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Enhancing protease enantioselectivity by ionic liquids based on chiral- or ω-amino acids

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Abstract—Ionic liquids (ILs) carrying anions of chiral- or ω-amino acids were prepared. The enzymatic hydrolysis of phenylalanine methyl ester was studied in aqueous solutions of these ILs. These ILs were found capable of stabilizing the protease activity and enantioselectivity at low concentrations. Interestingly, higher ees and yields of L-phenylalanine were generally observed in ILs based on D-amino acids rather than in those derived from L-isomers. The reason could be that D-amino acids are more kosmotropic than L-isomers. Meanwhile, the IL–D_2O solution was able to further enhance the enzymatic resolution, when comparing with that in an IL–H_2O system.

1. Introduction

Ions have a profound impact on the protein stabilization and enzyme activity.[1–5] Ion interactions with protein molecules can be specific (following the ion kosmotropicity, so called Hofmeister series[6,7]), or nonspecific (simply depending on the ionic strength).[1] In particular, the ion-specific interaction has been an attractive focus for many scientists who routinely work with proteins and enzymes. Such an interaction was recently adopted by our group to help explain the effect of ionic liquids (ILs) as new ionic solvents on the enzyme stability[8,9] and enantioselectivity.[10] Our studies suggested that in aqueous solutions, kosmotropic anions and chaotropic cations of ILs stabilize the enzyme activity and enantioselectivity since hydrophilic ILs dissociate into individual cations and anions in water. It is also important to note that the enzymatic catalysis in hydrophobic ILs containing low water contents seems to follow different mechanisms.[5,11–13]

Encouraged by these findings, our group has been searching for new ILs containing kosmotropic anions and chaotropic cations. It has come to our attention that most amino acids (α-, β-, or γ-) are actually so called compensatory (or compatible) solutes of proteins,[14–17] even though not all amino acids and short peptides stabilize the globular proteins (e.g., arginine is known as a protein denaturant).[18] There are several pieces of experimental evidence to strengthen this statement: (1) the effect of two amino acids (glycine and β-alanine) on the esterase activity of bovine carbonic anhydrase was observed close to that of kosmotropic acetate anion;[19] (2) the effect of amino acid salts on the pig heart mitochondrial dehydrogenase (*phm*-MDH) against temperature induced changes is in the order of stabilization of NaGlutamate, NaAspartate > NaGlycinate > lysine·HCl > arginine·HCl;[20] (3) glycine, alanine and proline showed nonperturbing or favorable effects on the enzyme-substrate and enzyme-cofactor complex formation, catalytic velocity and protein structural stability;[14] and (4) *N*γ-acetyldiaminobutyrate (NADA) displayed a greater ability in protecting rabbit muscle lactate dehydrogenase against thermal inactivation than ectoine or potassium diaminobutyrate.[15] Furthermore, Wiggins suggested that D-amino acids are kosmotropes while L-isomers are chaotropes.[21] Meanwhile, as reviewed by Baudequin et al.,[22] various chiral ILs have showed very promising applications in asymmetric synthesis. Therefore, it would be interesting to examine the enzyme enantioselectivity in chiral ILs based on D- or L-amino acids (AA), in the form of [$EMIM^+$][D-AA^-] or [$EMIM^+$][L-AA^-] ($EMIM^+$ is 1-ethyl-3-methylimidazolium). This is the first objective of this study. Fortunately, the preparation of these ILs based on racemic amino acids ([$EMIM^+$][DL-AA^-]) has already been reported by Fukumoto et al.[23]

It is very important to emphasize that some ω-amino acids are also known as compensatory solutes with a

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structure-enforcing effect.[24–26] It was observed that γ-amino acids are more structure-making than their α-isomers based on the more negative differential entropies of dilution in aqueous solutions of γ-amino acids than those of α- and β-ones.[27] The ω-amino acids, such as 4-aminobutanoic acid (4-ABA), 5-aminopentanoic acid (5-APA), 6-aminohexanoic acid (6-AHA), have high viscosity *B*-values and negative values of d*B*/d*T*, suggesting that these amino acids are strong kosmotropes.[28] Therefore, several ionic liquids containing ω-amino acids as anions ([EMIM$^+$][ω-AA$^-$]) were also prepared and used as media for the enzymatic resolution of phenylalanine. The last objective of this study is to examine the protease behavior in IL–heavy water (D_2O) solutions, because D_2O has showed a higher ability in stabilizing proteins against denaturation than ordinary water (H_2O).[29,30] Cioni and Strambini[31] observed a higher rigidity of most protein structures in D_2O than in H_2O, especially at higher temperatures. Based on the inverse relationship between the structural flexibility and stability to thermal denaturation,[32] they suggested that the folded state is more stable in D_2O than in H_2O.

2. Results and discussion

2.1. Effect of IL concentration

The enzymatic hydrolysis of phenylalanine methyl ester was conducted as a model reaction for examining protease activity and enantioselectivity. At first, we investigated the concentration effect of a promising IL ([EMIM][5-APA])[†] based on an ω-amino acid (5-aminopentanoic acid). The [EMIM]$^+$ cation was chosen because it is a chaotrope,[33] and stabilizes the enzyme.[8–10] As seen in Figure 1, a high enantiomeric excess (ee, 92.2%) and yield (97.2%) of L-phenylalanine were obtained in a low IL concentration (0.5 M), suggesting high enzyme activity in this medium. These values are very comparable with those in pure water (94.2% ee and 94.6% yield). However, with the increase of IL concentration, both ee and yield decreased, suggesting a lower enzyme activity and enantioselectivity. This observation is consistent with previous reports from our group[8,10] as well as others[34–37] indicating that high concentrations of hydrophilic ILs destabilize the enzyme.

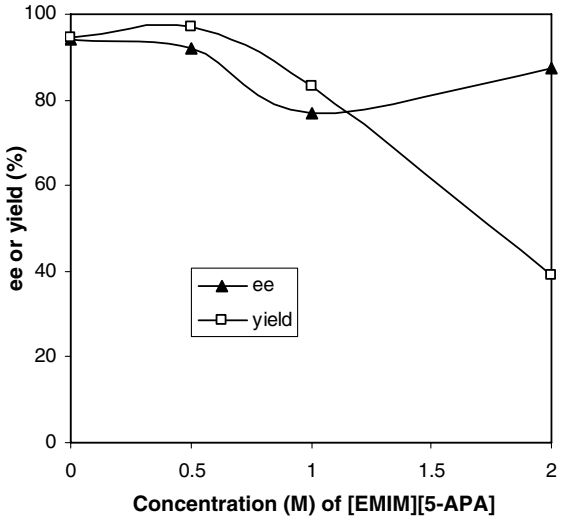

Figure 1. Effect of IL concentration on the protease enantioselectivity (40 min reaction time and 30 °C).

2.2. Effect of amino acid anions on the enzymatic resolution

We carried out the same enzymatic resolution in 0.5 M of other ILs containing anions of various chiral- or ω-amino acids (Fig. 2). The chiral amino acids proline, lysine, alanine, and glutamic acid were chosen because the former two (proline and lysine) are known as important compensatory solutes in nature with structure-making ability,[14,26,38–40] alanine showed favorable effects on the enzyme–substrate and enzyme–co-factor complex formation,[14] and sodium glutamate salt stabilized pig heart mitochondrial dehydrogenase (*phm*-MDH) against temperature induced changes.[20] Glycine was considered for the purpose of comparing the kosmotropicity of different amino acids.

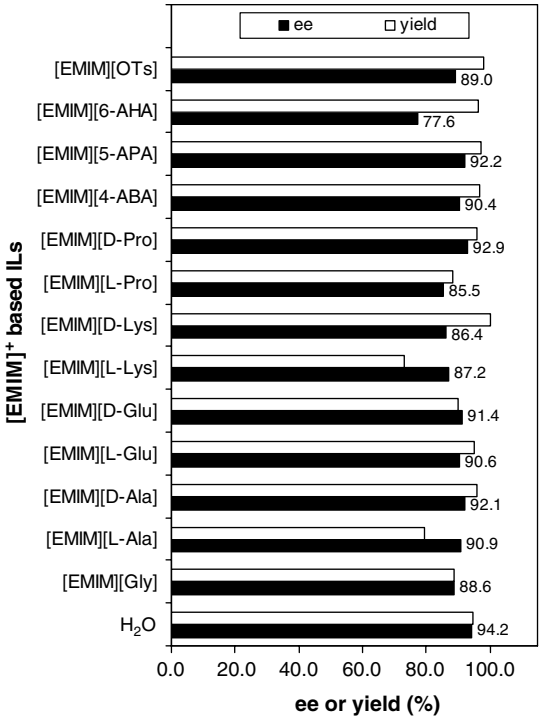

Figure 2. Comparison of the enzymatic resolution of phenylalanine methyl ester in 0.5 M ILs and water (40 min reaction time and 30 °C).

In general, a moderate to high enzyme activity and enantioselectivity were observed in most amino acid based ILs, suggesting that they are compatible solutes. Meanwhile, slightly higher ees were seen in D-amino acid based ILs than in those based on L-isomers (except very comparable ees in D- and L-lysine based ILs[‡]); higher yields were also

[†] The abbreviations of amino acids and ionic liquids are in Section 4.

[‡] The optimum resolution was actually achieved at 20 min of reaction time in both 0.5 M [EMIM][L-Lys] (94.1% ee and 74.6% yield) and 0.5 M [EMIM][D-Lys] (90.9% ee and 98.9% yield).

observed in the same trend with the exception of D/L-glutamate. A possible explanation is the following: since kosmotropic anions strongly stabilize the enzyme,[5] our observation is consistent with Wiggins' hypothesis that D-amino acids are usually more kosmotropic than their L-isomers.[21] On the other hand, relatively low ees were found in solutions of [EMIM][OTs] and [EMIM][Gly]. The reason for the former IL is that OTs^- was suspected to be a chaotrope based on our previous enzyme-stability studies.[8,9] The reason for the later IL could be explained by the kosmotropicity of amino acids. As discussed in detail by our recent review on this subject,[28] the viscosity *B*-coefficients of amino acids at 25 °C are 0.143 (glycine), 0.252 (alanine), 0.268 (proline), 0.29 (glutamic acid), 0.312 (4-ABA), 0.383 (5-APA), and 0.489 (6-AHA). Since a higher *B*-value suggests a higher kosmotropicity, glycine is least kosmotropic than other amino acids.

On the other hand, the ions of amino acids are more kosmotropic than zwitterionic amino acids (AA) in a decreasing order of anion > cation > AA;[28] for example, *B*-coefficients of glycine ions at 25 °C are in the order of 0.242 $(Gly^-) > 0.160$ $(Gly^+) > 0.143$ (Gly).[28] Therefore, amino acid anions are stronger enzyme stabilizers than zwitterionic amino acids. In the case of ω-amino acids, higher ees were observed in $[5\text{-APA}]^-$ than in $[4\text{-ABA}]^-$ due to higher kosmotropicity of the former anion. However, a lower ee was found in $[6\text{-AHA}]^-$ which should be the most kosmotropic anion in the series. This contradictory result might be due to the fact that an optimal stabilization of enzyme can usually be achieved through a balance between kosmotropic anions and chaotropic cations.[2,41,42] The anion $[6\text{-AHA}]^-$ could be too kosmotropic, breaking such a balance and causing a lower enzyme activity.

Figure 3 further demonstrates that with the increasing concentration of [EMIM][L-Glu] and [EMIM][D-Glu], the difference of ees in 1.0 M ILs (25.0% Δee) was more pronounced than that in 0.5 M ILs (0.8% Δee), although lower ee and yield were observed at a higher concentration (being similar to Fig. 1). These data confirmed that $[\text{D-Glu}]^-$ is more kosmotropic than $[\text{L-Glu}]^-$.

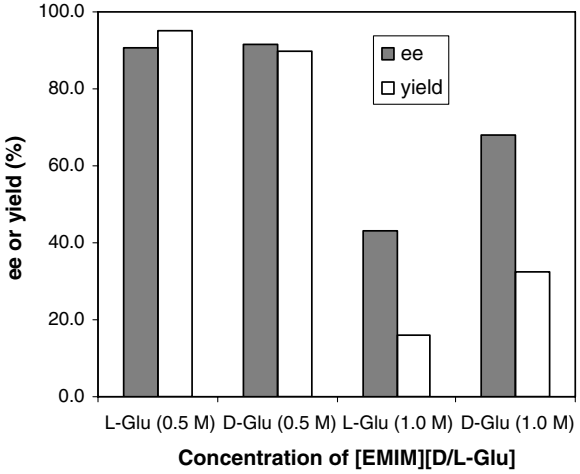

Figure 3. Effect of concentration of chiral ILs on the enzymatic resolution (40 min reaction time and 30 °C).

2.3. Effect of heavy water (D_2O) on the enzyme enantioselectivity

We next carried out the enzymatic reaction in D_2O solutions instead of ordinary H_2O solutions. As illustrated in Figure 4, ees obtained in pure H_2O and pure D_2O are not distinguishable, while a lower yield was found in D_2O. This observation suggests that the enzyme enantioselectivity is about the same in H_2O and D_2O, while a lower enzyme activity is observed in D_2O. The lower reaction rate in D_2O might be due to higher enzyme rigidity in D_2O than in H_2O,[31] and/or due to the enzyme being partially deuterated from the H/D exchange causing a slower reaction rate. However, when an IL (0.5 M [EMIM][5-APA]) was added, a higher ee (97.5%) was observed in D_2O solution than in H_2O solution (91.0%) although the yield is still lower in D_2O solution.

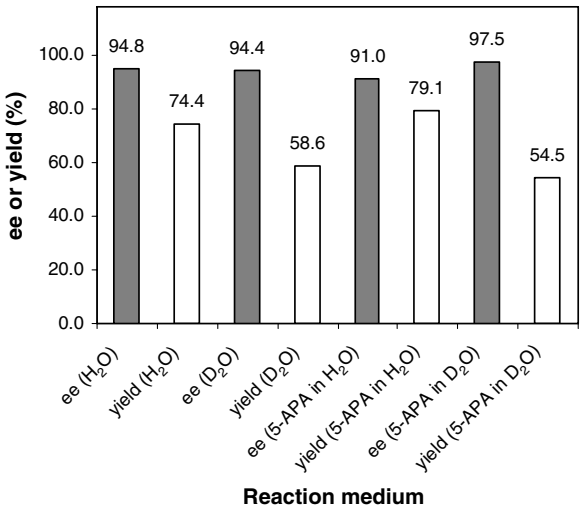

Figure 4. Effect of D_2O on the enzymatic resolution (20 min reaction time, [EMIM][5-APA] in 0.5 M, and 30 °C).

We suspected that the cation and/or anion hydration might be responsible for such a difference. As briefly discussed by Jenkins and Marcus,[43] negative *B*-coefficients of ions are more negative in D_2O than in H_2O, while there is little difference in the positive *B*-values in D_2O and H_2O. The *B*-coefficients of both $[EMIM]^+$ and $[5\text{-APA}]^-$ in H_2O are expected to be quite positive,[28,33] therefore, the hydration of these two ions in D_2O and H_2O should not be considerably different based upon their *B*-coefficients. However, the comparison made by Jenkins and Marcus was limited to several ions (including tetraalkylammonium cations). The hydration of large hydrophobic cations (such as $[EMIM]^+$) and anions (such as $[5\text{-APA}]^-$) in D_2O is not well understood.

Meanwhile, the hydrogen at the C2 position of the imidazolium cation is rather acidic ($pK_a = 21$–23) and is subject to deprotonation under basic conditions.[44–46] Since

our enzymatic reactions were conducted in a $NaHCO_3$ buffer, the H/D exchange of the imidazolium ring is likely to occur through carbene intermediates, especially on the C2 position.[46] The H/D exchange might also occur for the anion (e.g., H/D exchange of hydrogen atoms on the amine group).[47,48] These partially deuterated cations or anions have different properties (such as hydration behavior) in the solution, which could be responsible for the difference of enantioselectivity in D_2O and H_2O. However, the exact mechanism is not clear.

3. Conclusion

Generally, moderate to high enzyme enantioselectivities were observed in ILs based on chiral- or ω-amino acids. ILs based on D-amino acids are more favorable than those based on L-amino acids in terms of improving ee and yield. It seems that an enhanced enzymatic resolution could be achieved in the IL–D_2O solution rather than in the IL–H_2O solution.

4. Experimental

4.1. Materials

1-Ethyl-3-methylimidazolium bromide ([EMIM]Br), *Bacillus licheniformis* protease (subtilisin Carlsberg), DL-phenylalanine methyl ester hydrochloride, Amberlite® IRA-400 Cl resin, glycine (Gly), D- and L-alanine (Ala), D- and L-lysine (Lys), D- and L-glutamic acid (Glu), D- and L-proline (Pro), 4-aminobutanoic acid (4-ABA), 5-aminopentanoic acid (5-APA), 6-aminohexanoic acid (6-AHA), and other reagents were purchased from Sigma–Aldrich.

4.2. IL preparations

[EMIM][OH] was prepared according to a literature method[23] by using the anion exchange resin (Amberlite® IRA-400 Cl). A brief description of the procedures is the following: about 100 mL of the resin packed in a glass column was thoroughly washed with distilled water until no yellow color was observed in the eluting water and no precipitate could be detected by 0.1 M $AgNO_3$ solution. Next, the Cl^- ions on the resin were exchanged by OH^- ions through slow washing of the column with about 100 mL 3.0 M NaOH solution until no white precipitate (AgCl) could be detected by 0.1 M $AgNO_3$ solution; 20 g [EMIM]Br dissolved in 100 mL water was made to slowly drip through the column, and the eluting solution was monitored by 0.1 M $AgNO_3$ to ensure the absence of Br^-. If necessary, the resulting solution was run through the column (OH^- form) for the second time. Water was then removed from the resulting [EMIM][OH] solution through a rotary evaporator under vacuum at 60 °C.

The method for preparing the chiral- or ω-amino acid based ILs is based on a literature method (for preparing ILs based on racemic amino acids):[23] an aqueous solution of D-, L-, or ω-amino acid (1.2 equiv) was added dropwise into the [EMIM][OH] (1 equiv) solution. The mixture was stirred for 12 h (with gentle heat or microwave heating for some amino acids), followed by the removal of water under vacuum through a rotary evaporator at 60 °C. A mixed solution of acetonitrile and methanol (9:1, v/v) was added into the crude product. The mixture was then stirred vigorously to dissolve the IL. The excess amino acid (precipitate) was filtered off, and the solvents were removed from the filtrate through a rotary evaporator. The IL product was dried at 90 °C for 24 h. All product yields are greater than 80%. The IL purity was ensured by measurements of IR, ^1H NMR and HPLC. The thermal properties and ionic conductivity of most of these ILs have been reported in a recent paper.[23]

4.3. Enzymatic resolution

DL-Phenylalanine methyl ester (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. Samples were withdrawn from the reaction mixture periodically and analyzed by the HPLC. All experiments were run in duplicates. The averaged values are reported.

4.4. HPLC analysis

The samples were analyzed by a LC-10AT Schimadzu HPLC equipped with a SPD-10A UV–vis dual wavelength detector, and a Crownpak CR(+) chiral column (150 mm × 4.0 mm, particle size 5 μm). The mobile phase was 1.0 mL/min HPLC-grade water containing 0.1 M $HClO_4$. The detection wavelength is 210 nm (254 nm for reactions in pure water). The eluting sequence of four isomers is in the increasing retention time of D-acid < L-acid < D-ester < L-ester.

4.5. Calculations of ee and L-yield

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 calculated from comparing the current area of L-acid with that of complete conversion of the L-ester. The L-acid area for the complete conversion of the L-ester was measured by HPLC individually for each sample, typically at 2–3 h of reaction time.

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