nonporous. A major advantage of nonporous adsorbents is that significant intraparticle diffusion resistances are absent; this is particularly useful for the rapid adsorption of α-amylase.[28] Additionally, nonporous adsorbents have a positive effect on the kinetics of enzymatic reactions because the substrates and products do not have to diffuse inside the particle to reach the enzyme. The amount of MAPA in the mag-nano-poly(HEMA–MAPA) nanoparticles was determined by elemental analysis. The incorporation of MAPA was found to be 4.3×10^{-3} mmol/g of polymer with nitrogen stoichiometry. The specific surface area of the synthesized nanoparticles was calculated to be 580 m^2/g. From the electron spin resonance spectrum of mag-nano-poly(HEMA–MAPA), the g factor was calculated to be 2.21. The value showed that the polymeric structure had a local magnetic field of Fe_3O_4 in its structure.

Adsorption of α-amylase from aqueous solutions

Figure 1 shows α-amylase adsorption capacity at different pH values. The buffers were acetate for pH 3.0–5.0, phosphate for pH 6.0–8.0, and carbonate for pH 9.0. All of the buffers contained 1*M* Na_2SO_4 salt. The highest adsorption capacity was observed at pH 5.0 and was 705 mg/g. The isoelectric point of *B. licheniformis* α-amylase was 5.85,[29] and it is known that when the pH is close to a protein's isoelectric point, its net charge is zero, and hydrophobic interactions are maximum.[30] The maximum adsorption capacity was observed at pH 5.0, and this indicated that the binding of α-amylase on mag-nano-poly (HEMA–MAPA) should have involved hydrophobic interactions with a high percentage according to the other interactions. α-Amylase adsorption onto the magnetic nano-poly(2-hydroxyethyl methacrylate) [mag-nano-poly(HEMA)] nanoparticles was negligible (14 mg/g) at pH 5.0.

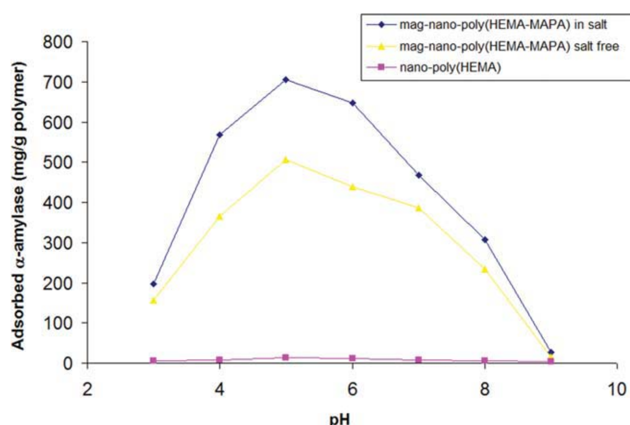

Figure 1 Effect of pH on α-amylase adsorption (α-amylase concentration = 0.5 mg/mL, incubation time = 2 h, temperature = 25°C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The α-amylase adsorption amount onto the mag-nano-poly(HEMA–MAPA) nanoparticles is shown in Figure 2. Note that one of the main requirements in affinity chromatography is the specificity of the adsorbent. The nonspecific interaction between the support, which is the mag-nano-poly(HEMA) nanoparticles in this case, and the molecules to be adsorbed, which are the α-amylase molecules here, should be minimum to consider the interaction as specific. As seen in this figure, nonspecific α-amylase adsorption was 14 mg/g. The amount of α-amylase adsorbed per unit mass of the mag-nano-poly (HEMA–MAPA) nanoparticles increased first with the initial concentration of α-amylase and then reached a plateau value, which represented saturation of the active adsorption sites (which were available and accessible for α-amylase) on the nanoparticle surfaces. This increase in the α-amylase binding capacity may have resulted from hydrophobic interactions caused by the hydrophobic MAPA monomer and by hydrophobic amino acids on the α-amylase molecules.

The effect of temperature on the adsorption of α-amylase on mag-nano-poly(HEMA–MAPA) nanoparticles is demonstrated Figure 3. The adsorption of α-amylase on the mag-nano-poly(HEMA–MAPA) nanoparticles increased significantly with increasing temperature; this indicated that hydrophobic interactions were much more significant in the adsorption. If the interaction between protein and the attached ligand involves a significant hydrophobic contribution, the contact surface area between the protein and the attached ligand should increase at higher temperature. This results is an increase in the affinity of α-amylase for the adsorbent higher temperatures. Increasing the temperature enhances the protein retention, and lowering the temperature generally promotes protein elution.[28,31] It is interesting to note that the van der Waals attraction forces, which operate in hydrophobic interactions, also increase with increasing temperature.

NaCl, Na_2SO_4, and $(NH_4)_2SO_4$ were used to investigate the effect of salt type on the adsorption capacity. The concentration of all of the salts studied here was 1.0*M*. The amounts of α-amylase adsorbed were 703.9, 639.1, 582.4, and 506.2 mg/g for Na_2SO_4, $(NH_4)_2SO_4$, NaCl, and salt-free magnetic nanoparticles, respectively. The highest amount of adsorbed α-amylase on the mag-nano-poly(HEMA–MAPA) nanoparticles was obtained by the addition of Na_2SO_4 salt to the adsorption medium. Salts such as Na_2SO_4, potassium sulfate, and $(NH_4)_2SO_4$ are the most effective salts to promote ligand–protein interactions because of their higher molal surface tension increment effects.[29] In Hydrophobic interaction chromatography (HIC), a high salt concentration in the

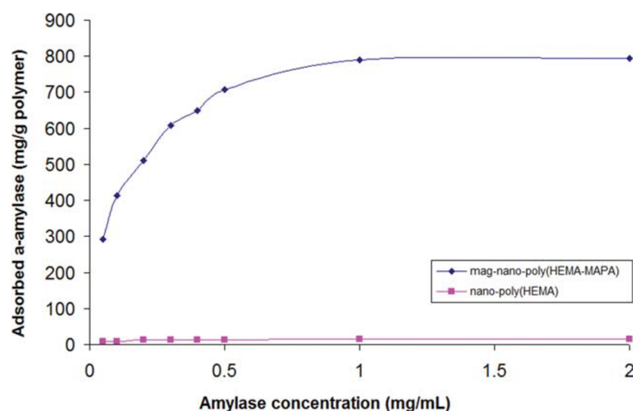

Figure 2 Effect of the α-amylase concentration on α-amylase adsorption [incubation time = 2 h, pH = 5.0, temperature = 25°C, salt (Na_2SO_4) concentration = 1.0*M*]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

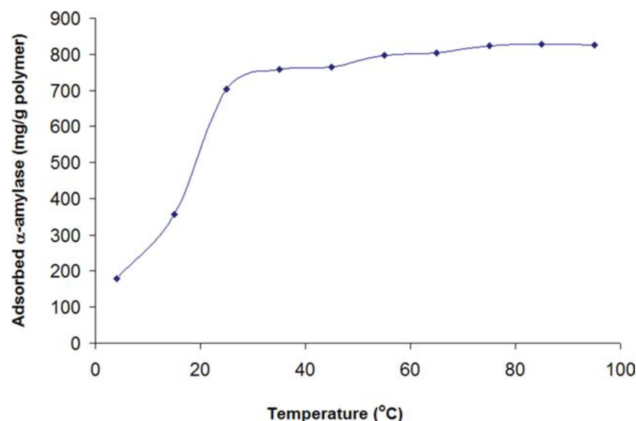

Figure 3 Effect of temperature on α-amylase adsorption [α-amylase concentration = 0.5 mg/mL, incubation time = 2 h, pH = 5.0, salt (Na_2SO_4) concentration = 1.0*M*]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I
Kinetic Parameters and Optimum pH Values and Temperatures for Free and Immobilized α-Amylase

Type of α-amylase	Optimum pH	Optimum temperature (°C)	V_{max} (U/mg)	K_m (mg/mL)	R^2
Free	7.0	85.0	0.179	181.82	0.9873
Immobilized	7.0	85.0	0.044	81.97	0.9916

equilibrium buffer and sample solution promotes the ligand–protein interactions and, consequently, the protein retention.[32,33] The amount of bound protein increases almost linearly with the enhancement in ionic strength and continues to increase in an exponential manner at still higher concentrations.[34]

Characterization of the free and immobilized α-amylase

The activity of free and immobilized α-amylase was compared as a function of pH, and we found that both free and bounded α-amylase had optimum activity at the same pH (7.0; Table I). The activity of free and immobilized α-amylase was assayed at various temperatures (4–95°C). The optimal temperature for both free and immobilized α-amylase to achieve the highest activity was 85.0°C (Table I).

Two kinetic parameters, the inverse of enzyme affinity (K_m) and the maximum reaction rate (V_{max}), which are Michaelis constants, were determined for the free and immobilized α-amylase with a Lineweaver–Burk plot. Starch was used as the substrate. The K_m values for free and immobilized α-amylase were 181.82 and 81.97 mg/mL, respectively. There was approximately a 2.2-fold decrease in the K_m value for the immobilized α-amylase. The K_m value is known as the affinity of the enzymes to substrates,[35] and lower values of K_m emphasize a higher affinity between enzymes and substrates.[24] The results show that the affinity of α-amylase to its substrate was increased by immobilization. V_{max} defines the highest possible velocity when all the enzyme is saturated with substrate; therefore, this parameter reflects the intrinsic characteristics of the immobilized enzyme but may be affected by diffusion constraints. The V_{max} value of free α-amylase (0.179 U/mg) was found to be higher than that of the immobilized α-amylase (0.044 U/mg) on the magnetic nanoparticles. The V_{max} values of enzymes usually demonstrate a decrease upon adsorption.[36]

Thermal, operational, and storage stability of the immobilized α-amylase

Thermal stability testing was carried out with free and immobilized α-amylases incubated in the absence of substrate at various temperatures. Figure 4(a,b) shows the heat inactivation curves between 55.0–95.0°C for the free and immobilized α-amylases, respectively. The immobilized α-amylase was inactivated at a much slower rate than the free form at high temperatures. These results show that the activity of the adsorbed preparation was more resistant than that of the soluble form against heat and denaturing agents. If the thermal stability of an enzyme were enhanced by immobilization, the potential utilization of such enzymes would be extensive. In principle, the thermal stability of an immobilized enzyme can be enhanced, diminished, or unchanged relative to its free counterparts.[19]

Unlike the free enzyme, the immobilized enzyme can easily be separated from the reaction mixture and reused. Hence, reusability or operational stability is an important criterion for the industrial use of

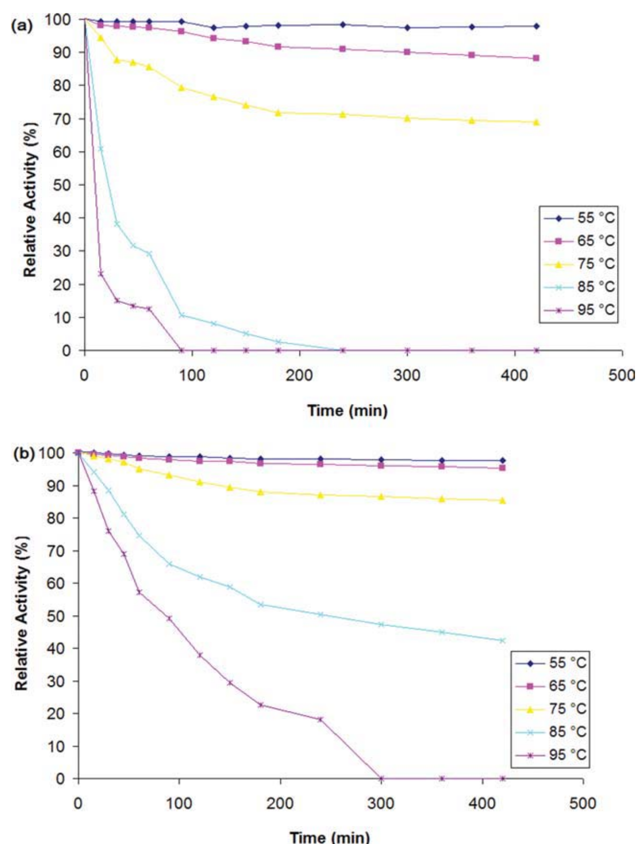

Figure 4 Thermal stability of (a) free and (b) immobilized α-amylase. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immobilized enzyme. The immobilized α-amylase retained a specific activity of 85% after 10 reuses.

One of the disadvantages in the use of free enzymes is that they are not stable during storage in solution, and their activity decreases gradually with time. The storage stabilities of the free and immobilized α-amylase were investigated by measurement of the enzyme activities for a period of 90 days. No enzyme release was observed. After 90 days, free α-amylase lost 36% of its initial activity, whereas the immobilized α-amylase retained 84% of its initial activity. This result showed that by immobilization, the enzyme gained more stable character than the free one.

Adsorption isotherms

Two important physicochemical aspects for the evaluation of the adsorption process as a unit operation are the kinetics and the equilibria of adsorption. The Langmuir model is based on the assumption of surface homogeneity, such as equally available adsorption sites, monolayer surface coverage, and no interaction between adsorbed species. The Freundlich isotherm is applicable to heterogeneous systems and reversible adsorption.[37] The Langmuir and Freundlich isotherms are presented in eqs. (2) and (3), respectively:

$$\frac{1}{q_e} = \left(\frac{1}{q_{\max}}\right) + \left(\frac{1}{q_{\max} b}\right)\left(\frac{1}{C_e}\right) \tag{2}$$

$$\ln q_e = \frac{1}{n}(\ln C_e) + \ln K_f \tag{3}$$

where q_e is the adsorbed amount of alpha-amylase at equilibrium (mg/g); q_{max} is maximum adsorption capacity of nanoparticles (mg/g); C_e is equilibrium alpha-amylase concentration (mg/mL); b is the Langmuir isotherm constant (mL/mg), K_f is the Freundlich constant (mg/g), and n is the Freundlich exponent. The value $1/n$ is a measure of the surface heterogeneity and ranges between 0 and 1, becoming more heterogeneous as its value gets closer to zero. The q_e ratio (mg/g) gives the theoretical monolayer saturation capacity of magnetic beads.[38] Some model parameters were determined by nonlinear regression with commercially available software and are shown in Table II. A comparison of all theoretical approaches used in this study showed that the Langmuir equation fit the experimental data best.

TABLE II
Kinetic Constants of the Langmuir and Freundlich Isotherms

Langmuir model			Freundlich model		
q_{max} (mg/g)	b (mL/mg)	R^2	K_f (mg/g)	$1/n$	R^2
833.3	17.1	0.9988	790.9	0.2147	0.9587

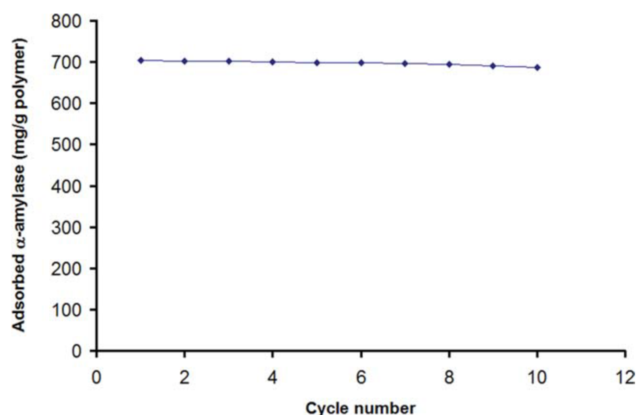

Figure 5 Repeated use of mag-nano-poly(HEMA–MAPA) nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Reusability of the mag-nano-poly(HEMA–MAPA) nanoparticles

To show the stability and reusability of the magnetic nanoparticles, the adsorption–desorption cycle was repeated 10 times with the same magnetic nanoparticles. Desorption of α-amylase was studied with a 50% ethylene glycol solution in a batch system. The recovery of α-amylase was 95%. At the end of 10 adsorption–desorption cycles, there was no remarkable reduction in the adsorption capacity (Fig. 5).

CONCLUSIONS

Compared to traditional microsized magnetic supports used in immobilization processes, nanosized magnetic carriers possess good performance because of their high specific surface area and the absence of internal diffusion resistance. In this work, mag-nano-poly(HEMA–MAPA) nanoparticles were prepared by the emulsion polymerization of HEMA and MAPA and were used for the adsorption of *B. licheniformis* α-amylase. The Langmuir adsorption model was found to be applicable in interpreting α-amylase adsorption onto magnetic nanoparticles. The maximum α-amylase capacity of the magnetic nanoparticles was 705 mg/g polymer at optimized conditions. It was seen that the thermal and storage stability of adsorbed α-amylase was greater than that of free α-amylase. Additionally, adsorbed α-amylase was desorbed with 95% recovery. This study showed that mag-nano-poly(HEMA–MAPA) nanoparticles could successfully be used for α-amylase adsorption.

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