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Immobilization of Enzyme on Hydrophobic Room Temperature Solid-Phase Ionic Liquids and Its Use for Biocatalytic Transesterification in Organic Solvent: Remarkably Enhanced Activity of Lipase

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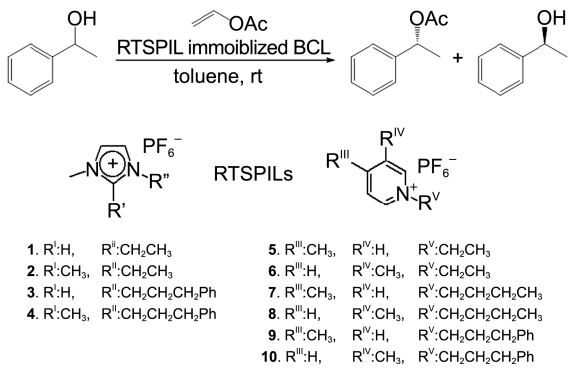
Nonaqueous biocatalysis provides a useful methodology in organic synthesis.[1] For example, lipase catalysis in organic solvents is of great use for the synthesis of optically active compounds such as chiral alcohols, acids, and their esters.[2] However, biocatalysis in nonaqueous media often reduces the activity, selectivity, and stability of the enzymes used.[3] Many approaches have been proposed to overcome this limitation. Among them, the use of ionic liquids as an alternative solvent for biotransformations in nonaqueous solvents has been found to enhance the activity, selectivity, and stability of enzymes.[4-10] In addition, a novel approach with ionic liquid-coated enzyme, which was readily prepared by mixing the enzyme powder with a room temperature solid-phase ionic liquid (RTSPIL) at an elevated temperature, has been reported to afford enhanced enantioselectivity and stability in organic solvents.[11] Furthermore, we have recently reported that coating of an enzyme by RTSPIL during lyophilization in an aqueous medium improves the enzyme activity. In particular, in the case of lipase, ionic liquids significantly increased the catalytic activity and selectivity for enzyme-catalyzed transesterifications.[12] Although the reason why ionic liquids improve activity and selectivity of an enzyme in biotransformations remains unclear, we speculate that the ionic environment provided by the liquids is responsible for the same.

On the other hand, 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] However, these immobilizations have been performed in nonionic environments; given the effectiveness of ionic liquids for biocatalysis, we expected that the immobilization of an enzyme on supporting materials in ionic environments would be more efficient than that in nonionic environments. Our research in this area has very recently lead us to report the synthesis of a new RTSPIL, [MOPMIM]-[PF_6] ([MOPMIM]$^+$ = 1-(3'-methacryloyloxypropyl)-3-methylimidazolium), by the modification of an imidazolium cation with a methacryloyl group and a hexafluorophosphate (PF_6) anion. In addition, we used this RTSPIL, which is an aggregate of small molecules, to immobilize lipase for transesterification of bulky secondary alcohol in an organic solvent, resulting in an about 2–11-fold increase in enantioselectivity compared to that of a nonimmobilized enzyme.[14] Recently, we found that an enzyme in buffer solution can be immobilized on bulk aggregates of hydrophobic RTSPILs based on alkyl imidazolium (or pyridinum) cation and PF_6 anion, which have not functional moieties such as methacryloyl group, *via* ionic attraction with surface of enzyme.

Herein, we further report the activity of an enzyme immobilized on various RTSPILs for the transesterification of *sec*-phenethyl alcohol with vinyl acetate in an organic solvent (Scheme 1). Interestingly, we found that the RTSPIL-immobilized enzyme exhibited an around two-orders-of-magnitude increase in catalytic activity and could be reused in an organic solvent.

The ten RTSPILs with PF_6 anion (**1**-**10**) used in this study were prepared according to the procedure reported previously: 1-ethyl-3-methylimidazolium ([EMIM], **1**, mp 60 °C), 1-(3-phenylpropyl)-3-methyl-imidazolium ([PPMIM], **2**, mp 52 °C), 1-(3-phenylpropyl)-2,3-dimethylimidazolium ([PPDMIM], **3**, mp 116 °C), 1-dodecyl-3-methylimidazolium ([C12MIM], **4**, mp 50 °C), 1-dodecyl-2,3-dimethylimidazolium ([C12DMIM], **5**, mp 69 °C), 1-ethyl-4-methylpyridinum ([E4MPy], **6**, mp 78 °C), 1-butyl-4-methylpyridinum ([B4MPy], **7**, mp 43 °C), 1-(3-phenylpropyl)-4-methylpyridinum ([PP4MPy], **8**, mp 80 °C), and 1-(3-phenylpropyl)-3-methylpyridinum ([PP4MPy], **9**, mp 74 °C).



Scheme 1. Schematic description of RTSPILs immobilized BCL catalyzed transesterification in anhydrous toluene.

These RTSPILs were composed of various imidazolium (or pyridinium) cation derivatives and a PF_6 anion, which provides the hydrophobic character to immobilize the enzyme in a buffer solution.[12]

As a representative enzyme for immobilization in an aqueous medium, the commercially available *Burkholderia cepacia* lipase (BCL; brand names: Lipase PS and Lipase PS-C) was chosen, since it has been frequently used for biotransformations in organic solvents. The RTSPIL-immobilized enzyme was prepared with a protocol similar to that reported previously.[14] The enzyme solution was prepared by dissolving crude BCL in a pH 7.2 sodium phosphate buffer and was filtered to remove insoluble contaminants. The synthesized RTSPIL was added to the enzyme solution, and then, the suspension was shaken at 170 rpm and 25 °C for 24 h. After the enzyme solution was filtered with filter paper, the filtrate was rinsed twice by pH 7.2 sodium phosphate buffer, to remove the weakly adsorbing enzyme, and dried to provide BCL/RTSPILs. The recovery yields of BCL/RTSPILs were around 95% in all cases based on the amount of RTSPIL. The protein contents of the BCL/RTSPILs were calculated from the results of Bradford assays. The activity of these BCL/RTSPILs was determined by initial rate of the transesterification reaction between *sec*-phenethyl alcohol and vinyl acetate in anhydrous toluene, and compared with that of crude BCL. The results are summarized in Table 1.

Although the hydrophobic RTSPILs used in this study do not have an attracting group such as a methacryloyl unit to interact with the functional moieties such as thiol, amine, or alcohol of the surface of BCL,[14] these RTSPILs can effectively work as efficient supporting materials for immobilization of enzyme, as shown in Table 1. It is hypothesized that the BCL is adsorbed on the bulky aggregates of the RTSPILs *via* charge interaction between the ionic motifs of both RTSPILs and BCL. Results indicate that the activity of **2**-, **3**-, **4**-, **5**-, **8**-, **9**-, and **10**-immobilized BCL was 1.7–6.3 times that of crude BCL (compare entry 1 with entries 3, 4, 5, 7, 9, 10, and 11 in Table 1), whereas the activity of **1**-, **6**-, and **7**-immobilized BCL less than that of crude BCL (compare entry 1 with entries 2, 7, and 8 in Table 1). However, variations in cationic motifs or side chain length of RTSPILs did not appear to influence the catalytic activity of the BCL/RTSPILs.

Next, we investigated the total activity of the BCL/RTSPILs, because the total activity can serve as an important guide in the practical application of a catalyst. The total activity factor could be determined through the activity of the obtained total BCL/RTSPILs amount versus the activity of total amount of crude BCL used before immobilizing. In particular, BCL/**5** showed a ~9.3-fold increment in total activity as well as a ~6.5-fold increment in initial activity compared to crude BCL (see entries 1 and 6). These results indicate that the immobilization of an enzyme using the hydrophobic RTSPILs might be one of the practical approaches for the preparation of activated biocatalysts.

As mentioned earlier, we also investigated the protein contents of the BCL/RTSPILs using the Bradford assay method. It was found that the amount of protein per unit mass of BCL/RTSPILs was very low. Despite this, however, all the BCL/RTSPILs seemed to show more activity in an organic solvent, with a remarkable 3–223-fold enhancement in specific activity compared to crude BCL.

Figure 1 shows the reusability of an enzyme immobilized on a representative RTSPIL, **3**, for transesterification of *sec*-phenethyl alcohol in toluene. As shown in Figure 1, the BCL/RTSPIL retained its activity even after being reused 5 times, showing good enantioselectivity without any denaturing of the enzyme during several reuses. These results indicate that the BCL/RTSPIL exhibited good stability.

In conclusion, we demonstrated that hydrophobic RTSPILs

Table 1. Catalytic activities of RTSPIL immobilized enzymes for the trasesterification of *sec*-phenethyl alcohol in toluene[a]

Entry	Enzyme	Initial activity (μmol/mg enzymeh)	Total acitivity[b] (μmol/h)	Total activation factor[c]	Protein content (μg/mg enzyme)[d]	Specific activity (μmol/mg enzymeh)[e]	Relative specific activity[f]
1	Crude BCL	0.80	1000	1.00	1.320[g]	6.10×10^1	1.00
2	**1**	0.41	779	0.78	0.054	7.59×10^3	1.24
3	**2**	3.37	6403	6.40	0.084	4.01×10^4	6.57×10
4	**3**	4.88	9272	9.27	0.038	1.28×10^5	2.10×10^2
5	**4**	1.54	2926	2.93	0.071	2.17×10^4	3.56×10
6	**5**	5.04	9576	9.58	0.037	1.36×10^5	2.23×10^2
7	**6**	0.20	380	0.38	0.065	3.08×10^3	5.05
8	**7**	0.07	133	0.13	0.038	1.84×10^3	3.05
9	**8**	1.37	2603	2.60	0.065	2.11×10^4	3.50×10
10	**9**	2.74	5206	5.20	0.047	5.83×10^4	9.52×10^4
11	**10**	2.52	4788	4.79	0.054	4.67×10^4	7.66×10

[a]The reactions were performed using enzyme (20 mg/mmol), 1-phenethyl alcohol (0.2 mmol), vinyl acetate (0.6 mmol) in anhydrous toluene (0.2 M) at 170 rpm and 25 °C, and analyzed by GC equipped with a chiral capillary column (Chiraldex B-PH, Altech). [b]Total activity of crude enzyme was determined on the basis of initial activity x the amount of crude enzyme to used for prepared RTSPIL immobilized enzyme, and total activity of RTSPIL immobilized enzyme was initial activity x the amount of obtained RTSPIL immobilized enzyme. [c]Total activation factor is $(\text{total activity})_{\text{BCL/RTSPIL}}/(\text{total activity})_{\text{crude BCL}}$. [d]Protein content was determined by Bradford assay method. [e]Specific activity of BCL/RTSPIL was calculated from the initial activity and the protein content per unit mass of BCL/RTSPIL. [f]Relative specific activity is $(\text{specific activity})_{\text{BCL/RTSPIL}}/(\text{specific activity})_{\text{crude BCL}}$. [g]Protein content of crude BCL was calculated from enzyme solution which was removed insoluble contaminants.

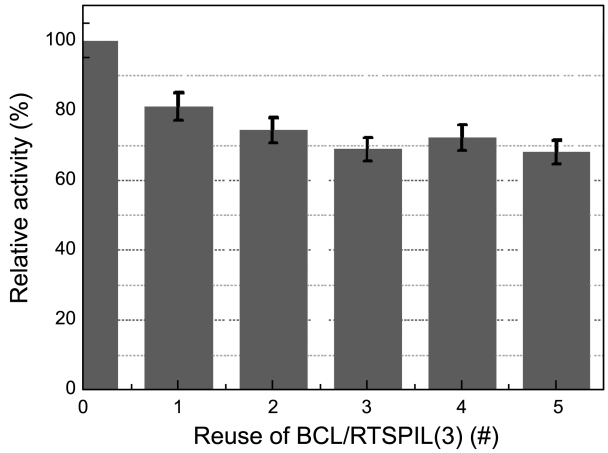


Figure 1. Reusability of representative RTSPIL, 3-immobilized enzyme for transesterification of sec-phenethyl alcohol in toluene.

are very effective and promising supporting materials for the immobilization of enzymes. The enzyme/RTSPILs exhibited superior performance as efficient and practical biocatalysts for use in an organic solvent, showing a better activity than the crude enzyme and having the advantage of being reusable several times without major losses in activity. The RTSPILs used as supporting materials were easily prepared *via* a simple and straightforward procedure with high yields. The proposed approach might provide a facile and useful protocol for increasing the activity and selectivity of enzymes. Ionic liquids are well known as "designer materials" that can be tailored for target applications, and further studies on various ionic liquids are therefore in progress to expand the scope of the utilization of the RTSPILs for immobilizing enzymes as well as improving their activity toward efficient biocatalysis in an organic solvent.

Experimental Section

BCL 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.

A suspension of crude BCL (1.25 g) in a 0.01 M phosphate buffer (5 mL, pH = 7.2) was stirred for 30 min at room temperature. After filtration through Celite, the filtrate was added to the ionic liquid (2.0 g), and the mixture was then shaken at 170 rpm for 24 h at room temperature. The mixture was filtered, and the resulting BCL/RTSPILs were dried and stored under suitable conditions. Water concentration of BCL/RTSPILs and crude BCL were determined by Karl-Fisher titration: all BCL/RTSPILs, < 0.5 w/w%; crude enzyme, 5 w/w%. Enhanced activity of enzyme was not affected by water concentration. When water content of solvent was 1.0 v/v%, the BCL/RTSPILs showed more enhanced activity than those in an anhydrous solvent.

Protein content was measured using a Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Company, USA) with bovine serum albumin as the standard. To prepare the unknown sample, BCL/RTSPILs were redissolved in the same volume of solvent mixture [800 μL, 50 v/v% dichloromethane, 50 v/v% phosphate buffer (0.01 M, pH = 7.2)]. The mixture was centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was removed and saved as the unknown sample.

The *sec*-phenethyl 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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