JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Synthesis and Properties of Immobilized Pectinase onto the Macroporous Polyacrylamide Microspheres

Zhongli Lei* and Qin Jiang

Key Laboratory of Applied Surface and Colloid Chemistry, School of Chemistry and Materials Science, Shaanxi Normal University, Ministry of Education, Xi'an 710062, People's Republic of China

ABSTRACT: Pectinase was covalently immobilized onto the macroporous polyacrylamide (PAM) microspheres synthesized via an inverse suspension polymerization approach, resulting in 81.7% immobilization yield. The stability of the macroporous PAM support, which has a large surface area, is not impeded by the adsorbed proteins despite the fact that up to 296.3 mg of enzyme is immobilized per gram of the carrier particles. The immobilized enzyme retained more than 75% of its initial activity over 30 days, and the optimum temperature/pH also increased to the range of 50−60 °C/3.0−5.0. The immobilized enzyme also exhibited great operational stability, and more than 75% residual activity was observed after 10 batch reactions. The kinetics of a model reaction catalyzed by the immobilized pectinase was finally investigated. Moreover, the immobilized pectinase could be recovered by centrifuging and showed durable activity at the process of recycle.

KEYWORDS: Covalent immobilization, pectinase, macroporous polyacrylamide (PAM) microspheres, large surface area

INTRODUCTION

As one of the important members of biocatalysts, pectinase has drawn much attention because of the numerous applications from the food industry for clarification of fruit and vegetable juices and wines by degrading pectin and reducing viscosity to the feed industry for the formulation of animal feeds with other hydrolases.[1,2] Despite excellent catalytic properties of pectinase, the native enzymes as biocatalysts always present some drawbacks, such as poor stability under operational conditions, difficulty of product recovery, and impossibility of multiple reuses in an industrial process.[3] To overcome these problems, enzyme immobilizations were involved to improve the catalytic features of enzymes against several forms of denaturation as well as to make the use of expensive enzymes economically viable, strengthening repeat use.[4−6] To date, pectinase has been immobilized on various supports by physical adsorption [7] or covalent immobilization.[8,9] In general, adsorption techniques are easy to perform; however, the bonding of the enzyme is often weak, and such biocatalysts generally lack the degree of stabilization achieved by covalent attachment, which has advantage of no loss of enzyme and long-term stability over adsorption immobilization. However, the final success depends upon the immobilization protocols and the supports.

The essential requirement for any carrier is the need to have a large surface area. In this respect, porous polymeric materials, which have obvious advantage of high internal surface areas, have being increasingly employed as the solid supports.[10,11] It has been found that the pore sizes and specific surface area play an important role in the enzyme loading and activity expression.[12−14] However, a very high loading may produce diffusion constraint, which is not favorable for enzyme immobilization. It is convenient to use supports with a very large specific surface, such as macroporous polyacrylamide (PAM), which provide substrate and product transport with the least diffusional restriction.

Macroporous PAM microspheres, a kind of macroporous amino resin,[15] were chosen as immobilization supports in our report because of their prominent advantages, such as availability of plentiful surface amino groups, perfect mechanical strength, large surface area,[16] amenable to chemical modifications, adjustable particle size, easy regeneration, low operational cost, high performance of antipollution, good selectivity, and favorable chemical stability. The advantages above may provide the pectinase immobilization: (i) a certain number of available binding sites and a very simple, mild, and time-saving process, (ii) the reuse support,[17] (iii) the reduction of immobilization costs. Moreover, their hydrophilic nature and high water content provide the enzyme with a microenvironment similar to that *In Vivo*. However, there was no report on immobilization using macroporous PAM microspheres.

In our present work, macroporous PAM microspheres were synthesized via an inverse suspension approach in the presence of toluene/carbon tetrachloride as porogenic agents. Pectinase was covalently immobilized on the macroporous PAM using glutaraldehyde (GA) as a crossing-linker, the most frequently used agent for covalent enzyme immobilization on amino-containing supports.[18] The previous studies stated that, with a support offering large surfaces to react with the enzyme and activated with groups, such as glyoxyl or epoxy, not only will the dissociation be prevented, but the overall rigidity of the protein will also be increased, promoting a further enzyme stabilization.[19] When the immobilization is carried out on preactivated supports, the primary amino groups of the enzyme would react with the aldehyde groups that have been introduced by modification of the amino groups of the support.[20] The cross-linking confers

Received: September 28, 2010
Accepted: January 27, 2011
Revised: December 25, 2010
Published: February 22, 2011

 © 2011 American Chemical Society

mechanical advantages to both support and enzyme, making the fragile materials become much more sturdy and robust. In fact, the covalent bonds created during the cross-linking reaction are stable, even in the presence of substrate or high ionic strength solutions.[20] Although partial enzyme inactivation because of chemical modification and the interactions between soluble enzymes is often unavoidable, in most cases, enough catalytic activity is retained.[21] In our report, 81.7% catalytic activity is retained.

The main objectives of this work were to evaluate the effect of the immobilization variables, such as contact time, enzyme concentration, enzyme loading, immobilization pH, and immobilization temperature of the immobilization process and to obtain the optimum conditions for immobilized pectinase. Finally, the properties of the immobilized enzyme, such as kinetic behavior, pH and temperature profile, and storage stability, were investigated.

MATERIALS AND METHODS

Materials. The commercial purified pectinase enzyme was polygalacturonase (PG or endo-PG; EC 3.2.1.15) with a molecular weight of approximately 38 kDa and an isoelectric point (PI) of ca. 3.6. It is a glycoprotein and active against pectin (polygalacturonate acid), obtained from the Fluka Chemical Co. Pectin (namely, polygalacturonic acid) with a molecular weight of approximately 30 000 from citrus peel was also supplied by Fluka Chemical Co. and used without further purification. GA was obtained from the Tianjin Chemical Factory (Tianjin, China). The monomer acrylamide (AM) was purified by recrystallization from a mixture of benzene and *n*-hexane and dried in vacuum. Cross-linker *N*,*N*′-methylenebisacrylamide (BA), initiator ammonium persulfate (APS), continuous-phase cyclohexane, suspension stabilizer Span-80 acetone, porogenic agent toluene/carbon tetrachloride (1:1, v/v), and anhydrous methanol were of analytical grade. All other reagents were purchased from commercial sources and used after the usual drying and/or distillation without further pretreatment unless otherwise indicated. Water used in the experiments was double-distilled.

Synthesis of Macroporous PAM Microsphere Supports. Macroporous PAM microspheres were synthesized as follows: 90 mL of cyclohexane, 5 mL of mixture of porogenic agent toluene/carbon tetrachloride (1:1, v/v), and 0.1 g of Span-80 were added to a 250 mL three-neck flask equipped with a mechanical stirrer and a nitrogen inlet. The mixture was stirred under nitrogen purging until the surfactant was uniformly dispersed. At the same time, 5.0 g of AM, 0.51 g of BA, and 0.05 g of APS dissolved into 30 mL of double-distilled water were added to the mixture. The mixture was then stirred (360 rpm) continuously under a nitrogen atmosphere at 68 °C for 5 h. The macroporous PAM microspheres were collected and washed alternatively with double-distilled water and methanol, and the white product was dried overnight under ambient conditions.

Modification of the Macroporous PAM Supports. Before immobilization, the macroporous PAM supports were modified with GA cross-linker agent. A total of 1 g of dry macroporous PAM supports was added to 10 mL of 0.25% (v/v) GA aqueous solution at room temperature for 4 h. The supports were washed with distilled water at least 3 times and dried overnight.

Enzyme Immobilization. A total of 100 mg of macroporous PAM support (dry weight) was added to 3 mL of either acetate buffer solution (pH 2.5−5.0) or phosphate (pH 6.0−8.0), containing different enzyme concentrations (between 2 and 12 units/mL). The immobilization reaction was carried out at 25 °C in a shaking water bath for a specific contact time (1−6 h). Afterward, the products were separated and the unbound enzyme was removed by centrifuging at 8000 rpm and washing 3 times with distilled water. The immobilized enzyme were used freshly or stored at 4 °C before reuses.

Determination of the Immobilized Protein Amount. The amount of protein in a solution was determined by the Lowry method.[22] The amount of immobilized protein was calculated by subtracting the amount of protein recovered in the solution at the end of the immobilization process and in the washing solutions from the amount of protein initially used for the immobilization.

Pectinase Activity Assay. The activity of free and immobilized pectinase was determined by the 3,5-dinitrosalicylic acid method,[23] using *d*-(+)-galacturonic acid monohydrate as the standard compound used for the calibration curve and pectin (namely, polygalacturonic acid) as a substrate. A total of 1 unit of pectinase activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of reducing sugar per minute under the described conditions. A total of 1 mL of 1 mg/mL free pectinase was added to 1 mL of 1% (w/v) pectin solutions prepared in acetate buffer (pH 4.0) and incubated at 50 °C for 30 min. The reaction was stopped by adding 1.5 mL of 3,5-dinitrosalicylic acid (DNS). Reaction mixtures were heated in a boiling water bath for 10 min and then immediately cooled to room temperature. After dilution with 6.5 mL of water, the reducing sugars were determined at an absorbance of 475 nm by the ultraviolet−visible (UV−vis) spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan) with the help of a calibration curve. A total of 1 mL of 0.4 g/mL immobilized pectinase was added to 1 mL of 1% (w/v) pectin solutions prepared in acetate buffer (pH 3.5) at 50 °C for the same incubation times. The resulting mixture was separated by centrifugation, and the eluate was collected to determine the amount of generated reducing sugars as the free enzyme.

Specific activity was defined as the enzyme units per milligram of protein. Activity retention was defined as the ratio of the activity of the amount of the enzyme coupled on the macroporous PAM microsphere supports to the activity of the same amount of free enzyme.

Optimization of the Immobilization Parameters. Immobilization time, pectinase concentration, temperature of the immobilization solution, and pH of the enzyme solution were investigated as immobilization parameters. Immobilization was carried out in a 3 unit/mL enzyme solution at pH 3.98 to determine times varying between 1 and 6 h at 25 °C. Different enzyme concentrations between 2 and 12 units/mL at pH 3.98 were used for immobilization to determine the proper enzyme concentration of the immobilization solution. Temperatures varying between 0 and 50 °C at pH 3.98 were tested to decide the proper immobilization temperature. To find the pH value of the immobilization medium at which maximum enzyme activity was obtained, different pH values between 2.5 and 8 were investigated.

Optimum pH and Temperature for Free and Immobilized Pectinase. The effect of pH on the activity of native and immobilized pectinase was studied at 50 °C by varying the pH of the reaction mixture in the range of 2.5−8.0 using either acetate buffer solution (pH 2.0−5.0) or phosphate (pH 6.0−8.0) sodium buffer. Other reaction conditions and enzyme activity monitoring were as described.

The optimum temperature of free and immobilized pectinase was determined by performing the reaction in the temperature range of 20−80 °C using sodium acetate at pH 4.0 and 3.5, respectively. Other reaction conditions and enzyme activity monitoring were as described.

Determination of the Storage Stability of Free and Immobilized Enzyme. The activity of the free and immobilized enzyme was measured daily at 30 °C for 60 days. The remaining percentage of immobilized enzyme activity was calculated in each determination.

Reusable Stability of the Immobilized Pectinase. The operational stability was assessed by carrying out at 50 °C and pH 4 using sodium acetate buffer. This procedure was performed in consecutive cycles while repeatedly reusing the enzyme. After each reaction period, the immobilized pectinase were removed from the reaction medium and washed with acetate buffer (pH 4) and water to remove any

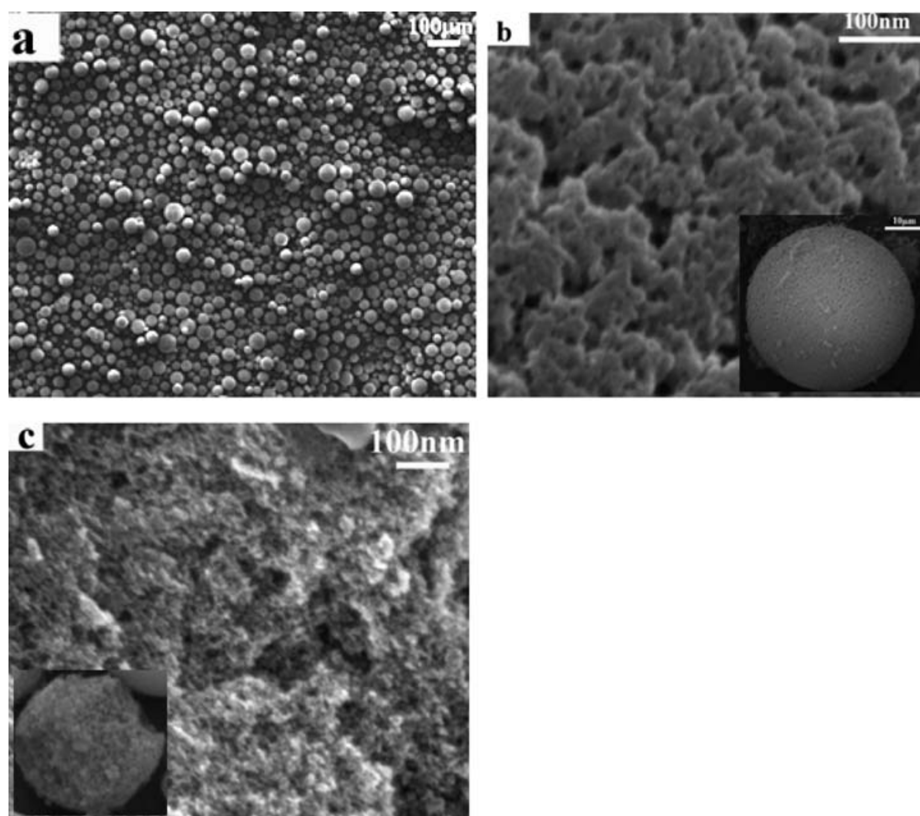

Figure 1. SEM images of (a) the holistic morphologies of the PAM macroporous microspheres, (b) the surface morphologies of the PAM macroporous microspheres, and (c) the internal morphologies of the PAM macroporous microspheres.

residual substrate within/on the immobilized enzyme. They were then reintroduced into fresh reaction medium. Each incubated solution was shaken for 30 min to measure the increase in the amount of reducing sugar for the determination of the enzyme activity.

Determination of Kinetic Parameters. Enzyme activities, in the free and immobilized forms, were evaluated using the classical Michaelis−Menten kinetics

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

where [S] is the substrate concentration, V_{max} is the maximum reaction rate attained at infinite substrate concentration, and K_m is the Michaelis−Menten constant.

The kinetic parameters of K_m and V_{max} were determined as follows: the substrate was pectin, and 0.20, 0.25, 0.30, 0.35, 0.40, 0.50, 0.60, 0.80, 1.0, 1.5, and 2.0 mL of 10 mg/mL pectin solution were added to different tubes. After the addition of acetate buffer (pH 3.98) into every tube with the columns at 4 mL in a water bath at 30 °C for 5 min, the immobilized pectinase or free enzyme solution was added to the tubes and shaken for the same time. Both the free and immobilized enzyme had the same concentrations.

Characterization. The morphologies of the macroporous PAM microspheres were examined by Philips scanning electron microscopy (SEM) using an accelerating voltage of 20 kV (the samples were coated with a thin layer of gold before measurement).

The infrared (IR) spectra were recorded on an AVTAR360 Nicolet Fourier transform infrared (FTIR) spectrometer using a KBr pellet.

The average diameter of the macroporous PAM microspheres was determined by a laser particle size distribution analyzer using a BI-90PLUS instrument. The particle distilled water solution of 3 mL (1 mg/mL) was put into a polystyrene latex cell and measured at a detector angle of 90° and a temperature of 20 °C.

The pore parameters related to macropores were determined by Barrett−Emmett−Teller (BET) and mercury intrusion porosimetry, respectively. Nitrogen adsorption measurements were performed, using a ZXF-06 instrument, using BET calculations for the surface area and pore diameter. Porosity measurements were performed by mercury porosimetry using AutoPore IV 9500, version 1.07, over a pressure range of 0.10−30 000.00 pounds per square inch absolute (psia).

Statistical Analysis. The data expressed in various studies were plotted using Origin-7.5. Each data represents the mean of three independent assays performed in duplicate with an average standard deviation of <5%, taking the control value as 100%.

RESULTS AND DISCUSSION

Morphology of the Macroporous PAM Supports. The SEM images of the resulting macroporous PAM microspheres are shown in Figure 1. It can be seen in Figure 1 that the macroporous PAM microspheres, after being washed with methanol, are perfect microspheres, with a diameter of less than 50 μm (see Figure 1a), and that their surfaces are smooth. The surface morphologies of the macroporous PAM microspheres exhibit porous structures (see Figure 1b). Their porous structures did not change much after being washed by methanol, and the diameter of their porous is about 25 nm. Figure 1c showed that the internal morphologies of the macroporous PAM microspheres exhibit porous structures.

FTIR Analysis of the Macroporous PAM Supports. The typical FTIR spectra of monomer AM and PAM microspheres was showed in Figure 2. The characteristic absorption bands for

AM and PAM were clearly observed in panels a and b of Figure 2, respectively. The characteristic peaks are at 1663 cm^{-1} ($\sigma_{C=O}$), 1541 cm^{-1} (σ_{-NH_2}), and 1447 and 3500−3300 cm^{-1} (σ_{-NH}). In comparison to Figure 2a, an obvious absorption band around 2927 cm^{-1} originating from −CH appeared and the absorption band between 900 and 1000 cm^{-1} originating from −C=C disappeared in Figure 2b. The spectra confirm that PAM was synthesized successfully.

Particle Size Distribution of the Macroporous PAM Supports. The average diameter of the macroporous PAM microspheres was determined by a laser particle size distribution analyzer. Drawn from the results of analysis, the PAM particles are concentrated at a majority at 25 μm (see Figure 3).

BET Analysis and Mercury Intrusion Porosimetry of the Macroporous PAM Supports. The pore parameters related to macropores were determined by mercury intrusion porosimetry and BET. The results are shown in Table 1 and panels a and b of Figure 4.

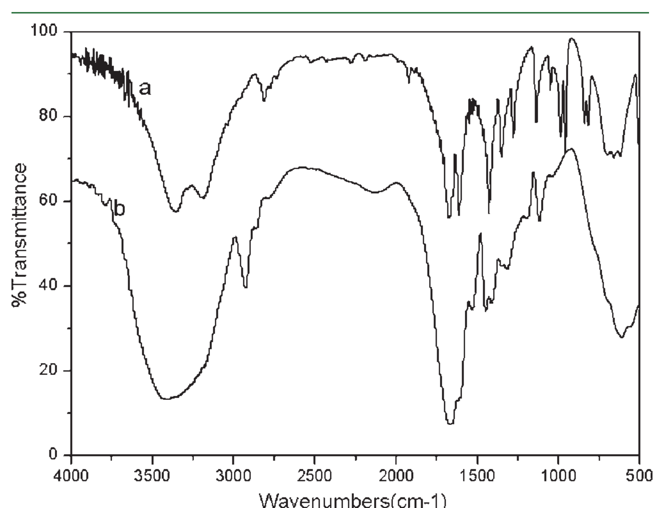

Figure 2. FTIR spectra of (a) AM and (b) PAM.

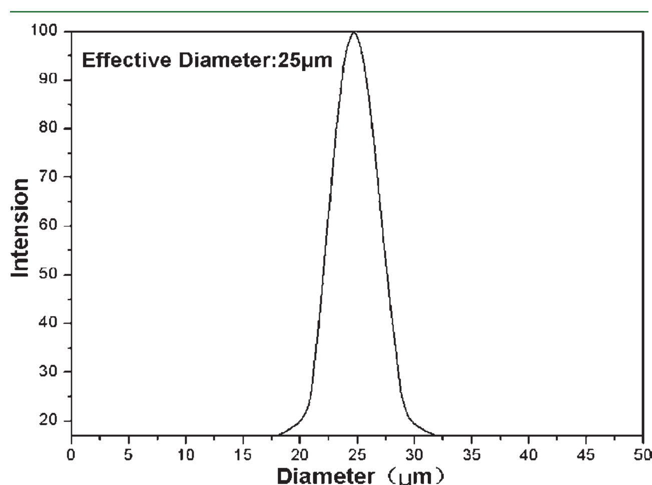

Figure 3. Particle size distribution of the PAM microspheres.

Table 1 showed the pore parameters determined by BET and mercury intrusion porosimetry. In comparison to the two groups of the pore parameters, the pore diameters, specific surface area, and porous volume were similar to each other. It can also be seen in Table 1 that the pore diameters present in the PAM microspheres confirm its fine porosity.

The pore diameter distribution curve of the macroporous PAM microspheres was shown in panels a and b of Figure 4. It obviously indicates that the pore sizes determined by BET and mercury intrusion porosimetry are 148.797 and 150.241 nm, respectively. The two determined methods are consistent with each other. This suggests that the most likely pore size is between 148 and 150 nm.

All of the above results indicate that the macropore PAM supports were synthesized successfully.

Immobilization of Pectinase on the Macropore PAM Supports. Table 2 summarizes the activity parameters of the free and immobilized pectinase under the optimum reaction conditions. The amount of bound protein is 296.3 mg/g, and the immobilized pectinase under its optimum reaction condition retains 81.7% in comparison to the free enzyme. The decrease in the specific activity of immobilized enzymes may be attributed to the diffusion effects caused by the more porous nature and fissured structure of the macroporous PAM support; this was the not the case in free enzymes. On the other hand, covalent immobilization methods may perturb the enzyme native structure and function.[24] Moreover, the interactions between the enzymes may have a very negative effect on the specific activity of immobilized enzymes.

Effect of Immobilization Parameters on Enzyme Activity. The following parameters that might influence the interaction between the enzyme and support were investigated: immobilization time, enzyme concentration, pH of enzyme solution, and immobilization temperature.

The effects of the contact time on enzyme immobilization are shown in Figure 5a. It was found that the enzymic activity was more stable after 4 h of immobilization time. This result was also

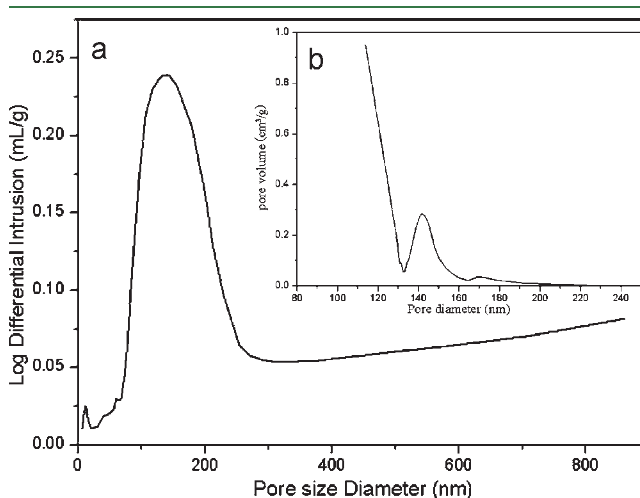

Figure 4. Pore diameter distribution curves of the PAM microspheres determined by (a) mercury intrusion porosimetry and (b) BET.

Table 1. Porous Parameters of the PAM Microspheres Determined by BET and Mercury Intrusion Porosimetry

porous parameters	average pore diameter (nm)	surface area (m^2/g)	porous volume (cm^3/g)
BET	148.797	29.3719	0.2873
mercury intrusion porosimetry	150.241	27.4279	0.2914

Table 2. Activity Parameters of the Free and Immobilized Pectinase under Optimum Reaction Conditions

samples	temperature (°C)	pH	bound protein (mg/g)	specific activity (unit/mg)	activity retention (%)
free pectinase	50	4.0		1.654	100
immobilized pectinase	50	3.5	296.3	1.349	81.7

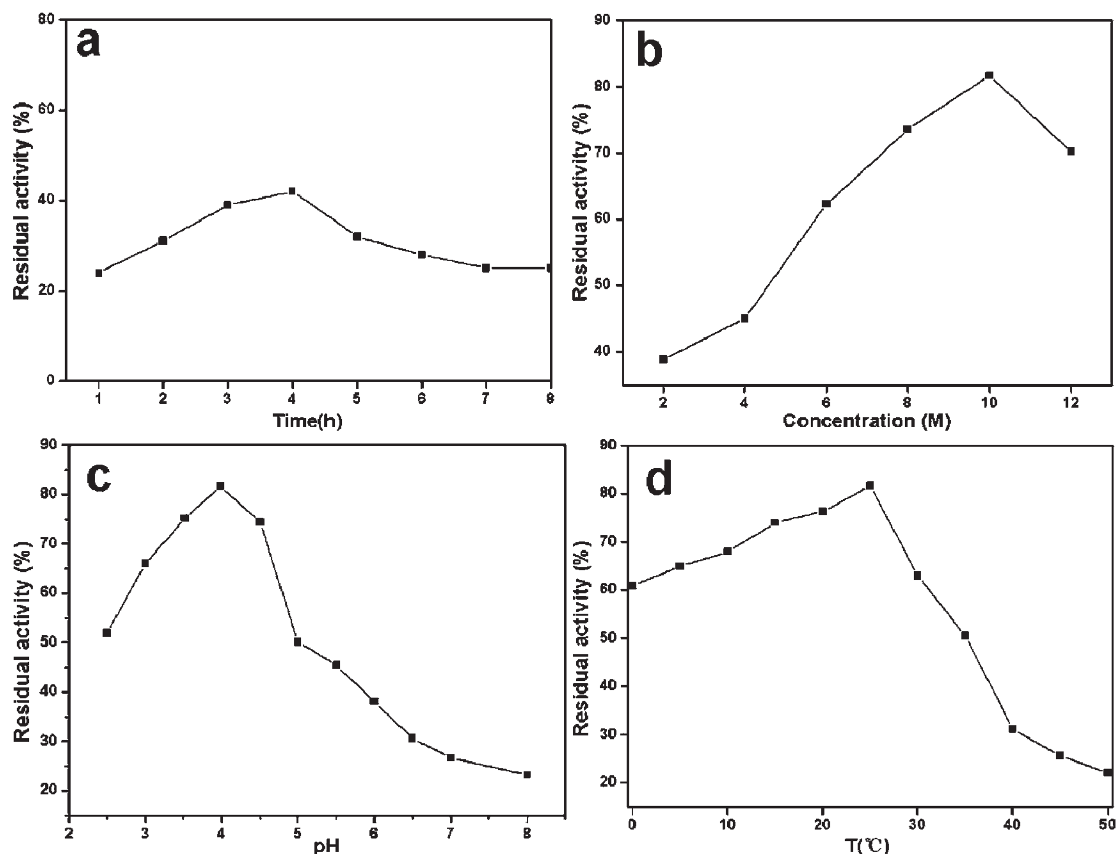

Figure 5. (a) Effect of the immobilization time on enzyme activity (pH, 3.98; *T*, 25 °C; enzyme concentration, 3 units/mL). (b) Effect of the enzyme concentration on enzyme activity (pH, 3.98; *T*, 25 °C). (c) Effect of the immobilization pH on enzyme activity (*T*, 25 °C; enzyme concentration, 10 units/mL). (d) Effect of the immobilization temperature on enzyme activity (pH, 3.98; enzyme concentration, 10 units/mL).

reported in our previous study,[8] and the same result was also obtained using a pectinase−alginate system by Li et al.[25]

The residual enzyme activity increased while the enzyme concentration increased from 1 to 10 units/mL (Figure 5b). However, it decreased above an enzyme concentration of 12 units/mL. This should be attributable to either oversaturation of the pore space of the macroporous PAM support with the enzyme, as a result of which relatively large substrate (pectin) diffusion limitations occur, or the presence of protein−protein interactions becoming more important, hindering the substrate conversions. It is considered that activity is more stable when the enzyme concentration is 10 units/mL, and the same result was also obtained by Lei et al.[7]

As shown in Figure 5c, the optimal immobilization temperature is 25 °C. Although low immobilization temperatures (4−10 °C) are preferred in general, immobilization processes at temperatures of 20−30 °C were also available.[7] One possible reason is that the rate of modified macroporous PAM support covalent immobilization with the enzyme at low temperatures is slower than that at 25 °C.

The activity of the immobilized enzyme was affected by immobilization pH significantly (Figure 5d). The residual activity of enzyme immobilization at low pH (3.0−4.0) was higher than that at high pH (5−10). The similar pH was obtained by Li et al. using sodium alginate support.[26] Pectinase was immobilized at pH 3.98, which is nearly the optimum pH for the free enzyme. In our experiments (data not shown), free pectinase used in this experiment was stable at a narrow pH range (3.0−4.5) and would lose most activity in alkaline solution. Moreover, the pI of pecinase (3.6) is very near the immobilization pH chosen, with this close relation between pI and optimum immobilization pH having previously been observed for pectinase immobilized on other supports.[8,26]

Effect of Immobilization on the Activity/pH Profile of Pectinase. The effect of pH on the activity of the free and immobilized pectinase was assayed in the range of pH from 2.5 to 8.0 (Figure 6a). The maximum pectinase activity was taken as 100%. It was observed that the optimal pH for free pectinase was obtained at 4.0, whereas the optimum pH of immobilized pectinase was 3.5, lower than that obtained for the free pectinase. The pH curve of immobilized enzyme shifted to the acidity side. In comparison to free pectinase, immobilized enzyme used in this experiment retained more than 80% activity in a wider pH range of 3.0−5.0. This result is similar to those published in ref 8. It means that the scope of pH stability of immobilized pectinase is

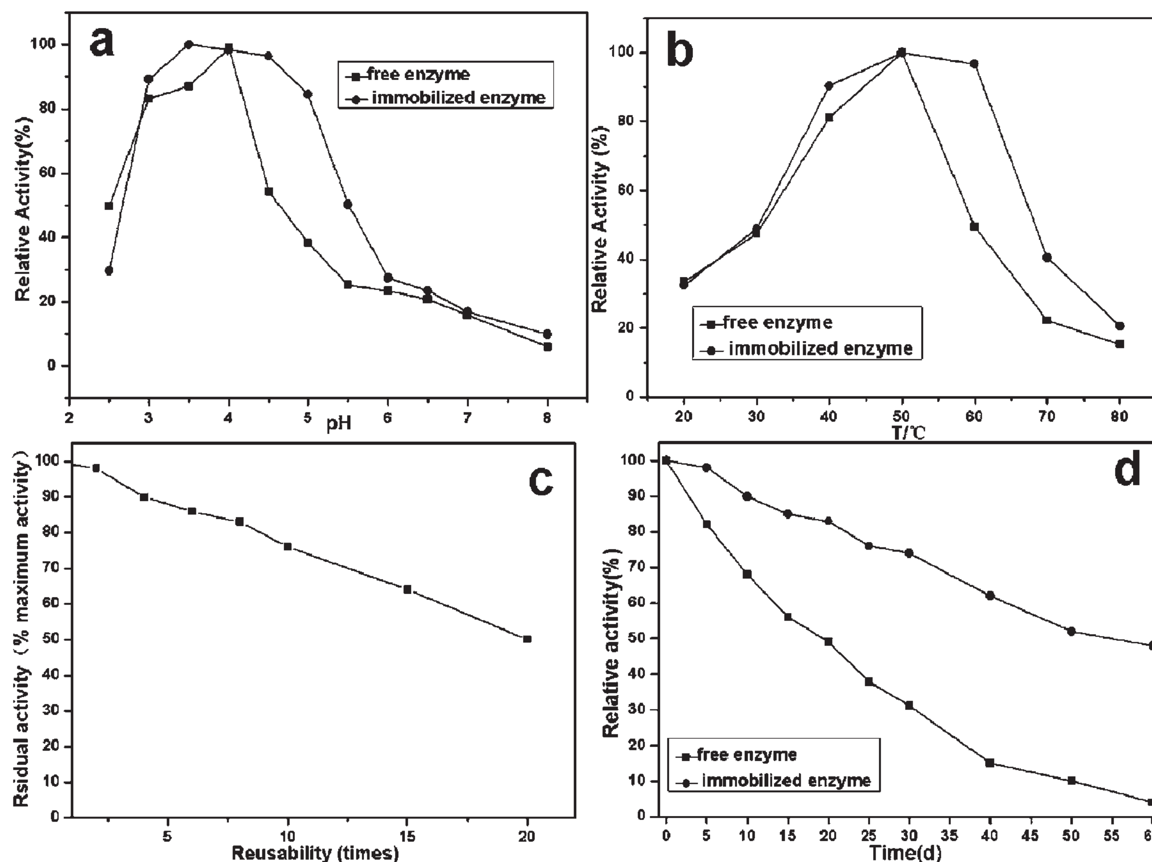

Figure 6. Relative activity of free and immobilized pectinase at different (a) pH values and (b) temperatures. (c) Reusability of immobilized pectinase on macroporous PAM supports. (d) Storage stability of free and immobilized pectinase at 30 °C.

significantly expanded, which should be attributed to the microenvironment of the immobilized enzyme on the macroporous PAM microsphere, and the immobilized enzyme was less affected from the acidity of the solution. On the other hand, PAM belongs to polycationic polymer, which causes the partitioning of protons between the bulk phase and the enzyme microenvironment, so that the optimum pH of the immobilized enzyme shifted slightly in the acidic region. The deviation in the optimal pH value of the enzyme depends upon the enzyme reaction as well as the structure and the charge of the matrix. The effect should be attributable to substrate diffusion limitations inside the pores of the support. If the intrinsic specific activity of the immobilized enzyme is high, the substrate concentration decreases inside the pores and the substrate may not reach enzyme molecules immobilized deep inside the pores. When the pH conditions are changed and the intrinsic enzymatic activity becomes lower, the substrate concentration gradient across the pores will be less steep. Moreover, the covalent bonds between enzyme and the macroporous PAM supports are stable enough that they can resist conformational changes and prevent the rate of the dissociation of the enzyme, even in the presence of substrate or high ionic strength solutions.[20] Therefore, the immobilized enzyme exhibits good adaptability to environmental acidity and reveals acceptable pH stabilities over a broad experimental range.

Thermal Stability of the Immobilized Pectinase. We have also investigated the effect of the temperature on the activities of free and immobilized enzyme in a range of 20−80 °C. The result is shown in Figure 6b. It was found that the optimum temperatures for free pectinase were obtained at 50 °C, whereas the optimum temperature of immobilized pectinase was in the range of 50−60 °C. The result is similar to the results of pectinase immobilized on a chitosan support by Li et al.[27] In comparison to free pectinase, immobilized pectinase exhibited an improved thermal stability. This is presumably the result of the newly prepared macroporous PAM microspheres, which result in enhanced resistance to unfolding in the thermal treatment. On the other hand, the covalent bond between the pectinase and macroporous PAM supports is capable of increasing the conformational rigidity of the enzyme and raising the activation energy of the thermal denaturation reaction. However, above 70 °C, the relative activity of immobilized and free enzyme dropped rapidly, because a certain temperature causes protein denaturation and, therefore, decreases the reaction rate.

Reusability of the Immobilized Pectinase. Reusability for the immobilized enzyme is very important in economics, and an improved stability can make the immobilized enzyme more advantageous than its free counterpart. To determine the reusability of immobilized pectinase, the activity of the immobilized pectinase was assayed in several batches and the same assay conditions were used for all batches. Activity of the first batch was taken as a reference (100%). The residual activity of immobilized pectinase after a number of uses is illustrated in Figure 6c, from which we found that the pectinase bound on PAM microspheres retained more than 75% of the original activity after use 10 times. This value was higher than that reported by Roy et al.[28] for pectinase entrapped in alginate, which exhibited a 55% loss in activity after four batch reactions. It is demonstrated that the immobilized pectinase has a better reusability. One possible

Table 3. Determination of Kinetic Parameters for Free and Immobilized Pectinase at 30 °C

kinetic parameters	immobilized enzyme	free enzyme
K_m (g of pectin/mL)	6.96	6.19
V_{max}[a]	2.82	3.27

[a] In units of μmol min^{-1} (mg of enzyme)$^{-1}$ and μmol min^{-1} (mg of particles)$^{-1}$ for free and immobilized enzymes, respectively.

reason for these results is that the amino group of PAM supports provides a certain number of available binding sites for immobilizing pectinase by covalent coupling, which avoids the enzyme from falling off the PAM microsphere support and denaturation of the enzyme molecule.

Storage Stability of the Immobilized Pectinase. The storage stability of the free and immobilized pectinase at certain time intervals and the results are shown in Figure 6d. Obviously, the activity of the immobilized pectinase decreases more slowly than that of the free pectinase. The free enzyme only maintained less than 30%, whereas the immobilized enzyme still remains more than 75% of its original activity after 30 days, which is higher than our previous report.[8] The free enzyme maintained less than 5% of its initial activity over 60 days, whereas the immobilized enzyme presented nearly 50%. It is found that the immobilized enzyme provides a prominent advantage stability over free enzyme. These results would be due to the covalent bonding between the pectinase molecule and the support PAM microspheres, avoiding the denaturation of the enzyme molecule at a long period. Thus, the immobilized pectinase exhibits higher storage stability than the free enzyme.

Kinetic Parameters of Immobilized Pectinase. The Michaelis constant (K_m) and maximal activities (V_{max}) for free and immobilized enzyme were determined through a Lineweaver—Burk plot at 30 °C. The Michaelis constant (K_m) reflects the effective characteristics of the enzyme and depends upon both partitioning and diffusional effects, and maximal activities (V_{max}) reflect the intrinsic characteristics of the immobilized enzyme but may be affected by diffusional constraints.

K_m and V_{max} of pectinase calculated from the equations of these plots are summarized at Table 3.

In this study, for pectinase, V_{max} values for free and immobilized enzymes demonstrate almost no change upon immobilization, which shows that the immobilized enzyme achieves the same velocity as the free enzyme when saturated with the substrate. The apparent Michealis—Menten constant for pectinase was affected after immobilization. The K_m value of the immobilization enzyme is slightly higher than that of the free enzyme, as expected. This result may due to the diffusion effects, caused by the three-dimensional structure of the support. Other reasons that may be invoked include the modeling of the process of adsorption.

In summary, pectinase was immobilized onto the macroporous PAM microspheres and properties of immobilized enzyme were compared to those of free pectinase. The immobilized pectinase exhibited higher relative activity and stability than the free enzyme in the solution. The optimum activity of the immobilized enzyme had wider pH and temperature ranges than that of the free enzyme. The storage stability and reusability of immobilized pectinase were also enhanced, as compared those of free pectinase. Furthermore, the immobilized enzyme can be recycled for further reaction by centrifugation. Thus, this immobilized enzyme strategy seemed to permit good results in terms of pectinase immobilization efficiency and demonstrated promising application potential for enzymatic catalysis in the food and agriculture industry.

AUTHOR INFORMATION

Corresponding Author

*Telephone: 86-29-8530-3952. Fax: 86-29-8530-7774. E-mail: zhllei@snnu.edu.cn.

REFERENCES

(1) Kashyap, D. R.; Vohra, P. K.; Chopra, S.; Tewari, R. Applications of pectinases in the commercial sector: A review. *Bioresour. Technol.* **2001**, *77*, 215–227.

(2) Alkorta, I.; Garbisu, C.; Llama, M. J.; Serra, J. L. Industrial applications of pectic enzymes. *Process Biochem.* **1998**, *33*, 21–28.

(3) Sheldon, R. A. Enzyme immobilization: The quest for optimum performance. *Adv. Synth. Catal.* **2007**, *349*, 1289–1307.

(4) Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and via immobilization techniques. *Enzyme Microb. Technol.* **2007**, *40*, 1451–1463.

(5) Jin, W.; Brennan, J. D. Properties and applications of proteins encapsulated within sol—gel derived materials. *Anal. Chim. Acta* **2002**, *461*, 1–36.

(6) Bukowski, R. M.; Ciriminna, R.; Pagliaro, M.; Bright, F. V. High-performance quenchometric oxygen sensors based on fluorinated xerogels doped with $[Ru(dpp)_3]^{+2}$. *Anal. Chem.* **2005**, *77*, 2670–2672.

(7) Lei, Z.; Bi, S. Preparation and properties of immobilized pectinase onto the amphiphilic PS-*b*-PAA diblock copolymers. *J. Biotechnol.* **2007**, *128*, 112–119.

(8) Lei, Z. L.; Ren, N.; Li, Y. L.; Li, N.; Mu, B. Fe_3O_4/SiO_2-*g*-PSStNa polymer nanocomposites microspheres (PNCMs) from a surface-initiated atom transfer radical polymerization (SI-ATRP) approach for pectinase immobilization. *J. Agric. Food Chem.* **2009**, *57*, 1544–1549.

(9) Liu, C.; Ohta, H.; Kuwahara, T.; Shimomura, M. Amperometric glucose-responding property of enzyme electrodes fabricated by covalent immobilization of glucose oxidase on conducting polymer films with macroporous structure. *Eur. Polym. J.* **2008**, *44*, 1114–1122.

(10) Li, Y.; Gao, F.; Wei, W.; Qu, J. B.; Ma, G. H.; Zhou, W. Q. Pore size of macroporous polystyrene microspheres affects lipase immobilization. *J. Mol. Catal. B: Enzym.* **2010**, *66*, 182–189.

(11) Blanco, R. M.; Terreros, P.; Fernandez-Perez, M.; Otero, C.; Diaz-Gonzalez, G. Functionalization of mesoporous silica for lipase immobilisation characterization of the support and the catalysts. *J. Mol. Catal. B: Enzym.* **2004**, *30*, 83–93.

(12) Das, R. D.; Maji, S.; Das, S.; Chaudhuri, C. R. Optimization of covalent antibody immobilization on macroporous silicon solid supports. *Appl. Surf. Sci.* **2010**, *256*, 5867–5875.

(13) Keeling-Tucker, T.; Brennan, J. D. Fluorescent probes as reporters on the local structure and dynamics in sol—gel-derived nanocomposite materials. *Chem. Mater.* **2001**, *13*, 3331–3350.

(14) Tsai, H. C.; Doong, R. A. Preparation and characterization of urease-encapsulated biosensors in poly(vinyl alcohol)-modified silica sol—gel materials. *Biosens. Bioelectron.* **2007**, *23*, 66–73.

(15) Liu, P.; Guo, J. Polyacrylamide grafted attapulgite (PAM-ATP) via surface-initiated atom transfer radical polymerization (SI-ATRP) for removal of Hg(II) ion and dyes. *Colloids Surf., A* **2006**, *282—283*, 498–503.

(16) Tang, Z. G.; Zhou, R. Q.; Duan, Z. T. Adsorption and desorption behaviour of taurine on macroporous adsorption resins. *J. Chem. Technol. Biotechnol.* **2001**, *76*, 752–756.

(17) Pessela, B. C. C.; Fernández-Lafuente, R.; Fuentes, M.; Vián, A.; Garcıa, J. L.; Carrascosa, A. V.; Mateo, C.; Guisán, J. M. Reversible immobilization of a thermophilic β-galactosidase via ionic adsorption on PEI-coated Sepabeads. *Enzyme Microb. Technol.* **2003**, *32*, 369–374.

(18) Milosavíc, N.; Prodanovíc, R.; Jovanovíc, S.; Vujčic, Z. Immobilization of glucoamylase on macroporous spheres. *Acta Period. Technol.* **2004**, *35*, 207–14.

(19) Fernandez-Lafuente, R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. *Enzyme Microb. Technol.* **2009**, *45*, 405–418.

(20) Walt, D. R.; Agayn, V. I. The chemistry of enzyme and protein immobilization with glutaraldehyde. *Trends Anal. Chem.* **1994**, *13*, 425–430.

(21) Migneault, I.; Dartiguenave, C.; Bertrand, M. J.; Waldron, K. C. Glutaraldehyde: Behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques* **2004**, *37*, 798–802.

(22) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(23) Miller, G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428.

(24) Durán, N.; Rosa, M. A.; D'Annibale, A.; Gianfreda, L. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: A review. *Enzyme Microb. Technol.* **2002**, *31*, 907–931.

(25) Li, T. P.; Wang, N.; Zhao, Q. C.; Guo, M.; Zhang, C. Y. Optimization of covalent immobilization of pectinase on sodium alginate support. *Biotechnol. Lett.* **2007**, *29*, 1413–1416.

(26) Li, T.; Li, S.; Wang, N.; Tain, L. Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. *Food Chem.* **2008**, *109*, 703–708.

(27) Li, Y.; Zhong, H.; Xiao, Y.; Ge, C.; Guo, H. Immobilization of pectinase on chitosan. *Food Sci.* **2002**, *23*, 50–53 (in Chinese).

(28) Roy, I.; Sardar, M.; Gupta, M. N. Evaluation of a smart bioconjugate of pectinase for chitin hydrolysis. *Biochem. Eng. J.* **2003**, *16*, 329–335.