Lipase-Catalyzed Incorporation of n-3 Polyunsaturated Fatty Acids into Vegetable Oils

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The ability of immobilized lipases IM60 from *Mucor miehei* and SP435 from *Candida antarctica* to modify the fatty acid composition of selected vegetable oils by incorporation of n-3 polyunsaturated fatty acids into the vegetable oils was studied. The transesterification was carried out in organic solvent with free acid and ethyl esters of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as acyl donors. With free EPA as acyl donor, IM60 gave higher incorporation of EPA than SP435. However, when ethyl esters of EPA and DHA were the acyl donors, SP435 gave higher incorporation of EPA and DHA than IM60. When IM60 and free acid were used, the addition of 5 μL water increased EPA incorporation into soybean oil by 4.9%. With ethyl ester of EPA as acyl donor, addition of 2 μL water increased EPA incorporation by 3.9%. For SP435, addition of water up to 2 μL resulted in increased EPA incorporation, but the incorporation declined when the added water exceeded this amount. The addition of water increased the EPA incorporation into Trisun 90 after 24 h reaction but not the reaction rate at early stages of the reaction.

KEY WORDS: *Candida antarctica*, lipases, *Mucor miehei*, n-3 PUFA, organic solvent, transesterification, vegetable oil.

Consumption of fish or fish oil rich in n-3 polyunsaturated fatty acids (PUFA) has been linked to improved cardiovascular and immune functions and may mediate inflammatory events. Not everyone likes to eat fish, but most people use vegetable oils and fats in food preparation. Therefore, vegetable oil triglycerides are considered as alternative molecules for carrying n-3 PUFA. Lipases are currently being used as biocatalysts for the hydrolysis, synthesis and modification of fats and oils (1-4). PUFA are easily oxidized at high temperature. Lipases are suitable to catalyze the incorporation of n-3 PUFA into vegetable oils because of the mild reaction conditions. Recently, Li and Ward (2) reported production of vegetable oils containing n-3 PUFA by using IM60 from *Mucor miehei* as biocatalyst. The free acid form of PUFA concentrate was transesterified with vegetable oils. Sridhar and Lakshminarayana (5) also reported the interesterification reaction between groundnut oil and n-3 PUFA concentrate methyl ester. In the present study, two immobilized lipases, IM60 from *M. miehei* and SP435 from *Candida antarctica*, were used as biocatalysts to modify the fatty acid composition of selected vegetable oils by incorporation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The effects of molar ratio of the substrates, time course, added water and the acyl donor type on the percent incorporation of n-3 PUFA were also studied.

EXPERIMENTAL PROCEDURES

Materials. Canola oil, peanut oil, soybean oil and hydrogenated soybean oil were obtained from Archer Daniels Midland Company (Decatur, IL). Trisun 80 and Trisun 90 were obtained from SVO Enterprises (Eastlake, OH). EPA 45 (45% EPA) was supplied by Callanish Ltd. (Scotland, United Kingdom). EPA and DHA ethyl ester (97 and 96% pure, respectively) were kindly provided by the United States Department of Commerce, National Fisheries Service (Charleston, SC). Immobilized 1,3-specific lipase IM60 and nonspecific lipase SP435 were kindly provided by Novo Nordisk Bioindustrial, Inc. (Danbury, CT). All organic solvents were obtained from Fisher Scientific (Norcross, GA).

Transesterification reaction. For general synthesis of modified vegetable oil, 100 mg vegetable oil was mixed with acyl donor at a molar ratio of vegetable oil to acyl donor of 1:2 (e.g., 68.3 mg for EPA, 73.6 mg for EPA ethyl ester and 79.5 mg for DHA ethyl ester)—and with immobilized lipase (10% combined weight of substrates) in 3 mL hexane. The mixture was incubated in an orbital shaking waterbath at 55°C for 24 h at 200 rpm. Molecular sieves 4Å were added after 2 h. All reactions were in duplicate.

Analysis of the products. The enzyme was filtered by passing through an anhydrous sodium sulfate column. An aliquot of 50 μL reaction product was applied to thin-layer chromatography plate and developed in petroleum ether/ethyl ether/acetic acid (90:10:1, vol/vol/vol) to isolate triacylglycerol (TAG). The TAG bands were visualized under ultraviolet (UV) radiation after spraying with 0.2% 2,7-dichlorofluorescein in methanol. The bands corresponding to TAG were scraped from the plate and methylated in 3 mL 6% HCl in methanol at 70–80°C for 2 h. The fatty acid methyl esters (FAME) were extracted with hexane twice, dried over sodium sulfate and concentrated under nitrogen. An HP 5890 Series II gas chromotograph (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 flame-ionization detector (FID), was used to analyze the samples. 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 used as the carrier gas at a total flow rate of 23 mL/min. The relative content of FAME as mol% was quantitated by an on-line computer with 17:0 as internal standard.

RESULTS AND DISCUSSION

Two lipases, IM60 and SP435, were investigated for their ability to incorporate n-3 PUFA into vegetable oils. The fatty acid composition of vegetable oils were remarkably changed after 24 h of incubation with enzyme and acyl donor (Tables 1 and 2). With EPA 45 free acid as the acyl donor, the lipase from *M. miehei* (IM60) gave higher incorporation of EPA (10.5%) than the *C. antarctica* lipase (SP435) (6.8%). However, when ethyl esters of EPA and DHA were the acyl donors, SP435 gave higher incorporation of EPA and DHA, respectively, than IM60. It appears that high levels of n-3 PUFA incorporation could be attained by using high-purity n-3 PUFA ethyl esters for both enzymes. The original concentration of EPA in ethyl ester

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TABLE 1

Fatty Acid Composition (in mol%[a]) of Soybean Oil Before and After Transesterification with Various Acyl Donors by *Mucor miehei* Lipase, IM60[b]

Fatty acids	Before transesterification	After transesterification with different acyl donor		
		EPA 45	EPA ethyl ester	DHA ethyl ester
14:0	0.2	0.9	0.6	0.7
14:1	0.1	1.4	1.1	1.3
16:0	11.6	11.8	9.6	12.1
16:1n-7	0.1	5.4	0.4	0.2
18:0	3.5	2.5	2.1	2.9
18:1n-9	21.9	19.1	15.5	19.0
18:2n-6	53.9	41.8	36.8	43.5
18:3n-6	0.3			
18:3n-3	8.4	5.6	5.1	
20:5n-3		10.5	29.2	
22:5n-3		0.4		
22:6n-3				14.6

[a]Mol% eicosapentaenoic acid (EPA) = (EPA mol/total fatty acid mol) × 100%. DHA, docosahexaenoic acid.
[b]Lipase, IM60 (Novo Nordisk Bioindustrial, Inc., Danbury, CT); EPA45 (Callanish Ltd., Scotland, United Kingdom); EPA and DHA ethyl ester (United States Department of Commerce, National Fisheries Service, Charleston, SC).

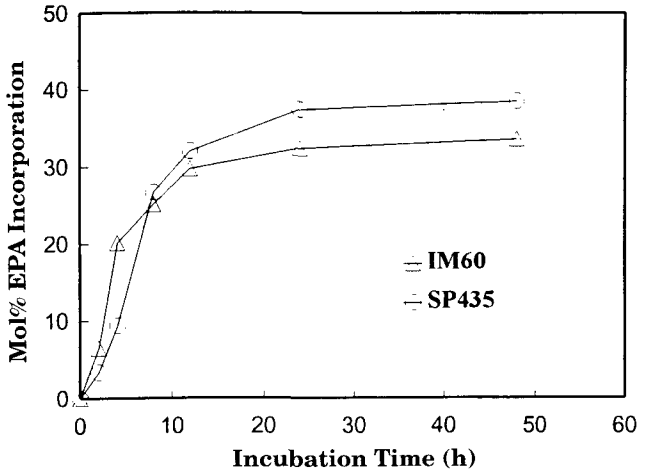

FIG. 1. Time course of lipase-catalyzed incorporation of eicosapentaenoic acid (EPA) into soybean oil with 1,3-specific (IM60) (Novo Nordisk Bioindustrial, Inc., Danbury, CT) and nonspecific (SP435) lipases (Novo Nordisk Bioindustrials, Inc.). The reaction mixture contained 100 mg soybean oil, 73.6 mg EPA ethyl ester, 17.4 mg of lipase and 3 mL hexane. The reaction mixture was incubated at 55°C in an orbital shaking waterbath at 200 rpm. Molecular sieves were added after 2 h.

TABLE 2

Fatty Acid Composition (in mol%[a]) of Soybean Oil Before and After Transesterification with Various Acyl Donors by *Candida antarctica* Lipase, SP435[b]

Fatty acids	Before transesterification	After transesterification with different acyl donor		
		EPA 45	EPA ethyl ester	DHA ethyl ester
14:0	0.2	0.7	0.2	0.1
14:1	0.1	0.4	0.1	0.1
16:0	11.6	11.9	6.9	7.3
16:1n-7	0.1	2.5	0.2	0.1
18:0	3.5	3.2	2.5	2.6
18:1n-9	21.9	21.6	15.7	16.2
18:2n-6	53.9	46.9	35.1	36.0
18:3n-6	0.3			5.0
18:3n-3	8.4	6.3	5.0	
20:5n-3		6.8	34.7	
22:5n-3				
22:6n-3				32.9

[a]Mol% EPA = (EPA mol/total fatty acid mol) × 100%.
[b]Lipase, SP435 (Novo Nordisk Bioindustrial, Inc., Danbury, CT). See Table 1 for other company sources and abbreviations.

form before transesterification was 2.16 times that of EPA 45, but the mol% incorporation was increased to 2.78 times for IM60 and 5.10 times for SP435. Thus, EPA ethyl ester appeared to be a better substrate for EPA incorporation into vegetable oils with SP435 as the biocatalyst.

Increasing the incubation time from 24 to 48 h only slightly increased the EPA incorporation into vegetable oil (Fig. 1). At an early stage of the reaction, higher incorporation was obtained with IM60, but after 8 h the incorporation with SP435 was higher and remained so for 48 h. Sridhar and Lakshminarayana (5) reported the incorporation of n-3 PUFA into groundnut with PUFA concentrate (34% EPA) methyl ester as acyl donor and lipase from *M. miehei* as biocatalyst in a magnetically stirred bioreactor. Ester interchange for 4 h resulted in the incorporation of 9.2 wt% of EPA and 7.4 wt% of DHA. For a 6-h reaction, incorporation of EPA was only marginally increased (5). Here, we used EPA ethyl ester (97% pure), and a higher EPA-to-TAG ratio in an orbital shaking waterbath. After 4 h reaction, EPA incorporation by IM60 increased to 20.1% (Fig. 1). EPA incorporation still increased after 24 h reaction. The inefficiency of mixing the reactants in the orbital shaking waterbath may contribute in part to the delayed equilibrium time. The different acyl donor type and higher EPA content in the reaction mixture may also contribute to the delayed equilibrium.

A trace amount of water is necessary for the enzyme function and for maintaining its three-dimensional structure (1), but too much water favors hydrolysis rather than synthesis. Yamane *et al.* (6) reported elevated diacylglycerol (DAG) level and lower TAG recovery with 7.9% water addition. We added various amounts of water at the beginning of the reaction, and molecular sieves were added 2 h later to remove water. No matter what the acyl donor type, the added water increased EPA incorporation for both IM60- and SP435-catalyzed reactions, but the incorporation declined when the added water exceeded 2 μL for SP435 (Fig. 2). For IM60-catalyzed reaction with EPA 45 as acyl donor, the addition of water up to 10 μL increased the incorporation of EPA by 5.1% (Fig. 2A). Compared to IM60, an increase of 4.9% EPA incorporation was obtained by the addition of 2 μL water to an SP435-catalyzed reaction, but resulted in zero incorporation when 10 μL water was added (Fig. 2A). A similar result was obtained when EPA ethyl ester was used (Fig. 2B). The EPA incorporation increased by 3.9% when 2 μL water was added to IM60 and remained the same up to 10 μL water, but when 10 μL water was added to the SP435 reaction mixture, the incorporation decreased from 35 to 4% (Fig. 2B).

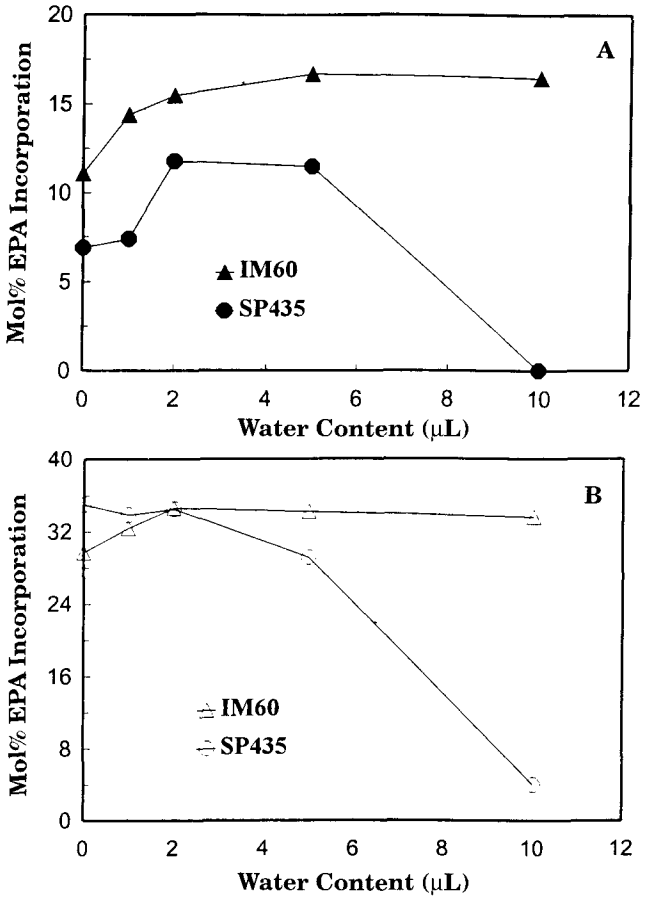

FIG. 2. Effect of added water on EPA incorporation with different lipases. Acyl donor: (A) with EPA45 (Callanish Ltd., Scotland, United Kingdom); (B) with EPA ethyl ester (United States Department of Commerce, National Fisheries Service, Charleston, SC). The reaction mixture contained 100 mg soybean oil, 73.6 mg EPA ethyl ester or 68.3 mg EPA45, 17.4 mg (16.8 mg with EPA45 as acyl donor) of lipase and 3 mL hexane. See Figure 1 for incubation conditions and abbreviations.

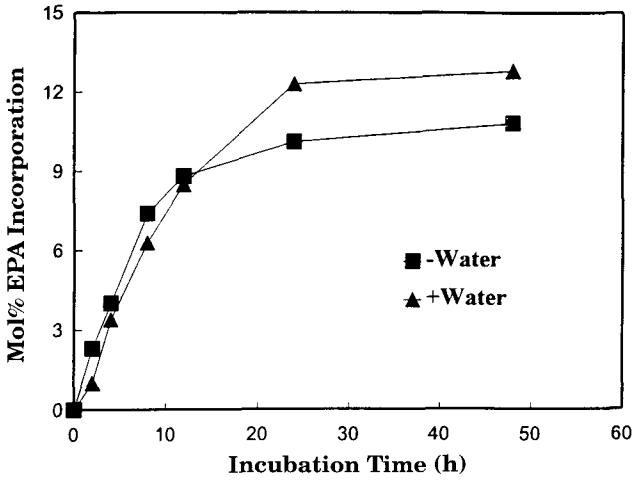

FIG. 3. Effect of added water or no water on the rate of EPA incorporation with IM60 as biocatalyst. The reaction condition was the same as in Figure 2. Trisun 80 (SVO Enterprises, Eastlake, OH) was transesterified with EPA45 in this reaction. See Figure 1 for other company sources and abbreviation.

Because the addition of water could increase the EPA incorporation in an IM60-catalyzed reaction, we became interested in whether the added water could reduce the reaction time. Yamane *et al.* (6) reported a lipase-catalyzed acidolysis for the production of n-3 PUFA-enriched fish oil in a nonsolvent system. The addition of water to the reaction mixture resulted in a higher reaction rate and shortened the reaction period at the early stage, but too much DAG formation did not allow conversion to TAG at a later stage (6). In our reaction, Trisun 80 and EPA 45 were incubated with IM60 with and without 10 μL water addition. The EPA incorporation was monitored at 0, 2, 4, 8, 12, 24 and 48 h after the beginning of the reaction (Fig. 3). The added 10 μL of water did not give higher EPA incorporation in the first 12 h of incubation compared to the reaction without water addition. The use of organic solvent as reaction medium was the major difference between our experiment and that of Yamane *et al.* (6). The polarity of solvent has a profound effect on the retention of enzyme-associated water and affects enzyme catalysis (1). We used a nonpolar organic solvent, hexane, for this reaction.

Canola oil, peanut oil, soybean oil, hydrogenated soybean oil, Trisun 80 and Trisun 90 were successfully transesterified with free EPA, EPA ethyl ester and DHA ethyl ester by IM60 (Table 3). The mol% EPA incorporation varied with different vegetable oils and acyl donor type. After the transesterification, canola oil gave the highest EPA incorporation up to 14.6% at the EPA-to-TAG molar ratio of 2. The lowest EPA incorporation was 6.1% for hydrogenated soybean oil. As the molar ratio was increased to 3, EPA incorporation was increased for most vegetable oils, except for peanut oil which remained unchanged. An average of 29.7% EPA incorporation was obtained when EPA ethyl ester was used. However, an average of only 13.2% DHA incorporation was obtained when DHA ethyl ester was used.

SP435 gave lower EPA incorporation into all vegetable oils with free acid as acyl donor at a molar ratio of 2 compared to IM60, except hydrogenated soybean oil (Table 4). EPA incorporation was only increased in canola oil and Trisun 90 as the molar ratio of EPA to vegetable oil TAG was increased from 2 to 3. The reason is unknown. This is probably due to the high water content because the addition of 10 μL water could completely disrupt the enzyme function (Fig. 2A). The average incorporations of EPA (29.7%) and DHA (28.7%) were only slightly different (Table 4). SP435 showed higher catalytic ability to incorporate n-3 PUFA into most of the selected vegetable oils with n-3 PUFA ethyl ester than IM60 (Tables 3 and 4). The reason for low incorporation of EPA into Trisun 80 and DHA into Trisun 90 by SP435 is not known.

Several research groups have successfully incorporated n-3 PUFA into vegetable oils (2,5). However, the factors that influence the reaction have not yet been studied. We found that lipase IM60 from *M. miehei* resulted in higher incorporation when free EPA was the acyl donor, and SP435 from *C. antarctica* performed better when EPA and

TABLE 3

Mol% n-3 PUFA in Triacylglycerol of Vegetable Oils After Transesterification with Various Acyl Donors by *Mucor miehei* Lipase, IM60

Acyl donor/molar ratio	Vegetable oils[a]					
	CO	PO	SO	HSO	T80	T90
EPA 45 molar ratio EPA/TAG = 2	14.6	12.0	10.5	6.1	8.8	8.0
EPA 45 molar ratio EPA/TAG = 3	18.8	12.0	17.4	16.4	10.2	11.8
EPA ethyl ester molar ratio EPA/TAG = 2	32.9	29.8	29.2	31.3	26.9	27.9
DHA ethyl ester molar ratio DHA/TAG = 2	18.1	14.9	14.6	10.9	14.0	6.9

[a]CO = canola oil; PO = peanut oil; SO = soybean oil; HSO = hydrogenated soybean oil; T80 = Trisun 80; T90 = Trisun 90. TAG; triacylglycerols; see Table 1 for other abbreviations and company sources.

TABLE 4

Mol% n-3 PUFA in Triacylglycerol of Vegetable Oils After Transesterification with Various Acyl Donors by *Candida antarctica* Lipase, SP435[a]

Acyl donor/molar ratio	Vegetable oils[a]					
	CO	PO	SO	HSO	T80	T90
EPA 45 molar ratio EPA/TAG = 2	3.7	9.5	6.8	8.8	1.8	5.3
EPA 45 molar ratio EPA/TAG = 3	12.6	7.2	6.8	8.1	1.3	9.4
EPA ethyl ester molar ratio EPA/TAG = 2	29.5	32.2	34.7	35.7	15.9	30.3
DHA ethyl ester molar ratio DHA/TAG = 2	29.0	31.6	32.9	32.8	31.2	14.7

[a]See Tables 1 and 2 for company sources and Tables 1 and 3 for abbreviations.

DHA ethyl esters were the acyl donors. EPA incorporation was improved by 5 μL water addition with IM60 as biocatalyst. The catalytic activity of SP435 was disrupted when the added water exceeded 2 μL. Water content must be carefully controlled when using SP435 as biocatalyst for the modification of oils. Overall, IM60 showed more consistency in most reactions.

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