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# Enhanced stability of *Candida antarctica* lipase B in ionic liquids

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**Abstract**−The activity and stability of lipase from *Candida antarctica* were investigated in the kinetic resolution of (*R,S*)-1-phenylethanol with vinyl acetate using ionic liquids (ILs) as reaction media. Among ILs tested, the highest activity of lipase was observed in [Edmim][$Tf_2N$]. In hydrophobic ILs such as [Edmim][$Tf_2N$], [Emim][$Tf_2N$] and [Pmim][$PF_6$], lipase could retain its activity after 5 times reuse, while the activity of lipase in hydrophilic ILs and organic solvents was drastically decreased. The activities of lipase in [Edmim][$Tf_2N$], [Emim][$Tf_2N$] and [Pmim][$PF_6$] were also well maintained after 1 day incubation at 80 °C. The lipase suspended in [Edmim][$Tf_2N$] could be successfully reused 6 times without loss of activity.

Key words: Ionic Liquid, Lipase, (*R,S*)-1-Phenylethanol, Reuse, Stability

## INTRODUCTION

Ionic liquids (ILs) are organic salts that melt below 100 °C. The interest in ILs stems from their potential as 'green solvents' [1]. Their non-volatile character and thermal stability make them attractive alternatives for volatile organic solvents. ILs also exhibit excellent physical characteristics including the ability to dissolve polar and nonpolar organic, inorganic, and polymeric compounds. Since the number of combinations of anions and cations encompassed by ILs is vast, their associated synthetic flexibility has led ILs being referred to as 'designer solvents' [2,3].

Several groups have reported that ILs can be used as alternative reaction media for biocatalysis. Remarkably higher enzyme activities and enantioselectivities were reported in ILs than in common organic solvents [4-7]. Stability studies revealed that the half-life of an immobilized esterase from *Bacillus stearothermophilus* significantly increased when the enzyme was incubated in [Bmim][$PF_6$] and [Bmim][$BF_4$] [8]. Lozano *et al*. reported an enhanced stability of free lipase from *Candida antarctica* in ILs containing substrates [9]. They showed that the half-life time of the enzyme was significantly increased more than 2,300 times when enzyme was operated with substrates in 2% (v/v) water-containing [Bmim][$PF_6$] compared to that in 2% (v/v) water-containing [Bmim][$PF_6$] in the absence of substrate. The enhanced thermal stability of *Candida rugosa* lipase in ILs was also reported [10]. Higher half-life times were obtained in [Bmim][$PF_6$] and [Omim][$PF_6$] compared to those in various organic solvents such as *n*-hexane, benzene, and dibutyl ether.

The enzyme suspended in [Bmim][$Tf_2N$] could be reused 3 times with less than 10% loss of activity per cycle [7]. Itoh *et al*. also studied the reuse of enzyme in ILs to develop a lipase-recycling system with vinyl acetate as acyl donor [11]. In this lipase-recycling system using vinyl acetate as acyl donor in [Bdmim][$BF_4$], the lipase was repeatedly used 10 times without any decrease in enantioselectivity and reactivity. However, the effect of ILs structure on the stability of enzyme has not been extensively studied so far.

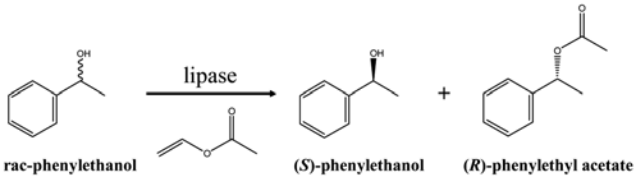


**Fig. 1. Lipase-catalyzed kinetic resolution of (*R,S*)-1-phenylethanol with vinyl acetate as a model reaction.**

In this work, the lipase-catalyzed enantioselective esterification of (*R,S*)-1-phenylethanol with vinyl acetate was selected as a model reaction to investigate the effect of ILs structure on the activity and stability of lipase from *Candida antarctica* (Fig. 1). The operational and thermal stabilities of lipase in ILs were also compared with those in organic solvents.

## MATERIALS AND METHODS

### 1. Materials

Novozym 435 (*Candida antarctica* type B lipase immobilized on acrylic resin) was provided by Novo Nordisk (Bagsvaerd, Denmark). All ILs were provided by C-TRI (Suwon, Korea) and had a residual chloride content of less than 30 ppm. (*R,S*)-1-phenylethanol and vinyl acetate were purchased from Aldrich (Steinheim, Germany). All other chemicals used in this work were of analytical grade and were used without further purification.

### 2. Kinetic Resolution of (*R,S*)-1-Phenylethanol

Substrate solutions were prepared by dissolving (*R,S*)-1-phenylethanol (0.1 mmol) and vinyl acetate (0.1 mmol) in 1 mL of various ILs and organic solvents. All ILs were dried in a vacuum oven at

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60 °C for several days prior to preparation of substrate solution. For each reaction, 1 mL of substrate solutions and 0.5 mg of Novozym 435 were added to glass vials. The reaction was carried out in a shaking incubator at 50 °C for 20 hrs. Periodically, 10 μL aliquots were taken. The substrate and product were extracted with 0.1 mL of mixture of *n*-hexane and isopropanol (90 : 10, v/v). The reaction conversion and the enantiomeric excess were evaluated by using the methods described in the following section.

**3. Enzyme Stability in Ionic Liquids**

The lipase-catalyzed transesterifications were started by adding 1 mL of substrate solution to glass vials containing Novozym 435 of 10 mg as described in section 2. For the determination of operational stability of lipase, lipase was recovered from ILs or organic solvents and washed with *n*-hexane. Then, reaction was started by adding new substrate solution. In order to measure thermal stability of lipase, 10 mg of Novozym 435 was incubated in 1 mL of ILs at 80 °C. After incubation, the residual lipase activity was measured by adding 0.1 mmol of (*R,S*)-1-phenylethanol and 0.1 mmol of vinyl acetate to the solution as described above.

**4. Reuse of Lipase Suspended in Ionic Liquid**

The mixture of 10 mg of Novozym 435 and 1 mL of [Edmim][$Tf_2N$] was used to carry out transesterification of (*R,S*)-1-phenylethanol (0.1 mmol) with vinyl acetate (0.1 mmol) for 5 hrs. The reaction course was monitored by HPLC analysis. Product and unreacted substrate were extracted with 20 mL mixture of *n*-hexane and isopropanol (95 : 5, v/v). The remaining ILs phase containing lipase was placed under reduced pressure for 1 hr to remove water and extraction solvent. Then, a mixture of (*R,S*)-1-phenylethanol (0.1 mmol) and vinyl acetate (0.1 mmol) was added to measure the residual activity of lipase suspended in ILs.

**5. HPLC Analysis**

The concentrations of (*R*)- and (*S*)-1-phenylethyl acetate, (*R*)- and (*S*)-1-phenylethanol were measured by HPLC. Separation was accomplished with a Shimadzu HPLC system (Model LC-10A, Japan) equipped with a Chiralcel OJ-H column (Daicel, USA) and a UV detector (205 nm). The mobile phase consisted of *n*-hexane/isopropanol/ethanol (965/30/5, volume ratio). Enantiomeric excesses of substrate ($ee_s$) and product ($ee_p$), conversion (c), and enantioselectivity (E) were calculated by using the following equations, respectively.

$$ee_s(\%) = \frac{[(S)_s] - [(R)_s]}{[(S)_s] + [(R)_s]} \times 100 \quad (1)$$

$$ee_p(\%) = \frac{[(R)_p] - [(S)_p]}{[(R)_p] + [(S)_p]} \times 100 \quad (2)$$

$$c(\%) = \frac{ee_s}{ee_s + ee_p} \times 100 \quad (3)$$

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \times 100 \quad (4)$$

where (R) and (S) represent the concentrations of the (R) and (S) form in substrate ($_s$) or product ($_p$), respectively.

## RESULTS AND DISCUSSION

**1. Activity and Enantioselectivity of Lipase in Ionic Liquids**

**Table 1. Activity and enantioselectivity of lipase in the kinetic resolution of (*R,S*)-1-phenylethanol with vinyl acetate in ionic liquids and organic solvents**

| Solvent | $ee_s$ | $ee_p$ | Conversion (c) | E |
|---|---|---|---|---|
| [Bmim][$BF_4$] | 2 | 91 | 2 | 22 |
| [Omim][$BF_4$] | 15 | >99 | 13 | >200 |
| [Emim][TfO] | 24 | >99 | 19 | >200 |
| [Bmim][TfO] | 5 | >99 | 5 | >200 |
| [Hmim][TfO] | 5 | >99 | 5 | >200 |
| [PPmim][TfO] | 68 | 97 | 41 | 157 |
| [Emim][MS] | 0 | ND[a] | 0 | ND |
| [Emim][$Tf_2N$] | 49 | >99 | 33 | >200 |
| [Bmim][$Tf_2N$] | 34 | >99 | 25 | >200 |
| [Hmim][$Tf_2N$] | 43 | >99 | 30 | >200 |
| [Omim][$Tf_2N$] | 34 | >99 | 25 | >200 |
| [Bzmim][$Tf_2N$] | 40 | >99 | 28 | >200 |
| [Edmim][$Tf_2N$] | >99 | >99 | 50 | >200 |
| [Bmim][$PF_6$] | 33 | >99 | 25 | >200 |
| [Hmim][$PF_6$] | 18 | >99 | 15 | >200 |
| [Omim][$PF_6$] | 57 | >99 | 36 | >200 |
| [Hmim][$SbF_6$] | 41 | >99 | 29 | >200 |
| [Empyr][$SbF_6$] | 0 | ND | 0 | ND |
| Toluene | >99 | >99 | 50 | >200 |
| DMSO | 0 | ND | 0 | ND |

[a]Not determined by low activity.

The activity and enantioselectivity of lipase from *Candida antarctica* were investigated in the kinetic resolution of (*R,S*)-1-phenylethanol with vinyl acetate using ILs as reaction media. Table 1 shows the conversion and enantioselectivity of lipase in various ILs and organic solvents. For 1-butyl-3-methylimidazolium based ILs, the activity of lipase was highly dependent on the anion identity. In [$Tf_2N$]-based imidazolium ILs, lipase usually showed higher activities and enantioselectivities. The highest activity of lipase was observed in [Edmim][$Tf_2N$] among ILs tested in this work. Table 1 also shows that the activity of lipase generally decreased with increasing alkyl chain length on cation in ILs. It may be caused by the limitation of mass transfer, because ILs with longer alkyl chain on cation have higher viscosity. Although the activities of lipase in most ILs were lower than that in toluene, which is used as a general organic solvent for biocatalysis, the enantioselectivities of lipase in most ILs showed higher values over 200.

**2. Stability of Lipase in Ionic Liquids**

The operational stabilities of lipase were evaluated in hydrophobic ILs, hydrophilic ILs and organic solvents. Fig. 2 shows the residual activity of lipase at each reuse cycle. The activities of lipase were well maintained after reuse in hydrophobic ILs such as [Edmim][$Tf_2N$], [Emim][$Tf_2N$] and [Bmim][$PF_6$], while the activities of lipase remarkably decreased in hydrophilic ILs and organic solvents. The stability of lipase in solvents can be usually understood by the log P value, the hydrophobicity of solvents [12]. It is believed that hydrophilic organic solvents strip essential water from the lipase, which leads to the unfolding of the molecule with exposure of the inner hydrophobic residues, while hydrophobic organic solvents keep lipase flexible and in active conformation [13]. It has been reported

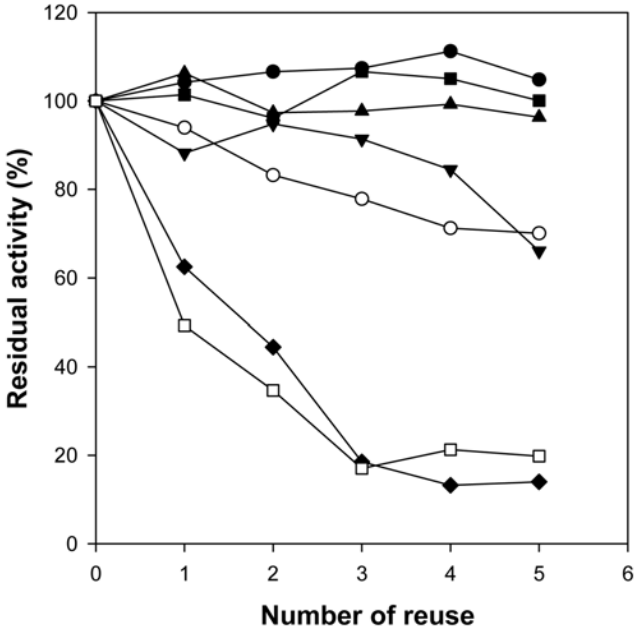


**Fig. 2. Operational stability of lipase in ILs. Reaction conditions: 0.1 mmol (*R,S*)-1-phenylethanol, 0.1 mmol vinyl acetate, 1 mL ILs, 10 mg Novozym 435, 50 °C (● [Edmim][$Tf_2N$], ■ [Emim][$Tf_2N$], ▲ [Bmim][$PF_6$], ▼ [PPmim][TfO], ◆ [Bmim][$BF_4$], ○ toluene, □ acetone).**

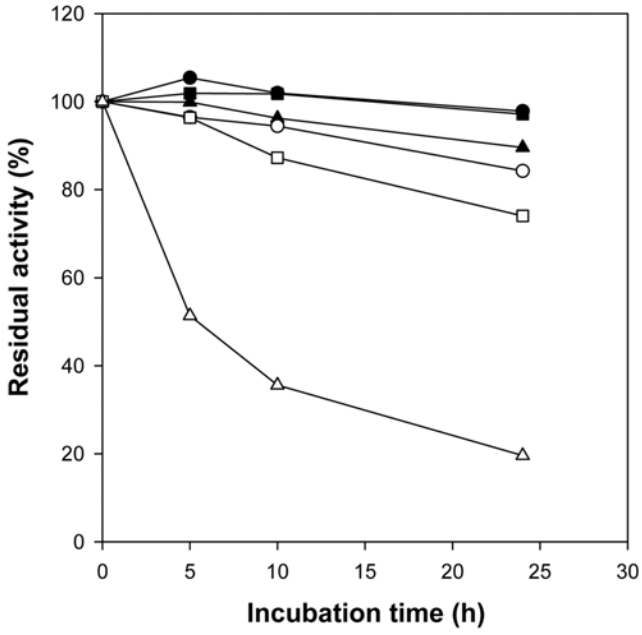


**Fig. 3. Thermal stability of lipase at 80 °C in ILs. Reaction conditions: 0.1 mmol (*R,S*)-1-phenylethanol, 0.1 mmol vinyl acetate, 1 mL ILs, 10 mg Novozym 435, 80 °C (● [Edmim][$Tf_2N$], ■ [Emim][$Tf_2N$], ▲ [Bmim][$PF_6$], ○ benzene, □ isooctane, △ 3-pentanone).**

that lipase can retain its activity in hydrophobic organic solvents such as toluene, hexane and isooctane. The log P values for [Emim][$Tf_2N$] (−1.18) and [Bmim][$PF_6$] (−2.06) are significantly lower than toluene (2.5) and similar to acetone (−0.23) [12]. Using above guidelines, it would suggest that ILs are highly hydrophilic in nature and would likely inactive lipase. However, our results showed that lipase retains its activity well in hydrophobic ILs such as [Emim][$Tf_2N$] and [Bmim][$PF_6$]. This implies that log P does not seem to act as a useful parameter to compare enzyme activity and stability in ILs with those in organic solvents.

The decrease in residual activity of lipase in [Bmim][$BF_4$] is similar to that in acetone because the log P value of [Bmim][$BF_4$] is −2.71 is much lower than that of hydrophobic ILs. Therefore, it can be partly understood that the hydrophobicity of ILs can influence the activity and stability of lipase in ILs. Recently, de Diego *et al*. showed that [Emim][$Tf_2N$] was able to stabilize the enzyme via the formation of a flexible and more compact 3D structure, being related to the preservation of the essential water shell [14].

The thermal stability of lipase in hydrophobic ILs was also studied. Fig. 3 shows the residual activity of lipase after incubation at 80 °C in various ILs and organic solvents, respectively. Lipase maintained more than 90% of its initial activity in [Edmim][$Tf_2N$] and [Emim][$Tf_2N$] even after 24 hrs incubation at 80 °C. The stabilities of lipase in these ILs were higher than those in common organic solvents such as benzene, isooctane, and 3-pentanone.

### 3. Reuse of Lipase Suspension in Ionic Liquids

In our study, it was observed that the activity of lipase usually increased with decreasing alkyl chain length on cation in ILs (Table 1). In addition, the stability of lipase in [$Tf_2N$]-based ILs was higher than that in ILs containing [$BF_4$] (Fig. 2). Therefore, [Edmim][$Tf_2N$]

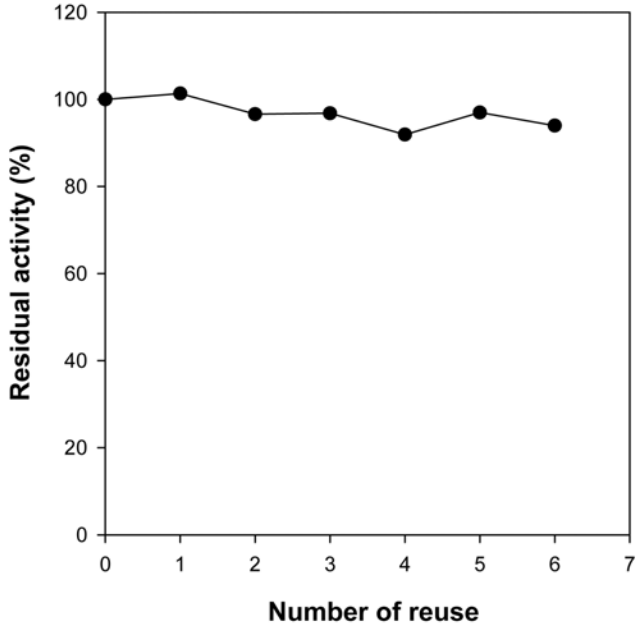


**Fig. 4. Residual activity of lipase after reuse of lipase suspended in [Edmim][$Tf_2N$]. Reaction conditions: 0.1 mmol (*R,S*)-1-phenylethanol, 0.1 mmol vinyl acetate, 1 mL [Edmim][$Tf_2N$], 10 mg Novozym 435, 50°C.**

was selected as the best ILs to investigate the reusability of lipase for the transesterification of (*R,S*)-1-phenylethanol with vinyl acetate. The substrate and product in ILs were extracted by using mixture of *n*-hexane and isopropanol, and then ILs were placed under reduced pressure for 1 hr to remove the extraction solvents. The

lipase suspended in [Edmim][$Tf_2N$] could be reused 6 times without the loss of lipase activity (Fig. 4). The activity determined by initial reaction rate and enantioselectivity in [Edmim][$Tf_2N$] still maintained 94% of initial activity and enantioselectivity over 200, respectively. High enzyme activity and enantioselectivity in [Edmim][$Tf_2N$] even after 6 times reuse can be explained by the decreased acidity caused by the removal of the proton at 2-position on imidazolium cation because the acidity of 2-position on imidazolium cation is very high [15]. Itoh *et al.* reported that [Bdmim][$BF_4$], which lacked hydrogen at the 2-position on the imidazolium cation, was found to be an excellent solvent for a lipase-recycling system using vinyl acetate as acyl donor [11]. They explained that no accumulation of an acetaldehyde oligomer in [Bdmim]-based ILs can induce the high reusability of lipase suspended in ILs.

## CONCLUSIONS

The activity and stability of lipase in ILs were highly dependent on the anion structure which determines the hydrophobicity. The activity of lipase usually increased with decreasing alkyl chain length on cation in ILs and the highest enzyme activity was achieved in [Edmim][$Tf_2N$]. The stabilities of lipase in hydrophobic ILs were higher than those in organic solvents. The activities of lipase were well maintained in these ILs after 5 times reuse. Our results suggest that hydrophobic ILs can be used as reaction media for biocatalysis instead of organic solvents. Specifically, [Edmim][$Tf_2N$] may work as the most useful reaction media for lipase-catalyzed reaction, because its acidity is lowered by removing the proton at 2-position on imidazolium cation which shows high acidity.

## ACKNOWLEDGMENTS

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## NOMENCLATURE

### Abbreviations

### Cations

[$Bmim]^+$ : 1-butyl-3-methylimidazolium;
[$Emim]^+$ : 1-ethyl-3-methylimidazolium;
[$Hmim]^+$ : 1-hexyl-3-methylimidazolium;
[$Omim]^+$ : 1-methyl-3-octylimidazolium;
[$PPmim]^+$: 1-phenylpropyl-3-methylimidazolium;
[$Bzmim]^+$: 1-benzyl-3-methylimidazolium;
[$Edmim]^+$: 1-ethyl-2,3-dimethylimidazolium;
[$Bdmim]^+$: 1-butyl-2,3-dimethylimidazolium;
[$Empyr]^+$ : 1-ethyl-1-methylpyrrolidinium;

### Anions

[$BF_4]^-$ : tetrafluoroborate;
[$PF_6]^-$ : hexafluorophosphate;
[$MS]^-$ : methylsulfate;
[$TfO]^-$ : trifluoromethanesulfonate;
[$Tf_2N]^-$ : bis[(trifluoromethyl)sulfonyl]amide

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