A Lipid-coated Lipase as a New Catalyst for Triglyceride Synthesis in Organic Solvents

Yoshio Okahata* and Kuniharu Ijiro

Department of Polymer Chemistry, Tokyo Institute of Technology, Ookayama, Meguro-ku, Tokyo 152, Japan

Lipid-coated enzyme (lipase) was prepared by mixing aqueous solutions of enzyme and synthetic dialkyl amphiphiles; it was insoluble in water but soluble in most organic solvents and showed a high activity for the synthesis of di- and tri-glycerides from monoglycerides and aliphatic acids in non-aqueous and homogeneous organic solvents.

In recent years there has been a considerable effort to try to mimic the catalytic action of enzymes in organic solvents.[1—3] For example, lipase has been used as a transesterification catalyst for lipophilic substances in 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;[3—5] ii, the direct dispersion of powdered enzyme in organic solvents;[6] iii, the surface modified enzyme with poly(ethylene glycol) (P.E.G.) which is soluble in organic solvents.[7]

We now report a new type of enzyme, which is organic solvent soluble, lipid-coated lipase. This is easily prepared and can catalyse efficiently the synthesis of di- and triglycerides from 1-monolaurin and lauric acid, compared with other enzyme systems in organic solvents.

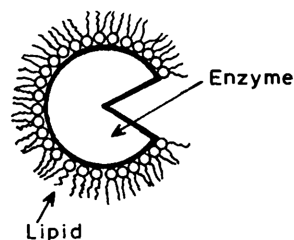
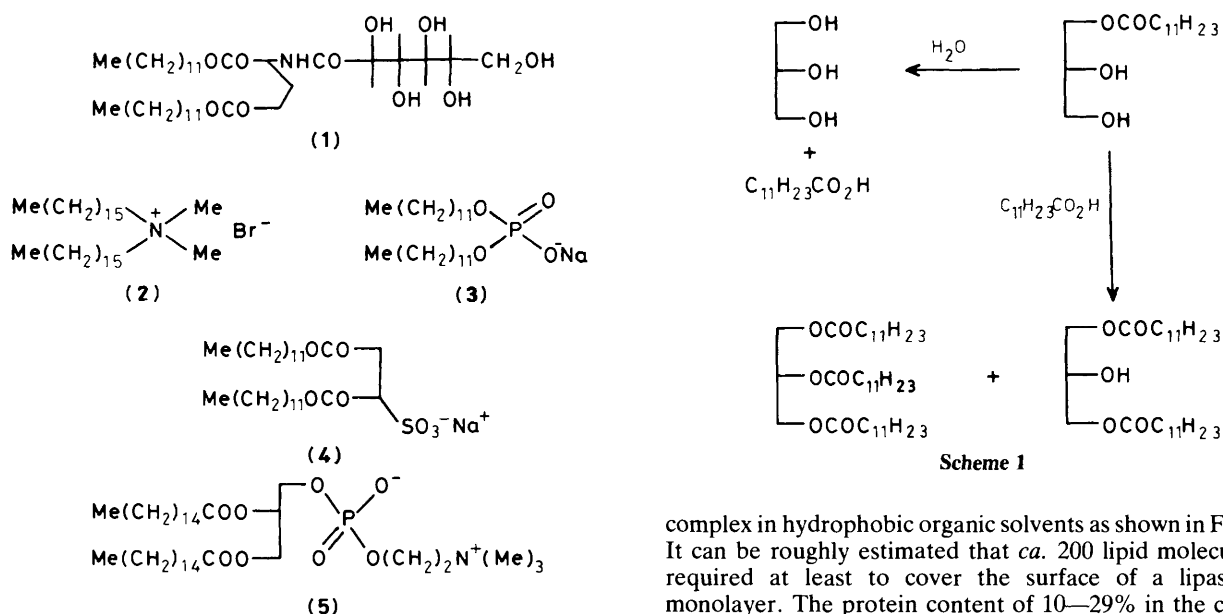


Figure 1

Table 1. Characterization and activity of lipase in organic solvents.

Enzyme system[a]	Yield of complex/mg[b]	Protein content in the complex /%wt[c]	Activity[d] Initial rate[e]	% Yield in 24 h	Solvent
Lipid–enzyme complex					
Lipase D + nonionic (**1**)	33.2	12.3	12.2	100	benzene
			15.4	100	isopropyl ether
			12.4	100	toluene
			13.9	100	n-hexane
Lipase D + cationic (**2**)	34.7	10.1	0.13	60	benzene
Lipase D + anionic (**3**)	0				
Lipase D + anionic (**4**)	0				
Lipase D + zwitterionic (**5**)	0				
Lipase B + nonionic (**1**)	20.6	28.6	1.85	100	benzene
Lipase N + nonionic (**1**)	30.0	17.4	0.20	70	benzene
P.E.G.-modified lipase B[f]			0.10	70	benzene
Lipase B in suspension[g]			<0.01	<5	benzene
Lipase B in reversed micelle[h]			<0.01	<5	benzene

[a] Lipase D from *Rhizopus delemar*; lipase B from *Pseudomonas fragi 22*; lipase N from *Phizopus niveus*. [b] Precipitated powder from the mixture of aqueous solution of 50 mg of lipid and 50 mg of lipase. [c] Average value obtained from the u.v. absorption and elemental analysis. [d] Synthesis of di- and triglycerides from monolaurin (50 mM) and lauric acid (500 mM) at 40 °C in the presence of 0.3 mg of protein in 2.5 ml of organic solvents. [e] [Di- and trilaurin] mM min^{-1} (mg of protein)$^{-1}$. [f] P.E.G. (mol wt. 5000) was surface-grafted on lipase according to Inada's method;[7] protein content 73%wt. [g] Native enzyme in suspension. [h] Aerosol OT was used as a surfactant.



Scheme 1

A typical preparation of lipid-coated lipase was as follows. An aqueous solution [25 ml, 0.01 M acetate buffer (pH 5.6)] of lipase (50 mg) and aqueous dispersion (25 ml) of diakyl amphiphiles (50 mg) were mixed, and the precipitates after incubation at 4 °C for 24 h were lyophilized. The pale yellow powder obtained was insoluble in 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 u.v. absorption of aromatic amino acid residues of proteins at 280 nm in chloroform in a similar manner as with an aqueous solution. Preparations of the lipid-enzyme complex are summarized in Table 1.

The nonionic (**1**) and cationic (**2**) lipids gave the complex in 20—35% yield probably due to hydrogen bonds and electrostatic interactions at head groups of lipids with the hydrophilic surface of lipase, respectively, independent of the origin of the lipase. Lipophilic tails of lipids are thought to solubilize the complex in hydrophobic organic solvents as shown in Figure 1. It can be roughly estimated that *ca.* 200 lipid molecules are required at least to cover the surface of a lipase as a monolayer. The protein content of 10—29% in the complex means that *ca.* 150—450 lipid molecules are attached around one lipase. The anionic (**3**), (**4**), and zwitterionic (phosphatidylcholine) (**5**) lipids, however, did not form the complex as precipitates because of the weak interaction with the negatively charged surface of lipase.

The catalytic activity of the lipid-coated lipase (0.3 mg of protein) was studied in the synthesis of di- and triglycerides from 1-monolaurin (50 mM) and an excess amount of lauric acid (500 mM) in homogeneous dry benzene (2.5 ml, water content: 83 p.p.m.) at 40 °C (Scheme 1). The production of di- and triglycerides and the disappearance of monoglycerides were followed by g.c. The diglycerides were obtained as a mixture of 1,2- and 1,3-isomers and it was difficult to separate and isolate the mixtures. Initial rates and yields at 24 h of the di- and triglyceride synthesis are summarized as an activity in Table 1. The complex from cationic lipids (**2**) showed very small activity compared with that from nonionic lipids (**1**), probably because the strong interaction with cationic lipids with the negatively charged lipase denatures protein

structures. The complex of lipase D (from *Rhizopus delemar*) with nonionic glycolipids (**1**) showed high activity [initial rates: 12—15 mM min^{-1} (mg of protein)$^{-1}$] independent of organic solvents: monolaurin was converted completely to dilaurin (25%) and trilaurin (75%) in 5 h, and trilaurin was obtained in 98% yield after 70 h.

The high activity and the complete conversion to triglyceride was observed in dry organic solvents such as benzene and n-hexane (water content: 0.0083%) in the presence of two pieces of 3 Å molecular sieve. In the absence of molecular sieves, the benzene solution became turbid as the reaction proceeded due to water production (0.1—0.2 wt%) and the yield of di- and triglycerides reached a plateau at 73%, with 27% of the monoglyceride unreacted. This means that even the small amount of water produced during the reaction inhibits the triglyceride synthesis. In fact, when the lipid-coated lipase was employed in water-containing organic solvent or water-in-oil microemulsion, the synthetic reaction of di- and triglycerides hardly occurred and only the reverse reaction (hydrolysis of monolaurin to glycerol) was observed.

The activity of the lipase–lipid complex was compared with other enzyme systems using the same lipase B (from *Pseudomonas fragi 22*) in the di- and triglycerides synthesis (Table 1). When the lipid-coated lipase B was dissolved in dry benzene, monoglyceride was converted completely to di- and triglycerides in 24 h, although the initial rate was reduced compared with that of the complex with lipase D. When the lipase B surface-grafted with poly(ethylene glycol)[7] (P.E.G.-lipase)† was employed in transparent benzene solution, the activity was relatively low [the initial rate: 0.10 mM min^{-1} (mg of protein)$^{-1}$] and the yield of di- and triglyceride reached a plateau at 70%. The poly(ethylene glycol) chains are amphiphilic and solubilize the water produced around the enzymes, which may inhibit the complete synthesis of triglycerides. The P.E.G.–lipase B, however, could catalyse completely the simple transesterification of lauric acid and butanol.[7] When lipase B or lipase D powder (1 mg of protein) was directly dispersed in benzene according to Klibanov's method,[1,6] the synthesis of di- and triglycerides hardly proceeded. The dispersion system requires a large amount of enzyme in a heterogeneous solution and lipases B and D may be not suitable for use in the dispersion system due to their origin. When the lipase B was dissolved in a water-in-oil microemulsion or reversed micellar systems, the synthetic reaction was not observed and only the hydrolysis of monolaurin to glycerol occurred.

In conclusion, the lipid-coated enzyme is easily prepared and is stable as a powder for at least a year and stable in an organic solvent for a long time *i.e.* the 80% activity remains after being kept in dry benzene for three months. The lipid-coated lipase could become a new catalyst for enzymatic synthetic reactions in organic solvents such as the synthesis of triglycerides from highly unsaturated fatty acids (E.P.A.) under mild reaction conditions.

† Presented by Sapporo Beer Co. Ltd., Tokyo.

Received, 15th April 1988; Com. 8/01463I

References

1 A. M. Klibanov, *Chem. Tech. (Leipzig)*, 1986, 355.
2 J. B. Jones, *Tetrahedron*, 1986, **42**, 3351.
3 P. L. Luisi, *Angew. Chem., Int. Ed. Engl.*, 1985, **24**, 439.
4 P. Luthi and P. L. Luisi, *J. Am. Chem. Soc.*, 1984, **106**, 7285.
5 M. D. Bednarski, H. K. Chenault, E. S. Simon, and G. M. Whitesides, *J. Am. Chem. Soc.*, 1987, **109**, 1283.
6 G. Kirchner, M. P. Scollar, and A. M. Klibanov, *J. Am. Chem. Soc.*, 1985, **107**, 7072.
7 K. Takahashi, H. Nishimura, T. Yoshimoto, Y. Saito, and Y. Inada, *Biochem. Biophys. Res. Commun.*, 1984, **121**, 261; T. Yoshimoto, K. Takahashi, H. Nishimura, A. Ajima, Y. Tamaura, and Y. Inada, *Biotech. Lett.*, 1984, **6**, 337; K. Takahashi, A. Ajima, T. Yoshimoto, M. Okada, A. Matsushima, Y. Tamaura, and Y. Inada, *J. Org. Chem.*, 1985, **50**, 3414.