Enzymatic Modification of Evening Primrose Oil: Incorporation of n-3 Polyunsaturated Fatty Acids

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ABSTRACT: Immobilized lipase SP435 from *Candida antarctica* was used as a biocatalyst for the modification of the fatty acid composition of evening primrose oil by incorporating n-3 polyunsaturated fatty acid (PUFA) and eicosapentaenoic acid (EPA). Transesterification (ester–ester interchange) was conducted in organic solvent or without solvent, with EPA ethyl ester (EEPA) as the acyl donor. Products were analyzed by gas–liquid chromatography (GLC). After 24-h incubation in hexane, the fatty acid composition of evening primrose oil was markedly changed to contain up to 43% EPA. The amount of 18:2n-6 PUFA was reduced by 32%, and the saturated fatty acid content was also reduced. The effects of incubation time, molar ratio, enzyme load, and reaction medium on mol% EPA incorporation were also studied. Generally, as the incubation time (up to 24 h), molar ratio, and enzyme load increased, EPA incorporation also increased. Evening primrose oil, containing EPA and γ-linolenic acid (18:3n-6) in the same glycerol backbone, was successfully produced and may be more beneficial for certain applications than unmodified oil.
JAOCS 73, 1059–1062 (1996).

KEY WORDS: *Candida antarctica*, evening primrose oil, immobilized lipase, γ-linolenic acid, n-3 PUFA, n-6 PUFA, transesterification.

Evening primrose oil is one of the commercial sources of γ-linolenic acid (GLA; 18:3n-6). GLA is an essential n-6 polyunsaturated fatty acid (PUFA) and a precursor of dihomo-γ-linolenic acid (20:3n-6) and arachidonic acid (20:4n-6) (1,2). GLA has the potential to increase tissue biosynthesis of 1-series prostaglandins and is known to play an important role in the treatment of diabetes (3), hypertension (4), thromboembolic disease (5), atopic eczema (6), and in the regulation of the inflammatory response (7). Essential fatty acids cannot be synthesized by the body and must therefore be ingested in the diet (2). The essential n-6 PUFA are required for physiological functions. Excess amounts of n-6 PUFA can cause vasoconstriction and platelet aggregation. The n-3 PUFA compete with n-6 PUFA and may reverse some of the putative adverse effects of excess n-6 PUFA (8). Eicosapentaenoic acid (EPA) is found in fish oil and is a common source of n-3 PUFA (9). EPA is an antagonist of the arachidonic acid cascade and competes with arachidonic acid for cyclooxygenase and lipoxygenase as substrates to produce eicosanoids. Recently, n-3 PUFA have been incorporated into vegetable oils (10), trilinolein (11), and melon seed oil (12) with immobilized lipases IM60 from *Rhizomucor miehei* and SP435 from *Candida antarctica* as biocatalysts.

The beneficial effects of both GLA and n-3 PUFA are attributed to eicosanoid synthesis. It was reported that eicosanoid production can be manipulated by diet (13–15). At present, n-3 PUFA-rich and GLA-rich oils have been used individually or as physical mixtures in feeding trials (13). Incorporation of EPA from fish oil into evening primrose oil would provide a unique specialty oil, eliminate the need for physical mixtures, and serve as a single rich source of both n-3 PUFA and GLA. One way to achieve this is through lipase-catalyzed reactions.

In this study, we report the incorporation of EPA into evening primrose oil by means of a *C. antarctica* lipase-catalyzed transesterification reaction. The effects of molar ratio, time course, enzyme load, and reaction medium on EPA incorporation are also reported.

MATERIALS AND METHODS

Materials. EPA (97% pure) was provided by the U.S. Department of Commerce, National Marine Fisheries Service (Charleston, SC). Immobilized nonspecific lipase SP435 was provided by Novo Nordisk Bioindustrial, Inc. (Danbury, CT). Evening primrose oil was obtained from Sigma Chemical Company (St. Louis, MO). All organic solvents were obtained from Fisher Scientific (Norcross, GA).

Enzymatic modification reaction. For general synthesis of modified evening primrose oil, 50 mg of evening primrose oil was mixed with EPA ethyl ester (EEPA) (56.7 mg) at a molar ratio of triacylglycerol (TAG)/n-3 PUFA of 1:3 in 1.5 mL hexane. Immobilized SP435 lipase (10.7 mg) was added at 10% by weight of reactants and 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 performed in duplicate. The general synthesis reaction described above was replicated six times on six separate days to study reproducibility or day-to-day variations in mol% EPA

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incorporation, n-6 PUFA changes, and to validate our enzymatic process.

Analysis of product. The enzyme was removed by passing the reaction mixture through an anhydrous sodium sulfate column. The reaction product was diluted 1:2 (one part reaction product to two parts hexane), and a 50-μL aliquot 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 being sprayed with 0.2% dichlorofluorescein in methanol. The bands corresponding to TAG were scraped from the TLC plate and methylated with 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 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), flame-ionization detector, and operated in a splitless mode. The injector and detector temperatures were 250 and 260°C, respectively. The column temperature was held isothermally at 205°C. 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 mol% was calculated by an on-line computer with 17:0 as internal standard.

RESULTS AND DISCUSSION

Figure 1 shows the time course of EPA incorporation into evening primrose oil. We used this to determine the shortest time necessary to obtain acceptable yields. EPA was successfully incorporated into evening primrose oil with SP435 lipase as a biocatalyst. After 24 h of incubation in hexane, 43% EPA was incorporated. EPA incorporation increased as incubation time increased up to 24 h. The mol% incorporation was highest at 24 h and decreased after 48 h. The largest increase in mol% incorporation occurred between 4 and 12 h, and the smallest increase occurred between 12 and 24 h. Subsequent reactions were then conducted for 24 h unless otherwise indicated. Based on previous work in our laboratory, we chose EEPA, water (2% by weight), and SP435 for this enzymatic modification because this combination produced a higher mol% EPA incorporation compared with EPA free acid and IM60 lipase from *R. miehei* (10).

The molar ratio of substrates (TAG/acyl donor) affected mol% EPA incorporated into the primrose oil. The mol% incorporation increased as the molar ratio increased (Fig. 2). In our previous report with SP 435 lipase, EEPA, and melon seed oil as substrates, EPA incorporation increased with an increase in molar ratio (12). The largest increase (16%) occurred between the molar ratios of 1:1 and 1:2, and the smallest increase (6%) occurred between the molar ratios of 1:2 and 1:3.

The effect of enzyme load is depicted in Figure 3. The greatest increase in mol% EPA incorporation occurred between the enzyme load of 5 and 10%. The smallest increase

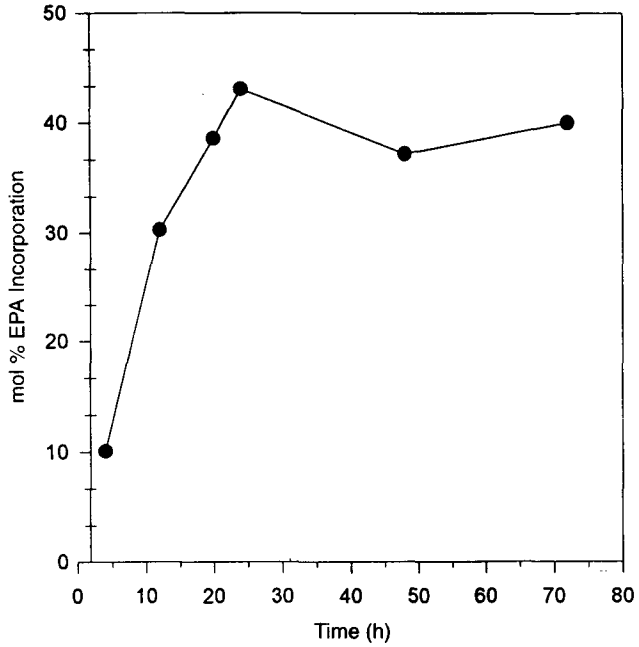

FIG. 1. Time course of SP435 (Novo Nordisk Bioindustrial, Inc., Danbury, CT) lipase-catalyzed modification of evening primrose oil in hexane by incorporation of eicosapentaenoic acid (EPA) with EPA ethyl ester (EEPA) as the acyl donor as determined by gas–liquid chromatography. Molar ratio of evening primrose oil to EEPA = 1:3. Incubation was at 55°C and 200 rpm. Samples were analyzed at 4, 12, 20, 24, 48, and 72 h in duplicate.

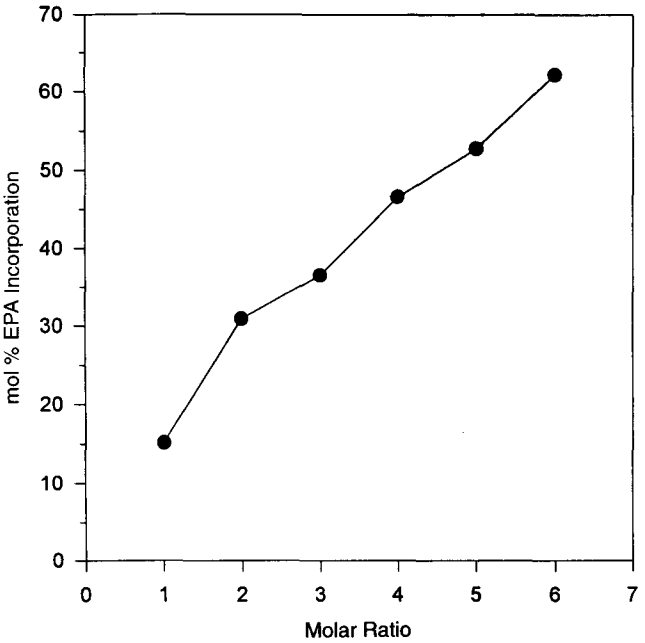

FIG. 2. Effect of molar ratio of substrates (evening primrose oil to EEPA) on EPA incorporation. The numbers 1–6 correspond to molar ratios of 1:1, 1:2, 1:3, 1:4, 1:5, and 1:6, respectively. Incubation was in hexane for 24 h at 55°C and 200 rpm. Enzyme amount was 10% by weight of reactants. See Figure 1 for company source, conditions, and abbreviations.

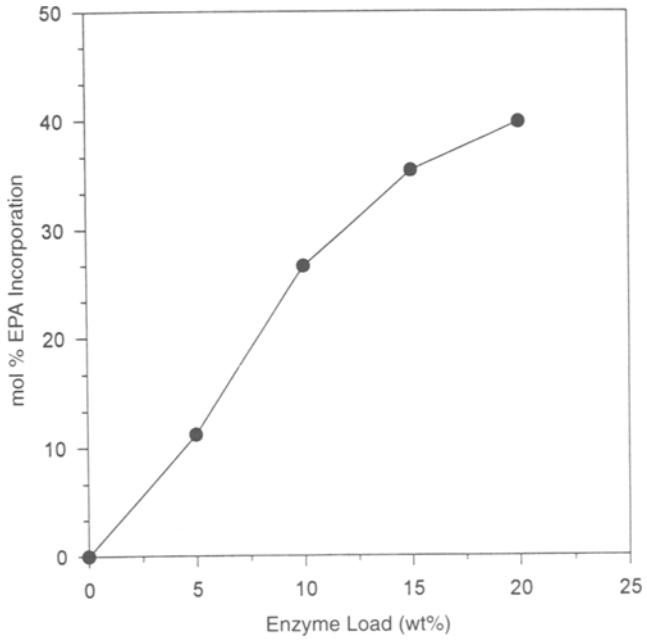

FIG. 3. Effect of enzyme load on EPA incorporation with SP435 as biocatalyst. Amount of enzyme was based on wt% of reactants. Molar ratio of evening primrose oil to EEPA = 1:3. Incubation was in hexane for 24 h at 55°C and 200 rpm. See Figure 1 for company source, conditions, and abbreviations.

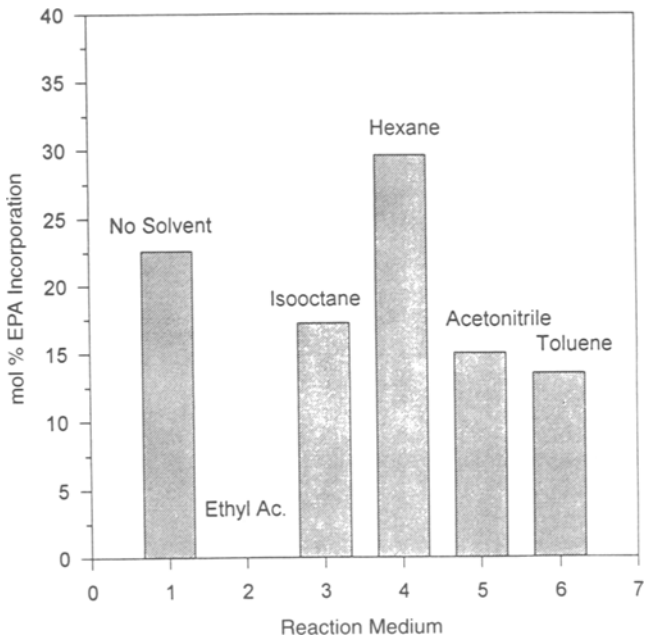

FIG. 4. Effect of different organic solvents as reaction media for the ester–ester interchange reaction catalyzed by SP435 lipase. The reaction mixture was incubated for 24 h at 55°C and 200 rpm. Log *P* values for ethyl acetate (Ethyl Ac.) = 0.68; isooctane = 4.5; hexane = 3.5; acetonitrile = −0.33; and toluene = 2.5 (Ref. 16). Molecular sieves were not used in the solvent-free incubations. See Figure 1 for company source and other abbreviation.

occurred between the enzyme load of 15 and 20%. By selecting proper reaction time and enzyme amount, it is possible to obtain high incorporation of EPA into evening primrose oil.

Polarity of solvents or hydrophobicity can have profound effects on the retention of enzyme-associated water necessary for enzyme catalysis (10). A good indicator of solvent polarity is the log *P* value, which is the partition coefficient between water and octanol (16). Various solvents were tested as reaction media to determine their effects on mol% incorporation (Fig. 4). The highest mol% EPA incorporation was achieved in hexane (30%, log $P = 3.5$) while isooctane (log $P = 4.5$) produced the second highest EPA incorporation. EPA was not incorporated when ethyl acetate (log $P = 0.68$) was used as the solvent. The solvent-free sample gave acceptable EPA incorporation (23%). In general, solvents with log $P \leq 2$ allow little enzyme activity, whereas those with log $P \geq 4$ allow high enzyme catalysis (16). We concluded that hexane was the best solvent for the ester–ester interchange reaction.

The fatty acid profiles of evening primrose oil before and after modification are given in Table 1. The fatty acid profile of evening primrose oil before modification was similar to reported values (17). Hill *et al.* (18) reported the enrichment of GLA content in evening primrose oil and docosahexaenoic acid in cod liver oil by selective lipase-catalyzed esterification in butanol. The 18:2n-6 fatty acid content of evening primrose oil was reduced by 32% after modification. GLA content was not significantly altered. The n-3/n-6 ratio changed from 0.01 to 0.60 after modification. The saturated fatty acid content was reduced by half.

The reproducibility of the transesterification process in our laboratory was established by completing six replicate incubations and analyses of the modified products fatty acid composition. The mean, standard deviation, and coefficient of variation (CV) for selected fatty acids, such as linoleic acid, GLA and EPA, after incubations on six separate days are given in Table 2. The CV for the three fatty acids were within 5.1–8.1%, indicating that our results are reproducible.

TABLE 1
Fatty Acid Composition (mol%) of Evening Primrose Oil Before and After Enzymatic Modification[a]

Major fatty acids	Before modification	After modification
16:0	8.8	4.7
18:0	2.1	0.8
18:1n-9	4.6	2.9
18:2n-6	75.6	51.0
18:3n-6	8.1	5.7
18:3n-3	0.8	ND
20:5n-3	ND	34.0
Total saturated	10.9	5.5
Total n-6	83.7	56.7
Total n-3	0.8	34
n-3/n-6 ratio	0.01	0.60

[a]ND = not detectable. Reaction was conducted in hexane with SP435 lipase (Novo Nordisk Bioindustrial, Inc., Danbury, CT) as biocatalyst. The reaction mixture was incubated at 55°C in an orbital shaking water bath for 24 h at 200 rpm. Molecular sieves 4 Å were added after 2 h.

TABLE 2
Mean, Standard Deviation, and Coefficient of Variation (%CV) of Selected Fatty Acids (mol%) from Six Replicate Incubations[a]

Fatty acid	Mean ± SD	%CV
18:2n-6	52.3 ± 2.7	5.1
18:3n-6	5.9 ± 0.4	6.3
20:5n-3	32.3 ± 2.6	8.1

[a]SP435 lipase-catalyzed modification of evening primrose oil in hexane by incorporation of eicosapentaenoic acid as determined by gas–liquid chromatography. Molar ratio of primrose oil/eicosapentaenoic acid ethyl ester = 1:3. The reaction mixture was incubated at 55°C in an orbital shaking water bath for 24 h at 200 rpm. Molecular sieves 4 Å were added after 2 h. Company source as in Table 1.

We have shown for the first time that evening primrose oil can be modified to incorporate EPA with SP435 as a biocatalyst. We have effectively increased the n-3 PUFA and lowered the n-6 PUFA concentrations of evening primrose oil. Modified evening primrose oil, containing both GLA and EPA, may be potentially more beneficial than unmodified evening primrose oil. The health and nutritional benefits of the modified oil should be investigated.

ACKNOWLEDGMENTS

Contributed by the Agricultural Experiment Station, College of Agricultural and Environmental Sciences, The University of Georgia. Research supported by Food Science Research.

REFERENCES

1. Gunstone, F.D., Gamma-Linolenic Acid—Occurrence and Physical and Chemical Properties, *Prog. Lipid Res. 31*:145–161 (1992).
2. Horrobin, D.F., Nutritional and Medical Importance of Gamma-Linolenic Acid, *Ibid. 31*:163–194 (1992).
3. Barcelli, U.O., M. Weiss, D. Beach, A. Motz, and B. Thompson, High Linoleic Acid Diets Ameliorate Diabetic Nephropathy in Rats, *Am. J. Kidney Dis. 16*:244–251 (1990).
4. Deferne, J-L., and A.R. Leeds, The Antihypertensive Effects of Dietary Supplementation with a 6-Desaturated Essential Fatty Acid Concentrate as Compared with Sunflower Seed Oil, *J. Human Hypertens. 6*:113–119 (1992).
5. Kernoff, P.B.A., A.L. Willis, K.J. Stone, J.A. Davies, and G.P. Nichols, Antithrombotic Potential of Dihomo-Gamma-Linolenic Acid in Man, *Br. Med. J. 2*:1441–1444 (1977).
6. Wright, S., and J.L. Burton, Oral Evening Primrose Seed Oil Improves Atopic Eczema, *Lancet 57*:1120–1122 (1982).
7. Kunkel, S.L., H. Ogawa, P.A. Ward, and R.B. Zurier, Suppression of Chronic Inflammation by Evening Primrose Oil, *Prog. Lipid Res. 20*:885–888 (1981).
8. Kinsella, J.E., Food Lipids and Fatty Acids: Importance in Food Quality, Nutrition, and Health, *Food Technol. 42*:124–145 (1988).
9. Johnston, P., Perspectives on Omega-3 Fatty Acids, *J. Am. Oil Chem. Soc. 64*:716–717 (1987).
10. Huang, K.-H., and C.C. Akoh, Lipase-Catalyzed Incorporation of n-3 Polyunsaturated Fatty Acids into Vegetable Oils, *Ibid. 71*:1277–1280 (1994).
11. Akoh, C.C., B.H. Jennings, and D.A. Lillard, Enzymatic Modification of Trilinolein: Incorporation of n-3 Polyunsaturated Fatty Acids, *Ibid. 72*:1317–1321 (1995).
12. Huang, K.-H., C.C. Akoh, and M.C. Erickson, Enzymatic Modification of Melon Seed Oil: Incorporation of Eicosapentaenoic Acid, *J. Agric. Food Chem. 42*:2646–2648 (1994).
13. Chapkin, R.S., S.D. Somers, L. Schumaker, and K.L. Erickson, Fatty Acid Composition of Macrophage Phospholipids in Mice Fed Fish or Borage Oil, *Lipids 23*:380–383 (1988).
14. Miller, C.C., V.A. Ziboh., T. Wong, and M.P. Fletcher, Dietary Supplementation with Oils Rich in n-3 and n-6 Fatty Acids Influences *in vivo* Levels of Epidermal Lipoxygenase Products in Guinea Pigs, *J. Nutr. 120*:36–44 (1990).
15. Fan, Y.-Y., and R.S. Chapkin, Mouse Peritoneal Macrophage Prostaglandin E_1 Synthesis Is Altered by Dietary Gamma-Linolenic Acid, *Ibid. 122*:1600–1606 (1992).
16. Laane, C., S. Boeren, K. Vos, and C. Veeger, Rules for Optimization of Biocatalysis in Organic Solvents, *Biotechnol. Bioeng. 30*:81–87 (1987).
17. Chapkin, R.S., V.A. Ziboh, and J.L. McCullough, Dietary Influences of Evening Primrose and Fish Oil on the Skin of Essential Fatty Acid-Deficient Guinea Pigs, *J. Nutr. 117*:1360–1370 (1987).
18. Hills, M.J., I. Kiewitt, and K.D. Mukherjee, Enzymatic Fractionation of Fatty Acids: Enrichment of Gamma-Linolenic Acid and Docosahexaenoic Acid by Selective Esterification Catalyzed by Lipases, *J. Am. Oil Chem. Soc. 67*:561–564 (1990).

[Received November 3, 1995; accepted April 3, 1996]