Lipase-Catalyzed Incorporation of n-3 PUFA into Palm Oil

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ABSTRACT: Two immobilized lipases, IM60 from *Rhizomucor miehei* and QLM from *Alcaligenes* sp., were used as biocatalysts for the modification of the FA composition of palm oil by incorporating n-3 PUFA. Acidolysis and interesterification reactions were conducted with hexane as organic solvent, and the products were analyzed by using GLC. After a 24-h incubation in hexane, there was an average incorporation of 20.8% EPA and 15.6% DHA into palm oil, respectively, while the percentages of palmitic and oleic acids in palm oil decreased by 28.8 and 11.8%, respectively. Higher EPA and DHA incorporation was obtained when EPAX (fish oil concentrate high in n-3 PUFA) was used in the ethyl ester form (interesterification reaction) than in the free acid form (acidolysis) in the presence of Lipozyme IM60 lipase. Lipase QLM was found to discriminate against EPA, and it showed slightly better catalytic activity for DHA in the free acid form than in the ethyl ester form. Generally, as the mole ratio of the acyl donor to TAG increased, the percentage incorporation of EPA and DHA increased; however, reactions catalyzed by Lipozyme IM60 did not show increases in the incorporation beyond a TAG/EPAX mole ratio of 3. When limitations due to mass transfer were not a factor, an increase in the reactant amount also gave an increase in the percentage incorporation of the n-3 PUFA. Palm oil containing EPA and DHA was successfully produced and may be beneficial in certain food and nutritional applications.

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KEY WORDS: Acidolysis, *Alcaligenes* sp. lipase, DHA, EPA, interesterification, Lipozyme IM60, palm oil, PUFA, *Rhizomucor miehei* lipase, structured lipid.

Palm oil (PO), obtained from the mesocarp of the fruit of the oil palm species, *Elaeis guineensis*, is a vegetable oil high in saturated FA content. PO consists of trisaturated (mainly PPP), disaturated (mainly POP), and monounsaturated (mainly POO) TAG (where P = palmitic, O = oleic) with a significant amount of the saturated FA, such as palmitic acid, at the *sn*-2 position of its TAG (1). At room temperature, PO exists in a semisolid form. It is an important source of edible oil in the food industry, but owing to its high saturation and solid fat content, PO is usually mixed with other oils/fats to impart plasticity, body, and better melting properties to the end product. Changes to the physical and chemical properties of mixtures of PO with other fats and oils can be brought about either through simple blending or by interesterification (2).

The n-3 PUFA such as EPA and DHA, which are abundant in fish oil, have potential health benefits such as the reduction of cardiovascular disease, immune disorders and inflammation, allergies, and diabetes (3,4). EPA and DHA also have been shown to promote the synthesis of beneficial nitric oxide in the endothelium. EPA reduces the level of very low density lipoprotein- and LDL-cholesterol in humans (5). EPA is an antagonist of the arachidonic acid (AA) cascade and competes with AA as a substrate for cyclooxygenase and lipoxygenase to produce certain eicosanoids (3). It acts as a precursor for the formation of series-3 prostaglandins, thromboxanes, and series-5 leukotrienes (3). Thus, the eicosanoids in dietary lipids are one of many possible mediators that play a role in influencing immune response.

The use of biocatalysts such as lipases to improve the FA composition of PO by incorporating EPA and DHA offers numerous health benefits by reducing the level of palmitic acid in the oil and optimizing the n-3 FA ratio for nutritional benefits. Work on the incorporation of n-3 PUFA into vegetable oils by using the immobilized lipases Lipozyme IM60 from *Rhizomucor miehei* and Lipase SP435 from *Candida antarctica* has been reported (6). The incorporation of n-3 PUFA into vegetable oils can be achieved by transesterification with the free or the methyl ester forms of the n-3 PUFA (7,8).

In our present study, two immobilized lipases, Lipozyme IM60 from *R. miehei* and Lipase QLM from *Alcaligenes* sp. were used as biocatalysts to modify the FA composition of PO by incorporating n-3 PUFA. The aim of this work was to obtain structured lipids with beneficial properties as well as to improve the FA composition of PO. The effects of mole ratio of the substrates, the acyl donor type, i.e., n-3 PUFA in FFA or ethyl ester (EE) form, and the reaction size on the n-3 PUFA incorporation were also studied.

EXPERIMENTAL PROCEDURES

Materials. Refined, bleached, and deodorized (RBD) cooking PO was a gift from the Malaysian Palm Oil Promotion Council (Selangor, Malaysia). EPAX 6000 EE form and EPAX 6000 FFA, which contains 60–70% PUFA, were obtained from Pronova Biocare (Sandefjord, Norway). Immobilized 1,3-specific lipase, Lipozyme IM60, from *R. miehei*, was provided by Novo Nordisk A/S (Bagsvaerd, Denmark), whereas the immobilized nonspecific but 1,3-positional preferential *Alcaligenes* sp. Lipase QLM was obtained from Meito Sangyo Co. Ltd.

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(Aichi, Japan). Silicic acid and silica gel 60 plates were purchased from Aldrich Chemical Company (Milwaukee, WI). All other reagents used were purchased from Fisher Scientific (Fairlawn, NJ).

Enzymatic modification reactions. For interesterification and acidolysis reactions, 200 mg of PO was mixed with the acid or ester in a 20-mL test tube at mole ratios of 1:1, 1:2, 1:3, and 1:4 TAG/EPAX by using 10 mL hexane/g of total reactants as organic solvent. Immobilized lipases, Lipozyme IM and Lipase QLM (10% by weight reactants), were added to the mixture and incubated in an orbital shaking water bath at 55°C for 24 h at 200 rpm. All reactions were performed in duplicate, and average values were reported.

Reactant ratios. The effect of reactant ratios on the incorporation of PUFA was compared by initially conducting the enzymatic modification reactions using 200 mg of reactants at a mole ratio of 1:1 to 1:4 TAG/EPAX. Subsequently, the mole ratio with the highest incorporation was selected, and the reaction was performed using 2000 mg of PO with a mole ratio of 1:3, 1:4, and 1:5 TAG/EPAX. In the design of the scaled-up version of the reaction, narrower 125-mL flasks and 5 mL hexane/g of total reactants were used. The agitation rate was increased to 250 rpm, and helper agitators made of glass were introduced into the flasks. All these were done to improve the mass transfer and yield of the reaction.

Analysis of products. The reactions were stopped by filtering the enzymes through a column of anhydrous sodium sulfate. The reaction products from the scaled-up experiments containing FAEE and TAG were separated by column chromatography on silicic acid and eluted with mixtures of hexane/ethyl ether. TAG was eluted with 99:1 vol/vol (95 mL) hexane/EE, whereas FAEE was eluted with 99:5 vol/vol (60 mL) of the same solvent at a flow rate of 2–3 mL/min. Aliquots of the reactants before and after enzyme reaction were then analyzed by TLC on silica gel 60 plates. These were developed with petroleum ether/diethyl ether/acetic acid (80:20:1, by vol) for the separation of partial acylglycerols, FFA, and TAG from acidolysis reactions and with petroleum ether/diethyl ether/acetic acid (90:10:1, by vol) for interesterification reactions. The bands were visualized under UV light after the plates were sprayed with 0.2% 2,7-dichlorofluorescein in methanol. The bands corresponding to TAG were scraped from the TLC plate and methylated in 3 mL of HCL in methanol at 75°C for 2 h (6). The FAME were extracted and analyzed by GLC as described previously (9). The relative content of FAME as a weight percentage was calculated by computer with 17:0 as the internal standard. Peaks less than 1% were considered to be negligible and labeled as "not detectable" (ND).

RESULTS AND DISCUSSION

The predominant FA in PO were palmitic and oleic acids, representing 44.1 and 38.7% of the total FA, respectively (Table 1). These results are very similar to the values reported by Noor Lida *et al.* (10). As expected, C20:5n-3 (EPA) and C22:6n-3 (DHA) were not detected in PO. The FA profiles of the commercial EPAX 6000 EE and EPAX 6000 FFA samples used in the reactions were analyzed and found to contain 40.4 and 40.5% of EPA, respectively, and 30.6 and 26.4% DHA, respectively (Table 1). Both acyl donors also contained oleic acid, 11.1 and 13.4%, respectively (Table 1).

TABLE 1
FA Composition (%) of Palm Oil and EPAX in the FFA and Ethyl Ester (EE) Form Before Enzymatic Modification

FA	Palm oil	EPAX FFA[a]	EPAX EE[b]
C16:0	44.1	8.0	3.4
C18:0	7.4	3.9	6.8
C18:1n-9	38.7	13.4	11.1
C18:2n-6	9.8	ND	3.3
C20:5n-3	ND	40.5	40.4
C22:6n-3	ND	26.4	30.6
Others	ND	7.8	4.4

[a]EPAX FFA, eicosapentaenoic free acid form.
[b]EPAX EE, eicosapentaenoic acid ethyl ester form.
[c]ND, not detectable; EPAX, fish oil concentrate high in n-3 PUFA.

Table 2 shows the percentage incorporation of EPA and DHA into PO after modification with Lipozyme IM60 and lipase QLM at a 1:1 mole ratio of TAG/EPAX. Lipase from Lipozyme IM60 showed a slightly higher catalytic activity for EPA and DHA in the EE form than in the FFA form. However, when EPA and DHA were present together in a mixture, lipase QLM was found to discriminate against EPA (incorporation less than 2%). Unlike Lipozyme IM60 lipase, lipase QLM showed a slightly better catalytic activity for DHA in the FFA form than in the EE form. On the basis of these results, we elected to study the effects of the mole ratios of EPAX in the EE and FFA form to TAG for Lipozyme IM60 and Lipase QLM, respectively.

Table 3 shows the incorporation of EPAX into PO at various TAG/EPAX mole ratios. For Lipozyme IM60, EPAX in the EE form was used in the reaction, whereas for Lipase QLM, the FFA form of EPAX was used since these were the forms that had the highest incorporation (Table 2). With EPAX EE and Lipozyme IM60, the incorporation increased as the mole ratio increased, but the incorporation did not increase beyond a mole ratio of TAG to EPAX EE of 3. With a higher mole ratio of TAG to EPAX EE, the ester interchange reaction between the two esters may compete with the product and possibly slow the reaction. Mutua and Akoh (11) reported a decreased EPA

TABLE 2
Incorporation (%) of EPA and DHA into Palm Oil After Modification with Lipozyme IM60 and Lipase QLM at a 1:1 Mole Ratio of TAG/EPAX

Enzymes[a]	EPAX form	EPA incorporated (%)	DHA incorporated (%)
Lipozyme IM60	FFA	9.7	9.4
Lipozyme IM60	EE	11.3	10.7
Lipase QLM	FFA	ND	10.3
Lipase QLM	EE	ND	9.9

[a]Immobilized Lipozyme IM60 (Novo Nordisk, Bagsvaerd, Denmark), from *Rhizomucor miehei*; immobilized Lipase QLM (Meito Sangyo Co. Ltd., Aichi, Japan), from *Alcaligenes* sp. ND, not detectable.

TABLE 3
Effect of TAG-to-EPAX Ratio on Percent EPA and DHA Incorporation After Modification with Lipozyme IM60 and Lipase QLM[a]

Enzyme	EPAX form	Mole ratio (TAG/EPAX, w/w)	EPA incorporated (%)	DHA incorporated (%)
Lipozyme IM60	EE	1:1	11.3	10.7
		1:2	18.2	15.5
		1:3	21.5	16.1
		1:4	18.3	14.3
Lipase QLM	FFA	1:1	ND	10.3
		1:2	1.7	10.4
		1:3	1.8	11.6
		1:4	3.0	13.4

[a]See Tables 1 and 2 for abbreviations, and Table 2 for manufacturers.

incorporation into biosurfactant by Lipozyme IM20 from *R. miehei* when the mole ratio of EPA to phospholipids exceeded 2:1. There was also no economic advantage in using high substrate mole ratios. Others have shown that a high mole ratio shortened reaction time, improved the reaction rate, and resulted in less acyl migration (12). With the EPAX FFA form and Lipase QLM, the incorporation increased as the mole ratio of TAG/EPAX FFA increased up to 4. However, the largest increase in incorporation occurred when the mole ratio of TAG/EPAX FFA increased from 3 to 4. No decrease in EPAX FFA incorporation into TAG was observed with a high TAG/EPAX FFA ratio by Lipase QLM in this study. Overall, however, the highest percent incorporation of EPA by Lipozyme IM60 was almost seven times higher than when Lipase QLM at a 1:4 mole ratio was used. For Lipozyme IM60, the percent incorporation of EPA was much higher (21.5%) than for DHA (16.1%). As for Lipase QLM, the opposite trend was observed. The percent incorporation of DHA (13.4%) was about four times higher than the percent incorporation of EPA (3.0%) (Table 3). This again confirms our earlier conclusion that Lipase QLM had a lower affinity toward EPA.

The change in the FA composition of the TAG pool following interesterification by Lipozyme IM60 at a 1:3 mole ratio of TAG/EPAX EE is shown in Table 4. The relative FA composition of both the PO and the EPAX before modification was different from the FA composition after modification. After 24 h incubation in hexane, there was an average of 20.8 and 15.6% incorporation of EPA and DHA into PO, respectively, whereas palmitic and oleic acids in PO decreased by 28.8 and 11.8%, respectively. As for the EPAX EE composition, the percentage of EPA and DHA decreased by 10.2 and 5.2, respectively, after modification, whereas palmitic and oleic acids increased by 10.1 and 7.4%, respectively. This indicates that interesterification generally was successful in modifying the FA composition of PO by incorporating the desired n-3 PUFA.

In our effort to avoid mass transfer limitations and to improve product yield, reactions involving a larger quantity of the reactants were prepared in a narrower flask, and higher TAG/EPAX EE mole ratios were used (Table 5). The incorporation of EPA increased slightly (27.6%) relative to smaller volume reactions (21.5%) (Table 3) at the same mole ratio of 1:3. However, there was not much change in terms of the percent incorporation of DHA. Increasing the mole ratio of EPAX EE from 3 to 4 increased the incorporation of both EPA and DHA by 11.3 and 6.5%, respectively, compared to when smaller quantities of the reactants were used (Table 3). However, when the mole ratio was further increased from 4 to 5, the percent incorporation decreased slightly (Table 5). This may be due to the effect of mass transfer limitations on product yield, or the reaction may have reached an equilibrium state. As the reactant quantities increased, so did the problem of mass transfer of the reactants to the enzymes. The FA compositions of the mod-

TABLE 4
Relative FA Composition (%) of Palm Oil/EPAX EE at a 1:3 Mole Ratio Before and After Modification with Lipozyme IM60[a]

	Palm oil			EPAX EE		
FA	Before	After	% Change	Before	After	% Change
C16:0	48.9	20.1	–28.8	3.2	13.3	+10.1
C18:0	6.1	7.2	+1.1	6.4	4.9	–1.5
C18:1n-9	36.4	24.6	–11.8	10.4	17.8	+7.4
C18:2n-6	8.6	4.5	–4.1	ND	3.4	+3.4
C20:5n-3	ND	20.8	+20.8	37.8	27.6	–10.2
C22:6n-3	ND	15.6	+15.6	28.7	23.5	–5.2
Others	ND	7.2	+7.2	13.5	9.5	–4.0

[a]See Tables 1 and 2 for abbreviations.

TABLE 5
Incorporation (%) of PA and DHA into PO by Interesterification with EPAX EE and Lipozyme IM60 (2 g palm oil reaction)[a]

Mole ratio (TAG/EPAX, w/w)	EPA incorporated (%)	DHA incorporated (%)
1:3	27.6	15.9
1:4	29.6	20.8
1:5	29.1	18.2

[a]See Table 1 for abbreviations.

TABLE 6
Relative FA Composition (%) of the TAG Fraction Before and After Interesterification by Lipozyme IM60 at a Palm Oil/EPAX EE Mole Ratio of 1:4 (2 g palm oil reaction)[a]

	FA composition (%)		
FA	Before	After	% Change
C16:0	47.2	12.1	–35.1
C18:0	5.9	5.3	–0.6
C18:1n-9	37.7	18.4	–19.3
C18:2n-6	9.1	3.6	–5.5
C20:5n-3	ND	29.6	+29.6
C22:6n-3	ND	20.8	+20.8
Others	ND	10.2	+10.2

[a]See Tables 1 and 2 for abbreviations.

ified PO and EPAX EE mixtures at a 1:4 mole ratio are given in Table 6. As expected, palmitic and oleic acids in the TAG fraction decreased by 35.1 and 19.3%, respectively, but the incorporation of EPA and DHA increased by 29.6 and 20.8%, respectively. This is because the lipase acts strongly on palmitic and oleic acids by removing them from the PO TAG while allowing the incorporation of incoming EPA and DHA into the TAG.

PO has a high content of saturated FA, mainly palmitic acid, with very little n-3 PUFA. In this work, we have shown that it is possible to change the FA composition of PO by using lipases as biocatalysts. Lipozyme IM60 from *R. miehei* and Lipase QLM from *Alcaligenes* sp. have very different activities toward EPA and DHA for the interesterification and acidolysis reactions studied. Using EPA EE, a high TAG/EPA FFA mole ratio, and optimal reactant sizes, one could manipulate the percent incorporation of the n-3 PUFA into PO. In future work, we may be able to incorporate other FA of interest to obtain a more balanced FA profile for PO and determine the location for the incorporation of the FA. Incorporation of EPA and DHA at the *sn*-2 position of the modified structured lipid is desirable as it may enhance their absorption, and the resultant structured lipids have the potential to be used as nutraceutical lipids.

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