On the background of enhanced stability and reusability of enzymes in ionic liquids

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Abstract
The ability of ILs (ionic liquids) to provide an environment of increased stability and, in this way, improve the recyclability of enzymes has been studied. The description of this phenomenon is not easy; there are several approaches for explanation. In this mini-review, the results from different research groups are summarized, with the aim of explaining the strong stability effect of ILs on several enzymes. Spectroscopic methods (e.g. fluorescence and CD, IR spectroscopy, mass spectroscopy and NMR) and investigations of polarity and kosmotropicity of ions are promising methods. Since higher stability means that we may be able to reuse enzymes more times, the recyclability of enzymes was also in the focus. From this point of view, the advantages and disadvantages of applying monophasic or biphasic systems are discussed too, presenting the coupled techniques as well.

Role of cations and anions of ILs (ionic liquids) in protein stability
Many research groups have reported that ILs are able to stabilize enzymes and increase their activity and selectivity [1–6].

Park and Kazlauskas [7] correlated these behaviours to the polarity of ILs characterized by Reichardt’s polarity scale and hydrogen bond basicity. Solvents with higher polarity increase the solubility of polar substrates, leading to faster reactions and changes in selectivity. But the relationship between ILs and enzymes seems to be more complicated. Several other factors of ILs such as anion nucleophilicity, hydrogen-bond basicity, excipients, impurities, pH and the overall enzyme–substrate–medium relationship strongly influence the stability and activity. Zhao [8] found that, in aqueous solutions of hydrophilic ILs, enzyme stability, like inorganic salts, follows the Hofmeister series, but, in the case of hydrophobic or anhydrous hydrophilic ILs, the situation is even more complicated [8].

Generally, cations and anions in hydrophilic ILs play an equally important role in stabilizing proteins and enzymes in the presence of water. A combination of chaotropic (weakly hydrated) cations and kosmotropic (strongly hydrated) anions is the best solution. This kosmotropicity order of ions could be quantified by the viscosity β-coefficients and by several other parameters (such as hydration entropies, hydration volumes, heat capacity, NMR β’-coefficients and ion mobility) [8].

Hydrophobic or anhydrous hydrophilic ILs do not follow the Hofmeister series in their stabilizing property. They are acting in three different ways: (i) interact with substrates and/or products, (ii) take the essential water away from the enzyme as organic solvents do and (iii) have electrostatic interactions with the enzyme [8].

Enhanced stability of proteins caused by a hydrogen bond network of ILs
Baker et al. [9] studied protein conformations and thermostability within ILs by fluorescence spectroscopy. Monellin was selected as a test protein, because it is small, has no disulfide bridges, but still highly structured. This protein showed extreme stability against thermal inactivation in [bmpy]Tf2N [1-butyl-1-methylpyrrolidinium bis(trifluoromethane sulfonyl)imide]. Unfolding temperature was 105°C in [bmpy]Tf2N IL compared with 40°C in bulk water [9].

Three aqueous imidazolium ILs were used in the study of Turner et al. [10], including oxygen-containing functional groups attached to the cationic portion of the solvent, having various degrees of hydrophobicity: [bmm]Cl (1-butyl-3-methylimidazolium chloride), [eoemim]Cl (1-ethoxyethanol-3-methylimidazolium chloride) and [hopmim]Cl (1-(2-hydroxypropyl)-3-methylimidazolium chloride). Both cellulase from Trichoderma reesei [11] and serum albumin (bovine and human) are denatured in the presence of hydrophilic [bmm]Cl, caused by either ionic strength or specific binding to the protein surface. CD results also showed the highest loss in secondary structure with this IL. The second highest intensity and stability were measured in [hopmim]Cl, having one oxygen molecule, and the highest in [eoemim]Cl, with two oxygen molecules. The latter IL provides the greatest opportunity for hydrogen-bond interaction, which proved that increasing the hydrogen-bond

Key words: biphasic reaction, kosmotropicity, hydrogen-bond network, ionic liquid, monophasic reaction, recycling.
Abbreviations used: [bmm][Cl], 1-butyl-3-methylimidazolium tetrafluoroborate; [bmm][X], 1-butyl-3-methylimidazolium chloride; [bmm][F], 1-butyl-3-methylimidazolium hexafluorophosphate; [bmpy][Tf]N, 1-butyl-1-methylpyrrolidinium bis(trifluoromethane sulfonyle)imide; CALB, Candida antarctica lipase B; DAI, 1,3-dialkyl imidazolium; [eoemim]Cl, 1-ethoxyethanol-3-methylimidazolium chloride; [hopmim]Cl, 1-(2-hydroxypropyl)-3-methylimidazolium chloride, HRP, horseradish peroxidase; IL, ionic liquid; s.c.CO2, supercritical CO2; THF, tetrahydrofuran.

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capacity enhanced the stability of the protein in aqueous solution.

Longer half-life ($t_{1/2}$) was obtained during the thermal stability investigations of *Candida rugosa* enzyme in IL solvents (12.3 h in [bmim]PF$_6$ (1-butyl-3-methylimidazolium hexafluorophosphate) and 10.6 h in [omim]PF$_6$ (1-octyl-3-methylimidazolium hexafluorophosphate)) than in organic solvents. From n-hexane, benzene and dibutyl ether, the longest time was measured in n-hexane (6.5 h), but it was still half of that observed in [bmim]PF$_6$ [3]. Machado and Saraiva [4] found improved thermal stability of HRP (horseradish peroxidase) when the aqueous buffer contained 5 and 10% (v/v) [bmim]BF$_4$ (1-butyl-3-methylimidazolium tetrafluoroborate). The HRP kinetics was studied in the range of 45–90°C in phosphate buffer [4]. The stability of an immobilized esterase from *Bacillus stearothermophilus* (now *Geobacillus stearothermophilus*) considerably increased at 40°C, when the enzyme was incubated in [bmim]PF$_6$ and [bmim]BF$_4$ compared with organic solvents. The measured $t_{1/2}$ of 240 h in [bmim]PF$_6$ was 30- and 3-fold higher than that in n-hexane and MTBE (methyl t-butyl ether) respectively [12]. Approximately 100 orders of magnitude longer $t_{1/2}$ was observed for free CALB (*Candida antarctica* lipase B) in [emim]BF$_4$ (1-ethyl-3-methylimidazolium tetrafluoroborate). The enzyme was incubated in the presence of substrates (vinyl-butyrate and 1-butanol) in the mentioned IL and the $t_{1/2}$ was 2300 times greater than that observed when the enzyme was incubated in the absence of substrate (3.2 h) [13]. Ulbert et al. [3] have found direct correlation between solvent polarity and thermal stability based on laboratory experiments and the literature [7,12]. The higher the polarity of the solvent, the lower was the stability decrease. Alkoholysis between vinyl acetate and 2-phenyl-1-propanol was investigated by Maruyama et al. [14] with lipases from *Pseudomonas cepacia* and *Pseudomonas fluorescens*. Kinetic study revealed that Michaelis constant for 2-phenyl-1-propanol in [bmim]PF$_6$ was only half of that found in n-hexane, suggesting that the IL stabilized the enzyme–substrate complex.

Dupont [15] has reported that 1,3-dialkyl imidazolium ILs have an extremely ordered three-dimensional structure, where the cations and anions (connected together by hydrogen bonds) are forming an extended network. In the two-dimensional monomeric unit, one imidazolium cation is surrounded by at least three anions and vice versa (Figure 1). The strongest hydrogen bond was forming with the most acidic hydrogen in position 2. The other two hydrogen atoms in the imidazolium ring and N-alkyl radicals have almost the same acidity. In the case of octahedral PF$_6^-$ ion, the equatorial F atoms are preferred in linkage, while in tetrahedral BF$_4^-$ anion only three of four F atoms are involved in the hydrogen-bonding. In the three-dimensional structure, chains of imidazolium rings were observed by X-ray spectroscopy. The surface was studied by recoil spectrometry. The average orientation of these chains is a vertical position with the plane of the ring, neither cation nor anion enriched on the surface (Figure 2). The strength of hydrogen bonds follows the order CF$_3$CO$_2^-$ > BF$_4^-$ > PF$_6^-$ > BPh$_4^-$ based on IR and MS. The pure imidazolium ILs were found to be well organized in solid state by NMR spectroscopy. They can be described as $[(DAI)_x(X)_y]^{x+}[(DAI)_z(X)_w]^{x-}$, where DAI is the 1,3-dialkyl imidazolium cation and X the anion. In liquid phase, analogous structural patterns were present; ion-ion or atom–atom distances were similar in both solid and liquid states. Even in gas phases, the clusters of this network were maintained [15].
Figure 2 | The surface of [bmim]PF₆ is rich in alkyl side chains and the orientation of rings is vertical

Chains of imidazolium rings can be seen in the three-dimensional arrangement. Also, Coulomb interactions and van der Waals forces are present in solid and liquid phases, which are not presented in this schematic view.

Figure 3 | Enzymes with a small amount of water are firmly trapped in the network of ILs

With this monomolecular layer of water, ILs are able to maintain the active structure of each individual enzyme for a long time.

Dynamic structure–function relationships in enzyme stabilization were investigated by Lozano et al. [1,16] by using structural investigating methods, such as intrinsic fluorescence and CD spectroscopic techniques. CALB and α-chymotrypsin enzymes were strongly stabilized in [emim]Tf₂N [1-ethyl-3-methylimidazolium bis(trifluoromethane sulfonyle)imide] and [btma]Tf₂N [butyltrimethylammonium bis(trifluoromethane sulfonyle)imide] ILs, where t½ increased greatly with respect to those obtained from n-propanol and n-hexane organic solvents. These spectroscopic studies for both enzymes correlated to the stabilization phenomena with the maintenance of the native structure of enzymes. The net of ILs is able to maintain the active structure of an enzyme and prevent it from classical thermal unfolding (Figure 3). Enzymes and other molecules and macromolecules are trapped in the hydrogen bond network, and nanostructures with polar and non-polar regions are generated. Stabilization of these inclusion compounds is mainly due to the electronic and steric effects provided by the nanostructures of \((\text{DAI}_n)(X)_{n-}\) and \((\text{DAI}_n(X)_n)^{±}\)-type ILs [15].

Monophasic reactions compared with biphasic reactions in the recycling of enzymes

Although enzymes show potentiality for recycle use in ILs [17–20], monophasic reactions have one disadvantage. After each cycle, the product and unchanged substrate(s) have to be extracted with some kind of organic solvent. Yuan et al. [17] extracted (±)-menthol from [bmim]PF₆ with hexane during the recycling process of C. rugosa lipase [17]. Moreover, in a trans-esterification reaction using vinyl acetate as the acyl donor, the inhibiting acetaldehyde oligomer accumulated in [bmim]PF₆ caused a gradual decrease in reactivity of CALB [18]. This problem was solved by changing the IL to [bdmim]BF₄ (1-butyl-2,3-dimethylimidazolium tetrafluoroborate). In this work, enzyme and IL were reused together, but the product and unchanged alcohol were also extracted with an organic solvent, diethyl ether [19]. Enzymes were much more thermostable in [bmim]BF₄ than in acetonitrile or THF (tetrahydrofuran) in hydroxynitrile lyase-catalysed monophasic trans-cyanation reactions. Both enzymes (from Prunus amygdalus and Manihot esculenta) were incubated in IL and organic solvents at 50 and 80°C. After 24 h at 80°C, the relative activity in [bmim]BF₄, acetonitrile and THF were 71, 21 and 9% respectively.

Biphasic reaction mixtures are widely used in biotransformations of hydrophobic compounds. In this system, usually an aqueous working phase and an organic extractive phase are present. Nowadays, the role of ILs in biphasic reactions is to replace the organic solvents [2,4,21–26], making the system environmentally friendly and enhancing the activity and selectivity of enzyme. A beneficial side effect is the increased stability, indicating longer t½. Just two research groups investigated the stability of enzymes in biphasic systems [4,21]; moreover, the reusability was studied only by Kafizik et al. [21]. In this work, the addition of 25% (v/v) [bmim]MeSO₄ (1-butyl-3-methylimidazolium methyl-sulfate) suppressed the secondary hydrolysis of the product N-acetyl-lactosamine and doubled the yield. The applied β-galactosidase from Bacillus circulans was able
to be reused several times without loss of activity in this trans-glycosylation reaction.

The function of IL was reversed in the research of Hernández et al. [26], where the IL was the working phase and s.c.CO₂ (supercritical CO₂) was the extractive phase. They have presented a biocatalytic process for CALB-assisted enzymatic synthesis of butyl propionate. The immobilized enzyme was coated with IL, which provided an adequate microenvironment for the enzyme, whereas s.c.CO₂ was applied as an effective extractant, protecting the product from any cross-contamination with IL. The IL-coated enzyme was immobilized on the surface of ceramic membranes. This combination of membrane technique with biocatalysis was successful, resulting in a recirculating enzymatic bioreactor.

A similar useful technique is demonstrated by our research [27], where [bmim]PF₆ and the excess of alcohol substrate formed a biphasic system. CALB was used as a catalyst in the enzymatic production of isoamyl acetate from acetic acid and 3-methylbutan-1-ol. The lower phase contained the IL and enzyme, whereas the main part of the product, the unchanged acetic acid, the excess of 3-methylbutan-1-ol and side product, water, were located in the upper phase. Both phases were working phases, but only the alcoholic phase was the extractive phase. The upper phase was removed for separation at the end of the reaction, whereas the lower phase was successfully reused ten times, by simply adding a fresh substrate mixture. That is why this process resulted in no loss in the weight of enzyme and IL, which were the most expensive compounds of the reaction mixture.

Conclusions

It is important to relate protein stability data to protein activity data and to understand the effects of various ILs on biomacromolecules. Polarity, kosmotropicity investigations and various spectroscopic studies were used to find an explanation for the strong stabilizing ability of ILs on enzymes. All of the methods proved that ILs are able to generate and maintain an excellent non-conventional environment for proteins. That is why they could be used as outstanding solvents in biocatalytic transformations. The stability, activity, selectivity and enantioselectivity of the enzyme are highly affected by the nature of IL, which can be fine tuned by choosing the appropriate cation and anion. Generally, they are used in monophasic reactions, but in few cases biphasic (mainly buffer–IL) systems are also applied. Biphasic and multiphase systems were proven to provide a higher possibility of more economic processes, by giving the chance for easy recovery of enzymes (and in some cases ILs). ILs are effective green alternatives for organic solvents, especially those having an ECOENG licence, which indicates their ecologically friendly character.

References


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