Enzymatic Modification of Melon Seed Oil: Incorporation of Eicosapentaenoic Acid

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Two immobilized lipases, IM60 from *Mucor miehei* and SP435 from *Candida antarctica*, were used as biocatalysts for the modification of the fatty acid composition of crude melon seed (*Citrullus colocynthis* L) oil by incorporating $n-3$ polyunsaturated fatty acid (PUFA). Higher eicosapentaenoic acid (EPA) incorporation was obtained by using EPA ethyl ester (97% pure) than by using EPA free acid (45% pure) for both enzyme-catalyzed reactions. IM60 required additional water to optimize the reaction condition, but any added water inhibited the catalytic activity of SP435. Increasing the molar ratio of acid or ester to triacylglycerol (TAG) significantly increased EPA incorporation, especially when EPA ethyl ester was used. The $n-6$ fatty acid content of melon seed oil was significantly lowered by using a high molar ratio of EPA ethyl ester to TAG. Incorporation of EPA into melon seed oil by immobilized lipase can increase the $n-3$ PUFA content, can help control the level of $n-6$ fatty acid, and may improve the nutritional quality of melon seed oil.

Keywords: *Eicosapentaenoic acid; melon seed oil; modification; lipases*

INTRODUCTION

Melon seeds can be used for oil production and as thickener in soup. The oil is used for frying and cooking in some African and Middle Eastern countries. Melon seed oil contains a large amount of linoleic acid (C18:2 $n-6$) and very little $n-3$ polyunsaturated fatty acids (PUFAs) (Akoh and Nwosu, 1992). Diets high in $n-6$ fatty acids, in particular C18:2 $n-6$, at the expense of $n-3$ fatty acids, may favor thrombotic effects mediated through thromboxane A_2 (TXA_2) (Jensen and Jensen, 1992). Eicosapentaenoic acid (EPA), C20:5 $n-3$, which is a competitor of arachidonic acid, is found in fish oil and has been shown to prevent cardiovascular diseases (Jensen and Jensen, 1992; Johnston and Hunter, 1987) and ameliorate acute inflammatory response by modulating eicosanoid production (Jensen and Jensen, 1992; Lefkowith, 1988).

The use of lipases as biocatalysts to modify melon seed oil fatty acid composition by incorporating EPA is viable and may help in controlling the level of linoleic acid in the oil and optimize the $n-3$ and $n-6$ fatty acid ratio for nutritional benefits. Recently, several laboratories have reported the incorporation of $n-3$ PUFAs into vegetable oils using immobilized lipase, IM60 from *Mucor miehei*, as biocatalyst. The production of vegetable oils containing $n-3$ PUFAs could be achieved by transesterification of the free acid form of $n-3$ PUFA concentrate (Li and Ward, 1993) or its methyl esters with vegetable oils (Sridhar and Lakshminarayana, 1992).

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 melon seed (*Citrullus colocynthis* L) oil by the incorporation of EPA. The effects of molar ratio of the substrates, added water, and the acyl donor type on EPA incorporation were also studied.

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MATERIALS AND METHODS

Materials. Dried melon seeds (*C. colocynthis* L.) were obtained from a local market in Onitsha, Nigeria. EPA 45 (45% eicosapentaenoic acid) was supplied by Callanish Ltd. (Scotland, U.K.). EPA ethyl ester (97% pure) was provided by the U.S. Department of Commerce, National Fisheries Service (Charleston, SC). Immobilized 1,3-specific lipase, IM60, and nonspecific lipase, SP435, were provided by Novo Nordisk Bioindustrial, Inc. (Danbury, CT). All organic solvents were from Fisher Scientific (Norcross, GA).

Melon Seed Oil Extraction. Melon seeds (20 g) were homogenized with a Waring blender. The melon seed oil was extracted in a Soxhlet with 200 mL of hexane for 8 h. Hexane was evaporated with a rotatory evaporator.

Enzymatic Modification Reaction. For general synthesis of modified melon seed oil, 100 mg of melon seed oil was mixed with the acid or ester at a molar ratio of triacylglycerol (TAG) of 1:2, i.e., 68.3 mg for EPA, 73.6 mg for EPA ethyl ester, and immobilized lipase (10% combined weight of substrates) in 3 mL of hexane. The mixture was incubated in an orbital shaking water bath at 55 °C for 24 h at 200 rpm. Molecular sieves (4 Å) were added after 2 h. All reactions were in duplicate.

Analysis of Product. The enzyme was filtered through an anhydrous sodium sulfate column. 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 v/v/v). The bands were visualized under ultraviolet 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 6% HCl in methanol at 70–80 °C for 2 h. The fatty acid methyl esters were extracted twice with 2 mL of 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 and 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 fatty acid methyl esters (FAME) as mole percent was quantitated by an on-line computer with 17:0 as internal standard.

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Table 1. Fatty Acid Composition (Mole Percent) of Melon Seed Oil Triacylglycerol before and after Modification

fatty acid	IM60 EPA[a]	IM60 EEPA[b]	SP435 EPA	SP435 EEPA	before modification
16:0	8.1	7.7	10.0	7.6	11.1
16:1 $n-7$	5.5	7.4	2.4	0.2	0.1
18:0	8.0	0.2	9.5	6.5	9.4
18:1 $n-9$	12.4	11.2	14.2	9.9	14.2
18:2 $n-6$	53.4	48.7	56.2	43.9	64.3
18:3 $n-6$	0.3	0.2	ND[c]	0.4	ND
18:3 $n-3$	0.3	0.1	0.2	0.1	0.1
20:5 $n-3$	8.7	24.0	5.2	31.2	ND
22:5 $n-3$	0.5	ND	0.5	ND	ND
others	2.8	0.5	1.8	0.2	0.8
total $n-3$	9.5	24.1	5.9	31.3	0.1
total $n-6$	53.7	48.9	56.2	44.3	64.3
saturated	16.1	7.9	19.5	14.1	20.5

[a] EPA, eicosapentaenoic acid. [b] EEPA, eicosapentaenoic acid ethyl ester. [c] ND, not detectable.

RESULTS AND DISCUSSION

Melon seeds have high oil content. Melon seeds used in this study contained up to 50% by weight crude melon seed oil. The predominant fatty acid of melon seed oil was linoleic acid, C18:2 $n-6$, representing 64.3% (mol %) of total fatty acid. This was very close to the value (63.4%) reported by Akoh and Nwosu (1992). C18:3 $n-3$ was the only detectable $n-3$ fatty acid in the crude extracts at a level of 0.1% of the total fatty acids.

Table 1 gives the fatty acid profile of TAG in melon seed oil before and after the modifications. By employing a good leaving group on the acyl donor (methyl or ethyl ester), high yields of ester synthesis can be achieved (Dordick, 1989). Both enzymes showed higher catalytic activity for EPA ethyl ester than EPA free acid. With IM60 as biocatalyst and EPA ethyl ester, EPA incorporation was 3 times greater than the levels obtained with the EPA free acid. With SP435, the levels of EPA were 6 times higher in EPA with ethyl ester than with the free acid. The purity of EPA ethyl ester (97%) is a possible reason for the higher EPA incorporation compared with EPA free acid (45% pure). IM60 showed higher catalytic activity than SP435 with EPA free acid, but SP435 had higher catalytic activity than IM60 with EPA ethyl ester. On the basis of these results, we elected to study other parameters using EPA free acid and EPA ethyl ester for IM60 and SP435 lipases, respectively. The $n-6$ fatty acid content was reduced by 31% by SP435 and by 24% by IM60 with EPA ethyl ester compared to unmodified oil. With EPA free acid, $n-6$ fatty acid was reduced by 13 and 16% by SP435 and IM60, respectively. The saturated fatty acid content of melon seed triacylglycerol was also decreased in all reactions.

The presence of water in the reaction mixture will thermodynamically favor the hydrolysis of TAG instead of transesterification, but some water is necessary for catalysis and for maintaining the three-dimensional structure of the enzyme. The need for water is obvious; however, the amount of water required for enzymatic activity is less clear (Dordick, 1989). Figure 1 shows the mole percent of EPA incorporation with 0, 6, 12, 30, and 60% (w/w of enzyme) added water in the reaction mixture. The incorporation of EPA decreased when water was added to the reaction mixture containing EPA ethyl ester and SP435. The incorporation of EPA increased by 4.3% when 6% water was added to the

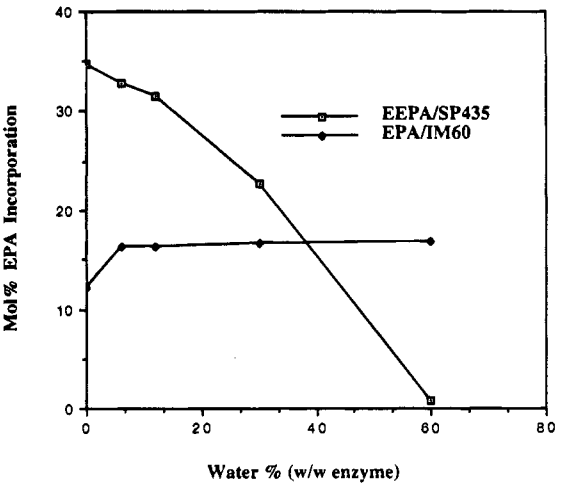

Figure 1. Effect of added water on the incorporation of EPA into melon seed oil. Water was added at the beginning of the reaction. Molecular seives (4 Å) were added after 2 h. EPA, eicosapentaenoic acid; EEPA, EPA ethyl ester.

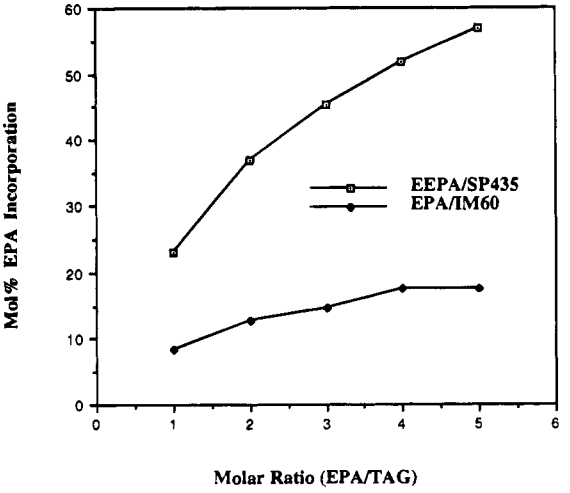

Figure 2. Effect of EPA to TAG ratio on EPA incorporation (see Figure 1 for legend).

reaction mixture containing EPA free acid and IM60 and only marginally increased with more water addition. Recently, the interesterification activity was shown to be maximum at a water activity of 0.25 for the lipase from *M. miehei*, but as the water activity of the enzyme increased, hydrolysis of the formed TAG increased (Cho and Rhee, 1993). Yamane et al. (1992) also reported that addition of some level of water resulted in higher reaction rates in the early stages of diacylglycerol (DAG) production, but when more water was added, incomplete conversion of the DAG to TAG was observed due to the competing hydrolysis of the formed TAG (Yamane et al., 1992). Here, we found that added water had the same effect on IM60 as previously reported (Cho and Rhee, 1993; Yamane et al., 1992), but less water was required by SP435. We found (Huang and Akoh, unpublished data) that SP435 required less water than IM60 for maximum incorporation of EPA into selected vegetable oils with EPA ethyl ester and EPA free acid, respectively.

Figure 2 shows the incorporation of EPA into melon seed oil at various EPA to TAG molar ratios. With EPA and IM60, the incorporation increased as the molar ratio of EPA to TAG increased, but the incorporation did not increase beyond a molar ratio of EPA to TAG of 4. With

EPA ethyl ester and SP435, the incorporation increased as the molar ratio of EPA ethyl ester (EEPA) to TAG increased up to 5. However, the largest increase in incorporation occurred when the molar ratio of EPA to TAG increased from 1 to 2. Longer reaction time may be required to incorporate EPA from ethyl ester into TAG and to reach an equilibrium. With a higher molar ratio of EEPA to TAG, the ester interchange reaction between the two esters may compete with the product and possibly slow down the reaction. Mutua and Akoh (1993) reported a decreased EPA incorporation into biosurfactant by IM20 from *M. miehei* when the molar ratio of EPA to phospholipid exceeded 2:1. However, no decrease in EPA incorporation into triacylglycerol was observed with high EPA to TAG ratio by IM60 in this study.

Melon seed oil has a high content of $n-6$ fatty acid with very little $n-3$ polyunsaturated fatty acids. We have shown that it is possible to change its fatty acid composition by using lipases as biocatalysts. IM60 from *M. miehei* and SP435 from *C. antarctica* have differing activities and water requirements for the transesterification reactions studied. Using EPA ethyl ester and high EEPA to TAG molar ratio, one could obtain high EPA incorporation into melon seed oil. In the future, it may be possible to incorporate other fatty acids of interest to obtain a more balanced fatty acid profile and improve the oxidative stability of modified melon seed oil.

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