Use of Thermostable *Fusarium heterosporum* Lipase for Production of Structured Lipid Containing Oleic and Palmitic Acids in Organic Solvent-Free System

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ABSTRACT: A newly developed 1,3-positionally specific thermostable lipase from *Fusarium heterosporum* (named R275A lipase) was immobilized on Dowex WBA for the production of structured lipid by acidolysis of tripalmitin (PPP) with oleic acid (OA). The immobilized catalyst was fully activated by pretreatment at 50°C in a PPP/OA mixture containing 2% water. The pretreatment caused concomitant hydrolysis, but the hydrolysis was repressed using a substrate without water in the subsequent reactions. The optimal reaction conditions were determined as follows: A mixture of PPP/OA (1:2, w/w) and 8% immobilized lipase catalyst was incubated at 50°C for 24 h with shaking at 130 oscillations/min. The acidolysis reached 50% under these conditions, and the contents of triolein, 1,3-dioleoyl-2-palmitoyl-glycerol, 1(3),2-dioleoyl-3(1)-palmitoyl-glycerol, 1(3),2-palmitoyl-3(1)-oleoyl-glycerol, 1,3-dipalmitoyl-2-oleoyl-glycerol, and PPP in the reaction mixture were 8, 36, 4, 28, 1, and 6 mol%, respectively. The stabilities of immobilized R275A lipase catalyst and two immobilized catalysts containing *Rhizopus delemar* or *Rhizomucor miehei* lipases were compared under the conditions mentioned above, with the catalysts being transferred to fresh substrate every 24 h. The half-life of the R275A lipase catalyst was 370 d, which was significantly longer than those of *Rhizopus* and *Rhizomucor* lipase catalysts.

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KEY WORDS: Acidolysis, *Fusarium heterosporum*, immobilized lipase, oleic acid, palmitic acid, structured lipid, thermostable lipase, tripalmitin.

Human milk fat contains 20–25% palmitic acid (PA, 16:0), and about 70% of this fatty acid is esterified to the 2-position of triacylglycerols (TAG) (1,2). In addition, the main component of the milk dienoic TAG is 1,3-dioleoyl-2-palmitoyl-glycerol (OPO). Gastric and pancreatic lipases hydrolyze TAG to free fatty acids and 2-monoacylglycerols (2-MAG), and the absorption of free PA liberated from the outer positions is lower than that of free unsaturated fatty acids (3). It has been therefore hypothesized that fat absorption is higher in infants fed fats with PA at the 2-position of TAG than the 1,3-positions (4). For this reason, OPO has been used as an ingredient of infant formula.

OPO can be produced by acidolysis of tripalmitin (PPP) with oleic acid (OA, 18:1) by using immobilized 1,3-positional specific lipase or by interesterification of PPP with ethyl oleate. In general, lipases act strongly on liquid-state substrates, but weakly on solid-state ones. If conducted in an organic solvent-free system, therefore, the reaction must be conducted above 50°C. Reactions in a system containing organic solvent progress efficiently even at lower temperatures because all substrates are in the liquid state. Because an organic solvent-free system is preferable from the industrial viewpoint, a thermostable 1,3-positional specific lipase is strongly desired.

Fusarium heterosporum produces a lipase that hydrolyzes ester bonds at the 1,3-positions of TAG (5). When the lipase cDNA was expressed in *Saccharomyces cerevisiae*, a 333-amino acid prepro-lipase was generated which was converted to a 301-amino acid mature lipase (lipase B) (6–8). The peptide bond between Arg275 and Asp276 was then cleaved by a trypsin-like protease, and lipase B was converted to lipase A. Lipase B was more stable than lipase A. A mutant lipase in which Arg275 was replaced by Ala (named R275A lipase) was not cleaved between 275- and 276-residues by the protease, and its thermostability increased to the level of lipase B (8,9). Thus we attempted the production of OPO using thermostable *Fusarium* R275A lipase.

This paper presents data that show that the anion exchange resin Dowex WBA is suitable as a support for the immobilization of R275A lipase expressed in *S. cerevisiae*, and that the immobilized lipase preparation is effective for the production of OPO by acidolysis of PPP with OA in an organic solvent-free system. It is also shown that the half-life of the lipase catalyst at 50°C is 1 yr, significantly longer than those of immobilized *Rhizopus delemar* and *Rhizomucor miehei* lipase catalysts.

MATERIALS AND METHODS

Reagents. Yeast Nitrogen Base (w/o amino acids) was purchased from Difco Laboratories (Detroit, MI), and yeast extract, peptone, and sucrose were from Wako Pure Chemical Co., Ltd. (Osaka, Japan). OA (purity: OA, 88 mol%; linoleic

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acid, 7 mol%; stearic acid, 3 mol%; PA, 1 mol%) and PPP (fatty acid composition: PA, 89 mol%; stearic acid, 8 mol%; myristic acid, 2 mol%) were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The anion exchange resins Dowex WBA, MWA, XUS, 66, and 77 were purchased from Muromachi Kagaku Kogyo Kaisha Ltd. (Tokyo, Japan), Duolite A7 and A568K were from Rohm and Haas Co. (Philadelphia, PA), and Amberlite IRA904 and IRA96SB were from Organo Co. (Tokyo, Japan). The ceramic supports Toyonite 200-M and SM-10 were obtained from Toyo Denka Co. (Kochi, Japan) and NGK Insulators (Aichi, Japan), respectively. The other reagents were of reagent grade.

Lipases. *Fusarium* wild-type lipase and thermostable R275A lipase were prepared from culture supernatants of *S. cerevisiae* SH1089 [pYGF2] (7) and *S. cerevisiae* SH1089 [pYGF2R275A] (9), respectively. The genotype of the host strain is [*MAT*a, *ura3-52*, *leu2-3*, *112*, *trp1*, *pho3-1*, *pho5-1* (*HIS4-lacZ*, *ura3-52*)]. Preculture of yeast was performed at 27°C in a 500-mL shaking flask containing 150 mL minimum medium (6.7 mg/mL Yeast Nitrogen Base, 5 mg/mL glucose, 20 μg/mL uracil, 20 μg/mL histidine, and 20 μg/mL leucine, pH 5.8) (7) for 24 h on a reciprocal shaker. The main culture was performed at 27°C for 3 d in a 500-mL shaking flask containing 170 mL YPSuc medium (3% yeast extract, 1% peptone, and 4% sucrose, pH 5.8) after inoculating with 3.4 mL preculture. Ammonium sulfate was added to the culture supernatant to give 75% saturation, and the resulting precipitate was dialyzed against water. The enzyme solution was concentrated by ultrafiltration and stored at −20°C. The ratio of *Fusarium* lipase A to B in the wild-type preparation was 3:1.

Rhizopus delemar lipase (Ta-lipase, 49000 units/g) was a gift from Tanabe Seiyaku Co. Ltd. (Osaka, Japan), and immobilized *R. miehei* lipase preparation (Lipozyme IM60) was purchased from Novo Nordisk (Bagsvaerd, Denmark).

Immobilization of lipase. *Fusarium* and *Rhizopus* lipases were immobilized as follows: After 10 g support was suspended in 8 mL *Fusarium* lipase solution (6,250 U/mL) or *Rhizopus* lipase solution (125 mg/mL), the immobilized lipase was prepared by drying under reduced pressure. Dowex WBA was used as a support for immobilization of *Rhizopus* lipase as described previously (10).

Reaction. Acidolysis of PPP with OA using immobilized lipase catalyst was performed in a screw-capped vessel. To activate immobilized *Fusarium* and *Rhizopus* lipase catalysts, the first-cycle reaction (pretreatment) was performed as follows: A mixture of PPP/OA (1:2, w/w), 8% immobilized lipase catalyst and 2% water was incubated at 50°C for 24 h with shaking at 130 oscillations/min. The subsequent reactions were conducted by transferring the activated lipase catalyst into the same amount of PPP/OA (1:2, w/w) mixture without water, followed by shaking under the same conditions as those for the pretreatment. Acidolysis was expressed as the OA content in acylglycerols. Immobilized *Rhizomucor* lipase catalyst was used without pretreatment in a substrate mixture containing water.

Extraction of acylglycerols from reaction mixture. Acylglycerols were extracted with 100 mL *n*-hexane after adding 70 mL 0.5 N KOH (20% ethanol solution) to 3–5 g of the reaction mixture. TAG in the resulting acylglycerols were purified by silica gel column chromatography. Acylglycerol mixture (2 g) was applied to a silica gel 60 (Merck, Darmstadt, Germany) column (200 × 15 mm) and eluted with a solvent mixture of *n*-hexane/ethyl acetate/acetic acid (98:2:1, vol/vol/vol).

Analysis. Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical) with 0.05 N KOH, according to our previous paper (11). The reaction was carried out at 30°C for 30 min with stirring at 500 rpm. One unit (U) of lipase was defined as the amount of the enzyme that liberated 1 μmol of fatty acid per minute. Fatty acid composition of acylglycerols was analyzed as described previously (12). The acylglycerols were methanolyzed using Na-methylate as a catalyst, and the resulting fatty acid methyl esters were analyzed by a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) equipped with a DB23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA).

Thin-layer chromatography (TLC) of acylglycerols was performed according to our previous paper (13). The acylglycerols were applied to a silica gel 60 plate (Merck), and then developed with a mixture of chloroform/acetone/acetic acid (96:4:1, vol/vol/vol). The contents of MAG, diacylglycerols (DAG), and TAG were measured by a TLC/flame-ionization detector (FID) analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol).

TAG were analyzed on two octadecyl silica (ODS) columns (4.6 × 150 mm; Cosmosil 5C18-AR; Nacalai Tesque Inc., Kyoto, Japan) connected with a high-performance liquid chromatography (HPLC) system (LC-9A; Shimadzu Co., Kyoto, Japan). The sample was eluted with a mixture of acetone/acetonitrile (3:1, vol/vol) at 40°C and 0.4 mL/min flow rate, and detected with a refractometer. OPO and 1(3),2-dioleoyl-3(1)-palmitoyl-glycerol (OOP), and 1(3),2-dipalmitoyl-3(1)-oleoyl-glycerol (PPO) and 1,3-dipalmitoyl-2-oleoyl-glycerol (POP) were analyzed by HPLC with Chrompak silver ion chromatography column (4.6 × 250 mm; Chrompak, Middelburg, the Netherlands). The sample was eluted with a mixture of isooctane/toluene/ethyl acetate (8:1:1, vol/vol/vol) at 40°C and 1 mL/min flow rate, and detected with a refractometer.

Regiospecific analysis. Fatty acid composition at the 2-position of transesterified TAG was determined according to AOCS Official Method Ch 3-91 (14) except for enzymatic hydrolysis. TAG (0.3 g) was hydrolyzed at 30°C for 25 min in 3 mL of 50 mM Na-acetate buffer (pH 5.6)/10 mM $CaCl_2$ with 250 U *Rhizopus* lipase. Under these conditions, hydrolysis reached 28%, and 1,3-DAG was not detectable.

RESULTS AND DISCUSSION

Suitable support for immobilization of Fusarium *lipase*. Anion exchange resins and ceramics have been well used as supports for the immobilization of lipases (10,15,16). It is

TABLE 1
Acidolysis Activity of *Fusarium* R275A Lipase Immobilized on Various Supports[a]

Support	Oleic acid content in acylglycerols (mol%)			
	First cycle	Second cycle	Third cycle	Sixth cycle
Dowex WBA[b]	47.6	51.4	50.5	50.8
Dowex MWA[b]	47.2	50.3	48.4	48.2
Dowex 77[b]	46.4	44.8	42.1	40.8
Duolite A7[c]	45.8	46.6	46.2	36.2
Amberlite IRA904[d]	45.1	40.2	41.3	28.7

[a]The first reaction was conducted in a mixture of tripalmitin/oleic acid (1:2, w/w), 8% immobilized lipase catalyst, and 2% water for the activation. The subsequent reactions were performed after transferring the lipase catalyst into a fresh substrate mixture without water.
[b]Muromachi Kagaku Kogyo Kaisha Ltd. (Tokyo, Japan).
[c]Rohm and Haas Co. (Philadelphia, PA).
[d]Organo Co. (Tokyo, Japan).

TABLE 2
Effect of Water Concentration on Activation of *Fusarium* R275A Lipase Immobilized on Dowex WBA[a]

Water (%)	Oleic acid content in acylglycerols (mol%)			
	First cycle	Second cycle	Third cycle	Sixth cycle
0	31.7	32.7	32.4	30.3
1	46.8	48.9	47.7	47.1
2	48.7	50.2	51.6	50.5
4	43.4	46.3	45.4	46.2
8	39.5	26.5	22.0	21.5

[a]The first reaction was conducted in a mixture containing water, and the subsequent reaction was conducted in a mixture without water. See Table 1 for company supplier and address.

also reported that the activity and stability strongly depend on the supports (10). We thus looked for a suitable support for thermostable R275A lipase among eight kinds of anion exchange resins and two kinds of ceramics mentioned in the Materials and Methods section. Immobilized lipase was prepared by drying under reduced pressure after 1 g of support was suspended in 0.8 mL of lipase solution (6250 U/mL). The resulting immobilized lipase catalyst was used for acidolysis of PPP with OA. Pretreatment for the activation of the catalyst (first-cycle reaction) was performed at 50°C for 24 h in a mixture of 4 g PPP/OA (1:2, w/w), 320 mg immobilized lipase catalyst, and 80 μL water. When the first-cycle reactions were conducted using the lipases immobilized on Dowex WBA, Dowex MWA, Dowex 77, Duolite A7, and Amberlite IRA904, the OA content in acylglycerols reached more than 45 mol%. These immobilized lipases were then transferred to a fresh substrate mixture without adding water, and the acidolyses were repeated under the same conditions as those of pretreatment (Table 1). The activities of lipases immobilized on Dowex 77, Duolite A7, and Amberlite IRA904 were decreased by repeating the reaction, but lipases immobilized on Dowex WBA and Dowex MWA maintained high activities even though the reactions were repeated for six cycles. Because the lipase immobilized on Dowex WBA showed a little higher activity than that on Dowex MWA, Dowex WBA was selected as a support for the following experiments.

To determine the lipase amount necessary for the immobilization, 1,000–10,000 U of R275A lipase was immobilized on 1 g of support. Acidolyses of PPP with OA using the resulting lipases showed that the activity reached a constant value at 5,000 U/g-support. Hence the amount of lipase for immobilization was fixed at 5,000 U/g-support.

Activation of immobilized lipase. We have reported immobilized *Rhizopus* lipase is activated by incubating in a substrate mixture containing 2% water (10,15). Because *Fusarium* R275A lipase required the pretreatment in a mixture containing a small amount of water for the activation in our preliminary experiment, the amount of water necessary for the activation was investigated. A mixture of 4 g PPP/OA (1:2, w/w), 320 mg immobilized lipase catalyst, and 0–320 μL water was shaken at 50°C for 24 h, and the OA content in acylglycerols was analyzed (Table 2). The subsequent reactions were repeated by transferring immobilized lipase to a fresh substrate mixture without adding water. The lipase catalyst showed 32% acidolysis in reactions conducted without water. Highest activity (49% acidolysis) was obtained by shaking in a mixture containing 2% water. When the pretreatment was conducted in a mixture containing less than 4% water, the activities in the subsequent reactions were not reduced. However, pretreatment in a mixture containing 8% water reduced the activities in the subsequent reactions. This result may be due to the release of the lipase from the support by an excessive amount of water. From these results, the pretreatment for activation was conducted in a mixture containing 2% water.

Pretreatment in a mixture containing water caused concomitant hydrolysis. The extent of the hydrolysis was evaluated by analyzing the contents of partial acylglycerols. Pretreatment in a mixture containing 2% water generated 36.6% partial acylglycerols (33.7% DAG, 2.9% MAG). The appearance of partial acylglycerols in the subsequent reaction was reduced by using a substrate without water, and the acidolysis activity was not reduced: 12.8% DAG and 0.5% MAG (second reaction); 4.3% DAG (third reaction); 3.5% DAG (sixth reaction); 3.4% DAG (fifteenth reaction). This result indicates that the concomitant hydrolysis was repressed by using a substrate without water after the pretreatment.

Effect of several factors affecting acidolysis of PPP with OA. The immobilized lipase catalyst that had been used in two cycles of reactions in a mixture without water after the pretreatment was chosen to investigate factors affecting the reaction. The enzyme was named activated lipase catalyst.

In general, lipases act weakly on solid-state substrates. Heating above 55°C is necessary for melting a mixture of PPP/OA (1:2, w/w), but the substrate mixture melted soon with the progress of the reaction even though the acidolysis was performed at 50°C. The extents of acidolysis after 3 and 24 h at 50 and 55°C were thus nearly the same: 50°C (3 h): 30.5% acidolysis; 55°C (3 h): 29.8%; 50°C (24 h): 50.3%; 55°C (24 h): 51.2%. Because the lower temperature is preferable for extending the life of the enzyme catalyst, the reaction temperature was fixed at 50°C.

To determine the effect of the amount of immobilized lipase, a mixture of 12 g PPP/OA (1:2, w/w) and 240 to 1200 mg of activated lipase catalyst was shaken at 50°C for 3 and 24 h, and the OA content in the acylglycerol fraction is shown in Figure 1. The acidolysis extent after 3 h increased with increasing lipase amount, showing that the acidolysis velocity depended on the amount of immobilized lipase catalyst. Meanwhile, because the acidolysis extent after 24 h was unchanged at levels of catalyst equal to and greater than 8%, the amount was fixed at 8%.

The effect of the OA amount in the substrate was examined. A mixture of 12 g PPP/OA (1:1 to 1:7, w/w) and 960 mg activated lipase catalyst was shaken at 50°C for 3 and 24 h (Fig. 2). The acidolysis extent after 3 h showed that the reaction was accelerated with increasing amounts of OA. The acidolysis extent after 24 h also depended on the ratio of PPP/OA and reached a constant value at the ratio of 1:5. Because an increase in the OA amount at constant total substrate levels leads to a decrease in the TAG amount in the reaction mixture, the ratio of OA to PPP was set at 2:1 (w/w) in this study.

A mixture of PPP/OA (1:2, w/w) and 8% activated lipase catalyst was shaken at 50°C, and a typical time course is shown in Figure 3. The PA content in acylglycerols rapidly decreased during the first 6 h of incubation, and gradually decreased thereafter. The increase in the OA content corresponded completely with the decrease in the PA content, and the reaction reached steady state after 18 h.

TAG composition of transesterified oil. Acylglycerols were extracted from 24-h reaction mixture with *n*-hexane, and TAG composition was analyzed by HPLC on an ODS column (Fig. 4). The components eluted between 13 to 17 min were DAG, as shown by TLC, and the components after 27 min were TAG. To identify the main TAG components fractionated by HPLC, the peaks numbered in Figure 4 were collected and their fatty acid compositions were analyzed (data not shown). On the basis of the molecular ratio of fatty acids, the structures of peaks I to IV were estimated as follows: Peak I, triolein (OOO); peak II, dioleoyl-palmitoyl-glycerol; peak III, dipalmitoyl-oleoyl-glycerol; peak IV, PPP. The contents of peaks I to IV were 8, 40, 29, and 6 mol%, respectively. The structures of the dioleoyl-palmitoyl-glycerols and dipalmitoyl-oleoyl-glycerols were further analyzed by silver ion chromatography. The results showed that dioleoyl-palmitoyl-glycerols were composed of 91 mol% OPO and 9 mol% OOP, and dipalmitoyl-oleoyl-glycerols were composed of 95 mol% PPO and 5 mol% POP. The results of HPLC showed that the contents of OOO, OPO, OOP, PPO, POP, and PPP are 8, 36, 4, 28, 1, and 6 mol%, respectively. From the composition of these TAG, the molar ratio of PA/OA at the 2-position of TAG was calculated to be 5.4.

TAG in a 24-h reaction mixture were purified by silica gel column chromatography, hydrolyzed with *R. delemar* lipase, and the fatty acid compositions of the resulting 2-MAG were determined. The result showed that fatty acid composition at the 2-position of transesterified TAG was 75.9 mol% PA, 13.9 mol% OA, 8.0 mol% stearic acid, and 1.3 mol% linoleic acid. The molar ratio of PA/OA was 5.5, which coincided well with

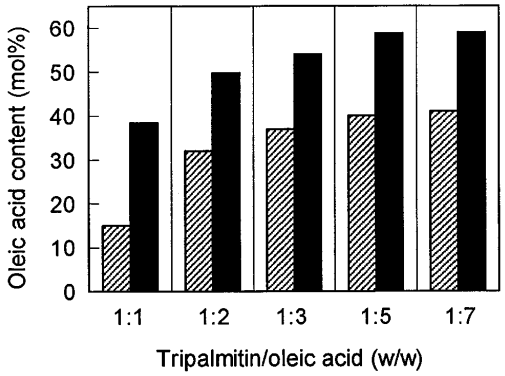

FIG. 2. Effect of oleic acid content on acidolysis of tripalmitin with oleic acid using immobilized R275A lipase catalyst. The reaction was performed for 3 and 24 h. Striped bar, oleic acid content in acylglycerols after 3-h reaction; solid bar, oleic acid content after 24-h reaction.

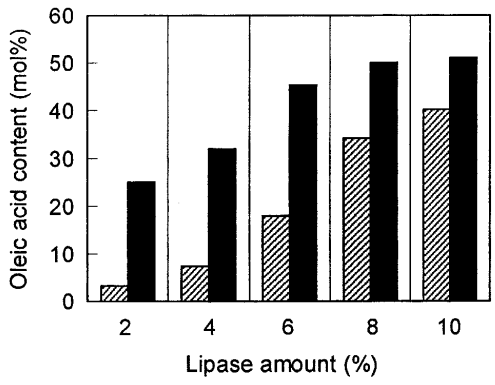

FIG. 1. Effect of amount of immobilized R275A lipase catalyst on acidolysis of tripalmitin with oleic acid. The reaction was performed for 3 and 24 h. Striped bar, oleic acid content in acylglycerols after 3-h reaction; solid bar, oleic acid content after 24-h reaction.

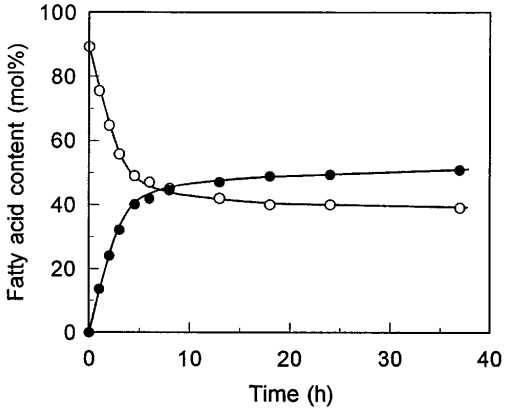

FIG. 3. Time course of acidolysis of tripalmitin with oleic acid using immobilized R275A lipase. ○, Palmitic acid content in acylglycerols; ●, oleic acid content.

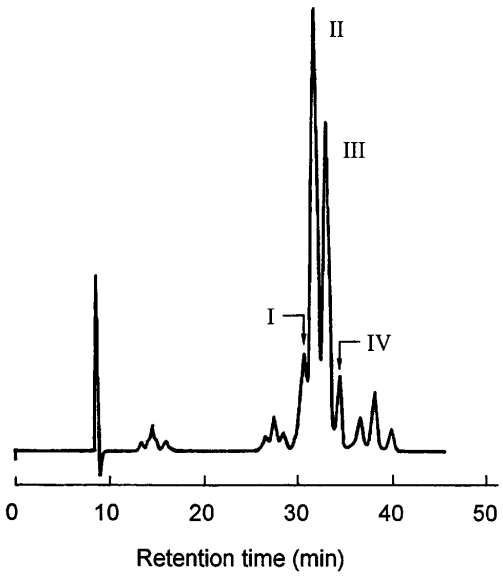

FIG. 4. Triacylglycerol components of transesterified fat. High-performance liquid chromatography was carried out as described in the Materials and Methods section. Peaks numbered in Figure were estimated as follows: Peak I, triolein; peak II, dioleoyl-palmitoyl-glycerol; peak III, dipalmitoyl-oleoyl-glycerol; peak IV, tripalmitin.

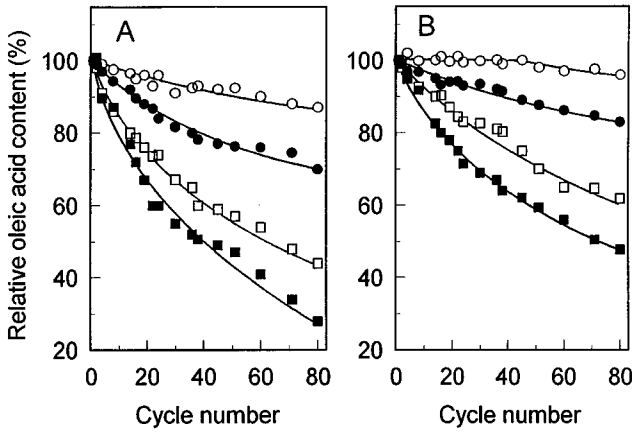

FIG. 5. Stability of immobilized *Fusarium heterosporum* R275A, *Fusarium* wild-type, *Rhizopus delemar* and *Rhizomucor miehei* lipase catalysts. Acidolysis of tripalmitin with oleic acid was repeated by transferring immobilized lipase to a fresh substrate mixture every 24 h. The oleic acid content in acylglycerols was expressed relative to that in the first-cycle reaction (the third-cycle in total). (A), Relative oleic acid content after 3-h reaction. The oleic acid contents in the first-cycle reactions: *Fusarium* R275A lipase, 34.2 mol%; *Fusarium* wild-type lipase, 29.7 mol%; *Rhizopus* lipase, 29 mol%; *Rhizomucor* lipase, 33.1 mol%. (B), Relative oleic acid content after 24-h reaction. The oleic acid contents in the first-cycle reactions: *Fusarium* R275A lipase, 50.1 mol%; *Fusarium* wild-type lipase, 50.1 mol%; *Rhizopus* lipase, 49.3 mol%; *Rhizomucor* lipase, 49.4 mol%. ○, *Fusarium* R275A lipase; ●, *Fusarium* wild-type lipase; □, *Rhizopus* lipase; ■, *Rhizomucor* lipase.

the ratio (5.4) calculated from TAG composition determined by HPLC. It was therefore confirmed that the TAG composition determined by HPLC was correct.

Generation of by-products; OOO, OOP, and POP. When tuna oil (15), borage oil (17), safflower oil (18), and linseed oil (18) were acidolyzed at 30°C with caprylic acid using immobilized *Rhizopus* lipase, tricaprylin and DAG were scarcely detected in the resulting transesterified oils. However, acidolysis of PPP with arachidonic acid at 40°C using the same lipase generated 7% triarachidonin and 5% DAG (19). The acidolysis at 50°C in this study also generated 8% OOO and 4% DAG. Because *Fusarium* lipase is a 1,3-positional specific lipase (5), and since the substrate (PPP) does not contain OA, OOO and DAG may have been generated through the following process: When the reaction was conducted above 40°C, hydrolysis progressed concomitantly due to the presence of a small amount of water (300–400 ppm). DAG with OA at the 1(3)-position and PA at the 2-position were generated, and the PA migrated spontaneously to the 3(1)-position. Diolein was then synthesized by acidolysis of the PA with OA. After OA at the 1(3)-position migrated to the 2-position, OOO was generated by esterification of free OA to the 1(3)-position. Other by-products, OOP and POP, could be synthesized through a similar process including the generation of DAG. This hypothesis is supported by the fact that main peak of DAG eluted at 14.5 min by HPLC contained 83 mol% of OA (Fig. 4), and also by the observation that a high concentration of DAG in an acidolysis reaction mixture can increase the degree of acyl migration, resulting in the appearance of by-products (20).

Stability of immobilized Fusarium *R275A lipase catalyst*. A mixture of 30 g PPP/OA (1:2, w/w) and 2.4 g activated lipase catalyst was shaken at 50°C, and the acidolysis was repeated by transferring the lipase to a fresh substrate mixture every 24 h. The stability of the immobilized lipase catalyst can be precisely evaluated by investigating the decrease in the reaction velocity, which can be determined from the OA content in acylglycerols after a 3-h reaction. Three grams of the reaction mixture was therefore taken out after 3 h, and the OA content in the acylglycerols was analyzed (Fig. 5A). In addition, the stability of R275A lipase was compared to the stabilities of *Rhizopus* lipase immobilized on Dowex WBA and *Rhizomucor* lipase (Lipozyme IM60), which often have been used to exchange fatty acids at the 1,3-positions of TAG, as well as to that of *Fusarium* wild-type lipase immobilized on Dowex WBA. The degree of acidolysis by R275A lipase after 80 cycles was 87% of that at the first cycle. Acidolysis by *Fusarium* wild-type lipase was only 68%. The half-lives of the R275A and wild-type lipase catalysts were estimated, respectively, to be 370 and 150 d by extending their logarithmic plots after 3-h reaction. Furthermore, the acidolyses by *Rhizopus* and *Rhizomucor* lipase catalysts after 80 cycles were 44 and 28% of those at the first-cycle reactions, respectively. Their half-lives were 65 and 45 d, respectively.

Figure 5B shows the OA contents in acylglycerols after 24-h acidolyses with the above four immobilized lipase catalysts. The acidolysis after 80 cycles by R275A lipase still maintained 96% of that at the first cycle, although acidolyses by *Rhizopus* and *Rhizomucor* lipase catalysts decreased to 62

and 48%, respectively. This result shows that the immobilized catalyst of thermostable R275A lipase can be used for long periods in the acidolysis of PPP at 50°C in an organic solvent-free system.

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