Lipase-Catalyzed Modification of Phospholipids: Incorporation of n-3 Fatty Acids into Biosurfactants

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Phospholipids were successfully modified by lipase-catalyzed transesterification to incorporate n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6). The phospholipid modification was carried out in organic media with lipase from *Mucor miehei* (lipozyme) as biocatalyst. The parameters studied were the effect of different solvents, enzymes, acyl donor type, phospholipid class, water, enzyme and substrate concentrations. Hydrolysis of phosphatidylcholine to yield lysophosphatidylcholine and the synthesis of phosphatidylcholine from lysophosphatidylcholine was also carried out. The optimal conditions for the modification of phospholipids by transesterification were obtained with phosphatidylcholine and free eicosapentaenoic acid EPA 45 as acyl donor in the presence of 15% w/w nonimmobilized *Mucor miehei* lipase (lipozyme) in hexane with no added water. The maximum incorporation of EPA 45 was 17.7 mol%. Hydrolysis was easily achieved with phospholipase A_2 in benzene and Tris-HCl buffer. The synthesis of phosphatidylcholine was difficult, and when it was achieved, not enough phosphatidylcholine was obtained for quantitation.

KEY WORDS: Biosurfactants, enzymatic modification, enzymatic synthesis, lipases, n-3 fatty acids, organic media, phospholipids, transesterification.

Phospholipids from natural sources contain several fatty acids, and their proportion depends on the source. For some practical applications it is desirable to have phospholipids that contain specific fatty acids. Phospholipids with specific fatty acid compositions can sometimes be obtained by fractionation of natural phospholipids, but the most common approach is to synthesize the desired compounds.

One promising synthetic approach is to use natural phospholipids as starting material and to replace the existing fatty acids with the desired ones (1). Enzymatic synthesis methods, which are characterized by mild reaction conditions and high selectivity, can be expected to be of great importance in the modification of phospholipids. The group of enzymes that are most likely to catalyze transesterifications of phospholipids are the phospholipases. Phospholipase A_1 and A_2 are specific for the hydrolysis of the fatty acids in the *sn*-1 and *sn*-2 positions, respectively. Other lipases, which in general are either nonspecific or are specific for the *sn*-1 and *sn*-3 positions, may also be useful in phospholipid modifications. The natural substrate for lipases are triglycerides, but many of these enzymes have broad substrate specificity and have been used for breaking and forming of ester bonds in a wide variety of compounds. Recent reports indicate that fatty acids of triglycerides can be exchanged by lipase-catalyzed interesterification reactions (2,3). Phospholipases and lipases whose purpose in nature is to catalyze hydrolytic reactions can also be manipulated to catalyze interesterification or synthetic reactions under defined conditions.

Tobani and Hara (4) reported the transesterification of soy phospholipid with fish oil by lipases from *Candida cylindracea* and *Rhizopus delemar*; the former resulted in greater incorporation of polyunsaturated fatty acids (PUFAs) than lipase from *R. delemar*. They were able to synthesize phosphatidylcholine containing 14.5% eicosapentaenoic acid (EPA) in a biphasic medium of hexane and water, but they reported a problem with the loss of the phosphatidylcholine fraction to lysophosphatidylcholine due to the competing hydrolytic reaction, which reduced the recovery of the phospholipids significantly. They attempted to suppress the hydrolytic reaction by substituting water with glycerine, which did increase the recovery of the phospholipid but decreased the degree of translipase fromification. Na *et al.* (5) suggested that phosphatidylcholine esterified with EPA or docosahexaenoic acid (DHA) at the *sn*-2 position could be more easily digested in the body and might be of value in nutritional and medical applications. The same authors reported the successful synthesis of n-3 fatty acid containing phosphatidylcholine by esterifying lysophosphatidylcholine with EPA and DHA in a reaction catalyzed by phospholipase A_2 in sodium *bis*(2-ethylhexyl)-sulfosuccinate-based microemulsions containing small amounts of water, but they were unable to synthesize the same phospholipid by transesterification of phosphatidylcholine.

The aim of this study was to synthesize and modify phospholipids to incorporate n-3 fatty acids by using lipase enzymes in organic media.

MATERIALS AND METHODS

Phospholipon 90 (90% phosphatidylcholine, 6% lysophosphatidylcholine) and Alcolec 678G (high in phosphatidylinositol) were kindly provided by American Lecithin Co. (Danbury, CT). Lysophosphatidylcholine, lysophosphatidylinositol, L-α phosphatidylcholine (95% pure), L-α phosphatidylethanolamine (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), L-α phosphatidylinositol (liver sodium salt) and L-α phosphatidylserine (brain, sodium salt) were purchased from Avanti Polar-Lipids Inc. (Alabaster, AL). ANS salt (8-anilino-1-naphthalenesulfonic acid ammonium salt, 97% pure) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Heptadecanoic acid (99% pure), Trizma hydrochloride (*tris* [hydroxymethyl]-aminomethane hydrochloride, reagent grade), Trizma base (*tris*[hydroxymethyl]-aminomethane, reagent grade) and calcium chloride dihydrate (99% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). EPA 45 (45% eicosapentaenoic acid) was supplied by Callanish Ltd., (Scotland, United Kingdom). EPA ethyl ester (97% pure) and DHA ethyl ester (96% pure) were kindly provided by the United States Department of Commerce, National Marine Fisheries Service (Charleston, SC). Immobilized nonspecific lipase SP382 (40 BIU/g) from *Candida* sp., IM20 (24.0 BIU/g) from *Mucor miehei* and phospholipase A_2 (12,000 IU/g)

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were kindly provided by Novo Nordisk Bioindustrials Inc. (Danbury, CT). BIU means Batch Interesterification Unit, *i.e.*, one μmole of incorporated palmitic acid into triolein per minute under standard conditions as defined by the manufacturer. Lipomod 200I (immobilized, unspecified activity) from *Candida cylindraceae*, nonimmobilized lipase from *Mucor miehei* (10,000 L/g) and phospholipase A_1 (unspecified activity) were supplied by Biocatalysts Ltd. (Wales, United Kingdom). Enzyco Lipopanc pancreatic lipase (double strength) was kindly provided by Biddle Sawyer Corp. (New York, NY). Lipase G (51,000 U/g) from *Penicillium cyclopium* and lipomod PC (unspecified activity, a broad spectrum mixed lipase) were kindly provided by Amano International Enzyme Co. Inc. (Troy, VA). Silica gel 60 thin-layer chromatography (TLC) plates were purchased from E. Merck (Darmstadt, Germany). All solvents high-performance liquid chromatography grade (HPLC) were obtained from Fisher Scientific (Norcross, GA).

Transesterification method. In a typical synthesis of modified phosphatidylcholine (MOD PC), 72.5 mg of phosphatidylcholine (P90) was combined with 124 mg of EPA (EPA 45), 29.48 mg (15% w/w) of nonimmobilized lipozyme (*Mucro miehei*) and 5 mL of solvent in a screw-cap test tube. The mixture was incubated in a rotary shaking water bath at 55°C for 72 h at 200 rpm, and the products were extracted. All the reactions were carried out in duplicate.

Hydrolysis of phospholipids. In a typical hydrolysis experiment, 100 mg of phosphatidylcholine (P90), 10 mg phospholipase A_2, 50 μL Tris-HCl buffer or water and 5 mL organic solvent (*e.g.*, benzene) were mixed in a screw-cap test tube. The mixture was incubated in an orbital shaking water bath at 55°C for 72 h at 200 rpm, and the products were extracted for analysis.

Synthesis of phosphatidylcholine from lysophosphatidylcholine. In a typical experiment, 5 mg of commercially obtained lysophosphatidylcholine was mixed with 22.8 mg EPA 45, 5.6 mg lipase (IM 20) and 3 mL of chloroform/methanol (1:1). The mixture was incubated in an orbital shaking water bath at 55°C for 72 h at 200 rpm, and the products were extracted for analysis.

Phospholipid analysis. The reaction products were cooled, the enzyme was filtered out and the filtrate was dried under N_2. The residue was redissolved in 1 mL of chloroform and dried over a column of anhydrous sodium sulfate. TLC was used to purify and isolate the phospholipid bands. A 50-μL aliquot of the reaction product was co-plated with phosphatidylcholine or phosphatidylinositol and a free fatty acid on pre-coated silica gel 60 plates activated by heating at 110°C for 1 h. The plates were developed with $CHCl_3$/MeOH/HOAc/H_2O (50:37.5:3.5:2.0, by vol), and the bands were visualized by examination under ultraviolet light after spraying with 0.1% ANS salt. The bands corresponding to lysophosphatidylcholine, phosphatidylcholine and free fatty acids (R_f = 0.08, 0.27 and 0.98, respectively) were scraped into a test tube to which a 10-mg solution of heptadecanoic acid (17:0) internal standard was added. Three milliliters of 6% HCl in methanol vol/vol was added, and the mixture was incubated at 70–80°C overnight. The fatty acid methyl esters were extracted twice with hexane, dried over anhydrous sodium sulfate, evaporated completely and redissolved in 75 μL of methylene chloride. One microliter of this extract was analyzed by gas-liquid chromatography. The hydrolysis samples were extracted with 1 mL chloroform, and 20 μL was injected into a Beckman System Gold Module HPLC System (Beckman Instruments, Fullerton, CA) fitted with a 20-μL loop, a silica normal-phase precolumn 4.6 mm × 4.5 mm and a silica normal-phase column 4.6 mm × 250 mm. Both columns were packed with 5μ ultrasphere-ODS (Altex, San Ramon, CA). Component phospholipids were detected at 206 nm by means of a Beckman 167 ultraviolet visible scanning detector. The mobile phase was acetonitrile/methanol/water (50:45:2, vol/vol) run isocratically, and the flow rate was 1.5 mL/min. Authentic phospholipid standards were prepared and analyzed by HPLC as described above for identification of the products and to optimize separation conditions. The retention times for phosphatidylcholine and lysophosphatidylcholine were 13.1 and 18.2 min, respectively. The relative amount of phospholipids as mol% were quantitated by an on-line computer based on total phospholipids.

RESULTS AND DISCUSSION

Effect of different enzymes. Nine different enzymes [phospholipase A_2, phospholipase A_1, nonimmobilized lipase (*Mucor miehei*), IM20 (*Mucor miehei*, lipozyme, immobilized), immobilized SP382 (*Candida* sp.), Lipomod 200I (*Candida cylindraceae*), pancreatic lipase, Lipomod PC (broad-spectrum mixed lipase) and lipase G (*Penicillium cylcopium)*] were used to incorporate EPA (EPA 45) into phosphatidylcholine in hexane by our transesterification method. The enzyme that gave the best incorporation was nonimmobilized lipase from *Mucor miehei* (17.7 mol%) followed by nonimmobilized phospholipase A_2 (17.2 mol%). Phospholipase A_1 gave 14.1 mol%; SP382 lipase, 8.5 mol%; IM20, 1.0 mol%; Lipomod 200I, 0.5 mol%; and pancreatic lipase 0.1 mol% incorporation of eicosapentaenoic acid, respectively. Lipomod PC and lipase G gave zero incorporation. Svensson *et al.* (1) reported the incorporation of heptadecanoic acid (18.6%) into phosphatidylcholine by transesterification reactions with immobilized lipase from *Mucor miehei* (lipozyme). Lipases from *Rhizopus arrhizus* and plant lipase extracted from potato tubers were also used, but they reported that the lipozyme was the most active enzyme. Yoshimoto *et al.* (6) used lipase from *C. cylindraceae* in benzene to obtain 8.5% incorporation of EPA into phosphatidylcholine, with hydrolysis being the predominant reaction. Brockerhoff *et al.* (7) reported that, with *sn*-1 specific lipase from *R. delemar*, the constituent fatty acids at the *sn*-1 position of diacylglycerophosphocholines could be exchanged against other fatty acids added as reaction partners. Mukherjee (8) reported the use of phospholipase A_2 to exchange fatty acids at the *sn*-2 position of diacylglycerophospholipids.

Effect of enzyme concentrations. Enzyme concentrations of 1.0, 5.0, 10.0, 15.0, 20.0 and 25.0% w/w of reactants were used in hexane with the transesterification method to catalyze the incorporation of eicosapentaenoic acid into phosphatidylcholine (Fig. 1). The result indicated 17.7 mol% incorporation at 15.0% w/w of nonimmobilized lipase (lipozyme) from *Mucor miehei*. Any further increase in the enzyme concentration did not result in an increase in incorporation of desired fatty acid, possibly

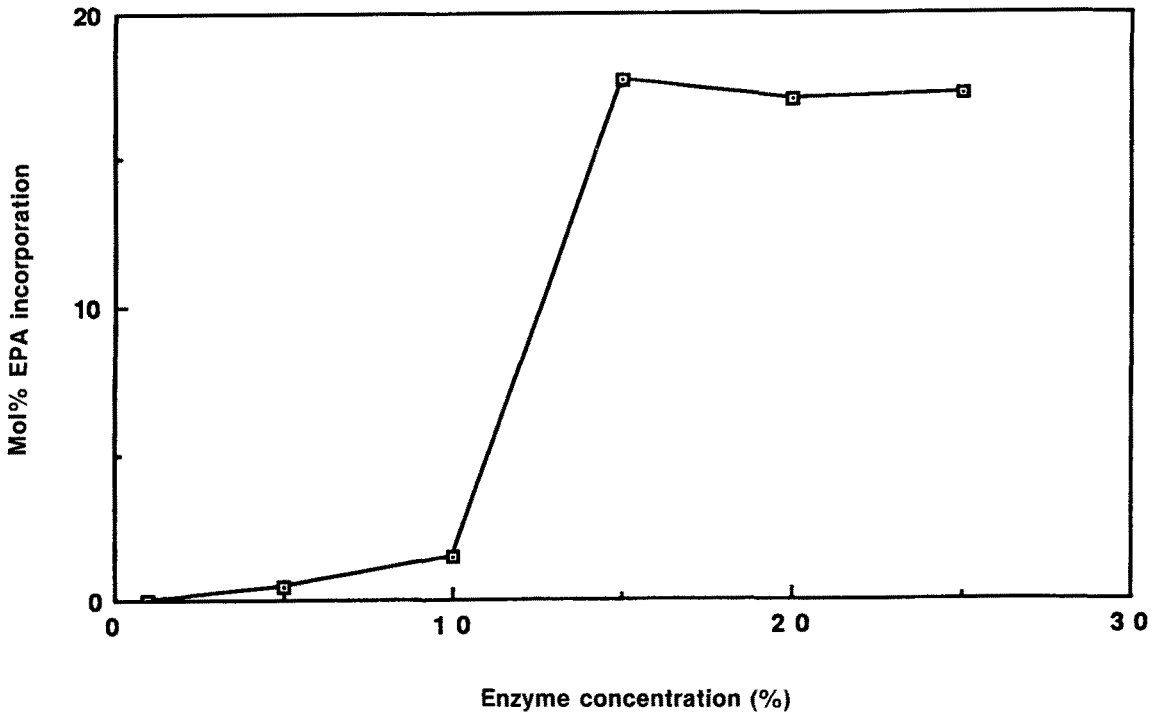

FIG. 1. Effect of enzyme concentration on incorporation of eicosapentaenoic acid (EPA) into phosphatidylcholine with EPA 45 (free EPA) and nonimmobilized lipase from *Mucor miehei* in hexane.

because the substrate concentration became a limiting factor in the face of an excess of active enzyme sites.

Effect of different phospholipids. Four different types of phospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine) were transesterified with EPA in hexane with our transesterification method. Phosphatidylcholine gave the highest EPA incorporation (17.7 mol%) followed by phosphatidylinositol (7.5 mol%), phosphatidylethanolamine (1.6 mol%) and phosphatidylserine (0.73 mol%). There are no available comparative data to date on the various phospholipid classes except for phosphatidylcholine.

Effect of different solvents. Six different solvents were compared as media for phospholipid modification with nonimmobilized *Mucor miehei* lipase. The highest EPA incorporation (17.3 mol%) was obtained with hexane (Table 1). All other solvents gave poor incorporation of EPA (<1.0 mol%). With water-saturated toluene and lipozyme, Svensson *et al.* (1) reported 24.1% and 18.8% incorporation of tetradecanoic acid and heptadecanoic acid, respectively, into phosphatidylcholine.

Effect of substrate concentrations. The molar ratio of phosphatidylcholine to EPA was varied from 1:1 to 1:4, and modification was carried out with nonimmobilized lipozyme (*Mucor miehei*) and our transesterification method in hexane. The data obtained indicated that the optimal ratio of phospholipid to fatty acid is 1:2 (Table 2). The amount of EPA incorporated onto phosphatidylcholine rapidly increased from 0.24 mol% when 1 mole of EPA was used to 17.3 mol% when 2 moles of the fatty acid was used. Increasing the ratio beyond 1:2 did not increase the amount of EPA incorporated. The reason for this is not clear. Svensson *et al.* (1) supplied fatty acids or fatty acid esters in amounts greatly in excess of those used in this work (10:1, fatty acid/phosphatidylcholine) and obtained incorporation ranging from 6.6% (tetradecanoic acid methyl ester) to 24.1% (tetradecanoic acid). Use of ethyl esters of EPA and DHA resulted in zero incorporation of these fatty acids into phosphatidylcholine.

TABLE 1

Effect of Different Solvents on the Incorporation of Eicosapentaenoic Acid (EPA) into Phosphatidylcholine with Nonimmobilized Lipase from *Mucor miehei* (lipozyme)

Solvent	Mol% incorporation of EPA
Hexane	17.30
n-Heptane	0.57
iso-Octane	0.25
Cyclohexane	0.25
Chloroform	0.12
Toluene	0.04

TABLE 2

Effect of Acyl Donor Concentration on Eicosapentaenoic Acid (EPA) Incorporation into Phosphatidylcholine with *Mucor miehei* Lipase (lipozyme) in Hexane

Moles EPA	Mol% incorporation
1	0.24
2	17.3
3	1.44
4	5.46

Effect of solvents on the hydrolysis of phosphatidylcholine by phospholipase A_2 were also studied. Experiments were carried out with phospholipase A_2 in organic solvents with Tris-HCl buffer pH 8.0 containing 5 mM of Ca^{2+} or with a lipase and water. When benzene was used as the solvent and buffer as the aqueous phase, a yield of 80.1% lysophosphatidylcholine was obtained. In *n*-heptane, the yield was 55.8%. Immobilized lipozyme (IM20) was also used in benzene and *n*-heptane to obtain yields of 21.1 and 20.1%, respectively. These yields were lower than those reported by Na *et al.* (5), and this could be attributed to the different media used for the hydrolytic experiments.

The synthesis of phosphatidylcholine containing a long-chain polyunsaturated acyl group at the *sn*-2 position with phospholipase A_2 was first reported by Na *et al.* (5). They performed the reactions with sodium *bis*(2-ethylhexyl)-sulfosuccinate (AOT)-based microemulsions containing small amounts of water, but they were not able to synthesize the same phospholipid by transesterification of phosphatidylcholine with the polyunsaturated acids in microemulsions. In our experiments we were able to synthesize EPA-containing phosphatidylcholine from lysophosphatidylcholine with phospholipase A_2 or IM20 lipozyme (*Mucor miehei*) in chloroform/methanol (1:1, vol/vol), but the quantities synthesized, though faintly detectable on TLC plates, were not large enough to be quantitated by HPLC.

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REFERENCES

1. Svensson, I., P. Adlercreutz and B. Mattiasson, *Appl. Micro. Biotech. 33*:255 (1990).
2. Macrae, A.R., *J. Am. Oil Chem. Soc. 60*:291 (1983).
3. Wisdom, R.A., P. Dunhill and M.D. Lilly, *Biotechnol. Bioeng. 29*, 1081 (1987).
4. Totani, Y., and S. Hara, *J. Am. Oil Chem. Soc. 68*:848 (1991).
5. Na, A., C. Eriksson, E. Osterberg and K. Holmberg, *Ibid. 67*:766 (1990).
6. Yoshimoto, T., M. Nakata, S. Yamagushi, T. Funada, Y. Saito and Y. Inada, *Biotech. Lett. 8*:771 (1986).
7. Brockerhoff, H., P.C. Schmidt, J.W. Fong and L.J. Tirri, *Lipids 11*:421 (1974).
8. Mukherjee, K.D., *Biocatalysis 3*:277 (1990).

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