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Effect of the physicochemical properties of binary ionic liquids on lipase activity and stability

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ABSTRACT

In the present study, the lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate is used as a model reaction to determine the activity and stability of *Candida rugosa* lipase in binary ionic liquids (ILs). The binary ILs consist of hydrophobic 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim]PF_6) and a small amount of hydrophilic 1-butyl-3-methylimidazolium nitrate ([Bmim]NO_3) or 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([Bmim]CF_3SO_3) or 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4). The activity and the stability of lipase are first correlated with the physicochemical properties of the binary ILs. In the three binary IL systems, both the hydrophilicity and the polarity of the systems increase with the increase of the content of hydrophilic ILs (HILs). At a fixed concentration of HIL, they vary in a descending order of [Bmim]PF_6/[Bmim]NO_3 > [Bmim]PF_6/[Bmim]CF_3SO_3 > [Bmim]PF_6/[Bmim]BF_4. This order is in contrast with the order of the lipase conformation stability, i.e., the higher the polarity of ILs, the more unstable the lipase conformation. However, both the activity and the stability of lipase depend on the type and the content of the HIL in binary ILs, showing a complex dependency. Analysis shows that the catalytic performance of lipase in the binary ILs is affected not only by the direct influence of the ILs on lipase conformation, but also through their indirect influence on the physicochemical properties of water. The present study helps to explore binary IL mixtures suitable for lipase-based biocatalysis.

1. Introduction

Room temperature ionic liquids (RTILs) are liquid organic salts at or below room temperature. Compared with molecular solvents, ionic liquids (ILs) have many advantages such as high chemical and thermal stability, low volatility and nonflammability [1,2]. As green solvents, ILs are able to dissolve many inorganic/organic compounds and macromolecular polymers [1–3]. The physicochemical properties of an IL depend on its component ions, and an appropriate combination of different ions could result in suitable media for the dissolution of a specific compound [4–6]. In addition, it is feasible to tune the physicochemical properties of an IL by introducing another IL [7–10]. Both strategies further demonstrate the advantage of ILs as "designable" media and extend the scope of the application of ILs as media. The aforementioned advantages of RTILs render them attractive in the fields of separation [11] and biocatalysis and biotransformation [12–14].

As early in 2000, Erbeldinger et al. reported thermolysin-catalyzed synthesis of *Z*-aspartame in 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim]PF_6) for the first time [15]. Since then, studies of enzyme-catalyzed reactions in ILs have attracted considerable attention [1,12–14]. Lipase belongs to hydrolases. It catalyzes the synthesis, hydrolysis, and transesterification of fatty acid esters [16,17]. The active site of lipase is constituted by the amino acid residues Ser, His, and Glu/Asp. Most lipases have a "lid" on their surface. Upon contacting with a hydrophobic surface or an oil–water interface, the "lid" opens and moves away for the access of a substrate to the active site, forming an acyl-enzyme intermediate. These lipases are therefore referred to surface activity enzymes [17,18]. In addition, lipase has rich Lys residues on its surface, which lays the foundation for the characterization of lipase conformation using spectral probe techniques [19]. For the catalytic performance of lipase in ILs, it has been shown that lipase is catalytically active in conventional hydrophobic ILs with anions being hexafluorophosphate (PF_6^-) or bis(trifluoromethanesulfonyl)imide (Tf_2N^-), but it cannot be dispersed on molecular level. In conventional hydrophilic ILs

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(HILs) with anions being trifluoromethanesulfonate ($CF_3SO_3^-$) and nitrate (NO_3^-), lipase usually has no activity even though it can be dissolved in them [20–23]. For example, Kaar et al. reported that lipase showed transesterification activity in [Bmim]PF_6 but no detectable activity was observed in imidazolium-based ILs containing acetate (Ac^-) and NO_3^-, respectively [20]. For lipase-catalyzed reactions, not only the compatibility between media and lipase, but also the solubility of substrates should be taken into account to enhance the efficiency of the reactions. In other words, a suitable IL should maximize the solubility of substrates in it while minimize its negative impact on lipase activity. Studies have shown that in addition to water-in-IL microemulsions [24–26], hydrophilic/hydrophobic IL mixtures could to some extent improve the efficiency of the enzymatic reactions. For instance, Lee et al. found that the yield of 6-*O*-lauroyl-*D*-glucose (catalyzed by lipase) could be improved when a mixture of 1-butyl-3-methylimizazolium trifluoromethanesulfonate ([Bmim]CF_3SO_3) and 1-butyl-3-methylimizazolium bis(trifluoromethanesulfonyl)imide ([Bmim]Tf_2N) (v/v, 1:1) was used as medium [27]. Coincidentally, Fischer et al. also found that in a binary IL mixture of 1-ethyl-3-methylimidazolium methanesulfonate ([Emim]CH_3SO_3) and 1-butyl-4-methylpyridinium hexafluorophosphate ([Bmpyr]PF_6), the lipase-catalyzed syntheses of 6-*O*-linoleyl-α-*D*-maltose and 6′-*O*-linoleyl-α-*D*-maltose result in better yields [28]. In these studies, however, the mechanism of the effect of binary ILs on the enzymatic reactions has not been addressed. So it is of great importance to investigate the composition dependent properties of binary IL mixtures and address how these properties influence enzyme, substrate, and enzymatic reaction.

In the present study, [Bmim]PF_6 as the main body was used to form binary IL mixtures with 1-butyl-3-methylimidazolium nitrate ([Bmim]NO_3), [Bmim]CF_3SO_3, or 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4). The hydrophilicity and the polarity of the binary mixtures were characterized. The activity and the stability of lipase in these binary IL mixtures were investigated based on the lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate (*p*-NPB). The relevant mechanisms were discussed in detail.

2. Experiment

2.1. Materials

Lipase from *Candida rugosa* (1170 U mg^{-1}), *p*-NPB and fluorescein isothiocyanate (FITC) were obtained from Sigma Co. (St. Louis, USA). ILs ([Bmim]PF_6, [Bmim]NO_3, [Bmim]CF_3SO_3, and [Bmim]BF_4) were purchased from Shanghai Chengjie Chemicals Co. Ltd., China. Pyrene-1-carboxaldehyde (PyCHO), tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (36%), ethanol, and dimethyl sulfoxide (DMSO) were provided by Sinopharm Chemical Reagent Co. Ltd., China. All chemical reagents were of analytical grade. All ILs were dried under vacuum before use. Triply distilled water was used throughout the experiments.

2.2. Methods

2.2.1. Characterization of hydrophilicity of binary ILs

To a binary IL mixture containing a certain amount of an HIL (its concentration in mol L^{-1} was defined as the mole number of the HIL divided by the total volume of the resulting binary system) was added triply distilled water drop by drop. After each addition of water, the resulting mixture was thoroughly mixed and then equilibrated at 30 °C. The above steps were repeated until the resulting system became turbid. The maximum water content (v/v, %) solubilized in the binary ILs was recorded.

2.2.2. Characterization of polarity of binary ILs

An aliquot of PyCHO (9.5 mM, prepared with ethanol) was added to a binary IL mixture. After thorough mixing, the mixture was dried in a vacuum oven at 70 °C for 24 h to remove ethanol. The final concentration of PyCHO was 4.8×10^{-5} M. Fluorescence measurements were carried out on an FL-4500 fluorescence spectrofluorimeter. The excitation wavelength was fixed at 365 nm. The slits for excitation and emission were 5.0 and 2.5 nm, respectively. The fluorescence spectra were collected over 400–480 nm with a scan speed of 600 nm/min.

2.2.3. Lipase activities in binary ILs

HIL concentration-dependent lipase activity. The lipase activity was assayed spectrophotometrically using *p*-NPB as a substrate. Specifically, an aliquot of lipase stock solution (prepared with 20 mM Tris–HCl buffer, pH 7.4) was added to a preprepared binary IL mixture. After thorough mixing, the resulting solution was equilibrated at 30 °C for 5 min. Then a 7.0 μL aliquot of pH 7.4, 20 mM Tris-HCl buffer and a 10.0 μL aliquot of *p*-NPB stock solution (dissolved in [Bmim]PF_6) were added to the solution to initiate the hydrolysis reaction. After quick mixing, the change in absorbance of the hydrolytic product *p*-nitrophenol (*p*-NP) with time at 340 nm ($\varepsilon = 5690\ L\ mol^{-1}\ cm^{-1}$) was recorded. The final concentrations of lipase, *p*-NPB and water were 0.9 μM, 4 mM, and 1 M, respectively. The reaction temperature was set at 30 °C.

Water content-dependent lipase activity. Briefly, an aliquot of lipase stock solution (prepared with 20 mM Tris-HCl buffer, pH 7.4) was added to a preprepared binary IL mixture containing a fixed concentration of an HIL. After thorough mixing, the resulting solution was equilibrated at 30 °C for 5 min. Then, a required amount of Tris-HCl buffer (pH 7.4, 20 mM) and a 10.0 μL aliquot of *p*-NPB stock solution (dissolved in [Bmim]PF_6) were added to the solution to initiate the hydrolysis reaction. After quick mixing, the sample was transferred into the chamber of the spectrophotometer to monitor the time-dependent change of the absorbance of *p*-NP at 30 °C. The final concentrations of lipase, *p*-NPB and water were 0.9 μM, 4 mM and 1 M, respectively.

2.2.4. Lipase labeling with FITC

An 80 μL aliquot of 0.0246 M of FITC solution (prepared with DMSO) was added to 1.0 mL of lipase solution (0.0164 g mL^{-1}, prepared with 20 mM, pH 7.4 Tris–HCl buffer). After mixing, the solution was kept in dark at 4 °C for 24 h. Subsequently, the solution was transferred to an ultrafiltering tube (10 kDa, Amicon Ultra-15, Millipore Corp. MA) to remove all small molecules under centrifugation (6000 rpm, 10 min). The upper layer concentrated solution was then washed with 20 mM, pH 7.4 Tris-HCl buffer, followed by centrifugation. The steps were repeated until the lower layer solution had no significant fluorescence signal. Finally, the FITC-lipase solution (the upper solution) was diluted to a final concentration of 0.24 mM and stored in dark at 4 °C.

2.2.5. Fluorescence spectroscopy of FITC-lipase

FITC-lipase incubated for 5 min. An aliquot of FITC-lipase stock solution was added to a preprepared binary IL mixture. After thorough mixing, the sample was equilibrated in dark at 30 °C for 5 min. The fluorescence measurements were carried out on an FL-4500 spectrofluorimeter. The final concentration of FITC-lipase was 0.9 μM. The excitation wavelength was set at 495 nm. The slits for excitation and emission were 5.0 and 10.0 nm, respectively. The emission spectra were collected over 510–600 nm with a scan speed of 600 nm/min.

FITC-lipase incubated for different time. To a series of preprepared binary IL mixtures containing a fixed concentration of an HIL was separately added an aliquot of FITC-lipase stock solution. The resulting solutions were incubated in dark at 30 °C for 0, 1, 2, 5,