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Preparation of a Lipid-Coated Lipase and Catalysis of Glyceride Ester Syntheses in Homogeneous Organic Solvents[1,2]

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A lipid-coated lipase was prepared by mixing aqueous solutions of lipase and dialkyl amphiphiles: A hydrophilic surface of the enzyme is covered with a lipid layer, of which lipophilic two-long-alkyl tails solubilize the enzyme in hydrophobic organic solvents. The lipid-coated lipase was prepared in various conditions by changing hydrophilic head groups of lipid molecules, a ratio of lipid/lipase, and an origin of lipase; the complex prepared from nonionic amphiphiles and lipase D from *Rhizopus delemar* showed a high stability and a high enzymatic activity in hydrophobic organic solutions. The lipid-coated lipase catalyzed di- and triacylglycerol syntheses from monoacylglycerols and aliphatic acids in the homogeneous and dry benzene solution in the presence of two pieces of molecular sieves (water: 80 ppm). The lipid-coated lipase could also catalyze ester exchange reactions in organic solvents with a small amount of water (250 ppm). Enzymatic activity was affected by a nature of organic solvents and a water content in organic solvents. The catalytic activity of the lipid-coated lipase was highly efficient compared with other enzyme systems such as the poly(ethylene glycol)-grafted lipase and the direct dispersion of lipase powder for glyceride syntheses in the dry organic solution.

In recent years there has been a considerable effort to mimic the catalytic action of enzymes in organic solvents.[3-8] For example, lipase has been used as a transesterification catalyst for lipophilic substances in hydrophobic organic solvents. To use enzymes in organic solvents it is necessary to avoid the deactivation or the denaturation of protein structures, and several approaches have been formulated: i) the water-in-oil microemulsion or the reversed micellar system in which enzymes are dissolved in the inner aqueous buffer solution,[9-11] ii) the direct dispersion of powdered enzymes in organic solvents,[12-17] and iii) the surface modified enzyme with poly(ethylene glycol) (PEG) which solubilizes the enzyme in organic solvents.[18-20] However, these methods have each disadvantage such as i) the hydrolysis of produced esters occurs due to a small amount of aqueous phase co-existed, ii) much amount of enzymes are required because of the heterogeneous reaction, and iii) it is difficult to prepare and purify the chemically-modified enzymes in good yield.

In this paper, we report a new type of organic-soluble enzyme, a lipid-coated lipase. It is easily prepared by mixing enzymes and lipid molecules in aqueous buffer solutions, and is soluble in most organic solvents but insoluble in aqueous solutions. The lipid-coated lipase was prepared in various conditions by changing hydrophilic head groups of lipid molecules, a ratio of lipid/lipase, and an origin of lipase, and was analyzed by a gel-permeation chromatography. The lipid-coated lipase can catalyze efficiently the syntheses of di- and triacyl-

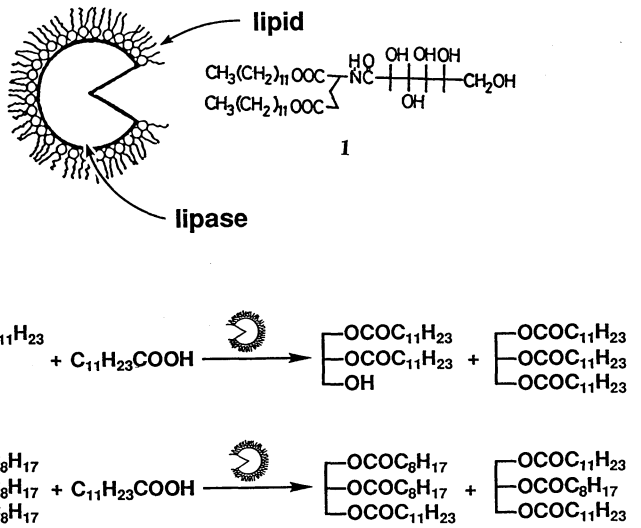


Fig. 1. A schematic illustration of a lipid-coated lipase and the reactions catalyzed by the lipid-coated lipase in organic solvents.

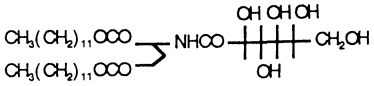
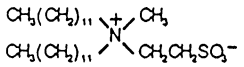
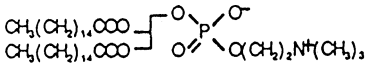
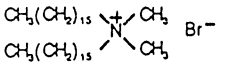
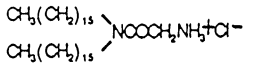
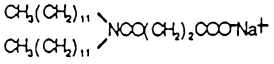
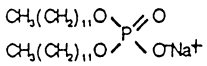
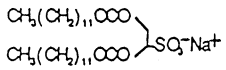
glycerols from 1-lauroylglycerol (1-monolaurin) and lauric acid or ester exchange reactions between aliphatic acids and triacylglycerols in organic solvents. A schematic illustration of the lipid-coated enzyme and reactions catalyzed by the lipid-coated lipase are shown in Fig. 1. Catalytic activities of the lipid-coated lipase were studied in different conditions: changing a nature of organic solvents, a water content in organic solvents, reaction temperatures, and a degree of unsaturation in fatty acid substrates. The catalytic activity of the lipid-coated lipase was compared with those of other enzyme systems in organic solvents: a water-in-oil emulsion or a reversed micellar system, a direct dispersion of powdered lipase, and a poly(ethylene glycol)-grafted lipase in organic solvents.

Experimental

Materials. Lipase D (from *Rhizopus delemar*), lipase B (from *Pseudomonas fragi* 22-39B), and lipase N (from *Rhizopus niveus*), lipase W (from *Wheat germ*), and lipase P (from *Pseudomonas fluorescens*) were purchased as a fine grade from Seikagaku Kogyo Co., Funakoshi Chemicals Co., and Amano Pharmaceutical Co., and used without further purification. Glycerides, aliphatic acids, unsaturated aliphatic acids, 1,2-dipalmitoyl-3-phosphatidylcholine (DPPC, **3**), dihexadecyldimethylammonium bromide (**4**), and sodium didodecylphosphate (**7**) were purchased as a finest grade from Tokyo Kasei Co., Tokyo, and Sogo Pharmaceutical Co., Tokyo. Preparations of dialkyl amphiphiles, didodecyl *N*-D-glucono-L-glutamate (**1**),[21] 2-(didodecylmethylammonio)ethanesulfonate (**2**),[21] 2-amino-*N*,*N*-dihexadecylacetamide hydrochloride (**5**),[22] sodium 3-(*N*,*N*-didodecylcarbamoyl)propionate (**6**),[22] and sodium 1,2-bis(dodecyloxycarbonyl)ethanesulfonate (**8**),[23] were reported elsewhere. Chemical structures of dialkyl amphiphiles (**1**—**8**) are shown in Table 1.

Preparations of a Lipid-Coated Lipase. A typical preparation method of a lipid-coated lipase is as follows. An aqueous solution (25 ml, 0.01 M acetate buffer (1 M=

Table 1. Effects of Hydrophilic Head Groups of Lipid Molecules on Preparations of Lipid-Coated Lipase D and Their Enzymatic Activity

Lipid		Preparations		Enzymatic activity[d]
		Yield[a]/mg	Protein content[b]/wt%	mM min^{-1} (mg of protein)$^{-1}$
	**1**	33.2	12.3 (12.5)[c]	12.2
	**2**	7.5	1.0 (0.85)[c]	2.02
	**3**	0	—	—
	**4**	34.7	10.1 (9.2)[c]	0.26
	**5**	36.7	4.8 (5.4)[c]	0.16
	**6**	0	—	—
	**7**	0	—	—
	**8**	0	—	—

a) Lipase D: 50 mg, lipid molecules: 50 mg. 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 di- and trilaurin syntheses from 1-monolaurin (50 mM) and lauric acid (500 mM) in the dry benzene (2.5 ml) at 40 °C catalyzed by the lipid-coated lipase D (0.5—10 mg, 0.12 mg of protein).

1 mol dm^{-3}), pH 5.6) of lipase (50 mg) was centrifuged at 4000 rpm for 5 min at 4°C to remove the insoluble impurity. The enzyme aqueous solution was mixed with an aqueous dispersion (25 ml) of dialkyl amphiphiles (50 mg) at 4°C, and then kept under stirring for 20 h at 4°C. Precipitates were gathered by centrifugation at 4°C (5000 rpm, 5 min.) and lyophilized. The obtained white or pale yellow powder was insoluble in any aqueous buffer solution or water but freely soluble in most nonaqueous organic solvents.

The protein content in the complex was estimated from both the elemental analysis (C, H, and N) and the UV absorption of aromatic amino acid residues in proteins at 280 nm in chloroform solution, in a similar manner as with an aqueous solution. 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^{-1}); 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 spectra (E_m: 450 nm, E_x: 320 nm).

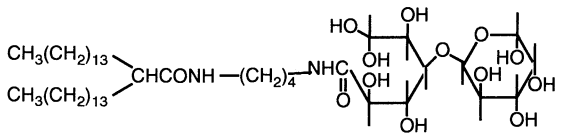
Catalytic Activity of a Lipid-Coated Lipase in Organic Solvents. A typical procedure is as follows. To the dry organic solution (2.5 ml) of a lipid-coated lipase (0.5—2 mg, 0.12 mg of protein), 1-monolaurin (34.5 mg, 50 mM) and an excess amount of lauric acid (250 mg, 500 mM) were added and stirred in the presence of two pieces of 3 Å molecular sieves at 40°C. Water content in the solution was measured to be 80 ppm during the reaction. With the prescribed time interval, the production of di- and triacylglycerols and the disappearance of 1-monolaurin 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 performed by comparing of the GC retention time and the GC peak area to those of the authentic samples, respectively. Diacylglycerols were obtained as a mixture of 1,2- and 1,3-isomers and it was difficult to separate these mixtures. Initial rates [mM min^{-1} (mg of protein)$^{-1}$] and/or total yield of di- and triacylglycerols in 24 h were mainly used as indicators of activities of enzymes.

Ester exchange reactions were also studied with tricaprylin (glyceryl trioctanoate, 24 mg, 20 mM) and an excess amount of lauric acid (200 mg, 400 mM) in the presence of a lipid-coated lipase (0.5—2 mg, 0.12 mg of protein) in the homogeneous benzene solution at 40°C. The production of glyceryl laurate dioctanoate and glyceryl dilaurate octanoate and the decrease of trioctanoate were followed by GC peak area comparing to those of their authentic samples. The water content in organic solutions was measured by Karl Fisher's method (instrument: Mitsubishi Chemical Co., Tokyo, type CA-05).

Results and Discussion

Characterization of Lipid-Coated Lipases. Effect of Hydrophilic Head Groups of Lipid Molecules. Lipid-coated lipases were prepared from lipase D (from *Rhizopus delemar*) and various kinds of dialkyl amphiphiles having cationic, anionic, zwitterionic, and nonionic hydrophilic head groups. The results are summarized in Table 1. Yields of a lipid-coated lipase were obtained as purified powder by weighing. The content of protein in the complex was estimated by the C/N ratio of elemental analyses and the UV absorption of aromatic amino acid residues in lipase. The protein contents obtained from both methods were consistent within experimental errors. An enzyme activity is shown as an initial rate of di- and trilaurin syntheses catalyzed by a lipid-coated lipase D from 1-monolaurin and lauric acid in the dry benzene at 40°C.

The lipid-coated lipase was obtained in a fair yield when nonionic (**1**), zwitterionic (**2**), and cationic (**4** and **5**) amphiphiles were employed, but not with the zwitterionic (**3**) and anionic amphiphiles (**6**, **7**, and **8**). Lipase has the negative charged surface at pH 5.6 (isoelectric point: 8.2) and the anionic amphiphiles seem not to interact with the lipase surface. Although the cationic amphiphiles (**4** and **5**) produce the complexes in a fair yield, their enzyme activities were very low compared with those of the complex with the nonionic amphiphiles (**1**). Probably because the strong electrostatic interaction between the cationic head groups of amphiphiles and the negatively charged surface of lipase denatures protein structures. As a result, the nonionic glycolipid (**1**) gave the complex (protein content: 10±1 wt%) in a good yield and the obtained complex showed a high enzymatic activity. It is due to the appropriate interaction through hydrogen bonds between the hydrophilic amino acid residues of a lipase surface and the hydroxyl head groups of the lipid (**1**). Lipase-lipid complexes were obtained in a good yield from the following synthetic glycolipids (**9** and **10**) with lipase D; the complex showed a high enzymatic activity in benzene as well as the complex from the lipid (**1**).



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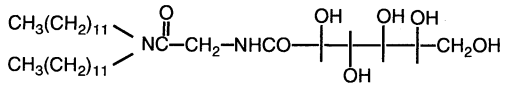

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The nonionic dialkyl amphiphile (**1**) was chosen as the lipid complexing with lipase in the following experiments.

Effect of an Origin of Lipase. The lipid-coated lipases were prepared with the nonionic glycolipid (**1**) with a different origin of lipases. The yield of the complex, the protein content in the complex, and the enzyme activity are shown in Table 2. When the glycolipid (**1**) was employed, the lipase-lipid complex was

Table 2. Effect of an Origin of Lipase on Preparations and Enzymatic Activities of the Lipid (**1**)-Coated Lipase

Origin of Lipse	Preparations		Enzymatic activity[c]
	Yield[a]/mg	Protein content[b]/wt%	mM min^{-1} (mg of protein)$^{-1}$
Lipase D from *Rhizopus delemar*	33.2	12.3	12.2
Lipase B from *Pseudomonas fragi* 22-39B	20.6	28.6	1.85
Lipase N from *Rhizopus niveus*	30.6	17.4	0.20
Lipase W from *Wheat germ*	33.6	24.6	0
Lipase P from *Pseudomonas fluorescens*	20.1	7.1	0

a) Lipase: 50 mg, lipid (**1**): 50 mg. b) Obtained from UV absorption of aromatic amino acid residues in the protein. c) Initial rates of di- and trilaurin syntheses from 1-monolaurin (50 mM) and lauric acid (500 mM) in the dry benzene (2.5 ml) at 40°C catalyzed by the lipid (**1**)-coated lipase (0.5—2 mg, 0.12 mg of protein).

obtained in a fair yield independent of an origin of lipase. However, the enzyme activity for glyceride syntheses in benzene was largely dependent on the origin: the complex from lipase D (from *Rhizopus delemar*) and lipase B (from *Pseudomonas fragi* 22-39B) showed the relatively high activity, but the complexes from lipase W (from *Wheat germ*) and lipase P (from *Pseudomonas fluorescense*) did not show the catalytic activity for glyceride syntheses in benzene.

The commercially available lipase D (from *Rhizopus delemar*) was chosen as a lipase mainly in the following experiments.

Characterization with Gel-Permeation Chromatography. In order to study the physical stability and the characterization of the lipase-lipid complex in organic solvents, we analyzed the lipid (**1**)-coated lipase D by gel-permeation chromatography (eluent, dichloromethane; detector, UV at 240 nm; molecular weight was calibrated using a standard polystyrene). A typical gel-permeation chromatograph is shown in Fig. 2. When fractions were followed by the UV absorption at 240 nm, two peaks were observed at the estimated molecular weight of $(13\pm2)\times10^4$ and at less than 10^3, respectively. The first peak showed the catalytic hydrolysis activity of 4-methyl-2-oxo-1-benzopyran-7-yl oleate in tetrahydrofurane–water solution, but not at the second peak of $M_w<10^3$. Since the molecular weight of a native lipase D and the lipid (**1**) are ca. 3.3×10^3 and 661, respectively, the first peak is estimated to be a lipid-coated lipase which is calculated to contain 150±30 lipid molecules per one lipase, and the second peak corresponds to the free lipid (**1**).

It can be roughly estimated from the molecular area of the lipid (0.45 nm^2) and lipase (diameter: ca. 3 nm) that ca. 150±50 lipid molecules are required to cover the surface of a lipase as a monolayer. The protein content in the lipid-lipase powder was obtained to be 12 wt% from both the elemental analysis (C/N ratio) and the UV absorption of aromatic amino acid residues in lipase (see Table 1). The protein content of 12 wt% indicates that 300—400 lipid molecules attached around a lipase surface. Thus, the obtained lipase-lipid powder contains a lipase coated strongly with one layer of 150±30 lipid molecules and other 150—250 lipid molecules

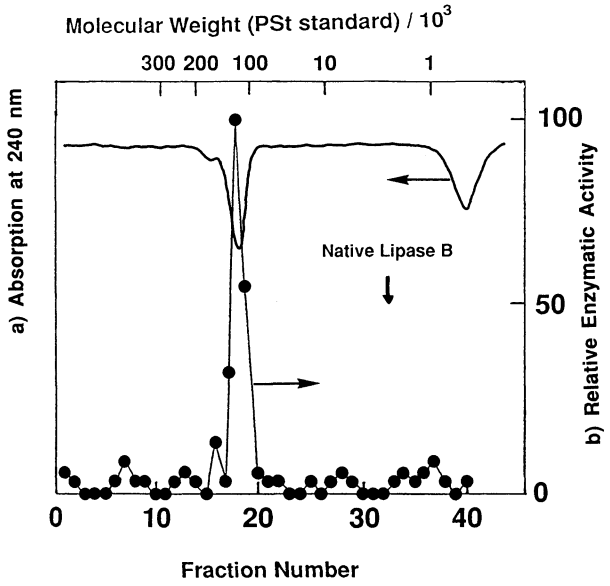


Fig. 2. A gel-permeation chromatograph of the lipid (**1**)-coated lipase D followed by a) UV absorption at 240 nm and b) hydrolytic enzyme activity. Eluent: dichloromethane, molecular weight was calibrated using a standard polystyrene. Enzyme activity of each fraction was measured by the catalytic hydrolysis of 4-methyl-2-oxo-2*H*-1-benzopyran-7-yl oleate in tetrahydrofurane-water solution followed by fluorescent spectra (E_m: 450 nm, E_x: 320 nm).

interact with the lipid monolayer-coated lipase with hydrophobic interactions. In gel-permeation chromatography, the lipid monolayer interacting with the enzyme surface does not remove from the complex, but the excess lipid molecules seem to remove easily in organic solvents.

We have observed ^1H NMR spectra (270 MHz) of the lipid (**1**)-coated lipase D and compared with those of the free lipid (**1**). The chemical shift of only hydrophilic OH groups of the lipid (**1**) in the complex moves to the down field (3.7→4.1 ppm) relative to that of the free lipids. It indicates that the hydroxyl head groups of the lipid (**1**) interact with the hydrophilic surface of lipase through hydrogen bonds and lipophilic tails of lipids are thought to solubilize the complex in hydrophobic organic solvents, as shown in an illustration of Fig. 1.

Table 3. Effect of Lipid/Lipase Ratio in Preparations on Protein Content and Enzymatic Activities of the Lipid (**1**)-Coated Lipase D

Lipid/Lipase ratio	Preparations		Enzymatic activity[d]
	Yield[a]/mg	Protein content[b]/wt%	mM min^{-1} (mg of protein)$^{-1}$
0.5/1	11.9	14.3 (11.2)[c]	14.7
1/1	37.5	12.3 (12.5)[c]	12.2
1.5/1	52.7	11.7 (10.6)[c]	8.83
2.0/1	66.3	9.2 (9.2)[c]	5.66

a) Lipase D: 50 mg, lipid (**1**): 50 mg. 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 di- and trilaurin syntheses from 1-monolaurin (50 mM) and lauric acid (500 mM) in the dry benzene (2.5 ml) at 40°C catalyzed by the lipid (**1**)-coated lipase D (ca. 1 mg, 0.12 mg of protein).

When the benzene solution (5 ml) of the lipid (**1**)-coated lipase D (50 mg) was extracted with 10 ml of water or an aqueous buffer solution (pH 5.6, 0.01 M acetate) several times for 12 h at 40°C, neither lipase nor lipid molecules were detected in the aqueous phase by thin-layer chromatography within experimental errors. The complex left in the organic phase still has the 90—100% of the original enzymatic activity. Thus, the interaction between lipid molecules and lipase is strong even in organic solvents and is not disturbed by aqueous phases. In enzymatic reactions in organic solutions, the lipid monolayer interacts with the lipase surface and the excess lipid molecules, which bind weakly to the complex, may remove from the complex in organic solutions.

Effects of a Ratio of Lipid/Lipase. When the enzyme–lipid complex was prepared from the lipid (**1**) and lipase D, the ratio of lipid/lipase was changed against the constant amount of lipase D (50 mg). Results are shown in Table 3. Although the yield of complex increased with increase of the ratio of lipid to lipase, the protein content in the complex and the enzyme activity per protein decreased with increase of the lipid ratio. Thus, an excess amount of lipids seems to bind around a lipase and decreases the enzyme activity per protein.

In the following enzymatic reactions, the lipid (**1**)-coated lipase D prepared by 1 : 1 weight ratio was used mainly.

Catalytic Syntheses of Glycerides in Organic Solvents. Lipase is known to synthesize esters using the reverse hydrolysis in an organic solvent by shifting the equilibria toward the condensation products.[3-8] Syntheses of di- and trilaurin from 1-monolaurin (50 mM) and lauric acid (500 mM) catalyzed by the lipid (**1**)-coated lipase D (1 mg, 0.12 mg of protein) were carried out in the homogeneous dry benzene in the presence of two pieces of 3 Å molecular sieves at 40°C. Figure 3 shows times courses of the decrement of 1-monolaurin and the production of dilaurin and trilaurin. The substrate, monolaurin, decreased promptly in 10 h and dilaurin was produced simultaneously, which was converted to trilaurin slowly in 100 h. If the reverse scheme of the hydrolysis can be applied for the condensation reaction in organic solvents, the following reaction is expected to

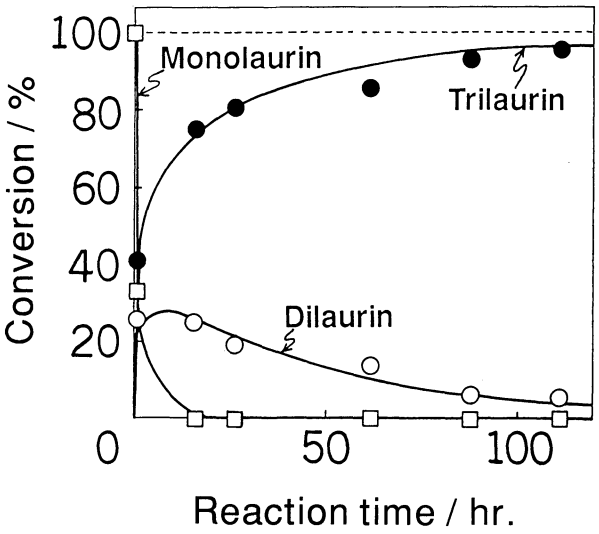
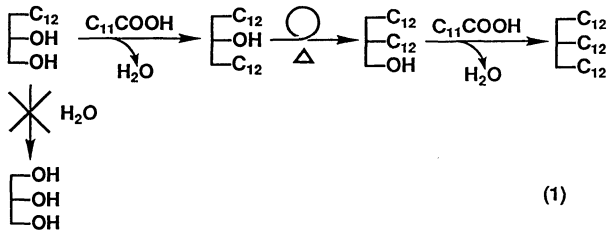


Fig. 3. Typical time courses in di- and trilaurin syntheses catalyzed by the lipid (**1**)-coated lipase D from 1-monolaurin and lauric acid in benzene in the presence of two pieces of molecular sieves at 40°C. [lipid-coated lipase]=1 mg (0.12 mg of protein), [1-monolaurin]=50 mM, [lauric acid]=500 mM, benzene: 2.5 ml.



(1)

occur. Since lipase D can recognize 1,2-acylglycerol but not 1,3-diacylglycerol,[24] 1,3-dilaurin prepared from 1-monolaurin will be thermally isomerized to 1,2-dilaurin which is then slowly converted to trilaurin with the enzymatic reaction. The slow production of trilaurin can be explained by the slow thermal isomerization from 1,3- to 1,2-diacylglycerol. Since monolaurin was converted to trilaurin almost in 100%, the hydrolysis to glycerol is concluded not to occur in the dry benzene solution. In the following discussion, we

Table 4. Effect of Organic Solvents on Solubility and Enzymatic Activity of the Lipid (**1**)-Coated Lipase D

Solvent	Solubility[a]	Initial rate of di- and trilaurin syntheses[b] mM min^{-1} (mg of protein)$^{-1}$
Water	−	0
Ethanol	−	0
Diisopropyl ether	+	15.4
Benzene	++	12.2
Toluene	++	12.4
Hexane	+	13.9
Chloroform	++	0
Dichloromethane	++	0
1,2-Dichloroethane	++	0

a) ++: easily soluble, +: fairly soluble, −: insoluble. b) [1-monolaurin]=50 mM, [lauric acid]=500 mM, [lipid-coated lipase]=1 mg (0.12 mg of protein), in each solvent (2.5 ml), at 40°C.

chose the initial rate of production of di- and triacylglycerols and the yield of di- and triacylglycerols after 24 h as indicators of enzyme activities.

Effect of Organic Solvents. The di- and triacylglycerol syntheses catalyzed by the lipid (**1**)-coated lipase D were carried out in various organic solvents. The solubility of the complex for solvents and the initial rate are shown in Table 4. The lipid-coated lipase was insoluble in hydrophilic solvents such as water, buffer solution (pH 5.6, 0.01 M acetate), and ethanol. In contrast, it was soluble in most hydrophobic organic solvents and showed a fairly high initial rate of di- and triacylglycerol syntheses in benzene, toluene, hexane, and diisopropyl ether. In halogenated solvents such as chloroform, dichloromethane, and 1,2-dichloroethane, the lipid-coated lipase did not show the catalytic activity although it is soluble. The lipid-coated lipase may be denatured in halogenated solvents.

Benzene was chosen as a standard solvent in the following experiments.

Effect of Water Content in an Organic Solvent. Effect of the addition of molecular sieves on the catalytic syntheses of di- and trilaurin with the lipid-coated lipase in benzene solution is shown in Fig. 4. In the presence of two pieces of molecular sieves in benzene solution (water content: 80±20 ppm), 1-monolaurin was completely converted to di- and trilaurin within 7 h with an initial rate (v_0) of 12.2 mM min^{-1} (mg of protein)$^{-1}$. In contrast, in the absence of molecular sieves in commercially available benzene (an initial water content: 0.025%, 250 ppm), the reaction stopped at 70% yield with the v_0=6.13 mM min^{-1} (mg of protein)$^{-1}$, and the benzene solution became turbid due to the produced water with the progress of the reaction. When two pieces of molecular sieves were added at the arrow in Fig. 4, the benzene solution became clear and the reaction proceeded completely within 10 h. Thus, the effective catalytic activity of the lipid-coated lipase for glyceride syntheses is shown in the dry organic solvent and the produced water inhibits the synthetic reaction.

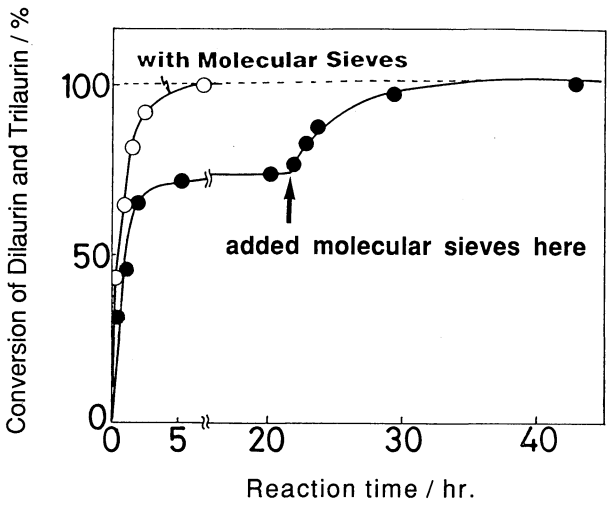


Fig. 4. Effect of dehydration in benzene solution with molecular sieves (2 pieces) on di- and trilaurin syntheses catalyzed by the lipid (**1**)-coated lipase D. Reaction conditions are the same as those in Fig. 3.

Figure 5 shows the effect of water content in benzene solution on initial rates of di- and triacylglycerol syntheses and of hydrolyses of monolaurin to glycerol. Glycerol is accumulated in the medium when the hydrolysis of 1-monolaurin occurs in the presence of much amount of water (see Eq. 1). The rate of ester syntheses was largest in the dry benzene in the presence of molecular sieves (water content: 80 ppm) and decreased with increasing the water content. When the benzene solution contains more than 0.1% of water (the solution is turbid), the hydrolysis to glycerol was observed and the conversion to di- and triacylglycerol decreased.

Although the lipid-coated lipase showed the highest synthetic activity in the dry benzene, it is difficult to know the true water content near the enzyme. After the lipid-coated lipase powder was dried completely in a vacuum desiccator (2—3 d at 0.01 mmHg, 1 mmHg=133.322 Pa), the weight of the enzyme hardly changed. The water content of the dry benzene solution did not change before

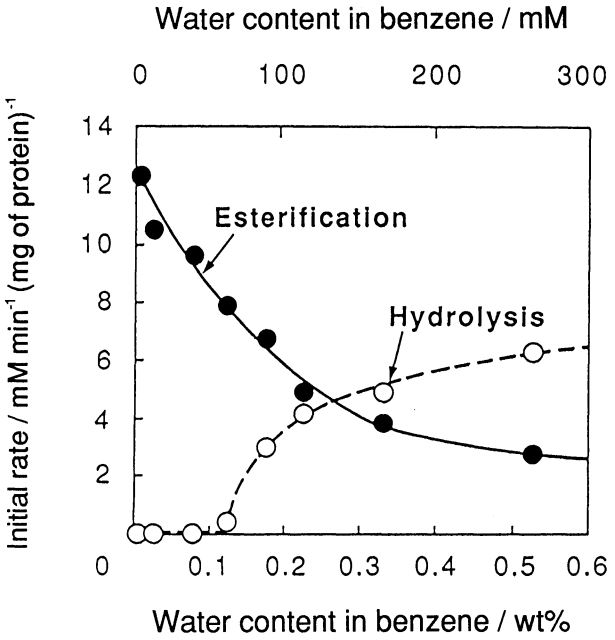


Fig. 5. Effect of water content in benzene solution on the initial rates of di- and trilaurin syntheses and of hydrolysis to glycerol at 40°C. Reaction conditions are the same as those in Fig. 3.

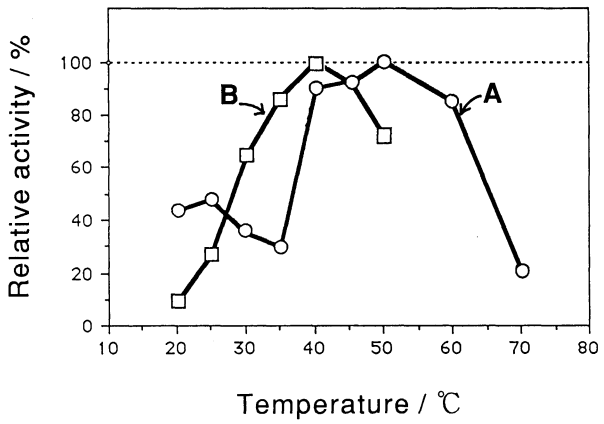


Fig. 6. Effect of temperature on the di- and trilaurin syntheses catalyzed by the lipid (**1**)-coated lipase D in the dry benzene and on the hydrolysis of olive oil catalyzed by a free lipase D in an aqueous buffer solution (pH 6.0). A) glyceride syntheses: [lipid-coated lipase]=1 mg (0.12 mg of protein), [1-monolaurin]= 50 mM, [lauric acid]=500 mM, benzene: 2.5 ml; B) hydrolyses: [lipase]=0.12 mg, [olive oil]=0.1 g, buffer solution: 2.5 ml.

and after the addition of the lipid-coated lipase (100 mg) in the solution (10 ml). This means that the lipid-coated enzyme may have a small amount of water near the interface between lipids and an enzyme surface, and that the water does not dissolve in the benzene solution during the reaction.

Effect of Temperature on Enzyme Activity. Trilaurin syntheses were carried out at various temperatures in the dry benzene in the presence of the lipid-coated lipase; the results are shown in Fig. 6. Effects of temperature on the hydrolysis of olive oil with a native lipase in an aqueous buffer solution are compared in the same figure. Although the hydrolysis activity of a native lipase decreased above temperatures at 40°C, the esterification activity of the lipid-coated lipase in benzene was kept at 60°C. When the native lipase was kept in a hot buffer solution at 50°C for 5 h, the hydrolytic activity decreased to ca. 10% of the original value. In contrast, the esterification activity of the lipid-coated lipase in benzene was maintained around 80—90% of the original value after being immersed at 60°C for 10 h. These results indicate that the surrounding lipid wall on the lipid-coated lipase may play important roles to keep the stability in organic solvents at higher temperatures.

Comparison with Other Enzyme Systems. Several approaches have been performed to use a lipase as a synthetic catalyst in organic solvents.[3-8] In addition to the water-in-oil emulsion or the reversed micellar system containing a small amount of water,[9-11] two elegant methods are reported by using the hydrophobic organic solvent as a medium: i) Inada and co-workers prepared a poly(ethylene glycol) (PEG)-grafted lipase which is soluble or swelled in hydrophobic organic solvents and catalyzes simple ester syntheses from aliphatic alcohols and acids[18-20] and ii) Klibanov and co-workers reported the direct dispersion method of powdered lipase in organic solvents, which can be used as an ester exchange catalyst in heterogeneous solution.[6-8,12-17]

We compared our lipid-coated lipase system with other systems in the same reaction conditions using the same lipase B (from *Pseudomonas fragi* 22-39B) as an enzyme. Di- and trilaurin syntheses from 1-monolaurin and lauric acid were carried out in the dry benzene with molecular sieves in the presence of the same amount (1 mg of protein) of lipid-coated lipase B, PEG-grafted lipase B, lipase B in w/o emulsion, and powdered lipase B. The time courses of the reactions are shown in Fig. 7. PEG-grafted lipase B was commercially available from Sapporo Beer Co., Tokyo, in which linear PEG (M_w: ca. 5000) was covalently immobilized on a lipase and protein content is 73%, prepared according to Inada's method.[18-20]

When the lipid-coated lipase B was employed, 1-monolaurin was completely converted to di- and trilaurins in the dry benzene within 7 h [initial rate: v_o=12.2 mM min^{-1} (mg of protein)$^{-1}$]. However, the direct dispersion method showed the very slow reaction rate (1/100 times) compared with that of the lipid-coated lipase [v_o<0.1 mM min^{-1} (mg of protein)$^{-1}$]. In the dispersion method, enzymes exist as a suspension in the substrate solution of organic solvents, therefore, much amount of enzymes may be required in order 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

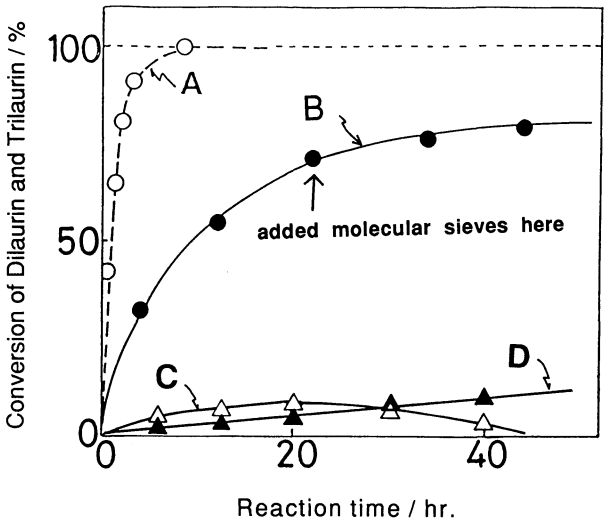


Fig. 7. Comparison of catalytic activities for di- and trilaurin syntheses of A) lipid-coated lipase B, B) PEG-grafted lipase B, C) lipase B in W/O emulsion, and D) dispersion of lipase B powder, in benzene solution at 40°C. Reaction conditions are the same as those in Fig. 3.

of enzyme was used. In the water-in-oil system, in which 1 mg of a native lipase B is solubilized in a buffer solution (pH 5.6) and emulsified in benzene, the rate of ester syntheses was very slow [v_o<0.1 mM min^{-1} (mg of protein)$^{-1}$] and decreased with increasing the reaction time. This is explained that the existence of a small amount of water in the w/o emulsion system caused the reverse hydrolysis reaction.

When PEG-grafted lipase B was employed, the esterification proceeded in a fair rate [v_o=6.0 mM min^{-1} (mg of protein)$^{-1}$, 0.5 times compared with that of the lipid-coated lipase B]. However, the conversion reached a plateau at 70% after 40—80 h in the dry benzene even in the presence of molecular sieves. As shown in Fig. 4, in di- and trilaurin synteses catalyzed by the lipid-coated lipase, the conversion of glycerides stopped at 70% in the absence of molecular sieves; the solution became turbid due to the water produced during the reaction. The conversion reached the 100% yield when the addition of two pieces of molecular sieves in order to remove water in the benzene solution. In the case of PEG–lipase B, the conversion also stopped around 70% both in the absence and presence of molecular sieves, however, the reaction medium was not turbid. After the addition of molecular sieves, the reaction did not proceed any more (see Fig. 7). This indicates that the PEG–lipase can keep the produced water near the amphiphilic PEG grafted chains so that the water molecules may not be removed by the addition of molecular sieves. The water solubilized near the lipase surface inhibits the ester synthesis in organic solvents. In contrast, the hydrophobic lipid-coated lipase can not solubilize the produced water and the glyceride synthesis proceeds completely when the produced water is removed by molecular sieves from the solution.

Thus, the lipid-coated lipase can catalyze effectively and completely the glyceride synthesis in dry organic solutions, as compared with other enzyme systems such as water-in-oil emulsion, enzyme dispersion, and PEG-grafted enzyme. Because the lipid-coated lipase is homogeneously soluble and stable in the dry organic solvents.

Esterification of Unsaturated Fatty Acids. Recently, highly unsaturated fatty acids such as icosapentaenoic acid (EPA) are thought to be converted to prostaglandin analogs in metabolic pathway and to act as a drug in our body. Since EPA is unstable due to the oxidation of double bonds in the air, it is expected that the conversion to glyceride ester increases the stability and the solubility into biological cell membranes. Therefore, it is important to study the catalytic esterification activity of the lipid-coated lipase for the unsaturated fatty acid. The esterification was performed from 1-monolaurin or 1-monostearin (50 mM) and various kinds of unsaturated fatty acids (500 mM) in the dry benzene or hexane in the presence of the lipid-coated lipase D or the lipid-coated lipase B at 40°C. The initial rate and the yield in 24 h are summarized in Table 5. The lipid-coated lipase B or D was found to convert highly unsaturated fatty acids such as EPA to di- and triacylglycerols in a fair yield independent of the degree of unsaturation in fatty acids. Thus, the lipid-coated lipase is effective as a esterification

Table 5. Di- and Triacylglycerol Syntheses from 1-Monoacylglycerol and Unsaturated Fatty Acids Catalyzed by the Lipid (**1**)-Coated Lipase D in Benzene Solution at 40°C[a)]

Fatty acid	Monoacylglycerol	Initial rate	Yield in 24 h
		mM min^{-1} (mg of protein)$^{-1}$	mol%
Stearic acid (18:0)	Monostearin	11.4	100
Oleic acid (18:1)	Monostearin	9.8	100
Linolic acid (18:2)	Monostearin	8.4	100
Linolenic acid (18:3)	Monostearin	3.8	100
Arachidic acid (20:4)	Monolaurin	11.6[b)]	82[b)]
Icosapentaenoic acid (20:5)	Monolaurin	7.8[c)]	70[c)]

a) [lipid-coated lipase]=1 mg (0.12 mg of protein), [Fatty acid]=500 mM, [Monoacylglycerol]=50 mM, benzene solution: 2.5 ml. b) In hexane solution (2.5 ml). c) In hexane solution, lipase B was used as a lipid-coated enzyme.

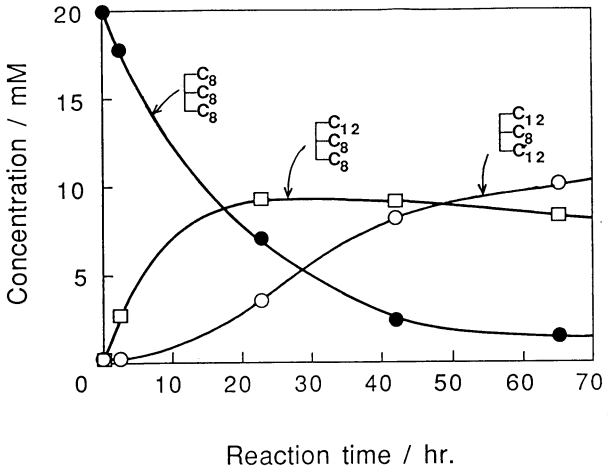


Fig. 8. Typical time courses of ester exchange reactions between tricaprylin and lauric acid catalyzed by the lipid (**1**)-coated lipase D in benzene at 40°C. [lipid-coated lipase]=1 mg (0.12 mg of protein), [tricaprylin]=20 mM, [lauric acid]=400 mM, benzene: 2.5 ml.

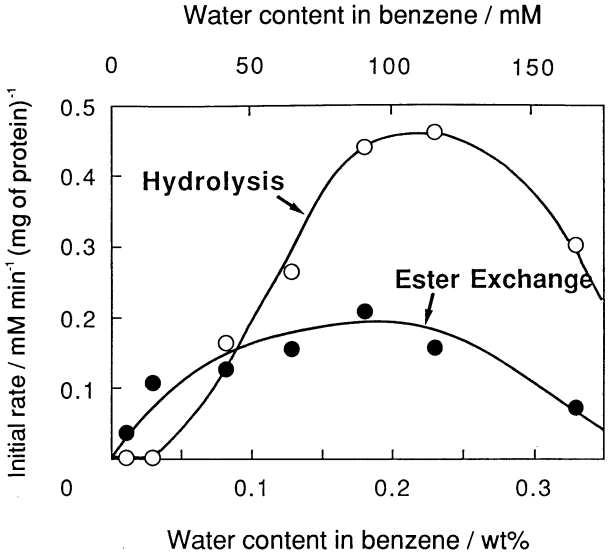


Fig. 9. Effect of water content in benzene solution on ester exchange reactions between tricaprylin and lauric acid catalyzed by the lipid (**1**)-coated lipase D at 40°C. Reaction conditions are the same as those in Fig. 8.

catalyst of unstable highly-unsaturated fatty acid in a mild condition.

Ester Exchange Reactions in Organic Solvents. The catalytic activities of the lipid-coated lipase D for ester exchange reactions between tricaprylin (glyceryl trioctanoate) and an excess amount of lauric acid are also studied in benzene. The decrease of tricaprylin and the production of glyceryl laurate dioctanoate and glyceryl dilaurate octanoate were followed with time; the results are shown in Fig. 8. Tricaprylin was converted to glyceryl laurate dioctanoate at the first stage and then to glyceryl dilaurate octanoate. Since trilaurin was not observed in the products, glyceryl 1,3-dilaurate 2-octanoate is not isomerized thermally to glyceryl 1,2-dilaurate 3-octanoate.

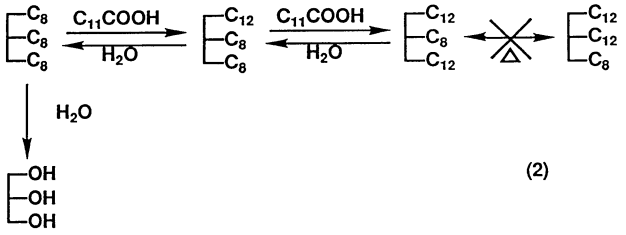


Figure 9 shows the effect of the water content in benzene on initial rates of the ester-exchange reaction and on the hydrolysis rate to glycerol. The ester exchanges hardly occurred in the dry benzene (80 ppm of water in the presence of molecular sieves). When commercially available benzene (water: 250 ppm, 0.025%) was employed, the ester-exchange reaction proceeds in a fair rate without the reverse hydrolysis reaction. When water was added to the solution (0.08%), the hydrolysis to glycerol was observed, the rate of which became larger than that of the ester-exchange reaction in the range of the water content of 0.08—0.33%.

It is interesting that the ester synthesis reaction occurred effectively in the dry benzene solution, but the ester exchange reaction hardly occurred in the dry benzene and the 0.02% of water is required to cause the reaction without the reverse hydrolysis (see Figs. 5 and 9). The small amount of water has been reported to be required near enzymes in order to use the enzyme as an effective catalyst in organic solvents.[3-8] Since the glyceride synthesis reaction from 1-monolaurin and lauric acid produces water molecules during the reaction, the small amount of water produced may absorb at the interface between the lipids and the enzyme surface and activates the catalytic reaction. The excess amount of water is dissolved in the solution and is absorbed with molecular sieves. In the case of the ester-exchange reaction, water is not produced during the reaction and the small amount of water should be added initially to keep the enzyme activity in organic solvents (for example, 0.02% of water in benzene). Thus, the lipid-coated lipase may require the small amount of water in order to keep the activity in hydrophobic organic solvents. It is difficult to know exactly how much amount of water exists in the lipid-coated lipase.

Summary

The lipid-coated lipase is easily prepared and homogeneously soluble in hydrophobic organic solvents. The enzyme is stable in organic solvents for a long time; i.e., an 80% activity remains after being kept in dry benzene for three months. Powder of the lipid-coated lipase is stable at least for a year. The lipid-coated lipase

showed the higher activity of glyceride syntheses than PEG-grafted lipase and the direct dispersion of powdered lipase, comparing under the same reaction condition.

The lipid-coated lipase could become a new type of enzymatic synthesis catalyst in organic solvents under a mild condition. Preparation techniques of the lipid-coated lipase can be widely applied for other enzymes to prepare organic solvent-soluble enzymes. For example, the lipid-coated enzyme can be easily transferred on a substrate using a Langmuir–Blodgett technique and act as a biosensor membrane.[1)]

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