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Incorporation of selected long-chain fatty acids into trilinolein and trilinolenin

Fayez Hamam [a], Fereidoon Shahidi [a,b,*]

[a] Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada AIB 3X9
[b] Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, Canada AIB 3X9

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Abstract

The effect of chain length, number of double bonds, the location and geometry of double bonds, the reaction conditions, and reactivity of different lipases on the incorporation of selected long-chain fatty acids (LCFA) into triacylglcerols, such as trilinolein (tri C18:2) and trilinolenin (tri C18:3) is examined. This study also discusses reasons behind different degrees of incorporation of selected LCFA into tri C18:2 or tri C18:3 on a molecular basis. Five lipases, namely *Candida antarctica* (Novozyme-435), *Mucor miehei* (Lipozyme-1M), *Pseudomonas* sp. (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30) were screened for their effect on catalyzing the acidolysis of trilinolein (tri C18:2) or trilinolenin (tri C18:3) with selected C18, C20 and C22 fatty acids (FA). Incorporation of a mixture of C18 FA into trilinolein, using *Pseudomonas* sp., the most effective lipase, was in the order of SA > OA > GLA > ALA > CLA. Meanwhile, the degree of n-6 FA incorporation into tri C18:2 with *Pseudomonas* sp. was in the order of GLA > AA > CLA. The order of incorporation of n-3 FA into trilinolein using lipases from *C. antarctica* and *M. miehei* was ALA > EPA > DPA > DHA.

Lipases from *M. miehei* and *Pseudomonas* sp. catalyzed better incorporation of a mixture of C18 FA into tri C18:3 while the remaining lipases catalyzed slight incorporation ($\leqslant$1%) of these FA into this oil. SA and CLA were the most and least reactive C18 FA, respectively. EPA was better incorporated into trilinolenin than DPA or DHA using the enzymes selected. Lipases from *Pseudomonas* sp., *C. rugosa*, and *M. miehei* catalyzed better the incorporation of a mixture of equimole quantities of n-6 FA into tri C18:3 than any of the other lipases tested; the lowest incorporation ($\leqslant$1%) was observed using *Candida antarctica* and *A. niger*, possibly due to the experimental conditions employed in this study which were suitable for these enzymes. The degree of incorporation of EPA into tristearin (tri C18:0), triolein (tri C18:1), and trilinolenin (tri C18:3) decreased as the number of double bonds increased from zero in tristearin to nine in trilenolenin. The same trends were observed for ALA and DHA.

Keywords: Acidolysis; Interesterification; Lipase; Tristearin (tri C18:0); Triolein (tri C18:1); Trilenolein (tri C18:2); Trilinolenin (tri C18:3); Structured lipids; Oleic acid; α-Linolenic acid; γ-Linolenic acid; Docosahexaenoic acid; Docosahexapentaenoic acid; Eicosapentaenoic acid; Conjugated linoleic acid; Stearic acid; Arachidonic acid; Linoleic acid

1. Introduction

Specialty lipids include a wide range of products amongst which structured lipids are a main class. Structured lipids (SL) are triacylglycerols (TAG) or phospholipids (PL) in which fatty acids have been placed in specific locations in the glycerol backbone and are produced using a chemical or enzymatic process. SL are new generation fats or oils with medical, nutraceutical and food applications.

Much attention is being paid to SL due to their potential biological functions, industrial applications and nutritional perspectives. Lipids can be restructured to meet essential fatty acid requirements or to incorporate specific fatty acids of interest into specific locations of the glycerol backbone of TAG. SL may offer the most efficient means of delivering target fatty acids for nutritive or therapeutic

* Corresponding author. Tel.: +1 709 737 8552; fax: +1 709 737 4000.
E-mail address: fshahidi@mun.ca (F. Shahidi).

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purposes as well as to alleviate specific disease and metabolic conditions.

The constituent fatty acids and their locations in the glycerol backbone determine the functional and physical features, the metabolic fate, and the health benefits of SL. Therefore, designing SL with selected fatty acids at specific locations in the TAG for medicinal applications has attracted much attention. The position of FA in the TAG molecules (*sn*-1, *sn*-2, and *sn*-3) will have a significant impact on their metabolism in the body. In general, FA at the terminal positions of TAG (*sn*-1 and *sn*-3) are hydrolyzed by pancreatic lipase and absorbed while those at the middle position of TAG (*sn*-2) remain unchanged and are used in the synthesis of new TAG. For example, it may be desirable to develop a SL containing polyunsaturated fatty acids (PUFA) at the *sn*-2 position with medium-chain fatty acids (MCFA) at the *sn*-1,3 positions for patients with mal-digestion as well as cystic fibrosis. IMPACT is a medical product containing SL and is made via interesterification of high-lauric acid (12:0) and high-linoleic acid oil. ACT is produced by Novartis Nutrition Company (Minneapolis, MN) and is used for patients suffering trauma or surgery, sepsis or cancer (Haumann, 1997). A SL containing MCFA and linoleic acid is more efficient in cystic fibrosis patients than safflower oil, which has about twice as much of linoleic acid (McKenna, Hubbard, & Aeri, 1985). The SL diet, IMPACT, with low amounts of linoleic acid resulted in decreased infection and shortened the length of stay in hospital as compared to other enteral formulas (Haumann, 1997).

SL have many industrial applications and have recently attracted the attention of food manufacturers for production of low-caloric lipids that are characterized by a mixture of short-chain fatty acids (SCFA) and/or MCFA and LCFA in the same glycerol moiety. Interest in such products stems from the fact that they contain 5-7 kCal/g energy compared to 9 kCal/g for usual fats and oils; this is because of the lower caloric content of SCFA or MCFA compared to LCFA. Reduced-calorie specialty lipids are intended for use in baking chips, dips, coatings, bakery and dairy products, or as a cocoa butter replacer. Examples of commercially available low-caloric lipids containing medium-chain fatty acids include Caprenin®and Neobee®.

Over the past two decades several research groups have successfully incorporated MCFA (caprylic or capric acids) into fish and marine oils containing PUFA (Akoh & Moussata, 2001; Jennings & Akoh, 1999; Kawashima et al., 2001, 2002; Senanayake & Shahidi, 2002a, 2002b; Shimada et al., 1996), into borage oil rich in γ-linolenic acid (Akoh & Moussata, 1998; Kawashima et al., 2002; Shimada, Suenaga, Sugihara, Seiichi, & Tominaga, 1996), and into single-cell oils (Hamam & Shahidi, 2004a, 2004b, 2004c, 2005; Iwasaki, Han, Narita, Rosu, & Yamane, 1999). However, these researchers have not discussed the reasons behind different degrees of incorporation of selected FA into different oils on a molecular basis. Thus, there is a noticeable gap in the existing scientific literature regarding the differences in the reactivity of various fatty acids that needs to be filled.

Structured lipids may be produced by incorporation of selected fatty acids into an oil. The degree of reactivity of different fatty acids may vary in different systems due to factors such as the lipase type, water activity, and other conditions (Yang, Xu, & Li, 2001). Many lipases have been shown to be more selective toward C18 FA with higher degrees of unsaturation in esterification and interesterification reactions (C18:0 < C18:1 < C18:2) (Ronne, Pederson, & Xu, 2005). Yang et al. (2001) compared incorporation of linoleic (LA) and conjugated linoleic (CLA) acids into tristearin (SSS) in a solvent-free system at 60 °C using 5% Lipozyme RM IM from *Rhizomucor miehei*. Incorporation of LA into SSS was higher than that of CLA and suggested that the rigidity of CLA might have been responsible for this observation (Yang et al., 2001). Tsuzuki (2005) screened ten lipases for their ability to catalyze acidolysis of triolein with SCFA (C2:0, C3:0, and C4:0) in organic solvents. Lipase from *Aspergillus oryzae* afforded the highest yields of products in the reaction of triolein with C2:0, C3:0, and C4:0. The results of this study indicated that as the chain length decreased, the degree of incorporation of SCFA into triolein increased. Paez, Medina, Rubio, Cerdan, and Grima (2003) reported that incorporation of caprylic acid (C8:0) into triolein was favored compared with that of oleic acid. Again chain length of the FA might play a role in the observed trends.

The synthesis of a modified oil via acidolysis of trilinolein (tri C18:2) with C8:0, using Lipozyme IM-60 as a biocatalyst has been reported (Sellappan & Akoh, 2001). Lipozyme IM-60 was found to be more effective for incorporation of LCFA than MCFA. Synthesis of SL by interesterification of trilinolein and tricaproin with *sn*-1,3-specific (IM 60) and nonspecific (SP 435) lipases was reported (Fomus & Akoh, 1998). IM 60 lipase produced 53.5 mol% dicaproyllinolein with a total carbon number (C_{33}) and 22.2% monocaproyldilinolein (C_{45}). SP 435 lipase produced 41% C_{33} and 18% C_{45}. When caproic acid (C6:0) was used in place of tri C6:0 as the acyl donor, the IM 60 lipase-assisted reaction produced 62.9% C_{33} (Fomus & Akoh, 1998). Akoh, Jennings, and Lillard (1995) used two immobilized lipases, IM 60 from *R. miehei* and SP435 from *Candida antarctica* to modify trilinolein (tri C18:2) with EPA and DHA, by using their ethyl esters as acyl donors. The total EPA product yields with *R. miehei* and *C. antarctica* lipases were 79.6% and 81.4%, respectively. However, no explanation was provided regarding higher EPA reactivity compared to DHA (Akoh & Huang, 1995).

In general, we found incorporation of selected LCFA into TAG (e.g. tristearin or triolein) may be affected by many factors, including chain length, number of double bonds, and the location and geometry of the double bonds as well as reaction conditions and reactivity and specificity of lipases employed. LA was more reactive than CLA due to the rigidity of the latter and/or specificity of the enzymes. EPA was more reactive than DHA, due to the

structural differences between the two (number of double bonds, chain length) (Hamam & Shahidi, 2007, in press). Lipases, Novozyme-435 enzyme from *C. antarctica* and AY-30 from *Candida rugosa*, might be considered as promising biocatalysts for acidolysis of tristearin and selected LCFA (Hamam & Shahidi, 2007). The high percent incorporation of FA into tristearin using lipase from *C. antarctica* or *C. rugosa* might be due to the experimental conditions employed in this study which were suitable for these two enzymes (Hamam & Shahidi, 2007). No systematic studies have been carried out for incorporating LCFA into TAG such as tristearin (tri C18:0); triolein (tri C18:1); trilinolein (tri C18:2); trilinolenin (tri C18:3), using different lipases. We have previously reported the acidolysis of tristearin (Hamam & Shahidi, 2007) and triolein (Hamam & Shahidi, in press) with LCFA. In this study, we examined the effect of the chain length, number of double bonds, and the location and geometry of double bonds on the incorporation of selected FA into tri C18:2 and tri C18:3. The work is expected to expand the existing knowledge, both basic and applied, in the area of lipid biotechnology. The information obtained in this work would allow the scientists and manufacturers to design and/or predict reaction results for incorporating different fatty acids of interest into triacylglycerols.

2. Materials and methods

2.1. Materials

Two lipases from *Candida antarctica* (Novozyme-435) and *M. miehei* (Lipozyme-IM) were acquired from Novozyme (Bagsvaerd, Denmark). Other lipases, namely *Pseudomonas* sp. (PS-30), *A. niger* (AP-12), and *C. rugosa* (AY-30), were obtained from Amano International Enzyme Com. Inc. (Troy, VA). All lipases used in this work were in the powder form and solvents were of analytical grade, and purchased from Fisher Scientific (Nepean, ON). Oleic acid (OA), linoleic acid (LA), γ-linolenic acid (GLA), α-linolenic acid (ALA), arachidonic acid (AA), trilinolein (tri C18:2), and trilinolenin (tri C18:3) were purchased from Nu-Chek Prep. Inc. (Elysian, MN). Eicosapentaenoic acid (EPA, >99% pure) was from Fuso Pharmaceutical Industries Ltd. (Osaka, Japan) and kindly provided by Dr. K. Miyashita. Algal oil containing DHA (docosahexaenoic acid) (40.0%) was kindly provided by Martek Biosciences Corporation (Columbia, MD). Docosapentaenoic acid (DPA) was prepared as a concentrate using a proprietary procedure.

2.2. Methods

2.2.1. Preparation of free fatty from algal oil and concentration of DHA by urea complexation

Preparation of free fatty acids from algal oil was conducted according to the urea complexation procedure method described by Wanasundara and Shahidi (1999).

2.2.2. Acidolysis of trilinolein (tri C18:2) or trilinolenin (tri C18:3) and selected long-chain FA

A combination of equimole quantities of C18 FA (SA + OA + CLA + GLA + ALA) at trilinolein (tri C18:2) or trilinolenin (tri C18:3) to C18 FA mole ratio of 1:3 was mixed in a screw-capped test tube. The enzyme amount, reaction temperature, and incubation time were 4%, 45 ± 2 °C, and 24 h, respectively. The same was done for a mixture of equimole amounts of n-3 FA (ALA + EPA + DHA + DPA) or n-6 FA (LA + GLA + AA).

2.2.3. Separation of acylglycerols after acidolysis

To terminate the reaction, after a given time period, a mixture of acetone and ethanol (20 mL; 1:1, v/v) was added to the reaction mixture. In order to neutralize free fatty acids, the reaction mixture was titrated against a 0.5 M NaOH solution, using phenolphathalein until the colour of the solution turned pink at pH 8.4. Hexane (25 mL) was added to the mixture to extract the acylglycerols. The mixture was thoroughly mixed and transferred into a separatory funnel. The two layers (aqueous and hexane) were allowed to separate, and the lower aqueous layer was discarded. The hexane layer was then passed through a bed of anhydrous sodium sulphate to remove any residual water. The hexane was evaporated using a rotary evaporator at 45 °C and the acylglycerol fraction was recovered. A portion of the acylglycerol fraction (5–10 mg) was transferred to 1 mL reacti-vial (Pierce, Rockford, IL) for further experimentation.

2.2.4. Preparation of fatty acid methyl esters (FAMEs)

Fatty acid profiles of products were determined following their conversion to the corresponding methyl esters. Transmethylation reagent (2.0 mL from a freshly prepared solution using 6.0 mL of concentrated sulfuric acid made up to 100 mL with methanol and 15 mg of hydroquinone as antioxidant) was added to the sample vial, followed by vortexing. The mixture was incubated at 60 °C for 24 h and subsequently cooled to room temperature. Distilled water (1 mL) was added to the mixture and after thorough mixing, a few crystals of hydroquinone were added to each vial to prevent oxidation; lipids were extracted three times, each with 1.5 mL of pesticide-grade hexane. The hexane layer was separated, combined and transferred to a clean test tube and then washed two times, each with 1.5 mL of distilled water. The hexane layer (the upper layer) was separated and evaporated under a stream of nitrogen. FAMEs were then dissolved in 1.0 mL of carbon disulfide and used for subsequent gas chromatographic analysis.

2.2.5. Analysis of FAMEs by gas chromatography

The FAMEs were analyzed using a Hewlett Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA) equipped with a Supelcowax-10 column (30 m length, 0.25 mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON). The oven temperature was set at 220 °C for 10.25 min and then raised to 240 °C at 30 °C/

min and subsequently held there for 15 min. An autosampler/injector HP7673 was used and the injector and detector (FID) temperatures were both set at 250 °C. Ultra high purity (UHP) helium was used as carrier gas at a flow rate of 1.2 mL/min; the split ratio was 1:167 and the injection volume was 2 :L. The data were treated using a Hewlett Packard 3365 Series II Chem Station Software (Agilent, Palo Alto, CA). The FAMEs were identified by comparing their retention times with those of authentic standard mixture GLC-461 from Nu-Chek Prep Inc. (Elysian, MN), and the results were presented as weight percentages.

2.2.6. Statistical analysis

All experiments were performed in triplicate. Data reported are mean ± standard deviation (SD). Analysis of variance and Tukey's test were carried out at a level of $p < 0.05$ to assess the significance of differences among mean values.

3. Results and discussion

3.1. Acidolysis of trilinolein (tri 18:2) and a combination of equimole quantities of C18 FA

Incorporation (%) of a mixture of equimole quantities of C18 FA into trilinolein, assisted by *Pseudomonas* sp., the most effective lipase, was in the order of SA > OA > GLA > ALA > CLA (Table 1). These results are consistent with expectations because stearic acid is a saturated FA with a straight chain. LA with two double bonds causes the chain to bend. ALA and GLA, with three double bonds, form a hooked shape which makes their incorporation into triolein more difficult than that of SA or LA. ALA and GLA shared the same chain length and number of double bonds, but the first double bond in ALA is located between C3 and C4 near the methyl end; in GLA, it is located between C6 and C7. From the carboxylic acid end group, the first double bond in ALA is located at C9 while in GLA it is positioned at C6. Therefore, location of these three double bonds in ALA on one side of the molecule, while their location in GLA in the middle of it may result in less bent shape of ALA compared to GLA. This explanation was supported when chemical models for ALA or GLA were constructed. Therefore, structural differences between ALA and GLA related to the location of double bonds as well as specificity of the enzymes used might lead to variation in their reactivity. On the other hand, the remaining enzymes catalyzed slight incorporation (<1%) of these FA into tri 18:2, the order being: OA > SA > GLA > ALA > CLA.

3.2. Acidolysis of trilinolein (tri 18:2) and a mixture of equimole quantities of n-3 FA

The effect of enzyme type on the incorporation of a combination of equimole quantities of n-3 FA into tri 18:2 is shown in Table 2. The order of incorporation of n-3 FA into trilinolein using lipases from *C. antarctica* and *M. miehei* was ALA > EPA > DPA > DHA; this is in agreement with expectations when parameters such as chain length, number of double bonds, steric hinderance and specificity of the enzymes used are considered. ALA is expected to be more reactive than other n-3 family members because it has a shorter chain length and a lesser number of double bonds than the remaining n-3 FA (EPA or DPA or DHA). EPA has a shorter chain length and a lesser number of double bonds than DPA or DHA; therefore EPA has a less bent shape compared to DPA or DHA. These differences (ie. the chain length and the number of double bonds), together with specificity of the enzymes for different fatty acids, lead to decreased incorporation of DPA or DHA into tri 18:2 compared to EPA. These results lend further support to the findings of Senanayake and Shahidi (1999) who reported that maximum incorporation of EPA (28.7–30.7%) in borage and evening primrose oils was obtained with lipase from *Pseudomonas* sp., and those of Akoh and Huang (1995) who found that EPA was more easily incorporated into trilinolein than DHA. Furthermore, the results presented in this study lend further support to our findings in the acidolysis reactions of selected FA and tristearin or triolein (Hamam & Shahidi, 2007, in press). In general, the extent of incorporation of EPA, using selected lipases, was higher than that of DHA. These results suggest that EPA was more reactive than DPA or DHA. Meanwhile, the rest of the lipases (i.e. *C. rugosa*, *Pseudomonas* sp. and *A. niger*) catalyzed incorporation (%) of these FA into trilinolein in the order of EPA > ALA > DPA ⩾ DHA. We are unable to offer any explanation for higher reactivity of EPA compared to ALA in the acidolysis reaction, but factors such as interactions between

Table 1
Effect of enzyme type on incorporation (wt%) of C18 fatty acids into trinlinolein

Enzyme source	SA	OA	CLA	GLA	ALA
Candida antarctica	0.40 ± 0.22	0.81 ± 0.17	ND	0.43 ± 0.07	0.32 ± 0.05
Mucor miehei	1.39 ± 0.10	1.68 ± 0.09	0.28 ± 0.02	0.60 ± 0.05	1.02 ± 0.05
Pseudomonas sp.	1.65 ± 0.01	1.66 ± 0.04	0.33 ± 0.01	1.69 ± 0.07	1.21 ± 0.05
Candida rugosa	0.55 ± 0.02	0.81 ± 0.04	ND	0.31 ± 0.02	0.29 ± 0.01
Aspergillus niger	0.42 ± 0.07	0.85 ± 0.01	ND	0.32 ± 0.02	0.30 ± 0.01

Symbols are as follows: SA, stearic acid; LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; and GLA,γ-linolenic acid. The reaction mixture contained trilinolein (100 mg), fatty acid to trilinolein at a mole ratio of 3:1, enzyme amount (4%, by weight of substrates), water (2%, by weight of enzyme and substrates), and 3.0 mL of hexane. The mixture was kept at 45 ± 1 °C for 24 h in an orbital shaking water bath at 250 rpm.

Table 2
Effect of enzyme type on incorporation of (wt%) n-3 fatty acids into trilinolein

Enzyme source	ALA	EPA	DPA	DHA
Trilinolein				
Candida antarctica	6.31 ± 0.24	5.79 ± 0.23	5.65 ± 0.31	4.22 ± 0.20
Mucor miehei	10.6 ± 0.48	10.3 ± 0.37	8.73 ± 0.39	6.85 ± 0.26
Pseudomonas sp.	5.79 ± 0.11	21.2 ± 1.75	2.91 ± 0.24	3.55 ± 0.25
Candida rugosa	2.36 ± 0.02	15.3 ± 0.29	1.95 ± 0.03	1.90 ± 0.02
Aspergillus niger	1.05 ± 0.03	17.7 ± 0.48	1.08 ± 0.01	1.27 ± 0.02
Trilinolenin				
Candida antarctica	–	11.3 ± 0.89	8.31 ± 0.03	8.44 ± 0.09
Mucor miehei	–	16.0 ± 0.08	14.8 ± 0.08	8.39 ± 0.26
Pseudomonas sp.	–	20.2 ± 0.33	8.06 ± 0.07	8.40 ± 0.00
Candida rugosa	–	24.3 ± 1.44	5.99 ± 0.12	7.30 ± 0.11
Aspergillus niger	–	23.7 ± 0.33	2.47 ± 0.07	1.63 ± 0.06

Symbols are as follows: ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; and DHA, docosahexaenoic acid. The reaction mixture contained trilinolein or trilinolenin (100 mg), fatty acid to trilinolein or trilinolenin at a mole ratio of 3:1, enzyme amount (4%, by weight of substrates), water (2%, by weight of enzyme and substrates), and 3.0 mL of hexane. The mixture was kept at 45 ± 1 °C for 24 h in an orbital shaking water bath at 250 rpm.

FA, and selectivity of lipases employed might lead to such results.

3.3. Acidolysis of trilinolein (tri 18:2) and a combination of equimole quantities of n-6 FA

The degree of incorporation of n-6 FA into tri 18:2 with *Pseudomonas* sp. was in the order of GLA > AA > CLA. Meanwhile, the remaining enzymes catalyzed a marginal degree of incorporation (<1%) of these FA into trilinolein and a better value was observed for AA while the lowest percent incorporation was for CLA (Table 3). GLA was more easily incorporated into tri 18:2 than AA for all lipases tested. The more double bonds the chain has in the *cis* configuration, the more bent the molecule will be. Since AA has four *cis* double bonds, it becomes quite curved compared to GLA and hence AA has more steric hindrance than GLA. Furthermore, specificity of lipases used for different fatty acid may be another factor affecting their different incorporation into the triacylglycerols examined.

3.4. Acidolysis of trilinolenin (tri 18:3) and a combination of equimole quantities of C18 FA

The extent of incorporation of C18 FA into trilinolenin with *M. miehei* was in the order of SA ⩾ LA > OA > GLA > CLA (Table 4). Other enzymes, namely *C. antarctica*, *C. rugosa*, *Pseudomonas* sp., and *A. niger* catalyzed incorporation of C18 FA into tri 18:3, in the order of LA > OA > SA > GLA > CLA. Lipases from *M. miehei* and *Pseudomonas* sp. catalyzed better incorporation of a mixture of C18 FA into trilinolenin while the remaining lipases (*C. antarctica*, *C. rugosa*, and *A. niger*) catalyzed slight incorporation (⩽1%) of these FA into trilinolenin. Therefore, structural differences between these molecules related to the number, the location and geometry as well

Table 3
Effect of enzyme type on incorporation (wt%) of n-6 fatty acids into trilinolein and trilinolenin

Enzyme source	CLA	LA	GLA	AA
Trilinolenin				
Candida antarctica	ND	0.63 ± 0.35	0.59 ± 0.01	0.75 ± 0.01
Mucor miehei	1.97 ± 0.16	6.58 ± 0.11	5.21 ± 0.33	6.98 ± 0.24
Pseudomonas sp.	2.30 ± 0.11	7.70 ± 0.05	9.47 ± 0.11	4.98 ± 0.04
Candida rugosa	1.69 ± 0.02	5.98 ± 0.05	7.61 ± 0.05	3.91 ± 0.07
Aspergillus niger	0.31 ± 0.01	1.33 ± 0.01	0.98 ± 0.02	1.12 ± 0.01
Trilinolein				
Candida antarctica	0.24 ± 0.01	–	0.56 ± 0.23	0.63 ± 0.03
Mucor miehei	0.62 ± 0.12	–	1.08 ± 0.02	1.77 ± 0.02
Pseudomonas sp.	1.23 ± 0.00	–	5.09 ± 0.01	2.37 ± 0.02
Candida rugosa	0.27 ± 0.01	–	0.50 ± 0.03	0.58 ± 0.03
Aspergillus niger	0.36 ± 0.02	–	0.79 ± 0.04	0.94 ± 0.06

Symbols are as follows: CLA, conjugated linoleic acid; LA, linoleic acid; GLA, γ-linolenic acid; and AA, arachidonic acid. The reaction mixture contained trilinolein or trilinolenin (100 mg), fatty acid to trilinolein or trilinolenin at a mole ratio of 3:1, enzyme amount (4%, by weight of substrates), water (2%, by weight of enzyme and substrates), and 3.0 mL of hexane. The mixture was kept at 45 ± 1 °C for 24 h in an orbital shaking water bath at 250 rpm.

Table 4
Effect of enzyme type on incorporation (wt%) of C 18 FA into trilinolenin

Enzyme source	SA	OA	CLA	LA	GLA
Candida antarctica	1.22 ± 0.01	1.45 ± 0.07	ND	1.48 ± 0.02	0.81 ± 0.01
Mucor miehei	3.85 ± 0.14	3.76 ± 0.11	0.62 ± 0.01	3.84 ± 0.13	1.63 ± 0.04
Pseudomonas sp	4.65 ± 0.03	4.85 ± 0.04	0.93 ± 0.02	5.48 ± 0.05	4.57 ± 0.04
Candida rugosa	1.06 ± 0.01	1.25 ± 0.01	ND	1.29 ± 0.01	0.76 ± 0.01
Aspergillus niger	0.69 ± 0.02	0.78 ± 0.03	ND	0.97 ± 0.02	0.46 ± 0.03

Symbols are as follows: SA, stearic acid; OA, oleic acid; LA, linoleic acid; CLA, conjugated linoleic acid; and GLA, γ-linolenic acid. The reaction mixture contained trilinolenin (100 mg), fatty acid to trilinolenin at a mole ratio of 3:1, enzyme amount (4%, by weight of substrates), water (2%, by weight of enzyme and substrates), and 3.0 mL of hexane. The mixture was kept at 45 ± 1 °C for 24 h in an orbital shaking water bath at 250 rpm.

as specificity of the enzymes used might lead to variation in their reactivity.

3.5. Acidolysis of trilinolenin (tri 18:3) and a combination of equimole quantities of n-3 FA

Incorporation of EPA into trilinolenin was better than that of DPA or DHA using all enzymes tested (Table 2). This observation might be due to the existing differences in the chain length and the number of double bonds in the respective molecules; thus, incorporation of DPA or DHA into tri 18:3 was more difficult than EPA, lending further support to the findings of Senanayake and Shahidi (1999, 2002a) who reported that incorporation of EPA into borage and evening primrose oils was better than that of DHA with lipase from *Pseudomonas* sp.

3.6. Acidolysis of trilinolenin (tri 18:3) and a combination of equimole quantities of n-6 FA

Lipases from *Pseudomonas* sp., *C. rugosa*, and *M. miehei* were able to better catalyze the incorporation of a mixture of equimole amounts of n-6 FA into trilinolenin than other lipases tested. The lowest incorporation (⩽1%) was observed using *C. antarctica* and *A. niger* (Table 3); possibly due to the experimental conditions employed in this study which were not suitable for these enzymes. Thus, further studies should be performed to verify the optimum conditions for each enzyme examined, perhaps using response surface methodology.

3.7. The effect of number and location of double bonds on n-3 FA incorporation into tristearin, triolein, trilinolein, and trilinolenin

Table 5 shows the effect of the number and location of double bonds on incorporation of n-3 FA into tristearin, triolein, trilinolein, and trilinolenin. As the number of double bonds increased from zero in tristearin to six in trilinolein, the degree of incorporation of ALA into tristearin, triolein, and trilinolein decreased from 13.7% to 5.79%. Tristearin is expected to have less steric hindrance than that of triolein or trilinolein and hence a high incorporation of n-3 FA into tristearin. The degree of incorporation of EPA into tristearin, triolein, trilinolein, and trilinolenin declined from 33.8% to 20.2% as the number of double bonds increased from zero in tristearin to nine in trilinolenin. Steric hindrance effect was in the order of trilinolenin > trilinolein > triolein > tristearin. The same trend was observed for DHA.

Table 5
Effect of double bonds number and location on incorporation (wt%) of equimole amounts of n-3 FA into tristearin, triolein, trilinolein, and trilinolenin

Oil type	ALA	EPA	DHA
Tristearin	13.7 ± 0.92	33.8 ± 2.53	26.0 ± 0.04
Triolein	10.3 ± 0.37	22.2 ± 0.05	5.06 ± 0.28
Trilinolein	5.79 ± 0.11	21.2 ± 1.75	3.55 ± 0.25
Trilinolenin	–	20.2 ± 0.33	1.63 ± 0.06

A combination of equimole quantities of n-3 fatty acids (ALA + EPA + DHA) at total n-3 to triacylglycerol mole ratio of 3:1 was mixed in a screw-capped test tube then *Pseudomonas* sp., lipase (4%) and water (2%) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 1 °C for 24 h in an orbital shaking water bath at 250 rpm.

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