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SCIENCE DIRECT
Food Chemistry 100 (2007) 1443–1452
Food Chemistry
www.elsevier.com/locate/foodchem

Chemoenzymatic synthesis of structured triacylglycerols with conjugated linoleic acids (CLA) in central position

P. Villeneuve [a,*], N. Barouh [a], B. Baréa [a], G. Piombo [a], M.C. Figueroa-Espinoza [a], F. Turon [c], M. Pina [a], R. Lago [a,b]

[a] *UMR IATE, Laboratoire de Lipotechnie, CIRAD, TA 40/16, 73 rue Jean François Breton, 34398 Montpellier Cedex 5, France*
[b] *Embrapa Labex, Avenue d'Agropolis, 34394 Montpellier Cedex 5, France*
[c] *Bertin SA, BP51, Route de Mareuil, 60330 Lagny le Sec, France*

Received 20 July 2005; received in revised form 9 December 2005; accepted 9 December 2005

Abstract

A chemoenzymatic process for the production of structured triacylglycerols (TAG) containing CLA at *sn2* position and lauric acid at external ones is proposed. First, castor bean oil was chemically dehydrated and isomerised to obtain a new modified oil with very high proportion of CLA (>95%). Then, this new oil was used for enzymatic transesterification allowing the grafting of lauric acid at external positions of the TAG backbone by using 1,3 regioselective enzymes. Among these, *Aspergillus niger* lipase was not satisfactory giving very low lauroyl incorporation (<5%) On the contrary, lipases from *Thermomyces lanuginosa* (Lipozyme TL IM) and from *Carica papaya* latex allowed good reaction yields. The effect of the type of acyl donor was studied. With alkyl esters *T. lanuginosa* lipase provided a final incorporation of 58.9% after 72 h corresponding to 88.4% transesterification yield. Concerning *C. papaya* lipase, incorporation of lauroyl residues was lower than Lipozyme TL IM. This lipase exhibited higher performance with lauric acid accounting for 44.7% lauroyl incorporation at the end of reaction for a 67.1% transesterification yield. The effect of the substrates mole ratio was also evaluated. It was observed that a 1:3 TAG/acyl donor mole ratio was the most efficient for both lipases. Finally, fatty acids regiodistribution of the newly formed structured TAG was determined. With Lipozyme TL IM, the proportion of lauric acid incorporated at the *sn2* position did not exceed 5.4% after 72 h while with *C. papaya* lipase a more pronounced incorporation of lauroyl residues at the central position (8.8%) was observed.

Keywords: Lipases; Structured TAG; CLA; Castor bean oil

1. Introduction

Conjugated linoleic acids (CLA) are a group of fatty acid isomers with 18 carbons chain length and two non-methylene interrupted double bonds. Depending on the double bonds positions and their configuration (*cis* or *trans*), different isomers can be found. For a few years now, CLA have been a subject of growing interest due to their suspected nutritional and therapeutic properties. Indeed, since the first study by Pariza, Ashoor, Chu, and Lund (1979) showing an inhibitory effect of CLA on rat liver cells mutagen formation, many other studies have demonstrated the beneficial effects of CLA isomers on human and animal health (Fernie, 2003), such as reduction of mammalian or skin tumors (Belury, Nickel, Bird, & Wu, 1996; Ip et al., 1996) and decrease of breast or prostate cancer risk (Lavillonnière & Bougnoux, 1999; Ochoa et al., 2004). Moreover, CLA isomers may also have some advantageous nutritional properties. A number of studies have shown that some isomers can contribute to the reduction of body fat (Blankson et al., 2000; Jahreis, Kraft, Tischendorf, Schone, & von Loeffelholz, 2000; Malpuech-Brugere et al., 2004; Petridou, Mougios, & Sagredos, 2003; Zambell

* Corresponding author. Tel.: +33 4 67 61 55 18; fax: +33 4 67 61 55 15.
E-mail address: villeneuve@cirad.fr (P. Villeneuve).

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doi:10.1016/j.foodchem.2005.12.018

et al., 2000) or have an action on immune functions preservation (Albers et al., 2003; Field & Schley, 2004; Kelley et al., 2002) or arthritis and inflammatory diseases (Bassaganya-Riera, Hontecillas, & Beitz, 2002; Cheng, Lii, Chen, Lin, & Liu, 2004; Shen, Dunn, Henry, Li, & Watkins, 2004; Watkins & Seifert, 2000) or atherosclerosis in animals (Kritchevsky, Tepper, Wright, Tso, & Czarnecki, 2000; Nagao et al., 2003; Yu, Correll, & Van den Heuvel, 2002). Endogenous production of CLA isomers by humans is very limited. Therefore, a very large proportion of CLA found in the body tissues are from dietary origin (Salminen, Mutanen, Jauhiainen, & Aro, 1998). At present, the interest in CLA as commercial nutritional complements is tremendous and different products are now commercially available (Saebo, 2003) as free fatty acids or alkyl ester mixtures. Only very few of them are obtained in triacylglycerol (TAG) form. Lately, different methods have been proposed for the lipase-catalyzed synthesis of structured TAG enriched in CLA isomers. For example, some authors studied the biocatalyzed synthesis of acylglycerols containing long chain omega 3 fatty acids and CLA by acidolysis of fish oil (Garcia, Arcos, Ward, & Hills, 2000; Torres, Barrios, & Hill, 2002; Torres, Nettekoven, & Hill, 2003). Garcia, Arcos, Keough, and Hill (2001) carried out acidolysis reactions of butter oil with CLA. In a similar study, the transesterification of CLA and tricaprylin was carried out (Kim, Yoon, & Lee, 2001) and the study of fatty acids regiodistribution of the final structured TAG showed that CLA were preferentially incorporated at the *sn*-1,3 positions with the assayed lipases. Acidolysis reactions were also used for the incorporation of CLA into tristearin (Torres, Munir, Lessard, & Hill, 2002). Here also, the regiodistribution of the final TAG product showed incorporation of CLA at the external positions. Finally, some more recent works have evaluated the production of CLA structured TAG by lipase-catalyzed acidolysis of safflower oil (Lee, Shin, Lee, & Lee, 2004). However, despite the interest of such research studies for the evaluation of lipase activities on non-methylene interrupted double bonds fatty acids, one can question the nutritional advantage of such structured TAG when considering the real bioavailability of their constitutive CLA located in this case only at the external positions. Indeed, it is well known that absorption and metabolism of fatty acids are highly dependent on their position on the TAG backbone the ones located at the central *sn-2* position being more effectively metabolised (Christensen, Hoy, Becker, & Redgrave, 1995; Haumann, 1997; Renaud, Ruf, & Petithory, 1995; Sadou et al., 1995). Consequently, we consider that some recent research works where structured TAG are obtained by multi enzymatic steps or chemoenzymatic processes are more attractive since such procedures can guarantee a complete control of the fatty acids regiodistribution. For instance, Halldorsson, Magnusson, and Haraldsson (2001) proposed a chemoenzymatic approach to obtain highly pure structured TAG possessing a medium-chain fatty acids at the primary positions and long chain PUFA at the secondary one. Similarly, various multi-step enzymatic procedures were elaborated for the production of pure structured TAG based on alcoholysis and re-esterification reactions (Schmid, Bornscheuer, Soumanou, McNeill, & Schmid, 1999; Soumanou & Bornscheuer, 2003; Wongsakul, Kittikun, & Bornscheuer, 2004). Finally, a method to produce structured TAG with caprylic acids and CLA, this latter being located at internal or external positions, was recently described (Kawashima et al., 2004). Similarly, we were interested in the production of high purity structured TAG containing CLA in central position and medium chain fatty acids at the external positions. Such MLM TAG (where M are medium-chain fatty acids in external position and L long chain unsaturated ones) would be attractive since allowing an optimal absorption of CLA due to their *sn-2* position while medium-chain fatty acids would guarantee a fast metabolisation of the TAG and would provide fast energy (Babayan, 1968; Bach, Storck, & Meraihi, 1998; Ingle, Driedger, Traul, & Nakhasi, 1999). Indeed, such compounds would be rapidly attacked by pancreatic enzymes to give CLA 2-monoacylglycerols which are efficiently absorbed (Bornscheuer, 1999). Based on our previous work where castor bean oil was dehydrated and isomerised to obtain TAG containing more than 95% CLA (Villeneuve et al., 2005) we describe herein a chemoenzymatic process to obtain such structured TAG.

2. Material and methods

2.1. Chemicals and enzymes

Sodium sulphate, HCl, phenolphthalein, methanol, ethanol, chloroform, acetone, diethyl ether, acetic acid and hexane, all of analytical grade, were from *Carlo Erba* (Montpellier, France). Lauric acid, methyl laurate, phosphoric acid, zinc, Wilkinson catalyst ($RhCl(PPh_3)_3$), $SnCl_2 \cdot 2H_2O$, sodium methylate and acetyl chloride were purchased from *Aldrich* (Saint Quentin, France). Castor bean oil (*Ricinus communis*) was purchased from Sigma (Saint Quentin, France). Its typical fatty acid composition was: C16:0 (1.2%), C18:0 (1.1%), C18:1 n − 9 *cis* (2.8%), C18:1 n − 9 *cis*, n − 7 OH (ricinoleic, 90.0%), 18:2 n − 6 *cis* (linoleic, 4.3%), C18:3 n − 6 (0.4%) and C20:0 (0.2%).

Different lipases or enzymatic preparations were tested: Lipozyme TL IM (Novozymes, A/S, Denmark), a silica granulated immobilised lipase from *Thermomyces lanuginosa* (100 KLU/g, According to supplier one KLU being the amount of enzyme which liberates 1 mmol per minute of tritrable butyric acid from tributyrin). *Carica papaya* lipase, commercialised as crude papain (ref P3375, *Sigma*) corresponds to a crude dried enzymatic preparation from *C. papaya* latex (Lipase activity: 1600 U/g, measured by the authors on tributyrin emulsion at pH 8.0 and 50 °C). Its powder was ground in a mortar before use. Its initial water activity (a_w) was adjusted to 0.22 by storage in a dessicator with the corresponding equilibrating salt and controlled at 25 °C with an FA-ST/1 instrument, GBX

Sc. Inst. (Romans, France) before use. *Aspergillus niger* lipase (12,000 U/g according to supplier) was purchased from Amano (Japan) as lipase AS. All tested lipases were used in this study at their optimal thermodynamic water activity (a_w) in order to guarantee their optimal syntheses activity. These a_w values were determined according to the procedure described by Caro et al. (2002) with butanolysis reaction as a model.

2.2. Methods

2.2.1. Castor bean oil dehydration

Castor bean oil (250 ml) was magnetically stirred (250 rpm) and heated in a 500 ml three-necked round-bottom flask placed in a mineral oil thermostated bath (280 °C) in the presence of zinc powder (1 g) as anti-polymerisation agent. After 20 min at 280 °C, phosphoric acid (0.1%) was added and the dehydration reaction was then initiated. Reaction medium was maintained under vacuum to allow the removal of water formed during the process and nitrogen was gently bubbled to limit oxygen presence and possible oxidation reaction. Samples (50 mg) were periodically taken from the reaction medium using a syringe and methylated for GC analysis and determination of fatty acid composition. After 5 h, reaction was stopped and cooled to room temperature. A typical FA composition of the dehydrated castor bean oil was C16:0 (1.2%), C18:0 (1.1%), C18:1n − 9 *cis* (2.8%), ricinoleic acid (<2%), non-conjugated linoleic acids (33%), C18:3 n − 6 (0.4%), C20:0 (0.2%) and total CLA (60%) with the following isomers distribution: 9-*cis*, 11-*trans* (61.0% isomer/total CLA), 10-*cis*,12-*cis* (21.7% isomer/total CLA), 9-*trans*, 11-*trans* (16.1% isomer/total CLA) and 10-*trans*, 12-*cis* (1.2% isomer/total CLA).

2.2.2. Isomerisation of dehydrated castor bean oil

In a hermetically sealed 50 ml round-bottom flask under nitrogen atmosphere, dehydrated castor bean oil (10 g) was mixed in ethanol in the presence of Wilkinson catalyst (50 mg) and $SnCl_2 \cdot 2H_2O$ (40 mg) at 60 °C. Samples were periodically removed from the reaction mixture and analyzed by GC for fatty acid composition. After 24 h, reaction was stopped and cooled to room temperature. Dehydrated/isomerised castor bean oil was then purified on column chromatography in order to eliminate the chemical catalyst and non-TAG fractions. Typically, the obtained dehydrated/isomerised castor bean oil had the following fatty acid composition: C16:0 (1.2%), C18:0 (1.1%), C18:1n − 9 *cis* (2.8%), ricinoleic acid (<2%), non-conjugated linoleic acids (<0.5%), C18:3 n − 6 (0.4%), C20:0 (0.2%) and total CLA (92.6%) with the following isomers distribution: 9-*cis*,11-*trans* (40.2% isomer/total CLA), 10-*cis*,12-*cis* (10.1% isomer/total CLA), 9-*trans*,11-*trans* (41.1% isomer/total CLA) and 10-*trans*,12-*cis* (7.8% isomer/total CLA). Dehydrated isomerised castor bean oil production yield (from native castor bean oil) was >95% and was made of >98% TAG (checked by TLC-FID).

2.2.3. Enzymatic reactions

Reactions were carried out in 25 ml hermetically sealed vials with different mole ratio of lauric acid or methyl laurate and dehydrated/isomerised castor bean oil in solvent free system in the presence of 10% (w/w of both substrates) of the tested biocatalyst. Vials were placed in an oven set to 60 °C and reaction mixtures were magnetically stirred at 300 rpm. Aliquot fractions (10 mg of total lipids) were periodically removed from the reaction for thin-layer chromatography, TAG purification and GC analysis.

2.3. Analysis

2.3.1. TLC analysis and TAG isolation

Aliquot fractions (3 drops) were removed periodically from the reactions mixtures and diluted in 1 ml of hexane and filtered (Millex 0.5 μm, Millipore, Bedford, MA, USA). Samples (500 μl) were applied with an automatic applicator (Linomat IV, Camag Ltd. Muttenz, Switzerland) on preparative silica plates (20 × 10 cm, Silica gel F254, 0.25 mm layer thickness, Merck, Darmstadt, Germany) and eluted. Development was carried out with *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v) in the case of TAG/methyl laurate mixtures or 60:40:1 (v/v/v) for TAG/lauric acid mixtures. Spots were visualised by spraying with 2,7-dichlorofluoresceine (Sigma). The TAG fraction was scrapped off and further used to analyze its FA composition and regiodistribution.

2.3.2. TLC-FID analysis

Lipids composition (monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG), free fatty acids (FFA), fatty acid methyl esters (FAME)) of the reactions medium were periodically checked using an Iatroscan MK6 TLC-FID system (Bionis, Paris, France). Samples (20 mg) were diluted in 1 ml Hexane. Samples (1 μl) were deposited on silica Chromarods using a semi-automatic applicator SES 3202. For reactions with free fatty acids as acyl donors, Chromarods were developed in toluene/chloroform/ acetic acid 60:24:0.8 (v/v/v). For reactions with free methyl esters as acyl donors, Chromarods were developed first, in hexane/diethyl ether/acetic acid 90:10:1 (v/v/v) and then in toluene/hexane 50:50 (v/v). After development, Chromarods were dried and analyzed by Iatroscan MK6 equipped with Chromstar 2Ea quantification software. DAG and MAG contents were expressed as weight percentage of MAG + DAG + TAG. The reported Data correspond to mean values of three determinations.

2.3.3. GC analysis

In 25 ml round-bottom flask, samples (10 mg) were added to 3 ml sodium methylate solution containing phenolphthalein. Reaction mixture was refluxed for 10 min and 3 ml methanolic HCl were added until phenolphthalein discoloration and the mixture was, then, refluxed again for 10 min and cooled to room temperature. Hexane (8 ml) and 10 ml water were added and the organic phase was

recovered, dried over anhydrous sodium sulphate and filtered for subsequent GC analysis: Agilent 6890 series GC apparatus provided with a Supelcowax 10 capillary column (SGE, Courtaboeuf, France) with the following characteristics: length, 30 m; internal diameter, 0.32 mm, film thickness 0.25 μm, was used. Fatty acid methyl esters were directly injected into the GC. Carrier gas was Helium with a flow rate of 2.2 ml/min, splitting ratio of 1/80. The injector temp was 250 °C and that of FID Detector was 270 °C. The temperature settings were as follows: 150 °C to 225 at 5 °C/min, and then held at 225 °C for 20 min. CLA isomers were identified by comparison with commercially available CLA standards.

2.3.4. Regiodistribution analysis

The regiodistribution analysis of isolated TAG was performed after conversion to partial acylglycerols with ethyl magnesium bromide (Turon, Bachain, Caro, Pina, & Graille, 2002; Turon, Bonnot, Caro, Pina, & Graille, 2003). The resulting mixture of partial acylglycerols was separated by preparative TLC (20 × 10 cm, Silica gel F254, 0.25 mm layer thickness, Merck, Darmstadt, Germany). The plate was developed with a chloroform/acetone/acetic acid solution (85:15:1, by vol). The α-monoacylglycerols (α-MAG) band at $R_f = 0.26$ was scraped off, the α-MAG were converted to the corresponding fatty acid methyl esters and analyzed by GC.

All assays concerning castor bean oil dehydration, isomerisation, and lipase interesterification or fatty acid composition and regiodistribution were run in triplicate with less than 3% deviation. Data presented in the table and figures correspond to the mean value of three determinations.

3. Results and discussion

3.1. Production of dehydrated/isomerised castor bean oil

The most common method to produce CLA is the alkaline isomerisation of linoleic acid. Generally, this reaction cannot be carried out on natural linoleic vegetable oils such as sunflower, soy or safflower, but instead on their corresponding soap, which, once conjugated through the action of a strong base, are then transformed into free fatty acid with a dilute acid. Reaction temperature is around 200–250 °C and the obtained free fatty acids are generally purified by distillation at the end of the process. Such isomerisation reactions can also be applied to methyl esters (Chin, Liu, Storkson, Ha, & Pariza, 1992). Nowadays, alkaline isomerisation of linoleic acid is often performed in propylene glycol in order to limit reaction temperature (<100 °C) and catalyst quantities (2%) (Delmonte, Roach, Mossoba, Losi, & Yurawecz, 2004). Dehydration of castor bean oil is another well-known process that had been extensively studied about sixty years ago (Radlove, Dejong, & Falkenburg, 1948; Terrill, 1950; Waheeduddin, Siva Samba, & Aggarwal, 1966). This oil contains about 85–90% ricinoleic acid (12-hydroxy-9-*cis* 18:1), which can be easily dehydrated at high temperatures and acid catalysis to form an additional double bond in the aliphatic chain. Depending on the dehydration conditions, this newly formed double bond can possibly be conjugated with that initially present, leading to various CLA isomers and non-conjugated linoleic acid. In a previous paper, we have shown that castor bean oil dehydration combined with further isomerisation was an attractive process for obtaining highly enriched CLA oil (Villeneuve et al., 2005). This process was applied herein to obtain pure enriched CLA TAG to be used as starting material for the enzymatic synthesis of CLA structured TAG. Castor bean oil was first dehydrated at 280 °C for 5 h using phosphoric acid (0.1%) and CLA production was followed by GC (Fig. 1). FA composition of the dehydrated oil is given in Table 1. A significant amount of CLA was obtained (60.0%). However, non-conjugated linoleic acid is also formed during the dehydration process (33.0%). Isomerisation was then carried out in the presence of Wilkinson catalyst and $SnCl_2 \cdot 2H_2O$ at 60 °C for 24 h in order to transform non-conjugated linoleic acid into CLA (Table 1) followed by GC analysis (Fig. 1). The FA composition of resulting isomerised dehydrated castor bean oil shows that the process allows the synthesis of highly enriched CLA oil containing about 92.6% of such fatty acids. Un-reacted ricinoleic acid (1.3%) and non-conjugated linoleic acid (0.4%) were present only in negligible quantities while the content of others FA remained unaltered C16:0 (1.2%), C18:0 (1.1%), C18:1n − 9 *cis* (2.8%), C18:3 n − 6 (0.4%), C20:0 (0.2%). This dehydrated/isomerised castor bean oil (DICBO) was then used as starting material for the enzymatic production of structured TAG containing CLA in central position and lauric fatty acid at external ones.

3.2. Screening of lipases

Three commercially available 1,3-regioselective lipases, namely Lipozyme TL IM (*T. lanuginosa*), *C. papaya* lipase, and *A. niger* lipase, were selected and evaluated for their ability to catalyze transesterification reactions between methyl laurate and DICBO. Reactions were carried out at 60 °C for 24 h using a 1:3 TAG/methyl ester mole ratio and 10% (w/w) of the various assayed enzymes (Fig. 2). It is worth noting that although the biocatalysts had different activities, as mentioned by their suppliers, we decided to compare these lipases at the same weight ratio in order to estimate the total costs of the process, the latter being obviously dependent on the final enzyme choice. The incorporation of lauroyl residues into TAG of DICBO was measured by GC after TLC purification and recovery of newly formed TAG. As these lipases are known to express a strong 1,3-regioselectivity in such reaction conditions, the theoretical maximum incorporation of lauroyl residues would be 66.6% corresponding to a 100% transesterification yield of the two external positions of the TAG backbone. Among the assayed enzymes, lipase AS showed a very weak activity with less than 5% lauroyl residue incorporation into TAG. This result is in accordance with others authors who found a similar lack of biocatalytic activity of

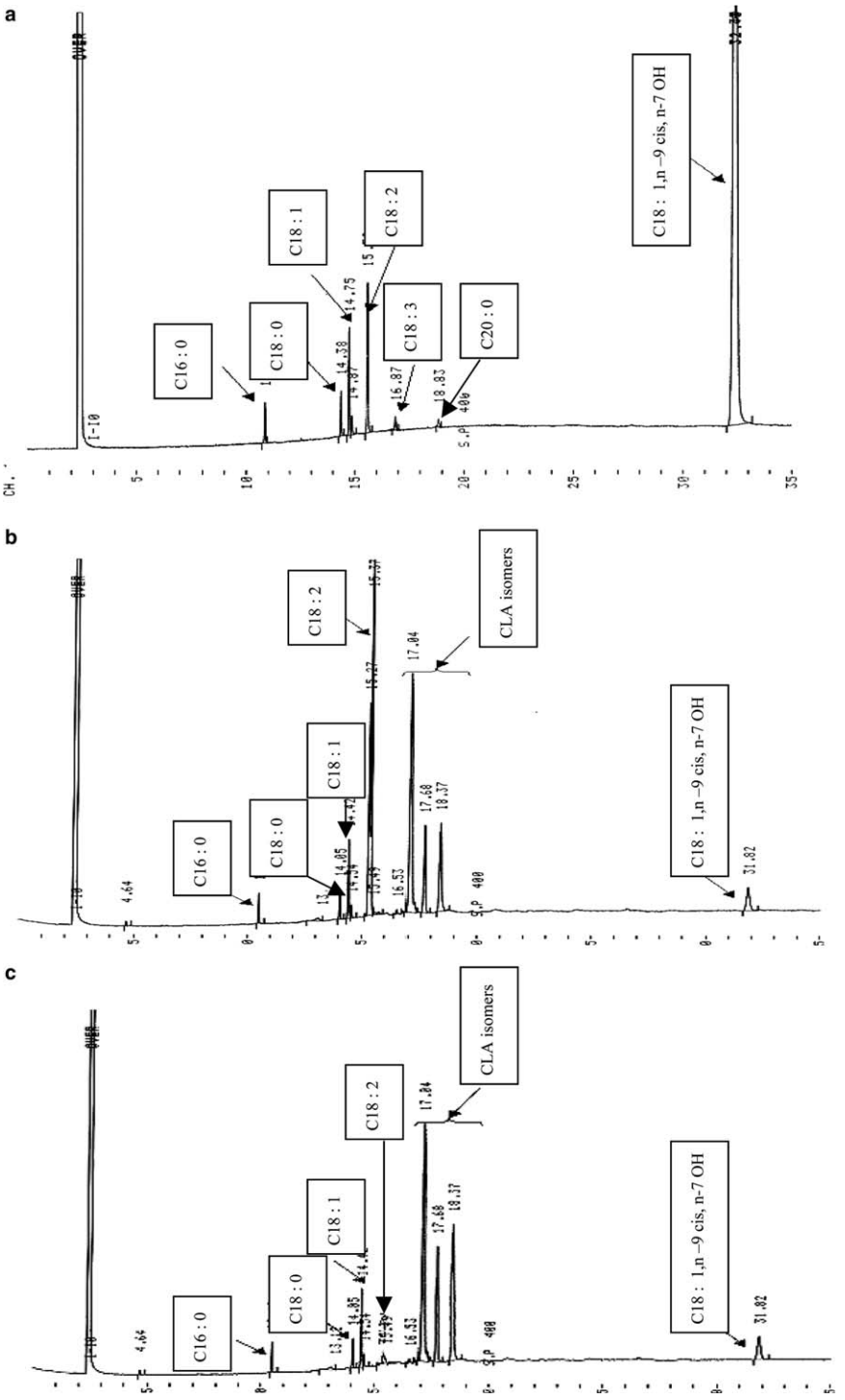

Fig. 1. Representative GC chromatograms obtained for unmodified castor bean oil (a), dehydrated castor bean oil (b) and dehydrated isomerised castor bean oil (c) (analysis parameters: carrier gas; helium debit 2.2 ml/min, splitting ratio: 1/80. Injector temp: 250 °C, FID detector temp: 270 °C. The temperature settings were as follows: 150 °C to 225 at 5 °C/min, 225 °C for 20 min. CLA isomers were identified in comparison with commercially available CLA standards).

this enzyme in transesterification reaction of CLA with tricaprylin (Kim et al., 2001). On the other hand, the degree of incorporation was more important for the two other lipases with 41.6% for Lipozyme TL IM and 35.4% for *C. papaya* after 24 h. Therefore, these two biocatalysts were selected for further experiments.

3.3. Effect of acyl donor type

The influence of the acyl donor type on the transesterification efficiency was evaluated with lauric acid or methyl laurate in reactions set at 60 °C using 1:3 TAG/acyl donor mole ratio and 10% (w/w) enzyme load. The incorporation

Table 1
Fatty acid composition (mol%) of initial castor bean oil (CBO), dehydrated castor bean oil (DCBO) and dehydrated/isomerised castor bean oil (DICBO) (dehydration parameters: phosphoric acid 0.1%, 280 °C, 5 h. Isomerisation parameters: Wilkinson catalyst (50 mg) and $SnCl_2 \cdot 2H_2O$ (40 mg), 60 °C, 24 h)

	C16:0	C18:0	C18:1 (n − 9 *cis*)	Ricinoleic Acid (C18:1 n − 9 *cis*, n − 7 OH)	Linoleic acid[a] C18:2 n − 6	Linolenic acid C18:3 n − 3	C20:0	Total CLA
CBO[b]	1.2	1.1	2.8	90.0	4.3	0.4	0.2	0.0
DCBO[b]	1.2	1.1	2.8	1.3	33.0	0.4	0.2	60.0
DICBO[b]	1.2	1.1	2.8	1.3	0.4	0.4	0.2	92.6

[a] Data corresponds to total methylene interrupted (non-conjugated) linoleic acid.
[b] CBO, castor bean oil; DCBO, dehydrated castor bean oil; DICBO, isomerised dehydrated castor bean oil.

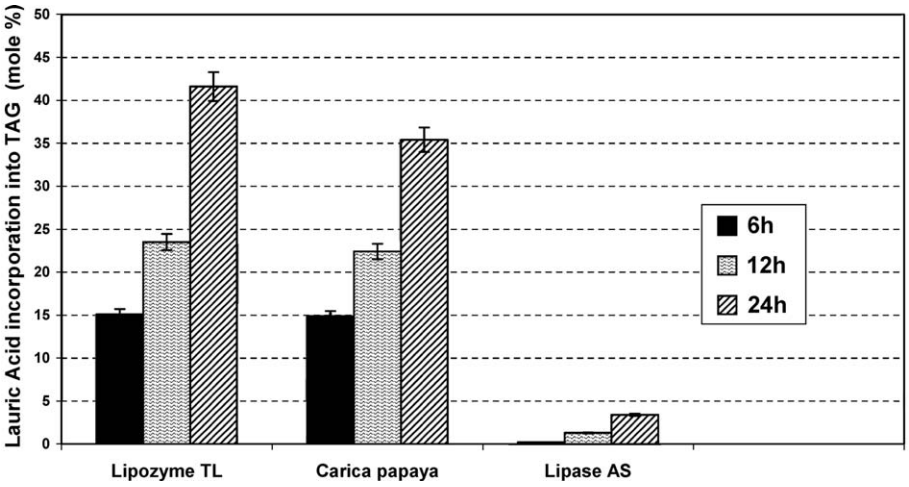

Fig. 2. Enzyme screening for the transesterification of DICBO with methyl laurate (reactions conditions: 1:3 TAG/methyl ester mole ratio, $t = 60$ °C, enzyme load 10% (w/w), reaction time 24 h).

of lauroyl residues into TAG of DICBO over reaction course was monitored and quantified by GC (Fig. 3). Best results were obtained with Lipozyme TL IM and methyl laurate as acyl donor with a final incorporation of 58.9% into TAG after 72 h corresponding to an 88.4% transesterification yield. Moreover, this reaction was the fastest one, reaching 19.5% incorporation after 4 h and 27.9% after 8 h. This same enzyme was slightly less efficient when the acyl donor was lauric acid. Whereas optimal incorporation was very comparable (57.2% after 72 h), reaction was slower with incorporations of 15.1% and 23.4% after 4 and 8 h, respectively. Concerning *C. papaya* lipase, incorpora-

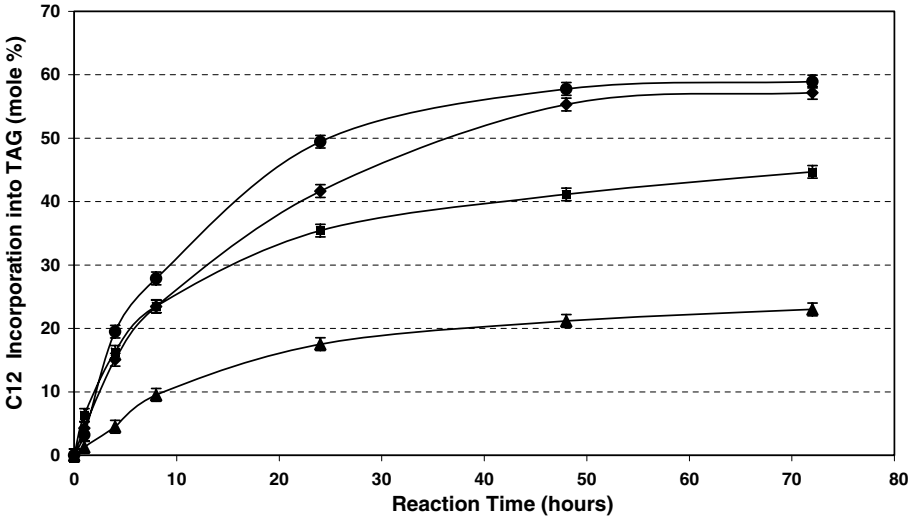

Fig. 3. Effect of acyl donor on the incorporation of lauric acid into TAG of DICBO: (◆) Lipozyme TL IM with lauric acid, (●) Lipozyme TL IM with methyl laurate, (■) *Carica papaya* lipase with lauric acid, (▲) *Carica papaya* lipase with methyl laurate (reactions conditions: 1:3 TAG/acyl donor mole ratio, $t = 60$ °C, enzyme load 10% (w/w)).

tion of lauroyl residues into TAG of DICBO was lower than that provided by Lipozyme TL IM whatever the type of acyl donor used. In fact, this lipase performed better with lauric acid as acyl donor resulting in 44.7% lauroyl final incorporation and 67.1% transesterification yield. When using methyl laurate as acyl donor C12 incorporation into TAG was lower (23.0% after 72 h) for a transesterification yield of 34.5%. This result is in accordance with that observed previously concerning the acyl donor preference of *C. papaya* latex (Villeneuve, Skarbek, Pina, Graille, & Foglia, 1997). Indeed, this lipase was shown to have a better catalytic activity with free fatty acids than alkyl esters in various transesterification reactions. The reasons explaining the weak affinity of *C. papaya* lipase towards alkyl esters substrates are still unclear. This could be attributed to a side competitive hydrolysis reaction of the alkyl substrates which would then release methanol. This highly polar compound could then have a strong denaturing effect on the lipase resulting in a lowering of its catalytic activity for the transesterification reaction. When the lipids profile of the medium was followed by TLC-FID over reaction course, the occurrence of side hydrolysis reaction was indeed observed (a maximum of 9.6% after 72 h).

3.4. Effect of substrates molar ratio

The effect of the substrates mole ratio was also evaluated regarding their influence on the reaction equilibrium in order to check whether higher proportions of acyl donors could result in a displacement of reaction equilibrium to favour the optimisation of the transesterification yields. Both Lipozyme TL IM and *C. papaya* lipase were assayed at different mole ratios TAG/acyl donor (1:3, 1:5, 1:10) at 10% (w/w) enzyme load. The incorporation of lauroyl residues into TAG was quantified by GC over the reaction course (Figs. 4a(a) and 4b). For both lipases increased

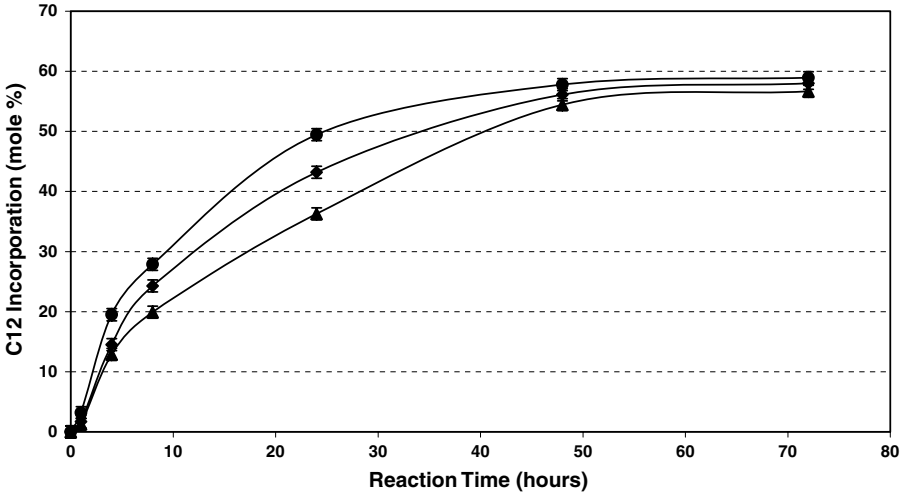

Fig. 4a. Effect of substrate mole ratio on the incorporation of lauroyl residues into TAG of DICBO with Lipozyme TL IM (●) 1:3 TAG/methyl laurate, (♦) 1:5 TAG/methyl laurate, (▲) 1:10 TAG/methyl laurate (reactions conditions: enzyme load: 10% (w/w), $t = 60$ °C).

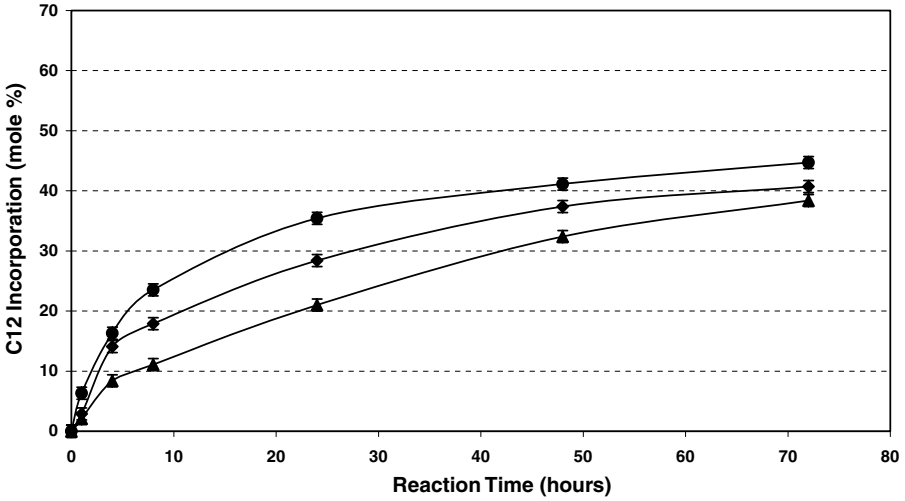

Fig. 4b. Effect of substrate mole ratio on the incorporation of lauroyl residues into TAG of DICBO with *Carica papaya* (●) 1:3 TAG/lauric acid, (♦) 1:5 TAG/lauric acid, (▲) 1:10 TAG/lauric acid (reactions conditions: enzyme load: 10% (w/w), $t = 60$ °C).

amounts of acyl donor (methyl laurate for Lipozyme TL IM and lauric acid for *C. papaya* lipase) did not result in a better incorporation of lauroyl residues into TAG. For Lipozyme TL IM, while optimal incorporations were globally comparable at the end of the reaction, fastest kinetics were obtained with 1:3 TAG/methyl laurate mole ratio (Fig. 4a). For 1:5 mole ratio, 43.2% incorporation was observed after 24 h reaching a maximum of 58.0% after 72 h. Slowest kinetics was obtained with 1:10 mole ratio resulting in 36.3% incorporation at 24 h. Concerning *C. papaya* lipase, a similar profile was obtained although transesterification rates were lower than those with Lipozyme TL IM (Fig. 4b). The optimum incorporation (44.7%) and fastest kinetics were also observed with 1:3 TAG/acyl donor mole ratio. Higher amounts of acyl donor resulted in slower reactions and lower C12 incorporation. For example, after 24 h, lauroyl residues incorporation was around 28.4% at 1:5 mole ratio, and 21.0% at 1:10 corresponding, respectively, to 43.2% and 31.5% transesterification rates. These results are unusual since it is generally accepted that increasing ratios of acyl donor/TAG result in better incorporation of fatty acids into the TAG. However, such behaviours were already observed where high amounts of acyl donors lead to a decrease of FA incorporation (Mutua & Akoh, 1993; Ramirez-Fajardo, Akoh, & Lai, 2003). In particular, this was already observed for *C. papaya* lipase (Lee & Foglia, 2000). In general, such phenomena are attributed to a deactivation effect of the acyl donor on the enzyme. For any further experiments, 1:3 TAG/acyl donor mole ratio was selected.

3.5. Regiodistribution of obtained structured TAG

Fatty acids located at the *sn-2* position of TAG are preferentially absorbed during the digestion process. Therefore, it was very important to confirm that lauroyl residues were only introduced in the external position of the TAG backbone while CLA fatty acids located at the central one were not affected by the transesterification reaction. Reaction was carried out under optimised conditions for both enzymes using their preferred acyl donors. Both the incorporation of lauroyl and the regiodistribution of fatty acids located at external or central positions of the transesterified TAG were evaluated. With Lipozyme TL IM, results showed that the degree of incorporation of lauroyl residues at the central position was limited for the first hours of reactions (<24 h) (Fig. 5a). Indeed, the proportion of lauric acid incorporated at the *sn-2* position did not exceed 2.7% with 74.2% transesterification yield. For prolonged reaction times (48 and 72 h), although rates were more satisfactory, it appeared that lauroyl residues located at the central position became more important (4.2% and 5.4%, respectively). It is difficult to determine whether the increasing of lauroyl residues at the *sn-2* position was due to a loss of the biocatalyst strict 1,3-regiospecificity or to acyl migration phenomenon that can always occur during such transesterification reactions. Such acyl migration are observed as partial acyl-

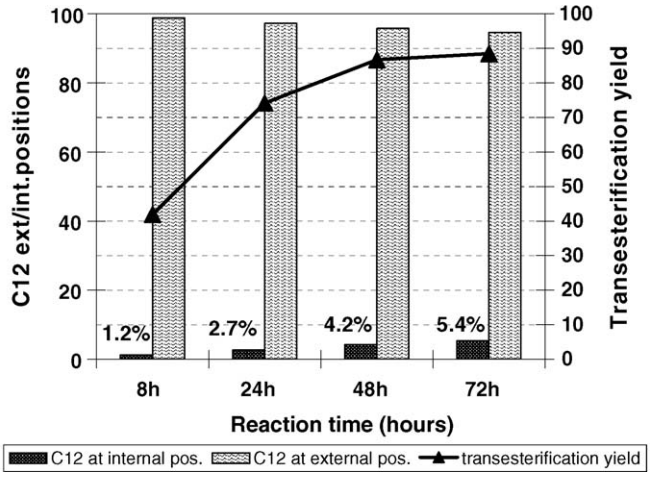

Fig. 5a. Proportions of lauroyl residues incorporated at central or external position during transesterification reaction of DICBO with methyl laurate using Lipozyme TL IM as biocatalyst (reactions conditions:enzyme load, 10% (w/w), $t = 60$ °C, TAG/methyl laurate mole ratio, 1:3).

glycerols which are formed as a side hydrolysis reaction during the process. Therefore, the formation of partial acylglycerols was checked throughout the reaction course using a TLC-FID technique. A maximum of 8.7% (w/w of total acylglycerols) after 72 h was obtained, confirming the fact that acyl migration was indeed possible. With *C. papaya* latex, a more pronounced incorporation of lauroyl residues at the central position was observed (Fig. 5b). While this proportion was around 3.7% at 24 h, it reached 8.8% when the reaction was stopped at 72 h with a transesterification rate of 67.1%. TLC FID analysis of the reaction medium showed that the amount of partial acylglycerols was slightly higher than that for the reaction with Lipozyme TL IM, which reached 9.6% (w/w of total acylglycerols) after 72 h. The differences found in terms of C12 incorporation at the *sn-2* position for the two enzymes could be attributed to the higher proportion of partial acylglycerols found in

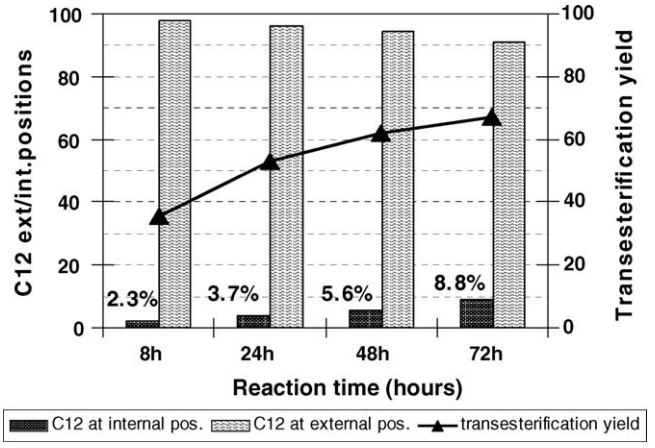

Fig. 5b. Proportions of lauroyl residues incorporated at central or external position during transesterification reaction of DICBO with lauric acid using *Carica papaya* lipase as biocatalyst (reactions conditions: enzyme load: 10% (w/w), $t = 60$ °C, TAG/lauric acid mole ratio, 1:3).

the *C. papaya* lipase reaction resulting in a more pronounced acyl migration phenomenon. However, it is also possible that such differences can be explained by a difference in the conservation of the 1,3-regiospecificity of the enzyme. Indeed, *C. papaya* lipase may express a greater loss of its 1,3-regiospecificity during the reaction course than Lipozyme TL IM.

In conclusion, the chemoenzymatic process used appears to be effective for the synthesis of structured TAG containing CLA isomers at the central position and lauric acids at external positions. This fatty acids regiodistribution guarantees an optimum absorption of CLA during the digestion process. Among the tested lipases, Lipozyme TL IM was shown to be the most appropriate. Using methyl laurate as acyl donor with optimised TAG/methyl ester mole ratio in the presence of Lipozyme TL IM, it was possible to obtain high transesterification rates at external positions with limited incorporation of lauroyl residues at the central position.

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