Determining the Regioselectivity of Immobilized Lipases in Triacylglycerol Acidolysis Reactions

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ABSTRACT: Lipase regioselectivity is the ability to distinguish between primary (i.e., *sn*-1,3) and secondary (*sn*-2) ester functionalities in a triacylglycerol molecule, which is of importance in the manufacture of structured lipids. Unlike existing methods of assessment, which utilize hydrolysis reactions, an alternative technique to assess the regioselectivity of lipases in triacylglycerol transesterification reactions has been developed. An acidolysis reaction is performed between triolein and decanoic, lauric, or stearic acids under conditions that minimize acyl migration, and products are analyzed by silver-ion complexation liquid chromatography, enabling detection of specific triacylglycerol positional isomers. From the rate of formation of these isomers the relative selectivity of the lipase for *sn*-2 and *sn*-1,3 ester bonds is determined. With lipases known to lack regioselectivity, the rate of reaction at *sn*-2 was similar to that at *sn*-1,3 from the start of the reaction. With *sn*-1,3 selective lipases, the formation of triacylglycerol isomers with decanoic acid in the secondary position was not detected at any point in the reaction. Regioselectivity as a function of reaction progress was monitored. Two lipases from the genus *Pseudomonas* exhibited activity toward all positions, but the rate at *sn*-2 was much reduced, and no incorporation of decanoic acid into this position was detectable until a high degree of conversion had been achieved.

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KEY WORDS: Acidolysis, assay, lipase, regioselectivity, selectivity, silver-ion high-performance liquid chromatography, *sn*-2.

Lipases are utilized as catalysts for an increasing number of commercial biotransformation reactions, such as the production of Betapol®, an infant formula fat with high *sn*-2 palmitic acid content, and isopropyl myristate, an effective biodegradable lubricant (1,2). The increase in use is largely a result of the ability of lipases to catalyze reactions under mild conditions, and more importantly, of their ability to exhibit selectivity for certain substrates and their stereoisomers. With regard to reactions involving triacylglycerols, lipases can be classified according to their selectivity for the acyl position on the glycerol backbone (i.e., regioselectivity). Generally, lipases catalyze reactions at the primary acyl positions of a triacylglycerol (*sn*-1,3) or at all positions (*sn*-1,2, and 3). A lipase with selectivity for the secondary (*sn*-2) position only does not appear to exist. Knowledge of the regioselectivity of a lipase is important to a lipid chemist as the nutritional and physical properties of fats can be manipulated by changing the constituent acyl chains at specific positions.

Classifying the regio- (and stereo-) selectivity of lipases has traditionally been carried out by use of hydrolytic assays, where a known substrate is partially hydrolyzed by a lipase and the products of the hydrolysis are characterized (3–8). These techniques are often laborious and there is a risk that acyl migration might occur, leading to false interpretation of data. Prior to the exploitation of lipase selectivity in a transesterification reaction, it is desirable to test for that selectivity in a similar microaqueous system. Methods to accomplish this have been reported, which involve the analysis of the product triacylglycerols by mass spectrometry, nuclear magnetic resonance, or following nonenzymic degradation to elucidate the positional character (9–11). A simpler analysis is available by starting with a triacylglycerol substrate of known positional composition (e.g., cocoa butter stearin, consisting mainly of palmitic acid/stearic acid in *sn*-1,3 and oleic acid in *sn*-2), which is then enzymically transesterified with a known fatty acid (e.g., lauric). The triacylglycerol products are analyzed by gas chromatography after transmethylation; the decrease in oleic acid content is taken as a measure of the nonselectivity of the lipase (12). Although this method is technically sound, an alternative assay is presented here that utilizes triolein and decanoic acid, both of which are readily available; their contrasting levels of saturation facilitate the characterization of the triacylglycerol products by silver-ion complexation high-performance liquid chromatography (HPLC) (13–15). An unexpected benefit of the assay is the ability to determine the onset of transesterification at the *sn*-2 position.

MATERIALS AND METHODS

All chemicals were obtained in the highest available purity from the Sigma-Aldrich group (Poole, United Kingdom) or Fisher Scientific (Loughborough, United Kingdom). Solvents were obtained in analytical grade or better from Sherman Chemicals (Sandy, United Kingdom) or Rathburns (Glasgow, United Kingdom). Lipases (EC 3.1.1.3) were obtained from various sources. Lipases from *Rhizopus niveus* (Lipase N, 4,500 LU/g), *R. delemar* (Lipase D, 650,000 LU/g, now reclassified as *R. oryzae*), and *Pseudomonas* sp. (Lipase P, 22,000 LU/g, now reclassified as *Burkholderia* sp.) were obtained from Amano Pharmaceuticals Co. Ltd. (Nagoya, Japan). Lipases from *Humicola lanuginosa* (Lipolase, 100,000 LU/mL, now reclassified as *Thermomyces lanuginosus*), *Candida*

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TABLE 1
Summary of Catalyst Activities Calculated for the Acidolysis Reaction at Positions *sn*-1,3 and *sn*-2 and the Relative Rates at *sn*-2 Expressed as a Percentage of the Rate at *sn*-1,3

Lipase source, carrier and loading ($LU/g_{support}$)	Catalyst activity[b] ($g_{TAG} \cdot h^{-1} \cdot g_{CAT}^{-1}$)		Relative percentage of *sn*-2 activity
	sn-1/3 positions	*sn*-2 position	
Rhizomucor miehei[a] (Lipozyme)/Duolite ES568 10,000 LU/g 3.8	3.8	Negligible	—
	4.0	Negligible	—
Rhizopus niveus (Lipase N)/Accurel EP100 12,800 LU/g	6.4	Negligible	—
Rhizopus oryzae (Lipase D)/Accurel EP100 41,000 LU/g	37.8	Negligible	—
Humicola sp.[a] (Lipolase)/ Accurel EP100 182,000 LU/g	28.2	Negligible	—
Pseudomonas sp. (Kurita KWI-56)/ Accurel EP100 22,000 LU/g	3.5	0.6	17
Pseudomonas sp. (Lipase P)/ Accurel EP100 74,000 LU/g	3.5	0.7	21
Arthrobacter sp. Accurel EP100 37,100 LU/g	6.8	6.8	100
Candida antarctica A[a] Accurel EP100 27,000 LU/g	0.8	0.9	116
	0.9	*1.1*	*123*

[a]This lipase has been expressed by a host organism (*Aspergillus oryzae*) encoded with the gene from the named organism using recombinant DNA technology.
[b]Italics refer to a reaction using stearic acid instead of decanoic acid. TAG, triacylglycerol; CAT, catalyst.

antarctica (A lipase, immobilized onto Accurel EP100), and *Rhizomucor miehei* (Lipozyme IM, immobilized onto Duolite ES568N anion exchange resin) were gifts from Novo Nordisk (Bagsværd, Denmark). Lipase from *Pseudomonas* sp. (code KWI-56, 500,000 LU/g) was a gift from Kurita Water Industries Ltd. (Osaka, Japan). Lipase from *Arthrobacter* sp. was a gift from Sumitomo Chemical Co. Ltd. (Tokyo, Japan, 180,000 LU/g). The lipases that were not supplied in the form of an immobilized catalyst were adsorbed onto macroporous polypropylene (Accurel EP100 from Akzo Nobel, Obernburg, Germany) before use, following the method of Bosley and Peilow (16). The hydrolytic activity of the free lipase preparations was determined. One Lipase Unit (LU) is defined as the amount of enzyme that liberates 1 μmol titratable butyric acid/min from an emulsion of tributyrin at pH 7.0. The lipase loadings of the immobilized preparations are stated in Table 1. Porcine pancreatic lipase for determination of *sn*-2 composition was purchased from Sigma (Type II, crude, 41,000 LU/g).

Acidolysis reaction. All reactions were carried out in duplicate. Single-acid substrates were selected to simplify the interpretation of chromatograms, although the results shown in Table 2 are from experiments with high oleic acid sunflower oil. Triolein (2 g, 2.3 mmol), decanoic acid (1.17 g, 6.8 mmol) or lauric acid (1.36 g, 6.8 mmol) or stearic acid (1.93 g, 6.8 mmol), and deionized water (6.3 μL, 0.2 % w/w) were mixed in a 6-mL sealed vial at 50°C until fully dispersed. When stearic acid was used, 10 mL of *n*-hexane was also added to the vial in order to solubilize stearic acid at 50°C. The reactions may be carried out at 30°C if the lipase is thermally labile, but this was not necessary for the lipases evaluated. Immobilized lipase catalyst was added to the reaction mixture to commence the transesterification reaction. The amount of catalyst that was required to complete the reaction in 2 h was predetermined. This was achieved by carrying out a prior reaction using an arbitrary amount of immobilized catalyst (e.g,. 0.1% w/w) to establish the catalyst activity and interpolating the quantity required for the regioselectivity tests. The experimental conditions were selected in order to

TABLE 2
The Effect of Lipase Source on the Degree of Incorporation of Lauric Acid into the *sn*-2 Position of High Oleate Sunflower Oil at High Degrees of Conversion[a]

Lipase source[a] and carrier[a]	Percentage of lauric acid incorporated into *sn*-2 at high degrees of conversion (≥90%)
Rhizomucor miehei (Lipozyme)/Duolite ES568	3
Rhizopus niveus (Lipase N)/Accurel EP100	7
Pseudomonas sp. (Kurita KWI-56)/ Accurel EP100	32
Pseudomonas sp. (Lipase P)/Accurel EP100	36

[a]Determined by Porcine Pancreatic Lipase Hydrolysis and Analysis of the 2-Monoacylglycerol.
[b]Lipase N and Lipase P were from Amano Pharmaceuticals Co. Ltd. (Nagoya, Japan), Lipozyme immobilized on Duolite ES568 was from Novo Nordisk (Bagsvaerd, Denmark), and Kurita KWI-56 was from Kurita Water Industries Ltd. (Osaka, Japan). Accurel EP100 was supplied by Akzo Nobel, (Obernburg, Germany).

minimize the risk of acyl migration, which has been shown to be dependent on reaction time, water content, enzyme concentration, and support type (17,18). Samples of reaction mixture (50 μL) were removed by syringe through a septum from the reaction vial at regular intervals. Free fatty acid was removed from the sample by elution from a column of aluminum oxide (Brockmann type II, 2 g; 5 mm i.d. column; Fisher) with diethyl ether (4 mL). After removal of the solvent by evaporation under a stream of nitrogen, the triacylglycerol was analyzed. No attempt was made to remove diacylglycerol components, which were chromatographically resolved from triacylglycerol during the analysis.

Positional analysis of the triacylglycerol by silver ion complexation HPLC. The distribution of saturated decanoic acid in triacylglycerol positions *sn*-1,3 and *sn*-2 was determined by silver ion complexation HPLC (Varian 5560 ternary liquid chromatograph fitted with a Varian 8300 autosampler; Palo Alto, CA), following the exact methodology developed by Jeffrey (14). A solution of the deacidified triacylglycerol (10–20 mg/mL in toluene) was injected onto a 100 × 4.6 mm i.d. silica column (Nucleosil 100-3; Machery-Nagel, Düren, Germany) impregnated with 10% (w/w) silver nitrate. The preparation of this column is detailed in Jeffrey's article (14). Separation of triacylglycerol isomers was based on the number and position of unsaturated double bonds within the molecule. This was achieved by using a programmed solvent gradient of increasing polarity (from toluene/*n*-hexane (1:1 vol/vol) to toluene/ethyl acetate (9:1 vol/vol) over 10 min (flow rate of 1.5 mL/min) then regenerating the column with 0.008% formic acid in toluene for 5 min. A flame-ionization detector (FID) was used (Tracor 945 transport-FID; Tracor Instruments Inc., Austin, TX). The entire column eluent was discharged onto a quartz belt, which rotated at 5 rpm. The belt then carried the eluent through a heated and evacuated chamber to evaporate the solvent at 180°C, then through a hydrogen FID (160 mL/min hydrogen; 400 mL/min air) to quantify the remaining high-boiling lipid fraction. The belt was then returned to a clean state by a hydrogen/oxygen cleaner flame (500 mL/min hydrogen; 260 mL/min oxygen). A fuller description of the Tracor detector can be found in Hammond's review of lipid chromatography techniques (19). The peaks on the chromatograms were positively identified by use of chemically synthesized standard acylglycerols. The detector response from medium chain-containing triacylglycerols was essentially identical to that for long-chain molecules. Where appropriate, internal standards (e.g., tripalmitin) were added to the sample, and the sample dilution was decreased in order to accurately quantify the smaller peaks.

Calculation of the degree of reaction conversion. By using peak area percentage data from the HPLC chromatograms the degree of conversion was determined. The degree of conversion (x) was expressed in terms of progress of the reaction toward a predicted equilibrium, where

x = molar percentage of decanoic acid incorporated into triacylglycerols/molar percentage of decanoic acid predicted to be incorporated into triacylglycerols at thermodynamic equilibrium [1]

From HPLC data

$$x = [\%\ DOO + (2 \cdot \%\ DOD) + (2 \cdot \%\ DDO) + (3 \cdot \%\ DDD) + \%\ ODO]/[\%\ DOO + (2 \cdot \%\ DOD) + (2 \cdot \%\ DDO) + (3 \cdot \%\ DDD) + \%\ ODO \text{ at thermodynamic equilibrium}] \quad [2]$$

where D = decanoic acid and O = oleic acid; e.g., DDO refers to the triacylglycerol species 1,2-didecanoyl-3-oleoyl-*rac*-glycerol.

The percentage of decanoic acid expected in the sample at thermodynamic equilibrium was calculated by application of known rules for acylglycerol distribution based on the molar concentrations of the reaction substrates. For *sn*-1,3-selective lipases this is the *1,3-random-2-random* rule and for nonselective lipases, the *random (1,2,3-random)* distribution rule (20). Partial regioselectivity presents a difficulty as neither of the above distribution rules is strictly correct but the *random (1,2,3-random)* rule has been applied here.

Calculation of relative lipase activity with respect to positions sn-*1,3 and* sn-*2.* From HPLC data, a degree of conversion (x) was calculated for the primary and secondary triacylglycerol positions at each time point, where

$$x_{(sn\text{-}1,3)} = [\%\ DOO + (2 \cdot \%\ DOD) + (2 \cdot \%\ DDO) + (3 \cdot \%\ DDD)]/[\%\ DOO + (2 \cdot \%\ DOD) + (2 \cdot \%\ DDO) + (3 \cdot \%\ DDD) \text{ at thermodynamic equilibrium}] \quad [3]$$

$$x_{(sn\text{-}2)} = [\%\ ODO + \%\ DDO + \%\ DDD]/[\%\ ODO + \%\ DDO + \%\ DDD \text{ at thermodynamic equilibrium}]. \quad [4]$$

From a linear first-order plot of $-\ln(1 - x)$ against time, the

catalyst activities (A) at *sn*-1,3 and *sn*-2 were calculated as

$$A = \text{slope/weight of catalyst} \quad [5]$$

where the weight of catalyst refers to the combined weight of lipase and support.

Analysis of the triacylglycerol sn-*2 composition by pancreatic lipase hydrolysis*. The method used for the hydrolysis of triacylglycerol and separation of the 2-monoacylglycerol product was that described by Christie (21). The isolated 2-monoacylglycerol was transmethylated with 0.5 M sodium methoxide in methanol at 30°C for 30 min in a sealed vial to avoid loss of volatile short-chain fatty acid methyl esters. The methyl esters were analyzed using a PerkinElmer 8500 gas–liquid chromatograph with programmed temperature vaporization on-column injection (Shelton, CT), equipped with a FAMEWAX column (0.53 mm i.d. × 30 m; Restek, Evry, France). Column-oven temperature was programmed from 80 to 180°C at 20°C/min. Components were identified by reference to a methyl ester standard (17A; Nu-Chek-Prep, Elysian, MN).

RESULTS AND DISCUSSION

The theory that silver-ion HPLC could be used as a method of quantifying the degree of incorporation of decanoic acid into the *sn*-2 position of triolein has been verified. Figure 1 shows a chromatogram in which the six expected triacylglycerol components from a nonselective acidolysis of triolein and decanoic acid have been resolved. Decanoic acid was selected for use to allow for reactions to be conducted at 30–50°C without the need to add solvent. Owing to the relative polarity of decanoic acid, triacylglycerols containing decanoic acid eluted from the column later than triacylglycerols containing longer-chain saturated acids (data not shown). However, this delay in elution did not affect either resolution or identification. A number of other acylglycerol species eluted after the six peaks of interest, and these were predominantly diacylglycerols (with a trace of polyunsaturated triacylglycerol impurity). Because diacylglycerols elute later than triacylglycerols on silica, all acylglycerol products were injected to avoid loss of the relatively polar decanoic acid-containing triacylglycerols during sample cleanup.

A range of lipases was selected for testing, including lipases known to be nonselective and those known to be *sn*-1,3 selective. Catalyst activity varies greatly depending on the lipase loading and its source, but the data were found to be reproducible within a 10% error range (typical for immobilized catalyst reactions).

Rhizopus, Rhizomucor, *and* Humicola *lipases.* No significant incorporation of decanoic acid into *sn*-2 was observed in reactions conducted with these lipases using the new assay (Fig. 2, Table 1). This finding was expected as the regioselectivity of these lipases, and the *R. miehei* lipase in particular, has been thoroughly tested by many research groups and all are accepted as being positionally *sn*-1,3 selective. However, at very high degrees of conversion, small quantities of triacylglycerols with decanoic acid in *sn*-2 were detected, and this observation was also made in comparative experiments where hydrolysis by pancreatic lipase was used to determine the *sn*-2 content of the triacylglycerol products of enzymatic interesterification (Table 2). Acyl migration is believed to be the cause of this *sn*-2 incorporation, resulting from the long reaction times required to achieve the high degree of conversion at which these products were obtained (>90% after 8–16 h of reaction).

The extent of acyl migration is known to be time dependent, and it is also known that the quantity of catalyst and use of an ionic support for lipases can promote acyl migration (17,18,22). However, when testing the new assay with *R. miehei* lipase immobilized on Duolite ion exchange resin, no significant quantity of triacylglycerols with decanoic acid in the *sn*-2 position could be detected in any assay that was concluded within 4 h (all reactions were catalyzed by less than 10% w/w catalyst). This appeared to justify the adoption of a short reaction time for the newly developed assay, to minimize effects of acyl migration. Further corroboration was

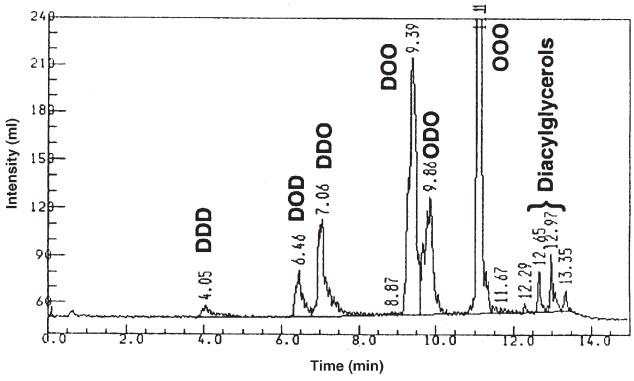

FIG. 1. Silver-ion complexation high-performance liquid chromatogram of triacylglycerol product after 2 h of reaction (catalyzed by *Candida antarctica* A lipase), where D = decanoic acid and O = oleic acid, e.g., DDO refers to the triacylglycerol species 1,2-didecanoyl-3-oleoyl-*rac*-glycerol.

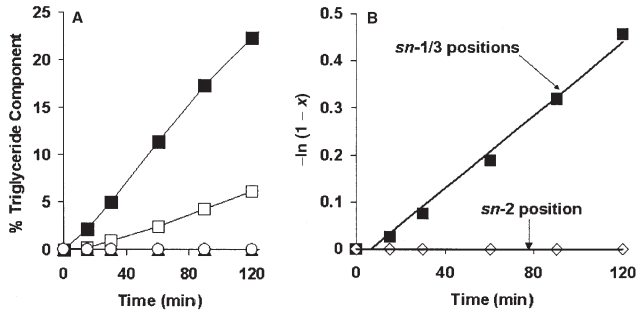

FIG. 2. Reaction composition (A) and first-order rate plots (B) for the acidolysis of triolein with decanoic acid catalyzed by *sn*-1/3 selective lipase (*Rhizopus niveus*), OOO, not shown; ◆, DDD; □, DOD; ▲, DDO; ■, DOO; ○, ODO. For abbreviations see Figure 1.

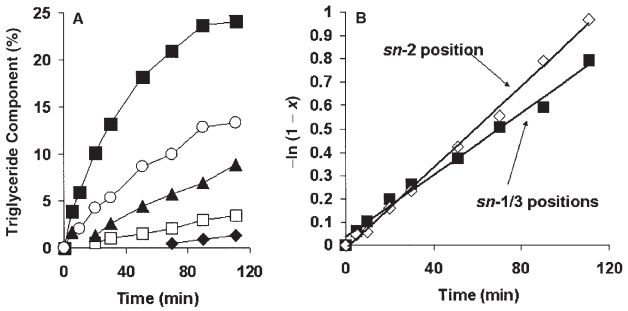

FIG. 3. Reaction composition (A) and first-order rate plots (B) for the acidolysis of triolein with decanoic acid catalyzed by nonregioselective lipase (*Candida antarctica* A). For abbreviations and key see Figures 1 and 2; OOO not shown.

available from Muderwha *et al.* (23) and Haraldsson *et al.* (24), in which no significant changes in *sn*-2 composition were observed in acidolysis reactions catalyzed by *R. miehei* lipase within 4 or 5 h. In these articles the composition of the *sn*-2 position in products of acidolysis and transesterification reactions was determined by either ethyl magnesium bromide or pancreatic lipase, and it was concluded that acyl migration was the likely cause of changes in the composition of the mid position. It is therefore assumed that if triacylglycerols containing decanoic (or stearic) acid in position *sn*-2 are detected in the new assay carried out under the specified conditions, they are likely to have resulted from direct lipase catalysis rather than acyl migration.

Candida antarctica *A lipase.* As expected, the A lipase from *C. antarctica* incorporated decanoic acid into the primary and secondary positions at similar rates (Fig. 3). However, a small preference for *sn*-2 was observed (16% higher rate than at *sn*-1,3). A small preference for position *sn*-2 by *C. antarctica* A was also reported by Rogalska *et al.* (4). By using stearic acid as the donor acid in the new assay, this slight preference for position *sn*-2 was also obtained (Table 1).

Pseudomonas *sp. lipases*. The broad *Pseudomonas* genus contains strains that produce lipases with different regioselectivities. However, partial regioselectivity for *sn*-2 was observed for the two *Pseudomonas* lipases that were tested (Lipase P and KWI-56; Fig. 4 and Table 1), where the rate of incorporation at *sn*-2 was approximately one-fifth of the rate at *sn*-1,3. At high degrees of conversion, a similar order of *sn*-2 incorporation was found for the two *Pseudomonas* lipases using pancreatic lipase hydrolysis to determine the composition of the *sn*-2 position (Table 2). Partial *sn*-2 selectivity of Lipase P was also reported by Matori *et al.* (3) and is a well-known characteristic for lipases produced by certain *Pseudomonas* sp. organisms. However, the most interesting observation that was made for reactions catalyzed by Lipase P and KWI-56 is that no decanoic acid incorporation into *sn*-2 was detected until an overall reaction conversion of 40% had been reached. To my knowledge this observation has not been previously reported for either of these *Pseudomonas* lipases. In their paper, Rogalska *et al.* (4) reported no significant *sn*-2 activity with four *Pseudomonas* sp. lipases but these measurements were taken at a degree of conversion of only 6% (4).

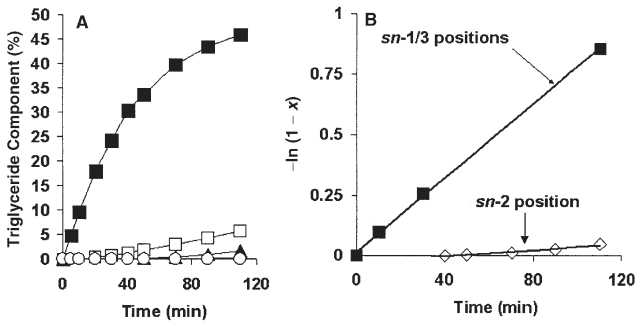

FIG. 4. Reaction composition (A) and first-order rate plots (B) for the acidolysis of triolein with decanoic acid catalyzed by partially regioselective lipase (Lipase P). For abbreviations and key see Figures 1 and 2; OOO, not shown.

Arthrobacter *sp. lipase.* This lipase was demonstrated to have nonregioselective behavior by using this assay. A small but significant delay was encountered before the detection of triacylglycerols containing *sn*-2 decanoate (reaction conversion of 4%).

Substrate acid chain length. Changing the acid in the new assay from decanoic to stearic had no significant effect on either the overall rate of reaction or the observed regioselectivity of *R. miehei* lipase and *C. antarctica* A lipase (Table 1). However, for some lipases the chain length of the substrate can have an effect on reaction rate, and this should be accounted for when assaying the regioselectivity of different lipases. It is reported that the lipases from *C. rugosa* and porcine pancreas exhibit a preference for shorter-chain substrates, whereas the lipase from *R. miehei* showed little significant difference in transesterification reaction rate with a range of C8–C18 esters (25). The effect of the chain length of the triacylglycerol substrate with respect to lipase regioselectivity has not been reported in many instances, but an effect of chain length on lipase stereoselectivity has been observed in previous studies (4,26).

The positional selectivity of an immobilized lipase in nonaqueous synthetic reactions can be determined by direct HPLC analysis of reaction products. Use of complex hydrolytic methods of analysis can therefore be avoided.

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