Biotechnol Lett (2009) 31:705–709
DOI 10.1007/s10529-009-9915-6

ORIGINAL RESEARCH PAPER

Enzymatic enrichment of egg-yolk phosphatidylcholine with α-linolenic acid

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Received: 29 October 2008 / Revised: 17 December 2008 / Accepted: 19 December 2008 / Published online: 23 January 2009

Abstract α-Linolenic acid (ALA) was incorporated at 28% into the *sn*-1 position of egg-yolk phosphatidylcholine using Novozyme 435 in one-step transesterification process. Using phospholipase A_2 in a two-step process gave 25% incorporation of ALA into the *sn*-2 position.

Keywords Egg-yolk phosphatidylcholine · α-Linolenic acid · Lipase · Phospholipase · Transesterification

Introduction

Phospholipids (PLs) are major constituents of cell membranes and play crucial role in the biochemistry and physiology of the cell. They are widely used as emulsifiers in foods, pharmaceutical and cosmetic products (Mead et al. 1986; Guo et al. 2005). Commercially available PLs are isolated from soybean oil, sunflower oil and egg yolk. The egg yolk lecithin comprises 78% (w/w) phosphatidylcholine (PC), 18% (w/w) phosphatidylethanolamine (PE) and 2% (w/w) cholesterol (Guo et al. 2005). Phospholipids from natural sources contain several acyl groups whose proportions depend on the source (Haraldsson and Thorarensen 1999; Reddy et al. 2005). A typical fatty acyl composition of PC isolated from egg yolk is: palmitic (16:0; 33%,), stearic (18:0; 11%), oleic (18:1; 32%) and linoleic (18:2; 9%). PC from egg yolk thus contains mainly saturated and there are no *n*-3 polyunsaturated fatty acids (PUFA).

Regular consumption of even small amounts of *n*-3 fatty acids may reduce the risk of coronary disease, may be helpful in the treatment and prevention of hypertension and could reduce cardiovascular risk, mainly risk of sudden cardiac death (Bigger and El-Sherif 2001). The recommended ratio of *n*-6/*n*-3 intake should be between 5:1 and 3:1. α-Linolenic acid [(ALA); 18:3 (9,12,15)] occurs naturally in many plants oils, such as linseed, soybean and rapeseed oils. In humans, α-linolenic acid is metabolized to other biologically active PUFA (EPA, DHA) by enzymatic desaturation and elongation of the chain. Increase of ALA concentration in human tissues by only 1% lowers the systolic blood pressure (SBP) by 5 mm Hg (Berry and Hirsch 1986). ALA is an essential fatty acid and therefore must be supplied in food. It would therefore be valuable from the nutritional point of view to incorporate ALA into the PL isolated from egg yolk.

Enzymatic methods to incorporate specific fatty acids into PC using lipases and phospholipases have been reported but most work pertains to the incorporation

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of medium-chain and long-chain saturated fatty acids, eicosapentaenoic (EPA), docosahexaenoic (DHA), and conjugated linoleic acid (CLA) (Svenson et al. 1993; Adlecreutz et al. 2003; Adlecreutz and Wehtje 2004; Reddy et al. 2005) mainly into the soya lecithin (Aura et al. 1995; Hara et al. 2002; Peng et al. 2002; Vikbjerg et al. 2005a, b). Only a few reports are available on the enzymatic modification of PC from egg yolks (Haraldsson and Thorarensen 1999; Doig and Diks 2003; Reddy et al. 2005) and no data are given about the enriching egg-yolk PC with ALA.

The incorporation of desired fatty acids in the *sn*-1 position is possible using phospholipase A_1 (PLA_1) or lipases because of their regiospecificity for the ester bond in the *sn*-1 position of the phospholipids. Modification of *sn*-2 position is possible with phospholipase A_2 (PLA_2) (Guo et al. 2005; Vikbjerg et al. 2005a, b). There are two general reaction routes for the modification of phospholipids. The first route is carried out in two steps: hydrolysis of PC to its lyso-form (LPC) and re-esterification of LPC with a fatty acid. The other route is conducted in one-step process—acidolysis or interesterification between PLs and fatty acids or their esters (for example fish oils), respectively.

To modify PC as food additive, and taking into consideration the lack of α-linolenic acid in PC from egg yolk, we decided to incorporate this in the *sn*-1 and *sn*-2 positions of phosphatidylcholine using enzymatic methods. Here we present the first results of our studies.

Materials and methods

Materials

The Lohmann Brown hens' eggs were the gift from Tronina factory. Phospholipase A_2 from porcine pancreas (Lecitase 10L, 10,000 U/ml) and immobilized *sn*-1,3-specific lipase from *Thermomyces lanuginosa* (Lipozyme TL IM, 250 U/g) were the gift from the Novozymes A/S. Lipase from *Candida antarctica* immobilized on acrylic resin (Novozym 435, >10,000 U/g) was obtained from Sigma-Aldrich. Immobilized *sn*-1, 3-specific lipase from *Mucor miehei* (Lipozyme, 37 U/g) and α-linolenic acid (content: 70% α-linolenic acid, 25% linoleic acid and 5% oleic acid) were purchased from Fluka.

Analysis

The progress of enzymatic reactions and the purity of products were monitored by TLC on silica gel-coated aluminium plates (chloroform/methanol/water, 65:25:4, by vol.) and the lipids visualized by I_2 vapor. The modified phospholipids were purified by means of column chromatography on silica gel (Kieselgel 60, 230–400 mesh) using the same eluent as mentioned above.

HPLC analysis of PLs were done using DIONEX UltiMate 3000 LC system equipped with a CORONA CAD detector (ESA Biosciences, Chelmsford, MA). A Waters Spherisorb S5 W column (150 × 4.6 mm) was used for analysis. HPLC gradient program (flow rate 1 ml min^{-1}): at 0 min 59/40/1 (%A/%B/%C), at 6 min 52/40/8, at 21 min 59/40/1, then holding for 5 min (A/B/C = water/hexane/2-propanol).

The fatty acid methyl esters (FAME) were analyzed by GC using 70% (v/w) cyanopropyl polysilphenylene-siloxane column (TR FAME, 30 m × 0.25 mm × 0.25 μm) and FID detector. The temperature parameters were as follows: injector 250°C, detector 280°C, column: 160°C (hold 3 min),160–220°C (rate 5°C/min), 220–260°C (rate 30°C/min), 260°C (hold 3 min). Total time: 19.33 min.

Isolation of PC from eggs

Fresh egg yolks 30 g were extracted with 95% (v/v) ethanol (4 × 50 ml). Combined ethanol extracts were condensed to 50 ml leaving two insoluble layers. The ethanol from the lower layer was removed and the residue was dissolved in hexane/ethanol/water (4:4:0.6 by vol.), shaken and left for 2 h for separation. The solvent was removed from the lower layer and the residue was chromatographed to obtain 1.9 g pure PC. PC was converted to FAME.

Enzymatic hydrolysis of phosphatidylcholine by phospholipase A_2

The reaction was carried out as described by Adlecreutz and Wehtje (2004). The yolk suspension (45 g) was hydrolyzed by phospholipase A_2 solution (3 ml) at room temperature. The mixture of products was extracted with 95% (v/v) ethanol (3 × 160 ml), the solvent was removed and the residue was washed with diethyl ether (2 × 100 ml) and dried under

reduced pressure. 0.7 g pure 1-acyl LPC was obtained after isolation and purification by column chromatography. LPC was converted to FAME.

Enzymatic esterification of 1-acyl LPC with ALA

The reaction was carried out using water-activity gradient and two different temperatures (Adlecreutz and Wehtje 2004). 1-Acyl-LPC (500 mg) and linolenic acid (2.5 g, 9 mmol) were dissolved in toluene (10 ml) and 5 g phospholipase A_2 immobilized on Supelite DAX-8 was added. The water activity was successively decreased from 0.43 to 0.22 and finally to 0.11 during the reaction. The temperature was 25°C during first 48 h and 40°C during next 48 h. The column chromatography of crude products mixture gave 0.12 g modified PC and 0.11 g unreacted 1-acyl LPC. Modified PC was converted to FAME.

The transesterification of PC with ALA by lipases

The transesterification processes were carried out using three different lipases, in N_2 atmosphere, at 52–55°C. The reaction was initiated by adding 220 mg enzyme (20% w/w of total substrates) to heptane (5 ml) containing egg yolk PC (0.5 mmol, 375 mg) and ALA (2.75 mmol, 760 mg). Modified PC (150 mg) was separated from the reaction mixture by column chromatography and converted to FAME.

Results and discussion

The material for our studies was PC from hen's eggs. The predominating FA were palmitic and oleic acid (Table 1). The former is located in *sn*-1 position whereas the *sn*-2 position is occupied by unsaturated acids from *n*-6 and *n*-9 family. We decided to exchange them to valuable α-linolenic acid from *n*-3 family PUFA through enzyme-catalyzed modification and obtain modified phospholipids. Three different lipases were used to modify the *sn*-1 position of PC in one-step transesterification process (Scheme 1): Lipozyme RM IM, Lipozyme TL IM and Novozym 435. Two first enzymes are classified as *sn*-1,3-regiospecific lipases, the third one (Novozym 435, *Candida antarctica* lipase B) is considered as non-specific enzyme but shows high selectivity towards the *sn*-1 position (Virto and Adlecreutz 2000). Immobilization of these enzymes makes possible to use them in the presence of solvent and at higher reaction temperatures.

The results in Table 1 showed that the best incorporation of ALA into the PC was achieved

Table 1 Relative fatty acid composition of PC before and after enzymatic modification

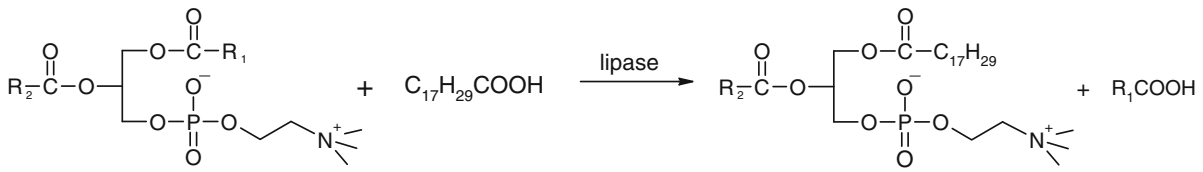
FA	Egg-yolk PC	1-acyl LPC	2-acyl LPC[a]	Enzymatically modified PC			
				Modification of *sn*-1 position[b]			Modification of *sn*-2 position[c]
				Novozym 435	Lipozyme RM IM	Lipozyme TL IM	Lecitase 10L
16:0	40	76	1	6	16	24	43
16:1	1	2	–	1	<1	<1	<1
18:0	17	18	1	3	6	11	17
18:1	27	3	41	30	27	28	4
18:2	12	1	34	30	26	23	10
18:3*	–	–	–	28	23	12	25
20:4	3	–	22	2	1	1	–

* 18:3 = α-linolenic acid (ALA, 18:3 n−3)

[a] Obtained by ethanolysis of PC with conditions: PC (0.1 g), Lipozyme RM IM (0.2 g) in 95% ethanol (2.5 ml), 5 h, room temperature

[b] Reaction conditions: 1:5.5 molar ratio of PC (0.5 mmol, 375 mg) to ALA (2.75 mmol, 760 mg), heptane (5 ml), lipase (20% w/w of total substrates, 220 mg), at 52–55°C for 48 h

[c] Reaction conditions: 1-acyl-LPC (500 mg), ALA (2.5 g, 9 mmol), toluene (10 ml), phospholipase A_2 (5 g), 25°C for 48 h and 40°C for next 48 h



Scheme 1

using immobilized *Candida antarctica* lipase (Novozyme 435). In this case, ALA accounted for 28% of all fatty acids in the modified PC. Using Lipozyme RM IM resulted in lower incorporation of ALA (23%). With Lipozyme TL IM only 12% ALA was in the PC. The FA composition in modified PC clearly reveals that mainly saturated FA, 16:0 and 18:0, were replaced by ALA during transesterification.

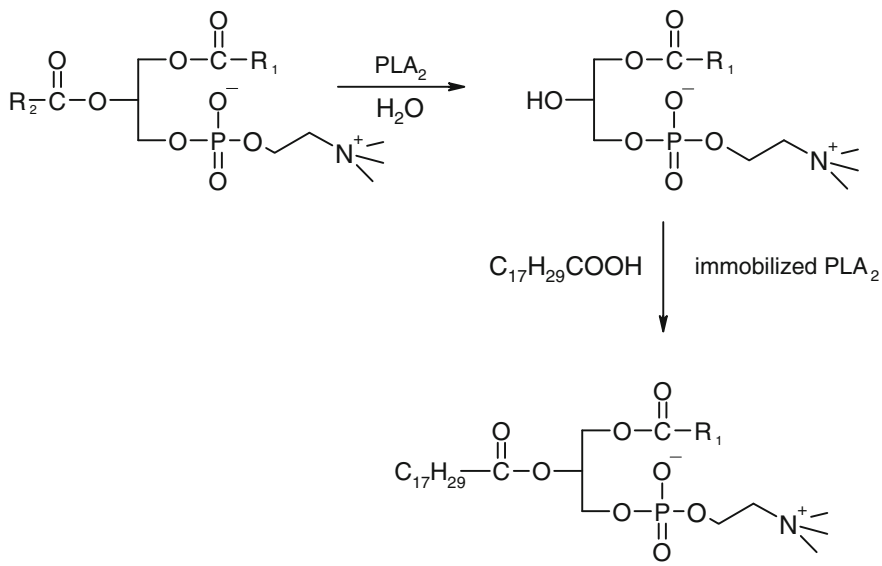
The incorporation of ALA into *sn*-2 position was carried out similar to method applied earlier for the introduction of hexanoic acid into the *sn*-2 position (Adlecreutz and Wehtje 2004). The process involved regioselective hydrolysis of PC to 1-acyl LPC and esterification of 1-acyl LPC with ALA in the second step (Scheme 2). Both steps were catalyzed by phospholipase A_2—the hydrolysis was carried out in egg-yolk suspension using the liquid enzyme preparation and before the esterification step the enzyme was immobilized on Supelite DAX-8.

After the hydrolysis and purification, 0.7 g 1-acyl LPC was obtained. The analysis of FA in 1-acyl LPC showed that saturated acids (16:0, 18:0) predominated in the *sn*-1 position (Table 1). HPLC analysis confirmed that there was no acyl migration in 1-acyl LPC. The second step of the process—esterification of 1-acyl LPC with ALA was carried out in toluene and with the water-activity gradient. After reaction, we obtained modified PC with a 25% content of ALA. Taking into consideration the regioselectivity of phospholipase A_2 the desired ALA was introduced exclusively in *sn*-2 position. In this case, mainly oleic acid from *sn*-2 position was replaced by ALA.

All lipases used in our studies are regiospecific but not specific for the incorporation of ALA. Linoleic and oleic acids present in the commercially available ALA preparation were simultaneously incorporated into *sn*-1 position of PC along with α-linolenic acid.

Summarizing the results, we introduced ALA in both (*sn*-1 and *sn*-2) position of PC using different enzymatic methods. The further optimalization of these methods will be carried out soon in order to increase incorporation rate of ALA into PC.

Scheme 2



Acknowledgements This work was supported by Ministry of Science and Higher Education, Grant no R05 049 02.

References

Adlecreutz D, Wehtje E (2004) An enzymatic method for the synthesis of mixed-acid phosphatidylcholine. J Am Oil Chem Soc 81:553–557

Adlecreutz P, Lyberg AM, Adlecreutz D (2003) Enzymatic fatty acid exchange in glycerophospholipids. Eur J Lipid Sci Technol 105:638–645

Aura A-M, Forssell P, Mustranta A, Poutanen K (1995) Transesterification of soy lecithin by lipase and phospholipase. J Am Oil Chem Soc 72:1375–1379

Berry EJ, Hirsch J (1986) Does dietary linolenic acid influence blood pressure? Am J Clin Nutr 44:36–340

Bigger JT, El-Sherif T (2001) Polyunsaturated fatty acids and cardiovascular events. A fish tale. Circulation 103:623–625

Doig SD, Diks RMM (2003) Toolbox for exchanging constituent fatty acids in lecithin. Eur J Lipid Sci Technol 105:368–376

Guo Z, Vikbjerg AF, Xu X (2005) Enzymatic modification of phospholipids for functional applications and human nutrition. Biotechnol Adv 23:203–259

Hara S, Hasuo H, Nakasato M et al (2002) Modification of soybean phospholipids by enzymatic transacylation. J Oleo Sci 51:417–421

Haraldsson GG, Thorarensen A (1999) Preparation of phospholipids highly enriched with n-3 polyunsaturated fatty acids by lipase. J Am Oil Chem Soc 76:1143–1149

Mead JF, Alfin-Slater RB, Howton DR, Popjak G (1986) Lipids: chemistry, biochemistry and nutrition. Plenum Press, New York

Peng L, Xu X, Mu H et al (2002) Production of structured phospholipids by lipase-catalyzed acidolysis: optimization using response surface methodology. Enzyme Microb Technol 31:523–532

Reddy JRC, Vijeeta T, Karuna MSL et al (2005) Lipase-catalyzed preparation of palmitic and stearic acid-rich phosphatidylcholine. J Am Oil Chem Soc 82:727–730

Svenson I, Adelcreutz P, Mattiasson B et al (1993) Phase behaviour of aqueous systems of enzymatically modified phosphatidylcholines with one hexadecyl and one hexyl or octyl chain. Chem Phys Lipids 66:195–197

Vikbjerg A, Mu H, Xu X (2005a) Lipase-catalyzed acyl exchange of soybean phosphatidylcholine in n-hexane: a critical evaluation of both acyl incorporation and product recovery. Biotechnol Prog 21:397–404

Vikbjerg AF, Peng L, Mu H, Xu X (2005b) Continuous production of structured phospholipids in a packed bed reactor with lipase from *Thermomyces lanuginosa*. J Am Oil Chem Soc 82:237–242