# Effects of Imprinting and Water Activity on Transesterification and Thermostability with Lipases in Ionic Liquid



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The effect of bio-imprinting and water activity on catalytic activities and the thermostability of lipases was investigated for transesterification using vinyl acetate and benzyl alcohol as substrates in ionic liquid, $[C_n mim][PF_6]$ (n=4,6,8), and benzene. The catalytic activities were enhanced by imprinting in benzene and $[C_4 mim][PF_6]$, and the relations between the transesterification activities and the water activity in both solvents were approximately bell shaped. The reactivity of the transesterification in benzene was higher than that in $[C_4 mim][PF_6]$. The effects of water activity and imprinting on the kinetic parameters in $[C_4 mim][PF_6]$ were examined. Without controlling the water content, the values of $K_{m,VA}$ and $K_{m,BA}$ (Michaelis constants of vinyl acetate and benzyl alcohol, respectively) decreased, and the values of $V_m$ (maximum rate) increased by imprinting. On the other hand, by controlling the water content in the organic media, the values of $V_m$, $K_{m,VA}$, and $K_{m,BA}$ increased by imprinting. The activities of lipase in ionic liquid are more strongly affected by water activity and imprinting than those in benzene. We observed effects of water activity on thermostability but none from imprinting.

*Keywords:*
lipase, ionic liquid, bio-imprinting, transesterification

## Introduction

Enzyme-catalyzed reactions in organic media are widely performed in industrial biotechnology[1]. Lipases are a typical enzyme used in the reactions in organic media[2]. Depending on their origin, lipases are generally stable in hydrophobic organic solvents[3]. Although many practical applications have been developed in organic media, usage of organic solvents must be reduced in the future[4] because of environmental contamination, physical hazards, toxicity, and volatility circumstances. Alternative media such as supercritical fluids, solvent-free systems, fluorinated solvents and ionic liquids[5] have been proposed. Among them, ionic liquids have emerged as a representative alternative because their physicochemical properties, including density, viscosity, solubility, and hydrophobicity, can be easily tuned for applications by satisfactorily combining cation and anion moieties. The stability and reactivity of lipase-catalyzed reactions in ionic liquids have also been reported[5,6]. The use of lipase in ionic liquids provides advantages, such as selectivity enhancement, enzyme stability enhancement, a higher conversion rate, and a better recovery system[4] over the conventional organic solvents. Enzymes in ionic liquids consisting of $BF_4^-$, $PF_6^-$, and $NTf_2^-$ anions showed better activity than those in organic solvents[4]. Previous studies showed that 1-butyl-3-methyl imidazolium hexafluorophosphate ($[C_4 mim][PF_6]$) significantly enhanced the transesterification activity of *Candida rugosa* lipase (CRL)[7–9]. However, CRL was inactive in other 1-butyl-3-methyl imidazolium based ionic liquids, such as $[C_4 mim][CH_3CO_2]$, $[C_4 mim][NO_3]$ and $[C_4 mim][CF_3CO_2]$[8]. These inhibitory ionic liquids were water-soluble, suggesting that enzyme activity in them depends on their anions and is correlated with the hydrophobicity of anions[8].

In this study, we examined how to enhance the performance of CRL in $[C_n mim][PF_6]$ (n = 4,6,8). One way is a bio-imprinting method, which includes the lyophilization of an enzyme loaded with a substrate analogue to form an enzyme-substrate complex and washing the substrate analogue in anhydrous media. However, the enzyme does not return to its original conformation due to its rigid structure[10]. This method was reported in enzymatic reactions in conventional organic media[11] because the structural memory of bio-imprinted enzyme is lost in aqueous media[12]. To the best of our knowl-

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edge, this is the first time a bio-imprinting method has been applied to enzymatic reactions in ionic liquids. Next, we evaluated the effect of water activity on enzymatic reactions in ionic liquids because water activity influences enzymatic reactions in organic media[13–17]. A small amount of water is essential to express the enzyme function. Most water molecules, which are associated with the enzyme surface, increase the flexibility of the enzyme in organic media. Conversely, high water content in organic media leads to undesirable conformational changes for enzymatic reaction[18], and an unwanted hydrolysis of products. Hence, there is optimum water activity for enzyme-catalyzed reaction in organic media. The optimum activity for CRL has been reported at water activities between 0.33 and 1[14–16,19,20]. Ulbert *et al.*[21] studied its effect on esterification with CRL in $[C_4mim][PF_6]$, and obtained the best stability and reactivity at water activity of 0.51 without determining the kinetic parameters. Lee *et al.*[22] found the maximum initial rate at water activity of 0.4 in the CRL-catalyzed transesterification in $[C_8mim][Tf_2N]$. The optimal water activity depends on the lipase origin, the solvent, and the reaction type[23]. Even though experimental data in many systems have been accumulated, understanding the effect of water activity on enzyme-catalyzed reactions remains limited. Moreover, few studies have investigated the effect of water activity on the thermostability of enzymes[13,21].

In the present work, the effects of bio-imprinting and water activity on transesterification rates and thermostability with CRL in ionic liquids, 1-alkyl-3-methyl imidazolium hexafluorophosphates, were investigated to enhance the performance of CRL. 1-Alkyl-3-methyl imidazolium hexafluorophosphates as an ionic liquid were used because they have been frequently used in lipase-catalytic reactions and lipases in 1-alkyl-3-methyl imidazolium hexafluorophosphates showed a high enzymatic activity[4]. Specifically, hexafluorophosphate is a less nucleophilic anion, which strongly coordinates to positively charged sites in the lipase and thus causes unfavorable conformation changes[24]. Although the instability of hexafluorophosphate toward hydrolysis forming toxic compounds is known[25], this study was carried out under a water-restricted environment.

## Materials and methods

### Materials

Lipase from *Candida rugosa* (specific activity of 706 olive oil units/mg, Type VII, Sigma-Aldrich) was used throughout the experiments. 1-Butyl-3--methyl, 1-hexyl-3-methyl and 1-octyl-3-methyl imidazolium hexafluorophosphates ($[C_nmim][PF_6]$, n = 4,6,8) were prepared by the method of Laszlo and Compton[26]. Vinyl acetate and benzyl alcohol were used as substrates in the transesterification. Benzene and $[C_4mim][PF_6]$, $[C_6mim][PF_6]$, and $[C_8mim][PF_6]$ were used as a medium for transesterification, and hexane was used in the pretreatment process, and dried with 3 Å molecular sieves prior to use. All the reagents were of analytical grade from Wako Pure Chem. Ind. (Osaka, Japan) and used without further purification.

### Bio-imprinting of lipase

The pretreatment of lipase with carboxylic acid (formic, acetic, and propionic acids) as an imprint molecule was identical to a method described in a previous paper[11]. Lipase (300 mg) was dissolved in 9 $cm^3$ of a phosphate buffer solution at pH 7. Formic acid (0.35 mmol) and Tween 20 (1000 mg) were dissolved in 10 $cm^3$ of ethanol. To the enzyme solution, 1 $cm^3$ of acid solution was added, and the mixed solution was incubated for 30 min at 25 °C. The mixed solution was then freeze-dried. The obtained powder was washed with hexane to remove the acid. The resultant lipase was filtered and dried in vacuo.

### Transesterification

The reactions were initiated by adding 1.0 mg of lipase powder pretreated with carboxy acid into 1 mL of ionic liquids and organic solvents containing both substrates (vinyl acetate and benzyl alcohol) in a vial tube at 37 °C[27–29] and 1000 rpm. At 5-minute intervals for 30 minutes, the vial tubes were removed from the thermomixer (Comport, Eppendorf), and immediately centrifuged. The extent of the reaction was monitored by measuring the benzyl acetate concentration using HPLC. The initial experimental conditions were as follows: a vinyl acetate concentration of [VA] = 30 mol $m^{-3}$ and a benzyl alcohol concentration of [BA] = 30 mol $m^{-3}$. The reaction rates were evaluated as the initial rate in the initial period. The values obtained were averaged over three measurements within 20 % error.

To examine the effect of water activity in the solvents, ionic liquids and organic solvents were pre-equilibrated with deionized water or a saturated aqueous solution of salt in a separate container[17,21]. The salts used were $KNO_3$ (water activity, $a_w$ = 0.924), KCl ($a_w$ = 0.8426), NaCl ($a_w$ = 0.7528), $CoCl_2$ ($a_w$ = 0.634), $MgCl_2$ ($a_w$ = 0.330), and LiCl ($a_w$ = 0.110)[17]. The values of the activities indicate those of the aqueous solution that were pre-equilibrated with the solvents.

### Thermostability of lipase

The lipases were incubated in benzene or [$C_4$mim][$PF_6$] (0.5 $cm^3$) at 60 °C. After the prescribed incubation time, the substrate solution (0.5 $cm^3$) was added and the reaction conducted at 37 °C and 1000 rpm for 10 min. Samples were taken to measure the enzyme activity, and the residual activities were defined as the ratio of the initial reaction rates with the incubated lipases of those with lipases without incubation.

### Analysis

Standard benzyl acetate solutions were used for preparing the calibration curves. The concentrations of the benzyl acetate in the organic solutions were determined by HPLC (Shimadzu LC-10ADvp) with a Wakosil-II 5C18AR column and an eluent solution (acetonitrile:water = 1:1) as a mobile phase (0.6 $cm^3$ $min^{-1}$). Esters were detected with an UV detector at 255 nm (Shimadzu SPD10AV).

## Results and discussion

### Effect of imprinting molecules on transesterification

Prior to the transesterification catalyzed by the imprinted lipase, the effect of the imprinting molecules on the transesterification of vinyl acetate and benzyl alcohol diluted in [$C_8$mim][$PF_6$] was examined. Fig. 1 shows the concentration of benzyl acetate produced at 5 min with various lipases (native, and carboxylic acid-pretreated). The activities of the pretreated lipase with carboxylic acids were higher than those of the native lipase, suggesting that the pretreatment of carboxylic acids is effective for transesterification. Among the carboxylic acids, the highest activity was obtained with lipase that was pretreated by formic acid as a substrate analogue. Increase in the alkyl chain length of the carboxylic acid as a substrate analogue reduced the reactivity. This suggests that the preferable conformational change of lipase for binding with vinyl acetate is initially induced by formic acid, and its conformation is retained after removing the formic acid in a process called bio-imprinting[10]. The cavity size of the lipase created by formic acid is best fitted for binding lipase with vinyl acetate. Thus, the optimal imprint molecule depends on the reaction system. The optimum molecules were octanoic acid[30] for the esterification of lauric acid and benzyl alcohol in toluene, and heptafluorobutyric acid[11] for the transesterification of vinyl acetate and benzyl alcohol in cyclohexane. In the following experiments, formic acid was used as an imprinting molecule.

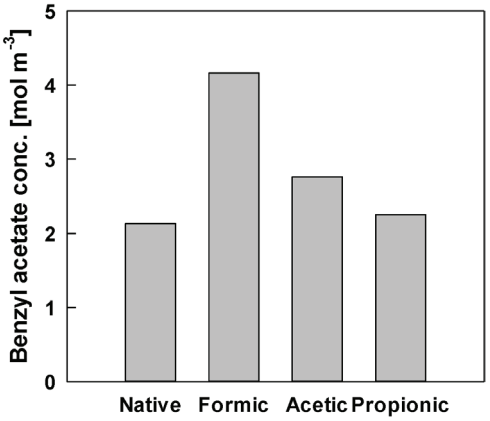


Fig. 1 – *Effect of carboxylic acids as bio-imprinting molecules on transesterification in [$C_8$mim][$PF_6$]. [VA] = [BA] = 30 mol $m^{-3}$.*

### Effect of solvent on transesterification

The effect of the reaction solvent on the transesterification of vinyl acetate and benzyl alcohol was examined. Table 1 shows the concentration of benzyl acetate produced at 5 min with various solvents and their log*P* values[31,32]. In conventional solvents, a water-miscible solvent, acetone, showed no activity because it stripped the essential water bound to the surface of lipase to exhibit both conformational flexibility and enzymatic activity[3]. In this reaction system, benzene gave the highest activity for the native and imprinted lipases. Although ionic liquids, [$C_n$mim][$PF_6$] had lower log*P* values than the acetone, [$C_n$mim][$PF_6$] were slightly soluble in water. Therefore, [$C_n$mim][$PF_6$] allowed the preservation of the essential water layer in the active site of the lipase. The activity of the lipase increased with increasing the alkyl chain length from C4 to C8 for both lipases. When the ionic liquids

Table 1 – *Effect of solvent on transesterification of vinyl acetate and benzyl alcohol*

| Solvent | Log*P* [31,32] | Native[a)] | Imprint[a)b)] |
|---|---|---|---|
| [$C_4$mim][$PF_6$] | –1.72 | 1.00 | 1.49 |
| [$C_6$mim][$PF_6$] | –1.20 | 1.71 | 2.58 |
| [$C_8$mim][$PF_6$] | –0.35 | 2.13 | 4.16 |
| Acetone | –0.23 | 0 | 0 |
| Benzene | 2.12 | 6.32 | 6.58 |
| Toluene | 2.73 | 2.14 | 3.09 |
| Ethylbenzene | 3.15 | 1.60 | 2.24 |
| Cyclohexane | 3.44 | 0.71 | 0.59 |

Vinyl acetate concentration = benzyl alcohol concentration = 30 mol $m^{-3}$

a) Benzyl acetate concentration (mol $m^{-3}$) produced at 5 min

b) Imprinted lipase by formic acid

contained a [$Tf_2N$] anion, the transesterification activity of lipase decreased with increasing the alkyl chain length because of the higher viscosity of the ionic liquids containing cations of longer alkyl chain lengths[22]. However, the most hydrophobic ionic liquid among the ionic liquids investigated, [$C_8$mim] [$Tf_2N$], gave the highest activity[22]. Conflicting results have been reported for the effect of alkyl chain length of ionic liquids containing [$BF_4$] or [$PF_6$][22]. The viscosity and the hydrophobicity of ionic liquids increased with increasing the alkyl chain length on the imidazolium cation. Although, in this study, the hydrophobicity of ionic liquids was more effective than their viscosities, these two conflicting factors may appear differently in different reaction systems.

### Effect of water activity on transesterification

Fig. 2 shows the effect of water activity in benzene and [$C_4$mim][$PF_6$] on transesterification. Both profiles were approximately bell-shaped despite a large difference in water solubility between benzene and [$C_4$mim][$PF_6$]. These bell -shapes were observed in previous studies on enzymatic esterification in conventional organic media and ionic liquids[18–21,23,33]. In our study, the reactivity of transesterification in benzene was higher than that in [$C_4$mim][$PF_6$], although the reactivity of butyl-2-chloropropionate ester in [$C_4$mim][$PF_6$] that was catalyzed by the same lipase from *Candida rugosa* was reported to be higher than that in benzene[21]. The difference in esterification and transesterification is probably due to higher water solubility in [$C_4$mim][$PF_6$] (18 g (L-[$C_4$mim][$PF_6$])$^{-1}$ [34]) than that in benzene (1.8 g (L-benzene)$^{-1}$). Because substrates are carboxylic acid in esterification and ester in transesterification, vinyl acetate ester as the substrate is more susceptible to hydrolysis as a side reaction in [$C_4$mim][$PF_6$].

In benzene, the effect of water activity is relatively small; the maximum activity is at an $a_w$ value of 0.7528. In [$C_4$mim][$PF_6$], the reactivity reached a maximum at an $a_w$ value of 0.8426 and a subsequent increase in water activity sharply reduced it. In higher water activity, vinyl acetate may be hydrolyzed. Maximum initial rates were reported to be at a water activity of 0.4 for the CRL-catalyzed transesterification in [$C_8$mim][$Tf_2N$][22] and at a water activity of 0.51 for esterification with CRL in [$C_4$mim][$PF_6$][21]. The values of the optimum water activity in this study were within the reported values for CRL (0.33–1)[19–21] for solvents including conventional organic solvents and ionic liquids. Although the shifts of optimal $a_w$ reflect the interaction between enzyme and ionic liquid, at present, this interaction cannot be rationally predicted.

In the following experiment, we used benzene ($a_w$ = 0.7528) and [$C_4$mim][$PF_6$] ($a_w$ = 0.8426).

### Effect of water activity and imprinting on transesterification kinetics

Fig. 3 shows the relative initial rates of transesterification with treated lipases based on those with native lipases. In benzene, there is little effect from the treatment of lipase. On the other hand, in [$C_4$mim][$PF_6$] the initial reaction rates with imprinted lipase at $a_w$ = 0.8426 are three times higher than those with native lipase. Fig. 4 shows the effect of vinyl acetate concentration on the initial rate of the transesterification for pretreated lipases. As is evident from Fig. 3, no substrate inhibitions were observed. The kinetic rate equation for transesterifica-

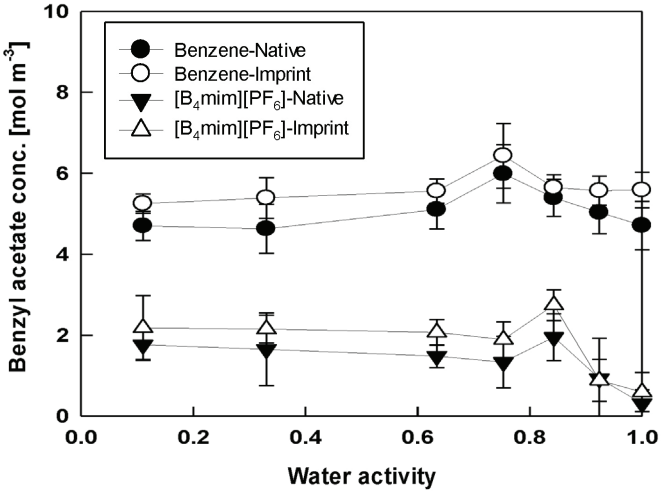


Fig. 2 – *Effect of water activity on transesterification activity. [VA] = [BA] = 30 mol m$^{-3}$.*

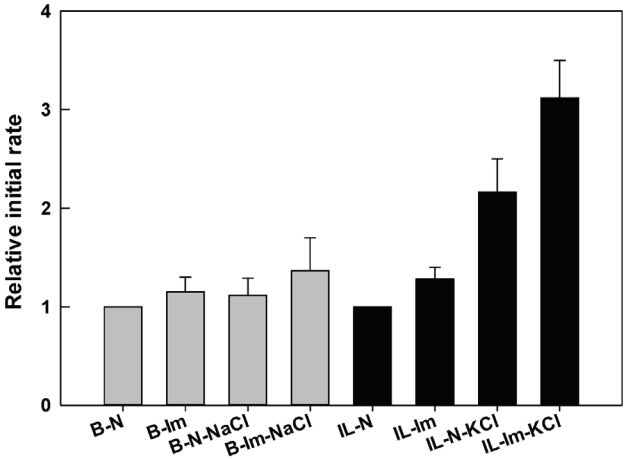


Fig. 3 – *Relative initial rates of transesterification with lipases based on those with native lipases. B: benzene, IL: [$C_4$mim][$PF_6$], N: native, Im: imprinted. NaCl and KCl denote solvents treated with saturated NaCl ($a_w$ = 0.7528) or KCl ($a_w$ = 0.8426) aqueous solution. [VA] = [BA] = 30 mol m$^{-3}$. Relative initial rates were defined as ratio of initial rates with treated lipase in treated organic media to those with native lipase in untreated organic media; relative initial velocities of B-N and IL-N are unity.*

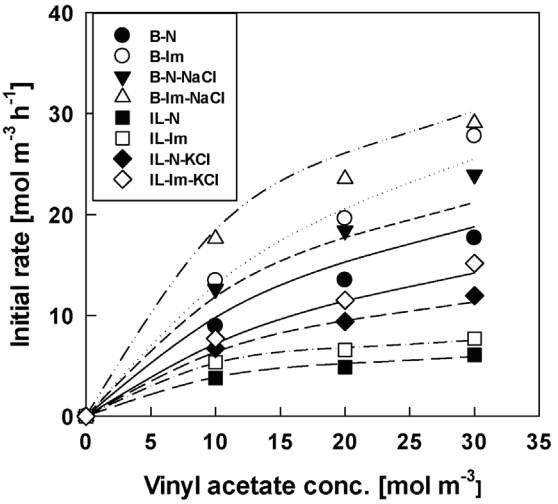


Fig. 4 – *Effect of vinyl acetate concentration and treatment of lipase on initial rate of transesterification; [BA] = 20 mol m$^{-3}$. Solid lines are calculated by Eq. (1). Key legends are identical to those in Fig. 3.*

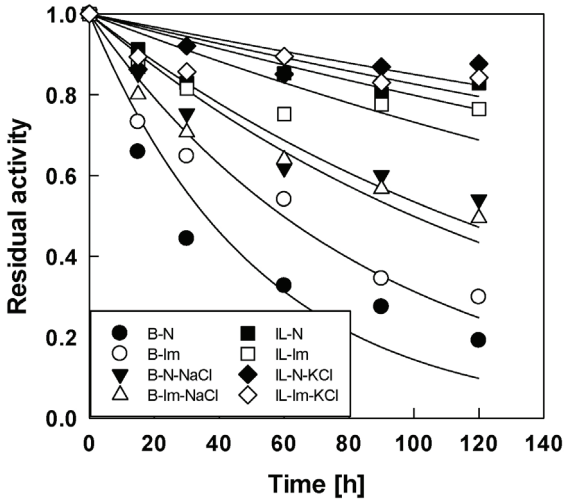


Fig. 5 – *Thermostability of treated lipases at 60 °C. Solid lines are calculated by Eq. (2). Key legends are identical to those in Fig. 3.*

tion by lipase can be written by the Ping-Pong Bi-Bi mechanism without substrate inhibition, as shown in Eq. 1[35,36].

$$V = \frac{V_m [VA][BA]}{K_{m,VA}[BA] + K_{m,BA}[VA] + [VA][BA]} \quad (1)$$

where $V_m$ is the maximum rate, [VA] and [BA] are the concentrations of vinyl acetate and benzyl alcohol, and $K_{m,VA}$ and $K_{m,BA}$ are the Michaelis constants of vinyl acetate and benzyl alcohol, respectively. The obtained kinetic parameters are listed in Table 2. In Fig. 4, the solid lines were calculated by these kinetic parameters, and agree well with the experimental data. From the data in Table 2 without controlling the water content, by pretreating with formic acid, the values of $K_{m,VA}$ and $K_{m,BA}$ decreased, that is, the affinity of the vinyl acetate and the benzyl alcohol increased, as did the values of $V_m$. The preferable conformation change of lipase for binding the vinyl acetate was initially induced by pretreating and contact with the hydrophobic solvent and substrate opened the lid to make the active site accessible. Similar behavior was reported for lipase-catalyzed solvent-free esterification[30]. By controlling the water content in the organic media, although the values of $V_m$ increased, the values of $K_{m,VA}$ and $K_{m,BA}$ also generally increased by pretreating with formic acid. A possible explanation is that solvents interact more strongly with active sites and function as a competitive inhibitor[37]. Thereby the values of $K_{m,VA}$ and $K_{m,BA}$ may increase. In benzene, the effects of water activity and imprinting on the kinetic parameters were relatively small. In [$C_4$mim][$PF_6$], both water activity and imprinting preferably affected the kinetic parameters. Because ionic liquids are more polar and hydrophilic than organic solvents[4], lipases in ionic liquid are subject to water activity and imprinting.

Table 2 – *Kinetic parameters for enzymatic transesterification and deactivation constant of lipase at 60 °C*

| | $V_m$ [mol m$^{-3}$ h$^{-1}$] | $K_{m,VA}$ [mol m$^{-3}$] | $K_{m,BA}$ [mol m$^{-3}$] | $10^2 k_d$ [min$^{-1}$] |
|---|---|---|---|---|
| Benzene (N) | 66.0 | 48.4 | 18.0 | 1.94 |
| Benzene (Im) | 78.9 | 36.8 | 10.9 | 1.16 |
| Benzene (N-NaCl) | 67.2 | 37.6 | 10.5 | 0.625 |
| Benzene (Im-NaCl) | 93.6 | 29.5 | 22.2 | 0.694 |
| [$C_4$mim][$PF_6$] (N) | 9.6 | 14.0 | 4.0 | 0.225 |
| [$C_4$mim][$PF_6$] (Im) | 11.3 | 9.6 | 3.6 | 0.311 |
| [$C_4$mim][$PF_6$] (N-KCl) | 20.6 | 21.3 | 2.2 | 0.162 |
| [$C_4$mim][$PF_6$] (Im-KCl) | 37.9 | 38.3 | 6.9 | 0.190 |

N: native, Im: imprinted. NaCl and KCl denote solvents treated with saturated NaCl ($a_w$ = 0.7528) or KCl ($a_w$ = 0.8426) aqueous solution. $K_{m,VA}$ and $K_{m,BA}$ are the Michaelis constants of vinyl acetate and benzyl alcohol.

## Effect of water activity and imprinting on thermostability of lipase

Fig. 5 shows the time-courses of the residual activities of lipases suspended in solvents at 60 °C. The native untreated lipase lost most of its original activity in 1 h[27]. Residual activity, $a$, is given by the following equation based on irreversible first-order deactivation kinetics

$$a = \exp(-k_d t) \quad (2)$$

where $k_d$ is a deactivation constant and $t$ is the elapsed time.

The deactivation constants obtained from Fig. 5 are also listed in Table 2. In Fig. 5, the solid lines were calculated by Eq. (2), and approximately fit the experimental data. Table 2 shows that the lipases in $[C_4mim][PF_6]$ were more stable than those with benzene. Ulbert *et al.*[21] described how the higher polarities of ionic liquids led to smaller deactivation. However, this result was obtained from two limited ionic liquids. The interaction between lipase and ionic liquid[24] may affect the thermostability as well as the catalytic activity.

Table 2 also shows that water activity greatly affected the thermostability of lipase. This result resembled those in previous studies[13,21], suggesting that controlling water activity, i.e., the hydration state of CRL, plays an important role in thermostability.

As described in our previous paper[11], in benzene the pretreatment of native lipase with carboxylic acid effectively stabilized lipase. In contrast, no enhancement of thermostability was observed by imprinting. We found that water activity greatly affected the thermostability of lipase but not from imprinting.

## Conclusions

The effects of water activity and bio-imprinting on the catalytic activities and the thermostability of lipases were investigated for transesterification in an ionic liquid. The activities were enhanced by imprinting in $[C_4mim][PF_6]$ where the reactivity reached a maximum at an $a_w$ value of 0.8426, and a subsequent increase in water activity sharply reduced the reactivity due to high water solubility. The effects of water activity and imprinting on the kinetic parameters were greater than were those in benzene because of the high polarity of the ionic liquid. We found that water activity greatly affected the thermostability of lipase but not from imprinting.

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