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Flexibility of Lipase Brought About by Solvent Effects Controls Its Enantioselectivity in Organic Media

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The behavior of the enantioselectivity of *Candida rugosa* lipase was investigated in the esterification of 2-(4-substituted phenoxy)propionic acids with 1-butanol in aliphatic, aromatic, and ethereal solvents. The observed enantioselectivity (*E* value) was greatly affected by the solvent nature. Two key solvent characteristics, dielectric constant (ε) and hydrophobicity (log *P*), failed to explain the solvent effects on the *E* value. On the other hand, the variation of the *E* value was found to be successfully correlated with the lipase flexibility brought about by the solvent effects, the flexibility of which was estimated by the ESR measurements of the spin-labeled lipase. Although organic chemists should often undertake a solvent screening step in the biocatalytic asymmetric resolutions, the obtained correlation will provide a useful guide in the solvent optimization step.

Since the pioneering study showing enzymatic activity even in organic solvents by Klibanov et al.,[1] a great number of papers have reported that the enantioselectivity and catalytic activity of enzymes can be affected by the nature of the organic solvents used.[2] Indeed, the enzymatic properties have been changed profoundly on switching from one solvent to another. A striking example of the solvent effects is the solvent-induced inversion in the enantioselectivity of enzyme-catalyzed reactions,[3] although the stereochemical preference of enzyme is considered to be its intrinsic property. Therefore, the choice of the reaction medium (solvent engineering) provides an attractive strategy for researchers seeking the improvement of the enzyme enantioselectivity, because an ultimate goal, especially for organic chemists, is to control rationally the enantioselectivity as a function of the reaction conditions. In spite of numerous studies of the solvent effects on enzyme properties, an understanding of what characteristics of the solvents are essential for the determination of the enzyme enantioselectivity is still elusive

Here, we report that the enantioselectivity for the lipase-catalyzed reaction in various organic solvents is closely correlated with the lipase flexibility estimated by the ESR measurement. Furthermore, the specific solvent effect, such as the formation of solvent-substrate complex, apart from altering the lipase flexibility, may be detected on the basis of the correlation obtained.

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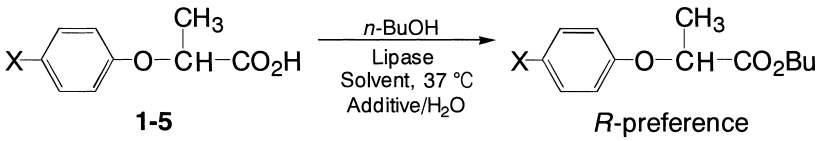
Results and Discussion

As a model reaction, we chose the esterification of 2-(4-substituted phenoxy)propionic acids with 1-butanol catalyzed by *Candida rugosa* lipase MY in aliphatic, aromatic, and ethereal organic solvents (Scheme 1); in the hydrophilic solvents such as tetrahydrofuran, lipase MY displayed extremely low reactivity and poor enantioselectivity. Lipase MY used here was semi-purified by dialyzing and lyophilizing from crude material. The MALDI-TOF MS spectrum of the semi-purified lipase showed a single parent peak, *m*/*z* 60.2 kD, which is consistent with the molecular weight of *Candida rugosa* lipase.[4] In the actual reactions, 0.3 vol% of water was added to the reaction medium, because lipase with the flexible conformation induced by water added should be affected by the solvent nature like a dielectric constant; without water, the enantioselectivity was poor and insensitive to the change of the solvents (data not shown). In a model reaction, lipase catalyzed preferentially the *R* enantiomer of all the substrates used.

Table 1 summarizes the results of the variation of the enantioselectivity (*E* value[5]) in the esterification of the substrates **1**–**3** by changing the solvents. It can be seen from the data of Table 1 that the solvent nature affects significantly the enantioselectivity of lipase. The observed *E* values appear to show some correlation with two key solvent characteristics, dielectric constant (ε) and hydrophobicity (log *P*), as listed in Table 2. The plots of the *E* value for **1** against ε and log *P* are illustrated in Figs. 1 and 2, respectively; some scatter diagrams were obtained. In a few studies, the behavior of the enzyme enantioselectivity in organic solvents has been found to corre-



1-5 *R*-preference

1: X = CH_3CH_2, **2**: X = CH_3, **3**: X = $(CH_3)_2CH$, **4**: X = $(CH_3)_3C$, **5**: X = H

Scheme 1. Lipase-catalyzed esterification of 2-(4-substituted phenoxy)propionic acids **1**–**5** with 1-butanol in aliphatic, aromatic, and ethereal solvents.

Table 1. Effects of Organic Solvents on the Enantioselectivity in the Lipase-Catalyzed Esterification of the Substrates **1**–**3** with 1-Butanol

Entry	Solvent	Substrate								
		1			**2**			**3**		
		Convn./%	ee/%	*E*	Convn./%	ee/%	*E*	Convn./%	ee/%	*E*
1	Cyclohexane	43.6	61.2	6.5	42.6	61.1	5.8	36.6	61.8	6.0
2	Hexane	43.0	66.3	8.0	46.0	61.6	7.0	36.5	63.2	6.3
3	Heptane	38.6	71.4	9.3	39.4	66.4	7.5	36.7	65.7	7.0
4	Toluene	40.5	54.5	5.5	44.6	42.8	4.0	38.6	56.3	4.4
5	Benzene	38.6	61.9	6.2	33.1	51.5	4.0	46.4	46.2	4.8
6	Chlorobenzene	44.2	62.0	5.8	36.2	48.6	3.4	46.7	44.2	4.5
7	Isooctane	35.7	80.0	13.9	45.3	66.6	8.6	39.0	74.6	10.9
8	Dibutyl ether	43.7	77.9	14.8	37.4	72.3	9.5	36.9	77.1	12.7
9	Diisopropyl ether	35.6	88.2	25.8	39.0	74.6	10.9	37.2	75.0	10.8
10	*t*-Butyl methyl ether	34.5	93.8	51.4	26.2	84.5	16.0	26.2	84.1	15.5

Table 2. Solvent Property (Dielectric Constant: ε and Hydrophobicity: log *P*) and the Value of Hi/(Ha + Hi) as a Direct Measure of the Lipase Flexibility Estimated from ESR Spectrum

Entry	Solvent	Solvent property		Lipase flexibility
		ε[a)]	log *P*[b)]	Hi/(Ha + Hi)
1	Cyclohexane	2.22	3.2	0.36
2	Hexane	1.88	3.5	0.41
3	Heptane	1.92	4.0	0.43
4	Toluene	2.38	2.5	0.47
5	Benzene	2.28	2.0	0.54
6	Chlorobenzene	5.62	2.8	0.62
7	Isooctane	1.95	4.5	0.63
8	Dibutyl ether	3.08	2.9	0.68
9	Diisopropyl ether	3.88	1.9	0.72
10	*t*-Butyl methyl ether	4.50	1.4	0.80

a) Taken from Ref. 19. b) Taken from Ref. 20.

late with one of two solvent parameters, ε and log *P*.[6] It is reasonable, however, to expect that the variation of the *E* values is brought about by the cooperative effects of these solvent properties.[7] Also, it is known that enzyme should become more flexible in solvents with high ε and/or large log *P* than in those with low ε and/or small log *P*.[6,8] From these facts, the cooperative solvent effects on lipase are assumed to bring about the alteration of its flexibility, sometimes accompanying a conformational change. In other words, we consider that the cooperative solvent effects on lipase may converge on its flexibility.

This assumption prompted us to estimate the lipase flexibility by the ESR measurements of the spin-labeled lipase under the same medium conditions as the model reaction. The active site (serine) of lipase was spin-labeled with 1-oxy-2,2,6,6-tetramethyl-4-piperidyl-ethoxyphosphorofluoridate (TEMPO-4-EPF). Figure 3 shows the typical ESR spectra of the spin-labeled lipase suspended in aliphatic, aromatic, and ethereal solvents, the spectrum of which is composed of two components, the isotropic signal (Hi) and the anisotropic signal (Ha) (see the spectrum in cyclohexane). In Fig. 3, on changing the solvent from cyclohexane to *t*-butyl methyl ether, the isotropic signal (Hi) was found to arise quickly and the spectral line narrowed in width. This change of the ESR spectrum signifies that the conformation of the lipase's active site around the spin label becomes more flexible.[9,10] According to our method,[11] the degree of the lipase flexibility was estimated by the change in the ratio of Hi to (Hi + Ha), where Hi and Ha represent the peak heights of the corresponding signal (Fig. 3). Table 2 also includes the value of Hi/(Hi + Ha) as a direct measure of the lipase flexibility altered by the change of the solvents.

To test the validity of the Hi/(Hi + Ha) value instead of the solvent parameters, the *E* values for **1** and **2** listed in Table 1

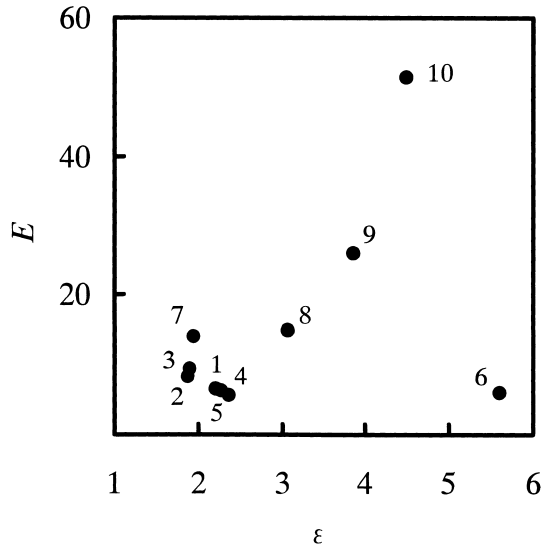


Fig. 1. Plot of the E value in lipase-catalyzed esterification of **1** against dielectric constant (ε). Numbering (1–10) indicates the corresponding solvents (see Table 1).

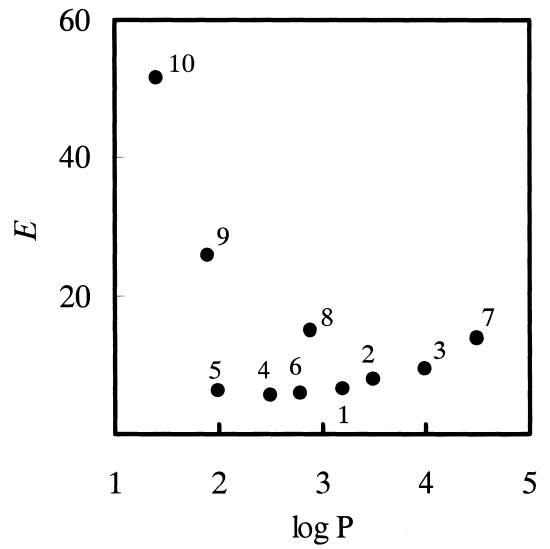


Fig. 2. Plot of the E value in the lipase-catalyzed esterification of **1** against hydrophobicity (log P). Numbering (1–10) indicates the corresponding solvents (see Table 1).

are plotted as a function of the Hi/(Hi + Ha) value in Fig. 4. As judged from Fig. 4, the variation of the E value is dominantly controlled by the lipase flexibility brought about by the cooperative solvent effects, except for the case of the aromatic solvents. Also, for the other two substrates, **4** with the bulky substituent (X = *t*-Bu) and **5** with the small substituent (X = H), the dependence of the lipase flexibility due to the solvent effects upon the E value is almost the same as the profile shown in Fig. 4, although the solvent sensitivity of the E value is relatively small (Table 3). This small solvent sensitivity can probably be attributed to a loose (X = H) or a tight (X = *t*-Bu) accommodation of the substrate into the lipase's active site. Thus, for a combination of lipase MY and the substrates **1–5** studied here, the enantioselectivity is found to be correlated

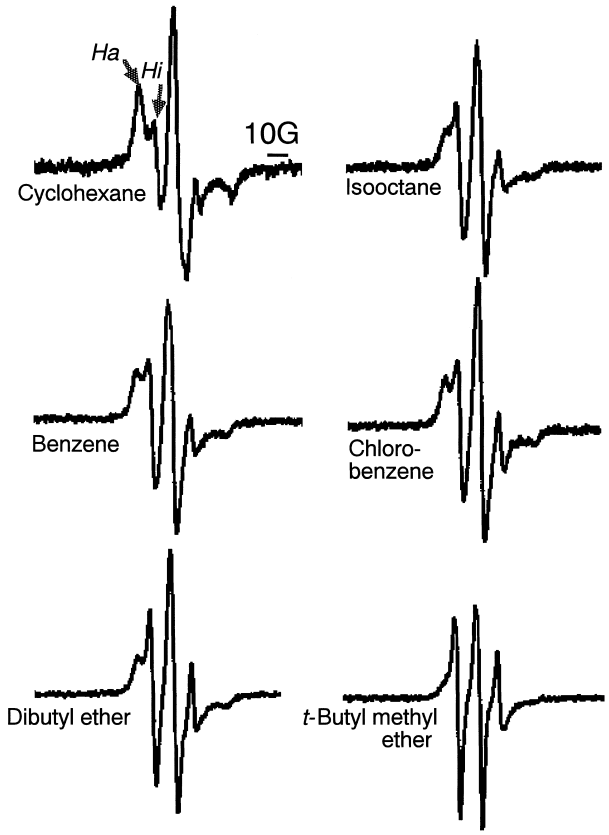


Fig. 3. Typical ESR spectra of the spin-labeled lipase suspended in aliphatic, aromatic, and ethereal solvents.

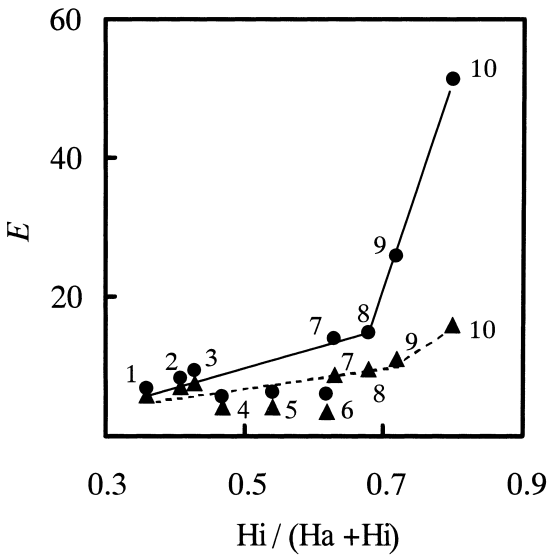


Fig. 4. Correlation between the Hi/(Ha + Hi) value as a direct measure of lipase flexibility and the E value in the lipase-catalyzed esterification of **1** (●) and **2** (▲). Numbering (1–10) indicates the corresponding solvents (see Table 1).

with the increased flexibility of lipase brought about by the solvent effects.

In contrast to our findings, there is a possibility that the in-

Table 3. Effects of Organic Solvents on the Enantioselectivity in the Lipase-Catalyzed Esterification of the Substrates **4** and **5** with 1-Butanol

Entry	Solvent	Substrate					
		4			**5**		
		Convn./%	ee/%	*E*	Convn./%	ee/%	*E*
1	Cyclohexane	37.2	44.2	3.2	33.9	46.9	3.5
2	Hexane	38.2	41.5	3.2	45.1	47.4	4.0
3	Heptane	42.8	50.2	4.1	35.5	50.1	3.9
4	Toluene	41.7	39.3	3.0	32.3	26.5	2.1
5	Benzene	35.7	41.6	3.0	34.1	24.8	1.9
6	Chlorobenzene	36.2	43.9	4.0	35.2	27.1	2.0
7	Isooctane	43.2	47.2	3.9	45.8	48.2	4.2
8	Dibutyl ether	44.6	55.6	5.4	40.9	49.4	4.1
9	Diisopropyl ether	38.3	68.2	7.9	38.3	64.5	6.8
10	*t*-Butyl methyl ether	22.1	70.1	8.2	20.9	67.2	7.1

creased flexibility of enzyme causes an acceleration of the reactivity of the incorrectly binding enantiomer relative to that of the correctly binding counterpart, thus leading to the loss of the *E* value. The direction in the effect of the enzyme flexibility on its enantioselectivity seems to depend on a special relationship between the structure of a given substrate and the stereochemical environment around the active site of a given enzyme. In any event, it is noteworthy that the enzyme enantioselectivity in organic solvents is mainly controlled by its flexibility and that the obtained correlation (Fig. 4) permits a prediction of the solvent effects on the *E* value.

As to the observed deviation of the aromatic solvents corresponding to numbers 4, 5, and 6 in Fig. 4, one of the possible explanations is a particular contribution due to the OH$\cdots\pi$ association[12,13] between the carboxylic proton of the substrate and the π electrons of the aromatic solvents or the local solvent-enzyme interaction at the close vicinity of enzyme active site reported by Nakamura et al.[14]

The direct measurements of enzyme flexibility in organic solvents are limited and are performed by solid state NMR,[15] ESR,[9] and time-resolved fluorescence anisotropy studies.[16] Among them, to our knowledge, only one study showed that the increased enantioselectivity of subtilisin Carlsberg in organic solvents was correlated with its increased flexibility.[16] Our results obtained for lipase are consistent with those for subtilisin.

In conclusion, the enantioselectivity of lipase in organic solvents is found to be closely correlated with the lipase flexibility brought about by the cooperative solvent effects rather than with a sole solvent property such as ε or log *P*. The success of the correlation should be of interest to organic chemists, because somewhat rational approaches to the solvent choice as the reaction medium can be made for enantioselective synthesis by use of enzyme.

Experimental

Materials. Lipase MY was supplied from Meito Sangyo Co., Ltd., and was semi-purified by dialyzing and lyophilizing from the crude material. Organic solvents were purchased from Wako Pure Chemical Industries, Ltd., Japan. Racemic 2-(4-substituted phenoxy)propionic acids **1**–**5** were prepared by the reaction of the corresponding 4-substituted phenol and ethyl 2-bromopropionate (Tokyo Kasei Kogyo Co., Ltd., Japan), according to a known method.[17]

Lipase-Catalyzed Esterification. The substrates **1**–**5** (0.036 mmol) and 1-butanol (1.08 mmol, 30 mol amt.) were dissolved in an organic solvent (2 mL). To the solution, 0.3 vol% of water was added, followed by ultrasonic dispersion, and then the semipurified lipase (2 mg) was added. The suspension was shaken (170 strokes/min) at 37 °C. The *E* value was calculated from the enantiomeric excess (ee) for the butyl ester produced, according to the literature.[5] The ee was measured by HPLC on a chiral column (Chiralcel OK, from Daicel Chemical Industries Co. Ltd., Japan).

MALDI-TOF MS and ESR Spectra. The MALDI-TOF MS spectrum of the sample prepared in a sinapic acid (matrix) was obtained with a Shimadzu AXIMA-CFR. The active site (serine) of the semi-purified lipase was spin-labeled with 1-oxy-2,2,6,6-tetramethyl-4-piperidyl-ethoxyphosphorofluoridate (TEMPO-4-EPF) purchased from SIGMA, according to the procedure reported by Morrisett and Broomfield.[18] It can be assumed that the spin label has attached to the active site, because the spin-labeled lipase showed a clear decrease in the enzymatic activity for the esterification of **1**. Typically about 35% of the active sites is considered to be labeled as calculated from the residual enzymatic activity. All the ESR measurements were carried out at room temperature (ca. 25 °C) on a Bruker EMX081 spectrometer at X-band frequency in organic solvents containing 0.3 vol% of water.

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7 For example, the solvent effects may be represented by $\alpha\varepsilon + \beta\log P + \gamma$ (other factors such as dipole moment), where α, β and γ are adjustment parameters depending on the reaction conditions such as enzyme, substrate, temperature, etc.

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