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Enhancing Catalytic Performance of Porcine Pancreatic Lipase by Covalent Modification Using Functional Ionic Liquids

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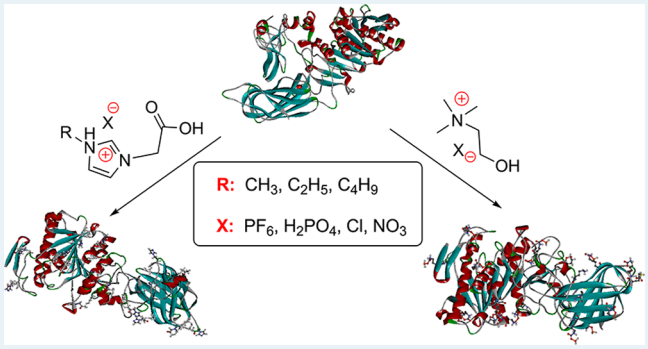
Supporting Information

ABSTRACT: Various functional ionic liquids (ILs) composed of different cations and anions were activated with carbonyldiimidazole and then covalently linked onto porcine pancreatic lipase (PPL) through lysine coupling. Catalytic performances, such as activity, thermostability, and enantioselectivity were improved successfully, as was investigated in *p*-nitrophenyl palmitate (pNPP) hydrolysis and racemic 1-phenethyl acetate hydrolysis reaction. The correlation between catalytic performance and modification of IL was studied by catalytic and spectroscopic data, which showed improvement of catalytic performances to a different extent. Hydrolytic activity was enhanced by ILs with chaotropic cations and kosmotropic anions (e.g., more than 4-fold with [choline]-[H_2PO_4]). Modifications by ILs bearing kosmotropic cations and chaotropic anions contribute to lipase thermostability and enantioselectivity (e.g., modification with [HOOCBMIm][Cl] showed a 12-fold thermostability increase at 60 °C and more than 7-fold enantioselectivity enhancement than native enzyme). The Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry experiments suggest that ILs bind with lipase protein. Conformation changes were confirmed by fluorescence spectroscopy, and circular dichroism spectroscopy.



KEYWORDS: *enzyme catalysis, ionic liquids, protein modifications, specific ion effects, structure−activity relationship*

INTRODUCTION

Over the past decade, scientific and technological advances have established biocatalysis as a practical and environmentally friendly alternative to traditional metallo- and organocatalysis in chemical reactions, both in the laboratory and on an industrial scale.[1] Lipases are important biocatalysts that catalyze various reactions, such as esterification, interesterification, aminolysis, and hydrolysis, which produce numerous important chemicals including alcohols, esters, acids, and amine.[2,3] Lipases tend to form bimolecular aggregates even at very low concentrations, these aggregates have very different properties when compared with the monomeric form of the lipases, this makes the study of the enzyme very complex and difficult to reproduce or understand the differences between different researches,[4−7] and dimers that may be broken by detergents (e.g., Triton X-100).[8] Porcine pancreatic lipase (PPL) is one of the most widely used lipases in biotransformation reactions because it is cheaper and more accessible compared with other commercial enzymes; however, low activity and stability of PPL are usually observed.[9]

Chemical modification is an important approach to alter protein function by introducing non-natural fragments into proteins.[10] This specific modification can expand the efficiency of native biocatalysts,[11,12] also enzyme properties may be improved via chemical modification compatible with a proper immobilization.[13,14] Several modifiers that improve the catalytic performance of PPL have been reported in recent years. Improving 20% thermostability was observed by grafting Z-proline on PPL in the temperature range from 20 to 50 °C with respect to native enzyme, with no significant increase in hydrolysis activity.[15] Onefold thermostability and 20% hydrolysis activity at 50 °C of PPL increased when modified with phthalic anhydride.[16] However, simultaneous and noticeable increase in both activity and stability were not obtained by the aforementioned modifiers nor was enantioselectivity assayed. Therefore, developing a novel and efficient chemical modification method is necessary to further improve the catalytic performance of PPL.

Ionic liquids (ILs), which act as solvents or additives, have been widely used in enzymatic catalysis and present enzymes with excellent activity and stability because of their environmentally friendly and desirable features.[17−19] In our previous

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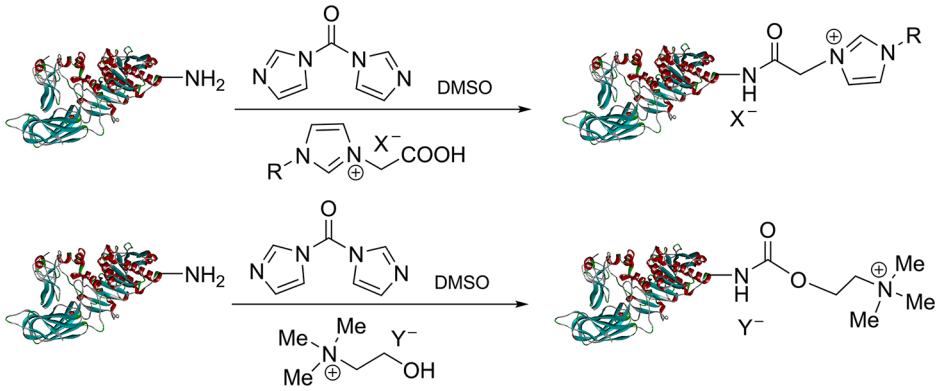
Scheme 1. Chemical Modification of PPL with Functional Ionic Liquids



studies, functional ionic liquids were synthesized and grafted onto the surface of mesoporous SBA-15; high activity and stability of immobilized PPL was obtained.[20−23] Recently, Bastien Doumèche et al. reported that IL covalently bound to formate dehydrogenase to improve its stability and activity in ILs, and found a higher modified degree from a more chaotropic cation, contributing to significantly improved activity and stability.[24,25]

In this study, PPL was first modified by a series of ILs composed of different cations and anions (Scheme 1). The modification degree, activity, kinetic parameters, thermal stability, and enantioselectivity were investigated. This study aims to develop a novel, efficient, and practical method to enhance the catalytic performance of lipase and propose useful guidelines for designing functional ILs applied to modify lipase.

EXPERIMENTAL SECTION

Unless otherwise noted, all reagents are of analytical grade or higher. PPL (type II), *p*-nitrophenyl palmitate (98%), *p*-nitrophenol (99.5%), and 2,4,6-trinitrobenzenesulfonic acid solution (5% w/v in H_2O) were purchased from Sigma-Aldrich China Inc. (*R*,*S*)-1-Phenethyl acetate was prepared according to standard procedure. Bisuccinic acid (BCA) protein assay reagent kit was obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). All seven functional ILs (99%, HPLC) were purchased from Shanghai Cheng Jie Chemical Co., Ltd. Carbonyldiimidazole (97%) was from Aladdin Chemistry Co., Ltd., matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectra were obtained using a BRUKER Auto FLEX-T2 apparatus.

Enzyme Preparation. The PPL solution was obtained by solubilizing the enzyme powders (2 g) in 50 mL of the deionized water, with magnetic stirring for 30 min at 4 °C. Solubilization was followed by centrifugation and subsequent concentration with ammonium sulfate precipitation. Finally, PPL was transferred into a 10 kDa dialysis membrane to remove excess salt. The purity of the enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Chemical Modification. Two milliliters anhydrous dimethyl sulfoxide (DMSO) containing IL (500 mM) and carbonyldiimidazole (500 mM) were stirring in dark for 2 h at 25 °C to activate the functional IL.[26] After that, 200 μL reactant without separation were added into 10 mL of PPL solution (100 μM) in deionized water (pH 7.0), with magnetic stirring for 8 h at 4 °C. The modified PPL was purified by ultrafiltration in deionized water using a 10 kDa membrane. Enzymes were stored in solution at 4 °C or used directly after modification. Enzyme concentration was determined via the BCA method using bovine serum albumin (BSA) as standard.[27] The modification degree was investigated using the trinitrobenzene sulfonate (TNBS) assay procedure.[28]

Enzyme Activity. The activity of the enzyme to catalyze the hydrolysis of *p*-nitrophenyl palmitate (pNPP) to *p*-nitrophenol (pNP) was assayed following the procedures described by Kordel et al.,[29] with slight modification. The substrate solution was prepared by mixing one volume of a solution of pNPP (16.5 mM) with 9 volumes of buffer (50 mM phosphate pH 7.5) containing 0.4% (w/v) Triton X-100 and 0.1% (w/v) Arabic gum. Then a volume of 10 μL of an appropriate dilution of the enzyme solution was added to 240 μL substrate solution to start the reaction at 25 °C, the pNP formation was monitored at 405 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). Under the conditions used, the extinction coefficient of *p*-nitrophenol was 8620 M^{-1} cm^{-1}. One unit of lipase activity was defined as the amount of lipase releasing 1 μmol of *p*-nitrophenol from pNPP per minute.

Enzyme Thermostability. The enzyme solution in deionized water was incubated at 30, 40, 50, and 60 °C, respectively. Samples of 30 μL were taken periodically for activity assay at 25 °C, as previously mentioned. The time-dependent loss in activity was used to calculate the half-life for each enzyme solution.

Kinetic Measurements. The reaction was performed as described in "Assay of Enzyme Activity". The substrate concentration in the reaction mixture varied between 0.08 and 0.83 mM at a constant enzyme concentration (0.1 mg/mL) for 4 min. The maximum rate (V_{max}) and the Michaelis constant (K_M) of the enzyme were determined by Hanes−Woolf plots.

Enantioselectivity Measurements. (*R*,*S*)-1-phenethyl acetate (60 mM) was added to 10 mL aqueous buffer (100 mM, pH 7.5), followed by ultrasonic dispersion for 10 min, before the enzyme (5 mg) was finally added. The mixture was stirred in 150 rpm at 25 °C. At an appropriate time interval, aliquots (0.2 mL) were withdrawn, then both the 1-phenylethanol-produced and the residual ester were extracted into ethyl acetate (0.2 mL). The organic layer was dried over Na_2SO_4, and then subjected to HPLC analysis on a chiral column (Chiralcel OZ-H, hexane/2-propanol = 98/2). In each case, the nonenzymatic reaction was subtracted from the total reaction.

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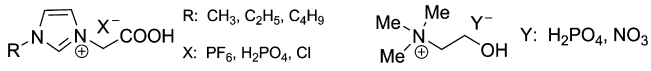
Table 1. ILs Used in This Study



number	ionic liquid	cation	anion	IUPAC name
1a	[HOOCMMIm][Cl]	chaotropic	chaotropic	3-(2-carboxymethyl)-1-methylimidazolium chloride
1b	[HOOCEMIm][Cl]	chaotropic	chaotropic	3-(2-carboxymethyl)-1-ethylimidazolium chloride
1c	[HOOCBMIm][Cl]	kosmotropic	chaotropic	3-(2-carboxymethyl)-1-butylimidazolium chloride
1d	[HOOCBMIm][H_2PO_4]	kosmotropic	kosmotropic	3-(2-carboxymethyl)-1-butylimidazolium dihydrophosphate
1e	[HOOCMMIm][PF_6]	chaotropic	chaotropic	3-(2-carboxymethyl)-1-methylimidazolium hexafluorophosphate
1f	[choline][NO_3]	chaotropic	chaotropic	choline nitrate
1g	[choline][H_2PO_4]	chaotropic	kosmotropic	choline dihydrophosphate

Fluorescence Spectroscopy. The fluorescence spectra of the dilute enzymes (0.5 μM) were monitored using a spectrofluorometer (PerkinElmer LS55, U.S.A.) at 25 °C and excited at 270 nm. The emission registered from 300 to 400 nm using a 5-nm bandwidth in both excitation and emission paths.

Circular Dichroism (CD) Spectroscopy. Circular dichroism (CD) (190−250 nm) spectra were recorded on a JASCO-J810 Spectropolarimeter (Jasco Co., Japan) in a cell with 1 cm light path length at 25 °C. The scanning rate was set at 50 nm/min and the spectra were the average of three readings of a highly dilute enzyme solution (0.5 μM). Baseline correction was automatically carried out with the deionized water spectrum during the complete collection time. The secondary structure parameters of the enzymes were computed using the Jwsse32 software according to the reference.[30]

RESULTS AND DISCUSSION

Design of Different Cations and Anions of ILs Used in This Study. Specific ion effects have been well-known in biology for over a century.[31] In recent years, numerous reports focused on the relationship between the ionic nature of ILs and the enzyme properties, with kosmotropic ions having high surface charge density and strong hydration, whereas the opposite conditions were observed in chaotropic ions.[32−34] In this study, seven kinds of ILs containing different typical kosmotropic/chaotropic ions were designed, as listed in Table 1. The chaotropicity of cations is in the order of choline$^+$> MMIm$^+$> EMIm$^+$> BMIm$^+$, with the first three being chaotropes and the last one being kosmotropic cation.[35] The chaotropicity of anions vary in the order of PF_6^-> NO_3^-> Cl^-, whereas $H_2PO_4^-$ is considered a kosmotrope.[36]

Modification of PPL with ILs. PPL is a globular protein composed of a single chain of 449 amino acids, with a molecular weight of 50−52 kDa. The open conformation of the lid domain in PPL is stabilized by hydrogen bonds between the lid and colipase, which allows the access of the substrate to the active site of the lipase. Colipase is a small protein cofactor with molecular weight of 10 kDa which anchors lipase to the bile salts to the lipid/water interface.[9] Also, commercial preparation of PPL contains several contaminant proteins with lipolytic activity, among them are three main components: the actual PPL, a lipase of 33 kDa and a protein with approximately 25 kDa, those may give very different results when they are used as biocatalysts.[37−39] So the purification of crude PPL is necessary before any modification (Supporting Information, Figures S1).

Various functional ILs were activated with carbonyldiimidazole before being covalently linked onto the lysine residues.[26] The modification degree was controlled by the amount (molar ratio) and time of the activated ILs grafted onto lysine residue. Figure 1A and 1B (taking PPL-1a for example) show that the modification degree was proportional to the molar ratio and

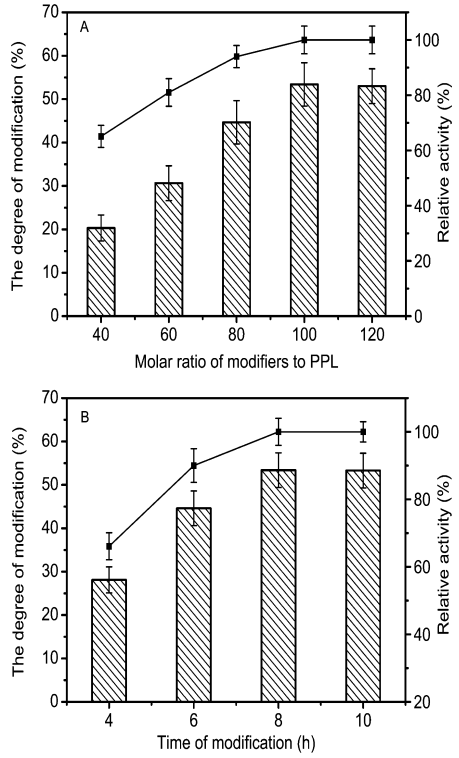


Figure 1. Modification degree and activity of PPL-1a (line + symbol, activity; column, modification degree). Reaction conditions: (A) different molar ratios of modifiers for 10 h and (B) different modification time at optimized molar ratio.

time of activating groups to amino groups, and plateau values were observed at the ratio of 100:1 and 8 h. The enzyme with a higher modification degree showed higher activity. The other ILs modified on PPL showed the same tendency (Supporting Information, Figures S2−S7). Therefore, considering both modification degrees and activities, the molar ratios of 100:1 and 8 h of activating ILs to PPL were the appropriate concentration and modification time, respectively.

MALDI-TOF mass spectra experiments were conducted to confirm the IL binding with the lipase protein. The number of modified lysine residues is determined in Figure 2. The mass spectrum (MS) of the native enzyme presents a single peak at m/z 51,725 (Figure 2A). The grafting of cation (IL-1a) onto PPL resulted in a heterogeneous population of seven proteins containing 7 to 13 modified lysine residues (Figure 2B). Each

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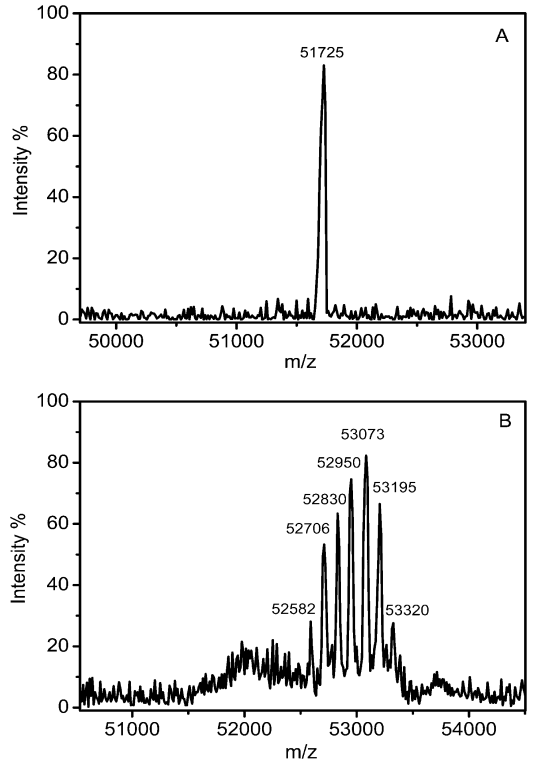


Figure 2. Results of MALDI-TOF mass spectra experiments on PPL (A) and PPL-1a (B).

cation (IL-1a) led to the increased mass of 123, and PPL contains 21 lysine residues (PDB ID 1ETH), numbers or degree of modification could be calculated with the data showed above; the other MS were shown in Supporting Information Figures S8−S10.

Table 2 shows the average modification degree investigated using TNBS assay procedure, which agree with the MS results.

Table 2. Degree of Amino Groups Modified on PPL Molecule

enzyme	numbers of amino groups modified (MS)	degree of amino groups modified (%) (TNBS)[a]
PPL-1a	7−13	53.4 ± 2.8
PPL-1b	5−10	36.8 ± 2.4
PPL-1c	3−6	22.6 ± 1.2
PPL-1d	3−7	27.9 ± 2.1
PPL-1e	6−11	41.2 ± 2.3
PPL-1f	7−12	46.3 ± 2.6
PPL-1g	9−16	65.3 ± 3.5

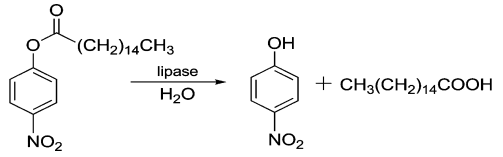
[a]Conditions: 0.25 mL of 0.01% TNBS was added to 0.75 mL phosphate buffer (0.025 mol/L, pH 8.2, containing 66 μg/mL enzymes) then incubation at 37 °C. After 2 h, 0.5 mL sodium dodecyl sulfate solution (10%) and 0.25 mL hydrochloric acid solution (1 mol/L) were added then measured at 335 nm in a UV-1200.

For the same anion, the more chaotropic cations resulted in higher modification degrees. These values were obvious for PPL-1a, PPL-1b, and PPL-1c, whose modification degrees were 53.4%, 36.8%, and 22.6%, respectively. Doumèche[24,25] reported that this higher number of grafted residues is partially explained by lower steric hindrances induced by the cation size and the alkyl chain flexibility.

Anions also have an important function in modification. For PPL-1a and PPL-1e, PPL-1c and PPL-1d, and PPL-1f and PPL-1g, their modified ILs have the same cations. A higher modification degree was obtained with a more kosmotropic anion. A microaqueous phase surrounds the enzyme molecules, and the modifier should penetrate this aqueous layer for direct contact with the enzyme for modification. Thus, the activated cation will be easy to graft onto the enzyme with a more kosmotropic anion by preferential hydration.

Hydrolytic Activity Assay. Table 3 shows that the activities of all seven modified enzymes compared with native

Table 3. Kinetic Parameters of Enzymes[a]



enzyme	V_{max} (U/g)	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
PPL	15.43 ± 1.56	0.297 ± 0.006	2.69
PPL-1a	53.52 ± 2.34	0.345 ± 0.007	8.02
PPL-1b	47.89 ± 2.57	0.315 ± 0.005	7.86
PPL-1c	22.17 ± 2.11	0.274 ± 0.005	4.19
PPL-1d	28.29 ± 2.03	0.286 ± 0.006	5.12
PPL-1e	39.98 ± 3.03	0.321 ± 0.005	6.44
PPL-1f	43.51 ± 2.36	0.332 ± 0.007	6.78
PPL-1g	64.18 ± 2.89	0.349 ± 0.008	9.51

[a]Conditions: substrate concentrations varied between 0.08 mM and 0.83 mM, protein content (0.1 mg/mL), sodium phosphate buffer (pH 7.5, 50 mM) were performed at 25 °C for 4 min.

enzyme in aqueous solution varied broadly. PPL was activated by the modification of all seven ILs. PPL was activated highest at 415.94% (by [choline][H_2PO_4]) and lowest at 143.68% (by [HOOCBMIm][Cl]) of the activity relative to native enzyme in pNPP hydrolysis. In the presence of ILs with the same anions, the enzyme activity decreased in the order of PPL-1a> PPL-1b> PPL-1c, whereas the order was PPL-1a> PPL-1e, PPL-1g> PPL-1f, PPL-1d> PPL-1c for ILs holding the same cations.

The results indicate that when owning the same anions, modified ILs with more chaotropic cations produce higher improvement of activity. This result is probably correlated with the higher modification of lysine residues compared with kosmotropic cation, which is suspected to prevent more efficient lipase unfolding. The specific ion effect of anions on lipase showed that the more kosmotropic the anion was, the higher the activity observed.[40] Similar results were obtained in this study. Kosmotropic anion possibly facilitates more cations to graft onto the enzyme surface with improved hydration, resulting in higher modification and activity. The K_M values were slightly altered, and K_M slightly increased after modification, except for PPL-1c and 1d. The catalytic efficiency (k_{cat}/K_M) of modified enzymes increased up to 1.56 to 3.54 than native enzyme because of the slight change in K_M values. A slight decrease in K_M of PPL-1c and 1d were observed compared with the other enzymes. These changes of lipase after ionic liquids modification could be attributing to the open-closed equilibrium of the lipase toward the active form.

Thermostability Assay. The thermostabilities of both native enzyme and modified enzymes were studied by measuring the residual activities of the enzymes after incubation

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in aqueous solution for appropriate periods at different temperatures (30, 40, 50, and 60 °C). Table 4 shows that

Table 4. Thermostability of Modified PPL Compared with Native Enzyme in Aqueous Solution

	half life (min)			
enzyme	30 °C	40 °C	50 °C	60 °C
PPL	75 ± 3	27 ± 2	10 ± 1	2 ± 0.5
PPL-1a	75 ± 4	50 ± 3	31 ± 2	10 ± 1
PPL-1b	85 ± 7	62 ± 3	32 ± 3	13 ± 2
PPL-1c	100 ± 6	73 ± 3	50 ± 3	24 ± 3
PPL-1d	85 ± 5	60 ± 2	35 ± 3	20 ± 1
PPL-1e	130 ± 10	93 ± 5	62 ± 4	31 ± 3
PPL-1f	105 ± 8	52 ± 4	31 ± 2	12 ± 2
PPL-1g	66 ± 4	41 ± 3	22 ± 2	10 ± 1

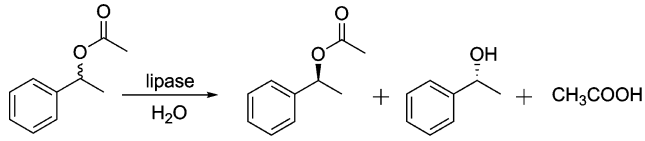
modified enzymes exhibited higher stability. The anions and cations significantly influenced the thermostabilities, rather than the modification degree, compared with the effect on hydrolytic activity. At each incubation temperature, the lipase stabilized in the presence of imidazolium ILs with the same anion varied in the order of: PPL-1c> PPL-1b> PPL-1a. For ILs with the same cations grafted on PPL, improved thermostability was observed with a more chaotropic anion.

When ILs were grafted covalently onto the enzyme surface, bulk water was perturbed by the cations. $BMIm^+$ is a kosmotropic ion with a high charge density that interacts more strongly with water than $MMIm^+$ and $EMIm^+$. Thus, $BMIm^+$ tends to protect essential water molecules, which stabilizes lipase. Anion has a large effect on enzyme stability.[41] The results emphasized that better thermostability can be observed, such as PPL-1a and PPL-1e, by changing only the anion to a more chaotropic one. Although $MMIm^+$ is a chaotropic cation, with a more chaotropic PF_6^-, this cation showed excellent thermostability, as previously found for lipases.[42,43]

Enantioselectivity Assay. All the eight enzymes have been tested in the hydrolysis of racemic 1-phenethyl acetate. The data in Table 5 show that using grafted ILs on PPL in the lipase-mediated resolution of racemic 1-phenethyl acetate can significantly enhance activity and enantioselectivity. The PPL-1a and PPL-1g showed high hydrolytic activity, achieving higher conversion with almost half time than native enzyme, in contrast, the PPL-1c and PPL-1d displayed a very low activity similar to native enzyme, and the results were in agreement with the consequence of hydrolysis of pNPP. From the data summarized in Table 5, seven different modified enzymes increased enantioselectivity to a different extent, PPL-1a and PPL-1g owned great hydrolytic activity with only slight increase of enantioselectivity, while PPL-1c which owned more than 7-fold enantioselectivity compared with that of native enzyme, furnishing (*R*)-1-phenylethanol in 98.3% ee_p, corresponding to an *E* value of 290, even though the hydrolytic activity without significant improvement.

All tested hydrolysis reactions of racemic 1-phenethyl acetate employing the seven different modified enzymes significantly increased enantioselectivity than catalyzing with native enzyme. The results of increased enantioselectivity may be the introduction of the ILs chemical modification on enzyme caused a conformational change on the enzyme and form the active open-lid conformation of the enzyme.[46−48] In the case of PPL-1a, PPL-1b, and PPL-1c, with the same anion, more

Table 5. Results of Lipase-Catalyzed Hydrolysis of (*R*,*S*)-1-Phenethyl Acetate[a]



enzyme	time (h)	ee_s (%)	ee_p (%)	conv. (%)[b]	*E* value[b]
PPL	5	75	89	46	39
PPL-1a	3	88	92	49	71
PPL-1b	4	85	93	48	76
PPL-1c	5	80	98	45	>200
PPL-1d	5	82	98	46	>200
PPL-1e	4	85	97	47	183
PPL-1f	4	86	95	48	113
PPL-1g	3	89	94	49	101

[a]Conditions: (*R*,*S*)-1-phenethyl acetate (60 mmol), enzymes (5 mg), and sodium phosphate buffer (10 mL, pH 7.5, 100 mM) were performed at 25 °C and 150 rpm. [b]Calculated by ee_p and ee_s. $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$, here *c* means conv., which was calculated by the following formula: $c = ee_s/(ee_s + ee_p)$.[44,45]

kosmotropic cation leads to higher enantioselectivity and the hydrophobicity of modified ILs increase with increasing length of the alkyl group attached on the cation. When grafting onto enzyme, cations have a highly important function in affecting enantioselective performance. Higher enantioselective efficiency was obtained with a more kosmotropic cation. Kosmotropic ion interacts more strongly with water, thus, this ion can help maintain the enzyme conformation and stabilized the enzyme.[49,50] Also, when grating more hydrophobic cations, they can make more contribution to the lid opening and active conformation forming,[51,52] those may be the reason that PPL-1c and PPL-1d own better enantioselectivity than the other modified enzymes. It was found that the enantioselectivity was enhanced by the ILs which acted as additives or solvents containing kosmotropic anions,[53,54] our experiments showed opposite results that chaotropic anions help increase enantioselectivity. The reason may be that ILs as modifiers and cations grating on enzyme are more crucial in affecting the enantioselectivity of the enzyme. PPL-1e also showed great enantioselectivity, the possible explanation is that modified IL contain PF_6^-, which is typically stabilizing enzyme and help achieve high enantioselectivity.[55]

Intrinsic Fluorescence Analysis. Investigation on any possible conformational changes induced by IL modification may shed some light on the effect of ILs on enzyme performance. Fluorescence spectroscopy is used to follow the changes in the maximal intensity of fluorescence (I_{max}) and the maximal emission wavelength (λ_{max}).[56] Figure 3A shows the fluorescence spectra of PPL and three differently modified enzymes in water. Figure 3B shows the spectra of enzymes (mentioned in Figure 3A) which incubated at 40 °C for 30 min. The maximum intensity for all the spectra has been normalized with respect to the initial spectrum obtained from PPL in water at 25 °C.

PPL exhibited an initial λ_{max} of 350 nm in water. After modification, it is noticeable how the fluorescence spectra of modified enzymes were clearly modified compared with the spectra of native enzyme. ILs with the same anion grafting on enzyme exhibited blue shift in λ_{max} and an increase in I_{max} (Figure 3A, Supporting Information Figure S11A). Chaotropic

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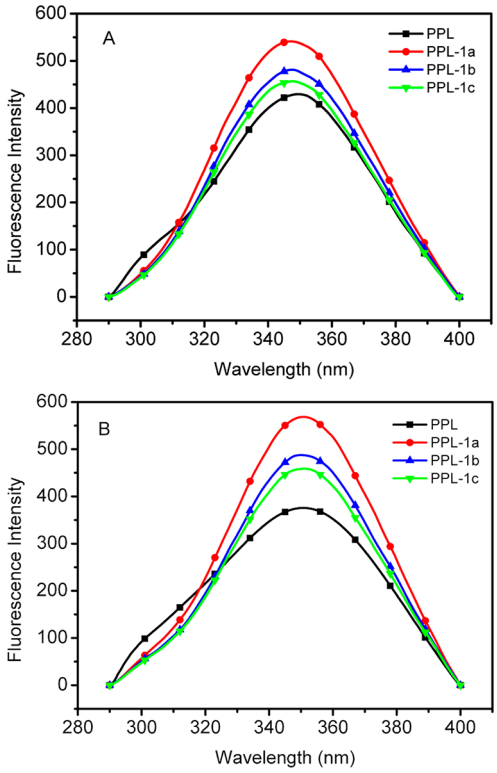

Figure 3. Fluorescence spectra: (A) three imidazolium ILs with Cl^- as the anion and (B) enzymes incubated at 40 °C for 30 min compared with (A).

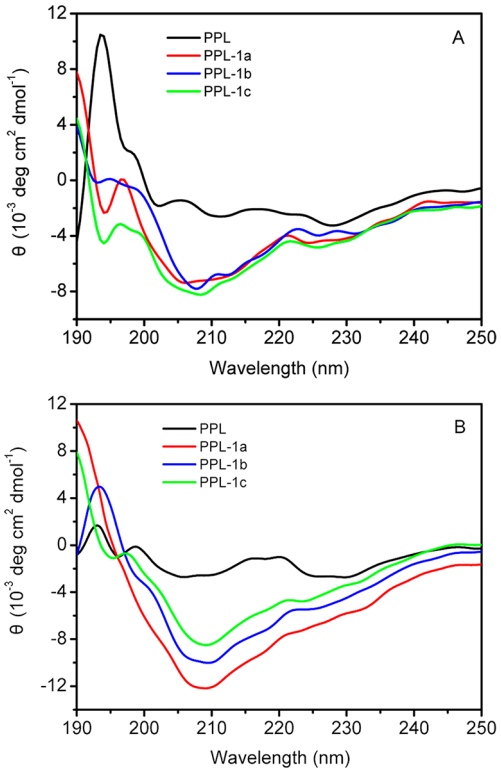

Figure 4. Far-UV CD spectra of PPL and modified PPL in water: (A) three imidazolium ILs with Cl- as the anion and (B) enzymes incubated at 40 °C for 30 min compared with (A).

cation grafting on enzyme resulted in a higher maximal intensity than kosmotropic ones, which could be the amount of modification degree, the results showed that protein changes to an active conformation with high activity.[57] After incubation at 40 °C for 30 min, the fluorescence spectra of native enzyme clearly shifted from the initial spectra, while spectra of modified enzymes had negligible changes (Figure 3B, Supporting Information Figure S11B). The decreased I_{max} and the red shift of λ_{max} observed in PPL spectra correspond to a classical unfolding process of proteins which were accompanied by an activity loss,[58] while little changes of spectra of modified enzymes showed the ability to stabilize enzyme (Table 4).

Circular Dichroism Analysis. Circular dichroism spectra is one of the most widely used techniques for determining the structure of proteins, making it possible to quantify conformational modifications in the 3D structure from changes in the CD spectra. In particular, the line shape analysis of far-UV CD spectra (190−250 nm wavelengths) may allow identifying modification-induced structural changes of α-helice, β-sheet, β-turn and random coil in proteins, respectively.[59] The far-UV CD spectra of the enzymes indicated that the protein has a particular distribution of the secondary structure in each condition. ILs with same anion grafted on PPL caused variation of protein conformation (Figure 4A, Supporting Information Figure S12A). After incubation at 40 °C for 30 min, the noticeable conformation shift of PPL occurred, as shown in Figure 4B. The conformations of modified enzymes changed differently, especially the spectra of PPL-1a. Compared with the other two, PPL-1c slightly shifted. This result can be attributed to the kosmotropicity of $BMIm^+$, which helps preserve critical water molecules in the microenvironment of the enzyme and maintain native conformation to a great extent, while $MMIm^+$ is chaotropic cation.[60]

The secondary structures from the CD spectra were computed using the Jwsse32 software following the method of CD-Yang.Jwr (Table 6). The secondary structure distribution of native enzyme was determined from an aqueous solution at 25 °C, which indicates that the α-helix content agrees with the reported data.[61] All the modified enzymes lower

Table 6. Secondary Structure Percentages of Enzymes from CD Spectra: 25 °C without Incubation (A) and Incubation at 40 °C for 30 min (B)

	α-helix (%)	β-sheet (%)	β-turn (%)	random (%)
		(A)		
PPL	27.4	28.0	17.1	27.5
PPL-1a	20.2	31.4	17.3	31.1
PPL-1b	18.2	32.3	18.8	30.7
PPL-1c	17.5	38.8	14.4	29.3
PPL-1d	18.1	34.8	16.9	30.2
PPL-1e	19.6	33.3	16.8	30.3
PPL-1f	21.4	30.2	17.5	30.9
PPL-1g	19.5	34.1	15.2	31.2
		(B)		
PPL	14.6	20.1	23.1	42.2
PPL-1a	12.7	33.9	11.5	41.9
PPL-1b	12.1	36.5	14.6	36.8
PPL-1c	16.2	39.8	13.8	30.2
PPL-1d	11.8	37.6	13.2	37.4
PPL-1e	12.3	33.9	18.2	35.6
PPL-1f	11.4	33.5	14.6	40.5
PPL-1g	12.8	35.5	13.4	38.3

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α-helix content but higher β-sheet content in comparison with native enzyme, especially PPL-1c. The analysis of the CD spectrum of the PPL incubated at 40 °C for 30 min (Figure 4B, Supporting Information Figure S12B) showed a decline in both α-helix and β-sheet secondary structures, which agree with an unfolded protein state. This loss of native enzyme conformation is clearly correlated with poor thermostability, as shown in Table 4. Enzyme modification preserves catalytic activity, which is reflected by the maintenance of the secondary structure of PPL. After modification, the structure did not change significantly. In the same way, the loss of α-helix content accompanies a significant increase in the β-sheet content of modified enzymes with time. These results show how the active enzyme conformation was protected by IL grafting against deactivation, displaying high stability and enantioselectivity as seen from a comparison with Tables 4 and 5.

CONCLUSION

In this study, chemical modifications of PPL by various functional ILs to improve activity, thermostability, and enantioselectivity were reported. Results showed that ILs with chaotropic cations and kosmotropic anions grafting on lipase significantly improved hydrolytic activity, which is attributed to high modification degree. Also, ILs with kosmotropic cations and chaotropic anions grafting on lipase contribute to excellent thermostability and enantioselectivity, which are attributed to protection of active conformations after modification. However, the specific ion effect is not the only factor controlling lipase performance. Further research should be conducted to investigate the cause of enhanced catalytic performance. Thus, promoting this feasible methodology will pave a way to using ILs in biocatalytic processes and the development of a novel, efficient, and practical biocatalyst.

ASSOCIATED CONTENT

Supporting Information

SDS-PAGE, optimal modification degrees, MALDI-TOF MS results, far-UV CD spectra, and fluorescence spectra. This information is available free of charge via the Internet at http://pubs.acs.org/.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. R.J. and Y.H. contributed equally.

Notes

The authors declare no competing financial interest.

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