Pergamon

Tetrahedron: Asymmetry Vol. 6, No. 6, pp. 1311-1322, 1995
Elsevier Science Ltd
Printed in Great Britain
0957-4166/95 $9.50+0.00

0957-4166(95)00165-4

Enhancing Enantioselectivity of a Lipid-coated Lipase *via* Imprinting Methods for Esterification in Organic Solvents[1]

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Abstract: Lipase OF from *Candida* showed only low enantioselectivity for esterification of *(R)*- or *S*-1-phenylethanol with lauric acid (v_R/v_S = 5.5). However, when lipase OF was imprinted with a substrate analogue such as *(R)*-1-phenylethanol and then coated with synthetic glycolipid molecules, the imprinted lipid-coated lipase shows a large enantioselectivity for the esterification in anhydrous isooctane (v_R/v_S = 77). When the native lipase OF was imprinted by the same procedure, the enantioselectivity hardly changed. The lipid coating was important to keep the imprinted structure as well as to solubilize enzymes in organic solvents. The improved enantioselectivity was confirmed from Michaelis-Menten kinetics as due to the intramolecular catalytic reaction and not the substrate binding process. The improved enantioselectivity reverts to the original non-imprinted value if kept in the organic solvents at high temperatures for days.

In recent years, interest in the use of hydrolytic enzymes as synthetic chiral catalysts has risen rapidly.[2-5] Since lipases are physically stable and have broad substrate specificity, they have been employed as catalysts in enantioselective esterification or transesterification reactions in monophasic organic solvents or water-organic solvent biphasic systems.[6] To use enzymes in organic media it is necessary to avoid deactivation or denaturation.

We have reported the preparation of a lipid-coated lipase whose surface is covered by a synthetic glycolipid monolayer and that is soluble in most of organic media.[7-10] The lipid-coated lipase B or P from *Pseudomonas* can catalyze enantioselective esterification from racemic alcohols and aliphatic acids in anhydrous isooctane in the presence of molecular sieves.[1,7] The catalytic activity of the lipid-coated lipase was 2-100 times higher than those of other systems such as lipase in water in oil (w/o) emulsion,[11-13] direct dispersion of powdered lipase,[14-19] and poly(ethylene glycol)-grafted lipase in organic solvents.[20-22] The lipid-coated lipase can prepare esters directly from acids and alcohols in anhydrous organic solvents. The enantioselectivity and substrate specificity of the lipid-coated lipase were the same as those of a native lipase.[1,7]

Although the enzyme activity can be improved by chemical modification of enzymes and changing of reaction media, the control of the enantioselectivity of the enzyme has been difficult and the enantioselectivity depends largely on the origin of lipase. There are only a limited number of commercial lipases that are suitable for the desired enantioselective reactions, and the discovery of new lipases with

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well-defined stereo chemical properties is difficult. Several approaches to improve the enantioselectivity of enzymes have been reported by the modification of the substrate molecules and enzymes,[23,24] the use of non-aqueous organic solvents,[25-27] and enantioselective inhibition.[28]

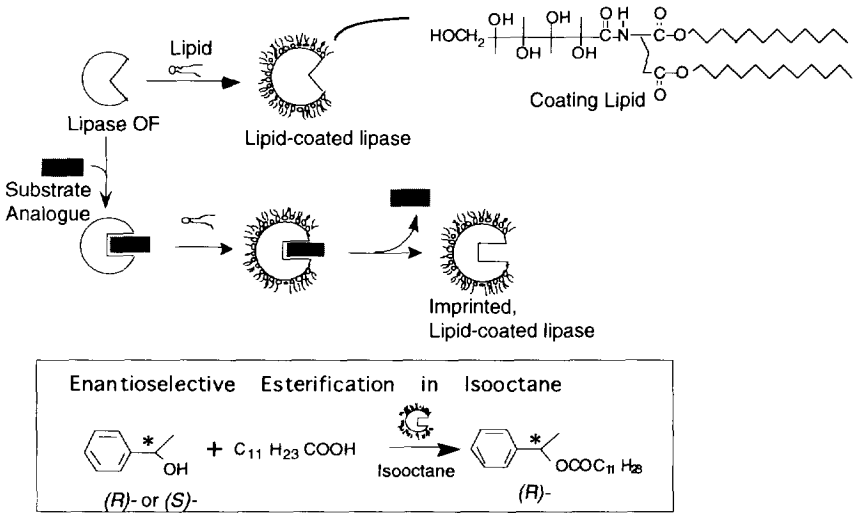


Figure 1. A schematic illustration of imprinting process of a lipid-coated lipase and enantioselective esterification in isooctane.

In this paper, we report a new strategy to improve the low enantioselectivity of an enzyme, which was imprinted by a substrate analogue (see Figure 1). We chose lipase OF from *Candida cylindracea* that shows only low enantioselectivity for ester hydrolysis in aqueous solution or ester synthesis in organic media.[23-27] When the lipid-coated lipase is prepared by mixing an aqueous solution of the lipase and glycolipids in the presence of a substrate analogue, the obtained imprinted, lipid-coated lipase showed a 10 times larger enantioselectivity for esterification of racemic alcohols and aliphatic acids in the dry isooctane than that for a native lipase or a lipid-coated lipase without imprinting process.

Experimental Section

Materials. Lipase OF (from *Candida cylindracea*) was purchased as a fine grade from Meito Industrial Co., Tokyo and Amano Pharmaceutical Co., Tokyo, and used without further purification. Preparations of a synthetic glycolipid, dioctadecyl N-D-glucono-L-glutamate, was reported elsewhere.[29] *(R)*- or *(S)*-1-phenylethanol and other chiral alcohols were a kind gift from Chisso Co. Ltd., Tokyo. Other chemicals and organic solvents were purchased as a fine grade from Tokyo Kasei Co., Tokyo.

Preparation of a Lipid-coated and/or Imprinted Lipase OF. A lipid-coated lipase OF imprinted with substrate analogues was prepared typically as follows. The preparation process of a lipid-coated lipase was the similar to previous papers.[1,7-10] To an aqueous solution (25 ml, 0.01 M acetate buffer, pH 5.6) of lipase OF (from *Candida cylindracea*, 50 mg), imprinting substrates (10 mM) such as

(R)-1-phenylethanol was added and maintained under stirring for 30 min. at room temperature. Hot acetone solution (0.5 ml) of glycolipid was added dropwisely into the enzyme aqueous solution at room temperature, and then kept under stirring for 20 h at 4 °C. Precipitates were gathered by centrifugation at 4 °C (5000 rpm for 5 min.) and lyophilized. The imprinting substrates added could be recovered in a liquid N_2 trap during lyophilization. The white powder obtained of the lipid-coated lipase was insoluble in water or any aqueous buffer solution but freely soluble in most organic solvents.

In the case of the imprinting of a native lipase, aqueous solution of lipase OF with imprinting substrates was kept for 30 min. at room temperature, and then lyophilized.

The protein content in the lipid-coated lipase was obtained to be 5.4 and 6.5 wt% from the elemental analysis (C, H, and N) and the UV absorption of aromatic amino acid residues in proteins at 280 nm in chloroform solution, respectively.

Gel-permeation chromatography of the lipid-coated lipase OF showed one peak at the estimated molecular weight of $(130 \pm 20) \times 10^3$ in dichloromethane as eluent (detector, UV at 240 nm; molecular weight was calibrated using a standard polystyrene). Since the molecular weights of a native lipase and the glycolipid were *ca.* 33,000 and 621, respectively, the lipid-coated lipase is calculated to contain 150 ± 50 lipid molecules per one lipase. It can be roughly estimated from the molecular area of the lipid (0.45 nm^2) and lipase (diameter, *ca.* 4 nm) that *ca.* 150 ± 50 lipid molecules are required to cover the surface of a lipase as a monolayer. These values are consistent with each other and with the protein content (6.0 ± 0.5 wt%) in the complex obtained from elemental analyses and UV measurements.

Catalytic Activity of a Lipid-coated Lipase in Organic Solvents. A typical procedure is as follows. To the anhydrous isooctane solution (5 ml) of a lipid-coated lipase (10-20 mg, 1.0 mg of protein), *(R)*- or *(S)*-1-phenylethanol (30.5 mg, 50 mM) and an excess amount of lauric acid (250 mg, 500 mM) were added and stirred in the presence of two pieces of 3 Å molecular sieves at 40 °C. Water content in the solution was monitored to be 60-80 ppm during the reaction by Karl-Fisher titration (instrument: Mitsubishi Chemical, Tokyo, type CA-05). With the prescribed time interval, the production of *(R)*- or *(S)*-esters and the disappearance of *(R)*- or *(S)*-1-phenylethanol were followed by gas chromatography (GC; instrument, Shimadzu GC-8APT; column, silicon GS-1+Uniport HP 100/120, f3.2 mm x 1 m glass tube; column temperature, 190-300 °C/16 °C min^{-1}; injection temperature, 320 °C; carrier gas, He). Identification and quantification of the substrates and the products were performed by comparing of the GC retention time and the GC peak area to those of the authentic *(R)*- or *(S)*-samples, respectively.

Results and Discussion

Figure 2 shows typical time courses of ester syntheses from *(R)*- and *(S)*-1-phenylethanol and excess lauric acid catalyzed by a native lipase OF and a lipid-coated lipase OF in anhydrous isooctane (60-80 ppm of H_2O) at 40 °C. When the native lipase was employed, the esterification reaction was very slow since native lipase is not soluble and dispersed in isooctane, and the enantioselectivity for 1-phenylethanol was also very low. It is well known that lipases from *Candida sylindracea* such as lipase OF and Lipase AY show generally low enantioselectivity compared with lipases from *Pseudomonas* and *Rhizopus delemer* such as lipase P, lipase D, and lipase B for enatioselective ester hydrolysis in aqueous and

aqueous-organic biphasic solvents.[6,7,23-28] When a lipid-coated lipase OF that was imprinted with *(R)*-1-phenylethanol was employed, the remarkable high reaction rates and enantioselectivity for the esterification of *(R)*-1-phenylethanol were obtained. The lipid-coated lipase is freely soluble in most of organic solvents such as isooctane and the reaction rate was very high compared with that of the dispersed native lipase.[1,7-10] It is interesting that enantioselectivity for the esterification of *(R)*-1-phenylethanol was largely improved by using a lipid-coated and imprinted lipase in organic solvents. Initial rates for *(R)*- and *(S)*-isomers obtained from slopes in Figure 2 and their ratio (v_R/v_S) are used mainly for the following discussion of enzyme activity and enantioselectivity.

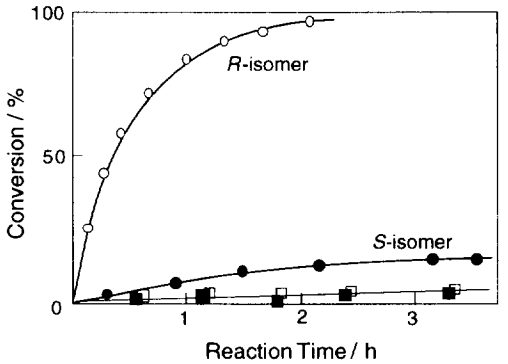


Figure 2. Typical time-courses of esterification of *(R)*- and *(S)*-1-phenylethanol (open and closed symbols, respectively, 50 mM) with lauric acid (500 mM) catalyzed by a lipid-coated lipase OF (1 mg of protein) imprinted with *(R)*-1-phenylethanol (○ and ●), and a native lipase OF dispersed (□ and ■) in anhydrous dry isooctane (5 ml, H_2O: 60-80 ppm) in the presence of molecular sieves at 40 °C

Effect of Imprinting procedures. Table 1 summarizes initial reaction rates and enantioselectivities for the esterification of *(R)*- or *(S)*-1-phenylethanol and lauric acid in the anhydrous isooctane by using lipases prepared by various imprinting methods. Since native lipase OF from *Candida Cylindracea* is known generally to show the low enantioselectivity for ester hydrolyses, it is reasonable that native lipase OF showed the low enantioselectivity ($v_R/v_S = 5.5$) for ester synthesis in organic solvents, and that the reaction rate was very small due to the dispersion state of water-soluble lipase in organic solvents. When native lipase OF was imprinted with substrate analogue, *(R)*-1-phenylethanol, by incubating in buffer solution and lyophilizing, the enantioselectivity was hardly changed, and the reaction rate was largely decreased probably due to denaturation during the lyophilizing process.

When a lipid-coated lipase was employed as a catalyst without imprinting, the enantioselectivity was slightly increased ($v_R/v_S = 16$) compared with that of native lipase. This is explained by the effect of coating lipids that may change the enantioselectivity slightly. When lipase was imprinted with *(R)*-1-phenylethanol and then coated with lipids, a very large enantioselectivity ($v_R/v_S = 77$) was obtained due to the large increase of the v_R value. On the contrary, lipase was first coated with lipids and then incubated with *(R)*-1-phenylethanol in buffer solution, the obtained lipid-coated lipase did not show the high enantioselectivity ($v_R/v_S = 22$) compared with that of the imprinted, then lipid-coated lipase. These results

indicate that coating lipids are important to memory the imprinting effect with incubating in the substrate analogue.

Table I. Preparations of Imprinted Lipase OF and their Enantioselectivity for Ester Synthesis in Anhydrous Isooctane[a]

Enzymes	Initial Rates / mM s^{-1} (mg of protein)$^{-1}$		Enantioselectivity
	v_R	v_S	v_R/v_S
Native lipase (not imprinted)	0.071	0.013	5.5
Imprinted native lipase[b]	0.0027	0.00046	5.9
Lipid-coated lipase (not imprinted)[c]	0.44	0.026	16
Imprinted, then lipid-coated lipase[d]	1.1	0.014	77
Lipid-coated, then imprinted lipase[e]	0.93	0.043	22

[a] Ester syntheses from *(R)*- or *(S)*-1-phenylethanol (50 mM) and lauric acid (500 mM) catalyzed by lipase OF (1.0 mg of protein) in the anhydrous isooctane (5 ml) at 40 °C. [b] Native lipase OF was incubated with *(R)*-1-phenylethanol in acetate buffer, then lyophilized. [c] Aqueous solution of Lipase OF was mixed with lipids and the precipitates were lyophilized. [d] Lipase OF was incubated with *(R)*-1-phenylethanol in acetate buffer, glycolipids were added, and the precipitates were lyophilized (see Experimental). [e] Lipid-coated lipase was incubated with *(R)*-1-phenylethanol in acetate buffer, then lyophilized.

Imprinting Effects for Other Reactions. Table II shows the results when lipase OF was imprinted with *(R)*-1-phenylethanol or *(R)*-2-nonanol and then coated with lipids, and employed as an esterification catalyst of *(R)*- or *(S)*-2-nonanol with lauric acid in the anhydrous isooctane. The enantioselectivity was hardly changed by imprinting with both *(R)*-substrates. This indicates that the lipase imprinted with *(R)*-1-phenylethanol having a large phenyl group and a small methyl group can increase the enantioselectivity for the esterification of the same alcohol enantiomers, but not for other substrates. *(R)*-2-nonanol having a long aliphatic chain was not a good imprinting substrate for the enantioselective esterification of the same alcohol. There must be a suitable combination between the imprinting substrate and the enantioselective reaction.

Table III shows that the imprinting effect of *(R)*-1-phenylethanol on the enantioselective esterification of *(R)*- or *(S)*-2-propionic acid with 1-nonanol in isooctane. The enantioselectivity for the carboxylic acid moiety was hardly changed by imprinting with the enantio-alcohol moiety. This indicates that 1-phenylethanol imprinted the alcohol binding pocket and did not affect the carboxylic acid binding pocket.

Table IV shows the imprinting effect of lipase OF on the catalytic hydrolysis catalyst of *(R)*- or *(S)*-1-phenylethanol acetate in the aqueous buffer solution. A native lipase OF and the lipid-coated lipase OF without imprinting process showed the low enantioselectivity also in the hydrolysis reaction. When lipase OF was imprinted with a substrate analogue *(R)*-1-phenylethanol and then coated with lipid molecules, the enantioselectivity was slightly increased. However, the imprinting effect was very small ($v_R/v_S = 11$) compared with the esterification of 1-phenylethanol in organic solvents ($v_R/v_S = 77$, see Table I). This indicates that the imprinting effect can be kept in the organic solvents, but the imprinted enzyme structure may be reversed to the original in the aqueous buffer solution.

Table II. Effect of Imprinting of Lipase OF on Enantioselective Esterification of 2-Nonanol in Isooctane at 40 °C[a]

Enzymes	Initial Rates / mM s^{-1} (mg of protein)$^{-1}$		Enantioselectivity
	v_R	v_S	v_R/v_S
Native lipase (not imprinted)	0.12	0.076	1.6
Lipid-coated lipase			
not imprinted	0.43	0.39	1.1
imprinted with *(R)*-1-phenylethanol[b]	4.0	2.3	1.7
imprinted with *(R)*-2-nonanol	0.87	0.54	1.6

[a]

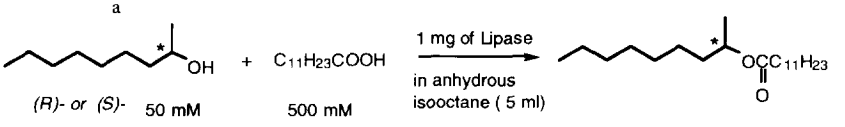


Table III. Effect of Imprinting of Lipase OF on Enantioselective Esterification of 2-Phenylpropionic acid in Isooctane at 40 °C[a]

Enzymes	Initial Rates[a] / mM s^{-1} (mg of protein)$^{-1}$		Enantioselectivity
	v_R	v_S	v_R/v_S
Native lipase (not imprinted)	0.0011	0.0026	0.42
Lipid-coated lipase			
not imprinted	0.0018	0.011	0.16
imprinted with *(R)*-1-phenylethanol	0.0046	0.026	0.21

[a]

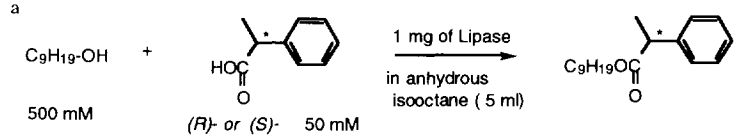


Table IV. Effect of Imprinting of Lipase OF on Enantioselective Ester Hydrolysis in Aqueous Solution at 40 °C[a]

Enzymes	Initial Rates[a] / mM s^{-1} (mg of protein)$^{-1}$		Enantioselectivity
	v_R	v_S	v_R/v_S
Native lipase (not imprinted)	0.45	0.16	2.8
Lipid-coated lipase			
not imprinted	0.081	0.013	6.2
imprinted with *(R)*-1-phenylethanol	0.25	0.023	11

[a]

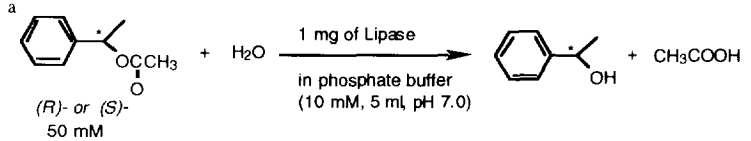
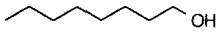
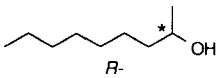
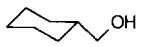
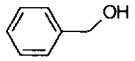
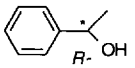
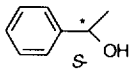
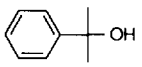
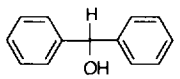
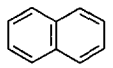
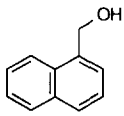


Table V. Effect of Chemical Structures of Imprinting Substrates on Enantioselectivity for esterification of *(R)*- or *(S)*-1-phenylethanol

Run	Imprinting Substrates	Initial rates[a] / mM s^{-1} (mg of protein)$^{-1}$ v_R	v_S	Enantioselectivity v_R / v_S
1		0.82	0.046	18
2		1.1	0.041	28
3		0.78	0.038	20
4		1.3	0.035	38
5		0.41	0.028	15
6		0.23	0.0048	45
7		1.1	0.014	77
8		1.2	0.038	31
9		0.99	0.024	41
10		0.22	0.013	17
11		0.82	0.054	15
12		0.82	0.031	27

[a] Initial rates of ester syntheses from *(R)*- or *(S)*-1-phenylethanol (50 mM) and lauric acid (500 mM) in the anhydrous isooctane (5 ml) at 40 °C catalyzed by the lipid-coated lipase OF (8 mg, 1 mg of protein) that was imprinted with the respective substrate.

Effect of Structures of Imprinted Substrates. After lipase OF was imprinted with the substrate analogue [*(R)*-1-phenylethanol] and then coated with lipids, the enantioselectivity of lipase OF was largely improved in the esterification of *(R)*- or *(S)*-1-phenylethanol with lauric acid in anhydrous isooctane. When chemical structures of the imprinting substrate were changed, effects on the enantioselectivity for the same esterification are summarized in Table V. When lipase OF was imprinted with simple hydrocarbons such as benzene, cyclohexane, and naphthalene (Runs 3, 5, and 11) or simple

aliphatic alcohol (Run 1), the enantioselectivity was hardly affected compared with the lipid-coated lipase without imprinting (v_R/v_S= 16, see Table I). On the contrary, when lipase OF was imprinted with alcohols having a sterically bulky group such as cyclohexylmethanol, benzylalcohol, *(R)*- and *(S)*-1-phenylethanol, 2-phenylpropanol, and naphthylmethanol (Runs 2, 4, 6-9, and 12, respectively), the enantioselectivity was substantially increased (v_R/v_S= 38-77). Interestingly, when the lipase was imprinted with *(S)*-1-phenylethanol, the lipase showed the *(R)*-selectivity as well as that imprinted with *(R)*-1-phenylethanol.

Lipases are understand to have two binding sites as shown in Figure 3: a wide pocket for alcohol and a long narrow pocket for carboxylic acid substrates.[1] Since lipase is originally a hydrolysis catalyst for triglycerides, two acyl chains of triglycerides are bound to the wide pocket and the other single acyl chain is bound to the long and narrow pocket. Lipase B from *Pseudomonas* and lipase P from *Rhizopus delemer* can recognize the enantiomer of secondary alcohols by the minute structure of the wide binding pocket and shows the high enantioselectivity for alcohols. On the contrary, lipase OF from *Candida sylindracea* has been shown the low enantioselectivity for alcohols probably due to the flat structure of the wide binding site for alcohols.[1,7] When lipase OF was imprinted with the substrate analogue, the minute structure of the wide binding pocket may be changed and the enantioselectivity will be increased. When alcohols having sterically bulky or branched groups were employed as imprinting substrates, the imprinting effect was large compared with the simple hydrocarbon and linear alcohols. Whether *(R)*- or *(S)*-1-phenylethanol was employed as an imprinting substrate, the imprinting effect to form the minute structure of the binding site seems to be the same although the direction of OH-group is different.

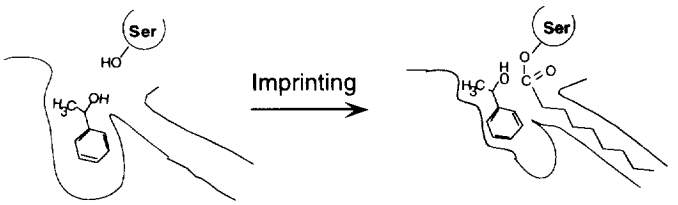


Figure 3. Schematic illustrations of the imprinting effect in the binding site of lipase OF.

Stability of Imprinted Effect. Figure 4 shows effects of reaction temperatures on the enantioselective esterification of *(R)*- or *(S)*-1-phenylethanol and lauric acid catalyzed by the imprinted or non-imprinted lipid-coated lipase OF. The improved enantioselectivity of the imprinted lipid-coated lipase could be kept constant below 40 °C. However, the enantioselectivity of the imprinted lipase was drastically decreased above 40 °C and reverted to that of the non-imprinted lipase around 60 °C. This is explained by the revert of the imprinted structure to the original non-imprinted one at high temperatures.

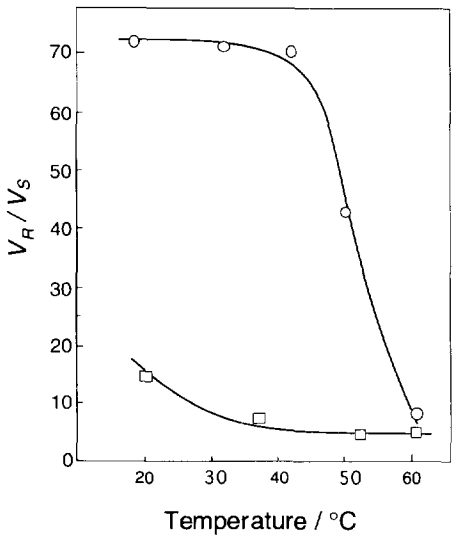


Figure 4. Effect of reaction temperatures on the enantioselectivity for the esterification of *(R)*- and *(S)*-1-phenylethanol with lauric acid in isooctane catalyzed by the lipid-coated lipase OF imprinted with *(R)*-1-phenylethanol (○) and the non-imprinted lipid-coated lipase OF (□). Reaction conditions are the same as those in Figure 2.

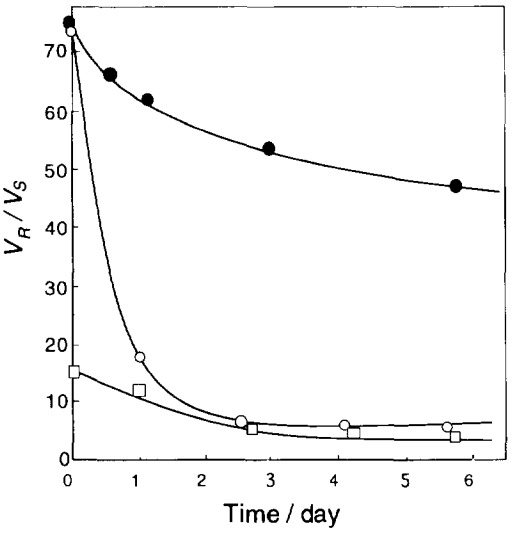


Figure 5. The enantioselectivity changes, after incubating in isooctane for the respective days at room temperature. (●): The imprinted lipid-coated lipase incubated in the presence of the substrate analogue [*(R)*-1-phenylethanol], (○): the imprinted lipid-coated lipase, and (□): the non-imprinted lipid-coated lipase. Reaction conditions are the same as those in Figure 2.

Figure 5 shows effects of incubation of the lipid-coated lipases in isooctane on the enantioselectivity for the esterification of *(R)*- or *(S)*-1-phenylethanol with lauric acid in isooctane. When the imprinted lipid-coated lipase was kept in isooctane for 1 or 2 days, the enantioselectivity was reverted to the original non-imprinted value. However, when the imprinted lipase was kept in the organic media in the presence of the imprinted substrate [*(R)*-1-phenylethanol, 50 mM], the improved enantioselectivity could be kept in v_S/v_R = 40-50 even after incubating for 6 days in isooctane. This indicates that the imprinting substrate is bound in the imprinted site and prevents it reverting to the original alcohol binding site. When the imprinted lipase was kept as a powder state in a refrigerator, the enantioselectivity was kept in the improved value for at least one month.

Michaelis-Menten Kinetics. The lipid-coated enzyme system is suitable for kinetic measurements because both enzyme and substrates are homogeneously solubilized in organic solvents. Figure 6A and 6B show typical saturation behavior of initial rates when the concentration of lauric acid or *(R)*-1-phenylethanol was increased to 120 mM at the constant concentration of the other substrate (50 mM) in the presence of the imprinted lipid-coated lipase OF. When the concentration of both substrates was high over 150 mM, the substrate inhibition was observed. In the case of esterification of *(S)*-isomers, the similar saturation kinetics were also observed although their absolute rates were very small.

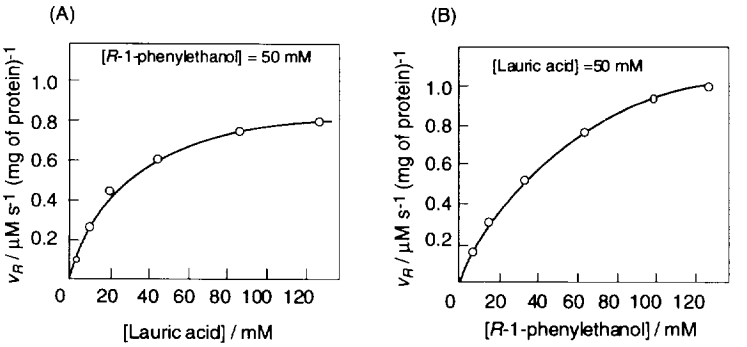


Figure 6. Effect of Substrate Concentrations on the esterification of *(R)*-1-phenylethanol and lauric acid catalyzed by the imprinted lipid-coated lipase OF (1 mg of protein) in isooctane at 40 °C

$$E + S_A \rightleftharpoons E S_A \quad (1)$$

$$E S_A + S_B \rightleftharpoons E S_A S_B \quad (2)$$

$$E S_A S_B \longrightarrow E + P \quad (3)$$

These results indicate clearly that both alcohol and acid substrates are incorporated in each binding site of enzyme followed by the intramolecular reaction according to two-substrates Michaelis-Menten kinetics. The two-substrate's reaction, however, shows relatively complicated mechanism to solve it: whether ping-pong or ordered mechanism is suitable, and which substrate (S_A or S_B) is bound first or simultaneously.[30,31] We obtained, therefore, simply $K_{m,RCCOH}$ and $k_{cat,RCOOH}$ values, and $K_{m,ROH}$ and $k_{cat,ROH}$ values from double reciprocal plots of Figure 6A and Figure 6B, respectively. The results obtained are summarized in Table VI, together with those values for non-imprinted lipid-coated lipase OF. The binding ability for alcohols was slightly decreased ($K_{m,ROH}$ was increased), but the binding for acids was not affected by imprinting. The effect of imprinting appeared in k_{cat} values: differences of $k_{cat,ROH}$ and $k_{cat,RCOOH}$ were increased 5-10 times by imprinting.

Table VI. Michaelis-Menten Kinetic Parameters in Esterification of *(R)*- or *(S)*-1-phenylethanol and lauric acid catalyzed by the Lipid-coated Lipase OF in Isooctane[a]

Reactions	$K_{m,RCOOH}$ / mM	$K_{m,ROH}$ / mM / mM	$k_{cat,RCOOH}$ / s^{-1} / s^{-1}	$k_{cat,ROH}$
Non-imprinted, lipid-coated lipase OF				
(R)-isomer	38	9.3	0.46	0.43
(S)-isomer	25	9.3	0.10	0.25
Imprinted, lipid-coated lipase OF				
(R)-isomer	24	34	1.2	0.95
(S)-isomer	25	26	0.065	0.020

[a] [lauric acid] = 0 - 120 mM, [1-phenylethanol] = 0 - 120 mM, in the anhydrous isooctane (5 ml) at 40 °C catalyzed by the lipid-coated lipase OF (8 mg, 1 mg of protein).

Thus, lipase OF showed originally the low enantioselectivity due to the small difference of k_{cat} values but not the binding process. The imprinting effect on the improvement of the enantioselectivity can be explained by the increase of the intramolecular reaction for the *(R)*-isomer, and the binding ability is slightly decreased by imprinting.

Conclusion

The lipid-coated lipase is easily prepared by mixing lipases with lipids, and is solubilized in most hydrophobic organic solvents and can catalyze the esterification of alcohols and acids in organic solvents. However, the enantioselectivity of the esterification depends largely on the origin of lipase. In the case of lipase OF from *Candida*, the lipid-coated lipase shows low enantioselectivity for the esterification as well as the hydrolysis. When, lipase OF was imprinted with the substrate analogue and then coated with lipid molecules, the enantioselectivity was substantially improved. The coating lipids and the reaction in the organic media were important to maintain the large imprinting effect. This imprinting effect disappeared at high temperatures and keeping in the organic solvents for days. The improved enantioselectivity was achieved by the catalytic reaction process (k_{cat}), and not the substrate binding process (K_m).

We believe that the combination of the imprinting method and the lipid-coating method can be widely applicable for improving the enantioselectivity esterification catalyzed by lipase in organic solvents.

Acknowledgments: We are grateful for Chisso, Co., Tokyo for their helpful gifts of chiral alcohols. The research was supported partially by a grant-in-aid for science research from Ministry of Education, Science and Culture Japan, and a grant pioneering research project in biotechnology from Ministry of Agriculture, Forestry and Fisheries Japan.

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(*Received in Japan* 17 *April* 1995)