Polyethylene Glycol Modification of *Candida rugosa* Lipase

Mary Welch Baillargeon* and **Philip E. Sonnet**
Eastern Regional Research Center, ARS/USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118

Polyethylene glycol (PEG) was covalently attached to lipase (EC 3.1.1.3) from *Candida rugosa* yielding a modified lipase of higher specific activity in hydrolytic and synthetic reactions in organic solvents. PEG of molecular weights 5000 and 1900 solubilized the lipase in selected organic solvents, but PEG of molecular weight 750 was too small to accomplish this completely. The modified lipase was 10 times more stable in water than native lipase, but was less stable in benzene. The selectivity of the modified lipase was also altered to favor reaction with oleic versus stearic acid.

Covalent attachment of polyethylene glycol (PEG) to free amino groups in proteins, as first described by Abuchowski et al. (1), causes some unique changes. PEG-modified enzymes have little or no immunogenicity and have extended circulating lifetimes in blood (2). Derivatized enzymes are soluble in water or in selected organic solvents due to the amphipathic nature of PEG. Inada et al. (3) demonstrate catalytic activity in organic solvents using a variety of PEG- derivatized enzymes, including lipoprotein lipase. Concurrent with our efforts, they have reported the PEG-derivatization of lipase from *Candida rugosa* (formerly *C. cylindracea*) by means of a carboxymethylated PEG (4,5). Unfortunately, the control experiment (reaction of *C. rugosa* lipase itself) is absent from their report; criticism backed by definitive experimental results has been offered by Klibanov (6).

PEG-derivatized lipase may be used to study kinetics in homogeneous organic solutions rather than in emulsions. Derivatization also may lead to the development of catalysts with altered properties [such as substrate selectivities, as recently reported for thermolysin (7)]. We report here the preparation of PEG-derivatized *C. rugosa* lipase using a PEG-triazine reagent. The effects of PEG chain length are noted, and a direct comparison of the activity, stability and selectivity of native with derivatized material is made.

EXPERIMENTAL

Materials. Lipase M from *C. rugosa*, lots K12484 and K15505, 30 units/mg, was a gift from Enzyme Development Corporation. Poly(ethylene glycol mono-methyl ether) of average mol wt 5000, 1900 and 750 was obtained from Polysciences, Inc. Cyanuric chloride was obtained from Aldrich Chemical Co.

Protein assays. Protein was measured in duplicate by the biuret method (8) using bovine serum albumin as standard. The biuret method was used because the presence of PEG does not interfere with the assay (1). To measure the modified protein only, PEG-lipase was dissolved in benzene, centrifuged, and the supernatant evaporated under N_2. The residue was redissolved in water and analyzed. Free amino groups were measured in triplicate by the trinitrobenzenesulfonic acid procedure of Habeeb (9).

Preparation of PEG-lipase. PEG was activated with cyanuric chloride according to the method of Nishimura et al. (10). After the appropriate reaction time, the mixture was brought to pH 6, concentrated by ultrafiltration, dialyzed against water and lyophilized. (Lipase covalently modified with activated polyethylene glycol is termed PEG-lipase. The average mol wt of the PEG used is indicated by subscripts, e.g. PEG_{5000}-lipase. "Activated PEG" is 2,4-bis[ω-methoxypoly-(ethyleneoxy)]-4,6-dichloro-*s*-triazine, $(PEG)_2Cl$, or 2-[ω-methoxypoly-(ethyleneoxy)]-4,6-dichloro-*s*-triazine, $(PEG)Cl_2$.)

Polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed under nondenaturing conditions according to Davis (11) on 5% acrylamide mini slab gels. PEG-lipase activity in samples dissolved as described above was detected as esterase activity in gels soaked in 0.03% α-naphthyl acetate, 0.05% Fast Blue RR salt, and 1% acetone in 0.1 M Tris, pH 7.4. The gel was incubated in this solution at room temperature with gentle agitation until activity bands were visible. Gels were scanned at 465 nm with a Shimadzu model CS-930 densitometer.

Lipase assay. Lipase activity was measured at room temperature by a modification of the initial rate assay of Parry et al. (12) using an unbuffered olive oil emulsion at pH 7.3 after purification and sonication according to the procedures of Linfield et al. (13,14). The rate of free fatty acid release was followed by addition of 0.0200 or 0.100 N NaOH under nitrogen using a Radiometer titration system in pH stat mode. Enzyme-free blanks showed no uptake of base.

Stability. The lipase solution was incubated in water or benzene at 45 C. Remaining lipolytic activity was measured by the olive oil assay. The half-life ($t_{1/2}$) was calculated from a first order plot obtained by linear regression.

Hydrolytic and synthetic reactions. Lipase-catalyzed hydrolysis in various solvents was studied by stirring 0.50 g olive oil, five ml solvent and 10 to 100 mg enzyme at room temperature for one hr. Esterification was measured by reacting lipase with one mmol each of lauric acid and 1-octanol in one ml water-saturated solvent for 16 hr at 30 C in a shaking water bath. In all cases, the release or uptake of free fatty acid was measured by automatic titration to pH 9.5.

Selectivity. The selectivity of lipase for oleic vs stearic acid was measured by esterifying one molar equivalent of each acid (0.091 M) with two equivalents of alcohol (0.182 M methanol or 1-octanol) at 30 C. The percent conversion to esters was determined by free fatty acid titration, and the ratio of the two esters formed was determined by gas chromatography at 240 C (methyl esters) or 280 C (octyl esters) using a fused silica SPB-1 capillary column (0.25 mm i.d. × 30 m). The ratio of the specificity constants, (V_{max}/K_m), for oleic and stearic acids, where V_{max} is the maximal velocity and K_m is the Michaelis constant, was calculated by the method of

*To whom correspondence should be addressed.

Chen et al. (15). Although originally derived to study relative rates during resolution of racemates, the method was equally useful here for identifying relative selectivity for substrates undergoing competitive enzymatic catalysis. Recently, similar competitive esterification reactions in organic solution were used successfully to study the mechanism of crude lipases from *Candida rugosa* and *Mucor meihei* and to predict kinetic separations and resolutions by the lipase (16).

RESULTS AND DISCUSSION

Preparation and characterization of PEG-lipase. C. rugosa lipase loses activity under the reaction conditions (37 C, pH 10.0 for 1 hr) used by Inada et al. (3) for lipoprotein lipase modification. In order to limit exposure of the *C. rugosa* lipase to basic conditions, the amount of time between dissolution and reaction is minimized, and reaction time is decreased. Twenty min reaction at room temperature at pH 7.5 results in a higher recovery of activity than longer reactions at pH 8.0. After an immediate pH change upon mixing of enzyme and activated PEG, little additional change occurs, indicating a rapid reaction.

Representative batches of PEG-lipase are compared with native lipase in Table 1. A tenfold molar excess of activated PEG to free amino groups was used initially in the derivation reactions, calculated using 35 free amino groups and a mol wt of 125,000/1000 amino acid residues (17). This results in a PEG_{5000}-lipase that rapidly dissolves in organic solvents such as benzene, toluene, chloroform and trichloroethane. However, PEG_{1900}-lipase made in this fashion only partly dissolves in the organic solvents, while PEG_{750}-lipase is apparently insoluble, judged by the presence of a benzene-insoluble fraction and by the inability of the benzene-soluble fraction to catalyze synthetic reactions. Increasing the molar excess of activated PEG, and thus the percent amino groups reacted, increases the solubility of the PEG_{1900}- and PEG_{750}-lipases.

The solubilities of PEG_{5000}- and PEG_{1900}-lipases in benzene are the same, within experimental error, as their solubilities in water. However, the solubility of PEG_{750}-lipase is ca. 50% lower in benzene than in water in spite of the excess of PEG used in its preparation (Table 1). It appears that PEG_{750} is too small in size compared to the lipase to completely solubilize it in benzene. The high protein content (6.4%) of PEG_{750}-lipase results from the lower mol wt of activated $(PEG_{750})_2Cl$ compared to $(PEG_{1900})_2Cl$ or $(PEG_{5000})_2Cl$ and, therefore, its more efficient removal by dialysis.

The activity of PEG-lipase decreases directly with increasing percent modification (Table 1). PEG-lipase activity and extent of modification is dependent upon pH. A momentary pH rise to 8.2 resulted in a PEG-lipase preparation with 71% modification (compared to 36% at pH 7.5) and reduced recovery and specific activity (Table 1).

The recovery of lipase activity in PEG-lipase ranges from 4 to 74%. A single experiment with $(PEG_{5000})Cl_2$ resulted in 4% recovery of activity; use of this activated PEG was discontinued.

Electrophoresis. PAGE electrophoresis with esterase-specific stain allows the modified lipase to be visualized. Figure 1 shows a gel scan of crude native lipase and PEG_{5000}-lipases of 36 and 71% modification. Both PEG-lipases show a decreased mobility compared to native lipase, as expected for higher mol wt species, with the 71% modified PEG-lipase showing the least mobility. Gels of PEG_{1900}- and PEG_{750}-lipase also show decreased mobility compared to native controls (data not shown).

Because of the heterogeneous character of both the activated PEG and the crude enzyme, broad bands of active lipase are observed. While the native lipase has two main peaks of activity, the 36% modified PEG-lipase shows one peak and a very broad smear, and the 71% modified species shows only a smear of activity near the top of the gel. Electrophoresis of benzene extracts of native lipase or native lipase with added PEG_{5000} shows no bands after staining. This indicates

TABLE 1

Characterization of PEG-Lipase

Lipase	Molar excess PEG	Modification (%)	M_r[a] ($\times 10^{-5}$)	Protein (%)		Activity (%)	
				Water	Benzene	Recovered	Relative specific
Native	-	0	1.2	12.4	0	100[d]	100[e]
$(PEG_{5000})_2Cl$	10	36	2.5	2.1	1.3	53	42
	10	71[b]	3.7	3.2	2.4	26	12
	50	67	3.6	0.5	1.1	22	29
$(PEG_{5000})Cl_2$	15	40	1.9	2.3	n.d[c]	4	11
$(PEG_{1900})_2Cl$	52	53	1.9	1.6	1.0	74	42
$(PEG_{750})_2Cl$	53	77	1.6	6.4	2.9	44	11

[a]Calculated mol wt.

[b]pH momentarily raised from 7.5 to 8.2.

[c]Not determined.

[d]100% is the total units of native lipase used per preparation.

[e]100% is based on the specific activity of native lipase carried through the PEG-derivatization procedure, which resulted in a 3-fold purification.

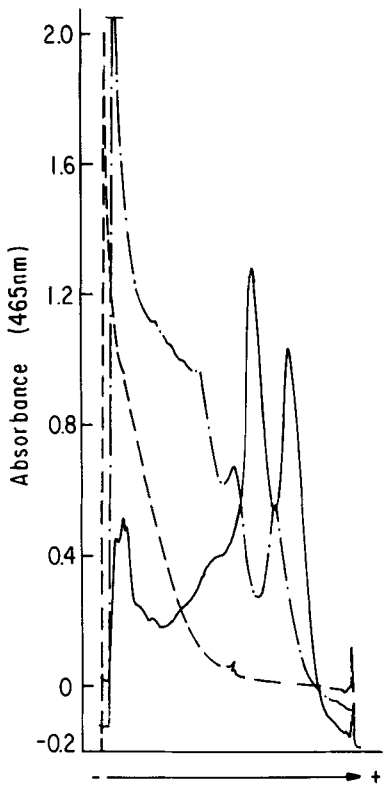

FIG. 1. Densitometer scan of native and PEG_{5000}-lipases. The gel was run under nondenaturing conditions and stained for esterase activity. The origin is at the cathode (negative electrode). ——, 66 μg native; -•-, 66 μg PEG_{5000}-lipase, 36% modified; - - -, 107 μg PEG_{5000}-lipase, 71% modified.

that native lipase is insoluble in benzene and that free PEG does not solubilize the enzyme.

Activity of PEG-Lipase. Table 2 presents the hydrolytic and synthetic activities of native and PEG_{5000}-lipase in water and organic solvents. PEG-lipase has 2.4 to 4.5 times greater specific activity than native lipase in all organic solvents tested, in spite of lower activity in water (Table 1). In organic solvents, the specific activity is ca. the same for hydrolysis and synthesis by either native or PEG-lipase. This effect has also been noted by Zaks and Klibanov (6) for native pancreatic lipase.

Both native and PEG-lipase show hydrolytic and synthetic activity in solvents in which they are insoluble. In fact, the specific activity of PEG-lipase is higher in some solvents in which it is insoluble (Table 2) than in solvents in which it dissolves. The choice of solvent is significant for lipase-catalyzed reactions in organics, confirming earlier observations (18).

Figure 2 shows the lipase-catalyzed esterification of lauric acid and 1-octanol in benzene. Only the benzene-soluble fraction of PEG-lipase was used, thereby eliminating activity from any remaining unmodified enzyme. Although slower initially, the PEG_{5000}-lipase reaches equilibrium faster than native enzyme. The fastest rate of reaction for native lipase is the initial rate, 19.5 μmol of free fatty acid (FFA) reacted/μg protein-hr. Interestingly, the fastest rate for the PEG-lipases occurs after an initial lag time. This may represent interaction

TABLE 2

Activity of Native and PEG-Lipase

Enzyme	Solvent	Specific activity (μmol FFA/min-mg protein) Hydrolytic	Synthetic
PEG_{5000}-lipase	H_2O	25.0	n.d.[a]
	iso-octane	1.09	1.53
	cyclohexane	1.04	1.34
	hexanes	0.75	n.d.
	trichloroethane	n.d.	0.68
	benzene	0.51	0.48
	n-octane	0.44	n.d.
Native	H_2O	75.0	n.d.
	iso-octane	0.28	0.38
	cyclohexane	n.d.	0.42
	trichloroethane	n.d.	0.15
	benzene	0.21	0.28

[a]Not determined.

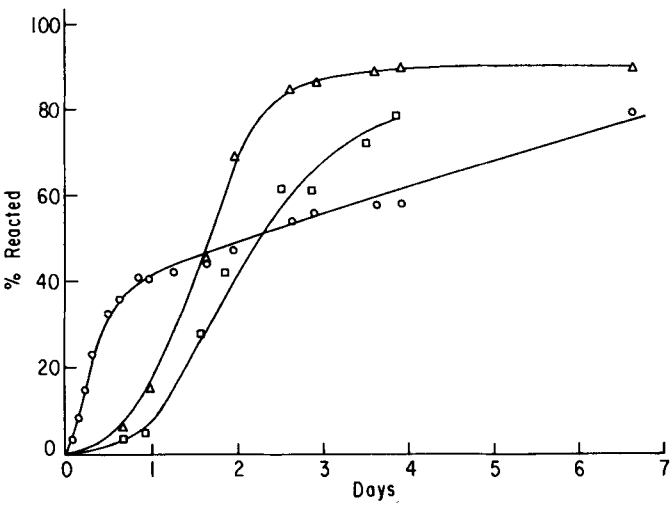

FIG. 2. Esterification time course. Millimolar lauric acid and 1-octanol reacted at 30 C in saturated benzene. O, native lipase, 1.2 mg protein/ml; △, PEG_{5000}-lipase, 0.49 mg protein/ml; □, PEG_{1900}-lipase, 0.20 mg protein/ml.

of the PEG-lipase with a small additional amount of water produced by the reaction. The PEG_{1900}-lipase, a high activity preparation (Table 1), and the PEG_{5000}-lipase (71% modified) have fastest rates of 63.9 and 33.9 μmol free fatty acid (FFA) reacted/μg protein-hr, respectively.

Stability. The stabilities of native and PEG-lipases at 45 C were measured in water and benzene. The loss of activity is first order, and the half-lives are listed in Table 3. PEG-lipase is, remarkably, 10 times more stable than native in water, but less stable in benzene. The stability in benzene of the insoluble native lipase vs the instability of the soluble PEG-lipase extends Zaks' and Klibanov's (19) observations on the stability of native lipases in organic solvents. They postulated that in organic solutions at low water concentrations, the enzyme molecule is insoluble, rigid and slow to denature. Solubilizing the enzyme with PEG would be expected to

TABLE 3

Stability and Selectivity Studies

	$t_{1/2}$ (hr)		$(V_{max}/K_m)_{18:1}/(V_{max}/K_m)_{18:0}$[a]	
Lipase	Water	Benzene	Methanol	1-Octanol
Native	7.03 ± .25	> 72	1.25	2.40
PEG_{5000}	79 ± 9	44 ± 4	1	33
PEG_{1900}	69 ± 3	36 ± 4	n.d.[b]	n.d.

[a]Relative specificity constants of oleic (18:1) to stearic (18:0) acid in benzene.

[b]Not determined.

introduce flexibility in organic solution and thus lower stability. Conversely, in water the PEG chains are solvated and may reduce the molecular motions that are intrinsic to denaturation.

Selectivity. Table 3 also shows results of selectivity studies measuring the reaction of 1:1 molar mixtures of oleic and stearic acids with methanol and/or 1-octanol. Control experiments demonstrated that: (i) equivalent results were obtained when methanol and 1-octanol were reacted separately or in combination and (ii) addition of free PEG_{5000} to the reaction mixture does not change native lipase selectivity. Native *C. rugosa* lipase slightly favors production of oleate esters in benzene and in hexane (data not shown). PEG_{5000}-lipase strongly favors esterification of oleic acid when reacting with 1-octanol although no preference is shown with methanol. The preferential production of 1-octyl oleate over the stearate ester suggests a subtle change in the lipase's active or binding site as a result of the derivatization. We plan to investigate this phenomenon further.

PEG-lipase from *C. rugosa* has modified characteristics which may be useful commercially. Its greatly increased stability in water could lead to use in hydrolysis of fats and oils under very mild conditions, especially if it can be recycled successfully. The PEG-lipase can be recovered from benzene with good yield by precipitation with hexane or ether (20). The altered selectivity of PEG-lipase favoring reaction with oleic vs stearic acid may also be useful.

REFERENCES

1. Abuchowski, A., T. van Es, N.C. Palczuk and F.F. Davis, *J. Biol. Chem. 252*:3578 (1977).
2. Abuchowski, A., J.R. McCoy, N.C. Palczuk, T. van Es and F.F. Davis, *Ibid. 252*:3582 (1977).
3. Inada, Y., H. Nishimura, K. Takahashi, T. Yoshimoto, A.R. Saha and Y. Saito, *Biochem. Biophys. Res. Comm. 122*:845 (1984).
4. Yoshimoto, T., M. Nakata, S. Yamaguchi, T. Funada, Y. Saito and Y. Inada, *Biotechnol. Lett. 8*:771 (1986).
5. Kodera, Y., K. Takahashi, H. Nishimura, A. Matsushima, Y. Saito and Y. Inada, *Ibid. 8*:881 (1986).
6. Zaks, A., and A.M. Klibanov, *Proc. Natl. Acad. Sci. USA 82*:3192 (1985).
7. Ferjancic, A., A. Puigserver and H. Gaertner, *Biotechnol. Lett. 10*:101 (1988).
8. Layne, E., in *Meth. Enzymol. Vol. III*, edited by S.P. Colowick and N.O. Kaplan, Academic Press, Inc., New York, 1957, pp. 450-451.
9. Habeeb, A.F.S.A., *Anal. Biochem. 14*:328 (1966).
10. Nishimura, H., K. Takahashi, K. Sakurai, K. Fujinuma, Y. Imamura, M. Ooba and Y. Inada, *Life Sciences 33*:1467 (1983).
11. Davis, B.J., *Ann. N.Y. Acad. Sci. 121*:404 (1964).
12. Parry, R.M. Jr., R.C. Chandan and K.M. Shahani, *J. Dairy Sci. 49*:356 (1966).
13. Linfield, W.M., D.J. O'Brien, S. Serota and R.A. Barauskas, *J. Am. Oil Chem. Soc. 61*:1067 (1984).
14. Linfield, W.Y., S. Serota and L. Sivieri, *Ibid. 62*:1152 (1985).
15. Chen, C-S., Y. Fujimoto, G. Girdaukas and C.J. Sih, *J. Am. Chem. Soc. 104*:7294 (1982).
16. Deleuze, H., G. Langrand, H. Millet, J. Baratti, G. Buono and C. Triantaphylides, *Biochim. Biophys. Acta 911*:117 (1987).
17. Tomizuka, N., Y. Ota and K. Yamada, *Agric. Biol. Chem. 30*:1090 (1966).
18. Kim, K.H., D.Y. Kwon and J.S. Rhee, *Lipids 19*:975 (1984).
19. Zaks, A., and A.M. Klibanov, *Science 224*:1249 (1984).
20. Yoshimoto, T., K. Takahashi, H. Nishimura, A. Ajima, Y. Tamaura and Y. Inada, *Biotechnol. Lett. 6*:337 (1984).

[Received February 23, 1988; accepted July 12, 1988]