Lipase-Catalyzed Preparation of Palmitic and Stearic Acid-rich Phosphatidylcholine

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ABSTRACT: Saturated FA enhance the oxidative stability of phospholipids. In the present study phosphatidylcholine (PC) rich in palmitic and stearic acids was prepared using lipase-catalyzed transesterification from PC isolated from egg and soybean lecithins. Two different lipases, namely, Novozym 435 and Lipozyme TL IM, were used for the transesterification. The reaction conditions were optimized by varying the lipase dosage, molar ratio of PC to FA, and reaction period. Palmitic acid could be incorporated up to 58.6 and 57.1% using Lipozyme TL IM and 56 and 61% using Novozym 435 in egg and soybean PC from an initial content of 37.4 and 16.8%, respectively. Similarly, stearic acid incorporation was up to 44.7 and 46.3% using Lipozyme TL IM and 37.2 and 55.8% using Novozym 435 in egg and soybean PC from an initial content of 8.6 and 2.1%, respectively.

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KEY WORDS: Lipozyme TL IM, Novozym 435, palmitic acid, phosphatidylcholine, stearic acid, transesterification.

Phospholipids (PL) are the main structural constituents of biological membranes and play a crucial role in the biochemistry and physiology of the cell (1). In addition, they are used for several important applications such as emulsifiers in food and pharmaceuticals and in the preparation of liposomes for cosmetics and drug delivery (2). Among the PL, diacyl-*sn*-glycero-3-phosphorylcholine is the most nearly ubiquitous. PC plays a vital role in liver and cell functions and is an alternative to choline chloride and choline bitartarate, which are commonly used in vitamin and nutritional supplements. Stearic or palmitic acid-rich PC can further improve the oxidative stability of PC. Stearic acid-rich PC is used as a lubricating oil additive, in chocolate formulations, and in emulsions for intravenous injections and liposomes (3). Dipalmitoyl PC is used to help normalize surface tension at the air–lung interface, improve respiratory function, and significantly increase neonatal survival (4).

PC is generally isolated from biological materials or plant sources and contains different molecular species with certain combinations of FA. It is very difficult to isolate individual molecular species of PC with a defined FA composition from any natural source. It is also cumbersome to synthesize PC chemically with a specified FA composition because it involves multistep procedures (5,6). Hydrogenation of PC isolated from soybean lecithin requires drastic reaction conditions and yields stearic acid-rich PC along with *trans* FA. According to the World Health Organization and Food and Agricultural Organization, human dietary fats should not contain more than 4% of *trans* FA (7). Introduction of saturated FA into PC by enzymatic transesterification does not lead to the formation of *trans* FA, as it does in the case of catalytic hydrogenation of vegetable oils (8). Enzyme-mediated synthesis of specific PL from natural sources has several advantages stemming from the mild reaction conditions and high stereochemical or positional specificities. Enzymatic methods to incorporate saturated and unsaturated FA into PC using lipases and phospholipases are reported in the literature. Most of the work reported in this direction pertains to the incorporation of hexanoic, octanoic, decanoic, lauric, heptadecanoic, octadecenoic, eicosapentaenoic, docosahexaenoic, and conjugated linoleic acids using lipases or phospholipase-mediated reactions (9–13).

Octanoic acid was incorporated into PC to an extent of 47% by using Lipozyme TL IM (9). The same authors compared the efficiency of Lipozyme TL IM and Novozym 435 for the incorporation of octanoic, eicosapentaenoic, docosahexaenoic, and CLA into PL and reported that Lipozyme TL IM exhibited better performance than Novozym 435. Maximal incorporation (58%) of EPA into PC was reported using a large excess (100%) of Lipozyme (14).

Even though palmitic and stearic acid-rich PC exhibit potential applications in biological and pharmaceutical areas, no attempt has been made to synthesize these PC using enzymatic approaches. In the present study, a lipase-catalyzed preparation of palmitic and stearic acid-rich PC from egg and soybean PC using Novozym 435 and Lipozyme TL IM lipases has been reported.

MATERIALS AND METHODS

Novozym 435 [*Candida antarctica* immobilized on macroporous polyacrylate resin beads with an activity of 10,000 propyl laurate units per gram (PLU/g)] and Lipozyme TL IM [granulated silica preparation of a microbial lipase from *Thermomyces lanuginosa* with an activity of 75 IUN/g (IUN is Interesterification Unit Novo/g)] were gift samples from M/s. Novozymes South Asia Pvt. Ltd. (Banglore, India). Palmitic and stearic acids (98% pure) were purchased from Qualigens Fine Chemicals (Mumbai, India). Common solvents and reagents were purchased from Spectrochem Pvt. Ltd. (Mumbai, India). All the reagents were of analytical grade and were used without further purification.

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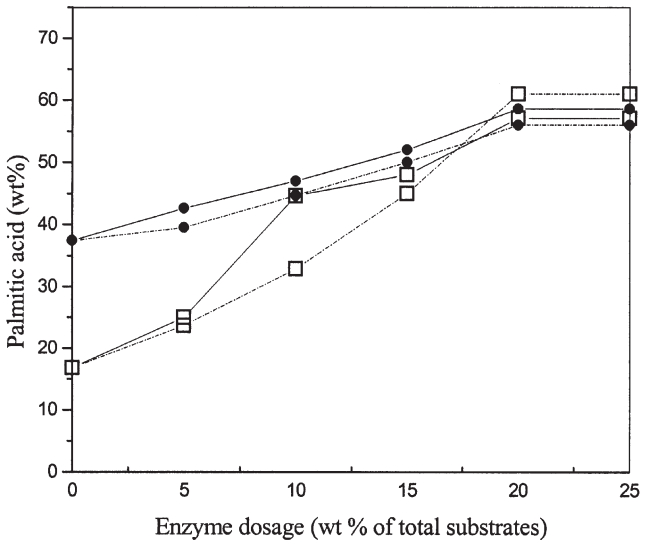

FIG. 1. Effect of enzyme dosage on incorporation of palmitic acid into PC at 65–70°C using Lipozyme TL IM (solid line) and at 50–55°C using Novozym 435 (dotted line) for 24 h using 1:5 molar ratio of PC (soybean , 387 mg, □; egg , 384 mg, ●) to palmitic acid (640 mg). Values are mean of three individual experiments. Enzymes were provided by M/s. Novozymes South Asia Pvt. Ltd. (Bangalore, India).

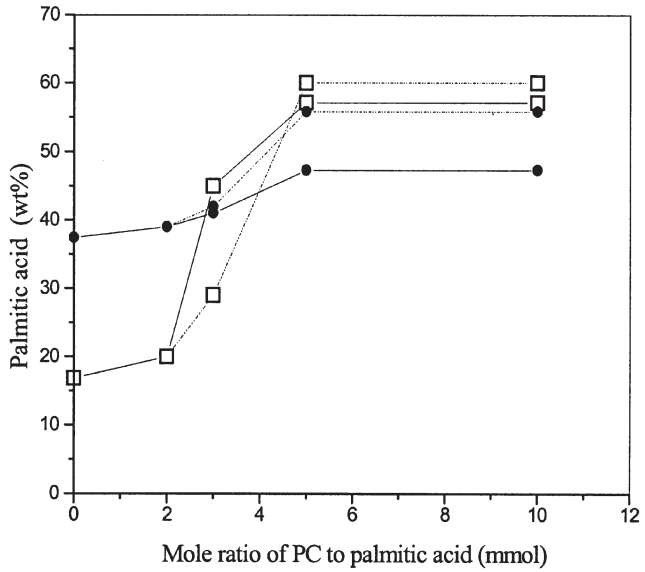

FIG. 2. Effect of molar ratio of palmitic acid to PC on its incorporation into PC (soybean, 387 mg, □; egg, 384 mg, ●) in the presence of Novozym 435 (dotted line) at 50–55°C and Lipozyme TL IM (solid line) at 65–70°C using 20 wt% of total substrates for 24 h. Values are mean of three individual experiments. For enzyme source see Figure 1.

Isolation of PC from egg and soybean lecithin. Crude soybean lecithin was procured from a local soybean oil refinery. Hen's egg yolk was obtained from eggs purchased locally. Crude soybean lecithin (52 g) was dispersed in 100 mL acetone and slowly added to cold (10°C) acetone at an acetone-to-lecithin ratio of 5 mL/g with stirring. The contents were centrifuged at 5–10°C at 7286 × *g*. The acetone-insoluble material was taken in ethanol at a ratio of 5 mL/g ethanol to acetone-insolubles while stirring magnetically for 1 h and centrifuged at 7286 × *g*. The solvent was removed from the ethanol-soluble material to obtain a PL mixture (11.3 g) enriched with PC. Pure PC (2.5 g) was isolated from this mixture by alumina column chromatography (15). Similarly, 5.18 g of pure PC was obtained from 140 g of hen's egg yolk using the above isolation procedure.

General procedure for the transesterification of PC with palmitic and stearic acids. Palmitic acid (2.5 mmol, 640 mg) and soybean PC (0.5 mmol, 387 mg) were placed in a screw-capped tube containing heptane (4 mL), and Lipozyme TL IM (205 mg, 20 wt% of total substrates) was added. The contents were magnetically stirred at 65–70°C after tightly closing the reaction tube. The reaction was also carried out with Novozym 435 (205 mg, 20 wt% of total substrates) at 50–55°C. After 24 h of reaction, lipase was separated by filtration and washed with hexane to recover the reaction product. Modified PC (169.8 mg, 45% yield in the case of Lipozyme TL IM; 176.4 mg, 47% yield in the case of Novozym 435) was separated from the unreacted palmitic acid by silica column chromatography, eluting with a chloroform/methanol mixture at a ratio of 80:20 and 60:40, vol/vol, respectively.

Palmitic acid (2.5 mmol, 640 mg) was also incorporated into egg PC (0.5 mmol, 384 mg) using Lipozyme TL IM and Novozym 435 to obtain modified PC (177 mg, 48% yield in the case of Lipozyme TL IM; 188.5 mg, 50% yield in the case of Novozym 435). Similarly, stearic acid (2.5 mmol, 710 mg) was incorporated into soybean PC (0.5 mmol, 387 mg) using Lipozyme TL IM and Novozym 435 to obtain modified PC (187.2 mg, 48% yield in the case of Lipozyme TL IM; 196.2 mg, 50% yield in the case of Novozym 435). Stearic acid (2.5 mmol, 710 mg) was also incorporated into egg PC (0.5 mmol, 384 mg) using Lipozyme TL IM and Novozym 435 to obtain modified PC (162.3 mg, 44% yield in the case of Lipozyme TL IM; 174.4 mg, 45% yield in the case of Novozym 435).

FA analysis. The FA composition of PC was determined after converting it to FAME by transesterification using 0.5 M sodium methoxide in methanol for about 30 min at 50°C (16). The GC analysis was carried out with an Agilent 6850 unit equipped with FID by using a nonbonded cyanosilicone column SP-2330 (30 m × 25 mm i.d. × 0.20 mm film thickness). The oven temperature was programmed from 170 to 220°C at 5°C/min, and the flow rate of carrier gas (N_2) was 1.5 mL/min. The injector and detectors were maintained at 250 and 275°C, respectively, and the area percentage was recorded with a Hewlett-Packard HP ChemStation data system.

RESULTS AND DISCUSSION

In spite of the fact that PC that are rich in saturated FA have several unique applications, not many groups have attempted to synthesize these compounds using lipase-catalyzed reactions. In the present study an attempt was made to incorporate palmitic and stearic acids into soybean and egg PC, using *sn*-1,3 specific (Lipozyme TL IM) and nonspecific (Novozym 435) lipases. The effects of lipase dosage, FA molar ratio with

TABLE 1
FA Composition[a] (wt%) of Egg and Soybean PC Before and After Incorporation of Palmitic and Stearic Acid Under Optimal Reaction Conditions (using Novozym 435/Lipozyme TL IM)[b]

	Egg					Soybean				
		Novozym 435		Lipozyme TL IM			Novozym 435		Lipozyme TL IM	
FA	PC	PA-rich PC	SA-rich PC	PA-rich PC	SA-rich PC	PC	PA-rich PC	SA-rich PC	PA-rich PC	SA-rich PC
16:0	37.4	56.0	23.0	58.6	18.8	16.8	61.0	4.0	57.1	12.2
16:1	2.4	2.0	1.5	1.2	1.6	—	—	—	—	—
18:0	8.6	3.0	37.2	4.3	44.7	2.1	2.1	55.8	2.1	46.3
18:1	34.2	27.4	26.0	23.0	28.8	20.3	6.0	17.2	6.0	20.3
18:2	12.9	10.0	8.7	9.9	5.0	57.0	27.9	21.0	31.0	20.1
18:3	—	—	—	—	—	3.8	3.0	2.0	3.8	1.1
20:4	4.5	1.6	3.6	3.0	1.1	—	—	—	—	—

[a]Values are mean of three individual experiments.
[b]Enzymes were provided by M/s. Novozymes South Asia Pvt. Ltd. (Bangalore, India). PA, palmitic acid; SA, stearic acid.

respect to substrate, and reaction periods were optimized to obtain maximal incorporation of palmitic and stearic acids by Lipozyme TL IM and Novozym 435, respectively.

Effect of lipase dosage. The effect of lipase on the incorporation of palmitic and stearic acids into egg and soybean PC was studied using both lipases independently by varying the dosage from 5 to 25% (wt% of total substrates) while maintaining the constant molar ratio of substrates (PC/FA, 1:5 w/w) at 65–70°C using Lipozyme TL IM and 50–55°C in the case of Novozym 435 for 24 h. As the Lipozyme dosage increased from 5 to 20%, the incorporation of palmitic acid reached up to 58.6 and 57.1% from an initial content of 37.4 and 16.8% in egg and soybean PC, respectively. Likewise, the incorporation of palmitic acid into egg and soybean PC was up to 56 and 61%, respectively, when using Novozym 435 (Fig.1).

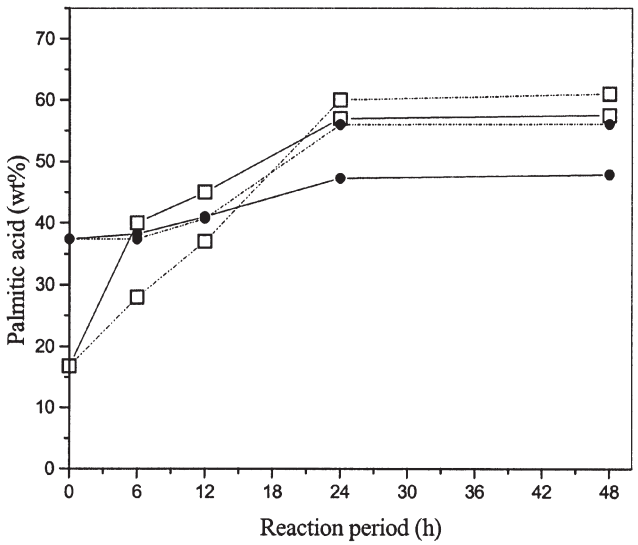

FIG. 3. Time course of the incorporation of palmitic acid into PC using a 1:5 molar ratio of PC (soybean, 387 mg, □; egg, 384 mg, ●) to palmitic acid (640 mg) in the presence of Lipozyme TL IM (solid line) at 65–70°C and Novozym 435 (dotted line) at 50–55°C using 20 wt% of total substrates for 24 h. Values are mean of three individual experiments. For enzyme source see Figure 1.

The incorporation of stearic acid into egg and soybean PC was also studied by varying the lipase dosage from 5 to 25% (wt% of total substrates) using the above reaction conditions. The maximal incorporation of stearic acid was 44.7 and 46.3% from an initial content of 8.6 and 2.1% in egg and soybean PC, respectively, using Lipozyme TL IM. With Novozym 435, stearic acid was incorporated up to 37.2 and 55.8% in egg and soybean PC, respectively. Further increase in the dosage of enzyme (25%) did not show any significant change in the incorporation of palmitic and stearic acids into the modified PC. Hence, the highest incorporation of palmitic/stearic acids into PC was achieved using 20% (w/w of total substrates) lipase and similar amounts of lipase in further reaction optimization studies.

Effect of FA molar ratio. Using 20% (wt% of total substrates) lipase, we optimized the molar ratio of PC to palmitic acid by varying it from 1:2 to 1:10 (Fig. 2). The PC to FA (both palmitic and stearic) molar ratio for maximal incorporation was 1:5. On further increase from 1:5 to 1:10 molar ratio, no significant change in the incorporation was observed.

Effect of reaction period. Using the above conditions of enzyme dosage and PC to FA molar ratio, we considered the effect of reaction period. The incorporation of palmitic (Fig. 3) and stearic acids using both the enzymes was maximal at a reaction period of 24 h. The reaction was continued for a further 24 h, and there was no additional incorporation of FA during this time.

Based on the present study, the best reaction conditions for the maximal incorporation of palmitic and stearic acids into egg and soybean PC were found to be 20% (w/w) of lipase dosage, and a 1:5 (w/w) molar ratio of PC to FA for a reaction period of 24 h. Table 1 shows the FA composition of modified PC (stearic/palmitic acid-rich) of egg and soybean PC, prepared using both lipases at our best reaction conditions.

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