J. Biochem. 2009;145(3)355–364
doi:10.1093/jb/mvn173

Importance of the Ionic Nature of Ionic Liquids in Affecting Enzyme Performance

Zhen Yang*, Ya-Jun Yue, Wen-Chao Huang, Xiao-Ma Zhuang, Zheng-Tao Chen and Miao Xing

College of Life Sciences, Shenzhen University, Shenzhen, Guangdong 518060, China

Received October 27, 2008; accepted December 8, 2008; published online December 27, 2008

The activity and stability of mushroom tyrosinase were studied in ionic liquid (IL)-containing aqueous systems. The effect of three ILs ([BMIm][PF_6], [BMIm][BF_4]), and [BMIm][$MeSO_4$], where [BMIm] = 1-butyl-3-methylimidazolium) and their inorganic salts ($KMeSO_4$, KPF_6, and $NaBF_4$) on the enzyme performance was investigated by comparing the kinetic (such as K_m, V_{max}, optimal pH and temperature, and activation energy) and thermostability parameters (including half-lives, deactivation constants, activation energies for enzyme deactivation, ΔG^*, ΔH^*, and ΔS^*) of the enzyme in the absence and presence of the ILs and their anions. Both the three ILs and their inorganic salts were able to trigger enzyme activation. The enzyme could be stabilized by addition of $KMeSO_4$ and $NaBF_4$ but destabilized by the presence of all the three ILs. The substrate selectivity of the enzyme was unchanged. The effect of ILs on enzyme performance can be largely attributed to their ionic nature via interaction with the enzyme structure, the substrate, and the water molecules associated with the enzyme, depending on their kosmotropocity, nucleophilicity, and H-bond basicity. The different influences brought from the ILs and their associated ions indicate the cooperative functioning of both cation and anion of the IL in affecting the enzyme performance.

Key words: Hofmeister series, ionic liquids, ionic nature, kosmotropicity, tyrosinase.

INTRODUCTION

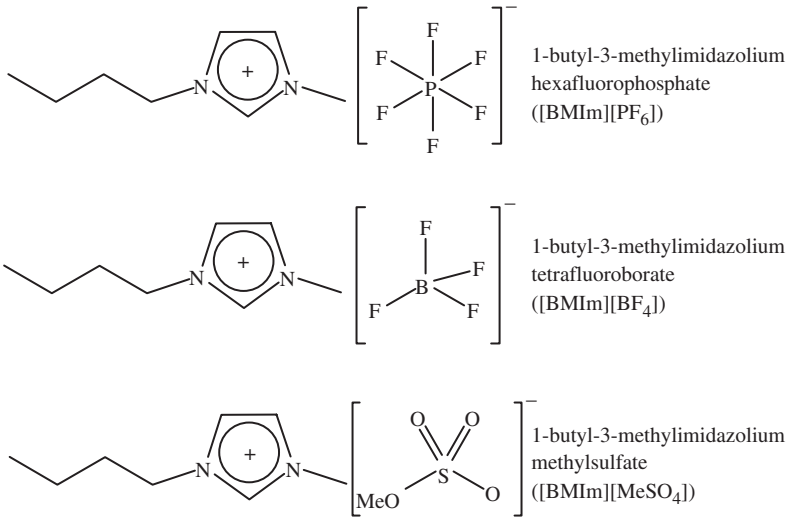
Ionic liquids (ILs) have been taken as a promising new class of solvents for biocatalytic processes, as they are capable of presenting high enzymatic efficiency, enzyme stability and selectivity (*1–3*). One of the major advantages of using ILs as the reaction media is that their chemical and physical properties can be finely tuned by modification of either the cation or anion or both. It is important to set out a detailed investigation on how the enzymatic performance is affected by changing the structure and composition of the ionic liquid.

Research conducted so far has clearly revealed that enzymes in ionic liquids basically follow the same catalytic mechanism as in water and in organic solvents. For instance, the enzyme shows a hyperbolic increase in the initial reaction rate upon the increase in substrate concentration, offering normal K_m and V_{max} values, and sometimes suffering substrate inhibition (*4*); the enzyme activity is dependent on the water activity in the same way as in organic solvents (*5*, *6*); and the enzyme is stabilized by ionic liquids as by hydrophobic organic solvents with the ability to maintain its compact native structure (*7*). Therefore, it can be inferred that similar to organic solvents, an enzyme in ionic liquids is also functioning with a microaqueous phase surrounding the enzyme molecules. Like organic solvents, ILs may also affect the enzyme performance via the interactions such as stripping off the essential water from the enzyme and interacting with the enzyme, the substrates, and the products (*3*). However, unlike conventional molecular organic solvents, when penetrating into the aqueous phase surrounding the enzyme molecules, ionic liquids actually dissolve and dissociate into individual cations and anions rather than exist as intact molecules. The presence of these ions may play an important role in affecting the enzyme performance in ILs, presumably by direct interaction with the enzyme molecules and/or by modifying the microenvironment in and around the enzyme molecules due to their different physicochemical properties. Indeed, it has been observed that the enzyme activity in ionic liquids is dependent on the IL anions (*8*), especially on their H-bond basicity and nucleophilicity (*3*). In organic solvents, an enzyme can be pronouncedly activated by pre-lyophilization with a series of inorganic salts, and this activation correlates well with the kosmotropicity/chaotropicity of the salts (*9*).

Taking these findings into consideration, in addition to examining enzyme catalysis in ionic liquids, a comprehensive study of enzyme performance in aqueous solution containing different ILs and their anions is necessary, which will provide valuable information to understand the behavior of ionic liquids in biocatalysis.

In this study, mushroom tyrosinase (polyphenol oxidase, EC 4.14.18.1) was employed as a model enzyme. It catalyzes the oxidation of a variety of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones. Due to its specific regioselectivity, tyrosinase has been one of the most synthetically useful

*To whom the correspondence should be addressed. Tel: +86-755-2653-4152, Fax: +86-755-2653-4274,
E-mail: zyang@szu.edu.cn



Scheme 1. **Molecular formula of the three ionic liquids.**

oxidoreductases (*10*), facilitating the formation of various products that are important in areas such as pharmaceutical and fine chemical industries (*11*). Our recent report has shown that this enzyme is also active in ionic liquids (*4*): Its activity in the hydrophobic [BMIm][PF_6] was comparable to that presented in chloroform and much higher than those obtained in the other two hydrophilic ILs, [BMIm][BF_4] and [BMIm][$MeSO_4$]. The three ILs (Scheme 1) that have been selected contain the same cation and different anions. The aim of our current study was to further investigate the IL effects by studying the activity and stability of mushroom tyrosinase in aqueous solution with addition of these three ILs and their anions.

MATERIALS AND METHODS

Materials—Fresh white mushrooms were purchased from a local supermarket in Shenzhen, China. The three substrates (catechol, 4-methylcatechnol, and 4-*tert*-butylcatechol), the three ILs ([BMIm][PF_6], [BMIm][BF_4], and [BMIm][$MeSO_4$]) and their inorganic salts ($NaBF_4$, KPF_6, and $KMeSO_4$) were purchased from Sigma Chemical Co. All other reagents used were of analytical grade.

Enzyme Preparation—The enzyme was obtained by extraction of fresh mushrooms (50 g) into 100 ml phosphate buffer (50 mM, pH 6.0) as detailed in ref. (*12*).

Enzyme Activity Assays—The activity of tyrosinase was determined by following the oxidation of 4-methylcatechol to 4-methyl-*o*-quinone. A typical reaction was carried out in a cuvette where 50 µl of the enzyme solution was added to 2 m1 of 50 mM pH 6.0 phosphate buffer containing 1 mM of the substrate and various amounts of an IL (0–20%, v/v) or its inorganic salt (0–0.15 M), with the pH readjusted to 6.0. The formation of the product at 30°C was followed spectrophotometrically by recording the absorbance at 400 nm with the Pharmacia Biotech Ultraspec 2000 UV/Vis spectrophotometer equipped with a thermostated cell. The K_m and V_{max} values of the enzyme were obtained from the Lineweaver–Burk plots. Optimum pH was determined by measuring tyrosinase activity at 30°C over a pH range of 5.0–8.0 in 50 mM phosphate buffer. Optimum temperature was determined by measuring the activity of tyrosinase in 50 mM phosphate buffer (pH 6.0) at various reaction temperatures (30–70°C), and the data were used to calculate the activation energy according to the Arrhenius equation. All the experiments were duplicated, being subject to less than 5% error for each data point.

Thermal Inactivation Studies—In order to study the effect of ILs on the enzyme stability, 1 ml of enzyme solution in phosphate buffer (pH 6.0, 50 mM) only, in the buffer containing 2% (v/v) [BMIm][BF_4] or [BMIm][$MeSO_4$], or in the buffer saturated with [BMIm][PF_6] was incubated in an Eppendorf thermomixer at different temperatures in the range of 40–70°C. Periodically, 50 µl of the enzyme solution was taken for activity assay in phosphate buffer (pH 6.0, 50 mM) at 30°C as noted above. The relative residual activity referred to the initial activity obtained above as compared to the one obtained prior to incubation. The first-order deactivation rate constants (k) were determined from the semi-logarithmic plot of residual activity as a function of time. Activation energies for the enzyme's thermal deactivation (E_a) were calculated from the Arrhenius plots of logarithms of the deactivation rate constants versus reciprocals of the absolute temperatures. Thermodynamic functions (ΔH^*, ΔG^*, ΔS^*) were obtained according to ref. (*13*). For studying the effect of IL anions on the enzyme stability, the enzyme was incubated either in phosphate buffer (pH 6.0, 50 mM) only or in the buffer containing 0.3 M of $NaBF_4$, KPF_6, or $KMeSO_4$ at different temperatures in the range of 40–60°C. The pH of all the buffer solutions was readjusted to 6.0 prior to test.

RESULTS

Effect of Ionic Liquids on Enzyme Activity and Kinetic Parameters—The effect of ionic liquids on enzyme activity was first studied by measuring the initial rates of reactions catalyzed by mushroom tyrosinase in aqueous

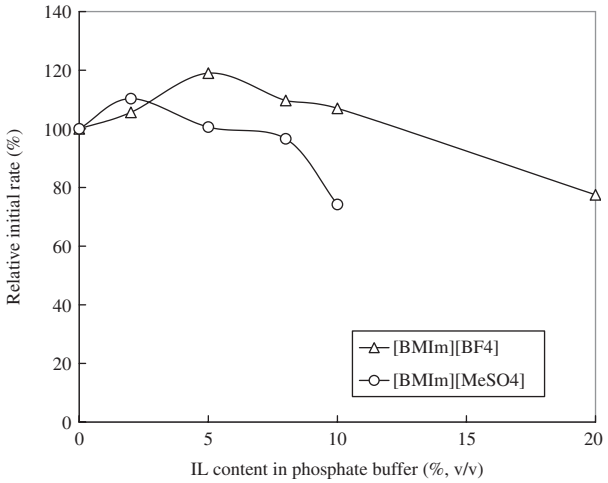


Fig. 1. **Effect of IL content on enzyme activity.** Tyrosinase activity was determined in the phosphate buffer (50 mM, pH 6.0) containing different amounts of ILs. The relative initial rate (%) refers to the percentage of the initial reaction rate obtained by the enzyme in the presence of the IL as compared to the one obtained in the IL-free buffer.

buffer solution with addition of different amounts of the three ILs. When tested in the presence of two water-miscible ILs (Fig. 1), the enzyme activity increased initially with an increase in the IL content up to 5% (v/v) for [BMIm][BF_4] and 2% (v/v) for [BMIm][$MeSO_4$], and the subsequent decline was more abrupt in the latter case. The maximum activities presented by the enzyme in the presence of both ILs were 119% and 110%, respectively, of the initial reaction rate obtained in the IL-free buffer. No further tests were done at higher IL contents simply because this would induce an increase in the viscosity and acidity [for [BMIm][BF_4] (*14*)] or alkalinity [for [BMIm][$MeSO_4$] (*15*)] of the reaction medium, which might interfere with our results. In the phosphate buffer saturated with the water-immiscible [BMIm][PF_6], the enzyme achieved an initial reaction rate which was 108% of that obtained in the IL-free buffer. The solubility of this IL in water at 30°C is 2.4% (w/w) (*16*), which can be translated to 1.7% (v/v).

The above initial data seemed to show that all the three selected ILs were able to trigger enzyme activation. Shipovskov *et al*. (*17*) have also reported that both [BMIm]Br and [BMIm][$N(CN)_2$] stimulated the activity of laccase in water when provided at concentrations between 10% and 20% and between 50% and 60% (v/v), respectively. However, a decline of enzyme activity in aqueous solution with an increase in IL content is usually observed (*18–20*). In order to get a deeper insight into how the ionic liquids affect the enzyme kinetics, K_m and V_{max} values of the enzyme were determined in the presence of each IL. The results for the two hydrophilic ILs were presented in Fig. 2. When the IL content increased, both K_m (Fig. 2A) and V_{max} (Fig. 2B) increased gradually (for [BMIm][$MeSO_4$] when IL content <8%, v/v), but their ratio V_{max}/K_m as a measure of the catalytic efficiency (Fig. 2C) showed a bell-shaped relationship with the IL content. This is consistent with

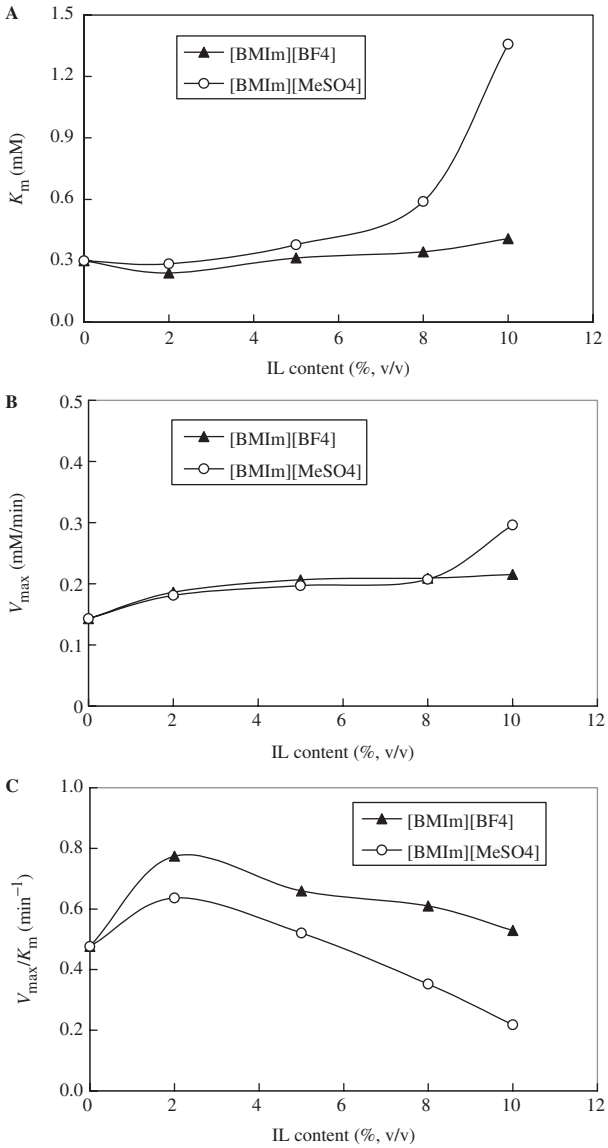


Fig. 2. **Effect of IL content on kinetic parameters of mushroom tyrosinase: (A) K_m, (B) V_{max}, (C) V_{max}/K_m.**

the results shown in Fig. 1, suggesting that the presence of ionic liquids affected both the K_m and V_{max} of the enzyme.

Comparatively, the kinetic variation induced by [BMIm][$MeSO_4$] was more severe, especially when its content in the aqueous buffer exceeded 8%. As a result, when the content of this IL was raised to 10%, the enzyme retained only 45.8% of its original catalytic efficiency (V_{max}/K_m) obtained prior to addition of any IL, whereas 111% of the original was obtained by the enzyme when the same content of [BMIm][BF_4] was present in the buffer. When placed in the phosphate buffer saturated with [BMIm][PF_6], the enzyme showed a decrease in K_m while keeping the V_{max} fairly constant, thus resulting in a 50% increase in the catalytic efficiency.

The above results have, therefore, manifested that the presence of ILs can trigger enzyme activation and that the activity of mushroom tyrosinase in IL-containing

aqueous solution varied in the order of [BMIm][PF_6] > [BMIm][BF_4] > [BMIm][$MeSO_4$]. This activity order agrees well with our recent report about the same enzyme suspended in ionic liquid media (*4*).

Effect of Ionic Liquids on pH and Temperature Optima—Variation of the enzyme activity upon the change of pH in the presence of different ILs was examined and depicted in Fig. 3. In buffer only, the enzyme showed an optimal pH of 6.0. It was right shifted to 6.5 when 2% of [BMIm][$MeSO_4$] was added. However, the enzyme activity did not seem to respond sensitively upon the change in pH when either [BMIm][PF_6] or [BMIm][BF_4] was present in the aqueous solution. This is consistent with our previous findings with tyrosinase suspended in [BMIm][PF_6] only (*4*): The enzyme presented an activity that was insensitive to the pH of the aqueous buffer from which the enzyme was prepared.

The effect of ILs on the optimal reaction temperature of the enzyme was also investigated. The activity–temperature profile did not seem to be altered to a great extent, but the presence of 2% [BMIm][BF_4] or a saturation level of [BMIm][PF_6] seemed to trigger a slight left shift in the optimal reaction temperature (data not shown).

Activation energy of the enzyme was determined by evaluating in more detail the enzymatic reaction rates in the temperature range of 30–50°C. In phosphate buffer with addition of 2% of either [BMIm][$MeSO_4$] or [BMIm][BF_4] or a saturated level of [BMIm][PF_6], the activation energy of the enzyme was 20.9, 23.0, 27.7 kJ/mol, respectively, all being lower than the one obtained by the enzyme in the IL-free phosphate buffer (30.5 kJ/mol). This further confirmed the activation effect of the ILs.

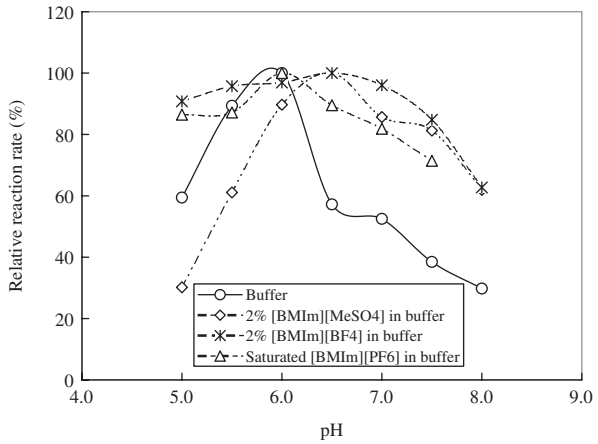


Fig. 3. **pH dependence of mushroom tyrosinase in phosphate buffer with and without addition of ILs.**

Effect of Ionic Liquids on the Enzyme Thermostability—The loss of enzyme activity at different temperatures (40–70°C) as a function of incubation time was detected and compared in phosphate buffer only, in the buffer containing 2% (v/v) [BMIm][BF_4] or [BMIm][$MeSO_4$], or in the buffer saturated with [BMIm][PF_6]. All the three ILs showed a destabilization effect on the enzyme as can be observed by comparing the half-life data (third entry, Table 1). [BMIm][$MeSO_4$] was not included in this table simply because the enzyme was so unstable in the solution containing this IL that the enzyme activity was lost immediately. Cytochrome *c* has also been found to retain negligible activity after 3 weeks of storage in [BMIm][$MeSO_4$] containing 20% H_2O (*21*). Therefore, the enzyme was most unstable in the presence of [BMIm][$MeSO_4$], less unstable when treated with [BMIm][PF_6], and most stable with [BMIm][BF_4].

Our thermostability data seemed to fit the first-order enzyme deactivation model, suggesting that this was the mechanism that the enzyme followed during its thermal inactivation process in the phosphate buffer with or without addition of the ILs. A typical example was shown in Fig. 4. The first-order deactivation rate constants were

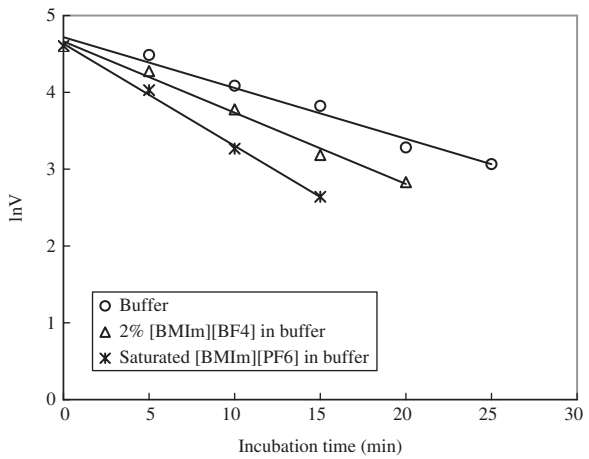


Fig. 4. **Enzyme deactivation at 60°C in phosphate buffer, in phosphate buffer containing 2% (v/v) [BMIm][BF_4], and in phosphate buffer saturated with [BMIm][PF_6].** *V* is the relative residual activity (%). A value of 100% refers to the activity obtained by the enzyme prior to incubation. Straight lines are the results of fitting the first-order deactivation model.

Table 1. **Thermal deactivation parameters for mushroom tyrosinase in aqueous solution with and without addition of ILs.**

Incubation medium	IL-free phosphate buffer				Buffer with 2% (v/v) [BMIm][BF_4]				Buffer saturated with [BMIm][PF_6]		
Incubation temperature (°C)	40	50	60	70	40	50	60	70	50	60	70
Half-life (min)	462	53	10	2	267	30	7	1.4	26	5	1.1
First-order deactivation rate constant (10^{-2} min^{-1})	0.15	1.3	6.6	31.5	0.26	2.3	9.3	49.4	2.6	13.3	64.5
Activation energy (kJ·mol^{-1})	158				153				147		
ΔG^* (kJ·mol^{-1})	104	102	101	99	103	100	100	98	100	99	97
ΔH^* (kJ·mol^{-1})	155	155	155	155	150	150	150	150	144	144	144
ΔS^* (J·mol^{-1}·K^{-1})	163	165	163	162	151	154	151	152	137	136	136

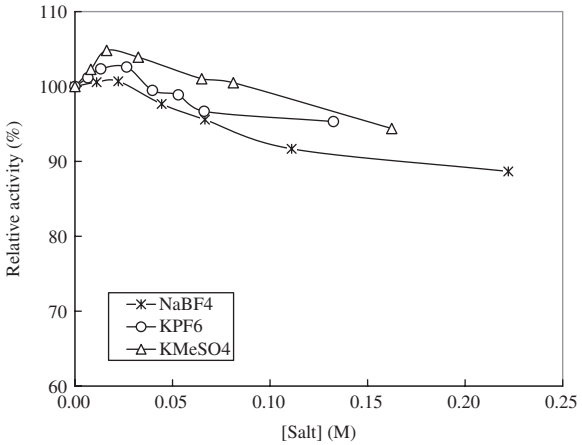


Fig. 5. **Variation of enzyme activity upon the change in the salt concentration**. Tyrosinase activity was determined in the phosphate buffer (50 mM, pH 6.0) containing different amounts of $NaBF_4$, KPF_6, or $KMeSO_4$. The relative initial rate (%) refers to the percentage of the initial reaction rate obtained by the enzyme in the presence of the above salts as compared to the one obtained in the buffer containing none of them.

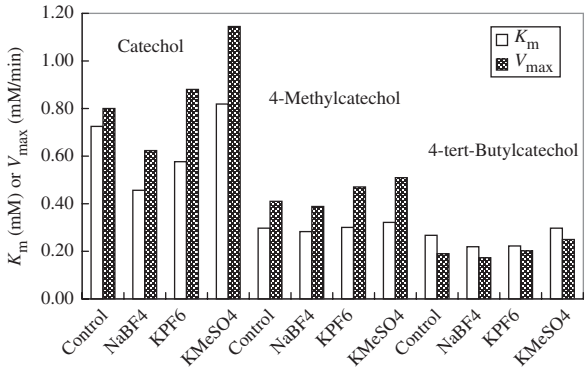


Fig. 6. **A comparison of the K_m, V_{max} values obtained by the enzyme with either catechol, 4-methylcatechol, or 4-*tert*-butylcatechol as the substrate in the presence of different salts (0.15 M).** The control refers to the buffer solution with no additional salts.

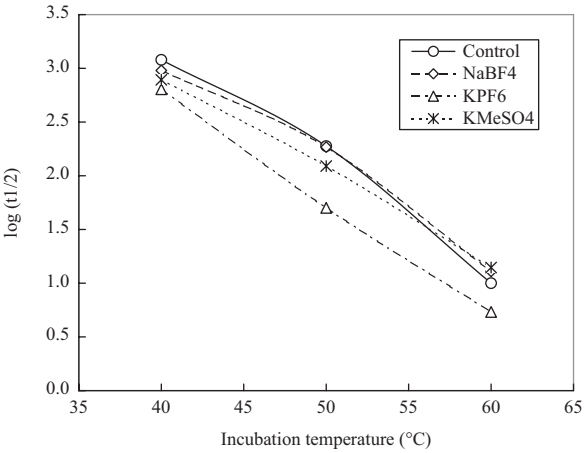


Fig. 7. **Effect of the IL anions on the thermal stability of the enzyme.** The half-lives (min) of the enzyme were determined in phosphate buffer (pH 6.0) containing 0.3 M of different salts ($NaBF_4$, KPF_6, or $KMeSO_4$) at different incubation temperatures. The control refers to the buffer solution containing none of the above salts.

rather temperature sensitive (fourth entry, Table 1). Within the temperature range evaluated, deactivation rate constants for the enzyme incubated in IL-free buffer were all apparently less than the results obtained by the enzyme in IL-containing solutions. In addition, the activation energies of enzyme deactivation were slightly lower for the enzyme incubated in IL-containing phosphate buffer as compared to the result obtained in the IL-free solution (fifth entry, Table 1), confirming the thermal destabilization effect of the ILs.

Effect of IL Anions on Activity and Stability of Mushroom Tyrosinase—Because the three ILs tested above contain the same cation but different anions and affect the enzyme performance differently, it is necessary to determine whether these IL anions have any specific effects on the enzyme. This was evaluated by testing the activity and stability of mushroom tyrosinase in the presence of sodium or potassium salts containing those IL anions, that is, $NaBF_4$, KPF_6, and $KMeSO_4$.

As shown in Fig. 5, although variation of salt concentration did not result in a significant change in enzyme activity, the enzyme showed a higher tendency of retaining its activity in the presence of $KMeSO_4$ than in the presence of KPF_6 and $NaBF_4$. A similar trend was also observed when comparing the K_m, V_{max} values of the enzyme with either catechol, 4-methylcatechol, or 4-*tert* butylcatechol as the substrate (Fig. 6). Addition of each inorganic salt (0.15 M) did not seem to alter the enzyme's substrate selectivity. Both pH and temperature optima were not altered by addition of these salts.

The half-lives of the enzyme in the phosphate buffer containing 0.3 M of each inorganic salt were compared in Fig. 7. Among the three salts tested, KPF_6 was the most destabilizing agent at all the incubation temperatures tested ranging between 30°C and 70°C. The stability of the enzyme was lower when incubated at 40°C in the $NaBF_4$-containing buffer as compared to that in the salt-free buffer, but the situation became reversed when the incubation temperature was getting higher. The same happened to $KMeSO_4$. As a result, when incubated at 60°C, the enzyme presented its stability in the order of $KMeSO_4 > NaBF_4 > control > KPF_6$; both $KMeSO_4$ and $NaBF_4$ showed the ability of stabilizing the enzyme.

DISCUSSION

Our study has demonstrated that both the activity and stability of mushroom tyrosinase in aqueous solution can be affected by addition of different ILs or their inorganic salts. In fact, the influences brought from ILs and their anions were very different. Because ionic liquids normally dissociate into individual ions when dissolved in water, a careful examination of their ionic nature and its impact on enzyme catalysis is very important, as it provides another means for us to investigate how the

structure and composition of ionic liquids affect the enzyme's catalytic performance.

Influence of salts and ions on protein stability in aqueous solution has been recognized since 1888, and the sequence of the ability of the ions in stabilizing proteins is well known as the Hofmeister series (*22*), although the mechanism involved is still not clearly known. Various physicochemical properties of the salts and their interactions with the protein have to be taken into account. The Hofmeister series of salts has been related to their kosmotropic/chaotropic properties (*23*), which may be important in elucidating the impact of the IL ions on enzyme activity and stability (*24*). Nucleophilicity and H-bond basicity of the IL ions may also play important roles in affecting the enzyme performance in IL-containing media (*3*).

The behavior of the enzyme in the presence of ILs and their anions can be explained by their kosmotropic/chaotropic properties. These properties have been characterized by the Jones–Dole viscosity B coefficient, a measure of the degree to which an additive increases or decreases the viscosity of water (*25*). It is assumed that the influence of the Hofmeister ion series on protein structure is mediated through the influence of the ions on water structure (*26*). Kosmotropes have positive values of the B coefficient and tend to increase the protein stability in solution due to their stronger interactions with water than does water with itself (*23*). The opposite is true of chaotropes.

Among the three IL anions tested in this study, BF_4^- and PF_6^- are chaotropic with negative B coefficients at 25°C (–0.093 and –0.21 dm^3/mol, respectively) (*25*). The B coefficients for $MeSO_4^-$ and the cation $[BMIm]^+$ are not available. But it is believed that $MeSO_4^-$ is a strong kosmotrope like SO_4^{2-} and $MeSO_3^-$ [with a B coefficient of +0.206 and +0.127, respectively (*25*)], and that $[BMIm]^+$ is a kosmotrope as well (*24*), much stronger than the other two cations used in this study, Na^+ (with a B coefficient of +0.085) and K^+ (with a B coefficient of –0.009).

When the effect of IL anions on tyrosinase was tested by introducing only their Na or K salts into the solution, both the activity and stability data for the enzyme seemed to follow the Hofmeister series. For all the three substrates tested, the enzyme always showed the highest K_m and V_{max} values when in the presence of $KMeSO_4$. At all the incubation temperatures tested, KPF_6 always yielded the lowest half-life, and at 60°C, the enzyme stability was exhibited in the order of $KMeSO_4 > NaBF_4 > KPF_6$.

It is believed that an enzyme could be stabilized by addition of a kosmotropic anion, primarily due to preferential hydration (*27*). Because of its strong interaction with water, a kosmotropic anion like $MeSO_4^-$ may take up quite some of the water molecules that are originally associated with the enzyme molecule, together being excluded from the enzyme surface. This results in a reduction in the average free energy of the bulk water to a value that more closely resembles neat water (*26*). Therefore, the native folded state of the enzyme molecule is favored due to the restored driving force of the hydrophobic effect, and the enzyme becomes more stable. This state is also catalytically more active, thus yielding a higher V_{max}. Because of the folding, however, this structure is more closed and hence less accessible by the substrate molecules, thus leading to a higher K_m.

Sgalla *et al*. (*20*) have demonstrated that the inhibition of horseradish peroxidase in $[BMIm][BF_4]/H_2O$ mixtures was due to the binding of fluoride anions released from BF_4^- anion to the heme iron. As tyrosinase is a copper-containing enzyme (*28*), possibility of fluoride ion binding to the copper ion at the enzyme's active site cannot be ruled out, and this might partly account for the low enzyme activity in the presence of BF_4^- and PF_6^-.

When the ILs (but not the inorganic salts containing their anions) were introduced into the aqueous solution, however, the enzyme presented its activity in the order that is opposite to the Hofmeister anion series, that is, $[BMIm][PF_6] > [BMIm][BF_4] > [BMIm][MeSO_4]$, and the enzyme was not only most inactive but also most unstable in the presence of $[BMIm][MeSO_4]$. This different behavior, as opposed to the situation presented by the enzyme in the presence of the IL anions, could possibly be attributed to the influence of the IL cation.

Although anions generally have a dominant effect relative to cations of the same charge, the kosmotropic behavior of anions could be lessened in the presence of kosmotropic cations (*26*). By monitoring the changes in apomyoglobin conformation with the aid of far-UV circular dichroism (CD), Eggers and Valentine (*26*) have demonstrated that the kosmotropic Li^+ and Mg^{2+} reduced the fractional content of α-helical structure of the protein, whereas the chaotropic K^+ and Cs^+ enhanced it. It was hypothesized that kosmotropic cations, as opposed to their chaotropic partners, have a higher tendency of ion-pairing with their counter-anions, thus reducing the abundance of the free anions in solution to play their kosmotropic role (*26*). This hypothesis could be used to account for our experimental results, as $[BMIm]^+$ is a kosmotropic cation, whereas Na^+ and K^+ are more chaotropic. In this view, therefore, it is no surprise that as compared to the stability of the enzyme in phosphate buffer only, tyrosinase could be stabilized by addition of $KMeSO_4$ and $NaBF_4$, but got destabilized in the presence of all the three ILs.

Indeed, the impact of the IL cations deserves serious attention. Constantinescu *et al*. (*29*) have demonstrated by employing differential scanning calorimetry that the cation series in decreasing the transition temperature (T_m) of RNase A was $K^+ > Na^+ > EMIm^+ > BMIm^+$, corresponding to the chaotropicity order of these cations. Zhao *et al*. (*18*) have also reported that a protease from *Aspergillus melleus* showed a higher stability in the presence of $[EMIm][CF_3COO]$ than in the presence of $[BMIm][CF_3COO]$ ($[BMIm]^+$ is believed to be more kosmotropic than $[EMIm]^+$). It has been demonstrated that *N*-methyl imidazole (*30*) and its cationic form $[BMIm]^+$ (*31*) had an ability of accelerating the transesterification reaction catalyzed by lipase in acetone. The reactivity of lipase in the presence of $[BMIm][BF_4]$ was lower than the one in the presence of *N*-methyl imidazole (*30*), simply due to the anion's destabilizing effect. Moreover, Itoh's group (*32*, *33*) has also observed a remarkable acceleration obtained by lipase in diisopropyl ether, accomplished by coating the enzyme with an imidazolium alkyl PEG sulfate ionic liquid, and the

result may be related to the direct binding of the IL cation with the enzyme, as has been demonstrated by using the MALDI–TOF mass spectrometry.

On the other hand, the nucleophilicity and H-bond basicity of the IL anions may also be the key determinants in affecting enzyme performance. Among the three IL anions tested, BF_4^- may be slightly higher than PF_6^- in both H-bond basicity (considering the negative charge spreading) and nucleophilicity (considering the polarity of B–F and P–F bonds), whereas $MeSO_4^-$ is the highest in both properties. Basically, the IL anions can interact with both the enzyme and substrate molecules to modify their reactivities. Low H-bond basicity allows the anion for a lower tendency to form hydrogen bonds so as not to interfere with the internal H-bonds of the enzyme molecule, whereas an anion with lower nucleophilicity will be less likely to interact with the positively charged residues of the enzyme molecules, hence protecting the enzyme structure from being altered.

With regard to mushroom tyrosinase, a close look at the active site structure and reaction mechanism will be helpful in understanding the influence of the nucleophilicity and H-bond basicity of the IL anions on the catalytic performance of this enzyme. At the active site of the enzyme, the oxygenated form (E_{oxy}) consists of two tetragonal Cu(II) atoms, each coordinated by three N_{His} ligands (two strong equatorial and one weaker axial). The exogenous oxygen molecule is bound as peroxide and bridges the two Cu centers. As shown in Scheme 2, the substrate (*o*-diphenol) is oxidized as the enzyme passes through five enzyme states: E_{deoxy}, E_{oxy}, $E_{oxy}D$, E_{met}, and $E_{met}D$ (*34*, *35*). V_{max} involves the two rate constants (k_2, k_4) that govern the formation of the quinone product, whereas K_m is dependent not only on these two rate constants but also on the two binding constants (K_1, K_3) that govern the nucleophilic attack of the substrate *o*-diphenol on the active site Cu(II) ion (*35*).

A nucleophilic anion will help to neutralize the Cu(II) ion at the active site. An anion with a high H-bond basicity will make the substrate (*o*-diphenol) a better nucleophile by H-bonding with the two hydrogen atoms from the hydroxyl groups of the substrate. Both situations favor the stabilization of the two reaction intermediates, $E_{met}D$ and $E_{oxy}D$, leading to an increase in the two rate constants, k_2 and k_4, and a reduction in the two binding constants, K_1 and K_3. According to the two equations listed in Scheme 2, V_{max} is increased whereas the change in K_m is not certain. This could possibly explain our findings that for all the three substrates tested, the enzyme always presented its highest V_{max} in the presence of $KMeSO_4$. The effect on the K_m value might presumably be due to the IL-induced change in the enzyme's conformation, followed by a stronger (in the case of [BMIm][PF_6]) or weaker (in the case of [BMIm][BF_4] and [BMIm][$MeSO_4$]) binding of the substrate to the enzyme's active site. Indeed, by examining the single-pass attenuated total reflection FT-IR and resonance Raman spectra, Fujita *et al*. (*20*) have demonstrated that cytochrome *c* may be subjected to a change in its secondary or tertiary structure due to the presence of different ionic liquids.

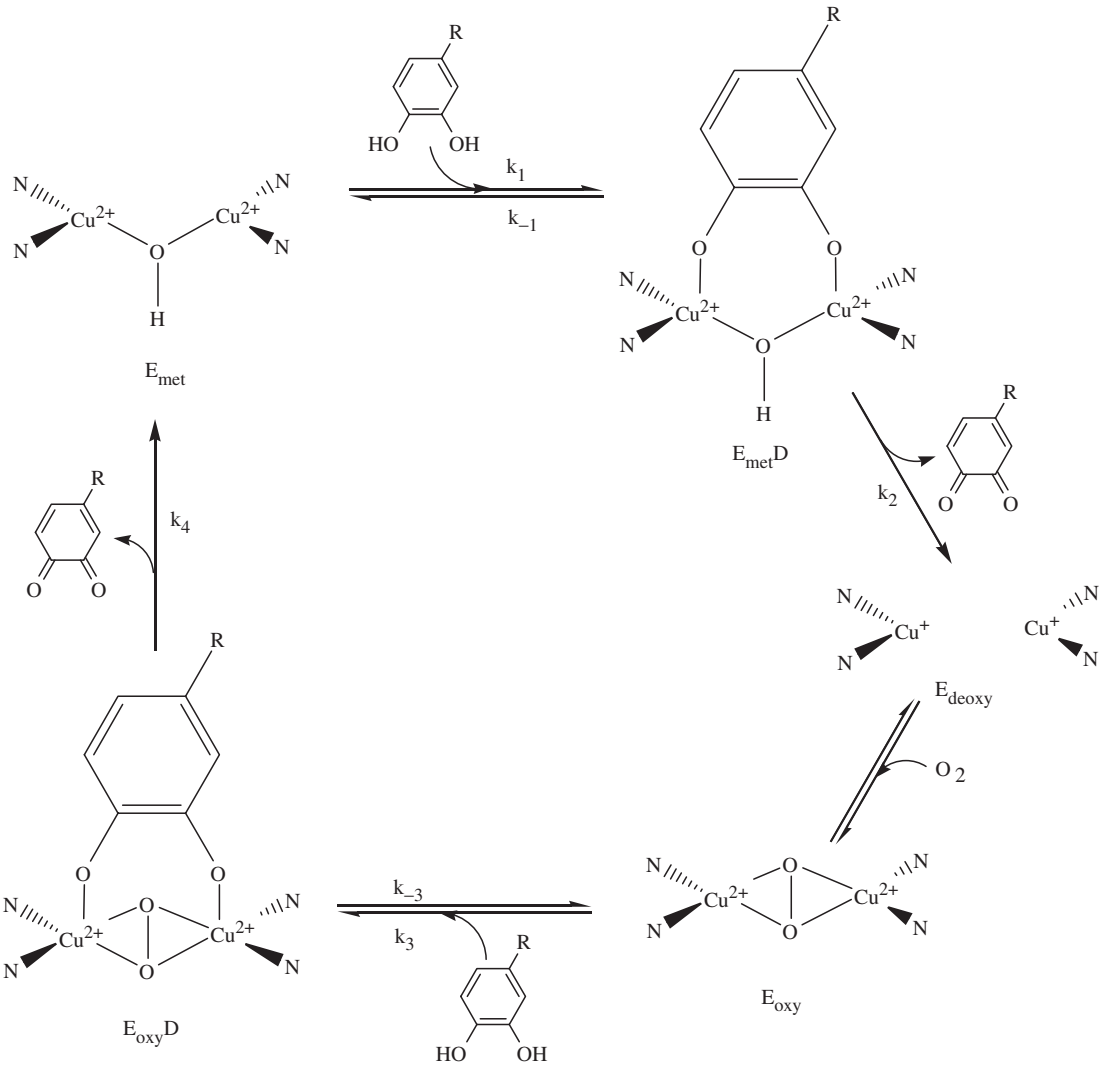
Interestingly, the activity of tyrosinase showed an insensitive response to pH, whether the enzyme was functioning in the IL ([BMIm][PF_6] or [BMIm][BF_4])-containing aqueous buffer system (Fig. 3) or in the IL ([BMIm][PF_6])-dominating reaction medium (*5*). This latter case seemed to be exceptional, as pH memory has been considered one of the novel properties exhibited by enzymes in nonaqueous media (*10*). Nevertheless, a combination of these results could make us speculate that [BMIm][PF_6] might play a special role in polishing the pH effect on the enzyme. The equilibrium between PF_6^- and PF_5 might partially account for this. The same explanation may also apply to [BMIm][BF_4].

[BMIm][$MeSO_4$], however, did not show the same pH buffering effect (Fig. 3). The right shift of the pH optimum for the enzyme in the presence of this IL could be explained by its charge effect. As the isoelectric point of mushroom tyrosinase is 4.8 (*28*), the overall charge of the enzyme molecule should be negative in the pH 6.0 phosphate buffer. Taking into account the bulkiness and the charge of the ionic liquid, the cation $[BMIm]^+$ would bind to the enzyme molecule directly, leaving the anion $[MeSO_4]^-$ in the immediate vicinity of the enzyme molecule. As compared to the pH of the phosphate buffer in the bulk phase, the local pH in the microenvironment where these anions locate might be lowered to some degree. Therefore, in order to have a real pH 6.0 reaching the active site of the enzyme molecule, the pH of the bulk phosphate buffer should be slightly raised accordingly.

The fact that tyrosinase was more thermolabile in the IL-containing buffer has been supported by the thermostability data such as half-lives, first-order deactivation rate constants, and activation energies of enzyme deactivation (entries 3–5 in Table 1). In particular, the calculated thermodynamic parameters over the temperature range studied (the last three entries in Table 1) indicated that when placed in an IL-containing rather than IL-free aqueous solution, the enzyme had a higher tendency of undergoing the denaturation process, although the process of transition to the state of its denatured form presented a more ordered structure. In fact, this more ordered structure can be taken as the result of the shielding effect from the ILs. Because of the strong interactions, the enzyme molecules are surrounded by the cations and anions of the IL that make the protein structure of the enzyme molecules highly organized.

The IL-induced thermal destabilization observed in this study should be significant, taking into account the minor IL contents that were present (2% for either [BMIm][BF_4] or [BMIm][$MeSO_4$], or saturated level (1.7%) of [BMIm][PF_6]). It is well accepted that a high ionic strength (caused by addition of sufficient counterions) is important in maintaining a stable protein structure (*36*). In our case, tyrosinase was able to get stabilized by the addition of some IL anions such as $MeSO_4^-$ and BF_4^-, but was destabilized by the three ILs present in the aqueous solution. This reinforced the importance of the IL cation in monitoring the enzyme's catalytic performance. Lower stability for enzymes in IL-containing aqueous solution has also been reported by some other authors (*18*, *37*).

Overall, the presence of ILs and their anions can significantly affect both activity and stability of



Scheme 2. **Catalytic cycle for oxidation of *o*-diphenols to *o*-quinones by tyrosinase.**

mushroom tyrosinase. By employing an optimal content, the three ILs and the inorganic salts containing their anions can trigger enzyme activation. Enzyme stabilization can also be obtained by addition of $KMeSO_4$ and $NaBF_4$. The effects of ILs and their associated ions can be largely attributed to their ionic nature. They can affect the enzyme activity and stability by interacting with the enzyme structure, the substrates, and the water molecules associated with the enzyme, depending on their kosmotropicity, nucleophilicity, and H-bond basicity. Both the cation and anion of the IL affect the enzyme performance cooperatively. The insensitive pH-dependence of the enzyme in aqueous solution containing $[BMIm][PF_6]$ or $[BMIm][BF_4]$ suggests a pH buffering effect offered by these two ILs. All these results will shed light on elucidating the fundamentals regarding the IL effects on biocatalysis.

It is worth noting that the impact of ILs on enzyme performance may be the result of numerous, complex interactions between the enzyme, the IL, water,

substrate, product, and the buffer system (*17*). Therefore, attempts of finding a few simple physical properties of the ILs to describe their effects on enzyme performance may not be appropriate (*3*). The related mechanism, especially that regarding the cooperative functioning of the cation and anion of the IL, is worth a further investigation, and it is being conducted in our laboratory.

ACKNOWLEDGEMENTS

This work was sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, Ministry of Education, PRC. We thank Prof. P.J. Halling for helpful discussions.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Park, S. and Kazlauskas, R.J. (2003) Biocatalysis in ionic liquids – advantages beyond green technology. *Curr. Opin. Biotechnol.* **14**, 432–437
2. van Rantwijk, F. and Sheldon, R.A. (2007) Biocatalysis in ionic liquids. *Chem. Rev.* **107**, 2757–2785
3. Yang, Z. and Pan, W. (2005) Ionic liquids: green solvents for nonaqueous biocatalysis. *Enz. Microb. Technol.* **37**, 19–28
4. Yang, Z., Yue, Y.-J., and Xing, M. (2008) Tyrosinase activity in ionic liquids. *Biotechnol. Lett.* **30**, 153–158
5. Laszlo, J.A. and Compton, D.L. (2001) α-Chymotrypsin catalysis in imidazolium-based ionic liquids. *Biotechnol. Bioeng.* **75**, 181–186
6. Berberich, J.A., Kaar, J.L., and Russell, A.J. (2003) Use of salt hydrate pairs to control water activity for enzyme catalysis in ionic liquids. *Biotechnol. Prog.* **19**, 1029–1032
7. de Diego, T., Lozano, P., Gmouh, S., Vaultier, M., and Iborra, J.L. (2004) Fluorescence and CD spectroscopic analysis of the α-chymotrypsin stabilization by the ionic liquid, 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide. *Biotechnol. Bioeng.* **88**, 916–924
8. Kaar, J.L., Jesionowski, A.M., Berberich, J.A., Moulton, M., and Russell, A.J. (2003) Impact of ionic liquid physical properties on lipase activity and stability. *J. Am. Chem. Soc.* **125**, 4125–4131
9. Lindsay, J.P., Clark, D.S., and Dordick, J.S. (2004) Combinatorial formulation of biocatalyst preparations for increased activity in organic solvents: salt activation of penicillin amidase. *Biotechnol. Bioeng.* **85**, 553–560
10. Klibanov, A.M. (2003) Asymmetric enzymatic oxidoreductions in organic solvents. *Curr. Opin. Biotechnol.* **14**, 427–431
11. Müller, G., Lang, A., Seithel, D.R., and Waldmann, H. (1998) An enzyme-initiated hydroxylation-oxidation Carbo Diels-Alder domino reaction. *Chem. Eur. J.* **4**, 2513–2522
12. Yang, Z. and Robb, D.A. (1993) Comparison of tyrosinase activity and stability in aqueous and nearly nonaqueous environments. *Enz. Microb. Technol.* **15**, 1030–1036
13. Gouda, M.D., Singh, S.A., Rao, A.G.A., Thakur, M.S., and Karanth, N.G. (2003) Thermal inactivation of glucose oxidase: mechanism and stabilization using additives. *J. Biol. Chem.* **278**, 24324–24333
14. Lou, W.-Y., Zong, M.-H., Liu, Y.-Y., and Wang, J.-F. (2006) Efficient enantioselective hydrolysis of D,L-phenylglycine methyl ester catalyzed by immobilized *Candida Antarctica* lipase B in ionic liquid containing systems. *J. Biotechnol.* **125**, 64–74
15. Sanfilippo, C., D'Antona, N., and Nicolosi, G. (2004) Chloroperoxidase from *Caldariomyces fumago* is active in the presence of an ionic liquid as co-solvent. *Biotechnol. Lett.* **26**, 1815–1819
16. Wong, D.S.H., Chen, J.P., Chang, J.M., and Chou, C.H. (2002) Phase equilibria of water and ionic liquids [emim][PF6] and [bmim][PF6]. *Fluid Phase Equilib.* **194–197**, 1089–1095
17. Shipovskov, S., Gunaratne, H.Q.N., Seddon, K.R., and Stephens, G. (2008) Catalytic activity of laccases in aqueous solutions of ionic liquids. *Green Chem.* **10**, 806–810
18. Zhao, H., Olubajo, O., Song, Z., Sims, A.L., Person, T.E., Lawal, R.A., and Holley, L.A. (2006) Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions. *Bioorg. Chem.* **34**, 15–25
19. Hinckley, G., Mozhaev, V.V., Budde, C.C., and Khmelnitsky, Y.L. (2002) Oxidative enzymes possess catalytic activity in systems with ionic liquids. *Biotechnol. Lett.* **24**, 2083–2087
20. Sgalla, S., Fabrizi, G., Cacchi, S., Macone, A., Bonamore, A., and Boffi, A. (2007) Horseradish peroxidase in ionic liquids. Reactions with water insoluble phenolic substrates. *J. Mol. Catal. B: Enzymatic* **44**, 144–148
21. Fujita, K., MacFarlane, D.R., Forsyth, M., Yoshizawa-Fujita, M., Murata, K., Nakamura, N., and Ohno, H. (2007) Solubility and stability of cytochrome *c* in hydrated ionic liquids: effect of oxo acid residues and kosmotropicity. *Biomacromolecules* **8**, 2080–2086
22. Hofmeister, F. (1888) Zur lehre der wirkung der salze. Zweite mittheilung. *Arch. Exp. Pathol. Pharmakol.* **24**, 247–260
23. Collins, K.D. (1997) Charge density-dependent strength of hydration and biological structure. *Biophys. J.* **72**, 65–76
24. Zhao, H. (2005) Effect of ions and other compatible solutes on enzyme activity, and its implications for biocatalysis using ionic liquids. *J. Mol. Catal. B: Enzymatic* **37**, 16–25
25. Jenkins, H.D.W. and Marcus, Y. (1995) Viscosity B-coefficients of ions in solution. *Chem. Rev.* **95**, 2695–2724
26. Eggers, D.K. and Valentine, J.S. (2001) Crowding and hydration effects on protein conformation: a study with sol-gel encapsulated proteins. *J. Mol. Biol.* **314**, 911–922
27. Timasheff, S.N. (1993) The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Annu. Rev. Biophys. Biomol. Struct.* **22**, 67–97
28. Robb, D.A. (1984) Tyrosinase in *Copper proteins and copper enzymes* (Lontie, R., ed.) Vol. II, pp. 207–241, CRC Press, Boca Raton, FL
29. Constantinescu, D., Weingärtner, H., and Herrmann, C. (2007) Protein denaturation by ionic liquids and the Hofmeister series: a case study of aqueous solutions of ribonuclease A. *Angew. Chem. Int. Ed.* **46**, 8887–8889
30. Liu, B.K., Wu, Q., Xu, J.M., and Lin, X.F. (2007) N-Methylimidazole significantly improves lipase-catalysed acylation of ribavirin. *Chem. Commun.* 295–297
31. Liu, B.K., Wang, N.W., Chen, Z.C., Wu, Q., and Lin, X.F. (2006) Markedly enhancing lipase-catalyzed synthesis of nucleoside drugs' ester by using a mixture system containing organic solvents and ionic liquid. *Bioorg. Med. Chem. Lett.* **16**, 3769–3771
32. Itoh, T., Matsushita, Y., Abe, Y., Han, S.-H., Wade, S., Hayase, S., Kawatsura, M., Takai, S., Morimoto, M., and Hirose, Y. (2006) Increased enantioselectivity and remarkable acceleration of lipase-catalyzed transesterification by using an imidazolium PEG–alkyl sulfate ionic liquid. *Chem. Eur. J.* **12**, 9228–9237
33. Abe, Y., Hirakawa, T., Nakajima, S., Okano, N., Hayase, S., Kawatsura, M., Hirose, Y., and Itoh, T. (2008) Remarkable

activation of an enzyme by (R)-pyrrolidine-substituted imidazolium alkyl PEG sulfate. *Adv. Synth. Catal.* **350**, 1954–1958
34. Sánchez-Ferrer, A., Rodríguez-López, J.N., García-Cánovas, F., and García-Carmona, F. (1995) Tyrosinase: a comprehensive review of its mechanism. *Biochim. Biophys. Acta* **1247**, 1–11
35. Espín, J.C., Varón, R., Fenoll, L.G., Gilabert, M.A., García-Ruíz, P.A., Tudela, J., and García-Cánovas, F. (2000) Kinetic characterization of the substrate specificity and mechanism of mushroom tyrosinase. *Eur. J. Biochem.* **267**, 1270–1279
36. Ibragimova, G.T. and Wade, R.C. (1998) Importance of explicit salt ions for protein stability in molecular dynamics simulation. *Biophys. J.* **74**, 2906–2911
37. Lutz-Wahl, S., Trost, E.-M., Wagner, B., and Fischer, M.L. (2006) Performance of D-amino acid oxidase in presence of ionic liquids. *J. Biotechnol.* **124**, 163–171