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Poly(ethylene glycol)-lipase complexes that are highly active and enantioselective in ionic liquids

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Lipase-catalyzed alcoholysis between vinyl acetate and 2-phenyl-1-propanol was investigated in dialkylimidazolium-based ionic liquids. Although native lipase powder exhibited very low activity in an ionic liquid, forming a poly(ethylene glycol) (PEG)-lipase complex improved the lipase activity in the ionic liquid. The activity of the PEG-lipase complex was higher in ionic liquids than in common organic solvents (*n*-hexane, isooctane and dimethylsulfoxide). Fluorescence measurements using 4-aminophthalimide revealed that the ionic liquids were more hydrophilic than the organic solvents used for non-aqueous enzymology. A kinetic study of lipase-catalyzed alcoholysis in an ionic liquid ([Bmim][PF_6]) revealed that the Michaelis constant (*K*m) for 2-phenyl-1-propanol in the ionic liquid was half that in *n*-hexane, suggesting that the ionic liquid stabilized the enzyme-substrate complex. Finally, we carried out enantioselective alcoholysis of 1-phenylethanol in ionic liquids employing the PEG-lipase complex, and obtained high enantioselectivity, comparable to that in *n*-hexane.

Introduction

Room temperature ionic liquids have emerged during the last decade as alternative reaction solvents.[1–3] Ionic liquids, formally called molten salts, consist of an organic cation and an inorganic anion. They are liquid over a broad temperature range, are almost involatile, and dissolve polar and non-polar organic, inorganic and polymeric compounds.[4,5] The physical and chemical properties of ionic liquids can be modified by altering the cation, the anion, and the attached substituents. Moreover, ionic liquids are simple to manufacture and easy to recycle.

Several research groups have recently used ionic liquids as reaction media for biocatalytic processes. Cull *et al*. were the first to use an ionic liquid for the two-phase biotransformation of 1,3-dicyanobenzene to 3-cyanobenzamide and 3-cyanobenzoic acid using nitrile hydratase.[2] Erbeldinger *et al*. reported that (*Z*)-aspartame synthesis could be catalyzed by thermolysin in an ionic liquid.[3] Park and Kazlauskas demonstrated that ionic liquids are fairly hydrophilic in comparison to the common organic solvents employed for non-aqueous enzymology, and performed enzymatic syntheses involving hydrophilic substrates in ionic liquids.[6] Furthermore, some research groups have reported that enantioselectivities of enzymatic reactions in ionic liquids are high in comparison to those in common organic solvents.[7–9] These studies demonstrate the applicability of ionic liquids as novel media for enzymatic reactions. There are, however, few reports on the activation of enzymes for catalysis in ionic liquids. Kaar *et al*. recently studied an enzymatic reaction in ionic liquids in detail.[10] They also investigated several activation methods, such as covalent poly(ethylene glycol) modification, for lipase in an ionic liquid, and immobilization in polyurethane foam, however, they could not obtain a large improvement in lipase activity in ionic liquids.

Improvement of enzyme activity in non-aqueous solvents has been of great significance in the field of non-aqueous enzymology. Therefore, a number of enzyme activation methods have been developed, such as enzyme immobilization, reverse micellar systems, surfactant-coating and bioimprinting. To date, we have demonstrated that surfactant-coating of enzyme molecules is very effective for improving and maintaining the catalytic activity of various enzymes in ordinary organic solvents.[11–14] Here, we used poly(ethylene glycol) (abbreviated as PEG) as the enzyme-coating amphiphile for preparation of the PEG-lipase complex, because PEG is soluble in ionic liquids. In the present study, we investigated alcoholysis catalyzed by the PEG-lipase complex in ionic liquids and compared these results to those in ordinary organic solvents. The kinetics and the enantioselectivity of the lipase-catalyzed reaction in ionic liquids are discussed.

Materials and methods

Lipase PS (from *Pseudomonas cepacia*) and lipase AK (from *Pseudomonas fluorescens*) were kindly provided by Amano Enzyme Inc. (Nagoya, Japan). 1-Methylimidazole, 1-chlorobutane, 1-chlorohexane, 1-chlorooctane and lithium bistrifluoromethanesulfonimidate were obtained from Aldrich Co. (Milwaukee, WI). All other chemicals were purchased from Wako Pure Chemicals Ltd., Japan.

Synthesis of ionic liquids

The synthesis of ionic liquids as described by Huddleston *et al.*[1] and Cull *et al.*[2] was modified. 1-Methylimidazole (103 g, 1.25 mol) and 1-chlorobutane (139 g, 1.5 mol) or 1-chlorohexane (189 g, 1.5 mol) or 1-chlorooctane (223 g, 1.5 mol), were mixed in a flask with gentle stirring. The flask was connected to a reflux condenser and nitrogen gas was passed over the contents. The flask was heated in an oil bath at 70 °C. The reaction was carried out under these conditions for 72 h. Excess chloroalkane (upper phase) was decanted and the residual chloroalkane was removed under reduced pressure with heating (110 °C). The product was washed three times with 250 ml of ethyl acetate. Residual ethyl acetate was removed under reduced pressure.

1-Butyl-3-methylimidazolium hexafluorophosphate [Bmim][PF_6], 1-hexyl-3-methylimidazolium hexafluorophosphate [Hmim][PF_6] and 1-octyl-3-methylimidazolium hexafluorophosphate [Omim] [PF_6]

Ammonium hexafluorophosphate (200 g, 1.3 mol) in 0.5 L water and [Bmim][Cl] (184 g, 1.0 mol) or [Hmim][Cl] (208 g, 1.0 mol) or [Omim][Cl] (231 g, 1.0 mol) in 0.5 L water were

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mixed, stirred vigorously for 2 h, and incubated at room temperature for 12 hours. The two phases formed were allowed to separate, and the upper aqueous phase was decanted. The lower organic phase was washed twice with 500 ml of water, then washed with a saturated sodium bicarbonate solution to remove free hexafluorophosphate ion. Ionic liquids were extracted from the mixture using dichloromethane (250 ml), and the organic phase was removed under reduced pressure at 110 °C. The remaining water in the ionic liquids was removed by lyophilization for 1 day.

1-Butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide [Bmim][Tf_2N]

[Bmim][Cl] (0.15 mol) was dissolved in 50 ml of water and then an equimolar amount of lithium bistrifluoromethanesulfonimidate was added. After mixing for 2 h, the generated lower phase was subjected to vacuum drying to remove water. Ionic liquids synthesized were confirmed by elemental analysis.

1-Butyl-3-methylimidazolium hexafluorophosphate. Anal. Calcd for [Bmim][PF_6]: C, 33.81; H, 5.32; N, 10.22. Found: C, 33.88; H, 5.27; N, 9.86%.

1-Hexyl-3-methylimidazolium hexafluorophosphate. Anal. Calcd for [Hmim][PF_6]: C, 38.41; H, 6.13; N, 9.00. Found: C, 38.5; H, 6.10; N, 8.94%.

1-Octyl-3-methylimidazolium hexafluorophosphate. Anal. Calcd for [Omim] [PF_6]: C, 42.35; H, 6.81; N, 8.23. Found: C, 42.43; H, 6.87; N, 8.20%.

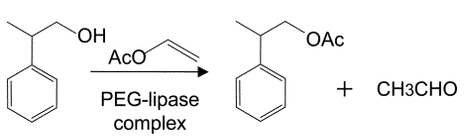
1-Butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]-imide. Anal. Calcd for [Bmim][Tf_2N]: C, 28.64; H, 3.61; N, 10.02. Found: C, 28.7; H, 3.57; N, 10.09%.

Preparation of poly(ethylene glycol) (PEG)-lipase complexes

The preparation of PEG-lipase complex was previously described.[14] Each lipase (10 mg) and poly(ethylene glycol) 20,000 (PEG) was dissolved in phosphate buffer (5 ml, pH 7.0). The typical molar ratio (PEG : lipase) was 10. For effective coating of the lipase surface,[13] toluene (13 ml) was added to the lipase solution and emulsified at 20,000 rpm for 3 min using a homogenizer (Polytron PT 3000, Kinematica AG, Switzerland) in an ice bath, to prepare water-in-oil (w/o) emulsions. The w/o emulsions were immediately frozen in liquid nitrogen, followed by lyophilization for 24 h using a freeze-drier. The PEG-lipase complexes were obtained as white powders. As a control, the native lipase was lyophilized from the same phosphate buffer solution.

Enzymatic reaction in ionic liquids

Typical alcoholysis between vinyl acetate (100 mmol l^{-1}) and 2-phenyl-1-propanol (100 mmol l^{-1}) to produce 2-phenyl-1-propyl acetate (Scheme 1) was conducted in 1 ml of an ionic liquid using native lyophilized lipases (1 mg) or PEG-lipase complexes (containing 1 mg of original lipase PS) at 45 °C. The biocatalyst was suspended in the reaction medium. Samples were periodically withdrawn from the reaction medium and directly subjected to HPLC analysis. The substrate and product were monitored at 254 nm using an HPLC system (JASCO 2000 series) on a 4.6 × 250 mm ODS column (Capcell Pak C18MG, Shiseido). A solvent system of acetonitrile/water (80 : 20) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. The initial production rate of 2-phenyl-1-propyl acetate was adopted as the lipase activity.



Scheme 1 Alcoholysis between 2-phenyl-1-propanol and vinyl acetate.

To investigate the enantioselectivity of lipase, alcoholysis between vinyl acetate (100 mmol l^{-1}) and 1-phenylethanol (100 mmol l^{-1}) was carried out in 1 ml of ionic liquids and *n*-hexane using the PEG-lipase complex (containing 1 mg of original lipase PS) at 45 °C. The substrate and product were monitored at 254 nm using the HPLC system on a 4.6 × 150 mm chiral column (Chiracel OD-RH, Daicel).

Fluorescence measurement using 4-aminophthalimide

To evaluate the hydrophobicity of ionic liquids, the fluorescence of 4-aminophthalimide in ionic liquids was measured at 25 °C using a fluorescence spectrophotometer (LS50B, Perkin Elmer).[15–18]

Results and discussion

Alcoholysis reaction catalyzed by PEG-lipase complex in ionic liquids

Many studies on enzymatic reactions in ionic liquids have employed enzymes as native powders or commercially available immobilized forms. In non-aqueous enzymology, enzyme preparation is very important for enzymatic performance in non-aqueous media. We firstly compared the activity of native lipase powder with that of the PEG-lipase complex in an ionic liquid. The lipase PS-catalyzed alcoholysis between vinyl acetate and 2-phenyl-1-propanol was carried out in [Bmim][PF_6] at 45 °C. As previously reported,[14] the lipase PS in a complex with PEG 20,000 catalyzed the reaction much more effectively than the native lipase did (Fig. 1). This result means that the methodology used for enzyme activation in common organic solvents is valid for activating enzyme in an ionic liquid. Very recently, Kaar *et al*. investigated the covalently-linked PEG-lipase complex in an attempt to improve lipase activity in ionic liquids, however, they found that covalent-PEG modification did not improve the lipase activity.[10] The major differences between their PEG-modified lipase and the one used in our present study were in the preparation procedure and the PEG employed for lipase modification. Lipase is an enzyme that works at oil-water interfaces and lipase activity is generally improved by the presence of an oil-water interface together with a conformational change in the lipase molecule.[19,20] Our preparation procedure for the PEG-lipase complex could provide a toluene/water interface for the lipase molecules, which probably induced the enhancement of the lipase activity in the ionic liquid. While Kaar *et al*. used the PEG specific for the covalent modification of the enzyme,[10] the present study employed common poly(ethylene glycol) with molecular weight 20,000. The PEG used in this study dissolves well in

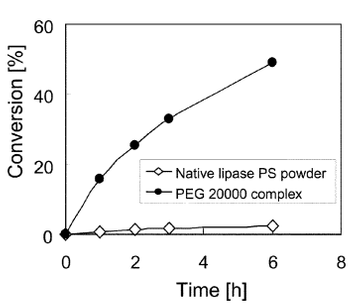


Fig. 1 Time course of alcoholysis catalyzed by lyophilized native lipase PS and PEG-lipase PS complex in [Bmim][PF_6]. The reaction between 2-phenyl-1-propanol (100 mmol l^{-1}) and vinyl acetate (100 mmol l^{-1}) was carried out in 1 ml of [Bmim][PF_6] at 45 °C employing 7.5 mg of the PEG-lipase PS complex (containing 1 mg of original lipase PS). No water was added.

Table 1 Fluorescent measurements of 4-aminophthalimide in various solvents

Solvent	Wavelength of emission/nm	Wavelength of excitation/nm
Water	543	367
Dimethylsulfoxide	475	370
[Bmim][PF_6]	470	362
[Hmim][PF_6]	464	365
[Omim][PF_6]	468	363
[Bmim][Tf_2N]	470	361
Acetonitrile	458	369
n-Hexane	416[a]	

[a] Ref. 17.

[Bmim][PF_6] (the solubility is greater than 100 g/l). The high solubility of the PEG 20,000 would help the dispersion of the PEG-lipase complex in the ionic liquid, resulting in the high lipase activity in the ionic liquid. It should be noted that the direct addition of the same amount of PEG to the ionic liquid did not enhance the activity of native lipase (data not shown) and that the elemental analysis confirmed that PEG molecules were not desorbed from the PEG-lipase complex in the ionic liquid. The measurement of absorbance at 280 nm of the filtered reaction medium also proved that the PEG-lipase complex was not solubilized in the ionic liquid.

Furthermore, we examined the effect of PEG molecular weight on the lipase activity in [Bmim][PF_6] (Fig. 2). The PEG with molecular weight 20,000 provided the highest activity to the lipase PS in the ionic liquid, presumably due to the good dispersion of the PEG-lipase complex in the ionic liquid, although the PEG-lipase complex was insoluble in the ionic liquid. The following experiments were carried out employing PEG with a molecular weight of 20,000 for the lipase modification.

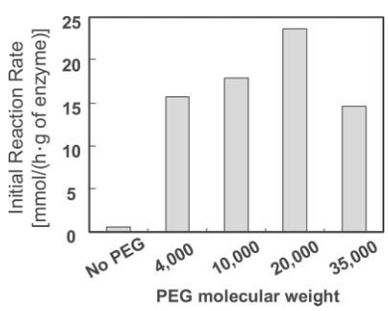


Fig. 2 Effect of the molecular weight of poly(ethylene glycol) on the lipase activity in [Bmim][PF_6]. The reaction conditions were the same as those in Fig. 1. The employed PEG-lipase complex contained 1 mg of original lipase PS.

Polarity of the ionic liquids and the effect of solvents on the lipase activity

The polarity or hydrophobicity of the reaction medium often influences enzymatic activity in organic solvents. Laane *et al.* reported that enzymatic activity in organic solvents was related to the hydrophobicity (octanol-water partition coefficient; log *P*) of the organic solvents.[21] Room temperature ionic liquids are relatively novel solvents, therefore, there is considerable uncertainty regarding the properties of ionic liquids. We studied the polarities of various ionic liquids and compared the values obtained in common organic solvents. Log *P* is an important solvatochromic parameter but we could not measure the log *P* values for the ionic liquids because of the low solubility of the ionic liquids in octanol. Instead, a polarity-sensitive fluorescent probe, 4-aminophthalimide, was used to determine the polarities of the ionic liquids. Because of the charge-transfer nature, the lowest excited state of 4-aminophthalimide is very sensitive to solvent polarity. The fluorescence maximum exhibits a shift as large as 115 nm on changing the solvent from ether to water.[15–18] Table 1 summarizes the emission wavelengths of 4-aminophthalimide in the ionic liquids along with those for 4-aminophthalimide in common organic solvents. The emission wavelengths of 4-aminophthalimide in polar solvents, such as acetonitrile and dimethylsulfoxide, were relatively long, while that in a non-polar solvent, *n*-hexane, was relatively short. Fluorescence measurements for the ionic liquids revealed that the ionic liquids were highly polar, similar to dimethylsulfoxide and acetonitrile, instead of reflecting the diversity of their structures. It should be noted that the ionic liquids are not water-miscible, unlike dimethylsulfoxide and acetonitrile.

We investigated the activity of the PEG-lipase complex in the ionic liquids and in common organic solvents. Under the experimental conditions, the PEG-lipase complex exhibited higher activities in the ionic liquids than in the ordinary organic solvents (Fig. 3). Laane *et al.* demonstrated that solvents with a log *P* less than two are hydrophilic in nature and likely to have an unfavorable effect on enzymatic reactions.[21] The ionic liquids tested here were very hydrophilic compared to acetonitrile (log *P*: −0.34) and dimethylsulfoxide (log *P*: −1.35), however, the PEG-lipase complex exhibited much higher activity in ionic liquids than in acetonitrile and dimethylsulfoxide. This is probably because the ionic liquids do not have an unfavorable effect on the lipase molecules while acetonitrile and dimethylsulfoxide have an inherent ability to deactivate enzymes. This result is consistent with previous reports.[6,22]

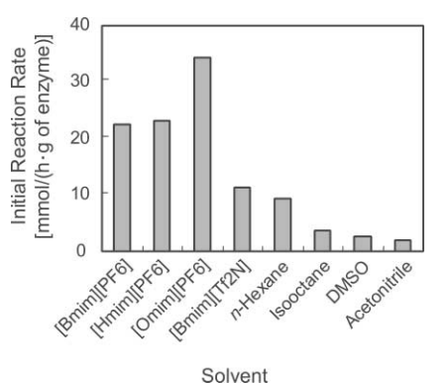


Fig. 3 Lipase activities in ionic liquids and organic solvents. The reaction conditions were the same as those in Fig. 1.

pH memory and optimum temperature in ionic liquids

In non-aqueous enzymology, enzymes are known to remember the pH of the buffer used during preparation, and an enzyme prepared at an optimum pH exhibits maximum activity in non-aqueous solvents; this is called "pH memory".[23] Here, we investigated the effect of the buffer pH used for preparation of the enzyme for lipase activity in [Bmim][PF_6] (Fig. 4). A pH 7 buffer afforded the highest activity for the PEG-lipase PS complex in [Bmim][PF_6], while native lipase PS also showed maximal activity around pH 7 in aqueous solution (data from the enzyme manufacturer). That is, we observed "pH memory"

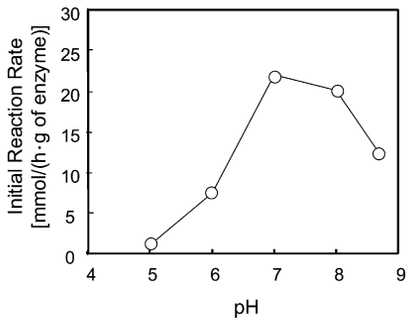


Fig. 4 Effect of pH during the preparation of the PEG-lipase complex on alcoholysis activity in [Bmim][[PF_6]. The reaction conditions were the same as those in Fig. 1.

in the lipase-catalyzed reaction in the ionic liquid as well as in organic solvents.

Many researchers have reported high thermal stability of enzymes in hydrophobic organic solvents and also demonstrated that the optimum reaction temperature for enzymatic reaction in the organic solvents is higher than that in an aqueous solution. The optimum reaction temperature depends on the type of solvent used as a reaction medium. Fig. 5 shows the effect of reaction temperature on the activity of the PEG-lipase complex in [Bmim][PF_6]. The optimum temperature for the PEG-lipase complex activity was found to be 45 °C, while the native lipase PS exhibited maximal activity in water around 50 °C (data from the enzyme manufacturer). In contrast to when hydrophobic organic solvents are used, the optimum temperature in the ionic liquid was almost the same as that in water. The high polarity of the ionic liquid would result in an optimum reaction temperature similar to that in water.

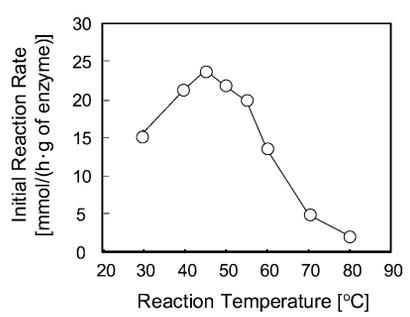


Fig. 5 Effect of reaction temperature on the activity of PEG-lipase PS in [Bmim][[PF_6]. Except for reaction temperature, the reaction conditions were the same as those in Fig. 1.

Effect of water content on lipase activity in ionic liquids

One of the most important parameters affecting the enzymatic activity in an organic solvent is the water content. We evaluated the effect of water content on the lipase activity in [Bmim][PF_6] by adding a small amount of water to the ionic liquid (Fig. 6). Dry [Bmim][PF_6], which has not had any water added, contains 590 ppm water.[24] The PEG-lipase complex exhibited the highest

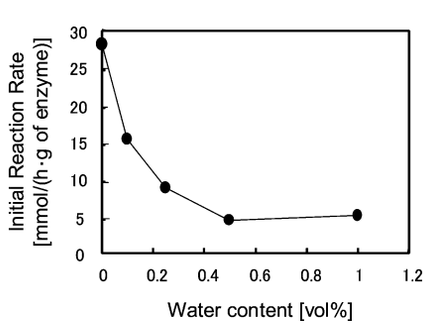


Fig. 6 Effect of water content on the activity of PEG-lipase PS in [Bmim][PF_6]. The reaction conditions were the same as those in Fig. 1.

Table 2 Kinetic parameters of alcoholysis reaction catalyzed by PEG-lipase complex in [Bmim][PF_6] and *n*-hexane

Solvent	*V*max/mmol (g·h)$^{-1}$	*K*m/mmol l^{-1}
[Bmim][PF_6]	55	89
n-Hexane	47	167

activity in dry [Bmim][PF_6]. Although the addition of a small amount of water usually facilitates enzymes in organic solvents,[25,26] the addition of water to [Bmim][PF_6] reduced the lipase activity. The water initially contained in the dry [Bmim][PF_6] is thought to be sufficient to facilitate the PEG-lipase complex. Further addition of water above 0.5 vol% did not affect the lipase activity. The alcoholysis in the present study is transesterification *via* acylated-lipase. The presence of water simultaneously causes hydrolysis of acylated-lipase to produce acetic acid. An excess amount of water produced acetic acid and would result in low alcoholysis activity.

Kinetics of lipase-catalyzed alcoholysis in ionic liquids

The higher activity of the PEG-lipase complex in ionic liquids than in the common organic solvents, as shown in Fig. 3, indicates some differences between the kinetic parameters in the ionic liquids and the organic solvents. We examined the kinetic parameters of the lipase-catalyzed alcoholysis in [Bmim][PF_6] and in *n*-hexane. Although lipase-catalyzed esterification in an organic solvent follows a Ping-Pong Bi-Bi mechanism with inhibition by an excess amount of alcohol,[27] to simplify the kinetic analysis, we assumed that the alcoholysis reaction conforms to a pseudo-first-order reaction in the presence of excess vinyl acetate. Figs. 7a and 7b show the effect of the concentration of 2-phenyl-1-propanol on the initial reaction rate in [Bmim][PF_6] and in *n*-hexane. The intercepts of the y-axis and the x-axis give the *V*max and *K*m for 2-phenyl-1-propanol, respectively. The kinetic parameters obtained are summarized in Table 2. The *V*max in [Bmim][PF_6] was slightly higher than that in *n*-hexane, but the *K*m in [Bmim][PF_6] was half that in *n*-hexane, which resulted in 2.2-fold enhancement of the specificity constant (*V*max/*K*m) in the ionic liquid compared to *n*-hexane. These results suggest that the enzyme-substrate

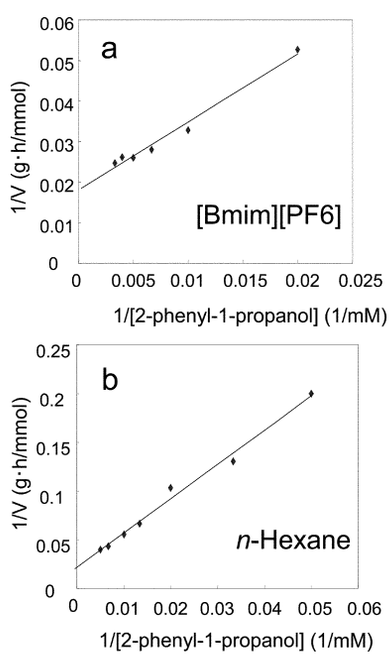


Fig. 7 Effect of the 2-phenyl-1-propanol concentration on the initial reaction rate in [Bmim][PF_6] and in *n*-hexane. The alcoholysis reaction was carried out in 1 ml of a solvent containing 2-phenyl-1-propanol, vinyl acetate (1.2 mol l^{-1}) and PEG-lipase complex (containing 1 mg of original lipase PS) at 45 °C.

Table 3 Enantiomeric excess (ee_p of *R*-isomer derivative [%]), conversion and *E* value (for *R*-isomer derivative [%]) in the alcoholysis of 1-phenylethanol and vinyl acetate catalyzed by the PEG-lipase complexes in ionic liquids and in *n*-hexane[a]

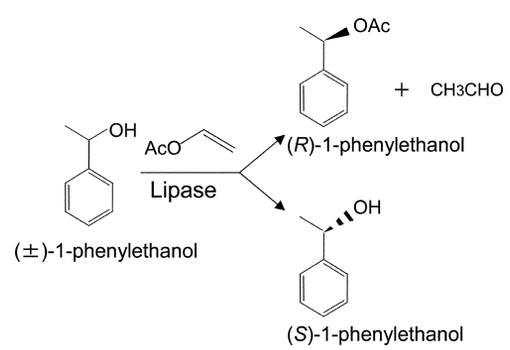
	Lipase PS			Lipase AK		
	ee_p	Conversion [%]	*E* value	ee_p	Conversion [%]	*E* value
[Bmim][Tf_2N]	98	15	120	98	47	280
[Bmim][PF_6]	80	43	17	80	42	16
[Omim][PF_6]	80	29	12	77	25	9.9
n-Hexane	80	17	11	83	53	37

[a] The results were obtained after 48 h.

(2-phenyl-1-propanol) complex is more likely to form in [Bmim][PF_6] than in *n*-hexane, and was stabilized more in the ionic liquid than in *n*-hexane. This is one of the reasons why the PEG-lipase complex exhibited higher activity in the ionic liquids than in the organic solvents. Solvation of substrates is also of great importance for enzymatic activity in non-aqueous media.[28–29] We are currently studying the solvation effect of various substrates on lipase activity in ionic liquids.

Enantioselective alcoholysis catalyzed by PEG-lipase complex in ionic liquids and *n*-hexane

Several recent studies reported that lipases exhibit higher enantioselectivities in ionic liquids than in common organic solvents.[7–9] In these studies, native lipase powder or immobilized lipase were employed for the enantioselective biotransformations. In the present study, we demonstrated that the PEG-lipase complex was highly active in ionic liquids, meaning that the PEG-lipase complex could serve as an active biocatalyst for enantioselective reactions. We examined the enantioselectivity of the alcoholysis between racemic 1-phenylethanol and vinyl acetate catalyzed by the PEG-lipase complex in ionic liquids and in *n*-hexane (Scheme 2). Table 3 lists the enantioselectivities and conversions after 48 h of reaction. In [Bmim][Tf_2N], lipases PS and AK in a complex form with PEG 20,000 exhibited high enantioselectivities for the *R*-isomer (the *E* values were 120 and 280.), while the *E* values in *n*-hexane were 17 and 37, respectively. The enantioselectivities of both PEG-lipase complexes were higher in [Bmim][Tf_2N] than in other ionic liquids. This result agreed with those reported by other research groups.[7–9] However, both PEG-lipase complexes showed enantioselectivities in [Bmim][PF_6] and [Omim][PF_6] comparable to that in *n*-hexane. These results showed that employing ionic liquids as reaction media does not always improve enantioselectivity in lipase-catalyzed reactions. Note that the catalytic activity of the native lipases was too low to determine the enantioselectivity in the ionic liquids.



Scheme 2 Alcoholysis between 1-phenylethanol and vinyl acetate.

Conclusions

We have demonstrated that the PEG-lipase complex is highly active in ionic liquids, comparable to, or more than, its activity in ordinary organic solvents, even though the ionic liquids were considerably polar. The promotion of the formation of the enzyme-substrate complex in ionic liquids contributed to the high activity of the PEG-lipase complex in ionic liquids. Similarly to enzymology in organic solvents, lipase pH memory was observed in ionic liquids. The enantioselectivity of the lipase-catalyzed reaction in ionic liquids was comparable to or higher than that in *n*-hexane. These results demonstrated that the PEG-lipase complex serves as a highly active and enantioselective biocatalyst in ionic liquids.

Acknowledgements

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