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# Ionization basis for activation of enzymes soluble in ionic liquids

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ABSTRACT

*Background:* The complex interactions between electrolytes and proteins have been studied for more than a century. However, understanding is not yet complete and does not provide a basis for predicting the activity of enzymes in ionic media. The use of ionic liquids (ILs) as reaction medium has opened up new opportunities for better understanding of the mechanism of enzymatic catalysis. Although a number of properties of ILs have been correlated with enzyme function, these relationships are not completely understood at a molecular level.
*Methods:* We propose that ILs must be able to promote ionization of protein ionizable groups in order to dissolve active enzymes. The biocompatible IL need to possess a functional group with large donor number and acceptor number in both cationic and anionic units, each of which is based on a high dielectric constant lead structure. We designed and synthesized two series of ILs and determined their ionizing–dissociating abilities and activities of lipases soluble in these new ILs.
*Results:* The results showed that the ionizing–dissociating abilities of ILs paralleled the catalytic activity trend of lipases dissolved in the ILs. The activities of lipases soluble in the newly designed ILs were comparable to those in water.
*Conclusions:* We can conclude that ionizing–dissociating abilities of an IL can be used as a basis for predicting the activity of enzymes soluble in the IL.
General significance
Ionization basis for activation of enzymes gives a deeper understanding of the behavior of enzymes in non-aqueous media at a molecular level.



## 1. Introduction

The complex interactions between electrolytes and proteins have been studied for more than a century [1,2]. However, understanding is not yet complete and does not provide a basis for predicting the activity of enzymes in ionic media. The use of ionic liquids (ILs) as reaction medium has opened up new opportunities for better understanding of the mechanism of enzymatic catalysis. Up to now, a number of properties of ILs have been correlated with enzyme function, such as anion nucleophilicity [3,4], hydrophobicity [3–6], and kosmotropicity [7,8], but these relationships are not completely understood at a molecular level. Therefore, it is necessary to develop a theoretical basis for predicting activity of enzymes in ILs.

To understand the behavior of enzymes in ILs at a molecular level, it is first desirable to dissolve enzymes in the solvents. For soluble catalysts, there are many relatively simple spectroscopic and other techniques for obtaining accurate information at a molecular level. Parker et al. [9] reported that cation solvating power of solvents is well measured by solvent donor properties (donor number, DN), while anion solvating power of solvents is well measured by solvent acceptor properties (acceptor number, AN). Water is a strongly amphoteric solvent with large DN and AN values (DN = 33.0, AN = 54.8) [10]. Enzymes are polyelectrolytes and can hence readily dissolve in water. And we might expect that active enzymes will tend to dissolve in water-like solvents with a hydroxyl-functionality. Walker and Bruce [11,12] were the first to describe the design of an IL in which the cation contained a hydroxyalkyl group to stabilize the dissolved enzyme. The incorporation of a hydroxyl-functionality in the solvents for dissolving enzymes with modest to good catalytic activity was also demonstrated by several groups [13–15] including our own [16–18]. ILs based on other functional groups such as $NO_3^-$, lactate, $EtSO_4^-$, and $CH_3COO^-$ may also dissolve enzymes, however, most of them cause severe enzyme deactivation [3, 19–21]. What causes activation or deactivation of enzyme dissolved in ILs?

The behavior of enzymes is strongly dependent on the protonation state of their ionizable groups [17,22–27]. Enzymes catalyze reactions using a variety of ionizable groups functioning as electrophiles, nucleophiles, or general acid/base catalysts. For an ionizable group (like —COOH), the ionization process can be broken conceptually into two steps (Fig. 1). In the first step, solvent molecules act as a Lewis base (electron pair donor) to the H atom and as a Lewis acid (electron pair

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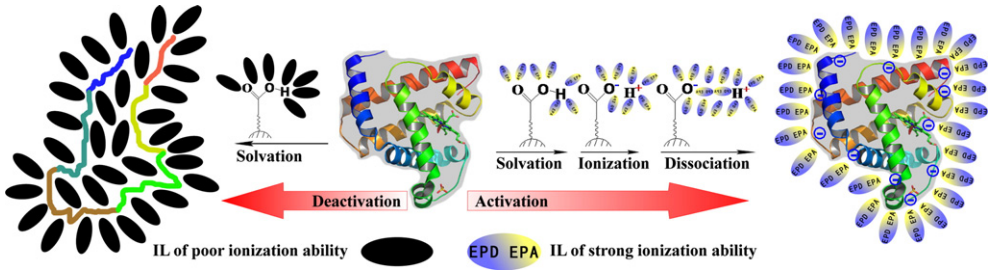



**Fig. 1.** Activation and deactivation of enzyme dissolved in IL.

acceptor) to the O atom, and ionize the H and O atoms, resulting in the breaking of the O—H covalent bond and the formation of an ion pair $(O^-,H^+)_{solv}$ in which both ions now interact with solvent. The ionization step is a function of the DN and AN of the solvent. Higher values mean that solvent has higher ability to ionize polar O—H covalent bond and to stabilize the formed ions. In other words, strongly amphoteric solvents are good ionizing solvents. In the second step, the ion pair $(O^-,H^+)_{solv}$ dissociates into free ions. The dissociation process is easy if the solvent has a high relative permittivity (dielectric constant, $\varepsilon_r$).

According to the above analysis, solvents need to have water-like ionizing–dissociating abilities in order to dissolve active enzymes (In this paper we will refer to such solvents as "biocompatible"). Recently, we have proposed basic principles for biocompatible organic solvent design [18], which involve the introduction of hydroxyl groups into high dielectric constant compounds. The main objective of the solvent design is to improve ionizing–dissociating abilities of the studied solvent. The great success in designing biocompatible organic solvents in our previous studies [17,18] inspired us to design and synthesize biocompatible ILs. The basic principles for biocompatible IL design are:

1. The design process starts with a known high dielectric compound used as a lead, so that the new IL has strong dissociating ability. Among common organic compounds, we found three classes of compounds that meet the demand, which can be used as lead compounds for construction of ILs. They are represented by dimethyl sulfone ($\varepsilon_r$ = 47.4 at 383 K), triethanolamine ($\varepsilon_r$ = 29.4), and imidazole ($\varepsilon_r$ = 23.0) [28].

2. Starting from the structure of the selected lead, the IL is designed to have strong ionizing ability. This is the functionalization stage, and a functional group with large values of both DN and AN is built into the molecular structure of the lead compound. Hydroxyl group possesses high DN and AN values, therefore, hydroxyl-functionalization of the lead with high dielectric constant value may be advantageous for ionizing and dissociating enzyme functional groups. The incorporation of a hydroxyl-functionality in the high dielectric organic solvents for ionizing and dissociating enzymes with high catalytic activity was demonstrated by our group [17,18].

3. The acid-base properties of the IL are important issues to consider because protonation changes caused by Brønsted-acidic or basic ions may result in enzyme deactivation [29]. Thereby, the acid-base properties of the newly designed IL need proper control for enzymes by selection of appropriate cation and anion types.

Biocompatible ILs were reported by several groups [13,30–38] including our own [16]. Cations of these ILs do have a functional group with high AN and DN values, but their anions do not. Generally, both the cation and anion of an IL function cooperatively to affect the enzyme activity. We speculate that incorporation of a hydroxyl-functionality in both cationic and anionic units with high dielectric constant values may be advantageous for dissolving and ionizing enzymes. In addition, the newly designed ILs need to provide suitable acid-base environment for the enzymes. Enzymes tested in this work are lipases from *Candida antarctica* (CAL) and *Pseudomonas cepacia* (PCL) functioning in neutral environment, therefore, the newly designed ILs should be neutral. Following this rationale, we designed and synthesized two series of ILs (Scheme 1), hydroxyalkyl imidazolium hydroxyalkyl sulphonate ($[C_mOHMIM][HOC_nSO_3]$) and hydroxyalkyl tri(hydroxyethyl) ammonium hydroxylalkyl sulphonate ($[C_mOHTEA][HOC_nSO_3]$). We then determined their ionizing–dissociating abilities and their compatibility with lipase activity, in order to test the above hypothesis.

(A) Anions based on sulfoxide (B) imidazolium-type cations and (C) Ammonium-type cations.

## 2. Materials and methods

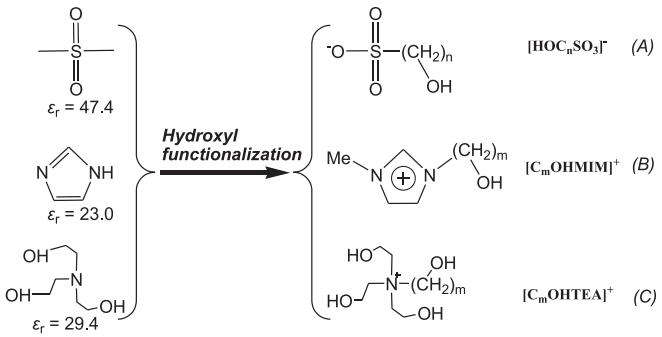
### 2.1. General

*Candida antarctica* lipase (CAL, 1.5 U $mg^{-1}$) and *Pseudomonas cepacia* lipase (PCL, 30 U $mg^{-1}$) were purchased from Sigma and used as supplied. All other chemicals and reagents were of analytical grade from Sigma. Ethyl butyrate, 1-butanol, 1-methylimidazole, triethanolamine, 2-chloroethanol, 3-chloro-1-propanol were dried by 3A molecular sieves before use. $[BMIM]NO_3$ was synthesized according to published procedures and checked for the absence of chloride and acid [16].

The $^1H$ NMR and $^{13}C$ NMR spectra were obtained on a Brüker AV-400 or AV-600 Fourier transform NMR spectrometer. NMR spectra were referenced to tetramethylsilane. The mass spectra were analysed on the Agilent 6130 Dual Source ESI/APCI mass spectrometer or AB SCIEX Triple TOF 5600+.

### 2.2. Synthesis of ILs

Synthesis of 1-(2-Hydroxyethyl)-3-methyl-imidazolium chloride ($[C_2OHMIM]Cl$): $[C_2OHMIM]Cl$ was prepared by the method as described in the literature with minor modifications [39]. 2-Chloroethanol (64 mL, 0.95 mol) and 1-methylimidazole (50 mL, 0.63 mol) were added to a round-bottomed flask fitted with a reflux condenser for 24 h at 353 K with stirring. Ethyl acetate (50 mL) was added to extract the excess 2-chloroethanol with thorough mixing and white crystals were obtained. The crystals were washed by ethyl acetate twice and then dried at 323 K under a vacuum for 2 d. (yield 92.5%). $^1H$ NMR (400 MHz, $D_2O$, 298 K): 3.92–3.94 (overlapped, 5H), 4.34 (t, J = 5.38 Hz, 2H), 7.50 (s, 1H), 7.55 (s, 1H), 8.80 (s, 1H); $^{13}C$




**Scheme 1.** Design of biocompatible ILs.