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Buffer-mediated activation of *Candida antarctica* lipase B dissolved in hydroxyl-functionalized ionic liquids

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ABSTRACT

Ionic-liquid buffer having phosphate anion was synthesized for the development of buffered enzymatic ionic liquid systems. Both the conformation and transesterification activity of *Candida antarctica* lipase B (CALB) dissolved in the hydroxyl-functionalized ionic liquids were buffer dependent. Intrinsic fluorescence studies indicated that the CALB possessed a more compact conformation in the medium consisted of ionic liquid buffer having phosphate anion and hydroxyl-functionalized ionic liquids like 1-(1-hydroxyethyl)-3-methyl-imidazolium tetrafluoroborate and 1-(1-hydroxyethyl)-3-methyl-imidazolium nitrate. High activity and outstanding stability could be obtained with the CALB enzyme in the buffered ionic liquids for the transesterification.

1. Introduction

Enzymes catalyze a wide variety of reactions best in aqueous environments and at physiological pH with exquisite selectivity and stereospecificity [1,2]. Over the years, much effort, using both experimental and theoretical approaches, has been devoted to the studies of enzyme stability [3–5]. Several parameters such as hydrophobic interactions [3,6], electrostatic interactions [7,8], hydrogen bonding [9,10], packing [6,11], and entropic effect [12] have been identified as major contributions to the stability of an enzyme. Despite of these, much work is still needed to understand the dominant forces that stabilize the enzymes, especially in ionic liquid (IL) media.

The use of ILs as novel reaction media may offer a convenient solution to both the solvent emission and the catalyst recycling problem [13,14]. Because active enzymes are charged biomacromolecules with a hydrophilic nature, they are usually insoluble in common ILs. Enzymatic reactions in ILs are basically limited to the use of suspended enzymes [15–19] or immobilized enzymes forms [15,16,20]. However, it is of interest to develop ILs that can dissolve enzymes because an enzyme solution in IL enables detailed investigation of enzyme structure and function by various spectroscopic methods [21,22]. It was reported that ILs containing nitrate [23], acetate or lactate [24], and chloride [25], can dissolve enzymes but also cause severe enzyme deactivation. Turner and co-workers reported that incorporation of a hydroxyl-functionality in the cation of an imidazolium based IL has the potential to stabilize proteins while keeping them soluble [26]. The potential of the IL with a cation 1-(1-hydroxypropyl)-3-methyl-imidazolium ([C_3OHMIM]) for dissolving enzymes without affecting their catalytic activity was demonstrated by Walker and Bruce [27,28]. Bermejo et al. recently found that when fresh CALB dissolved in the IL 1-(1-hydroxypropyl)-3-methyl-imidazolium nitrate, [C_3OHMIM][NO_3], 35% initial activity lost as compared to its aqueous solution. After three months of storing the CALB-IL solution, the enzyme maintained about 80% of its activity with respect to the fresh solution [29]. Obviously, not all ILs are suitable for biocatalysis. The development of dissolved and active enzymes in IL media still remains a challenge.

It is well known that buffers affect enzyme activity in aqueous system. There are, however, few reports concerned with the control of ionization state of enzymes in non-aqueous media. Some types of buffers have been used to tune the ionization state of enzymes in organic solvents [30–32]. Unfortunately, they were found to have only limited solubility in non-aqueous media. To overcome this restriction, we have synthesized a new class of ILs with buffering behaviour that are referred to as IL buffers, which are miscible with polar solvents such as methanol, DMF, and dichloromethane and also with ILs like [BMIM][PF_6] and [BMIM][BF_4] [33]. Remarkable buffer dependence of the catalytic activities has been observed in hydrogenation of olefins [33] and selective hydrogenation of unsaturated aldehyde [34] in non-aqueous media.

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Our strategy for solubilizing enzymes in ILs is to select ILs with hydroxyl group that have high affinity for protein molecules. We have reported that common ILs such as [BMIM][BF_4] and [BMIM][PF_6] showed a weak power to solvate PAMAM dendrimers, which are known to be good mimics of globular proteins. Whereas the IL 1-(1-hydroxyethyl)-3-methyl-imidazolium tetrafluoroborate, [C_2OHMIM][BF_4], could dissolve the dendrimers well. The charged dendrimer-encapsulated Pd nanoparticles in this IL showed high activity and reusability for hydrogenation of styrene [35]. Herein, we report a novel IL system consisting of IL buffer having phosphate anion and hydroxyl-functionalized IL like [C_2OHMIM][BF_4] and 1-(1-hydroxyethyl)-3-methyl-imidazolium nitrate, [C_2OHMIM][NO_3], for the dissolvation and activation of *Candida antarctica* lipase B (CALB). To understand the activation and denaturation phenomena of the enzyme in the designed IL media, the buffer effects on dissolved CALB were investigated and Fluorometric measurements were applied to analyze changes in enzyme conformation.

2. Materials and methods

2.1. Materials

C. antarctica lipase B (CALB, ~9 units mg^{-1}) was purchased from Fluka. Tryptophan and tyrosine was purchased from Sigma–Aldrich. Ethyl butyrate and *n*-butanol were analytical reagents and were dried by 3A molecular sieves before use. All other chemicals and reagents were of analytical grade.

2.2. Synthesis of ionic liquids

[BMIM][BF_4] [36], [BMIM][PF_6] [36], [C_2OHMIM][BF_4] [37], [C_2OHMIM][NO_3] and [BMIM][NO_3] [21] were synthesised according to published procedures and checked for the absence of chloride and acid. ILs were passed through a neutral alumina column, dried at 50 °C under reduced pressure for more than 18 h, and stored under dry N_2.

2.3. Synthesis of IL buffer having phosphate anion

An aqueous solution of [BMIM]OH was prepared by passing the corresponding imidazolium halide ([BMIM]Cl) through a column filled with anion exchange resin, as described in the literature [33,34]. The aqueous [BMIM]OH was then neutralized with phosphoric acid in a beaker and the pH of the solution was adjusted to 6.86. The solution was evaporated at 50 °C under reduced pressure to give a viscous liquid, which was then vacuum dried at 50 °C for 18 h to afford the ionic liquid buffer product. mp 48–53 °C. NMR spectra of IL buffer having phosphate anion were shown in Fig. S1. ^{1}H NMR (400 MHz, D_2O) δ: 8.555 (s, 1H, im-H), 7.306 (s, 1H, im-H), 7.265 (s, 1H, im-H), 4.022 (t, 2H, $-CH_2$), 3.721 (s, 3H, im-CH_3), 1.651–1.688 (m, 2H, $-CH_2$), 1.108–1.164 (m, 2H, $-CH_2$), 0.722–0.759 (t, 3H, $-CH_3$); ^{13}C NMR (400 MHz, D_2O) δ: 135.754, 123.392, 122.098, 49.147, 35.534, 31.161, 18.632, 12.538. ^{31}P NMR (400 MHz, D_2O) δ: 1.324. Mass spectra of IL buffer having phosphate anion were shown in Fig. S2. MS (*m*/*z*) calcd for [BMIM]$^+$ of the IL buffer 139.1 obsd 139.1; MS (*m*/*z*) calcd for $H_2PO_4^-$ of the IL buffer 97.0 obsd 97.2.

2.4. Characterizations

Each dried IL was diluted with anhydrous methanol to reduce viscosity, then its water content was measured by Karl–Fischer titration (Metrohm Ltd., model 787 KF Titrino). Water contents of the dry [BMIM][BF_4], [BMIM][PF_6], [C_2OHMIM][BF_4], [C_2OHMIM][NO_3], and [BMIM][NO_3] were calculated by subtracting that of anhydrous methanol, all of which were less than 0.19%. The ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were obtained on a Brüker AV-400 Fourier transform NMR spectrometer. ^{1}H NMR spectra were referenced to tetramethylsilane in $CDCl_3$. ^{31}P NMR spectra were referenced to phosphoric acid. Mass spectra were acquired using a Finnigan LCQ mass spectrometer. Thermal gravimetric analysis (TGA) was performed on a Netzsch TG 209F1 instrument in N_2 flow with a heating speed of 10 K min^{-1}.

2.5. General procedures of enzymatic transesterification

CALB (1.2 mg) was dissolved in 500 μL of IL medium (30 mg of IL buffer having phosphate anion was added in the case of buffered medium) in a 5 mL flask. 110 μL (0.83 mmol) ethyl butyrate and 110 μL (1.21 mmol) *n*-butanol and 50 μL nonane (internal standard) were added. The reaction mixture was stirred at 40 °C in oil bath for 3 h. After the reaction was complete, the products were decanted from [C_2OHMIM][BF_4]. In the cases of [BMIM][NO_3] and [C_2OHMIM][NO_3] as reaction media, the products were extracted with cyclohexane. The organic phase was analyzed with a gas chromatograph equipped with an FID and a capillary column (SE-30, 30 m × 0.32 mm × 0.25 μm). The residual reactant mixture in IL phase was removed in vacuum at 40 °C for more than 1 h. The new cycle was restarted by addition of fresh substrate. This process was repeated 9 times.

2.6. Fluorescence spectroscopy

Fluorescence spectra were monitored on a fluorescence spectrophotometer (FP-6200, Jasco, Japan) at room temperature (20 °C). Enzyme samples were excited at 290 nm and emission registered from 300 to 450 nm using 5 nm band width in both the excitation and emission path. The final concentration in all the media was 0.5 mg mL^{-1} of CALB or Trp. The fluorescence spectra of CALB were obtained by subtracting the spectra of the ILs without the enzyme or Trp to discount the influence of the imidazolium ring fluorescence on the fluorescence spectrum.

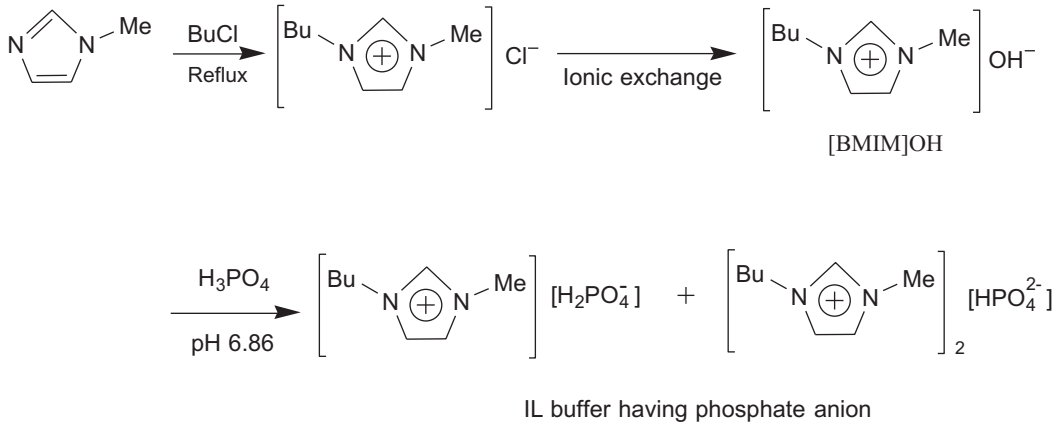
3. Results and discussion

3.1. Synthesis and characterization of IL buffer having phosphate anion

The synthesis pathway of IL buffer having phosphate anion is shown in Scheme 1. The IL buffer was synthesized as previously reported [33,34] and characterized by NMR, MS and TGA. All the characterization data are consistent with the expected compositions and structures (Figs. S1–S3). Buffer value (number of moles of strong base required to change the pH of one liter of solution by one unit) and dilution value (change of pH on dilution with an equal volume of water) of the aqueous solution of mixed phosphate IL buffer at 0.025 mol L^{-1} and 20 °C are 0.026 and 0.10, respectively, which buffering behaviors are exactly in agreement with those of the inorganic counterpart, mixed phosphate buffer [38].

3.2. Enzymatic transesterification in the presence of IL buffer having phosphate anion

To test the activity of CALB lipase in dissolved form in ILs, we examined the CALB-catalyzed transesterification of ethyl butyrate with *n*-butanol. All the reactions were performed under the same conditions, 40 °C and 300 rpm, in this study to eliminate any temperature or mixing effects. The transesterification efficiencies of CALB in dried water-miscible ILs, [BMIM][NO_3], [C_2OHMIM][NO_3], and [C_2OHMIM][BF_4], are shown in Table 1. It can be found



Scheme 1. Synthesis of IL buffer having phosphate anion.

that CALB was inactive in pure and buffered [BMIM][NO_3], and also afforded lower substrate conversion in pure [C_2OHMIM][BF_4] (8.7%) and [C_2OHMIM][NO_3] (6.8%). In contrast, CALB exhibited high levels of transesterification activity in the buffered [C_2OHMIM][BF_4] and [C_2OHMIM][NO_3], indicating that the buffer was responsible for the rate enhancement.

The above results showed that the enzyme activity is greatly affected by the solvent and buffer. It has long been know that ionization equilibrium of an acid or a base is affected by a solvent change, not only because of the acicity or basicity of the solvent, but also because of its dielectric constant, and the ability of the solvent to solvate the various species of the equilibrium equation. The ionization constant of ionizable groups is greatly affected by the solvent [39]. Thomazeau et al. reported that the acid $HNTf_2$ is more acidic in [BMIM][NTf_2] and [BMIM][BF_4] than in water [40]. In aqueous systems, enzymes have an optimum pH or pH range in which their activity is maximal, and at higher or lower pH their activity decreases. Drastic changes in pH often lead to denaturation. Hydrogen ion concentration affects enzymes in several ways. First, catalytic activity is related to the ionic state of active site. Changes in hydrogen ion concentration can affect the ionization of active site groups. Secondly, changes in ionizable groups may change the tertiary structure of the enzyme. Ionic interactions occur between charged amino and carboxyl groups in the side chains of some amino acids in the folded protein. If the ionization state of the enzyme is changed, some of those functional groups will gain or lose a proton and, therefore, will lose their charge or become charged, depending on which way the ionization state of the enzyme is changed and by how much. That will eliminate some, perhaps many, of the ionic interactions that were necessary for maintenance of the active enzyme conformation. Russell and Fersht reported that changing the surface charge of subtilisin by site-directed mutagenesis produces enzymes with significantly shifted pH-activity profiles, higher catalytic activities and altered specificities [7]. Based on the above analyses, enzyme-benign ILs needs to have water-like physicochemical properties, one of which involves the introduction of hydroxyl group into ILs to obtain higher dielectric constant and salvation ability to protein molecules. And an appropriate buffer is needed to closely regulate the ionization state of ionizable groups of the enzyme. One can conclude that enzyme activity in ILs is also buffer dependent as in aqueous systems.

The recycling ability of the CALB solubilized in buffered [C_2OHMIM][BF_4] under the above reaction conditions is illustrated in Fig. 1. After each reaction, the reactant mixture in the upper layer and CALB-IL in the lower layer were separated by decantation. The CALB-IL phase was then evacuated to remove residual reactant mixture and charged for the next reaction without adding any new enzyme, IL and buffer. The reaction conversion can maintain 75% of its initial value after 10 runs, indicating that the dissolved CALB in the buffered [C_2OHMIM][BF_4] is a kind of very stable biocatalyst system.

Table 1
Buffer dependence of transesterification activities of CALB in different ILs.[a]

Reaction medium (IL)	Conversion/%[b]	
	In pure IL medium	In buffered IL medium
[BMIM][NO_3]	0	0.3
[C_2OHMIM][NO_3]	6.8	15.6
[C_2OHMIM][BF_4]	8.7	44.5

[a] The reaction conditions are the same as Fig. 1.
[b] Conversion refers to ethyl butyrate. In the absence of CALB, no conversion was observed in the IL solvent systems studied.

3.3. Intrinsic fluorescence studies

Structural analyses of the enzyme had provided much insight into the enzyme activity and the mechanism of enzymatic reactions. The solubility of CALB in hydroxyl-functionalized ILs provided the opportunity to investigate the enzyme conformation by various spectroscopic methods. To better understand the effects of the IL buffer on the conformation of CALB, fluorescence emis-

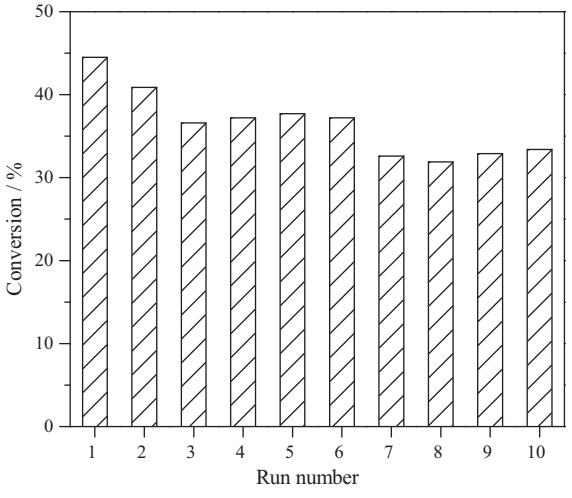


Fig. 1. Stability of CALB in buffered [C_2OHMIM][BF_4] during recycling. Reaction conditions: 1.2 mg of CALB; 30 mg of mixed phosphate IL buffer; 500 μL of IL; 110 μL of ethyl butyrate (0.83 mmol); 110 μL of *n*-butanol (1.21 mmol); stirring speed = 300 rpm; temperature = 40 °C; reaction time = 3 h.

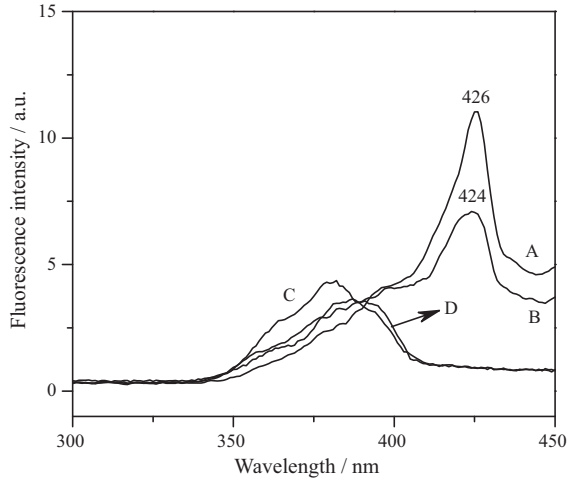


Fig. 2. Fluorescence emission spectra of CALB in several ILs. (A) Buffered $C_2OHMIM][NO_3]$, (B) pure $[C_2OHMIM][NO_3]$, (C) pure $[BMIM][NO_3]$, and (D) buffered $[BMIM][NO_3]$.

sion spectra of CALB are obtained under dilute conditions where protein–protein interactions are minimal. Fig. 2 indicated that $[BMIM][NO_3]$ completely quenched the fluorescence of the tryptophan (Trp) residues in CALB while a maximum emission at near 425 nm was observed in both pure and buffered $[C_2OHMIM][NO_3]$. Compared with the emission contours of the 'naked' Trp in bulk $[C_2OHMIM][BF_4]$ (Fig. 3), the peak at wavelength *ca.* 425 nm in Fig. 2 could be assigned to the fluorescence of Trp residues in CALB.

Indole, Trp, and their derivatives are uniquely sensitive to quenching by electron scavengers such as protons, NO_3^-, and fumarate, which are collisional quenchers [41]. For collisional quenching to occur, the quencher must collide with the fluorophore during the lifetime of the excited state, causing the fluorophore to return to the ground state without emission of a photon. Consequently, if the Trp residue is buried inside the protein, quenching is not expected to occur. If the Trp residue is on the protein surface, then quenching is expected. Native CALB has two surface Trp residues and three buried Trp residues [42]. The Trp residues in CALB are almost completely quenched in $[BMIM][NO_3]$. This suggests that under this condition all the Trp residues are accessible to NO_3^- of the solvent, indicating the complete unfolding of the protein. While in the case of $[C_2OHMIM][NO_3]$, the Trp residues are

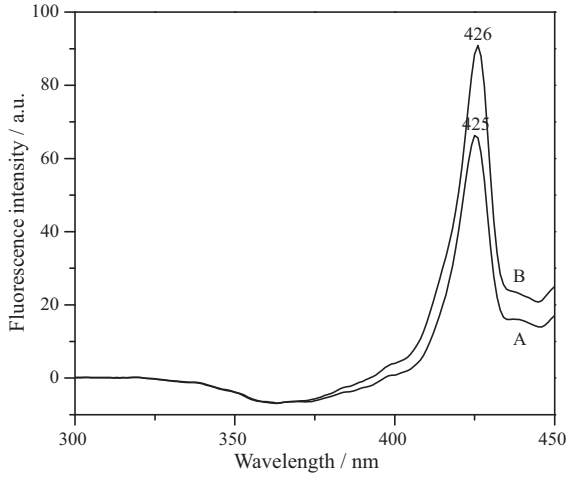


Fig. 3. Fluorescence emission spectra of Trp. (A) Trp in $[C_2OHMIM][BF_4]$, (B) addition of Tyr to Trp IL solution.

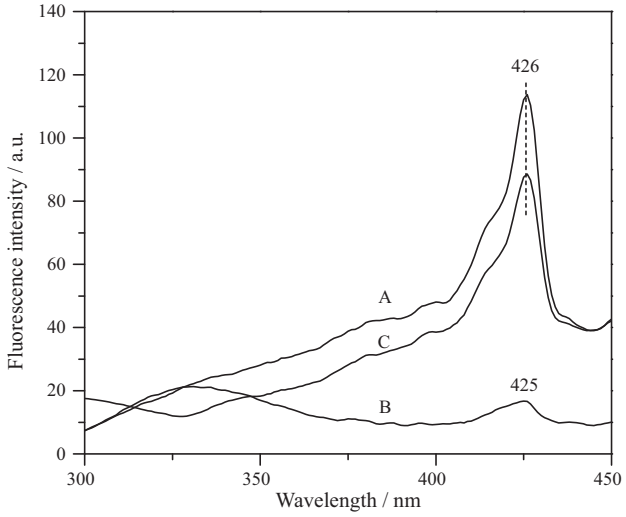


Fig. 4. Fluorescence emission spectra of CALB in $[C_2OHMIM][BF_4]$. (A) Buffered IL, (B) pure IL, and (C) buffered IL containing $[C_2OHMIM][NO_3]$.

partially quenched, suggesting that CALB still preserves its compact conformation.

In the presence of mixed phosphate IL buffer, the fluorescence emission spectra of CALB showed an increase in fluorescence intensity (curve A in Figs. 2 and 4) compared with the spectra in pure hydroxyl-functionalized ILs (curve B in Figs. 2 and 4). A decrease in fluorescence intensity at λ_{max} is generally observed when proteins unfold. The observed changes in the Trp intensity are most likely due to Förster-resonance energy transfer (FRET). The most commonly observed resonance energy transfer in proteins is from tyrosine (Tyr) to Trp. The rate of energy transfer $k_T(r)$ is given by Eq. (1),

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \quad (1)$$

where τ_D is decay time of donor in absence of the acceptor, R_0 is the Förster distance, and r is the distance between donor and acceptor. Any changes in donor-acceptor distance will affect the rate of energy transfer. If a suitable acceptor fluorophore is nearby, then non-radiative energy transfer between the donor and acceptor can occur. CALB possesses 5 Trp residues (2 on the surface and 3 in the interior) and 9 Tyr residues. Tyr 61, Tyr 91, and Tyr 234 make a "cage" around Trp 52, whereas Tyr 82, Tyr 135, and Tyr 300 surround Trp 113 [42]. Efficient FRET may occur if the distance between Tyr and Trp is below the Förster distance 9–18 Å [41,43]. It was observed that addition of Tyr in Trp $[C_2OHMIM][BF_4]$ solution resulted in intensity increase of the Trp (Fig. 3). It is likely that a significant part of the observed intensity increase at wavelength 425 nm in buffered hydroxyl-functionalized ILs is due to a smaller Tyr–Trp distance in CALB and thus a more efficient energy transfer, which may reflect a more compact enzyme conformation. Changes in the activity of CALB caused by the buffer were found to correlate well with the compaction of the specific three-dimensional structure or conformation of CALB.

Fluorescence quenching experiments have been proved to give precise information about local conformational fluctuation around Trp residues. We thus used this approach to investigate the conformation of CALB in buffered $[C_2OHMIM][BF_4]$. The selective ion NO_3^- was used as a quencher to preferentially probe Trp residues near the surface of the protein. The results are shown in Fig. 4. In the presence of quencher, $[C_2OHMIM][NO_3]$, the fluorescence emission spectrum showed a decrease in fluorescence intensity (curve C in Fig. 4) compared with the spectrum of CALB in the absence of $[C_2OHMIM]$ $[NO_3]$ (curve A in Fig. 4). In this case, the surface Trp

residues are quenched and the buried ones are not. The water soluble quencher does not readily penetrate the hydrophobic regions of CALB. This suggests that CALB keeps the compact conformation in buffered [C_2OHMIM][BF_4].

4. Conclusions

In summary, we have, for the first time, found that both the conformation and transesterification activity of CALB solubilized in the hydroxyl-functionalized ILs like [C_2OHMIM][BF_4] and [C_2OHMIM][NO_3] were buffer dependent. Intrinsic fluorescence studies showed that CALB possessed a more compact conformation in the buffered [C_2OHMIM][BF_4] or [C_2OHMIM][NO_3], resulting in an enhancement of transesterification activity with highly stability during recycles. The knowledge gained in this study might be applied to homogeneous enzymatic catalysis in organic solvents with hydroxyl group. The research is currently underway of progress and will be reported in due course.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data, including characterization data [NMR spectra (^{1}H, ^{13}C, and ^{31}P), MS spectra, TGA traces] and other physicochemical properties for ILs, associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.09.013.

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