J Am Oil Chem Soc (2009) 86:427–435
DOI 10.1007/s11746-009-1372-9

ORIGINAL PAPER

Selective Synthesis of Diacylglycerols of Conjugated Linoleic Acid

Estela Hernández-Martín · Charles G. Hill Jr ·
Cristina Otero

Received: 2 July 2008 / Revised: 23 December 2008 / Accepted: 26 January 2009 / Published online: 7 April 2009
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Abstract Quasi-quantitative selective production of diacylglycerols (DAG) rich in polyunsaturated fatty acids (PUFA) was demonstrated using a *Penicillium camembertii* lipase. Under optimal initial conditions [60 °C, 10% (w/w) biocatalyst based on total reactants, 5:1 molar ratio of free conjugated linoleic acid (CLA) to hydroxyl groups in partial glycerides consisting of ca. 90% (w/w) monoacylglycerols (MAG) and ca. 10% (w/w) diacylglycerols (DAG)], reaction for only 4.5 h gave 98.62% DAG and 1.38% MAG. The DAG contained >95% unsaturated fatty acid residues. Predominant DAG were LnLn, LnL and LL, although LO and LP were also significant (Ln = linolenic; L = linoleic; O = oleic; P = palmitic). Effects of the acylating agent (free CLA), solvent, and temperature on undesirable side reactions were determined. Reaction selectivities were similar in *n*-hexane and solvent-free media. The re-esterified products contained less than 7% saturated fatty acids and a higher ratio of unsaturated to saturated fatty acid residues (19.00) than the precursor soybean oil (5.22). The biocatalyst retained 55% of its initial activity after use in three consecutive reaction/extraction cycles.

Keywords Acylglycerides · Acylglycerols · Soybean oil · Lipase · *Penicillium camembertii* · Polyunsaturated fatty acids · CLA · Structured lipids · Nutraceuticals

E. Hernández-Martín · C. Otero (✉)
Departamento de Biocatálisis,
Instituto de Catálisis y Petroleoquímica,
CSIC, C/Marie Curie 2, Cantoblanco,
28049 Madrid, Spain
e-mail: cotero@icp.csic.es

C. G. Hill Jr
Department of Chemical and Biological Engineering,
University of Wisconsin, Madison,
WI 53706-1691, USA

Introduction

Structured lipids are triglycerides (TAG), 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. Polyunsaturated fatty acids (PUFA) such as conjugated linoleic acid (CLA) and omega-6 and omega-3 fatty acids are nutraceuticals whose ingestion produces physiological benefits in humans. Daily ingestion of these PUFA can act to reduce the incidence of coronary heart disease, certain forms of cancer, diabetes, high blood pressure, and a variety of other disease conditions [1].

DAG are naturally occurring minor edible constituents of fats and oils. Both DAG and MAG are widely employed as emulsifiers in processed foods. Researchers in Japan have recently developed a cooking oil containing 80% (w/w) DAG that is now commercially available [2, 3]. This DAG is similar to other fats and oils, but has the advantages that absorption of this substance in the intestinal tract is virtually complete and that normal metabolic processes for fats are modified to cause decreased storage of fats in the liver [4]. Consequently, oils with high DAG content are of interest for the prevention of obesity and related diseases despite having an energy value and digestibility similar to TAG.

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Chemical syntheses of DAG via glycerolysis reactions have been previously studied [5]. Moreover, different strategies for the synthesis of DAG involving enzymatic catalysis have been reported: solvent-free preparation of DAG by lipase-catalyzed esterification or transesterification of MAG and glycerol with rapeseed oil [6]; synthesis of 1,3-DAG containing CLA via lipase-catalyzed esterification [7]; and enzymatic esterification of glycerol with CLA in hexane in order to obtain DAG and MAG, as well as TAG.

Use of lipases as biocatalysts permits the use of mild reaction conditions which facilitate control of the reaction and yield both short reaction times and high specificity with respect to the type and the position of the fatty acid residues on the glycerol backbone. The optimum reaction conditions (e.g., molar ratio of substrates, enzyme loading and temperature) and the chemical composition of the reaction products were also determined.

The purpose of this research was to develop a selective synthetic route for production of DAG rich in PUFA in the presence of a commercially available *Penicillium camembertii* free lipase in both organic solvents and solvent-free media.

In our research DAG were synthesized using the following multi-step process:

1. Preparation of partial glycerides (MAG and DAG) enriched in PUFA via optimized enzymatic ethanolysis of soybean oil [8].
2. Purification of partial glycerides and removal of fatty acid ethyl esters (FAEE) by solid phase extraction (SPE).
3. Re-esterification of purified MAG and DAG with CLA in its free fatty acid (FFA) form.

Materials and Methods

Materials

Soybean oil was purchased from Biolasi (Ordizia, Spain) while free CLA was provided by Natural Asa (Hovdebygda, Norway). The fatty acid composition of the free CLA was as follows: 80% CLA, 15% C18:1 and 5% others (mainly C16:0 and a mixture of C18 species). As reported by the vendor, the GC area percentages of the different isomers of free CLA were: 0.42% c9c12, 42.73% c9t11, 44.24% t10c12, 1.33% c9c11, 1.35% c10c12 and 1.19% (t10t11 + t10t12). The lipase Novozym® 435 (*Candida antarctica* fraction B lipase immobilized on a macroporous resin) was a generous gift from Novozymes A/S (Bagsvaerd, Denmark). Lipase G 50 (*Penicillium camembertii* free lipase) was purchased from Amano Enzyme Inc. (Nagoya, Japan). This enzyme is sold in powdered form but is free, rather than immobilized. 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 obtained from Aldrich (Madrid, Spain). Ethyl acetate, *n*-hexane, acetone, acetonitrile, methanol, ethanol, and 2-propanol were HPLC grade from Scharlau (Barcelona, Spain) whereas dichloromethane was obtained from Panreac (Barcelona, Spain). Methanolic-base and a fatty acid standard Supelco 37 Component FAME Mix were purchased from Supelco (Bellefonte, PA, USA). Finally acylglycerol standards (triolein, diolein and monoolein) and molecular sieves (8–12 mesh beads) were obtained from Sigma (Madrid, Spain).

Methods

Synthesis of Partial Glycerides

Optimized enzymatic ethanolysis of soybean oil was used to produce partial glycerides enriched in PUFA as described by Hernández-Martín et al. [8]. The reaction conditions were as follows: 2 g soybean oil, 1.5 g ethanol ([EtOH]/[FA residues in the soybean oil] = 4.5 (mol/mol)), 50% Novozym® 435 (w/w with respect to the oil), 25 °C, 200 rpm, 1 h reaction time. The reaction products prepared from 10 g soybean oil, were dissolved in chloroform (ca. 20 mg/mL) after removal of the immobilized enzyme by filtration.

Purification of Partial Glycerides

FAEE as well as TAG were removed from the 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 partial glycerides from the FAEE and the extracted compounds is shown in Table 1. Isopropanol was eliminated by bubbling nitrogen through the liquid mixture until it was dry. Next, the resulting mixture of DAG and MAG was subjected to a re-esterification reaction with free CLA. SPE technology was employed instead of molecular distillation in order to avoid migration of acyl moieties during the extraction/separation process.

Re-esterification of Partial Glycerides with Free CLA

0.5 g of partial glycerides from soybean oil, 0.05 g (0.22 mmol) of hexadecane as an internal standard and 10% molecular sieves (w/w with respect to total reactants) were placed in 50-mL stoppered batch reactors at temperatures ranging from 25 to 60 °C in an orbital shaker

Table 1 Elution scheme used to purify the lower glycerides

Step	Solvent	Volume (mL)	Eluted compounds
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	–

operating at 200 rpm. The molar ratio of free CLA to OH groups in the partial glycerides was varied from 1 to 10 (mol/mol).

Variable amounts (5–25%) of native Lipase G 50 were employed. Some reactions were carried out in the presence of 5 mL hexadecane or 5 mL acetone. 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 in order to remove the enzyme.

Compositional Analyses of the Reaction Products by Gas Chromatography (GC)

Gas chromatography was employed to determine the composition of the FA residues incorporated in the product acylglycerols after partial derivatization of the samples.

Partial Derivatization of the Glycerides To quantify the total fatty acid residues in the glycerides as fatty acid methyl esters (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 were added. The resulting solutions were extracted with two 1-mL portions of *n*-hexane and then dried with sodium sulphate for at least 2 h. Hexane solutions (ca. 4 mg/mL) were subjected to GC analyses.

Analyses of FAME by GC Analyses of FAME were conducted by GC. One microliter of sample was injected into an Agilent (Palo Alto, CA) gas chromatograph (model 6890N) fitted with both a flame ionization detector (FID) and a Zebron ZB-WAX capillary column (30 m × 0.25 mm × 0.25 μm film thickness) purchased from Phenomenex (Torrance, CA). 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 °C 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 comparisons with a Supelco 37 Component FAME Mix.

Analyses of FAME by GC/MS The products of the re-esterification reactions were also analyzed by a GC coupled to a mass spectrometer in order to confirm the peak assignments. Re-esterification reactions of 0.5 g of the partial glyceride mixture derived from soybean oil were conducted under conditions such that the molar ratio of free CLA to OH groups was 5 (mole/mole). These reactions were conducted at 60 °C in the presence of 10% Lipase G 50 (w/w with respect to total reactants), and proceeded for 4.5 h in an orbital shaker operating at 200 rpm. Samples were then treated and methylated as described above. For these analyses, a Varian 3800 gas chromatograph was fitted with a VF-5ms 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 was scanned from 40 to 500 amu in order to detect an extensive number of compounds. The temperature program employed was identical to that described earlier for the GC analyses.

Compositional Analyses of the Reaction Products by HPLC

Normal Phase HPLC (NP-HPLC) Analyses Reaction products were analyzed by NP-HPLC to separate and quantify the acylglycerols. 600 μL of the chloroform solutions were diluted with 1000 μL chloroform and subjected to HPLC analyses. The Merck–Hitachi HPLC apparatus was equipped with both 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 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 a acetonitrile/dichloromethane solvent system [75% acetonitrile/25% dichloromethane from 0 to18 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 DAG 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 the 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 a 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 by the mass analyzer system. Mass spectrometer parameters were adjusted so as to obtain appropriate conditions. The values selected for the capillary voltage, gas temperature, drying gas, and nebulizer pressure were 3500 V, 200 °C, 5 L/min and 2.4 bar, 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 native biocatalyst Lipase G 50, we conducted replicate trials of reactions under optimum conditions [10% biocatalyst (w/w with respect to total reactants), 5:1 molar ratio of free CLA to OH groups of the partial glycerides, 60 °C, 200 rpm] by recovering and transferring the enzyme to a fresh substrate mixture. After a time corresponding to one complete reaction cycle (4.5 h), 20 mL chloroform were added to the reaction mixture and the resulting suspension was then filtered to recover the enzyme. The enzyme was then washed with 10 mL chloroform in order to remove residual oil and then dried in ambient air for ca. 2 h to remove the chloroform. The remaining activity was measured in terms of changes in the DAG peak area for each cycle.

Results and Discussion

Our previous studies of enzymatic re-esterification of mixtures of partial glycerides derived from soybean oil with CLA [9] have demonstrated that Lipase G 50 (*Penicillium camembertii* free lipase) has the ability to produce virtually quantitative conversions of these mixtures [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG] to DAG by reaction with free CLA. *Penicillium camembertii* lipase is known to be effective for esterification of FFA with glycerol to produce MAG [10]. This research focuses on both the study of the optimum reaction parameters for re-esterification of a mixture of partial glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG] with CLA and the compositional, physical and functional properties of the resulting products.

Effects of the Loading of the Biocatalyst on the Temporal History of Re-esterification Reactions

Penicillium camembertii lipase requires a small amount of water for full expression of its activity. However, the enzyme can be activated fully even by a small amount of water generated during esterification [10]. In our studies, we used molecular sieves to prevent undesirable hydrolysis side reactions involving the water generated by the re-esterification reaction.

Several trials were conducted to elucidate the effect of the loading of the biocatalyst (Lipase G 50) on the conversion, the initial reaction rate, and the time course of the process in a batch reactor subjected to orbital agitation. The reactions were carried out at 60 °C using a molar ratio of free CLA to OH groups in the initial mixture of partial glycerides of 5:1 (mol/mol). In all these trials, product formation (measured in terms of the increase in the DAG peak area) increased as the enzyme loading increased from 10 to 25% (w/w with respect to total reactants) (Fig. 1).

The progress of the re-esterification reaction was monitored until quasi-equilibrium was approached. Figure 2 depicts the time courses of the reaction at three different enzyme loadings (panel a: 5%, panel b: 10% and panel c: 25%). Quasi-equilibrium conditions were reached more rapidly as the enzyme loading increased. Thus virtually complete conversions to DAG were achieved at 0.35–0.40 and 4.5 h for enzyme loadings of 25 and 10% (w/w with respect to total reactants) respectively (Fig. 2b, c). In both these trials rapid depletion of the MAG peak area was observed; in addition, rapid growth of the primary product DAG was noted in both trials. When 5% Lipase G 50 was

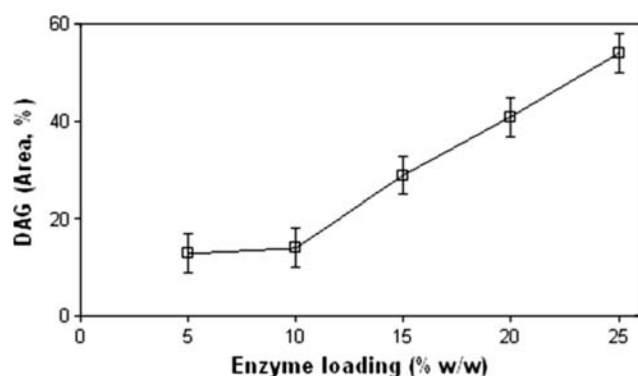

Fig. 1 Effects of the loading of Lipase G 50 on the rate of re-esterification. Conditions: 0.5 g lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g free CLA (5 mol CLA/mol OH groups in lower glycerides), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 60 °C, 200 rpm, reaction for 0.50 h

employed (Fig. 2a, considerably more than 7 h was required to attain the quasi-equilibrium state. In none of these re-esterification trials was any TAG formed, regardless of the enzyme loading. Because only DAG were synthesized, Lipase G 50 is selective for reactions leading to DAG, but is not active for formation of TAG.

We selected a Lipase G 50 loading of 10% (w/w) for use in subsequent experiments for several reasons: (1) at this loading, conversions approached 100% DAG in a reasonable time (4.5 h); (2) uniform suspensions of the enzyme could be maintained in the reaction medium; and (3) this biocatalyst was highly selective for formation of DAG.

Effect of the Molar Ratio of Free CLA to OH Groups in Partial Glycerides

Re-esterification reactions of partial acylglycerols derived from soybean oil with CLA were conducted 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 using 0.5 g partial glycerides [ca. 90% (w/w) MAG and ca. 10% (/w) DAG], 165 g molecular sieves (10% w/w with respect to total reactants), 10% Lipase G 50 (w/w with respect to total reactants). The DAG peak area increased as the initial molar ratio of free CLA to OH groups increased regardless of the reaction time (3, 5 and 11% DAG at 0.25 h or 9, 12 and 33% at 0.50 h, using 1:1, 5:1 and 10:1 mole ratio, respectively). When the reaction was allowed to proceed for several hours, the resulting distribution of acylglycerols depended on the amount of free CLA present initially. For an equimolar ratio of reactants (1:1, mol/mol), more than 7 h was needed for the re-esterification reaction to approach a quasi-equilibrium composition. At 7 h, the reaction mixture was composed of 83% DAG and 17% MAG. Inspection of Fig. 2b indicates that for a molar ratio

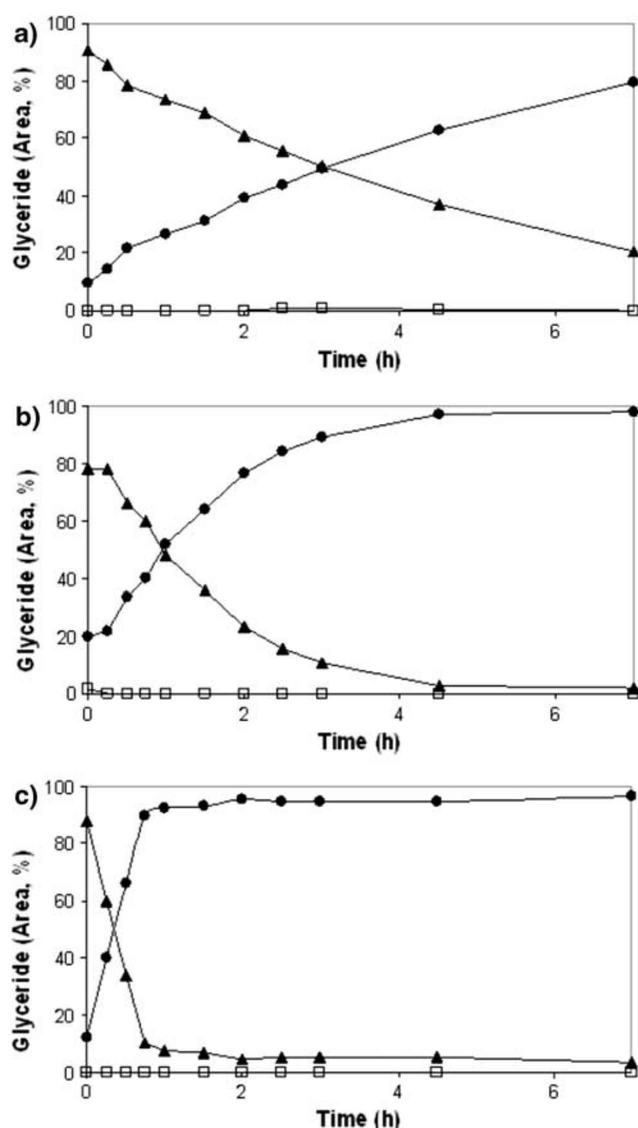

Fig. 2 Time courses of the re-esterification reactions mediated by Lipase G 50. Conditions: 0.5 g lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g free CLA (5 mol CLA/mol OH groups in lower glycerides), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 60 °C, 200 rpm. **a** 5%; **b** 10%; **c** 25% (w/w with respect to total reactants) or **a** 0.0825 g; **b** 0.165 g; **c** 04,125 g Lipase G 50. *Filled triangles*, MAG; *filled circles*, DAG; *open squares*, TAG

of free CLA to OH groups of 5:1 (mol/mol) the reaction reached a quasi-equilibrium composition (100% DAG) after 4.5 h (see "Effects of the loading of the biocatalyst on the temporal history of re-esterificationreactions"). By contrast, when the molar ratio of reactants was 10:1 (free CLA to OH groups, mol/mol), only 2 h was necessary to achieve nearly 100% DAG. However, significant increases were observed in the area of the TAG peak area for times greater than 3 h (5% at 3 h, 6% at 4.5 h and 14% at 7 h). Thus the presence of excess acylating agent (free CLA)

may lead to non-selective behavior of the enzyme, because TAG were formed in the reactor. Acyl migration may have occurred, as excess acid is widely known to catalyze acyl migration of DAG and especially MAG. When the concentration of TAG in the reaction medium increased, the maximum amount of DAG that could be present decreased. Moreover, the presence of a large excess of acylating agent (free CLA) favored undesirable acidolysis reactions of the C18:2 species [(c10, c12) and (t10, t12)] present in the precursor glycerides of soybean oil. For example, 16, 33 and 62% C18:2 (c10, c12) and (t10, t12) were observed after 2 h of reaction in trials involving initial mole ratios of free CLA to OH groups of 1:1, 5:1 and 10:1, respectively.

For subsequent trials, we selected a molar ratio of free CLA to OH groups in the partial glycerides of 5:1 (mol/mol) because in reasonably short times (4.5 h) one can achieve quasi-quantitative and selective production of DAG in which no TAG are present.

Effect of the Solvent

Addition of a solvent to the reaction mixture was explored in order to ascertain if improved results could be obtained. Several trials were conducted in solvents that are acceptable for the preparation of food-grade products and additives: *n*-hexane (non-polar) and acetone (polar). The use of an organic medium in re-esterification processes involves several advantages, such as the ability to reverse hydrolytic side reactions and better contact of the reagents with the biocatalyst, thereby obtaining enhanced conversions [11]. On the other hand, the use of organic solvents in enzymatic reactions is seriously limited because many of these materials are known to inactivate or denature biocatalysts.

Inspection of Fig. 3a reveals that hexane was a good solvent for the reaction when Lipase G 50 was the biocatalyst because the initial increase in the DAG peak area was much greater than that obtained in absence of any solvent under otherwise identical reaction conditions (Fig. 2b). This result may be a consequence of a conformational change of the enzyme when hexane is used as a solvent for the re-esterification reactions. Quasi-equilibrium compositions were approached within only 3 h; by contrast, 4.5 h were required to achieve this composition in the solvent-free process. However, acetone was not a suitable solvent for re-esterification of partial glycerides to produce DAG when Lipase G 50 was used to mediate the reaction (Fig. 3b). The increase in product DAG was lower than that obtained in the corresponding solvent-free process. These results are consistent with those obtained in an earlier study in our laboratory in which different immobilized enzymes (Novozym® 435, Lipozyme® TL IM and Lipozyme® RM IM) were employed as biocatalysts for re-esterification reactions [9].

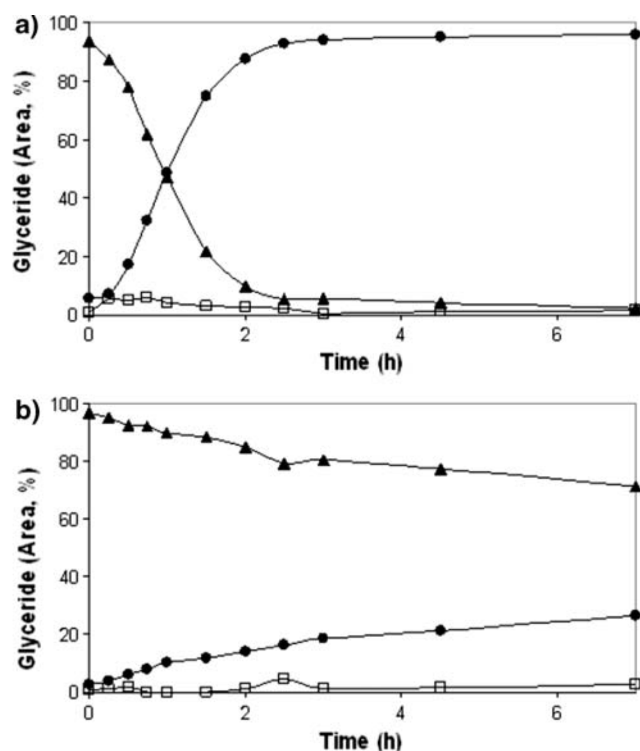

Fig. 3 Effect of the solvent in the rate of re-esterification. Conditions: 0.5 g lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g free CLA (5 mol CLA/mol OH groups in lower glycerides), 10% Lipase G 50 (w/w with respect to total reactants), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 60 °C, 200 rpm. **a** 5 mL hexane; **b** 5 mL acetone. *Filled triangle*, MAG; *filled circle*, DAG; *open square*, TAG

A detailed analysis of side-reactions (hydrolysis and acidolysis) of the precursor partial glycerides indicated that the common peak associated with C18:2 (c10, c12) and (t10, t12) arising from the precursor soybean oil diminishes much more slowly in the medium containing acetone than in the medium containing *n*-hexane. Because the area associated with this fatty acid isomer varied during the course of the re-esterification reaction in a similar manner for both the *n*-hexane and solvent-free systems, this solvent was believed to affect both the desired and undesired reaction via the same mechanism. The reaction selectivity was similar in *n*-hexane and the solvent-free system. In fact, in both the presence and the absence of *n*-hexane, the ratios of unsaturated to saturated fatty acid residues in the product were quite similar (19.00 and 20.97, respectively, see Table 2).

If a solvent is needed, the best non-aqueous reaction media for enzymatic modification of hydrophobic substrates are those containing hydrophobic water-immiscible solvents like hexane [12]. Enzymes placed in these solvents tend to retain a layer of essential water which allows them to maintain their native conformation, as well as sufficient flexibility, and hence catalytic activity. By contrast, polar solvents such as acetone tend to distort the essential water–

Table 2 Compositions of the FA residues of the soybean oil, the partial glycerides used as precursor for the re-esterification reaction and the product of re-esterification with CLA

FA residue (mol%)	Soybean oil	Lower glycerides	Product at 4.5 h
C16:0	11.39	2.00	5.00
C18:0	4.35	0	n.d.
C18:1	20.82	20.47	16.20
C18:2 (c10, c12), (t10, t12)[a]	53.82	63.74	22.35
C18:3n3	9.28	7.16	2.58
C18:2 (c9, t11)	0	3.28	25.52
C18:2 (t10, c12)	0	3.35	25.14
Others (unsaturated)	0.34	0	3.21
Total saturated residues (mol%)	16.08	2.00	5.00
Total unsaturated residues (mol%)	83.92	98.00	95.00
Unsaturated/saturated (mol/mol)	5.22	49.00	19.00

Conditions: 0.5 g partial glycerides, 1.15 g free CLA (5 mol CLA/mol OH groups in lower glycerides), 10% Lipase G 50 (w/w with respect to total reactants), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 60 °C, 200 rpm

n.d. not detected

[a] Linoleic acid was present in the lower glycerides of soybean oil that was used as a starting material

biocatalyst interactions responsible for the conformation of the enzyme, thereby inactivating or denaturing the protein. Nonetheless, it has been widely demonstrated that some lipases (especially Novozym® 435) are very stable and robust in presence of polar organic solvents such as ethanol [13].

Effects of Temperature

The effects of temperature on reaction rates and the composition of the final mixture were also determined. Several trials involving re-esterification of the partial glycerides derived from soybean oil were conducted using an enzyme loading of 10% (w/w with respect to total reactants) and a molar ratio of free CLA to OH groups of 5:1 (mol/mol). These trials were conducted at 25, 35, 45, 55 and 60 °C.

The amount of DAG produced within 1 h of reaction and the amount of acylating agent consumed in this period increased as the temperature rose from 25 to 60 °C (Fig. 4a). Figure 4b depicts the increases in DAG peak area observed as a function of time for trials at several different temperatures. Much more DAG was obtained in the trial at 45 °C than at lower temperatures (25 and 35 °C). At 45 °C, quasi-equilibrium was approached in 4.5 h. Even faster reactions were observed at higher temperatures (55–60 °C). In the trials at 55 and 60 °C, the composition versus time profiles were very similar. In these trials, quasi-equilibrium was approached after only 3 h of reaction. Thus Lipase G 50 is a native enzyme which exhibits high activity for the re-esterification reaction at elevated temperatures.

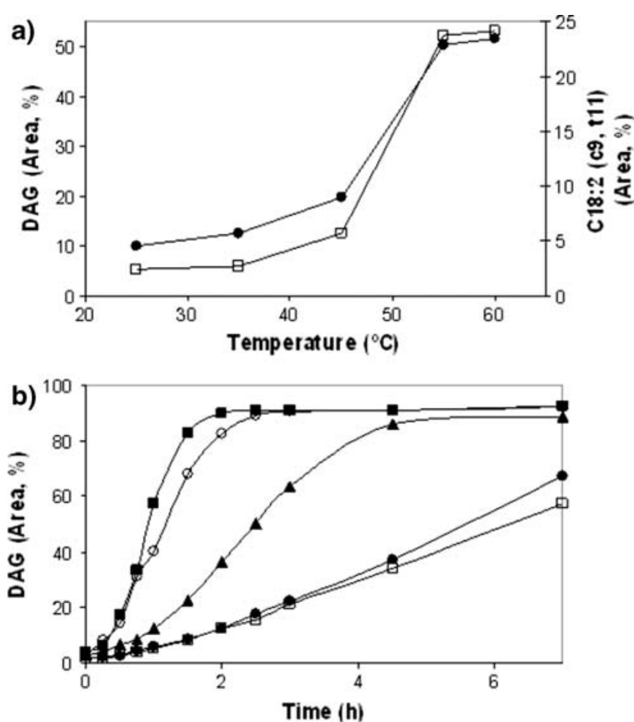

Fig. 4 **a** Increase in DAG after 1 h of re-esterification with free CLA and incorporation of C18:2 (c9, t11) present in the acylating agent after a 1-h reaction. *Open squares*, DAG; *filled circles*, C18:2. **b** Effect of temperature on the course of the re-esterification reaction. *Open squares*, 25 °C; *filled circles*, 35 °C; *filled triangles*, 45 °C; *filled squares*, 55 °C; *open circles*, 60 °C. Conditions: 0.5 g lower glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g free CLA (5 mol CLA/mol OH groups in lower glycerides), 10% Lipase G 50 (w/w with respect to total reactants), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 200 rpm

On the large scale, the best process is the one that involves minimal disruptions at the plant and makes a consistently high quality of product at the best price. It is desirable to reach these conditions while still obtaining the highest conversion in the shortest time. Moreover, the melting points of MAG containing unsaturated FA residues are lower than those of other components of the reaction mixture (14 °C for mono-linolein, 35 °C for mono-olein) [8]. Operation at temperatures above these melting points serves to liquefy the MAG, thereby facilitating the reaction. Moreover the viscosity of the reaction mixture decreases as the temperature increases. For these reasons, we selected 60 °C for use in subsequent experiments.

Compositional Analyses of Fatty Acid Residues (GC-MS)

The compositions of the original soybean oil, the partial glycerides derived from soybean oil as well as the products

obtained during re-esterification of these partial glycerides with free CLA (for 4.5 h) are summarized in Table 2 in terms of the overall component FA residues. The percentage of saturated FA residues was almost negligible in both the reactants and the re-esterified products. The re-esterified product had a higher saturated FA content than the precursor partial glycerides (5 vs. 2%). These species originate as minor constituents in the acylating agent (free CLA). Inspection of Table 2 demonstrates that the increase in C18:2 was appreciable as a consequence of the large amount of free CLA participating in the re-esterification reaction. The re-esterified product had a higher ratio of unsaturated to saturated FA residues (19.00) than the original soybean oil (5.22) (Table 2).

Compositional Analyses of the Acylglycerides (HPLC-MS and HPLC-ELSD)

The molecular structures of the acylglycerol species present in the re-esterified oils produced under near equilibrium conditions were determined using reverse phase HPLC/APCI-MS. When optimum reaction conditions were employed [10% Lipase G 50 (w/w with respect to total reactants), 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 4.5 h], the reaction products were composed of 1.38% MAG and 98.62% DAG. The normal phase HPLC-ELSD analyses did not reveal the presence of any TAG species in the products when quasi-equilibrium conditions were approached (4.5 h). The chemical structures and the area percentages (by reverse phase HPLC-ELSD) determined for all the DAG present in the re-esterified oils are summarized in Table 3. Examination of this table indicates that the predominant DAG species are LL, and LnL + LL, although LO, LnLn, and LP + LO were also present in readily quantifiable proportions.

Both 1,3-DAG and 1,2-DAG were present when normal phase HPLC-ELSD was used to separate the acylglycerols of the product mixtures. The predominant DAG species were 1,3-DAG (60% of the peak area). Watanabe et al. [10] have reported that *Penicillium* lipase seems to be *sn*-1,3-regiospecific. However, our findings indicate that migration of acyl groups from the *sn*-2 position to the *sn*-1,3-positions can occur in partial glycerides because of the high temperature of the process (60 °C) and the reaction time (4.5 h).

Table 3 Acylglycerol composition of the re-esterified products formed in 4.5 h under optimal conditions

Acylglycerol species	Area (%)
MAG (Ln, L, O)	1.09
LnLn	2.63
LnL + LL	48.76
LL	39.16
LO	7.06
LP (+ LO)	0.81
LP	0.09
OO	0.15
OO + OP	0.25

Conditions: 0.5 g partial glycerides, 1.15 g free CLA (5 mol CLA/mol OH groups in lower glycerides), 10% Lipase G 50 (w/w with respect to total reactants), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 60 °C, 200 rpm

Ln linolenic, *L* linoleic, *O* oleic, *P* palmitic

Enzyme Reuse—Multiple Use of the Native Enzyme

Several re-esterification trials involving multiple use of the same free Lipase G 50 biocatalyst were carried out in order to ascertain the stability of this enzyme when exposed to reaction conditions. Figure 5 is a plot of the activity of the free lipase as a function of the number of reaction/extraction cycles carried out in the batch reactor. The point corresponding to cycle 0 corresponds to the activity of the fresh biocatalyst used in the first reaction trial. For each cycle, the activity of the biocatalyst was calculated on the basis of the conversion achieved in 4.5 h reaction. The activity of the fresh lipase employed for the first reaction trial was taken as 100%. For each trial the optimum operating conditions were employed (see the legend of Fig. 5).

The activity of Lipase G 50 had diminished by ca. 55% after the third cycle (after it had been used to carry out a total of four reaction trials). The loss of activity can be attributed to several factors: (1) free Lipase G 50 exhibits

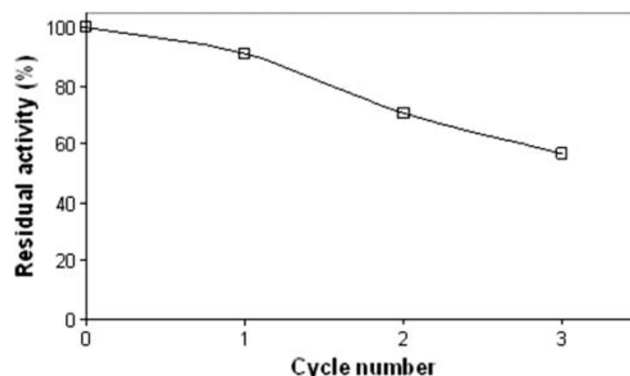

Fig. 5 Operational stability of Lipase G 50. Conditions: 0.5 g partial glycerides [ca. 90% (w/w) MAG and ca. 10% (w/w) DAG], 1.15 g CLA FFA (5 mol CLA/mol OH groups in lower glycerides), 10% Lipase G 50 (w/w with respect to total reactants), 0.165 g molecular sieves (10%, w/w with respect to total reactants), 0.22 mmol hexadecane, 60 °C, 200 rpm, reaction for 4.5 h

low thermal stability; and (2) some of the enzyme protein might be lost during the recovery process involving extraction and washing with chloroform [14]. Experience in our laboratory with several immobilized enzymes leads to the working hypothesis that exposure of the Lipase G 50 to chloroform is probably not responsible for the loss of lipase activity. Improved results might be obtained if the enzyme were immobilized on an appropriate support (silica, resin or a polymeric matrix, etc.).

Acknowledgments This work was supported by the Spanish CICYT (MAT2007-6666-C02-02). A predoctoral fellowship for E. Hernández-Martín was provided by the Spanish Research Council, CSIC.

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