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Hofmeister series of ionic liquids: kosmotropic effect of ionic liquids on the enzymatic hydrolysis of enantiomeric phenylalanine methyl ester

Hua Zhao,* Sophia M. Campbell, Lee Jackson, Zhiyan Song and Olarongbe Olubajo

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

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Abstract—The kinetic hydrolysis of enantiomeric phenylalanine methyl ester catalyzed by *Bacillus licheniformis* protease was performed in aqueous solutions of several hydrophilic ionic liquids (ILs). The protease enantioselectivity was found related to the kosmotropicity of individual cations and anions of ILs. The ion effectiveness in enhancing the enzyme enantioselectivity follows the Hofmeister series: kosmotropic anions and chaotropic cations stabilize the enzyme. In this application, the Hofmeister series of ILs was established in an order of decreasing effectiveness for anions: PO_4^{3-} > citrate^{3-}, CH_3COO^-, $EtSO_4^-$, CF_3COO^- > Br^- > OTs^-, BF_4^- and for cations: $[EMIM]^+$ > $[BMIM]^+$ > $[HMIM]^+$. The overall IL kosmotropicity was quantified by the δ value (difference in the Jones–Dole viscosity *B*-coefficients of anion and cation). In general, a high enzyme enantioselectivity was observed in a solution of IL with a high δ value.

1. Introduction

Over the past several years, a great deal of excitement has been generated by the awareness and use of ionic liquids (ILs) as novel reaction media in a variety of enzymatic reactions.[1–6] There have been some mechanistic discussions as to why enzymes either are active or inactive in certain ILs. Several factors of ILs seem to be responsible for the enzyme activity and stability, including IL polarity,[1] hydrogen-bond basicity[7,8] and anion nucleophilicity.[9] For example, Park and Kazlauskas correlated the enzyme (*Pseudomonas cepacia* lipase) activity with the IL polarity, observing higher conversions in more polar ILs.[10] However, many other studies[5,9,11,12] have not yet established a simple correlation between the enzyme activity and IL polarity. The other two factors (hydrogen-bond basicity and anion nucleophilicity) have the same problem in terms of lacking a general relationship between the enzyme activity and IL properties. Therefore, the current understanding of the IL effect on enzyme activity is still in its infancy.

In hydrophobic ILs [such as PF_6^- and $(CF_3SO_2)_2N^-$ salts], enzymes have shown very high stabilities in a number of applications.[9,13–18] The enzyme activity is related to the solvent hydrophobicity (in terms of log *P* values, the partition coefficients between water and octanol) and thermodynamic water activity (a_w) as discussed in our recent review.[19] Enzymes were found to be more stable in solvents with a larger log *P* (>3) (such as hexane, which has a log *P* of 3.9) than lower log *P* (such as ethanol, which has a log *P* of −0.24).[20] The reason is because the hydrophobic solvents have a lesser tendency of taking away the 'essential' (or 'critical') water from the enzyme's surface.[21–23] It is known that enzyme activity is determined by the water bound to the enzyme ('essential' water, a few monolayers of water), rather than the bulk-water content in the system.[24–26] To further examine the IL effect on protein structures from a molecular level, the circular dichroism (CD) spectrum of α-chymotrypsin in hydrophobic ILs was found closer to that in water; the spectrum also revealed that the β-strand of the protein secondary structures was considerably increased in ILs.[15,27,28] Another aspect to interpret the enzyme stability in ILs is based on the observation that ILs may form the so called organized 'nano-structures' (hydrogen-bonded polymeric supramolecules, just like water molecules) with polar and nonpolar regions in solid, liquid, and solution states, or even in the gas phase.[29,30] In his review, Dupont[30] pointed out that the aqueous solution of free

* Corresponding author. E-mail: zhaoh@savstate.edu

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enzyme might be embedded in the IL network, which could protect the essential water of proteins and the solvophobic interactions, that is critical for maintaining the native structure.

On the other hand, when hydrophilic ILs are dissolved in an aqueous solution, they dissociate into individual cations and anions. These individual ions may affect the enzyme behavior in aqueous environments. As a matter of fact, the effect of individual ions on protein stabilization is well known (the Hofmeister series):[31,32] kosmotropic anions[†] and chaotropic cations[†] stabilize proteins, while chaotropic anions and kosmotropic cations destabilize them.[33–37] Our recent review[19] indicated that the same ion effect was applicable to enzyme activity, specificity and stability as supported by numerous experimental data. Therefore, the effect of ILs on the enzyme activity not only relied on the overall solvent properties (such as polarity), but also depended on the individual ion contributions.

The structural stabilization of proteins and enzymes by ILs has recently gained some attention. An early study by Summers and Flowers[38] investigated a room-temperature IL, ethylammonium nitrate (EAN, or $[EtNH_3][NO_3]$), in the protein refolding of hen egg white lysozyme (HEWL). Their results confirmed that EAN is a denaturant (since it is a structure-breaking electrolyte as suggested by its volumetric behavior during hydration;[39] meanwhile, both $[EtNH_3]^+$ (*B*-coefficient:[40] 0.132) and NO_3^- (−0.043) are chaotropic anions).[41] However, EAN was able to prevent the aggregation of denatured protein. It was suspected that the interaction between the ethyl group of EAN and the hydrophobic surfaces of the protein protects EAN from intermolecular association. Meanwhile, electrostatic interactions between the ions and the charged portion of the protein could stabilize the secondary structure.[42] Another recent study examined the activity of HEWL, and the renaturation of HEWL and single-chain antibody fragment ScFvOx in 1-alkyl and 1-(ω-hydroxyalkyl)-3-methylimidazolium chlorides.[43] The protein refolding by these ILs was also explained as the suppression of aggregate formation by these organic salts. Both the activity of HEWL in ILs and the ability of ILs in refolding the HEWL and ScFvOx followed the Hofmeister series (in a decreasing order): $[OH–EMIM]^+ > [EMIM]^+$, $[OH–PMIM]^+ > [BMIM]^+$, $[OH–HMIM]^+ > [HMIM]^+$.[‡] The cation kosmotropicity is in an increasing order because the ion hydrophobicity increases upon lengthening of alkyl chain.[41] The cations containing hydroxyl groups are less hydrophobic, and are thus less kosmotropic when compared with those containing no hydroxyl groups, but with the same carbon-chain length. Our recent studies[12,44] on the protease activity in IL aqueous solutions also revealed that enzyme stability followed the Hofmeister series in an order of decreasing effectiveness for anions: CH_3COO^-, $CF_3COO^- > Cl^-$, $Br^- > OTs^- > BF_4^-$ (decreasing kosmotropicity), and for cations: $[EMIM]^+$, $[BuPy]^+ > [BMIM]^+ > [EtPy]^+$ (increasing kosmotropicity in general).

Based on the above pioneering work, we explored further the effect of IL kosmotropicity on the enantioselectivity of a protease in this study. More specifically, we investigated the enzymatic hydrolysis of enantiomeric phenylalanine methyl ester in aqueous solutions of various ILs. The enzyme, *Bacillus licheniformis* protease (subtilisin Carlsberg), was chosen because it is cofactor-independent and active in low-water environments (~9% water/dry enzyme, wt/wt), its physiological function is to hydrolyze water-soluble proteins, and its structures, catalytic mechanism and properties are well understood.[21,45] Herein, we report the correlation between the Hofmeister series of ILs and the enzyme stabilization, which would enable us to use the series as an empirical guideline in designing ILs for specific enzymatic applications.

2. Results

2.1. Effect of buffer and IL concentrations on the kinetic hydrolysis

The enzymatic hydrolysis rate of L-phenylalanine methyl ester is faster than that of D-ester. Figure 1 illustrates the effect of buffer concentration (hence the pH value) on the hydrolysis reaction. The enantiomeric excess (ee) of L-phenylalanine decreased with an increase

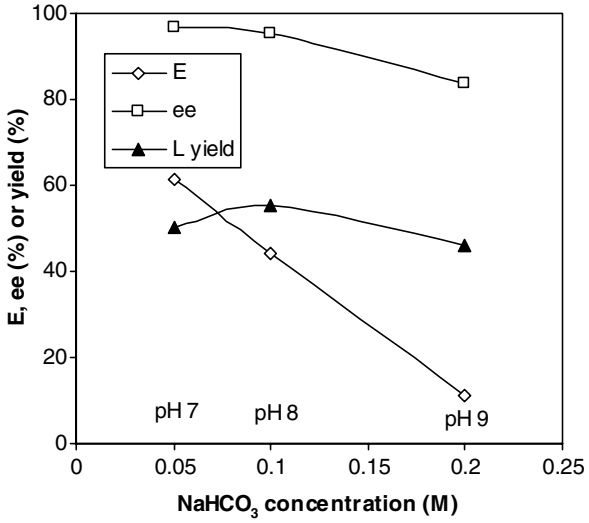

Figure 1. Effect of buffer ($NaHCO_3$) concentration and pH on the enzymatic hydrolysis of phenylalanine methyl ester in 0.5 M $[EMIM][EtSO_4]$ (40 min reaction time).

[†] 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^+, OH^-, etc. 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), $(CH_3)_4N^+$ (tetramethylammonium), and others.

[‡] OH–EMIM, 1-(2-hydroxyethyl)-3-methylimidazolium; EMIM, 1-ethyl-3-methylimidazolium; OH–PMIM, 1-(3-hydroxypropyl)-3-methylimidazolium; BMIM, 1-butyl-3-methylimidazolium; OH–HMIM, 1-(6-hydroxyhexyl)-3-methylimidazolium; HMIM, 1-hexyl-3-methylimidazolium.

of $NaHCO_3$ concentration, causing a dramatic decrease of the enantiomeric ratio (E value). However, the highest yield of L-phenylalanine was observed in 0.1 M $NaHCO_3$ solution, where the ee retained a relatively high value. Therefore, we selected 0.1 M $NaHCO_3$ as the buffer solution for the following study.

The IL, [EMIM][$EtSO_4$], was chosen because a high enzyme activity was observed in this organic salt (Fig. 2). Meanwhile, this IL, known as ECOENG™ 212, showed no irritation to skin and eyes, and had a low toxicity (LD_{50} value > 2000 mg/kg [rat, Wistar strain], and EC_{50} value > 100 mg/L [*Daphnia magna*]).[46]

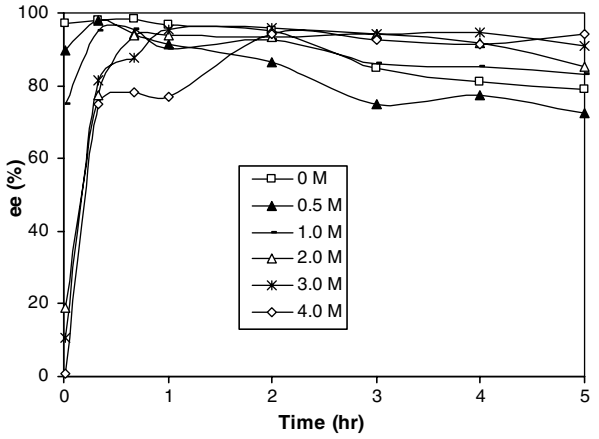

Figure 2. Effect of [EMIM][$EtSO_4$] concentration on the enantiomeric excess (ee) of L-phenylalanine.

An inspection of Figures 2 and 3 afforded the conclusion that the protease enantioselectivity is IL concentration dependent. The IL concentrations investigated in this study ranged from 0 to 4.0 M (~80%, v/v). Figure 2 illustrated that within a 5 h period, the enantioselectivity increased as the IL concentration increased, with most

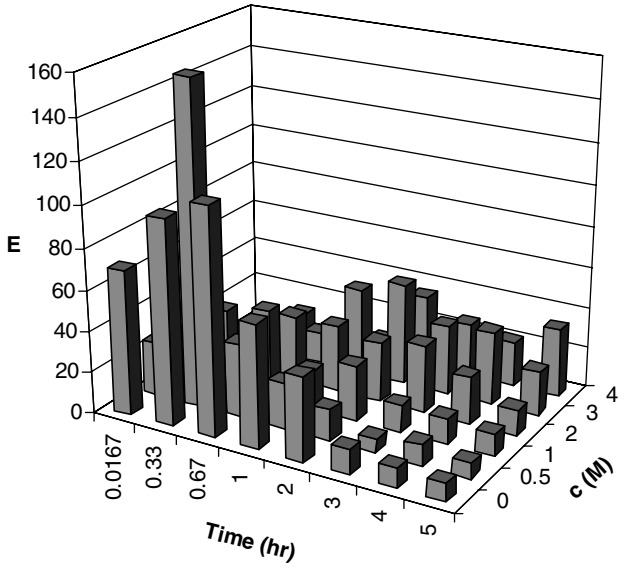

Figure 3. The enantiomeric ratio (E) as a function of reaction time and [EMIM][$EtSO_4$] concentration.

ee values being moderately high (above 80%). This finding demonstrated that $EtSO_4^-$ is a kosmotropic anion and [EMIM][$EtSO_4$] is an enzyme-'friendly' ionic solvent. However, extremely high ee values (>90%) were normally observed within 40 min of the hydrolysis time because when the reaction time was prolonged further, the hydrolysis rate of the L-ester was slower while that of the D-ester became faster. Such an observation was also reflected by the E values in Figure 3 where the highest E (98% ee and 42% yield for L-phenylalanine) was achieved in 0.5 M [EMIM][$EtSO_4$], when the reaction time was 20 min. This E value was even higher than those obtained in pure water, indicating that this IL concentration was able to activate the enzyme. Figure 3 also indicated that with an increase of IL concentration, a longer reaction time was needed to achieve the optimum E value for each concentration. This was probably due to the slow substrate dissolution causing the diffusional limitation in viscous IL solutions (especially when the concentration is greater than 3.0 M).

2.2. Effect of kosmotropicity of anions and cations on the enantioselectivity

The effect of anion kosmotropicity on the enzyme enantioselectivity was demonstrated in Figure 4. High ee values were obtained in IL solutions (0.5 M) containing kosmotropic anions, such as PO_4^{3-}, citrate^{3-}, CH_3COO^-, $EtSO_4^-$, and CF_3COO^-. A moderately high ee was observed when the weakly chaotropic anion Br^- was present, while very low enantioselectivities were seen when strong chaotropic anions (OTs^- and BF_4^-) were involved. Out of curiosity, we examined further

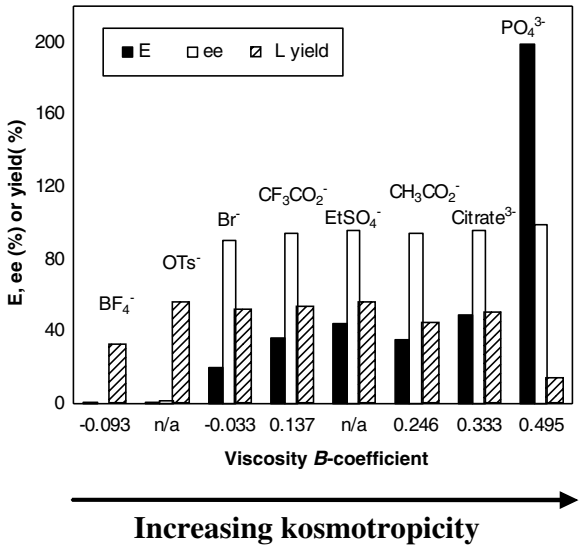

Figure 4. The effect of anion kosmotropicity of ILs on the enzymatic hydrolysis (0.5 M $[EMIM]^+$ based ILs; 40 min reaction time; B-coefficients from Marcus's selections;[40] *n/a* means the B-coefficient is not available for that ion; the kosmotropicity of CF_3COO^- is known between CH_3COO^- and Cl^-,[41] the B value of CF_3COO^- is roughly estimated from the NMR B'-coefficients of CF_3COO^- (0.10) and CH_3COO^- (0.18)[47] based on the simple assumption that the B-coefficient is proportional to the B'-coefficients; the B-coefficient of citrate was calculated from Ref. 48 using the simplified Jones–Dole equation $\eta/\eta_0 = 1 + B \times c$).

the enzymatic hydrolysis in higher concentrations of citrate based ILs (up to 2.0 M, ~75% v/v IL). As shown in Figure 5, high enantioselectivities were also achieved in concentrated solutions of citrate based ILs; the yield was slightly decreased with an increase of IL concentration due to the diffusional limitation in viscous solutions. Figure 6 reported the effect of IL cations on the enzymatic hydrolysis. The ee and E values decreased with the increase of the alkyl chain length of cations.

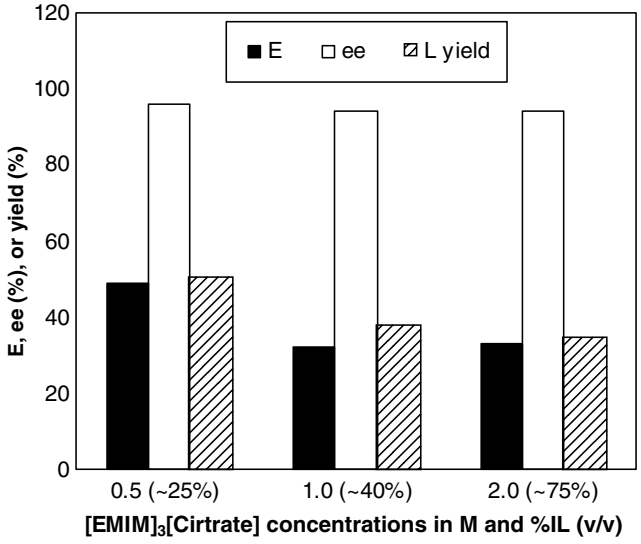

Figure 5. Enzymatic hydrolysis of phenylalanine methyl ester in citrate based ILs of various concentrations (40 min reaction time).

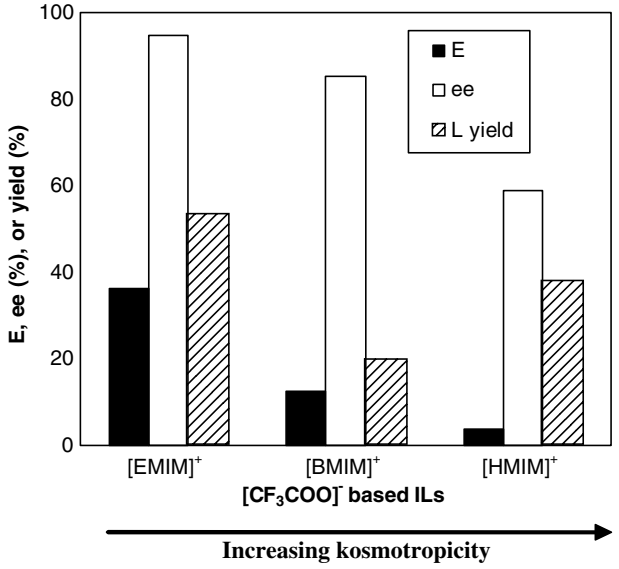

Figure 6. Effect of cation kosmotropicity of ILs on the enzymatic hydrolysis of phenylalanine methyl ester (0.5 M $[CF_3COO]^-$ based ILs; 40 min reaction time).

2.3. Correlation between the enantioselectivity and δ values

Figure 7 correlates the protease enantioselectivity with the δ values (differences in the Jones–Dole viscosity B-coefficients of anion and cation) of N-alkylpyridinium based ILs. The general relationship between the enantioselectivity and the δ values is thus: the ee and E increase with an increase of δ value. One exception was the [BuPy]Cl salt, the reason behind it being that BF_4^- is a much stronger chaotrope than Cl^- while the kosmotropicity difference between $[EtPy]^+$ and $[BuPy]^+$ is not significantly large.[41] Unfortunately, the B-coefficients of imidazolium and many other organic cations are not yet available for more correlations.

3. Discussion

The buffer chosen for the enzymatic hydrolysis was sodium bicarbonate ($NaHCO_3$). The Jones–Dole viscosity B-coefficients for Na^+ and HCO_3^- were 0.085 and 0.031, respectively;[40] since a large positive B value means a strong kosmotrope while a large negative B value means a strong chaotrope,[41] both the cation and anion can be classified as borderline ions.[§] Therefore, both of them have minimum kosmotropic effects on the enzyme activity. If a phosphate buffer was used instead, the ions present in buffer may influence the enzyme stability since HPO_4^{2-} anion (B value:[40] 0.382) is a kosmotrope and $H_2PO_4^-$ anion (0.340) is considered as a borderline ion, while Na^+ and K^+ (−0.009) (if present) are in the range of borderline ions.[51]

Figure 4 suggests that the protease enantioselectivity follows the Hofmeister series, that is, the kosmotropic anions stabilize the enzyme while chaotropic anions destabilize it. The anion ability in improving the enzyme enantioselectivity is in an order of decreasing effectiveness: $PO_4^{3-} > citrate^{3-}$, CH_3COO^-, $EtSO_4^-$, $CF_3COO^- > Br^- > OTs^-$, BF_4^-. Figures 2 and 5 concluded that the enzyme enantioselectivity is still very high in high concentrations of ILs containing kosmotropic anions (such as $EtSO_4^-$ and citrate). However, a lower substrate conversion was also observed in ILs with very strong kosmotropic anions (such as PO_4^{3-}). This could probably be explained by the reactivity–selectivity principle, that is, the less the reactivity of a species, the greater selectivity it will be.

As explained in our recent review,[41] the hydrophobicity of cations increases with the cation sizes, yielding a higher kosmotropicity. Since the high kosmotropic cations destabilize the enzyme based on the Hofmeister series, the cation ability in improving the enzyme enantioselectivity is in an order of decreasing effectiveness: $[EMIM]^+ > [BMIM]^+ > [HMIM]^+$ (Fig. 6).

The δ value reflects the overall kosmotropic effect of cations and anions of ILs (Fig. 7): high kosmotropicity of anion and low kosmotropicity of cation seem desirable for the enzyme stabilization. A balance between these

[§] A different B value for HCO_3^- is 0.130,[40] however, based on the slightly negative value of structural entropy and the zero value of ΔG_{HB} (defined in the literature),[50] this ion was classified as a borderline ion.[41,51]

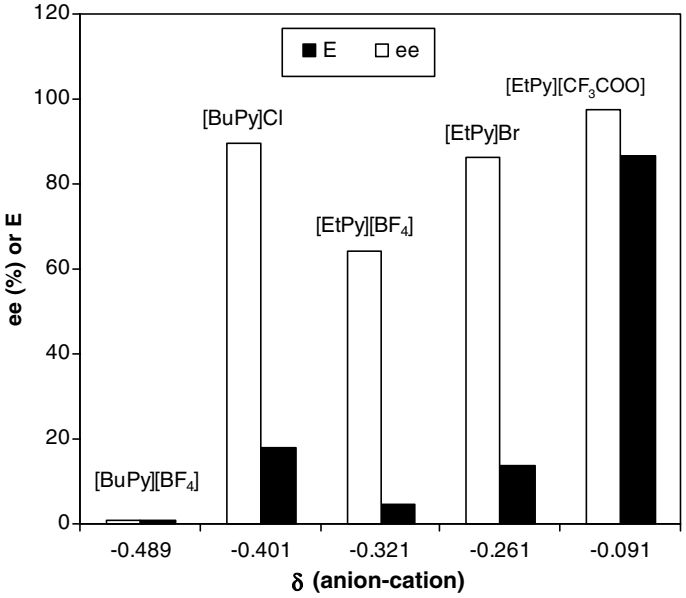

Figure 7. Correlation between the enantioselectivity and the difference in the Jones–Dole viscosity *B*-coefficients of anion and cation (δ is defined[40,49] as $\sum v_i B$(anion) − $\sum v_i B$(cation) where v_i is mole fraction of the ion; *B*-coefficients of anions are from Marcus's selections;[40] *B*-coefficients of *N*-alkylpyridinium cations are from our recent review;[41] reaction time is 40 min).

two could enable an optimal stabilization of biological macromolecules (including enzymes).[35,37,52] Lindsay et al.[49] correlated the reactivity of penicillin amidase formulations with the δ values of inorganic salts and their mixtures (used in the lyophilization of the enzyme). Similarly, they observed that the enzyme activity increased with the δ value.

4. Conclusion

Both the buffer concentration (inorganic salt concentration) and the IL concentration (organic salt concentration) considerably modify the enzymatic hydrolysis rate of enantiomeric phenylalanine methyl ester. The protease enantioselectivity was greatly enhanced by using IL solutions containing kosmotropic anions and chaotropic cations because the effect of ions on the enzyme stabilization follows the Hofmeister series. The δ value is an empirical scale for evaluating the overall kosmotropicity of an IL. Generally, high enzyme enantioselectivities were observed in ILs with high δ values. The ion ability in improving the enzyme enantioselectivity is in an order of decreasing effectiveness for anions: PO_4^{3-} > citrate^{3-}, CH_3COO^-, $EtSO_4^-$, CF_3COO^- > Br^- > OTs^-, BF_4^- and for cations: $[EMIM]^+$ > $[BMIM]^+$ > $[HMIM]^+$. However, for different biological applications, ions do not necessarily stabilize or destabilize the enzyme in exactly the same Hofmeister order.[19,52–54]

5. Experimental

5.1. Materials

N-Ethylpyridinium bromide ([EtPy]Br) and *N*-*n*-butylpyridinium chloride ([BuPy]Cl) were obtained from the Alfa Aesar Company. 1-Ethyl-3-methylimidazolium bromide ([EMIM]Br), 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM][$EtSO_4$]), 1-ethyl-3-methylimidazolium tosylate ([EMIM][OTs]), 1-butyl-3-methylimidazolium bromide ([BMIM]Br), 1-hexyl-3-methylimidazolium chloride ([HMIM]Cl), silver acetate, *B. licheniformis* protease (subtilisin Carlsberg), D-phenylalanine methyl ester hydrochloride, L-phenylalanine methyl ester hydrochloride, and other reagents were purchased from the Sigma–Aldrich.

5.2. IL preparations

Table 1 summarizes the source and appearances of the ILs used in this study. [EMIM]$_3$[PO_4] was prepared by the stoichiometric titration of phosphoric acid with [EMIM][OH], which was prepared according to a literature method[55] by using the anion exchange resin (Amberlite® IRA-400 Cl). [EMIM]$_3$[citrate] was prepared by mixing a [EMIM]Br aqueous solution with slightly excess equimolar silver citrate hydrate (white powder, insoluble) with a gentle heat; the yellow precipitate (AgBr) formed in the solution; the completeness of the reaction was monitored by taking samples from the solution and reacting with $AgNO_3$ solution; the precipitates (AgBr and excess silver citrate) were removed when the solution was cooled by an ice bath; the possible presence of silver citrate in the mother liquid was checked by a [EMIM]Br solution. Other ILs were prepared in our laboratory by the silver metathesis method, as explained in the literatures.[10,56] Charcoal was used for an effective removal of color and impurities from all crude ILs.[57] The silica gel column was further used to remove trace impurities. Water was evaporated through a rotary evaporator under vacuum at 50 °C. [EMIM]$_3$[PO_4] is a brown and viscous liquid, and other ILs prepared are slightly viscous and colorless liquids. The prepared ILs were examined by 0.1 M $AgNO_3$ and 0.1 M HCl solutions, respectively,

Table 1. Ionic liquids (ILs) investigated in this study

IL	Molar mass (g mol^{-1})	Appearance	Source
[EMIM]Br	191.07	Solid, slightly yellow	Sigma–Aldrich
[EMIM][$EtSO_4$]	236.29	Viscous liquid, slightly yellow	Sigma–Aldrich
[EMIM][CH_3COO]	170.19	Slightly viscous liquid, colorless	Prepared
[EMIM][CF_3COO]	224.19	Slightly viscous liquid, colorless	Prepared
[EMIM][OTs]	282.36	Solid, white	Sigma–Aldrich
[EMIM][BF_4]	197.98	Slightly viscous liquid, colorless	Prepared
[EMIM]$_3$[citrate]	522.63	Viscous liquid, colorless	Prepared
[EMIM]$_3$[PO_4]	428.48	Viscous liquid, brown	Prepared
[BMIM]Br	219.12	Solid, slightly yellow	Sigma–Aldrich
[BMIM][CF_3COO]	252.24	Slightly viscous liquid, colorless	Prepared
[HMIM]Cl	202.72	Very viscous liquid, yellow	Sigma–Aldrich
[HMIM][CF_3COO]	280.30	Slightly viscous liquid, colorless	Prepared
[EtPy]Br	188.07	Solid, slightly yellow	Alfa Aesar
[EtPy][CF_3COO]	221.19	Slightly viscous liquid, colorless	Prepared
[EtPy][BF_4]	194.98	Slightly viscous liquid (sometimes crystals), colorless	Prepared
[BuPy]Cl	171.66	Solid, slightly yellow	Alfa Aesar
[BuPy][BF_4]	223.02	Slightly viscous liquid (sometimes crystals), colorless	Prepared

to ensure the absence of halides and Ag^+ impurities. ^{1}H NMR, FT-IR, and HPLC data confirmed that the prepared ILs are free of measurable impurities including water.

5.3. Enzymatic hydrolysis of phenylalanine methyl esters

D- (or L-) Phenylalanine methyl ester (20 mg) was dissolved in 2 mL solvent consisting of an IL and 0.1 M $NaHCO_3$ buffer (pH 8.0). Enzyme (1 mg) was added to the reaction mixture at time zero. The reaction was maintained at 25 ± 1 °C. The hydrolysis progress was periodically measured by the HPLC analysis. All experiments were run in duplicates. The average values were reported. The relative errors were normally less than 5%.

5.4. HPLC analysis

The samples were analyzed by a LC-10AT Schimadzu HPLC equipped with a SPD-10A UV–vis dual wavelength detector and a Schimadzu Premier C18 column (150 mm × 4.6 mm, particle size 5 μm). The flow rate is 1.0 mL/min with water/MeOH ratio of 90/10. The detection wavelength is 254 nm.

5.5. Calculations of ee, L yield and *E*

The ee of L-phenylalanine was calculated from the HPLC integration area as (L area − D area)/(L area + D area) × 100%. The yield of L-acid (maximum is 100% for a complete conversion of L-ester) was computed by comparing the area of L-enantiomer with that of standard samples. The enantiomeric ratio (E) was calculated from the following formula as defined by Chen et al.[58]

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

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 concentration, respectively; P and Q are concentrations of products of A and B, respectively.

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