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Mesoporous Material SBA-15 Modified by Amino Acid Ionic Liquid To Immobilize Lipase via Ionic Bonding and Cross-Linking Method

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ABSTRACT: Novel supported task-specific amino acid ionic liquids have been developed for the first time via the ionic-pair coupling of imidazolium cation of the modified mesoporous material SBA-15 with L-lysine anion. The material functionalized by amino acid ionic liquid (AA-SBA) as a novel carrier was used to immobilize porcine pancreas lipase (PPL) through ionic bonding interaction and cross-linking method (PPL-AA-SBA). To investigate the stability changes of the lipase immobilized onto novel carrier AA-SBA via special methods, the enzymatic properties of PPL-AA-SBA were studied in the triacetin hydrolysis reaction. The results indicated that the immobilization efficiency of PPL was up to 98% and the stability of lipase immobilized onto AA-SBA was dramatically improved. Especially the leaching investigation in consecutive use showed that special immobilization method inhibited the enzyme leaching obviously, and immobilization efficiency was above 96% after five recycles.

■ INTRODUCTION

Lipases (E.C.3.1.1.3) are one kind of enzyme of considerable industrial significance because of their broad application in many reactions and chemical industries. A lot of useful chemical products including alcohols, esters, acids, and amine are produced via esterification, deacylation, and hydrolysis reactions under mild reaction conditions.^{1–3} However, free lipases are unstable, and the reuse from reaction system is usually difficult. Immobilization has been a widely employed technique in industrial applications of lipases for enhancing thermal stability, imparting reusability, making enzyme-based processes cost-effective and continuous operation available.^{4,5}

A great variety of carriers and methods have been used for the enzyme immobilization. Usually, the activities and stabilities of immobilized enzyme mainly depend on the textural and surface chemical property of the support.^{6–8} Ordered mesoporous silicas such as SBA-15 have attracted particular interest because of their large specific surface area, controlled pore size, and pore volume.^{9–12} However, enzymes immobilized on pure SBA-15 usually exhibit rapid loss of activity attributed to enzyme leaching. Another special characteristic of SBA-15, abundant surface silanol groups, is then significant. Various functional groups via reacting with silanol groups can be incorporated into the surface of SBA-15 to adjust adsorption interactions with the enzymes and subsequently attain a highly active and stable biocatalyst.^{13–15}

Recently, we reported an efficient method to attain highly active immobilized enzyme.¹⁶ We synthesized designable functionalized ionic liquid in which lipase usually exhibits good activity. Also, ionic liquid was grafted onto the surface of SBA-15, decreasing the consumption amount and high cost.^{16,17} The carrier modified by C₁/BF₄ (1-methyl-3-(3-(trimethoxysilyl)propyl) imidazolium tetrafluoroborate) was subjected to immobilizing porcine pancreas lipase by simple physical adsorption. By optimization reaction conditions, we found that specific activity of immobilized enzyme improved 5.72-fold after C₁/BF₄ modification. Nevertheless, the physical adsorption method suffered from low immobilization efficiency

(only 53%) and reusability (only 35% of initial activity in the fifth reuse).¹⁷

It is known that the immobilization method is another essential factor to determine the properties of immobilized enzyme.¹⁸ Up to now, enzymes have been immobilized on carriers by several methods including cross-linking, covalent attachment, physical entrapment, and physical adsorption. As compared to physical adsorption and entrapment, cross-linking and covalent attachment could enhance the stabilities of enzymes obviously.^{19–25} In this work, we resolved the existing problem by adjusting immobilization methods. First, amino acid ionic liquid 1-methyl-3-(3-(trimethoxysilyl)propyl) imidazolium lysinate that was both to adjust the surface properties of the carriers via introducing imidazole cation and to alter lipase scaffolds through anion covalent modification was grafted onto the SBA-15 (AA-SBA).^{26,27} Second, lipase was originally immobilized onto the surface of AA-SBA via ionic-binding and cross-linking methods, which were expected to enhance the stability (Scheme 1). Ultimately, the prepared carriers and immobilized lipase were characterized to explain the changes of carrier properties and enzyme structure. The enzymatic properties of PPL-AA-SBA, including optimum temperature, optimum pH, thermal stability, and reusability, were studied in the triacetin hydrolysis reaction. We hope that PPL-AA-SBA would be a rather stable and efficient biocatalyst, which could resolve the problem of low immobilization efficiency and stability in previous works.

■ MATERIALS AND METHODS

Materials. Parent SBA-15 was purchased from Novel Chemical Technology Co., Ltd. (Shanghai, China). The relative crystallinity was higher than 95%, and the catalyst residue was

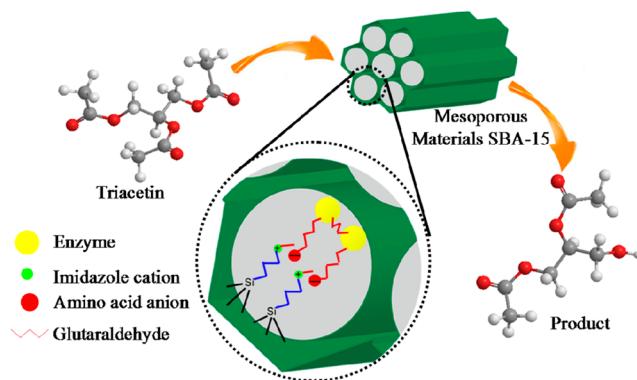
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Scheme 1. Structure of Amino Acid Ionic Liquid-Modified SBA-15 and Immobilized Lipase



less than 2%. (3-Chloropropyl) trimethoxysilane, 1-methylimidazole, glutaraldehyde, and aminotrimethylolmethane (Tris) were purchased from Sigma-Aldrich Chemistry Co. Lipase (E.C.3.1.1.3) from porcine pancreas was purchased from Sigma and stored at 0–4 °C. The protein content of lipase is 17%, and the activity is 228 U/g PPL (using triacetin at pH 7.0 and 35 °C). Other reagents all used in this work were analytical purity.

Carriers' Preparation. In a well-dried flask, (3-chloropropyl) trimethoxysilane (19.87 g, 0.1 mol) was added to 1-methylimidazole (8.21 g, 0.1 mol), and the prepared system was programmed heated. After being stirred at 95 °C for 26 h under nitrogen atmosphere, the resulting mixture was cooled to room temperature, extracted with dry ethyl acetate three times, and concentrated by a rotary evaporator. The obtained product C denoted as [Smim]⁺Cl⁻ was ionic liquid precursor (Scheme 2). In a dry flask, the synthesized ionic liquid precursor (2.8 g, 0.01 mol) was dissolved in 150 mL of chloroform, and then 2 g of SBA-15 was added into the system under constant stirring.^{28–31} After reaction at 61 °C for 24 h, the obtained solid was isolated by filtration and repeatedly washed with chloroform and ether, then Soxhlet-extracted with dichloromethane for 20 h to obtain ionic liquid-modified SBA-15 ([Smim]⁺Cl⁻-SBA). The resultant solid and NaOH (0.1 g, 2.5 mmol) were suspended in 1000 mL of water to ionic exchange for 4 h under atmosphere with constant stirring. The product named [Smim]⁺OH⁻-SBA was washed with distilled water three times, Soxhlet-extracted with dichloromethane for 20 h,

and then subjected to application in the next step.³² Afterward, 3 g of lysine was added into the synthesized ionic liquid-modified SBA-15, and a neutralization reaction was conducted on the mixture in 100 mL of distilled water under atmosphere temperature for 24 h. The resulting mixture was filtered, washed with distilled water and ethanol thoroughly, and the final product was amino acid ionic liquid-modified carrier (AA-SBA).^{26,33}

Immobilization of PPL on AA-SBA. 0.125 g of PPL was added into 25 mL of Tris-HCl buffer solution (0.025 M, PH 7.0). After the PPL was completely dispersed, 0.5 g of SBA-15 (or AA-SBA) and 25 mL of distilled water were introduced, and the mixture was further stirred at 30 °C and 30 rpm for 2 h until the samples reached adsorption equilibrium. Afterward, 4 mL of 1% glutaraldehyde solution was added, and the mixture was treated for another 2 h (the volume 4 mL was optimum value measured from 0 to 5 mL, relative activities decreased obviously, and protein loading increased slightly when the volume was more than 4 mL). After standing at room temperature for 10 min, the suspension was centrifuged (8000 rpm, 3 min), filtered, washed with buffer solution three times, and dried in a freeze-dryer.³⁴ The obtained lipase immobilized onto parent SBA-15 via cross-linking was named PPL-CRO-SBA. Lipase immobilized onto the surface of AA-SBA via ionic-binding and cross-linking methods was denoted as PPL-AA-SBA.

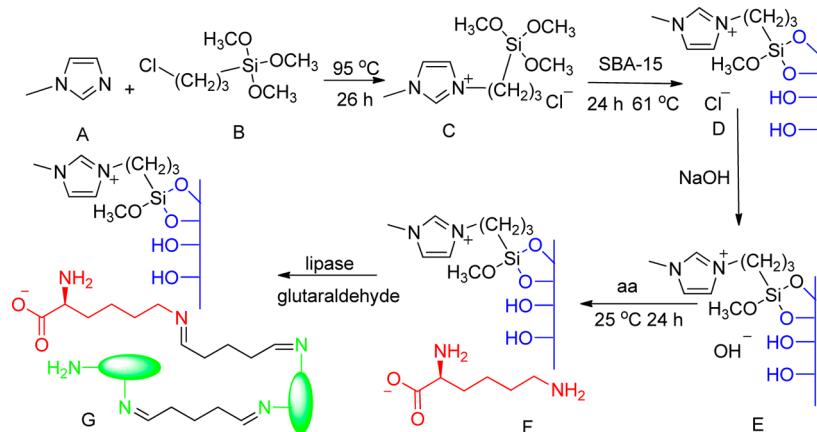
Protein Assay. The immobilization efficiency was deduced according to the protein amount measured by Bradford's method.^{35,36} The amount of protein was assayed indirectly by comparing the difference between the amount of protein offered before immobilization and the amount of protein both in the filtrate solutions and in the supernatant separated from immobilized mixture. The immobilization yield (IY) was calculated according to the formulas:

$$IY (\%) = \frac{C_i - C_f}{C_i} \times 100\% \quad (1)$$

C_i and C_f represent the amount of the lipase protein initially and finally in the immobilization medium (mg).

Assay of Lipase Activity. The lipase activity of the immobilized enzyme was determined on the basis of triacetin hydrolysis by the pH-stat method, using the 718stat titrino device.³⁴ The immobilized enzyme (0.25 g) was added to 25

Scheme 2. Synthesis of Ionic Liquid-Modified SBA-15 and Immobilized Enzyme^a



^a aa: amino acid.

mL of 2.73% homogenized triacetin. The reaction mixture was incubated at 30–55 °C in a shaking incubator at a speed of 30 rpm for 10 min. The pH was maintained at 6.5–8.5 by titrating the acetic acid produced in the triacetin hydrolysis reaction with 0.05 M NaOH. One lipase unit (1 U) was defined as the amount of lipase required to consume 1 μmol of triacetin per minute.¹⁶ All measurement experiments were carried out at least three times. Diffusion limitation for substrate and product was negligible in the titrating process due to the small carrier sizes (around 400 nm).^{16,34}

$$\text{lipase activity (U/g immobilized PPL)} = \frac{(V - V_0) \times 50}{10 \times 0.25} \quad (2)$$

$$\text{specific activity (U/g PPL)} = \frac{\text{activity of immobilized PPL}}{\text{amount of PPL loaded}} \quad (3)$$

where V was the sodium hydroxide consumption when the reaction was catalyzed by enzymes (mL), V_0 was the volume of sodium hydroxide when titrating the acetic acid without enzymes (mL), 10 was reaction time (min), and 0.25 was the amount of immobilized PPL (g). 50 was the concentration of sodium hydroxide to titrate the acetic acid (μmol/mL).

Assay of Thermal Stability. PPL-CRO-SBA and PPL-AA-SBA were incubated in 25 mL of phosphate buffer (0.025 M, pH 7.5) at 60 °C for different times from 1 to 8 h. After incubation, the phosphate buffer was removed, and the fresh homogenized triacetin incubating at optimum condition was added. The activity of immobilized enzyme was measured according to the procedure described above. The activity of the lipase without incubation was considered as 100% relative activity.³⁶

Leaching Assay and Reusability. Concrete procedures of leaching assay were followed. 0.25 g of immobilized enzyme was introduced to a 100 mL flask, and 25 mL of phosphate buffer (0.025 M, pH 7.5) was added to the flask. The prepared system was stirred for 10 min under optimal reaction condition. Protein leaching was measured according to the procedure described above. The immobilized PPL was recycled by filtration for the same test again.

The reusability of immobilized lipase was measured as follows: 0.25 g of immobilized enzyme was introduced into a 100 mL flask, and 25 mL of triacetin was added to the flask. Enzymatic activity was obtained according to the procedure described in activity assay process. The immobilized PPL was recycled and dried in a freeze drier for the same test again.

Kinetic Constants of Immobilized PPL. The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the immobilized enzyme were obtained by measuring the reaction rates at optimum reaction conditions for 3 min. For this purpose, immobilized PPL was added to various concentrations of triacetin emulsification solution, and the reaction rates were determined by the $[S]$ – V plots. K_m and V_{max} were calculated from the scatchard plot through the Michaelis–Menten kinetic equations measured by the pH-stat.^{25,37}

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (4)$$

$$\frac{V}{[S]} = \frac{V_{max}}{K_m} - \frac{V}{K_m} \quad (5)$$

where V was the enzymatic reaction rate (mg/mL·min), and $[S]$ was the substrate concentration (mg/mL). The slope of the line was $-1/K_m$, the intercept on the y -axis was V_{max}/K_m , and the intercept on the x -axis was V_{max} (mg·min⁻¹·g PPL⁻¹).

Characterization of Immobilized Enzyme and Carriers. FT-IR spectra were collected via a Nicolet 670 spectrophotometer using the standard KBr disk method. Materials were obtained in the 400–4000 cm⁻¹ wavenumber range. A total of 512 scans at 2 cm⁻¹ resolution in the range of 1600–1700 cm⁻¹ were averaged to obtain each spectrum of free and immobilized lipase. The low-temperature N₂ adsorption was carried out using a Micromeritics ASAP 2020 apparatus at –196 °C. The pore diameter was calculated on the adsorption isotherm using the Barrett–Joyner–Halenda (BJH) method, and the surface area was calculated using the Brunauer–Emmett–Teller (BET) method. The thermo analytical curves were obtained using a Perkin Elmer TGA7 Thermo balance instrument. The operation conditions were adjusted to a temperature heating range of 50–700 °C with a heating rate of 5 °C min⁻¹ and a flow rate of 20 mL min⁻¹ in pure nitrogen atmosphere. Element analysis was obtained using a Perkin Elmer 240 analyzer. Temperature-programmed desorption (TPD) of carbon dioxide was obtained with BELCAT-B-82 equipment. The sample was first calcined at 150 °C for 3 h and subsequently cooled to 100 °C under a helium flow (30 mL min⁻¹). The CO₂-TPD was performed at a rate of 10 °C/min to 300 °C, and kept for 90 min at 300 °C. Transmission electron micrographs (TEM) were performed on a JEOL JEM-2010 microscope operating at 100 kV.

RESULTS AND DISCUSSION

Characterization of Carriers and Immobilized Lipase. Figure 1 reported the nitrogen adsorption–desorption

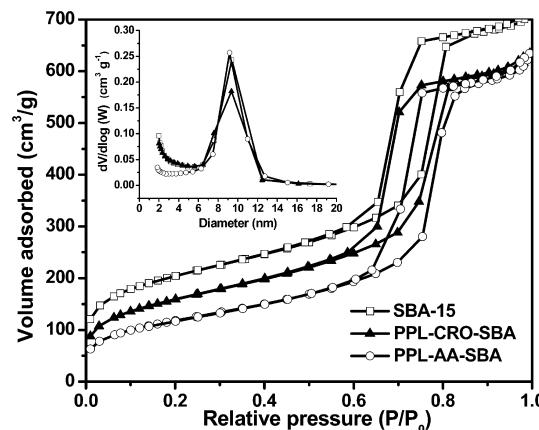


Figure 1. Nitrogen adsorption desorption isotherms of SBA-15, PPL-CRO-SBA, and PPL-AA-SBA.

isotherms and corresponding pore size distribution of carriers and immobilized lipases. Both carriers and lipases exhibited type IV isotherms with H₁ type hysteresis loop. The H₁ type hysteresis was caused by condensation–evaporation, which took place in cylindrical pore openings of SBA-15.^{19,38} These behaviors reflected the regular array of rather cylindrical pore structure of SBA-15 materials. It was indicated that amino acid ionic liquid modification did not cause material construction collapse obviously. It was shown in Figure 1 that the area of hysteresis loop of SBA-15 was larger than that of AA-SBA. This

observation indicated that the mesopores and macropores in SBA-15 were slightly larger than AA-SBA. The volume adsorbed (Y-axis Figure 1) reflected that the surface area of AA-SBA was significantly lower than that of SBA-15. The drop in S_{BET} after modification was attributed to the partial collapse of mesoporous domains. These results could also be confirmed by Table 1. After modification, the pore diameter and pore

Table 1. Pore Structure Parameters of Carriers and Immobilized PPL

sample	S_{BET}^a ($\text{m}^2 \text{ g}^{-1}$)	volume ($\text{cm}^3 \text{ g}^{-1}$)	diameter (nm)	loading of ionic liquid ^b (mg/g SBA-15)
SBA-15	728	1.14	9.5	
PPL-CRO-SBA	574	1.00	9.3	
AA-SBA	430	0.99	9.3	98
PPL-AA-SBA	410	0.96	9.1	

^aAll of the samples were degassed at 80 °C for 12 h before the N₂ adsorption measurement. ^bThe data were calculated from TG profile.

volume decreased from 9.5 nm and 1.14 cm³ g⁻¹ for SBA-15 to 9.3 nm and 0.99 cm³ g⁻¹ for AA-SBA, respectively (Table 1). In comparison with SBA-15, SBA-15 immobilized PPL (PPL-CRO-SBA) showed a marked decrease in the specific surface area (from 728 to 574 m² g⁻¹) and pore volume (from 1.14 to 1.00 m³ g⁻¹). These results also suggested that the modification of AA and immobilization of PPL in the channels of SBA-15 have occurred.

Figure 2A presented the FT-IR spectra of SBA-15 and modified SBA-15 samples. The FT-IR transmittance spectra of materials were obtained in the 400–4000 cm⁻¹ wavenumber range. The typical bands around 1081, 794, and 457 cm⁻¹ were assigned to the stretching vibrations of Si—O—Si moiety and observed on all samples.¹⁶ The presence of peaks at 2950 and 1470 cm⁻¹ corresponded to aliphatic C—H vibrations and imidazole ring stretching, respectively. The IR band at 1665 and 1570 cm⁻¹ can be attributed to C=N and C=C stretching vibrations of imidazole rings.³⁹ For AA-SBA, the C=O adsorption band of —COOH was around 1720 cm⁻¹.⁴⁰ These behaviors indicated that AA has been grafted onto the surface of SBA-15. It is hard to confirm the mode of glutaraldehyde interaction in the FT-IR. It may be that the typical bands of glutaraldehyde were overlapped with the strong broad band of lipase due to the small amount of glutaraldehyde as compared to PPL loading. The FT-IR spectra of amide I and II regions were usually used to study the protein conformation. The amide I region (1600–1700 cm⁻¹) assigned to the stretching mode of C=O vibrations has been widely used to quantify the individual elements of the secondary structure.^{18,24} The amide II band near 1550 cm⁻¹ was obviously weaker than amide I and was not discussed here because its changes were minor after PPL immobilization. The percentage of α -helix near 1650 cm⁻¹ calculated from the amide I region was applied to study the structural perturbations of PPL when attached onto SBA-15. Figure 2B showed FT-IR spectra of free lipase and immobilized PPL in the amide I region. The peaks in the range from 1650 to 1660 cm⁻¹ were assigned to the α -helix of secondary structure. Its relative amount was determined by computing the areas under the assigned bands. The higher content of α -helix demonstrated better retention of 3D structure of lipase. Gaussian deconvolution of the spectrum revealed α -helix

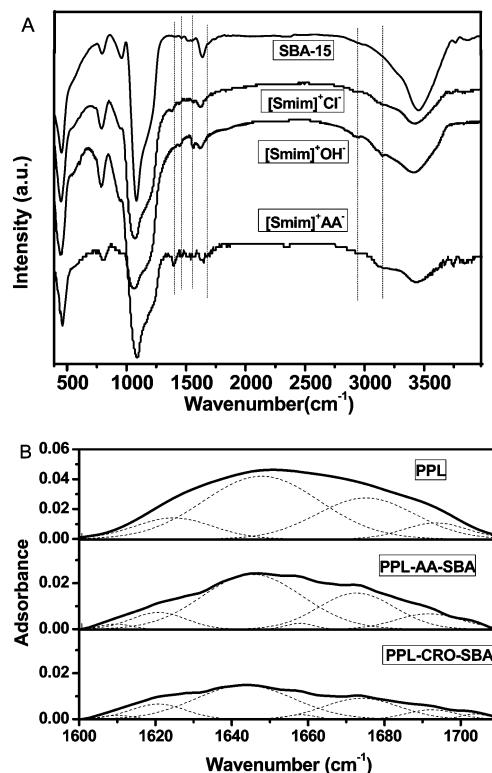


Figure 2. (A) FT-IR spectra of mesoporous materials (a) SBA-15, (b) [Smim]⁺Cl⁻-SBA, (c) [Smim]⁺OH⁻-SBA, and (d) AA-SBA. (B) Fourier self-deconvoluted FT-IR spectra and Gaussian curve-fitting of free lipase and immobilized PPL: (a) free PPL, (b) PPL-AA-SBA, and (c) PPL-CRO-SBA.

content of 25% for PPL-AA-SBA as compared to 16% for PPL-CRO-SBA, corresponding to the results that the activity of PPL-AA-SBA was higher than PPL-CRO-SBA (Table 2).

The CO₂-TPD profiles for (a) [Smim]⁺Cl⁻-SBA, (b) [Smim]⁺OH⁻-SBA, and (c) AA-SBA were shown in Figure 3. It was proved that AA-SBA was successfully synthesized. As shown in Figure 3, there was mainly one desorption peak in the CO₂-TPD profiles around 225 °C. The base amount of Smim⁺Cl⁻-SBA, Smim⁺OH⁻-SBA, and AA-SBA changed significantly in the range of our research. The base amount of Smim⁺OH⁻-SBA was 20.17 mmol/g and increased dramatically as compared to Smim⁺Cl⁻-SBA (1.17 mmol/g). It was deduced that OH⁻ groups existed on the surface of Smim⁺OH⁻-SBA. Meanwhile, the base amount of AA-SBA was 3.19 mmol/g and more than Smim⁺Cl⁻-SBA due to the special CO₂ absorption capacity of amino acid ionic liquid.⁴¹

The thermal gravimetric analysis of the ionic liquid-modified carrier was used to evaluate the loading of amino acid ionic liquid. The thermal analysis curves in Figure 4 revealed that the ionic liquid-modified carrier AA-SBA was stable up to 252 °C. A gradual weight loss was observed at higher temperature. The loading of ionic liquid was determined by the difference in AA-SBA mass before and after thermal desorption process.³¹ In Figure 4, the first weight loss of 3.6% occurring at 73 °C should be due to water release. The second weight loss from 252 °C should be due to decomposition of AA-SBA and the weight loss is 9.8%, and so it was deduced that the loading of ionic liquid was 98 mg/g SBA-15. Analysis calculated for amino acid ionic liquid C₁₄H₂₈N₄O₅Si was C, 46.65%; N, 15.54%; C/N, 3.0. The C/N analysis of ionic liquid [Smim]⁺OH⁻ and [Smim]⁺Cl⁻

Table 2. Results of Immobilization Process of PPL

sample	enzyme immobilization ^a		enzyme assay ^b		
	immobilization efficiency (%)	lipase content (mg lipase/g immobilized PPL)	expressed activity U/g immobilized PPL	specific activity U/g PPL	relative activity (%)
PPL-CRO-SBA	94 ± 2	190	30 ± 2	158	69
PPL-AA-SBA	98 ± 1	197	48 ± 3	244	107

^aImmobilization conditions: time 4 h, temperature 30 °C, pH 7.0 Tris-HCl buffer solution. ^bReaction conditions: optimum temperature and pH, respectively. Activity of free PPL: 228 U/g; relative activity of free PPL was defined as 100%.

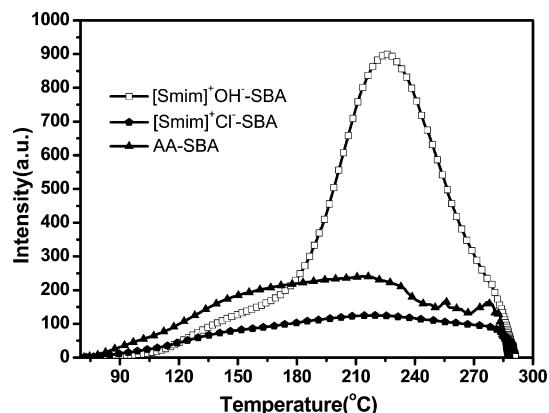


Figure 3. CO₂-TPD profiles for (a) [Smim]⁺Cl⁻-SBA, (b) [Smim]⁺OH⁻-SBA, and (c) AA-SBA.

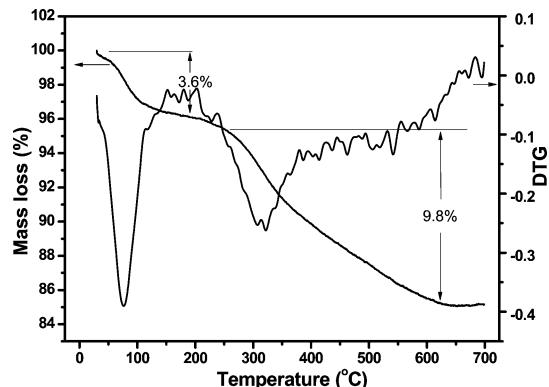


Figure 4. Thermal analysis of AA-SBA to assay the loading of ionic liquid.

was 3.9. From the element analysis results, we found amino acid ionic liquid C₁₄H₂₈N₄O₅Si in AA-SBA was C, 4.24%; N, 1.32%; C/N, 3.2. We deduced that the purity of amino acid ionic liquid C₁₄H₂₈N₄O₅Si in AA-SBA was 77.8%. The loading of amino acid ionic liquid combined element analysis with thermal gravimetric analysis was 76 mg/g SBA-15 (Table 1).

The TEM micrographs shown in Figure 5a directly noted the arrays of long-range mesopore channels of parent SBA-15. Figure 5b, [Smim]⁺OH⁻-SBA, and Figure 5c, AA-SBA, showed that the ordered hexagonally arrangement and size uniformity became more vague than SBA-15, but they were still observed. It was clearly indicated that the modification of amino acid ionic liquid onto the surface of SBA-15 did not destroy the long-range mesopore channels and Si—O—Si network of the support materials, consistent with the result of N₂ adsorption and FT-IR.¹⁷

Activity Measurements and Kinetic Constants. Figure 6 shows the reaction temperature profiles for PPL-CRO-SBA

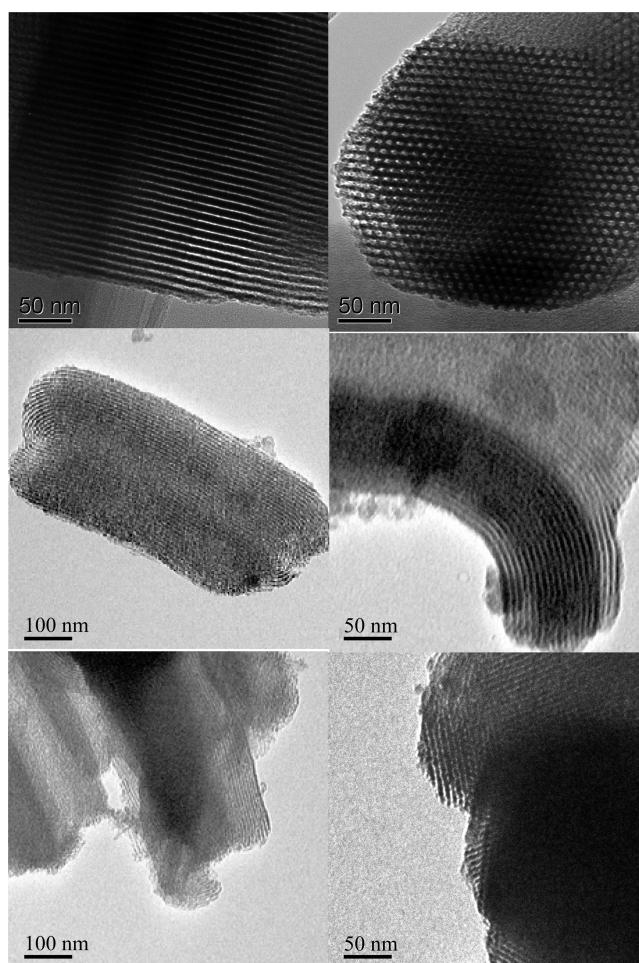


Figure 5. TEM images of (a) SBA-15, (b) [Smim]⁺OH⁻-SBA, and (c) AA-SBA.

and PPL-AA-SBA. As compared to PPL-AA-SBA, the relative activity of PPL-CRO-SBA fluctuated dramatically. We could find that PPL-AA-SBA was insensitive to temperature changes, and its relative activity was always up 90%. The optimum temperature value for PPL-CRO-SBA was 45 °C, and that of PPL-AA-SBA was heightened to 50 °C. The temperature increasing of PPL-AA-SBA would be suitable for the conformational adjustment of PPL immobilized onto AA-SBA by cross-linking and ionic-binding.⁴² Furthermore, increasing temperature facilitated substrate diffusion into meso-channels of the support, which is helpful to increase the relative activity.

The optimum pH values of PPL-CRO-SBA and PPL-AA-SBA were shown in Figure 7. Their responses to optimum pH were similar with maximal activities at pH 7.5. However, we could find that PPL-AA-SBA was more stable in the range pH

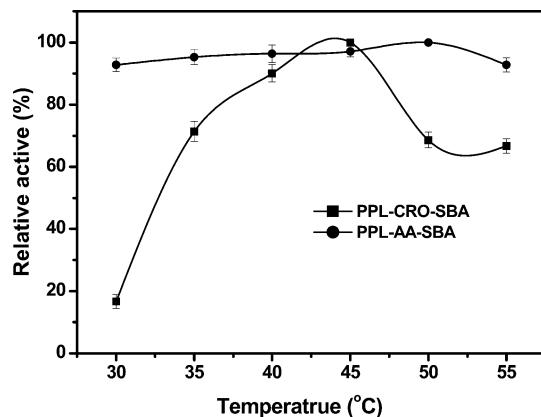


Figure 6. Effect of reaction temperature on enzyme activity.

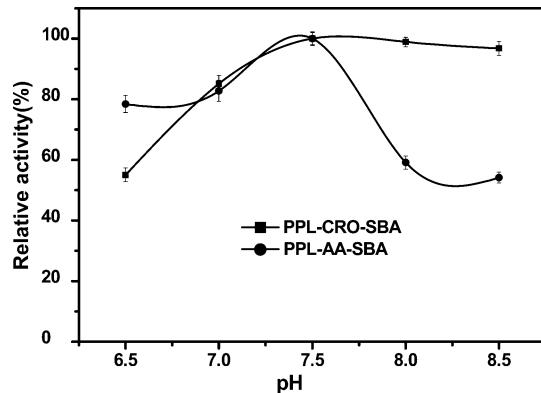


Figure 7. Effect of reaction pH on enzyme activity.

6.5–7.5 and could retain above 78% of the highest enzyme activity. It may be because that lipase was modified by ionic liquid anion via its free amino groups, that grafting of ionic liquid provided the enzyme with a more polyanionic character. Moreover, the little electrostatic repulsion at pH 7.0–7.5 between the amino acid residues provided PPL a benign environment.

Kinetic constants of lipase, including K_m and V_{max} values (Table 3), were determined by using triacetin emulsification

Table 3. Kinetic K_m and V_{max} of Immobilized PPL

sample	V_{max}^a mg/min·g PPL	K_m mg/mL	V_{max}/K_m^b
PPL-CRO-SBA	242	22.8	10.6
PPL-AA-SBA	332	23.1	14.4

^a K_m and V_{max} were calculated from the scatchard plots using $[S]-V$ data measured by pH-stat. ^bCatalytic efficiency was defined as the ratio of V_{max}/K_m .

solution as substrate. The affinity change of the PPL to its substrate reflected by K_m was probably caused by opening the active site of enzyme after ionic liquid modification and by higher accessibility of the substrate to surface of AA-SBA. The activities of the immobilized PPL were plotted in the form of $[S]-V$ plots, as shown in Figure 8, and V_{max} and K_m values were calculated from the intercepts on x - and y -axes, respectively. Only a minor change in K_m was observed for PPL-CRO-SBA and PPL-AA-SBA. Yet the apparent V_{max} value of PPL-AA-SBA was higher than that of PPL-SBA. It was about 159% of that of the PPL-CRO-SBA (Table 3). The catalytic efficiency (V_{max}/K_m)

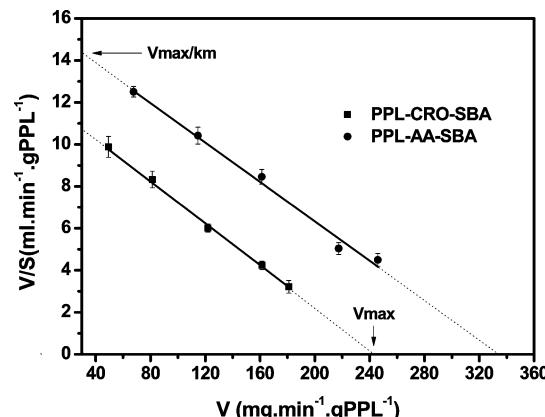


Figure 8. Scatchard plot of kinetic parameters of PPL-CRO-SBA and PPL-AA-SBA (immobilized PPL (0.25 g) was added into triacetin emulsification solution with different concentrations (0.5%, 1%, 2%, 4%, and 6% (w/v)).

K_m) of the AA-SBA immobilized PPL was higher than PPL-CRO-SBA, which was due to the unique polarity, amphiphilicity of the AA-SBA;³⁷ the detailed results were given in Table 3.

Operational Stability of Immobilized Lipases. The operational stabilities were important indexes for biocatalysts. The thermal stability, protein leaching, and reusability of PPL-CRO-SBA and PPL-AA-SBA were investigated in this work by monitoring the relative activity.

As shown in Figure 9, the thermal stability of PPL-CRO-SBA and PPL-AA-SBA was investigated by incubating the lipase at

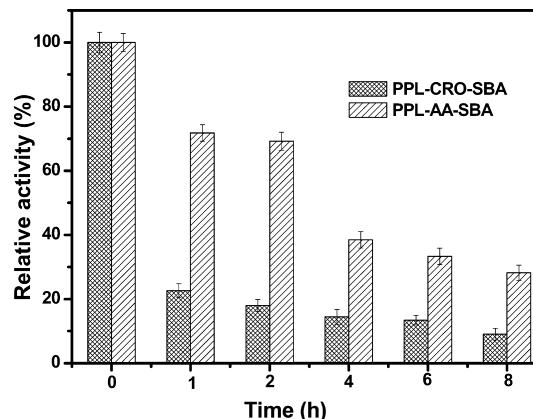


Figure 9. The thermal stability of PPL-CRO-SBA and PPL-AA-SBA. All reactions were carried out after incubation at 60 °C and pH 7.5 for different time (activity assay condition: 25 mL of 2.73% (w/v) triacetin emulsification solution at respective optimum reaction condition for 10 min; the initial activity before incubation was defined as 100% relative activity).

60 °C for different times. The relative activity of PPL-AA-SBA remained comparatively stable and holds over 69% of its initial hydrolysis activity after 2 h, while that of the PPL-CRO-SBA decreased quickly with the increased treatment time and retains only 17% of its initial activity. It is indicated that the PPL-AA-SBA held much more excellent heat resistance than PPL-CRO-SBA. The same results could be obtained from the optimal temperature experiment (Figure 6). Comparing between PPL-CRO-SBA and PPL-AA-SBA, PPL-AA-SBA exhibited better thermal stability. The optimum reaction temperature for PPL-

CRO-SBA was 45 °C, but that for PPL-AA-SBA was heightened to 50 °C. The relative activity of PPL-CRO-SBA was 67% at 55 °C, while the relative activity of the PPL-AA-SBA was 93% at the same temperature. One possible reason was that the PPL-AA-SBA was less susceptible to denaturation in the channel of AA-SBA. It could be interpreted that modified carrier could keep enzyme from injuring due to the biocompatibility of amino acid ionic liquid.^{33,37} However, probably attributing to the low thermal stability nature of amino acid ionic liquid (Figure 4), PPL-AA-SBA's thermal stability was not very high and needs further improvement.

Figure 10 showed the PPL leaching amount of PPL-CRO-SBA was 7% (from 94% to 87%) after five cycles (left Y-axis).

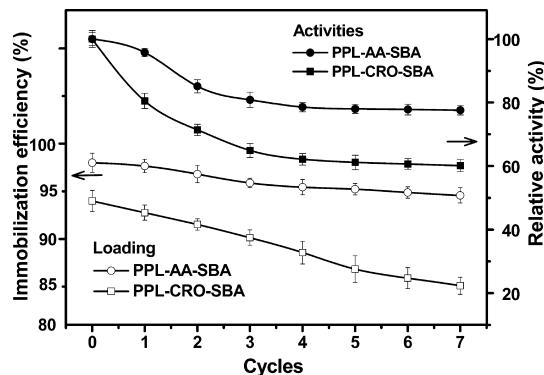


Figure 10. Residual immobilization efficiency (left Y-axis) and reusability (right Y-axis) of PPL-CRO-SBA and PPL-AA-SBA in five recycle runs. Error bars represent deviation from the mean for two separate experiments (assay condition: optimum reaction condition for 10 min, respectively; relative activity without recycle was defined as 100%).

However, the PPL leaching amount of PPL-AA-SBA was only 2% (from 98% to 96%). It is indicated that immobilization only by cross-linking was not strong enough to prevent PPL leaching.⁴³ In this work, lipase immobilized onto the surface of AA-SBA via ionic-binding and cross-linking methods prevented PPL leaching and enhanced the PPL stability. Figure 10 also showed the reusability of PPL-CRO-SBA and PPL-AA-SBA (right Y-axis). It was observed that PPL-CRO-SBA retained only 61% of initial activity after the fifth reuses, whereas PPL-AA-SBA was still above 78% retention. The relative activities and protein loading after the fourth cycle were changed slightly. These results further confirmed that PPL-AA-SBA could be a rather stable and efficient biocatalyst, which successfully resolved the problem of low immobilization efficiency and stability in previous works. Lower relative activity of PPL-CRO-SBA was mainly attributed to protein denaturation and PPL leaching from the domain of the carrier (Figure 10). The thermal stability, multiple soaking, separation, and washing steps also affected the enzymatic activity in the successive experiments.⁴²

CONCLUSION

In this work, mesoporous material SBA-15 was first functionalized by amino acid ionic liquid (AA-SBA). The prepared material AA-SBA as a novel carrier was used to immobilize PPL through ionic bonding interaction and cross-linking methods (PPL-AA-SBA). The results indicated that AA-SBA makes PPL more resistant to temperature increment. Immobilization efficiency of PPL was up to 98% via ionic bonding and cross-

linking, as compared to 53% of previous physical adsorption, and the loading was dramatically improved. The leaching investigation in consecutive use showed that AA-SBA surface inhibited the enzyme leaching to some extent and immobilization efficiency was always above 96% after five cycles. Because of high immobilization efficiency, PPL immobilization on AA-SBA was still above 78% retention of initial activity after five reuses. These results illustrated that with the combination of the advantages of novel carrier AA-SBA and special immobilization method, PPL-AA-SBA was a rather stable and efficient biocatalyst, which successfully resolved the problem of low immobilization efficiency and stability in previous works. Although the stability of lipase immobilized onto AA-SBA was dramatically improved as compared to physical adsorption, further research is ongoing to enhance the activity.

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Notes

The authors declare no competing financial interest.

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