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Room Temperature Solid-Phase Ionic Liquid-Immobilized Enzyme for Biocatalysis in Organic Solvent: Markedly Enhanced Enantioselectivity†

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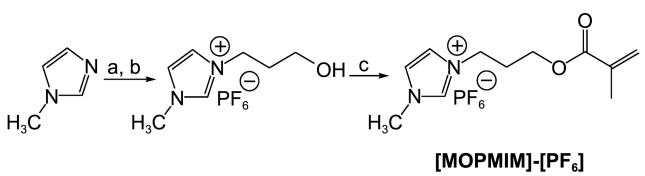
Nonaqueous biocatalysis provides a useful component of methodology in organic synthesis.[1] For example, lipase catalysis in organic solvents is of great use for the synthesis of optically active compounds such as chiral alcohols, acids, and their esters.[2] However, biocatalysis in nonaqueous media often suffers from reduced activity, selectivity or stability of enzymes.[3] To overcome these limitations, many approaches have focused on the development of more efficient enzymes. Among them, the use of ionic liquids as an alternative solvent for the biotransformations in nonaqueous solvents enhanced the activity, selectivity, and stability of enzymes.[4-10] And novel approach with ionic liquid-coated enzyme (ILCE), which was readily prepared by mixing the enzyme powder with a room temperature solid phase ionic liquid (RTSPIL) at elevated temperature, was previously reported showing enhanced enantioselectivity and stability in organic solvents.[11] Also, we have recently reported that the enzyme coated by RTSPIL during lyophilization in aqueous medium exhibited better activity.[12] Although it was difficult to clarify the reason that why ionic liquids increase significantly the catalytic activity as well as the selectivity of the lipase for enzyme-catalyzed transesterifications, we have speculated that the ionic environments by ionic liquid could improve activity and selectivity of the enzyme in biotransformations.

On the other hands, numerous methods have been developed for the immobilization of enzymes onto various supports such as ceramic, polymer, gels, membranes, and microcapsules to increase the stability and recyclability of the biocatalyst in organic syntheses.[13] To extend our knowledge of the application with ionic liquids for biocatalysis, we have expected that the immobilized enzyme in ionic environments could be more efficient during immobilizing of enzymes on supporting materials compared to that in non-ionic environments. Herein, we wish to report the synthesis of new RTSPIL composed of imidazolium cation modified with the methacryloyl group and hexafluorophosphate (PF_6) anion and the biocatalysis in an organic solvent using lipase immobilized on this RTSPIL, which is an aggregate of small molecules.

The novel RTSPIL, [MOPMIM]-[PF_6], ([MOPMIM]$^+$ = 1-(3'-methacryloyloxypropyl)-3-methylimidazolium) was synthesized according to the following procedure[11] reported previously to immobilize the lipase, which was modified with methacryloyl group that can readily interact with the functional moieties such as thiol, amine, and alcohol on the surface of lipase.(Scheme 1). [MOPMIM]-[PF_6] is very stable in moisture and melts at temperature of 70 °C. The remarkable property of [MOPMIM]-[PF_6] is low solubility in water due to hydrophobic character of PF_6 anion, which lets us immobilize the lipase on RTSPIL in a buffer solution.[12] Indeed, *Burkholderia cepacia* lipase (BCL, native) was immobilized on [MOPMIM]-[PF_6] through interaction between [MOPMIM]-[PF_6] and BCL in a phosphate buffer.

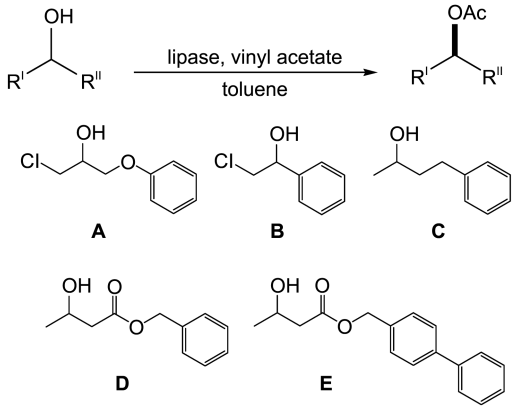
The enantioselectivity of BCL/[MOPMIM]-[PF_6] was examined in the transesterification reaction of bulky secondary alcohols (**A**-**E**) with vinyl acetate in toluene at 25 °C (Scheme 1). For comparison, the same reactions were carried out with non-immobilized BCL. After the reaction reached 10-50% completion, the enzymes were removed by filtration and the resulting solution was concentrated. The organic residues were subjected to silica gel chromatography to obtain unreacted substrate and acetylated product. Their optical purities were then determined by high-performance liquid chromatography (HPLC) using a chiral column, which allowed us to measure the enantiomeric excess (ee) up to > 99.5%. The E values were calculated using the equation, $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$, were $c = ee_s/(ee_s + ee_p)$.[14] The results are described in Table 1.

The data from Table 1 indicate that the transesterification of **A**-**E** catalyzed by BCL/[MOPMIM]-[PF_6] proceeded with better enantioselectivity in toluene than by BCL (Table 1, entry 1-10). The best enantioselectivity (E = 1194) was ob-



Scheme 1. Synthesis of [MOPMIM]-[PF_6] 2d. a) 3-chloro-1-propanol, 70°C, 1d. b) hexafluoro phosphoric acid, rt., 6h. c) methacryloyl chloride. rt., 1d.

†This paper is dedicated to Professor Eun Lee on the occasion of his honourable retirement.



Scheme 2. Lipase-catalyzed Transesterification of various bulky secondary alcohols.

served for the transesterification reaction of **A** substrate by BCL/[MOPMIM]-[PF_6]. The enantioselectivity enhancement by the use of BCL/[MOPMIM]-[PF_6] is about 2-11 fold compared to that by BCL. The results are comparable to those reported previously for the ionic liquid coated enzyme (ILCE) and ionic liquid co-lyophilized enzyme (ILCoE), suggesting that the RTSPIL enhances the enantioselectivity of the enzyme in a similar fashion to the ILCE and ILCoE. However, it is not clear why the enantioselectivity of the enzyme is improved by RTSPIL in this study. We speculate that these high enantioselectivity of BCL/[MOPMIM]-[PF_6] may be caused by an ionic environment, which is provided by the support, RTSPIL around the BCL. That may be due to more favorable structural adaptation of the enzyme in a polar ionic environment. This interpretation could be supported by the results that the co-lyophilization of the enzyme with ionic salts have resulted in increasing enzymatic activities in organic solvents.[15-16]

We also investigated the BCL/[MOPMIM]-[PF_6]-catalyzed transesterification in various organic solvents. The results were summarized in Table 2. The BCL/[MOPMIM]-[PF_6] exhibited better enantioselectivity in non-polar solvent such as toluene and *t*BuOMe, but did not work in polar solvents such as THF and $CHCl_3$. These note that BCL/[MOPMIM]-[PF_6] can be denatured in polar solvents because the [MOPMIM]-[PF_6] is well-soluble in polar organic solvents. Therefore, the BCL/[MOPMIM]-[PF_6] should be used in non-polar solvents such as toluene and *t*BuOMe.

The stability of the biocatalyst immobilized on RTSPIL was investigated by recycling of the enzyme in transesterification of the substrate C (Table 3). The data in Table 3 indicate that BCL/[MOPMIM]-[PF_6] could be reusable without a big loss of activity and enantioselectivity more than 8 times.

In summary, this work demonstrated that RTSPIL can serve as the support for immobilization of lipase with an advantage of enhancing the enantioselectivity. The RTSPIL has enough of the capacity for the support, which can immobilize the lipase, and the BCL/[MOPMIM]-[PF_6]-catalyzed transesterifications takes place effectively in organic medium with impressively high enantioselectivity. Although there needs more study regarding the interaction between [MOPMIM]-[PF_6]

Table 1. The Enantioselectivities for the Lipase-Catalyzed Transesterification[a]

entry	substrate	lipase	ee_s	ee_p	E
1	**A**	BCL	0.418	0.965	84.7
2		BCL/[MOPMIM]-[PF_6]	0.887	0.995	1194
3	**B**	BCL	0.420	0.981	158
4		BCL/[MOPMIM]-[PF_6]	0.548	> 0.995	> 450
5	**C**	BCL	0.188	0.986	171
6		BCL/[MOPMIM]-[PF_6]	0.517	> 0.995	> 670
7	**D**	BCL	0.632	0.959	92
8		BCL/[MOPMIM]-[PF_6]	0.486	0.988	269
9	**E**	BCL	0.676	0.972	143
10		BCL/[MOPMIM]-[PF_6]	0.485	0.989	293

[a]On the basis analyses by HPLC using a chiral column. Analytical condition: chiralcel OD, hexane/2-propanol system.

Table 2. The Enantioselectivities for the Lipase-Catalyzed Transesterification in Various Organic Medium

entry	solvent	enzyme	ee_s	ee_p	E
1	toluene	BCL	0.428	0.965	85
2		BCL/[MOPMIM]-[PF_6]	0.887	> 0.995	> 1194
3	*t*BuOMe	BCL	0.643	0.991	434
4		BCL/[MOPMIM]-[PF_6]	0.940	0.991	793
5	THF	BCL	0.420	0.980	150
6		BCL/[MOPMIM]-[PF_6]		trace	
7	$CHCl_3$	BCL	0.208	0.992	305
8		BCL/[MOPMIM]-[PF_6]		trace	

Table 3. Recycling of BCL/[MOPMIM]-[PF6] in transesterification of substrate **C**

run	time	ee_s	ee_p	C (%)[a]	E [b]
1	12h	0.279	> 0.995	23	> 524
2	12h	0.329	> 0.995	25	> 551
3	12h	0.254	> 0.995	20	> 512
4	12h	0.155	> 0.995	13	> 465
5	12h	0.181	> 0.995	15	> 624
6	12h	0.243	> 0.995	20	> 506
7	12h	0.238	> 0.995	19	> 504
8	12h	0.446	> 0.995	31	> 620

[a] $C(\%) = ee_s/(ee_s+ee_p) \times 100$, [b] $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$, where $c = ee_s/(ee_s+ee_p)$.

and BCL, we believe that they can be applied to biotechnology. It also has potential as a novel area for biotechnology, and the immobilization of the lipase on the assembly of small molecules such as RTSPIL now opens up a new field in immobilization technology.

Experimental Section

Burkholderia cepacia lipase as crude enzyme was available from some commercial suppliers such as Fluka, Roche, and Amano. We used the one provided by Amano. And all reagents were purchased from Aldrich. Thin-layer chromatography was performed in Merck silica gel 69F245 and column chromatography was performed using a Merck silica gel 60. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-300 instrument with peak referenced to tetramethyl silane in $CDCl_3$. Mass spectroscopy was recorded using a KRATOS Ms 25RFA (70 eV, EI). HPLC from SpectraSYSTEM (P2000) and GC from HEWLETT PACKARD (HP6890) were used for determining the enantioselectivity and reactivity of enzymes.

The RTSPILs used in this study were prepared according to the following procedure reported previously[11]: 3-Chloro-1-propanol (0.125 mol) was dissolved in 1-methylimidazole (0.125 mol) and then refluxed for 24 h at 70 °C. Water (100 mL) and HPF_6 (0.15 mol) were added to the reaction mixture and stirred vigorously at room temperature. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and washed with saturated $NaHCO_3$ (2 × 100 mL). The organic layer was dried and concentrated *in vacuo*. The methacryloyl chloride (0.2 mol) was added to the reaction product, the 1-(3'-hydroxypropyl)-3-methlyimidazolium hexafluorophosphate (0.1 mol) and stirred vigorously for 24 h at room temperature. The final product, [MOPMIM]-[PF_6] was obtained in good yields (> 80%).

A suspension of crude BCL (Amano PS) in a phosphate buffer (10 mL, pH = 7) was stirred for some time at room temperature. After filtration through Celite, to the filtrate was added RTSPIL (200 mg) and the mixture was then stirred for 20-24 h at room temperature. The mixture was filtered, and the resulting lipase-supporting RTSPIL was dried and stored under suitable conditions.

The bulky secondary alcohol substrate (20 mg, 0.1 mmol), vinyl acetate (28 mL, 0.3 mmol), and lipase (0.5 mg/mmol) were added in toluene (0.5 mL), and the resulting semi-homogeneous mixture was shaken at 170 rpm and 25 °C for 8 h. Then, the enantiomeric purities were determined by HPLC using a chiral column.

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