# Transesterification of Phospholipids in Different Reaction Conditions

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**Transesterification of synthetic dimyristoyl phosphatidylcholine with oleic acid by commercial lipase preparations from *Aspergillus niger* and *Rhizomucor miehei* was studied in the presence and absence of solvent. A high-performance liquid chromatography method for determination of the modified phosphatidylcholine was developed. Under solvent-free conditions, transesterification could be carried out as efficiently as in toluene, and the degree of hydrolysis was lower than in toluene. Transesterification was influenced by the water content as well as by the fatty acid concentration in the reaction mixture. The optimum water content for transesterification in solvent-free reaction medium was higher than in toluene with both lipases. The yield of modified phosphatidylcholine increased, and the degree of hydrolysis decreased with increasing fatty acid concentration. The maximum yield of modified phosphatidylcholine, 35% of the original phospholipid, was obtained with *R. miehei* lipase.**

KEY WORDS: Lipases, phospholipids, phospholipases, transesterification.

Phospholipids with specific fatty acid composition are desirable for medical and nutritional applications. By exchanging fatty acids in the phosphatidylcholine (PC) molecule, new physical properties can be obtained. Solvent fractionation as well as chemical and enzymatic modifications are being studied to produce different types of phospholipids (1). Enzymatic conversion is advantageous because of its high regiospecificity and mild reaction conditions.

Phospholipases $A_1$ and $A_2$, which are specific for the hydrolysis of fatty acids at the 1- and 2-position of the phospholipids, are most likely to catalyze the transesterification reaction of phospholipids. However, transesterification reactions with phospholipases have not been reported. There are several reports on interesterification reactions of triglycerides with lipases (2), and recently, lipases have also been found to be efficient in the transesterification of phospholipids.

Lipase-catalyzed transesterification is an opposing reaction, which requires a low water content. By using organic solvents, an optimum low water content necessary to diminish the hydrolysis reaction can be obtained. The most common solvents used are hexane and toluene (3,4).

Microbial lipases from *Rhizopus, Candida* and *Rhizomucor* have recently been used for transesterification of phospholipids with different fatty acids (5–10) and edible oils (11). Yoshimoto *et al.* (5) used polyethyleneglycol-modified *Candida cylindracea* lipase, which is soluble in organic solvents, in the transesterification of dipalmitoyl PC with eicosapentaenoic acid in water-saturated benzene. The yield of eicosapentaenoyl palmitoyl PC obtained was low, about 3%. A hexane/buffer two-phase system was used by Yagi *et al.* (6) for transesterification of PC and phosphatidylethanolamine with different fatty acids by *Rhizopus delemar* lipase. The yields were rather low, between 11 and 37%. Totani and Hara (11) studied the preparation of polyunsaturated phospholipids by transesterification of soybean lecithin with sardine oil by *R. delemar* and *C. cylindracea* lipases in a water/hexane two-phase system. The total percentage of polyunsaturated fatty acids incorporated was 18.4%. In these systems, the predominant problems were the hydrolysis reactions and consequent low yields. Higher yields were obtained in the transesterification of egg PC with heptadecanoic acid in toluene by *R. arrhizus* lipase immobilized on polypropylene (8). A yield of 60% with almost 50% incorporation of heptadecanoic acid was obtained. With *Rhizomucor miehei* lipase, the incorporation varied between 33 and 45% (8).

Transesterification by lipases has been shown to occur mainly at the 1-position of the phospholipid (6,8,11). Transesterification at the 2-position of the phospholipid by lipases or by phospholipase $A_2$ has not been presented hitherto. On the other hand, 1-acyl-lysophosphatidylcholine can be esterified with a desired fatty acid by phospholipase $A_2$ (12).

The determination of modified phospholipid has mostly been based on thin-layer (TLC) and gas–liquid chromatographic (GLC) methods. TLC has been used for separation of modified phospholipid from the reaction mixture and GLC for determination of the fatty acid composition of the phospholipid. This method is both laborious and coarse.

We have previously studied the modification of phospholipids by hydrolysis and transesterification (13,14). The aim of this work was to compare the efficiency of commercially available lipase preparations in the transesterification of synthetic dimyristoyl PC (PC–MM) with oleic acid, and to determine the suitability of a solvent-free system as compared with toluene as a reaction medium. The reactions were monitored by high-performance liquid chromatography (HPLC) analysis for the appearance of modified PC.

## EXPERIMENTAL PROCEDURES

*Enzyme sources.* Lipases from *Aspergillus niger* (Biocatalysts Ltd., Pontypridd, Great Britain), *C. cylindracea* (Biocatalysts Ltd.), *Penicillium cyclopium* (Biocatalysts Ltd.) and *R. miehei* immobilized on anion exchange resin (Lipozyme; Novo Industri A/S, Bagsvaerd, Denmark), and phospholipases $A_1$ and $A_2$ from *A. niger* (Biocatalysts Ltd.) were used in this work.

*Materials.* PC–MM (P 0888) and oleic acid (0 1630) were purchased from Sigma Chemical Co. (St. Louis, MO). The support used for immobilization of enzymes was Celite No. 535 (Johns-Manville Corporation, Denver, CO).

*Immobilization. Aspergillus niger* lipase was immobilized on diatomaceous earth (Celite) by adsorption. Celite was washed with distilled water and dried in an oven at 105°C for 20 h before immobilization. Crude lipase powder (250 mg) was suspended in 20 mL 20 mM acetate buffer, pH 4.5, containing 20 mM $CaCl_2$, and centrifuged for 5 min at 2000 rpm to remove the insoluble material. The enzyme solution was mixed with Celite (2 g), and the suspension was stirred for 24 h at room temperature. The support, containing immobilized lipase, was filtered with

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suction, washed with distilled water and dried by lyophilization.

*Enzyme assays.* Phospholipase activity was determined with 2% soybean lecithin (Sigma P-5638) as substrate. Lecithin was suspended in water with a magnetic stirrer. $CaCl_2$ (10 mM) was added to the suspension, and the pH was adjusted to 4.5. The suspension was homogenized for 60 s. The reaction mixture, consisting of 25 mL of substrate and 1 mL of the enzyme solution, was incubated for 30 min at 37°C with magnetic stirring. The liberated fatty acids were titrated immediately with 0.05 N sodium hydroxide solution in an automatic titrator. One unit of phospholipase activity (nkat) was defined as the amount of enzyme that liberated 1 nmol of fatty acid per second under the assay conditions.

Lipase activity was assayed by the olive oil emulsion method (15). One unit of lipase activity (nkat) was defined as the amount of enzyme that liberated 1 nmol of fatty acid per second under the assay conditions.

Protein contents in the enzyme preparations were determined by the method of Lowry *et al.* (16) with bovine serum albumin as standard.

*HPLC analysis.* The chromatographic system consisted of an M-6000A pump, 717 automatic injector and RCM 8 × 10 column module, which was normally thermostated to 35°C, and an M-411 refractive index detector. The data were handled, and the chromatographic system was controlled by a Milenium computer program. The equipment was from Waters (Milford, MA). The column used was a Nova-Pak C18 (8 × 100 mm, 4 μm particles; Waters) and methanol, containing 100 mM tetramethylammonium chloride, was used as the eluent. The standard curves [PC–MM and dioleyl phosphatidylcholine (PC–OO)] were linear in the concentration range of 10–5000 mg/L with correlation coefficients better than 0.99.

*Water content.* Water contents of the reactants were determined by Karl Fischer titration (ISO 760).

*Transesterification.* The reaction mixture contained 100 mM PC–MM, 400 mM oleic acid in toluene (1 mL) and 150 mg immobilized lipase or phospholipase preparation in screw-capped tubes unless otherwise indicated. In reaction mixtures without organic solvent, PC–MM was dissolved in oleic acid (1 mL). The tubes were incubated in a water bath at 40°C with magnetic stirring (150 rpm) for 24 h. After incubation, the immobilized enzymes were filtered out and washed with methanol to remove the adsorbed reaction products. The combined solvents were removed by evaporation. The residues were redissolved in 2 mL of methanol for HPLC analysis.

## RESULTS AND DISCUSSION

*Enzyme preparations.* Commercial lipase preparations from *A. niger, R. miehei, C. cylindracea* and *P. cyclopium* and two phospholipases ($A_1$ and $A_2$) from *A. niger* were first tested for their ability to catalyze the transesterification reaction. *Penicillium cyclopium* and phospholipase $A_2$ were inactive, whereas the other enzymes exhibited measurable activities (results not shown). The most active *R. miehei* and *A. niger* lipases were selected for further studies. In addition to lipase, these two enzymes also contained phospholipase activity.

1,3-Specific *R. miehei* lipase (Lipozyme), immobilized on anion exchange resin (Duolite), is marketed for esterification and transesterification reactions of triglycerides. This preparation showed rather low hydrolytic lipase and phospholipase activities in the assay methods used (Table 1). *Aspergillus niger* lipase preparation is also 1,3-specific in hydrolyzing the ester linkages of triglycerides (17). The protein content of the powdered *A. niger* lipase preparation was 5%. It contained 133000 nkat/g lipase activity and 45000 nkat/g phospholipase activity.

Immobilization of lipases on suitable carriers protects the enzymes from the solvent environment and enhances their stability. Therefore, the free *A. niger* lipase was immobilized by adsorption on diatomaceous earth for the transesterification experiments. The activity yields of phospholipase and lipase activities of the *A. niger* lipase preparation in immobilization were about 36 and 30%, respectively. After immobilization, the phospholipase activity of the preparation was 2000 nkat/g, which was 20 times higher than that of the *R. miehei* enzyme (Table 1).

*Transesterification of PC.* The transesterification reaction of PC–MM with oleic acid was chosen as a model reaction because of good resolution of PC–MM, oleyl myristoyl PC (PC–OM) and PC–OO in the HPLC analysis (Fig. 1). The HPLC method developed was rugged because the reaction mixture could be injected directly into the system without any preparation. The experiments were carried

**TABLE 1**

**Phospholipase and Lipase Activities of the Immobilized Enzyme Preparations**

| Enzyme | Phospholipase activity (nkat/g) | Lipase activity (nkat/g) |
|---|---|---|
| *Aspergillus niger* lipase[a] | 2000 | 5000 |
| *Rhizomucor miehei* lipase[b] (Lipozyme) | 100 | 700 |

[a]Immobilized on Celite.
[b]Commercially available, immobilized on Duolite.

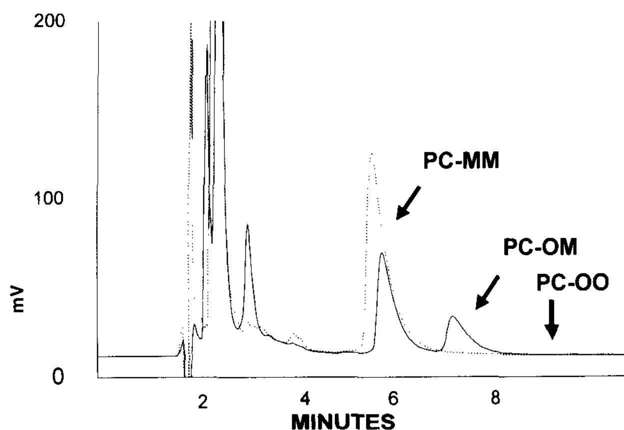


**FIG. 1. High-performance liquid chromatography chromatograms of the reaction mixture of 100 mM dimyristoyl phosphatidylcholine (PC–MM) and 400 mM oleic acid after transesterification reaction of 24 h in toluene at 40°C in the absence (---) and in the presence of *Rhizomucor miehei* lipase (——). PC–OO, dioleyl phosphatidylcholine; PC–OM, oleyl myristoyl phosphatidylcholine.**

out both without solvent and in toluene. Toluene was selected as a reaction solvent because it has previously been shown to be appropriate for transesterification reactions of phospholipids (8). In solvent-free reaction medium, PC–MM was dissolved in oleic acid (3000 mM).

The effects of enzyme, substrate and water concentration and reaction time were studied with immobilized lipases from *A. niger* and *R. miehei* at a constant reaction temperature (40°C).

*Effect of water content.* Transesterification of PC–MM with oleic acid was first studied at various enzyme concentrations (50–450 mg/reaction mixture). A suitable enzyme concentration for the transesterification reaction was 150 mg in toluene as well as in solvent-free reaction medium.

In solvent-free reaction medium, the optimum water content for the transesterification reaction was higher than in toluene. The optimum water contents for *A. niger* and *R. miehei* lipases in the transesterification reaction of 100 mM PC–MM with 400 mM oleic acid in toluene were about 0.5 and 1%, respectively (Fig. 2a). The corresponding values in solvent-free medium were 1 and 2.5% (Fig. 2b). The difference between these lipases could be largely due to the different water sorption isotherms of the carriers. The Duolite carrier of *R. miehei* lipase, with a larger specific surface area, was more water-adsorbing than diatomaceous earth.

Various water concentrations have been used in transesterification reactions of phospholipids. Very high water content (37–50%) was used in the transesterification of soybean phospholipids with fish oil (11) and in the transesterification of dipalmitoyl PC with oleic acid (6). No water was added to the reaction mixture, and the water content was not controlled in the transesterification reaction in organic solvent reported by Yoshimoto *et al.* (5) and Mutua and Akoh (10). Svensson *et al.* (8) expressed the water content as thermodynamic water activity.

The yield of PC–OM from the original amount of PC–MM by *R. miehei* lipase was 19% in toluene at a water content of 1 and 17% in solvent-free medium at a water content of 2.5% (Fig. 2a). The amounts of residual PC (PC–MM + PC–OM) were 27 and 70%, respectively. With *A. niger* lipase, the yield of PC–OM was 12% in toluene at a water content of 0.5 and 13% in solvent-free medium at a water content of 1% (Fig. 2b). The amounts of residual PC from the original PC–MM were 25 and 72%, respectively.

The transesterification reaction occurred in parallel with hydrolysis. The extent of hydrolysis increased with increasing water content and was much higher in toluene than in solvent-free medium. The mechanism of transesterification involves LPC as an intermediate product, followed by incorporation of a new free fatty acid to the LPC (8). As the amount of water was increased, more PC–MM was hydrolyzed without subsequent reesterification. However, at low water content (below 0.5%), the transesterification was retarded.

It has been reported that lipases catalyze the acyl exchange reaction only at the 1-position of the phospholipids (6,8,11). However, possible phospholipase activity in the lipase preparations used in these studies was not determined. On the basis of the cited reports, the transesterified PC produced in our experiments was probably 1-oleyl 2-myristoyl PC. Formation of PC–OO was not observed.

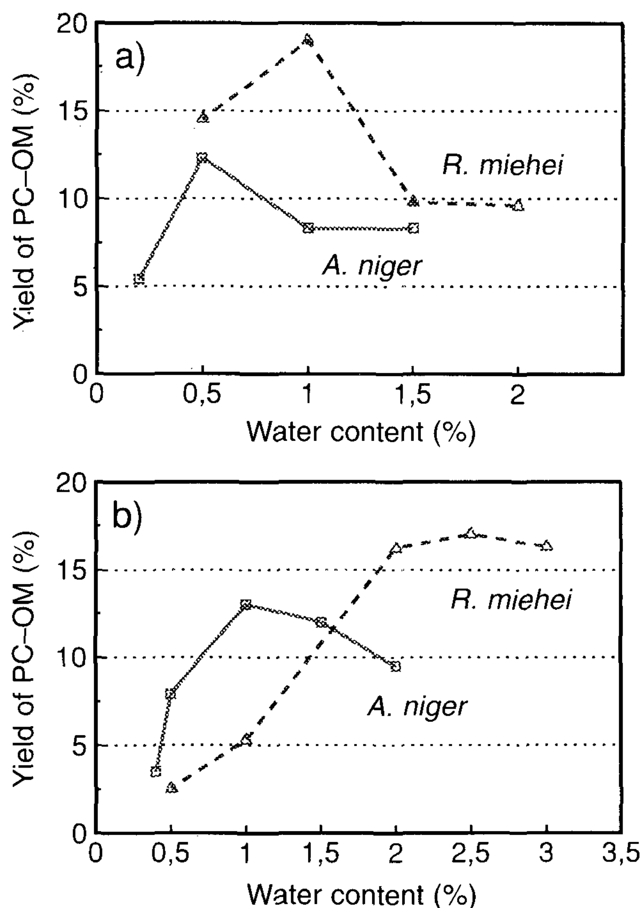


**FIG. 2. Effect of water content on the yield of PC–OM in the transesterification reaction of PC–MM (100 mM) with oleic acid (400 mM) by *Rhizomucor miehei* and *Aspergillus niger* lipases after 24 h at 40°C in (a) toluene and in (b) solvent-free medium. See Figure 1 for abbreviations.**

*Effect of substrate concentration.* The effects of PC–MM and oleic acid concentrations on the extent of the transesterification reaction in toluene and in solvent-free reaction medium were compared. At a constant oleic acid concentration (400 mM) with *A. niger* lipase in toluene, an almost linear increase of PC–OM was observed with increasing PC–MM concentration from 60 to 150 mM, although the enzyme/substrate ratio decreased with increasing PC–MM concentration (Fig. 3a). The maximum yield of PC–OM, as measured from the original PC–MM, was 12% at a water content of 1%. With *R. miehei* lipase, the amount of PC–OM increased with increasing PC–MM concentration from 60 to 100 mM but decreased above 100 mM. The maximum yield of PC–OM of the original PC–MM was 19% at a water content of 1% (Fig. 3a). The degree of hydrolysis decreased with increasing PC–MM concentration. The amounts of residual PC remaining, when using *A. niger* and *R. miehei* lipases and a PC–MM concentration of 150 mM, were about 30 and 50%, respectively.

Comparable data were obtained when working with increased PC–MM concentration in solvent-free reaction medium (3000 mM oleic acid) (Fig. 3b). The amount of

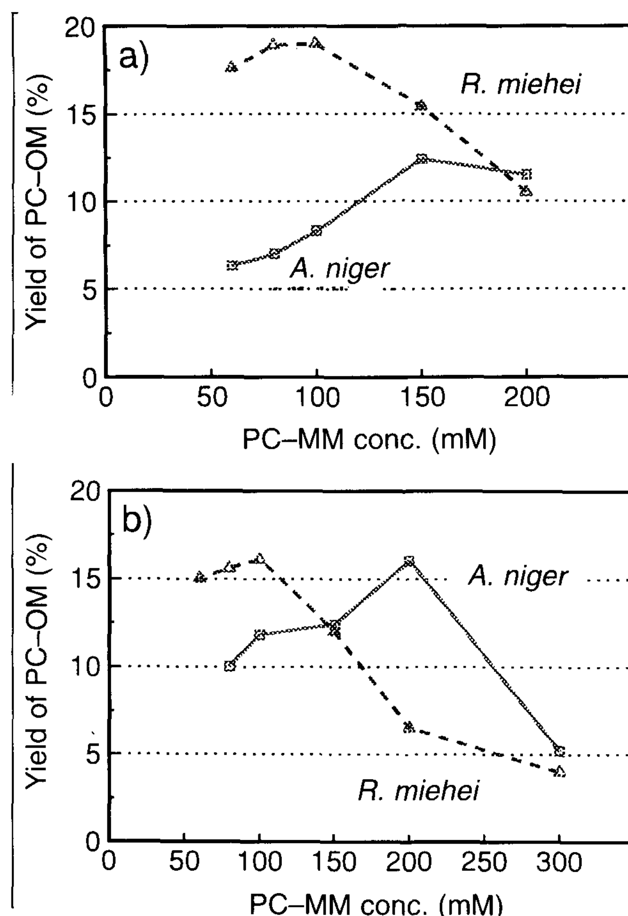


**FIG. 3. Effect of PC-MM concentration at constant oleic acid content (400 mM) on the yield of PC-OM in the transesterification reaction by *Rhizomucor miehei* and *Aspergillus niger* lipases after 24 h at 40°C in (a) toluene and in (b) solvent-free medium. See Figure 1 for abbreviations.**

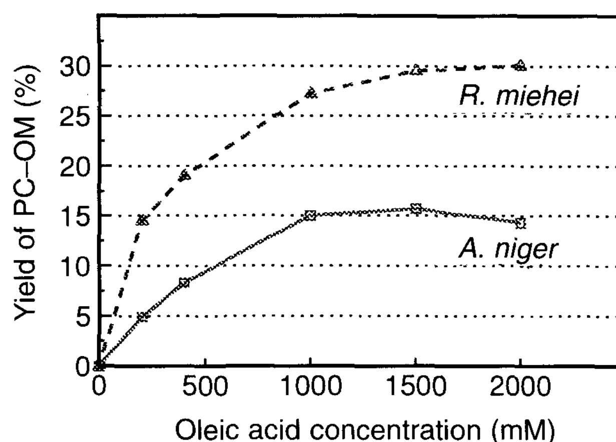


**FIG. 4. Effect of oleic acid concentration at constant PC-MM content (100 mM) on the yield of PC-OM in the transesterification reaction in toluene by *Rhizomucor miehei* and *Aspergillus niger* lipases after 24 h at 40°C. See Figure 1 for abbreviations.**

PC-OM formed by *A. niger* lipase increased linearly up to 200 mM PC-MM at a water content of 1%. The maximum yield of PC-OM was about 16%. The optimum PC-MM concentration for *R. miehei* lipase was 100 mM at a water content of 1%. The maximum yield of PC-OM was 16%. The ratio of oleic acid to PC-MM concentration was 15:1 for *A. niger* lipase and 30:1 for *R. miehei* lipase. The degree of hydrolysis was much lower in solvent-free medium than in toluene. It decreased with increasing PC-MM concentration. The amount of residual PC was 77% with *A. niger* lipase and 80% with *R. miehei* lipase at the optimum PC-MM concentration.

The yield of PC-OM could be increased by increasing the oleic acid concentration in the toluene-containing reaction mixture. Furthermore, the presence of high concentrations of oleic acid had an inhibitory effect on the hydrolysis reaction in toluene with both lipases. This could be due to the inhibition of lipases by free fatty acids, which is a well-known phenomenon in emulsion systems (18). The optimal oleic acid concentration for *A. niger* lipase was 1500 mM at a PC-MM concentration of 100 mM and at a water content of 1%. The yield of PC-OM was 15%. The corresponding value for *R. miehei* lipase was about 2000 mM. The yield of PC-OM was 30% from the original PC-MM (Fig. 4). The optimum ratio of oleic acid to PC-MM concentration was 15:1 for *A. niger* lipase and 20:1 for *R. miehei* lipase. The amount of residual PC was 36% with *A. niger* lipase and 53% with *R. miehei* lipase at the optimum oleic acid concentration.

*Effect of reaction time.* The degree of transesterification by both lipases increased during the first 16 h of the reaction in toluene. The PC-OM content remained approximately constant after 16 h of incubation (Fig. 5a). The transesterification reaction in solvent-free reaction medium proceeded only slowly after 16 h (Fig. 5b). The degree of hydrolysis increased with reaction time in both systems (Fig. 6). The extent of hydrolysis at the reaction time of 24 h with both lipases was lower in solvent-free medium, 28% (*R. miehei*) and 35% (*A. niger*), than in toluene (43 and 52%, respectively).

*The transesterification activity of phospholipase $A_1$.* The activity of *A. niger* phospholipase $A_1$ preparation was compared with that of *R. miehei* and *A. niger* lipases in the transesterification reaction. In addition to phospholipase (6700 nkat/mL), this liquid enzyme preparation suitable for food applications also contained high lipase activity (14260 nkat/mL). After immobilization on diatomaceous earth, it contained higher activities than *A. niger* and *R. miehei* lipases. Phospholipase activity was 3000 nkat/g and lipase activity 6500 nkat/g.

Phospholipase $A_1$ showed similar activity to that of *A. niger* lipase in the transesterification reaction of 100 mM PC-MM with oleic acid (400 mM) at a water content of 0.5%. The yield of PC-OM and the amount of residual PC in toluene were 12 and 67%, respectively. The corresponding values in solvent-free reaction medium were 10 and 73%. In conclusion, transesterification of PC-MM with oleic acid by *A. niger* and *R. miehei* lipases was as efficient in solvent-free reaction medium as in the presence of toluene. Transesterification was markedly affected by water concentration. In solvent-free medium, the optimum water content for the transesterification reaction was higher, and the degree of hydrolysis was lower than in toluene. The oleic acid concentration in the reaction mix-

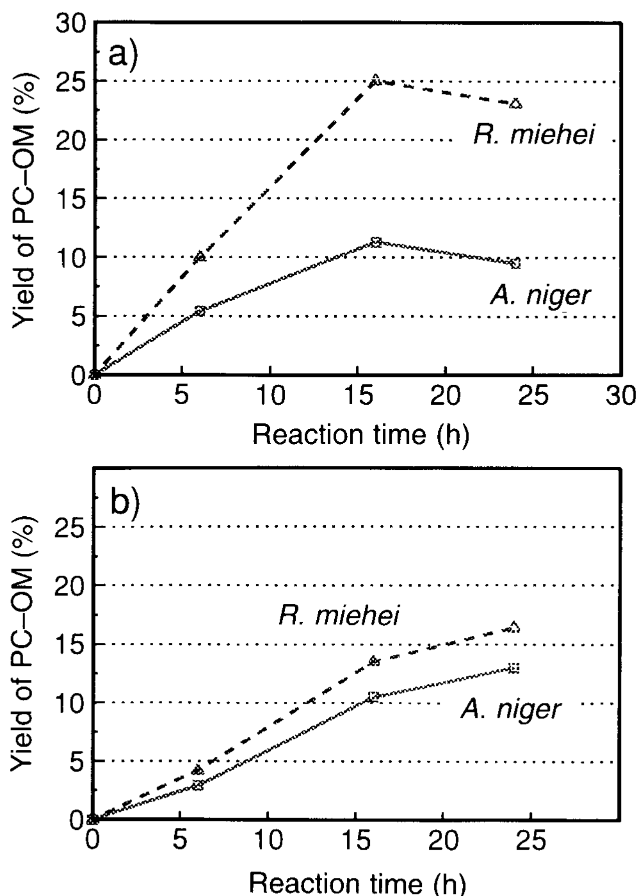


**FIG. 5. Effect of reaction time on the yield of PC–OM in the transesterification reaction of PC–MM (100 mM) with oleic acid (400 mM) by *Rhizomucor miehei* and *Aspergillus niger* lipases at 40°C in (a) toluene in (b) solvent-free medium. See Figure 1 for abbreviations.**

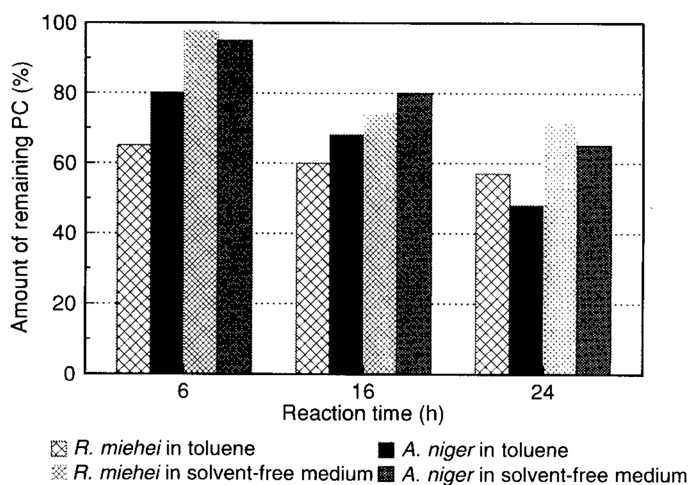


**FIG. 6. Effect of reaction time on the amount of residual PC in the transesterification reaction of PC–MM (100 mM) with oleic acid (400 mM) in toluene and in solvent-free medium by *Rhizomucor miehei* and *Aspergillus niger* lipases at 40°C. See Figure 1 for abbreviations.**

ture influenced the extent and the yield of transesterification. With increasing fatty acid concentration, the yield of transesterified phospholipid increased and the degree of hydrolysis decreased. The phospholipase activities of the immobilized enzymes did not correlate with the transesterification activities. Although the phospholipase and lipase activities of the *R. miehei* lipase preparation were low, it was more active in transesterification than the *A. niger* lipase preparation with higher hydrolytic activities.

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