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# Enhanced activity and stability of ionic liquid-pretreated lipase

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**Abstract**

The activity and stability of *Mucor javanicus* lipase pretreated with various ionic liquids (ILs) were investigated. The results show that the activity and stability of lipase pretreated with ILs were higher than those of untreated lipase for the hydrolysis reaction in an aqueous medium. The activities of lipase pretreated with ILs such as [Bmim][$PF_6$], [Emim][$Tf_2N$], [Bmim][$BF_4$] and [Emim][$BF_4$] were 1.81, 1.66, 1.56 and 1.60 times higher than that of untreated lipase, respectively. Furthermore, activities of lipase in ILs were well maintained even after 7 days of incubation in ILs at 60 °C, while untreated lipase in phosphate buffer was fully inactivated only after 12 h of incubation at the same temperature. These results suggest that pretreatment of lipase with ILs might form IL-coated lipase which causes the structural change of lipase, and thus, enhances the activity and stability of lipase in aqueous solution.


*Keywords:* Lipase; Ionic liquids; Hydrolysis; Activity; Stability

## 1. Introduction

It has been reported that enzyme pretreated with organic solvents (hexane, toluene, chloroform, *etc*.) led to an enhancement in enzyme activity and stability [1,2]. It was also found that organic solvent-pretreated enzymes do not lose their activities even at higher temperature compared to those at their optimum temperature in water. However, organic solvents have several unfavorable characteristics such as evaporable, flammable, toxic properties, *etc*. As a result, ionic liquids (ILs) were paid attention as an alternative [3,4]. ILs were known as salts and therefore are entirely composed of ions which are liquids below 100 °C or typically close to room temperature. The interest as green chemicals resides in their high thermal stability and very low vapor pressure, which can be used to resolve the problem of volatile organic solvent emission into the atmosphere [4,5].

Recently, several papers regarding the utilization of ILs in bioconversion have been published [6–8]. Erbeldinger et al. used [Bmim][$BF_4$] for the thermolysin-catalyzed synthesis of Z-aspartame which has shown high enzyme activity and stability in [Bmim][$BF_4$] [9]. Kaar et al. have examined the lipase-catalyzed transeseterification of divinyl adipate and 1,4-butadiol [10]. It was shown that the activity of free lipase in [Bmim][$PF_6$] was higher than in hexane. Lozano et al. have investigated the stability enhancement of free *Candida antartica* lipase in ILs. They showed that the half-life time of the enzyme in ILs was increased compared to that in the assayed organic solvents [11]. Furthermore, enzyme stability was significantly increased more than 2300 times when enzyme was operated with substrates in 2% (v/v) water-contained [Bmim][$PF_6$] compared to that observed in 2% (v/v) water-contained [Bmim][$PF_6$] in the absence of substrate. It was explained by the prevention of the rapid enzyme deactivation through the specific interaction of substrates with the active site of the enzyme. Ulbert et al. also reported the enhancement in thermal stability of *C. rugosa* lipase in ILs [12]. Higher half-life times of lipase were observed in [Bmim][$BF_4$] and [Omim][$BF_4$] compared to those obtained in the common organic solvents such as hexane, benzene and dibutylether, at the same enzyme hydration.

*Abbreviations:* [Bmim][$BF_4$], 1-butyl-3-methylimidazolium tetrafluoroborate; [Emim][$BF_4$], 1-ethyl-3-methylimidazolium tetrafluoroborate; [Bmim][$PF_6$], 1-butyl-3-methylimidazolium hexafluorophosphate; [Emim][TfO], 1-ethyl-3-methylimidazolium trifluoromethanesulfonate; [Hmim][TfO], 1-hexyl-3-methylimidazolium trifluoromethanesulfonate; [Emim][$Tf_2N$], 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide; DMF, dimethylformamide; PB, phosphate buffer

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In the present study, the activity and stability of *Mucor javanicus* lipase pretreated with various ILs were investigated for *p*-nitrophenyl butyrate hydrolysis reaction in an aqueous medium.

## 2. Experimental

### 2.1. Materials

Lipase from *M. javanicus* was purchased from Sigma. [Bmim][$BF_4$] was purchased from Merck (Germany) and [Emim][$BF_4$], [Bmim][$PF_6$], [Emim][TfO], [Hmim][TfO] and [Emim][$Tf_2N$] were kindly supplied by C-Tri (Korea). *p*-Nitrophenyl butyrate, used as the substrate in the hydrolysis reaction, and dimethylformamide (DMF) were purchased from Sigma–Aldrich. Sodium phosphate was purchased from Oriental Chemical Industry (Korea). Water bath (Vision Company, Korea), circulator (Cole-Parmer Instrument Company, USA) and UV/vis spectronic (Milton Roy Company, USA) instrument were used for this experiment.

### 2.2. Treatment of lipase with ionic liquids

Lipase of 10 mg was suspended in 1 ml of various ILs for 20 min. Then, the mixture of lipase and ILs was used in the hydrolysis reaction.

### 2.3. Enzyme activity

Enzyme activity was determined by measuring the increase in absorbance at 400 nm produced by the release of *p*-nitrophenol during the hydrolysis of 0.5 mM *p*-nitrophenyl butyrate in 20 mM phosphate buffer (PB) at pH 6.5 and 37 °C. The reaction was started by adding 10 μl of lipase solution or lipase/ILs mixture which contains 0.02 mg of lipase to 990 μl of substrate solution and carried out at 37 °C in water bath with shaking at 200 rpm or in circulator with stirring at 300 rpm. To investigate the effect of different concentrations of ILs in the reaction medium on enzyme activity, the reaction was started by adding 2, 5, 10, 20 and 30 μl of lipase/ILs into substrate solution to make 0.2, 0.5, 1, 2, and 3% (v/v) of lipase/ILs in 1 ml of the reaction medium. Lipase concentration in reaction medium was kept at 0.02 mg/ml throughout the experiment.

### 2.4. Enzyme stability

Lipase was suspended in PB or various ILs, and then incubated at 60 °C. At regular time intervals, the enzyme was withdrawn and the activity was measured as described above.

## 3. Results and discussion

The hydrolytic activities of IL-pretreated lipase in aqueous media with various concentrations of [Emim][$BF_4$] are shown in Fig. 1. To compare enzyme activities between [Emim][$BF_4$]-pretreated lipase and untreated lipase with [Emim][$BF_4$] in reaction, free lipase in the reaction medium with different concentrations of [Emim][$BF_4$] was prepared by adding lipase solution and [Emim][$BF_4$] into the reaction medium as additives. The activities of IL-pretreated lipase were much higher than those of free lipase at various ILs concentrations (0.2–3%) in the reaction medium. The highest activity was obtained when [Emim][$BF_4$]-pretreated lipase was dissolved in the reaction medium with 0.5% (v/v) of [Emim][$BF_4$]. Since there was no separation of enzyme from ILs after treatment in this study, the effect of ILs on the hydrolysis reaction was examined. ILs generally are more viscous than water and common organic solvents ([Emim][$BF_4$] 66.5 cP at 20 °C, [Bmim][$BF_4$] 154 cP at 20 °C; $H_2O$ 0.9 cP at 25 °C; toluene 0.59 cP) [13]. The high viscosity limits the rate of diffusion of solute molecules, and thus, may limit the rate constants of bimolecular chemical reactions. To find out whether the viscosity is related to lower enzyme activities in higher ILs concentration, the reaction was carried out in the reaction block with stirrer to provide perfect mixing. As shown in Table 1, there were little differences in enzyme activities in reaction media with over 1% of [Emim][$BF_4$] in between the water bath and the reaction block as reaction system (2.66% and 4.20% for 1% and 3% of [Emim][$BF_4$]), respectively. How-

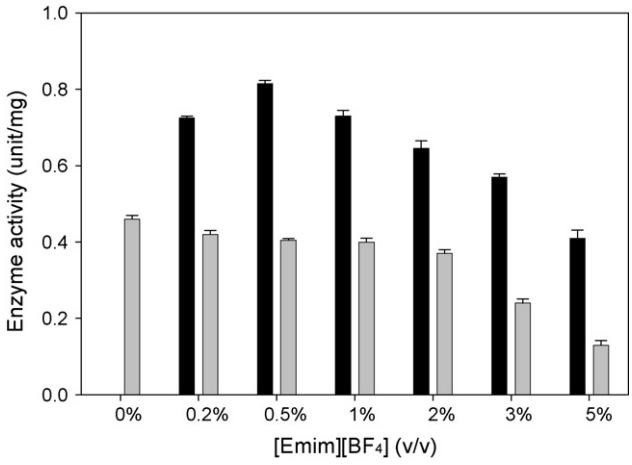


Fig. 1. The effect of [Emim][$BF_4$] concentration in the reaction medium on activity of [Emim][$BF_4$]-pretreated enzyme ([Emim][$BF_4$]-pretreated lipase (black bar), free lipase with [Emim][$BF_4$] in reaction mixture as additive (grey bar)).

Table 1
Activity of lipase pretreated with [Emim][$BF_4$] or [Bmim][$BF_4$] at different concentrations of ILs in the hydrolysis reaction

| Ionic liquids | Concentration (% (v/v)) | Enzyme activity (unit/mg) | |
|---|---|---|---|
| | | Water bath | Circulator |
| [Emim][$BF_4$] | 0.2 | 0.73 | 0.89 |
| | 0.5 | 0.82 | 0.90 |
| | 1.0 | 0.73 | 0.75 |
| | 2.0 | 0.65 | 0.64 |
| | 3.0 | 0.57 | 0.60 |
| [Bmim][$BF_4$] | 0.2 | 0.79 | 0.86 |
| | 0.5 | 0.80 | 0.88 |
| | 1.0 | 0.77 | 0.84 |
| | 3.0 | 0.45 | 0.62 |

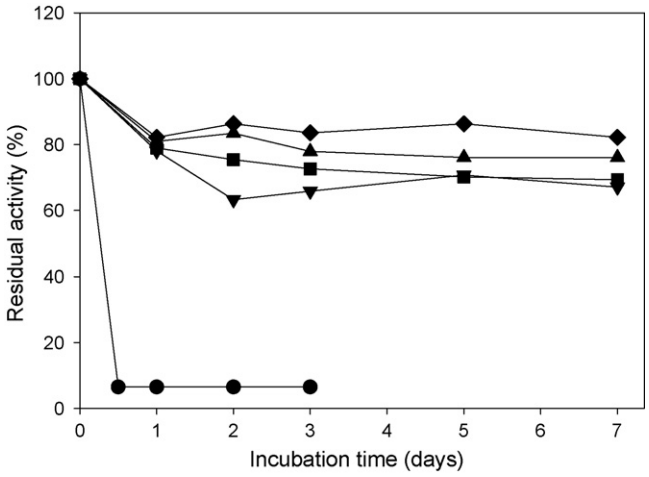


Fig. 2. Stability of [Emim][$BF_4$]-pretreated lipase at 0.5% (▲), 1% (♦), 3% (■) and 5% (▼) of [Emim][$BF_4$] in reaction mixture and untreated lipase in phosphate buffer (●) at 60 °C. The enzyme activity was measured in aqueous solution after incubating lipase in pure [Emim][$BF_4$] at 60 °C for the selected times.

ever, much higher enzyme activity (26% increase) was observed in the reaction block compared to that in the water bath at 3% of [Bmim][$BF_4$] in the reaction medium. These results imply that the viscosity of ILs does not seriously affect enzyme activity in the range of IL concentrations in reaction media tested except 3% of [Bmim][$BF_4$]. Table 1 also shows that the highest enzyme activity was obtained at 0.5% of ILs in the reaction medium as observed in Fig. 1. It implies that there exists an optimum concentration of ILs (0.5% in this case) in reaction medium for enzyme activity.

The thermal stability of lipase in ILs was investigated by incubating *M. javanicus* lipase in [Emim][$BF_4$] at 60 °C. Fig. 2 shows that the residual activities of lipase were well maintained around 78%, 83%, 72%, and 68% at 0.5%, 1%, 3%, and 5% (v/v) of [Emim][$BF_4$] in reaction medium, respectively, even after 7 days of incubation in [Emim][$BF_4$] at 60 °C. That of untreated lipase was 6% only after 12 h of incubation at 60 °C which indicates the denaturation of untreated lipase by high temperature (Fig. 2). A possible explanation for enhanced activity and stability of [Emim][$BF_4$]-pretreated lipase is the change on the secondary structure of lipase in ILs. De Diego et al. have demonstrated that the stabilization of lipase by ILs seems to be related to the observed evolution of α-helix and β-sheet secondary structure of the enzyme, leading to a more compact enzyme conformation able to exhibit more activity and stability [14].

Since the optimal concentrations of [Emim][$BF_4$] in reaction medium for the activity and stability of lipase were found to be 0.5% and 1% (v/v), respectively, lipase was pretreated with various ILs to investigate the effect of change in ILs structure on enzyme activity. Fig. 3 shows that the activity of lipase pretreated with ILs were higher than that of untreated lipase in the hydrolysis reaction in an aqueous medium. The activities of lipase pretreated with [Bmim][$BF_4$], [Bmim][$PF_6$], [Emim][$BF_4$] and [Emim][$Tf_2N$] were 1.56, 1.81, 1.60 and 1.66 times higher than that of untreated lipase in phosphate buffer, respectively. The activities of lipase pretreated with hydrophobic ILs ([Bmim][$PF_6$] and [Emim][$Tf_2N$]) showed a little higher than those in hydrophilic ILs ([Bmim][$BF_4$] and [Emim][$BF_4$]). It means that hydrophobic ILs may be favorable for enzyme activity in hydrolysis reaction rather than hydrophilic ILs.

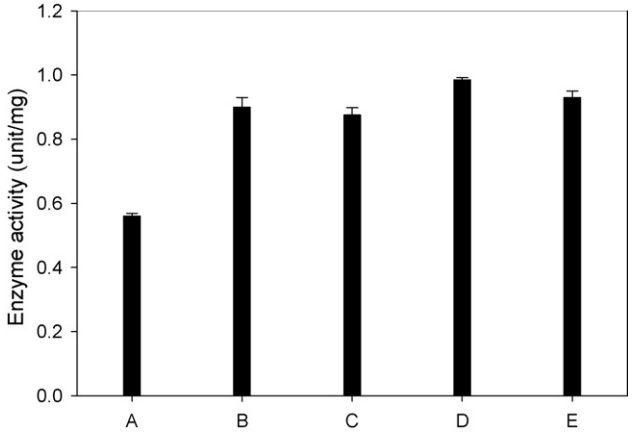


Fig. 3. Activities of lipase pretreated with various ILs in hydrolysis reaction ((A) unpretreated; (B) [Emim][$BF_4$]; (C) [Bmim][$BF_4$]; (D) [Bmim][$PF_6$]; (E) [Emim][$Tf_2N$]).

The thermal stability of lipase in various ILs was also studied by incubating lipase in ionic liquids at 60 °C. Fig. 4 shows the residual activity of lipase pretreated with hydrophobic ILs such as [Bmim][$PF_6$] and [Emim][$Tf_2N$] were well maintained at 85% and 82% even after 7 days of incubation at 60 °C, respectively. Hydrophilic ILs ([Bmim][$BF_4$] and [Hmim][TfO])-pretreated lipase also retained their initial activity more than 80% even after 7 days of incubation at 60 °C. However, [Emim][TfO]-pretreated lipase whose activity was significantly decreased after 3 days of incubation as shown in Fig. 5. One possible explanation of high thermal stability of enzyme in ILs is that the structure of enzyme may be compact in ILs which prevents the thermal denaturation of enzyme under the temperature condition tested. This enhancement of thermal stability of lipase in ILs was a coincidence with the results of

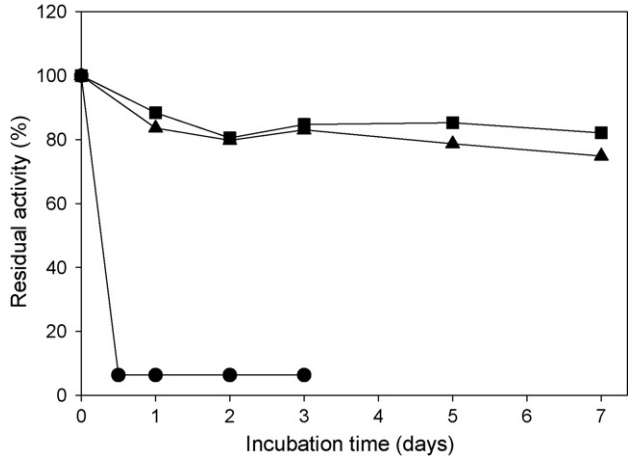


Fig. 4. Stability of lipase in hydrophobic ILs at 60 °C (1% [Bmim][$PF_6$] (■), 1% [Emim][$Tf_2N$] (▲), and phosphate buffer (●)). The enzyme activity was measured in aqueous solution after incubating lipase in pure ILs at 60 °C for the selected times.

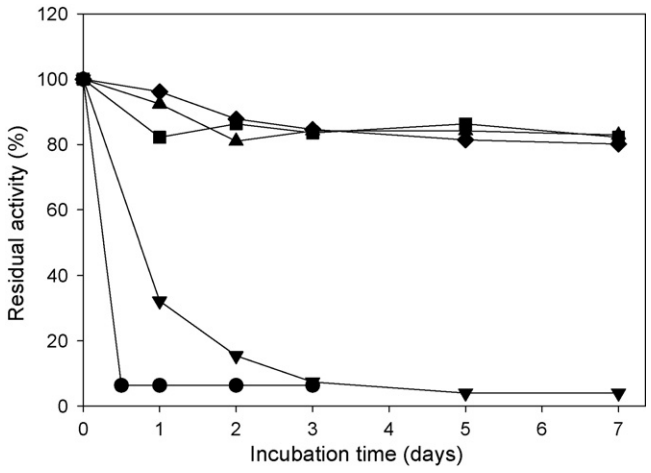


Fig. 5. Stability of lipase in hydrophilic ILs at 60 °C (1% [Emim][$BF_4$] (■), 1% [Bmim][$BF_4$] (▲), 1% [Emim][TfO] (▼), 1% [Hmim][TfO] (♦), and phosphate buffer (●)). The enzyme activity was measured in aqueous solution after incubating lipase in pure ILs at 60 °C for the selected times.

Erbeldinger et al. [9]. ILs might stabilize enzyme to a greater extent than the commonly used organic solvents. Lozano et al. have also shown that ILs could stabilize the enzyme and increase enzyme half-life greatly [15].

## 4. Conclusions

In the hydrolysis reaction, the activity and stability of IL-pretreated lipase were investigated with hydrophobic and hydrophilic ILs. Activities of IL-pretreated lipase were obviously higher than that of untreated lipase. This may be explained by the change on the secondary structure of lipase in ILs, which gives rise to the enhancement in the interaction between substrate and active site. Moreover, the activities of lipase in ILs were well maintained after 7 days of incubation at 60 °C compared with the stability of free lipase. These results suggest that pretreatment of lipase with ILs might form IL-coated lipase which causes the structural change of lipase, and thus, enhance the activity and stability of lipase in aqueous solution.

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