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Enzymatic Incorporation of Selected Long-Chain Fatty Acids into Triolein

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Abstract Acidolysis of triolein (tri C18:1) with selected long-chain fatty acids (LCFA) was carried out using *Candida antarctica* (Novozym 435), *Rhizomucor miehei* (Lipozyme RM IM), *Pseudomonas* sp. (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30). A better incorporation of stearic acid (SA), α-linolenic acid (ALA), γ-linolenic acid (GLA), arachidonic acid (AA), and docosapentaenoic acid (DPA) was achieved using lipase from *Rhizomucor miehei*. Lipase from *Pseudomonas* sp. catalyzed a better incorporation of linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) into triolein. Thus, *Rhizomucor miehei* and to a lesser extent *Pseudomonas* sp. might be considered as providing the most effective enzymes for acidolysis of triolein with selected LCFA. In general, incorporation of LCFA into triolein (tri C18:1) may be affected by 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 used. As the ratio of the number of moles of a mixture of equimole quantities of C18 FA to triolein changed from 1 to 3, incorporation of C18 FA into triolein increased accordingly with *Rhizomucor miehei* lipase. Similarly, incorporation of n-3 FA into triolein increased when ALA, DPA, DHA, and EPA were used. The same trend was noticed for a mixture of n-6 FA (LA + GLA + AA) and triolein.

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Introduction

Structured lipids may be produced by incorporation of selected fatty acids into 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 [1]. Many lipases have been shown to be more selective toward C18 fatty acids with higher degrees of unsaturation in esterification and interesterification reactions (C18:0 < C18:1 < C18:2) [2].

Yang et al. [1] 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*. Acyl incorporation of LA was higher than that of CLA. Furthermore, it was suggested that LA was more reactive than CLA probably due to the rigidity of the latter [1].

Tsuzuki [3] screened ten lipases for their ability to catalyze acidolysis of triolein and short-chain FA (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 which were 86, 71, 60%, respectively. The results of this study indicated that as the chain length decreased, the degree of incorporation of short-chain fatty acids into triolein increased. Paez and coworkers [4] reported that incorporation of caprylic acid (C8:0) into triolein was favored compared with that of oleic acid. Again chain length of FA might play a role in the observed trends. Furthermore, acidolysis

reached a maximal level at a caprylic acid to triolein mole ratio of 4-6:1. Lipase IM 60 from *Rhizomucor miehei* was most effective in SL production from acidolysis of caprylic acid and triolein. The products contained 57.4 mol% monocaprylоolein with a total carbon number of 47 (C_{47}), 35.4 mol% dicaprylоolein (C_{57}), and 5.3 mol% unreacted triolein (C_{57}). The optimal conditions included an oil-to-caprylic acid mole ratio of 1:4, at 55 °C over 24 h and 10% enzyme concentration [5]. Lipase IM 60 from *Rhizomucor miehei* was most effective in catalyzing acidolysis of triolein and short-chain FA (caproic acid, C6:0; butyric acid, C4:0) to produce low calorie SL. The SL so produced contained 49 mol% disubstituted (SLS), 38 mol% monosubstituted (SLL), and 13 mol% unreacted triolein (LLL), at triolein to caproic and butyric acids of 1:4:4 mole ratio, at 55 °C over 24 h [6]. No reasons were given for different incorporation levels of caproic and butyric acids into triolein. In another study, Huang and Akoh [7] successfully produced a SL via transesterification of caprylic acid ethyl ester and triolein using eight lipases. Among the enzymes tested, immobilized lipase IM 60 from *Rhizomucor miehei* converted most of the triolein into SL dicaprylоolein (41.7%) and monocaprylоolein (46.0%). However, lipase SP 435 from *Candida antarctica* catalyzed the conversion of triolein into dicaprylоolein (62.0%) and monocaprylоolein (33.5%) at 55 °C.

Akoh and Moussata [8] modified fish and canola oils with caprylic acid using Lipozyme RM IM from *Rhizomucor miehei*. Their results showed a higher level of caprylic acid incorporation (40.1%) into canola oil than into fish oil (29.5%). The total polyunsaturated FA (PUFA, EPA and DHA) of fish oil remained unchanged after the modification while PUFA of canola oil were reduced from 29.6 to 21.2%. Monoenes, particularly 18:1n-9 and 16:1n-7, were remarkably reduced by caprylic acid incorporation into fish oil. Indeed, oleic acid was completely replaced by caprylic acid in fish oil whereas it was reduced to 34.7% in canola oil. Lipozyme RM IM from *Rhizomucor miehei* catalyzed incorporation of caprylic acid, up to 70%, in the *sn*-1,3 positions of the modified fish oil [9]. In another study, Xu et al. [10] produced SL upon acidolysis of menhaden oil with caprylic acid which contained 40% caprylic acid and 35% EPA and DHA with less than 3% caprylic acid at the *sn*-2 position using Lipozyme RM IM in a solvent-free system. Lipase-catalyzed interesterification between fish oil and medium-chain TAG (containing 60 mol% caprylic and 40 mol% capric acids) has been studied in a packed-bed reactor using a lipase from *Thermomyces lanuginosa* [11]. The results showed that the degree of reaction progress was significantly correlated to the flow rate and reached equilibrium at 30–40 min at 60 °C. Fatty acids distribution analysis showed that PUFA, particularly EPA and DHA, remained in the *sn*-2 position in the enzymatically interesterified product compared to that produced chemically [11].

Little attention has been paid to the incorporation of selected LCFA into triolein using lipases from different sources, such as bacteria, fungi, and yeasts. Thus, this study aimed to examine 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 LCFA into triolein. It also sought reasons behind different degrees of incorporation of selected LCFA into tri C18:1 on a molecular basis in order to fill an important gap in the existing scientific literature regarding the differences in the reactivity of different fatty acids. 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.

Materials and Methods

Materials

Two lipases from *Candida antarctica* (Novozym 435) and *Rhizomucor miehei* (Lipozyme RM IM) were acquired from Novozymes A/S (Bagsvaerd, Denmark). Other lipases, namely from *Pseudomonas* sp. (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30), were obtained from Amano Enzyme (Troy, VA, USA). Lipases from *Pseudomonas* sp., *Aspergillus niger*, and *Candida rugosa* used in this work were in the powder form, while those from *Candida antarctica* and *Rhizomucor miehei* were in the granular form. All solvents used were of analytical grade and purchased from Fisher Scientific (Nepean, ON, USA). Oleic acid (OA), linoleic acid (LA), γ-linolenic acid (GLA), α-linolenic acid (ALA), arachidonic acid (AA), and triolein (C18:1) were purchased from Nu-Chek (Elysian, MN, USA). 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 provided by the Martek Biosciences Corporation (Columbia, MD, USA). Docosapentaenoic acid (DPA) was prepared as a concentrate using a proprietary procedure.

Preparation of Free Fatty acids 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 [12].

Acidolysis of Triolein (tri C18:1) and Selected Long-Chain FA

Triolein (100 mg) was mixed with different fatty acids (SA, GLA, LA, CLA, ALA, AA, EPA, DPA, and DHA) at a mole ratio of acid to triolein of 3:1 in a screw-capped test tube, then lipase (4% by weight of substrates) and water (2% by weight of substrates and enzyme) were added in hexane (3.0 mL). The mixture was incubated at 45 ± 2 °C for 24 h in a shaking water bath at 250 rpm. In another set of experiments, a mixture of equimole amounts of C18 FA (SA + LA + CLA + GLA + ALA) at C18 FA to triolein ratios of 1:1, 2:1, and 3:1 was used to investigate the effect of substrate mole ratio on incorporation of these fatty acids into triolein. One mole of triolein was mixed with 0.2 mol of each of the five C18 FA (triolein to total C18 FA of 1:1). For a mole ratio of tri C18:1 to C18 FA of 1:2, 1 mol of triolein was mixed with 0.4 mol of each of the 5 C18 FA and for mole ratio of 1:3, this was 0.6 for each FA to 1 mol of triolein. The experimental conditions were the same as those mentioned earlier. Similarly, a combination of equimole quantities of unsaturated C18 FA (LA + CLA + GLA + ALA) at C18 FA to triolein ratios ranging from 1:1 to 3:1 was prepared. The enzyme amount, reaction temperature, and incubation time were 4%, 45 ± 2 °C, and 24 h, respectively.

A similar experiment was carried out using a mixture of equimole amounts of n-3 FA (ALA + EPA + DHA + DPA) or n-6 FA (LA + GLA + AA). This was to examine and compare their reactivity in the acidolysis reaction.

Indeed, the catalyst was aliquoted on a weight basis, not on activity basis, and that different results may have been obtained had uniform amounts of activity, not masses of catalyst, been used.

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 a phenolphthalein indicator) until the color of the solution turned pink. 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 for separation, and the lower aqueous layer was discarded. The hexane layer was then passed through a bed of anhydrous sodium sulfate 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 fraction (5–10 mg) was transferred to a special transmethylation vial.

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, freshly prepared 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 time 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.

Analysis of FAMEs by Gas Chromatography

The FAMEs were analysed using a Hewlett Packard 5890 Series II gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a Supelcowax-10 column (30-m length, 0.25-mm diameter, 0.25 μm film thickness; Supelco Canada Ltd., Oakville, ON, USA). The oven temperature was first set at 220 °C for 10.25 min and then raised to 240 °C at 30 °C/min and held there for 15 min. The injector and detector (FID) temperatures were both set at 250 °C split ratio was 1:167. Ultra high purity (UHP) helium was used as a carrier gas at a flow rate of 1.2 mL/min. The data were treated using a Hewlett Packard 3365 Series II Chem Station Software (Agilent, Palo Alto, CA, USA). The FAMEs were identified by comparing their retention times with those of authentic standard mixture GLC-461 from Nu-Check (Elysian, MN, USA), and the results were presented as weight percentages.

Results and Discussion

Acidolysis of Triolein (tri C18:1) and C18 FA

Table 1 shows the degree of incorporation of C18 FA, namely SA, LA, CLA, ALA, and GLA into triolein. LA was more easily incorporated into triolein than CLA using the selected enzymes. Although, CLA and LA have the same carbon and double bond numbers, the different positions of double bonds and existing differences in their geometrical configurations result in their different chemical, physical, and biological properties [1].

Table 1 Effect of different lipases on incorporation of (wt %) C18 fatty acids into triolein

Enzyme source	SA	LA	CLA	ALA	GLA
Candida antarctica	14.6 ± 1.00	33.8 ± 3.70	11.9 ± 0.00	19.2 ± 0.25	12.9 ± 0.41
Rhizomucor miehei	31.8 ± 2.40	36.4 ± 1.75	17.3 ± 2.65	41.6 ± 2.85	25.4 ± 0.55
Pseudomonas sp.	31.8 ± 0.20	40.2 ± 1.75	18.9 ± 1.35	27.2 ± 1.75	19.2 ± 0.75
Candida rugosa	15.9 ± 0.95	21.4 ± 2.6	14.9 ± 1.35	8.39 ± 0.11	8.19 ± 0.39
Aspergillus niger	8.64 ± 0.35	23.6 ± 2.08	5.03 ± 0.62	9.83 ± 3.07	8.65 ± 0.15

The reaction mixture contains triolein (100 mg), fatty acid at mole ratio 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 water bath at 250 rpm

LA linoleic acid; *CLA* conjugated linoleic acid; *ALA* α-linolenic acid; *GLA* and γ-linolenic acid

Table 2 Effect of different lipases on incorporation of (wt%) C20 fatty acids into triolein

Enzyme source	AA	EPA
Candida antarctica	15.5 ± 0.90	44.5 ± 3.35
Rhizomucor miehei	34.1 ± 0.15	47.5 ± 0.10
Pseudomonas sp.	20.3 ± 2.20	59.3 ± 0.65
Candida rugosa	10.4 ± 1.23	56.9 ± 1.00
Aspergillus niger	11.2 ± 0.70	52.0 ± 0.70

The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein 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 water bath at 250 rpm

AA arachidonic acid; *EPA* eicosapentaenoic acid

The acidolysis of triolein (OOO) with linoleic (LA) and conjugated linoleic (CLA) acids can be written into the following equations for the initial reactions (the initial rate of an enzyme reaction is the rate at the earliest time that the reaction can be measured after mixing the reactants), respectively:

$$\mathrm{LA} + \mathrm{OOO} \quad \rightleftharpoons \quad \mathrm{LAOO} + \mathrm{O} \tag{1}$$

$$\mathrm{CLA} + \mathrm{OOO} \quad \rightleftharpoons \quad \mathrm{CLAOO} + \mathrm{O} \tag{2}$$

ΔG can be calculated at a particular instant in time [13]. Therefore, at any instance in time:

$$\Delta G\ \mathrm{LA} \rightleftharpoons \mathrm{GLAOO} + \mathrm{G\,O} - \mathrm{G\,OOO} - \mathrm{G\,LA} \tag{3}$$

$$\Delta G\,\mathrm{CLA} \rightleftharpoons \mathrm{G\,CLAOO} + \mathrm{G\,O} - \mathrm{G\,OO} - \mathrm{G\,CLA} \tag{4}$$

Where ΔG LA and ΔG CLA are free energy changes for the acidolysis of LA and CLA with triolein, respectively. *G* LAOO, *G* OOO, *G* O, *G* LA, *G* LA, and *G* CLA are the free energies of LAOO, OOO, O, LA, CLAOO, and CLA, respectively. March [14] and Barrow [15] described the relationship between the chemical structure and the free energy; the more extended shape of CLA, due to the conjugation of its double bonds, results in less stability of CLAOO compared to LAOO. Therefore, *G* LAOO is less than *G* CLAOO. The magnitude and sign of the free energy determine the direction in which the reaction proceeds. Reactions will always proceed in a direction that tends to bring the ΔG of the components on one side of the equation equal to those on the other side. When the free energies on both sides of the reaction are equal, equilibrium is reached [13]. The lower ΔG for a reaction, the higher will be the completion of that reaction [1, 13]. Since *G* LAOO is less than *G* CLAOO, the reaction between LA and triolein occurs more favorably than that between CLA and triolein. The results reported in this study agree with those of Yang et al. [1] who reported LA incorporation of up to 50 mol% into tristearin, whereas CLA incorporation was only 28 mol% in the same acidolysis reaction catalyzed by Lipozyme RM IM from *Rhizomucor miehei*. Furthermore, these authors suggested that LA was more reactive than CLA, probably due to the rigid structure of the latter because of the conjugation of double bonds. The rigidity and hindrance of CLA could produce obstacles to the access of CLA to the active site of a lipase, and hence lead to its low incorporation into triolein. The results presented in this study demonstrated that incorporation of CLA into the glycerol backbone of tri C18:1 was more difficult than that of LA, lending further support to the finding of Yang et al. [1].

Acidolysis of Triolein (tri C18:1) and C20 FA

Table 2 shows the effect of different lipases on percent incorporation of C20 (AA and EPA) fatty acids into triolein. Among lipases tested, PS-30 lipase from *Pseudomonas* sp. catalyzed the highest incorporation of EPA (59.3%) into triolein, whereas Lipozyme RM IM from *Rhizomucor miehei* catalyzed the highest incorporation of AA (34.1%) into triolein whereas the remaining enzymes were almost active as *Pseudomonas* sp. lipase. EPA from the n-3 family was more easily incorporated into triolein than AA from the n-6 family for all lipases tested. The more double bonds the chain has in the *cis* configuration, the more bent the molecule will be. Since EPA has five *cis* double bonds, it

Table 3 Effect of different lipases on incorporation (wt%) of C22 fatty acids into triolein

Enzyme source	DPA	DHA
Candida antarctica	6.25 ± 0.09	8.64 ± 1.96
Rhizomucor miehei	28.2 ± 1.45	22.4 ± 0.70
Pseudomonas sp.	11.8 ± 0.55	21.4 ± 0.12
Candida rugosa	5.72 ± 0.96	4.31 ± 0.09
Aspergillus niger	5.16 ± 0.36	4.16 ± 0.05

The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein 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 water bath at 250 rpm

DPA docosapentaenoic acid; *DHA* docosahexaenoic acid

becomes quite curved compared to AA and hence EPA has more steric hindrance than AA. Therefore, structural differences between AA and EPA related to the location of double bonds as well as specificity of the enzymes used might lead to variation in their reactivity. Nonetheless, EPA was more reactive than AA when the selected lipases were examined. The exact reason behind the higher reactivity of EPA compared to that of AA needs further investigation.

Acidolysis of Triolein (tri C18:1) and C22 FA

Table 3 shows variation in reactivity among the n-3 fatty acids (DPA and DHA) into triolein. The highest incorporation of DPA (28.2%) into tri C18:1 was catalyzed by Lipozyme RM IM from *Rhizomucor miehei*, while the lowest DPA incorporation (5.16%) into tri C18:1 was obtained with lipase from *Aspergillus niger*. Lipase from *Pseudomonas* sp. catalyzed the highest incorporation of DHA (21.4%) into triolein, whereas the lowest incorporation (4.16%) was observed for acidolysis reaction catalyzed by *Aspergillus niger* lipase. DPA was more reactive than DHA except for the reaction catalyzed by *Pseudomonas* sp., possibly due to the fact that DPA has one less double bond and hence a less bent structure than that of DHA as well as specificity of the enzymes used might lead to variation in their reactivity. Reasons behind higher incorporation of DHA into triolein using *Pseudomonas* sp. are unclear, but might be attributed to the selectivity of this enzyme toward DHA compared to DPA. Therefore, further research should be conducted to verify this assumption.

Acidolysis of Triolein and a Combination of Equimole Quantities of C18 FA

A mixture of equimole quantities of C18 FA (SA + LA + CLA + GLA + ALA) at triolein to C18 FA ratios of 1:1, 1:2, and 1:3 was used to investigate the effect of the substrate mole ratio on the incorporation of these FA into tri C18:1 (Table 4). As the mole ratio of C18 FA to triolein changed from 1 to 3, incorporation of C18 FA into triolein increased accordingly for the reaction catalyzed by Lipozyme RM IM from *Rhizomucor miehei* lipase. On the contrary, incorporation of C18 FA into triolein decreased when the mole ratio of triolein to C18 FA was altered from 1:1 to 1:3 when the remaining lipases were used. One possible explanation might be acidification of the enzyme layer by an excess of the free FA which might affect the three-dimensional structure of the enzyme and hence lower its catalytic activities. An alternative explanation for a decrease in the incorporation of C18 FA into triolein at high FA content could be related to the water activity. The solubility of water in the FA decreases as the chain length of the FA increases [4]. Therefore, as the amount of FA increases, the overall water activity is expected to decrease, and hence may reduce the degree of incorporation of these FA into triolein. These results are consistent with those obtained by Paez et al. [4] who reported that increased caprylic acid content leads to decreased water activity and thus decreased rate of incorporation of caprylic acid into triolein. Similarly, the extent of capric acid (10:0) incorporation (26.3%) into borage oil was much higher than that of EPA (10.2%) using Lipozyme IM 60 from *Rhizomucor miehei* [16]. It was demonstrated that FA chain length had a role in FA reactivity. In contrast, incorporation of stearic acid (18:0) was higher than that of caprylic acid (8:0) during transesterification of trilinolein and stearic or caprylic acids using 10% lipase from Lipozyme IM 60 from *Rhizomucor miehei*, at 55 °C over 32 h, and a mole ratio of trilinolein/stearic acid/caprylic acid 1:4:4. The chain length, relative polarity of stearic acid and/or selectivity of this lipase (*Rhizomucor miehei*) were suggested as being responsible for differences in the incorporation of these FA [17].

Lipases from *A. niger* and *C. rugosa* failed to allow CLA to participate in the acidolysis reaction, while the remaining FA, namely SA, LA, GLA, and ALA were only slightly incorporated into triolein. *Pseudomonas* sp. and *Rhizomucor miehei* lipases catalyzed better incorporation of C18 FA into tri C18:1 at each mole ratio examined. Thus, these two lipases might be considered as promising biocatalysts for acidolysis reactions of triolein with C18 FA. *Candida antarctica* lipase catalyzed slight incorporation of C18 FA into triolein except at a mole ratio of triolein to C18 fatty acids of 1 to 1.

In general, the order of C18 FA incorporation into triolein for the most effective biocatalysts (*Pseudomonas* sp. and *Rhizomucor miehei*) was obtained at a mole ratio of triolein to C18 FA of 1 to 3: SA ≥ LA > ALA ≥ GLA > CLA. These results are consistent with expectation because stearic acid is a saturated FA with a straight chain and thus less sterically hindered. LA with two double

Table 4 Effect of the mole ratio of substrates on incorporation (wt %) of C18 FA into triolein

Mole ratio (Triolein/FA)	Enzyme source	SA	LA	CLA	GLA	ALA
1:1	*Candida antarctica*	2.45 ± 0.03	2.50 ± 0.23	0.95 ± 0.06	2.43 ± 0.16	2.69 ± 0.32
	Rhizomucor miehei	4.42 ± 1.05	4.88 ± 0.44	2.34 ± 0.04	1.40 ± 0.37	4.88 ± 0.59
	Pseudomonas sp.	4.33 ± 0.35	6.09 ± 0.54	2.11 ± 0.26	4.68 ± 0.49	5.42 ± 0.44
	Candida rugosa	0.83 ± 0.26	0.91 ± 0.44	ND	0.97 ± 0.71	ND
	Aspergillus niger	1.34 ± 0.43	1.51 ± 0.61	ND	0.79 ± 0.43	1.74 ± 0.89
1:2	*Candida antarctica*	1.06 ± 0.30	0.73 ± 0.21	ND	0.62 ± 0.17	0.64 ± 0.24
	Rhizomucor miehei	5.17 ± 0.46	5.86 ± 0.23	1.54 ± 0.05	2.34 ± 0.28	5.02 ± 0.47
	Pseudomonas sp.	2.29 ± 0.07	3.09 ± 0.04	0.70 ± 0.04	2.64 ± 0.18	2.76 ± 0.08
	Candida rugosa	0.56 ± 0.14	0.39 ± 0.07	ND	0.23 ± 0.04	0.24 ± 0.07
	Aspergillus niger	0.44 ± 0.03	0.36 ± 0.03	ND	0.30 ± 0.03	0.24 ± 0.05
1:3	*Candida antarctica*	0.55 ± 0.08	0.62 ± 0.10	ND	0.60 ± 0.11	0.51 ± 0.09
	Rhizomucor miehei	8.65 ± 0.37	6.75 ± 0.14	3.12 ± 0.09	2.27 ± 0.03	6.29 ± 0.05
	Pseudomonas sp.	1.13 ± 0.01	0.92 ± 0.01	0.35 ± 0.02	1.07 ± 0.01	0.73 ± 0.01
	Candida rugosa	0.37 ± 0.10	0.46 ± 0.07	ND	0.32 ± 0.03	0.25 ± 0.04
	Aspergillus niger	0.39 ± 0.09	0.49 ± 0.03	ND	0.32 ± 0.04	0.29 ± 0.05

One mole of triolein was mixed with 0.2 mol of each of the five C18 FA (triolein to total C18 FA of 1:1). For a mole ratio of tri C18:1 to C18 FA of 1:2, 1 mol of triolein was mixed with 0.4 mol of each of the five C18 FA and for mole ratio of 1:3, this was 0.6 for each FA to 1 mol of triolein. The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein of 1:1, 2:1, and 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 water bath at 250 rpm

SA stearic acid; *LA* linoleic acid; *CLA* conjugated linoleic acid; *ALA* α-linolenic acid; *GLA* γ-linolenic acid; *FA* fatty acids; *ND* not detected

bonds causes the chain to bend which would then produce hindrance to the access of LA to the active site of the lipase. 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 these two molecules (ALA or GLA) related to the location of double bonds as well as specificity of the lipases examined might lead to variation in their reactivity. Although these structural differences might lead to variations in the incorporation of ALA and GLA into triolein, further work needs to be carried out to further shed light into these findings.

Acidolysis of Triolein and a Combination of Equimole Quantities of n-3 FA

The effect of the mole ratio of substrates on the incorporation of a mixture of equimole amounts of n-3 FA, namely ALA, EPA, DPA, and DHA, into tri C18:1 is shown in Table 5. When the mole ratio of triolein to n-3 FA increased from 1:1 to 1:3, incorporation of n-3 FA increased accordingly. EPA was more reactive than ALA, DPA, and DHA during acidolysis using the enzymes tested. *Aspergillus niger* and *Candida rugosa* lipases catalyzed slight incorporation (almost 1%) of ALA, DPA, and DHA into triolein, possibly due to the experimental conditions employed in this study which might not be adequate for these two enzymes. Thus, further studies should be conducted to verify the optimum conditions for each enzyme examined, perhaps using response surface methodology. In general, thelipase from *Rhizomucor miehei* might be considered the most active catalyst in the acidolysis reactions of triolein and a mixture of equimole amounts of n-3 FA. DPA was more reactive than DHA except for the acidolysis reaction catalyzed by *Pseudomonas* sp. DPA and DHA have the same chain length and both belong to the n-3 family, but DPA has one less double bond than DHA and hence less steric hindrance. In general, incorporation of n-3 FA into triolein was in the order of EPA > ALA > DPA > DHA. Theoretically, ALA incorporation into triolein is expected to be higher than that of EPA when considering factors such as chain length, number of double bonds, and steric hindrance. These differences (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 18:1 compared to EPA.

Table 5 Effect of mole ratio of substrates on incorporation (wt%) of n-3 fatty acids into triolein

Mole ratio (Triolein/FA)	Enzyme source	ALA	EPA	DPA	DHA
1:1	*Candida antarctica*	5.12 ± 0.02	4.23 ± 0.03	5.37 ± 0.11	4.86 ± 0.47
	Rhizomucor miehei	7.36 ± 0.41	7.61 ± 0.67	5.19 ± 0.49	2.83 ± 0.04
	Pseudomonas sp.	6.36 ± 0.86	8.88 ± 0.92	3.07 ± 0.51	3.86 ± 0.53
	Candida rugosa	2.55 ± 0.12	10.7 ± 1.22	1.18 ± 0.14	1.09 ± 0.16
	Aspergillus niger	1.26 ± 0.20	11.6 ± 2.22	1.16 ± 0.37	0.91 ± 0.32
1:2	*Candida antarctica*	5.24 ± 0.08	4.24 ± 0.69	4.39 ± 0.47	4.04 ± 0.39
	Rhizomucor miehei	10.0 ± 0.47	9.56 ± 0.94	8.73 ± 0.33	4.72 ± 0.17
	Pseudomonas sp.	6.57 ± 0.43	14.6 ± 1.50	4.16 ± 1.00	4.21 ± 0.64
	Candida rugosa	1.54 ± 0.51	16.0 ± 0.70	1.51 ± 0.38	1.34 ± 0.38
	Aspergillus niger	0.95 ± 0.02	17.8 ± 1.95	1.06 ± 0.03	0.82 ± 0.01
1:3	*Candida antarctica*	8.52 ± 0.64	8.70 ± 0.14	8.08 ± 0.62	6.57 ± 0.30
	Rhizomucor miehei	11.5 ± 0.80	14.5 ± 4.25	9.77 ± 0.19	3.95 ± 0.21
	Pseudomonas sp.	10.3 ± 0.37	22.2 ± 0.05	4.68 ± 0.09	5.06 ± 0.28
	Candida rugosa	1.81 ± 0.17	25.7 ± 0.05	1.74 ± 0.03	1.24 ± 0.19
	Aspergillus niger	1.28 ± 0.12	25.9 ± 1.75	1.47 ± 0.12	0.98 ± 0.09

One mole of triolein was mixed with 0.25 mol of each of the four n-3 FA (triolein to total n-3 FA of 1:1). For a mole ratio of tri C18:1 to n-3 FA of 1:2, 1 mol of triolein was mixed with 0.5 mol of each of the four n-3 FA and for mole ratio of 1:3, this was 0.75 for each FA to one mole of triolein. The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein of 1:1, 2:1, and 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 water bath at 250 rpm

ALA α-linolenic acid; *EPA* eicosapentaenoic acid; *DPA* docosapentaenoic acid; *DHA* docosahexaenoic acid

However, at this stage, we are unable to offer any explanation for the observed trend.

Acidolysis of Triolein and a Combination of Equimole Quantities of n-6 FA

Table 6 shows the effect of mole ratio of substrates on the incorporation of a mixture of equimole amounts of n-6 FA into triolein. As the number of moles of a mixture of n-6 FA (LA + GLA + AA) was changed from 1 to 3, incorporation of n-6 FA increased accordingly except for the reaction assisted by lipase from *Candida antarctica.* LA was more reactive than GLA or AA during acidolysis using enzymes tested in this study. This result might be attributed to the existing structural differences related to the number of double bonds in the fatty acids examined, together with specificity of the lipases examined for different FA. In general, Lipozyme RM IM from *Rhizomucor miehei* catalyzed the highest incorporation of LA and AA into triolein at each mole ratio of triolein to a mixture of n-6 FA (LA, GLA, and AA) examined. Meanwhile, Lipases from *Pseudomonas* sp. assisted the highest incorporation of LA (15.1%) and GLA (13.7%) into triolein at a mole ratio of triolein to a mixture of n-6 FA of 1:3.

Incorporation of n-6 FA into triolein for the reactions catalyzed by *Pseudomonas* sp. at all mole ratios employed was in the order of LA > GLA > AA. However, the order of incorporation of these FA into tri C18:1 using the remaining lipases (*Candida antarctica*, *Rhizomucor miehei*, *Aspergillus niger*, and *Candida rugosa*) was: LA > AA > GLA. In both groups, LA was the most reactive FA among n-6 FA. The reasons for the higher incorporation of AA than GLA are not clear, but GLA has a shorter chain length than AA and fewer double bonds (3 vs. 4).

While lipases are derived from different sources, such as bacteria, fungi, and yeasts, they all tend to have similar three-dimensional structures. Recent research, and success in crystallizing proteins, has led to the determination of the structures of many lipases by X-ray crystallography, including lipase from *Rhizomucor miehei* [19, 20], lipase B from *Candida antarctica* [21] and *Candida rugosa* lipase [22]. In general, lipases have a polypeptide chain folded into two domains, the C-terminal domain and the N-terminal domain. The latter contains the active site with a hydrophobic tunnel from the catalytic serine to the surface that can accommodate a long fatty acid chain. A general structural characteristic among lipases is the α/β structure; alternating β-pleated sheets and α-helices [19, 21]. Peptide sequence of lipases from a number of prokaryotic and eukaryotic organisms are known [19]. It has been revealed that they all contain a Gly–X–Ser–X–Gly/Ala sequence, where X can be any amino acid [19]. The catalytic site of lipases consist of serine, aspartate/glutamate and histidine, which is in the reverse order compared to that in serine proteases. All lipases characterized to date contain a serine

Table 6 Effect of mole ratio of substrates on incorporation (wt %) of n-6 fatty acids into triolein

Mole ratio (Triolein/FA)	Enzyme source	LA	GLA	AA
1:1	*Candida antarctica*	2.68 ± 0.41	1.99 ± 0.13	2.81 ± 0.01
	Rhizomucor miehei	7.12 ± 1.09	4.09 ± 0.66	6.54 ± 0.75
	Pseudomonas sp.	6.35 ± 1.35	4.84 ± 1.04	3.12 ± 1.12
	Candida rugosa	3.85 ± 0.14	1.31 ± 0.18	1.52 ± 0.15
	Aspergillus niger	2.39 ± 0.30	1.36 ± 0.05	1.87 ± 0.09
1:2	*Candida antarctica*	2.49 ± 0.03	1.61 ± 0.25	2.54 ± 0.02
	Rhizomucor miehei	9.76 ± 1.45	4.83 ± 0.58	8.51 ± 0.97
	Pseudomonas sp.	9.45 ± 0.49	7.58 ± 0.80	3.86 ± 0.08
	Candida rugosa	5.87 ± 1.59	1.64 ± 0.08	1.86 ± 0.08
	Aspergillus niger	3.62 ± 0.07	1.39 ± 0.02	2.22 ± 0.31
1:3	*Candida antarctica*	1.42 ± 0.10	0.77 ± 0.02	1.36 ± 0.13
	Rhizomucor miehei	14.2 ± 0.15	4.77 ± 0.12	13.2 ± 0.50
	Pseudomonas sp.	15.1 ± 0.25	13.7 ± 0.40	6.23 ± 0.27
	Candida rugosa	8.96 ± 0.46	2.03 ± 0.36	1.67 ± 0.18
	Aspergillus niger	2.83 ± 0.40	1.14 ± 0.15	1.67 ± 0.09

One mole of triolein was mixed with 0.33 mol of each of the three n-6 FA (triolein to total n-6 FA of 1:1). For a mole ratio of tri C18:1 to n-6 FA of 1:2, 1 mol of triolein was mixed with 0.66 mol of each of the three n-6 FA and for mole ratio of 1:3, this was 1.0 for each FA to 1 mol of triolein. The reaction mixture contains triolein (100 mg), fatty acid at mole ratio of an acid to triolein of 1:1, 2:1, and 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 water bath at 250 rpm

LA linoleic acid; *GLA* γ-linolenic acid; *AA* arachidonic acid

as their nucleophilic residue [21]. The active site (Ser–His–Asp) is buried under the surface loops and folded into the triad [19]. Klein et al. [23] reported the crystal structure of *Rhizopus delemar* (*Rd*) lipase (now known as *Rhizopus oryzae*). The molecular structure of the enzyme consists of a single globular domain containing mainly parallel β-sheets with conserved α-helices packed on either side. The active center consists of a triad of Ser–His–Asp. Furthermore, the FA specificity of the *Rhizopus delemar* was enhanced significantly and depended on the pH of the substrate emulsion [23]. For example, at neutral pH, this lipase displayed strong preference (>10-fold) for hydrolysis of FA with chain length ranging from C_8 to C_{14} but no hydrolysis for C_4 to C_6 fatty acids and little activity manifested toward FA with 16 or more carbon atoms [23].

The oxidative stability of the modified triolein in comparison with the original oil, as indicated in both conjugated dienes (CD) and the 2-thiobarbituric acid reactive substances (TBARS) values (results not shown), showed that the unmodified oil remained unchanged during storage for 72 h. The modified oils with n-3 FA were more susceptible to oxidation than those modified with n-6 FA, when considering both CD and TBARS values. We have previously demonstrated that both degree of unsaturation as well as removal of endogenous tocopherols, at least in part by their esterification, are the main causes for compromised stability of structured lipids [18].

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