Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 17 (2006) 2491–2498

Using ionic liquid [EMIM][CH_3COO] as an enzyme-'friendly' co-solvent for resolution of amino acids

Hua Zhao,* Lee Jackson, Zhiyan Song and Olarongbe Olubajo

Chemistry Program, Savannah State University, Savannah, GA 31404, USA

Received 25 July 2006; accepted 7 September 2006

Abstract—An ionic liquid (IL), 1-ethyl-3-methylimidazolium acetate [EMIM][CH_3COO], was used in 0–4.0 M (~60% IL, v/v), as a non-volatile organic medium for the enzymatic resolution of amino acids. When DL-phenylalanine methyl ester was studied as a model substrate, high enantiomeric excesses (ee) of L-amino acid were obtained in all ionic concentrations; however, lower yields were observed at high IL concentrations. This IL is more enzyme-'friendly' than the hydrophilic organic solvent acetonitrile and those ILs containing chaotropic anions (such as [EMIM][OTs]). Among three proteases and two lipases investigated, lyophilized *Bacillus licheniformis* protease exhibited the best enantioselectivity and activity. Highly enantioselective resolutions were also produced for several other amino acids in 2.0 M IL. Interestingly, high ee were also found in deuterium oxide (D_2O) rather than in ordinary water, and a further enhancement was achieved with the co-existence of [EMIM][CH_3COO]. The heavy water effect was explained in terms of protein stabilization by D_2O. The secondary structural changes of enzyme in various media were interpreted by the second derivatives of FT-IR spectra.

1. Introduction

Ionic liquids (ILs) are relatively new organic solvents consisting of ions. Their increasing applications in organic reactions including biocatalysis (see reviews[1–6]) are not solely due to their favorably low volatility thus potentially being 'green', but more importantly, owe to their unique solvent properties, such as tunable polarity, viscosity, and hydrophobicity through modifying the combination of cations and anions.[7–10] At present, most IL-mediated enzymatic reactions are carried out in hydrophobic ILs (e.g., PF_6^- or $(CF_3SO_2)_2N^-$ salts) (see more discussion in our recent review[11]). These hydrophobic ILs can be as advantageous as hydrophobic organic solvents in enzymatic reactions,[12–14] particularly their lower tendency to strip the 'essential' water from enzymes.[15,16] The enzyme activity, stability, and enantioselectivity have been interpreted in terms of IL properties including polarity,[1] hydrogen-bond basicity,[17,18] anion nucleophilicity,[19] and viscosity.[5] However, since the enzyme is suspended rather than dissolved in hydrophobic ILs, its activity in ILs is expected to be lower than that in aqueous solutions due to similar observations in hydrophobic organic solvents.[12,20] Although some hydrophilic ILs might dissolve certain enzymes, different enzyme activities are observed[21] using a pure or very concentrated IL as the reaction medium can be disadvantageous because the high IL viscosity usually leads to a slow dissolution of substrate(s) and a reduction of reaction rates.

To overcome these drawbacks, we have been exploring the uses of aqueous solutions of hydrophilic ILs as enzymatic media.[22–24] In diluted IL aqueous solutions, the enzyme is typically well hydrated; also, the medium viscosity and solution dissolution ability (towards substrates) are adjustable through varying the IL content. However, since hydrophilic ILs dissociate into cations and anions in water, the overall solvent properties (such as polarity) do not always account for the enzymatic behaviors in these media. In other words, the mechanism of solvent effect on the enzyme function in IL aqueous solutions is different from that in nearly dry ILs. Our recent studies[22–24] suggested that the individual ion's kosmotropicity,[†] following the

* Corresponding author. Tel.: +1 912 353 5290; fax: +1 912 691 6839; e-mail: zhaoh@savstate.edu

[†] Kosmotropes are strongly hydrated species and thus called water 'structure-makers'. Kosmotropic ions include CH_3COO^-, SO_4^{2-}, HPO_4^{2-}, Mg^{2+}, Ca^{2+}, Li^+, H^+, and OH^-. Chaotropes are weakly hydrated species and thus called water 'structure-breakers'. They include SCN^-, I^-, NO_3^-, BF_4^-, Cs^+, K^+, $(NH_2)_3C^+$ (guanidinium), and $(CH_3)_4N^+$ (tetramethylammonium). The kosmotropicity measures how strong a species (ion or neutral compound) is as a kosmotrope.

0957-4166/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2006.09.009

Hofmeister ion series of protein stabilization,[11,25–27] is responsible for the enzyme stability and enantioselectivity in IL aqueous solutions. The general conclusion was that ILs consisting of a kosmotropic anion and chaotropic cation can stabilize or even activate the enzyme in aqueous solutions. One explanation of the kosmotropic effect is that although both a kosmotropic cation and an anion (e.g., Ca^{2+} and SO_4^{2-}) strongly salt out nonpolar groups, the cation (Ca^{2+}) strongly salts in (and interacts with) the peptide group (causing destabilization) while the anion (SO_4^{2-}) does not.[28]

Through previous studies,[22,23] we have identified several room-temperature ILs (such as 1-ethyl-3-methylimidazolium $[EMIM]^+$ salts of acetate, trifluoroacetate, ethyl sulfate, and citrate) that are considered enzyme-'friendly' in terms of enabling high enzyme activities and enantioselectivities. It is because each of these ILs contains a kosmotropic anion and a chaotropic cation.[29] In addition, $[EMIM][CH_3COO]$ has a relatively low viscosity (determined in this study as 11.2 cP, see Section 4.6),[‡] and does not contain a fluorine atom as many other ILs do (thus being 'greener'). For these reasons, we focused on the use of this IL in a current study for the enzymatic resolution of amino acids. It is well recognized that chiral amino acids are becoming increasingly important intermediates for chiral drug synthesis.[31–33]

Meanwhile, we are very interested in examining the effect of heavy water (deuterium oxide, D_2O) on enzymatic resolution. Heavy water (92%D) has been known to be lethal to tadpoles, guppy fish, and worms although a low concentration (30%) seems safe for tadpoles.[34] However, an early study[35] suggested that the rate of ovalbumin denaturation by urea in heavy water is one-third that in ordinary water; it was suspected that the strong hydrogen bonds with deuterium stabilize the protein. Another study[36] indicates that a protein named tubulin is unstable when existing in solutions, but could be stabilized by D_2O against inactivation at both 4 and 37 °C; it was explained that D_2O might be involved in the conformational step that influences the hydrophobic forces. The hydrogen bonds are essential for enzymes to maintain the protein tertiary structures. Hydrogen bond with deuterium is slightly stronger than that with a regular hydrogen, therefore, the rigidity of most protein structures can be increased by using D_2O instead of H_2O, especially at higher temperatures, as demonstrated by Cioni and Strambini.[37] They also suggested that the folded state is more stable in D_2O than in H_2O based on the inverse relationship between structural flexibility and stability to thermal denaturation.[38] However, a contradictory result based on the Gibbs energy of protein unfolding at 25 °C demonstrated that the stabilities of ribonuclease A in D_2O and H_2O are quite close; however, the stabilities of horse cytochrome *c* and hen egg lysozyme in H_2O are higher than their respective ones in D_2O.[39] In this paper, we discuss some preliminary data of the effect of heavy water on enzyme activity, with or without the co-presence of an IL.

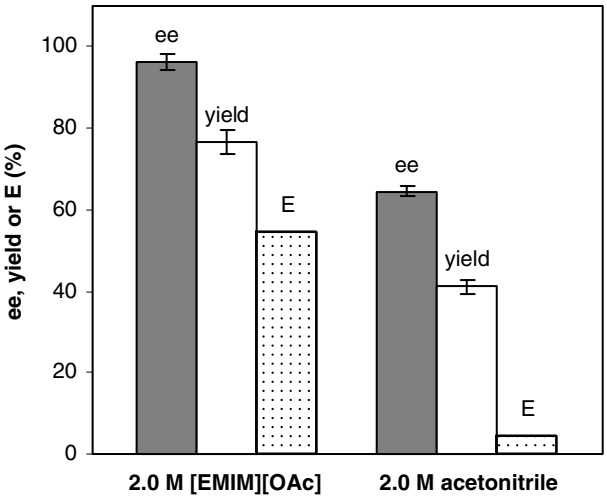

Figure 1. Comparing the effect of IL and organic solvent on the resolution of DL-phenylalanine (lyophilized *Bacillus licheniformis* protease, 40 min reaction time, 30 °C, the error bars indicating percentage errors).

2. Results and discussion

2.1. Solvent effect on the enzymatic reaction

The enzymatic resolution of DL-phenylalanine methyl ester was performed in 2.0 M $[EMIM][CH_3COO]$ and 2.0 M acetonitrile, respectively (Fig. 1). It is not surprising that low enantioselectivity and activity were observed in the organic solution (especially at high concentrations). The reason is that polar organic solvents tend to deactivate enzymes due to their interactions with proteins.[40,41] However, considerably higher ee and yield (or in terms of enantiomeric ratio, *E*) were obtained in 2.0 IL solution, suggesting that this IL does not have a strong interaction with the protein and can stabilize the enzyme.

In order to understand the structural changes of proteins in the above media, we measured the infrared spectra of a protease in these solvents and calculated their second derivatives (Fig. 2). It is well established that the second derivatives of the IR spectra between 1600 and 1700 cm^{-1} (so-called the amide I band)[§] can reveal the protein secondary structures (α-helix, β-sheets, β-turns, and nonordered or irregular structures).[42,43] The α-helical structures are normally observable between 1650 and 1658 cm^{-1}, and β-sheets are between 1620 and 1640 cm^{-1}.[43] As illustrated in Figure 2, after 40 min of incubation at 30 °C, the protease in 2.0 M $[EMIM][CH_3COO]$ contains essentially the same structural elements (α-helix, β-sheets, and β-turns) as that in water, suggesting that the protein's secondary structures were well maintained in the IL solution. However, when the enzyme was kept in 2.0 M acetonitrile for

[‡] The viscosities of other common ILs are usually much higher, for example,[30] $[EMIM][BF_4] = 43$ cP (30 °C) and $[EMIM][Tf_2N] = 28$ cP (25 °C).

[§] This band is due to the in-plane C=O stretching vibration of the amide group, coupled with C–N stretching and in-plane N–H bending.[42]

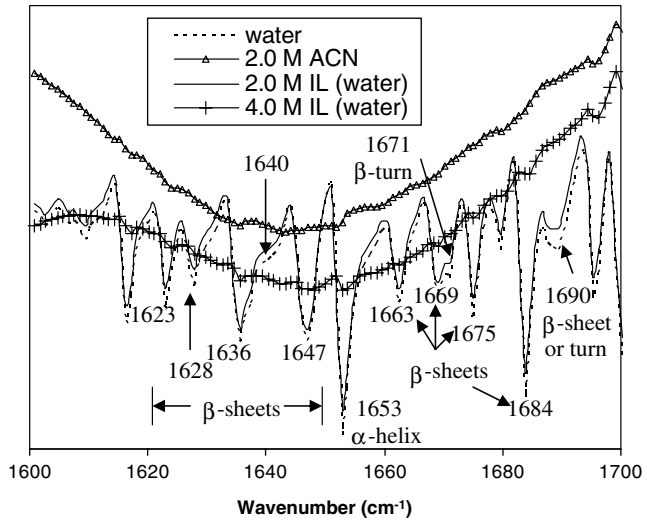

Figure 2. The second derivative spectra (FT-IR) of *Bacillus licheniformis* protease after incubation in various media for 40 min at 30 °C (the peak assignment was based on the literature as discussed in our recent paper,[24] while assigning the 1671 cm^{-1} peak as a β-turn was based on a different article[70]).

40 min, most peaks basically disappeared indicating that the protein structures were seriously disrupted by the organic solvent.

Figure 3 further illustrated the effect of two different ILs: one bearing a kosmotropic anion (CH_3COO^-) while the other containing a chaotropic anion (OTs^-).[29] A better enzymatic resolution (higher ee and *E* values) was achieved in [EMIM][CH_3COO] solution than in [EMIM][OTs] solution. This observation is consistent with our previous studies:[11,22–24] kosmotropic anions stabilize the enzyme while chaotropic ones destabilize it. Since [EMIM][CH_3COO] has a unique combination of a kosmotropic anion and a chaotropic cation, it is quite favorable for the protein stabilization and enzyme activation. The effect of IL kosmotropicity on enzyme activity has been discussed in detail in our above-mentioned studies.

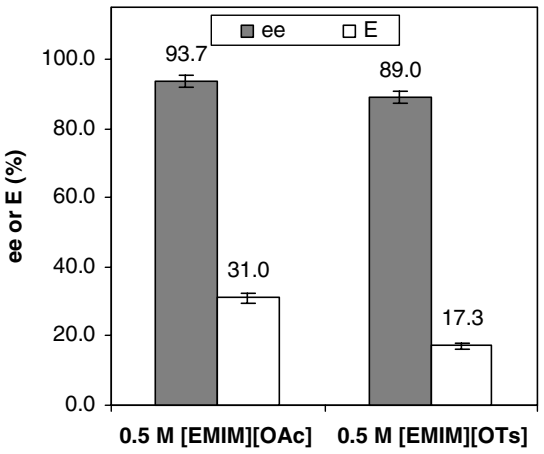

Figure 3. Effect of IL kosmotropicity on the resolution of DL-phenylalanine (lyophilized *Bacillus licheniformis* protease, 40 min reaction time, 30 °C, the error bars indicating percentage errors).

2.2. Effect of IL concentrations

To further examine the compatibility of [EMIM][CH_3COO] with proteases, we conducted the enantioseparation of DL-phenylalanine methyl ester in different concentrations of this IL. The IL concentrations studied range from 0 (pure water) to 4.0 M (~60% IL, v/v). As illustrated in Figure 4, at 40 min of reaction time, very high ee (>90%) of L-amino acid could be achieved in all IL solutions (up to 4.0 M). Considering the margins of error, the ee does not seem to strongly depend on the IL concentration, which implies that unlike many inorganic salts[44,45] or other hydrophilic ILs,[22,46] [EMIM][CH_3COO] does not strongly interact with proteins and destabilize the enzyme at relatively high concentrations. This observation further confirms that this organic salt is an enzyme-'friendly' IL.

Figure 4 also suggests that the yield of L-phenylalanine is independent of the IL content at low concentrations (from 0 to 1.0 M), but decreases dramatically when the IL content is greater than 2.0 M (suggesting slower reaction rates and lower enzyme activities). At first, we suspected that it was because of mass-transfer limitations caused by the high viscosity of concentrated IL solutions. Therefore, we measured the viscosities of IL solutions at 30 °C (Fig. 5). The viscosity of IL solution was found to be almost linearly increasing with the IL concentration. However, the yield was not linearly decreasing with the IL concentration, especially in the 0–1.0 M range. On the other hand, the increase of viscosity from 0.5 to 4.0 M is less than 0.2 cP, which is a very small increment when compared with the viscosity of pure IL (11.2 cP). Therefore, it is not very likely that the viscosity is the major factor in determining the enzyme activity in IL solutions (at least up to 4.0 M). We also

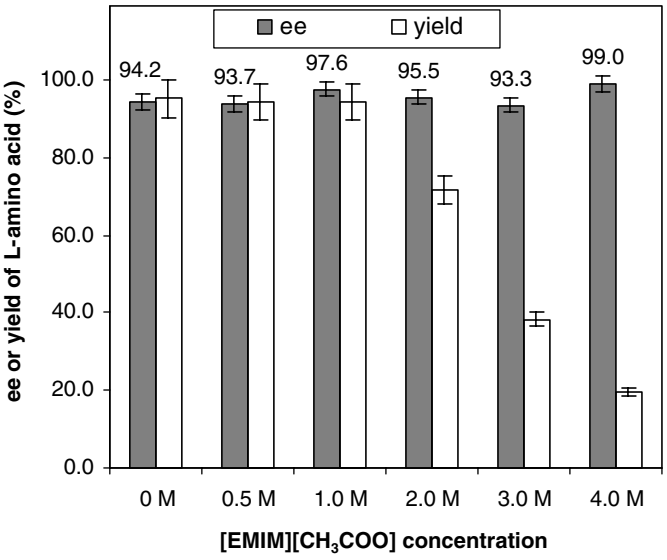

Figure 4. Effect of [EMIM][CH_3COO] concentration on the ee and yield of L-phenylalanine (lyophilized *Bacillus licheniformis* protease, 40 min reaction time, 30 °C, the error bars indicating percentage errors).

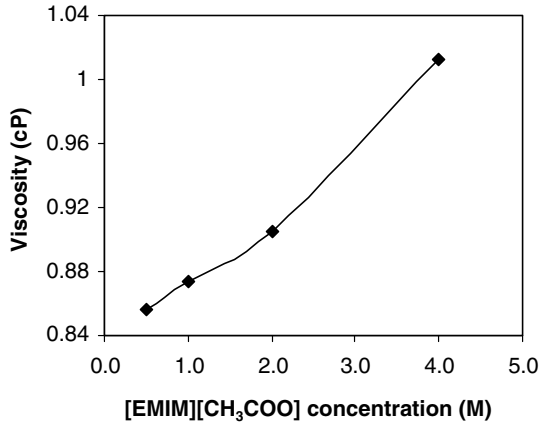

Figure 5. Viscosities of [EMIM][CH_3COO] aqueous solutions at 30 °C.

conducted the same reaction in pure IL at 30 °C, and no considerable resolution was detected within 24 h. This is consistent with the decreasing trend of L-yield with the IL concentration. To further examine the mass-transfer concerns, we compared the kinetic resolution at two different substrate concentrations in 4.0 M [EMIM][CH_3COO] (Fig. 6). The L-yield is almost doubled when the substrate concentration is doubled, suggesting that the overall reaction rate is proportional to the substrate concentration (i.e., $r \propto [S]$). Therefore, the mass transfer due to viscosity is not the limiting step (at least up to 4.0 M IL). However, in other more viscous media, the viscosity may impact the enzyme activity.[5] In addition, Figure 6 also indicates that the enzyme selectivity is lower when the substrate concentration is increased. This could be explained by the reactivity–selectivity principle: the substrate is more reactive at a higher concentration, thus a lower selectivity is expected.

One explanation of the decreasing yield (Fig. 4) is that high concentrations of hydrophilic ILs (especially those carrying strongly hydrated ions such as acetate) tend to 'absorb' more water molecules to hydrate themselves, leading to a 'dehydrated' enzyme and lower enzyme flexibility. A similar observation was found in the case of amino acids (as model compounds of proteins): high concentrations of kosmotropic salts dehydrate amino acids.[47] When the IL concentration is very high, severe dehydration may lead to protein unfolding.[42] Figure 2 demonstrated that when the protease was incubated in 4.0 M IL for 40 min, the characteristic protein peaks are very weak compared with that in water, suggesting a considerable structural change of protein molecules. Meanwhile, since water is also the substrate in this reaction, a high IL concentration results in a low water activity,[48] causing a slow substrate (amino acid ester) conversion. In summary, a high IL concentration may change the native environment of the enzyme, or induce the substrate ground-state stabilization.

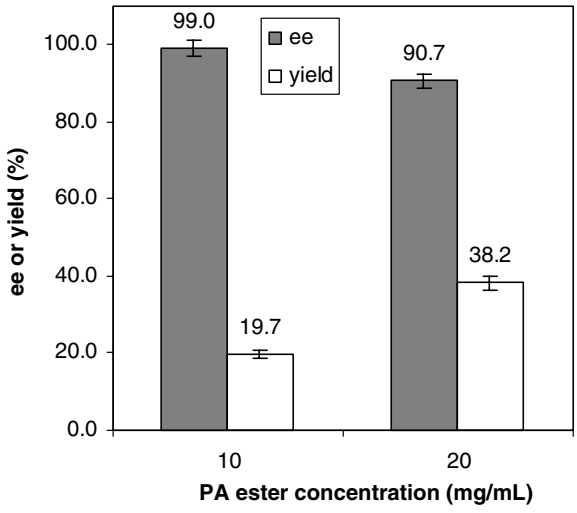

Figure 6. Effect of substrate concentration (phenylalanine methyl ester, or PA ester) on ee and L-yield at 30 °C (40 min of reaction time, 4.0 M [EMIM][CH_3COO]).

Dupont[49] proposed a different explanation for the enzyme stabilization by ILs using the structural organization of imidazolium ILs. Based on a number of experiments, he suggested the structure of pure 1,3-dialkylimidazolium ILs (in solid, liquid, or even gas phase) to be similar to those of hydrogen-bonded polymeric supramolecules. In solutions of ILs, the hydrogen-bonded nano-structures with polar and nonpolar regions can be formed. Therefore, the structured IL network embraces the hydrated enzyme, protecting it from losing essential water and from thermal denaturation. This theory has been used to explain the enzyme-stabilization by ILs.[50] In our case, at higher IL concentrations, the structure of IL network is more enhanced and the enzyme is more tightly 'included' in the network. As a result, the enzyme is less accessible by the substrate, resulting in a lower yield.

Overall, considering that high ee and moderately high yield could be achieved in 2.0 M [EMIM][CH_3COO], and the 2.0 M (~25%, v/v) solution has a relatively strong dissolution power towards hydrophobic substrates, we continued to investigate this concentration for further study of other enzymes and amino acids. One might suspect the advantage of using 2.0 M IL instead of water as a reaction medium, since the same high resolution could be achieved in water. However, pure water may not be suitable for the resolution of hydrophobic substrates although it is perfectly fine for phenylalanine methyl ester, since it is water soluble. By using a reasonable amount of IL, we can increase the dissolution ability of the media, especially when the hydrophobic substrates are present.

2.3. IL effect on different enzymes

In order to compare the enantioselectivities of different enzymes in [EMIM][CH_3COO], we selected several other enzymes frequently employed in enzymatic resolutions, including two more proteases (alcalase and Amano P6) and two lipases (PPL and Novozyme 435/immobilized CAL-B). These enzymes were chosen based on the following considerations: alcalase showed high enantioselectivities in the kinetic resolution of amino acids conducted in organic solutions[51–55] and in low-IL solutions;[56] Amano P6 maintained high stabilities in 0.7 M solutions of pyridinium and imidazolium based ILs including [EMIM][CH_3-

COO];[22] a highly enantioselective resolution of amino acids was achieved in phosphate buffer using PPL;[57] Novozyme 435 (or CAL-B) has been widely applied in many enzymatic reactions performed in ILs, with high enzyme activities being reported.[19,58–62] However, low enzyme enantioselectivities of alcalase and Novozyme 435 were also seen in some IL systems.[62]

The data listed in Table 1 clearly illustrate that three proteases produced higher ee than two lipases. Among the proteases, the resolution catalyzed by the lyophilized protease (*Bacillus licheniformis*) exhibited the best enantioselectivity and yield. In concentrated [EMIM][CH_3COO] solutions, the lyophilized protease may not be completely solvated as in water because the kosmotropic anion (acetate) tends to pull water molecules away to hydrate itself[29,47] and thus allow the protein to refold.[63] Therefore, being similar to the situation of lyophilized enzymes in organic solvents, proteins are more rigid in IL solutions and aggregates of protein molecules may exist, which allows for possible control of substrate specificity and protects enzymes from thermal or chemical denaturation.[14,64–66] On the other hand, alcalase is an aqueous form of *B. licheniformis* protease. Compared with lyophilized enzyme, the alcalase molecules have a higher flexibility before dissolving in IL solutions. Once alcalase is mixed with IL solutions, this enzyme produces fewer aggregates than the lyophilized enzyme, which permits protein molecules having more freedom to unfold in a high IL environment. The third protease (Amano P6), however, is a different type of protease obtained from *Aspergillus* melleus, which might be liable from its different activity. The poor resolutions by lipases might be due to the insolubility of enzymes in IL aqueous solutions, causing poor interactions between substrates and enzyme active sites, especially when the lipase is immobilized (Novozyme 435). Furthermore, our results indicate that the optimal IL concentration for one particular enzyme (lyophilized protease) may not always be the best concentration for other enzymes, because different biological macromolecules are stabilized by different degrees of ion kosmotropicity.[44,67,68]

Table 1. Enzymatic resolution of phenylalanine in 2.0 M [EMIM][CH_3-COO] by various enzymes at 30 °C

Enzyme	Optimum reaction time (min)	ee (%) (L-acid)	Yield (%) (L-acid)
Lyophilized protease	90	96.3	>99
Alcalase	20	78.6	45.2
Amano protease P6	40	69.8	26.7
PPL	20	47.6	11.3
Novozyme 435	20	1.4	7.6

Note: The amount of enzymes used is as follows: alcalase 20 mg, Novozyme-435 1.0 mg and other enzymes 0.5 mg.

2.4. Enzymatic resolution of different amino acids

In order to demonstrate the optimum conditions (2.0 M IL and lyophilized protease) on more substrates, we further conducted the resolution reactions of several other amino acids. Table 2 indicates that at optimum reaction times, a poor resolution (70.0% ee and 33.9% yield) of phenylalanine was produced in 2.0 M acetonitrile solution, while much higher ee were observed in 2.0 M [EMIM][CH_3COO] solutions (of H_2O: 96.3% ee, or in D_2O: 98.3 % ee; the heavy water effect is explained in Section 2.5). In general, moderately high to very high resolutions of amino acids were obtained in 2.0 M [EMIM][CH_3COO] solutions at different optimum reaction times. These results suggest that this IL system could be adopted by other similar enzymatic reactions.

Table 2. Enzymatic resolution of DL-amino acid esters in 2.0 M [EMIM][CH_3COO] at 30 °C

Amino acid ester	Optimum reaction time (h)	ee (%) (L-acid)	Yield (%) (L-acid)
Phenylalanine methyl ester (in 2.0 M acetonitrile)	0.33	70.0	33.9
Phenylalanine methyl ester (2.0 M IL in H_2O)	1.5	96.3	>99
Phenylalanine methyl ester (2.0 M IL in D_2O)	2.0	98.3	>99
4-Chlorophenylalanine ethyl ester	1.0	95.5	>99
Methionine methyl ester	0.33	91.4	31.0
Phenylglycine methyl ester	24	94.8	36.4
p-Hydroxyphenylglycine methyl ester	3.0	81.6	16.1

Note: 0.5 mg of *Bacillus licheniformis* protease lyophilizate was used in all reactions.

2.5. Heavy water effect of water on the enzymatic reaction

Figure 7 and Table 2 showed that the ee of L-amino acid achieved in D_2O solutions are very comparable with (or even slightly higher than) their respective ones in H_2O

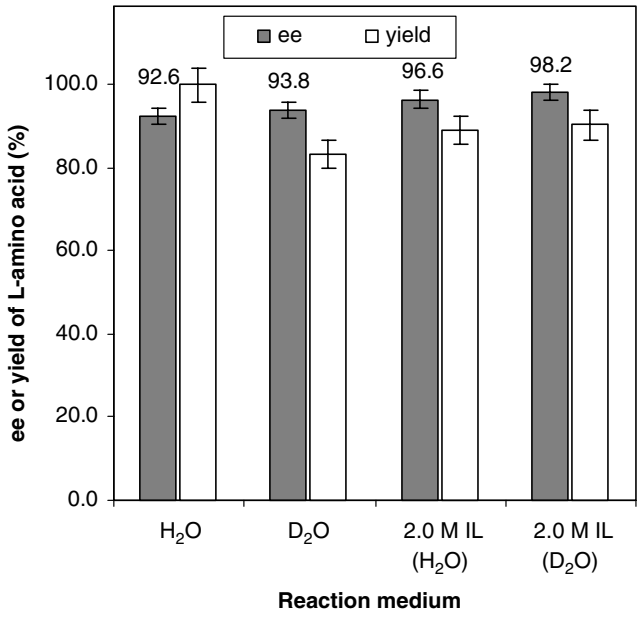

Figure 7. Effect of heavy water on the enzymatic resolution of phenylalanine methyl ester (IL is [EMIM][CH_3COO], lyophilized *Bacillus licheniformis* protease, 1 h reaction time, 30 °C, the error bars indicating percentage errors).

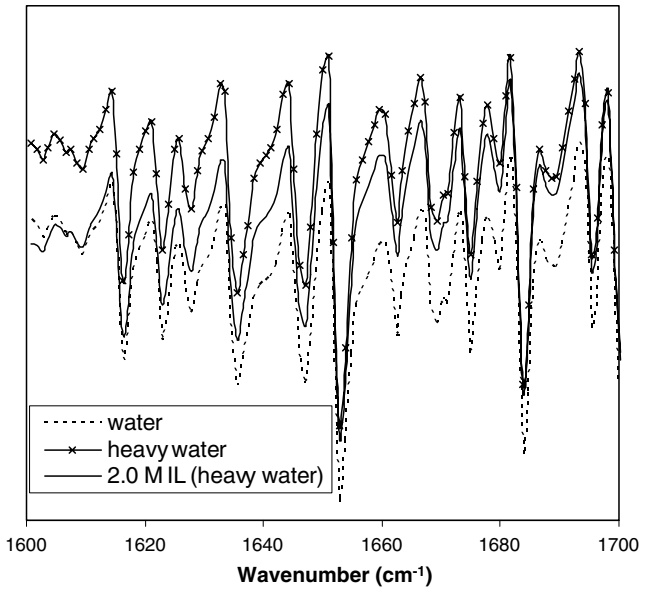

Figure 8. The second derivative spectra (FT-IR) of *Bacillus licheniformis* protease after incubation in various media (H_2O or D_2O) for 40 min at 30 °C.

solutions (with or without [EMIM][CH_3COO]), implying that the enzyme is stabilized by D_2O. Furthermore, enhanced ee was observed with the addition of IL, suggesting that [EMIM][CH_3COO] activates the enzyme. A similar observation was found in the D_2O solution of another IL named [EMIM][5-APA] (where 5-APA is 5-aminopentanoate).[69] However, a lower yield was obtained in pure D_2O comparing with that in pure H_2O, indicating a slower reaction rate in D_2O. It is known that deuterium oxide decreases cellular metabolism because its higher molecular mass (than H_2O) reduces the reaction rates. The rate reduction is due to two reasons: (1) the enzyme is more rigid in D_2O than in H_2O,[37] and (2) the H/D exchange of peptide NH protons modifies the protein properties.[70] However, very comparable yields were achieved in both H_2O and D_2O with the presence of IL (2.0 M [EMIM][CH_3COO]).

As shown in Figure 8, the second derivative spectra of protease in H_2O, D_2O and 2.0 M IL (in D_2O) are not quite distinguishable, suggesting that all these solvents are capable of preserving the protein secondary structures and enabling high enzyme activities. The quantitative analyses of second derivative spectra by Dong et al.[70] suggest that although the H/D exchange does not change the secondary structures of proteins, the structural elements (α-helix, β-sheets, β-turns and unordered) of proteins in H_2O and D_2O are different, possibly resulting in different enzyme conformation and catalytic activity.[59,71]

On the other hand, we also suspected that the hydration of $[EMIM]^+$ in D_2O might be different from that in H_2O, affording different kosmotropicity. The $[EMIM]^+$ cation in D_2O may experience H/D exchange. The C-2 hydrogen is the most acidic hydrogen ($pK_a = 21$–23) among all hydrogens on the imidazolium ring, and may be deprotonated under basic conditions.[72–74] Because our enzymatic reaction was performed in a basic buffer, the H/D exchange of the imidazolium cation could occur at the C-2 position.[74] Through ^1H NMR determination, Nguyen et al.[75] observed such a H/D exchange occurring in imidazolium cations. As a result, the imidazolium cation becomes partially deuterated, and thus its hydration behavior and kosmotropicity in D_2O are expected to be different from those in H_2O.

3. Conclusion

The lyophilized *B. licheniformis* protease showed a very high enantioselectivity and activity in up to 4.0 M [EMIM][CH_3COO], qualifying this IL as an enzyme-'friendly' solvent. The high enantioselectivity is almost independent of the IL concentration, while lower yields were observed in high ionic media. High enantioselective separations of several other amino acids were also observed in 2.0 M [EMIM][CH_3COO]. Deuterium oxide (D_2O) is able to stabilize the enzyme, especially with the presence of [EMIM][CH_3COO], producing high ee and yield.

4. Experimental

4.1. Materials

The following chemicals and enzymes were purchased from Sigma–Aldrich: 1-ethyl-3-methylimidazolium bromide ([EMIM]Br), 1-ethyl-3-methylimidazolium tosylate ([EMIM][OTs]), silver acetate, *B. licheniformis* protease lyophilizate (subtilisin Carlsberg, 12 U/mg, product number 85968), alcalase (*B. licheniformis* protease, $\geqslant$2.4 U/g, product number P4860), procine pancreas lipase (PPL, 30–90 U/mg using triacetin, product number L3126), Novozyme 435 immobilized on acrylic resin (lipase B from *Candida antarctica* so-called CAL-B, product number 537322), DL-phenylalanine methyl ester hydrochloride, DL-methionine methyl ester, 4-chlorophenylalanine ethyl ester, DL-*p*-hydroxyphenylglycine methyl ester, deuterium oxide (99.96 atom% D), and other reagents. Protease P 'Amamo' 6 (produced from a selected strain of *Aspergillus* melleus, 60 U/mg) was a kind gift from the Amano Enzyme USA.

4.2. IL preparations

1-Ethyl-3-methylimidazolium acetate ([EMIM][CH_3COO]) was synthesized by a slow addition of an aqueous [EMIM]Br solution into an equimolar Ag(CH_3COO) solution. The reaction was covered by the aluminum foil to prevent the photodegradation of silver acetate. The reaction mixture was stirred at room temperature for 2 h, followed by a removal of AgBr precipitate through filtration. Charcoal was added to the filtrate to remove color and impurities overnight. After filtering off the charcoal, water was removed from the filtrate through rotary evaporation under vacuum at 60 °C. The resulting IL is a slightly viscous and colorless liquid at room temperature. The absence of Br^- and Ag^+ in the IL was examined by 0.1 M $AgNO_3$ and 0.1 M HCl solutions, respectively. The IR, HPLC, and

^{1}H NMR measurements confirmed that the prepared IL contains no measurable impurities, including water.

4.3. Enzymatic resolution

DL-Amino acid (10 mg) was dissolved in 1.0 mL solvent consisting of an IL and 0.2 M $NaHCO_3$ buffer. Immediately, 0.5 mg of enzyme was added to the reaction mixture at time zero. The reaction was shaken and maintained at 30 ± 1 °C. The samples were withdrawn from the reaction mixture periodically and analyzed by a chiral HPLC. All experiments were run in duplicates. The averaged values are reported.

4.4. HPLC analysis

A Schimadzu LC-10AT HPLC is equipped with a SPD-10A UV–Vis dual wavelength detector, and a Crownpak CR(+) chiral column (150 mm × 4.0 mm, particle size 5 μm). The flow rate was 1.0 mL/min of 0.1 M $HClO_4$ solution. The detection wavelength is 254 nm. The typical eluting sequence of four isomers is in the increasing retention times of D-acid < L-acid < D-ester < L-ester.

4.5. Calculations of ee, L-yield and *E*

The ee of L-amino acid (so-called 'eep,' ee of product) was calculated from the HPLC integration area as (L area − D area)/(L area + D area) × 100%. The percentage yield of L-acid (shorten as yield or L-yield, its maximum is 100% for a complete conversion of L-ester) was calculated from comparing the current area of L-acid with that of complete conversion of L-ester. The L-acid area for the complete conversion of L-ester was determined individually for each sample by HPLC, typically at 2–3 h of extended reaction time. The enantiomeric ratio (E) was calculated from the following formula as defined by Chen et al.[76]

$$E = \frac{\ln[1 - c(1 + \mathrm{ee}(P))]}{\ln[1 - c(1 - \mathrm{ee}(P))]}$$

where $c = 1 - (A + B)/(A_0 + B_0)$ and $\mathrm{ee}(P) = (P - Q)/(P + Q)$. A and B are concentrations of a pair of enantiomers, A_0 and B_0 are their initial concentrations.

4.6. Measurement of viscosity

The viscosity of IL or IL aqueous solution was determined by a Cannon–Fenske Routine (CFR) viscometer. The viscometer was incubated in a water bath maintained at 30 ± 1 °C. The distilled water was used as a standard compound ($\eta = 0.7977$ at 30 °C).[77] Triplicates were measured for each sample. The relative errors are less than 3%. The viscosity of pure [EMIM][CH_3COO] (dried) was determined as 11.2 ± 0.3 cP.

4.7. FT-IR measurements

The method is a modification of a literature method:[71] 0.5 mg of enzyme was incubated in 1.0 mL of 0.2 M $NaHCO_3$ aqueous solution of IL or organic solvent at 30 °C. The solution was periodically withdrawn and placed between two CaF_2 windows (Aldrich, 25 mm × 2mm). A Shimadzu FT-IR 8300 equipped with a dTGS detector was used to measure the infrared spectra (% transmittance) through averaging 32 scans at 2 cm^{-1} resolution (using the Happ-Genzel apodization). The instrument was controlled by the Shimadzu software IRsolution 1.20, and second derivative spectra were calculated via the 9-point Savitsky-Golay function provided by the same software.

Acknowledgments

This project was supported by NSF HBCU-UP 2006 Research Program, and a CASTME Research Project under the Title III grant from the Department of Education.

References

1. van Rantwijk, F.; Madeira Lau, R.; Sheldon, R. A. *Trends Biotechnol.* **2003**, *21*, 131–138.
2. Park, S.; Kazlauskas, R. J. *Curr. Opin. Biotechnol.* **2003**, *14*, 432–437.
3. Kragl, U.; Eckstein, M.; Kaftzik, N. *Curr. Opin. Biotechnol.* **2002**, *13*, 565–571.
4. Sheldon, R. A.; Lau, R. M.; Sorgedrager, M. J.; van Rantwijk, F.; Seddon, K. R. *Green Chem.* **2002**, *4*, 147–151.
5. Yang, Z.; Pan, W. *Enzyme Microb. Technol.* **2005**, *37*, 19–28.
6. *Ionic Liquids in Synthesis*; Wasserscheid, P., Welton, T., Eds.; Wiley-VCH: Weinheim, 2003.
7. Seddon, K. R. *J. Chem. Technol. Biotechnol.* **1997**, *68*, 351–356.
8. Zhao, H.; Malhotra, S. V. *Aldrichim. Acta.* **2002**, *35*, 75–83.
9. Tokuda, H.; Hayamizu, K.; Ishii, K.; Susan, M. A. B. H.; Watanabe, M. *J. Phys. Chem. B* **2004**, *108*, 16593–16600.
10. Tokuda, H.; Hayamizu, K.; Ishii, K.; Susan, M. A. B. H.; Watanabe, M. *J. Phys. Chem. B* **2005**, *109*, 6103–6110.
11. Zhao, H. *J. Mol. Catal. B: Enzym.* **2005**, *37*, 16–25.
12. Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192–3196.
13. Bell, G.; Halling, P. J.; Moore, B. D.; Partridge, J.; Rees, D. G. *Trends Biotechnol.* **1995**, *13*, 468–473.
14. Gupta, M. N. *Eur. J. Biochem.* **1992**, *203*, 25–32.
15. Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194–3201.
16. Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81–87.
17. Anderson, J. L.; Ding, J.; Welton, T.; Armstrong, D. W. *J. Am. Chem. Soc.* **2002**, *124*, 14247–14254.
18. Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. *J. Am. Chem. Soc.* **2002**, *124*, 4974–4975.
19. Kaar, J. L.; Jesionowski, A. M.; Berberich, J. A.; Moulton, R.; Russell, A. J. *J. Am. Chem. Soc.* **2003**, *125*, 4125–4131.
20. Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017–8021.
21. van Rantwijk, F.; Secundo, F.; Sheldon, R. A. *Green Chem.* **2006**, *8*, 282–286.
22. Zhao, H.; Olubajo, O.; Song, Z.; Sims, A. L.; Person, T. E.; Lawal, R. A.; Holley, L. A. *Bioorg. Chem.* **2006**, *34*, 15–25.
23. Zhao, H.; Campbell, S.; Jackson, L.; Song, Z.; Olubajo, O. *Tetrahedron: Asymmetry* **2006**, *17*, 377–383.
24. Zhao, H.; Campbell, S.; Solomon, J.; Song, Z.; Olubajo, O. *Chin. J. Chem.* **2006**, *24*, 580–584.
25. Collins, K. D.; Washabaugh, M. W. *Quart. Rev. Biophys.* **1985**, *18*, 323–422.

26. Boström, M.; Williams, D. R. M.; Ninham, B. W. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 48–52.
27. Wiggins, P. M. *Microbiol. Rev.* **1990**, *54*, 432–449.
28. Nandi, P. K.; Robinson, D. R. *J. Am. Chem. Soc.* **1972**, *94*, 1299–1308.
29. Zhao, H. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 877–891.
30. Huddleston, J. G.; Visser, A. E.; Reichert, W. M.; Willauer, H. D.; Broker, G. A.; Rogers, R. D. *Green Chem.* **2001**, *3*, 156–164.
31. Rhodes, V. *Chim. Oggi (Chem. Today)* **2002**, *20*.
32. Chorghade, M. S.; Gurjar, M. K.; Talukdar, A. *Chim. Oggi (Chem. Today)* **2002**, *20*.
33. Stinson, S. C. *Chem. Eng. News* **2001**, *79*, 79–97.
34. *Science News.* 1933, October 14 Issue. Online, http://www.sciencenews.org/articles/20031018/timeline.asp (accessed April 2006).
35. Maybury, R. H.; Katz, J. J. *Nature* **1956**, *177*, 629–630.
36. Chakrabarti, G.; Kim, S.; Gupta, M. L., Jr.; Barton, J. S.; Himes, R. H. *Biochemistry* **1999**, *38*, 3067–3072.
37. Cioni, P.; Strambini, G. B. *Biophys. Chem.* **2002**, *82*, 3246–3253.
38. Tang, K. E. S.; Dill, K. A. *J. Biomol. Struct. Dyn.* **1998**, *16*, 397–411.
39. Makhatadze, G. I.; Clore, G. M.; Gronenborn, A. M. *Nat. Struct. Biol.* **1995**, *2*, 852–855.
40. Chin, J. T.; Wheeler, S. L.; Klibanov, A. M. *Biotechnol. Bioeng.* **1994**, *44*, 140–145.
41. Klibanov, A. M. *Trends Biotechnol.* **1997**, *15*, 97–101.
42. Carpenter, J. F.; Prestrelski, S. J.; Dong, A. *Eur. J. Pharm. Biopharm.* **1998**, *45*, 231–238.
43. Surewicz, W. K.; Mantsch, H. H.; Chapman, D. *Biochemistry* **1993**, *32*, 389–394.
44. von Hippel, P. H.; Schleich, T. *Acc. Chem. Res.* **1969**, *2*, 257–265.
45. *Biotransformations in Organic Chemistry*; Faber, K., Ed.; Springer: New York, 2000.
46. Turner, M. B.; Spear, S. K.; Huddleston, J. G.; Holbrey, J. D.; Rogers, R. D. *Green Chem.* **2003**, *5*, 443–447.
47. Zhao, H. *Biophys. Chem.* **2006**, *122*, 157–183.
48. Magnusson, D. K.; Bodley, J. W.; Evans, D. F. *J. Solution Chem.* **1984**, *13*, 583–587.
49. Dupont, J. *J. Braz. Chem. Soc.* **2004**, *15*, 341–350.
50. Lozano, P.; De Diego, T.; Gmouh, S.; Vaultier, M.; Iborra, J. L. *Biocatal. Biotransform.* **2005**, *23*, 169–176.
51. Kijima, T.; Ohshima, K.; Kise, H. *J. Chem. Technol. Biotechnol.* **1994**, *59*, 61–65.
52. Chen, S.-T.; Wang, K.-T.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1986**, 1514–1516.
53. Chen, S.-T.; Huang, W.-H.; Wang, K.-T. *Chirality* **1994**, *6*, 572–576.
54. Chen, S.-T.; Chen, S.-Y.; Hsiao, S.-C.; Wang, K.-T. *Biotechnol. Lett.* **1991**, *13*, 773–778.
55. Zhao, H.; Luo, R. G.; Wei, D.; Malhotra, S. V. *Enantiomer.* **2002**, *7*, 1–3.
56. Zhao, H.; Malhotra, S. V. *Biotechnol. Lett.* **2002**, *24*, 1257–1260.
57. Houng, J.-Y.; Wu, M.-L.; Chen, S.-T. *Chirality* **1998**, *8*, 418–422.
58. Husum, T. L.; Jorgensen, C. T.; Christensen, M. W.; Kirk, O. *Biocatal. Biotransform.* **2001**, *19*, 331–338.
59. De Diego, T.; Lozano, P.; Gmouh, S.; Vaultier, M.; Iborra, J. L. *Biomacromolecules* **2005**, *6*, 1457–1464.
60. Kim, K. W.; Song, B.; Choi, M. Y.; Kim, M. J. *Org. Lett.* **2001**, *3*, 1507–1509.
61. Park, S.; Kazlauskas, R. J. *J. Org. Chem.* **2001**, *66*, 8395–8401.
62. Liu, Y.-Y.; Lou, W.-Y.; Zong, M.-H.; Xu, R.; Hong, X.; Wu, H. *Biocatal. Biotransform.* **2005**, *23*, 89–95.
63. Schiffer, C. A.; Dotsch, V. *Curr. Opin. Biotechnol.* **1996**, *7*, 428–432.
64. Dabulis, K.; Klibanov, A. M. *Biotechnol. Bioeng.* **1993**, *41*, 566–571.
65. Dordick, J. S. *Biotechnol. Prog.* **1992**, *8*, 259–267.
66. Yang, L.; Dordick, J. S.; Garde, S. *Biophys. J.* **2004**, *87*, 812–821.
67. Wiggins, P. M. *Cell. Mol. Biol.* **2001**, *47*, 735–744.
68. von Hippel, P. H.; Schleich, T. The effects of neutral salts on the structure and conformational stability of macromolecules in solution. In *Structure and Stability of Biological Macromolecules*; Timasheff, S. N., Fasman, G. D., Eds.; Marcel Dekker: New York, 1969; pp 417–574.
69. Zhao, H.; Jackson, L.; Song, Z.; Olubajo, O. *Tetrahedron: Asymmetry* **2006**, *17*, 1549–1553.
70. Dong, A.; Matsuura, J.; Allison, S. D.; Chrisman, E.; Manning, M. C.; Carpenter, J. F. *Biochemistry* **1996**, *35*, 1450–1457.
71. Lau, R. M.; Sorgedrager, M. J.; Carrea, G.; van Rantwijk, F.; Secundo, F.; Sheldon, R. A. *Green Chem.* **2004**, *6*, 483–487.
72. Amyes, T. L.; Diver, S. T.; Richard, J. P.; Rivas, F. M.; Toth, K. *J. Am. Chem. Soc.* **2004**, *126*, 4366–4374.
73. Magill, A. M.; Yates, B. F. *Aust. J. Chem.* **2004**, *57*, 1205–1210.
74. Dupont, J.; Spencer, J. *Angew. Chem., Int. Ed.* **2004**, *43*, 5296–5297.
75. Nguyen, H.-P.; Kirilov, P.; Matondo, H.; Baboulène, M. *J. Mol. Catal. A. Chem.* **2004**, *218*, 41–45.
76. Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–7299.
77. Lide, D. R. *CRC Handbook of Chemistry and Physics*, 84th ed.; CRC Press: New York, 2003; Section 6, p 3.