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Enzymatic Re-esterification of Lower Glycerides from Soybean Oil with Conjugated Linoleic Acid (CLA)

ESTELA HERNÁNDEZ-MARTÍN AND CRISTINA OTERO*

Department of Biocatalysis, Institute of Catalysis and Petroleochemistry, CSIC, Cantoblanco, 28049 Madrid, Spain

New structured lipids essentially free of saturated fatty acids and rich in essential fatty acids and CLA were prepared from soybean oil using a selective enzymatic procedure of synthesis. Saturated fatty acids originally present in the oil were selectively removed in a selective alcoholysis step. Enzymatic re-esterification with conjugated linoleic acid (CLA) of lower glycerides (di- and monoglycerides) obtained from soybean oil was studied using several commercially available lipases. Higher maximum reaction conversions were obtained with the free acid form of CLA than with the corresponding ethyl ester. Reactions with Novozym 435 were fastest, producing 84% diacylglycerols (DAG) in 0.5 h of reaction at 60 °C. For Lipozyme RM IM the maximum yields of triacylglycerols (TAG) were achieved at 60 °C (43% in 4.5 h) and 25 °C (43% TAG in 7 h). The dominant species in DAG-rich products were LnL and LL. TAG-rich products obtained with Lipozyme RM IM had LLL and LLO/OLO as their predominant TAG. Quantitative conversions to DAG were obtained in 3−4.5 h with 10% (w/w) Lipase G 50 at 60 °C and a molar ratio of free CLA to hydroxyl groups of 5:1.

KEYWORDS: Acylglycerides; soybean oil; lipase; polyunsaturated fatty acids; CLA; structured lipids; nutraceuticals

INTRODUCTION

Structured lipids are triglycerides (TAG) and lower glycerides [i.e., diglycerides (DAG) and monoglycerides (MAG)] that have been modified, either chemically or enzymatically, to obtain lipids that contain specific fatty acid (FA) residues and/or are characterized by particular distributions of these residues along the glycerol backbone. Some structured lipids are used in the formulation of nutraceuticals because they confer either preventative or therapeutic medicinal benefits.

Enzymatic reactions provide a viable technology for obtaining glycerides having structures that have been optimized with respect to the physiological and therapeutic properties associated with particular fatty acid residues. One thus minimizes undesirable effects associated with these residues and maximizes the beneficial health effects of the modified glycerides. Use of lipases as biocatalysts permits one to utilize mild reaction conditions, which facilitate control of the reaction, require short reaction times (which minimize both migration of acyl groups and partial oxidation processes), and improve selectivity with respect to both the type and positions of fatty acid residues on the glycerol backbone.

Various strategies for the synthesis of structured lipids based on the use of enzymatic catalysis have been studied. These biocatalytic processes include enrichment of salmon oil with polyunsaturated fatty acid (PUFA) residues via lipolysis and enzymatic re-esterification reactions (*1*), preparation of lower glycerides by enzymatic esterification of glycerol with CLA (*2*), and lipase-catalyzed synthesis of structrured TAG from 1,3-DAG (*3*), among others.

Conjugated linoleic acid (CLA) refers to a mixture of positional and geometric isomers of linoleic acid. Linoleic acid is an essential PUFA with an 18-carbon chain with two olefinic bonds in positions 9 and 12. At least 13 different isomers of CLA have been reported. The olefinic bonds of the isomers are conjugated. CLA is a substance that occurs naturally in dairy products and meat. Ingestion of CLA produces physiological benefits in humans, stimulating the immune system, protecting against cancer and atherosclerosis, and having positive effects on cardiovascular health (*4*).

The purpose of this investigation is to use lower glycerides derived from soybean oil to prepare new structured lipids essentially free of saturated fatty acids and rich in essential fatty acids and CLA, through the combined use of two selective enzymatic steps: one that selectively reduces the content of saturated fatty acids, followed by another step in which maximal incorporation of CLA and minimal removal of unsaturated fatty acids is obtained. The synthesis protocol involved the following steps: (i) Preparation of lower glycerides enriched in PUFA was performed via optimized enzymatic ethanolysis of soybean oil (*5*). This step permits one to selectively eliminate saturated FA residues from the TAG constituting soybean oil. (ii) Purification of glycerides and removal of fatty acid ethyl esters (FAEE) from

* Address correspondence to this author at the Instituto de Catálisis, C/Marie Curie 2, 28049 Cantoblanco, Madrid, Spain (e-mail cotero@icp.csic.es; telephone 34 91 5854805 or 34 91 5854800; fax 34 91 5854760).

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the product mixture was performed using solid phase extraction (SPE). (iii) The purified MAG and DAG were re-esterified with CLA.

The synthetic route described above employs enzyme-based biotechnology for incorporation of a beneficial PUFA (CLA) residue in a mixture of MAGs and DAGs to convert soybean oil into an oil that is essentially free of saturated fatty acid residues. In the present study, conditions for the re-esterification of lower glycerides using commercially available immobilized and native lipases as biocatalysts for reaction in both an organic solvent and a solvent-free medium were investigated. The reaction parameters for optimum operating conditions and the chemical compositions of the PUFA-rich reaction products were also determined. In addition, conditions for maximum production of TAGs and DAGs were explored.

MATERIALS

Soybean oil was purchased from Biolasi (Ordizia, Spain). The free fatty acid (FFA) form of CLA and the ethyl esters of CLA (FAEE) were provided by Natural Asa (Hovdebygda, Norway). The composition of the CLA FFA was as follows: 80% CLA, 15% C18:1, and 5% others (mainly C18 and C16:0). As reported by the vendor, GC area percentages of the different isomers of CLA (FFA) were 0.42% c9c12, 42.73% c9t11, 44.24% t10c12, 1.33% c9c11, 1.35% c10c12, and 1.19% t10t11 + t10t12. The fatty acid composition of the CLA FAEE was 85% CLA, 10% C18:1, and 5% others (mainly C18 and C16:0). The lipases Novozym 435 (*Candida antarctica* fraction B lipase immobilized on a macroporous resin), Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on a granulated silica), and Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on an anion-exchange resin) were a generous gift from Novozymes A/S (Bagsvaerd, Denmark). Lipase PS (*Burkholderia cepacia* free lipase), Lipase PS-C I (*B. cepacia* lipase immobilized on ceramic), Lipase PS-C II (*B. cepacia* lipase immobilized on ceramic), Lipase PS-D I (*B. cepacia* lipase immobilized on diatomaceous earth), Lipase AK (*Pseudomonas fluorescens* free lipase), Lipase AY 30 (*Candida rugosa* free lipase), Lipase AY 30 G (*C. rugosa* free lipase), Lipase AYS (*C. rugosa* free lipase), and Lipase G 50 (*Penicillium camembertii* free lipase) were purchased from Amano Enzyme Inc. (Nagoya, Japan). Spe-ed SPE cartridges (silica gel, 10 g, 60 mL) were obtained from Applied Separations (Allentown, PA). Hexadecane, formic acid, and HPLC grade chloroform were from Aldrich (Madrid, Spain). *n*-Hexane, 2-propanol, ethyl acetate, acetone, acetonitrile, methanol, and ethanol were of HPLC grade from Scharlau (Barcelona, Spain), whereas dichloromethane was obtained from Panreac (Barcelona, Spain). Methanolic-base and fatty acid standard Supelco 37 Component FAME Mix were purchased from Supelco (Bellefonte, PA). Acylglycerol standards (triolein, diolein, and monoolein) and molecular sieves (8−12 mesh beads) were obtained from Sigma (Madrid, Spain).

METHODS

Synthesis of Lower Glycerides. Lower glycerides enriched in PUFA were prepared using optimized conditions for the enzymatic ethanolysis of soybean oil as described by Hernández-Martín et al. (*5*). The reaction conditions were as follows: 2 g of soybean oil, 1.5 g of ethanol ([EtOH]/[FA] = 4.5 mol/mol), 50% Novozym 435 (w/w with respect to the oil), 25 °C, 200 rpm, and 1 h of reaction time. The reaction products prepared from 10 g of soybean oil were dissolved in chloroform (ca. 20 mg/mL) after removal of the enzyme by filtration.

Purification of Lower Glycerides. The FAEE products as well as the remaining TAGs were removed from the oil samples by SPE, using silica gel cartridges and a PrepSep 12-Port SPE vacuum manifold from Fischer Scientific (Supelco, Bellefonte, PA). The elution scheme used to separate the lower glycerides from the FAEE and the extracted compounds are shown in **Table 1**. Isopropanol was eliminated using a stream of flowing nitrogen until dry. Next, an isolated mixture of DAG and MAG was subjected to a re-esterification reaction with CLA. SPE technology was employed instead of molecular distillation to avoid migration of acyl moieties during the extraction/separation process.

Table 1. Elution Protocol Employed for Purification of Lower Glycerides

step	solvent	vol (mL)	eluted compd
conditioning	$CHCl_3$	20	
sample loading	$CHCl_3$	20	
extraction	$CHCl_3$	20	FAEE, TAG
	$CHCl_3$/acetone/MeOH (95:4.5:0.5 v/v)	20	FAEE, TAG
	2-PrOH	20	DAG, MAG
washing	EtOH	20	
	acetone	20	

Re-esterification of Lower Glycerides with CLA. Half a gram of lower glycerides from soybean oil, 0.05 g of (0.22 mmol) of hexadecane as an internal standard, and 10% (w/w with respect to total reactants) molecular sieves were placed in 50 mL stoppered batch reactors incubated at temperatures ranging from 25 to 60 °C in an orbital shaker operating at 200 rpm. The molar ratio of either the free fatty acid form of CLA or the corresponding ethyl ester (CLA FAEE) to OH groups present in the lower glycerides was varied from 1:1 to 10:1 (mol/mol). Variable amounts of different lipases from Novozymes A/S (Novozym 435, Lipozyme TL IM, and Lipozyme RM IM) and Amano Enzyme Inc. (Lipase PS, Lipase PS-C I, Lipase PS-C II, Lipase PS-D I, Lipase AK, Lipase AY 30, Lipase AY 30 G, Lipase AYS, and Lipase G 50) were employed as biocatalysts. Where it is so indicated, some of the reaction trials were carried out in the presence of hexane (2.5 or 5 mL) or acetone (5 mL). Aliquots of the reaction mixture were withdrawn at periodic intervals and subjected to HPLC and GC (after methylation) analyses. The reactions were allowed to proceed for a total time of 7 h. Reaction products (20 μL) were dissolved in 1000 μL of chloroform and then filtered through a 0.45 μm nylon syringe filter to remove the enzyme.

Compositional Analyses of the Reaction Products by GC. Gas chromatography (GC) was employed to determine the composition of the FA residues incorporated in the glycerol backbone of the acylglyceride products. Prior to these analyses, aliquots of the product mixture were derivatized to convert the residues present in the various glycerides to fatty acid methyl esters (FAME). All of the samples were analyzed in duplicate.

Derivatization of the Glycerides. To quantify the fatty acid residues in the glycerides as FAME, 400 μL aliquots of the chloroform solutions were methylated by adding 1 mL of 0.2 N methanolic NaOH and maintaining the solution at 60 °C for 30 min. After this time, 200 μL of distilled water was added. The resulting solutions were extracted with two 1 mL portions of *n*-hexane and then dried with sodium sulfate for at least 2 h. Hexane solutions of the dried derivatives (ca. 4 mg/mL) were subjected to GC analyses.

Analyses of FAME by GC. One microliter of the hexane solution of each sample was injected into an Agilent (Palo Alto, CA) gas chromatograph (model 6890N) fitted with a Zebron ZB-WAX capillary column (30 m × 0.25 mm × 0.25 μm film thickness) purchased from Phenomenex (Torrance, CA) and a flame ionization detector (FID). Injector and detector temperatures were 250 and 300 °C, respectively. The temperature program was as follows: starting at 50 °C for 2 min and then heating to 220 at 30 °C/min, holding at 220 °C for 18 min. Finally, the temperature was raised to 250 °C and held at this temperature for 7 min. Identification of the various fatty acids was based on a Supelco 37 Component FAME Mix.

Analyses of FAME by GC-MS. The products of the re-esterification reactions were also analyzed by GC-MS to confirm the peak assignments. These analyses focused on the products of the re-esterification reactions described above in which 0.5 g samples of the lower glycerides of soybean oil were combined with free CLA using a molar ratio of free CLA to OH groups in the glycerides of 5:1 (mol/mol), mediated by 10% Novozym 435, Lipozyme TL IM, or Lipozyme RM IM (w/w with respect to total reactants). Trials were conducted for 7 h at 25 °C in an orbital shaker operating at 200 rpm. Samples of the products were then treated and methylated as described above. For these analyses, a Varian 3800 gas chromatograph was fitted with a VF-5 ms Factor IV capillary column (30 m × 0.25 mm × 0.25 μm film thickness) from Varian and coupled to a 1200 L triple-quadrupole mass spectrometer, which operated in an electron ionization mode. The quadrupole

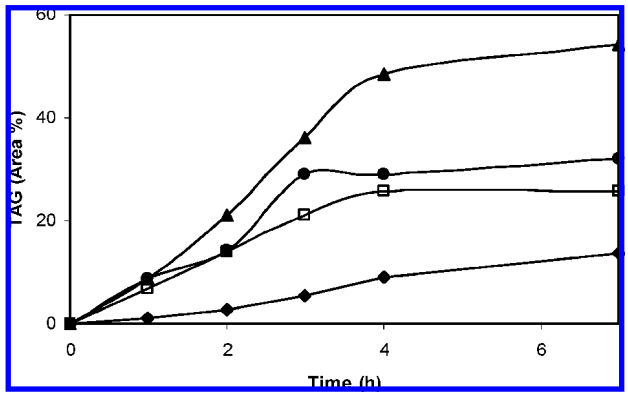

Figure 1. Effect of acylating agent on the rate of re-esterification. Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm. Symbols: ◆, 5:1 mol of free CLA/mol of OH groups in lower glycerides; ▲, 5:1 mol of free CLA/mol of OH groups in lower glycerides; ●, 1:1 mol of CLA FAEE/mol of OH groups in lower glycerides; □, 5:1 mol of CLA FAEE/mol of OH groups in lower glycerides. Experimental values correspond to the mean of at least two independent experiments. Experimental errors were below 5%.

was scanned from 40 to 500 amu to detect an extensive number of compounds. The temperature program used was that described above for the GC analyses.

Compositional Analyses of the Reaction Products by HP-LC. *Normal Phase HPLC (NP-HPLC) Analyses.* Reaction products were analyzed in duplicate by NP-HPLC to separate and quantify the acylglycerols. Six hundred microliters of the chloroform solutions was diluted with 1000 μL of chloroform and subjected to HPLC analysis.

The Merck-Hitachi HPLC apparatus was equipped with a Kromasil silica column (250 mm × 4.6 mm × 5 μm) from Análisis Vínicos (Ciudad Real, Spain) maintained at 25 °C and a Sedex 55 evaporative light scattering detector (ELSD from SEDERE, France). The ELSD drift tube temperature was 70 °C, and the air pressure was 2 bar. Two mobile phases were employed: phase A consisted of hexane, 2-propanol, ethyl acetate, and formic acid (80:10:10:0.1, v/v), and phase B consisted of hexane and formic acid (100:0.2, v/v). The flow rate was 1.5 mL/min, and the injection volume was 10 μL. The protocol employed for the mobile phase involved linear elution gradients of 1% (v/v) A increasing to 98% (v/v) A in 20 min. The final mixture (98:2, v/v, A/B) was employed for 3 min. Next, the system was restored to its initial condition by passing the 1:99 (v/v) A/B mixture through the column for 10 min.

Reversed Phase HPLC (RP-HPLC) Analyses. RP-HPLC was used to separate and quantify the TAG and DAG families present in samples of the reaction products. The column was a Kromasil C18 column (250 mm × 4.6 mm × 5 μm) from Análisis Vínicos maintained at 25 °C. The ELSD drift tube temperature was 70 °C, and the air pressure was 2 bar. The flow rate of the eluant was 0.75 mL/min, and the injection volume was 10 μL. The protocol employed for the mobile phase involved linear elution gradients for an acetonitrile/dichloromethane solvent system (75% acetonitrile/25% dichloromethane from 0 to 18 min, followed by 65% acetonitrile/35% dichloromethane until 75 min).

Chemical Characterization of Glycerides by HPLC−Mass Spectrometry (HPLC-MS) Analyses. The acylglycerol species present in the reaction mixture were analyzed using reversed phase HPLC coupled to a mass spectrometer through an atmospheric pressure chemical ionization source (RP-HPLC/APCI-MS). Compositional analyses of the glycerides by HPLC-MS were performed using a quadrupole mass analyzer (LC/MS G6120 A from Agilent Technologies) with an APCI interface coupled to an HPLC apparatus (LC/MSD mass selective detector from Agilent Technologies). Samples (10 μL) were injected in an HPLC apparatus containing a Kromasil C18 column (250 mm × 4.6 mm × 5 μm) from Análisis Vínicos. The flow rate of the eluant was 0.7 mL/min for an acetonitrile/dichloromethane solvent system (75% acetonitrile/25% dichloromethane from 0 to 18 min, followed by 65% acetonitrile/35% dichloromethane until 75 min). Detection was accomplished using the mass analyzer system. Mass spectrometer parameters were adjusted to obtain appropriate conditions. The values selected for the capillary voltage, gas temperature, flow rate of the drying gas, and nebulizer pressure were 3500 V, 200 °C, 5 L/min, and 35 psi, respectively. The vaporizer temperature, the corona current, and the voltage of the fragmentor were 200 °C, 5 μA, and 100 V, respectively. The quadrupole was scanned from 100 to 1500 amu to detect an extensive number of compounds. The spectra were recorded in the positive APCI mode.

Stability Study. To assess the stability of the lipases Novozym 435, Lipozyme TL IM, and Lipozyme RM IM, we conducted replicate trials of reactions under optimum conditions (10% biocatalyst, molar ratio of free CLA to OH groups in the lower glycerides equal to 5:1, 60 °C, 200 rpm) by repeated transfers of the enzyme to a fresh mixture of substrates. After a time corresponding to one complete reaction cycle (4 h), 20 mL of chloroform was added to the reaction mixture, and the resulting suspension was then filtered. Prior to reuse, the enzyme was washed with 10 mL of chloroform to remove oil from the previous trial and then dried. The remaining activity was measured in terms of the increase in DAG peak area for each cycle.

RESULTS AND DISCUSSION

Effect of the Acylating Agent: FFA versus FAEE. The effect of the acylating agent on the rate of the re-esterification reaction was studied in a series of trials in which either free CLA or CLA FAEE was used as the acylating agent. Inspection of **Figure 1** indicates that for the same reaction conditions the initial rates (t < 1 h) were quite similar, regardless of which acylating agent (CLA FAEE or free CLA) was employed. However, acylation with a 5 times molar excess of free CLA produced greater maximum conversion to TAG (in area percent) than the reaction with CLA FAEE. Reports in the literature indicate that incorporation of CLA in the castor oil is greater when free CLA rather than CLA FAEE is used as the acylating agent (*6*).

When FAEE is employed as acylating agent, ethanol is a byproduct and lipase-mediated ethanolysis reactions take place concurrently in the reaction medium. Elimination of the ethanol from the reaction mixture (for example, by bubbling a stream of flowing nitrogen through the liquid phase in a batch reactor) should circumvent this undesirable side reaction and shift the equilibrium position toward greater production of TAG, thereby increasing the efficiency of the process (*7*). For reaction times of 3 h or more, conversion of the lower glycerides to TAG was greater when an equimolar ratio of CLA FAEE to OH groups was employed than when this ratio was 5:1. The use of an excess of the FAEE relative to the OH groups in the lower acylglycerols is accompanied by negative effects, specifically, losses of fatty acid residues from the TAG via interesterification reactions.

We chose to employ the CLA in FFA form for subsequent experiments because this material permits one to achieve high conversions in reasonable times and because the byproduct water formed is less hazardous and does not require subsequent treatment for removal as does ethanol. Moreover, the extents of side reactions can be minimized by simply using molecular sieves in laboratory-scale experiments. Similar industrial-scale processes may be conducted under reduced pressure (partial vacuum) to largely eliminate water and to reduce the required reaction time.

Effect of the Type of Biocatalyst. The results obtained in screening experiments to assess the effectiveness of different lipases on the kinetics of re-esterification reactions are shown in **Figure 2**. During the first hour of reaction for the large majority of the biocatalysts, the increase in DAG content is

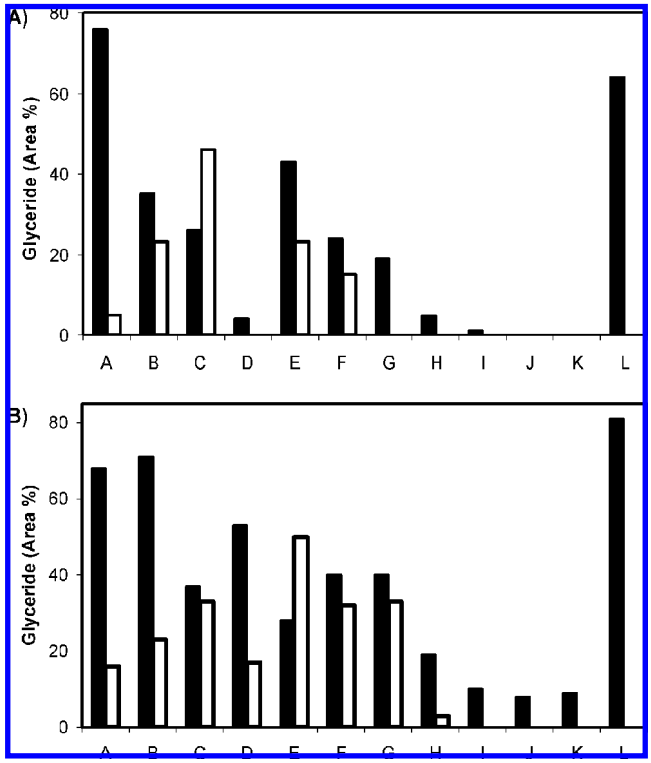

Figure 2. Screening of lipases employed in the re-esterification of lower glycerides: (**A**) 1 h; (**B**) 7 h. Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm. Black bars, DAG; white bars, TAG; A, Novozym 435; B, Lipozyme TL IM; C, Lipozyme RM IM; D, Lipase PS; E, Lipase PS-C I; F, Lipase PS-C II; G, Lipase PS-D I; H, Lipase AK; I, Lipase AY 30; J, Lipase AY 30 G; K, Lipase AYS; L, Lipase G 50. Experimental values correspond to the mean of at least two independent experiments. Experimental errors were below 5%.

significantly greater than that of TAG content (see panel **A**). As the reaction proceeds for an additional 6 h (7 h total for reaction, see panel **B**), increased amounts of TAG are formed. By contrast, the amount of DAG in the reaction decreased in some cases and increased in others. This situation can be interpreted as corresponding to sequential addition of FFA to the glycerol backbone, according to the scheme: MAG → DAG → TAG, where the FFA reactant and the byproduct water molecules have been omitted to simplify the schematic. For both reaction times, Novozym 435, Lipozyme TL IM, and Lipozyme RM IM produced major increases in the peak areas corresponding to DAG and TAG. Lipozyme RM IM produced the highest amount of TAG in 1 h (*1*). The trial involving catalysis by lipase PS was characterized by a low initial rate of reaction, but the increases in product yields observed after a total of 7 h of reaction were unexpectedly high. Lipase PS-C I was the only lipase that produced more TAG than DAG in 7 h. For the 7 h trials, Lipase PS-C II and Lipase PS-D I produced results similar to those for Lipozyme RM IM. Lipases AK, AY 30, AY 30 G, and AYS all failed to produce appreciable yields of products, even after 7 h of reaction. By contrast, Lipase G 50 was an efficient catalyst with a very high specificity for the production of DAG as shown by the results of the trials at both 1 and 7 h. This behavior suggests that Lipase G 50 could be employed to synthesize pure DAG rich in CLA, provided that the composition of the starting material contains minimal amounts of TAG.

Table 2. Extent of Incorporation of Free CLA in Precursor Lower Glycerides at the Indicated Times[a]

	esterified FA (mmol)					
	Novozym 435		Lipozyme TL IM		Lipozyme RM IM	
free CLA/OH groups (mol/mol)	0.25 h	2 h	0.25 h	2 h	0.25 h	2 h
1	0.92	1.29	0.20	0.82	0.39	0.91
5	0.80	1.48	0.22	1.13	0.63	1.10
10	0.53	0.73	0.28	1.02	1.01	1.55

[a] Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm. The uncertainties in the reported values of the extents of reaction are ±0.01 mmol.

Table 3. Composition of the FA Residues in the Re-esterified Reaction Products[a]

		re-esterified products		
FA residue (mol %)	lower glycerides	Novozym 435	Lipozyme TL IM	Lipozyme RM IM
C16:0	2.00	5.06	2.28	5.26
C18:0	0	1.71	n.d.	1.35
C18:1	20.47	15.31	19.82	14.06
C18:2 (c10, 1c12), (t10, t12)[b]	63.74	18.89	57.72	20.34
C18:3n3	7.16	1.87	6.24	2.17
C18:2 (c9, t11)	3.28	27.37	7.80	26.04
C18:2 (t10, c12)	3.35	27.66	6.14	25.06
others (unsaturated FA residues)	0	2.13	0	5.72

[a] Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm, 7 h. [b] Linoleic acid was present in the lower glycerides of soybean oil that was used as a starting material. The composition values correspond to the total values of the indicated isomer species of C18:2.

Quantitative yields of DAG (ca. 100% DAG) were obtained in 3−4.5 h of reaction when 10% (w/w with respect to total reactants) Lipase G50 was employed under the same reaction conditions used in the other trials designed to elucidate the effects of the type of biocatalyst employed. The glyceride substrate consisted solely of ca. 90% MAG and ca. 10% DAG; the molar ratio of free CLA to OH groups was 5:1, and other conditions were 10% molecular sieves, 60 °C, and 200 rpm.

Effect of the Molar Ratio of Free CLA to OH Groups on the Glycerol Backbone. The re-esterification reactions of the mixture of lower glycerides produced from soybean oil with free CLA were studied using different molar ratios of free CLA to OH groups (1:1, 5:1, and 10:1 mol/mol). Trials were conducted at 60 °C with three immobilized lipases. Reactions were carried out using 10% lipase (w/w with respect to total reactants) (**Table 3**). For short reaction times (i.e., 0.25 h), these three different enzymes exhibited contrasting behaviors. For the trials involving the use of Novozym 435 as the biocatalyst, the number of FA residues in the glyceride products decreased when the molar ratio of free CLA to OH groups in the glycerol backbone increased. By contrast, when the other two lipases were employed as biocatalysts, the number of residues in the products increased as the indicated ratio increased. Analyses of the results obtained at the time corresponding to maximum production of TAG (2 h, see **Table 2**) indicate that catalysis by Lipozyme RM IM leads to glyceride products containing increased quantities of FA residues (1.55 mmol) relative to those

produced using either Novozym 435 (1.48 mmol) or Lipozyme TL IM (1.13 mmol). However, for Lipozyme RM IM one must employ a much greater excess of the acylating agent (a molar ratio of free CLA to OH groups in the glycerol backbone of 10:1 versus a ratio of only 5:1 for the other two lipases). Moreover, the quantity of FA residues in the product synthesized using Novozym 435 (1.48 mmol) is very close to that in the product obtained employing Lipozyme RM IM (1.55 mmol).

Analyses of the peak areas corresponding to the acylating agent indicate that the maximum amount of free CLA is incorporated in the product glycerides as a residue when the molar ratio of this agent to OH groups is 5:1, independent of the biocatalyst employed (data not shown). The smallest extent of reaction of the free CLA was obtained when equimolar proportions of reagents were employed.

In addition to the above-mentioned differences, the three biocatalysts also differ with respect to the extent to which the free CLA engages in undesired acidolysis reactions with the lower glycerides used as a starting material. For all three lipases the GC peak corresponding to the C18:2 (c10, c12) and (t10, t12) acids originating in the precursor soybean oil did not diminish when free CLA was employed in equimolar proportions with the number of OH groups in the starting lower glycerides. However, when molar ratios (free CLA to OH groups) of 5:1 and 10:1 were employed, the peak area of the indicated two isomers of C18:2 diminished by 26 and 37% for trials with Novozym 435, by 2 and 4% for trials with Lipozyme TL IM, and by 8 and 0.5% for trials with Lipozyme RM IM, respectively.

We selected a molar ratio of 5:1 for use in subsequent experiments because this ratio represents a reasonable balance between the desires to achieve maximum incorporation of the acylating agent and to minimize the extent of acidolysis, as well as providing a relatively short reaction time. Other authors have employed equimolar proportions of FFA and glycerides in studies conducted under vacuum (*8*). When the substrate for the esterification reaction is glycerol, authors prefer to work with low concentrations of the acylating agent (e.g., a mole ratio of FA to glycerol of 1:1 or 2:1) (*2*). By contrast, mixtures of MAG and DAG from fish oils are often esterified using an excess of FFA (e.g., 5:1 mol of FFA/mol of OH groups) (*9*).

Effects of the Loading of the Biocatalyst. To elucidate the effects of the loading of the biocatalyst on re-esterification reactions in a batch reactor subjected to orbital agitation, we conducted several trials using different immobilized lipases from Novozymes A/S. These trials were carried out using a molar ratio of free CLA to OH groups present in lower glycerides of 5:1 (mol/mol).

The effects of the loading of the three immobilized lipases on the rate of re-esterification are depicted in **Figure 3A**. In these trials the extent of consumption of the free acid in a fixed time increased as the enzyme loading increased from 5 to 25% (w/w with respect to total reactants) biocatalyst. The results of these trials were used as a basis for choosing an enzyme loading of 10% (w/w) for use in subsequent experiments because (i) this loading produces substantive reaction rates; (ii) with the agitation provided by the orbital shaker, one cannot maintain a uniform suspension of the immobilized enzyme at higher loadings; and (iii) when one increases the loading of the biocatalyst, the extents of undesirable side reactions (hydrolysis, acidolysis) may also increase (see **Figure 3B**). At an enzyme loading of 10% (w/w) and a molar ratio of free CLA to OH groups equal to 5:1, the amounts of FA esterified in 0.25 h ranked in the order Novozym 435 > Lipozyme RM IM > Lipozyme TL IM. At the same loading of enzyme, the re-esterification reaction catalyzed by Novozym 435 occurs most rapidly. This enzyme produced the greatest amount of incorporation of free FA in the glycerol backbone, is nonspecific with respect to the position at which incorporation occurs, is very stable in the reaction medium, and exhibits high activity at elevated temperatures. As received from the vendor, Lipozyme TL IM contained much more water than either Novozym 435 or Lipozyme RM IM. The high water content of Lipozyme TL IM led to diminished production of TAG and DAG species because this biocatalyst also promoted undesirable hydrolysis reactions. Moreover, Lipozyme TL IM had low activity for the re-esterification reaction (see **Figure 4**).

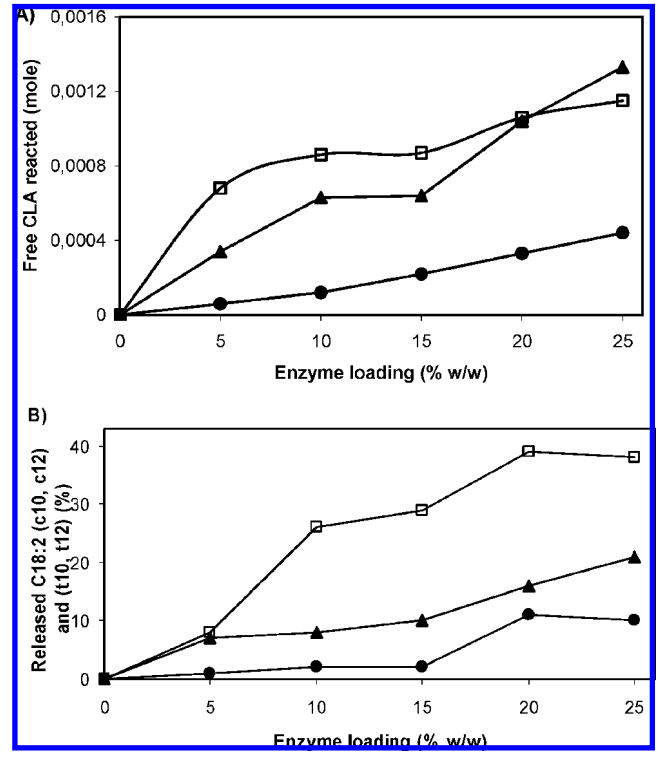

Figure 3. Effects of enzyme loading on (**A**) the rate of re-esterification and (**B**) the acidolysis side reaction of lower glycerides. Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm, 0.25 h reaction time. Symbols: □, Novozym 435; ▲, Lipozyme RM IM; ●, Lipozyme TL IM. Experimental values correspond to the mean of at least two independent experiments. Experimental errors were below 5%.

Trajectories of the Re-esterification Reactions. Trajectories of the re-esterification reactions were studied using three different biocatalysts. **Figure 4** depicts the trajectories of the reactions mediated by Novozym 435 (panel **A**), Lipozyme TL IM (panel **B**). and Lipozyme RM IM (panel **C**). The data for the initial stages of the reactions are consistent with the results depicted in **Figure 3**. The trial involving Novozym 435 (panel **A**) was characterized by the fastest initial rate. For this trial, the acylglyceride composition corresponding to a total reaction time of 7 h was 74% DAG and 22% MAG. However, after only 0.5 h of reaction, the liquid mixture is composed of 84% DAG. At the same loading of the three biocatalysts, Lipozyme TL IM produced the slowest initial reaction rate, although the composition of the reaction mixture after 7 h of reaction was similar to that obtained at the same time with Novozym 435 (71% DAG and 25% TAG). Finally, the trial with Lipozyme

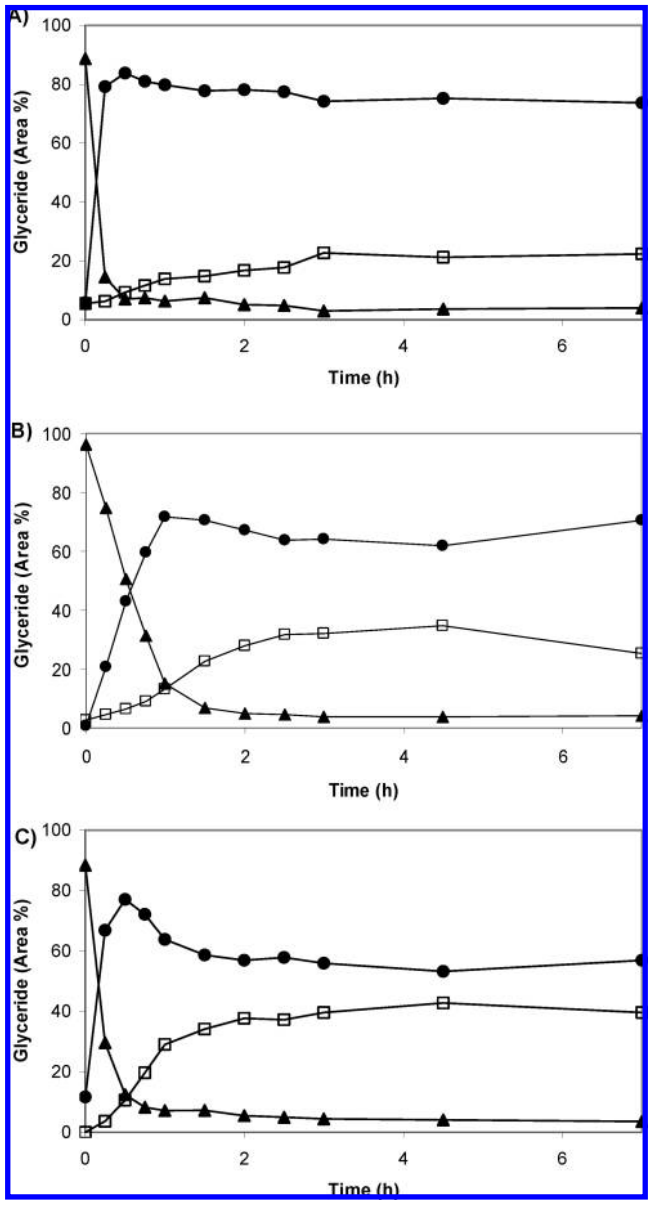

Figure 4. Trajectories of the re-esterification reactions with time: (**A**) Novozym 435; (**B**) Lipozyme TL IM; (**C**) Lipozyme RM IM. Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm. Symbols: ▲, MG; ●, DG; □, TG. Experimental values correspond to the mean of at least two independent experiments. Experimental errors were below 5%.

RM IM was characterized by a high initial reaction rate, but the acylglyceride composition at 7 h (57% DAG and 40% TAG) was quite different from that observed with the other two enzymes. In none of the trials involving the three biocatalysts was the amount of TAG greater than the amount of DAG. Oils with high DAG content are of interest for the manufacture of oils for the prevention of obesity and related diseases. They have energy values and digestibilities similar to those of TAG (*10*).

Inspection of **Figure 4** demonstrates that for each of the three immobilized lipases the maximum TAG content is approached in only 2 h of reaction. After 2 h of reaction, no significant increases in the concentration of CLA residues were observed. These results may reflect the influence of several factors (*3*):

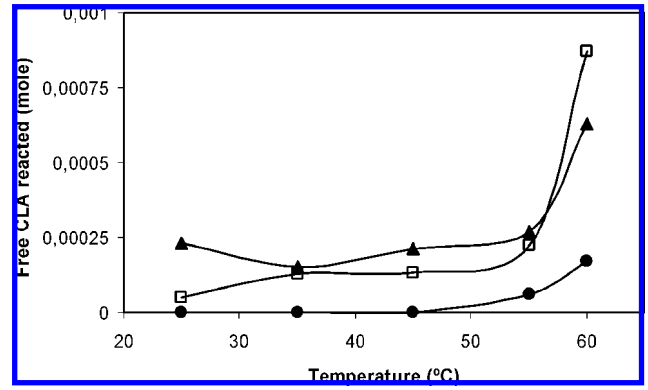

Figure 5. Effect of temperature on the re-esterification reaction: effect on incorporation of C18:2 (c9, t11) and C18:2 (t10, c12) from the precursor free CLA. Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 200 rpm, 0.25 h reaction time. Symbols: □, Novozym 435; ▲, Lipozyme RM IM; ●, Lipozyme TL IM. Experimental values correspond to the mean of at least twto independent experiments. Experimental errors were below 5%.

(i) failure of the orbital shaker to provide agitation sufficient to maintain uniform suspension of the solid immobilized enzymes; (ii) agglomeration of suspended particles; and (iii) limitations on the rates of mass transfer of reactants or products. Because these three lipases exhibited high levels of residual activity after several reaction cycles (especially Novozym 435 and Lipozyme RM IM) (see Enzyme Stability), extensive deactivation of these biocatalysts did not occur under the conditions employed.

As the reaction proceeded, CLA residues [primarily C18:2 (c9, t11) and (t10, c12)] were created along the glycerol backbone. Small amounts of C18:2 (c10, c12) and (t10, t12) from the linoleic acid originally present in the starting oil were released as indicated by the GC analyses (see **Table 3**). The final products are thus rich in CLA isomers.

Effects of Temperature. The effects of temperature on the initial reaction rate and the final composition of the mixture were also studied. Several trials involving re-esterification of the lower glycerides of soybean oil were conducted using an enzyme loading of 10% (w/w with respect to total reactants), a molar ratio of free CLA to OH groups of 5:1, and temperatures of 25, 35, 45, 55, and 60 °C.

Figure 5 reflects the variation of the peak area corresponding to the amount of free CLA reacted in 0.25 h with temperature for reactions catalyzed by three immobilized lipases (Novozym 435, Lipozyme TL IM, and Lipozyme RM IM). The initial reaction rates ranked in the order Novozym 435 > Lipozyme RM IM > Lipozyme TL IM. The amount of free CLA reacted increased when the reaction temperature was increased. However, the effect of temperature on the initial rate was much greater at temperatures above 55 °C than it was below this temperature. This very marked effect can be attributed to the following factors: (i) specific glycerides can react only when temperatures above the melting points of these glycerides are employed; and (ii) the viscosity of the reaction mixture decreases as the temperature increases, thereby enhancing mass transfer rates.

For economic reasons, the optimum process is the one that achieves the highest conversion in the shortest time. Consequently, the reaction should be conducted at an elevated temperature. In choosing this temperature, one must recognize this temperature should be sufficiently high that all components

Table 4. Effect of Temperature on the Maximum Production of DAG and TAG via Re-esterification of Lower Glycerides Derived from Soybean Oil[a]

	Novozym 435		Lipozyme TL IM		Lipozyme RM IM	
temp (°C)	DAG (area %)	TAG (area %)	DAG (area %)	TAG (area %)	DAG (area %)	TAG (area %)
25	92 (4.5)	5 (7)	18 (7)	0	80 (1)	43 (7)
35	91 (7)	3 (7)			84 (1)	23 (3)
45	76 (4.5)	19 (7)	21 (7)	1 (4.5)	70 (1)	32 (4.5)
55	83 (4.5)	23 (7)	33 (7)	0	78 (0.5)	25 (3)
60	75 (4.5)	21 (4.5)	71 (7)	35 (4.5)	77 (0.5)	43 (4.5)

[a] Entries in parentheses are the corresponding times (in h). Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 200 rpm.

of the reaction mixture are liquefied in order to avoid mass transfer limitations on the rate. Hence, we selected 60 °C for use in subsequent experiments involving each of the lipases studied.

The maximum yields of DAG and TAG and the corresponding time requirements at various reaction temperatures are summarized in **Table 4**. Use of Novozym 435 at 25 °C permits one to obtain a mixture of glycerides (92% DAG) rich in PUFA in 4.5 h. Lipozyme RM IM can be used at 35 °C to prepare glyceride products with lower DAG content (84%) in a significantly shorter time (1 h). To obtain products richer in TAG, one might employ Lipozyme RM IM at 60 °C (43% TAG in 4.5 h) or at 25 °C for a longer time (43% TAG in 7 h).

Compositional Analyses of the Fatty Acid Residues via GC-MS. The compositions of the lower glycerides derived from soybean oil that were employed as the starting material, as well as the glyceride products of the re-esterification reactions (obtained in trials involving near-equilibrium conditions, ca. 7 h) are summarized in **Table 3** in terms of their constituent FA residues. The percentages of saturated FA residues were very low in both the starting materials and the re-esterified products (<7%). The precursor mixture of lower glycerides is high in both C18:2 (70.37%) and C18:1 (20.47%) residues, but contains only 2% saturated FA residues. The product obtained using the Novozym 435 biocatalyst contains the most C18:2 (c9, t11) (27.37%) and C18:2 (t10, c12) (27.66%). These residues originate in the acylating agent. By contrast, the product synthesized using Lipozyme TL IM contains lower percentages of C18:2 (c9, t11) (7.80%) and C18:2 (t10, c12) (6.14%) and higher proportions of C18:2 (c10, c12) and (t10, t12) (57.72%) (see **Table 3**). These residues originate in the soybean oil.

Compositional Analyses of the Acylglycerides via HPLC-MS and HPLC-ELSD. The molecular structures of the acylglycerol species present in the re-esterified oils under near-equilibrium conditions were determined using reverse phase HPLC/APCI-MS. The products were synthesized under reaction conditions involving 10% enzyme loading (w/w with respect to total reactants), a molar ratio of free CLA to OH groups of 5:1 (mol/mol), 10% molecular sieves (w/w with respect to total reactants), 60 °C, 200 rpm, and 7 h. When Novozyme 435 was employed as the biocatalyst, the quasi-equilibrium product mixture was composed of 0.85% MAG, 81.19% DAG, and 17.76% TAG. A similar product mixture (1.98% MAG, 80.76% DAG, and 17.16% TAG) was obtained using Lipozyme TL IM. By contrast, when Lipozyme RM IM was used to effect the reaction, the quasi-equilibrium product mixture contained 0.16% MAG, 57.84% DAG, and 42.00% TAG. This difference may be attributed to differences in the specificities of these lipases, although to our knowledge such differences have not previously been reported in the literature. The chemical structures and the area percentages determined by HPLC-ELSD for all of the DAG and TAG present in the enriched oils are summarized in **Table 5**. Analysis of the tabular entries indicates that regardless of the biocatalyst employed, the predominant DAG species present in the oils are LnL and LL, although LO is also present at a significant level. For trials involving 7 h of reaction, the trial mediated by Lipozyme RM IM gave a product with a higher TAG content (42.00%) than the trials involving Novozym 435 (17.96%) and Lipozyme TL IM (17.16%). For all three immobilized lipases, the predominant TAG species were LLL and LLO/OLO.

Table 5. Composition of the Re-esterified Products in Terms of Acylglycerol Species in Area (Percent)[a]

acylglycerol species	Novozym 435	Lipozyme TL IM	Lipozyme RM IM
MAG (Ln, L, O)	0.85	1.98	0.16
LnLn	9.64	9.22	5.77
LnL + LL	24.95	32.85	18.13
LL	35.02	28.00	24.41
LO	9.14	7.60	7.70
LP + LO	1.12	0.94	0.83
LP	0.19	0.15	0.12
OO	0.70	0.93	0.52
OO + OP	0.17	0.23	0.11
OS	0.09	0.37	0.06
SP	0.18	0.58	0.19
LnLL + LLnL	0.38	0.54	1.09
LLL	12.30	10.36	26.78
LLO + OLO	4.27	5.32	12.13
LLP + LPL	1.01	0.94	2.00

[a] Conditions: 0.5 g of lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g of free CLA (5:1 mol of free CLA/mol of OH groups in lower glycerides), 10% biocatalyst (w/w with respect to total reactants), 0.165 g of molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol of hexadecane, 60 °C, 200 rpm, 7 h. Ln, linolenic; L, linoleic; O, oleic; S, stearic; P, palmitic.

Both 1,3-DAG and 1,2-DAG were detected in the product mixtures when normal phase HPLC-ELSD was used to separate the acylglycerols (TAG, DAG, and MAG). The predominant DAG species were 1,3-DAG, regardless of the biocatalyst. On the basis of the total amount of DAG in the sample, the area percentages corresponding to 1,3-DAG (from HPLC-ELSD analyses) were 70% for the trial with Novozym 435; 60% for Lipozyme TL IM; and 58% for Lipozyme RM IM (w/w with respect to the total area of DAG present in the products). Novozym 435 is a nonspecific lipase, whereas Lipozyme TL IM and Lipozyme RM IM are generally regarded as *sn*-1,3-regiospecific enzymes. Consequently, migration of acyl groups from the *sn*-2 position to the *sn*-1,3 positions must have occurred in the lower glycerides. Migration is favored at high temperature (60 °C) and long reaction times (7 h) and by thermodynamic considerations (1,3-DAG are more stable than 1,2-DAG).

Enzyme Stability—Multiple Use of the Immobilized Enzymes. Several trials involving multiple reuse of the Novozym 435, Lipozyme TL IM, and Lipozyme RM IM biocatalysts were carried out to ascertain the stability of these biocatalysts when exposed to reaction conditions. The residual activity of each of the immobilized lipases as a function of the number of reaction/washing cycles carried out in a batch reactor was determined. Residual activities were determined using the increase in the DAG peak area observed during 4 h of reaction under optimum operating conditions for the enzyme of interest.

After three reaction/washing cycles, the activities of Novozym 435 and Lipozyme RM IM both remain constant within the

uncertainties in the data. These biocatalysts are relatively resistant to deactivation when employed under conditions that are optimal for production of TAG via esterification of lower glycerides with free CLA. Moreover, both lipases are thermally stable when used at 60 °C in sequential trials.

By contrast, the residual activity of Lipozyme TL IM diminished by >50% during three reaction/washing cycles. The loss of activity can be attributed to the following factors: (i) Lipozyme TL IM is characterized by low thermal stability; and (ii) some of the immobilized enzyme protein can be desorbed or its molecular configuration altered during either reaction or washing (*8*). It has been demonstrated that the activity loss of Lipozyme TL IM is not a consequence of multiple washes with chloroform in a series of cycles (*11*).

ABBREVIATIONS USED

CLA, conjugated linoleic acid; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; FA, fatty acid; PUFA, polyunsaturated fatty acid; FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; SPE, solid phase extraction; GC, gas chromatography; MS, mass spectrometry; HPLC, high-performance liquid chromatography; RP, reversed phase; NP, normal phase; ELSD, evaporative light scattering detector; APCI, atmospheric pressure chemical ionization; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; P, palmitic acid.

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