

## Lipase Modified for Solubility in Organic Solvents

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### INTRODUCTION

Covalent attachment of polyethylene glycol (PEG) to free amino groups in proteins, as first described by Abuchowski *et al.*,<sup>1</sup> causes some unique changes. PEG-modified enzymes have little or no immunogenicity and have extended circulating lifetimes in blood.<sup>2</sup> Inada *et al.*<sup>3</sup> have used the amphipathic nature of PEG to introduce solubility in organic solvents into a variety of enzymes, including lipoprotein lipase. Concurrent with our efforts, there have been reports of the PEG derivatization of *Candida rugosa* (formerly *Candida cylindracea*) lipase by means of carboxymethylated PEG.<sup>4,5</sup> Herein, we report on the preparation of a PEG-derivatized *C. rugosa* lipase using the original PEG-triazine reagent. The effects of PEG chain length are noted and a direct comparison of the original with the derivatized material is made.

### MATERIALS AND METHODS

Lipase M (EC 3.1.1.3) from *C. rugosa* (lot nos. K12484 and K15505), 30 units/mg, was a gift from the Enzyme Development Corporation. Poly(ethylene glycol mono-methyl ether) of average molecular weights of 5000, 1900, and 750 was obtained from Polysciences. Cyanuric chloride was obtained from Aldrich Chemical.

Protein was measured in duplicate by the biuret method<sup>6</sup> using bovine serum albumin as the standard. To measure the modified protein only, PEG-lipase<sup>a</sup> was dissolved in benzene and centrifuged; then, the supernatant was evaporated under N<sub>2</sub> and redissolved in water. Free amino groups were measured in triplicate by the trinitrobenzenesulfonic acid procedure of Habeeb.<sup>7</sup>

#### *Preparation of PEG-Lipase*

PEG was activated with cyanuric chloride according to the method of Nishimura *et al.*<sup>8</sup> After 20 minutes reaction with lipase at room temperature, pH 7.5, the mixture was brought to pH 6, concentrated by ultrafiltration, dialyzed, and lyophilized.

<sup>a</sup>Lipase covalently modified with activated polyethylene glycol is termed PEG-lipase. The average molecular weight of the PEG used is indicated by subscripts, for example, PEG<sub>5000</sub>-lipase. "Activated PEG" is 2,4-bis[ $\omega$ -methoxypoly-(ethyleneoxy)]-6-chloro-*s*-triazine, (PEG)<sub>2</sub>Cl, or 2-[ $\omega$ -methoxypoly-(ethyleneoxy)]-4,6-dichloro-*s*-triazine, (PEG)Cl<sub>2</sub>.

## BAILLARGEON & SONNET: LIPASE MODIFIED FOR SOLUBILITY

### *Polyacrylamide Gel Electrophoresis (PAGE)*

Electrophoresis was performed under non-denaturing conditions according to Davis<sup>9</sup> on 5% acrylamide minislab gels. PEG-lipase samples were dissolved in benzene and redissolved in water as described above. Esterase activity was visualized on gels with an  $\alpha$ -naphthyl acetate stain of 0.03%  $\alpha$ -naphthyl acetate, 0.05% Fast Blue RR salt, and 1% acetone in 0.1 M Tris, pH 7.4. 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.*<sup>10</sup> using an olive oil emulsion at pH 7.3 after purification and sonication according to the procedures of Linfield *et al.*<sup>11,12</sup> The rate of free fatty acid formation was followed by automatic titration.

### *Stability*

The lipase solution was incubated in water or benzene at 45 °C. The 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.

### *Selectivity*

The selectivity of lipase for oleic versus 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 % conversion to esters was determined by free fatty acid titration, and the ratio of the two esters formed was determined by gas chromatography. These data allow calculation of E, the ratio of the specificity constants,  $(V_{\max}/K_m)_{18:1}/(V_{\max}/K_m)_{18:0}$ , for oleic and stearic acids, using the method of Chen *et al.*<sup>13</sup>

## RESULTS AND DISCUSSION

### *Preparation and Characterization of PEG-Lipase*

Representative batches of PEG-lipase are compared with native lipase in TABLE 1. A tenfold molar excess of activated PEG<sub>5000</sub> to free amino groups (calculated using 35 free amino groups and a molecular weight of 125,000 per 1000 amino acid residues<sup>14</sup>) results in a PEG<sub>5000</sub>-lipase that dissolves rapidly in organic solvents such as benzene, toluene, chloroform, and trichloroethane. However, increasing the molar excess of activated PEG is required to prepare soluble PEG<sub>1900</sub>- and PEG<sub>750</sub>-lipases.

The solubilities of PEG<sub>5000</sub>- and PEG<sub>1900</sub>-lipases in benzene are the same, within experimental error, as their solubilities in water. In contrast, it appears that PEG<sub>750</sub> is too small in size as compared to the lipase to solubilize it successfully in benzene.

PEG-lipase activity decreases with increasing pH and extent of modification. The % modification is negatively correlated with the % relative specific activity (correlation coefficient =  $-0.96$ ).

### Electrophoresis

FIGURE 1 shows a gel scan of crude native lipase and PEG<sub>5000</sub>-lipases of 36% and 71% modification. Both PEG-lipases show a decreased mobility in comparison to native lipase, as expected for higher molecular weight species. Gels of PEG<sub>1900</sub>- and PEG<sub>750</sub>-lipases also show an increased molecular weight (data not shown).

### Activity of PEG-Lipase

TABLE 2 presents the activities of native lipase and PEG<sub>5000</sub>-lipase in water and organic solvents in both the hydrolytic and synthetic modes. Despite a lower activity in water (TABLE 1), PEG-lipase has 2.4 to 4.5 times greater specific activity than native lipase in all organic solvents tested.

FIGURE 2 shows the lipase-catalyzed esterification of lauric acid and 1-octanol in benzene. Only the benzene-soluble fraction of PEG-lipase has been used, thereby eliminating activity from any remaining unmodified enzyme. Although slower initially, the PEG<sub>5000</sub>-lipase reaches equilibrium faster than the native enzyme.

### Stability

The half-lives of native lipase at 45 °C are  $7.0 \pm 0.3$  hours in water and  $>72$  hours in benzene. PEG<sub>5000</sub>-lipase is ten times more stable than native lipase in water ( $t_{1/2} = 79 \pm 9$  hours), but less stable in benzene ( $t_{1/2} = 44 \pm 4$  hours). Similar results are obtained with PEG<sub>1900</sub>-lipase. Solubilizing the enzyme with PEG would be

TABLE 1. Characterization of PEG-Lipase

Lipase	Molar Excess PEG	Modification <sup>a</sup> (%)	$M_r^b$ ( $\times 10^{-5}$ )	Protein <sup>c</sup> (%)		Activity <sup>d</sup> (%)	
				Water	Benzene	Recovered	Relative Specific
Native	—	0	1.2	12.4	0	100	100
(PEG <sub>5000</sub> ) <sub>2</sub> Cl	10	36	2.5	2.1	1.3	53	42
	10	71 <sup>e</sup>	3.7	3.2	2.4	26	12
	50	67	3.6	0.5	1.1	22	29
(PEG <sub>5000</sub> )Cl <sub>2</sub>	15	40	1.9	2.3	n.d. <sup>f</sup>	4	11
(PEG <sub>1900</sub> ) <sub>2</sub> Cl	52	53	1.9	1.6	1.0	74	42
(PEG <sub>750</sub> ) <sub>2</sub> Cl	53	77	1.6	6.4	2.9	44	11

<sup>a</sup>Trinitrobenzenesulfonic acid assay.

<sup>b</sup>Calculated molecular weight.

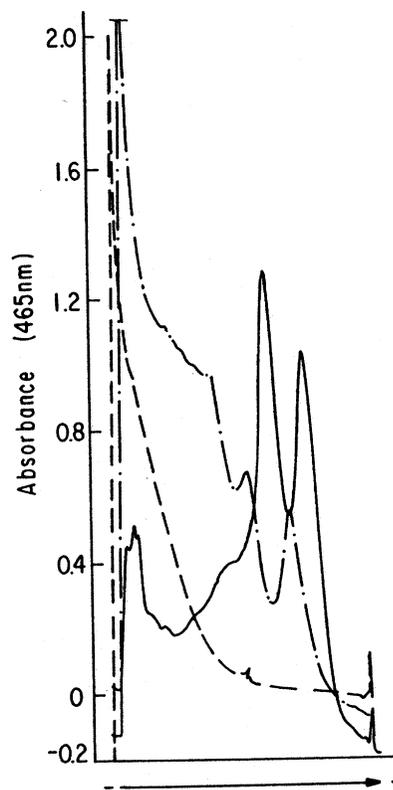
<sup>c</sup>Biuret assay: expressed in % by weight of dry powder.

<sup>d</sup>Initial rate lipase assay using an olive oil-gum arabic emulsion in water.

<sup>e</sup>pH momentarily raised from 7.5 to 8.2.

<sup>f</sup>Not determined.

**FIGURE 1.** Densitometer scan of native lipase and PEG<sub>5000</sub>-lipase. The gel was run under non-denaturing conditions and stained for esterase activity. The origin is at the cathode (negative electrode): (—) 66  $\mu$ g native; (- - -) 66  $\mu$ g PEG<sub>5000</sub>-lipase, 36% modified; (---) 107  $\mu$ g PEG<sub>5000</sub>-lipase, 71% modified.



expected to 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*

Native *C. rugosa* lipase slightly favors production of oleate esters in benzene and in hexane ( $E = 1.25$  to  $3.3$ ). PEG<sub>5000</sub>-lipase strongly favors esterification of oleic acid when reacting with 1-octanol in benzene ( $E = 33$ ), although no preference is shown with methanol ( $E = 1$ ). 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.

#### CONCLUSIONS

PEG-lipase from *C. rugosa* has modified characteristics that may be commercially useful. Its greatly increased stability in water could lead to use in hydrolysis of fats and

## BAILLARGEON & SONNET: LIPASE MODIFIED FOR SOLUBILITY

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.<sup>15</sup> The altered selectivity of PEG-lipase favoring reaction with oleic versus stearic acid also may be useful.

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