Technical News Feature

Lipase Made Active in Hydrophobic Media[1]

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Enzymes are distinguished from other catalysts by their high substrate specificity. This is a great asset when one wants to apply them for syntheses of various compounds. Their usage, however, generally is limited to hydrophilic reaction media, because they usually are not soluble and active in hydrophobic media. Recently, we have been able to make various enzymes soluble and active in highly hydrophobic organic solvents. The key to this success is the chemical modification of enzymes with an amphipathic synthetic polymer, polyethylene glycol. The activated polymers can be attached to enzymes in aqueous buffer solutions, and once enzymes are modified they become soluble and active in various organic solvents such as benzene, toluene and cholorinated hydrocarbons and exhibit high enzymic activities in these organic solvents. Modified hydrolytic enzymes catalyzed the reverse reaction of hydrolysis in organic solvents. The modified lipase catalyzed various ester synthesis reactions. Because the reactions were conducted in the pure solvent system, it also was possible to study the kinetics and the substrate specificity for ester synthesis reaction. It also catalyzed the polymerization of a hydroxy group containing carboxylic acid due to the bifunctional nature. The modified lipase catalyzed ester exchange between an ester and an alcohol, between an ester and a carboxylic acid and between two esters in organic solvents. When the two substrates for ester exchange were liquid, the reaction could take place without organic solvents. The modified lipase catalyzed an ester exchange reaction between trilaurin and triolein when dissolved in these substrates. Dilauroyl-monooleoyl-glycerol and monolauroyl-dioleoyl-glycerol were formed from these two substrates in the presence of the modified lipase. The modified enzyme was extremely thermostable in its substrates. In the ester synthesis and ester exchange reactions, a trace amount of water was necessary for expression of the enzymic activity. It is suggested that the amphipathic polymer molecules retained water in close proximity to the enzyme.

Proteins can be modified by chemically binding synthetic or natural macromolecule to the surface of the molecule (1). This can counter some of the drawbacks of native proteins and improve their properties. Polyethylene glycol has been used to reduce immunogenicity of antigenic proteins or to suppress IgE production in response to allergenic proteins, with a possibility of application to new drugs (2).

Our interest also lies in making enzymes soluble and active in hydrophobic environments such as organic solvents. They would allow us to study enzymic reactions in hydrophobic environments. They would become indispensable tools for syntheses of complicated organic compounds in the biotechnical field.

Enzymes such as lipase, chymotrypsin, catalase and peroxidase have been made soluble and active in organic solvents such as benzene by covalently attaching an amphipathic molecule (polyethylene glycol, PEG) to the surface of enzymes. The hydrophilic nature of PEG makes it possible to modify enzymes in aqueous solution, and its hydrophobic nature enables modified enzymes to function in a hydrophobic environment.

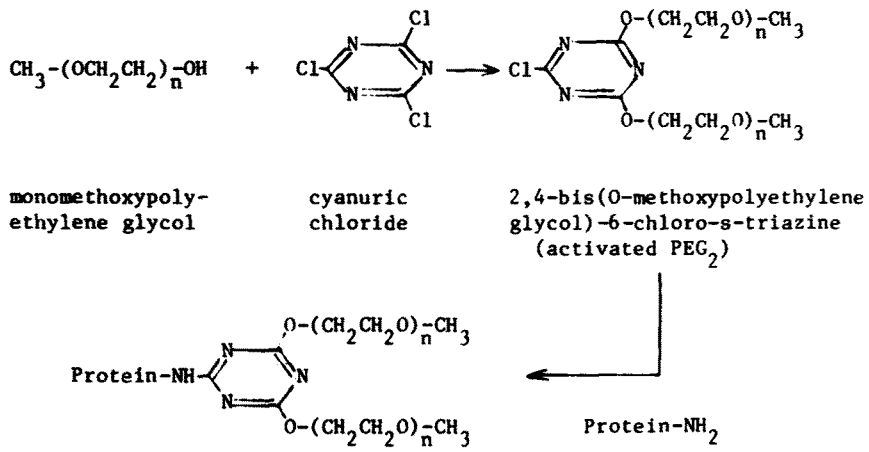

FIG. 1. Synthesis of organic solvent-soluble enzymes.

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TABLE 1

Solubility of Modified Catalase in Benzene

Degree of modification (%)	Solubility (mg/ml)
0	0.00
21	0.14
34	0.25
44	0.64
55	>2.00

SYNTHESIS OF CHEMICALLY MODIFIED ENZYMES

Chemically binding synthetic macromolecules to the surface of protein molecules can alter the proteins' physicochemical and biological properties without drastically changing their conformation. The method for preparing organic solvent-soluble enzymes is shown in Figure 1.

Monomethoxypolyethylene glycol (with a molecular weight of about 5000) and cyanuric chloride were reacted to synthesize 2,4-bis(0-methoxypolyethylene glycol)-6-chloro-s-triazine (activated PEG_2) (3): two of the three chlorine atoms in cyanuric chloride molecule were replaced with PEG-chains. Owing to the third chlorine atom remaining on the activated PEG_2, it can react with amino groups of N-terminal and/or lysine residues on the surface of enzyme molecules. Therefore, two PEG-chains can be attached to each amino group through the triazine ring. In general, the coupling reaction should be performed under mild conditions (at room temperature and neutral pH in an aqueous solution) to avoid denaturation of enzymes. The degree of modification can be controlled by changing the molar ratio of activated PEG_2 to protein in reaction systems.

Bovine liver catalase has a molecular weight of 248,000 and consists of four subunits, each containing a haem group. Dissociation of the molecule into its subunits causes complete loss of enzyme activity. Each subunit contains 28 amino groups and one haem. Catalase molecule was coupled with activated PEG_2 to produce modified catalase (4). The native enzyme was insoluble in benzene. It became increasingly soluble with the modification of amino groups with activated PEG_2 (Table 1). More than 2 mg of the modified catalase, in which 55% of the amino groups in the molecule were coupled with activated PEG_2, could be dissolved in 1 ml of benzene.

Lipases catalyze hydrolysis of highly hydrophobic triglycerides to release fatty acids. They also catalyze hydrolysis of water-insoluble esters of fatty acids and long-chain alcohols. Lipase from *Pseudomonas fluorescens* consists of two peptide chains linked by two disulfide bridges. It has a molecular weight of 33,000 and contains seven amino groups in each molecule. To prepare lipase that was soluble in organic solvent, about half of the seven amino groups in the molecule were coupled with activated PEG_2 (5). This PEG-modified lipase preparation had 80% of the original activity of native lipase measured in an emulsified aqueous system and did not contain native lipase as a contaminant. The modified lipase was readily soluble in organic solvents such as benzene, toluene, chloroform and 1,1,1-trichloroethane, and it catalyzed reactions in these organic solvents. Table 2 shows enzymic activities of PEG-modified enzymes in various organic solvents (6). The reactions occured in pure organic solvent, rather than in an emulsified system.

ESTER SYNTHETIC REACTIONS CATALYZED WITH POLYETHYLENE GLYCOL-MODIFIED LIPASE (PEG-LIPASE)

PEG-lipase from *Pseudomonas fluorescens* catalyzed the ester synthesis reaction in organic solvents. The rate of ester synthesis increased linearly with increasing concentration of the modified enzyme, indicating that the reaction was catalyzed in the pure benzene solution. Because the reactions were conducted in pure solvent system, it was possible to study kinetics for substrate specificity in benzene for ester synthesis reaction (Fig. 2, Table 3) (8). V_{max} was enhanced by increasing the carbon chain length of either fatty acid or alcohol, but Km was hardly affected (Km = 0.1–0.3 M). Fatty acids with a branched carbon chain at the position close to the carboxyl group, such as 2)methyl pentanoic acid (Ki = 0.22 and 0.27 M, respectively). Tertiary alcohols such as 1,1-dimethylpropyl alcohol were not able to serve as substrate of ester synthesis.

TABLE 2

Enzymic Activity of Polyethylene Glycol-modified Enzyme in Organic Solvents at 25 C

Solvent	Relative activity (%)[a]			
	Catalase (Ref. 4)	Chymotrypsin (Ref. 6)	Peroxidase (Ref. 7)	Lipase (Ref. 5)
Benzene	100	100	100	100
Toluene	38	—	—	87
Chloroform	90	—	—	67
1,1,1-Trichloroethane	128	152	—	359
Trichloroethylene	30	114	—	33
Perchloroethylene	33	30	—	33

[a]Activity of each enzyme in benzene was defined as 100%. Modified catalase, chymotrypsin, peroxidase and lipase catalyzed hydrogen peroxide decomposition, acid-amid bond formation, oxidation with hydrogen peroxide and ester synthesis.

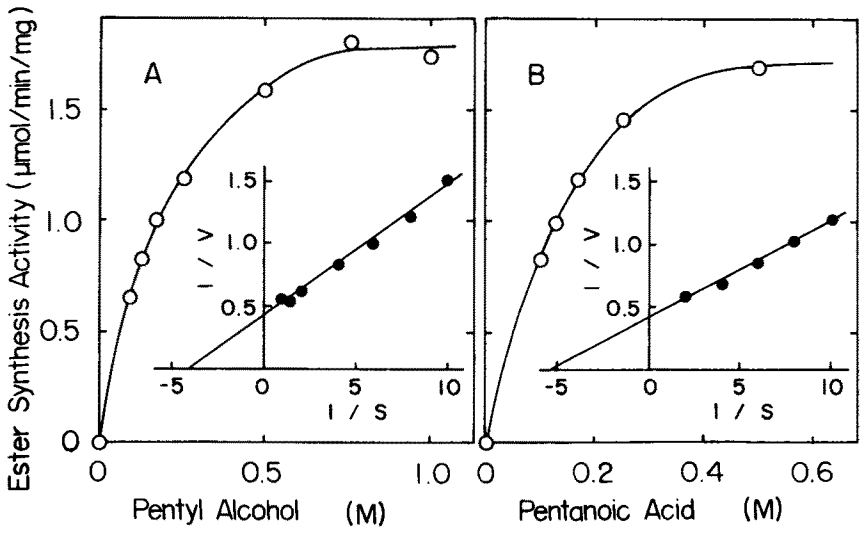

FIG. 2. Kinetic study of ester synthesis by PEG-lipase (*Pseudomonas fluorescens*) in benzene at 20 C when the concentrations of two substrates, pentyl alcohol (A) and pentanoic acid (B), were changed. Curves A and B were obtained at a fixed concentration of 0.5 M pentyl alcohol and 0.75 M pentyl alcohol, respectively.

TABLE 3

Substrate Specificity of Modified Lipase (*Pseudomonas fluorescens*) for Ester Synthesis in Benzene at 25 C

Substrate	K_m (M)	K_i (M)	V_{max} (μmol/min/mg protein)
A. Carboxylic acid (0.75 M pentyl alcohol)			
Pentanoic acid	0.17		2.8
Hexanoic acid	0.22		8.0
Octanoic acid	0.14		8.0
Dodecanoic acid	0.31		10.3
2-Methylpentanoic acid[a]	—	0.22	—
3-Methylpentanoic acid[a]	—	0.27	—
4-Methylpentanoic acid	0.19		0.2
Benzoic acid[a]	—	0.10	—
3-Phenylpropionic acid	0.18		7.0
4-Phenylbutyric acid	0.15		7.3
B. Alcohol (0.5 M pentanoic acid)			
Pentyl alcohol	0.23		2.6
Hexyl alcohol	0.25		2.2
Octyl alcohol	0.24		5.4
Dodecyl alcohol	0.33		8.0
2-Methylpentyl alcohol	0.21		2.5
3-Methylpentyl alcohol	0.62		2.0
4-Methylpentyl alcohol	0.19		1.0
1-Methylbutyl alcohol	0.31		0.1
1,1-Dimethylpropyl alcohol[b]	—	—	—

[a]These substances acted as inhibitors, but not substrates.

[b]This acted neither as an inhibitor nor as a substrate.

As mentioned above, PEG-lipase from *Pseudomonas fluorescens* did not catalyze ester synthesis when carboxylic acids had branching at 2- or 3-position. Such carboxylic acids were indeed competititve inhibitors for ester synthesis. Another lipase form, *Candida cylindracea*, could be chosen to synthesize ester from alcohol and 2- and 3-substituted carboxylic acids in benzene (9). We examined the case of synthesis of methyl benzoate from methyl alcohol and benzoic acid (2-substituted carboxylic acid), for which PEG-lipase (*Candida*) would have to be used instead of PEG-lipase (*Pseudomonas*). The results are shown in Figure 3. Curve A represents methyl benzoate synthesis in benzene with PEG-lipase (*Candida*). The amount of methyl benzoate was increased with reaction-time up to 30 hr and thereafter remained constant. The yield of product

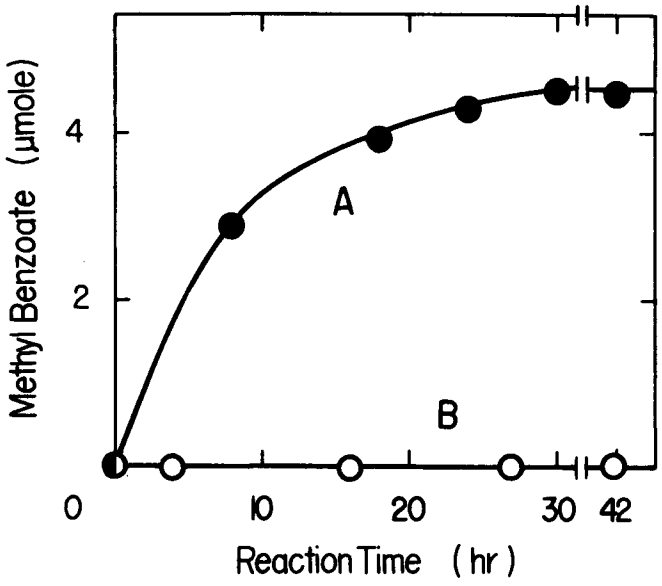

FIG. 3. Methyl benzoate synthesis in benzene (0.3 ml) with PEG-lipase at 25 C from methyl alcohol (0.05M) and benzoic acid (0.2 M). Curves A and B were obtained with PEG-lipase (*Candida*) and PEG-lipase (*Pseudomonas*), respectively. The concentrations of the enzymes were 0.45 mg/ml.

was approximately 30% based on methyl alcohol. No methyl benzoate synthesis took place with the other lipase, PEG-lipase (*Pseudomonas*) (Curve B). Similarly, methyl retinoate was synthesized in benzene from methyl alcohol and retinoic acid (2-substituted carboxylic acid) only with PEG-lipase (*Candida*).

A hydroxyl group-containing carboxylic acid, 10-hydroxydecanoic acid, was polymerized by ester bond formation with PEG-lipase (*Pseudomonas*) in a transparent benzene solution (10). It was linearly elongated under quite mild conditions (Table 4).

To further expand the experiment, ester synthesis was conducted with combinations of various kinds of terpene alcohols and carboxylic acids using PEG-lipase (11). The results are shown in Table 5. As is clear from the table, yields of esters synthesized except ones with acetic acid were very high, ranging from 52% to 95% after eight hr of incubation at 25 C. Even with terpenyl acetate, the yield ranged from 81% to 91% when the incubation time was prolonged to 48 hr. Even if substrates have many double bonds, the modified enzyme permitted the synthesis of esters without oxidation of the products.

TABLE 4

Polymerization of 10-Hydroxydecanoic Acid Catalyzed by PEG-lipase (*Pseudomonas fluorescens*) in Benzene

Reaction time (hr)	Relative amount (%)					
	Substrate $HOC_9H_{18}COOH$	Polymers $HO(C_9H_{18}COO)_nH$				
		n=2	n=3	n=4	n=5	n>5
0	100	0	0	0	0	0
0.33	84	6	5	5	0	0
3	39	32	15	4	1	9
44	0	18	21	13	8	40

The reaction mixture containing 22.5 mM 10-hydroxydecanoic acid and 0.1 mg/ml of the modified lipase was incubated at 25 C.

ESTER-EXCHANGE REACTIONS CATALYZED WITH POLYETHYLENE GLYCOL-MODIFIED LIPASE (*PSEUDOMONAS*)

The *Pseudomonas fluorescens* PEG-lipase catlayzes ester-exchange reactions between an ester and an alcohol, between an ester and an acid, and between two esters (13). These reactions occur not only in organic solvents, but also in straight hydrophobic substrates. This suggests that the modified lipase is useful for many practical applications. One of them is the reformation of fat to oil (14). PEG-lipase efficiently catalyzed ester-exchnage reaction between trilaurin and triolein without any solvent. As seen in Table 6, amounts of two substrates, trilaurin and triolein, were reduced by increasing the reaction time, while those of two products, dilauroyl-monooleoyl-glycerol and monolauroyl-dioleoyl-glycerol, were increased with time. At 65 hr incubation, approximately 30% of the substrates were transformed to products. The melting temperature of the reaction mixture was lowered from 33–36 C to 11–13 C, due to fatty acid-exchange between two triglycerides. Similar ester-exchange reactions took place between fat and oil comprised of triglycerides, accompanied by the decrease in the melting temperature of the reaction mixture.

Vitamin A is stored in the liver as retinyl palmitate. Retinyl esters are more stable than vitamin A such as retinol, retinal and retinoic acid. Retinyl esters

TABLE 5

Synthesis of Ester from Terpene Alcohol and Carboxylic Acid by PEG-lipase (*Pseudomonas fragi* 22.39B) in Benzene

Terpene alcohol	Yield (%)			
	Acetic acid	Propionic acid	n-Butyric acid	Valeric acid
Citronellol	18(81)	52	88	74
Geraniol	19(84)	81	94	83
Farnesol	19(82)	78	90	81
Phytol	29(91)	86	95	92

Yields were measured after eight hr-incubation, but ones in parentheses were after 48 hr. All concentrations of alcohols and carboxylic acids were 100 μM, and PEG-lipase was used at 0.33 mg/ml.

TABLE 6

Triglyceride Composition and Melting Temperature of the Mixture of Trilaurin and Triolein During the Incubation with Modified Lipase (*Pseudomonas fluorescens*) 58 C

Incubation time (hr)	Melting temp. (°C)	Triglyceride-composition* (μmol)			
		LLL	LLO	LOO	OOO
0	33-36	180	0	0	154
17	23-26	94	33	28	94
40	12-16	89	42	37	87
65	11-13	79	58	44	71

*, LLL, trilaurin (trilauroyl-glycerol); LLO; dilauroyl-monooleoyl-glycerol; LOO, monolauroyl-dioleoyl-glycerol; OOO, triolein (trioleoyl-glycerol).

with long chain fatty acids produced by organic synthesis, which usually includes refluxing at high temperature, however, have high peroxide value and are very unstable (15). The merit of using PEG-lipase is that the synthesis proceeds under a quite mild condition. When substrates have double bonds and are unstable, the synthesis with PEG-lipase is much more reliable than a conventional organic synthesis. We have succeeded in synthesis of retinyl palmitate and retinyl oleate with small peroxide value by the ester-exchange between acetate and palmitic acid or oleic acid, using PEG-lipase in benzene with good yields (Fig. 4). As shown in Table 7, enzymically synthesized retinyl palmitate (yield being about 85%) had lower POV than the one obtained by the conventional organic synthesis (yield being about 30%) regardless of the presence of air; 2.5 or 4.1 vs 43 meq/kg. When we used retinyl acetate and oleic acid, which also has one double bond, the same clear difference was observed. POVs of products obtained by the enzymic synthesis were as small as 5%-10% of those obtained by organic syntheses, though the same batch of intitial substrates was used.

MAGNETIC POLYETHYLENE GLYCOL-MODIFIED LIPASE (MAGNETIC LIPASE)

In biotechnology, removing enzymes from reaction mixture often is critically important. The idea of immobilizing enzymes on various gels and solid surfaces was introduced for this purpose. However, efficiency of catalysts usually is reduced by immobilization. One of the most useful and attractive alternatives for this approach is to make use of magnetic force.

As discussed above, we made lipase soluble and active in organic solvents such as benzene by covalently attaching an amphipathic macromolecule, PEG, to the surface of the enzyme molecules. PEG-modified lipase was further endowed with magnetic property. Magnetic lipase was prepared by coupling of PEG-modified lipase with magnetite formed by reacting with Fe^{+2} and Fe^{+3} ions at pH 8.0-8.5 and at 25 C, according to the following equation (16):

$$FE^{+2} + Fe^{+3} + \text{PEG-Lipase} \partial \text{Magnetite-PEG-Lipase.}$$

PEG-lipase was not released from magnetite by extensively dialyzing against water, indicating that PEG-lipase is very tightly bound to magnetite particles. Weight percentages of magnetite (Fe^3O^4), polyethylene glycol and protein in magnetic lipase were 31%, 44% and 25%, respectively. Magnetic lipase was dispersed into not only aqueous solution but also organic solvents such as benzene, indicating that the amphipathic polyethylene glycol chain was exposed on the surface. It made a very stable colloid in benzene; no precipitate was formed even after centrifugation at 4500 × g for 15 min. The average particle size of the magnetic lipase was 120 ± 60 nm.

Magnetic lipase catalyzed ester synthesis reactions, the reverse reaction of hydrolysis, in organic solvents such as benzene and 1,1,1-trichloroethane. The synthesis of lauryl laurate from lauryl alcohol (0.45 M) and lauric acid (0.45 M) proceeded in benzene, and its enzymic activity was 1.2 μmol/min/mg protein. Even higher activity was observed in 1,1,1-trichloroethane (17).

Magnetic lipase, which was dispersed and exerted enzymic activity in benzene, was completely recovered

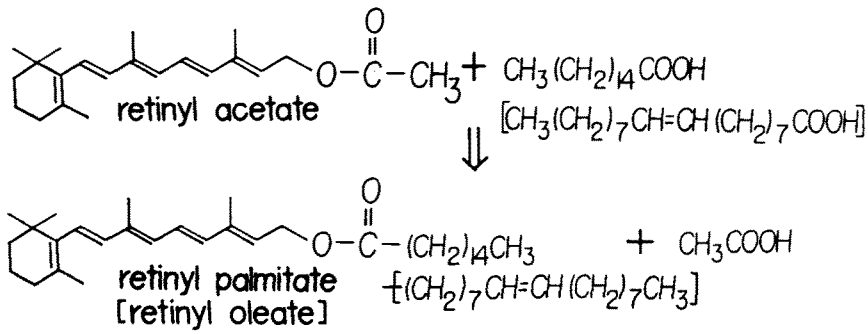

FIG. 4. Ester-exchange between retinyl acetate and palmitic acid (or oleic acid).

TABLE 7

Peroxide Value (POV) of Retinyl Esters Synthesized with PEG-lipase (*Pseudomonas fluorescens*) in Benzene at 25 C

Method		POV (meq/kg)	
		Retinyl palmitate	Retinyl oleate
Enzymic synthesis	under N_2 gas	2.5 ± 0.3	9.0 ± 0.4
	under air	4.1 ± 0.3	11 ± 0.1
Organic synthesis	under N_2 gas	43 ± 4.3	200 ± 16

TABLE 8

Magnetic Separation of Magnetic Lipase from Benzene

Solution	Activity of enzyme (μmol/min/ml)
Colloidal solution of magnetic lipase	2.35
Residual solution after magnetic separation	0.01
Magnetic lipase recovered by magnetic separation	2.36

The magnetic separation was carried out at the magnetic field of 6000 Oe for five min in benzene (1 ml) containing 7.9 mg of the magnetic lipase. The enzymic activity for ester synthesis was determined using lauryl alcohol (0.45 M) and lauric acid (0.45 M).

in five min in a magnetic field of 6000 Oe (Table 8). The ester synthesis activity was not detected in benzene after the magnetic separation. Recovered magnetic lipase was dispersed again in benzene without any loss of enzymic activity.

We can use this kind of magnetic enzymes as effective catalysts in organic solvents as well as aqueous solution and allow magnetic separation of enzymes from the reaction mixture. This approach will open a new avenue for the development of biochemistry and biotechnology.

FUTURE PROSPECTS

Modified lipase exhibited good storage stability in benzene: after storage for three months, modified lipase retained about 50% of the initial activity for ester synthesis (amyl laurate) and, after 140 days, about 40% of the intial activity (18). Although this review describes mainly lipase, we can consider modifying chemically various biological materials, e.g. enzymes, proteins, drugs and biologically active substances with low molecular weight (19). This broad application of the modification concept may have an important contribution to the fields of medicine, pharmacy, technology and agriculture.

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