# Fluorometric Detection of Interaction Between Lipase and Glyceride

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**ABSTRACT:** Previously, we devised the efficient modification of lipase, which can be dissolved and still maintain its activity in organic solvents. In this work, the fluorescence of the modified lipase could be detected in chloroform. When glycerides were added to the modified lipase solution, the intrinsic tryptophan fluorescence of the modified lipase decreased, which suggests that the environment of the tryptophan residue was affected by the substrate. The interaction between the modified lipase and glyceride was studied kinetically in terms of fluorescence intensity of the tryptophan residue. Because glyceride is not subject to hydrolysis in nonaqueous solution, the dissociation constant of the enzyme–substrate complex could be determined. Thus, insight into the direct interaction between enzyme and substrate provided some structural information regarding the active site of lipase.

*JAOCS 75*, 535–538 (1998).

**KEY WORDS:** Fluorometry, glyceride, interaction, modified lipase, tryptophan.

Although lipase is a water-soluble enzyme, most of its substrates are insoluble in water. This discrepancy brings both complexity and interest into lipase research. Because lipase reacts with its substrate at the oil/water interface, the steric structure around the active site seems to be important. The specificity of lipase has been studied by structural and chemical methods. The X-ray structure of human or microbial lipase suggests that the active site is covered by a surface loop, and that the conformational change of this surface loop makes the active site accessible to substrates at the water/oil interface (1–5). Structural analysis revealed the existence of a tryptophan residue in the surface loop moiety of human and microbial lipase (1,3,5,6). Fluorescence intensity of tryptophan is affected by its environment. Previously, it was reported that fluorescence of tryptophan in human lipase changed when the lipase was inactivated by a water-soluble inhibitor, such as tetrahydrolipstatin (7). It was suggested that the surface loop might open before an inhibitor could enter the active center of the lipase (7). Therefore, fluorometric techniques are suitable to monitor the conformational changes around the active site of lipase.

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We have developed an efficient preparation of the organic solvent-soluble lipase modified with synthetic detergent (8). This modified lipase is only slightly denatured by organic solvents (9), making it a powerful tool in investigating the interaction with insoluble substrates in nonaqueous solution. Glycerides, which are inherent substrates of lipase, are subject to hydrolysis in buffer. However, they are impossible to hydrolyze in nonaqueous solutions. If the modified lipase is introduced to a nonaqueous solution of glycerides, the substrate can interact with the modified lipase but will not be hydrolyzed. This means that the dissociation of glycerides with the modified lipase in organic solvent can be estimated like that of inhibitors with native lipase in buffer.

Previously, the kinetics of human lipase and a water-soluble inhibitor were analyzed fluorometrically in buffer (7). In this work, this fluorometric method was applied to the nonaqueous solvent system. Lipase P and lipase B, which have a tryptophan residue identified in the surface loop (6), were modified to be soluble in an organic solvent. The intrinsic tryptophan fluorescence of this modified lipase was monitored with added glycerides. The interaction between modified lipase and glycerides in a nonaqueous solution could be characterized by the fluorometric method.

## MATERIALS AND METHODS

*Reagents*. Lipase P from *Pseudomonas* sp. was purchased from Amano Pharmaceutical (Nagoya, Japan), and Lipase B from *Pseudomonas fragi* 22.39B was obtained from Wako Pure Chemical Co. (Osaka, Japan). Didodecyl glucosyl glutamate was synthesized according to the method described previously (10). Tetrahydrofuran (THF), without a stabilizer, and chloroform were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals of analytical grade were purchased from Wako Pure Chemical Co. and Kanto Chemical Co. (Tokyo, Japan).

*Preparation of modified lipase*. Lipase was modified with a synthetic detergent as follows. Lipase (10 mg) in water (2 mL) was added to a solution of didodecyl glucosyl glutamate (50 mg) in THF (4 mL). The mixture was stirred vigorously at 4°C for 24 h. After evaporation of the solvent, the precipitate was collected by centrifugation, washed with water to eliminate the unmodified lipase, and lyophilized overnight.

The powder thus obtained, which contained the modified lipase and free detergent, was stored at 4°C until use.

To separate the modified lipase from free detergent, the modified lipase powder (5 mg), dissolved in chloroform (0.5 mL), was applied to a Sephadex LH-20 column (Pharmacia) (1.2 cm i.d. × 10 cm in length) and eluted with chloroform. Each fraction (1 mL) was monitored at 280 nm to detect the protein. The fraction containing protein was collected and used for the fluorometric measurement.

*Fluorometric measurement.* Static measurements of fluorescence spectra and titration of the enzyme with glyceride were carried out with a JASCO FP-770 spectrofluorometer (Tokyo, Japan) at room temperature. Both excitation and emission spectra from monochrometers were set at 4-nm slit widths. Spectra were obtained in 1-cm quartz cells at an excitation wavelength of 280 nm. Fluorescence emission spectra were corrected by subtraction of the spectra of an incubation mixture without lipase.

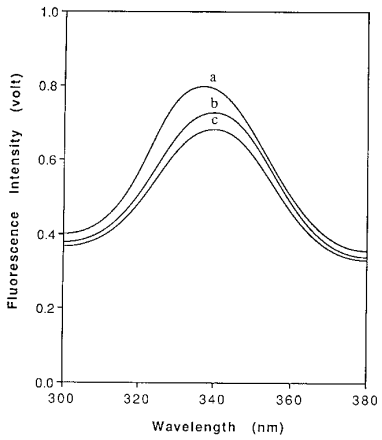


**FIG. 1.** Fluorescence emission spectra of the modified lipase (Lipase P) in chloroform (a) with an excitation wavelength of 280 nm, with 2 mM of tripalmitin (b) and 10 mM of tripalmitin (c).

## RESULTS AND DISCUSSION

*Intrinsic fluorescence of modified lipase in chloroform*. Fluorescence emission spectra of the modified lipase (Lipase P) in chloroform are shown in Figure 1, for which the excitation light length was set at 280 nm. The native lipase in chloroform had no fluorescence emission, because of its insolubility. The modified lipase shows maximal emission at 337 nm (Fig. 1a). The spectrum of the modified lipase in chloroform is in good agreement with that of the native lipase in buffer (7). No changes could be detected in the spectrum of the modified lipase for 6 h. These results suggest that the environment around the tryptophan residue of the modified lipase might be similar to that of the native material. The structural stability of the modified lipase in chloroform was also confirmed from the long-range constancy of the fluorometric spectrum.

*Fluorometric change of modified lipase in interacting with glycerides*. The interaction between the modified lipase and glyceride was studied by fluorometry. When the lipase was incubated with glyceride, maximal emission of fluorescence of the modified lipase was 3 nm red-shifted, and the emission intensity was partially decreased (Fig. 1b, c). The fluorescence quenching, produced by the addition of glyceride, suggests that some changes occur in the microenviroment of the tryptophan residue. The intrinsic fluorescence might be a superior indicator to predict the interaction between lipase and glyceride.

*Estimation of dissociation constant between modified lipase and glyceride in chloroform*. Based on the fluorescence change of the tryptophan residue, the dissociation constant between the modified lipase and glyceride was determined. Figure 2 shows the decrease of fluorescence intensity at 340 nm (ΔF), which depends on the glyceride concentration. In the past, the dissociation constant ($K_d$) for gluconolactone of glucoamylase was determined by a fluorometric method (11). This method was applied to evaluate the $K_d$ value for glycerides of the modified lipase in this work. Although glycerides are inherent substrates of lipase, they are not hydrolyzed by lipase in nonaqueous solution. Under this condition, glycerides are thought to behave like inhibitors or substrate analogs. This makes it possible to estimate $K_d$ values for the substrate–lipase complex. The equilibrium of the interaction between the modified lipase and glyceride in chloroform is as follows:

$$E + S \leftrightarrow ES \qquad [1]$$

where E, S, and ES represent the enzyme, the substrate, and the enzyme–substrate complex, respectively. Under the conditions employed, the total concentration of the enzyme [E] is negligible in comparison with the substrate concentration [S]. In Equation 2,

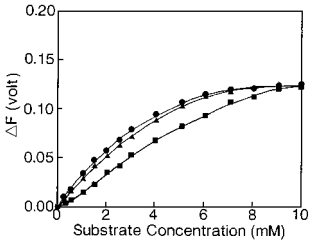


**FIG. 2.** Fluorescence decrement of the modified lipase P at 340 nm (ΔF) as function of the concentration of glyceride. Tripalmitin (●), 1,2-dipalmitin (▲), and 1,3-dipalmitin (■) were used as substrates.

$$\Delta F = F_{max}[S]/(K_d + [S]) \quad [2]$$

where $F_{max}$ is the maximal fluorescence decrease observed when the modified lipase is saturated by glyceride. $K_d$ values for glycerides of the modified lipase were determined as shown in Table 1. From the result of the fluorometric estimation of $K_d$ values, some properties of the modified lipase have been revealed: (i) The $K_d$ values for tripalmitin, triolein, and trilinolein were compared by using the modified lipase B and lipase P. Small differences among these values suggest that the structural difference in the side chain of the glyceride has little effect on the dissociation of glycerides. (ii) $K_d$ values depended significantly on the acylated position in the glycerol moiety of the glyceride. (iii) Comparing modified lipase B with modified lipase P, considerable difference was found between the $K_d$ values of 1,2-dipalmitin and those of 1,3-dipalmitin. For lipase B, the $K_d$ value for tripalmitin was similar to that for 1,3-dipalmitin and was remarkably smaller than that for 1,2-dipalmitin. On the other hand, the $K_d$ value of modified lipase P for tripalmitin did not correspond with that for 1,3-dipalmitin, but with that for 1,2-dipalmitin.

The results of (i) and (ii) suggest that the glycerol moiety of the glyceride might affect the affinity of the modified lipase to the glyceride, with little participation by the side chains of the glyceride. In (iii), comparison of the $K_d$ values between lipase B and lipase P shows the species specificity. The acylation at the second position of the glyceride affects the affinity of lipase P. For lipase B, the acylation at the third position of the glyceride might be responsible for the affinity of enzyme to substrate. This difference may characterize a structural diversity around the active site between lipase B and lipase P.

The modified lipase has encouraged us to investigate the catalytic properties in organic solvents because of its solubility and its activity in organic solvents. Under these nonaqueous conditions, glycerides are not degraded. Previously, lipase has been used in organic solvents as a catalyst. However, kinetic properties of the lipase in organic solvents have not been studied fully. In this work, $K_d$ values for glycerides of lipase were determined in a nonaqueous solution. Studies of the behavior of lipase in a nonaqueous solvent system provide us with a prediction of the product catalyzed by lipase.

In the previous work, fluorometric analysis could detect the interaction between lipase and a water-soluble inhibitor (7). In this work, fluorescence of the tryptophan residue was useful for detection of the conformational change of lipase, even in organic solvents. The accessibility of glyceride to lipase might induce a decrease of the fluorometric intensity, which reflects the steric structural change. One of the tryptophan residues is in the surface loop moiety of lipase. This residue is conserved in many lipases. The decrease of fluorescence intensity may depend on such a tryptophan residue. Although amino acid residues that constitute active sites of some lipases have been identified, the reactive specificities of these lipases can not be accounted for satisfactorily. Not only the active site but also the steric structure that surrounds the active site affects the complicated substrate specificity of lipase. The structural analysis of lipase is in progress with various substrate derivatives.

This procedure, combining the modification of enzyme and fluorometric detection, could possibly be applied to other hydrolysis enzymes that contain esterase, as well as to insoluble proteins, such as membrane-bound proteins.

**TABLE 1**
**$K_d$ Values for the Interaction Between Glycerides and Solvent-Soluble Lipase in Chloroform[a]**

| Enzyme | Glyceride | $K_d$ (mM) |
|---|---|---|
| Lipase P | Tripalmitin | 2.0 |
| | 1,2-Dipalmitin | 2.2 |
| | 1,3-Dipalmitin | 3.6 |
| | 1-Monopalmitin | 10.8 |
| | 2-Monopalmitin | 23.6 |
| | Tripalmitin | 2.0 |
| | Triolein | 2.0 |
| | Trilinolein | 2.0 |
| Lipase B | Tripalmitin | 1.7 |
| | 1,2-Dipalmitin | 3.4 |
| | 1,3-Dipalmitin | 1.8 |
| | 1-Monopalmitin | 11.3 |
| | 2-Monopalmitin | 25.5 |
| | Tripalmitin | 1.7 |
| | Triolein | 1.7 |
| | Trilinolein | 1.7 |

[a]$K_d$, dissociation constant.

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[Received March 10, 1997; accepted October 29, 1997]