Diacylglycerol Synthesis by Enzymatic Glycerolysis: Screening of Commercially Available Lipases

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ABSTRACT: Seven lipases were screened for their ability to synthesize DAG in the glycerolysis of rapeseed oil. In batch reactions with free glycerol, the lipase carrier was of great importance for catalysis. Catalysis did not take place in reactions with lipases having hydrophilic carriers. The best DAG yield (approx. 60 wt%) was achieved with Novozym 435 and Lipase PS-D after 7 h, and an equilibrium was obtained. Stepwise addition of glycerol allowed catalysis with Novozym CALB L (immobilized) to take place in spite of the hydrophilic carrier; however, the DAG yield was only 19 wt%. This result suggests that glycerol forms a layer around the hydrophilic lipase particles, limiting contact between the lipases and the hydrophobic oil phase. With glycerol absorbed on silica gel, all lipases catalyzed the glycerolysis reaction. Faster conversion of TAG was obtained with Lipase PS-D, Lipase AK, and Lipase F-AP15 than in reactions with free glycerol, but the DAG yield remained approximately 60–65 wt%. Nonspecific lipases yielded more 1,3-DAG early in the reaction.

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KEY WORDS: Diacylglycerol, glycerol, glycerolysis, lipase carrier, lipases, silica gel, specificity of lipases.

The use of DAG as nonionic emulsifiers in the food, cosmetic, and pharmaceutical industries is well known, and often a mixture of MAG and DAG is used for these applications because of the lower cost and the proper performance of the mixture (1,2). DAG is also a minor natural component of various edible oils (3). Today, DAG oil is manufactured as a functional cooking oil in Japan, where it has been on the market since 1999. In January 2005, DAG cooking oil was launched nationwide in the United States. This oil contains approximately 80 wt% DAG, the rest being TAG (4). Recent studies on the nutritional properties and dietary effects of DAG oil have demonstrated some beneficial effects. In comparisons of DAG and TAG oils, studies on animals and humans have shown a decrease in postprandial TAG levels in serum and a suppressed accumulation of TAG in body fat and liver after the intake of DAG oil (5–8). DAG is present in two isomeric forms, *sn*-1,2(2,3)-DAG and *sn*-1,3-DAG, with a natural isomeric ratio of approximately 3:7. The 1,3-DAG form is responsible for the beneficial effects because it is metabolized along a different metabolic pathway than TAG and 1,2-DAG (5).

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A mixture of MAG and DAG oils can be produced chemically using high temperatures (220–260°C) and an inorganic catalyst, such as sodium, potassium, or calcium hydroxide (9). Another approach is to use lipase-catalyzed reactions, which provide several advantages such as increased selectivity, higher product purity and quality, energy conservation, and the omission of toxic catalysts.

The lipase-catalyzed synthesis of partial acylglycerols has been studied thoroughly, mainly with the aim of obtaining MAG in high yield (10–12). However, few reports are available on the synthesis of DAG as the primary target. Esterification reactions with and without the use of an organic solvent have been tested for the synthesis of DAG (13–15). Glycerolysis reactions also have been used for the synthesis of DAG (16–18). In this reaction, DAG can be formed both by removal of an acyl moiety from the TAG molecule and by acylation of the MAG formed during the reaction.

The most recent method for the production of high-purity 1,3-DAG in the industry involves two reactions: the partial hydrolysis of a fat or oil to obtain a partial hydrolysate with a high FFA content, followed by 1,3-specific lipase-catalyzed esterification of the FFA in the hydrolysate with glycerol (19).

In this work we chose the solvent-free glycerolysis of TAG oil for DAG production since it is a simple process industrially. The oil can be used directly for the production of DAG because there is no need to liberate FFA. No solvents are used, which avoids time-consuming and costly solvent recovery; the intent to use the DAG product in foods also makes the use of solvents undesirable. In the present study, seven commercially available lipases were screened to find the one best suited for further optimization and large-scale production of DAG oil with a high content of 1,3-DAG. *Candida antarctica* lipase B was immobilized on a macroporous anion-exchange resin (trivial name, Duolite) to obtain a food-grade preparation. Two protocols were tested: In the first, all the glycerol was added at the beginning of the reaction (batch principle); in the second, glycerol was added stepwise during the reaction (fed-batch principle). The batch reactions also were tested with glycerol absorbed on silica gel. This method was applied to reveal the interactions between the lipase carrier material and glycerol.

EXPERIMENTAL PROCEDURES

Materials. Rapeseed oil was provided by Aarhus United (Aarhus, Denmark). Glycerol (99%) was supplied by BHD

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TABLE 1
Lipase Source[a], Carrier, Specificity, and Reaction Temperature

Lipase	Carrier	Specificity	Temperature (°C)
Lipase PS-D (*Pseudomonas cepacia* lipase)	Celite, acid-washed	Nonspecific	55
Lipase AK (*P. fluorescens* lipase)	Nonimmobilized (powdered form)	Nonspecific	55
Lipase F-AB15 (*Rhizopus oryzae* lipase)	Nonimmobilized (powdered form)	1,3-Specific	40
Novozym 435 (*Candida antarctica* lipase)	Macroporous acrylic resin	Nonspecific	65
Lipozyme TL IM (*Thermomyces lanuginosa* lipase)	Granulated silica	1,3-Specific	65
Lipozyme RM IM (*Rhizomucor miehei* lipase)	Macroporous anion-exchange resin[b]	1,3-Specific	65
Novozym CALB L (imm.) (*C. antarctica* lipase)	Macroporous anion-exchange resin[b]	Nonspecific	65

[a]Lipozyme RM IM, Lipozyme TL IM, Novozym 435, and Novozym CALB L (see Experimental Procedures section) were from Novozymes A/S (Bagsvaerd, Denmark). Lipase PS-D, Lipase F-AP15, and Lipase AK were from Amano Enzyme Inc. (Aichi, Japan).
[b]Trivial name, Duolite.

Laboratory Suppliers (Poole, England). Silica gel 60H was from Merck KGaA (Darmstadt, Germany). Duolite A568 was kindly provided by Novozymes A/S (Kalundborg, Denmark). Lipozyme RM IM, Lipozyme TL IM, Novozym 435, and Novozym CALB L (nonimmobilized, liquid form) were generously supplied by Novozymes A/S (Bagsvaerd, Denmark). Lipase PS-D, Lipase F-AP15, and Lipase AK were kindly supplied by Amano Enzyme Inc. (Aichi, Japan). All lipase sources and their carrier materials are listed in Table 1.

Standards for HPLC and GC analysis (tripalmitin, 1,2-dipalmitin, 1,3-dipalmitin, 2-monopalmitin, and propyl laurate) were purchased from Sigma Inc. (St. Louis, MO), and lauric acid was from Merck Schuchardt OHG (Hohenbrunn, Germany). The solvents used for analysis—heptane, ethyl acetate, and 2-propanol from Rathburn Chemicals Ltd. (Walkerburn, Scotland); chloroform from Lab-Scan (Dublin, Ireland); acetic acid and 1-propanol from Merck KGaA; and acetone from BHD Laboratory Suppliers—were all of HPLC grade.

Absorption of glycerol on silica gel. Glycerol was absorbed onto silica gel as described by Berger *et al.* (13). Equal amounts of glycerol and silica gel were used.

Immobilization of Novozym CALB L. Novozym CALB L was immobilized on Duolite A568 using a modification of the method described by Arcos and Otero (20). A 45-g quantity of Novozym CALB L solution was added to 42 mL of ice-cold buffer (Tris, 0.1 M, pH 7.5) under magnetic stirring. Then 30 g of Duolite A568 was added and stirring was continued on an ice bath for 1.5 h. Cold acetone (7.5 mL) was added and the solution was stirred for another 10 min, followed by filtration. The precipitate was washed twice with 75 mL of cold acetone and filtered after each wash. The washed precipitate was dried under vacuum for 3 h at 30°C. This preparation is referred to as "Novozym CALB L (imm.)."

Reactions and sampling. For the glycerolysis reactions, a set of standard parameters was applied: 30 g of rapeseed oil and a molar ratio of 1:2 oil/glycerol. The amount of lipase used was 5 wt% of the oil mass, and the glycerol contained 3.5 wt% distilled water. Glycerolysis reactions (batch principle) were run with all lipases in 200-mL Erlenmeyer flasks with glass-slip stoppers, and magnetic stirring was applied (approx. 250 rpm). The temperature of all reactions was the optimal temperature for the lipase used (Table 1).

In fed-batch glycerolysis reactions, one-fourth of the total mass of glycerol was added at the following times: 0, 3.5, 7, and 23 h. Glycerolysis using the fed-batch principle was run with Novozym 435 and Novozym CALB L (imm.), and the lipases made up 10 wt% of the oil mass. All other parameters were standard parameters.

For glycerolysis reactions (batch principle) with the glycerol absorbed onto silica gel, the same content of glycerol was used as in reactions with nonabsorbed glycerol, and all lipases were tested.

Samples were withdrawn from the batch-principle glycerolysis reactions at 1, 2, 4, 7, 23, and 28 h. For reactions using the fed-batch principle, samples were withdrawn at 3.5, 7, 23, and 28 h (prior to glycerol addition). A 2-mL quantity of the reaction mixture was withdrawn and centrifuged (2900 × *g* for 15 min).

Normal-phase HPLC analysis. The analysis was performed on equipment described by Mu *et al.* (21) using ELSD. The separation of TAG, 1,3- and 1,2-DAG, and MAG was performed on a Hypersil silica column (length, 10 cm; i.d., 2.1 cm; particle size, 5 µm) from Thermo Hypersil-Keystone (Bellefonte, PA) using heptane (solvent A) and heptane/ethyl acetate/2-propanol/acetic acid (solvent B) (80:10:10:1, by vol). Solvent B was increased from 2 to 35% over 10 min at a flow rate of 0.50 mL/min, to 98% for 1 min, and maintained for 6 min before reverting to 2%, all at a flow rate of 1 mL/min.

Calibration curves were constructed, and the results are given as the weight percentage of total lipids.

Activity measurements. Activity measurements were performed according to the Novozymes method "Lipase Activity

Based on Ester Synthesis—PLU, Propyl-Laurate Synthesis."

1-Propanol and lauric acid (40 mmol each) were mixed with 0.03 g of catalyst (dry wt), and water (3 wt%) was added to the substrate. The reaction was incubated at 60°C for 20 min with shaking (approx. 150 rpm), and lauric acid and propyl laurate were determined by GC. A gas chromatograph with a capillary column was used (Zebron ZB-FFAP, 30 m, i.d. 0.32 mm, 0.25 mm film; Phenomenex, Torrance, CA). The injector was 275°C and was used in the split mode with a split ratio of 75:1. The oven temperature was held at 60°C for 1.5 min, then raised to 250°C at 20°C/min and held for 9.5 min. The temperature of the detector (flame ionization) was 300°C. Response factors for lauric acid and propyl laurate were determined from standards. One PLU unit was defined as 1 μmol of propyl laurate formed per minute by the enzyme at standard conditions.

RESULTS AND DISCUSSION

Glycerolysis with nonabsorbed glycerol. The seven reaction mixtures showed very different physical behaviors. In reactions with Lipozyme RM IM, Lipozyme TL IM, and Novozym CALB L (imm.), enzyme particles tended to clump or adhere to the walls of the reaction flask together with the glycerol phase, as observed by others (22,23). The different lipase carrier materials might have been responsible for the differences in dispersion among the glycerolysis mixtures. The carrier materials for Lipozyme TL IM, Lipozyme RM IM, and Novozym CALB L (imm.) were hydrophilic (Table 1), which explains their tendency to stick to the hydrophilic glycerol phase. Lipase AK and Lipase F-AP15 dispersed easily in the reaction mixture. These lipase preparations were in powdered form and no carrier was used. Novozym 435 and Lipase PS-D, which had a hydrophobic and a neutral carrier, respectively, also dispersed easily in the reaction mixture.

The best DAG yield (Fig. 1) was achieved in reactions catalyzed by Novozym 435 and Lipase PS-D. Almost 60 wt% DAG was obtained at 7 h of incubation with Lipase PS-D, and 62 wt% DAG was obtained at 23 h with Novozym 435. The acylglycerols seem to have reached equilibrium at 7 to 23 h of incubation for all reactions. Reactions took place only in the reaction mixtures in which the lipases were properly dispersed. This can be explained by the formation of a glycerol layer on the immobilized lipase particles when a hydrophilic carrier was used, thereby restricting contact with the hydrophobic oil phase (23,24).

When four lipases were tested in the solvent-free glycerolysis of commercially available fish oil (78 wt% TAG, 22 wt% DAG), the immobilized *C. antarctica* lipase (Chirazyme L2) was the most efficient one and yielded the highest conversion of TAG into DAG (increased to 44 wt%) (16). We obtained a higher DAG yield with one-fourth the dose of catalyst. Novozym 435 and Lipozyme RM IM were tested previously in the glycerolysis of olive oil (17). Only Novozym 435 catalyzed the glycerolysis reaction, reaching equilibrium at 7 h with approximately 50 wt% DAG in the product. We obtained 10 wt% higher DAG and 10 wt% lower MAG in this work. Solvent engineering was applied previously to optimize the yield of 1,3-DAG in the glycerolysis of triolein catalyzed by Lipozyme IM-77 (18). The maximum yield was approximately 40% 1,3-DAG; the use of solvents did not cause a higher 1,3-DAG yield compared with this work.

Stepwise addition of glycerol. In glycerolysis reactions with a stepwise addition of glycerol, the small amount of glycerol present in the first 3.5 h slowed the glycerolysis reaction catalyzed by Novozym CALB L (imm.) (Fig. 2). The stoichiometric molar ratio of oil to glycerol for DAG synthesis by glycerolysis is 2. Therefore, the slow reaction was not due to an insufficient amount of glycerol but rather to partial blockage of the lipase particles by the glycerol. After 23 h, a mixture of acylglycerols containing 19 wt% DAG was obtained. Because partial acylglycerols were formed, the mixing of the hydrophilic glycerol phase and the increasingly less hydrophobic oil phase may have been more pronounced, facilitating rapid catalysis. This explanation accounts for the time course of the reaction with Novozym CALB L (imm.) (Fig. 2).

With Novozym 435, the DAG content increased from 52 to 59 wt% from 3.5 h, where the molar ratio of oil/glycerol was 2, to 28 h, where the ratio was 0.5 (Fig. 2). This result suggests that

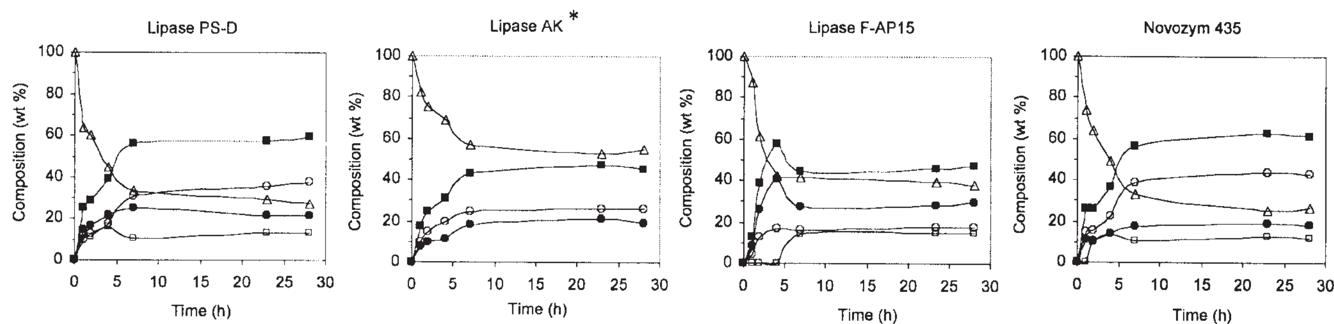

FIG. 1. Time courses for lipase-catalyzed glycerolysis reactions (batch principle). (△) TAG, (○) 1,3-DAG, (●) 1,2-DAG, (□) MAG, (■) total DAG. *No MAG detected. No reactions occurred with Lipozyme TL IM, Lipozyme RM IM, and Novozym CALB L (imm.). Lipozyme RM IM, Lipozyme TL IM, Novozym 435, and Novozym CALB L (see Experimental Procedures section) were from Novozymes A/S (Bagsvaerd, Denmark). Lipase PS-D, Lipase F-AP15, and Lipase AK were from Amano Enzyme Inc. (Aichi, Japan).

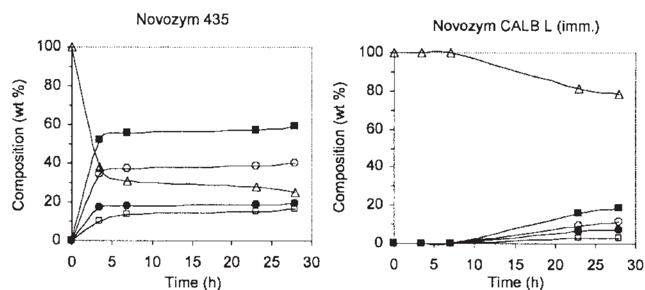

FIG. 2. Time courses for fed-batch glycerolysis reactions. Glycerol was added at 0, 3.5, 7, and 23 h just after sampling. (△) TAG, (○) 1,3-DAG, (●) 1,2-DAG, (□) MAG, (■) total DAG. For enzyme suppliers, see Figure 1.

the amount of glycerol does not have an effect on the equilibrium level of DAG when a hydrophobic carrier is used and that excess glycerol is not necessary at a molar ratio higher than 1.

A high DAG yield was achieved faster in the fed-batch reaction than in the batch reaction of Novozym 435 (*cf.* Figs. 1, 2). Although there were early differences, the DAG content in the two reactions was similar at 7 h due to equilibrium between the acylglycerols in the reaction mixture, which did not shift because of a higher lipase load and/or the stepwise addition of glycerol in the fed-batch reaction.

The nonabsorbed glycerol reactions showed that the lipase carrier is very important in DAG production. Novozym 435 and Novozym CALB L (imm.) carried the same lipase on different carriers. Only Novozym 435 (hydrophobic carrier) yielded DAG in a batch reaction. Novozym CALB L (imm.) was able to produce DAG with the stepwise addition of glycerol, but only at a low yield (Fig. 2).

Glycerolysis with glycerol absorbed on silica gel. Glycerolysis using glycerol absorbed on silica gel was used with all lipases (13,25). A fine dispersion of silica gel-absorbed glycerol and the lipase preparations in the oil was visible in all reactions. The lipase-catalyzed synthesis took place with all lipases (Fig. 3). With this approach, a faster conversion of TAG occurred with Lipase PS-D, Lipase AK, and Lipase F-AP15 than in reactions with nonabsorbed glycerol. The maximum DAG yield (60–65 wt%) was achieved after 2–4 h. Novozym 435 displayed a slower conversion and slightly lower DAG yield compared with the reaction using nonabsorbed glycerol. Lipozyme TL IM and Lipozyme RM IM gave quick conversions of TAG and high DAG yields. Novozym CALB L (imm.) gave a very slow conversion of TAG, and the DAG yield was only approximately 50 wt% after 23 h. Hence, this lipase was the least fit for DAG synthesis.

Faster conversion rates have been reported in esterification reactions when silica gel was used (13,26). However, Torres *et al.* (16) did not observe any improvement in the yield or reaction rate when silica gel was used for glycerol absorption in a glycerolysis reaction with a *C. antarctica* lipase (Chirazyme L2). We obtained the same result with a similar lipase, Novozym 435.

The fact that all tested lipases catalyzed the glycerolysis reaction between rapeseed oil and glycerol when glycerol was absorbed on silica gel suggests that the free glycerol forms a layer around the lipase particles when a hydrophilic carrier is used.

Silica gel was added to the mixture of oil and glycerol with

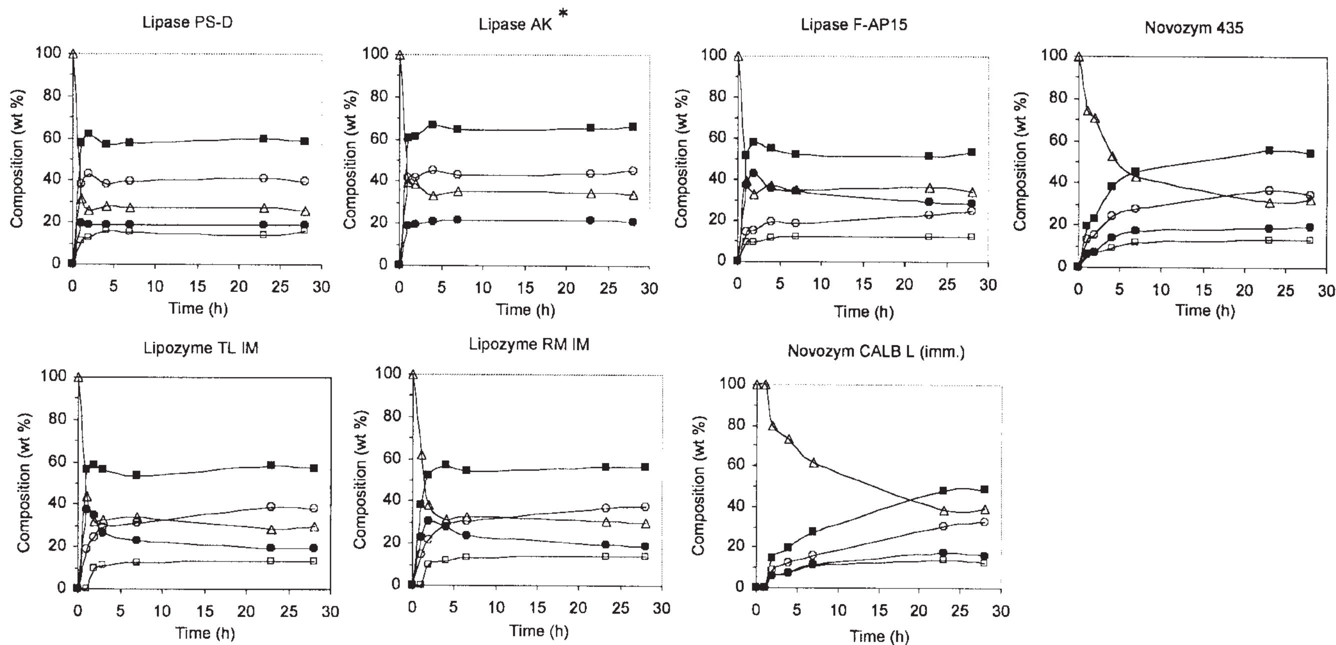

FIG. 3. Time courses for lipase-catalyzed glycerolysis reactions with glycerol absorbed on silica gel. (△) TAG, (○) 1,3-DAG, (●) 1,2-DAG, (□) MAG, (■) total DAG. *No MAG detected. For enzyme suppliers, see Figure 1.

TABLE 2
Activity of the Seven Lipases (expressed as PLU/g)

Lipases	Activity (PLU/g)[a]
Lipase PS-D	305 ± 48.0
Lipase AK	310 ± 0.7
Lipase F-AP15	2531 ± 495.4
Novozym 435	7922 ± 161.6
Lipozyme TL IM	693 ± 32.2
Lipozyme RM IM	3744 ± 17.4
Novozym CALB L (imm.)	5580 ± 345.1

[a]Average ± SD, $n = 4$. For enzyme suppliers, see Table 1.

Lipase PS-D, as demonstrated in an esterification reaction in *n*-hexane by Kwon *et al.* (27). In our study, Lipase PS-D showed similar rates of TAG conversion and a similar DAG yield when silica gel was added to the reaction substrate as when glycerol was preabsorbed on the silica gel (data not shown). Glycerol appears to absorb to silica gel in the reaction mixture automatically, which can explain the inactivation of Lipozyme TL IM in reactions with free glycerol, since the carrier material is granulated silica. Moreover, the absorption of glycerol may be the mechanism responsible for inactivating the tested lipases having hydrophilic carriers when free glycerol was used.

Activity measurements. The esterification activity of the seven lipases was determined to compare the glycerolysis results with lipase activities. Large differences were observed between the activities of the lipases (expressed as PLU/g) (Table 2). These differences were not reflected in any of the glycerolysis results. The physical nature of the reaction medium may have affected the lipase preparations differently and thereby have had great influence on the catalysis. The ester synthesis activity of the lipases was unrelated to the glycerolysis activity, as also shown in the literature (28).

1,3-DAG vs. 1,2-DAG contents. A high content of the 1,3-DAG isomer was desired because of its nutritional benefits. The specificity of the lipase, the lipase carrier material, and the temperature of the reaction contributed to the isomeric DAG ratio in the reaction mixture. Nonspecific lipases produced more 1,3-DAG in a short reaction time compared with 1,3-specific lipases (Fig. 4A). This was expected because of the ability of nonspecific lipases to remove an acyl moiety from the *sn*-2 position of the TAG molecule, preferably forming 1,3-DAG or 1(3)-MAG. Hence, with nonspecific lipases any acyl moiety of the TAG molecules can be transesterified to any position in the glycerol. With 1,3-specific lipases, only acyl moieties in the *sn*-1 and *sn*-3 positions of TAG molecules can be transeserified to the *sn*-1 and *sn*-3 positions of the glycerol. In this case, TAG molecules can be transesterified only to 1,2-DAG and 2-MAG during glycerolysis and the glycerol molecules only to 1,3-DAG and 1(3)-MAG. At longer reaction times, acyl migration becomes important and the amount of the 1,3-DAG isomer increases in the glycerolysis mixture. Acids, bases, and heat can promote acyl migration (29). Accordingly, the enzyme carrier can also affect acyl migration if it has an acidic or basic surface, such as silica, celite, and ion-exchange resins. In the screened reactions, all lipases except Lipase F-AP15 resulted in a higher 1,3-DAG content at 28 h (Fig. 4B). The lower reaction temperature and the lack of an enzyme carrier may explain the high 1,2-DAG content for Lipase F-AP15. Fureby *et al.* (22) reported that equilibrium between 1,2-DAG and 1,3-DAG in a glycerolysis reaction at 40°C with the 1,3-specific lipase from *Rhizopus arrhizus* was reached in 72 h. Hence 1,3-DAG might have become the dominant isomer in the reaction mixture with Lipase F-AP15 had a longer reaction time been applied.

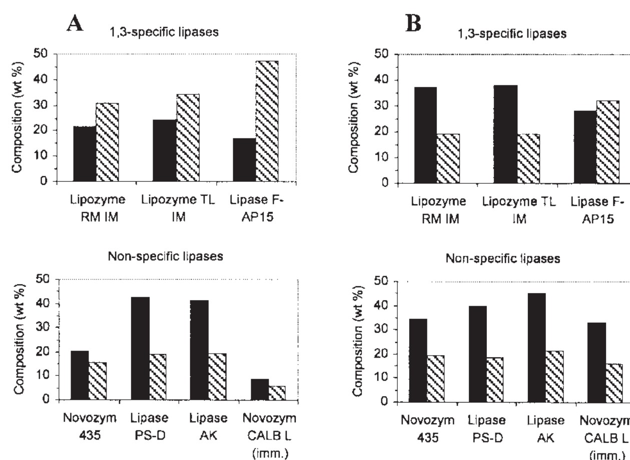

FIG. 4. Isomeric ratio of 1,3-DAG and 1,2-DAG in the reaction mixture for 1,3-specific lipases and nonspecific lipases, respectively. (A) 2 h of incubation; (B) 28 h of incubation. (black bars) 1,3-DAG, (crossed-hatched bars) 1,2-DAG. For enzyme suppliers, see Figure 1.

In the large-scale production of DAG at high yields, short reaction times and low temperatures are of economic interest, so a nonspecific lipase is recommended.

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