# Kinetics of Organic Solvent-Soluble and Native Lipase

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**ABSTRACT:** Organic solvent-soluble lipase was prepared with a synthetic detergent. The solvent-soluble lipase was presumed to be a complex of the enzyme and the detergent. To investigate the effect of the detergent attachment to the enzyme on the reaction properties, the kinetics of the solvent-soluble lipase and of the native lipase were estimated by using glycerides in a homogeneous solution of buffer and tetrahydrofuran. Analysis of the direct interaction between the enzyme and the substrate could serve to characterize the steric structure around the active site of the lipase. The $K_m$ values also differentiated the solvent-soluble lipase from the native lipase. These findings show for the first time that the detergent surrounding the enzyme molecule may affect not only the solubility of the enzyme, but its kinetics as well.

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**KEY WORDS:** Aqueous THF solution, didodecyl glucosyl glutamate, fluorometric HPLC, glyceride, hydrophobicity, kinetics, $K_m$, lipase, solvent-soluble lipase, $V_{max}$.

Recently, several investigators have studied modification of enzymes to dissolve and maintain their activity in organic solvents by using detergents, polyethyleneglycol, etc. (1–8). The purpose of these approaches has focused on the efficient production of useful compounds in organic solvents by enzymes. Actually, modified enzymes can synthesize ester derivatives under nonaqueous conditions (1–3). Furthermore, they reported that the behavior of these modified enzymes in organic solvents was considerably different from that of native enzymes (5–8). However, details of the differences are not clear because of the diversity of properties between the modified and the native enzyme, such as solubility, modification, and sensitivity to the organic solvent. Especially, differences in their solubilities have led to slow progress of studies on the relationship between reaction properties and modification in both types of the enzyme. Studying the influence of the modification on the reaction properties can resolve some structural problems, such as binding the modifier to the enzyme and the interaction of the substrate to the enzyme. Furthermore, the changes in reaction properties of the enzyme modified with other modifiers may be predicted. These interpretations at the molecular level will be a clue to the artificial modification of enzymes.

To these purposes, the kinetics of the modified and native lipases were compared with Lipase P (Amano Pharmaceutical Co., Ltd., Nagoya, Japan), which resisted several organic solvents. A series of glycerides were hydrolyzed by both types of lipase in a homogeneous solution of buffer and tetrahydrofuran (THF). Detection of the direct interaction between the enzyme and the substrate caused the resolution of the reaction characteristics of Lipase P. Furthermore, by comparing the kinetic parameters of the solvent-soluble lipase and the native lipase, the effects of the detergent attached to the enzyme molecule on the enzymatic reaction could be discussed.

## MATERIALS AND METHODS

*Reagents.* Didodecyl glucosyl glutamate was synthesized according to the method of Okahata (7). All of the glycerides used were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO). 9-Bromomethyl acridine (9BMA), a fluorescent labeling reagent for released fatty acids, and tetraethylammonium carbonate (TEAC), a catalyst for the labeling reaction, were synthesized according to a previously published method (9,10). THF without stabilizer was purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). All other chemicals of analytical grade were purchased from Wako Pure Chemical Co., Ltd., and from Kanto Chemical Co., Ltd. (Tokyo, Japan).

*Preparation of organic solvent-soluble lipase.* Modification of Lipase P to the organic solvent-soluble type was as follows: Lipase P (10 mg) in water (2 mL) was added to didodecyl glucosyl glutamate (10 mg) in THF (4 mL) and stirred vigorously at 4°C for 24 h. After evaporation of the solvent at 4°C, the precipitates were collected by centrifugation, washed with water to eliminate the unmodified lipase, and lyophilized overnight. The powder thus obtained, which contained the organic solvent-soluble lipase and free detergent, was stored at 4°C until use.

*Lipase assay.* For the determination of lipase activity, all hydrolysis reactions of the glycerides by either the solvent-soluble lipase or the native lipase were conducted in a homogeneous solvent system of 200 μL, a mixture of THF and buffer [Britton-Robinson buffer (25 mM of phosphoric acid, acetic acid, boric acid, and 15 mM of sodium hydroxide), pH 8.0, 7:3, vol/vol]. The mixture was incubated at 37°C for 30

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min for the routine assay. After hydrolysis by lipase, 200 μL each of 9BMA [5 mM in dimethylsulfoxide (DMSO)] and TEAC (2.5 mM in DMSO) was added to the reaction mixture, which was then allowed to stand at room temperature for more than 40 min. The enzyme was denatured, and the hydrolysis reaction was terminated by the addition of DMSO solution of 9BMA and TEAC. The labeling reaction was completed within 30 min. The fluorescence intensity was stable for more than 4 h (11). A 20-μL aliquot was injected into the high-performance liquid chromatography (HPLC). Fatty acid as a standard for quantitation also was labeled by the same procedure. The HPLC apparatus used was an EYELA Model PLC-5D equipped with a loop-type sample injection (Tokyo Rika Kikai Co. Ltd., Tokyo, Japan). The separation column was TSK-gel ODS 80Tm (Tosoh Co. Ltd., Tokyo, Japan) with a solvent system of ethanol/acetonitrile/water (95:95:10, vol/vol/vol) at a flow rate of 0.8 mL/min. For detection, a JASCO Model FP-820 spectrofluorometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) was used to monitor the fluorescence intensity at 425 nm (excitation at 365 nm). The data were produced with a Chromatopack C-R1A integrator (Shimadzu Seisakusho, Kyoto, Japan), and the peak area was used for quantitation.

*Protein content.* To estimate the activity of lipase, the content of protein in the solvent-soluble lipase or in the native lipase was measured by using a protein assay kit (Bio-Rad Laboratories Co., Ltd., Richmond, CA). For the solvent-soluble lipase, the protein content was determined after extraction from the complex by a method described previously (12).

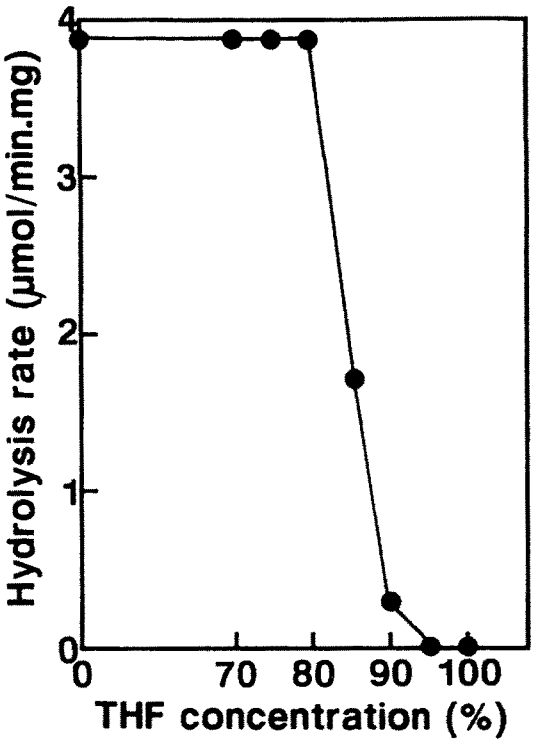


**FIG. 1.** Effect of tetrahydrofuran (THF) on lipase activity. The specific activity of the native Lipase P (Amano, Nagoya, Japan) was measured at various THF concentrations, with 1 mM tripalmitin as a substrate.

## RESULTS AND DISCUSSION

*Resistance of Lipase P to THF.* To investigate the inactivation of Lipase P by THF, the specific activity of the native Lipase P was measured in solution at THF concentrations ranging from 0 to 100% (vol/vol). As shown in Figure 1, no changes of specific activity were detected in solutions that contained less than 80% THF concentration. The remarkable decrease of hydrolysis activity in the >80% THF region suggests that native Lipase P was inactivated. Kinetic analysis of the native lipase should be conducted under conditions that avoid denaturation. Considering the solubility of the solvent-soluble lipase, the addition of THF to the reaction mixture is necessary. Therefore, when the reactive properties of the native lipase and the solvent-soluble lipase were compared, the THF concentration was set at 70%, at which the native Lipase P was scarcely inactivated, and the solvent-soluble Lipase P was well dissolved.

*Hydrolysis of glycerides.* To differentiate the solvent-soluble lipase from the native one, a series of glycerides were hydrolyzed in aqueous THF solution (70% THF). All substrates were dissolved at a concentration of 1 mM. The solvent-soluble lipase powder, which was prepared according to the method described in the Materials and Methods section, contained not only the complex of the lipase and detergent, but also some free detergent. The solvent-soluble lipase can be separated from the free detergent by column chromatography. Because the prepared complex powder was used without further purification in these experiments, there was free detergent in the reaction mixture of the solvent-soluble lipase. The effect of the detergent attached to the enzyme molecule on the reaction properties should be distinguished from that of free detergent. Free detergent was added to the native lipase solution to make it the same composition ratio of protein and detergent as that in the prepared complex powder. The composition of protein and detergent in the prepared complex powder had already been investigated (4). The glycerides were hydrolyzed by either the native lipase alone or the native lipase with the free detergent or the solvent-soluble lipase in 70% THF, and hydrolysis rates were determined.

At first, glycerides with various lengths of saturated side chains were hydrolyzed (Fig. 2). Both the native lipase and the solvent-soluble lipase hydrolyzed triacylglycerides with short side chains more rapidly than those with long side chains. However, there was a notable difference in the hydrolysis rates of triacylglyceride with long side chains between the solvent-soluble and the native lipase. The effect of the length of side chains on the hydrolysis rate by the solvent-soluble lipase is smaller than that by the native lipase. Thus, the level of relative activity for hydrolysis of tristearin by the solvent-soluble lipase was maintained at 74%, compared with 100% for trilaurin. With native lipase, only 35% of the activity remained. The presence of free detergent in the reaction mixture scarcely affected the hydrolysis rate of the native lipase.

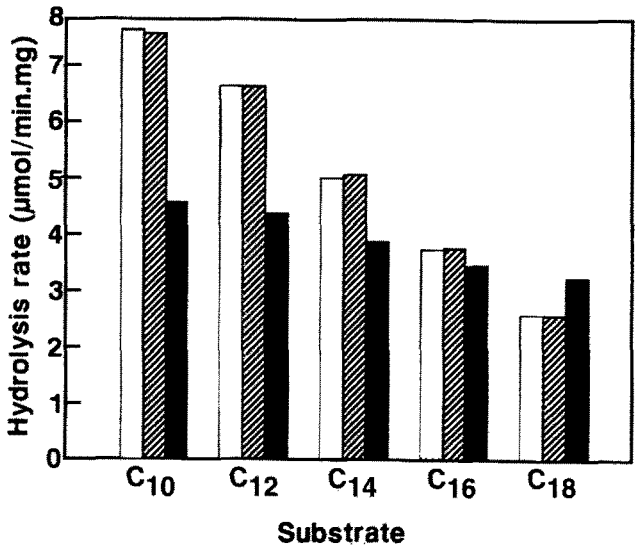


**FIG. 2.** Hydrolysis rates of various triacylglycerides. Each glyceride was hydrolyzed at 37°C for 30 min by the native lipase without detergent (open bar) or with detergent (cross-hatched bar) or by the solvent-soluble lipase (closed bar).

Next, glycerides that were acylated at various positions in the glycerol moiety were used as substrates. As shown in Figure 3, the hydrolysis rates of both lipases were significantly different, depending on the acylated position. The rate of hydrolysis by each lipase increased in the following order, 2-monopalmitin, 1-monopalmitin, 1,3-dipalmitin, 1,2-dipalmitin, and tripalmitin. The native lipase with free detergent hydrolyzed these glycerides in the same way as that without free detergent. However, differences were detected in hydrolysis rates between the native lipase and the solvent-soluble lipase. The level of relative activity for the hydrolysis of 1-monopalmitin by the solvent-soluble lipase was maintained at only 6%, compared with 100% for tripalmitin. As for the native lipase, 11% of the activity remained. This suggests that the detergent surrounding the lipase molecule affected the substrate specificity of lipase.

Finally, the glycerides with unsaturated side chains were used as substrates to examine the effect of the three-dimensional structure of the glyceride side chain on lipase activity. Tristearin ($C_{18:0}$), triolein ($C_{18:1}$), and trilinolein ($C_{18:2}$) were hydrolyzed by each type of lipase. As shown in Figure 4, no differences could be detected in the hydrolysis rates among the three glycerides with these three types of lipase.

By comparison with the hydrolysis properties of the native lipase, the solvent-soluble lipase could be characterized as follows: (i) Although the hydrolysis properties of the solvent-soluble lipase are not completely the same as those of the native lipase, the inherent substrate specificity of Lipase P itself was not changed dramatically in the solvent-soluble lipase. The detergent attached to the lipase molecule hardly modifies the three-dimensional structure of the active site. (ii) No differences were detected in the hydrolysis rates of the native lipase with or without free detergent. This indicates that the unique properties of the solvent-soluble lipase resulted from the attached detergent.

*Kinetic analysis.* To clarify the characteristics of the solvent-soluble lipase, the kinetic parameters for the interaction between glycerides and the lipase were determined in aqueous THF (70% THF). The reactivity of the native Lipase P in 100% buffer was not considered as a control because of the insolubility of the substrates. The values for the maximum velocity ($V_{max}$) and the Michaelis constant ($K_m$) are summarized in Table 1. A comparison of the kinetic parameters for these triacylglycerides showed that the length of the side chains of triacylglycerides did not impact the values of $V_{max}$, but $K_m$ varied. The longer the side chains of the glyceride, the larger the $K_m$ value of both types of lipase became. However,

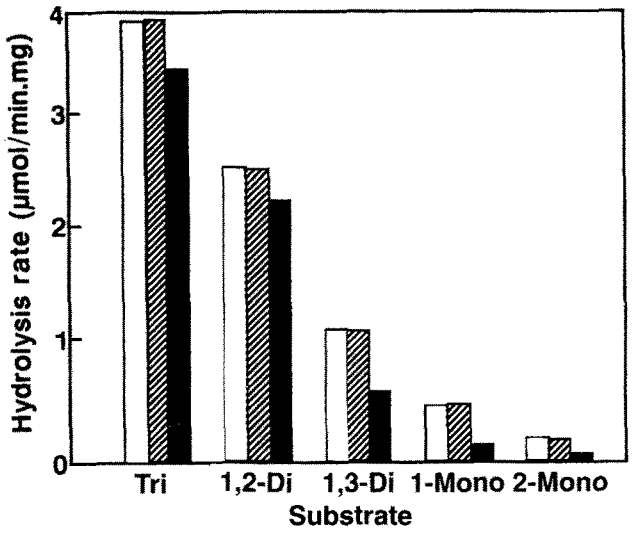


**FIG. 3.** Hydrolysis rates of glycerides acylated at various positions in the glycerol moiety. Each glyceride was hydrolyzed at 37°C for 30 min by the native lipase without detergent (open bar) or with detergent (cross-hatched bar), or by the solvent-soluble lipase (closed bar).

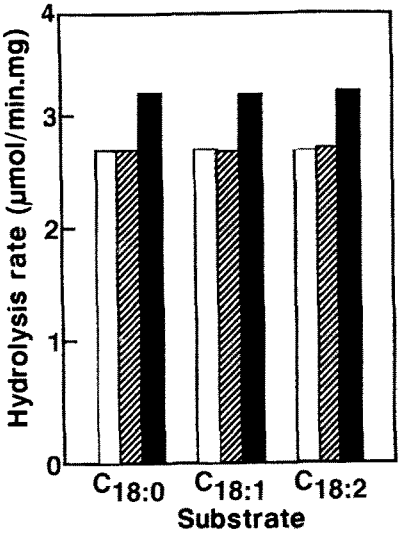


**FIG. 4.** Hydrolysis rates of glycerides with unsaturated side chains. Each glyceride was hydrolyzed at 37°C for 30 min by the native lipase without detergent (open bar) or with detergent (cross-hatched bar), or by the solvent-soluble lipase (closed bar).

**TABLE 1**
**Kinetic Constant for Glycerides and Lipase P in Aqueous Tetrahydrofuran Solution[a]**

| Enzyme | Substrate | Acylated position of glycerol | Number of carbons in fatty acid[b] | $K_m$ (mM) | $V_{max}$ (μmol/min) |
|---|---|---|---|---|---|
| Complex | Tricaprin | 1,2,3 | 10(0) | 5.56 ± 0.13 | 33.3 |
| | Trilaurin | 1,2,3 | 12(0) | 6.17 ± 0.17 | 32.3 |
| | Trimyristin | 1,2,3 | 14(0) | 6.25 ± 0.15 | 31.3 |
| | Tripalmitin | 1,2,3 | 16(0) | 7.25 ± 0.17 | 32.1 |
| | Tristearin | 1,2,3 | 18(0) | 9.09 ± 0.20 | 31.5 |
| Native | Tricaprin | 1,2,3 | 10(0) | 3.05 ± 0.08 | 34.4 |
| | Trilaurin | 1,2,3 | 12(0) | 3.82 ± 0.09 | 33.4 |
| | Trimyristin | 1,2,3 | 14(0) | 5.62 ± 0.12 | 33.6 |
| | Tripalmitin | 1,2,3 | 16(0) | 7.04 ± 0.18 | 33.2 |
| | Tristearin | 1,2,3 | 18(0) | 10.87 ± 0.26 | 33.4 |
| Complex | Tripalmitin | 1,2,3 | 16(0) | 7.25 ± 0.17 | 32.1 |
| | 1,3-Dipalmitin | 1,3 | 16(0) | 55.56 ± 1.58 | 32.5 |
| | 1,2-Dipalmitin | 1,2 | 16(0) | 11.36 ± 0.25 | 31.9 |
| | 1-Monopalmitin | 1 | 16(0) | 226.14 ± 7.57 | 32.2 |
| | 2-Monopalmitin | 2 | 16(0) | 525.10 ± 18.25 | 31.3 |
| Native | Tripalmitin | 1,2,3 | 16(0) | 7.04 ± 0.18 | 33.2 |
| | 1,3-Dipalmitin | 1,3 | 16(0) | 20.83 ± 0.51 | 32.7 |
| | 1,2-Dipalmitin | 1,2 | 16(0) | 8.47 ± 0.21 | 33.4 |
| | 1-Monopalmitin | 1 | 16(0) | 83.67 ± 2.27 | 33.1 |
| | 2-Monopalmitin | 2 | 16(0) | 220.75 ± 7.33 | 32.9 |
| Complex | Tristearin | 1,2,3 | 18(0) | 9.09 ± 0.20 | 31.5 |
| | Triolein | 1,2,3 | 18(1) | 8.97 ± 0.21 | 31.1 |
| | Trilinolein | 1,2,3 | 18(2) | 9.11 ± 0.20 | 31.8 |
| Native | Tristearin | 1,2,3 | 18(0) | 10.87 ± 0.26 | 33.4 |
| | Triolein | 1,2,3 | 18(1) | 11.02 ± 0.28 | 32.8 |
| | Trilinolein | 1,2,3 | 18(2) | 10.91 ± 0.28 | 32.9 |

[a]Lipase P from Amano (Nagoya, Japan). [b]Number of double bonds in parentheses.

the difference in the $K_m$ values of the solvent-soluble lipase (5.56–9.0 mM) was no larger than that of the native lipase (3.05–10.87 mM). Especially, in the case of tristearin, the $K_m$ value of the solvent-soluble lipase (9.09 mM) became smaller than that of the native lipase (10.87 mM). The $K_m$ value of the solvent-soluble lipase may possibly be smaller than that of the native lipase, for a substrate whose hydrophobicity is stronger than tristearin. The detergent surrounding the lipase molecule might be responsible for this characteristic $K_m$ value of the solvent-soluble lipase.

The $K_m$ values of both lipases were affected depending on the acylated position in the glycerol moiety. The $K_m$ values of both lipases for tripalmitin, 1,2-dipalmitin, 1,3-dipalmitin, 1-monopalmitin, and 2-monopalmitin increased in that order (Table 1). Most interesting is that the $K_m$ values for 1,2-dipalmitin were smaller than for 1,3-dipalmitin, and close to that for tripalmitin, in spite of the considerably large $K_m$ values for 2-monopalmitin. Acylation at the 2-position in the glycerol moiety might possibly be important for the interaction between the lipase and the glycerides. In our previous work, Lipase B from *Pseudomonas fragi* 22.39B (Wako Pure Chemical Co., Ltd.) was modified to become organic solvent-soluble with didodecyl glucosyl glutamate, and the $K_m$ values between each glyceride and the solvent-soluble lipase were determined (10). The kinetics of the native Lipase B was not analyzed in that study. $K_m$ values of the solvent-soluble Lipase B for tripalmitin, 1,3-dipalmitin, 1,2-dipalmitin, and 1-monopalmitin were 5.34, 5.85, 11.69, and 19.35 mM, respectively (10). The $K_m$ values for tripalmitin were similar to those for 1,3-dipalmitin, and were considerably smaller than those for 1,2-dipalmitin. On the other hand, the results of this work indicate that the $K_m$ value of Lipase P for tripalmitin did not correspond with that for 1,3-dipalmitin, but with that for 1,2-dipalmitin. This difference may possibly depend on the structural characteristics of the active site between Lipase B and Lipase P.

Compared to native Lipase P, the $K_m$ values of the solvent-soluble Lipase P for monoacylglyceride were considerably large. It indicates that the detergent of the solvent-soluble lipase affects its reactivity.

The $K_m$ and $V_{max}$ values for tristearin, triolein, and trilinolein were determined. These glycerides were the same in terms of the length of the side chains and the positions acylated in the glycerol moiety, although there were differences in the number of double bonds in the side chains. As shown in Table 1, no differences could be detected in the $K_m$ and

$V_{max}$ values for the three glycerides by either the solvent-soluble lipase or by the native lipase. The $K_m$ values were remarkably different for glycerides acylated at various positions on the glycerol moiety. However, the $K_m$ values were not influenced by the structural diversities in the side chains. These findings suggest that the lipase may recognize the conformation of the glycerol moiety of glycerides rigidly, with rough recognition of the structure in the side chains.

Recently, the structure of lipase catalytic site has been proposed based on kinetic analysis with ester compounds as substrates (13–16). In this study, the structural properties of lipase were characterized from detecting the interaction with glycerides, which are the inherent substrates for lipase. Introduction of the glycerides brought some new information about the active site of lipase, as described previously.

A comparison of the kinetic parameters of solvent-soluble lipase with that of native lipase could characterize the solvent-soluble lipase. When the substrates were considerably hydrophobic, such as tristearin, the $K_m$ values of the solvent-soluble lipase were smaller than those of the native lipase. In other words, selectivity of the solvent-soluble lipase toward hydrophobic substrates was increased. The hydrophobicity around the enzyme molecule might possibly be enhanced by the attached detergent. It demonstrated, for the first time, that the detergent attached to the enzyme molecule changed its substrate specificity in relation to $K_m$ values. Previously, the specificities of lipase have been altered by substitution of amino acid in the catalytic site by means of a genetic technological method (17–21). This work shows that modification with detergent may possibly be another method to improve its reactive properties. Further studies on the relationship between molecular structure of the detergent and changes of the reactive properties are in progress with other lipases and several detergents.

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