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ORIGINAL PAPER

Evaluation of *Rhizopus oryzae* Lipase for the Determination of Regiodistribution in Triacylglycerols with Medium Chain Fatty Acids

Marlène Pérignon · Jérôme Lecomte · Michel Pina · Anne Renault · Camille Simonneau-Deve · Pierre Villeneuve

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Abstract The nutritional profile and rheological behaviors of lipids is both due to fatty acid composition and regiodistribution on external and internal positions of triacylglycerol. Actual methods for regiodistribution analysis having some restrictions, there is still a need for investigating a safe, simple and environmentally friendly method for the *sn*-2 position analysis that could especially be used for the analysis of fats containing medium and short chain fatty acids. The objective of this study was to evaluate the 1,3-selectivity and typoselectivity of *Rhizopus oryzae* lipase in the presence of short/medium chain fatty acids in partial hydrolysis conditions used for regiodistribution analysis. Structured triacylglycerols containing eight-carbon-chain length fatty acids in the *sn*-2 position were chemically synthesized using DCC/DMAP coupling agent and purification steps by flash-chromatography. The final product showed very high purity and was used as the substrate for 1,3-selectivity evaluation. Typoselectivity was assessed by investigating partial hydrolysis of equimolar blends of homogeneous TAG. This study confirmed the 1,3-selectivity of *Rhizopus oryzae* lipase in the hydrolysis conditions used, and revealed that this lipase was less influenced by fatty acids chain length than pancreatic lipase. Considering this, *Rhizopus oryzae* lipase appeared to be a good candidate for regiodistribution analysis of fats containing medium and short chain fatty acids.

M. Pérignon · J. Lecomte · M. Pina · P. Villeneuve (✉)
CIRAD, UMR Ingénierie des Agro-polymères et Technologies Emergentes, Campus Supagro/INRA, 2 place Viala, 34060 Montpellier, France
e-mail: pierre.villeneuve@cirad.fr

A. Renault · C. Simonneau-Deve
St Hubert, 13-15 rue du Pont des Halles, 94526 Rungis, France

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Introduction

There is currently a significant interest in chemical or enzymatic processes to modify the composition and/or regiodistribution of oils and fats to obtain new lipids, known as structured triacylglycerols (structured TAG) [1]. Those trends are mainly due to a growing concern for nutritional impact and FA bioavailability which are in fact governed both by the overall fatty acid (FA) composition and the stereospecific distribution of FA in TAG molecules [2]. This determination of FA regiodistribution on TAG requires methods that can be accurately employed on a wide range of oils and fats substrates which may greatly differ in term of fatty acid composition. Many various methods have then been developed such as chemical procedures using the action of Grignard reagents that react in a non-specific way on ester functions of triacylglycerols. Thus, Brockerhoff et al. [3] analyze the $\alpha\beta$-diacylglycerols ($\alpha\beta$-DAG) obtained after partial deacylation by Grignard reagent. However, the subsequent calculation of internal position uses factors which overemphasize the experimental error [4]. Therefore, Turon et al. [5] developed a modification of the Brockerhoff procedure in which the analysis is carried out on α-monoacylglycerols which are easier to collect than diacylglycerols isomers after subsequent TLC elution. However, methods involving Grignard reagents suffer various drawbacks. Indeed, these methods use an ethyl magnesium bromide which is a toxic chemical, highly reactive and very sensitive to water. Furthermore, those chemical methods are tedious and their accuracy is reliant on the limitation of the

acyl migration occurring during deacylation. Other methods have been developed with the use of ^{13}C-NMR or LC–MS equipment [6–8]. Although these methods are efficient they impose the use of expensive tools which are not readily available. Finally, probably the most widely used method for the determination of triacylglycerol regiodistribution is an enzymatic one, involving the use of pancreatic lipase [9]. This enzyme being known as strictly 1,3 regioselective, its use in such determinations consists of the partial hydrolysis of TAG substrate leading to the formation of acylglycerols and in particular β-monoacylglycerols (β-MAG) which are representatives of the native TAG. Then, the analysis of this β-MAG allows us to determine the FA composition of the internal position of native TAG. However, due to the pancreatic lipase selectivity for some FA, this method shows some restrictions. Actually, short-chain FA are more readily hydrolyzed by pancreatic lipase than longer chain ones, what leads to the formation of non representative β-MAG and consequently incorrect estimation of FA positional distribution. Thus, this method cannot be used for the analysis of fat containing a high content of medium and short-chain FA such as milk fat.

Consequently, although this enzymatic method is satisfactory due to its relative simplicity, there is still a need in identifying new lipases with limited acyl selectivity that could be an alternative to pancreatic lipase. Indeed, the development of a new enzymatic method involving a lipase which acts similarly on short chain or long chain fatty acids would broaden the range of application of such a method, allowing its use on substrates containing short aliphatic chains like hard stock used in margarine formulation. Recently, two 1,3 regiospecific lipase, namely *Rhizopus oryzae* [10, 11] and *Rhizopus arrhizus* [12, 13] have been proposed, but these studies were limited to TAG containing medium and long chain lengths. However, in order to evaluate the possibility of one of these lipases as an alternative to pancreatic lipase, it is necessary to assess its activity toward a wider range of substrates and especially those with short chains fatty acids down to eight carbons. Accordingly, in the present paper we have studied the behavior of *Rhizopus oryzae* lipase on the hydrolysis of model structured triacylglycerols containing caprylic acid in the central *sn*-2 position and compare its 1,3 regioselectivity and typoselectivity to the one of pancreatic lipase.

Materials and Methods

Chemicals and Lipases

Caprylic acid (≥99%), oleic acid (>90%), triolein (≥99%), tricaprylin (≥99%), trilaurin (≥99%), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), sodium borohydride ($NaBH_4$) and solvents were purchased from Sigma-Aldrich (St Quentin Fallavier, France). 1,3-Dihydroxyacetone was purchased from VWR (Fontenay sous Bois, France). Tris buffer 1 M was prepared from tris-(hydroxymethyl)aminomethane and its pH was adjusted by HCl 2 M. Lipase from *Rhizopus oryzae* (55 U/mg, 1 U corresponds to the amount of enzyme which liberates 1 μmol fatty acid from triacylglycerols per minute at pH 7.2 and 37 °C, olive oil as substrate) and lipase from porcine pancreas (Type II, 100–400 U/mg protein, one unit will hydrolyze 1.0 microequivalent of fatty acid from olive oil in 1 h at pH 7.7 at 37 °C.) were purchased from Sigma-Aldrich.

Analysis

Thin Layer Chromatography (TLC) Densitometry

Analytical TLC was performed on pre-coated silica-gel TLC plates (TLC Silica gel 60, 10 × 20 cm, Merck, Fontenay sous bois, France). After development in a suitable elution solvent (as detailed in each section), TLC-plates were analyzed by densitometry. Densitometric evaluations were performed with a TLC-scanner 3 (CAMAG, Switzerland) in absorbance mode at $\lambda = 190$ nm. Data were processed with the software winCATS.

Flash-Chromatography

Purification steps were performed by flash liquid chromatography using a CombiFlash Companion (Teledyne Isco, USA) and RediSep prepacked columns. Column size, flow rate, solvents and gradient used are detailed in each purification section.

^{1}H- and ^{13}C-NMR Spectroscopy

^{1}H-NMR spectra were recorded on a Bruker Avance DRX 400 spectrometer at 400 MHz in deuterochloroform ($CDCl_3$) as solvent. Chemical shifts (δ) are given in parts per million (ppm) and the coupling constant (J) in Hertz (Hz). Each resonance is shown according to the following convention: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), number of hydrogens, assignment). ^{13}C-NMR spectra were recorded on the same spectrometer at 100 MHz in $CDCl_3$ as solvent. For each ^{13}C signal, the number of carbon nuclei is indicated in parentheses when there is more than one carbon responsible for the peak.

Synthesis of Model TAG

Pure 1,3-dioleoyl-2-capryloyl-*sn*-glycerol (OCO) was chemically synthesized in 3 steps.

Synthesis of 1,3-Dioleoylpropane-2-one

Oleic acid (0.05 mol) and 4-DMAP (0.05 mol) were added to a solution of 1,3-dihydroxyacetone (0.025 mol) in 50 ml of chloroform. A solution of DCC (0.05 mol) in 25 ml of chloroform was then added dropwise under stirring at room temperature. Formation of 1,3-dioleoylpropane-2-one was monitored by TLC in hexane/ethyl acetate (80:20, v/v). After overnight reaction, the precipitated dicyclohexylurea was removed by filtration and the solvent eliminated under vacuum (rotavapor).

The 1,3-dioleoylpropane-2-one was then purified by flash chromatography according to the following procedure: 4 g of filtrate were adsorbed on silica and placed in a solid load cartridge. Elution was then carried out on a 80 g silica-gel column with a gradient of hexane to hexane/ethyl acetate (70:30, v/v) (initial 100% hexane was held for 15 min and increase of ethyl acetate content was achieved in 40 min) at a flow rate of 30 ml/min. Separation was followed by UV-detection at 200 nm and fractions of 10 ml were collected and analyzed by TLC-densitometry. TLC plates were developed in hexane/ethyl acetate 80:20 (v/v) and scanned at $\lambda = 190$ nm. Fractions of pure 1,3-dioleoylpropane-2-one ($R_f = 0.55$) were combined and the solvent evaporated under vacuum.

Synthesis of 1,3-Dioleate

1,3-dioleoylpropane-2-one (3.5 mmol) was dissolved in THF (45 ml) and distilled water (3 ml). This heterogeneous solution was cooled to 5 °C in a water bath under magnetic stirring, and sodium borohydride (~8 mmol) was carefully added. Reaction was followed by TLC in solvent system hexane/diethyl ether/acetic acid 70:30:1 (v/v/v). After 30 min, 1,3-dioleoylpropane-2-one was totally reduced in 1,3-dioleate and excess borohydride was neutralized by dropwise addition of glacial acetic acid (1 ml). The solution was diluted in 100 ml of chloroform, washed with water (~100 ml), aqueous sodium bicarbonate (~100 ml) and water (~100 ml), dried over anhydrous sodium sulfate, filtered and the solvent evaporated under vacuum.

Due to acyl migration, the unwanted isomer (1,2(2,3)-dioleate) can also be formed during the reaction. This isomerization was monitored by TLC-densitometry: TLC plates were developed in hexane/diethyl ether/acetic acid (70:30:1, v/v/v) and scanned at $\lambda = 190$ nm. 1,2(2,3)-dioleate and 1,3-dioleate had a R_f of 0.25 and 0.34, respectively.

Purification of 1,3-dioleate was achieved by flash chromatography according to the following procedure: the above dioleate was adsorbed on silica and placed in a solid load cartridge. Elution was then carried out on a 40 g silica-gel column with a gradient of chloroform to chloroform/acetone (95:5, v/v) achieved in 45 min at a flow rate of 10 ml/min. Fractions of 3 ml were collected and analyzed by TLC-densitometry as described above. Fractions of pure 1,3-dioleate ($R_f = 0.34$) were combined and the solvent evaporated. Final yield = 85%.

Synthesis of Pure 1,3-Dioleoyl-2-Capryloyl-sn-Glycerol (OCO)

Caprylic acid (10 mmol) in 4 ml of chloroform and 4-DMAP (3 mmol) were added to a solution of the previously synthesized 1,3-dioleate (3 mmol) in 30 ml of chloroform. The reaction mixture was heated at 30 °C under magnetic stirring and a solution of DCC (10 mmol) in 20 ml of chloroform was then added in small portions (~30 min). Formation of triacylglycerols (TAG) was followed by GC on a Thermo instrument (Courtaboeuf, France), GC 8000 series, equipped with a flame ionization detector and Supelco's Equity 1 capillary column (15 m × 0.32 mm, film thickness 0.1 μm). Helium was used as carrier gas at a flow rate of 5 ml/min. The operating conditions were: 1 μl injection on-column, oven temperature programmed from 80 to 330 °C at 10 °C/min with a final hold of 10 min, detector at 350 °C.

On completion of the reaction, the precipitated dicyclohexylurea was removed by filtration and the solvent evaporated. Formation of 1,2(2,3)-dioleate during the reaction was monitored by TLC-densitometry as described above.

The TAG contained in the filtrate was finally purified by flash-chromatography according to the following procedure: 3.7 g of filtrate was adsorbed on silica and placed in a solid load cartridge. Elution was then carried out on a 12 g silica-gel column with a gradient of hexane to hexane/diethyl ether (70:30, v/v) achieved in 60 min at a flow rate of 10 ml/min. The effluent was monitored by UV-absorption at $\lambda = 200$ nm. Fractions of 5 ml were collected and analyzed by TLC-densitometry in hexane/diethyl ether/acetic acid (70/30/1, v/v/v). Fractions of pure TAG ($R_f \approx 0.55$) were collected and combined. Final yield = 50%. ^{1}H NMR δ 5.40–5.30 (m, 4H, =C–*H*), 5.30–5.27 (m, 1H, CH_2–C*H*–CH_2), 4.33–4.29 (dd, J = 11.9 Hz, 2H, C*H*$_2$–CH–CH_2), 4.19–4.14 (dd, $J = 11.9$ Hz, 2H, CH_2–CH–C*H*$_2$), 2.35–2.31 (m, 6H, C*H*$_2$–CO), 2.08–2.00 (m, 8H, C*H*$_2$–CH=CH), 1.64–1.61 (m, 6H, C*H*$_2$–CH_2–CO), 1.32–1.29 (m, 48H, –C*H*$_2$), 0.92–0.88 (m, 9H, –C*H3*). ^{13}C NMR δ 174.65 (2, αC=O), 174.26 (1, βC=O), 131.62–129.31 (4, C=C), 70.29 (1, CH), 63.52 (2, OCH_2), 36.08–23.99 (34, CH_2), 15.52–15.47 (3, CH_3).

Lipase Specificities Measurement

1,3-Regioselectivity

Twenty milligrams of pure OCO in 0.1 ml of hexane and 2 ml of 1 M tris-buffer, pH 7 were heated at 40 °C for 5 min. 100 µl of the *Rhizopus oryzae* (RO) lipase solution (20 mg/ml) were added and the mixture was manually shaken at 40 °C for 1 min, then shaken vigorously for 1 min, followed by shaking at 40 °C for 1 min. After 3 min of hydrolysis, 1 ml of 2 M HCl solution was added to stop the reaction. Then 2 ml of diethyl ether was added and the medium was vigorously shaken to extract the reaction products. After centrifugation at 790 g for 5 min, the upper layer of the organic phase was collected and separated by semi-preparative TLC: 250 µl of the organic phase were spotted on a silica gel plate which was then developed with a hexane/diethyl ether/acetic acid solution (50:50:1 v/v/v). After a short drying time, a part of the plate was cut and the lipid spots were detected by immersion in a solution of saturated copper sulfate/phosphoric acid (50:50, v/v) and heating at 180 °C for 10 min. The β-MAG band ($R_f \approx 0.08$) was scraped off from the plate and its FA composition was determined by GC after conversion into methyl esters according to the following procedure: 3 ml sodium methylate solution containing phenolphthalein were added directly to the silica gel. The reaction medium was refluxed for 10 min. Then 3 mL chlorohydric methanol (acetyl chloride in methanol, 1 M) were added until phenolphthalein discoloration and the mixture was refluxed again for 10 min and then cooled to room temperature. Finally, 3 ml hexane and 10 ml water were added and the organic phase containing fatty acids methyl esters (FAME) was recovered for subsequent analysis. FAME were analyzed by GC (Agilent 6890 series, Bios Analytique, France) equipped with a flame ionization detector, and a Supelcowax capillary column (SGE, Courtaboeuf, France) with the following characteristics: 30 m × 0.32 mm; film thickness 0.25 µm. Oven was heated from 100 to 225 °C at 5 °C/min and held at 225 °C for 5 min. Detector and injector temperatures were 250 and 270 °C, respectively, and helium was used at a flow rate of 1 ml/min. Peak area percentages obtained with the integrator were divided by the molecular weight of individual FAME to yield mole percent of fatty acids. Experiments were performed in triplicate, and results given correspond to the mean values of three determinations.

Typoselectivity

Chain length selectivity of RO lipase was assessed by investigating partial hydrolysis of equimolar blends of pure homogeneous triacylglycerols. The first blend was prepared from tricaprylin (329.4 mg, 0.7 mmol), triolein (619.7 mg, 0.7 mmol) and hexane to obtain a 100 mg/ml solution. After vigorous shaking, 1 ml of the blend was taken, dried under nitrogen and used as substrate for subsequent hydrolysis. A second blend was prepared in the same conditions from trilaurin (319.7 mg, 0.5 mmol) and triolein (443.7 mg, 0.5 mmol). The hydrolysis procedure was the same than described above but adding 0.5 ml of lipase solution (20 mg/ml).

Hydrolysis by Pancreatic Lipase

Partial hydrolysis using pancreatic lipase was achieved on OCO and on the equimolar blends of homogeneous TAG as described in the official method for regiodistribution analysis [9].

Results and Discussion

Synthesis of Model TAG

Evaluation of *Rhizopus oryzae* lipase 1,3-selectivity was based on the hydrolysis of pure 1,3-dioleoyl-2-capryloyl-*sn*-glycerol (OCO) (Fig. 1). FA composition and regiodistribution of this substrate has to be perfectly known in order to predict hydrolysis products and conclude regarding the lipase selectivity. Various strategies have been described for the synthesis of highly pure triacylglycerols. These methods have been recently reviewed by Wijesundera et al. [14, 15] and generally require numerous chemical or enzymatic steps and tedious subsequent purification. The main difficulty to overcome during such synthesis is to avoid acyl migration phenomena which can easily occur depending on many parameters (e.g., alkalinity of the medium, temperature) [16–18]. Therefore, we aimed our strategy at the synthesis of pure OCO via a 1,3-dioleate intermediate of high purity and minimum formation of 1(3),2-DAG isomers. Actually, long-chain DAGs typically display a 1:2 ratio of 1,2(2,3)-DAG to 1,3-DAG at equilibrium [18]. Purification of 1,3-dioleate is required to

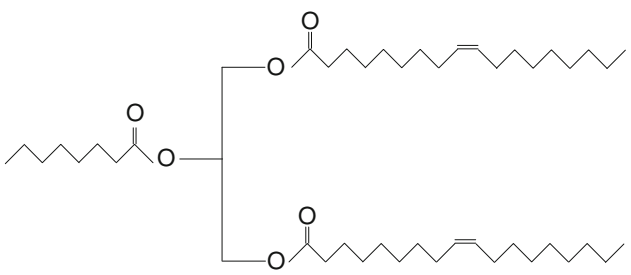
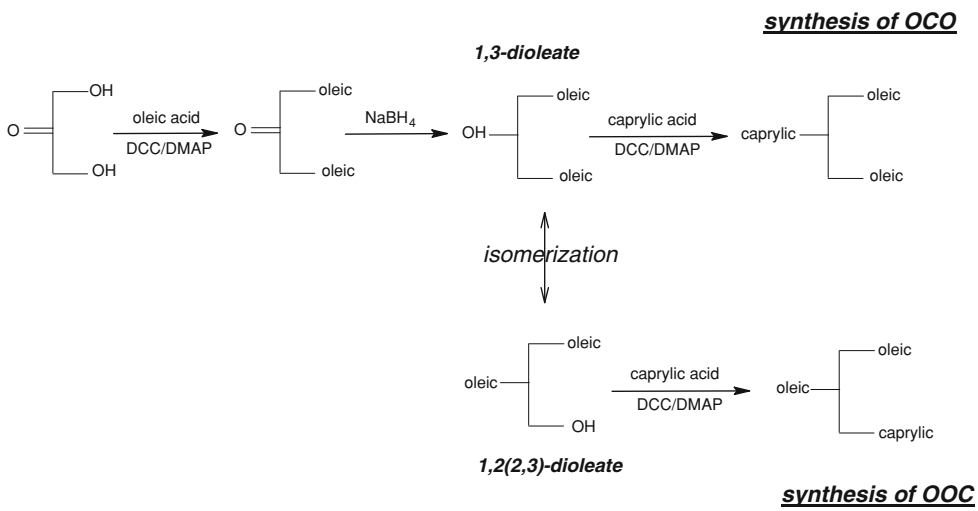


Fig. 1 Structured triacylglycerol OCO. O is oleic acid (C18:1) and C is caprylic acid (C8:0)

Fig. 2 Overall synthesis



avoid the formation of the unwanted isomer 1,2-dioleoyl-3-octanoyl-*sn*-glycerol (OOC) from 1,2(2,3)-dioleate. Accordingly, 1,3-dioleoyl-2-capryloyl-*sn*-glycerol (OCO) was synthesized in three steps as displayed in Fig. 2. The first step, adapted from the method described by Kodali et al. [19], consisted of the synthesis of 1,3-dioleoylpropane-2-one by acylation of dihydroxyacetone. This had the advantage of starting from a cheap and easily available material. 1,3-dioleoylpropane-2-one was obtained in high purity after flash chromatography and used in the second step, as described by Bentley et al. [20]. This step consisted in the reduction of the central keto group of 1,3-dioleoylpropane-2-one by action of sodium borohydride to form 1,3-dioleate. At this stage, acyl migration occurred and led to the formation of the isomer 1,2(2,3)-dioleate. Before purification, the ratio 1,3-/1,2(2,3)-dioleate obtained was 86/14 (Table 1). After purification by flash-chromatography, no 1,2(2,3)-dioleate was detected in the final product, neither by densitometric evaluation at $\lambda = 190$ nm nor by detection after immersion in a solution of saturated copper sulfate/phosphoric acid (50:50, v/v) and heating at 180 °C for 10 min. The final step, adapted from the method described by Kodali et al. [19] and Adlof et al.[21], consisted in the synthesis of OCO by acylation of 1,3-dioleate. Isomerization was also monitored during this step. No 1,2(2,3)-dioleate was detected, what allowed us to expect high purity for OCO.

Regiodistribution of the final product was analyzed by the pancreatic lipase method. Due to the strict 1,3-selectivity of this lipase, partial hydrolysis led to the formation of β-MAG. The analysis of their FA composition showed that MAG resulting from the hydrolysis of the final product were almost exclusively made of monocaprylin. Actually, recovered MAG were constituted at 99.2% of caprylic acid (Table 2). Thus, the internal position was contaminated by only 0.8% of oleic acid due to acyl migration during the last steps of synthesis. It has to be reminded that hydrolysis by pancreatic lipase is influenced by FA chain length due to lipase typoselectivity. This selectivity concerns short and medium chain FA which means that in the case of OCO/OOC isomers, and taking also the strict 1,3-selectivity into account, pancreatic lipase would tend to hydrolyze caprylic acid of the external positions faster than oleic acid. Thus, if caprylic acid occurs on external positions, corresponding to the presence of the unwanted isomer OOC, the typoselectivity will induce an increase of the formation of monoolein. Consequently, typoselectivity of pancreatic lipase can lead to an overestimation of the oleic content of MAG formed, and, as a result, to an overestimation of the OOC content. This means that the purity of the OCO isomer estimated from the FA composition of MAG resulting from hydrolysis by pancreatic lipase will be

Table 1 Purity of the intermediate and final products of OCO synthesis

Synthesis step	Purity of the isomer	Purity evaluation method
Synthesis of 1,3-dioleate		
Before purification	86% of 1,3-DAG	TLC-densitometry
After purification	No 1,2(2,3)-DAG detected	TLC-densitometry
Synthesis of OCO	>99.2%	Pancreatic lipase method

Table 2 Fatty acids composition of MAG resulting from hydrolysis of OCO by RO and pancreatic lipases

Mol (%)	RO lipase	Pancreatic lipase
C8:0	99.6 ± 0.2	99.2 ± 0.6
C18:1	0.4 ± 0.2	0.8 ± 0.6

underestimated. Consequently, according to these considerations and the results obtained, we can say that purity of the 1,3-dioleoyl-2-capryloyl-*sn*-glycerol synthesized was higher than 99.2% (Table 1).

Lipase Selectivity Evaluation

The official method for regiodistribution analysis involves the action of pancreatic lipase. This method especially uses the strict 1,3-selectivity of the lipase under partial hydrolysis conditions which leads to the formation of *β*-MAG representative of native TAG. Subsequent analysis of this *β*-MAG allows direct determination of the FA composition of internal position. However, due to the specificity of pancreatic lipase, this method cannot be used for the analysis of fats containing a significant quantity of FA with 12 carbons or less [4, 9]. Actually, pancreatic lipase tends to hydrolyze these FA faster than longer chain ones leading to the formation of unrepresentative MAG. Consequently, overcoming these restrictions by finding an alternative to pancreatic lipase for regiodistribution analysis amounts to finding a lipase both 1,3-selective and with limited acyl selectivity toward FA chainlength.

Our main objective was to estimate whether *Rhizopus oryzae* lipase exhibits a strict 1,3-selectivity when catalyzing the hydrolysis of TAG containing C8 carbon chain length fatty acids. For this, 1,3-dioleoyl-2-capryloyl-*sn*-glycerol (OCO) appeared to be particularly appropriate due to the location of this fatty acid at the central *sn*-2 position of this structured substrate. Indeed, investigating the partial hydrolysis of OCO can give valuable information regarding the conservation of the 1,3-regioselectivity of the tested lipase and its competition with any potential typoselectivity (acyl selectivity for C8 FA). The conditions of hydrolysis were adapted from the method described by Kosugi et al. [10] and a strict regio preference for the two external position of the triglycerides backbone of OCO would result in the release of only oleic acid as free fatty acid and concomitantly the appearance of MAG exclusively made of caprylic acid. Results obtained for FA composition of the newly formed MAG showed that they were almost exclusively made of monocaprylin as caprylic acid accounting for 99.6% of the fatty acid composition of recovered MAG (Table 2). This result showed that *Rhizopus oryzae* lipase maintains its strict 1,3-regioselectivity even though C8 fatty acids are present in the triglyceridic structure of the substrate.

For typoselectivity evaluation, partial hydrolysis by *Rhizopus oryzae* lipase was carried out on equimolar blends of tricaprylin/triolein (CCC/OOO) and trilaurin/triolein (LLL/OOO) and results were compared with those obtained for hydrolysis of the same blends by pancreatic lipase. FA compositions of the initial CCC/OOO blend and of MAG released from hydrolysis by the two lipases are shown in Fig. 3. These results revealed that MAG formed by hydrolysis of CCC/OOO by RO lipase had the same FA composition than the initial blend showing that the lipase hydrolyzed the TAG without any FA chain length preference. Considering results obtained for hydrolysis of OCO, it also showed that, in this hydrolysis conditions, RO lipase keeps its 1,3-selectivity even in presence of short-chain fatty acids in central position of the TAG. On the contrary, MAG formed by hydrolysis of CCC/OOO by pancreatic lipase consisted of more than 80% of caprylic acid (Fig. 3) meaning that pancreatic lipase hydrolyzed tricaprylin more preferentially than triolein, due to a typoselectivity toward caprylic acid or at least short chain FA typoselectivity.

FA compositions of the initial LLL/OOO blend and of MAG released from hydrolysis by the two lipases are

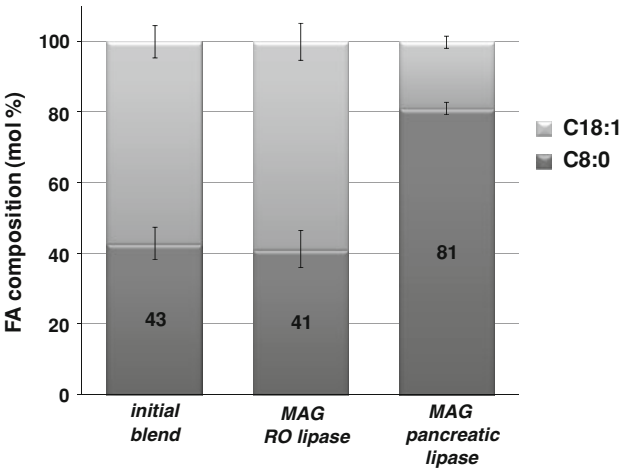


Fig. 3 FA composition of initial blend CCC/OOO and of MAG resulting from hydrolysis of the blend by RO and pancreatic lipases

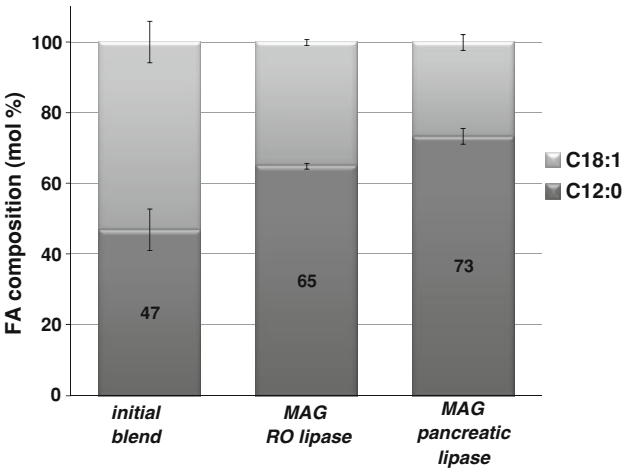


Fig. 4 FA composition of initial blend LLL/OOO and of MAG resulting from hydrolysis of the blend by RO and pancreatic lipases

shown in Fig. 4. These results show that both lipases hydrolyzed trilaurin more preferentially than triolein. For pancreatic lipase, it confirms its typoselectivity for medium chain FA. Concerning RO lipase results tend to show a typoselectivity for lauric acid with 65% of the newly formed MAG being made of monolaurin. However, we cannot exclude the hypothesis that this preferential hydrolysis is due to a modification of the emulsion. Actually, hydrolysis forms MAG and DAG which are known for their emulsifying properties. The hydrophilic/lipophilic balance of partial acylglycerols is function of the chain length of FA esterified on the glycerol [22]. MAG and DAG resulting from the hydrolysis of trilaurin (12-MAG and 12-DAG) have a better hydrophilic/hydrophobic balance than MAG and DAG resulting from hydrolysis of triolein (18-MAG and 18-DAG). Thus, whereas 18-MAG and 18-DAG would tend to form micelles or stay in the oil phase because of their high hydrophobicity, 12-MAG and 12-DAG would tend to emulsify TAG droplets. By placing themselves at the oil/water interface where the enzymatic reaction takes place [23], 12-DAG are more readily hydrolyzed, what leads to an increase in 12-MAG content.

However, for hydrolysis of both blends, the RO lipase was less influenced by fatty acid chain length than pancreatic lipase. It means that MAG resulting from the hydrolysis by RO lipase are more representative of the initial TAG than MAG from hydrolysis by pancreatic lipase. Considering also its 1,3-specificity, the RO lipase turns out to be a better candidate for the regiodistribution analysis of fat containing medium and short chain FA.

Conclusion

Synthesis of the structured triacylglycerol 1,3-dioleoyl-2-caprylyol-*sn*-glycerol (OCO) was achieved by action of DCC/DMAP coupling agent and purification steps by flash-chromatography. The final product showed a very high purity with less than 1% of the unwanted isomer OOC.

This structured TAG was used as substrate to evaluate the regioselectivity of the *Rhizopus oryzae* lipase. Results showed a strict 1,3-selectivity of the lipase even in presence of short chain FA in central position. Typoselectivity of the lipase was evaluated by hydrolyzing equimolar blends of homogeneous TAG containing 8 or 12 carbons chain lengths. It turned out that the RO lipase was less influenced by FA chain length than the pancreatic lipase generally used for regiodistribution analysis.

Considering these results of lipase selectivity evaluation, it appeared that *Rhizopus oryzae* lipase should be preferentially used for the determination of regiodistribution in TAG containing short and medium chain FA.

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References

1. Xu X (2000) Production of specific-structured triacylglycerols by lipase-catalyzed reactions: a review. Eur J Lipid Sci Technol 102:287–303
2. Mu H, Porsgaard T (2005) The metabolism of structured triacylglycerols. Prog Lipid Res 44:430–448
3. Brockerhoff H (1967) Stereospecific analysis of triglycerides: an alternative method. J Lipid Res 8:167–169
4. Turon F (2003) Clarification on the analysis of the regiodistribution in fatty acids of triacylglycerols. Ocl-Oleag Corps Gras Lipides 10:144–149
5. Turon F, Bachain P, Caro Y, Pina M, Graille J (2002) A direct method for regiospecific analysis of TAG using alpha-MAG. Lipids 37:817–821
6. Mottram HR (2005) Regiospecific analysis of triacylglycerols using high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. In: Byrdwell WC (ed) Modern methods for lipid analysis by liquid chromatography/mass spectrometry and related techniques. AOCS Press, Champaign, pp 276–297
7. Vlahov G (1998) Regiospecific analysis of natural mixtures of triglycerides using quantitative C-13 nuclear magnetic resonance of acyl chain carbonyl carbons. Magn Reson Chem 36:359–362
8. Blaise P, Wolff R, Farines M (1997) Regiospecific study of vegetable oil triacylglycerols by chemical cleavage and high-resolution NMR C-13. Ocl-Oleag Corps Gras Lipides 4:135–141
9. AFNOR (2000) Determination of the composition of fatty acids in the 2-position of the triglycerides molecules. In: AFNOR (ed) Corps gras, graines oléagineuses, produits dérivés Tome 1: corps gras et produits dérivés, pp 189–202
10. Kosugi Y, Oshima A, Koike S, Fukatsu M, Minami K, Miyake Y, Masui K (2002) Determination of fatty acid composition at *sn*-2 acyl position in triacylglycerol by capillary gas chromatography with lipase from *Rhizopus delemar*. J Oleo Sci 51:599–605
11. Kosugi Y, Oshima A, Koike S, Fukatsu M, Minami K, Miyake Y, Masui K (2004) Use of *Rhizopus delemar* lipase as compared with other lipases for determination of *sn*-2 fatty acids in triacylglycerol (vol 81, pg 235, 2004). J Am Oil Chem Soc 81:523
12. Amate L, Ramirez M, Gil A (1999) Positional analysis of triglycerides and phospholipids rich in long-chain polyunsaturated fatty acids. Lipids 34:865–871
13. Arcos JA, Garcia HS, Hill CG (2000) Regioselective analysis of the fatty acid composition of triacylglycerols with conventional high-performance liquid chromatography. J Am Oil Chem Soc 77:507–512
14. Wijesundera C (2005) Synthesis of regioisomerically pure triacylglycerols containing n-3 very long-chain polyunsaturated fatty acids. Eur J Lipid Sci Technol 107:824–832
15. Haraldsson GG, Halldorsson A, Kulas E (2000) Chemoenzymatic synthesis of structured triacylglycerols containing eicosapentaenoic and docosahexaenoic acids. J Am Oil Chem Soc 77:1139–1145
16. Halldorsson A, Magnusson CD, Haraldsson GG (2003) Chemoenzymatic synthesis of structured triacylglycerols by highly regioselective acylation. Tetrahedron 59:9101–9109
17. Li W, Du W, Li Q, Sun T, Liu D (2010) Study on acyl migration kinetics of partial glycerides: dependence on temperature and water activity. J Mol Catal B: Enzym 63:17–22

18. Laszlo JA, Compton DL, Vermillion KE (2008) Acyl migration kinetics of vegetable oil 1,2-diacylglycerols. J Am Oil Chem Soc 85:307–312
19. Kodali DR, Atkinson D, Redgrave TG, Small DM (1987) Structure and polymorphism of 18-carbon fatty acyl triacylglycerols: effect of unsaturation and substitution in the 2-position. J Lipid Res 28:403–413
20. Bentley PH, McCrae W (2002) Synthesis of symmetrical 1,3-diglycerides. J Org Chem 35:2082–2083
21. Adlof RO, List GR (2008) Synthesis and physical properties of symmetrical and non-symmetrical triacylglycerols containing two palmitic fatty acids. J Am Oil Chem Soc 85:99–104
22. Gunstone F, Harwood J, Dijkstra A (2007) The lipid handbook with CD-ROM, 3rd edn. CRC Press, Boca Raton
23. Fickers P, Destain J, Thonart P (2008) Lipases are atypical hydrolases: Principal characteristics and applications. Biotechnol Agron Soc Environ 12:119–130