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PAPER

Specific ion effects of ionic liquids on enzyme activity and stability

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Catalytic performance of two model enzymes, *Penicillium expansum* lipase (PEL) and mushroom tyrosinase, was examined in aqueous solution with addition of 14 different ionic liquids (ILs) and has been found to correlate well with the IL's kosmotropic/chaotropic properties, which are assessed by the viscosity B coefficients (B_+ for cations and B_- for anions). The activity and stability of PEL were similarly correlated with B_+ values but not with B_- of the ILs tested. PEL can be activated and stabilized by addition of ILs (*e.g.*, 5.2-fold activation and 1.4-fold stabilization in the presence of 0.63 M [choline][Ac] and 0.27 M [$NHMe_3$][$MeSO_3$], respectively). Choline ILs activated PEL but imidazolium ones deactivated it. The results indicate that the IL cations play a crucial role in affecting the enzyme performance and that ammonium ILs composed of chaotropic cations (favorably with H-bonding capability) and kosmotropic anions are favored for enzyme catalysis. The Hofmeister effect of ILs on PEL was confirmed by the kinetic and thermostability studies and structural analysis on tyrosinase. Our investigations on both enzymes have thus demonstrated that ILs can affect the enzyme functioning through the Hofmeister effect, and the mechanisms have been discussed in terms of the influence of the IL cations and anions on the surface pH, active site conformation, and catalytic mechanism of each specific enzyme, following the Hofmeister series.

Introduction

Ionic liquids have been regarded as a promising new type of nonaqueous solvents for biocatalysis because of their unique solvent properties and their ability to present excellent enzymatic performance.[1–4] One of the major attractions of utilizing ILs in place of organic solvents is their 'designer solvent' property: combinations of different cations and anions can make up different ILs with different physical and chemical properties. However, the prerequisite for taking this advantage depends on our understanding of the effects of ILs on the enzymatic performance. It has been recently proposed that in addition to the factors including polarity, hydrophobicity, nucleophilicity, and H-bond basicity, the Hofmeister ion effects may also offer a reasonable explanation for this.[5–7]

It has long been realized that enzyme performance in aqueous solution can be altered by different inorganic salts and ions following a common order known as the Hofmeister series.[8] It is believed to be correlated with the kosmotropic/chaotropic properties of the ions, which can be characterized by the Jones–Dole viscosity B coefficients.[9] The presence of a salt composed of a kosmotropic anion and a chaotropic cation is usually found to favor enzyme functioning.[5,6,10] Based on the comparison of a limited number of ILs, a few enzymes have been found to have their catalytic behavior in IL-containing aqueous systems responding to the Hofmeister effect,[5,6] while disobeying examples have also appeared in literature.[11,12]

Meanwhile, there has been a growing demand for developing 'greener' ILs.[13,14] Ionic liquids based on alkylammonium as the cation represent promising biocompatible ones which can be prepared from natural sources.[7] Indeed, *Candida antarctica* lipase B has been found to dissolve in [Et_3MeN][$MeSO_4$] while maintaining its activity and structure,[15] so did cytochrome c in [choline][H_2PO_4][16] and subtilisin in diethanolammonium chloride ([DEA][Cl]).[17] Among the ammonium ILs, the protic ones, with a proton attached to the cation nitrogen, possess special characteristics including the capability of H-bonding as well as the ionic and hydrophobic characters. Although protic ILs are currently under intense study in a variety of applications,[18] their utility in biocatalysis has not been much assessed yet.

In this study, both activity and stability of two model enzymes, *Penicillium expansum* lipase (PEL) and mushroom tyrosinase, were investigated in aqueous solution with addition of 14 different ILs. The aims of this study were: (1) to systematically explore the specific ion effect of ILs on enzyme performance and to verify its generality; (2) to assess the feasibility of using

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ammonium ILs, especially the protic ones, in biocatalysis; and (3) to propose a few useful guidelines for designing green and biocompatible ILs based on the above results. The two selected model enzymes belong to different classes. PEL is a hydrolase effective for catalyzing hydrolytic and transesterification reactions including biodiesel production, being much more active in the IL [BMIm][PF_6] than in commonly used organic solvents;[19] while mushroom tyrosinase is an oxidoreductase catalyzing the hydroxylation of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones, and its action in both aqueous solution, organic solvents, and ionic liquids has been extensively investigated.[12,20,21]

Experimental

Materials

Penicillium expansum lipase was kindly donated by Shenzhen Leveking Bioengineering Co. Ltd., China. All 14 ionic liquids (99%, HPLC) were purchased from ShangHai Cheng Jie Chemical Co. Ltd. *p*-Nitrophenyl palmitate (pNPP), *p*-nitrophenol (pNP), and 3,4-dihydroxyl-phenylalanine (L-DOPA) were purchased from Sigma–Aldrich China Inc. All other reagents used were of analytical grade from local manufacturers in China.

Enzyme preparation

PEL solution was obtained by solubilizing the enzyme powders (3.75 g) in 15 ml of the sodium phosphate buffer (50 mM, pH 6.5) with magnetic stirring for 30 min at 4 °C followed by centrifugation.[19] Mushroom tyrosinase was purified by extraction of fresh mushrooms into the phosphate buffer (50 mM, pH 6.0) and subsequent concentration with $(NH_4)_2SO_4$, following by gel filtration on a HiPrep 16/60 Sephacryl S-200 HR prepacked column (eluted with 50 mM, pH 6.0 phosphate buffer), ion-exchange chromatography on a DEAE Sephadex column (eluted with a linear gradient of NaCl (0–1.0 M) in the phosphate buffer), and gel filtration chromatography again. The purity of the enzyme was confirmed by SDS-PAGE.

Activity assays for PEL

The activity of PEL to catalyze the hydrolysis of pNPP to pNP was assayed following the procedures described in[19] with minor modification. The enzyme (0.2 ml) was added to the reaction mixture containing 2.07 ml sodium phosphate buffer (50 mM, pH 6.5, with addition of 2% (w/v) Triton X-100) and 0.23 ml pNPP solution (20 mM in isopropanol) to start the reaction (at 37 °C), the formation of pNP was monitored spectrophotometrically, and the molar extinction coefficient for pNP under each condition was determined as described in ref. 19. To study the IL effects, a specified amount of each IL was added to the phosphate buffer (50 mM, pH 6.5, containing 2% (w/v) Triton X-100) to reach a final concentration of 0–15% (w/v), and the pH of each such solution was measured. These buffer solutions were readjusted to pH 6.5 with HCl or NaOH before being used for the activity tests. All the experiments throughout this study were at least duplicated, being subject to less than 5% error for each data point.

Stability tests for PEL

The PEL solution in IL-containing phosphate buffer (50 mM, pH 6.5, final IL content 4.17%, w/v) was incubated at 35 °C, and 0.2 ml sample was taken periodically for activity assay at 37 °C as noted above. The time-dependent loss in activity was used to calculate the half-life for each enzyme solution.

Activity assays for tyrosinase

The activity of tyrosinase was measured spectrophotometrically by following the oxidation of L-DOPA to dopachrome.[20] The purified enzyme (0.05 ml) was added to a mixture containing 1.2 ml phosphate buffer (50 mM, pH 6.0) and 0.8 ml L-DOPA (10 mM, prepared in the same buffer) to start the reaction at 30 °C. For the reactions to study the IL effects, the phosphate buffer was prepared with addition of a specified amount of each IL. After pH measurement, the buffer solution was readjusted to pH 6.0 before being used for the activity tests. For K_m, V_{max} measurements, substrate solutions (air-saturated) with various concentrations of L-DOPA (0–9.8 mM) were prepared in the IL-containing phosphate buffer, and the apparent K_m and V_{max} values were obtained from the Lineweaver–Burk plots.

Stability tests for tyrosinase

Purified tyrosinase solution was mixed with a phosphate buffer (50 mM, pH 6.0) containing 10% (w/v) of each IL in a ratio of 1 : 1 (final IL content 5.0%, w/v), and the mixture was incubated at a specified temperature (30 °C, 40 °C, 50 °C). Periodically, 0.1 ml sample was taken for activity assay at 30 °C as noted above. The half-life for each enzyme solution was determined, and the first-order deactivation rate constants were obtained by fitting the thermoinactivation data to the classical first-order deactivation model.

Fluorescence spectroscopy

Fluorescence measurements for tyrosinase were carried out using a Hitachi F4500 spectrofluorimeter. Enzyme samples were prepared by dissolving the purified tyrosinase solution in the phosphate buffer (50 mM, pH 6.0) in the presence of different ILs (0–0.5 M IL, final pH 6.0, final protein content 0.09 mg ml^{-1}). The denatured enzyme sample was prepared by incubating the enzyme solution in a boiling water bath for 10 min and then cooling down to room temperature (no activity was detectable after such a treatment). The fluorescence was recorded for emission from 290–450 nm with excitation at 280 nm. A blank medium without enzyme was subtracted to discount the influence of the fluorescence of the ILs and buffer components on the enzyme fluorescence spectrum.

Results and discussion

Kosmotropicity/chaotropicity of the IL cations and anions used in this study

All the 14 ILs and the available viscosity B coefficients of their cations and anions are listed in Table 1. The kosmotropicity of the IL anions used in this study varied in the order of $H_2PO_4^- > Ac^- > MeSO_3^- \sim MeSO_4^- > NO_3^-$; the first three are kosmotropes

Table 1 Fourteen ionic liquids used in this study

IL No.	Ionic liquid		B_+[a]	B_-[a]
IL-1	[MMIm][$MeSO_4$]	1,3-dimethylimidazolium methylsulfate		
IL-2	[EMIm][$MeSO_4$]	1-ethyl-3-methylimidazolium methylsulfate	+0.49[b]	
IL-3	[BMIm][$MeSO_4$]	1-butyl-3-methylimidazolium methylsulfate	+0.61[b]	
IL-4	[choline][H_2PO_4]	choline dihydrophosphate		+0.34
IL-5	[choline][Ac]	choline acetate		+0.246
IL-6	[choline][$MeSO_3$]	cholate methylsulfonate		+0.127
IL-7	[choline][NO_3]	choline nitrate		–0.043
IL-8	[NMe_4][Ac]	tetramethylammonium acetate	+0.123	+0.246
IL-9	[NBu_4][Ac]	tetrabutyllammonium acetate	+1.275	+0.246
IL-10	[NBu_4][$MeSO_3$]	tetrabutyllammonium methylsulfonate	+1.275	+0.127
IL-11	[$NHMe_3$][$MeSO_3$]	trimethylammonium methylsulfonate	+0.117	+0.127
IL-12	[$NHMe_3$][H_2PO_4]	trimethylammonium dihydrophosphate	+0.117	+0.34
IL-13	[$NHEt_3$][H_2PO_4]	triethylammonium dihydrophosphate	+0.385	+0.34
IL-14	[$NHBu_3$][H_2PO_4]	tributylammonium dihydrophosphate		+0.34

[a] B_+ and B_- are the viscosity B coefficients of the cation and anion, respectively, obtained from ref. 23 except those labeled with 'b'. [b] The B_+ values for the two imidazolium cations were obtained as estimated by Zhao[22] based on the linear relationship between the viscosity B coefficients and ion hydration radii.

while the last one is a chaotrope. With respect to the IL cations, most of their viscosity B coefficients are not available. But based on the earlier studies on the hydration behavior of quaternary ammonium salts, more hydrophobic ammonium cations (with longer alkyl chains) experience hydrophobic hydration and are considered as more kosmotropic, and the same is true to the imidazolium cations.[22] The positive B coefficients for the limited number of ammonium cations listed in[23] can be taken as a good support for this. Two typical examples[24,25] have clearly illustrated this cation effect, confirming that consistent with the effect of inorganic cations, proteins are also stabilized by chaotropic cations and destabilized by kosmotropic ones from ILs.

It is worth mentioning that the pH of the aqueous buffer can be significantly affected by addition of ILs. For instance, pH lowered by more than 1 unit was observed in the presence of [choline][H_2PO_4] and particularly the last 4 protic ILs listed in Table 1 (all 5% w/v in the 50 mM, pH 6.5 phosphate buffer). This may to some extent be connected to the Hofmeister series but can be better understood in view of the acidity/basicity of the used ILs. Therefore, in order to avoid the IL-induced interference in the buffer pH, for the later reactions the IL-containing phosphate buffers were all readjusted to the required pH prior to use.

Effect of ILs on the activity and stability of PEL

Fig. 1A shows the activities of PEL in aqueous solution with addition of all the 14 ILs (4.14% w/v), which varied in a very broad range: The enzyme can be activated up to 316.4% (by [$NHMe_3$][$MeSO_3$]) and deactivated down to 36.4% (by [BMIm][$MeSO_4$]) of the rate obtained by the enzyme in the IL-free solution. The ILs that can stimulate the enzyme activity include [$NHMe_3$][$MeSO_3$], [NMe_4][Ac], [choline][Ac], [choline][$MeSO_3$], [choline][NO_3], and [$NHMe_3$][H_2PO_4]. A closer look at the graph can tell us that these activity data follow the Hofmeister series very well. In the presence of the ILs holding the same anions, the enzyme activity decreased in the order of [MMIm][$MeSO_4$] > [EMIm][$MeSO_4$] > [BMIm][$MeSO_4$], [NMe_4][Ac] > [NBu_4][Ac], [$NHMe_3$][$MeSO_3$] > [NBu_4][$MeSO_3$], and [$NHMe_3$][H_2PO_4] > [$NHEt_3$][H_2PO_4] > [$NHBu_3$][H_2PO_4]; while for the ILs holding the same cations, [choline][Ac] > [choline][$MeSO_3$] > [choline][NO_3], [NBu_4][Ac] > [NBu_4][$MeSO_3$]. When incubated in the phosphate buffer containing different ILs (4.17% w/v), the enzyme also exhibited variant half-lives as shown in Fig. 1B. The stability order resembles the activity one, except for the choline ILs showing the op-

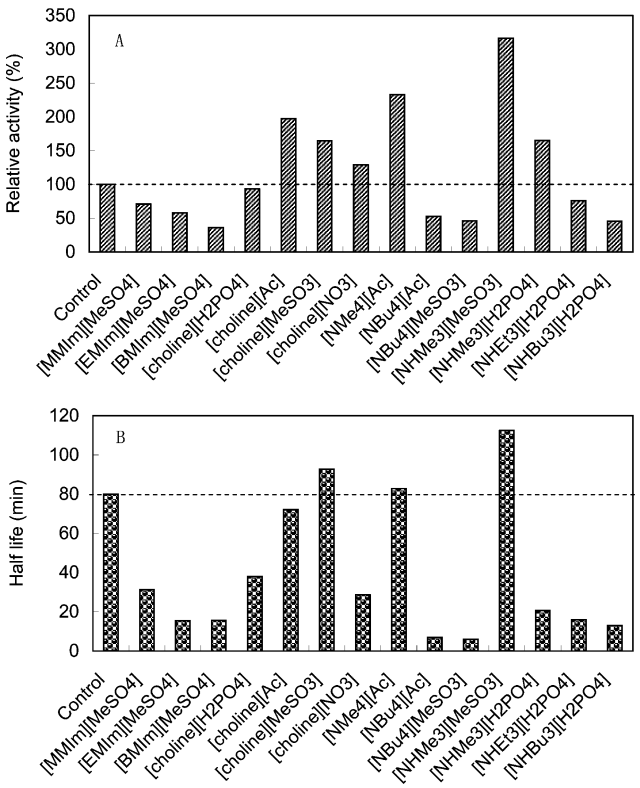


Fig. 1 Activity (A) and stability (B) of PEL in phosphate buffer (50 mM, pH 6.5) containing different ILs (4.14% and 4.17%, w/v, respectively). The relative activity (%) refers to the percentage of the initial reaction rate obtained by the enzyme in the IL-containing aqueous solution relative to the one obtained in the IL-free system (control, 7.21 μM min^{-1}).

posite trend. The enzyme can be stabilized by a few ILs including [$NHMe_3$][$MeSO_3$], [choline][$MeSO_3$], and [NMe_4]-[Ac], which are also listed among the ILs that can activate the enzyme.

The correlation between the enzyme performance and the Hofmeister series can be better illustrated by plotting the activity and stability data against the viscosity B coefficients of the anions (B_-) and cations (B_+) of the ILs (Fig. 2). Interestingly, the two plotting sets resemble each other again: Both activity and stability show no obvious relationship with B_- (Panels A and C) but are well correlated with B_+ (Panels B and D). This seems to give an indication of a more severe impact from the IL cations instead of from the anions, which is in contrast to the situation generally observed in the study of the effect of inorganic salts on enzyme performance.[6]

In order to confirm the influences of both the IL cations and anions, two sets of ILs (3 imidazolium ILs holding the same anion, $MeSO_4^-$, and 3 ammonium ILs holding the same cation, choline) were further investigated (Fig. 3). The enzyme activity was depressed gradually with addition of all the 3 imidazolium ILs. But comparatively, the more kosmotropic cation elicited a lower reaction rate, in the order of $BMIm^+ < EMIm^+ < MMIm^+$ (Fig. 3A). On the other hand, the enzyme was activated by all the 3 choline ILs, and under each IL level the reaction rate was higher in the presence of a more kosmotropic anion, *i.e.*, in the order of $Ac^- > MeSO_3^- > NO_3^-$ (Fig. 3B). The tests on these two sets of ILs have thus confirmed our notion that a high chaotropicity in the IL cation and a high kosmotropicity in the IL anion facilitate enzyme functioning. Moreover, the activation by all the choline ILs and depression by all the imidazolium ILs have exemplified the IL cation's dominating effect, and it is reasonable to assume that imidazolium cations are much more kosmotropic than the choline cation. Unfortunately, the viscosity B coefficients for these two types of cations are not available yet.

Therefore, our results have clearly substantiated that the PEL activity and stability in aqueous solution can be affected by addition of various ILs, following the Hofmeister series, which is in line with the impact of inorganic salts and ions on enzymes in aqueous solution. The anion, $H_2PO_4^-$, seems to be anomalous in this case, because both the enzyme activity and stability were unexpectedly low in the presence of all the ammonium ILs holding it as the anion, in spite of its highly positive viscosity B coefficient (+0.34). In fact, the B coefficient of an ion does not always agree well with its kosmotropicity,[22] and $H_2PO_4^-$ has once been considered as a borderline ion.[26] Besides, this anion may be further ionized in the buffer solution to produce a fraction a HPO_4^{2-}, which may also contribute to some extent to the observed effect. The fairly good activity and stability obtained in the presence of all the choline ILs can be attributed to the hydroxylated choline cation. Recently we have pointed out that ILs with hydroxylated cations (such as choline) facilitate the enzyme action because they hold the H_2O-mimicking property and H-bonding functionality, so as to help the enzyme resume its flexible and active conformation,[6,7] and this idea has been supported by previous experiments.[16,27,28] Our observation that

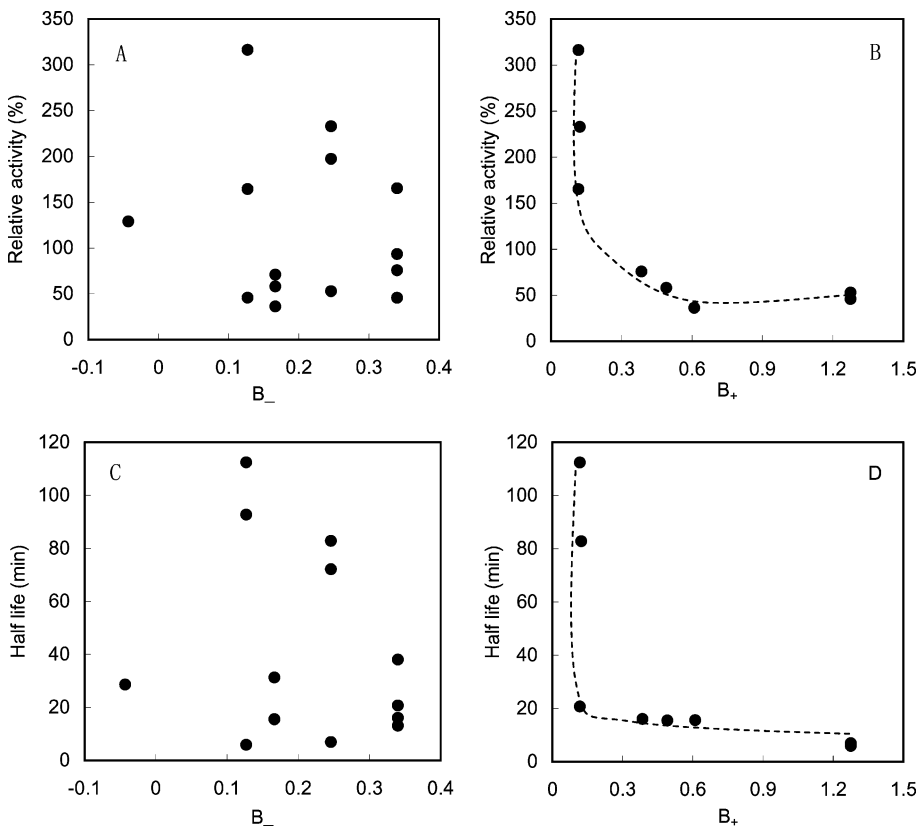


Fig. 2 Enzyme activity (top, A and B) and stability (bottom, C and D) of PEL (data from Fig. 1) as a function of the viscosity B coefficients of the anions (B_-) and cations (B_+) of the ILs used in this study. The dashed lines are drawn to direct the variation trend.

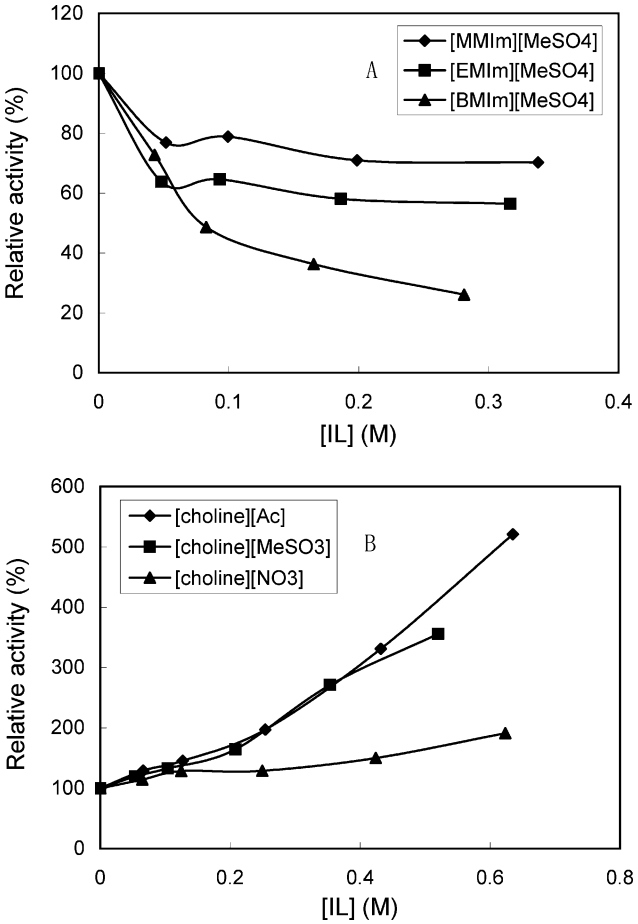


Fig. 3 Initial rate of the PEL-catalyzed hydrolysis reaction as a function of the IL concentration: (A) 3 imidazolium ILs with $MeSO_4^-$ as the anion; (B) 3 ammonium ILs with choline as the cation. The relative activity (%) refers to the percentage of the initial reaction rate obtained by the enzyme in the IL-containing aqueous solution relative to the one obtained in the IL-free system (11.0 μM min^{-1}).

PEL was activated by the 3 choline ILs but deactivated by the 3 imidazolium ones (Fig. 3) can also be taken as a support for this. The superior activating and stabilizing effect presented by the protic IL $[NHMe_3][MeSO_3]$ can be attributed to both the chaotropicity of the IL cation (in combination with a kosmotropic anion) and to its H-bond donating ability.

In our recent study of the Hofmeister effects on alkaline phosphatase, we have concluded that while the effect of inorganic salts and ions on the enzyme stability is general, their effect on activity is enzyme-specific.[29] A brief review of what is known about the structure and mechanism of lipases can help us understand better the specific effect of ILs on the PEL's activity and stability.

Lipases are carboxyl-esterases acting on hydrolysis or transesterification of long-chain acylglycerides, following a mechanism similar to the well-established acyl-enzyme mechanism for serine proteases.[30] At the active site of the free enzyme, the three critical amino acid residues (Ser, His, and an acid residue Glu or Asp) are linked in the form of Glu/Asp-His-Ser by H-bonding, and both Glu/Asp and His are in their deprotonated states. The first step in lipase catalysis involves the nucleophilic attack of the hydroxyl group of Ser to the carbonyl carbon on the ester substrate to form an enzyme-substrate tetrahedral intermediate. The His residue functions as a general base to remove the serine proton, thus making the Ser more nucleophilic, and the positive charge that forms on the His residue is stabilized by the negatively charged Glu/Asp. The detrimental effect of $H_2PO_4^-$ to PEL observed in this study can thus be explained by its strong H-bonding interactions with Glu/Asp and His, hence weakening the ability of these critical amino acid residues in assisting the catalysis at the active site. Based on the structural studies on *Candida rugosa* lipase, the active site of the enzyme is shielded from the solvent by a part of the polypeptide chain, the flap, which is hydrophilic on the side exposed to the aqueous milieu outside and hydrophobic on the side facing the protein, interacting with the hydrophobic residues surrounding the active site. The lipase molecule experiences transition between two conformations, active or inactive, depending on whether this flap is open or not, respectively.[31,32]

The lipase used in this study was produced from a special strain, PF898, of *Penicillium expansum*. With 258 amino acid residues and a molecular mass of 28 kDa, this lipase has a sequence homology of 39–49% as compared to other lipases from fungi and a high percentage of hydrophobic residues (51.4%) in the N-terminal region.[33,34] It is reasonable to suspect that this enzyme holds a similar active site structure and catalytic mechanism as noted above, although the details have not been reported yet.

In this study, both activity and stability of PEL were highly sensitive to the choice of IL cations (Panels B and D of Fig. 2). A few enzymes have been found to be less active[35–37] and less stable[25,27] in the presence of ILs holding cations with larger and longer hydrophobic alkyl chains, indicative of higher kosmotropicity and hydrophobicity. PEL provides one more example to support this. The depressing effect caused by these IL cations can be attributed to:[6] (1) their strong ion-pairing interactions with the kosmotropic moieties of the enzyme molecule, such as the carboxylic groups, on the enzyme surface, according to the "law of matching water affinity",[38] which states that a cation and an anion with equal water affinity, *i.e.*, both kosmotropic or both chaotropic, are expected to have a strong propensity to ion-pair with each other in aqueous solution; and (2) their strong hydrophobic interactions with the inner hydrophobic moieties of the enzyme molecule, leading to the disruption of the enzyme's native conformation.

There are some more reasons specific for PEL to account for its sensitive response to the IL cations. An IL cation with a long alkyl chain is hydrophobic and kosmotropic as stated before. It tends to open the flap that covers the active site and to enter the active site for its favorable interactions with the hydrophobic moieties inside, thus resulting in a serious distortion in the enzyme's active site conformation. The molecular dynamics simulations conducted by Klähn *et al.*[39] have also suggested the diffusion of the long alkyl chain of IL cations (such as $BMIm^+$) into the active site of *Candida antarctica* lipase B (CALB), which may obstruct the substrate molecules to get access to the active site. Once inside, the IL cation also has a high tendency of interacting with the critical Glu (or Asp) of the catalytic triad, in view of the "law of matching water affinity".[38] This surely disables the stabilizing role played by this amino acid residue in the catalytic triad, as described earlier. As a result, a reduction

in the enzyme activity is expected. Moreover, the opening of the flap triggers the exposure of the active site to the solvent, thus making the enzyme more vulnerable to the surrounding environments and in turn a lower stability. In contrast, a more chaotropic IL cation may help to protect the enzyme in its closed conformation, thus making it more stable. Even if accessing the active site, this cation has a low affinity for the critical Glu (or Asp) but can help to stabilize the negatively charged enzyme-substrate tetrahedral intermediate. This will lower the activation energy and hence lead to a higher enzyme activity. The above discussion can thus explain well why PEL was both active and stable in the presence of a chaotropic IL cation and both inactive and unstable with a kosmotropic one (Panel B and D of Fig. 2).

On the other hand, IL anions can also affect the enzyme performance by following the Hofmeister series. Because of its strong interaction with water, a kosmotropic anion is used to competing effectively for the water molecules originally associated with the enzyme molecule,[40] and hence may render the flap that covers the enzyme's active site to be more open, taking the hydrophilic character of its upper side into account. This may cause the formation of the enzyme's active conformation, leading to, on the one hand more substrate molecules to be available to the enzyme's active site and hence a higher enzyme activity, and on the other hand more exposure of the active site to the solvent and thus a lower enzyme stability. This can then account for the opposite responses of the activity and stability upon the addition of the choline ILs (Fig. 1).

However, a chaotropic anion has not only a low affinity for water but also a high polarizability, preferring to bind to the protein/water interface and to interact with the chaotropic cationic moieties (such as amino groups) on the enzyme surface.[6] This will cause more H^+ ions to be accumulated on the surface of the enzyme, leading to a reduction in the surface pH.[29,41] Boström *et al.*[42] have developed a modified ion-specific double-layer model to demonstrate that the surface pH of a protein is dependent on the salt concentration and on the ionic species following the Hofmeister series. A low surface pH would force the Glu (or Asp) and His at the active site to be protonated, thereby weakening their ability to assist the Ser residue in the nucleophilic attack to the carbonyl group of the substrate. As a result, the enzyme activity is reduced. The adsorption of the chaotropic anion on the enzyme surface and its strong interaction with the enzyme can also render the enzyme structure to be distorted, thus leading to a lower stability.

Effect of ILs on the activity and stability of mushroom tyrosinase

In order to verify whether the above results are applicable to other enzymes, we extended our study to a different type of enzyme, mushroom tyrosinase. The screening of its activity in the presence of all the 14 ILs (Fig. 4) shows some similar results (although none of the ILs tested can stimulate the tyrosinase activity): in the presence of the ILs holding the same anion, the initial rate of the tyrosinase-catalyzed oxidation reaction showed a decreasing order of [MMIm][$MeSO_4$] > [EMIm][$MeSO_4$] > [BMIm][$MeSO_4$], [NMe_4][Ac] > [NBu_4][Ac], and [$NHMe_3$][H_2PO_4] > [$NHEt_3$][H_2PO_4]. This suggests that the variation of tyrosinase activity also followed the kosmotropicity/chaotropicity of the IL cations. But for the ILs holding

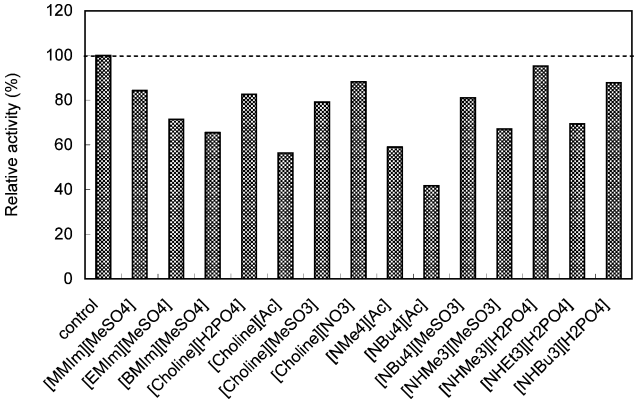


Fig. 4 Initial rates of tyrosinase-catalyzed oxidation of L-DOPA in the presence of different ILs (5.85%, w/v) relative to the rate obtained in the IL-free buffer solution (which was 0.104 mM min^{-1} and taken as 100 for comparison).

the same cation (such as choline$^+$ and NBu_4^+), the correlation between the activity data and the kosmotropicity of the anion was somewhat opposite to what we expected. The fact that the activity responded well upon the choice of the IL cation but not upon that of the anion may be taken as a support again to our earlier statement that the cation of an IL plays a more dominant role than its anion in affecting the enzyme performance. Another feature that is very different from the result obtained by PEL is that the tyrosinase activity is relatively high in the presence of all the ILs holding $H_2PO_4^-$ as the anion, suggesting that although detrimental to PEL, this anion is favored by tyrosinase.

In the interest of achieving some insights into the IL effects, a further investigation was carried out to assess the influence of the 3 imidazolium ILs on both the kinetics and thermostability of the enzyme. The kinetic studies reveal that for each IL tested, upon an increase in the IL content the K_m and V_{max} values of the enzyme increased and decreased, respectively, resulting in a reduction in V_{max}/K_m. But at any fixed IL levels within the tested range, the V_{max} is altered following the sequence of [MMIm][$MeSO_4$] > [EMIm][$MeSO_4$] > [BMIm][$MeSO_4$] (Fig. 5), again consistent with the Hofmeister cation effect, whereas

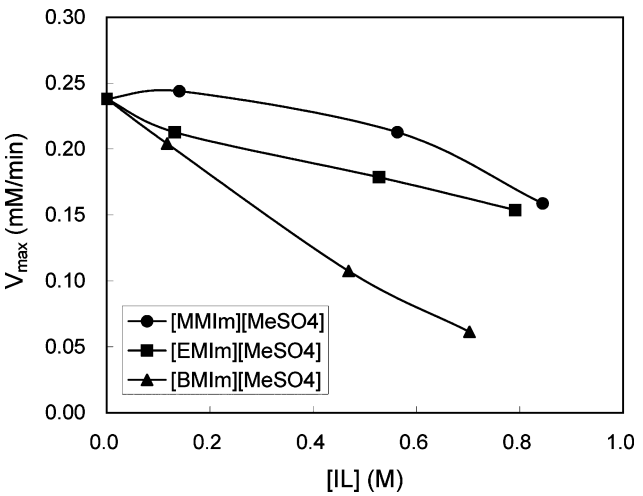


Fig. 5 V_{max} obtained by mushroom tyrosinase in phosphate buffer containing 3 imidazolium ILs with different concentrations (the protein content in the reaction system was 6.58 μg ml^{-1}).

Table 2 Thermostability of mushroom tyrosinase in the presence of the three imidazolium ILs (5% w/v) as compared to the control in the absence of any IL

	Incubation temperature	Control	[MMIm]-[$MeSO_4$]	[EMIm]-[$MeSO_4$]	[BMIm]-[$MeSO_4$]
Half life (min)	30 °C	856.9	347.4	152.7	124.0
	40 °C	119.7	31.5	22.5	8.3
	50 °C	14.9	4.6	3.8	2.1
First-order deactivation rate constant (10^{-3} min^{-1})	30 °C	1.0	2.0	3.6	4.2
	40 °C	4.0	22.0	28.4	n.a.[a]
	50 °C	44.9	n.a.	n.a.	n.a.

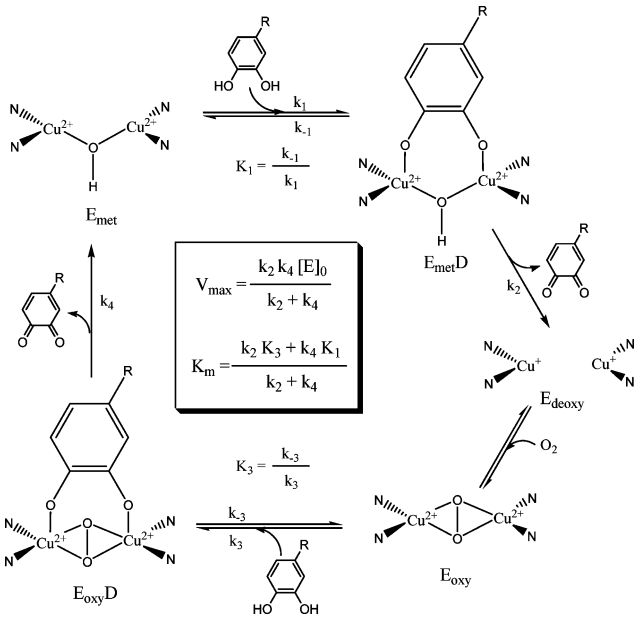
[a] 'n.a.' means 'not applicable', referring to the situations that significantly deviated from the classical first-order kinetics, in which case the deactivation rate constants were not determined.

no such correlation was found for either K_m or V_{max}/K_m values. Lou *et al.*[37] have reported that when papain was used to catalyze hydrolysis in aqueous buffer containing a series of imidazolium ILs ([C_nMIm][BF_4], n=2~6, 15% v/v), a reduction in V_{max}, along with an increase in K_m, was also observed with a longer alkyl chain attached on the IL cation.

The thermostability data are listed in Table 2. At each incubation temperature, the enzyme stability in the presence of the 3 imidazolium ILs varied in the same order as above: [MMIm][$MeSO_4$] > [EMIm][$MeSO_4$] > [BMIm][$MeSO_4$], although none of these 3 ILs showed the ability of stabilizing the enzyme. In our previous study,[12] tyrosinase in aqueous solution has been found to be destabilized by the three imidazolium ILs holding the same cation, namely [BMIm][PF_6], [BMIm][BF_4], and [BMIm][$MeSO_4$]. The stability order observed in this study confirms this and reinforces the fact that a more kosmotropic IL cation is less favorable for maintaining the enzyme stability. The deviations from first-order deactivation kinetics at a higher temperature (50 °C) and in the presence of a more kosmotropic IL cation ($BMIm^+$) suggest that under these conditions, the enzyme might undertake a more complex mechanism for its deactivation. It is actually not surprising to have observed these deviations, simply because the first-order kinetics model is oversimplified.

A closer look at the active site structure and catalytic mechanism of tyrosinase is necessary for us to understand how ILs affect the enzyme activity through the Hofmeister effect. Mushroom tyrosinase is a metalloenzyme consisting of two tetragonal Cu^{2+} atoms at the active site, as shown in Scheme 1.[43,44] V_{max} is dependent on the two rate constants (k_2, k_4) that govern the formation of the quinone product, whereas K_m is associated not only with these two rate constants but also with the two dissociation constants (K_1, K_3) that govern the nucleophilic attack of the substrate *o*-diphenol on the active site Cu^{2+} ion.[44]

Cu^{2+} ion is a strong kosmotrope with a highly positive viscosity B coefficient of +0.376.[23] Based on the "law of matching water affinity",[38] a kosmotropic anion has a strong power of ion-pairing with this metal ion, thus stabilizing the ground state of the enzyme and diminishing the ability of the copper ion in accepting the nucleophilic attack from the substrate, *o*-diphenol. As a result, the activation energy of the enzymatic reaction is increased and the reaction rate is lowered. This can explain the unexpected activity order obtained by the ILs holding



Scheme 1 Catalytic cycle of the tyrosinase-catalyzed oxidation from *o*-diphenol to *o*-quinone.

the same cation (Fig. 4): [choline][Ac] < [choline][$MeSO_3$] < [choline][NO_3] and [NBu_4][Ac] < [NBu_4][$MeSO_3$].

The above discussion can also be used to explain why tyrosinase activity was always lower than the control when conducting catalysis in the presence of the three imidazolium ILs. This is simply because the IL anion, $MeSO_4^-$, is a strong kosmotrope. In addition, a highly kosmotropic IL cation like $BMIm^+$ may compete with the similarly kosmotropic Cu^{2+} cation at the enzyme's active site for the substrate, *o*-diphenol, thereby preventing the latter from nucleophilic attack to the Cu^{2+} ion for proceeding the catalysis. This offers a plausible explanation for the sensitive response of the tyrosinase activity to the IL cation in the order of control > $MMIm^+$ > $EMIm^+$ > $BMIm^+$. The interaction between the imidazolium cation and the substrate leads to a reduction in the two rate constants, k_2 and k_4, and an increase in the two dissociation constants, K_1 and K_3. According to the two equations listed in Scheme 1, V_{max} is decreased whereas the change in K_m is either increased or decreased, depending on the balance between the two rate constants and the two dissociation constants. This can readily explain why it is the V_{max}, rather than the K_m, that varied by following the Hofmeister effect of the IL cation (Fig. 5).

Structural study of mushroom tyrosinase

Investigation on any possible conformational changes induced by ILs may shed some light on the effect of ILs on enzyme performance. Fluorescence spectroscopy can be used to follow the changes in protein conformation, such as unfolding. This is reflected by both the decrease in the maximal intensity of the fluorescence (I_{max}) and the red-shift in the maximal emission wavelength (λ_{max}), both resulting from the increased polarity experienced by the fluorophore residues (particularly Trp) of the protein globule.[45]

Fig. 6 shows the fluorescence spectra of tyrosinase in phosphate buffer with addition of two sets of ILs holding the same

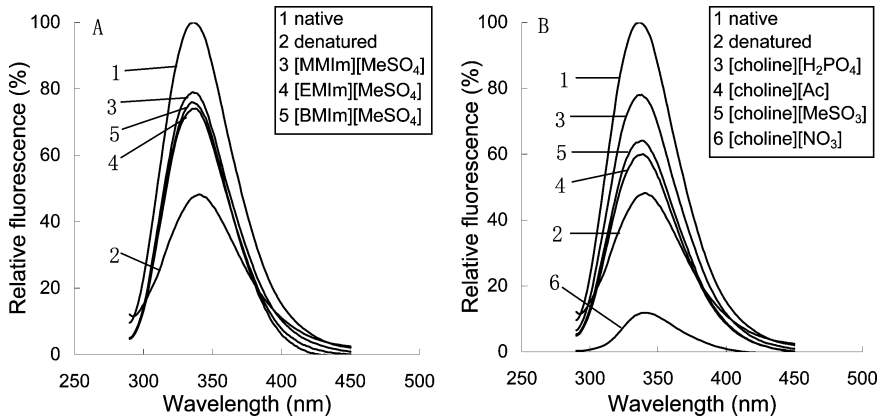


Fig. 6 Fluorescence spectra of tyrosinase in phosphate buffer (50 mM, pH 6.0) in the absence and presence of different ILs (2.93%, w/v): (A) 3 imidazolium ILs with $MeSO_4^-$ as the anion; (B) 4 ammonium ILs with choline as the cation. The data are normalized with respect to the maximal fluorescence intensity obtained at 336 nm in the IL-free buffer solution.

anion (A) and cation (B), respectively. As compared to its native form the fully denatured enzyme exhibited a red-shift in λ_{max} (from 336 nm to 340 nm) and a significant drop in I_{max} (from 100% to 48.2%), confirming the unfolding of the protein structure leading to the exposure of the tryptophan residues to the solvent. Addition of both groups of the ILs resulted in a noticeable reduction in I_{max}, which is also shown in Fig. 7, and negligible shift in λ_{max}. This suggests that although the tryptophan residues were not very much exposed, a more loosely packed conformation of the protein was triggered by all the ILs tested, in concert with the reduced activity assayed in the presence of these ILs (Fig. 4). Upon IL addition the fluorescence intensity of the enzyme decreased following the increase in the kosmotropicity of the IL cation (Fig. 7A) and the decrease in the kosmotropicity of the IL anion (Fig. 7B). A severe situation was observed in the presence of [choline][NO_3], which holds a chaotropic anion obviously detrimental to the protein structure:[6] A red-shift of 9 nm in λ_{max} and a 99.5% reduction in I_{max} were caused when the concentration of this IL was increased from 0 to 0.53 M. All these strongly substantiate that the impact of ILs on the protein structure follows the Hofmeister series very well.[6] The IL-induced variation in the protein structure basically agrees with the activity data, particularly in the case of the imidazolium ILs holding the same anion. Interestingly, the order of the fluorescence intensity ([choline][Ac] ~ [choline][$MeSO_3$] > [choline][NO_3], Fig. 7B) was inverse to the activity order shown in Fig. 4, and in the presence of [choline][NO_3] the enzyme presented a relatively high activity while yielding a poor fluorescence intensity, which is even weaker than the one obtained by the enzyme totally denatured by heat. It has been proposed that the folded state does not always induce enzyme activation,[46,47] and our results seem to agree with this.

Conclusions

This work has clearly demonstrated that specific ion effect of ILs plays an important role in affecting enzymatic performance. Both activity and stability of the two model enzymes in aqueous solution are controlled directly or indirectly by the Hofmeister effect of the ILs, as has been supported by the structural study with fluorescence spectroscopy. Specific impacts of ILs may be associated with the influence of their cations and anions on the surface pH, active site conformation, and catalytic mechanism of each specific enzyme, following the Hofmeister

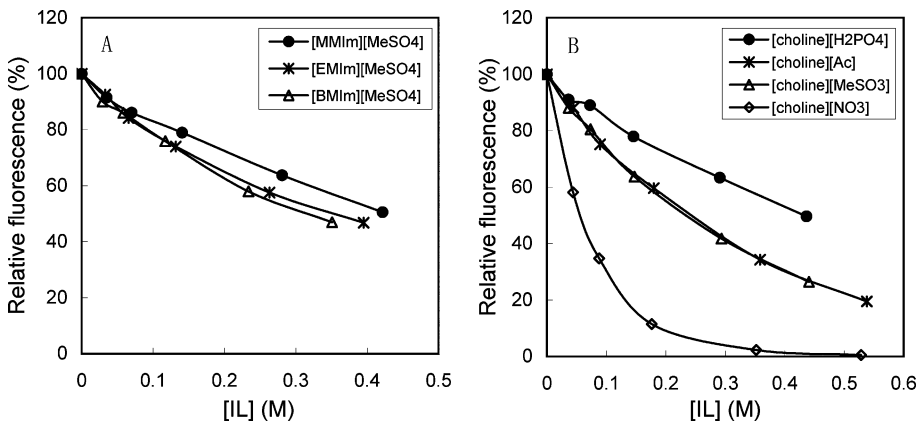


Fig. 7 Effect of ILs and their concentrations on the maximal fluorescence intensity of tyrosinase (obtained at 336 nm), relative to the maximal fluorescence intensity obtained by the enzyme at 336 nm in the IL-free buffer solution. (A) 3 imidazolium ILs with $MeSO_4^-$ as the anion; (B) 4 ammonium ILs with choline as the cation.

series. Ammonium ILs, especially those composed of chaotropic cations and kosmotropic anions, offer potential advantages in facilitating enzyme functioning. A few ammonium ILs such as [$NHMe_3$][$MeSO_3$] (which is a protic IL) and [choline][Ac] (which possesses a hydroxylated cation) are effective in promoting enzyme activity and stability, and the plausible reason may be related to both the H-bonding capability and chaotropicity of these IL cations (in combination with a kosmotropic anion). The IL cations may play a predominant role over their counter-anions in affecting the enzyme behavior. By testing 79 ILs of different types in their acetylcholinesterase (AchE) inhibition screening assay, Arning *et al.*[48] have already observed a more profound effect elicited by the IL cations, in terms of their head groups and side chains, whereas the vast majority of the anion species exhibited no impact on AchE.

However, it has to be stated that the specific ion effect is not the only factor controlling enzyme performance in the IL systems, and that the behavior of an enzyme in IL-containing aqueous solution may be somewhat different from that in an IL-dominating system. As has been demonstrated,[49] the catalytic activity is highly dependent on the characteristics of the IL, the structure of the substrate, and the nature of the enzyme and the support. Further research has to be carried out in order to advance our knowledge about biocatalysis in ILs, thus promoting the development of green biotransformation processes in such promising nonaqueous media.

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