Lyophilization of Lipase Together with Ionic Compounds Generates Highly Enantioselective and Solvent-sensitive Lipase in Organic Solvents

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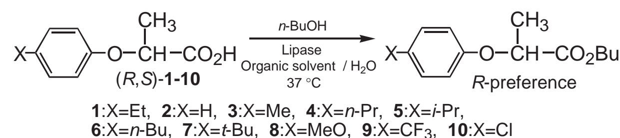
The lyophilization of lipase from aqueous enzyme solution containing ionic compounds such as disodium hydrogenphosphate resulted in a dramatic improvement of its enantioselectivity for esterification in organic solvents, compared with the ionic compound-free lipase. The enantioselectivity of the ionic compound-coated lipase was found to be fairly sensitive to the change of the solvents.

Although lipases are most frequently used as an important tool in organic synthesis of optically pure compounds,[1] native lipases are often poor enantioselective toward non-natural substrates such as pharmaceuticals, agricultural, and other specialty chemicals. This is mainly because their structures are widely different from those of natural substrates, triacylglycerols. To overcome this difficulty, a variety of strategies were proposed for controlling the enzyme enantioselectivity.[2] Further developments, however, are awaited with interest as the efficient approach for exploring the enzyme enantioselectivity, because of the increasing need of various fine chemicals and intermediates with high stereochemical purity. Our continuing interest in seeking the enzyme enantioselectivity[3] led us to examine a new method based on an easy preparation procedure.

Here, we wish to report that the enantioselectivity of *Candida rugosa* lipase-catalyzed esterification of 2-(4-substituted phenoxy)propanoic acids **1**–**10** is markedly improved by use of lipase lyophilized in the presence of ionic compounds such as disodium hydrogenphosphate (Na_2HPO_4) or L-alanine. As another remarkable feature of the lipase prepared, its *E* value is found to be much more sensitive to the change of the dielectric constant of the solvent as the reaction medium than that of native lipase.

In a typical enzymatic reaction, the substrates **1**–**10** (0.36 mmol) prepared from the known method[4] and a three-fold excess of *n*-BuOH (1.08 mmol) were dissolved in 2 mL of isopropyl ether containing 0.75 vol % of water. To the solution, *Candida rugosa* lipase (6.1 mg) resulting from lyophilization in the presence of the ionic compound was added and the suspension was shaken (150–170 strokes/min) at 37 °C. Lipase sample used here was prepared by lyophilizing an aqueous enzyme solution (100 mL) containing semi-purified lipase[5] (100 mg) and Na_2HPO_4 (14.6 mmol/g of enzyme) or L-alanine (500 mmol/g of enzyme). The ee was measured by HPLC on a chiral column (Chiralcel OK, from Daicel Chemical Industries).

For the model reaction (Scheme 1), the lyophilized lipase catalyzed preferentially the *R* enantiomer of all the substrates used. Initially, various ionic compounds were tested as the additives in the lyophilization of lipase to investigate the enhancement effect of the enantioselectivity (*E* value)[7] for the model reaction using **1** (Table 1). As can be seen from the *E* value listed in Table 1, Na_2HPO_4 and L-alanine in the lipase preparation brought about ca. 20-fold jump in the *E* value in isopropyl ether, as compared with native lipase. On the other hand, the enhancement effect in the *E* value is small when the same concentrations of these ionic compounds were only added to the reaction mixture (for example, $E = 22$ at 0.4 vol % of aq Na_2HPO_4, $E = 23$ at 0.75 vol % of aq Na_2HPO_4, and $E = 13$ at 1.1 vol % of aq Na_2HPO_4).



Scheme 1. Lipase catalyzed esterification of 2-(4-substituted phenoxy) propanoic acids **1**–**10** in organic media.

Table 1. Enantioselectivity (*E* value) for esterification of **1** in isopropyl ether catalyzed by lipase lyophilized in the presence of various ionic compounds

Ionic compound[a]	Time/h	Conv./%	ee/%	*E* value
None[b]	27	38.2	85.0	21
Na_2HPO_4[c] (14.6 mmol)	24	39.9	99.1	463
$Na_5P_3O_{10}$[d] (5.84 mmol)	24	37.5	97.9	170
DNA (ca.0.04 mmol)	117	37.4	91.6	40
L-Ala (500 mmol)	21	35.4	99.3	520
D-Ala (500 mmol)	21	33.0	98.2	175
L-Leu (200 mmol)	17.5	35.9	98.4	211
D-Leu (200 mmol)	17.5	35.4	98.0	169
L-Phe (50 mmol)	18	36.6	96.7	104
D-Phe (50 mmol)	18	36.6	96.5	98
Gly (500 mmol)	18	38.2	92.0	43
$NH_2–(CH_2)_4–CO_2H$[e] (500 mmol)	210	2.32	77.3	7.9

[a]The value in the parenthesis indicates the optimal concentration (mmol/g of enzyme) to maximize the *E* value, among their different concentrations examined (for example, $E = 202$ at 7.3 mmol of Na_2HPO_4 and $E = 252$ at 29.2 mmol of Na_2HPO_4). [b]Semi-purified native lipase. [c]Disodium hydrogenphosphate. [d]Sodium tripolyphosphate. [e]5-aminovaleric acid.

To our knowledge, this is the first example of the dramatic enhancement of the lipase enantioselectivity caused by the incorporation of the ionic compounds during lyophilization of native lipase,[8] although there have been several interesting reports on the effect of simple inorganic salts on the activation of enzymes.[9] Furthermore, the different behavior in the *E* value between L- and D-amino acids may be mainly attributed to a different fashion in the chiral interaction between each enantiomer of the amino acid with dipolar ion and the charges on the surface of lipase (see Table 1).

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Table 2. Enantioselectivity (E value) for esterification of **2–10** in isopropyl ether catalyzed by lipase lyophilized with Na_2HPO_4 (14.6 mmol/g of enzyme) or L-Ala (500 mmol/g of enzyme)

	X	Ionic compound	Time/h	Conv./%	ee/%	E value
2	H	None[a]	119	38.2	59.5	5.6
		Na_2HPO_4				—[b]
		L-Ala	690	26.4	79.9	12
3	Me	None	50	36.7	80.6	15
		Na_2HPO_4	50	32.9	99.0	327
		L-Ala	49	34.9	98.0	169
4	n-Pr	None	19.5	38.2	71.2	9.1
		Na_2HPO_4	22	41.4	96.8	129
		L-Ala	26	37.3	95.9	85
5	i-Pr	None	24	35.6	89.1	28
		Na_2HPO_4	22	41.2	98.3	240
		L-Ala	24	39.9	98.1	204
6	n-Bu	None	310	37.9	62.6	6.3
		Na_2HPO_4	47	42.4	88.6	33
		L-Ala	120	38.2	94.8	68
7	t-Bu	None	435	34.9	53.2	4.3
		Na_2HPO_4	60	39.9	66.3	7.5
		L-Ala	552	34.9	88.4	26
8	MeO	None	50	39.8	84.4	21
		Na_2HPO_4	138	9.5	86.2	15
		L-Ala	59	35.6	96.8	104
9	CF_3	None	192	41.8	53.1	4.7
		Na_2HPO_4	60	34.9	91.2	35
		L-Ala	310	35.5	92.1	40
10	Cl	None	96	37.0	64.1	6.6
		Na_2HPO_4	210	26.3	95.0	54
		L-Ala	310	35.9	92.0	40

[a]Semi-purified native lipase. [b]The reaction rate was too late to obtain a reliable E value.

The scope of this enhancement effect in lipase enantioselectivity was evaluated by the model reaction using the other substrates **2–10** with a wide variety of substituents (Table 2). The data summarized in Table 2 shows that the E value enhancement brought about by the lipase preparation in the presence of Na_2HPO_4 or L-alanine was observed for all the substrates, except for **2** and **8** using Na_2HPO_4, although a few substrates displayed a lowering of the reaction rate.

We then investigated the effect of the ionic compound on initial rate for each enantiomer of **1** (Table 3). In the presence of the lyophilized lipase with Na_2HPO_4, the initial rate for the R enantiomer (correctly reacting substrate) was largely accelerated, whereas the S enantiomer (incorrectly reacting one) was decelerated, relative to native lipase. The direction of the initial rate observed is explained by assuming that the electrostatic association between the ionic compound and the charges on lipase surface may influence the conformation and/or the local polarity of the lipase active site allowing an easier access for the R enantiomer, and not for the S enantiomer. Thus, the opposite direction in their initial rates is ascribed to the marked enhancement of the enantioselectivity.

Table 3. Initial rate of esterification of each enantiomer of **1** in isopropyl ether catalyzed by native lipase and lipase lyophilized with Na_2HPO_4 (14.6 mmol/g of enzyme)

Ionic compound	Initial rate (μmol $h^{-1}mg^{-1}$)		V_R/V_S
	V_R[b]	V_S[b]	
None[a]	8.0	0.45	18
Na_2HPO_4	41.0	0.14	293

[a]Semi-purified native lipase. [b]The V_R and V_S denote the initial rates of the R and S enatniomers, respectively.

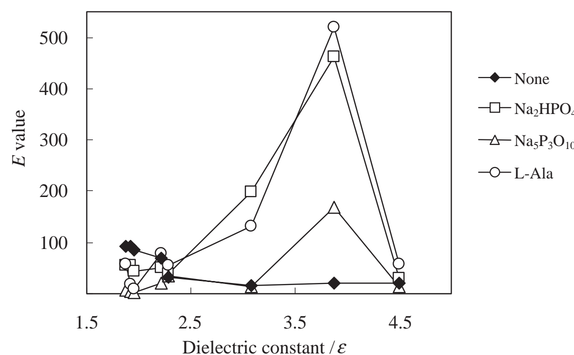

Figure 1. Plot of E value for lipase-catalyzed esterification against dielectric constant (ε) of solvent. The values of dielectric constant of the solvent examined were taken from Ref. 10: n-hexane (1.88), n-heptane (1.92) isooctane (1.95), cyclohexane (2.22), benzene (2.28), dibutyl ether (3.08), isopropyl ether (3.88), and t-butyl methyl ether (4.5).

This electrostatic association would be strongly controlled by the bulk property, dielectric constant, of the solvent as the reaction medium. Indeed, as is seen in Figure 1, the enantioselectivity of the ionic compound-coated lipase is found to be changed profoundly on switching the solvent from n-hexane ($E = 55$ for lipase with Na_2HPO_4) to isopropyl ether ($E = 463$ for lipase with Na_2HPO_4); this large solvent effect is thought to be significantly amplified by the electrostatic association appended to lipase surface.

In conclusion, the advantage of the ionic compound-coated lipase is that its enantioselectivity can be greatly altered by the combination effects of the ionic compound and the solvent.

References and Notes

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5 The semi-purified lipase was previously prepared by dialyzing and lyophilizing from crude material (Sigma Chemical Co.). The MALDI-TOF MS spectrum of the lipase showed a single parent peak, m/z 60.2 kD, which is consistent with the molecular weigh of *Candida rugosa* lipase.[6]
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8 The water contents of native lipase and Na_2HPO_4-coated lipase estimated by the Karl Fischer method under the reaction conditions were 573 and 769 μg, respectively. This difference of the water content seems not to be the main factor for enhancing the E value dramatically.
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