Metal ions dramatically enhance the enantioselectivity for lipase-catalysed reactions in organic solvents

Takashi Okamoto,[a] Ebara Yasuhito[b] and Shin-ichi Ueji*[b]

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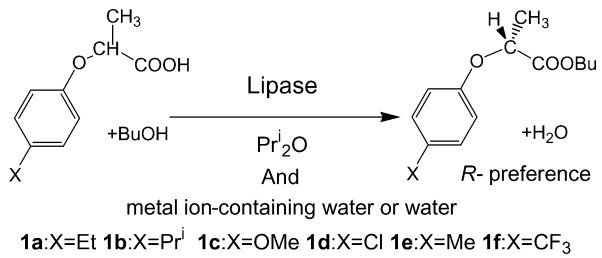
We propose a simple and a powerful method to enhance the enantioselectivity for lipase-catalysed transformations in organic solvents by an addition of metal ion-containing water to the reaction mixture. In this paper, various metal ions such as LiCl or $MgCl_2$ are tested to improve the enantioselectivity for the model reactions. The enantioselectivities obtained are dramatically enhanced, the *E* values of which are about 100-fold as compared with the ordinary conditions without a metal ion, for example, $E = 200$ by addition of LiCl. Furthermore, lowering the reaction temperature led to an almost perfect enantioselectivity of lipase in the presence of a metal ion, for example, $E = 1300$ by addition of LiCl. Also, a mechanism for the drastic enhancement by metal ions is discussed briefly on the basis of the EPR spectroscopic study and the initial rate for each enantiomer of the substrate.

Introduction

Since the basic discovery showing enzymatic activity even in organic solvents,[1] a number of applications of enzyme-catalysed enantioselective transformations have appeared in the field of organic synthesis to obtain useful chiral compounds.[2] For the enzymatic reactions, their catalysis in organic solvents has several potential advantages compared with aqueous media, such as the increased solubility of substrates, the suppression of undesirable side reactions, and an increased stability of the enzyme to thermal denaturation. Also, among these enzymes applied to organic synthesis, lipases have been used most frequently, because they accept rather broad substrate specificities with high enantioselectivities.[3] In these enzymatic reactions in organic solvents, however, controlling the enantioselectivity toward a given substrate by the reaction conditions still remains a major problem for organic chemists and enzymologists.

Therefore, several approaches to improve the enantioselectivity for the lipase-catalysed reactions have been proposed: for example, optimization of the nature of the solvent[4] and of the water content,[5] chemical and non-covalent modifications of enzymes,[6] temperature[7] and additive effects,[5,8] and protein engineering.[9] In these approaches, the method of additives seems to be attractive in view of its simplicity of use. Indeed, various types of additives are known to control the enantioselectivity of the lipases.[8] Most of them, however, are focused on the lipase-catalysed hydrolysis in aqueous media.

Here, we wish to report that the enantioselectivity for lipase-catalysed esterification of 2-(4-substituted phenoxy) propionic acids **1a**–**1f** as a model reaction (Scheme 1) is improved dramatically by the addition of aqueous solutions of alkaline metal ions (*e.g.* LiCl) and alkaline earth metal ions (*e.g.* $MgCl_2$) directly to the isopropyl ether of the reaction medium.[10] Furthermore, the mechanism of the enhanced enantio-recognitions induced by the metal ions will be discussed briefly on the basis of the initial rate obtained for each enantiomer of **1a** and the behaviour of the flexibility of lipase estimated by EPR measurements.



Scheme 1

Results and discussion

Metal ions enhance the enantioselectivity for lipase-catalysed esterification in an organic solvent

We investigated the lipase MY- or AY-catalysed esterfication of the substrates **1a**–**1f** having various substituents with n-butanol in dry isopropyl ether as the model reaction (Scheme 1). Lipase MY differs from lipase AY in its catalytic features,[11] although they are originated from the same micro-organism, *Candida rugosa*. For the model reaction (Scheme 1), the *R* enantiomer of the butyl esters was preferentially produced for all of the substrates. The enantiomeric ratio (*E* value) was calculated from the enantiomeric excess (ee) for the butyl ester produced, according to the literature.[12] 2-Phenoxypropionic acids are well-known herbicides and also have other biological activities.[13] The *R* enantiomers are known to be active in these cases.

As shown in Table 1, under no-additive conditions (condition A), all of the substrates **1a**–**1f** show low enantioselectivities (*E* values) for both lipases, except for substrate **1a** with lipase AY. This result is consistent with the observations that the

[a]*The Graduate School of Science and Technology, Kobe University, Nada, Kobe 657-8501, Japan*
[b]*Division of Natural Environment and Bioorganic Chemistry, Faculty of Human Development and Sciences, Kobe University, Nada, Kobe 657-8501, Japan. E-mail: ueji@kobe-u.ac.jp; Fax: +81-78-803-7761*

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Table 1 Enantioselectivity for lipase MY- or AY-catalysed esterification of 2-(4-substituted phenoxy) propionic acids **1a**–**1f** under various conditions

	X	Lipase	Additive[a]	*t*/h	Conv. (%)	Ee (%)	*E* value
1a	Et	MY	A	120	25.0	52.3	3.8
			B	6	41.7	90.9	41
			C	16.5	39.5	98.1	200
			D	9	39.2	98.6	270
1b	Pr^i	MY	A	240	67.4	30.7	3.4
			B	12	43.5	84.1	22
			C	48	30.9	97.9	150
			D	24	40.7	98.7	310
1c	OMe	MY	A	168	6.65	17.8	1.5
			B	16	43.6	82.1	20
			C	168	33.9	92.8	43
			D	24	29.7	97.7	130
1d	Cl	MY	A	168	8.82	15.1	1.4
			B	24	38.1	61.4	6.0
			C	168	9.75	87.7	17
			D	96	34.0	98.7	29
1e	Me	MY	A	168	17.4	20.4	1.6
			B	9.5	40.4	84.8	22
			C	72	34.3	94.1	53
			D	24	37.0	97.5	140
1f	CF_3	MY	A	168	12.6	10.6	1.3
			B	24	40.8	74.6	11
			C	168	19.7	90.9	26
			D	168	38.6	94.7	67
1a	Et	AY	A	120	19.1	84.9	15
			B	24	43.9	84.7	24
			C	48	34.8	96.4	90
			D	38	41.8	97.6	170
1b	Pr^i	AY	A	200	5.5	53.8	3.4
			B	12	39.7	94.8	71
			C	120	37.1	97.9	170
			D	48	40.4	96.6	110
1c	OMe	AY	A	168	2.56	35.5	2.1
			B	62	48.8	82.7	25
			C	122	40.4	92.6	49
			D	48	35.2	93.6	51
1d	Cl	AY	A	168	2.89	17.9	1.4
			B	62	38.0	80.3	15
			C	168	33.9	87.1	23
			D	96	3.3	90.6	32
1e	Me	AY	A	168	3.43	44.0	2.6
			B	24	40.7	93.0	54
			C	62	38.4	96.6	110
			D	48	40.4	96.7	120
1f	CF_3	AY	A	168	1.93	11.6	1.3
			B	62	39.0	90.1	34
			C	168	26.3	95.0	56
			D	168	37.7	92.2	43

[a] A: no additive; B: 0.5 vol% water; C: 0.5 vol% aq. LiCl (2.4 M); D: 0.5 vol% aq. $MgCl_2$ (1.2 M).

lipase-catalysed asymmetric transformations, the esterification of 2-aryloxypropionic acids and the hydrolysis of the corresponding methyl esters generally resulted in low enantioselectivities.[14]

To improve the enantioselectivity of lipase in organic solvents, we first added a small amount of water to our model reaction, because the addition of a small amount of water is known to improve the enantioselectivity of the lipase-catalysed reaction in organic solvent.[5] As seen in comparison of the enantioselectivity values obtained for the no-additive conditions (condition A) and water-additive conditions (condition B) listed in Table 1, a small amount of the water allowed the enantioselectivities to be boosted by several times for lipase MY- or lipase AY-catalysed esterification of all the substrates **1a**–**1f**. The *E* value brought about by the water addition, however, would not be sufficient for an effective resolution.

Next, a new approach employing aqueous solutions containing a small amount of metal ions (2.4 M for LiCl, NaCl or KCl, and 1.2 M for $MgCl_2$, $CaCl_2$ or $BaCl_2$) as additive, instead of water alone, was tested for our model reaction. Fig. 1 and Fig. 2 show the variation of the *E* value for the lipase MY-catalysed esterification of **1a** at *ca.* 40% conversion as a function of the amount of metal ion-containing water. Interestingly, the presence of a small amount of the metal ion is found to cause a drastic enhancement of the enantioselectivity at around 0.5 vol% of each additive, the effect of which is much superior to the known procedure of a small amount of the water alone. In particular, upon the addition of 0.5 vol% of

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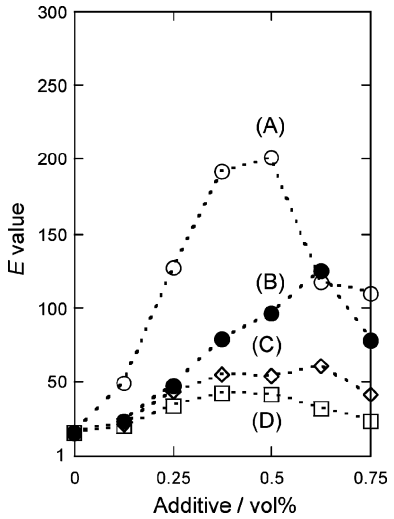


Fig. 1 Variation of the *E* value for the lipase MY-catalysed esterification of **1a** at *ca.* 40% conversion as a function of the amount of each additive: (A) LiCl, (B) NaCl, (C) KCl, (D) water.

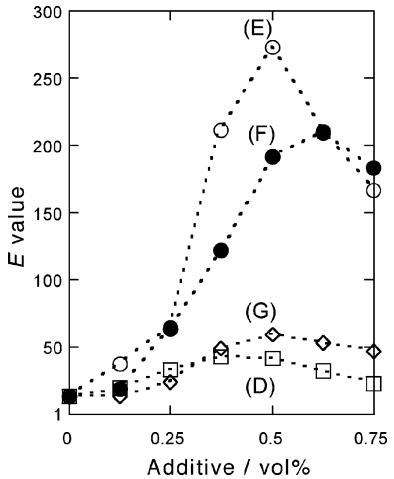


Fig. 2 Variation of the *E* value for the lipase MY-catalysed esterification of **1a** at *ca.* 40% conversion as a function of the amount of each additive: (E) $MgCl_2$, (F) $CaCl_2$, (G) $BaCl_2$, (D) water.

$MgCl_2$-containing water, lipase MY displays the highest *E* value (*ca.* 270) for the esterification of **1a**, the value of which is about 70× and 7× larger than the no-additive conditions (condition A) and the water-additive conditions (condition B), respectively. In order to investigate the scope of the enantioselectivity enhancement brought about by the metal ions, the other substrates **1b**–**1f** with a wide variety of substituents and the other lipase AY were submitted to the model reaction in the presence of 0.5 vol% (optimum amount conditions) of 2.4 M LiCl or 1.2 M $MgCl_2$ aqueous solution. Table 1 also summarises the enantioselectivity of these esterifications (see condition C and condition D). Thus, it is found from the data listed in Table 1 that the metal ion as a new additive dramatically increases the enantioselectivity for all the substrates and for both lipases MY and AY. This is the first example of an alkaline metal ion or alkaline earth metal ion enhancing the enantioselectivity of lipase in an organic solvent.

As shown in Fig. 1 and Fig. 2, the enhancement of the enantioselectivity is likely to depend on the species of the metal ion: the increased order of the *E* value is LiCl > NaCl > KCl for the alkaline metal ions and $MgCl_2$ > $CaCl_2$ > $BaCl_2$ for the alkaline earth metal ions, respectively. Furthermore, there was an optimum concentration of LiCl to give the maximum *E* value toward **1a** (Fig. 3). Above 2.4 M of the optimum concentration of LiCl, the enantioselectivity was decreased with a marked inhibition of the activity. The decrease of the *E* value may be attributed to a conformational change of the lipase arising from an excess amount of metal ions; under the conditions of 0.5 vol% of 4.8 M LiCl in isopropyl ether, only 5.7% of the butyl ester of **1a** was produced within 96 h with a lower enantioselectivity ($E = 25$) compared with the water-additive conditions ($E = 41$).

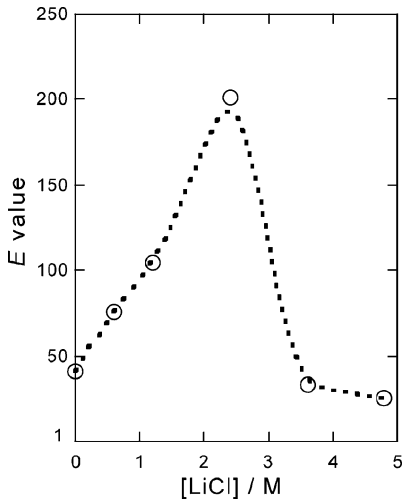
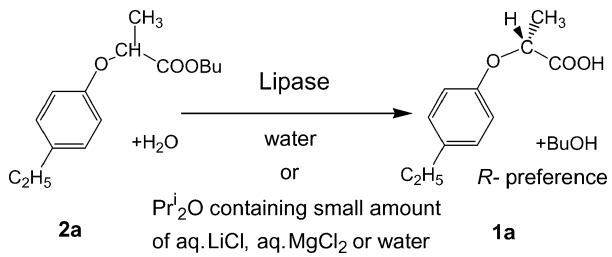


Fig. 3 Variation of *E* value for the lipase-catalysed esterification of **1a** as a function of the concentration of LiCl-containing water (0.5 vol%).

Metal ions enhance the enantioselectivity for lipase-catalysed hydrolysis in an organic solvent

The drop in *E* value which can be seen from the bell-shaped plots in Fig. 1 and Fig. 2 was produced by the addition of above 0.5 vol% of metal ion-containing water or water to isopropyl ether. This can be explained by assuming that an excess of water molecules hydrated to the metal ion should cause the hydrolysis (the reverse reaction) of the corresponding ester **2a**, thus leading to the reduction in the *E* value, probably because the sites of the lipase molecule associated with the water molecule are restricted. In fact, the addition of 0.75 vol% of 2.4 M LiCl or 1.2 M $MgCl_2$ solutions was found to cause highly enantioselective hydrolysis of **2a**–**2d** catalysed by lipase MY or AY in isopropyl ether (Scheme 2 and Table 2). In particular, by the use of 1.2 M $MgCl_2$ with lipase MY (condition H), the excellent



Scheme 2

Table 2 Enantioselectivity for lipase MY- or AY-catalysed hydrolysis of butyl 2-(4-substituted phenoxy) propionates **2a**–**2d** under the various conditions

	X	Lipase	Additive[a]	*t*/h	Conv. (%)	Ee (%)	*E* value
2a	Et	MY	E	0.5	51.2	23.1	2.0
			F	4	42.2	93.9	64
			G	4	41.6	99.3	610
			H	4	40.7	99.4	680
		AY	E	0.5	49.7	74.1	15
			F	2.2	30.7	98.2	170
			G	2.2	33.4	98.6	240
			H	2.2	33.7	98.9	300
2b	Pr^i	MY	E	0.5	52.5	50.5	5.2
			F	4	36.9	93.3	50
			G	4	32.3	99.0	330
			H	4	34.9	99.4	590
2c	OMe	MY	E	0.5	46.2	17.5	1.6
			F	6	37.2	94.1	57
			G	6	34.7	99.4	610
			H	6	34.7	99.0	320
2d	Cl	MY	E	1	47.3	14.2	1.5
			F	6	35.1	79.1	13
			G	6	23.9	98.8	230
			H	6	28.0	97.8	130

[a] E: aqueous solution; F: isopropyl ether containing 0.75 vol% water; G: isopropyl ether containing 0.75 vol% aq. LiCl (2.4 M); H: isopropyl ether containing 0.75 vol% aq. $MgCl_2$ (1.2 M).

E value toward **2a** was obtained, the value of which was increased by 340-fold as compared with the hydrolysis in water (condition E). This technique also proves to be one of the useful tools for improving the enantioselectivity of lipase-catalysed hydrolysis.

Initial rate for each enantiomer of the subustrate catalysed by lipase MY in an organic solvent

To find the reason for the remarkable enhancement of lipase's enantioselectivity brought about by the action of metal ions, we first investigated the initial rate for each enantiomer of **1a** for lipase MY-catalysed esterification under three additive conditions in *n*-hexane: no additive, water additive (0.5 vol%) and 2.4 M LiCl-containing water additive (0.5 vol%) (Table 3). As can be seen from the data listed in Table 3, there was a marked difference between the behaviour of the initial rate for the addition of water alone and that for the addition of aqueous LiCl. Thus, for the former additive, the initial rates for both *R* and *S* enantiomers are accelerated, and its acceleration for the *R* enantiomer (correctly binding substrate) is more pronounced than that for the *S* enantiomer (incorrectly binding substrate) as compared with the no-additive conditions. In contrast, for the latter additive, the initial rate for the *R* enantiomer is accelerated, whereas that for the *S* enantiomer is totally suppressed. Therefore, the larger value of the quotient (V_R/V_S), arising from the opposite trend of each initial rate, is responsible for the significant enhancement of lipase's enantioselectivity.

Table 3 Initial rate (V_R, V_S) for semi-purified lipase MY-catalysed esterification of each enantiomer of **1a** under three additive conditions in *n*-hexane

	Water			aq. LiCl[a]		
Additive[b]	V_R[c]	V_S[c]	V_R/V_S	V_R[c]	V_S[c]	V_R/V_S
0 (None)	0.014	0.011	1.2	0.014	0.011	1.2
0.1	0.16	0.062	2.5	0.21	0.0027	78
0.2	0.87	0.23	3.7	1.4	0.0022	670
0.3	1.1	0.21	5.2	1.4	0.0072	190
0.4	1.3	0.19	6.6	1.2	0.011	120
0.5	1.4	0.19	7.0	1.3	0.010	120

[a] Additive concentration: 2.4 M. [b] Units: vol%. [c] V_R and V_S denote the initial rates for the *R* and *S* enantiomers, respectively, in μmol min^{-1} mg^{-1}.

Correlation between the flexibility of lipase estimated by EPR measurements and its enantioselectivity

Next, to investigate a relationship between the flexibility of lipase and its enantioselectivity, we measured the EPR spectra of lipase in *n*-hexane† in the absence and the presence of the additive. The active site (serine) of the semi-purified lipase MY was spin-labelled with 1-oxy-2,2,6,6,-tetramethyl-4-piperidinyl-ethoxyphosphoro-fluoridate (TEMPO-4-EPF).[15] Fig. 4 shows the typical EPR spectra of the spin-labelled lipase suspended in *n*-hexane under three additive conditions. Upon the addition of

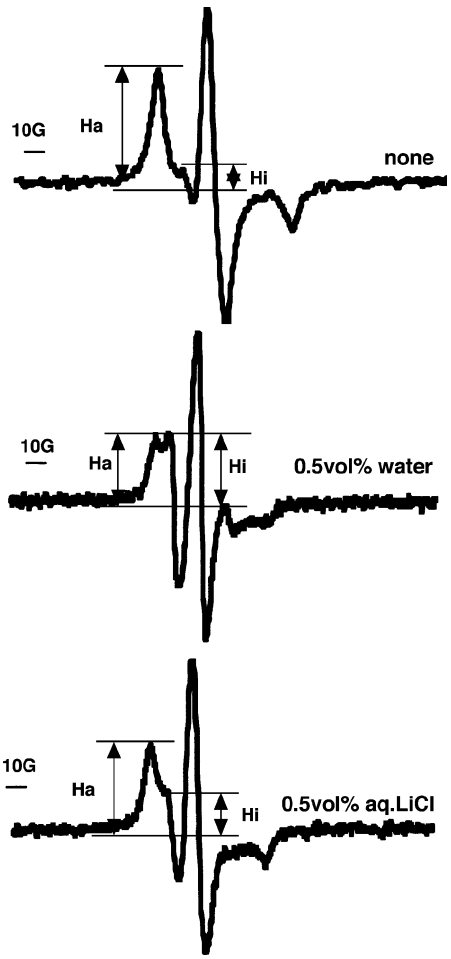


Fig. 4 Typical EPR spectra of the spin-labelled lipase under three additive conditions: no water additive (0 vol%), water additive (0.5 vol%), and 2.4 M LiCl-containing water additive (0.5 vol%).

† In isopropyl ether containing a small amount of water, a marked decrease in the *Q* value was observed, resulting in the poor reproducibility of the spectrum. For this reason, the solvent used in the EPR measurements and the initial rate was changed to *n*-hexane.

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Table 4 Secondary structure of lyophilized lipase MY suspended in *n*-hexane under the two additive conditions

Additive	α-Helix (%)	β-Sheet (%)
Water (0.5 vol%)	28.5	38.6
Aq. LiCl[a] (0.5 vol%)	25.8	37.6

[a] Additive concentration: 2.4 M.

water (0.5 vol%), a new isotropic signal was found to arise and the spectral lines narrowed in width as compared with the no-additive conditions. As for the LiCl-containing water-additive conditions (0.5 vol%), the isotropic signal also arose and the spectral lines narrowed, the extent of which seems to be smaller as compared with the water-additive conditions (0.5 vol%). This change of the EPR spectrum signifies an increase in the average motion of the TEMPO ring with respect to its environment, suggesting that the conformation of lipase's active site surrounding the TEMPO ring becomes flexible. This spectral result is similar to that observed for the EPR spectrum of the spin-labelled subtilisin Carlsberg suspended in tetrahydrofuran.[16] Additionally, as to the effects of LiCl on the structure of lipase, FT-IR measurement was carried out. All the IR spectra in the amide III region of lipase MY in *n*-hexane with 0.5 vol% of water and 0.5 vol% of aqueous LiCl were deconvoluted and assigned to the individual secondary structure elements according to the literature[17] (Table 4). It was found from the comparison of the data in Table 4 that the native secondary structure of the lipase is held even in the presence of LiCl.

Then, the degree of the lipase's flexibility can be monitored by the change in the ratio of H_i to $(H_i + H_a)$, where H_i and H_a represent the peak height of the isotropic signal and the anisotropic signal of the spin-label, respectively (Fig. 4).[18] As judged from Fig. 5, for both additives the $H_i/(H_i + H_a)$ value as a measure of the lipase's flexibility increased smoothly with an increase of the amount of the additive. The presence of an ion, however, reduced the $H_i/(H_i + H_a)$ value as compared with water alone.

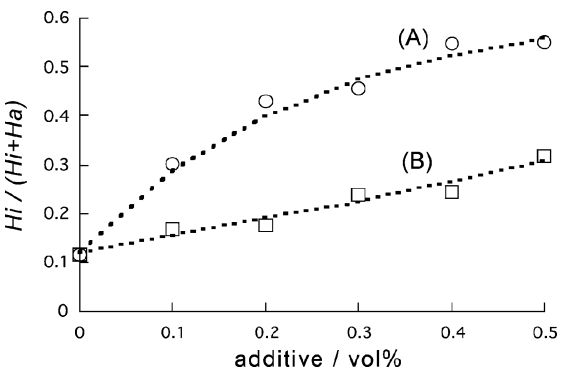

Fig. 5 Plot of lipase flexibility $[H_i/(H_i + H_a)$ value] against the amount of each additive: (A) water, (B) LiCl.

To obtain an insight into the mechanism of the lipase's enantio-recognition based on its flexibility observed, the initial rate for each enantiomer of **1a** (Table 3) was plotted as a function of the $H_i/(H_i + H_a)$ value (Fig. 6 and Fig. 7). The increase in the lipase's flexibility shows a great beneficial effect on the initial rate for the *R* enantiomer (correctly binding substrate) in both additives. In

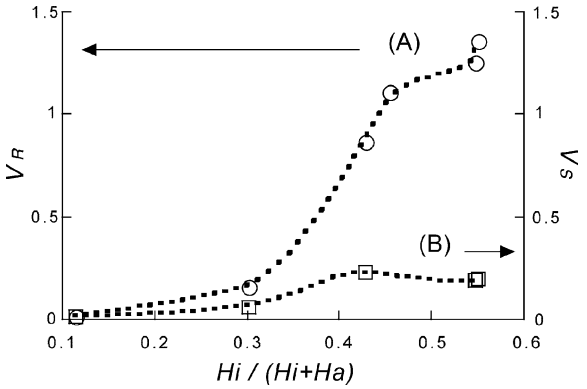

Fig. 6 Initial rate (V_R, V_S) for each enantiomer of **1a** as a function of lipase flexibility $[H_i/(H_i + H_a)$ value] under the water-additive conditions: (A) V_R, (B) V_S.

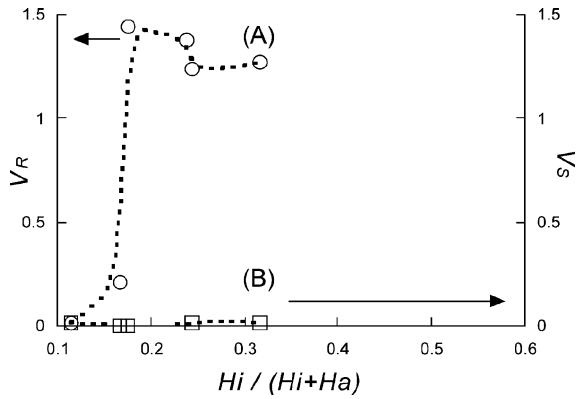

Fig. 7 Initial rate (V_R, V_S) for each enantiomer of **1a** as a function of lipase flexibility $[H_i/(H_i + H_a)$ value] under the LiCl-containing water additive conditions: (A) V_R, (B) V_S.

sharp contrast, the initial rate for the *S* enantiomer (incorrectly binding substrate) in the presence of LiCl cannot accept the benefit of lipase's flexibility, thus resulting in the almost complete suppression of the V_S value. Some conformational change of lipase caused by the metal ion may prevent its active site from accepting the *S* enantiomer, the incorrect binding substrate. The addition of the metal ion would partially neutralise the charges on the lipase's surface. This may alter the lipase conformation coupled with its flexibility, because enzyme conformation is based on a subtle balance of its net charge due to hydrogen bondings, electrostatic attractions, hydrophobic forces, *etc*.

Combined effects of metal ion and lowering reaction temperature on lipase-catalysed esterification in an organic solvent

To improve further the enantioselectivity of lipase MY-catalysed esterifications in isopropyl ether, we used a combination of the metal ion as an additive and low reaction temperature (Table 5). This is because the low temperature may increase the strength of the association between the metal ion and the surface charges of the lipase. In fact, it was found from the *E* values listed in Table 5 that the enantioselectivity for LiCl is quickly enhanced with the lowering of the reaction temperature from 50 to 10 °C, whereas for the addition of water alone the *E* value is not affected with the change of temperature. When the reaction temperature is lowered

Table 5 Enantioselectivity for the lipase MY-catalysed esterification of **1a** at various temperatures

T/°C	Lipase	Additive[a]	t/h	Conv. (%)	Ee (%)	E value
10	MY	A	24	2.1	81.0	10
		B	24	39.4	93.4	55
		C	48	38.5	99.7	1300
20	MY	A	24	4.0	71.5	6.2
		B	15	40.2	92.0	49
		C	24	37.8	99.1	400
37	MY	A	120	25.0	52.3	3.8
		B	6	41.7	90.9	41
		C	16.5	39.5	98.1	200
50	MY	A	48	7.3	10.6	1.3
		B	4	39.9	91.6	43
		C	48	11.1	83.0	12

[a] A: none; B: 0.5 vol% water; C: 0.5 vol% aq. LiCl (2.4 M).

to 10 °C, lipase MY with metal ions (condition C) displays an almost perfect enantioselectivity ($E = 1300$), the value of which is 130 × larger when compared with the no-additive conditions (condition A).

Conclusions

We have developed a simple and a powerful method based on the addition of a small amount of an aqueous metal ion-containing solution to the organic solvent as the reaction medium to achieve a remarkably enhanced enantioselectivity for lipase-catalysed transformations. Furthermore, the addition of metal ions and lowering the reaction temperature synergetically enhanced the enantioselecitivity of lipase and led to an almost perfect enantioselectivity ($E = 1300$). On the basis of the EPR measurements and the initial rate for each enantiomer of the substrate, the enantioselectivity enhancement brought about by the metal ions was found to be attributed to the opposite trend for the initial rate: acceleration for the correctly binding *R* enantiomer and serious deceleration for the incorrectly binding *S* enantiomer. Our method of metal ion addition will form a useful tool to obtain optically pure compounds.

Materials and methods

Materials

Lipases MY and AY originated from *Candida rugosa* were supplied from Meito Sangyo Co., Ltd., Japan and Amano Pharmaceutical Industries, Ltd., Japan, respectively. Solvents used in the reaction were purchased from Wako Pure Industries, Ltd., Japan and were dried prior to use by molecular sieves. 1-Oxy-2,2,6,6,-tetramethyl-4-piperidinyl-ethoxyphosphoro-fluoridate (TEMPO-4-EPF) was purchased from SIGMA. Semi-purified lipase MY was prepared by dialysing and lyophilizing from crude lipase MY.

Preparation of substrates

According to the known method,[13a] substrates **1a**–**1f** were prepared from the corresponding 4-substituted phenols and ethyl 2-bromopropionate, followed by the hydrolysis of the esters, and **2a**–**2d** were prepared from the corresponding 4-substituted phenols and butyl 2-bromopropionate. All of the products were purified by silica gel column chromatography (*n*-hexane–ethyl acetate = 10 : 1). Furthermore, the acids **1a**–**1f** obtained were recrystallised from *n*-hexane. The *R* or *S* enantiomer of **1a** (*ca.* 99% ee) was prepared from our method.[10a]

Lipase-catalysed esterification of 1a–1f

For the esterification, the racemic 2-(4-substituted phenoxy) propionic acid (0.36mmol) and 3-fold molar excess of 1-butanol (1.08 mmol) were dissolved in 2 ml of isopropyl ether. To the solution, a small amount of water (0–0.75 vol%) or (0.6–6 M) metal ion-containing water (0–0.75 vol%) was added followed by ultrasonic dispersion, and then powdered lipase MY or AY, originated from *Candida rugosa* (30 mg), was added. The suspension was shaken (170–200 strokes min^{-1}) at 10–50 °C until 40% of the substrate had reacted to the corresponding butyl ester. The enantiomeric ratio (E value) was calculated from the enantiomeric excess (ee) for the butyl ester produced, according to the literature.[12] The reaction was monitored by HPLC (using *n*-hexane–isopropanol = 97 : 3 as an eluent with the addition of 0.1 vol% trifluoroacetic acid) on a Chiralcel OK column (Daicel Chemical Industries Co.Ltd., Japan). The ee value for the product was also determined with HPLC on a Chiralcel OK column.

Lipase-catalysed hydrolysis of 2a–2d in organic solvent or aqueous solution

The racemic butyl 2-(4-ethylphenoxy) propionate (0.036mmol) was dissolved in water or isopropyl ether followed by ultrasonic dispersion and then powdered lipase MY or AY (30 mg) was added. The suspension was shaken (170–200 strokes min^{-1}) at 37 °C until 40% of the substrates **2a**–**2d** had reacted to the corresponding acids **1a**–**1d**.

Initial rate for lipase MY-catalysed esterification of 1a

R- or *S*-2-(4-Ethylphenoxy)propionic acid (0.036mmol) and a 30-fold molar excess of 1-butanol (1.08 mmol) were dissolved in 2 ml of *n*-hexane. To the solution, a small amount of 2.4 M LiCl-containing water (0–0.5 vol%) or water (0–0.5 vol%) was added followed by ultrasonic dispersion, and then powdered semi-purified lipase MY (2 mg) was added. The suspension was shaken (170–200 strokes min^{-1}) at 37 °C.

Spin-labelling to the lipase active site and EPR measurements

The active site (serine) of the semi-purified lipase was spin-labelled with 1-oxy-2,2,6,6,-tetramethyl-4-piperidinyl-ethoxyphosphoro-fluoridate (TEMPO-4-EPF) purchased from SIGMA, according to the procedure reported by Morrisett and Broomfield.[15] The extent of labelling was calculated from the residual activity for the esterification of **1a**. Typically, about 66% of the active site was labelled. All of the EPR measurements were carried out at room temperature (*ca.* 25 °C) on a Bruker EMX081 spectrometer at X-band frequency under the water-additive conditions ranging from 0 to 0.5 vol%, because an excess of the water addition leads to a marked decrease in the Q value.

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FT-IR measurements of lipase MY suspended in *n*-hexane and secondary structure analysis

All the FT-IR measurements were carried out at room temperature (*ca.* 25 °C) on a Horiba FT-720 spectrometer, equipped with KRS-5 attenuated total reflectance (ATR) accessory. The spectrometer was purged with dry N_2 air and was operated at a resolution of 2 cm^{-1}. The spectral data obtained were converted into ASCII format and identification of band position was carried out by second-order derivatisation of the FT-IR absorption spectra with the use of Microcal ORIGIN 5.0. The spectra were deconvoluted by the use of the ORIGIN 5.0 peak-fitting module. A half-bandwidth of 20 cm^{-1} was used to obtain the spectral deconvolution. The band assignment in the amide III region was performed as described in the literature:[17] α-helix, 1328–1289 cm^{-1}; unordered, 1288–1256 cm^{-1}; β-sheets, 1255–1224 cm^{-1}.

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