Alcoholysis of Palm Oil Mid-Fraction by Lipase from *Rhizopus rhizopodiformis*

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ABSTRACT: A mycelial lipase from *Rhizopus rhizopodiformis* was prepared in fragment form. The lipase was examined to catalyze the alcoholysis of palm oil mid-fraction (PMF) in organic solvents. High percentage conversions of PMF to alkyl esters were achieved when methanol or propanol was used as acyl acceptor. Of the two most prevalent fatty acids in PMF, palmitic acid seemed to be preferred over oleic acid in the formation of methyl and propyl esters. The optimal ratio of oil to methanol in the alcoholysis reaction is 1 to 2 moles. The lipase exhibited high alcoholysis activities in nonpolar solvents (log $P > 2$), such as hexane, benzene, toluene, and heptane. The enzyme showed exceptionally high thermostability.
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KEY WORDS: Alcoholysis, mycelial lipase, solvents, stability.

Alcoholysis of vegetable oils and animal fats is an important reaction that produces fatty acid alkyl esters that are valuable intermediates in oleochemistry, and methyl and ethyl esters, which are excellent substitutes for diesel fuel (1). Industrially, alcoholysis is usually carried out by heating vegetable oils in alcohols at 100 to 200°C. Because of the high energy cost of the conventional chemical process and the anticipated lower prices of enzymes, industrial application of lipase in the oleochemical industry has become more attractive (2). However, despite its greater potential industrial application, vegetable oil alcoholysis with lipases has not been investigated extensively.

Palm oil is a mixture of triglycerides of various degrees of unsaturation and carbon number. Through industrial processes, palm oil is fractionated into the main products of olein and stearine, and the lesser palm oil mid-fraction (PMF). In this study, the inexpensive PMF is used as the substrate for the alcoholysis reaction.

Mycelial (intracellular) lipases have great potential in the transformation of fats and oils because they catalyze more specific reactions (3,4). We have isolated a thermophilic *Rhizopus rhizopodiformis* from palm oil mill effluent (5). The extracellular enzyme harvested from the fungus has been purified and characterized (6). In this paper, the ability of the mycelial lipase to catalyze alcoholysis reactions is reported. The alcoholysis activities in various organic solvents were examined. The effects of temperature, amount of enzyme used, mole ratio of substrates, and thermostability of the enzyme were investigated.

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EXPERIMENTAL PROCEDURES

Materials. PMF was obtained from the Chemistry and Technology Laboratory, PORIM (Kuala, Lumpur, Malaysia). The fatty acid composition was: myristic acid 1%, palmitic acid 53%, stearic acid 7%, oleic acid 35%, and linoleic acid 4%. From that composition, an average molecular mass of 844 was determined. All other reagents were of analytical grade. The organic solvents and substrate fatty acids and alcohols were dried over molecular sieve 3 Å before use.

Production of mycelial lipase from R. rhizopodiformis. *R. rhizopodiformis* (thermophilic) was isolated and grown in our laboratory (5). After 72 h, the mycelia were collected by filtering the culture and washing the mycelia twice with 100 mL distilled water and 100 mL acetone, each time. The mycelia were then lyophilized in the cold and ground to fragments of about 1 mm size.

Hydrolysis. The reaction system consisted of PMF (2.50 g), $CaCl_2$ solution (20 µL, 0.02 M), and lipase (0.05 g). The mixture was incubated at 37°C for 30 min with continuous shaking at 150 rpm in a horizontal shaker waterbath. The reaction was terminated by dilution with 3.5 mL of ethanol/acetone (1:1, vol/vol). The amount of fatty acids produced was determined by titrating the mixture with 0.05 M NaOH in an automatic titrator (ABU 90; Radiometer, Copenhagen, Denmark) to an end point of pH 10. Specific activity of the enzyme is expressed as µmol free fatty acid produced/min/mg protein.

Esterification reaction. The reaction system consisted of oleic acid or palmitic acid (0.8 mmol), methanol (1.2 mmol), hexane (1.0 mL), and lipase (0.05 g). The mixture was incubated at 37°C for 16 h with continuous shaking at 150 rpm in a horizontal shaker waterbath. The reaction was terminated by dilution with 3.5 mL of ethanol/acetone (1:1, vol/vol), and the remaining free fatty acid in the reaction mixture was de-

termined by titration with 0.05 M NaOH in an automatic titrator (ABU 90; Radiometer) to an end point of pH 10. Specific activity of the enzyme is expressed as µmol of fatty acid used/min/mg protein.

Alcoholysis reaction. The reaction mixture consisted of hexane (3.0 mL), PMF (0.5 mmol), alcohol (1.0 mmol unless otherwise stated), and lipase (0.05 g). The mixture was incubated at 37°C for 16 h (unless otherwise stated) with continuous shaking at 150 rpm in a horizontal shaker waterbath. The reaction was terminated by separating the enzyme from the mixture by centrifugation. The sample was analyzed by injecting 1 µL of reaction mixture and internal standard into a gas chromatograph, equipped with a 30-m fused-silica capillary column, Nukol TM (0.32 mm i.d.) from Supelco Inc. (Bellefonte, PA). Helium was used as carrier gas. The split ratio was 50:1. The injector and detector temperatures were set at 250°C and the column temperature at 200°C. Methyl pentadecanoate and heptadecanoate esters were used as internal standards for methyl esters and propyl esters, respectively.

Thermostability. To investigate the effect of temperature on the stability of the enzyme, the mycelia were incubated in hexane at various temperatures (20, 30, 40, 50, 60, and 70°C) for 1 h in sealed vials. After the incubation, the enzyme mixtures were cooled to room temperature, and the residual alcoholysis activities were determined. The activities were expressed as percentages of the residual activities at different temperatures relative to the activity of the untreated enzyme (kept at room temperature). The stability of the enzyme in hexane, at 70 and 40°C, with respect to time was also investigated. The residual activities were expressed as percentages of the activity of the enzyme at zero time.

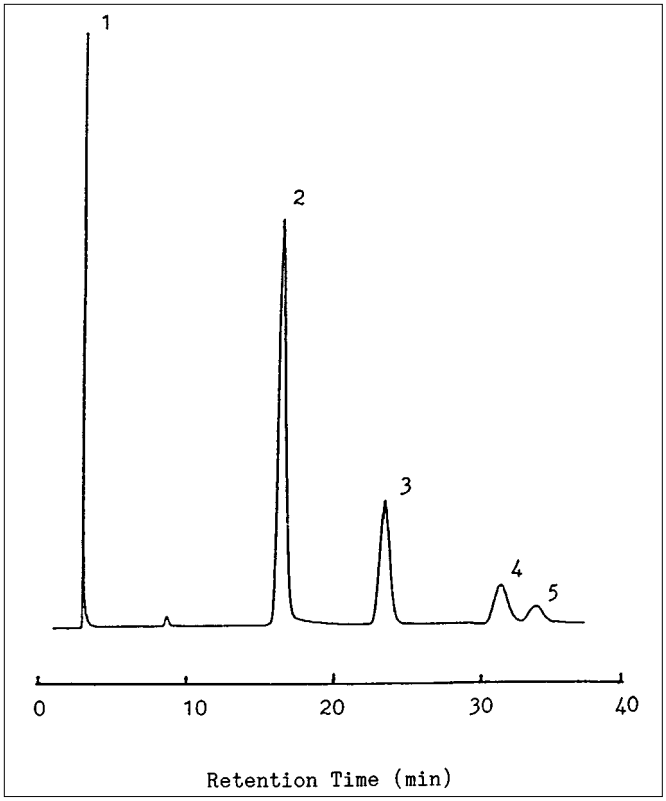

FIG. 1. The chromatogram of products of alcoholysis of palm oil mid-fraction with methanol. Symbols: hexane (1), methyl palmitate (2), methyl pentadecanoate (internal standard) (3), methyl oleate (4), methyl stearate (5); gas chromatography conditions: see the Experimental Procedures section.

RESULTS AND DISCUSSION

Hydrolysis and esterification. Alcoholysis is a transesterification reaction, in which the ester bond of triglycerides is cleaved by lipase to produce fatty acyls that consequently react with the alcohols to form alkyl esters. Initially, lipase from *R. rhizopodiformis* was tested for both hydrolysis and esterification reactions. The hydrolysis and esterification activities of the lipase were 0.22 µmol fatty acid produced/min/mg enzyme and 0.102 µmol fatty acid used/min/mg enzyme, respectively. The result showed that lipase from *R. rhizopodiformis* mycelial preparation catalyzed hydrolysis and esterification reactions and consequently might catalyze the alcoholysis of PMF.

Alcoholysis of PMF. A typical chromatogram of the products of the alcoholysis reaction of PMF and methanol is shown in Figure 1. The effect of time on the alcoholysis reaction by mycelial lipase from *R. rhizopodiformis* to yield the respective esters is shown in Figure 2. After 10 h of reaction, the conversion of PMF to methyl palmitate was more than 60% whereas the conversion of PMF to methyl oleate was about 10%. In this investigation, the palmitic acid in PMF produced higher conversions to the respective esters than oleic acid. This result is in agreement with Wang *et al.* (7),

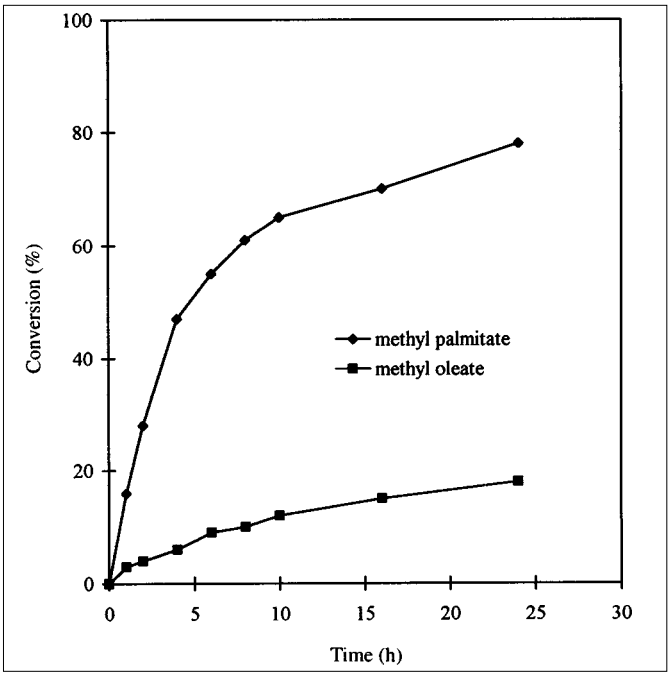

FIG. 2. Progress of alcoholysis of palm oil mid-fraction with methanol by lipase from *Rhizopus rhizopodiformis.*

who showed that lauric acid as the major component of palm kernel oil exhibited a higher yield conversion in alcoholysis than the other fatty acids.

It seemed that the yields of esters were also affected by the type of alcohol used. A different reaction rate was observed between methanolysis and propanolysis of PMF. The percentages of conversion of PMF to propyl palmitate and propyl oleate after 10 h were 85 and 15%, respectively (Fig. 3). Propanol was preferred as co-substrate as compared to methanol. A similar result was reported by Mittelbach (1), wherein the alcoholysis activity of lipase from *Pseudomonas fluorescens* increased with alcohols of higher chainlength.

Effect of molar ratio of substrates on alcoholysis. The optimal molar ratio of oil/methanol in the alcoholysis reaction is 1:2 (Fig. 4). This finding correlates with the mechanism of alcoholysis by lipase with 1,3-positional specificity in which 1 mole of oil needs 2 moles of alcohol to react. It indicates that mycelial lipase from *R. rhizopodiformis* has similar regiospecificity as the extracellular lipase (6). Increasing the mole ratio of propanol to PMF decreased alcoholysis activity. This observation may reflect the ability of the excess methanol (polar solvent) to distort the essential water layer that stabilizes the enzyme (8). Kanasawud *et al.* (9) showed that an excessive amount of alcohol affects the rate but not the equilibrium of the reaction. Contrary to this, increasing the methanol-to-oil ratio resulted in increasing the yield of methyl ester until the optimal level was achieved (2).

Alcoholysis in the presence of organic solvents. Alcoholysis activities of lipase from *R. rhizopodiformis* in various organic solvents are shown in Table 1. The lipase exhibited high alcoholysis activities in such nonpolar solvents (log $P > 2$) as hexane, benzene, toluene, and heptane. When polar organic solvents (log $P < 2$), such as pyridine and dimethylformamide, were used, no activity was detected. However, the considerable activity in acetone (log $P = -0.23$) could not be explained on that basis. Hexane appeared to be the best solvent for the alcoholysis reaction. This finding was in agreement with those of Laane *et al.* (8) who demonstrated that the activities of lipase were enhanced by changing the reaction

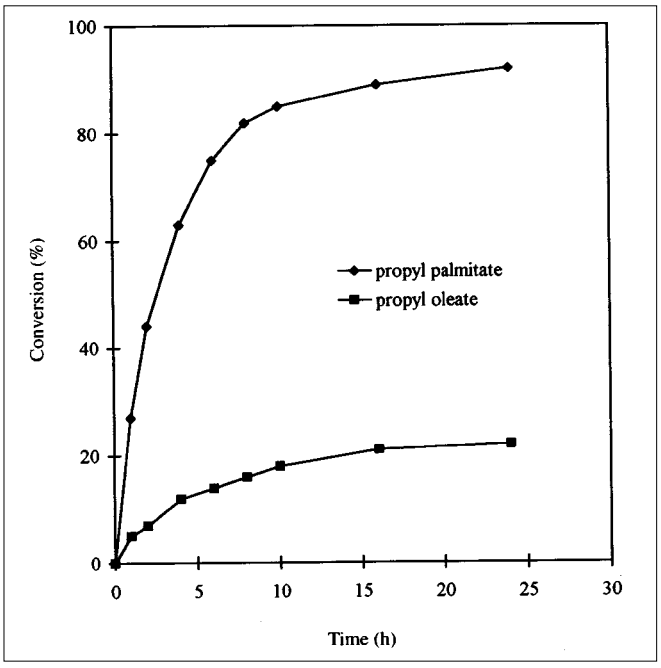

FIG. 3. Progress of alcoholysis of palm oil mid-fraction with propanol by lipase from *Rhizopus rhizopodiformis*.

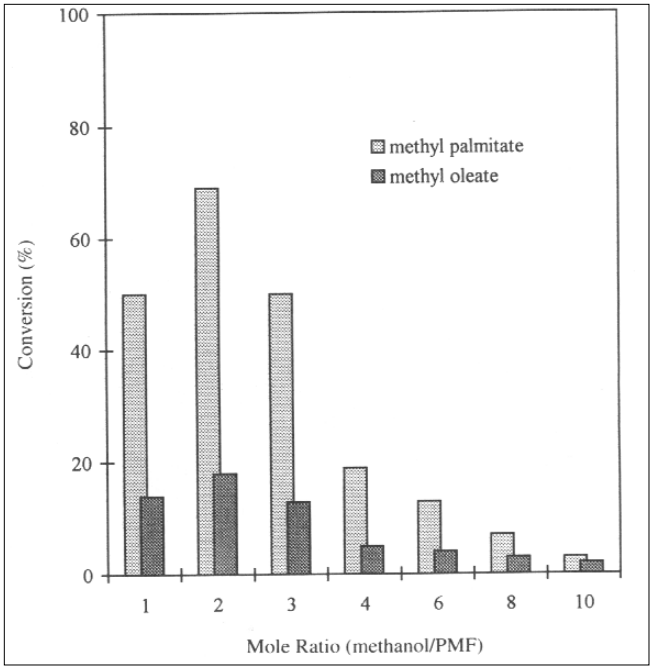

FIG. 4. The effect of mole ratio of substrates on the alcoholysis of palm oil mid-fraction (PMF) with methanol and propanol.

TABLE 1
Alcoholysis Activities of Lipase in Various Organic Solvents

Solvent	log P[a]	Activity[b] (μmoles/min/g enzyme)	(%)[c]
DMF	–1.00	0	(0)
Acetone	–0.23	10.6	(39)
Pyridine	0.71	0	(0)
Chloroform	2.0	0	(0)
Benzene	2.0	25.5	(96)
Toluene	2.5	20.6	(77)
Hexane	3.5	26.6	(100)
Heptane	4.0	21.4	(81)
Octane	4.5	17.2	(65)
Nonane	5.1	16.1	(61)
Decane	5.6	10.1	(38)
Undecane	6.1	10.9	(41)
Dodecane	6.6	7.8	(29)
Hexadecane	8.8	9.1	(34)

[a]From Laane *et al.* (8).
[b]Activities are expressed as μmol ester/min/g enzyme. The alcoholysis reaction is followed by the rate of appearance of esters from the reaction mixture containing propanol and palm oil mid-fraction.
[c]Percentage activities are expressed relative to the alcoholysis reaction of lipase in hexane; DMF, dimethylformamide.

medium. There is no activity of the lipase in chloroform, although log $P = 2.0$. This is probably due to the inability of the solvent to dissolve PMF under the reaction conditions. In solvents of high log *P,* such as dodecane and hexadecane, the activities were also relatively lower. This decreased alcoholysis could be due to the relatively high viscosity of the solvents, which hindered efficient interaction between the catalyst and the substrates.

Thermostability of lipase from R. rhizopodiformis *in alcoholysis reaction*. Mycelial lipase from *R. rhizopodiformis* exhibited high stability when incubated for 1 h at temperatures of 20 to 70°C (Fig. 5). The residual activities were high (above 80%) when it was incubated at 60°C. The residual activity was 70% when incubated at 70°C. Figure 6 shows the residual activity of lipase incubated at 70 and 40°C with respect to time. The half-life of lipase at 70°C was 5 h. At 40°C, the residual activity remained above 70%, even after 24 h of incubation. The high thermostability of the lipase is expected because the enzyme is immobilized in its native environment, the mycelial wall, which could protect the enzyme from deactivation by heat. Studies by Okumura *et al.* (10) showed that immobilization of an enzyme in its native, mycelial environment gave low deactivation rates compared to free enzymes. Hirano *et al.* (11) found that amino acylase immobilized onto the amino acylase-producing microorganism *Aspergillus ochroceus* was more efficient than when immobilized on other supports.

ACKNOWLEDGMENT

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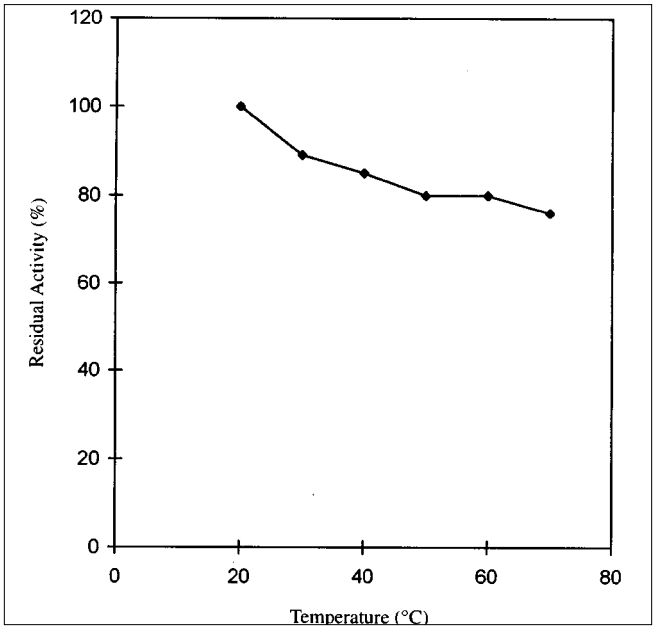

FIG. 5. Thermostability of lipase from *Rhizopus rhizopodiformis* incubated for 1 h.

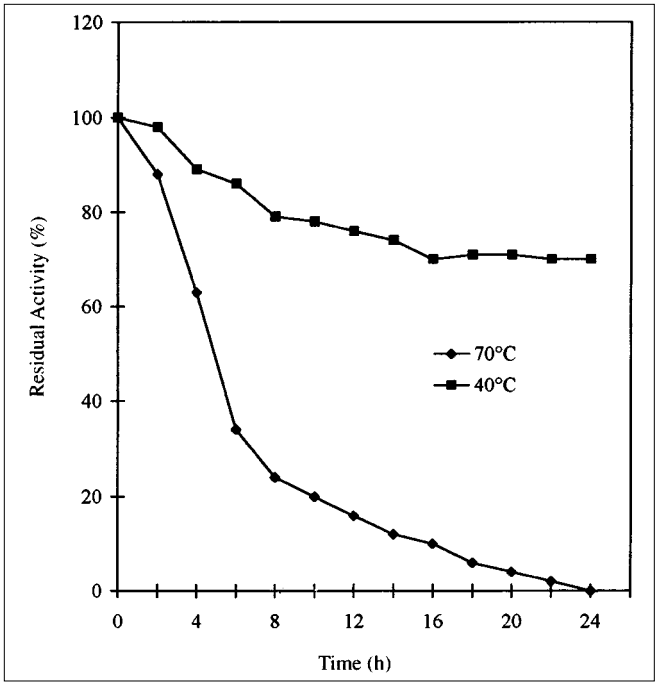

FIG. 6. Thermostability of lipase from *Rhizopus rhizopodiformis* with respect to time at 40 and 70°C.

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