A Lipid-Coated Lipase as an Enantioselective Ester Synthesis Catalyst in Homogeneous Organic Solvents[1,2]

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A lipid-coated lipase was prepared, in which hydrophilic head groups of lipids interact with the hydrophilic surface of the enzyme and lipophilic alkyl chains extend away from its surface and solubilize the enzyme in hydrophobic organic solvents. Enantioselective esterification of (*R*)- or (*S*)-1-phenylethanol with aliphatic acid was studied in the presence of the lipid-coated lipase, solubilized homogeneously in organic solvents, by varying lipase origin, coating lipid molecules, reaction media, and substrate structures. The lipid-coated lipase prepared from the glycolipid (**1**) and lipase B from *Pseudomonas fragi 22*-39B showed both a high catalytic activity and enantioselectivity for the esterification of (*R*)-1-phenylethanol with long-chain aliphatic acid in dry isooctane, relative to other enzyme systems such as poly(ethylene glycol)-grafted lipase, lipase powder directly dispersed in organic solvents, and a water/organic emulsion system. The coating lipids are found to not affect the enzyme reactions and to act simply as lipophilic tails in organic media. The lipid-coated lipase is suitable for studying the reaction mechanism of the enzyme in organic solvents since the reaction is carried out in homogeneous media. It has been found in studying Michaelis–Menten kinetics that the lipid-coated lipase (or native lipase) has two binding sites for long-chain aliphatic acids and for enantiomorphic secondary alcohols having a small methyl and a large phenyl side chain. Aliphatic acid is bound first and then alcohol. The enantioselectivity of the esterification is determined by the nucleophilic attack of the enantiomorphic alcohol, but not in the binding process of the enantiomorphic alcohol.

Introduction

In recent years, much research has centered on the conduct of enzyme reactions in organic solvents.[3-6] It is now well established that hydrolytic enzymes can catalyze esterification and transesterification reactions in monophasic organic solvents and in water–organic biphasic systems.[7] For example, lipase, the prevalent hydrolytic enzyme, has been used as an enantioselective transesterification catalyst for lipophilic substances in hydrophobic organic solvents. Of prime importance to the use of enzymes in organic media is the necessity to avoid enzyme deactivation or denaturation.

We have recently reported the preparation of a lipid-coated lipase as a result of mixing aqueous solutions of lipase and synthetic glycolipids. The coated lipase is soluble in most organic media.[1,2,8-11] Lipid-coated lipase D can catalyze triglyceride syntheses from 1-monoglyceride and aliphatic acid in dry benzene solution in the presence of molecular sieves.[1,8] The catalytic activity of the lipid-coated lipase was 2–100 times higher than that of other systems, including lipase in organic/aqueous emulsion,[12-14] dispersed powdered lipase,[15-20] and poly(ethylene glycol)-grafted lipase in organic solvents.[21-23] The lipid-coated lipase can prepare esters directly from acid and alcohol without requiring the reaction between activated esters and alcohols.

In this paper, we report that the lipid-coated lipase acts as an enantioselective esterification catalyst in homogeneous organic solvents. This activity can be exploited for the resolution of racemic alcohols as is shown in Figure 1. Enzymatic activity and enantioselectivity were studied under various conditions of lipase origin, organic solvent, and enantiomorphous alcohol and acid structure. We also examined Michaelis–Menten kinetics in organic media. In conventional lipase catalyses, reactions are usually carried out in biphasic systems as exemplified by the hydrolysis of lipophilic esters in organic/aqueous emulsion and transesterification in a dispersion of enzyme in organic solvent. The substrate selectivity and Michaelis–Menten kinetic parameters are not readily

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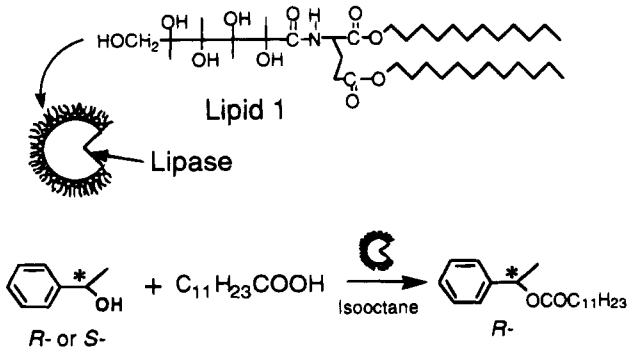


Figure 1. A schematic illustration of a lipid-coated lipase and enantioselective esterification in isooctane.

determined in these heterogeneous systems. The lipid-coated lipase system, in which both enzyme and substrate are soluble in homogeneous organic media, permits the study of the catalytic mechanism.

Experimental Section

Materials. Lipase B (from *Pseudomonas fragi 22*-39B), lipase P (from *Pseudomonas fluorescens*), lipase D (from *Rhizopus delemar*), lipase N (from *Rhizopus niveus*), lipase AY (from *Candida cylindracea*), and lipase AP (from *Aspergillus niger*) were purchased in a fine grade from Seikagaku Kogyo Co., Tokyo, Funakoshi Chemicals Co., Tokyo, and Amano Pharmaceutical Co., Tokyo, and were used without further purification. Preparations of dialkyl amphiphiles, didodecyl *N*-D-glucono-L-glutamate (**1**),[24] didodecyl *N*-D-gluconyl-D-glutamate (**2**),[24] dioctadecyl *N*-D-gluconyl-L-glutamate (**3**), bis-(perfluoroalkyl) *N*-D-gluconyl-L-glutamate (**4**), and sodium 1,2-bis(dodecyloxycarbonyl)ethanesulfonate (**7**),[25] were reported elsewhere. 1,2-Dipalmitoyl-3-phosphatidylcholine (DPPC, **5**) and dioctadecyldimethylammonium bromide (**6**) were purchased as the finest grade from Tokyo Kasei Co., Tokyo, and Sogo Pharmaceutical Co., Tokyo. Chemical structures of dialkyl amphiphiles (**1**-**7**) are shown in Scheme 1. (*R*)- or (*S*)-1-Phenylethanol and other chiral alcohols were kindly provided by Chisso Co. Ltd., Tokyo. Other chemicals and organic solvents were purchased from Tokyo Kasei Co., Tokyo.

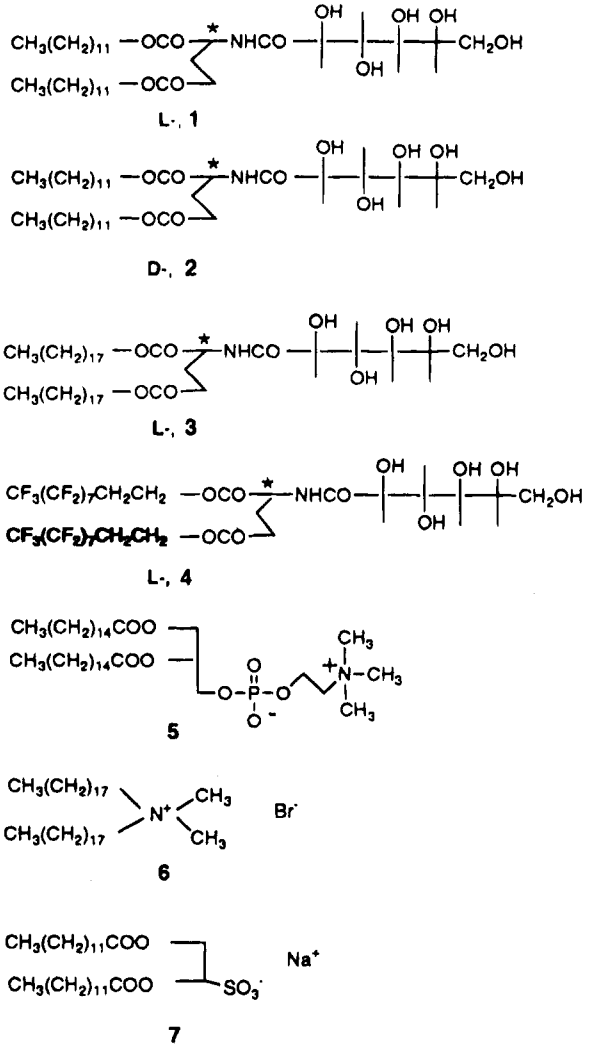
Preparation of a Lipid-Coated Lipase. A typical preparation of a lipid-coated lipase follows. An aqueous solution (25 mL, 0.01 M acetate buffer, pH 5.6) of lipase (50 mg) was centrifuged at 4000 rpm for 5 min at 4 °C to remove any insoluble impurities. The aqueous enzyme solution was mixed with an aqueous dispersion (25 mL) of dialkyl amphiphiles (50 mg) at 4 °C and was stirred for 20 h at 4 °C. Precipitates were gathered by centrifugation at 4 °C (5000 rpm, 5 min) and lyophilized. The resulting white powder was insoluble in water or any aqueous buffer solution but freely soluble in most organic solvents.

The protein content in the complex was estimated from both the elemental analysis (C, H, and N) and the UV absorption by aromatic amino acid residues of proteins at 280 nm in chloroform solution, in a manner similar to that in an aqueous solution.[1] Results are summarized in Table 1.

Lipid-coated lipases were analyzed by gel-permeation chromatography [instrument, Tokyo Rika, Co., Type PLC-5D; column, TSK-Gel G-400LH, ϕ 3.8 mm × 30 cm, Toso, Co., Tokyo; eluent, dichloromethane (0.4 mL/min); detector, UV at 240 nm; molecular weight calibration, a standard polystyrene]. The enzyme activity of eluents was monitored by hydrolysis of 4-methyl-2-oxo-2*H*-1-benzopyran-7-yl oleate in tetrahydrofuran–water solution at 40 °C followed by fluorescent spectroscopy (Em: 450 nm, Ex: 320 nm).[11]

Catalytic Activity of a Lipid-Coated Lipase in Organic Solvents. A typical procedure is as follows. To the dry isooctane solution (5 mL) of a lipid-coated lipase (10–20 mg, 1.0 mg of protein) were added (*R*)- or (*S*)-1-phenylethanol (30.5 mg, 50 mM) and an excess amount of aliphatic acid (100–300 mg, 500 mM), and the mixture was 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, ϕ 3.2 mm × 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 made by comparison of the GC retention time and the GC peak area to those of authentic *R*- or *S*-samples, respectively.

Scheme 1



Results and Discussion

Figure 2 shows typical time courses of ester syntheses from (*R*)-, (*S*)-, and racemic 1-phenylethanol and excess lauric acid catalyzed by a lipid (**1**)-coated lipase B in dry isooctane (60–80 ppm of H_2O) at 40 °C. When racemic alcohol was employed, the esterification reached equilibrium near 50% conversion, within 2 h. The *R*-isomer was completely converted to ester, but the *S*-isomer scarcely reacted with lauric acid. This indicates clearly that a lipid-coated lipase can recognize the (*R*)-1-phenylethanol and convert it to ester, but not the *S*-isomer.

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Table 1. Effects of Coating Lipid Structures on Preparation of Lipid-Coated Lipase B and Its Enzymatic Activity

lipids	preparation: yield[a], mg	preparation: protein content,[b] wt %	enzymatic activity,[d] μM s^{-1} (mg of protein)$^{-1}$: v_R	enzymatic activity,[d] μM s^{-1} (mg of protein)$^{-1}$: v_S	enantioselectivity: v_R/v_S
L-glycolipid **1**	25.4	12 (12)[c]	50	0.20	250
D-glycolipid **2**	20.0	12 (13)[c]	58	0.25	230
L-glycolipid **3**	14.1	12 (11)[c]	29	0.17	170
L-glycolipid **4**	3.6	2.4 (2.6)[c]	23	0.55	42
zwitterionic lipid **5**	7.5	1.0 (1.8)[c]	17	0.21	80
cationic lipid **6**	34.7	10 (12)[c]	2.2	0.44	5.0
anionic lipid **7**	0	–	–	–	–

[a] Both aqueous solution of Lipase B (50 mg) and lipids (50 mg) were mixed and precipitates were lyophilized. [b] Obtained from UV absorption of aromatic amino acid residues in the protein. [c] Obtained from C/N ratio of elemental analyses. [d] Initial rates of ester syntheses from (*R*)- or (*S*)-1-phenylethanol (50 mM) and lauric acid (500 mM) in dry isooctane (5 mL) at 40 °C catalyzed by the lipid-coated lipase B (8–20 mg, 1 mg of protein).

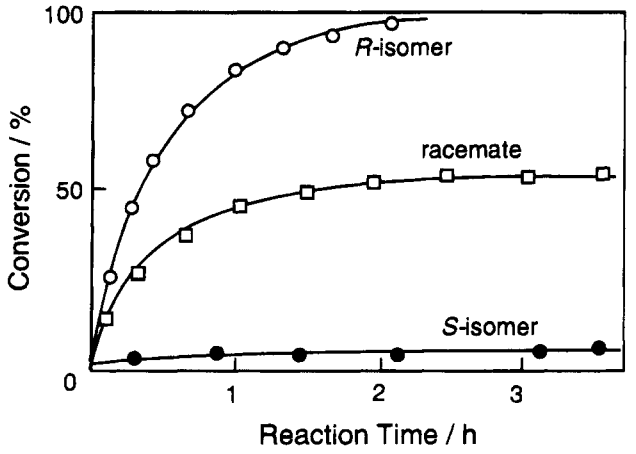

Figure 2. Typical time-courses of esterification of (*R*)-, (*S*)-, or racemic-1-phenylethanol (50 mM) with lauric acid (500 mM) catalyzed by a lipid (**1**)-coated lipase B (1 mg of protein) in the dry isooctane (5 mL, H_2O: 60–80 ppm) in the presence of two pieces of molecular sieves at 40 °C.

Initial rates [v_R or v_S/μM s^{-1} (mg of protein)$^{-1}$] were primarily used as indicators of enzyme activity. Enantioselectivity (v_R/v_S) was expressed as the ratio of initial rates for *R*- and *S*-isomers.

Effect of Coating Lipids. Lipid-coated lipases were prepared from lipase B (from *P. fragi*) and several kinds of dialkyl amphiphiles having cationic, anionic, zwitterionic, and nonionic hydrophilic head groups. The results are summarized in Table 1. Chemical structures of lipid molecules are shown in Scheme 1. Yields of a lipid-coated lipase were determined for the purified powder by weighing. The content of protein in the complex was estimated by the C/N ratio from elemental analyses and from the UV absorption of aromatic amino acid residues in the lipase. The protein contents obtained from the two methods were consistent within experimental errors. Enzyme activity is shown as initial rates (v_R and v_S) of the esterification catalyzed by a lipid-coated lipase B from (*R*)- or (*S*)-1-phenylethanol and lauric acid in dry isooctane at 40 °C. Enantioselectivity is expressed as v_R/v_S.

The lipid-coated lipase was obtained in a fair yield when nonionic (**1**–**3**), zwitterionic (**5**), and cationic (**6**) amphiphiles were employed, but not when anionic amphiphiles (**7**) were used. Although the cationic amphiphiles (**6**) produce complexes in fair yield, the enzyme activity and enantioselectivity were very low compared to those of complexes with nonionic and zwitterionic amphiphiles (**1**–**5**). This is probably because the strong electrostatic interaction between the cationic head groups of amphiphiles (**6**) and the hydrophilic surface of the lipase denatures the protein structure. With nonionic amphiphiles having perfluoroalkylated chains (**4**), the yield and protein content of the complex were low due to the low solubility of fluorinated lipid molecules.

The nonionic glycolipids **1**–**3** gave a complex (protein content: 12 ± 1 wt %) in good yield which showed both high enzymatic activity and enantioselectivity independent of alkyl chain length and the stereochemisty of the lipids (D- or L-glutamate linkages). This is due to appropriate interaction, through hydrogen bonds, between the hydrophilic amino acid residues of lipase surface and the hydroxyl head groups of the glycolipids. The stereochemistry of the coating lipids of **1** and **2** hardly affected the enantioselectivity of the ester synthesis. The coating lipid seems to not influence the enzymatic enantioselectivity but does impart the affinity with organic media to the enzymes.

When the lipid (**1**)-coated lipase B was solubilized in dry isooctane without any substrates at 40 °C for 1 day, no byproducts from coating lipids were observed within experimental error. Although coating lipids **1** bear some functional groups such as esters, amides, and alcohols, these did not react as substrates.

The nonionic dialkyl amphiphile **1** was chosen as the standard coating lipid for lipase in the following experiments.

Effect of Origin of Lipase. Lipid-coated lipases of different origin were prepared with the nonionic glycolipid (**1**) and the results are summarized in Table 2. When the glycolipid **1** was employed, the lipase–lipid complex was obtained in fair yield independent of lipase origin (20 ± 5 mg in yield and 10 ± 5 wt % in protein content). However, the enzyme activity and enantioselectivity were highly dependent on the origin: the complexes from lipase B (from *P. fragi 22*-39B), lipase P (from *P. fluorescens*), and lipase D (from *R. delemar*) showed both the relatively high activity and enantioselectivity, but the complexes from lipase N (from *R. niveus*), lipase AY (from *C. cylindracea*), lipase CES (from *Pseudomonas sp*), and lipase AP (from *A. niger*) did not show the remarkable catalytic activity for enantioselective esterification in isooctane.

Lipase B (from *P. fragi 22*-39B) was selected for use in the following experiments.

Characterization of a Lipid-Coated Lipase. The molecular weight of a lipid (**1**)-coated lipase was determined by gel permeation chromatography with dichloromethane eluent to be (13 ± 2) × 10^4 as described previously, indicating that 150 ± 30 lipid molecules bind per one lipase B (MW: 3.3 × 10^4).[11] It can be roughly estimated from the molecular area of the lipid (0.45 nm^2)

Table 2. Effect of Origin of Lipase on Enantioselective Esterification Catalyzed by the Lipid (1)-Coated Lipase

origin of lipase	initial rates,[a] μM s^{-1} (mg of protein)$^{-1}$ v_R	v_S	enantioselectivity: v_R/v_S
lipase B from *Pseudomonas fragi* 22-39B	50	0.2	250
lipase P from *Pseudomonas fluorescens*	22	0.05	440
lipase D from *Rhizopus delemar*	13	0.015	70
lipase N from *Rhizopus niveus*	0.14	0.002	70
lipase AY from *Candida cylindracea*	0.019	0.0082	2.3
lipase CES from *Pseudomonas sp.*	0.026	0.00026	10
lipase AP from *Aspergillus niger*	0.086	0.0022	4.0

[a] Initial rates of ester syntheses from (*R*)- or (*S*)-1-phenylethanol (50 mM) and lauric acid (500 mM) in dry isooctane (5 mL) at 40 °C catalyzed by the lipid (**1**)-coated lipase (10–100 mg).

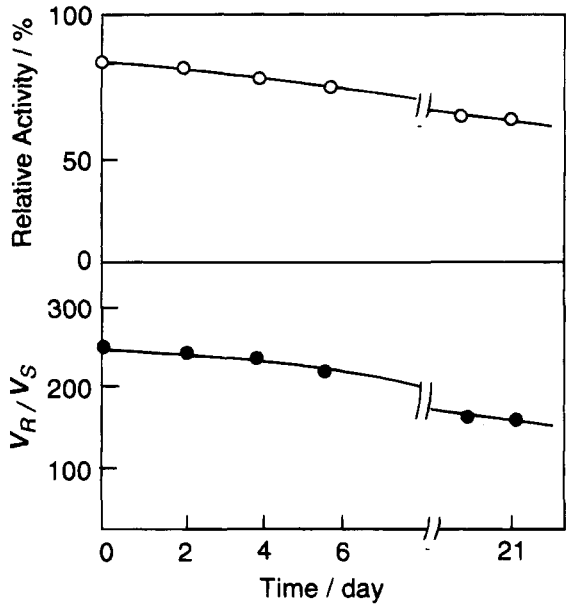

Figure 3. Enzymatic activity and enantioselectivity of the lipid (**1**)-coated lipase B, after incubation in isooctane for the respective days at room temperature. The reaction conditions of esterification of (*R*)-1-phenylethanol and lauric acid are the same as those in Figure 2.

and lipase (diameter: *ca.* 3 nm) that *ca.* 150 ± 50 lipid molecules are required to cover the surface of a lipase with a monolayer. The protein content of the lipid–lipase powder was determined to be 12 wt % from both the elemental analysis (C/N ratio) and the UV absorption of aromatic amino acid residues (see Table 1). A protein content of 12 wt % indicates that 300–400 lipid molecules are attached around a lipase surface. Thus, in the lipase–lipid powder, a lipase is coated strongly with one layer of 150 ± 30 lipid molecules and another 150–250 lipid molecules interact with the lipid monolayer-coated lipase hydrophobically.

We have compared ^{1}H-NMR spectra (270 MHz, solvent: $CDCl_3$) of the lipid (**1**)-coated lipase D with those of the free lipid **1**. The signals of only the hydrophilic OH groups of the lipid **1** complex appear downfield (3.7 → 4.1 ppm) of those of the free lipids. This indicates that the hydroxyl head groups of lipid **1** interact with the hydrophilic surface of the lipase through hydrogen bonds. The lipophilic tails of the lipids are thought to solubilize the complex in hydrophobic solvents, as illustrated in Figure 1.

Stability of a Lipid-Coated Lipase. When the isooctane solution (5 mL) of the lipid (**1**)-coated lipase B (50 mg) was extracted with 10 mL of water or an aqueous buffer solution (pH 5.6, 0.01 M acetate) several times over 12 h, neither lipase nor lipid molecules were detected in the aqueous phase by thin-layer chromatography within experimental error. The complex remaining in the organic phase still had 90–100% of its original enzymatic activity. Thus, the interaction between lipid molecules and lipase is strong even in organic solvent and is not disturbed by mixing with aqueous phases.

Table 3. Effect of Organic Solvents on Enantioselective Esterification Catalyzed by the Lipid (1)-Coated Lipase B

solvent	initial rates,[a] μM s^{-1} (mg of protein)$^{-1}$ v_R	v_S	enantioselectivity: v_R/v_S
isooctane	50	0.2	250
isopropyl ether	7.8	0.042	186
toluene	0.94	0.015	63
benzene	0.13	0.0026	50
tetrahydrofuran	0.036	0.0056	6.4
chloroform	0	0	–
dimethylformamide	0	0	–
acetone	0	0	–

[a] Initial rates of ester syntheses from (*R*)- or (*S*)-1-phenylethanol (50 mM) and lauric acid (500 mM) in the dry solvent (5 mL) at 40 °C catalyzed by the lipid (**1**)-coated lipase B (8 mg, 1.0 mg of protein).

Figure 3 shows the enzymatic activity and enantioselectivity of the lipid (**1**)-coated lipase B for ester synthesis from (*R*)- or (*S*)-1-phenylethanol and lauric acid in isooctane, after incubation of the lipid-coated lipase in isooctane for the indicated number of days. Both enzyme activity and enantioselectivity diminish gradually with incubation time, probably because of denaturation of the lipid-coated enzymes by organic solvents. After incubation of the lipid-coated enzyme in isooctane for 3 weeks, however, approximately 70% of the original reactivity and the enantioselectivity remain. When the lipid-coated lipase was kept in a powdered state, the reactivity was hardly changed after a year.

Effect of Organic Solvents. The enantioselective ester syntheses catalyzed by lipid (**1**)-coated lipase B were carried out in various organic solvents. Initial rates for *R*- and *S*-alcohols, and the enantioselectivity are shown in Table 3. The lipid-coated lipase was not soluble in hydrophilic solvents such as buffer solution (pH 5.6, 0.01 M acetate) and ethanol, but is soluble in all organic solvents listed in Table 3. The lipid-coated lipase showed fairly high initial rates and enantioselectivity in nonpolar solvents such as isooctane, isopropyl ether, and toluene. Both the enzyme activity and enantioselectivity decreased in somewhat polar solvents such as benzene and tetrahydrofuran. In halogenated or water-miscible solvents such as chloroform, dimethylformamide, and acetone, the lipid-coated lipase showed hardly any catalytic activity although it is soluble. It might be easily denatured in halogenated solvents. In water-miscible organic solvents, enzymes may be denatured due to the removal of essential water from the enzyme to the bulk solution.

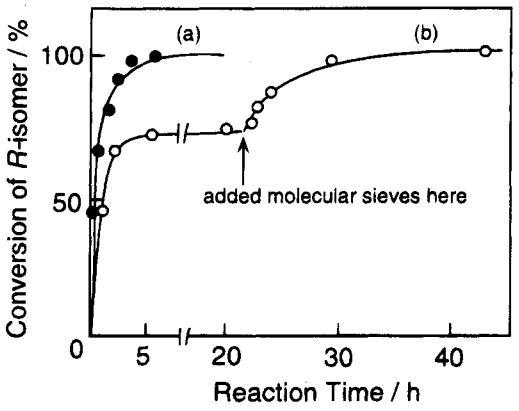

Figure 4. Effect of dehydration in isooctane solution (a) with and (b) without molecular sieves (two pieces) on the esterification conversion catalyzed by the lipid (**1**)-coated lipase B. The reaction conditions of esterification of (*R*)-1-phenylethanol and lauric acid are the same as those in Figure 2.

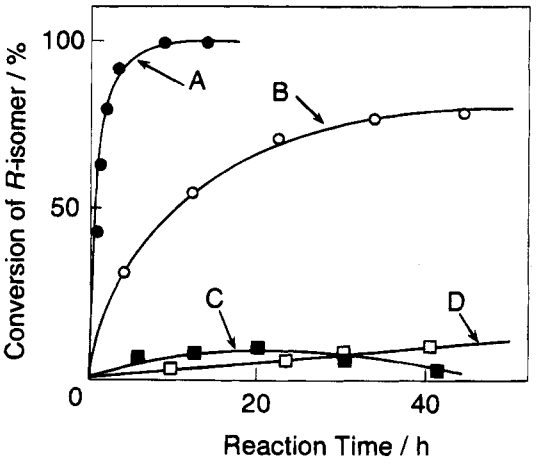

Figure 5. Comparison of catalytic activities for ester synthesis of (*R*)-1-phenylethanol and lauric acid in the dry isooctane at 40 °C catalyzed by (A) lipid-coated lipase B, (B) PEG-grafted lipase B, (C) lipase B in w/o emulsion, and (D) direct dispersion of lipase B powder. Reaction conditions are the same as those in Figure 2.

However, CD and UV spectra of the lipid-coated lipase scarcely different in these polar solvents and in isooctane.

Isooctane was the chosen solvent for the following experiments.

Effect of Water Content in Organic Media. Enantioselective esterification of (*R*)-1-phenylethanol with lauric acid was completed within 3 h with an initial rate of v_R = 50 μM s^{-1} (mg of protein)$^{-1}$] in 5 mL of dry isooctane (water content: 70 ± 20 ppm), in the presence of two pieces of 3 Å molecular sieves to remove the H_2O produced during the reaction (see curve a in Figure 4). In contrast, in commercially available isooctane without molecular sieves (initial water content: 250 ppm), the reaction with the v_R = 23 μmol s^{-1} (mg of protein)$^{-1}$ stopped at 70% conversion, and the isooctane solution became turbid due to the water produced in the course of the reaction. When two pieces of molecular sieves were added at the arrow of curve b in Figure 4, the isooctane solution became clear and the reaction proceeded to completion within 10 h. Thus, the highest catalytic activity of the lipid-coated lipase for ester syntheses is achieved only in dry organic solvent, and the product water inhibits the synthetic reaction.

Although the lipid-coated lipase showed the highest synthetic activity in dry isooctane, it is difficult to know the true water concentration around the enzyme. After the lipid-coated lipase powder was dried completely in a vacuum desiccator (2–3 days at 0.01 mmHg), the weight of the enzyme had hardly changed. The water content of the dry isooctane solution was not different before and after the addition of the lipid-coated lipase (100 mg) to the solution (10 mL). This means that if the lipid-coated enzyme has a small amount of water at the interface between the lipids and the enzyme surface, water does not dissolve in the isooctane solution during the reaction.

Comparison with Other Enzyme Systems. Several approaches have been made to use a lipase as a synthetic catalyst in organic solvents.[3–7] In addition to the water-in-oil emulsion and the reversed micellar system containing a small amount of water,[12–14] two elegant, previously-reported use both hydrophobic and hydrophilic organic solvents as a medium: (i) Klibanov and co-workers reported the direct dispersion of powdered lipase in organic solvents, to produce an ester exchange catalyst for heterogeneous solutions,[6,15–20] and (ii) Inada and co-workers prepared a poly(ethylene glycol)(PEG)-grafted lipase that is soluble or swelled in hydrophobic organic solvents and catalyzes simple ester syntheses from aliphatic alcohols and acids.[21–23]

We compared our lipid-coated lipase system with other systems under the same reaction conditions using the same lipase B (from *P. fragi 22*-39B). Enantioselective ester syntheses from (*R*)- or (*S*)-1-phenylethanol and lauric acid were carried out in the dry isooctane with molecular sieves in the presence of the same amount (1 mg of protein) of the lipid-coated lipase B, the PEG-grafted lipase B, the lipase B in water/isooctane emulsion, and the powdered dispersion of lipase B. The time courses of these reactions are shown in Figure 5. PEG-grafted lipase B was commercially available from Sapporo Beer Co., Tokyo, in which linear PEG (MW: *ca.* 5000) was covalently immobilized on a lipase, and protein content is 73%, prepared according to Inada's method.[21–23]

When the lipid-coated lipase B was employed (curve A), (*R*)-1-phenylethanol was completely converted to the ester within 3 h with high enantioselectivity [v_R = 50 μM s^{-1} (mg of protein)$^{-1}$, v_R/v_S = 250]. Although the direct dispersion method showed high enantioselectivity (v_R/v_S = 290), it gave a very slow reaction rate (1/100 times) compared with that of the lipid-coated lipase [v_R = 0.5 μM s^{-1} (mg of protein)$^{-1}$]. In the dispersion method (curve D), enzymes exist as a suspension in the substrate solution in organic solvents; therefore, a great amount of enzyme may be required to get a fairly high reaction rate. In other words, the homogeneously soluble lipid-coated lipase has much higher activity than the dispersed lipase when the same amount of enzyme is used. However, we should consider that the activity of the enzyme dispersion system is sometimes affected before or during dispersion by such things as pH-adjustments, moisture, and fineness of powder.

In the water-in-oil system (curve C), in which 1 mg of native lipase B is solubilized in a buffer solution (pH 5.6) and emulsified in isooctane, the rate of ester synthesis was very slow [v_R < 0.1 μM s^{-1} (mg of protein)$^{-1}$, v_R/v_S = 80–120] and decreased with increasing reaction time.

Table 4. Substrate Selectivity for Carboxylic Acid Moieties on Enantioselective Esterification with (*R*)- or (*S*)-1-Phenylethanol Catalyzed by the Lipid-Coated Lipase B in Isooctane

	initial rates,[a] μM s^{-1} (mg of protein)$^{-1}$			dispersed native lipase[b]	
carboxylic acids	v_R	v_S	enantioselectivity: v_R/v_S	v_R	v_R/v_S
$(CH_3)_3CCOOH$	0	0	–	0	–
PhCOOH	0	0	–	0	–
$Ph(CH_2)_3COOH$	1.5	0.010	150	0.01	180
CH_3COOH	0	0	–	0	–
CH_3CH_2COOH	1.1	0.056	2.0	0.01	180
$CH_3(CH_2)_4COOH$	14	0.056	250	0.2	180
$CH_3(CH_2)_{10}COOH$	50	0.20	250	0.5	290
$CH_3(CH_2)_{17}COOH$	106	0.32	390	1.2	380

[a] Initial rates of ester syntheses from (*R*)- or (*S*)-1-phenylethanol (50 mM) and the respective acid (500 mM) in dry isooctane (5 mL) at 40 °C catalyzed by the lipid (**1**)-coated lipase B (8 mg, 1 mg of protein). [b] The same esterification was catalyzed by dispersed native lipase B (500 mg) in isooctane [v_R, μM s^{-1} (mg of protein)$^{-1}$].

Table 5. Substrate Selectivity for Alcohol Moieties on Enantioselective Esterification with Lauric Acid Catalyzed by the Lipid-Coated Lipase B in Isooctane

	initial rates,[a] μM s^{-1} (mg of protein)$^{-1}$				native dispersed lipase[b]	
carboxylic acids	v_{rac}	v_R	v_S	enantioselectivity: v_R/v_S	v_{rac}	v_R/v_S
Ph*CH(CH_3)OH	26	50	0.20	250	0.25	290
Ph*CH(C_2H_5)OH	0			0	–	
Ph*CH($CH_2CH_2CH_3$)OH	0				0	–
$CH_3(CH_2)_5$*CH(CH_3)OH	46	70	20	4	0.50	3
$CH_3(CH_2)_5$*CH(CH_2Cl)OH	52	40	64	0.6	0.30	0.3
$CH_3(CH_2)_6$*CH(CH_3)OH	40	72	6.6	11	0.20	15
$CH_3(CH_2)_5$*CH(C_2H_5)OH	18				0.008	–
$CH_3(CH_2)_4$*CH($CH_2CH_2CH_3$)OH	2.2				0.02	–
$CH_3(CH_2)_3$*CH($CH_2CH_2CH_2CH_3$)OH	1.8				0.009	–

[a] Initial rates of ester syntheses from the respective racemic, *R*-, or *S*-alcohol (50 mM) and lauric acid (500 mM) in dry isooctane (5 mL) at 40 °C catalyzed by the lipid (**1**)-coated lipase B (8 mg, 1 mg of protein). [b] The same esterification was carried out by using dispersed native lipase B (500 mg) in isooctane [$v_{rac.}$, μM s^{-1} (mg of protein)$^{-1}$].

It seems that the existence of a small amount of water in the water/isooctane emulsion system caused the reverse hydrolysis reaction.

When the PEG-grafted lipase B was employed (curve B), the esterification proceeded at a fair rate with high enantioselectivity [v_R = 30 μM s^{-1} (mg of protein)$^{-1}$, v_R/v_S = 280] in the initial stages. However, the conversion reached a plateau at the 70% yield after 40 h in dry isooctane even in the presence of molecular sieves, and the reaction medium was not turbid. This suggests that the PEG-lipase retains the produced water near the amphiphilic PEG grafted chains such that the water molecules cannot be removed even by the molecular sieves. Water held near the lipase surface inhibits the ester synthesis in organic solvents. In contrast, the hydrophobic lipid-coated lipase does not retain the water produced, and the ester synthesis proceeds completely when this water is removed by molecular sieves from the solution.

Thus, the lipid-coated lipase can effectively and completely catalyze the ester synthesis in dry organic solutions without changing the enzyme enantioselectivity, unlike the other enzyme systems, because the lipid-coated lipase is homogeneously soluble and stable in dry organic solvents.

Substrate Selectivity. The lipid-coated lipase is a suitable system for studying substrate selectivity of ester synthesis, because both lipase and substrates are homogeneously soluble in organic solvents. Table 4 shows the effect of the chemical structures of carboxylic acid moieties on enantioselective esterification of (*R*)- or (*S*)-1-phenylethanol in isooctane. The same reaction was carried out in isooctane by dispersing native lipase instead of the lipid-coated lipase, and the initial rates for *R*-isomer conversion (v_R) and enantioselectivities (v_R/v_S) are found in Table 4. When carboxylic acid moieties have a sterically bulky group in the α-position, such as pivalic acid and benzoic acid, esterification is not observed. In the case of linear aliphatic acids, both esterification rate and enantioselectivity increased with increasing chain length. This is consistent in that lipase is an enzyme that hydrolyzes triglycerides of long-chain aliphatic acids. Although chemical structures of carboxylic acid moieties changed the reaction rates, they hardly affected the enantioselectivity for 1-phenylethanol. Thus, lipase can effectively recognize long-chain aliphatic acids in its cavity. When native lipase was employed in a dispersion form under the same reaction conditions, the same tendency for enzyme activity and enantioselectivity was observed although the absolute reactivity was greatly depressed. This indicates that the substrate selectivity for the acid moiety does not change with the coating lipid.

Table 5 shows the effect of the chemical structure of alcohol moieties on the enzyme activity and the enantioselectivity in the esterification with lauric acid. When 1-phenylethanol having a large phenyl group and a small methyl group was employed, *R*-isomer was esterified enantioselectively (v_R/v_S = 250). When the small methyl group of the alcohol was changed to an ethyl or propyl group (1-phenylpropanol and 1-phenylbutanol), the reactivity was much depressed. A similar tendency was found with the secondary aliphatic alcohols such as 2-octanol, 1-chloro-2-octanol, 2-nonanol, 3-nonanol, and 4-nonanol. The enantioselectivity for the secondary aliphatic alcohols was very low compared to that for 1-phenylethanol. The same tendency was observed when the dispersion of native lipase B was employed as a catalyst. These results indicate that lipase B can enantioselectively recognize a secondary alcohol having a large (phenyl) and a small (methyl) side chain and that both

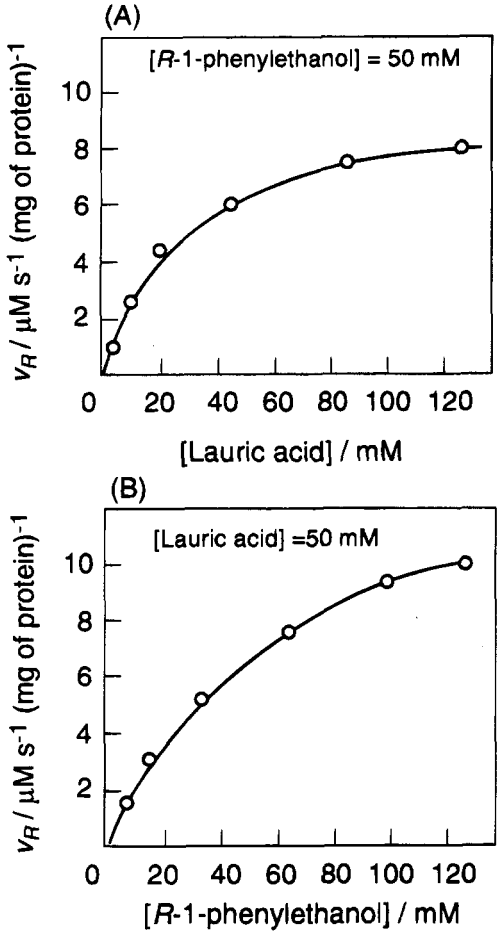

Figure 6. Effect of Substrate Concentrations on the esterification of (*R*)-1-phenylethanol and lauric acid catalyzed by the lipid (**1**)-coated lipase B (1 mg of protein) in the dry isooctane at 40 °C.

the enantioselectivity and the substrate selectivity are unaffected by the coating lipid molecules.

Michaelis–Menten Kinetics. The lipid-coated enzyme system is suitable for kinetic measurements because both enzyme and substrates are homogeneously soluble in organic solvents. Figure 6 shows the typical saturation of the initial rates when the concentration of either lauric acid or (*R*)-1-phenylethanol is increased to 120 mM with a constant concentration of the other substrate (50 mM). When the concentration of both substrates was over 150 mM, substrate inhibition was observed. In the case of esterification of *S*-isomers, similar saturation kinetics were also observed although the absolute rates were very small. These results indicate clearly that both alcohol and acid substrates are incorporated at each binding site of the enzyme followed by the intramolecular reaction according to two-substrate Michaelis–Menten kinetics.

A two-substrate reaction, however, presents several questions: whether a ping-pong or an ordered mechanism is operative, and which substrate (S_A or S_B), if either, is bound first.[26,27] We obtained, therefore, simply $K_{m,RCCOH}$ and $v_{max,RCOOH}$ values, and $K_{m,ROH}$ and $v_{max,ROH}$ values from double reciprocal plots of Figure 6A and Figure 6B, respectively. The obtained results are summarized in Table 6, together with those values for other chiral and racemic substrate combinations.

Table 6. Michaelis–Menten Kinetic Parameters in Esterification Catalyzed by the Lipid-Coated Lipase B in Isooctane[a]

esterification with lauric acid	$K_{m,RCOOH}$, mM	$K_{m,ROH}$, mM	$v_{max,RCOOH}$, mol s^{-1}	$v_{max,ROH}$, mol s^{-1}
1-nonanol	67	63	109	123
2-nonanol				
R-	100	69	26	10
S-	9.0	40	2.2	2.2
racemic	45	44	10	6.5
1-phenylethanol				
R-	18	125	8.1	20
S-	5.7	63	0.16	0.30
racemic	8.3	102	4.9	11.9

[a] [Lauric acid] = 0–120 mM, [alcohol] = 0–120 mM, in dry isooctane (5 mL) at 40 °C catalyzed by the lipid (**1**)-coated lipase B (8 mg, 1 mg of protein).

Binding ability of aliphatic acids ($K_{m,RCCOH}$) is greatly dependent on both chemical structure and chirality of alcohol. In the esterification of nonanols with lauric acid, the reactivity of 1-nonanol was greater than that of 2-nonanol. This is due to the higher intramolecular reaction rates (v_{max}) of 1-nonanol than 2-nonanol, but not due to their binding abilities (K_m). In the enantioselective reactions of 2-nonanol and 1-phenylethanol, the binding ability of *R*-isomers is weaker (larger $K_{m,ROH}$ values) than that of *S*-isomers and both $v_{max,RCOOH}$ and $v_{max,ROH}$ values for *R*-isomers were quite larger than those of *S*-isomers. Thus, the enantioselectivity for the *R*-isomer is mainly determined in the intramolecular reaction process, but not in the binding process. When racemic 1-phenylethanol and 2-nonanol were employed, K_m and v_{max} values showed average values in between those of the *R*- and *S*-isomers. Thus, the *R*- and *S*-isomers do not act as inhibitors of each other in the enantioselective esterification.

Conclusion

The lipid-coated lipase is easily prepared by mixing enzymes with lipids and is stable in hydrophobic organic media for several weeks. The lipid-coated lipase can act as an effective resolution catalyst of racemic alcohol by virtue of using esterification reactions in organic solvents. The lipid-coated lipase showed greater activity for ester synthesis than other systems such as the PEG-grafted lipase and the powder dispersion system. Since both the lipid-coated enzyme and substrates are soluble in organic solvents, this system is suitable for studies in substrate selectivity and enzyme kinetics in organic media. The coating lipid molecules act as lipophilic tails to be solubilized in organic media, but do not affect the enzyme selectivity. We believe the lipid-coated enzyme system can be widely applicable to other enzymes whose substrates are lipophilic.

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