Transesterification of Oil by Fatty Acid-Modified Lipase

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Fatty acid was covalently attached to lipase (EC 3.1.1.3.) from *Phycomyces nites*, yielding a modified lipase of higher specific activity in hydrolytic and synthetic reactions in organic solvents. Attached long-chain fatty acids solubilized the lipase in organic solvent and, therefore, promotion of dispersibility in organic solvent resulted in much higher reactivity. The initial rate of transesterification by modified lipase was almost 40 times that of native lipase in organic solvent. The specificities and selectivity of the modified lipase depended on the kind of attached fatty acid.

KEY WORDS: Dispersibility, fatty acid-modified lipase, organic solvent, selectivity, transesterification.

Lipase catalyzes the hydrolysis of lipids in water. However, in organic solvent lipases catalyze the reversible reaction, synthesis of ester and transesterification (1), by the equilibrium shift. However, the stability and dispersibility of native lipase in organic solvent is much poorer than in water. It seems reasonable to attempt to prepare more stable enzymes in organic solvents to overcome this problem.

Covalent attachment of polyethylene glycol (PEG) to free amino groups in protein was at first described by Abuchowski *et al.* (2). Inada *et al.* (3) have prepared some amphipathic enzymes by conjugating polyethylene glycol. They could be dissolved in organic solvent, and such conjugated lipases effectively catalyzed the esterification of lipids in organic solvent. Baillargeon *et al.* (4) reported the effects of PEG chainlength on selectivity. Okumura *et al.* (5) and Dordick *et al.* (6) have prepared modified lipase with alkenyl succinic anhydride and palmitic acid.

We have developed a new method to introduce fatty acid (FA) groups to amino groups in lipase by using the water-soluble acylating reagent, dimethylsulfoniophenyl (DSP) ester (Fig. 1) (7). We found that modified lipase was able to catalyze esterification of triglyceride in *n*-hexane. This chemical modification method with a water-soluble acylating reagent enables chemical modification to be performed under mild conditions with less damage to the enzyme. Moreover, the desired modifying group can be easily introduced to the enzyme. We report here the preparation of FA-modified lipase by using an FA-DSP ester and the effects of FA chainlength and unsaturation on transesterification.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *Phycomyces nites* was obtained from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). The synthetic procedures for the water-soluble acylating reagents have been described elsewhere (8). Fatty acids, triolein and tripalmitin were obtained from Kanto Chemical Co. (Tokyo, Japan).

Dispersibility. To measure the dispersibility of modified lipase in organic solvent, FA-lipase was dissolved in *n*-hexane and centrifuged, and the supernatant was evaporated under N_2. The evaporated residue was redissolved in water and analyzed in duplicate by the Biuret method (9).

Preparation of FA-lipase. Lipase (14 mg) was dissolved in 5 mL of 0.1 M $Na_2B_4O_7$-HCl (borate buffer, pH 7.9), and the intended amount of water-soluble acylating reagent was added. The reaction mixture was allowed to stand at 4°C for 20 h. To remove the precipitate, the reaction mixture was centrifuged at 20,000 × *g* for 10 min before dialyzing the supernatant against deionized water for 72 h with a dialysis membrane (Spectra/Por 1, 8,000 molecular weight cut-off; Spectrum Medical Industries, Los Angeles, CA). After the dialysis, the supernatant was lyophilized, and the lyophilized powder was used for subsequent enzymatic reactions. The modifying groups used are listed in Table 1.

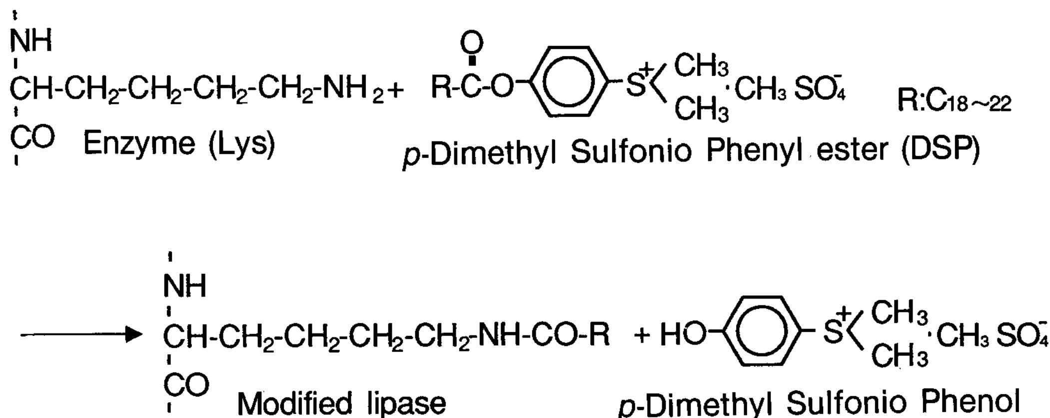

FIG. 1. Preparation of modified lipase.

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TABLE 1

Modifying Group Used for the Modification

Fatty acid	Modification ratio (%)
Stearic acid (C18:0)	38
Stearic acid (C18:0)	52
Stearic acid (C18:0)	72
Stearic acid (C18:0)	81
Oleic acid (C18:1)	50
Linoleic acid (C18:2)	48
Linolenic acid (C18:3)	50
Arachidic acid (C20:0)	47
Behenic acid (C22:0)	51

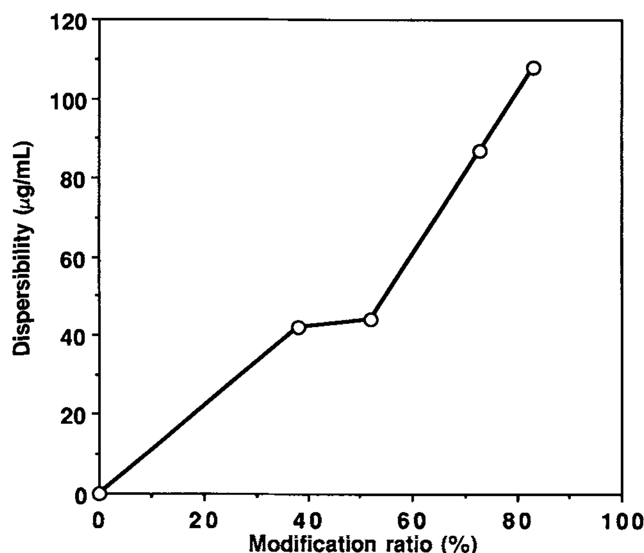

FIG. 2. Effect of modification ratio on dispersibility of C18:0-modified lipase in hexane. Dispersibility was defined as the concentration of protein in supernatant liquid. Modification ratio was defined as the ratio of the modified lysin residues.

Determination of the modification ratio. The modification ratio was determined on the basis of the amount of free amino group within the modified lipase, which was measured by using sodium 2,4,6-trinitrobenzenesulfonate (10). Modified lipase (1 mg), or unmodified lipase as a control, was dissolved in 1 mL water. The solution was mixed with 6 mL of 0.15 M borate buffer (pH 9.2) and incubated at 50°C for 1 h. After cooling the reaction mixture to room temperature, the absorbance was determined at 425 nm. The modification ratio was then calculated as:

$$\text{modification ratio (\%)} = (1 - A/B) \times 100 \qquad [1]$$

where A is A_{425} of the modified lipase and B is A_{425} of the unmodified lipase.

Transesterifications. Lipase-catalyzed transesterification was undertaken in organic solvent by stirring triolein (5 mg), linoleic acid (50 mg) and enzyme (1 mg) in water-saturated *n*-hexane (1 mL) at 37°C. Conversion was measured by the method of Ergan *et al.* (11) and defined by the following formula:

$$\text{conversion (\%)} = \frac{\text{transesterified FA}}{\text{all FA in triglyceride}} \times 100 \qquad [2]$$

Selectivity. The selectivity of the modified lipase for stearic, oleic, linoleic and linolenic acid was measured by transesterifying an equal weight mixture of four kinds of fatty acids (12.5 mg × 4: total 50 mg) with tripalmitin (5 mg) at 37°C for 48 h. The percent conversion to triglyceride was determined by gas-liquid chromatography (GLC) at 175°C with a fused-silica SP-2380 capillary column (0.25 mm i.d. × 30 m). The reaction mixture was first separated by thin-layer chromatography (TLC) on silica gel plate (Type 60, Merck, Darmstadt, Germany). It was developed in a mixture of diethylether/hexane/acetic acid (80:20:1, vol/vol/vol). After visualization with iodine vapor, the band for triglyceride was carefully scraped from the TLC plate and extracted by diethyl ether. The extract was converted to FA methyl esters by 2 N MeOH-KOH for measurement by GLC.

RESULTS AND DISCUSSION

Preparation and characterization. The reagent (*p*-dimethylsulfoniophenol) and FA chloride were reacted to synthesize DSP-FA ester. The activated ester reacted with amino groups of N-terminal and lysine residues on the surface of the enzyme molecules (Fig. 1). The coupling reaction was performed in an aqueous solution under mild conditions, such as low temperature and neutral pH to avoid denaturation of enzymes. The lipase from *P. nites* contained 11 lysine and N-terminal residues (12), and fatty acid was derivatized to them. The modification ratio can be controlled by changing the molar ratio of DSP ester and reaction time to protein in the reaction system.

Figure 2 shows the effect of modification ratio on dispersibility. The dispersibility of four kinds of stearic acid (C18:0)-modified lipase and unmodified lipase (native lipase) in *n*-hexane were measured. It is clear that the dispersibility increased linearly with increasing modification ratio.

Activity of FA-lipase. Figure 3 represents the transesterification activities of unmodified and two kinds of modified lipase in organic solvent. Activities of transesterification in organic solvent were examined with triolein and linoleic acid as substrate in hexane. The transesterification activity of C18:0-modified lipase was much higher than that of unmodified. The initial rate of transesterification by C18:0-modified (modification ratio: 50%) lipase was about 10 times higher than unmodified, and the initial rate by further modified lipase (modification ratio: 80%) was about 40 times higher than for unmodified enzyme. In case of stearic acid modifying, the initial rate increased exponentially with increasing dispersibility (Fig. 4).

Figure 5 shows the effect of FA chainlength of modified lipase on transesterification. Two longer fatty acids were introduced to lipase to obtain arachidic acid (C20:0)-modified lipase and behenic acid (C22:0)-modified lipase, which were supposed to be more hydrophobic than C18:0-modified lipase. The modification ratio of modified lipases was adjusted to about 50%. However, those lipases did not show higher activity in transesterification than C18:0-modified lipase.

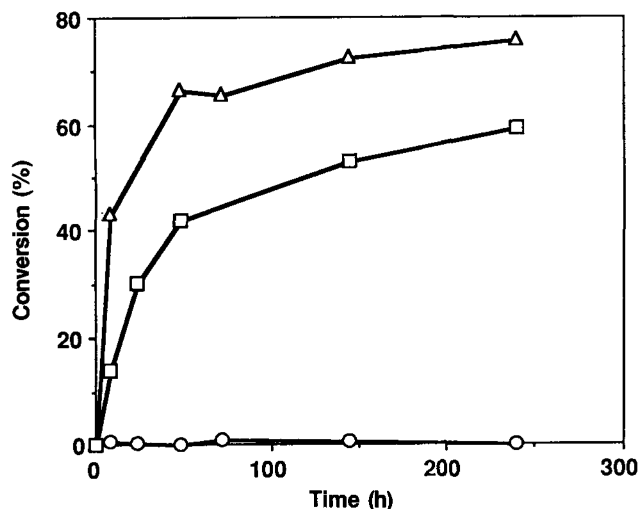

FIG. 3. Effect of modification on transesterification by C18:0-modified lipase in hexane. ○, 0% modified; □, 50% modified; △, 80% modified.

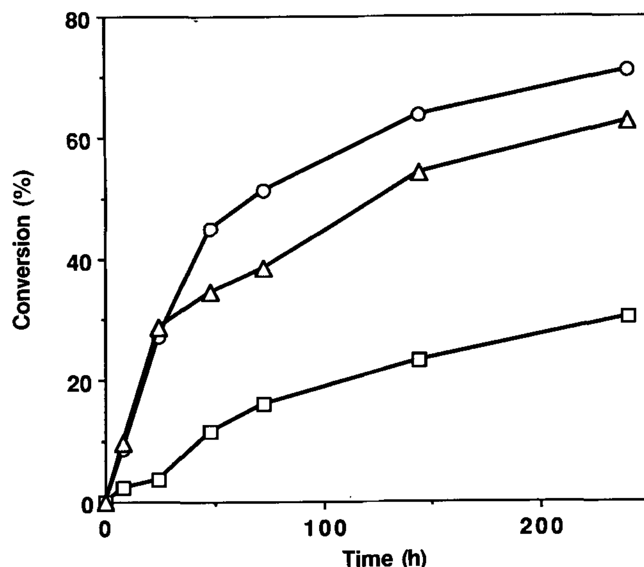

FIG. 5. Effect of the fatty acid chainlength of modified lipase on transesterification in hexane. ○, C18:0-modified lipase; □, C20:0-modified lipase; △, C22:0-modified lipase.

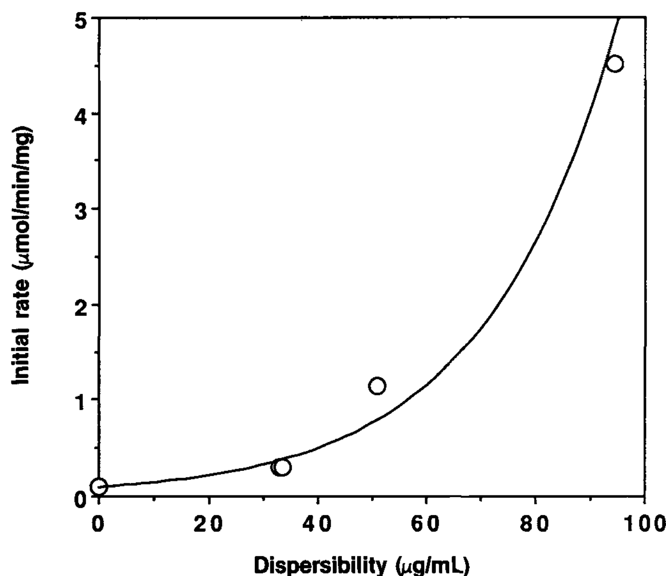

FIG. 4. Effect of dispersibility on the initial rate of transesterification.

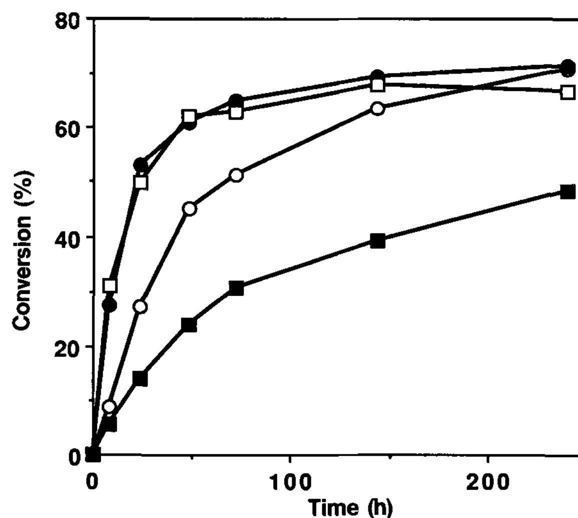

FIG. 6. Effect of degree of unsaturation on transesterification in hexane. ○, C18:0-modified lipase; □, C18:0-modified lipase; ●, C18:2-modified lipase; ■, C18:3-modified lipase.

Figure 6 shows the effect of the degree of unsaturation of fatty acid on transesterification. The FA used, from C18:0 to linolenic acid (C18:3), had the same chainlength but different degrees of unsaturation. Activities of oleic acid (C18:1)- and linoleic acid (C18:2)-modified lipase behaved similarly and were unexpectedly higher than those of C18:0- and C18:3-modified lipase. The initial rate of C18:1- and C18:2-modified lipase was higher than for C18:0- and C18:3-modified lipase when the conversion of all modified lipase finally reached 70%. The results showed that the modified lipase with C18:1 or C18:2 obtained the highest activity to promote transesterification between oleic acid and linoleic acid.

Selectivity. Figure 7 illustrates results of selectivity for the reaction of an equal-weight mixture of stearic, oleic, linoleic and linolenic acids with tripalmitin. The selectivities of each C18:0-, C18:1-, C18:2- and C18:3-modified lipase were obtained. Although C18:1-modified lipase reacted on the average with all four kinds of fatty acids as a substrate, C18:0 modified lipase reacted selectively with stearic acid. Further, C18:2-modified lipase reacted with linolenic acid, and C18:3-modified lipase reacted with linoleic acid selectively. The specificities and selectivity of the modified lipase depend on the kind of attached FA. The tendency appears to be that the modified lipase attached to saturated FA transesterified saturated FA and

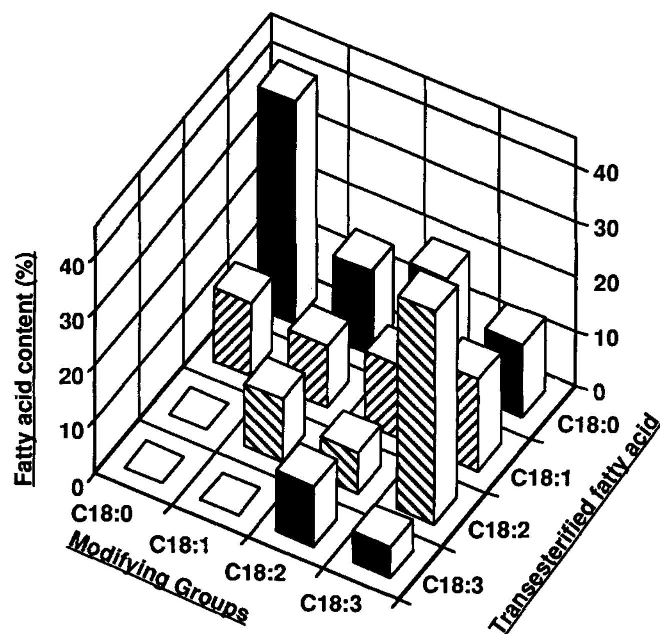

FIG. 7. Effect of degree of unsaturation on the selectivity of transesterification in hexane.

triglyceride, and the modified lipase with unsaturated fatty acid promoted the reaction between unsaturated FA and triglyceride. The method reported here is also useful for the transesterification of other FAs.

FA-modified lipase from *P. nites* has modified characteristics that may be useful commercially. Its greatly increased activity in an organic solvent could lead to use in esterification of fats and oil under mild conditions. The altered selectivity of FA-lipase also has potential.

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