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Ionic liquid co-lyophilized enzyme for biocatalysis in organic solvent: Remarkably enhanced activity and enantioselectivity

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ABSTRACT

The room temperature solid-phase ionic liquid (RTSPIL) co-lyophilized enzyme exhibited markedly enhanced activity in organic solvent. The enzyme co-lyophilized with a dodecyl-imidazolium salt was 660-fold more active compared to its RTSPIL-free counterpart. The activity enhancement by RTSPILs was mainly attributable to the reduced particle sizes and improved dispersion of enzymes suspended in organic solvent. Also, the RTSPIL co-lyophilized enzyme displayed significantly enhanced enantioselectivity. Its enantioselectivity was 2.5-fold higher than that of its RTSPIL-free counterpart.

1. Introduction

Nonaqueous biocatalysis provide 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 suffer from reduced activity, selectivity or stability of enzyme [3]. To overcome these limitations, many approaches have focused on the development of more efficient enzymes. One of them is the lyophilization of enzyme in the presence of lyoprotectant such as inorganic salt, crown ether, and cyclodextrin, which enhanced the activity, selectivity, or stability of enzyme [4–7]. Also the use of ionic liquids as an alternative solvent in the biotransformations enhanced the activity, selectivity, and stability of enzyme [8–15].

Previously, we have reported that ionic liquid-coated enzyme (ILCE), which was readily prepared by mixing enzyme powder with a room temperature solid phase ionic liquid (RTSPIL) at elevated temperature, exhibited enhanced enantioselectivity and stability in organic solvent [16]. This method, however, was unable to enhance the activity of enzyme. To overcome this limitation, we thought that if the enzyme were coated by ionic liquid during lyophilization in aqueous medium, it would exhibit better activity. We herein wish to report a room temperature solid-phase ionic liquid co-lyophilized enzyme, exhibited remarkably enhanced activity and enantioselectivity.

The room temperature ionic liquids (RTILs) and solid-phase ionic liquids (RTSPILs), which become liquid at elevated temperature, are very attractive materials. They are organic salts and their various properties, such as solubility in solvents, melting point, and conductivity are tunable. Particularly, RTSPILs led us to envisage that they might be suitable as lyoprotectants for the lyophilization-induced activation of enzyme, as well as the coating materials for operational practicality. RTSPILs are commercially available or easily synthesized in good yields [17]. We used ionic liquids composed of imidazolium or pyridinium cation and hexafluorophosphate anion (Scheme 1).

2. Experimental

2.1. General remarks

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

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1. R^{I} : H, R^{ii} : CH_2CH_3
2. R^{I} : H, R^{ii} : $CH_2CH_2CH_2Ph$
3. R^{I} : CH_3, R^{ii} : $CH_2CH_2CH_2Ph$
4. R^{I} : H, R^{ii} : $CH_2(CH_2)_{10}CH_3$
5. R^{I} : CH_3, R^{II} : $CH_2(CH_2)_{10}CH_3$

6. R^{IIi} : CH_3, R^{IV} : H, R^{V} : CH_2CH_3
7. R^{III} : CH^3, R^{IIV} : H, R^{V} : $CH_2CH_2CH_2CH_3$
8. R^{III} : CH_3, R^{IV} : H, R^{V} : $CH_2CH_2CH_2Ph$
9. R^{III} : H, R^{IV} : CH_3, R^{V} : $CH_2CH_2CH_2Ph$

Scheme 1. Structures of room temperature solid-phase ionic liquids (RTSPILs).

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.

2.2. Synthesis of room-temperature solid-phase ionic liquids (RTSPILs)

The RTSPILs used in this study were prepared according to the following procedure reported previously [16]: alkyl halide (0.125 mol) was dissolved in 1-methylimidazole (or 3(or 4)-methyl pyridine) (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. Various RTSPILs containing PF_6 anion were obtained in good yields (>90%): 1-ethyl-3-methylimidazolium ([EMIM], **1**, mp: 60 °C), 1-(3′-phenylpropyl)-3-methylimidazolium ([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).

2.3. Preparation of RTSPIL co-lyophilized enzyme

An enzyme solution was prepared by dissolving crude lipase (1.25 g) in a 10 mM sodium phosphate buffer (5 ml) and filtered to remove insoluble contaminant. The RTSPIL (wt% IL/enzyme) in THF was added to the enzyme solution, then the homogeneous solution was titrated to pH 7.0 by HCl and frozen at −78 °C, followed by lyophilization at around 60 Pa and −53 °C for 48 h to provide. The buffer content in final preparation was 0.1 wt%. We determined the particle sizes of IL-colyophilized enzyme using a particle size analyzer (Malvern Instrument) according to the following method; the IL-colyophilized enzyme (1 g) was suspended in toluene (1 L), which was used as organic solvent for the biocatalytic reaction in this study, and then analyzed by means of laser diffraction to determine the average particle sizes.

2.4. RTSPIL co-lyophilized enzyme catalyzed transesterification for reactivity

The lipase (1 mg/mmol), 1-phenethyl alcohol (0.2 mmol), vinyl acetate (0.6 mmol) were mixed with anhydrous toluene (0.2 M), and the resulting mixture was shaken at 170 rpm and 25 °C. The reaction mixture was sampled several times to determine the reactivity using GC analysis.

2.5. RTSPIL co-lyophilized enzyme catalyzed transesterification for enantioselectivity

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.

3. Results and discussion

3.1. Catalytic activity

As a representative enzyme for lyophilization in presence of ionic liquid, the crude *Burkholderia cepacia* lipase was chosen since it had been frequently used for biotransformations in organic solvents. The ionic liquid co-lyophilized enzyme samples[1] were prepared by lyophilizing the THF-buffer solution (pH 7.0) containing different amounts of enzyme, ionic liquid and sodium phosphate buffer for 48 h. The enzymatic activities of these samples were determined as the initial rates of the transesterification reaction between *sec*-phenethyl alcohol and vinyl acetate in anhydrous organic solvents, and compared with those of crude, salt-free, and KCl co-lyophilized enzymes. The results were described in Table 1.

The data from Table 1 indicate that the enzymatic activities were dependent on the ionic liquid contents of lyophilized enzyme preparations. Because the ionic liquids having hexafluorophosphate anion are hydrophobic and insoluble in water, THF was employed as the cosolvent with aqueous buffer. It was observed

[1] The water concentration of lyophilized enzyme was determined by the Karl–Fisher coulometer. All ionic liquid and KCl co-lyophilized enzymes contained 0.4–0.5 wt% of water and both crude and salt-free enzymes contained 5 wt% of water.

Table 1
Initial activity of lipase for transesterification reaction of secondary alcohol in toluene[a].

Entry	Excipient	Initial activity μmol (mg enzyme)$^{-1}$ h^{-1})	Activation factor[d]	Total activation factor[e]
1	Crude[b]	0.8	–	1
2	None	0.8	1.0	0.1
3	None[c]	1.0	1.3	0.1
4	KCl	37	46	5
5	**1**	151	189	19
6	**2**	50	63	6
7	**3**	60	75	8
8	**4**	530	663	66
9	**5**	384	480	48
10	**6**	347	434	43
11	**7**	237	396	30
12	**8**	306	384	38
13	**9**	234	293	29

[a] The reactions were performed using PCL (1 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] Crude PCL before purified.
[c] Lyophilization in THF-buffer medium.
[d] Activation factor is (initial activity)$_{excipient}$/(initial activity)$_{none}$.
[e] Total activation factor is (total activity)$_{excipient}$/(total activity)$_{crude}$.

that the co-medium had little effect on the activity of lyophilized enzymes (entry 3).

Previously, it was reported that the enzymatic activities were dramatically enhanced in case proteases such as subtilisin were colyophilized with 98% (w/w) KCl salts [6]. In this work, 98% (w/w) KCl co-lyophilized lipase displayed 40-fold enhanced activity compared to its salt-free counterpart. Surprisingly, 98% (w/w) ionic liquid co-lyophilized enzyme preparations were significantly more active than the 98% (w/w) KCl-lipase preparation. The former displayed 63–663-fold enhanced activities (entry 4–13). It was notable that the largest activity enhancement was achieved with ionic liquid **4**. All the data from Table 1 thus indicate that ionic liquids are more efficient lyoprotectant than KCl for lipase. We could not find any dependency of activation on the structure or side chain length of ionic liquid. Until now, there is no conclusive explanation for the activation of enzyme by lyoprotectants, ligands, or inorganic salts as KCl. However many researcher have typically explained the activation of enzyme by co-lyophlization as lyoprotectant, imprinting, or matrix effects. For example, Dordick and Clark have hypothesized that the activation of protease by KCl salts is due to a protective effect afforded by the salt matrix against deactivation by direct contact with the organic solvent [6]. Initially, we also assumed that ionic liquid matrix might protect enzyme because most ionic liquids are insoluble in toluene. However, it was observed that the enzyme colyophilized with the organic soluble ionic liquids, **4** and **5**, exhibited the best activities, which is against the matrix effect. Accordingly, other factor should be responsible for the ionic liquid-induced activation of enzyme. Because the activity of enzyme is dependent on the ionic liquid content of lyophilized preparation, we investigated the correlation between the ionic liquid contents and the particle sizes of ionic liquid **4** co-lyophilized enzymes in toluene. The ionic liquid content varied from 0 to 98 (w/w%). The

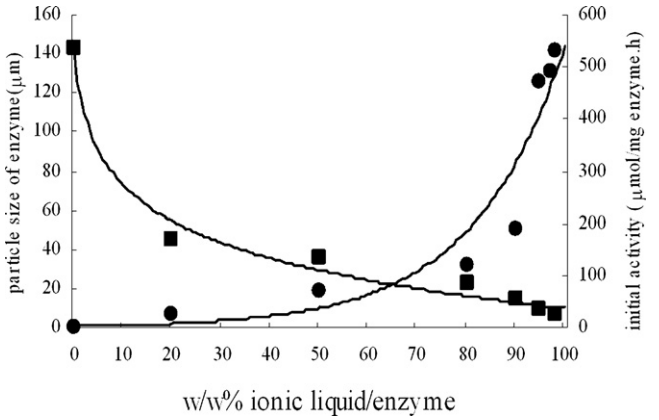


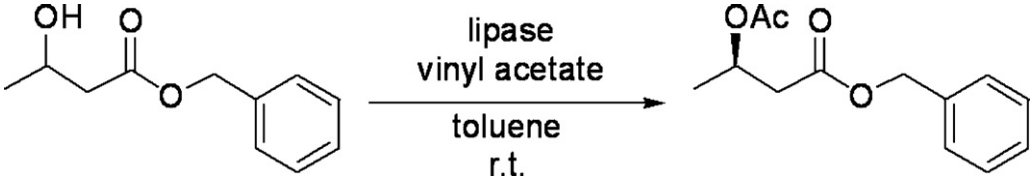
Fig. 1. Correlation between particle size (■) and initial activity (●) of RTSPIL (**4**) co-lyophilized enzyme in toluene.

data from Fig. 1 indicate that the average particle sizes of ionic liquid-enzyme preparations decrease with increasing the ionic liquid content. This observation therefore suggests that the enhanced enzymatic activity should result from the decreased particle sizes of insoluble enzyme aggregates. Consequently, we note that the ionic liquid-induced activation is mainly the size effect rather than the matrix effect.

For the preparation of ionic liquid-colyophilzed enzymes, we used water-soluble enzymes (about 10% of total weight) prepared from the commercially available crude enzymes. It was found that the water-soluble enzymes had the same specific activity as that of the crude enzymes. But the total activity of soluble enzymes was only 10% of that of crude enzymes used (entry 2, Table 1). This implies that the purification of soluble enzymes from the crude enzymes is not practical from a total activity point of view. This problem can be overcome by the lyophilization of soluble enzymes in the presence of ionic liquids, which enhances dramatically the enzymatic activity. The total activitiy of ionic liquid-activated enzymes was 6–66-fold higher than that of the crude enzyme (entries 4–13). On the basis of the results, we believe that the lyophilization of soluble enzymes in presence of ionic liquid provide a very practical method for the activation of enzymes for use in organic solvent.

3.2. Enantioselectivity

The enantioselectivity of ionic liquid (**4**)-co-lyophilized enzyme was examined in the transesterification reaction of a bulky secondary alcohol in the presence of vinyl acetate at 25 °C (Scheme 2). For comparison, the same reactions were carried out with both crude and salt-free lyophilized enzymes. The results were summarized in Table 2. The ionic liquid co-lyophilized enzyme displayed better enantioselectivity than the crude and salt-free enzymes. The enantioselectivity enhancement by the use of RTSPIL is about 2.5-fold (compare entries 1–2 and 3). The results are comparable to those reported previously for the ionic liquid coated enzyme (ILCE) [16], suggesting that the RTSPIL enhances the enantioselectivity of enzyme in a similar fashion to the ILCE. However, it is not clear why



Scheme 2. Lipase-catalyzed transesterification of secondary alcohol in toluene.

Table 2
The enantioselectivities in the lipase-catalyzed transesterifications in toluene[a],[b].

Entry	Lipase	ee_s	ee_p	E[c]
1	Crude	0.459	0.971	107
2	Salt-free lyophilized	0.214	0.977	106
3	**4** co-lyophilized	0.549	0.987	266

[a] Experimental procedure: the ionic liquid co-lyophilized PCL-catalyzed reaction is described as a representative procedure. Substrate, **11** (20 mg, 0.1 mmol), vinyl acetate (28 μL, 0.3 mmol), and enzyme (0.5 mg/mmol) were mixed with toluene (0.5 mL), and the resulting semi-homogeneous mixture was shaken at 170 rpm and 25 °C for 8 h.

[b] The optical purities were determined by HPLC using a chiral column. Analytical conditions: alcohol, **10** (Chiralcel OD, hexane/2-propanol: 95/5, flow rate: 1.0 ml/min, UV: 217 nm), acetate, **11** (Whelk-O1, hexane/2-propanol: 96/4, flow rate: 1.0 mL/min, UV: 217 nm).

[c] The E values were calculated using the equation, $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$, where $c = ee_s/(ee_s + ee_p)$.

the enantioselectivity is improved by RTSPILs. We speculate that it may be due to more favorable structural adaptation of enzyme in polar ionic environment.

4. Conclusion

In conclusion, this work has demonstrated that the RTSPIL co-lyophilized enzyme is an efficient and practical catalyst for use in organic solvents. In case using a dodecyl immidazolium salt as RTSPIL, it is 660-fold more active compared to the ionic liquid-free enzyme. Also, it is superior to its KCl-activated counterpart. The enhancement of enzymatic activity by RTSPILs is mainly attributable to the better dispersion of enzymes in organic solvent because the average particle sizes of enzymes suspended in organic solvent are significantly reduced by co-lyophilization with RTSPIL. The RTSPIL co-lyophilized enzymes also display better enantioselectivity than the ionic liquid-free enzymes. Further studies to broaden the scope of ionic liquid co-lyophilized enzymes as well as to apply them in synthetic transformations are in progress at this laboratory.

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