Lipase Made Active in Hydrophobic Media by Coupling with Polyethylene Glycol[1]

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Lipases from various microorganisms were chemically modified with polyethylene glycol derivatives: 2,4-*bis*[*O*-methoxypoly(ethylene glycol)]-6-chloro-*s*-triazine (activated PEG_2, a chain-shaped polymer) and copolymer of polyoxyethylene allyl methyl diether and maleic anhydride (activated PM, a comb-shaped polymer). Because each polymer is amphipathic, the modified lipases become soluble not only in aqueous solution but also in hydrophobic media. They exhibit potent catalytic actions for ester synthesis and ester exchange reactions, the reverse reaction of hydrolysis, in transparent organic solvents and also in oily substrates without organic solvents. With PEG_2-lipases, macrocyclic lactone and gefarnate (geranyl farnesylacetate) were synthesized in high yields from 16-hydroxy-hexadecanoic acid ethyl ester and from farnesylacetic acid and geraniol in organic solvents, respectively. The modified lipase catalyzed the esterification preferentially with the *(R)*-isomer of secondary alcohols. Because the ester synthesis reactions with modified lipase proceeded in the transparent benzene system, the kinetic parameters (Michaelis constant and maximum velocity) were obtained by reciprocal plotting according to the Michaelis equation. With comb-shaped polymer as modifier, PM-lipase catalyzed effectively the reverse reaction of hydrolysis in organic solvents. The properties of each modified lipase are discussed in relation to those of the nonmodified lipase.

KEY WORDS: Activated PEG_2, activated PM, esterification, heat-stability, lactonization, lipase, optical resolution, organic solvents, polyethylene glycol.

Chemical modification of proteins with polyethylene glycol (PEG) has been extensively studied for the purpose of applying the proteins to biomedical and biotechnological processes. Advantages of PEG-protein drugs are reduction of immunoreactivity, prolongation of clearance time and high stability (1). In fact, PEG-adenosine deaminase is the first PEG-protein drug that was approved by the Food and Drug Administration as an orphan drug. Since 1984, we have been able to solubilize various kinds of enzymes and to make them active in hydrophobic organic solvents (2). The key to this success is the chemical modification of enzymes with amphipathic PEG derivatives. The activated modifiers can be attached to enzymes in aqueous buffer solution, and once modified, the enzymes become soluble and active in various organic solvents, such as benzene, toluene and chlorinated hydrocarbons.

We present the recent findings concerning PEG-lipase, since our research work with PEG-enzymes, done in 1984–1987, has been published already in this (3) and other (4, 5) journals.

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PEG DERIVATIVES (PEG_2 AND PM)

PEG (mw: 5000) is activated by various schemes to couple with side chains of a protein, such as the amino group, carboxylic acid and sulfhydryl group. Figure 1 shows the structure of activated PEG_2 [2,4-*bis*(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine]. This compound reacts directly with ε-amino groups of lysine residues and/or *N*-terminal amino groups in a protein molecule under mild conditions (at low temperature, neutral pH and in aqueous solution). Activated PEG_2 is more effective than activated PEG_1 [2-(*O*-methoxypolyethylene glycol)-4,6-dichloro-*s*-triazine] because two PEG chains react with one amino group in the protein molecule. Recently, we synthesized activated PEG_2 in a homogeneous state with zinc oxide as catalyst (6). Figure 2 shows gel filtration chromatograms during the course of activated PEG_2

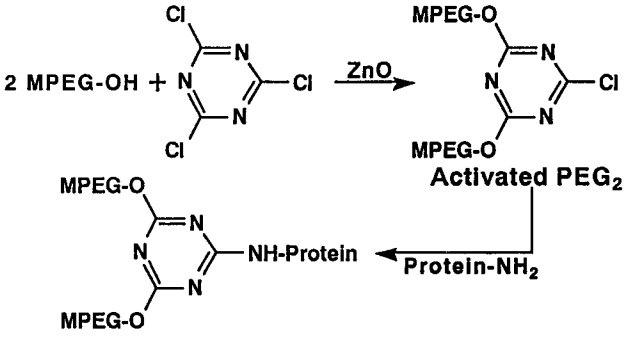


FIG. 1. Synthesis of activated 2,4-*bis*(*O*-methoxy polyethylene glycol)-6-chloro-*s*-triazine (PEG_2) and the modification of protein with the product. MPEG represents methoxypolyethylene glycol, CH_3-$(OC_2H_4)_n$-.

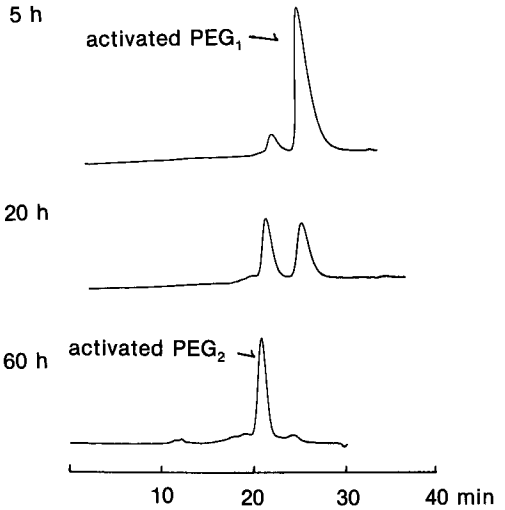
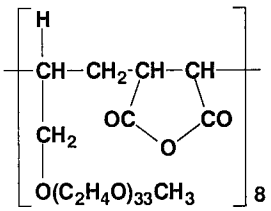


FIG. 2. Gel filtration high-performance liquid chromatography analysis during the course of activated PEG_2 synthesis with zinc oxide as catalyst. See Figure 1 for abbreviation. PEG_1, 2-(*O*-methoxypolyethylene glycol)-4,6-chichloro-*s*-triazine.



SCHEME 1

synthesis. At the first stage of the reaction, mainly activated PEG_1 is formed. After a 60-h reaction, activated PEG_2 with a molecular weight of 10,000 was obtained without contamination of activated PEG_1 and its polymerized forms.

A new PEG derivative with comb-shaped form was recently synthesized by us: Chemical structure of activated PM (mw: 13,000) (Scheme 1). The modifier, activated PM, is a copolymer of polyethylene allyl methyl diether and maleic anhydride (7). Activated PM couples with amino groups in a protein molecule at pHs ranging from 7 to 10 and at temperatures from 4 to 40°C. Carboxyl groups formed by the hydrolysis of the modifier after coupling with proteins do not serve as a substrate for PM-lipase in organic solvents.

PREPARATION OF MODIFIED LIPASE (PEG_2- AND PM-LIPASE)

Lipase from *Pseudomonas fragi* (8) or *P. cepacia* (9) was dissolved in borate buffer (pH 9.5), and then activated PEG_2 was added stepwise. The reaction proceeded for 5 h at 25°C. During the reaction, the degree of modification of amino groups increased gradually. After the reaction was completed, the mixture was ultrafiltrated against borate buffer (pH 8) and dialyzed against water. PEG_2-lipase was obtained after lyophilization. To lipase from *P. fluorescens* dissolved in 0.5 M borate buffer, pH 8.5, activated PM was added, and the mixture was incubated for 1 h at 4°C. Then, PM-lipase was obtained (10) after ultrafiltration, dialysis and lyophilization. The olive oil hydrolytic activity and lauryl stearate synthetic activity were measured by the method of Yamada *et al.* (11) and Inada *et al.* (12), respectively. Protein concentration and the degree of modification were determined by the biuret method and trinitrobenzenesulfonate method, respectively.

ESTER SYNTHESIS WITH PM-LIPASE

Figure 3 shows the chemical modification of lipase with activated PM. Increasing the molar ratio of activated PM to amino groups in lipase increased the degree of modification, which tended to reach a constant level of about 60%. PM-lipase, as well as PEG_2-lipase, is soluble and active in organic solvents, e.g., lauryl stearate was synthesized in benzene. The ester synthesis activity is increased by increasing the degree of modification, although the activity is not observed with nonmodified lipase. Additionally, the esterase activity of PM-lipase in an emulsified system is 1.3 times higher than that of nonmodified lipase.

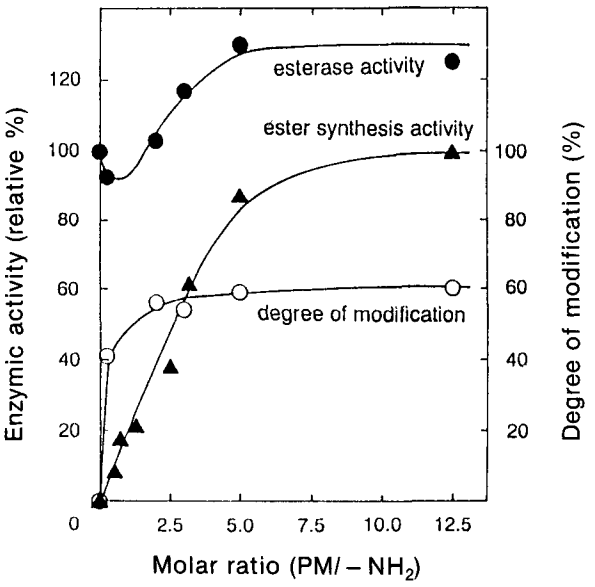


FIG. 3. Degree of modification together with esterase activity in an emulsified system and ester synthesis activity in benzene. The horizontal axis shows the molar ratio of activated copolymer of polyoxyethylene allyl methyl diether and maleic anhydride (PM) to an amino group in the lipase molecule. The coupling reaction was performed at 4°C for 1 h.

LACTONE SYNTHESIS WITH PEG_2-LIPASE

Lactone is a component of fragrances and macrolide antibiotics. PEG_2-lipase from *P. cepacia* catalyzed lactone synthesis from 16-hydroxyhexadecanoic acid ethyl ester in 1,1,1-trichloroethane (9). The optimum temperature for the lactone yield is 65–70°C. Because lactone synthesis occurs by intramolecular transesterification, the efficiency of the reaction depends on the substrate concentration. At 1 mM concentration, 92% of the substrate was converted into the lactone (Fig. 4). As the substrate concentration was increased, the yield of lactone was gradually

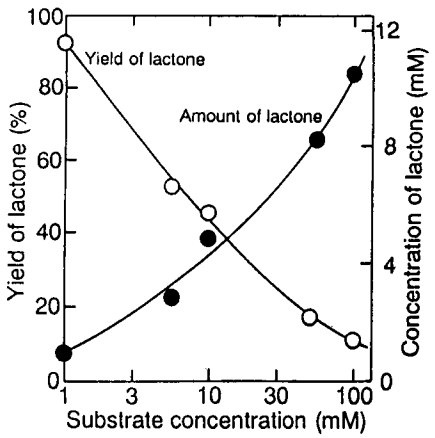


FIG. 4. Effect of substrate concentration on lactone synthesis. The reaction mixture contained 16-hydroxyhexadecanoic acid ethyl ester and PEG_2-lipase (1 mg protein/mL) in 1,1,1-trichloroethane and was incubated for 24 h at 65°C. See Figure 1 for abbreviation.

reduced, while the concentration of lactone synthesized was markedly increased. Lactone concentrations were 4.8 mM (48% yield) and 11 mM (11% yield) when 10 and 100 mM substrate were used, respectively.

GEFARNATE SYNTHESIS WITH PEG_2-LIPASE

Gefarnate (geranyl farnesylacetate) is used as an antipeptic drug. We tried to synthesize gefarnate from farnesylacetic acid and geraniol in 1,1,1-trichloroethane with PEG_2-lipase from *P. fragi* (8). The ester synthesis reaction proceeds effectively in transparent 1,1,1-trichloroethane at 25°C, and the yield of gefarnate was approximately 87% at 1 mg/mL PEG_2-lipase after a 50-h incubation (Fig. 5). By using PEG_2-lipase at higher concentrations (2.5 and 5.0 mg/mL), the same yields were obtained with only 25- and 10-h incubations, respectively. The PEG_2-lipase can be reused more than three times without any loss of enzymic activity by recovering the PEG_2-lipase as a precipitate from the reaction mixture after adding hexane.

OPTICAL RESOLUTION WITH PEG_2-LIPASE

PEG_2-lipase from *P. fragi* can recognize the chirality of alcohols (13). Figure 6 shows the kinetic study of ester synthesis with *(R)*- or *(S)*-2-octanol. The left panel shows that increasing the substrate concentration of chiral alcohol increased the rate of ester synthesis. But the reaction rates for *(S)*-isomer were markedly low. As the reaction proceeded in transparent benzene solution, the Michaelis constant (K_m) and maximum velocity (V_{max}) values were obtained by plotting 1/V against 1/S according to the Michaelis equation.

Table 1 shows the K_m and V_{max} values of chiral secondary alcohols. In the case of *(R)*-isomer, K_m and V_{max} values are hardly affected by changing the substrates. On the contrary, increasing the carbon chainlength of the *(S)*-isomer increased K_m values and decreased V_{max} values. PEG_2-lipase catalyzes esterification preferentially with *(R)*-isomer of secondary alcohols. *(S)*-Isomers with bulky side chains are not suitable substrate for the PEG_2-lipase.

So, we tried an optical resolution of racemic α-phenylethanol by esterification catalyzed with PEG_2-lipase. Figure 7 shows the time-course of α-phenylethyl dodecanoate synthesis from *(RS)*-α-phenylethanol and dodecanoic acid. The amount of the substrate, α-phenylethanol, decreased with time and tended to approach a constant level of 50%. On the contrary, the amount of the product, α-phenylethyl dodecanoate, which is only *(R)*-isomer, increased with time and approached 50%. The optical purity of nonreacted alcohol, *(S)*-α-phenylethanol, increased and reached 99% enantiomeric excess in 7 h. These results indicate that PEG_2-modified *P. fragi* lipase has the potential to discriminate *(R)*-isomer of α-phenylethanol from *(S)*-isomer and to synthesize *(R)*-α-phenylethyl dodecanoate.

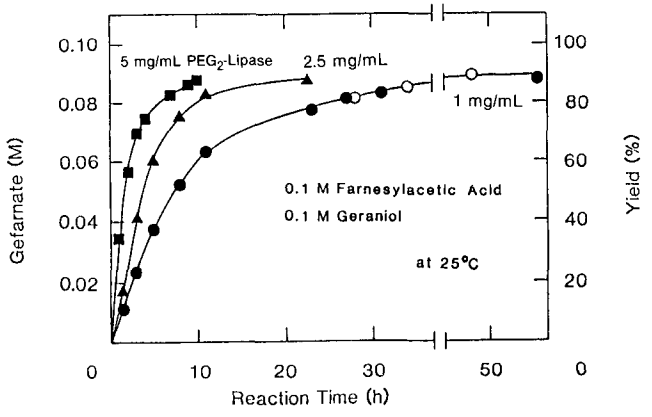


FIG. 5. Gefarnate synthesis from farnesylacetate and geraniol with PEG_2-lipase in 1,1,1-trichloroethane at 25°C. Closed circle: Fresh PEG_2-lipase. Open circle: PEG_2-lipase recovered three times. See Figure 1 for abbreviation.

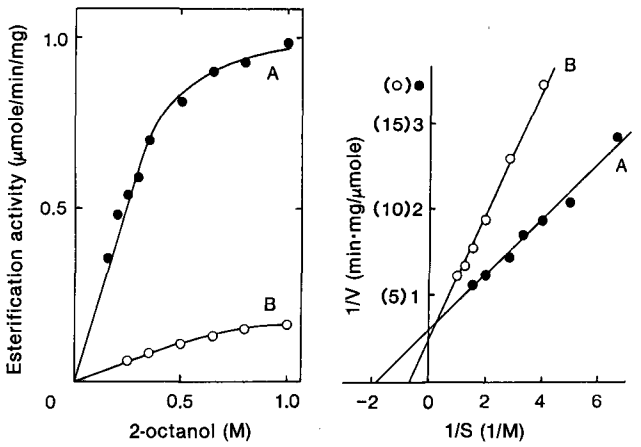


FIG. 6. Kinetics of the esterification of *(R)*- or *(S)*-2-octanol with dodecanoic acid by PEG_2-lipase in benzene at 25°C. Left panel: Plot of esterification activity against various concentrations of 2-octanol. Right panel: Reciprocal plot of reaction rate (1/V) against reciprocal of substrate concentration (1/S). Curves A and B: *(R)*- and *(S)*-2-octanol, respectively. See Figure 1 for abbreviation, PEG, polyethylene glycol.

HEAT-STABILITY OF PM-LIPASE

We expected that lipase from *P. fluorescens* modified with activated PM may have high stability toward heat owing to the unique structure of activated PM. Figure 8 shows the heat stability of PM-lipase (10). The PM-lipase, as well as nonmodified lipase, were incubated at 55°C in aqueous solution. While the hydrolytic activity of nonmodified lipase is completely lost after 150 min of incubation, that

TABLE 1

K_m and V_{max} Values of Esterification of Secondary Alcohols with 0.5 M Dodecanoic Acid in Benzene by PEG_2-Lipase

	K_m (M)		V_{max} (μmol/min/mg)	
Substrate	*(R)*	*(S)*	*(R)*	*(S)*
2-Butanol	0.43	0.43	1.18	1.23
2-Pentanol	0.43	1.34	1.25	0.45
2-Octanol	0.54	1.50	1.68	0.42
2-Nonanol	0.50	1.66	1.37	0.30
α-Phenylethanol	0.47	—[a]	0.90	—[a]

[a]Did not serve as substrate. Abbreviations: K_m, Michaelis constant; V_{max}, maximum velocity; PEG_2, 2,4-*bis*(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine.

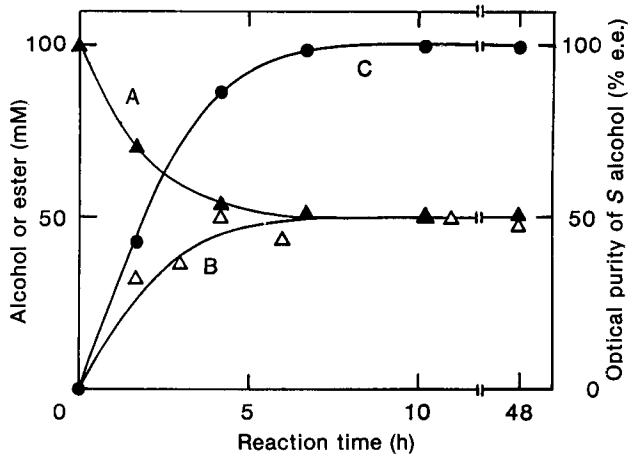


FIG. 7. Time-course of the optical resolution of α-phenylethanol by the esterification catalyzed with PEG_2-lipase. *(RS)*-α-Phenylethanol, dodecanoic acid and PEG_2-lipase were incubated in 1,1,1-trichloroethane at 25°C. Curves A and B: Concentrations of *(RS)*-α-phenylethanol and *(R)*-α-phenylethyl dodecanoate, respectively. Curve C: Optical purity of nonreacted alcohol, *(S)*-α-phenylethanol. e.e., Enantiomeric excess, other abbreviation as in Figure 1.

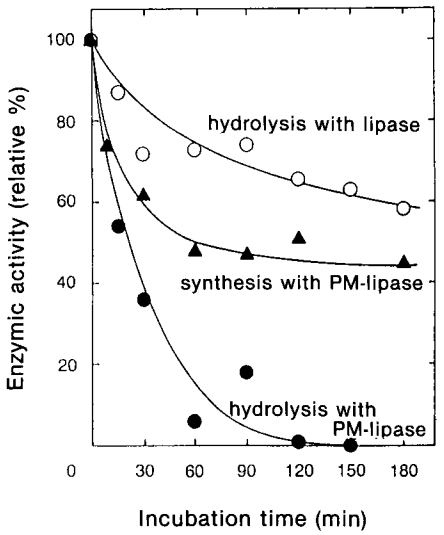


FIG. 8. Heat-stability of PM-lipase in which 60°C of the total amino groups in the molecule were modified. Enzyme solution was incubated at 55°C. See Figure 3 for abbreviation.

of PM-lipase is retained by 60%. Similarly, 50% of the ester synthesis activity of PM-lipase is retained in benzene. From these results it may be concluded that activated PM may serve to stabilize the conformation of the lipase molecule. This seems to be due to the interactions between the modifier and the surface of the lipase molecule, probably on account of acid-amide bonds and hydrogen or hydrophobic bonds. Now we are exploring various sizes of activated PMs, changing both the length of the PEG-chain and the degree of polymerization. We hope that various enzymes can be stabilized by coupling with activated PMs in the future.

Enzymes modified with PEG derivatives exhibit the following advantages in biotechnological processes: (i) The modified enzyme becomes soluble and active in hydrophobic media, so that the reverse reaction of hydrolysis proceeds effectively; (ii) hydrophobic materials can serve as substrates; (iii) unstable compounds can be synthesized as the enzymic reaction proceeds under mild conditions; (iv) PEG-lipase catalyzes stereospecific reactions in organic solvents; (v) PEG-lipase is highly heat-stable in aqueous solution and in benzene; (vi) PEG-lipase can be recovered from the reaction mixture as a precipitate without any loss of activity by adding hexane; and (vii) PEG-lipase conjugated with magnetite can be recovered easily by magnetic force (14).

This technique may lead to new biotechnological and biomedical processes because the number of reports concerning the chemical modification of proteins and enzymes with PEG are increasing year by year.

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