Enzymatic Modification of Trilinolein: Incorporation of n-3 Polyunsaturated Fatty Acids

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ABSTRACT: Two immobilized lipases, IM60 from *Mucor miehei* and SP435 from *Candida antarctica*, were used as biocatalysts for the modification of trilinolein with n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by using their ethyl esters as acyl donors (EEPA and EDHA, respectively). Transesterification (ester–ester interchange) reactions were carried out in organic solvent. The products were analyzed according to their equivalent carbon number and polarity by reverse-phase high-performance liquid chromatography, and the fatty acid profiles were determined by gas–liquid chromatography. Modified triacylglycerol products contained 1 or 2 molecules of n-3 PUFA. With EEPA as the acyl donor, the total EPA product yields with IM60 and SP435 as biocatalysts were 79.6 and 81.4%, respectively. However, with EDHA as the acyl donor and IM60 and SP435 as biocatalysts, the total DHA product yields were 70.5 and 79.7%, respectively. Effects of reaction parameters, such as type of solvent, enzyme load, time course, and molar ratio of substrates on the n-3 PUFA incorporation, were followed with SP435 as the biocatalyst. High yields were obtained, even in the absence of organic solvent. These lipids do hold promise for specialty nutrition and other therapeutic uses.
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KEY WORDS: *Candida antarctica*, ester–ester interchange, lipases, *Mucor miehei*, n-3 PUFA, organic solvent, transesterification, trilinolein.

Consumption of fish or fish oil that are rich in n-3 polyunsaturated fatty acids (PUFA) has been linked to improved cardiovascular and immune functions (1). The essential n-6 PUFA (for example, linoleic acid, 18:2n-6) are required in the diet to maintain certain functions. However, excess or imbalance of n-6 PUFA can lead to arachidonic acid production and may favor thrombotic effects that are mediated through thromboxane A_2 (TXA_2). TXA_2 is a potent vasoconstrictor and promoter of platelet aggregation. However, ingestion of n-3 PUFA shifts the physiological balance in the direction of vasodilation and antiaggregation. The right balance of n-3/n-6 ratio is required for combating or ameliorating some of these acute episodic events. n-3 PUFA are competitors of the arachidonic acid cascade and exert their action by modulating eicosanoid production (2,3).

The use of lipases as biocatalysts to modify trilinolein composition by incorporating eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) or both is viable and may help control the level of linoleic acid (18:2n-6) and optimize the n-3 and n-6 fatty acid ratio for nutritional benefits or clinical intervention studies. Recently, we reported the incorporation of n-3 PUFA into vegetable oils (4) and melon seed oil (5) with immobilized lipases—immobilized 1,3-specific lipase (IM60), and immobilized nonspecific lipase SP435—as biocatalysts. The production of vegetable oils that contain n-3 PUFA also could be achieved by transesterification of the free acid form of n-3 PUFA concentrate (5,6) or its methyl and ethyl esters with vegetable oils (4,7).

In the present study, two immobilized lipases, IM60 from *Mucor miehei* and SP435 from *Candida antarctica*, were used as biocatalysts to restructure the trilinolein fatty acid composition by incorporating EPA, DHA, or EPA–DHA into the trilinolein molecule. The effects of molar ratio of the substrates, added water, acyl donor type, solvents, enzyme load, and time course on n-3 PUFA incorporation also were studied.

MATERIALS AND METHODS

Materials. EPA and DHA ethyl esters (97 and 96% pure, respectively) were provided by the United States Department of Commerce, National Marine Fisheries Service (Charleston, SC). IM60 and SP435 were provided by Novo Nordisk Bioindustrial, Inc. (Danbury, CT). Trilinolein (99% pure) was purchased from Sigma Chemical Company (St. Louis, MO). All organic solvents were from Fisher Scientific (Norcross, GA).

Enzymatic modification reaction. For general synthesis of modified trilinolein, 100 mg of trilinolein was mixed with the eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) ethyl ester at a molar ratio of triacylglycerol (TAG)/n-3 PUFA of 1:2; that is, 75.2 mg for EPA ethyl ester (EEPA) and 81.0 mg for DHA ethyl ester (EDHA), and immobilized lipase (10% combined weight of substrates) in 3 mL hexane. The mixture was incubated in an orbital shaking water bath at 55°C for 24 h at 200 rpm. Molecular sieves 4Å were added after 2 h. All reactions were in duplicate.

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Analysis of product. The enzyme was removed by passing the reaction medium through an anhydrous sodium sulfate column. The column was subsequently washed with hexane to elute the reaction product. A 50-μL aliquot of the reaction product was analyzed by thin-layer chromatography (TLC) on silica gel 60 plates, developed with petroleum ether/ethyl ether/acetic acid (90:10:1, vol/vol/vol). The bands were visualized under ultraviolet light after spraying with 0.2% 2,7-dichlorofluorescein in methanol. The bands that corresponded to TAG were scraped from the TLC plate and methylated in 3 mL 6% HCl in methanol at 70–80°C for 2 h (4). The fatty acid methyl esters (FAME) were extracted twice with 2 mL hexane, dried over sodium sulfate, and concentrated under nitrogen. The gas chromatograph was an HP 5890 Series II (Hewlett-Packard, Avondale, PA) equipped with a DB-225 fused-silica capillary column 30 m × 0.25 mm i.d. (J&W Scientific, Folsom, CA) and an FID detector, and operated in a splitless mode. The injector and detector temperatures were 250 and 260°C, respectively. The column temperature was held at 205°C for 20 min, then programmed to 215°C at 20°C/min. Helium was the carrier gas, and the total gas flow rate was 23 mL/min. The relative content of FAME as mol% was quantitated by an on-line computer with 17:0 as internal standard.

HPLC analysis. To each aliquot of 85 μL reaction product was added 15 μL of internal standard solution (tricaprin, 100 mg/mL), which was then analyzed by high-performance liquid chromatography (HPLC). HPLC was carried out with a Hewlett-Packard Model 1090 Win liquid chromatograph equipped with a Vectra 486 computer and a Sedex 45 evaporative light-scattering mass detector (ELSD) (Richard Scientific, Novato, CA). The ELSD was set to 40°C, a nebulizer gas pressure of 2.1, and a gain of 5 for the nonaqueous reverse-phase system. A Hewlett-Packard 35900 digital A/D analog interface connected the mass detector electronically to the Vectra 486 computer. Triacylglycerol mixtures were analyzed by nonaqueous reverse-phase HPLC on a Beckman/Altex (San Ramon, CA) Ultrasphere ODS 5 μm (4.6 mm × 25 cm) column. The analysis procedure was based on our earlier method (8). Briefly, separations were obtained with acetonitrile (solvent A) and acetone (solvent B) as eluent, using the following gradient profile: initial condition 50:50 (A/B), hold 4 min at a flow rate of 1.8 mL/min; 5:95 (A/B), hold 8.5 min at a flow rate of 2.0 mL/min; return to original conditions.

RESULTS AND DISCUSSION

Table 1 gives the percent incorporation of n-3 PUFA (20:5n-3, EPA and 22:6n-3, DHA) into the trilinolein molecule when catalyzed by two different immobilized lipases, IM60 and SP435, with ethyl esters of EPA and DHA as the acyl donors. The reverse-phase HPLC separations were based on total carbon number and polarity. The order of elution was: C-61 and C-59 for EPA-containing TAG and C-65 and C-61 for DHA-containing TAG species. In either case, the unreacted trilinolein, C-57, eluted last. SP435 gave higher disubstitution (30.4 and 29.6%) of EPA and DHA than IM60, which gave 27.7 and 13.2%, respectively. The amounts of monosubstituted products were about the same (50.1–56.8%) in both products. Overall, SP435 gave the best total yield (sum of mono- and disubstituted products) of EPA (81.4%) and DHA-containing TAG (79.7%). Therefore, this enzyme was chosen for subsequent experiments. The fatty acid profile of the modified trilinolein is shown in Table 2. With EEPA as the acyl donor, approximately one-third (34%) of the fatty acids was 20:5n-3 and the n-3/n-6 ratio was 0.5. With EDHA as acyl

TABLE 1
Mol% Incorporation of n-3 PUFA into Trilinolein as Determined by HPLC

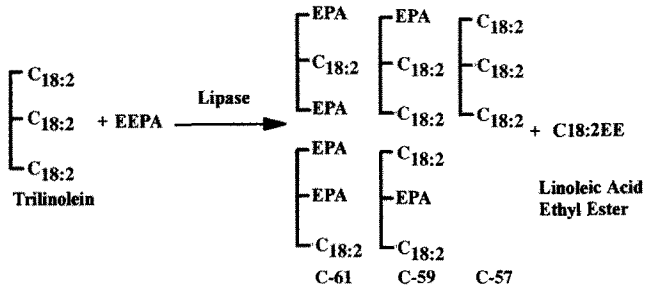
Enzyme	Triacylglycerol total carbon number			Total triacylglycerol containing n-3 PUFA
	C-61[b]	C-59[c]	C-57[d]	EPA[e]
IM60[a]	27.7	51.9	20.4	79.6
SP435[a]	30.4	51.0	18.6	81.4
	C-65[f]	C-61[g]	C-57	DHA[h]
IM60	13.2	56.8	30.0	70.0
SP435	29.6	50.1	20.3	79.7

[a]Novo Nordisk Bioindustrial, Inc. (Danbury, CT).
[b]Triacylglycerol containing two EPA molecules (dieicosapentaenoyllinolein); C-61 = triacylglycerol total carbon number.
[c]Triacylglycerol containing one EPA molecule (monoeicosapentaenoyllinolein), C-59 = total carbon number.
[d]Unreacted trilinolein; C-57 = total carbon number.
[e]EPA = eicosapentaenoic acid.
[f]Triacylglycerol containing two DHA molecules (didocosahexaenoyllinolein); C-65 = total carbon number.
[g]Triacylglycerol containing one DHA molecule (monodocosahexaenoyllinolein); C-61 = total carbon number.
[h]DHA = docosahexaenoic acid; PUFA, polyunsaturated fatty acids; HPLC, high-performance liquid chromatography.

TABLE 2
Fatty Acid Composition of Enzymatically Modified Trilinolein

Acyl donor/fatty acid	Mol%	
EEPA as acyl donor[a]	IM60	SP435
18:2n-6	65.1	65.5
20:5n-3	34.9	34.5
n-3/n-6 ratio	0.5	0.5
EDHA as acyl donor[a]		
18:2n-6	81.0	73.2
22:6n-3	19.0	26.8
n-3/n-6 ratio	0.2	0.4
EEPA and EDHA as acyl donors[b]		
18:2n-6		64.5
20:5n-3		20.2
22:6n-3		15.3
n-3/n-6 ratio		0.6

[a]Molar ratio of acyl donor/trilinolein = 2:1. All reactions were in hexane with no added water. The products contain unreacted trilinolein and different levels of substitution; United States Department of Commerce, National Marine Fisheries Service (Charleston, SC). See Table 1 for other company source.
[b]Molar ratio of EEPA/EDHA/trilinolein = 1:1:1; EEPA = ethyl ester of eicosapentaenoic acid; EDHA = ethyl ester of docosahexaenoic acid.



SCHEME 1

donor, IM60 gave a modified lipid with a lower content of 22:6n-3 (19%) compared to SP435-catalyzed product (26.8%). The n-3/n-6 ratio changed from 0.2 to 0.4 when SP435 was the biocatalyst, indicating again that this was the better enzyme for the acyl exchange reaction. The fatty acid profile of these modified lipids, which were scraped as the triacylglycerol band on the TLC plates, also contains unreacted trilinolein (C-57). Overall, HPLC and gas–liquid chromatography (GLC) data showed a high degree of agreement. We wanted to synthesize a modified lipid product that contained both 20:5n-3 and 22:6n-3. As shown in Table 2, the total n-3 PUFA was about 35.5%, which agrees with the incorporation obtained with SP435 as biocatalyst and EEPA alone as the acyl donor (34.5%). However, the n-3/n-6 ratio increased to 0.6. It is therefore possible to synthesize modified lipids with defined composition and desired n-3/n-6 ratio for nutrition or therapeutics.

Scheme 1 shows the reaction and possible products of the lipase-catalyzed ester–ester interchange (transesterification) reaction between trilinolein and EEPA. The total carbon number of the triacylglycerol species are also shown. Figure 1 shows the time course of SP435 lipase-catalyzed modification of trilinolein by incorporation of n-3 PUFA. Total EPA-containing TAG species (Fig. 1A) increased to 66.5% in 12 h and essentially reached equilibrium (71.5%) in 24 h, after which further incorporation was minimal. The same trend was observed for both monoeicosapentaenoyllinolein (C-59) and dieicosapentaenoyllinolein (C-61). Similar results were obtained when SP435 was used to incorporate EPA into soybean oil (4). DHA-containing TAG (Fig. 1B), however, reached equilibrium (71.3%) in 12 h, which was faster than the EPA-containing TAG species. Increasing the incubation time from 12 to 24 h in the case of DHA-containing products only slightly increased the amount of DHA incorporated. There was a slight increase in total DHA incorporation after 36 h as opposed to the slight decrease observed with EPA incorporation. The time course helps us follow product formation and reactant decrease or disappearance with time and gives an indication on when to stop the reaction, depending on the desired product.

Figure 2 shows the total incorporation of EPA and DHA into trilinolein at various n-3 PUFA to trilinolein molar ratios. The enzyme amount was kept constant at 10% by weight of

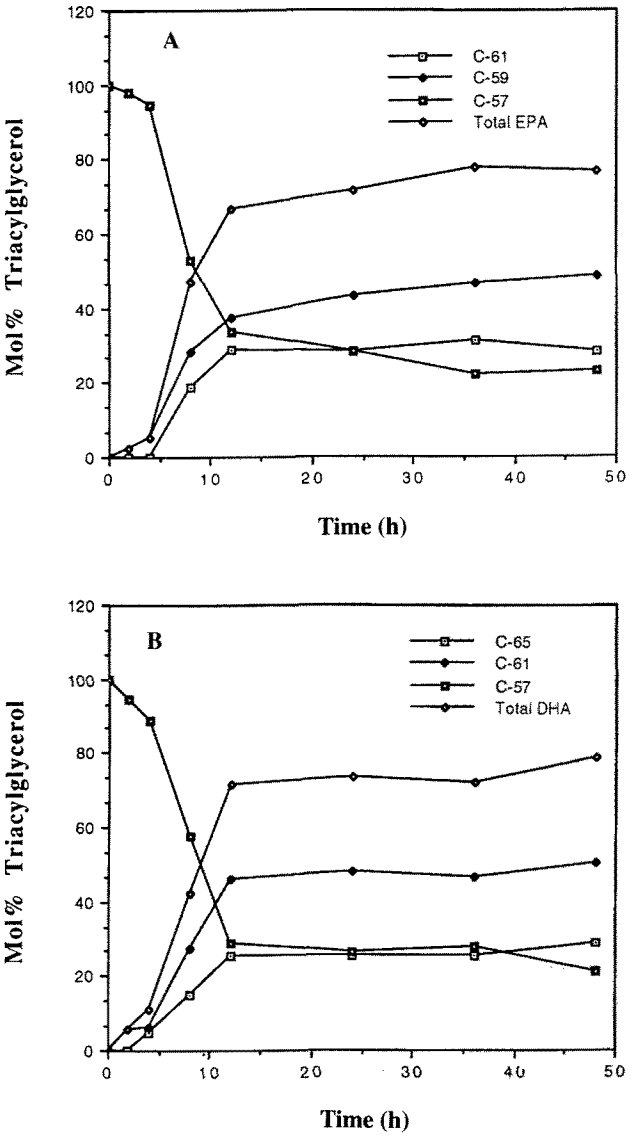


FIG. 1. Time course of SP435 (Novo Nordisk Bioindustrial, Inc., Danbury CT) lipase-catalyzed modification of trilinolein by incorporation of n-3 polyunsaturated fatty acids as determined by high-performance liquid chromatography. A = eicosapentaenoic acid (EPA) and B = docosahexaenoic acid (DHA) incorporation. Molar ratio of n-3 polyunsaturated fatty acids/trilinolein = 1:2. One microliter of water was added during the incubation. Samples were analyzed at 0, 2, 4, 8, 12, 24, 36, and 48 h in duplicate. EE, ethyl ester; EEPA, eicosapentaenoic acid ethyl ester. See Tables 1 and 2 for legends.

total reactants. With EEPA and EDHA as the acyl donors, the n-3 PUFA–containing TAG species increased as the molar ratio increased. For EEPA, the incorporation did not increase beyond a molar ratio of EEPA/trilinolein of 4. However, for EDHA, the incorporation increased as the molar ratio increased up to 5. The steep part of the curve occurred when the molar ratios of n-3 PUFA to trilinolein increased from 1 to 2 (for example, for EEPA, the increase was from 45 to 72%). This confirms our earlier observations on the molar ratio studies with melon seed oil (5). Subsequent parameters were then

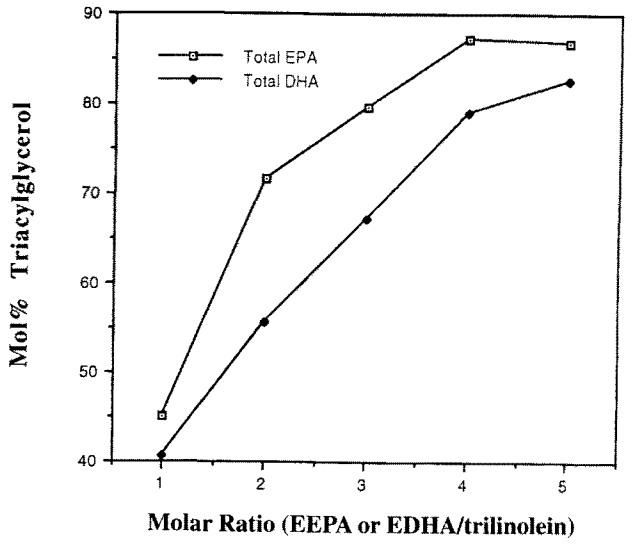


FIG. 2. Effect of EEPA or docosahexaenoic acid ethyl ester (EDHA) to trilinolein ratio on total n-3 polyunsaturated fatty acids incorporation with SP435 as biocatalyst (see Table 2 for legend). Water was not added, and the enzyme amount was 10% by weight of reactants. See Figure 1 for company source and other abbreviations.

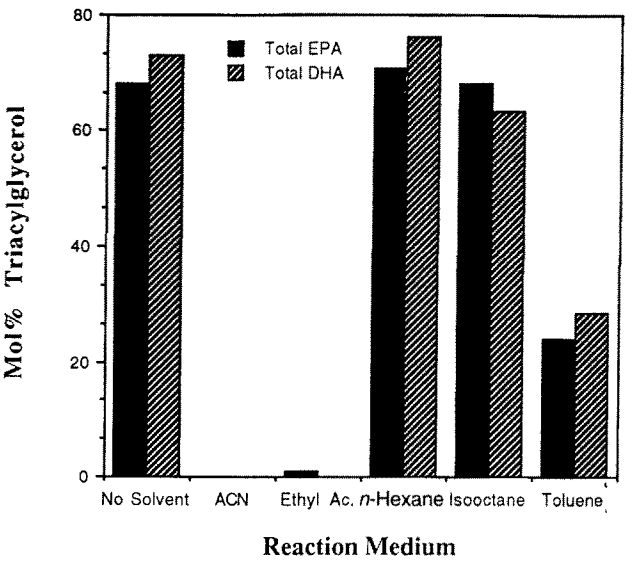


FIG. 4. Effect of different organic solvents as reaction media for the ester–ester interchange reaction catalyzed by SP435 lipase. The incubation included: No solvent; ACN = acetonitrile; Ethyl Ac. = ethyl acetate; *n*-hexane; isooctane; and toluene. For no solvent, molecular sieve was not added. Water was not added in this experiment. See Figure 1 for company source and other abbreviations.

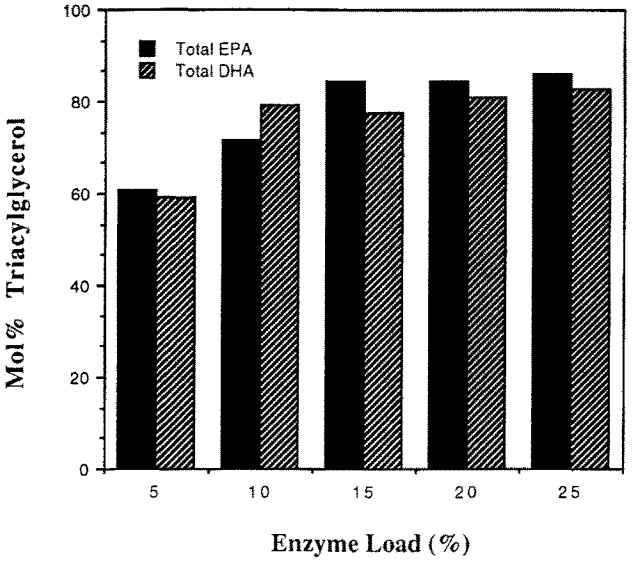


FIG. 3. Effect of enzyme load on the total n-3 polyunsaturated fatty acids (PUFA) incorporation into trilinolein with SP435 as biocatalyst. Amount of enzyme was based on wt% of reactants. Water was not added, and the molar ratio of n-3 PUFA to trilinolein = 1:3. See Figure 1 for company source and other abbreviations.

determined at an n-3 PUFA/trilinolein molar ratio of 2, or as otherwise stated. Figure 3 shows the effect of enzyme load or amount on the total incorporation of n-3 PUFA with SP435 as biocatalyst. At 5% by weight of enzyme, only about 60% incorporation was obtained. Beyond enzyme load of 10% by weight, there were no significant increases in the total EPA or DHA incorporated into the modified TAG species.Overall, more EPA was incorporated at the various enzyme loads tested except at 10 wt%. Depending on the desired yield, it seems that there was no obvious advantage of using more than 10% by weight enzyme, as recommended by the manufacturer, for batch reactions (9). Besides, because they are immobilized enzymes, they can be reused several times to cut cost (10,11).

Figure 4 shows the effect of different organic solvents and no solvent as the reaction medium for ester–ester interchange reaction catalyzed by SP435 lipase. Little or no acyl exchange was observed when ethyl acetate or acetonitrile was the reaction medium. Toluene gave up to 24 and 28.4% of EPA- and DHA-containing TAG species, respectively. The best solvents were *n*-hexane and isooctane, which gave total yields ranging from 63.2 to 76.2%. SP435 lipase also performed well when the reaction was carried out in the absence of any organic solvents, giving 68.2 and 72.9% total yields for EPA and DHA, respectively. Claon and Akoh (10) have demonstrated that this enzyme works well in the solvent-free synthesis of primary terpene acetates and poorly in acetonitrile.

We demonstrated that it is possible to modify the fatty acid profile of trilinolein by incorporating potentially beneficial n-3 PUFA into the TAG molecules. This reaction is possible in both organic solvents and in the absence of any solvent, which is more desirable from the food point of view. The products can be tailored to contain different n-3/n-6 PUFA ratios, which, in turn, may be used to target certain disease conditions while supplying the needed essential fatty acid as well as the n-3 PUFA that are antagonists of the arachidonic acid (20:4n-6) pathway.

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